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The Role of microRNA Biomarkers to Predict Complications of Gallstones

Thesis

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ABSTRACT

Gallstones are the most common cause for abdominal pain requiring an emergency hospital admission. About 5.5 million people in the UK have the condition and over 60,000 cholecystectomies are performed every year. At present there are no specific biomarker tests that can predict whether an individual patient with gallstones has severe inflammatory, infective or obstructive complications requiring inpatient management and urgent surgical intervention; or has symptoms without complications that can be managed as an outpatient or day-case basis. Building on evidence that microRNA (miRNA) biomarkers can predict acute cholestatic liver injury, and have been shown to be superior to alanine aminotransferase (ALT), this study hypothesised that miRNA biomarkers might be used to predict gallstone complications in emergency surgical patients and help to inform clinical decision-making. The specific aim of this research is to determine whether microRNA (miR) -122 and miR-210 measured at first presentation to hospital can predict complications of gallstone diseases and differentiate those patients who require acute inpatient admission.

Patients admitted to the Royal Infirmary of Edinburgh from 3rd September 2014 to 12th May 2015 with a differential diagnosis of gallstone disease on index presentation were recruited to this study and a blood sample was taken for miRNA analysis in the plasma fraction. A pilot study was carried out after 6 months of patient recruitment with a third of the samples collected. miR-122 and -210 were quantified by PCR. All analyses were performed blinded to the clinical data. Each patient was followed up for a minimum period of one month during which investigation reports, operation notes and pathology results were obtained.

A total of 232 patients were recruited to the study. Eight-two random samples were analysed in the pilot study. As the pilot study did not show any significant differences in miR-210 concentration between different patient groups ($P=0.365$), the main study only focused on miR-122 analysis. There was a significant difference in plasma miR-122 concentration between patients with gallstone (Median: 0.039 [95% CI: 0.027 , 0.059]) and non-gallstone diseases (Median: 0.011 [95% CI: 0.0079 , 0.015]) ($P<0.001$); and between uncomplicated (Median: 0.062 [95% CI: 0.039 , 0.11]) and complicated (Median: 0.030 [95% CI: 0.022 , 0.047]) gallstone diseases ($P=0.040$). Plasma miR-122 was significantly lower in patients with cholecystitis (Median: 0.023 [95% CI: 0.017 , 0.032]) ($P=0.006$) and significantly higher in patients with choledocholithiasis (Median: 0.099 [95% CI: 0.054 , 0.17]) ($P<0.001$). The PPV and NPV for plasma miR-122 in detecting (a) gallstone diseases were 78.0% and 41.9%

respectively; (b) complicated gallstone diseases were 81.0% and 32.0% respectively; and (c) choledocholithiasis were 63.0% and 75.9% respectively.

In conclusion, relative miR-122 concentration in plasma at presentation to hospital is significantly different in patients with complications of gallstone diseases that may require acute in-patient admission. When these tests are developed for rapid analysis or made suitable for a near-patient assay platform, and if these findings are validated in an independent cohort, there exists the potential to substantially reduce emergency hospital admissions by identifying low risk patients suitable for direct discharge from the Emergency Department and further management in an outpatient setting.

LAY SUMMARY

Gallstones are the most common cause for abdominal pain requiring an emergency hospital admission. Approximately 5.5 million people in the UK have gallstones. Gallstones can cause a number of problems while they are within the gallbladder and also if they pass along the tube carrying bile from the liver to the bowels. This can result in further complications, for example severe inflammation, infection and obstruction of the bile duct. At present there are no specific blood tests that can predict whether a person with gallstones will have the associated complications that will require inpatient treatment in hospital. Consequently, patients that could be managed as an outpatient or day-case are being admitted to hospital.

microRNAs (miRNAs) are small segments of genetic material that might be able to predict complications from gallstones. In previous research projects by us and other scientists, miRNAs can predict complications of liver inflammation. The specific aim of my research was to determine whether testing the levels of miR-122 and miR-210 in blood when patients with symptoms from gallstones arrive at hospital can predict complications of gallstone diseases and identify those patients who require acute inpatient admission.

We asked patients at the Royal Infirmary of Edinburgh if they would participate in our project after obtaining all the necessary ethical permissions and after gaining their consent. This phase of the study lasted from 3rd September 2014 to 12th May 2015. Participants gave a blood sample and we measured the concentration of miRNA in the blood. We compared the concentrations with the final diagnosis and the findings at gallbladder surgery, if they had it.

There were 232 participants. By measuring miRNA levels we could tell the difference between patients with gallstones and those with no gallstones, and could also tell the difference between patients with gallbladder inflammation and also patients with gallstones stuck in the bile duct.

If we can confirm and validate our findings in a second larger group of patients, this test might become a useful part of the process in deciding how best to offer treatment to people with gallstones.

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ETHICS APPROVAL AND FUNDING

This study was assessed by the South East Scotland Research Ethics Service of National Health Service (NHS) Lothian and was given ethical approval under the terms of the Governance Arrangements for Research Ethics Committees (Harmonised Edition). Lothian R&D project number 2014/0224; REC number 14/EM/0211. This study has been approved to recruit a total of 300 patients over a period of two years. This research is funded by the University of Edinburgh and NHS Lothian. This study is still currently ongoing. Permission to reuse illustrations in this study have been obtained from the journals and/or lead authors of the referenced articles.

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LIST OF ABBREVIATIONS

Ago	Argonaute
AlkP	Alkaline phosphatase
ALT	Alanine aminotransferase
ASGBI	Association of Surgeons of Great Britain and Ireland
BDL	Bile duct ligated
bp	Base pair
CBD	Common bile duct
CI	Confidence intervals
CRF	Clinical Research Facility
CRP	C-reactive protein
CT	Computed tomography
CYP7A1	Cholesterol 7 α -hydroxylase
DNA	Deoxyribonucleic acid
ERCP	Endoscopic retrograde cholangiopancreatography
GGT	Gamma-glutamyl transpeptidase
HIF	Hypoxia inducible factor
HNF	Hepatocyte nuclear factor
IQR	Interquartile range
LETf	Liver-enriched transcription factors
LFTs	Liver Function Tests
miR-122/-210	microRNA-122/-210
miRNA	microRNA
MRCP	Magnetic resonance cholangiopancreatography
mRNA	Mature ribonucleic acid
NAFLD	Non-alcoholic fatty liver disease
NHS	National Health Service
NPV	Negative predictive value
NSAP	Non-specific abdominal pain

nt	Nucleotides
PMH	Past medical history
Pol	Polymerase
PPV	Positive predictive value
QMRI	Queen Margaret Research Institute
r	Spearman's correlation coefficient
RCS	Royal College of Surgeons
RIE	Royal Infirmary of Edinburgh (Hospital)
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RUQ	Right upper quadrant
SIRS	Systemic inflammatory response syndrome
USS	Ultrasound/Ultrasonographic scan
UTR	Untranslated region
WCC	White Cell Count

CHAPTER 1: INTRODUCTION

The acute presenting symptom of abdominal pain accounts for up to 50% of all emergency admissions under the General Surgery specialty in the United Kingdom¹. Common causes for this symptom include biliary colic, which can be defined as transitory right-sided abdominal pain due to gallstones usually without acute inflammation; and cholecystitis, which is defined as acute inflammation of the gallbladder, usually in the presence of gallstones. A cluster of symptoms without a clear cause, termed non-specific abdominal pain (NSAP) makes up a significant proportion (approximately 40%) of the remainder. Gallstone disease is one of the most common and costly gastrointestinal disease in the country¹. However, as acute abdominal pain is common and clinical signs are relatively non-specific, only about 50% of patients with acute abdominal pain are correctly diagnosed on their index presentation¹. Furthermore, current available laboratory tests have their limitations. Imaging investigations have good sensitivity and specificity, but can be costly and time-consuming, and hence may delay patient management. Thus, current clinical, laboratory and imaging limitations pose a clinical diagnostic challenge. As a result of diagnostic uncertainty, patients are sometimes admitted unnecessarily for further ‘wait and see’ management, further imaging and/or serial blood tests. These cases represent a substantial, expensive and potentially avoidable inpatient burden¹. Hence, new diagnostic tools with better sensitivity and specificity in distinguishing different patients with abdominal pain would be of benefit – both clinically, in enhancing patient management with faster and more directive disease management; and with respect to improving healthcare demographics by creating more acute beds for more appropriate patients.

Over the last decade, microRNAs (miRNAs) have been emerging as potential biomarkers in aiding diagnosis, prognosis and treatment response in certain diseases. As novel biomarkers, the role of miRNAs in individual diseases is still not fully understood^{2,3}. With regards to biliary diseases, miRNAs have been extensively studied in liver diseases, but not with gallbladder or gallstone diseases. The two miRNAs of interest in this study are miR-122 and miR-210. The main reason for this is that both miRs have been shown to be associated with cholestatic induced liver injury and that they both correlated significantly with blood test, alanine aminotransferase (ALT), which is a classic marker of liver injury^{4,5}. miR-122 is liver specific and is known to play important roles in liver cholesterol metabolism and hence bile pool^{6,7}; whereas miR-210 is a hypoxamir and may play a role in gallbladder motility^{8,9}. As the roles that these miRNAs play are key pathogenic factors for gallstone formation, this study hypothesized that both miRNAs could be potential biomarkers for gallstone diseases, and

depending on their expression levels, could be used as biomarkers for the different types of gallstone diseases.

1.1 GALLSTONE DISEASE

1.1.1 Background

Abdominal pain is the most common cause for a surgical referral, accounting for up to 50% of all emergency admissions to the General Surgery specialty in the United Kingdom¹. Common sub-acute conditions causing this symptom include non-specific abdominal pain (NSAP), biliary colic and cholecystitis. Nevertheless, only about 50% of these patients are correctly diagnosed upon first presentation to hospital, thus leading to a higher number of hospital admissions than required¹. Hence, more sensitive and specific methods of distinguishing gallstone diseases from less urgent causes of abdominal pain such as NSAP; and in diagnosing acute presentations of different gallstone diseases would be beneficial. Consequently, earlier targeted management of gallstones could be delivered, and more efficient and effective healthcare service delivery could be achieved.

Gallstone related disease is one of the most common and costly gastrointestinal diseases in Western countries¹⁰. About 10-20% of the Western population have the condition¹¹ (about 5.5 million people in the UK have gallstones)¹², and about 1-4% of asymptomatic patients with gallstones are estimated to develop symptoms annually¹³. The prevalence of having gallstones is about 10-15% in males and 20-25% in females of all ages¹, and these statistics are expected to increase as life expectancy increases¹¹. The UK Hospital Episode Statistics' data for 2003-2004 showed that 25,743 patients were admitted with an acute gallbladder disease during that period, and these numbers are rising annually¹⁴. This is particularly an important issue in our aging population as the elderly population accounts for 50-70% of acute gallbladder cases requiring an emergency admission¹⁵. In a cadaveric study carried out in the United Kingdom, the reported incidence of gallstones in women 50 to 59 years of age was 24%, increasing to 30% in the ninth decade. In 50 to 59 year old men, the rate was 18%, with an increase to 29% in the ninth decade¹⁶.

1.1.2 Pathogenesis and Aetiology

Three important mechanisms in the formation of gallstones (cholelithiasis) are cholesterol supersaturation; gallbladder hypomotility; and pro-nucleating or anti-nucleating factors¹⁷. During the development of gallstones, solid conglomerates of cholesterol monohydrate crystals, mucin gel, calcium bilirubinate and other proteins accumulate, and increase in size within the gallbladder. See **Figure 1**. Gallstones can be classed according to their chemical composition – mainly pure cholesterol, pure pigment or mixed stones¹⁸. The colour of pigment stones depends on the aetiology: black in metabolic causes, i.e. in patients with haemolytic conditions or cirrhosis causing increased levels of unconjugated bilirubin; or brown (infectious) in patients with biliary infections or infestations, and this is more prevalent in the Asian population¹⁹. Mixed stones contain some amounts of bilirubin salts and calcium. In developed countries, cholesterol gallstones represent the major type of gallstones, accounting for about 75% of stones; black pigment stones for 20%; and brown pigment stones for about 5%¹⁸.

1.1.3 Risk Factors

Risk factors for gallstone development can be classed into non-modifiable and modifiable factors. Non-modifiable factors include increasing age, female gender, ethnicity and family history (genetic traits). In the Framingham study in which subjects 30-59 years of age were followed up for 10 years, the risk of gallstone formation was highest in subjects of age 55-62 years and the incidence in females was more than twice than that in males in any age range²⁰. The risk of gallstone formation is increased in those who have family members or identical twins with gallstones²¹.

Modifiable factors include obesity, rapid weight loss (>1.5 kg/week), sedentary lifestyle, and digestive disorders such as Crohn's disease²¹⁻²³. It is postulated that obesity causes increased hepatic secretion of cholesterol which results in an increased risk of gallstone formation. Gallstone disease has been reported to be asymptomatic in almost 80% of cases and physical exercise appears to have a protective role in the development of symptoms from the disease²⁴. Oestrogen also seems to play a role as pregnancy, parity, and oestrogen replacement therapy all increase the risk of gallstones²¹. The prevalence of gallstones is three times higher in people with liver cirrhosis, ranging between 25% to 30% more, than those who are without

cirrhosis²⁵⁻²⁷. Previous alcohol abuse and smoking are also independent risk factors for gallstone formation^{28,29}.

1.1.4 Presentation

Gallstone disease encompasses a wide spectrum of diseases including symptomatic cholelithiasis within the gallbladder (biliary colic), cholecystitis, gallstone pancreatitis, choledocholithiasis (stones within the common bile duct (CBD)) and ascending cholangitis (infection of the CBD)³⁰. See **Figure 2**. The acute onset of severe right upper quadrant (RUQ) pain is most commonly associated with gallstone diseases. The abdominal pain can last for several hours with minimal systemic upset (biliary colic) or can be more prolonged with systemic symptoms, most likely indicating localised gallbladder inflammation (cholecystitis)¹. According to the classification by the Royal College of Surgeons (RCS) and the Association of Surgeons of Great Britain and Ireland (ASGBI)¹, both biliary colic and cholecystitis are classed as simple acute biliary diseases. Conversely, the severe abdominal pain can be associated with jaundice and biliary duct dilatation on imaging, indicating likely choledocholithiasis or associated gallstone pancreatitis. These are classed as complex biliary diseases. Early radiological imaging, initially with an abdominal ultrasound scan (USS) and biochemical liver function tests (LFTs) are essential for the initial triage of acute biliary patients¹. Further imaging modalities, such as magnetic resonance cholangiopancreatography (MRCP) may be used if USS has not detected choledocholithiasis in the presence of dilated bile ducts and/or abnormal LFTs³¹. Patients with biliary colic also normally have LFTs within normal ranges and no biliary duct dilatation on imaging¹.

1.1.5 Gallstone Disease Spectrum (GDS)

Among patients who have been diagnosed with gallstones, 35% will eventually experience recurrent symptoms or complications leading them to have a cholecystectomy³². The risk of further gallstone complications is high during the first few years after initial diagnosis of gallstones, about 4% per year, and then it decreases, to less than 2% per year after 10 years³³⁻³⁵.

1.1.5a GDS: Background of Cholecystitis

Acute cholecystitis is one of the most frequent reasons for an emergency admission³⁶. It accounts for 3-10% of abdominal pain episodes needing hospitalisation and surgery³⁷⁻³⁹. Most cases of cholecystitis are due to gallstones. Other attributable factors include ischaemia, gallbladder motility disorders, infections, collagen disease and allergic reactions⁴⁰. Alternatively, acute acalculous cholecystitis, i.e. cholecystitis without gallstones, accounts for 2-15% of acute cholecystitis⁴¹ with potential risk factors of surgery; trauma; infection; thermal burn; parenteral nutrition; biliary stasis; and gallbladder ischaemia⁴¹⁻⁴³.

In more than 90% of cholecystitis cases, there is an obstruction of the cystic duct or the neck of the gallbladder by gallstones resulting in increased gallbladder pressure^{15,36}. Thereafter, the progression to cholecystitis depends on two factors – the degree and duration of the obstruction. See **Figure 3**. If the obstruction is complete and for a long duration, the patient develops acute cholecystitis; otherwise the patient experiences ‘biliary colic’. About 20% of patients with biliary colic will progress to develop cholecystitis if left untreated^{44,45}. Patients with acute cholecystitis normally experience pain for over 24 hours with systemic upset (pyrexia, tachycardia), have deranged inflammatory markers (white cell count (WCC) and/or C-reactive protein (CRP)), and on USS imaging demonstrate an oedematous thick-walled gallbladder, often with a gallstone stuck in the gallbladder neck or cystic duct^{33,46}. LFTs are commonly within the normal limits unless the gallstone is compressing the CBD or common hepatic duct resulting in a biliary obstruction (Mirizzi syndrome). The mortality rate of acute cholecystitis is about 1%⁴⁶.

The most severe form of cholecystitis is gangrenous cholecystitis. It can be defined as acute cholecystitis with transmural inflammation, loss of mucosa and necrosis of the gallbladder wall, which is primarily due to compromise of a terminal blood vessel (the cholecystic artery), leading to ischaemia and hypoxia of the gallbladder^{47,48}. Gangrenous cholecystitis affects 2-36% of patients with cholecystitis and the mortality rate of this complication is 15-50% higher than patients with other forms of cholecystitis⁴⁹⁻⁵⁶. Hence, early diagnosis is important in order to minimise the morbidity and mortality associated with further complications of the disease. Although medical imaging modalities are useful in distinguishing gangrenous cholecystitis from other less severe forms of cholecystitis, the preoperative diagnosis remains challenging as clinical and laboratory presentations are not specific^{53,57}.

More advanced potentially fatal complications include gallbladder perforation, abscess formation, biliary peritonitis, pericholecystic abscess and biliary fistula^{40,58}.

1.1.5b GDS: Background of Choledocholithiasis and Cholangitis

CBD stones or choledocholithiasis occurs in 10-15%⁵⁹ of patients with cholelithiasis and the incidence is higher in patients with cholecystitis (up to 20%)⁶⁰. When gallstones are passing along the CBD it can result in a non-obstructive choledocholithiasis, or a biliary obstruction resulting in an increase in intraductal pressure. This pressure, if unrelieved iatrogenically or naturally by passing through the CBD on its own, can be high enough to cause cholangiovenous or cholangiolymphatic reflux, ultimately resulting in a systemic inflammatory response syndrome (SIRS) and/or cholangitis⁶¹. The onset of acute cholangitis involves infection in the bile duct which may lead to more fatal conditions such as hepatic abscess and sepsis⁴⁶. Therefore, 'choledocholithiasis' could be 'aseptic' (with or without minimal inflammation), or infected with or without severe inflammation in the case of 'cholangitis'.

Patients with choledocholithiasis and cholangitis normally have a variable duration of abdominal pain with systemic upset, deranged LFTs and have dilated biliary tree on ultrasound¹. The main cause of the deranged LFTs from this biliary obstructive disease is the resultant increased hepatocellular membrane permeability from the increased intraductal pressure, the increase in amino acid transcription enzymes and subsequent hepatocellular toxicity of the retained bile acids^{62,63}. One of the diagnostic criteria for acute cholangitis is the presence of Charcot's triad – RUQ abdominal pain, fevers or rigors, and jaundice^{61,64}. It is also essential to identify the presence of concurrent acute cholecystitis as there are several management options to manage both diseases⁶⁵. The proportion of cases of cholangitis that becomes severe is 12.3%⁴⁶. It can occasionally be life threatening as the acute disease inadvertently can lead to septic shock, organ failure and disseminated intravascular coagulation (DIC)⁴⁶. The mortality rate is about 2.7-10%^{46,66,67}.

1.1.5c GDS: Background of Gallstone Pancreatitis

Gallstone pathology is the most common cause of acute pancreatitis in the Western countries⁶⁸. In up to 40% of all gallstone related cases, pancreatitis is the first manifestation⁶⁹. It is still unclear as to why certain gallstone cases cause pancreatitis but it has been associated

with small gallstones, excess cholesterol crystals and good gallbladder emptying which promotes gallstone migration to the bile ducts^{70,71}. Thereafter, bile reflux into the pancreatic duct, pancreatic duct hypertension or functional obstruction at the sphincter of Oddi could induce pancreatic duct injury. Subsequently, a cascade of activated pancreatic enzymes are released into the glandular interstitium which triggers cytokine release, resulting in pancreatitis⁶⁹.

These patients normally present with epigastric or RUQ pain that radiates to the back (in 50% of cases)⁷², has systemic upset, raised serum amylase or lipase, and may have deranged LFTs and inflammatory markers. Gallstone pancreatitis can normally be diagnosed with acute upper abdominal pain and hyperamylasaemia of more than 3 times the upper limit of normal, with gallstones and/or dilated bile ducts on USS^{68,69}. Serum amylase is elevated in at least 75% of cases and it remains elevated for 5-10 days⁷³. The severity of the disease can be predicted by a prognostic scoring system such as the Modified Glasgow, or Acute Physiology and Chronic Health Evaluation II (APACHE II)⁷⁴. Severe pancreatitis occurs in 20% of cases⁶⁸ and complications that can be associated with this disease include early renal or pulmonary failure, and later infected pancreatic necrosis which occurs in about 15-25% of patients⁷⁵. Hence, patients normally require a Computed Tomography (CT) imaging between the third and tenth day of admission to determine the presence of pancreatic necrosis¹⁵. The reported mortality rate of gallstone pancreatitis is about 2-17%⁶⁹, and up to 40% with acute necrotising pancreatitis⁶⁹.

1.1.6 Management and Evidence-based Medicine (EBM)

The majority of operations carried out on the gallbladder (cholecystectomy) are now with the laparoscopic technique. This technique was first carried out in 1985 by Mühe⁷⁶ and since then there has been a large increase in the number of cholecystectomies carried out worldwide. This less invasive technique generates better aesthetic results with lower surgical risk and postsurgical complications, and shorter postsurgical recovery period compared to the conventional procedure^{76,77}.

1.1.6a Management and EBM of Biliary Colic and Cholecystitis

According to the ASGBI commissioning guide¹, patients with suspected biliary colic are suitable for early discharge or ambulatory care if the inflammatory markers are not

deranged. Patients with biliary colic who are medically fit can be offered an elective laparoscopic cholecystectomy, ideally within 6 weeks¹.

Patients with evidence of acute cholecystitis on imaging investigation should be admitted to have fluid resuscitation, antibiotics and analgesia. These patients can be treated conservatively followed by elective cholecystectomy or by early cholecystectomy during their index admission especially if the pain onset is of less than 5 days. However, surgeons should take into account that about 10% of patients being managed conservatively will not settle and will eventually require a cholecystectomy during that episode. Several independent studies have highlighted the clinical and prognostic importance of early surgical intervention in patients with cholecystitis, and also the associated benefits to the healthcare service and expenditure. Early cholecystectomy during the patients' index admission avoids 10-15% of patients being re-admitted with an acute biliary disease, and it has been shown to be safe and cost effective¹. Recent studies^{78,79} in patients with acute cholecystitis have shown that early surgery (within 24 hours of admission) was associated with shorter total hospital stay, less total costs and a better quality of life with no significant impact on morbidity and mortality⁸⁰⁻⁸³ compared to delayed surgery (after 6-8 weeks of conservative treatment). The associated shorter hospital stay for each patient also means that more acute surgical beds can be created for other acute patients. Moreover, early laparoscopic cholecystectomy reduces the risk of recurrent cholecystitis that occurs in the 10-15% of patients¹. These patients with recurrent acute symptoms would then require an emergency cholecystectomy, which is incurred with an increased risk of conversion to an open procedure⁸⁴. When acute cholecystitis is compared to chronic cholecystitis, it is thought that the oedematous inflammation in acute cholecystitis creates a plane around the gallbladder, thus facilitating its dissection from the surrounding structures. The advancing maturation of the inflammation and organisation of the adhesions which result in scarring and contraction, may later create challenges during a delayed cholecystectomy⁷⁸.

Among the elderly population, cholelithiasis is the most common indication for abdominal surgery with a prevalence of 27.5% in individuals over 70 years old⁸⁵. Laparoscopic cholecystectomy should also be the gold standard approach for acute cholecystitis even in the extremely elderly patients (>80 years) as this has been shown to be safe and feasible⁸⁶. Studies within the elderly population are of relevance as global life expectancy is continuing to increase and therefore, so will the prevalence of gallstones⁸⁶.

1.1.6b Management and EBM of Choledocholithiasis and Cholangitis

In general, stable patients with choledocholithiasis can be managed as an outpatient and/or day case, whereas patients with cholangitis should be admitted for antibiotics, analgesia and fluids. Patients with temporarily deranged LFTs with no biliary dilatation on imaging most likely indicates a passed choledocholithiasis. These patients are suitable for early inpatient cholecystectomy or delayed elective cholecystectomy, ideally within 6 weeks of hospital discharge. Patients with biliary dilatation on ultrasound should undergo MRCP to determine the presence of choledocholithiasis, which frequently is associated with cholelithiasis. If choledocholithiasis is confirmed, and the deranged LFTs and abdominal pain are resolving, the patient can be discharged with outpatient ERCP prior to cholecystectomy, or cholecystectomy with intraoperative CBD exploration, depending on the expertise available. However, if the patient's abdominal pain persists and LFTs are deteriorating, this may indicate that the patient is at high risk of developing cholangitis; or if clinical observations and infection markers are deranged, confirm cholangitis. Hence, these patients should be managed as an inpatient with urgent ERCP and/or later inpatient or elective cholecystectomy¹. Occasionally, severely septic patients with cholangitis will require urgent biliary drainage either by ERCP or percutaneous transhepatic cholangiography (PTC)¹. In this study, patients with choledocholithiasis have been subdivided into those who were clinically aseptic, and those with cholangitis.

1.1.6c Management and EBM of Gallstone Pancreatitis

Patients with this potentially fatal disease should be admitted, resuscitated and be considered for management in a critical care setting depending on the severity of the disease. Management is mainly conservative and supportive. Patients with mild gallstone pancreatitis should be considered for early ERCP if there is concomitant biliary obstruction or cholangitis, and early cholecystectomy, if indicated. There is currently no evidence to support the surgical approach in severe gallstone pancreatitis^{87,88}.

1.1.7 Healthcare Expenses in Gallstone Diseases

Gallstone related diseases accounts for a large expenditure within the United Kingdom's National Health Service (NHS) – approximately 60,000 cholecystectomies are performed each year⁸⁹. The incidence of gallstone diseases is also likely to increase over the

next few years due to the increasing global life expectancy and obesity rates, amongst several known risk factors for the development of cholelithiasis⁷⁷. The overall cost from patient referral to the General Surgeons, up to point of hospital discharge is about £4,697 ($\pm$ £2,007) per patient, ranging from £3,406 to £12,011⁹⁰. For patients who undergo surgeries, the main modifiable difference in cost per patient episode is largely the inpatient cost and not the cost of the surgery or the imaging investigations carried out⁹⁰. Hence, changes in practice that can reduce the duration of inpatient stay could therefore also reduce the overall NHS expenditure in patients who could be managed as an outpatient and/or day case.

