

BERLINER

KLINISCHE WOCHENSCHRIFT.

Organ für practische Aerzte.

Mit Berücksichtigung der preussischen Medicinalverwaltung und Medicinalgesetzgebung

nach amtlichen Mittheilungen.

Redacteur: Professor Dr. C. A. Ewald. Verlag von August Hirschwald in Berlin.

Montag, den 27. November 1882. № 48. Neunzehnter Jahrgang.

Inhalt: I. Langenbuch. Ein Fall von Exstirpation der Gallenblase wegen chronischer Cholelithiasis. Heilung. — II. [illegible] — III. Günta. Ueber die Schwäche des weiblichen Schambeins. — IV. Hartmann. Supraorbitalneuralgie, hervorgerufen durch Muttermilch. — V. Schenk. Carbolharnen. — VI. Referate (Cohnheim. Vorlesungen über allgemeine Pathologie. — Mauthner. Glaucom.) — VII. Verhandlungen Ärztlicher Gesellschaften (Berliner medicinische Gesellschaft). — VIII. Feuilleton (Die 55. Versammlung deutscher Naturforscher und Ärzte zu Eisenach. — Die Krankenhäuser Berlins. — Tagesgeschichtliche Notizen). — IX. Amtliche Mittheilungen. — Inserate.

I. Ein Fall von Exstirpation der Gallenblase wegen chronischer Cholelithiasis. Heilung.

Mitgetheilt von

Dr. **Carl Langenbuch**,

dirig. Arzt am Lazarus-Krankenhause in Berlin.

Die Gallenblase ist nach einstimmigem Urtheil der modernen Autoren, wie Frerichs[1]), Schüppel[2]) u. A., der eigentliche *Bildungsherd der Steine* und nur verhältnissmässig selten entstammen diese den Gallengängen. Lediglich die Gallenblasensteine sollen uns hier interessiren. Es ist von ihnen sogleich zu beachten, dass neuere Forschungen zufolge solche von 1 Ctm. Durchmesser den Ductus cysticus wahrscheinlich kaum mehr zu passiren vermögen (Schüppel[2]) und dass die Concremente überhaupt, wenn Fiedler[3]) in dieser Ansicht nicht zu weit geht, die Gallenblase meist bereits auf dem Ver-

noch Fälle, in denen wir eben kein gutes Resultat zu erzielen vermögen. Den solchen zugehörenden Patienten, und ihrer befindet sich wohl der eine oder andere in der Clientel eines jeden beschäftigten Arztes, drohen die obengenannten Gefahren, ihr Leiden ist durchaus inoperös und es fragt sich hier immer wieder, ob sie ihrem Schicksale überlassen bleiben sollen, oder ob man nicht noch eine Radicalheilung in der Weise erzielen kann, dass man den Bildungsherd der Steine, also die Gallenblase selbst entfernt. Dies wäre natürlich nur auf chirurgischem Wege anzustreben.

Die Chirurgie der Gallenblase ist noch in den Anfängen der Entwicklung, aber sie existirt und die Namen Petit, Thudichum, M. Sims, Kocher, G. Brown, Lawson Tait, König u. A. sind in rühmlicher Weise mit ihr verknüpft. Freilich hat sie auch bis jetzt nur so gewinnt durch die Steinbildung bedingte

Frontispiece of the paper announcing the first cholecystectomy for gallstones carried out by Karl Langenbuch in Berlin, 1882.

RELATIONSHIPS AMONGST BILE STASIS, SEPSIS AND CHOLEDOCHOLITHIASIS: CLINICAL AND EXPERIMENTAL STUDIES

by

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A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in the
FACULTY OF MEDICINE
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June 1992.

ABSTRACT

These studies were undertaken to clarify the biological mechanisms bringing to the formation of primary common bile duct stones which are commonly associated with stasis and sepsis. This has been explained by the possibility of deconjugation of bilirubin diglucuronide in bile by a beta-glucuronidase of bacterial origin. Free bilirubin then combines with calcium ions and precipitates as calcium bilirubinate. Another proposed mechanism is the enzymatic transformation of biliary lecithins to lysolecithins with formation of calcium palmitate.

An original canine model of chronic incomplete biliary obstruction was established in eight dogs. The presence of incomplete obstruction was validated by serial retrograde cholangiograms. Bile was collected regularly for microbiology and biochemistry. Biopsies of liver and bile duct were performed for histological and histochemical examination. Experimentally produced primary ductal stones were found in two dogs. Bile was also collected for microbiology and biochemistry from 174 patients undergoing biliary surgery, as in the experimental study. In addition x-ray diffractometry of common bile duct stones was performed. A relationship between bile microbiology and clinical infection was also demonstrated in these patients. In a further series of patients, histochemical studies of liver and common bile duct tissues were carried out to assess betaglucuronidase and to correlate this with bile microbiology and biochemistry.

In summary, we have produced a novel canine model of chronic incomplete biliary obstruction, in which changes in bile microbiology and biochemistry may be studied repeatedly over a prolonged period. In both the experimental and clinical studies bacterial colonization of bile increased with time. However, neither in the experimental nor in the

clinical studies was the presence of bacteria in bile associated with changes in betaglucuronidase activity or in unconjugated bilirubin levels, even in presence of experimentally produced or naturally occurring common bile duct stones. Similarly, no changes were observed in phospholipase activity.

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1.3 LIST OF ABBREVIATIONS

(This list summarizes the more common abbreviations. However some more complex tables are provided with separate footnote abbreviations).

AB/no-AB	= Antibiotics/no-antibiotics receiving patients
BA	= Bile Acids
BetaGLU	= Betaglucuronidase
BS	= Blood sample
C	= degrees Centigrade
CBD	= Common bile duct
CDS	= Choledochoduodenostomy
Conj./Unconj	= Conjugated/unconjugated bilirubin Bil.
Epith	= Loss of bile canaliculi epithelium
fist	= fistoulous tract excision
g	= grammes
gammaGT	= Gamma Glutamyl transpeptidase
GB	= Gallbladder
GOT	= Glutamate-oxalacetate transaminases
GPT	= Glutamate pyruvate transaminase
Gran tiss	= granulation tissue
HCC	= Hilar cholangiocarcinoma
Hist	= Clinical history (of gallstone disease)

i.m./i.v	= intramuscular/intravenous
Inflam	= Inflammatory changes
l	= litres
LFT(s)	= Liver function tests
mM	= millimoles
nM	= nanomoles
PL	= Phospholipase
PIP	= Piperacillin
PM	= Post mortem
Probd	= Proliferation bile ducts
Prol	= glandular proliferation
PST	= Papillosphyncterotomy
RF	= Risk factors (for biliary infections)
RPMS	= Royal Postgraduate Medical School
Sa	= Sacrifice
TOB	= Tobramycin
UI	= International Units
uM	= micromoles
Tot.	= Total
wks	= weeks

2 PREFACE

2.1 SCOPE

Although endoscopic papillosphincterotomy was first described by Classen & Demling in 1974, in the late seventies the treatment of Common Bile Duct (CBD) stones remained a surgical matter in many hospitals, where expert endoscopic facilities were not yet available. This supported different views on the approach to CBD stones, often based on personal views between supporters of either treatment, rather than on a rigorously scientific basis. At that time the papers of Madden et al. (1968, 1970) stressed the importance of identifying the primary or secondary origin of a stone found in the CBD in order to have a correct approach to the treatment of this pathological condition.

Suggested pathogenetic mechanisms of the formation of primary CBD or brown pigment stone have been the possible role of a betaglucuronidase of bacterial origin which could cleave bilirubin diglucuronide. The free bilirubin formed could combine with Ca++, precipitating as calcium bilirubinate (Maki, 1966). In addition the role of a phospholipase in hydrolyzing biliary lecithins and of decreased total bile acids in bile have been proposed.

This thesis has been therefore designed to investigate the existence, incidence, pathogenesis and characteristics of CBD stones of primary ductal origin.

2.2 MAJOR RESULTS

In order to reproduce the conditions of bile stasis and infection which are reported in the literature to be associated with the occurrence of primary CBD stones, we have developed a model of chronic incomplete biliary stricture in the dog. This has been achieved by passing a nylon sling around the common bile duct after cholecystectomy. The ends of the sling were passed through a length of a polyethylene tube in the abdomen, before being secured in a subcutaneous pouch. A Thomas cannula was then introduced into the duodenum, facing the papilla of Vater for periodical cholangiography and bile sampling for microbiology and biochemistry. In addition liver and CBD tissues sampled intraoperatively were processed for histology and for contents of tissue betaglucuronidase.

All the dogs developed an incomplete stricture. Primary CBD stones were experimentally produced on two occasions. Studies on bile microbiology and biochemistry demonstrated that bile infection regularly increased with the duration of the obstruction. Conversely no changes were observed in bilirubin and bile acid levels, nor in betaglucuronidase and phospholipase activities in the presence or absence of bacteria in bile or in the presence or absence of stones. In addition, the presence of betaglucuronidase granules in liver or CBD tissues did not correlate with changes in unconjugated bilirubin or betaglucuronidase activity in bile.

Bile and CBD stones were also collected from 174 patients undergoing surgery for biliary disease in Ospedale Cardarelli, Napoli, Italy. Microbiology and biochemical assays were carried out as in the experimental study. No changes were demonstrated in bilirubin and bile acid levels nor in betaglucuronidase or phospholipase activity in presence or absence of infected bile or in the presence or absence of stones. Furthermore, an increased CBD size was found associated with higher bile infection rates. CBD stones were

studied by X-ray diffractometry in 15 cases. Calcium palmitate, which is reported as characteristic of brown pigment or primary CBD stones, was found in five of these, whereas 10 had no calcium palmitate. Bile infection and increased CBD size were found to be associated with the presence of CBD stones, this being further enhanced when calcium palmitate containing stones were present.

In a further clinical study carried out in London no changes in bilirubin levels and betaglucuronidase activity in bile were found in relation to the presence of betaglucuronidase in liver, CBD or gallbladder (GB) tissues.

2.3 MAJOR CONCLUSIONS

From the above results we can confirm that the occurrence of primary CBD stones is correlated with stasis and bacterial colonization of bile. However, these phenomena are not correlated to the deconjugation of bilirubin by a betaglucuronidase of either bacterial or tissue origin, nor to phospholipase-mediated hydrolysis of biliary lecithins, nor are these accompanied by changes in total bile acid levels. Calcium palmitate containing stones have occurred in 33% of patients in our population and have been associated with increased CBD size and bile infection.

3 ACKNOWLEDGEMENTS

This thesis has developed during a period of 10 years and work has been carried out in two different countries. Thus I have met in this time a number of people who have given help with their material work or with teaching, or also with their support and encouragement in several difficult steps in the development of this work. I therefore report them all below here, chronologically.

I wish to thank S.M. Aloj, Professor of Molecular Pathology at the University of Napoli. He has a deep knowledge of the Anglo- Saxon academic world and first encouraged me to undertake this programme, suggesting that this would be certainly repaid in terms of solid training and contributions.

When I first arrived in London Professor L.H. Blumgart was Professor of Surgery at the RPMS. Visiting his unit I developed my interest for hepatobiliary surgery and I learned that a scientific reputation can only be built on the basis of an everyday hardworking attitude and on the exercise of rigorous self-criticism. He also accepted my request to undertake this academic programme, despite the unusual way of collecting data in two different places, and acted as supervisor in an early phase. I am enormously grateful to him for all this. I wish also to acknowledge the help which I have always had from the staff of the Wellcome Library at the RPMS.

I have grateful feelings for the memory of the late Professor A. Trapani, head of the XVI Divisione Ospedale Cardarelli, Napoli. He brought me along the first steps of my career, stimulated me to study the role of primary CBD stones, and was happy to allow me to use his clinical material for the purposes of this thesis. Together with him I wish to thank the members of his surgical staff, who all collaborated in the collection of bile and stones in the clinical study. I wish to acknowledge the help that I had from Professor I. Covelli,

Professor of Microbiology at the University of Napoli and his co-workers Dr. G. Amato and Dr. E. Nani who cheerfully undertook the microbiological assays carried out on 174 patients operated in Napoli and from Professor E. Franco, Professor of Mineralogy at University of Napoli who agreed to perform the X-ray analysis of the CBD stones. I wish also to acknowledge the help received from Mr. V. Margarita, who taught me the first steps of computing and generously allowed me to use his computer facilities in Napoli.

I am happy to thank Mrs. Caroline Eggs, Mr. Benjamin's former secretary at RPMS, for her kind secretarial assistance and for typing the first parts of the manuscript. I wish to thank also Mr. D. Wilson, responsible for the animal house at RPMS, who has taken care of the animals under experiment even in the periods of my absence and has taught me a number of things concerning the management of experimental animals, and Mr. M. Cussen, responsible for the experimental surgical theatre, whose anaesthetic expertise I enjoyed during my operations on dogs.

I thank Dr. Carol Record, consultant radiologist at Northwick Park Hospital in London, who managed the cholangiographic studies on dogs and I am particularly grateful to Dr. J.I. Blenkharn, Microbiologist at the RPMS who has taught to me many things concerning the bacteriology of bile, and has carried out the microbiological assays for the studies in London. I am also deeply grateful to Mr. Kang Li of Surgical Sciences Unit at the RPMS. He patiently illustrated to me the biochemical assays techniques, discussed with me the methods and performed all LFT and bile biochemical assays reported in this thesis. Together with them I wish to thank Dr. C Foster, who performed histology and histochemistry studies and Mrs. S. Van Noorden who arranged the material for these studies.

I am deeply grateful to my colleague and friend Dr M. Ansalone. He took over our surgical practice during my prolonged absences from Napoli bearing most of my duties

towards my own hospital. During his own stay in London, he has collected a great part of the material for the histochemistry study, has helped me in the performance of the experimental study and has given to me cheerful hospitality in his house.

I am grateful to Dr. N. Bevilacqua for his expert advise and help in preparing the last form of the manuscript and for discussing with me the statistics and to Dr. I. Sessa who has enthusiastically accepted to re-edit with me the last editions of the manuscript.

Towards my supervisor, Professor I.S. Benjamin, I owe an incalculable debt. It needs to be underlined that when I first arrived in London I knew little of biliary surgery and of English and nothing about research. Professor Benjamin since then has spent hours and hours with me in order to bring me to the position of writing the initial project, carrying out the research work, analyzing the results, writing and revising the manuscript. He did all this always without underlining my deficiencies or discouraging me in the face of the difficulties that arose during the development of this work, but stimulating my sense of responsibility and independence. He also supported me in every possible way in the UK. as well as in Italy, and gave me warm hospitality in his house. It is really hard for me to imagine that a supervisor has ever done more for a student.

Finally my thanks and deep appreciation to Annamaria, my wife. She encouraged me to undertake what was for me a formidable task, patiently tolerated my prolonged absences, also taking care of house and children for me and always demonstrated her confidence in my possibility of arriving to this submission. This thesis is dedicated to her.

4 INTRODUCTION

4.1 SURGICAL BACKGROUND

The presence of stones in the common bile duct (CBD) is an important and common clinical condition which poses a great challenge to the surgeon. The frequent association with infection, the possibility of recurrence of stones, the associated pathological changes, the different operations proposed to relieve this condition and the increasing availability of non-surgical therapeutic options make the whole approach to the problem and the choice of treatment a matter still open to debate.

4.1.1 Historical perspective

Several different operations for CBD stones were carried out for the first time around the turn of the century. Choledochoduodenostomy was first advocated by Riedel (1888), although Sprengel (1891) gave a full description of the circumstances leading to this operation. The closure of a choledochotomy over a T-tube after CBD exploration was described by Thornton (1891) and subsequently popularized by Kehr (1913), whereas Halstead (1897) advocated "primary" closure of the bile duct after choledochotomy and suggested that if decompression was necessary cannulation of the cystic duct stump could be used. Richter and Bushbinder (1919) described the use of transduodenal sphincterotomy in cases of distal bile duct obstruction or chronic pancreatitis.

Today the problem of the treatment of CBD stones is one of considerable magnitude since gallstones affect approximately 10% of the adult population. According to Motson & Way (1982) and McSherry (1982) each year 800,000 new cases presented and 400,000 cholecystectomies were performed in the USA, and the number is certainly higher now.

Since the overall incidence of CBD stones in large collected series is 9.6% (Girard & Legros, 1988), we can assume that CBD stones are removed each year from some 38,000 patients undergoing cholecystectomy in the United States.

4.1.2 Identification

CBD stones may be suspected on clinical grounds by preoperative investigation or by intraoperative assessment. Clinically their presence is usually associated with transient episodes of jaundice, fever and rigors or colicky biliary pain. Preoperative assessment such as ultrasound or intravenous cholangiography may detect stones. Percutaneous transhepatic or endoscopic retrograde cholangiography bring probably the highest success rate in the detection of stones (Blumgart et al., 1977; Benjamin et al., 1978). However, while endoscopic retrograde cholangiography is largely used because of its high accuracy with low complication rates (2%, Cotton, 1977; 1.1% Classen & Phillip, 1988) and also because it allows the simultaneous endoscopic removal of CBD stones, percutaneous transhepatic cholangiography is usually restricted to patients with established biliary obstruction because of the 5% reported morbidity rates (Mueller et al., 1982; Gibson, 1988).

While the presence of palpable CBD stones at operation in experienced hands has given acceptable results (Way et al., 1972; Kakos et al., 1972), this is not the optimal method for detecting or excluding stones. Intraoperative cholangiography (Mirizzi, 1937; Tompkins & Pitt, 1982; Stubbs et al, 1983), biliary manometry (Caroli, 1945; Puglionisi, 1978), choledochoscopy (Berci, 1961; 1988) or more recently operative ultrasound techniques (Eiseman 1965; Jakimowitz, 1985), allow accurate identification of stones in the CBD.

Lacaine et al. (1980) found the association of increased CBD size, raised alkaline phosphatase and a history of jaundice to be highly predictive of CBD stones. Lygidakis

correlated CBD dilatation not only with the presence of stones in the duct, but also with age, previous biliary surgery, infections and a long history of biliary disease.

However, despite the techniques described, considerable rates of retained stones are reported in the literature, ranging from 4.3% (Glenn, 1975) to 9% (Velacott & Powell, 1979). Girard & Legros (1988) report that the incidence of retained stones, being rare after simple cholecystectomy, ranges between 1.3 and 7% after CBD exploration. Tweedle & Blumgart (1988) underline that with the use of postexplorative cholangiography and/or choledochoscopy the reported retained stone rate should be 4% or less. According to Girard & Legros (1988), 50% of stones are retained in the CBD because their removal from the CBD was technically impossible, 23% are overlooked because of technically inadequate post-exploratory cholangiography, 19% are overlooked despite good quality post-exploratory cholangiography and 8% because they are misinterpreted as air bubbles on post- exploratory cholangiography.

4.1.3 Management

Several different operations have been proposed for the treatment of common duct stones: the use of a T-tube after supraduodenal choledochotomy for CBD exploration has been supported because of its safety (Glenn, 1958; Trapani et al., 1981; Falcione et al., 1983) but others have found it unnecessary and associated with higher infective complication rates (Chande & Devitt, 1973; Keighley et al., 1976).

Choledochoduodenostomy has been advocated by some for its safety and efficacy, especially in elderly patients with dilated ducts (Madden et al., 1968; 1970; Degenshein, 1974; Lygidakis, 1981), but others have reported that it is associated with high rates of postoperative cholangitis, or responsible for the so called "sump syndrome" related to the filling of the blind pouch below the stoma with food fragments, vegetable fibres or calculi

(Smith, 1964; Stock & Fung, 1964; Jones, 1973; Tondelli & Cyr, 1978). A CBD calibre of at least 15 mm has been considered an essential prerequisite for the operation. When the duct is smaller, or when a stone is impacted in the ampulla, the performance of transduodenal sphincteroplasty may be preferred (Thioas et al., 1971; White, 1973). This operation has been extensively used by some Italian surgical teams (Stefanini et al, 1974; 1977; Speranza et al., 1982;) although others have criticized it for carrying high morbidity and mortality rates (Blumgart, 1978; Imrie et al., 1978).

The introduction of endoscopic sphincterotomy (Classen & Demling, 1974; Kawai et al., 1974) generated great enthusiasm for this non-surgical approach to the problem of choledocholithiasis and during the last two decades trends for the treatment of CBD stones have changed radically. Excellent results have been reported in most series (Safrany, 1981; Reiter et al., 1984; Cotton & Vallon, 1981), with an overall success rate in stone removal of 85%. Some critical overviews of these data (Blumgart & Lygidakis, 1982; Tweedle & Blumgart, 1988) underline the presence of much higher failure rates in some isolated series (30%, Sloof et al., 1980), or in collected surveys from non-specialist centres (24%, Cotton & Vallon, 1981). Kullman et al. (1991) reported an overall success rate in duct clearance of 88%, with a 15.5% complication rate and mortality rate over 4%. In the past decade failure rates as high as 38% in patients with stones larger than two cm were reported (Safrany, 1978; Cotton, 1980) and on the basis of these results some authors suggested a careful selection of patients either for surgery or for endoscopic removal of stones. Now this technique has become more widely practised and its indications undoubtedly are increasing: series report a CBD clearance rate of up 98% when this technique is associated with mechanical lithotripsy (Vladimirof et al., 1990).

Stain et al. (1991) underline that in patients with stones both in GB and CBD, the duct clearance rate was significantly higher when patients were treated only by surgery rather than by endoscopic sphincterotomy plus surgery, with better results also in terms of

morbidity and costs. In contrast it has been recently suggested that when GB stones are associated with CBD stones, simple endoscopic removal of CBD stones in many cases may be adequate therapy, since the persistence of symptoms related to the presence of stones in the GB is reported to range between 5% (Hill et al., 1991) and 11% (Kullman et al, 1991). The use of endoscopic papillosphincterotomy has been recommended even for previous gallstone pancreatitis, with stones present only in the GB (May et al., 1991) in place of cholecystectomy.

As the use of endoscopic sphincterotomy is progressively extended, it has also been recommended for those patients presenting with sphincter of Oddi dysfunction. The simple presence of biliary pain after injection of contrast medium at ERCP has not proven to be consistently associated with sphincteric functional impairment (Schmaltz et al., 1990). However, selection based on dynamic scintigraphy (Fullarton et al., 1990), has produced more reliable results.

The indications for and results of endoscopic sphincterotomy were recently reviewed in a consensus conference: figures of 10% morbidity and 3% risk of death were agreed (Cotton et al., 1991). In this respect it seems worthy to report here the recommendations published by Moreaux (1990) who reports an experience of 25 years treatment of CBD stones, surgical until 1981 and endoscopic and surgical thereafter. This author suggests that the choice between surgical or endoscopic treatment of CBD stones should be based upon considerations such as the age of the patient, the presence of associated complications such as jaundice or cholangitis, the presence of the GB in situ, predicted difficulties for endoscopy or for surgery and the relative experience of the endoscopic or surgical teams.

In the presence of retained stones, mechanical extraction through the T-tube tract was suggested 20 years ago (Burhenne, 1973; Mazzariello, 1973). The use of dissolution agents such as mono- octanoin (Leuschner et al., 1979) has long been debated and more

recently the use of methyl-tert-butyl ether through naso- biliary catheters seems to achieve more interesting results (Murray et al., 1988; Neoptolemos et al., 1990). However these studies have underlined that this agent is effective only against pure cholesterol stones and have emphasized that this technique may potentially carry complications related to the high toxicity of the agent and should only be adopted in specialized units.

The present clinical studies were carried out in the XVI Divisione Ospedale Cardarelli, Napoli, Italy, in the years between 1969 and 1983. In this unit, the surgical policy adopted for common duct stones aimed to define precise indications for each operation. This was done by selecting the patients according to the degree of dilatation of the common bile duct as found at operation: for ducts up to 18mm, temporary external drainage was utilized via a T-tube or trans-cystic ductal tube; for ducts over 18mm diameter a biliodigestive anastomosis, a choledochoduodenostomy in the vast majority of the cases, was performed. Furthermore, according to the suggestion of Madden (1968), at operation we also tried to establish whether a stone was of primary or secondary origin on the basis of its macroscopic appearance using the classification proposed by Aschoff (1924). These criteria, although based upon rather empirical grounds, have allowed satisfactory results carrying acceptable morbidity and mortality rates in a series of over 400 patients (Trapani et al., 1983).

It is important to distinguish the CBD stone which has originated in the GB (secondary CBD stone) and the so-called "primary" common duct stone, the stone which forms primarily in the common bile duct. As well as having a different pathogenesis and physical and chemical characteristics, the differentiation of these stones in fact is a question of major surgical significance. Madden et al.(1968) suggested that when a stone forms within the common duct it means that in that duct the biochemical conditions exist which gave rise to the formation of the stone and therefore surgery should modify such conditions by mean of a bilio-enteric anastomosis in order to relieve bile stasis. By contrast in the presence of

a secondary stone, choledochotomy and stone extraction is adequate therapy, with no need to modify the pathophysiological status of the duct.

The object of this thesis was therefore to assess the incidence and to clarify the pathogenesis of primary CBD stones. A detailed review of the literature will be reported in each separate section of the work. In this background section the status of our knowledge before the start of the study is reported, with special reference to previous studies which have influenced the major questions of this thesis.

4.2 BIOCHEMISTRY OF BILE

Bile secretion is one of the major functions of the liver. Bile is an aqueous solution of organic and inorganic compounds. The major organic components are conjugated bile acids or related salts, bile pigments, cholesterol and phospholipids. Small amounts of protein are also present. Inorganic electrolytes are present in bile in concentrations similar to plasma.

Cholesterol, bile salts and phospholipids are excreted in macromolecular complexes called mixed micelles. The precise site for their formation has not yet been determined (Bouchier et al, 1988). In normal gallstone-free bile these compounds are maintained in a micellar solution; should the amount of cholesterol increase - absolutely, from increased dietary intake, or proportionally, from decreased content of bile salts - cholesterol will tend to form crystals. These relationships have been expressed by Admirand & Small (1968) in a phase diagram, where cholesterol, phospholipids and bile salts are plotted on triangular coordinates. Interestingly, not all bile supersaturated with cholesterol will form crystals: the presence of a metastable zone is described in which bile is supersaturated with cholesterol, possibly in a liquid crystalline phase, which does not form crystals. A

metastable solution will precipitate either spontaneously in the presence of further markedly increased content of cholesterol (homogeneous nucleation) or if nucleating factors are introduced (heterogeneous nucleation) (Mufson, 1974, Carey & Small, 1978). Other factors, such as the speed of the process, molecular proximity and impaired balance between pronucleating and nucleating agents have also been recently considered (Strasberg & Harvey, 1990)

In contrast to the enormous amount of information available on the pathogenesis of cholesterol gallstones, much less is known about the pathogenesis of pigment stones. Black or brown pigment stones differ in composition, aetiology and clinical associations. The major component of these stones is calcium bilirubinate and recently (Moore et al., 1982) the role of ionized calcium has been recognized and needs further elucidation. Bilirubin is present in bile as di- and mono- glucuronide. The latter is particularly unstable and therefore easily converted to free bilirubin, and is present in bile associated with micellar lipids.

4.3 GALLSTONE FORMATION

4.3.1 Classification

Madden stressed the importance of recognising whether a CBD stone originates from the gallbladder or from the CBD. To achieve this he used the classification published by Aschoff in 1924. This author classified biliary stones according to their origin as (1) inflammatory; (2) metabolic; (3) mixed; and (4) stasis (see Table 4.1).

The so-called stasis stone arises primarily in the common bile duct and, according to Madden, is therefore truly recurrent, since it may reform once it has been removed. It is described as yellow-brownish, ovoid, soft, conforming in shape to the size of the bile duct, easily crushable with the fingers, generally solitary but surrounded by biliary mud and sludge (Fig. 4.1).

More recently a different and simpler system has been used (Trotman and Soloway, 1982) which classifies stones according to their composition as cholesterol stones or pigment stones (see Table 4.1). The latter are further divided into black and brown pigment stones. These three types of stones differ in appearance, epidemiology, significance, clinical associations, and aetiology. Cholesterol and black pigment gallstones arise more frequently in Western countries where people typically eat a high protein, high fat, low fibre diet. The male:female ratio is 1:2. They are usually found in the gallbladder and are not associated with infection. Cholesterol gallstones are due to bile saturation of cholesterol, mostly from increased dietary consumption. In contrast black pigment stones are often due to increased bilirubin content of bile and therefore may be associated with haemolytic anaemia.

Table 4.1 *

CLASSIFICATIONS OF BILIARY STONES

A. according to their origin (Aschoff, 1924)

1. Inflammatory

2. Metabolic

3. Mixed

4. Stasis (ovoid, soft, generally single, conforming in shape to the contour of the CBD; readily crushable with fingers into multiple fragments of biliary mud. Brown, central nidus with yellow laminated periphery).

B. according to their composition (Trotman & Soloway, 1982)

Cholesterol Stones

Pigment Stones
(a) BLACK Pigment Stones
(b) BROWN Pigment Stones

* also reported as Table 6.1



Fig.4.1 A recurrent or "primary" CBD stone, yellow-brownish in colour and surrounded by biliary mud.

Brown pigment gallstones have been typically described in countries with a low protein, low fat, high fibre diet, such as in the Far East. Interestingly, these stones were extremely common in Japan before or soon after the Second World War when a low protein, low fat, high fibre diet was common. However the incidence has decreased in recent years since Japanese people have adopted Western dietary habits and the incidence of cholesterol stones has correspondingly increased in that country. There are also social characteristics for these stones since they have been described more commonly in people from a rural environment than in those from large cities (Maki, 1961). The male:female ratio is 1:1. In the Far East they may arise in the gallbladder as well as in the common bile duct and their origin is still unclear even though they have been found often to be associated with infection. In addition these stones have been found in Japan and in other parts of the Far East (Tung et al., 1960) associated with biliary parasites such as Ascaris Lumbricoides or round worm. This parasite penetrates the papilla, enters the biliary tract and may obstruct bile flow into the duodenum. Furthermore the passage of the round worm through the papillary apparatus causes a forced papillary dilatation and inflammation with subsequent fibrosis, stasis, infection and stone formation. Finally parasites are described to act as a nidus which facilitates precipitation of calcium compounds and stone formation. Sato (1955) has produced experimental stones by keeping the carcass of a round worm in the gallbladder of a living dog for one year.

Brown pigment stones are therefore rare in the Western world where usually people eat a high protein, high fat, low fibre diet and biliary parasitic infestation is exceptional. In this respect Aschoff (1924) described such stones as "stasis stones" and classified them as of primary ductal origin, associated with infection and bile stasis.

4.3.2 Cholestasis

There is evidence in the literature that primary common bile duct stones are associated with stasis and bacterial colonization of bile (Imamoglu et al., 1957; Maki, 1961; Glenn, 1964; Maki, 1966; Madden, 1968; Keighley, 1976; Saharia et al., 1978; Cetta, 1983; Lygidakis, 1983; Lygidakis, 1984). However, the biological pathways which correlate these three phenomena are still unclear and indeed it is difficult to distinguish causes from effects.

Benjamin (1983) has classified biliary obstruction into four types: complete, intermittent, chronic incomplete and segmental. In the presence of CBD stones usually a type II or intermittent biliary obstruction takes place. However, when CBD stones are associated with papillary stenosis (Madden et al., 1968), or in patients with long standing gallstone disease such as most of the patients operated on for biliary stones in south Europe and in general in the Mediterranean area (Lygidakis, 1984), the obstruction may change to type III or chronic incomplete obstruction.

Organic impairment of papillary function may be primary in a small percentage of cases (Arianoff, 1968). More commonly it is secondary to inflammatory processes, to trauma related to the passage of small calculi through the sphincteric apparatus, or to iatrogenic trauma caused by surgical probing of the papilla (Blumgart, 1978; Imrie et al., 1978; Hunt & Blumgart, 1980). Such papillary stenosis results in dilatation of the CBD and has been found to be significantly associated with the presence of primary CBD stones (Madden, 1968).

Finally, in recent years endoscopic manometry studies of the sphincter of Oddi have been undertaken (Toouli, 1984; 1988) and this has allowed the distinction to be made between structural stenosis and true dyskinesia of the sphincter. Papillary stenosis, irrespective of

its origin, results in a dilatation of the CBD and is significantly associated with the presence of primary CBD stones (Madden et al., 1968).

4.3.3 Infection

Normal bile is sterile. The presence of bacteria in bile was first described by Welch (1891). Since that time this field of research has been continuously explored and more recent observations report a 30-50% incidence of bacterial colonization of bile sampled during cholecystectomy (Kune and Schultz, 1974; Goswitz, 1974; Keighley 1977). Benign biliary strictures and choledocholithiasis are associated with an incidence of infection greater than 80% (Scott and Khan, 1967; Pitt et al, 1980; Jackaman et al, 1980), whereas in obstruction due to malignancy bacterial colonization is generally less than 20% (Keighley et al, 1976; Blenkharn et al, 1984). In 37 patients undergoing long-standing external biliary intubation, with a mean duration of 18 days, McPherson et al. (1982) reported a 27% bile infection rate at first culture. By the time of operation this rose to 54.5% due to external contamination, with polymicrobial bacterial growth in 42.4%.

The organisms most commonly isolated from bile are enterobacteria: E.coli, Klebsiella, Pseudomonas, Proteus and Streptococcus faecalis predominate amongst the aerobes, while Bacteroides fragilis and Clostridia are the most frequently isolated anaerobes (Nielsen & Justensen, 1976).

The route of infection of the bile is generally regarded as haematogenous with direct spread of organisms through the gallbladder wall and common bile duct wall (Scott & Kahn, 1967; Nielsen & Justensen, 1976). However, ascending infection from the duodenum (Keighley, 1977) and the possibility of an entero- hepatic cycle of bacteria (Glenn, 1973) have also been suggested.

Post-operative infections are usually caused by organisms originating in bile (Mason 1968; Keighley et al, 1977; Keighley 1982). Commonly recognized risk factors for infections following biliary tract surgery are age greater than 70 years, obstructive jaundice, a history of cholangitis, previous biliary tract surgery, emergency operation and the presence of CBD stones (Keighley et al., 1976).

Antibiotic prophylaxis in biliary surgery has been long debated, but is now coming closer to consensus. For many years there was no agreement about the ideal pharmacokinetics for an antibiotic for prophylaxis in biliary tract surgery (Paradisi et al, 1967; Bevan and Williams, 1961; Strachan, 1978; Keighley et al, 1976), nor about its spectrum of activity (Mason, 1968; Morran et al, 1984; Willis et al, 1984; Blenkharn and Blumgart, 1985). More recently it has been agreed that antibiotics capable of high plasma concentrations and active against enterobacteria, S. faecalis and anaerobes will afford the best protection in biliary tract surgery. However, it is still open to debate whether antibiotic prophylaxis should be given to all patients undergoing biliary surgery (Stubbs et al, 1983; Murray & Bradley, 1983; Morran et al, 1984; Burdon et al, 1984), or only to those selected according to criteria capable of predicting patients at highest risk of infection (Keighley et al, 1976b).

4.3.4 Biochemical changes

Several biochemical changes have been reported in the bile of patients with pigment CBD stones: these changes have been extensively investigated in the last 30 years in order to correlate the composition of bile and of CBD stones with possible associated infection.

The key hypothesis to explain the association of primary CBD stones with infected bile was proposed by Maki and his co-workers in Tohoku University, Sendai, Japan, and this work will be repeatedly referred to in this thesis. In a series of studies (Maki, 1961, Maki

et al, 1962, 1964; Matsushiro et al, 1965, Maki, 1966) these workers stated that in the presence of bacterial colonization of bile, bilirubin diglucuronide is hydrolyzed by a betaglucuronidase of bacterial origin. Unconjugated free bilirubin then combines with calcium ions, precipitating as calcium bilirubinate (fig. 4.2).

To support this theory Maki and his co-workers recalled the experiment of Becmeister (1908) who noticed a precipitate of cholesterol crystals forming in vitro in bile from patients with cholesterol gallstones. In 1966 Maki reported an experiment where betaglucuronidase of bacterial origin was added to human bile in vitro. Biliary concrements were formed, both in the presence of betaglucuronidase and in bile infected with E.coli. By contrast no precipitate was seen in the presence of sterile bile. In addition Maki searched for physical factors which could have enhanced the ability to coagulate biliary precipitates. He added to the bile electrolytes, such as calcium ions, to check the action of electrical attraction, and found that stones were formed particularly at an acid pH. Finally Maki added substances

with a high molecular weight in order to investigate the bridging properties related to their complex molecular structure, or applied physical movement to the tubes containing bile and obtained the formation of stones in all cases.

However, betaglucuronidase activity in bile has been reported also in the absence of bacteria (Wosiewitz et al, 1979). This has suggested the possibility that the enzyme may also originate in liver parenchyma and/or in the bile duct wall and the possibility of bilirubin deconjugation by betaglucuronidase of non-bacterial origin has been proposed in several papers (Ostrow, 1984b; Ho, 1985; Holmberg et al, 1986).

Although based on in-vitro demonstration, the Maki theory has previously been widely accepted in the scientific community. However, while a number of papers have supported

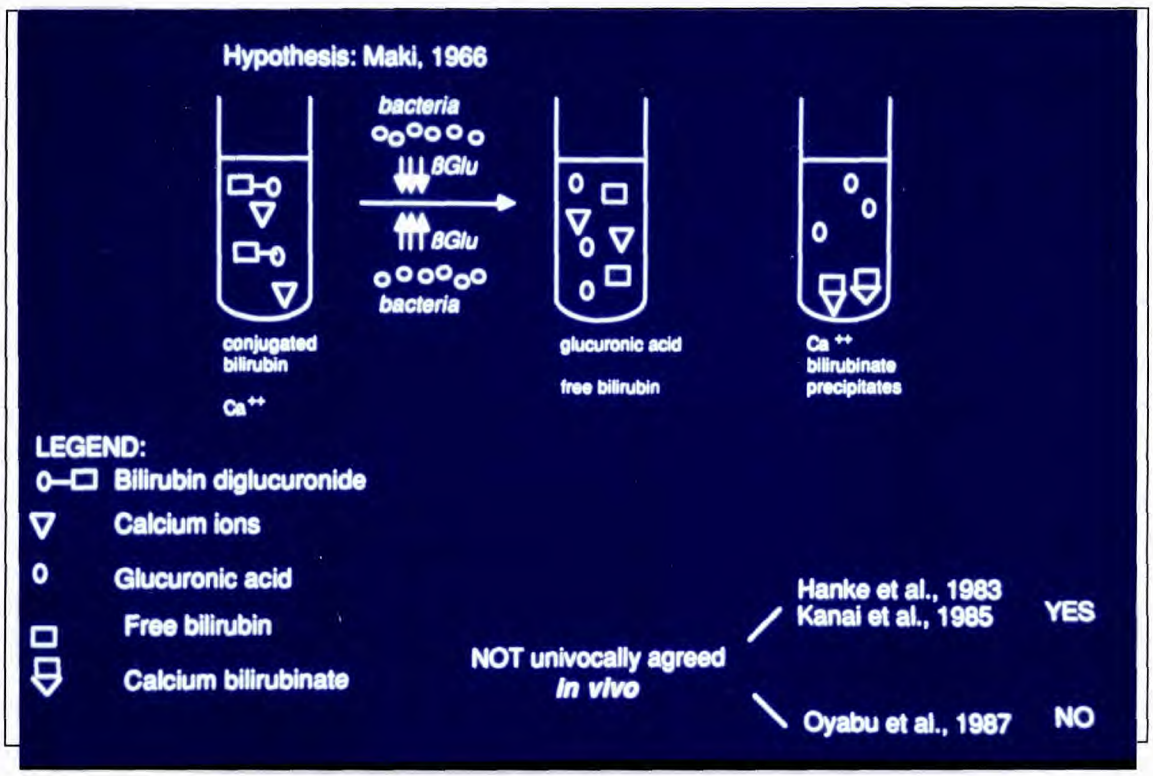


Fig.4.2 Graphic representation of the Maki's hypothesis.

it on the basis of clinical and experimental data (Hanke & Marklein, 1983; Kanai, 1985), others authors have challenged or clearly rejected it (Masuda & Nakayama, 1978; Wosieweitz, 1979; Soloway, 1982; Ostrow, 1984; Oyabu et al., 1987).

The role of biliary pH has not yet been elucidated, but is probably related to the optimal conditions for betaglucuronidase or other enzyme activity in bile. Some have reported that biliary pH in CBD bile in the presence of recurrent stones is lower than that in the presence of residual stones (Cetta, 1983): it may be that the frequent presence of bacteria in the bile of these patients might lower the biliary pH and consequently increase the activity of endogenous betaglucuronidase (Ostrow, 1984).

The finding of large amounts of calcium palmitate in brown pigment stones and in recurrent CBD stones (Sutor & Wooley, 1971; Cetta, 1983) led to the hypothesis that biliary lecithins might be transformed to lysolecithin. By the action of a phospholipase of bacterial or pancreatic origin. The subsequently formed palmitic acid might combine with calcium forming a crystalline compound of calcium palmitate. In addition lecithins attacked by phospholipase are converted to lysolecithin (with release of palmitic acid): this compound has been shown to play a role in the pathogenesis of acute cholecystitis (Sjoodal et al., 1978) as an inflammatory mediator with the production of mucoprotein complexes which might act as nucleation agents. However, this hypothesis also appears rather controversial since, while some authors identify a definite place for phospholipase in primary CBD stone formation (Cetta et al., 1984), others have found no phospholipase activity in bile (Goswani and Frey, 1974).

The bile acids in hepatocytes and in bile canaliculi are the sum of those returning from the intestinal tract and those newly synthesized in the liver. The proportion of the bile acids in normal subjects is 40% cholic acid, 40% chenodeoxycholic acid and 20% deoxycholic acid. Cholesterol gallstones may be associated with a decreased bile acid pool size, which

induces relative cholesterol supersaturation and precipitation of cholesterol crystals. However, there is no evidence in the literature to suggest a similar mechanism for the presence of pigment, calcium bilirubinate stones: indeed it has been reported (Shimada et al, 1984) that bacterial infection in bile might enhance the formation of free bile acid, but no correlation has been demonstrated with the presence of common bile duct stones.

4.4 STRUCTURAL CHANGES IN BILE DUCT AND LIVER

The presence of stones in the common bile duct is often associated with structural changes in bile duct and liver, which are related to the condition of chronic incomplete biliary obstruction (Benjamin, 1983). Following an initial proliferation of biliary canaliculi in size, number, and length, deposition of collagen reticulin takes place in the periportal areas, causing fibrosis which in turn aggravates cholestasis. In addition the extrahepatic ducts develop mucosal atrophy and squamous metaplasia, with fibrosis of the subepithelial layers, particularly in the presence of infection. The evolution of such changes is rather slow and for this reason they are found mainly in the presence of chronic incomplete obstruction associated with stones in the CBD or a benign biliary stricture. These changes have obvious clinical relevance, since while fibrotic changes are described initially, a progressive evolution to irreversible biliary cirrhosis is seen in patients with iatrogenic strictures, leading to portal hypertension (Blumgart et al, 1984).

4.5 GENERAL PLAN OF THE WORK

We have seen in this introduction that although the presence of CBD stones is a common clinical condition there is no agreement in the literature about the incidence and pathogenesis of primary CBD stones. To establish whether a CBD stone is of primary or

secondary origin represents a major surgical question, since simple stone extraction represents an inadequate therapy for primary CBD stones.

We have seen that the stasis stone described by Aschoff (1924) associated with bile stasis and infection corresponds to the brown pigment stone (Trotman & Soloway 1982) which is reported frequently from Far Eastern countries associated with parasitic infestation, stasis and infection. The possible role of bacterial betaglucuronidase and phospholipase in the formation of calcium bilirubinate and palmitate stones respectively has been described above. Animal models of cholesterol stones and black pigment stones have been described respectively in dogs fed with high cholesterol diets (Bouchier, 1971)) or in mice with hereditary haemolytic anaemia (Trotman et al., 1981). However, much less is known about the pathogenesis of brown pigment stones and no satisfactory animal models have been reported so far. This was one of the principal aims of this work.

In order to study these relationships under clinical conditions we have used the population of patients who underwent biliary surgery in the Department of Surgery at Ospedale Cardarelli, Napoli, Italy, between February 1983 and January 1985. A total of 174 patients were studied prospectively and bile was collected for microbiology and biochemistry, as well as measurement of CBD size and evaluation of the characteristics of CBD stones, if present.

Since the initial results of this part of the work failed to confirm the suggested relationships between the presence of bacteria in bile and the formation of brown pigment stones by way of a betaglucuronidase of bacterial origin, we carried out histochemical studies of betaglucuronidase in liver and bile duct tissues available from both experimental and clinical material.

This work therefore consists of three separate but related studies, with the following general aims:

1. To develop an appropriate experimental model of long-standing incomplete biliary obstruction in which the microbiological, biochemical and structural changes associated with primary CBD stone formation may be studied.

2. To use this model to examine the reported correlations amongst stasis, infection and CBD stone formation.

3. To examine in the experimental model and in man the relationships between infection and stone formation and specifically to review (a) the levels of bilirubin diglucuronide, betaglucuronidase activity and bacteria in bile; (b) phospholipase activity; (c) levels of total bile acids in bile.

4. To assess the possible role of a non-bacterial, tissue betaglucuronidase in the formation of primary CBD stones.

5. To examine in man the relationships between CBD size and infection and the macroscopic classification of CBD stones as proposed by Aschoff (1924).

6. To draw from the answers to the above questions conclusions which may be valuable in clinical practice.

5 CANINE BILIARY OBSTRUCTION STUDY

5.1 INTRODUCTION

In order to study the changes which are at the origin of the association between bile stasis, infection and stone formation there was a clear need for an animal model of biliary obstruction. Many models have been described, but the majority are models of acute complete obstruction and/or of artificial colonization of bile, while models of chronic long-standing biliary obstruction and gallstone formation have been reported much more infrequently.

5.1.1 Models of acute biliary duct obstruction.

Dawson, 1968, in order to study the effects of obstructive jaundice on renal function, developed a model of acute biliary obstruction by ligating the common bile duct in rats and in dogs. The rats were kept alive for one week and the dogs for up to four weeks. In this paper the author also quotes previous studies which used models of acute bile duct obstruction (MacGregor, 1953; Williams et al., 1960; Haber, 1962; Wright & Braithwaite, 1962; Dawson, 1964;).

Cardoso et al (1982) reported a model of acute biliary obstruction in dogs: animals were divided in two groups. In the first at laparotomy the cystic duct was ligated and the CBD was cannulated. In the second group the CBD was also ligated. Two weeks later animals were reoperated and after observation of the state of obstruction bacteria were inoculated

into the mesenteric vein and thereafter serial sampling of bile and portal blood were carried out.

5.1.2 Models of chronic biliary obstruction and stone formation.

Few such models have been reported. Imamoglu et al. (1957) quote in their paper two previous models of chronic incomplete biliary tract obstruction. The first was that of Westphal & Gleichman (1931) from Hannover, Germany. In one series of operations on dogs they ligated the cystic duct. In an other series they established a model of incomplete biliary tract obstruction by ligating the mid portion of CBD with catgut. In a third series of dogs complete CBD occlusion was obtained with a ligature just proximal to its entrance into the duodenum. In some dogs in all these series the authors injected bacteria: in some animals what they describe as "bilirubin fall-out" was found, while in some animals from the second series, stones were found in the gallbladder. In the majority of animals bacteria were isolated from bile. In the second study Bisgard & Baker (1940) established a model of "temporary incomplete and long-standing complete" biliary tract obstruction by ligating the CBD in the goat above or below the confluence with the pancreatic duct, permitting the reflux of pancreatic juice. No stones were observed in the first group, but stones were found in the gallbladder in three out of five experiments in the second group, suggesting to the authors a role for pancreatic juice in gallstone formation.

A solid calcium bilirubinate stone was experimentally produced by Sato (1955), by keeping a carcass of Ascaris Lumbricoides for about 10 months in the gallbladder of a living dog. One year later Atsumi (1956), from the same laboratory, obtained a precipitate of calcium bilirubinate around eggshells, a few days after injection of a saline suspension of eggshells into the gallbladder of a dog.

Models of chronic biliary tract obstruction are difficult to produce, since to prolong the condition of biliary obstruction, the obstruction itself must remain incomplete. The model described by Imamoglu et al (1957) was valuable in the planning of the present study. These authors applied a tape of cellophane - a substance capable of stimulating fibrosis - around the lower part of the common bile duct in rabbits, dogs and monkeys. At post mortem incomplete obstruction of the common bile duct was found in the majority of animals, but several were found to have complete obstruction. Primary CBD stones were experimentally produced in ten out of eighteen animals with incomplete obstruction and in one out of five animals with complete obstruction. The stones were all described as having a typical soft consistency, so that their composition probably included calcium palmitate.

Huang et al. (1969) described a model of incomplete biliary obstruction in dogs, obtained by ligating the common bile duct around a silver probe of 1 mm diameter inserted via a duodenal incision. After the ligature, the probe was removed and the duodenotomy was closed. Cholecystectomy was also performed and a thin-walled Teflon catheter inserted in the cystic duct stump, connected to an external pressure transducer, in order to record changes in biliary pressures. Six weeks later, E.coli was injected into the cystic duct stump in order to induce acute cholangitis. This study demonstrated the presence of bacteria in thoracic duct lymph only with biliary pressures in the CBD above 200 mm H2O and positive blood cultures with biliary pressures above 250 mm H2O.

Miranda et al. (1981) reported a model of intermittent obstruction of the bile duct in dogs, obtained by introducing a Teflon catheter through the cystic duct into the duodenum. The catheter was obliterated at its midpoint and the ends were brought out from the fundus of the GB and duodenal wall. The catheter was provided with side holes and both ends were connected to a pressure recording system. Thomas cannulae were placed in the GB fundus and duodenal wall to study biliary pressures in conscious dogs. The authors concluded that GB fasting pressure is lower than CBD pressure. No motor activity was demonstrated

in the CBD. A high pressure zone was found at the level of the choledochoduodenal junction. No pressure differences were found between conscious and anaesthetized animals.

These previously reported studies suffer from a number of problems. In none of them was a clear demonstration made of the presence and degree of incomplete biliary obstruction. None of them have reported microbiological or biochemical studies on bile made on regular basis. This has led us to devise a model where an incomplete bile duct obstruction could be consistently achieved and evaluated radiologically and which gives easy access to the CBD for serial sampling of bile for microbiology and biochemistry.

5.1.3 The choice of the animal

Several considerations were of importance in the choice of animal. Firstly, we had to choose an animal of adequate size capable of undergoing complex operative procedures and with anatomy reasonably comparable with the human hepatobiliary apparatus. Secondly, it was important to maintain the animal in clean surroundings, easily approachable for handling during a long period of obstruction. These considerations led us to exclude animals such as pigs, whose habits include lying in fecal material and which therefore are very difficult to keep clean in a long-standing experiment. Cats tolerate poorly a long period of nursing with a Thomas cannula, which we planned to use in our model. We have also considered to set up our model in sheeps or goats, but the digestive system of these ruminants appeared to us to represent a less suitable model of biliary obstruction.

The above considerations have led us to chose the dog for our experimental model. Greyhounds were in regular use in the Royal Postgraduate Medical School for most of the experimental work on dogs, for reasons of local availability and standardization. These dogs are of reasonable size and good temperament and easy for research workers

and animal house staff to handle. The disadvantage of the greyhound is its lack of fat reserves and its deep chest wall. Other commonly used laboratory breeds such as the beagle were considered but these are difficult to obtain and particularly short in the legs, and therefore not suitable for managing a long period of cannulation. Another option was the labrador, a breed particularly fit for cannula work, but expensive and, again, difficult to obtain.

5.2 METHODS

5.2.1 Animals and housing

Ten adult female greyhounds were studied, weighing 24.4 kg to 30.6 kg, (mean 26.7 kg). The dogs were kept in the animal house for at least two weeks before the start of the experiment. On admission they were weighed and bathed with antiseptic shampoo. Faeces were macroscopically assessed for the presence of worms: in the presence of round worms, Diethylcarbamide citrate (Banocide, Wellcome) 200 mg per ten pounds of body weight was given. In the presence of tapeworm, Niclosamide 0.5 mg (Yomesan, Bayer) one tablet per ten pounds of body weight was given.

The dogs were fed daily with one pound of meat, 1.5 lb. of rice and biscuits with added vitamins and minerals, though the nutritional regimen was usually tailored according to the dog's weight. Greyhounds are slim, racing dogs and it was important that the ideal body weight was well maintained during the experiment: in particular the dogs were overfed before surgery, so that the nutritional status remained satisfactory during the postoperative fasting period. When systemic blood was needed it was taken from a vein in the foreleg with a 19 gauge butterfly needle, fixed by tape, after shaving the skin with an electric clipper.

5.2.2 Production of the stricture

* Anaesthesia

The bladder was catheterized with a 14-16 gauge Foley catheter. A foreleg vein was cannulated and blood sampled for liver function tests, and anesthesia was induced with Penthotal diluted to 5%. Dogs were intubated with a size 11 Magill rubber tube, using a straight bladed Magill laryngoscope. Anaesthesia was maintained with halothane 12% plus mixed 02, 3 liters/min. and N02, 4 liters/min. During the operation dextrose 5% and normal saline solutions were given alternately up to about one liter for each solution.

* Surgery

The dog was placed supine and abdominal hair shaved with an electric clipper. The procedure was performed by the author with one or two assistants. All operations were carried out under strict asepsis. The skin was prepared with povidone iodine and an upper abdominal mid line incision was made. A self-retaining retractor was placed and a large bladed Mikulicz retractor gently used to lift the liver. Marginal liver biopsies were taken, sampling enough material for both ordinary histology and histochemical assays for betaglucuronidase; haemostasis was secured with transfixing stitches and diathermy. Bile was taken with sterile syringes from the gallbladder and CBD for bacteriology and biochemistry, using an insulin needle. For sampling bile from the CBD a gentle negative pressure in the syringe was needed, in order to avoid collapsing of the walls of the duct. The duodenal loop was turned leftwards to expose the portal vein and blood sampled for culture. Cholecystectomy was then performed in an antegrade fashion, transfixing the cystic duct with a Vicryl stitch. The mid portion of the common bile duct was dissected and a nylon sling (Ethilon size 0) placed around it.

The next step of the operation was the positioning of a Thomas cannula (Thomas, 1959, Fig. 5.1) in the duodenum to allow repeated access to the biliary tract. The level of the entrance of the common bile duct to the duodenum was determined by inspecting the lower course of the duct: a duodenotomy was then performed along the ante mesenteric margin of the duodenum. This is one of the most critical steps of the operation since a mistake in the positioning of the cannula would result in difficult cannulation of papilla of Vater for radiology. Initially, a short longitudinal opening was therefore made in the duodenal wall, at the level of the supposed entrance of the common bile duct, to allow the identification of the papilla from the inside. The duodenotomy was then enlarged accordingly to allow insertion of the Thomas cannula. The duodenotomy was then closed around the cannula with a double 00 silk purse string suture. An additional longitudinal abdominal incision was then made at about three finger breadths to the right of the midline incision. The longitudinal position of this incision and therefore of the Thomas cannula in the abdominal wall is an other important step in the operation. The cannula must be positioned neither so high as to cause pain by touching the costal margin nor so low as to produce an excessive traction of the duodenum to the abdominal wall and possible duodenal obstruction. It has been our policy to ask the unscrubbed anaesthetic assistant to release the legs of the animal from the fixed position before deciding where to place the incision in the abdominal wall. A skin incision of about 4 cm is necessary, following which the fascial planes were opened longitudinally, the muscles split and the peritoneum incised. Margins were then retracted and the external flange of the cannula pulled through the incision from inside to outside. Once the position of the cannula in the abdominal wall was checked again, the muscles were closed around the cannula with interrupted nylon stitches. The free margin of the omentum was finally pulled around the cannula between the duodenal serosa and the peritoneum to prevent possible leaks, and fixed with interrupted sutures (2/0 silk).

