

**GENE EXPRESSION AND MICRO
RNA PROFILING IN ACUTE
PANCREATITIS**

by

Miss Kiran Altaf

(MBBS, MRCS)

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Abstract

Introduction

Prognostication in Acute pancreatitis (AP) remains a challenging issue. This study aimed to measure gene expression of the peripheral blood in patients with acute pancreatitis and identify an expression signature that would accurately stratify patients into either mild or severe disease groups. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and play important roles in a variety of cellular functions. These have emerged as major potential biomarkers in a variety of sepsis related disorders and cancers. Alongside, gene expression profiling, the prognostic potential of micro RNA at the time of admission in context of acute pancreatitis was also explored.

Methods

This observational study was conducted at Royal Liverpool University Hospital, NHS Foundation Trust, in collaboration with University Hospital Aintree, NHS Foundation Trust. For gene expression profiling, Affymetrix HGU133 Plus 2.0 microarrays were utilised. Total RNA extracted from whole blood samples of patients collected at the time of admission was included. A pilot study was conducted in the first instance to enable sample size calculations. Analysis was performed using Partek Genomics Suite software. Expression level data were quantile normalised and ANOVA was used with batch hybridization effects.

For microRNA analysis, a similar pilot study was undertaken to estimate sample size. Total RNA was extracted from plasma samples of patients obtained at the time of admission. RNA were hybridised to GeneChip™ miRNA 2.0 arrays. Analysis was performed using appropriate packages in R/Bioconductor and using Partek Genomics Suite software.

Expression level data were invariant set normalized. Differential expression of miRNAs in severe compared to mild pancreatitis was detected using ANOVA with batch hybridization effects removed. Severity was defined in line with Atlanta criteria for both the parts of the study.

Results

58 patients were included (23 severe, 35 mild). After adjusting for batch effects, setting power at 80%, fold change at 1.5 and significance at 0.05, 98 genes were identified that were differentially expressed between mild and severe disease. More specifically, 49 genes were up-regulated in severe form and 49 were down-regulated when compared to the mild disease. Canonical pathway analysis revealed signalling in T cells and lymphocytes to be the most significant pathway involved in the process. These were specifically down-regulated in severe form of the disease. While a lot of processes associated with the cellular immune response were down-regulated in severe pancreatitis, the innate response didn't change and the humoral response might be stimulated. Nuclear Factor of Activated T cells (NFAT) was noted to be the most significantly implicated gene in the dataset, being a key molecule implicated in at least 12 different pathways.

Nineteen patients were included (severe 9, mild 10) for the miRNA profiling. Keeping the FDR $p < 0.05$, 45 micro RNA were found to be differentially expressed between mild and severe pancreatitis. Out of these, only 23 were annotated in IPA – 22 were novel discoveries. Interestingly, 19 small nucleolar RNA (snoRNAs) were identified to be differentially expressed between the two groups.

Conclusions

Understanding more about the pathophysiology and genomic regulation in acute pancreatitis will provide us with potential prognostic biomarkers and targets for therapy. This study has selected a series of gene expression features which could act as biomarkers to accurately stratify patients into mild and severe groups. Appropriate therapy can then be chosen earlier to improve outcomes in the disease. MiRNA and snoRNA can predict severity of acute pancreatitis at the time of admission. These can also be developed to predict specific complications of the disease, including organ failure and pancreatic necrosis, as early as, at the time of admission. Once developed, this could fill in the gap that currently exists in prognostication arena in acute pancreatitis.

Acknowledgements

This thesis is truly a team effort and the list of people who have contributed to it, through their valuable time and efforts, grows by the day.

Liverpool NIHR Pancreas Biomedical Research Unit (PBRU) revolutionised my journey into the unknown world of transcriptomics, for which, I will forever be grateful. Jane, Brian and Lucille held my hand and taught me the complexities of human research and mind boggling microarray analyses. All the members of the unit worked round the clock to ensure we captured all the precious patient samples that would go on to form basis of so many wonderfully successful projects which have put PBRU right in the centre of the envy of all the world renowned pancreatic centres.

I am indebted to Professor Robert Sutton for inspiring me along the way. I was not sure if research was for me in the beginning. He truly has taught me by example. His constant encouragement has seen me through some really difficult times, something I will always be obliged for. I definitely look up to him for all my academic and clinical aspirations.

I think behind every successful woman are two men – in my case, it is my father – who dreamt a lot bigger for me than I could possibly have for myself. If there is one thing I want from my life, it is to make him very proud of me. My husband is the next in line; who made my dreams his own, my aspirations his objective and my struggle his passion. I would truly be lost without him.

Finally, I would like to dedicate this work to my mother, without whose prayers and good wishes, I would be nothing. Whatever I have achieved or hope to in future, it's all because of her. She continues to be my number one fan and I hope to never let her down.

Outputs

This project has led to following papers, abstracts and presentations.

1. Prediction of the severity of acute pancreatitis on admission by carboxypeptidase-B activation peptide: A Systematic review and meta-analysis

Clinical Biochemistry 2015 Jul;48(10-11):740-6

Deng L, Wang L, Yong F, Xiong J, Jin T, De La Iglesia-Garcia D, Bharucha S, **Altaf K**, Huang W, Xia Q.

2. Prediction of the severity of acute pancreatitis on admission by urinary trypsinogen activation peptide: a meta-analysis

World Journal of Gastroenterology 2013 Jul 28;19(28):4607-15.

Wei Huang, **Kiran Altaf**, Tao Jin, Jun-Jie Xiong, Li Wen, Muhammad A. Javed, Ping Xue, Christopher M. Halloran, Qing Xia

3. Meta-Analysis of the Role of C-Reactive Protein in Predicting Severity of Acute Pancreatitis

Pancreas Volume 41, Number 8, November 2012

T. Jin, **K. Altaf**, JJ Xiong, MA Javed, W Huang, R Sutton, Q Xia

4. Urinary Trypsinogen Activation Peptide (TAP) and Carboxypeptidase B Activation Peptide (CABAP) in Severity Stratification of Acute Pancreatitis: a Meta-analysis

Pancreas Volume 41, Number 8, November 2012

T. Jin, **K. Altaf**, J.J. Xiong, L. Wen, M.A. Javed, X.N. Yang, P. Xue, W. Huang, R. Sutton, Q. Xia

5. Gene Expression profiling in acute pancreatitis

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6. Micro RNA profiling in acute pancreatitis

Pancreas Volume 42, Number 8, November 2013

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7. Gene expression profiling in acute pancreatitis. American Pancreatic Association (APA) Annual Conference. November 2013. Poster Presentation

8. Micro RNA profiling in acute pancreatitis. American Pancreatic Association (APA) Annual Conference. November 2013. Poster Presentation

9. Gene expression profiling in acute pancreatitis. Pancreatic society of Great Britain and Ireland (PSGBI) annual conference. December 2013. Oral Presentation

10. Micro RNA profiling in acute pancreatitis. Pancreatic society of Great Britain and Ireland (PSGBI) annual conference. December 2013. Poster Presentation

11. Trypsinogen activation peptide and Carboxypeptidase B activation peptide in severity stratification in Acute Pancreatitis – A meta-analysis. American Pancreatic Association (APA) Annual Conference. November 2012. Poster Presentation

12. Meta-Analysis of the Role of C-Reactive Protein in Predicting Severity of Acute Pancreatitis. American Pancreatic Association (APA) Annual Conference. November 2012. Poster Presentation

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Declarations

All the work described in this thesis has been undertaken by me, with few exceptions.

- Microarray experiments were performed by Dr. Lucille Rainbow
- Bioinformatic analyses were conducted by Dr. Brian Lane
- A substantial number of samples were collected by myself. However, recruitment to Acute Pancreatitis Biobank (where the samples have been used from) was undertaken by clinical staff of the unit according to a pre-planned rota.
- Clinical Data was retrieved from Acute Pancreatitis Biobank Database, which I set up, populated and was responsible for the maintenance of.

This study and my fellowship was funded by National Institute of Health Research (NIHR).

Abbreviations

AP – Acute Pancreatitis

miRNA – Micro RNA

RNA – Ribonucleic Acid

HGU133 – Human Genome U133

ANOVA – Analysis of Variance

NFAT – Nuclear Factor of Activated T Cells

FDR – False Discovery Rate

snoRNA – Small Nucleolar RNA

NIHR – National Institute of Health Research

PBRU – Pancreas Biomedical Research Unit

PP – Pancreatic Polypeptide

MODY – Mature Onset Diabetes of the young

PDAC – Pancreatic Adenocarcinoma

VIPoma – Vasoactive Peptide oma

MEN1 – Multiple Endocrine Neoplasia type 1

MAP – Mild Acute Pancreatitis

SAP – Severe Acute Pancreatitis

CP – Chronic Pancreatitis

ERCP – Endoscopic Retrograde Cholangiopancreatography

HIV – Human Immunodeficiency Virus

UK – United Kingdom

SIRS – Systemic Inflammatory Response Syndrome

CT – Computed Tomography

EPR necrosis – Extra-Pancreatic Retroperitoneal necrosis

ARDS – Acute Respiratory Distress Syndrome

DIC – Disseminated Intravascular Coagulopathy

HUS – Haemolytic Uraemic Syndrome

MARPN – Minimal access Retroperitoneal Pancreatic Necrosectomy

ER stress – Endoplasmic Reticulum Stress

TNF – Tumour Necrosis Factor

IL – Interleukin

PG – Prostaglandin

PAF – Platelet Activating Factor

CRP – C Reactive Protein

MODS – Multi-Organ Dysfunction Syndrome

CARS – Compensatory Anti-inflammatory Response Syndrome

HLA-DR – Human Leukocyte Antigen – antigen D Related

MCP – Monocyte Chemo-attractant Protein

MIP – Macrophage Inflammatory Protein

RANTES – Regulated upon Activation, Normal T Expressed and Secreted

CINC – Cytokine Induced Neutrophil Chemo-attractant (CINC)

NF- κ β – Nuclear Factor - κ β

STAT – Signal Transducer and Activators of Transcription

MAPK – Mitogen Activated Protein Kinase

AP-1 – Activating Factor 1

SOCE – Store Operated Calcium Entry

MPTP – Mitochondrial Permeability Transition Pore

ATP – Adenosine Triphosphate

IP₃ – Inositol Triphosphate

STIM1 – Stromal Interaction Molecule 1

PMNs – PolyMorphonuclear Leukocytes

RBCs – Red Blood Cells

ET – Endotoxin Tolerance

LPS – Lipopolysaccharide

NO – Nitric Oxide

PPV – Positive Predictive Value

APACHE – Acute Physiology and Chronic Health Evaluation

NPV – Negative Predictive Value

AST – Aspartate Transaminase

ALT – Alanine Transaminase

MPM – Mortality Probability Model

AC – Atlanta Classification

ICU – Intensive Care Unit

SOFA – Sequential Organ Failure Assessment

CTSI – CT Severity Index

TAP – Trypsinogen Activation Peptide

ELISA - Enzyme-Linked Immunosorbent Assay

SAA – Serum Amyloid A

CAD – Coronary Artery Disease

mRNA – Messenger RNA

PBMCs – Peripheral Blood Mononuclear Cells

SOP – Standard Operating Procedure

COSHH - Control of Substances Hazardous to Health

QA – Quality Assessment

RLUH – Royal Liverpool University Hospital

GCLP – Good Clinical and Laboratory Practice

LIMS – Laboratory Information and Management System

EDTA - Ethylenediaminetetraacetic acid

BRT – Blood RNA Tube

CECT – Contrast Enhanced Computed Tomography

OF – Organ Failure

CVS – Cardiovascular System

CNS – Central Nervous System

GCS – Glasgow Coma Scale

DBC – Determinant Based Classification

MCT – Microcentrifuge Tube
PSC – PAXgene Shredder spin Column
PRC – PAXgene RNA spin Column
DNA – Deoxyribonucleic Acid
IVT – In Vitro Transcription
PCR – Polymerase Chain Reaction
cDNA – complementary DNA
aRNA – antisense RNA
GCOS - GeneChip® Operating Software
AGCC – Affymetrix GeneChip Command Console
RPM – Rotations Per Minute
DMSO – Dimethyl Sulfoxide
QC – Quality Control
ASSIST – Alcohol, Smoking and Substance Involvement Screening Test
HDU – High Dependency Unit
SMV – Superior Mesenteric Vein
PNA – Peptide Nucleic Acid
cRNA – complementary RNA
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
CV – Coefficient of Variance
rRNA – ribosomal RNA
pm/mm – perfect match/mismatch
REL – Relative Expression Level
PCA – Principle Component Analysis
MMP – Matrix Metalloproteinase
OLAH – Oleoyl-ACP hydrolase
ADAMST – A Disintegrin And Metalloproteinase with thrombospondin motifs
HPGD – Hydroxy prostaglandin dehydrogenase 15
PPAR – Peroxisome Proliferator-Activated Receptors

CRISP3 - Cysteine- Rich Secretory Protein 3

NOV – Neuroblastoma overexpressed

TRAT - T cell Receptor Associated Transmembrane Adaptor

TRDV - T cell Receptor Delta Variable

TRAV - T cell Receptor Alpha Variable

TRAC - T cell Receptor Alpha constant

TCR – T Cell Receptor

B-H – Benjamini-Hochberg

PLC – Phospholipase C

CRAC – Calcium Release Activated Calcium

IPA – Ingenuity Pathway Analysis

MUC – Mucin

GLUT1 – Glucose Transporter 1

GPC3 – Glypican 3

ABCA1 - ATP-Binding Cassette Transporter

SLE – Systemic Lupus Erythematosus

ncRNA – non coding RNA

IFN – Interferon

GDF – Growth Differentiation Factor

PTX3 – Pentraxin 3

APPRENTICE - Acute Pancreatitis Patient Registry to Examine Novel Therapies in Clinical Experience

Tregs – Regulatory T cells

ARRE-2 - Antigen Receptor Response Element – 2

ALI – Acute Lung Injury

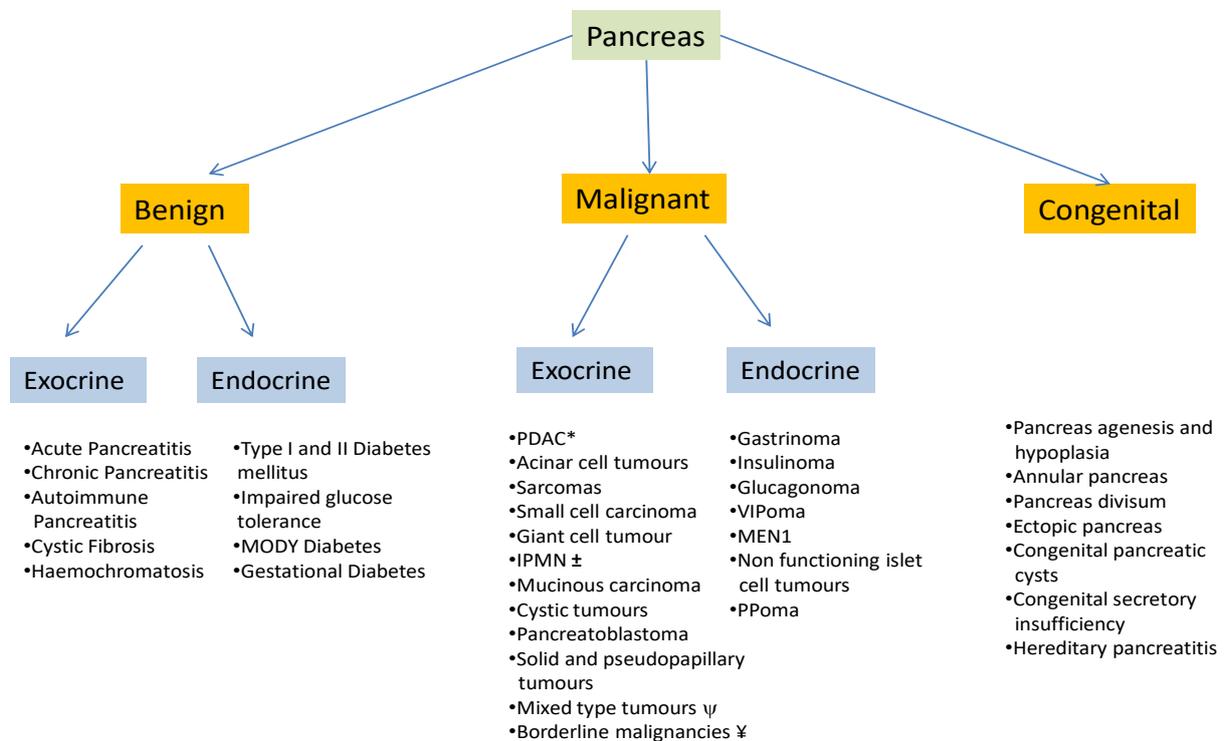
CRC – Colorectal Cancer

CHAPTER 1: ACUTE PANCREATITIS – CLINICAL COURSE AND COMPLICATIONS

1.1 Pancreas as a gland

Pancreas is a retroperitoneal lobulated organ which serves, both, as an endocrine and exocrine gland. Various types of endocrine cells (α , β , δ , and PP), arranged as islets of Langerhans, produce different peptides, including glucagon, insulin, somatostatin, pancreatic polypeptide, which are invariably delivered to other parts of the body via the blood stream. Acinar exocrine cells are essentially of one type, although each one contains a myriad of different substances, helping to secrete a potent mixture of digestive enzymes in response to food intake. These enzymes are synthesised and secreted by the acinar cells, whereas, columnar epithelial cells lining the ductal system secrete bicarbonate. However, the acinar cell itself is not the functional unit in the exocrine pancreatic tissue because acinar cells are organized into acini which are made up of many acinar cells and are linked by numerous gap-junctional channels that help to allow direct chemical and electrical intercellular communication.

Morphological and functional disorders affecting pancreatic tissue, both exocrine and endocrine, lead to a whole spectrum of clinical conditions (figure 1.1). Benign diseases of the pancreas are often complex and can prove to be challenging, both diagnostically and therapeutically. Despite advances, cancer of the pancreas still carries a dismal prognosis.



MODY – Maturity Onset Diabetes of the Young

*Pancreatic Ductal AdenoCarcinoma

±Intraductal Papillary Mucinous Neoplasm

ψDuctal-endocrine/acinar-endocrine

¥Mucinous cystic tumour with dysplasia/IPMN with dysplasia

MEN1 - Multiple Endocrine Neoplasia Type 1

PPoma – Pancreatic Polypeptide oma

Figure 1.6 – Pathological conditions affecting pancreas

1.2 Acute pancreatitis – an overview

“Most terrible of all calamities that occur in connection with the abdominal viscera. The suddenness of its onset, the illimitable agony which accompanies it, and the mortality attendant upon it render it all the most formidable of catastrophes”.

- **Moynihan 1925** ^[1].

The incidence of acute pancreatitis is increasing in Europe, with significant medical, surgical and financial implications. The overall incidence of acute pancreatitis is 150 to 420 cases per million population in UK with a hospital admission rate of 9.6 per year per 100,000 population in the United Kingdom [2, 3].

1.2.1 Classification

Acute pancreatitis may be classified based on pathology, aetiology, severity of disease or the presence of necrosis.

Clinically, acute pancreatitis can be classified as mild or severe.

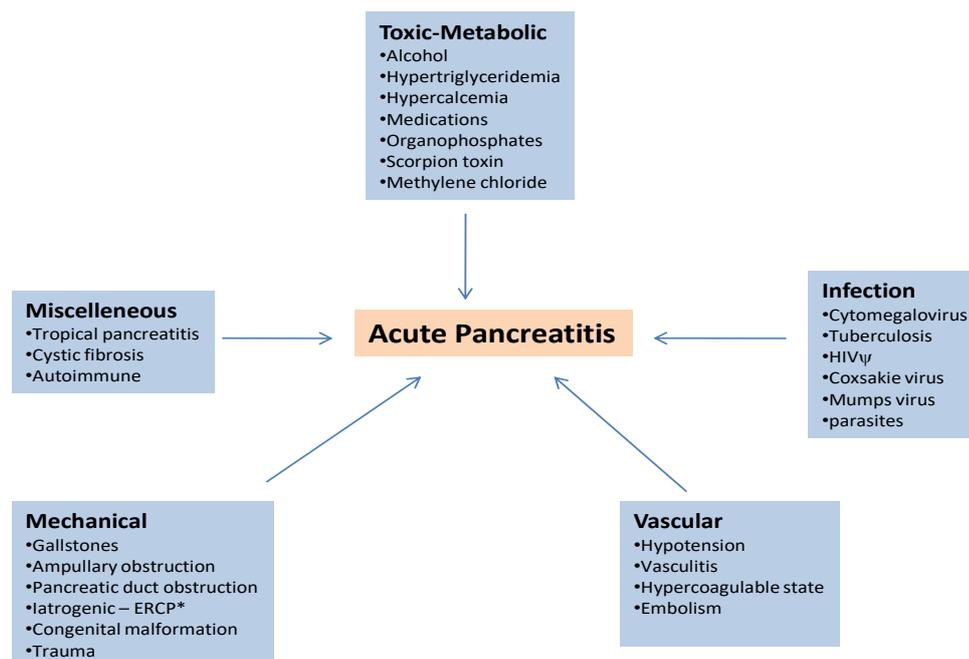
Mild oedematous pancreatitis (MAP) usually presents with minimal organ dysfunction and leads to uneventful recovery. In this state, the pancreas shows oedematous swelling and multiple tiny spots of fatty tissue necrosis, mainly on the surface of the gland but also to some extent in the interlobular fatty tissue that infiltrates the pancreas [4]. The mild form only rarely progresses to severe pancreatitis [5], since the interstitial oedema, which is rich in pancreatic enzymes, is usually resolved by macrophages within a few days and does not lead to secondary changes. The fate of fat necrosis depends on its size. A small focus of fat necrosis (<1 cm) on the surface of the pancreas, as seen in mild pancreatitis, resolves entirely. The necrotic material, rich in lipids, is phagocytised by macrophages, which are transformed to foam cells. Later such an area may show a small focus of fibrotic tissue.

Severe acute pancreatitis (SAP) is associated with organ failure and/or local complications such as necrosis, abscess, or pseudocyst. The peri-pancreatic tissue shows numerous large and often confluent areas of fat necrosis. In addition, there are also necrotic foci within the pancreas, although these are usually small compared with the extra-pancreatic alterations. Intra-pancreatic necrosis also develops in the interlobular fatty tissue, where it is dependent on the amount of fat in the pancreas. Where fat necrosis comes into contact with a blood vessel, it damages the vessel wall, leading to thrombosis and eventually, vessel necrosis, rupture, and haemorrhage. Focal destruction of single interlobular ducts and peripheral acinar cells are further sequelae of expanding fat necrosis. Fat necrosis can become infected, usually with the gut derived gram negative bacteria, adding to the mortality of the disease. Infection of the peritoneal cavity leads to purulent peritonitis. Necrotic areas within the pancreas

resolve slowly and may induce interlobular fibrosis [6, 7]. If this process, termed the “necrosis–fibrosis sequence” [8], takes place repeatedly because of recurrent attacks of severe acute pancreatitis and also involves the large interlobular ducts or the main duct, acute pancreatitis may evolve into chronic pancreatitis (CP) [7, 9-11].

1.2.2 Aetiology

Gallstones and alcohol excess continue to be the main causative agents (figure 1.2). In approximately 10–20% of patients, no aetiology is identified. Some of these patients may have microlithiasis as the aetiology of AP. With the increasing knowledge and understanding of the role of genetic abnormalities in hereditary and idiopathic chronic pancreatitis (CP), it is possible that these abnormalities will be implicated in idiopathic AP. Furthermore, polymorphisms in inflammatory mediators may influence disease severity [12].



*Endoscopic Retrograde Cholangio-Pancreatography

ψHuman Immunodeficiency Virus

Figure 1.7 - Conditions leading to acute pancreatitis

Gallstone (Biliary) pancreatitis – Gallstones are implicated in approximately 80% of all cases of acute pancreatitis [13, 14]. Although gallstones are common, they rarely cause pancreatitis. It is estimated that over a 20- to 30-year period, the risk of developing biliary pancreatitis in patients with asymptomatic gallstones is approximately 2%. Various theories have been proposed about the possible mechanism of development of pancreatitis secondary to gallstones, most of these arising out of works of Opie [15].

Detailed studies of patients with “idiopathic” acute pancreatitis suggest that a high proportion of these cases are due to microlithiasis, which may be detected by endoluminal ultrasound (EUS) if performed early enough after the onset of disease. Such studies suggest that up to 80% of “idiopathic” cases are actually due to gallstones [16-18].

Alcoholic pancreatitis - Alcoholic pancreatitis can present as acute pancreatitis, although in most patients, it occurs in the presence of already established chronic pancreatitis. It is the most common cause of recurrent pancreatitis. In UK, approximately 20-30% cases of acute pancreatitis are thought to be due to alcohol abuse [19]. It is of interest to note that the incidence of alcohol-induced acute pancreatitis may have been increasing over the past decade. A study in the UK has reported an increase in the crude incidence rate of alcohol-induced acute pancreatitis from 14.5 cases per 100 000 population in 1989–90 to 20.7 per 1,00,000 in 1999–2002 [19]. The incidence of alcoholic pancreatitis is low (about 5%) in alcohol abusers [20, 21]. As reported by Dreiling and Koller [20], given 100 alcoholics, five will develop clinical acute pancreatitis, 15 will develop alcoholic cirrhosis, while only one will develop clinical evidence of both diseases. This estimate suggests that in addition to alcohol ingestion, other factors, such as genetic background or environmental influences, may affect patient susceptibility. Several major physiological mechanisms may contribute to the development of alcoholic pancreatitis, including abnormal sphincter of Oddi spasm, obstruction of the small ducts by proteinaceous material, and direct toxic effect of alcohol and its metabolites. Despite the fact that alcoholic pancreatitis can be complicated by severe disease, it is a less common cause of fatal pancreatitis.

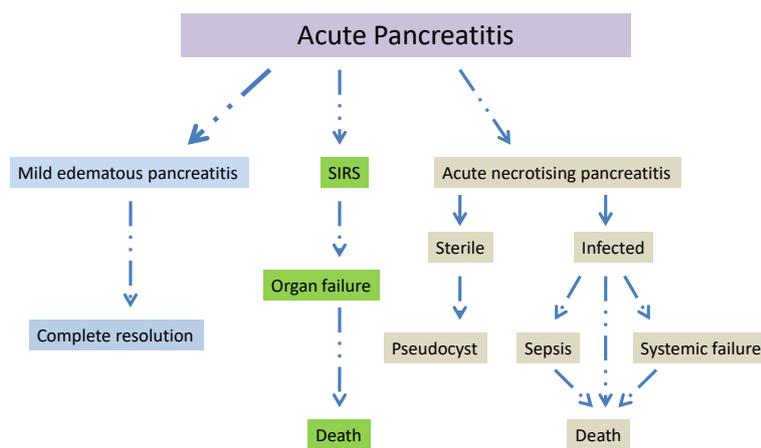
Other aetiologies - Other potential causes identified include pancreatic cancer in 1% of cases, post-ERCP in 2–3%, medications in 1%, miscellaneous causes in 2%, and unknown causes in 15–23% of first attacks of acute pancreatitis.

Recurrent Acute Pancreatitis - Bouts of recurrent acute pancreatitis are most commonly alcohol-related (60%); other aetiologies include unknown causes (17%) and untreated gallstones (19%). Recurrent acute pancreatitis appears to be relatively benign and is associated with a low mortality rate.

1.2.3 Clinical course

The majority of patients with mild pancreatitis recover uneventfully and once the etiological factor is identified and removed, there are no long-term complications or recurrences. Approximately 10–20% of patients with acute pancreatitis develop severe disease and have a complicated hospital course.

In general, severe pancreatitis develops in two phases (Figure 1.3).



■ early phase
■ late phase

Figure 1.8 - Clinical course of Acute Pancreatitis

The first two weeks after onset of symptoms are characterised by the systemic inflammatory response syndrome (SIRS) (Figure 1.4). Release of pro-inflammatory mediators is thought to contribute to the pathogenesis of SIRS associated pulmonary, cardiovascular, and renal insufficiency [22, 23]. Similarly, pancreatic necrosis develops within the first four days after the onset of symptoms to its full extent in most of the cases [24], although late onset pancreatic necrosis has also been reported in literature [25]. Although SIRS in the early phase

of severe pancreatitis may be found in the absence of significant pancreatic necrosis, the majority of patients with severe early organ dysfunction will have pancreatic necrosis on computed tomography (CT) scan [26]. Late deterioration of organ dysfunction occurs most commonly in the second to third week after admission [24], and is usually the result of secondary infection of pancreatic or peripancreatic necrosis.

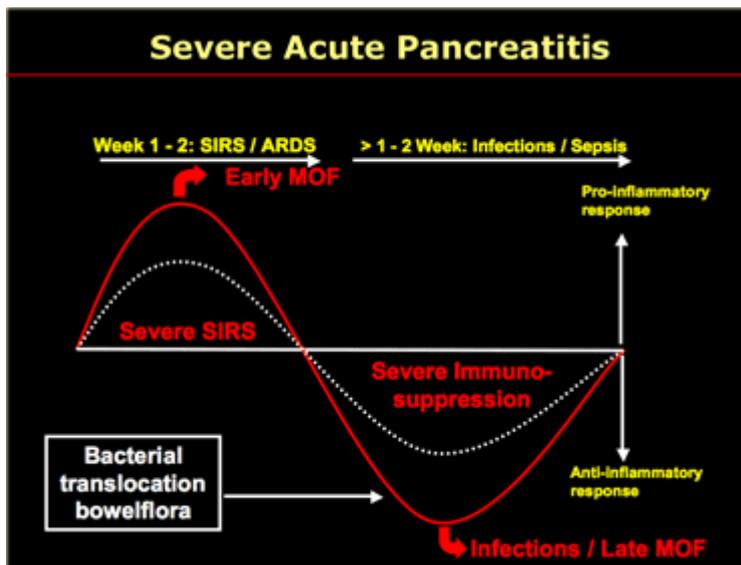
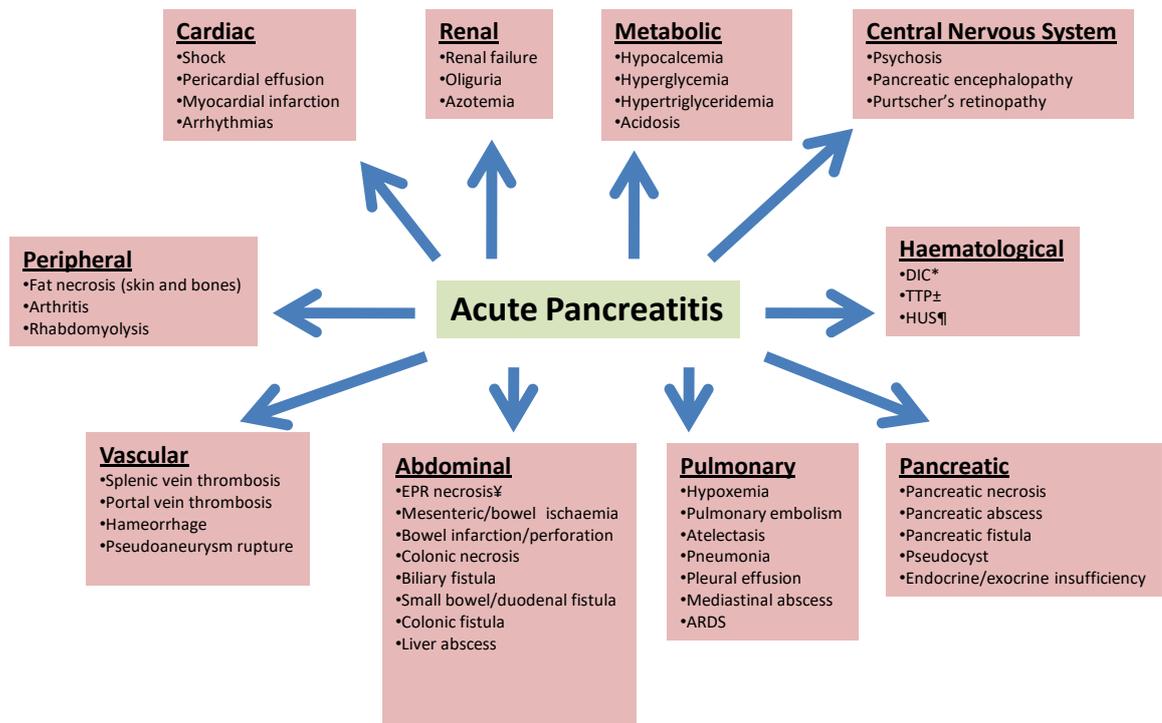


Figure 1.9 - Course of severe acute pancreatitis – (adapted with permission)

1.2.4 Complications associated with the severe disease

Since acute severe pancreatitis is a systemic disease, it not only results in local complications, but can also lead to organ failure (figure 1.5).



EPR necrosis – Extra-pancreatic retroperitoneal necrosis, ARDS – Acute Respiratory Distress Syndrome, DIC – Disseminated Intra-vascular Coagulopathy, TTP – Thrombotic Thrombocytopenic Purpura, HUS – Haemolytic Uraemic Syndrome

Figure 1.10 – Complications of Acute Pancreatitis

Respiratory dysfunction is the most critical of all extra-pancreatic manifestations. It occurs in almost 75% of cases and ranges from hypoxemia to Acute Respiratory Distress Syndrome (ARDS). Course of pulmonary dysfunction and subsequent failure happens in three stages; Stage 1 deals with pulmonary manifestations in the form of tachypnoea, mild respiratory alkalosis and hypoxemia without any noticeable changes radiologically; stage 2 emphasises radiologic changes observed in the form of pulmonary infiltrates or atelectasis, pleural effusions, and pulmonary oedema and stage 3 is hallmarked by ARDS, characterised by severe dyspnoea and extreme hypoxemia refractory to a high inspired oxygen concentration.

Vascular complications in pancreatitis are well recognized and occur in approximately one fourth of the patients [27]. The most common complications are haemorrhage into a

pseudocyst, erosions of the upper gastrointestinal arteries, thromboses of the portal venous system, formation of varices or pseudoaneurysms and rupture of a pseudoaneurysm.

Pancreatitis in combination with vascular complications is dangerous and potentially lethal. The survival of patients with pancreatitis and vascular complications depends on the early diagnosis of these complications.

Acute renal failure is one of the most common complications in patients with severe acute pancreatitis. Its incidence rate ranges from 14% to 42% in the literature [28, 29]. The history of renal disease, acute coronary syndrome (ACS), and hypoxemia are the significant risk factors for it [30].

Pancreatic pseudocysts are well known sequelae of acute pancreatitis with a prevalence rate ranging from 6% to 18.5% [5, 31]. Complications arising out of pseudocysts, influencing patient morbidity and mortality include rupture into gastro-intestinal tract, peritoneal cavity or vascular system [32], haemorrhage due to erosion into a major vessel [33], infection [34] and obstructive jaundice arising from pressure on the common bile duct [35, 36].

Pancreatic and gastrointestinal tract fistulas (gastric, enteric, duodenal, and colonic) are common complications of severe necrotizing pancreatitis. Mortality in these patients parallels the mortality for severe necrotizing pancreatitis [37, 38].

Pancreatic endocrine and exocrine insufficiencies are well reported follow-up complications of acute necrotizing pancreatitis. Pancreatic endocrine insufficiency is diagnosed on the basis of abnormal oral glucose tolerance test, after recovery from acute illness. Pancreatic exocrine insufficiency is diagnosed on the basis of clinical symptoms of steatorrhea [39-41].

In contemporary pancreatic practice, the diagnosis and management of **colonic complications** of pancreatitis remains a relatively difficult management problem. These roughly include colonic necrosis [42-44], colonic fistula [42], colonic stricture/stenosis [45], colonic obstruction and colonic haemorrhage [44]. In 1989, Aldridge and colleagues reported 36 cases of large bowel involvement in acute necrotising pancreatitis and discovered a spectrum of changes in the resected colons ranging from pericolicitis through to ischaemic necrosis, suggesting at least two possible mechanisms [43]. Kriwanek *et al* found colonic

necrosis to be an independent prognostic factor for death in his patient cohort with an associated mortality of 53% [46]. Hence, colonic necrosis is a potentially lethal complication of acute pancreatitis with a mortality of 54% and an overall mortality of 35% for patients with colonic complications of pancreatitis [44].

1.2.5 Mortality in acute pancreatitis

The mortality of acute pancreatitis is reported in the literature as being between 1.3 and 10%. A range of 2–5% likely represents a true mortality because the higher rates are indicated in studies from referral centres and probably do not include patients with mild disease. Overall, studies suggest a reduction in mortality in the last decade. The mortality rate takes a big leap in severe acute pancreatitis, where the reported figures are between 14 and 30%.

Approximately half of this mortality is seen in the first two weeks [47-49]. Mortality appears to be influenced by age, aetiology (higher in patients with idiopathic, post-ERCP pancreatitis, and gallstone), presence of organ failure on admission and the presence of pancreatic necrosis. Additionally, patients with severe pancreatitis transferred to tertiary care facilities for management have higher mortalities [50]. Most studies suggest that approximately 10–20% of fatal pancreatitis is missed with the diagnosis only being made at autopsy. The missed diagnosis appears in patients who present without abdominal pain, with acute respiratory failure or neurological changes, and/or normal serum enzymes or pancreatic imaging.

1.2.6 Available treatment modalities

For a disease of this magnitude, it is unfortunate that there is no pancreatitis specific pharmacotherapy available to treat acute pancreatitis. Therefore, the management of the disease largely remains supportive. The primary objective in the initial treatment of acute pancreatitis is to provide supportive therapy and to treat specific complications as these arise. It is also of paramount importance to make efforts to limit both the pancreatic inflammation and invariably, pancreatic necrosis and the systemic inflammatory response by specifically interrupting their pathogenesis.

Fluid resuscitation and rehydration - Maintaining an adequate intravascular volume is probably the most essential therapeutic measure in the treatment of acute pancreatitis. If not

achieved, it is also the most consequential mistake. Patients with acute pancreatitis can sequester large amounts of fluid into the retroperitoneal space, intraperitoneal cavity (pancreatic ascites), gut and the pleural space. Haemodilution to a haematocrit of around 30% with dextran 60 has been shown to improve pancreatic microcirculation and oxygenation in experimental acute pancreatitis [51]. There is also increasing evidence that oxygen supplementation to maintain an arterial saturation of 95% is associated with the resolution of organ failure, since it ensures optimal oxygen transport [52].

Nutritional supplementation – Severe acute pancreatitis creates a catabolic stress state, exacerbating the pathological progression by promoting systemic inflammatory response and nutritional deterioration [53]. This, combined with absence of oral intake, promotes persistent negative nitrogen balance that appears to be associated with a higher mortality rate as a result of loss of function and structural integrity of vital organs [5, 54, 55]. Increasing evidence suggests that enteral feeding is not only safe but can also reduce complications by helping to maintain the intestinal barrier function and intestinal blood flow, and by preventing or reducing bacterial translocation from the gut. Furthermore, enteral nutrition eliminates some of the complications of parenteral nutrition, such as catheter sepsis, as well as other less common complications.

Recent meta-analyses have supported use of enteral nutrition in patients with severe pancreatitis requiring nutritional support over total parenteral nutrition. It has also been found that patients receiving enteral nutrition are less likely to suffer from multiple organ failure, systemic infections, operative interventions and more importantly, death [56-58].

Treatment of pain – Inflammatory mediators released as a result of pancreatic inflammation and necrosis can have a direct effect on sensory nerve fibres in the celiac plexus (spinal cord level T5–T9) and therefore mediate visceral pain, which is often excruciating. Adequate pain relief is therefore one of the most important and urgent treatment goals. The systemic administration of intravenous procaine hydrochloride has long been advocated as an alternative to opiates, but has now been shown to be completely ineffective for the treatment of pain in patients with acute pancreatitis [59, 60]. Concerns that morphine analogues might negatively affect the course of pancreatitis because of their inhibitory effect on the sphincter of Oddi are unwarranted [61]. Some centres have begun to use thoracic epidural analgesia to treat pain in acute pancreatitis patients [62]. This medication not only leads to rapid pain

relief [63] but often abolishes the need for opiates. Although shown to be effective in a few studies, well designed randomised multicentre trials are required to establish its efficacy.

