

**THE PATHOGENESIS AND NON-SURGICAL TREATMENT OF  
GALLSTONES: CLINICAL AND LABORATORY STUDIES**

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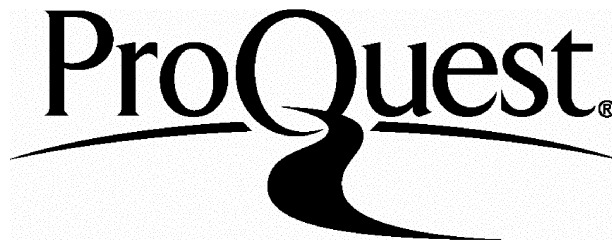
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## ABSTRACT

**Background:** Gallstone disease is common and affects between 10% and 20% of the world's population. This thesis addresses selected aspects of the non-surgical treatment of patients with symptomatic gallbladder stones, and the pathogenesis of primary and recurrent gallstone formation. **Methods:** Pretreatment gallstone characteristics that predict the speed and completeness of non-surgical treatments were analysed, together with the reasons for non-response in patients with incomplete gallstone dissolution, and the composition of recurrent gallstones. In three distinct patient groups with, or at risk of developing, gallstones, various aspects of bile composition were also examined: i) bile physical chemistry and cholesterol microcrystal nucleation times in patients with 'conventional' cholesterol-rich gallstone disease, before and after successful medical dissolution therapy, (ii) the effects of octreotide on biliary phospholipid and mucin glycoprotein concentrations in paired, before and during treatment studies in acromegalic patients, and (iii) alterations in plasma and biliary phospholipids, biliary bile acids and non-mucin glycoproteins, in patients with active inflammatory bowel disease or following bowel resection. **Results and Discussion:** In patients with gallbladder stones undergoing medical dissolution therapy, the pretreatment computed tomography attenuation score predicted both the speed and completeness of gallstone dissolution. Irrespective of the original gallstone composition, recurrent stones were usually cholesterol-rich. Gallbladder bile from acromegalic patients treated with octreotide had multiple abnormalities of bile composition that were comparable with those seen in conventional cholesterol gallstone disease, with increases in biliary deoxycholic acid, arachidonic acid-rich phospholipids, mucin glycoprotein and cholesterol saturation — supporting the hypothesis that a rise in biliary deoxycholic acid, secondary to prolongation of intestinal transit and/or impaired gallbladder emptying, leads to the formation of lithogenic bile and cholesterol-rich gallstones. In contrast, in patients with ileal disease/resection, the presence of high biliary bilirubin concentrations and low % deoxycholic acid may favour the formation of mixed, pigment-rich gallstones.

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## TABLE OF CONTENTS

|                                  |    |
|----------------------------------|----|
| Abstract.....                    | 2  |
| Acknowledgements .....           | 3  |
| Table of Contents .....          | 4  |
| List of Tables and Figures ..... | 9  |
| List of Abbreviations .....      | 14 |
| Thesis Plan.....                 | 15 |

## ***PART 1. BACKGROUND***..... 16

### **CHAPTER 1. GALLBLADDER STONE DISEASE** ..... 17

|                                                                   |    |
|-------------------------------------------------------------------|----|
| 1.1 INTRODUCTION .....                                            | 17 |
| 1.2 EPIDEMIOLOGY .....                                            | 17 |
| 1.2.1 Risk factors for conventional gallstones.....               | 17 |
| 1.2.2 Slow intestinal transit.....                                | 20 |
| 1.2.3 Octreotide-induced gallstones.....                          | 21 |
| 1.2.4 Gallstones in inflammatory bowel disease.....               | 23 |
| 1.2.5 Pigment gallstones .....                                    | 24 |
| 1.2.6 Biliary sludge .....                                        | 25 |
| 1.3 BILE COMPOSITION AND PHYSICAL CHEMISTRY .....                 | 27 |
| 1.3.1 Cholesterol .....                                           | 27 |
| 1.3.2 Cholesterol carriers .....                                  | 28 |
| 1.3.3 Phospholipids.....                                          | 29 |
| 1.3.4 Bile acids.....                                             | 34 |
| 1.4 GALLSTONE PATHOGENESIS.....                                   | 36 |
| 1.4.1 Supersaturation of bile with cholesterol .....              | 37 |
| 1.4.2 Cholesterol microcrystal nucleation and crystal growth..... | 38 |
| 1.4.3 Role of biliary phospholipids.....                          | 39 |
| 1.4.4 Mucin glycoprotein.....                                     | 42 |
| 1.4.5 Non-mucin promoters and inhibitors of nucleation.....       | 45 |
| 1.4.6 Gallbladder motor dysfunction .....                         | 46 |

### **CHAPTER 2. NON-SURGICAL TREATMENT OF GALLSTONES** ..... 49

|                                        |    |
|----------------------------------------|----|
| 2.1 INTRODUCTION .....                 | 49 |
| 2.2 NATURAL HISTORY OF GALLSTONES..... | 49 |
| 2.2.1 Asymptomatic gallstones .....    | 49 |
| 2.2.2 Symptomatic gallstones .....     | 51 |
| 2.3 INVESTIGATIONS .....               | 52 |
| 2.3.1 Ultrasonography.....             | 52 |
| 2.3.2 Oral cholecystography .....      | 53 |
| 2.3.3 Computed tomography.....         | 54 |
| 2.3.4 Magnetic resonance imaging ..... | 55 |
| 2.4 MEDICAL MANAGEMENT .....           | 55 |
| 2.4.1 Oral bile acid therapy.....      | 56 |

|       |                                                   |    |
|-------|---------------------------------------------------|----|
| 2.4.2 | Reasons for incomplete gallstone dissolution..... | 58 |
| 2.4.3 | Extracorporeal shock-wave lithotripsy .....       | 59 |
| 2.4.4 | Instillation of contact solvents .....            | 60 |
| 2.4.5 | Percutaneous cholecystolithotomy.....             | 62 |
| 2.5   | MANAGEMENT OF RECURRENT GALLSTONES .....          | 62 |
| 2.6   | GALLSTONE PREVENTION.....                         | 63 |
| 2.6.1 | Diet.....                                         | 63 |
| 2.6.2 | Ursodeoxycholic acid.....                         | 64 |
| 2.6.3 | Aspirin and NSAIDs .....                          | 64 |
| 2.6.4 | Prokinetic agents .....                           | 66 |

## ***PART II. NON-SURGICAL TREATMENT OF GALLSTONES: CLINICAL STUDIES..... 68***

### **CHAPTER 3. PERCUTANEOUS CHOLECYSTOLITHOTOMY: RISKS, BENEFITS AND LONG-TERM OUTCOME..... 69**

|       |                                               |    |
|-------|-----------------------------------------------|----|
| 3.1   | INTRODUCTION .....                            | 69 |
| 3.2   | METHODS .....                                 | 69 |
| 3.2.1 | Patients .....                                | 69 |
| 3.2.2 | PCCL technique .....                          | 70 |
| 3.2.3 | Patient follow-up .....                       | 71 |
| 3.3   | RESULTS.....                                  | 71 |
| 3.3.1 | Procedure-related complications .....         | 73 |
| 3.3.2 | Long-term follow-up.....                      | 73 |
| 3.3.3 | Characteristics of recurrent gallstones ..... | 74 |
| 3.4   | DISCUSSION .....                              | 75 |

### **CHAPTER 4. GALLSTONE DISSOLUTION WITH ORAL BILE ACID THERAPY: IMPORTANCE OF PRE-TREATMENT CT SCANNING AND REASONS FOR NON-RESPONSE..... 78**

|       |                                                                        |    |
|-------|------------------------------------------------------------------------|----|
| 4.1   | INTRODUCTION .....                                                     | 78 |
| 4.2   | METHODS .....                                                          | 79 |
| 4.2.1 | Patient selection .....                                                | 80 |
| 4.2.2 | Assessment of patients with no/arrested gallstone dissolution .....    | 81 |
| 4.3   | RESULTS.....                                                           | 81 |
| 4.3.1 | Pre-treatment gallstone characteristics .....                          | 81 |
| 4.3.2 | The value of pre-treatment CT in predicting dissolution response ..... | 82 |
| 4.3.3 | Reasons for no/arrested gallstone dissolution.....                     | 84 |
| 4.4   | DISCUSSION .....                                                       | 85 |

### **CHAPTER 5. GALLSTONE RECURRENCE AFTER MEDICAL TREATMENT: DO GALLSTONES RECUR TRUE TO TYPE?..... 89**

|       |                                                   |    |
|-------|---------------------------------------------------|----|
| 5.1   | INTRODUCTION .....                                | 89 |
| 5.2   | METHODS .....                                     | 89 |
| 5.2.1 | Gallstone dissolution/removal and recurrence..... | 90 |
| 5.2.2 | Statistical analysis .....                        | 91 |

|                                                         |    |
|---------------------------------------------------------|----|
| 5.3 RESULTS.....                                        | 91 |
| 5.3.1 Pre-treatment gallstone characteristics .....     | 91 |
| 5.3.2 Recurrent stone number and size .....             | 93 |
| 5.3.3 Indirect assessment of gallstone composition..... | 93 |
| 5.3.4 Treatment of the recurrent stones .....           | 95 |
| 5.4 DISCUSSION .....                                    | 96 |

### ***PART III. THE PATHOGENESIS OF GALLSTONES: LABORATORY STUDIES***..... 100

#### **CHAPTER 6. BILE PHYSICAL CHEMISTRY IN PATIENTS WITH ARRESTED GALLSTONE DISSOLUTION DURING ORAL BILE THERAPY** ..... 101

|                                                         |     |
|---------------------------------------------------------|-----|
| 6.1 INTRODUCTION .....                                  | 101 |
| 6.2 METHODS .....                                       | 102 |
| 6.2.1 Patients .....                                    | 102 |
| 6.2.2 Storage and handling of samples .....             | 102 |
| 6.2.3 Cholesterol microcrystal nucleation times.....    | 103 |
| 6.2.4 Total bile acids.....                             | 104 |
| 6.2.5 Total phospholipids.....                          | 105 |
| 6.2.6 Biliary cholesterol concentrations.....           | 107 |
| 6.2.7 Cholesterol saturation indices .....              | 108 |
| 6.2.8 Measurement of biliary bile acid conjugates ..... | 109 |
| 6.3 RESULTS.....                                        | 112 |
| 6.4 DISCUSSION .....                                    | 114 |

#### **CHAPTER 7. THE ROLE OF PHOSPHOLIPIDS AND MUCIN GLYCOPROTEIN IN THE PATHOGENESIS OF OCTREOTIDE-INDUCED GALLSTONES**..... 116

|                                                                                 |     |
|---------------------------------------------------------------------------------|-----|
| 7.1 INTRODUCTION .....                                                          | 116 |
| 7.2 METHODS .....                                                               | 117 |
| 7.2.1 Patients .....                                                            | 117 |
| 7.2.2 Bile sampling .....                                                       | 118 |
| 7.2.3 Biliary lipid and bile acid composition.....                              | 118 |
| 7.2.4 Fölch extraction of biliary phospholipids .....                           | 118 |
| 7.2.5 Separation of phospholipid classes.....                                   | 119 |
| 7.2.6 Separation of molecular species of phosphatidylcholine .....              | 121 |
| 7.2.7 Purification of gallbladder mucin by gel filtration .....                 | 122 |
| 7.2.8 Quantification of immunoreactive mucin by ELISA.....                      | 126 |
| 7.2.9 Statistical analysis .....                                                | 127 |
| 7.3 RESULTS.....                                                                | 128 |
| 7.3.1 Phosphatidylcholine species before and during octreotide .....            | 128 |
| 7.3.2 Relationships between phosphatidylcholine and cholesterol saturation..... | 129 |
| 7.3.3 Biliary deoxycholic acid .....                                            | 130 |
| 7.3.4 Mucin glycoprotein concentrations .....                                   | 131 |
| 7.4 DISCUSSION .....                                                            | 131 |

#### **CHAPTER 8. BILE COMPOSITION IN INFLAMMATORY BOWEL DISEASE: ILEAL DISEASE AND COLECTOMY, BUT NOT COLITIS, INDUCE LITHOGENIC BILE** ..... 136

|                                                                                                               |            |
|---------------------------------------------------------------------------------------------------------------|------------|
| 8.1 INTRODUCTION .....                                                                                        | 136        |
| 8.2 METHODS .....                                                                                             | 137        |
| 8.2.1 Patients .....                                                                                          | 137        |
| 8.2.2 Aspiration of gallbladder bile .....                                                                    | 139        |
| 8.2.3 Cholesterol microcrystal nucleation times .....                                                         | 139        |
| 8.2.4 Bile physical chemistry .....                                                                           | 139        |
| 8.2.5 Bile acid conjugates and phosphatidylcholine species .....                                              | 140        |
| 8.2.6 Statistical analysis .....                                                                              | 141        |
| 8.3 RESULTS.....                                                                                              | 141        |
| 8.3.1 Bile lipids and cholesterol saturation indices .....                                                    | 141        |
| 8.3.2 Cholesterol microcrystal nucleation times .....                                                         | 143        |
| 8.3.3 Biliary bilirubin levels.....                                                                           | 144        |
| 8.3.4 Bile acid conjugates and phosphatidylcholine species .....                                              | 145        |
| 8.4 DISCUSSION .....                                                                                          | 147        |
| <b>CHAPTER 9. BILIARY NON-MUCIN GLYCOPROTEINS IN INFLAMMATORY BOWEL DISEASE .....</b>                         | <b>153</b> |
| 9.1 INTRODUCTION .....                                                                                        | 153        |
| 9.2 METHODS .....                                                                                             | 154        |
| 9.2.1 Patients .....                                                                                          | 154        |
| 9.2.2 Aspiration of gallbladder bile .....                                                                    | 156        |
| 9.2.3 Gel filtration of biliary lactoferrin .....                                                             | 156        |
| 9.2.4 Lactoferrin ELISA .....                                                                                 | 158        |
| 9.2.5 Electrophoresis and Western Blotting .....                                                              | 159        |
| 9.2.6 Myeloperoxidase ELISA .....                                                                             | 161        |
| 9.2.7 Biliary bile acid concentrations .....                                                                  | 161        |
| 9.2.8 Cholesterol saturation indices and microcrystal nucleation times.....                                   | 161        |
| 9.2.9 Statistical analysis .....                                                                              | 162        |
| 9.3 RESULTS.....                                                                                              | 162        |
| 9.3.1 Biliary lactoferrin in ulcerative colitis .....                                                         | 162        |
| 9.3.2 Lactoferrin in Crohn's disease .....                                                                    | 163        |
| 9.3.3 Bile acid concentrations.....                                                                           | 164        |
| 9.3.4 Myeloperoxidase in ulcerative colitis .....                                                             | 165        |
| 9.3.5 Relationship between biliary lactoferrin and bile lithogenicity .....                                   | 166        |
| 9.4 DISCUSSION .....                                                                                          | 166        |
| <b>CHAPTER 10. PLASMA ARACHIDONIC ACID-RICH PHOSPHOLIPIDS IN CROHN'S DISEASE: RESPONSE TO TREATMENT .....</b> | <b>171</b> |
| 10.1 INTRODUCTION .....                                                                                       | 171        |
| 10.2 METHODS .....                                                                                            | 172        |
| 10.2.1 Experimental design and evaluation of response to treatment .....                                      | 172        |
| 10.2.2 Plasma phosphatidylcholine species .....                                                               | 173        |
| 10.2.3 Statistical analysis .....                                                                             | 173        |
| 10.3 RESULTS .....                                                                                            | 173        |
| 10.3.1 Clinical response to treatment.....                                                                    | 174        |
| 10.3.2 Plasma lactoferrin .....                                                                               | 174        |
| 10.3.3 Plasma phosphatidylcholine species .....                                                               | 176        |
| 10.4 DISCUSSION .....                                                                                         | 177        |

|                                                             |                |
|-------------------------------------------------------------|----------------|
| <b>CHAPTER 11. GENERAL DISCUSSION AND CONCLUSIONS .....</b> | <b>181</b>     |
| 11.1 NON-SURGICAL TREATMENT OF GALLSTONES .....             | 181            |
| 11.2 GALLSTONE PATHOGENESIS.....                            | 182            |
| 11.2.1 Octreotide-induced gallstones.....                   | 182            |
| 11.2.2 Gallstones in inflammatory bowel disease .....       | 183            |
| 11.3 GALLSTONE PREVENTION .....                             | 185            |
| <b>REFERENCES.....</b>                                      | <b>187</b>     |
| <br><b>APPENDIX. PAPERS ARISING FROM THIS THESIS....</b>    | <br><b>230</b> |
| First-author papers.....                                    | 230            |
| Co-authored articles .....                                  | 231            |

## LIST OF TABLES AND FIGURES

|             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |    |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1  | The structure of native somatostatin and octreotide. From Hussaini 1995.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 21 |
| Figure 1.2  | Gallstones from a patient with octreotide-induced gallstones. The stones are multiple, smooth and faceted. The cut surface shows a pigmented centre with a radiating lattice composed of crystalline cholesterol. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | 23 |
| Figure 1.3  | Hepatic cholesterol metabolism. Cholesterol is taken up into the liver from plasma lipoproteins. The boxes indicate the rate limiting enzymes for: (i) de novo cholesterol synthesis: HMG CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, (ii) cholesterol ester synthesis (ACAT: acyl coenzyme A cholesterol acyltransferase), (iii) cholesterol ester hydrolysis (CEH: neutral cholesterol ester hydrolase), and (iv) bile acid synthesis (7 $\alpha$ hydroxylase).....                                                                                                                                                                                                                 | 28 |
| Figure 1.4  | The phospholipid classes.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 30 |
| Figure 1.5  | Schematic diagram of phosphatidylcholine, the predominant biliary phospholipid. <i>Sn-1</i> is typically a saturated or monosaturated fatty acid, e.g. palmitic acid or stearic acid, while <i>sn-2</i> is usually a polyunsaturated fatty acid, e.g. linoleic acid or arachidonic acid. ....                                                                                                                                                                                                                                                                                                                                                                                                    | 31 |
| Figure 1.6  | Postulated mechanism of mdr2 P-glycoprotein-mediated lipid secretion. Phosphatidylcholine (PC) is supplied to the membrane mainly via PC-transfer protein which inserts PC into the inner leaflet. Mdr2 P-glycoprotein ‘flips’ PC to the outer leaflet into PC-rich microdomains. Luminal bile acid micelles and/or monomers further destabilise these domains and this leads (via an unknown mechanism) to the formation and release of vesicular structures. In the absence of PC-rich domains, luminal bile acid micelles can still solubilise cholesterol but are unable to extract PC from the outer leaflet of the membrane. From Oude Elferink and Groen 1999. ....                       | 34 |
| Figure 1.7  | Scheme of hepatic and intestinal metabolism of bile acids and the enterohepatic circulation. Primary bile acids undergo bacterial 7 $\alpha$ dehydroxylation to form the secondary bile acids, lithocholic acid and deoxycholic acid. Unlike deoxycholic acid, lithocholic acid is not absorbed from the intestine to any appreciable extent.....                                                                                                                                                                                                                                                                                                                                                | 35 |
| Figure 1.8  | Venn diagram illustrating the three components of the classical ‘triple defect’ predisposing to cholesterol gallstone formation.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 37 |
| Figure 1.9  | Simplified schematic diagram of the arachidonic acid pathway. PG = prostaglandin.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 40 |
| Figure 1.10 | A schematic representation of the current hypothesis for the nucleation of cholesterol microcrystals. Cholesterol (CH) is co-secreted into the biliary canaliculus with phospholipids (PL) as stable unilamellar vesicles with a low CH:PL molar ratio. Bile acid monomers solubilise more vesicular PL than CH to form mixed micelles, with preferential transfer of arachidonic acid-rich phospholipid species from vesicles to micelles. Thus, when bile is supersaturated with CH, the residual vesicles become unstable due to their high CH:PL molar ratio. These unstable vesicles then fuse to form multi-lamellar vesicles from which cholesterol nucleates to form microcrystals. .... | 41 |
| Figure 1.11 | Postulated initial gallbladder events during cholesterol lithogenesis. Mucosal hydrolysis of adsorbed PC 16:0-20:4 releases free arachidonic acid that enters the prostanoid synthetic cascade, resulting in gallbladder mucin hypersecretion and a reduced contractile response of the gallbladder to regulatory peptides. Adapted from Carey and Cahalane 1988. ....                                                                                                                                                                                                                                                                                                                           | 42 |
| Table 1.1   | Clinical studies of the role of biliary mucin glycoprotein in gallstone pathogenesis.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 44 |
| Figure 2.1  | Radiolucent, cholesterol-rich gallstones seen at oral cholecystography. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 53 |

|            |                                                                                                                                                                                                                                                                                                                                                                                            |     |
|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 2.2 | Computed tomography of the gallbladder, showing a CT-dense gallstone with an attenuation score of 193 Hounsfield Units. ....                                                                                                                                                                                                                                                               | 54  |
| Table 2.1  | Recommended selection criteria for oral bile acid therapy and extracorporeal shock-wave lithotripsy (ESWL) ± adjuvant oral bile acids. OCG = oral cholecystography.....                                                                                                                                                                                                                    | 58  |
| Figure 2.3 | Diagram depicting the technique of contact dissolution of gallbladder stones using contact solvents.....                                                                                                                                                                                                                                                                                   | 61  |
| Table 2.2  | Animal studies of the effects of NSAIDs on bile physical chemistry, gallbladder contractility, cholesterol microcrystal nucleation and gallstone formation.....                                                                                                                                                                                                                            | 65  |
| Table 2.3  | Clinical studies of the effects of NSAIDs on bile physical chemistry, gallbladder contractility, cholesterol microcrystal nucleation and gallstone formation.....                                                                                                                                                                                                                          | 67  |
| Figure 3.1 | Multiple, radiolucent, pigment stones seen in an opacified gallbladder, before and after removal via an Amplatz sheath inserted into the gallbladder at the time of percutaneous cholecystolithotomy.....                                                                                                                                                                                  | 70  |
| Figure 3.2 | Short-term outcome in 21 patients undergoing PCCL. GB = gallbladder; ERCP = endoscopic retrograde cholangiopancreatography; GA = general anaesthesia; CBD = common bile duct. ....                                                                                                                                                                                                         | 72  |
| Figure 3.3 | Long-term outcome in the 17 patients rendered stone-free by PCCL. Patients were followed up clinically, and by serial ultrasound, every 3-6 months for a median of 35 (range, 4-62) months. Biliary sludge was defined as gravitating, echogenic, but non-shadowing material seen at ultrasound.....                                                                                       | 74  |
| Figure 3.4 | Actuarial or life-table analysis of gallstone, and combined stone and sludge, recurrence rates. Results are means (± SEMs). The number of patients 'at risk' and the number of recurrences of stones or sludge, and combined stone/sludge recurrence rate, are shown for each time period, below. ....                                                                                     | 75  |
| Table 4.1  | Pre-treatment patient and gallstone characteristics in the stone-free and no/arrested dissolution groups. ....                                                                                                                                                                                                                                                                             | 82  |
| Table 4.2  | Gallstone dissolution with oral bile acids ± ESWL as a function of the pretreatment CT attenuation score. *p < 0.01 v combined data of the other three subgroups.....                                                                                                                                                                                                                      | 82  |
| Figure 4.1 | Mean time (in months) to confirmed complete gallstone dissolution, by pre-treatment CT attenuation score.....                                                                                                                                                                                                                                                                              | 83  |
| Figure 4.2 | Reasons for no/arrested gallstone dissolution in 43 patients.....                                                                                                                                                                                                                                                                                                                          | 84  |
| Figure 4.3 | CT attenuation scores of gallstones, measured before and during 12-29 months oral bile acid therapy, in nine patients with no/arrested gallstone dissolution associated with acquired stone calcification. ....                                                                                                                                                                            | 85  |
| Table 5.1  | Characteristics of the primary gallstones by the four treatment modalities. OBAs = oral bile acids.....                                                                                                                                                                                                                                                                                    | 92  |
| Figure 5.1 | Comparison of primary and recurrent gallstones by stone number.....                                                                                                                                                                                                                                                                                                                        | 93  |
| Figure 5.2 | CT attenuation scores of primary and recurrent stones, in patients treated with a) medical dissolution therapy, or b) PCCL. Stone composition was assessed indirectly from the maximum Hounsfield Unit (HU) score by localised CT of the gallbladder: A CT score of < 100 HU suggested the stones were cholesterol-rich, while a score of ≥ 100 HU was indicative of calcified stones..... | 94  |
| Figure 5.3 | Treatment outcome in the 21 patients with recurrent gallstones.....                                                                                                                                                                                                                                                                                                                        | 95  |
| Figure 6.1 | Diagram depicting transhepatic fine-needle puncture of the gallbladder. ....                                                                                                                                                                                                                                                                                                               | 102 |
| Figure 6.2 | Typical rhomboid cholesterol microcrystals as viewed through a polarising light microscope.....                                                                                                                                                                                                                                                                                            | 104 |

|             |                                                                                                                                                                                                                                                                                                                                                                                                            |     |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 6.3  | Example of a standard curve for the bile acid assay. Standard solutions of glycochenodeoxycholic acid in methanol were assayed using the 3 $\alpha$ dehydrogenase method.....                                                                                                                                                                                                                              | 105 |
| Figure 6.4  | Standard curves for the phospholipid assay performed on four consecutive days. Total phospholipid concentrations are plotted against optical density of the solution following the assay reaction.....                                                                                                                                                                                                     | 106 |
| Figure 6.5  | Example of a standard curve for the cholesterol assay.....                                                                                                                                                                                                                                                                                                                                                 | 108 |
| Figure 6.6  | An example of a pen recorder trace following the injection (100 $\mu$ l) of a methanol solution containing 10 conjugated bile acid standards onto the HPLC column. The trace shows the clear separation of 10 distinct peaks, with retention times corresponding to those of the individual bile acid standards. ....                                                                                      | 110 |
| Figure 6.7  | Concentration curves for taurine- and glycine-conjugated bile acid species measured by HPLC.....                                                                                                                                                                                                                                                                                                           | 111 |
| Figure 6.8  | Biliary ursodeoxycholic acid (% of total bile acids) and cholesterol saturation indices in six patients with no/arrested dissolution of cholesterol-rich (CT score < 100 HU) gallstones in a 'functioning' gallbladder and who claimed full compliance with oral bile acid therapy, during continued treatment.....                                                                                        | 113 |
| Figure 6.9  | Cholesterol microcrystal nucleation times in the stone-free and no/arrested dissolution groups. The broken horizontal line at 10 days represents the lower limit of normal for cholesterol microcrystal nucleation. Note that, at the time of the study, all five stone-free patients had discontinued oral bile acids while the six in the no/arrested dissolution group continued to take UDCA+CDCA..... | 114 |
| Figure 7.1  | Flowchart of phospholipid analysis. ....                                                                                                                                                                                                                                                                                                                                                                   | 119 |
| Figure 7.2  | Separation of phospholipid classes by HPLC according to flow rate. Phosphatidylcholine (PC) eluted after approximately 40 min at a flow rate of 2 ml/min. ....                                                                                                                                                                                                                                             | 120 |
| Figure 7.3  | Thin layer chromatography of cholesterol and phospholipid standards (lanes 1-7), bile before and after Fölch extraction (lanes 8-10), and after separation of PC from bile by HPLC (right lane). ....                                                                                                                                                                                                      | 121 |
| Figure 7.4  | Separation of phosphatidylcholine species by HPLC in bile, red blood cell (RBC) membranes and plasma from a patient with gallstones. ....                                                                                                                                                                                                                                                                  | 122 |
| Figure 7.5  | CAM 17.1 ELISA of Sepharose CL-2B fractions from the bile of an acromegalic patient before and during octreotide therapy. The shaded bar (2.5-5.5 ml) represents the void volume for mucin glycoprotein. ....                                                                                                                                                                                              | 123 |
| Figure 7.6  | Calibration of the Superose 6 fast performance liquid chromatography (FPLC) column using molecular weight standards (upper panel). There was an inverse linear relationship between retention times on the column, expressed as ratios to that of blue dextran, and the log <sub>10</sub> molecular weights of the standards (lower panel). ....                                                           | 124 |
| Figure 7.7  | Superose 6 FPLC of mucin-enriched bile after Sepharose CL-2B gel filtration. The shaded bar at 11-13 ml represents the void volume for biliary mucin glycoprotein.....                                                                                                                                                                                                                                     | 125 |
| Figure 7.8  | CAM 17.1 ELISA of Superose 6 fractions from an acromegalic patient before and during octreotide therapy.....                                                                                                                                                                                                                                                                                               | 126 |
| Figure 7.9  | Example of a standard curve for the CAM 17.1 ELISA, using Superose 6-purified human colonic mucin glycoprotein as standard. ....                                                                                                                                                                                                                                                                           | 127 |
| Figure 7.10 | Effect of octreotide therapy on the distribution of phosphatidylcholine species in gallbladder bile (means $\pm$ SEM). The two arachidonic acid-rich phosphatidylcholine species were the only species which increased significantly during treatment. ....                                                                                                                                                | 128 |

|             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |     |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 7.11 | The proportions of a) PC 16:0-20:4 and b) PC 18:0-20:4, in gallbladder bile before and during octreotide treatment. The closed circles are the individual values, the lines connect the paired data, while the shaded bars show the means. One patient in the paired studies (*) developed gallbladder stones during treatment. ....                                                                                                                                                                                                                                                                                                                                                                                                          | 129 |
| Figure 7.12 | Pearson correlations for the % biliary PC 16:0-20:4 and a) the cholesterol saturation indices, b) % vesicular cholesterol, and c) the % deoxycholic acid in gallbladder bile (O = no octreotide, ◆ = during octreotide treatment). ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 130 |
| Figure 7.13 | Biliary mucin glycoprotein concentrations before and during octreotide treatment.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 131 |
| Table 8.1   | Clinical data of the patients with Crohn's disease and ulcerative colitis. * Mean (range) † "Disease duration" (from the time of diagnosis to the time of the study): median (range) .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 138 |
| Table 8.2   | Bile physical chemistry in the patients with Crohn's disease and ulcerative colitis. All data are expressed as means ± SEM. *Individual bile acid concentrations were corrected to a standard bile acid concentration of 100 mM. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 142 |
| Figure 8.2  | Cholesterol microcrystal nucleation times in the patients with Crohn's disease and ulcerative colitis. The broken horizontal line (at 10 days) represents the limit above which nucleation time is normal. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | 144 |
| Table 8.3   | Group data (means ± SEM) for biliary bile acid composition and the molecular species (fatty acid composition) of phosphatidylcholine in the patients with Crohn's disease and cholesterol-rich gallbladder stones (positive controls). ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 146 |
| Table 9.1   | Clinical data of the patients studied.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 155 |
| Figure 9.1  | Protein assay of Sepharose CL-2B fractions after elution of purified human biliary lactoferrin (n = 2 patients). The shaded bar (6.5-11.0 ml) represents the void volume for lactoferrin. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 157 |
| Figure 9.2  | Principle of the lactoferrin ELISA.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 158 |
| Figure 9.3  | Lactoferrin ELISA standard curve derived from assays performed on six consecutive days (mean ± SD). ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 159 |
| Figure 9.4  | Western blot of: bile (10 µl) (i) before and (ii) after Sepharose CL-2B gel chromatography, (iii) human lactoferrin (LF) standard (1 µg), and (iv) pre-stained molecular weight standards (Bio-Rad). SDS-PAGE separations were performed in duplicate on a 4%-15% gradient gel and the protein blotted onto a PVDF membrane. One half of the membrane was stained with Coomassie blue, while the other half was immunoblotted using rabbit anti-human lactoferrin and horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin antibodies. Note the presence of a protein band in bile corresponding to the 76 kDa human lactoferrin standard, and the absence of cross-reactivity of the antibody with other biliary proteins..... | 160 |
| Figure 9.5  | Example of a myeloperoxidase standard curve.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | 161 |
| Figure 9.6  | Biliary lactoferrin concentrations in patients with active and inactive ulcerative colitis. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | 163 |
| Figure 9.7  | Biliary lactoferrin concentrations in patients with Crohn's disease. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 164 |
| Figure 9.8  | Biliary myeloperoxidase concentrations in patients with active and inactive ulcerative colitis. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 165 |
| Figure 9.9  | Scatterplot of biliary lactoferrin concentrations versus cholesterol saturation indices (CSIs) in patients with active ulcerative or Crohn's colitis (O), or who were post-colectomy (◆). ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 166 |
| Table 10.1  | Markers of disease activity in the 13 patients with Crohn's disease.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 174 |

|             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |     |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 10.1 | Plasma lactoferrin concentrations in control subjects (n=17) and patients with Crohn's disease (n=13), before treatment, and after two and eight weeks of therapy. At each time point, the mean lactoferrin concentration was significantly higher ( $p < 0.0001$ ) than that of the control subjects.....                                                                                                                                                                                                                                          | 175 |
| Figure 10.2 | Molecular species (fatty acid composition) of plasma phosphatidylcholine in 20 control subjects and 13 patients with Crohn's disease. Values are expressed as means with SEMs represented by vertical bars. Before treatment, the proportions of both polyunsaturated species, PC 16:0-22:6 and PC 16:0-20:4, in the Crohn's disease patients were significantly higher than those in the control subjects.....                                                                                                                                     | 176 |
| Figure 10.3 | Relative proportions of the major arachidonic acid-rich phospholipid, PC 16:0-20:4, in the plasma of the control subjects and the patients with Crohn's disease, before treatment, and after two and eight weeks of therapy. The mean % PC 16:0-20:4 in the plasma of patients with active Crohn's disease was significantly higher ( $p < 0.01$ ) than that in the control subjects, but returned to normal values after eight weeks of therapy. ....                                                                                              | 177 |
| Figure 11.1 | Hypothesis for the formation of 'conventional' and octreotide-induced cholesterol-rich gallstones. Prolongation of intestinal transit leads to increased conversion of cholic to deoxycholic acid. The increase in biliary deoxycholic acid not only induces biliary cholesterol supersaturation but also the rapid nucleation of cholesterol microcrystals, which are retained in the gallbladder due to impaired gallbladder emptying and mucin glycoprotein hypersecretion, predisposing to the development of cholesterol-rich gallstones. .... | 183 |

## LIST OF ABBREVIATIONS

|          |                                                            |
|----------|------------------------------------------------------------|
| CA       | cholic acid                                                |
| CAM      | carbohydrate antigen mucin                                 |
| CDCA     | chenodeoxycholic acid                                      |
| CSI      | cholesterol saturation index                               |
| CT       | computerised tomography                                    |
| DCA      | deoxycholic acid                                           |
| ELISA    | enzyme-linked immunosorbent assay                          |
| ERCP     | endoscopic retrograde cholangiopancreatography             |
| ESR      | erythrocyte sedimentation rate                             |
| ESWL     | extracorporeal shock-wave lithotripsy                      |
| FPLC     | fast protein liquid chromatography                         |
| HU       | Hounsfield Units                                           |
| HPLC     | high performance liquid chromatography                     |
| MRCP     | magnetic resonance cholangiopancreatography                |
| MTBE     | methyl- <i>tert</i> butyl ether                            |
| PBS      | phosphate-buffered saline                                  |
| PC       | phosphatidylcholine                                        |
| PCCL     | percutaneous cholecystolithotomy                           |
| PUFAs    | polyunsaturated fatty acids                                |
| PVDF     | polyvinylidene difluoride                                  |
| SICDA    | simple index of Crohn's disease activity                   |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| UDCA     | ursodeoxycholic acid                                       |

## THESIS PLAN

This thesis addresses selected aspects of the pathogenesis and non-surgical treatment of gallstones, and is divided into three parts.

The *background* section reviews the epidemiology of gallbladder stone disease, the known changes in bile physical chemistry and cholesterol microcrystal nucleation associated with cholesterol-rich gallstones, and the medical management of symptomatic gallstones.

The *clinical studies* section is composed of three published papers addressing selected aspects of the non-surgical treatment of gallstones, including the problem of gallstone recurrence after medical treatment.

The third section contains *laboratory studies of the pathogenesis of gallstones* in patients with: i) ‘conventional’ cholesterol-rich gallstones, (ii) acromegaly with and without octreotide-associated gallstones, and (iii) active and inactive inflammatory bowel disease. In this section, the techniques used to analyse the biliary cholesterol saturation, cholesterol microcrystal nucleation times, phospholipid and bile acid composition, and mucin glycoprotein concentrations, are described and validated. In a series of five original papers, these parameters are studied in each of the three patient groups, including: (i) changes in bile physical chemistry in patients with ‘conventional’ cholesterol-rich gallstones, (ii) the effects of octreotide on biliary phospholipid and mucin glycoprotein concentrations in paired, before and during treatment studies in acromegalic patients, and (iii) alterations in plasma and biliary phospholipids, biliary bile acids and non-mucin glycoproteins, in patients with active inflammatory bowel disease or following bowel resection. In the final chapter, the results arising from the thesis are summarised and discussed.

## ***PART 1. BACKGROUND***

## **CHAPTER 1. GALLBLADDER STONE DISEASE**

### **1.1 INTRODUCTION**

In Europe and North America, more than 75% of gallbladder stones are cholesterol-rich — defined, arbitrarily, as those containing at least 70-90% cholesterol by weight, on chemical analysis (Dowling 2000a). This chapter reviews the epidemiology and pathogenesis of gallstones, including the changes in bile composition and physical chemistry which determine biliary cholesterol supersaturation and cholesterol microcrystal nucleation — important factors in cholesterol gallstone formation.

### **1.2 EPIDEMIOLOGY**

#### **1.2.1 Risk factors for conventional gallstones**

Gallstone disease is common and affects between 10% and 20% of the world's population. There are marked geographical variations, with a high prevalence in Chile, <sup>who have a higher prevalence than any other group in the world (Bateson 1999)</sup> the Scandinavian countries and among Native Americans, and a relatively low risk in populations residing in sub-Saharan Africa and some parts of Asia (Donovan 1999). <sup>Gallstones tend to be a disease of more affluent societies, but there are clearly racial differences (Bateson 1999).</sup> Gallstones occur more frequently in women than in men by a factor of approximately two — attributed to the effects of oestrogen-induced cholesterol hypersecretion and impairment of gallbladder motility by endogenous progesterone (Everson *et al* 1991). Most series in the West report a gallstone prevalence in women of 5% to 25% between the ages of 20 and 50, and 20% to 40% after the age of 50 (Bilhartz and Horton 1998). In men, the prevalence appears to be increasing. In a 10-year prospective study of 8,563 autopsies in England (Bates *et al* 1992), the prevalence of gallstones or cholecystectomy in women remained relatively constant (26% to 27%), but in men aged 50-59 years, it rose from 7% during the first three years of the study, to 18% in the last three years. In men aged 60-69, the prevalence increased from 12% to 20% and in this age group, the proportion of women to men with stones was 0.8 : 1. Cholecystectomy had been performed in 12% of the autopsies and was three times more common in women than in men. Similarly, in a recently published

epidemiological study from Scotland of 9,634 autopsies performed in 1953-73 and 7,219 autopsies completed in 1974-98 (Bateson 2000), the prevalence of gallstones increased with time for both sexes over the age of 50 years, with the increase most marked in men. In men aged 50-59 years, the autopsy prevalence of gallstones increased from 6% to 12% during the two time periods, compared with a rise from 16% to 25% in women in the same age group. In subjects over 90 years of age, gallstone disease was present in 24% of men and 35% of women in 1953-73, compared with figures of 45% and 48%, respectively, in 1974-98.

Genetic factors implicated in the pathogenesis of gallstone disease remain poorly defined and are multifactorial (Lammert *et al* 2001). *Animal studies have implicated several genes in gallstone pathogenesis, but their role in human disease is largely unknown* Transgenic knockout mice in which the multiple drug resistant gene *Mdr-2* has been deleted (*mdr2*  $-/-$ ) are incapable of secreting phospholipid (and cholesterol) in bile, even though their bile acid secretion remains normal (Smit *et al* 1993). Studies following on from this work have shown that the *Mdr-2* gene encodes for a canalicular protein that acts as a 'flippase', translocating phospholipids from the inner, to the outer, leaflet of the canalicular membrane, and the corresponding defect in the human *Mdr-3* gene has, occasionally, been reported in patients with hepatolithiasis (Rosmorduc *et al* 2001) and with familial intrahepatic cholestasis (Deleuze *et al* 1996; de Vree *et al* 1998). Another family of putative genes implicated in gallstone formation are the so-called *Lith* genes, which determine, in part, cholesterol supersaturation, gallbladder size and mucin gel accumulation (Wang *et al* 1999). When mice are fed a lithogenic diet, susceptible strains develop gallstones with varying frequency, depending on whether they have the *Lith1*, *Lith2* or *Lith3* genes (Khanuja *et al* 1995; Wang *et al* 1997; Wang *et al* 1999). In humans, the apolipoprotein E locus (a genetically polymorphic protein influencing lipoprotein metabolism) may also play a role in gallstone pathogenesis. Apolipoprotein E exists as three different alleles, *apoE2*, *apoE3* (the wild-type allele) and *apoE4*. In patients who express the *apoE4* allele, the cholesterol content of gallstones (Juvonen *et al* 1993), rate of cholecystectomy (Bertomeu *et al* 1996), and risk of stone recurrence after extracorporeal shock-wave lithotripsy (Portincasa *et al* 1996), are all increased. One or more of the above factors may explain, in part, the observation that first-degree

relatives of patients with gallstones have at least a two-fold increased prevalence over the general population (Gilat *et al* 1983).

Pregnancy and parity are other risk factors for cholelithiasis. In a study of 980 Chilean women who underwent ultrasound immediately after giving birth (Valdivieso *et al* 1993), the prevalence of gallstones was 12.2% compared with a figure of 1.3% in 150 nulliparous, age-matched controls. Stones formed during pregnancy are composed mostly of cholesterol, are usually asymptomatic and in up to 30% of women, disappear spontaneously after delivery. *There is an increased risk of stones with increasing parity, especially in younger women.* (Everson *et al* 1991; Ko *et al* 1999).

Obesity also predisposes to gallstone formation. In the U.S. Nurses' Health Study of 90,302 women followed up from 1980 to 1988 (Stampfer *et al* 1992), the annual incidence of symptomatic gallstones in women with a body mass index  $> 30 \text{ kg/m}^2$  was more than 1% — four times that seen in non-obese women. Those with the highest body mass index ( $> 45 \text{ kg/m}^2$ ) had a seven-fold risk of developing symptomatic gallstones. Obesity is associated with enhanced total body cholesterol synthesis and increased secretion of cholesterol into bile, secondary to increased activity of the rate-limiting enzyme for cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase. Obese patients on very low calorie diets or following jejunoileal bypass also have a high incidence of cholesterol gallstone formation, of up to 32% during the first one to four months of rapid weight loss (Broomfield *et al* 1988; Shiffman *et al* 1995; Sugerman *et al* 1995). Dietary factors implicated in gallstone formation include a high energy intake, increased consumption of unrefined carbohydrate, and a low intake of fibre (Heaton 2000).

Other conditions associated with gallstones include hypertriglyceridaemia, diabetes mellitus and hepatic cirrhosis (Attili *et al* 1997; Conte *et al* 1999). Bacterial infection and parasitic infestation of the biliary tree are important factors in the development of pigment stones in Asia but less so in the West. Patients with chronic haemolysis related to hereditary spherocytosis, sickle-cell disease and thalassaemia, have an increased prevalence of pigment, rather than cholesterol-rich, stones (Ko and Lee 1999).

### 1.2.2 Slow intestinal transit

Known risk factors are present in only half of all patients with gallstones. Marcus and Heaton (1986b) showed that, by prolonging whole-gut transit times in normal individuals with loperamide, the percent biliary deoxycholate increased and their bile became supersaturated with cholesterol. Thus, abnormally slow whole gut transit may contribute to gallstone pathogenesis. One hypothesis for this effect is that an increase in whole-gut transit time leads to increased  $7\alpha$ -dehydroxylation by colonic-type bacteria of cholic acid to form deoxycholic acid which, after passive absorption across the colonic mucosa, induces hepatic cholesterol hypersecretion (Marcus and Heaton 1988). To test this hypothesis, Heaton *et al* (1993) identified fifteen non-obese women (BMI < 25 mg/kg<sup>2</sup>) with gallstones from an ultrasound survey of 1058 women aged 25-69 years. In these women and in age-matched non-obese controls with healthy gallbladders, whole gut transit times were measured by following the rate of appearance, in two consecutive stools, of four different orally administered radio-opaque shapes, or estimated indirectly from a formula based on defaecation frequency and stool consistency. In the women with gallstones, the mean whole gut transit time was significantly longer (82 v 63 h), and the mean stool weight significantly lower (74 v 141 g/24 h), than in the matched controls. Subsequently, the Bristol group found that gallstones were also common in non-obese Ladakhi women from Northern India whose stool characteristics (lumpy or hard) 'implied' that they had slow intestinal transit (Spathis *et al* 1997). Further indirect evidence for a role of intestinal transit in the pathogenesis of cholesterol gallstone formation comes from a large epidemiological study conducted in southern Italy, in which the use of laxatives, considered a proxy for constipation and slow transit, was an independent risk factor for cholelithiasis (Misciagna *et al* 1996). Conversely, in a postal questionnaire of 79,829 American women aged 36-61 years, Dukas *et al* (Dukas *et al* 2001) did not find an association between infrequent bowel movements or laxative use and risk of symptomatic gallstone during a 14-year follow-up period.

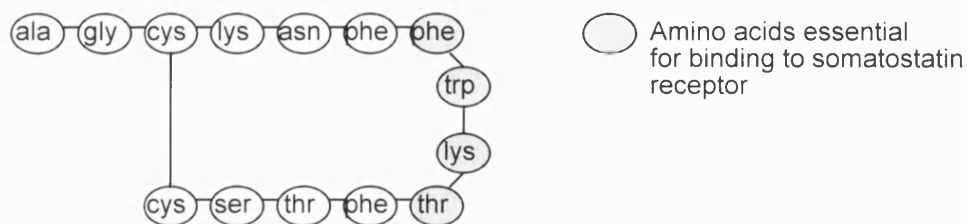
Studies from Japan, Sweden (Shoda *et al* 1995) and Italy (Azzaroli *et al* 1999) also suggest that gallstone carriers have significantly prolonged small bowel transit times compared with controls. In the study by Shoda *et al* (1995), gallstone carriers had a

two-fold increase in the percentage of deoxycholic acid in bile and had supersaturated bile, in contrast to the unsaturated bile found in the gallstone-free controls. Similarly, Azzaroli *et al* (1999) reported that gallstone patients, in association with a prolonged oro-caecal transit, had a higher mean % biliary deoxycholic acid (23% v 15%) and cholesterol saturation index (1.2 v 0.7) than healthy controls, and that there was a linear relationship between biliary deoxycholic acid and small bowel transit time.

### 1.2.3 Octreotide-induced gallstones

Support for the hypothesis that prolongation of intestinal transit may be important in gallstone pathogenesis also comes from studies of acromegalic patients treated with the long-acting somatostatin analogue, octreotide (Figure 1.1). Octreotide induces gallbladder stones in 10% to 60% (mean 29%) of patients after 1-2 years of treatment (Dowling 2000a), with most of the stones being cholesterol-rich (Hussaini *et al* 1995a). Octreotide acts by suppressing circulating levels of growth hormone and insulin-like growth factor-1, and it also inhibits meal-stimulated cholecystokinin release from the small intestine. As a result, both acute and chronic octreotide treatment effectively abolish meal-stimulated gallbladder emptying (Hussaini *et al* 1996).

#### Native somatostatin



#### Octreotide

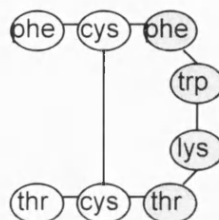


Figure 1.1 The structure of native somatostatin and octreotide. From Hussaini 1995.

Until recently, suppression of cholecystokinin release and meal-stimulated gallbladder emptying was considered to be the principal mechanism for the induction of octreotide-induced gallbladder stones. However, in control subjects and patients with the irritable bowel syndrome, octreotide also prolongs mouth-to-caecum transit times by a factor of three (Dowling *et al* 1992). Furthermore, in acromegalic patients, octreotide has been shown to alter bile physical chemistry — by increasing the relative amount of biliary deoxycholic acid, cholesterol saturation, the % of total biliary cholesterol transported in vesicles and the cholesterol : phospholipid molar ratio in the vesicles, and by shortening the nucleation time (Hussaini *et al* 1994).

One mechanism for the increased proportion of biliary deoxycholic acid may be prolongation of intestinal transit, leading to increased bacterial deconjugation and 7- $\alpha$  dehydroxylation by colonic-type bacteria of the parent bile acid, cholic acid, to form deoxycholate. In both control subjects and acromegalic patients (Hussaini *et al* 1996; Veysey *et al* 1999; Veysey *et al* 2001a), octreotide markedly prolongs oro-caecal transit times (measured by the lactulose breath hydrogen test (Bond *et al* 1975; Pereira *et al* 1991; Pereira *et al* 1992)) as well as large bowel transit times — as assessed by recording the transit of radio-opaque markers through the intestine (Metcalf *et al* 1987; Veysey *et al* 2001a). Veysey and colleagues (1999; Veysey *et al* 2001a) showed that octreotide not only increased the percentage of deoxycholic acid in fasting serum (a surrogate marker for the proportion in bile), but it also increased the pool size and formation rate of deoxycholic acid. After passive absorption across the colonic mucosa, the resultant increased proportion of biliary deoxycholate may induce cholesterol hypersecretion and destabilisation of biliary cholesterol carriers leading to cholesterol microcrystal nucleation and gallstone formation (Hussaini *et al* 1994) (Figure 1.2). However, not all investigators have documented a higher proportion of deoxycholic acid in the bile of gallstone patients than in controls (Jungst *et al* 1999), and some conditions associated with an increased risk of gallstones (such as cirrhosis and Crohn's disease) tend to have low proportions of biliary deoxycholic acid, so that clearly other factors in gallstone pathogenesis also play a role (Barry Zisser).



Figure 1.2 Gallstones from a patient with octreotide-induced gallstones. The stones are multiple, smooth and faceted. The cut surface shows a pigmented centre with a radiating lattice composed of crystalline cholesterol.

#### **1.2.4 Gallstones in inflammatory bowel disease**

In patients with Crohn's disease involving the small intestine, the risk of gallbladder stone development is higher than in the general population (Heaton and Read 1969; Cohen *et al* 1971; Hill *et al* 1975; Whorwell *et al* 1984). A direct relationship between the extent of ileal resection and subsequent gallstone prevalence, has also been observed (Andersson *et al* 1987; Kangas *et al* 1990; Lorusso *et al* 1990).

The increased frequency of gallstones in ileal Crohn's disease has been attributed to decreased active bile acid absorption in the terminal ileum, thereby reducing the bile acid pool within the enterohepatic circulation and increasing the ratio of hepatic cholesterol : bile acid secretion, resulting in supersaturation of bile with cholesterol (Rutgeerts *et al* 1987; Santavirta *et al* 1990; Akerlund *et al* 1991). In patients with ileal disease/resection, a high biliary cholesterol saturation index has been reported in most (Dowling *et al* 1972; Rutgeerts *et al* 1986; Rutgeerts *et al* 1987; Heubi *et al* 1992), but

not all (Lapidus and Einarsson 1991), studies. Other proposed mechanisms of cholesterol gallstone formation in Crohn's disease include: (i) impaired gallbladder contractility (Murray *et al* 1992c), and (ii) the development of biliary sludge following surgery (Hutchinson *et al* 1994) or total parenteral nutrition (Roslyn *et al* 1983).

Although there are few controlled studies available, there may also be an increased prevalence of gallstones in patients with colonic Crohn's disease or ulcerative colitis (Hill *et al* 1975; Bluth *et al* 1984; Kurchin *et al* 1984). In a case-control ultrasonographic study of 159 patients with inflammatory bowel disease, Lorusso *et al* (1990) found a similar increased risk of gallstones in patients with Crohn's colitis or ulcerative colitis (odds ratio = 2.5; 95% CI 1.2-5.2) over that of the general population. In patients with ileal Crohn's disease, the relative risk of gallstone development was 4.5 (95% CI 1.5-14.1) (Lorusso *et al* 1990). Other studies have also reported an increased risk of gallstones in ileostomy patients (Hill *et al* 1975; Kurchin *et al* 1984), but in this group both the colon and a segment of terminal ileum is usually removed.

An alternative explanation for gallstone formation in Crohn's disease is that bile acids that are malabsorbed in the small intestine solubilise unconjugated bilirubin in the colon and increase its enterohepatic cycling, which in turn increases the rate of bilirubin secretion into bile and the risk of pigment gallstone formation (Feverly 1999). In support of this hypothesis, Brink *et al* found a twofold increase in bilirubin secretion rates into bile of ileectomised, but not jejunectomised, rats (1996) and up to a threefold increase in total bilirubin concentrations in the gallbladder bile of patients with extensive (> 50 cm) ileitis or previous ileectomy (1999). However, to date there have been no large studies of gallstone composition in Crohn's disease, and it remains unclear as to whether such patients are at increased risk of developing pigment or cholesterol-rich gallstones.

#### **1.2.5 Pigment gallstones**

Bile pigment stones are formed predominantly of polymerised bilirubin and calcium bilirubinate, and contain < 10-30% cholesterol. They are usually small and multiple, and about half are radio-opaque (Ko and Lee 1999).

Pigment stones can be classified as either brown and black stones. The soft, friable, brown pigment stones are especially common in the Far East and are associated with chronic low-grade infection or inflammation in the biliary tree — conditions that increase activity of the enzyme  $\beta$ -glucuronidase. This enzyme is produced by bacteria such as *Escherichia coli*, *Bacteroides* and clostridia within the biliary tract, but it is also present in the biliary epithelium and can be identified in uninfected bile (Ho and Ho 1988). In bile,  $\beta$ -glucuronidase deconjugates bilirubin mono- and di-glucuronide to form free unconjugated bilirubin. Nonenzymatic hydrolysis of bilirubin may also occur (Spivak *et al* 1987). In the setting of biliary stasis, unconjugated bilirubin combines with calcium to form the sparingly soluble calcium bilirubinate, which precipitates. Brown pigment stones are also associated with duodenal diverticula and are more likely to form *de novo* in the biliary tree than are other types of stones (Sandstad *et al* 1994). Microscopically, brown stones contain cytoskeletons of bacteria (Ko and Lee 1999).

The incidence of black or pure pigment stones increases with age and they are found with increased frequency in the gallbladders of patients with cirrhosis or chronic haemolytic disorders, such as sickle cell anaemia or thalassaemia. In situations where hepatic function is impaired or there is excessive haemolysis, the fraction of bilirubin escaping conjugation into mono- and di-glucuronides in the liver increases, resulting in an excess of unconjugated bilirubin in bile, which is vulnerable to polymerisation and/or co-precipitation with free ionized calcium. Black pigment stones consist predominantly of polymerised bilirubin but may also contain calcium bilirubinate, together with calcium carbonate and phosphate, and calcium salts of fatty acids. All pigment stones also contain a large amount of mucin glycoprotein matrix (Goresky *et al* 1995; Ko and Lee 1999; Dowling 2000b).

#### **1.2.6 Biliary sludge**

Biliary sludge is defined ultrasonographically as echogenic, gravitating material in the gallbladder which does not produce acoustic shadowing. It consists of cholesterol microcrystals, calcium bilirubinate granules, and a high concentration of mucin glycoprotein (Ko *et al* 1999). Groups at risk of sludge formation include critically ill

patients in intensive care units, patients with high spinal cord injuries and those receiving total parenteral nutrition (Murray *et al* 1992a; Nakano *et al* 1992; Rubin *et al* 1992). During intravenous feeding, the absence of exogenous luminal nutrients leads to impaired release of meal-stimulated peptide hormones such as cholecystokinin, resulting in stagnation of bile acids within the enterohepatic circulation (Quigley *et al* 1993). Increases in both the cholesterol saturation index and the vesicular cholesterol concentration, together with rapid cholesterol microcrystal nucleation times, have been documented within 48 h of starting total parenteral nutrition (Nakano *et al* 1992; Rubin *et al* 1992). Gallbladder sludge, in turn, may occur after as little as three weeks of total parenteral nutrition, and up to 40% of patients will develop gallstones after four months of continued treatment (Pitt *et al* 1983).

or 'gravel'

In a minority of patients, biliary sludge<sup>or 'gravel'</sup> may cause symptoms and precede gallstone formation. In a study of 96 patients with biliary sludge who were followed prospectively for a mean of 38 months (Lee *et al* 1988), 8% formed asymptomatic gallstones and 6% developed symptomatic stones requiring cholecystectomy. Six patients with biliary sludge also required cholecystectomy for severe biliary pain and/or recurrent acute pancreatitis. In 60% of the patients, the sludge disappeared and subsequently reappeared, while in 18% it resolved completely. In another study of 286 patients with biliary sludge (Janowitz *et al* 1994), gallbladder stones or complications such as acute cholecystitis occurred in 20% of patients during 20 months follow-up.

Conventional transabdominal ultrasonography is relatively insensitive in detecting the presence of biliary sludge or individual stones < 2 mm in diameter. In an early study of 31 patients with acute pancreatitis considered to be idiopathic (no gallstones on ultrasound and no other causes found) (Lee *et al* 1992), microscopic examination of duodenal bile revealed bilirubinate granules or cholesterol crystals in 23 (86%), but sludge was seen by ultrasound in only 48%. In a more recent prospective study of 45 patients with acute pancreatitis or suspected choledocholithiasis but two consecutive 'normal' transabdominal gallbladder ultrasounds (Dahan *et al* 1996), the sensitivity of *endoscopic* ultrasound for detecting gallbladder microlithiasis was 96%, compared with a sensitivity of 67% for cholecystokinin-induced duodenal bile.

### 1.3 BILE COMPOSITION AND PHYSICAL CHEMISTRY

Between 500 ml and 1000 ml of bile is secreted by the human liver every day. Bile is composed predominantly of water (97%), with the major lipid components being bile acids (approximately 67% of total solute mass), phospholipids (22%) and cholesterol (4%). Proteins (4.5%) and bilirubin conjugates (0.3%) account for a small percentage of the total solute mass (Carey and Cahalane 1988).

#### 1.3.1 Cholesterol

*cellular* Cholesterol is synthesized primarily <sup>in the cells of</sup> the liver and small intestine. The rate-limiting enzyme for cholesterol production is 3-hydroxy-3-methoxyglutaryl coenzyme A reductase, which catalyses the first step towards non-esterified (free) cholesterol being secreted into bile. Cholesterol is also transported to the liver as chylomicron remnants after intestinal absorption of dietary cholesterol, or via low-density lipoproteins from the peripheral tissues (Bilhartz *et al* 1989). Hepatic uptake of lipoproteins occurs via the low-density lipoprotein receptor, the low density lipoprotein receptor-related protein and the scavenger receptor B1. This transported cholesterol contributes to both the free cholesterol pool and cholesterol ester, which can be stored as such or converted to the unesterified form by one of several cholesterol esterases (Donovan 1999).

The hepatic metabolism of cholesterol is summarised in Figure 1.3. The major route of hepatic cholesterol disposal is through bile acid synthesis via the rate-limiting enzyme, cholesterol 7 $\alpha$  hydroxylase. Small amounts of hepatic cholesterol are also converted to cholesterol esters by the microsomal enzyme, acyl coenzyme A cholesterol acyltransferase, or secreted into bile unaltered (Donovan 1999).

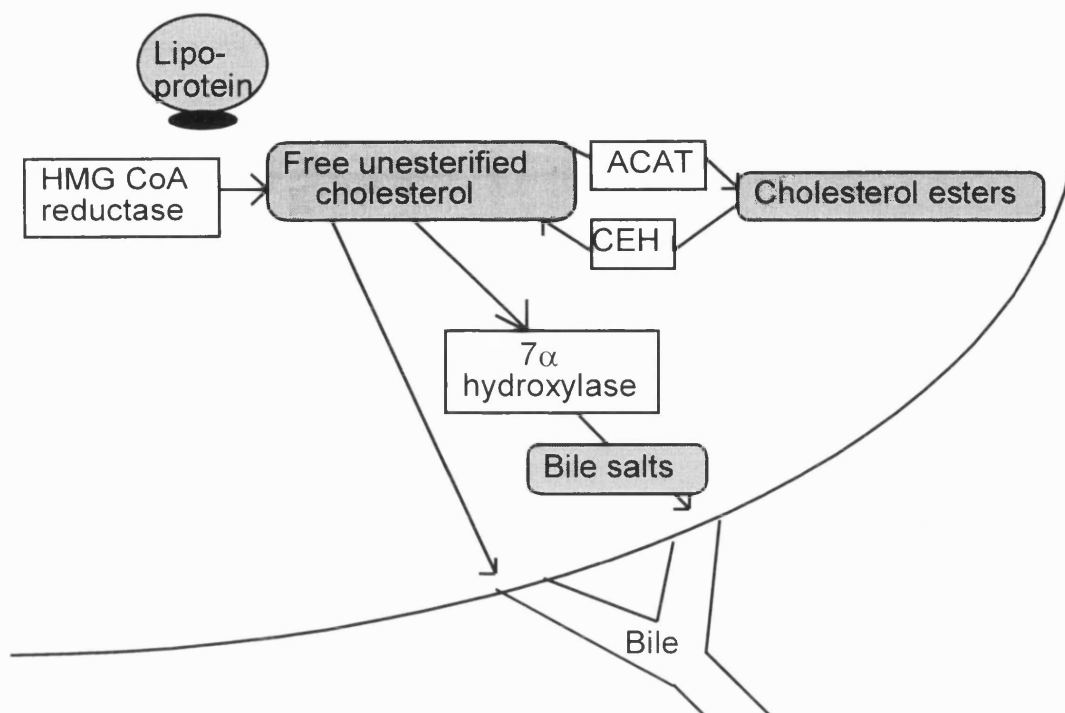


Figure 1.3 Hepatic cholesterol metabolism. Cholesterol is taken up into the liver from plasma lipoproteins. The boxes indicate the rate limiting enzymes for: (i) de novo cholesterol synthesis: HMG CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, (ii) cholesterol ester synthesis (ACAT: acyl coenzyme A cholesterol acyltransferase), (iii) cholesterol ester hydrolysis (CEH: neutral cholesterol ester hydrolase), and (iv) bile acid synthesis (7 $\alpha$  hydroxylase).

### 1.3.2 Cholesterol carriers

Since cholesterol is virtually insoluble in water (Renshaw *et al* 1983; Armstrong and Carey 1987), a variety of cholesterol-solubilising lipid aggregates (cholesterol carriers) are required to facilitate cholesterol transport in aqueous bile. The results of early studies suggesting that the lipophilic cholesterol in bile was solubilised as mixed cholesterol-phospholipid-bile acid micelles (Admirand and Small 1968), was reappraised following the observations of Somjen and Gilat (1983). These authors, and other groups using a variety of techniques including quasi-elastic light scattering, gel filtration, electron microscopy and microscope laser light scattering spectroscopy (Mazer and Carey 1983; Pattinson 1985; Halpern *et al* 1986; Ulloa *et al* 1987; Cohen and Carey 1990; Mockel *et al* 1995), showed that cholesterol is cosecreted with phospholipids into the biliary canaliculus as unilamellar vesicles, while bile acids are

secreted separately as monomers. These vesicles measure between 600-800 Å and, in dilute hepatic bile, have a low cholesterol : phospholipid molar ratio (Somjen and Gilat 1983; Ulloa *et al* 1987; Peled *et al* 1988). In the post-canalicular biliary tree, water and electrolytes are absorbed, in particular by the gallbladder epithelium (Carey and Cahalane 1988), leading to a rise in the total lipid concentration of bile above that of the critical micellar concentration. Thus, unilamellar vesicles of cholesterol and phospholipid may be solubilised by bile acid monomers to form simple cholesterol-bile acid micelles and mixed micelles consisting of cholesterol, phospholipids and bile acids. Bile acid hydrophobicity not only determines the rate of biliary bile acid secretion but also whether unilamellar vesicles are solubilised to form mixed micelles alone (hydrophobic bile acid-rich biles) or mixed micelles plus multilamellar vesicles (hydrophilic bile acid-rich biles) (Cohen *et al* 1992). The micellar capacity to solubilise phospholipids is approximately four-fold greater than the micellar capacity to solubilise cholesterol (Kibe *et al* 1985; Harvey *et al* 1987).

Other cholesterol carriers in the form of phospholipid lamellae have also been described (Somjen *et al* 1990; Somjen *et al* 1991; Corradini *et al* 1995). These lamellae appear to be stacked in model biles with high concentrations of biliary cholesterol (> 10 moles %) and bile acid : cholesterol ratios of < 10, although their contribution to cholesterol solubilisation *in vivo* remains controversial (Cohen *et al* 1993).

### **1.3.3 Phospholipids**

Phospholipids are classified into two main groups: phosphoglycerides and sphingophospholipids. The phosphoglycerides are amphipathic compounds composed of a glycerol backbone esterified with two fatty acid chains at the *sn-1* (*substitution-1*) and *sn-2* (the second carbon) positions, with a phosphoric acid on the third carbon. This forms the basic structure of a phospholipid (phosphatidic acid), which can be esterified at the R<sub>3</sub> phosphate position with a specific alcohol to form the main phospholipid classes (Figure 1.4). Sphingophospholipids such as sphingomyelin contain sphingosine instead of glycerol as the backbone component (Vance 1990).



The human canalicular membrane contains substantial amounts of a variety of phospholipid classes, including sphingomyelin ( $\pm 20\%$ ), phosphatidylethanolamine ( $\pm 20\%$ ) and phosphatidylserine ( $\pm 20\%$ ), in addition to phosphatidylcholine (PC) (Coleman and Rahman 1992). In contrast, PC is by far the predominant class in bile, accounting for more than 95% of secreted phospholipids, the remainder being mostly phosphatidylethanolamine with small amounts of phosphatidylserine and phosphatidylinositol (Hay and Carey 1990).

30

(Angelico *et al* 1992) (Figure 1.5). Palmitoyl-linoleoyl PC (PC 16:0-18:2) accounts for about half of the PCs in normal bile, followed by palmitoyl-oleoyl-PC (PC 16:0-18:1) and palmitoyl-arachidonyl PC (PC 16:0-20:4). Together, these three major species make up approximately 75% of biliary PC (Ahlberg *et al* 1981; Cantafora *et al* 1983; Hay *et al* 1993). In comparison, PC within the canalicular membrane contains predominantly arachidonate (20:4) in the *sn*-2 position (Coleman and Rahman 1992).

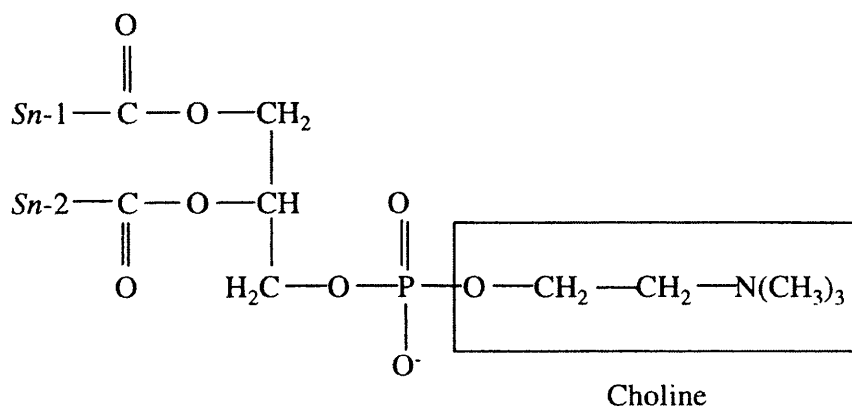


Figure 1.5 Schematic diagram of phosphatidylcholine, the predominant biliary phospholipid. *Sn-1* is typically a saturated or monosaturated fatty acid, e.g. palmitic acid or stearic acid, while *sn-2* is usually a polyunsaturated fatty acid, e.g. linoleic acid or arachidonic acid.

The reasons for these differences in phospholipid composition between the canalicular membrane and bile are complex and remain poorly understood. Only 3-5% of phospholipids are synthesised in the liver, the rest being derived from preexisting microsomal and extrahepatic pools originating from dietary sources (Robins and Brunengraber 1982; Patton *et al* 1994). PC was initially thought to be transported to the canalicular membrane via PC-rich vesicles derived from the endoplasmic reticulum, where the lipid undergoes remodeling via fatty acid transacylation, reacylation from lyosPC or, to a minor extent, is synthesised *de novo* (Robins and Brunengraber 1982; Verkade *et al* 1991). In order to explain the striking differences between the composition of biliary phospholipids and that of the canalicular membrane, an early hypothesis proposed a specific hepatic phospholipid pool residing in PC-rich microdomains within the canalicular membrane that was destined for biliary secretion (Gregory *et al* 1975; Kawamoto *et al* 1980). According to this theory, the more

hydrophilic PC species are selected for secretion as a consequence of passive extraction from the canalicular membrane by micellar bile acids in the canalicular lumen (Cohen *et al* 1990; Robins *et al* 1990; Coleman and Rahman 1992).

However, the results of other studies have suggested that the bulk of biliary PC is supplied from the endoplasmic reticulum to the canalicular cell membrane, apparently not by lipid transport vesicles, but most likely by protein-mediated cytosolic transfer (Reynier *et al* 1992; Cohen *et al* 1994). Although definite proof for this mechanism is still lacking (Oude Elferink and Groen 1999), PC appears to be supplied directly to the inner leaflet of the canalicular membrane by an asymmetric process involving PC-transfer protein. This protein is abundantly present in liver cytosol and shows a substrate-preference for biliary-type PC species, with an affinity for PC binding decreasing in the order 16:0-18:2 > 16:0-18:1 > 16:0-20:4 (Wirtz 1991).

The activity of PC-transfer protein is thought not to be the sole determinant of biliary PC composition (Kasurinen *et al* 1990). A second key factor in phospholipid secretion into bile is the translocation of phospholipid molecules across the canalicular membrane. The necessity of this step was demonstrated by the absence of biliary phospholipid and cholesterol secretion despite normal bile acid secretion in mice with a disrupted *Mdr2* gene (*mdr2* *-/-* mice) (Smit *et al* 1993) and by subsequent evidence that phospholipids are translocated (flipped) by its gene product, *mdr2* P-glycoprotein (Crawford *et al* 1997), from the inner to the outer leaflet of the canalicular membrane. Furthermore, bile of mice heterozygous for *Mdr2* gene disruption (*mdr2* *+/-* mice) had a 40% decreased phospholipid content in bile, but cholesterol secretion was preserved and dependent on the secretion rate and hydrophobicity of endogenous bile acids (Oude Elferink *et al* 1995; Oude Elferink *et al* 1996).

These observations demonstrated that *mdr2* P-glycoprotein — a member of the family of adenosine triphosphate (ATP)-binding cassette (ABC) transporters (Arrese *et al* 1998) with high homology to human *mdr3* P-glycoprotein (Oude Elferink and Groen 1999) — catalyzes a primary, rate-controlling step in the biliary secretion of phospholipids. In the absence of PC translocation activity, PC in the outer leaflet of the canalicular membrane is highly resistant to the detergent action of high concentrations of micellar bile acids present in the canalicular lumen, although bile acid micelles are

still able to extract cholesterol directly from the outer leaflet of the canalicular membrane. Conversely, in the presence of normal PC translocation activity, biliary bile acids destabilise the microdomains of translocated PC together with cholesterol in the outer leaflet, possibly by the formation of inverted micelles in the membrane. Through the ongoing translocation of PC, the domains grow into vesicular structures, which may pinch off to yield cholesterol-phospholipid vesicles (Oude Elferink and Groen 1999). According to this model, all lipids that are translocated are also excreted into the lumen as vesicles, so that the selective excretion of bile-type PC is determined largely by the substrate specificity of mdr2 P-glycoprotein (Figure 1.6).

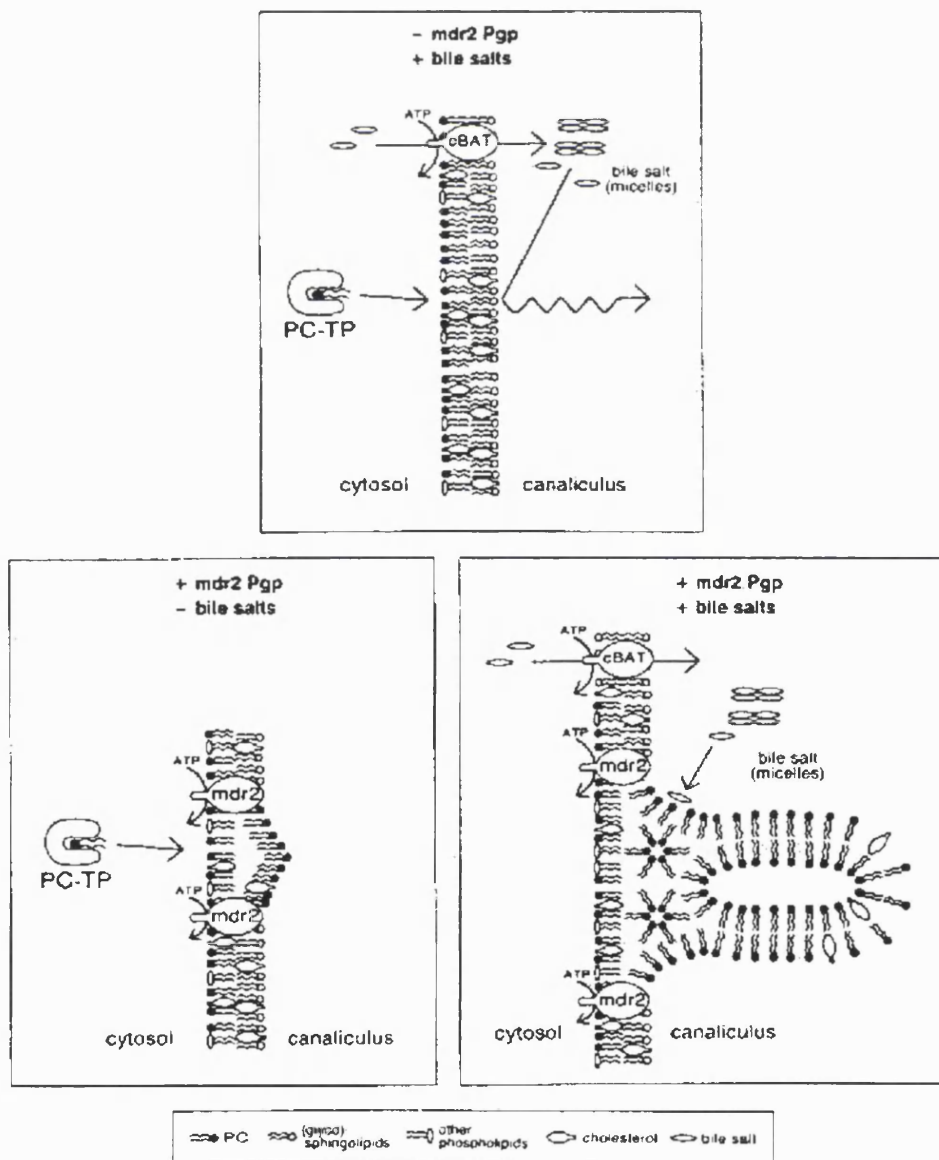


Figure 1.6 Postulated mechanism of mdr2 P-glycoprotein-mediated lipid secretion. Phosphatidylcholine (PC) is supplied to the membrane mainly via PC-transfer protein which inserts PC into the inner leaflet. Mdr2 P-glycoprotein 'flips' PC to the outer leaflet into PC-rich microdomains. Luminal bile acid micelles and/or monomers further destabilise these domains and this leads (via an unknown mechanism) to the formation and release of vesicular structures. In the absence of PC-rich domains, luminal bile acid micelles can still solubilise cholesterol but are unable to extract PC from the outer leaflet of the membrane. From Oude Elferink and Groen 1999.

#### 1.3.4 Bile acids

The hepatic synthesis of the primary bile acids, cholic acid and chenodeoxycholic acid, from cholesterol is controlled by the enzymatic action of hepatic cholesterol 7 $\alpha$  hydroxylase, followed by 12 $\alpha$  hydroxylation and side chain cleavage with carboxylation occurring at the C<sub>24</sub> position (Hofmann 1999). Cholic acid and chenodeoxycholic acid are then conjugated with glycine and taurine in an approximate ratio of 3:1 (Garbutt *et al* 1969).

Under normal conditions, more than 95% of the bile acids excreted into bile are reabsorbed, principally in the terminal ileum, and pass back to the liver via the portal venous system. The remainder enters the colon where the secondary bile acids, deoxycholic acid and lithocholic acid, are derived from primary bile acids by intestinal bacterial metabolism (Figure 1.7). Chenodeoxycholic acid (hydroxyl groups in the 3 $\alpha$  and 7 $\alpha$  position of the four ring steroid molecule) and deoxycholic acid (3 $\alpha$  and 12 $\alpha$ ) are di-hydroxy bile acids, whereas lithocholic acid (3 $\alpha$ ) is a mono-hydroxy bile acid. The fifth major bile acid found in trace amounts in human bile is ursodeoxycholic acid, which is a 7 $\beta$  epimer of chenodeoxycholic acid. Ursodeoxycholic acid is formed from chenodeoxycholic acid via bacterial oxidation in the intestine (Miwa *et al* 1986), and is identical in structure to chenodeoxycholic acid except that the hydroxy group at C-7 is in a  $\beta$  rather than an  $\alpha$  configuration (Hofmann 1999).

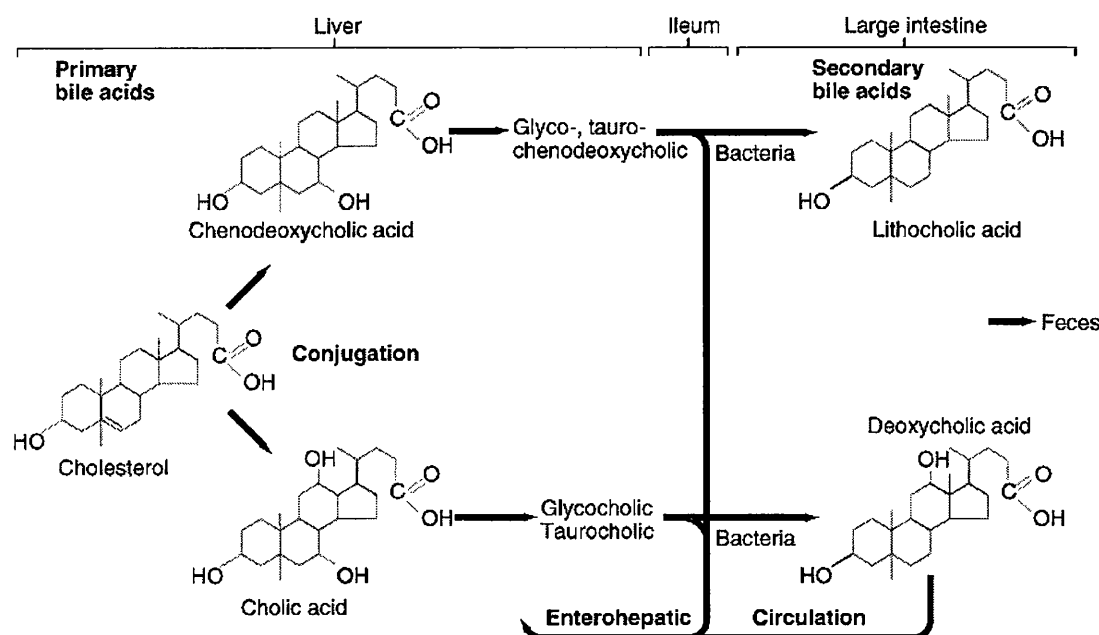


Figure 1.7 Scheme of hepatic and intestinal metabolism of bile acids and the enterohepatic circulation. Primary bile acids undergo bacterial 7 $\alpha$  dehydroxylation to form the secondary bile acids, lithocholic acid and deoxycholic acid. Unlike deoxycholic acid, lithocholic acid is not absorbed from the intestine to any appreciable extent.