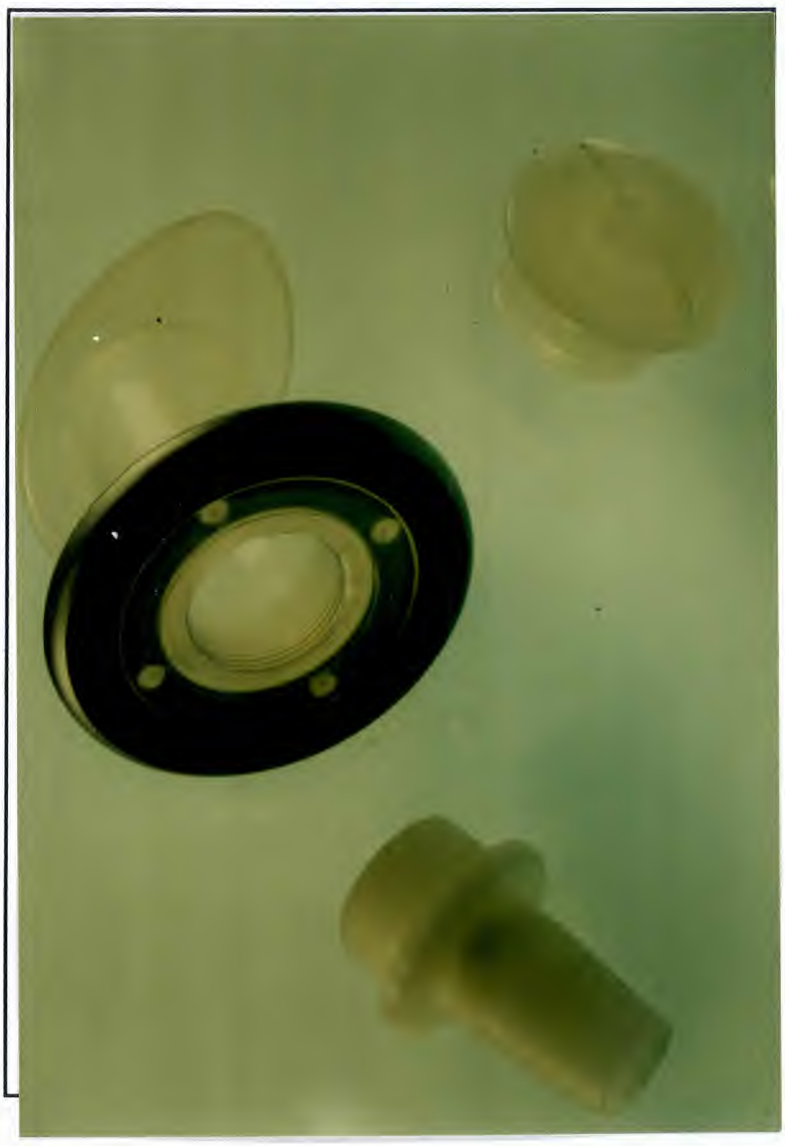


Fig.5.1 The Thomas cannula.

In the last step of the operation, the ends of the nylon sling around the common bile duct were pulled through a 6-8 cm length of polyethylene nasogastric tube, passed through the abdominal wall via a separate incision, and left in a subcutaneous pouch tied to a short piece of polyethylene tube.

The tension of this nylon loop around the common bile duct depends upon the length of the polyethylene tube and the tightness of the nylon sling around the common bile duct. The polyethylene tube has therefore to be cut short enough not to distort the direction of the common bile duct, and so produce complete obstruction, but long enough to allow the creation of incomplete obstruction by tightening the sling postoperatively under fluoroscopic control. For practical purposes the nylon loop around the common bile duct was loose enough to allow the introduction of tips of an artery forceps inside the loop close to the common bile duct. Fig. 5.2 shows the nylon sling passing through the polyethylene tube as prepared for producing the obstruction

Finally the abdominal wall was closed in one layer of continuous nylon suture and the skin wounds with interrupted silk sutures.

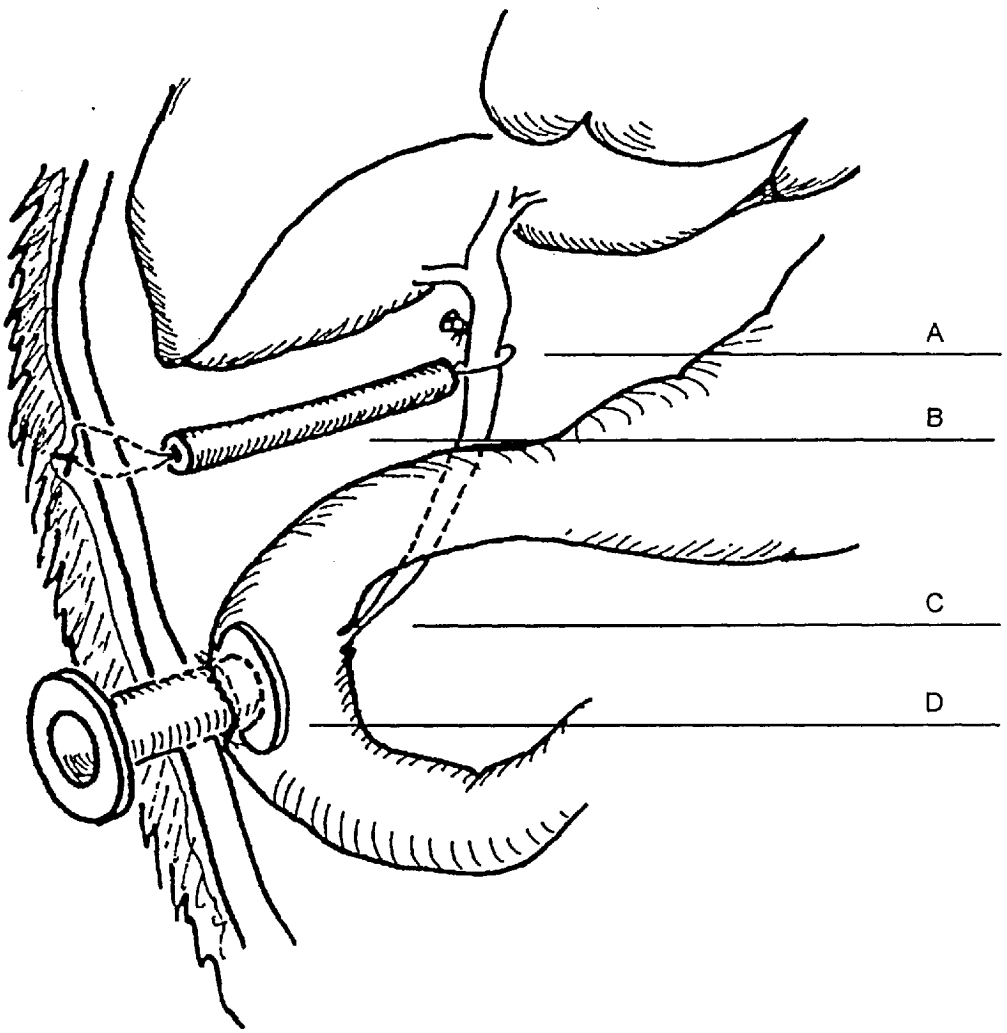
The model is represented in Fig. 5.3.

* Aftercare.

The dogs were nursed postoperatively in a recovery room warmed to 75 F by infra-red and ultra-violet lamps. Rectal temperature was measured twice a day. Parenteral piperacillin was started intra-operatively after bile sampling was finished and continued in a dose of 1 g twice a day for two days. Broad spectrum penicillin was continued after this time when indicated, depending on the temperature. Electrolyte solutions and 5% dextrose were given each day, the amount being determined by



Fig.5.2 The system used to produce the incomplete biliary stricture.



A = nylon sling around CBD and capped subcutaneously
B = polyethylene tube
C = papilla
D = Thomas cannula inserted into duodenum

Fig. 5.3 The model of chronic incomplete biliary obstruction.

monitoring the fluid balance and checking the hydration of the oral mucosa. After three days, when there was no evidence of vomiting, oral nutrition was gradually re-established with small, frequent liquid feeds. This was generally well tolerated and the animals gradually returned to normal feeding within a few days.

The stitches were removed after eight to ten days. The Thomas cannula was cleaned daily, washing the surrounding skin with saline, drying with swabs and protecting the skin with aluminum and zinc oxide paste. When reddened, the skin around the cannula was also protected by a barrier cream. Periodically the cap of the cannula was unscrewed and the cannula flushed inside.

5.2.3 Bile sampling and verification of the stricture.

The aim of these studies was to check or adjust the degree of incomplete obstruction of the CBD and to sample bile for microbiology and biochemistry. The x-ray procedures were all carried out at Northwick Park Hospital in collaboration with the staff of the MRC Experimental Radiology Unit.

Dogs were fasted for one day before this procedure in order to empty the stomach and duodenum. Systemic venous blood was taken at the beginning of the procedure for liver function tests. The dogs were sedated with acylpromazine (1.5-2 ml i.m.). On occasions it was necessary to repeat this dosage.

The sedated dog was placed on its left side on the radiology table and the cap of the cannula was removed using a large bladed screwdriver. Despite fasting before the procedure it was usual to find the cannula as well as the duodenum filled with food fragments and vegetable fibers, which were removed gently with forceps before flushing with saline and drying with cotton wool balls. The ampulla was then identified using an

ENT head light and gently manipulating the cannula with the hand in cases of difficulty until bile could be seen issuing from the orifice. The ampulla was then gently cannulated with a ureteric catheter. Patience was sometimes necessary to await relaxation of spasm of the sphincter caused by the pressure of the catheter. A guide wire was sometimes necessary to achieve cannulation, and this was removed once deep cannulation was achieved. A 21 gauge butterfly needle was attached to the external end of the catheter and bile slowly aspirated using gentle suction with a 20 ml disposable syringe. Once enough bile was collected for microbiology and biochemical assays (not less than 4 ml) the x-ray procedure was performed.

Fluoroscopy time was approximately two minutes per case. An initial plain x-ray was exposed after cannulation, but before injecting any contrast. Contrast medium was then injected slowly under fluoroscopic screening, using only enough contrast to fill but not to distend the intrahepatic ducts. Up to 18 ml of Meglumine Iothalamate 60%, 280 mg/ml (Conray 280 from May & Baker Ltd.) diluted to 50% with normal saline solution were used. After slow injection of contrast, serial pictures were taken. Lateral x-rays were taken routinely, but oblique projections were required in one case to separate the overlapping hepatic ducts. Two final pictures were usually taken soon after the catheter was removed and also about 30 minutes later to document the obstruction of the bile flow. At the end of the procedure the cap of the cannula was screwed back in place and the dog was returned to the Animal House. Piperacillin 1g i.m. was given twice in the following 24 hours.

5.2.4 Reversal of the stricture.

* Procedure

The aim of this experiment was to reverse the biliary obstruction at variable time intervals and to sample systemic blood, bile, portal blood, liver and common bile duct tissues and, when present, biliary stones.

Anaesthesia was induced as for the primary procedure and the abdomen shaved with an electric clipper. The operation was usually performed by the surgeon with one assistant. The upper midline incision was re-opened. The first step was to remove the Thomas cannula, either (in the earliest dogs) through a separate abdominal incision, or (in the last three dogs) by including the cannula tract in a right paramedian incision. Intra-abdominal adhesions were divided, the external end of the cannula pushed inside, and the duodenum dissected from the abdominal wall. The double silk purse string was cut, the cannula removed and the duodenotomy closed in a transverse fashion with a two layer interrupted inverting chromic catgut suture.

A wedge biopsy of the liver edge was obtained. Approximately four ml of bile was sampled from the common bile duct using a 10 ml syringe, sampling being easier at this procedure because of the presence of the biliary obstruction. The duodenal loop was then gently turned leftwards and about 1 ml of portal blood withdrawn for microbiology.

The bile duct was then mobilized using the previously placed polyethylene tube (Fig. 5.4) and the nylon sling placed around the CBD (Fig.5.5) as a guide . As reported in the results section, in two cases (J299 and J769) the polyethylene tube was not found in the expected place. When the common bile duct was dissected free from the adhesions and the site of obstruction was clearly identified (Fig. 5.6), a double silk tie was placed on the duct below

the stricture and the duct divided between the ties. The proximal stump was then retracted upwards, carefully freeing the adhesions between the duct and the portal vein until the common hepatic duct well above the stricture was reached. A swab was positioned behind the dissected common bile duct to catch any stones or debris, a small opening made in the anterior wall and a 20 ml syringe inserted in the hole to aspirate bile and possible biliary concrements. Two 2/0 silk stay sutures were then placed in the anterior layer and the posterior wall of the duct divided and held in two further stay sutures. The isolated segment of the hepatic duct was now removed and opened longitudinally (Fig. 5.7) and specimens prepared for ordinary histology and histochemistry.

A Roux-en-Y loop of 35 cm was prepared at about 20 cm from the duodenojejunal junction. The blind end of the jejunum was closed with a single layer of Vicryl and the jejunojejunal anastomosis was performed in two layers of continuous 00 chromic catgut. A transverse incision about 1 cm length was then performed close to the blind end of the Roux loop, and approximated to the stump of the hepatic duct. The abdominal anatomy of the dog usually does not require that the Roux loop is passed through the mesocolon. Hepaticojejunostomy was performed according to the standard technique used in clinical practice at Hammersmith Hospital (Blumgart & Kelly, 1984) with interrupted stitches of 3/0 chromic catgut. The anterior wall sutures were placed first from inside to outside on the hepatic duct. The posterior wall stitches were placed on the jejunum from inside to outside and the hepatic duct from outside to inside. The posterior wall was tied and the anterior row now placed in the jejunum from outside to inside and then tied. This technique provides a reliable inverting mucosa- to-mucosa apposition with full thickness sutures in both duct and jejunum.

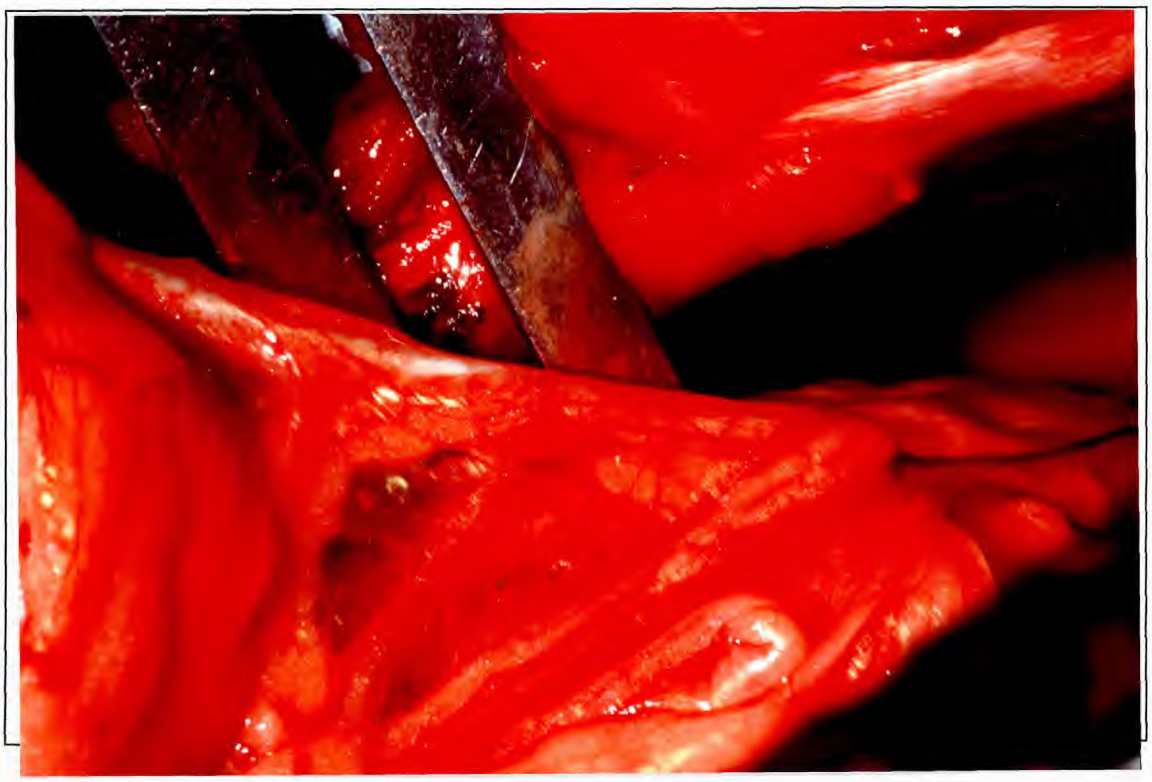


Fig.5.4 Second operation: the polyethylene tube driving to the identification of the CBD.

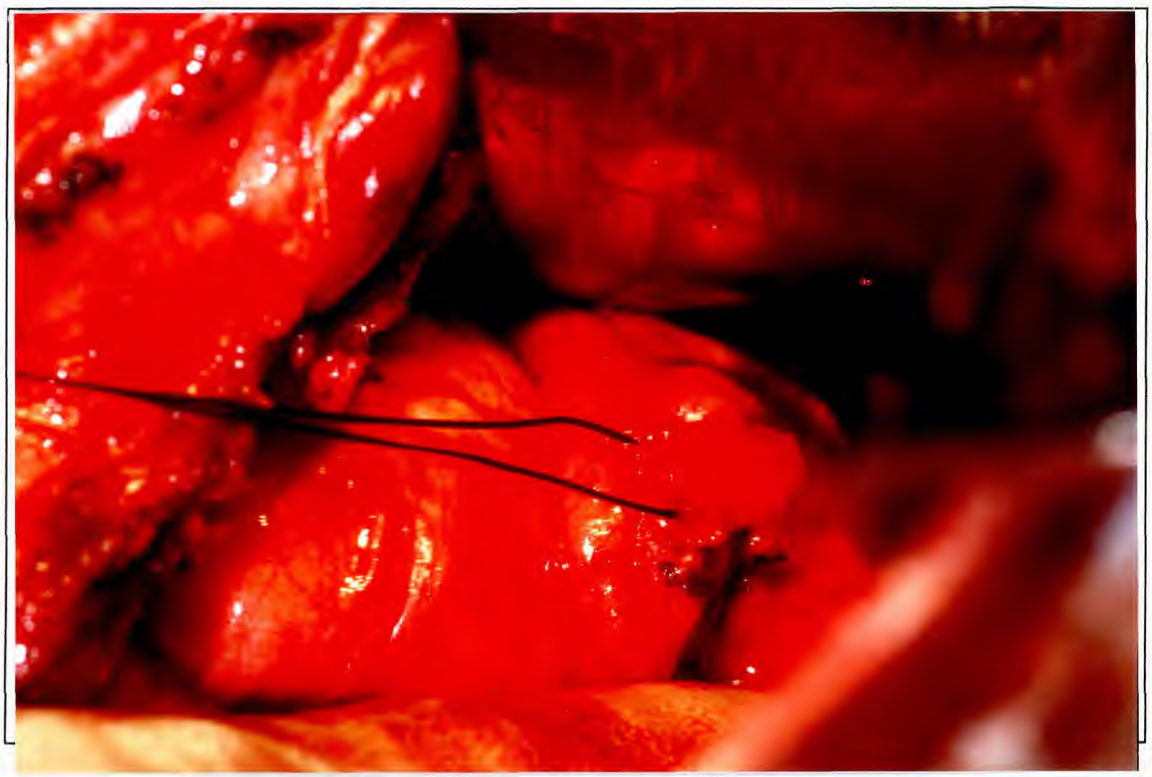


Fig.5.5 Second operation: the nylon sling placed around the CBD to produce the stricture.

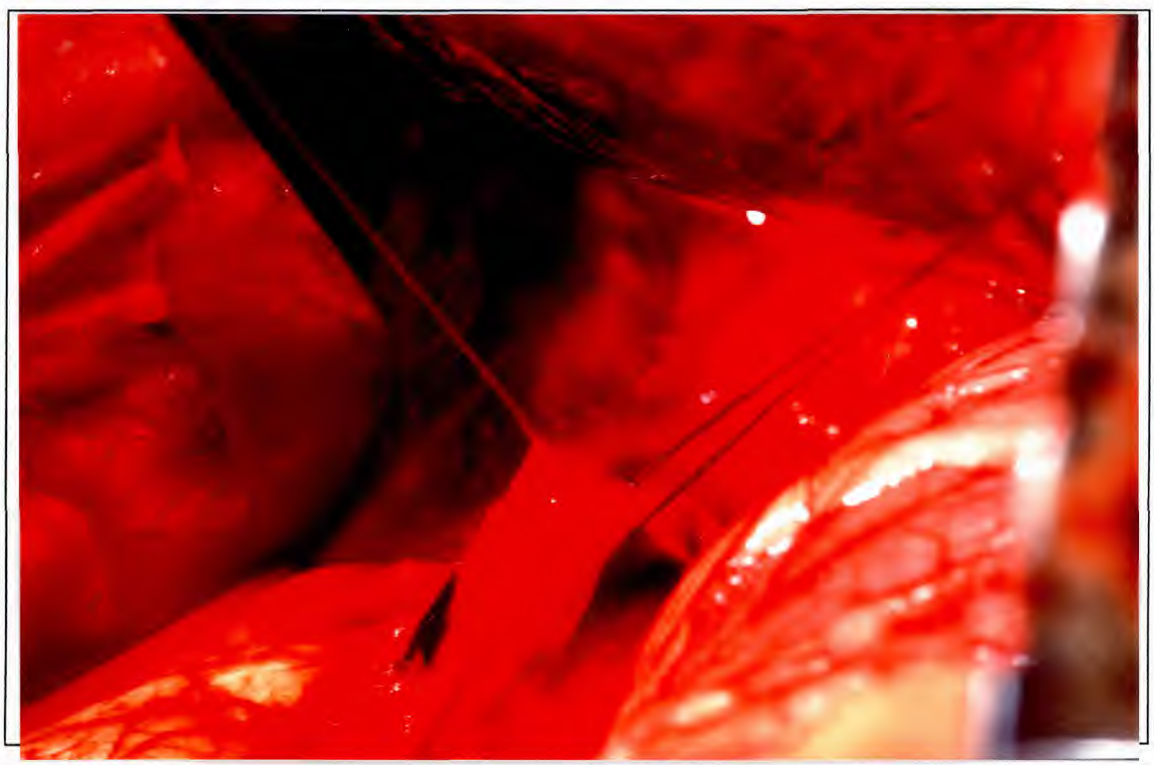


Fig.5.6 Second operation: the dissected CBD is held by stay sutures.



Fig.5.7 Second operation: the site of the CBD stricture has been removed and opened longitudinally. Note the dilatation above.

After haemostasis was achieved and the peritoneal cavity washed with hydrogen peroxide and saline solution, the abdomen was closed in one layer of continuous nylon. Piperacillin 1 g was given intravenously soon after sampling.

* Aftercare.

The aftercare procedures are similar to those described after the first operation. Fluid replacement and antibiotics were pursued more aggressively, as a result of increased operative losses and heavy bacterial contamination.

5.2.5 Sacrifice

A foreleg vein was cannulated and blood sampled for liver function tests. Anaesthesia was induced with Sodium pentobarbitone diluted to 5%. and maintained throughout the procedure in same way as previously described. A formal median laparotomy was carried out and adhesions freed. Portal blood was sampled. The previous bilio-enteric anastomosis at the porta hepatis was dissected and bile sampled from the hepatic duct. The anastomosis was then disconnected, the ducts dissected further upwards to a level above the confluence of hepatic ducts and the bile duct excised. A liver biopsy was then taken for ordinary histology and histochemistry. The abdomen was closed and the animal killed with an overdose of Sodium pentobarbitone.

5.2.6 Handling the Samples.

Blood samples were collected without anticoagulants, serum separated and stored at -20 C until assayed. Portal blood was stored in a sterile container for microbiological assays.

Bile samples (approximately 4 ml) were separated in two aliquots in separate sterile containers, one stored at 1 to 4 C for microbiological assay and one at -20 C for measurement of conjugated and unconjugated bilirubin, total bile acids, and activities of betaglucuronidase and phospholipase.

Liver biopsies were cut into pieces no thicker than 5 mm. One part was placed in ordinary formalin and stored at 1 to 4 C. The other specimen was stored in cold formol calcium for 3-12 hours and then transferred to gum sucrose, before being processed for histochemical staining for betaglucuronidase. Similarly bile duct tissue sampled at the second operation was split longitudinally into two sections stored in the same way for histology and histochemistry.

5.2.7 Laboratory methods

The laboratory methods used in this study are listed here consecutively. A full account of the methods used is given in Appendix 1.

* Blood LFT

Bilirubin in blood was measured with an automated version of the Michaelssohn method (Simmons, 1968; Billing et al, 1971). Alkaline phosphatase was calculated according to the procedure of Bessey, Lowry & Brock, as reported by Tietz (1970). Serum GOT and GPT were measured according to the method described by Reitman & Frankel (1957); gamma GT was assayed according to the method of Rosalky et al. (1970) and serum proteins were determined by the methods of Reinhold (1953) for total proteins and by the manual method of Doumas (1971), as modified by Spencer & Price (1977) for albumin.

* Bile biochemistry

Conjugated and unconjugated bilirubin levels were measured by the automated version of Michaelssohn's method (Simmons, 1968; Billing et al, 1971). Total bile acids were measured according to the method of Mashige et al. (1978). Betaglucuronidase was measured according to the method of Fishman (1955), as modified by Sato (1962). Phospholipase assay was initially attempted by measuring phospholipase C according to the method described by Molby & Wadstrom (1973). However, since little or no activity was detected in a number of samples, in the latter part of the study phospholipase A activity was assayed by the method described by Goswamy & Frey (1974). (The reason for this change in the method will be discussed later).

* Microbiology

The specimens of bile and portal blood were cultured semi- quantitatively on blood agar, neomycin blood agar, McConkey agar, and Cetrimide agar. All isolates were identified by standard techniques, according to Cowan (1975) and Holdeman et al. (1977). In selected cases, on the basis of initial Gram film observation and of the results of the cultures, specimens were additionally examined by gas liquid chromatography.

* Histology

Tissues were fixed in formalin from 4 up to 24 hours, then enblocked in paraffin wax, cut and stained with hematoxylin-eosin (Drury et al, 1967). Tissues were examined with particular reference to the most common changes which may be present in liver and CBD structures, such as the loss of epithelium, inflammatory changes, proliferation of bile ducts, proliferation of blood vessels (granulation tissue) and fibrotic changes. For each of these

aspects samples were classified according to a grading ranging from 1 (normal findings) to 5 (maximum severity of changes).

* Histochemistry

Following fixation in formol calcium and gum sucrose frozen sections were cut, as described by Holt et al. (1960). The activity and distribution of betaglucuronidase were detected by the method of Gossrau (1973), as reported by Lojda et al.

The presence of betaglucuronidase in the liver was analysed semi- quantitatively: to achieve this a calibrated microscope eyepiece graticule was used and the granules of the enzyme were counted in 10 squares scattered in the fields: the sum of these figures is reported as the amount of liver tissue betaglucuronidase.

The presence of betaglucuronidase in GB and CBD tissues was evaluated qualitatively: even a very small amount of enzyme granules seen in the sample was considered positive.

5.3 RESULTS

The timing of the various procedures in ten dogs is shown in Table 5.1. Two dogs (765 & 821) died soon after the first operation, one from massive gastric dilatation and the other for unexplained reasons, probably related to generalized sepsis. Two dogs died respectively 58 and 32 weeks after the first operation, five dogs died within 48 hours after the second operation, and one dog survived both the operations and was sacrified 21 weeks after the second (Table 5.1). Amongst the eight dogs who survived the first operation, three experienced transient episodes of cholangitis with fever and jaundice in the early postoperative course while the remaining ones had a rapid and uneventful recovery. Every dog had some leakage of duodenal juice around the Thomas cannula, but this was easily controlled and improved with time.

With the exception of the two dogs who died shortly after the first operation, the remaining eight dogs all underwent retrograde cholangiography on at least one occasion. Four dogs had one x-ray assessment, three dogs were assessed twice and one dog three times. Two of the four dogs who underwent only one x- ray assessment had undergone another attempt at cannulation which was in fact unsuccessful. All the tests performed showed the presence of an incomplete stricture of the CBD (fig. 5.8-5.21) and it was never necessary to tighten the nylon sling placed around the CBD as planned in the original protocol.

Data from the two dogs who died after the first operation have been analyzed only in respect of the basal blood liver function tests for one dog (765) and not analysed at all for the other (821). For the first dog (765) bile was taken at operation, but the samples were lost in the bacteriology department. The other dog (821) who died a few days after the first operation, had heavy colonization of both GB and CBD bile (107 colonies of Streptococcus faecalis). This unique finding in bile under basal conditions, together with

the unexpected death of the animal in the early postoperative period, suggested that this dog was atypical and should be excluded entirely from the analysis.

The polyethylene tube containing the ends of the nylon sling passed around the CBD was found in its correct place in six out of eight dogs where a chronic biliary obstruction was obtained. In the first dog operated in this series (299), who died after 58 weeks of obstruction, the tube with the sling were not found at postmortem, neither in their correct place nor elsewhere in abdomen or in the chest, despite a careful search. Furthermore, in another dog (769) at the second operation the tube was found inside the common bile duct above the stricture, where it had slowly penetrated during 39 weeks of obstruction.

Cholangiography demonstrated the presence of an incomplete obstruction in every case, and delayed pictures always demonstrated the presence of bile stasis (fig. 5.8 - 5.21). At operation the isolated CBD uniformly showed the presence of a hard, fibrotic strictured segment. The bile duct proximal to the stricture was dilated, with a thickened wall (Fig. 5.7).

Experimentally produced primary CBD stones were recovered from two dogs (737, 769, Table 5.1) after respectively 21 and 40 weeks of incomplete obstruction. In one case several very small granules of black-greyish material were found (Fig. 5.22) floating in the bile, while in the dog in which the polyethylene tube and the nylon sling were found inside the duct the tube was surrounded by a muddy, yellow-brownish, soft, greasy substance (Fig. 5.23) similar to the typical descriptions of primary CBD stones (Aschoff, 1924; Madden et al., 1968).

Table 5.1

Summary of the procedures carried out in all the dogs
(The recording number of the experimental surgery unit at RPMS has been used)

	Duration of Obstruction (weeks)																							
Dog number	0	1	7	8	9	10	13	20	21	22	27	31	32	33	35	37	40	42	47	48	49	52	58	61	72
299	O											X									XX		PM		
300	O												X							XX					OO
520	O										X		PM												
731	O			X,BS	BS					XX,BS								OO							
737	O		X,BS					BS	OOs,PM																
758	O						X,BS							XX					XXX			OO			
765	O																								
769	O	BS							BS						X		OOs							SA	
820	O					X		BS								OO									
821	O																								

Keys:
- O 1st operation
- OO 2nd operation
- OOs 2nd operation, stones within CBD
- X 1st x-ray
- XX 2nd x-ray
- XXX 3rd x-ray
- BS Blood Sample
- SA Sacrifice
- PM Post-mortem



Fig.5.8 X-ray picture of the incomplete stricture. Dog 299.



Fig.5.9 X-ray picture of the incomplete stricture. Dog 300.



Fig.5.10 X-ray picture of the incomplete stricture. Dog 520.



Fig.5.11 X-ray picture of the incomplete stricture. Dog 520. This picture, taken after withdrawing the catheter, underlines the presence of biliary obstruction.



Fig.5.12 X-ray picture of the incomplete stricture. Dog 731.



Fig.5.13 X-ray picture of the incomplete stricture. Dog 731. Late phase (about 30 minutes after withdrawal).



Fig.5.14 X-ray picture of the incomplete stricture. Dog 737.



Fig.5.15 X-ray picture of the incomplete stricture. Dog 737. Late phase (about 30 minutes after withdrawal).

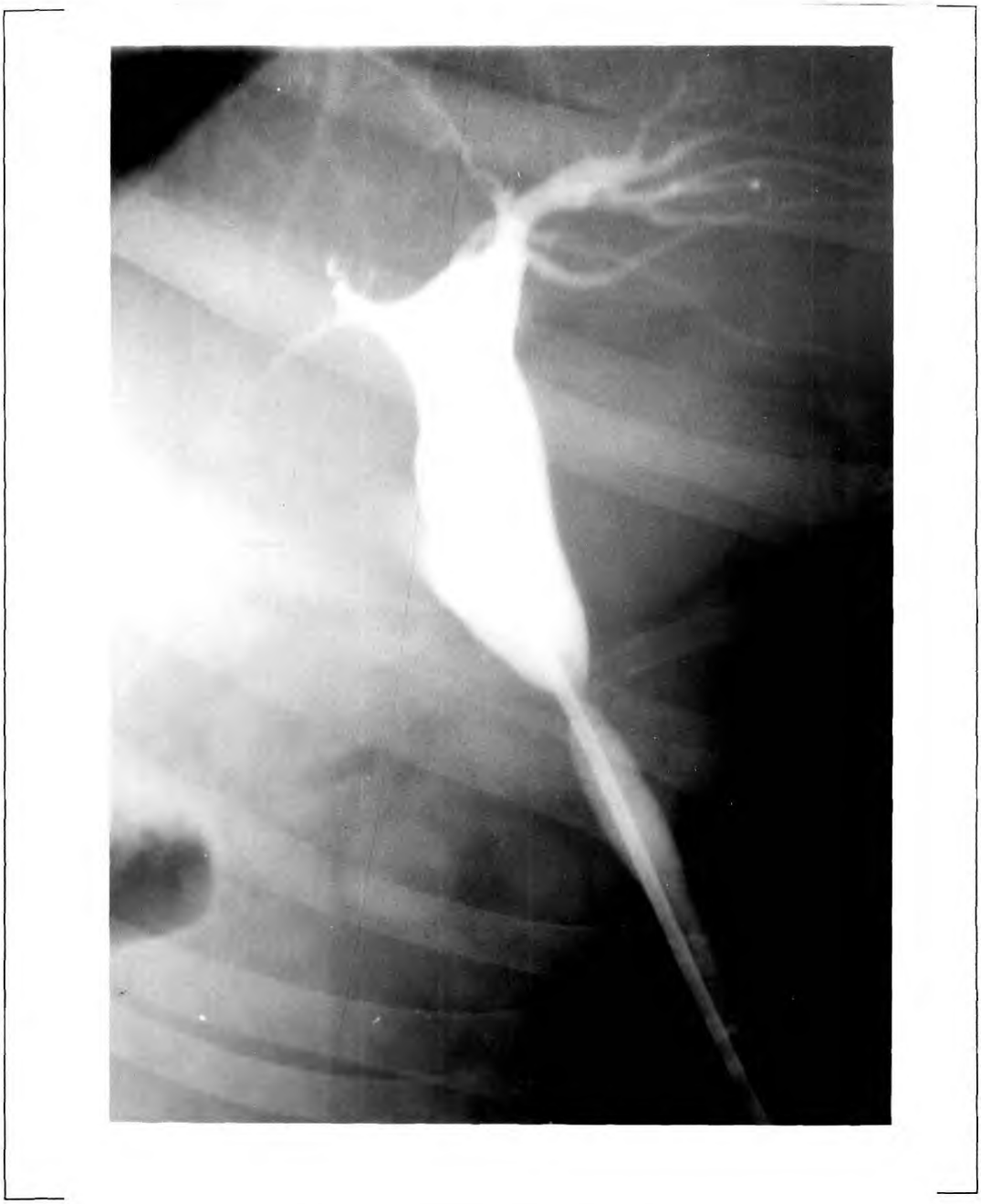


Fig.5.16 X-ray picture of the incomplete stricture. Dog 758.



Fig.5.17 X-ray picture of the incomplete stricture. Dog 758. Late phase (about 30 minutes after withdrawal).



Fig.5.18 X-ray picture of the incomplete stricture. Dog 769.



Fig.5.19 X-ray picture of the incomplete stricture. Dog 769. Late phase (about 30 minutes after withdrawal).



Fig.5.20 X-ray picture of the incomplete stricture. Dog 820.



Fig.5.21 X-ray picture of the incomplete stricture. Dog 820. Late phase (about 30 minutes after withdrawal).



Fig.5.22 Primary CBD stones experimentally produced in dog 737.



Fig.5.23 Polyethylene tube of dog 769 migrated into the duct and found at second operation surrounded by biliary mud and sludge.

Since not all studies were performed at uniform time points, due to constraints of the availability of departmental facilities such as radiology, I have found it useful to aggregate data and express them in periods of 10 weeks. In the following tables when the data were not normally distributed they are presented as medians and ranges. The full experimental data are reported in Table 13.1, Appendix 2.

5.3.1 Blood Liver Function Tests (Tables 5.2-5.4.).

Blood liver function tests were carried out on 28 occasions, in 19 cases immediately before other procedures (see Table 5.1).

Simple linear regression analysis of liver function tests with the actual duration of obstruction from the first operation showed no correlation, even though plasma proteins demonstrated a trend towards an increase with the duration of obstruction ($r=0.6$).

Table 5.2

Blood bilirubin, blood proteins and duration of the obstruction

TEST	Tot. Bil (uM/l)	Conj. Bil (uM/l)	Tot Prot (g/l)	Albumin (g/l)	A/G Ratio
OBSTR (wks) (n)					
0 (basal) MD (n=6) RG	6.6 2.2 - 16.2	1.7 0.5 - 8.4	53.6 47.5 - 58.3	27.8 20.8 - 35.4	1.1 0.8 - 2.6
(1 - 20) MD (n=10) RG	2.2 1.2 75.5	1.2 0.2 - 35.4	53.3 48.6 - 59.5	28.3 21.4 - 31.5	1.2 0.7 - 1.6
(21 - 30) MD (n=4) RG	3 2.2 - 4	0.9 0.3 - 1.9	54.8 54.5 - 62.5	29.8 22 - 25.4	1.1 0.7 - 1.6
(31 - 40) MD (n=4) RG	3.1 2.5 - 5.2	1 0.4 - 1.7	60.9 52.5 - 62.6	24 22 - 25.4	0.7 0.5 - 0.9
(41 - 50) MD (n=2) RG	2.5 , 3.1	0.2 , 1.1	59.8 , 60	22.5 , 33	0.7 , 1.2
(> 50) MD (n=2) RG	4.4 , 8.3	1.2 , 3.7	51.3 , 76.1	24.7 , 25.9	0.5 , 1

Table 5.3

Alk Phosphatase, Transaminases, gamma GT and duration of the obstruction

TEST	Alk Phos (UI/l)	GOT (UI/l)	GPT (UI/l)	gamma GT (g/l)
OBSTR (wks) (n)				
0 (basal) MD (n=6) RG	19.5 10.3 - 56.8	14 6 - 28	13.5 6 - 21	7 5 - 10
(1 - 20) MD (n=10) RG	21.2 15 - 503.8	11 6 - 49	10 5 - 169	10.5 8 - 70
(21 - 30) MD (n=4) RG	17.2 10.2 - 33.2	11 10 - 13	9 8 - 17	11.5 3 - 14
(31 - 40) MD (n=4) RG	22.4 11.8 - 35.3	10.5 9 - 12	8 7 - 12	7 7 - 8
(41 - 50) MD (n=2) RG	33.4 , 67.4	9 , 13	8 , 29	6 , 12
(> 50) MD (n=2) RG	17.8 , 25.7	10 , 12	15 , 32	9 , 12

In 18 out of 28 procedures where blood liver function tests were recorded, the CBD bile was also cultured. Organisms were cultured from bile in 11 procedures in this subgroup, whereas in seven the bile was sterile. Table 5.4 shows the values of blood liver function tests found in the presence of positive and negative bile culture. It is worthy of note that in the presence infected bile the production of albumin and the albumin/globulin ratio in the blood were significantly lower, irrespective of the duration of the obstruction and that positive bile cultures were associated with higher values of alkaline phosphatase, though this did not reach statistical significance.

Table 5.4

Blood LFT values and bile cultures in 18 procedures where both values were obtained

Test	-VE cult		+Ve cult	ALL
	(n=7)		(n=11)	(n=18)
TOTAL BIL (uM/l)				
median	2.6		3.1	3.1
(range)	(1.4-16.2)		(1.9-8.3)	(1.4-16.2)
CONJ. BIL (uM/l)				
median	0.6		1.2	1.1
(range)	(0.3-8.4)		(0.2-3.7)	(0.2-8.4)
TOTAL PROT (g/l)				
median	54.6		59.8	54.6
(range)	(47.5-58.3)		(48.6-76.1)	(47.5-76.1)
ALBUMIN(g/l)				
median	29.2	*	24.7	25.8
(range)	(20.8-35.4)		(21.4-33)	(20.8-35.4)
A/G RATIO				
median	1.3	**	0.7	0.9
(range)	(0.8-2.6)		(0.5-1.3)	(0.5-2.6)
AP(UI/l)				
median	17.4		25.7	21.1
(range)	(10.3-32.4)		(11.8-67.4)	(10.3-67.4)
GOT(UI/l)				
median	11		11	11
(range)	(6-28)		(9-18)	(6-28)
GPT(UI/l)				
median	10		12	11
(range)	(6-21)		(7-32)	(6-32)
GAMMAGT(UI/l)				
median	8		8	8
(range)	(5-14)		(6/12)	(5-14)

* P = 0.038 Mann-Whitney test
** P = 0.015 Mann-Whitney test

5.3.2 Microbiology (Table 5.5. Fig.5.24)

* Portal blood

Portal blood was cultured in 11 cases, five at the first and six at the second operation. Portal cultures at the first operation were always sterile, while one out of six cultures at the second operation (at 52 weeks) was infected with E.coli and S.faecalis 104. In this dog the same species 107 were cultured from CBD bile.

* Bile cultures

Gallbladder bile was cultured at first operation in seven out of eight dogs for whom microbiological data were available and was sterile in every case.

CBD cultures were carried out at 28 procedures in eight dogs. The overall positive bile culture rate was 18/28 (64.3%). E. coli was the organism most frequently isolated (15), followed by S. faecalis (11), P. aeruginosa (2), P. mirabilis (2), Klebsiella (2), and Clostridia genera (2). Other coliforms, S.aureus, Bacteroides and coagulase positive Staphylococci were isolated on one occasion each. S epidermidis was also isolated on three occasions, probably as a result of external contamination. The positive bile culture rate was significantly associated with the presence of biliary obstruction ($P < 0.001$, Chi square test, Table 5.5). Moreover the duration of obstruction was significantly correlated with the number of bacterial species isolated ($r= 0.69$. $P < 0.001$, Fig 5.24).

On both occasions on which primary CBD stones were found there was a positive bile culture, and both showed polymicrobial bacterial growth. The comparable figures in the absence of stones were respectively 16/26 (61.5%) cases of positive bile culture and 10/26 (38.5%) cases of multiple isolates.

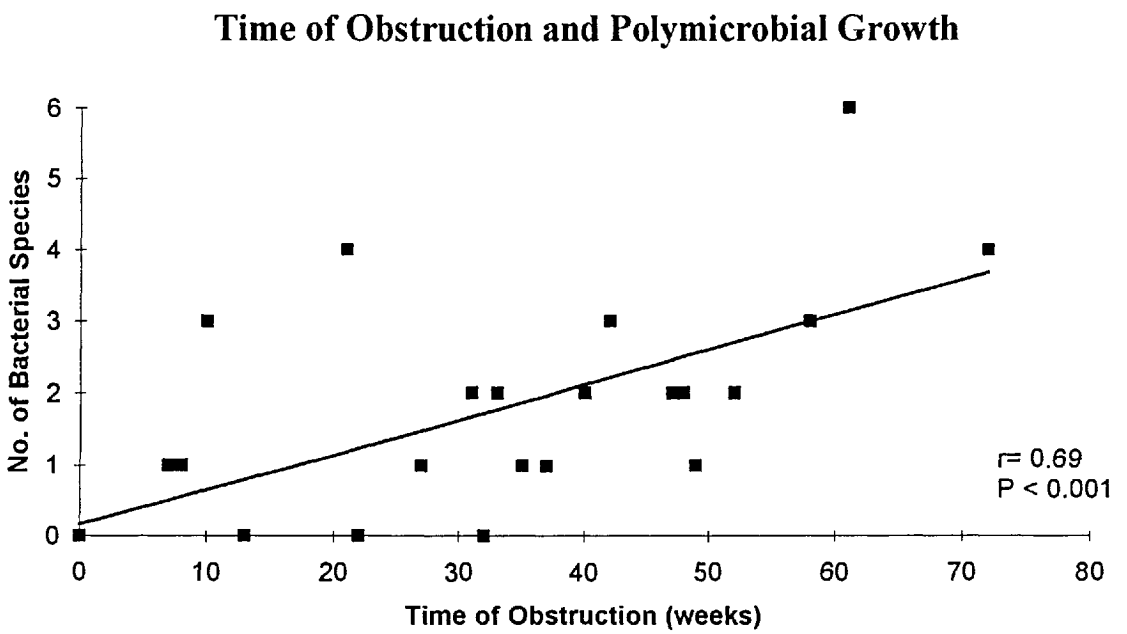


Fig.5.24 Correlation between the duration of the experimental biliary obstruction and the presence of multiple isolates in bile.

Table 5.5

CBD bile infection rate and biliary obstruction.

OBSTRUCTION	N	+VE CULTURE N	(%)
NO (basal)	7	0	(0)
YES (1-72 wks)	21	18	(85.7)

$P < 0.001$, Chi Square test

5.3.3 Biochemical studies (Tables 5.6, 5.7. Fig. 5.25)

Twenty four samples of CBD bile were available for biochemical assay. In all these cases bile for culture was also sampled. No correlation was found between duration of obstruction and any of the biochemical tests performed. Furthermore, there was no significant correlation between betaglucuronidase activity and unconjugated bilirubin levels ($r = 0.11$; Fig. 5.25).

Bile cultures were negative in eight cases and positive in 16. Biochemical assays were compared amongst these subgroups and no statistical significance was shown (Table 5.6). The number of isolated species did not correlate with betaglucuronidase or phospholipase activities or unconjugated bilirubin levels.

Primary CBD stones were found in two out of 24 procedures. Table 5.7 shows the results of the biochemical assays in these two dogs, and the summary data for the animals without stones.

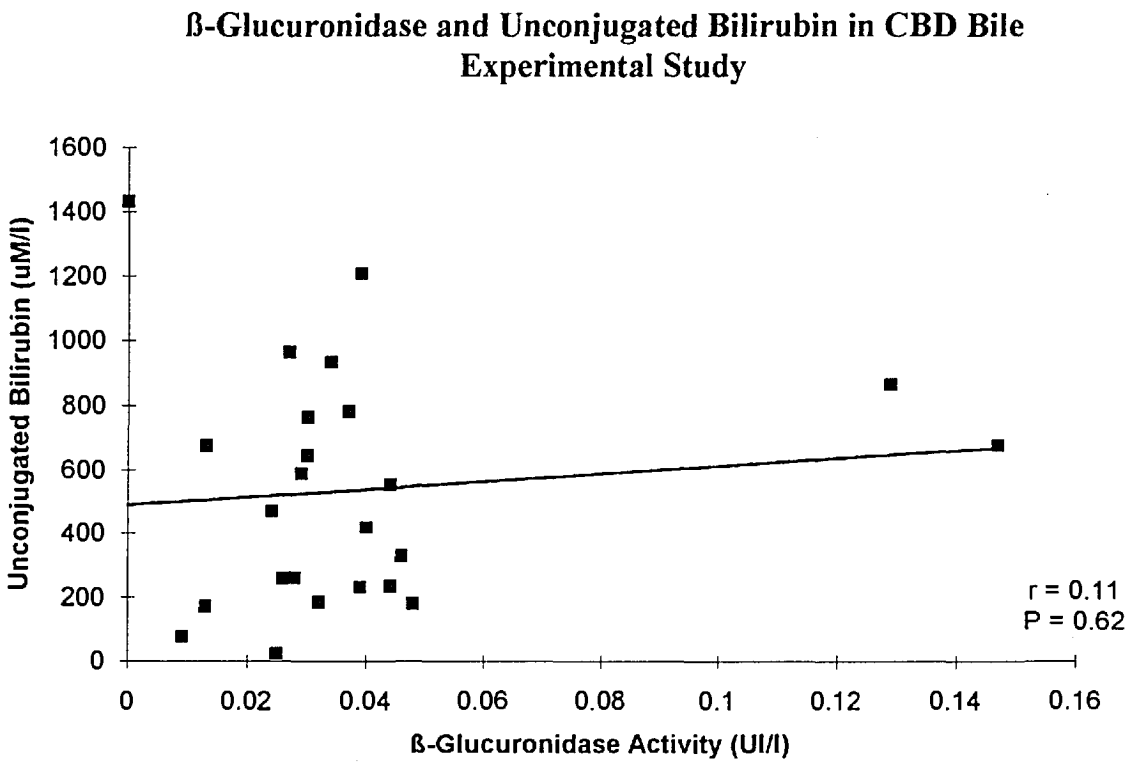


Fig.5.25 Betaglucuronidase activity and unconjugated bilirubin in CBD bile.

Table 5.6

CBD bile Bilirubin, Betaglucuronidase, Phospholipase, Bile Acid and bile cultures (in 24 procedures carried out in 8 dogs)

Test	-VE culture (n=8)	+Ve culture (n=16)	ALL (n=24)
TOTAL.BIL.(uM/l)	P = 0.761*		
median	1198	1853.1	1918.4
(range)	(1136.3-3866.6)	(730.3-6853)	(730.3-6853)!
CONJ.BIL.(uM/l)	P = 0.464*		
median	1219	1481.9	1366.5
(range)	(618.3-3222.1)	(496.6-5423)	(496.6-5423)
UNCON.BIL.(uM/l)	P = 0.087*		
median	659.5	294	512.6
(range)	(260.2-1208.8)	(27-1430)	(27-1430)
BetaGLU.(UI/l)	P = 0.693*		
median	0.03	0.03	0.03
(range)	(0.01-0.13)	(0-0.15)	(0-0.15)
PHOSPHOL.(UI/l)	P = 0.880*		
median	0	0	0
(range)	(0-0.20)	(0-0.13)	(0-0.13)
BILE ACID(mM/l)	P = 0.393*		
median	47.6	54.2	54.2
(range)	(13.4-114)	(11.6-170.2)	(11.6-170.2)

* Mann-Whitney Test

Table 5.7
CBD bile Bilirubin, Betaglucuronidase, Phospholipase, Bile Acid and CBD stones
(in 24 procedures carried out in 8 dogs)

Test	No STONES (n=22)	STONES (n=2)	ALL (n=24)
TOTAL.BIL. (uM/l)	P = 0.061*		
median	1948		1918.4
(range)	(1057-6853)	(730.3 , 1268)	(730.3-6853)
CONJ.BIL. (uM/l)	P = 0.077*		
median	1481.9		1366.5
(range)	(618.3-5423.0)	(496.6 , 1036.7)	(496.6-5423)
UNCON.BIL. (uM/l)	P = 0.212*		
median	572.2		512.6
(range)	(27-1430)	(231.3 , 233.7)	(27-1430)
BetaGLU.(UI/l)	P = 0.253*		
median	0.03		0.03
(range)	(0-0.15)	(0.04 , 0.04)	(0-0.15)
PHOSPHOL.(UI/l)	P = 0.680*		
median	0		0
(range)	(0-0.13)	(0 , 0.20)	(0-0.20)
BILE ACID (mM/l)	P = 0.254*		
median	64.6		54.2
(range)	(11.6-170.2)	(17.2 , 25.8)	(11.6-170.2)

* Mann-Whitney Test

5.3.4 Histology (Tables 5.8-5.13. Figs. 5.26-5.30)

* Liver

Liver tissues were sampled for histology on 10 occasions: in four cases tissues were sampled at first operation whereas in four cases at second operation and in two cases at post-mortem examination. When tissues were sampled at surgery, blood liver function tests were always performed, as well as sampling of bile for microbiology and biochemistry. Specimens sampled at the first operation demonstrated normal findings in all but one case (dog 769) where a marked inflammation of portal spaces was found. After a variable time of obstruction, histological patterns demonstrated in some cases the presence of fibrosis, inflammation or loss of the bile canaliculi epithelium. Examples of liver histological patterns are shown in Fig. 5.26-5.28.

The relationships between histological findings and duration of obstruction are reported in Table 5.8: the frequency of pathological findings did not increase significantly in the presence of obstruction ($P > 0.05$ Chi Square test).

Tables 5.9 displays data related to liver histology and blood LFT. Since there are only eight observations and no normal distribution of the data only median and ranges are reported. The related figures are all displayed in Table 13.1 in Appendix 13.2. A statistical comparison has been possible only for data concerning inflammation and no significant differences have been demonstrated amongst the groups (Neuman-Keuls multiple comparisons test). Similarly, data related to the correlation between liver histology with bile microbiology and biochemistry are displayed in Table 5.10: no association was demonstrated when comparing liver histology with bile microbiology (Chi Square test), nor when comparing inflammatory changes in liver tissue with biochemical

assays in bile (Neuman-Keuls multiple comparisons test). For the whole set of the related figures see also Table 13.1 in Appendix 13.2.

Table 5.8

Liver histology and duration of the obstruction

event	obstr (weeks)	epith	inflam.	prolif.BD	gran.tiss.	fibrosis
1st	0	-	-	-	-	-
op.	0	-	-	-	-	-
(n=4)	0	-	+	-	-	-
	0	-	-	-	-	-
2nd	40	-	+	-	-	+
op.	42	-	-	-	-	-
(n=4)	52	-	-	-	-	-
	72	+	+	-	-	-
PM	21	-	-	-	-	+
(n=2)	58	+	-	-	-	+

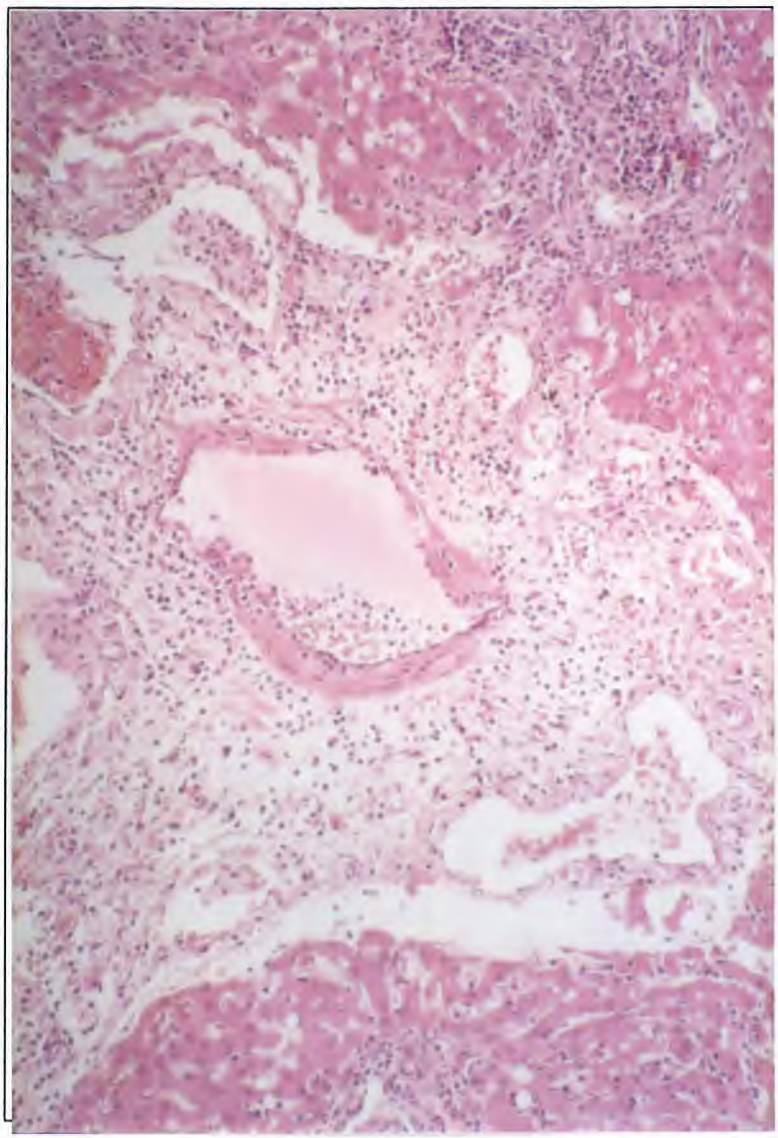


Fig.5.26 Liver histology at second operation (40 weeks of obstruction). Marked inflammation and portal fibrosis.