Role of antibiotics in acute pancreatitis – Infected pancreatic necrosis increases mortality risk to more than 40% and requires surgical debridement to improve clinical outcome [24, 64, 65]. The infecting agents, usually gut-derived bacteria [66], and organisms are thought to migrate from nearby bowel via intervening lymphatics. Gut mucosal defences against bacterial translocation become impaired in severe acute pancreatitis [67, 68]. Infected pancreatic necrosis worsens pre-existing multi-organ failure and may lead to development of new organ failure or other signs of systemic sepsis [5]. There appears to be a window of opportunity of around 1-2 weeks during which infection may be prevented by administering antibacterial therapy with agents active against enteric organisms [24, 69]. Recent meta-analyses have reported conflicting results with regards to efficacy of prophylactic antibiotic therapy in acute pancreatitis. While some of them have not shown any reduction in mortality or protection against infected necrosis or frequency of surgical intervention [70, 71], others have strongly advocated prophylactic use of antibiotics in settings of acute necrotising pancreatitis [72, 73][74].

1.2.6.1 Interventional management

Endoscopic sphincterotomy - There has been much interest and debate on the early endoscopic removal of gallstones retained in the common bile duct in patients with acute biliary pancreatitis. Although ERCP has no role in the initial diagnosis of acute pancreatitis, there is good evidence that early endoscopic sphincterotomy with the aim of removing obstructing gallstones is the procedure of choice in patients with cholangitis or with impacted stones [75]. In cases of mild acute biliary pancreatitis without signs of cholestasis, cholangitis or clinical deterioration, monitoring of the clinical progress alone suffices, without requiring any interventional modality. A randomised controlled trial has shown that patients with predicted severe pancreatitis, but without biliary obstruction, do not benefit from endoscopic sphincterotomy [76].

Surgical procedures – Management of pancreatic necrosis has taken a shift from very aggressive open surgery to more conservative intensive care approach in the recent years. Surgery is now indicated in acute necrotising pancreatitis only for infected pancreatic tissue,

diagnosis of which is made on the evidence of bacterial or fungal growth on the fine needle aspiration or presence of gas in the retroperitoneum on CT scan. Aim of a standard surgical treatment is to control the focus so that further complications are avoided by restricting the progression of infection and release of pro-inflammatory mediators. Although a generally accepted principle of organ preserving surgical approach is the gold standard, four principle methods have been advocated.

- Necrosectomy combined with open packing [77]
- Planned staged re-laparotomies with repeated lavage [78]
- Closed continuous lavage of the lesser sac and retroperitoneum [79]
- Closed packing [80]

Open necrosectomy has been associated with high rates of morbidity (34-95%) and mortality (11-39%), along with the risk of long term pancreatic insufficiency [39, 81-83], morbidity is low in techniques which provide postoperative exit channels for further slough and infected debris [84].

Advent of minimally invasive interventional procedures has revolutionised the surgical management of acute necrotising pancreatitis. The rationale is to minimise peri- and postoperative stress in critically ill septic patients, suffering from multi-organ failure. These procedures, including percutaneous drainage, endoscopic drainage, or minimally invasive surgery play a role as a temporary measure to bridge the critical early time after onset of acute pancreatitis to a later optimal time point for definitive intervention. The Dutch multicentre randomised controlled trial demonstrated that a minimally invasive step-up approach, which consisted of percutaneous/endoscopic drainage of infected fluid collection as the first step and minimally invasive retroperitoneal necrosectomy as the next step if required, reduced the rate of the major complications and death among patients with necrotising pancreatitis and infected necrotic tissue, as compared with open necrosectomy [85].

The pancreatic unit at the Royal Liverpool University Hospital has been performing Minimal Access Retroperitoneal Pancreatic Necrosectomy (MARPN) since 1998.

The technique involves using a nephroscope over a guidewire that is placed under CT guidance into the necrotic tissue and can be performed under local anaesthetic as well.

Various publications outlining the unit's experience with the technique and comparing it with the traditional open necrosectomy (ON) have shown superiority of MARPN over the former. ON was found to be associated with increased post-operative multi-organ failure and mortality when compared with MARPN [39, 86-89].

**CHAPTER 2: WHAT HAPPENS IN ACUTE
PANCREATITIS - COMPLEX
PATHOGENESIS SURROUNDING THE
DISEASE**

Acute pancreatitis is a disorder that has numerous causes and an obscure pathogenesis. The spectrum of the disease can range from mild and self-limiting to severe and fatal. Several aspects of the condition remain poorly understood or controversial, although recent advances have improved our understanding in many areas, such as predisposing risk factors, pathology, and biochemical events within the pancreas, through a large number of clinical and experimental studies. However, the earliest changes in the pancreas, the so-called initiator or trigger mechanisms, await discovery. The clinical disease varies in severity, but the basic response of the pancreas to the injurious stimulus appears to be limited to one or two sequences of events, giving credence to the idea of a "common pathway" for the initiation of pancreatitis, despite the disparate predisposing factors. The latter must lead to similar cellular alterations, which manifest as clinically similar forms of pancreatitis. Any encompassing theory of the pathogenesis of pancreatitis must explain the diversity of the initiating stimuli, the varying clinical and pathologic pictures and the systemic manifestations of the disease.

2.1 The Trypsin Centred Theory

This widely popular theory rests on the common knowledge that most experimental models of AP have shown pathological activation of trypsinogen to trypsin [90-92]. This invariably establishes a cause-effect relationship [91, 93]. However, new emerging evidence questions this very notion [91].

- the detectable premature trypsinogen activation – may not be pathologically significant quantitatively
- Protective responses within the acinar cell may be adequate to clear the response efficiently
- It may lead to some pathologic acinar cell inflammatory signalling without incurring any direct cell damage
- Alternatively, it may cause some direct cell damage, consequently, triggering protective and pathologic cellular pathways such as autophagy and endoplasmic reticulum (ER) stress, and activate pathological inflammatory signalling
- Premature activation may act as a protective response leading to rapid cell death [94, 95]

- Trypsinogen knock out mice experiments have successfully shown development of local and systemic inflammation comparable to that seen in wild type mice in the absence of significant pathological trypsinogen activation [96, 97].
- Other, alternative, trypsin-independent pathological events may be present in sufficient degree to cause pancreatitis independently of or in conjunction with trypsin-related injury [96].

Thus, premature trypsinogen activation seems to contribute to a component of local injury but inflammation, both local and systemic, progresses independently of trypsinogen activation during acute pancreatitis and therefore, this does not necessarily translate into pathogenicity [96-98]. Although some recent work into hereditary pancreatitis with identification of mutant genes (PRSS1, CFTR, SPINK1, CTSC and CASR) [99-102] has supported the trypsin centred theory, it is not without its flaws [103-109] and this remains open to speculations.

Regardless of the exact mechanisms leading to the development of pancreatitis, once the inciting agent/pathway activates the inflammatory cascade, the pancreatic inflammation is driven by release of active pancreatic enzymes and inflammatory mediators into the blood stream [110, 111]. TNF- α , IL-1, IL-6, IL-8, IL-10, PGE₂, C reactive protein and PAF especially are crucial in this process [112-118]. The release of these interleukins and TNF- α from macrophages triggers an inflammatory cascade, resulting in necrosis of the acini and pancreatic islets, interstitial fat necrosis, necrotising vasculitis and systemic inflammatory response syndrome (SIRS) [23]. SIRS may develop into ARDS and multi-organ dysfunction syndrome (MODS). TNF- α induced response also damages the intestinal barrier leading to bacterial translocation (from small bowel or colon) and pancreatic infection. It is an important factor for complications, increases mortality by several folds [119-121] and may be a feature of early phase of the disease [122].

2.2 Inflammatory versus anti-inflammatory response in Acute Pancreatitis

The initial events in acute pancreatitis lead to disruption of the acinar cells, following on from intracellular co-localisation of digestive and lysosomal enzymes [123-125]. When the natural

defence mechanisms fail to contain the inflammatory process to the local site, uncontrolled activation of inflammatory cells and mediators leads to an inflammatory rampage that leads to SIRS [126]. Cytokines are poured out into the circulation [127], leading to activation of other pro-inflammatory signals, synthesis of acute phase proteins and activation of vascular endothelium. This leads to increased permeability, copious leakage from the capillaries and migration of leukocytes into the tissues. Coagulation cascade is activated consequently. Vascular endothelium in addition to being activated, also suffers damage from the proteolytic enzymes and oxygen radicals released from circulating activated neutrophils and monocytes [128]. This dysfunction of endothelium results in impairment of microcirculation, lack of oxygen to organs and subsequently leads to organ dysfunction and failure [129, 130].

The emerging concept of compensatory anti-inflammatory response syndrome (CARS) has provided useful explanations for some of the aspects of the pathophysiology of the disease [131, 132]. Although, a lot of it still hides in the shrouds of mystery, it is widely agreed upon that in acute pancreatitis, it is observed and leads to effects in the later part of the disease – secondary infections due to excessive immunosuppression [24]. Some studies have provided useful data and hence, established evidence that in AP decreased HLA-DR expression of monocytes, resultant from impaired antigen presentation capacity [133], is positively associated with development of secondary infections [132], organ failure [134] and mortality [135]. Another important contributor to CARS is IL-10. It not only decreases monocyte HLA-DR expression but has also been found to predict development of organ failure in the early part of the disease [136]. IL-6 and IL-1ra are also important as IL-6 prevents synthesis of TNF α and IL 1 β .

2.3 Inflammatory Role of Acinar Cells

It has only transpired recently that pancreatic acinar cells act as inflammatory cells, releasing inflammatory signals and provoking immune responses when noxious stimuli incite injury within them. These are also capable of activating signalling pathways, giving way to expression of various inflammatory mediators [137, 138].

Cytokine production by acinar cells in the context of AP has been well established by various studies which have shown production, release and response to TNF α , IL-6, IL-10 and IL-1 β

in *in vivo* and *in vitro* models [139-144]. These cytokines not only accelerate pancreatic inflammation, through induction of regulatory genes in acinar cells, but also play a pivotal role in determining severity of acute pancreatitis by inducing apoptosis in acinar cells [139, 145].

Chemokines have also been laid out to be involved in the early inflammatory response mounted by acinar cells. Monocyte chemo-attractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , regulated upon activation, normal T expressed and secreted (RANTES), IL-8, cytokine induced neutrophil chemo-attractant (CINC), Mob-1 (the homologue of human IP-10) and MIP-2 have all been identified as being secreted by acinar cells during AP in animal models [140, 146-151].

Acinar cells regulate the pathological functioning of cytokines and chemokines, in the context of acute pancreatitis, through four major inflammatory pathways - Nuclear Factor- κ B (NF- κ B), Signal Transducers and Activators of Transcription (STAT), Mitogen Activated Protein Kinase (MAPK) and Activating Factor 1 (AP-1). These pathways accentuate the inflammatory process, through induction of genes responsible for expression of cytokines and chemokines, leading to overproduction [137-139, 142, 143, 150-157].

2.4 Calcium Signalling

Works from Liverpool NIHR Pancreas Biomedical Research unit have clearly demonstrated prolonged elevations in cytosolic calcium to be the trigger of AP [158], which leads to store-operated calcium entry dependent (SOCE) pancreatic acinar cell injury and necrosis [159-164]. The concept is undoubtedly old but there has been increasing evidence in the recent times. The abnormal elevations of calcium lead to molecular events that play a crucial role in the pathogenesis of AP. These include premature intracellular enzyme activation, mitochondrial dysfunction, impaired autophagy and vacuolization, to name a few [165]. Mitochondrial dysfunction, likely due to intracellular calcium overload, is now recognised to be at the heart of the pathogenesis of AP, being the principle mechanism of injury [159, 166-171]. Mitochondrial matrix calcium overload drives opening of the Mitochondrial Permeability Transition Pore (MPTP) – a non-specific channel that forms in the inner mitochondrial membrane that has already been demonstrated to be crucial to AP by

mediating impaired ATP production, defective autophagy, zymogen activation, inflammatory responses and necrosis. Cell death occurs when the ability to produce ATP has been critically affected due to significant damage to the mitochondria consequent to the cytosolic calcium overload [172]. Additionally, many studies have indicated the importance of inositol triphosphate (IP₃) [173-175] and ryanodine [161, 176] stimulated calcium release channels on internal stores to pancreatic injury induced by AP precipitants. Concurrently, recent evidence has demonstrated a role for the STIM1-Orai complex as the principal calcium entry channel in pancreatic acinar cells [160, 164, 177]. Indeed, inhibition of Orai1 channels has been shown to cause reduced pathological calcium entry into murine pancreatic acinar cells, necrotic cell death and trypsin activation, suggesting therapeutic potential of calcium release activated calcium channel blockade to ameliorate AP [160].

2.5 Acute Pancreatitis as a model of sepsis

AP has now been well established in the literature as a clinical example of immune response in sepsis and indeed, the complications and outcomes of the two closely resemble each other – organ dysfunction and failure [178]. This suggests the intricate and absolute involvement of pro-inflammatory cascade, initiated by perhaps, different aetiologies to be the main driving force.

As mentioned earlier, SIRS is likely to be the main contributor towards early mortality of the disease, with MODS occurring as a complication of the SAP in 20-80% of the cases [179]. Distinction between SIRS and sepsis can often be tricky, as the presentation may be very similar. The involved pathophysiological mechanisms are complex and similar and comprise of activation of several inflammatory mediators and components of the complement cascade. Polymorphonuclear leukocytes (PMNs) not only contribute towards the initiation of the pathological response but also extend the inflammatory process. Ischaemic injury is incited by cytotoxic insult and sequestration of platelets, PMNs and red blood cells (RBCs) in the microcirculation, leading to systemic hypotension, low peripheral resistance and a hyperdynamic circulation. This eventually translates into anaerobic metabolism and subsequent lactic acidosis, culminating into tissue injury and organ failure.

Another important consideration into the two disease states is the ability of the triggering factor to activate the host inflammatory response in an uncontrolled fashion with resultant dissemination into the system, leading to multiple systemic effects – organ dysfunction/failure. TNF α , IL-1 and nitric oxide are the most common mediators involved in this.

Coagulation abnormalities are an important contributor to the inflammatory process in both sepsis and AP. These are initiated, in part, by the pro-inflammatory cytokines and activated endothelial cells [180] and can lead to widespread thrombosis, culminating in disseminated intra-vascular coagulopathy (DIC) and hence, organ failure [181]. Indeed, these microcirculatory disorders affecting the capillary blood flow, capillary permeability and leukocyte endothelial interaction have been demonstrated in the colon, liver, and lungs, in addition to the pancreas, in studies [129].

A landmark study in 2004 also implicated genetic polymorphisms for cytokines to be associated with prognosis, both in sepsis and AP. This especially applies to the polymorphisms involving glutathione levels in acinar cells. This may lead to increased oxidative stress and further clinical deterioration [12].

A recent interesting concept has also endeavoured to explain the pathophysiological similarities between SAP, SIRS and Sepsis. This is tied in by the ‘endotoxin tolerance’ (ET). Essentially, it is the diminished capacity of the host or macrophage/monocyte to respond to a Lipopolysaccharide (LPS) challenge after a first exposure to this stimulus [182, 183]. Experimental works have verified, to an extent, the hypothesis that the innate immune system of patients with SAP, SIRS and sepsis has characteristics of endotoxin tolerance. Works of Vasilescu and others [184] have placed merit on the role of TNF α related mechanisms as the underlying cause behind the phenomenon. They showed that the TNF α releasing capacity of the whole blood is lower in SAP than in trauma patients but higher than in diffuse peritonitis. Similar findings have previously been recorded by Ertel et al [185]. These TNF α releasing mechanisms form basis of the pathophysiological similarities in SAP and severe sepsis. There has been increasing evidence to place more significance on ET in response of innate immunity in SIRS, SAP and sepsis, assuming the role of a pivotal component of immune dysregulation that sits at the heart of clinical evolution of patients with SAP. Further studies on the matter will elaborate on the more intricate and complex components of the process but

the concept that the immune depression of patients with SAP, SIRS and sepsis is based on a mechanism similar to endotoxin tolerance, and that in SAP, the amplitude of the immune depression is of an intermediate degree between SIRS and sepsis, presents a very plausible theory, rendering SAP a very good clinical model for these conditions.

2.6 Oxidative Stress in Acute Pancreatitis

Our understanding of role of oxygen metabolites in inflammation has improved considerably and perhaps, dramatically over the last decade or so. These are important contributors towards release of inflammatory mediators and their sequestration within tissues, development of pancreatic oedema and also play an important role in controlling extent of necrosis at cellular level, both in acinar and non-acinar cells. Imbalance of free radicals release-clearance mechanisms and reduced anti-oxidative capability, demonstrated in both patients and animal models, has been proposed to be at the centre of the disease process in AP and SIRS. [186-189]. Animal models and human studies have placed significant value on oxidative stress as the common pathway in the pathogenesis of AP [190-194]. Experimental data have also suggested for a very early involvement of pancreatic oxidative stress in the disease process of AP. These assumptions and notions are supported by further studies on the topic, which have demonstrated conveniently, decreased antioxidant levels and increased release of peroxidation by-products in AP.

Although some studies have shown excessive production of free radicals to be associated with increased cell damage and necrosis and pancreatic edema [195], a landmark study by Booth et al [187] showed for the first time that in animal model, free radical production led to apoptosis and inhibition of the same caused necrosis. These findings suggest that free radical generation within acinar cells may be a protective response during pancreatitis. It was also postulated in the same study that oxidative stress in the neutrophils activated during inflammatory response to acinar injury may be responsible for further propagation of local and systemic inflammation. As it materialises, oxidative stress may well have a dual role in pancreatic injury.

Hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen all cause destruction of lipid membranes and lysosomal membranes by peroxidation of fatty acids, leading to cellular

and endothelial injury in AP. These act on arachidonic acid cascade and through their platelet aggregation and vasoconstricting effects, lower tissue circulation [196]. These also exert their pro-inflammatory influence and promote activation of leukocytes and lysosomal discharge [197]. A vicious cycle develops when the activated leukocytes cause respiratory burst, culminating in more free radical formation and hence, more tissue and acinar cell damage [139, 198, 199]. This oxidative stress in the neutrophils, accentuating the further propagation of local and systemic inflammation [187], may well be the driving force providing synergy between pro-inflammatory cytokines and oxidative stress in AP, perhaps through activation of mitogen activated protein kinases and nuclear factor-kappa B (NF-kB) and inactivation of protein phosphatases [200-203].

Nitric oxide (NO), on the other hand, has a dual role. Its protective role is shown by its ability to relax smooth muscle, especially sphincter of Oddi and to improve pancreatic microvascular blood flow by vasodilation. Pancreatic damage is caused due to its free radical action and its association with bacterial translocation, putting endotoxemia in the centre of pathogenesis of MOF and septic complications of AP. Indeed high levels of NO have been correlated with sepsis and mortality in AP [204]. These different roles can be explained by difference in concentration and synthesis of NO at different stages of the disease. Early phase of AP is characterised by low concentration of NO, which only causes muscle relaxation and vasodilation and hence, is protective. The concentration increases as the disease progresses and NO starts acting as a free radical participating in oxidative reactions [205].

Whether the oxygen free radical species are the initiators of the tissue damage in AP remains to be elucidated but these have been proven to be important mediators in the early and later courses of AP, correlating with clinical severity in many studies [206, 207].

CHAPTER 3: PROGNOSTICATION IN ACUTE PANCREATITIS

3.1 Significance of severity stratification in acute pancreatitis

Acute pancreatitis resolves spontaneously in approximately 80% of the patients, without requiring any intervention. However, a subset of the patients goes on to develop the severe form of the disease, including local and systemic complications and require intensive management. These patients should be monitored and managed in high dependency/critical care units as their condition demands. Unfortunately, it is not possible to accurately identify such patients when they present with the disease. Similarly, it is not feasible to manage all the patients who are diagnosed with acute pancreatitis to be managed in the critical care settings. Current scoring and predicting systems have their short comings and fail to achieve what is required. Therefore, it becomes difficult to allocate the health resources to the patients who need them the most. If patients, who potentially will develop severe acute pancreatitis in the course of their disease, can be accurately identified immediately on admission through a scoring system/biochemical parameter, it will not only enable the medical team to single out these patients and provide them with the best care possible, it will also ensure that the health resources are appropriately and efficiently used.

3.2 An ideal biomarker does not exist

An ideal prognostic system/marker doesn't exist, and current approaches fall short of what is needed when dealing with individual patients. It has been 35 years since Ranson et al. [208] showed that it was possible to stratify patients with acute pancreatitis according to their risk of dying. Since then, there have been thousands of articles promoting hundreds of prognostic markers and systems. The fact that very few of these have actually found place in the clinical practice goes on to show that we still haven't found what we are looking for.

An ideal or desirable detection scoring system/biomarker should:

- Have high sensitivity and positive prediction value (PPV).
- Be able to predict necrosis early (<48 hours)
- Be performed rapidly (<4 hrs)
- Be available in most hospitals
- Be relatively inexpensive

- Be objective and not observer-dependent [209].

The extent to which different clinical, laboratory and radiological detection methods satisfy these requirements vary greatly and show distinct limitations peculiar to each individual system.

3.3 Current scoring methods

3.3.1 Scoring systems

APACHE II (Acute Physiology and Chronic Health Evaluation II) is a classification system designed to measure the severity of a disease for acutely unwell adult patients [210]. In the prediction of mortality for acute pancreatitis, it has been analysed in various studies and has been found to have a sensitivity of 65-81%, specificity of 77-91%, PPV of 23-69% and NPV of 86-99% [211]. Some studies have found a significant association of APACHE II with mortality, but conflicting results were found within these as well [212-214]. Moreover, it relies very heavily on age. In fact, a 76-year-old (6 points) would require very little physiologic disruption to be classified in the category of 'severe acute pancreatitis' (>8 points). This has been one of the arguments all along of defined number of APACHE II points when the age is high. Hence, the need for more accurate predictors of mortality.

Ranson's Criteria were initially introduced in 1974 for the assessment of severity of acute pancreatitis [208]. It is an objective indicator of diseases severity and is particularly useful at the two ends of the scale. Pancreatitis is mild when the score is ≤ 2 , whereas pancreatitis is severe when the score is > 6 . The correlation of severity of disease or development of necrosis in patients with a score of 3-5 (which is a common occurrence) is deficient [215, 216]. Moreover, the system requires the completion of 11 measurements, which necessitates a total of 48 hrs of observation for proper evaluation [217]. Although a modification of the Ranson's scoring was introduced by Blamey et al [218], the overall sensitivity of these numeric systems in the initial staging of an attack of pancreatitis remains 65%, with specificity of 70%, PPV of 20-63% and NPV of 86-94% [211]. Some studies have found conflicting results between patients who died compared with those who survived [208, 209, 219, 220].

The **Glasgow Criteria** were originally presented in 1978 [221] and the original system used nine data elements. This was subsequently modified to eight data elements, by the removal of the contribution of transaminase levels (either AST or ALT (>100 U/l) [218]. The Glasgow criteria have been analysed in multiple studies and the sensitivity was found to be 94%, specificity 28%, PPV 18-66% and NPV 86-100% [211]. The score, however, is not valid for repeated measurements beyond 48 hrs and like Ranson's criteria, has not been validated for use in children.

Other scoring systems like, **Mortality Probability Model (MPM)** [222] and **APACHE III** [223] (a modified version of APACHE II including the prior site of health care- i.e. hospital floor, emergency room etc. – and additional physiological parameters – urine output, blood urea nitrogen, albumin, bilirubin, glucose) at 96 hrs have been calculated and brought forward but without creating much influence on the current clinical practice.

3.3.2 Organ failure scores

It has been shown in various studies that development of organ failure in the first week of acute pancreatitis is not only marker of poor prognosis but also translates into local complications later on [224]. According to Atlanta classification (AC) [5], patients with persistent organ failure in the first week are likely to have severe acute pancreatitis. Various organ failure scoring systems have been validated and are in clinical use. Ideally for ICU use, these scoring systems can be used to assess patients outside the intensive care environment as well. These include Sequential Organ Failure Assessment (SOFA) [225], Marshall's scoring system [226] and Goris system [227], among others.

3.3.3 Radiology scores

Unlike clinical scores, **radiological scores** are based on local anatomic changes in the pancreatic and peri-pancreatic tissues. Contrast-enhanced CT scans can be used to assess pancreatic necrosis, since loss of perfusion consequent upon necrosis results in a reduced enhancement. In 1985, Balthazar et al [228] proposed a scoring system for the grading of disease severity based on the CT findings. The severity of pancreatitis is divided into 5 distinct groups, from A to E. Most patients with severe acute pancreatitis (SAP) exhibit one or more

pancreatic fluid collections and are classified as grades D or E according to the Balthazar score. Furthermore, when the score is >5 , this is defined as SAP on the CT severity index (CTSI) – a development of the Balthazar score in which is included the pancreatic necrosis. Even in these cases, as with the clinical scores, the commonly used radiological scores are particularly useful in predicting survival in the absence of peri-pancreatic fluid collections (Balthazar score) or collections/pancreatic necrosis (CTSI), but when these abnormalities are present, the scores may miss up to 50-59% of patients who eventually die from the disease [211].

From the above mentioned facts, it is evident that there is no one most reliable predictor of mortality and all scores and parameters seem to show a good NPV but a relatively low PPV. The ability to confidently exclude a large cohort of patients with a low risk of mortality is of value in the management of patients with acute pancreatitis. However, all scoring systems and prognostic factors suffer from a low PPV and patients scoring highly on such parameters do not necessarily progress to a fatal outcome.

3.3.4 Biochemical parameters

Much effort has been directed to develop a single, simple, rapid, affordable and reliable laboratory test for the prediction of severity and to monitor disease progression. So far, several single biological parameters, which represent important steps in the pathophysiology of acute pancreatitis, have been evaluated, and several tests have been developed.

Trypsinogen Activation Peptide (TAP) is the most studied activation peptide in acute pancreatitis. It is specifically related to the onset of acute pancreatitis and therefore, seem to be a good marker for it [229-231]. It is rapidly cleared by the kidneys and excreted into the urine [232], so that its detection in urine is easier than in serum. However, 30% of all patients with acute pancreatitis have normal TAP values in urine on admission [229]. Similarly, urinary TAP has not been able to predict mild pancreatitis following ERCP [233]. Two multicentre studies investigated the predictive value of urinary TAP [230, 234]. The American study showed a very high sensitivity of 100% and a specificity of 85% within 48 hrs, while the European trial demonstrated a sensitivity of only 58% and a specificity of 73% within 24 hrs after the onset of symptoms. Likelihood ratios for a positive urinary TAP assay at 48 hrs showed a small increase in the positive likelihood of severe acute pancreatitis from 20% to 35% [235]. This finding may limit the clinical usefulness of urinary TAP measurements for the prediction of

disease severity. The general accuracy of TAP alone does not qualify for clinical decision-making. The elevated TAP concentration in severe cases decreases quickly in serum and within 72 hrs in urine, so that the prognostic accuracy declines rapidly [229-231, 234]. Moreover, the TAP-ELISA is still too complex and too expensive to be used as a routine test in everyday practice.

In patients with acute pancreatitis, elevated **Tumour Necrosis Factor (TNF)** levels have been well documented [236]. However, TNF measurements are inconsistent even in severe disease because of intermittent release and rapid clearance by the liver before it reaches the general circulation.

The importance of **Interleukin-6 (IL-6)** in the acute phase response has been confirmed by the observation that it stimulates the synthesis of acute phase proteins, including C reactive protein (CRP), from hepatocytes *in vitro* and *in vivo* [237, 238]. Its concentrations peak already 24 hrs after the onset of the disease, but decrease to baseline within four days [239]. A large series of patients with acute pancreatitis confirmed that IL-6 levels on admission correlate well with the subsequent course of pancreatitis [240]. But due to its rapid decline, IL-6 does not seem to be a useful marker for monitoring disease progression. Similarly, **Interleukin-8 (IL-8)** levels have been shown to increase even earlier than IL-6, with a peak detected 12 hours after the onset of acute pancreatitis [241]. However, similar to IL-6, IL-8 decreases rapidly within 3-5 days [242] and consequently, disease progression cannot be recorded with these markers.

The most famous acute-phase reactant and most commonly used serum parameter for the staging of acute pancreatitis is **C-Reactive Protein (CRP)**. The importance of CRP in predicting severity of acute pancreatitis has been shown in numerous clinical studies over the past years [243-245]. Increased levels of CRP are a direct effect of hepatocytes stimulation by cytokine release. This also explains the chronological delay of increased CRP levels compared to IL-6 and IL-8 concentrations, with peak levels occurring 48-96 hrs after symptom onset. Pezzilli et al [242] found the highest sensitivity and specificity for predicting severity of acute pancreatitis for IL-6 and IL-8 on days 1 and 2, while on day 3 CRP surpassed both of these mediators. This is due to the relative rapid decrease of the two cytokines. Although, CRP is a valuable parameter for predicting severity of acute pancreatitis more than 48 hrs after the onset of the disease and for monitoring disease progression, it does not help with prediction within the first 48 hours of the disease onset.

Serum Amyloid A (SAA) proteins constitute a family of apolipoproteins mainly synthesized in liver in response to cytokines [246]. SAA has been found to be of similar value for the discrimination between oedematous and necrotising pancreatitis as CRP [247, 248]. However, SAA was less accurate than CRP in predicting infection, multi-organ failure and death. Moreover, in contrast to CRP, SAA does not have an accepted cut-off value. The European multicentre trial [248] was able to demonstrate that SAA distinguishes mild from severe acute pancreatitis already at the onset of the disease and that this difference was significant for the whole observation period of five days. However, the other trial could not demonstrate the early predictive value of SAA [247].

It is very clear from the discussion above that despite the proliferation of scoring systems and biochemical parameters for grading acute pancreatitis, there is still no single system or biomarker which is completely reliable in accurately predicting the severity of the disease within the first 48 hrs.

CHAPTER 4: GENE EXPRESSION AND MICRO RNA PROFILING

4.1 Circulatory blood (peripheral) gene expression profiling

Gene expression profiling represents a new and promising tool which could enable us to come up with a more comprehensive management of human diseases and disease risk factors. In particular, microarray based transcriptional portraits (transcriptomes) of disease can identify disease-specific signatures [249, 250], which will ultimately be translated into clinically useful molecular biomarkers. Recently, investigators have measured gene expression of the pancreas in experimental pancreatitis and have identified several novel genes which are likely to be important in the development and severity of acute pancreatitis [251, 252]. Ji et al [251] utilized microarrays to identify genes commonly induced in rat pancreatic acinar cells within 1-4 hours in two *in vivo* models (caerulein and taurocholate) and observed that a complex programme of gene expression was rapidly initiated early in the course of the disease. Their data suggested a model in which early signalling events led to activation of transcription factors which regulated the expression of specific cellular and secreted molecules. These secreted factors were hypothesised to be useful prognostic indices for the disease. Similarly, some researchers have identified genes likely to be involved in the endogenous self-protection mechanisms in acute pancreatitis using experimental animal models [253], while others have studied changes in the population of leukocytes in pancreatitis and have emphasized on a pathogenetic role of immune system in the development of acute pancreatitis [254].

Although the early acinar cell expression is important for the subsequent severity of acute pancreatitis and could provide insight into the mechanisms involved in the initiation of acute pancreatitis [251], obtaining pancreatic tissue to analyse acinar cell gene expression is not a practical option in human patients. This difficulty in obtaining pancreatic samples has clearly impeded transcriptomic research in this area.

Circulating blood is easily accessible and has been suggested as an alternative to tissue samples for molecular profiling of human disease and disease risk. This is based on the capacity of peripheral blood to reflect pathological changes in the body and the identification of these alterations as a blood molecular signature. Since sample acquisition is a daunting challenge for the application of molecular profiling as a diagnostic tool or for the identification of candidate risk factors, possibility of utility of peripheral blood cells as 'surrogate' for biopsy tissue for profiling in humans [255] appears to be an attractive option. Blood sampling enables relatively large samples to be collected and is highly amenable to standardisation of technical procedures. Thus, blood represents an attractive alternative sample for profiling human diseases and discovering new biomarkers.

Recent studies have shown that the peripheral blood leukocytes have the ability to respond differentially to varying environmental, physiological and pathological insults or perturbations occurring anywhere in the body. Indeed, these cells have been found to share more than 80% of the transcriptome with each of the nine tissues tested in one of these studies, namely: brain, colon, heart, kidney, liver, lung, prostate, spleen and stomach [256]. It also showed that these blood cells expressed organ specific genes, such as β -myosin heavy chain (heart) and insulin (pancreas).

Early profiling studies of the peripheral blood leukocytes involved analysis of haematological malignancies where these cells displayed disease-specific gene-expression signatures accurate enough to identify clinically relevant patient subgroups (i.e. subgroups associated with response to treatment, prognosis and novel malignancy subtypes) [257]. Others profiled gene expression of peripheral blood leukocytes in a wide range of non-haematological disorders and demonstrated that monitoring gene expression in blood results in distinct transcriptional signatures for more than 35 different conditions in humans [256], including, osteoarthritis [258], schizophrenia [259], cardiovascular disease and various types of cancers: bladder [260], colorectal [261] and breast [262].

In the recent years, the peripheral blood leukocyte gene expression in cardiovascular disease has been the target of extensive investigations. In one of the initial studies, Ma et al identified significant differences between blood samples from coronary artery disease (CAD) patients as compared to healthy controls [263]. Some of these changes could be interpreted in terms of potential contribution of peripheral blood leukocytes to the pathogenesis of CAD [263]. In another study, peripheral blood leukocytes responded to changing plasma lipid levels by regulating a network of genes, including genes involved in immune responses and inflammation and in lipid and fatty acid metabolisms [264]. This study provided further data on the inert relationships between plasma lipid levels and leukocytes in the atherogenic process. It also suggested that peripheral blood leukocyte gene expression profiling was not only useful for identifying disease biomarkers but might also shed light on disease pathways involved. Similarly, Chon et al [265] studied changes in mRNA levels in leukocytes from untreated or treated hypertensive patients. Their findings indicated that microarray gene expression profiling of peripheral leukocytes can distinguish hypertensive patients from age- and sex-matched controls.

In oncology studies, Twine et al [266] tested for surrogate transcriptional markers in peripheral blood mononuclear cells of patients with renal cell carcinoma; an eight-gene classifier set was identified to best predict renal cell carcinoma versus normal. Subsequently, the same group showed significant correlation with overall survival and progression-free survival in patients in a phase II study of CCI-779 [267]. In another study, Sharma et al [262] reported a 37-gene signature that detected breast cancer correctly in more than 80% of cases. In this study, female patients without cancer but with other comorbidities were classified correctly as ‘non-cancer’, highlighting the specificity of the breast-cancer signature identified from blood.

Many other studies support the power of blood leukocyte gene-expression profiling in disease detection, classification and diagnosis. Such studies have investigated profiles in asthma [268], severe lupus erythematosus [269], rheumatoid arthritis [270], Crohn’s disease and ulcerative colitis [271], pulmonary arterial hypertension [272], ischemic stroke and Alzheimer’s and Huntington’s diseases [273, 274].

4.1.1 Peripheral blood gene expression profiling in acute pancreatitis

Based on all the above mentioned evidence, it can be confidently stated that microarray based peripheral blood leukocyte gene-expression profiling holds great promise. But to date, no one has actually studied the role of blood derived inflammatory cells as markers for pancreatic inflammation. It is hypothesised, for this project, that the peripheral blood leukocytes can serve as a ‘reporter function’ as biomarkers of acute pancreatitis, and as such would express unique genes during pancreatitis. Further, as discussed before, these cells represent an easily accessible, non-invasive source of material. This easy availability of blood leukocytes to provide reporter function for solid organ disease will likely prove useful in pancreatitis. It is therefore, postulated, for the reasons stated above, that the response to pancreatitis and potentially the prognostic indicator of disease severity, rests in the peripheral blood leukocytes. These are the cells which mediate the immunologic reaction to pancreatitis. The genetic map of these leukocytes during this disease has not been looked into before. Recently, Bluth et al [275] looked into the gene expression profiles in cells of peripheral blood in a rat model of experimental pancreatitis (sodium taurocholate) and used a model of septic shock and saline controls as controls to identify new molecular markers of acute pancreatitis using microarray technology. They found 140 genes which showed unique significant changes in expression of

PBMCs during the acute phase of acute pancreatitis, but not in sepsis. They concluded that pancreatitis related genes are induced in cells outside the pancreas – in peripheral blood mononuclear cells during necrotising pancreatitis. It should not be surprising that such genes are induced since these genes were originally identified from inflamed pancreatic tissue which likely already had similar peripheral blood mononuclear cells infiltrating them.

4.1.2 Gene expression Microarrays

Gene expression microarrays have revolutionised the advances in genomics. From analysis of 378 arrayed lysed bacterial colonies in 1982 [276] to complete eukaryotic genome expression on a microarray in 1997 [277], the microarray technology has come a long way in developing our understanding of molecular mechanisms underlying normal and pathological biological phenomena. It has enabled us to measure expression of the whole human genome with a single biological sample and provide a snapshot of all the transcriptional activity in it. Microarrays have facilitated the discovery of totally novel and unexpected functional roles of genes, as opposed to study of a single gene or a subset of genes which was the only possible molecular biology application available in the past. These tools have helped molecular biologists in discovering novel disease subtypes, developing new diagnostic tools, and identifying underlying mechanisms of disease or drug response.

4.2 MicroRNA Profiling

MicroRNA (miRNA) have emerged as the most significant scientific discovery of the decade. Their role in understanding pathophysiology of many disease processes, as diagnostic, prognostic and predictive tissue specific biomarkers, is only just beginning to be unravelled [278-288].

These are small (18-22 nucleotide), non-coding RNA that arise from a longer precursor RNA, exhibiting a hairpin structure. The active 22 nucleotide moiety then gets incorporated into the composite machinery – this results in translational silencing when it forms a partial duplex with the 3' untranslated regions of the targeted mRNA [289, 290]. Other mechanism proposed for its gene expression control is by degradation of mRNA.

MicroRNA regulate gene expression and are implicated in many biological and pathological processes, including modulation of haematopoietic lineage differentiation, insulin secretion, apoptosis, stress response and cell and organ development [291-295]. A single miRNA can regulate an average of 200 mRNA targets and concurrently, a single mRNA can be regulated by several miRNA, leading to a very complex genomic regulation of the disease process [289].

MicroRNA have been particularly appealing due to their stability in various tissues samples, even post-fixation. These can still be extracted and assessed with incredible efficiency and reliability [296]. Since circulating nucleic acids usually occur in very small amounts in the body fluids and are very difficult to extract and detect reliably [297], this miRNA stability can certainly be exploited into development of practical detection methods, rendering them almost perfect to act as clinical biomarkers. Recent studies have also revealed the possible superiority of miRNA over mRNA in terms of accuracy as biomarkers since mRNA has to be translated into a protein for it to exert its effect, whereas, miRNA are an active moiety in themselves, impacting the expression of multiple genes and therefore, are more likely to emulate the real biological and pathological events. Also, it is postulated that powerful and yet simple approaches to miRNA characterisation and quantification will far outweigh traditional, more cumbersome and inconvenient proteomic biomarker discovery techniques in terms of biomarker validation [298]. Furthermore, miRNA are more likely to predict clinical behaviour, prognosis and therapeutic response in pathological states with more accuracy since these influence the behaviour and phenotype of the tissue these are expressed from. Additionally, it has been established in some well-designed recent studies that miRNA enter circulation and stay in it in unaltered form. The reasons behind this remarkable stability are predicated to be them being packaged within exosomes secreted from cells [299] or found in protein associations – Argonaute [300] or Nucleophosmin [301, 302]. This protects them from degradation from endogenous RNAses, contributing towards their inherent stability and making them an ideal candidates for serum/plasma based biomarkers. This also makes them best suited for affecting the distant cells in way of novel signalling molecules and mediating short and long range inter-cellular communication as extra-cellular messengers.

4.2.1 MicroRNA profiling in sepsis and acute pancreatitis

Sepsis usually results from malfunction of regulatory mechanisms, precipitating loss of control of inflammation, immunosuppression and tissue/organ damage [303-305]. MiRNA have been found to play a pivotal role in sepsis – these strictly regulate the expression of pro and anti-inflammatory genes involved in disease process, mainly correlated with cytokines [306] and therefore, their aberrant expression can be investigated to be used as diagnostic, prognostic and predictive marker.