The majority of bile acid reabsorption takes place through active transport processes within the terminal ileum (Mok *et al* 1977). The transport system has a greater affinity for: (i) conjugated bile acids than for unconjugated bile acids, (ii) tri-hydroxy than for di-hydroxy bile acids, which is in turn greater than for mono-hydroxy bile acids, and (iii) taurine conjugates compared with glycine conjugates (Hofmann 1999). This enterohepatic circulation of bile acids takes place several times daily. As a result of efficient active and passive reabsorption of bile acids in the small intestine (Hepner *et al* 1972a; Hepner *et al* 1972b; Hepner *et al* 1973), bile acids within the enterohepatic circulation are normally well conserved, with < 5% of the total bile acid pool being excreted in the faeces. Compensatory bile acid synthesis is controlled by the negative feedback of bile acids returning in the portal venous blood, which downregulate hepatic 7 $\alpha$ -hydroxylase activity.

The bacterial conversion of primary to secondary bile acids occurs mainly in the caecum (Morris *et al* 1973; Yahiro *et al* 1980; Macdonald *et al* 1983). Approximately

30-50% of the deoxycholate and lithocholate formed by bacterial  $7\alpha$  dehydroxylation is absorbed passively from the colon and returned to the liver (Samuel *et al* 1968; Morris and Heaton 1974). Deoxycholic acid is conjugated and excreted into the bile and, in most adults, constitutes < 20% of the biliary bile acids. Lithocholic acid is conjugated with glycine or taurine and excreted into bile but, in contrast to the conjugates of primary bile acids and of deoxycholic acid, these double conjugates (sulpholithocholylglycine and sulpholithocholyltaurine), <sup>as a result of sulphation,</sup> are not efficiently absorbed from the small intestine. Consequently, lithocholate is poorly conserved and never accounts for more than a few percent of total biliary bile acids (Hofmann 1999).

#### 1.4 GALLSTONE PATHOGENESIS

Stones composed predominantly of cholesterol account for more than 75% of all gallstones in Europe and North America. Pure cholesterol stones do occur, but most stones are mixed and contain at least 70% cholesterol in a matrix of mucin glycoprotein, calcium bilirubinate and calcium phosphate. These cholesterol-rich stones are usually multiple, hard and faceted, and are layered on cross-section.

Cholesterol gallstones develop when at least three abnormalities co-exist; the so-called triple defect (Dowling 2000a): (i) supersaturation of bile with cholesterol, secondary to hepatic cholesterol hypersecretion and/or a decrease in the total bile acid pool — a precondition for crystallisation of biliary cholesterol, (ii) abnormally rapid appearance in bile of cholesterol microcrystals (the so-called nucleation defect), and (iii) retention of crystals within the gallbladder, as a result of (a) crystal trapping within an excess mucin glycoprotein surface gel (which is itself preceded by increases in biliary arachidonic acid-rich phospholipid concentrations and mucosal prostaglandin synthesis) adherant to the gallbladder mucosa, and (b) stasis due to gallbladder motor dysfunction — primarily impaired gallbladder emptying (Figure 1.8).

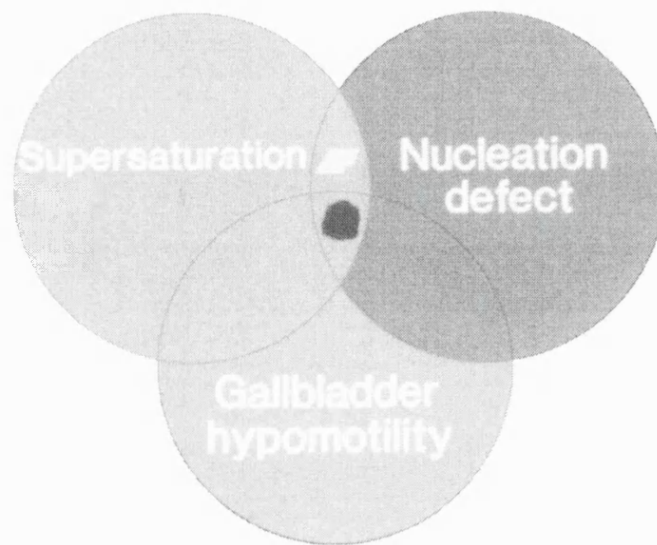


Figure 1.8 Venn diagram illustrating the three components of the classical 'triple defect' predisposing to cholesterol gallstone formation.

#### 1.4.1 Supersaturation of bile with cholesterol

Small and colleagues (Small *et al* 1966; Bourges *et al* 1967) were the first to demonstrate, in model bile solutions, that the limits of cholesterol solubility in aqueous solution were dependent on the relative quantities of cholesterol, phospholipid, total bile acids and water. This relationship was later expressed as a polynomial equation, based on the molar percentages of phospholipids and bile acids (Thomas and Hofmann 1973). Relating this maximum theoretical solubility to the actual molar percentage of cholesterol present in bile allowed a cholesterol saturation index (CSI) to be derived, with a value of  $> 1.0$  being defined as supersaturated, while bile with a CSI of  $\leq 1.0$  was termed unsaturated. An oxobiocholic acid - corrected lithogenic index has also been proposed (Botson *et al* 1980).

Cholesterol hypersecretion is essential for the development of cholesterol gallstones. Either a reduction in the total bile acid pool size or changes in bile acid composition (particularly an increase in the percent deoxycholic acid in bile (which, in turn, increases biliary cholesterol secretion) may also contribute to the formation of bile supersaturated with cholesterol. In studies of non-obese patients with gallstones, a combination of biochemical defects in the hepatic turnover of cholesterol have been documented, with an overall tendency towards reduced cholesterol  $7\alpha$  hydroxylase

activity and increased 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (Salen *et al* 1975; Key *et al* 1980; Maton *et al* 1980). In obese subjects, cholesterol supersaturation is predominantly due to increased biliary cholesterol secretion (Valdivieso *et al* 1979). Conversely, a reduction in the bile acid pool may be due to defective synthesis of bile acids (Vlahcevic *et al* 1973; Carey and Cahalane 1988) or excessive gastrointestinal losses secondary to ileal disease or bowel resection (Dowling *et al* 1971; Hofmann *et al* 1991). However, in general, gallstone patients have normal bile acid secretion rates, and even in those with a small bile acid pool, a compensatory increase in the enterohepatic cycling rate may occur so that biliary bile acid secretion remains in the normal range (Northfield and Hofmann 1975; Mok *et al* 1977).

#### **1.4.2 Cholesterol microcrystal nucleation and crystal growth**

Cholesterol supersaturation is a prerequisite for the precipitation of biliary cholesterol monohydrate crystals, but it is also frequently present in healthy stone-free individuals (Holzbach *et al* 1973; Holan *et al* 1979; Burnstein *et al* 1983). In contrast, in bile samples studied *in vitro* by light microscopy, the number of days taken for cholesterol crystals to appear (the cholesterol microcrystal nucleation time) discriminates between patients with gallstones and normal controls (Holan *et al* 1979; Sedaghat and Grundy 1980). This difference in nucleation time has been attributed to an imbalance between nucleation promoting and inhibiting factors in bile (Harvey and Strasberg 1993).

The appearance of cholesterol microcrystals represents a chain of events including the transfer of cholesterol and phospholipids between micelles and vesicles, the aggregation and fusion of unstable vesicles, and the formation of sub-microscopic cholesterol crystals which then aggregate and grow to form visible cholesterol microcrystals. In bile which is supersaturated with cholesterol, bile acids solubilise phospholipids more than cholesterol from unilamellar vesicles, to form mixed micelles. As a result, the vesicles that remain after micelle formation have an increased cholesterol : phospholipid molar ratio (Carey and LaMont 1992). These remnant vesicles, unstable by virtue of a high molar ratio, are prone to aggregate and fuse to

form large multilamellar vesicles (liquid crystals) from which cholesterol microcrystals nucleate (Harvey and Strasberg 1993). In the presence of a net excess of pronucleating proteins over antinucleating factors, cholesterol microcrystal nucleation occurs. Cholesterol precipitation starts with the formation of helical, filamentous or tubular micro-structures (crystalline forms of anhydrous cholesterol), to form the classical rhomboid-shaped cholesterol monohydrate microcrystals visible by polarised light microscopy (Konikoff *et al* 1992).

#### **1.4.3 Role of biliary phospholipids**

Changes in biliary phospholipid composition may play an important role in gallstone pathogenesis. In an early study of hamsters fed a lithogenic diet (Kajiyama *et al* 1980), bile became supersaturated with cholesterol and there was progressive replacement of 18:2 fatty acid-rich phospholipids by 18:1 and, to a lesser extent, arachidonic (20:4) fatty acid-rich phospholipids. Similarly, in an early study of Swedish gallstone patients (Ahlberg *et al* 1981), the proportions of 18:1 and 20:4 fatty acid-rich phospholipids in gallbladder bile was increased, and 18:2 phospholipids decreased, compared with stone-free controls. Other groups (Berr *et al* 1992; Hatsushika *et al* 1993) have also reported an increased proportion of the unsaturated molecular species of phosphatidylcholine, particularly arachidonic acid-rich (20:4) phospholipids, in cholesterol gallstone disease compared with controls.

An increase in biliary arachidonic acid-rich phospholipids may favour gallstone formation by a number of mechanisms. The secretion of biliary cholesterol is normally coupled tightly to that of phosphatidylcholine (PC), the principal biliary phospholipid (Berr *et al* 1997; Crawford *et al* 1997). In cholesterol gallstone disease, an increase in % biliary deoxycholate is associated with hepatic cholesterol hypersecretion (Berr *et al* 1992), as well as an increased proportion of unsaturated molecular species of PC, particularly arachidonic acid-rich species, in bile (Angelico *et al* 1992; Berr *et al* 1992; Hatsushika *et al* 1993).

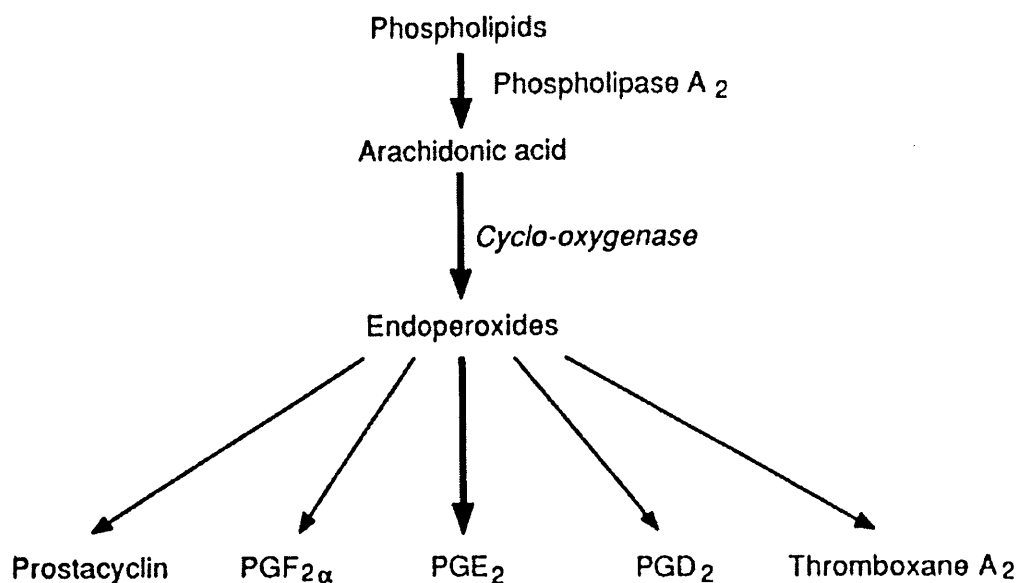


Figure 1.9 Simplified schematic diagram of the arachidonic acid pathway. PG = prostaglandin.

Data from model bile systems (Cohen and Carey 1991; Halpern *et al* 1992; Halpern *et al* 1993), and from studies of human gallbladder bile (Booker *et al* 1992), indicate that the molecular species of PC are distributed asymmetrically between micelles and vesicles. Partitioning between these two carriers is determined by *sn-1* fatty acid chain length and the degree of unsaturation of the fatty acids at both the *sn-1* and *sn-2* positions (Cohen and Carey 1991; Booker *et al* 1992). Arachidonic acid-rich PCs, by virtue of the looser packing constraints imposed by a longer chain length and particularly by a higher degree of unsaturation, partition less well into highly ordered biliary vesicles, than into micelles. They are, therefore, found in higher concentrations in mixed micelles than in vesicles (Cohen and Carey 1991; Booker *et al* 1992; Halpern *et al* 1993). The fraction of cholesterol carried in mixed micelles is also increased (Cohen and Carey 1991; Angelico *et al* 1992; Halpern *et al* 1993). As less PC is available for vesicle formation, fewer cholesterol-rich vesicles will form and these will have a higher cholesterol : phospholipid molar ratio (Cohen and Carey 1991), thus favouring vesicle aggregation, fusion and cholesterol microcrystal nucleation (Figure 1.10).

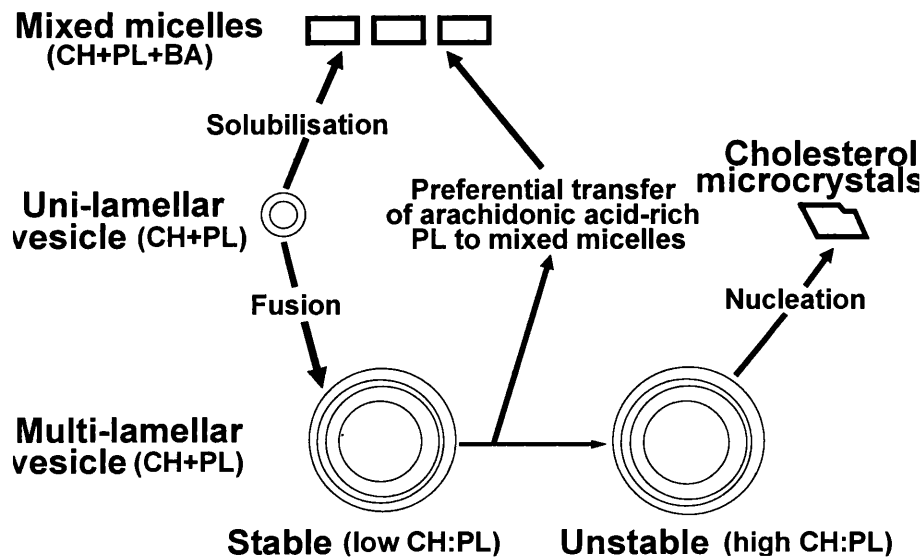


Figure 1.10 A schematic representation of the current hypothesis for the nucleation of cholesterol microcrystals. Cholesterol (CH) is co-secreted into the biliary canaliculus with phospholipids (PL) as stable unilamellar vesicles with a low CH:PL molar ratio. Bile acid monomers solubilise more vesicular PL than CH to form mixed micelles, with preferential transfer of arachidonic acid-rich phospholipid species from vesicles to micelles. Thus, when bile is supersaturated with CH, the residual vesicles become unstable due to their high CH:PL molar ratio. These unstable vesicles then fuse to form multi-lamellar vesicles from which cholesterol nucleates to form microcrystals.

In addition to enhancing cholesterol secretion into bile and destabilising the cholesterol carriers, arachidonic acid-rich PC species may also play a role in gallstone formation by providing the substrate for prostaglandin synthesis within the gallbladder mucosa, resulting in: (i) impairment of gallbladder motility (Nakata *et al* 1981; Kotwall *et al* 1984), and (ii) mucin glycoprotein hypersecretion by the gallbladder mucosa (Carey and Cahalane 1988; Myers and Bartula 1992) (Figure 1.11). In support of this hypothesis, Marks *et al* (1997) found that cholesterol saturation, biliary arachidonate, prostaglandin E<sub>2</sub> and total glycoprotein were all increased in postmenopausal women with cholesterol microcrystals compared with women with either unsaturated bile or saturated bile without crystals. The mRNA expression and protein mass of group II phospholipase A<sub>2</sub> — a secretory 14 kDa low-molecular-weight protein which hydrolyses the fatty acid at the *sn*-2 position of the glycerol moiety to leave 1-acyl

lysophospholipid (Sunami *et al* 2001) — are also increased in the gallbladder mucosa of patients with cholesterol gallstones, and are associated with concomitant increases in phospholipase A<sub>2</sub>, free arachidonate, prostaglandin E<sub>2</sub> and mucin glycoprotein, in gallbladder bile (Shoda *et al* 1997; Kano *et al* 1998).

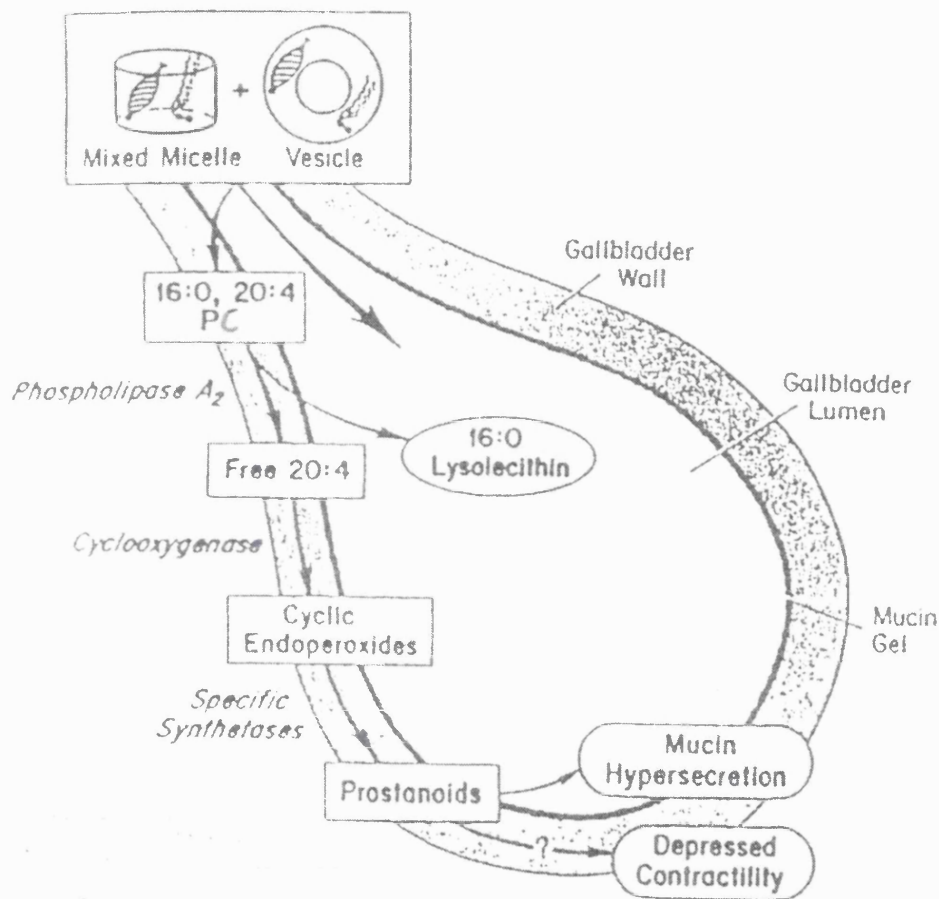


Figure 1.11 Postulated initial gallbladder events during cholesterol lithogenesis. Mucosal hydrolysis of adsorbed PC 16:0-20:4 releases free arachidonic acid that enters the prostanoid synthetic cascade, resulting in gallbladder mucin hypersecretion and a reduced contractile response of the gallbladder to regulatory peptides. Adapted from Carey and Cahalane 1988.

#### 1.4.4 Mucin glycoprotein

Gallbladder mucin, a high molecular weight glycoprotein, is the major secretory product of the gallbladder epithelium which, together with water, electrolytes,

immunoglobulins and non-mucin proteins, makes up gallbladder mucus. The viscous and gel-forming properties of gallbladder mucus are related predominantly to the amount of mucin glycoprotein secreted by the gallbladder epithelial cells (Wahlin *et al* 1974).

There are several lines of evidence to suggest that gallbladder mucin is important in gallstone formation (Table 1.1). Mucin is encoded by distinct mucin core polypeptide genes (the MUC genes), some of which show increased expression in the gallbladders of cholesterol gallstone patients compared with those of controls (Kim and Gum 1995; Kano *et al* 1998). Mucin can bind biliary lipids and accelerate the trapping and nucleation of cholesterol monohydrate crystals, both in supersaturated model and native biles (Lee 1991). The addition of human gallbladder mucin to model bile (Levy *et al* 1984) and native gallbladder bile (Gallinger *et al* 1985) accelerates cholesterol microcrystal nucleation, and this effect is lost if the mucin is first treated with pronase, a bacterial enzyme that digests the mucin protein core (Gallinger *et al* 1985). In several animal models, the hypersecretion of mucin glycoprotein antedates the formation of biliary cholesterol crystals (Lee *et al* 1981b; MacPherson *et al* 1987). Similarly, during rapid weight loss in the obese, mucin glycoprotein hypersecretion precedes cholesterol microcrystal formation and is accompanied by an increase in the CSI (Marks *et al* 1992; Shiffman *et al* 1992). Increased quantities of gallbladder mucin are also found in patients with cholesterol-rich gallstones (Lee *et al* 1979; Lee and Nicholls 1986), and the centres of the stones contain mucin complexed with bilirubin (Smith and LaMont 1985). Biliary sludge, of which gallbladder mucin is an important component (Lee and Nicholls 1986), precedes gallstone formation in approximately 20% of patients (Lee *et al* 1988).

Increases in mucin glycoprotein secretion are themselves preceded by increases in mucosal prostaglandin synthesis (Myers and Bartula 1992). In obese patients on a very low calorie diet (520 kcal/d), Marks *et al* (1992) showed that biliary cholesterol saturation and the concentrations of free arachidonic acid, prostaglandin E<sub>2</sub> and mucin glycoprotein all increased within four weeks of beginning the diet. The order in which these events occurred, however, was of particular interest. The increase in biliary arachidonate levels antedated the increase in prostaglandin E<sub>2</sub> which, in turn, preceded

the increase in glycoprotein — supporting the hypothesis that biliary arachidonic acid-rich phospholipid species provide the substrate for prostaglandin synthesis and thereby increase gallbladder mucin glycoprotein synthesis and secretion.

| Reference                       | Patient groups                                                                                   | Results                                                                                            |
|---------------------------------|--------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| (Bouchier 1966)                 | Cholesterol gallstones<br>Stone-free controls                                                    | Increase in gallbladder bile hexosamine content<br>Increase in bile viscosity                      |
| (Harvey <i>et al</i> 1986)      | Cholesterol gallstones (n=17)<br>Pigment gallstones (n=6)<br>Stone-free controls (n=11)          | No difference in mucin glycoprotein conc.<br>No correlation of mucin with CSI                      |
| (Swobodnik <i>et al</i> 1991b)  | Cholesterol gallstones (n=10)<br>Stone-free controls (n=10)                                      | No difference in mucin glycoprotein conc.<br>Positive correlation of mucin with c-AMP              |
| (Marks <i>et al</i> 1992)       | Obese patients undergoing rapid weight reduction (n=7)                                           | Increase in mucin conc. preceded by increases in prostaglandin E <sub>2</sub> and arachidonic acid |
| (Hatsushika <i>et al</i> 1993)  | Cholesterol gallstones (n=21)<br>Non-cholesterol gallstones (n=25)<br>Stone-free controls (n=56) | Increase in mucin glycoprotein conc. in patients with cholesterol-rich gallstones                  |
| (Shiffman <i>et al</i> 1993)    | Obese patients with gallstones after gastric bypass (n=11)                                       | Increase in mucin glycoprotein conc.                                                               |
| (van Wijland <i>et al</i> 1994) | Cholesterol gallstones (n=60)<br>Stone-free controls (n=20)                                      | No difference in mucin conc.<br>No difference in lectin binding by mucin                           |
| (Kano <i>et al</i> 1998)        | Cholesterol gallstones (n=80)<br>Stone-free controls (n=24)                                      | Increase in free arachidonate, mucin glycoprotein conc., and bile viscosity                        |

Table 1.1 Clinical studies of the role of biliary mucin glycoprotein in gallstone pathogenesis.

However, the pathophysiological role of mucin glycoprotein in cholesterol gallstone formation remains controversial. Some workers have failed to show an effect of mucin glycoprotein on cholesterol microcrystal nucleation in model or human bile (Whiting and Watts 1985), or an alteration in nucleation time following the removal of mucin glycoprotein from abnormal biles by ultracentrifugation and ultrafiltration (Gallinger *et al* 1985). Furthermore, in some studies of gallstone patients and controls (Harvey *et al*

1985), quantitative or qualitative differences in gallbladder mucin composition have not been demonstrated. Overall, however, the available evidence supports the hypothesis that cholesterol crystal trapping by a mucin layer adherent to the gallbladder wall, in conjunction with impaired gallbladder emptying, predisposes to cholesterol gallstone formation.

#### **1.4.5 Non-mucin promoters and inhibitors of nucleation**

Approximately 4% of biliary solutes consist of a protein mass (Carey and Cahalane 1988), which may be divided into mucin glycoprotein, as described above, and non-mucin proteins. Although the strongest evidence incriminates mucin glycoprotein as a pronucleator (Dowling 2000b), there are accumulating data that some non-mucin proteins act as nucleating promoters or inhibitors of cholesterol nucleation in bile, and that it is the relative imbalance between these nucleating factors that predisposes to cholesterol gallstone formation.

In 1983, Burnstein and colleagues (1983) reported that the nucleation time of supersaturated gallbladder bile from subjects without cholesterol gallstones, was shortened by the addition of small amounts of gallbladder bile from patients with rapidly nucleating bile, suggesting the presence of so-called nucleation promoting factors. In 1987, Gallinger *et al* (1987) reported that non-mucin proteins purified from the bile of patients with cholesterol gallstones accelerated microcrystal nucleation *in vitro*, whereas biliary protein from controls had little effect. The following year, Groen and co-workers (1988) reported on their use of Concanavalin A (a plant lectin that binds to carbohydrates) to obtain biliary glycoproteins free of bile pigment, biliary lipids and mucin glycoprotein. These authors demonstrated that T-tube bile had both pro- and anti-nucleating activity in the same sample. The Dutch group then went on to isolate a potent pronucleating non-mucin glycoprotein of molecular weight  $150 \pm 30$  kDa (Groen *et al* 1990), which was later characterised as aminopeptidase N (Offner *et al* 1994). Similarly, Teramen *et al* (1995) isolated six different concanavalin A-bound glycoproteins (40, 50, 58, 80, 98 and 143 kDa), all of which either shortened the nucleation time, accelerated the growth of cholesterol microcrystals, or both. Other

putative promoters of nucleation include immunoglobulins A and M (Harvey *et al* 1991b), phospholipase C (Pattinson and Willis 1991), fibronectin (Chijiwa *et al* 1991), a small (< 10 kDa) acidic protein (Lafont *et al* 1997) and a Concanavalin A-binding 42 kDa glycoprotein (Abei *et al* 1993).

Conversely, Holzbach and co-workers (1984) proposed that bile from stone-free controls contained inhibitors of cholesterol nucleation, following the observation that bile from stone-free controls had a longer nucleation time than model biles of similar lipid composition or of native bile in which the protein fraction had been removed by solvent extraction. The addition of the protein fraction from the delipidated native bile prolonged the nucleation time of model bile. Similarly, Kibe *et al* (1984) showed that apolipoproteins A-I and A-II inhibited the nucleation of model bile by a factor of 1.4 to 2.7 times that of controls, although Sewell *et al* (1983) found no difference in the concentrations of apolipoproteins A-I or Apo A-II in the bile of gallstone patients and controls. Ohya *et al* (1993), using *Helix pomatia* lectin affinity binding columns, isolated a 120 kDa heterodimeric human biliary glycoprotein with subunits of 63 and 58 kDa which inhibited cholesterol crystal growth, but it is not known whether this glycoprotein is present in lower concentrations in the bile of patients with gallstones compared with controls.

#### **1.4.6 Gallbladder motor dysfunction**

Under normal conditions, the gallbladder contracts in response to sham feeding (Fisher *et al* 1986), liquid meals containing fat (Forgacs *et al* 1984) or mixed liquid-solid phase meals (Cano *et al* 1986). After sham feeding, up to 65% of the gallbladder volume may be expelled. A minimum or nadir gallbladder volume of 5-10 ml is reached 30-45 min after a liquid meal stimulus (Forgacs *et al* 1984), although following a mixed phase meal, gallbladder volume may take up to 4 hours to reach a nadir (Cano *et al* 1986). The gallbladder also contracts periodically in the interdigestive state, expelling approximately 10% of gallbladder contents every one to two hours (Cano *et al* 1986; Lanzini *et al* 1987). This periodic gallbladder emptying coincides with phase

III migrating motor complexes (Toouli *et al* 1986; Marzio *et al* 1988), which probably originate in the antrum (Stolk *et al* 1993).

The feeding of a lithogenic diet (0.4-1.2% cholesterol) to prairie dogs results in the formation of cholesterol gallstones (Brenneman *et al* 1972). This model has been used to demonstrate that a reduction of gallbladder emptying antedates the formation of cholesterol microcrystals and gallstones (Roslyn *et al* 1979; Doty *et al* 1983). In the ground squirrel, *in vitro* gallbladder contractility in response to cholecystokinin-8 was reduced by 42% before gallstones appeared and by 65% after the formation of gallstones (Fridhandler *et al* 1983). Similarly, Poston *et al* (Poston *et al* 1992) showed that isolated guinea pig gallbladder muscle strips had decreased sensitivity to cholecystokinin stimulation in the early stages of stone formation induced by a lithogenic diet, related in part to a lower concentration of cholecystokinin receptors on the gallbladder smooth muscle. Carey and Cahalane (1988) postulated that these changes in gallbladder contractility may be mediated, in part, by an increase in gallbladder wall prostaglandin synthesis, as has been observed in the prairie dog model (Chapman *et al* 1989).

Human studies also support the hypothesis that reduced gallbladder emptying precedes stone formation. In an early study of 21 stone-free subjects (van der Linden 1974), 12 were found to be weak contractors by oral cholecystography, seven of whom developed gallstones up to 14 years later. In another study of 36 patients with post-prandial upper abdominal pain and nausea who had no evidence of gallstones by oral cholecystography or ultrasound of the gallbladder (Brugge *et al* 1986), cholesterol microcrystals were present in the duodenal bile of 16 (44%). In this group, the mean post-prandial gallbladder ejection fraction was 26%, compared with 60% in those without crystals.

Gallbladder stones do not appear to compromise gallbladder emptying significantly, since impaired gallbladder motility persists after stone clearance with extracorporeal lithotripsy (Spengler *et al* 1989) and is not dependent on stone size or number (Pomeranz and Shaffer 1985). Nonetheless, in large series, approximately 50% of patients with gallstones have reduced meal- or cholecystokinin-stimulated gallbladder emptying (Forgacs *et al* 1984; Pomeranz and Shaffer 1985; Kishk *et al* 1987). In these

so-called 'poor contractors', any cholesterol microcrystals that form within the gallbladder may not be expelled during gallbladder contraction, leading to retention of cholesterol microcrystals and stone formation. Conversely, in those with normal gallbladder emptying rates, fasting and post-stimulus residual gallbladder volumes are increased (Kishk *et al* 1987; Festi *et al* 1990), which may also favour gallstone formation by providing the time for cholesterol microcrystal nucleation and aggregation to occur.

There are several lines of evidence to suggest that the impaired gallbladder motility seen in cholesterol gallstone disease is secondary, in part, to increased biliary cholesterol saturation. As described earlier, when the prairie dog (Doty *et al* 1983) or ground squirrel (Fridhandler *et al* 1983) is fed a lithogenic diet, the development of supersaturated bile is associated with gallbladder hypomotility before stone formation. In both normal subjects and patients with gallstones, there is an inverse correlation between the degree of postprandial gall bladder emptying and biliary cholesterol saturation (Fisher *et al* 1982; Pomeranz and Shaffer 1985; Festi *et al* 1990). Studies in patients with cholesterol gallstone disease suggest that the site of gallbladder motor dysfunction is localised to the sarcomere cell membrane and is not a result of defective intracellular contractility mediated by second messengers such as inositol triphosphate (Behar *et al* 1989). One proposed explanation for a sarcolemmal defect is that biliary cholesterol diffuses passively into the sarcomere cell membrane, causing increased membrane rigidity (Li *et al* 1990).

## **CHAPTER 2. NON-SURGICAL TREATMENT OF GALLSTONES**

### **2.1 INTRODUCTION**

Before the advent of laparoscopic cholecystectomy in 1987, many patients with symptomatic gallbladder stones were reluctant to undergo cholecystectomy. As a result, in a selected minority of symptomatic patients with specific, gallstone-related symptoms and a patent cystic duct, a variety of different management options were developed. The purpose of this chapter is to review selected aspects of the non-surgical management of gallbladder stones, and to discuss the residual role for these treatments in the era of laparoscopic cholecystectomy.

### **2.2 NATURAL HISTORY OF GALLSTONES**

Non-specific upper abdominal symptoms occur with equal frequency in gallstone carriers and gallstone-free subjects. An estimated 20-30% of patients referred with the diagnosis of biliary symptoms are judged, clinically, to have either a colonic motility disorder or non-specific dyspepsia unrelated to their gallbladder stones. Conversely, some studies suggest that only about three-quarters of patients with symptomatic gallstone disease actually seek medical attention because of episodic abdominal pain (Fenster *et al* 1995).

#### **2.2.1 Asymptomatic gallstones**

It has been estimated that primary gallbladder stones grow at a rate of 1-4 mm per year, and usually do not cause symptoms until at least 2-7 years after their formation (Mok *et al* 1986; Wolpers and Hofmann 1993). The results of epidemiological studies indicate that in 66-80% of gallstone carriers, the stones remain silent or asymptomatic. Thus, only a minority of patients will ever develop specific, gallstone-related symptoms such as biliary colic — arbitrarily defined as a steady epigastric and/or right upper quadrant pain lasting more than 30 min that is unrelated to bowel movements (Pereira 2002). An even smaller proportion present with ‘surgical’ complications — due mainly

to obstruction of the cystic duct (acute cholecystitis  $\pm$  empyema formation or the Mirizzi syndrome) or common bile duct (cholestasis/jaundice, cholangitis or pancreatitis) (Dowling 1992).

In most of the initial long-term studies of patients with asymptomatic gallstones — often with follow-up of 20 years or more — the annual rates of developing biliary pain or gallstone complications varied from approximately 1% to 4% (Friedman 1993). In contrast, in the American National Cooperative Gallstone Study of 193 gallstone patients who had been asymptomatic in the previous 12 months, the average annual rate of developing biliary pain over the next two years was 17% and of undergoing cholecystectomy, 1.3% (Thistle *et al* 1984). These higher figures may have been due to the close follow-up of the patients, and the possibility that some had had symptoms more than 12 months beforehand and were not truly asymptomatic. Overall, in patients with gallstones which are discovered incidentally, most studies suggest that symptoms or complications will develop in only a few percent per year. Asymptomatic stones usually become symptomatic before they cause complications, and the longer the stones remain quiescent after an initial attack of biliary colic, the less likely it is that complications will occur (Friedman 1993).

Based on the evidence above, prophylactic treatment of gallbladder stones in <sup>in native Americans who appear to have a rate of gallstone-associated gallbladder cancer that is sufficiently high to justify prophylactic cholecystectomy. Another is,</sup> asymptomatic patients is rarely indicated. One exception is choledocholithiasis in patients with asymptomatic gallbladder stones. Alternatively, in patients who are poor risks for surgery and who do not have acute cholecystitis, endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy without cholecystectomy is often used to treat common bile duct stones. In one study published more than a decade ago (Ingoldby *et al* 1989), only 18 of 186 elderly patients treated in this manner required later cholecystectomy during an average follow-up of 32 months. However, the results of more recent prospective randomised studies have questioned the rationale of adopting a wait and see strategy (Tham and Carr-Locke 1999). In a study of 98 elderly patients (mean age 80 years) randomised to either open cholecystectomy with bile duct exploration or endoscopic sphincterotomy alone (Targarona *et al* 1996), there were no significant differences in immediate morbidity (23% v 16%) or mortality (4% v 6%). However, during a mean follow-up of 17 months, biliary symptoms recurred in 10

of the sphincterotomy group (20%), seven of whom required surgery. In a similarly designed study of middle-aged and elderly patients with more than five years follow-up (Hammarstrom *et al* 1995), 13 of 35 (37%) in the sphincterotomy group required later surgery compared with two of 41 in the open cholecystectomy group. There are fewer comparative data on laparoscopic cholecystectomy versus sphincterotomy, but in high-risk patients 'lap chole' has been shown to carry less morbidity and mortality than open cholecystectomy (Deziel *et al* 1993). In a recently reported multicentre trial of 98 patients aged 18-80 years who were randomised to laparoscopic cholecystectomy or a 'wait and see' policy after endoscopic sphincterotomy and bile duct clearance (Boerma *et al* 2001), 40% of the expectantly managed patients developed recurrent symptoms and 30% required cholecystectomy within two years. Thus, bile duct stone clearance followed by laparoscopic cholecystectomy remains the standard treatment for such patients.

### **2.2.2 Symptomatic gallstones**

Patients with symptomatic gallstones have a higher risk of developing recurrent biliary colic or gallstone complications than those with asymptomatic gallstones. In the U.S. National Cooperative Gallstone Study (Thistle *et al* 1984), of 112 patients who had experienced biliary pain in the 12 months before entry, the average annual rate of developing recurrent biliary colic over the next two years was 44% and of undergoing cholecystectomy, 3.2% — more than double the corresponding rate in previously asymptomatic patients. For the reasons discussed in the previous section, these figures are also higher than those of most other long-term follow-up studies of patients with mildly symptomatic gallstones. In these series, the average annual rates of developing severe pain (usually requiring cholecystectomy) ranged from 1% to 8%, with complications (acute cholecystitis, choledocholithiasis, pancreatitis) occurring in only 1% to 3% per year (Friedman 1993). These figures suggest that cholecystectomy or medical dissolution therapy should be offered to patients only after significant biliary symptoms develop. In those with mild, non-specific symptoms, or who have had a single attack of mild biliary colic, simple observation alone may be appropriate, since

as many as 30-50% of patients who have had one episode of pain will not have a recurrent episode.

## **2.3 INVESTIGATIONS**

### **2.3.1 Ultrasonography**

Transabdominal ultrasound is the most accurate of the non-invasive imaging techniques for detecting the presence of stones in the gallbladder, with a reported sensitivity of 92-96% and specificity close to 100% if a hyperechoic image with acoustic shadowing is seen in the gallbladder. It cannot reliably detect individual particles measuring < 2 mm in diameter, and small stones located in the infundibulum may also be difficult to visualise (Shea *et al* 1994; Dahan *et al* 1996). Ultrasound can also detect the presence of biliary sludge, but it is less reliable for stones in the common bile duct, where the sensitivity of detecting choledocholithiasis or a dilated common bile duct ranges from 50-70%, compared with a sensitivity of about 95% for ERCP. Endoscopic ultrasonography has a comparable sensitivity to ERCP and specificity approaching 100% in detecting bile duct stones, and in some centres is now used routinely in the investigation of patients with a low or intermediate pre-test probability of having ductal stones, with associated reductions in costs and morbidity compared with ERCP (Palazzo 1997; Brugge 1998).

In patients with symptomatic but uncomplicated cholelithiasis who are eligible for cholecystectomy, transabdominal ultrasound is usually the only imaging study required. However, in patients who are being considered for medical dissolution therapy, localised computed tomography of the gallbladder (to determine stone composition) and oral cholecystography (to assess patency of the cystic duct), are also indicated for the reasons given below. Alternatively, a significant reduction of gallbladder volume detected in response to a cholecystokinetic stimulus, such as a fatty meal or intravenous cholecystokinin, during ultrasonography predicts patency of the cystic duct with acceptable accuracy (Brakel *et al* 1991).

### 2.3.2 Oral cholecystography

Although this was the standard method of investigating the gallbladder for many years, it has now been largely superseded by ultrasonography. A biliary contrast medium based on triiodobenzoic acid, which is primarily excreted by the liver, is given orally. It is absorbed from the small bowel into the portal venous system, transported across hepatocytes into bile, and concentrated in the gallbladder, where it becomes visible on a radiograph (Figure 2.1). Opacification of the gallbladder during oral cholecystography confirms that the cystic duct is patent — a prerequisite for non-surgical treatment of gallstones. Patients are then given a fatty meal; a subsequent gallbladder contractile response of  $> 30\%$  is a predictor of complete gallstone dissolution after oral bile acids  $\pm$  shock-wave lithotripsy (Sackmann *et al* 1993). Conversely, failure of the gallbladder to opacify is usually indicative of gallbladder disease, providing that the contrast material has been absorbed and the patient is not jaundiced. The examination also has an occasional role in patients who present with gallbladder symptoms but in whom the gallbladder remains contracted despite fasting or is difficult to visualise by ultrasonography.



Figure 2.1 Radiolucent, cholesterol-rich gallstones seen at oral cholecystography.

### 2.3.3 Computed tomography

Radiolucent stones are usually, but not always, cholesterol-rich. Thus, 14-20% of stones that appear lucent by conventional radiology (plain x-ray + oral cholecystogram) are non-cholesterol in type, while approximately 50% of stones which are lucent by conventional radiology appear dense by computed tomography (CT) of the gallbladder. The maximum gallstone attenuation score, measured by CT *in vivo*, predicts stone composition and dissolvability, and is cost-effective in selecting patients for oral dissolution treatment. Although there remains some controversy about the most appropriate cut-off point in the maximum gallstone attenuation score which should be used to exclude patients from dissolution therapy, values  $\geq 100$  Hounsfield Units predict calcium-containing, non-dissolvable stones (Ell *et al* 1991; Caroli *et al* 1992). The best results with medical dissolution therapy are obtained with stones with low CT attenuation scores of  $< 50$ -70 HU, or which are isodense with bile and not visualised at all by CT (Dowling 1992).

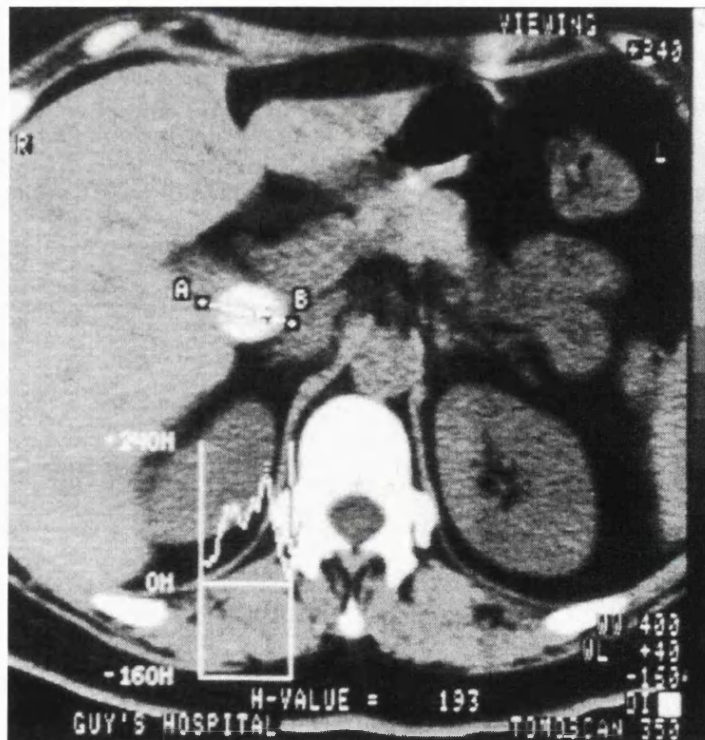


Figure 2.2 Computed tomography of the gallbladder, showing a CT-dense gallstone with an attenuation score of 193 Hounsfield Units.

### **2.3.4 Magnetic resonance imaging**

The use of heavily T2-weighted, fat-suppressed sequences to isolate out signal from stationary fluid was first described by Japanese and American groups in 1989. At that time, image acquisition was slow and the resultant images were motion-degraded despite respiratory gating. Improvements in both software and hardware have resulted in breath-hold abdominal imaging, better fat suppression and rapid K space sampling such that magnetic resonance cholangiopancreatography (MRCP) has become an established clinical tool. The major advantage of MRCP over ERCP is that it is entirely noninvasive, and requires only a co-operative non-claustrophobic patient who can lie flat and breath-hold for less than 20 seconds on request. No contrast is injected.

Compared with the gold standard of ERCP, MRCP has a reported sensitivity of 85-100% and specificity of 83-100% in the detection of choledocholithiasis, with an overall accuracy of 89-97%. The sensitivity appears to decrease according to stone size: 33-71% for bile duct stones < 6 mm in size, 89-94% for stones measuring 6-10 mm, and 67-100% for stones greater than 10 mm in size. A stone impacted at or near the ampulla, without surrounding fluid, may also go undetected on MRCP (Barish *et al* 1999).

## **2.4 MEDICAL MANAGEMENT**

The results of epidemiological studies from Italy have shown that when gallbladder stones are diagnosed by ultrasound and the carriers are studied further by oral cholecystography, approximately 30% of patients with silent or asymptomatic gallstones have a non-visualisable gallbladder (Attili *et al* 1995). In symptomatic gallstone patients with a blocked cystic duct, there is generally no role for medical therapy and the decision usually lies between laparoscopic or open abdominal removal of the gallbladder. However, in patients with a patent cystic duct who decline or are unfit for surgery, cholesterol-rich (radio- and CT-lucent) gallstones can be removed or dissolved from the gallbladder and bile ducts in a number of ways, as outlined below. These techniques avoid the discomfort and small risks of general anaesthesia and surgical exploration of the abdomen and bile ducts.

Before the advent of laparoscopic cholecystectomy, some of the reasons given by patients for declining conventional open cholecystectomy included fear of anaesthesia/surgery, adverse reactions of the patient or relatives/friends to previous surgery, loss of income during hospitalisation and convalescence, and a ‘nothing to lose’ philosophy about a trial of medical treatment (Dowling 1992). Although some of these reasons also apply in the era of laparoscopic cholecystectomy, the cosmetic objection to a traditional surgical scar no longer applies. The time required in hospital and for subsequent convalescence is also much shorter after laparoscopic than conventional cholecystectomy, with day-case ‘lap choles’ now being performed routinely in many centres.

In general, for reasons of clinical- as well as cost-effectiveness, the non-surgical treatments should be reserved for patients with mild, uncomplicated gallstone symptoms who decline surgery or in whom the risk of cholecystectomy is high. In a carefully selected group of patients, these medical approaches work moderately well but they are relatively expensive, require long-term surveillance and are associated with a gallstone recurrence rate of approximately 50% at five years.

#### **2.4.1 Oral bile acid therapy**

Oral bile acid therapy is the slowest, safest, and best documented of all the non-surgical/minimally invasive alternatives to cholecystectomy. Although the reported dissolution rates for gallstones vary widely, up to 90% of patients with radio- and CT-lucent stones and a patent cystic duct will progress to confirmed complete gallstone dissolution during oral bile acid therapy — given alone, or together with extracorporeal shock-wave lithotripsy (ESWL). Until recently, the preferred oral bile acid regime in most centres was combination therapy with chenodeoxycholic acid (5-7.5 mg/kg/day) and its 7- $\beta$ -hydroxy epimer, ursodeoxycholic acid (UDCA; 5-7.5 mg/kg/day). These bile acids, normal constituents of bile, act by reducing the hepatic synthesis and biliary excretion of cholesterol, resulting in cholesterol desaturation of bile and the leaching out of cholesterol from gallstones. UDCA is also associated with a decrease in some of the nucleation promoters and their crystallisation-promoting

activity in bile (Van Erpecum *et al* 1996). UDCA has advantages over chenodeoxycholic acid in that it rarely induces diarrhoea and does not cause elevations of serum transaminases. In the UK, chenodeoxycholic acid is no longer available, and UDCA monotherapy is given at a dose of 10-12 mg/kg/day, with gallstone dissolution rates similar to that of combination therapy (Sackmann *et al* 1991a; Petroni *et al* 2001). In patients who have had a previous sphincterotomy, oral bile acid therapy is relatively ineffective in dissolving cholesterol-rich gallbladder stones because of reduced concentrations of the prescribed bile acid within the underfilled gallbladder (Dowling 1992).

Gallstone dissolution usually requires at least six months, and up to two years, of oral bile acid therapy — depending on stone size, number and composition. During treatment, ultrasonography is usually performed every three to six months until the gallbladder is clear of stones, followed by a repeat test one to three months later to confirm complete gallstone dissolution, at which time UDCA therapy is stopped. In most cases, treatment should also be discontinued if there is no evidence of partial gallstone dissolution (a decrease in the number and/or size of the stones) after one year, or incomplete dissolution after two years.

Recommended selection criteria for oral bile acid therapy are shown in Table 2.1. The stones should be small (ideally  $\leq 5$  mm), so that there is a high surface-to-volume ratio, and cholesterol-rich — as determined by a CT attenuation score of the stones of  $< 100$  Hounsfield Units. The cystic duct should also be patent (e.g., reduction in gallbladder volume by more than 30% after cholecystokinin or fatty meal stimulation), and the patient not be morbidly obese, so that enrichment of the bile with the prescribed bile acid and cholesterol desaturation can be accomplished. Unfortunately, these selection criteria account for only about 15% of the gallstone population (Strasberg and Clavien 1992). Oral bile acid therapy has also been occasionally used for common bile duct stones, with reported dissolution/disappearance rates of about 50% (Salviali *et al* 1983).

| Oral Bile Acids                                           | ESWL ± Oral Bile Acids                                    |
|-----------------------------------------------------------|-----------------------------------------------------------|
| Stones ≤ 5 mm<br>(≤ 15 mm acceptable)                     | Single stone ≤ 20 mm<br>(≤ 3 stones ≤ 30 mm acceptable)   |
| Cholesterol-rich stones<br>(CT score < 100 HU)            | Cholesterol-rich stones<br>(calcified rim acceptable)     |
| Patent cystic duct<br>(OCG or ultrasound with fatty meal) | Patent cystic duct<br>(OCG or ultrasound with fatty meal) |
| Nonobese patient                                          | Nonobese patient                                          |
| Mild biliary symptoms                                     | Mild biliary symptoms                                     |
| Patient declines or is unfit for<br>Cholecystectomy       | Patient declines or is unfit for<br>cholecystectomy       |

Table 2.1 Recommended selection criteria for oral bile acid therapy and extracorporeal shock-wave lithotripsy (ESWL) ± adjuvant oral bile acids. OCG = oral cholecystography.

#### 2.4.2 Reasons for incomplete gallstone dissolution

By ensuring that only those with potentially dissolvable stones are considered for treatment, up to 90% of those given medical dissolution therapy can achieve confirmed, complete gallstone dissolution (by life table analysis) at 18 months. However, even when the stones are lucent by conventional radiology and have CT scores of < 100 HU, no/arrested gallstone dissolution (no ultrasonographic response to the oral bile acids after one year or partial, but arrested, dissolution after two years of treatment) still occurs in 10-35% of patients (Strasberg and Clavien 1992; Pereira *et al* 1997).

The reasons for incomplete gallstone dissolution, in patients selected for treatment using the optimal criteria of a patent cystic duct and a low gallstone CT attenuation score, are poorly defined. Possible causes include the development of either a blocked cystic duct or impaired gallbladder emptying during treatment. In a study of 126

patients treated with UDCA alone, cystic duct obstruction developed in approximately 20% of patients after up to four years of continued treatment (Gleeson *et al* 1990). Another reason for incomplete gallstone dissolution is acquired stone calcification, which has been reported in 9-21% of patients with no/arrested dissolution during oral bile acid therapy (Bateson 1981; Lirussi *et al* 1993; Pereira *et al* 1997). Acquired gallstone calcification may also occur spontaneously (Bazzoli *et al* 1995; Pereira *et al* 1997), and is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (Plaisier *et al* 1994b; Bazzoli *et al* 1995).

#### **2.4.3 Extracorporeal shock-wave lithotripsy**

The best results with extracorporeal shock-wave lithotripsy (ESWL) are obtained in patients with a patent cystic duct and solitary, radio- and CT-lucent stones measuring < 20 mm in diameter — although acceptable fragmentation rates are also achieved in patients with up to three large stones  $\leq$  30 mm in diameter. Such patients represent a highly selected subset — an estimated 15% of all symptomatic gallstone patients — although only about half of this subgroup will have optimal selection criteria of a solitary radiolucent stone < 20 mm diameter in a functioning gallbladder (Strasberg and Clavien 1992). Given the high capital cost of the ESWL equipment and its maintenance costs, this treatment remains confined to a few specialised centres — usually where the ESWL machine is also used in the treatment of renal stone disease.

Most reports suggest that cholesterol-rich gallstones can be targeted and fragmented to a maximum diameter of < 10 mm in 80-100% of patients (Dowling 1992; Sauter *et al* 1997). However, it is not always possible to target gallbladder stones in the obese and in those with small contracted or high, subhepatic gallbladders masked by the costal margin. Following fragmentation, oral bile acid therapy is usually given until complete gallstone dissolution is confirmed by serial ultrasound. In general, cholesterol-rich stone fragments measuring < 10 mm in diameter dissolve readily — although the best results are obtained in those with even smaller stone fragments (< 5 mm in diameter). Adverse effects of ESWL are usually minor, but include biliary colic, skin petechiae, and microscopic haematuria.

Giant or impacted common bile duct stones that cannot be removed after endoscopic sphincterotomy and mechanical lithotripsy are other appropriate targets for ESWL. If the stones are targeted successfully, the fragments can then be extracted by conventional means. Alternative forms of treatment of difficult bile duct stones include electrohydraulic contact lithotripsy and laser lithotripsy. The latter technique involves passage of a fine fiberoptic bundle capable of transmitting laser energy via a T-tube or by the endoscopic route into the common bile duct. In three published series using a pulsed dye laser with an optical stone detection system designed to improve stone targeting (Wilkinson 1998), 103 of 114 patients (90%) achieved complete stone clearance. In a German study of 60 patients randomised to ESWL or intracorporeal laser lithotripsy for difficult bile duct stones (Neuhaus *et al* 1998), bile duct clearance was achieved in 22 (73%) in the ESWL group and 29 (97%) of the laser lithotripsy group, after a maximum of three lithotripsy sessions. Next-generation, pulsed solid-state lasers with optical stone detection systems are currently under investigation.

#### **2.4.4 Instillation of contact solvents**

In symptomatic patients with a functioning gallbladder, cholesterol-rich stones can be rapidly dissolved by the direct instillation into the gallbladder of cholesterol solvents such as methyl *tert*-butyl ether (MTBE) or ethyl propionate (Leuschner *et al* 1991; Dowling 1992). This is usually performed via a percutaneous, transhepatic catheter placed under local anaesthesia (Figure 2.3). However, some patients experience considerable pain during the placement of the pig-tail catheter despite intravenous sedation and analgesia, and the procedure is at the limits of acceptability as a technique suitable for local anaesthesia.

Despite the initially rapid dissolution rates achieved with contact solvents over 6-12 h, gallstone dissolution is often incomplete. In an early study from the Mayo Clinic (Thistle *et al* 1989), 51 of 75 patients were left with residual gallstone debris. The residual particles usually disappear completely with oral bile acid therapy, but the need for weeks or months of adjuvant oral bile acids partly defeats the aim of rapid, complete gallstone dissolution by this relatively invasive technique. MTBE has

sedative-anaesthetic properties and it can cause other transient side-effects such as oedema of the gallbladder mucosa and duodenitis. Ethyl propionate seems to lack many of these disadvantages but experience with it remains limited (Esch *et al* 1993). There may still be a small place for contact solvent dissolution of gallstones in patients at prohibitive risk for general anaesthesia, but in only a few specialised centres where there is a large experience with the technique.

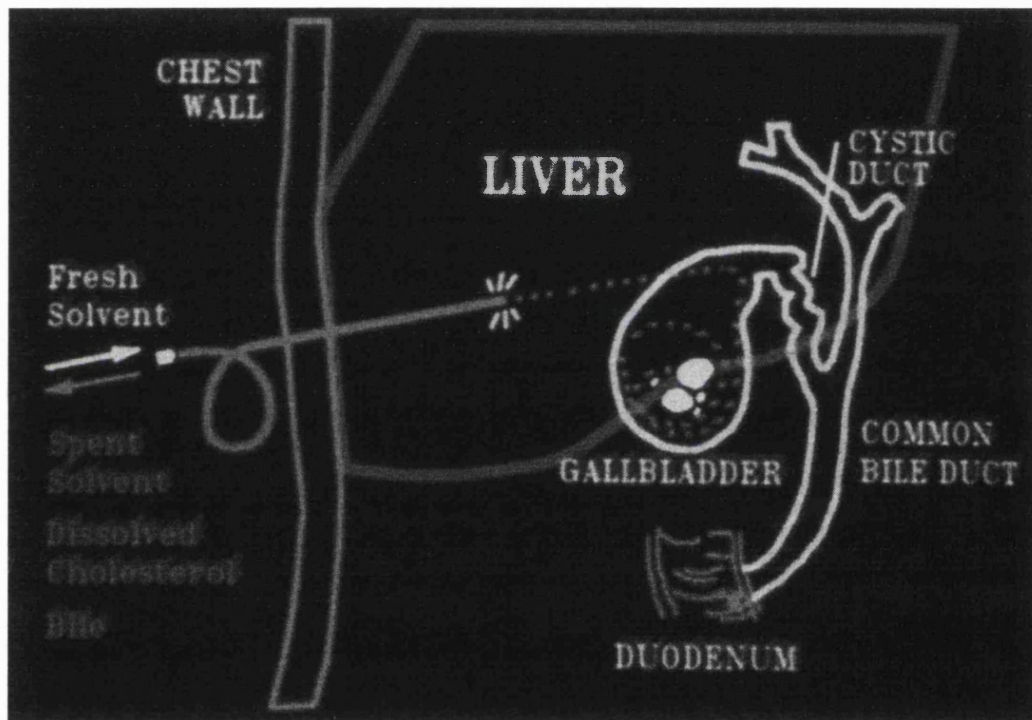


Figure 2.3 Diagram depicting the technique of contact dissolution of gallbladder stones using contact solvents.

An alternative to the percutaneous approach is endoscopic retrograde catheterisation of the gallbladder through the cystic duct (Soehendra *et al* 1990). In skilled hands, this technique is possible in up to 75-80% of patients but even with the use of steerable-tip catheters, the limiting factor is often the calibre and plasticity of the cystic duct and the tortuosity of its spiral valve of Heister. Once the naso-cholecystic catheter is in place, cholesterol-rich gallstones may be dissolved with contact solvents as described above for the percutaneous route.

#### **2.4.5 Percutaneous cholecystolithotomy**

This technique was based on experience gained by interventional radiologists in the emergency drainage of acutely inflamed gallbladders by cholecystostomy. The procedure is usually carried out under general anaesthesia. The gallbladder is punctured percutaneously, usually by the sub-hepatic route, under ultrasound and/or fluoroscopic control. A guidewire is then inserted into the gallbladder, and a track is serially dilated until it is wide enough to admit a 22 Fr Amplatz tube through which an endoscope is passed and the stones extracted under direct vision. Under favourable circumstances, the tract can be dilated in 20-30 min and, depending on their size and number, the stones removed over about the same time. The patients usually stay in hospital until the following day, with a self-retaining Foley catheter left in the gallbladder for a further 10-14 days to minimise the chances of a bile leak (Dowling 1992; Gillams *et al* 1992).

Since the stones are to be removed, rather than dissolved, they can be of any composition. The technique has had a particular role, therefore, in symptomatic patients with radio-opaque, non-dissolvable stones (those with attenuation scores of  $\geq 100$  HU) who are not suitable for the less invasive non-surgical modalities. However, the total in-patient time and cost compare unfavourably with that required for laparoscopic cholecystectomy, which has now largely displaced percutaneous cholecystolithotomy from the list of management options.

### **2.5 MANAGEMENT OF RECURRENT GALLSTONES**

The risk of developing recurrent gallstones is now well-documented after oral bile acid therapy and ESWL, although there are fewer data on gallstone recurrence after the more invasive techniques of MTBE or PCCL (Cheslyn-Curtis *et al* 1992; Pauletzki *et al* 1995). In general, when oral bile acid treatment is withdrawn after complete dissolution, gallstones recur at a rate of 10-15% per annum, reaching a cumulative actuarial plateau between 40% and 70% (mean around 50%) after 5-10 years (Dowling 1992). Gallstone recurrence after successful shock-wave lithotripsy and adjuvant oral bile acids is at least 7% at one year and increases to 30-69% after more than five years (Sackmann *et al* 1994; Cesmeli *et al* 1999; Carrilho-Ribeiro *et al* 2000). The lower

recurrence rate after ESWL reported in some studies is probably due to the fact that some 90% of patients selected for lithotripsy initially have solitary stones — the recurrence rate in such patients is approximately one-third of that in patients who, before treatment, had had multiple stones (Sackmann *et al* 1994; Petroni *et al* 2000)

The development of recurrent gallbladder stones, like that of primary gallstones, involves at least a triple defect: (i) cholesterol supersaturation, (ii) abnormally rapid nucleation in bile of cholesterol microcrystals, and (iii) impaired gallbladder emptying. After gallstones have been dissolved or cleared and bile acid treatment is withdrawn, the gallbladder bile reverts to being supersaturated with cholesterol in one to four weeks (Iser *et al* 1977). The gallbladder motor defect, which characterises untreated gallstone disease, also persists in most patients (Spengler *et al* 1989; Festi *et al* 1990).

After confirmed complete dissolution of primary cholesterol-rich gallstones, patients should normally undergo annual ultrasonography to exclude gallstone recurrence — at least for the first few years. Since calcification of primary cholesterol-rich gallstones is a function of both stone size and age, early detection of recurrent stones explains why, in most studies, even patients who originally had calcified gallstones develop small, cholesterol-rich stones on recurrence, which are usually readily dissolvable with oral bile acid therapy (Petroni *et al* 1991).

## **2.6 GALLSTONE PREVENTION**

When cholesterol-rich gallbladder stones have been dissolved completely and treatment is withdrawn, ultimately stones recur in 40-70% (mean approximately 50%) of patients (Lanzini *et al* 1986; Ruppini *et al* 1986; Villanova *et al* 1989; Cheslyn-Curtis *et al* 1992; Hood *et al* 1993). Gallbladder stone recurrence needs to be prevented if non-surgical methods of treating gallbladder stones are to become more established.

### **2.6.1 Diet**

The related factors of obesity, physical inactivity, hypertriglyceridaemia and low serum levels of high density lipoproteins are all associated with an increased risk of

gallstone disease. However, apart from general advice about exercising regularly and eating a diet naturally rich in fibre and low in sugar and fat, as recommended for the prevention of heart disease and cancer, consistent evidence for particular dietary factors in gallstone formation is lacking (Heaton 2000).

### 2.6.2 Ursodeoxycholic acid

The cost of UDCA means that long-term, full-dose treatment is not a practical long-term management option to prevent primary or recurrent gallstone formation. The results of most studies also suggest that low-dose oral bile acid treatment reduces, but does not completely prevent, the risk of gallstone formation or recurrence (Dowling *et al* 1994). <sup>However,</sup> In the controlled British-Belgian gallstone dissolution trial (Hood *et al* 1993), UDCA in one-third the full therapeutic dose (3 mg/kg/day) <sup>did not significantly reduce</sup> the rate of gallstone recurrence after successful dissolution. Short-term UDCA prophylaxis has been recommended in patients at high risk of gallstone formation, such as obese patients undergoing acute weight reduction as a result of gastroplasty or low (520 kcal/day) calorie dieting (Shiffman *et al* 1995; Sugerman *et al* 1995). In one study of 1004 obese subjects enrolled in a 16-week weight loss programme (Shiffman *et al* 1995), 28% of the placebo-treated group developed gallstones, compared with 8% of those treated with 150 mg UDCA twice daily ( $p < 0.001$ ). Moreover, this prevention of gallstone formation by UDCA was dose-dependent — only 3% of those randomised to 600 mg/day, and 2% of those taking 1200 mg/day, developed stones.

### 2.6.3 Aspirin and NSAIDs

Several lines of evidence suggest that aspirin and other NSAIDs can prevent experimental, primary and recurrent gallstone formation. Prairie-dogs fed a lithogenic diet (one which is rich in cholesterol and promotes stone formation) develop cholesterol supersaturation of gallbladder bile, cholesterol microcrystals and gallstones (Lee *et al* 1981b). Increases in gallbladder mucin glycoprotein synthesis occur before the formation of the crystals (Lee *et al* 1981b). Studies in the same model show that the increases in mucin secretion are themselves preceded by increases in mucosal

prostaglandin synthesis (LaMorte *et al* 1986). When aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) are given together with the lithogenic diet, some, but not all, studies (Table 2.2), suggest that they inhibit gallbladder mucosal prostaglandin synthesis and prevent mucin glycoprotein hypersecretion (which has been implicated both in nucleation (Levy *et al* 1984) and in the trapping of cholesterol microcrystals (Malet *et al* 1989)), as well as microcrystal nucleation and gallstone formation (Lee *et al* 1981a).

| Study population                                               | Drug<br>(70 kg equiv.)   | Treatment<br>(weeks) | End-points                                       | Result                 |
|----------------------------------------------------------------|--------------------------|----------------------|--------------------------------------------------|------------------------|
| 15 prairie dogs on lithogenic diet<br>(Lee <i>et al</i> 1981a) | Aspirin<br>7 g/d         | 2                    | Mucin glycoprotein conc<br>Gallstone formation   | Decreased<br>Decreased |
| 12 guinea pigs<br>(Brotschi <i>et al</i> 1984)                 | Indomethacin<br>350 mg/d | 6-12                 | Gallbladder volume<br>Gallstone formation        | Decreased<br>No change |
| 36 prairie dogs, hamsters<br>(Cohen <i>et al</i> 1991)         | Aspirin<br>7 g/d         | 2-4                  | Cholesterol formation<br>Gallstone formation     | No change<br>No change |
| 5 prairie dogs<br>(O'Leary <i>et al</i> 1991)                  | Indomethacin<br>84 mg/d  | 3                    | Mucin glycoprotein conc<br>Cholesterol crystals  | No change<br>No change |
| 28 prairie dogs<br>(Li <i>et al</i> 1994)                      | Aspirin<br>1 g/d         | 2                    | Gallbladder contractility<br>Gallstone formation | No change<br>No change |

Table 2.2 Animal studies of the effects of NSAIDs on bile physical chemistry, gallbladder contractility, cholesterol microcrystal nucleation and gallstone formation.

Studies of gallstone prevention in humans have largely supported the results in experimental animals, although the only placebo-controlled study of low-dose aspirin in preventing gallstone complications lacked sufficient power to detect small differences between the active treatment and placebo groups (Kurata *et al* 1991). In an early study of obese individuals during acute weight reduction (Broomfield *et al* 1988), the incidence of developing microcrystals or gallstones was significantly reduced in patients given high-dose aspirin (1300 mg/day). Similarly, in a retrospective study of 75 post-dissolution patients, Hood *et al* (1988b) showed that none of the 12 who regularly

took NSAIDS developed recurrent stones, while there were 20 recurrences in the 63 who had never, or only occasionally, taken NSAIDS. In 55 gallstone patients scheduled for elective cholecystectomy, Rhodes *et al* (1992) showed that pre-treatment for one week with 300 mg aspirin/day significantly reduced (by an average of 50%) the *in vitro* synthesis of mucin glycoprotein by strips of freshly excised gallbladders. In a more recent study of 47 obese patients taking very low calorie weight reduction diets (Marks *et al* 1996), ibuprofen in a dose of 1600 mg/day for 12 weeks countered the adverse effects of the diet on biliary cholesterol saturation, microcrystal nucleation and growth, and gallbladder contraction (Table 2.3).

Taken together, the results of these studies provide strong evidence that by inhibiting gallbladder mucosal prostaglandin synthesis, aspirin and other NSAIDs may reduce experimental, primary and recurrent gallstone formation. Aspirin and other NSAIDs may have additional effects on the gallbladder, such as improving gallbladder contractility (O'Donnell *et al* 1992), but whether they can prevent primary or recurrent gallstone formation in high risk individuals, remains unclear.

#### **2.6.4 Prokinetic agents**

In patients at risk of sludge and stone formation, such as those in intensive care or receiving total parenteral nutrition — prokinetic drugs such as cholecystokinin, cisapride and erythromycin can prevent the development of gallstones (Dowling *et al* 1997). These drugs stimulate gallbladder emptying, but they are also prokinetic to the intestine and may have an effect through decreased 7 $\alpha$ -dehydroxylation by colonic bacteria of cholic acid to form deoxycholate. Supportive evidence for this hypothesis comes from early studies of constipated people given laxatives, resulting in their bile becoming depleted of deoxycholic acid and less saturated with cholesterol (Marcus and Heaton 1986b). Similarly, in acromegalic patients on long-term octreotide, oral cisapride shortens large bowel transit times and reduces the proportion of serum (and presumably biliary) deoxycholic acid to normal (Veysey *et al* 2001b). However, as yet there have been no formal prospective studies of the efficacy of intestinal prokinetic agents in preventing cholesterol formation in high-risk groups.

| Study population                                                           | Drug                    | Treatment (wks) | End-points                                                                      | Result                              |
|----------------------------------------------------------------------------|-------------------------|-----------------|---------------------------------------------------------------------------------|-------------------------------------|
| 22 obese subjects on very low calorie diets (Broomfield <i>et al</i> 1988) | Aspirin<br>1300 mg/d    | 16              | Cholesterol saturation<br>Crystals or gallstones<br>Mucin glycoprotein conc     | No change<br>Decreased<br>Decreased |
| 12 patients post-stone dissolution (Hood <i>et al</i> 1988b)               | Any NSAID               | 130             | Gallstone recurrence (63 no NSAIDs)                                             | Decreased                           |
| 2267 patients post-AMI (Kurata <i>et al</i> 1991)                          | Aspirin<br>1 g/d        | 144             | Gallstone complications                                                         | No change                           |
| 10 obese subjects on very low calorie diets (Marks <i>et al</i> 1991)      | Aspirin<br>1300 mg/d    | 4               | Biliary deoxycholic acid<br>Biliary arachidonic acid<br>Mucin glycoprotein conc | No change<br>Decreased<br>No change |
| 7 normal subjects (Murray <i>et al</i> 1992b)                              | Indomethacin<br>125 mg  | 1 tablet        | Gallbladder contractility                                                       | No change                           |
| 7 gallstone patients (O'Donnell <i>et al</i> 1992)                         | Indomethacin<br>75 mg/d | 1               | Gallbladder contractility                                                       | Increased                           |
| 27 gallstone patients (Rhodes <i>et al</i> 1992)                           | Aspirin<br>300 mg/d     | 1               | Mucin glycoprotein conc<br>Glycoprotein synthesis                               | No change<br>Decreased              |
| 8 gallstone patients (von Ritter <i>et al</i> 1993)                        | Indomethacin<br>75 mg/d | 1               | Mucin glycoprotein conc<br>Bile viscosity                                       | No change<br>Decreased              |
| 45 patients stone-free after ESWL (Adamek <i>et al</i> 1994)               | Aspirin<br>100 mg/d     | 80              | Gallstone recurrence                                                            | No change                           |
| 20 healthy volunteers<br>30 gallstone patients (Das <i>et al</i> 1995)     | Aspirin<br>350 mg/d     | 2               | Gallbladder contractility                                                       | No change<br>Decreased              |
| 47 obese subjects on very low calorie diets (Marks <i>et al</i> 1996)      | Ibuprofen<br>1600 mg/d  | 12              | Cholesterol saturation<br>Microcrystal nucleation<br>Gallbladder contractility  | Decreased<br>Decreased<br>Increased |
| 25,584 individuals screened by US (Attili <i>et al</i> 1997)               | Any NSAID               | —               | Gallstone prevalence                                                            | No effect                           |
| 432 gallstone patients & controls (Pazzi <i>et al</i> 1998)                | Any NSAID               | —               | Gallstone prevalence                                                            | No effect                           |

Table 2.3 Clinical studies of the effects of NSAIDs on bile physical chemistry, gallbladder contractility, cholesterol microcrystal nucleation and gallstone formation.

***PART II. NON-SURGICAL  
TREATMENT OF GALLSTONES:  
CLINICAL STUDIES***

## **CHAPTER 3. PERCUTANEOUS CHOLECYSTOLITHOTOMY: RISKS, BENEFITS AND LONG-TERM OUTCOME**

### **3.1 INTRODUCTION**

Laparoscopic cholecystectomy is now the option of choice for the surgical management of symptomatic gallstone disease. Nonetheless, there remains a small number of patients who opt for non-surgical treatment of their gallbladder stones — usually with oral bile acids (May *et al* 1993) or extracorporeal shock wave lithotripsy (ESWL) with or without adjuvant oral bile acids (Sackmann *et al* 1991b).

The technique of percutaneous extraction of gallbladder stones was first described in 1985 by Akiyama *et al* (1985) and Kerlan *et al* (1985). In 1988, Kellett and colleagues (1988) reported their experience with PCCL performed as a single-stage procedure, using a transperitoneal approach and a modified percutaneous nephrolithotomy technique. Other centres have also shown that PCCL is safe and effective (Cheslyn-Curtis *et al* 1992), but there are few data on the long-term recurrence of gallbladder stones after PCCL.

The aim of the present study was to determine the efficacy, complications and frequency of gallstone recurrence after PCCL in 21 patients.

### **3.2 METHODS**

#### **3.2.1 Patients**

Over a five-year period, approximately 350 patients were referred to the Guy's Hospital Gallstone Clinic for non-surgical management of their gallbladder stones. Of these, only symptomatic patients with a patent cystic duct (opacification of the gallbladder during oral cholecystography) and cholesterol-rich stones (defined as CT scores < 100 Hounsfield Units; HU) were offered treatment with either oral bile acid therapy, ESWL with adjuvant oral bile acids, or methyl-*tert*-butyl ether (MTBE) (Dowling 1992). Symptomatic patients with a patent cystic duct and calcified (CT score  $\geq$  100 HU) stones were offered the choice of either PCCL or cholecystectomy.

Over a two-year period, 21 symptomatic patients with a median of five gallbladder stones (range, 1-200) in an opacifying gallbladder during oral cholecystography, declined elective cholecystectomy and opted for PCCL. There were 17 women and four men, with a mean age of 51 (range, 27-62) years. All 21 had CT-dense stones with maximum gallstone attenuation scores of 100-969 HU (median, 202 HU).

### 3.2.2 PCCL technique

The technique used was similar to that described by van Heerdan *et al* (1991) and Gillams *et al* (1992). In brief, under general anaesthesia, and using fluoroscopic  $\pm$  ultrasound guidance, the gallbladder was punctured with a 22-G spinal needle using the subhepatic transperitoneal approach, and opacified with radiographic contrast. The gallbladder was then re-punctured with an 18-G Kellett needle and a guide-wire was introduced, which remained coiled within the gallbladder throughout the procedure. The needle was removed, the tract dilated with Teflon or metal dilators to 24-30 F, and an Amplatz sheath of the same dimension inserted (Figure 3.1).

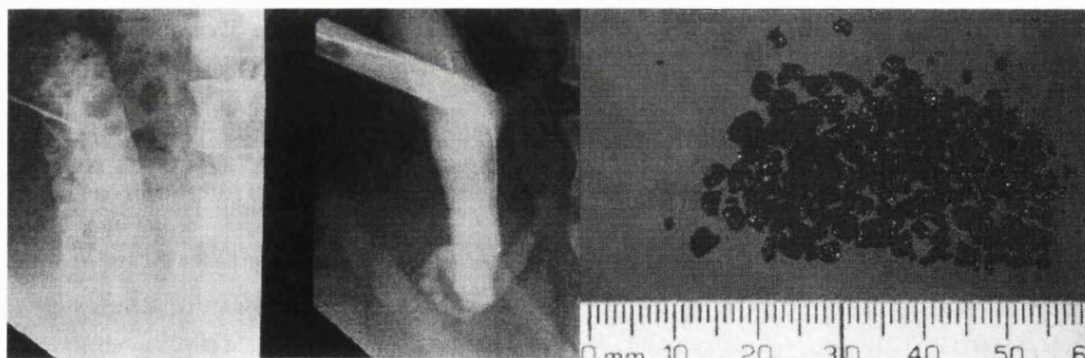


Figure 3.1 Multiple, radiolucent, pigment stones seen in an opacified gallbladder, before and after removal via an Amplatz sheath inserted into the gallbladder at the time of percutaneous cholecystolithotomy.

A rigid, 24-F side-viewing Wickham nephroscope (Wolf UK) was inserted through the Amplatz sheath, and the stones extracted under direct vision, using 'alligator' or grasping forceps (with or without prior electrohydraulic contact lithotripsy) or, in the case of small stones, flushed out by saline irrigation. When there were multiple stones,

a larger stone was temporarily wedged in the entrance to the cystic duct to act as a plug and prevent smaller stones being flushed into the duct. This larger stone was then removed after all the other stones had been extracted. Visual confirmation of complete stone extraction was improved later in the series by using a modified flexible cystoscope (Olympus UK).

A 14- or 16-F Foley catheter was then introduced into the gallbladder, and a catheter cholecystogram ('tubogram') performed to confirm complete stone removal, after which the guidewire was removed and the catheter balloon inflated under fluoroscopic control. Gentle traction was applied to the Foley catheter as the Amplatz sheath was removed, to minimise bile leakage from the puncture site.

Patients were usually discharged from hospital within 48 h of the procedure, to return 10-14 days later for repeat catheter cholangiography. If no gallstones were seen at that time, the Foley catheter was removed and the patient observed overnight to ensure that there was no evidence of a bile leak.

### **3.2.3 Patient follow-up**

After PCCL, patients were assessed clinically every 3-6 months, and with serial ultrasounds of the gallbladder annually, to exclude sludge or recurrent stone formation. If recurrent gallstones were detected, oral cholecystography (to assess patency of the cystic duct) and localised CT of the gallbladder (as a means of predicting stone composition and dissolvability (Walters *et al* 1992)) were performed before further treatment was considered.

## **3.3 RESULTS**

The short term outcome of PCCL in the 21 patients is summarised as a flow diagram in Figure 3.2.

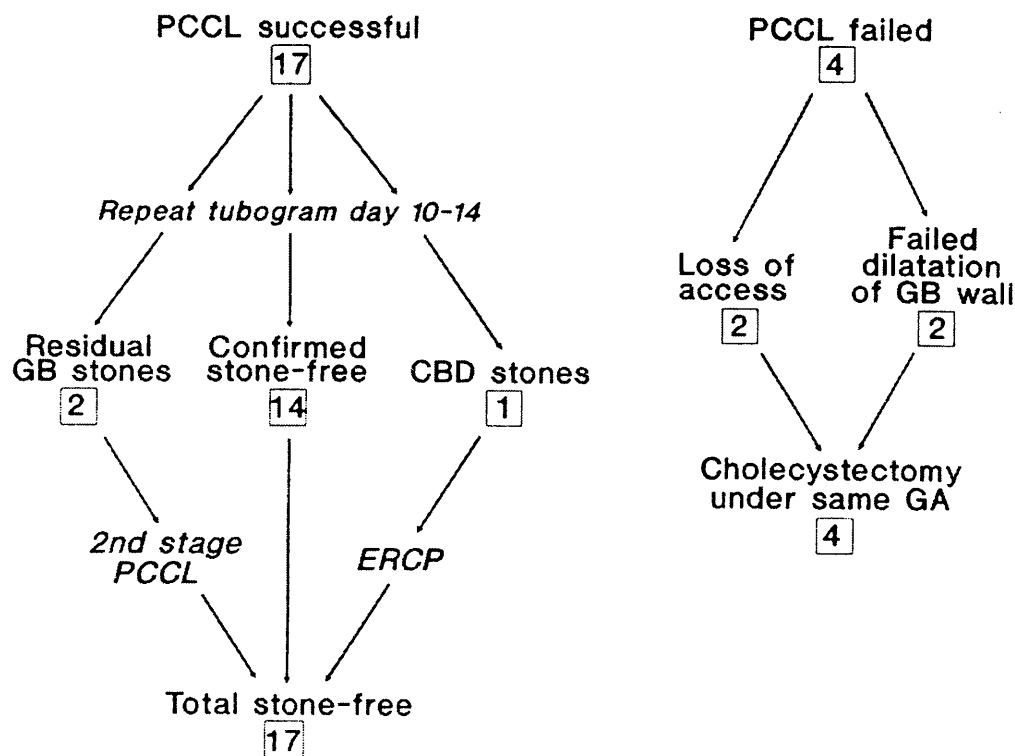


Figure 3.2 Short-term outcome in 21 patients undergoing PCCL. GB = gallbladder; ERCP = endoscopic retrograde cholangiopancreatography; GA = general anaesthesia; CBD = common bile duct.