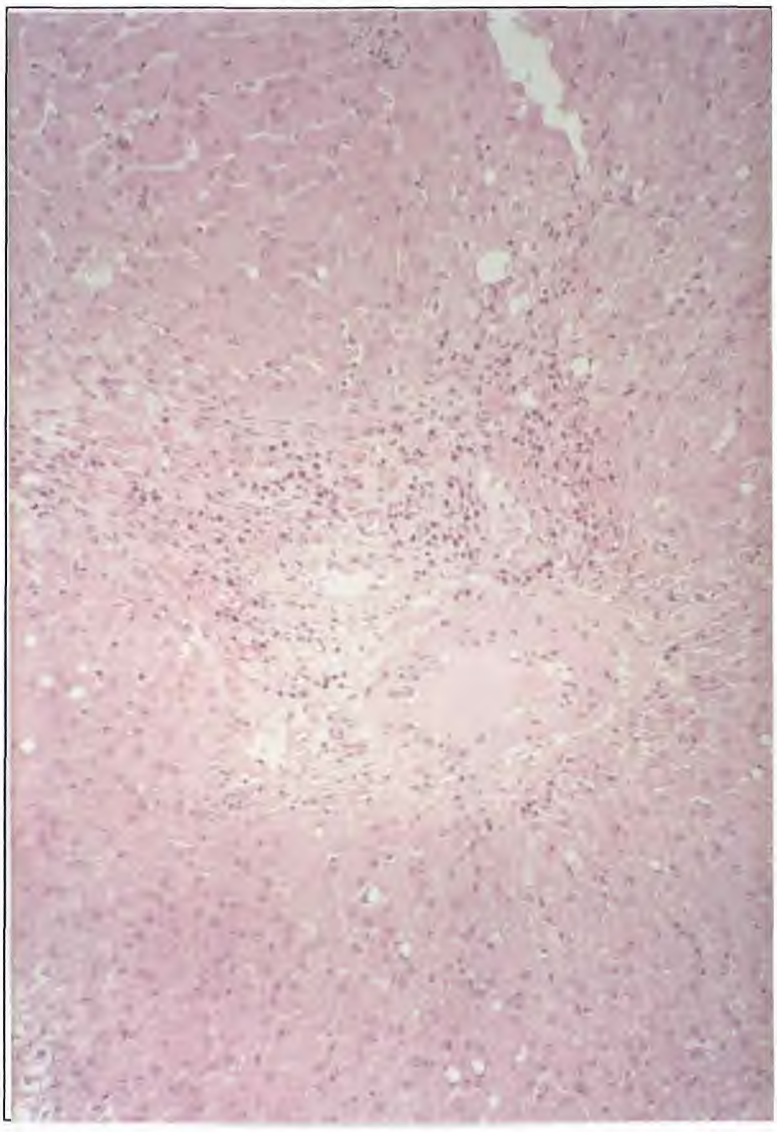


Fig.5.27 Liver histology at second operation (72 weeks of obstruction). Moderate inflammatory reaction. Early fibrosis.

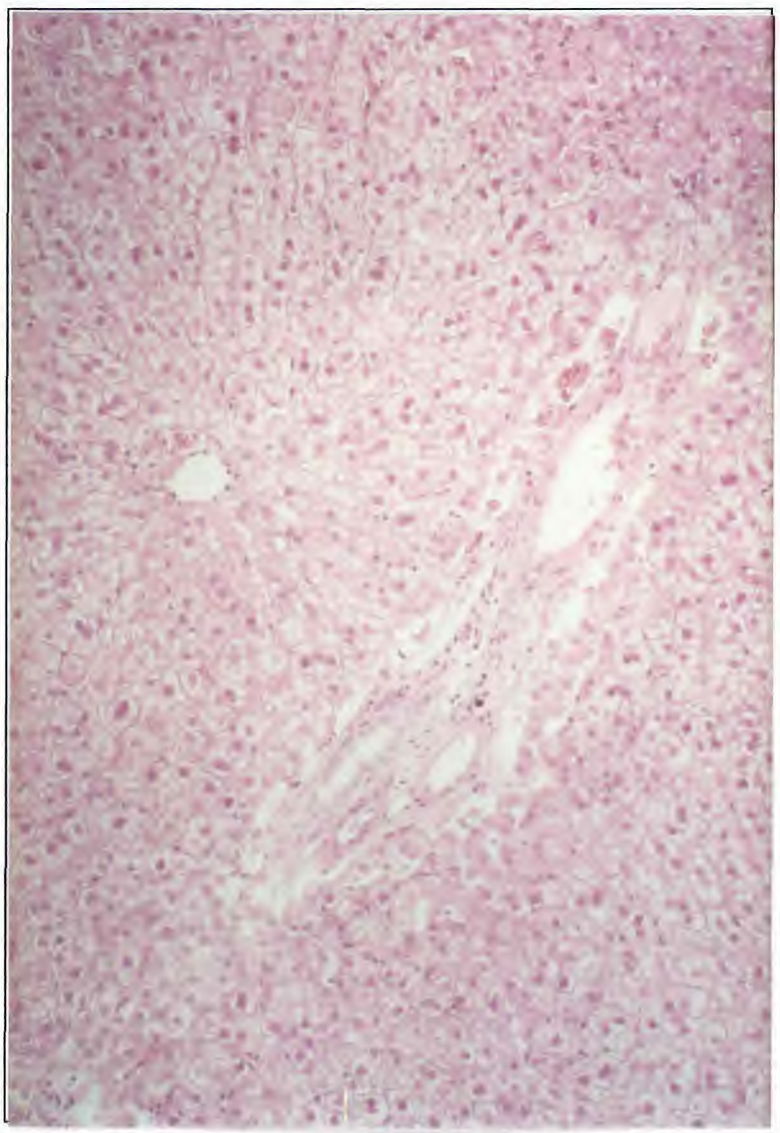


Fig.5.28 Liver histology at second operation (42 weeks of obstruction). Normal portal space. Early swelling of bile ducts epithelium.

Table 5.9

Liver histology and blood LFT (8 procedures)

	Tot B (uM/l)	Con B (uM/l)	TotPro (g/l)	Album (g/l)	A/G	AP (UI/l)	GOT (UI/l)	GPT (UI/l	gGT (UI/l)
EPITHELIUM									
gr.1 MD	8.3	1.7	57	27.6	1	17.8	12	10	7
n=7 RG	(2.2-	(0.2-	(47.5-	(20.8-	(0.7-	(11.8-	(6-	(6-	(5-
	-16.2)	-8.4)	-60.9)	-33)	-1.3)	-33.4)	-28)	-17)	-10
gr.3	2	1	36.4	10.7	0.4	29.8	14	12	0
n=1									
INFLAM.									
gr.1 MD	9.9	3.7	51.3	27.6	1	21.6	12	10	6
n=5 RG	(3.1-	(0.2-	(47.5-	(20.8-	(0.8-	(17.4-	(6-	(6-	(5-
	-16.2)	-8.4)	-60)	-33)	-1.3)	-33.4)	-28)	-17)	-9)
gr.3 n=1	2	1	36.4	10.7	0.4	29.8	14	12	0
gr.5 n=2	2.2,	0.6,	57,	29.2,	1.1,	13.2,	18,	12,	10,
	5.2	1.7	60.9	24	0.7	11.8	12	8	7
PROLIF. BD									
gr.1 MD	6.7	1.7	54.1	26.7	0.9	19.7	12.5	11	6.5
n=8 RG	(2-	(0.2-	(36.4-	(10.7-	(0.4-	(11.8	(6-	(6-	(0-
	-16.2)	-8.4)	-60.9)	-33)	-1.3)	-33.4)	-28)	-17)	-10)
GRAN.TISS									
gr.1 MD	6.7	1.7	54.1	26.7	0.9	19.7	12.5	11	6.5
n=8 RG	(2-	(0.2-	(36.4-	(10.7-	(0.4-	(11.8	(6-	(6-	(0-
	-16.2)	-8.4)	-60.9)	-33)	-1.3)	-33.4)	-28)	-17)	-10)
FIBROSIS									
gr.1 MD	8.3	2.7	51.3	27.6	1	21.6	13	12	6
n=7 RG	(2-	(0.2-	(36.4-	(10.7-	(0.4-	(13.2-	(6-	(6-	(0-
	-16.2)	-8.4)	-60)	-33)	-1.3)	-33.4)	-28)	-17)	-10)
gr.5 n=1	5.2	1.7	60.9	24	0.7	11.8	12	8	7

Table 5.10

Liver histology and CBD bile microbiology and biochemistry (8 procedures)

+vecult/n			tot bil (uM/l)	conj. bil (uM/l)	un bil (uM/l)	BetaGLU (UI/l)	phosphol (UI/l)	BA (mM/l)
EPITHELIUM								
gr.1	3/7	MD	1933.7	1094.8	782	0.04	0	17.2
		RG	(730.3-	(496-	(233.7-	(0.03-	(0-	(11.6-
			-3076)	-1867.2)	-1208.8)	-0.13)	-0.2)	-114)
gr.3	1/1		1973	1946	27	0.02	0.07	81.5
INFLAM.								
gr.1	2/5	MD	1933.7	1094.8	782	0.04	0	16.4
		RG	(1255.5-	(834.1-	(421.4-	(0.03-	(0-	(11.6-
			-2323.1)	-1389.7)	-933.4)	-0.13)	-0.20)	-114)
gr.3	1/1		1973	1946	27	0.02	0.07	81.5
gr.5	1/2		3076,	1867.2,	1208.8,	0.04,	0,	21.4,
			730.3	496.6	233.7	0.04	0	17.2
PROLIF. BD								
gr.1	4/8	MD	1948	1219	686.2	0.04	0	19.3
		RG	(730.3-	496.6-	(27-	(0.02-	(0-	(11.6-
			-3076)	-1946)	-1208)	-0.13)	-0.20)	-114)
GRAN.TISS.								
gr.1	4/8	MD	1948	1219	686.2	0.04	0	19.3
		RG	(730.3-	496.6-	(27-	(0.02-	(0-	(11.6-
			-3076)	-1946)	-1208)	-0.13)	-0.20)	-114)
FIBROSIS								
gr.1	3/7	MD	1962.4	1343.3	782	0.04	0	21.4
		RG	(1255.5-	(834.1-	(27-	(0.02-	(0-	(11.6-
			-3076)	-1946	-1208)	-0.13)	-0.20	-114)
gr.5	1/1		730.3	496.6	236.7	0.04	0	17.2

* CBD

Tissues for histology were also available from the CBD in five cases sampled at the second operation and in one case at post-mortem examination (Fig. 5.29, 5.30). Appearances of fibrosis, inflammation and glandular proliferation were seen in some cases. Results are displayed in Table 5.11: the frequency of pathological changes did not increase significantly with the duration of the biliary obstruction.

In four out of six procedures where CBD histology was performed, routine LFT were also carried out. Microbiology and biochemistry of bile were otherwise carried out in five out of six cases. Bile cultures were positive in every case. Tables 5.12 and 5.13 show respectively the data related to blood LFT and to bile biochemistry. Data are summarized only as median and ranges because of the presence of wide variances. No statistical correlation was demonstrated when comparing CBD histology with blood LFT (Table 5.12), nor with bile biochemistry (Table 5.13) (Mann-Whitney & Neuman-Keuls multiple comparison tests). The complete set of figures is displayed in Table 13.1, in Appendix 13.2.

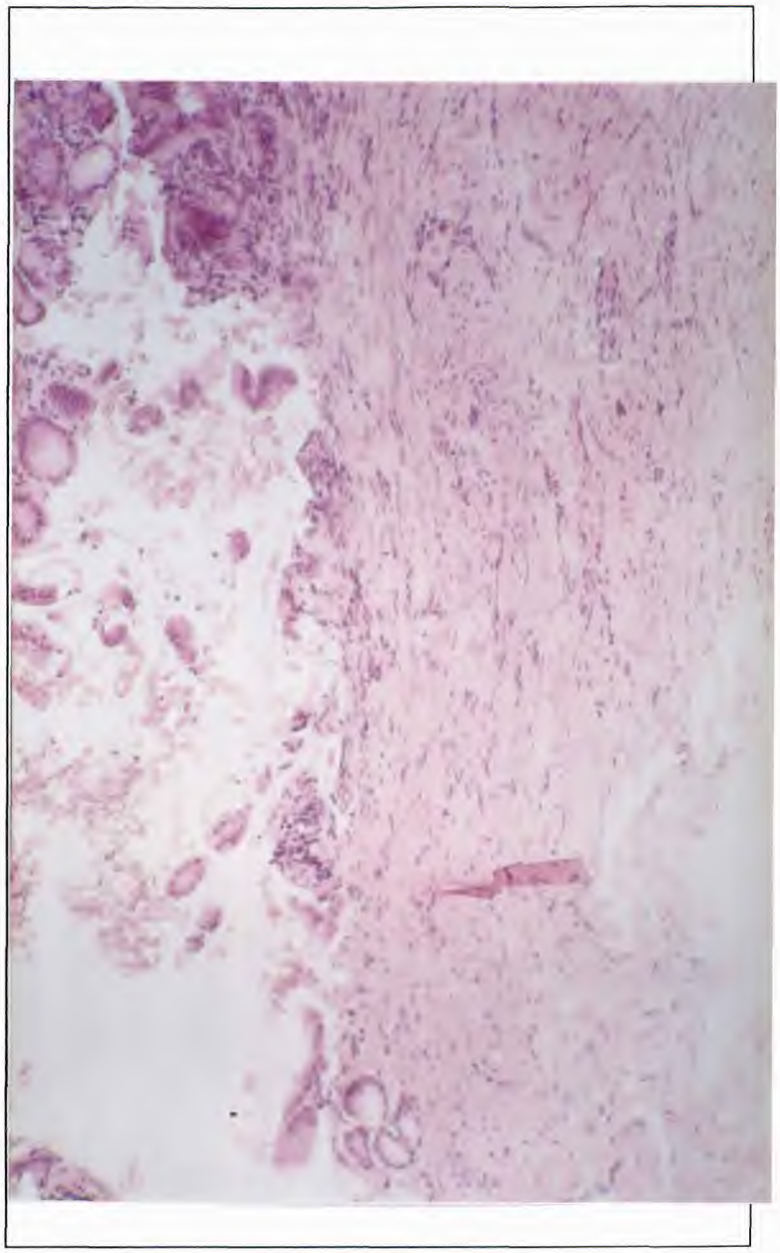


Fig.5.29 CBD histology after 40 weeks obstruction. Marked fibrotic changes. Inflammation with erosion of bile duct epithelium.

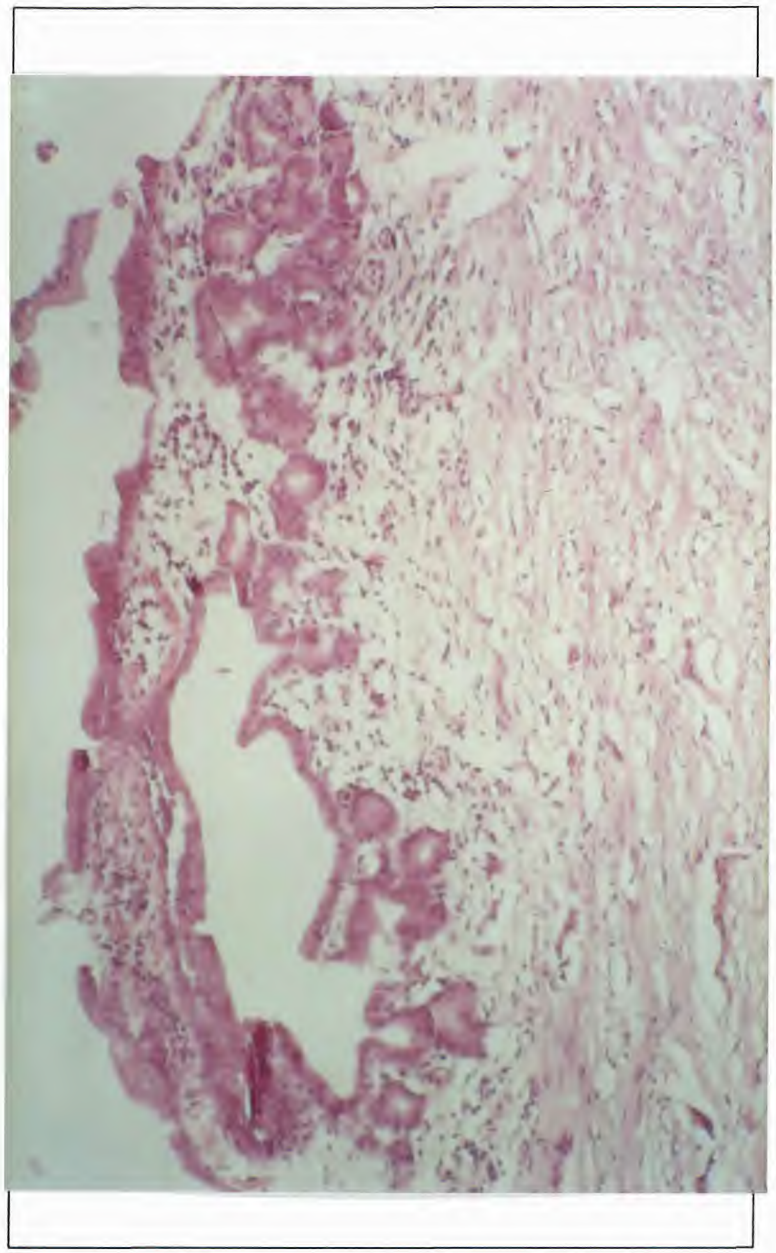


Fig.5.30 CBD histology after 42 weeks of obstruction. Moderate inflammation and fibrosis: early glandular proliferation.

Table 5.11

CBD histology and duration of the obstruction

event	weeks of obstr.	epith.	inflam.	prolif. BD	gran.t.	fibrosis
2nd op.	21	-	+	+	+	-
(n=5)	40	-	+	+	-	+
	42	+	+	+	-	+
	52	+	+	+	+	-
	72	-	+	+	-	-
PM	58	-	-	-	-	-

Key:
obstr. = duration of obstruction
epith. = loss of bile canaliculi epithelium
inflam. = inflammatory changes
prolif.BD = proliferation of bile ducts
gran.t. = granulation tissue

Table 5.12

CBD histology and blood LFT (4 procedures)

			Tot B (uM/l)	Con B (uM/l)	TotPro (g/l)	Album (g/l)	A/G	AP (UI/l)	GOT (UI/l)	GPT (UI/l	gGT (UI/l)
EPITHELIUM											
gr.1	n=2		2, 5.2	1, 1.7	36.4, 60.9	10.7, 24	0.4, 0.7	29.8, 11.8	14, 12	12, 8	0, 7
gr.3	n=2		3.1, 8.3	0.2, 3.7	60, 51.3	33, 25.9	1.2, 1	33.4, 17.8	13, 12	8, 15	6, 9
INFLAM.											
gr.3	n=3	MD	3.1	1	51.3	25.9	1	29.8	13	12	6
		RG	(2-	(0.2-	(36.4-	(10.7-	(0.4-	(17.8-	(12-	(8-	(0-
			-8.3)	-3.7)	-60)	-33)	-1.2)	-33.4)	-14)	-15)	-9)
gr.5	n=1		5.2	1.7	60.9	24	0.7	11.8	12	8	7
PROLIFER.											
gr.3	n=2		2, 3.1	1, 0.2	36.4, 60	10.7, 33	0.4, 1.2	29.8, 33.4	14, 13	12, 8	0, 6
gr.5	n=2		8.3, 5.2	3.7, 1.7	51.3, 60.9	25.9, 24	1, 0.7	17.8, 11.8	12, 12	15, 8	9, 7
GRAN.TISS.											
gr.1	n=3	MD	3.1	1	60	24	0.7	29.8	13	8	6
		RG	(2-	(0.2-	(36.4-	(10.7-	(0.4-	(11.8-	(12-	(8-	(0-
			-5.2)	-1.7)	-60.9)	-33)	-1.2)	-33.4)	-14)	-12)	-7)
gr.3	n=1		8.3	3.7	51.3	25.9	1	17.8	12	15	9
FIBROSIS											
gr.1	n=2		2, 8.3	1, 3.7	36.4, 51.3	10.7, 25.9	0.4, 1	29.8, 17.8	14, 12	12, 15	0, 9
gr.3	n=2		3.1, 5.2	0.2, 1.7	60, 60.9	33, 24	1.2, 0.7	33.4, 11.8	13, 12	8, 8	6, 7

Table 5.13

CBD histology and CBD bile microbiology and biochemistry (5 proc.)

			tot bil (uM/l)	conj.bil (uM/l)	un bil (uM/l)	betaglu (UI/l)	phosphol (UI/l)	BA (mM/l)
EPITHELIUM								
gr.1	n=3	MD	1268	1036.7	231.3	0.04	0	25.8
		RG	(730.3-	(496.6-	(27-	(0.02-	(0-	(17.2-
			-1973)	-1946)	-233.7)	-0.04)	-0.07)	-81.5)
gr.3	n=2		1255.5,	834.1,	421.4,	0.04,	0,	11.6,
			2323.1	1389.7	933.4	0.04	0	26.8
INFLAM.								
gr.3	n=3	MD	1973	1389.7	421.4	0.03	0	26.8
		RG	(1255.5-	(834.1-	(27-	(0.02-	(0-	(11.6-
			-2323.1)	-1946)	-933.4)	-0.04)	-0.07)	-81.5)
gr.5	n=2		1268,	1036.7,	231.3,	0.04,	0,	25.8,
			730.3	496.6	233.7	0.04	0	17.2
PROLIFER.								
gr.3	n=3	MD	1268	1036.7	231.3	0.04	0	25.8
		RG	(1255.5-	(834.1-	(27-	(0.02-	(0-	(11.6-
			-1973)	-1946)	-421.4)	-0.04	-0.07)	-81.5)
gr.5	n=2		2323.1,	1389.7,	933.4,	0.04,	0,	26.8,
			730.3	496.6	233.7	0.04	0	17.2
GRAN.TISS								
gr.1	n=3	MD	1255.5	834.1	233.7	0.04	0	17.2
		RG	(730.3-	(496.6-	(27-	(0.02-	(0-	(11.6-
			-1973)	-1946)	-421.4)	-0.04)	-0.07)	-81.5)
gr.3	n=2		1268,	1036.7,	231.3,	0.04,	0,	25.8,
			2323.1	1389.7	933.4	0.04	0	26.8
FIBROSIS								
gr.1	n=3	MD	1973	1389.7	231.3	0.03	0	26.8
		RG	(1268-	(1036.7-	(27-	(0.02-	(0-	(25.8-
			-2323.1)	-1946)	-933.4)	-0.04)	-0.07)	-81.5)
gr.3	n=2		1255.5,	834.1,	421.4,	0.04,	0,	11.6,
			730.3	496.6	233.7	0.04	0	17.2

5.3.5 Histochemistry (Tables 5.14, 5.15. Figs. 5.31-5.33)

* Liver

Histochemical assays of tissue betaglucuronidase were carried out on liver tissues in 10 procedures (Table 5.14). The presence of the enzyme in liver tissue was calculated by use of a calibrated eyepiece graticule and counting the betaglucuronidase granules in 10 squares scattered in the field. The sum of all the granules counted represents the amount of betaglucuronidase reported.

Betaglucuronidase granules were present on nine occasions whereas no enzyme was found in the tenth. No correlation was demonstrated between the duration of the obstruction and the presence of betaglucuronidase in the liver.

The presence of betaglucuronidase granules in liver tissues was also compared with the presence of bacteria in bile: lower tissue betaglucuronidase values were demonstrated in the presence of bacteria in bile, this difference not reaching statistical significance (Fig. 5.31).

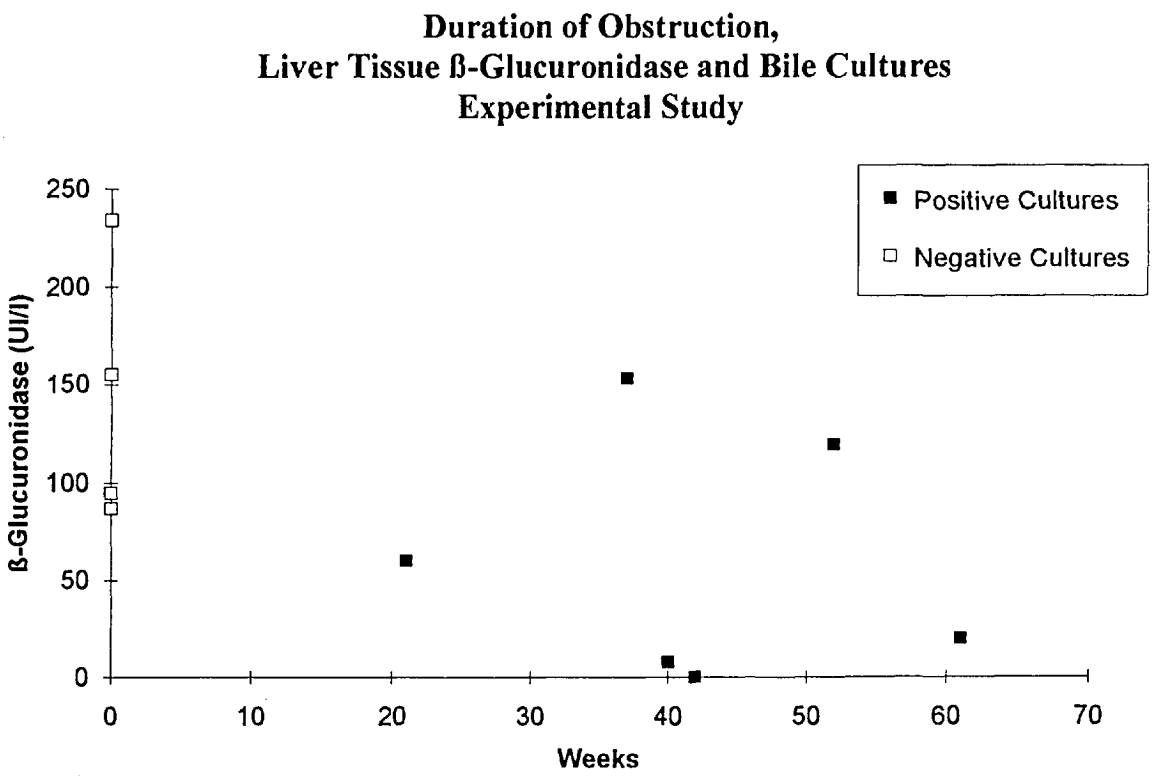


Fig.5.31 Time of obstruction, liver tissue betaglucuronidase and CBD bile cultures.

Table 5.14

Betaglucuronidase in liver tissues (10 procedures)

procedure	obstruction (weeks)	BetaGLU count	LFT	bile cult	bile bioch
1st op.	0	87	Y	Y	Y
(n=4)	0	234	Y	Y	Y
	0	155	Y	Y	Y
	0	95	Y	Y	Y
2nd op.	21	60	N	Y	Y
(n=5)	37	153	Y	Y	Y
	40	8	Y	Y	Y
	42	0	Y	Y	Y
	52	119	Y	Y	Y
SA (n=1)	61	20	Y	Y	N

In nine out of 10 procedures where liver tissues were assayed for the presence of betaglucuronidase, blood liver function tests were also performed. However, linear regression analysis has shown no statistical relationship for all these comparisons.

Finally the presence of tissue betaglucuronidase was compared with total, conjugated and unconjugated bilirubin levels and betaglucuronidase activity in bile and again no correlation was demonstrated (simple linear regression analysis: $r=0.02$, Fig. 5.32 for betaglucuronidase; $r=0.64$, Fig. 5.33 for bilirubin).

* CBD

Tissue betaglucuronidase was qualitatively assessed also in CBD tissues, in six procedures: however, in no case was the enzyme found in CBD tissues (Table 5.15).

Table 5.15

Betaglucuronidase in CBD tissues (6 procedures)

procedure	obstruction (weeks)	BetaGLU count	LFT	bile cult	bile bioch
2nd op.	21	N	N	Y	Y
(n=5)	37	N	Y	Y	Y
	40	N	Y	Y	Y
	42	N	Y	Y	Y
	52	N	Y	Y	Y
SA (n=1)	61	N	Y	Y	N

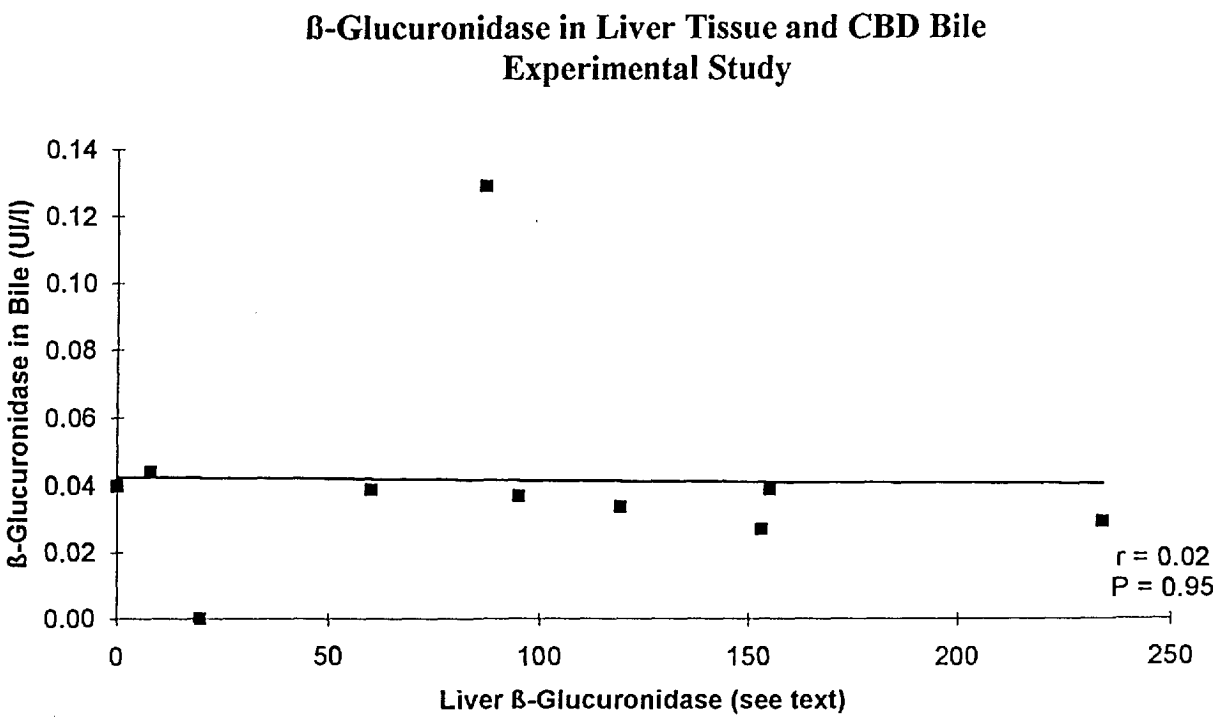


Fig.5.32 Betaglucuronidase activity in liver tissue and in CBD bile.

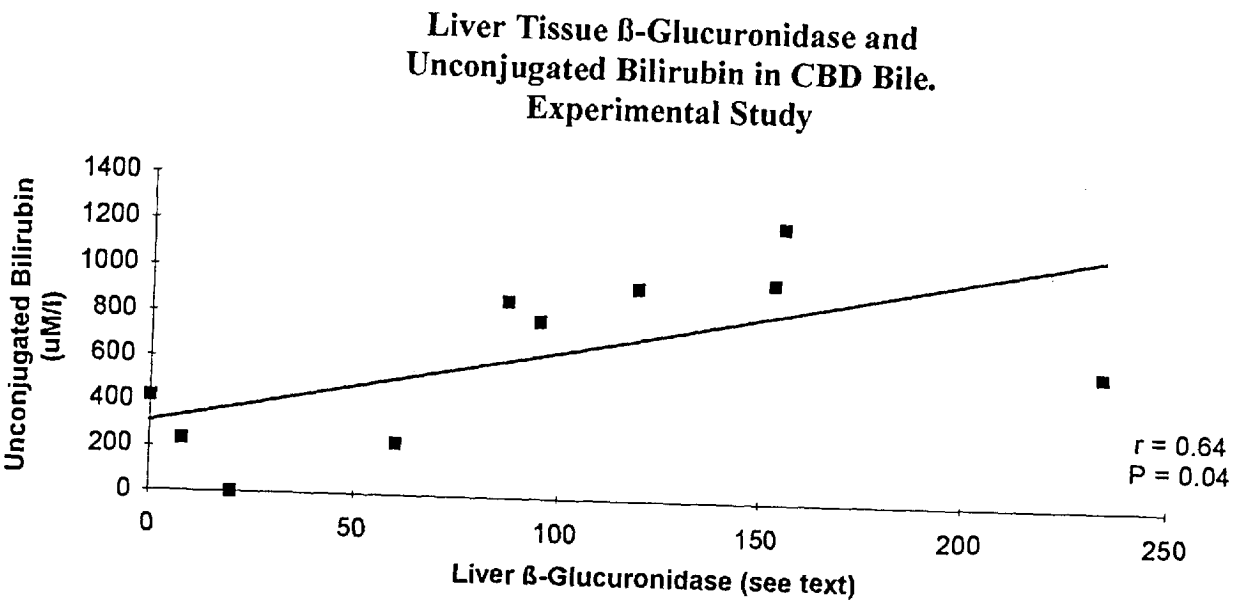


Fig.5.33 Liver tissue betaglucuronidase and unconjugated bilirubin in CBD bile.

5.4 DISCUSSION

We have succeeded in developing a model of chronic incomplete CBD obstruction, radiologically confirmed with bile sampling for microbiology and biochemistry, and the production of primary CBD stones in two cases. This represents an advance on the models available at the start of this work.

5.4.1 Models of experimental biliary obstruction published after the start of this work.

Several new studies reporting models of acute or chronic biliary obstruction have since been published. Abedin et al. (1989) studied changes in biliary calcium and bile acid composition in the presence of cholesterol and pigment gallstones. In prairie dogs the cystic duct was ligated and the gallbladder removed. The CBD was ligated and cannulated with a silastic catheter. Total bile acid concentration did not differ in four different groups of animals. Chenodeoxycholic acid increased in animals fed by cholesterol or carbohydrate-rich diet. In GB bile calcium concentration showed a linear relationship with total bile acids and taurochenodeoxycholic acid and an inverse relationship with taurocholic acid. These data indicate that diet-induced alterations in bile acid composition may modify calcium solubility in GB bile.

Snowball et al (1989) produced biliary sludge and pigment phosphate stones in guinea pigs treated with lincomycin by intraperitoneal injection, this being related to epithelial inflammation induced by lincomycin.

Hancke and Marklein (1983) report a model of chronic, partial occlusion of the cystic duct in mongrel dogs, with subsequent injection of E.coli in the spleen or in the colon

and demonstrated gallbladder stone formation. Hardison et al (1983) described a model of choledochal fistulae in the rat, connecting externally two polythene catheters placed in the common bile duct and in the superior vena cava via the right internal jugular vein. According to the authors this model allowed complete retention of biliary contents without impairment of bile flow.

Konturas et al (1985) used a biliary obstruction model described by Accattino (1979); the common bile duct was cut and cannulated with a polyethylene cannula: its distal end was buried in a subcutaneous space and kept closed for a time ranging from 2-28 days and then opened externally to study the post-cholestatic characteristic of bile flow. Tanaka et al. (1985) described a model of common bile duct ligation and excision of a length of 5-8 mm associated with intrabiliary injection of bacteria and with splenectomy.

A model of chronic biliary fistula has been reported by Rotolo et al (1988) who studied the regulation of biliary protein secretion in mongrel dogs. The experiment was carried out by performing cholecystectomy, ligating the lesser pancreatic duct and inserting a Thomas cannula into the duodenum through which bile was sampled periodically by introducing a ureteric catheter into the papilla.

More recently (1990) Xu reported the formation of black pigment gallstones after partial CBD ligation in guinea pigs and Liu (1991) described the formation of pigment gallstones in the gallbladder of germ-free guinea pigs .

We have already noted that bile pigment stones had been experimentally produced on several occasion (Westphal & Gleichmann, 1931; Bisgard & Baker, 1940; Sato et al., 1955; Atsumi, 1956; Imamoglu et al., 1957). However, with the exception of the last, all produced gallbladder stones. Furthermore, amongst models which produced gallbladder stones, all but one, the Bisgard & Baker (1940) model, needed the addition of external

substances (bacteria, Ascaris Lumbricoides, a suspension of eggshells). The data most recently published by the Chinese workers now report the experimental production of pigment gallstones in germ-free guinea pigs (Liu, 1991) and the production of black pigment gallstones after partial CBD ligation in guinea pigs (Xu, 1990).

5.4.2 Comments on our model

The work of Imamoglu et al (1957) is the only one to our knowledge in which primary CBD stones have been experimentally produced by biliary stasis alone without the use of any additional substance. When we developed the idea of our model we attempted to reproduce conditions similar to those obtained by these workers, but placed a Thomas cannula into the duodenum in order to allow periodical sampling of bile and also cholangiographic confirmation of the development and persistence of an incomplete stricture. This original procedure has made it possible to follow changes in the microbiology and biochemistry of bile during the development of chronic incomplete biliary tract obstruction. The formation of primary CBD stones makes this model valuable for future studies on the pathophysiology of the obstructed biliary tree and the pathogenesis of its stones.

The above description of the model itself is sufficiently detailed to underline most of the difficulties encountered in this experiment; however several other points are worthy of more detailed discussion. The position of the Thomas cannula is crucial. It is of paramount importance to choose the right position of the duodenal incision with respect to the papilla, otherwise considerable difficulties will arise at time of retrograde papillary cannulation for x-ray and sampling. Equally important is the position of the cannula in the abdominal wall, since a cannula placed too low may distort and obstruct the duodenum: one of dogs who died soon after the first operation (765) showed at PM examination a massive gastric dilatation probably due to malposition of the cannula. By contrast a

cannula placed too high will bring the risk of interfering with the costal margin with painful consequences for the animal during the long period of cannulation.

It is also advisable to snug carefully the double duodenal purse string around the cannula and to keep the cannula tract in the abdominal wall as tight as possible in order to avoid spillage of duodenal contents around the cannula and inflammation of the surrounding skin. In this respect we found it useful to keep the lateral incision of the abdominal wall short, to hold it temporarily with retractors at time of the passage of the cannula and to place a pedicle of omentum around the cannula in order to reduce the leakage of duodenal juice.

Another important point is the choice of the right length of the polyethylene tube, through which the nylon sling is passed after encircling the CBD. Too long a tube may displace the CBD medially with a risk of perforation. This was the case in dog 769, in which the polyethylene tube was found inside the CBD surrounded by biliary concrements. Conversely, a too short polyethylene segment will make it impossible to produce the stricture either spontaneously or by pulling on the ends during postoperative radiology.

It is worthy of comment that the nylon sling whose ends were placed at the first operation never needed to be tightened under cholangiographic control in order to produce the incomplete stricture, this being always present at the first cholangiography. This finding surprised us at first, but proved to be quite reliable and consistent, presumably due to the fibrosis induced in the CBD by the dissection followed by the presence of nylon. This is similar to the results reported by Imamoglu et al (1957) who found an incomplete CBD obstruction in most of the animals following passage of a cellophane tape around the duct.

In verification of the stricture the most difficult manoeuvre is to identify and cannulate the papilla. An operator with previous experience in ERCP may not find the same difficulties.

However I had no previous experience of endoscopic retrograde cholangiography in clinical practice, and had two unsuccessful early cannulation attempts in a total of 15 procedures. Both of these dogs had successful cannulation later. It is important to identify the papilla precisely by inspection before attempts to pass the cannula, since the first attempt has the best chance, while subsequent oedema and spasm of the ampulla will progressively frustrate further attempts. It is also important to push the catheter gently well above the stricture before sampling bile from the obstructed portion of the CBD, and to aspirate bile slowly.

At the second operation we have found it useful in the last three dogs (758, 769, 820) to include the cannula tract in the principal incision rather than making a double incision as in the earlier dogs. This change in technique has resulted in easier dissection of the duodenum from the abdominal wall, decreased blood loss, less danger of tears in the duodenal wall, and faster and more precise closure of the duodenotomy.

Unfortunately, only one dog survived the second procedure of bilio-jejunal anastomosis. It may be that with more experience results of this operation would improve and allow long term study of the liver and bile ducts during the recovery period.

Stones have been experimentally produced in two dogs in this series: it is interesting to comment that on one occasion biliary concrements were found around the polyethylene tube migrated inside the duct: this recalls the experiments of Sato (1955) and Atsumi (1956) who produced experimental gallbladder stones around carcass of Ascaris Lumbricoides or a suspension of eggshells and confirms the role of the so called heterogeneous nucleation in this type of stones.

5.4.3 Problems encountered in this study.

One of the major deficiencies of this thesis is the lack of an analysis of the composition of the experimentally produced stones. As explained in detail in the clinical study, this is due to the loss of the material in the laboratory of Professor Wosiewitz, Department of Gastroenterology, University of Frankfurt am Main, Germany.

It should be emphasized that in the clinical study, which started before the experimental and histochemistry studies, in order to assess the possible role of a phospholipase of bacterial origin, we assayed phospholipase C activity (Molby et al., 1973). Later, extending our study of the literature, we read the paper of Cetta et al. (1984) and learned that these authors searched for phospholipase A, using the method described by Goswami & Frey (1974). We therefore elected to search for this enzymatic fraction in the experimental study. Thus, after an early phase in which phospholipase C was measured with no positive results (dogs 299, 300 & 520, Table 5.1), we changed to the method of Goswami & Frey for the assays performed in the final part of the experimental study (dogs 731, 737, 758, 765, 769, 820, 821, Table 5.1) and for the whole histochemical study. Nonetheless, phospholipase values remained extremely low with either method.

5.4.4 Comments on the results of this study

* Blood LFT

The finding of reduced blood albumin and albumin/globulin ratio in the presence of infected bile in our experimental work is in keeping with data reported by Blumgart et al. (1984) who noticed a significant relationship between the presence of liver disease and a history of major infections in their series of patients with benign biliary strictures. This finding also indirectly confirms data published by Halliday et al. (1987) who reported a

quick recovery of plasma prealbumin levels in jaundiced patients treated with preoperative transhepatic biliary drainage plus a nutritional regimen of at least 3000 calories per day, only in the presence of sterile bile.

* Microbiology

Portal cultures were found positive only in one out of eleven cases, and in this case the same organisms were recovered from the bile. However, the dog is not a reliable model of the human situation with respect to portal bacteraemia. Orloff et al. (1958) found portal cultures in man to be predominantly sterile. By contrast, Schatten et al. (1955) studied the bacteriological characteristics of portal blood in man in 25 patients undergoing laparotomy and found 32% positive portal cultures rate.

The data concerning bile bacteriology, however, are consistent with problems related to biliary tract obstruction and bile infection in man. Bacteria isolated from bile were similar to those reported in the literature from clinical studies on bile microbiology. All basal bile cultures were sterile, as in man (Edlund et al., 1959; Kune & Schultz, 1974).

The incidence of bacteria in the bile rose significantly with increasing duration of obstruction, similar to the findings in benign biliary strictures in man (Jackaman et al., 1980; Blumgart et al., 1984). It will be noted later that in the clinical study in this thesis there was an increased bile colonization rate in the presence of a long duration of gallstone disease.

In both cases where stones were found we have demonstrated bile infection and this is also in keeping with clinical studies (Edlund et al., 1959; Goswitz, 1970; Kune & Schultz, 1974; Keighley et al., 1974, Keighley & Blenkharn, 1988). Recent electron microscopic studies have also shown bacterial casts inside brown pigment stones (Stewart et al., 1987;

Kaufman et al., 1989). Imamoglu et al. (1957), however, found infected bile only in one out of six cases where stones were produced in rabbits.

* Bile Biochemistry

Measurements of bilirubin levels and betaglucuronidase activity do not support the hypothesis suggested by Maki (1966) of bilirubin deconjugation by bacterial betaglucuronidase and the well demonstrated association between bile stasis, infection and primary CBD stone formation, confirmed in this experimental study, does not seem to rest upon this biochemical correlation.

No correlation was found between bilirubin or betaglucuronidase and the duration of obstruction. Similarly no correlation was found between betaglucuronidase activity and bilirubin levels in the presence or absence of bacteria in bile, nor in the presence or absence of stones nor between unconjugated bilirubin and betaglucuronidase. These findings are also against the possibility of bilirubin deconjugation by a non-bacterial, tissue betaglucuronidase (Wosiewitz, 1979; Trotman & Soloway, 1982; Kouroumanlis et al., 1983; Ho, 1985; Ho et al., 1986). This aspect, however, will be discussed later in detail.

Maki et al. (1962) found heavy bacterial colonization in patients with pigment gallstones, and very low or zero colonization in patients with cholesterol stones, as well as lower betaglucuronidase activity in pigment stone patients. To support his theory Maki (1966) quotes the papers of Miyake (1900) and Westphal & Gliedman (1931) who observed the presence of biliary concrements in the gallbladder of dogs, after prolonged bile stasis and injection of a suspension of E coli. However this does not demonstrate that the formation of stones in the presence of bacteria in bile is the consequence of an enzymatic deconjugation of bilirubin in bile.

Boonapysit et al (1978) reported increased unconjugated bilirubin levels in patients carrying pigment stones, but did not perform betaglucuronidase assays. In addition it is not clear whether these authors assayed bile from patients with black or brown pigment stones, but the fact that the stones were collected in a series of patients from United States and that no bacteria were found in the bile suggests that there were black pigment stones, rather than brown ones, which are uncommon in western countries and usually associated with bile infection. Also Masuda et al. (1979) do not exclude the presence of bilirubin hydrolysis in absence of bacteria, though reporting a correlation between positive cultures and unconjugated bilirubin and presence of stones.

Spivak et al. (1987) demonstrated a non-enzymatic hydrolysis of bilirubin in guinea pigs: this would be in keeping with the lack of a relationship that we have found in our study between betaglucuronidase activity and unconjugated bilirubin values and Xu (1990), who produced experimental pigment gallstones after CBD ligation, reports no changes in betaglucuronidase activity between animals where gallstones were produced and controls, stating that is hard to explain the production of stones with Maki's theory. However, no microbiological data have been reported in this study.

Generally phospholipase activity was extremely low in this study, with mean values not exceeding 0.02 UI/l. No differences were found in the presence of infected or sterile bile or in the presence or absence of stones.

These data are in agreement with those reported by Goswamy & Frey (1974) who reported no phospholipase activity in bile in their assays. However, Cetta et al. (1986) found phospholipase activity in 80% of patients with recurrent CBD stones, although in this paper only qualitative estimations of phospholipase activity are reported, which may be insufficient to support a role for phospholipase A in the production of primary CBD stones. This low phospholipase activity in bile may be due to sequestration of enzyme

granules inside lysosomes (Sjodal et al., 1978) or by variation of activity in bile due to the intermittent reflux of pancreatic juice into bile (Anderson et al., 1979).

It is well established that some cholesterol gallstone patients have a decreased total bile acid pool size (Vlahcevic et al., 1970; Grundy et al., 1974). In our animal model, total biliary bile acid levels did not change significantly with the duration of obstruction, nor in the presence of sterile or infected bile, nor did they correlate with the presence or absence of experimentally produced primary CBD stones. Almond et al, (1973) also found no changes in total biliary bile acids in subjects measured before and after cholecystectomy, and Gleeson et al. (1986) found no difference between patients with cholesterol or calcified stones. By contrast our data differ from those published by Nakano et al. (1989), who report significantly lower total bile acid levels in the presence of primary CBD stones and cholangitis. Also Akyoshi et al (1990) report significantly lower levels of total bile acids in the presence of primary CBD stones, these changes being explained by an increased absorption of bile acids by epithelium of an inflamed biliary tract in the presence of infection. Moreover these authors report an increase of free bile acids, in agreement with Shimada et al. (1985) who suggested that the presence of free bile acids may initiate an inflammatory process leading to the formation of pigment stones. However our data cannot address this question since we have measured only total bile acid levels in this study.

* Histology

Histological changes in the liver and CBD in this model were not associated with the duration of obstruction, with changes in blood LFTs or with changes in bile microbiology or biochemistry. This is in contrast with Koyama et al. (1981) who reported lower values of total plasma proteins and albumin in the presence of experimental acute bile duct obstruction. However, it should be underlined that this study reports changes in liver structure in complete acute biliary obstruction and that it is so far unclear whether these

and other changes such as modifications in bile canaliculi structure followed by bile duct proliferation and fibrosis (Benjamin, 1988) may also take place in presence of a longer, incomplete biliary obstruction. In this respect Flinn et al (1977) have reported pathological signs of acute inflammation in the presence of choledocholithiasis, but our data do not confirm this finding. It should be also underlined that Schaffner & Popper (1985) described in the initial phase of complete biliary obstruction changes in bile canaliculi such as microvilli distortion and swelling. However, since this study is based on electron microscopy observations we cannot comment this on the basis of our data.

* Histochemistry

No betaglucuronidase activity at all was found in CBD tissues and no relationship was demonstrated between liver tissue betaglucuronidase and the duration of obstruction. Similarly no statistical correlation was found comparing results of blood LFTs with hepatic betaglucuronidase or the values of the enzyme in the presence of sterile or infected bile, or correlating them with the values of unconjugated bilirubin. This stands against data reported by Sussi & Rubaltelli (1968) who suggested a relationship between extrahepatic cholestasis and liver betaglucuronidase, and by Boonapysit et al. (1978), who found no correlation between bacterial betaglucuronidase activity and stone formation and suggested a possible role of non-bacterial activity of betaglucuronidase. Conversely our findings are in keeping with data reported by Masuda et al., (1978) who raise the possibility of a non-bacterial betaglucuronidase, but report in their study a significant association between bacteria and brown pigment gallstones and by Ho et al., (1986), who remark as in their own as well as in other studies that betaglucuronidase activity is inconsistent. These authors try to explain such irregular findings with the possibility that betaglucuronidase granules may be partially sequestered into mixed micelles and lose its activity, the measured betaglucuronidase activity being in fact only an apparent activity.

5.4.5 Conclusions

What conclusions can we draw from this experimental study?

1. An original and reproducible experimental model of chronic incomplete biliary obstruction has been established in the dog. Animals have been kept alive to a maximum of 72 weeks, and the presence of incomplete biliary obstruction has been validated by periodical retrograde cholangiography. In addition bile has been sampled serially for microbiology and biochemical assays of bilirubin, total bile acids, betaglucuronidase and phospholipase activities.

2. In two cases primary CBD stones were experimentally produced, in the presence of stasis and heavy bacterial colonization of bile. However, no specific biochemical changes were demonstrated in the presence of stones.

3. Positive bile culture rates rose significantly with the duration of obstruction. However, no significant changes were demonstrated in bilirubin, betaglucuronidase, phospholipase or bile acid values in relation to the duration of obstruction, in the presence or absence of bacteria in bile, or in the presence or absence of stones. In addition, levels of unconjugated bilirubin showed no correlation with betaglucuronidase activity in bile.

4. Liver tissue betaglucuronidase showed no correlation with duration of obstruction, nor with the presence of bacteria in bile or with unconjugated bilirubin levels. No betaglucuronidase was found in ductal epithelium.

5. The presence of bacteria in bile has proved to be associated with significantly lower levels of plasma albumin.

Thus the main conclusions of this study are that the primary CBD stone exists and is associated with bile stasis and infection. However, the causal link between these associations is not necessarily represented by the deconjugation of bilirubin in bile by way of a betaglucuronidase of bacterial or tissue origin, in contrast with the hypothesis suggested by Maki (1966), nor by the hydrolysis of lecithins by the action of a phospholipase nor by changes in total bile acids.

6 CLINICAL STUDY

6.1 INTRODUCTION

Biliary stones are known to have affected human beings for several thousand years, since gallstones have been found in an Egyptian mummy (Glenn, 1971; Praderi, 1987). Today gallstones are reported in approximately 10% of the adult population and in 20% of people over 40 years (Orloff, 1978/b). CBD stones are reported in about 10% of patients undergoing cholecystectomy.

Common bile duct stones are classified as primary when originating in the CBD and secondary when originating in the GB and migrating to the CBD. Secondary stones are sometimes termed "residual" if they have been overlooked at cholecystectomy (Glenn, 1972).

6.1.1 Incidence of primary CBD stones

The existence of primary CBD stones has been proved by the reports of ductal stones in patients with congenital absence of the gallbladder (Villareal, 1948; Rains, 1951; Gerwig et al. 1961). However, despite the high incidence of choledocholithiasis, there is no agreement in the surgical community about the incidence of primary CBD stones (Millbourn, 1950; Madden et al, 1968; Bartlett, 1972; Saharia et al., 1978), the criteria to for classifying stones as primary, or their pathogenesis. Madden (1968) first called attention to the importance of identifying at operation the primary or secondary origin of a CBD stone, since this may influence surgical strategy. This author, using the classification published by Aschoff in 1924 (Table 6.1), reported that amongst patients with CBD stones, 56% had stones of primary ductal origin. Because of this high

incidence Madden stressed the use of choledochoduodenostomy as a drainage procedure in this situation. By contrast, Saharia et al. (1978) suggested that the Aschoff classification should be used together with other clinical criteria, such as a history of cholecystectomy followed by a symptom-free interval of at least two years and no evidence of a long cystic duct remnant. Using these criteria the authors found a "primary" stone in only 4% of the patients operated for choledocholithiasis. In view of this low incidence of primary CBD stones, these authors felt that the choice of treatment must be carefully considered in each case.

6.1.2 Bile Stasis.

Primary CBD stones are usually reported as associated with bile stasis. Aschoff (1924) in his classification defined the primary or recurrent CBD stone as a "stasis" stone. Madden et al. (1968) reported papillary stenosis and CBD dilatation in 46.6% of primary and in 10% of secondary stones in his series and Saharia et al. (1978) stated that CBD dilatation was often present in patients with primary CBD stones.

Stasis may be related to chronic impairment of papillary function, either primary (Arianoff, 1968) or secondary to surgical probing of the papilla (Hunt & Blumgart, 1980) or other surgical manoeuvres on the CBD (Glenn, 1972). In addition, in patients from Far Eastern countries, similar stones have been reported not only from the CBD but also from the GB, usually associated with biliary infection and infestation: parasites, especially Ascaris Lumbricoides, not only act as a nidus for stone precipitation but also induce biliary stasis (Maki, 1961).

Table 6.1 *

CLASSIFICATIONS OF BILIARY STONES

A. according to their origin (Aschoff, 1924)

1. Inflammatory

2. Metabolic

3. Mixed

4. Stasis (ovoid, soft, generally single, conforming in shape to the contourof the CBD; readily crushable with fingers into multiple fragments of biliary mud. Brown, central nidus with yellow laminated periphery).

B. according to their composition (Trotman & Soloway, 1982)

Cholesterol Stones

Pigment Stones
(a) BLACK Pigment Stones
(b) BROWN Pigment Stones

* also reported as Table 4.1

6.1.3 Infection

Welch (1891) first described bacterial colonization of bile, but the significance of this observation has only been accurately evaluated more recently. Bile infection rates vary largely. Normal bile is sterile (Edlund et al, 1959; Kune & Schultz, 1974; Nielsen & Justensen, 1976), but in presence of GB stones incidences ranging from 4% (Goswitz, 1970) to 13% (Kune & Schultz, 1974) to 50% (Edlund et al., 1959) are reported. These figures rise in presence of acute cholecystitis (54%, Kune & Schultz, 1974; 60%, Goswitz, 74;), while Keighley et al. (1977) report figures as high as 25% in elective operations rising to 94% in emergencies.

Positive bile culture rates up to 100% have been found in the presence of CBD stones (Goswitz, 1970). However when separate figures are reported, much higher rates are found in the presence of CBD stones and biliary obstruction (30% versus 76%, Kune & Schultz, 1974; 90%, Keighley et al, 1974). High infection rates are also reported in the presence of benign biliary strictures (100%, Kune & Schultz, 1974), and Jackaman et al. (1980) reported a positive bile culture rate rising from 73% in patients at the first attempted repair of benign biliary stricture to 100% in patients at the fourth, fifth and sixth operation.

The presence of stones in the CBD is a risk factor for predicting biliary infection (Keighley, 1976). Glenn (1964) pointed out that postoperative infection was more likely to occur in the presence of biliary obstruction due to stones than in the presence of neoplastic lesions, and Saharia et al. (1978) reported the frequent association of primary CBD stones with cholangitis.

In order to identify before surgery patients at risk of developing infection in biliary surgery some authors reported that advanced age, need of emergency operation, biliary

stasis, jaundice and a history of cholangitis were associated with increased risk of bacteria in bile (Edlund et al., 1959; Mason, 1968; Haw & Gunn, 1973; Nielsen & Justensen, 1976). Keighley et al., (1976) undertook a multivariate analysis of factors capable of predicting infections following biliary surgery and concluded that patients with age greater than 70 years, jaundice, a history of cholangitis, the need of emergency operation, previous biliary surgery and CBD stones were more likely to carry bacteria in bile and therefore to develop infective complications.

In addition to these conventionally recognized risk factors for predicting infection, a long history of biliary disease may be important. Madden et al. (1968) observed that patients with primary CBD stones had a significantly higher number of previous biliary operations and Glenn (1972) stated that patients with a longer history of biliary disease had higher risk of CBD stones. Similarly Saharia et al., (1978) reported an increased incidence of CBD stones in patients with longer duration of symptoms. Lygidakis (1982) found that patients with primary CBD stones were characterized by older age, longer duration of disease, increased CBD size and higher bile infection rate. By contrast Edlund et al. (1959) reported that length of history was not related to presence of bacteria in bile. Similarly Haw & Gunn (1973) and Nielsen & Justensen (1976) found no association between a long clinical history of biliary disease and the presence of bacteria in bile.