1.1.8 Pitfalls of Current Diagnostic Modalities for Gallstone Diseases

Acute gallstone diseases are usually diagnosed in the emergency setting where the only available data is limited to clinical history, laboratory data, and/or USS findings⁹¹. As the demographics of the population is slowly changing, so is the prevalence of the disease as well as the challenges that are associated in managing the disease. Clinical signs and laboratory investigations are diagnostically helpful but not definitive in diagnosing complications of gallstone disease⁹². The diagnosis of gallstone diseases is especially challenging in the elderly population as they often present as complicated diseases with atypical symptoms⁹³, for example, abdominal pain may be absent⁶⁹. Furthermore, with the prevalence of obesity exponentially rising worldwide, there is increasing challenges in obtaining clear diagnostic value from medical imaging modalities⁹⁴. Nevertheless, the diagnosis of gallstone complications is important in deciding the need for urgent surgical treatment to avoid further morbidities as earlier discussed⁹⁵⁻⁹⁸.

Normally, more advanced imaging modalities such as Computed Tomography (CT), MRCP and ERCP are carried out if non-invasive simple transabdominal USS findings are inconclusive. MRCP, although safe as it is non-invasive, not requiring an intravenous contrast agent or ionising radiation, is however contraindicated in severely obese, claustrophobic and/or patients with implanted magnetic devices⁹⁹. On the other hand, ERCP is invasive, provides little information about other solid organs of the abdomen, involves ionising radiation and can be associated with post-ERCP complications such as pancreatitis, perforation, bleeding, cholangitis, sepsis, and death¹⁰⁰. It is associated with a morbidity rate of 3% and mortality rate of 0.2%¹⁰¹. Overall, although inflammatory markers, LFTs and transabdominal USS are the standard investigations for gallstone diseases, they are not without their limitations.

1.1.8a Diagnostic Pitfalls for Cholecystitis

Clinically, only about 32% to 53% of patients with cholecystitis have fever on presentation. Not only is leukocyte count of little value in discriminating severe from non-severe cholecystitis⁹¹, leucocytosis is only present in about 51% to 53% of patients with cholecystitis^{102,103}. Deranged LFTs in acute cholecystitis, without CBD stones, have also been shown to not only be associated with the hepatocellular injury from the severe inflammation of the gallbladder, but also the presence of fatty liver and its severity in radiological findings¹⁰⁴⁻¹⁰⁶. Conversely, more than 97% of patients undergoing laparoscopic cholecystectomy have normal liver function tests¹⁰⁷. Overall, the use of USS is an accurate test for cholelithiasis but has a high rate of false negative values for acute cholecystitis - positive predictive values (PPV) of 37%-88% and negative predictive values (NPV) of 38%-86%¹⁰⁸⁻¹¹². The additional use of CT with USS has been shown not to improve the NPV¹¹³. The sensitivity of USS in diagnosing acute cholecystitis is about 80%¹¹⁴.

It is critical to diagnose the more severe form of cholecystitis, gangrenous cholecystitis, during its earlier phase as this disease is associated with increased morbidity and mortality. Although variables of blood White Cell Count (WCC), C-reactive protein (CRP) and albumin, USS findings of thick walled gallbladder, and age have all been shown to be potential diagnostic components for gangrenous cholecystitis, only CRP has been shown to be significant in predicting this disease – with a NPV of 100% when CRP cut-off is 200¹¹⁵. Nevertheless, other studies have only shown limited data on this^{51,116}. The only laboratory test that has consistently been reported to be associated with this disease is a raised WCC of $14 \times 10^9/\text{L}$ or greater, which is caused by the severe inflammatory reaction from the necrotic changes rather than an infection^{50,53,55,117}. Further challenges in differentiating this life-threatening complication from uncomplicated acute cholecystitis is that there are currently no pathologic or ultrasonographic criteria⁵⁵. Current studies so far have not been able to consistently demonstrate ultrasonography findings that are associated with gangrenous cholecystitis^{50,53,58,118}.

1.1.8b Diagnostic Pitfalls for Choledocholithiasis and Cholangitis

Early diagnosis and immediate treatment is undoubtedly important in preventing morbidity and mortality associated with acute cholangitis. However, only between 42%-70% of patients with cholangitis present with the Charcot's triad^{52,53}. Moreover, in the presence of

SIRS or sepsis, the diagnosis of acute cholangitis can be missed in up to 25% of cases especially when signs and symptoms are not specific¹¹⁹. Currently available LFTs have the most utility in excluding choledocholithiasis pathology⁸⁶. Nevertheless, the PPV of abnormal LFTs has been reported to be only about 15-50%^{106,120,121}. False positive factors include Gilbert's Syndrome, spontaneous passage of choledocholithiasis, and general illness. Partial obstruction of the CBD by a gallstone is a false negative factor. These factors limit the use of LFTs as predictors for choledocholithiasis¹⁰³. Another challenge that clinicians face is to determine the presence of choledocholithiasis in patients with cholecystitis and deranged LFTs since only about 42% of such cases have choledocholithiasis¹²².

The 'normal' CBD diameter is about 3 to 6 mm. A CBD dilatation of greater than 8 mm on imaging usually indicates biliary obstruction^{106,123}. However, the upper normal limit of the CBD normally increases with age¹²⁴, and this poses more challenges in diagnosing choledocholithiasis, especially in the elderly. Depending on the experience of the investigator and gallstone size, bile duct stones are often difficult to be visualised on USS due to the air-containing intestinal loops in front of it (low sensitivity 27-43% and high specificity 99-100%)¹²⁵. The prevalence of isolated CBD dilatation on an USS carried out in acute presentations of cholecystitis and choledocholithiasis is <1%¹²⁶ since both can manifest clinically prior to biliary dilatation¹²⁷. Small stones of <8mm in size can pass through the duodenal papilla¹²⁸ which may also contribute to non-dilated bile ducts. In the early stages of biliary obstruction there may be disproportionate increases in the different LFT components. The clinical diagnosis may therefore be confused with viral hepatitis and this may delay the appropriate management of patients⁶⁸.

Other diagnostic modalities have better sensitivities compared to USS: CT scan (65-88%) and MRCP (85-92%)^{106,129,130}. However, MRCP's sensitivity is lower if the stones are small in size (<5mm) and/or bile ducts are strongly dilated^{68,131}; but MRCP has a good specificity of 93%-95% in detecting choledocholithiasis¹³²⁻¹³⁴. Nevertheless, the waiting time for obtaining an MRCP investigation is likely to delay patient care by about 2.9 days⁶⁵. A randomised trial carried out by Nathanson et al¹³⁵ demonstrated that 20-60% of patients who underwent ERCP had no visible stones but were still exposed to the risks associated with ERCP.

1.1.8c Diagnostic Pitfalls for Gallstone Pancreatitis

In some pancreatitis patients, LFTs may be normal even in the presence of gallstones. In the Western world, with a prevalence of gallstones of 10% in the general population and as high as 40% in the elderly, the mere presence of gallstones does not necessarily prove that the pancreatitis is of biliary origin⁶⁸. This is of particular interest as elderly people have been shown to respond poorly to this acute insult¹³⁶. A serum ALT of more than 150 U/L is only 48% sensitive for diagnosing gallstone pancreatitis⁷¹. Both amylase and lipase lack specificity for pancreatitis, albeit lipase is more specific, as they can also be elevated in other disorders such as renal failure, intestinal obstruction and perforated ulcer, to name a few^{137,138}. Although lipase is more specific than amylase, the latter remains the first line of investigation as lipase is more expensive¹³⁹.

During an acute pancreatitis attack, USS sensitivity can be reduced to as low as 60%^{140,141} due to increased dilatation of bowel loops from ileus (compared to the USS sensitivity of 90-95% when carried out in patients without pancreatitis)⁶⁸. USS may also fail to detect stones that are smaller than 4mm; small stones are a known risk factor for gallstone pancreatitis¹⁴². CT scans can also be normal in 15-20% of patients with mild pancreatitis¹⁴³.

1.1.9 The Need for Novel Biomarkers

In summary, stable patients with uncomplicated biliary colic and aseptic choledocholithiasis may be safely discharged without the need for inpatient management and be offered an outpatient procedure or day case surgery. Conversely, patients with complications from gallstones such as cholecystitis, cholangitis and gallstone pancreatitis should be managed as an inpatient with antibiotics and urgent cholecystectomy and/or other suitable investigations and procedures. However, currently available laboratory blood tests are limited in sensitivity and specificity in discriminating biliary colic and aseptic choledocholithiasis from other complications of gallstone disease needing an acute hospital admission. As a result of this, most patients have to be admitted for further assessment and investigations. Although imaging modalities have a relatively acceptable sensitivity and specificity, they are relatively costly, less readily available, and have associated complications especially if they are invasive. Furthermore, obtaining imaging investigations often takes time and also in getting the results reported. This may mean a delay in the accurate diagnosis and management of the patient's disease. With a more sensitive and specific novel biomarker for

the disease spectrum associated with gallstones, not only can the earlier delivery of essential directive treatment be achieved, but there is also a potential to create more acute beds within the healthcare system for clinically more appropriate patients. Over the past few years, microRNAs (miRNAs) have been emerging as potential novel biomarkers and independent studies in different diseases have shown promising results in using miRNAs as potential diagnostic, prognostic and therapeutic tools. However, these molecules are still not fully understood especially in their association with gallstone related diseases. This study hence investigated the potential of biomarkers for early detection of gallstone complications with an aim to enhance patient management.

1.2 MICRORNA (miRNA)

1.2.1 Background

MicroRNAs (miRNAs) are single-stranded, endogenous, small non-protein-coding ribonucleic acids (RNAs) that are approximately 18-25 nucleotides (nt) in length. They function by negatively regulating gene expression post-transcriptionally through RNA interference^{144,145}. Their genes correspond to 1-3% of all genes of the genome¹⁴⁶. These small molecules can cause either messenger RNA degradation by binding with complete complementarity to its target mature RNA (mRNA), or inhibit translation by binding to their targets with incomplete complementarity, often in the 3' untranslated region (UTR) of the mRNA¹⁴⁷. The first miRs, lin-4, were first discovered in the worm *Caenorhabditis elegans* in 1993 by Ambros' group and later on, let-7, by Ruvkuv's group in 2000^{148,149}. miRNAs were later found to be evolutionarily conserved^{148,150}. miRNAs have been identified in 206 organisms, ranging from microbes to the animal species including humans¹⁵¹. Until relatively recently, little was known about these tiny miRNAs and the significant roles they play in human physiologic and pathologic processes. Subsequently, more miRNAs were rapidly being discovered and it was found that miRNA-mediated gene effects occur in both animals and plants¹⁵². Thus far, more than 2,500 miRNAs have been identified in humans¹⁵¹.

1.2.2 Biogenesis and Maturation of miRNAs

miRNA genes can be found throughout the genome with their own promoter region, or can be located within introns of premature miRNAs, or in exons of non-coding RNAs^{153,154}.

Current theories for miRNA transcription and processing suggest that the compartmentalized stepwise process first occurs in the nucleus and thereafter in the cytoplasm. See **Figure 4**. When in the nucleus, the majority of intergenic miRNAs are transcribed as longer primary transcripts, primary miRNA (Pri-miRNAs), or gene hosts by RNA polymerase (Pol) II. Some interspersed repetitive sequence-derived miRNA genes are transcribed by Pol III. Pri-miRNAs have a hairpin structure containing a 5' methyl guanosine cap and 3' polyadenylated tail. The pri-miRNAs are then processed by enzyme Drosha (ribonuclease, RNase III) that works in concert with protein complex Di George syndrome critical region 8 (DGCR 8). The Drosha/DGCR8 complex cleaves the pri-miRNA at precisely 11 base pairs corresponding to one turn of a double stranded RNA helix from the base of the double-stranded RNA loop and RNA stem's junction. This process produces a double stranded hairpin structure with an asymmetric 3' overhang that is about 60-70 nt long precursor called the pre-mature miRNA (pre-miRNA). Pre-miRNAs are later exported out of the nucleus via a Ran-dependent 4 enzyme, Exportin 5, into the cytoplasm. The Exportin 5 does so by recognising the pre-miRNA's double stranded loop and 3' overhang^{145,153,155-157}.

When in the cytoplasm, the pre-miRNAs are cleaved by another RNase enzyme, Dicer. This process produces the mature, approximately 22 nt, RNA duplexes called the miRNAs. The mRNAs are assembled with Argonaute protein (Ago 2) into an RNA-induced silencing complex (RISC), and can then be relocated to the nucleus where they may regulate transcription, or they can be secreted from the cell via different transport mechanisms such as exosomes, microvesicles, lipoproteins and autosomal bodies¹⁵⁸. The RISC then binds to the 3' UTR of the target mRNAs, and depending upon the degree of complementarity, this may result in post-transcriptional inhibition of mRNA expression either by cleavage of the target mRNA or by translational repression. If the miRNA matches the mRNA target with almost a near perfect complementarity, the mRNA will be cleaved; but in majority of the cases, the complementarity is not perfect and translational repression of mRNA occurs. miRNAs have also been shown to regulate their targets by binding to the 5' UTR, and that target-interactions may also stimulate the expression of target genes^{144,155,156,158}.

1.2.3 miRNAs and Disease Biology

miRNAs vary greatly in their expression levels and patterns during development. A single miRNA can have several target genes since the minimal requirement of pairing consists of about 7 nt within the 5' proximal part of the miRNA¹⁵⁹. This means that when miRNAs are

altered they can result in profound effects within a cell. Conversely, a single gene can be targeted by multiple miRNAs, implying that it may be safeguarded against alteration in the expression levels of a particular miRNA. There may likely be a delicate balance of miRNA levels and complex interactions among the different types of miRNAs in a cell, tissue or organ level. This balance allows for regulation of essential life processes including cellular differentiation, apoptosis, metabolism, immune response, just to name a few^{152,160,161}.

As most miRNAs are tissue specific, it has been suggested that miRNAs may be involved in determining and maintaining tissue identity. It has been demonstrated that alteration in miRNA expression might contribute to disease processes including cancer, genetic and infectious diseases. Some diseases have been shown to be associated with altered enzymes regulating miRNA biogenesis; and some associated with an altered modulation of miRNA expression or genetic alterations of genes, including deletions and single-nucleotide polymorphisms, that encode the miRNAs or their targets. Ultimately, these may lead to the gain or loss of miRNA-target interaction¹⁶²⁻¹⁶⁴. It has been estimated that 30%-60% of genes in humans are targeted by miRNAs^{152,159}. Nevertheless, it is widely known that gene expression is mainly controlled by regulation of transcription factors and epigenetic regulation, and that miRNAs are thought to 'fine-tune' these gene expressions¹⁶⁵.

Some miRNAs have the propensity to target genes with associated functions which can provide an insight into the biological roles of these miRNAs¹⁶⁶. For example, miR-17 to miR-92 cluster tend to target genes involved in growth control¹⁶⁷, and they also exhibit oncogenic properties¹⁶⁸. However, this is not always the case even for those miRNAs with very striking expression specificities as they are not always statistically enriched for specific functions or processes. Nevertheless, most of the broadly conserved miRNAs each represses genes with a wide variety of biological and molecular functions¹⁶⁶.

In the research field, quantification and qualification of miRNA expression is essential for functional studies and diagnostic approaches. However, the assignment of the function of a miRNA-target interaction is challenging. With so many targets for each miRNA, the monitoring of protein changes after miRNA knockout or knockdown cannot be concluded in confidence that indirect effects had not taken place. A strategic approach to this is to disrupt only the interaction of interest and to observe the phenotypic significance of the altered interaction¹⁶⁶. There are several methods to carry this out, for example, by preventing miRNA pairing with the use of antisense reagents that hybridize to the target site within the 3' UTR¹⁶⁹,

by mutating the miRNA sites¹⁷⁰, or by perturbing an endogenous site through homologous recombination¹⁷¹.

1.2.4 Application of miRNAs Clinically

Although the significance of miRNAs are currently not fully understood, these small molecules have recently emerged as promising candidates for biomarker research. This is mainly because of the stability of the miRNAs in biofluids as they are resistant to action of ribonucleases², and their abundance in body fluids reflect their abundance in the abnormal cells causing the disease. Moreover, as some of the miRNAs exhibit tissue-specific distribution^{2,4,165}, they may be regarded as blood-based fingerprints of particular tissues^{172,173}. Hence, both tissue and circulating miRNA expression profiles have the potential to be used as biomarkers for diagnostic, prognostic and therapeutic purposes. See **Figure 5**.

miRNAs were first recognised in patients with cancer¹⁷⁴ and they are currently potential diagnostic and prognostic biomarkers in for example, chronic lymphocytic leukaemia, prostate cancer, breast cancer, non-small cell lung cancer and colorectal cancer¹⁷⁵⁻¹⁷⁸. Studies have also revealed that miRNAs are directly released from damaged cells or through active processes such as apoptosis, for example in myocardial infarction and atherosclerosis^{158,179}. miR-122 inhibitor (miravarsen) and mir-34 mimic (MRX34) are the first miRNA-based molecules to enter clinical trials as therapy against chronic hepatitis C virus infection and liver cancer respectively^{180,181}.

The association between miRNAs and gallstone diseases has not been fully explored. The formation of gallstones, in brief, occurs when the concentration of cholesterol in bile exceeds the ability of bile to hold it in solution form, thus resulting in crystallization and development into gallstones¹⁸². Although the exact role of miRNAs in biliary diseases is still unclear, they may regulate the tightly controlled homeostatic mechanisms that affect bile synthesis and secretion¹⁸³. Indeed, miRNAs, including miR-122,-370 and -33, have been demonstrated to have major influences on cholesterol homeostasis^{144,166,184}, which when impaired contributes to the pathogenesis of gallstones. On the other hand, miR-210 has been shown to be associated with diseases via hypoxia pathways during inflammation¹⁸⁵, and in enzymes that may affect gallbladder motility¹⁸⁶. Other miRNAs, such as miR-190 and -743, have also been shown to be specific for cholestasis of both obstructive and hepatocellular type¹⁸⁷.

Both miR-122 and miR-210 were chosen in this study as both have been demonstrated to strongly correlate with certain LFTs, especially ALT^{4,188}; both miRNAs have previously been studied mainly in animal models with induced cholestasis, and had shown promising results; both miRNAs have previously been demonstrated that they may play important roles in the pathogenesis of gallstones; and both miRNAs, to our best knowledge, have not been studied in humans with the different diseases of the gallstone disease spectrum.

1.2.5 microRNA-122 (miR-122)

1.2.5a Background of miR-122

miR-122 was first discovered by Taylor's team¹⁸⁹ in the late 1980s when they were investigating woodchuck liver tumours. It is the most abundantly expressed miRNA in the liver, with more than 50,000 copies per cell. It constitutes 70% and 52% of the whole hepatic miRNome in the adult mouse and human respectively^{155,190-192}. miR-122 is a conserved liver-specific miRNA and has not been shown to be expressed in other non-liver tissues except the brain and thymus – but with much less expression than that shown in the liver by 185-fold and 332-fold respectively¹⁹³⁻¹⁹⁶. miR-122 plays a central role in liver functions, development, differentiation, and homeostasis. Its expression is driven by liver-enriched transcription factors (LETfs) including hepatocyte nuclear factor 6 and 4a, which have also been shown to fine-tune miR-122 dosage during liver development *in vivo*¹⁹⁷⁻¹⁹⁹. It has been suggested that the expression of both miR-122 and LETfs help regulate the proper balance between cell proliferation and differentiation in both hepatocyte and cholangiocyte lineages^{197,198}, which is of significance as miR-122 promotes hepatobiliary segregation along with the acquisition and maintenance of hepato-specific phenotype^{197,198,200}.

miR-122 participates in the regulation of post-translational gene expression in diverse physiological and pathological processes²⁰¹. Apart from binding to partially complementary sequences in the 3' UTR of target mRNAs, miR-122 may also bind to the 5' UTR of the RNA genome of, for example, hepatitis C virus thus promoting viral replication²⁰². It also plays a role in apoptosis, cell growth, carcinogenesis²⁰³, the regulation of hepatic iron and in cholesterol metabolism²⁰⁴⁻²⁰⁶. Over the past few years, miR-122 has been indicated as a potential novel biomarker for diseases such as acute coronary syndrome²⁰⁷, drug-induced liver injury²⁰⁸, gallbladder²⁰⁹ and hepatocellular carcinoma²¹⁰.

1.2.5b Role of miR-122 in Cholesterol Synthesis and Bile Acid Pool

Bile acids are physiological detergents and signalling molecules. They facilitate the absorption, transportation and distribution of dietary fats, sterols and lipid-soluble vitamins, and the disposal of toxic metabolites and xenobiotics. They also activate cell signalling pathways to regulate lipid, glucose and energy homeostasis²¹¹. Bile, which is secreted by hepatocytes into the canalicular lumen, is constantly being modified during its transit flow along the biliary tree and gallbladder. This involves both absorptive and secretative processes resulting in alkalinisation and fluidization of the bile²¹². Several enzymes and transporters are involved in maintaining the bile acid pool including cholesterol 7 α -hydroxylase (CYP7A1) and canalicular bile salt export pump, both which have protagonist roles²¹³. This process can be impaired by different pathological conditions leading to a reduction in the amount of bile reaching the duodenum (cholestasis), and in severe cases, simultaneous retention of substances that are normally secreted into bile, in the liver and eventually into the blood. Persistent cholestasis (the stopping or slowing of bile flow) ultimately leads to hepatocyte and bile duct epithelial cell injury and inflammation, and later, progression to fibrosis, cirrhosis and death²¹⁴. Mechanical obstructive cholestasis of the intra- or extrahepatic biliary tree and gallbladder is commonly caused by gallstones, whereas hepatocellular cholestasis may be due to impaired secretory mechanisms of hepatocytes and/or cholangiocytes²¹⁵. miR-122 has been reported to directly target human CYP7A1 mRNA⁶ and affect the regulation of hepatic cholesterol and bile acid homeostasis⁷. See **Figure 6**.

Similar reduction in the stability of CYP7A1 mRNA by miR-122 was also demonstrated in a study on human hepatocytes⁶. This effect lead to an inhibition of protein translation and the eventual bile acid synthesis. In this same study⁶, miR-122 antagomirs, which are chemically modified antisense oligonucleotides (ASO) that interfere with miRNA function¹⁹⁴, also stimulated bile acid synthesis thus suggesting that miR-122 may reduce bile acid synthesis and thus may encourage cholesterol accumulation in the gallbladder. Indeed, miR-122 inhibition by an antagomir was associated with more than 30% reduction in cholesterol levels and 40% reduction in triglyceride levels in mice^{194,204}. The mice also exhibited increased hepatic fatty acid oxidation, reduced levels of hepatic fatty acids and reduction in cholesterol synthesis. These were partly due to a reduction in the expression of 3-hydroxy-3-methylglutaryl-CoA-reductase (Hmgcr) which is a rate limiting enzyme in the endogenous biosynthesis of cholesterol¹⁹⁴. However, this is an indirect reaction and the direct

target of miR-122 affecting plasma cholesterol levels remain unclear. Similar results were also obtained in a non-human primate study²⁰⁶ with miR-122 inhibition in absence of apparent liver toxicity, which suggests that firstly, miR-122 inhibition in humans might be feasible; secondly, that other studies on miR-122 in animals may also be inferred in humans; and lastly, further studies with human subjects would be of benefit in further determining miR-122's role. Nevertheless, miR-122's roles in altering the expression factors that regulate cholesterol formation and lithogenic bile secretion suggest that miR-122 may be associated with the pathogenesis of gallstone formation.

1.2.5c Potential Roles of miR-122 in Biliary Diseases

miR-122, also a known circulating biomarker of necrosis²¹⁴, has been demonstrated to act as a tumour suppressor in the hepatocytes of hepatocellular carcinoma tissues^{203,216,217}. Several studies have shown that miR-122 is a more sensitive and specific biomarker than Alanine Aminotransferase (ALT) in several disease pathologies and this is of clinical significance as current screening methods that utilise ALT can have limitations⁴. In patients with non-alcoholic fatty liver disease (NAFLD)²¹⁸, miR-122 was demonstrated to be strongly correlating to ALT activity and was superior to ALT as concentration levels of miR-122 became elevated earlier in the associated liver injury^{219,220}. Similar findings were also shown in patients with drug-induced liver injury with an earlier rise and with greater increase in miR-122 concentrations when compared to ALT levels. Overall, miR-122 was proven to be more superior than ALT with respect to specificity and sensitivity²²¹ in hepatocellular diseases.

Early studies with cholestasis-induced mice models have demonstrated that miR-122 levels were again elevated in a more responsive manner, with an earlier rise and in higher magnitude compared to circulating ALT^{4,214}. It was indicated that the mode of cell death in bile duct ligated (BDL) rat or mice models were mainly due to oncotic necrosis and not apoptosis^{214,222,223}. BDL caused necrosis by progressively recruiting neutrophils into the liver causing extensive oxidant stress and cell death^{224,225}. ALT levels were shown to rise steadily through the first 24 hours after BDL injury prior to subsiding. Conversely, serum miR-122 levels increased progressively up to 48 hours prior to declining thereafter²¹⁴. This feature of miR-122 may be of clinical benefit especially for patients who do not seek medical attention on initial onset of symptoms.

Subsequently, a study by Zhang's team⁴ with cholestatic mice models had demonstrated that there was no proven time-dependent change for ALT levels, especially from 3 days after liver injury was induced in the mice, whereas miR-122 showed a significant increase 1 day after liver injury was induced, reaching its peak at day 3 and remaining significantly increased until 14 days after. The time-course between ALT and miR-122 was similar, indicating that miR-122 may be employed as a useful, more diagnostically sensitive biomarker for cholestatic liver injury than ALT. miR-122 levels were also shown to differ significantly between the severity groups of cholestasis. The same study also demonstrated that serum miR-122 levels were significantly higher ($P < 0.001$) in patients with gallstone-induced liver injury compared to healthy controls. Serum miR-122 exhibited significant diagnostic value for patients with gallstones with a 77.4% sensitivity and 96.4% specificity compared to the healthy controls, at a cut-off value of 659.28.

Another study²⁰⁹ carried out in patients with gallbladder carcinoma demonstrated an increase in circulating miR-122 concentration levels compared to healthy human subjects, although this was not statistically significant. As gallstones, chronic cholecystitis and cholangitis are key pathogenic events leading to gallbladder carcinoma, there might be an association between these complicated gallstone diseases and miR-122. In summary, all these studies suggest that miR-122 may play an important role in the pathogenesis of gallstones mainly by affecting bile acid homeostasis. miR-122 could be a more specific and sensitive biomarker than current available blood tests in detecting gallstone complications, such as cholestasis caused by choledocholithiasis, for patients presenting with an acute abdominal pain.