Sepsis provides a good model for acute pancreatitis, where many patho-physiological processes are similar and the critical driving immune responses are identical. Therefore, it doesn't come as a surprise that the prognostic role of miRNA in acute pancreatitis has been investigated on the similar lines.

Current literature and body of research has identified at least three potential roles for miRNA in the setting of acute pancreatitis - as a diagnostic/prognostic marker, as a marker of pathophysiological processes in the pancreatic tissue and as a marker of mediation of end-organ/distant organ failure due to their ability to act as inter-cellular signalling molecules. The last one is especially intriguing since the evidence on distant organ failure in acute pancreatitis is not as well defined as local complications.

4.3 Messenger RNA and Micro RNA interaction

The complex genomic relationship that results from mRNA and miRNA interactions translates into a stringent control that is exerted by miRNA on cellular protein output and function. Needless to say, learning more about mRNA and miRNA interactions is absolutely crucial in identifying important cellular pathways that are pivotal to patho-physiology of the disease.

Currently, there are few methods in place to identify regulatory miRNAs. The most traditional method remains based on microarray expression data, using prediction algorithms such as TargetScanS [307], PicTar [308] and miRanda [309] and gene set testing [310]. Another newer and perhaps a better method has been put forward which is based on Odds Ratio [311]. This methods takes expression data from both miRNA and mRNA into consideration and determines if there is an association between miRNA expression and its predicted targets. If the association is found to be statistically significant, the miRNA is

considered to be regulatory. Some of the other publications have also put forward varying integrative methods but these are not without their limitations – these do not take into consideration the fact that miRNA may co-regulate a group of mRNAs [311]; these may not provide any information on miR-mR pairs of interest [312, 313]; expression data correlation between miRNA and mRNA is not taken into account [312]; Identification of the modules is always based on negative correlation between miRNA and mRNA [314] which is not always true, as has been recently shown in a study [315]. These limitations have been overcome and dealt with in a new statistical model proposed [316] that identifies regulatory miRNA clusters regulating mRNA clusters and estimates the association between the two, since both the steps are crucial for obtaining more useful biological information.

With this in mind, the plan is to take a systems biology approach to attain better understanding of the biological processes and the disease. This will be in line with the key components already outlined for this approach – measurement of the molecules, integration of biological information, identification of molecular responses to the disease process, formulating models to be tested and consequent refinement of these models through hypothesis testing. These models can then be utilised to predict disease responses, identify new time points in the course of the disease and add to our understanding of the key pathophysiological events [317].

CHAPTER 5: HYPOTHESIS AND AIMS

5.1 Hypothesis

It is hypothesised that mild and severe acute pancreatitis are two distinct entities, associated with different disease courses.

Gene expression and miRNA profiling can identify gene and miRNA signature/s which may be exploited to act as potential prognostic markers to predict severity of the disease at the time of admission.

5.1.1 Aims

- To establish protocol and Standard operating procedures for collection of samples from patients with acute pancreatitis and establish and an Acute Pancreatitis Biobank, associated with Acute Pancreatitis Database, housing demographic and clinical information for the recruited patients.
- To develop a gene expression profile of peripheral blood in patients with acute pancreatitis and identify differentially expressed genes between mild and severe acute pancreatitis.
- To develop a micro RNA profile of peripheral blood in patients with acute pancreatitis and find a micro RNA signature which will accurately stratify patients in mild and severe groups.

5.1.2 Objectives

- To measure gene expression profiles in patients with mild and severe acute pancreatitis, using RNA extracted from peripheral blood collected within the first 24 hours of disease presentation.

- To measure micro RNA profiles in patients with mild and severe acute pancreatitis, using RNA extracted from plasma samples of patients collected within the first 24 hours of disease presentation.
- To develop a protocol which not only suits the needs and demands of the project but is also robust and reproducible for the main study.

**CHAPTER 6: LIVERPOOL PANCREAS
RESEARCH UNIT ACUTE PANCREATITIS
BIOBANK, DATABASE, SAMPLE
COLLECTION AND CLINICAL DATA**

Acute pancreatitis research is severely limited by the availability of biological samples at appropriate and clinically relevant time points. The first 24 hours are the most crucial in terms of diagnosing, predicting the severity of the attack and intervening in the right patient group. For all of this to happen, it is fundamental that the biological processes during this time period are properly understood at the cellular and molecular level.

The Liverpool NIHR Pancreas Biomedical Research Unit (PBRU) was established in 2008 with the mandate to translate basic pancreatic research into a clinical setting, in order to improve the treatment of patients with acute pancreatitis, chronic pancreatitis and pancreatic cancer.

The three main themes of research are:

- Drug discovery and development of new interventions
- Application of new diagnostic and imaging strategies
- Validation of new biomarkers and screening protocols

In 2010, the Liverpool NIHR Pancreas Biomedical Research Unit (PBRU) Acute pancreatitis (AP) Biobank was established to enable us to create a pool of patient samples which will contribute towards various research projects in the fields of proteomics, genomics, metabolomics and pharmacology. This was established under the ethical approval awarded by the Sheffield Ethics Committee (reference number 10/H1308/31).

The PBRU Biobank is managed on the basis of set of rules and regulations governing the collection, storage and future use of these samples. The samples for the PBRU AP Biobank are obtained and stored from donors for research purposes in compliance with the Human Tissue Act, 2004. All individual studies, where the biobanking of samples is required, have to have the approval of the Executive Director and full ethical approval before the study begins. All studies have to be transparent, auditable, and allow the chain of custody of all samples to be followed at all times. Samples are only taken from donors following full, informed consent given by the donor.

Standard Operating Procedures (SOPs) are in place to control all aspects of sample storage.

SOPs contain details and follow procedures concerning:-

- Donor identification
- Obtaining consent
- Sample anonymisation
- Sample collection
- Sample processing
- Sample storage (including database storage of sample information and coding)
- Sample analysis (samples to be analysed according to ethically approved sample protocols for the study)
- All procedures are COSHH (Control of Substances Hazardous to Health Regulations) and risk assessed and obey University of Liverpool (and where applicable NHS) Health and Safety rules
- SOPs are signed off by the author, with two signatories to approve the SOP and Quality Assessment (QA) review, which ensures that only approved SOPs that comply with the applicable regulatory requirements for the study are produced
- All SOPs are version controlled (by the Liverpool Cancer Trials Unit Document Management System) and only issued to designated copyholders
- The location of the freezers where the samples are to be stored, and the electronic database are clearly identified
- A Disaster Recovery Plan is in place in the event of equipment failure

The Biobank stores samples obtained from patients who present and are admitted to Royal Liverpool University Hospital (RLUH) with provisional diagnosis of acute pancreatitis. After obtaining informed written consent, these samples are collected at specific time points – 24 hours, 48 hours, week one, two and four of patient's hospital stay. Once a patient is discharged, no follow-up is undertaken and patients are not contacted for further samples/information. The whole blood collected from these patients is taken to PBRU laboratory immediately and processed according to specific Good Clinical and Laboratory Practice (GCLP) approved Standard Operating Procedures (SOPs) to render plasma, serum and white cell pellets. Whole blood is collected into PAXgene Blood RNA tubes and stored in -80°C freezer for RNA isolation to be undertaken later on.

Clinical data from these patients is recorded in the Acute Pancreatitis database. It consists of two linked databases, first of which is an identifiable database of demographic data that includes name, date of birth, address, General Practitioner details and hospital unit number, with a coded link to a second pseudo-anonymised clinical database. The identifiable demographic database is stored on a password-protected, separate Royal Liverpool University Hospital network drive. The separate pseudo-anonymised clinical database is maintained on a password-protected, separate University of Liverpool network drive. This specifically designed clinical database includes details of each coded patient's presenting features, diagnostic tests, clinical course, interventions, complications and outcome, obtained predominantly from patient medical records, and includes a coded link to the patient's banked research samples. The banked research samples are also coded and are not identifiable without reference to the pseudo-anonymised Clinical Database and from that to the demographic database. The clinical database contains all relevant Good Clinical Practice data on every research sample, including when taken, when processed and where stored. Only PBRU staff have access to the full data set and are able to link samples back to individual patients.

6.1 Sample collection

All samples for the project were obtained from the Liverpool NIHR PBRU Acute Pancreatitis Biobank. These samples have been collected following the SOP 'Collection of Samples for the Acute Pancreatitis Biobank in the PBRU' (version 3, document reference SOP 051). A brief overview of the process is described below.

Patients with probable diagnosis of acute pancreatitis are identified first through biochemistry department of RLUH, where a list is generated of all patients who have presented with raised amylase levels (>450 U/l, three times the normal range) in the last 24 hours. These patients are then reviewed for their suitability for inclusion (clinical and biochemical picture compatible with the diagnosis). After obtaining written informed consent, blood samples are collected using premade kits with pre labelled tubes, in line with the Laboratory Information

Management System (LIMS). Kits are used according to the time point at which patient is being approached for the sample collection, e.g., Kit A is used for the first 24 hours samples, Kit B for the 48 hours, week 1, 2 and 4 samples and Kit C for all other time points.

Approximately 20 mls of blood is drawn into two BD Vacutainer® K₂ EDTA tubes (10 and 4 mls) for plasma and cell pellet extraction, BD Vacutainer® SST™ tube for serum and PAXgene Blood RNA Tube (BRT) for RNA extraction to be undertaken later on. These samples are then taken to the PBRU laboratory and processed according to the SOP 'Processing of Samples for the Acute Pancreatitis Biobank in PBRU'.

PAXgene BRTs were collected at 24 hours, 48 hours, week 1, 2 and 4 time points. These were either stored within 10 minutes of being collected in -80°C freezer in the GCLP laboratory (time 0 hour samples) or incubated at room temperature for two hours after being collected before being stored at -80°C (time 2 hours samples). For the sake of this project, only PAXgene BRTs collected within the first 24 hours of patient admission were included and RNA extractions undertaken from these. Since this part of the study was majorly intended for sample size calculations and for assessing baseline variability among our patient population, only patients with mild pancreatitis were included.

The definitions of mild and severe acute pancreatitis are in accordance with the Atlanta Classification for acute pancreatitis [5]. Presence of organ failure (renal, respiratory, cardiovascular, hepatic, nervous system) within the first week of admission or development of pancreatic parenchymal necrosis after that is considered as severe form of the disease. The severity of the disease was estimated only when patients had been discharged or had died. Data pertaining to these would be derived from the PBRU Acute pancreatitis database which

is a prospective database storing all the clinical and demographic information on our patients who have donated samples to us.

In order to determine if there was a difference in the gene expression of the samples stored at different time points from the time of collection, two PAXgene BRTs were collected from six patients, one stored within 10 minutes of being collected (time 0 hour samples) and the other one after two hours of incubation at room temperature (time 2 hours samples). All these pair matched samples were stored in the -80°C freezer in the GCLP laboratory.

6.2 Clinical Data

Clinical characteristics were collected for all included patients. These were retrieved from the Liverpool NIHR PBRU Acute Pancreatitis Database that houses information on patients who are recruited to the Acute Pancreatitis Biobank. This information includes their demographic data and detailed account of their hospital admission. These are as follows:-

- Details of hospital admission (symptom onset, date and time of admission, length of stay, aetiology)
- Details of co-morbidities (in the form of Charlson's scoring), past surgical and specifically, biliary surgery and in use medication
- Details of haematology and biochemistry (blood tests) and scoring systems (APACHE II)
- Details of imaging (ultrasound, CT scans) and microbiology
- Details of surgical procedures (if undertaken)
- Details of intensive care admission – supportive treatments used

- Details of complications and outcomes

These data were used to label these patients as mild and severe according to Atlanta classification (1992) for the purpose of data analysis. During the course of the project, revised Atlanta classification was published, which was soon followed by determinant based classification. Since this project was conceived and started, with samples collected, before the new classifications were proposed, it was deemed best to continue with the old Atlanta classification and therefore, data analysis was undertaken according to that.

Keeping in mind the objectives of this study, it was obvious that Atlanta classification [5] would be used to include/exclude patients for this study. Accurate characterisation of the patient cohort would be of paramount importance. Sample size calculations had indicated a sample size of 40 in mild and severe categories. Patient characteristics were retrieved from the Acute Pancreatitis Database. This is a database set up in 2010 in line with the Acute Pancreatitis biobank. The aim was to collect phenotypic information on all the samples being collected for research so that genotype could be correlated with the phenotype of these patients. The database contains comprehensive data on clinical features and outcomes of these patients who have been recruited to donate samples to the biobank.

Diagnosis of acute pancreatitis was based on two of the following three features: 1) abdominal pain suggestive strongly of acute pancreatitis, 2) serum amylase activity at least three times greater than the upper limit of normal 3) characteristic findings of acute pancreatitis on trans-abdominal ultrasonography or on Contrast Enhanced CT (CECT) scan.

As indicated, patients who developed organ failure (OF) lasting for more than 48 hours (i.e., organ failure recorded at least once during each of three consecutive days) or developed >30% pancreatic necrosis were classified as severe patients. The first hospital day was designated as day 1; day 2 started at 8 AM on the following day and lasted for 24 hours.

Persistent evidence of organ failure (one or more organ systems) on at least one occasion on three consecutive days was recorded for each patient. Patients with no complications or transient OF were classified and included in the mild group.

6.2.1 Definitions of organ failure

This was defined according to Sepsis-related Organ Failure Assessment (SOFA) scoring [225]. As per AC, only respiratory, renal and CVS failures were considered for severity assessment. A score of ≥ 2 was considered. Although Atlanta classification advises use of Marshall Scoring system for organ failure, SOFA score was chosen for the ease of recording on patients. Marshall scoring requires assessment of cardiovascular status by taking into consideration patient’s response to fluid resuscitation. This can only be undertaken prospectively, while one attends to the patient. Accurate quantification of the same may become problematic and potentially, impossible retrospectively. SOFA is easy to use and record and takes six organ systems into consideration. It is also important to note that point criteria and hence points scored on both the scoring systems are more or less the same, therefore, organ failure for the purpose of this project was assessed through SOFA scoring. Definitions are detailed in table 1.

Table 6.2 - SOFA Scoring System

System	1	2	3	4
Respiratory PaO ₂ /FiO ₂ mm Hg	<400	<300	<200*	<100*
Coagulation x 10 ³ mm ³	<150	<100	<50	<20
Liver (bilirubin)	20-32	33-101	102-204	>204

μmol/L				
Cardiovascular Hypotension	MAP < 70 mm Hg	Dopamine ≤ 5, Dobutamine any dose ^a	Dopamine >5 Epinephrine/Norepinephrine ≤ 0.1	Dopamine >15 Epinephrine/Norepinephrine > 0.1
CNS (GCS)	13-14	10-12	6-9	<6
Renal Creatinine μmol/L	110-170	171-299	300-440	>440

*with respiratory support

^aAdrenergic agents administered for atleast 1 hr

It has to be borne in mind that Liver score wasn't taken into consideration in these patients, as bilirubin can be raised due to gallstone disease.

6.2.2 Definition of Outcome/Severity Assessment

CECT findings were used for definitive diagnosis and extent of the necrosis. Quantification of the necrosis was undertaken using a morphometric technique which is traditionally used by histopathologists to qualitatively and quantitatively assess tissue. A grid would traditionally be placed over a histological image and the number of points within the region of interest would be counted. The number of points within the image as a whole would also be counted and the area of the region of interest expressed as a fraction of the area of the histological image as a whole. When applied over consecutive images, it allows the 3-dimensional quantification of a tissue from 2-dimensional axial slices. This method was adapted for axial CT images with a 2mm grid placed over consecutive axial slices covering the pancreas. The grid was applied using *COREL® PHOTO-PAINT™ X4*. This software is not specifically

designed for use in morphometry. It is primarily used as an editing package for digital photographs. However, it contained several features which allowed it to be used for manipulating the CE-CT images for the morphometric analysis. It also allowed for the magnification of the images if required. The whole of the pancreas was clearly identified, outlined and divided into head, body and tail before the point-counting was commenced. Both viable and non viable areas in each part of the pancreas were assessed using this method and this was expressed as a fraction of the area of the pancreas as a whole. This process was repeated for all axial slices of the abdomen which included the pancreas (average of 60 slices per CE-CT scan) and the average of each consecutive fraction was calculated as the percentage necrosis of the pancreas. The percentage necrosis was assessed for each part of the pancreas for each CT scan included in the study.

Axial slices obtained in the portal phase of contrast enhancement (approximately 70 seconds) were reviewed.

There is no consensus on the exact boundaries of the different parts of the pancreas. Major anatomy [318] and pancreas [319] textbooks, although divide pancreas in to head, neck, body and tail, do not define the boundaries between these major parts. For the purpose of this project, it was imperative that we defined these anatomic divisions precisely. According to 'Radiology of Pancreas [320] (a textbook on dedicated pancreatic radiology), the head of the pancreas along with the uncinate process is taken as the part of the pancreas which is seen on the right of the mesenteric and portal veins. Neck is constricted portion of the pancreas lying to the left of the right border of the superior mesenteric vein and to the right of its left border. According to the classification proposed by the Japanese Pancreas Society, portion of the gland left to the left border superior mesenteric vein is bisected into the body (the right half) and the tail (the left half). However, it was important that these boundaries would be visible on the CE-CT too.

Pancreatic necrosis was defined as the loss of normal enhancement of the pancreas when compared with the liver and spleen as well as loss of the normal lobulated pancreatic outline. Reduced enhancement without loss of normal architecture was not considered to be necrosis. For the purposes of this study, pancreatic pseudocyst, phlegmon and peripancreatic abscesses were noted but not included in the pancreatic necrosis estimation. Pancreatic abscesses were quantified in a similar manner but separate to necrosis.

Pancreatic pseudocysts and peri-pancreatic abscesses were noted when present.

6.3 Classifications of severity of acute pancreatitis

When the project was conceived, Atlanta classification [5] was taken as the guide for defining the severity of the disease. Definitions considered are as follows:-

Mild acute pancreatitis is defined as the absence of organ failure or the presence of organ failure that does not exceed 48 hours in duration (Transient organ failure).

Severe acute pancreatitis was defined as persistence of organ failure – organ failure documented at least once in three consecutive days (Persistent organ failure) and/or presence of >30% pancreatic necrosis.

However, during the course of the project, at least two more clinically relevant and internationally agreed upon classifications were published and widely discussed. These were:-

Determinant Based Classification (DBC) [321] – This one hypothesised that the spectrum of the disease was too wide to be classified into just two categories and therefore, more classes were needed for more accurate description of the disease as the outcomes varied with each. This classification proposed four different groups:-

Mild – No peri-pancreatic necrosis or organ failure

Moderate – Sterile pancreatic necrosis and/or transient organ failure

Severe – Infected pancreatic necrosis or persistent organ failure

Critical – Infected pancreatic necrosis and persistent organ failure

Revision of Atlanta Classification [322] – Like DBC, this web based consensus also recognised the short-comings of the old AC and aimed to improve the assessment of severity through sensible integration of new concepts of the pathology in with the existing information which should then consequently, help with standardised reporting of the data and of the disease management across the globe. Their revision classified the disease into three different categories:-

Mild – No organ failure/local or systemic complications

Moderately severe – Transient organ failure, local complications or exacerbation of a comorbid disease.

Severe – Persistent organ failure

Local complications have been defined as peri-pancreatic fluid collections, pancreatic and peri-pancreatic necrosis (sterile or infected), pseudocyst and walled-off necrosis (sterile or infected).

As mentioned and explained above, this project only concerns the old Atlanta classification.

6.4 Clinical Data

During the time of my PhD, I managed to recruit 315 patients with acute pancreatitis to the Acute Pancreatitis Biobank. These included patients who had primarily presented to Royal Liverpool University Hospital (RLUH) with the disease and also the ones who had been transferred from other hospitals for more definitive and ongoing management of their complicated disease course. I obtained and processed samples from them at different time

points, for the biobank. These were at the time of admission, 48 hours, 72 hours, week 1, 2 and 4. This amounted to approximately 800 samples in 18 months duration.

For this project, I only included fifty eight patients, who had presented primarily to RLUH and their samples were collected within 24 hours of hospital presentation. They had been recruited prospectively to Acute Pancreatitis Biobank from June 2010 to June 2012 and samples were collected prospectively at the time of admission. Baselines characteristics were also recorded for each patient and clinical details observed for the length of the stay. Severity assessment was then undertaken retrospectively (at the end of the clinical episode), using Atlanta Classification to see if the patient had a severe or mild attack. Baseline demographic characteristics are given below in table 6.1.

Table 6.2 – Demographic characteristics of included patients

Characteristics	Mild	Severe
	Median	Median
Number	35	23
Gender (Male/Female)	20:16	14:9
Age (years)	64	63
Aetiology		
Gallstones	20	16
Alcohol	9	5
Post-ERCP	2	1
Idiopathic	4	1
Duration (Symptoms to admission) (hours)	6	4
Duration (Admission to sampling) (hours)	14.6	15.5
Duration (Symptoms to sampling) (hours)	22.75	20.25
History of acute pancreatitis	9	5
Length of hospital stay (days)	10	16

Clinical co-morbidities were also taken into consideration for the recruited patients using Charlson’s Co-morbidity index. Smoking and alcohol history was ascertained using World Health Organisation’s recommended Alcohol, Smoking and Substance Involvement Screening Test (ASSIST) (Version 3). These data are presented in table 6.3.

Table 6.3 – ASSIST and Charlson’s scores for included patients

Parameters	Mild	Severe
	Median	Median
Smoking	20	15
ASSIST score - Smoking	25	20
ASSIST score - Alcohol	27	26
Charlson’s score	2	3

Prediction of severity of the disease was recorded using Glasgow’s criteria, and CRP values at the time of admission, at 48 hours and at the end of week one. These values are illustrated in table 4. Glasgow and APACHE II scores were not found to be of significance in our dataset, with non-discriminatory values between the two groups. Only CRP values at admission, 24 and 48 hours were significantly different between mild and severe groups.

Table 6.4 – Prediction of Severity of included patients

	Mild	Severe
	Median	Median
Glasgow score on admission	1	2
Glasgow score at 48 hours	1	2
CRP on admission (mg/dl)	31	78
CRP at 48 hours (mg/dl)	130	216
CRP at 72 hours (mg/dl)	136	272
APACHE II score on admission	7	8
APACHE II score at 48 hours	7	8

Approximately 76% of MAP patients underwent ultrasound scanning to assess for presence of gallstones. This amounted to 56% in SAP patients. Almost the same number in both the

groups had CT scan to assess severity of the disease. CT severity index (CTSI) was understandably higher in SAP than in MAP group, largely due to severe localised disease in the form of fluid collections in SAP. Also, extent of pancreatic parenchymal necrosis was more prevalent in SAP than in their counterparts. While similar number of patients in both the groups had two CT scans, slightly more patients went on to have a third and then a fourth scan in SAP group (3 vs. 5; 1 vs. 3 respectively). Extra-pancreatic complications, identified on the CT scans, included, gastric outlet obstruction, ascites, pleural effusion, thromboses (portal vein, splenic vein, superior mesenteric vein), splenic infarcts and liver abscesses. Details of radiologic findings are summarised in the table below.

Table 6.5 – Imaging details of included patients

	Mild	Severe
	Median	Median
Ultrasound undertaken	26	13
CT undertaken	19	20
CTSI	4	6
Balthazar Score 2	14	10
Balthazar Score 4	3	9
No Pancreatic necrosis	13	10
Pancreatic necrosis < 30%	15	2
Pancreatic necrosis >30%	0	6

Only five patients needed intubational feeding. Interestingly, two belonged to MAP and three to the SAP group. The duration of the feeding averaged to five days. None of the patients required total parenteral nutrition. Only two patients underwent minimal access retroperitoneal pancreatic necrosectomy (MARPN). One patient required two procedures, whereas, adequate debridement was achieved after three procedures in the other. Another patient required open necrosectomy. One patient in the MAP group and two in the SAP needed percutaneous drainage of peri-pancreatic collections.

Thirteen (56%) SAP patients needed intensive care admission and care – mainly for respiratory and cardiovascular support, in the form of inotropes and ventilation. The average ITU/HDU stay was eight days. As anticipated, none of the MAP patients needed such input and were all managed on the wards, without additional organ support.

Table 6.6 shows the daily SOFA scores between the two groups. The scores are distinctly higher for the SAP patients – this becomes more evident and significant from day four onwards when the scores become almost normal for the MAP, but increase or are consistently the same for the SAP. It would not be wrong to say that the discriminatory value of SOFA scores increases after 72 hours, based on our dataset.

Table 6.6 – SOFA scoring of included patients

	Mild	Severe
	Median	Median
SOFA score Day 1	1	2
SOFA score Day 2	1	2
SOFA score Day 3	1	3
SOFA score Day 4	0	3
SOFA score Day 5	0	4
SOFA score Day 6	0	3
SOFA score Day 7	0	3

Table 6.7 shows the overall and organ specific complications in our dataset. While all SAP patients, by definition, had complications, 12 out of 35 MAP also had various complications but not fitting the SAP criteria. These included pleural effusion (n=3), acute coronary syndrome (n=2), Portal vein thrombosis (n=1), cardiac arrhythmias (n=1) and pancreatic parenchymal necrosis of less than 30% (n=4). Five of the SAP (21%) had acute circulatory failure for more than 48 hours. Vascular complications in four SAP patients comprised portal, splenic and superior mesenteric vein (SMV) thromboses and pseudo-aneurysm. Almost half of all the SAP patients developed respiratory complications – five had respiratory failure for

more than 48 hours; others had pleural effusion (n=5), atelectasis (n=4) and hypoxia (n=2).

Approximately 49% of all SAP patients were found to have pancreatic parenchymal necrosis of more than 30%, categorising them into the severe category. Pseudocysts were identified in four cases, along with a liver abscess and a small bowel fistula.

None of the included patients had any neurological complications.

Seven patients (30%) with SAP died during the course of the disease, accounting for an overall mortality of 12%.

Table 6.7 – Complications of included patients

	Total	Mild	Severe
	Number of patients	Number of patients	Number of patients
Complications developed	35/58	12	23
Cardiac	10/58	3	7
Vascular	5/58	1	4
Respiratory	16/58	4	12
Abdominal	18/55	5	13
Death	7/58	0	7

Specific complications of the patients with SAP are listed in table. Nine patients only had local complications, whereas, nine patients had isolated organ failure. Five patients had both.

Table 6.8 – Local and systemic complications in SAP patients

ID	Local complications	Systemic complications/Organ Failure	Mortality
AP09	>50% pancreatic necrosis	Respiratory failure in the first week	
AP011		Respiratory failure in the first week	
AP29		Circulatory and respiratory failure in the first week	Yes
AP34	45% pancreatic necrosis		
AP46	Pancreatic abscess > 3 cm		

AP51	>50% pancreatic necrosis, SMV thrombosis, splenic infarcts		
AP57	40% pancreatic necrosis		
AP58	Peri-pancreatic fluid collections	Respiratory failure in the first week	
AP63		Respiratory failure in the first week	
AP66	Peri-pancreatic fluid collections	Circulatory failure in the first week	Yes
AP81		Circulatory failure in the first week	Yes
AP99	35% pancreatic necrosis		
AP103	>50% pancreatic necrosis		
AP117	Peri-pancreatic fluid collections, splenic and SMV thromboses, small bowel fistulae	Respiratory failure in the first week	
AP144	30% pancreatic necrosis	Respiratory failure in the first week	
AP168		Respiratory and circulatory failure in the first week	Yes
AP177		Respiratory failure in the first week	
AP187		Respiratory and circulatory failure in the first week	Yes
AP192		Respiratory, renal and circulatory failure in the first week (multi-organ)	Yes
AP234	40% pancreatic necrosis		
AP273	75% pancreatic necrosis		
AP307	50% pancreatic necrosis		
UHA08		Respiratory and circulatory failure in the first week	Yes

CHAPTER 7: GENE EXPRESSION PROFILING

7.1 Sample preparation – the RNA extraction

The RNA samples to be used for the project were manually extracted from the PAXgene BRTs using the PAXgene Blood RNA kit (Qiagen). All samples were processed following the same protocol.

Before the procedure – All PAXgene BRTs were taken out of the -80 °C freezer and incubated at room temperature (15-25° C) for 2 hrs after thawing.

- PAXgene BRTs were centrifuged for 10 minutes at 4000 x g using a swing-out rotor.
- Supernatant was removed by decanting. RNase-free water (4 mls) was added to the pellet.
- Mixture was vortexed until the pellet was visibly dissolved, and then, centrifuged for 10 minutes at 4000 x g using a swing-out rotor. Entire supernatant was discarded.
- Buffer BR1, which is a resuspension buffer, (350µl) was added and vortexed until the pellet was visibly dissolved.
- Sample was pipetted into a 1.5 ml microcentrifuge tube (MCT). Buffer BR2, a binding buffer (300µl) and proteinase K (40 µl) were added to the sample and mixed by vortexing for 5 seconds, and incubated for 10 minutes at 55°C using a shaker–incubator at 400 rpm. Proteinase K was added to ensure adequate protein digestion.
- Lysate was then pipetted directly into a PAXgene Shredder spin column (PSC) and centrifuged for 3 minutes at 17,000 x g to homogenise the cell lysate and remove residual cell debris. Entire supernatant of the flow-through fraction was transferred to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
- Ethanol (350 µl - >99%) was added to the supernatant to adjust binding conditions and mixture was then mixed by vortexing, and centrifuged briefly (one second at 700 x g) to remove drops from the inside of the tube lid.

- 700 μ l of the sample was pipetted into the PAXgene RNA spin column (PRC) and centrifuged for one minute at 17,000 g. Spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
- The remaining sample was pipetted into the PAXgene RNA spin column, and centrifuged for one minute at 17,000 x g. Spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
- 350 μ l of Buffer BR3, a wash buffer, was pipetted into the PAXgene RNA spin column and centrifuged for one minute at 17,000 x g. Spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
- DNase I incubation mix was then prepared by mixing gently DNase I stock solution to DNA Digestion Buffer in a 1.5 ml microcentrifuge tube.
- DNase I incubation mix (80 μ l) was then directly pipetted onto the PAXgene RNA spin column membrane, and placed on the bench top (20–30°C) for 15 minutes to ensure removal of trace amounts of bound DNA.
- PAXgene RNA spin column was again washed with Buffer BR3, and centrifuged for one minute at 17,000 x g. Spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
- 500 μ l of Buffer BR4 was pipetted to the PAXgene RNA spin column for another wash, and centrifuged for one minute at 17,000 x g. Spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
- Wash with Buffer BR4 was repeated and centrifuged for 3 minutes at 17,000 x g.
- The tube containing the flow-through was discarded. PAXgene RNA spin column was placed in a new 2 ml processing tube and centrifuged for 2 minute at 17,000 x g to dry off the ethanol.

- The tube containing the flow-through was discarded. RNA was eluted by pipetting 40 µl of elution buffer BR5 into the PAXgene RNA spin column placed in a 1.5 ml microcentrifuge tube and centrifuged for 1 minute at 17,000 g to elute the RNA.
- This 1st elute was stored in two aliquots, 15 µl each, and the rest was reserved for qualitative and quantitative assessments.
- PAXgene spin column was placed in a fresh 1.5 ml microcentrifuge tube and 40 µl of elution buffer was pipetted on to elute the remaining RNA. It was centrifuged for one minute at 17,000 x g.
- This was stored as one aliquot and called 2nd elute.
- All the extracted samples were labelled and stored in the -80°C freezer until further processing.

7.2 Quality control Assessments

Aliquots for quality control assessments were taken at the time of extraction. Concentration of the samples was analysed using Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the qualitative assessment was undertaken using Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit.

Since 100 ng of RNA was required in a total volume of 3 µl, concentration of the sample was adjusted accordingly. Highly concentrated samples were diluted with RNase free water and diluted samples were speed vacuumed to achieve uniform concentration throughout. Samples were renamed for feasibility with further analyses.

7.2.1 Qualitative assessment of RNA samples using Agilent 2100 Bioanalyzer

Before starting: All reagent and reagent mixes, except RNA ladder, were kept at 4°C.

Reagents were taken out of the fridge to warm at room temperature for at least 30 minutes.

The dye and gel-dye mix was wrapped in aluminium foil and put in the drawer to protect from light.

Gel matrix was prepared by adding 550 µl of gel matrix to a spin filter and spun for 10 minutes at 4000 rpm.

Electrode cleaners were used, one with RNase Zap and the other with RNase free water (350 µl). These were put in machine and run for 1 minute each.

Gel-dye matrix was prepared by adding 1 µl of dye to a 65 µl tube of gel matrix and vortexed. Mix was then centrifuged at maximum speed at 14000 rpm for 10 minutes.

9 µl of gel-dye matrix was loaded into the well marked G. Another 9 µl of the gel dye matrix was pipetted in the two additional wells marked G. 5 µl of marker was then pipetted into the well marked ladder and each of the 12 RNA wells

The RNA samples and RNA ladder were denatured by incubating in the 70° C heating block for 2 minutes and put on ice for 5 minutes.

1 µl of ladder was loaded into the well marked “ladder” and 1 µl of RNA into each of the 12 wells.

After being placed in the adapted vortexer and mixed for one minute at 2400 rpm, the loaded chip was placed in the Bioanalyzer and run for 30 minutes to obtain the traces.

Once it was ensured that the samples were of high quality, as assessed by the Bioanalyzer traces, these were processed through for preparation for the microarray experiments.

7.3 Protocol A – 3' IVT Express protocol

Since HG U133 Plus 2.0 arrays were intended to be utilised for the project, the 3' IVT Express protocol was used for preparation of the targets.

The overview of the protocol is summarised in figure 7.1.

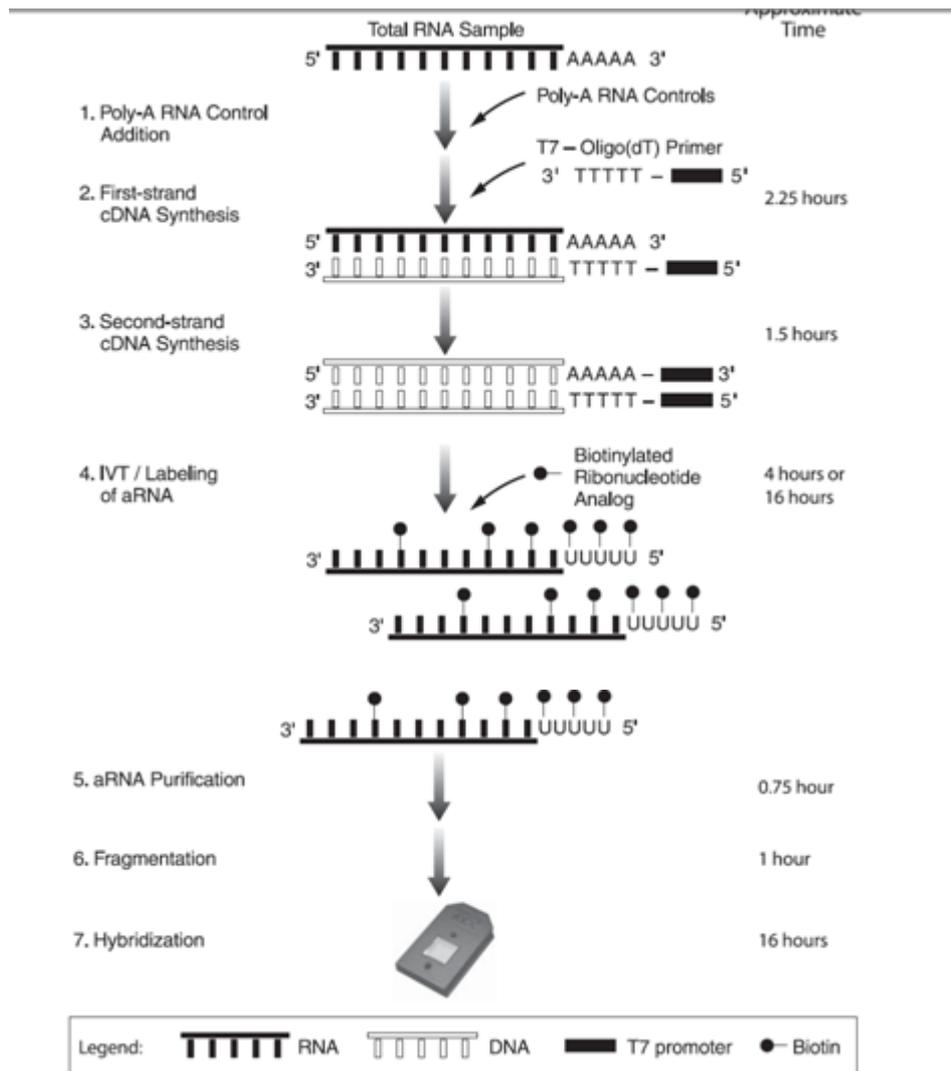


Figure 7.1 - 3' In vitro Transcription protocol (Adapted with permission)

7.3.1 Step 1 – Preparation of Poly-A RNA control mixture

Poly-A RNA control mixture was prepared. Poly-A controls are prokaryotic genes which are absent in eukaryotic samples (*dap, thre, phe, lys*). These are *in vitro* synthesised and the polyadenylated transcripts for *B. subtilis* genes are premixed at staggering concentrations. Examining the hybridization intensities of these controls on the Genechip arrays helps to monitor the labelling process independently from the quality of the starting RNA.

- Serial dilutions required to prepare poly A control mixture depend on the amount of the starting material. Since I used 100 ng as the input amount of RNA, the serial dilutions selected were: 1:20 (1st dilution), 1:50 (2nd dilution), 1:50 (3rd dilution), 1:10 (4th dilution).
- For 1st dilution, 2 µl of Poly A control stock was added to 38 µl of Poly A control dilution buffer. 2 µl of this solution was added to 98 µl of dilution buffer to produce the 2nd dilution. 2 µl of the 2nd dilution was mixed with 98 µl of dilution buffer to produce the 3rd dilution. Finally, 4 µl of the solution was mixed with 36 µl of dilution buffer to produce the 4th dilution.
- 2 µl of the 4th dilution was added to 3 µl of each RNA sample to make a 5 µl solution, ready for the 1st strand synthesis.

7.3.2 Step 2 – 1st strand cDNA synthesis (using reverse transcription)

- First strand master mix was prepared by mixing 4 µl of buffer mix with 1 µl of enzyme mix for each sample.
- 5 µl of the master mix was aliquotted to 12 PCR tubes.
- 5 µl of the Total RNA/poly-A Control Mixture (from step 1) was added to each aliquot of the first strand master mix to achieve a final volume of 10 µl.
- Samples were incubated for two hours at 42 °C in a thermal cycler.

7.3.3 Step 3 – 2nd strand cDNA synthesis

- Second strand master mix was prepared by mixing 5 µl of buffer mix, 13 µl of nuclease free water and 2 µl of enzyme mix for each sample.
- 20 µl of the master mix was transferred to each (10 µl) cDNA sample.
- Samples were placed in a 16 °C thermal cycler block and incubated for an hour followed by 10 minutes at 65°C.

7.3.4 Step 4 – synthesis of labelled aRNA (using In Vitro Transcription IVT)

- IVT master mix was prepared by mixing together IVT biotin label (4 µl), labelling buffer (20 µl) and enzyme mix (6 µl) for each sample.
- IVT Master Mix (30 µl) was transferred to each (30 µl) double-stranded cDNA sample and the reactions were incubated for 16 hours at 40 °C in thermal cycler.

7.3.5 Step 5 – Purification of aRNA

After synthesis, the aRNA was purified to remove enzymes, salts, and unincorporated nucleotides.

- aRNA Binding Mix was prepared by mixing RNA binding beads (10 µl) and aRNA binding buffer concentrate (50 µl) for each sample.
- 60 µl of aRNA Binding Mix was added to each sample and the resultant 120 µl was transferred to the U-bottom plate.
- 120 µl of 100% ethanol was added to each sample and mixed adequately to allow aRNA to bind to the RNA binding beads.

- Magnetic beads were captured using the magnetic stand. Once capture was complete and RNA binding beads had formed pellets, supernatant was discarded and plate removed from the stand.
- RNA binding beads were washed using aRNA Wash Solution (100 µl/sample) and shaken at a moderate speed for 1 min on lab-line titre plate shaker. Magnetic beads were captured using the magnetic stand. Once capture was complete and RNA binding beads had formed pellets, supernatant was discarded and plate removed from the stand.
- Another wash was performed following the same procedure and the plate shaken at very high speed for one min on lab-line titre plate shaker to evaporate residual ethanol.
- Purified aRNA was eluted from the RNA binding beads by adding 50 µl of preheated (50–60 °C) aRNA Elution Solution to each sample. The plate was then shaken vigorously for 3 minutes on the lab-line titre plate shaker. RNA binding beads were captured by moving the plate to the magnetic stand. Supernatant (containing the eluted aRNA) was transferred to a nuclease free PCR tube.