The Organisms most frequently isolated from bile are aerobic Gram negative genera such as E. coli, Klebsiella spp,. Pseudomonas spp., Proteus, and S.faecalis (Nielsen & Justensen, 1976; Keighley, 1982). Anaerobic genera are also isolated in bile, even though less frequently in patients with uncomplicated biliary disease. The Anaerobes most frequently isolated are Clostridia and B.fragilis (Keighley, 1982). Anaerobes were reported in 56% of patients with previous biliary anastomosis and in 24% of patients with CBD stones or strictures (Nielsen & Justensen, 1976). Finegold (1979) found anaerobes more common after multiple biliary procedures and followed by higher postoperative

infection rates. Multiple isolates were found in 8% of patients (Haw & Gunn 1973), while Nielsen & Justensen (1976) found polymicrobial growth significantly higher in patients with previous biliary-enteric anastomosis.

Goswitz (1970) reported a 22% overall postoperative infection rate, and Keighley (1977) reports 20% overall wound sepsis rate: 41% in patients undergoing emergency surgery and 18% after elective operations. Furthermore wound infections and positive blood cultures were significantly more frequent after CBD exploration than in patients operated with cholecystectomy alone.

A close relationship between bile and postoperative positive cultures has also been reported by many authors (61% Mason, 1968; 100% Goswitz, 1970; 75% Kune & Schultz, 1974), while Keighley et al. (1977) and Pitt et al. (1982) reported a significant increase of postoperative infection rates in the presence of infected bile.

6.1.4 Biochemical changes in bile

The central biochemical aim of this study has been to assess under clinical and experimental conditions Maki's theory on the pathogenesis of calcium bilirubinate stone by way of a betaglucuronidase of bacterial origin which was reported to hydrolyze the diglucuronide of bilirubin into glucuronic acid and free bilirubin, the latter combining with Ca++ and precipitating as calcium bilirubinate (Maki, 1966). This enzyme was first isolated in 1934 from bovine kidney by Masamune at Tohoku University during the studies of this author on carbohydrates metabolism. In 1966 Maki reported intense betaglucuronidase activity at pH 7 in bile infected with E.coli. This author reported studies from Miyake (1900) and Westphal et al. (1931) who found calcium bilirubinate concrements in dogs after prolonged bile stasis and E.coli injection. To reproduce this finding and recalling the experiment of Bacmeister (1908) for cholesterol stones, Maki

stored bile in three test tubes: normal bile in the first, bile from patients with calcium bilirubinate stones in the second and bile with added betaglucuronidase in the third. Biliary concrements were produced in the latter two and the author concluded that bacterial betaglucuronidase would play a central role in calcium bilirubinate stone formation. In addition the author added high molecular weight substances or poly-electrolytes or applied physical movement to the system to enhance coagulation.

In the following years many papers were published supporting or refusing this hypothesis. While Trotman et al. (1974) found no unconjugated bilirubin in bile of patients with pigment gallstones, others (Soloway et al. 1977; Boonyapisit et al. 1978) reported higher hydrolysis of bilirubin in bile of patients with pigment gallstones.

Some authors do not relate the hydrolysis of conjugated bilirubin to the presence of bacteria in bile, but suggest the role of a betaglucuronidase of non-bacterial, tissue origin Soloway et al., 1977; Boonyapisit et al., 1978; Masuda et al., 1979; Trotman et al., 1982).

Factors inhibiting betaglucuronidase activity have also been taken into account. Maki (1966) reported studies of Matsushiro on the inhibiting effect of substances such as glucuronic acid, copper ions, glucaro 1-4 lactone. The increased dietary consumption of such compounds would remarkably reduce betaglucuronidase activity. In 1977 Matsushiro reported that glucaro 1-4 lactone is produced by the liver as a result of glucaric acid metabolism and that while other betaglucuronidase inhibitors, such as Cu++ or glucuronic acid, have no relevant effect, oral administration of glucaro 1-4 lactone reduces the activity of betaglucuronidase in bile. He concluded that increased consumption of this substance resulting from the adoption of a high protein, high fat "western" diet, together with decreased parasitic infestation, has played an important role in decreasing the incidence of pigment stones in Japan.

The role of a phospholipase of pancreatic or bacterial origin has been postulated in the process of pigment gallstone formation. In normal bile phospholipids, namely lecithin, are kept in suspension with cholesterol and bile acids. The common finding of calcium palmitate in the composition of calcium pigment stones (Sutor & Wooley, 1971; Trotman et al., 1982) from GB and CBD has suggested the possible hydrolysis of lecithin to lysolecithin, with formation of palmitic acid and subsequent precipitation of calcium palmitate.

Goswami & Frey (1974) studied the role of phospholipase A in catalyzing the conversion of lecithin to lysolecithin and fatty acids. Thirty out of 40 patients had no bacteria in bile, while 10/40 had. No lysolecithin was detected and this suggested the absence of phospholipase A in acute or chronic cholecystitis. This led the authors to conclude that phospholipase A does not play a role in gallstone formation. In this respect it has been suggested that phospholipase activity in human GB might be restricted into membrane-limited elements such as lysosomes: under pathologic conditions the enzyme, released by lysosomes, may catalyze the reaction to lysolecithin (Sjodal et al., 1978).

Pancreatic reflux into CBD is known to occurr both in animals and in man (Anderson et al., 1979) and both pancreatic amylase and lipase are present in bile in concentrations higher than in serum. Another proposed explanation of such low phospholipase activity is the temporary closure of the sphincter of Oddi: the enzyme levels are intermittently high when multiple measurements are reported. It has also been suggested that phospholipase A, by decreasing phospholipids in bile, indirectly enhances cholesterol supersaturation, favouring precipitation and cholesterol gallstone formation (Anderson et al., 1979).

In cholesterol gallstone formation cholesterol supersaturation may occur in bile due to increased cholesterol or decreased bile acids. It is unclear, however, whether changes in bile acid metabolism take place also in pigment gallstone formation. Almond et al. (1973)

studied ten patients before and after cholecystectomy and found no differences in bile acid pool. However bile acid pool was markedly reduced in patients with cholesterol gallstones. Soloway et al. (1977) reported a lower total bile acid concentration in gallbladder bile in the presence of stones. Cohn et al. (1979) performed sphincteroplasty in six adult mongrel dogs and found that after operation the concentration of phospholipids and bile acids decreased in GB bile and increased in CBD bile, while cholesterol concentration was unchanged in GB bile and decreased in CBD bile. This demonstrates a trend towards an increased bile lithogenicity in GB bile and decreased bile lithogenicity in CBD bile.

6.1.5 CBD stones

In background section it has been noted that while Aschoff (1924) classified biliary stones according to their origin, more recently Trotman & Soloway (1982) classified biliary stones according to their composition (Table 6.1). This classification divides stones into cholesterol and pigment stones, the latter being in turn divided in black and brown pigment stones. Patients with these three different types of stones differ markedly in geographical, social, dietary characteristics as well as in sex, age, association with infection and parasitic infestation.

Miyake (1913) noticed that pigment stones were much more frequent in Japan than in the western world. Maki (1961) underlined that most gallstones collected in western areas were cholesterol stones, whereas stones collected in Japan were calcium pigment stones; according to this author the incidence of calcium pigment stones was at that time 60-80% including those of parasitic origin. Furthermore while the majority of stones in western areas originated in the gallbladder, in Japan at that time the majority of gallstones originated in the bile ducts. Miyake & Johnston (1968) reported that stones from the USA contained over 70% of cholesterol, while stones from Hong Kong contained less than 20%.

Stones from Japan are in the middle of this range. More recently Trotman & Soloway (1982) confirmed that while cholesterol and black pigment stones are more frequent in western areas, brown pigment stones are found more commonly in the Far East.

Maki (1961) pointed out the importance of social class. He observed that pigment stones are more frequent in laboring group and cholesterol stones in the professional group; moreover, calcium pigment stones are usually found in people from rural districts rather than from urban areas (Maki, 1966; Miyake & Johnston, 1968; Soloway et al., 1977).

It has been emphasized that pigment stones arise more frequently in people eating a low protein, low fat, high fibre diet, whereas cholesterol stones are usually reported in populations using high protein, high fat diet (Maki, 1966). Calcium pigment stones were reported in Japan more frequently before or soon after World War II when a low protein, low fat, high fibre diet was commonly used in that country, especially in people of suburban areas and /or low social status, than in recent years, when their incidence has decreased and that of cholesterol stones has increased in parallel with the adoption of a western high fat, high protein diet (Miyake & Johnston, 1968; Matsushiro et al., 1977; Trotman & Soloway, 1982).

Calcium pigment stones have been described in association with parasitic infestation (Maki, 1966). Parasite infestation of over 70% was reported in Japan soon after World War II (15% in large cities and 30-69% in rural districts, Maki, 1961). Similar rates have been reported from Vietnam (Tung et al., 1969). Such high incidences are decreasing in recent years with the changes of social and dietary habits in Japan (Matsushiro et al., 1977). It has already been noted that parasites may act as a nidus for stone precipitation in the process of calcium pigment stones formation (Sato, 1955) and that may induce papillary fibrosis and bile stasis (Maki, 1961).

Cholesterol and black pigment stones in patients from western countries are reported up to six times more frequently in females than in males, whereas calcium bilirubinate stones in Japan have an equal sex incidence. Similarly, Sutor & Wooley (1971) in their study of composition of stones of patients from eight countries report an incidence of cholesterol stones significantly higher amongst females and an incidence of calcium palmitate stones significantly higher amongst males.

Cholesterol stones are usually reported in people with age over 30 years, even in patients from Japan, and calcium bilirubinate stones in patients under 25 (Maki, 1961). By contrast, Sutor & Wooley (1971) reported calcium bilirubinate stones more commonly in males in aged male patients.

Brown pigment stones are usually associated with infection. Maki reported in 1961 a series in which bacterial colonization of bile was present in 44% of patients with cholesterol stones and in 88% of calcium bilirubinate stones and five years later (Maki 1966) a group of patients with calcium bilirubinate stones whose bile was always infected with E.coli. Interestingly Goodhart et al. (1978) report an incidence of 1/26 infected bile in cholesterol stone and 1/9 infected biles in pigment stone amongst 35 patients undergoing cholecystectomy for gallstone disease and conclude that pigment stone disease in America is not associated with bacterial infection as in Japan. Tabata et al. (1981) found a lower incidence of bacterial colonization in cholesterol and black pigment stones, and a higher bacterial infection rate in brown pigment stones. Furthermore these authors found high bacterial infection rates in the presence of CBD stones, irrespective of stone composition and concluded that in the presence of cholesterol or black pigment CBD stones bacterial colonization was a secondary phenomenon, whereas in the presence of brown pigment stones bacterial colonization was a primary phenomenon. These authors also underlined that brown pigment stones were always associated with cholangitis or previous biliary surgery and followed by a high postoperative infective complication rate.

Aschoff's classification of biliary stones (1924) identifies the so called stasis stone as the only primary or true "recurrent" CBD stone. The morphological characteristics of this stone (Table 6.1) closely resemble those of brown pigment stones later described by Maki (1961) and classified by Trotman & Soloway (1982). The presence of this stone is related to bile stasis, to infection, to particular dietary habits typical of Far East areas especially until some decades ago and to biliary parasites. It is interesting to note that Aschoff, the German pathologist, who had the opportunity of carrying out autopsy observations on European subjects, found calcium bilirubinate (or brown pigment) stones only as primary CBD stones since the conditions of bile stasis and infection are common in this area almost exclusively in the CBD. By contrast in the Far East this type of stone is found in the GB as well as in the CBD since bile stasis and sepsis may arise also in GB, probably in connection with parasitic infestation. In this respect Maki (1961) interestingly reports that when in 1913 Aschoff visited Professor Miyake's clinic at Kyushu University in Japan, he was particularly impressed to observe the typical pigmented calcium as stones in the GB. Professor Miyake later sent him a few such stones as a souvenir !!

An other aspect that still remains unclear about primary CBD stones is their composition. Indeed these stones have been reported as calcium bilirubinate or calcium palmitate stones, calcium pigment stones, "earthy" or stasis stones. Sutor & Wooley (1971) studied the stone composition of 331 patients from eight countries and found cholesterol in 71% of stones, calcium palmitate in 36% and calcium carbonates such as calcite, vaterite and aragonite in 15%. In this paper calcium palmitate was found in stones coming from Kuwait and from South Africa and was reported for the first time from outside Japan.

Trotman & Soloway, (1982) underlined that brown pigment stones are composed of calcium salts of bilirubin calcium bilirubinate and calcium palmitate and minor amounts of cholesterol in a glycoprotein matrix, whereas black pigment stones are composed of calcium bilirubinate in a polymerized form and calcium phosphates in a glycoprotein

matrix. No calcium palmitate is found in these stones. Hence we may argue that the presence of calcium palmitate is the main difference in composition in these two different types of calcium pigment stones.

Other clinical associations of pigment stones have also been reported. Maki (1961) found CBD dilatation more frequently in patients with calcium pigment than in cholesterol stones, even in patients with GB stones. This author also underlines that jaundice is reported in 20-50% of western series and in 50-70% in japanese patients. By contrast silent stones are more frequent in western series, being reported in 5-39% patients with cholesterol stones and in no patient with pigment stones. Soloway et al., (1977) report that pigment stones in the USA are associated in some patients with cirrhosis, malaria and haemolytic anaemia, and in others with none of these associations but with parasites and stasis. Trotman & Soloway (1982) reported the association of black pigment stones with haemolysis and cirrhosis and a concomitant decrease in bile salts, and of brown pigment stones with stasis, infection and increased activity of betaglucuronidase of bacterial or tissue origin.

6.1.6 Aims of the Clinical Study.

In order to address in a clinical situation the questions mentioned above a population of patients attending the XVI Divisione Ospedale Cardarelli, Naples, Italy, in 1983 and 1984 has been used. This population of patients has been a most valuable one for the purposes of this study, since in common with many patients from Southern Europe and the Mediterranean area, most of these patients had a long history of biliary disease, allowing us to study the long-term effects of the presence of stones on the microbiology and biochemistry of bile.

In this group of patients we recorded clinical data and, at operation, CBD size. Bile was sampled for microbiology and biochemical assays of conjugated and unconjugated

bilirubin, total bile acid levels, betaglucuronidase and phospholipase activities, as in the experimental study on dogs. In addition, common duct stones of 15 patients which were macroscopically classified at operation according to the Aschoff criteria, were analyzed with X-ray diffractometry.

6.2 METHODS (TABLES 6.2, 6.3)

6.2.1 Population of patients

One hundred and seventy four patients undergoing elective biliary surgery in the XVI Divisione Ospedale Cardarelli, Naples, Italy, between February 1983 and January 1985 were studied prospectively.

6.2.2 Antibiotics

It is important to explain the reasons behind the selection of patients for use of antibiotic prophylaxis in this study, because the situation is a rather unusual one for current western practice. Furthermore, the observation that amongst the patients who did not receive antibiotic prophylaxis there was a significantly higher number of patients at risk of developing infective complications (Table 6.7), makes the problem still more unclear.

The study contains two subgroups of patients. The first group (from February to September 1983) was treated according to the general antibiotic policy in the surgical department of Ospedale Cardarelli at that time. In 1983 it was the usual policy for most general surgical operations in that hospital not to start any antibiotic administration before surgery, but to start "prophylaxis" immediately after the operation. When I started the collection of data for the study on the pathogenesis of primary CBD stones (February 1983) I elected to include in the study patients whose clinical characteristics suggested the possible presence of CBD stones and these patients were therefore managed using this general "no-antibiotic prophylaxis" policy. This group therefore contains a number of "higher risk" patients who did not receive any preoperative antibiotics. In October 1983, a randomized prospective trial of antibiotic prophylaxis was undertaken. The decision of the

hospital was to perform a trial of prophylaxis in patients at "low risk" of infection. 104 pts were enterd into this trial between October 1983 and January 1985, 57 receiving Piperacillin (2gr) and 47 receiving Tobramycin (100 mg). I collected data from patients in this antibiotic prophylaxis trial in uncomplicated biliary surgery and also from patients at "high risk" of CBD stones, who continued to be treated without pre-operative antibiotics. The total number of patients in this group was 70 (Table 6.2).

Since patients at risk of carrying CBD stones (those falling into the no-antibiotic prophylaxis group of patients) are often also jaundiced or with a history of cholangitis, and therefore are also at risk of developing infection following biliary surgery, I have paradoxically recorded a higher number of high risk patients in the no-antibiotic group. Conversely, because the antibiotic prophylaxis trial related to uncomplicated biliary surgery, most patients in the antibiotic subgroup were in fact at low risk of developing infective complications.

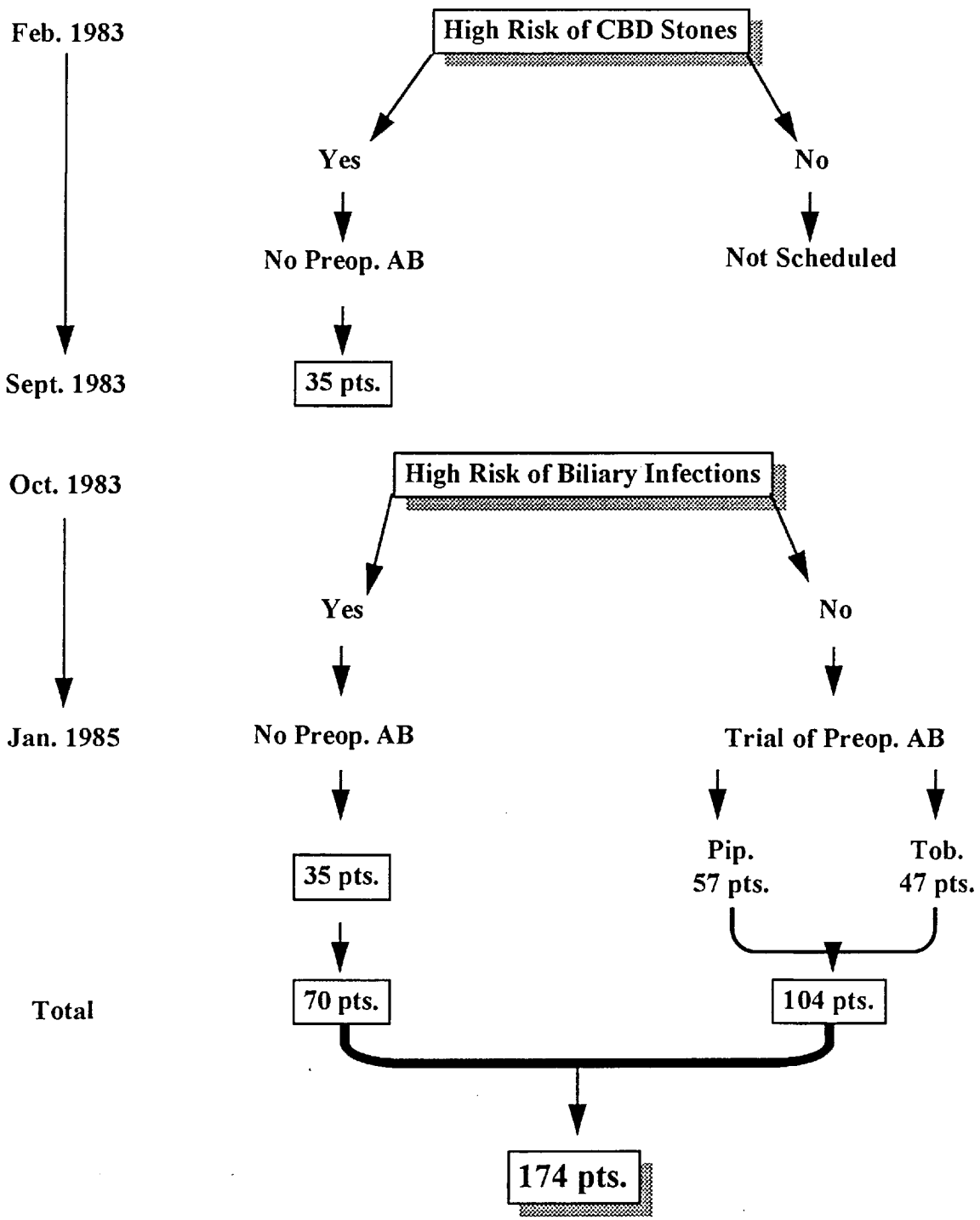
This paradoxical and somewhat anomalous situation must be clearly recognized in order to understand some of the unusual data presented here, but it must also be clear that the author does not assume respopnsability for, or endorse the clinical practices in use at that time in the hospital where patients were available for study.

6.2.3 Clinical features

On admission, patients underwent routine clinical examination. The duration of the disease was defined by a careful clinical history, with particular attention to the known presence of gallstones, determined on the basis of positive oral cholecystogram or on

Table 6.2

Antibiotics Selection



the presence of episodes of cholangitis with the characteristic Charcot's triad. The presence of risk factors conventionally recognized as capable of predicting infection (age over 70 years, obstructive jaundice, history of cholangitis, previous biliary operations, known stones in the common bile duct) was also recorded (Keighley et al., 1976/b).

Stones in the GB were detected with oral cholecystogram and/or ultrasound scanning. Intravenous cholangiography was carried out in every patient whose liver function tests did not show biochemical features of biliary obstruction. In the presence of suspected biliary obstruction direct cholangiography (percutaneous or retrograde) was performed.

All clinical and operative data, as well as laboratory results, were recorded preoperatively on a standard proforma (Table 13.4, appendix 13.2). Data were subsequently stored and analysed on an IBM-compatible personal computer, using the dBaseIII+ data-base.

6.2.4 Surgical approach

All operations were carried out by nine surgeons, all members of the Professor Trapani's surgical team.

Patients with gallbladder stones were treated by cholecystectomy, which was carried out by a standardized technique: the hilus of the gallbladder was first dissected, identifying the cystic duct, ligating and dividing the cystic artery, and removing the gallbladder from the gallbladder fossa, starting from the fundus.

Operative cholangiography was performed in selected cases: criteria for selection were a history of jaundice, cholangitis or previous biliary surgery or a preoperative intravenous cholangiogram demonstrating a filling defect, CBD dilation or delayed emptying of the CBD.

CBD exploration was always carried out in a supraduodenal manner. The CBD was explored through the cystic duct or after it was opened, depending upon the circumstances. The incision in the CBD wall was performed in a transverse direction in the presence of a dilated CBD. Otherwise, when a normal sized CBD was present, the opening was carried out longitudinally. When CBD stones were found at duct exploration, a choledochoduodenostomy or external biliary drainage via T-tube was carried out. Only in three cases a papillosphincterotomy was added to cholecystectomy and in two of these external biliary drainage via T-tube was also carried out. Choledochoduodenostomy was performed when the external size of the CBD was 18 mm or greater or in the presence of a "primary" stone according to Aschoff's criteria (1924). Otherwise T-tubes were placed and left in place for 12-13 days.

Hepaticojejunostomy Roux-en-Y was carried out according to the technique used at Hammersmith Hospital Hepatobiliary Unit (Blumgart & Kelly, 1984).

An intraperitoneal tube drain was used in all cases, placed in the subhepatic space, behind the hepatoduodenal ligament. When a Roux-en-Y loop was prepared, an additional tube drain was also positioned close to the jejuno-jejunal anastomosis. Tubes were always brought outside through a separate incision.

6.2.5 Postoperative manegement

Postoperative recovery was conducted in a routine fashion: until bowel function was re-established patients were supported with glucose and saline solutions. In patients undergoing complex biliary procedures amino-acids, lipids, fresh plasma and blood transfusions were also given when indicated. Nasogastric tubes were used routinely in patients with a bilio-enteric anastomosis or those who had a bile duct exploration, until bowel function was re-established. In patients undergoing only cholecystectomy, a nasogastric tube was positioned only in cases of transitory delayed gastric emptying. Urinary bladder catheters were used only in patients undergoing complex procedures, or in patients with specific urinary problems or those considered at risk of developing postoperative renal failure.

Free intraperitoneal drains were kept under swab coverage. Such coverage was changed every 2 days, after gentle aspiration from the tube with a 20 ml syringe. When there was active drainage the tubes were connected to a collection bag which was changed daily. Following cholecystectomies, tubes were removed on the fifth postoperative day. In patients undergoing external T-tube drainage, the intraperitoneal drains were removed one day after removal of the T-tube. In patients treated with bilio-enteric anastomosis tubes were removed on the 9th postoperative day.

6.2.6 T-tubes

T-tubes were connected to a collection bag for five days postoperatively, allowing free external biliary drainage. The collection bag was then progressively elevated in the following days and the tube was removed after 12-13 days. T-tube cholangiography was always performed before removal.

6.2.7 Sampling

CBD size was measured by a metal caliper at operation at the level of the cystic duct junction.

Bile was collected under aseptic conditions from the gallbladder when present, usually after removal, and from the CBD whenever possible. CBD bile was collected via the cholangiography cannula through the cystic duct, before performing cholangiography, when there was no need for surgical exploration of the duct. Otherwise, it was aspirated by direct puncture of the duct.

Ductal stones were removed with a Dormia basket catheter (Burhenne, 1988) or with a biliary Fogarty balloon catheter (Fogarty et al., 1968) and collected in a sterile container.

On the fifth postoperative day, a clinical assessment for the evidence of infection was carried out by the author: wounds were inspected and classified according to a grading ranging from zero (wound perfectly healed) to five (large purulent collection with impairment of general status) (Table 6.3). The development of evident infection was also followed through the hospital stay or at follow up. Patients were considered to have distant infections if they developed pneumonia, bacteraemia, or urinary tract infections. The presence of fever over 38 C. was also recorded.

Table 6.3

Clinical wound infection grading

0. Wound perfectly healed.
1. Wound slightly reddened.
2. Wound markedly reddened; infiltration.
3. Little serosal discharge.
4. Discharge of purulent material.
5. Large purulent collection with impairment of general status.

A specimen from the swabs covering the wounds was also taken, irrespective of the clinical status of the wound, as well as from fluids aspirated from intraperitoneal tube drains for aerobic and anaerobic cultures. In patients with T-tubes, bile was sampled every two days in the postoperative period.

6.2.8 Handling the samples

The minimum amount of bile needed to perform the requested assays ranged from three to four ml. However it was not always possible to sample sufficient material for all studies. Usually 0.5 to 1 ml, depending on the circumstances, was used for microbiology, the remainider being used for biochemistry.

Roughly 50% of the bile used for microbiology was put in a vacuum container for anaerobic culture, the remainder being left in the syringe for aerobic processing. The specimens were transported rapidly to the microbiology laboratory, in the Istituto di Microbiologia della Universita' di Napoli, II Facolta' di Medicina.

Bile for biochemical assays was immediately frozen at -20 C. Specimens were transferred in batches to the laboratories of the Royal Postgraduate Medical School in London. For the transfer large polystyrene boxes were used, with the test tubes totally encircled by dry ice,

which completely filled the box. The overall transfer time never exceeded 12 hours and all samples remained frozen on arrival, before transfer to a -20 C freezer.

Stones were collected at operation from the CBD whenever possible. They were stored in a large syringe or in a plastic box. In cases in which stone composition analysis was undertaken by X-ray diffractometry stones were transferred to the Istituto di Mineralogia, Facolta' di Scienze, Universita' di Napoli, Italy.

6.2.9 Laboratory methods

* Microbiology

A detailed account of methods used for bile and postoperative cultures in this study is reported in Appendix 13.1, paragraph 13.1.3.

* Biochemistry

The measurement of conjugated and unconjugated bilirubin, total bile acid levels, betaglucuronidase and phospholipase activities in bile were carried out in the laboratories of the Department of Surgery at the Royal Postgraduate Medical School. The methods are described in detail in Appendix 13.1, paragraph 13.1.3. In this clinical study phospholipase C was assayed in all samples, while in the experimental study the same fraction was assayed in all samples tested before September 1988 and phospholipase A thereafter. In the histochemistry study phospholipase A was assayed in all cases. The reason for this change in the methods is explained in the discussion of the experimental study.

* X-ray diffractometry

This is a rapid, non-destructive method for the identification of crystalline substances. Amorphous material cannot be identified by this method. X-rays are passed through a sample of a crystalline compound and the diffraction pattern is recorded on a photographic film placed a few centimetres away. Stones were washed in distilled water and vacuum dried. They were then split and material was sampled from inside and powdered. When a layered structure was shown, the analysis was performed layerwise. Powders were examined by a Philips infractometer at a wave length of Cu K alpha 1,54178. The diffraction patterns were read against standards. Cholesterol monohydrate was read against the standard published by Bogren & Larsson (1963). Anhydrous Cholesterol was reported in the ASTM (American Society for Testing Materials) powders diffraction file. Calcite was compared with standards published by Sutor & Scheidt (1968), whereas Cholesterol II and calcium palmitate were calculated from Sutor & Wooley (1971).

6.3. RESULTS

6.3.1 Population of patients

One hundred and seventy four patients are considered in this section of the work, consisting of the subgroups defined in Table 6.2. Because the presence of antibiotics might affect the microbiology of the bile, the characteristics of the antibiotic and no-antibiotic groups will be detailed separately hereafter whenever this difference is relevant.

The mean age was 50 years (range 18-81). Thirty seven patients were male, with a mean age of 52 (28-76) years and 137 female, with a mean age of 50 (18-81) years. No statistical differences were found in sex and age distribution (Table 6.4).

The diagnoses are shown in Table 6.5. One hundred and thirteen patients were admitted for gallbladder stones only, whereas 12 had stones in their common bile duct and 26 in the common bile duct as well as in gallbladder. Four patients were admitted for benign stenosis of the extrahepatic biliary tract, which was in fact due to papillary stenosis in three cases (two of them following cholecystectomy) and to a postoperative iatrogenic stricture of the common bile duct associated with stones above the stricture in one case.

Fifteen patients had a neoplastic lesion: four had a tumour in the gallbladder, four in the pancreas, one in the ampulla of Vater and six in the common bile duct or at the confluence of the hepatic ducts. In addition, four of the neoplastic patients had stones in their gallbladder and two also in the common bile duct. One patient presented with sclerosing cholangitis and three patients with biliary fistula (one external, one bilio-gastric and one bilio-duodenal) all associated with biliary stones. There was a higher proportion of patients with gallbladder stones in the antibiotic group, while patients with CBD stones

were significantly more frequent in the no antibiotic group. The reason for this distribution is discussed above, in the methods section.

The procedures performed are listed in Table 6.6. One hundred and two patients underwent cholecystectomy and cholecystectomy was combined with external biliary drainage via a T-tube in 31 patients, and with a choledochoduodenostomy in 19 patients. Three patients underwent only external biliary T-tube drainage. One patient had a choledochoduodenostomy and a T tube insertion and seven had choledochoduodenostomy alone. Three patients underwent papillosphincterotomy, in one case associated with cholecystectomy and in two with cholecystectomy plus T-tube drainage. In one patient cholecystectomy was associated with the excision of an external fistulous track and in another with choledochoduodenostomy plus the closure of a cholecysto-duodenal fistula. In three patients another form of biliary bypass was carried out: one had an hepaticoduodenostomy, another received an hepaticojejunostomy at the confluence of the hepatic ducts and a third was treated with hepaticojejunostomy to the segment III duct. Finally two patients had a local resection of hilar cholangiocarcinoma, one associated with cholecystectomy, and one had a Whipple operation. As previously noted simple cholecystectomy was significantly more frequent in the antibiotic group, while T-tube drainage and choledochoduodenostomy when associated with cholecystectomy was significantly more frequent in the no-antibiotic group.

The presence of conventional risk factors for infective complications was assessed in every patient. Eighty five patients had one or more risk factors, whereas 89 had none (Table 6.7). In addition a known history of gallstone disease longer than three years was recorded in 97 patients.

Table 6.4

Population of Patients

groups (n)		n (174)	antibiotic (104)	No-antibiotic (70)
AGE	Mean	50	49	52
	(range)	(18-81)	(18-81)	(19-80)
SEX	M(mean age)	37 (52)	19 (49)	18 (56)
	F(mean age)	137(50)	85 (49)	52 (51)

Table 6.5

Diagnoses

groups	n	antibiotic	no-antibiotic
GB stones	113	84**	29
CBD stones	12	3*	9
GB & CBD stones	26	5**	21
Benign strictures	4	1	3
Sclerosing cholangitis	1	0	1
Biliary fistulae	3	3	0
GB tumours	4	3	1
Pancreas tumours	4	0	4
Ampullary tumours	1	1	0
CBD/hilar tumours	6	4	2
Total	174	104	70

** $P < 0.001$ Chi Square test
* $P < 0.05$ Chi Square test

Table 6.6

Procedures

groups	n	antibiotic	no-antibiotic
Cholecystectomy alone	102	82**	20
Cholecystectomy + Ttube	31	7**	24
Cholecystectomy + CDS	19	5*	14
Cholecystectomy + CDS+fist.	1	1	0
Cholecystectomy + fist.	1	1	0
T tube		3	1 2
CDS		7	3 4
CDS + T tube		1	0 1
PST + cholecystectomy	1	0	1
PST + cholecystectomy + Ttube	2	1	1
Bypass (other than CDS)	3	2	1
Local resection of HCC	2	0	2
Whipple operation	1	1	0
Total	174	104	70

** $P < 0.001$ Chi Square test
* $P = 0.004$ Chi Square test

Key: CDS = choledochoduodenostomy; HCC =hilar cholangiocarcinoma
PST = papillosphincterotomy

Table 6.7

Risk Factors

groups (n)	n (174)	antibiotic (104)	no antibiotic (70)
No risk factors	89	69**	20
age >70	14	6	8
jaundice	42	15	27
cholangitis	41	22	19
Previous surgery	19	7	12
CBD stones	43	11	32
One or more conventional R.F.	85	35**	50
History > 3 yrs	97	65*	32

** $P < 0.001$ Chi Square test
* $P < 0.05$ Chi Square test

6.3.2 Microbiology

* Bile cultures (Tables 6.8 - 6.15)

Bile was collected from every patient. The results of bile cultures and the organisms isolated are displayed in Table 6.8. In the antibiotic group positive bile cultures were recorded in 27 out of 104 patients (26%). Six of these (5.8%) had polymicrobial growth E.coli, Klebsiella, Pseudomonas, Enterobacter, and Proteus amongst the aerobes and S. intermedius and bacteroides amongst the anaerobes were the organisms most frequently isolated in this group.

In the no-antibiotic group bacteria were cultured in 31/70 patients (44.3%), while 11/70 (15.7%) presented more than one organism in bile. E. coli was the predominant organism, followed by other enterobacteria such as Klebsiella, Pseudomonas, S. faecalis, E. cloacae

and S. typhi. Anaerobes were isolated in 16.1% of cases. S.Intermedius was the anaerobic species most commonly isolated.

Table 6.8

Bacterial Isolates from Bile

	antibiotic n=104		no-antibiotic n=70	
	n	(% of +ve cultures)	n(%	of +ve cultures)
+ve cultures	27	(26% of total)	31	(44.3% of total)
Polymicrobial growth	6	(22.2)	11	(35.5)
E. coli	8	(29.6)	13	(41.9)
Klebsiella	5	(18.5)	7	(22.6)
Pseudomonas	5	(18.5)	4	(12.9)
Enterobacter	4	(14.8)		
Proteus	3	(11.1)		
Acromonas Hydrofila	2	(7.4)		
S. faecalis	1	(3.7)	3	(9.7)
Diphteroides	1	(3.7)		
Flavobacterium	1	(3.7)		
E. Cloacae			1	(3.2)
S. Typhi			1	(3.2)
Anaerobes	2	(7.4)	5	(16.1)
S. intermedius	1	(3.7)	3	(9.7)
Bacteroides	1	(3.7)		
P. magnus			2	(6.4)
C. perfrigens			1	(3.2)

The finding of positive bile cultures according to the presence or absence of pre-operative risk factors is shown in Table 6.9. In the antibiotic group positive bile cultures were recorded in 15/35 patients with risk factors (42.8%) and in 12/69 patients without (17.4%, P=0.01). Multiple isolates were found in similar proportions in high risk (2/35, 5.7%) and low risk patients (4/69, 5.8%). In the no-antibiotic group 28/50 patients (56%) with one or

more risk factors had infected bile, with more than one organism in 9 cases (18%). By contrast, amongst patients with no risk factors only three out of 20 (15%) patients had bacteria in bile ($P<0.05$) and two out of 20 (10%) polymicrobial bacterial growth.

Table 6.9

Bile Cultures and Risk Factors

		antibiotic (104 Pts)		no-antibiotic (70 Pts)	
+ve cultures	RF	15/35	(42.8%) **	28/50	(56%) *
	No RF	12/69	(17.4%)	3/20	(15%)
polymicrobial growth	RF	2/35	(5.7%)	9/50	(18%)
	No RF	4/69	(5.8%)	2/20	(10%)

** P = 0.01 Chi Square test
* P < 0.05 Chi Square test

We have examined the effects of a history > 3 years on the biliary infection rate, in patients with or without conventional risk factors (Tables 6.10, 6.11). There were 95 such patients in the antibiotic group. This analysis was restricted to those with gallstone disease. Table 6.10 demonstrates that bile culture rates were statistically higher in presence of conventional risk factors in gallstones patients. The presence of a long clinical history of gallstone disease was also associated with higher infection rates, though this was not statistically significant. However we note that no polymicrobial bacterial growth in bile was recorded in presence of a clinical history shorter than three years.

In the no-antibiotic group with gallstone disease (60 patients), conventionally risk factors were associated with significantly higher bile infection rates, while a history longer than three years showed no association with the presence of bacteria in bile, either considered as

an independent factor or when considered together with conventional risk factors (Table 6.11).

Table 6.10

Clinical History over 3 years and Bile Infection in 95 patients with Gallstone Disease (antibiotic group)

	Bile inf.		polymicrobial	
	n	(%)	n	(%)
Conventional RF	11/27	(40.7)*	1/27	(3.7)
Hist.>3yrs	10/23	(43.5)	1/23	(4.3)
Hist.<3yrs	1/4	(25)	0/4	
NoConventional RF	11/68	(16.2)*	3/68	(4.4)
Hist.>3yrs	8/39	(20.5)	3/39	(7.7)
Hist.<3yrs	3/29	(10.3)	0/29	

* P = 0.02 Chi Square test

Table 6.11

Clinical History over 3 years and Bile Infection in 60 patients with Gallstone Disease (no-antibiotic group)

	+ve cultures		polymicrobial	
	n	(%)	n	(%)
Conventional RF	24/40	(60)*	8/40	(20)
Hist.>3yrs	14/20	(70)	5/20	(25)
Hist.<3yrs	10/20	(50)	3/20	(15)
No conventional RF	3/20	(15)*	2/20	(10)
Hist.>3yrs	1/9	(11.1)	0/9	
Hist.<3yrs	2/11	(18.2)	2/11	(18.2)

* P = 0.001 Chi Square test

In the antibiotic group, positive bile cultures were found in 17/84 (20.2%) patients with GB stones, whereas bacteria were found in bile of 4/8 (50%) patients with ductal stones, and 5/8 (62.5%) patients with tumours. Table 6.12 shows the detailed figures.

In the no-antibiotic group positive bile cultures were recorded in 1/7 (14.3%) patients with tumours, 8/29 (27.6%) patients with gallbladder stones, 19/30 (63.3%) patients with common bile duct stones and 3/3 (100%) patients with benign biliary strictures. Multiple isolates had a similar pattern of incidence (Table 6.13).

Table 6.12

Bile Cultures and Diagnosis
antibiotic group: n=104

Diagnosis		patients	+ve cultures	polymicrbial	
	n	n	(%)	n	(%)
GB stones	84	17	(20.2)*	3	(3.6)
CBD stones	8	4	(50)	1	(12.5)
CBD	3	2	(66.7)	1	(33.3)
GB + CBD	5	2	(40)	0	
Benign strictures	1	0		0	
Biliary fistula	3	1	(33.3)	0	
Tumours 8	5		(62.5)* 2		(25)
GB	3	\ 2	(66.7)	1	(33.3)
Ampulla of Vater	1	0		0	
CBD + HCC	4	\\ 3	(75)	1	(25)

* P = 0.008 Chi Square test
\ Both also had GB stones
\\ 2 had previous bilio-enteric anastomosis

Table 6.13

Bile Cultures and Diagnosis
no-antibiotic group: n=70

Diagnosis	patients	+ve cultures		polymicrobial	
	n	n	(%)	n	(%)
GB Stones	29	8	(27.6)*	4	(13.8)
CBD Stones	30	19	(63.3)*	6	(20)
CBD	9	6	(66.7)	2	(22.2)
GB + CBD	21	13	(61.9)	4	(19)
Benign Strictures	3	3	(100)*	1	(33.3)
Sclerosing Cholangitis	1	0		0	
Tumours 7	1		(14.3) 0		
GB	1	0		0	
Pancreas	4	0		0	
CBD and HCC	2	1	(50)	0	

* P = 0.022 Chi Square test

Bile duct diameter was recorded in 101 patients in the antibiotic group. In 89 patients with stones the median bile duct size was 10 mm (range 6-20). In the presence of sterile bile, the median bile duct size was 10 mm (range 10-18) and in the presence of infected bile was 10 (range 6-20). CBD dilatation was more common in patients with polymicrobial bacterial growth in bile (Table 6.14).

Bile duct diameter was also measured in 57 patients affected by gallstone disease in the no-antibiotic group. Sterile bile was associated with a median common bile duct size of 10.5 mm (range 9-25), whereas in presence of infected bile the median size was 15 mm (range 10-28) and in presence of more than one organism 16.5 mm (10-28); the difference in CBD size values between patients with sterile or infected bile was statistically significant (P = 0.007 Mann-Whitney U test). Moreover, bile duct dilatation was significantly associated with the presence of bacteria in bile (table 6.15).

Table 6.14

Bile Cultures and CBD size
(CBD size recorded from 89 patients of antibiotic group)

CBD Size (mm)	n	+ve cultures		polymicrobial	
		n	(%)	n	(%)
<= 12	77	9	(11.7)*	1	(1.3)
> 12	12	4	(33.3)*	1	(8.3)
13 - 18	11	3	(27.3)	0	
> 18	1	1	(100)	1	(100)

* P = 0.049 Chi Square test; P = 0.070 Fisher's exact test

Table 6.15

Bile cultures and CBD size
no-antibiotic group: 57 pts

CBD Size (mm)		+ve cultures		polymicrobial	
	n	n	(%)	n	(%)
<= 12	23	3	(13)*	1	(4.3)
> 12	34	18	(52.9)*	5	(14.7)
13 - 18	22	9	(40.1)	1	(4.5)
> 18	12	9	(75)	4	(33.3)

* P = 0.002 Chi Square test

* Postoperative infection (Tables 6.16 - 6.29)

Postoperative infection was assessed in 89 patients in the antibiotic group. One or more infections were recorded in 31 patients (34.8%). Eighteen patients (20.2%) presented wound infection, in 17 (19.1%) cases on clinical examination and as positive culture from the wound in one case (1.1%). Bacteria were cultured in intraperitoneal drain fluids of nine patients (10.1%). Fourteen patients (14.7%) had distant infections: two had pneumonia, one a subphrenic collection, one had urinary tract infection and 10 had postoperative fever of unexplained origin. These postoperative infection rates did not differ significantly between patients with positive or negative bile cultures at the time of surgery (Table 6.16). Four out of nine patients with positive post-operative cultures (44.4%) had the same organisms cultured from bile at the time of surgery. (E. coli 4, P. aeruginosa 2, E. cloacae 1 and S. aureus 1). Two patients were found to carry anaerobes postoperatively: S.intermedius in one case and P. magnus in an other.

In the no-antibiotic group postoperative infections were assessed in 21 patients. Eleven patients (52.4%) had one or more infections. Five patients had a wound infected at clinical (four) and/or microbiological (two) assessment. Five patients had positive cultures from intraperitoneal drain fluid. Organisms isolated postoperatively were E.coli, Klebsiella spp, Pseudomonas spp, S.faecalis, Proteus, S.typhi and S.aureus. Six paticnts presented postoperative fever.

Postoperative infection rate has shown a slight increase in patients with positive bile cultures at time of surgery (Table 6.17). Moreover in four out of six patients (66.6%) who had positive postoperative cultures, the same organism had been already cultured from bile.

Table 6.16

Bile Cultures and Postoperative Infections
antibiotic group: n=104
(postoperative infections assessed in 89 patients)

Infections (%)	n	any	wound	drain	distant
+ve bile cultures	23	7 (30.4)	3 (13)	4 (17.4)	5 (21.7)
-ve bile cultures	66	24 (36.4)	15 (22.7)	5 (7.8)	9 (13.6)

Table 6.17

Bile Cultures and Postoperative Infections
no-antibiotic group: n=70
(postoperative infections recorded in 21 patients)

Infections (%)	n	any	wound	drain	distant
+ve bile cultures	8	5 (62.5)	2 (25)	3 (37.5)	2 (25)
-ve bile cultures	13	6 (46.1)	3 (23.1)	2 (15.4)	4 (30.8)

In the antibiotic group the overall post-operative infection rate was also similar in patients with one or more conventional risk factors (36.4%) and in patients without (33.9%). There were more positive drain cultures in this group amongst high risk patients (P=0.002, Chi Square test; Table 6.18).

In the no-antibiotic group postoperative infections were present in 10/19 (52.6%) patients with one or more conventional risk factors and in 1/2 (50%) patients with no risk factor (Table 6.19).

Table 6.18

Risk Factors and Postoperative Infections
antibiotic group: n=104
(postoperative infections assessed in 89 patients)

Infections (%)	n	any	wound	drain	distant
one or more RF	33	12 (36.4)	7 (21.2)	8 (24.2)	4 (12.1)
No RF	56	19 (33.9)	11 (19.6)	1 (1.8)	10 (17.8)

* P = 0.002 Chi Square test

Table 6.19

Risk Factors and Postoperative Infections
no-antibiotic group n=70
(postoperative infections recorded in 21 patients)

Infections (%)	n	any	wound	drain	distant
One or more RF	19	10 (52.6)	5 (26.3)	5 (26.3)	5 (26.3)
No RF	2	1 (50)	0	0	1 (50)

Postoperative infections were also assessed separately in patients carrying gallstones. In the antibiotic group no difference was found in postoperative infection rate between patients with conventional risk factors for infection and patients without. However, in the subgroup of 55 patients without conventional risk factors, a clinical history of gallstone disease longer than three years was found to be significantly associated with a higher overall postoperative infection rate (Table 6.20).

In the no-antibiotic group conventional risk factors were not associated with a significant increase of post-operative infections, nor was a long history of gallstone disease either when associated with other conventional risk factors or alone. (Table 6.21).

Table 6.20

Risk Factors and Postoperative Infections in Gallstones Patients
antibiotic group: n=104
(postoperative infections assessed in 81 patients)

Infections (%)	n	any	wound	drain	distant
One or more RF	26	9 (34.6)	5 (19.2)	6 (23.1)**	3 (11.5)
Hist>3yrs	22	8 (36)	5 (22.7)	6 (27)	2 (9)
Hist<3yrs	4	1 (25)	0	0	1 (25)
No RF	55	18 (32.7)	10 (18.2)	1 (1.8)**	9 (16.3)
Hist>3yrs	33	15 (45)*	8(24)	1 (3)	8 (24)
Hist<3yrs	22	3 (14)*	2 (9)	0	1 (4)

** P = 0.006 Chi Square test
* P = 0.030 Chi Square test

Table 6.21

Risk Factors and Postoperative Infectionsin Gallstones Patients
no-antibiotic group n=70
(postoperative infections assessed in 19 patients)

Infections (%)	n	any	wound	drain	distant
One or more RF	17	9 (52.9)	4 (23.5)	5 (29.4)	5 (29.4)
Hist.>3yrs	7	5 (71.4)	3 (42.8)	3 (42.8)	3 (42.8)
Hist.<3yrs	10	4 (40)	1 (10)	2 (20)	2 (20)
No RF	2	1 (50)	0	0	1 (50)
Hist>3yrs	1	0	0	0	0
Hist<3yrs	1	1 (100)	0	0	1 (100)

Tables 6.22 to 6.25 show the postoperative infection rates in relation to diagnosis and to operative procedure. In neither the antibiotic nor the no-antibiotic group there was any difference in infection rates related to diagnosis or to the procedure undertaken.

Table 6.22

Postoperative Infections and Diagnosis
antibiotic group: n=104
(postoperative infections recorded in 89 patients)

Infections (%)	n	any	wound	drain	distant
GB stones	70	21 (30)	12 (17.1)	2 (2.8)	10 (14.2)
CBD stones	8	4 (50)	2 (25)	4 (50)	0
Benign str	1	1 (100)	1 (100)	0	0
Tumour	7	3 (42.8)	2 (28.5)	2 (28.5)	2 (28.5)

Table 6.23

Postoperative Infections and Diagnosis
no-antibiotic group: n=70
(postoperative infections recorded in 21 patients)

Infections (%)	n	any	wound	drain	distant
GB stones	8	3 (37.5)	1 (12.5)	1 (12.5)	3 (37.5)
CBD stones	10	7 (70)	3 (30)	4 (40)	3 (30)
Tumour	2*	1 (50)	1 (50)	0	0

* both patients treated with hepaticojejunostomy

Table 6.24

Postoperative Infections and Procedures
antibiotic group: n=104
(postoperative infections assessed in 89 patients)

Infections (%)	n	any	wound	drain	distant
Cholecystectomies	68	22 (32.3)	12 (17.6)	2 (2.9)	12 (17.6)
T-tube	8	2 (25)	2 (25)	1 (12.5)	0
CDS and other bypass	9	5 (55.5)	2 (22.2)	4 (44.4)	1 (11.1)
Whipple op.	1	0	0	0	0

Table 6.25

Postoperative Infections and Procedures
No Antibiotic group: n=70
(postoperative infections assessed in 21 patients)

Infections (%)	n	any	wound	drain	distant
Cholecystectomy	6	2 (33.3)	0	0	2 (33.3)
T-tube	10	5 (50)	3 (30)	2 (20)	4 (40)
CDS	2	2 (100)	1 (50)	2 (100)	0
local resection of Hil. CC	2	1 (50)	1 (50)	0	0

Finally, in patients with gallstone disease, postoperative infections have been examined in relation to CBD size and to the presence of stones in the common bile duct. In the the antibiotic group the overall postoperative infection rate increased significantly in the presence of a dilated common bile duct, (Table 6.26). The postoperative positive drain culture rate was also significantly higher in patients with stones in the common bile duct (Table 6.28). Similar trends were found in the no- antibiotic group though without statistical significance (Tables 6.27, 6.29).

Table 6.26

Postoperative Infections and CBD size
antibiotic group n=104
(Postoperative infections recorded in 81 pts carrying stones.
CBD size recorded in 80 pts)

Infections (%)	n	any	wound	drain	distant
CBD size(mm)					
<= 12	68	19 (27.9)*	10 (14.7)	1 (1.5)**	10(14.7)
> 12	12	7 (58.3)*	4 (33.3)	5 (41.6)**	2(16.6)
13 - 18	10	6 (60)	3 (30)	4 (40)	1 (10)
> 18	2	1 (50)	1 (50)	1 (50)	1 (50)

* P = 0.039 Chi Square test
** P < 0.001 Chi Square test

Table 6.27

Postoperative Infections and CBD size
no-antibiotic group: n=70
(Postoperativeinfections recorded in 21 pts.)

Infections (%)	n	any	wound	drain	distant
CBD size (mm)					
<= 12	11	5 (45.4)	2 (18.2)	2 (18.2)	4 (36.3)
> 12	10	6 (60)	3 (30)	3 (30)	2 (20)
13 - 18	6	3 (50)	2(33)	0	2(33.3)
> 18	4	3 (75)	1 (25)	3 (75)	0

Table 6.28

Postoperative Infections and CBD Stones
antibiotic group n=104
(Postoperative infections recorded in 81 pts carrying stones)

Infections (%)	n	any	wound	drain	distant
CBD stones	9	5 (55.5)	3 (33.3)	5 (55.5)*	1 (11.1)
No CBD ston.	72	22 (30.5)	12 (16.6)	2 (2.7)*	11 (15.2)

* $P < 0.001$ Chi Square test

Table 6.29

Postoperative Infections and CBD Stones
no-antibiotic group: n=70
(Postoperative infections recorded in 21 pts)

Infections (%)	n	any	wound	drain	distant
No CBD stones	10	3 (30)	2 (20)	1 (10)	2 (20)
CBD stones	11	8 (72.7)	3 (27.3)	4 (36.4)	4 (36.4)

6.3.3 CBD size (Tables 6.30 - 6.32)

Bile duct size was recorded in 146 patients with gallstones. A dilated CBD was significantly related to the presence of CBD stones. Patients over 70 years had more frequent CBD dilatation, though without statistical significance; however, simple linear regression analysis showed a direct correlation between these two variables (r= 0.2). Finally no association was demonstrated with a long clinical history of gallstone disease (Table 6.30).

Table 6.30

CBD Stones, Age, Length of History and CBD Size
(CBD size measured in 146 patients with gallstones)

CBD size (mm)	n	CBD stones n (%)	Age>70yrs n (%)	Hist>3yrs n (%)
<= 12	100	5 (5)*	5 (5)	59(59)
> 12	46	32 (69.5)*	3 (6.5)	26(56.5)
13-18	33	19 (57.6)	0	18 (54.5)
> 18	13	13 (100)	3 (23.1)	8 (61.5)

* $P < 0.001$ Chi Square test

These trends did not differ between the antibiotic and no- antibiotic groups (Tables 6.31, 6.32).

Table 6.31

CBD Stones, Age, Length of History and CBD Size
antibiotic group: n=104
(CBD size measured in 89 patients with gallstones)

CBD sizen (mm)	n	CBD stones n (%)	Age>70yrs n (%)	Hist>3yrs n (%)
<= 12	77	2 (2.6)*	3 (3.9)	49 (63.6)
> 12	12	5 (41.6)*	1 (8.3)	9 (75)
13-18	11	4 (36.4)	0	8 (72.7)
> 18	1	1 (100)	1 (100)	1 (100)

* P < 0.001 Chi Square test

Table 6.32

CBD Stones, Age, Length of History and CBD Size
no-antibiotic group: 70 pts
(CBD size measured in 57 patients with gallstones)

CBD size (mm)	n	CBD stones n (%)	Age>70yrs n (%)	Hist>3yrs n (%)
<= 12	23	3 (13)*	2 (8.7)	10(43.5)
> 12	34	27 (79.4)*	2 (5.8)	17(50)
13-18	22	15 (68)	0	10(45.4)
> 18	12	12 (100)	2 (16.7)	7 (58.3)

* P< 0.001 Chi Square test.

6.3.4 Biochemical studies

* GB bile (Tab. 6.33-6.35.Fig 6.1, 6.2)

The results of the biochemical assays in GB bile are shown in Tables 6.33, 6.34. No statistical differences were observed in the presence or absence of infection in both antibiotic and no-antibiotic group.

Table 6.33

GB bile Bilirubin, Betaglucuronidase,Phospholipase, Bile Acids and Bile Cultures
antibiotic group: n=104
(biochemical assays in 56 pts)

Bile cultures	-ve(n=45)	+ve(n=11)	all(n=11)
CONJ. BILIRUBIN (uM/l)			
mean	1396	928	1304
median	1121	140	875
(range)	(1-6014)	(10-5405)	(1-6014)
UNCONJ. BIL. (uM/l)			
mean	317	471	347
median	140	60	133
(range)	(1-2109)	(10-2755)	(1-2755)
BETA-GLUCURON. (UI/l)			
mean	0.483	0.572	0.500
median	0.058	0.118	0.064
(range)	(0.005-2.781)	(0.007-1.759)	(0.005-2.781)
PHOSPHOLIPASE (UI/l)			
mean	0.115	0.153	0.122
median	0.083	0.104	0.083
(range)	(0.001-0.603)	(0.001-0.601)	(0.001-0.603)
TOT. BILE ACIDS (mM/l)			
mean	69.04	36.40	62.63
median	44.22	11.99	37.38
(range)	(0.26-241.20)	(1.28-113.12)	(0.26-241.20)

Table 6.34

GB bile Bilirubin, Betaglucuronidase, Phospholipase, Bile Acids and Bile Cultures
no-antibiotic group: 70 pts
(biochemical assays in 30 pts)

bile cultures	-ve (n=17)	+ve (n=13)	all
CONJ.BIL. (uM/l)			
mean	584	477	537
median	228	200	214
(range)	(1-3384)	(38-2109)	(1-3384)
UNCONJ.BIL. (uM/l)			
mean	302	240	275
median	150	76	112
(range)	(1-1443)	(10-1311)	(1-1443)
Beta GLU. (UI/l)			
mean	0.460	0.729	0.576
median	0.080	0.187	0.128
(range)	(0.021-2.108)	(0.017-2.568)	(0.017-2.568)
PHOSPHOLIPASE (UI/l)			
mean	0.186	0.170	0.179
median	0.164	0.050	0.104
(range)	(0.001-0.621)	(0.001-0.674)	(0.001-0.674)
TOT.BILE ACIDS (mM/l)			
mean	61.56	28.33	47.17
median	57.51	20.10	36.38
(range)	(2.22-208.52)	(3.38-108.400)	(2.22-208.52)

Biochemical assays were also calculated in the presence (26 patients) or absence (4 patients) of gallbladder stones (no- antibiotic group). No significant differences were shown in conjugated bilirubin, phospholipase or total bile acids values. However unconjugated bilirubin was significantly lower (P=0.016) and betaglucuronidase greater (P=0.011) in patients with stones (Table 6.35).