The gallbladder was punctured in all 21 patients, but PCCL failed in four (19%) because the gallbladder wall could not be dilated ( $n = 2$ ) or because the tract became lost during dilatation ( $n = 2$ ). In all four the procedure was converted to open cholecystectomy under the same general anaesthetic. In the remaining 17, after PCCL and catheter drainage of the gallbladder for 10-14 days, a second catheter cholecystogram confirmed that the gallbladder was stone-free in 15 but showed retained gallbladder stones in two, both of whom underwent a successful second-stage PCCL. In one of the 15 patients, a 59-year-old woman, the tubogram revealed common bile duct stones. These had not been apparent at the time of gallbladder stone extraction 10 days previously, nor had they been predicted clinically, by ultrasonography or on the basis of liver function tests. She was treated successfully by endoscopic sphincterotomy

plus stone extraction. Thus, complete gallstone clearance was ultimately achieved in 17 patients (81%).

### **3.3.1 Procedure-related complications**

Three patients developed a transient (< 24 h) low-grade (< 38.0°C) fever within 48 h of the procedure, which responded to antibiotic therapy. Two other patients were found at intra-operative catheter cholecystography to have developed a minor leak of contrast material, but by ultrasonography there was no evidence of a subhepatic collection and they were discharged on the third postoperative day. In a further patient, a 28-year-old woman, the colon was inadvertently punctured and she developed a small cholecystocolonic fistula. She was treated in hospital with a low-residue diet and Foley catheter drainage of the gallbladder for two weeks, after which a repeat catheter cholecystogram showed spontaneous closure of the fistula.

### **3.3.2 Long-term follow-up**

After clearance, the 17 patients were followed up clinically, and with serial ultrasounds, for a median period of 35 (range, 4-62) months. Nine remained symptom-free and stone-free. However, in four biliary sludge was detected seven, 30, 32 and 35 months after PCCL. Initially, none of the four had any symptoms. In one, the sludge disappeared spontaneously four months after it was first detected. In a second, the sludge persisted for 23 months. In two others, 4-6 months after the sludge was first detected and 36-38 months after PCCL, multiple (3-10) small (2-4 mm) gallbladder stones were seen at ultrasonography. These two patients both developed biliary colic four and seven months after the recurrences were first diagnosed, and underwent laparoscopic cholecystectomy.

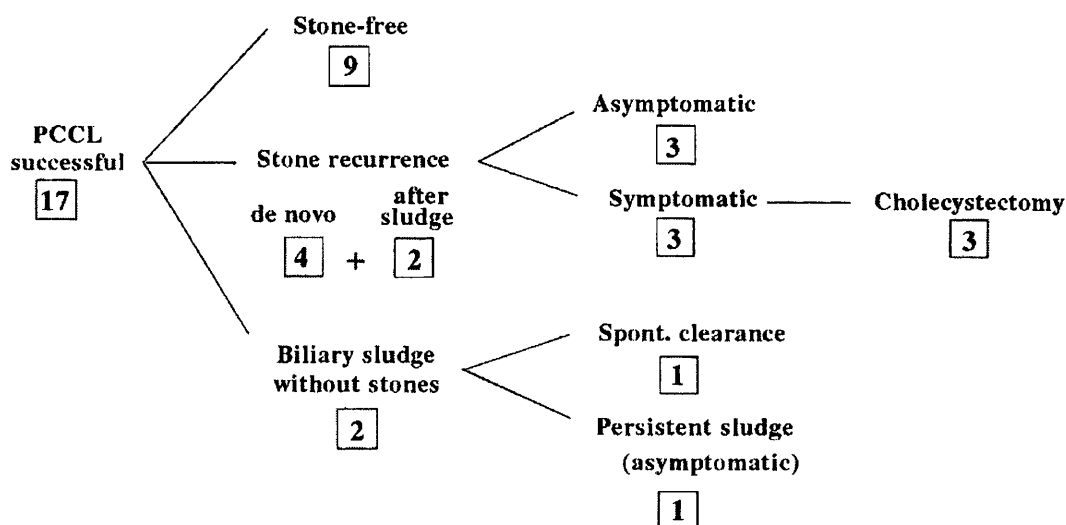


Figure 3.3 Long-term outcome in the 17 patients rendered stone-free by PCCL. Patients were followed up clinically, and by serial ultrasound, every 3-6 months for a median of 35 (range, 4-62) months. Biliary sludge was defined as gravitating, echogenic, but non-shadowing material seen at ultrasound.

In four other patients, gallstones recurred without evidence of preceding biliary sludge, at nine, 16, 19 and 27 months (symptomatic in one who underwent cholecystectomy three months after stone recurrence, and silent in three) (Figure 3.3). By actuarial (life-table) analysis, the gallstone recurrence rates were  $7.7 \pm \text{SEM } 7.4\%$  at 12 months,  $25.4 \pm 12.8\%$  at 24 and  $53.4 \pm 15.1\%$  at 36 months. The corresponding combined stone/sludge recurrence rates were  $15.4 \pm 10.0\%$ ,  $31.7 \pm 13.1\%$  and  $63.4 \pm 13.5\%$ , respectively (Figure 3.4).

### 3.3.3 Characteristics of recurrent gallstones

Initially, five of the 17 patients who underwent PCCL had had only one or two gallstones. All five remained stone-free after a median follow-up of 19 (range, 4-37) months. However, of the 12 patients who underwent PCCL for multiple stones, no less than six developed multiple recurrent stones. In these six, the recurrent gallstones were smaller in number (median 2, range 1-10) and size (2-6 mm) than the original stones

(median 10, range 4-30; size 5-14 mm) — probably because they were detected early as a result of regular surveillance.

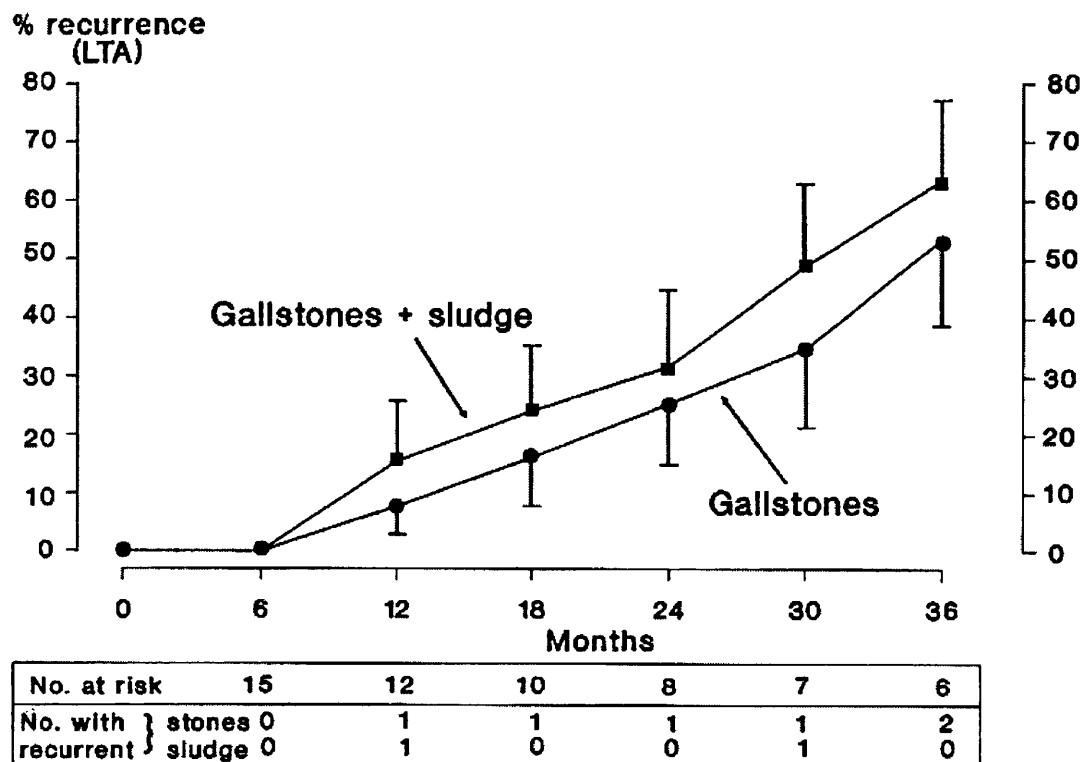


Figure 3.4 Actuarial or life-table analysis of gallstone, and combined stone and sludge, recurrence rates. Results are means ( $\pm$  SEMs). The number of patients 'at risk' and the number of recurrences of stones or sludge, and combined stone/sludge recurrence rate, are shown for each time period, below.

### 3.4 DISCUSSION

Before the advent of laparoscopic cholecystectomy, PCCL was developed for the management of patients with symptomatic non-dissolvable gallbladder stones who wished to retain their 'functioning' gallbladders and avoid the 5-20% morbidity associated with open cholecystectomy (Clavien *et al* 1992). Several reports suggest that, in patients with non-acute gallstone disease, complete stone clearance can be achieved by PCCL in over 90% of patients (Gibney *et al* 1989; Chiverton *et al* 1990; van Heerden *et al* 1991; Cheslyn-Curtis *et al* 1992; Clavien *et al* 1992; Picus *et al*

1992). In the same reports, complications of bile leak or subhepatic bile collections occurred in 3-7% (Chiverton *et al* 1990; Cheslyn-Curtis *et al* 1992; Picus *et al* 1992), and there have been at least two reported cases of inadvertent colonic puncture (Chiverton *et al* 1990; van Heerden *et al* 1991). All these complications were treated conservatively, and to date there have been no reported deaths after elective PCCL.

These figures accord with the present findings of an overall stone clearance rate of 81%, and a complication rate for bile leak or colonic puncture of 14%. Although this complication rate, and that of others (van Heerden *et al* 1991; Picus *et al* 1992), is similar to that reported for open cholecystectomy (Clavien *et al* 1992), it is higher than the reported morbidity after laparoscopic cholecystectomy (Deziel *et al* 1993).

As discussed below, there are advantages and disadvantages of leaving the gallbladder behind. One obvious disadvantage is the risk of stone recurrence. In patients with cholesterol-rich stones treated successfully with oral bile acid therapy, the risk of stone recurrence is approximately 10-15% per annum, rising to 50% at 5 years — after which it tends to plateau (Villanova *et al* 1989; Hood *et al* 1993). However, the risk of recurrence is up to three times higher in patients who initially had multiple stones, than in those who originally had solitary stones (Villanova *et al* 1989; Pelletier *et al* 1992; Hood *et al* 1993). Recurrence rates as low as 11-15% over three years have been reported in patients with solitary stones treated with oral bile acids (Villanova *et al* 1989; Hood *et al* 1993) or ESWL (Villanova *et al* 1989; Pelletier *et al* 1992; Hood *et al* 1993; Schneider *et al* 1993; Sackmann *et al* 1994).

Data on stone recurrence rates after PCCL are limited, but reported rates have ranged from 10-27% at 14-18 months (Gibney *et al* 1989; Cheslyn-Curtis *et al* 1992), to 31% at a mean follow-up of 26 months (Donald *et al* 1994). In the present series, with a median follow-up of 35 months, four patients developed biliary sludge, which is a precursor of gallstones in approximately 20% of patients (Lee *et al* 1988; Ko *et al* 1999). Two of these subsequently developed recurrent gallstones, while four others developed recurrent gallstones *de novo* — giving a combined recurrence rate of 35% and an actuarial rate of  $53.4 \pm 15.1\%$  at 36 months — rates similar to those reported after oral bile acid therapy in patients with multiple stones. In the present series, none of the

five patients who originally had only one or two stones developed recurrent stones or sludge after a median follow-up of 19 months.

The advantages of PCCL over laparoscopic cholecystectomy are that the patient is left with one small abdominal scar — as opposed to the four or five required for laparoscopic cholecystectomy — and he/she retains a functioning gallbladder (Dowling 1992). However, these advantages are marginal, particularly when the risk of gallstone recurrence suggested by the present results, and those of others (Gibney *et al* 1989), is taken into account. In specialised centres, PCCL remains a valid management for a selected minority of patients — generally those who are a high operative risk — but it has largely been replaced by laparoscopic cholecystectomy.

**CHAPTER 4. GALLSTONE DISSOLUTION WITH ORAL BILE ACID  
THERAPY: IMPORTANCE OF PRE-TREATMENT CT SCANNING AND  
REASONS FOR NON-RESPONSE**

#### **4.1 INTRODUCTION**

In symptomatic patients with cholesterol-rich gallbladder stones and a patent cystic duct, complete stone clearance rates of 65-90% may be achieved with oral bile acids — given alone (Strasberg and Clavien 1992; May *et al* 1993), or together with extracorporeal shock-wave lithotripsy (ESWL) (Ell *et al* 1991; Sackmann *et al* 1991b; Strasberg and Clavien 1992; Elewaut *et al* 1993; May *et al* 1993). Because calcium-containing gallstones respond poorly to treatment (radio-opaque stones contain significant amounts of calcium (Bell *et al* 1975; Trotman *et al* 1975), oral cholecystography has been used to screen patients before treatment. However, approximately 50% of stones that appear lucent by conventional radiology are visibly dense on computed tomography (CT) and are unlikely to dissolve completely with oral bile acids (Brakel *et al* 1990). Although there is controversy about the most appropriate cut-off point in the maximum gallstone attenuation score which should be used to exclude patients from dissolution therapy (Brakel *et al* 1990; Ell *et al* 1991; Caroli *et al* 1992; Walters *et al* 1992; Brink *et al* 1994; Petroni *et al* 1995), in our Unit CT attenuation values  $\geq 100$  Hounsfield Units (HU) predict calcium-containing, non-dissolvable stones (Walters *et al* 1992). However, even when the stones are lucent by conventional radiology and have CT scores of  $< 100$  HU, no/arrested gallstone dissolution (no cholecystosonographic response to the oral bile acids after one year or partial, but arrested, dissolution after two years of treatment) still occurs in up to 35% of patients (Caroli *et al* 1992; Walters *et al* 1992).

In patients selected for treatment using the optimal criteria of a patent cystic duct and a low gallstone CT attenuation score, the reasons for incomplete gallstone dissolution are poorly defined. Therefore, the aims of the present study were to determine whether the pre-treatment stone characteristics, including maximum gallstone CT attenuation scores, could predict the speed and completeness of

dissolution with oral bile acids  $\pm$  ESWL, and to assess, in those with no/arrested dissolution, the reasons for the poor response.

## 4.2 METHODS

Since 1986, localised CT scanning of the gallbladder has been performed routinely in all patients referred to the Guy's Hospital Gallstone Clinic for non-surgical treatment of their gallbladder stones. Patients were selected for treatment with oral bile acids (ursodeoxycholic acid alone in a dose of 10 mg/kg/day or the combination of ursodeoxycholic acid 5 mg/kg/day + chenodeoxycholic acid 7.5 mg/kg/day), with or without adjuvant lithotripsy, only if: (i) specific, gallstone-related symptoms (mainly biliary colic) were present, (ii) the cystic duct was patent (as judged usually by opacification of the gallbladder during oral cholecystography), (iii) the gallstones were radiolucent by conventional radiology (plain abdominal x-ray and oral cholecystography), and (iv) the stones had maximum CT attenuation scores of  $< 100$  HU (Walters *et al* 1992). After commencing treatment with oral bile acids  $\pm$  ESWL, gallstone dissolution was assessed every three to six months by ultrasonography. Confirmed complete gallstone dissolution was defined by two consecutive ultrasonographic examinations, at least one month apart, during continued oral bile acid therapy. If sludge was detected in the gallbladder, the patient was not regarded as being stone-free and oral bile acids were continued.

In those without confirmed complete gallstone dissolution, treatment was usually withdrawn: (i) at one year in patients who were compliant in taking their prescribed dose of oral bile acids but who showed no radiological evidence of gallstone dissolution, or (ii) after a total of two to three years of oral bile acids in those who had unequivocal evidence of partial gallstone dissolution (defined as a 50% decrease in gallstone number or volume, or both (Walters *et al* 1992)) at one year but who had not progressed to complete gallstone dissolution despite continued, full-dose oral bile acid therapy. To investigate the reasons for the non-response or arrested gallstone dissolution in these patients, our policy was to repeat the oral cholecystogram and CT and, in selected patients, to recommend ultrasound-guided, percutaneous, transhepatic,

fine-needle aspiration of gallbladder bile (Swobodnik *et al* 1991a; Hussaini *et al* 1995c) for assessment of bile acid composition and bile physical chemistry.

#### **4.2.1 Patient selection**

Two groups of patients were studied: (i) a stone-free group of 43 patients (33 women, 10 men; mean age 47, range 19-78 years) who had become stone-free after 4-27 (median 9) months of oral bile acids — alone (n = 18) or with ESWL (n = 25), and (ii) a no/arrested dissolution group of 43 age- and sex-matched patients (33 women, 10 men; mean age 49, range 21-73 years) whose stones had not dissolved despite 12-36 (median 22) months of oral bile acids alone (n = 23) or with adjuvant lithotripsy (n = 20).

In these two groups, pre-treatment gallstone characteristics were compared as follows. Ultrasound and oral cholecystogram films (including the preliminary pre-contrast x-ray) were reviewed ‘blindly’ by a senior radiologist (Dr Colette Kennedy) to assess the number and size of the gallbladder stones and their x-ray lucency, and to determine whether or not the cystic duct was patent. Gallstone composition was estimated indirectly by stone attenuation value at localised CT of the gallbladder (5 mm contiguous slices). A CT appearance of the stones was also assigned, as follows: (i) dense, homogenous calcification, (ii) faintly homogenous calcification, (iii) rim calcification, and (iv) isodense with bile (not visible on contiguous 5 mm CT slices). If the gallstones were isodense with bile, the highest HU reading for bile was taken as the representative value for the stones (Brink *et al* 1994; Petroni *et al* 1995).

In the present study, patients were selected for treatment if the pre-treatment CT attenuation scores of the stones were < 100 HU (Walters *et al* 1992). In order to determine whether or not this cut-off point was too high a threshold, the efficacy of oral bile acid therapy was compared in patients with pre-treatment gallstone CT attenuation scores of < 25 HU, 25-49 HU, 50-74 HU, and 75-99 HU, respectively.

#### **4.2.2 Assessment of patients with no/arrested gallstone dissolution**

In the patients whose gallstones remained incompletely dissolved despite 12-36 months oral bile acids  $\pm$  ESWL, oral cholecystography and localised CT scan of the gallbladder were repeated, in an attempt to determine the reasons for no/arrested dissolution. The development of a blocked cystic duct or poor gallbladder contractility was assessed by repeat oral cholecystography with fatty meal stimulation. The presence of radio- and CT-lucent but presumed non-cholesterol stones, or the development of acquired calcification during treatment, was evaluated by oral cholecystography and CT. The prescription of a suboptimal bile acid dose (although in the present study, the doses of UDCA and CDCA were prescribed according to body weight), or poor compliance in taking the prescribed oral bile acids, was documented by review of the case notes.

### **4.3 RESULTS**

#### **4.3.1 Pre-treatment gallstone characteristics**

The demographic data and gallstone characteristics of the patients in the stone-free and no/arrested dissolution groups, are given in Table 4.1.

|                                         | <b>Stone-free<br/>(n = 43)</b> | <b>No/arrested dissolution<br/>(n = 43)</b> |
|-----------------------------------------|--------------------------------|---------------------------------------------|
| <b><u>Patient characteristics</u></b>   |                                |                                             |
| Sex                                     | 33F, 10M                       | 33F, 10M                                    |
| Age (range) (yr)                        | 47 (19-78)                     | 49 (21-73)                                  |
| Weight (kg)                             | 69                             | 74                                          |
| <b><u>Stone characteristics</u></b>     |                                |                                             |
| Median stone no.                        | 3                              | 3                                           |
| Max stone size (mm)                     | 9 (3-35)                       | 10 (3-32)                                   |
| X-ray lucency <sup>Median (range)</sup> | n = 43                         | n = 43                                      |
| Mean CT score (HU)                      | 35 (10-76)                     | 39 (4-93)                                   |

Table 4.1 Pre-treatment patient and gallstone characteristics in the stone-free and no/arrested dissolution groups.

Before treatment, all 86 patients had patent cystic ducts, gallbladder stones that appeared lucent by both abdominal x-ray and oral cholecystogram, and maximum CT attenuation scores of < 100 HU. There were no significant differences between the stone-free and no/arrested dissolution groups in initial gallstone number, the diameter of the largest stone or in maximum CT attenuation score.

#### 4.3.2 The value of pre-treatment CT in predicting dissolution response

The efficacy of oral bile acid therapy as a function of the pre-treatment CT attenuation score is given in Table 4.2. In the first three subgroups (< 25 HU, 25-49 HU, 50-74 HU), the numbers of patients in the stone-free and incomplete dissolution groups were similar. However, of the nine patients with pre-treatment scores of > 75 HU, only one (11%) became stone-free, compared with 42 of 77 (55%) with scores of < 75 HU ( $p < 0.01$ ). In fact, in the one patient with a pre-treatment gallstone attenuation score of the stones of > 75 HU who became stone-free, the actual value was 76 HU.

|           | Stone-free | No/arrested dissolution |
|-----------|------------|-------------------------|
| < 25 HU   | 15         | 12                      |
| 25-49 HU  | 20         | 15                      |
| 50-74 HU  | 7          | 8                       |
| 75-99 HU* | 1          | 8                       |

Table 4.2 Gallstone dissolution with oral bile acids  $\pm$  ESWL as a function of the pretreatment CT attenuation score. \* $p < 0.01$  v combined data of the other three subgroups.

The visual appearance of the stones on the pre-treatment CT films also predicted the dissolution response. Thus, 79% of the 59 patients whose stones were isodense with gallbladder bile became stone-free, compared with 58% of the 27 patients whose stones were visible on the CT scans ( $\chi^2$ :  $p = 0.06$ ). Of the 43 patients in the stone-free group, nine (21%) had stones that were visible at CT (faint homogenous calcification in two and rim calcification in seven), compared with 19 (44%) in the no/arrested dissolution group (dense homogenous calcification in two, faint homogenous calcification in 11, and rim calcification in six) ( $\chi^2 = 4.3$ ,  $p < 0.05$ ).

In the 43 patients who became stone-free, the pre-treatment CT scores also predicted the speed of dissolution — the median times to complete gallstone disappearance being seven months in those with scores of  $< 25$  HU, nine months with scores of 25-49 HU and 13 months with scores of 50-76 HU (Figure 4.1). Moreover, in patients with stones that were isodense with bile before treatment ( $n = 34$ ), the median time to confirmed complete gallstone dissolution was only 10 months, compared with 15 months in those whose stones were visible on the pre-treatment CT scans ( $n = 9$ ).

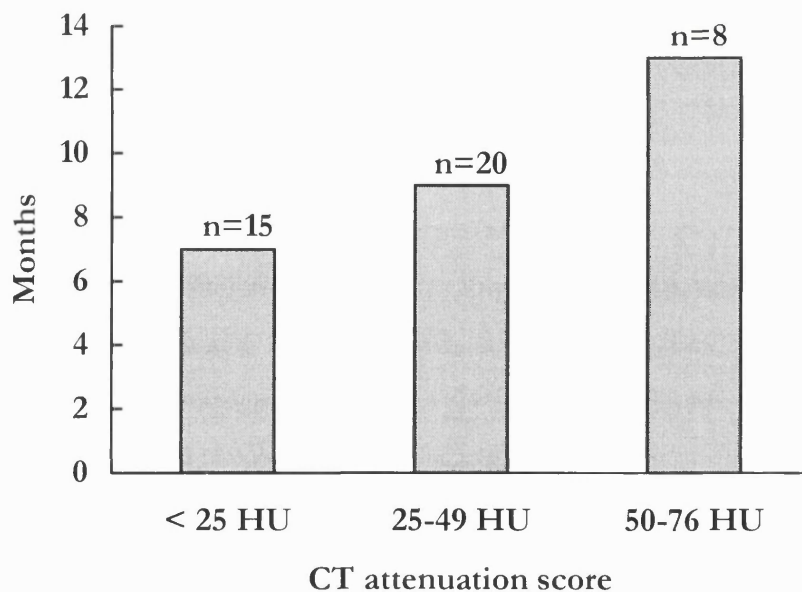


Figure 4.1 Mean time (in months) to confirmed complete gallstone dissolution, by pre-treatment CT attenuation score.

### 4.3.3 Reasons for no/arrested gallstone dissolution

In the 43 patients with no/arrested dissolution, repeat oral cholecystography at 12-36 months revealed that all but one patient still had stones which were lucent by conventional radiology, and that gallbladder emptying had become markedly impaired in 16 (37%). The contractile response of the gallbladder to a fatty meal was classified as poor (ejection fraction < 25%) in 11 patients, while the gallbladder did not opacify in five, indicating a blocked cystic duct. Three of these five had had a poor contractile response to a fatty meal during the pre-treatment cholecystogram. (Figure 4.2).

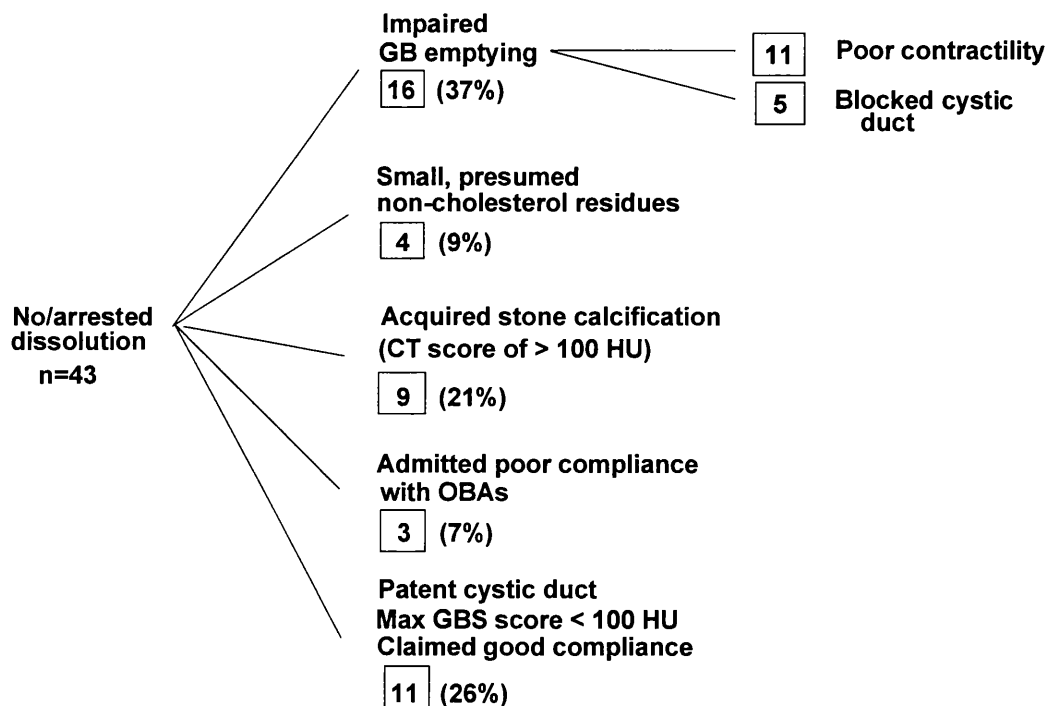


Figure 4.2 Reasons for no/arrested gallstone dissolution in 43 patients.

After treatment, four of the 43 patients (9%) were left with multiple, small (2-5 mm), radio- and CT-lucent, presumed non-cholesterol residues, while nine (21%) developed acquired stone calcification. By virtue of the selection criteria, before treatment all patients had maximum gallstone attenuation scores of < 100 HU, with a median of 65 HU (range, 15-93 HU). However, after 12-29 (median 24) months treatment with UDCA ± CDCA, the median score had increased to 132 HU (range,

100-446 HU). Only one of these patients (a 55-year-old woman with a maximum gallstone attenuation score of 446 HU) had stones that were radio-opaque by conventional radiology (Figure 4.3).

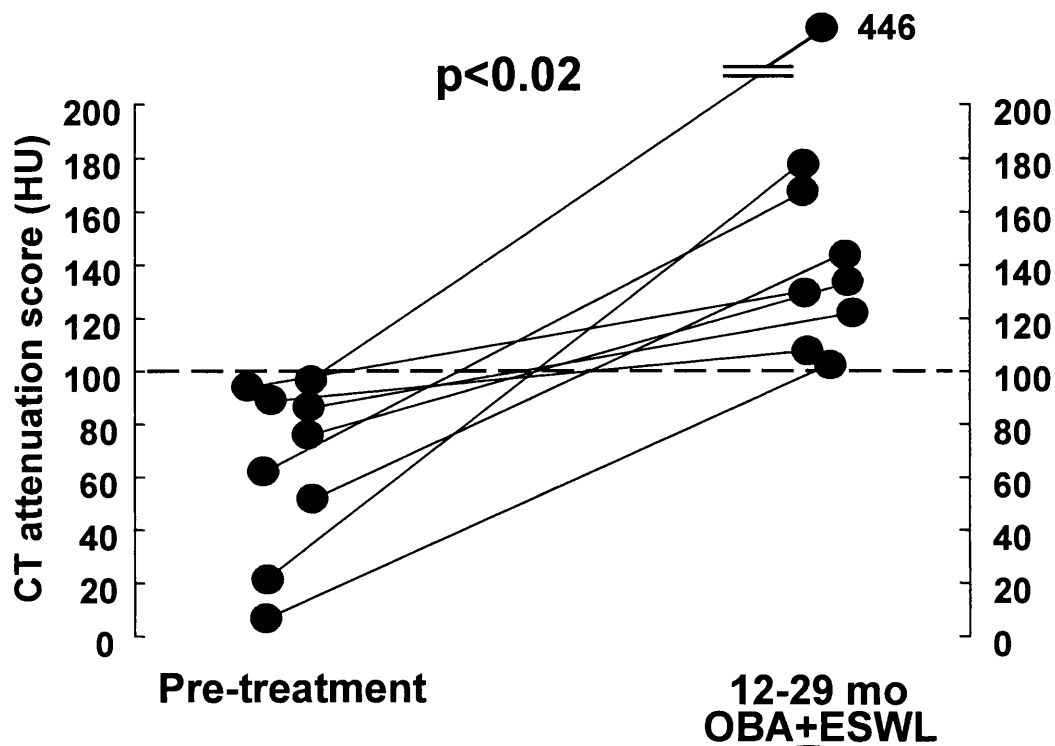


Figure 4.3 CT attenuation scores of gallstones, measured before and during 12-29 months oral bile acid therapy, in nine patients with no/arrested gallstone dissolution associated with acquired stone calcification.

#### 4.4 DISCUSSION

Oral bile acid dissolution therapy, with or without adjuvant extracorporeal shock-wave lithotripsy, is a safe and moderately effective treatment for selected patients with symptomatic cholesterol gallstone disease. Although the reported dissolution rates for gallstones vary widely, approximately 60% of patients with radiolucent stones and a patent cystic duct will progress to complete gallstone dissolution during oral bile acids, given alone (May *et al* 1993), or together with ESWL (Strasberg and Clavien 1992). The use of pre-treatment CT screening of the stones results in higher rates of confirmed

complete gallstone dissolution by oral bile acids  $\pm$  ESWL (Ell *et al* 1991; Caroli *et al* 1992; Walters *et al* 1992; Petroni *et al* 1995), but there is a need to refine selection criteria so that dissolution rates can be further improved.

In previous work from our Unit, Walters *et al* (1992) reported that patients with a pre-treatment maximum gallstone CT attenuation score of  $< 100$  HU had significantly better dissolution rates (50% confirmed complete gallstone dissolution at one year) than those not screened in this way (12% dissolution at one year). These results were similar to those of a subsequent study by Caroli *et al* (1992), who used a lower maximum gallstone attenuation score cutoff of 60 HU but also reported complete dissolution rates at one year of 50%. The results of the present study extend these earlier findings by showing that, in patients selected for treatment by a stone CT attenuation score of  $< 100$  HU, gallstones which are isodense with bile and/or have CT scores of  $< 75$  HU, dissolve more readily than those with scores of 75-99 HU. Furthermore, the lower the CT score, the faster the rate of gallstone dissolution.

A novel finding of the present study was that over two-thirds of the 86 patients with gallstone attenuation scores of  $< 100$  HU, had stones which were not visible on CT scanning of the gallbladder. Therefore, the stones were considered to be isodense with bile, with representative CT scores of the bile ranging from 2-44 (median 20) HU. One possible explanation for the high proportion of isodense gallstones is that they were actually missed at CT. However, this is unlikely since 5-mm contiguous CT 'slices' of the gallbladder were performed and the median size of the stones was  $> 9$  mm. Furthermore, of the 29 patients who had stones  $< 5$  mm in diameter, 25 (86%) had multiple stones. Although the two-thirds prevalence of isodense stones is higher than the 14-57% reported by others (Baron *et al* 1988; Bova *et al* 1992; Marzio *et al* 1992), in the present study the patients were selected for oral bile acid therapy if the CT attenuation value of the stones was  $< 100$  HU. Thus, patients with gallstones which contained significant amounts of calcium (CT scores  $\geq 100$  HU (Walters *et al* 1992)) were excluded from dissolution treatment. The pre-treatment CT score also predicted the speed of gallstone dissolution during oral bile acid therapy — the times to confirmed complete gallstone dissolution being shortest in those patients with low stone attenuation scores, or whose stones were isodense at CT. In other words, the

lower the calcium content of the stones, assessed indirectly by CT, the faster the dissolution response. Indeed, there is a strong correlation between gallstone CT attenuation scores *in vivo* and cholesterol/calcium carbonate composition of the stones determined by chemical analysis (Baron *et al* 1988; Brakel *et al* 1990; Bova *et al* 1992; Marzio *et al* 1992).

Irrespective of the selection criteria, it is likely that the efficacy of oral bile acid treatment in achieving a goal of a symptom-free, stone-free patient, will never be 100%. In the present study, over a third of the 43 patients with no/arrested dissolution developed either a blocked cystic duct or markedly impaired gallbladder emptying. Before commencing oral bile acids, the cystic duct had been patent in all patients but three had had a poor contractile response of the gallbladder to fatty meal stimulation — all three of whom later developed a blocked cystic duct during treatment. In an earlier study of 126 patients treated with UDCA alone from our Unit (Gleeson *et al* 1990), cystic duct obstruction developed in approximately 20% (by life-table analysis) of patients after four years of treatment. Oral bile therapy itself is unlikely to be responsible for the development of cystic duct obstruction. The results of the American National Cooperative Gallstone Study (NCGS) have shown that the incidence of acquired cystic duct obstruction during oral bile acid therapy is not different from that in a matched control group (Schoenfield *et al* 1981).

The present results also suggest that the reduced gallbladder emptying in response to a fatty meal seen in some patients with gallstones (Rothstein *et al* 1993; Sackmann *et al* 1993; Portincasa *et al* 1994), may become further impaired during oral bile acid therapy (Forgacs *et al* 1984; Festi *et al* 1990; Rothstein *et al* 1993). Increases in the fasting gallbladder volume and postprandial residual volume during UDCA therapy have also been reported (Forgacs *et al* 1984; Festi *et al* 1990; Rothstein *et al* 1993; Pauletzki *et al* 1994). The mechanism whereby UDCA affects gallbladder emptying is unknown, but it is possible that oral bile acid treatment itself may be a factor in the development of incomplete gallstone dissolution, as well as the reported reduction in the frequency and/or severity of biliary colic noted during therapy (Forgacs *et al* 1984; Janowitz *et al* 1991).

A further finding of this study was that 21% of patients with no/arrested dissolution had evidence of acquired stone calcification, with a significant increase in the median CT attenuation score from 65 HU before treatment, to 132 HU after a median of 22 months oral bile acid therapy. This frequency of acquired stone calcification is higher than the 9-15% incidence reported in most (Bateson 1981; Tint *et al* 1982; Lirussi *et al* 1993), but not all (Gleeson *et al* 1990), earlier studies — although previous workers have employed oral cholecystography, rather than the more sensitive technique of CT (Baron *et al* 1988; Brakel *et al* 1990; Marzio *et al* 1992; Brink *et al* 1994), to assess the presence of acquired stone calcification. One explanation for acquired stone calcification may be the bicarbonate-rich choleresis induced by UDCA administration, resulting in the precipitation of  $\text{CaCO}_3$  in bile (Hofmann 1994). However, acquired gallstone calcification has also been reported in CDCA-treated patients (Whiting *et al* 1980; Schoenfield *et al* 1981; Bazzoli *et al* 1995), and to occur spontaneously (Schoenfield *et al* 1981; Freilich *et al* 1986). Moreover, spontaneous stone calcification is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (Bell *et al* 1975; Lirussi *et al* 1993; Plaisier *et al* 1994a; Bazzoli *et al* 1995). Therefore, it remains unclear whether or not UDCA itself is responsible for the development of acquired stone calcification.

In conclusion, cholesterol-rich gallstones which are isodense with bile and/or have CT attenuation values of  $< 75$  HU, dissolve more readily and at a faster rate than stones with high CT attenuation scores. Moreover, the lower the pre-treatment CT score the more rapid the rate of gallstone dissolution. In those patients whose stones do not dissolve completely despite up to two years of oral bile acid therapy, the combination of oral cholecystography and CT will determine the underlying reason for no/arrested stone dissolution in most, but not all, cases — the main causes for failure being impaired gallbladder contractility and acquired stone calcification.

## **CHAPTER 5. GALLSTONE RECURRENCE AFTER MEDICAL TREATMENT: DO GALLSTONES RECUR TRUE TO TYPE?**

### **5.1 INTRODUCTION**

Before treatment, primary gallbladder stones may be: (i) solitary or multiple, and (ii) radiolucent or radio-opaque (Plaisier *et al* 1994b). Radiolucent stones are usually, but not always, cholesterol-rich. Thus, 14-20% of stones that appear lucent by conventional radiology are non-cholesterol in type (Bell *et al* 1975; Trotman *et al* 1975; Brink *et al* 1994), while approximately 50% of stones which are lucent by conventional radiology, appear dense by computed tomography (CT) of the gallbladder (Brakel *et al* 1990; Janowitz *et al* 1990; Walters *et al* 1992). The maximum gallstone attenuation score, measured by CT *in vivo*, predicts stone composition and dissolvability (Caroli *et al* 1992; Walters *et al* 1992). Therefore, CT scanning can be used to select patients for oral dissolution treatment. Although there is controversy about the most appropriate cut-off point in the maximum gallstone attenuation score which should be used to exclude patients from dissolution therapy (Brakel *et al* 1990; Ell *et al* 1991), we (Pereira *et al* 1997) and others (Ell *et al* 1991; Caroli *et al* 1992) have shown that values of  $\geq 75$ -100 Hounsfield Units (HU) predict calcium-containing, non-dissolvable stones.

While there have been many studies of primary gallstone composition, there are few data on the physical characteristics of recurrent stones, or their response to medical dissolution therapy (Pelletier *et al* 1992; Schneider *et al* 1993; Sackmann *et al* 1994). The aims of this study, therefore, were to determine whether gallstones recur 'true-to-type' — in terms of composition and stone number — and to assess the dissolvability of the recurrent stones with oral bile acid therapy.

### **5.2 METHODS**

Since 1986, all patients referred to the Guy's Hospital Gallstone Clinic for non-surgical treatment of their gallbladder stones have undergone localised CT scanning of the gallbladder, in addition to ultrasonography and oral cholecystography.

Data on gallstone characteristics were collected in patients who had developed stone recurrence after: (i) confirmed complete gallstone dissolution with oral bile acids, extracorporeal shock-wave lithotripsy (ESWL) + adjuvant oral bile acids, or contact dissolution with methyl *tert*-butyl ether (MTBE), or (ii) complete stone removal by percutaneous cholecystolithomy (PCCL). Only patients with well-characterised (ultrasonography, oral cholecystography and localised CT of the gallbladder) primary and recurrent gallstones, were included.

Ultrasound was used to assess the number and size of both the primary and the recurrent gallstones. Patency or blockage of the cystic duct was assessed by opacification of the gallbladder during oral cholecystography. All x-ray films were also reviewed 'blindly' by a senior radiologist (Dr Colette Kennedy), to assess radiolucency of the stones and the contractile response of the gallbladder to fatty meal stimulation. Localised CT scanning of the gallbladder (5 mm contiguous slices) was used to estimate gallstone composition indirectly, by measuring the stone attenuation scores in HU. When the gallstones were isodense with bile, the highest HU reading for bile was taken as the representative value for the stones.

### **5.2.1 Gallstone dissolution/removal and recurrence**

For patients who were treated with oral bile acids (Gleeson *et al* 1990) or ESWL + oral bile acids (Hood *et al* 1988a), the gallstone dissolution response was assessed every three to six months by ultrasonography. Confirmed complete stone dissolution was accepted only if there were two normal cholecystosonograms, at least one month apart, during continued oral bile acid treatment. If sludge was detected in the gallbladder, the patient was not regarded as being stone-free and oral bile acid therapy was continued.

In patients treated with MTBE, gallstone dissolution was assessed by catheter cholecystography during therapy, and by an ultrasound performed the next day. If residual stones or biliary sludge were detected, adjuvant oral bile acid therapy was commenced, and continued until complete gallstone dissolution was achieved.

For patients treated with PCCL (Pereira *et al* 1995b), complete gallstone clearance was assessed by catheter cholecystography immediately after the procedure, and confirmed by a repeat 'tubogram' 10-14 days later.

After confirmed gallstone dissolution or removal, all patients were followed up clinically, and with serial ultrasounds, at least once every 12 months. If recurrent stones were detected, a repeat oral cholecystogram and localised CT scan of the gallbladder were performed, to assess patency of the cystic duct and stone composition, before considering further treatment.

### **5.2.2 Statistical analysis**

Data processing was performed using a Paradox database (Borland International, Scotts Valley, California) and CRISP (CRunch Interactive Statistical Package; Crunch Software Corporation, San Francisco, California). A two-tailed *t* test was used for continuous variables, the  $\chi^2$  test for proportions, and the Mann-Whitney test for discontinuous variables.

## **5.3 RESULTS**

### **5.3.1 Pre-treatment gallstone characteristics**

Twenty-one patients who had developed stone recurrence after confirmed complete gallstone dissolution/removal, and who had well-characterised primary and recurrent stones, were studied. There were 18 women and three men, with a mean age of 49 (range, 21-69) years.

The characteristics of the primary (pre-treatment) gallstones are given in Table 5.1. As a result of selection, all 15 patients treated with one of the dissolution approaches had stones that were radiolucent by plain abdominal x-ray and oral cholecystography, and had maximum CT attenuation values of < 100 HU.

In the four patients treated with oral bile acids, complete gallstone dissolution was confirmed by ultrasonography after a mean of 16 (range, 13-20) months treatment. In

the eight patients treated with lithotripsy plus adjuvant oral bile acids, stone fragmentation (maximum diameter of fragments < 10 mm) occurred after one to four (mean two) sessions of ESWL. Complete gallstone dissolution/clearance of the resultant fragments was achieved with adjuvant oral bile acids given for 12-23 (mean 17) months. In the three patients treated with MTBE, the stones did not dissolve completely with the contact solvent and all three required adjuvant oral bile acids for 4-17 (mean 9) months before complete clearance of the stones was confirmed.

The six patients who underwent PCCL all had stones with CT attenuation scores  $\geq$  100 HU. Therefore, by our criteria, none of these patients was eligible for any of the dissolution approaches. By conventional radiography, one of the six patients had radiolucent stones, a second had rim/surface stone calcification, while the remaining four had radio-opaque stones. Following PCCL, intra-operative catheter cholecystography showed that complete gallstone clearance had been achieved in all patients, and this was confirmed by the second 'tubogram' 10-14 days later.

|                                | OBA<br>(n = 4) | ESWL+OBA<br>(n = 8) | MTBE<br>(n = 3) | PCCL<br>(n = 6)  | Total<br>(n = 21) |
|--------------------------------|----------------|---------------------|-----------------|------------------|-------------------|
| Patients with:                 |                |                     |                 |                  |                   |
| Single stones                  | 1              | 4                   | 0               | 0                | 5 (24%)           |
| Multiple stones                | 3              | 4                   | 3               | 6                | 16 (76%)          |
| Stone characteristics:         |                |                     |                 |                  |                   |
| Median diameter<br>(mm; range) | 5<br>(4-6)     | 20<br>(5-22)        | 20<br>(4-27)    | 9<br>(6-18)      | 11<br>(4-27)      |
| X-ray-lucent                   | 4              | 8                   | 3               | 2                | 17 (81%)          |
| Median CT score<br>(HU; range) | 41<br>(32-84)  | 33<br>(10-79)       | 17<br>(10-50)   | 351<br>(100-969) |                   |

Table 5.1      Characteristics of the primary gallstones by the four treatment modalities. OBAs = oral bile acids.

### 5.3.2 Recurrent stone number and size

In the 21 patients, recurrent gallbladder stones were detected 5-74 months (mean 26  $\pm$  SEM 4 months) after confirmed complete stone dissolution/removal. Of the 16 patients who originally had had multiple stones, 11 developed multiple recurrent stones, while five had solitary recurrent stones. Of the five patients with solitary primary gallstones, all five developed multiple recurrent stones (Figure 5.1).

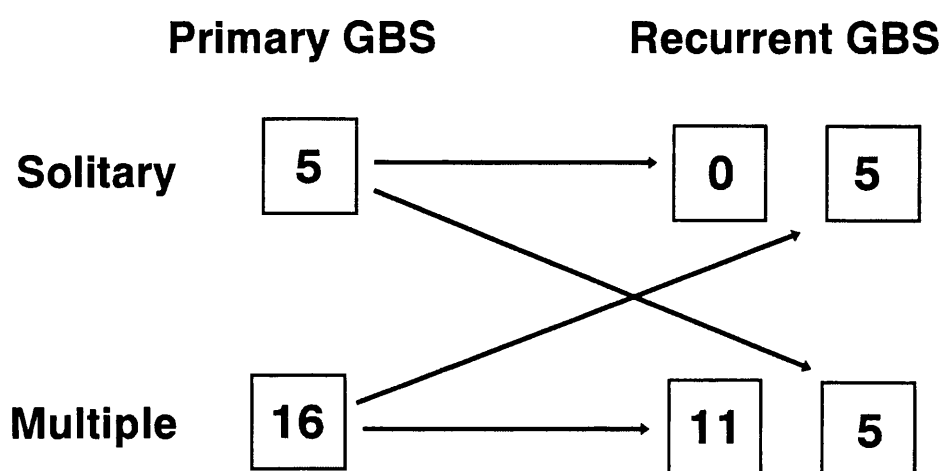


Figure 5.1 Comparison of primary and recurrent gallstones by stone number.

In the 21 patients, the size of the recurrent stones (median four mm, range 2-11 mm) was smaller ( $n = 17$ ), or the same as ( $n = 4$ ), that of the primary stones (median nine, range 4-27 mm) — probably because, as a result of regular surveillance, the recurrent stones were detected at an early stage of their development.

### 5.3.3 Indirect assessment of gallstone composition

In the 15 patients who underwent dissolution treatment, the mean attenuation score of the primary stones was 45 (range, 10-84) HU, while that of the recurrent stones was 62 HU (NS). In 13 of these 15 patients, the recurrent stones were again CT-lucent, with maximum scores of 6-88 HU, but two developed stones with scores of  $\geq 100$  HU (118

and 176 HU) — suggesting that the recurrent stones contained at least some calcium salts (Figure 5.2).

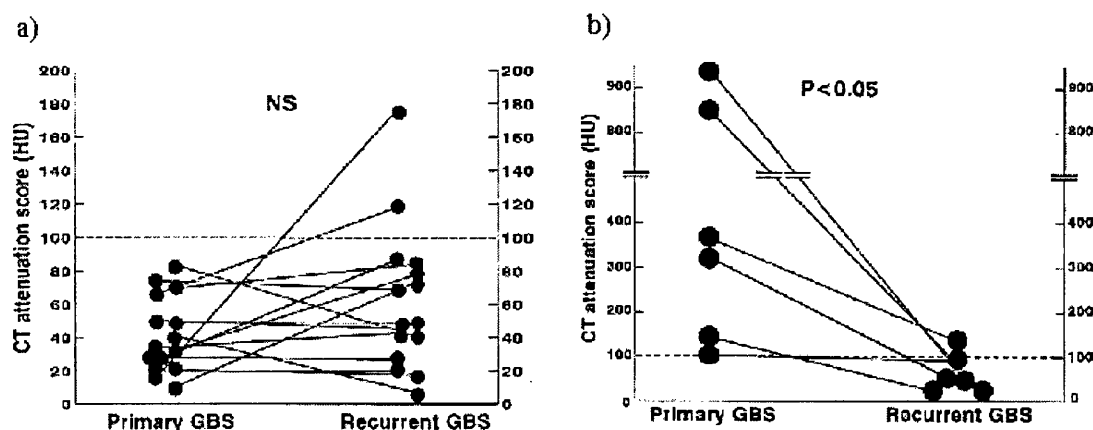


Figure 5.2 CT attenuation scores of primary and recurrent stones, in patients treated with a) medical dissolution therapy, or b) PCCL. Stone composition was assessed indirectly from the maximum Hounsfield Unit (HU) score by localised CT of the gallbladder: A CT score of < 100 HU suggested the stones were cholesterol-rich, while a score of  $\geq$  100 HU was indicative of calcified stones.

By plain abdominal x-ray and oral cholecystography, none of the primary or recurrent stones in the 15 patients was radio-opaque, although one patient developed a solitary recurrent stone with faint rim calcification. Both before treatment and after recurrence, the cystic duct remained patent in all patients, although in one patient with recurrent stones, the gallbladder opacified only faintly during oral cholecystography.

In the six patients who developed gallstone recurrence after PCCL, oral cholecystography again confirmed that the cystic duct remained patent in every case and showed no evidence of calcification in the recurrent stones. By CT, however, one of the six had calcium-containing recurrent stones with a maximum HU score of 140, while a second had stones with a score of 98 HU — close to our arbitrary cut-off point of 100 HU. In the other four patients, the recurrent stones were isodense with gallbladder bile, which had representative values of 16-50 HU, again suggesting that the recurrent stones were cholesterol-rich (Figure 5.2).

### 5.3.4 Treatment of the recurrent stones

The management of the patients with recurrent gallstones, and their response to oral bile acid treatment ( $\pm$  ESWL), is summarised as a flow diagram in Figure 5.3.

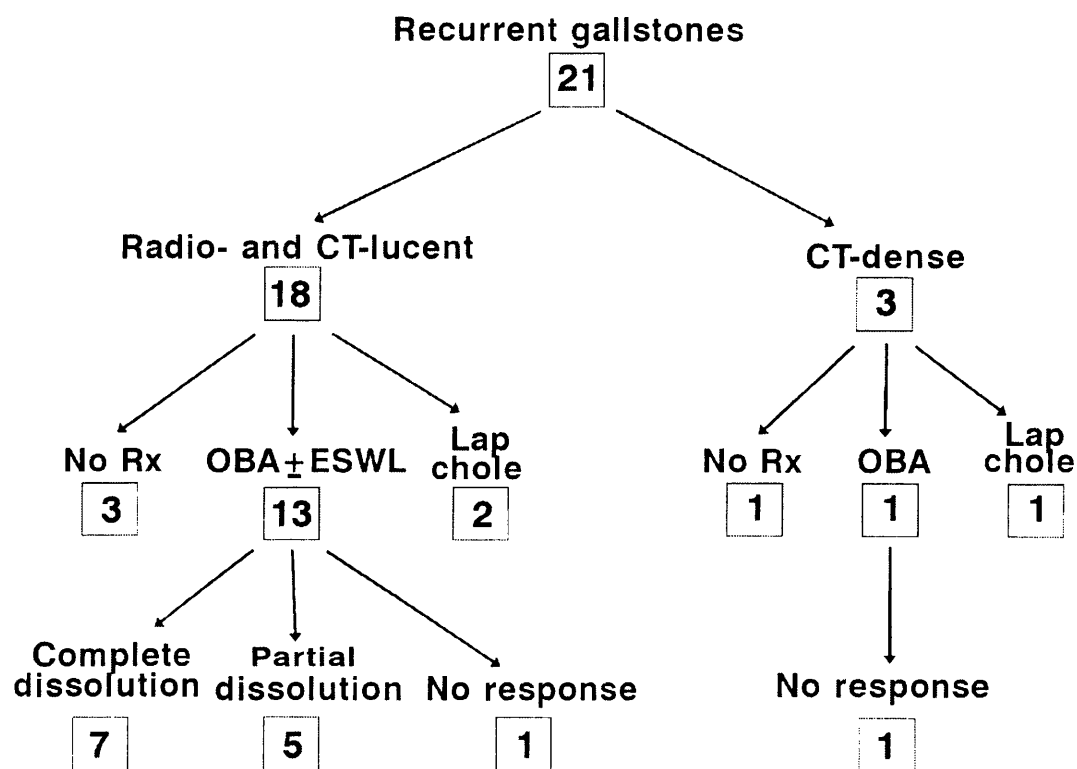


Figure 5.3 Treatment outcome in the 21 patients with recurrent gallstones.

Thirteen of the 18 patients with recurrent stones that were lucent by conventional radiology and CT, were treated with oral bile acids ( $n = 11$ ) or lithotripsy + oral bile acids ( $n = 2$ ). After a mean duration of nine months oral bile acid treatment (range, 2-15 months), dissolution of the recurrent stones was complete in seven and partial in five. In those with partial gallstone dissolution, the stone load decreased from a median of four stones with a maximum diameter of six mm, to a median of two with a maximum diameter of four mm — suggesting that at least parts of the recurrent stones were cholesterol-rich. The patient with recurrent stones in a poorly opacifying gallbladder, showed no evidence of stone dissolution after 22 months oral bile acid therapy.

Two other patients with plain x-ray-lucent and CT-lucent recurrent stones were lost to follow-up and one received no further treatment, while the remaining two developed biliary colic and underwent laparoscopic cholecystectomy. Samples of the gallbladder bile in these two patients (obtained during surgery): (i) contained cholesterol microcrystals, (ii) were supersaturated with cholesterol (saturation indices of 1.5 and 1.3) and (iii) had rapid microcrystal nucleation times (one and three days, respectively) (33) — thus providing further strong, albeit indirect, evidence that their recurrent stones were cholesterol-rich.

Of the three patients with CT-dense, calcium-containing recurrent stones, one remained asymptomatic and untreated, a second developed biliary colic and underwent laparoscopic cholecystectomy (her stones were not available for analysis), while the third, whom we elected to treat with oral bile acids despite the fact that her maximum gallstone attenuation score was 118 HU, showed no dissolution response after 11 months therapy with ursodeoxycholic acid 5 mg/kg/d + chenodeoxycholic acid 7.5 mg/kg/d.

## 5.4 DISCUSSION

The results of the present study show that in terms of stone number or composition, gallbladder stones often do not recur true-to-type. However, irrespective of the composition of the original stones, recurrent stones are usually lucent by conventional x-ray and CT, presumed cholesterol-rich, and potentially dissolvable with medical therapy. These findings have important implications for the treatment of recurrent gallstone disease. They also provide clues about gallstone pathogenesis in the high-risk group of patients whose stones have been removed or dissolved, but who retain their functioning gallbladders.

The development of recurrent gallbladder stones, like that of primary gallstones, involves at least a triple defect: (i) cholesterol supersaturation, (ii) abnormally rapid nucleation in bile of cholesterol microcrystals, and (iii) impaired gallbladder emptying (Dowling *et al* 1994).

In patients treated with oral bile acids, when complete dissolution of the primary gallstones has been confirmed and the bile acid treatment withdrawn, the gallbladder bile reverts to being supersaturated with cholesterol in one to four weeks (Iser *et al* 1977). Furthermore, in patients whose gallstones have been cleared with the combination of shock-wave lithotripsy plus oral bile acids, the impaired gallbladder emptying, found in many patients with primary gallstones (Forgacs *et al* 1984; Pomeranz and Shaffer 1985; Festi *et al* 1990), persists (Spengler *et al* 1989; Festi *et al* 1990). Given that these defects persist or recur in up to 100% of patients rendered stone-free by dissolution or extraction, while gallstones recur in only 50%, these two components of the triple defect (cholesterol supersaturation and impaired gallbladder motility) have low predictive value for the development of gallstone recurrence. By implication, therefore, the component most likely to distinguish between those who will, and those who will not, develop recurrence is abnormal nucleation — whether due to persistence or recurrence of an excess of pronucleating factors or a deficiency of antinucleating proteins.

In primary cholesterol gallstone disease, the cholesterol microcrystal nucleation time of gallbladder bile, measured *ex vivo*, is considerably shorter in patients with multiple stones, than in those with solitary stones (Groen *et al* 1990; Jungst *et al* 1992). One explanation for this observation is that cholesterol-nucleating proteins are present at higher titres in bile samples from patients with multiple gallstones, than in those with solitary stones (Groen *et al* 1990). Indirect evidence that these pronucleating factors persist, or recur, after complete stone dissolution is provided by: (i) reports of rapid nucleation of cholesterol microcrystals in gallbladder bile (Hussaini *et al* 1995b) and bile-rich duodenal fluid (Berr *et al* 1994) in approximately 50% of patients after complete gallstone dissolution, and (ii) the fact that there is a three-fold higher risk of stone recurrence in patients who originally had multiple stones, than in those who initially had solitary stones (Villanova *et al* 1989; Pelletier *et al* 1992; Hood *et al* 1993).

There is conflicting evidence that gallstones recur true-to-type in terms of stone number. In the British/Belgian Gallstone Study Group's post-dissolution trial (Hood *et al* 1993) — in which 21 of 82 patients developed stone recurrence between 12 and 42

months after complete stone dissolution — there was complete concordance between primary and recurrent stone number. However, apart from the results of one other recent study (Petroni *et al* 1991), our findings, and those of other groups (Schneider *et al* 1993; Berr *et al* 1994; Sackmann *et al* 1994), suggest that there is a poor correlation between primary and recurrent stone number.

In studies of gallstone recurrence, it has often been suggested (Lanzini *et al* 1986; O'Leary and Johnson 1993; Schneider *et al* 1993) that both the initial 'clearance rate' and, therefore, the subsequent recurrence rate, are overestimated. That is, the imaging techniques used to diagnose and confirm complete gallstone disappearance, may not be sufficiently sensitive to detect small, incompletely dissolved, residual particles. Ultrasonography can detect biliary sludge, but cannot reliably detect individual particles measuring < 2 mm in diameter (Janowitz *et al* 1994; Ko *et al* 1999). Furthermore, in the present study, complete stone dissolution/removal was confirmed in all patients by two consecutive ultrasounds, at least one month apart. In over three-quarters of the patients, the recurrent gallstones were not detected until at least 12 months after gallstone dissolution. It is unlikely, therefore, that small residual particles in the gallbladder would have remained undetected despite three or more ultrasonographic examinations, before re-growing into echographically visible stones.

There are few data on the composition of recurrent stones. Most previous studies (Thistle *et al* 1989; Donald *et al* 1994) have relied on oral cholecystography to assess the presence or absence of calcification in the recurrent stones. In one report (Petroni *et al* 1991), of 34 patients who developed stone recurrence after complete dissolution of their radiolucent primary stones, only two (6%) had cholecystographic evidence of calcification in their recurrent stones. This observation, suggesting that recurrent stones are almost always non-calcified, is consistent with the present findings. In the present study, the recurrent stones were radiolucent (by plain abdominal x-ray and oral cholecystography) in 20 of the 21 (95%) patients. Furthermore, indirect assessment of recurrent stone composition, by localised CT scanning, suggested that the stones were indeed cholesterol-rich in 18 of the 21 (86%) patients — including five of the six who originally had calcified gallstones.

Calcification of primary cholesterol-rich gallstones is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (Bell *et al* 1975). Early detection of recurrent gallstones may explain why, in the present study, patients who originally had calcified gallstones developed small, cholesterol-rich stones on recurrence. It has been estimated that primary gallbladder stones grow at a rate of 1-4 mm per year (Mok *et al* 1986; Wolpers and Hofmann 1993), and do not cause symptoms until two to seven years after their formation (Mok *et al* 1986). Therefore, it is likely that the primary stones in the present study had been present for several years before their detection. In contrast, as a result of regular ultrasonographic surveillance (at least once a year), the recurrent stones were detected within months of their formation.

Patients with 'post-stone gallstone disease' are at high risk (10-15% per annum) of developing new stones (Lanzini *et al* 1986; Villanova *et al* 1989; Hood *et al* 1993; Donald *et al* 1994; Pauletzki *et al* 1995). Provided that the pathogenesis of these recurrent stones is similar to that of the primary stones, studies of recurrent gallstone formation are relevant to patients at risk of primary gallstone formation. However, the present results have shown that gallstones do not recur true-to-type — suggesting that the pathogenetic factors which determine primary and recurrent stone size and composition, cannot be constant. Nonetheless, independent of their original composition, recurrent gallstones are usually lucent by conventional x-ray and CT, presumed cholesterol-rich, and dissolvable with oral bile acid therapy.

***PART III. THE PATHOGENESIS OF  
GALLSTONES: LABORATORY  
STUDIES***

## **CHAPTER 6. BILE PHYSICAL CHEMISTRY IN PATIENTS WITH ARRESTED GALLSTONE DISSOLUTION DURING ORAL BILE THERAPY**

### **6.1 INTRODUCTION**

By ensuring that only patients with cholesterol-rich gallbladder stones (as assessed by CT attenuation values of < 100 Hounsfield Unit (Walters *et al* 1992; Pereira *et al* 1997)) and a patent cystic duct are considered for treatment with oral bile acids — given alone (May *et al* 1993), or together with extracorporeal shock-wave lithotripsy (ESWL) (Strasberg and Clavien 1992), confirmed complete gallstone dissolution (by life-table analysis) can be achieved in 65-90% of patients (Caroli *et al* 1992). However, no/arrested gallstone dissolution (no cholecystosonographic response to the oral bile acids after one year or partial, but arrested, dissolution after two years of treatment) still occurs in up to 35% of patients (Caroli *et al* 1992; Walters *et al* 1992).

In a previous study from our Unit described in Chapter 6 (Pereira *et al* 1997), over a third of 43 patients with no/arrested dissolution developed either a blocked cystic duct or markedly impaired gallbladder emptying, and a further 21% had evidence of acquired stone calcification. However, an explanation for no/arrested dissolution could not be found in 11 of the 43 patients (26%) whose stones had not dissolved despite 12-36 (median 22) months of oral bile acids alone (n = 23) or with adjuvant lithotripsy (n = 20). These patients: (i) claimed good compliance in taking the prescribed dose of oral bile acids (ursodeoxycholic acid 5 mg/kg/day + chenodeoxycholic acid 7.5 mg/kg/day) regularly, (ii) had a patent cystic duct at repeat oral cholecystography, and (iii) had no evidence of acquired stone calcification at repeat CT.

To investigate the reasons for the non-response or arrested gallstone dissolution in this latter group, in consenting patients we performed ultrasound-guided, percutaneous, transhepatic, fine-needle aspiration of gallbladder bile (Swobodnik *et al* 1991a; Hussaini *et al* 1995c) for assessment of bile acid composition and bile physical chemistry.

## 6.2 METHODS

### 6.2.1 Patients

Of the 11 patients with no/arrested gallstone dissolution who claimed good compliance, had a patent cystic duct and no CT evidence of acquired stone calcification, six (55%) consented to ultrasound-guided, percutaneous, transhepatic, fine-needle, gallbladder puncture (Swobodnik *et al* 1991a; Hussaini *et al* 1995c) during continued treatment (Figure 6.1). There were four women and two men, with a mean age of 42 years (range 27-65 years). The results of bile analysis in these six patients were compared with those from five patients (four women, one man; mean age 46, range 31-65 years) who had become stone-free after 4-28 (median seven) months of oral bile acids — alone (n=3) or with ESWL (n=2). These patients underwent gallbladder puncture at least four weeks after complete gallstone dissolution had been confirmed, and oral bile acid therapy withdrawn.

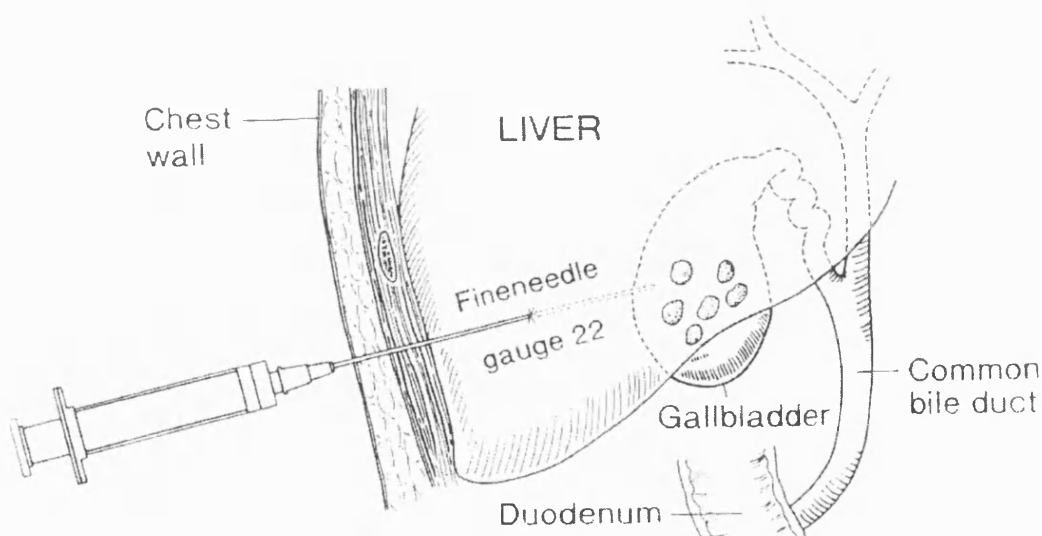


Figure 6.1 Diagram depicting transhepatic fine-needle puncture of the gallbladder.

### 6.2.2 Storage and handling of samples

Following collection, samples of gallbladder bile were stored immediately in a sterile plastic universal container, and transported in water at 37°C. Aliquots of bile were also taken for aerobic and anaerobic culture of bacteria, and for examination by

polarised light microscopy (x10 magnification) for the presence or absence of cholesterol microcrystals.

For analysis of total biliary lipids, gallbladder bile samples were diluted 1:20 in methanol (for total bile acid assay) or 1:10 in isopropanol (for cholesterol and phospholipid assays). Samples were then either analysed immediately or stored overnight at -4 °C and assayed within 24 h. If stored, bile samples were allowed to reach room temperature before assay. Before analysis, all samples were mixed thoroughly and then centrifuged for 15 min at 3000 rpm (700 g) to remove insoluble debris.

### **6.2.3 Cholesterol microcrystal nucleation times**

The nucleation time assay was devised by Holan *et al* (1979) as a method of measuring the rate of *de novo* cholesterol microcrystal formation in gallbladder bile, although it is really a measure of nucleation and growth of cholesterol crystals to a size detectable by microscopy (Harvey and Strasberg 1993).

To determine the cholesterol microcrystal nucleation time, a 2-ml sample of fresh gallbladder bile was ultracentrifuged at 100,000 g for 2 h at 37°C. (Beckman L8-70M ultracentrifuge, 70 Ti rotor). This process separated bile into three phases, which were examined by polarised light microscopy. The upper layer consisted of liquid crystals (recognised by their Maltese cross appearance), while the lower phase contained solid crystals and cellular debris, and the middle phase appeared clear. This 'isotropic phase' was then incubated in a water bath at 37°C.

Aliquots of the incubated bile were examined daily. A drop of bile was placed onto a microscope slide and examined for cholesterol microcrystals by polarised light microscopy on a slide stage heated to 37°C. Cholesterol microcrystals were recognised by their classical rhomboid plate-like appearance, as shown in Figure 6.2. The time, in days, taken for cholesterol microcrystals to appear was defined as the nucleation time.

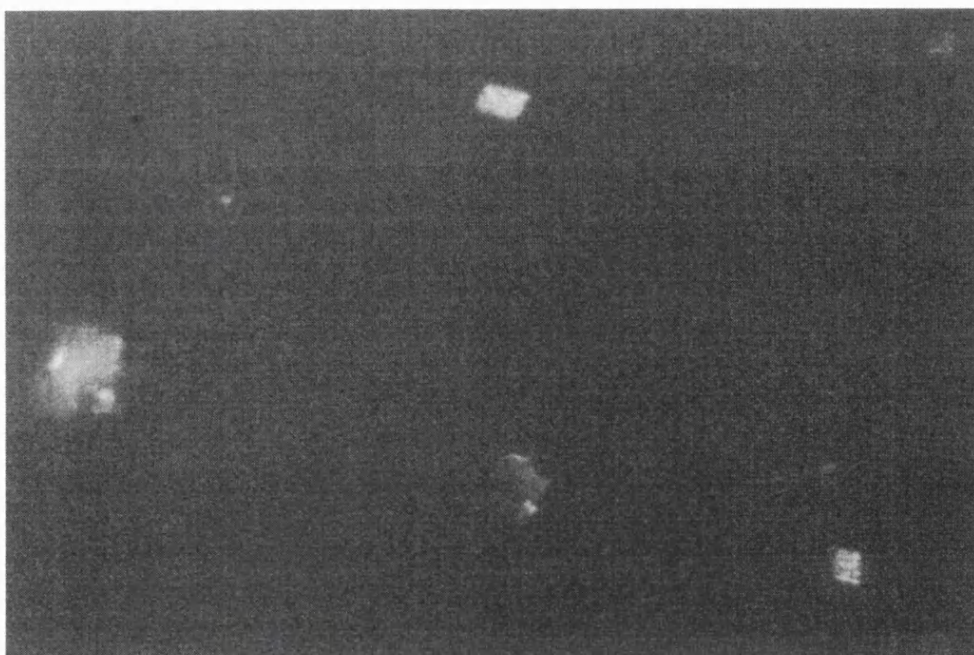


Figure 6.2 Typical rhomboid cholesterol microcrystals as viewed through a polarising light microscope.

#### 6.2.4 Total bile acids

Total bile acids were determined using a commercially available kit (Sigma Chemical Co., Poole, UK) based on the  $3\alpha$  hydroxy steroid dehydrogenase method first described by Talalay (1960). The common bile acids have a  $3\alpha$  hydroxyl group which, in the presence of nicotinic adenine dinucleotide (NAD), is oxidised by  $3\alpha$  hydroxy dehydrogenase to generate nicotinamide adenine dinucleotide dehydrogenase (NADH), which can be estimated spectrophotometrically.

- (i) Inactive reagent. This comprised 0.153 mg NAD, 100 ml (0.1 M pH 9.5) glycine, 150 ml (1.0 M pH 9.5) hydrazine hydrate, and 10 ml of tris (0.03 M tris in 0.001 M ethylenediaminetetraacetic acid: EDTA) buffer, adjusted to a pH of 9.5.
- (ii) Active reagent. To make the active reagent, 1-2 mg of  $3\alpha$  hydroxysteroid dehydrogenase (activity 0.5-2.0 units/mg) was added to the inactive reagent.
- (iii) Standard solutions. Standard solutions of glycochenodeoxycholic acid 0-5 mM were made up in methanol.

### ***Assay procedure***

100  $\mu$ l of bile was added to four tubes (two tests, two blanks). Three ml of active reagent were added to the duplicate test tubes, and three ml of inactive reagent added to the blanks. All tubes were incubated at room temperature for 50 min and then the solutions in the tubes were read in a spectrophotometer at 340 nm. A typical standard curve is shown in Figure 6.3.

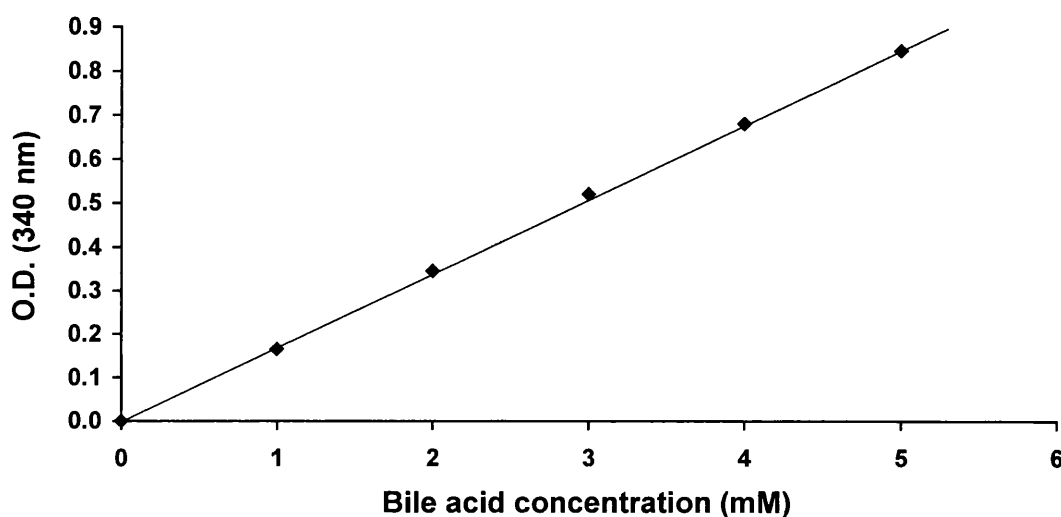


Figure 6.3 Example of a standard curve for the bile acid assay. Standard solutions of glycochenodeoxycholic acid in methanol were assayed using the  $3\alpha$  dehydrogenase method.

### **6.2.5 Total phospholipids**

Biliary phospholipids were measured using a commercially available kit developed by Wako Pure Chemicals Ltd (supplied by Alpha Laboratories Ltd, Eastleigh, Hants, UK). This assay is based on an enzymatic method whereby phosphatidylcholine is broken down by phospholipase D to phosphoric acid ester and choline (Qureshi *et al* 1980). The free choline is then oxidised by choline oxidase to betaine and hydrogen peroxide. The liberated hydrogen peroxide then couples 4-aminoantipyrine and phenol to produce a chromogen which can be measured spectrophotometrically.

- (i) Buffer solution. 0.05 M Tris buffer solution (pH 8.0) containing 0.05% phenol.
- (ii) Colour Reagent. 0.4 U/ml phospholipase D, 2 U/ml choline oxidase, 5 U/ml peroxidase and 0.015% 4-aminoantipyrine made up to 45 ml with buffer solution.
- (iii) Standard solutions. A solution containing 0.54 mg/ml choline chloride and 0.1% phenol corresponding to 3.85 mM phospholipid content was diluted with isopropanol to make standard solutions with phospholipid concentrations in the range 0-5 mM. A typical standard curve is shown in Figure 6.4.

#### ***Assay procedure***

Aliquots (20  $\mu$ l) of diluted bile (1:10) were added to 3 ml of colour reagent in duplicate. Blanks were prepared by adding isopropanol alone (20  $\mu$ l) to the colour reagent. Samples were mixed, covered and left to stand overnight for 16 h at room temperature. The optical density of the resultant coloured solution was then read in a spectrophotometer at 505 nm.

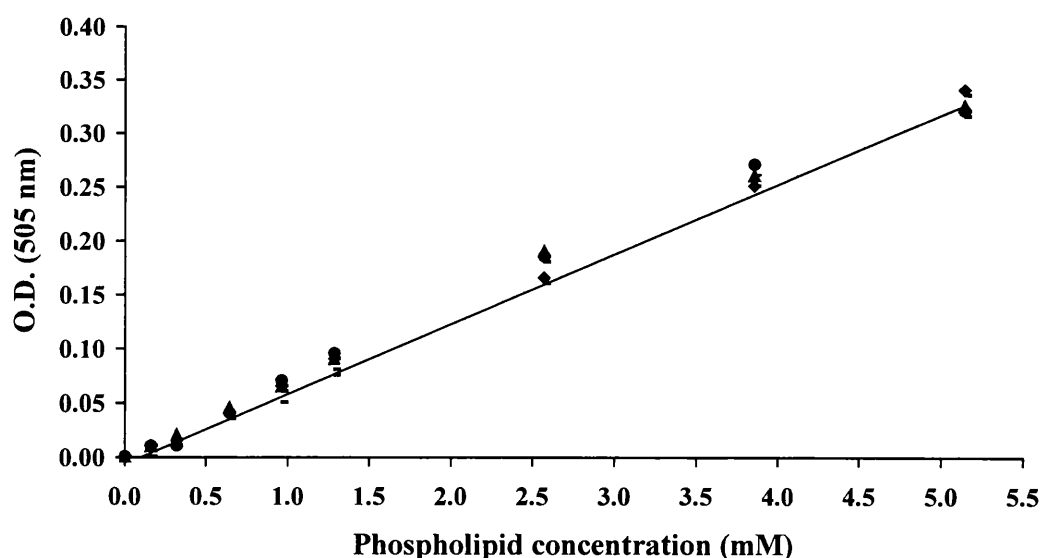


Figure 6.4 Standard curves for the phospholipid assay performed on four consecutive days. Total phospholipid concentrations are plotted against optical density of the solution following the assay reaction.

### 6.2.6 Biliary cholesterol concentrations

This commercial assay (Sigma Chemical Co., Poole, UK) is based on the method described by Roschlau *et al* (1973). Any cholesterol esters present in bile are hydrolysed by cholesterol esterase to cholesterol, which is then oxidised by cholesterol oxidase to 4-cholestanone with the production of hydrogen peroxide. The methanol and hydrogen peroxide combine by the action of the catalase, producing formaldehyde which combines with acetylacetone and ammonia to form 3,5-diacetyl-1-4-dihydrolutidine, which can be measured spectrophotometrically.

- (i) Ammonium orthophosphate/catalase buffer (Reagent 1). 100 ml of ammonium orthophosphate (121.86 mg/ml) was mixed with 190 ml of monobasic ammonium phosphate (69 mg/ml) and the pH adjusted to 7.0. Bovine liver catalase (250,000 U) was then added to and mixed with the solution.
- (ii) Acetylacetone solution (0.42 M; Reagent 2). 20 ml of methanol was mixed with 10.5 ml acetylacetone and made up to 250 ml with water.
- (iii) Enzyme preparation. Cholesterol esterase (700 U/ml) and cholesterol oxidase (4 U/ml) were made up using the ammonium phosphate buffer solution from reagent 1.
- (iv) Working reagent. 100 ml of reagent 1 was added to 5 ml of reagent 2 and 0.8 ml of cholesterol esterase. Triton X-10 (2 g) was then added and the solution was thoroughly mixed.
- (v) Standard solution. A standard solution of cholesterol was prepared in isopropanol with dilutions ranging from 0-2.5 mM. A typical standard curve is shown in Figure 6.5.

#### ***Assay procedure***

Aliquots of bile (100 µl) were placed in four tubes (two tests, two blanks), followed by the addition of 1 ml of working reagent to each tube. Then, 20 µl of cholesterol oxidase was added to the active tubes and all tubes were thoroughly mixed. The tubes were incubated at 37°C for 1 h and then 1 ml of distilled water was added to terminate the reaction. The samples were mixed and then read at 410 nm in a spectrophotometer.

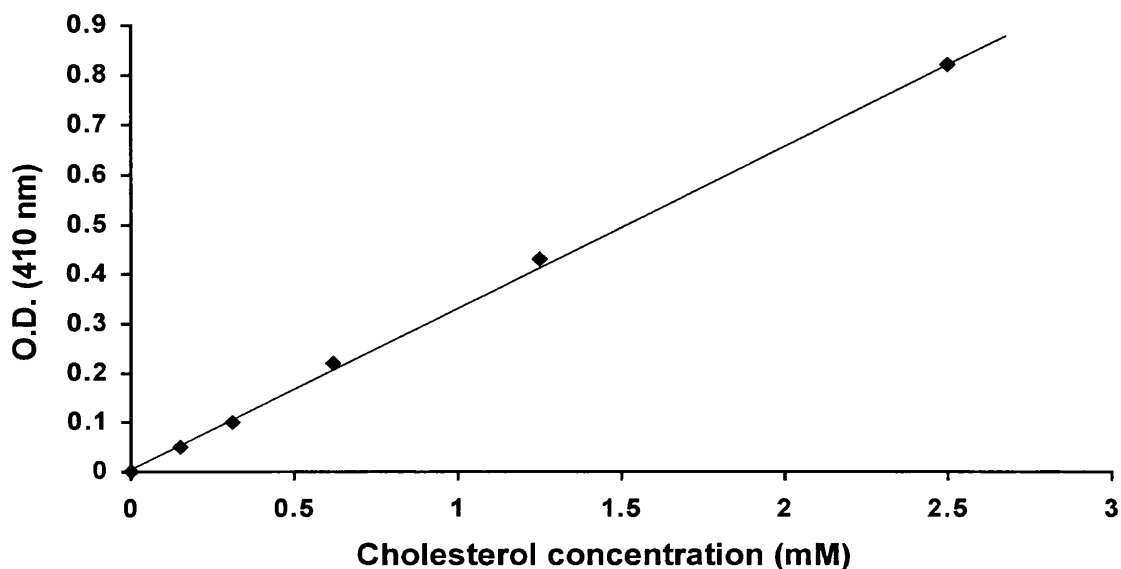


Figure 6.5 Example of a standard curve for the cholesterol assay.