1.2.6 microRNA-210 (miR-210)

1.2.6a Background of miR-210

Over the past few years, independent studies have demonstrated that a specific set of miRNAs are upregulated by hypoxia through hypoxia inducible factors (HIF), and these miRNAs have been termed as 'hypoxamirs'²²⁶⁻²³³. They appear to control a network of processes during hypoxic response²²⁶. miR-210 has been identified as one of the most consistent and robust hypoxamirs in both tumour and normal cells^{234,235}, and is known as the 'master miRNA' of hypoxic response²³⁶. miR-210 is 6-8 nt long²³⁷ and is regulated via the HIF-1 and -2 pathways in response to hypoxia^{238,239}. Its expression is controlled by inflammatory signals that can be immunological or oncological in origin²⁴⁰. miR-210 is

associated with the control of a wide range of cellular responses that influence normal developmental physiology, including metabolism, survival, proliferation, apoptosis, migration and angiogenesis^{237,239,241,242} (See **Figure 7**); and in hypoxia-dependent disease states such as inflammation, tissue ischaemia and solid tumorigenesis¹⁸⁵. The dynamic regulation of miR-210 is linked directly to low oxygen levels rather than other mechanisms that are secondarily associated with hypoxia such as oxidative stress and inflammation¹⁸⁵. The upregulation of miR-210 is dose-dependent with the decline in oxygen levels^{243,244} suggesting that there is a direct dependence on hypoxia for miR-210 expression. It has been established that miR-210 levels are specifically regulated in hypoxia-induced human diseases such as pre-eclampsia^{245,246}.

As hypoxic states can induce angiogenesis, upregulation of miR-210 has been associated with increased angiogenic and invasion potential in hypoxic cells^{247,248}. This is of importance as metastasis was observed more frequently in cancer cells with overexpressed miR-210²⁴⁹. Indeed, miR-210 has been proposed as a novel biomarker for diseases such as breast cancer²²⁸, diffuse B-cell lymphoma²⁵⁰, pancreatic cancer²⁵¹, colorectal cancer²⁵² and glioma²⁵³. As miR-210 is differently expressed in the different neoplasms, it may be cell-type specific^{227,250,254-257}. miR-210 is also associated with other non-malignant hypoxia related states such as in cerebral ischaemia, where miR-210 had been demonstrated to also induce neurogenesis²⁵⁸, and in congestive heart failure²⁵⁹.

1.2.6b Potential Roles of miR-210 in Biliary Diseases

A study on hepatitis B patients demonstrated that both liver and serum miR-210 correlated significantly with ALT and bilirubin levels¹⁸⁸, which suggests that serum miR-210 may serve as a molecular biomarker for liver disease and biliary-related liver injury. In mice models with induced chronic cholestasis, upregulation of miR-210 accelerated the progression of cholangiocarcinoma via the HIF-2 α -miR-210-Mnt pathways⁵. Since chronic cholestasis is an important pathogenic factor for gallstones, this study predicted that miR-210 may play a role in gallstone diseases.

In a study by Yang et al¹⁸⁶, miR-210 expression was significantly increased in the gallbladder mucosa of patients with gallstones compared to patients without gallstones. This study also demonstrated that miR-210 was inversely linked to ATP11A, an ATPase which transports compounds across membranes, which is essential for the maintenance of the

phospholipid asymmetry in lipid bilayers of the membranes^{8,9}. Consequently, this may affect gallbladder mobility¹⁸⁶, another important pathogenic factor for gallstone formation. Furthermore, metabolic pathways, such as bile secretion, were also observed to be enhanced in this study.

In summary, these studies demonstrate miR-210's role in hypoxic inflammatory conditions, and that miR-210 could play a role in gallbladder motility. Like miR-122, miR-210 also has the potential to be a more sensitive biomarker than ALT for cholestasis. Hence, miR-210 could be a diagnostic tool for different gallstone diseases depending on their aetiology.

1.2.7 miR-122 and miR-210 in This Study

A constantly increasing number of studies are suggesting that miRNA profiles from peripheral blood samples can be used as predictive indicators for the development of certain pathologies^{260,261}. This means that miRNAs possess the potential as novel biomarkers to be early diagnostic and prognostic investigative tools for different diseases. Both miR-122 and miR-210 were selected due to their pathophysiological relation to gallstones and/or biliary diseases. We hypothesized that the level of plasma miR-122 and miR-210 may be used to detect gallstone diseases and the complications associated with gallstones. This is of clinical significance especially in patients with gallstone diseases who are presenting with atypical symptoms or with inconclusive laboratory blood results and/or investigations. This study investigated the utility of both miRNAs as a potential novel biomarker for early detection of patients with gallstone complications, with an aim to enhance patient management.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Design and Pilot Study

This ‘proof of concept’ research study will inform the power calculation for a multi-centre qualification study. This study was conducted in a single masked manner such that all miRNA laboratory analyses were carried out blinded to the patients’ clinical data including patients’ diagnoses and hospital laboratory blood results. The study design incorporated an early milestone with a clear go or no go criterion to mitigate risk if there had not been a strong biomarker signal for miR-122 or miR-210. This study has been approved to recruit a total of 300 patients over a period of two years, and this thesis reflects recruitment and analysis of the first year of patient sampling.

In the pilot study, at least a third of the random samples collected underwent analyses for both miRNAs at 6 months after commencement of the study to determine if there was any association between the miRNAs and the different patient groups; and if the miRNAs correlated with patient’s admission ALT and total bilirubin results. Both ALT and total bilirubin levels were chosen for initial analysis in this pilot study as studies^{4,188} have consistently proven their significant correlations with both miRNAs. Thereafter, a decision whether or not to continue analyses of both miRNAs in the main study was made depending on the presence of a significant difference in miRNA expressions between the different patient groups.

The ‘clinical’ aspect of the study was performed in the General Surgery Department at the Royal Infirmary of Edinburgh (RIE), which is under NHS Lothian Trust. Patients were approached while they were in the unit to obtain their informed consent and thereafter a blood sample taken for miRNA analysis if consent was given. The ‘laboratory’ aspect of the study was carried out in the Clinical Research Facility (CRF) in the RIE and the Queen’s Medical Research Institute (QMRI) which is adjacent to the RIE.

2.2 Study Population and Data Collection

Patient recruitment period for the study was for 36 calendar weeks from 3rd September 2014 to 12th May 2015. The study aimed to recruit at least 5 patients per week. Suitable patients were identified from the on-call General Surgical team’s daily handover sheet. Eligible

patients were then approached, given information - both verbally and with a Patient Information Sheet (See **Appendix 1**), and were given adequate time of at least 30 minutes to consider whether or not to participate in the study. Thereafter, the patients were given the opportunity to ask the recruiter any questions that they may have had. Once informed consent had been obtained, patient's demographic information and admission laboratory blood results were recorded. Patient's blood sample was then acquired for the study to analyse miRNAs. Patient information were obtained from the patient's account verbally, from the written medical notes and the electronic national patient record system, the TRAK system. Each patient was then followed up through their electronic record for a minimum of one month. Patients who were waiting for surgery at a later date (longer than a month) were followed up until the end of the study period. Information obtained during the follow up period were all carried out electronically from TRAK. All patients were followed up until 15th June 2015.

The blood samples obtained from the General Surgical patients were collected in a 9 mL ethylenediaminetetraacetic acid (EDTA) tube and transported in an ice box to the CRF to be centrifuged as soon as possible after collection. Samples were centrifuged at 1,000 x g for 15 minutes at 4°C. The plasma layer was then pipetted and stored in aliquots at -80°C in a freezer. Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were carried out in the QMRI at a later date.

2.3 Inclusion and Exclusion Criteria

All adult patients who had been referred to the on-call General Surgeons with acute abdominal pain, irrespective of the characteristics of the pain, and with a differential diagnosis of gallstone disease upon index presentation were included in the study. To be eligible, patients must have presented to hospital within 24 hours prior to the recruitment period. Patients who were unable to give an informed consent, who refuse to participate in the study or who were younger than 16 years of age were excluded from the study. Patients transferred from a hospital out with the NHS regional trust (NHS Lothian) were not eligible as presentation blood results and information for the follow-up period would not be obtainable from the TRAK system. Patients who had been indicated as being unsuitable subjects by the medical or nursing staff in the unit due to patients' clinical condition, and patients who were in high dependency unit (HDU) or intensive care unit (ICU) were also deemed not suitable for participation in the study.

2.4 Variables

The following information were obtained from individual patient's histories: the site of abdominal pain; the estimated time of onset of the abdominal pain; if the patient had a prior past medical history of gallstones or excess alcohol consumption; if the patient had ingested any alcohol 24 hours prior to hospital admission; and if the patient had experienced jaundice over the last month, or rigors over the last 24 hours prior to hospital presentation (**Appendix 2**). The time at which the blood samples were taken for this study, was recorded. Information about alcohol intake was obtained as this may affect LFTs and likely miRNA expression.²⁶² Information on the presence of jaundice and rigors were collected to aid in determining patient's final diagnosis, for example, in cholangitis.

Patient demographics (date of birth and sex) and clinical observations within the first 24 hours of hospital presentation –body temperature, heart rate, and respiratory rate, were obtained from patient's written medical notes (observation charts). These clinical parameters are part of the criteria for systematic inflammatory response syndrome (SIRS) and/or sepsis as defined by the American College of Chest Physicians and Society of Critical Care Medicine (ACCP/SCCM)²⁶³.

Laboratory blood results upon hospital presentation and the peak values were obtained from TRAK. Blood results recorded were white cell count (WCC), C-reactive protein (CRP), creatinine, amylase and liver function tests ((LFTs), comprising total bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (AlkP), and gamma-glutamyl transferase (GGT)). Information on imaging investigations – Ultrasonography (USS), Computed Tomography (CT) and Magnetic Resonance Cholangiopancreatography (MRCP), were obtained from TRAK. The date of cholecystectomy, macroscopic operative findings, pathology examination results of the gallbladder specimen and any microbiology results for blood cultures were obtained electronically.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the imaging modalities were determined in the patients who had underwent an operation, and hence diagnoses were accurately based on histological and/or operative findings. The presence of cholesterosis, or features of gangrene or necrosis in gallbladder specimens were also recorded from histopathological findings, if available. Information on the presence of fatty liver was obtained electronically from either operative findings (macroscopic

appearance) or by imaging findings during that hospital episode and/or from previous investigations. Cholesterolosis and fatty liver were recorded as miR-122 is known to be associated with cholesterol metabolism⁶, and miR-122 expression has been shown to be affected by fatty liver diseases²¹⁸; whereas presence of gangrenous or necrotic gallbladder specimens was recorded as miR-122 is associated with necroinflammation²⁶⁴, and miR-210 with hypoxic conditions²²⁶.

2.5 Definitions

The duration of pain, in hours, was calculated from the onset of the abdominal pain to time the patient's blood sample was obtained for this study. 'Peak' laboratory blood results – WCC, CRP creatinine, amylase and LFTs, were defined as the highest value for each variable during the patient's hospital episode for that index presentation. A 'hospital episode' was that defined by the TRAK system – patients who had been listed as 'Discharged' from the General Surgeons were considered to have reached their endpoint of that hospital episode and was not further followed up; patients who were still being seen as an outpatient for further management of their index presenting symptom(s) were easily distinguishable on TRAK and these patients were remotely followed up until they were discharged from the General Surgeons and/or the end of this study.

The final diagnosis of the patients were determined by histopathology results, Surgeon's operation findings, imaging investigations and/or discharge scripts, following that order. Patients with a 'NSAP' diagnosis had normal investigation results and the cause of their abdominal pain was not found, irrespective of their blood results. Patients with gallstone diseases were grouped into 'Uncomplicated' and 'Complicated' cases according to their clinical management, with respect to the need for urgent inpatient assessment and treatment. Patients with an 'Uncomplicated' gallstone disease had either biliary colic (cholelithiasis in gallbladder) or aseptic choledocholithiasis (choledocholithiasis without clinical or microbiological evidence of severe inflammation or sepsis). These subgroup of patients could be managed as an outpatient and/or a day case procedure¹. Patients with a 'Complicated' gallstone disease were patients with cholangitis (septic choledocholithiasis), cholecystitis or gallstone pancreatitis. These group of patients should be managed as an inpatient due to the associated morbidity and mortality¹. Patients with gallstones can have an overlap of different gallstone diseases. For such patients in this study, their diagnosis for allocation into patient group was based on the diagnosis of their acute index presentation as this would most likely

represent the concentration of plasma miRNA (miRNA blood samples were obtained within 24 hours of hospital presentation). ‘Gallstone pancreatitis’ was determined by the standard amylase rise of at least 3 times the upper normal limit^{69,265}.

Patient’s presentation to hospital with an evidence of SIRS was defined according to the ACCP/SCCM’s definition for SIRS²⁶³ - at least two of the following recorded observations within 24 hours since index presentation: temperature less than 36°C or more than 38°C; heart rate of more than 90 beats per minute; respiratory rate of more than 20 breaths per min; or WCC less than $4 \times 10^9/L$ or more than $12 \times 10^9/L$.

2.6 Total RNA Extraction

The stored frozen plasma samples were first fully defrosted at room temperature. miRNA was then extracted from the plasma using the miRNeasy Serum/Plasma Kit (Qiagen, Venlo, Netherlands) according to manufacturer’s protocol (**Appendix 3**)²⁶⁶. 50 μL of plasma sample was diluted in 150 μL of RNase-free water, followed by addition of 1 mL of QiAzol Lysis Reagent (Qiagen). The solution was then vortexed and incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Thereafter, 3.5 μL of synthetic miR-39 (at 1.6×10^8 copies/ μL) was added as a Spike-in control. miR-39 is expressed in *C. elegans* but not in humans and it was used to normalise for differences in extraction efficiency between samples²⁶⁷. After the addition of 200 μL of chloroform and 15 minutes of centrifugation at 4°C, the upper aqueous phase was added with 100% ethanol in a MinElute spin column (Qiagen, Venlo, Netherlands). Subsequently, on column washing steps, with intermittent centrifugation, were performed with RWT and RPE Buffers. The total RNA containing the miRNA fraction was then eluted in 15 μL RNase-free water.

2.7 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

miRNAs were measured with Taqman-based quantitative PCR. Small RNA elutes were reverse transcribed using specific stem-loop reverse-transcription RT primers (Applied Biosystems, Foster City, CA, USA) for each target miRNA species, following the manufacturer’s instructions (**Appendix 4**)²⁶⁸. The stem-loop qRT-PCR assay is highly specific, as it is able to distinguish homologous miRNA sequences, and it is highly sensitive: it has a wide linear dynamic range of over 7 logs with detection limits in the lower picogram range²⁶⁹. Briefly, TaqMan MicroRNA Reverse Transcription Kit was used in the reverse transcription

reaction to detect mature single strand miRNAs²⁷⁰⁻²⁷². The RT reactions were performed in a total volume of 7.5 μ L, each containing 1 μ L of RNA eluate. The resulting first strand cDNA was subsequently used for qRT-PCR. No template controls (NTCs) were included to test for miRNA contamination.

At the qPCR amplification stage, master mixes with specific primers (Applied Biosystems, Foster City, CA) for each miRNA were prepared and volumes of 9 μ L were added to a 384-well PCR plate in duplicates for each sample. Subsequently 1 μ L of first strand cDNA product from each sample including NTCs were added, resulting in a total reaction volume of 10 μ L. The plates were run on a Light Cycler 480 (Roche, Basel, Switzerland). Cycle threshold (ct) values were determined using the fluorescent signal produced from the TaqMan probes. The relative expressions of miR-39, miR-122 and miR-210 were analysed using the standard $2^{-\text{dct}}$ method²⁷³. The quantities of miR-122 and miR-210 were normalized to the Spike-in control, miR-39.

2.8 Statistical analysis

Continuous variables are presented as medians and interquartile ranges (IQR). Categorical variables are presented as frequencies. The Pearson Chi-squared test was used to examine the association between categorical variables. The Kolmogorov-Smirnov test was used to compare the distribution of non-parametric data against the Normal distribution. The Kruskal-Wallis test was used to compare non-parametric variables and later, the Mann-Whitney U test was utilised if a significant difference was present, to analyse between-group (2 x 2 contingency) comparisons. The sensitivity and specificity for imaging and laboratory blood investigations used in this study were calculated. 95% confidence intervals (CI) were calculated for all continuous variables.

miRNA expression data analysis was performed according to the $2^{-\text{dct}}$ method. The Kruskal-Wallis test was used to find the differentially expressed concentrations of miRNAs between the groups of patients. The focus on using the miRNAs as a potential diagnostic biomarker for complicated gallstone disease utilised PPV as the most relevant measure of accuracy. NPV was calculated and implicated as the most appropriate measure for predicting the accuracy of the miRNAs as an exclusion marker for complicated gallstone disease. Sensitivity and specificity of the miRNAs were also calculated. Receiver operating characteristic (ROC) curve analysis of miRNAs were plotted for the different patient groups

to provide a robust test of sensitivity and specificity. The area under the ROC curves were calculated with 95% CI, and were compared between the indicated groups. Sensitivity, PPV and NPV of miRNAs of interest were obtained at 90% specificity. For correlation analysis, Spearman's Rank Correlation test was utilised for non-parametric variables, with correlation coefficient (r) and 95% CI for comparison. All statistical tests were based on a two-sided α -value of 0.05. Statistical analysis was performed using IBM SPSS Statistics Version 19.0 (IBM Corp., Armonk, NY, USA), and G*Power 3.1 (Universität Düsseldorf, Germany). Figures were designed, and correlation analyses were carried out using GraphPad Prism Version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER 3: RESULTS

3.1 Pilot Study

As part of the study's protocol, a third of random samples underwent miRNA analyses after 6 months of patient recruitment. Eighty-two samples were chosen blinded to clinical data. **Table 1(a)** shows the demographics of the patients in this pilot study. The variables in this study did not have a normal distribution (Kolmogorov-Smirnov test $P < 0.05$). There was a significant difference in gender distribution between the four patient groups (Chi-squared test, 2 d.f., $P = 0.003$). This significant difference was also seen between patients with NSAP and both, complicated gallstone disease and other diseases in 2x2 contingency tables (Chi-squared test, 1 d.f., $P = 0.002$ and $P = 0.007$ respectively); and also between patients with uncomplicated gallstone disease and both, complicated and other diseases ($P = 0.014$ and $P = 0.044$ respectively). Patients with NSAP were also significantly younger than patients with complicated gallstone disease (Median: 37.5 years [IQR: 26.5-60.7 years] versus Median: 55.1 years [IQR: 44.8-68.5 years]; Mann Whitney U test, 1 d.f., $P = 0.020$). Similar significant difference in age was also seen between patients with uncomplicated and complicated gallstone disease (Median: 46.4 years [IQR: 27.9-52.7 years] vs. Median: 55.1 years [IQR: 44.8-68.5 years]; $P = 0.011$). In the 82 samples, the median duration from the onset of patients' abdominal pain to the time miRNA blood samples were obtained was 72.0 hours (IQR: 27.8-120.0 hours) [95% CI: 48.0 , 96.0].

Total bilirubin levels were significantly different between patients with NSAP and both uncomplicated and complicated gallstone diseases (Mann Whitney-U test, 1 d.f., $P = 0.004$ and $P = 0.009$ respectively). ALT levels also showed similar significant differences between NSAP and rest of the three remaining groups (Vs. Uncomplicated, $P = 0.001$; Complicated, $P < 0.001$; and Other, $P < 0.001$). Interestingly, there was no significant difference in bilirubin and ALT levels between patients with uncomplicated and complicated gallstone diseases ($P = 0.569$ and $P = 0.453$ respectively) in this pilot study. There was a significant difference in miR-122 concentration between the four patient groups (Kruskal-Wallis test, 2 d.f., $P = 0.001$). See **Table 1(b)** and **Figure 8**. This significant difference was also seen when patients with NSAP were compared to patients with uncomplicated and complicated gallstone disease (Mann Whitney-U test, 1 d.f., $P < 0.001$ and $P = 0.003$ respectively); and when patients with uncomplicated gallstone disease were compared with those with an other non-gallstone disease ($P = 0.012$). Correlation of miR-122 levels with total bilirubin and ALT levels are illustrated in

Table 1(c). miR-122 had a stronger correlation with ALT compared to total bilirubin levels. (Spearman's r value: 0.640 [95% CI: 0.487-0.756; $P < 0.0001$]; vs. Spearman's r value: 0.342 [95% CI: 0.128-0.525]; $P = 0.0017$).

However, there was no significant difference in miR-210 levels between the four different patient groups (Kruskal-Wallis test, 2 d.f., $P = 0.365$) as shown in **Table 1(b)**. **Figure 9** clearly depicts the comparable median values of miR-210 between the four groups. Comparison of miR-210 levels between patients with and without gallstones revealed a P value of 0.101 (Gallstones: Median: 0.0024 [IQR: 0.0016-0.0036] vs. Non-gallstones: Median: 0.0020 [IQR: 0.0015-0.0025]). Correlation of miR-210 with total bilirubin and ALT levels are illustrated in **Table 1(c)**. Of the 82 patients, thirty-eight patients (46.3%) underwent cholecystectomy, of which 9 gallbladder specimens (23.7%) revealed necrotic and/or gangrenous pathology. However, there was no significant difference in plasma miR-210 levels between patients with necrotic and/or gangrenous gallbladder specimens ($n = 9$) and the remaining gallbladder specimens ($n = 29$) (Mann-Whitney U test, 1.d.f., $P = 0.279$). Following these insignificant findings with miR-210 between the different disease pathologies, further analyses of plasma samples for miR-210 expression were halted, and this study only focused on the analysis of miR-122 and its relationship with gallstone-related diseases.

3.2 Study Population

A total of 237 patients have been recruited to the study throughout the 36 calendar weeks. See **Figure 10**. Five patients were excluded from the study for the following reasons: blood samples were not obtained from two patients as they were transferred out of hospital soon after consenting to participate in the study, one patient was under 16 years of age, one had difficulty during the venepuncture process in obtaining a blood sample and another was transferred from a different hospital more than 24 hours prior recruitment to the study. Excluded patients were informed either verbally or by mail as to why they had to be omitted from the study. So far, there has been no negative feedback or complaints from the patients who had been informed regarding their exclusion from the study.

Of the 232 patients included in the study, 41 (17.7%) patients had 'non-specific' abdominal pain (NSAP), 43 patients (18.5%) had 'uncomplicated' gallstone disease, 100 patients (43.1%) had a 'complicated' gallstone disease and 48 patients (20.7%) had an 'other' non-gallstone related disease. **Figure 10** shows a further breakdown of the different diagnoses

within each patient group and those who underwent an operation (cholecystectomy). All the non-parametric variables in this study were not normally distributed: Kolmogorov-Smirnov <0.05 .

The demographics of the study subjects are illustrated in **Table 2**. There was a significant difference in the gender (Pearson Chi-Square test, 2 d.f., $P<0.001$) and age (Kruskal-Wallis test, 2 d.f., $P<0.001$) distribution between the four study groups. There was a significant difference in gender distribution between patients with NSAP, and patients with complicated gallstone disease (Pearson Chi-Square test, 1 d.f., $P<0.001$) and other diseases ($P<0.001$); and when patients with uncomplicated gallstone disease were compared to patients with complicated disease ($P<0.001$) and other diseases ($P=0.008$). A significant difference in age distribution was observed when patients with NSAP were compared to patients with complicated gallstone disease (Mann-Whitney U test, 1 d.f., $P<0.001$), and when patients with uncomplicated gallstone disease were compared to patients with complicated gallstone disease ($P<0.001$); and with other disease ($P=0.048$).

There was no difference in the proportion of patients presenting with right upper quadrant abdominal pain and those who had a past medical history (PMH) of excess alcohol intake between the different groups of patients (Pearson Chi-Square test, 2 d.f., $P=0.350$; and $P=0.452$ respectively). The significant difference ($P=0.018$) in the proportion of patients with a PMH of gallstones between the four groups was also observed when patients with NSAP were compared with those of uncomplicated gallstone disease (Pearson Chi-Square test, 1 d.f., $P=0.038$); and when patients with uncomplicated gallstone disease were compared with patients with other diseases ($P=0.002$).

Although there was no significant difference in the proportion of patients with parameters that met the SIRS criteria within 24 hours of hospital presentation (Pearson Chi-Square test, 2.d.f., $P=0.091$) (See **Appendix 5**), there was a significant difference in the proportion of patients who had a blood culture sample obtained (Pearson Chi-Square test, 2.d.f., $P=0.001$). This difference was seen between patients with NSAP and both groups: uncomplicated gallstone ($P<0.001$) and other diseases ($P=0.001$); and between patients with uncomplicated and complicated gallstone disease ($P=0.025$). Of the 49 patients who met the SIRS criteria within 24 hours of hospital presentation, only 20 patients (41%) had a blood culture sample obtained throughout their patient episode in hospital: One patient (5%) with NSAP, 2 patients (10%) with uncomplicated disease, 12 patients (60%) with complicated

disease and 5 patients (25%) with an other (non-gallstone) disease. There were 3 unavailable data for patient's time of onset of abdominal pain as all 3 patients could not recall when their pain started. There was no difference in the duration from the onset of patients' abdominal pain to the time patients' blood samples were obtained for miRNA analysis between the four study groups ($P=0.447$).

3.3 Laboratory Blood Results

Of the 232 patients included in this study, forty-two patients (18.1%) did not have presentation CRP levels, 6 patients (2.6%) without presentation Amylase and 71 patients (30.6%) without presentation GGT. There was no specific reasons for this. **Table 3(a)** illustrates the presentation blood results and **Table 3 (b)** the peak blood results of the study group patients. All the blood tests of interest (WCC, CRP, Creatinine, Bilirubin, ALT, AlkP, GGT), except for Amylase, had demonstrated a significant difference in levels between the four different patient groups (Kruskal-Wallis test, 2 d.f., $P<0.005$). This was true on both occasions - upon hospital presentation and also in their peak values during the patient's hospital episode. Of interest, WCC levels upon hospital presentation only differed significantly between patients with NSAP, and both complicated gallstone disease (Mann-Whitney U test, 1 d.f., $P<0.001$) and other diseases ($P=0.006$). Interestingly, there was no statistical difference in presentation WCC levels between the two gallstone disease subgroups (Uncomplicated vs. Complicated, $P=0.063$). As expected, presentation CRP levels were significantly lower in patients with NSAP compared to the other three groups ($P<0.05$); but was also significantly different between patients with uncomplicated and complicated gallstone diseases ($P=0.001$).

Presentation bilirubin levels were significantly different between patients with gallstone and non-gallstone diseases ($P<0.05$), but was not statistically different between patients with NSAP and patients with other diseases ($P=0.230$). Interestingly, there was no significant difference in total bilirubin levels between patients with uncomplicated and complicated gallstone diseases ($P=0.761$). See **Figure 11(a)**. Presentation ALT levels were significantly lower in patients with NSAP compared to the three other patients groups ($P<0.001$), but a statistical difference in level was not observed between the two gallstone disease subgroups (Uncomplicated vs. Complicated; $P=0.176$), as depicted in **Figure 11(b)**. Similarly, presentation AlkP and GGT levels were significantly different in patients with NSAP compared to the three other patient groups ($P<0.05$); but there was no significant

difference in presentation AlkP and GGT between the two gallstone disease subgroups ($P=0.094$ and $P=0.461$ respectively). See **Figure 11(c)-(d)**.