7.3.6 Step 6 – Analysis of aRNA size

The size distribution of aRNA was evaluated using Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit. Concentration of aRNA was assessed using Thermo Scientific NanoDrop™ 1000 Spectrophotometer to ensure adequacy of the amplification process.

7.4 Protocol B – Ovation whole blood protocol

The overview of the protocol is summarised in figure 7.2.

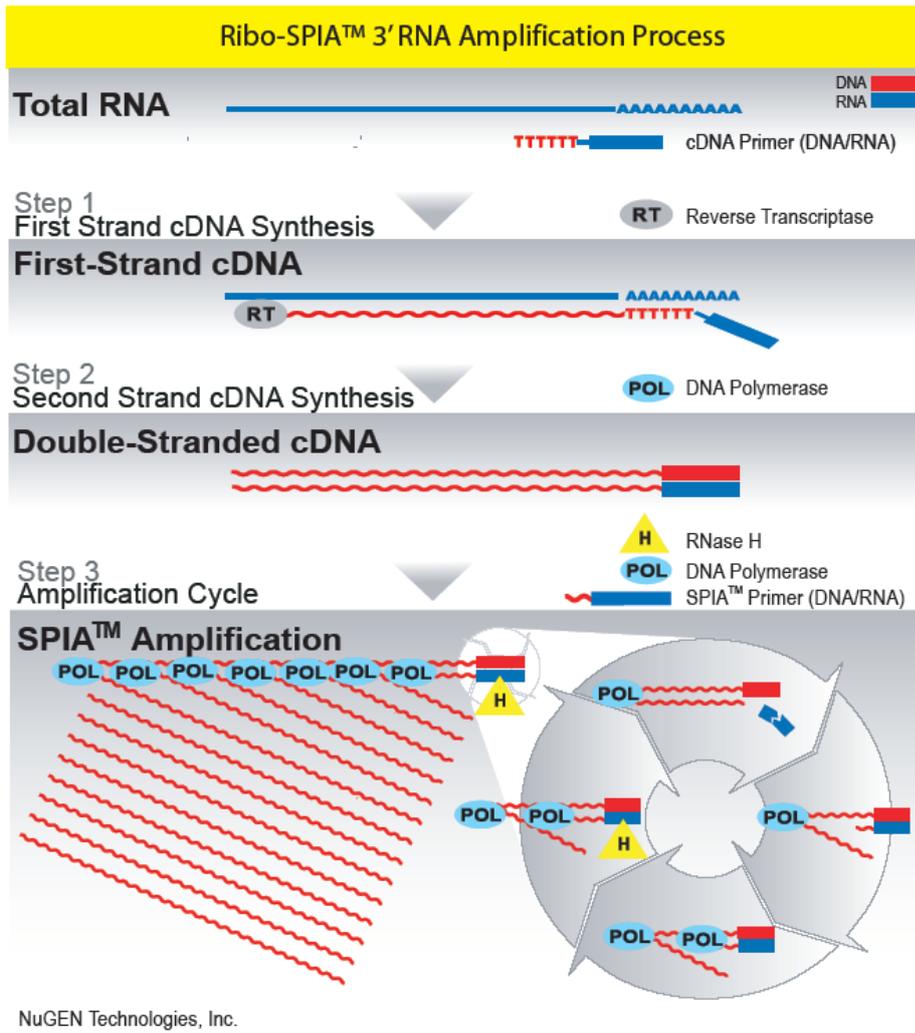


Figure 7.2 - Ribo-SPIA™ 3' RNA Amplification Protocol (Adapted with permission)

Before proceeding with the protocol, concentration of RNA samples was analysed using Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the qualitative assessment was undertaken using Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit.

7.4.1 Step 1 – Preparation of Poly-A RNA control mixture

Since serial dilutions required to prepare poly A control mixture depend on the amount of the starting material and the starting RNA input for this protocol was 40 ng, the serial dilutions selected for this protocol were : 1:20 (1st dilution), 1:50 (2nd dilution), 1:50 (3rd dilution), 1:25 (4th dilution).

For 1st dilution, 2 μ l of Poly-A control stock was added to 38 μ l of Poly A control dilution buffer. 2 μ l of this solution was added to 98 μ l of dilution buffer to produce the 2nd dilution. 2 μ l of the 2nd dilution was mixed with 98 μ l of dilution buffer to produce the 3rd dilution. Finally, 2 μ l of the solution was mixed with 48 μ l of dilution buffer to produce the 4th dilution (total volume of 50 μ l).

2 μ l of the 4th dilution was added to 3 μ l of each RNA sample to make a 5 μ l solution, ready for the 1st strand synthesis.

7.4.2 Step 2 - 1st strand cDNA synthesis

- 2 μ l of first strand primer mix was mixed with 5 μ l of the RNA sample to allow for primer annealing.
- 1st strand master mix was prepared by mixing buffer mix (12 μ l) and enzyme mix (1 μ l). 13 μ l of master mix was added to each reaction to make up a final volume of 20 μ l.
- Samples were incubated at 48°C for 60 minutes, at 70°C for 15 minutes and cooled to 4°C in thermal cycler.

7.4.3 Step 3 - 2nd strand cDNA synthesis

- Second strand master mix was prepared by combining buffer mix (18 µl) and enzyme mix (2 µl). 20 µl of the mix was added to each first strand reaction tube.
- Samples were incubated at 37 °C for 30 minutes, at 75 °C for 15 minutes and then cooled to 4 °C.

7.4.4 Step 4 - SPIA® Amplification

- Master mix was made by combining sequentially the SPIA buffer mix (72 µl), SPIA primer mix (2 µl), nuclease free water (4 µl) and SPIA enzyme mix (40 µl).
- 118 µl of the SPIA master mix was added to the entire volume of the 2nd strand reaction.
- Resultant 158 µl reaction volume was split into two 79 µl volume in new PCR tubes.
- 24 PCR tubes were left to incubate at 48 °C for 30 minutes and then cooled to 4 °C.
- 3 µl of Ovation whole blood reagent was added to each 79 µl of SPIA reaction.
- All reactions were incubated at 48 °C for 30 minutes, at 95°C for 5 minutes and then cooled to 4 °C.

7.4.5 Step 5 - Purification of amplified cDNA

This was undertaken using the Qiagen QIAquick® PCR Purification Kit.

- 160 µl of amplified cDNA product (82 µl aliquots pooled together) was added to PCR tubes containing 800 µl of PB buffer each and mixed by vortexing.
- 480 µl of sample was loaded onto the QIAquick® spin column and centrifuged for one minute at 13,000 rpm.

- Remaining 480 µl of the sample was loaded onto the same column and centrifuged for one minute at 13,000 rpm.
- 700 µl of 80% ethanol was added to the column and centrifuged for one minute at 13,000 rpm.
- Ethanol treatment was repeated another time and to remove the remaining liquid, column was centrifuged for one additional minute at 13,000 rpm.
- Nuclease-free water (30 µl) was added to the centre of each column and allowed to stand for five minutes at room temperature to elute purified cDNA.
- Columns were then centrifuged at 13,000 rpm for one minute to collect 25 µl of purified cDNA.

Concentration of the purified amplified cDNA was analysed using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Since 4.4 µg of RNA was required in a final volume of 25 µl for further labelling and fragmentation reactions, samples were diluted in nuclease free water to achieve desired concentration.

7.4.6 Step 6 – Fragmentation of amplified cDNA

- Fragmentation master mix was prepared by mixing together fragmentation buffer (5 µl) and enzyme mix (2 µl) together for each sample.
- 25 µl of the purified SPIA™ cDNA into a PCR tube was added to 7 µl of the Fragmentation Master Mix.
- After adequate mixing, tubes were incubated at 37 °C for 30 minutes, at 95 °C for 2 minutes and cooled to 4 °C.

7.4.7 Step 7 - Biotin Labelling

- Fragmentation of amplified cDNA was immediately followed by the Biotin labelling.
- Labelling master mix was prepared by combining together labelling buffer (15 µl), reagent (1.5 µl) and enzyme mix (1.5 µl).
- 18 µl of labelling master mix was added to each sample (32 µl), making up a total volume of 50 µl for the hybridization reaction.
- Samples were mixed thoroughly by vortexing and spinning and incubated in a pre-warmed thermal cycler at 37 °C for 60 minutes, at 70 °C for 10 minutes and then cooled to 4 °C.

The fragmentation success and the size distribution of the final fragmented and biotinylated product was viewed on Agilent 2100 Bioanalyzer on an RNA 6000 Nano LabChip®.

7.4.8 Step 8 - Hybridization

The Affymetrix Genechip® Human Genome U133 Plus 2.0 arrays (HG-U133 Plus 2.0) were used. This particular array is comprised of 1,300,000 unique oligonucleotide features covering over 47,000 transcripts and variants, which, in turn, represent approximately 39,000 of the best characterized human genes. The probe sets used for the design of the array have been selected from Genbank®, dbEST and RefSeq. Oligonucleotide probes are synthesized in situ complementary to each corresponding sequence. Eleven pairs of oligonucleotide probes are used to measure the level of transcription of each sequence represented.

- Hybridization Master Mix was prepared by adding together Oligo B2 (3.7 μ l), 20 x hybridization controls (11 μ l), 2 x hybridization buffer (110 μ l), 100% DMSO (22 μ l) and nuclease free water (25.3 μ l) for each sample.
- 48 μ l of fragmented cDNA was added to 172 μ l of hybridization master mix to render a final volume of 220 μ l.
- Hybridization cocktail (master mix + fragmented cDNA) was heated to 99° C for 2 minutes in a heat block and shifted to 45° C heat block for 5 minutes. It was later centrifuged at maximum speed for 5 minutes just prior to loading.
- 200 μ l of pre-hybridization mix was added to each chip and these were rotated in the hybridization oven for 10 minutes at 45°C. The mix was removed from each chip prior to sample loading.
- 200 μ l of sample was added to each chip and hybridized for 16 hours in the oven at 45° with a speed of 60 rpm.

7.4.9 Step 9 - Washing, staining and scanning

After 16 hours of incubation, chips were taken out of hybridization oven and placed at room temperature.

- Prime fluidics station was primed using the GeneChip® Operating Software (GCOS) on computer, which took approximately seven minutes.
- Hybridization cocktail was pipetted out of the chip and stored in an eppendorf at 20°C.
- Each chip was then filled with wash buffer A (~300 μ l).
- Stain cocktails 1 and 2 (600 μ l each) and Array Holding Buffer (800 μ l) were aliquotted and inserted appropriately (sample holder 1, 2 and 3 respectively) in the Fluidics Station 450/250.

- Using the AGCC Fluidics Control Software, washing and staining of the gene chips was undertaken.
- Once the protocol was completed, the arrays were ready to scan on the GeneChip Scanner 3000

Scanning: Affymetrix Command Console software was used to scan the genechips.

- Genechips were inserted into the scanner.
- Once scanning was completed, Affymetrix Expression Console was used to generate the quality control metrics to ensure adequate and appropriate processing of the arrays.

7.5 Statistics

Data analysis was undertaken using R software (R Development Core Team, 2006; Version 2.10.1), Bioconductor (version 2.10) and Partek Genomics Suite software. Other packages used were simpleaffy (for generating QC metrics), affy (for generating probe level data from CEL files), Limma (to perform between-array normalisation and to construct and perform volcano plot) and siggenes (for performing power calculations). Mas5 transformation was used to convert probe level data into expression level data. Expression level data were quantile normalised before differential expression analysis of mRNAs of severe versus mild acute pancreatitis was performed using ANOVA with batch hybridization effects. The Benjamini and Hochberg multiple testing correction was applied to the resulting p-values.

7.6 Optimisation and selection of protocol and pilot study

Since the HU 133 Plus 2.0 arrays were used for this particular project, we used the 3' IVT express protocol (Protocol A), which is the complementary protocol to these arrays, to carry out the amplification and labelling processes. However, when the size of the resultant amplified unfragmented RNA was analysed, abnormal peaks were observed on the trace. A normal amplified RNA looks like the following figure 11.1.

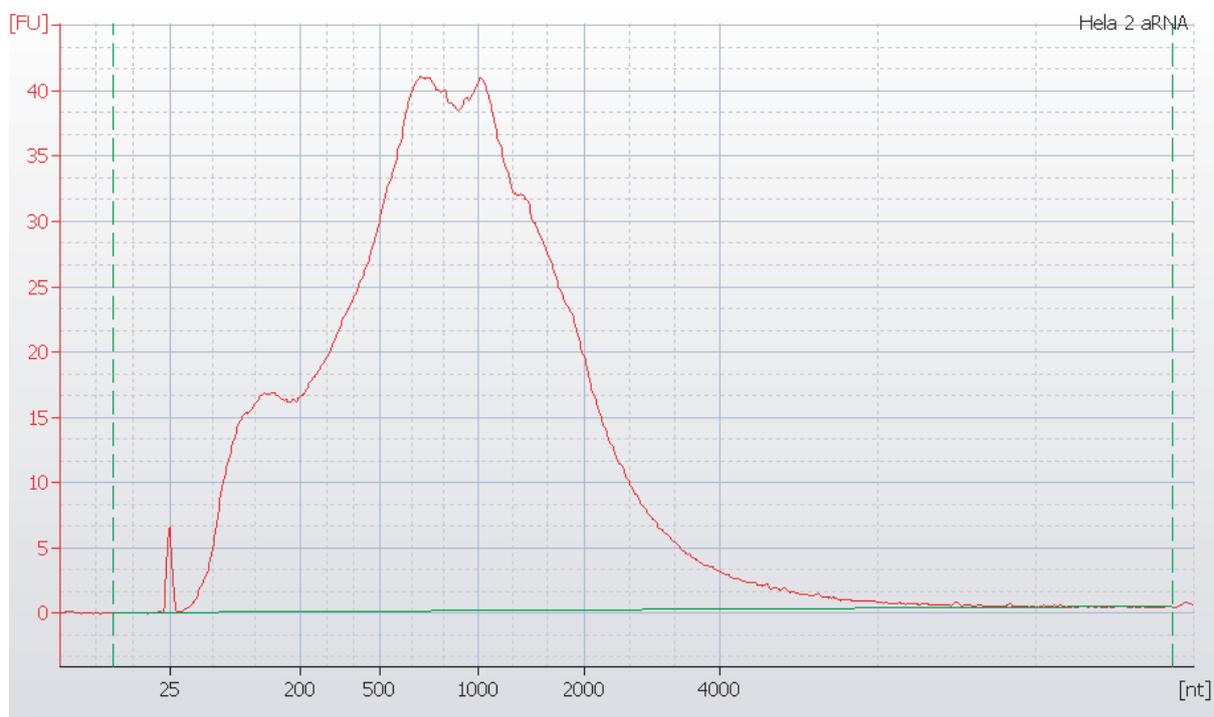


Figure 7.3 – Normal amplified RNA trace

The unexpected peaks were observed throughout all the samples. Similarly, the electrophoresis gel image also showed abnormal bands in the region of 700-800 nucleotides. These abnormal findings were thought to be due to very high levels of haemoglobin mRNA in the samples. This is not an uncommon finding, especially when dealing with human whole blood samples.

The degree of globin message representation in mRNA in whole blood can vary widely, with globin mRNA species constituting up to 70% of whole blood mRNA in some patients [323] and can interfere with assessment of other genes.

Increases in globin mRNA contributes to decreased sensitivity of detection of transcripts on microarrays [324]. It has been shown by several groups [325-327] that excessive level of globin transcripts can induce a data artefact through promiscuous cross hybridization to microarray probes. Consistent with this, both scale factor and percentage present calls are negatively impacted by increasing amounts of globin.

Conversely, Globin reduction improves the detection of sensitivity of the microarrays by increasing the call rates from higher signal intensities and lower signal to noise ratios and permitting detection of additional 'masked' genes [326, 328].

Therefore, it was decided that the Ovation whole blood protocol would be used to reduce globin levels. A justification of this is given below.

7.6.1 Globin mitigation protocols

To improve the laboratory assays and increase discovery power, several commercially available solutions have been developed to reduce or mitigate the effects of excess globin transcripts on microarray hybridization signal in the last few years. These can be classified into two strategies.

Globin clear approach: The first approach focuses on minimizing the amplification of globin specific messages in amplified cRNA. These methods include physically removing

globin transcripts from total RNA by hybridization to anti-globin oligonucleotides affixed to magnetic beads (GLOBINclear™) [328]. Because of sample manipulation, GLOBINclear has the potential to adversely affect the integrity of total RNA [329], is difficult to scale up and requires species-specific reagents.

Globin reduction PNA approach: This method blocks the amplification of globin transcripts by using oligonucleotides of nucleic acid analogs (PNA, LNA), which when bound to a transcript prevents its amplification by reverse transcriptase [330]. The PNA approach was once recommended by Affymetrix but has subsequently been withdrawn from the market. The PNA-based technique was simple and scalable, but PNA design was difficult and costly to expand for other species. Theoretically, the PNA-based process seemed to fit better into the established target preparation process because it was performed in the same tube as the cDNA synthesis reaction. However, the stability of PNA in the long term was unknown and the method required taking measures to prevent PNA aggregation and precipitation. The method had been shown by the manufacturers to increase detection of transcripts and reduce biological variation between samples. However, it was not known how it would transform a gene expression profile, or to what extent it could generate a profile similar to that of samples without high levels of globin mRNA.

Disadvantages of Globin clear and PNA techniques: Despite globin reduction protocols, globin depletion is ~75%. These techniques are mostly efficient at reducing globin mRNA from cRNA but can induce RNA type-dependent changes in quality of RNA during sample preparation and quality of microarray results. Both techniques generate a hybridization target composed of cRNA and rely on the post-RNA isolation manipulation of the samples prior to or at the first step of mRNA amplification, leading to potential processing bias in gene expression data. The globin reduction steps introduce particular transformations to the

original gene expression profiles, and even after globin removal, it would not be possible to recover a profile equivalent to that of an identical RNA sample without globin mRNA excesses.

A study [326] compared these two methods (GLOBINclear and GeneChip Globin-Reduction PNA Kit) in a Jurkat cell line and showed that neither method could recover a profile equivalent to that of an identical RNA sample without globin mRNA excess. Therefore, none of these protocols could completely remove globin gene mRNA and the remaining globin genes in the blood samples still continue to drive the main eigenvector in the data set.

Thus, despite the application of globin reduction protocols, overrepresentation of globin genes on the microarrays can be a confounder for biomarker discovery, particularly in diseases with subtle gene expression signature.

7.6.2 cRNA versus cDNA targets

Several groups have detected widespread cross-hybridization in microarray measurements [331, 332], and on the order of 10% of the probes on a common oligonucleotide array platform were predicted to be susceptible to cross-hybridization [332]. High level of promiscuity in DNA-RNA hybridisations underlies widespread cross hybridization in microarrays. This cross hybridisation can be reduced by using cDNA targets instead of cRNA.

From its inception, microarray technology took advantage of either of two types of biochemical entities as the labelled target, cRNA [333] or cDNA [334]. Although in many aspects these two types of labelled target are considered to be equivalent for the purpose of microarray analysis, the use of cRNA has held an important methodological advantage.

Because RNA polymerase does not require a primer, it was rather straightforward to design a near-linear target amplification method, which has been extremely useful in experiments with small amounts of starting RNA [333], including most clinical studies. Even so, there are hints in the literature indicating that DNA-RNA hybridization might be less specific than DNA-DNA hybridization and hence, more susceptible to cross hybridization. One such relevant observation is that in free solution DNA-RNA hybrids, certain mismatch ‘wobble’ base pairs are more stable than complementary base pairs [335]. This effect is, however, absent in DNA-DNA hybrids [336], indicating that targets made of DNA might be a more specific alternative to standard RNA targets. These characteristics also support the use of DNA as a means of mitigating the effects of globin, and potentially other highly abundant interfering transcripts. cDNA hybridizations have greater intensity (low Scale Factor) and better discrimination between true signal and background. Not only are there improvements in hybridization metrics, but the deleterious effects of globin cross-hybridization are reduced. Another study concluded that the cDNA target is better able to discriminate between the correct probe and similar but incorrect probes and that it is unlikely that the increased specificity using cDNA targets instead of cRNA targets comes at the price of decreased sensitivity [337].

7.6.3 The Ovation Whole blood protocol

Ovation whole blood technique is a new single primer, isothermal linear amplification method, according to which, small amounts of total RNA are reverse transcribed into cDNA using a chimeric RNA/DNA primer containing oligo (dT) and a unique RNA sequence tag at the 5’ end. A single linear isothermal amplification reaction rapidly generates sufficient single stranded cDNA for multiple hybridization reactions.

This method does not specifically restrict amplification of globin transcripts; rather it relies on the high specificity of DNA-based hybridization [337, 338]. During this procedure, all transcripts, including globin, are amplified to produce complementary cDNA. It is believed that the high specificity of DNA-DNA interactions reduces cross hybridization signal due to excess globin, thereby reducing artifactual signals. The methodology amplifies target mRNA using a novel template generation and isothermal strand displacement strategy [338, 339]. It has recently been improved with the addition of the Whole Blood reagent (WB) that optimizes the amplification for whole blood samples.

A recent study by Parrish et al [325] compared and quantified the impact of various mitigation technologies by employing the cDNA hybridization and PNA protocol. Their results reiterated the fact that globin related cross hybridization is the main source of artefact. They also found that the cDNA amplification (ovation whole blood protocol) improved the percent present calls by 25%, as compared to 10% by the PNA treatment and also exhibited more uniform detection and discrimination of low expressed genes by increasing expression signal across a wider range of low intensity probes. Another important characteristic of the cDNA targets was found to be the reduction of background intensity.

In a nutshell, the Ovation whole blood protocol is highly reproducible and sensitive, fast and simple to operate. Both whole transcript amplification and 3' initiated RNA amplification reproducibly generate high quality cDNA amplification products suitable for undertaking transcriptomic studies on GeneChip microarrays. The system provides a means for global gene expression analysis from very small samples and is particularly useful for clinical research where supplies of biologic samples are limited.

7.7 Quantitative and Qualitative Analyses

There were 6 paired samples for the pilot study. Prior to the amplification process, it was ensured that the concentration of total RNA was adequate for the downstream application.

Table 11.1 lists the final concentrations of the samples.

Table 7.1 – Concentration of RNA samples prior to amplification process

Sample ID	New ID	Concentration (ng/ μ l)	Final concentration (ng/ μ l)
AP040 – 0 hr	1	31.6*	50.2
AP040 – 2 hrs	2	41.6	41.6
AP041 – 0 hr	3	41	41
AP041 – 2 hrs	4	67.3	67.3
AP042 – 0 hr	5	45.3	45.3
AP042 – 2 hrs	6	243.9 \pm	50.08
AP043 – 0 hr	7	36.1	36.1
AP043 – 2 hrs	8	30.2*	45
AP044 – 0 hr	9	137.5 \pm	50.9
AP044 – 2 hrs	10	123.4 \pm	51.4
AP045 – 0 hr	11	36.5	36.5
AP045 – 2 hrs	12	20.5*	43

*these samples were speed vacuumed to achieve a concentration of >40 ng/ μ l

\pm these samples were diluted to achieve a concentration of >50 ng/ μ l

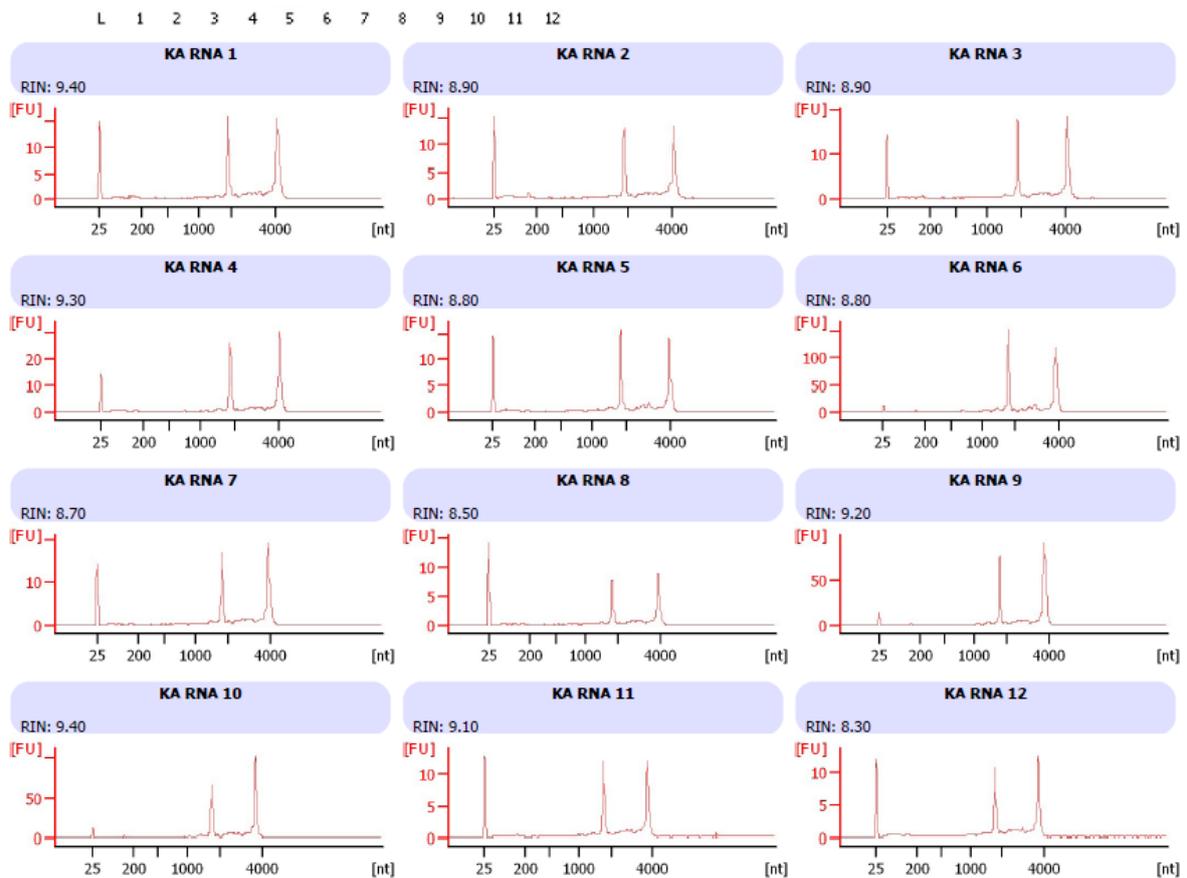


Figure 7.4 – Bioanalyzer traces of the Total RNA prior to Amplification

Amplified RNA (Protocol A) was found to be in adequate concentration as can be seen in the table 7.2.

Table 7.2 – Concentration of amplified RNA

ID	Concentration (ng/μl)
1	969.6
2	1045
3	1039

4	1114.7
5	1080.2
6	884.5
7	965.3
8	892.6
9	558.3
10	515.9
11	866
12	874

For Protocol B, cDNA was synthesized and quantified. It was found to have amplified adequately and efficiently as is reflected in the concentration and Bioanalyzer traces.

Table 7.3 – Concentration of amplified cDNA

ID	Concentration (ng/ μ l)	Volume of sample required (μ l)*	Volume of nuclease free water required (μ l) \pm	Final volume (μ l)
1	351.5	12.60	12.4	25
2	320.7	13.72	11.8	25
3	298.7	14.75	10.25	25
4	272.1	16.20	8.8	25
5	251.1	17.55	7.45	25
6	290.8	15.15	9.85	25
7	296	14.90	10.1	25
8	264	16.67	8.33	25

9	290.3	15.16	9.84	25
10	270	16.30	8.70	25
11	255.4	17.23	7.70	25
12	234	18.82	6.18	25

*4400/concentration = volume of sample required

±25-volume of sample required = volume of nuclease free water require

The qualitative assessment was undertaken using Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit.

7.8 Data Analysis

Table 7.4 shows the various parameters looked at for the HU133 plus 2.0 arrays.

Table 7.4 – Quality control metrics

QC metrics for HG U133 Plus 2.0 arrays												
	1	2	3	4	5	6	7	8	9	10	11	12
Scale												
Factor	1.51	1.652	1.39	1.417	1.658	1.62	2.222	1.604	1.293	1.372	1.746	1.618
Backgro	Avg:											
und	28.46	31.33	30.78	32.66	28.44	30.44	27.47	29.55	30.22	30.56	28.18	30.80
Corner	Avg:											
+	191	193	208	205	159	209	111	127	130	136	111	123
	Avg:											
	1330	Avg:										
Corner-	7	16526	9921	18193	13220	14850	10296	9593	9034	5890	7465	7848

	Avg:											
	1338	Avg:										
Central-	6	16870	10469	18377	14220	15135	11638	10366	8681	6339	7475	7939
	2963											
Number	0	28667	33152	32677	31571	32714	30964	32153	33664	33202	30174	32135
Present:	54.4%	52.4%	60.6%	59.8%	57.7%	59.8%	56.6%	58.8%	61.6%	60.7%	55.2%	58.8%
Internal												
Control	Sig(3'	Sig(3'/										
s	/5')	5')	5')	5')	5')	5')	5')	5')	5')	5')	5')	5')
GAPDH	1.38	1.41	1.41	1.35	1.58	1.61	1.42	1.55	1.47	1.64	1.65	1.88
Beta												
Actin	2.35	2.08	2.32	2.68	2.34	2.78	2.06	2.81	2.81	3.07	2.84	4.72
Spike												
Control												
s												
Probe	Sig											
Set	(3')	Sig(3')										
	3739.	3781.2	3215.5	2838.3	3273.2	3575.2	3378.7	3185.7	2739.5	2769.6	3441.7	3546.0
bioB	3 (P)	(P)	6 (P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)
	7623.	7632.2	7116.9	6300.6	7128.4	7907.3	6865.1	6887.7	5783.4	5895.1	7465.1	7338.3
bioC	7 (P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)
	2271											
	6.3	20763.	20295.	18121.	20232.	21844.	21408.	20077.	16512.	17844.	21153.	20629.
bioD	(P)	8 (P)	7 (P)	5 (P)	0 (P)	7 (P)	7 (P)	6 (P)	8 (P)	9 (P)	8 (P)	1 (P)
	3384											
	0.7	33171.	32022.	27645.	32237.	34509.	35189.	30979.	26253.	29001.	33241.	33513.
cre	(P)	3 (P)	7 (P)	7 (P)	7 (P)	6 (P)	8 (P)	0 (P)	1 (P)	9 (P)	7 (P)	6 (P)
	6300.	6850.4	6861.1	6919.9	9441.5	8453.6	8245.1	7505.8	5677.2	7502.0	8677.3	7639.4
dap	8 (P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)
	942.3	913.3	1103.6	934.2	1741.0	1278.2	1145.2	1202.8	885.4	1306.8	1395.6	1973.5
lys	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)
	731.0	924.6	874.5	963.5	1423.6	1584.1	989.0	1343.0	995.0	1265.9	1769.6	1283.9
phe	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)

	969.5	1037.1	982.5	1511.2	2249.3	1742.5	1824.1	1658.0	1172.2	1431.4	2006.4	1933.6
thr	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)	(P)

All arrays scanned for 54675 probe sets. Scale factor was found to be the same through all the samples, depicting uniformity in the quality of these. Background was found to be within a range of 27-33. Although there are no official guidelines regarding background, Affymetrix has found that typical Average Background values range from 20-100 for arrays scanned with the GeneChip® Scanner 3000. This may point towards a low probability of inappropriate hybridization and therefore, more accurate results. All 12 arrays had comparable background values. Note that the low background has shifted the mean intensity towards the left side. This is graphically represented in figure 7.5, (generated from the [simpleaffy](#) package).

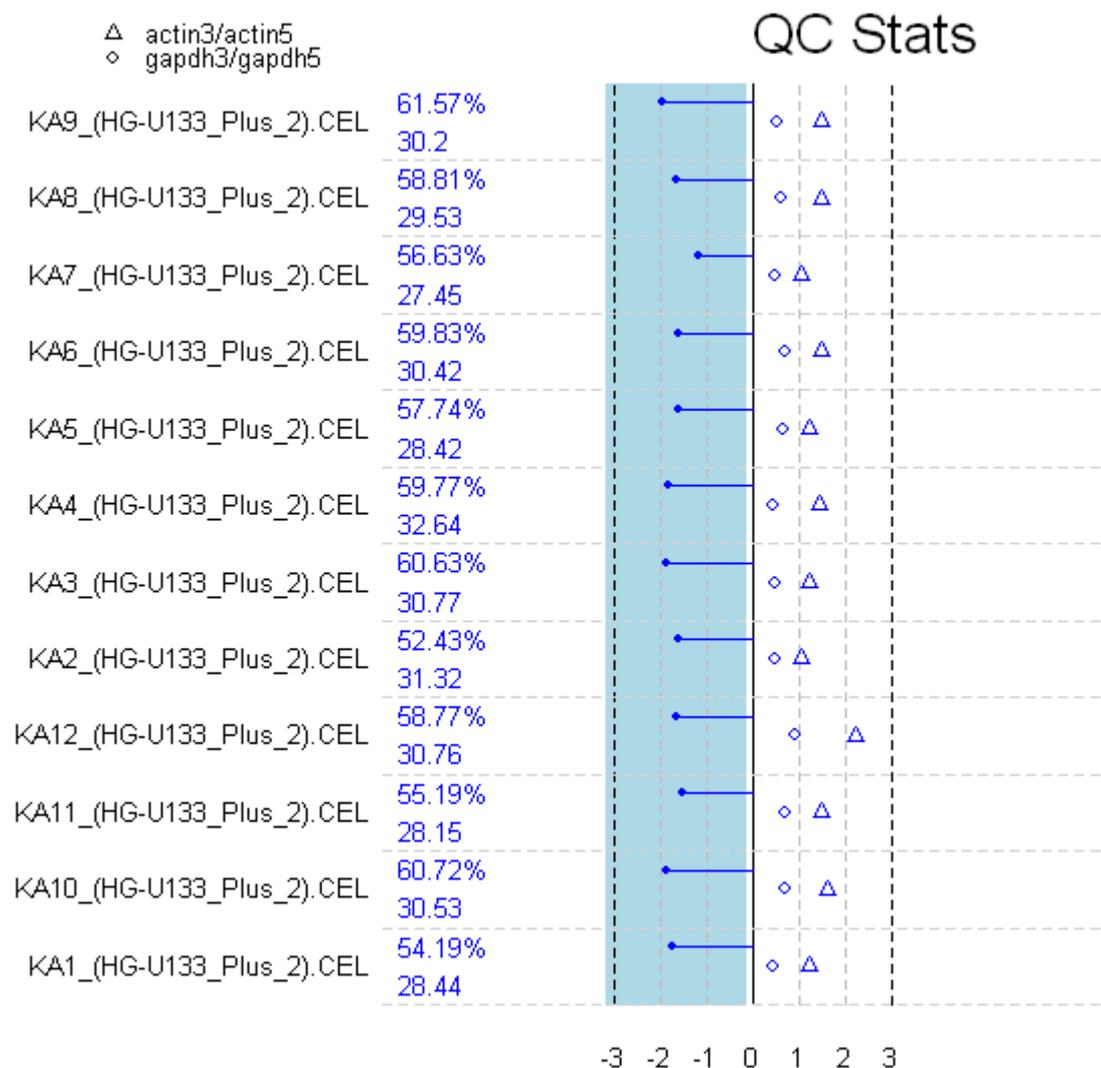


Figure 7.5 – Quality control Statistics

Corner⁺, Corner⁻ and Central⁻ values indicate adequate alignment of the grid to the microarray slide. Percentage of present calls varied from 51 to 62%, which is considerably high as compared to usual microarray results, where it averages around 30-40%.

Several internal controls were checked for. B2 oligo, which serves as a positive hybridization control, was shown to have performed adequately as no grid misalignment was observed. Poly A control genes (*dap*, *lys*, *phe*, *thre*) were used to monitor the entire target labelling process. These were all called present with increasing signal values in the order of *lys*, *phe*,

thr, *dap*. Hybridization controls, *bioB*, *bioC*, *bioD* and *cre* were used to evaluate sample hybridization efficiency on eukaryotic gene expression arrays. All of these were called present with increasing signal values, reflecting their relative concentrations. Internal control genes, β -actin and GAPDH, were used to assess RNA sample and assay quality. The signal values of the 3' probe sets for actin and GAPDH were compared to the signal values of the corresponding 5' probe sets (3'/5' ratios). These were all within range (less than 3, since this was a one-cycle assay).

The affy package was used to generate probe level data from CEL files for the 12 arrays and is shown in figure 7.6.

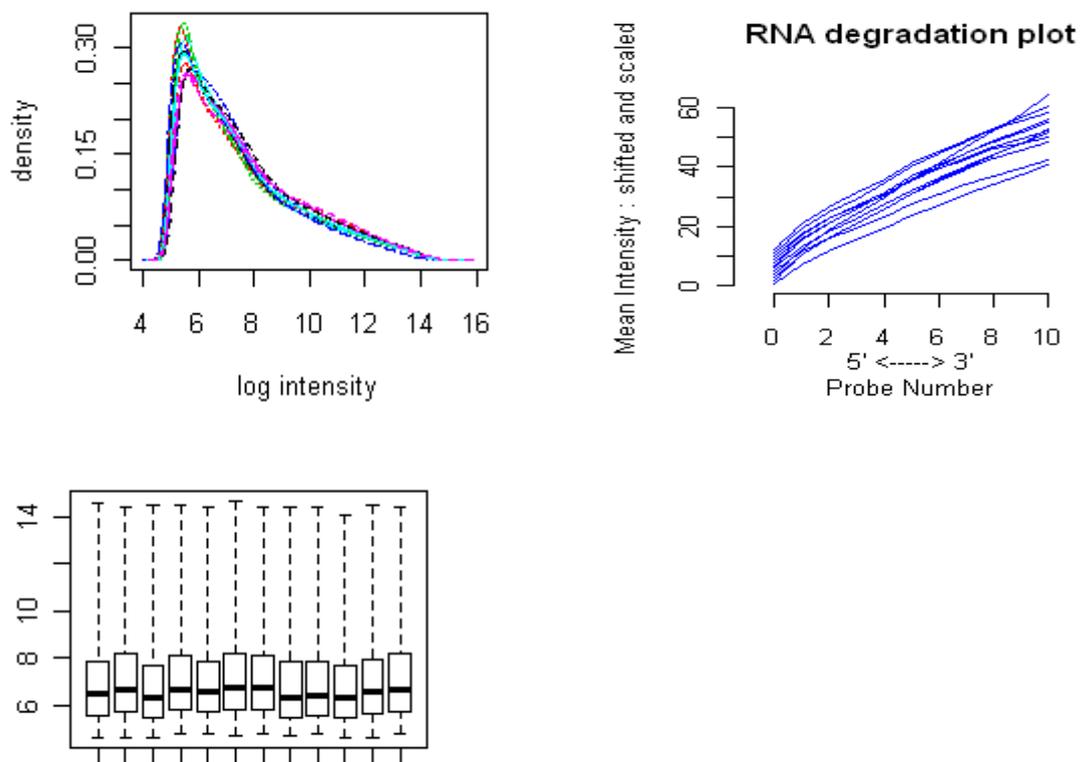


Figure 7.6 Probe level data and RNA degradation plot

As can be seen from the figure, probe level intensities overlap and have a similar distribution. RNA degradation plots showed similar pattern across all the arrays. Expression level data were generated from probe level data using the mas5 transformation and is represented in a box plot below (figure 7.7).