#### ***Reliability of biliary lipid analysis***

The precision of the assays for total bile acids, phospholipids and cholesterol was assessed from the duplicate results for 23 different bile samples. The coefficient of variations for the total bile acids, phospholipids and cholesterol assays were 1.8%, 4.3% and 4.0%, respectively.

#### **6.2.7 Cholesterol saturation indices**

The cholesterol saturation index was calculated from the molar concentrations of total bile acids, phospholipids and cholesterol, using a formula devised by Thomas and Hofmann (1973). The formula is based on a rectangular transformation of the pooled data of cholesterol solubility in model bile acid-phospholipid solutions described by Hegardt and Dam (1971) and Holzbach *et al* (1973).

First, the ratio of phospholipid : bile acid + phospholipid was determined (x), and the molar percent of cholesterol for a saturated solution (y) derived from the formula:

$$y = 3.082 - 0.804x + 117.05x^2 - 204.94x^3$$

The saturation index was calculated as the molar percentage of cholesterol in the sample divided by the calculated value of the molar percentage of cholesterol at saturation (y), where a saturated solution has a value of 1.0.

#### **6.2.8 Measurement of biliary bile acid conjugates**

The concentrations of the individual bile acid conjugates were determined using reverse-phase high-performance liquid chromatography (HPLC) (Wildgrube *et al* 1983), and expressed as a percentage of total bile acids. In reverse-phase HPLC, the mobile phase is an aqueous organic solvent mixture containing samples of bile diluted in methanol, with different affinities for the polar mobile phase and nonpolar stationary phase. Ion-pair chromatography uses an ion-pairing agent (e.g. tetrabutylammonium phosphate) to bind to the more ionised bile acid conjugates to improve the separation of individual bile acids, though the theoretical basis for this action is not fully understood. The net result is that polar forces determine the retention time of bile acid conjugates, so that the more hydrophobic bile acids are retained longer and the most hydrophilic bile acids elute first.

The chromatographic column was a RP 'Brownlee' C18 (5  $\mu$ m) column from Applied Biosystems Ltd (Warrington, Cheshire, UK). Injections were made using a 100  $\mu$ l syringe into a Rheodyne valve with a 100  $\mu$ l sample loop. A model UV 50 detector (Varian Inc, Warrington, UK) set at 205 nm was used and peak heights measured from a pen recorder.

**Mobile phase solvent.** 3.0 g of sodium dihydrogen-phosphate (BDH Laboratory Supplies, Poole, UK) and 0.29 g of tetrabutylammonium phosphate (Sigma Chemical Co., Poole, UK) were added to 320 ml of double distilled water. This was then made up to 1000 ml with 650 ml HPLC grade methanol and 30 ml of tetrahydrofuran (Sigma-Aldrich Co. Ltd, Poole, UK). The pH of the solution was adjusted to 5.4 with phosphoric acid and degassed through a 'Millex' filter.

**Extraction of bile acids.** 1 ml of bile was alkalinised with 10 ml of 0.1 M sodium hydroxide to which 5 ml of hexane was added, mixed and then allowed to partition into aqueous and hexane phases. The upper hexane phase was discarded and 1 ml of XAD 2

resin (washed in distilled water) was added to the remaining lower aqueous phase. The XAD 2 resin and aqueous solution containing the bile acid conjugates was then left on a rotary mixer for 1 h. The XAD beads were allowed to settle and the supernatant discarded. The beads were washed in 10 ml of 0.01 M sodium hydroxide and roller mixed for a further 15 min, after which the supernatant was again discarded. 10 ml of absolute methanol was then added, the stoppered tube roller mixed for another 15 min and the supernatant collected. This process was repeated three times with a further 5 ml of methanol on each occasion and the supernatants pooled. The methanol solution of bile acids was then evaporated to dryness on a rotary evaporator. Finally, the sample was reconstituted to 1 ml with HPLC-grade methanol. The recovery of bile acids after extraction was assessed by comparing total bile acid concentrations before and after extraction in eight bile samples, and was  $> 85\%$  in all cases (mean  $91.2\% \pm \text{SEM } 2.1\%$ ).

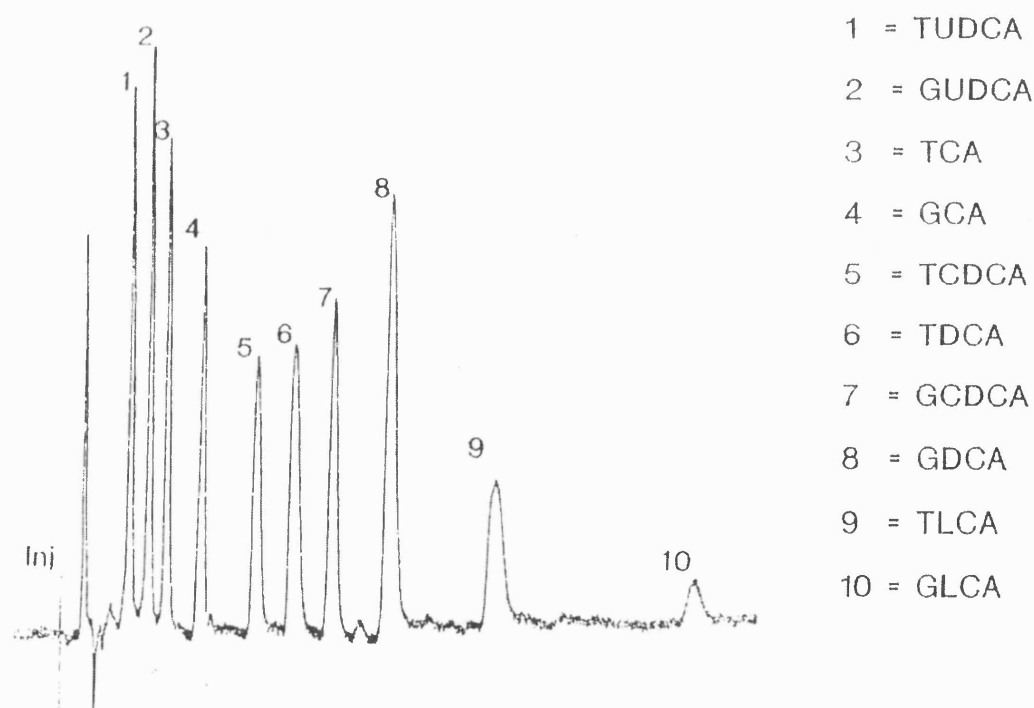


Figure 6.6 An example of a pen recorder trace following the injection (100  $\mu\text{l}$ ) of a methanol solution containing 10 conjugated bile acid standards onto the HPLC column. The trace shows the clear separation of 10 distinct peaks, with retention times corresponding to those of the individual bile acid standards.

**Chromatographic analysis.** The flow rate of the mobile phase was 1.1 ml/min. The detector was set at a sensitivity of 0.05 Absorbance Units and wavelength of 205 nm. The pen recorder sensitivity was 2 mV/mm with a paper feed rate of 2 mm/min. Once a stable baseline was obtained, 100  $\mu$ l of bile diluted in HPLC grade methanol was injected into the sampling valve. The height (mm) and retention time of each peak was recorded. Peak height measurements were linearly related to area under the curve measurements, although the former were more reproducible. Therefore, peak height was used as a measure of bile acid concentration. An example of a pen-recorder trace depicting the resolution of ten bile acid standards following separation by HPLC is shown in Figure 6.6.

Standard bile acids (Sigma Chemical Co., Poole, UK) were weighed and dissolved in 20 ml of methanol, and the concentration of each standard solution determined using the 3 $\alpha$  dehydrogenase method. Standard curves of bile acid concentration versus peak height for each bile acid were performed in duplicate and shown to be linear over a deflection scale range of 30-150 mm (Figure 6.7).

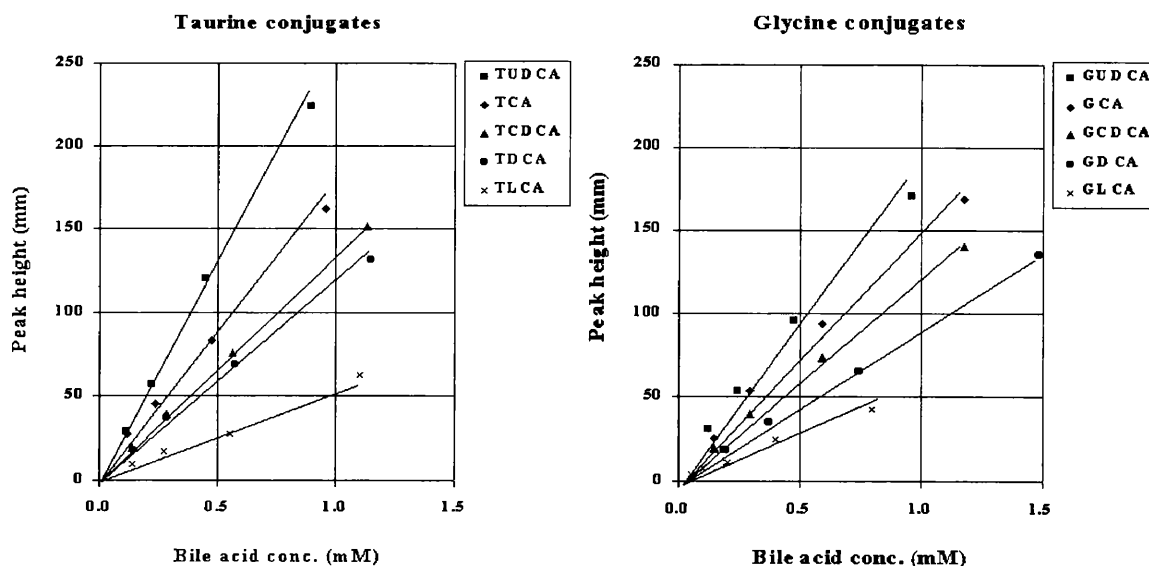


Figure 6.7 Concentration curves for taurine- and glycine-conjugated bile acid species measured by HPLC.

**Reliability criteria.** The lower limit of sensitivity for each bile acid was arbitrarily taken as that which gave a minimal deflection of 30 mm. The precision of each injection of 100  $\mu$ l of sample was examined by measuring the peak height of six sequential 2.3 mM injections of taurocholic acid onto the column. The coefficient of variation was  $< 2.1\%$ .

### 6.3 RESULTS

Of the 6 patients in whom an explanation could not be found for no/arrested gallstone dissolution and who consented to percutaneous, fine-needle puncture of the gallbladder during continued chenodeoxycholic acid (CDCA) + ursodeoxycholic acid (UDCA) therapy, the on-treatment amounts of CDCA, expressed as a percentage of total bile acids, were  $52.8 \pm 6.3\%$  (range, 30.7-72.0%), while those of UDCA were  $16.2 \pm 3.8\%$  (range, 1.0-25.3%). The mean ( $\pm$  SEM) CSI was  $1.0 \pm 0.11$ , with a range of 0.67-1.4. In the three patients with unsaturated bile, the proportion of biliary UDCA was at least 15% of total bile acids — a lesser degree of enrichment than would be expected with full dose monotherapy (Hofmann 1994). However, despite the presence of unsaturated bile, the stones had not dissolved, suggesting that they were not cholesterol-rich. In contrast, two of the three patients with CSIs of  $> 1$  had low levels of UDCA, suggesting either that they were not taking or that they were not absorbing the prescribed dose of UDCA. In all six patients, the cholesterol microcrystal nucleation times during treatment were  $> 10$  days, with a median of 18 (range, 13-21) days (Figure 6.8).

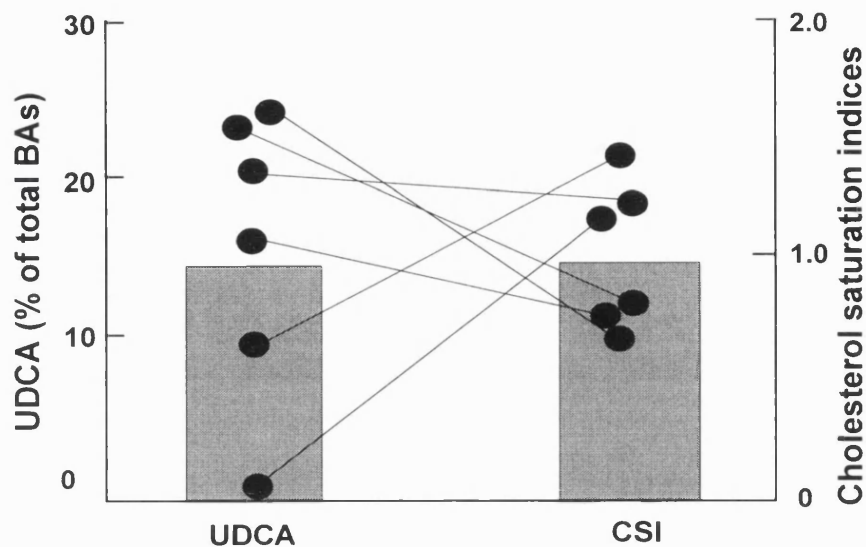


Figure 6.8 Biliary ursodeoxycholic acid (% of total bile acids) and cholesterol saturation indices in six patients with no/arrested dissolution of cholesterol-rich (CT score < 100 HU) gallstones in a 'functioning' gallbladder and who claimed full compliance with oral bile acid therapy, during continued treatment.

In comparison, the results of bile analysis in five patients who became stone-free after 4-28 months oral bile acids  $\pm$  ESWL, were similar to those reported previously in untreated patients with cholesterol-rich gallstones (Hofmann *et al* 1982; Tazuma *et al* 1989; Hussaini *et al* 1994). Thus, all of the patients in the present study had bile which was supersaturated with cholesterol (mean CSI 1.43, range 1.25-1.69), with pathologically short cholesterol microcrystal nucleation times of  $\leq 10$  days (median 5, range 2-10 days) (Figure 6.9). Moreover, their chenodeoxycholic acid levels of 36.3-53.5% (mean  $44.5 \pm 2.5\%$ ) of total bile acids were similar to those of the no/arrested dissolution group. UDCA was present in small amounts (mean  $0.7 \pm 0.3\%$ , range 0-1.4%;  $p < 0.05$  v no/arrested dissolution group). The proportions of the other two major bile acids in gallbladder bile, cholic acid ( $31.4 \pm 4.6\%$  v  $20.3 \pm 5.7\%$ ; NS) and deoxycholic acid ( $23.4 \pm 4.8\%$  v  $9.9 \pm 4.7\%$ ; NS) were similar in the stone-free and no/arrested dissolution groups, respectively.

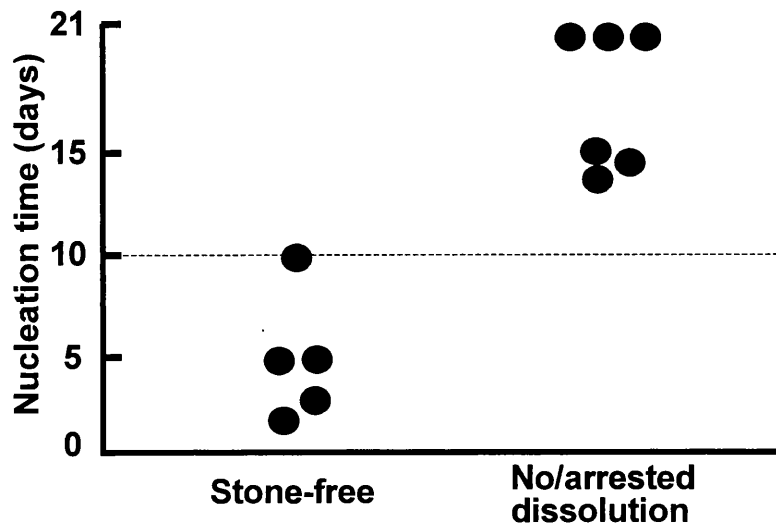


Figure 6.9 Cholesterol microcrystal nucleation times in the stone-free and no/arrested dissolution groups. The broken horizontal line at 10 days represents the lower limit of normal for cholesterol microcrystal nucleation. Note that, at the time of the study, all five stone-free patients had discontinued oral bile acids while the six in the no/arrested dissolution group continued to take UDCA+CDCA.

#### 6.4 DISCUSSION

The effects of oral bile acid therapy on biliary cholesterol saturation and bile acid composition in patients with gallstones, have been studied extensively (Bachrach 1982; Lanzini and Northfield 1988; Tazuma *et al* 1989; Janowitz *et al* 1991; Zuin *et al* 1991; Galatola *et al* 1992; Walters *et al* 1992; Mizuno *et al* 1993; Hofmann 1994; Tudyka *et al* 1994). However, there have been few studies of gallbladder bile composition in patients with no/incomplete dissolution despite prolonged oral bile acid therapy (Janowitz *et al* 1991). The present results show that the bile composition and physical chemistry in five patients who became stone-free after oral bile acids  $\pm$  ESWL, and who had been off treatment for at least one month, were similar to those reported previously in patients with cholesterol-rich gallstones (Hofmann *et al* 1982; Tazuma *et al* 1989; Hussaini *et al* 1994). In contrast, the results of biliary bile acid composition and cholesterol saturation in the six patients with incomplete dissolution fell into two categories: (i) those with enrichment of their bile with the prescribed bile acids, low

cholesterol saturation indices, but incomplete dissolution of presumed non-cholesterol residues, and (ii) those with low bile acid enrichment and supersaturated bile — possibly because of poor compliance to, or malabsorption of, the prescribed bile acids. In patients with gallstones, the degree of enrichment of bile with UDCA conjugates during chronic ingestion of UDCA is proportional to the bile acid dose, although there is a considerable range (Masson *et al* 1966; Hofmann 1994).

Although gallbladder bile was obtained from only a small number of patients, the results of the bile analyses suggest that, in selected patients, determination of biliary bile acid composition and cholesterol saturation (either in gallbladder bile obtained by fine-needle puncture (Hussaini *et al* 1995c) or in bile-rich duodenal fluid (Janowitz *et al* 1991)), may be helpful in determining the reason for no/arrested gallstone dissolution. Conversely, in the present study, the cholesterol microcrystal nucleation time was of poor predictive value, being consistently prolonged to > 10 days in all six patients with incomplete dissolution. Both UDCA and CDCA are known to reduce cholesterol secretion into bile, alter the biliary concentrations of nucleation promoting/inhibiting factors (Tazuma *et al* 1989; Mizuno *et al* 1993; Tudyka *et al* 1994), and prolong the cholesterol microcrystal nucleation time (Tazuma *et al* 1989; Mazzella *et al* 1991; Mizuno *et al* 1993). Furthermore, the combined administration of UDCA and CDCA appears to be more effective in dissolving cholesterol-rich gallbladder stones than either agent alone (Janowitz *et al* 1991; Zuin *et al* 1991) — although this claim is debated (Sackmann *et al* 1991a; Petroni *et al* 2001).

In patients whose gallbladder stones do not dissolve completely despite up to two years of oral bile acid therapy, we have shown previously that the main causes for failure are impaired gallbladder contractility and acquired stone calcification — as assessed by oral cholecystography and CT, respectively (Pereira *et al* 1997). In those patients with no/arrested dissolution despite the presence of CT-lucent stones in a ‘functioning’ gallbladder, analysis of gallbladder bile will determine the underlying cause of no/arrested stone dissolution in most, but not all, of the remaining cases — the main reasons being either: (i) incomplete dissolution of presumed non-cholesterol residues, or (ii) poor compliance to, or malabsorption of, the prescribed bile acids.

**CHAPTER 7. THE ROLE OF PHOSPHOLIPIDS AND MUCIN**  
**GLYCOPROTEIN IN THE PATHOGENESIS OF OCTREOTIDE-INDUCED**  
**GALLSTONES**

## **7.1 INTRODUCTION**

Octreotide, a long-acting analogue of somatostatin, is an effective treatment for acromegaly. However, it induces gallbladder stone formation in 10-63% of patients after one to two years of treatment (Redfern and Fortuner 1995; Dowling *et al* 1997). The mechanisms for the formation of these iatrogenic stones are complex. Octreotide inhibits meal-stimulated cholecystokinin release from the small intestine, which is the principal, but not the sole, reason for the associated reduction in gallbladder emptying (Dowling *et al* 1997). However, octreotide also induces multiple lithogenic changes in the composition of gallbladder bile, including: (i) supersaturation of bile with cholesterol, (ii) partitioning of the excess biliary cholesterol into vesicles which have a high cholesterol : phospholipid molar ratio, (iii) rapid nucleation of cholesterol microcrystals, and (iv) an increase in the proportion of the hydrophobic bile acid, deoxycholic acid (Hussaini *et al* 1994). Although controversial (Jungst *et al* 1999), it has been suggested that by inducing cholesterol hypersecretion (Carulli *et al* 1984; Berr *et al* 1992), the increased proportion of biliary deoxycholic acid may be the main pathogenetic mechanism responsible for the induction of supersaturated gallbladder bile and the development of octreotide-induced gallstones (Hussaini *et al* 1994).

Biliary cholesterol secretion is normally coupled tightly to that of phospholipids, and particularly to that of the principal biliary phospholipid, phosphatidylcholine (PC) (Berr *et al* 1997; Crawford *et al* 1997). Previous studies have suggested that arachidonic acid-rich phospholipids may also induce cholesterol supersaturation and the rapid nucleation of cholesterol microcrystals in gallbladder bile by promoting cholesterol hypersecretion (Berr *et al* 1992; Hatsushika *et al* 1993), inducing the preferential transfer of arachidonic acid-rich phospholipids from vesicles to micelles (Cohen and Carey 1991; Booker *et al* 1992; Halpern *et al* 1992; Halpern *et al* 1993), and

stimulating mucin glycoprotein secretion by the gallbladder mucosa (Lee 1991; Marks *et al* 1992; Shiffman *et al* 1992).

The aims of this study were to determine whether chronic octreotide treatment of acromegalic patients alters the concentration of arachidonic acid-rich phospholipids in gallbladder bile and if so, to relate changes in biliary phospholipid composition to: (i) the cholesterol saturation, (ii) the vesicular cholesterol : phospholipid molar ratio, (iii) the mucin glycoprotein concentration, and (iv) the percent deoxycholic acid, in gallbladder bile.

## **7.2 METHODS**

### **7.2.1 Patients**

Gallbladder bile was obtained from two groups of acromegalic patients — those untreated and those treated with octreotide.

The untreated group comprised eight patients (4 men and 4 women, with a mean age of 49; range, 29-64 years), who had received no octreotide for at least two months and were gallstone-free by ultrasonography. Although these were not normal controls, Hussaini *et al* (1994) showed previously that bile composition and physical chemistry in this group of patients are not significantly different from those in healthy controls (Holan *et al* 1979; Rossi *et al* 1987; Sahlin *et al* 1991).

The octreotide-treated group consisted of nine acromegalic patients (5 men, 4 women; mean age 53, range 36-64 years), who received 100-200 ug octreotide three times daily by subcutaneous injection for at least three months. Five of the nine were studied twice — once before octreotide treatment when all five were stone-free (untreated group), and again after 3-24 (mean 8) months octreotide therapy. At the time of the second study, four of the five remained stone-free, but one, a 59 year old woman, had developed asymptomatic octreotide-induced gallbladder stones after three months treatment. The four patients who were studied only once had not undergone pre-treatment ultrasonography, but were all found to have multiple gallstones after 3-66 months octreotide treatment.

### **7.2.2 Bile sampling**

On 16 of the 17 occasions, samples of fresh gallbladder bile were obtained by ultrasound-guided percutaneous transhepatic fine needle puncture, as previously described (Hussaini *et al* 1995c). However, in the paired studies, one of the patients declined repeat gallbladder puncture. Therefore on the second occasion, fasting bile-rich fluid was obtained by duodenal intubation, after stimulating gallbladder contraction with intravenous cholecystokin. All biles were stored at -20°C until analysed. The use of ultrasound-guided gallbladder puncture was approved by the Research Ethics Committee of St Bartholomew's Hospital and by the Ethics Committee of Guy's Hospital. All patients gave their written informed consent.

### **7.2.3 Biliary lipid and bile acid composition**

Total biliary bile acids (Talalay 1960), phospholipids (Qureshi *et al* 1980) and cholesterol (Roschlau *et al* 1973) were determined by standard enzymatic assays, and the cholesterol saturation index (CSI) derived (Thomas and Hofmann 1973). The concentrations of the individual bile acid conjugates were determined using reverse-phase high-performance liquid chromatography (Wildgrube *et al* 1983) and expressed as a percentage of total bile acids, as described in Chapter 6.

### **7.2.4 Fölch extraction of biliary phospholipids**

HPLC-grade methanol, isopropanol, hexane, acetonitrile and ethanol were obtained from Rathburn Chemicals Ltd (Walkerburn, UK). Phospholipid class standards and PC molecular species standards were obtained from Sigma Chemical Co. (Poole, UK) and Avanti Polar Lipids (Alabaster, Alabama, USA).

Phospholipids were extracted from bile according to the method of Fölch *et al* (1957), as follows. Two ml of bile was mixed with 18 ml chloroform : methanol (2:1 v/v) and 4 ml 0.05M sodium chloride, followed by centrifugation for 10 min at 3000 g to reveal a clear biphasic solution consisting of approximately 9.5 ml water-soluble upper phase and 18.5 ml lipid-soluble lower phase. The lower phase was removed

using a glass pipette and placed into a separate tube. The remaining contents were then mixed with chloroform : methanol and centrifuged on two further occasions, and the lower phase containing the lipid-soluble phospholipids collected. Using this technique, the amount of phospholipids lost into the water-soluble upper phase (as assessed by the total phospholipid assay) was  $3.0 \pm 0.7\%$  ( $n=6$  runs). Following extraction, the lower phase was placed into a quickfit tube together with 2 ml methanol, evaporated to dryness and reconstituted in 2 ml hexane-isopropanol-ethanol (3.7:5:1 v/v).

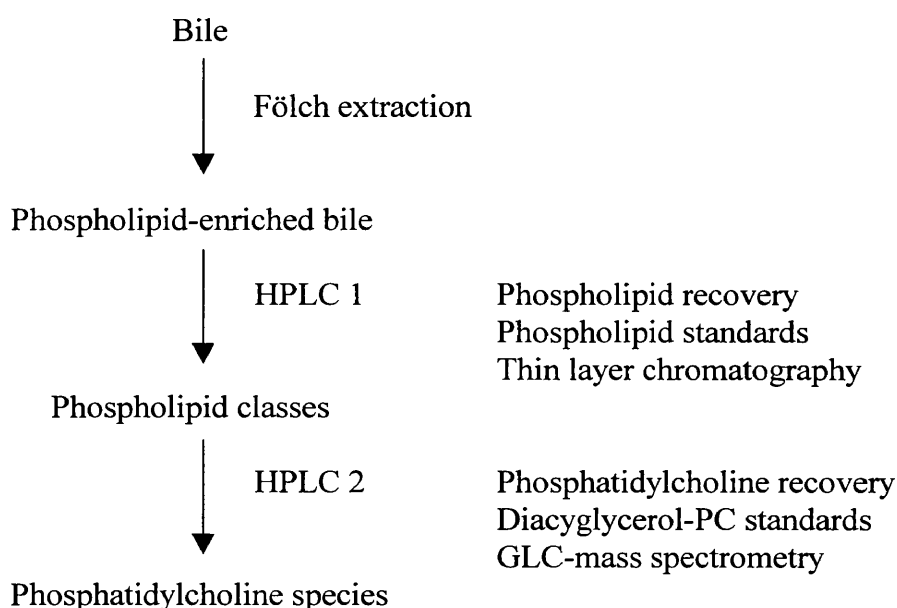


Figure 7.1 Flowchart of phospholipid analysis.

### 7.2.5 Separation of phospholipid classes

After Fölch extraction of the biliary lipids (Fölch *et al* 1957), 200  $\mu$ l aliquots of the lipid extract were loaded onto a 4.6 x 250 mm octadecyl Kromasil column packed with 10  $\mu$ m silica (Hichrom Ltd, Theale, UK), and eluted at a flow rate of 2 ml/min, using a mobile phase of hexane-isopropanol-ethanol-phosphate buffer-acetic acid 367:495:100:57:0.3 (Patton *et al* 1982). This technique of adsorption chromatography results in the solutes eluting from the polar stationary phase (silica bonded to hydroxyl groups) in order of increasing polarity. Phospholipid peaks were detected at an

absorbance of 205 nm, with detector conditions of 50 mV and 0.01 Absorbance Units (Figure 7.2).

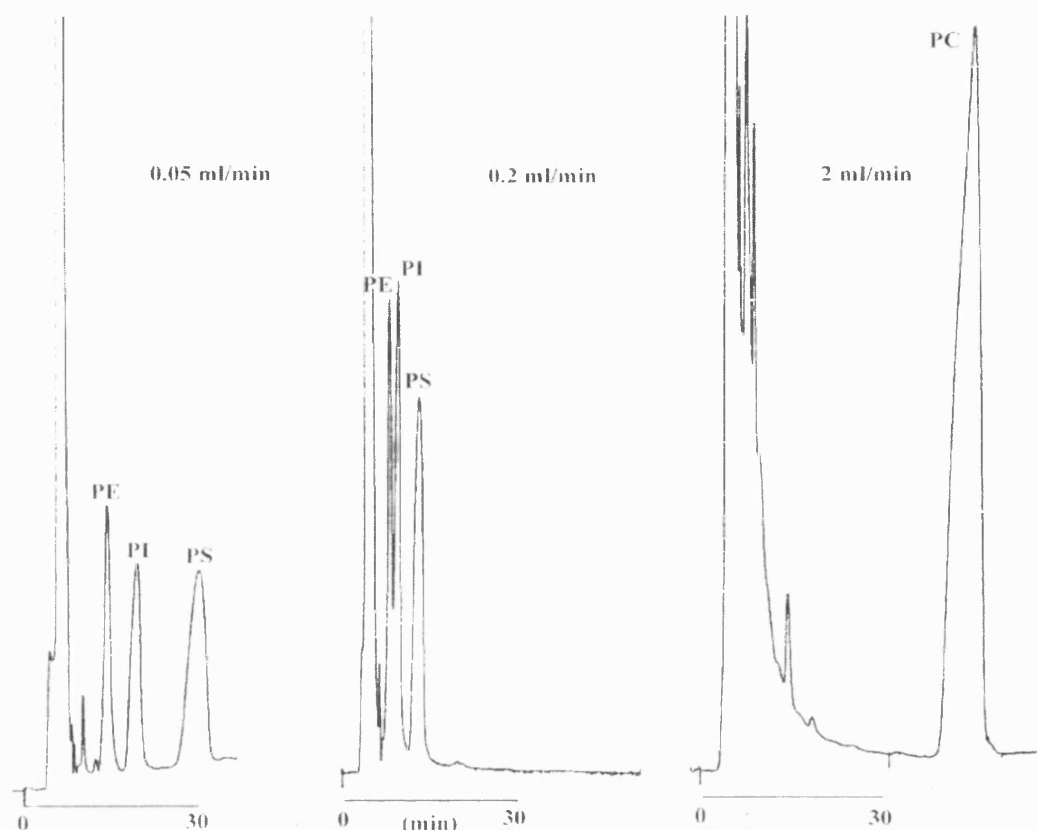


Figure 7.2 Separation of phospholipid classes by HPLC according to flow rate. Phosphatidylcholine (PC) eluted after approximately 40 min at a flow rate of 2 ml/min.

The PC peak was identified by comparison with the retention time of standard bovine PC and confirmed by thin layer chromatography (Bradova *et al* 1990). In brief, 10 x 10 cm silica gel plates were activated in an oven at 110°C for 10 min, followed by loading of 20 µl aliquots of samples and standards. The plate was then placed into a chromatank containing chloroform-methanol-isopropanol-0.25% KCl-ethyl acetate (30:9:25:6:18 v/v) approximately 1 cm in depth. The plate was left to run for a few hours until the solvent front approached the top of the plate, and then dried at room temperature before being exposed to iodine vapour for 15 min. On thin layer chromatography, the retention time of PC isolated from bile by Fölch extraction and HPLC resulted in a single band corresponding to the PC standard (Figure 7.3).

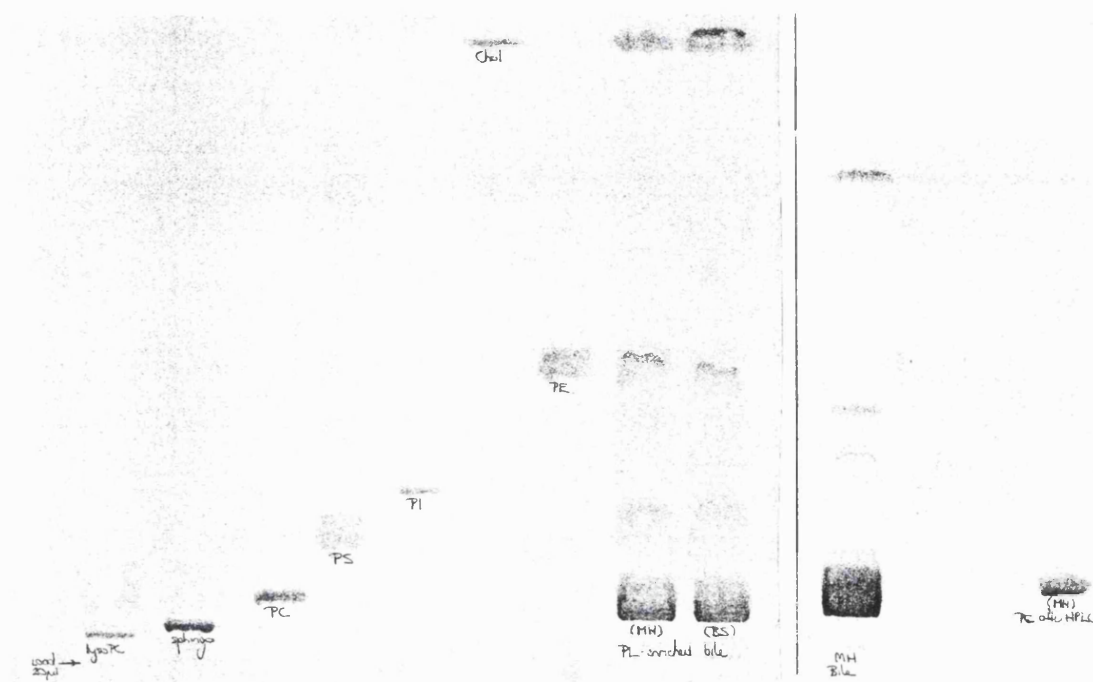


Figure 7.3 Thin layer chromatography of cholesterol and phospholipid standards (lanes 1-7), bile before and after Fölch extraction (lanes 8-10), and after separation of PC from bile by HPLC (right lane).

#### 7.2.6 Separation of molecular species of phosphatidylcholine

Next, the PC peak collected from the previous HPLC stage was dried, reconstituted in 1 ml methanol-acetonitrile-water (80:5:15 v/v), and loaded onto a 4.6 x 250 mm Nucleosil column packed with 5  $\mu$ m diameter beads of octadecylsilane bonded phase (Hichrom Ltd, Theale, UK). In this technique, the stationary phase, silica, is chemically bonded to alkylsilyl groups to give a non-polar, hydrophobic surface, and a polar eluant is used. Solute retention is due to the hydrophobic interactions between the solutes and the hydrocarbon stationary phase, so that polar molecules have little affinity for the hydrophobic stationary phase and are eluted in order of decreasing polarity.

Elution was performed at 1.5 ml/min, with a mobile phase of methanol-acetonitrile-water 80:5:15 (Sotirhos *et al* 1985) and detector conditions of 2 mV and 0.1 Absorbance Units (Figure 7.4). Individual peaks were detected at an absorbance of 492 nm, identified by comparing their retention times with those of standard PC molecular species (Sigma Chemical Co.), and confirmed by mass spectrometry (Davis 1997). All samples were run in duplicate.

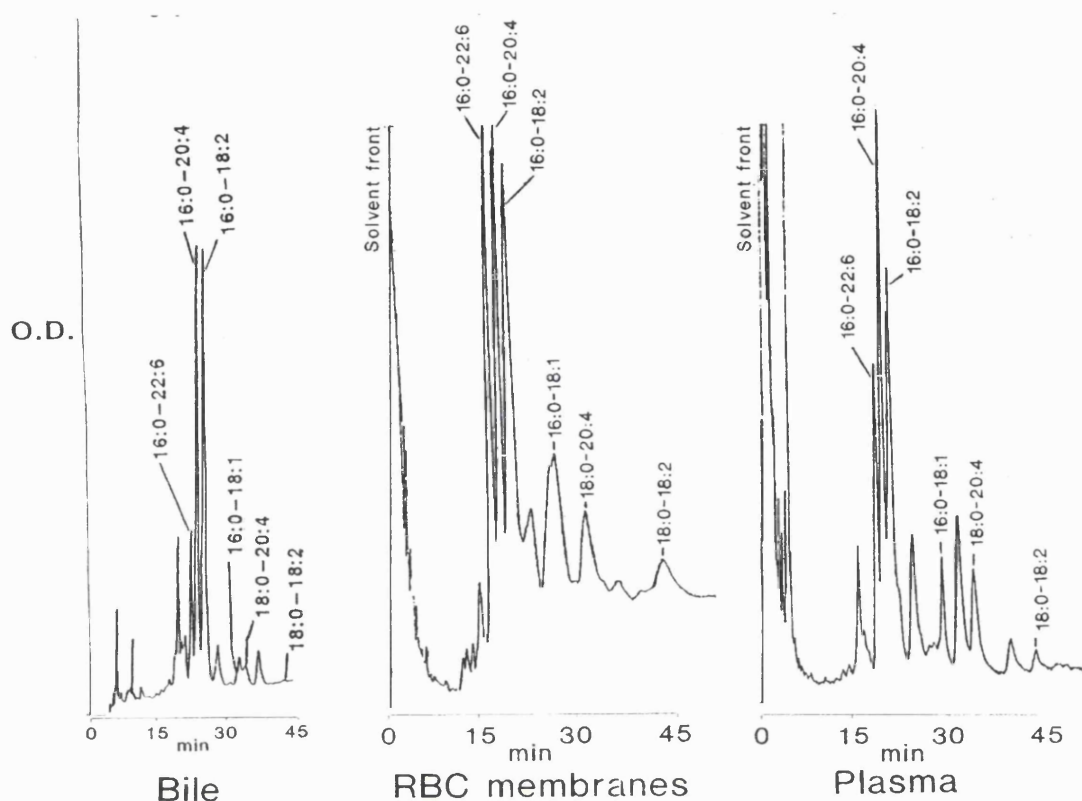


Figure 7.4 Separation of phosphatidylcholine species by HPLC in bile, red blood cell (RBC) membranes and plasma from a patient with gallstones.

### 7.2.7 Purification of gallbladder mucin by gel filtration

Gallbladder mucin was purified by a two-step gel filtration method, as previously described (Parker *et al* 1993; Finnie *et al* 1995). First, 200  $\mu$ l aliquots of gallbladder bile were loaded onto Sepharose CL-2B mini-columns (5 x 1.5 cm; Pharmacia: Uppsala, Sweden) and eluted with 0.1 M Tris-HCl pH 8. Twenty-four x 0.5 ml fractions were collected and monitored by ELISA using a CAM 17.1 monoclonal anti-mucin antibody (Raouf *et al* 1991) (Figure 7.5), as described in the following section. The mucin-containing fractions (2.5-5.5 ml elution volume) were then pooled, frozen at -70°C, lyophilised overnight and reconstituted in 600  $\mu$ l double-distilled water.

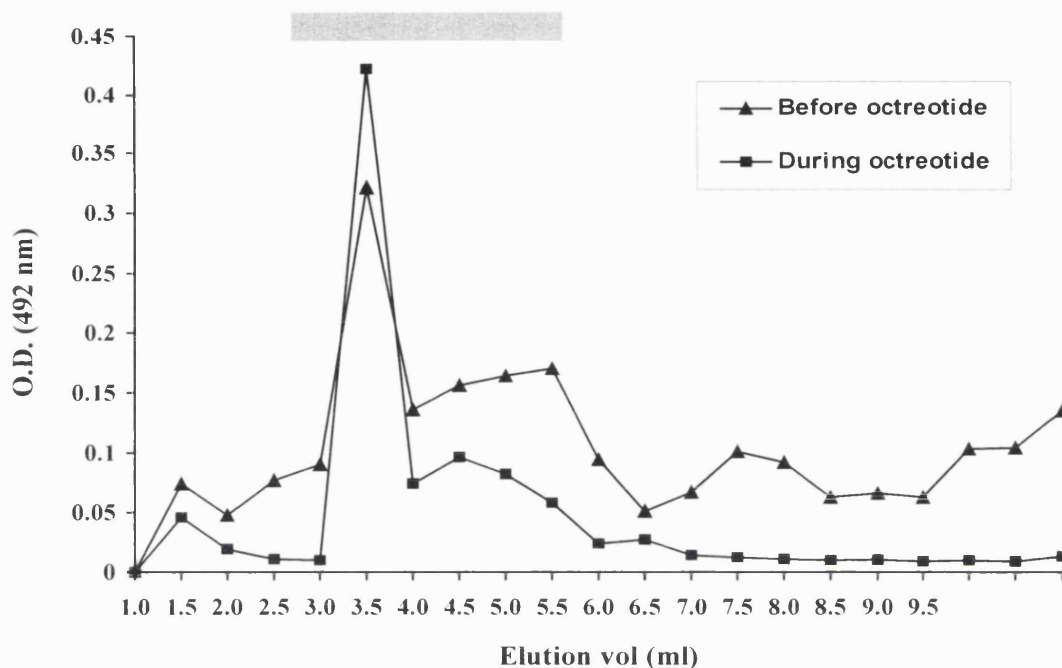


Figure 7.5 CAM 17.1 ELISA of Sepharose CL-2B fractions from the bile of an acromegalic patient before and during octreotide therapy. The shaded bar (2.5-5.5 ml) represents the void volume for mucin glycoprotein.

Next, the mucin was purified further by fast protein liquid chromatography (FPLC). Samples (200  $\mu$ l) of the reconstituted mucin were loaded onto a 30 x 1 cm Superose 6 column (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M tris-HCl pH 8 (degassed and 0.2  $\mu$ m filtered), using a Pharmacia Pump P-500 and Single Path Monitor UV-1. Thirty 1-ml fractions were collected at a flow rate of 0.25 ml/min and monitored at an absorbance of 280 nm. Calibration of the Superose 6 FPLC column was performed daily using high molecular weight standards from a gel filtration calibration kit (Pharmacia), at concentrations of 2.5-5 mg/ml (Figure 7.6).

# MOLECULAR WEIGHT STANDARDS ON SUPEROSE 6 FPLC

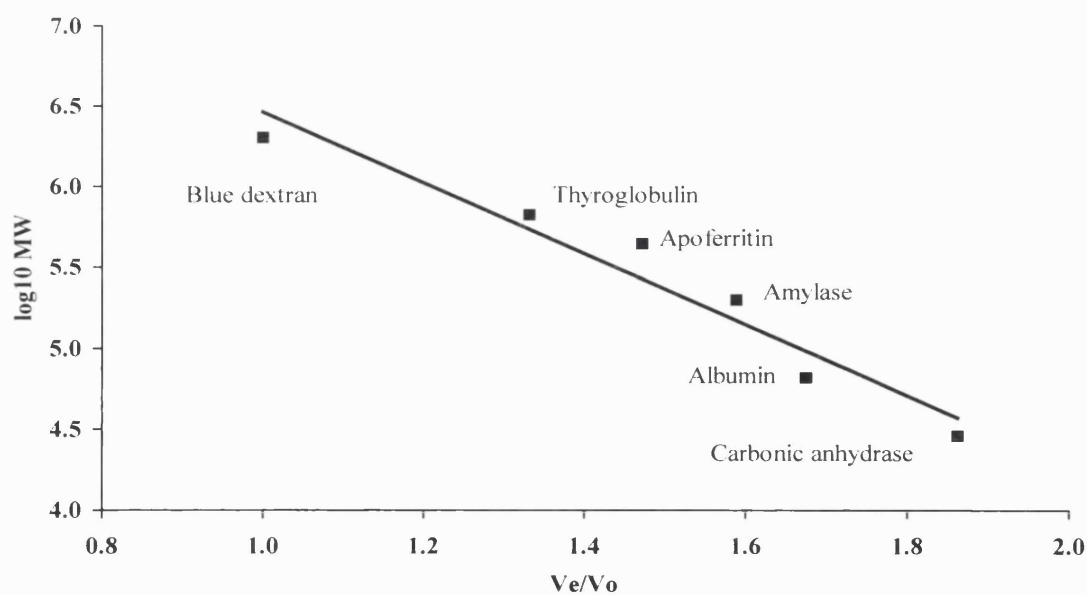
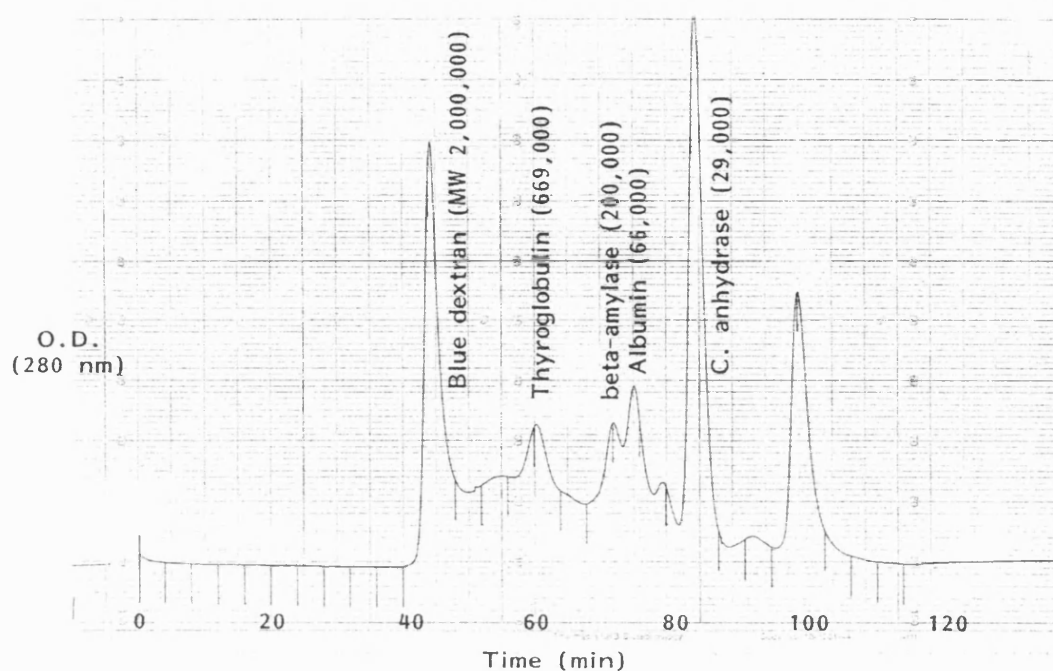


Figure 7.6 Calibration of the Superose 6 fast performance liquid chromatography (FPLC) column using molecular weight standards (upper panel). There was an inverse linear relationship between retention times on the column, expressed as ratios to that of blue dextran, and the  $\log_{10}$  molecular weights of the standards (lower panel).

As shown in Figure 7.7, the high-molecular-weight biliary mucin glycoprotein eluted from the column at 11-13 ml (40-52 min) under the conditions described above, with a similar retention time to that of blue dextran (molecular weight 2,000,000) and bovine submaxillary mucin (Sigma).

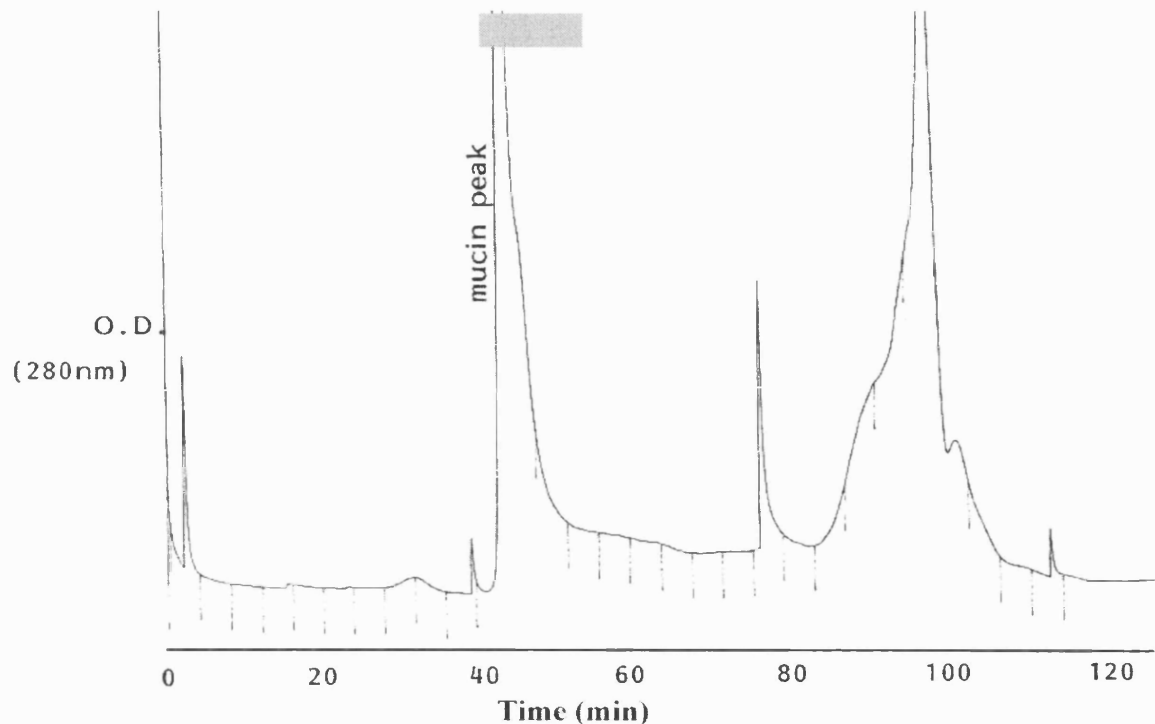


Figure 7.7 Superose 6 FPLC of mucin-enriched bile after Sepharose CL-2B gel filtration. The shaded bar at 11-13 ml represents the void volume for biliary mucin glycoprotein.

The void volume (11-13 ml fractions) was confirmed to contain mucin glycoprotein using a CAM 17.1 ELISA (Figure 7.8), as described in the following section, and to be free of concanavalin A-binding non-mucin glycoproteins, as assessed by a *Concanavalin A* enzyme-linked lectin-binding assay (data not shown). The concanavalin A assay was performed in a similar manner to the CAM 17.1 ELISA, except that a peroxidase-conjugated lectin, concanavalin A (Vector, Peterborough, UK), diluted 1:250 in phosphate-buffered saline (PBS)-Tween, was added instead of the CAM 17.1 + rabbit antimouse antibody.

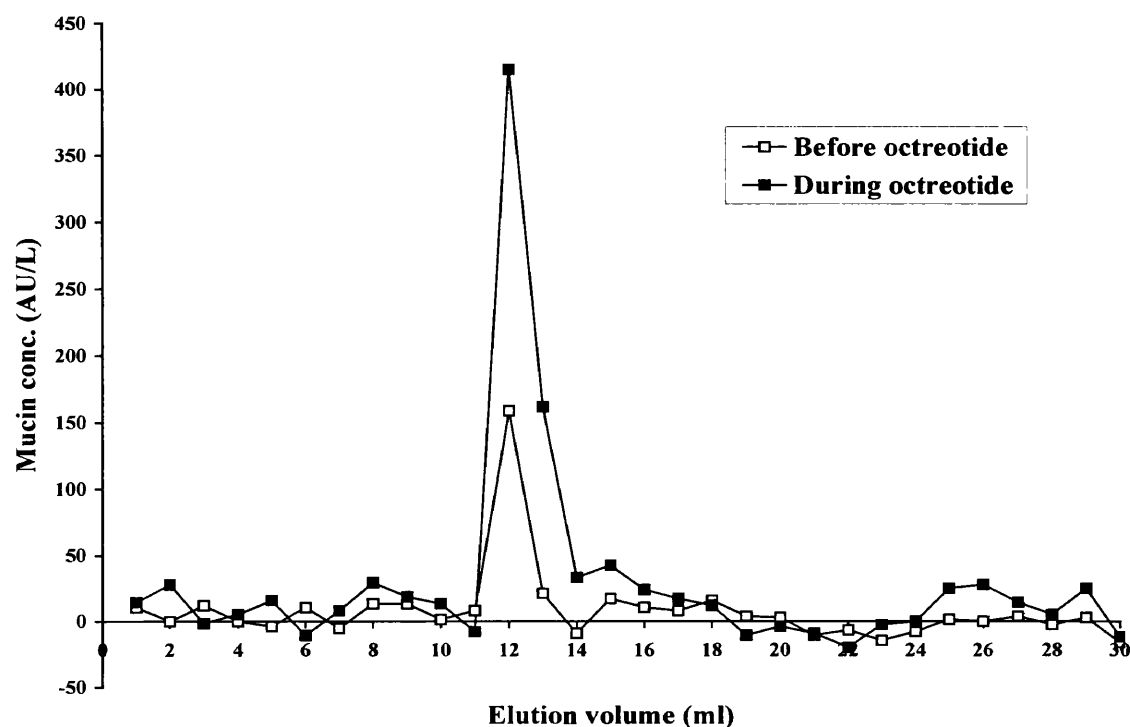


Figure 7.8 CAM 17.1 ELISA of Superose 6 fractions from an acromegalic patient before and during octreotide therapy.

### 7.2.8 Quantification of immunoreactive mucin by ELISA

CAM 17.1 is an immunoglobulin M antibody which binds the sialylated blood group I antigen, and has been shown immunohistochemically and serologically to be highly specific for intestinal mucus, particularly in the colon, small intestine, biliary tract and pancreas (Makin 1986; Parker *et al* 1992; Parker *et al* 1993).

Aliquots (50  $\mu$ l) from each Superose 6 fraction, which contained the mucin glycoprotein, were diluted 1:1 in 0.05 M carbonate buffer (pH 9.6) and coated onto ELISA plates for 16 h at 4°C. The plates were washed and blocked with PBS (pH 7.2) + Tween 20 (0.1%), followed by incubation with 100  $\mu$ l of CAM 17.1 (diluted 1 in 10 in PBS/Tween) for 2 h at 37°C. After further washing, 100  $\mu$ l aliquots of peroxidase-conjugated rabbit anti-mouse immunoglobulin (diluted 1/600 in PBS/Tween) were added to the plate and incubated at 37°C for a further 2 h. The plate was then developed

with 100  $\mu$ l of peroxidase substrate (0.4 mg/ml *o*-phenylenediamine + 0.04  $\mu$ l/ml H<sub>2</sub>O<sub>2</sub> in 0.05 M citrate/phosphate buffer, pH 5), and the reaction stopped after 5 min by adding 100  $\mu$ l of 4 M H<sub>2</sub>SO<sub>4</sub>. The plate was read at 492 nm (Parker *et al* 1992). Biliary mucin glycoprotein concentrations were then derived from standard curves using Superose 6-purified human colonic mucin glycoprotein as standard (Figure 7.9).

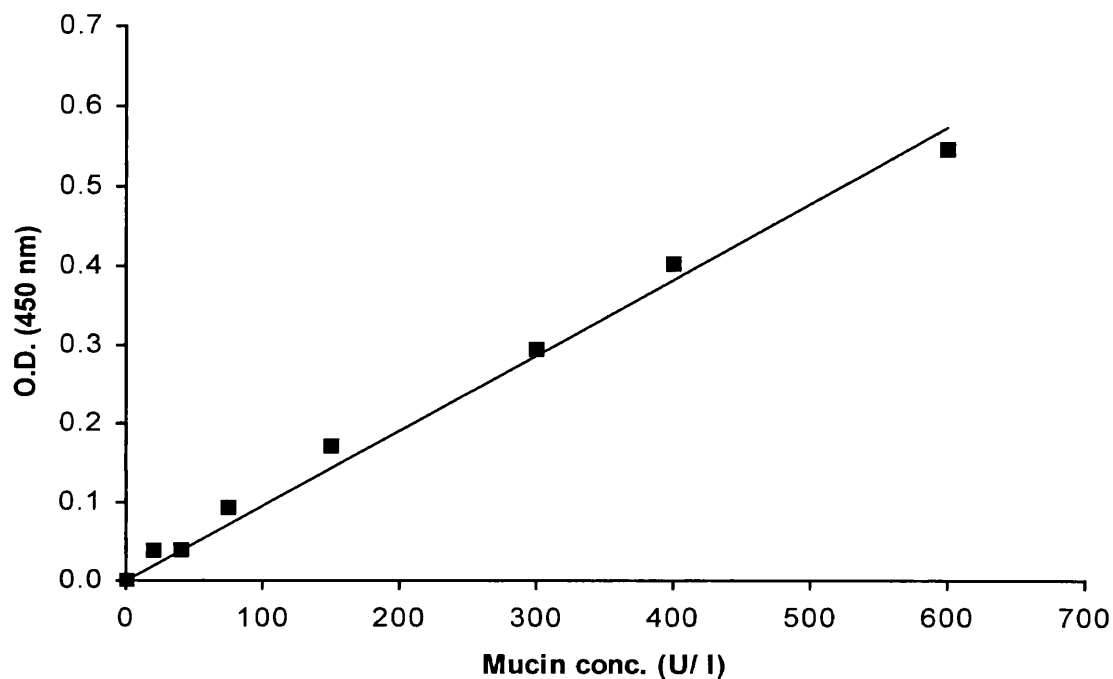


Figure 7.9 Example of a standard curve for the CAM 17.1 ELISA, using Superose 6-purified human colonic mucin glycoprotein as standard.

### 7.2.9 Statistical analysis

All results are expressed as means (SEMs). For comparisons of data between the groups, the Student's *t* test (two-tailed) or the Mann-Whitney non-parametric method was used, as appropriate. Differences in proportions between two groups were compared using the  $\chi^2$  test. Values of  $p < 0.05$  were considered to be significant.

## 7.3 RESULTS

### 7.3.1 Phosphatidylcholine species before and during octreotide

The distribution profile of the different species of phosphatidylcholine (PC), expressed as percentages of the total PC in the two groups of acromegalic patients, is shown in Figure 7.10. The paired and non-paired data for the two arachidonic acid-rich PCs in the acromegalic patients untreated with octreotide and in those given long-term octreotide, is shown in Figure 7.11.

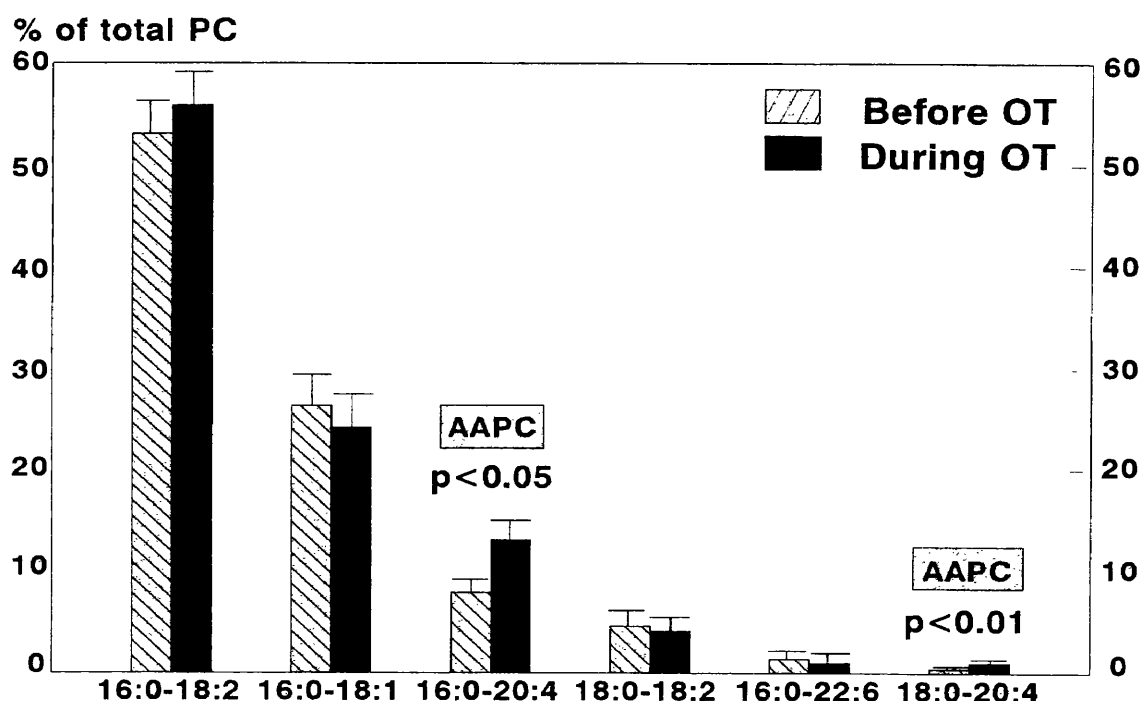


Figure 7.10 Effect of octreotide therapy on the distribution of phosphatidylcholine species in gallbladder bile (means  $\pm$  SEM). The two arachidonic acid-rich phosphatidylcholine species were the only species which increased significantly during treatment.

Overall, the relative proportions of the major PC molecular species, 16:0-18:2 and 16:0-18:1, were similar before and during octreotide. However, the arachidonic acid-rich PC 16:0-20:4 increased from a mean of  $8.1 \pm \text{SEM } 1.3\%$  (range, 3.6-13.4%) of total biliary PC before, to  $12.5 \pm 1.0\%$  (range, 8.5-16.6%) during octreotide ( $p < 0.01$ ), while PC 18:0-20:4 rose from  $0.37 \pm 0.11\%$  to  $0.74 \pm 0.09\%$  ( $p < 0.02$ ). In the five

patients in whom paired data were obtained, PC 16:0-20:4 increased in every case, from a mean of  $8.0 \pm 1.7\%$  before, to  $12.7 \pm 1.6\%$  during, octreotide ( $p < 0.05$ ), while PC 18:0-20:4 rose from  $0.4 \pm 0.18\%$  to  $0.8 \pm 0.09\%$  ( $p < 0.02$ ). The patient who developed gallstones after only three months of octreotide treatment, also had the steepest rise in the proportion of AAPLs during treatment (Figures 7.11a & b).

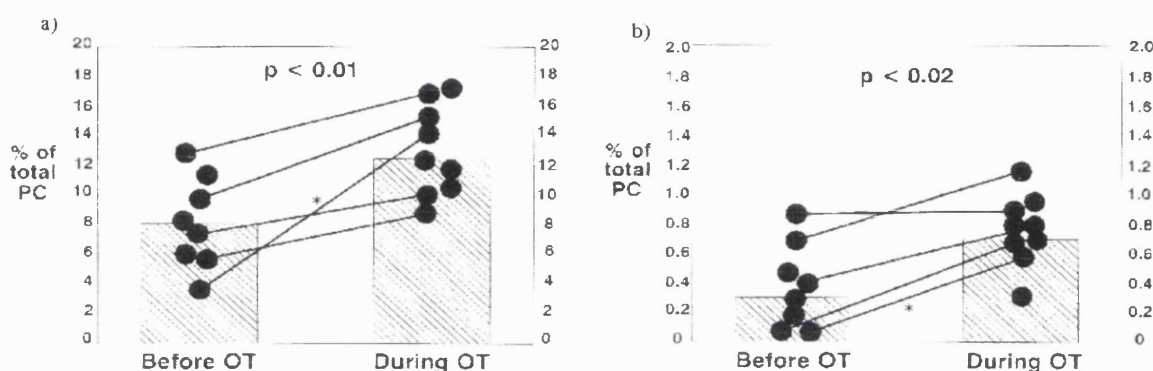


Figure 7.11 The proportions of a) PC 16:0-20:4 and b) PC 18:0-20:4, in gallbladder bile before and during octreotide treatment. The closed circles are the individual values, the lines connect the paired data, while the shaded bars show the means. One patient in the paired studies (\*) developed gallbladder stones during treatment.

### 7.3.2 Relationships between phosphatidylcholine and cholesterol saturation

The increase in the proportion of biliary arachidonic acid-rich phosphatidylcholine was associated with a rise in biliary cholesterol saturation, with the CSI increasing from  $0.90 \pm 0.05$  before treatment to  $1.2 \pm 0.05$  ( $p < 0.01$ ) during octreotide therapy. In the paired studies, the biliary CSIs rose in all five patients by a mean of 26%, from  $0.89 \pm 0.06$  to  $1.1 \pm 0.03$  ( $p < 0.02$ ). Similar rises were also seen in the % vesicular cholesterol (from  $37.7 \pm 3.5\%$  to  $59.4 \pm 6.2\%$ ;  $p < 0.02$ ) and the cholesterol : phospholipid molar (from  $0.52 \pm 0.05$  to  $0.73 \pm 0.11$ ;  $p < 0.01$ ), respectively. Furthermore, there were significant positive correlations between the most abundant arachidonic acid-rich phosphatidylcholine species, PC 16:0-20:4, and both the cholesterol saturation index ( $r = 0.57$ ,  $p < 0.02$ ) and % vesicular cholesterol ( $r = 0.67$ ,  $p < 0.01$ ) (Figures 7.12a & b).

### 7.3.3 Biliary deoxycholic acid

Before octreotide treatment, the proportion of biliary deoxycholic acid, expressed as a percentage of total biliary bile acids in the gallbladder bile of the eight stone-free acromegalic patients, was  $13.9 \pm 1.4\%$ . In the nine patients treated with octreotide, it increased to  $24.9 \pm 2.7\%$  ( $p < 0.01$ ). There was also a significant positive correlation between the % deoxycholic acid and PC 16:0-20:4, in gallbladder bile ( $r = 0.48$ ,  $p < 0.05$ ) (Figure 7.12c).

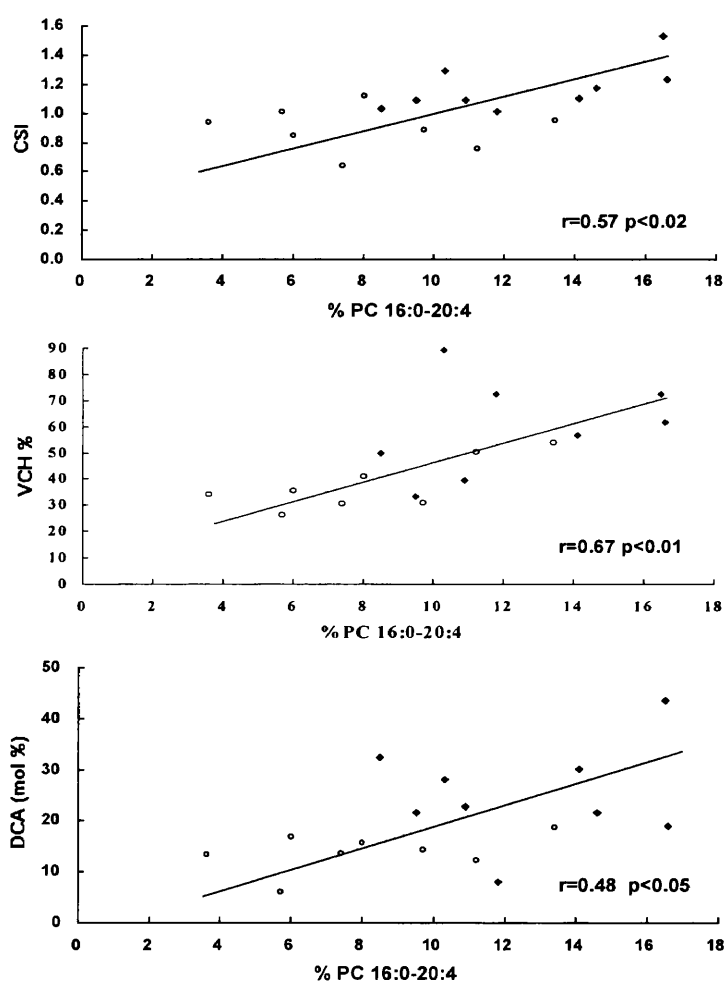


Figure 7.12 Pearson correlations for the % biliary PC 16:0-20:4 and a) the cholesterol saturation indices, b) % vesicular cholesterol, and c) the % deoxycholic acid in gallbladder bile (○ = no octreotide, ◆ = during octreotide treatment).

#### 7.3.4 Mucin glycoprotein concentrations

During octreotide treatment, there was a 121% increase in mean gallbladder mucin concentration, from  $2.8 \pm 1.2$  to  $6.2 \pm 2.2$  U/l, but this difference was not statistically significant (Figure 7.13). Moreover, there was no significant relationship between gallbladder mucin concentration and either the cholesterol saturation index or the % distribution of different PC species, in gallbladder bile.

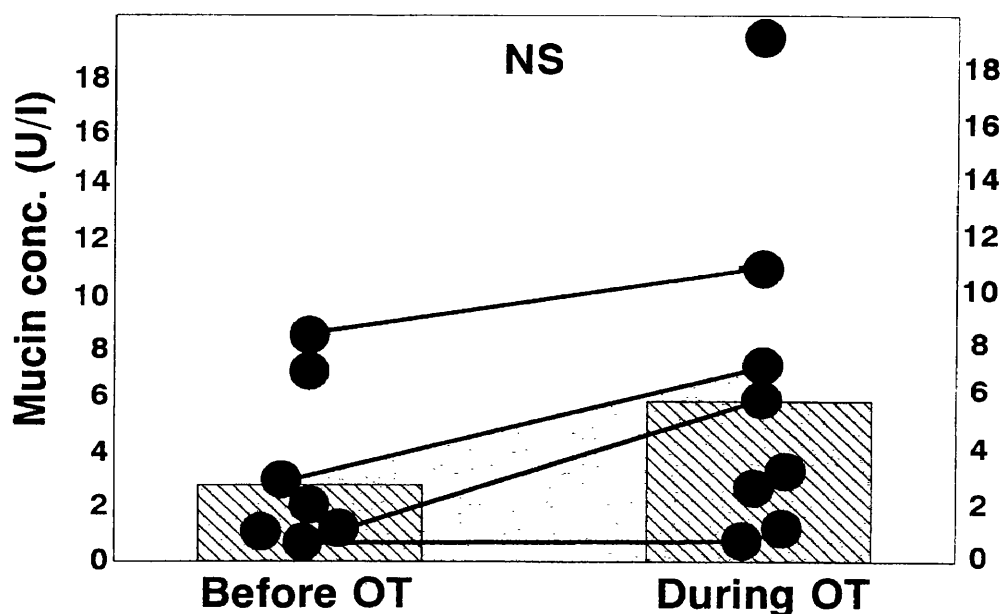


Figure 7.13 Biliary mucin glycoprotein concentrations before and during octreotide treatment.

#### 7.4 DISCUSSION

Several previous studies have shown that octreotide inhibits meal-stimulated cholecystokinin release from the intestine (Creutzfeldt *et al* 1987; Lembcke *et al* 1987; Stolk *et al* 1993) and effectively abolishes meal-stimulated gallbladder emptying (Lembcke *et al* 1987; van Liessum *et al* 1989; Catnach *et al* 1993; Stolk *et al* 1993; Moschetta *et al* 2001). Although impaired gallbladder contractility is undoubtedly one factor in the development of octreotide-induced gallbladder stones, the results of the present study extend our previous observations (Hussaini *et al* 1994) by showing that

octreotide also induces marked changes in bile composition and physical chemistry. In acromegalic patients treated with octreotide for at least three months, the proportion of the two arachidonic acid-rich phospholipid species in gallbladder bile (PC 16:0-20:4 and PC 18:0-20:4) both increased significantly. There were associated significant rises in: (i) the CSI, (ii) the vesicular cholesterol and cholesterol : phospholipid molar ratio, and (iii) the proportion of deoxycholic acid, and a non-significant doubling of mucin glycoprotein levels in gallbladder bile.

These findings provide further insight into the pathogenesis of octreotide-induced gallstones but they may also be relevant to that of 'conventional' cholesterol-rich gallstones. In cholesterol gallstone disease, hepatic cholesterol hypersecretion is associated with an increased proportion of unsaturated molecular species of PC, particularly arachidonic acid-rich (20:4) species, in bile (Berr *et al* 1992; Hatsushika *et al* 1993). Furthermore, results of studies using model bile systems (Cohen and Carey 1991; Halpern *et al* 1992; Halpern *et al* 1993; Ochi *et al* 1996) and human gallbladder bile (Booker *et al* 1992) indicate that the molecular species of PC are distributed asymmetrically between micelles and vesicles. Partitioning of cholesterol between these two lipid carriers is determined by the fatty acid chain length in substitution one (*sn-1*) position, and the degree of unsaturation of the fatty acids at both the *sn-1* and *sn-2* positions of PC (Cohen and Carey 1991; Booker *et al* 1992). By virtue of the looser packing constraints imposed by their long chain length, and particularly by their high degree of unsaturation, arachidonic acid-rich PCs partition less well into highly ordered biliary vesicles than into micelles. Therefore, arachidonic acid-rich phospholipids are found in higher concentrations in mixed micelles, than in vesicles (Cohen and Carey 1991; Booker *et al* 1992; Halpern *et al* 1992). Although we did not measure the phospholipid composition of the mixed micelles and vesicles, we did find significant positive correlations between the main arachidonic acid-rich phospholipid species, PC 16:0-20:4, and the CSI, the % vesicular cholesterol, and the cholesterol : phospholipid molar ratio. These findings are consistent with the hypothesis that, as less PC becomes available for vesicle formation, fewer cholesterol-rich vesicles form. However, those which do form will have an increased cholesterol : phospholipid molar ratio (Cohen

and Carey 1991) — thus favouring aggregation and fusion of vesicles, and cholesterol microcrystal nucleation.

In addition to enhancing cholesterol secretion into bile and destabilising the cholesterol carriers, arachidonic acid-rich PC species may also play a role in stimulating mucin glycoprotein production by the gallbladder mucosa. Gallbladder mucin, a high molecular weight glycoprotein, is a major secretory product of the gallbladder epithelium. It can bind biliary lipids (Lee and Smith 1989; Nunes *et al* 1999) and accelerate the trapping and nucleation of cholesterol microcrystals, in both supersaturated model, and native, biles (Levy *et al* 1984; Smith 1987; Afdhal *et al* 1993; Yamasaki *et al* 1993). In obese subjects on very low calorie diets, who are at high risk of cholesterol gallstone formation, increases in mucin glycoprotein secretion are preceded by increases in biliary arachidonic acid concentrations and mucosal prostaglandin synthesis (Marks *et al* 1992; Marks *et al* 1997). In the prairie dog model, when aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) are given together with a lithogenic diet, they inhibit gallbladder mucosal prostaglandin synthesis and prevent mucin glycoprotein hypersecretion, as well as microcrystal and gallstone formation (Lee *et al* 1981a). Similar findings were reported in a study of obese individuals treated with high-dose (1300 mg per day) aspirin during acute weight reduction (Broomfield *et al* 1988), although epidemiological studies of the effect of chronic NSAID ingestion on the incidence of gallstones have yielded conflicting results (Hood *et al* 1993; Pazzi *et al* 1998; Sterling and Shiffman 1998). The present results, showing a significant increase in biliary arachidonic acid-rich phospholipids and a two-fold rise in mucin glycoprotein concentration during octreotide treatment, are consistent with these earlier findings. They provide indirect support for the hypothesis that biliary arachidonic acid-rich phospholipid concentrations may influence biliary mucin glycoprotein levels through the conversion of arachidonic acid to prostaglandins.

The mechanisms whereby increased proportions of biliary deoxycholic acid favour gallstone formation are complex. We (Hussaini *et al* 1995b) and others (Hofmann *et al* 1982; Angelico *et al* 1992; Berr *et al* 1992; Shoda *et al* 1995) have found that there is a linear relationship between the % deoxycholic acid, and both the CSI and moles % cholesterol in bile. Furthermore, the present results are consistent with those of

previous studies (Cantafora *et al* 1983; van Berge Henegouwen *et al* 1987; Hatsushika *et al* 1993), which showed that, in patients with cholesterol-rich gallstones, the % deoxycholic acid in bile correlates positively with the proportion of arachidonic acid-rich phospholipids in bile.

We showed previously that in individuals with rapid nucleation of cholesterol microcrystals (< 5 days), the mean % deoxycholic acid in bile was approximately twice as great as that in those with normal nucleation times (> 10 days) (Hussaini *et al* 1994). Whether this difference in nucleation time is due directly to the % deoxycholic acid in bile, or indirectly to secondary increases in biliary cholesterol saturation, mucin glycoprotein hypersecretion and/or cholesterol microcrystal nucleation, is unclear. Nonetheless, there is strong indirect evidence that the principal mechanism for the increased % deoxycholic acid in the bile of patients with octreotide-associated gallstones, is prolongation of intestinal transit. Thus, several previous studies have reported that both native somatostatin (Efendic and Mattsson 1978; Johansson *et al* 1978; Konturek 1978; Johansson *et al* 1981), and its analogue octreotide (Fuessl *et al* 1987; Lembcke *et al* 1987; Moller *et al* 1988; O'Donnell *et al* 1990; Hussaini *et al* 1996; Veysey *et al* 1999), prolong mouth-to-caecum transit times. In paired, before and during treatment, studies of acromegalic patients, Veysey *et al* (1999) showed that the mean colonic transit time increased by approximately 15 h, with an associated linear increase in serum deoxycholate (expressed as a percentage of serum total bile acids). Subsequent studies from our Unit found that prolonged colonic transit times, increased bacterial 7 $\alpha$ -dehydroxylation activity in caecal aspirates, and transit-induced increases in distal colonic luminal pH, were all independent risk factors for high proportions of deoxycholic acid in serum (and by implication in bile) (Thomas *et al* 2000; Thomas *et al* 2001b). Thus, octreotide-induced changes in intestinal transit, leading to an increased proportion of deoxycholic acid, may induce a complex series of changes in bile composition and physical chemistry which favour gallstone formation (Hussaini *et al* 1996; Dowling 2000a).

In summary, in acromegalic patients treated with octreotide for at least three months, there are increases in the proportion of both arachidonic acid-rich phospholipid species in gallbladder bile. Based on the present results, and those of earlier studies from our

Unit (Hussaini *et al* 1994; Hussaini *et al* 1996; Veysey *et al* 1999) and by others (Hofmann *et al* 1982; Angelico *et al* 1992; Berr *et al* 1992; Shoda *et al* 1995), the increase in biliary deoxycholic acid may be at least partly responsible for this rise in arachidonic acid-rich phospholipids. These changes, in turn, contribute to cholesterol supersaturation and mucin glycoprotein hypersecretion which, in conjunction with the abolition of meal-stimulated gallbladder emptying induced by octreotide, predispose to the rapid nucleation of microcrystals and cholesterol gallstone formation.