There were 76 missing peak blood values: 51 peak GGT values, 3 peak Amylase and 22 peak CRP values, for unknown reasons. See **Table 3(b)**. The peak blood values demonstrated similar significant profiles as their presentation counterparts between the patient groups. Of interest, peak WCC and CRP levels were significantly different between the two gallstone disease subgroups ($P=0.011$ and $P<0.001$ respectively). However, peak LFTs (Bilirubin, ALT, AlkP, GGT) did not show any statistical difference between the two gallstone groups ($P=0.883$, $P=0.422$, $P=0.545$ and $P=0.743$ respectively). See **Figure 11(e)-(h)**.

3.4 Imaging Investigations

Table 4 illustrates the imaging investigations (USS, CT, MRCP) that patients had received. There was a higher proportion of patients with an ‘other’ non-gallstone disease (91.7%) who underwent an ultrasound scan compared to patients with gallstones: versus Uncomplicated disease (65.1%, Pearson Chi-Square test, 1 d.f., $P=0.002$); Vs. Complicated disease (77.0%, $P=0.033$). Similar significant differences were also revealed for patients who underwent a CT scan: Other diseases vs. NSAP/Uncomplicated/Complicated disease; $P<0.05$. Interestingly, although there was a significantly higher proportion of patients with gallstone diseases (both uncomplicated and complicated) who underwent MRCP compared to patients with non-gallstone diseases (NSAP and ‘Other’) ($P<0.05$), there was no statistical difference between the two gallstone disease subgroups (Uncomplicated vs. Complicated, $P=0.984$).

Of the 11 patients who did not undergo any imaging investigations, 10 patients had a PMH of having gallstones, of which 5 underwent a cholecystectomy, 2 (who had a previous cholecystectomy) were diagnosed with NSAP when bloods were within normal ranges and abdominal pain self-resolved, 1 patient with biliary colic was for conservative management due to co-morbidities, one underwent an ERCP for choledocholithiasis, and one had a recurrent episode of idiopathic pancreatitis. The remaining patient without a PMH of gallstones self-discharged prior undergoing further investigations for unknown reasons.

3.5 Operative and Histological Findings

In this study, a total of 101 (43.5%) patients underwent a cholecystectomy (**Table 5**). Not only was the significant difference in the proportion of patients who underwent an

operation observed between patients with gallstone and non-gallstone diseases (Pearson Chi-Square test, 2 d.f., $P < 0.001$), but was also evident between patients with uncomplicated and complicated gallstone diseases ($P < 0.001$). Among those who underwent a cholecystectomy, there was no difference in the time period (in Days) from patient's index hospital presentation to the day of operation (Kruskal-Wallis test, 2 d.f., $P = 0.715$). All 101 patients had gallbladder specimen pathology results. There was a significant difference in the proportion of patients with gallbladder cholesterosis between patients with complicated gallstone disease and an other (non-gallstone) disease ($P = 0.046$). There were in total 230 patients (99.1%) in the study who had an imaging investigation performed and/or had Surgeon's operation findings and/or relevant PMH and previous investigations recorded to be able to determine the fatty nature of patients' liver. Two patient data, both with NSAP, on this variable were missing – one patient with no PMH (and previous imaging) self-discharged for unknown reasons and the other patient's abdominal pain resolved and was discharged prior to undergoing further investigations. There was no difference in the proportion of patients with fatty liver disease between the four groups ($P = 0.847$).

3.6 Evaluation of Imaging Results against Pathology Results and/or Surgeon's Findings

Table 6 shows the overall sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of the imaging modalities used in this study. Overall, the sensitivity and specificity of the imaging modalities used in this study to diagnose presence of gallstones was 94.4% and 37.5% respectively, and was 52.5% sensitive and 82.9% specific in diagnosing cholecystitis. As a diagnostic tool for detecting gallstones, in this study, MRCP had the highest PPV of 96.9% (with NPV of 33.3%) and CT had the highest NPV of 66.7% (with PPV of 86.7%). Conversely, USS had the highest PPV and NPV for cholecystitis, 85.3% and 56.2% respectively.

3.7 miR-122 in Gallstone Diseases and Non-Gallstone Diseases

Table 7 illustrates the median miR-122 concentration values ($2^{-\text{dct}}$ values), normalised to Spike-in control miR-39 for patients with and without gallstones. Of the 232 patients included in the study, 143 (61.6%) patients had a gallstone pathology. There was significant difference in miR-122 concentrations between patients with and without gallstones (Median: 0.039, IQR: 0.012-0.13; vs. Median: 0.011, IQR: 0.0046-0.041; Mann-Whitney U test, 1 d.f.,

$P < 0.001$). See **Figure 12(a)**. A Receiver Operating Characteristic (ROC) curve analysis (**Figure 12(b)**) of miR-122 against patients with and without gallstones yielded an area under the curve (AUC) of 0.65, with miR-122 sensitivity of 22.2% (95% CI: 13.3-33.6) at 90% specificity; $P = 0.001$. The calculated PPV and NPV at this specificity were 78.0% and 41.9% respectively. See **Table 8**. Again, patients with gallstones were subdivided into ‘uncomplicated’ (biliary colic and aseptic choledocholithiasis) and ‘complicated’ (cholecystitis, cholangitis and gallstone pancreatitis) subgroups; whereas patients without gallstones were subdivided into patients with ‘NSAP’ and with an ‘other’ disease. Median miR-122 levels for each of the four groups is shown in **Table 9** and **Figure 13(a)**. There was significant difference (Kruskal-Wallis test, 2.d.f., $P < 0.001$) in miR-122 concentrations between the four patient groups. This significant difference was not only seen between patients with gallstone and non-gallstone related diseases ($P < 0.001$) but also between the two gallstone disease subgroups (Uncomplicated vs. Complicated, $P = 0.040$). A ROC Curve analysis (**Figure 13(b)**) of miR-122 against both subgroups of gallstone disease produced an AUC of 0.61 with sensitivity of 17% (95% CI: 10.2%-25.8%) at 90% specificity; $P = 0.04$. The calculated PPV and NPV of miR-122 in distinguishing complicated gallstone diseases from uncomplicated gallstone diseases were 81.0% and 32.0% respectively (**Table 8**).

3.8 miR-122 in the Gallstone Disease Spectrum

Table 10(a) illustrates the median plasma miR-122 concentration in different gallstone diseases: cholecystitis, all choledocholithiasis (both aseptic and cholangitis), aseptic choledocholithiasis, cholangitis, and gallstone pancreatitis. The number of patients in each disease is expressed as a percentage of patients with gallstone disease ($n = 145$). There was significant difference in the concentration of miR-122 between patients with cholecystitis and patients with other aetiologies of gallstone diseases (Median: 0.023, IQR: 0.0089-0.079 vs. Median: 0.062, IQR: 0.014-0.16; Mann-Whitney U test, 1 d.f., $P = 0.006$). Patients with a choledocholithiasis pathology (both aseptic and cholangitis subtypes) had significantly higher miR-122 concentrations (Median: 0.099, IQR: 0.036-0.25 vs. Median: 0.024, IQR: 0.0088-0.079; $P < 0.001$). See **Figure 14(a)**. ROC curve analysis of miR-122 against patients with choledocholithiasis and other gallstone diseases produced an AUC of 0.72 with sensitivity of 37.0% (95% CI: 27.6-47.2) at 90% specificity; $P < 0.0001$, as illustrated in **Figure 14(b)**. When this group was further divided into its subgroups, aseptic and cholangitis, the former had a significantly higher expression of plasma miR-122 compared to other gallstone pathology subjects ($P < 0.001$); however, this significance was not demonstrated in cholangitis patients

($P=0.722$). There was also no difference in miR-122 between patients with gallstone pancreatitis and other gallstone pathologies. **Figure 15(a)** depicts the concentrations of miR-122 for the different gallstone diseases. A ROC curve analysis for the significant differences in miR-122 values in the other respective gallstone diseases (cholecystitis and aseptic choledocholithiasis) were plotted in **Figure 15(b)-(c)** and detailed in **Table 10(b)**. Patients with cholecystitis yielded an AUC of 0.63 with sensitivity of 16.7% (95% CI: 9.4-26.4) at 90% specificity; $P=0.0058$. Plasma miR-122 had a PPV of 55.6% and NPV of 59.2% in detecting cholecystitis. Patients with choledocholithiasis of any aetiology yielded an AUC of 0.72 with sensitivity of 37.0% (95% CI: 27.6-47.2), PPV of 63.0% and NPV of 75.9% at 90% specificity; $P<0.0001$; whereas patients with only aseptic choledocholithiasis had an AUC of 0.76 with sensitivity of 54.9% at 90% specificity; $P<0.0001$. Of particular interest to this study, presentation ALT was further analysed between the different complicated gallstone disease groups (**Table 10(c)**) for comparison with miR-122 levels. Also, specifically for choledocholithiasis cases, ROC curve analysis of presentation LFT values were carried out to evaluate their strength in determining presence of choledocholithiasis (**Table 10(d)**).

3.9 miR-122 in Other Relevant Pathologies

Further analyses of miR-122 between other different pathologies of interest were carried out as shown in **Table 11**. There was no significant difference in concentration of miR-122 between patients with pancreatitis of gallstone aetiology from other aetiologies ($P=0.437$). Of the patients who underwent an operation, there was no significant difference in miR-122 concentrations between specimens that demonstrated necrotic or gangrenous changes in comparison to other gallbladder specimens with cholecystitis ($P=0.964$); and between specimens with and without cholesterosis ($P=0.498$). Patients with a proven fatty liver also did not have a statistical difference in miR-122 concentrations from patients with no evidence of a fatty liver pathology ($P=0.569$).

3.10 Correlation of miR-122 with Laboratory Blood Tests

Correlation analyses on plasma miR-122 were performed against the laboratory blood results obtained on hospital presentation. See **Table 12(a)**. The miR-122 samples were obtained within 24 hours of hospital presentation. All the LFTs and serum creatinine correlated significantly with miR-122 values ($P<0.0001$ and $P=0.029$ respectively). These are illustrated in **Figure 16(a)-(e)**. The correlation coefficients (r) were 0.432, 0.654, 0.425 and 0.488 for the

LFTs (Total bilirubin, ALT, AlkP and GGT, respectively) and -0.144 for serum creatinine. There was no significant correlation between plasma miR-122, and both presentation WCC and CRP levels ($P>0.30$).

Correlation analyses for the plasma miR-122 obtained within 24 hours of hospital presentation were then performed against the peak laboratory blood results during individual patient's hospital episode (**Table 12(b)**). miR-122 concentrations revealed significant correlations with the LFTs ($P<0.0001$). See **Figure 16(f)-(i)**. The correlation coefficients (95% CI) for Total Bilirubin, ALT, AlkP and GGT were 0.509 (0.404-0.601), 0.697 (0.622-0.759), 0.469 (0.359-0.566) and 0.541 (0.425-0.639) respectively.

CHAPTER 4: DISCUSSION

Gallstone-related diseases are some of the most common gastrointestinal diseases in Western countries¹⁰ and the prevalence is expected to increase in line with the aging population¹¹. Gallstone diseases comprise a spectrum of diseases. Some patients with gallstones can be managed non-urgently as an outpatient or by day-case surgery for uncomplicated cases, while others require inpatient management and/or urgent surgery or procedures in the presence of complications¹. Currently available laboratory tests, clinical examination and imaging investigations have their limitations in discriminating between these groups of patients, thus leading to unnecessary inpatient admissions¹ and delayed patient management^{64,67}. Based on current knowledge that liver-specific miR-122 plays a role in cholesterol metabolism⁶ (and hence bile acid pool⁷); that hypoxamir miR-210 may affect gallbladder motility; and that both miRNAs have been demonstrated to be associated with cholestasis as a more superior marker than ALT^{4,5,214}; this study hypothesized that both miR-122 and miR-210 have the potential to be early diagnostic investigation tools in differentiating patients with uncomplicated from complicated gallstone diseases.

4.1 The Pilot Study and miR-210

The pilot study demonstrated a significant difference in miR-122 concentrations between the 4 study groups but no statistical difference was found for miR-210 concentrations. miR-122 also had stronger correlations with total bilirubin and ALT levels compared to those observed with miR-210. Yang et al¹⁸⁶ had demonstrated an upregulation of miR-210 levels in subjects with gallstones compared to those without gallstones. However, the miR-210 expressions in their study were measured from the gallbladder mucosal tissue, rather than from circulating mediums like in this present study. It could be that miR-210 in this study may not yet have been released into the circulation from the inflamed gallbladder. Indeed, most studies that have demonstrated miR-210 induction under hypoxia were carried out on organ cells, for example, pancreatic cells^{235,274}, non-small cell lung cancer²⁷⁵, hepatocellular carcinoma²⁷⁶, colorectal carcinoma²⁷⁷, paragangliomas²⁷⁸, oral tumours²⁷⁹, and cholangiocarcinoma⁵, rather than on circulating biological matrices such as plasma or serum in humans. Moreover, most of these proven associations were with tumour tissues, rather than with inflammatory-only tissues, and hence other molecular pathways inducing miR-210 expression cannot be ruled out. Another explanation for the comparable concentration levels of miR-210 between the four patient groups in this current study could be that the miRNA concentrations may have tailed

off very soon after the initial onset of symptoms. A study by Fasonara et al²⁴³ demonstrated that the elevated expression of miR-210 induced by hypoxia remained upregulated for 72 hours after the insult prior to its reduction in concentration levels. However, the median time period from onset of symptoms to time when miR-210 sample was obtained in this study was 72.0 hour, so is unlikely the case, but still could be a possibility given that this variable had an interquartile range of 27.8-120.0 hours. Alternatively, the expression of miR-210 in gallstone diseases may be more localised to the gallbladder tissue, or may be associated with increased miR-210 concentrations that are too insignificant to affect circulating concentrations. This possibility of localised hypoxic and inflammatory effects of the gallbladder could be supported by this study's non-significant difference in plasma concentrations of miR-210 in patients with gangrenous and/or necrotic gallbladder specimens compared to those without. From these statistically non-significant findings in the pilot study, the analysis of miR-210 in the remaining plasma samples were halted.

4.2 The Main Study and miR-122

This main study thereafter focused on miR-122 analysis only. This study demonstrated that plasma miR-122 concentrations were significantly higher in patients with gallstone diseases than in non-gallstone diseases; higher in patients with clinically uncomplicated gallstone diseases than in complicated gallstone diseases; and higher in patients with choledocholithiasis compared to patients with other gallstone pathologies. miR-122 concentrations were also shown to be significantly lower in patients with cholecystitis compared to patients with other gallstone pathologies.

In this study, there was no significant difference in the proportion of patients who presented to the hospital with parameters meeting the SIRS criteria²⁶³ between the four groups of patients (NSAP, uncomplicated and complicated gallstone diseases, and other diseases) ($P=0.091$). This highlights the unreliability of clinical parameters on initial acute presentations, even for patients with NSAP. The missing information for CRP, GGT and Amylase levels could be because they are currently not the routine blood tests when ordering laboratory investigations of Full Blood Count and LFTs within our NHS Trust. Blood tests carried out upon presentation to hospital are important diagnostics tools especially in acute settings¹. However, this study revealed no significant differences between presentation WCC and LFT values in both gallstone disease subgroups (uncomplicated and complicated cases). Furthermore, the similar time frame (median values) from index presentation to operation

between both gallstone disease subgroups could indicate that most of the uncomplicated cases (mainly biliary colic cases) were treated as urgent cases with a potential differential diagnosis of cholecystitis. It could also be that since the RIE has a dedicated emergency theatre list every day, most of the biliary colic patients had been managed to be placed onto the operating list for the day, but this is unlikely the case as the statistics⁸⁸ evidently demonstrates the exponential increase in demand on the NHS services. These initial findings in this study further supports the need for a novel biomarker to aid in the diagnosis of gallstone diseases (from other non-gallstone diseases), and to distinguish uncomplicated from complicated gallstone diseases in order to enhance patient management.

The presentation CRP values in this study were significantly higher in complicated than in uncomplicated diseases, which is in keeping with Mok et al's¹¹⁵ study findings that CRP is a good predictor for severity of gallstone cases (in the latter study, cholecystitis were compared). In this study, the PPV and NPV of USS for cholecystitis were 85.3% and 56.2% which is similar to findings reported by other studies^{108,112,113}. However, the sensitivity of USS for cholecystitis was 59.2%, which is lower than that reported by Kiewiet et al¹¹⁴ (80%) but similar to that by Bingener et al.¹¹⁰ (60%). The CT used in this current study had an NPV of 41.7% for cholecystitis, similar to that reported by Hwang et al¹¹³, who also stated that the additional use of CT with USS did not improve the NPV in diagnosing cholecystitis. The sensitivity and specificity of MRCP and CT used in this study to diagnose cholecystitis were lower than those of USS. Nevertheless the sample size for patients who had undergone MRCP (35.3%) and CT (22.8%) investigations were smaller compared to those who had an USS (78.0%), and hence an accurate evaluation cannot be made.

miR-122 is widely known to play a crucial role in the regulation of cholesterol and fatty acid metabolism¹⁴⁵. Under certain conditions, miR-122 may reduce CYP7A1 mRNA stability to inhibit bile acid synthesis and to reduce serum cholesterol and triglycerides, thus affecting the bile acid pool. As these are key contributors to the pathogenesis of gallstones, subsequently, this study aimed to determine the association between plasma miR-122 and gallstone diseases, and also its differential expression within the gallstone disease spectrum. If novel biomarkers are sufficiently sensitive and specific in distinguishing both subgroups of gallstone diseases, earlier directive patient management can be enhanced; as can the proportion of unnecessary patient admissions, and hence healthcare expenditure⁸⁹, be reduced. The resultant efficient and effective patient management could also create extra acute beds and also reduce the inpatient cost per patient episode⁸⁸.

This present study demonstrated that plasma miR-122 was significantly increased in patients with gallstones ($P < 0.001$). These findings are similar to those by Huang et al⁴ who compared their disease cohort with healthy control groups using serum miR-122. However, their study yielded a higher AUC on the ROC analysis, 0.931 with 77.4% sensitivity and 96.4% specificity at cut off value of 659.28; and even at a specificity of 96.4%, the sensitivity of plasma miR-122 in this current study was remarkably lower (at 5.6%). This could be that our comparison group was with non-gallstone related disease cases (Other diseases and NSAP), who most likely had deranged inflammatory markers and/or LFTs, rather than with healthy subjects. Hence, miR-122 concentration levels were likely to be affected in this comparison group, thus reducing the miRNA expression gap between patients with and without gallstones in this study. The difference in the evaluation results of miRNA between Huang et al's⁴ study and this current study could also be explained by the different circulating mediums used in both studies. Recent studies show that many miRNAs in plasma can combine with proteins such as Argonaute 2 and high-density lipoproteins, thereby generating high stability state^{280,281}. McDonald et al²⁸² found higher concentrations of circulating endogenous miRNAs in plasma samples compared to serum samples. Indeed, studies carried out on hepatocellular carcinoma patients with serum miR-122^{283,284} and with plasma miR-122²⁸⁵ demonstrated inconsistent findings, which could be explained by the heterogeneity of the samples used²¹⁰. In contrary, Pirola et al²¹⁸ observed that extracellular Argo2-miR-122 complexes represent only a small fraction in the serum of NAFLD patients, and other experimental studies have been shown that miR-122 mostly circulates in Argo2-free complexes²¹⁹. Nevertheless, serum and plasma samples have been shown to be strongly correlating, although being expressed at different levels³. Only a handful of studies have carried out miR-122 evaluation in both tissue and circulating mediums for comparison in gallbladder diseases. A study by Li et al²⁰⁹ demonstrated that miR-122 expression, although at differing levels in both gallbladder tissue and plasma, were both upregulated in cholangiocarcinoma. This suggests that expression of miR-122 in plasma is likely to be representative of that in the gallbladder tissue.

There are currently no known previous studies from literature banks that has investigated miR-122 expression in different complications associated with gallstones. This study showed more than 2.5 fold reduction in plasma miR-122 concentration in patients with cholecystitis compared to patients with other gallstone diseases. This could possibly be explained by the anti-inflammatory and anti-fibrotic properties of miR-122, as demonstrated in studies on mouse liver^{286,287}. These same properties have also been shown in human studies:

reduction in miR-122 levels were associated with non-alcoholic steatohepatitis (NASH)¹⁵² and with rapid progression to liver fibrosis in hepatitis patients²⁸⁸. Indeed, patients in this current study with the more severe form of cholecystitis, necrotic or gangrenous cholecystitis, were shown to have lower plasma miR-122 concentrations compared to other forms of cholecystitis. This comparison is relatively robust since the diagnosis of the presence of necrotic or gangrenous features of cholecystitis were based on histological findings. In contrary, Pirola et al's²¹⁸ study suggested that lower expression of liver miR-122 is a consequence of high rate of release into the circulation rather than a down-regulation of miRNA expression. Multiple other studies investigating liver injury of different aetiologies (including acute viral hepatitis, drug-induced liver injury and liver carcinoma) have also validated the opposite expression pattern between circulating and tissue miRNA^{200,289,290}. However, this finding of reduced plasma miRNA expression for cholecystitis is interesting, as it conflicts with the outcome of increased miRNA expression found for all gallstone diseases in general. One possibility could be that the reduction in anti-inflammatory and anti-fibrotic properties of miRNA outweighed the properties in gallstone pathology that normally increases miRNA expression; or because ALT levels in patients with cholecystitis were significantly lower than patients without cholecystitis and this may have affected miR-122 expression. Another reason could be that cholecystitis may regulate miR-122 as a completely different entity to other gallstone diseases via a different biomolecular pathway that is currently still unknown.

miR-122 has been consistently demonstrated to be associated with cholestasis-induced liver injury in independent studies^{4,209,214}. miR-122 has previously been used as a circulating biomarker of bile acid induced necrosis in mice's serum, and its upregulation has been demonstrated in cholestasis and bile acid induced tissue injuries in mice models with bile duct ligations (BDL)^{4,189,214}. Zhang's team⁴ also reported that serum miR-122 was associated with gallstone-induced cholestatic liver injury in humans. However, there was no separation of the different gallstone pathologies against miR-122 in the latter study and they only had 31 patients in their study. Nevertheless, in this present study, the expression of plasma miR-122 in patients with choledocholithiasis, a common cause of cholestasis²¹⁵, was proven to be significantly higher ($P < 0.001$) when compared to patients with other types of gallstone diseases.

The mechanism of the alteration of miR-122 expression in presence of cholestasis and/or bile acid injury is still being debated. Enzymes such as AlkP and GGT that are located at the apical membranes of hepatocytes and cholangiocytes are released into the serum during

injury of these cells. Hence they are used as routine tests to assess biliary function¹⁸³. Similarly, ALT is a metabolic enzyme mainly enriched in the liver and is released into the serum during liver injury²⁹¹. Studies^{4,291} have shown that miR-122 exhibited strong correlations with ALT activity in the blood, and that changes in miR-122 concentrations were in a similar time course profile to ALT in mice with cholestatic liver injury and in humans with drug induced liver injury. These suggest that miR-122 could also be released from the injured cells, in a similar way as ALT; and also that miR-122 has the potential to serve similar functions as ALT in disease assessments. The phenomenon of miRNAs being released from injured cells could explain the significantly increased miR-122 concentration by more than 4-fold in all choledocholithiasis cases in this present study by causing cholestasis-induced hepatocyte and/or cholangiocyte injury. miR-122 expression was also most significantly correlated with ALT compared to other LFTs. However, surprisingly, although plasma miR-122 was higher in patients with cholangitis, this was not significantly different compared to patients with other gallstone diseases, in contrast to aseptic choledocholithiasis. The higher miR-122 levels in aseptic choledocholithiasis could be explained with the higher ALT levels compared to cholangitis, suggesting a higher degree of hepatocellular injury; or the reduction in miR-122 levels in inflammatory conditions (cholangitis), as discussed above, may have offset the increase in miR-122 in cholestatic-induced liver injury conditions. Nevertheless, sample size in these subgroups are relatively small.

Studies investigating cholestasis in mouse models^{4,214} have also demonstrated that the change in miR-122 appeared earlier in the circulation and at concentrations with higher magnitudes than ALT. This was also demonstrated in studies with human viral²⁸⁹, drug^{221,290} and alcohol-induced liver injury^{219,220}. It is still not known whether the earlier detection of miR-122 is because of an earlier release of miRNA into the circulation by an active process or if it is due to a more sensitive method of detection (qRT-PCR)²²¹. In this present study, presentation ALT was not able to distinguish between patients with uncomplicated and complicated gallstone diseases, between uncomplicated and other non-gallstone diseases, and between patients with complicated and other non-gallstone diseases; whereas plasma miR-122 were statistically significant between these comparison groups despite the samples being obtained at a later time (within 24 hours) than patients' presentation laboratory blood samples. This may indicate that miR-122 might have been expressed in the circulation at an earlier time than ALT, or miR-122 were initially present in the circulation at higher concentrations than ALT. Indeed, Huang et al's⁴ study with cholestatic liver injury in mice demonstrated that miR-122 concentrations remained significantly increased at 14 days and miR-122 was suggested as

a more diagnostically sensitive marker for cholestasis at any time point. Additionally, plasma miR-122 levels in this current study were more strongly correlated to peak LFTs compared to their counterparts on presentation, which further supports that plasma miR-122 levels were affected at an earlier time than current laboratory LFTs, hence rendering plasma miR-122 a more sensitive marker of liver injury associated with gallstones.

Although ALT is the classical marker for liver injury, it is still not perfect as ALT activity is not organ specific and can be affected by the kidney, heart, skeletal muscles and the pancreas. Moreover, increased release of hepatocellular contents of ALT can occur during normal hepatocyte turnover which could result in false positive signals²⁹²⁻²⁹⁴. miR-122 is not only organ (liver) specific, but also very abundant, stable in plasma and can be detected using small amounts of sample volume (50 μ L of plasma sample was used in this study)²⁶⁷. Furthermore, overexpression of miR-122 has been shown to enhance ALT activity without a change in transcriptional level of the coding gene and rather, was involved in the translation activity of ALT²¹⁸; which could suggest that ALT activity can be affected by miR-122 but may not be conversely true. If this was indeed the case, miR-122 would potentially be a more specific biomarker than ALT. As the exact role of miR-122 in gallstone diseases is still not clear, most of the current suggested mechanisms are linked to studies done specifically in liver diseases.

Serum miR-122 has been shown to be upregulated in patients with fatty liver diseases^{218,295}. However, this was not demonstrated in this present study, and rather, plasma miR-122 concentrations were lower compared to patients with no evidence of a fatty liver. Furthermore, patients with cholesterosis on their gallbladder specimens also had lower plasma miR-122 concentrations compared to those that don't. Nonetheless, plasma miR-122 were not statistically different in both pathologies. These findings, in comparison to the increased miR-122 levels in gallstone diseases, are contrary since they share a similar pathology of dysregulated cholesterol and/or fatty acid metabolism. However, this would suggest that miR-122 expression in these diseases might be modulated via independent pathways or a common pathway currently still unidentified. Like with other associations made and suggestions discussed in this study, one should also bear in mind that one miRNA normally targets a great amount of mRNAs and one mRNA can be potentially regulated by multiple miRNAs²⁹⁶. Therefore, there could be other genes that might be regulating miR-122 involved in gallstone diseases, which again could be different for every gallstone disease in the spectrum depending on their biomolecular pathology.