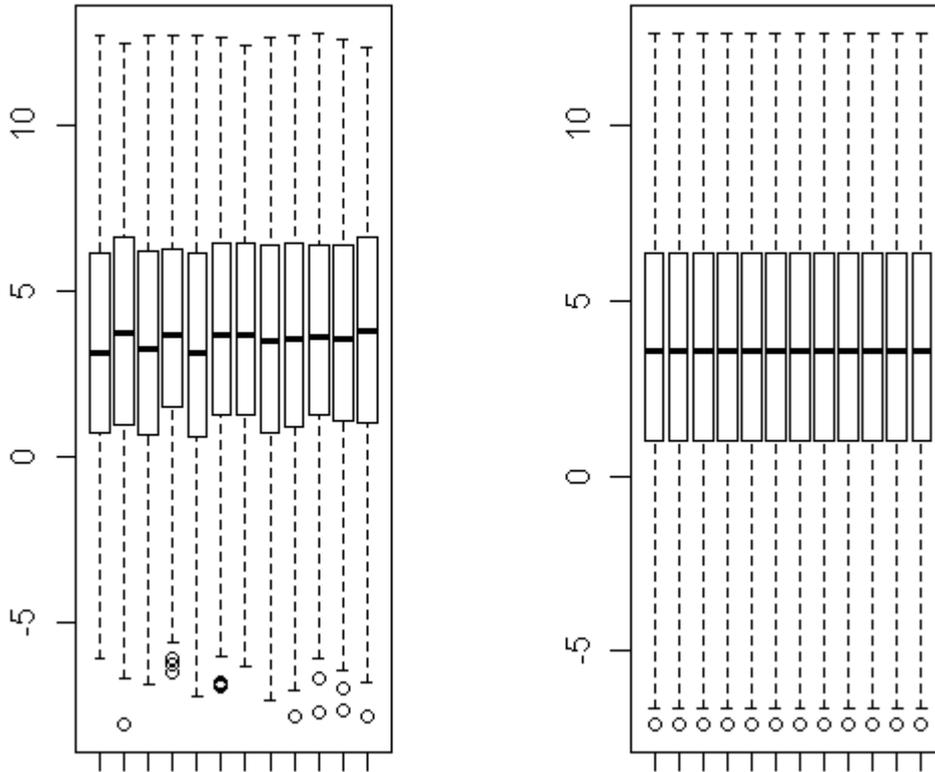


Figure 7.7 – Expression level data

A between-array normalisation was performed (from the limma package) to remove inter-array differences. Data were filtered to remove control probes, probes flagged “Absent” in all samples and probes with a CV across all arrays greater than the 90th quantile.

One of the objectives of the study was to see the variability between the samples stored immediately after collection (time 0 hour) and those stored after two hours of incubation at room temperature (time 2 hours). These treatments were used as factors in a fixed term linear

model of gene expression in paired patient samples which was constructed using limma, outputting ‘moderated t-statistics’ and associated FDR corrected p-values in the form of a volcano plot (plots log-fold changes versus log-odds of differential expression).

Limma, a commonly used package for microarray analysis in BioConductor provides a ‘moderated t-statistic’ as part of the test for significantly changed genes. This moderately t-statistic borrows variability information from other genes on the array in order to calculate the t-statistic, rather than considering each gene in isolation. This moderated t-statistic is derived from the empirical Bayes method. The approach in limma incorporates average differential expression and a measure of variability similar to that used in a t-statistic (i.e. a standard error), but the gene wise standard errors are modified.

Volcano plot in figure 7.8 fails to demonstrate any significant difference in the differential expression between the two treatment time points. Since there is no difference between the differential expression at these time points, these samples can be treated equally and used together.

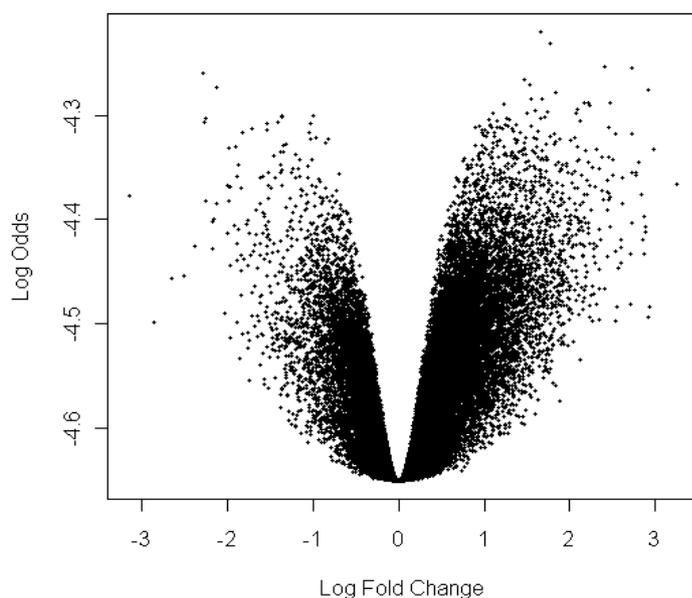


Figure 7.8 – Volcano plot to demonstrate difference between the two groups

Power calculations were performed using the Siggenes package. Power was set at 80%, specificity (significance cut-off) at 0.05 and minimum fold change at 2. Figure 7.9 shows the minimum number of samples required in each group to achieve 80% power (in time 2 hours group) to be approximately 40.

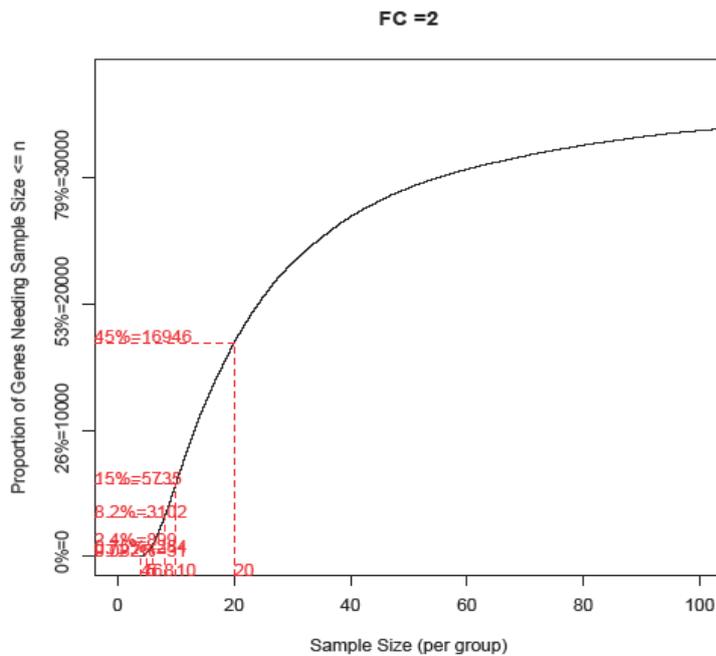


Figure 7.9 – Sample size calculation in time 2 hours group

Figure 7.10 shows the same calculation performed with fold change increased to 4, giving a sample size of 20 in each group.

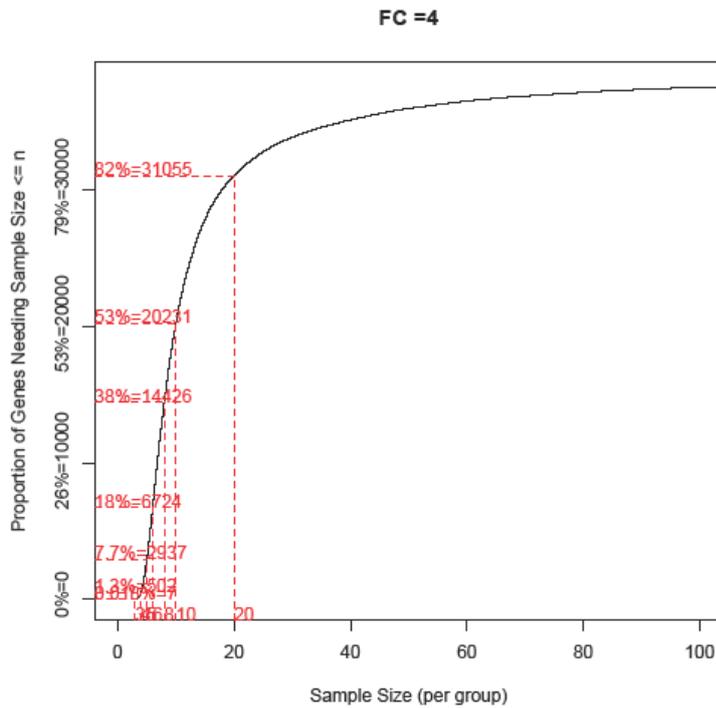


Figure 7.10 – Sample size calculation in time 2 hours group with fold change of 4

The same calculations were repeated for the other treatment group (time 0 hour) with varying fold changes (2 and 4 in figures 7.11 and 7.12, respectively).

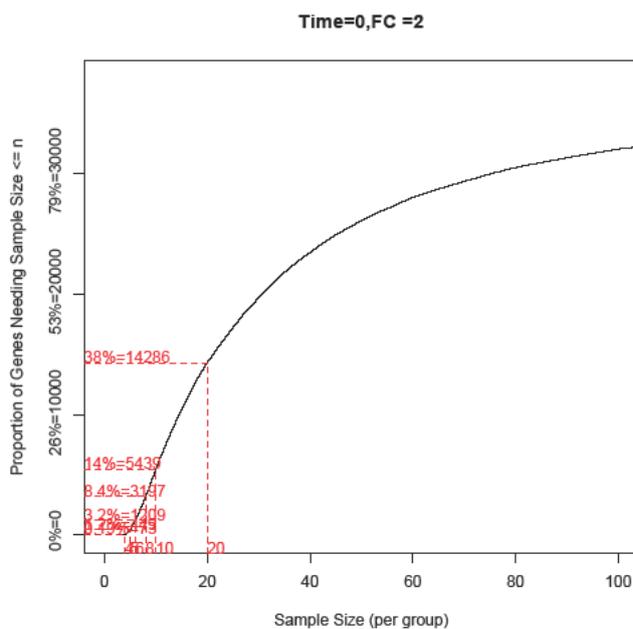


Figure 7.11 – Sample size calculation in time 0 hour group with fold change of 2

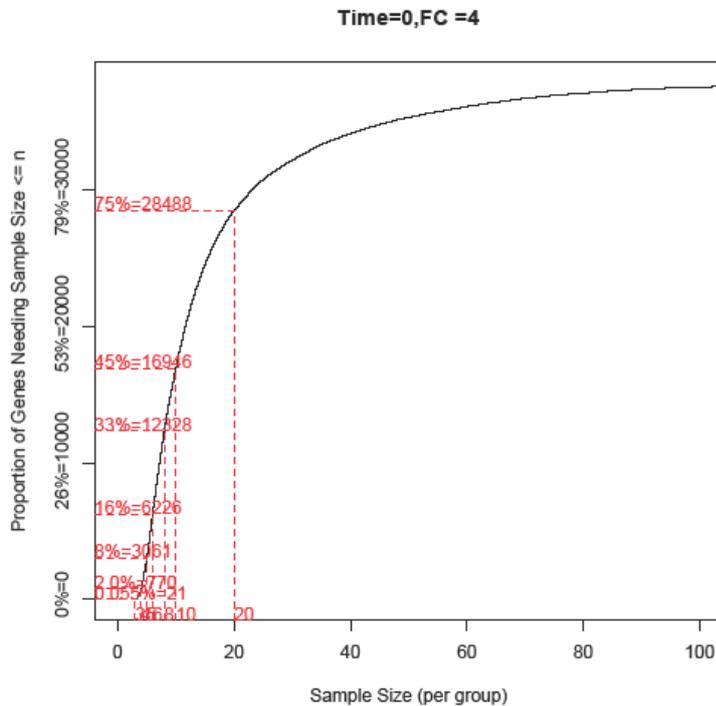


Figure 7.12 – Sample size calculation in time 0 hour group with fold change of 4

Solely using samples from time 0 hour group will require an unrealistically large number of patient samples for each group, but this number could be significantly reduced by using samples from time 2 hours group. This does point towards a varying difference in the differential expression of these groups which may mean that although there was no significant difference in the differential expression of the two groups on the volcano plot, there could be a difference in gene expression variance in these groups. In the light of these results, samples from time 2 hours group will be used for the main study, with 40 samples in each group (mild and severe).

One of the other objectives of this pilot study was to establish and refine a protocol for processing samples and data analysis for the main study. Experience with the 3' IVT express kit had shown an abundance of globin mRNA leading to potential problems with the processing and subsequent data analyses. A method was sought to remove haemoglobin from mRNA prior to hybridisation. The Ovation whole blood protocol was then used to check if it

neutralised the abundance of globin mRNA in our samples. Figure 7.13 shows a box plot of expression levels data with alpha and beta haemoglobin probes overlaid following treatment with the Ovation whole blood protocol.

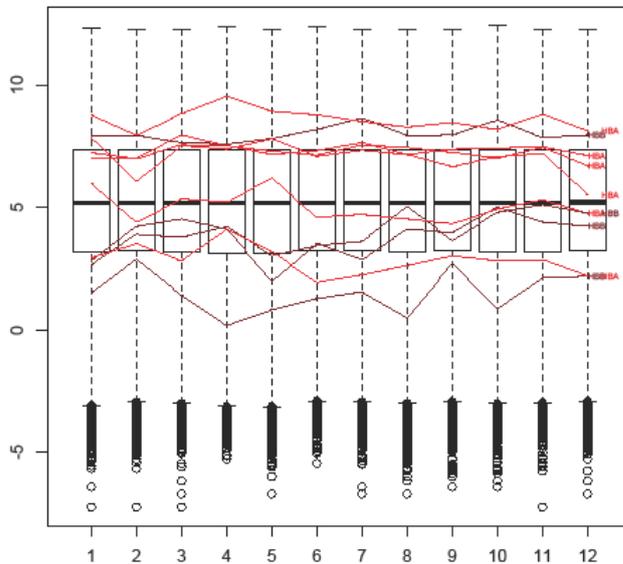


Figure 7.13 – Expression levels of haemoglobin probes

All haemoglobin alpha- and beta-probes fall within the upper or lower quantiles of expression level, indicating that the abundance of globin mRNA has fallen to within expected limits.

This provided the confidence that the Ovation whole blood system protocol not only solved the problem of abundance of globin mRNA in the samples, it also suited the project since a small amount of starting RNA was required and the procedure was very user friendly and highly reproducible. This protocol was therefore used for the main study.

7.9 Qualitative Assessments of the sample preparation and processing

Qualitative checks were undertaken at three important steps:-

- After RNA extraction – to assess quality and quantity of the RNA to be used for further experiments
- After amplification of the resultant cDNA – to assess adequacy of the amplification process and of the quantity of the resultant amplified product
- After fragmentation – to assess adequacy of the resultant fragment and its suitability for the hybridization process

7.9.1 Qualitative and Quantitative Analyses after the RNA extractions

All 58 samples were processed in a total of five batches so as to minimise batch effects. Care was taken to have a constant proportion of mild and severe patients in each batch so that there was homogeneity among the samples and any effects observed were due to the disease process and not due to the way samples were processed or handled. Details of the batches are given below:-

Table 7.5 – Samples in each batch

<p>Batch 1</p> <p>AP034 AP047 AP052 AP079 AP065 AP051 AP059 AP046 AP055 AP091 AP029 AP048</p>	<p>Batch 2</p> <p>AP067 AP062 AP072 AP0100 AP088 AP081 AP097 AP066 AP103 AP063 AP058 AP105</p>
<p>Batch 3</p> <p>AP057 AP117 AP135 AP077 AP124 AP137 AP078 AP128 AP144 AP099 AP129 AP149</p>	<p>Batch 4</p> <p>AP164 AP191 AP230 AP168 AP192 AP234 AP177 AP203 AP300 AP187 AP206</p>
<p>Batch 5</p>	

AP009	AP227	AP273	
AP011	AP232	AP307	
AP021	AP251	UHA-08	
AP211	AP267		

Red highlighted samples are severe acute pancreatitis

PAXgene Blood RNA kit (Qiagen) was used to extract RNA from stored PAXgene Blood RNA Tubes, as described in the methods section. Following table demonstrates the concentrations of the extracted RNA using Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the bioanalyzer traces using Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit are also shown below. Nanodrop Traces are provided in Appendix 1.

Table 7.6 – RNA concentrations

Sample	Concentration (µg)	RIN	Sample	Concentration	RIN
AP034	40.2	8.90	AP057	126.7	7.70
AP079	110.2	8.40	AP077	36	8.80
AP059	274.5	7.90	AP078	50.6	8.50
AP091	144.4	7.80	AP099	46.4	8.60
AP047	235.7	8.70	AP117	58.8	8.50
AP065	50.4	8.10	AP124	122.3	8.70
AP046	225.8	8.30	AP128	41.6	8.40
AP029	41.4	8.40	AP129	59.4	8.40
AP052	61	2.50	AP135	160	8.40
AP051	58.9	7.80	AP137	176	8.40
AP055	331.1	8.10	AP144	78.2	NA
AP048	165.7	8.40	AP149	44.3	8.20
AP067	106.3	8.40	AP164	293.4	8.0
AP100	84.5	8.10	AP168	54.2	8.7
AP097	133	8.60	AP177	47.2	8.0
AP063	112.9	8.60	AP187	111.1	8.3
AP062	64.2	8.50	AP191	8.4	NA
AP088	1109	7.80	AP192	134.5	7.50
AP066	72.5	8.50	AP203	108.1	NA

AP058	49.6	NA	AP206	58.4	8.7
AP072	107.4	NA	AP230	112.7	8.0
AP081	138.6	8.30	AP234	37	7.80
AP103	45.5	8.10	AP300	43.9	7.70
AP105	45	NA			
AP009	155	8.4			
AP011	126	8.5			
AP021	84.5	8.9			
AP211	35.4	7.40			
AP227	92.2	9.10			
AP232	27.6	7.90			
AP251	16.1	6.60			
AP267	135.4	8.50			
AP273	115.4	8.50			
AP307	58.1	8.80			
UHA-08	45.2	NA			

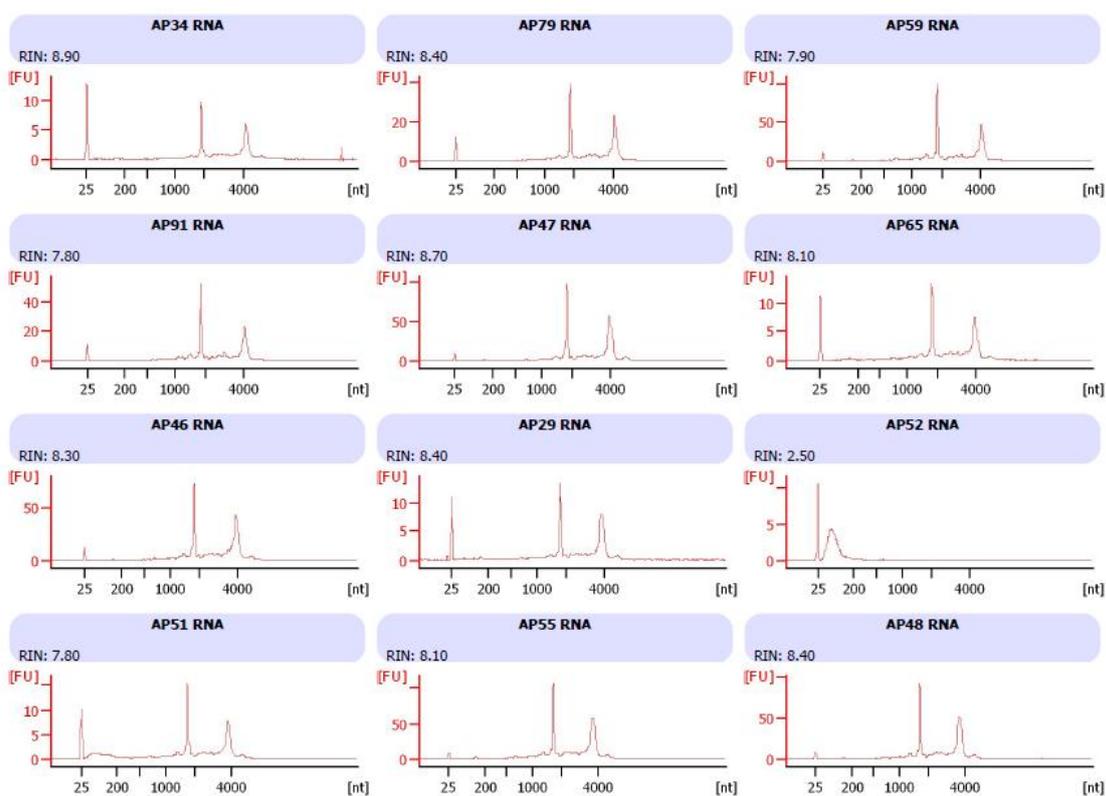


Figure 7.14 – Bioanalyzer trace for extracted RNA (Batch 1)

Since AP052 didn't show the usual 28S and 18S peaks, this was repeated to ensure good quality of the sample. This is shown in Figure 12.2.

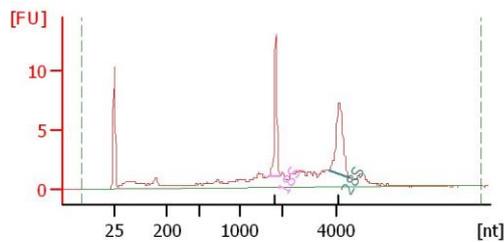


Figure 7.15 – Repeat processing of AP052

Additional peaks were seen in the traces for at least four samples in batch 2 (AP105, AP072, AP058 and AP066), four samples in batch 3 (AP099, AP117, AP144 and AP149), three samples in batch 4 (AP191, AP203 and AP300) and two samples in batch 5 (AP273 and UHA-08).

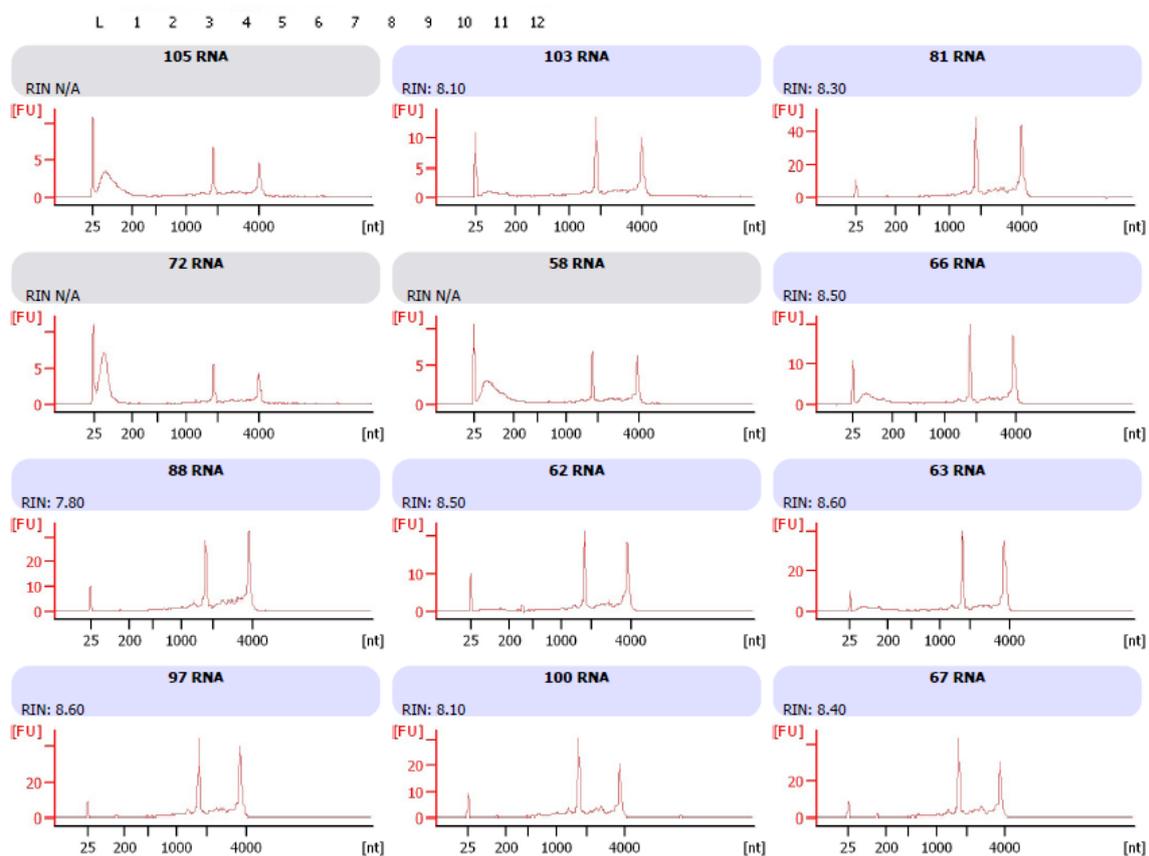


Figure 7.16 – Bioanalyzer trace for extracted RNA (Batch 2)

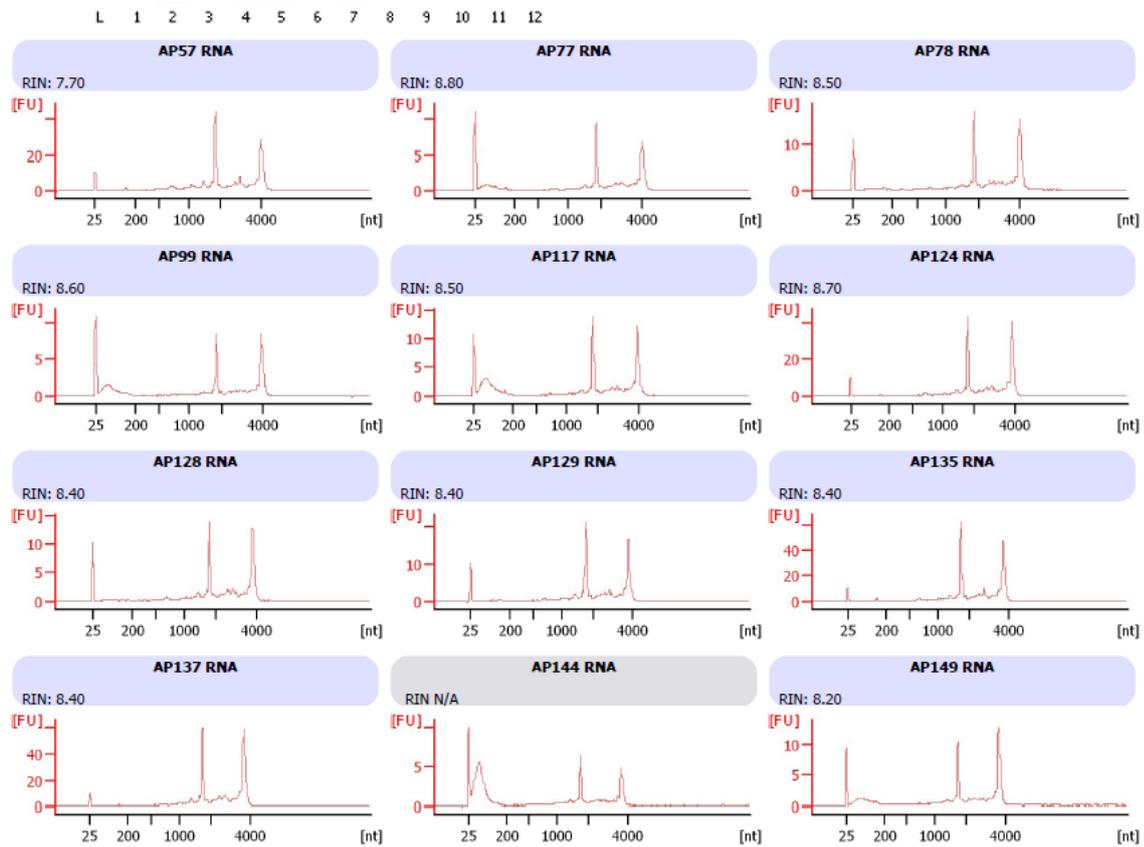


Figure 7.17 – Bioanalyzer trace for extracted RNA (Batch 3)

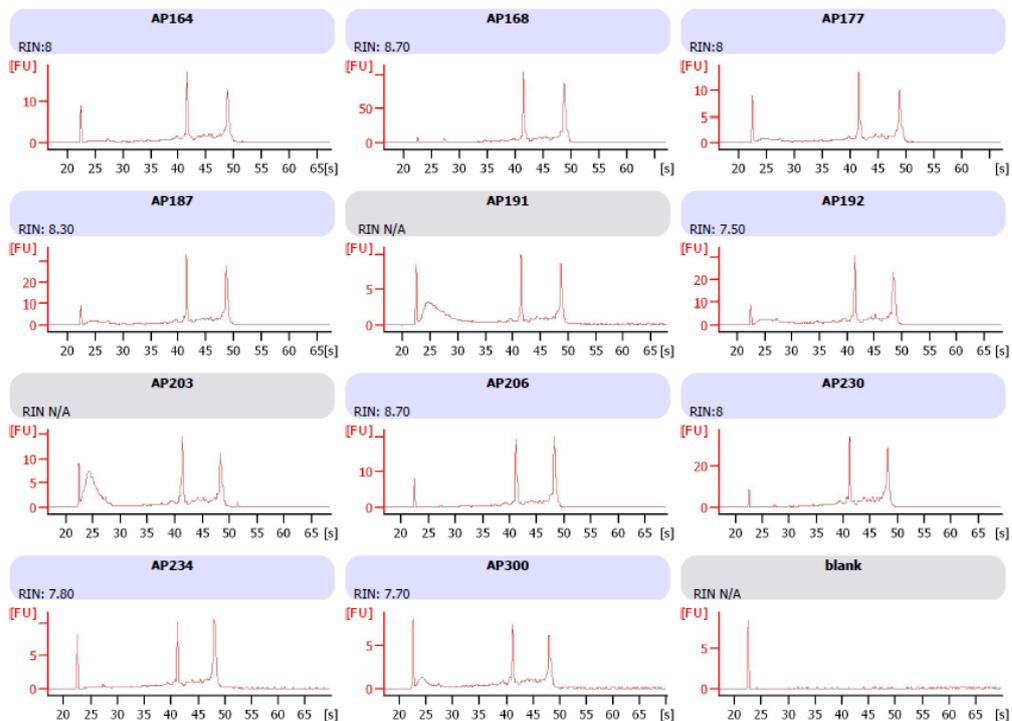


Figure 7.18 – Bioanalyzer trace for extracted RNA (Batch 4)

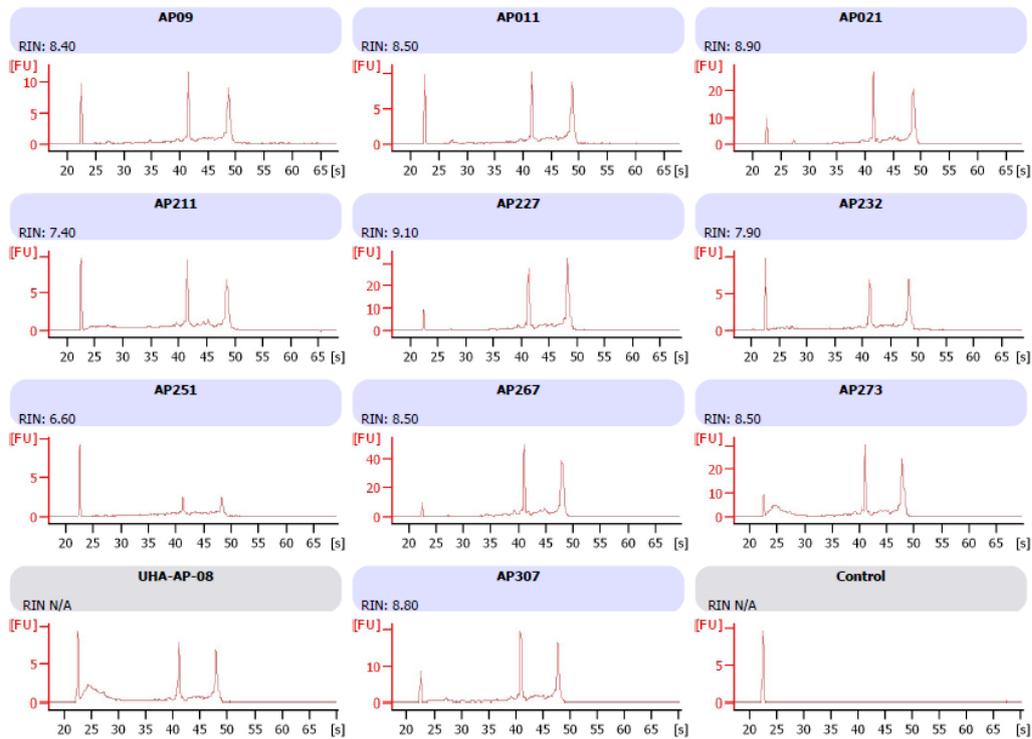


Figure 7.19 – Bioanalyzer trace for extracted RNA (Batch 5)

These additional peaks didn't appear to be due to RNA degradation which is characteristically a different trace, without the presence of distinct 18S and 28S bands (figure 12.7).

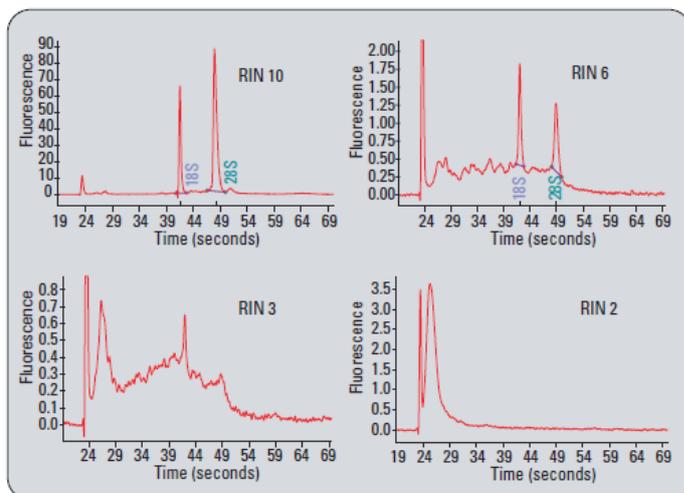


Figure 7.20 – Bioanalyzer traces demonstrating traces relating to varying RINs and hence degradation of RNA

To assess this better, another application called degradometer (Version 1.41) was used to ascertain the quality of these ‘affected’ samples. It provides quantitative data about the integrity and concentration of eukaryotic total RNA. The ‘DegFact’ represents the degradation – the higher the number, the more the degradation. T18S shows the timing of the 18S rRNA peak; usually approximately 41.4 seconds, whereas T28S is the timing of the 28S rRNA peak; usually 48 seconds. 18S is the signal intensity of the 18S rRNA peak and 28S is the signal intensity of the 28S rRNA peak. Finally, 28S/18S is the ratio of 28S/18S signal heights. This number decreases in apoptosis. A DegFact value of <8 is considered satisfactory and is indicative of minimal degradation. Following tables (7.7, 7.8, 7.9) show results from the first 3 batches. These provided enough assurance that the RNA was of high quality and without significant degradation and therefore, it was decided to proceed with the samples.

Table 7.7 – Results of Degradometer Batch 1

Sample	DegFact	RNA Concentration	t18S	t28S	18S	28S	28S/18S
		ng/μl					
AP34	4.27	25.9	41.05	48	40.7	29.84	0.73
AP46	5.02	201.2	41.1	48	39.01	26.92	0.69
AP47	4.03	214.4	41.05	48	43.75	34.29	0.78
AP48	3.56	221.2	41.2	48	45.27	30.13	0.67
AP51	5.67	44.1	41.15	48	36.7	22.21	0.61
AP52	9.58	49.1	40.95	48	27.07	19.77	0.73
AP55	4.83	285.6	41.15	48	40.51	25.74	0.64
AP59	4.66	217.1	41.1	48	48.99	27.33	0.56
AP65	6.69	40.9	41.05	48	33.69	23.3	0.69
AP79	4.56	94.2	41.1	48	44.61	30.85	0.69
AP91	5.11	110.9	41.1	48	46.42	26.76	0.58

Table 7.8 – Results of Degradometer Batch 2

Sample	DegFact	RNA Concentration	t18S	t28S	18S	28S	28S/18S
		ng/μl					
AP58	7.85	24.5	41.2	48	28.75	32.6	1.13
AP62	4.47	54.3	41.25	48	41.69	43.06	1.03
AP63	3.78	94	41.2	48	45.6	46.51	1.02
AP66	4.17	49.3	41.2	48	42.4	43.82	1.03
AP67	3.86	97.6	41.25	48	48.3	39.54	0.82
AP72	5.51	16.8	41.2	48	34.9	32.25	0.92
AP81	4.64	124.9	41.1	48	42.23	44.64	1.06
AP88	6.94	94.6	41.2	48	30.37	42.46	1.4
AP97	4.22	114.2	41.25	48	42.59	44.51	1.05
AP100	4.74	71.9	41.25	48	46.08	36.32	0.79
AP103	6.74	38.3	41.1	48	32.02	33.05	1.03
AP105	7.29	23.6	41.1	48	31.71	24.63	0.78

Table 7.9 – Results of Degradometer Batch 3

Sample	DegFact	RNA Concentration	t18S	t28S	18S	28S	28S/18S
		ng/μl					
AP57	5.9	147.7	41.1	48	44.17	33.32	0.75
AP77	4.46	30.8	41.1	48	39.62	38.43	0.97
AP78	5.23	65.5	41.1	48	35.94	39.96	1.11
AP99	4.21	30.1	41.15	48	41.55	48.3	1.16
AP117	4.74	49.1	41.2	48	37.27	42.05	1.13
AP124	3.97	143.2	41.15	48	44.11	47.86	1.09
AP128	6.04	53.7	41.25	48	34.1	40.17	1.18
AP129	4.28	69.7	41.25	48	44.46	41.56	0.93
AP135	3.98	189.6	41.25	48	47.75	42.36	0.89
AP137	3.99	204.4	41.3	48	42.9	48.6	1.13
AP144	6.75	26.6	41.3	48	35.36	31.31	0.89
AP149	6.36	50.5	41.35	48	30.86	42.5	1.38

7.9.2 Qualitative and Quantitative Analyses after amplification of cDNA

Nanodrop readings indicated the amount of amplified cDNA and the bioanalyzer trace depicted the efficacy of the amplification process, as is shown below. Bioanalyzer traces are shown below. Nanodrop traces are included in supplementary data.