A significant inverse linear relationship was found between unconjugated bilirubin levels and betaglucuronidase activity in both antibiotic (r=0.36, P=0,006; Fig. 6.1) and no-antibiotic group (r=0.38, P=0,037; Fig 6.2. No statistical relationship was shown between any of the biochemical assays and age for either the antibiotic) groups.

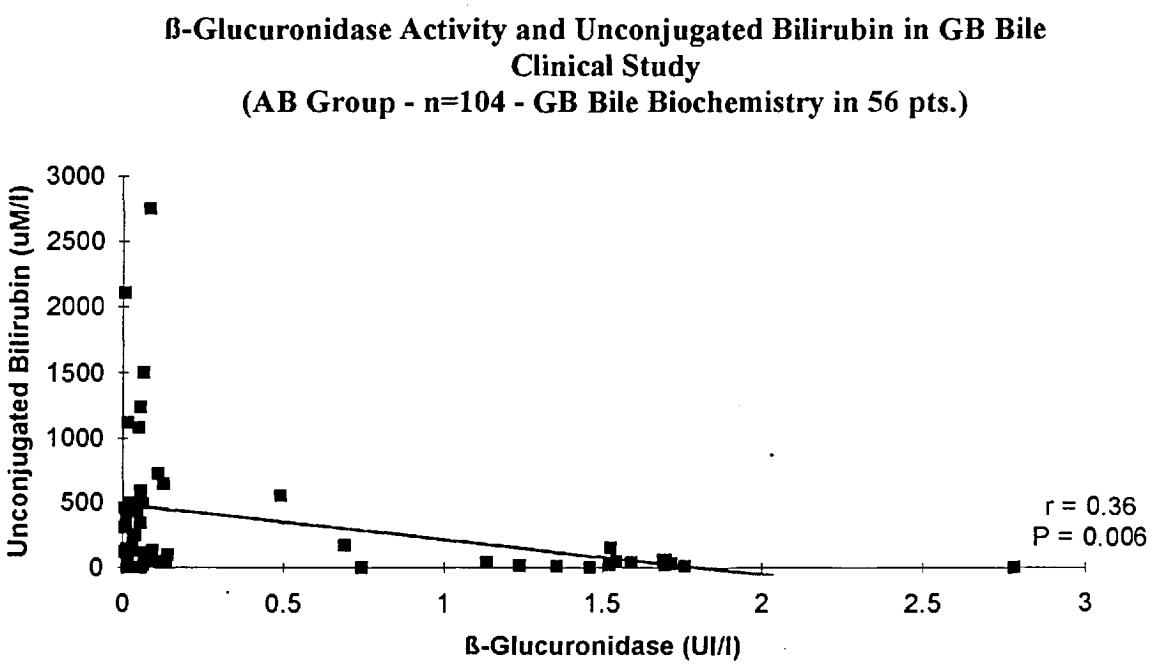


Fig.6.1 Betaglucuronidase activity and unconjugated bilirubin in GB bile (antibiotic group).

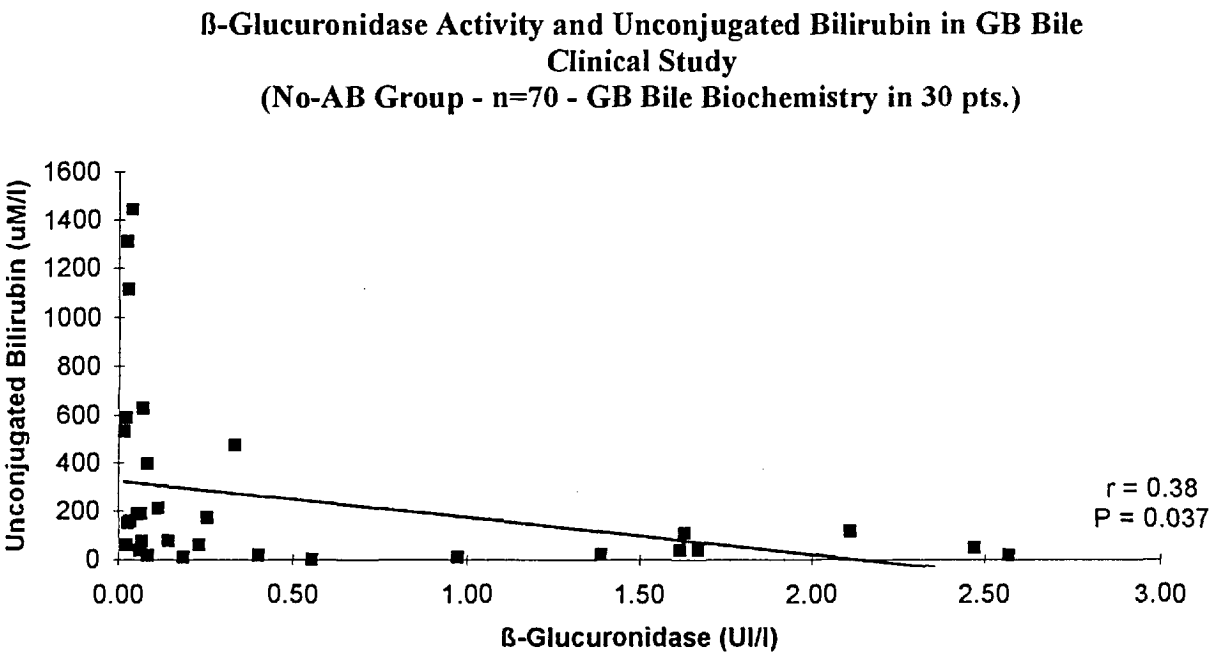


Fig.6.2 Betaglucuronidase activity and unconjugated bilirubin in GB bile (no-antibiotic group).

Table 6.35

GB bile Bilirubin, Betaglucuronidase Phospholipase, BileAcids and GB Stones
no-antibiotic group: 70 pts
(biochemical assys in 30 pts)

GB Stones	stones(n=26)		no stones(n=4)	all
CONJ. BIL. (uM/l)				
mean	534		560	537
median	176		323	214
(range)	(1-3384)		(76-1519)	(1-3384)
UNCONJ.BIL. (uM/l)				
mean	190	*	827	275
median	76		852	112
(range)	(1-1311)		(160-1443)	(1-1443)
Beta GLU (UI/l)				
mean	0.660	**	0.030	0.576
median	0.209		0.031	0.128
(range)	(0.017-2.568)		(0.021-0.037)	(0.017-2.568)
PHOSPHOLIPASE (UI/l)				
mean	0.191		0.104	0.179
median	0.115		0.016	0.104
(range)	(0.001-0.674)		(0.001-0.381)	(0.001-0.674)
TOT.BILE ACIDS (mM/l)				
mean	44.01		67.60	47.17
median	33.58		45.28	36.38
(range)	(2.22-208.52)		(32.43-147.40)	(2.22-208.52)

* P=0.016 Mann-Whitney test
** P=0.011 Mann-Whitney test

* CBD Bile (Tab.6.36 - 6.39. Fig. 6.3, 6.4)

The results of the biochemical assays in CBD bile are shown in Tables 6.36 - 6.39. No statistical differences were observed in the presence or absence of infection in the no-antibiotic group, while total bile acids were lower in presence of infected bile in the antibiotic group. Biochemical assays were also calculated in the presence or absence of CBD stones in both groups. In the antibiotic group there were 7 patients with CBD

stones and 19 without. In the no-antibiotic group 19 patients had CBD stones and 12 had not. No significant differences were shown in biochemical assays in the antibioic group, whereas in the no- antibiotic group conjugated bilirubin levels and phospholipase activity were lower in presence of stones.

No relationship was found between unconjugated bilirubin levels and betaglucuronidase activity in either the antibiotic (r=0.01, P=0,95; Fig. 6.3) or the no-antibiotic group (r=0.19, P=0,30; Fig 6.4). No statistical relationship was shown between any of the biochemical assays and age for either group.

Table 6.36

CBD bile Bilirubin,Betaglucuronidase, Phospholipase, BileAcids and Bile Cultures
antibiotic group: 104 pts
(biochemical assays in 26 pts)

Bile Cultures	-ve (n=17)		+ve (n= 9)	all
CONJ. BILIRUBIN (uM/l)				
mean	467		373	434
median	418		270	385
(range)	(100-1349)		(120-330)	(100-1349)
UNCONJ. BIL. (uM/l)				
mean	357		238	316
median	330		170	235
(range)	(80-950)		(50-590)	(50-950)
BETA-GLU (UI/l)				
mean	0.054		0.057	0.055
median	0.017		0.119	0.017
(range)	(0.004-0.347)		(0.006-0.262)	(0.004-0.347)
PHOSPHOLIPASE (UI/l)				
mean	0.050		0.020	0.040
median	0.017		0.001	0.001
(range)	(0.001-0.228)		(0.001-0.073)	(0.001-0.228)
TOT. BILE ACIDS (mM/l)				
mean	18.13	*	10.08	15.34
median	17.24		9.47	(11.75)
(range)	(3.80-61.90)		(4.37-16.96)	(3.80-61.90)

* P = 0.031 Mann Whitney test

Table 6.37

CBD bile Bilirubin,Betaglucuronidase,Phospholipase, Bile Acids and Bile Cultures
no-antibiotic group: 70 pts
(biochemical assays in 31 pts)

Bile Cultures	-ve (n=17)	+ve (n=14)	all
CONJ. BIL. (uM/l)			
mean	324	329	326
median	323	275	300
(range)	(50-700)	(50-920)	(50-920)
UNCONJ. BIL (uM/l)			
mean	327	265	299
median	190	180	190
(range)	(40-1501)	(30-900)	(30-1501)
Beta GLU (UI/l)			
mean	0.073	0.056	0.065
median	0.027	0.032	0.032
(range)	(0.002-0.338)	(0.004-0.208)	(0.002-0.338)
PHOSPHOLIPASE (UI/l)			
mean	0.054	0.049	0.052
median	0.014	0.038	0.022
(range)	(0.001-0.243)	(0.001-0.188)	(0.001-0.243)
TOT.BILE ACIDS (mM/l)			
mean	21.44	17.74	19.77
median	14.57	13.76	14.47
(range)	(5.02-128.11)	(2.77-53.86)	(2.77-128.11)

Table 6.38

CBD bile Bilirubin, Betaglucuronidase,Phospholipase, BileAcids and CBD Stones
antibiotic group: 104 pts
(biochemical assays in 26 pts)

CBD Stones	no stones (n=19)	stones (n=7)	all
CONJ. BIL (uM/l)			
mean	442	414	434
median	410	360	385
(range)	(100-1349)	(100-830)	(100-1349)
UNCONJ. BIL (uM/l)			
mean	343	241	316
median	240	150	235
(range)	(80-950)	(50-510)	(50-950)
BETA-GLU (UI/l)			
mean	0.043	0.090	0.055
median	0.017	0.027	0.017
(range)	(0.004-0.347)	(0.004-0.281)	(0.004-0.347)
PHOSPHOLIPASE (UI/l)			
mean	0.050	0.010	0.040
median	0.018	0.001	0.001
(range)	(0.001-0.228)	(0.001-0.050)	(0.001-0.228)
TOT. BILE ACIDS (mM/l)			
mean	16.29	12.76	15.34
median	11.05	13.84	11.75
(range)	(3.80-61.90)	(5.26-17.36)	(3.80-61-90)

Table 6.39

CBD bile Bilirubin,Betaglucuronidase, Phospholipase, Bile Acids and CBD Stones
no-antibiotic group: 70 pts
(biochemical assays in 31 patients)

CBD Stones	no stones(n=12)		stones(n=19)	all
CONJ. BIL. (uM/l)				
mean	430	*	261	326
median	381		270	300
(range)	(80-920)		(50-570)	(50-920)
UNCONJ. BIL.(uM/l)				
mean	459		197	299
median	200		150	190
(range)	(40-1501)		(30-720)	(30-1501)
BETA GLU (UI/l)				
mean	0.074		0.060	0.065
median	0.030		0.032	0.032
(range)	(0.002-0.320)		(0.004-0.338)	(0.002-0.338)
PHOSPHOLIPASE (UI/l)				
mean	0.080	**	0.033	0.052
median	0.063		0.001	0.022
(range)	(0.001-0.243)		(0.001-0.138)	(0.001-0.243)
TOT.BILE ACIDS (mM/l)				
mean	28.54		14.22	19.77
median	15.72		14.36	14.47
(range)	(5.52-128.11)		(2.77-34.78)	(2.77-128.11)

* P = 0.032 Mann-Whitney test
** P = 0.046 Mann-Whitney test

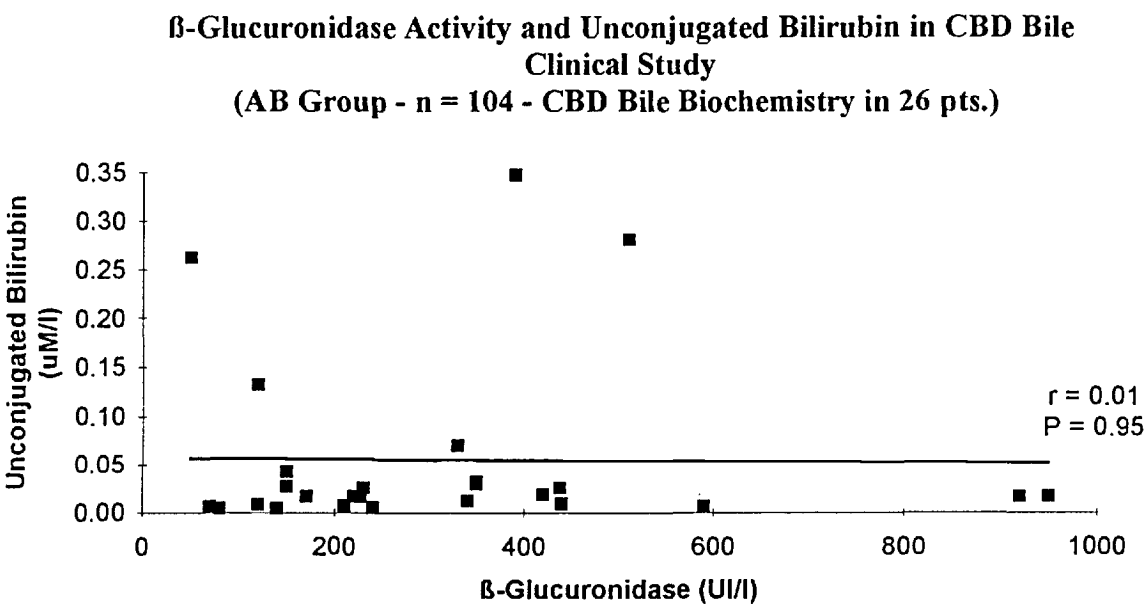


Fig.6.3 Betaglucuronidase activity and unconjugated bilirubin in CBD bile (antibiotic group).

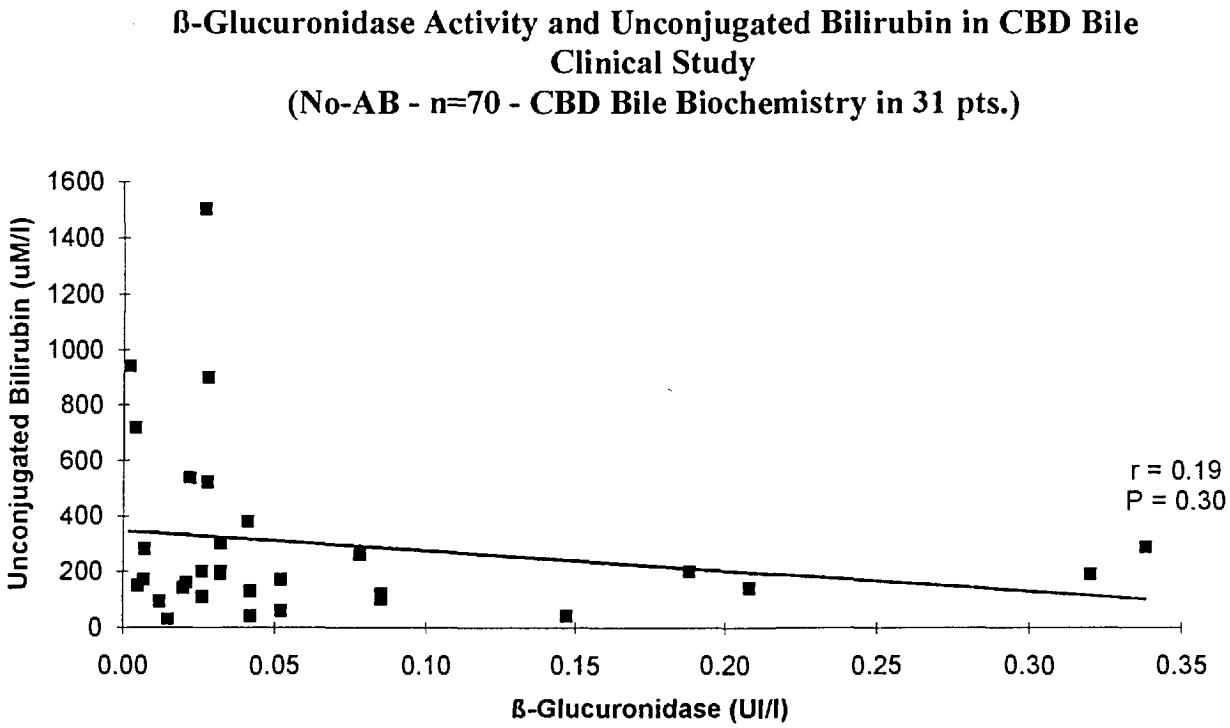


Fig.6.4 Betaglucuronidase activity and unconjugated bilirubin in CBD bile (no-antibiotic group).

6.3.5 CBD stone composition (Tables 6.40 - 6.44)

X-ray diffraction analysis was carried out in a total of 15 patients in this group. Table 6.40 shows the composition for all stones analyzed. Five patients had stones containing calcium palmitate, whereas ten patients had no calcium palmitate detected in their stones.

The mean age in the whole series of 174 patients was 50 years, 53 years in 43 patients with CBD stones and 49 years in 131 patients with no CBD stones. In the subset of 15 patients whose stones underwent analysis there was no differences in average age of patients with calcium palmitate stones (57 years) and that of patients with no-calcium palmitate stones (53 years) ($P>0.05$ Mann-Whitney test).

Neither the presence nor the composition of stones in the CBD seem to be correlated with a long clinical history (Tables 6.41, 6.42).

Table 6.40

X-ray Diffraction Analysis of CBD Stones
Whole series: n=174
(stone composition analysis in 15 pts)

patient number	CBD stones composition
9005	An. Chol., Ca. Palm.
9007	Chol. Monoh., Ca. palm.
9010	Chol. Monoh., Calcite
9016	Chol. Monoh.
9018	Chol. Monoh.
9025	An. Chol., Ca. palm.
9031	Chol. Monoh.
9032	Chol. Monoh.
9033	Chol. Monoh.
9034	Chol. Monoh.
9039	An. Chol., Chol. Monoh.
9050	Chol. Monoh., Ca. Palm.
9077	Chol. Monoh.
9078	Chol Monoh.
9152	Ca. Palm.

KEYS:

An. Chol = Anhydrous Cholesterol
Chol. Monoh = Cholesterol Monohydrate
Ca. Palm. = Calcium Palmitate

Table 6.41

CBD Stones, CBD Stone Composition & Average Clinical History of gallstone disease
Whole series: n=174
(stone composition analysis in 15 pts)

(Average length of history in the whole group: 69 months)		
Patients	N	AverageHistory (months)
No CBD stones	131	65
CBD stones	43	82
Ca palmitate stones	5	38
Other stones	10	86

Table 6.42

CBD Stones, CBD Stones Composition & Length of Clinical History of gallstone disease
Whole series: n=174
(stone composition analysis in 15 pts)

Patients		History > 3 yrs	
	N	N	(%)
No CBD stones	131	69	(52.7)
CBD stones	43	28	(65.1)
Ca palmitate stones	5	2	(40)
Other stones	10	8	(80)

The correlations between CBD stone composition and bile infection rates have been evaluated in the subgroup of 13 patients in the no-antibiotic group who had stone composition analysis carried out. A significant increase of bile infection rate has been demonstrated in patients with CBD stones (21/32, 65.6%) when compared to patients with no stones in the CBD (10/38, 26.3%, P=0.002). Similar infection rates were found in patients with stones containing calcium palmitate and in patients with stones not containing calcium palmitate. Multiple isolates have shown a similar pattern, though with no statistical significance (Table 6.43).

Table 6.43

CBDStones,CBDStonecomposition & Bile Infection
No antibiotic group: n=70
(stone composition analysis in 13 pts)

Patients		+ve bile cult.		polymic	
	N	N	(%)	N	(%)
No CBD stones	38	10*	(26.3)	4	(10.5)
CBD stones	32	21*	(65.6)	7	(21.9)
Ca palmitate stones	5	4	(80)	3	(60)
Otherstones	8	6	(75)	2	(25)

* P= 0.002 Chi Square test.

Bile duct size was measured in 146 patients with gallstones disease. The average CBD size was 12 millimeters. In the subgroup of 109 patients with no stones in the common bile duct the average CBD size was 11 millimeters, whereas amongst 37 patients with common bile duct stones the average CBD size was 17 millimeters ($P<0.001$ Mann-Whitney test). The average CBD size in 12 patients in whom stone composition was assessed by x-ray diffraction analysis was 18 millimeters. Furthermore CBD size averaged 16 millimeters in the subgroup of eight patients carrying stones not containing calcium palmitate and 20 millimeters in the group of four patients whose stones contained calcium palmitate ($P>0.05$ Mann-Whitney test).

Seven out of 10 patients (70%) carrying stones not containining calcium palmitate had had their CBD stones classified at operation as not of primary ductal origin, whereas four out of five patients (80%) who presented stones containing calcium palmitate had had their stones classified at operation as primary CBD stones.

Table 6.44 summarizes the values of each biochemical assay in the whole group of patients, in patients with or without stones, in all patients whose stones were analysed with x-ray diffractometry, and in those with stones containing or not containing calcium palmitate. Statistical evaluation with Mann- Whitney test showed no statistical differences between any group.

Table 6.44

Mean Bilirubin, Betaglucuronidase, Phospholipase and Total Bile Acids in CBD bile in the whole group, in patients with or without stones, in the whole group of patients whose stones were analysed by x-ray diffractometry, and in patients with stones containing or not containing Ca palmitate.

Assay	wh. sr.	no st.	stones	st.comp	-ve c.plm	+ve c.plm
(N)	(58)	(32)	(26)	(7)	(5)	(2)
CONJ.BIL	378	439	302	206	210	84
UNCONJ.BIL	304	382	209	140	158	52
BetaGLU	.060	.053	.068	.029	.022	.020
PHOSPHOL	.045	.060	.027	.029	.040	0
Tot. BA	17.76	20.96	13.83	16.08	16.55	15.97

6.4 DISCUSSION

6.4.1 Introductory

There is evidence in the literature that primary CBD stones are associated with bile stasis and infection (Imamoglu et al., 1957; Maki, 1966; Madden, 1968; Cetta, 1983). However, the biological pathways which correlate these three phenomena are still unclear. In particular it is uncertain whether stasis is a primary or secondary phenomenon and what is its pathogenesis. The status of the knowledge on this matter before the start of this study was reported in the introduction. However, a number of other studies have been published during the performance of this work.

6.4.2 Review of the papers published after the start of this study.

* Cholestasis

The association between stasis, infection and primary CBD stone formation has been further underlined in recent years. Lygidakis (1983) published a study on 3,802 operations carried out for calculous biliary tract disease in 15 years and reported that the incidence of primary CBD stones rose from 35% in patients with CBD size of 1.5 cm progressively to 100% in patients with CBD size of 5 cm. Moreover this author reports a rise in the incidence of positive bile culture rate from 16% in patients with a CBD size of 1.5 cm, to 100% in patients with CBD size of 5 cm and a bile infection rate of 16.3% in patients with secondary CBD stones and of 100% in patients with primary CBD stones. In this paper also an association between age and the occurrence of primary CBD stones is reported.

The same author (Lygidakis 1984) correlated CBD dilatation not only with the presence of stones in the duct but also with age, previous biliary surgery, infections and a long clinical history of biliary disease.

At ultrasounds studies (Hunt & Scott 1989) greater CBD size has been found in patients undergoing CBD exploration both before and after operation, irrespective of the presence of ductal stones. These authors report also studies by other workers about a small, not significant increase of CBD size with time elapsed from operation and with age (Goransson et al., 1980; Faris et al., 1975; Mueller et al., 1981).

* Infection:

Bile infection incidence

More recent studies report overall bile infection rates of 17% (Willis et al. 1984) to 19% (Wells et al. 1989). Interstingly Keighley & Blenkharn (1988) reported a bile infection rate in malignant biliary obstruction ranging from 29 to 32 %. rising to 90% in presence of benign biliary strictures.

Risk Factors

In order to identify patients at risk of developing infection after biliary surgery, Murray & Bradley (1983) compared wound infection rates in 50 patients who received unselective antibiotic prophylaxis with 50 patients who received selective antibiotic prophylaxis according to intraoperative Gram staining. In the unselected group no patients developed wound infection, whereas amongst patients who received selective antibiotic prophylaxis wound infection was 18% ($P<0.001$). On the basis of these results the authors concluded that antibiotic prophylaxis should be started before anaesthesia if it is to be effective.

The role of risk factors for predicting infections following biliary surgery has been studied retrospectively by Stubbs (1983) who found significant differences between patients who received prophylactic antibiotics and patients who did not, in both low and high risk patient groups. This author concluded that it is difficult to identify a true group of patients at low risk of infection in biliary surgery. Similar conclusions were drawn by Morran et al. (1984) who compared prospectively patients who received antibiotic prophylaxis and patients who did not and found a significantly lower wound infection rate in antibiotic treated patients.

Lygidakis(1983) and Armstrong et al (1985) identified age as a significant risk factor for infection. Willis et al. (1984) found a significantly higher positive bile culture rate in elderly patients or in the presence of jaundice, emergency operations, CBD exploration and CBD stones.

The significance of a history of cholangitis has been stressed by Pitt et al. (1982) who reported age, incidence of jaundice, leukocytosis, CBD stones, bacterial colonization of bile, the presence of anaerobes, renal impairment and prolonged hospital stay associated with this feature and by Lewis et al. (1987) who found a 96% bile infection rate with 12% anaerobes incidence in presence of cholangitis. Finally Lygidakis (1983) found a correlation between bile infection and the number of previous biliary operations.

Organisms

Bacteria most commonly isolated from bile have been largely reported in the background section. More recent studies have emphasized an increased presence of staphylococci in bile (Morran et al., 1984) and the presence of faecal streptococci in 5-15% of all positive bile cultures (Blenkharn & Blumgart, 1985).

Postoperative infections

A significant increase in the postoperative infection rate in patients with infected bile has been reported by Armstong et al. (1985) and by Wells et al. (1989), who also showed a higher postoperative infection rate in patients undergoing CBD exploration. Armstrong et al. (1985) isolated the same organism in bile and postoperative cultures in 80% of cases while Willis et al. (1984) reported that only 2/21 patients with positive wound cultures had same organisms in bile. Both Armstrong et al. (1985) and Sheridan et al. (1987) recorded only a slight reduction of postoperative septic complications in presence of antibiotic prophylaxis, with no statistical significance.

* Biochemistry:

Bilirubin Deconjugation

This hypothesis has been extensively investigated in recent years. Some authors have underlined the presence of bacterial strains producing betaglucuronidase, especially C. perfrigens (Sakagucki et al., 1983) or E coli, B fragilis and Clostridia (Skar et al., 1986). Feretis et al. (1984) reported significantly higher values of unconjugated bilirubin in patients with infected bile up to six months after operation. However these authors did not perform betaglucuronidase assay and concluded that the relationship between bacteria, betaglucuronidase, unconjugated bilirubin and pigment stone formation was not obvious on the basis of their study. Ho et al. (1986) detected betaglucuronidase in some but not in all patients carrying bacteria in bile and suggested that betaglucuronidase activity may be affected by other factors such as ionic strength, presence of glucaro 1-4 lactone and electrolytes. In addition they suggested that the enzyme may be sequestered into mixed micelles and lose activity, so that the expressed activity of betaglucuronidase should be considered as apparent activity and not as a quantitative measure. Oyabu et al. (1987)

constantly found a greater proportion of unconjugated bilirubin in GB bile than in CBD bile, regardless of the presence of bacteria. They correlated this with stasis of the bile in the GB and concluded that the values of unconjugated bilirubin may be expected to increase with bile stasis. Finally these authors reported that addition of Glucaro 1-4 lactone suppressed hydrolysis of conjugated bilirubin. Nakano et al. (1988) reported a positive correlation between the presence of bacteria in bile and the proportion of unconjugated bilirubin in patients with a history of acute cholangitis, whereas in patients without such a history no correlation was found. Furthermore, in patients with a history of cholangitis the proportion of unconjugated bilirubin was significantly higher in the presence of betaglucuronidase-producing bacteria: after relief of biliary obstruction the proportion of unconjugated bilirubin returned to normal within few days. These findings led the authors to conclude that cholangitis facilitates the hydrolysis of bilirubin and that bouts of cholangitis may produce repeated episodes of precipitation, which explain the layered structure of brown pigment stones.

Spivack et al. (1987) experimentally demonstrated in GB bile the existence of non-enzymatic hydrolysis of bilirubin. Oyabu et al. (1987) demonstrated changes in total bilirubin composition after incubating bile with polymorphonuclear leukocytes. Skar et al. (1989) suggested that a non-enzymatic hydrolysis of bilirubin takes place in CBD bile.

Ho (1986) found that the proportion of conjugated bilirubin decreased progressively from control patients (89%) to cholesterol stone patients (86%) to black pigment stone patients (79%) to brown pigment stone patient (56%). He concluded that bilirubin deconjugation was greater in brown pigment stone patients than in black pigment stone or cholesterol patients and greater in the GB than in the CBD. Finally this author stressed that the optimal pH was 7 for bacterial and 5.2 for endogenous betaglucuronidase. Nakano et al. (1988) found that in the presence of cholangitis the bile pH is lowered towards the optimum for bacterial betaglucuronidase and subsequent hydrolysis of bilirubin.

Phospholipase

Cetta et al., (1984) observed that calcium palmitate represents about 15% of dry weight of recurrent stones and less than 1% of other stones. They proposed that palmitic acid may form as product of hydrolysis of lecithin subsequent to the action of a phospholipase of possible bacterial origin. These authors studied 30 patients, 10 with GB stones, 10 with GB and secondary CBD stones, five patients with black pigment CBD stones and five patients with brown pigment CBD stones. In a qualitative assay phospholipase was detected in in 4/5 patients with recurrent brown pigment stones and in 0/25 others. Furthermore bacterial colonization of bile was found in 5/5 patients with recurrent stones and 2/25 others. Groen et al, (1989) assessing the role of phopholipase in the transformation of lecithin as the initial step of cholesterol gallstone formation, found extremely low activity of phospholipase in both patients with cholesterol gallstones and controls.

Bile Acids

Work on changes in bile acid levels in bile in patients with gallstone disease have been reported in the introduction. However in recent years this topic has been further investigated. Gleeson et al. (1986) report that GB bile of patients with radiolucent stones did not differ from that of calcified stones with regard to bile acids or total calcium. By contrast several studies have reported an increased proportion of free bile acids in the presence of brown pigment stones and infected bile. Shimada et al. (1984) reported the presence of free bile acids in patients with anaerobic biliary tract infections and an increase of pigment stones in patients with free bile acids in bile. These authors conclude suggesting that the presence of free bile acids may produce damage to the biliary epithelium which initiate an inflammatory process which in turn leads to pigment stone formation. Nakano et al., (1989) reported total bile acid levels in bile significantly lower in

the presence of brown pigment stones and cholangitis. Akyoshi et al. (1990) reported that bile acids were only a minor constituent of cholesterol stones, but represented nearly 34% in brown and black pigment stones. In brown pigment stones mostly free bile acids were formed and moreover the total bile acid concentration in patients with brown pigment stones was significantly lower than in controls. They explained this by increased absorption of bile acids from an inflamed biliary tract. The proportion of free bile acids to total bile acids was significantly greater in brown pigment stones, independently of the bile acid deconjugating activity of bacteria in bile. The concentration of free bile acid was significantly greater in the presence of bacteria with bile acids deconjugating activity. Free bile acids in brown pigment stones were 6-10 times when compared to bile from same patients whereas glycine and taurine conjugates are reduced. It is concluded that bacteria in bile of patients with brown pigment stones possess capacity of hydrolyzing bile acids and bilirubin conjugates.

The role of biliary calcium

The role of biliary calcium in the pathogenesis of calcium pigment stones has been investigated only in recent years. Moore (1985) suggested that calcium represents an important constituent of pigment stones and, since the nidus of cholesterol stones is mostly composed of calcium bilirubinate, it is possible that calcium also has a role as nucleating agent in cholesterol stones. Any measure reducing free calcium will therefore reduce bile lithogenicity. Magnuson et al. (1989) found a significantly higher incidence of pigment stones in prairie dogs fed on a calcium supplemented diet. Abedin et al. (1989) reported a positive linear relationship between calcium concentrations and total bile acids in GB bile, but found no such relationship in hepatic bile. The authors conclude that diet induced alterations in bile acid composition may modify calcium solubility or GB function contributing to cholesterol or pigment stone formation. Magnuson et al. (1990) report biliary sludge formation induced by oestrogen administration in prairie dogs. All the

animals which received oestrogens developed cholesterol crystals and 4/6 developed calcium bilirubinate granules. Radiolabelled activity indicated GB stasis and a significant decrease in total bilirubin suggested precipitation of calcium bilirubinate.

* Stones

The characteristics of brown pigment stones, their macroscopic appearance and their clinical associations have been already reported in the background section and in the introduction to this clinical study. In recent years interest has developed further and several of studies on this topic has been reported.

Epidemiology

Ostrow (1984) underlined that the incidence of brown pigment stones is much lower in western countries where these stones usually arise primarily in the CBD.

The role of foreign bodies.

The frequent association of brown pigment stones with foreign bodies within the biliary tract has been long recognized. In the large majority of cases in patients from western countries the foreign body is suture material related to previous biliary operations. Wosiewitz et al. (1983) reported that suture material was found much more frequently in patients with pigment CBD stones than in patients with cholesterol CBD stones and correlated this with the possible primary ductal origin of pigment stones and the gallbladder origin of cholesterol stones. Also Ostrow (1984) reported brown pigment stones with a nucleus of non-absorbablesuture material and Cetta (1986) found suture material in one of two cases of recurrent brown pigment stones.

In patients from Japan parasites such as Ascaris lumbricoides penetrate the papilla and biliary tract and eggs or carcases of the parasite act as a kernel, inducing precipitation of calcium pigment stones (Maki et al., 1984). In addition the papillary orifice is expanded by the passage of the parasite, resulting later in papillary fibrosis, which in turn produces bile stasis and bacterial colonization. In this respect it is interesting to note that when a larva of Clonorchis sinensis, a parasite too small to induce papillary dilatation and subsequent fibrosis, is found within the biliary tract, it is not usually associated with brown pigment stones. Interestingly Csendes et al. have recently reported (1989) a study by Yamamoto who studied patients undergoing liver resection for intrahepatic calculi. Multiple glandular formations in intrahepatic ducts of these patients and mucous substances produced by these glands may play a role as nucleating factors in intrahepatic stones formations.

Clinical associations

Several clinical associations are reported for pigment stones. Kaufman et al. (1989) found that patients with brown pigment stones were predominantly male and significantly older, whereas black pigment stones were found in significantly younger patients association with haemolytic anemia, ileal disorders, obesity, or parenteral nutrition.

Association with infection.

Infection is commonly reported associated with brown pigment stones. Smith et al. (1989) studied gallstone composition by scanning electron microscopy and found bacteria in 4.5% of cholesterol stones, 68% of pigment stones and 80% of composite stones. These authors defined such stones as "infectious" and found them to be commoner in the CBD and significantly associated with cholangitis. Three patients in this series needed intensive care and all had "infectious" stones. Infective complications were significantly associated with

the presence of infectious stones. Interestingly these authors conclude suggesting the use of a biliary drainage procedure not only in the presence of CBD dilatation, but in every case in which the presence of an infectious stone is recognized. Cetta (1983) found positive bile cultures in 100% of patients affected by primary CBD stones and in 43% of patients affected by secondary CBD stones. The same author later underlined that he never identified substantial amounts of calcium palmitate - which is a well recognized constituent of brown pigment stones - in the absence of bile infection (Cetta, 1986). Ostrow (1984) found positive bile cultures in 85% of cases of brown pigment stones and Lombardo et al. (1992) have recently reported a 100% incidence of E coli in patients with carrying calcium palmitate-containing CBD stones.

Stewart et al. (1987) studied gallstones in 85 patients with scanning electron microscopy. Fresh pigment stones had bacterial microcolonies on their surface in 92% of cases: the only patients without microcolonies were those affected by haemolysis. By contrast only one patient with cholesterol stones had bacterial surface microcolonies. However, bacteria were noted in the pigmented centre of 33% of cholesterol stones. The authors conclude that bacteria are responsible for the formation of the majority of black as well as brown pigment stones in the orient as well as in western countries. Leung et al. (1989) studied with scanning electron microscopy stones removed endoscopically from 38 patients with acute cholangitis. Positive bile cultures were found in 91% of patients and similar organisms were found in the stones. Furthermore scanning electron microscopy demonstrated the presence of bacteria in all layers of the stones suggesting that bacteria are involved in the stone formation process from an early stage.

Kaufman et al. (1989) found a signifcantly higher positive bile culture rate in patients with brown pigment stones. Smith et al (1989) reported that bacteria were found in the bile of 97% of patients with infectious stones but also in 31% of patients with non infectious

stones. Similarly Vitetta et al (1989) reported positive bile cultures in 100% of patients with brown pigment stones and in 10-33% of patients with cholesterol stones.

Macroscopic appearance.

Ostrow (1984) described black pigment stones as brown to black, most commonly found in presence of haemolysis or cirrhosis and arising primarily in the GB; brown pigment stones were brown to orange, laminated and of soft greasy consistency. By contrast Stewart et al. (1987) stated that while it is easy distinguish between cholesterol and pigment stones by their macroscopic appearance, the distinction between black and brown pigment stones bas been somewhat over-estimated in the literature, since stones rather than falling neatly into one group, exibit a spectrum of appearances from the crumbly milk chocolate brown stone at one extreme to the hard, black, obsidian-like stone at the other.

Composition

Understanding of gallstone composition has improved in recent years by a number of studies. Wosiewitz et al. (1983) found inorganic calcium salts more frequent in GB stones whereas calcium bilirubinate and calcium salts of fatty acids were more frequent in CBD stones. These two compounds were often observed in combinations indicating co-precipitation. Black pigment stones are found more frequently in the GB. A glycoprotein has been identified in pigmented areas of cholesterol stones and suture material found associated with pigment stones, suggesting the occurrence of heterogeneous nucleation in the precipitation of calcium bilirubinate stones. Also Cetta et al. (1983) found that calcium palmitate always occurred in recurrent CBD stones. By contrast only small amounts of cholesterol were found in primary stones, but large amounts in secondary stones. Maki et al. (1984) reported that the bilirubin content of calcium bilirubinate (brown pigment) stones is over 30%, whereas in black stones it averages 12%; by contrast the final

amorphous residue in calcium bilirubinate stones is about 9% whereas in black pigment stones it is very abundant. Similarly Ostrow (1984) reported large amounts of an insoluble black polymer pigment in black pigment stones, whereas brown pigment stones are formed mainly by calcium soaps of fatty acids, deriving from lecithin, with smaller proportion of polymer and a significant proportion of cholesterol. Oyabu et al. (1987) reported that cholesterol stones are formed of 70 % of cholesterol, brown pigment stones are formed mainly of calcium bilirubinate whereas black pigment stones are mainly formed by unextractable residue, probably a bilirubinoid polymer. However, Suzuki (1985) stated that the black material was calcium bilirubinate.

The use of scanning electron microscopy of stones has revealed other relevant aspects of pigment gallstone composition and has proposed new explanations of stone formation. Stewart et al. (1987) carried out scanning electron microscopy of brown pigment stones and concluded that bacteria represent a substantial proportion of such stones and not just a remote source of betaglucuronidase. They possess specific adhesive properties and the adhesion is mediated by a charged glycoprotein, bacterial glycocalyx, that extends from the bacterial surface. Speer et al. (1988) studied with light and electron microscopy the material that frequently blocks biliary stents, an event which is uniformly accompanied by bile stasis and infection. This material was composed of an extracellular bacterial protein, mixed with crystals of calcium palmitate, cholesterol and granules of calcium bilirubinate. Bacterial cells were found attached to the stents suggesting that the initial event is the adherence of bacterial biofilms, following which bacterial enzymatic activity with phospholipase and betagucuronidase leads to crystal deposition. Leung et al. (1989) in Hong Kong studied with scanning electron microscopy stones obtained after endoscopic sphincterotomy from 38 patients with cholangitis. Only 10.5% of these stones contained cholesterol, in amounts ranging from 11 to 30%; concentric layers of alternating calcium palmitate and calcium bilirubinate were present, mixed with a granular substance moulded into an amorphous matrix. Kaufman et al. (1989) studied stones from 67 patients with

scanning electron microscopy. Bacteria were uniformly found in brown pigment stones and never in black pigment stones. However, the cholesterol content of bile was higher in in patients with brown than in those with black pigment stones, though significantly lower than in patients with cholesterol stones. Inorganic calcium salts such as calcium phosphate and calcium carbonate were present only in patients with black pigment stones. Moreover these authors most interestingly underlined that they did not demonstrate bacteria in cholesterol and black pigment stones, despite the presence of considerable amounts of calcium bilirubinate in the latter. Finally Vitetta et al. (1989) studied gallstones from 225 patients, classifying the stones by gross inspection and infrared spectroscopy. Cholesterol stones were found to contain cholesterol, whereas pigment stones were composed of calcium palmitate and calcium bilirubinate. Positive bile cultures were found in 100% of brown pigment stones and in 10-33% of cholesterol stones. Both types of stones contained at least one species of betaglucuronidase-producing bacteria. The authors suggest that betaglucuronidase may play a role not only in brown pigment stones but also in cholesterol stones.

6.4.3 Comments on our results.

* Clinical examination

The clinical examination of patients entering the study has been carried out by the author in every case in order to afford uniformity in the collection of data. We calculated the duration of the disease on the basis of episodes of cholangitis or ultrasound or x-ray assessment in order to obtain an objective baseline for the start of symptoms independent of the personal interpretation of the examining physician.

* Selection for antibiotics

The presence of two groups of patients who received antibiotic prophylaxis or not and the criteria adopted for selection have been explained in details in the methods section and do not need to be re-discussded here. It should be otherwise underlined that the group of 70 patients not receiving preoperative antibiotic prophylaxis, even in the presence of risk factors for infection, represents something rather unusual in the recent literature and has allowed us to study the effects of the presence of bacteria in bile, the related perioperative complications and the association with CBD stones without the possible influence of high antibiotic levels in blood or bile.

* Surgery

All operations were performed by members of the same surgical team which had been working together for several years before the start of this study. Thus, the surgical techniques adoptedwere well standardized and should have no significant influence on the results. Also the sampling manoeuvres were adjusted after few initial cases and became standardized. The recording of precise CBD size was achieved by repeating several times until the measurements gave constant results.

* Problems encounterd with methods

The difficoulties encountered in the performance of the phospholipase assay and the reasons for changing the method are reported in the discussion of the experimental study.

Difficulties were also found in the study of gallstone composition. In the papers of Wosiewitz et al., (1983) and Cetta et al., (1983) we learned about the possibility of undertaking qualitative analysis of gallstone composition by way of X-ray diffraction

analysis. We therefore set up a cooperative project with the staff of Istituto di Mineralogia, Facolta' di Scienze, Universita' di Napoli. However, the scientists in this institution work mainly on inorganic material from common minerals, and were not familiar with the interpretation of the spectra of biliary stones. In order to get an experienced reader of the spectra of CBD stones and to learn the appropriate method, in 1987 the author made contact with professor Wosiewitz and travelled twice to University of Frankfurt am Main in Germany. Unfortunately on neither of these occasions was it possible to meet Professor Wosiewitz, nor to gain any information about the method for reading the X-ray diffraction analysis spectra of CBD stones. Moreover the primary CBD stones experimentally produced in two dogs in the experimental study carried out in London were delivered to Professor Wosiewitz's department in Frankfurt for assay, but these specimens were in fact lost and no information was obtained about the composition of this unique material. We therefore agreed with mineralogists in University of Naples to read the clinical spectra according to the published standards, as reported in the methods section and in this way we have obtained qualitative information about CBD stone composition.

* Bile microbiology:

Incidence

Overall positive bile cultures rates of 26% in the antibiotic group and 44% in the no antibiotic group are similar to those reported in the literature (Goswitz, 1970, Keighley et al., 1974; Keighley et al, 1977; Willis et al., 1984; Wells et al., 1989), and we note that in both groups patients considered at risk of developing infection had a significantly higher positive bile culture rate (Table 6.9): this, combined with the significantly higher number of high risk patients in the no- antibiotic group (Table 6.7), also indirectly explains the slightly higher positive bile culture rate in the no-antibiotic group.

Organisms

Bacterial species isolated in bile in our patients are similar to those described in the literature (Mason, 1968; Nielsen & Justensen 1976, Keighley, 1982 see ref). S. faecalis was isolated in bile in 3.7% of positive cultures in the antibiotic group and in 9.7% of the no-antibiotic group (Table 6.8). These figures agree with those reported by Blenkharn & Blumgart (1985), who called attention to a 5 to 15% incidence of S. faecalis in positive bile cultures in patients undergoing complex biliary procedures. By contrast our data do not confirm those reported by Morran et al (1984), who underlined an increased presence of Staphylococci in positive bile cultures. Polymicrobial bacterial growth ranged between 5.8% in the antibiotic group to 15.7% in the no-antibiotic group. While figures in the antibiotic group are similar to those reported in the literature (Haw & Gunn, 1973), the no-antibiotic group rate is considerably higher: however, it is unclear from our data whether this difference is related to preoperative antibiotic administration or to the presence of more high risk patients in the no-antibiotic group.

Risk factors

It should be also considered that no significant differences have been reported in multiple isolates incidence between high and low risk patients in both groups (Table 6.9): this suggests that the presence of risk factors for predicting infection has not affected significantly the incidence of polymicrobial growth and therefore that the relatively low presence of multiple isolates in antibiotic group is related to the antibiotic prophylaxis.

Diagnosis

In the no-antibiotic group the positive bile culture rate rose from 14.3% in patients with malignant disease to 100% in patients with benign biliary strictures (Table 6.13), in

accord with other results in the literature (Edlund et al., 1959; Goswitz, 1970; Kune & Schultz, 1974; Keighley et al., 1974; Goswitz, 1974; Nielsen & Justensen, 1976; Jackaman & Smith, 1980; Keighley & Blenkharn, 1988). However, a similar relationship did not apply to the antibiotic group (Table 6.12) who demonstrated a surprisingly high incidence of 62.5% of positive cultures in patients with a neoplastic lesion. However, two of five neoplastic patients with bile infection had previous biliary- enteric anastomoses and a third patient had GB stones associated with a neoplastic lesion.

* Postoperative infections:

Incidence

The overall postoperative infection rate was 34.8% in the antibiotic group and 52.4% in the no-antibiotic group. These figures are higher than those reported in the literature (Goswitz, 1970; Keighley, 1977). This may be partly because in this study we classified as postoperatively infected patients with positive cultures from wound swabs or drain fluids, those with clinical infection of the wound or distant infection and also those with only a persistent postoperative fever. No significant correlation was shown between bile cultures and overall postoperative infections: only in the no-antibiotic group was a slight increase of overall postoperative infection rate shown in patients with infected bile (62.5%) when compared to patients with sterile bile (46.1%). However, positive postoperative drain cultures were more than doubled in patients with infected bile in both groups, although without statistical significance (Tables 6.16 & 6.17). This finding accords with that reported by Haw & Gunn (1973) and Keighley et al., (1974) who reported increased positive postoperative culture rates in patients with infected bile. The same organisms were isolated in bile and postoperative cultures in 44.4% of cases in the antibiotic group and in 66.6% in the no-antibiotic group: this is well within the wide range reported by the literature which varies from a minimum of 9.5% reported by Willis et al. (1984) to 100% in the study of Goswitz (1970). Such a wide range in the reported data is probably due to the effect of antibiotic prophylaxis used in recent studies.

Risk factors

The preoperative clinical characteristics conventionally considered as predictive factors for infection in biliary tract surgery (Keighley et al., 1976) were associated with a significant increase of positive bile cultures in both the antibiotic and no-antibiotic groups (Table 6.9).

By contrast no correlation was found in this study between the presence of conventional risk factors and multiple isolates in bile (Tables 6.9) nor between conventional risk factors and postoperative infections (Tables 6.18, 6.19, 6.20, 6.21) in either the antibiotic and no-antibiotic group.

Duration of history

The significance of a long clinical history of gallstone disease has been carefully assessed in this study. While several authors have shown that prolonged disease is associated with bile infection, CBD stones and increased infective complications (Madden et al., 1968; Glenn, 1972; Saharia et al., 1976), others had found no such correlation (Edlund et al., 1959; Haw & Gunn, 1973; Nielsen & Justensen, 1976). Lygidakis (1982, 1983) has recently stressed the frequent occurrence of this condition in patients from the Mediterranean countries and reported an association between long clinical history, older age, bile infection and primary CBD stones (Lygidakis, 1982) or between bile infection and the number of previous biliary operations (Lygidakis, 1983). However, this problem has not previously been studied prospectively. The results of our study confirm that patients from the south of Italy as well as those from other Mediterranean areas frequently have a long history of gallstones disease. In the whole series of 174 patients we have found a clinical history longer than three years in 97/174 cases (Table 6.7), 95/159 in patients with gallstone disease, with an average duration of symptoms of 69 months in the whole series and a duration of symptoms of over 30 years in several cases. However, the interpretation of the possible correlations between a long clinical history and bile infection or postoperative complications is more difficult. In the antibiotic group positive bile cultures were more frequent in the presence of a long clinical history of gallstone disease, whether in association with other conventional risk factor or not, but this difference did not reach statistical significance. Polymicrobial bacterial growth showed a similar pattern (Table 6.10) and we note that no patient with a history < 3 years had multiple bacterial

species isolated in bile. This is probably due to the use of repeated courses of antibiotics to control episodes of cholangitis in patients with a long history of biliary stones. In addition a long clinical history was found to be significantly associated with postoperative infections, even in the absence of other, conventional risk factors (Table 6.20). By contrast, in no-antibiotic patients, a long history was associated with increased positive bile culture (Table 6.11) or postoperative infection rates (Table 6.21) only in presence of one or more conventional risk factors and this did not reach statistical significance. No association was seen between a long clinical history as an independent risk factor and postoperative infection rates (Table 6.21).

Different diagnosis affected overall postoperative infection rate which in antibiotic patients changed from 30% in cases with GB stones to 42% in neoplastic patients, to 66.7% in presence of CBD stones, to 100% in patients carrying benign biliary strictures. These differences did not reach statistical significance. No- antibiotic patients showed a similar pattern.

The performance of a biliodigestive anastomosis was associated with increased postoperative infection rate, whereas simple CBD exploration with subsequent external biliary drainage gave postoperative infection rates closer to those for cholecystectomy alone (Tables 6.24 & 6.25). This differs from Keighley's report (1977) of a significant increase of wound infection and positive blood culture rates after CBD exploration. Wells et al. (1989) also found an increased wound infection rate after CBD exploration, but similar wound infection rates after cholecystectomy and biliary bypass.

On the basis of this study we can conclude that the identification of a clearly defined group of patients at high risk of infection in biliary surgery is actually difficult and is related to particular characteristics for every series of patients, inclusing regional influences. This makes the selective administration of preoperative antibiotic prophylaxis

unreliable, a conclusion which has been drawn also by other authors (Stubbs, 1983; Murray & Bradley, 1983; Morran et al., 1984).

* CBD Size

The significance of CBD dilatation and its associations has been carefully assessed in this study. CBD size was associated with an increased positive bile culture rate and with an increased occurrence of polymicrobial growth in both antibiotic and no- antibiotic group of patients. However, while in no-antibiotic group increased CBD size was significantly associated with positive bile cultures (p=0.002) (Table 6.15), in antibiotic group this difference did not reach statistical significance for bile cultures (P=0.07), while was not calculated for polymicrobial growth because of a too small number of cases with this feature who had also calculated CBD size (table 6.14). This difference between the groups is probably related to some influence of antibiotic prophylaxis on bile cultures.

Glenn (1964) underlined an association between infections in biliary surgery and biliary obstruction due to CBD stones. However evident correlations between CBD size and postoperative infections have been not reported so far. In this study a significant correlation was shown between an increased CBD size and incidence of overall postoperative infections in antibiotic group (Tables 6.26), while Table 6.27 demonstrated no significant association in no-antibiotic group though showing a similar trend. We found a significant association between CBD dilatation and the presence of CBD stones and a direct correlation between CBD dilatation and age. By contrast no association was demonstrated between CBD dilatation and a history of gallstone disease (Table 6.30). This supports in our opinion the possibility that CBD dilatation may depend on primary papillary fibrosis (Arianoff, 1968) with subsequent impairment of papillary function in older people, while the absence of correlation between CBD dilatation and a long history of gallstone disease stands against the hypothesis of a papillary fibrosis secondary to the

passage of microcalculi (Hunt & Blumgart, 1980) or to surgical manoeuvres during previous attempts at papillary dilatation (Glenn, 1972 Imrie et al 1978). Moreover the association between CBD dilatation and the presence of CBD stones should alert the surgeon to the possible occurrence of overlooked stones and should probably suggest the performance of a drainage procedure.

* Biochemical studies:

Bilirubin deconjugation

We have found no correlation between bacteria, betaglucuronidase activity and unconjugated bilirubin levels in this study in GB or in CBD, in either the antibiotic or no-antibiotic group, or in the presence or absence of stones.

In GB bile there was no statistical correlation between conjugated or unconjugated bilirubin levels or in betaglucuronidase activity in patients with sterile or infected bile in both the antibiotic and the no-antibiotic groups (Tables 6.33 & 6.34). In the no-antibiotic group we found that betaglucuronidase activity was significantly higher in patients with stones, whereas unconjugated bilirubin was significantly higher in patients without stones (Table 6.35). However, in evaluating this striking finding it should be taken into account that only four patients fell into the subgroup without stones and that this quite small number of cases make formal statistical analysis impossible.

Biochemical assays of CBD bile demonstrated no correlation of betaglucuronidase activity and conjugated or unconjugated bilirubin levels in patients with positive or negative bile cultures (Tables 6.36 & 6.38), in patients with or without stones in the CBD (Tables 6.37 & 6.39), or in either antibiotic and no-antibiotic group. In addition linear regression

analysis showed no correlations with age or with CBD size, nor correlation between betaglucuronidase activity and unconjugated bilirubin levels.

It is not possible from these results to reject Maki's theory on pigment gallstone formation by way of a betaglucuronidase of bacterial origin. However, the data from this study do not support this hypothesis, either in the presence or absence of bile infection and with or without GB or CBD stones. Even in Maki's papers there are some points which do not support his own theory. Maki et al. (1962) report higher betaglucuronidase activity in patients with cholesterol stones than in patients with pigment stones. They explain this finding by a possible role played by tissue betaglucuronidase. The authors report also high betaglucuronidase activity in patients with neoplastic lesions and underline that these enzymes from different sources reach optimum activity at a different pH, 4.2 for tissue betaglucuronidase and 7.0 for bacterial betaglucuronidase. Moreover, Maki's "in vitro" experiment (1966) offers several points for discussion: the author reports that he stored bile in three test tubes, normal bile in the first, bile containing E coli in the second and bile with added betaglucuronidase in the third, and that biliary concrements were produced in the latter two. The finding of biliary precipitates in bile containing E. coli for some time does not of course establish a major role for the biological pathway suggested by the authors. Indeed, even in our study pigment stones have been found associated with higher bile bacterial colonization rate (Table 6.43) but this does not mean that these two phenomena are linked by the hydrolysis of bilirubin by a betaglucuronidase of bacterial origin.