Some important limitations have been recognised in this present study. Foremost, the sample size is relatively small. This was further highlighted when the study groups were further divided into subgroups, for example patients with cholangitis. Nevertheless, thus far as a ‘proof of concept’ study, the results are promising. As this study has been approved to recruit a total of 300 patients, recruitment of patients is still currently ongoing to enable further evaluation of plasma miR-122 with a larger sample size. The time at which blood samples were obtained for miR-122 analysis was different (within 24 hours) from those obtained for the presentation laboratory samples. Furthermore, the time when the presentation and peak laboratory blood samples were taken were not recorded; and miRNA sampling was taken as a one off rather than as a series throughout patient’s hospital episode. These hinder the study to be able to evaluate the change in concentrations of miR-122 with respect to time progression and also for comparison with LFT activities throughout the course of the disease. Laboratory blood tests were also obtained in retrospect, thus restricting the availability of all the tests of interest. Only plasma samples were used to investigate miR-122 concentrations and it would be of value if serum levels were also analysed to further evaluate expression profiles in both mediums. The findings in this study are limited by the demographic differences and small sample size especially for patients with only one gallstone pathology. This is however realistically difficult, as proven by the overlapping diagnoses in this study, for example, patients with choledocholithiasis are also likely to also have stones in the gallbladder. Therefore, further studies with a larger sample size; serial blood sampling of miRNA samples at the same time as routine laboratory samples; and the application of multivariate logistic regression to correct for patient demographics, and investigation and disease characteristics, are needed to validate the diagnostic value of plasma miR-122 for the spectrum of gallstone diseases.

miR-122 as a potential biomarker also has its limitations. Firstly, as a novel biomarker there are currently no consensus regarding whether plasma or serum is preferable for use as a sample²¹⁰. Although organ specific, circulating miRNA expressions may also be affected by various physiologic modulations and pathologic disruptions²¹⁰, and these situations cannot be strictly excluded in clinical studies. Standard references of miRNA values are also currently not in place which makes it difficult to explore their current clinical application²⁹⁷. Also, like with other miRNAs, there is currently a need for validated protocols to enable for a standard miRNA normalization²⁹⁸. As studies have consistently demonstrated the potential uses of miR-122 in clinical practice and its association with gallstone-related complications (cholestasis), further large scale studies are needed to reveal the network between miR-122, and other

targeted genes and biomolecular pathways to provide a more detailed insight in understanding the mechanisms of miR-122 in gallstone diseases.

The recognised strengths of this research study include the type of study design – a pilot study was carried out and the study was carried out in a blinded manner, with analysis of miRNA samples carried out blinded to clinical data, thus reducing potential bias. The study was restricted to only include patients who had presented to hospital within 24 hours, thus ensuring that miRNA concentration levels would be as closely representative of the patients presenting condition as possible. Potential technical biases in RNA extraction and PCR analyses were recognised and efforts were made to minimise, for example, batch to batch variability and reliability of fluid transfer into microwells²⁹⁹. The study was hence limited to only two personnel being involved in the laboratory analyses of the samples – one was assigned for RNA extraction and one for PCR analysis. The laboratory kits used, including the pipettes, were the same throughout the whole study, thus minimizing potential inter-observer and instrumental biases.

CHAPTER 5: CONCLUSION

This current study has demonstrated that relative plasma miR-122 concentration is significantly higher in patients presenting acutely with signs and symptoms suggesting a gallstone pathology; and in patients with choledocholithiasis when compared to patients with other gallstone pathologies. However, whether these statistical differences translate to clinical differences, would require further developments. Nevertheless, miR-122's application in a clinical setting is promising, and may be beneficial in diagnosing the different complications of gallstone diseases as a supplement to current standard blood tests, if not used in isolation.

Figure 1

Figure 1: Pathogenic factors in cholesterol gallstone formation³⁰⁰. Cholesterol homeostasis in bile plays a key role in the pathogenesis of cholesterol gallstones. Pathologies, including certain genetic factors, resulting in hepatic hypersecretion of biliary cholesterol can lead to physiological supersaturation of cholesterol within the gallbladder bile. At the enterocytes (of the small intestine), absorption of cholesterol is enhanced. In bile, accelerated phase transitions of cholesterol occur which are facilitated by prolonged gallbladder stasis from impaired gallbladder motility and immune-mediated gallbladder inflammation. If persistent, conglomerates of cholesterol monohydrate crystals, mucin gel, calcium bilirubinate and other proteins accumulate, crystallise and form gallstones³⁰⁰. *Figure reproduced from reference 300.*

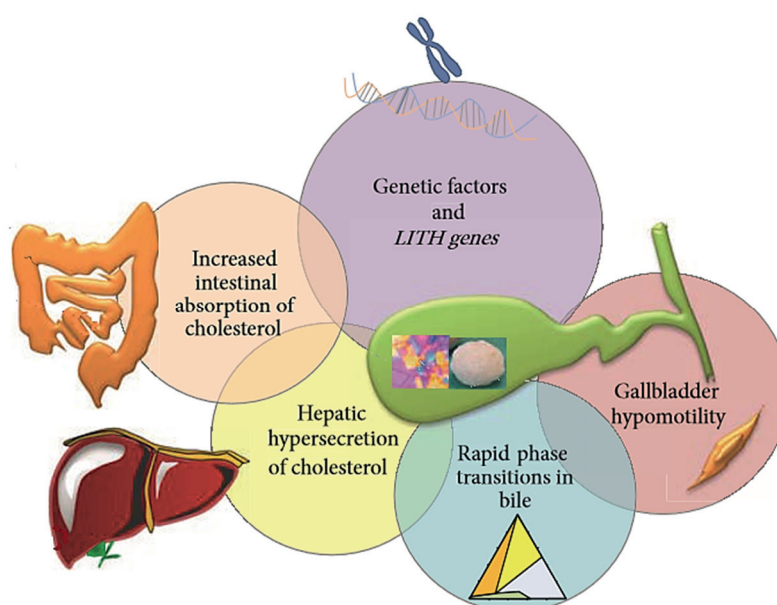


Figure 2

Figure 2: The biliary tree depicting the gallstone disease spectrum at each level³⁰¹. About 80% of gallstones are asymptomatic²⁴ and about 1-4% of these become symptomatic annually¹³. Cholelithiasis with minimal gallbladder inflammation and minimal systemic upset causes biliary colic pain that can last for several hours. However, the gallbladder can be acutely inflamed causing more prolonged abdominal pain and systemic symptoms in cholecystitis. More severe forms of cholecystitis, if left untreated, can lead to empyema and perforation of the gallbladder. Empyema occurs when bile is unable to drain through the cystic duct for a prolonged period and an abscess forms within the gallbladder lumen due to bacterial proliferation and exudation of neutrophils³⁰¹. Gallstones can also migrate from the gallbladder to the biliary tree and to the common bile duct (CBD). Choledocholithiasis can be non-obstructive or obstructive, leading to increased intraductal pressure, increased hepatocellular membrane permeability and ultimately, retention of bile acids causing jaundice (obstructive jaundice). Choledocholithiasis can also be infected resulting in acute cholangitis. Some gallstones, especially small gallstones, can pass along the CBD near to the pancreatic duct junction, resulting in bile reflux into the pancreatic duct thus activating pancreatic enzyme release and causing pancreatitis³⁰¹. *Figure reproduced from reference 301.*

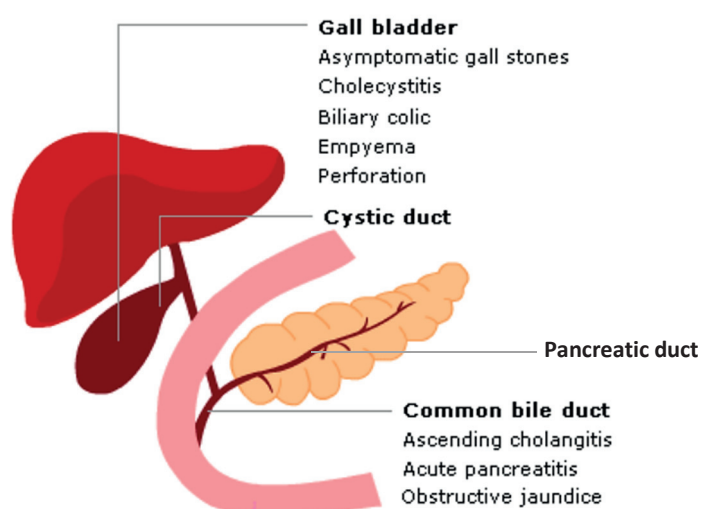


Figure 3

Figure 3: Pathogenesis of acute cholecystitis (Secondary to impacted gallstone in the cystic duct)³⁰². White arrows indicate interaction of the ischaemic mucosa with bile resulting in inflammation. More than 90% of cholecystitis cases are caused by an obstruction of the cystic duct or the neck of the gallbladder by gallstones. This results in increased gallbladder pressure thus reducing blood flow to the mucosa and impairs the defences. Thereafter, bile damages the mucosa leading to inflammation and oedema (cholecystitis)³⁰². *Figure reproduced from reference 302.*

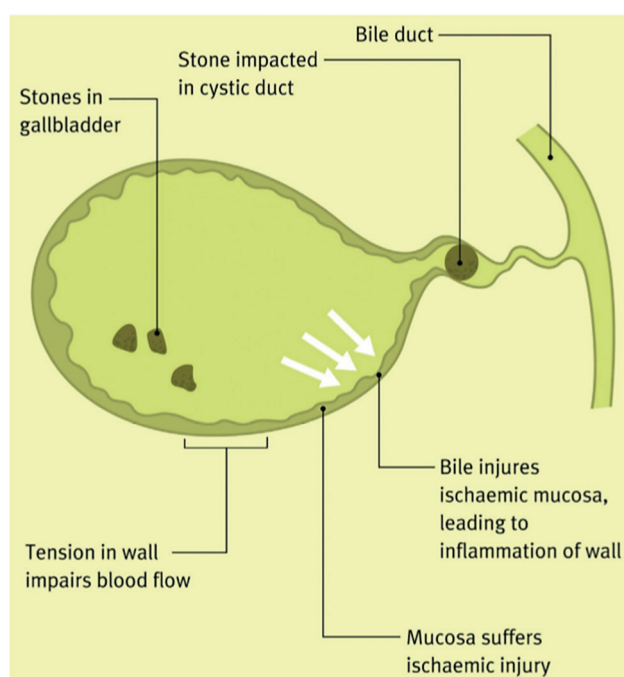


Figure 4

Figure 4: miRNA biosynthesis pathway¹⁵⁵. miRNA transcription and processing first occurs in the nucleus and thereafter in the cytoplasm. When in the nucleus, the majority of intergenic miRNAs are transcribed as longer primary transcripts (pri-miRNAs) by RNA polymerase (Pol) II. The pri-RNAs are then processed by Drosha /DGCR8 complex to form premature miRNA (pre-miRNA). Pre-miRNAs are later exported out of the nucleus via enzyme Exportin 5 into the cytoplasm and then processed by another enzyme, Dicer, to form mature miRNA (mRNA). mRNAs are assembled into an RNA-inducing complex (RISC) that inhibits mRNA expression post-transcriptionally by binding to the 3' UTR of the target mRNA, or cause sequestration or degradation of mRNA²⁶⁴. *Figure reproduced from reference 155.*

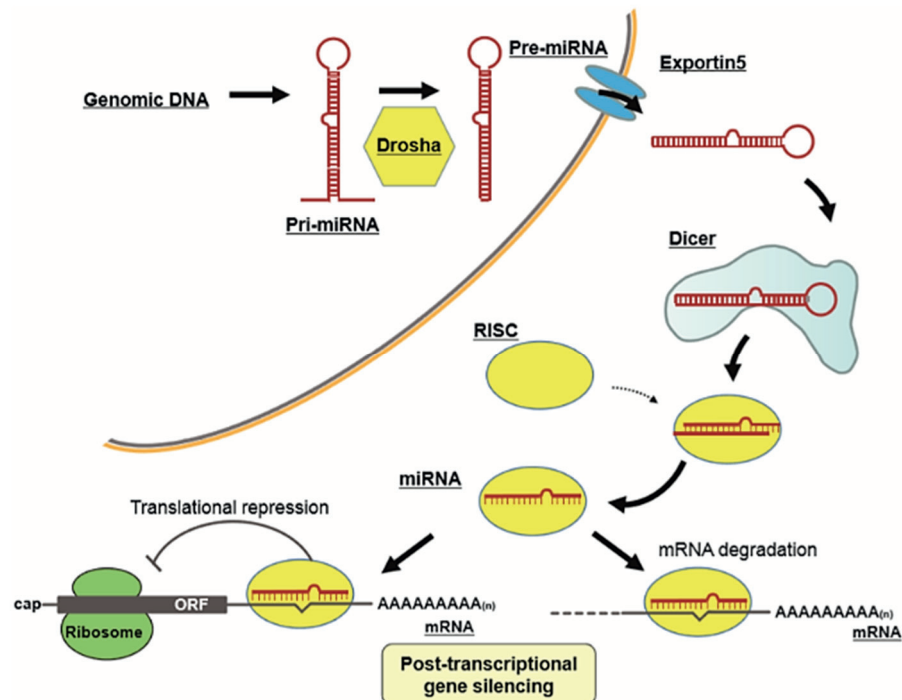


Figure 5

Figure 5: miRNA-based biomarkers³⁰³. As some miRNAs exhibit tissue-specific distribution^{2,4,165}, they may be regarded as blood-based fingerprints of particular tissues^{172,173}. Hence, expression profiles have the potential to be used as biomarkers for diagnostic, prognostic and therapeutic purposes. The schematic diagram shows that miRNA secretion into body fluids can occur through microvesicles, protein complexes, lipoproteins, apoptotic bodies and passive release. The secreted miRNA can be extracted with RNA extraction kits and be analysed with different quantitative approaches. The profile of miRNA populations can be used as diagnostic and/or prognostic biomarkers to aid clinical decisions for management of diseases³⁰³. *Figure reproduced from reference 303.*

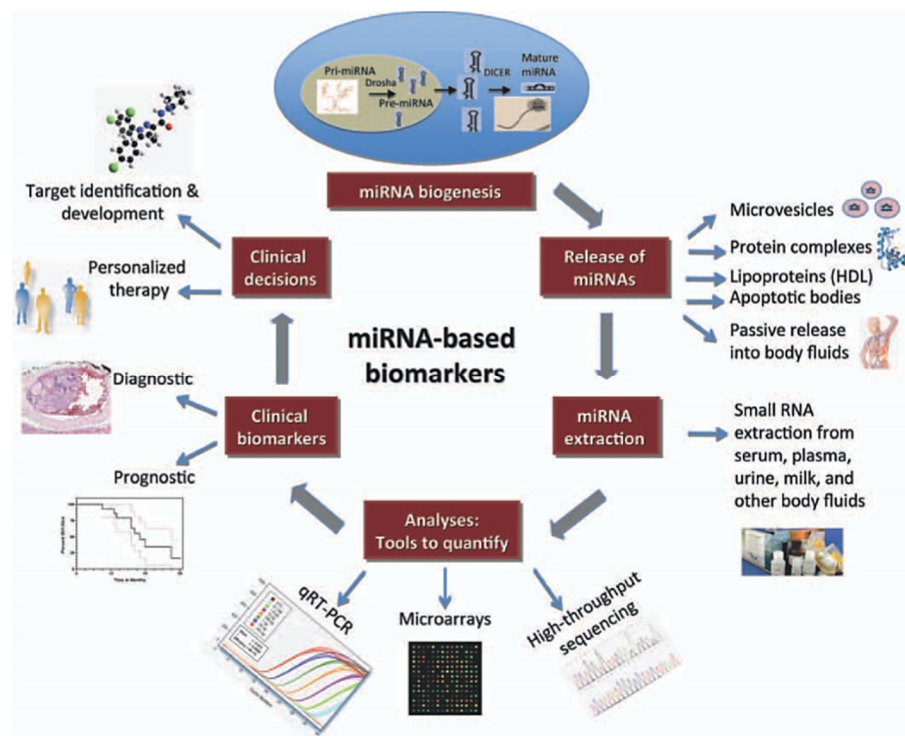


Figure 6

Figure 6: The bile salt-cholesterol-phospholipid diagram and the different pathways of cholesterol solubilisation or precipitation, or both, in bile¹⁰. The three axes of the triangle represent the concentrations of the three lipids: cholesterol, phospholipids, and bile salts. This diagram highlights the importance of relative amounts of bile salts and phospholipids needed to solubilise biliary cholesterol. Cholesterol precipitates quickly with excess bile salts; the phospholipids solubilise cholesterol into vesicles and crystal formation is slower or absent. The one-phase zone is in green (only micelles); and the other three zones are with cholesterol supersaturation. Bile from patients with cholesterol gallstones plots within the left orange zone and the centre red zone. If hydrophilic bile salt is present, the right yellow zone (without crystals) expands towards the right thus preventing cholesterol crystallisation and gallstone formation. Conversely, in the presence of high concentrations of hydrophobic bile salts, the right yellow zone expands to the left at the expense of the orange and red zones (with crystals)¹⁰. *Figure reproduced from reference 10.*

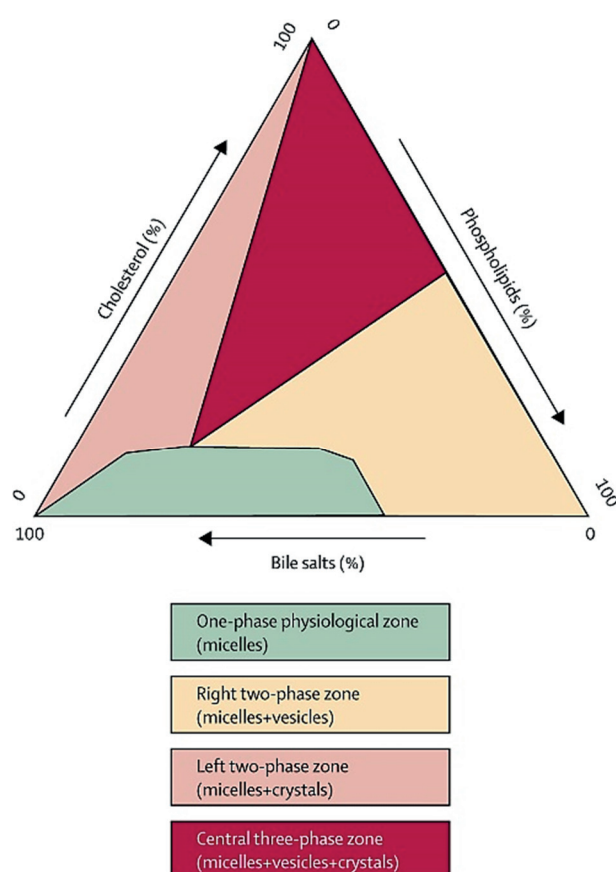


Figure 7

Figure 7: mir-210 directly represses transcripts associated with cellular functions¹⁸⁵. Currently, at least 35 unique transcripts (denoted with their official gene symbol on the diagram) have been identified as direct targets of repression by miR-210. These transcripts are linked to a variety of fundamental cellular processes (indicated in the grey ovals in the diagram). These listed cellular processes are critical to hypoxic survival and adaptation of the mammalian cell which is keeping with miR-210's role as a hypoxamir¹⁸⁵. *Figure reproduced from reference 185.*

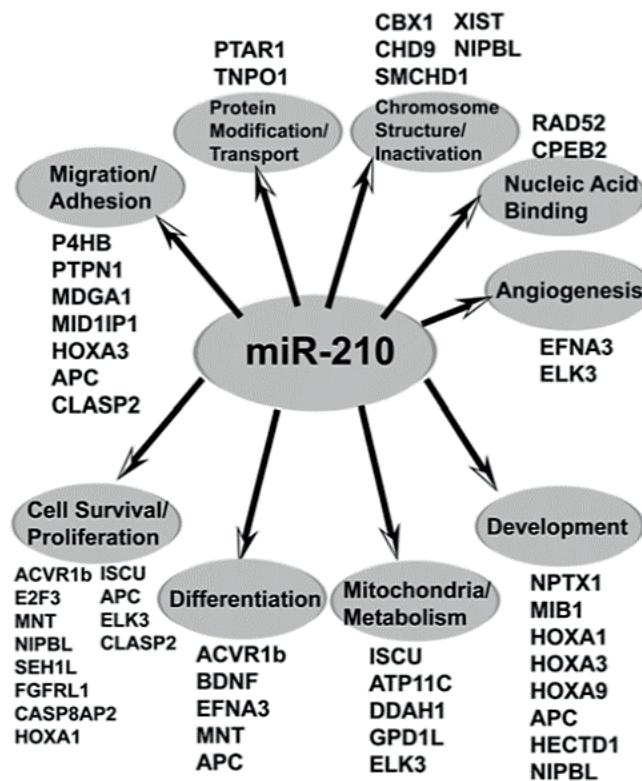


Figure 8

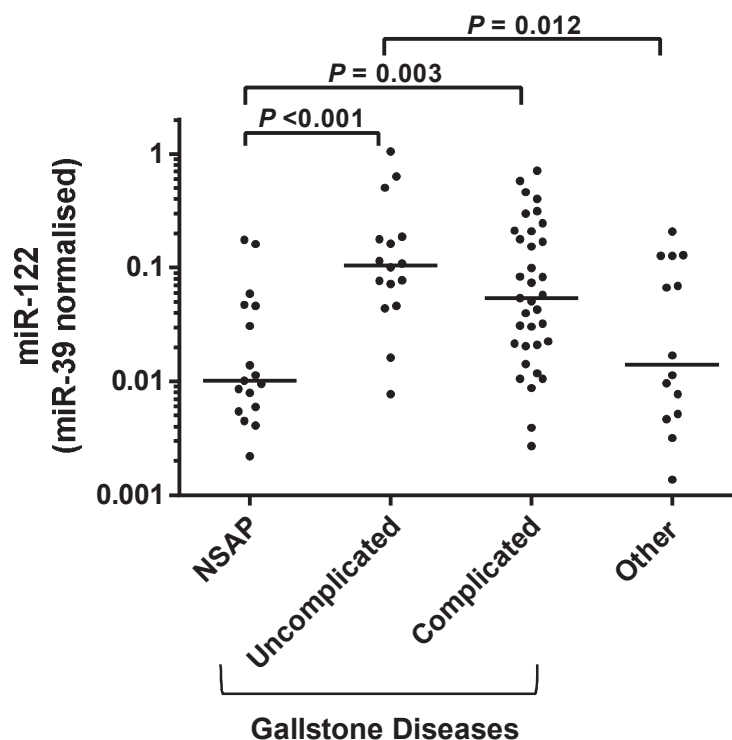


Figure 8: Pilot Study: miR-122 concentrations for each patient group. miR-122 concentrations expressed as $\log_{10} 2^{-dct}$. Each data point represents an individual patient and the horizontal line within each patient group represents the median miR-122 concentration. 'NSAP': Non-specific abdominal pain; 'Other': Non-gallstone diseases of different pathologies. miR-122 concentration was significantly different between the four patient groups (Kruskal-Wallis test, 2 d.f., $P=0.001$). P values are shown in the figure for Mann-Whitney U test (2-tailed) where significant in group comparisons.

Figure 9

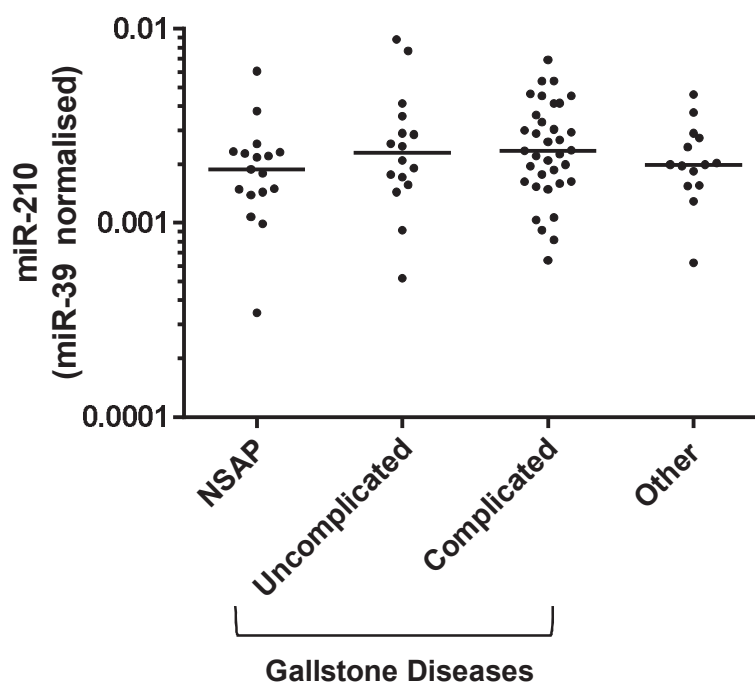


Figure 9: Pilot study: miR-210 concentrations for each patient group. miR-210 concentrations expressed as $\log_{10} 2^{-\text{dct}}$. Each data point represents an individual patient and the horizontal line within each patient group represents the median miR-210 concentration. 'NSAP': Non-specific abdominal pain; 'Other': Non-gallstone diseases of different pathologies. There was no significant difference in miR-210 concentrations between the four groups (Kruskal-Wallis test, 2 d.f., $P=0.365$).