**CHAPTER 8. BILE COMPOSITION IN INFLAMMATORY BOWEL  
DISEASE: ILEAL DISEASE AND COLECTOMY, BUT NOT COLITIS,  
INDUCE LITHOGENIC BILE**

## **8.1 INTRODUCTION**

In patients with Crohn's disease involving the small intestine, the prevalence of gallbladder stones is higher than that in the general population (Heaton and Read 1969; Cohen *et al* 1971; Lorusso *et al* 1990; Hutchinson *et al* 1994; Lapidus *et al* 1999). One hypothesis for this increased risk is that bile acid malabsorption, secondary to impaired active bile acid transport as a consequence of ileal disease/resection, leads to a reduction in the total bile acid pool and an increase in biliary cholesterol saturation (Dowling *et al* 1972; Rutgeerts *et al* 1986; Rutgeerts *et al* 1987; Santavirta *et al* 1990; Heubi *et al* 1992). In patients with ulcerative or Crohn's colitis, or who have undergone colectomy, the bile acid malabsorption is less than in those with ileal dysfunction or resection, but the risk of gallstone formation is still increased — allegedly by the same mechanism (Lorusso *et al* 1990; Santavirta *et al* 1990; Hylander *et al* 1991; Natori *et al* 1992). However, supersaturated bile has been reported in only a minority of patients who have undergone colonic resection (Rutgeerts *et al* 1986; Makino *et al* 1994) — in contrast to most (Dowling *et al* 1972; Rutgeerts *et al* 1986; Rutgeerts *et al* 1987; Heubi *et al* 1992), but not all (Lapidus and Einarsson 1991), studies of patients with ileal disease/resection.

An alternative explanation for gallstone formation in small bowel Crohn's disease is that bile acids which are malabsorbed in the small intestine, spill into the colon where they solubilise unconjugated bilirubin and increase its enterohepatic cycling. In turn, this increases the rate of bilirubin secretion into bile and the risk of pigment gallstone formation. In support of this hypothesis, Brink *et al* (Brink *et al* 1999) found a two- to three-fold increased concentration of total bilirubin in gallbladder bile samples obtained intraoperatively from 23 patients with Crohn's ileitis or previous ileectomy, compared with that in those with Crohn's colitis (n=6) or ulcerative colitis (n=19) — with the highest values being in the four patients with extensive (>50 cm) ileal involvement.

However, only one of the 23 patients with Crohn's ileitis/ileectomy developed pigment gallstones whereas two others had cholesterol-rich gallstones by chemical analysis and five had cholesterol monohydrate crystals detected microscopically. To date, there have been no large studies of gallstone composition in such patients and there are few data on other factors known to be important in 'conventional' cholesterol gallstone disease — such as the rate of cholesterol microcrystal nucleation or changes in biliary phospholipid composition.

The aims of the present study, therefore, were to determine the cholesterol saturation indices, microcrystal nucleation times and total bilirubin concentration, in the bile of patients with ileal or colonic Crohn's disease and those who had undergone colonic resection, and to compare these values with those from a group of patients with active ulcerative colitis or who were post-colectomy. In the patients with Crohn's disease, we also determined the biliary bile acid composition and molecular species of phosphatidylcholine (PC; the principal biliary phospholipid), and compared these values with those from a group of patients with 'conventional' cholesterol-rich gallstones.

## **8.2 METHODS**

### **8.2.1 Patients**

The patients with inflammatory bowel disease were recruited for the study by the Academic Department of Surgery at the Queen Elizabeth Hospital, Birmingham. The clinical details of the patients are given in Table 8.1.

The site and extent of disease (small bowel, colon or both) were determined by endoscopic and radiological methods, together with information from surgical exploration/resection and histological examination. The results of liver function tests were normal in all patients, and none had received total parenteral nutrition. None of the patients had undergone cholecystectomy and all were free of gallbladder stones, as assessed by pre-operative ultrasonography (Hutchinson *et al* 1994) and direct palpation of the gallbladder at the time of surgery.

Twenty-three patients (15 men, eight women) had Crohn's disease, with a mean age of 40 (range 20-76) years. On the basis of their clinical characteristics, the patients were divided into three groups. In 12 patients, the Crohn's disease was confined to the colon, of whom (i) seven had active colitis and were admitted for colectomy, and (ii) five had undergone previous colectomy (n=2) or defunctioning loop ileostomy (n=3) and had inactive disease. The third group consisted of 11 patients with ileal (n=5) or ileocolonic (n=6) disease who were admitted for small bowel resection. Apart from one patient with steroid-resistant Crohn's colitis who required urgent colectomy, all were admitted for elective surgery after they had failed to respond to medical treatment, and underwent laparotomy after an overnight fast.

| Patient group                           | Sex<br>(M/F) | Age*<br>(yr)  | Disease<br>duration† (yr) | Previous surgery                                                              | Medical treatment                  |
|-----------------------------------------|--------------|---------------|---------------------------|-------------------------------------------------------------------------------|------------------------------------|
| <b>Crohn's disease (n=23)</b>           |              |               |                           |                                                                               |                                    |
| Active colitis (n=7)                    | 4/3          | 34<br>(23-65) | 2<br>(1-5)                | Loop ileostomy (n=1)                                                          | Steroids (n=3)<br>Mesalazine (n=1) |
| Active ileitis or<br>ileocolitis (n=11) | 7/4          | 37<br>(20-61) | 12<br>(3-20)              | Ileal resection (n=4)<br>Right hemicolectomy (n=3)<br>Colonic resection (n=3) | Steroids (n=6)                     |
| Post-colectomy<br>(n=5)                 | 4/1          | 49<br>(34-76) | 5<br>(2-12)               | Colectomy (n=2)<br>Loop ileostomy (n=3)                                       | Nil                                |
| <b>Ulcerative colitis (n=31)</b>        |              |               |                           |                                                                               |                                    |
| Active colitis (n=14)                   | 11/3         | 42<br>(16-75) | 2.5<br>(1-10)             | Nil                                                                           | Steroids (n=11)                    |
| Post-colectomy<br>(n=17)                | 13/4         | 40<br>(16-66) | 5<br>(1-21)               | Colectomy + ileostomy<br>(n=17)                                               | Nil                                |

Table 8.1 Clinical data of the patients with Crohn's disease and ulcerative colitis. \* Mean (range) † "Disease duration" (from the time of diagnosis to the time of the study): median (range)

Thirty-one patients had ulcerative colitis, of whom 24 were men and seven women, with an age range of 16-75 (mean 41) years. The patients either: (i) had active colitis (n=14; admitted for total colectomy because of failed medical treatment), or (ii) were

admitted for J-pouch ileo-anal anastomosis, following colectomy with formation of an ileostomy and rectal stump 1-2 months earlier (n=17). In six of the 31 patients, paired bile samples were obtained — first during active colitis and again 1-2 months after total colectomy.

### **8.2.2 Aspiration of gallbladder bile**

The technique of intra-operative sampling of gallbladder bile was approved by the Ethics Committee of Guy's Hospital and the South Birmingham Ethical Committee. Immediately after opening the abdomen and before manipulation of the bowel, bile was sampled by the technique of fine-needle gallbladder puncture described by Strasberg *et al* (1990a). To avoid possible sampling errors as a result of stratification of bile, the gallbladder contents were aspirated as completely as possible. No clinical evidence of bile leakage occurred, and there was no apparent morbidity associated with the procedure. Bile samples thus obtained were stored immediately at -20°C until analysed.

### **8.2.3 Cholesterol microcrystal nucleation times**

Immediately after obtaining the samples, aliquots of gallbladder bile were centrifuged at 100,000 g for 2 h. This process separated bile into three phases; an upper layer consisting of liquid crystals, a lower phase containing solid crystals and cellular debris, and a clear middle phase. This 'isotropic phase' was then incubated at 37°C and examined daily with polarised light microscopy for up to 21 days (Holan *et al* 1979), as described in Chapter 6.2.3. The time taken in days for cholesterol microcrystals to appear was defined as the nucleation time. If there had been no nucleation of cholesterol microcrystals by 21 days, the observations were abandoned.

### **8.2.4 Bile physical chemistry**

Total biliary bile acids, phospholipids and cholesterol were determined by standard enzymatic assays (Talalay 1960; Roschlau *et al* 1973; Qureshi *et al* 1980) (Chapter 6).

Total lipid concentration was calculated from the sum of the bile acid, phospholipid and cholesterol concentrations, and expressed in mM and grams per decilitre. The cholesterol saturation indices (CSIs) were derived using the polynomial equation described by Thomas and Hofmann (1973).

Total bilirubin concentrations were determined enzymatically using the bilirubin oxidase method (Perry *et al* 1986). Preliminary studies in our laboratory showed that results obtained using this assay correlated strongly (n=10 bile samples, r=0.997) with those obtained using the Jendrassik-Grof diazo method, which has been reported to be less susceptible to interference by photobilirubin (Ihara *et al* 1990).

All assays were performed in duplicate. Since varying dilutions of gallbladder bile could, theoretically, have influenced the pattern of biliary bilirubin results, the results for total bilirubin concentrations were expressed both as uncorrected data and, after standardisation to a total bile acid concentration of 100 mM, as corrected data.

### **8.2.5 Bile acid conjugates and phosphatidylcholine species**

In 16 of the 23 patients with Crohn's disease, sufficient bile was obtained to assess both bile acid composition and the molecular species (fatty acid composition) of PC, the principal biliary phospholipid. The concentrations of the individual bile acid conjugates were determined using reverse-phase high-performance liquid chromatography (HPLC) (Wildgrube *et al* 1983), and expressed as a percentage of total conjugated bile acids. Following Fölch extraction of the lipid phase of gallbladder bile (Fölch *et al* 1957), PC was separated from the other biliary lipids by HPLC (Patton *et al* 1982), and the molecular species of the separated PC determined by reverse phase HPLC (Sotirhos *et al* 1985), as described in Chapter 7. Results were compared with those obtained from a group of 13 patients (10 women, 3 men; mean age 45 years, range 31-69 years) with 'conventional' cholesterol-rich gallstone disease, in whom gallbladder bile was obtained by either fine-needle aspiration during elective cholecystectomy (n=4) or by ultrasound-guided percutaneous transhepatic fine-needle puncture (n=9), as previously described (Hussaini *et al* 1995c). In these patients, evidence that the stones were indeed cholesterol-rich was based either on chemical

analysis of stones (> 70% cholesterol by weight) retrieved at cholecystectomy (4 patients) or indirectly by a maximum gallstone attenuation score of < 100 Hounsfield Units at localised computed tomography (CT) of the gallbladder (9 patients) (Pereira *et al* 1997).

#### **8.2.6 Statistical analysis**

All results are expressed as means  $\pm$  SEM. A two-tailed Student's *t* test was used to determine the significance of differences between two groups, and the  $\chi^2$  test or Fisher's exact test for differences in proportions between groups. P values < 0.05 were considered to be significant.

### **8.3 RESULTS**

#### **8.3.1 Bile lipids and cholesterol saturation indices**

The group data for total lipid concentration, biliary bile acids, phospholipids and cholesterol (means  $\pm$  SEMs), and for the derived CSIs, in the patients with Crohn's disease and ulcerative colitis are given in Table 8.2.

In the patients with Crohn's disease, the mean biliary bile acid, phospholipid and cholesterol concentrations (expressed in mM, and as moles percent of the total lipid concentration, respectively) were similar between the three groups. Total lipid concentrations in the two ulcerative colitis groups were also comparable, but the mean bile acid concentration in the post-colectomy ulcerative colitis group was significantly higher than that in both the active Crohn's colitis ( $p < 0.01$ ) and Crohn's post-colectomy groups ( $p < 0.05$ ). The mean cholesterol concentration in the post-colectomy ulcerative colitis patients was also higher than that in the patients with active Crohn's colitis ( $p < 0.01$ ).

|                               | Crohn's disease            |                                      |                            | Ulcerative colitis         |                              |                                                                          |
|-------------------------------|----------------------------|--------------------------------------|----------------------------|----------------------------|------------------------------|--------------------------------------------------------------------------|
|                               | Active colitis (n=7)       | Active ileitis or ileocolitis (n=11) | Post-colectomy (n=5)       | Active colitis (n=14)      | Post-colectomy (n=17)        |                                                                          |
| Total lipid concentration: MM | 167.2 ± 22.3               | 201.7 ± 29.5                         | 173.5 ± 41.2               | 228.5 ± 20.9               | 229.6 ± 21.9                 |                                                                          |
| Total bile acids: MM          | 112.2 ± 17.6 <sup>1</sup>  | 136.8 ± 20.9                         | 116.1 ± 27.3 <sup>2</sup>  | 160.5 ± 15.3               | 162.0 ± 9.2 <sup>1,2</sup>   | <sup>1</sup> p=0.01, <sup>2</sup> p=0.05                                 |
| mol %                         | 66.8 ± 4.1                 | 67.4 ± 3.4                           | 67.3 ± 1.8                 | 70.2 ± 1.5                 | 67.8 ± 1.6                   |                                                                          |
| Phospholipid: MM              | 45.7 ± 7.2                 | 50.4 ± 8.9                           | 43.1 ± 10.4                | 53.9 ± 5.3                 | 55.5 ± 5.4                   |                                                                          |
| mol %                         | 27.6 ± 3.5                 | 25.2 ± 2.7                           | 24.9 ± 1.2                 | 23.6 ± 0.9                 | 24.0 ± 1.1                   |                                                                          |
| Cholesterol: MM               | 9.3 ± 1.6 <sup>1</sup>     | 14.5 ± 2.3                           | 14.3 ± 4.1                 | 14.2 ± 2.2                 | 19.4 ± 2.6 <sup>1</sup>      | <sup>1</sup> p<0.01                                                      |
| mol %                         | 5.6 ± 0.8 <sup>2</sup>     | 7.4 ± 0.8                            | 7.8 ± 0.8                  | 6.2 ± 0.7                  | 8.2 ± 0.8 <sup>2</sup>       | <sup>2</sup> p<0.05                                                      |
| Cholesterol saturation index: | 1.1 ± 0.1 <sup>1,2,3</sup> | 1.5 ± 0.1 <sup>1,4,5</sup>           | 1.6 ± 0.1 <sup>2,6,7</sup> | 0.8 ± 0.1 <sup>4,6,8</sup> | 1.2 ± 0.1 <sup>3,5,7,8</sup> | <sup>1,5</sup> p<0.05, <sup>2,3,7,8</sup> p<0.01, <sup>4,6</sup> p<0.001 |
| Total bilirubin: (mM)         | 4.6 ± 1.2 <sup>1</sup>     | 10.5 ± 1.9 <sup>1,2</sup>            | 3.0 ± 1.6 <sup>2</sup>     | 7.7 ± 1.4                  | 6.1 ± 1.3                    | <sup>1,2</sup> p<0.05                                                    |
| (mM/100 mM bile acids)*       | 5.3 ± 1.9                  | 9.3 ± 2.4 <sup>1</sup>               | 2.5 ± 0.9 <sup>1</sup>     | 5.1 ± 0.9                  | 3.5 ± 0.7                    | <sup>1</sup> p<0.01                                                      |

Table 8.2 Bile physical chemistry in the patients with Crohn's disease and ulcerative colitis. All data are expressed as means ± SEM. \*Individual bile acid concentrations were corrected to a standard bile acid concentration of 100 mM.

The mean values and distribution of individual data points for the CSIs are given in Table 8.2 and Figure 8.1, respectively.

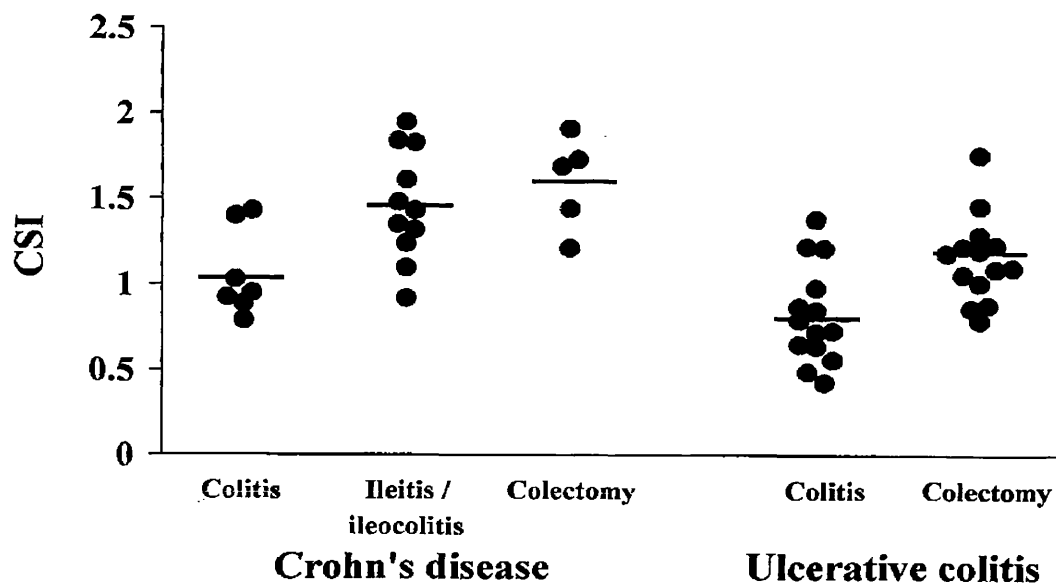


Figure 8.1 The distribution of individual data points for the cholesterol saturation indices (CSIs). The horizontal bars represent the mean values. The broken horizontal line represents the limit above which bile is supersaturated with cholesterol. P values are given in Table 8.2.

In the Crohn's patients whose disease was confined to the colon, the bile was significantly less supersaturated with cholesterol (mean CSI  $1.1 \pm 0.1$ ) than that from patients with ileal or ileocolonic disease ( $1.5 \pm 0.1$ ;  $p < 0.01$ ) or who were post-colectomy ( $1.6 \pm 0.1$ ;  $p < 0.01$ ). In the 14 patients with active ulcerative colitis, the mean CSI of  $0.8 \pm 0.1$  was also significantly lower ( $p < 0.01$ ) than the value of  $1.2 \pm 0.1$  in the 17 patients who had undergone colectomy, with the fashioning of an ileostomy and rectal stump, 1-2 months earlier. Thus, only three of the 14 patients with active ulcerative colitis had supersaturated bile, compared with 11 of the 14 post-colectomy patients in whom CSIs were measured ( $p < 0.01$ ). Similarly, in the six patients with ulcerative colitis in whom paired bile samples were obtained, the CSI increased in every case, from a mean of  $0.8 \pm 0.1$  before, to  $1.2 \pm 0.1$  after, colectomy ( $p < 0.001$ ). There were no significant differences in biliary cholesterol saturation indices between the Crohn's colitis and ulcerative colitis patients, or between the two post-colectomy groups, but all other CSI comparisons between the Crohn's disease and ulcerative colitis groups were statistically significant.

### 8.3.2 Cholesterol microcrystal nucleation times

The time in days for cholesterol microcrystals to nucleate and grow to a detectable size in bile is shown in Figure 8.2.

In all seven patients with Crohn's colitis, and in 11 of the 14 with ulcerative colitis, there had been no nucleation of cholesterol microcrystals by 21 days, at which time the observations were abandoned. In those with Crohn's colitis, the median nucleation time of 21 days was significantly longer than the median value of 5 days in the post-colectomy group ( $p < 0.01$ ), with an intermediate time of 14 days in those with Crohn's ileitis or ileocolitis ( $p < 0.05$  v colitis alone). In the patients with ulcerative colitis, three of the 14 with active disease had short ( $\leq 10$  days) nucleation times, compared with

nine of the 14 post-colectomy patients in whom nucleation times were measured (p<0.05).

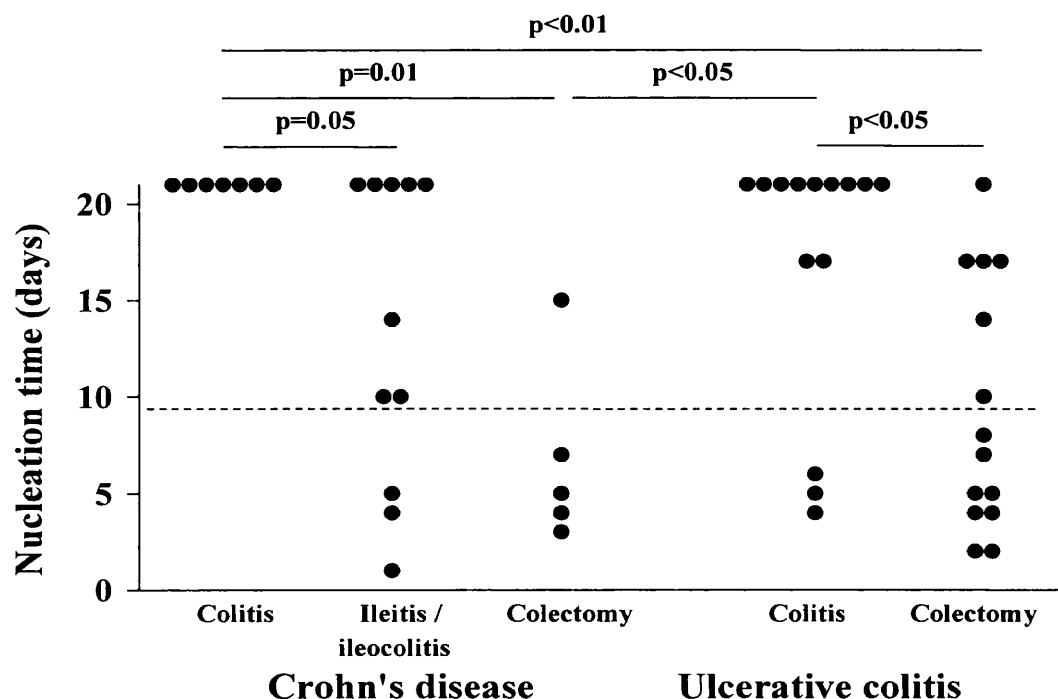


Figure 8.2 Cholesterol microcrystal nucleation times in the patients with Crohn's disease and ulcerative colitis. The broken horizontal line (at 10 days) represents the limit above which nucleation time is normal.

### 8.3.3 Biliary bilirubin levels

In the 11 patients with active Crohn's ileitis or ileocolitis, the mean total biliary bilirubin concentration (uncorrected for bile acid concentration) was  $10.5 \pm 1.9$  mM, which was approximately double that in the seven patients with active Crohn's colitis alone ( $4.6 \pm 1.2$  mM;  $p < 0.05$ ) and more than three-fold higher than that in the Crohn's post-colectomy group ( $3.0 \pm 1.6$  mM;  $p < 0.03$ ) (Table 2). Correction of biliary bilirubin concentrations to a standard bile acid concentration of 100 mM increased the significance of differences between the groups only in the active ileitis/ileocolitis and post-colectomy patients ( $9.3 \pm 2.4$  v  $2.5 \pm 0.9$  mM/100 mM bile acids;  $p < 0.01$ ). In the subgroup of seven patients with Crohn's ileitis/ileocolitis who had previously undergone intestinal resection (ileal resection in four, right hemicolectomy in three), the mean total bilirubin level of  $11.7 \pm 1.2$  mM was similar to the mean value in the other

four patients in that group, but was significantly higher ( $p < 0.05$ ) than that in the Crohn's colitis and post-colectomy groups. All seven patients who had undergone small bowel resection had supersaturated bile (CSI range, 1.10-1.95) and three of the seven had rapid cholesterol microcrystal nucleation times ( $\leq 10$  days).

In the 14 patients with active ulcerative colitis, the mean uncorrected biliary bilirubin concentration of  $7.7 \pm 1.4$  mM was similar to that in the 17 post-colectomy patients ( $6.1 \pm 1.3$  mM). In the six patients in whom paired bile samples were obtained (during active colitis and again after resection of the colon), total biliary bilirubin levels were also similar ( $7.5 \pm 1.6$  mM v  $4.0 \pm 0.8$  mM). Furthermore, total bilirubin concentrations did not differ between the Crohn's colitis and ulcerative colitis groups, nor between the two post-colectomy groups, and standardisation of biliary bilirubin concentrations had no significant effect on the distribution of the data or quoted  $p$  values.

#### **8.3.4 Bile acid conjugates and phosphatidylcholine species**

The distribution of the glycine and taurine conjugates of the major bile acids, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA), expressed as percentages of total biliary bile acids, are given in Table 8. 3. The mean % DCA in each of the three Crohn's disease groups was lower than that in the disease controls (patients with cholesterol-rich gallbladder stones), although this difference was significant only in the patients with active ileitis or ileocolitis ( $14.2 \pm 4.8\%$  v  $22.3 \pm 1.9\%$  of total bile acids;  $p < 0.05$ ). There was a reciprocal pattern of results for the proportion of UDCA in bile. Thus, in all three Crohn's disease groups, the mean % UDCA was significantly greater ( $p < 0.001$ ) than that in the positive controls. Lithocholic acid was present in negligible amounts in all groups. The combined bile acid data from the Crohn's disease patients revealed a significant negative correlation between the biliary proportions of the primary bile acid, CA, and its  $7\alpha$ -dehydroxylated derivative, DCA ( $r = -0.835$ ,  $p < 0.001$ ). However, there was no relationship between the % CDCA and lithocholic acid or the  $7\beta$ -hydroxyepimer of CDCA, UDCA.

|                                  | Crohn's disease        |                                     |                        | Positive controls          |                          |
|----------------------------------|------------------------|-------------------------------------|------------------------|----------------------------|--------------------------|
|                                  | Active colitis (n=4)   | Active ileitis or ileocolitis (n=8) | Post-colectomy (n=4)   | Gallbladder stones (n=14)  |                          |
| Bile acid conjugates (%):        |                        |                                     |                        |                            |                          |
| Cholic acid                      | 35.2 ± 7.3             | 37.0 ± 4.3                          | 44.6 ± 4.7             | 36.1 ± 2.8                 |                          |
| Chenodeoxycholic acid            | 37.2 ± 5.8             | 39.8 ± 3.6                          | 37.6 ± 1.3             | 40.5 ± 2.0                 |                          |
| Deoxycholic acid                 | 18.8 ± 11.0            | 14.2 ± 4.8 <sup>1</sup>             | 15.3 ± 4.1             | 22.3 ± 1.9 <sup>1</sup>    | <sup>1</sup> p=0.05      |
| Ursodeoxycholic acid             | 8.8 ± 3.0 <sup>1</sup> | 9.0 ± 1.8 <sup>2</sup>              | 2.5 ± 2.5 <sup>3</sup> | 0.7 ± 0.2 <sup>1,2,3</sup> | <sup>1,2,3</sup> p<0.001 |
| Phosphatidylcholine species (%): |                        |                                     |                        |                            |                          |
| 16:0-22:6                        | 4.0 ± 0.6              | 5.5 ± 0.7                           | 5.5 ± 1.0              | 5.6 ± 0.7                  |                          |
| 16:0-20:4                        | 13.6 ± 1.9             | 11.3 ± 0.9                          | 15.1 ± 2.8             | 12.7 ± 0.8                 |                          |
| 16:0-18:2                        | 50.4 ± 3.9             | 50.0 ± 2.2 <sup>1</sup>             | 50.0 ± 5.2             | 55.8 ± 1.6 <sup>1</sup>    | <sup>1</sup> p<0.05      |
| 16:0-18:1                        | 25.4 ± 1.4             | 28.5 ± 1.6                          | 25.1 ± 2.8             | 22.9 ± 2.0                 |                          |
| 18:0-20:4                        | 0.8 ± 0.3              | 0.7 ± 0.1                           | 1.1 ± 0.1 <sup>1</sup> | 0.7 ± 0.1 <sup>1</sup>     | <sup>1</sup> p<0.01      |
| 18:0-18:2                        | 5.8 ± 1.8              | 4.5 ± 0.3                           | 3.7 ± 0.4              | 5.9 ± 0.6                  |                          |

Table 8.3 Group data (means ± SEM) for biliary bile acid composition and the molecular species (fatty acid composition) of phosphatidylcholine in the patients with Crohn's disease and cholesterol-rich gallbladder stones (positive controls).

The distribution profile of the different species of PC, expressed as percentages of total biliary PC, in 16 patients with Crohn's disease and 13 patients with cholesterol-rich gallstones, is also shown in Table 8.3. The relative proportions of the major PC molecular species, 16:0-18:2 and 16:0-18:1, were similar between the three Crohn's disease subgroups, although the mean % 16:0-18:2 in the ileitis/ileocolitis group was significantly lower than that in the positive controls ( $p < 0.05$ ). Overall, there were no significant differences between the proportions of the arachidonic acid-rich PCs, 16:0-20:4 and 18:0-20:4, in the Crohn's disease groups and positive controls, apart from the finding that PC 18:0-20:4 was significantly higher in the post-colectomy group than in the patients with gallstones ( $1.1 \pm 0.1\%$  v  $0.7 \pm 0.1\%$ ;  $p < 0.01$ ). There was a significant positive correlation between the two main arachidonic acid-rich phosphatidylcholine species, PC 16:0-20:4 and 18:0-20:4 ( $r = 0.643$ ,  $p < 0.01$ ), but there was no relationship between the proportions of either of the two arachidonic acid-rich PC species and: (i) the CSI, (ii) DCA, or (iii) the other bile acid conjugates, in gallbladder bile.

## 8.4 DISCUSSION

The results of the present study have shown that the bile lipid composition, cholesterol saturation indices and microcrystal nucleation times are abnormal in patients with inflammatory bowel disease and that they vary with the site of the disease. In general, the bile of patients with ulcerative or Crohn's colitis was only marginally saturated with cholesterol and almost 90% of patients had normal microcrystal nucleation times. In contrast, in the two colectomised groups, all but three patients (84%) had supersaturated bile and over two-thirds of patients had short nucleation times of  $\leq 10$  days. Similarly, all but one of the 11 patients with Crohn's ileitis or ileocolitis had supersaturated bile, with the highest CSIs being in those with previous ileal resection, and five of the 11 also had short nucleation times.

These changes in bile physical chemistry are consistent with the strong epidemiological association between Crohn's disease and gallbladder stones (Heaton and Read 1969; Cohen *et al* 1971; Whorwell *et al* 1984), and the reported relationship between the extent (Andersson *et al* 1987; Kangas *et al* 1990; Lorusso *et al* 1990) and number (Lapidus *et al* 1999) of previous small bowel resections and subsequent gallstone formation. There are no large reports of gallstone composition in inflammatory bowel disease to indicate whether such patients develop cholesterol-rich or pigment stones. As early as 1969, Heaton and Read (Heaton and Read 1969) showed that up to 40% of gallstones in patients with Crohn's disease are radio-opaque by oral cholecystography and therefore likely to be rich in calcium. However, radio-opacity of gallstones is due usually to the presence of calcium carbonate (and less frequently to calcium phosphate) — rather than to calcium bilirubinate or calcium fatty acid soaps (Dowling 2000b). Furthermore, secondary calcification has been reported in 9-21% of patients with cholesterol-rich stones (Pereira *et al* 1997), and is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (Bell *et al* 1975; Lirussi *et al* 1993; Plaisier *et al* 1994a; Bazzoli *et al* 1995). In the few small reports of the chemical composition of stones obtained at cholecystectomy from patients with Crohn's disease, both pigment stones (Magnuson *et al* 1989; Brink *et al* 1999) and cholesterol-rich stones (Makino *et al* 1994; Brink *et al* 1999) have been reported.

Our data on bile composition and physical chemistry suggest that patients with ileal disease/resection are at risk of developing gallstones through at least two different mechanisms. First, our findings of biliary cholesterol supersaturation and altered bile acid composition in Crohn's disease are consistent with those of previous studies which have reported reduced active bile acid absorption in the diseased or resected terminal ileum and a reduction in the bile acid pool within the enterohepatic circulation (Rutgeerts *et al* 1986; Rutgeerts *et al* 1987; Santavirta *et al* 1990). One possible consequence is that there is upregulation of hepatic low density lipoprotein uptake (Akerlund *et al* 1991; Stahlberg *et al* 1991) and the activation of the rate-determining enzymes of cholesterol and bile acid synthesis (3-hydroxy-3-methylglutaryl coenzyme A and 7 $\alpha$ -hydroxylase, respectively) (Akerlund *et al* 1991), so that the ratio of biliary cholesterol : bile acid secretion increases (Rutgeerts *et al* 1987; Akerlund *et al* 1994). However, this hypothesis of a reduced bile acid pool within the enterohepatic circulation in Crohn's disease has been criticised as there is considerable reserve bile acid synthetic capacity in the liver, so that in the chronic state bile acid secretion rates may not fall until faecal bile acid losses rise severalfold (Ferezou *et al* 1993). Nevertheless, in the present study, we found a significantly lower % biliary DCA in the patients with Crohn's ileitis/ileocolitis than in the disease controls. Conversely, biliary UDCA was increased significantly in all three of the Crohn's disease subgroups — similar findings to those of other investigators (Vantrappen *et al* 1977; Nishida *et al* 1982; Lapidus and Einarsson 1998). Ursodeoxycholic acid, the 7 $\beta$ -hydroxyepimer of CDCA, is derived from CDCA via oxidation by intestinal bacteria to 7-ketolithocholic acid. In the intestine, 7-ketolithocholic acid is converted mainly to UDCA rather than being reduced back to CDCA. In patients with Crohn's disease, the increased conversion of CDCA to UDCA and marked increase in biliary UDCA levels is poorly understood, but may be due to a combination of bile acid malabsorption and increased colonisation of the small and large intestine by bacteria responsible for 7 $\beta$ -hydroxyepimerisation (Miwa *et al* 1986; Lapidus and Einarsson 1991).

The present results, and those of another recent study (Keulemans *et al* 1999), indicate that many patients with small bowel Crohn's disease also have abnormally rapid cholesterol microcrystal nucleation times — a prerequisite for cholesterol

gallstone formation. To date, there have been very few studies of nucleation inhibitors or promoters in the bile of patients with inflammatory bowel disease. Keulemans *et al* (1999), using the technique of Concanavalin A-Sepharose affinity fractionation, reported increased cholesterol crystallisation-promoting activity in the bile of 10 out of 11 patients with Crohn's disease, whereas bile from all eight patients with ulcerative colitis contained a non-mucin glycoprotein that inhibited cholesterol crystallisation.

Case-control ultrasonographic studies from Italy (Lorusso *et al* 1990) and Sweden (Lapidus *et al* 1999), of gallstone prevalence in normal controls and patients with inflammatory bowel disease, have revealed a significantly increased risk of gallstones both in patients with ileal Crohn's disease — with reported odds ratios of 1.8 (Lapidus *et al* 1999) to 4.5 (Lorusso *et al* 1990)) — and in those with ulcerative or Crohn's colitis (odds ratio = 2.5 (Lorusso *et al* 1990)). We also found that bile physical chemistry was abnormal in some patients with colitis alone — consistent with the findings of some (Harvey *et al* 1991a; Makino *et al* 1994; Hakala *et al* 1997), but not all (Galatola *et al* 1995; Akerlund and Einarsson 2000), earlier studies of bile composition in such patients. Bile acids are normally well conserved within the enterohepatic circulation, with less than 5% of the circulating bile acid pool being excreted each day in the faeces. The only intestinal site of active bile acid absorption is the terminal ileum, but bile acids can also be absorbed by passive nonionic diffusion after bacterial deconjugation — as occurs normally in the colon and abnormally in the contaminated small bowel (Pereira and Dowling 1995; Hofmann 1999). In patients with a terminal ileostomy or an ileo-anal pouch, the faecal output of bile acids is higher than that in normal volunteers (Santavirta *et al* 1990; Hylander *et al* 1991; Natori *et al* 1992). Thus, increased faecal bile acid losses may be one explanation for the increase in biliary cholesterol saturation seen in some of the colitis patients and in most of those who were post-colectomy.

An alternative hypothesis for gallstone formation in Crohn's disease is that patients with ileal disease or resection are at particular risk of developing pigment gallstones, as a consequence of increased colonic bile acid levels solubilising unconjugated bilirubin and promoting its absorption from the large intestine, thus increasing the enterohepatic cycling of bilirubin (Feverly 1999). In support of this theory, Brink *et al* observed a

twofold increase in biliary bilirubin secretion rates in ileectomised, but not jejunectomised, rats (Brink *et al* 1996), and up to a threefold increase in total bilirubin concentrations in the gallbladder bile of patients with extensive (>50 cm) ileitis or previous ileectomy (Brink *et al* 1999). However, in the clinical study (Brink *et al* 1999), only one of the 23 patients with Crohn's ileitis or previous ileectomy had pigment gallstones (16% cholesterol by weight) while two developed cholesterol-rich gallstones (92% and 73% cholesterol by weight, respectively) and five had cholesterol monohydrate crystals detected microscopically in bile samples obtained intraoperatively. Given that cholesterol-rich gallstones are common in the general population (Dowling 2000b), it remains unclear whether patients with ileal disease or resection are at increased risk of cholesterol or pigment gallstone disease, or both. In the present study, total biliary bilirubin levels in the patients with small bowel Crohn's disease were two- to three-fold higher than in those with colitis or who were post-colectomy. However, all but one of the patients with ileal disease also had supersaturated bile and almost half had abnormally rapid cholesterol microcrystal times. We did not measure the individual amounts of unconjugated bilirubin or of mono- or di-glucuronides of bilirubin in bile, but Brink *et al* (Brink *et al* 1999) found that the increased levels of these components in the bile of patients with Crohn's disease correlated with total biliary bilirubin concentrations. Another potential pathophysiological role of increased biliary bilirubin in these patients is that mucin glycoprotein and bilirubin are frequently identified at the centre of human cholesterol gallstones, suggesting that mucin-bilirubin complexes might function as nucleating agents for cholesterol gallstones (Smith and LaMont 1983). Furthermore, biliary sludge (defined ultrasonographically as echogenic, gravitating material within the gallbladder which does not produce acoustic shadowing), which consists of cholesterol microcrystals, calcium bilirubinate granules, and mucin glycoprotein, is a known precursor to cholesterol-rich gallbladder stones (Ko *et al* 1999).

There have been few previous studies of the molecular species of biliary phospholipids in inflammatory bowel disease. The hepatic secretion of cholesterol is normally coupled tightly to that of PC (the principal biliary phospholipid). In 'conventional' cholesterol gallstone disease, an increase in the % biliary DCA is

associated with cholesterol supersaturation (Berr *et al* 1992; Hussaini *et al* 1994), and also with an increased proportion of unsaturated molecular species of PC, particularly arachidonic acid (Angelico *et al* 1992; Berr *et al* 1992; Hatsushika *et al* 1993). In turn, this increase in arachidonic acid-rich phospholipids has been shown to promote biliary cholesterol supersaturation, destabilise cholesterol carriers and induce mucin glycoprotein synthesis (Cohen and Carey 1991; Lee 1991; Booker *et al* 1992).

In the present study, the proportions of arachidonic acid-rich PC species in the bile of the patients with active Crohn's disease were similar to those in patients with cholesterol-rich gallstones, and higher than the levels found in the bile of historical controls (stone-free patients with acromegaly untreated with octreotide) that we have reported previously (Pereira *et al* 2001). We did not detect a difference in PC composition between the three Crohn's disease subgroups although, given their relatively small size, it is possible that this may represent a type II error. Furthermore, unlike some previous reports of a positive correlation between biliary DCA and arachidonic acid-rich phospholipids in patients with cholesterol-rich gallstones (van Berge Henegouwen *et al* 1987) or octreotide-induced gallstones (Pereira *et al* 2001), we found no such relationship in the current study — similar findings to two comparative studies of patients with pigment and cholesterol-rich gallstones (van Erpecum *et al* 1991; Jungst *et al* 1999). In patients with Crohn's disease, an increased proportion of arachidonic acid-rich phospholipids has been described both in the plasma (Farkkila *et al* 1987; Esteve Comas *et al* 1992; Esteve Comas *et al* 1993; Pereira *et al* 1996) and in intestinal mucosal biopsies (Pacheco *et al* 1987; Buhner *et al* 1994). It is possible, therefore, that the increase in biliary arachidonic-acid rich species seen in the present study may simply reflect a more generalised inflammatory response. An alternative explanation is that the biliary secretion of arachidonic-acid rich PCs in Crohn's disease is increased selectively, independent of bile acid-mediated solubilisation of lipids from the outer leaflet of the canalicular membrane. The hepatic secretion of biliary phospholipids is poorly understood, but PC and cholesterol appear to be co-transported from the smooth endoplasmic reticulum to the canalicular plasma membrane, partly by microtubule-mediated vesicular movement (Marzolo *et al* 1990), but predominantly by cytosolic lipid transfer proteins (Reynier *et al* 1992; Cohen *et al*

1994). Under experimental conditions whereby the hepatic synthesis of cholesterol is increased during a controlled rate of bile acid secretion (Berr *et al* 1997), there is a linear increase in the biliary % of both PC 16:0-18:1 and PC 16:0-20:4. There is indirect evidence that this coupling of particular PC species with the amount of cholesterol supplied for biliary secretion occurs at the level of the endoplasmic reticulum (Berr *et al* 1997).

In conclusion, we have shown that patients with small bowel Crohn's disease, as well as those who have undergone colectomy, have supersaturated bile and an increased tendency to form cholesterol microcrystals compared with patients with ulcerative or Crohn's colitis, and thus appear to be at increased risk of forming cholesterol-rich rather than pigment gallstones. In those with ileal dysfunction/resection, total biliary bilirubin levels were also increased, but it is unclear whether this may predispose to pigment stone formation or contribute to cholesterol microcrystal nucleation. However, reports of impaired gallbladder contractility (Murray *et al* 1992c) and increased concentrations of cholesterol microcrystal nucleation-promoting factors (Keulemans *et al* 1999), in Crohn's disease indicate that such patients fulfil the 'triple defect' of cholesterol gallstone formation. Furthermore, the increase in arachidonic acid-rich PC species in the presence of low % DCA in the gallbladder bile of these patients suggests that the pathogenesis of gallstones may differ from that of patients with 'conventional' cholesterol-rich gallstones, in whom prolonged colonic transit times (Heaton *et al* 1993) and increased 7 $\alpha$ -dehydroxylating colonic bacteria (Wells *et al* 2000) may lead to increased biliary % DCA which, in turn, induces cholesterol hypersecretion, destabilisation of biliary cholesterol carriers and cholesterol microcrystal nucleation (Dowling 2000b).

## **CHAPTER 9. BILIARY NON-MUCIN GLYCOPROTEINS IN INFLAMMATORY BOWEL DISEASE**

### **9.1 INTRODUCTION**

Neutrophil accumulation and degranulation in the inflamed intestinal mucosa are a prominent feature of inflammatory bowel disease, and contribute to tissue damage at sites of inflammation (Lee and Kaplan 1995). One such neutrophil degranulation product is lactoferrin — a 76 kDa iron-binding glycoprotein related in structure to transferrin (Sanchez *et al* 1992). Lactoferrin is present in small amounts in exocrine secretions — such as milk, from which the name derives (Masson *et al* 1966; Hegnhøj *et al* 1986). However, the major, if not the sole, source of circulating lactoferrin is the secondary (specific) granules of neutrophils (Birgens 1985; Furmanski and Li 1990). In control subjects and in patients with inactive inflammatory bowel disease, plasma concentrations of lactoferrin are low (Bennett and Kokocinski 1978; Adeyemi and Hodgson 1991; Antonsen *et al* 1993; Pereira *et al* 1996). However, in active inflammatory bowel disease, both circulating (Adeyemi and Hodgson 1991; Pereira *et al* 1996) and faecal (Uchida *et al* 1994; Sugi *et al* 1996; Dwarakanath *et al* 1997) lactoferrin concentrations are increased. Plasma lactoferrin levels correlate well with both serum C-reactive protein concentrations and clinical indices of disease activity (Adeyemi and Hodgson 1991; Pereira *et al* 1996).

In addition to being a marker of disease activity, lactoferrin has been implicated in the pathogenesis of inflammatory bowel disease as well as in cholesterol gallstone formation. Thus, when lactoferrin is infused into rat mesenteric arteries, it increases colonic mucosal permeability, induces neutrophil recruitment into the mucosa and causes a frank colitis (Kurose *et al* 1994). Furthermore, lactoferrin may be a major antigen for perinuclear-staining anti-neutrophil cytoplasmic antibodies (Gross *et al* 1993; Mulder *et al* 1993; Peen *et al* 1993; Skogh and Peen 1993; Ellerbroek *et al* 1994). These antibodies have been reported in the sera of 13-89% of patients with inflammatory bowel disease (Gross *et al* 1993; Mulder *et al* 1993; Ellerbroek *et al* 1994; Seibold *et al* 1994; Vecchi *et al* 1994; Aisenberg *et al* 1995) and from 65-82% of

patients with primary sclerosing cholangitis (Duerr *et al* 1991b; Klein *et al* 1991; Lo *et al* 1992; Seibold *et al* 1992; Seibold *et al* 1994) — a chronic cholestatic liver disease which occurs in 2.5-7.5% of patients with ulcerative colitis (Chapman 1991; Fausa *et al* 1991; Olsson *et al* 1991; Lo *et al* 1992), and in approximately 1% of those with Crohn's disease — predominantly those with colonic involvement (Schrumpf and Gjone 1982; Mir-Madjlessi *et al* 1987; Saxon *et al* 1990).

There have been no previous studies of biliary lactoferrin concentrations in inflammatory bowel disease. In theory, however, high concentrations of lactoferrin within the hepatobiliary tract could play a role in the initiation of primary sclerosing cholangitis or in cholesterol microcrystal nucleation (Braganza and Worthington 1995). Thus, if there were an enterohepatic circulation of lactoferrin, or of proteins such as *N*-formyl peptides which induce biliary neutrophil degranulation (Ferry *et al* 1989; Fittschen and Henson 1994), biliary concentrations of lactoferrin should be high during active inflammatory bowel disease and fall to normal levels during disease remission. To study this, we compared the concentrations of lactoferrin in gallbladder bile from patients with active and inactive inflammatory bowel disease. In addition, to determine whether lactoferrin is released selectively from neutrophil secondary granules in active inflammatory bowel disease (Dwarakanath *et al* 1995), or is merely a non-specific marker of neutrophil degranulation, we compared the concentrations of lactoferrin with those of myeloperoxidase (stored in neutrophil primary granules (Fittschen and Henson 1994)) in gallbladder bile.

## **9.2 METHODS**

### **9.2.1 Patients**

The patients were recruited for the study by the Academic Department of Surgery at the Queen Elizabeth Hospital, Birmingham. All required surgical management of their inflammatory bowel disease. The clinical details are given in Table 9.1.

| Patient group                         | Sex (M/F) | Age (yr) (mean, range) | Disease duration (median, range) | Previous surgery                                                               | Medical treatment                      |
|---------------------------------------|-----------|------------------------|----------------------------------|--------------------------------------------------------------------------------|----------------------------------------|
| <b>Ulcerative colitis (n = 42)</b>    |           |                        |                                  |                                                                                |                                        |
| Active colitis (n = 14)               | 11/3      | 42 (16-75)             | 2.5 (1-10)                       | Nil                                                                            | Steroids (n = 11)                      |
| Post-colectomy (n = 17)               | 13/4      | 40 (16-66)             | 5 (1-21)                         | Colectomy + ileostomy with mucous fistula (n = 17)                             | Nil                                    |
| Active pouchitis (n = 11)             | 7/4       | 36 (19-66)             |                                  | Colectomy + ileoanal anastomosis (n = 11)                                      | Nil                                    |
| <b>Crohn's disease (n = 21)</b>       |           |                        |                                  |                                                                                |                                        |
| Active colitis (n = 7)                | 4/3       | 34 (23-65)             | 2 (1-5)                          | Loop ileostomy (n = 1)                                                         | Steroids (n = 3)<br>Mesalazine (n = 1) |
| Post-colectomy (n = 5)                | 4/1       | 49 (34-76)             | 5 (2-12)                         | Colectomy (n = 2)<br>Loop ileostomy (n = 3)                                    | Nil                                    |
| Active ileitis or ileocolitis (n = 9) | 6/3       | 36 (22-53)             | 12 (3-20)                        | Ileal resection (n = 3)<br>Ileorectal anastomosis (n = 2)<br>Ileostomy (n = 3) | Steroids (n = 5)                       |

Table 9.1 Clinical data of the patients studied.

Forty-two patients had ulcerative colitis. There were 11 women and 31 men, and their ages ranged from 16 to 75 (mean 40) years. The patients were subdivided into three groups: (i) active colitis (n=14; admitted for proctocolectomy), (ii) post-colectomy (n = 17; admitted for J-pouch ileo-anal anastomosis following proctocolectomy 1-2 months earlier), and (iii) pouchitis (n = 11; admitted for J-pouch resection/repair because of clinical pouchitis or anastomotic breakdown. In six patients, paired bile samples were obtained, first during active colitis and again 1-2 months after proctocolectomy.

Twenty-one patients had Crohn's disease. There were seven women and 14 men, with a mean age of 40 (range, 22-76) years. Twelve patients had colonic disease alone, of whom seven had active colitis requiring colectomy and five had inactive disease (previous colectomy or defunctioning loop ileostomy). The remaining nine had active ileal (n = 4) or ileocolonic (n = 5) disease and were admitted for small bowel resection.

None of the patients with ulcerative colitis or Crohn's disease had undergone cholecystectomy and all were free of gallbladder stones, as assessed by pre-operative ultrasonography (Hutchinson *et al* 1994) and direct palpation of the gallbladder at the time of laparotomy. The results of serum liver function tests were normal in all patients.

### **9.2.2 Aspiration of gallbladder bile**

The technique of intra-operative sampling of gallbladder bile was approved by the Ethics Committee of Guy's Hospital, and the South Birmingham Ethical Committee. At the start of surgery, and before manipulation of the bowel, bile was sampled using standard techniques of gallbladder puncture (Strasberg *et al* 1990b). To avoid possible sampling errors as a result of stratification of bile, the gallbladder contents were aspirated as completely as possible. No episodes of bile leakage occurred, and there was no morbidity associated with the procedure. Bile samples thus obtained were stored immediately at -20°C until analysis.

### **9.2.3 Gel filtration of biliary lactoferrin**

Biliary lactoferrin was separated from high-molecular-mass glycoproteins by sepharose CL-2B gel chromatography, as described in Chapter 7.2.7. In brief, aliquots of gallbladder bile (200 µl) were loaded onto sepharose CL-2B mini-columns (5 x 1.5 cm; Pharmacia, Uppsala, Sweden), and eluted with 0.1 M Tris-HCl (pH 8.0) in 24 x 0.5 ml fractions.

In order to determine the void volume for lactoferrin, the bicinchoninic acid protein assay, as described by Smith *et al* (1985), was used. This assay is available as a commercial kit (Sigma, Poole, UK), and is based on the principle that proteins reduce Cu (II) to Cu (I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu (I) forming a purple complex with an absorbance maximum at 562 nm.

The reagents were:

1. Bicinchinonic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N sodium hydroxide (pH 11.25).
2. Copper (II) sulphate pentahydrate 4% solution (w/v).

The above solutions were mixed immediately before use in the proportion reagent 1 : reagent 2, 50:1. Next, 0.1 ml of the test sample was added to 2 ml of the reagent mixture, vortexed and incubated at 37°C for 30 min. After cooling to room temperature, absorbance was measured at 562 nm on a Beckman DU-62 spectrophotometer. A calibration curve was prepared with 20, 40, 60, 80 and 100 µg of bovine serum albumin. The assay had an inter-assay coefficient of variation of 5.5% (n=10) and a lower limit of detection of 5 µg/ml.

Preliminary experiments showed that lactoferrin purified from human breast milk (Sigma Chemical Co., Poole, UK), and lactoferrin in gallbladder bile, both eluted at 6.5-11 ml (Figure 9.1). Therefore, these fractions were combined for the lactoferrin ELISA.

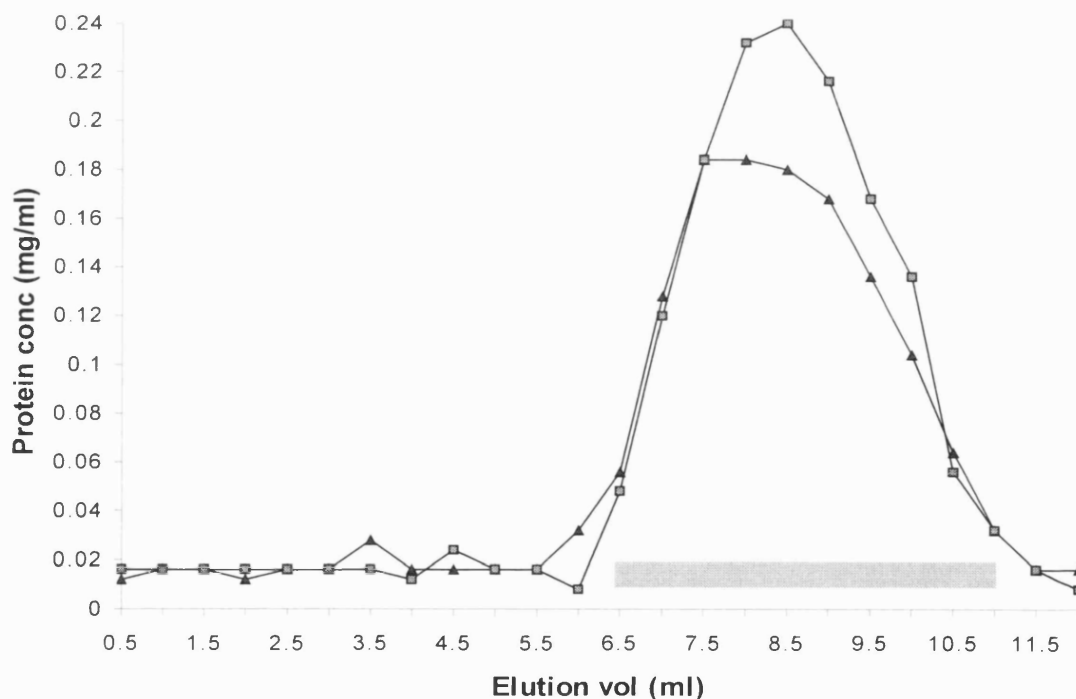


Figure 9.1 Protein assay of Sepharose CL-2B fractions after elution of purified human biliary lactoferrin (n = 2 patients). The shaded bar (6.5-11.0 ml) represents the void volume for lactoferrin.

#### 9.2.4 Lactoferrin ELISA

The lactoferrin ELISA was based on that described by Hegnhøj and Schaffalitzky de Muckadell (1985) (Figure 9.2). Briefly, 100 µl aliquots of a polyclonal rabbit anti-human lactoferrin (Sigma Chemical Co., Poole, UK), diluted 1:2000 in 0.1 M carbonate buffer, were incubated overnight at 4°C on an ELISA plate (Immulon 4, Dynatech, Billingshurst, UK). Next, 100 µl aliquots of human lactoferrin standard (Sigma Chemical Co., Poole, UK), and the pooled sepharose samples (diluted 1:125 in 0.1 M PBS/1% Tween), were incubated on the ELISA plates for 2 h at 37°C. Aliquots (100 µl) of peroxidase-conjugated rabbit anti-human lactoferrin (diluted 1:1000 in PBS-Tween) were then added, and incubated for 2 h at 37°C. Between each step, the plate was washed four times with PBS-Tween. After a final wash, colour was developed using 100 µl aliquots of peroxidase substrate (*o*-phenylenediamine dihydrochloride + 30% hydrogen peroxide + 0.05 M phosphate citrate buffer, pH 5), and the reaction stopped after 15 min by the addition of 4 M sulphuric acid. The absorbances of the samples and standards were read at 490 nm and the concentration of lactoferrin calculated from standard curves (Figure 9.3).

Add rabbit anti-human lactoferrin IgG 1:2000

Incubate overnight at 4°C



Wash x4 with PBS/Tween

Add sample containing lactoferrin (ng/ml)

Incubate 2 h at 37°C



Wash x4 with PBS/Tween

Peroxidase-conjugated anti-lactoferrin IgG 1:1000

Incubate 2 h at 37°C



Wash x4 with PBS/Tween

Add *o*-phenylenediamine dihydrochloride/H<sub>2</sub>O<sub>2</sub> substrate

Stop colour development at 15 min with 4 M H<sub>2</sub>SO<sub>4</sub>

Figure 9.2 Principle of the lactoferrin ELISA.

All assays were performed in quadruplicate. The sensitivity of the lactoferrin ELISA was 0.5 ng/ml, the intra-assay coefficient of variation was 3.4% and inter-assay coefficient of variation was 8.0% (n = 20). There was no cross-reactivity of the lactoferrin ELISA with pasteurised milk, which contains small quantities of bovine lactoferrin (Magnuson *et al* 1990). Subsequent experiments showed that the lactoferrin ELISA gave similar results when either native whole bile, or the pooled sepharose fractions, were analysed.

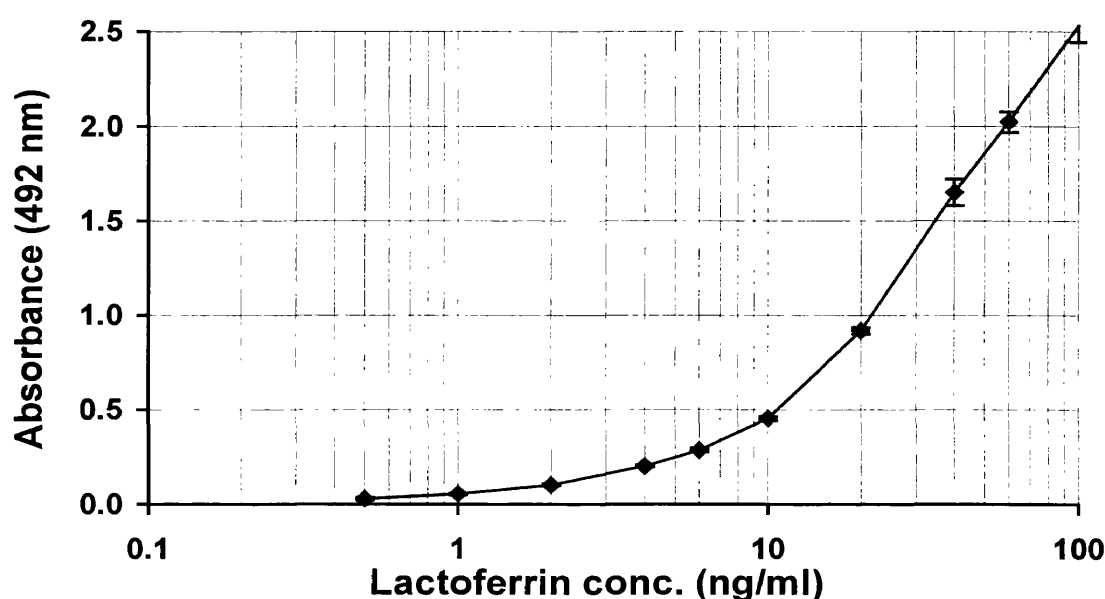


Figure 9.3 Lactoferrin ELISA standard curve derived from assays performed on six consecutive days (mean  $\pm$  SD).

### 9.2.5 Electrophoresis and Western Blotting

To assess cross-reactivity of the polyclonal rabbit anti-human lactoferrin antibody to other biliary proteins, bile samples and the human lactoferrin standard were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-11% gradient gels (Bio-Rad Laboratories, Hertfordshire, UK), using the buffer system of Laemmli (1970). Thereafter, the gels were blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA), using a semi-dry transfer system (LKB Multiphor II Electrophoresis Unit; Pharmacia Biotech, Hertfordshire, UK). The membrane was blocked overnight at 4°C with 2% casein in

0.1 M phosphate-buffered saline (PBS). It was then incubated for 1 h at 37°C in rabbit anti-human lactoferrin, diluted 1:1000 in PBS + 0.1% Tween-20 (PBS-Tween), followed by a further 1 h incubation at 37°C with mouse anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Dako A/S, (Dako, High Wycombe, UK) (diluted 1:500). Between each step, the membrane was washed (5 x 5 min) with PBS-Tween. Colour was developed by the 3-3'-diaminobenzidine reaction.

As shown by SDS-PAGE and immunoblotting, the polyclonal rabbit anti-human lactoferrin antibody was specific for human lactoferrin and did not bind to any of the other proteins in gallbladder bile (Figure 9.4).

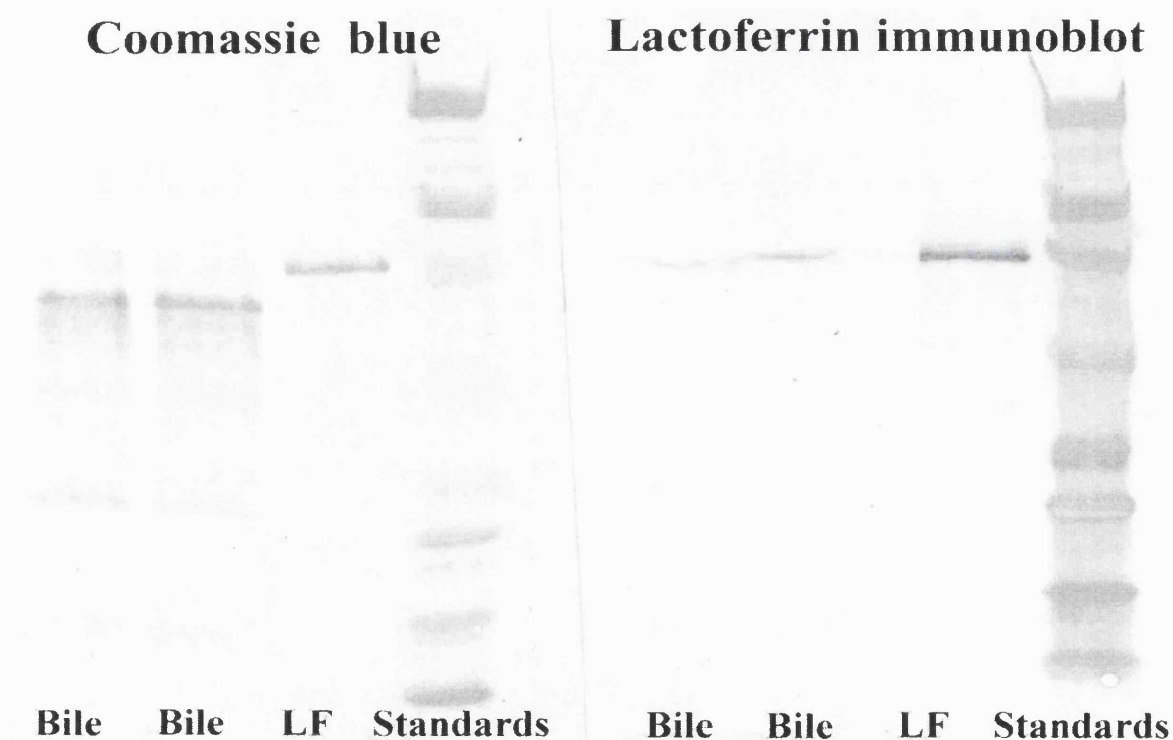


**Figure 9.4** Western blot of: bile (10 µl) (i) before and (ii) after Sepharose CL-2B gel chromatography, (iii) human lactoferrin (LF) standard (1 µg), and (iv) pre-stained molecular weight standards (Bio-Rad). SDS-PAGE separations were performed in duplicate on a 4%-15% gradient gel and the protein blotted onto a PVDF membrane. One half of the membrane was stained with Coomassie blue, while the other half was immunoblotted using rabbit anti-human lactoferrin and horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin antibodies. Note the presence of a protein band in bile corresponding to the 76 kDa human lactoferrin standard, and the absence of cross-reactivity of the antibody with other biliary proteins.

### 9.2.6 Myeloperoxidase ELISA

Biliary myeloperoxidase concentrations were determined using a commercially available ELISA kit from Bioxytech, Bonneuil sur Marne, France (Klebanoff 1992). The sensitivity of the assay was 0.5 ng/ml, and the intra- and inter-assay coefficients of variation were both < 5% (n = 12).

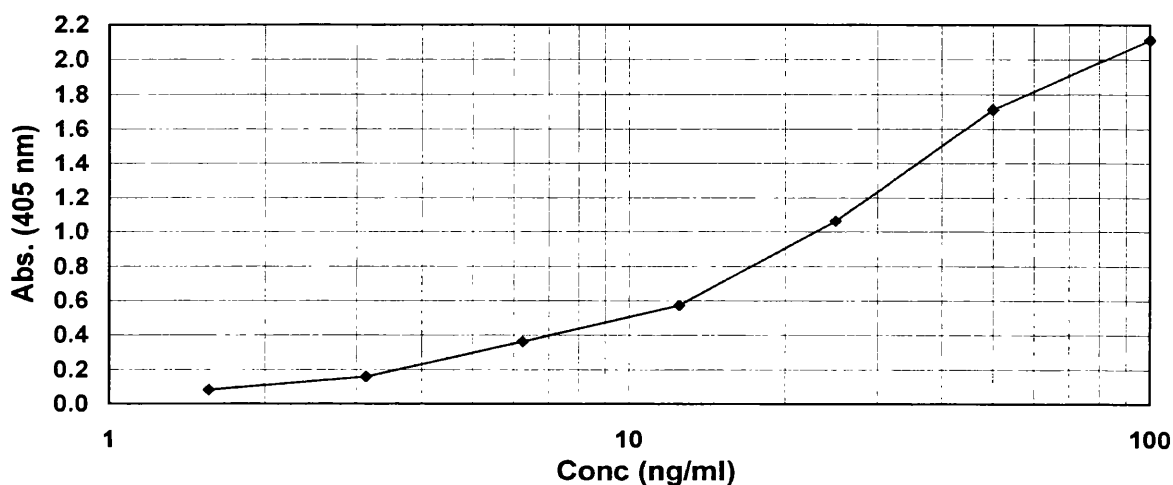


Figure 9.5 Example of a myeloperoxidase standard curve.

### 9.2.7 Biliary bile acid concentrations

To correct for the effect of varying dilutions of gallbladder bile on lactoferrin and myeloperoxidase concentrations, total biliary bile acid concentrations were determined by enzymatic assay (Talalay 1960), as described in Chapter 6.2.4. Lactoferrin and myeloperoxidase concentrations were then expressed both as 'raw' data and, after normalisation to a total bile acid concentration of 100 mM, as standardised data.

### 9.2.8 Cholesterol saturation indices and microcrystal nucleation times

As part of a separate study described in Chapter 8, biliary cholesterol saturation indices and microcrystal nucleation times were also determined, and the values

correlated with the concentrations of lactoferrin and myeloperoxidase, in gallbladder bile.

### **9.2.9 Statistical analysis**

The significance of differences in results between groups was tested with the Student's *t* test (two-tailed) or the Mann-Whitney non-parametric method, as appropriate.

## **9.3 RESULTS**

### **9.3.1 Biliary lactoferrin in ulcerative colitis**

In the 14 patients with active ulcerative colitis, the mean ( $\pm$  SEM) lactoferrin concentration (unrelated to bile acid concentration) in gallbladder bile was  $2.8 \pm 0.40$  mg/l (range, 1.2-5.8 mg/l). This value was significantly higher ( $p < 0.0001$ ) than that in the bile of the 17 post-colectomy patients (mean  $1.2 \pm 0.11$  mg/l, range 0.32-1.8 mg/l). In the six patients with colitis in whom paired bile samples were obtained (during active colitis and again after resection of the colon), biliary lactoferrin concentrations fell after colectomy in five, with a significant decrease in the mean concentration from  $2.1 \pm 0.32$  to  $1.1 \pm 0.16$  mg/l ( $p < 0.05$ ).

In the 11 patients who had pouchitis, or an anastomotic breakdown of their J-pouches, the mean biliary lactoferrin concentration of  $1.8 \pm 0.34$  mg/l (range, 0.56-4.4 mg/l) was intermediate between that of the other two groups (Figure 9.6).

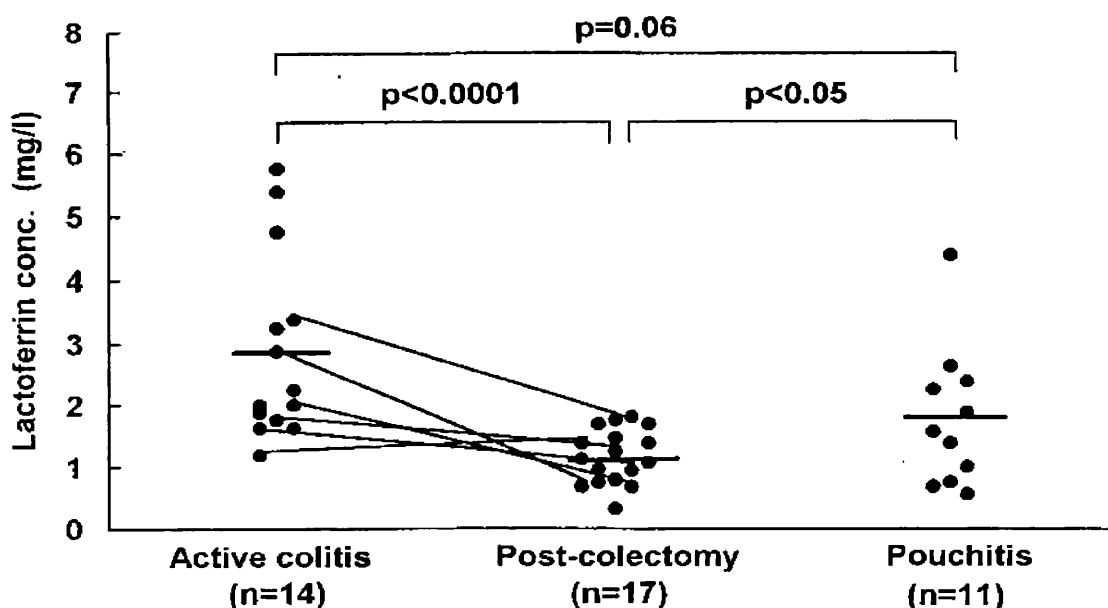


Figure 9.6 Biliary lactoferrin concentrations in patients with active and inactive ulcerative colitis.

### 9.3.2 Lactoferrin in Crohn's disease

In the seven patients with active Crohn's colitis (but no evidence of small bowel disease) and the nine with active ileitis or ileocolitis, the mean uncorrected biliary lactoferrin concentrations were similar, with values of  $3.7 \pm 0.9$  mg/l (range, 0.58-8.4 mg/l) and  $3.1 \pm 0.71$  mg/l (range, 0.21-6.3 mg/l), respectively. Both these values were significantly higher than the mean of  $1.1 \pm 0.24$  mg/l (range, 0.75-2.0 mg/l) in the Crohn's post-colectomy group ( $p < 0.05$  v Crohn's colitis;  $p = 0.06$  v ileitis/ileocolitis) (Figure 9.7).

When the mean concentrations of biliary lactoferrin in the patients with ulcerative colitis or Crohn's disease were compared, there was no significant difference between the two active colitis or post-colectomy groups, or between those with ulcerative pouchitis or active Crohn's ileitis/ileocolitis.

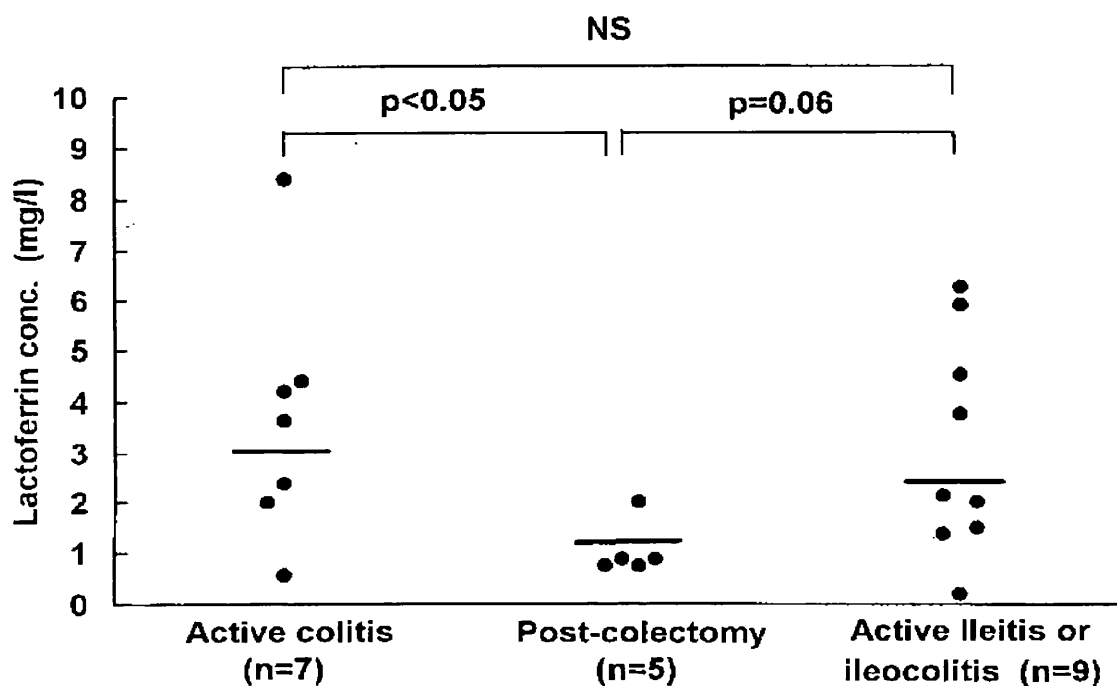


Figure 9.7 Biliary lactoferrin concentrations in patients with Crohn's disease.

### 9.3.3 Bile acid concentrations

In the patients with ulcerative colitis, the mean ( $\pm$  SEM) biliary bile acid concentrations in the active colitis, post-colectomy and pouchitis subgroups were  $136 \pm 13.4$  mM,  $171 \pm 8.7$  mM ( $p < 0.05$  v active colitis group) and  $163 \pm 19.9$  mM (NS), respectively. In those with Crohn's disease, the mean biliary bile acid concentrations were  $112 \pm 17.6$  mM (active colitis),  $116 \pm 27.3$  mM (post-colectomy or defunctioning loop ileostomy) and  $137 \pm 20.9$  mM (active ileitis or ileocolitis). There was a significant difference in biliary bile acid concentrations between the post-colectomy ulcerative colitis patients and those with active Crohn's colitis ( $p < 0.01$ ); all other comparisons between the ulcerative colitis and Crohn's disease groups were non-significant.

In the patients with ulcerative colitis, normalisation of biliary lactoferrin concentrations to a standard bile acid concentration of 100 mM increased the significance of differences in mean lactoferrin levels between the three subgroups. Thus, in the 14 patients with active colitis, the mean ( $\pm$  SEM) corrected lactoferrin concentration in gallbladder bile was  $2.4 \pm 0.48$  mg/l, compared with a value of

0.69±0.07 mg/l in the post-colectomy patients ( $p < 0.0005$ ), and 1.1±0.14 mg/l in those with pouchitis ( $p < 0.05$  and  $p < 0.01$  v the active colitis and pouchitis groups, respectively). In the patients with Crohn's disease, however, normalisation of biliary lactoferrin concentrations had no significant effect on the distribution of data or quoted  $p$  values.

### 9.3.4 Myeloperoxidase in ulcerative colitis

In the three groups of patients with ulcerative colitis, the concentrations of myeloperoxidase in gallbladder bile were also measured. On average, myeloperoxidase concentrations were approximately 300 times lower, on a molar basis, than those of lactoferrin. Furthermore, in contrast to the lactoferrin results, there were no significant differences in mean myeloperoxidase concentrations between the three groups. Thus, the mean ( $\pm$  SEM) myeloperoxidase levels in the active colitis, post-colectomy and pouchitis groups were 12.0±4.4  $\mu\text{g/l}$ , 11.9±2.5  $\mu\text{g/l}$  and 9.2±2.8  $\mu\text{g/l}$ , respectively, with an overall range of 0.2-60  $\mu\text{g/l}$  (Figure 9.8). In the 42 patients with ulcerative colitis, there was no significant correlation between biliary lactoferrin and myeloperoxidase levels in any of the patient subgroups, or when the raw data were combined or normalised to a standard bile acid concentration of 100 mM.

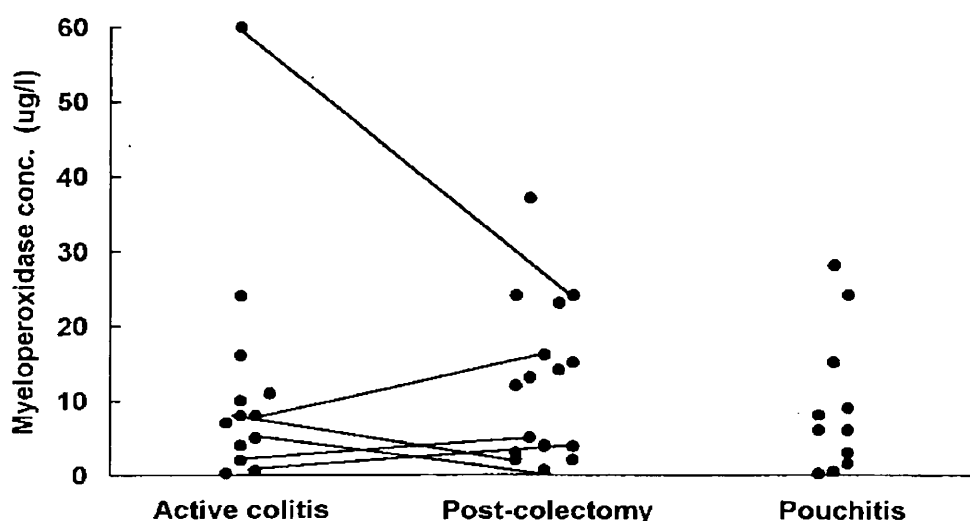


Figure 9.8 Biliary myeloperoxidase concentrations in patients with active and inactive ulcerative colitis.

### 9.3.5 Relationship between biliary lactoferrin and bile lithogenicity

In the patients with active Crohn's disease or ulcerative colitis, or who had undergone previous colectomy, there were no significant correlations between biliary lactoferrin concentrations and cholesterol saturation indices in any of the subgroups (Figure 9.9). Moreover, as described in Chapter 8.3.2, cholesterol microcrystal nucleation times were normal ( $> 10$  days) in 26 of the 32 patients with active Crohn's disease or ulcerative colitis — the groups with the highest biliary lactoferrin concentrations — whereas 12 of the 19 post-colectomy patients had pathologically short nucleation times in the presence of low lactoferrin levels. Furthermore, there was no significant relationship between biliary myeloperoxidase concentrations and the lithogenicity of bile.

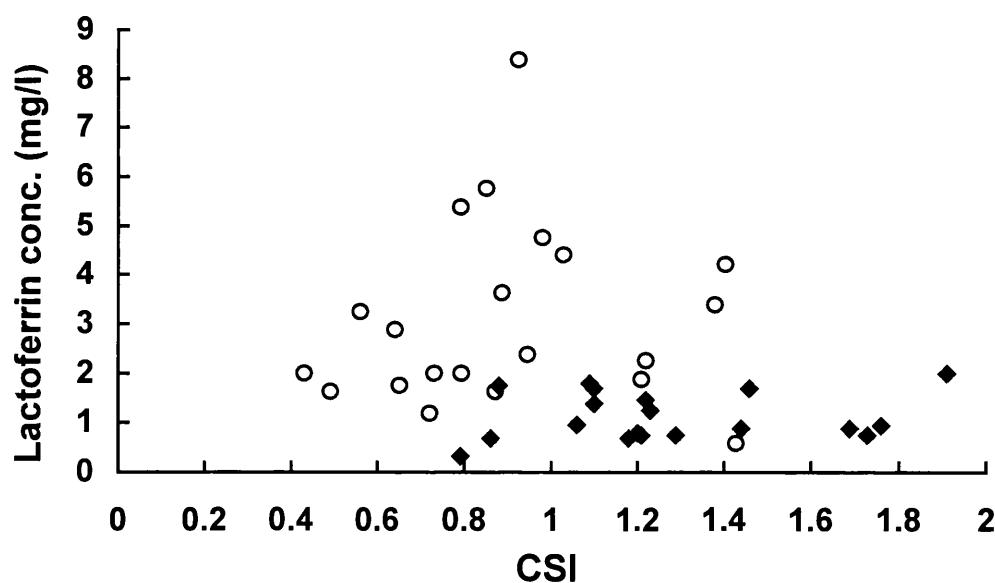


Figure 9.9 Scatterplot of biliary lactoferrin concentrations versus cholesterol saturation indices (CSIs) in patients with active ulcerative or Crohn's colitis (O), or who were post-colectomy (◆).

## 9.4 DISCUSSION

The results of this study show that there is a marked difference between biliary lactoferrin concentrations in patients with active and inactive inflammatory bowel disease. Thus, in the 21 patients with active Crohn's colitis or ulcerative colitis, the

mean lactoferrin concentration in gallbladder bile was more than 3 mg/l — similar to that in the 20 patients with Crohn's ileitis or ulcerative pouchitis. In contrast, in the 22 patients with inactive inflammatory bowel disease (post-colectomy or defunctioning loop ileostomy with no evidence of active disease), biliary lactoferrin levels were significantly lower than in the other two groups, with a mean concentration of approximately 1 mg/l.