Several other authors have re-examined Maki's theory. Soloway et al., (1977) reported hydrolysis of bilirubin in 40% of patients with pigment stones and Boonyapisit et al. (1978) reported elevated concentrations of unconjugated bilirubin in patients with pigment stones. It should be noted however that some years later Oyabu et al. (1987) underlined the presence of higher rates of unconjugated bilirubin in GB when compared to CBD bile and

concluded suggesting that this could be related to stasis of bile which normally takes place in gall bladder and that bile stasis itself could enhance bilirubin deconjugation. In this respect our study showed slight decrease of conjugated bilirubin in patients with larger CBD size in both groups, though without statistical significance. Similarly, unconjugated bilirubin has showed unchanged values even in presence of increased CBD size. Other studies have tried to find alternative explanations to Maki's theory: Masuda et al (1979) reported higher pigment stone rates in patients with positive bile cultures, but noticed that bilirubin deconjugation may also take place in absence of bacteria. Ho et al. (1986) observed betaglucuronidase activity only in some patients carrying bacteria and concluded that the enzyme may be sequestered into mixed micelles and therefore the activity measured is only the apparent enzymatic activity. Again, Oyabu et al. (1987) demonstrated hydrolysis of bilirubin in presence of polymorphonuclear leukocytes, and Nakano et al. (1988) found increased unconjugated bilirubin levels only after episodes of cholangitis, caused by betaglucuronidase producing bacteria.

Another factor that may affect this process and that may be in some way responsible for the apparently different results in different studies is the presence of substances inhibiting betaglucuronidase activity. Glucaro-1-4-lactone (Matsushiro, 1977) is produced by liver as result of glucaric acid metabolism: its increased consumption in parallel with the adoption of western diet in Japan has contributed to the decrease in pigment stones in that country: hence the hypothesis of high dietary consumption of this substance in our patients fed with a western diet and a possible explanation of the disagreement with the theory of enzymatic deconjugation of bilirubin.

Phospholipase

Phospholipase activity in GB and CBD bile showed no significant differences between patients with sterile or infected bile in both the antibiotic and no-antibiotic groups, while

phospholipase activity was significantly lower in patients with CBD stones when compared to patients with no CBD stones in the no-antibiotic group. These data correlate with those reported by Goswami & Frey (1974) who found an extremely low phospholipase activity both in patients with sterile and infected bile and by Groen (1989) who found extremely low levels of phospholipase both in cholesterol stone patients and in controls. These data do not disagree with those reported by Sjodal et al. (1978) who hypothesized that phospholipase could in normal conditions be contained within lysosomes and be delivered only under some pathologic conditions. Anderson et al. (1979) also report different values of phopholipase at different measurements and hypothesized a correlation of this phenomenon with phases of activity of the sphincter of Oddi. Finally data from our study do not agree with the study reported by Cetta (1984) who found a significant increase of phospholipase activity in patients with infected bile and brown pigment stones when compared to those with different black pigment, cholesterol or no stones and sterile bile.

Bile Acids

Total bile acids in GB bile showed no significant differences in patients with sterile or infected bile as well as in patients with or without stones and in CBD bile for the no-antibiotic group. In the antibiotic group total bile acids were significantly lower in patients with infected bile and a significant inverse correlation was found with age. Almond et al. (1973) reported a reduced bile acid pool in patients with stones and Soloway et al. (1977) reported lower bile acid concentrations in patients with cholesterol stones. However, possible relationships with bile infection were not reported. More recently Nakano et al. (1989) and Akyoshi et al. (1990) report a significantly lower total bile acid concentration in patients with brown pigment stones, which are frequently associated with bacteria in bile. A possible explanation could be that of an increased absorption of bile acids through the epithelium of an inflamed biliary tract. However, our data do not confirm this finding (Table 6.44).

* CBD Stones

There is wide agreement in the literature that brown pigment stones contain calcium palmitate whereas cholesterol and black pigment stones do not (Sutor & Wooley, 1971; Wosiewitz, 1983; Cetta et al., 1983; Ostrow et al., 1984; Maki et al., 1984; Cetta, 1986; Oyabu et al., 1987; Speer et al., 1988; Vitetta et al., 1989; Cetta, 1991). The presence of other substances such as cholesterol or calcium bilirubinate is not specific for a particular type of stone. Thus since brown pigment stones have been reported in western countries only as stones primarily originating in the CBD, in order to identify the incidence of primary CBD stones in our series we elected to classify as brown pigment stones only those which contained calcium palmitate.

Clinical Associations

The presence of CBD stones containing calcium palmitate was not significantly correlated with age. The clinical history of gallstone disease was slightly longer in patients carrying CBD stones, but was shorter in patients with stones containing calcium palmitate in contrast to the data of Lygidakis (1983) who found primary CBD stones associated with older age and a long clinical history.

Infection

In order to research the reported association between primary CBD stones and bile infection we have studied 13 patients in the no- antibiotic group whose stones underwent x-ray diffraction analysis. We have excluded the two patients in the antibiotic group whose stones were analysed because the sample is too small for comparison. Our data are in agreement with the literature since stones in the common duct have been found significantly associated with bile infection, this being further enhanced in the presence of

calcium palmitate stones (Table 6.43). These data confirm the correlation between infection and primary CBD stones, even though the pathways between these phenomena remain unclear.

CBD size; "Aschoff" grading

We found the mean CBD size significantly higher in patients with CBD stones than in patients without and also a greater CBD size in patients with stones containing ca palmitate than in patients with other stones. Furthermore the intraoperative allocation of CBD stones according to the Aschoff classification has proved to be reliable in 70% of no-calcium palmitate stones and in 80% of calcium palmitate-containing stones. These data are in agreement with those reported by Madden in 1968 and later by Lygidakis (1983) about the association of primary CBD stones with increased CBD size and about the reliability of the Aschoff classification of gallstones at least for distinguishing primary from secondary CBD stones (Madden et al., 1968). These data are of surgical significance since they suggest that the association between these characteristics may be used by the surgeon to recognize intraoperatively the presence of a primary CBD stone and therefore to undertake the proper surgical procedure.

7 BILIARY HISTOCHEMISTRY STUDY

7.1 INTRODUCTION

During the development of the two studies which have been reported so far, we were increasingly surprised by the data which did not confirm deconjugation of bilirubin by way of a bacterial betaglucuronidase as the initial step of primary CBD stone formation. Both before and during the period of these studies we found that several other authors found similarly conflicting results, and some tried to explain them by the possibility of non-bacterial hydrolysis of bilirubin in bile (Soloway et al., 1977; Boonyapisit et al., 1978; Masuda et al., 1979; Trotman & Soloway, 1982; Ho, 1985; Ho et al., 1986; Oyabu et al., 1987). We therefore elected to assess in a clinical study the possible role of a non-bacterial, tissue betaglucuronidase. We studied a small group of 19 patients with complex biliary and pancreatic disease attending the HPB Surgery Unit at Hammersmith Hospital, with the aim of correlating microbiological and biochemical findings in bile with histological and histochemical patterns in liver, gallbladder and bile duct.

6.4.4 Conclusions.

Several conclusions can be drawn from this clinical study.

1. Conventional risk factors for predicting infections in biliary surgery were associated with increased positive bile culture rates, but they were not associated with the presence of multiple isolates in bile or with increased postoperative infection rates. Increased postoperative infection rates were found in the presence of a long history of gallstone disease. In the subgroup of patients who received antibiotic prophylaxis this relationship was statistically significant. Furthermore, postoperative infection rates were not related to the diagnosis or to the procedures undertaken.

2. In this series increased CBD size was significantly associated with positive bile and postoperative cultures and also with age and the presence of CBD stones. The latter association was enhanced in presence of calcium palmitate containing stones.

3. Betaglucuronidase activity in bile did not correlate with the presence of infected bile, nor with stones, nor with changes in unconjugated bilirubin levels, in contrast with the hypothesis suggested by Maki. Similarly phospholipase activity did not correlate with the presence of stones or bile infection.

4. Total bile acids in GB bile did not differ between patients with sterile or infected bile, whether in the presence or absence of stones, whereas for CBD bile in the antibiotic subgroup they were significantly lower in presence of infected bile.

7.2 METHODS

7.2.1 Patients

Nineteen patients attending the Hammersmith Hospital HPB Surgical Unit form the object of this study. The characteristics of the population of patients are summarized in Table 7.1

Five patients presented with stones, three in the gallbladder, one in the common bile duct and one in both gallbladder and common bile duct. Eight patients presented with a neoplastic lesion, three in the head of the pancreas, one had a pancreatic carcinoid tumour, one an ampullary carcinoma, one a gallbladder carcinoma invading the common bile duct and two an hilar cholangiocarcinoma. One patient presented with an adenomyoma of the gallbladder. Finally one patient presented with a benign biliary stricture, one with chronic pancreatitis, two with choledochal cysts and one with recurrent cholangitis (Table 7.2.).

All patients received Piperacillin 4 g. IV on induction of anaesthesia.

Table 7.1

Population of patients

	male (n=9)	female (n=10)
Mean age (range)	54 (42-72)	48 (27-73)

5. Calcium palmitate containing stones were associated with older patients in this series. Bile infection and increased CBD size were significantly associated with the presence of stones in the CBD, this being further enhanced in the presence of calcium palmitate containing stones. Finally the macroscopic classification of stones proposed by Aschoff (1924) has demonstrated a reliability of 70% true negativity and 80% true positivity in recognizing calcium palmitate containing or primary CBD stones.

Table 7.2

Diagnosis

STONES	5	
GB		3
CBD		1
GB & CBD		1
BENIGN STRICTURES	1	
RECURRENT CHOLANGITIS	1	
CHRONIC PANCREATITIS	1	
CHOLEDOCHAL CYSTS	2	
GB ADENOMYOMA	1	
TUMOURS	8	
GB		1
HEAD OF THE PANCREAS		3
PANCREATIC CARCINOID		1
AMPULLA		1
HILAR CC		2

7.2.2 Sampling

Bile was collected with a sterile syringe from the gallbladder when present and from the common bile duct whenever possible, for microbiology and biochemical assays of conjugated and unconjugated bilirubin levels, and betaglucuronidase activity. Bile was usually collected from the gallbladder after removal, whereas it was collected from the common bile duct before opening the duct. Needle biopsies were taken from the liver and full- thickness open biopsies from the cut edge of the common bile duct. On occasion gallbladder tissues were also sampled. Tissues were processed for ordinary histology as well as for histochemical assay of betaglucuronidase.

7.2.3 Handling the samples and laboratory methods.

Samples of bile and tissues were processed as described in the experimental study (Sections 5.2.6 & 5.2.7). In order to classify results obtained from histological and histochemical assays we used the same criteria referred to in the methods section of the experimental study (Section 5.2.7).

7.3 RESULTS

7.3.1 Microbiology

GB bile was sampled for culture in 13 patients and was infected in 3 (23.1%). CBD bile was sampled in 15 patients and was infected in 3 (20%).

7.3.2 Biochemistry (Tables 7.3, 7.4; Fig. 7.1,7.2)

Biochemical assays in GB bile were carried out in 12 patients. Table 7.3 shows the results of GB bile biochemistry in patients with sterile or infected bile: no significant statistical correlations were found. In particular, there was no significant correlation between betaglucuronidase activity and unconjugated bilirubin levels (Fig 7.1).

Biochemistry of CBD bile was carried out in 15 patients. Table 7.4 shows the comparisons of biochemical assays in patients with sterile or infected bile, with no statistical differences amongst the groups. Similarly, no statistical correlation was found between betaglucuronidase activity and unconjugated bilirubin levels (Fig. 7.2).

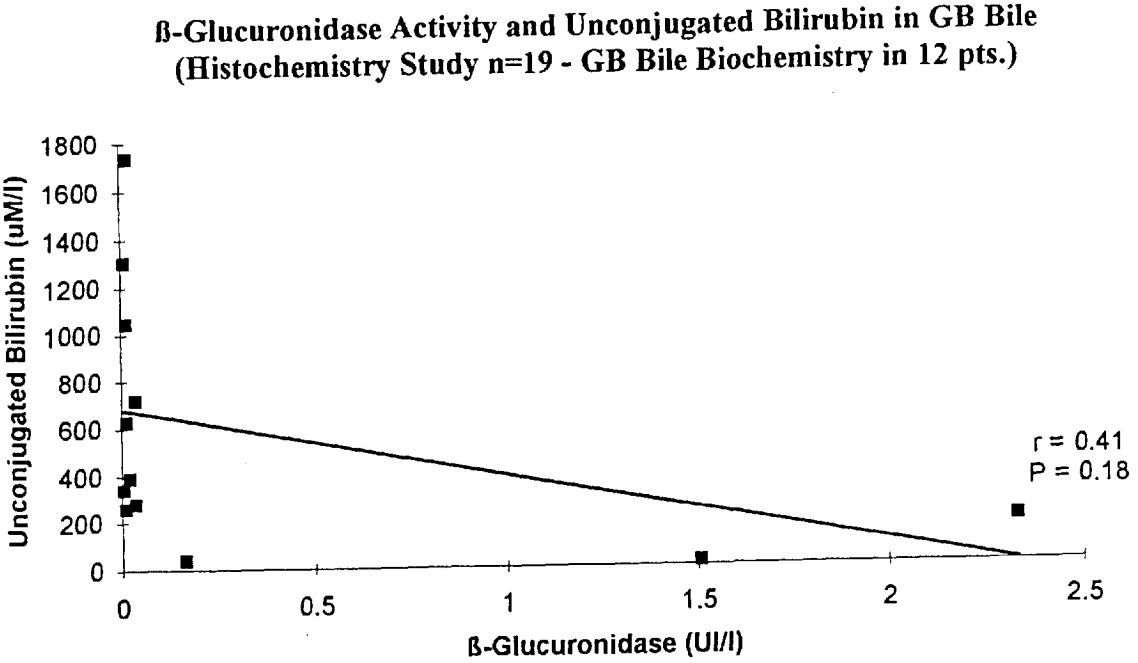


Fig.7.1 Betaglucuronidase activity and unconjugated bilirubin in GB bile.

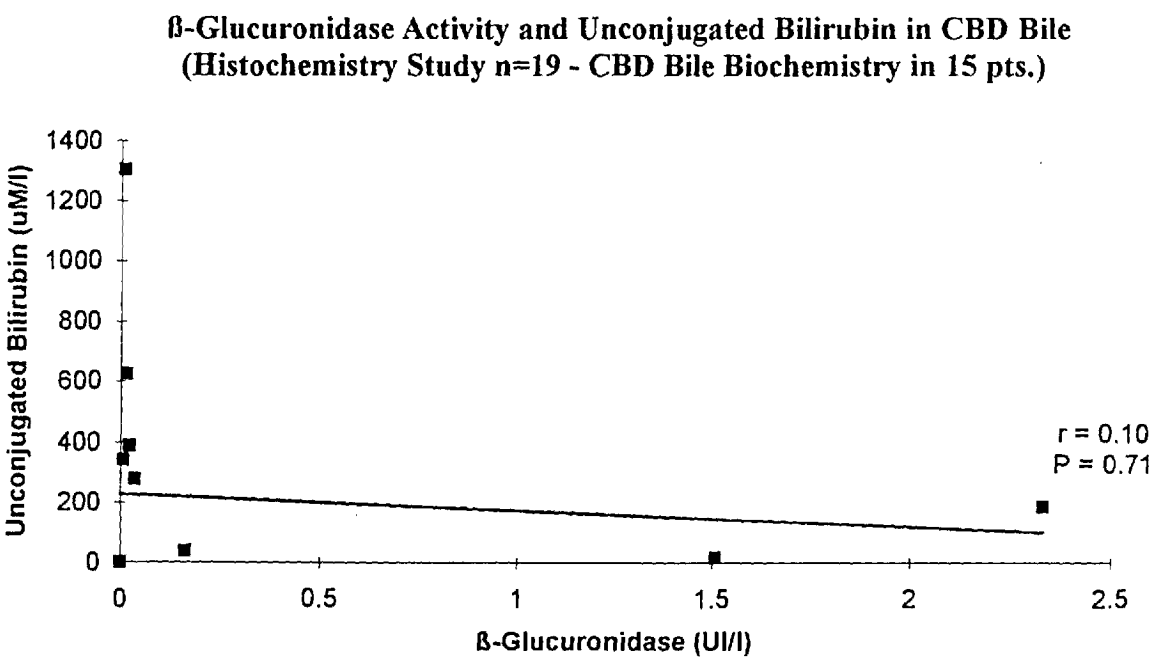


Fig.7.2 Betaglucuronidase activity and unconjugated bilirubin in CBD bile.

Table 7.3

Biochemical assays in GB bile (12 patients)

CULTURES	-VE (n=9)	+VE (n=3)	ALL (n=12)
TOTAL BILIRUBIN (uM/l)			
median	1976.1	965.8	1526.1
(range)	(85.2-7557.6)	(56-1048)	(56-7557.6)
CONJ. BILIRUBIN (uM/l)			
median	1300	704.7	1021
(range)	(46.4-5821.6)	(39-768)	(39-5821.6)
UNCONJ. BILIRUBIN (uM/l)			
median	624.4	261.1	366.3
(range)	(38.8-1736)	(17-280)	(17-1736)
BetaGLU. (UI/l)			
median	0.02	0.04	0.02
(range)	(0.01-2.33)	(0.01-1.50)	(0.01-2.33)

Table 7.4

Biochemical assays in CBD bile (15 patients)

CULTURES	-VE (n=9)	+VE (n=3)	ALL (n=12)
TOTAL BILIRUBIN (uM/l)			
median	353.7	596	381
(range)	(169.2-4588)	(380.7-752)	(169.2-4588)
CONJ. BILIRUBIN (uM/l)			
median	259.5	471	278.9
(range)	(99.2-2624)	(229.9-548.8)	(99.2-2624)
UNCONJ. BILIRUBIN (uM/l)			
median	101.4	150.8	105.3
(range)	(46-1964)	(125-203.2)	(46-1964)
BETAGLU. (UI/l)			
median	0.04	0.01	0.03
(range)	(0.01-0.47)	(0.01-0.05)	(0.01-0.47)

7.3.3 Histology (Tables 7.5-7.7; Fig.7.3-7.8)

Tissues were sampled for histological studies from liver, GB and CBD. Liver tissues were sampled from eight patients. In all these patients bile was also sampled for microbiological and biochemical assays. Bile cultures were positive in 1/8 of these patients and negative in 7/8 patients, this making impossible any statistical correlation with histological findings. Biochemical assays on CBD bile were also undertaken in all the eight patients who had liver histology done. Table 7.5 shows the correlations between histological findings and bilirubin levels or betaglucuronidase activity in CBD bile: no statistical association was demonstrated.

CBD tissues were sampled from seven patients and all had also CBD bile sampled for microbiology and biochemistry. CBD bile cultures were positive in two cases and negative in five. No association was demonstrated between the presence of infected bile and histological findings in CBD wall or between histological findings and changes in bilirubin levels or betaglucuronidase activity in CBD bile (Table 7.6). Similarly GB tissues were obtained in eight patients all having also microbiological and biochemical assays on GB bile done. GB bile cultures were positive in two cases and negative in six. Changes in GB wall histology were not associated with the presence of infected bile. Similarly bilirubin levels or betaglucuronidase activity showed no association with histological findings (Table 7.7).

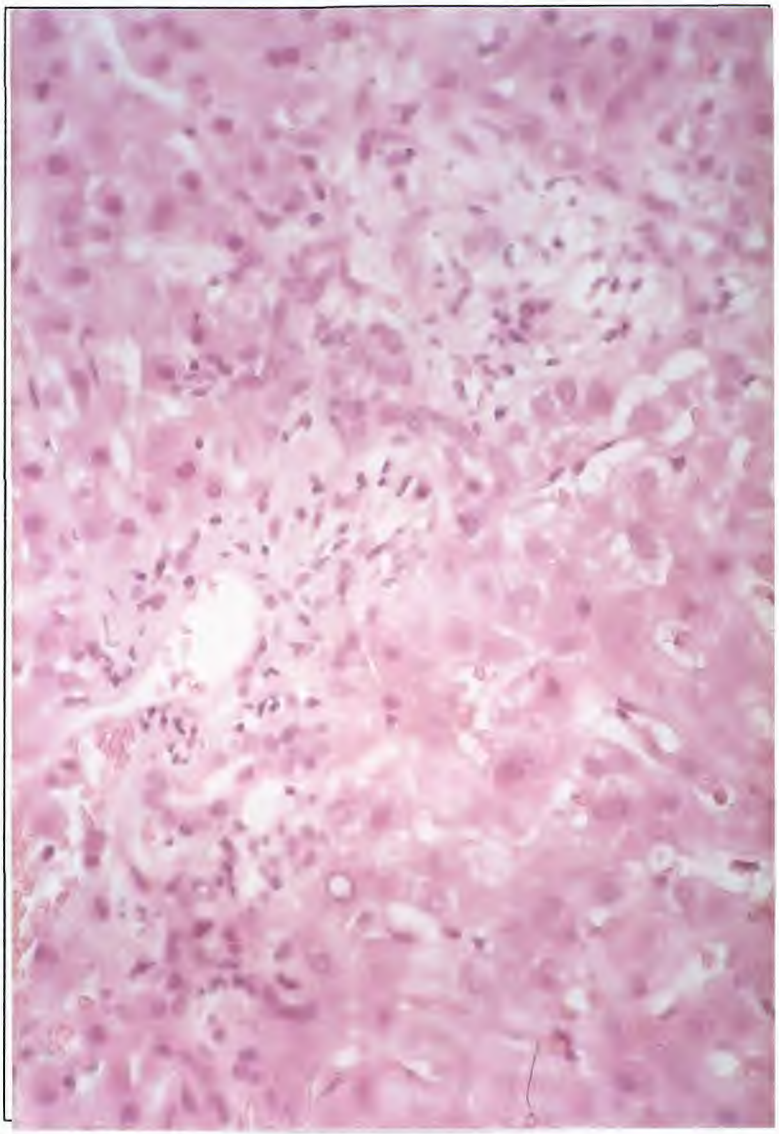


Fig.7.3 Liver histology: early fibrosis in portal space. Moderate inflammation and proliferation of bile ducts.

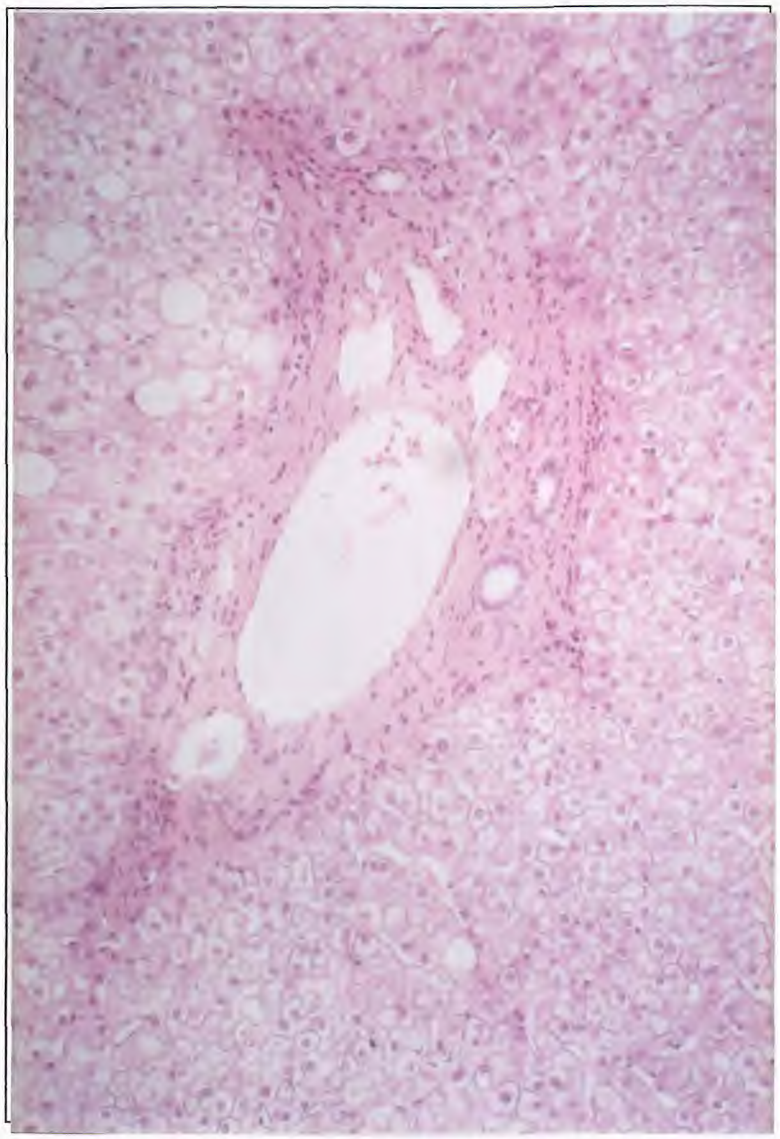


Fig.7.4 Liver histology: enlargement of the portal spaces with fibrosis. Moderate inflammation and bile duct proliferation.

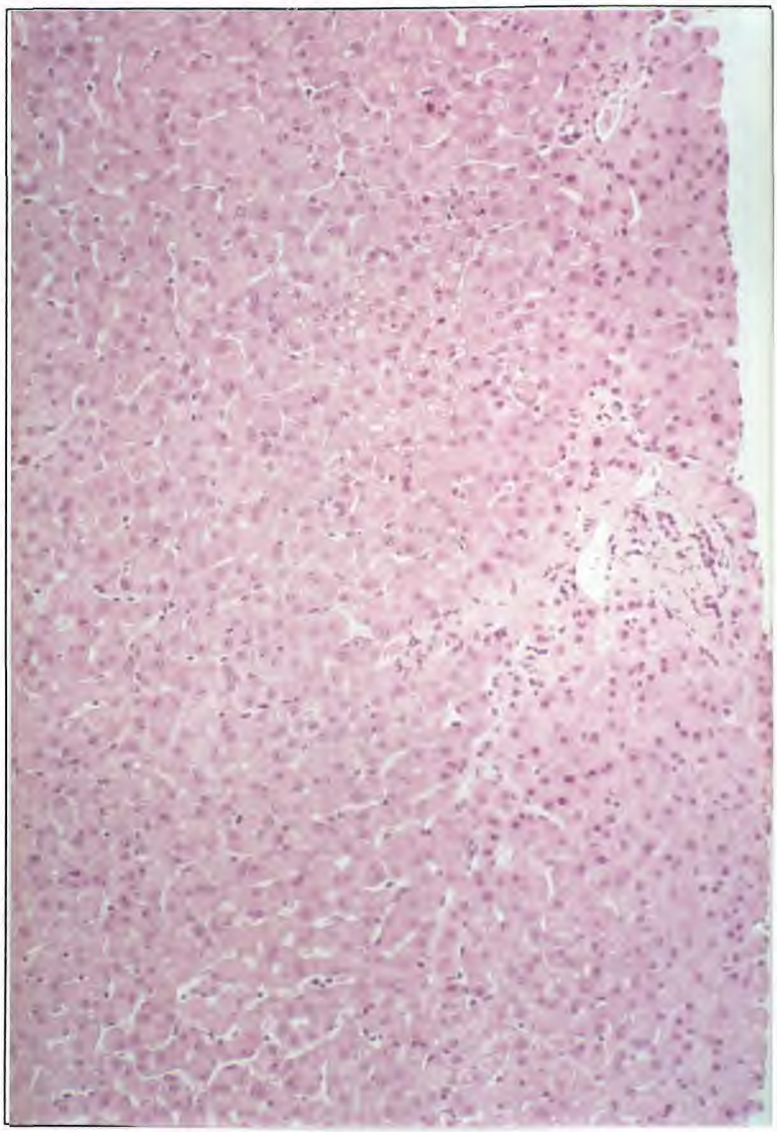


Fig.7.5 Liver histology: portal space with normal findings. Early fibrosis. No evidence of inflammation.

Table 7.5

Liver histology and CBD bile biochemistry (8 patients)

grade	n		tot.bil (uM/l)	conj.bil (uM/l)	unconj.bil (uM/l)	BetaGLU (UI/l)
EPITHELIUM						
gr.1	n=7	MD	381.5	279.8	101.7	0.03
		RG	(240-	(121-	(73-	(0.01-
			-2135.5)	-1461.9)	-6783.6)	-0.27
gr.3	n=0					
gr.5	n=1		169.2	119	50.2	0.04
INFLAM.						
gr.1	n=2		240,	121,	119,	0.27
			2135.5	1461.9	673.6	0.06
gr.3	n=6	MD	353.7	260	93.4	0.03
		RG	(169.2-	(119-	(50.2-	(0.01-
			-752)	-548.8)	-203.2)	-0.08)
gr.5	n=0					
PROLIFER.						
gr.1	n=2		240,	121,	119,	0.27
			2135.5	1461.9	673.6	0.06
gr.3	n=6	MD	353.7	260	93.4	0.03
		RG	(169.2-	(119-	(50.2-	(0.01-
			-752)	-548.8)	-203.2)	-0.08)
gr.5	n=0					
GRAN.TISS						
gr.1	n=7	MD	381.5	279.8	101.7	0.03
		RG	(240-	(121-	(73-	(0.01-
			-2135)	-1461.9)	-673.6)	-0.27)
gr.3	n=1		169.2	119	50.2	0.04
gr.5	n=0					
FIBROSIS						
gr.1	n=6	MD	386.5	299.2	110.3	0.04
		RG	(169.2-	(119-	(50-	(0.01-
			-2135.5)	-1461.9)	-673.6)	-0.27)
gr.3	n=1		325.9	240.2	85.7	0.03
gr.5	n=1		291.6	190.4	101.2	0.08

Table 7.6

CBD histology and CBD bile microbiology and biochemistry (7 pts.)

		n+ VE		tot.bil (uM/l)	conj.bil (uM/l)	unconj.bil (uM/l)	BetaGLU (UI/l)
EPITHELIUM							
gr.1	n=2	1		596, 169.2	471, 119	125, 50.2	0.05, 0.04
gr.3	n=1	0		391.6	318.6	73	0.01
gr.5	n=4	1	MD RG	566.7 (325.9- -2135.5)	414.3 (240.2- -1461.9)	152.4 (85.7- -673.6)	0.03 (0.01- -0.06)
INFLAM.							
gr.1	n=2	0		169.2, 381.5	119, 279.8	50.2, 101.7	0.04, 0.03
gr.3	n=4	1	MD RG	571.8 (325.9- -2135.5)	433.7 (240.2- -1461.9)	144.4 (73- -673.6)	0.02 (0.01- -0.06)
gr.5	n=1	1		596	471	125	0.05
PROLIFER.							
gr.1	n=3	0	MD RG	381.5 (169.2- -2135.5)	279.8 (119- -1461.9)	101.7 (50.2- -673.6)	0.04 (0.03- -0.06)
gr.3	n=2	1		752, 325.9	548, 240.2	203.2, 85.7	0.01, 0.03
gr.5	n=2	1		596, 391.6	471, 318.6	125, 73	0.05, 0.01
GRAN.TISS.							
gr.1	n=1	0		596	471	125	0.05
gr.3	n=3	1	MD RG	!391.6 (381.5- -752)	318.6 (279.8- -548.8)	101.7 (73- -203.2)	0.01 (0.01- -0.03)
gr.5	n=3	1	MD RG	596 (325.9- -2135.5)	471 (240.2- -1461.9)	125 (85.7- -673.6)	0.05 (0.03- -0.06)
FIBROSIS							
gr.1	n=1	0		169.2	119	50.2	0.04
gr.3	n=3	2	MD RG	596 (391.6- -752)	471 (318.6- -548.8)	125 (73- -203.2)	0.01 (0.01- -0.05)
gr.5	n=3	0	MD RG	381.5 (325.9- -2135.5)	279.8 (240.2- -1461.9)	101.7 (85.7- -673.6)	0.03 (0.03- -0.06)

Table 7.7

GB histology and GB bile microbiology and biochemistry (8 pts.)

		n+ VE		tot.bil (uM/l)	conj.bil (uM/l)	unconj.bil (uM/l)	BetaGLU (UI/l)
EPITHELIUM							
gr.1	n=4	0	MD	3120.8	2426.7	694.1	0.01
			RG	(85.2-	(46.4-	(38.8-	(0.01-
				-7557.6)	-5821.6)	-1736)	-0.16)
gr.3	n=3	2	MD	965.8	704.7	261.1	0.01
			RG	(56-	(39-	(17-	(0.01-
				-1926.4)	-1300)	-626.4)	-1.51)
gr.5	n=1	0		317.9	129.7	188.2	2.3
INFLAM.							
gr.1	n=3	0	MD	1125.8	783.1	342.7	0.02
			RG	(317.9-	(129.7-	(188.2-	(0.01-
				-7557.6)	-5821.6)	-1736)	-2.33)
gr.3	n=4	1	MD	1005.8	673.2	332.6	0.09
			RG	(56-	(39-	(17-	(0.01-
				-5115.9)	-4070.4)	-1045.5)	-1.50)
gr.5	n=1	1		965.8	704.7	261.1	0.01
PROLIFER.							
gr.1	n=4	1	MD	2716.9	2100	616.8	0.76
			RG	(56-	(39-	(17-	(0.01-
				-7557.6)	-5821.6)	-1736)	-2.33)
gr.3	n=3	0	MD	1125.8	783.1	342.7	0.01
			RG	(85.2-	(46.4-	(38.8-	(0.01-
				-1926.4)	-1300)	-626.4)	-0.16)
gr.5	n=1	1		965.8	704.7	261.1	0.01
GRAN.TISS.							
gr.1	n=5	0	MD	1125.8	783.1	342.7	0.02
			RG	(85.2-	(46.4-	(38.8-	(0.01-
				-7557.6)	-5821.6)	-1736)	-2.33)
gr.3	n=3	2	MD	965.8	704.7	261.1	0.01
			RG	(56-	(39-	(17-	(0.01-
				-1926.4)	-1300)	-626.4)	-1.51)
gr.5	n=0						
FIBROSIS							
gr.1	n=7	1	MD	1125.8	783.1	342.7	0.02
			RG	(56-	(39-	(17-	(0.01-
				-7557.6)	-5821.6)	-1736)	-2.33)
gr.3	n=1	1		965.8	704.7	261.1	0.01
gr.5	n=0						

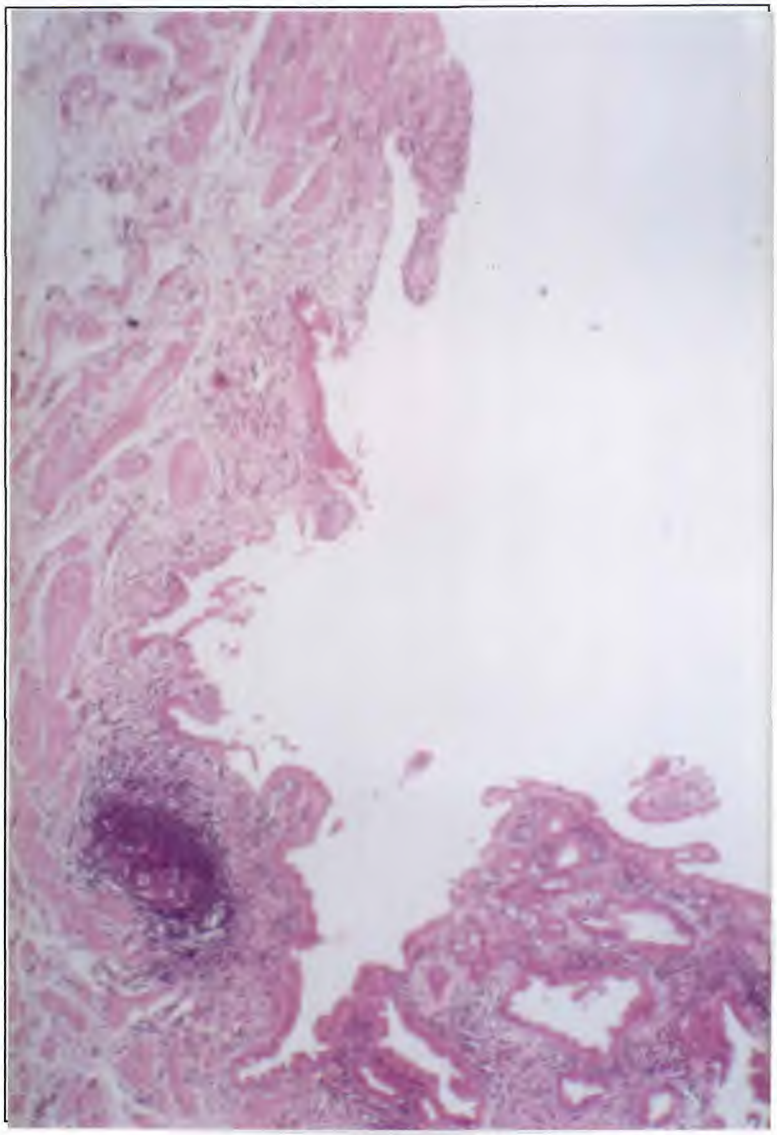


Fig.7.6 GB histology: GB wall of a patient with choledochal cyst. Moderate inflammation and loss of epithelium. Early fibrosis.

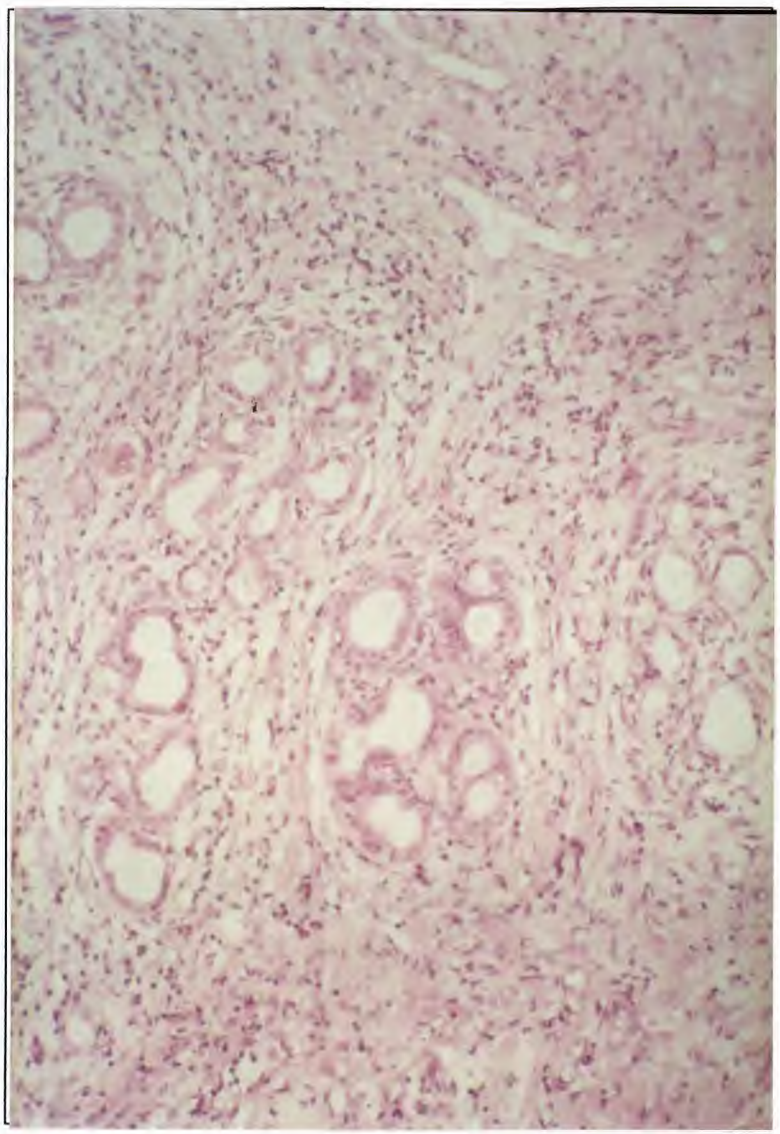


Fig.7.7 CBD histology: CBD wall of a patient with neoplastic biliary obstruction. Glandular proliferation with severe inflammation and production of granulation tissue. Moderate fibrosis.

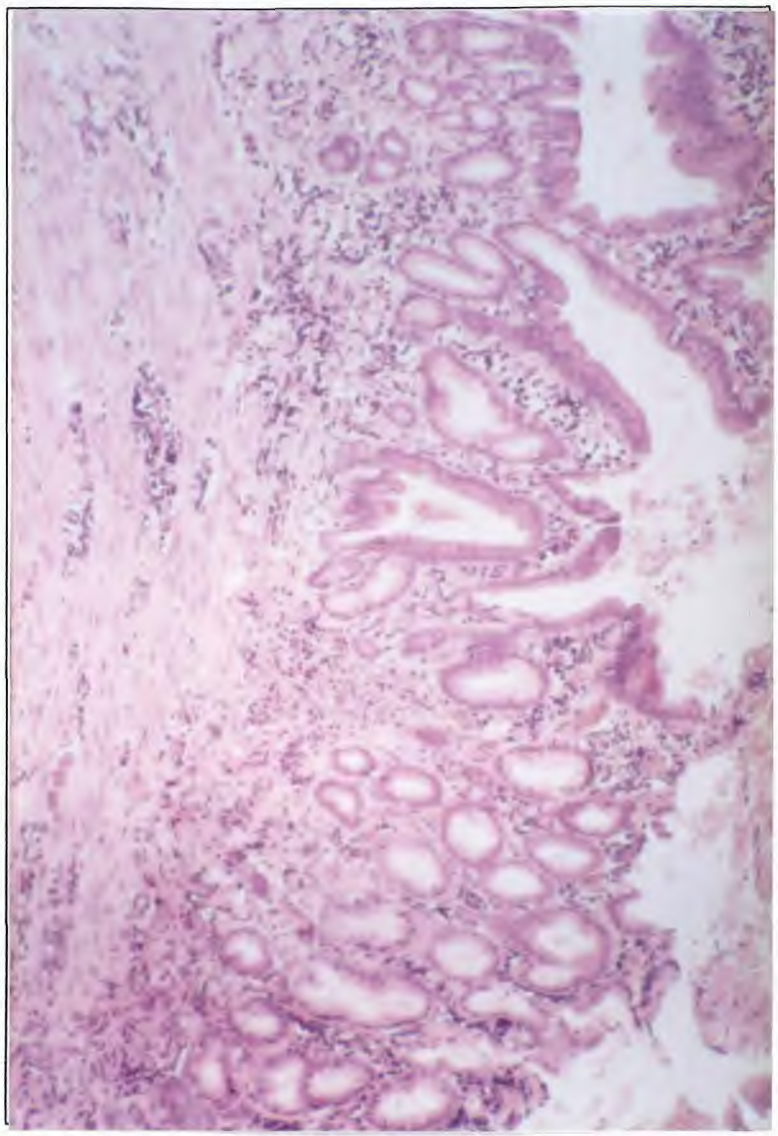


Fig. 7.8 CBD histology: CBD wall in a patient carrying CBD stones. Marked proliferation, moderate inflammation and fibrosis with hyperplasia of ductal epithelium.

7.3.4 Histochemistry (Tables 7.8-7.10. Fig. 7.9-7.12)

Histochemical assays of tissue betaglucuronidase were undertaken on liver, CBD and GB tissues. Liver tissues for histochemical assays were sampled from eight patients. All these patients also had CBD bile sampled for microbiology and biochemistry. Bile was sterile in seven patients in this subgroup and was infected in one: the presence of betaglucuronidase granules in liver tissues was similar in presence of sterile or infected bile (Table 7.8). Moreover, no correlation was found between the presence of betaglucuronidase in liver tissues and betaglucuronidase activity or unconjugated bilirubin levels in bile (Fig. 7.11, 7.12).

Table 7.8

Liver tissue Betaglucuronidase and CBD bile infection (8 pts.)

bile culture	-VE (n=7)	+VE (n=1)
MEDIAN	92	65
(RANGE)	(3-226)	

The presence of betaglucuronidase in CBD tissues was assessed in 10 patients (Fig. 7.10). CBD bile was sampled for microbiology and biochemistry in all these patients. No difference was found in the presence of tissue betaglucuronidase in the CBD wall between patients with sterile or infected bile (respectively 5/8 & 1/2). Similarly no differences in bilirubin levels or betaglucuronidase activity were found between patients who had tissue betaglucuronidase in CBD wall and in patients who had not (Table 7.9)

Table 7.9

Betaglucuronidase in CBD tissues and bilirubin and betaglucuronidase in CBD bile

	NO tiss. BETAGLU (n=4)	tiss. BETAGLU (n=6)
TOTAL BILIRUBIN (uM/l)		
median	571.8	300.9
(range)	(179.5-2135.5)	(169.2-596.0)
CONJ.BILIRUBIN (uM/l)		
median	433.7	235.1
(range)	(99.2-1461.9)	(119-471)
UNCON. BILIRUBIN (uM/l)		
median	141.7	93.7
(range)	(73-673)	(46-125)
BETAGLU. (UI/l)		
median	0.03	0.04
(range)	(0.01-0.06)	(0.02-0.27)

Betaglucuronidase in GB tissues was assessed in nine patients who all had bile sampled also from GB for microbiology and biochemistry. The presence of tissue betaglucuronidase did not differ between patients with sterile or infected bile (5/7 & 2/2 respectively). Betaglucuronidase activity and unconjugated bilirubin levels were higher in the presence of tissue betaglucuronidase (Table 7.10)

Table 7.10

Betaglucuronidase in GB tissues and bilirubin and betaglucuronidase in GB bile

	NO tiss. BETAGLU (n=2)	tiss. BETAGLU (n=7)
TOTAL BILIRUBIN (uM/l)		
median		965.8
(range)	1125.8,7557.6	(56-6465)
CONJ. BILIRUBIN (uM/l)		
median		704.7
(range)	783.1,5821.6	(39-5161)
UNCONJ. BILIRUBIN (uM/l)		
median		261.1
(range)	342.7,1736	(17-1304)
BETAGLU. (UI/l)		*
median		0.01
(range)	0.01,0.02	(0.01-2.33)

* P = 0.031 Mann-Whitney test

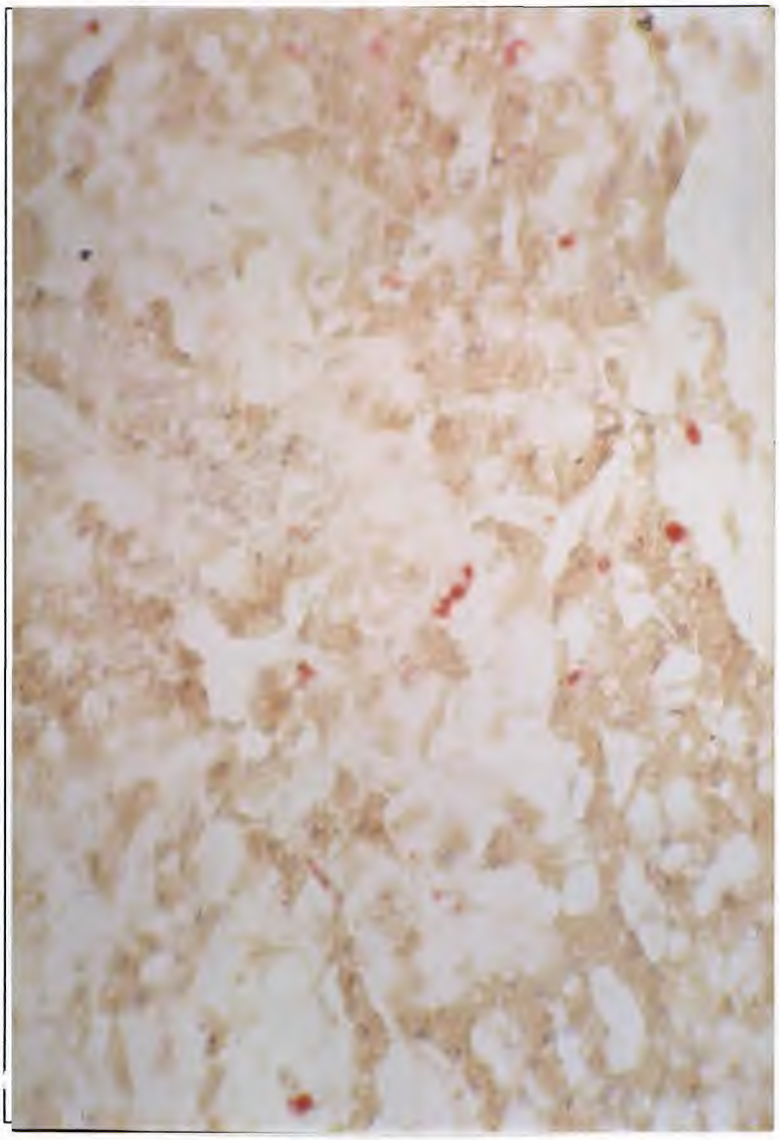


Fig.7.9 Liver histochemistry: betaglucuronidase granules are stained red.

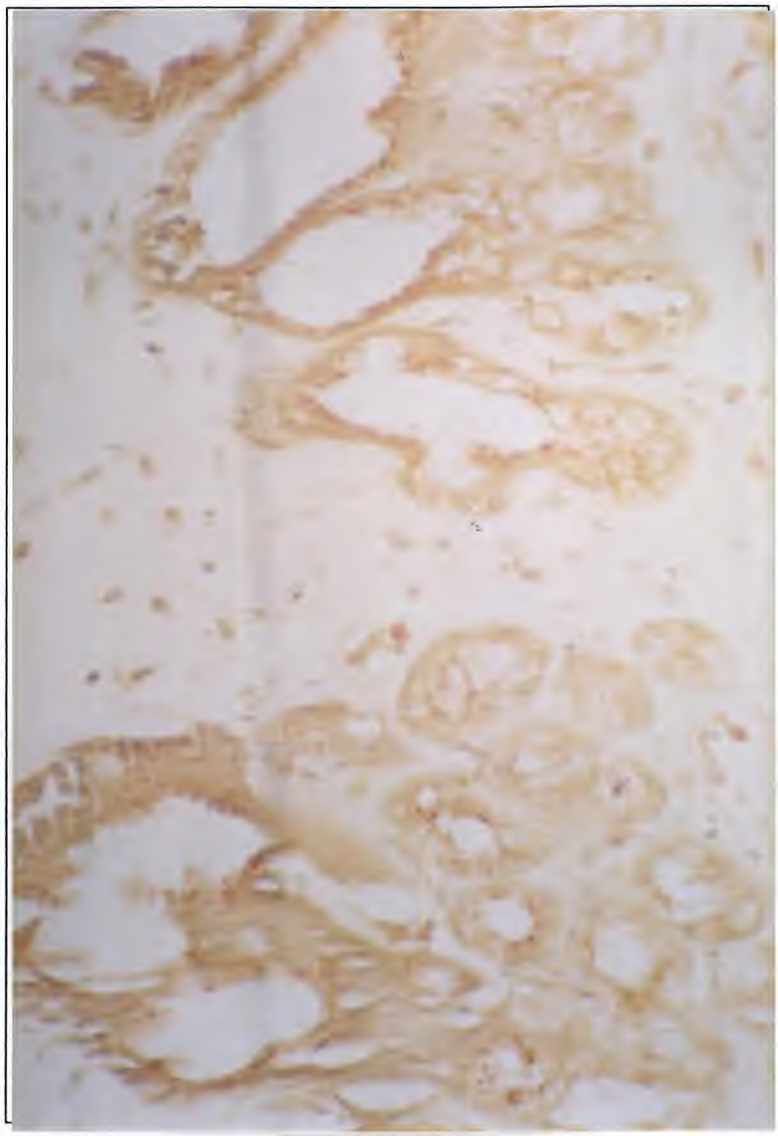


Fig.7.10 CBD histochemistry: betaglucuronidase granules are stained red.

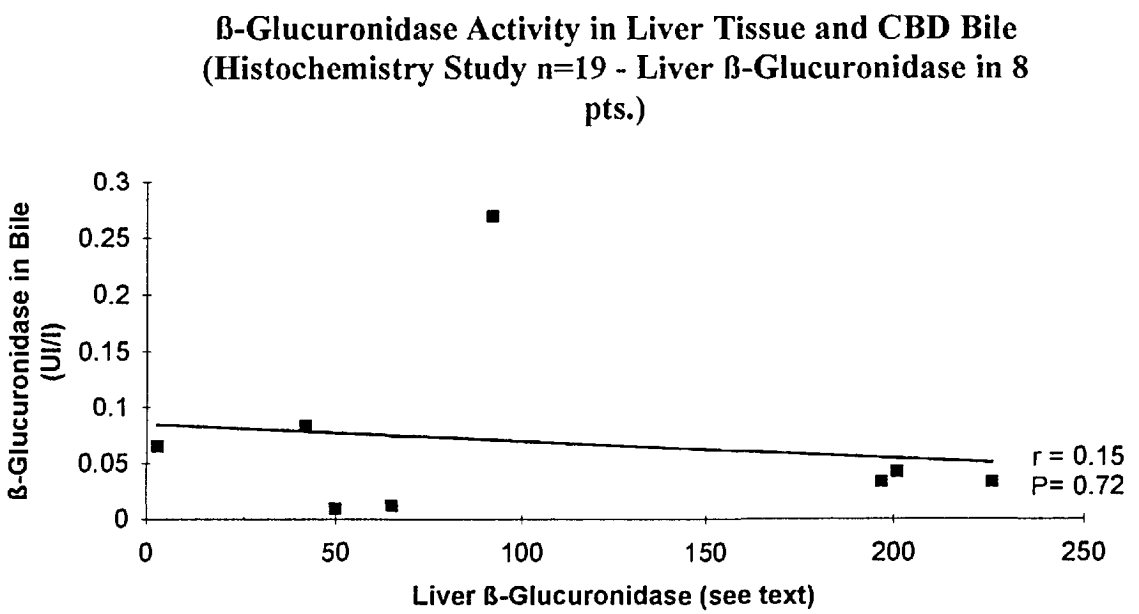


Fig.7.11 Betaglucuronidase activity in liver tissue and CBD bile.

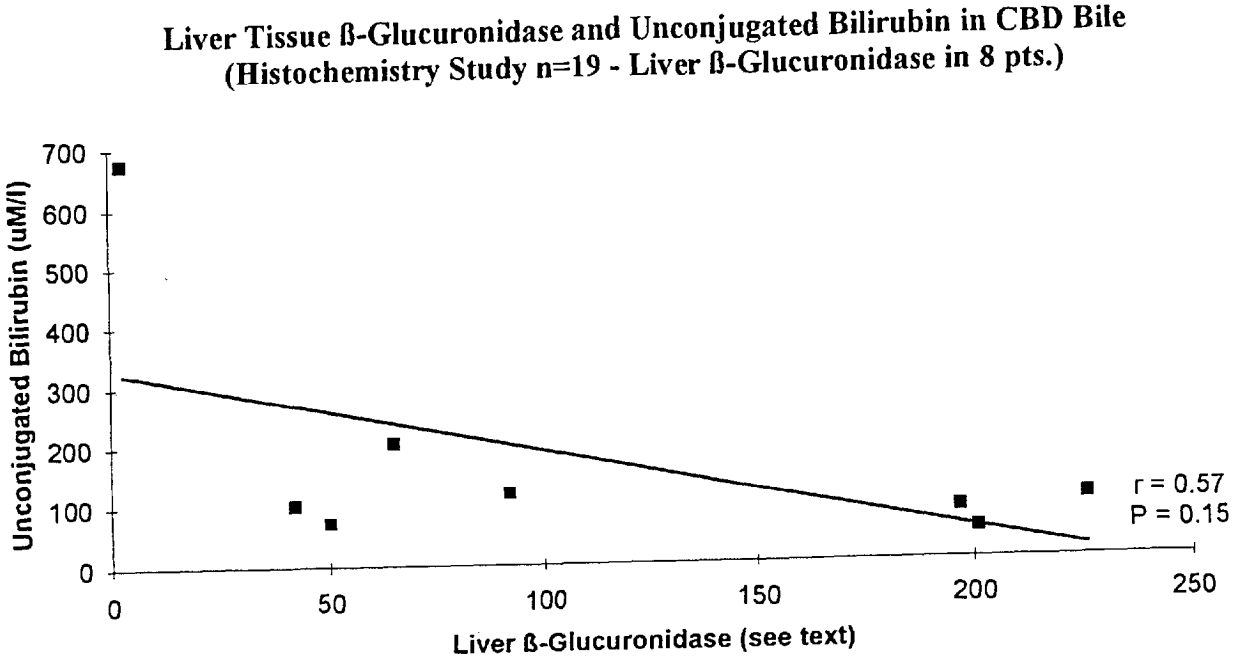


Fig.7.12 Betaglucuronidase in liver tissue and unconjugated bilirubin in CBD bile.