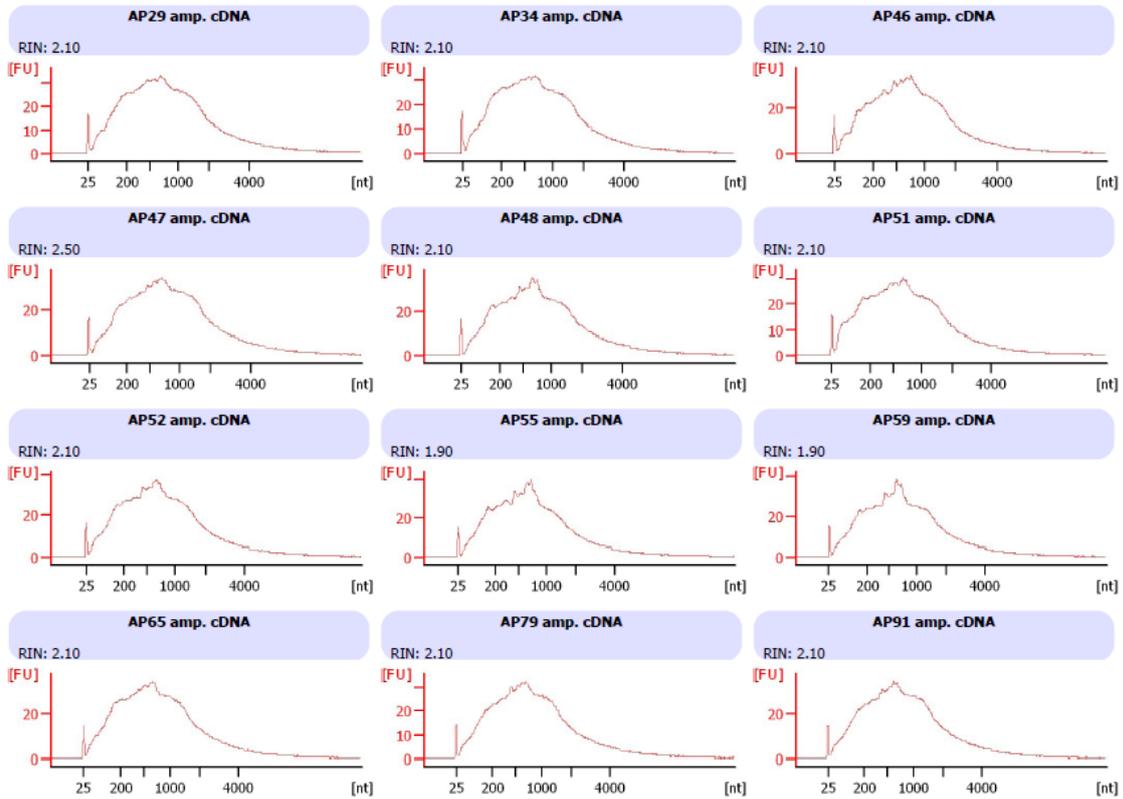


Figure 7.21 –Bioanalyzer traces of amplified cDNA (Batch 1)

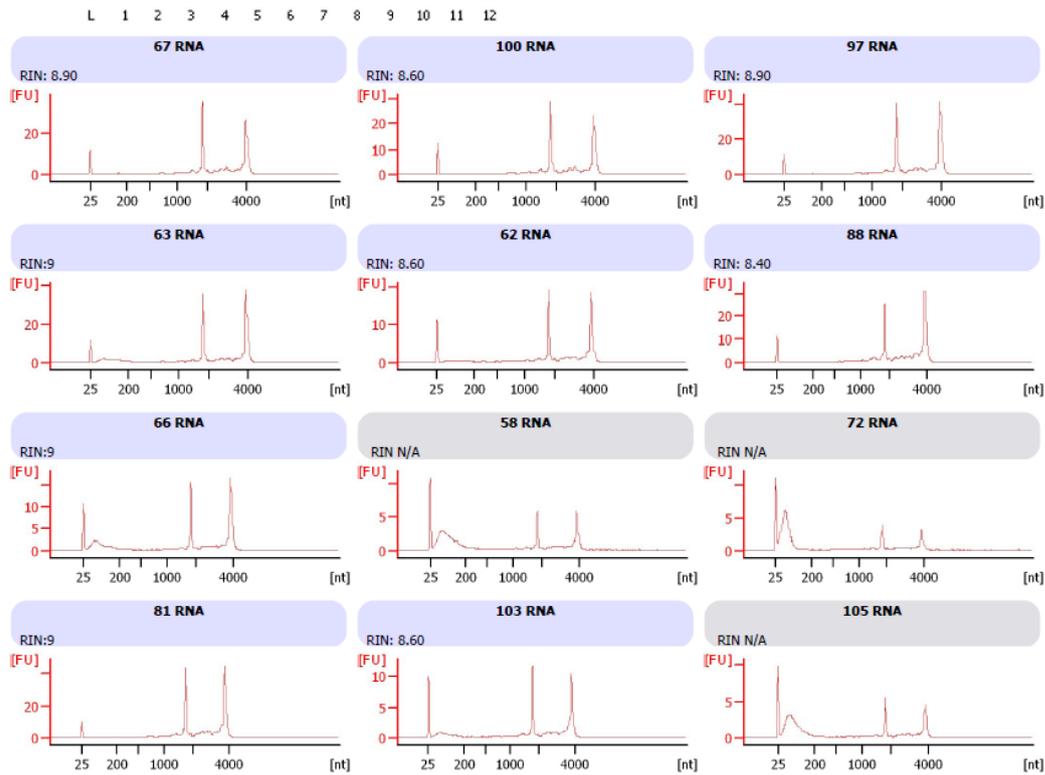


Figure 7.22 –Bioanalyzer traces of amplified cDNA (Batch 2)

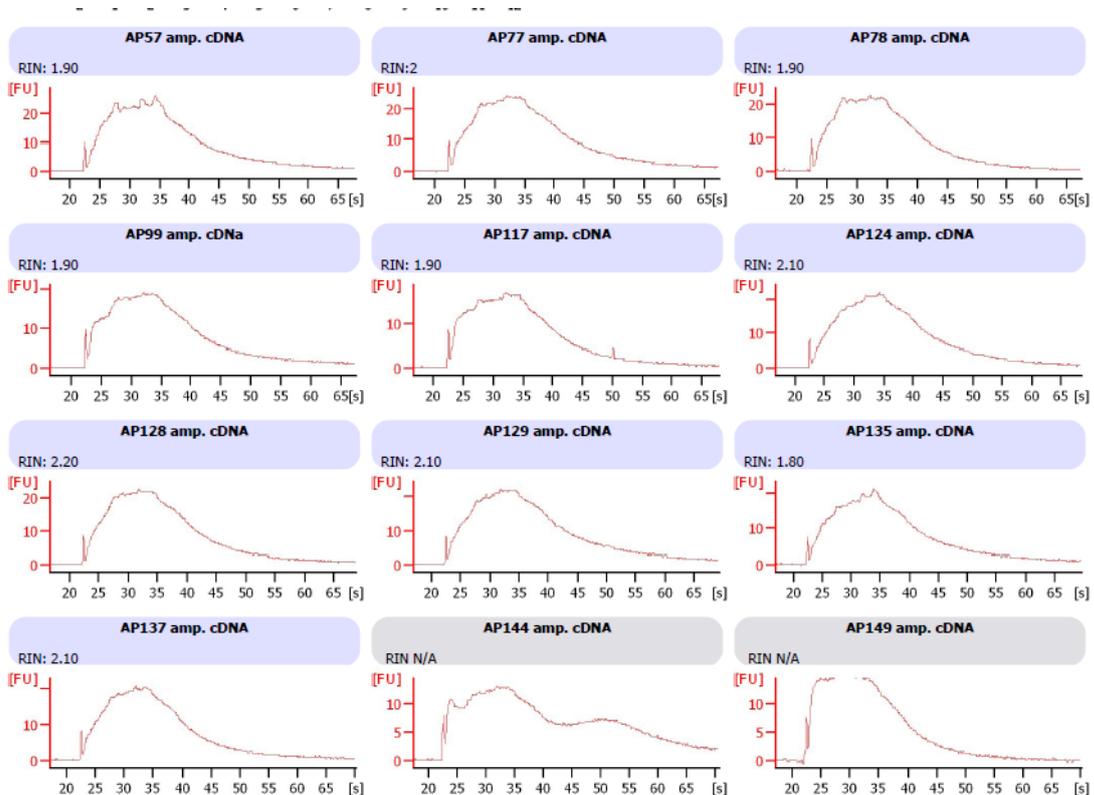


Figure 7.23 –Bioanalyzer traces of amplified cDNA (Batch 3)

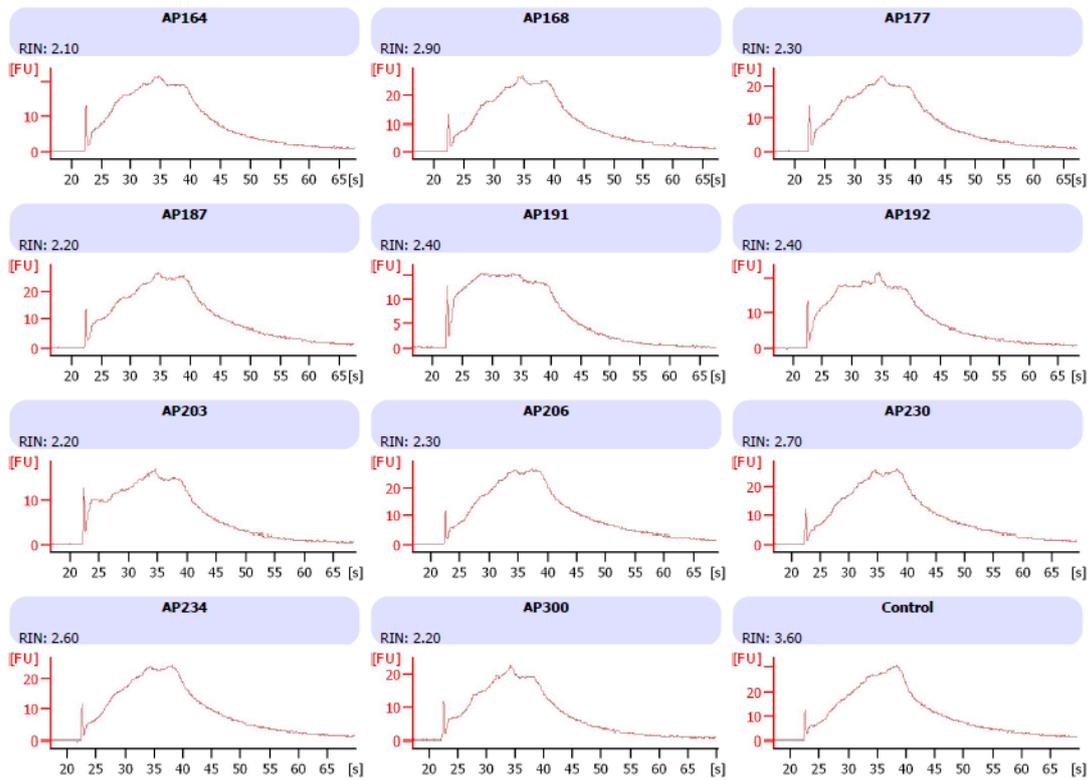


Figure 7.24 –Bioanalyzer traces of amplified cDNA (Batch 4)

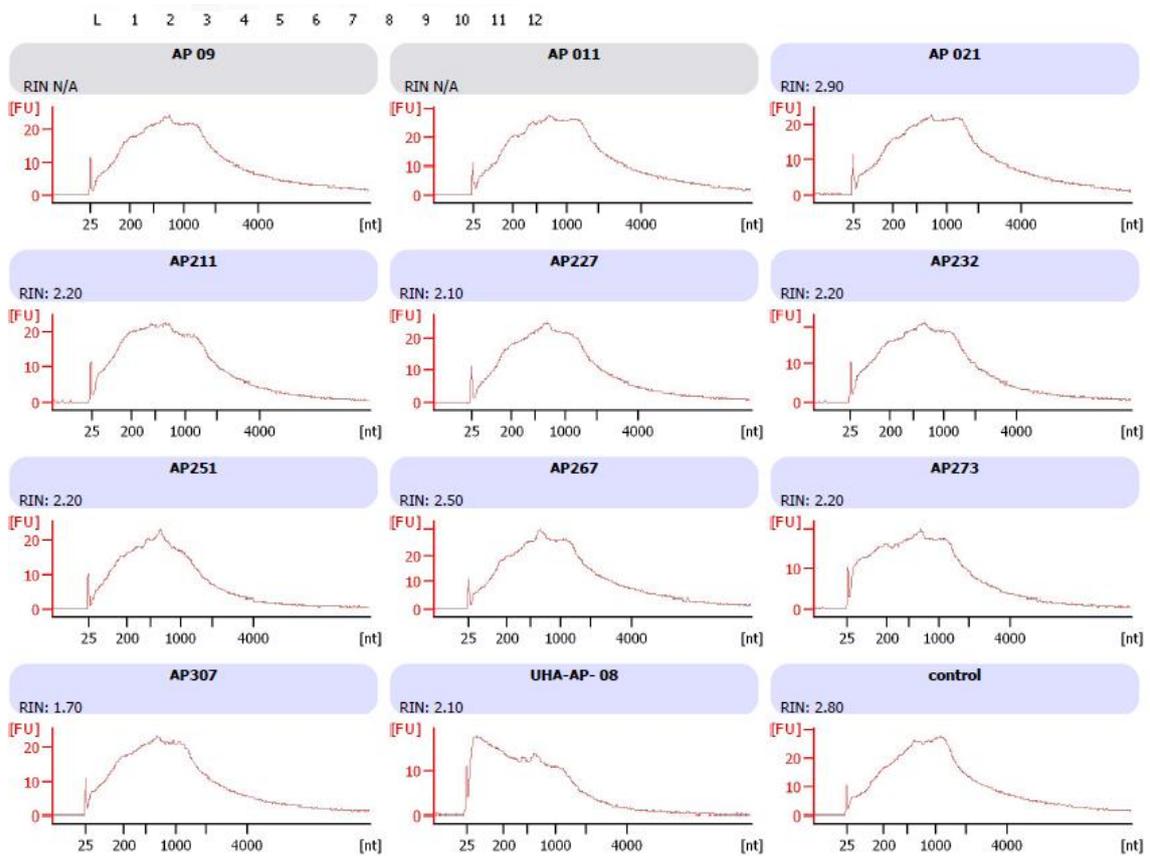


Figure 7.25 –Bioanalyzer traces of amplified cDNA (Batch 5)

7.9.3 Qualitative and Quantitative Analyses after fragmentation of cDNA

cDNA was later fragmented to achieve fragments of ~ 90-120 nt. This would ensure appropriate size that would facilitate hybridization. All fragmented cDNA revealed adequate size. This is shown in figures 7.26, 7.27, 7.28, 7.29 and 7.30.

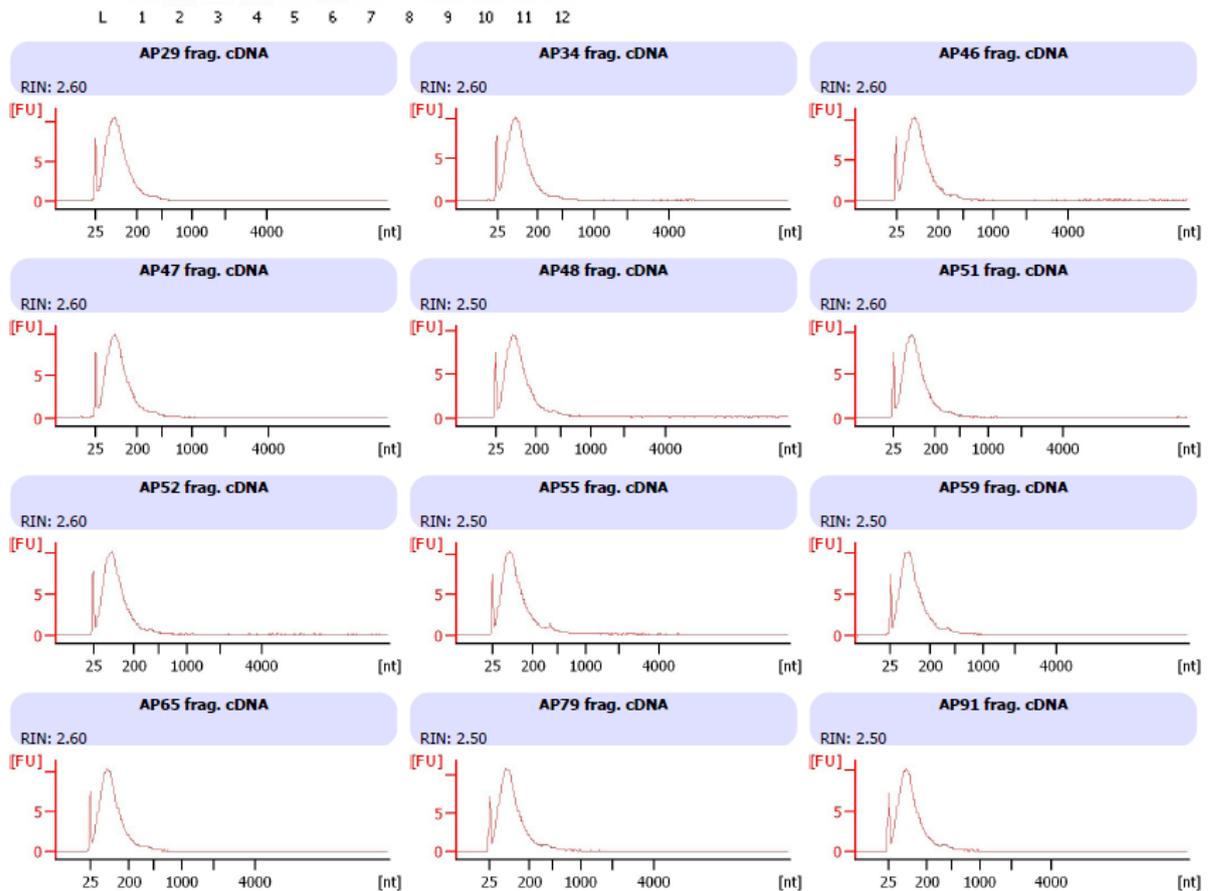


Figure 7.26 – Bioanalyzer traces of fragmented cDNA (Batch 1)

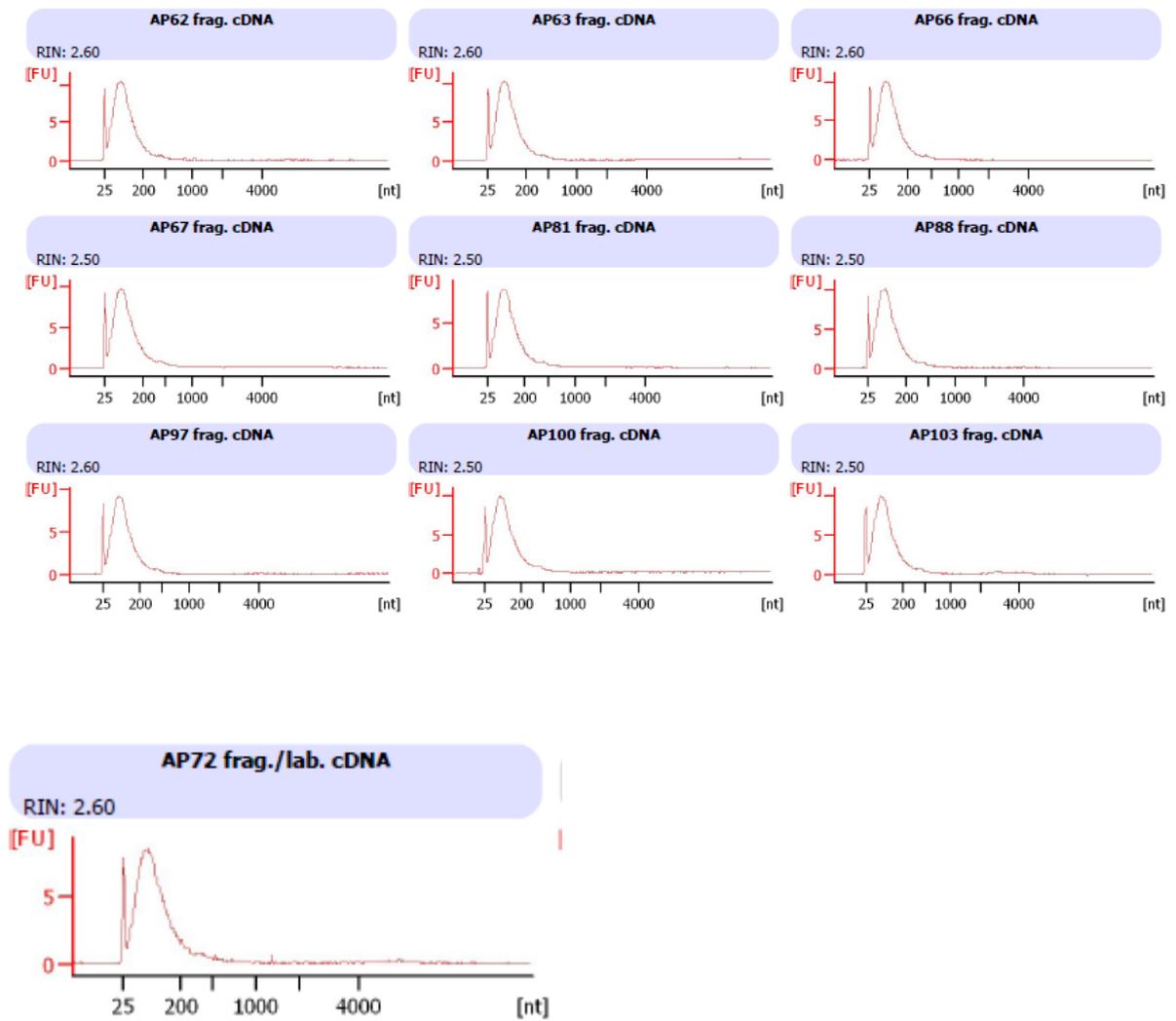


Figure 7.27 – Bioanalyzer traces of fragmented cDNA (Batch 2)

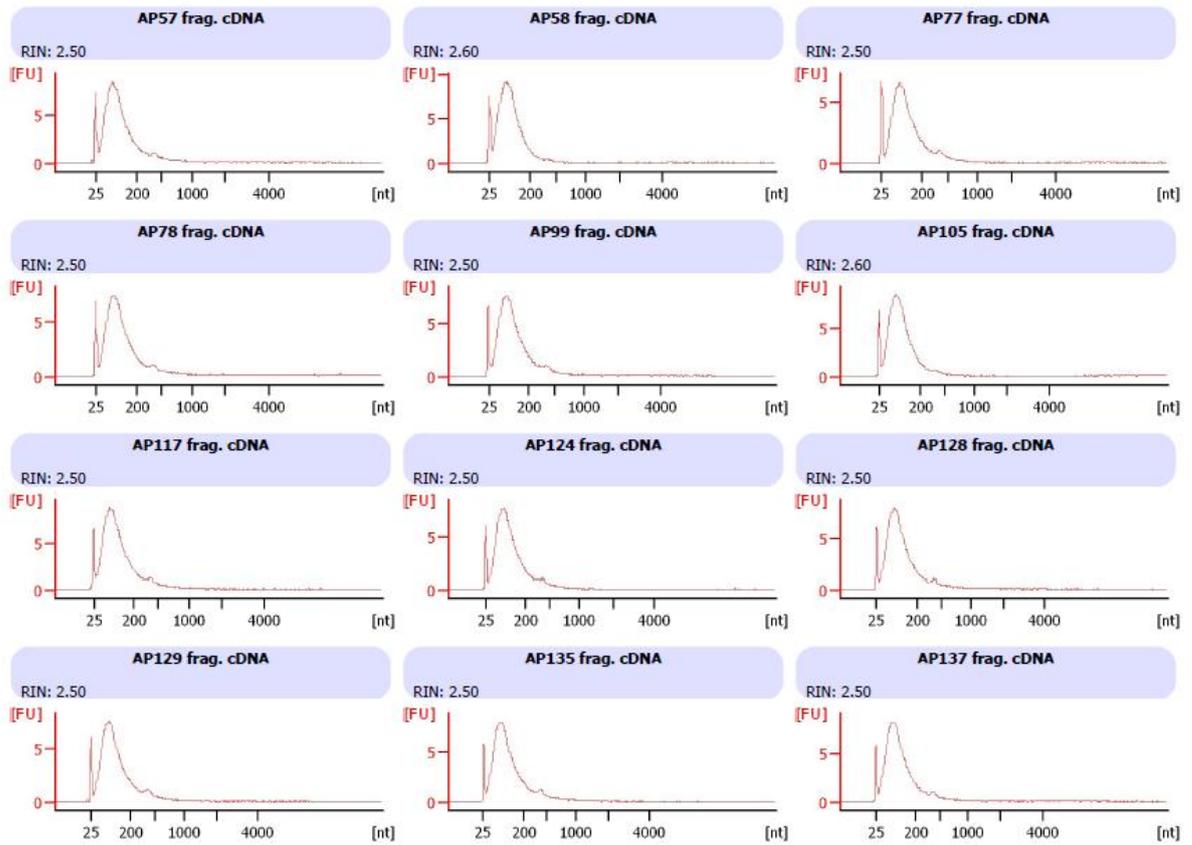


Figure 7.28 – Bioanalyzer traces of fragmented cDNA (Batch 3)

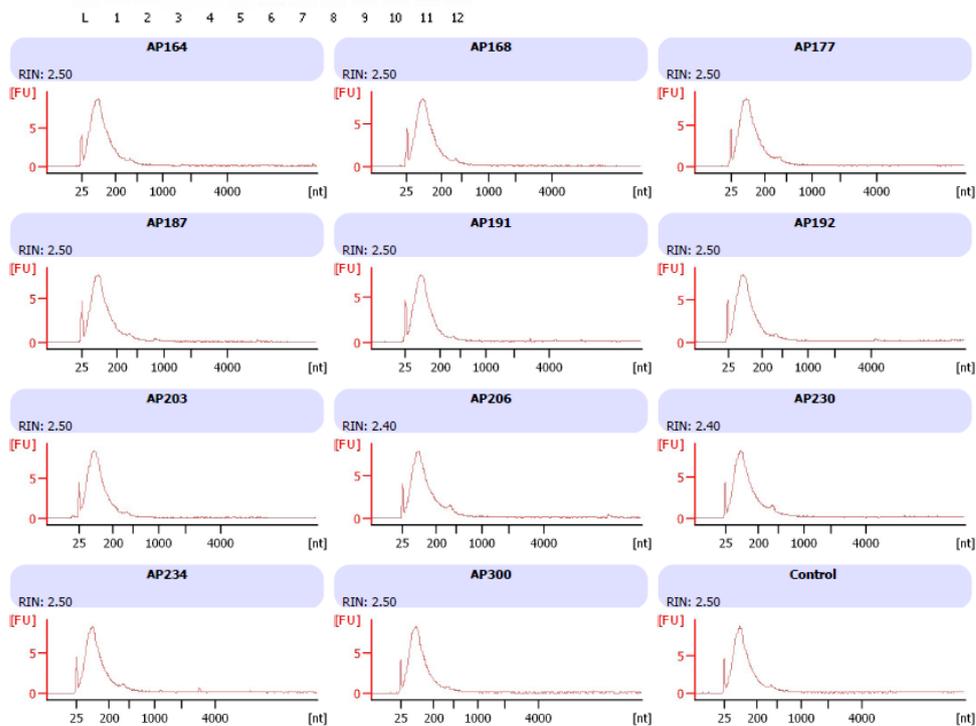


Figure 7.29 – Bioanalyzer traces of fragmented cDNA (Batch 4)

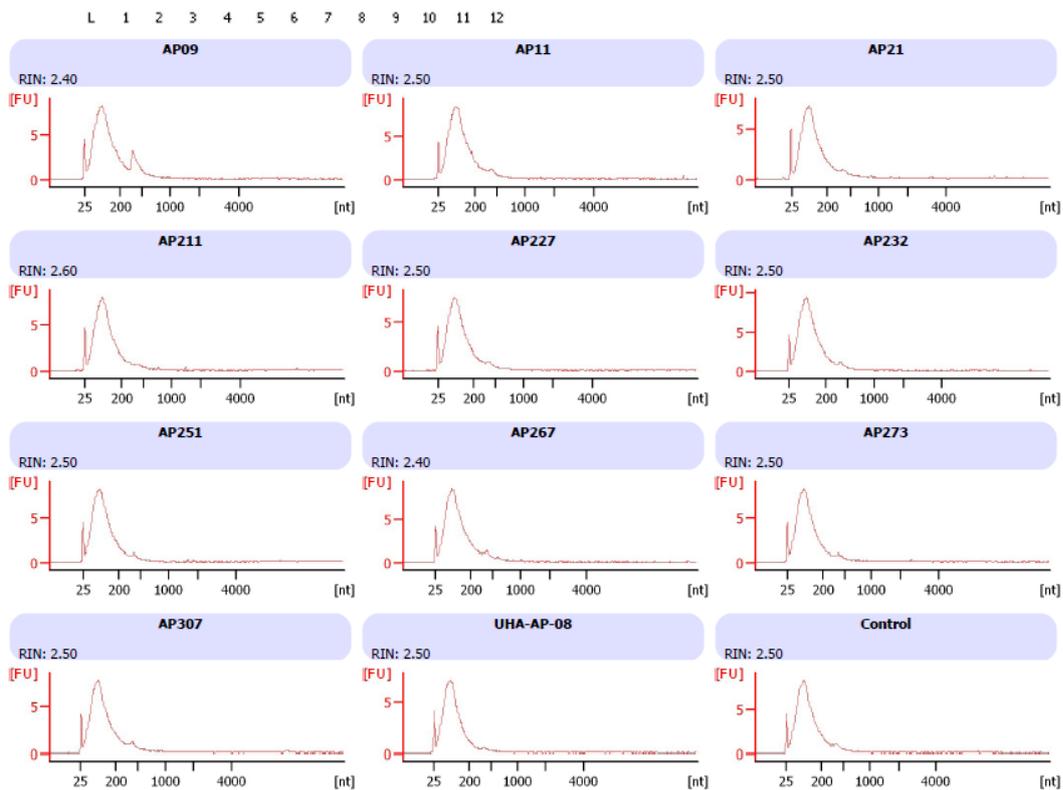


Figure 7.30 – Bioanalyzer traces of fragmented cDNA (Batch 5)

7.10 Quality Control metrics for Hybridization

High 3'5' ratios for house-keeping genes were noticed. Typically, a normal value for 3'/5' ratio for the GAPDH is up to 3 and up to 20 for beta actin, since this gene is 2.2 kb long. This can be explained by the fact that the 5' probes for the human actin gene are approximately 1.7 kb from the 3' polyA tail of the message. QPCR analysis has revealed that the Ovation amplified cDNA material is long enough to provide good transcript representation out to 1 kb from the polyA tail (Figures 7.31 and 7.32). This is completely adequate for use on the current generation of Affx arrays as the probes for the arrays are designed to be within 600 bases of the polyA tail.

Table 7.10 – Quality control metrics for batch 1 hybridizations

Sample Name	AP029	AP034	AP046	AP047	AP048	AP051
Scale Factor (SF):	0.2942	0.31752	0.3356	0.2865	0.3668	0.3385
Background:	27.1639	29.7466	27.684	29.506	28.704	28.947
Total Probe Sets:	54675	54675	54675	54675	54675	54675
Number Present:	56.3457	60.2451	61.652	62.219	59.076	55.02
Housekeeping Controls:						
AFFX-HUMGAPDH/M33197	11.7952	11.6848	11.679	11.429	11.81	11.876
AFFX-HSAC07/X00351	11.9694	12.0084	12.246	11.903	12.34	12.138
Spike Controls:						
Probe Set	Sig(3')	Sig(3')	Sig(3')	Sig(3')	Sig(3')	Sig(3')
AFFX-r2-Ec-bioB	9.11221	9.29811	9.4174	9.0247	9.3237	9.2846
AFFX-r2-Ec-bioC	10.2282	10.3554	10.469	10.198	10.448	10.371
AFFX-r2-Ec-bioD	11.7378	11.8621	11.958	11.708	12.019	11.944
AFFX-r2-P1-cre	12.5358	12.6011	12.758	12.436	12.821	12.708

Sample Name	AP052	AP055	AP059	AP065	AP079	AP091
Scale Factor (SF):	0.3278	0.3678	0.2841	0.3864	0.3276	0.28361
Background:	28.576	28.3763	30.198	28.351	29.332	28.7078
Total Probe Sets:	54675	54675	54675	54675	54675	54675
Number Present:	58.345	58.9886	59.546	56.913	59.497	60.225
Housekeeping Controls:						
AFFX-HUMGAPDH/M33197	11.792	11.7199	11.557	12.126	11.641	11.3354
AFFX-HSAC07/X00351	12.162	12.2479	11.95	12.343	11.914	11.8933
Spike Controls:						
Probe Set	Sig(3')	Sig(3')	Sig(3')	Sig(3')	Sig(3')	Sig(3')
AFFX-r2-Ec-bioB	9.2079	9.25516	8.9654	9.3981	9.1323	9.16866
AFFX-r2-Ec-bioC	10.369	10.3948	10.162	10.468	10.326	10.2459
AFFX-r2-Ec-bioD	11.869	11.9666	11.754	12.062	11.739	11.7761
AFFX-r2-P1-cre	12.655	12.7418	12.511	12.836	12.536	12.4713

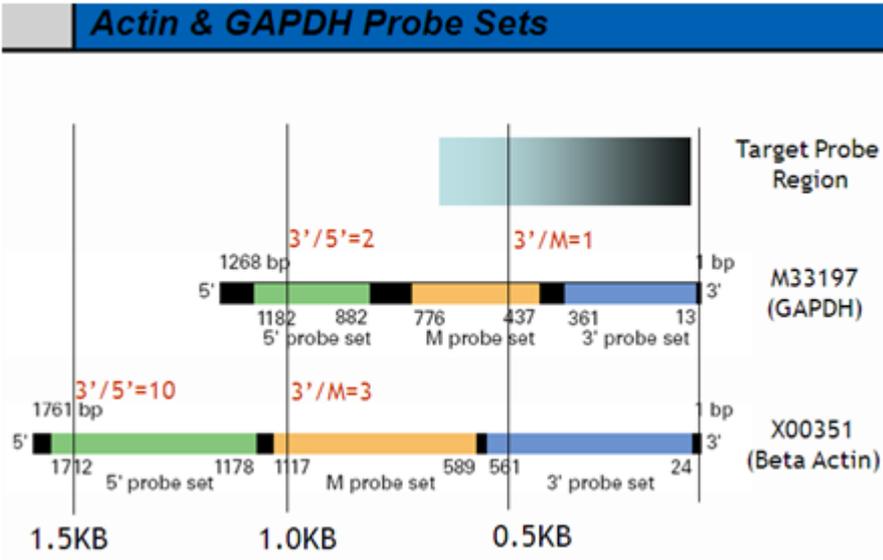


Figure 7.31 – Actin and GAPDH probe sets

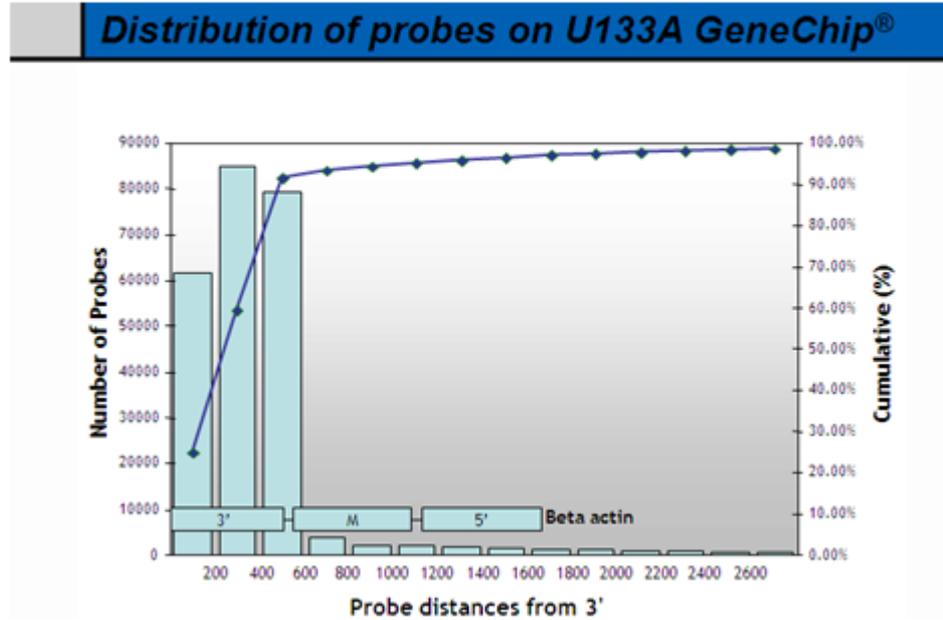


Figure 7.32 – Distribution of probes on U133A genechip

Having said that, it has to be kept in mind that the QC metrics by Affymetrix have been optimized for aRNA probes, not cDNA, so their recommended values may not be applicable on the selected ovation protocol treated hybridizations. Besides, the percentage of present

calls was found to be quite high in the samples, along with improved scaling factor and low background.

Keeping all this in the context, these QC metrics were considered adequate and reliable.

Diagrammatic representation is presented below in figures, including RNA degradation plot (Figures 7.33 and 7.34)

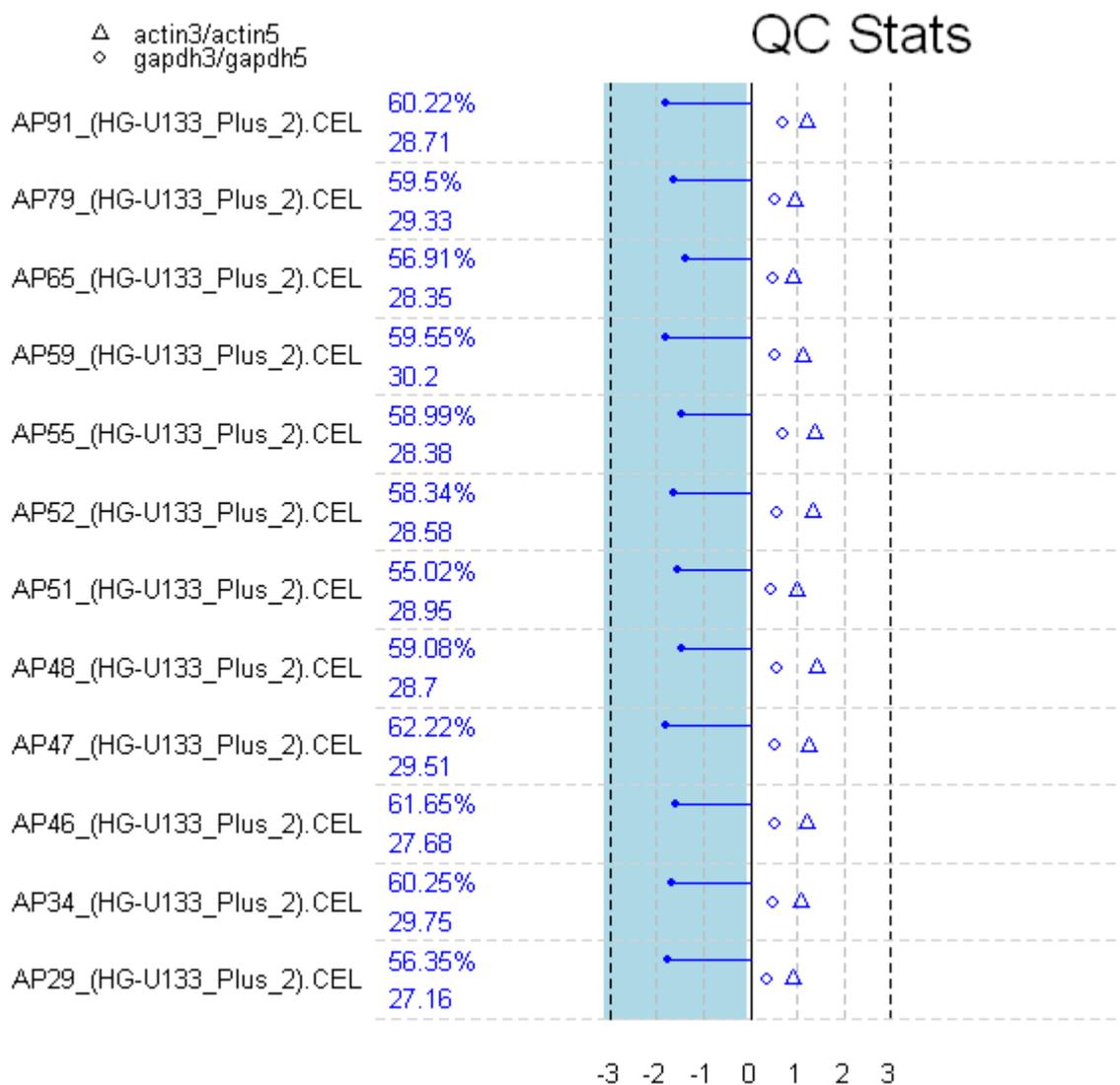


Figure 7.33 – Quality control metrics of the house keeping genes

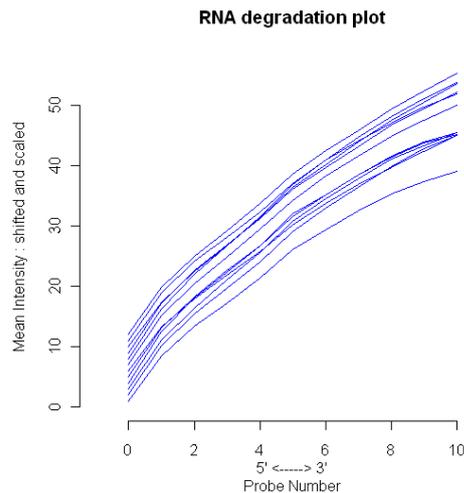


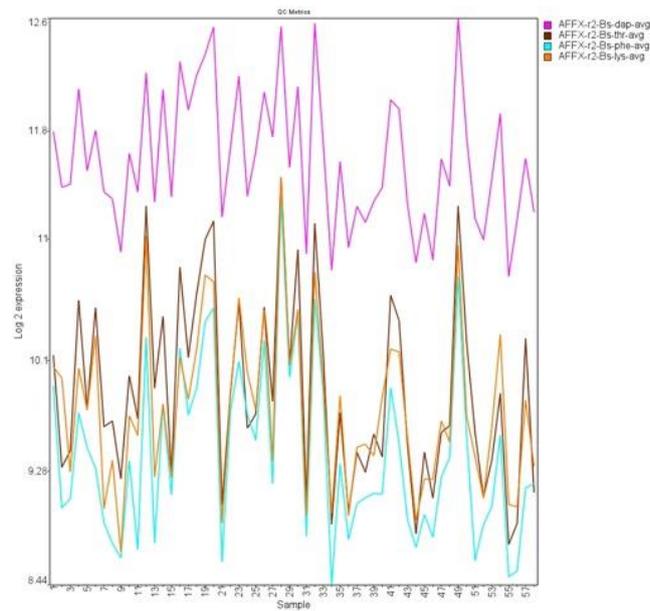
Figure 7.34 – RNA degradation plot

7.11 Qualitative Analyses

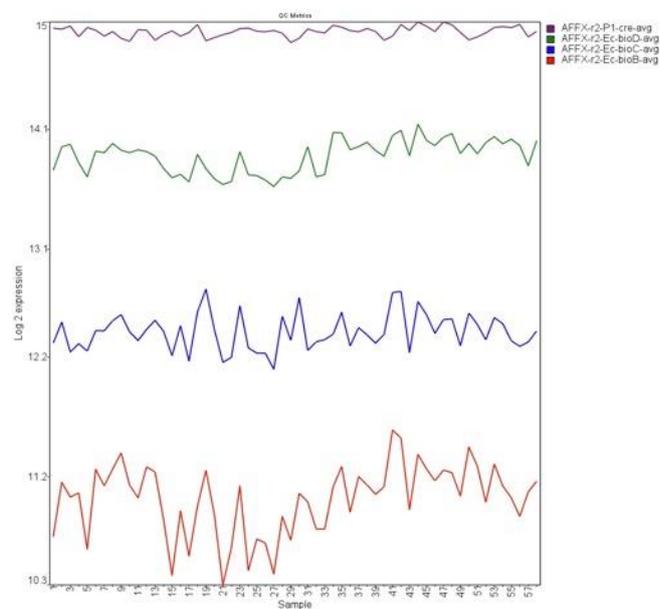
Quality control metrics (QC) were performed to ensure efficient hybridizations. These can be broadly classified into within arrays, between arrays and between batches. It is of paramount importance to perform quality checks at all these points so as to minimise any systematic bias. For ‘within array’ QCs, assessment of 'housekeeping genes' is undertaken (approximately 100 on standard affymetrix chips). These act as controls and there are several probe sets corresponding to controls. These are easily identified by the letters ‘AFFX’ at the beginning of the probe identifier. Some of them are spiked-in control genes. The matching targets are added in precise quantities and at different steps in the labelling and hybridization process. These transcripts have predictable expression levels and so the probe sets play an important quality control role in the MAS algorithm. Controlled input results in predictable outcomes, enabling discovery and correction of sources of error, if any. The chips are re-scaled so the average values of these housekeeping genes are equal across all chips. This is much better than using a single housekeeping gene, and probably adequate for about 80% of chips in practice. Figure (7.35) below depict the QCs for labelling, hybridization, 3’-5’ and pm/mm respectively. These demonstrate that the intensity of the house keeping genes was predominantly consistent

through all the samples. There was some variability but it was not significant enough for the sample to be labelled as an outlier and therefore, all samples were included in the final analysis.