In the present study, gallbladder bile was not obtained from control subjects. Nonetheless, it is likely that biliary lactoferrin concentrations in those with inactive inflammatory bowel disease represent basal or 'normal' levels of lactoferrin in gallbladder bile. Indeed, the biliary lactoferrin levels in patients with inactive inflammatory bowel disease were similar to those that we (Pereira *et al* 1996), and others (Adeyemi and Hodgson 1991; Antonsen *et al* 1993), have reported in the plasma of control subjects and patients with inactive inflammatory bowel disease. In contrast, in those with active disease, the mean biliary lactoferrin concentration was similar to the high levels reported in the systemic circulation of such patients (Adeyemi and Hodgson 1991; Pereira *et al* 1996).

These results are consistent with either the presence of an enterohepatic circulation of lactoferrin, or selective degranulation of neutrophils within the biliary tract by circulating bacterial *N*-formyl peptides. In the rat, bacterial chemotactic peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine, have been shown to undergo an enterohepatic circulation (Ferry *et al* 1989; Anderson *et al* 1992) and to induce selective release of neutrophil secondary granule products *in vitro* (Fittschen and Henson 1994). Therefore, in active inflammatory bowel disease, possibly as a result of decreased protease activity within the intestinal lumen (Chadwick *et al* 1990) and/or increased intestinal mucosal permeability (Peeters *et al* 1994; Oriishi *et al* 1995), biliary concentrations of bacterial oligopeptides may also rise and induce lactoferrin release from neutrophils within the hepatobiliary tract. Previous work from our group (Dwarakanath *et al* 1997) has suggested that, in active inflammatory bowel disease, lactoferrin is released selectively from neutrophil secondary granules. Conversely, if biliary lactoferrin concentrations were merely a marker of non-specific neutrophil degranulation, we would expect that biliary concentrations of myeloperoxidase (stored

in neutrophil primary granules) would also vary with disease activity. However, in the present study, there were no significant differences in mean myeloperoxidase concentrations between the different groups — supporting indirectly the hypothesis of selective neutrophil degranulation in active inflammatory bowel disease.

An alternative hypothesis is that lactoferrin itself undergoes an enterohepatic circulation. Lactoferrin is transferred from the plasma into the hepatocyte largely by an active transport mechanism (McAbee *et al* 1993; Regoeczi *et al* 1994; Meilinger *et al* 1995), and is probably routed to the bile canaliculus by vesicular transport (Regoeczi *et al* 1985; Regoeczi *et al* 1994). Although only a small proportion of lactoferrin that is transferred from the plasma escapes degradation within the liver (Bennett and Kokocinski 1979; Regoeczi *et al* 1994), the present results may also be explained by the presence of an enterohepatic circulation of lactoferrin. If so, then biliary lactoferrin concentrations would also be expected to be high in other inflammatory conditions in which systemic lactoferrin concentrations are increased (Baynes *et al* 1986; Adeyemi *et al* 1990). Indeed, high plasma lactoferrin concentrations have been reported in active rheumatoid arthritis and systemic lupus erythematosus (Adeyemi *et al* 1990) — conditions also associated with the presence of serum antineutrophil antibodies (Nassberger *et al* 1994; Gross and Csernok 1995). In inflammatory bowel disease, 22–89% of patients with ulcerative colitis (Saxon *et al* 1990; Duerr *et al* 1991a; Lo *et al* 1992; Seibold *et al* 1992; Mulder *et al* 1993; Ellerbroek *et al* 1994; Oudkerk Pool *et al* 1994; Seibold *et al* 1994; Vecchi *et al* 1994; Aisenberg *et al* 1995; Castellino *et al* 1995) and up to 34% of patients with Crohn's disease (Duerr *et al* 1991a; Lo *et al* 1992; Seibold *et al* 1992; Mulder *et al* 1993; Oudkerk Pool *et al* 1994; Seibold *et al* 1994; Castellino *et al* 1995) have antineutrophil cytoplasmic antibodies in their sera. Recent evidence suggests that lactoferrin (but not myeloperoxidase (Saxon *et al* 1990; Peen *et al* 1996)) may be a major target antigen for these antibodies in inflammatory bowel disease (Gross *et al* 1993; Mulder *et al* 1993; Skogh and Peen 1993; Ellerbroek *et al* 1994; Peen *et al* 1996). Walmsley *et al* (1997) detected serum anti-lactoferrin antibodies in only two of 52 patients with ulcerative colitis, but in another study, immunoglobulin G anti-lactoferrin antibodies were found in the sera from 50% of patients with ulcerative colitis and/or primary sclerosing cholangitis (Peen *et al* 1993).

These antibodies may be of pathogenic relevance since, *in vitro*, intact immunoglobulin G anti-lactoferrin antibodies bind to vascular endothelial cells and stimulate neutrophils to produce free oxygen radicals (Peen *et al* 1996).

Although increased concentrations of plasma or biliary lactoferrin in patients with inflammatory bowel disease do not prove that it has a pathogenetic role, nonetheless, *in vitro*, lactoferrin acts as a potent neutrophil chemotactic factor (Oseas *et al* 1981; Boxer *et al* 1982; Sanchez *et al* 1992; Kurose *et al* 1994), promotes hydroxyl radical production (Grisham 1994) and enhances monocyte cytotoxicity (Birgens 1991). Lactoferrin also has potent heparin-neutralising activity (Wu *et al* 1995) — of interest in view of the pro-thrombotic state associated with active inflammatory bowel disease (Souto *et al* 1995), and recent observations that heparin itself may induce remission in steroid-resistant disease (Dwarakanath *et al* 1995; Gaffney *et al* 1995). Furthermore, in animal models, mesenteric arterial infusion of lactoferrin (Kurose *et al* 1994), or colonic instillation of bacterial *N*-formyl peptides (LeDuc and Nast 1990), results in mucosal neutrophil recruitment and the development of a frank colitis. Although there are few data in humans, the present results suggest that the proposed relationship between biliary and systemic lactoferrin concentrations, antineutrophil cytoplasmic antibodies and the development of primary sclerosing cholangitis in patients with inflammatory bowel disease, warrants further study.

This is also the first study to examine the relationship between biliary neutrophil degranulation products and bile lithogenicity, as determined by cholesterol saturation indices and cholesterol microcrystal nucleation times, in patients with inflammatory bowel disease. Although there have been no studies of the effect of lactoferrin on cholesterol microcrystal nucleation *in vitro*, Braganza and Worthington (1995) have proposed that gallstones may stem from insufficiency of micronutrient antioxidants relative to the load of oxidants and/or oxidation-prone substrates within hepatocytes in such a way that ancillary hepatobiliary resources, including bilirubin with lactoferrin and mucin, are mobilized to combat oxidative stress and also thereby promote lithogenesis. However, in the present study, we found no significant correlation between biliary lactoferrin concentrations and bile lithogenicity, and in fact the patients with the lowest biliary lactoferrin levels (those who had undergone colectomy 1-2

months earlier) had the highest cholesterol saturation indices and abnormally rapid cholesterol microcrystal nucleation times. It is conceivable that biliary lactoferrin may act as a pronucleating factor in cholesterol gallstone formation, but its effects *in vivo* may be overshadowed by the consequences of reduced bile acid absorption and increased biliary secretion of cholesterol, arachidonic acid-rich phospholipids and mucin glycoprotein.

## **CHAPTER 10. PLASMA ARACHIDONIC ACID-RICH PHOSPHOLIPIDS**

### **IN CROHN'S DISEASE: RESPONSE TO TREATMENT**

#### **10.1 INTRODUCTION**

Arachidonic acid, incorporated into the second fatty acid position (*sn*-2) of phospholipids, is an integral component of cell membranes. Arachidonic acid metabolites, formed via the cyclo-oxygenase and lipoxygenase pathways, are also important mediators of inflammation (Spector and Yorek 1985). In active Crohn's disease, the tissue concentrations of arachidonic acid (Nishida *et al* 1987; Pacheco *et al* 1987; Buhner *et al* 1994), as well as those of several arachidonic acid-derived eicosanoids (such as prostaglandin E<sub>2</sub>, thromboxane B<sub>2</sub> and the leukotrienes), are increased, and are important mediators of inflammation within the intestinal mucosa (Lauritsen *et al* 1988; Hommes *et al* 1996).

Raised plasma concentrations of arachidonic acid, as well as other polyunsaturated fatty acids (PUFAs), have been described in patients with active Crohn's disease (Esteve Comas *et al* 1992; Royall *et al* 1994). These studies used the technique of gas liquid chromatography to determine the proportions of total (both free and bound) fatty acids in plasma. However, although triglycerides, cholesterol esters and free fatty acids all contribute to the plasma total fatty acid profile, quantitatively the most important source of both circulating and membrane PUFAs, is the phospholipids (Cabre *et al* 1992). Furthermore, alterations in the composition of triglycerides and the other minor fatty acid carriers induced by diet or disease, occur in parallel with changes in the fatty acid composition of plasma phospholipids (Farkkila *et al* 1987). Therefore, in active Crohn's disease, the reported increases in total plasma PUFAs (Esteve Comas *et al* 1992; Royall *et al* 1994) may actually represent rises in the proportion of PUFAs (such as arachidonic acid) incorporated into the *sn*-2 position of phospholipids. By acting as a substrate for eicosanoid synthesis (Stubbs and Smith 1984; Spector and Yorek 1985; Minami *et al* 1992; Minami *et al* 1994), an increase in the PUFA composition of plasma phospholipids may be relevant to the pathogenesis of Crohn's disease and to the increased risk of gallstones in these patients, as discussed in Chapter 8. However, there

have been no previous reports of the fatty acid composition (molecular species) of plasma phospholipids in Crohn's disease.

Therefore, we compared the molecular species of plasma phosphatidylcholine (PC; the principal plasma phospholipid (Banerji *et al* 1989)) in control subjects, with those in patients with Crohn's disease studied before and during treatment, and related the results to markers of disease activity.

## 10.2 METHODS

Fasting plasma samples were obtained from 17 healthy laboratory staff (12 men and five women; mean age 31, range 22-55 years). None of these control subjects had ever had inflammatory bowel disease.

Thirteen patients (six men and seven women; mean age 33, range 23-54 years) with symptomatic small bowel Crohn's disease, with or without colonic involvement, were also studied. All had active disease — as judged by clinical history and by high ( $\geq 6$ ) simple indices of Crohn's disease activity (SICDA; Harvey and Bradshaw (1980)), increased serum C-reactive protein levels and/or high erythrocyte sedimentation rates (ESR).

### 10.2.1 Experimental design and evaluation of response to treatment

As part of a separate study, in which the efficacy of a semi-elemental diet was compared with that of oral prednisolone in inducing disease remission (Engelman *et al* 1993), the patients were treated for two weeks with either: (i) oral prednisolone (0.5 mg/kg/d decreasing to nil over eight weeks, together with an unrestricted diet), or (ii) a flavoured liquid peptide diet ('Peptamen', Clintec Nutrition, Chicago, IL, 30-35 kcal/kg/d), with resumption of a normal diet by eight weeks.

Clinical data and fasting blood samples were obtained before, and two and eight weeks after, entry into the study. Patients were regarded as being in clinical remission when the SICDA score became  $< 6$  and when they had resumed a normal diet without the need for drugs or surgery.

Serum C-reactive protein concentrations and ESRs were measured before, and two and eight weeks after, commencing treatment. Plasma lactoferrin concentrations — which also correlate positively with Crohn's disease activity (Adeyemi and Hodgson 1991) — were determined by ELISA, as described in Chapter 9.2.4.

### **10.2.2 Plasma phosphatidylcholine species**

HPLC-grade methanol, isopropanol, hexane, acetonitrile and ethanol were obtained from Rathburn Chemicals Ltd (Walkerburn, UK). Standards for the different phospholipid classes (PC, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine) and the major molecular species of PC, were obtained from Sigma Chemical Co. (Poole, UK) and Avanti Polar Lipids (Alabaster, Alabama, USA). All other solvents or chemicals were of analytical grade.

After Fölch extraction of plasma lipids (Fölch *et al* 1957), plasma PC was separated from the other phospholipid classes by high performance liquid chromatography (HPLC) (Patton *et al* 1982), and the molecular species of the PC determined by reverse-phase HPLC (Sotirhos *et al* 1985), as described in Chapter 7.2.

### **10.2.3 Statistical analysis**

All results are expressed as means (SEMs). A two-tailed Student's *t* test (paired) was used to determine the significance of differences within groups. The  $\chi^2$  test was used to test for differences in proportions, and the Mann-Whitney test for discontinuous variables.

## **10.3 RESULTS**

Of the 13 patients with active Crohn's disease, nine had ileal disease alone while four had ileocolonic disease — as judged by barium studies and/or colonoscopy  $\pm$  biopsy. In the past, three patients had undergone limited (< 60 cm) ileal resections and one had had a colectomy plus terminal ileostomy.

### 10.3.1 Clinical response to treatment

At entry, the 'Peptamen' and prednisolone groups were comparable with respect to sex and age, and to the site and severity of their disease. Between the two groups, there were no significant differences in the clinical response to treatment, markers of disease activity, or plasma PC species. Therefore, at each time point the results in the two groups of patients were combined.

Before treatment, the Crohn's disease was mildly active, with a mean SICDA of  $9.9 \pm \text{SEM } 0.8$ , an ESR of  $26.4 \pm 6.5$  mm/h and a C-reactive protein of  $2.8 \pm 0.4$  mg/l (normal  $< 1$  mg/l).

After two weeks treatment, the SICDA score fell to  $< 6$  in all but one patient (who scored eight), with a mean value in the 13 patients of  $3.2 \pm 0.6$  ( $p < 0.0001$  v pre-treatment value). There were corresponding falls in both the ESR (to  $12.6 \pm 2.7$  mm/h:  $p < 0.05$ ) and C-reactive protein (to  $1.7 \pm 0.3$  mg/l:  $p < 0.05$ ).

At eight weeks, the clinical remission was sustained in 10 patients, but in two there had been a relapse with SICDAs of 8 and 10, respectively. For all 13 patients, the mean SICDA ( $3.5 \pm 0.9$ ), ESR ( $11.3 \pm 2.6$  mm/h) and C-reactive protein ( $1.6 \pm 0.3$  mg/l) results at eight weeks were not statistically different from those at two weeks (Table 10.1).

|            | Pre-treatment          | Week 2                 | Week 8         |                       |
|------------|------------------------|------------------------|----------------|-----------------------|
| SICDA      | $9.8 \pm 0.7^*$        | $2.8 \pm 0.6^*$        | $3.5 \pm 1.0$  | $^*p < 0.0001$        |
| ESR (mm/h) | $26.4 \pm 6.5^\dagger$ | $12.3 \pm 2.8^\dagger$ | $10.7 \pm 2.4$ | $^\dagger p < 0.05$   |
| CRP (mg/l) | $2.8 \pm 0.4^\ddagger$ | $1.6 \pm 0.3^\ddagger$ | $1.5 \pm 0.3$  | $^\ddagger p < 0.005$ |

Table 10.1 Markers of disease activity in the 13 patients with Crohn's disease.

### 10.3.2 Plasma lactoferrin

The plasma lactoferrin levels in the control subjects, and in the patients with Crohn's disease studied before and during treatment, are shown in Figure 10.1.

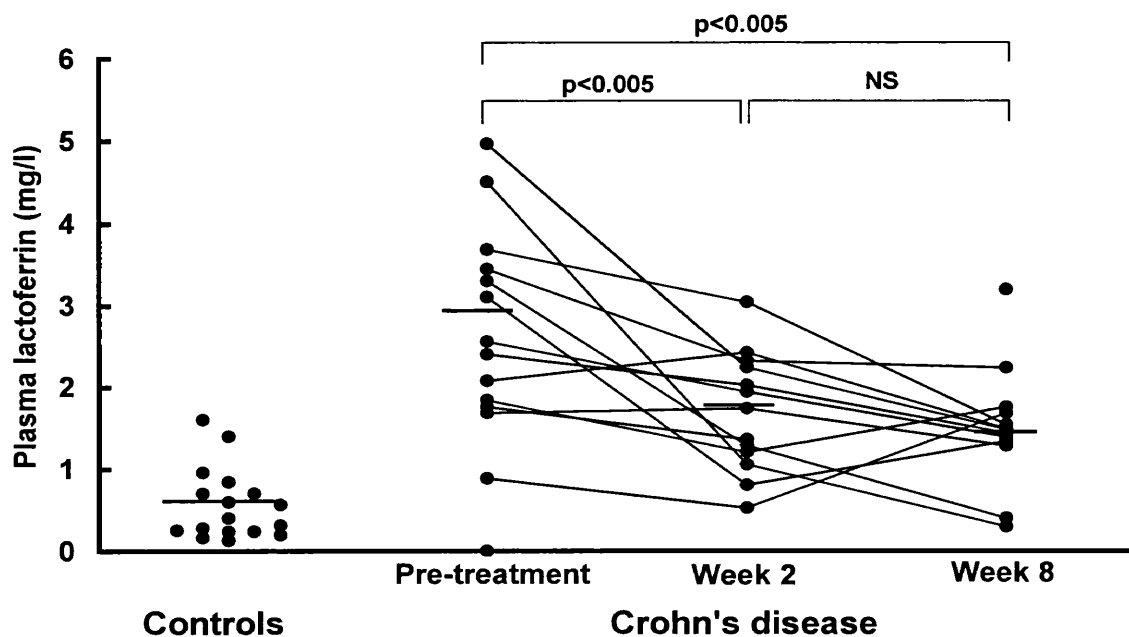


Figure 10.1 Plasma lactoferrin concentrations in control subjects (n=17) and patients with Crohn's disease (n=13), before treatment, and after two and eight weeks of therapy. At each time point, the mean lactoferrin concentration was significantly higher ( $p<0.0001$ ) than that of the control subjects.

In the Crohn's disease patients, the mean lactoferrin concentration before treatment was  $2.9\pm0.32$  mg/l (range 0.9-5.0 mg/l), which was significantly higher than that in the controls ( $0.55\pm0.11$  mg/l, range 0.13-1.6 mg/l;  $p<0.0001$ ). Two and eight weeks after commencing treatment, the mean concentrations had fallen to  $1.7\pm0.20$  mg/l ( $p<0.005$  compared with the pre-treatment concentration) and  $1.4\pm0.14$  ( $p<0.005$ ), respectively. Both these on-treatment values, however, remained significantly higher ( $p<0.0001$ ) than that of the control subjects.

Before treatment, plasma lactoferrin concentrations correlated significantly with the C-reactive protein ( $r=0.65$ ,  $p<0.05$ ), but not with the ESR ( $r=0.53$ ,  $p=0.07$ ) or the SICDA ( $r=0.10$ ).

### 10.3.3 Plasma phosphatidylcholine species

The relative proportions of the six major molecular species of phosphatidylcholine for the control subjects and the patients with Crohn's disease, are shown in Figure 10.2.

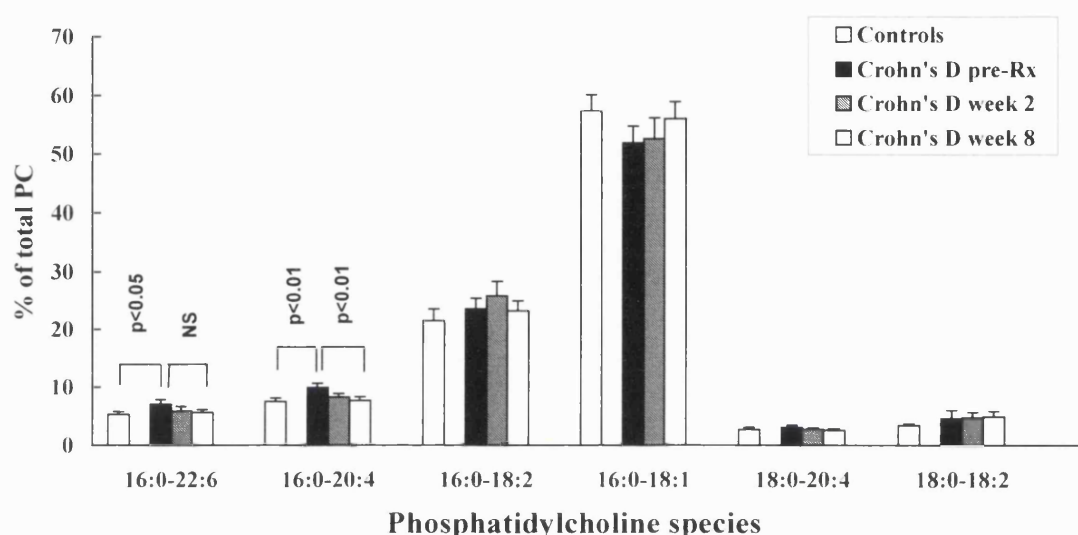


Figure 10.2 Molecular species (fatty acid composition) of plasma phosphatidylcholine in 20 control subjects and 13 patients with Crohn's disease. Values are expressed as means with SEMs represented by vertical bars. Before treatment, the proportions of both polyunsaturated species, PC 16:0-22:6 and PC 16:0-20:4, in the Crohn's disease patients were significantly higher than those in the control subjects.

In the control subjects and the patients with active Crohn's disease, the predominant plasma PC species were 16:0-18:1 ( $57.2 \pm 2.9\%$  and  $51.8 \pm 2.9\%$  of total PC, respectively; NS) and 16:0-18:2 ( $21.4 \pm 2.1\%$  v  $23.5 \pm 1.8\%$ ; NS). Before treatment, however, the proportions of both polyunsaturated species, PC 16:0-20:4 ( $10.0 \pm 0.7\%$ ) and PC 16:0-22:6 ( $7.1 \pm 0.8\%$ ) in the Crohn's disease patients were significantly higher than those in the control subjects ( $7.6 \pm 0.5\%$ ,  $p < 0.01$ ; and  $5.3 \pm 0.5\%$ ,  $p < 0.05$ ; respectively).

The relative proportions of the major arachidonic acid-rich phospholipid, PC 16:0-20:4 for the control subjects and the patients with Crohn's disease, are shown in Figure 10.3. After eight weeks treatment of the Crohn's disease patients, there was a fall to

normal values of PC 16:0-20:4 (to  $7.7 \pm 0.6\%$ ;  $p < 0.01$  v pre-treatment value) and of PC 16:0-22:6 (to  $5.7 \pm 0.5\%$ ; NS). The proportions of the other plasma phospholipid species measured did not change significantly during treatment.

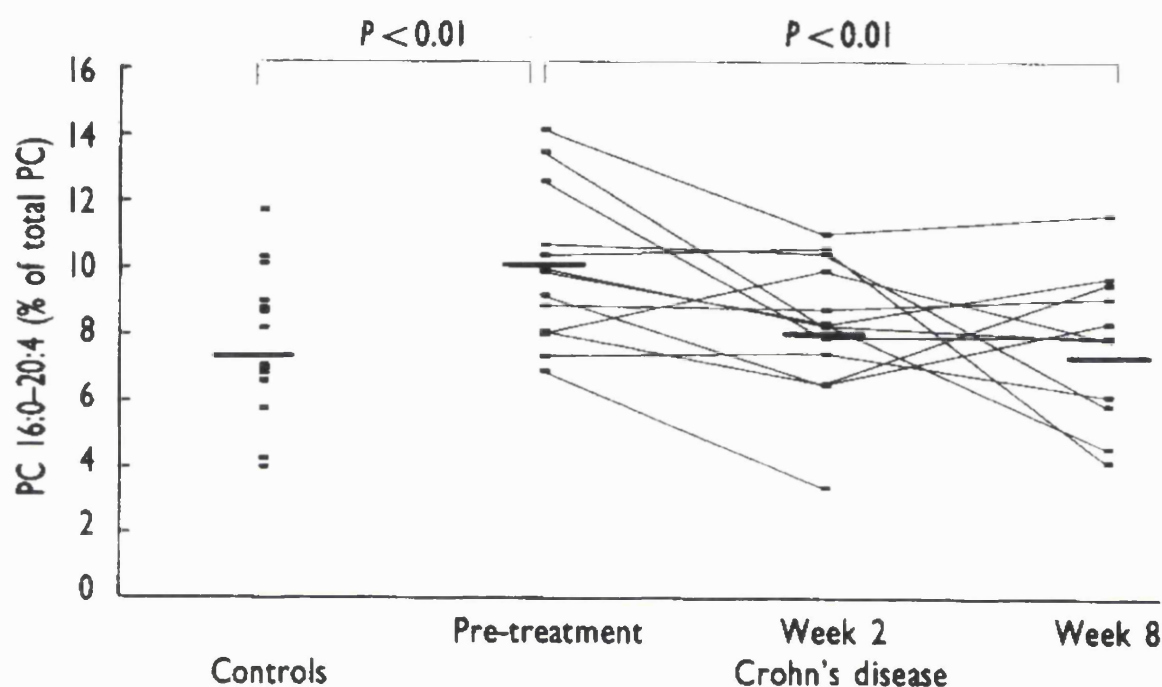


Figure 10.3 Relative proportions of the major arachidonic acid-rich phospholipid, PC 16:0-20:4, in the plasma of the control subjects and the patients with Crohn's disease, before treatment, and after two and eight weeks of therapy. The mean % PC 16:0-20:4 in the plasma of patients with active Crohn's disease was significantly higher ( $p < 0.01$ ) than that in the control subjects, but returned to normal values after eight weeks of therapy.

#### 10.4 DISCUSSION

The present study has shown that the proportions of polyunsaturated PC molecular species — in particular, palmitoyl-arachidonoyl (16:0-20:4, n-6) PC and palmitoyl-docosahexaenoyl (16:0-22:6, n-3) PC — are increased in the plasma of patients with active Crohn's disease. However, after two to eight weeks treatment with either

prednisolone or a peptide-based diet, the distribution of plasma PC species changed and became similar to that of the control subjects. By this time, most patients were in clinical remission and the standard markers of disease activity had fallen towards normal levels.

These alterations in the molecular species of PC may be relevant to the pathogenesis of Crohn's disease, and to the increased risk of gallstones in these patients. Phosphatidylcholine, the predominant plasma phospholipid, is the major source of PUFAs in the plasma (Banerji *et al* 1989), and in cell membranes (Zachowski 1993). In active Crohn's disease, increased proportions of PUFAs in the plasma (Esteve Comas *et al* 1992; Royall *et al* 1994), peripheral blood mononuclear cells (Bloomfield *et al* 1984) and intestinal mucosa (Nishida *et al* 1987; Pacheco *et al* 1987; Buhner *et al* 1994), have all been reported. Therefore, the present results suggest that the reported increases in plasma PUFAs actually represent rises in the proportion of PUFA-containing phospholipids.

In active inflammatory bowel disease, the concentrations of arachidonic acid (20:4, n6) and docosahexanoic acid (22:6, n3) in the mucosa of the inflamed bowel are increased (Nishida *et al* 1987; Pacheco *et al* 1987). Furthermore, in active Crohn's disease, inflammatory cell recruitment into the intestinal mucosa correlates well with tissue PUFA composition (Pacheco *et al* 1987). Disease remission, in turn, is associated with a fall in neutrophil (Mansfield *et al* 1995) and PUFA (Nishida *et al* 1987) content of the intestinal mucosa. Therefore, infiltration of neutrophils and mononuclear cells (which are rich in arachidonic acid-rich phospholipids (Cockcroft and Allan 1984)) into the intestinal mucosa, may be responsible, at least in part, for the rise in PUFA content of the intestinal mucosa in active Crohn's disease. This increase in the PUFA content of phospholipids is accompanied by a rise in phospholipase A<sub>2</sub> activity within the intestinal mucosa (Olaison *et al* 1988; Olaison *et al* 1989; Minami *et al* 1994), resulting in increased hydrolysis of the fatty acyl ester bond at the *sn*-2 position of phospholipids. In turn, this results in the release of free PUFAs, enhanced eicosanoid synthesis and further mucosal inflammation (Zifroni *et al* 1983; Sharon and Stenson 1984).

Although there have been no previous studies of plasma phospholipid species in Crohn's disease, the present results are consistent with earlier reports of plasma fatty acid composition in Crohn's disease (Esteve Comas *et al* 1992; Esteve Comas *et al* 1993; Royall *et al* 1994). In patients with both inactive (Esteve Comas *et al* 1993) and mildly active (Esteve Comas *et al* 1992) Crohn's disease, Esteve-Comas *et al* reported that both the proportion (% of total fatty acids) and the concentration (measured in  $\mu\text{mol/l}$ ) of arachidonic acid in the plasma was higher than that in controls, and there was a highly significant increase in docosahexanoic acid. Conversely, in patients with active Crohn's disease, the proportions of arachidonic acid and docosahexanoic acid in the plasma fell with increasing severity of disease. The authors postulated that, in active inflammatory bowel disease, increased PUFA biosynthesis coexists with increased fatty acid consumption. In mildly active disease, an enhanced PUFA biosynthesis predominates. However, when disease activity becomes more severe, PUFA hypermetabolism occurs, in order to meet the needs for cellular repair and energy (Esteve Comas *et al* 1992). In patients with extensive disease or resection of the small intestine, essential fatty acid deficiency may also play a role, resulting in low concentrations of PUFAs in plasma phospholipids, cholesterol esters and triacylglycerols (Shimoyama *et al* 1973; Press *et al* 1974; Farkkila *et al* 1987).

The present finding of a decrease with treatment of plasma PUFA-containing phospholipids to normal levels, is consistent with most (Esteve Comas *et al* 1992; Royall *et al* 1994), but not all (Esteve Comas *et al* 1993), previous reports. In the present study, there were parallel falls in the standard biochemical markers of inflammation, as well as in plasma lactoferrin concentrations. Lactoferrin, an iron-binding protein released from the secondary granules (as opposed to the primary granules) of neutrophils (Furmanski and Li 1990), plays a role in neutrophil-endothelial adherence and margination (Oseas *et al* 1981), and mucosal injury (Kurose *et al* 1994). In contrast to the one previous report of plasma lactoferrin concentrations in Crohn's disease (Adeyemi and Hodgson 1991), the results of the present study show that plasma lactoferrin concentrations remained higher in the treated patients than in the control subjects. This was despite the fact that treatment with "Peptamen" or prednisolone was successful in most patients — as judged by clinical response and falls in the ESR and

C-reactive protein concentrations. These findings suggest that there is either a primary defect in neutrophil secondary granule release in Crohn's disease, or that plasma lactoferrin may be a more sensitive marker of persisting neutrophil recruitment and intestinal mucosal inflammation, than standard biochemical markers of disease activity. Further studies of the relationship of lactoferrin to neutrophil recruitment and PUFA content of intestinal mucosa, and of its potential role in gallstone pathogenesis, in inflammatory bowel disease appear to be warranted.

## **CHAPTER 11. GENERAL DISCUSSION AND CONCLUSIONS**

### **11.1 NON-SURGICAL TREATMENT OF GALLSTONES**

The three studies of the non-surgical treatment of gallstones contained in this thesis have shown that, in patients selected the optimal criteria of low CT attenuation scores (which predict both the speed and completeness of gallstone dissolution) and a patent cystic duct, these treatments work best in patients with cholesterol-rich gallstones which are isodense with bile and/or have CT attenuation values of  $< 75$  HU. In those whose stones do not dissolve completely despite up to two years of oral bile acid therapy, the main causes for failure are impaired gallbladder contractility, acquired stone calcification and incomplete dissolution of presumed non-cholesterol residues (Pereira *et al* 1997). Furthermore, even if complete gallstone removal or dissolution is achieved, there is a high risk of gallstone recurrence so that these non-surgical approaches are effective in only a minority of patients overall (Pereira *et al* 1995b; Pereira *et al* 1995a). Irrespective of the original gallstone composition, recurrent stones are usually cholesterol-rich and are therefore potentially dissolvable with oral bile therapy. However, in terms of clinical- and cost-effectiveness, laparoscopic cholecystectomy remains the preferred treatment for symptomatic gallstones in almost all patients (Pereira *et al* 1993; Pereira 2002).

The availability of an effective and safe treatment to prevent gallstone recurrence after medical dissolution therapy would make conservative approaches more attractive and therefore more competitive with laparoscopic cholecystectomy. Gallstone dissolution/removal does not alter any of the three major predisposing factors — supersaturation, hypernucleation and gallbladder stasis — so that therapies aimed at correcting these factors would be a logical approach for future studies. For example, an improved understanding of the chemical structure and mechanism of action of pro- and anti-nucleating factors, and of the aetiology of impaired gallbladder motility in patients with, or at risk of developing, cholesterol-rich gallstones, are of potential importance. With respect to prevention of gallstone recurrence, low-dose ursodeoxycholic acid reduces, but does not prevent, this risk (Villanova *et al* 1989; Hood *et al* 1993), while

other drugs such as aspirin or prokinetic agents have either not been shown to be effective or have not been tested adequately in controlled trials.

## **11.2 GALLSTONE PATHOGENESIS**

### **11.2.1 Octreotide-induced gallstones**

Octreotide induces gallstone formation by a complex series of events that affect gallbladder and intestinal motility, bile composition and physical chemistry. The results of this thesis have shown that gallbladder bile from acromegalic patients treated with octreotide for at least three months had multiple abnormalities of bile composition that were comparable to those seen in conventional cholesterol gallstone disease. Specifically, there were significant increases in the proportions of biliary deoxycholate and arachidonic acid-rich phospholipid species, with associated rises in cholesterol saturation and mucin glycoprotein levels, and pathologically short cholesterol microcrystal nucleation times (Pereira *et al* 2001). These findings support other studies from our Unit (Hussaini *et al* 1994; Hussaini *et al* 1996; Veysey *et al* 1999; Thomas *et al* 2000; Thomas *et al* 2001a; Thomas *et al* 2001b; Veysey *et al* 2001a) and by others (Hofmann *et al* 1982; Angelico *et al* 1992; Berr *et al* 1992; Shoda *et al* 1995) which suggest that it is the increase in biliary deoxycholic acid, secondary to octreotide-induced prolongation of intestinal transit and/or abolition of meal-stimulated gallbladder emptying, which leads to the formation of lithogenic bile and cholesterol-rich gallstone formation.

Thus, acromegalic patients treated with octreotide appear to be a model for cholesterol gallstone pathogenesis. As in octreotide-induced gallstone disease, prolonged colonic transit times (Heaton *et al* 1993) and increased 7 $\alpha$ -dehydroxylating colonic bacteria (Wells *et al* 2000) have also been reported in patients with 'conventional' cholesterol-rich gallstones. Further studies of patients treated with octreotide could provide valuable insights into the pathogenesis of cholesterol gallstones in general and the prevention of primary and secondary gallstone formation. Possible strategies for changing deoxycholate kinetics to prevent octreotide-induced

stone formation could include the use of bran or wheat fibre supplements (Pomare *et al* 1976; Marcus and Heaton 1986a; Wechsler *et al* 1987), the administration of prokinetic agents that promote intestinal transit such as cisapride (Veysey *et al* 2001b) or senna (Marcus and Heaton 1986b; Lewis and Heaton 1997), and/or the reduction of colonic luminal pH with agents such as lactulose (Thornton and Heaton 1981; El Oufir *et al* 1996).

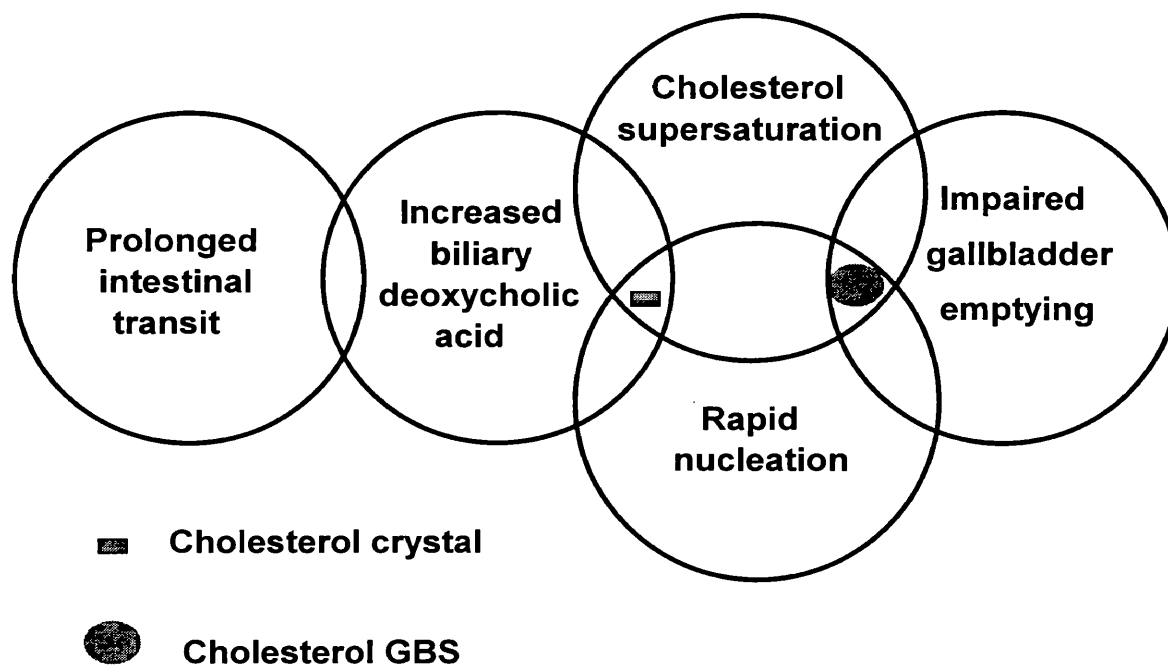


**Figure 11.1** Hypothesis for the formation of ‘conventional’ and octreotide-induced cholesterol-rich gallstones. Prolongation of intestinal transit leads to increased conversion of cholic to deoxycholic acid. The increase in biliary deoxycholic acid not only induces biliary cholesterol supersaturation but also the rapid nucleation of cholesterol microcrystals, which are retained in the gallbladder due to impaired gallbladder emptying and mucin glycoprotein hypersecretion, predisposing to the development of cholesterol-rich gallstones.

### **11.2.2 Gallstones in inflammatory bowel disease**

Patients with inflammatory bowel disease have an increased risk of developing gallbladder stones, but there have been no large studies of gallstone composition in such patients and there are few data on factors known to be important in ‘conventional’

cholesterol gallstone disease — such as the rate of cholesterol microcrystal nucleation or changes in biliary phospholipid composition.

The results of this thesis have shown that patients with small bowel Crohn's disease, as well as those who have undergone colectomy, have supersaturated bile and an increased tendency to form cholesterol microcrystals. In contrast, the bile of patients with ulcerative or Crohn's colitis was only marginally saturated with cholesterol and almost all had normal microcrystal nucleation times (Pereira *et al* 2002). The proportions of arachidonic acid-rich PC species in the bile of the patients with active Crohn's disease were also similar to those in patients with cholesterol-rich gallstones, and higher than the levels found in the bile of historical controls that we have reported previously (Pereira *et al* 2001). Plasma arachidonic acid-rich phospholipids, and both plasma and biliary concentrations of lactoferrin — a putative cholesterol microcrystal nucleation-promoting factor (Braganza and Worthington 1995) — were also increased and correlated with disease activity (Pereira *et al* 1996; Pereira *et al* 1998). It is possible, therefore, that the increase in biliary arachidonic-acid rich species and rapid nucleation times seen in these patients may be partly secondary to disease activity in Crohn's disease. Furthermore, the increase in cholesterol saturation and arachidonic acid-rich PC species occurred in the presence of low proportions of biliary deoxycholate — suggesting that the pathogenesis of gallstones in inflammatory bowel disease may differ from that of patients with 'conventional' or octreotide-induced cholesterol-rich gallstones. Indeed, our findings are consistent with those of previous studies in Crohn's disease which have implicated hepatic cholesterol hypersecretion, secondary to reduced bile acid absorption in the diseased or resected bowel (Rutgeerts *et al* 1986; Rutgeerts *et al* 1987; Santavirta *et al* 1990), as an important predisposing factor. Reports of impaired gallbladder contractility (Murray *et al* 1992c) and increased concentrations of cholesterol microcrystal nucleation-promoting factors (Keulemans *et al* 1999), in Crohn's disease indicate that such patients fulfil the 'triple defect' of cholesterol gallstone formation.

An alternative hypothesis for gallstone formation in Crohn's disease is that patients with ileal disease/resection are at particular risk of developing pigment gallstones, as a consequence of increased colonic bile acid levels solubilising unconjugated bilirubin

and promoting its absorption from the large intestine, thus increasing the enterohepatic cycling of bilirubin (Fevery 1999). Indeed, total biliary bilirubin levels were two- to three-fold higher in the patients with small bowel Crohn's disease than in those with colitis or who had undergone colectomy, but all but one of the patients with ileal disease also had supersaturated bile and almost half had abnormally rapid cholesterol microcrystal times (Pereira *et al* 2002). Other potential pathophysiological roles of increased biliary bilirubin in these patients are that mucin-bilirubin complexes in bile may function as nucleating agents for cholesterol gallstones (Smith and LaMont 1983), and biliary sludge — which consists of cholesterol microcrystals, calcium bilirubinate granules and a high concentration of mucin glycoprotein — is a known precursor to cholesterol-rich gallbladder stones (Ko *et al* 1999; Fracchia *et al* 2001). Given that cholesterol-rich gallstones are common in the general population, it remains unclear whether patients with ileal disease/resection are at increased risk of developing mixed, pigment-rich, rather than cholesterol-rich, stones, and further studies of gallstone composition in these patients are required.

### 11.3 GALLSTONE PREVENTION

The ultimate goal in gallstone research must be to prevent rather than to treat gallbladder stones, either medically or surgically. It is expected that more precise knowledge concerning the genetic determinants and molecular mechanisms underlying cholesterol cholelithiasis will accelerate future strategies for gallstone prevention and may provide the basis for identification of multiple key proteins as new targets for rational drug design and dietary interventions (Lammert *et al* 2001; Gilat *et al* 2001), and this remains a potential area for pharmacological intervention in humans. In principle, a number of 'wellness' recommendations — as advocated for the prevention of heart disease — may also decrease cholesterol gallstone formation. These would include: (i) the elimination of obesity (to reduce excessive cholesterol biosynthesis), (ii) consumption of a high-fibre, high-calcium, low saturated fatty acid diet and avoidance of constipation (which would decrease the biliary input of deoxycholic acid and may decrease cholesterol microcrystal nucleation), and (iii) a regular eating pattern, in order

to promote gallbladder emptying (Hofmann 1993). To date, there have been few controlled clinical trials aimed at the primary prevention of gallstones in high-risk individuals, but given the increasing evidence that changes in intestinal transit play a role in the pathogenesis of 'conventional' gallstone disease, this is a logical area for future research. With an understanding of the role of the gut in gallstone pathogenesis should come better planning of dietary and pharmacological interventions and improved advice on prevention.

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## **APPENDIX: PAPERS ARISING FROM THIS THESIS**

### **First-author papers**

#### ***Chapter 1 & 2:***

**Pereira SP**, Hussaini SH, Dowling RH (1993). 'Gallstones.' *Curr Opin Gastroenterol* **9**: 800-9.

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#### ***Chapter 3:***

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#### ***Chapter 5:***

**Pereira SP**, Hussaini SH, Kennedy C, Dowling RH (1995). 'Gallbladder stone recurrence after medical treatment: do gallstones recur true to type?' *Dig Dis Sci* **40**: 2568-75.

#### ***Chapter 7:***

**Pereira SP**, Hussaini SH, Murphy GM, Wass JAH, Dowling RH (2001). 'Octreotide increases the proportions of arachidonic acid-rich phospholipids in gallbladder bile.' *Aliment Pharmacol Therap* **15**: 1435-44.

### **Chapter 8:**

**Pereira SP**, Bain IM, Kumar D, Dowling RH (2002). 'Bile composition in inflammatory bowel disease: ileal disease and colectomy, but not colitis, induces lithogenic bile.' (*submitted*).

### **Chapter 9:**

**Pereira SP**, Rhodes JM, Campbell BJ, Kumar D, Bain IM, Murphy GM, Dowling RH (1998). 'Biliary lactoferrin concentrations are increased in active inflammatory bowel disease: a factor in the pathogenesis of primary sclerosing cholangitis?' *Clin Sci* **95**: 637-44.

### **Chapter 10:**

**Pereira SP**, Cassell TB, Engelman JL, Murphy GM, Sladen GE, Dowling RH (1996). 'Plasma arachidonic acid-rich phospholipids in Crohn's disease.' *Clin Sci* **91**: 509-12.

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Hussaini SH, **Pereira SP**, Dowling RH, Wass JAH (1993). 'Slow intestinal transit and gallstone formation.' *Lancet* **341**: 638.

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# Gallstones

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The past year has seen contributions to our understanding of gallstone pathogenesis from epidemiologic data and from studies of bile physical chemistry and the nucleation of cholesterol microcrystals. The importance of biliary bile acids, phospholipid composition, and mucus glycoprotein secretion in stone formation was investigated further, as was the role of biliary calcium, a component of both cholesterol-rich and pigment stones. Finally, more information on the natural history of biliary sludge, a precursor of gallstones, has come to light.

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## Epidemiology of gallstones

### Prevalence

Gallstones are more common in women than in men by a factor of approximately two. This difference in prevalence is attributed to effects of female sex hormones on biliary cholesterol secretion and gallbladder contractility. Recent data from the southeast of England, however, suggest that the prevalence of gallstones in men is increasing [1\*].

In a 10-year prospective study, Bates *et al.* [1\*] determined the prevalence of gallstones or cholecystectomy in autopsies performed on 8563 adults. Over a 10-year period, the prevalence of gallstone disease in women remained relatively constant (25% to 27%), but in men 50 to 59 years old, it rose from 7% during the first 3 years of the study, to 18% over the last 3 years. In men 60 to 69 years old, the prevalence increased from 12% to 20% and in this age group, the ratio of women to men with gallstone disease was 0.8:1. Cholecystectomy had been performed in 12% of the autopsies and was three times more common in women than in men.

In both sexes, there had been a slight increase in the mean body mass index (BMI) during the period of study. National data on dietary intake suggest that there has been a large reduction in saturated fats and a slight increase in polyunsaturated fats over the same period. Therefore, if the observed increase in the prevalence of gallstone disease in men is real, dietary factors may well be important in their pathogenesis.

### Pregnancy

Pregnancy is a well-known risk factor for gallstones [2\*,3\*]. In 980 Chilean women studied by ultrasound immediately after giving birth, Valdivieso *et al.* [4\*\*] found that the prevalence of gallstones was 12.2% compared with 1.3% in nulliparous, age-matched controls. Bile samples obtained by duodenal intubation from women within 48 hours of giving birth were supersaturated with cholesterol with a mean cholesterol saturation index (CSI) of 1.30 compared with a mean of 0.89 in nulliparous controls. A unique finding in this study was that when bile from 19 women with small stones was examined several weeks after delivery, it was unsaturated with cholesterol (CSI 0.89). This may explain why, in 41 puerperal women with stones measuring under 10 mm in diameter who were followed-up using ultrasound for 3 to 24 (mean 8.7) months, all remained asymptomatic and the stones disappeared spontaneously in 12 (29%). The biliary cholesterol supersaturation seen in pregnancy, therefore, is a reversible phenomenon. Stones formed during pregnancy are composed mostly of cholesterol; are usually asymptomatic, and in a proportion of women will disappear spontaneously after delivery.

### Obesity and rapid weight loss

Obesity is also a recognized risk factor for gallstones [5\*,6\*]. Two large prospective studies of the incidence of newly diagnosed symptomatic gallstones in American men of Japanese ancestry [7\*\*] and in obese American women [8\*\*], involving 152,831 and 607,104

### Abbreviations

BMI—body mass index; CSI—cholesterol saturation index.

person-years of observation respectively, have been published during this past year. In American men of Japanese ancestry, the relative risk of developing gallstones in those in the highest BMI quartile ( $\text{BMI} > 25.8 \text{ kg/m}^2$ ) was 1.6 times greater than those in the lowest quartile ( $\text{BMI} < 21.65$ ). In the Nurses' Health Study, the yearly incidence of symptomatic gallstones in women with a BMI greater than  $30 \text{ kg/m}^2$  was more than 1%, four times that seen in women with a BMI under  $24 \text{ kg/m}^2$ . However, in both studies, gallbladder ultrasonography was performed only in symptomatic individuals. Therefore, no conclusions about the incidence of prevalence of asymptomatic gallstones can be drawn from these studies. In fact, the results of epidemiologic studies show that 66% to 80% of gallstone carriers have "silent" or asymptomatic stones [9,10].

In both of these studies [7,8], cigarette smoking and a history of reduced caloric intake were also associated with a moderately increased risk of developing symptomatic gallstones. This is consistent with the observation that obese patients on very low calorie diets have a high incidence of cholesterol gallstone formation—up to 25% during the first 1 to 4 months of rapid weight loss [11–13]. In 457 obese subjects who enrolled in a weight control program and who were shown by ultrasonography to be stone-free before entry into the study, Yang *et al.* [13] found that the incidence of gallstones was 10.9% after 16 weeks of rapid weight loss. Men and women were at similar risk of forming stones. The risk factors for gallstone development were the initial BMI, the rate of weight loss, and serum triglyceride levels, although their combined sensitivity for predicting the formation of gallstones was only 12%.

### Slow intestinal transit

Known risk factors are present in only half of all patients with gallstones, Marcus and Heaton [14] have shown previously that, by prolonging whole-gut transit time, the percent biliary deoxycholate is increased in normal individuals and their bile becomes supersaturated with cholesterol. In the absence of other risk factors, therefore, abnormally slow intestinal transit may contribute to gallstone pathogenesis. To test this hypothesis, Heaton *et al.* [15] identified 15 nonobese women ( $\text{BMI} < 25 \text{ kg/m}^2$ ) with gallstones, from an ultrasound survey of 1058 women 25 to 69 years old. In these women and in age-matched nonobese controls with healthy gallbladders, whole-gut transit time was measured by following the rate of appearance, in two consecutive stools, of four different orally administered radiopaque shapes. In some cases, the whole-gut transit time was estimated indirectly from a formula based on defecation frequency and stool consistency. The authors found that in women with gallstones, the mean whole-gut transit time was significantly longer (82 vs 63 hours) and the mean stool weight was significantly

greater (74 vs 141 g/24 h) than in the matched controls (Fig. 1).

Support for the hypothesis that prolongation of intestinal transit may be important in gallstone pathogenesis comes from studies of acromegalic patients treated with the long-acting somatostatin analogue, octreotide. Octreotide induces gallbladder stones in up to 50% of patients. It is known to inhibit meal-stimulated cholecystokinin release and gallbladder contractility [16–18]. However, in control subjects and patients with the irritable bowel syndrome it also prolongs mouth-to-cecum transit time by a factor of three [19]. Furthermore, in acromegalic patients, octreotide also alters bile physical chemistry (by increasing the biliary cholesterol saturation, the percent of total biliary cholesterol transported in vesicles, and the cholesterol-to-phospholipid molar ratio in the vesicles and by shortening the nucleation time), with an increase in the relative amount of biliary deoxycholic acid [20]. The mechanism for the increased proportion of biliary deoxycholic acid is unknown, but it may be due to prolongation of intestinal transit, leading to increased bacterial deconjugation and  $7\alpha$ -dehydroxylation of the

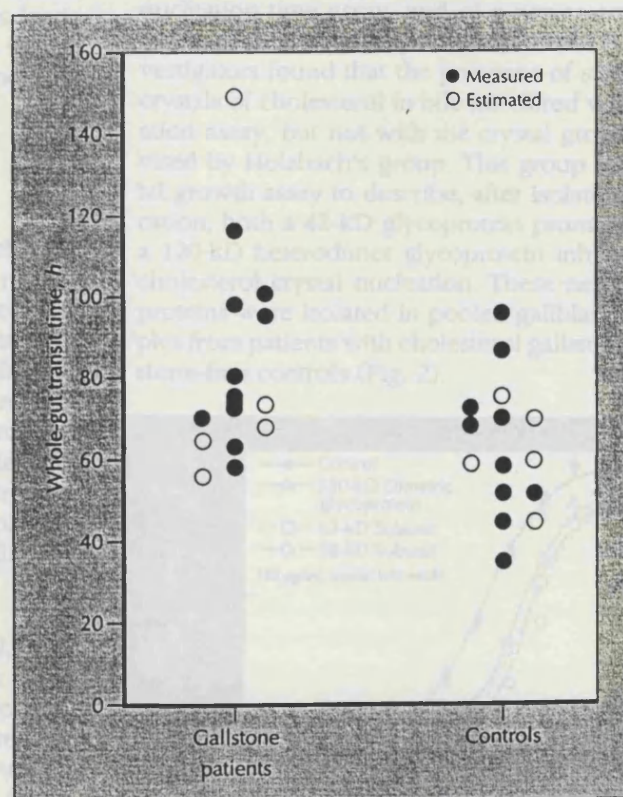


Fig. 1. Whole-gut transit time (WGTT) in normal-weight women with gallstones and in age-matched controls. WGTT was measured by the four-marker, two-stool method, or estimated indirectly from a formula based on defecation frequency and stool consistency. The WGTT of women with gallstones was, on average, 19 hours longer than that of their controls (95% confidence interval, 2 to 37). (From Heaton *et al.* [15]; with permission.)

parent bile acid, cholic acid, to form deoxycholate. Further studies in this area are awaited with interest.

## Bile physical chemistry and composition

In industrialized societies, most gallstones are cholesterol-rich. Cholesterol stones are thought to form in the presence of a triple defect: supersaturation of bile with cholesterol, abnormally rapid appearance in bile of cholesterol microcrystals (the so-called nucleation defect), and retention of crystals within the gallbladder. The range of pathogenic factors responsible for the formation of gallstones was reviewed by several authors during the year [21•–26•]. Carey and Lamont's comprehensive two-part review [22•,23•] dealt in detail with the pathobiology of biliary lipid secretion and lipid aggregates (cholesterol carriers), the role of biliary bile acid and phospholipid composition, the nucleation of cholesterol microcrystals, and the processes leading to the retention of cholesterol microcrystals within the gallbladder, such as mucus hypersecretion and gallbladder motor dysfunction.

### Formation of cholesterol microcrystals

An early event in the formation of cholesterol-rich gallbladder stones is the precipitation of cholesterol microcrystals in the gallbladder bile. Further evidence that cholesterol is co-secreted with phospholipids into the biliary canaliculus as unilamellar vesicles, came from a study in the bile fistula prairie dog [27•]. The results of this study also suggested that bile acid hydrophobicity not only determines the rate of biliary bile acid secretion but also whether unilamellar vesicles are solubilized to form mixed micelles alone (hydrophobic bile acid-rich biles) or mixed micelles plus multilamellar vesicle (hydrophilic bile acid-rich biles).

In bile that is supersaturated with cholesterol, bile acids solubilize phospholipids from unilamellar vesicles more than cholesterol, to form mixed micelles. As a result, the remaining vesicles have an increased cholesterol-to-phospholipid molar ratio. These unstable vesicles are prone to aggregate and fuse, forming multilamellar vesicle (liquid crystals) from which cholesterol crystals nucleate [24•]. A study from Japan [28•] confirmed that the major cholesterol carrier in bile, from which cholesterol microcrystals nucleate, is the large vesicle, with a molecular weight of 300 kD or more. When bile from patients with cholesterol gallstones was filtered through a 300-kD cut-off membrane, the nucleation time became prolonged. The authors of this study showed, by gel filtration chromatography, that the 300-kD filter removed vesicles, rather than micelles.

The same group [29•] demonstrated increased uptake of radiolabeled cholesterol from isotropic bile into a polyethylene disc ("cholesterol activity") associated with cholesterol crystallization. These findings applied to both the vesicular and micellar phases of bile from gallstone patients, but not from stone-free controls. They suggest that cholesterol activity represents the amount of thermodynamically unstable cholesterol in bile.

A new technique to monitor the formation of cholesterol microcrystals was reported by Konikoff *et al.* [30•]. They used time-lapse photography to show that cholesterol precipitation starts with the formation of helical, filamentous, or tubular microstructures (crystalline forms of anhydrous cholesterol), which pre-date the formation of the familiar, plate-like rhomboid crystals of cholesterol monohydrate.

The validity of the "conventional" cholesterol crystal nucleation time assay, and of a newer crystal growth assay, was examined by de Bruijn *et al.* [31•]. These investigators found that the presence of submicroscopic crystals of cholesterol in bile interfered with the nucleation assay, but not with the crystal growth assay devised by Holzbach's group. This group used the crystal growth assay to describe, after isolation and purification, both a 42-kD glycoprotein promoter [32•] and a 120-kD heterodimer glycoprotein inhibitor [33•] of cholesterol crystal nucleation. These newly described proteins were isolated in pooled gallbladder bile samples from patients with cholesterol gallstones, and from stone-free controls (Fig. 2).

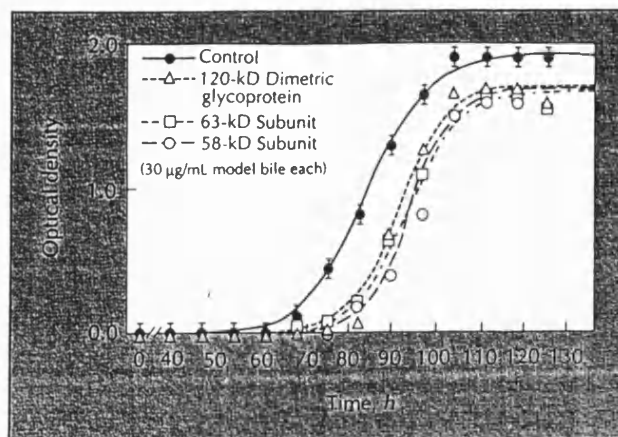


Fig. 2. Crystal growth curve of model bile (cholesterol saturation index = 1.5), demonstrating that a 120-kD heterodimer glycoprotein and the 63-kD and 58-kD subunits are inhibitors of cholesterol crystal nucleation and growth. (From Ohya *et al.* [33•]; with permission.)

In an accompanying editorial, Harvey and Strasberg [24•] highlighted the problems in determining the pro- and antinucleating factors that may play a physiologic

role in influencing nucleation time. The authors draw attention to the fact that the nucleation time assay—that is, the time taken for cholesterol microcrystals to appear in isotropic crystal-free aliquots of bile—is not only a measure of cholesterol nucleation but also of crystal growth to a size detectable by light microscopy.

From the hepatic bile of patients with cholesterol gallstone disease, Miquel *et al.* [34<sup>\*</sup>] isolated six different nonmucin glycoproteins with molecular weights of 52 to 200 kD, which were associated with cholesterol-rich vesicles and also had potent pronucleating activity. The implication from their data is that nucleating proteins act on vesicles, rather than on micelles, to promote nucleation.

The observation that, following nonsurgical clearance of gallbladder stones, patients with multiple gallstones at higher risk of gallstone recurrence than those patients with solitary stones, may be partially explained by the fact that patients with multiple gallstones have shorter nucleation times than those with solitary stones [35<sup>\*</sup>]. This observation by Jungst *et al.* [35<sup>\*</sup>] confirms the results of an earlier study by Groen *et al.* [36].

#### Biliary bile acid composition

Either a reduction in the total bile acid pool size or an increase in the percent deoxycholic acid in bile (which, in turn, increases biliary cholesterol secretion) may contribute to the formation of bile supersaturated with cholesterol. Berr *et al.* [37<sup>\*\*</sup>] found that in nonobese women with cholesterol gallstones, the size of the primary bile acid pool was reduced, while that of deoxycholic acid was normal or even increased (in those with a nonopacifying gallbladder). These changes were associated with an increased turnover of bile acids. The authors speculate that this may be due to rapid small bowel transit, because gallbladder emptying was either normal or reduced in the gallstone carriers whereas bile acid synthesis was the same in both the patients and the stone-free controls. However, as noted above, in another study of nonobese women with cholesterol gallstones [15<sup>\*\*</sup>], whole-gut transit time was prolonged. As yet, therefore, there is little consensus regarding the relationship between gallstone formation and intestinal motility.

Mixed, cholesterol-rich gallstones occasionally contain calcium salts of bile acids, which are more likely to precipitate within the biliary tract, in the presence of relatively uncommon secondary bile acids such as lithocholate, murideoxycholate, and allodeoxycholate [38<sup>\*</sup>]. These precipitates may not only contribute to gallstone formation but also to biliary sludge, which is a cause of idiopathic pancreatitis and a precursor of gallstones.

#### Biliary phospholipids and mucus glycoprotein

The secretion of biliary cholesterol is normally coupled tightly to that of phosphatidylcholine, the principal biliary phospholipid. Bile is especially rich in molecular species of phosphatidylcholine with 16:0 in the *sn*-1 position (palmitic acid with 16 carbons and no double bonds) and either 18:2 or 18:1 in the *sn*-2 position [39<sup>\*</sup>]. In cholesterol gallstone disease, an increase in percent biliary deoxycholate is associated with hepatic cholesterol hypersecretion [40<sup>\*</sup>], and also with an increased proportion of unsaturated molecular species of phosphatidylcholine, particularly arachidonic acid-rich (20:4) species, in bile [41<sup>\*</sup>]. Data from model bile systems [42,43<sup>\*</sup>,44<sup>\*</sup>] and from studies of human gallbladder bile [45<sup>\*\*</sup>], indicate that the molecular species of phosphatidylcholine are distributed asymmetrically between micelles and vesicles. Partitioning between these two carriers is determined by *sn*-1 fatty acid chain length and the degree of unsaturation of the fatty acids at both the *sn*-1 and *sn*-2 positions [42,45<sup>\*\*</sup>]. Arachidonic acid-rich phosphatidylcholines, by virtue of the looser packing constraints imposed by a longer chain length and particularly by a higher degree of unsaturation, partition less well into highly ordered biliary vesicles than into micelles. They are, therefore, found in higher concentrations in mixed micelles than in vesicles [42,43<sup>\*</sup>,45<sup>\*\*</sup>]. The fraction of cholesterol carried in mixed micelles is also increased [39<sup>\*</sup>,42,43<sup>\*</sup>]. As less phosphatidylcholine is available for vesicle formation, fewer cholesterol-rich vesicles will form and these will have a higher cholesterol-to-phospholipid molar ratio [42], thus favoring vesicle aggregation, fusion, and cholesterol microcrystal nucleation.

In addition to enhancing cholesterol secretion into bile and destabilizing the cholesterol carriers, arachidonic acid-rich phosphatidylcholine species may also play a role in stimulating mucus glycoprotein production by the gallbladder mucosa. Gallbladder mucin, a high molecular weight glycoprotein, is the major secretory product of the gallbladder epithelium. It can bind biliary lipids and accelerate the trapping and nucleation of cholesterol monohydrate crystals, both in supersaturated model and native biles [46]. During rapid weight loss in the obese, mucus glycoprotein hypersecretion precedes cholesterol microcrystal formation and is accompanied by an increase in the CSI [47<sup>\*\*</sup>,48<sup>\*</sup>].

Increases in mucus glycoprotein secretion are themselves preceded by increases in mucosal prostaglandin synthesis [49<sup>\*</sup>]. A recent study by Marks *et al.* [47<sup>\*\*</sup>] examines the sequence of events leading to gallstone formation in this patient group. In seven obese patients, repeated duodenal "bile" sampling by Entero-Test string (HDC Corp., Palo Alto, CA) was performed during the first 56 days of a very low calorie diet (520 kcal/d). Biliary cholesterol saturation and the

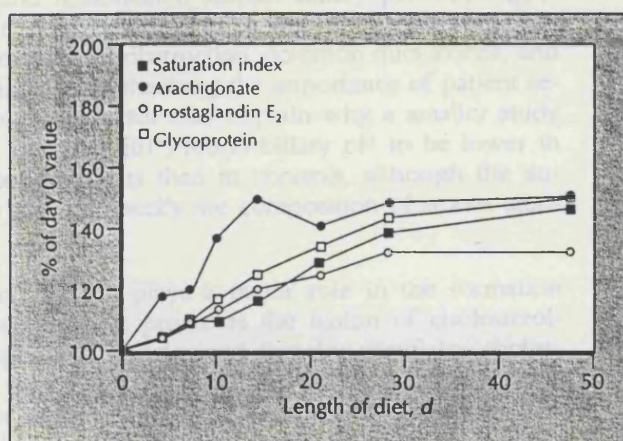


Fig. 3. Mean percent change in stimulated duodenal bile, in seven obese patients during a very low calorie diet (520 kcal/d). Increases first reached significance ( $P < 0.05$ ) for arachidonate at days 10 to 12, saturation index at days 14 to 15, prostaglandin E<sub>2</sub> at days 14 to 15, and glycoprotein at days 21 to 22. (From Marks *et al.* [47••]; with permission.)

concentrations of free arachidonic acid, prostaglandin E<sub>2</sub>, and mucus glycoprotein all increased within 4 weeks of beginning the diet. The order in which these events occurred, however, was of particular interest. The increase in biliary arachidonate levels antedated the increase in prostaglandin E<sub>2</sub> which, in turn, preceded the increase in glycoprotein. Although the number of patients studied was small, the data support the hypothesis that biliary arachidonate concentrations may influence biliary glycoprotein levels through the conversion of arachidonate to prostaglandins (Fig. 3).

There has been continuous interest in the role of aspirin and other nonsteroidal anti-inflammatory drugs in the prevention of experimental, primary, and recurrent gallstone formation. In animal models there is strong evidence that, by inhibiting gallbladder mucosal prostaglandin synthesis, aspirin and other nonsteroidal anti-inflammatory drugs reduce mucus glycoprotein synthesis [46]. However, in patients with cholesterol gallstones, evidence that mucus glycoprotein synthesis is prostaglandin-dependent is conflicting [50•–52•].

Rhodes *et al.* [51•] showed that, in gallstone patients scheduled for elective cholecystectomy, pretreatment for 1 week with aspirin 300 mg/d significantly reduced (by an average of 50%) the *in vitro* synthesis of mucus glycoprotein by strips of freshly excised gallbladders. Whether aspirin or other nonsteroidal anti-inflammatory drugs have other effects on the gallbladder, such as improving gallbladder contractility [53•], or whether they can prevent gallstone formation or recurrence in high-risk individuals, remains unclear.

### Gallbladder absorption and secretory function

The hypothesis that abnormal cholesterol metabolism within the gallbladder mucosa may contribute to cholesterol supersaturation in bile was examined by Sahlin *et al.* [54•]. These authors found no difference in the activities of the enzymes 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA R) and acyl coenzyme A:acetyltransferase (ACAT) in the gallbladder mucosa of patients with and patients without gallstones.

Igimi *et al.* [55••] investigated the secretory function of the gallbladder in 35 patients who had recovered from percutaneous transhepatic gallbladder drainage performed for acute cholecystitis. After an overnight fast, gallbladder bile was dark and had high concentrations of both bilirubin and bile acids. However, 2 hours after a meal, the bile had become clear and opalescent, and had the composition of an extracellular fluid. In the same paper, they demonstrated that sodium flux in canine gallbladder epithelial tissue cultures was stimulated by secretin. Therefore, the authors suggest that in the postprandial state, the gallbladder mucosa secretes a dilute electrolyte solution, possibly modulated by secretin.

In prairie dogs fed a lithogenic diet, changes in mucin secretion and gallbladder motility antedate the formation of stones. In this animal species, Conter *et al.* [56•] showed that gallbladder mucosal blood flow increased markedly during the early stages of cholesterol stone formation in the presence of supersaturated, as opposed to unsaturated, bile. They suggested that alterations in gallbladder mucosal blood flow, influenced by the presence or absence of lithogenic bile, may play a role in cholesterol gallstone formation.

### Biliary pH and calcium

The flux of calcium and hydrogen ions across the gallbladder mucosa is important in determining the solubility of calcium salts in bile. In the past, acidification of gallbladder bile was attributed primarily to the absorption of biliary bicarbonate. However, active hydrogen ion secretion, through a Na<sup>+</sup>-H<sup>+</sup> exchange mechanism, was demonstrated recently in human gallbladder explants [57••]. The authors of this study speculated that H<sup>+</sup> ion secretion by the gallbladder mucosa may protect against the precipitation of calcium salts in bile. Indeed, Gleeson *et al.* [58••] showed that the pH of gallbladder bile was higher in patients with surface gallstone calcification than in those with noncalcified stones, suggesting that impaired acidification of bile is one cause of calcified gallstone formation. This observation was confirmed by Knyrim *et al.* [59••]. However, Magnuson *et al.*

[60•] could find no differences in free ionized calcium carbonate saturation in patients with cholesterol gallstones, black pigment stones, or stone-free controls, although they did not stratify their results for calcified and noncalcified stones. Biliary pH was higher than normal, however, in patients with acute cholecystitis, cystic duct obstruction, common duct stones, and cholangitis, emphasizing the importance of patient selection. This factor may explain why a smaller study from Denmark [61•] found biliary pH to be lower in gallstone patients than in controls, although the authors did not specify the composition of stones studied.

Biliary calcium plays a major role in the formation of gallstones. It promotes the fusion of cholesterol-phospholipid vesicles and thereby stimulates cholesterol crystal growth. In gallbladder bile, calcium exists either as free  $\text{Ca}^{2+}$  or in bound form. Shiffman *et al.* [62•] measured total and free ionized calcium levels in gallbladder bile from 50 obese and nonobese patients undergoing cholecystectomy for gallstones, and in 45 stone-free controls. They found that, as the bile acid concentration increased, there was a linear increase in both total and free ionized calcium concentration. Furthermore, at any given bile acid concentration, the biliary-free  $\text{Ca}^{2+}$  concentration was higher in patients with stones than in the controls. The authors speculate that this increase in  $[\text{Ca}^{2+}]$  may be due to a Gibbs-Donnan effect exerted by excess mucin secretion. Although this finding was supported by the results of the Danish study [61•], Gleeson *et al.* [58•] found no correlation between free ionized  $[\text{Ca}^{2+}]$  and bile acid concentrations. The different pattern of results in the studies by Shiffman *et al.* [62•] and Gleeson *et al.* [58•] was the subject of an editorial comment [63•], but the discrepancies between the results from these two laboratories remain unresolved. They may relate to methodologic differences in measuring free ionized  $\text{Ca}^{2+}$  activity with two different calcium ion-selective electrodes.

In order for Gibbs-Donnan forces to play a significant role in determining the concentration of free ionized calcium concentration in bile, calcium must be freely permeable across the gallbladder mucosa. Evidence that the gallbladder mucosa is moderately permeable to calcium comes from studies of  $\text{Ca}^{2+}$  transport across the guinea pig gallbladder epithelium [64•].

The precipitation of calcium salts, like that of cholesterol, may be regulated by promoters and inhibitors of nucleation, Okido *et al.* [65•] isolated a 5-kD acidic protein from the bile of seven patients with calcium-containing black pigment stones, similar to that found in patients with cholesterol stones. This protein inhibited the initiation and growth of calcium carbonate crystals. The presence of calcium-

dependent antinucleating activity in gallbladder bile from controls was also found in the Munich study [59•].

## Biliary sludge

Biliary sludge is defined ultrasonographically as echogenic, gravitating material in the gallbladder that does not produce acoustic shadowing. It consists of cholesterol microcrystals, calcium bilirubinate granules, and a high concentration of mucin glycoprotein [66•]. There is increasing evidence that biliary sludge may cause symptoms and, in some patients, it may precede gallstone formation [66•,67•]. The results of two recent studies suggest that biliary sludge is responsible for most cases of idiopathic pancreatitis [68,69•]. Several risk factors for sludge formation have been identified [66•]. These include critically ill patients in intensive care units [70•] and those receiving total parenteral nutrition [71•–73•]. Murray *et al.* [70•] found that biliary sludge developed in 17 of 36 patients (47%) after a mean of 5.5 days in intensive care. Biochemical evidence of cholestasis developed in five of 16 non-sludge-forming patients and in 13 of 17 patients who developed sludge. However, eight of the sludge-forming patients also received total parenteral nutrition within a few days of admission, so it is difficult to know whether the sludge or the total parenteral nutrition was responsible for the abnormal liver function tests. During total parenteral nutrition, the absence of exogenous luminal nutrients leads to stagnation of bile acids within the enterohepatic circulation with relative cholestasis [74•]. Impaired gallbladder emptying also occurs in patients being nourished parenterally, and increases in both the CSI and the vesicular cholesterol concentration, with shortening of the nucleation time, have been documented after as little as 48 hours of intravenous hyperalimentation [72•,73•].

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## 39. Gallstones

STEPHEN P. PEREIRA

The purpose of this chapter is to review selected aspects of the non-surgical management of gallbladder stones, and to discuss the role for these treatments in the era of laparoscopic cholecystectomy.

### EPIDEMIOLOGY

Gallstone disease is **common**, although there are marked geographical variations, with a high prevalence in Chile and Scandinavia and among native Americans, and a low prevalence in sub-Saharan Africa and some parts of Asia. It affects between 10 and 20% of the world's population. Gallstones occur more frequently in women than in men by a factor of approximately two; this is attributed to the effects of female sex hormones on biliary cholesterol secretion and gallbladder contractility. Most studies report a prevalence of gallstones in women of 5–25% between the ages of 20 and 50 years, and 20–40% after the age of 50 years.

**First-degree relatives** of patients with gallstones have a prevalence of the disease that is increased at least twofold compared with that in the general population. Genetic factors implicated in the pathogenesis of gallstone disease remain poorly defined and are multifactorial.

**Pregnancy and parity** are other risk factors for cholelithiasis. In a study of 980 Chilean women who underwent ultrasound immediately after giving birth, the prevalence of gallstones was 12.2%, compared with a figure of 1.3% in 150 nulliparous, age-matched controls. Stones that form during pregnancy are composed mostly of cholesterol, are usu-

ally asymptomatic and, in up to 30% of women, disappear spontaneously after delivery.

**Obesity** also predisposes to gallstone formation. In the Nurses' Health Study of 90 302 women followed from 1980 to 1988, the annual incidence of symptomatic gallstones in women with a body mass index greater than 30 kg/m<sup>2</sup> was more than 1% — four times that seen in non-obese women. Those with the greatest body mass index (>45 kg/m<sup>2</sup>) had a sevenfold risk of developing symptomatic gallstones. Obesity is associated with enhanced total body cholesterol synthesis and increased secretion of cholesterol into bile, secondary to increased activity of the rate-limiting enzyme, hydroxymethyl glutaryl coenzyme A reductase. Obese patients consuming very low energy diets or having undergone jejunoileal bypass also have a high incidence of cholesterol gallstone formation, of up to 25% during the first 1–4 months of rapid weight loss.

**Crohn's disease and terminal ileal resection** are associated with up to a fourfold increase in the prevalence of gallstones. Their increased frequency in ileal Crohn's disease has been attributed to reduced active bile acid absorption in the terminal ileum, thereby reducing the bile acid pool within the enterohepatic circulation. As a consequence, the hepatic cholesterol:bile acid secretion ratio is increased, resulting in supersaturation of bile with cholesterol. However, a high biliary cholesterol saturation index has been reported in most, but not all studies of patients with ileal disease/resection and in only a minority of post-colectomy and colitis patients, so that this hypothesis remains controversial. An alternative explanation is that malabsorbed

bile acids solubilize unconjugated bilirubin in the colon and increase its enterohepatic cycling, which in turn increases the secretion of bilirubin pigments into the bile and subsequent pigment gallstone formation.

**Dietary factors** implicated in gallstone formation include a high energy intake, increased consumption of unrefined carbohydrate, and diets low in fibre (1). Other associated conditions include hypertriglyceridaemia, diabetes mellitus, and hepatic cirrhosis. Bacterial infection and parasitic infestation of the biliary tree are important factors in the development of pigment stones in Asia, but less so in the West. Patients with haemolytic anaemia caused by hereditary spherocytosis, sickle-cell disease, and thalassaemia also have an increased prevalence of pigment stones.

## GALLSTONE COMPOSITION AND MECHANISM OF FORMATION

### Cholesterol gallstones

Stones composed predominantly of cholesterol account for about 75% of all gallstones in Europe and North America. Pure cholesterol stones do occur, but most stones are mixed, and contain at least 70% cholesterol in a matrix of calcium bilirubinate, calcium phosphate, and mucin glycoprotein. Mixed stones are usually multiple, hard, and faceted, and have a layered structure when seen in cross-section.

Cholesterol stones form in the presence of supersaturation of bile with cholesterol secondary to hepatic cholesterol hypersecretion, an abnormally rapid appearance of cholesterol microcrystals (the so-called nucleation defect), or crystals retained within the gallbladder, secondary to mucin glycoprotein hypersecretion and gallbladder hypomotility.

### Pigment gallstones

Pigment stones can be brown or black, are formed predominantly of calcium bilirubinate, and contain less than 25% of cholesterol. They are usually small and multiple, and about half are radioopaque.

The soft, friable, brown pigment stones are especially common in populations in the Far East, and are associated with **parasitic infestations** and with *Escherichia coli*, *Bacteroides* spp., and clostridial colonization of the biliary tract. These bacteria

contribute to stone formation by deconjugating bilirubin diglucuronide to form free, unconjugated bilirubin, which combines with calcium to form sparingly soluble calcium bilirubinate. Microscopically, brown stones contain cytoskeletons of bacteria. Brown pigment stones are also associated with duodenal diverticula and are more likely to form *de novo* in bile ducts than are other types of stones.

The incidence of black or pure pigment stones increases with age, and they are found in the gallbladders of patients with cirrhosis or haemolytic disorders. Black pigment stones contain an insoluble black pigment, calcium bilirubinate, together with calcium carbonate and phosphate, calcium salts of fatty acids, and bile acids. All pigment stones also contain a large amount of mucin glycoprotein matrix.

### Biliary sludge

Biliary sludge is defined ultrasonographically as echogenic, gravitating material in the gallbladder that does not produce acoustic shadowing. It consists of cholesterol microcrystals, calcium bilirubinate granules, and a high concentration of mucin glycoprotein. Groups at risk of sludge formation include critically ill patients in intensive care units, patients with high spinal-cord injuries, and those receiving total parenteral nutrition (TPN). During TPN, the absence of exogenous luminal nutrients leads to reduced meal-stimulated release of peptide hormone and stagnation of bile acids within the enterohepatic circulation. Gallbladder emptying is also impaired. Increases in both the cholesterol saturation index and the vesicular cholesterol concentration, with shortening of the nucleation time, have been documented within 48 h of the commencement of TPN. Gallbladder sludge, in turn, can occur after as little as 3 weeks, and up to 40% of patients will develop gallstones after 4 months of continued treatment.

In a minority of patients, biliary sludge may cause symptoms and precede gallstone formation. In a study of 96 patients with biliary sludge who were followed prospectively for a mean of 38 months, 8% formed asymptomatic gallstones and 6% developed symptomatic stones requiring cholecystectomy. In 60% of the patients, the sludge disappeared and subsequently reappeared, whereas in 18% it resolved completely. Six patients with biliary sludge underwent cholecystectomy for severe biliary pain, recurrent acute pancreatitis, or both. In another study

of 286 patients with biliary sludge followed for 20 months, gallbladder stones or complications such as acute cholecystitis occurred in 20% of patients.

Transabdominal ultrasonography is relatively insensitive in detecting biliary sludge or stones that are less than 2 mm diameter. In an early study of 31 patients with acute pancreatitis that was considered to be idiopathic (no gallstones on ultrasound and no other causes found), microscopical examination of duodenal bile revealed bilirubinate granules or cholesterol crystals in 23 (86%), but sludge was seen by ultrasound in only 48%. In contrast, in a recent prospective study of 45 patients with acute pancreatitis or suspected choledocholithiasis but two consecutive "normal" transabdominal gallbladder ultrasound examinations, the sensitivity of **endoscopic ultrasound** for detecting gallbladder microlithiasis was 96%, compared with a sensitivity of 67% for cholecystokinin-induced duodenal bile (compared with the gold standard of cholecystectomy). Thus, biliary sludge/microlithiasis may be responsible for most cases of "idiopathic" acute pancreatitis and, in experienced hands, endoscopic endosonography is currently the most sensitive non-surgical method for detecting microlithiasis.