7.4 DISCUSSION

Although Maki's theory of deconjugation of bilirubin by way of bacterial betaglucuronidase is still widely accepted, a number of papers have arisen in recent years which indirectly challenge this hypothesis (Soloway et al., 1977; Boonyapisit et al., 1978; Masuda & Nakayama, 1979; Trotman & Soloway, 1982; Ho, 1985, Ho et al., 1986). Most of them, however try to explain the formation of calcium bilirubinate stones by the action of a betaglucuronidase of tissue origin. Masuda & Nakayama, (1979) conclude their study by suggesting that the hydrolysis of bilirubin diglucuronide may take place even in the absence of bacteria. Wosiewitz (1979) hypothesized a possible role of betaglucuronidase produced by hepatocytes or biliary epithelium: the betaglucuronidase activity could be enhanced by an acidification of biliary pH in presence of bacteria. Kouroumalis et al. (1983) searched for the presence of tissue betaglucuronidase in gall bladder epithelium in various pathological conditions and found higher enzymatic activity in the presence of acalculous cholecystitis. These authors concluded that in the presence of either cholesterol or calcium pigment gallstones it is possible to hypothesize a role for tissue betaglucuronidase in deconjugating bilirubin: the calcium bilirubinate could represent a considerable portion of pigment gallstones and also the nucleus of cholesterol stones. Shinya et al. (1985), in a paper from Maki's group, reported a high hydrolysis rate at pH 8 even in the presence of low bacterial betaglucuronidase activity and hypothesized a role of tissue betaglucuronidase in patients with tumours. Ho (1985) suggests the possibility that the considerable amounts of endogenous betaglucuronidase normally present in human liver may be activated at pH 7.0 by the catalytic action of some conjugated bile acids. The same workers (Ho et al., 1986) suggested that betaglucuronidase activity may be influenced by several external factors such as ionic strength, electrolytes, and Glucaro-1 - 4- lactone. The enzyme may also be sequestered in micelles and lose activity so that it is possible to measure only its apparent activity. Finally

Holmberg et al. (1986) found increased enzymatic activity in rats with acute biliary obstruction, both in hepatocytes and in Kuppfer cells, and also in the spleen.

In this study the overall positive bile culture rate was 23% for GB bile and 20% for CBD bile: this is in keeping with data usually reported in the literature. No statistical differences were found in biochemical assays in presence of sterile or infected bile. Similarly no correlation was found between betaglucuronidase activity and unconjugated bilirubin levels. These findings are similar to those reported in the other studies.

In this study histological findings were not associated with the presence of bacteria in bile. Similarly bilirubin levels and betaglucuronidase activity did not correlate with histological changes in liver, CBD or GB tissues. Other authors have suggested the possible release of betaglucuronidase from within the lysosomes during the development of an inflammatory process (Holmberg et al., 1986; Ho et al., 1986) : however, data in our study do not support this hypothesis.

The presence of tissue betaglucuronidase in liver and CBD was not associated with bile infection or with changes in bile betaglucuronidase activity or conjugated or unconjugated bilirubin levels and no association was found between infected GB bile and the presence of betaglucuronidase granules in GB tissues. In the presence of betaglucuronidase in GB tissues, higher betaglucuronidase activity ($P = 0.031$) and unconjugated bilirubin levels were found. However, only two patients fall in the subgroup of patients who had no tissue betaglucuronidase and therefore we consider this number too small to draw any conclusion. This finding however needs further elucidation.

The results of this study do not support a role for tissue betaglucuronidase in the deconjugation of bilirubin in bile. This is also supported in our opinion by the lack of correlation between the values of betaglucuronidase (of any origin) and unconjugated

bilirubin in bile. Other studies have supported this possibility, although most have not measured betaglucuronidase activity and unconjugated bilirubin levels in relation to the presence of brown pigment gallstones (Soloway et al., 1977; Boonyapisit et al., 1978; Masuda & Nakayama, 1979). When such measurements have been carried out they give rise to contradictory results. Thus Ho et al. (1986) stated that "high betaglucuronidase activity could be detected in some, but not in all of the patients with brown pigment stones and occasionally in patients with other types of stones. There are a host of factors that might contribute to such inconsistent observation of the betaglucuronidase activity...". Those observations therefore remain incompletely explained.

8 GENERAL DISCUSSION.

8.1 INTRODUCTORY

This thesis was therefore undertaken to achicve three principal aims:

1. To develop an experimental model of long-standing biliary obstruction.

2. To use this model to study the correlations amongst bile stasis, infection and stone formation.

3. To test the same relationships in man, in a surgical population.

Our dog model allowed predictable creation of a biliary stricture analogous to that found in the human situation, with long-standing partial obstruction. It afforded us the possibility of repeated cholangiographic checks and sampling of bile, as well as final examination of liver and biliary tissues.

In addition we had the possibility of collecting bile and stones for the same microbiological and biochemical studies from patients undergoing biliary surgery in Napoli.

Finally, during the development of this work, because of negative results in relation to the possible role of a betaglucuronidase of bacterial origin in deconjugation of bilirubin, we elected to study also the role of a non-bacterial tissue betaglucuronidase in liver, gall bladder and CBD. This was carried out both in the last part of our experimental study and in an additional clinical study carried out in London.

8.2 CHOLESTASIS.

Bile stasis in our studies both clinical and experimental was associated with infection. Whether this phenomenon is due to ascending infection from the small bowel or to a haematogenous route we cannot answer on the basis of our data. However, in our experimental study portal cultures were negative in all but one case, which would not support an entero-hepatic cycle of bacteria via the portal vein (Schatten et al., 1955).

Bile stasis has also been demonstrated to be associated with primary CBD stones. Our experimental CBD stones were produced by inducing a condition of incomplete biliary obstruction and in the clinical study an increased CBD size was associated with the presence of stones (primary or secondary) in the CBD. Moreover, the CBD size was greatest in the presence of calcium palmitate containing stones. This is perhaps the most important point of this work, since Aschoff in 1924 defined primary CBD stones as "stasis stones" and Imamoglu (1957) produced the only experimental primary CBD stones reported so far by inducing bile stasis in his animals.

8.3 INFECTION

In our study patients with CBD stones or benign strictures had significantly higher bile infection rates. Similarly, heavy bacterial colonization was always found when primary CBD stones were produced in dogs. Furthermore, infected bile was found in 80% of stones containing calcium palmitate and multiple organisms in 60%, this being also in keeping with figures recently published by Lombardo et al. (1992).

The conventional risk factors failed to predict post-operative infections in our clinical study, though they were significantly associated with the presence of bacteria in bile. Moreover the presence of bacteria in bile at operation in this study was not associated with increased postoperative infection rates as reported in other studies (Keighley et al., 1977). This may be due to the wide criteria that we have adopted for defining postoperative infections, which included not only positive postoperative cultures but also distant infections or postoperative fever. Another possible explanation is the presence of a long-standing history of gallstone disease in many of our patients. Even in our antibiotic treated patients this was found to be significantly associated with higher postoperative infection rates.

8.4 BIOCHEMICAL CHANGES IN BILE

Several studies have challenged Maki's hypothesis and the reasons for this have been extensively reviewed above. In our opinion it is possible that the role of enzymatic deconjugation of bilirubin by bacterial betaglucuronidase in the formation of brown pigment stones has been overestimated.

Phospholipase activity was extremely low in all the sections of this work, irrespective of the change in the enzyme fraction measured, in keeping with the findings of some other authors (Goswami & Frey, 1974).

Some others have also found lower total bile acid levels in the presence of CBD stones and cholangitis or have found a decreased total bile acid pool size with an increased fraction of free bile acids. Our studies do not support changes in the total bile acid pool size though we cannot comment on individual bile acid composition. Possible changes in total bile acid

behaviour in patients with clinical acute cholangitis together with stones, or in bile acids composition remain to be investigated further.

8.5 STRUCTURAL CHANGES

In our studies histological changes, was found not associated with impaired LFT nor with bile infection, in contrast with other authors who have reported definite changes such as bile duct proliferation or periportal fibrosis in presence of complete bile duct obstruction (Flinn et al., 1976; Koyama et al., 1981; Benjamin et al., 1988). However, our experimental model has been designed to produce an incomplete obstruction and we cannot exclude that this in longer time may produce structural changes similar to those reported by others.

The finding of reduced plasma albumin in the presence of bile infection is in agreement with data reported by Blumgart et al. (1984) about impaired liver function in patients with benign biliary strictures and an associated history of major infections. Moreover the presence of an impaired serum albumin has been reported by others (Dixon et al., 1983; Blamey et al., 1983) as a significant prognostic factor in biliary surgery and its recovery has been correlated with the presence of sterile bile (Halliday et al., 1987).

In this work no association has been demonstrated between tissue betaglucuronidase in liver, CBD and GB tissues with values of bilirubin and betaglucuronidase in bile, in the presence of infection or in the presence of stones. Thus, our data do not support the suggested role of a betaglucuronidase of non- bacterial, tissue origin in stone formation. This is confirmed further by the failure to find a correlation between unconjugated bilirubin levels and the activity of betaglucuronidase of either origin in bile.

8.6 STONES

This work has confirmed that primary CBD stones have been found both in clinical and experimental conditions associated with bile stasis and heavy bacterial colonization. Our data do not suggest that this possible link is represented by the deconjugation of bilirubin in bile, nor by changes in biliary lecithins related to the activity of phospholipase nor by changes in total bile acids composition.

The impossibility of analyzing the experimentally produced stones, for the reasons that we have explained above, represents a major gap in this work. However to reproduce experimentally the model and the primary CBD stones, with an adequate organization to obtain stone composition analysis and electron microscopy, has to be considered the most important objective of the future prosecution of this research.

Experimentally produced primary CBD stones have confirmed the typical macroscopical appearance previously reported (Aschoff, 1924; Madden et al., 1968; Trotman & Soloway, 1982), presenting with a yellow brownish colour, soft, greasy consistency, easily crushable with fingers, associated with biliary mud and sludge. In the clinical study, on the basis of Aschoff criteria we were able to classify as secondary stones seven out of 10 stones whose diffraction analysis demonstrated no calcium palmitate. These criteria also allowed to us to recognize a primary stone in 80% of cases confirming their clinical reliability.

8.7 SURGICAL PERSPECTIVE

What is the surgical message that comes from this work? Langenbuch, who in 1882 performed the first cholecystectomy explained the reasons for his "revolutionaire" operation suggesting that "the Gallbladder has to be taken out not because it contains stones, but because it produces stones" (Langenbuch, 1883). Similarly we have tried in this study to establish whether the CBD produces stones, when it produces them and how it produces them. We should now explain what behaviour the surgeon should adopt in the presence of such stones in the light of this study.

Primary CBD stones do exist, usually associated with a large sized common bile duct and a history of clinical infections. Furthermore in an acceptable number of cases it can also be recognized macroscopically by the surgeon at operation. Its incidence was 33% of the analysed stones in our population of patients. In these cases a permanent drainage operation should be recommended since we have seen in this study that stasis and bile infection in time led to the formation of such primary CBD stones.

However, what should one do in the presence of secondary CBD stones, which by definition are only "contained" and not "produced" by the CBD, and which represent the majority cases (67%) of the analysed stones in our series? On strict pathophysiological criteria in these cases there is no need for a permanent drainage procedure, and simple choledochotomy and stone extraction are adequate therapy. This was the usual policy adopted in XVI Divisione Ospedale Cardarelli, where I collected the data for this clinical series. On the basis of these results we may conclude that this surgical attitude was entirely appropriate in that historical context.

Now the times have changed and indeed there is mounting clinical experience (Vladimirof et al., 1990; Cotton et al., 1991) of series of successful endoscopic treatment of CBD stones. This may support the option of endoscopic rather than surgical drainage of the CBD in the presence of primary CBD stones and challenges even the concept of simple surgical stone removal in the presence of secondary CBD stones. This choice however rests also upon considerations of clinical strategy and is therefore outside the scope of this work.

9 CONCLUSIONS.

Trotman & Soloway (1982) have reported the results of a workshop on Pigment gallstones disease held at University of Pennsylvania in May 1981. That meeting ended indicating the future guidelines for research on this topic. The scientists involved suggested, amongst other issues, the following tasks:

a. investigate the structural and chemical composition of stones.

b. elucidate the role of stasis and/or infection in the formation of brown pigment stones.

c. undertake controlled epidemiological studies to define risk factors in various populations.

d. use animals models to provide mechanism of stone formation.

I had not yet read this paper when I planned the present study, but I was pleased to observe that the major questions that we have put to ourselves in the beginning of this work were very close to the issues suggested by the major experts on this topic. What then are the main conclusions which we can draw from this study ?

1. An original, reproducible model of chronic incomplete biliary obstruction has been developed in the dog, which allows cholangiographic examination of the persistence of the incomplete biliary obstruction as well as sampling of bile for microbiology and biochemistry.

2. Primary common bile duct stones have been experimentally produced on two occasions, associated with bile stasis and heavy bacterial colonization.

3. Primary CBD stones collected in the clinical study were also found to be associated with bile stasis and infection.

4. In the clinical study conventional risk factors for infection in biliary surgery were not found to be associated with increased postoperative infection rates; similarly no association was demonstrated between the presence of bacteria in bile and postoperative infection. By contrast, in a subgroup of patients a clinical history of gallstone disease longer than three years was found to be significantly associated with increased postoperative infection even in the absence of conventional risk factors.

5. Biochemical studies have demonstrated no changes in conjugated and unconjugated bilirubin and total bile acid levels and no changes were demonstrated in betaglucuronidase and phospholipase activities. Similarly, no correlations were demonstrated between betaglucuronidase activity and unconjugated bilirubin levels.

6. The presence of tissue betaglucuronidase in liver, GB or CBD was not found to be associated with changes in biliary betaglucuronidase or bilirubin.

7. The presence of chronic incomplete biliary obstruction was not associated with structural changes in liver or CBD tissues. However, impaired serum albumin levels were demonstrated in presence of bacteria in bile.

10 FUTURE PROPOSAL.

A major unresolved question remains about the composition of primary CBD stones. X-ray diffractometry should be undertaken on stones eventually produced again in our dog model. In addition it would be valuable to undertake scanning electron microscopy on these experimentally produced primary CBD stones. Other questions about biochemical changes in bile surrounding stones remain to be clarified. The role of Glucaro-1-4-lactone and its inhibitory effect on betaglucuronidase activity needs further elucidation, particularly under clinical conditions in a western population. Similarly the reported increased fraction of unconjugated bile acids (Akioshy, 1990) await further investigations: these studies would be most valuable in the presence of experimentally produced primary CBD stones.

The experimental model itself should be developed further: it is a common finding that endoscopically or percutaneously placed biliary stents for long-standing intubation of benign and malignant biliary strictures soon become obstructed by biliary sludge or mud, this obstruction being enhanced by the surface adhesive properties of bacteria present in an obstructed biliary tree, which form a bacterial biofilm on the surface of the obstructed stents (Speer et al., 1988). This has generated the need for self-expandable endoprostheses which in time could increase the size of the obstructed biliary tract improving the drainage of bile. However while few experimental studies have been carried out in the normal biliary tree to support the use of such devices, no report exists on its use in the presence of an experimental biliary stricture. The possibility of using our model for this purpose deserves some further attention.

Finally it is desirable to attempt to reproduce this model in a smaller animal such as rabbit, mini-pig, rat or guinea pig: it is unclear whether such a sophisticated model, inclusive of biliary obstruction and Thomas cannula and necessitating periodic cholangiography to validate the presence of the stricture, could be easily moved into other animals.

Nevertheless, this option deserves attention in the future, in order to find an animal which is easily managed, has low maintenance costs and equal reliability to the dog, which would allow the performance of further studies on the pathophysiology of a chronically incompletely obstructed biliary tree.

11 SUMMARY

These studies were undertaken in order to clarify under clinical and experimental conditions the incidence and the pathogenesis of the primary CBD stone.

Primary CBD stones are associated with bile stasis and infection, but the biological mechanisms which correlate these three phenomena are still unclear. One proposed possibility, based on "in vitro" work, is the deconjugation of bilirubin by a betaglucuronidase of bacterial origin. Free bilirubin combines with Ca++ and precipitates as calcium bilirubinate (Maki, 1966). However clinical and experimental studies have given rise to conflicting results. Another mechanism was suggested by the frequent observation that primary CBD stones invariably contained calcium palmitate, which might be precipitated as a result of hydrolysis of lecithin to lysolecithin by a biliary phospholipase. Finally it was unclear whether the total bile acid pool in bile also played a role in the process of primary CBD stone formation, as in the formation of cholesterol stones in the GB.

In order to reproduce the conditions of bile stasis and infections, we set up a model of chronic incomplete biliary obstruction in the dog. In eight dogs cholecystectomy was performed and a nylon sling passed around the common bile duct. A Thomas cannula was placed into the duodenum for periodic cholangiography and bile sampling for microbiology and biochemistry. Retrograde cholangiographies were carried out in every dog and demonstrated the presence of incomplete bile duct obstruction. At variable times dogs underwent a second operation for sampling of bile and tissues from liver, GB and CBD for histological and histochemical studies. Blood liver function tests were also carried out before every procedure.

All dogs studied developed an incomplete biliary stricture. Bile infection rates increased with the duration of the obstruction and reduced plasma protein levels were observed. Primary CBD stones were produced in two dogs in the presence of incomplete obstruction and heavy bacterial colonization of bile. However, no changes were observed in bilirubin and bile acids levels, nor in betaglucuronidase and phospholipase activities. Similarly no correlation was found between tissue betaglucuronidase in liver or bile ducts and betaglucuronidase activity or bilirubin levels in bile.

In a parallel clinical study we have also collected bile from 174 patients undergoing biliary surgery in Napoli, for microbiology and biochemistry as in the experimental study. Conventional risk factors for predicting infections in biliary surgery were associated with higher bile infection rates, but were not associated with increased postoperative infection rates. Post- operative infection rates were higher in a subgroup of patients with a clinical history of biliary disease longer than three years. Biochemical studies on bile demonstrated no changes in bilirubin and bile acids levels nor in betaglucuronidase or phospholipase activity in the presence of bile infection or stones, in agreement with the findings of the experimental study.

Finally, in a further clinical study carried out in London on 19 patients, the presence of betaglucuronidase in liver, CBD and GB tissues was not found to be associated with changes in bilirubin or betaglucuronidase in bile, in agreement with the results of the histochemical study in dogs.

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13 APPENDICES

13.1 METHODS

13.1.1 Introductory

This work is formed by three different studies, for which data have been collected in London as well as in Naples. While for bile biochemistry all the specimens have been assayed in London, for bile microbiology the specimens from the clinical study have been assayed in Naples and therefore the different methods are reported here accordingly.

13.1.2 Microbiology (Experimental study, RPMS, London)

* Bacterial Culture

Reference:
Cowan, 1986.

Culture Media:

1.Blood Agar Base, CM55 (Oxoid Ltd, Basingstoke), supplemented with 5% v/v defibrinated horse blood.
2.Schaedler Agar, CM437 (Oxoid Ltd, Basingstoke), supplemented with 5% v/v defibrinated horse blood and 25 microg./ml.neomycin sulphate.
3.MacConkey Agar, CM7 (Oxoid Ltd, Basingstoke)
4.Sabouraud Dextrose Agar, CM41 (Oxoid Ltd, Basingstoke), supplemented with 400microg./ml chloramphenicol.
5.Thioglycollate Medium USP (Oxoid Ltd, Basingstoke).

Method:

Gram stained films of each specimen were examined for bacteria. A minimum of 5 microl from each specimen was plated on to the following media using a standardized radial plating technique.

media	atmosphere	min.incub.time(hrs)
Blood Agar	aerobic	24
Blood Agar	anaerobic	48
Neom.Bl.Agar	anaerobic	48
MacConkey Agar	aerobic	24
Sabouraud Agar	aerobic	48

Additionally, the residual portion of each specimen was inoculated into a 50ml volume of thioglycollate broth and incubated for at least 48 hours prior to subculture on to agar media. The amount of growth was recorded semiquantitatively according to the following scheme

1+<10(3) colony forming units/ml
2+ 10(3)-10(5) colony forming units/ml
3+ 10(5)-10(7) colony forming units/ml
4+>10(7) colony forming units/ml

All isolates were subcultured for identification according to standard criteria (Cowan, 1986).

* Gas-Liquid Chromatography of Volatile Fatty Acids

Reference:

Holdeman et al, 1977.

Principle:

Short chain volatile fatty acids (VFAs), typical of the growth and metabolism of obligately anaerobic bacteria, are extracted into diethyl ether and separated by gas liquid chromatography. The presence of any combination of VFAs (propionic, isobutyrric, isovaleric, valeric and isocapronic acids) is considered indicative of the presence of obligatory anaerobic bacteria. Genus and species level identification of pure cultures of obligately anaerobic bacteria is facilitated by the qualitative and semiquantitative detection of VFAs from overnight peptone yeast extract-glucose broths, together with data from appropriate fermentation reaction (Holdeman et al, 1977).

Apparatus:

Pye Unicam series 104 gas chromatograph

Column:

2.5m. X 3mm glass column packed with 25% neopentyl glycol adipate on 20% phosphoric acid treted 100-120 mesh Celite

Column temp:	120 C.
Carrier gas:	Nitrogen (oxygen free)
Detector:	Flame ionization
Detector temp:	140 C.

Reference solutions:

acetic acid	CH3COOH
propionic acid	CH3CH2COOH
isobutyrric acid	(CH3)2CHCOOH
butyrric acid	CH3CH2CH2COOH
isovaleric acid	(CH3)CHCH2COOH
valeric acid	CH3CH2CH2CH2COOH
3-methyl valeric acid	(CH3)2CH2CHCH2COOH

Technique:

Specimen preparation and extraction procedure.
One ml of the specimen (blood, bile tissues etc) is mixed thoroughly with an excess of sterile distilled water . To this is added a few drops of 25% v/v aqueous orthophosphoric acid (H3PO4) and of saturated aqueous sodium chloride solution. An equal volume of diethyl ether ((C2H5)2 O)is added and the two immiscible phases mixed gently for 1 minute. After brief centrifugation to aid separation,of ethereal phase, this is removed, dried over magnesium sulphate (MgSo4.H2O) and evaporated to approximately 50microl under nitrogen.

Gas liquid chromatography:

Two microl of the dried ether extract of each test specimen was injected onto the column. After elution of each compound the identity and relative concentration were determined by comparison of the peak height and retention time, with the chromatogram obtained from the reference standard.

Interpretation:
The presence of volatile fatty acids identical (by retention time) to those in the reference solution , but with the exception of the acetic acid was considered indicative of the presence of the anaerobic bacteria in the specimen. For genus and species identification chromatograms were compared with the data of Holdeman et al., (1977).

13.1.3 Microbiology (Clinical study,Ospedale Cardarelli, Napoli)

Reference:

Holdeman et al., 1977.
Aldridge et al., 1978.

Culture media:

1.Tripticase Soy Agar (TSA) (Becton- Dickinson, Milano, Italy) supplemented with 5% v/v tup blood.
2.MacConkey Agar(Becton-Dickinson, Milano, Italy).
3.SabouraudDextrose Agar(Becton- Dickinson, Milano, Italy).
4.Wilkins-Chalgren Agar (Oxoid Ltd, Basingstoke) supplemented with 5% v/v tup blood.

Method:

Aliquots of at least 10 ul of bile were cultured in the above media and incubated as follows:

media	atmosphere	min.incub.time (h)
TSA	aerobic	48
MacConkey Agar	aerobic	48
Sabouraud Agar	aerobic	48
Wilk.Chal.Agar	anaerobic	72

Colonies were identified by the following systems:

Staphylococci	coagulase production (Sclavo Spa,Siena)
	API 20 Sthaph. (Bio-Merieux, Roma)
Streptococci	Streptex (Wellcome Ltd, Dartford)
	API 20 Strept. (Bio-Merieux, Roma)
Gram-ve aerobes	API 20 NE & API 20 E (Bio-Merieux, Roma)
Anaerobes	API 20 A (Bio-Merieux, Roma)

13.1.4 Blood Liver Function Tests (Experimental study, RPMS, London)

* Bilirubin

References:
Simmonds, 1968; Billing et al, 1971.

Principle
(Van der Bergh reaction): automated version of Michaelsson method
Bilirubin + diazoreagent---------->
------>Diazobil. + red purple (in acid medium) or intense blue purple in alkaline conditions.

Reagents:
1.Caffeine reagent
To approximately 400ml of water at 50-60 C add 37.5g caffeine, 57g. sodium benzoate and 94.5g. sodium acetate. Mix, allow to cool and dilute to 1 L. with water. Stable for at least 6 months.
2.Sulphanilic acid stock solution
Add 10g. of Sulphanilic acid and 15 ml concentrated HCl to water. Mix and dilute to 1 L.
3.Sodium nitrite 0.5%
Dissolve 5g sodium nitrite in water and dilute to 1 L.. Store in a refrigerator in a stoppered container. Stable for 1 month.
4.Diazo reagent
Mix 2.5 ml 0.5% sodium nitrite with 100ml sulphanilic acid stock solution. Make up fresh.
5.Ascorbic acid 4%
Dissolve 1g. ascorbic acid in water and dilute to 25 ml. Make up fresh.
6.Alkaline Tartrate buffer
Dissolve 50 g. NaOH and 175g potassium sodium tartrate in water and dilute to 1L.. Stable at least for 6 months.
7.HCl 0.05M
8.Bilirubin standards
Frozen dried commercially prepared bilirubin standards were used for standard curve calibration.

Apparatus:

Technicon Auto analyzer AA1

Procedure:
The sample line aspirates 0.16ml/min so that about 0.3 ml is required for three runs, total conjugated bilirubin and sample blank. Samples are run at up to 60/hours. As reagents are aspirated in a different order for each of the three,

changing the order is facilitated if the manifold is mounted on platter with lines numbered. When a change is made, wash water through rapidly for about 10 minutes. For specimens with bilirubin concentration more than 250microM./L (about 15mg/100ml) dilute the serum and re-run. Protect samples from light during preparation and analysis. A blackened cover plate is desirable as bilirubin is sensitive to light.

* Plasma proteins

Reference:

Reinhold, 1953.

Principle:
When a solution of protein is treated with Cu ions in a moderately alkaline medium, a colored chelate complex of unknown composition is formed betweeen Cu ion and the Carbonyl (- C=O). and the (=N-H) of the peptide bonds. The intensity of the color produced is proportional to the number of peptide bonds undergoing reaction. Thus the biuret reaction can be used as a basis for a simple and rapid colorimetric method for determining protein.
Reagents:
1.Sodium chloride (0.85%)
2.Standard protein
3.Biuret diluent KI 0.5% in 0.25M NaOH
4.Alkaline tartrate, Potassium sodium tartrate in biuret diluent
5.Biuret reagent
3g Cupric sulphate in 70 ml water.
9g Potassium sodium tartrate in 700ml biuret diluent. Mix together and filter.

Procedure:

	Test	Test blnk	Reagent blnk
saline	1ml	1ml	1.1ml
sample	100ul	100ul	-
biuret	4ml	-	4ml
alk.tartr.	-	4ml	-

mix; allow colour to develop for 30 min. at room temperature. Read absorbance at 550nm using either water or the reagent blank as blank. Set up standards and read concentration of protein samples against standard curve.

*Albumin

Reference:

Doumas et al., 1971.

Principle:

Determination of Albumin using bromocresol green manual method.

Reagents:

1.Succinate buffer, pH 4.10
2.BCG dye solution 10 mmol/l. Dissolve 1.75g BCG in 5ml 1 mol/l sodium hydroxide and make up to 250 ml with water.
3. 25% Brij 35
Working BCG solution: 100 ml succinate buffer, 8 ml BCG preparation, 2.5 ml Brij 35 and made up to one litre.

Procedure:
Add 20 ul sample to 4 ml working dye solution, mix, stand at room temperature for 10 minutes and read the extinction at 632 nm against a blank of working dye solution. Put up standard curve using bovine albumin in water.

*Alkaline Phosphatase

Reference:

N.W. Tietz, 1970

Principle:
p-nitrophenylphosphate + alk.phosphatase--->
---->phosphate ion + p-nitrophenylate
(the latter at alkaline pH changes from the colorless benzoid form to the yellow quinoid form)

Reagents:

1.Glycine buffer, 0.10 M containing Mg++, pH of 10.3 to 10.4 at 37C. Dissolve 7.50g of highest purity glycine and 0.203 g of MgCl2 6h20 in about 750ml of water. To this solution add 85 ml of 1.0 N NaOH. Adjust the pH at 10.5-10.7 at 25C. (or at 10.3-10.4 if measured at 37C.) by adding more NaOH or 1.0 N HCl (Glycine buffer has a large temperature coefficient).Dilute the solution to 1 liter. A few drops of chloroform may be added as a preservative.
2.Stock substrate. A freshly prepared solution of disodium salt of paranitrophenyl phosphate, concentration = 4.0mg./ml=15.2 uM/ml. Prepare just enough to provide a two weeks' supply of the buffered substrate (reagent 3). The chemical is unstable, but high purity, stabilized preparations are available from a number of commercial sources in gram lots and in the form of 100 mg. capsules.The solid should be kept dry and stored in the dark at below 4 C.
3.Buffered substrate. Prepare the buffered substrate by mixing equal portions of glycine buffer and stock substrate. Check the pH as before, and adjust the pH if necessary. Pipett 1.0 ml.portionts into a series of 15X125mm. test tubes. Stopper the test tubes, store them in a freezer and use as needed. Alternately prepare enough for the day's work and use immediately. Buffer concentration = 0.050M; Mg++=5X10-4M;p-nitrophenyl phosphate= 7.6 micromol./ml.
4.Sodium Hydroxide, 0.02 N. Prepare by diluting any standardized stock NaOH solution.
5.Stock standard. Use 0.010 M p-nitrophenol in water. Dissolve 0.3479 gm. of pure crystals in 250 ml of water.Specially purified crystals are available from several commercial sources. The standard is stable for six months if kept frozen and in the dark.
6.Hydrochloric acid, concentrated (12 N).

Procedure:

wawe length 405nM
glass cuvette 405 light path
temperature of incubation at 37 C

	test	blank
substrate	1ml	1ml
sample	1ml	-

mix,incubate at 37 C for exactly 30min. Add:

NaOH	10ml	10ml
sample	-	0.1ml

mix, read absorbance of text agaist blank.
calculation: mU/ml=E*200.

*Glutamate-Oxalacetate-Transaminase (GOT)

Reference:

Reitman & Frankel, 1956.

Principle:

After a fixed time the 2,4dinitrophenylhydrazone of the reaction product, formed oxaloacetate, is determined colorimetrically in alkaline solution. Some of the oxaloacetate spontaneously dacarboxylates to pyruvate. The assay mixture therefore contains oxaloacetate, pyruvate and 2-oxoglutarate, all of which form 2,4-dinitrophenylhydrazones with maximal absorption at different wavelengths. During the reaction the concentration of 2- oxoglutarate decreases while those of oxaloacetate and pyruvate increase. To keep low the contribution of the 2-oxoglutarate hydrazone to the colour, the measurements are made at wavelengths higher than the maximal absorption which allows the greatest differentiation between the extintions of the three hydrazones.

Reagents:

1.Buffer/substrate (0.1 M phosphate buffer, pH 7.4; 0.1 M aspartate; 2mM 2-oxoglutarate.
2. 2,4-dinitrophenylhydrazine, 1mM, 20mg of the chemical dissolved in 1 M HCl and make up to 100 ml.
3.Sodium hydroxide (0.4 M).
4.Sodium Pyruvate (2 mM).

Procedure:

One ml buffer/substrate warmed to 37 C in water bath. 0.2 ml serum was added, mixed and incubate for exactly 60 minutes in the water bath. At the end of the incubation, one ml reagent 2 was added, mixed and allowed to stand for 20 minutes at room temperature. Ten ml reagent 3 added, mixed and allowed to stand for 5 minutes. The optical density was read against blank.
The method could be standardized with optical densities related to that of control sera of known GOT activities. However, calibration curve was constructed using standards with different amounts of pyruvate but a constant sum of pyruvate + 2-oxoglutarate.

Test Tube (n)	Na pyruv sol. (ml)	reagent 1 (ml)
1	0.00	1.00
2	0.05	0.95
3	0.10	0.90
4	0.15	0.85
5	0.20	0.80
6	0.25	0.75

Pipette into each tube 0.2 ml distilled water and one ml reagent 2, mix and after 20 minutes add 10ml reagent 3. Mix and after five minutes read optical densities of tubes 2-6 against tube 1. Plot the optical density against mU/ml and by comparison with the UV method :

Tube no. 2 = 10.5 U/l serum
Tube no. 3 = 21 U/l serum
Tube no. 4 = 32 U/l serum
Tube no. 5 = 48 U/l serum
Tube no. 6 = 70 U/l serum

By direct comparison of the two methods the following table was constructed for measurements at 546 nm:

optical density	U/l
0.020	4
0.040	8
0.060	12
0.080	16
0.100	20
0.120	24
0.140	30
0.160	35
0.180	41
0.200	50
0.220	59
0.240	70
0.260	83

* Glutamate-Pyruvate-Transaminase (GPT)

Reference:

Reitman & Frankel, 1956.

Principle:

As in GOT assays, GPT activity can be measured by use of two reference points. After a fixed time the pyruvate formed from L- alanine and 2-oxoglutarate isdetermined by treating the 2,4-nitrophenylhydrazone with alkali.

Reagents:
1.Buffer/substrate solution (0.1 M phosphate buffer, pH 7.4; 0.2 M DL-alanine; 2 mM 2-oxoglutarate).
2. 1 mM 2,4-dinitrophenylhydrazine.
3. 0.4 M NaOH.
4. Pyruvate solution 2 mM.

Procedure:

The procedure is the same as for GOT except that the incubation time was 30 minutes instead of 60 minutes. Calibration standard curve prepared as before for GOT.
On comparison with the UV method:
Tube no. 2 = 13 U/l serum
Tube no. 3 = 28 U/l serum
Tube no. 4 = 48 U/l serum
Tube no. 5 = 78 U/l serum
Tube no. 6 = 102 U/l serum
The following table was constructed for measurements at 546 nm:

optical density	U/l
0.025	2.5
0.050	5.5
0.075	9
0.100	12
0.125	17
0.150	21
0.175	25
0.200	30
0.225	35
0.250	41
0.275	47
0.300	54
0.325	61
0.350	70
0.375	80

*Gamma-Glutamyl-Transpeptidase (GGT)

Reference:

Rosalki et al., 1970.

Principle:

Gamma-Glutamyl-Transpeptidase (GGT) catalyses the transfer of the gamma-glutamyl group from gamma-glutamyl peptides to another peptide or to L-aminoacids or the removal of the glutamyl group from some compounds wich contain it. The kidneys are richest in this enzyme with apreciable amounts in liver, pancreas and prostate and with little in other organs.

Reagents:

1.Buffer-substrate:dissolvefreshly for use 6.25 mM L-gamma-glutamyl-p-nitroanilide/l in this buffer, 100 mM/l containing 50 mM glycylglycine/l adjusted to pH 9.0 at 25 C.
2.10% glacial acetic acid.
3.Standard p-Nitroaniline solution , 100 uM/l.
Procedure:
Add 50 ul serum to 450 ul pre-warmed buffer-substrate, incubate at 37 C for 30 minutes, then add 2.5 ml acetic acid to stop the reaction and read at 405 nm against a blank prepared by adding 50 ul serum to 450 ul buffer-substrate and 2.5 ml acetic acid. A calibration curve can be prepared from the p- nitroaniline.

$$\text{Serum GGT (U/l)} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200$$

13.1.5 BileBiochemistry.

(Experimental and Histochemistry study, RPMS, London; Clinical study, Ospedale Cardarelli, Napoli)

* Bilirubin

Reference:

Simmonds, 1968;
Billing et al., 1971.

It has been used the same method and apparatus described for the measurement of bilirubin in blood, as described in the appropriate section. Since the concentration of bilirubin in bile is much higher than in blood, bile has been diluted from 1/10 to 1/100, according to the experience.

* Betaglucuronidase

Reference:

Fishman, 1955
Sato, 1962

Principle:

Glucuronide-R + H2O>------>Glucuronic acid + R-OH
(R = aglycone-phenolphthalein, p-nitrophenol, 8-hydroxyquinoline).

Reagents:

1.Phenolphthalein glucuronide (10mM) used as purchased.
2.Acetate buffer (0.2 M, pH 11.7) 11.58 g sodium acetate. 3H2O in water, add 6.72 ml acetic acid and dilute to 1 L.
3.Glycine (0.22 M, ph 11.7) 16.3 g. of glycine and 12.65 g NaCl in 500 ml distilled water, adjust to pH 11.7 with 50% NaOH and dilute to 1 litre with distilled water.
4.Phenolphthalein in standard solution (0.1mg/ml, 0.315 mM). Dissolve 5mg phenolphthalein in 25ml 95% alcohol and dilute to 50 ml with water.
5.Activated charcoal 100mg in 150 ml ethanol (98%).

Procedure:

Centrifuge about 200 ul of bile for five minutes at 3000 r.p.m. Pipette in microfuge tubes:
400 ul acetate buffer
50 ul bile
50 ul phenolphthalein glucuronide substrate
1 or 2 drops of toluene.
Put up a test blank (replace the substrate with 50 ul of buffer). Incubate both tests and blanks for 36 hours or more at 37 C. Add 150 ul charcoal in ethanol and mix well. Centrifuge at 2000 r.p.m. for 5 minutes, transfer the supernatant to LP4 tubes(a), wash pellet with 150 ul ethanol, mix and centrifuge. Add 1 ml glycine buffer and 1.2 ml water. Read at 540 nm. Set up standard curve using phenolphtalein (0.1 mg/ml)

concentration nM	std ul	buffer ul	ethanol ul	glycine ml	water ml
3.15	10	500	298	1	1.2
6.3	25	500	275	1	1.2
12.6	50	500	250	1	1.2
25.2	100	500	200	1	1.2
37.8	150	500	150	1	1.2
50.4	200	500	100	1	1.2

and read off concentration of phenolphtalein ofsample from graph and calculate unit per litre (U/l) of betaglucuronidase activity from data. Minimum detection limit has been 0.5 nmol of phenolphtalein which is equivalent to 0.003 U/l when the samples are incubated for 48 hours

* Phospholipase A

Reference:

Goswami & Frey, 1974

Principle:

lecithin+H2O>------phospholipaseA2
----->lysolecithin + free fatty acid

Reagents:

1.pure lecithin, lysolecithin and glycine
2.boric acid
3.potassium hydroxide
4.sodium deoxycholate
5.chloroform
6.methanol
7.buffer diluent: 0.1 M glycine in 0.05M borate buffer, pH 8.45 (0.6184 g boric acid dissolved in 100 ml water, 1.502 g glycine, add potassium hydroxide until adjust pH at 8.45 and make up to 200 ml)
8.substrate: 25 mg lecithin, 20 mg sodium deoxycholate in 4ml of glycine borate buffer

Procedure:

prepare:

tube	buffer	substrate	bile
1.reagent blank	2ml	4ml	-
2.test	1ml	4ml	1ml
3.test blank	1ml+4ml	-	1ml

Preheat tubes to 55 C for 30 minutes. Add 4ml of subtrate in tube 1 and tube 3, 4 ml of buffer in tube 2 and continue incubation for 60 minutes. Phospholipid is extracted from 1 ml of incubation mixture with 19 ml of chloroform/methanol (2:1). The chloroform phase then evaporates to dryness at 40 C. The lipid redissolves immediately in 0.2 ml of chloroform. 0.04 ml is spotted in duplicate on thin layer chromatographic plate to form a band 2 cm wide. Chromatograms are developed by means of chloroform/ methanol/water (65:25:4) after drying in the oven. Lecithin and lysolecothin are identified by running standards and spraying the plate with copper- molybdenum spray reagent.
Spray detection of phospholipids by thin- layer chromatography.

Reagent:

1. 0.08 g metallic copper.

2.0.25 g ammonium molybdate in 1 ml distilled water. The mixture is chilled.
3. 1 ml concentrated sulphuric acid

Procedure:

The deep blue solution is shaken. This mixture is kept for two hours at room temperature with occasional shaking. 40ml of distilled water are then added and shaken; the color changes from deep blue to light brown. The copper metal is then removed and 3.2 ml concentrated sulphuric acid is added. The solutions to be tested are applied on precoated thin layer chromatography plate and sprayed with reagent. The plate is then kept in oven at 65-70 C for five minutes, removed, sprayed again with reagent and kept for an additional five-six minutes in the oven. Phospholipids stain blue against a light blue background. Overheating produces a pink coloration of the cholesterol, which ultimately turns to greenish/grey against blak-blue background. The sensivity of the phosphate spray is determined with commercially purchased lecithin. The solution is applied to a thin-layer plate as spotts of 4ml diameter. As little 1 ug can be detected.

*Phospholipase C

Reference:

Molby et al., (1973)

Principle:

Lecithin $+H_2O$-----phospholipase---C----> ---
>phosphorylcholine-diglyceride
diglyceride+2 H_2O---> 2Fatty acids+ glycerol
glycerol+ATP--->glycerolkinase-->glycerol-3-P+
+ ADP
FEP+ADP --->PK--->pyruvate + ATP
Pyruvate+NADH + H^+--->LDH--->Lactate + NAD^+

Reagents:

1.triethanolamine buffer 0.1M; pH 7.5; 20mM $CaCl_2$
2.lecithin suspension (dayly prepared)
3.lipase suspension in 3.2M ammonium sulphate solution
4.tricloroacetic acid 7.5 M
5.triethanolamine buffer 0.3M; pH7.6
6.ATP/NADH/PEP solution
7.lactate dehydrogenase LDH crystalline suspension
8.pyruvate kinase, PK, crystalline suspension
9.glycerokinase crystalline suspension.

Procedure:

pipette into centrifuge tubes:
triethanolamine buffer(1)
lecithin suspension
redistilled water
lipase suspension
sample
mix and incubate for exactly 15 min at 37 C. stop the reaction with the addition of trichloroacetic acid.
mix, heat for 2 min in boiling water bath cool and centrifuge prtecipitate. take the supranatant for determination of glycerol.
pipette into cuvette:
triethanolamine buffer (5)
supernatant
mix well:
ATP/NADH/FEP solution
LDH suspension
PK suspension
mix, read after 2min (end the primary reaction)start the next reaction with the addition of glycerokinase suspension.
Wavelength 340 nm. inc. temperature 37 C. inc volume 287 ml. read against blank.

*Bile Acids

Reference:

Mashige et. al., 1978

Principle:

the measurement is based on the following steps:
1.Liberation of bile acids from serum by dialysis at alkaline pH.
2. 3-alpha-hydroxysteroid + NAD+ >----(3 alpha HSD)----------->3-oxo-steroid + NADH + H+
3. Measurement of NADH by fluorometry with excitation at 360 nm and emission 460 nm.
The modification used: as the activity of the enzyme 3 alpha-hydroxy-steroid dehydrogenase decreases with time, sucrose is used to stabilize the enzyme and the whole determination is adapted into autoanalyser method.

Reagents:

1.glycine buffer

glycine	7.57	g.
hydrazine sulphate	5.205	g.
brij 35 (30%)	0.5	ml.
EDTA	0.146	g.

Adjust pH to 9.4 and make up to 1 litre
2.enzyme
3a hydroxysteroid dehydrogenase 0.1U/ml (1 part of enzyme + 70 parts of sucrose) in buffer
3b-NAD 33mg/10ml buffer
4. standard
deoxycolic acid 10mmol/L
(0.4145g/100ml) dissolved in 2% Na2CO3.
Dilute 1/10 before use equivalent to 1mmo/l
5.standard range 0.1 to 1 mmol/L.

13.1.6 Histology.

(Experimental and Histochemistry study, RPMS, London)

Reference:

Drury & Wallington, 1967.

Materials:

1.formalin (10% formol saline or neutral phosphate buffered formalin)
2.graded alcohols, 70%, 90%, 100%
3.xylene
4.paraffin wax (Fibrowax from B.D.H.)

Apparatus:

1.automatic tissue processors: Shonen
2.cutting sections machine: Sledge Microtome

Procedure:

sections preparation:
fix tissues in formalin four up to 24 hours.
Dehydrate through graded alcohols 70%, 90%, 100% X 2, to solvent such as xylene (two changes), one or two hours in each solution, with agitation.
Impregnate in paraffin wax at 56 C, two changes of two hours each.
Embed in fresh paraffin wax.
Cut from blocks 3-4 um sections and mount on albuminized slides.

Haematoxylin & Eosin staining:
dewax sections in solvent;
rehydrate through graded alcohols/water;
stain in haematoxylin (Harris or Coles), up 10 minutes;
differentiate briefly in 1% HCl in 70% alcohol (to remove background staining of cytoplasm);
rinse in running tap water for 10 minutes to "blue" the nuclei or, if local water is insufficiently alkaline, "blue" in Scott's tap water substitute, 1 minute;
rinse in water;
stain in 1% eosin, five minutes;
rinse in water;
dehydrate through graded alcohols;
clear in solvent (xylene);
mount in synthetic permanent mountant.

13.1.7 Histochemistry

(Experimental and Histochemistry study, RPMS, London)

Reference:

Lojda et al, 1979

Principle:

Beta-glucuronide+H2O<----->glucuronate+aglycon

Method:

Azocoupling and indigogenic procedures.

Fixing:

Formol calcium:
Formalin 50 ml (commercial solution, 36-40%)
Calcium acetate (monohydrate) 10g.
Distilled water 450 ml
final pH should be 7.1. Keep at 4 C.
Fix tissues (small pieces)for 2-4 hours at 4 C; then transfer to Gum sucrose at 4 C for storage.
If storage solution grows mouldy, transfer tissues in a freshly made solution.

Gum sucrose:
sucrose 30 g
gum arabic (gum acacia) 1g;
mix well; add distilled water to 100 cc;
warm and stir to dissolve; cool to 4 C before use.

Incubation media:

NaphtolAs-Bi beta glucuronide, 3-4 mg
(Sigma, U.K.);
dissolve in N,N-dimethylformamide 0.25 ml;
buffer with hexazonium-p-rosaniline or new fuchsine 20 ml (made up of 18.2-19.4 ml 0.2M sodium acetate and 0.6-1.8 ml hexazonium-p-rosaniline or new fuchsine; adjust pH to 5 with 1 N and 0.1 N NaOH);
mix and filter.

Incubation:

3min-3h at 37C or overnight in a refrigerator at +4C; after fixation of tissue blocks free floating incubation is also appropriate.

Treatment:

Pour off incubation medium;

rinse in distilled water;
place in 4% formaldehyde (to reduce formation of gas bubbles in sections) for several hours at room temperature;
rinse in tap water;
where appropriate counterstain nuclei with hematoxylin;
mount in glycerin jelly or apathy's gum syrup.

Results:

Enzyme-active sites are stained red.

13.1.8 Xray diffractometry of Common Bile Duct stones

(Clinical study, Ospedale Cardarelli, Napoli)

Reference:

Sutor & Wooley, 1971.

Principle:

If a cristalline substance is irradiated with X-rays, the intensities and direction of the diffracted rays depend on the atomic arrangement and are therefore characteristic of the substance.

Standards:

Calcite: Sutor & Scheidt, 1968
Cholesterol monohydrate: Bogren & Larsson, 1963
Anhydrouscholesterol: ASTM (American Society for Testing and Materials) powder diffraction file.
Calcium palmitate: Sutor & Wooley, 1971.

Method:

Stones were splitted and specimens were taken from the exposed surfaces and powdered. In presence of a layered structure the analysis was carried out layerwise. The specimens were then photographed by a Philips infractometer at a wave length of Cu K alpha 1,54178.a Nonius-Guinier type camera. The diffraction patterns were matched against standards comparing the intensities and spacings of the lines on the resulting photograph.

Table 13.1
Experimental Study - Synopsis

Dog Number	Event Type (t)	Weeks of Obstruction	Total Bilirubin	Conjugated Bilirubin	Total Proteins	Albumin	A/G Ratio	Alkaline Phosfatase	GOT	GPT	Gamma GT	GB Bile For Culture	GB Bile E.coli (log)	GB Bile S.faecalis (log)	GB Bile P.aeruginosa (log)	GB Bile P.mirabilis (log)	GB Bile Klebsiella (log)	GB Bile Coliforms (log)	GB Bile Clostridia (log)	GB Bile S.aureus (log)	GB Bile S.epidermidis (log)	CBD Bile For Culture	CBD Bile E.coli (log)	CBD Bile S.faecalis (log)	CBD Bile P.aeruginosa (log)	CBD Bile P.mirabilis (log)	CBD Bile Klebsiella (log)	CBD Bile Coliforms (log)	CBD Bile Clostridia (log)	CBD Bile S.aureus (log)	CBD Bile S.epidermidis (log)	CBD Bile Others	N° of Species from CBD Bile	CBD Bile Higest log
299	O											No										Yes										0		
299	X	31	6,0	4,0	47,8	19,8	0,7	32	10	11		No										Yes	3	1								0	3	2
299	XX	49	5,5	1,5	57,5	23,3	0,7	34	14	5		No										Yes									8	0	8	1
299	PM	58										No										Yes	2	2		3						no	3	3
300	O											No										No										no		
300	X	32	3,0	1,0	50,3	19,3	0,6	27	14	12		No										Yes										no		
300	XX	48	4,0	2,0	50,3	19,3	0,6	30	18	12		No										Yes	8				5					no	8	2
300	OO	72	2,0	1,0	36,4	10,7	0,4	30	14	12		No										Yes	5	5			5		5			no	5	4
520	O		10,0	6,2	62,2	26,6	0,8	186	91	45		No										Yes										no		
520	X	27	2,5	1,4	56,2	30,8	1,2	38	13	8		No										Yes	1									no	1	1
520	PM	32										No										No										no		
731	O		2,6	0,5	54,6	35,4	2,6	10	24	21	7	No										Yes										no		
731	X	8	4,2	3,2	48,6	27,6	1,3	15	18	14	8	No										Yes	4									no	4	1
731	BS	8	1,3	1,1	49,5	29,0	1,4	15	8	8	9	No										No										no		
731	BS	9	4,5	1,5	59,5	31,5	1,1	50	6	5	8	No										No										no		
731	XX	22	2,6	0,6	54,7	33,8	1,6	16	10	9	14	No										Yes										no		
731	BS	22	2,2	1,9	54,5	33,1	1,5	18	10	9	12	No										No										no		
731	OO	42	3,1	0,2	60,0	33,0	1,2	33	13	8	6	No										Yes	4	4				4				no	4	3
737	O		9,9	1,7	47,5	20,8	0,8	32	28	17	5	No										Yes										no		
737	X	7	1,9	1,3	54,5	25,8	0,9	33	18	26	12	No										Yes									2	no	2	1
737	BS	1	1,7	0,6	55,6	26,7	0,9	42	6	7	13	No										No										no		
737	BS	20	1,2	0,6	53,5	26,1	1,0	18	12	19	12	No										No										no		

Dog Number	Event Type (1)	Weeks of Obstruction	Total Bilirubin	Conjugated Bilirubin	Total Proteins	Albumin	A/G Ratio	Alkaline Phosfatase	GOT	GPT	Gamma GT	GB Bile For Culture	GB Bile E.coli (log)	GB Bile S.faecalis (log)	GB Bile P.aeruginosa (log)	GB Bile P.mirabilis (log)	GB Bile Klebsiella (log)	GB Bile Coliforms (log)	GB Bile Clostridia (log)	GB Bile S.aureus (log)	GB Bile S.epidermidis (log)	CBD Bile For Culture	CBD Bile E.coli (log)	CBD Bile S.faecalis (log)	CBD Bile P.aeruginosa (log)	CBD Bile P.mirabilis (log)	CBD Bile Klebsiella (log)	CBD Bile Coliforms (log)	CBD Bile Clostridia (log)	CBD Bile S.aureus (log)	CBD Bile S.epidermidis (log)	CBD Bile Others	N° of Species from CBD Bile	CBD Bile Higest log
737	OO	21										No										Yes	5	5	4					3		no	5	4
737	PM	21										No										No											no		
758	O		9,9	8,4	48,5	27,6	1,3	17	6	6	5	No										Yes											no		
758	X	13	1,4	0,3	50,6	30,8	1,6	21	11	10	10	No										Yes											no		
758	BS	13	2,5	1,6	51,6	31,0	1,5	21	11	10	10	No										No											no		
758	XX	33	3,2	0,4	52,5	25,4	0,9	32	10	12	7	No										Yes	4	4									no	4	2
758	XXX	47	2,5	1,1	59,8	22,5	0,7	67	9	29	12	No										Yes	8	6									no	8	2
758	OO	52	8,3	3,7	51,3	25,9	1,0	18	12	15	9	No										Yes	7	7									no	7	2
765	O		3,3	1,8	52,6	27,7	1,1	57	10	15	7	No										No													
769	O		2,2	0,6	57,0	29,2	1,1	13	18	12	10	No										Yes											no		
769	BS	1	75,5	55,4	53,1	30,3	1,3	504	49	169	70	No										No											no		
769	BS	21	3,4	0,3	62,5	26,5	0,8	33	13	17	3	No										No											no		
769	X	35	2,5	0,5	60,9	24,0	0,7	35	9	8	7	No										Yes	8										no	8	1
769	OO	40	5,2	1,7	60,9	24,0	0,7	12	12	8	7	No										Yes	6										coag-staph 4	6	2
769	SA	61	4,4	1,2	76,1	24,7	0,5	26	10	32	12	No										Yes	6	5	3				4		4		bacteroides 4	6	6
820	O		16,2	5,2	58,3	27,9	0,9	22	10	10	8	No										Yes											no		
820	X	10	2,7	0,2	53,7	21,4	0,7	22	10	7	11	No										Yes	4	6		3							no	6	3
820	BS	21	4,0	1,1	54,9	22,9	0,7	10	12	8	11	No										No											no		
820	OO	37	3,1	1,5	62,6	22,0	0,5	13	11	7	8	No										Yes		7									no	7	1

(1) - Event Type

0,OO	1st and 2nd Operation
X, XX, XX	1st, 2nd and 3rd X-Ray
BS	Blood Sample
SA	Sacrifice
PM	Post Mortem

Dog Number	Portal Blood Positive Culture	Portal Blood Bacteria	GB Total Bilirubin	GB Conjugated Bilirubin	GB β-Glucuronidase	GB Phospholipase	GB Bile Acids	CBD Total Bilirubin	CBD Conjugated Bilirubin	CBD Unconjugated Bilirubin	CBD β-Glucuronidase	CBD Phospholipase	CBD Bile Acids	Liver Bile Canaliculi Epithelium	Liver Inflammation	Liver Proliferation Bile Ducts	Liver Granulation Tissue	Liver Fibrosis	Liver β-Gluc. Granules Count	CBD Epithelium	CBD Inflammation	CBD Proliferation	CBD Granulation Tissue	CBD Fibrosis	CBD β-Glucuronidase	CBD Stones Presence
299		no																								No
299		no						1260	1080	180	0,048		53													No
299		no						1803	1632	171	0,013	0,095	48													No
299		no												3	1	1	1	5		1	1	1	1	1		No
300		no																								No
300		no																								No
300		no						6853	5423	1430		0,135	119													No
300		no						1973	1946	27	0,025	0,069	82	3	3	1	1	1		1	3	3	1	1		No
520		no	6526	5485	0,721		167	1136	665	471	0,024		74													No
520		no						1410	1226	185	0,032		170													No
520		no						3796	2471	1324	0,031		52													No
731		no						1293	618	675	0,013		19													No
731		no						1903	1574	329	0,046		23													No
731		no																								No
731		no																								No
731		no						3867	3222	645	0,030		87													No
731		no																								No
731		no						1256	834	421	0,040		12	1	1	1	1	1	O	3	3	3	1	3	No	No
737		no	3135	2382	0,019	0,022	102	1962	1095	868	0,129	0,200	114	1	1	1	1	1	87							No
737		no						2368	1690	677	0,147		12													No
737		no																								No
737		no																								No

Dog Number	Portal Blood Positive Culture	Portal Blood Bacteria	GB Total Bilirubin	GB Conjugated Bilirubin	GB β-Glucuronidase	GB Phospholipase	GB Bile Acids	CBD Total Bilirubin	CBD Conjugated Bilirubin	CBD Unconjugated Bilirubin	CBD β-Glucuronidase	CBD Phospholipase	CBD Bile Acids	Liver Bile Canaliculi Epithelium	Liver Inflammation	Liver Proliferation Bile Ducts	Liver Granulation Tissue	Liver Fibrosis	Liver β-Gluc. Granules Count	CBD Epithelium	CBD Inflammation	CBD Proliferation	CBD Granulation Tissue	CBD Fibrosis	CBD β-Glucuronidase	CBD Stones Presence
737		no						1268	1037	231	0,039		26						60	1	5	3	3	1	No	Yes
737		no												1	1	1	1	3								No
758		no	8952	7289	0,070		128	1934	1343	590	0,029		13	1	1	1	1	1	234							No
758		no						2357	2096	260	0,028		88													No
758		no																								No
758		no						2756	2202	554	0,044		124													No
758		no						5271	4508	763	0,030		144													No
758		eco4 sfae4						2323	1390	933	0,034		27	1	1	1	1	1	119	3	3	5	3	1	No	No
765		no	6668	5458	0,057		137	1663	1349	313	0,081		23													No
769		no	5582	4804	0,025		120	3076	1867	1209	0,039		21	1	5	1	1	1	155							No
769		no																								No
769		no																								No
769		no						1057	979	78	0,009		55													No
769		no						730	497	234	0,044		17	1	5	1	1	5	8	1	5	5	1	3	No	Yes
769		no																	20						No	No
820		no	6666	5878	0,044		134	1708	926	782	0,037		16	1	1	1	1	1	95							No
820		no						1450	1191	259	0,026		103													No
820		no																								No
820		no						4563	3598	965	0,027		149						153						No	No