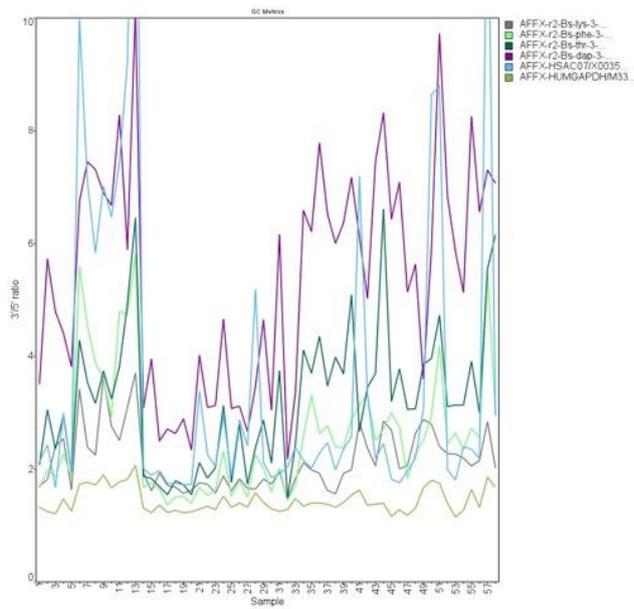
Labelling



Hybridization



3'/5' ratio



Perfect match/mismatch repair

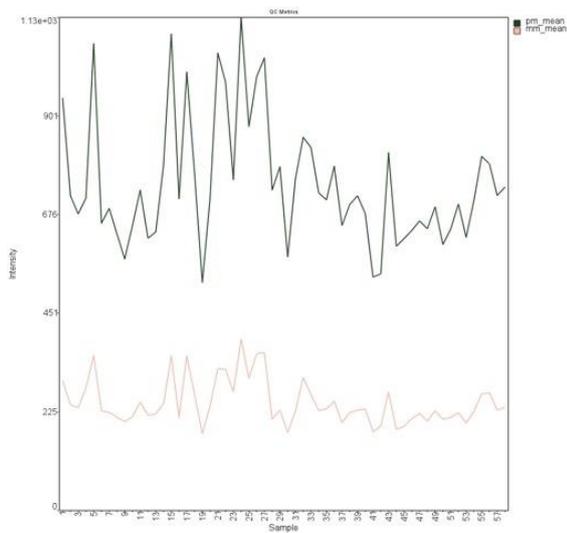
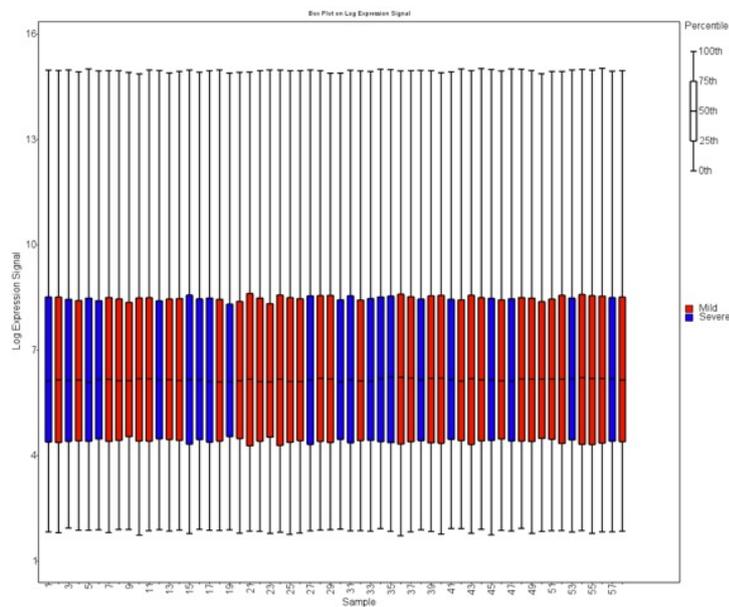
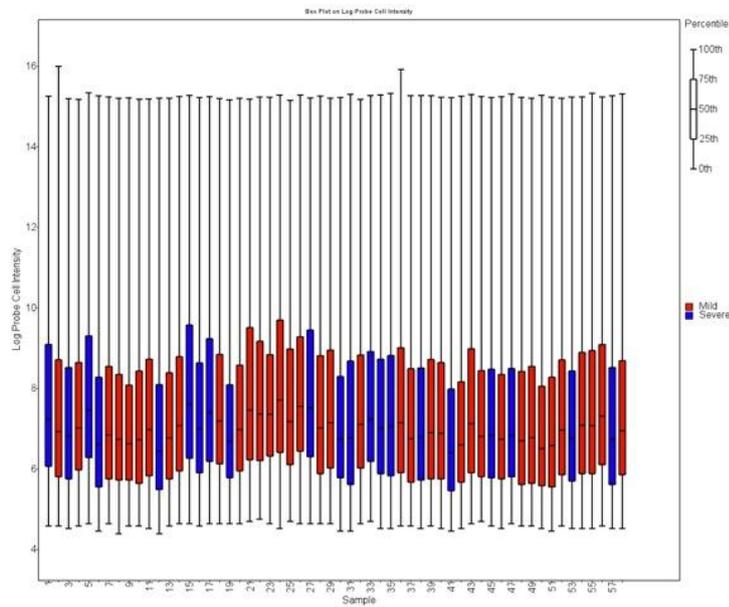


Figure 7.35 – Quality Control Metrics for labelling, hybridisation, 3'-5' and pm/mm

Quality control for 'between arrays' was performed by looking at probe level, expression level and relative expression level (REL) data.

Box plots show a picture of the overall distribution of probe intensities found on each array.

As it can be seen in the figures below (7.36), that the probe level, expression level and REL intensities overlap and have similar distribution.



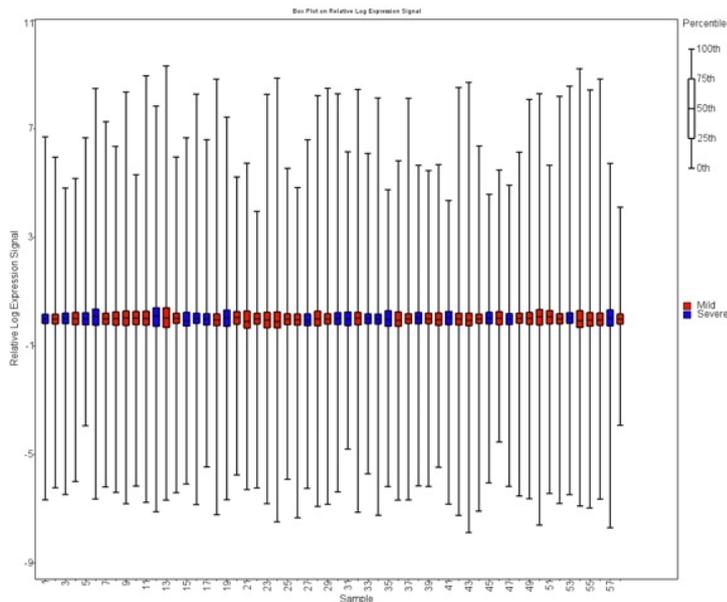


Figure 7.36 – Quality control metrics for probe level, expression level and REL intensities

Data obtained from the probes (probe level data) was transformed to expression estimates using MAS5. This is a standard affymetrix algorithm which performs background correction and normalisation algorithm. It converts the values to log10 and then averages across genes and samples. It helps to equalise the contribution of different probes as well.

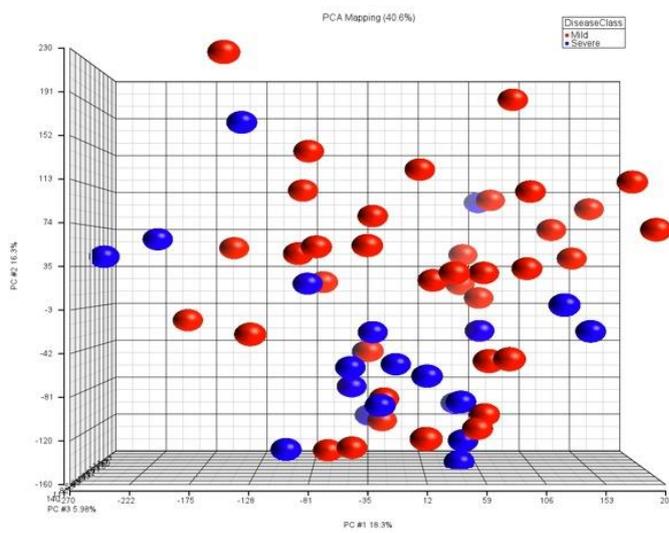
7.12 Principle Component Analysis

Batch effects are technical sources of variation that have been added to the samples during handling. The importance of removing these cannot be over-emphasised. Their presence decreases statistical power, since it adds variation to the data, may act as a confounder and induce differences between the study groups. It is absolutely critical that this type of technical variation does not confound with the biology, ensuring that biological treatment groups do not overlap with technical groups. This is often evaluated using explorative

approaches, involving distance measures, clustering and spatial methods. Principle Component Analysis (PCA) was used in this study to illustrate this and Analysis of Variance (ANOVA) to assess and correct for batch effects.

Figure 7.37 shows differences between the two study groups on the basis of their disease (mild vs. severe) with and without the batch effects, respectively.

With batch effects



Without batch effects

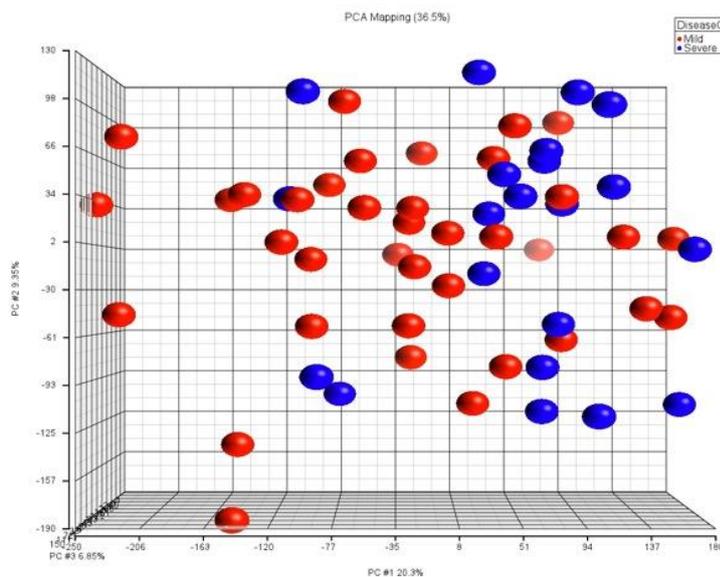
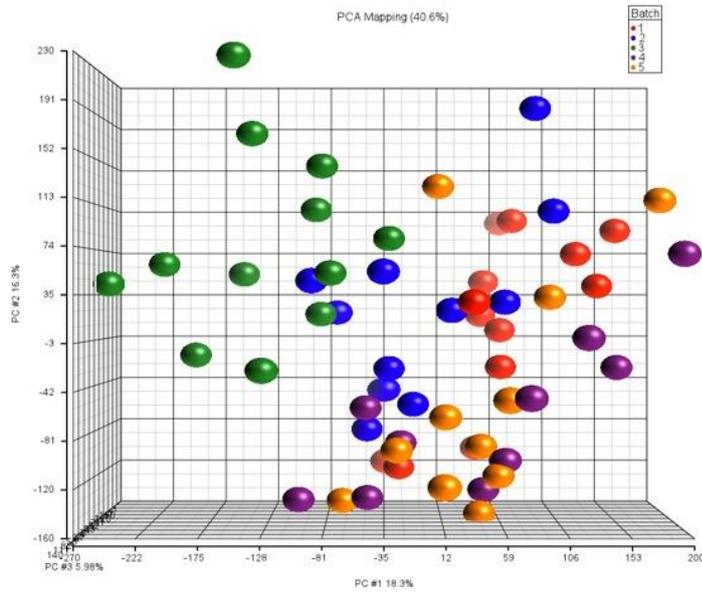


Figure 7.37 – Principle Component Analysis demonstrating difference between MAP and SAP, with and without batch effects

Another difference to assess for was between the different batches. Since all the samples were processed in five batches, these were classified according to that and batch effects removed. Figure 7.38 depicts the different batches before and after removal of batch effects, respectively.

With batch effects



Without Batch Effects

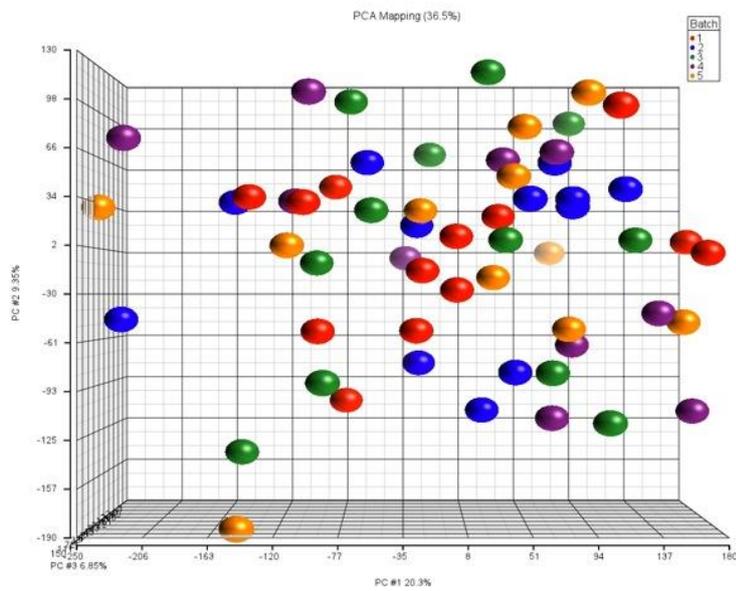


Figure 7.38 – Principle Component Analysis demonstrating difference between different batches

7.13 Hierarchical Clustering

As the next step, gene expression analysis was performed by using two way ANOVA (with CV <30%, IQR >0.5 and fold change of 2.0). ANOVA not only improves the estimate of disease effects but also quantitates both batch and disease effects per gene, using only the disease specific effects for inference. Based on that, 98 genes were identified that are differentially expressed between mild and severe pancreatitis. Based on those 98, the groups separate out quite well as can be seen in hierarchical clustering plot below. Both the gene and sample distances were Euclidean and average linkage was used for clustering; the images are scaled so that mean is 0 and standard deviation is 1.

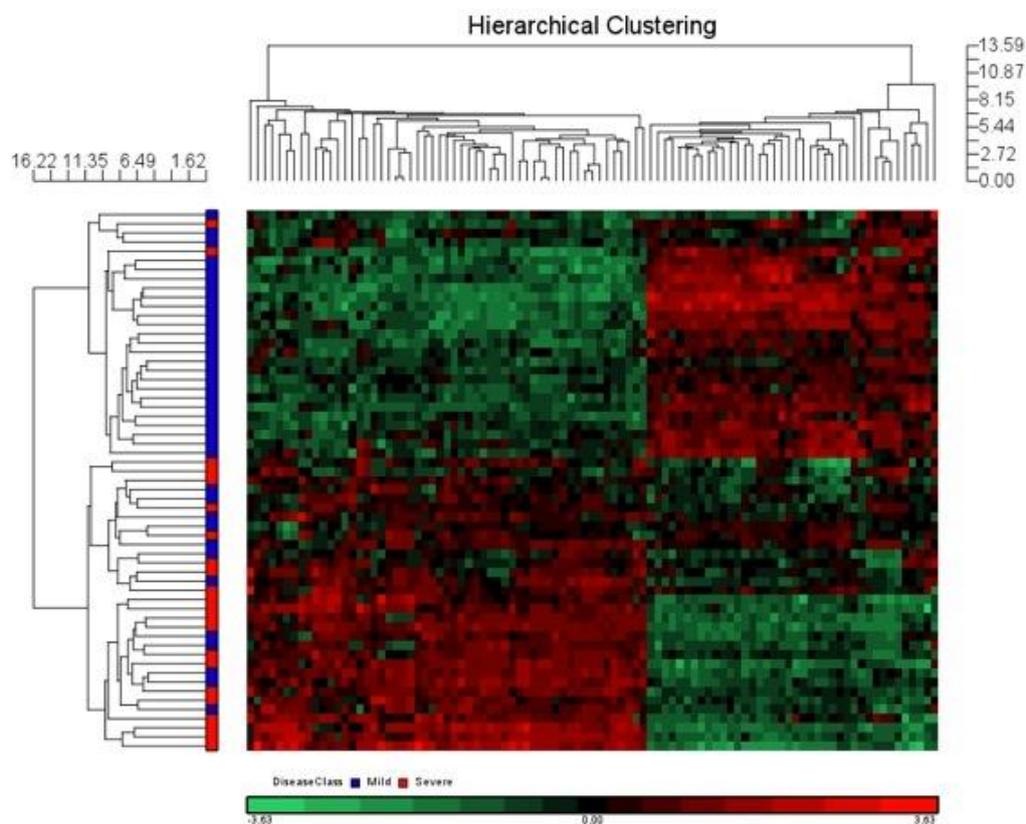


Figure 7.39 - Hierarchical Cluster Analysis of differential gene expression data from mild and severe acute pancreatitis patients. Heat map depicting the gene expression patterns in 58 patients (35 mild and 23 severe acute pancreatitis). Columns represent individual samples and rows represent each gene. Each

cell in the matrix represents the expression level of a gene feature in an individual sample. Blue represents mild and red illustrates severe patients. Upregulated (high expression level) genes are coloured red whereas downregulated (low expression level) genes are shown as green, as indicated in the scale bar (log₂-transformed scale).

The above figure shows the genes clustering to specific disease groups. While there are a few samples that are intermediate in their expression, the fact that clustering is clearly appreciated translates into a clear distinction between the groups – mild and severe. This provides evidence that based on gene expression, these are two different disease groups where different mechanisms are at play, leading to different clinical pictures and hence the outcomes.

It also raises an extremely pertinent point of the intermediate patients – where the expression lies somewhere between the two, eluding to presence of a different entity which may have different clinical presentation and may represent a different category altogether.

7.14 Overall gene expression differences between severe and mild acute pancreatitis

98 genes were differentially expressed (FC 1.5, FDR < 0.05) in severe acute pancreatitis (SAP) in comparison to mild acute pancreatitis (MAP). Table 7.11 lists the most significantly differentially expressed (upregulated) genes between all SAP and the corresponding FC and p-values obtained from comparisons with MAP. Details are provided in appendix 2. The single most up-regulated gene was MMP8 (Matrix Metalloproteinase 8) (FC=4.49), which codes for MMP 8 protein - a neutrophil collagenase, actively involved in breaking down of the extracellular matrix. Its role in cancer is very well established where it is involved in proliferation, invasion, epithelial-to mesenchymal transformation, metastasis and

angiogenesis. OLAH and ADAMST genes code for enzymes that are heavily involved in the extracellular matrix remodelling and development. Another important upregulated gene was found to be HPGD (Hydroxy prostaglandin dehydrogenase 15). This enzyme converts proinflammatory PGE2 to anti-inflammatory PGE2. In a recent study, HPGD is found to be protective against Lipopolysaccharide induced liver injury by activating PPAR- γ in Kupffer cells and concurrently, inhibiting their ability to produce inflammatory cytokines [340]. It is safe to assume a similar role is played by HPGD upregulation in the setting of acute pancreatitis as well. Cysteine- rich secretory protein 3 (CRISP3) gene was found to be upregulated in our dataset as well. It is defence-associated molecule (due to its expression in B lymphocytes) with predominant expression in the salivary gland, pancreas and prostate. It has been shown to be involved in pathophysiology of chronic pancreatitis [341]. CRISP-3 seems to play an important role in acinar cell degeneration and ductal cell proliferation in the course of the tissue destruction and remodelling in chronic pancreatitis and has also been found to be expressed in specific granules of neutrophils in response to pathogen infections [342, 343]. This would explain its source and potential role in the context of AP where neutrophils are the hallmark cellular component.

Table 7.11. Top 50 differentially expressed genes (upregulated) in severe and mild acute pancreatitis

Gene Symbol	ID	P value	Fold Change
MMP8	231688_at	0.000108	4.49
OLAH	222945_x_at	0.0005	3.419
ADAMTS2	226311_at	0.0000419	3.095
RETN	220570_at	0.000028	3.085
HPGD	211548_s_at	0.00263	3.077
SLC1A3	202800_at	0.000335	3.072
OLAH	233126_s_at	0.00108	2.918
HPGD	203914_x_at	0.00316	2.915

OLAH	219975_x_at	0.0028	2.87
GPR84	223767_at	0.000604	2.847
SLC51A	229230_at	0.000368	2.739
CRISP3	207802_at	0.000132	2.701
HPGD	211549_s_at	0.000838	2.624
DAAM2	212793_at	0.0018	2.551
CD163	216233_at	0.0000173	2.538
FGF13	205110_s_at	0.00332	2.537
ANKRD22	239196_at	0.000477	2.534
VSIG4	204787_at	0.000163	2.452
BPI	205557_at	0.0000131	2.445
PFKFB2	226733_at	0.000208	2.398
TIMP4	206243_at	0.0000661	2.391
ARG1	231663_s_at	0.000666	2.335
SH3PXD2B	231823_s_at	0.00036	2.315
HGF	210997_at	0.000874	2.31
HGF	209960_at	0.000334	2.302
PTGES	210367_s_at	0.000518	2.252
SAMSN1	1555638_a_at	0.000349	2.238
TMEM45A	219410_at	0.0000832	2.227
CEACAM1	210610_at	0.000094	2.221
DLC1	224822_at	0.00183	2.21
TCN1	205513_at	0.0000795	2.196
DHRS9	224009_x_at	0.000477	2.195
SEMA6B	223567_at	0.000799	2.182
LCN2	212531_at	0.00161	2.165
CYP1B1-AS1	1553829_at	0.00273	2.157
ADAMTS2	214535_s_at	0.0000261	2.151
DHRS9	223952_x_at	0.000497	2.149
LTF	202018_s_at	0.00292	2.134
CEACAM1	206576_s_at	0.00104	2.124
CRISP2	210262_at	0.000751	2.111
HGF	210998_s_at	0.000224	2.103
C1QC	225353_s_at	0.00269	2.1
THBS1	235086_at	0.000875	2.094
CEACAM1	211889_x_at	0.00129	2.09
SIRT5	219185_at	0.0000365	2.073
DHRS9	219799_s_at	0.000357	2.06
FGF13-AS1	1560537_at	0.000345	2.053
ERLIN1	202444_s_at	0.0000247	2.048
PDE4D	1554717_a_at	0.000131	2.027
KL	205978_at	0.000595	2.012

The most differentially expressed genes (upregulated) in severe and mild acute pancreatitis, (FC 1.5 and FDR < 0.05) and the corresponding FC and p-values. Abbreviations: FC = fold changes (FC); FDR = False discovery rate.

Table 7.12 lists the most differentially expressed (downregulated) genes between all SAP and the corresponding FC and p-values obtained from comparisons with MAP.

Table 7.12 - Top 50 differentially expressed genes (down regulated) in severe and mild acute pancreatitis

Gene Symbol	ID	P value	Fold Change
NELL2	203413_at	0.000450	-2.594
NOV	214321_at	0.000285	-2.519
YME1L1	234849_at	0.000218	-2.407
YME1L1	234013_at	0.00125	-2.405
TRDV3	216191_s_at	0.0000328	-2.398
YME1L1	217143_s_at	0.0000358	-2.386
RORA	226682_at	0.0000236	-2.364
P2RY10	236280_at	0.0000354	-2.321
THEMIS	1558972_s_at	0.00018	-2.299
INPP4B	235046_at	0.000166	-2.292
TRAT1	217147_s_at	0.00037	-2.283
KIAA1671	225525_at	0.000529	-2.254
GZMK	206666_at	0.0000878	-2.235
YME1L1	213830_at	0.0000298	-2.214
BCL11B	219528_s_at	0.000125	-2.208
RPS6KA5	1557113_at	5.51E-06	-2.201
GBP4	235175_at	0.000101	-2.178
TRAV9-2	217412_at	0.000319	-2.165
GATA3	209602_s_at	0.0000954	-2.159
CD28	206545_at	0.000194	-2.139
CAMK4	229029_at	0.00103	-2.113
TBC1D4	203387_s_at	0.0000443	-2.104
CD160	207840_at	0.000213	-2.091
TRAV24	234398_at	0.000694	-2.09
BEX2	224367_at	0.00321	-2.082
PRKCQ-AS1	1561004_at	0.000382	-2.075
IFNLR1	244261_at	0.000122	-2.068
TRAC	210972_x_at	0.00035	-2.03
DPP4	211478_s_at	0.000147	-2.026
RPS6KA5	204635_at	0.0000249	-2.022
GRAMD1C	219313_at	0.000717	-2.02
ATP8B2	226771_at	0.0000993	-2.016
ETV7	224225_s_at	0.00165	-2.012
ISM1	235182_at	0.00174	-2.007
LOC105369609	244798_at	0.00274	-2.003
CAMK4	241871_at	0.000522	-2
THEMIS	1558971_at	0.000189	-1.984
CD3D	213539_at	0.000425	-1.982
PYHIN1	240413_at	0.000401	-1.977

RORA	236266_at	0.000103	-1.975
LCK	204891_s_at	0.000231	-1.972
BCL11B	222895_s_at	0.000208	-1.97
YME1L1	211902_x_at	0.000521	-1.968
CXCR3	207681_at	0.0000454	-1.966
CD40LG	207892_at	0.000941	-1.954
MYBL1	213906_at	0.000183	-1.954
NR3C2	205259_at	0.000664	-1.939
GBP5	238581_at	0.000391	-1.936
TRAC	209671_x_at	0.000479	-1.935
RPS6KA5	204633_s_at	0.000152	-1.931

The most differentially expressed genes (downregulated) in severe and mild acute pancreatitis, (FC 1.5 and FDR < 0.05) and the corresponding FC and p-values. Abbreviations: FC = fold changes (FC); FDR = False discovery rate.

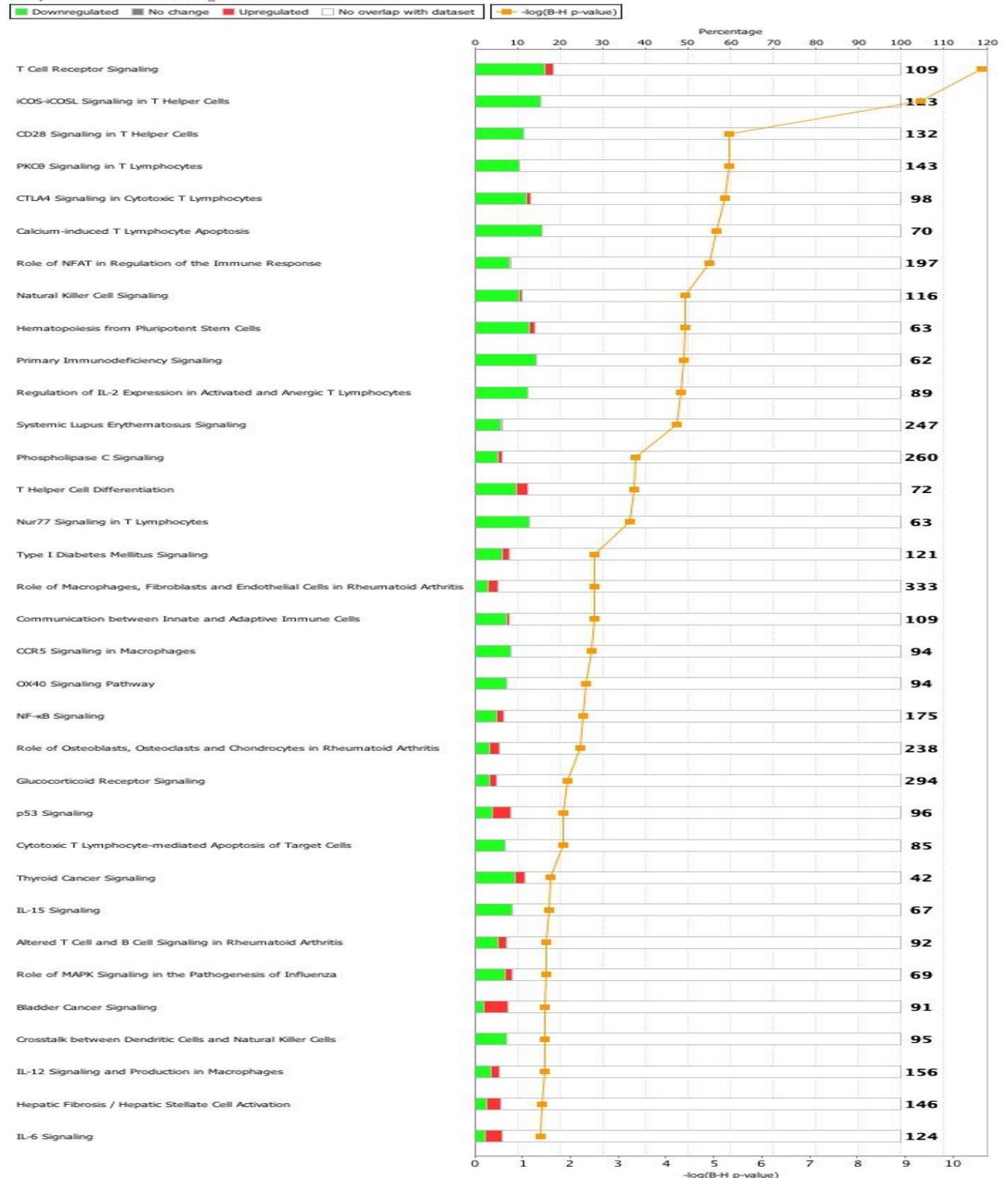
NOV (Neuroblastoma overexpressed) was found to be one of the most downregulated genes in our dataset. NOV is involved in regulating multiple cellular activities, including migration, proliferation, differentiation and survival. It has also been found to be involved in causing Ca²⁺ influx. Recently, regulatory T cells were identified to mediate oligodendrocyte differentiation in regeneration of myelin following damage. NOV was thought to be a key player in facilitating this process.

YME1L1 encodes for ATPases and is involved in mitochondrial organisation and protein metabolism. Pancreas is known to be one of the only organs in the body to have high concentrations of its protein due to high concentration of mitochondria. YME1L1 controls the accumulation of respiratory chain subunits and is required for apoptotic resistance, cristae morphogenesis, and cell proliferation.

T cell Receptor Associated Transmembrane Adaptor 1 (TRAT1), T cell Receptor Delta Variable 3 (TRDV3), T cell Receptor Alpha Variable 9-2 and 24 (TRAV 9-2 and TRAV24), T cell Receptor Alpha constant (TRAC) are all responsible for stabilising the T cell receptor (TCR) and are particularly involved in NFAT pathway. These were found to be most

significantly downregulated in SAP, indicating their dysregulation to be a key event in the disease process. This was further confirmed and is highlighted in the description of canonical pathways.

Analysis: FilteredSevereMild1_5 - 2012-09-03 09:14 AM



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Figure 7.40 - Top canonical pathways differentially expressed in SAP and MAP

The above figure (7.40) lists top 34 pathways differentially expressed in severe acute pancreatitis when compared with mild acute pancreatitis. The stacked bar chart displays the percentage of genes that were upregulated (red), downregulated (green), and genes not overlapping with our data set (white) in each canonical pathway. The numerical value at the top of each bar represents the total number of genes in the canonical pathway. The secondary x-axis shows the $-\log$ of P-value calculated by the Benjamini-Hochberg (B-H) method; the B-H method was used to adjust the right-tailed Fisher’s exact test P-value, which indicates the significance of each pathway.

Adjusting the rate helps to control for the fact that sometimes small p-values (less than 5%) happen by chance, which could lead to incorrect rejection of the true null hypotheses. In other words, the B-H procedure helps to avoid type I errors (false positives).

Details of the top canonical pathways identified are listed in table 14.3.

Table 7.13 - Top canonical pathways differentially expressed in SAP and MAP

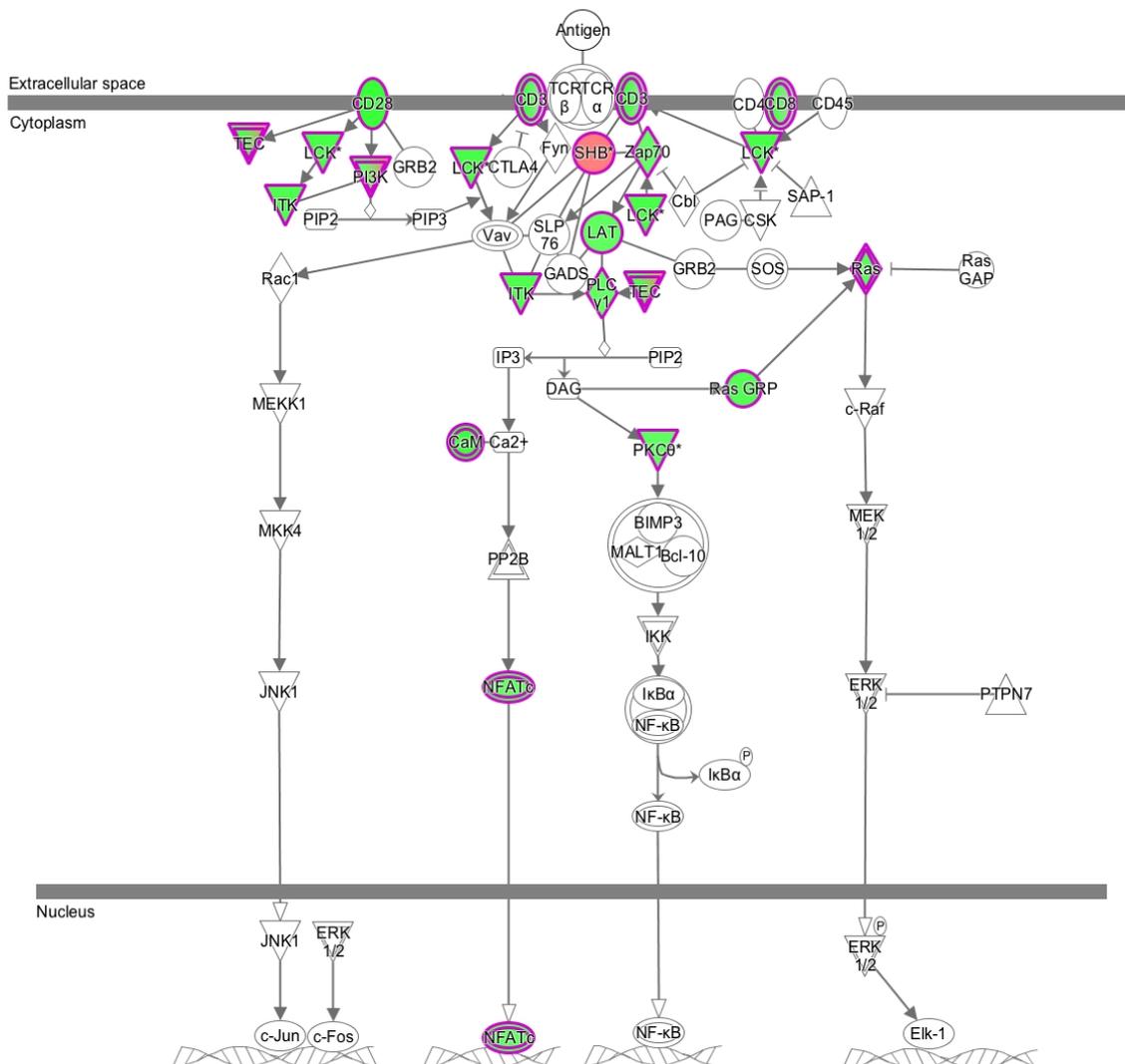
Ingenuity Canonical pathways	P value	Downregulated	Upregulated	No overlap with the data set
T Cell Receptor Signalling	10.6	18/109 (17%)	2/109 (2%)	89/109 (82%)
iCOS-iCOSL Signalling in T Helper Cells	9.31	19/123 (15%)	0/123 (0%)	104/123 (85%)
CD28 Signalling in T Helper Cells	5.32	15/132 (11%)	0/132 (0%)	117/132 (89%)
PKCθ Signalling in T Lymphocytes	5.32	15/143 (10%)	0/143 (0%)	128/143 (90%)
CTLA4 Signalling in Cytotoxic T Lymphocytes	5.24	12/98 (12%)	1/98 (1%)	85/98 (87%)
Calcium-induced T Lymphocyte Apoptosis	5.04	11/70 (16%)	0/70 (0%)	59/70 (84%)
Role of NFAT in Regulation of the Immune Response	4.91	16/197 (8%)	1/197 (1%)	180/197 (91%)
Natural Killer Cell Signalling	4.4	12/116 (10%)	1/116 (1%)	103/116 (89%)
Hematopoiesis from Pluripotent Stem Cells	4.4	8/63 (13%)	1/63 (2%)	54/63 (86%)
Primary Immunodeficiency Signalling	4.37	9/62 (15%)	0/62 (0%)	53/62 (85%)

Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	4.31	11/89 (12%)	0/89 (0%)	78/89 (88%)
Systemic Lupus Erythematosus Signalling	4.21	15/247 (6%)	1/247 (0%)	231/247 (94%)
Phospholipase C Signalling	3.37	14/260 (5%)	3/260 (1%)	243/260 (93%)
T Helper Cell Differentiation	3.32	7/72 (10%)	2/72 (3%)	63/72 (88%)
Nur77 Signalling in T Lymphocytes	3.24	8/63 (13%)	0/63 (0%)	55/63 (87%)
Type I Diabetes Mellitus Signalling	2.51	8/121 (7%)	2/121 (2%)	111/121 (92%)
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.51	10/333 (3%)	8/333 (2%)	315/333 (95%)
Communication between Innate and Adaptive Immune Cells	2.51	8/109 (7%)	1/109 (1%)	100/109 (92%)
CCR5 Signalling in Macrophages	2.45	8/94 (9%)	0/94 (0%)	86/94 (91%)
OX40 Signalling Pathway	2.34	7/94 (7%)	0/94 (0%)	87/94 (93%)
NF-κB Signalling	2.26	9/175 (5%)	3/175 (2%)	163/175 (93%)
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	2.2	8/238 (3%)	6/238 (3%)	224/238 (94%)
Glucocorticoid Receptor Signalling	1.94	10/294 (3%)	5/294 (2%)	279/294 (95%)
p53 Signalling	1.85	4/96 (4%)	4/96 (4%)	88/96 (92%)
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	1.85	6/85 (7%)	0/85 (0%)	79/85 (93%)
Thyroid Cancer Signalling	1.6	4/42 (10%)	1/42 (2%)	37/42 (88%)
IL-15 Signalling	1.56	6/67 (9%)	0/67 (0%)	61/67 (91%)
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	1.49	5/92 (5%)	2/92 (2%)	85/92 (92%)
Role of MAPK Signalling in the Pathogenesis of Influenza	1.49	5/69 (7%)	1/69 (1%)	63/69 (91%)
Bladder Cancer Signalling	1.48	2/91 (2%)	5/91 (5%)	84/91 (92%)
Crosstalk between Dendritic Cells and Natural Killer Cells	1.48	7/95 (7%)	0/95 (0%)	88/95 (93%)
IL-12 Signalling and Production in Macrophages	1.47	6/156 (4%)	3/156 (2%)	147/156 (94%)
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.42	4/146 (3%)	5/146 (3%)	137/146 (94%)
IL-6 Signalling	1.39	3/124 (2%)	5/124 (4%)	116/124 (94%)

Figure below (7.41) depicts the genes involved in TCR signalling in our dataset. The downregulated genes are highlighted in green. This demonstrates the process of TCR activation which promotes a number of signalling cascades, resulting in cytokine production and progression of inflammation. Lymphocyte protein tyrosine kinase (Lck) and Zeta-chain associated protein kinase (Zap-70) become activated and further lead to recruitment of

inducible T cell kinase (Itk). This phosphorylates phospholipase C γ 1 (PLC γ 1) which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP₃). IP₃ triggers the release of Ca²⁺ from endoplasmic reticulum, which promotes entry of extracellular Ca²⁺ into cells through calcium release-activated Ca²⁺ (CRAC) channels. Calcium-bound calmodulin (Ca²⁺/CaM) activates the phosphatase calcineurin, which promotes IL-2 gene transcription through the transcription factor NFAT.