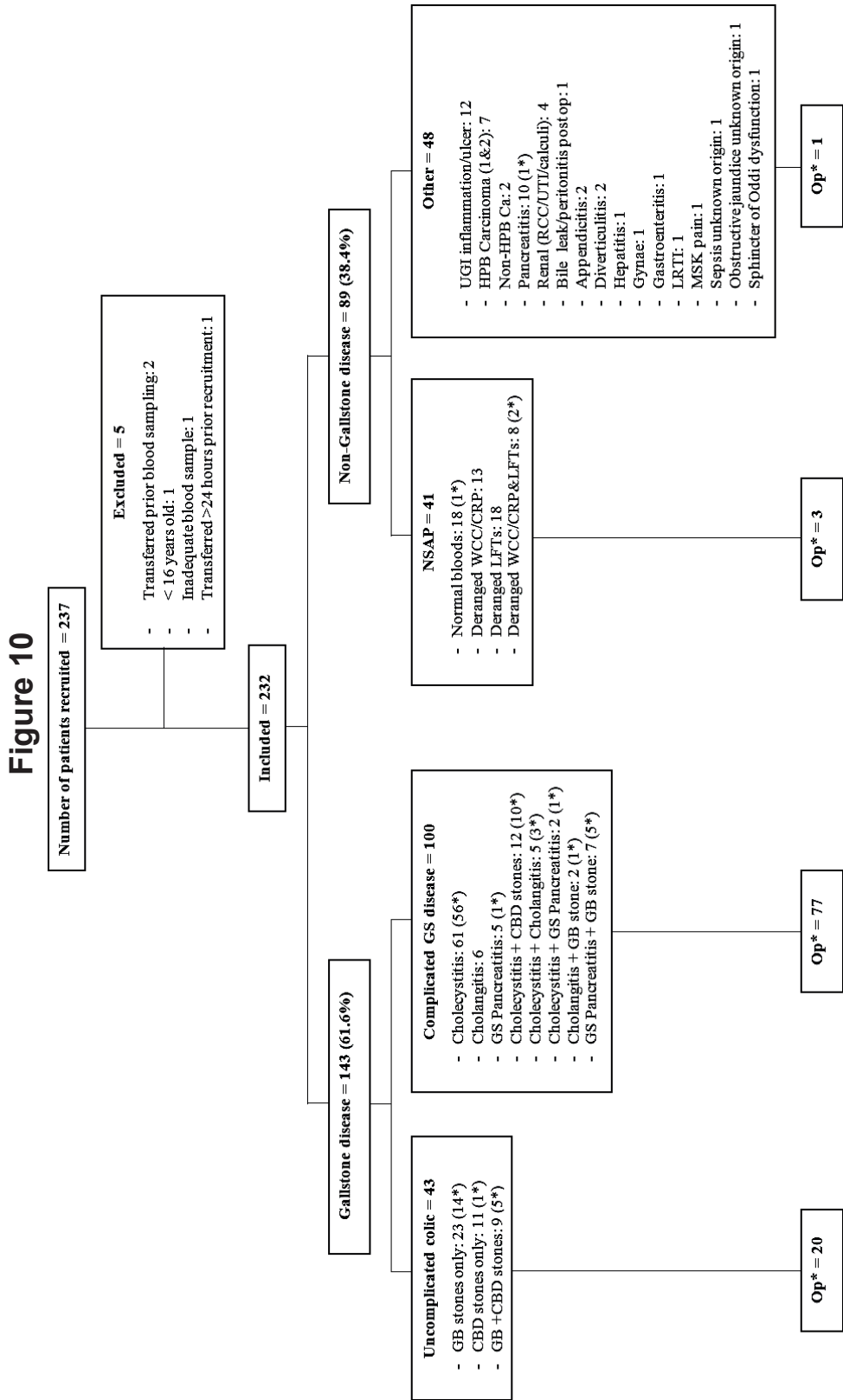


Figure 10: CONSORT diagram for the main study. The study was carried out for 36 calendar weeks. Patient numbers are shown. The number of patients who underwent cholecystectomy within each group is illustrated in brackets with an asterisk (X*).

Figure 11(a)-(d)

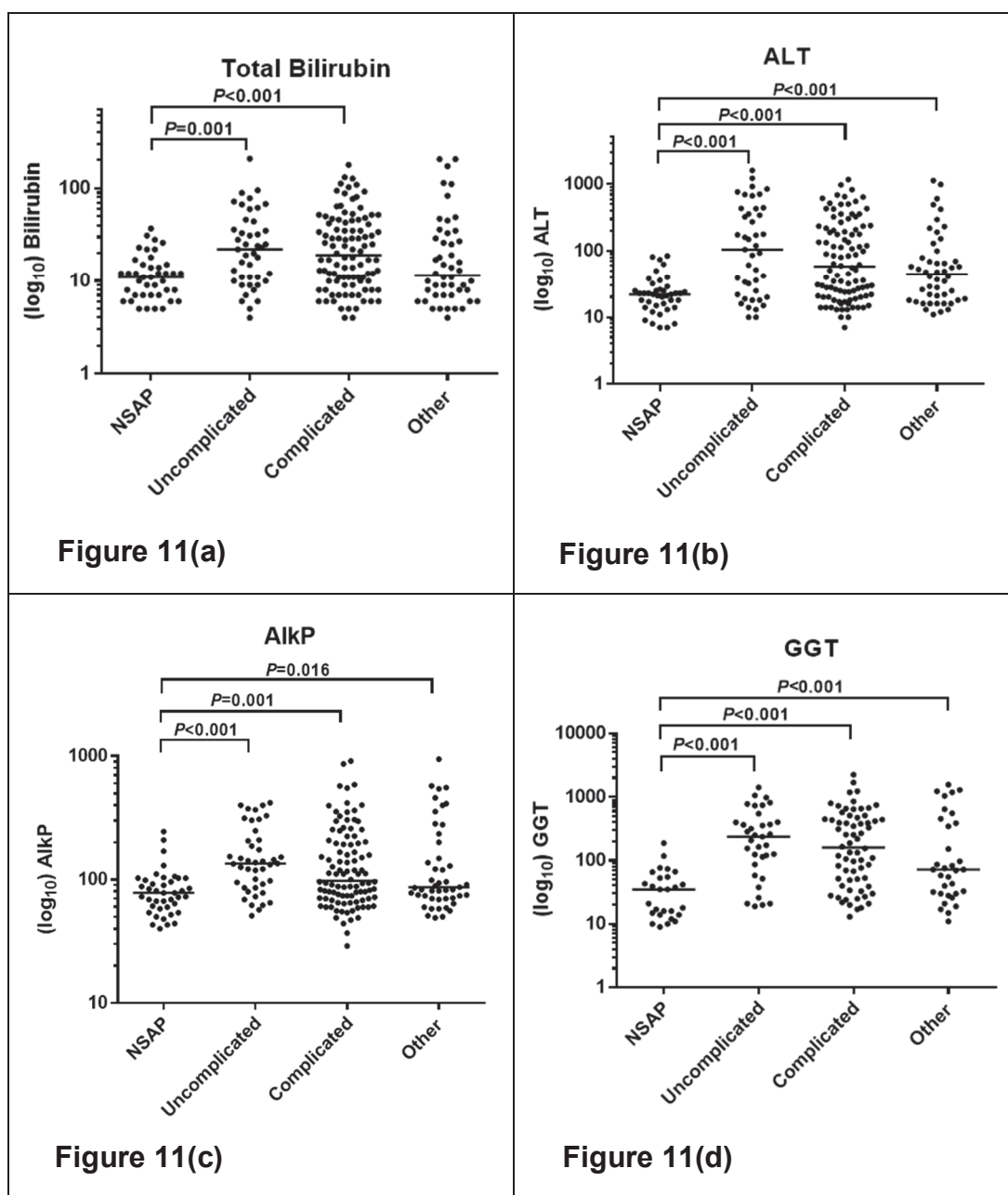


Figure 11(a)-(d): Presentation LFTs (Total Bilirubin, ALT, AlkP and GGT) by patient group. Each data point represents an individual patient and the horizontal line within each patient group represents the median value. 'NSAP': Non-specific abdominal pain; 'Uncomplicated' and 'Complicated': Subgroups of gallstone diseases; 'Other': Non-gallstone diseases of different pathologies. There were significant differences in LFTs between the four patient groups (Kruskal-Wallis test; 2 d.f., $P < 0.01$). Statistical significances (P value) by Mann Whitney U test (2-tailed) are shown for between group comparisons.

Figure 11(e)-(h)

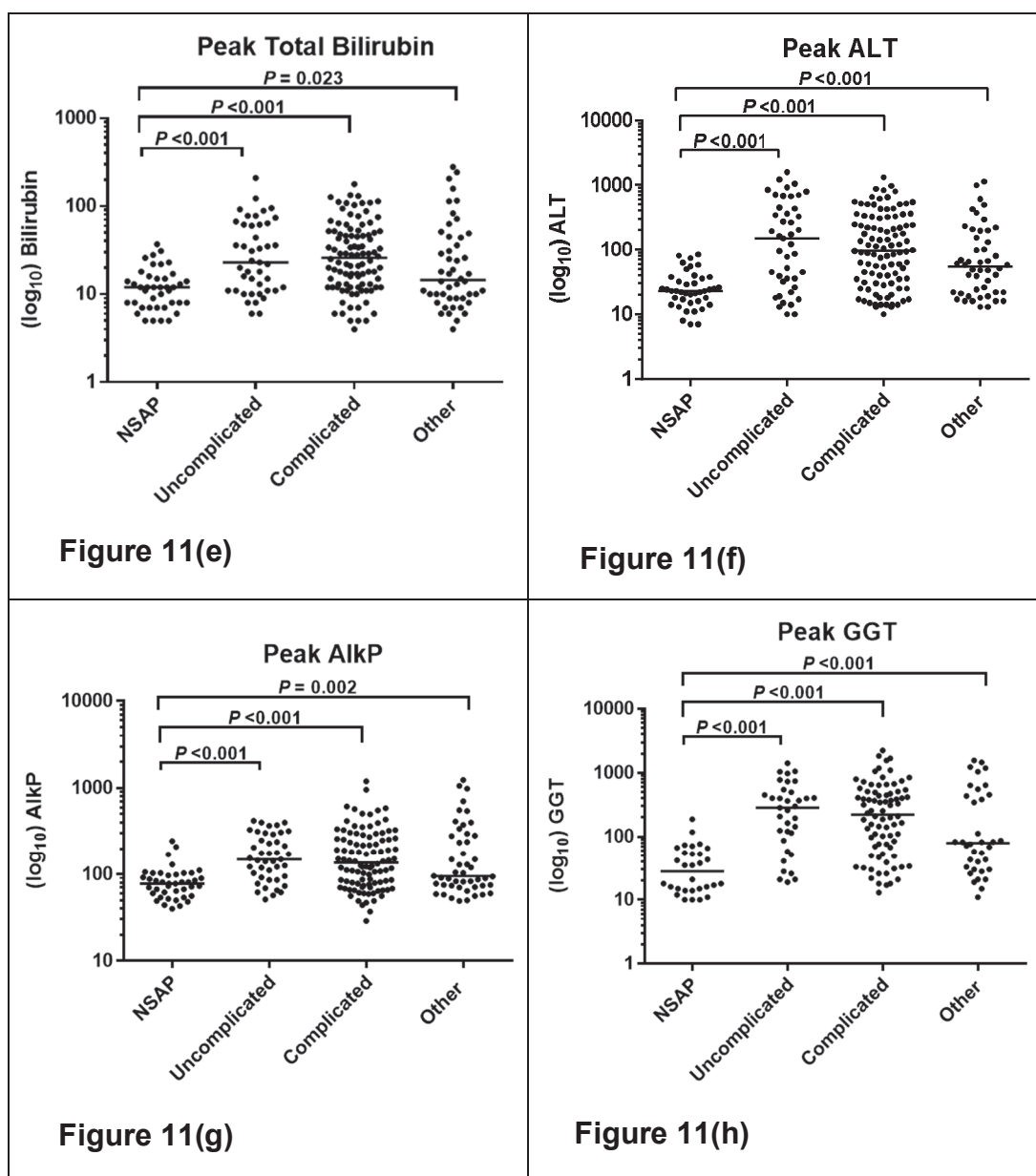


Figure 11(e)-(h): Peak LFTs (Total Bilirubin, ALT, AlkP and GGT) by patient group. Each data point represents an individual patient and the horizontal line within each patient group represents the median value. 'NSAP': Non-specific abdominal pain; 'Uncomplicated' and 'Complicated': Subgroups of gallstone diseases; 'Other': Non-gallstone diseases of different pathologies. There were significant differences in LFTs between the four patient groups (Kruskal-Wallis test; 2 d.f., $P < 0.01$). Statistical significances (P value) by Mann Whitney U test (2-tailed) are shown for between group comparisons.

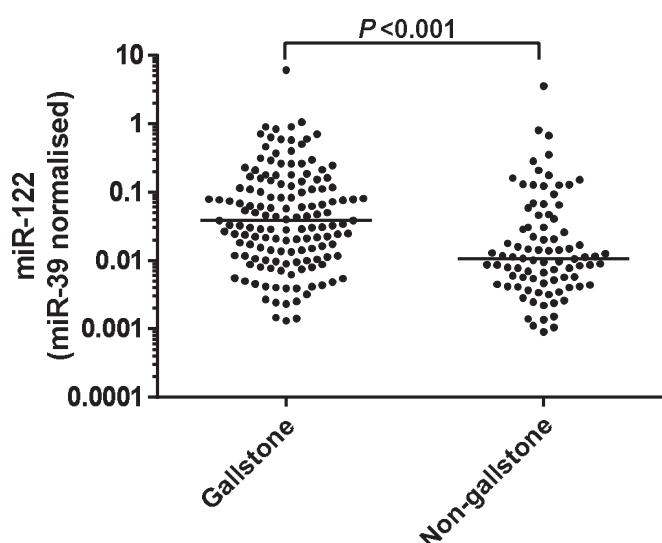
Figure 12(a)

Figure 12(a): miR-122 concentration in gallstone and non-gallstone diseases. miR-122 concentrations (log₁₀ 2^{-dct}) were normalised to Spike-in control miR-39. There were 143 patients (61.6%) with gallstones and 89 patients (38.4%) without gallstone pathology. Each data point represents an individual patient and the horizontal line within each patient group represents the median concentration. Statistical significance (P value) by Mann Whitney U test (2-tailed) is shown.

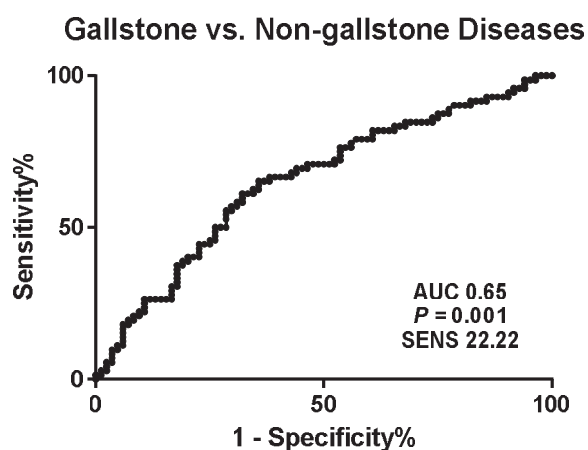
Figure 12(b)

Figure 12(b): Receiver Operating Characteristic (ROC) curve of miR-122 against gallstone and non-gallstone diseases. Area under the curve (AUC) yielded was 0.65 (95% CI: 0.57-0.74) with sensitivity of 22.22% (95% CI: 13.27-33.56) at 90% specificity; P-value of 0.001. The calculated PPV was 78.0% and NPV was 41.9% at this specificity.

Figure 13(a)

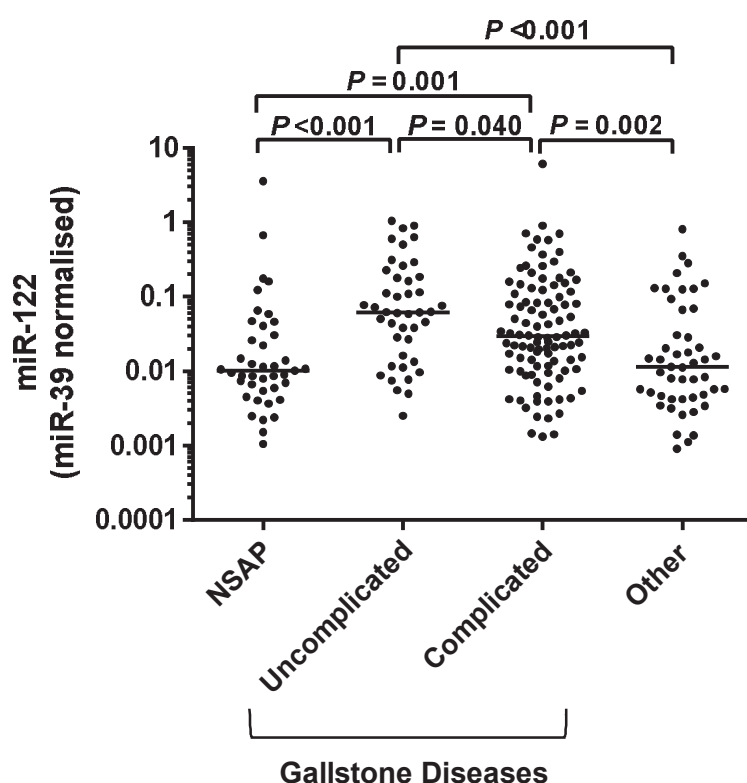


Figure 13(a): miR-122 concentrations for the main patient groups. miR-122 concentrations ($\log_{10} 2^{-\text{dct}}$) were normalised to Spike-in control miR-39. Patients with uncomplicated gallstone diseases had biliary colic and choledocholithiasis without sepsis; whereas complicated gallstone diseases were patients with cholecystitis, cholangitis and/or gallstone pancreatitis. There were 41 patients (17.7%) with NSAP, 43 patients (18.5%) with uncomplicated gallstone disease, 100 patients (43.1%) with complicated gallstone disease and 48 patients (20.7%) with an other (non-gallstone) disease. Each data point represents an individual patient and the horizontal line within each patient group represents the median value. 'NSAP': Non-specific abdominal pain; 'Other': Non-gallstone diseases of different pathologies. There was a significant difference in miR-122 concentrations between the four groups (Kruskal-Wallis test, 2 d.f., $P < 0.001$). Statistical significances (P value) by Mann Whitney U test (2-tailed) are shown in the figure for between group comparisons.

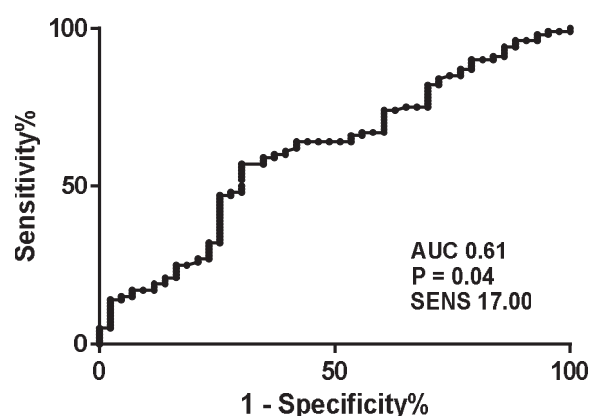
Figure 13(b)**Complicated vs. Uncomplicated Gallstone Diseases**

Figure 13(b): ROC curve of miR-122 concentration against complicated and uncomplicated gallstone diseases. Patients with uncomplicated gallstone diseases were patients who had biliary colic and/or choledocholithiasis without sepsis; whereas complicated gallstone diseases were patients with cholecystitis, cholangitis and/or gallstone pancreatitis. Area under the curve (AUC) yielded was 0.61 (95% CI: 0.51-0.71) with sensitivity of 17.0% (95% CI: 10.23-25.82) at 90% specificity; P-value 0.0398. The calculated PPV was 81.0% and NPV was 32.0% at this specificity.

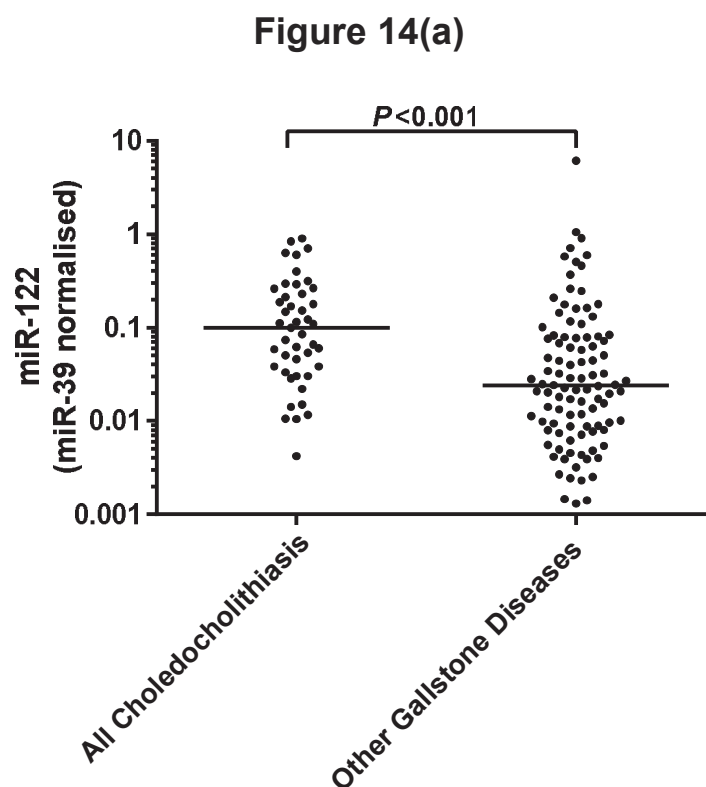


Figure 14(a): miR-122 concentrations in choledocholithiasis and other gallstone diseases. miR-122 concentrations ($\log_{10} 2^{-dct}$) were normalised to Spike-in control miR-39. Forty-five patients (31.5%) had a choledocholithiasis pathology (both aseptic and cholangitis cases) and 98 patients (68.5%) had different gallstone pathologies. Each data point represents an individual patient and the horizontal line within each patient group represents the median value. Statistical significance (P value) by Mann Whitney U test (2-tailed) is shown.

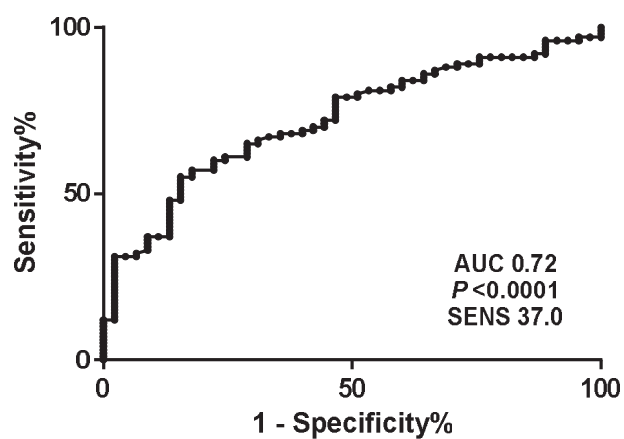
Figure 14(b)**All Choledocholithiasis vs. Other Gallstone diseases**

Figure 14(b): ROC curve of miR-122 against all choledocholithiasis and other gallstone diseases. There were 45 patients (31.5%) with choledocholithiasis and 98 patients (68.5%) with other gallstone pathologies. AUC yielded was 0.72 (95% CI: 0.64-0.81) with sensitivity of 37.0% (95% CI: 27.56-47.24) at 90% specificity; P-value <0.0001. The calculated PPV was 63.0% and NPV was 76.3% at this specificity.

Figure 15(a)

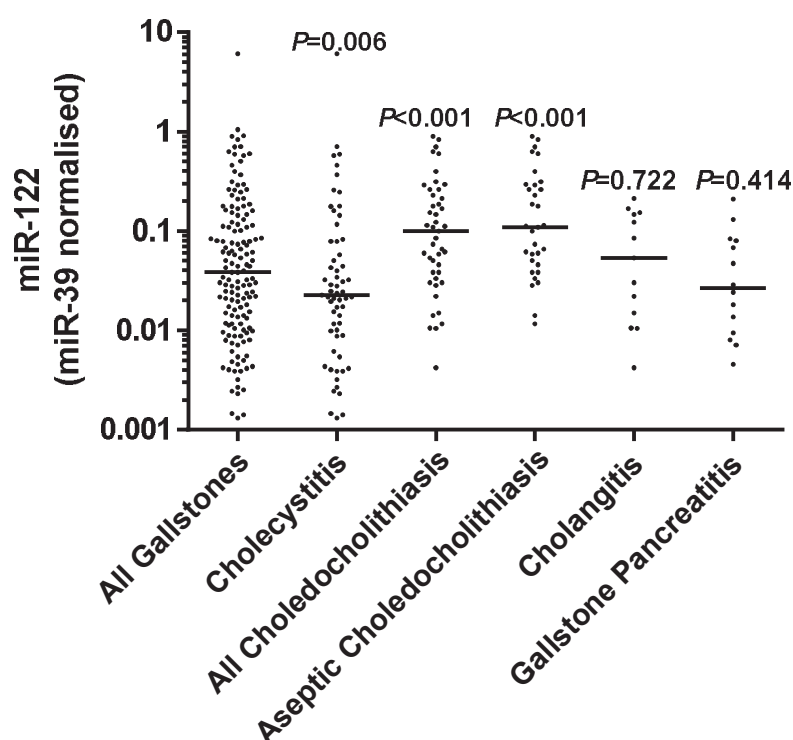


Figure 15(a): Plasma miR-122 concentrations for different gallstone diseases. miR-122 blood samples were obtained within 24 hours of patient presentation to hospital, and values were normalised to Spike-in control miR-39. A total of 143 patients (143/232, 61.6%) in the study had a gallstone pathology. Of this, 61 patients had cholecystitis, 45 patients had a choledocholithiasis (32 aseptic and 13 cholangitis patients), and 14 patients had gallstone pancreatitis. Each data point represents an individual patient and the horizontal line within each patient group represents the median miR-122 concentration. Patients with the listed gallstone diseases were compared to patients with other different gallstone pathologies; the P values by Mann Whitney U test (2-tailed) are shown.