Table 13.2
Clinical Study - Synopsis

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 2 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Antibiotics (7)	Timing (8)
9001	M	55	Yes	Yes	Yes	Yes	120	No	2	5	0	9	0	4	19	0	0	0,000	0,000	0,00	0	0	0,000	0,000	16,72	Yes	5	0	0	0	No	0	A
9002	F	66	No	No	No	No	5	No	1	2	7	1	8	0	13	76	19	2,568	0,616	15,12	250	160	0,021	0,014	14,57	No	0	0	0	0	No	0	A
9003	F	30	No	Yes	No	Yes	120	No	3	2	3	1	6	6	15	46	46	2,468	0,001	11,55	160	110	0,022	0,000	11,86	No	0	0	0	0	No	0	A
9004	F	46	No	No	No	Yes	60	No	1	2	1	1	8	0	14	38	38	1,616	0,164	3,57	510	200	0,000	0,000	44,57	No	0	0	0	0	No	0	A
9005	M	56	Yes	Yes	No	No	24	No	3	3	4	4	8	5	20	360	210	0,113	0,001	6,22	30	70	0,004	0,000	7,76	No	0	0	0	0	Yes	0	A
9006	F	29	Yes	No	No	Yes	84	No	3	9	1	1	1	1	25	152	114	2,108	0,209	3,88	270	110	0,026	0,001	5,02	No	0	0	0	0	No	0	A
9007	F	62	No	Yes	Yes	Yes	84	No	4	6	0	9	0	4	20	0	0	0,000	0,000	0,00	0	0	0,000	0,000	42,23	No	0	0	0	0	Yes	0	A
9008	F	57	No	No	Yes	No	24	No	2	5	0	1	0	8	20	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9009	F	36	No	No	No	Yes	48	No	1	1	1	1	8	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9010	F	49	No	No	Yes	Yes	96	No	2	4	0	5	0	1	15	0	0	0,000	0,000	0,00	50	30	0,015	0,096	34,78	No	0	0	0	0	No	0	A
9011	F	35	No	No	No	No	36	No	1	1	1	1	8	0	12	38	38	0,060	0,189	63,80	10	10	0,030	0,000	2,84	No	0	0	0	0	No	0	A
9012	M	40	No	No	No	No	24	No	1	1	1	1	7	0	12	76	19	0,400	0,195	108,38	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9013	F	61	No	No	No	Yes	72	No	1	2	1	1	8	0	15	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9014	M	35	Yes	No	Yes	Yes	110	No	2	4	0	3	0	4	14	0	0	0,000	0,000	0,00	10	40	0,000	0,000	4,51	No	0	0	0	0	No	0	A
9015	F	78	Yes	No	No	No	20	Yes	8	3	1	5	0	0	18	0	0	0,000	0,000	0,00	560	980	0,000	0,000	17,33	No	0	0	0	0	No	0	A
9016	M	66	No	Yes	No	Yes	60	No	3	3	9	4	1	4	25	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9017	F	68	No	No	No	Yes	300	No	1	2	1	1	7	0	14	0	0	0,000	0,000	0,00	46	92	0,000	0,000	23,15	No	0	0	0	0	No	0	A
9018	F	68	No	No	No	Yes	240	No	3	2	3	1	8	1	14	38	76	0,066	0,674	108,40	37	74	0,000	0,000	11,23	No	0	0	0	0	No	0	A
9019	F	63	No	Yes	No	No	9	No	1	1	3	3	1	0	14	0	0	0,000	0,000	0,00	80	100	0,004	0,000	37,37	No	0	0	0	0	No	0	A
9020	F	62	No	No	No	No	24	No	1	1	1	1	3	0	13	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Timing (8)	Antibiotics (7)
9021	F	28	No	No	No	No	12	No	1	1	7	1	5	0	12	0	0	0,000	0,000	0,00	20	70	0,021	0,000	23,21	No	0	0	0	0	No	A	0
9022	F	40	No	No	No	No	36	No	1	1	1	1	7	0	9	266	19	0,084	0,246	64,70	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9023	F	21	No	No	No	No	1	No	1	1	1	1	7	0	10	228	190	0,064	0,621	62,99	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9024	F	39	No	No	No	No	24	No	1	1	1	0	3	0	10	1	1	0,245	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9025	F	52	Yes	No	Yes	No	36	No	2	5	0	9	0	4	28	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	Yes	A	0
9026	M	55	Yes	Yes	No	No	1	No	1	1	9	4	5	0	14	76	76	1,877	0,001	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9027	F	21	Yes	No	No	No	1	No	2	2	1	1	0	4	14	0	0	0,000	0,000	0,00	60	200	0,026	0,138	9,54	No	0	0	0	0	No	A	0
9028	F	62	Yes	No	Yes	Yes	120	No	4	5	0	5	0	0	20	0	0	0,000	0,000	0,00	170	190	0,032	0,082	24,28	No	0	0	0	0	No	A	0
9029	F	65	Yes	No	No	No	1	Yes	6	3	1	1	0	0	25	296	1443	0,037	0,381	147,40	80	200	0,188	0,001	16,77	No	0	0	0	0	No	A	0
9030	F	38	No	Yes	No	No	36	No	1	2	1	1	6	0	9	456	0	0,029	0,177	118,95	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9031	F	49	Yes	No	No	No	3	No	3	2	1	1	1	1	15	0	0	0,000	0,000	0,00	160	150	0,005	0,001	11,97	No	0	0	0	0	No	A	0
9032	M	60	No	No	No	Yes	84	No	2	3	3	3	0	4	15	350	160	0,033	0,001	33,06	300	170	0,052	0,001	14,47	No	0	0	0	0	No	A	0
9033	F	47	Yes	No	Yes	Yes	180	No	3	2	3	3	8	6	14	70	10	0,187	0,001	27,46	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9034	F	42	No	No	No	Yes	48	No	3	9	8	8	1	4	13	969	532	0,017	0,140	39,70	440	300	0,032	0,083	10,09	No	0	0	0	0	No	A	0
9035	F	36	No	No	No	No	24	No	1	1	1	1	7	0	11	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9036	F	61	No	No	No	Yes	240	No	1	1	1	1	7	0	10	1	1	0,555	0,001	2,22	0	0	0,000	0,000	0,00	No	0	0	0	0	No	A	0
9037	M	54	No	No	No	Yes	48	No	1	1	1	0	5	0	10	1653	456	0,008	0,001	190,24	0	0	0,000	0,000	0,00	Yes	4	1	0	1	No	T	B
9039	F	63	No	No	No	Yes	60	No	1	1	1	1	7	0	12	0	0	0,000	0,000	0,00	540	350	0,032	0,001	25,86	No	1	1	1	1	No	T	B
9039	F	69	Yes	Yes	No	Yes	72	No	3	9	1	1	8	1	18	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	1	3	3	1	No	P	B
9040	F	58	No	No	No	Yes	360	No	1	1	1	0	7	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	0	0	0	No	0	A
9043	M	28	No	No	No	No	15	No	1	1	1	1	6	0	10	3330	2109	0,005	0,179	166,86	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9044	F	61	No	No	No	Yes	50	No	1	1	1	1	1	0	11	0	0	0,144	0,001	2,54	200	140	0,000	0,000	23,37	No	1	1	1	1	No	P	B
9045	F	51	No	No	No	Yes	60	No	1	1	1	0	7	0	10	1463	1083	0,049	0,064	104,86	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9046	F	45	No	No	No	Yes	90	No	1	1	1	0	7	0	10	114	57	1,692	0,581	12,01	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	P	B
9047	F	60	No	No	No	Yes	40	No	1	1	1	0	7	0	10	741	399	0,080	0,243	70,56	0	0	0,000	0,000	0,00	No	2	0	1	6	No	P	A
9048	F	61	No	No	No	Yes	72	No	1	1	2	1	3	0	10	76	19	1,696	0,055	11,99	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Antibiotics (7)	Timing (8)
9049	F	47	No	No	No	Yes	50	Yes	5	1	8	0	5	0	10	10	10	1,759	0,001	1,28	0	0	0,000	0,000	0,00	Yes	3	1	1	4	No	T	B
9050	M	74	Yes	Yes	No	Yes	40	No	3	3	4	9	1	4	20	57	38	1,669	0,050	5,56	60	100	0,085	0,001	9,98	No	0	0	0	9	Yes	P	A
9051	F	53	No	No	No	Yes	60	No	1	1	9	0	5	0	10	10	0	0,061	0,050	69,35	0	0	0,000	0,000	0,00	No	0	0	0	0	No	T	B
9052	F	43	No	No	No	Yes	60	No	1	1	1	1	6	0	10	760	342	0,054	0,055	34,17	0	0	0,000	0,000	0,00	No	2	1	1	2	No	T	B
9054	F	40	No	No	No	Yes	60	No	1	1	1	0	5	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9055	F	73	Yes	No	No	Yes	144	No	1	1	1	0	5	0	10	475	589	0,055	0,603	196,49	0	0	0,000	0,000	0,00	No	1	1	1	1	No	T	B
9056	M	35	No	No	No	Yes	55	No	1	1	1	0	6	0	14	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	5	1	No	T	B
9057	M	33	Yes	No	No	No	3	No	2	2	1	1	0	1	17	0	0	0,000	0,000	0,00	50	60	0,052	0,072	17,68	No	1	1	1	1	No	P	A
9058	F	25	No	No	No	No	24	No	1	1	1	0	6	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9059	F	62	No	No	No	No	24	No	1	1	1	0	7	0	10	2090	1121	0,016	0,101	30,09	0	0	0,000	0,000	0,00	Yes	4	1	1	1	No	T	B
9061	F	61	Yes	No	No	Yes	60	No	1	1	3	0	5	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9062	F	80	Yes	Yes	No	Yes	240	No	9	2	1	0	7	0	7	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	A
9063	F	25	No	No	No	No	18	No	1	1	1	0	6	0	10	228	38	1,133	0,146	112,56	0	0	0,000	0,000	0,00	No	2	1	1	6	No	P	B
9064	F	72	Yes	Yes	No	Yes	48	No	1	1	1	0	3	0	10	1672	646	0,126	0,082	22,11	0	0	0,000	0,000	0,00	No	2	1	5	1	No	T	B
9065	M	56	No	No	No	Yes	120	No	3	2	1	1	3	8	10	40	20	1,522	0,001	10,05	590	510	0,281	0,050	17,36	No	2	1	1	1	No	P	B
9066	F	41	No	No	No	No	12	No	1	1	1	0	6	0	10	1045	399	0,012	0,091	142,79	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9067	M	46	No	No	No	Yes	90	No	1	1	1	0	3	0	10	1691	627	0,069	0,073	208,52	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	A
9068	F	41	No	No	No	No	18	No	1	1	1	0	7	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	1	0	No	T	B
9069	F	57	No	No	No	Yes	40	No	1	1	8	8	8	0	0	0	0	0,000	0,000	0,00	540	280	0,004	0,000	23,93	No	0	0	0	0	No	P	B
9072	M	47	No	Yes	No	Yes	84	No	3	3	3	3	8	8	20	1235	475	0,333	0,001	48,24	0	0	0,000	0,000	0,00	No	1	1	0	6	No	T	A
9073	F	22	No	Yes	No	No	8	No	1	1	1	0	6	0	10	399	133	0,032	0,037	53,27	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9074	M	41	Yes	No	No	No	2	Yes	5	17	0	1	0	0	22	0	0	0,000	0,000	0,00	180	130	0,042	0,048	5,52	No	1	1	1	1	No	T	A
9075	F	33	No	No	No	No	24	No	1	1	1	3	1	0	10	310	0	0,018	0,028	64,32	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9076	F	45	No	No	No	No	24	No	1	1	1	0	6	0	10	2300	500	0,023	0,044	120,60	0	0	0,000	0,000	0,00	No	0	1	1	1	No	P	B
9077	F	42	No	No	No	No	18	Yes	5	2	1	1	3	3	15	76	38	1,590	0,083	2,83	100	140	0,004	0,017	11,42	No	1	1	1	1	No	P	B
9078	F	38	No	No	No	Yes	60	No	3	2	1	1	3	3	0	900	150	0,026	0,069	104,52	0	0	0,000	0,000	0,00	No	0	0	5	1	No	0	A

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Timing (8)	Antibiotics (7)
9079	F	40	No	No	No	Yes	120	No	1	1	1	1	1	0	10	4	1	2,781	0,028	0,26	100	210	0,007	0,028	3,80	No	2	1	1	6	No	T	B
9080	F	62	Yes	No	No	No	1	Yes	8	3	1	1	3	0	30	3	1	0,742	0,021	0,30	670	920	0,017	0,006	12,08	No	1	1	1	1	No	P	B
9081	F	31	No	No	No	No	36	No	1	1	1	0	7	0	10	820	140	0,013	0,111	64,32	0	0	0,000	0,000	0,00	No	0	0	0	0	No	P	B
9082	F	29	No	No	No	No	36	No	1	1	1	0	7	0	10	270	50	1,544	0,138	24,12	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9083	F	36	No	Yes	No	Yes	120	No	1	1	1	1	1	0	10	270	10	1,356	0,194	40,20	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9084	F	18	No	No	No	No	24	No	1	1	4	0	6	0	10	1490	120	0,007	0,083	88,44	0	0	0,000	0,000	0,00	No	1	1	1	1	No	T	B
9085	F	63	No	No	No	Yes	60	No	9	39	4	4	0	4	20	76	38	0,137	0,601	6,12	0	0	0,000	0,000	0,00	Yes	4	1	4	6	No	T	B
9086	M	69	No	No	No	No	6	No	1	1	4	0	3	0	10	170	30	1,714	0,145	10,71	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9087	F	53	No	No	No	No	36	No	1	1	1	0	1	0	10	1121	19	0,065	0,152	66,30	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9088	M	55	No	No	No	Yes	444	No	1	1	1	1	1	0	10	0	0	0,000	0,000	0,00	510	440	0,009	0,017	17,24	No	2	1	1	1	No	P	B
9089	F	39	No	No	No	No	36	No	1	1	1	0	3	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9090	F	77	Yes	Yes	No	No	12	Yes	8	3	4	4	0	0	25	140	60	0,015	0,159	5,10	260	220	0,017	0,001	4,37	No	2	1	1	1	No	T	B
9091	F	55	No	No	No	Yes	120	No	1	1	1	0	3	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9092	F	64	No	No	No	Yes	120	No	1	1	1	0	5	0	10	2945	95	0,140	0,194	152,76	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9093	M	59	No	No	Yes	Yes	72	No	1	1	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9094	F	59	No	No	No	No	4	No	1	2	1	1	1	0	0	0	0	0,000	0,000	0,00	230	90	0,000	0,000	30,67	No	1	0	0	1	No	P	A
9095	F	71	No	Yes	No	Yes	360	No	1	1	4	4	4	0	6	140	40	0,118	0,083	13,52	0	0	0,000	0,000	0,00	No	1	1	1	1	No	T	B
9096	F	42	No	No	No	No	36	No	1	1	1	1	5	0	10	1570	310	0,012	0,083	81,60	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9097	F	43	No	Yes	No	Yes	60	No	1	1	1	0	6	0	10	5177	124	0,017	0,152	241,20	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9098	F	79	No	No	No	No	1	No	1	1	1	8	7	0	10	1862	0	0,090	0,215	76,50	800	200	0,032	0,188	53,86	No	2	1	1	1	No	O	A
9099	M	36	No	No	No	No	36	No	1	1	1	0	3	0	10	60	20	1,521	0,201	1,69	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9100	F	19	No	No	No	No	3	No	3	2	1	1	1	1	18	418	171	0,255	0,046	57,29	380	170	0,007	0,001	10,76	No	2	1	1	6	No	T	A
9101	F	54	Yes	Yes	No	Yes	60	No	1	2	3	3	5	0	11	2109	1311	0,023	0,023	46,09	920	900	0,028	0,022	7,33	No	2	1	1	1	No	T	A
9102	F	50	Yes	Yes	No	Yes	84	No	1	1	1	3	3	0	10	133	57	1,697	0,059	30,75	222	74	0,000	0,000	7,45	No	0	0	0	2	No	T	B
9103	F	62	No	No	No	Yes	120	No	1	1	1	0	3	0	10	1339	447	0,032	0,055	128,01	0	0	0,000	0,000	0,00	No	1	1	1	1	No	T	B
9104	F	46	No	Yes	No	Yes	276	No	1	1	1	0	6	0	10	1	3	0,015	0,001	0,39	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Timing (8) Antibiotics (7)	
9105	F	21	No	No	No	No	10	No	1	2	1	1	5	0	10	1501	494	0,043	0,050	112,56	1349	437	0,026	0,221	61,90	No	1	1	1	1	No	T	B
9106	F	67	No	No	No	Yes	60	No	3	2	1	3	7	4	12	1273	494	0,063	0,087	44,22	830	350	0,030	0,001	14,14	No	1	1	1	1	No	T	B
9107	F	47	No	No	No	No	20	No	1	1	1	1	3	0	10	931	722	0,111	0,023	91,94	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9108	M	41	No	No	No	Yes	144	No	1	1	1	0	5	0	10	6014	1240	0,052	0,152	129,96	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	T	B
9109	M	37	No	No	No	Yes	60	No	1	1	1	0	7	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9110	F	75	No	No	No	Yes	180	No	4	3	3	3	1	0	18	70	110	1,628	0,519	3,40	210	40	0,147	0,094	13,06	No	0	1	1	0	No	P	A
9111	F	27	No	No	No	Yes	96	No	1	1	1	0	5	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	1	1	0	No	P	B
9112	M	46	Yes	Yes	No	No	24	No	1	2	1	3	5	0	13	2033	551	0,491	0,194	29,73	0	0	0,000	0,000	0,00	No	2	1	1	1	No	P	B
9113	F	41	No	Yes	No	Yes	60	No	1	1	1	3	3	0	10	2831	247	0,034	0,076	143,37	0	0	0,000	0,000	10,30	No	2	1	1	1	No	T	B
9114	F	56	No	No	No	Yes	60	No	1	1	1	0	1	0	10	2033	304	0,007	0,111	82,53	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	P	B
9115	M	52	Yes	No	No	No	1	No	3	2	1	3	1	1	10	60	20	1,386	0,464	6,43	50	720	0,004	0,061	7,65	No	2	1	3	1	No	P	A
9116	F	58	No	No	No	Yes	96	No	1	1	3	0	7	0	10	38	2755	0,083	0,104	102,17	0	0	0,000	0,000	0,00	No	2	1	1	6	No	T	B
9117	F	51	No	Yes	No	No	36	No	1	1	1	1	1	0	10	683	5	1,459	0,042	4,79	530	230	0,026	0,033	20,73	No	2	1	1	6	No	P	B
9118	M	32	No	Yes	No	No	3	No	1	1	1	1	3	0	10	0	0	0,000	0,000	115,14	400	300	0,009	0,000	15,44	No	1	1	1	1	No	P	B
9119	F	45	No	No	No	No	12	No	1	1	1	0	1	0	10	220	60	0,022	0,069	0,00	0	0	0,000	0,000	0,00	No	2	0	0	1	No	T	A
9120	F	63	No	No	No	Yes	144	No	1	1	2	2	3	0	10	5405	1504	0,062	0,228	113,12	0	0	0,000	0,000	0,00	No	1	1	1	6	No	P	B
9121	F	51	Yes	Yes	No	No	1	No	3	9	3	3	1	1	20	0	0	0,000	0,000	0,00	50	120	0,085	0,001	2,77	No	2	1	3	1	No	T	A
9122	F	62	No	No	Yes	No	12	Yes	8	7	0	1	0	0	16	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	P	A
9123	F	50	No	No	No	Yes	40	No	1	1	1	1	3	0	10	1	0	0,083	0,104	0,12	20	20	0,064	0,000	3,45	Yes	4	1	1	1	No	P	B
9124	F	18	No	No	No	No	20	No	1	1	1	0	3	0	13	1748	247	0,040	0,159	20,24	0	0	0,000	0,000	0,00	No	0	1	1	0	No	P	B
9126	F	42	No	No	No	No	6	No	1	1	1	1	1	0	10	0	0	0,000	0,000	0,00	540	230	0,000	0,000	17,18	No	2	1	1	1	No	P	B
9127	F	33	No	No	No	No	18	No	1	1	1	0	1	0	10	3384	188	0,053	0,118	48,99	0	0	0,000	0,000	0,00	No	2	1	1	6	No	P	A
9128	F	64	No	No	No	No	2	No	1	1	1	0	5	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	T	B
9130	F	50	No	No	No	Yes	130	No	1	2	1	1	1	0	13	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	3	1	1	1	No	T	B
9131	F	39	No	No	No	Yes	48	No	1	1	1	1	7	0	10	1293	47	0,100	0,215	33,18	110	90	0,000	0,000	6,38	Yes	3	1	1	1	No	T	B
9132	F	63	No	No	No	Yes	84	No	1	1	1	1	8	0	10	1	1	0,058	0,083	0,75	0	0	0,000	0,000	0,00	No	1	1	1	6	No	T	B

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Timing (8)	Antibiotics (7)
9133	F	59	No	Yes	No	Yes	120	Yes	5	3	4	4	7	4	25	460	170	0,688	0,222	9,57	360	150	0,027	0,001	10,37	Yes	4	1	4	2	No	T	B
9134	M	70	Yes	No	No	No	24	No	3	2	1	1	1	1	15	0	0	0,000	0,000	0,00	570	260	0,078	0,006	14,36	No	2	1	1	1	No	T	A
9135	M	37	No	No	No	No	2	No	1	1	1	0	7	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	1	1	0	No	P	B
9136	F	71	No	No	No	No	24	No	3	3	3	3	5	4	25	200	60	0,231	0,083	3,38	250	140	0,208	0,001	5,05	No	0	1	9	0	No	T	A
9137	F	37	No	No	No	Yes	70	No	1	1	8	8	7	0	10	0	0	0,000	0,000	0,00	180	170	0,017	0,034	9,47	No	1	1	1	1	No	T	B
9138	F	33	No	No	No	Yes	72	No	1	1	1	3	7	0	10	1520	114	0,070	0,201	44,68	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9139	F	44	Yes	No	Yes	Yes	168	No	2	4	0	1	0	8	0	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	3	1	9	1	No	P	B
9140	F	63	No	No	No	Yes	60	No	1	1	1	0	8	0	10	0	0	0,000	0,000	0,00	480	190	0,005	0,001	18,63	No	2	1	1	1	No	P	B
9141	F	68	Yes	No	No	Yes	120	No	3	3	1	1	8	8	18	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	2	1	No	P	B
9142	F	46	Yes	No	Yes	Yes	168	No	1	2	0	1	4	0	10	0	0	0,000	0,000	0,00	450	190	0,320	0,243	14,68	Yes	4	1	3	6	No	P	A
9143	F	69	No	Yes	No	Yes	168	No	3	3	1	1	6	6	20	0	0	0,000	0,000	0,00	171	114	0,000	0,000	12,54	No	1	1	3	1	No	T	A
9144	F	67	Yes	No	Yes	Yes	204	No	2	5	0	5	0	4	16	0	0	0,000	0,000	0,00	270	70	0,006	0,001	16,96	No	1	1	1	1	No	P	B
9145	F	81	No	No	Yes	Yes	60	No	4	5	0	1	0	0	22	0	0	0,000	0,000	0,00	160	80	0,004	0,001	7,62	Yes	4	1	1	1	No	P	B
9146	M	45	No	Yes	No	Yes	65	No	1	1	1	0	3	0	10	11	17	1,237	0,118	0,26	0	0	0,000	0,000	0,00	Yes	4	1	1	1	No	P	B
9147	M	71	No	No	No	No	24	No	1	1	1	0	1	0	10	40	10	0,971	0,111	3,32	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	A
9151	F	59	No	Yes	No	Yes	96	No	1	2	5	1	6	0	14	0	1	0,176	0,021	0,01	290	240	0,005	0,001	10,51	No	1	1	1	1	No	T	B
9152	F	42	No	Yes	No	No	5	No	3	2	1	1	6	4	14	0	0	0,000	0,000	0,00	330	90	0,012	0,001	19,87	No	2	0	0	1	Yes	T	A
9153	F	63	No	Yes	No	Yes	90	No	1	1	1	1	6	0	14	817	133	0,092	0,069	30,30	240	150	0,042	0,044	20,92	Yes	3	1	1	3	No	P	B
9154	F	49	No	No	No	Yes	60	No	1	1	1	0	7	0	10	2375	95	0,018	0,076	36,36	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9155	F	32	No	No	No	No	5	No	1	1	1	1	4	0	13	0	0	0,000	0,000	0,00	418	228	0,016	0,181	19,24	No	1	1	1	1	No	P	B
9156	F	77	No	Yes	Yes	Yes	50	No	2	5	0	9	0	4	20	0	0	0,000	0,000	0,00	630	420	0,019	0,001	13,84	No	1	1	1	1	No	P	B
9157	F	45	No	No	No	No	4	No	1	1	1	1	1	0	10	969	0	0,038	0,001	38,38	320	120	0,008	0,018	23,06	No	1	1	1	1	No	P	B
9158	M	55	Yes	No	No	No	1	Yes	7	8	1	1	0	0	18	456	209	0,000	0,042	0,61	410	330	0,070	0,001	8,89	No	1	1	1	1	No	T	B
9166	M	68	Yes	No	No	No	1	Yes	6	3	1	1	0	0	19	76	589	0,021	0,001	32,43	323	1501	0,027	0,146	128,11	No	0	0	0	0	No	P	A
9167	F	39	No	No	No	Yes	90	No	1	1	1	1	1	0	12	3097	152	0,032	0,009	110,55	490	390	0,347	0,001	10,48	No	1	1	1	1	No	T	B
9170	M	59	Yes	Yes	No	No	1	Yes	6	3	1	1	0	0	20	0	0	0,000	0,000	0,00	440	940	0,002	0,048	27,73	No	0	0	0	0	No	P	A

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB ß-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD ß-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Antibiotics (7)	Timing (8)
9171	F	64	No	No	No	No	12	No	9	9	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	1	1	No	P	B
9172	M	64	No	No	No	Yes	48	No	9	1	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	1	1	6	No	T	B
9173	F	44	Yes	Yes	Yes	Yes	120	No	1	1	4	1	1	1	10	0	0	0,000	0,000	0,00	440	290	0,338	0,114	16,81	No	1	1	1	6	No	T	A
9174	F	39	No	No	No	Yes	60	No	1	1	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	3	1	1	6	No	T	B
9175	F	26	No	No	No	Yes	60	No	1	1	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	Yes	4	1	1	6	No	T	B
9176	F	51	No	Yes	No	Yes	264	No	1	1	1	1	5	0	10	0	0	0,000	0,000	0,00	900	950	0,017	0,001	25,98	No	2	1	1	1	No	T	B
9177	F	70	No	Yes	No	Yes	40	No	3	3	9	9	5	4	22	627	76	0,143	0,096	20,10	470	280	0,007	0,001	18,27	Yes	4	9	9	1	No	T	A
9178	F	35	No	No	No	Yes	72	No	1	1	1	0	3	0	10	817	152	1,525	0,005	26,75	0	0	0,000	0,000	0,00	No	1	1	1	1	No	T	B
9179	F	37	Yes	No	No	No	6	No	3	2	3	3	6	6	17	0	0	0,000	0,000	0,00	520	380	0,041	0,001	21,69	No	1	1	1	1	No	P	A
9180	F	40	No	No	No	Yes	60	No	1	1	3	3	3	0	10	2204	437	0,011	0,005	38,40	550	590	0,006	0,073	8,12	No	1	1	1	1	No	P	B
9181	M	53	No	No	No	Yes	72	No	1	1	1	1	7	0	10	4495	434	0,045	0,027	133,73	0	0	0,000	0,000	0,00	No	1	0	0	1	No	P	B
9183	F	70	No	No	No	Yes	180	No	1	1	1	0	1	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	0	0	1	No	P	B
9184	F	63	No	No	No	No	6	No	1	1	1	0	6	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	0	1	1	1	No	P	B
9187	F	41	No	No	No	Yes	204	No	1	1	1	0	8	0	10	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	2	1	1	1	No	T	B
9188	F	35	No	No	No	No	6	No	1	1	1	0	1	0	0	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	0	0	1	No	T	B
9189	F	22	No	No	No	Yes	48	No	1	2	3	1	8	0	12	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	0	0	3	No	P	A
9191	M	37	Yes	Yes	Yes	No	18	Yes	8	6	0	3	0	0	16	0	0	0,000	0,000	0,00	0	0	0,000	0,000	0,00	No	1	0	0	1	No	P	B
9192	F	61	Yes	Yes	No	Yes	240	No	3	3	3	3	1	1	18	0	0	0,000	0,000	0,00	120	50	0,262	0,001	5,26	No	1	1	3	1	No	P	B
9193	F	68	No	Yes	No	No	2	No	2	2	4	4	0	1	13	0	0	0,000	0,000	0,00	320	140	0,020	0,055	25,06	Yes	1	4	1	6	No	P	A
9194	F	49	No	No	No	No	24	No	1	1	1	1	3	0	10	0	0	0,000	0,000	0,00	330	340	0,012	0,228	11,05	No	1	1	1	1	No	T	B
9196	M	64	Yes	Yes	Yes	No	12	Yes	8	6	0	9	0	0	25	0	0	0,000	0,000	0,00	160	120	0,132	0,066	8,22	No	1	1	4	1	No	P	B
9197	F	35	Yes	No	No	No	24	No	1	1	1	1	7	0	10	0	0	0,000	0,000	0,00	640	540	0,022	0,001	11,85	No	1	1	1	1	No	T	A
9198	F	62	No	No	Yes	Yes	420	No	3	2	1	1	6	5	11	0	0	0,000	0,000	0,00	180	40	0,042	0,001	14,41	Yes	3	1	1	6	No	T	A
9199	M	76	Yes	No	No	No	1	Yes	6	3	1	1	0	0	30	1519	1116	0,028	0,032	57,51	700	520	0,028	0,077	24,77	No	0	0	0	0	No	T	A

Number	Sex	Age	Previous Jaundice	Previous Cholangitis	Previous Bil. Surgery	History over 3 Years	Months of History	Tumor	Diagnosis (1)	Procedure (2)	GB Bile Cultures (3)	CBD Bile Cultures (3)	GB Stones Type (4)	CBD Stones Type (4)	CBD Size	GB Conj. Bilirubin (*)	GB Unconj. Bilirubin (*)	GB R-Glucuronidase (*)	GB Phospholipase (*)	GB Bile Acids (*)	CBD Conj. Bilirubin (*)	CBD Unconj. Bilirubin (*)	CBD b-Glucuronidase (*)	CBD Phospholipase (*)	CBD Bile Acids (*)	Wound Infection	Clinical Infection	Wound Culture (3)	Drain Culture (3)	Distant Infections (5)	Primary Stone (6)	Antibiotics (7)	Timing (8)

(*) - For Biochemical Tests 0 = not done

(1) - Diagnosis

1 = GB Stones
2 = CBD Stones
3 = GB + CBD Stones
4 = Benign Strictures
5 = GB Tumors
6 = Pancreas Tumors
7 = Ampullary Tumors
8 = CBD and Hilar Tumors
9 = Others

(2) - Procedures

1 = Cholecistectomy
2 = Cholecistectomy + T Tube
3 = Cholecistectomy + CDS
4 = T Tube
5 = CDS
6 = Biliary By-pass (no CDS)
7 = Local Resection HCC
8 = Whipple
9 = Others

(3) - Positive Cultures

0 = Not Done
1 = Sterile
2 = Any Bacteria
3 = Enterobacteria
4 = E.coli
5 = Anaerobes
6 = Polymicrobial Growth
7 = Polymicrobial Including Anaerobes
8 = Polymicrobial Including Enterobacteria
9 = Polimicrobial Including E.coli

(4) - Stones Type (Aschoff code)

0 = Absent
1 = 1
3 = 3
4 = 4
5 = 2A
6 = 2B
7 = 2C
8 = Unknown

(5) - Distant Infections

0 = Not Done
1 = Normal
2 = Respiratory
3 = UTI
4 = Abscess
5 = Bacteriaemia
6 = Post Operative Fever
7 = Others

(6) - Primary CBD Stones (Ca-palmitate Containing)

(7) - Antibiotic Prophylaxis

0 = Not done
P = Piperacillin
T = Tobramicin

(8) - Timing

A = After
B = Before

Table 13.3
Histochemistry Study - Synopsis

Number	Age	Sex	Months of History	Previous Bil. Surgery	Previous Jaundice	Previous Cholangitis	CBD Stones	Jaundice	Fever	GB Bile for Culture	GB Bile E.coli (log)	GB Bile S.faecalis (log)	GB Bile P.aeruginosa (log)	GB Bile P.mirabilis (log)	GB Bile Klebsiella (log)	GB Bile Coliforms (log)	GB Bile Anaerobes (log)	GB Bile Others	GB Bile Highest log	N° of Species from GB Bile	CBD Bile for Culture	CBD Bile E.coli (log)	CBD Bile S.faecalis (log)	CBD Bile P.aeruginosa (log)	CBD Bile P.mirabilis (log)	CBD Bile Klebsiella (log)	CBD Bile Coliforms (log)	CBD Bile Anaerobes (log)	CBD Bile Others	CBD Bile Highest log	N° of Species from CBD Bile
0001	57	M	30	Yes	No	Yes	Yes	No	No	No											Yes										
0002	52	M		No	No	No	Yes	No	No	Yes											Yes										
0003	37	F	240	Yes	Yes	Yes	No	No	No	Yes	2								2	2	Yes										
0004	47	M	1	No	Yes	No	No	No	No	Yes	3	3							3	3	Yes	1								1	1
0005	54	F	3	No	No	No	Yes	No	No	Yes											Yes										
0006	40	M	30	No	No	No	No	No	No	Yes											No										
0007	43	F	3	Yes	No	No	No	No	No	Yes											Yes										
0008	72	M	3	No	No	No	No	Yes	No	No											Yes										
0009	47	F		Yes	Yes	No	No	No	Yes	Yes											Yes										
0010	73	F	24	Yes	No	No	No	No	No	No											Yes	2	1							2	2
0011	56	M	1	No	No	No	No	Yes	No	No											Yes										
0012	42	M	2	No	No	No	No	Yes	No	Yes											Yes										
0013	57	M		No	No	No	No	No	No	Yes											No										
0014	38	F	10	No	No	No	No	No	No	Yes											No										
0015	40	F	6	Yes	Yes	No	No	Yes	No	No											Yes										
0016	64	F	4	Yes	Yes	No	No	No	No	No											Yes										
0017	63	M	4	Yes	No	No	Yes	No	No	Yes											Yes	3					3	3		3	3
0018	60	F	36	No	No	No	No	No	No	Yes							4	3		4	4	No										
0019	27	F	1	No	No	No	No	Yes	No	Yes											Yes										

Number	GB Bile Total Bilirubin	GB Bile Conj. Bilirubin	GB Bile Unconj. Bilirubin	GB Bile β-Glucuronidase	CBD Bile Total Bilirubin	CBD Bile Conj. Bilirubin	CBD Bile Unconj. Bilirubin	CBD Bile β-Glucuronidase	Liver Bile Canalicuil Epithelium	Liver Inflammation	Liver Granulation Tissue	Liver Proliferation Bile Ducts	Liver Fibrosis	Liver β-Gluc. Granules Count	CBD Epithelium	CBD Inflammation	CBD Proliferation	CBD Granulation Tissue	CBD Fibrosis	CBD Tissue β-Glucuronidase	GB Epithelium	GB Inflammation	GB Proliferation	GB Granulation Tissue	GB Fibrosis	GB Tissue β-Glucuronidase
0001					276	230	46	0,017												Yes						O
0002	6465	5161	1304	0,009	4588	2624	1964	0,010												O						Yes
0003	56	39	17	1,507	240	121	119	0,269	1	1	1	1	1	92						Yes	3	3	1	3	1	Yes
0004	1048	768	280	0,036	596	471	125	0,051							1	5	5	5	3	Yes						O
0005	1126	783	343	0,006	292	190	101	0,083	1	3	3	1	5	42						O	1	1	3	1	1	No
0006	1976	1259	717	0,036																Yes						O
0007	2825	2435	390	0,022	698	298	400	0,009												O						O
0008					180	99	80	0,045												No						O
0009	1926	1300	626	0,014	2136	1462	674	0,065	1	1	1	1	1	3	5	3	1	5	5	No	3	3	3	3	1	Yes
0010					752	549	203	0,012	1	3	3	1	1	65	5	3	3	3	3	No						O
0011					169	119	50	0,042	5	3	3	3	1	201	1	1	1	1	1	Yes						O
0012	318	130	188	2,331	382	280	102	0,033	1	3	3	1	1	226	5	1	1	3	5	Yes	5	1	1	1	1	Yes
0013	5116	4070	1046	0,013																O	1	3	1	1	1	Yes
0014	7558	5822	1736	0,018																O	1	1	1	1	1	No
0015					326	240	86	0,033	1	3	3	1	3	197	5	3	3	5	5	Yes						O
0016					392	319	73	0,009	1	3	3	1	1	50	3	3	5	3	3	No						O
0017	85	46	39	0,163	381	230	151	0,015												O	1	3	3	1	1	Yes
0018	966	705	261	0,010																O	3	5	5	3	3	Yes
0019					384	279	105	0,472												O						O

Table 13.4
Reproduction of the Form Used to Record Data

Case Number [_____]

Admission Date _____
Operation Date _____
Hospital n° _____
Ward/Bed n° _____

Antibiotic (timing) _____

Name _____ Age _____
Address _____
Diagnosis _____

History

Duration [___] y. [___] m.

Prev. Jaundice [Y | N]
Prev. Cholangitis [Y | N]
Prev. Biliary Surgery [Y | N]
Prev. Gallstones [Y | N]

Clinical Examination

Pain [Y | N]
where [_____]
Fever [Y | N]
Jaundice [Y | N]
Sign : Murphy [Y | N]
Courvoisier [Y | N]

Radiology

_____

_____

Procedure

I.O. Cholangiography [Y | N]
findings _____
CBD Size (mm) _____
Aschoff Type _____

Bile Biochemistry

Bilirubin Total _____
Conjugated _____
Unconjugated _____

ß-Glucuronidase _____
Phospholipase _____
Total Bile Acids _____

Cultures

Bile

GB Bile _____
CBD Bile _____

Postoperative

Swabs _____
Drain Fluids _____

5th Post-Op. Day _____
(clinical assessment)

Wound Status _____

Distant Infections _____
1

Stones X-Ray Diffractometry

_____

_____

Dig Surg 1988;5:185–188

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0253–4886/88/0054–0185$2.75/0

Length of Clinical History as a Risk Factor for Infection following Surgery for Gallstone Disease

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Key Words. Bile · Infection · Gallbladder · Common bile duct · Biliary tract infection · Biliary tract disease · Antibiotics · Risk factors

Abstract. The role of risk factors for predicting infection in surgery for gallstone disease has been evaluated in 96 patients. Conventionally recognized risk factors were present in 43 patients, whereas 53 patients had none. Four patients had polymicrobial bacterial growth in bile and all of them had a clinical history longer than 3 years. There was no statistical difference in postoperative infection rates between the groups (37 and 30%, respectively). In the low-risk group, however, the patients with a clinical history of over 3 years had a postoperative infection rate significantly higher (p = 0.0001 Fisher's exact test).

Introduction

Infective complications of bilary tract surgery still pose a great challenge to the surgeon. The incidence of bacteria in bile sampled at operation, during uncomplicated biliary surgery, ranges from 30 to 50% [1–3] and the incidence of wound infection for elective cholecystectomy varies from 11 to 21% [4, 5]. However, this figure rises well above 80% for bile infection [6, 7] in the presence of biliary obstruction due to stones or benign strictures. In the presence of obstruction due to malignancy, bacterial colonization is less, ranging from 0 [8] to 20% [9]. The commonest isolates in bile are the aerobic organisms *Escherichia coli, Klebsiella, Pseudomonas, Proteus* and *Streptococcus faecalis* and the anaerobes *Bacteroides fragilis* and *Clostridia* [10]. However, one recent report found a high incidence of staphylococci [11].

[1] GA and ISB were supported by NATO grant No. 0029/87.

In the present study we examine positive bile cultures and postoperative infective complications relating to the presence of preoperative risk factors, in a prospective randomized trial of antibiotic prophylaxis in biliary surgery and emphasize the role played by a little recognized risk factor: a long clinical history of gallstone disease.

Patients and Methods

Ninety-six patients attending the 'Divisione A. Trapani', Ospedale Cardarelli, Napoli, Italy, with gallstone disease since March 1983 to June 1984 are considered in this paper. Sixteen were males and eighty females, the mean age being 48.5 years (range 18–81). On admission, the duration of the disease was defined by a full clinical history, paying careful attention to the duration of the known presence of gallstones, determined on the basis of an oral cholecystogram or on the length of clinical history indicated by the presence of cholangitis with characteristic Charcot's triad. The presence of conventionally recognized risk factors, as defined by Keighley et al. [8] – age over 60 yeras, jaundice, cholangitis, previous surgery, common bile duct (CBD) stones – was also determined for each case.

Fifty-two patients were randomized to receive preoperative antibiotic prophylaxis with piperacillin (2 g i.m.) and 44 with tobramycin (100 mg i.m.). Cholecystectomies were carried out in a routine fashion. Intraoperative cholangiography was performed in selected cases: criteria for selection were a history of jaundice, cholangitis or previous biliary surgery or a preoperative intravenous cholangiogram demonstrating abnormalities in the CBD, such as filling defects, increased size or delayed emptying. Operative cholangiography was performed both before and after CBD exploration which was always carried out in a supraduodenal manner. After duct exploration, a choledochoduodenostomy or an external biliary drainage via t-tube was carried out. Choledochoduodenostomy was performed when the CBD was more than 18 mm in size or in the presence of a 'primary' stone according to the Aschoff's criteria [12]. Otherwise t-tubes were brought out through a separate incision and were left in place for 12–13 days. The tubes remained completely open for 5 days allowing free external biliary drainage, then progressively closed elevating the bag for bile collection for the following days. T-tube cholangiography was always performed before removal.

Bile was collected in every patient, from the gallbladder when present and from the CBD whenever possible. Organisms were identified by conventional methods. An intraperitoneal drain was left in place in each patient, draining the subhepatic space and brought out through a separate stab incision.

On the 5th postoperative day, cultures were taken from intraperitoneal fluids and wound swabs. At the same time, a clinical assessment for the presence of infection was also carried out: wounds were classified according to a standard grading: 0 = wound perfectly healed; 1 = wound slightly reddened; 2 = wound markedly reddened; infiltration; 3 = little serosal discharge; 4 = increased discharge of purulent material; 5 = large purulent collection, with impairment of general status. The development of later infection was also determined during the hospital stay and at follow-up. Patients were considered to have a distant infection if they had pneumonia, urinary tract infection or bacteraemia. The presence of fever over 38 °C for longer than 48 h postoperatively was also recorded.

Results

Conventionally recognized risk factors [8] were found in 43 out of 96 patients. Twenty-seven had age >60 years, 9 obstructive jaundice, 19 a history of cholangitis, 6 previous biliary surgery and 11 CBD stones. No patients in this series underwent emergency surgery. In addition, 63 out of 96 patients had a clinical history longer than 3 years.

Ninety-two patients underwent cholecystectomy alone or combined with choledochoduodenostomy (4 cases) or CBD exploration via a t-tube drainage (7 cases). Three cases with a previous cholecystectomy had a choledochoduodenostomy and 1 patient had an external biliary drainage via a t-tube. No patient in this series had residual stones detected at t-tube cholangiography.

Twenty-three patients out of 96 (23.9%) had positive intraoperative bile cultures. Patients treated with piperacillin had a slightly lower positive bile culture rate. *E. coli, Klebsiella, Pseudomonas, Enterobacter, S. faecalis* were the organisms most frequently recovered from bile. Anaerobic bacteria *(S. intermedius)* were isolated from bile in one patient.

Thirty-two patients showed the presence of one or more postoperative infections: 17 demonstrated a wound clinically infected at postoperative examination; one had a positive culture from the wound swab; 8 positive drain cultures; 3 developed distant infections and 15

Table I. Correlation between length of clinical history, bile infection and polymicrobial bacterial growth (p values: Fisher's exact test)

	Bile infection	Polymicrobial growth
History >3 years (n = 63)	19	4
History <3 years (n = 33)	4	0
Significance of difference	$p > 0.05$	$p > 0.05$
Total (n = 96)	23	4

Table II. Correlation between length of history and postoperative infections (p values: Fisher's exact test)

Risk factors	Infections			
	overall	local	drain	general
Conventional risk factors (n = 43)	16 (37.2)	9	6	9
No conventional risk factors (n = 53)	16 (30.2)	9	2	7
Significance of difference	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
No risk factors and history >3 years (n = 29)	15 (51.7)	9	1	7
No risk factors and history <3 years (n = 24)	1 (4.2)	0	1	0
Significance of difference	$p = 0.001$	$p = 0.005$	$p > 0.05$	$p = 0.02$

Figures in parentheses are percentages.

patients presented postoperative fever. Eight patients developed positive postoperative cultures: in 3 cases (37.5%) these cultures were due to organisms previously cultured in bile *(E. coli, Pseudomas aeruginosa, Enterobacter cloacae); Straphylococcus aureus* was isolated from the wound swab in 1 patient; in 2 cases anaerobic bacteria were isolated *(S. intermedius, P. magnus);* in the remaining two cases, *E. coli* and *P. aeruginosa* were isolated from the wound, despite sterile bile at operation.

Table I shows the relationship between length of clinical history and bile infection and polymicrobial bacterial growth: the rates of positive bile cultures increase with the duration of disease although without statistical significance. Moreover it should be noted that in 3 out of 4 patients with polymicrobial bacterial growth, no conventional risk factor was detected.

The relationships between preoperative risk factors and postoperative infections are shown in table II. The infection rate is surprisingly similar in this series in patients with or without commonly recognized risk factors (37.2 and 30.2%, respectively). However, when the group of patients at low risk of infections is divided into those patients with a clinical history longer or shorter than 3 years, there is a highly significant difference in postoperative infection rate ($p < 0.001$, Fisher's exact test). Furthermore, this correlation is found both for wound infections together with postoperative cultures and distant infections together with postoperative fever.

Discussion

The importance of risk factors for predicting infections in patients undergoing biliary tract surgery has been emphasized by many authors [13–15]. More recently [8], such risk factors have been more accurately defined. There is now general agreement [7, 11, 16] that age > 60 years, obstructive jaundice, a history of cholangitis, previous biliary surgery, CBD stones and emergency operations are well established risk factors for predicting the presence of bacteria in bile.

The incidence of positive bile cultures in our patients (23.9%) was similar to that reported in the literature [1–3]. By contrast anaerobic bacteria were isolated from bile much less (1%) than in other series [7, 10], probably because most of our patients underwent surgery for uncomplicated gallbladder stone disease which, as already hypothesized by others [16], does not predispose to the growth of anaerobic bacteria in bile. Genera most frequently isolated from bile were similar to those commonly reported in the literature [10].

We have defined as postoperatively infected both patients showing a clinically infected wound and those presenting postoperative fever even in the absence of positive postoperative cultures: this wide classification explains the high overall postoperative infection rate (33.3%). We have found in this study a close relationship between bacteria isolated from bile at time of surgery and organisms responsible for postoperative wound infections: this is in agreement with data reported by others [15, 16] and should be taken into account in the choice of antibiotic prophylaxis.

A clinical history longer than 3 years is uncommon in most countries, and this has not been identified in previous published series as a significant risk factor. In contrast, in the south of Italy, and probably in other Mediterranean countries, a higher number of patients are found with this feature. Sixty-three patients have been found in this study with a history of gallstone disease longer than 3 years and 28 of them with a history longer than 6 years, with a mean length of 6.2 years; the importance of this long history has been clearly demonstrated in this study: bacterial colonization of bile is higher in patients carrying gallstone disease for a longer time and this is in keeping also with data from experimental work in dogs [17]. Furthermore, the association of a long clinical history with polymicrobial bacterial growth in bile may be due to previous attacks of cholangitis to which a patient with long-standing gallstone disease is more susceptible. The need for numerous courses of antibiotic therapy might increase the emergence of resistant strains.

The relationship between bile infection and postoperative infection is well established [3, 11]. By contrast there is still debate whether prophylactic antibiotic coverage is indicated for all patients undergoing biliary surgery or only for high-risk patients selected by conventionally accepted criteria [18, 19]. In one recent report, however [20], conventional risk factors failed to fulfil their proposed predictive role, as in the present series. Morran et al. [11] and Stubbs [21] have also emphasized how difficult it may be to identify a true group at low risk of infection in biliary surgery. This may be due to failure to recognize some other factor, such as the length of clinical history, as seen in our series. The selective administration of antibiotics in patients undergoing surgery for gallstone disease can only be accepted if a full evaluation of all factors which may predict infection is undertaken. A long clinical history may well be one such factor.

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Received: April 11, 1988
Accepted: May 16, 1988

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Antibiotic Prophylaxis for Biliary Tract Surgery: Selection of Patient and Agent

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Reprinted from INTERNATIONAL SURGERY
Vol. 76 - No. 2 - pp. 131-134 (April-June 1991)

EDIZIONI MINERVA MEDICA - TORINO

INT SURG 1991; 76:131-134

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One hundred and three patients undergoing biliary surgery have been entered in a randomized prospective trial for antibiotic prophylaxis. Fifty six received piperacillin (2 gr) and 47 tobramycin (100 mg). Cultures were taken from bile at operation and a clinical and microbiological assessment for infection was carried out postoperatively. Overall infection rate was significantly lower in the piperacillin group ($P < 0.05$). The positive bile culture and wound infection rates were also lower in the piperacillin group, though this did not reach statistical significance. *Anaerobic bacteria* (three patients) and *Streptococcus faecalis* (one patient) were isolated in the tobramycin group. Overall postoperative infection rate was similar in patients with risk factors for infection and in those without. In conclusion, piperacillin did not significantly improve bile and wound infection rates compared to tobramycin. However piperacillin showed a more appropriate spectrum of activity. Antibiotic prophylaxis is suggested for all patients undergoing biliary surgery.

KEY WORDS: Antibiotics - Bile - Infection - Gallbladder - Common bile duct - Biliary tract disease - Risk factors.

It is almost a century since Welch first described the presence of bacteria in bile and in the centre of gallstones.[1] Recent observations report a 30-50% incidence of bacteria in bile sampled at the time of biliary tract surgery.[2-4] The incidence of positive bile cultures rises above 80% in the presence of biliary obstruction due to stones or iatrogenic strictures. In obstruction due to malignancy, however, positive bile cultures have been reported in as few as 20% of patients.[5]

Commonly recognized risk factors associated with a high incidence of bacteria in bile are: age greater than 60 years, obstructive jaundice, previous biliary tract surgery, emergency operations, choledocholithiasis and a history of cholangitis.[5] Postoperative infections may be caused either by coagulase positive staphylococci or by organisms originating from bile [4] such as *Escherichia coli, Klebsiella species, Proteus species* and *Streptococcus faecalis*. Anaerobic bacteria, particularly *Bacteroides fragilis* and *Clostridium* species are less commonly isolated from bile, unless previous bilio-enteric anastomosis has been performed.[6,7] The judicious use of antibiotic prophylaxis in the prevention of sepsis following biliary surgery is widely advocated. Antibiotic selection must be based upon spectrum of activity and pharmacokinetics and upon controlled clinical study of their efficacy.

In the present study we compare the results of antibiotic prophylaxis with piperacillin or tobramycin in patients undergoing surgery for biliary tract disease.

Material and methods

One-hundred and three patients undergoing biliary surgery in "Divisione A. Trapani", Ospedale Cardarelli, Naples, Italy, over a period of 16 months, between March 1983 and June 1984, were entered into a randomized prospective trial. After informed consent was obtained, 56 (Group 1) were randomly allocated to receive piperacillin (2 G IM) and 47 (Group 2) to receive tobramycin (100 mg IM) one hour before surgery. Antibiotic administration was repeated at 12 hourly intervals for 48 hours.

On admission a detailed history and clinical examination was obtained for each patient recording the presence of all accepted risk factors for infection (Table I). Patients were defined as jaundiced when total serum bilirubin was 1.3 mg/dl (20 μmol/L). The operations performed in each group are listed in Table II. Cholecystectomy was carried out in 96 of 103 patients. Choledochoduodenostomy was undertaken in nine patients (in

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TABLE V.—*Antibiotic concentration in bile and serum (mg/l).*

	Piperacillin (n=50) mean (range)	Tobramycin (n=45) mean (range)
Serum	21.6 (1-100)	7.7 (1-32)
Gallbladder bile	66.3 (1-2000)	2.7 (0-8)

Antibiotic Assays

Results of antibiotic assays in serum and bile from 95 patients are reported in Table V. Serum concentration of both tobramycin and piperacillin were generally therapeutically adequate. In some individual patients, however, the serum concentration of antibiotic was below the likely minimal inhibitory concentration of normally susceptible organisms. Bile levels of piperacillin were adequate (mean 66.3 mg/l) in the majority of patients studied, whereas levels of tobramycin were much lower (mean 2.7 mg/l) and in one patient no drug was detectable in bile.

Risk Factors

We have also studied the relationship of risk factors identifiable in the preoperative period and the development of postoperative infections. One or more of the conventional risk factors, as defined by Keighley *et al.*,[5] were noted in 50 of 103 patients, whereas 103 had none (Table I). The overall postoperative infection rate was similar in those patients in whom no risk factors could be identified (17 of 53, 32.1%), and in those in whom one or more risk factors were noted (18 of 50, 36%).

Discussion

The role of antibiotic prophylaxis in biliary surgery is well recognized. Keighley *et al.*[8] have clearly demonstrated the efficacy of gentamicin, an agent giving adequate serum levels but low concentrations in bile, in the prevention of infection, when compared to rifamide, an agent excreted in high concentration in bile.

When we started this study we had three questions in our mind:

1) In the presence of adequate serum levels do high bile levels of antibiotic allow a better protection against infections in biliary tract surgery?

2) Which spectrum of activity should an antibiotic have to prevent these infections?

3) Should antibiotic prophylaxis be used in all patients undergoing biliary tract surgery rather than only in patients selected according to conventionally accepted criteria?

We therefore compared tobramycin, an aminoglycoside antibiotic similar in spectrum of activity and pharmacokinetics to gentamicin, with piperacillin, an acylureidopenicillin achieving good activity in serum and bile.[9-11] There was no significant difference between the groups in bile cultures and postoperative wound, drain and distant infections. However, the overall incidence of postoperative infective problems ($P=0.04$) and of postoperative fever ($P=0.02$) was lower in the piperacillin group.

No anaerobes were isolated from bile or postoperative cultures of patients receiving piperacillin, whose spectrum of activity includes anaerobic bacteria,[12] compared with three patients receiving prophylaxis with tobramycin, which lacks activity against these genera. Streptococcus faecalis was isolated in only one patient in this series, a positive bile culture in a patient receiving tobramycin. Others have reported a high incidence of Streptococcus faecalis in bile [13] and have found it to be a significant pathogen in patients undergoing complex biliary procedures.[14]

The clear association between bacteria found in bile at the time of surgery and organisms responsible for postoperative septic complications underlines the importance of basing the choice of antibiotic on the usual microbiology of the bile in the population under treatment. The selective administration of antibiotic to those patients in whom one or more risk factors may be identified preoperatively has been advocated.[5 15] However, in the present study conventional criteria failed to identify all patients at risk of infection and this finding is supported by several other authors.[16-18] Although there was no untreated control group in this study, these data suggest that antibiotic prophylaxis may be appropriate for all patients undergoing biliary tract surgery.

On the basis of our data we therefore conclude that: 1) to prevent infections in biliary tract surgery an antibiotic should be capable of high serum concentrations: the simultaneous presence of high bile levels does not seem to reduce significantly the incidence of bile and postoperative local and general infections; 2) a spectrum of activity which includes anaerobic bacteria, as shown by piperacillin in our study, as well as enterobacteria and faecal streptococci, seems to be more effective in preventing infections in biliary tract surgery; and 3) the difficulty in identifying a true group of 'high risk patients' suggests the use of antibiotic prophylaxis for all patients undergoing biliary tract surgery.

Acknowledgement.—. Dr. G. Aloj and Mr. I. S. Benjamin were supported by the NATO Scientific Affairs Division, Grant n. 0029/87.
We are also grateful to Lederle Italia for their support during this study.

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