## NATURAL HISTORY OF GALLSTONES

### Asymptomatic gallstones

It has been estimated that primary gallbladder stones grow at a rate of 1–4 mm per year, and usually do not cause symptoms until at least 2–7 years after their formation. Stones remain silent or asymptomatic in 66–80% of gallstone carriers. Thus only a minority of patients will ever develop specific, gallstone-related symptoms such as biliary colic — arbitrarily defined as a steady epigastric or right upper-quadrant pain lasting more than 30 min that is unrelated to bowel movement. An even smaller proportion present with "surgical" complications that are mainly the result of obstruction of the cystic duct (acute cholecystitis with or without empyema formation, or even the rare Mirizzi syndrome) or common bile duct (cholestasis/jaundice, cholangitis, or pancreatitis).

In most long-term studies of patients with asymptomatic gallstones, the annual rates of developing biliary pain or gallstone complications vary from approximately 1% to 4%. Asymptomatic stones usually become symptomatic before they cause

complications, and the longer the stones remain quiescent after an initial attack of biliary colic, the less likely it is that complications will occur (2).

### *Choledocholithiasis in patients with asymptomatic gallbladder stones*

On the basis of the evidence above, prophylactic treatment of gallbladder stones in asymptomatic patients is rarely indicated. One exception is choledocholithiasis in patients with asymptomatic gallbladder stones. In patients who are poor risks for surgery and who do not have acute cholecystitis, **endoscopic retrograde cholangiopancreatography (ERCP)** and **sphincterotomy without cholecystectomy** are often used to treat stones in the common bile duct. In one study published more than a decade ago, only 18 of 186 patients treated in this manner required later cholecystectomy, during an average follow-up of 32 months. However, more recent prospective randomized studies have questioned the rationale of adopting a "wait and see" strategy. In one study (3) of 98 elderly and other high-risk patients (mean age 80 years) assigned randomly to undergo either open cholecystectomy with bile-duct exploration or endoscopic sphincterotomy alone, there were no significant differences in immediate morbidity (23% compared with 16%) or mortality (4% compared with 6%). However, during a mean follow-up of 17 months, biliary symptoms recurred in 10 of the sphincterotomy group (20%), seven of whom required later surgery. In a similar study (4) with more than 5 years follow-up, 13 of 35 patients (37%) in the sphincterotomy group required later surgery, compared with two of 41 in the open-cholecystectomy group. There are fewer data comparing laparoscopic cholecystectomy with sphincterotomy, but the former has been shown to have less morbidity and mortality than open cholecystectomy in high-risk patients. In patients who have had a previous sphincterotomy, oral bile-acid treatment is less effective in dissolving cholesterol-rich gallbladder stones because of reduced concentrations of the prescribed bile acid within the underfilled gallbladder.

### Symptomatic gallstones

Patients with symptomatic gallstones have a greater risk of developing recurrent biliary colic or gallstone complications than those with asymptomatic

gallstones. The average annual rates of developing severe pain (usually requiring cholecystectomy) ranges from 1–8%, with complications (acute cholecystitis, choledocholithiasis, pancreatitis) occurring in 1–3% per year. These figures suggest that treatment should be offered to patients only after significant biliary symptoms develop. In those with mild, non-specific symptoms, or who have had a single attack of biliary colic, simple observation alone may be appropriate, because as many as 30–50% of patients who have had one episode of pain will not have a recurrent episode.

## INVESTIGATIONS

### Ultrasonography

Transabdominal ultrasound has a reported sensitivity of 92–96% and specificity close to 100% if a hyperechoic image with acoustic shadowing is seen in the gallbladder. It can not reliably detect individual particles measuring less than 2 mm in diameter, and small stones located in the infundibulum may also be difficult to visualise. Ultrasound can also detect biliary sludge, but is less reliable for stones in the common bile duct, where the sensitivity of detecting choledocholithiasis or a dilated common bile duct ranges from 50 to 70%, compared with a sensitivity of about 95% for ERCP. Endoscopic ultrasonography has a sensitivity comparable to that of ERCP and a specificity approaching 100% in detecting bile-duct stones, and is likely to play an increasing role in the investigation of patients with a low or intermediate pre-test probability of having ductal stones, with associated reductions in costs and morbidity compared with ERCP.

In patients with symptomatic but uncomplicated cholelithiasis who are eligible for cholecystectomy, transabdominal ultrasound is usually the only imaging study required. However, in patients who are being considered for medical dissolution therapy, localized computed tomography of the gallbladder (to determine stone composition) and oral cholecystography (to assess patency of the cystic duct), are also indicated, for the reasons given below. Alternatively, a significant reduction of gallbladder volume detected in response to a cholecystokinetic stimulus, such as a fatty meal or intravenous cholecystokinin, during ultrasonography predicts patency of the cystic duct with acceptable accuracy.

### Computed tomography (CT)

Although radiolucent stones are usually cholesterol-rich, 14–20% of stones that appear lucent by plain X-ray are non-cholesterol in type, whereas at least 50% of stones that are lucent by conventional radiology appear dense by computed tomography (CT) of the gallbladder. The **maximum gallstone attenuation score**, measured by CT *in vivo*, predicts stone composition and dissolvability, and is cost effective in selecting patients for treatment by oral dissolution of the stones; values greater than 100 Hounsfield Units (HU) predict calcium-containing, non-dissolvable stones. The best results with medical dissolution treatment are obtained with stones that have low CT attenuation scores (less than 50–70 HU), or those that are isodense with bile and not visualized at all by CT.

### Cholescintigraphy

Although more commonly used in the evaluation of patients with suspected acute cholecystitis or post-operative biliary leak, **isotope scanning** of the gallbladder has been used in the detection of patients with chronic acalculous biliary pain. A technetium-99m-labelled derivative of iminodiacetic acid (e.g. hydroxy iminodiacetic acid) is administered intravenously and images are then recorded by gamma-camera. Serial scans after injection normally show radioactivity in the gallbladder, common bile duct, and small bowel within 30–60 min. An abnormal, or positive, scan is defined as non-visualization of the gallbladder with preserved excretion into the common bile duct and small bowel. False positive results may occur in patients with chronic liver disease, the critically ill, and those maintained on total parenteral nutrition, in whom the gallbladder is atonic. In patients who fail to have the gallbladder visualized within 60 min, the use of intravenous morphine sulphate (which increases pressure within the sphincter of Oddi and induces flow into the gallbladder unless the cystic duct is obstructed) reduces this false positive rate.

### Magnetic resonance imaging

**Magnetic resonance cholangiopancreatography** (MRCP) has the advantage over both ultrasound and ERCP of being completely non-invasive and is also highly specific in detecting bile-duct stones,

but recently reported sensitivities have ranged from less than 60% to over 90% — generally because of failure of MRCP to detect small stones (<3–6 mm) in a non-dilated biliary tree (5). Improvements in image acquisition, together with prospective studies to define in which clinical contexts MRCP may obviate the need for ERCP, are awaited.

## NON-SURGICAL TREATMENT OF GALLSTONES

### Medical treatment

In patients with symptomatic gallstones in whom there is a blocked cystic duct (30% of patients with gallstones diagnosed on ultrasound have a non-visualizing gallbladder on oral cholecystography), there is generally no role for medical treatment and the decision for the specialist usually lies between laparoscopic or open-abdominal removal of the gallbladder. However, in patients with a patent cystic duct who decline or are unfit for surgery, cholesterol-rich (radio- and CT-lucent) gallstones can be removed or dissolved from the gallbladder and bile ducts in a number of ways. These techniques avoid the discomfort and small risks of general anaesthesia and surgical exploration of the abdomen and bile ducts.

In general, for reasons of both clinical and cost effectiveness, the non-surgical treatments should be reserved for patients with mild, uncomplicated gallstone symptoms who decline surgery or in whom the risk of cholecystectomy is high. In a carefully selected group of patients, these medical approaches work moderately well, but they are relatively expensive, require long-term surveillance, and have a rate of recurrence of gallstones of approximately 50% at 5 years.

### Oral bile acid therapy

Oral bile acid therapy is the slowest, safest, and best documented of all the non-surgical or minimally invasive alternatives to cholecystectomy. Although the reported rates of dissolution of gallstones vary widely, up to 80% of patients with radio- and CT-lucent stones and a patent cystic duct will progress to confirmed complete dissolution of their gallstones during oral bile acid therapy — given alone, or together with extracorporeal shock-wave lithotripsy (ESWL). Until recently, the preferred oral bile acid regimen in most centres was combination therapy with chenodeoxycholic acid (5–7 mg/kg per day) and its 7- $\beta$ -hydroxy epimer, ursodeoxycholic acid (UDCA; 5–7 mg/kg per day). These bile acids, normal constituents of bile, act by reducing the hepatic synthesis and biliary excretion of cholesterol, resulting in cholesterol desaturation of bile and the leaching out of cholesterol from gallstones. In the UK, chenodeoxycholic acid is no longer available, and UDCA monotherapy is given at a dose of 10–12 mg/kg per day.

Gallstone dissolution usually requires at least 6 months, and up to 2 years, of oral bile acid therapy — depending on the size, number, and composition of the stones. Ultrasonography should be performed every 3–6 months until the gallbladder is clear of stones, followed by a repeat test 3 months later to confirm complete gallstone dissolution, at which time UDCA treatment is stopped. In most patients, treatment should also be discontinued if there is no evidence of partial gallstone dissolution (a decrease in the number or size of the stones) after 1 year, or incomplete dissolution after 2 years.

Recommended selection criteria for oral bile acid therapy are given in Table 39.1. The stones should be small (ideally no larger than 5 mm), so that there is a high surface:volume ratio, and

**Table 39.1.** Selection criteria for oral bile acid therapy and ESWL with or without adjuvant oral bile acids.

| Oral bile acids                                           | ESWL $\pm$ oral bile acids                                                |
|-----------------------------------------------------------|---------------------------------------------------------------------------|
| Stones $\leq 5$ mm<br>( $\leq 15$ mm acceptable)          | Single stone $\leq 20$ mm<br>(up to three stones $\leq 30$ mm acceptable) |
| Cholesterol-rich stones<br>(CT score <50–75 HU)           | Cholesterol-rich stones<br>(calcified rim acceptable)                     |
| Patent cystic duct<br>(OCG or ultrasound with fatty meal) | Patent cystic duct<br>(OCG or ultrasound with fatty meal)                 |
| Non-obese patient                                         | Non-obese patient                                                         |
| Mild biliary symptoms                                     | Mild biliary symptoms                                                     |
| Patient declines or is unfit for cholecystectomy          | Patient declines or is unfit for cholecystectomy                          |

cholesterol-rich as determined by a CT attenuation score less than 100 HU. In addition, the cystic duct should be patent (e.g. emptying of more than 30% after cholecystokinin or fatty-meal stimulation) and the patient should not be morbidly obese, so that enrichment of the bile with the prescribed bile acid and cholesterol desaturation can be accomplished. Unfortunately, these selection criteria account for only about 15% of the population with gallstones, with an estimated rate of 80–90% for complete dissolution of the gallstones at 2 years.

#### *Extracorporeal shock-wave lithotripsy*

The best results with ESWL are obtained in patients with a patent cystic duct and solitary, radio- and CT-lucent stones measuring less than 20 mm in diameter, although acceptable fragmentation rates are also achieved in patients with up to three large stones no more than 30 mm in diameter. Such patients represent a highly selected subset — an estimated 15% of all patients with symptomatic gallstones — although, only about 7% will have optimal selection criteria of a solitary radiolucent stone less than 20 mm in diameter in a functioning gallbladder. Given the high capital cost of the equipment and its depreciation and maintenance costs, this treatment remains confined to a few specialized centres — usually where the ESWL machine is also used in the treatment of renal stone disease (6).

Most reports suggest that cholesterol-rich gallstones can be targeted and fragmented to less than 10 mm in maximum diameter in 80–100% of patients. However, it is not always possible to target gallbladder stones in the obese and in those with small contracted or high, intrahepatic gallbladders masked by the costal margin. After fragmentation of the stones, oral bile acids are given until complete dissolution of the gallstones is confirmed by serial ultrasound. In general, cholesterol-rich stone fragments measuring less than 10 mm in diameter dissolve readily, although the best results are obtained in those with even smaller stone fragments (less than 5 mm in diameter). Adverse effects of ESWL are usually minor, but include biliary colic, skin petechiae, and microscopic haematuria.

Giant or impacted common bile duct stones that can not be removed after endoscopic sphincterotomy and mechanical lithotripsy are other appropriate targets for ESWL. If the stones are targeted

successfully, the fragments can then be extracted by conventional means. Alternative forms of treatment of difficult bile-duct stones are **electrohydraulic lithotripsy** and **laser lithotripsy**. The latter technique involves passage of a fine fiberoptic bundle capable of transmitting laser energy via a T-tube or by the endoscopic retrograde route into the common bile duct. In three published series using a pulsed-dye laser with an optical stone-detection system designed to improve stone targeting, complete stone clearance was achieved in 103 of 114 patients (90%). In a German randomized study comparing ESWL with intracorporeal laser lithotripsy for difficult bile duct stones, bile duct clearance was achieved in 22 of 30 patients (73%) in the ESWL group and in 29 of 30 (97%) in the laser lithotripsy group, after a maximum of three lithotripsy sessions. Next-generation, less costly, pulsed solid-state laser systems are currently under investigation, and may become available in some units in the UK.

#### *Instillation of contact solvents*

In symptomatic patients with a functioning gallbladder, cholesterol-rich stones can be rapidly dissolved by the direct instillation into the gallbladder of **methyl *tert*-butyl ether (MTBE)** or **ethyl propionate**, via a percutaneous, transhepatic catheter — usually performed under local anaesthesia. However, some patients experience considerable pain during the placement of the pig-tail catheter, despite intravenous sedation and analgesia, and the procedure is at the limits of acceptability as a technique suitable in conjunction with local anaesthesia.

Despite the initially rapid dissolution rates achieved with contact solvents over 6–12 h, gallstone dissolution is often incomplete. In an early study from the Mayo Clinic, 51 of 75 patients were left with residual gallstone debris. The residual particles usually disappear completely with oral bile-acid therapy, but the need for weeks or months of adjuvant oral bile acids partly defeats the aim of rapid, complete gallstone dissolution by this relatively invasive technique. MTBE has sedative–anaesthetic properties and it can cause other transient side effects, such as oedema of the gallbladder mucosa and duodenitis. Ethyl propionate seems to lack many of these disadvantages, but experience with it remains limited. There is probably still a small place for contact solvent dissolution of gallstones in patients at prohibitive

risk for general anaesthesia, but only in a few specialized (non-UK) centres where there is a large experience with the technique.

#### *Percutaneous cholecystolithotomy*

Percutaneous cholecystolithotomy (PCCL) is usually carried out under general anaesthesia. The gallbladder is punctured percutaneously, usually by the subhepatic route, under ultrasound or fluoroscopic control, or both. A guide-wire is then inserted into the gallbladder, and the track is serially dilated until it is wide enough to admit a 22 Fr Amplatz tube, through which an endoscope is passed; the stones are extracted under direct vision.

Because the stones are removed, rather than dissolved, they can be of any composition. The technique has had a particular role, therefore, in symptomatic patients with radioopaque, non-dissolvable stones (those with attenuation scores greater than 100 HU) who are not suitable for the less invasive non-surgical modalities. However, the total in-patient time and cost compare unfavourably with those associated with laparoscopic cholecystectomy, which has largely displaced PCCL from the list of management options.

#### **Reasons for incomplete dissolution of gallstones**

Complete dissolution of gallstones (by life table analysis) at 18 months can be achieved in up to 80% of those selected as suitable for medical dissolution treatment. However, even when the stones are lucent by conventional radiology and have CT scores of less than 100 HU, arrested gallstone dissolution (no ultrasonographic response to the oral bile acids after 1 year or partial, but arrested, dissolution after 2 years of treatment) still occurs in 20–35% of patients.

The reasons for incomplete gallstone dissolution in patients selected for treatment using the optimal criteria of a patent cystic duct and a low gallstone CT attenuation score include:

1. *Blocked cystic duct or impaired gallbladder emptying:* These may develop during treatment. In a study of 126 patients treated with UDCA alone, cystic duct obstruction developed in approximately 20% of patients after 4 years of treatment. Oral bile acid therapy itself is unlikely to be responsible for the development of

cystic duct obstruction, as the (American) National Cooperative Gallstone Study showed that the incidence of acquired cystic duct obstruction during oral bile acid therapy was not different from that in a matched, untreated group of patients with gallstones.

2. *Acquired stone calcification:* this has been reported in 9–21% of patients with no or arrested dissolution of gallstones during oral bile acid therapy, and is attributed to the bicarbonate-rich choleretic induced by the administration of UDCA, which results in the precipitation of calcium carbonate in bile. Acquired gallstone calcification may also occur spontaneously, and is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age.

#### **MANAGEMENT OF RECURRENT GALLSTONES**

When oral bile acid treatment is withdrawn after complete dissolution, gallstones recur at a rate of 10–15% per annum, reaching a cumulative actuarial plateau of between 40 and 70% (mean around 50%) after 5–10 years. Gallstone recurrence after successful shock-wave lithotripsy and adjuvant oral bile acids is approximately 7% at 1 year and increases to about 30% at 5 years. The lower recurrence rate after ESWL is probably attributable to the fact that some 90% of patients selected for lithotripsy initially have solitary stones — the recurrence rate in such patients is less than that in patients who, before treatment, had had multiple stones.

After confirmed complete dissolution of primary cholesterol-rich gallstones, patients should normally undergo annual ultrasonography to exclude recurrence of their gallstones — at least for the first few years. As calcification of primary cholesterol-rich gallstones is a function of both stone size and age, early detection of recurrent stones explains why, in most studies, even those patients who originally had calcified gallstones develop small, cholesterol-rich stones on recurrence. In two recent reports, recurrent stones were radiolucent by plain abdominal X-ray in approximately 95% of patients, and CT-lucent (attenuation score less than 100 HU) in almost 90%. Thus recurrent gallstones are usually small and cholesterol-rich, and readily dissolvable with oral bile acid therapy.

## PREVENTION OF GALLSTONES

### Diet

The related factors of obesity, hypertriglyceridaemia, reduced concentrations of high-density lipoproteins and physical inactivity are all associated with an increased risk of gallstone disease, and measures to improve these should be recommended to patients. However, apart from general advice about eating a diet naturally rich in fibre and low in sugar and fat, as recommended for the prevention of heart disease and cancer, consistent evidence for particular dietary factors in gallstone formation is lacking.

### Ursodeoxycholic acid

The cost of UDCA means that full-dose treatment is not a practical long-term management option in the prevention of primary or recurrent gallstone formation. The results of most studies also suggest that low-dose oral bile acid treatment reduces, but does not completely prevent, the risk of gallstone formation or recurrence. In the controlled British-Belgian gallstone dissolution trial, UDCA in one-third the full therapeutic dose (3 mg/kg per day) halved the rate of gallstone recurrence after successful dissolution. Short-term UDCA prophylaxis has been recommended in patients at high risk of gallstone formation, such as obese patients undergoing rapid weight loss as a result of gastroplasty or low (2184 kJ/day) energy dieting. In one study, 23% of the placebo-treated group developed gallstones over the 16-week period of the trial, compared with 6% of those treated with 150 mg UDCA twice daily. Moreover, this prevention of gallstone formation by UDCA was dose-dependent, only 2.8% of those taking 600 mg/day, and 1.6% of those taking 1200 mg/day, developed stones.

### Aspirin and non-steroidal anti-inflammatory drugs

When aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) are given to experimental animals together with a lithogenic diet, they inhibit gallbladder synthesis of mucosal prostaglandin and prevent hypersecretion of mucin glycoprotein (which has been implicated both in nucleation and in the trapping of cholesterol microcrystals), in addition to the formation of microcrystals and gallstones.

Subsequent studies in humans have largely confirmed these results in experimental animals, although the only controlled study of low-dose aspirin in preventing the complications of gallstones lacked sufficient power to detect a difference. In an early study of obese individuals during acute weight reduction, the incidence of developing microcrystals or gallstones was significantly reduced if patients were given high-dose aspirin (1300 mg/day). In a retrospective study of 75 patients who had undergone dissolution of gallstones, none of 12 patients who regularly took NSAIDs developed recurrent stones, whereas 20 recurrences occurred among the 63 who had never, or only occasionally, taken NSAIDs. These data provide insights into the pathogenesis of gallstone disease, but do not yet justify the use of, for example, low-dose aspirin for the prevention of primary or recurrent gallstones in high-risk individuals.

### Prokinetic agents

In patients at risk of sludge and stone formation, such as those in intensive care or receiving TPN, prokinetic drugs such as cholecystokinin, cisapride, and erythromycin can prevent the development of gallstones. These drugs stimulate gallbladder emptying, but they are also prokinetic to the intestine and may have an effect through decreased 7 $\alpha$ -dehydroxylation (by colonic bacteria) of cholic acid to form deoxycholate. Supportive evidence for this hypothesis comes from early studies of constipated people who were given laxatives, with the result that their bile became depleted of deoxycholic acid and less saturated with cholesterol. Similarly, in acromegalic patients receiving long-term treatment with octreotide, oral cisapride shortens large-bowel transit times and reduces the proportion of serum (and presumably biliary) deoxycholic acid to normal. However, as yet there have been no formal prospective studies of the efficacy of intestinal prokinetic agents in preventing cholesterol formation in high-risk groups, and cisapride has now been withdrawn from the market.

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# Percutaneous Cholecystolithotomy: Risks, Benefits, and Long-Term Outcome

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Pereira SP, Ellul JPM, Keightley A, Kennedy C, Dick J, Dowling RH. Percutaneous cholecystolithotomy: risks, benefits, and long-term outcome. *Scand J Gastroenterol* 1995;30:484-488.

**Background:** For symptomatic patients with gallbladder stones and a patent cystic duct who wish to retain their 'functioning' gallbladders, percutaneous cholecystolithotomy (PCCL) offers an alternative to open or laparoscopic cholecystectomy. However, there are few data on the risks and benefits of this approach or on the long-term outcome. **Methods and Results:** In 21 patients with symptomatic calcified gallstones, PCCL was successful (gallstone clearance) in 17 (81%). Four to 62 (median, 35) months after clearance 9 of the 17 remained symptom-free and stone-free, whereas 4 developed biliary sludge at 7, 30, 32, and 35 months, 2 of whom subsequently developed gallstones. In four other patients gallstones recurred without evidence of preceding biliary sludge at 9, 16, 19, and 27 months, corresponding to an actuarial gallstone recurrence rate at 36 months of  $53.4 \pm \text{SEM } 15.1\%$ , and a combined stone/sludge recurrence rate of  $63.4 \pm 13.5\%$ . **Conclusions:** PCCL is moderately effective but, because of the frequency of complications and sludge/stone recurrence, is likely to have only a limited residual role in the era of laparoscopic cholecystectomy.

**Key words:** Cholecystitis; gallbladder; gallstone recurrence; percutaneous cholecystolithotomy

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Laparoscopic cholecystectomy is now the option of choice for the surgical management of symptomatic gallstone disease (1). Nonetheless, there remains a small but significant number of patients who opt for non-surgical treatment of their gallbladder stones with oral bile acids (2), extracorporeal shockwave lithotripsy (ESWL), usually with adjuvant oral bile acids (3), contact solvents such as methyl *tert*-butyl ether (MTBE) (4) or ethyl propionate (5), or percutaneous cholecystolithotomy (PCCL) (6-9).

The technique of percutaneous extraction of gallbladder stones was first described in 1985 by Akiyama et al. (6) and Kerlan et al. (7). In 1988, Kellett et al. (8) reported their experience with PCCL performed as a single-stage procedure, using a transperitoneal approach and a modified percutaneous nephrolithotomy technique. More recently, other centres have also shown that PCCL is safe and effective (9), but there are few data on the long-term recurrence of gallbladder stones after PCCL.

We performed PCCL in 21 patients and record here our experience, with emphasis on efficacy, complications, and gallstone recurrence.

## PATIENTS AND METHODS

### Patients

Since 1988 approximately 350 patients have been referred to the Guy's Hospital Gallstone Clinic for non-surgical management of their gallbladder stones. Of these, only sympto-

matic patients with a patent cystic duct (opacification of the gallbladder during oral cholecystography) and cholesterol-rich stones (defined as those with computed tomography (CT) scores < 100 Hounsfield Units (HU)) were offered treatment with either oral bile acid therapy, ESWL with adjuvant oral bile acids, or MTBE (11-14). Between 1988 and 1991 symptomatic patients with a patent cystic duct and calcified (CT scores > 100 HU) stones were offered the choice of either PCCL or cholecystectomy.

Over a 2-year period 21 symptomatic patients, with a median of 5 gallbladder stones (range, 1-200) in an opacifying gallbladder during oral cholecystography, declined elective cholecystectomy and opted for PCCL. There were 17 women and 4 men with a mean age of 51 (range, 27-62) years. All 21 had CT-dense stones with maximum gallstone attenuation scores of 100-969 HU (median, 202 HU).

### PCCL technique

The technique used (15) was similar to that described by van Heerdan et al. (16) and Gillams et al. (17). In brief, under general anaesthesia, and using fluoroscopic  $\pm$  ultrasound guidance, the gallbladder was punctured with a 22-G spinal needle, using the subhepatic transperitoneal approach, and opacified with a double dose of cholecystographic contrast medium. The gallbladder was then repunctured with an 18-G Kellett needle, and a guidewire was introduced, which remained coiled within the gallbladder throughout the procedure. The needle was removed, the

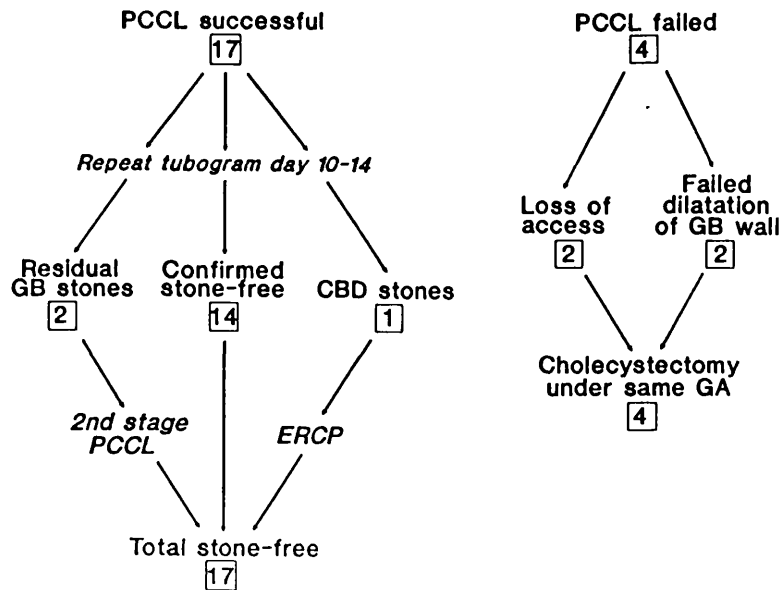


Fig. 1. Short-term outcome in 21 patients undergoing percutaneous cholecystolithotomy (PCCL). GB = gallbladder; ERCP = endoscopic retrograde cholangiopancreatography; GA = general anaesthesia; CBD = common bile duct.

tract dilated with Teflon or metal dilators to 24–30 F, and an Amplatz sheath of the same dimension inserted.

A rigid, 24-F side-viewing Wickham nephroscope (Wolf UK) was inserted through the Amplatz sheath, and the stones extracted under direct vision, using 'alligator' or grasping forceps (with or without prior electrohydraulic contact lithotripsy) or, in the case of small stones, flushed out by saline irrigation. When there were multiple stones, a larger stone was temporarily wedged in the entrance to the cystic duct to act as a 'plug' and prevent smaller stones from being flushed into the duct. This larger stone was then removed after all the other stones had been extracted. Visual confirmation of complete stone extraction was improved later in the series by using a modified flexible cystoscope (Olympus UK).

A 14- or 16-F Foley catheter was then introduced into the gallbladder, and a catheter cholecystogram ('tubogram') was performed to confirm complete stone removal, after which the guidewire was removed and the catheter balloon inflated under fluoroscopic control. Gentle traction was applied to the Foley catheter as the Amplatz sheath was removed, to minimize bile leakage from the puncture site.

Patients were usually discharged from hospital within 48 h of the procedure, to return 10–14 days later for repeat catheter cholangiography. If no gallstones were seen at that time, the Foley catheter was removed and the patient observed overnight to ensure that there was no evidence of bile leak.

#### Patient follow-up

After PCCL, patients were assessed clinically every 3–6

months, and with serial ultrasounds of the gallbladder annually, to exclude sludge or recurrent stone formation. If recurrent gallstones were detected, oral cholecystography (to assess patency of the cystic duct) and localized CT of the gallbladder (as a means of predicting stone composition and dissolvability (10)) were performed before further treatment was considered.

#### RESULTS

The short-term outcome of PCCL in the 21 patients is summarized as a flow diagram in Fig. 1.

The gallbladder was punctured in all 21 patients, but PCCL failed in 4 (19%) because the gallbladder wall could not be dilated ( $n = 2$ ) or because the tract became lost during dilatation ( $n = 2$ ). In all four the procedure was converted to open cholecystectomy under the same general anaesthetic. In the other 17, after PCCL and catheter drainage of the gallbladder for 10–14 days, a second catheter cholecystogram confirmed that the gallbladder was stone-free in 15 but showed retained gallbladder stones in 2, both of whom underwent a successful second-stage PCCL. In 1 of the 15 patients, a 59-year-old woman, the tubogram showed common bile duct stones. These had not been apparent at the time of gallbladder stone extraction 10 days previously, nor had they been predicted clinically, by ultrasonography, or on the basis of liver 'function' tests (18). She was treated successfully by endoscopic sphincterotomy plus stone extraction. Thus, complete gallstone clearance was ultimately achieved in 17 patients (81%).

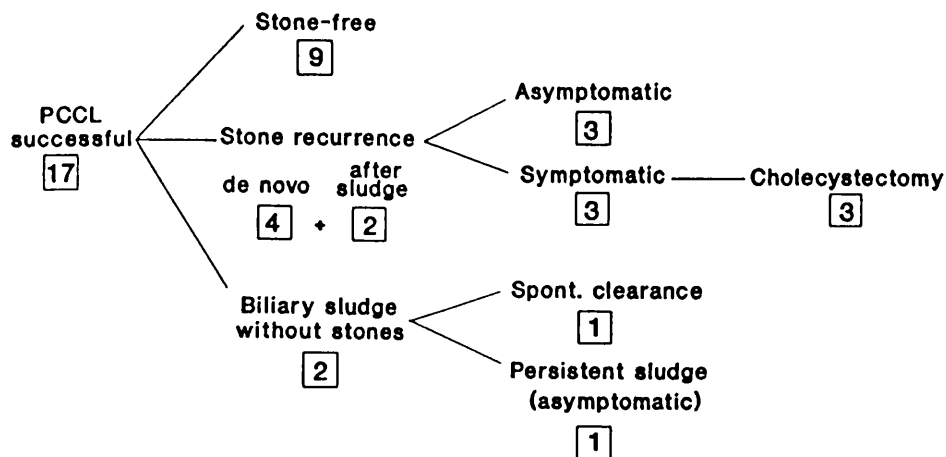


Fig. 2. Long-term outcome in the 17 patients rendered stone-free by percutaneous cholecystolithotomy (PCCL). Patients were followed up clinically, and by serial ultrasound, every 3–6 months for a median of 35 (range, 4–62) months. Biliary sludge was defined as gravitating, echogenic, but non-shadowing material seen at ultrasound.

#### Procedure-related complications

Three patients developed a transient (< 24 h) low-grade (< 38.0°C) fever within 48 h of the procedure, which responded to antibiotic therapy. Two other patients were found, at intra-operative catheter cholecystography, to have developed a minor leak of contrast material, but by ultrasonography there was no evidence of a subhepatic collection, and they were discharged on the 3rd postoperative day. In a further patient, a 28-year-old woman, the colon was inadvertently punctured, and she developed a small cholecystocolonic fistula. She was treated in hospital with a low-residue diet and Foley catheter drainage of the gallbladder for 2 weeks, after which a repeat catheter cholecystogram showed spontaneous closure of the fistula.

#### Long-term follow-up

After clearance, 17 patients have been followed up clinically, and with serial ultrasounds, for a median period of 35 (range, 4–62) months. Nine remain symptom-free and stone-free. However, in four biliary sludge was detected 7, 30, 32, and 35 months after PCCL. Initially, none of the four had any symptoms. In one the sludge disappeared spontaneously 4 months after it was first detected. In a second the sludge has persisted for 23 months. In two others, 4–6 months after the sludge was first detected and 36–38 months after PCCL, multiple (3–10) small (2–4 mm) gallbladder stones were seen at ultrasonography. These two patients both developed biliary colic 4 and 7 months after the recurrences were first diagnosed and underwent laparoscopic cholecystectomy.

In four other patients gallstones recurred without evidence of preceding biliary sludge at 9, 16, 19, and 27 months (symptomatic in one who underwent cholecystectomy 3 months after stone recurrence, and silent in three) (Fig. 2). By actuarial (life-table) analysis, the gallstone recurrence

rates were  $7.7 \pm \text{SEM } 7.4\%$  at 12 months,  $25.4 \pm 12.8\%$  at 24 months, and  $53.4 \pm 15.1\%$  at 36 months. The corresponding combined stone/sludge recurrence rates were  $15.4 \pm 10.0\%$ ,  $31.7 \pm 13.1\%$ , and  $63.4 \pm 13.5\%$ , respectively (Fig. 3).

#### Characteristics of recurrent gallstones

Initially, 5 of the 17 patients who underwent PCCL had had only 1 or 2 gallstones. All five remain stone-free after a median follow-up time of 19 (range, 4–37) months. However, of the 12 patients who underwent PCCL for multiple stones, no less than 6 developed multiple recurrent stones. In these 6 the recurrent gallstones were fewer (median, 2; range, 1–10) and smaller (2–6 mm) than the original stones (median, 10; range, 4–30; size, 5–14 mm)—probably because they were detected early as a result of regular surveillance.

#### DISCUSSION

Before the advent of laparoscopic cholecystectomy, PCCL was developed for the management of patients with symptomatic non-dissolvable gallbladder stones who wished to retain their 'functioning' gallbladders and avoid the 5–20% morbidity associated with open cholecystectomy (19–21). Recent reports suggest that, in patients with non-acute gallstone disease, complete stone clearance can be achieved by PCCL in more than 90% of patients (9, 16, 22–24). In the same reports, complications of bile leak or subhepatic bile collections occurred in 3–7% (9, 23, 24), and there have been at least two previous cases of inadvertent colonic puncture (16, 23). All these complications were treated conservatively, and to date there have been no reported deaths after elective PCCL.

These figures accord with our findings of an overall stone

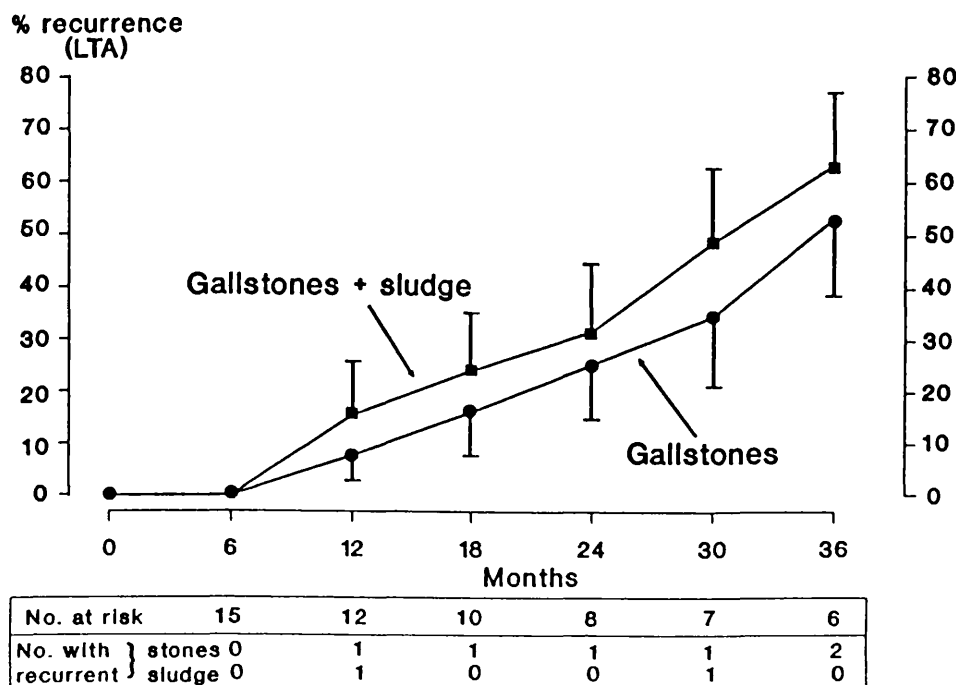


Fig. 3. Actuarial or life-table analysis (LTA) of gallstone, and combined stone and sludge, recurrence rates. Results are means ( $\pm$ SEMs). The number of patients 'at risk' and the number of recurrences of stones or sludge, and the combined stone/sludge recurrence rate, are shown for each time period, below.

clearance rate of 81%, and a complication rate for bile leak or colonic puncture of 14%. Whereas our complication rate, and that of others (16, 24), is similar to that reported for open cholecystectomy (19–21), it is higher than the reported morbidity after laparoscopic cholecystectomy (25–29).

As discussed below, there are advantages and disadvantages to leaving the gallbladder behind. One obvious disadvantage is the risk of stone recurrence. In patients with cholesterol-rich stones treated successfully with oral bile acid therapy, the risk of stone recurrence is approximately 10–15% per annum, rising to 50% at 5 years—after which it tends to plateau (30–32). However, the risk of recurrence is up to three times higher in patients who initially had multiple stones than in those who originally had solitary stones (31–33). Recurrence rates as low as 11% to 15% over 3 years have been reported in patients with solitary stones treated with oral bile acids (31, 32) or ESWL (33–36).

Data on stone recurrence rates after PCCL are limited, but reported rates have ranged from 10–27% at 14–18 months (9, 22) to 31% at a mean follow-up of 26 months (37). In the present series, with a median follow-up of 35 months, four patients developed biliary sludge, which is a precursor of gallstones in approximately 20% of patients (38). Two of the four with sludge subsequently developed recurrent gallstones, whereas four others developed recurrent gallstones 'de novo'—giving a combined recurrence rate of 35%, which corresponds to an actuarial rate of  $53.4 \pm 15.1\%$  at 36 months—rates similar to those reported after oral bile acid therapy in patients with multiple stones.

In the present series none of the five patients who originally had only one or two stones developed recurrent stones or sludge after a median follow-up time of 19 months.

The advantages of PCCL over laparoscopic cholecystectomy are that the patient is left with one small abdominal scar—as opposed to the four or five required for laparoscopic cholecystectomy—and he/she retains a functioning gallbladder (11, 39). However, these advantages are marginal, particularly when the risk of gallstone recurrence, suggested by the present results and those of others (22), is taken into account. In specialized centres, PCCL remains a valid management option for a selected minority of patients who have symptomatic gallstones and wish to retain their 'functioning' gallbladders or who are a high operative risk. However, there is a learning curve (17) and a significant morbidity associated with the procedure. We continue to offer other non-surgical techniques—oral bile acids, ESWL + oral bile acids, and, only occasionally now, contact stone dissolution with MTBE (32, 40, 41)—to symptomatic patients with cholesterol-rich gallbladder stones, but in our unit PCCL has been replaced by laparoscopic cholecystectomy.

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# Gallstone Dissolution with Oral Bile Acid Therapy

## Importance of Pretreatment CT Scanning and Reasons for Nonresponse

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In patients with cholesterol-rich gallbladder stones and a patent cystic duct, complete stone clearance rates of 65–90% have been reported with oral bile acids (OBAs) alone or with adjuvant lithotripsy (extracorporeal shock-wave lithotripsy; ESWL). The aims of the present study were to analyze pretreatment gallstone characteristics that predict the speed and completeness of dissolution with OBAs  $\pm$  ESWL, and to assess, in patients with incomplete dissolution, the reasons for the poor response. We compared pretreatment gallstone characteristics in 43 patients who became stone-free after a median of 9 months OBAs  $\pm$  ESWL with those in 43 age- and sex-matched patients whose stones failed to dissolve after two years of treatment. In those with incomplete gallstone dissolution, we repeated the oral cholecystogram and computed tomogram (CT) and, in selected patients, obtained gallbladder bile by percutaneous fine-needle puncture. In patients who became stone-free, those with stones that were isodense with bile and/or had CT scores of  $<75$  Hounsfield units had the fastest dissolution rates. In the 43 nonresponders, the main causes for treatment failure were impaired gallbladder contractility and acquired stone calcification. CT-lucent, noncholesterol stones, or failure of desaturation of bile with the prescribed bile acids, occurred in a minority. We conclude that the pretreatment CT attenuation score predicts both the speed and completeness of gallstone dissolution. In patients with incomplete stone dissolution, the combination of oral cholecystography, CT, and analysis of gallbladder bile will determine the underlying reasons for treatment failure in most, but not all, cases.

**KEY WORDS:** cholesterol saturation index; computed tomography; extracorporeal shock-wave lithotripsy; gallstones; oral bile acids; oral cholecystography.

In symptomatic patients with cholesterol-rich gallbladder stones and a patent cystic duct, complete stone clearance rates of 65–90% may be achieved with oral bile acids (OBAs) given alone (1–3) or together with extracorporeal shock-wave lithotripsy

(ESWL) (3–6). Because calcium-containing gallstones respond poorly to treatment [radiopaque stones contain significant amounts of calcium (7, 8)], oral cholecystography has been used to screen patients before treatment. However, approximately 50% of stones that appear lucent by conventional radiology are visibly dense on computed tomography (CT) and are unlikely to dissolve completely with oral bile acids (9, 10). Although there is controversy about the most appropriate cutoff point in the maximum gallstone attenuation score that should be used to

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exclude patients from dissolution therapy (4, 10–16), in our unit CT attenuation values  $>100$  Hounsfield units (HU) predict calcium-containing, nondissolvable stones (13). However, even when the stones are lucent by conventional radiology and have CT scores of  $<100$  HU, no/arrested gallstone dissolution (no cholecystosonographic response to the OBAs after one year or partial, but arrested, dissolution after two years of treatment) still occurs in up to 35% of patients (13, 14).

In patients selected for treatment using the optimal criteria of a patent cystic duct and a low gallstone CT attenuation score, the reasons for incomplete gallstone dissolution are poorly defined. Therefore, the aims of the present study were to determine, retrospectively, whether the pretreatment stone characteristics, including maximum gallstone CT attenuation scores, could predict the speed and completeness of dissolution with OBAs  $\pm$  ESWL, and to assess, in those with no/arrested dissolution, the reasons for the poor response.

## MATERIALS AND METHODS

Since 1986, localized CT scanning of the gallbladder has been performed routinely in all patients referred to the Guy's Hospital Gallstone Clinic for nonsurgical treatment of their gallbladder stones. Patients are selected for treatment with OBAs (ursodeoxycholic acid alone in a dose of 10 mg/kg/day or the combination of ursodeoxycholic acid 5 mg/kg/day + chenodeoxycholic acid 7.5 mg/kg/day), with or without adjuvant lithotripsy, only if: (1) specific, gallstone-related symptoms are present; (2) the cystic duct is patent (as judged usually by opacification of the gallbladder during oral cholecystography); (3) the gallstones are radiolucent by conventional radiology (plain abdominal x-ray and oral cholecystography); and (4) the stones have maximum CT attenuation scores of  $<100$  HU (13). After commencing treatment with OBAs  $\pm$  ESWL, gallstone dissolution is assessed every three to six months by ultrasonography. Complete gallstone dissolution is confirmed by two consecutive ultrasonographic examinations, at least one month apart, during continued OBA therapy. If sludge is detected in the gallbladder, the patient is not regarded as being stone-free and OBAs are continued.

In those without confirmed complete gallstone dissolution, treatment is usually withdrawn: (1) at one year in patients who have been compliant in taking their prescribed dose of OBAs but who have shown no radiological evidence of gallstone dissolution, or (2) after a total of two to three years of OBAs in those who have shown unequivocal evidence of partial gallstone dissolution (defined as a 50% decrease in gallstone number or volume, or both (13)) at one year but who have not progressed to complete gallstone dissolution despite continued, full-dose OBA therapy. To investigate the reasons for the nonresponse or arrested gallstone dissolution in these patients, our policy has been to repeat the oral cholecystogram and CT and, in selected

patients, to recommend ultrasound-guided, percutaneous, transhepatic, fine-needle aspiration of gallbladder bile (17, 18) for assessment of bile acid and bile lipid composition and bile physical chemistry.

**Patient Selection.** Two groups of patients were studied: (1) a stone-free group of 43 patients (33 women, 10 men; mean age 47, range 19–78 years) who had become stone-free after 4–27 (median 9) months of OBAs—alone ( $N = 18$ ) or with ESWL ( $N = 25$ ), and (2) a no/arrested dissolution group of 43 age- and sex-matched patients (33 women, 10 men; mean age 49, range 21–73 years) whose stones had not dissolved despite 12–36 (median 22) months of OBAs alone ( $N = 23$ ) or with adjuvant lithotripsy ( $N = 20$ ).

In these two groups we compared, retrospectively, pretreatment gallstone characteristics as follows. Ultrasound and oral cholecystogram films (including the preliminary precontrast x-ray) were reviewed “blindly” by a senior radiologist (C.K.) to assess the number and size of the gallbladder stones and their x-ray lucency and to determine whether or not the cystic duct was patent. Gallstone composition was estimated indirectly by maximum attenuation value of the stones at localized CT of the gallbladder, using 5-mm contiguous slices. A CT appearance of the stones was also assigned, as follows: (1) dense, homogenous calcification; (2) faintly homogenous calcification; (3) rim calcification; and (4) isodense with bile (not visible on contiguous 5-mm CT slices). If the gallstones were isodense with bile, the highest HU reading for bile was taken as the representative value for the stones (15, 16).

In the present study, patients were selected for treatment if the pretreatment CT attenuation scores of the stones were  $<100$  HU (13). In order to determine whether or not this cutoff point was too high a threshold, the efficacy of OBA therapy was compared in patients with pretreatment gallstone CT attenuation scores of  $<25$  HU, 25–49 HU, 50–74 HU, and 75–99 HU, respectively.

**Assessment of Patients with No/Arrested Gallstone Dissolution.** In the patients whose gallstones remained incompletely dissolved despite 12–36 months of OBAs  $\pm$  ESWL, we repeated the oral cholecystogram and localized CT scans of the gallbladder, in an attempt to determine the reasons for no/arrested dissolution. The development of a blocked cystic duct or impaired gallbladder contractility was assessed by repeat oral cholecystography with fatty meal stimulation. The presence of radio- and CT-lucent but presumably noncholesterol stones, or the development of acquired calcification during treatment, was evaluated by oral cholecystography and CT. The prescription of a sub-optimal bile acid dose (although in the present study, the doses of UDCA and CDCA were prescribed according to body weight) or poor compliance in taking the prescribed OBAs was also documented.

**Gallbladder Bile Analysis.** In consenting patients with no/arrested gallstone dissolution who: (1) claimed good compliance in taking the prescribed dose of OBAs regularly, (2) had a patent cystic duct at repeat oral cholecystography, and (3) had no evidence of acquired stone calcification at repeat CT, bile was obtained during continued treatment by the technique of ultrasound-guided, percutaneous, transhepatic, fine-needle, gallbladder puncture (17, 18). In the bile, we determined the cholesterol microcrystal

## GALLSTONE DISSOLUTION WITH ORAL BILE ACID THERAPY

TABLE 1. PRETREATMENT PATIENT AND GALLSTONE CHARACTERISTICS IN STONE-FREE AND NO/ARRESTED DISSOLUTION GROUPS

|                                | Stone-free<br>(N = 43) | No/arrested dissolution<br>(N = 43) |
|--------------------------------|------------------------|-------------------------------------|
| <b>Patient characteristics</b> |                        |                                     |
| Sex                            | 33F, 10M               | 33F, 10M                            |
| Age (yr)                       | 47 (19-78)             | 49 (21-73)                          |
| Weight (kg)                    | 69                     | 74                                  |
| <b>Stone characteristics</b>   |                        |                                     |
| Median stone no.               | 3                      | 3                                   |
| Maximum stone size (range), mm | 9 (3-35)               | 10 (3-32)                           |
| X-ray lucency                  | n = 43                 | n = 43                              |
| Mean CT score (range), HU      | 35 (10-76)             | 39 (4-93)                           |

nucleation times, cholesterol saturation indices (CSIs), and bile acid composition, as previously described (19). The results in these patients were then compared with those from consenting patients in the stone-free group who underwent gallbladder puncture at least four weeks after complete gallstone dissolution had been confirmed and OBA therapy withdrawn.

### RESULTS

**Pretreatment Gallstone Characteristics.** The demographic data and gallstone characteristics of the patients in the stone-free and no/arrested dissolution groups, are given in Table 1. Before treatment, all 86 patients had patent cystic ducts, gallbladder stones that appeared lucent by both abdominal x-ray and oral cholecystogram, and maximum CT attenuation scores of <100 HU. There were no significant differences between the stone-free and no/arrested dissolution groups in initial gallstone number, the diameter of the largest stone, or in maximum CT attenuation score.

**Value of Pretreatment CT in Predicting Dissolution Response.** The efficacy of OBA therapy as a function of the pretreatment CT attenuation score is given in Table 2. In the first three subgroups (<25 HU, 25-49 HU, and 50-74 HU), the numbers of patients in the stone-free and incomplete dissolution groups were similar. However, of the nine patients

TABLE 2. GALLSTONE DISSOLUTION WITH OBAS ± ESWL AS A FUNCTION OF PRETREATMENT CT ATTENUATION SCORE

| HU      | Stone-free | No/arrested dissolution |
|---------|------------|-------------------------|
| <25     | 15         | 12                      |
| 25-49   | 20         | 15                      |
| 50-74   | 7          | 8                       |
| 75-100* | 1          | 8                       |

\*P < 0.01 vs combined data of the other three subgroups.

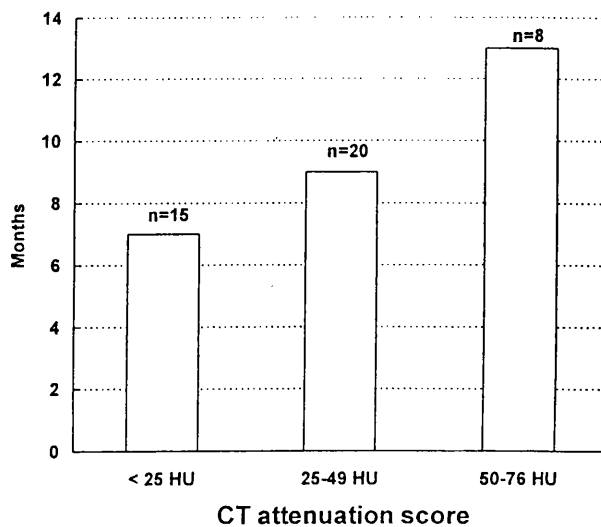


Fig 1. Mean time (in months) to confirmed complete gallstone dissolution, by pretreatment CT attenuation score.

with pretreatment scores of >75 HU, only one (11%) became stone-free, compared with 42 of 77 (55%) with scores of <75 HU ( $P < 0.01$ ) (Table 2). In fact, in the one patient with a pretreatment gallstone attenuation score of >75 HU who became stone-free, the actual value was 76 HU.

The visual appearance of the stones on the pretreatment CT films also predicted the dissolution response. Thus, 79% of the 59 patients whose stones were isodense with gallbladder bile became stone-free, compared with 58% of the 27 patients whose stones were visible on the CT scans ( $\chi^2$ ;  $P = 0.06$ ). Of the 43 patients in the stone-free group, 9 (21%) had stones that were visible at CT (faint homogenous calcification in two and rim calcification in seven), compared with 19 (44%) in the no/arrested dissolution group (dense homogenous calcification in two, faint homogenous calcification in 11, and rim calcification in six) ( $\chi^2 = 4.3$ ;  $P < 0.05$ ).

In the 43 patients who became stone-free, the pretreatment CT scores also predicted the speed of dissolution—the median times to complete gallstone disappearance being seven months in those with scores of <25 HU, nine months with scores of 25-49 HU, and 13 months with scores of 50-76 HU (Figure 1). Moreover, in patients with stones that were isodense with bile before treatment ( $N = 34$ ), the median time to confirmed complete gallstone dissolution was only 10 months, compared with 15 months in those whose stones were visible on the pretreatment CT scans ( $N = 9$ ).

**Reasons for No/Arrested Gallstone Dissolution.** In

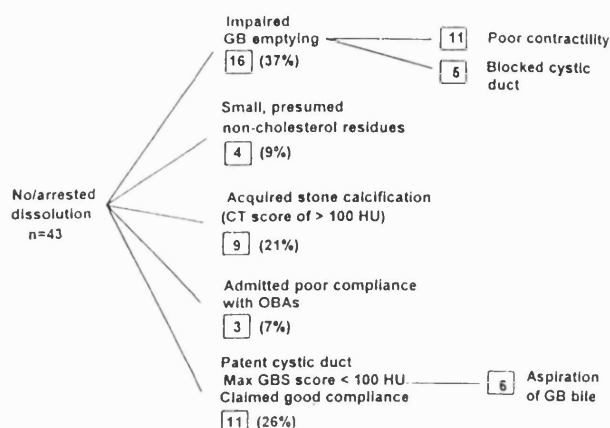


Fig 2. Reasons for no/arrested gallstone dissolution in 43 patients.

the 43 patients with no/arrested dissolution, repeat oral cholecystography at 12–36 months revealed that all but one patient still had stones that were lucent by conventional radiology and that gallbladder emptying had become markedly impaired in 16 (37%). The contractile response of the gallbladder to a fatty meal was classified as poor (ejection fraction <25%) in 11 patients, while the gallbladder did not opacify in five, indicating a blocked cystic duct. Three of these five had also had a poor contractile response to a fatty meal during the pretreatment cholecystogram (Figure 2).

Four of the 43 patients (9%) were left after treatment with multiple, small (2 to 5-mm), radio- and CT-lucent, presumably noncholesterol residues, while nine (21%) developed acquired stone calcification. By virtue of the selection criteria, before treatment all patients had maximum gallstone attenuation scores of <100 HU, with a median of 65 HU (range 15–93 HU). However, after 12–29 (median 24) months of treatment with UDCA  $\pm$  CDCA, the median score had increased to 132 HU (range 100–446 HU). Only one of these patients (a 55-year-old woman with a maximum gallstone attenuation score of 446 HU) had stones that were radiopaque by conventional radiology (Figure 3).

**Analysis of Gallbladder Bile.** Three of the 43 patients with no/arrested dissolution, in whom treatment was withdrawn at 15, 18, and 23 months, admitted to poor compliance in taking the prescribed dose of OBAs. However, in 11 other patients—all of whom had a patent cystic duct, x-ray- and CT-lucent stones, and claimed good compliance with OBA therapy—no explanation for the incomplete dissolution could be found. Six of these 11 consented to percutaneous,

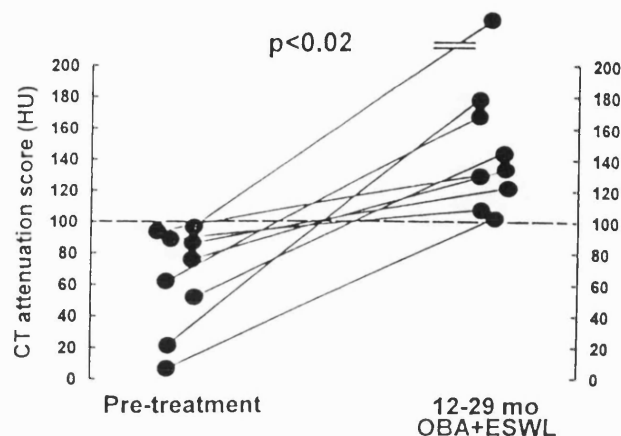


Fig 3. CT attenuation scores of gallstones, measured before and during 12–29 months oral bile acid therapy, in nine patients with no/arrested gallstone dissolution associated with acquired stone calcification.

fine-needle puncture of the gallbladder during continued UDCA + CDCA therapy.

In these six, the on-treatment amounts of CDCA, expressed as a percentage of total bile acids, were  $52.8 \pm \text{SEM } 6.3\%$  (range 30.7–72.0%), while those of UDCA (Figure 4) were  $16.2 \pm 3.8\%$  (range 1.0–25.3%). The mean CSI was  $1.0 \pm 0.11$ , with a range of 0.67–1.40 (Figure 4). In the three patients with unsaturated bile, the proportion of biliary UDCA was at least 15% of total bile acids—a lesser degree of enrichment than would be expected with full dose monotherapy (20, 21). However, despite the presence of unsaturated bile, the stones had not dissolved, suggesting that they were not cholesterol-rich. In contrast, two of the three patients with CSIs of greater

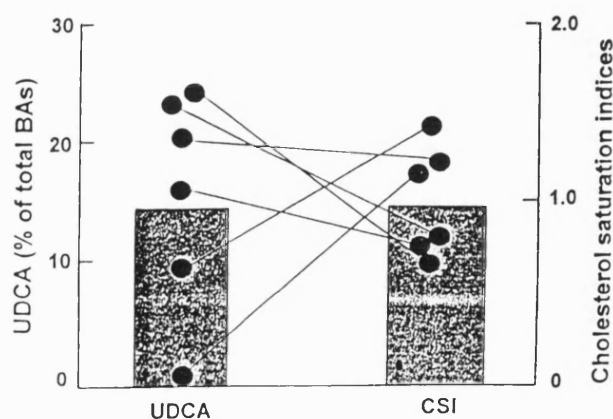


Fig 4. Biliary ursodeoxycholic acid (% of total BAs) and cholesterol saturation indices in six patients with no/arrested dissolution of cholesterol-rich (CT score <100 HU) gallstones in a "functioning" gallbladder and who claimed full compliance with OBA therapy, during continued treatment.

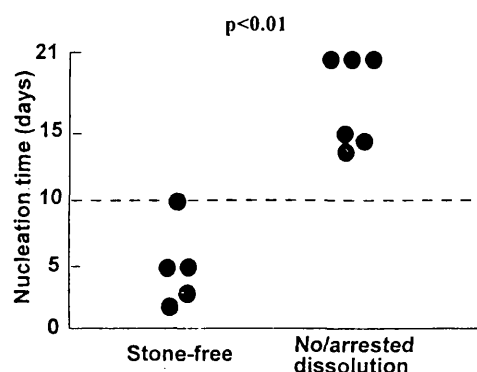


Fig 5. Cholesterol microcrystal nucleation times in the stone-free and no/arrested dissolution groups. The broken horizontal line at 10 days represents the lower limit of normal for cholesterol microcrystal nucleation. Note that, at the time of study, all five stone-free patients had discontinued oral bile acids while the six in the no/arrested dissolution group continued to take UDCA + CDCA (see text).

than 1.0 had low levels of UDCA, suggesting either that they were not taking or that they were not absorbing the prescribed dose of UDCA. In all six patients, the cholesterol microcrystal nucleation times during treatment were greater than 10 days, with a median of 18 (range 13–21) days (Figure 5).

The results of bile lipid analysis in five patients who became stone-free after 4–28 months of OBAs  $\pm$  ESWL, were similar to those reported previously in untreated patients with cholesterol-rich gallstones (19, 22, 23). Thus, all of the patients in the present study had bile that was supersaturated with cholesterol (mean 1.43, range 1.25–1.69) and pathologically short cholesterol microcrystal nucleation times of  $\leq 10$  days (median 5, range 2–10 days) (Figure 5). Moreover, their chenodeoxycholic acid levels of 36.3–53.5% (mean  $44.5 \pm 2.5\%$ ) of total bile acids were similar to those of the no/arrested dissolution group; UDCA was present in small amounts (mean  $0.7 \pm 0.3\%$ , range 0–1.4%). The other two major bile acids in gallbladder bile, cholic acid ( $31.4 \pm 4.6\%$  vs  $20.3 \pm 5.7\%$ ; NS) and deoxycholic acid ( $23.4 \pm 4.8\%$  vs  $9.9 \pm 4.7\%$ ; NS) were present in similar proportions in the stone-free and no/arrested dissolution groups, respectively.

## DISCUSSION

Oral bile acid dissolution therapy, with or without adjuvant extracorporeal shock-wave lithotripsy, is a safe and moderately effective treatment for selected patients with symptomatic cholesterol gallstone disease. Although the reported dissolution rates for gallstones vary widely, approximately 60% of patients

with radiolucent stones and a patent cystic duct will progress to complete gallstone dissolution during OBA therapy—given alone (2, 24) or together with ESWL (3). The use of pretreatment CT screening of the stones results in higher rates of confirmed complete gallstone dissolution by OBAs  $\pm$  ESWL (4, 13, 14, 16), but there is a need to refine selection criteria so that dissolution rates can be further improved.

In previous work from our unit, Walters et al (13) reported that patients with a pretreatment maximum gallstone CT attenuation score of  $<100$  HU had significantly better dissolution rates (50% confirmed complete gallstone dissolution at one year) than those not screened in this way (12% dissolution at one year). These results were similar to those of a subsequent study by Caroli et al (14), who used a lower maximum gallstone attenuation score cutoff of 60 HU but also reported complete dissolution rates at one year of 50%. The results of the present study extend these earlier findings they show that, in patients selected for treatment by a stone CT attenuation score of  $<100$  HU, gallstones that are isodense with bile and/or have CT scores of  $<75$  HU, dissolve more readily than those with scores of 75–100 HU. Furthermore, the lower the CT score, the faster the rate of gallstone dissolution.

A novel finding of the present study was that over two thirds of the 86 patients with gallstone attenuation scores of  $<100$  HU, had stones that were not visible on CT scanning of the gallbladder. Therefore, the stones were considered to be isodense with bile, with representative CT scores of the bile ranging from 2 to 44 (median 20) HU. One possible explanation for the high proportion of isodense gallstones is that they were actually missed at CT. However, this is unlikely since 5-mm contiguous CT slices of the gallbladder were performed and the median size of the stones was greater than 9 mm. Furthermore, of the 29 patients who had stones  $<5$  mm in diameter, 25 (86%) had multiple stones. Although the two-thirds prevalence of isodense stones is higher than the 14–57% reported by others (16, 25–27), in the present study the patients were selected for oral bile acid therapy if the CT attenuation value of the stones was  $<100$  HU. Thus, patients with gallstones that contained significant amounts of calcium [CT scores  $\geq 100$  HU (9, 13)] were excluded from dissolution treatment. The pretreatment CT score also predicted the speed of gallstone dissolution during OBA therapy—the times to confirmed complete gallstone dissolution being shortest in those patients with low stone attenuation scores, or whose stones were isodense at CT. In other

words, the lower the calcium content of the stones, assessed indirectly by CT, the faster the dissolution response. Indeed, there is a strong correlation between gallstone CT attenuation scores *in vivo* and cholesterol/calcium carbonate composition of the stones determined by chemical analysis (9, 10, 25–27).

Irrespective of the selection criteria, it is likely that the efficacy of oral bile acid treatment in achieving the goal of a symptom-free, stone-free patient, will never be 100%. In the present study, over a third of the 43 patients with no/arrested dissolution developed either a blocked cystic duct or impaired gallbladder emptying. Before commencing OBAs, the cystic duct had been patent in all patients, but there had been a poor contractile response of the gallbladder to fatty meal stimulation in three—all of whom later developed a blocked cystic duct during treatment. In an earlier study from our unit of 126 patients treated with UDCA alone, cystic duct obstruction developed in approximately 20% (by life-table analysis) of patients after four years of treatment (28). Oral bile therapy itself is unlikely to be responsible for the development of cystic duct obstruction. The results of the American National Cooperative Gallstone Study (NCGS) showed that the incidence of acquired cystic duct obstruction during OBA therapy was not different from that in a matched, untreated group of patients with gallstones (29).

In the present study, we used oral cholecystography, rather than ultrasonography with fatty meal stimulation, to assess patency of the cystic duct and to detect the presence or absence of impaired gallbladder contractility. The OCG results suggest that the reduced gallbladder emptying seen in response to a fatty meal in some patients with gallstones (30–32), may become further impaired during OBA therapy (30, 33, 34). Increases in the fasting gallbladder volume and postprandial residual volume during UDCA therapy have also been reported (30, 33–35). Therefore, it is possible that OBA treatment itself may be a factor in the development of incomplete gallstone dissolution, as well as the reported reduction in the frequency and/or severity of biliary colic noted during therapy (33, 36).

A further finding of this study was that 21% of patients with no/arrested dissolution had evidence of acquired stone calcification, with a significant increase in the median CT attenuation score from 65 HU before treatment, to 132 HU after a median of 22 months OBA therapy. This frequency of acquired stone calcification is higher than the 9–15% incidence reported in most (37–40), but not all (28), earlier

studies—although previous workers have employed oral cholecystography, rather than the more sensitive technique of CT (9, 10, 15, 25, 27), to assess the presence of acquired stone calcification. One explanation for acquired stone calcification may be the bicarbonate-rich choleresis induced by UDCA administration, resulting in the precipitation of  $\text{CaCO}_3$  in bile (21, 41). However, acquired gallstone calcification has also been reported in CDCA-treated patients (29, 42–44) and to occur spontaneously (29, 43, 45). Moreover, spontaneous stone calcification is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (7, 44, 46, 47). Therefore, it remains unclear whether or not UDCA itself is responsible for the development of acquired stone calcification.

The effects of oral bile acid therapy on biliary cholesterol saturation and bile acid composition in patients with gallstones have been studied extensively (1, 13, 21, 23, 36, 48–52). However, there have been few studies of gallbladder bile composition in patients with no/incomplete gallstone dissolution, despite prolonged OBA therapy (36). The present results show that the bile composition and physical chemistry in five patients who became stone-free after OBAs  $\pm$  ESWL, and who had been off treatment for at least one month, were similar to those reported previously in untreated patients with cholesterol-rich gallstones (19, 22, 23). In contrast, the results of biliary bile acid composition and cholesterol saturation indices in the six patients with incomplete dissolution, fell into two categories: (1) those with enrichment of their bile with the prescribed bile acids, low cholesterol saturation indices, but incomplete dissolution of presumed noncholesterol residues; and (2) those with low bile acid enrichment and supersaturated bile—possibly because of poor compliance with, or malabsorption of, the prescribed bile acids. In patients with gallstones, the degree of enrichment of bile with UDCA conjugates during chronic ingestion of UDCA is proportional to the bile acid dose (20, 21).

Although gallbladder bile was obtained from only a small number of patients, the results of the bile analyses suggest that, in selected patients, determination of biliary bile acid composition and cholesterol saturation [either in gallbladder bile obtained by fine-needle puncture (18) or in bile-rich duodenal fluid (36)], may be helpful in determining the reason for no/arrested gallstone dissolution. Conversely, in the present study, the cholesterol microcrystal nucleation time was of poor predictive value, being consistently prolonged to greater than 10 days in all six patients

with incomplete dissolution. Both UDCA and CDCA are known to reduce cholesterol secretion into bile, alter the biliary concentrations of nucleation promoting/inhibiting factors (23, 51, 52), and prolong the cholesterol microcrystal nucleation time (23, 51, 53). Furthermore, the combined administration of UDCA and CDCA appears to be more effective in dissolving cholesterol-rich gallbladder stones than either agent alone (24, 36, 49)—although this claim is debated (54).

In conclusion, cholesterol-rich gallstones that are isodense with bile and/or have CT attenuation values of <75 HU dissolve more readily and at a faster rate than stones with high CT attenuation scores. Moreover, the lower the pretreatment CT score, the more rapid the rate of gallstone dissolution. In those patients whose stones do not dissolve completely despite up to two years of oral bile acid therapy, the combination of oral cholecystography, CT, and analysis of gallbladder bile will determine the underlying reason for no/arrested stone dissolution in most, but not all, cases—the main causes for failure being impaired gallbladder contractility and acquired stone calcification.

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# Gallbladder Stone Recurrence After Medical Treatment

## Do Gallstones Recur True to Type?

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Medical treatments that dissolve or remove gallbladder stones but leave the gallbladder in situ have the disadvantage of gallstone recurrence. Little is known about the composition of recurrent stones or whether they recur true to type. In 21 patients with recurrent stones detected 5–74 months (mean  $\pm$  SEM,  $26 \pm 4$  months) after being rendered stone-free with dissolution therapy ( $N = 15$ ) or percutaneous cholecystolithotomy ( $N = 6$ ), we compared pretreatment and postrecurrence gallstone number, maximum gallstone attenuation scores measured by computed tomography (CT) and, in 13, the dissolvability of the recurrent stones with oral bile acids  $\pm$  extracorporeal shock-wave lithotripsy. Before treatment, five patients had solitary and 16 had multiple stones but on recurrence, the gallstones differed in number from the primary stones in 10 of the 21 patients. As a result of patient selection, before dissolution, the primary stones were all radiolucent with maximum CT scores of  $<100$  Hounsfield units (HU) (mean 45, range 10–84 HU). On recurrence, the stones were again CT-lucent in 13 of the 15 patients but were CT-dense in the remaining two (118 and 176 HU). Initially, all six patients treated by percutaneous cholecystolithotomy had radio-opaque stones, with a mean CT score of 459 (range 100–969) HU. However, on recurrence, only one had calcified stones (HU 140); the remaining five had CT-lucent stones (16–98 HU,  $P < 0.05$ ). Of the 13 patients whose recurrent, plain x-ray-lucent and CT-lucent stones were treated with oral bile acids  $\pm$  lithotripsy, 12 (92%) showed evidence of gallstone dissolution. We conclude that gallbladder stones do not recur true to type in up to two thirds of patients. However, irrespective of original gallstone composition, recurrent stones are usually radio- and CT-lucent, presumed cholesterol-rich, and therefore potentially dissolvable with oral bile acids.

**KEY WORDS:** bile acids; cholelithiasis; cholesterol; computed tomography; gallstone recurrence.

Before treatment, primary gallbladder stones may be solitary or multiple and radiolucent or radiopaque (1, 2). Radiolucent stones are usually, but not always, cholesterol-rich. Thus, 14–20% of stones that appear

lucent by conventional radiology are noncholesterol in type (3–6), while approximately 50% of stones that are lucent by conventional radiology appear dense by computed tomography (CT) of the gallbladder (7–10). The maximum gallstone attenuation score, measured by CT *in vivo*, predicts stone composition and dissolvability (10, 11). Therefore, CT scanning can be used to select patients for oral dissolution treatment. Although there is controversy about the most appropriate cutoff point in the maximum gallstone attenuation score that should be used to exclude patients

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## DO GALLBLADDER STONES RECUR TRUE TO TYPE?

TABLE 1. CHARACTERISTICS OF PRIMARY GALLSTONES BY FOUR TREATMENT MODALITIES\*

|                             | OBA<br>(N = 4) | ESWL + OBA<br>(N = 8) | MTBE<br>(N = 3) | PCCL<br>(N = 6) | Total<br>(N = 21) |
|-----------------------------|----------------|-----------------------|-----------------|-----------------|-------------------|
| Patients with               |                |                       |                 |                 |                   |
| Single stones               | 1              | 4                     | 0               | 0               | 5 (24%)           |
| Multiple stones             | 3              | 4                     | 3               | 6               | 16 (76%)          |
| Stone characteristics:      |                |                       |                 |                 |                   |
| Median diameter (mm; range) | 5 (4-6)        | 20 (5-22)             | 20 (4-27)       | 9 (6-18)        | 11 (4-27)         |
| X-ray-lucent                | 4              | 8                     | 3               | 2               | 17 (81%)          |
| Median CT score (HU; range) | 41 (32-84)     | 33 (10-79)            | 17 (10-50)      | 351 (100-969)   |                   |

\*OBA, oral bile acids; ESWL, extracorporeal shock-wave lithotripsy; MTBE, methyl *tert*-butyl ether; PCCL, percutaneous cholecystolithotomy; CT, computed tomography; HU, Hounsfield units.

from dissolution therapy (8, 12), values of >100 Hounsfield units (HU) predict calcium-containing, nondissolvable stones (10, 11).

While there have been many studies of primary gallstone composition, there are few data on the physical characteristics of recurrent stones or their response to medical dissolution therapy (13-16). The aims of this study, therefore, were to determine whether gallstones recur true to type—in terms of composition and stone number—and to assess the dissolvability of the recurrent stones with oral bile acid therapy.

### MATERIALS AND METHODS

Since 1986, all patients referred to the Guy's Hospital Gallstone Clinic for nonsurgical treatment of their gallbladder stones have undergone localized CT scanning of the gallbladder in addition to ultrasonography (US) and oral cholecystography (OCG).

Data on gallstone characteristics were collected in patients who had developed stone recurrence after: (1) confirmed complete gallstone dissolution with oral bile acids (OBA), extracorporeal shock-wave lithotripsy (ESWL) + adjuvant OBAs, or contact dissolution with methyl *tert*-butyl ether (MTBE); or (2) complete stone removal by percutaneous cholecystolithotomy (PCCL). Only patients with well-characterized (US, OCG, and localized CT of the gallbladder) primary and recurrent gallstones, were included.

US was used to assess the number and size of both the primary and the recurrent gallstones. Patency or blockage of the cystic duct was assessed by opacification of the gallbladder during OCG. All OCG films were also reviewed "blindly" by a senior radiologist (C.K.) to assess radiolucency of the stones and the contractile response of the gallbladder to fatty meal stimulation. Localized CT scanning of the gallbladder (5 mm contiguous slices) was used to estimate gallstone composition indirectly, by measuring the maximum stone attenuation scores in HU. When the gallstones were isodense with bile, the highest HU reading for bile was taken as the representative value for the stones.

**Gallstone Dissolution/Removal and Recurrence.** For patients who were treated with OBA (17-20) or ESWL + OBA (21-24), the gallstone dissolution response was assessed every three to six months by ultrasonography. Con-

firmed complete stone dissolution was accepted only if there were two normal cholecystosonograms, at least one month apart, during continued oral bile acid treatment. If sludge was detected in the gallbladder, the patient was not regarded as being stone-free and OBA therapy was continued.

In patients treated with MTBE (25-29), gallstone dissolution was assessed by catheter cholecystography during therapy and by an US performed the next day. If residual stones or biliary sludge were detected, adjuvant OBA therapy was commenced and continued until complete gallstone dissolution was achieved.

For patients treated with PCCL (30-32), complete gallstone clearance was assessed by catheter cholecystography immediately after the procedure and confirmed by a repeat "tubogram" 10-14 days later.

After confirmed gallstone dissolution or removal, all patients were followed up clinically and with serial ultrasounds at least once every 12 months. If recurrent stones were detected, a repeat OCG and localized CT scan of the gallbladder were performed, to assess patency of the cystic duct and stone composition, before considering further treatment.

**Statistical Analysis.** Data processing was performed using a Paradox data base (Borland International, Scotts Valley, California) and CRISP (CRunch Interactive Statistical Package; Crunch Software Corporation, San Francisco, California). A two-tailed *t* test was used for continuous variables, the  $\chi^2$  test for proportions, and the Mann-Whitney test for discontinuous variables.

### RESULTS

**Pretreatment Gallstone Characteristics.** Twenty-one patients who had developed stone recurrence after confirmed complete gallstone dissolution/removal and who had well-characterized primary and recurrent stones were studied. There were 18 women and three men, with a mean age of 49 (range 21-69) years.

The characteristics of the primary (pretreatment) gallstones are given in Table 1. As a result of selection, all 15 patients treated with one of the dissolution approaches had stones that were radiolucent by plain

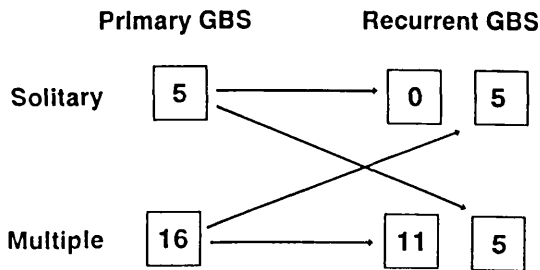


Fig 1. Comparison of primary and recurrent gallstones by stone number.

abdominal x-ray and OCG and had maximum CT attenuation values of <100 HU.

In the four patients treated with OBAs, complete gallstone dissolution was confirmed by ultrasonography after a mean of 16 (range 13–20) months treatment. In the eight patients treated with lithotripsy plus adjuvant oral bile acids, stone fragmentation (maximum diameter of fragments <10 mm) occurred after one to four (mean two) sessions of ESWL. Complete gallstone dissolution/clearance of the resultant fragments was achieved with adjuvant OBAs given for 12–23 (mean 17) months. In the three patients treated with MTBE, the stones did not dissolve completely with the contact solvent, and all three required adjuvant OBAs for 4–17 (mean 9) months before complete clearance of the stones was confirmed.

The six patients who underwent PCCL all had stones with maximum CT attenuation scores >100 HU. Therefore, by our criteria, none of these patients was eligible for any of the dissolution approaches. By conventional radiography, one of the six patients had radiolucent stones, a second had rim/surface stone calcification, while the remaining four had radio-opaque stones. Following PCCL, intraoperative catheter cholecystography showed that complete gallstone clearance had been achieved in all patients, and this was confirmed by the second “tubogram” 10–14 days later.

**Recurrent Stone Number and Size.** In the 21 patients, recurrent gallbladder stones were detected 5–74 months (mean  $\pm$  SEM,  $26 \pm 4$  months) after stone dissolution/removal. Of the 16 patients who originally had multiple stones, 11 developed multiple recurrent stones, while five had solitary recurrent stones. Of the five patients with solitary primary gallstones, all five developed multiple recurrent stones (Figure 1). From the total of 21 patients, the size of the recurrent stones (median four mm, range 2–11 mm) was smaller ( $N = 17$ ) or the same as ( $N = 4$ ) that

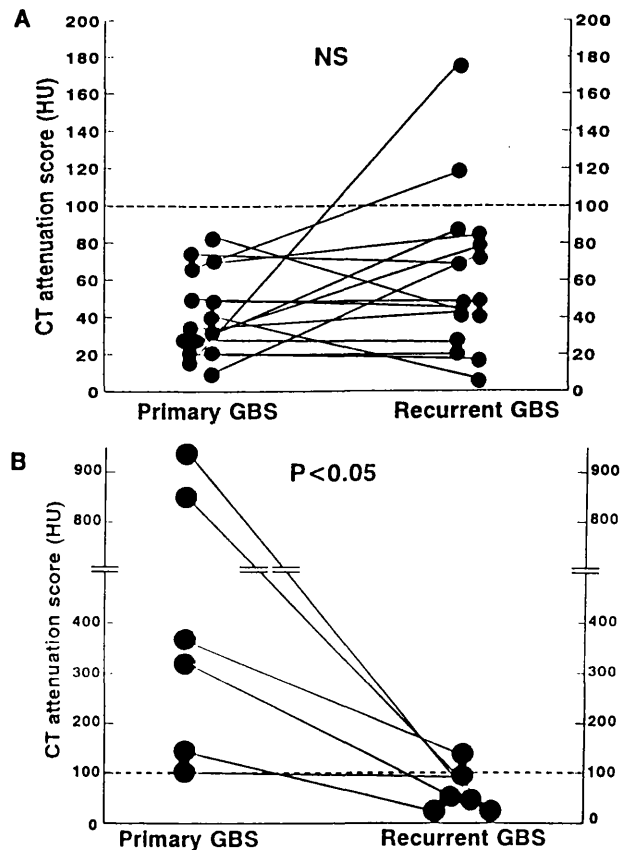


Fig 2. CT attenuation scores of primary and recurrent stones, in patients treated with (A) medical dissolution therapy, or (B) PCCL. Stone composition was assessed indirectly from the maximum Hounsfield unit (HU) score by localized CT of the gallbladder: A CT score of <100 HU suggested the stones were cholesterol-rich, while a score of  $\geq 100$  HU was indicative of calcified stones (10).

of the primary stones (median 9, range 4–27 mm)—probably because, as a result of regular surveillance, the recurrent stones were detected at an early stage of their development.

**Indirect Assessment of Gallstone Composition.** In the 15 patients who underwent dissolution treatment, the mean attenuation score of the primary stones was 45 (range 10–84) HU, while that of the recurrent stones was 62 HU (NS). In 13 of these 15 patients, the recurrent stones were again CT-lucent, with maximum scores of 6–88 HU, but two developed stones with scores of >100 HU (118 and 176 HU)—suggesting that the recurrent stones contained at least some calcium salts (Figure 2).