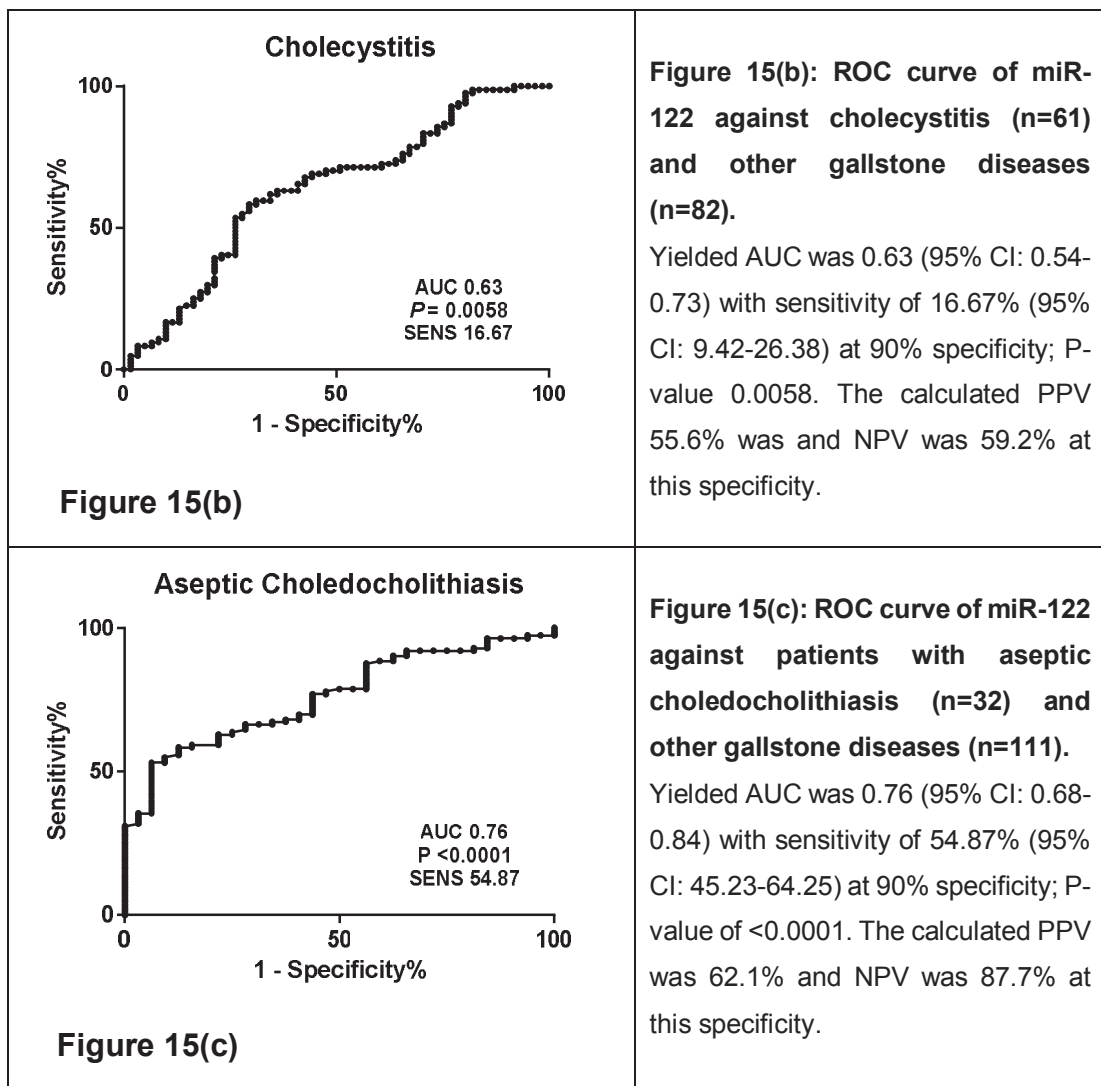
Figure 15(b)-(c)

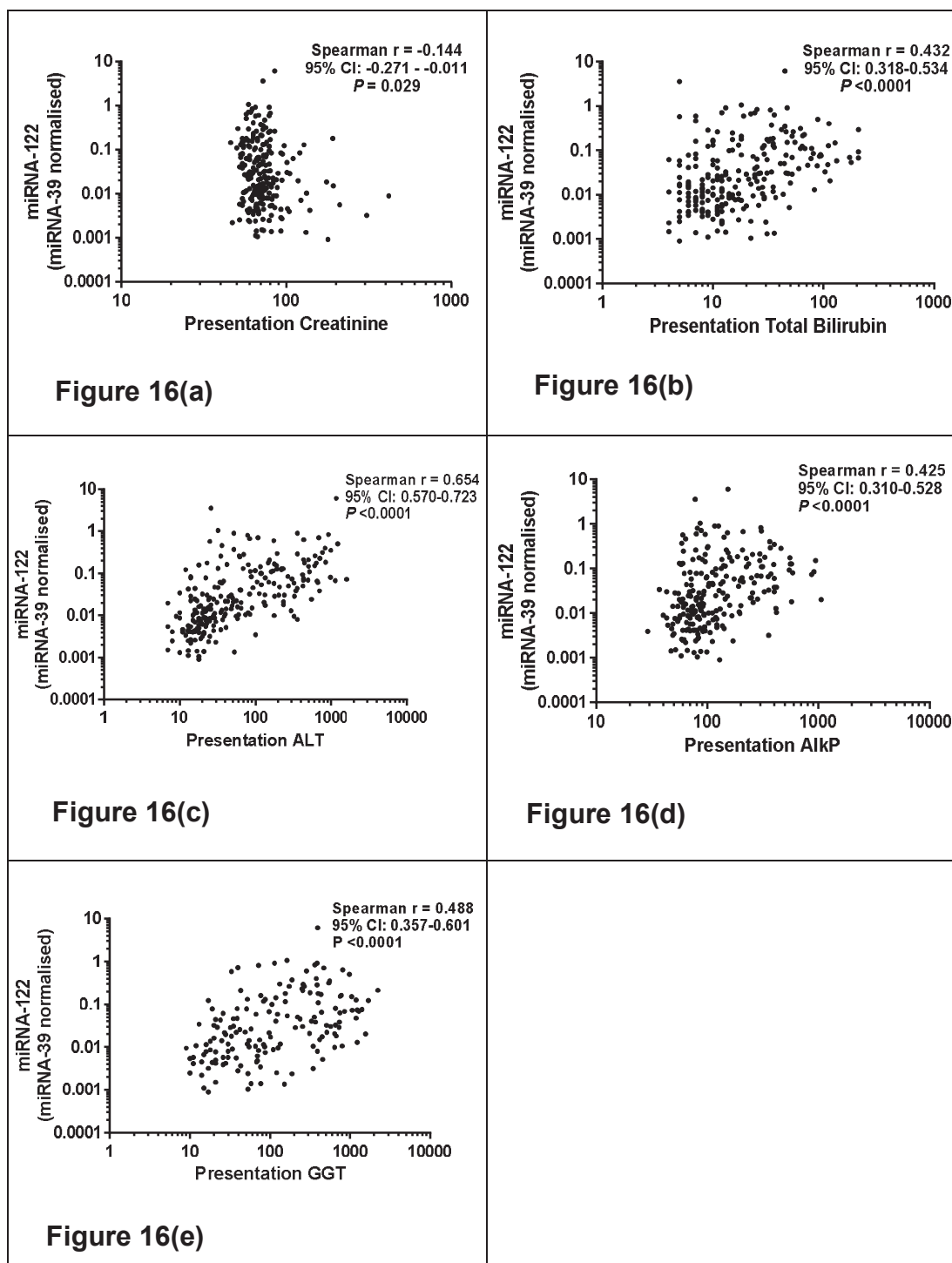
Figure 16(a)-(e)

Figure 16(a)-(e): Correlation of presentation (a) Creatinine; (b) Total Bilirubin; (c) ALT; (d) AlkP; (e) GGT, with plasma miR-122.

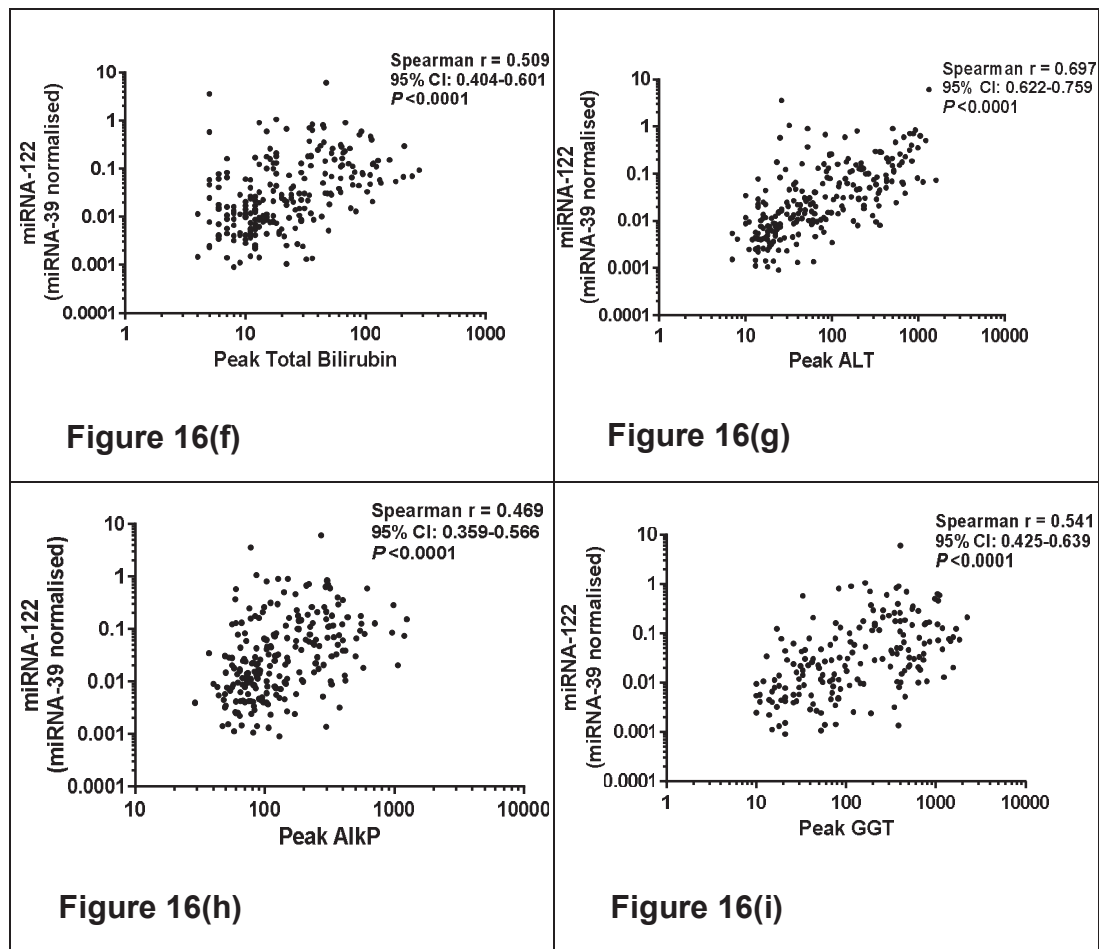
Figure 16(f)-(i)

Figure 16(f)-(i): Correlation of peak (f) Total Bilirubin; (g) ALT; (h) AlkP; (i) GGT, with plasma miR-122.

Table 1(a)

	Overall Sample	NSAP ¹	Uncomplicated ²	Complicated ³	Other ⁴	P-value
n (%)	82 (100)	17 (20.7)	16 (19.5)	35 (42.7)	14 (17.1)	
Gender (n, (%))						
Male	20 (24.4)	0	1 (6.3)	14 (40)	5 (35.7)	0.003 ⁵
Female	62 (75.6)	17 (100)	15 (93.7)	21 (60)	9 (64.3)	
Age (Years)						
Median	50.3	37.5	46.4	55.1	44.2	0.031 ⁶
IQR	32.0-66.8	26.5-60.7	27.9-52.7	44.8-68.5	27.4-65.5	
95% CI	45.0,54.6	26.7,53.9	27.2,53.2	51.3,67.7	25.8,66.4	
Total Bilirubin (umol/L)						
Median	17.0	10.0	30.0	18.0	23.5	0.030 ⁶
IQR	8.8-37.3	7.0-16.0	15.3-60.0	10.0-48.0	8.0-64.5	
95% CI	12.0,28.0	7.0,15.0	15.0,68.0	13.0,41.0	8.0,111.0	
Alanine Aminotransferase, ALT (U/L)						
Median	54.5	23.0	140.5	88.0	54.5	<0.001 ⁶
IQR	23.0-190.5	13.5-270	21.5-441.5	31.0-194.0	28.0-222.3	
95% CI	32.0,108.0	14.0,25.0	18.0,444.0	43.0,176.0	25.0,232.0	

¹ Non-Specific Abdominal Pain² Biliary colic, Aseptic choledocholithiasis³ Cholecystitis, Cholangitis, Gallstone pancreatitis⁴ Non-gallstone diseases⁵ Pearson Chi-Square test (Asymptotic significance, 2-sided)⁶ Kruskal-Wallis test for independent samples**Table 1(a): Pilot study: Demographics.**

Table 1(b)

	Overall sample	NSAP ¹	Uncomplicated ²	Complicated ³	Other ⁴	P-value ⁵
miR-122 ⁶						
Median	0.047	0.010	0.11	0.054	0.014	0.001
IQR	0.011-0.16	0.0060-0.047	0.053-0.18	0.021-0.21	0.0050-0.13	
95% CI	0.023,0.076	0.0060,0.046	0.040,0.19	0.030,0.15	0.0050,0.13	
miR-210 ⁶						
Median	0.0021	0.0019	0.0023	0.0024	0.0020	0.365
IQR	0.0016-0.0030	0.0014-0.0023	0.0016-0.0034	0.0016-0.0036	0.0016-0.0028	
95% CI	0.0020,0.0020	0.0010,0.0020	0.0020,0.0040	0.0020,0.0030	0.0020,0.0030	

¹ Non-Specific Abdominal Pain

² Biliary colic and Choledocholithiasis

³ Cholecystitis, Cholangitis, Gallstone Pancreatitis

⁴ Non-gallstone diseases

⁵ Kruskal-Wallis test for independent samples

⁶ 2^{-dct} values

Table 1(b): Pilot study: Plasma miRNA concentrations. miRNA blood samples were obtained within 24 hours of presentation laboratory bloods. miRNAs were normalised to spike-in control miR-39. miRNA concentrations are expressed to 2 significant figures.

Table 1(c)

miRs	Laboratory tests ¹	Correlation Coefficient (r) ²	95% CI	P-value
-122	Total bilirubin	0.342	0.128-0.525	0.0017
	ALT	0.640	0.487-0.756	<0.0001
-210	Total bilirubin	0.104	(-0.115)-0.314	0.351
	ALT	0.336	0.128-0.516	0.002

¹ Obtained upon patient presentation to hospital

² Spearman's rank correlation test

Table 1(b): Pilot study: Correlation of miRNAs with presentation Total Bilirubin and ALT.

miRNA blood samples were obtained within 24 hours of presentation laboratory bloods. miRNAs were normalised to spike-in control miR-39.

Table 2

	Overall	NSAP ¹	Uncomplica- ted ²	Complica- ted ³	Other ⁴	P-value
n (%)	232 (100)	41 (17.7)	43 (18.5)	100 (43.1)	48 (20.7)	
Gender (n, (%))						
Male	71 (30.6)	1 (2.4)	5 (11.6)	48 (48.0)	17 (35.4)	<0.001 ⁶
Female	161 (69.4)	40 (97.6)	38 (88.4)	52 (52.0)	31 (64.6)	
Age (Years)						
Median	50.0	42.2	43.2	55.9	48.2	<0.001 ⁷
IQR	36.3-66.2	29.8-57.4	27.2-56.4	44.7-68.6	36.4-66.2	
95% CI	46.4,53.4	31.8 , 53.8	31.4 , 49.4	51.5 , 64.7	40.5 , 61.8	
Right Upper Quadrant Abdominal Pain (n, (%))						
Yes	181 (78.0)	32 (78.0)	35 (81.4)	73 (73.0)	41 (85.4)	0.350 ⁶
No	51 (22.0)	9 (22.0)	8 (18.6)	27 (27.0)	7 (14.6)	
Past Medical History of Alcohol Excess (n, (%))						
Yes	11 (4.7)	0	2 (4.7)	6 (6.0)	3 (6.3)	0.452 ⁶
No	221 (95.3)	41 (100)	41 (95.3)	94 (94.0)	45 (93.7)	
Past Medical History of Gallstones (n, (%))						
Yes	74 (31.9)	11 (26.8)	21 (48.8)	33 (33.0)	9 (18.8)	0.018 ⁶
No	158 (68.1)	30 (73.2)	22 (51.2)	67 (67.0)	39 (81.2)	
Presented with SIRS ⁵ (n, (%))						
Yes	49 (21.1)	5 (12.2)	6 (14.0)	23 (23.0)	15 (31.2)	0.091 ⁶
No	183 (78.9)	36 (87.8)	37 (86.0)	77 (77.0)	33 (68.8)	
Blood Cultures (n, (%))						
Yes	48 (20.7)	1 (2.4)	5 (11.6)	29 (29.0)	13 (27.1)	0.001 ⁶
No	184 (79.3)	40 (97.6)	38 (88.4)	71 (71.0)	35 (72.9)	
Duration from Onset of Abdominal Pain to miRNA Sample Obtained for this Study (Hours)						
Median	60.0	72.0	48.0	60.0	63.0	0.447 ⁷
IQR	25.9-120.0	24.0-180.0	23.0-74.3	28.8-106.9	28.0-168.0	
95% CI	48.0,72.0	30.0 , 120.0	30.0 , 72.0	43.9 , 84.0	48.0 , 96.0	

¹ Non-specific abdominal pain² Biliary colic, Aseptic choledocholithiasis³ Cholecystitis, Cholangitis, Gallstone pancreatitis⁴ Non-gallstone diseases⁵ Systemic Inflammatory Response Syndrome⁶ Pearson Chi-Square test (Asymptotic significance, 2-sided)⁷ Kruskal-Wallis test for independent samples**Table 2: Demographics of the main study cohort.**

Table 3(a)
[Part 1]

Present- ation Bloods	Overall (n=232)	NSAP ¹ (n=41)	Uncompli- cated ² (n=43)	Complica- ted ³ (n=100)	Other ⁴ (n=48)	P- value ⁵
White Cell Count, WCC (x 10 ⁹ /L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	0.002
Median	9.7	8.2	8.8	10.8	10.7	
IQR	7.3-13.2	6.9-9.7	6.5-12.2	8.4-13.6	7.6-14.5	
95% CI	9.0,10.5	7.2 , 9.0	7.3 , 10.9	9.4 , 11.3	8.7 , 13.0	
C-reactive Protein, CRP (mg/L)						
n (%)	190 (81.9)	31 (75.6)	34 (79.1)	84 (84.0)	41 (85.4)	<0.001
Median	10.5	3.0	6.0	17.5	11.0	
IQR	3.0-53.8	2.0-12.0	3.8-17.3	7.0-127.8	3.0-62.0	
95% CI	8.0,15.0	2.0 , 9.0	4.0 , 12.0	12.0 , 42.0	4.0 , 24.0	
Serum Creatinine (umol/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	69.0	67.0	63.0	73.0	71.5	
IQR	61.0-79.0	60.0-71.0	57.0-75.0	63.3-83.0	64.3-77.5	
95% CI	67.0,71.0	61.0 , 69.0	60.0 , 68.0	69.0 , 78.0	67.0 , 72.0	
Total Bilirubin (umol/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	0.001
Median	15.0	11.0	22.0	19.0	11.5	
IQR	8.3-34.0	7.0-15.0	10.0-44.0	10.0-44.5	7.0-32.0	
95% CI	12.0,18.0	8.0 , 12.0	12.0 , 32.0	13.0 , 29.0	9.0 , 19.0	
Alanine Aminotransferase, ALT (U/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	38.5	22.0	105.0	58.0	44.5	
IQR	19.3-174.3	14.5-27.5	22.0-430.0	21.3-217.0	18.3-94.8	
95% CI	30.0,53.0	18.0 , 25.0	36.0 , 273.0	32.0 , 113.0	25.0 , 65.0	
Alkaline Phosphatases, AlkP (U/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	94.0	78.0	135.0	98.0	86.5	
IQR	70.0-173.8	59.0-101.0	86.0-209.0	70.3-200.8	70.3-226.5	
95% CI	86.0,106.0	67.0 , 90.0	101.0 , 154.0	84.0 , 127.0	75.0 , 122.0	
Gamma-glutamyl Transpeptidase, GGT (U/L)						
n (%)	161 (69.4)	27 (65.9)	35 (81.4)	66 (66.0)	33 (68.8)	<0.001
Median	97.0	35.0	238.0	160.5	72.0	
IQR	32.5-390.0	14.0-55.0	87.0-402.0	46.8-455.0	30.0-422.0	
95% CI	70.0,152.0	15.0 , 53.0	124.0 , 364.0	86.0 , 347.0	33.0 , 152.0	
Amylase (U/L)						
n (%)	226 (97.4)	40 (97.6)	42 (97.7)	98 (98.0)	46 (95.8)	0.795
Median	49.0	52.0	49.0	48.0	47.0	
IQR	34.0-73.0	39.5-72.3	33.8-62.0	33.0-78.5	31.0-86.3	
95% CI	44.0,53.0	45.0 , 59.0	38.0 , 59.0	40.0 , 57.0	37.0 , 67.0	

Table 3(a)
[Part 2]

- ¹ Non-specific abdominal pain
- ² Biliary colic, Aseptic choledocholithiasis
- ³ Cholecystitis, Cholangitis, Gallstone pancreatitis
- ⁴ Non-gallstone diseases
- ⁵ Kruskal-Wallis test for independent samples

Table 3(a): Presentation laboratory blood results.

Table 3(b)
[Part 1]

Peak Blood Tests	Overall (n=232)	NSAP ¹ (n=41)	Uncom- plicated ² (n=43)	Complica- ted ³ (n=100)	Other ⁴ (n=48)	P- value ⁵
White Cell Count, WCC (x 10 ⁹ /L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	10.3	8.2	9.7	11.3	11.5	
IQR	7.8-14.3	6.9-9.8	7.1-13.6	8.9-15.2	8.4-14.7	
95% CI	9.6,11.2	7.4 , 9.2	7.5 , 11.5	10.3 , 13.1	9.1 , 14.0	
C-reactive Protein, CRP (mg/L)						
n (%)	210 (90.5)	33 (80.5)	36 (83.7)	96 (96.0)	45 (93.8)	<0.001
Median	16.5	4.0	6.5	45.0	46.0	
IQR	5.0-107.3	2.0-16.5	4.0-27.0	10.3-173.8	35.0-167.0	
95% CI	12.0,24.0	3.0 , 12.0	5.0 , 21.0	17.0 , 91.0	9.0 , 114.0	
Serum Creatinine (umol/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	70.0	67.0	65.0	73.5	72.0	
IQR	62.3-80.8	60.5-71.0	59.0-75.0	63.3-87.8	67.0-87.3	
95% CI	68.0,71.0	62.0 , 69.0	62.0 , 70.0	70.0 , 79.0	68.0 , 79.0	
Total Bilirubin (umol/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	0.001
Median	18.0	12.0	23.0	26.0	14.5	
IQR	11.0-44.0	7.5-11.5	11.0-62.0	12.3-51.5	9.0-44.8	
95% CI	15.0,22.0	8.0 , 14.0	15.0 , 36.0	18.0 , 34.0	10.0 , 26.0	
Alanine Aminotransferase, ALT (U/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	58.5	23.0	15.0	97.0	54.5	
IQR	22.0-217.5	15.5-37.0	32.0-449.0	32.0-315.5	22.0-184.5	
95% CI	45.0,80.0	18.0 , 29.0	45.0 , 324.0	63.0 , 147.0	33.0 , 80.0	
Alkaline Phosphatases, AlkP (U/L)						
n (%)	232 (100)	41 (100)	43 (100)	100 (100)	48 (100)	<0.001
Median	110.5	78.0	149.0	137.0	95.0	
IQR	73.0-235.8	58.0-101.0	92.0-293.0	78.3-263.0	74.0-282.3	
95% CI	97.0,127.0	67.0 , 90.0	119.0 , 215.0	110.0 , 183.0	82.0 , 149.0	
Gamma-glutamyl Transpeptidase, GGT (U/L)						
n (%)	181 (78.0)	30 (73.2)	35 (81.4)	80 (80.0)	36 (75.0)	<0.001
Median	114.0	28.0	285.0	222.0	79.0	
IQR	35.0-412.5	14.8-57.8	87.0-498.0	68.5-506.8	30.8-450.0	
95% CI	77.0,189.0	16.0 , 53.0	124.0 , 402.0	141.0 , 371.0	43.0 , 345.0	
Amylase (U/L)						
n (%)	229 (98.7)	40 (97.6)	43 (100)	99 (99.0)	47 (97.9)	0.805
Median	51.0	52.0	50.0	51.0	55.0	
IQR	36.5-81.5	39.5-72.3	36.0-79.0	34.0-89.0	37.0-147.0	
95% CI	47.0,57.0	45.0 , 59.0	41.0 , 60.0	41.0 , 62.0	42.0 , 68.0	

Table 3(b)
[Part 2]

- ¹ Non-specific abdominal pain
- ² Biliary colic, Aseptic choledocholithiasis
- ³ Cholecystitis, Cholangitis, Gallstone pancreatitis
- ⁴ Non-gallstone diseases
- ⁵ Kruskal-Wallis test for independent samples

Table 3(b): Peak laboratory blood results during individual patient's hospital episode.

All patients were followed up and blood results were obtained electronically from patient archives.

Table 4

	Overall (n=232)	NSAP ¹ (n=41)	Uncompli- cated ² (n=43)	Complica- ted ³ (n=100)	Other ⁴ (n=48)	P- value ⁵
Ultrasonography, USS (n, (%))						
Yes	181 (78.0)	32 (78.0)	28 (65.1)	77 (77.0)	44 (91.7)	0.024
No	51 (22.0)	9 (22.0)	15 (34.9)	23 (23.0)	4 (8.3)	
Computed Tomography, CT (n, (%))						
Yes	53 (22.8)	8 (19.5)	7 (16.3)	17 (17.0)	21 (43.8)	0.002
No	179 (77.2)	33 (80.5)	36 (83.7)	83 (83.0)	27 (56.3)	
Magnetic Resonance Cholangiopancreatography, MRCP (n, (%))						
Yes	82 (35.3)	8 (19.5)	19 (44.2)	44 (44.0)	11 (22.9)	0.006
No	150 (64.7)	33 (80.5)	24 (55.8)	56 (56.0)	37 (77.1)	
Number who underwent ≥1 imaging						
n (%)	221 (95.3)	38 (92.7)	40 (93.0)	96 (96.0)	47 (97.9)	0.583
Gallstones on imaging (n, (%)) ⁶						
Yes	127 (57.5)	2 (5.3)	33 (82.5)	86 (89.6)	6 (12.8)	<0.001
No	94 (42.5)	36 (94.7)	7 (17.5)	10 (10.4)	41 (87.2)	
Cholecystitis on imaging (n, (%)) ⁶						
Yes	50 (22.6)	0	3 (7.5)	45 (46.9)	2 (4.3)	<0.001
No	171 (77.4)	38 (100)	37 (92.5)	51 (53.1)	45 (95.7)	

¹ Non-specific abdominal pain² Biliary colic, Aseptic choledocholithiasis³ Cholecystitis, Cholangitis, Gallstone pancreatitis⁴ Non-gallstone diseases⁵ Pearson Chi-Square test (Asymptotic significance, 2-sided)⁶ As a percentage of the number of patients who underwent ≥ 1 imaging in that patient group**Table 4: Imaging investigations.**

Table 5

	Overall	NSAP ¹	Uncompli- cated ²	Complica- ted ³	Other ⁴	P- value
Underwent Operation (n, (%))						
Yes	101 (43.5)	3 (7.3)	20 (46.5)	77 (77.0)	1 (2.1)	<0.001 ⁷
No	131 (56.5)	38 (92.7)	23 (53.5)	23 (23.0)	47 (97.9)	
Duration from index presentation to Operation (Days)						
Median	2.0	1.0	2.0	2.0	4.0	0.715 ⁸
IQR	1.0-6.0	-	1.0-6.3	1.0-6.0	4.0-4.0	
95% CI	1.0 , 3.0	0 , 4.0	1.0 , 4.0	1.0 , 3.0	-	
Cholesterolosis (n,(%)) ⁵						
Yes	20 (19.8)	2 (66.7)	5 (25.0)	12 (15.6)	1 (100)	0.024 ⁷
No	81 (80.2)	1 (33.3)	15 (75.0)	65 (84.4)	0	
Gangrenous/necrotic gallbladder (n, (%)) ⁵						
Yes	20 (19.8)	0	0	19 (24.7)	1 (100)	0.063 ⁷
No	81 (80.2)	3 (100)	20 (100)	58 (75.3)	0	
Fatty liver (n, (%)) ⁶						
Yes	38 (16.5)	5 (12.8)	8 (18.6)	18 (18.0)	7 (14.6)	0.847 ⁷
No	192 (83.5)	34 (87.2)	35 (81.4)	82 (82.0)	41 (85.4)	

¹ Non-Specific Abdominal Pain

² Biliary colic, Aseptic choledocholithiasis

³ Cholecystitis, Cholangitis, Gallstone Pancreatitis

⁴ Non-gallstone diseases

⁵ As a percentage of the number patients who underwent an operation within each patient group

⁶ As a percentage of the number of patients who had at least one imaging carried out and/or Surgeon's findings on operation and/or is known to have fatty liver from previous investigations (i.e. Total number of patients in NSAP = 39; Uncomplicated = 43; Complicated = 100; Other = 48)

⁷ Pearson Chi-Square test (Asymptotic significance, 2-sided)

⁸ Kruskal-Wallis test for independent samples

Table 5: Operative and histological characteristics.

Table 6

Imaging Investigations	Sensitivity (%)	Specificity (%)	PPV¹ (%)	NPV² (%)
Overall Sample (n=97)				
Gallstones	94.4	37.5	94.4	37.5
Cholecystitis	52.5	82.9	84.2	50.0
Ultrasonography, USS (n=80)				
Gallstones	93.2	28.6	93.2	28.6
Cholecystitis	59.2	83.9	85.3	56.2
Computed Tomography, CT (n=18)				
Gallstones	92.9	50.0	86.7	66.7
Cholecystitis	41.7	83.3	83.3	41.7
Magnetic Resonance Cholangiopancreatography, MRCP (n=35)				
Gallstones	93.9	50.0	96.9	33.3
Cholecystitis	41.7	72.7	76.9	36.4

¹ Positive predictive value² Negative predictive value

Table 6: Evaluation of imaging investigations. Analyses were carried for patients who had a cholecystectomy. Pathology results and/or Surgeon's findings (macroscopic) on operation were used to determine the final diagnosis of patients, for comparison against imaging results.

Table 7

	Ratio (%)	miR-122 ¹			P-value ²
		Median	IQR	95% CI (Lower , Upper)	
Gallstone Pathology					
Yes	143/232 (61.6)	0.039	0.012-0.13	0.027 , 0.059	<0.001
No	89/232 (38.4)	0.011	0.0046-0.041	0.0079 , 0.015	

¹ 2^{-dct} values, normalised to miR-39

² Mann-Whitney U test for independent samples

Table 7: Plasma miR-122 concentration between gallstone and non-gallstone diseases. Values are normalised to spike-in control miR-39.

Table 8

	ROC AUC (95% CI)	Sensitivity, % (95% CI)	PPV¹ (%)	NPV² (%)	P- value
Gallstone vs. Non-gallstone³ diseases	0.65 (0.57-0.74)	22.22 (13.27-33.56)	78.0	41.9	0.001
Complicated⁴ vs. Uncomplicated⁵ gallstone diseases	0.61 (0.51-0.71)	17.0 (10.23-25.82)	81.0	32.0	0.0398

¹ Positive predictive value

² Negative predictive value

³ NSAP and Other (non-gallstone) diseases

⁴ Cholecystitis, Cholangitis, Gallstone pancreatitis

⁵ Biliary colic, Aseptic choledocholithiasis

Table 8: Evaluation of plasma miR-122 from ROC curve analysis. Positive predictive value (PPV) and negative predictive value (NPV) calculated for group comparisons. Sensitivity, PPV and NPV values obtained at 90% specificity. P-values of AUC are significant at 0.05.

Table 9

	Overall (n=232)	NSAP ¹ (n=41)	Uncomplicated ² (n=43)	Complicated ³ (n=100)	Other ⁴ (n=48)	P-value ⁵
miR-122⁶						
Median	0.022	0.010	0.062	0.030	0.011	<0.001
IQR	0.0080-0.098	0.0057-0.036	0.016-0.19	0.011-0.12	0.0045-0.058	
95% CI	0.016 , 0.031	0.0074 , 0.015	0.039 , 0.11	0.022 , 0.047	0.0057 , 0.018	

¹ Non-specific abdominal pain

² Biliary colic, Aseptic choledocholithiasis

³ Cholecystitis, Cholangitis, Gallstone pancreatitis

⁴ Non-gallstone diseases

⁵ Kruskal-Wallis test for independent samples (2-tailed)

⁶ 2^{-dct} values, normalised to miR-39

Table 9: Plasma miR-122 concentrations for main patient groups. Plasma samples obtained for miR-122 analysis were obtained within 24 hours of patient's presentation to hospital. Expression of the miRNA was normalised to Spike-in control miR-39. miR-122 concentrations are expressed to 2 significant figures.

Table 10(a)

	Ratio (%) ¹	miR-122 ²			P- value ³
		Median	IQR	95% CI (Lower , Upper)	
Cholecystitis only					
Yes	61/143 (42.7)	0.023	0.0089-0.079	0.017 , 0.032	0.006
No	82/143 (57.3)	0.062	0.014-0.16	0.039 , 0.083	
All Choledocholithiasis ⁴					
Yes	45/143 (31.5)	0.099	0.036-0.25	0.054 , 0.17	<0.001
No	98/143 (68.5)	0.024	0.0088-0.079	0.017 , 0.034	
Aseptic choledocholithiasis ⁵					
Yes	32/143 (22.4)	0.11	0.047-0.30	0.059 , 0.26	<0.001
No	111/143 (77.6)	0.024	0.0092-0.083	0.018 , 0.040	
Cholangitis					
Yes	13/143 (9.1)	0.054	0.013-0.15	0.011 , 0.15	0.722
No	130/143 (90.9)	0.036	0.012-0.12	0.025 , 0.059	
Gallstone pancreatitis					
Yes	14/143 (9.8)	0.027	0.0091-0.081	0.0080 , 0.083	0.414
No	129/143 (90.2)	0.039	0.012-0.15	0.027 , 0.061	

¹ As a percentage of number of patients with gallstone disease (n=143)

² 2^{-dct} values, normalised to miR-39

³ Mann-Whitney U test for independent samples

⁴ Aseptic choledocholithiasis and/or Cholangitis

⁵ Choledocholithiasis without clinical or microbiological evidence of severe inflammation or sepsis

Table 10(a): miR-122 concentrations for different gallstone diseases. All cases were expressed as a percentage as the total number of patients with a gallstone pathology (n=143). Mann Whitney U test for P values are significant at 0.05 (2-tailed). Patients with all types of choledocholithiasis (aseptic and cholangitis) were further divided into its subgroups and compared individually against other patients with gallstones. miR-122 concentration values are expressed to 2 significant figures.

Table 10(b)

Gallstone Disease¹	ROC AUC (95% CI)	Sensitivity, % (95% CI)	PPV (%)²	NPV (%)³	P-value
Cholecystitis	0.63 (0.54-0.73)	16.67 (9.42-26.38)	55.6	59.2	0.0058
All Choledocholithiasis⁴	0.72 (0.64-0.81)	37.00 (27.56-47.24)	63.0	75.9	<0.0001
Aseptic Choledocholithiasis⁵	0.76 (0.68-0.84)	54.87 (45.23-64.25)	62.1	87.7	<0.0001

¹ Diseases listed as a comparison to other types of gallstone pathologies

² Positive predictive value

³ Negative predictive value

⁴ Aseptic choledocholithiasis and cholangitis cases

⁵ Choledocholithiasis without clinical or microbiological evidence of severe inflammation or sepsis

Table 10(b): Evaluation of plasma miR-122 from ROC curve analysis for gallstone diseases. Sensitivity, PPV and NPV values were obtained at 90% specificity. P-values of AUC are significant at 0.05.

Table 10(c)

Complicated Gallstone Diseases ¹	Presentation Alanine Aminotransferase, ALT (U/L)			P-value ²
	Median	IQR	95% CI	
Cholecystitis				
Yes	28.0	17.5-82.0	21.0 , 40.0	<0.001
No	173.5	35.3-429.8	90.0 , 241.0	
All Choledocholithiasis ³				
Yes	241.0	115.0-511.0	175.0 , 359.0	<0.001
No	32.0	18.0-119.0	25.0 , 42.0	
Aseptic Choledocholithiasis ⁴				
Yes	333.5	155.5-663.0	175.0 , 614.0	<0.001
No	36.0	19.0-141.0	28.0 , 56.0	
Cholangitis				
Yes	178.0	72.5-255.0	60.0 , 269.0	0.121
No	51.5	20.3-263.5	35.0 , 105.0	
Gallstone Pancreatitis				
Yes	279.0	34.3-446.8	32.0 , 500.0	0.033
No	56.0	20.0-203.0	35.0 , 105.0	

¹ Diseases listed as a comparison to other types of gallstone pathologies

² Mann-Whitney U test for independent samples (2-tailed)

³ Aseptic choledocholithiasis and cholangitis cases

⁴ Choledocholithiasis without clinical or microbiological evidence of severe inflammation or sepsis

Table 10(c): Analysis of presentation ALT values in complicated gallstone diseases.**Table 10(d)**

Presentation Liver Function Tests (LFTs)	ROC AUC (95% CI)	Sensitivity, % (95% CI)	P-value
Total Bilirubin	0.76 (0.68-0.84)	34.00 (24.82-44.15)	<0.0001
Alanine Aminotransferase, ALT	0.80 (0.721-0.87)	64.95 (54.59-74.36)	<0.0001
Alkaline Phosphatase, AlkP	0.83 (0.76-0.90)	61.86 (51.43-71.53)	<0.0001
Gamma-glutamyl Transpeptidase, GGT	0.72 (0.62-0.82)	54.10 (40.85-66.94)	<0.0001

Table 10(d): Evaluation of presentation liver function tests (LFTs) from ROC curve analysis in choledocholithiasis. Analysis includes aseptic and cholangitis cases, compared against other gallstone pathologies. Sensitivity values were obtained at 90% specificity. P-values of AUC are significant at 0.05.

Table 11

	Ratio (%)	miR-122 ¹			P-value ²
		Median	IQR	95% CI (Lower , Upper)	
Gallstone (GS) pancreatitis vs. Non-gallstone (Non-GS) pancreatitis					
GS ³	14/24 (58.3)	0.027	0.0091-0.081	0.0080 , 0.083	0.437
Non-GS ³	10/24 (41.7)	0.011	0.0040-0.19	0.0034 , 0.35	
Cholesterolosis ⁴					
Yes	20/101 (19.8)	0.030	0.010-0.080	0.010 , 0.068	0.498
No	81/101 (80.2)	0.031	0.012-0.15	0.021 , 0.066	
Necrotic/gangrenous cholecystitis ⁵					
Yes	17/56 (30.4)	0.022	0.010-0.10	0.012 , 0.083	0.964
No	39/56 (69.6)	0.024	0.010-0.058	0.017 , 0.040	
Fatty liver ⁹					
Yes	38/230 (16.5)	0.021	0.0095-0.053	0.013 , 0.028	0.569
No	192/230 (83.5)	0.026	0.0080-0.11	0.016 , 0.040	

¹ 2^{-dct} values, normalised to miR-39

² Mann-Whitney U test for independent samples

³ As a percentage of the total number of patients with pancreatitis of any aetiology

⁴ As a percentage of the total number of patients who underwent an operation

⁵ As a percentage of the total number of patients were diagnosed with cholecystitis on Pathology

⁶ As a percentage of the number of patients who had at least one imaging carried out and/or Surgeon's findings on operation and/or has a PMH of fatty liver (i.e. n = 230)

Table 11: Plasma miR-122 concentrations in other pathologies. Patients with gallstone pancreatitis and non-gallstone pancreatitis were expressed as a ratio of number of patients with pancreatitis of any aetiology in the study (n=24). Proportion of patients with cholesterolosis on gallbladder pathology results was expressed as the total number of patients who underwent an operation and hence, had pathology results (n=101). Ratio of patients with evidence of necrotic and/or gangrenous gallbladder on pathology was expressed against the total number of patients who was diagnosed with having cholecystitis on Pathology (n=56). Fatty nature of patient's liver was defined by imagings from that hospital episode and/or had Surgeon's operative findings (macroscopic) and/or previous history of fatty liver either in notes or previous imagings (n=230). Two NSAP patients did not have any imagings – one self-discharged, and one's pain self-resolved and was discharged prior further investigations. Mann Whitney U test for P values are significant at 0.05 (2-tailed). miR-122 concentrations are expressed to 2 significant figures.

Table 12(a)

Presentation Blood Tests	Correlation Coefficient (r) ¹	95% CI	P-value
White Cell Count, WCC	-0.068	(-0.199) – 0.065	0.301
C-reactive Protein, CRP	0.061	(-0.081) – 0.202	0.385
Serum Creatinine	-0.144	(-0.271) – (-0.011)	0.029
Total Bilirubin	0.432	0.318 – 0.534	<0.0001
Alanine Aminotransferase, ALT	0.654	0.570 – 0.723	<0.0001
Alkaline Phosphatase, AlkP	0.425	0.310 – 0.528	<0.0001
Gamma-glutamyl Transpeptidase, GGT	0.488	0.357 – 0.601	<0.0001
Amylase	-0.114	(-0.245) – 0.020	0.087

¹ Spearman's Rank Correlation test

Table 12(a): Correlation of plasma miR-122 with presentation laboratory blood tests.

miR-122 was normalised to spike-in control miR-39. miR-122 samples were obtained within 24 hours of hospital presentation.

Table 12(b)

Peak Blood Tests	Correlation Coefficient (r) ¹	95% CI	P-value
White Cell Count, WCC	-0.007	(-0.140) – 0.126	0.915
C-reactive Protein, CRP	0.114	(-0.025) – 0.250	0.099
Serum Creatinine	-0.111	(-0.240) – 0.022	0.092
Total Bilirubin	0.509	0.404 – 0.601	<0.0001
Alanine Aminotransferase, ALT	0.697	0.622 – 0.759	<0.0001
Alkaline Phosphatase, AlkP	0.469	0.359 – 0.566	<0.0001
Gamma-glutamyl Transpeptidase, GGT	0.541	0.425 – 0.639	<0.0001
Amylase	0.020	(-0.114) – 0.153	0.769

¹ Spearman's Rank Correlation test

Table 12(b): Correlation of plasma miR-122 with peak laboratory blood tests.

miR-122 was normalised to spike-in control miR-39. miR-122 samples were obtained within 24 hours of hospital presentation.

APPENDIX 1

Appendix 1: Patient information sheet. *Three pages.*

(Page 1)



Participant Information Sheet

Stratification of gall stone disease using circulating liver specific microRNA-122 - (GB122 study)

You are being invited to take part in a research study. Before you decide whether or not to agree, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We have a new blood test that promises to identify those people who need to have urgent surgery for their gall stones and allow safe discharge of patients who can wait for out-patient treatment. We wish to collect a blood sample from you and see if our new test predicts your eventual diagnosis.

Why have I been asked to take part?

Doctors suspect that your current symptoms might be a result of having gall stones. Therefore we are inviting you to take part in this study.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Deciding not to take part or withdrawing from the study will not affect the healthcare that you receive, or your legal rights.

What will happen if I take part?

We would like to take a blood sample now. If available we will also collect your blood sample, which has already been taken, from our hospital lab.

We will record some simple information about you such as age, sex, symptoms and routine blood test results. Then we will check your medical records in around 1 month to establish your diagnosis by reviewing the scans and other tests performed during your hospital stay. This information will be kept secure within the NHS. Your information will be linked to the blood sample by a four digit number so it will not be possible for anyone to identify you from the other study participants.

Our new blood tests measure the levels of liver proteins and RNA in the blood. We will not be taking or measuring DNA. This study will create a bank of samples that we can use to develop our new tests for liver irritation. The samples will be carefully stored in a freezer for a maximum of 10 years and, with appropriate ethical approval,

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these samples may be shared with other clinical, academic and commercial researchers in the UK and worldwide.

What are the possible benefits of taking part?

There are no direct benefits to you from taking part in this study, but the results from this study might inform the future healthcare of other patients. The results of this study may be used for the future commercial development of a new test. Your participation in this study will not entitle you to benefit financially from the company developing the product, treatment or test.

What are the possible disadvantages and risks of taking part?

The only disadvantage is you may have one extra needle for our blood sample. We will try to take any routine blood samples at the same time. However, this may not always be possible. We will take great care that the blood sample cannot be identified.

What if there is a problem?

If you have a concern about any aspect of this study please contact the Edinburgh Clinical Research Facility, Dr Francesca Th'Ng or Dr James Dear who will do their best to answer your questions.

In the unlikely event that something goes wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against NHS Lothian but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

If you wish to make a complaint about the study please contact:

NHS Lothian Customer Relations & Feedback Team
Waverley Gate
2 - 4 Waterloo Place
Edinburgh
EH1 3EG
Tel: 0131 536 3370
craft@nhslothian.scot.nhs.uk

What happens when the study is finished?

At the end of the research we will store your blood samples for a maximum of 10 years. This will allow other researchers in our field the chance to benefit by using these samples.

Will my taking part in the study be kept confidential?

All the information we collect during the course of the research will be kept confidential and there are strict laws which safeguard your privacy at every stage. All identifiable records (for example your hospital number) will be kept exclusively within the NHS and will be protected by a password. Any information that leaves the NHS system will be anonymised so you cannot be identified.

With your consent we will inform your GP that you are taking part.

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To ensure that the study is being run correctly, we will ask your consent for responsible representatives from the Sponsor and NHS Institution to access your records and data collected during the study, where it is relevant. The Sponsor is responsible for overall management of the study and providing insurance and indemnity.

What will happen to the results of the study?

The study will be written up in medical publications and shared with other doctors at national and international meetings. If you are interested we will share our results with you.

Who is organising the research and why?

This study is being organised/sponsored by the University of Edinburgh and NHS Lothian.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee (REC). A favourable ethical opinion has been obtained from the East Midlands REC. NHS management approval has also been obtained.

If you have any further questions about the study please contact Dr James Dear on 0131 242 9236 or email: james.dear@ed.ac.uk

If you would like to discuss this study with someone independent of the study please contact:

**Professor Michael Eddleston
M.Eddleston@ed.ac.uk**

Thank you for taking the time to read this information sheet.

APPENDIX 2

Appendix 2: Patient data collection sheets. *Two pages.*

(Page 1)

E141403 GB122 Study	
	Stratification of gallstone disease using circulating liver microRNA and markers of cell necrosis
Participant Number:	CHI:
Sex: ☐ Male ☐ Female	
CONSENT	
Date of consent: / /	
AT PRESENTATION WITHIN 24 HOURS	
Age years	Temperature >38°C? ☐ Yes ☐ No
Known history of gall stones? ☐ Yes ☐ No	Jaundice within previous month? ☐ Yes ☐ No
Long term alcohol excess? ☐ Yes ☐ No	Rigors? ☐ Yes ☐ No
Alcohol unit intake in previous 24 hours?	
Abdominal pain? ☐ Yes ☐ No	Pulse > 90 bpm? ☐ Yes ☐ No
RUQ pain? ☐ Yes ☐ No	RR >20 breaths per minute? ☐ Yes ☐ No
Hours since pain started? hours	
BLOOD SAMPLE	
Blood Sample taken:	☐ Yes ☐ No
Time sample was taken:	:
Date sample was taken:	/ /
Initial:	
AT PRESENTATION	PEAK HOSPITAL STAY
WCC 10 ⁹ /L	WCC 10 ⁹ /L
Creatinine μmol/L	Creatinine μmol/L
ALT U/L	ALT U/L
Alk Phos U/L	Alk Phos U/L
GGT U/L	GGT U/L
Bilirubin μmol/L	Bilirubin μmol/L
Amylase U/L	Amylase U/L
CRP mg/L	CRP mg/L

(Page 2)

E141403 GB122 Study



Stratification of gallstone disease using circulating liver microRNA and markers of cell necrosis

Participant Number: CHI: Sex: ☐ Male ☐ Female

AT DISCHARGE

Discharge Diagnosis: _____

Ultrasound Result: **Stones in Gall bladder?** ☐ Yes ☐ No **Thick Walled Gall bladder?** ☐ Yes ☐ No
Fatty liver: ☐ Yes ☐ No
CBD Dilated >8mm? ☐ Yes ☐ No **Cholecystitis?** ☐ Yes ☐ No
 CT/MRI Result: _____ **CBD Stone?** ☐ Yes ☐ No **Pancreatitis?** ☐ Yes ☐ No
 Surgical Specimen: ☐ Yes ☐ No Histology Diagnosis? _____
 Blood Culture ☐ Positive ☐ Negative Organism: _____

FINDINGS AT OPERATION

Normal Gall bladder: ☐ Yes ☐ No **Acute Cholecystitis:** ☐ Yes ☐ No
Empyema: ☐ Yes ☐ No **Mucocele:** ☐ Yes ☐ No
CBD Stone on IOC: ☐ Yes ☐ No **Perforated Gall bladder/Gangrene:** ☐ Yes ☐ No
Fatty Liver: ☐ Yes ☐ No **Cirrhosis:** ☐ Yes ☐ No

Researcher Signature: _____

Date: _____

Please update subject log.

APPENDIX 3

Appendix 3: microRNA extraction from blood plasma using Qiagen miRNeasy

Step 1.

Use 50 μ l of sample and add 150 μ l of RNase free water. Add 5 volumes (1ml) QIAzol Lysis Reagent. Mix by vortexing or pipetting up and down. Incubate the homogenate at room temperature (15–25°C) for 5 min.

Step 2.

Add 3.5 μ l miRNeasy Serum/Plasma Spike-In Control (at 1.6×10^8 copies/ μ l).

Step 3.

Add chloroform of an equal volume to the starting sample and cap tube securely (e.g., for 200 μ l sample, add 200 μ l chloroform). Shake vigorously for 15 s. Incubate at room temperature for 2–3 min.

Step 4.

Centrifuge for 15 min at $12,000 \times g$ at 4°C.

Step 5.

Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transferring any interphase. Add 1.5 volumes of 100% ethanol (e.g., for 600 μ l aqueous phase, add 900 μ l ethanol). Mix thoroughly by pipetting.

Step 6.

Pipet up to 700 μ l sample, including any precipitate, into an RNeasy MinElute spin column in a 2 ml collection tube. Close the lid and centrifuge at $\geq 8000 \times g$ for 15 s at room temperature. Discard the flow-through. Repeat using the remainder of the sample.

Step 7.

Add 700 μ l Buffer RWT to the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at $\geq 8000 \times g$. Discard the flow-through.

Step 8.

Pipet 500 μ l Buffer RPE onto the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at $\geq 8000 \times g$. Discard the flow-through.

Serum/Plasma-Kit. *(Extracted from Reference 266).***Step 9.**

Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid, and centrifuge for 2 min at $\geq 8000 \times g$. Discard the flow-through and the collection tube.

Step 10.

Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and the collection tube.

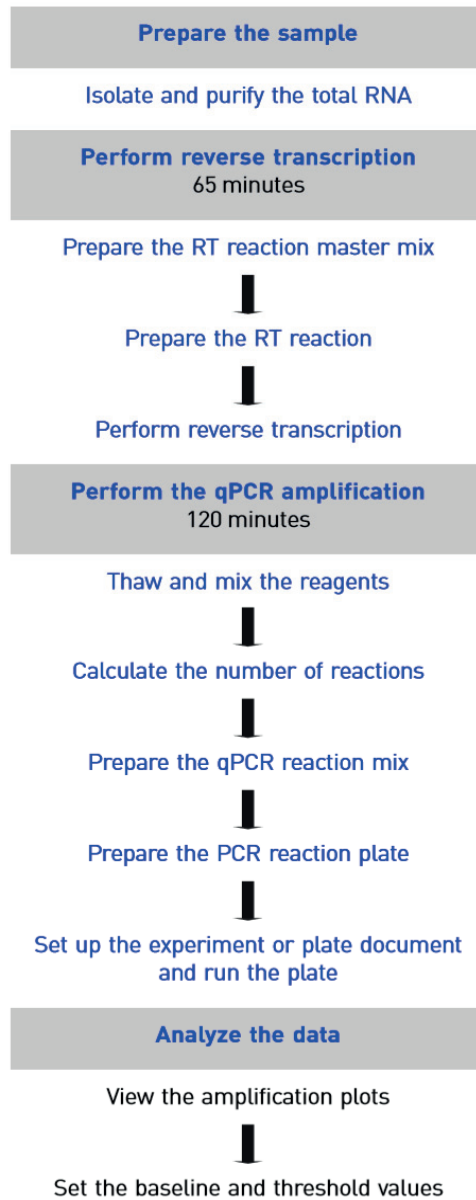
Step 11.

Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid. Spin for 1 minute.

APPENDIX 4

Appendix 4: Procedural overview of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis. (Extracted from Reference 268).

Procedural overview



APPENDIX 5

Appendix 5: Other demographics of the main study.

	Overall	NSAP ¹	Uncompli- cated ²	Compli- cated ³	Others ⁴	P-value
Jaundice						
Yes	37 (15.9)	0	6 (14)	24 (24.0)	7 (14.6)	0.005 ⁶
No	195 (84.1)	41 (100)	37 (86)	76 (76.0)	41 (85.4)	
Rigors						
Yes	75 (32.3)	14 (34.1)	10 (23.3)	36 (36.0)	15 (31.3)	0.508 ⁶
No	157 (67.7)	27 (65.69)	33 (76.7)	64 (64.0)	33 (68.8)	
Temp <36°C or >38°C ⁵						
Yes	29 (12.5)	1 (2.4)	3 (7.0)	20 (20.0)	5 (10.4)	0.016 ⁶
No	203 (87.5)	40 (97.6)	40 (93.0)	80 (80.0)	43 (89.6)	
Heart Rate >90 bpm ⁵						
Yes	67 (28.9)	13 (31.7)	11 (25.6)	24 (24.0)	19 (39.6)	0.238 ⁶
No	165 (71.1)	28 (68.3)	32 (74.4)	76 (76.0)	29 (60.4)	
Respiratory Rate >20 breaths per minute ⁵						
Yes	15 (6.5)	1 (2.4)	2 (4.7)	9 (9.0)	3 (6.3)	0.494 ⁶
No	217 (93.5)	40 (97.6)	41 (95.3)	91 (91.0)	45 (93.8)	
Alcohol intake in last 24 hours prior miRNA blood sampling						
Median	0	Only two patients had ingested alcohol within 24 hours prior to hospital presentation. One patient (2 units of alcohol) with NSAP and one patient (1 unit of alcohol) with Other disease				0.731 ⁷
IQR	0					
95% CI	0					

¹ Non-Specific Abdominal Pain

² Biliary colic, Aseptic Choledocholithiasis

³ Cholecystitis, Cholangitis, Gallstone Pancreatitis

⁴ Non-gallstone diseases

⁵ Part of SIRS criteria

⁶ Pearson Chi-Square test (Asymptotic significance, 2-sided)

⁷ Kruskal-Wallis test for independent samples

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