By plain abdominal x-ray and OCG, none of the primary or recurrent stones in the 15 patients was radio-opaque, although one patient developed a solitary recurrent stone with faint rim calcification. Both

## DO GALLBLADDER STONES RECUR TRUE TO TYPE?

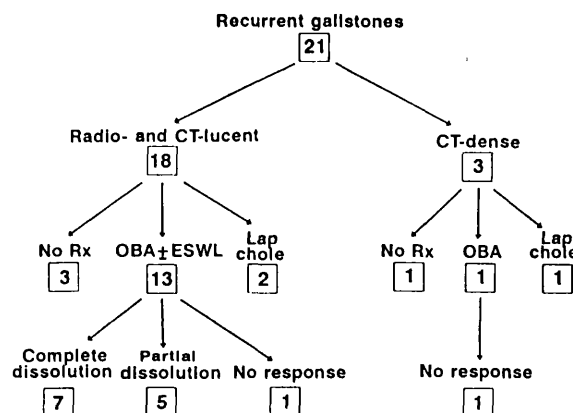


Fig 3. Treatment outcome in the 21 patients with recurrent gallstones.

before treatment and after recurrence, the cystic duct remained patent in all patients, although in one patient with recurrent stones, the gallbladder opacified only faintly during OCG.

In the six patients who developed gallstone recurrence after PCCL, oral cholecystography again confirmed that the cystic duct remained patent in every case and showed no evidence of calcification in the recurrent stones. By CT, however, one of the six had calcium-containing recurrent stones with a maximum HU score of 140, while a second had stones with a score of 98 HU—close to our arbitrary cutoff point of 100 HU. In the other four patients, the recurrent stones were isodense with gallbladder bile, which had representative values of 16–50 HU, again suggesting that the recurrent stones were cholesterol-rich (Figure 2).

**Treatment of Recurrent Stones.** The management of the patients with recurrent gallstones and their response to oral bile acid treatment ( $\pm$ ESWL) are summarized as a flow diagram in Figure 3.

Thirteen of the 18 patients with recurrent stones that were lucent by conventional radiology and CT were treated with OBAs ( $N = 11$ ) or lithotripsy + OBAs ( $N = 2$ ). After a mean duration of nine months OBA treatment (range 2–15 months), dissolution of the recurrent stones was complete in seven and partial in five. In those with partial gallstone dissolution, the stone “load” decreased from a median of four stones with a maximum diameter of 6 mm, to a median of two with a maximum diameter of 4 mm—suggesting that at least parts of the recurrent stones were cholesterol-rich. The patient with recurrent stones in a poorly opacifying gallbladder showed no evidence of stone dissolution after 22 months OBA therapy.

To date, two other patients with plain x-ray-lucent and CT-lucent recurrent stones have been lost to follow-up and one has received no treatment, while the remaining two developed biliary colic and underwent laparoscopic cholecystectomy. Samples of the gallbladder bile in these two patients (obtained during surgery): (1) contained cholesterol microcrystals, (2) were supersaturated with cholesterol (saturation indices of 1.5 and 1.3) and (3) had rapid microcrystal nucleation times (one and three days, respectively) (33)—thus providing further strong, albeit indirect, evidence that their recurrent stones were cholesterol-rich.

Of the three patients with CT-dense, calcium-containing recurrent stones, one remains asymptomatic and untreated, a second developed biliary colic and underwent laparoscopic cholecystectomy (her stones were not available for analysis), while the third, whom we elected to treat with OBAs despite the fact that her maximum gallstone attenuation score was 118 HU, has shown no dissolution response after 11 months therapy with ursodeoxycholic acid 5 mg/kg/d + chenodeoxycholic acid 7.5 mg/kg/d.

## DISCUSSION

The results of the present study show that in terms of stone number or composition, gallbladder stones often do not recur true to type. However, irrespective of the composition of the original stones, recurrent stones are usually lucent by conventional x-ray and CT, presumed cholesterol-rich, and potentially dissolvable with medical therapy. These findings have important implications for the treatment of recurrent gallstone disease. They also provide clues about gallstone pathogenesis in the high-risk group of patients whose stones have been removed or dissolved, but who retain their functioning gallbladders.

The development of recurrent gallbladder stones, like that of primary gallstones, involves at least a triple defect: (1) cholesterol supersaturation, (2) abnormally rapid nucleation in bile of cholesterol microcrystals, and (3) impaired gallbladder emptying (34).

In patients treated with oral bile acids, when complete dissolution of the primary gallstones has been confirmed and the bile acid treatment withdrawn, the gallbladder bile reverts to being supersaturated with cholesterol (35, 36) in one to four weeks (36). Furthermore, in patients whose gallstones have been cleared with the combination of shock-wave lithotripsy plus oral bile acids, the impaired gallbladder

emptying, found in many patients with primary gallstones (37–41), persists (40, 42). Given that these defects persist or recur in up to 100% of patients rendered stone-free by dissolution or extraction, while gallstones recur in only 50%, these two components of the triple defect (cholesterol supersaturation and impaired gallbladder motility) have low predictive value for the development of gallstone recurrence. By implication, therefore, the component most likely to distinguish between those who will, and those who will not, develop recurrence is abnormal nucleation—whether due to persistence or recurrence of an excess of pronucleating factors or a deficiency of antinucleating factors.

In primary cholesterol gallstone disease, the cholesterol microcrystal nucleation time of gallbladder bile, measured *ex vivo*, is considerably shorter in patients with multiple stones than in those with solitary stones (43–46). One explanation for this observation is that cholesterol-nucleating proteins are present at higher titers in bile samples from patients with multiple gallstones, than in those with solitary stones (45, 47). Indirect evidence that these pronucleating factors persist, or recur, after complete stone dissolution is provided by: (1) reports of rapid nucleation of cholesterol microcrystals in gallbladder bile (48) and bile-rich duodenal fluid (49) in approximately 50% of patients after complete gallstone dissolution, and (2) the fact that there is a threefold higher risk of stone recurrence in patients who originally had multiple stones than in those who initially had solitary stones (13, 50, 51).

There is conflicting evidence that gallstones recur true to type in terms of stone number. In the British/Belgian Gallstone Study Group's (BBGSG) postdissolution trial (51)—in which 21 of 82 patients developed stone recurrence between 12 and 42 months after complete stone dissolution—there was complete concordance between primary and recurrent stone number. However, apart from the results of one other recent study (16), our findings and those of other groups (14, 15, 49, 52, 53) suggest that there is a poor correlation between primary and recurrent stone number.

In studies of gallstone recurrence, it has often been suggested (14, 54–56) that both the initial clearance rate and, therefore, the subsequent recurrence rate, are overestimated. That is, the imaging techniques used to diagnose and confirm complete gallstone disappearance, may not be sufficiently sensitive to detect small, incompletely dissolved, residual particles. Ultrasonography can detect biliary sludge (58, 59), but

cannot reliably detect individual particles measuring less than 2 mm in diameter (57). Furthermore, in the present study, complete stone dissolution/removal was confirmed in all patients by two consecutive ultrasounds, at least one month apart. In over three quarters of the patients, the recurrent gallstones were not detected until at least 12 months after gallstone dissolution. It is unlikely, therefore, that small residual particles in the gallbladder would have remained undetected despite three or more ultrasonographic examinations, before regrowing into echographically visible stones.

There are few data on the composition of recurrent stones. Most previous studies have relied on oral cholecystography to assess the presence or absence of calcification in the recurrent stones. In one report (16), of 34 patients who developed stone recurrence after complete dissolution of their radiolucent primary stones, only two (6%) had cholecystographic evidence of calcification in their recurrent stones. This observation, suggesting that recurrent stones are almost always noncalcified, is consistent with the present findings. In the present study, the recurrent stones were radiolucent (by plain abdominal x-ray and OCG) in 20 of the 21 (95%) patients. Furthermore, indirect assessment of recurrent stone composition, by localized CT scanning, suggested that the stones were indeed cholesterol-rich in 18 of the 21 (86%) patients—including five of the six who originally had calcified gallstones.

Calcification of primary cholesterol-rich gallstones is a function of both stone size (large gallbladder stones are more likely to be calcified than small stones) and stone age (3, 60). Early detection of recurrent gallstones may explain why, in the present study, patients who originally had calcified gallstones developed small, cholesterol-rich stones on recurrence. It has been estimated that primary gallbladder stones grow at a rate of 1–4 mm per year (61–63) and do not cause symptoms until two to seven years after their formation (62). Therefore, it is likely that the primary stones in the present study had been present for several years before their detection. In contrast, as a result of regular ultrasonographic surveillance (at least once a year), the recurrent stones were detected within months of their formation.

Patients with poststone gallstone disease are at high risk (10–15% per annum) of developing new stones (32, 50, 51, 53, 55, 64, 65). Provided that the pathogenesis of these recurrent stones is similar to that of the primary stones, studies of recurrent gallstone formation are relevant to patients at risk of

## DO GALLBLADDER STONES RECUR TRUE TO TYPE?

primary gallstone formation. However, the present results have shown that gallstones do not recur true to type—suggesting that the pathogenetic factors which determine primary and recurrent stone size and composition cannot be constant. Nonetheless, independent of their original composition, recurrent gallstones are usually lucent by conventional x-ray and CT, presumed cholesterol-rich, and dissolvable with oral bile acid therapy.

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## Octreotide increases the proportions of arachidonic acid-rich phospholipids in gall-bladder bile

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### SUMMARY

**Background and aims:** Octreotide treatment of acromegalic patients induces cholesterol gallstone formation, in part by impairing cholecystokinin release and gall-bladder contraction. However, there are few data on the effect of octreotide on biliary arachidonic acid-rich phospholipids or mucin glycoprotein, factors which also influence cholesterol gallstone formation.

**Methods:** In acromegalic patients studied before and during 3 months of octreotide treatment, we measured mucin glycoprotein concentrations and the molecular species of phosphatidylcholine, and related the results to the cholesterol saturation and percentage of deoxycholic acid in gall-bladder bile.

**Results:** The relative proportions of the major arachidonic acid-rich phosphatidylcholine species, PC

16:0–20:4 and PC 18:0–20:4, increased significantly during octreotide treatment. These changes were associated with a rise in the cholesterol saturation index and a non-significant twofold increase in mucin glycoprotein concentration. There were significant correlations between PC 16:0–20:4 and the cholesterol saturation index, percentage of vesicular cholesterol and percentage of deoxycholic acid in gall-bladder bile.

**Conclusions:** In acromegalic patients, octreotide increases the proportions of arachidonic acid-rich phospholipids, with associated rises in: (a) the cholesterol saturation index and percentage of vesicular cholesterol, and (b) the percentage of deoxycholic acid in gall-bladder bile—changes similar to those found in patients with cholesterol-rich gall-bladder stones.

### INTRODUCTION

Octreotide, a long-acting analogue of somatostatin, is an effective treatment for acromegaly. However, it induces gall-bladder stone formation in 10–63% of patients after 1–2 years of treatment.<sup>1, 2</sup> The mechanisms for the formation of these iatrogenic stones are complex. Octreotide inhibits meal-stimulated cholecystokinin release from the small intestine, which is the principal, but not the sole, reason for the associated

reduction in gall-bladder emptying.<sup>1</sup> However, octreotide also induces multiple lithogenic changes in the composition of gall-bladder bile, including: (a) supersaturation of bile with cholesterol; (b) partitioning of the excess biliary cholesterol into vesicles which have a high cholesterol to phospholipid molar ratio; (c) rapid nucleation of cholesterol microcrystals; and (d) an increase in the proportion of the hydrophobic bile acid, deoxycholic acid.<sup>3</sup> Although controversial,<sup>4</sup> it has been suggested that, by inducing cholesterol hypersecretion,<sup>5, 6</sup> the increased proportion of biliary deoxycholic acid may be the main pathogenetic mechanism responsible for the induction of supersaturated gall-bladder bile and the development of octreotide-induced gall-bladder stones.<sup>3</sup>

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Biliary cholesterol secretion is normally coupled tightly to that of phospholipids, and particularly to that of the principal biliary phospholipid, phosphatidylcholine.<sup>7, 8</sup> Previous studies have suggested that arachidonic acid-rich phospholipids may also induce cholesterol supersaturation and the rapid nucleation of cholesterol microcrystals in gall-bladder bile by promoting cholesterol hypersecretion,<sup>6, 9</sup> inducing the preferential transfer of arachidonic acid-rich phospholipids from vesicles to micelles<sup>10–13</sup> and stimulating mucin glycoprotein secretion by the gall-bladder mucosa.<sup>14–16</sup>

The aims of this study were to determine whether chronic octreotide treatment of acromegalic patients alters the concentration of arachidonic acid-rich phospholipids in gall-bladder bile and, if so, to relate the changes in biliary phospholipid composition to: (a) the cholesterol saturation; (b) the vesicular cholesterol to phospholipid molar ratio; (c) the mucin glycoprotein concentration; and (d) the percentage of deoxycholic acid in gall-bladder bile.

## MATERIALS AND METHODS

### *Patients*

Gall-bladder bile was obtained from two groups of acromegalic patients—those untreated and those treated with octreotide.

The untreated group comprised eight patients (four men and four women; mean age, 49 years; range, 29–64 years), who had received no octreotide for at least 2 months and were gallstone-free by ultrasonography. Although these were not normal controls, we have previously shown<sup>3</sup> that bile composition and physical chemistry in this group of patients are not significantly different from those in healthy controls.<sup>17–19</sup>

The octreotide-treated group consisted of nine acromegalic patients (five men and four women; mean age, 53 years; range, 36–64 years), who received 100–200 µg octreotide t.d.s. by subcutaneous injection for at least 3 months. Five of the nine were studied twice—once before octreotide treatment when all five were stone-free (untreated group), and again after 3–24 months (mean, 8 months) of octreotide therapy. At the time of the second study, four of the five remained stone-free, but one, a 59-year-old woman, had developed asymptomatic octreotide-induced gall-bladder stones after 3 months of treatment. The four patients who were studied only once had not undergone

pre-treatment ultrasonography, but were all found to have multiple gall-bladder stones after 3–66 months of octreotide treatment.

### *Bile sampling*

On 16 of the 17 occasions, samples of fresh gall-bladder bile were obtained by ultrasound-guided percutaneous transhepatic fine needle puncture, as previously described.<sup>20</sup> However, in the paired studies, one of the patients declined repeat gall-bladder puncture. Therefore, on the second occasion, her fasting bile-rich fluid was obtained by duodenal intubation, after stimulating gall-bladder contraction with intravenous cholecystokinin. All biles were stored at –20 °C until analysis. The use of ultrasound-guided gall-bladder puncture was approved by the Research Ethics Committee of St Bartholomew's Hospital and by the Ethics Committee of Guy's Hospital. All patients gave their written informed consent.

### *Biliary lipid and bile acid composition*

As part of a previous study by our group,<sup>3</sup> total biliary bile acids,<sup>21</sup> phospholipids<sup>22</sup> and cholesterol<sup>23</sup> were determined by standard enzymatic assays, and the cholesterol saturation index was derived.<sup>24</sup> The concentrations of the individual bile acid conjugates were determined using reverse-phase high-performance liquid chromatography,<sup>25</sup> and expressed as a percentage of total bile acids. Some of these data have been presented previously,<sup>3</sup> and were used in the present study to look for correlations with biliary phosphatidylcholine species and mucin glycoprotein concentrations (see below).

### *Separation of molecular species of phosphatidylcholine*

High-performance liquid chromatography grade methanol, isopropanol, hexane, acetonitrile and ethanol were obtained from Rathburn Chemicals Ltd (Walkerburn, UK). Phospholipid class standards and phosphatidylcholine molecular species standards were obtained from Sigma Chemical Co. (Poole, UK) and Avanti Polar Lipids (Alabaster, Alabama, USA).

After Fölch extraction of the biliary lipids,<sup>26</sup> 200-µL aliquots of the lipid phase were loaded on to a 4.6 × 250 mm octadecyl Kromasil column packed with 10 µm silica (Hichrom Ltd, Theale, UK), and eluted at a

flow rate of 2 mL/min, using a mobile phase of hexane–isopropanol–ethanol–phosphate buffer–acetic acid 367:495:100:57:0.3.<sup>27</sup> Phospholipid peaks were detected at an absorbance of 205 nm. The phosphatidylcholine peak was identified by comparison with the retention time of standard bovine phosphatidylcholine, and confirmed by thin layer chromatography.<sup>28</sup>

The phosphatidylcholine was then separated into its molecular species on a 4.6 × 250 mm Nucleosil column packed with 5 µm diameter beads of octadecylsilane bonded phase (Hichrom Ltd, Theale, UK). Elution was performed at 1.5 mL/min, with a mobile phase of methanol–hexane–water 80:5:15.<sup>29</sup> Individual peaks were detected at an absorbance of 492 nm, identified by comparing their retention times with those of standard phosphatidylcholine molecular species, and confirmed by mass spectrometry.

#### *Purification of gall-bladder mucin*

Gall-bladder mucin was purified using a two-step gel filtration method, as previously described.<sup>30, 31</sup> First, 200-µL aliquots of gall-bladder bile were loaded on to Sepharose CL-2B mini-columns (5 × 1.5 cm; Pharmacia, Uppsala, Sweden) and eluted with 0.1 M tris-HCl pH 8. Twenty 0.5-mL fractions were collected and monitored by enzyme-linked immunoabsorbent assay (ELISA) using a monoclonal antimucin antibody (CAM 17.1).<sup>32</sup> The mucin-containing fractions (elution volume, 2.5–5.5 mL) were then pooled, lyophilized and reconstituted in water.

Next, the mucin was purified further by fast protein liquid chromatography. Samples (200 µL) of the reconstituted mucin were loaded on to a 30 × 1 cm Superose 6 column (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M tris-HCl pH 8 at 15 mL/h. Thirty 1-mL fractions were collected and monitored at an absorbance of 280 nm, as well as by CAM 17.1 ELISA and a concanavalin A enzyme-linked lectin-binding assay. The concanavalin A assay was performed in a similar manner to the CAM 17.1 ELISA, except that a peroxidase-conjugated lectin concanavalin A (Vector, Peterborough, UK), diluted 1 : 250 in phosphate-buffered saline (PBS)–Tween, was added instead of the CAM 17.1+ rabbit antimouse antibody. Fractions which contained mucin glycoprotein, and which were free of concanavalin A-binding non-mucin glycoproteins (elution volume, 1–13 mL), were defined as the void volume.

#### *Quantification of immunoreactive mucin by ELISA*

Aliquots (50 µL) from each Superose 6 fraction, which contained the mucin glycoprotein, were diluted 1:1 in 0.05 M carbonate buffer (pH 9.6) and coated on to ELISA plates for 16 h at 4 °C. The plates were washed and blocked with PBS (pH 7.2) + Tween 20 (0.1%), followed by incubation with 100 µL of CAM 17.1 (a monoclonal antibody against the sialated I antigen of mucin glycoprotein<sup>33</sup>), diluted 1 : 10 in PBS–Tween, for 2 h at 37 °C. After further washing, 100-µL aliquots of peroxidase-conjugated rabbit antimouse immunoglobulin (diluted 1 : 600 in PBS–Tween) were added to the plate and incubated at 37 °C for a further 2 h. The plate was then developed with 100 µL of peroxidase substrate (0.4 mg/mL *o*-phenylenediamine + 0.04 µL/mL H<sub>2</sub>O<sub>2</sub> in 0.05 M citrate–phosphate buffer, pH 5), and the reaction was stopped after 5 min by adding 100 µL of 4 M H<sub>2</sub>SO<sub>4</sub>. The plate was read at 492 nm.<sup>34</sup>

#### *Statistics*

All results are expressed as means (S.E.M.). For comparisons of data between groups, we used Student's *t*-test (two-tailed) or the Mann–Whitney non-parametric method, as appropriate. Differences in proportions between two groups were compared using the chi-squared test. Values of *P* < 0.05 were considered to be significant.

## RESULTS

#### *Phosphatidylcholine species before and during octreotide therapy*

The distribution profile of the different species of phosphatidylcholine, expressed as percentages of the total phosphatidylcholines in the two groups of acromegalic patients, is shown in Figure 1. The paired and non-paired data for the two arachidonic acid-rich phosphatidylcholines in the acromegalic patients untreated with octreotide and in those given long-term octreotide are illustrated in Figure 2(A,B).

Overall, the relative proportions of the major phosphatidylcholine (PC) molecular species, PC 16:0–18:2 and 16:0–18:1, were similar before and during octreotide therapy. However, the arachidonic acid-rich phosphatidylcholine PC 16:0–20:4 increased from a

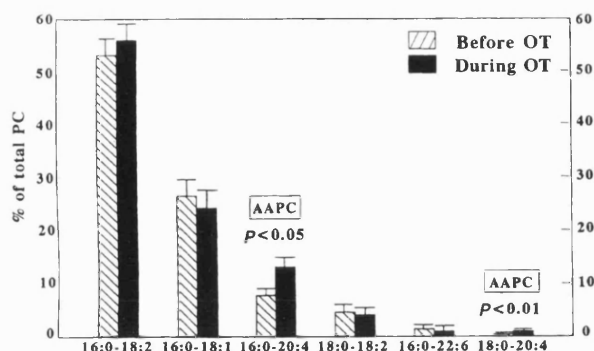


Figure 1. Effect of octreotide (OT) therapy on the distribution of phosphatidylcholine species in gall-bladder bile (means  $\pm$  S.E.M.). The two arachidonic acid-rich phosphatidylcholine (AAPC) species were the only species which increased significantly during treatment.

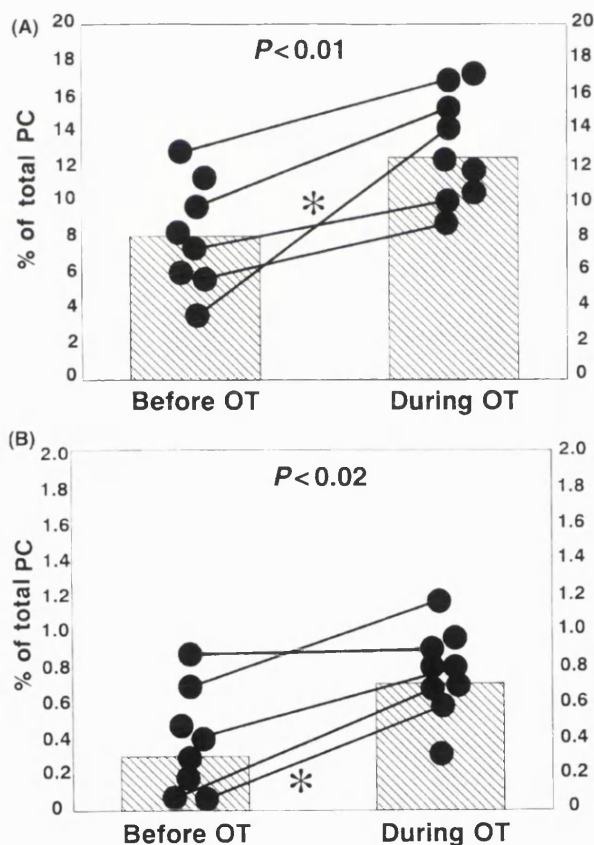


Figure 2. The proportions of PC 16:0–20:4 (A) and PC 18:0–20:4 (B) in gall-bladder bile before and during octreotide (OT) treatment. The filled circles are the individual data, the lines connect the paired data and the shaded bars show the means. One patient in the paired studies (\*) developed gall-bladder stones during treatment.

mean of  $8.1 \pm 1.3\%$  (range, 3.6–13.4%) of total biliary phosphatidylcholine before therapy to  $12.5 \pm 1.0\%$  (range, 8.5–16.6%) during octreotide therapy ( $P < 0.01$ ), while PC 18:0–20:4 rose from  $0.37 \pm 0.11\%$  to  $0.74 \pm 0.09\%$  ( $P < 0.02$ ). In the five patients in whom paired data were obtained, PC 16:0–20:4 increased in every case, from a mean of  $8.0 \pm 1.7\%$  before therapy to  $12.7 \pm 1.6\%$  during octreotide therapy ( $P < 0.05$ ), while PC 18:0–20:4 rose from  $0.4 \pm 0.18\%$  to  $0.8 \pm 0.09\%$  ( $P < 0.02$ ). The patient who developed gallstones after only 3 months of octreotide treatment also had the steepest rise in the proportion of arachidonic acid-rich phospholipids during treatment (Figure 2A,B).

#### *Relationships between phosphatidylcholine species and cholesterol saturation in gall-bladder bile*

The increase in the proportion of biliary arachidonic acid-rich phosphatidylcholine was associated with a rise in biliary cholesterol saturation, with the cholesterol saturation index increasing from  $0.90 \pm 0.05$  before treatment to  $1.2 \pm 0.05$  during octreotide therapy ( $P < 0.01$ ). In the paired studies, the biliary cholesterol saturation indices rose in all five patients by a mean of 26%, from  $0.89 \pm 0.06$  to  $1.1 \pm 0.03$  ( $P < 0.02$ ). Similar rises were also seen in the percentage of vesicular cholesterol (from  $37.7 \pm 3.5\%$  to  $59.4 \pm 6.2\%$ ;  $P < 0.02$ ) and the cholesterol to phospholipid molar ratio (from  $0.52 \pm 0.05$  to  $0.73 \pm 0.11$ ;  $P < 0.01$ ). Furthermore, there were significant positive correlations between the most abundant arachidonic acid-rich phosphatidylcholine species, PC 16:0–20:4, and both the cholesterol saturation index ( $r = 0.57$ ,  $P < 0.02$ ) and the percentage of vesicular cholesterol ( $r = 0.67$ ,  $P < 0.01$ ) (Figure 3A,B).

#### *Biliary deoxycholic acid*

Before octreotide treatment, the proportion of biliary deoxycholic acid, expressed as a percentage of total biliary bile acids in the gall-bladder bile of the eight stone-free acromegalic patients, was  $13.9 \pm 1.4\%$ . In the nine patients treated with octreotide, it increased to  $24.9 \pm 2.7\%$  ( $P < 0.01$ ). There was a significant positive correlation between the percentage of deoxycholic acid and PC 16:0–20:4 in gall-bladder bile ( $r = 0.48$ ,  $P < 0.05$ ) (Figure 3C).

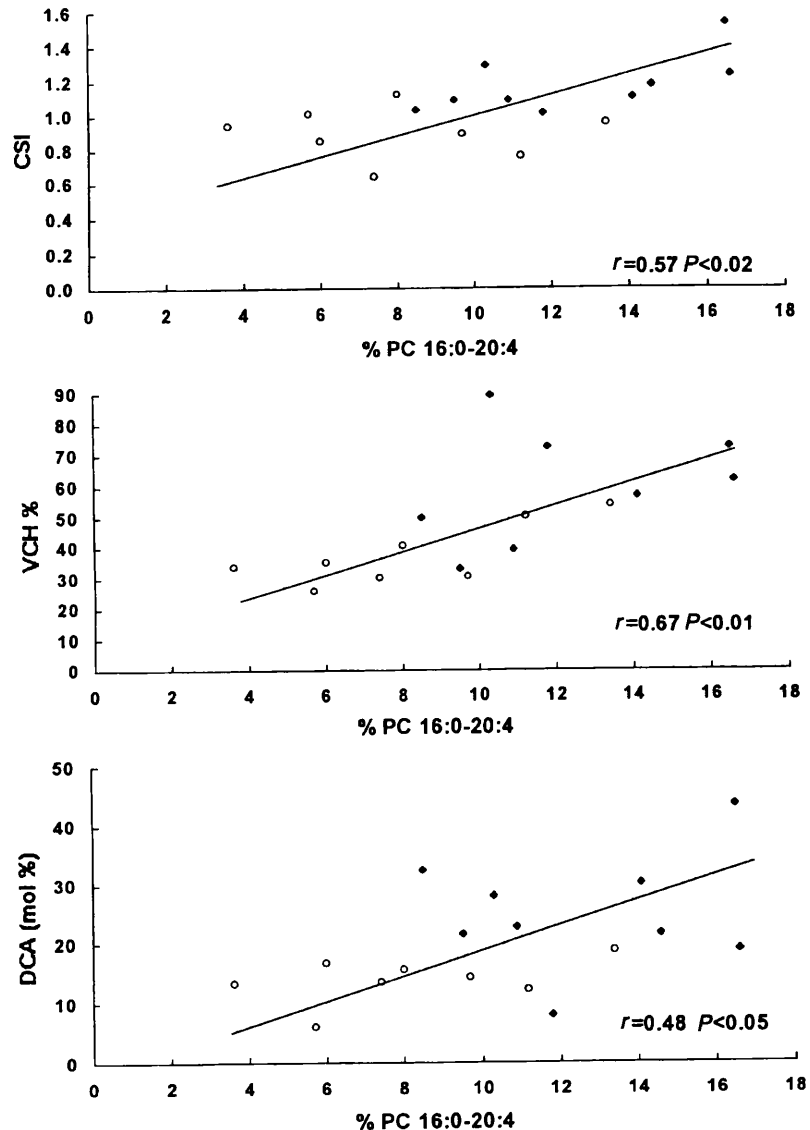


Figure 3. Pearson correlations for the percentage of biliary PC 16:0-20:4 and the cholesterol saturation indices (CSI) (A), the percentage of vesicular cholesterol (VCH) (B) and the percentage of deoxycholic acid (DCA) in gall-bladder bile (C) (○ = no octreotide; ◆ = during octreotide treatment).

#### Mucin glycoprotein concentration

During octreotide treatment, there was a 121% increase in mean gall-bladder mucin concentration, from  $2.8 \pm 1.2$  to  $6.2 \pm 2.2$  U/L, but this difference was not statistically significant (Figure 4). Moreover, there was no significant relationship between gall-bladder mucin concentration and either the cholesterol saturation index or the percentage distribution of different phospholipid species in gall-bladder bile.

#### DISCUSSION

Several previous studies have shown that octreotide inhibits meal-stimulated cholecystokinin release from the intestine<sup>35-37</sup> and effectively abolishes meal-stimulated gall-bladder emptying.<sup>35, 36, 38-40</sup> Although impaired gall-bladder contractility is undoubtedly one factor in the development of octreotide-induced gall-bladder stones, the results of the present study extend our previous observations<sup>3</sup> by showing that octreotide also induces marked changes in bile composition and

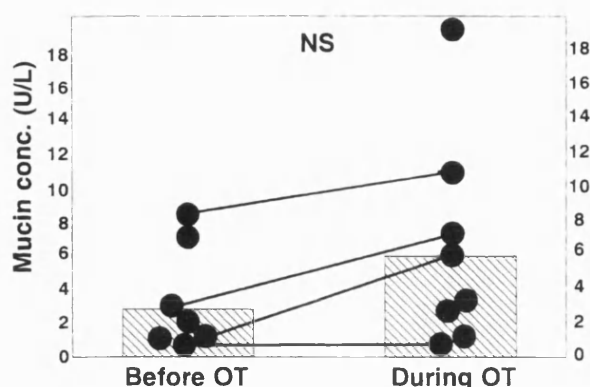


Figure 4. Biliary mucin glycoprotein concentrations before and during octreotide (OT) treatment.

physical chemistry. In acromegalic patients treated with octreotide for at least 3 months, the proportions of the two arachidonic acid-rich phospholipid species in gall-bladder bile (PC 16:0–20:4 and PC 18:0–20:4) increased significantly. There were associated significant rises in: (a) the cholesterol saturation index; (b) the vesicular cholesterol and cholesterol to phospholipid molar ratio; and (c) the proportion of deoxycholic acid; there was also a non-significant doubling of mucin glycoprotein levels in gall-bladder bile.

These findings provide further insights into the pathogenesis of octreotide-induced gall-bladder stones, but they may also be relevant to 'conventional' cholesterol-rich gall-bladder stones. In cholesterol gallstone disease, hepatic cholesterol hypersecretion is associated with an increased proportion of unsaturated molecular species of phosphatidylcholine, particularly arachidonic acid-rich (20:4) species, in bile.<sup>6, 9</sup> Furthermore, results of studies using model bile systems<sup>10–12, 41</sup> and human gall-bladder bile<sup>13</sup> indicate that the molecular species of phosphatidylcholine are distributed asymmetrically between micelles and vesicles. Partitioning of cholesterol between these two lipid carriers is determined by the fatty acid chain length in substitution one (sn-1) position, and the degree of unsaturation of the fatty acids at both the sn-1 and sn-2 positions of phosphatidylcholine.<sup>10, 13</sup> By virtue of the looser packing constraints imposed by their long chain length, and particularly by their high degree of unsaturation, arachidonic acid-rich phosphatidylcholines partition less well into highly ordered biliary vesicles than into micelles. Therefore, arachidonic acid-rich phospholipids are found in higher concentrations in mixed micelles than in vesicles.<sup>10, 11, 13</sup> Although we did not measure

the phospholipid composition of the mixed micelles and vesicles, we did find significant positive correlations between the main arachidonic acid-rich phospholipid species, PC 16:0–20:4, and the cholesterol saturation index, the percentage of vesicular cholesterol and the cholesterol to phospholipid molar ratio. These findings are consistent with the hypothesis that, as less phosphatidylcholine becomes available for vesicle formation, fewer cholesterol-rich vesicles form. However, those which do form will have an increased cholesterol to phospholipid molar ratio,<sup>10</sup> thus favouring aggregation and fusion of vesicles, and cholesterol microcrystal nucleation.

In addition to enhancing cholesterol secretion into bile and destabilizing the cholesterol carriers, arachidonic acid-rich phosphatidylcholine species may play a role in stimulating mucin glycoprotein production by the gall-bladder mucosa. Gall-bladder mucin, a high molecular weight glycoprotein, is a major secretory product of the gall-bladder epithelium. It can bind biliary lipids<sup>42, 43</sup> and accelerate the trapping and nucleation of cholesterol microcrystals in both supersaturated model and native biles.<sup>44–47</sup> In obese subjects on very low calorie diets who are at high risk of cholesterol gallstone formation, increases in mucin glycoprotein secretion are preceded by increases in biliary arachidonic acid concentrations and mucosal prostaglandin synthesis.<sup>15, 48</sup> In the prairie dog model, when aspirin or NSAIDs are given together with a lithogenic diet, they inhibit gall-bladder mucosal prostaglandin synthesis and prevent mucin glycoprotein hypersecretion, as well as microcrystal and gallstone formation.<sup>49</sup> Similar findings were reported in a study of obese individuals treated with high-dose (1300 mg/day) aspirin during acute weight reduction,<sup>50</sup> although epidemiological studies of the effect of chronic NSAID ingestion on the incidence of gallstones have yielded conflicting results.<sup>51–53</sup> Our results, showing a significant increase in biliary arachidonic acid-rich phospholipids and a twofold rise in mucin glycoprotein concentration during octreotide treatment, are consistent with these early studies. They provide indirect support for the hypothesis that biliary arachidonic acid-rich phospholipid concentrations may influence biliary mucin glycoprotein levels through the conversion of arachidonic acid to prostaglandins.

The mechanisms whereby increased proportions of biliary deoxycholic acid favour gallstone formation are complex. We<sup>54</sup> and others<sup>6, 55–57</sup> have found that there is a linear relationship between the percentage of

deoxycholic acid and both the cholesterol saturation index and the molar percentage of cholesterol in bile. Furthermore, the present results are consistent with those of previous studies,<sup>9, 58, 59</sup> which showed that, in patients with cholesterol-rich gallstones, the percentage of deoxycholic acid in bile correlates positively with the proportion of arachidonic acid-rich phospholipids in bile.

We showed previously that, in individuals with rapid nucleation of cholesterol microcrystals (< 5 days), the mean percentage of deoxycholic acid in bile was approximately twice as great as that in individuals with normal nucleation times (> 10 days).<sup>3</sup> Whether this difference in nucleation time is due directly to the percentage of deoxycholic acid in bile, or indirectly to secondary increases in biliary cholesterol saturation, mucin glycoprotein hypersecretion and/or cholesterol microcrystal nucleation, is unclear. Nonetheless, there is strong indirect evidence that the principal mechanism for the increased percentage of deoxycholic acid in the bile of patients with octreotide-associated gall-bladder stones is prolongation of intestinal transit. Thus, several previous studies have reported that both native somatostatin<sup>60–63</sup> and its analogue octreotide<sup>35, 64–68</sup> prolong mouth-to-caecum transit times. In paired, before and during treatment, studies of acromegalic patients, we also showed<sup>67</sup> that the mean colonic transit time increased by approximately 15 h, with an associated linear increase in serum deoxycholic acid (expressed as a percentage of serum total bile acids). A subsequent study from our Unit<sup>69</sup> found that the colonic transit time, the activity of the 7 $\alpha$ -dehydroxylation reaction and the distal colonic luminal pH were all independent risk factors for high proportions of deoxycholic acid in serum (and by implication in bile). Thus, we speculate that octreotide-induced changes in intestinal transit, leading to an increased proportion of deoxycholic acid, may induce a complex series of changes in bile composition and physical chemistry which favour gallstone formation.<sup>68, 70</sup>

In summary, we have shown that, in acromegalic patients treated with octreotide for at least 3 months, the proportions of both arachidonic acid-rich phospholipid species in gall-bladder bile increase. Based on the present results, and those of earlier studies by ourselves<sup>3, 67, 68</sup> and others,<sup>6, 55–57</sup> the increase in biliary deoxycholic acid may be at least partly responsible for this rise in arachidonic acid-rich phospholipids. These changes, in turn, contribute to the cholesterol supersaturation of bile and mucin glycoprotein hypersecre-

tion which, in conjunction with the abolition of the meal-stimulated gall-bladder emptying induced by octreotide, predisposes to the rapid nucleation of microcrystals and cholesterol gallstone formation.

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# Biliary lactoferrin concentrations are increased in active inflammatory bowel disease: a factor in the pathogenesis of primary sclerosing cholangitis?

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## ABSTRACT

1. One hypothesis for the link between inflammatory bowel disease and primary sclerosing cholangitis is that neutrophil activators, such as bacterial chemotactic peptides or neutrophil granule products themselves, pass from the inflamed colon to the liver via an enterohepatic circulation. However, there are no data on biliary concentrations of neutrophil granule products in patients with active and inactive inflammatory bowel disease.
2. Gall bladder bile was obtained at laparotomy from 42 patients with ulcerative colitis and 21 patients with Crohn's disease. Biliary lactoferrin and myeloperoxidase concentrations were quantified by ELISA.
3. In active ulcerative colitis, the mean lactoferrin concentration in gall bladder bile of  $2.8 \pm 0.40$  mg/l was higher than that seen after colectomy ( $1.2 \pm 0.11$  mg/l;  $P < 0.0001$ ) or in patients with pouchitis ( $1.8 \pm 0.34$  mg/l;  $P = 0.06$ ). In active Crohn's colitis, the mean lactoferrin concentration was  $3.7 \pm 0.9$  mg/l, compared with  $1.1 \pm 0.24$  mg/l in the post-colectomy group ( $P < 0.05$ ) and  $3.1 \pm 0.71$  mg/l in those with active ileitis or ileocolitis. In contrast, biliary myeloperoxidase concentrations were low and comparable in all groups, with a mean concentration in the 42 patients with ulcerative colitis of  $11.2 \pm 1.9$  µg/l.
4. In contrast to myeloperoxidase, biliary lactoferrin concentrations are increased in active ulcerative colitis and Crohn's disease, and fall with colectomy and with disease remission. These findings indirectly support the hypothesis that bacterial chemotactic peptides (which induce selective degranulation of neutrophil secondary granules), and/or lactoferrin itself, undergo an enterohepatic circulation.

## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease which occurs in 2.5–7.5 % of patients

with ulcerative colitis [1–4], and in approximately 1 % of those with Crohn's disease – predominantly those with colonic involvement [5–7]. Conversely, approximately 70 % of patients with PSC also have inflammatory bowel

**Key words:** bile, inflammatory bowel disease, lactoferrin, myeloperoxidase, primary sclerosing cholangitis.

**Abbreviations:** ANCA, anti-neutrophil cytoplasmic antibody; PSC, primary sclerosing cholangitis.

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disease – usually ulcerative colitis [3]. Although the aetiology of PSC is unknown, one hypothesis is that in patients with inflammatory bowel disease, there is an enterohepatic circulation of chemotactic factors (such as *N*-formyl peptides [8,9]) or of autoantigens [10,11], which may be responsible for the close association of inflammatory bowel disease and PSC.

Neutrophil accumulation and degranulation in the inflamed intestinal mucosa are prominent features of inflammatory bowel disease, and contribute to tissue damage at sites of inflammation [12]. One such neutrophil product is lactoferrin – a 76-kDa iron-binding glycoprotein related in structure to transferrin [13]. Lactoferrin is present in small amounts in exocrine secretions – such as milk, from which the name derives [14,15]. However, the major, if not the sole, source of circulating lactoferrin is the secondary (specific) granules of neutrophils [16,17]. In control subjects, and in patients with inactive inflammatory bowel disease, plasma concentrations of lactoferrin are low [18–21]. However, in active inflammatory bowel disease, both circulating [19,21] and faecal [22–24] lactoferrin concentrations are increased. Plasma lactoferrin levels correlate well with both serum C-reactive protein concentrations and clinical indices of disease activity [19,21].

In addition to being a marker of disease activity, lactoferrin has been implicated in the pathogenesis of inflammatory bowel disease. Thus, when lactoferrin is infused into rat mesenteric arteries, it increases colonic mucosal permeability, induces neutrophil recruitment into the mucosa and causes a frank colitis [25]. Furthermore, lactoferrin is one of the antigens for perinuclear staining anti-neutrophil cytoplasmic antibodies (p-ANCA) [26–32]. These antibodies have been reported in the sera of 13–89% of patients with inflammatory bowel disease [3,7,29,31–38] and 65–82% of patients with PSC [4,29,36,39–41].

There have been no previous studies of biliary lactoferrin concentrations in inflammatory bowel disease. In theory, however, high concentrations of lactoferrin within the hepatobiliary tract could play a role in the initiation of pericholangitis or PSC. Thus, if there was an enterohepatic circulation of lactoferrin, or of proteins such as *N*-formyl peptides which induce biliary neutrophil degranulation [9,42], biliary concentrations of lactoferrin should be high during active inflammatory bowel disease and fall to normal levels during disease remission. To study this, we compared the concentrations of lactoferrin in gall bladder bile from patients with active and inactive inflammatory bowel disease. Next, to determine whether lactoferrin is released selectively from neutrophil secondary granules in active inflammatory bowel disease [23], or is merely a non-specific marker of neutrophil degranulation, we compared the concentrations of lactoferrin with those of myeloperoxidase (stored in neutrophil primary granules [42]) in gall bladder bile.

## METHODS

### Patients

The patients were recruited for the study by the Academic Department of Surgery at the Queen Elizabeth Hospital, Birmingham. All required surgical management of their inflammatory bowel disease. The clinical details are given in Table 1.

Forty-two patients had ulcerative colitis. There were 11 women and 31 men, and their ages ranged from 16 to 75 (mean 40) years. The patients were subdivided into three groups: (i) active colitis ( $n = 14$ ; admitted for proctocolectomy), (ii) post-colectomy ( $n = 17$ ; admitted for J-pouch ileo-anal anastomosis after proctocolectomy 1–2 months earlier), and (iii) pouchitis ( $n = 11$ ; admitted for J-pouch resection/repair because of clinical pouchitis or anastomotic breakdown). In six patients, paired bile samples were obtained, first during active colitis and again 1–2 months after proctocolectomy.

Twenty-one patients had Crohn's disease. There were 7 women and 14 men, with a mean age of 40 (range 22–76) years. Twelve patients had colonic disease alone, of whom seven had active colitis requiring colectomy and five had inactive disease (previous colectomy or defunctioning loop ileostomy). The remaining nine had active ileal ( $n = 4$ ) or ileocolonic ( $n = 5$ ) disease and were admitted for small bowel resection.

None of the patients with ulcerative colitis or Crohn's disease had undergone cholecystectomy and all were free of gall bladder stones, as assessed by preoperative cholecystosonography [43] and direct palpation of the gall bladder at the time of laparotomy. The results of serum liver function tests were normal in all patients.

### Aspiration of gall bladder bile

The technique of intra-operative sampling of gall bladder bile was approved by the Ethics Committee of Guy's Hospital, and the South Birmingham Ethical Committee. At the start of surgery, and before manipulation of the bowel, bile was sampled using standard techniques of gall bladder puncture [44]. To avoid possible sampling errors as a result of stratification of bile, the gall bladder contents were aspirated as completely as possible. No episodes of bile leakage occurred, and there was no morbidity associated with the procedure. Bile samples thus obtained were stored immediately at  $-20^{\circ}\text{C}$  until analysis.

### Gel filtration of gall bladder bile

As part of a separate study of biliary mucin glycoprotein [45], biliary lactoferrin was separated from high-molecular-mass glycoproteins by sepharose CL-2B gel chromatography as follows. Aliquots of gall bladder bile (200  $\mu\text{l}$ ) were loaded on to sepharose CL-2B mini-columns (5  $\times$  1.5 cm; Pharmacia, Uppsala, Sweden), and eluted with 0.1 M Tris-HCl (pH 8.0) in 24  $\times$  0.5-ml

**Table 1** Clinical data of the patients studied

Values for age are expressed as mean and range, and for disease duration as median and range.

| Patient group                                    | Sex (M/F) | Age (years) | Disease duration (years) | Previous surgery                                                                                       | Medical treatment                                      |
|--------------------------------------------------|-----------|-------------|--------------------------|--------------------------------------------------------------------------------------------------------|--------------------------------------------------------|
| Ulcerative colitis ( <i>n</i> = 42)              |           |             |                          |                                                                                                        |                                                        |
| Active colitis ( <i>n</i> = 14)                  | 11/3      | 42 (16–75)  | 2.5 (1–10)               | Nil                                                                                                    | Steroids ( <i>n</i> = 11)                              |
| Post-colectomy ( <i>n</i> = 17)                  | 13/4      | 40 (16–66)  | 5 (1–21)                 | Colectomy + ileostomy<br>with mucus fistula ( <i>n</i> = 17)                                           | Nil                                                    |
| Active pouchitis ( <i>n</i> = 11)                | 7/4       | 36 (19–66)  |                          | Colectomy + ileoanal<br>anastomosis ( <i>n</i> = 11)                                                   | Nil                                                    |
| Crohn's disease ( <i>n</i> = 21)                 |           |             |                          |                                                                                                        |                                                        |
| Active colitis ( <i>n</i> = 7)                   | 4/3       | 34 (23–65)  | 2 (1–5)                  | Loop ileostomy (reversed) ( <i>n</i> = 1)                                                              | Steroids ( <i>n</i> = 3)<br>Mesalazine ( <i>n</i> = 1) |
| Post-colectomy ( <i>n</i> = 5)                   | 4/1       | 49 (34–76)  | 5 (2–12)                 | Colectomy ( <i>n</i> = 2)<br>Defunctioning loop<br>ileostomy ( <i>n</i> = 3)                           | Nil                                                    |
| Active ileitis or<br>ileocolitis ( <i>n</i> = 9) | 6/3       | 36 (22–53)  | 12 (3–20)                | Ileal resection ( <i>n</i> = 3)<br>Ileorectal anastomosis ( <i>n</i> = 2)<br>Ileostomy ( <i>n</i> = 3) | Steroids ( <i>n</i> = 5)                               |

fractions. Preliminary experiments showed that lactoferrin purified from human breast milk (Sigma Chemical Co., Poole, U.K.), and lactoferrin in gall bladder bile, both eluted at 6.5–11 ml. Therefore, these fractions were combined for the lactoferrin ELISA.

### Lactoferrin ELISA

The lactoferrin ELISA was a modification of that described by Hegnhøj et al. [46]. Briefly, 100 µl of a polyclonal rabbit anti-human lactoferrin (Sigma Chemical Co.), diluted 1:2000 in 0.1 M carbonate buffer, was incubated overnight at 4 °C on an ELISA plate (Immulon 4, Dynatech, Billingshurst, U.K.). Next, 100-µl aliquots of human lactoferrin standard (Sigma Chemical Co.) and the pooled sepharose samples (diluted 1:125 in 0.1 M PBS/1% Tween), were incubated on the ELISA plates for 2 h at 37 °C. Aliquots (100 µl) of peroxidase-conjugated rabbit anti-human lactoferrin (diluted 1:1000 in PBS–Tween) were then added, for 2 h at 37 °C. Between each step, the plate was washed four times with PBS–Tween. After a final wash, colour was developed using 100-µl aliquots of peroxidase substrate (*o*-phenylenediamine dihydrochloride + 30% hydrogen peroxide + 0.05 M phosphate citrate buffer, pH 5), and the reaction stopped after 15 min by the addition of 4 M sulphuric acid. The absorbances of the samples and standards were read at 490 nm and the concentration of lactoferrin calculated from standard curves. All assays were performed in quadruplicate.

### Electrophoresis and Western Blotting

To assess cross-reactivity of the polyclonal rabbit anti-human lactoferrin antibody to other biliary proteins, bile samples and the human lactoferrin standard were subjected to SDS–PAGE on 4–11% gradient gels (Bio-Rad

Laboratories, Herts, U.K.), using the buffer system of Laemmli [47]. Thereafter, the gels were blotted on to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA, U.S.A.), using a semi-dry transfer system (LKB Multiphor II Electrophoresis Unit; Pharmacia Biotech, Herts, U.K.). The membrane was blocked overnight at 4 °C with 2% casein in 0.1 M PBS. It was then incubated for 1 h at 37 °C in rabbit anti-human lactoferrin, diluted 1:1000 in PBS + 0.1% Tween-20 (PBS–Tween), followed by a further 1 h incubation at 37 °C with mouse anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Dako A/S, Dako, High Wycombe, U.K.) (diluted 1:500). Between each step, the membrane was washed (5 × 5 min) with PBS–Tween. Colour was developed by the 3-3'-diaminobenzidine reaction.

### Myeloperoxidase ELISA

Biliary myeloperoxidase concentrations were determined using a commercially available ELISA kit from Bioxytech, Bonneuil sur Marne, France [48].

### Biliary bile acid concentrations

To correct for the effect of varying dilutions of gall bladder bile on lactoferrin and myeloperoxidase concentrations, total biliary bile acid concentrations were determined by enzymic assay [49]. Lactoferrin and myeloperoxidase concentrations were then expressed both as 'raw' data and, after normalization to a total bile acid concentration of 100 mM, as standardized data.

### Statistical analysis

The significance of differences in results between groups was tested with Student's *t*-test (two-tailed) or the Mann–Whitney non-parametric method, as appropriate.

## RESULTS

The sensitivity of the lactoferrin ELISA was 0.5 ng/ml, the intra-assay coefficient of variation was 3.4% and inter-assay coefficient of variation was 8.0% ( $n = 20$ ). There was no cross-reactivity of the lactoferrin ELISA with pasteurized milk, which contains small quantities of bovine lactoferrin [50]. Subsequent experiments showed that the lactoferrin ELISA gave similar results when either native whole bile, or the pooled sepharose fractions, were analysed. As shown by SDS-PAGE and immunoblotting, the polyclonal rabbit anti-human lactoferrin antibody was specific for lactoferrin and did not bind to any of the other proteins in gall bladder bile.

### Biliary lactoferrin in ulcerative colitis

In the 14 patients with active ulcerative colitis, the mean ( $\pm$ S.E.M.) lactoferrin concentration (unrelated to bile acid concentration) in gall bladder bile was  $2.8 \pm 0.40$  mg/l (range 1.2–5.8 mg/l). This value was significantly higher ( $P < 0.0001$ ) than that in the bile of the 17 post-colectomy patients (mean  $1.2 \pm 0.11$  mg/l, range 0.32–1.8 mg/l). In the six patients with colitis in whom paired bile samples were obtained (during active colitis and again after resection of the colon), biliary lactoferrin concentrations fell after colectomy in five, with a significant ( $P < 0.05$ ) decrease in the mean concentration from  $2.1 \pm 0.32$  to  $1.1 \pm 0.16$  mg/l.

In the 11 patients who had pouchitis, or an anastomotic breakdown of their J-pouches, the mean biliary lactoferrin concentration of  $1.8 \pm 0.34$  mg/l (range 0.56–4.4 mg/l) was intermediate to those of the other two groups (Figure 1).

### Biliary lactoferrin in Crohn's disease

In the seven patients with active Crohn's colitis (but no evidence of small bowel disease) and the nine with active ileitis or ileocolitis, the mean uncorrected biliary lactoferrin concentrations were similar, with values of  $3.7 \pm 0.9$  mg/l (range 0.58–8.4 mg/l) and  $3.1 \pm 0.71$  mg/l (range 0.21–6.3 mg/l) respectively. Both these values

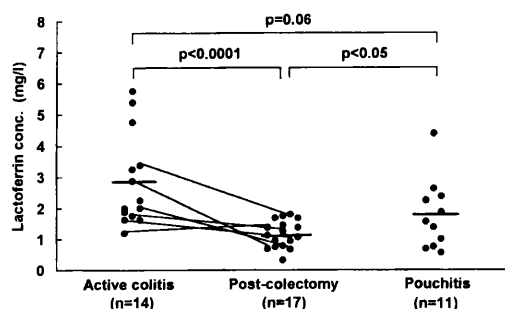


**Figure 1** Biliary lactoferrin concentrations in patients with active and inactive ulcerative colitis

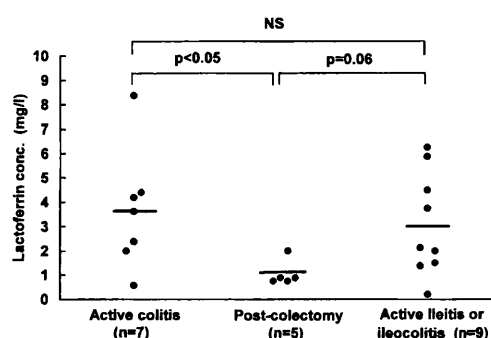


**Figure 2** Biliary lactoferrin concentrations in patients with Crohn's disease

were higher than the mean of  $1.1 \pm 0.24$  mg/l (range 0.75–2.0 mg/l) in the Crohn's post-colectomy group ( $P < 0.05$  versus Crohn's colitis;  $P = 0.06$  versus ileitis/ileocolitis) (Figure 2).

When the mean concentrations of biliary lactoferrin in the patients with ulcerative colitis or Crohn's disease were compared, there was no significant difference between the two active colitis or post-colectomy groups, or between those with ulcerative pouchitis or active Crohn's ileitis/ileocolitis. Eleven of the 14 patients with active ulcerative colitis, and three of the seven with active Crohn's colitis, had been treated with steroids for a median of 1 week (range 1–10 weeks) before laparotomy. In the patients treated with steroids, the mean biliary lactoferrin concentration was higher ( $3.32 \pm 0.55$  mg/l) than in the untreated patients ( $2.7 \pm 0.52$  mg/l,  $P < 0.05$ ).

### Biliary bile acid concentrations

In the patients with ulcerative colitis, the mean ( $\pm$ S.E.M.) biliary bile acid concentrations in the active colitis, post-colectomy and pouchitis subgroups were  $136 \pm 13.4$  mM,  $171 \pm 8.7$  mM ( $P < 0.05$  versus active colitis group) and  $163 \pm 19.9$  mM (not significant) respectively. In those with Crohn's disease, the mean biliary bile acid concentrations were  $112 \pm 17.6$  mM (active colitis),  $116 \pm 27.3$  mM (post-colectomy or defunctioning loop ileostomy) and  $137 \pm 20.9$  mM (active ileitis or ileocolitis). There was a significant difference in biliary bile acid concentrations between the post-colectomy ulcerative colitis patients and those with active Crohn's colitis ( $P < 0.01$ ); all other comparisons between the ulcerative colitis and Crohn's disease groups were non-significant.

In the patients with ulcerative colitis, normalization of biliary lactoferrin concentrations to a standard bile acid concentration of 100 mM increased the significance of differences in mean lactoferrin levels between the three subgroups. Thus, in the 14 patients with active colitis, the mean ( $\pm$ S.E.M.) corrected lactoferrin concentration in gall bladder bile was  $2.4 \pm 0.48$  mg/l, compared with a value of  $0.69 \pm 0.07$  mg/l in the post-colectomy patients

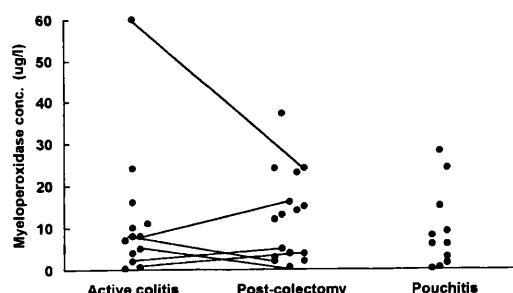


**Figure 3** Biliary myeloperoxidase concentrations in patients with active and inactive ulcerative colitis

( $P < 0.0005$ ), and  $1.1 \pm 0.14$  mg/l in those with pouchitis ( $P < 0.05$  and  $P < 0.01$  versus the active colitis and pouchitis groups respectively). In the patients with Crohn's disease, however, normalization of biliary lactoferrin concentrations had no significant effect on the distribution of data or quoted  $P$  values.

### Biliary myeloperoxidase in ulcerative colitis

In the three groups of patients with ulcerative colitis, the concentrations of myeloperoxidase in gall bladder bile were also measured. The sensitivity of this commercial assay was confirmed to be  $0.5 \mu\text{g/l}$ , with intra- and inter-assay coefficients of variation of  $< 5\%$  ( $n = 12$ ). On average, myeloperoxidase concentrations were approximately 300 times lower, on a molar basis, than those of lactoferrin. Furthermore, in contrast to the lactoferrin results, there were no significant differences in mean myeloperoxidase concentrations between the three groups. Thus, the mean ( $\pm$  S.E.M.) myeloperoxidase levels in the active colitis, post-colectomy and pouchitis groups were  $12.0 \pm 4.4 \mu\text{g/l}$ ,  $11.9 \pm 2.5 \mu\text{g/l}$  and  $9.2 \pm 2.8 \mu\text{g/l}$  respectively, with an overall range of  $0.2$ – $60 \mu\text{g/l}$  (Figure 3). In the 42 patients with ulcerative colitis, there was no significant correlation between biliary lactoferrin and myeloperoxidase levels in any of the patient subgroups, or when the raw data were combined or normalized to a standard bile acid concentration of  $100 \text{ mM}$ .

## DISCUSSION

The results of this study show that there is a marked difference between biliary lactoferrin concentrations in patients with active and inactive inflammatory bowel disease. Thus, in the 21 patients with active Crohn's colitis or ulcerative colitis, the mean lactoferrin concentration in gall bladder bile was more than  $3 \text{ mg/l}$  – similar to that in the 20 patients with Crohn's ileitis or ulcerative pouchitis. In contrast, in the 22 patients with inactive inflammatory bowel disease (post-colectomy or

defunctioning loop ileostomy with no evidence of active disease), biliary lactoferrin levels were significantly lower than in the other two groups, with a mean concentration of approximately  $1 \text{ mg/l}$ .

In the present study, gall bladder bile was not obtained from control subjects. Nonetheless, it is considered likely that biliary lactoferrin concentrations in those with inactive inflammatory bowel disease represent basal or 'normal' levels of lactoferrin in gall bladder bile. Indeed, the biliary lactoferrin levels in patients with inactive inflammatory bowel disease were similar to those that we [21] and others [19,20], have reported in the plasma of control subjects and patients with inactive inflammatory bowel disease. In contrast, in those with active disease, the mean biliary lactoferrin concentration was similar to the high levels reported in the systemic circulation of such patients [19,21].

The present results are consistent with either the presence of an enterohepatic circulation of lactoferrin or selective degranulation of neutrophils within the biliary tract by circulating bacterial  $N$ -formyl peptides. In the rat, bacterial chemotactic peptides, such as  $N$ -formyl-methionyl-leucyl-phenylalanine, have been shown to undergo an enterohepatic circulation [8,9] and to induce selective release of neutrophil secondary granule products *in vitro* [42]. Therefore, in active inflammatory bowel disease, possibly as a result of decreased protease activity within the intestinal lumen [51] and/or increased intestinal mucosal permeability [52,53], biliary concentrations of bacterial oligopeptides may also rise and induce lactoferrin release from neutrophils within the hepatobiliary tract. Previous work from our group [23] has suggested that, in active inflammatory bowel disease, lactoferrin is released selectively from neutrophil secondary granules. Conversely, if biliary lactoferrin concentrations were merely a marker of non-specific neutrophil degranulation, we would expect that biliary concentrations of myeloperoxidase (stored in neutrophil primary granules) would also vary with disease activity. However, in the present study, there were no significant differences in mean myeloperoxidase concentrations between the different groups, supporting indirectly the hypothesis of selective neutrophil degranulation in active inflammatory bowel disease.

An alternative hypothesis is that lactoferrin itself undergoes an enterohepatic circulation. Lactoferrin is transferred from the plasma into the hepatocyte largely by an active transport mechanism [54–56], and is probably routed to the bile canaliculus by vesicular transport [55,57]. Although only a small proportion of lactoferrin that is transferred from the plasma escapes degradation within the liver [55,58], the present results may also be explained by the presence of an enterohepatic circulation of lactoferrin. If so, then biliary lactoferrin concentrations would also be expected to be high in other inflammatory conditions in which systemic lactoferrin

concentrations are increased [59,60]. Indeed, high plasma lactoferrin concentrations have been reported in active rheumatoid arthritis and systemic lupus erythematosus [60] – conditions also associated with the presence of serum anti-neutrophil antibodies [61,62]. In inflammatory bowel disease, approximately 22–89% of patients with ulcerative colitis [4,7,28,29,31–38] and up to 34% of patients with Crohn's disease [4,28,32–34,36,41,63] are p-ANCA positive. As yet, the antigens that stimulate p-ANCA production are poorly defined. Nonetheless, evidence suggests that lactoferrin (but not myeloperoxidase [7,29]) may be a major target antigen for the p-ANCA of inflammatory bowel disease [27–31]. Walmsley et al. [32] detected serum anti-lactoferrin antibodies in only 2 of 52 patients with ulcerative colitis, but in another study, immunoglobulin G anti-lactoferrin antibodies were found in the sera from 50% of patients with ulcerative colitis and/or PSC [29]. These antibodies may be of pathogenic relevance since, *in vitro*, intact immunoglobulin G anti-lactoferrin antibodies bind to vascular endothelial cells and stimulate neutrophils to produce free oxygen radicals [64].

Although increased concentrations of plasma or biliary lactoferrin in patients with inflammatory bowel disease do not prove that it has a pathogenic role, nonetheless, *in vitro*, lactoferrin acts as a potent neutrophil chemotactic factor [13,25,65–67], promotes hydroxyl radical production [12] and enhances monocyte cytotoxicity [68]. Lactoferrin also has potent heparin-neutralizing activity [69] – of interest in view of the pro-thrombotic state associated with active inflammatory bowel disease [70], and recent observations that heparin itself may induce remission in steroid-resistant disease [71,72]. Furthermore, in animal models, mesenteric arterial infusion of lactoferrin [25], or colonic instillation of bacterial N-formyl peptides [73], results in mucosal neutrophil recruitment and the development of a frank colitis. Although there are few data in humans, the present results suggest that the proposed relationship between biliary and systemic neutrophil granule products, p-ANCA positivity and the development of PSC in patients with inflammatory bowel disease, warrants further study.

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## Plasma arachidonic acid-rich phospholipids in Crohn's disease: response to treatment

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1. Increased concentrations of plasma polyunsaturated fatty acids have been implicated in the pathogenesis of Crohn's disease. However, it is not known whether there are corresponding changes in circulating phospholipids – the major source of fatty acids in the plasma.

2. Fasting plasma samples were obtained from 17 control subjects and 13 patients with active Crohn's disease [Simple Index of Crohn's Disease Activity (SICDA) >6] before, and 2 and 8 weeks after, treatment with either a peptide diet or oral prednisolone.

3. Before treatment, the Crohn's disease patients had mildly active disease (SICDA  $9.9 \pm 0.8$ , erythrocyte sedimentation rate  $26.4 \pm 6.5$  mm/h, serum C-reactive protein  $2.8 \pm 0.4$  mg/l). The proportions of the polyunsaturated phosphatidylcholine species, 16:0–20:4 ( $10.0 \pm 0.7\%$ ) and 16:0–22:6 ( $7.1 \pm 0.8\%$ ), were both significantly higher than those in healthy controls ( $7.6 \pm 0.5\%$ ,  $P < 0.01$  and  $5.3 \pm 0.5\%$ ,  $P < 0.05$  respectively).

4. After 2 weeks treatment, the SICDA in the Crohn's disease patients decreased to  $3.2 \pm 0.6$  ( $P < 0.0001$  compared with the pretreatment value), and there were corresponding falls in the erythrocyte sedimentation rate (to  $12.6 \pm 2.7$  mm/h,  $P < 0.05$ ) and C-reactive protein concentration (to  $1.7 \pm 0.3$  mg/l,  $P < 0.05$ ) – these improvements being maintained at 8 weeks. There was also a fall to normal values in 16:0–20:4 (to  $7.7 \pm 0.6\%$ ,  $P < 0.01$  compared with the pretreatment value) and in 16:0–22:6 (to  $5.7 \pm 0.5\%$ ,  $P$  not significant), by week 8.

5. The proportions of polyunsaturated phosphatidylcholine molecular species were increased in the plasma of patients with active Crohn's disease, but fell to normal levels during disease remission. These observations are consistent with the theory that, in active Crohn's disease, the mucosal phospholipids containing polyunsaturated fatty acids are increased, contribute to eicosanoid synthesis and 'spill' into the plasma.

### INTRODUCTION

Arachidonic acid, incorporated into the second

fatty acid position (*sn*-2) of phospholipids, is an integral component of cell membranes. Arachidonic acid metabolites, formed via the cyclo-oxygenase and lipoxygenase pathways, are also important mediators of inflammation [1]. In active Crohn's disease, the tissue concentrations of arachidonic acid [2–4], as well as those of several arachidonic acid-derived eicosanoids (such as prostaglandin  $E_2$ , thromboxane  $B_2$  and the leukotrienes), are increased, and are important mediators of inflammation within the intestinal mucosa [5–8].

Raised plasma concentrations of arachidonic acid, as well as other polyunsaturated fatty acids (PUFAs), have been described in patients with active Crohn's disease [9, 10]. These studies used the technique of GLC to determine the proportions of total (both free and bound) fatty acids in plasma. However, although triacylglycerols, cholesterol esters and free fatty acids all contribute to the plasma total fatty acid profile, quantitatively the most important source of both circulating and membrane PUFAs is the phospholipids [11, 12]. Furthermore, alterations in the composition of triacylglycerols and the other minor fatty acid carriers induced by diet or disease, occur in parallel with changes in the fatty acid composition of plasma phospholipids [11, 13]. Therefore, in active Crohn's disease, the reported increases in total plasma PUFAs [9, 10] may actually represent rises in the proportion of PUFAs (such as arachidonic acid) incorporated into the *sn*-2 position of phospholipids (Fig. 1). By acting as a substrate for eicosanoid synthesis [1, 14–16], an increase in the PUFA composition of plasma phospholipids may be relevant to the pathogenesis of Crohn's disease. However, there have been no previous reports of the fatty acid composition (molecular species) of plasma phospholipids in Crohn's disease.

Therefore, we compared the molecular species of plasma phosphatidylcholine (PC; the principal plasma phospholipid [17, 18]) in control subjects, with those in patients with Crohn's disease studied before and during treatment, and related the results to markers of disease activity.

**Key words:** arachidonic acid, Crohn's disease, fatty acids, phospholipids.

**Abbreviations:** ESR, erythrocyte sedimentation rate; PC, phosphatidylcholine; PUFAs, polyunsaturated fatty acids; SICDA, Simple Index of Crohn's Disease Activity.

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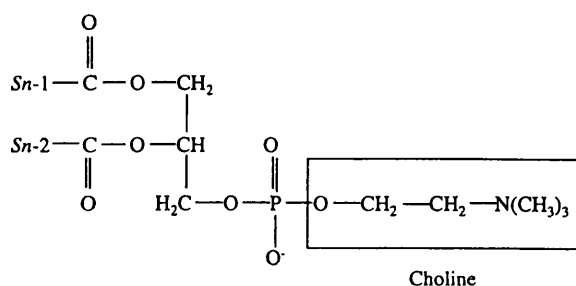


Fig. 1. Schematic diagram of PC, the predominant plasma phospholipid. Sn-1 is typically a saturated or monosaturated fatty acid, e.g. palmitic acid or oleic acid, while sn-2 is usually a polyunsaturated fatty acid, e.g. linoleic acid or arachidonic acid.

## PATIENTS AND METHODS

Fasting plasma samples were obtained from 17 healthy laboratory staff [12 men and five women; mean age 31 (22–55) years]. None of these control subjects had ever had inflammatory bowel disease.

Thirteen patients [six men and seven women; mean age 33 (23–54) years] with symptomatic small bowel Crohn's disease, with or without colonic involvement, were also studied. All had active disease – as judged by clinical history and by high (>6) simple indices of Crohn's disease activity (SICDA; Harvey and Bradshaw [19]), increased serum C-reactive protein levels and/or high erythrocyte sedimentation rates (ESR).

### Experimental design and evaluation of response to treatment

As part of a separate study, in which the efficacy of a semi-elemental diet was compared with that of oral prednisolone in inducing disease remission [20], the patients were treated for 2 weeks with either: (i) oral prednisolone (0.5 mg day<sup>-1</sup> kg<sup>-1</sup> decreasing to nil over 8 weeks, together with an unrestricted diet), or (ii) a flavoured liquid peptide diet ('Peptamen', 30–35 kcal day<sup>-1</sup> kg<sup>-1</sup>; Clintec Nutrition, Chicago, IL, U.S.A.), with resumption of a normal diet by 8 weeks.

Clinical data and fasting blood samples were obtained before, and 2 and 8 weeks after, entry into the study. Patients were regarded as being in clinical remission when the SICDA score became less than six and when they had resumed a normal diet without the need for drugs or surgery.

Serum C-reactive protein concentrations and ESRs were measured before, and 2 and 8 weeks after, commencing treatment.

### Plasma PC species

HPLC-grade methanol, isopropanol, hexane, acetonitrile and ethanol were obtained from Rathburn Chemicals Ltd (Walkerburn, U.K.). Standards for the different phospholipid classes (PC, phosphatidylinositol, phosphatidylserine) and the major molecular species of PC were obtained from Sigma Chemical Co (Poole, U.K.) and Avanti Polar Lipids

(Alabaster, AL, U.S.A.). All other solvents or chemicals were of analytical grade.

After Fölch extraction of plasma lipids [21], plasma PC was separated from the other phospholipid classes by HPLC [22], and the molecular species of the PC determined by reverse-phase HPLC [23], by the following methods.

Aliquots (200 µl) of the lipid extract were loaded onto a 4.6 × 250 mm octadecyl 'Kromasil' column packed with 10 µm of silica (Hichrom Ltd, Theale, U.K.), and eluted at a flow rate of 2 ml/min, using a mobile phase of hexane–isopropanol–ethanol–phosphate buffer–acetic acid (367:495:100:57:0.3) [22]. Phospholipid peaks were detected at an absorbance of 205 nm. The PC peak was identified by comparison with the retention time of standard bovine PC, and confirmed by TLC.

Next, the PC was separated into its molecular species on a 4.6 × 250 mm 'Nucleosil' column packed with octadecylsilane bonded phase, of 5 µm diameter (Hichrom Ltd). Elution was performed at a rate of 1.5 ml/min, with a mobile phase of methanol–hexane–water (80:5:15) [23]. Individual peaks were detected at an absorbance of 492 nm, and identified by MS and by comparing retention times with those of standard PC molecular species.

### Statistical analysis

All results are expressed as means (SEMs). A two-tailed Student's *t*-test (paired) was used to determine the significance of differences within groups. The  $\chi^2$  test was used to test for differences in proportions, and the Mann–Whitney test for discontinuous variables.

## RESULTS

Of the 13 patients with active Crohn's disease, nine had ileal disease alone while four had ileocolonic disease – as judged by barium studies and/or colonoscopy ± biopsy. In the past, three patients had undergone limited (<60 cm) ileal resections and one had had a colectomy plus terminal ileostomy.

### Clinical response to treatment

At entry, the 'Peptamen' and prednisolone groups were comparable with respect to sex and age, and to the site and severity of their disease. Between the two groups, there were no significant differences in the clinical response to treatment, markers of disease activity, or plasma PC species. Therefore, at each time point the results in the two groups of patients were combined.

Before treatment, the Crohn's disease was mildly active, with a mean SICDA of  $9.9 \pm 0.8$ , an ESR of  $26.4 \pm 6.5$  mm/h and a C-reactive protein concentration of  $2.8 \pm 0.4$  mg/l (normal <1 mg/l).

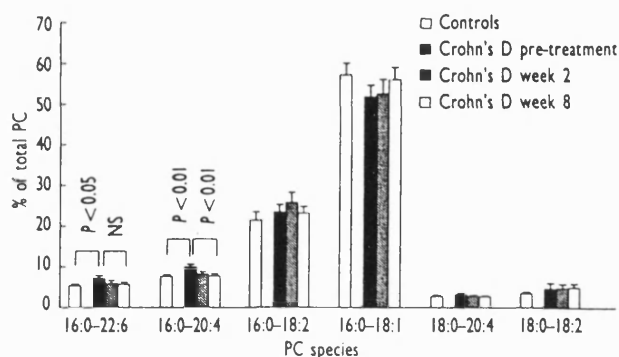


Fig. 2. Molecular species (fatty acid composition) of plasma PC in 17 control subjects and 13 patients with Crohn's disease. Values are expressed as means with SEMs represented by vertical bars. Before treatment, the proportions of both polyunsaturated species, PC 16:0-22:6 and PC 16:0-20:4, in the Crohn's disease patients were significantly higher than those in the control subjects.

After 2 weeks treatment, the SICDA score fell to  $<6$  in all but one patient (who scored 8), with a mean value in the 13 patients of  $3.2 \pm 0.6$  ( $P < 0.0001$  compared with the pretreatment value). There were corresponding falls in both the ESR (to  $12.6 \pm 2.7$  mm/h,  $P < 0.05$ ) and C-reactive protein level (to  $1.7 \pm 0.3$  mg/l,  $P < 0.05$ ).

At 8 weeks, the clinical remission was sustained in 10 patients, but in two there had been a relapse with SICDAs of 8 and 10, respectively. For all 13 patients, the mean SICDA ( $3.5 \pm 0.9$ ), ESR ( $11.3 \pm 2.6$  mm/h) and C-reactive protein ( $1.6 \pm 0.3$  mg/l) results at 8 weeks were not significantly different from those at 2 weeks.

### Plasma PC species

The relative proportions of the six major molecular species of PC for the control subjects and the patients with Crohn's disease are shown in Fig. 2.

In the control subjects and the patients with active Crohn's disease, the predominant plasma PC species were 16:0-18:1 ( $57.2 \pm 2.9\%$  and  $51.8 \pm 2.9\%$  of total PC, respectively;  $P$  not significant) and 16:0-18:2 ( $21.4 \pm 2.1\%$  compared with  $23.5 \pm 1.8\%$ ;  $P$  not significant). Before treatment, however, the proportions of both polyunsaturated species, PC 16:0-20:4 ( $10.0 \pm 0.7\%$ ) and PC 16:0-22:6 ( $7.1 \pm 0.8\%$ ) in the Crohn's disease patients were significantly higher than those in the control subjects [ $7.6 \pm 0.5\%$  ( $P < 0.01$ ) and  $5.3 \pm 0.5\%$  ( $P < 0.05$ ) respectively].

The relative proportions of the major arachidonic acid-rich phospholipid, PC 16:0-20:4 for the control subjects and the patients with Crohn's disease, are shown in Fig. 3. After 8 weeks treatment of the Crohn's disease patients, there was a fall to normal values of PC 16:0-20:4 (to  $7.7 \pm 0.6\%$ ,  $P < 0.01$  compared with pretreatment value) and PC 16:0-22:6 (to  $5.7 \pm 0.5\%$ ,  $P$  not significant). The proportions of the other plasma phospholipid species measured did not change significantly during treatment.

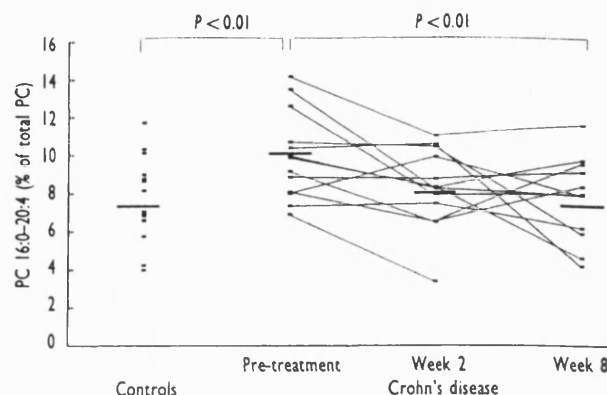


Fig. 3. Relative proportions of the major arachidonic acid-rich phospholipid, PC 16:0-20:4, in the plasma of the control subjects and the patients with Crohn's disease, before treatment, and after 2 and 8 weeks of therapy. The mean % PC 16:0-20:4 in the plasma of patients with active Crohn's disease was significantly higher ( $P < 0.01$ ) than that in the control subjects, but returned to normal values after 8 weeks of therapy.

### DISCUSSION

The present study has shown that the proportions of polyunsaturated PC molecular species – in particular, palmitoyl-arachidonoyl (16:0-20:4, n-6) PC and palmitoyl-docosahexanoyl (16:0-22:6, n-3) PC – are increased in the plasma of patients with active Crohn's disease. However, after 2 to 8 weeks treatment with either prednisolone or a peptide-based diet, the distribution of plasma PC species changed and became similar to that of the control subjects. By this time, most patients were in clinical remission and the standard markers of disease activity had fallen towards normal levels.

These alterations in the molecular species of PC may be relevant to the pathogenesis of Crohn's disease. PC, the predominant plasma phospholipid, is the major source of PUFAs in the plasma [17, 18], and in cell membranes [24]. In active Crohn's disease, increased proportions of PUFAs in the plasma [9, 10], peripheral blood mononuclear cells [25] and intestinal mucosa [2-4], have all been reported. Therefore, the present results suggest that the reported increases in plasma PUFAs actually represent rises in the proportion of PUFA-containing phospholipids.

In active inflammatory bowel disease, the concentrations of arachidonic acid (20:4, n-6) and docosahexanoic acid (22:6, n-3) in the inflamed colonic mucosa are increased [2-4]. Furthermore, in active Crohn's disease, inflammatory cell recruitment into the intestinal mucosa correlates well with tissue PUFA composition [3]. Disease remission, in turn, is associated with a fall in neutrophil [26] and PUFA [2] content of the intestinal mucosa. Therefore, infiltration of neutrophils and mononuclear cells (which are rich in arachidonic acid-rich phospholipids [27]) into the intestinal mucosa may be

responsible, at least in part, for the rise in PUFA content of the intestinal mucosa in active Crohn's disease. This increase in the PUFA content of phospholipids is accompanied by a rise in phospholipase A<sub>2</sub> activity within the intestinal mucosa [16, 28, 29], resulting in increased hydrolysis of the fatty acyl ester bond at the *sn*-2 position of phospholipids. In turn, this results in the release of free PUFAs, enhanced eicosanoid synthesis and further mucosal inflammation [6, 30].

Although there have been no previous studies of plasma phospholipid species in Crohn's disease, the present results are consistent with earlier reports of plasma fatty acid composition in Crohn's disease [9, 10, 31]. In patients with both inactive [31] and mildly active [9] Crohn's disease, Esteve-Comas et al. reported that both the proportion (% of total fatty acids) and the concentration (measured in  $\mu\text{mol/l}$ ) of arachidonic acid in the plasma was higher than that in controls, and there was a highly significant increase in docosahexaenoic acid. Conversely, in patients with active Crohn's disease, the proportions of arachidonic acid and docosahexaenoic acid in the plasma fell with increasing severity of disease. The authors postulated that, in active inflammatory bowel disease, increased PUFA biosynthesis coexists with increased fatty acid consumption. In mildly active disease, an enhanced PUFA biosynthesis predominates. However, when disease activity becomes more severe, PUFA hypermetabolism occurs, in order to meet the needs for cellular repair and energy [9]. In patients with extensive disease or resection of the small intestine, essential fatty acid deficiency may also play a role, resulting in low concentrations of PUFAs in plasma phospholipids, cholesterol esters and triacylglycerols [13, 32, 33].

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