T Cell Receptor Signaling : FilteredSevereMild1_5 : Expr Fold Change

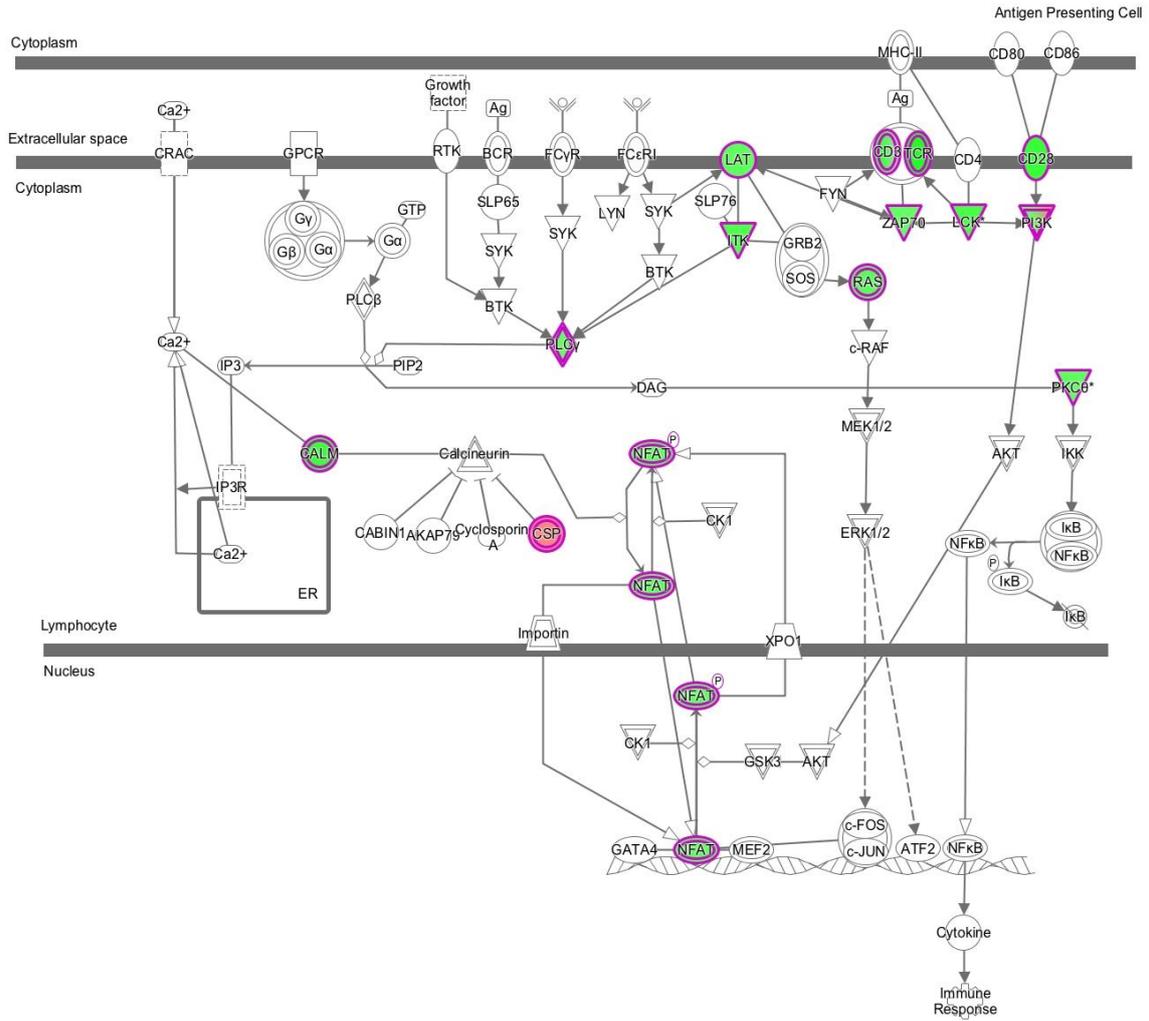


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Figure 7.41 - T Cell Receptor Signalling from the canonical pathways

Interestingly, NFAT was found to be the most abundantly expressed molecule in the differentially expressed canonical pathways. Out of the top 25, at least 12 pathways had NFAT as a key component, making it a crucial player in the pathogenesis.

As is evident from the figure below (7.42), on antigen engagement of lymphocyte receptors, phospholipase C- γ (PLC- γ) becomes activated and hydrolyses phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5trisphosphate (IP3) and diacylglycerol. IP3 then binds to specific receptors on the endoplasmic reticulum and drives calcium release from the endoplasmic reticulum into the cytoplasm, which triggers STIM1 and Orai1-mediated opening of calcium release activated calcium channels. As a result of increased intracellular calcium, the calcineurin enzyme becomes active and dephosphorylates NFAT, allowing NFAT translocation into the nucleus and subsequent regulation of gene expression. It should be noted, however, that NFAT must ultimately bind to additional transcription factors, such as AP1 to regulate gene expression [344, 345].



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Figure 7.42 – Role of NFAT in regulating immune response from the canonical pathways

CHAPTER 8: MICRO RNA PROFILING

8.1 Samples Used

Plasma samples obtained from patients with acute pancreatitis were used for this part of the project. These were obtained from patients at the time of admission and retrieved from Liverpool NIHR Pancreas Biomedical Research Unit Acute Pancreatitis Biobank, where these were stored as 1 ml aliquots.

Multiple experiments were designed to optimise the RNA extraction protocol to ensure maximum concentration of total RNA. Following is the final optimised protocol.

8.2 Total RNA extraction from Plasma samples

One aliquot of 1 ml plasma was used for each patient. Protocol used was Plasma/Serum Circulating RNA Purification Kit from Norgen Biotek Corporation.

8.2.1 Prior to extraction

All centrifugation steps were performed at room temperature.

All solutions were at room temperature prior to use.

Wash Solution I was prepared beforehand by adding 14.5 mL of 95% ethanol to the bottle containing the concentrated RNA Wash Solution.

Wash Solution II was also prepared by adding 10 mL of 95% ethanol to the bottle containing the concentrated RNA Wash Solution.

1 ml aliquots were speed vacuumed to 500 µl to concentrate the RNA and maximise the yield.

8.2.2 Procedure of RNA extraction

One mL of Lysis Solution was added to 500 μ l of concentrated plasma sample. This was vortexed for 15 seconds.

The lysate was incubated for 10 minutes at 60°C.

1.5 mls of 96 - 100% Ethanol was added to the plasma sample. This was mixed by vortexing for 10 seconds.

Lysate was then transferred to the Maxi Column assembly and vortexed for 15 seconds.

The Maxi Column assembly was centrifuged for 2 minutes at 3,000 RPM, after which, the collection tube was removed and flow-through discarded. The Maxi Column was reassembled with the collection tube and process repeated till all the lysate was applied.

700 μ L of Wash Solution I was then applied to the column and centrifuged for 2 minute at 3,000 RPM, the flow-through discarded and Maxi Column with the collection tube reassembled. The process was repeated one more time.

700 μ L of Elution Solution I was applied to the column and centrifuged for 2 minutes at 500 RPM, followed by 3 minutes at 3,000 RPM. Maxi Column was discarded from the conical tube.

One mL of Binding Solution was added to the eluted RNA contained within the conical tube and vortexed for 15 seconds.

0.5 mL of 96 - 100% ethanol was added to the resultant mixture. This was then mixed by vortexing for 10 seconds.

700 μ L of this mixture was applied onto the Mini Spin Column and centrifuged for one minute at 14,000 RPM.

This was repeated to load the rest of the sample.

400 μ L of Wash Solution II was applied to the column and centrifuged for one minute at 14,000 RPM.

This was repeated a second time to wash the column.

The column was centrifuged for 2 minutes at 14,000 RPM in order to thoroughly dry the resin.

The column was placed into a fresh 1.7 ml Elution tube and 100 μ L of Elution Solution II was added to the column. It was centrifuged for 3 minutes at 2,000 RPM, followed by two minutes at 14,000 RPM.

The resultant total RNA was then used for further assessment.

8.3 Quantification of extracted RNA

Aliquots of total RNA for quality control assessments were taken at the time of extraction.

Concentration of the samples was analysed using Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

8.4 Poly A tailing and ligation

Following figure provides diagrammatic representation of the process of Poly A tailing and ligation of the extracted RNA (Figure 8.1).

FlashTagHSR: Procedure Overview

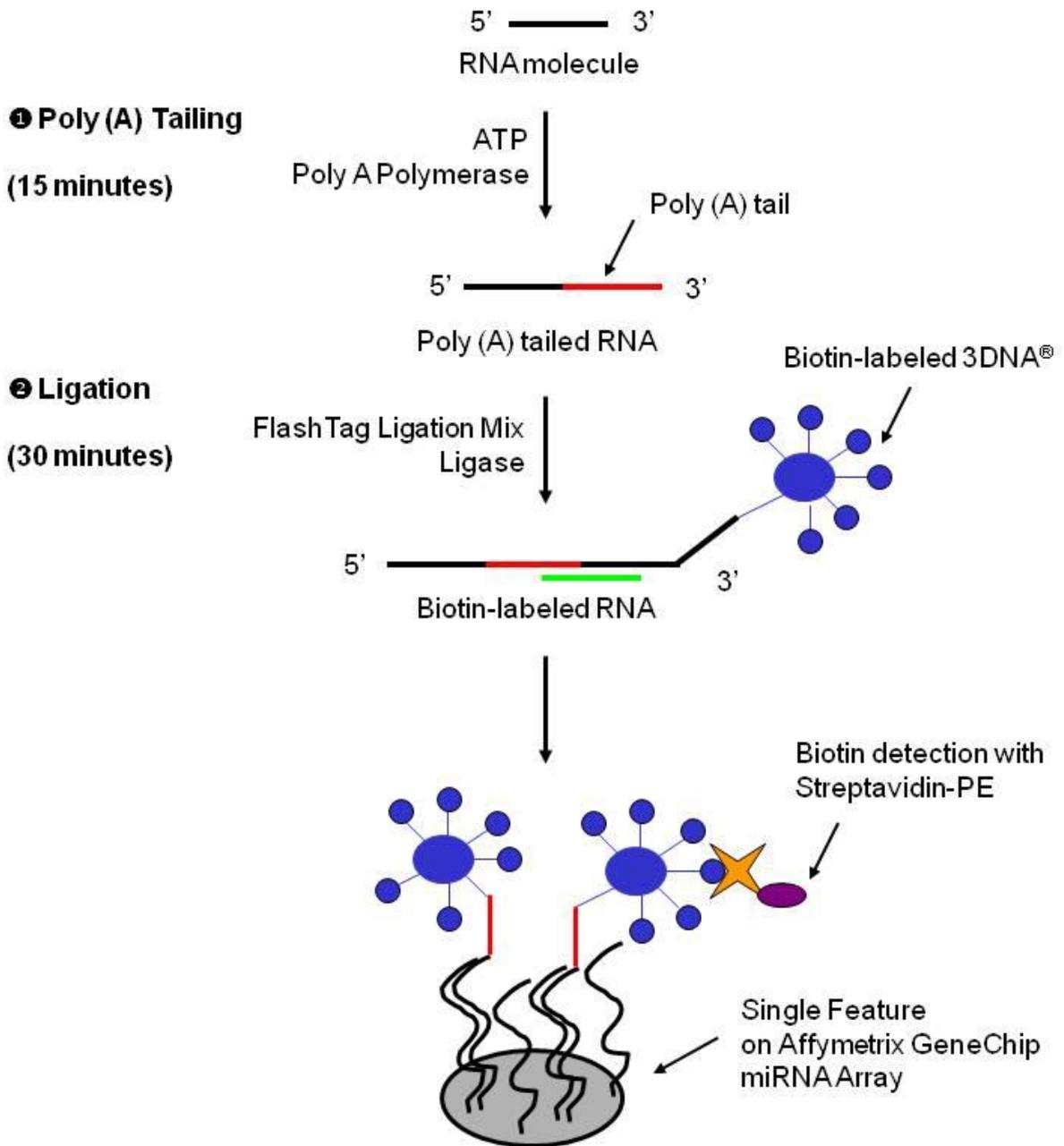


Figure 8.1 – Poly-A Tailing and ligation (Adapted with permission)

8.4.1 Poly (A) Tailing

Protocol used was FlashTag® Biotin HSR RNA Labeling Kit for Affymetrix® GeneChip® miRNA Arrays.

The total RNA was in a final volume of 80 µl. This was speed vacuumed to a volume of 8 µl.

All samples were heat denatured at 80°C for 10 minutes.

ATP mix was diluted in 1mM Tris in a ratio of 1:500.

2µl RNA Spike Control Oligos was added to the RNA sample, taking the total volume to 10 µl.

Following components were added to the 10µl RNA/Spike Control Oligos, for a volume of 15µl and microfuged:-

1.5µl 10X Reaction Buffer

1.5µl 25mM MnCl₂

1.0µl diluted ATP Mix

1.0µl PAP Enzyme

The mixture was incubated in a 37°C heat block for 15 minutes.

8.4.2 FlashTag Biotin HSR Ligation

15µl of tailed RNA was briefly microfuged and placed on ice.

4µl 5X FlashTag Biotin HSR Ligation Mix and 2 µl of T4 DNA Ligase was added. The resultant mixture was gently mixed and microfuged.

This was incubated at 25°C (room temperature) for 30 minutes.

The reaction was stopped by adding 2.5µl HSR Stop Solution. The 23.5µl of ligated sample was mixed and microfuged.

8.5 Affymetrix GeneChip miRNA Array Procedure

8.5.1 Hybridization

The 20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre from GeneChip Eukaryotic Hybridization Control Kit) were completely thawed and then heated for 5 minutes at 65°C.

The following components were added to the biotin-labelled sample in order to prepare the array hybridization cocktail:-

50µl 2X Hybridization Mix

15µl 27.5% Formamide

10µl DMSO

5 μ l 20X Eukaryotic Hybridization Controls

1.7 μ l Control Oligonucleotide B2, 3nM

The resultant volume was 103.2 μ l. This was incubated at 99°C for 5 minutes, then 45°C for 5 minutes.

100 μ l was aspirated and injected into an array (miRNA_2.0 array from Affymetrix).

The pipette tip was removed from the upper right septum of the array and both septa were covered with 1/2" Tough-Spots to minimize evaporation and/or prevent leaks.

Affymetrix Hybridization Oven 640 was turned on and preheated at the temperature of 48°C and RPM of 60.

The arrays were placed into hybridization oven trays and loaded into the hybridization oven.

The arrays were then incubated at 48°C and 60 rpm for 16 hours.

8.5.2 Washing and Staining

After 16 hours of hybridization, the arrays were removed from the oven and the Tough-Spots taken off.

The hybridization cocktail was extracted from each array and transferred to a new tube in order to save the hybridization cocktail and stored at –80°C for long-term storage.

Each array was completely filled with Array Holding Buffer.

The arrays were allowed to equilibrate to room temperature before washing and staining.

Vials were placed into sample holders on the fluidics station:-

One (amber) vial containing 600µl Stain Cocktail 1 in sample holder 1.

One (clear) vial containing 600µl Stain Cocktail 2 in sample holder 2.

One (clear) vial containing 800µl Array Holding Buffer in sample holder 3.

These were washed and stained with Fluidics Station 450 using fluidics script FS450_0003.

Post Hyb Wash #1 - 10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C.

Post Hyb Wash #2 - 8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C.

1st Stain: The probe array was stained for 10 minutes with Stain Cocktail 1 at 25°C.

Post Stain Wash - 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C.

2nd Stain: The probe array was stained for 10 minutes with Stain Cocktail 2 at 25°C.

3rd Stain: The probe array was stained for 10 minutes with Stain Cocktail 1 at 25°C.

Final Wash - 15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C.

The probe array was completely filled with Array Holding Buffer.

Once it was ensured that there were no air bubbles, both septa were covered with 3/8" Tough-Spots. The array glass surfaces were inspected for dust and other particulates prior to scanning.

8.5.3 Scanning

Once the protocol was completed, the arrays were ready to scan on the GeneChip Scanner 3000. Affymetrix Command Console software was used to scan the genechips.

Genechips were inserted into the scanner. Once scanning was completed, Affymetrix Expression Console was used to generate the quality control metrics to ensure adequate and appropriate processing of the arrays.

8.6 Analysis

The miRNA QC Tool software was used for data summarisation, normalisation, and quality control.

Analysis was performed using appropriate packages in R/Bioconductor and using Partek Genomics Suite software. Expression level data were invariant set normalised. Differential expression of miRNAs in severe versus mild acute pancreatitis was detected using ANOVA with batch hybridization effects removed. The Benjamini and Hochberg multiple testing correction was applied to the resulting p-values.

8.7 Quantitative Assessments

There were 20 samples included, ten of each mild and severe patients. These were processed in 4 batches with equal mix of both the groups to avoid batch effects and to ensure homogeneity among the samples.

Samples in each batch are as summarised below.

Table 8.1 – Samples included

<p>Batch 1</p> <p>AP040 AP041 AP042 AP043 AP044 AP045 AP046</p>	<p>Batch 2</p> <p>AP051 AP063 AP065 AP066</p>
<p>Batch 3</p> <p>AP077 AP103 AP117 AP144 AP192</p>	<p>Batch 4</p> <p>AP048 AP091 AP168 AP177 AP187</p>

Red highlighted samples are severe acute pancreatitis

Following table (8.2) provides concentrations of the total RNA extracted from plasma of each patient for microarray experiments.

Table 8.2 – Total RNA concentration of the samples included

Sample	Concentration (ng/μl)
AP040	3.3
AP041	2.7
AP042	1.5
AP043	2.3
AP044	0.9
AP045	2.3
AP051	3.9
AP063	1.5
AP065	2.4
AP066	2.3
AP077	4.4
AP103	3.0
AP117	2.0
AP144	3.2
AP192	3.6
AP048	4.7
AP091	6.2
AP168	6.4
AP177	4.6
AP187	6.1

The first step post hybridization and scanning of the arrays is the generation of quality control (QC) metrics to ensure efficient hybridization. Following are the QC metrics for the included samples.

Table 8.3 - Quality Control Metrics of the hybridizations

Samples	Mean Intensity	Mean Background Intensity	Detected Probes*	Detected Probe sets [‡]
AP040	86.63517	39.03065	19149	3273
AP041	85.28208	39.56137	12894	1823
AP042	74.03575	36.58618	13356	1792
AP043	84.91063	39.05802	12305	1906

AP044	90.64921	39.60151	12422	1987
AP045	87.27647	40.36115	13691	2088
AP051	90.4346	51.05437	13940	2193
AP063	89.57281	52.92628	12599	1954
AP065	76.81561	38.08247	14702	2406
AP066	85.99326	41.41285	14468	2391
AP077	80.21484	42.16008	13756	2231
AP103	71.31986	38.12845	13346	1994
AP117	73.73982	38.61769	12687	1577
AP144	139.3286	47.36808	21823	3313
AP192	83.2539	40.13697	12677	1867
AP048	80.46622	39.51514	12555	1992
AP091	84.81396	40.57718	17584	3018
AP168	76.28916	38.0399	12608	1949
AP177	75.0023	39.34704	11931	1598
AP187	83.39607	45.20448	13611	2361

* Out of total 202752 probes

¥ Out of total 20643 probe sets

The above table indicates uniformity and homogeneity among the samples that hybridized to accepted standards, making the data retrieved from them reliable and of biological importance.

This was then further investigated in the form of box plots. These are as follows:-

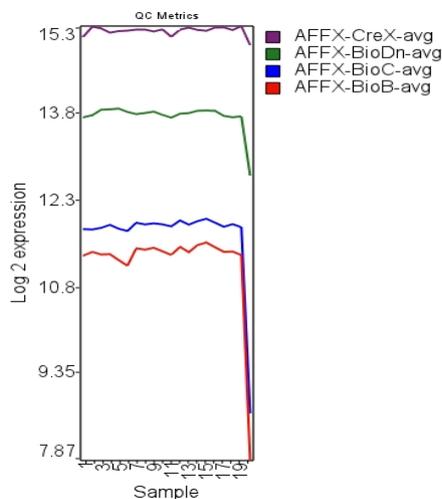


Figure 8.2 – Quality Control Metrics of Probe intensities of the House keeping genes

This figure (8.2) shows the log₂ expression of the probe intensities of the house keeping genes. This appeared to be fairly evenly distributed across all the samples, but sample 20 seemed to be an outlier.

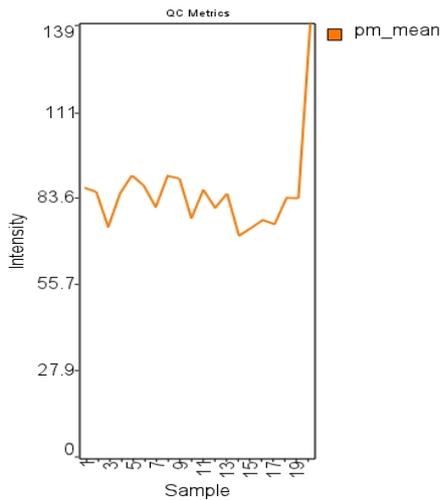


Figure 8.3 – Quality Control Metrics of Perfect match intensities

This was further confirmed on ‘perfect match’ intensities as well. But the expression level boxplot (see below) didn’t demonstrate this as clearly. Therefore, it was decided to proceed to principle component analysis for batch effects with 20 samples.

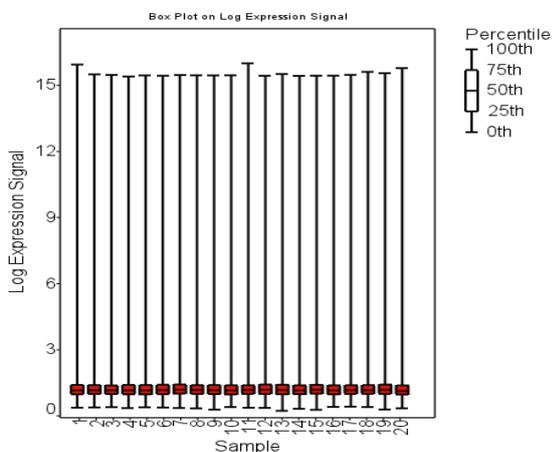


Figure 8.4 - QC metrics box plot on Log Expression Signal

8.8 Principle Component Analysis

Principle Component Analysis (PCA) was then performed to look for and correct batch effects and also to analyse differential expression. This was done using Partek. While it showed grouping of the samples into mild and severe rather elegantly, sample 20 was an obvious outlier. Therefore, it was decided to remove this from the group and performed the rest of analyses with 19 samples (10 MAP, 9 SAP). Also, it was noted on the PCA plots that when classified according to batch dates, reassuringly, no significant clustering was observed.

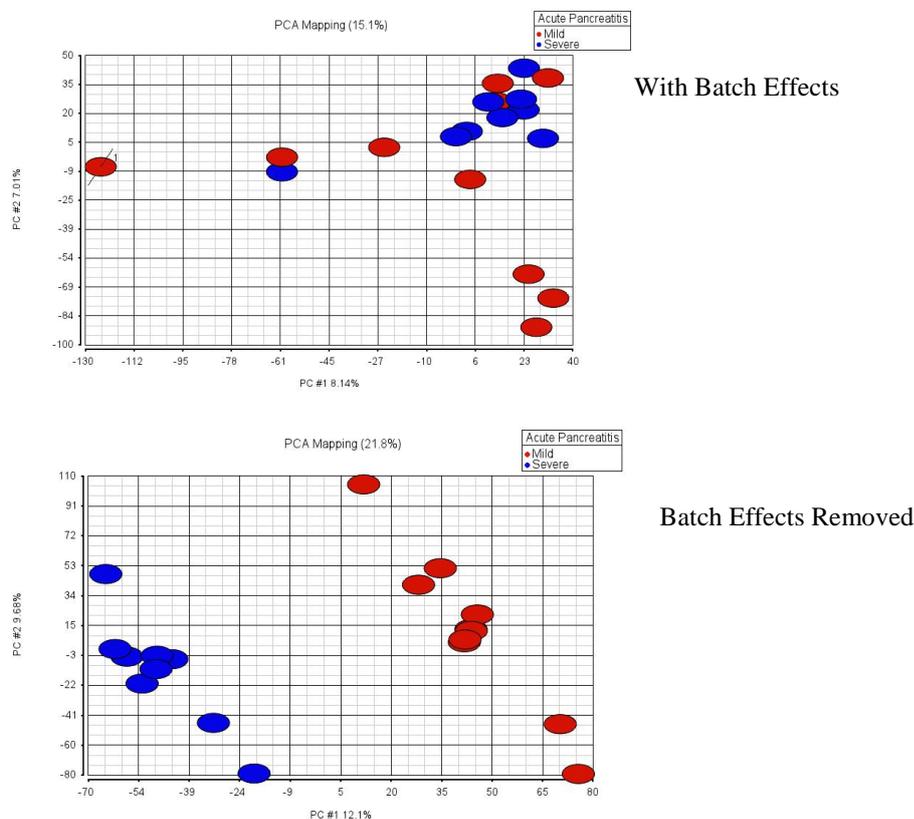
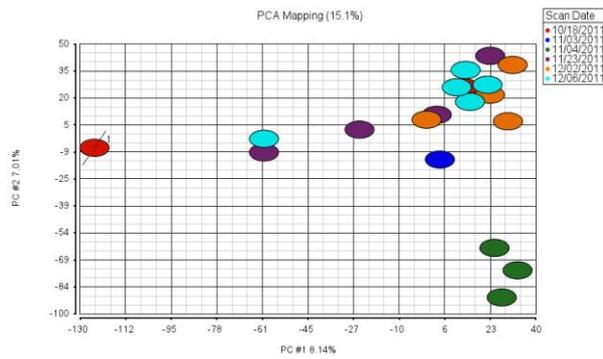
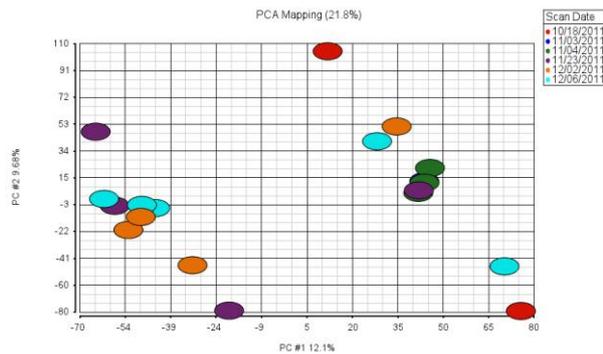


Figure 8.5 – Principle component analysis according to disease severity



With Batch Effects



Batch Effects removed

Figure 8.6 – Principle Component Analysis according to date of sample processing

8.9 Hierarchical Clustering and differentially expressed miRNA

Based on $FDR < 0.05$, 45 probes were identified that were differentially expressed in the two groups. These were all human probes.

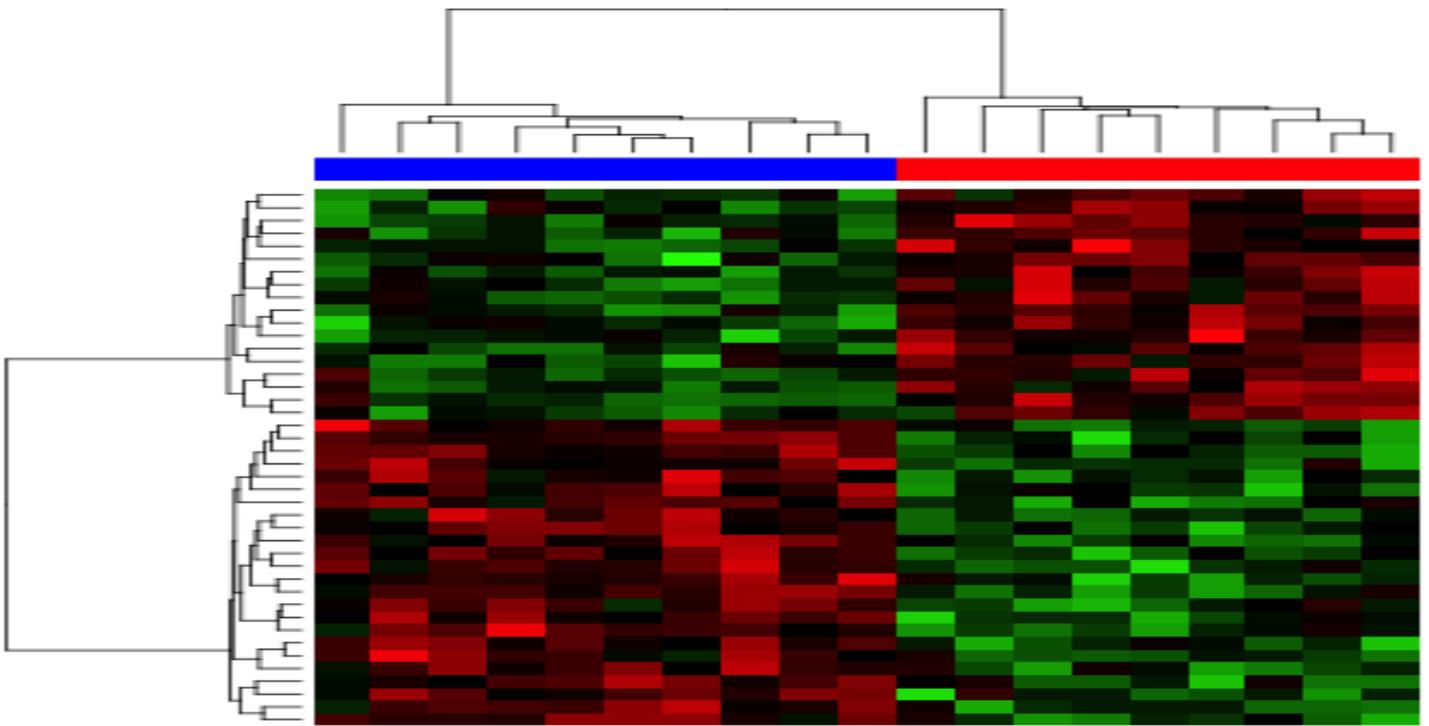


Figure 8.7 - Hierarchical Cluster Analysis of differentially expressed miRNA in mild and severe acute pancreatitis patients. Heat map depicting the miRNA expression patterns in 19 patients (10 mild and 9 severe acute pancreatitis). This was calculated keeping the FDR $p < 0.05$. 45 differentially expressed miRNA were identified, out of which only 23 were annotated in IPA/MetaCore. Columns represent individual samples and rows represent each gene. Each cell in the matrix represents the expression level of a gene feature in an individual sample. Blue represents mild and red illustrates severe patients. Upregulated (high expression level) genes are coloured red whereas downregulated (low expression level) genes are shown as green.

Here is the list of top 20 differentially expressed miRNA between the two groups in order of their ranking based on p value and the fold change. Complete list is provided in appendix 3.

Table 8.4 – List of top 20 differentially expressed miRNA between MAP and SAP.

Affy ID	Adjusted p Value	Fold Change
hp_hsa-mir-631_st	0.013909811	-1.86365
hp_hsa-mir-3129_x_st	0.013909811	-1.58772
ENSG00000238824_st	0.013909811	-1.29649
hp_hsa-mir-1291_s_st	0.018768512	1.813645
ACA50_st	0.018768512	-1.31933
ENSG00000212611_x_st	0.019781918	-1.26774
hsa-miR-592_st	0.021760076	1.7757
ACA9_x_st	0.021760076	-1.55509
ENSG00000239005_x_st	0.021760076	-1.45394
ENSG00000238450_x_st	0.022441665	1.509782
hp_hsa-mir-3157_st	0.022441665	-1.4312
ENSG00000238615_st	0.022441665	1.341408
hp_hsa-mir-548n_x_st	0.024523767	-1.77023
hsa-miR-302e_st	0.026509154	-1.52089
hsa-miR-513c_st	0.026509154	1.452108
hsa-miR-548j_st	0.028097164	-1.45883
ENSG00000206898_st	0.03542224	-1.24917
hsa-miR-758_st	0.03572517	1.638581
hsa-miR-3187_st	0.03572517	-1.58219
v49_ENSG00000200237_st	0.037128192	-1.12136

Role of **miR-631** is well studied in prostate cancer. It is avowed to inhibit the migration and invasion of prostate cancer cells. Related canonical pathways are metabolism and glycosaminoglycan metabolism.

miR-1291 (derived form SNORA34) has has been implicated in pathogenesis of various related and unrelated malignancies and disease processes. It was downregulated in clinical pancreatic ca samples and human pancreatic cancer cell lines and was also found to have significantly lower expression in oesophageal squamous cell carcinoma by acting as a tumour suppressor by targeting Mucin 1 (MUC1). High circulating levels have also been related to acute myocardial infarction. Loss of tumour repressive miR-1291 enhances renal cell carcinoma proliferation, migration and invasion through targeting SLC2A1/GLUT1. It is also

a biologically relevant regulator of GPC3 expression in hepatoma cells and acts through silencing of the endoplasmic reticulum stress sensor IRE1alpha.

miR-758-5p is another miRNA that has been associated with varying disease processes but its role is mainly defined in cholesterol metabolism. It decreases lipid accumulation of foam cell by regulating CD36-mediated cholesterol uptake and its expression is significantly upregulated in hyper-cholesterolemic plaques, perhaps through regulating Cholesterol-Efflux Regulatory Protein (ABCA1). It has also been suggested that it may serve as a potential diagnostic biomarker for lupus nephritis in patients with systemic lupus erythematosus (SLE). It also mediates Hepatitis C virus infection induced changes in Toll like Receptor 3 and 7.

Small nucleolar RNA **SNORA70** (U70) is a non-coding RNA (ncRNA) molecule which functions in the biogenesis (modification) of other small nuclear RNAs (snRNAs).

miR-592 has been linked to progress of various malignant processes. It has been laid out to be a novel and a potential carcinogen-initiated and metastasis-related biomarker in colorectal cancer, and down-regulation of miR-592 has been given due consideration as a potentially significant molecular treatment strategy for these patients [346, 347]. Overexpression of miR-592 has been shown to reduce cell proliferation, migration and invasion and induce cell arrest at G1/G0 phase in glioma tissues and cell lines. It was also demonstrated to yield smaller tumour volume and weight, specifying a promising application for its use in glioma treatment [348]. In addition to glioma, miR-592 is being considered as a promising therapeutic target for non-small cell lung cancer, due to its function as a tumour suppressor by suppressing SOX-9 [349].

miR-513 is expressed in human cholangiocytes and is down-regulated upon IFN- γ exposure. It regulates B7-H1 (a molecule that plays a critical role in immunomodulation of the cell

mediated immune response) translation and is involved in IFN- γ induced B7-H1 expression in the cholangiocytes. This emphasises role for miRNA mediated gene silencing in the regulation of cholangiocyte response to IFN- γ [350].

Small Nucleolar RNA (SnoRNA) U13 (SnoU13) was first described in 1989 [351]. It has been well characterized in 35 species by both functional assay and prediction. It is involved in the nucleolytic cleavage at the 3' end of 18S rRNA where it works as a trans-acting factor. SnoRNAs, in general, are poorly understood. While it is well known that they form the majority of non- coding RNAs, their functional value and role in cellular interactions is still largely unknown. Traditionally, snoRNAs have always been thought to be exclusively inside the nucleolus and Cajal bodies, involved in maturation of ribosomal RNA (rRNA) and small nuclear RNA (snRNA) locally [351, 352]. A recent study has however demonstrated that while more than 99% of the U13 snoRNAs are nuclear in their location in unstressed cells, cytoplasmic presence of snoRNA has been manifested by qPCR and RNA-seq, in response to stress, where their cytoplasmic abundance as a class is dynamically regulated by oxidative tone and is very rapid and significant. Because the nuclear snoRNAs are in vast excess to the cytoplasmic snoRNAs, even small changes in the nuclear pool would be expected to create large relative changes in the cytoplasmic pool. This study also suggests that cells have robust mechanisms for maintaining the cytoplasmic levels of snoRNAs and that detection of snoRNAs in the cytoplasm is not simply a “mass effect” in which some proportion of snoRNAs spill over into the cytoplasm [353]. It also entertains the possibility of anti-sense interactions of U13 snoRNA and the cytoplasmic mRNAs which could result in nucleotide modifications. It is safe to say that U13 snoRNA potentially could exert far reaching effects on cytoplasmic functions, a lot more than previously thought to be.

Small Nucleolar RNA U30 thought to act as a 2'-O-ribose methylation guide for ribosomal RNA [354].

Small Nucleolar RNA ACA 9 (SnoRA9) guides the sites of modifications of uridines to pseudouridines [355].

Small nucleolar RNA SNORA50 (also known as ACA50) is a non-coding RNA which affects modification of other small nuclear RNAs (snRNAs). ACA50 was originally cloned from HeLa cells and belongs to the H/ACA box class of snoRNAs. SnoRNA ACA50 is predicted to guide the pseudouridylation of U34 and U105 of 18S ribosomal RNA (rRNA) [355].

CHAPTER 9: DISCUSSION

In this observational, translational study, including 58 patients with acute pancreatitis, it has been identified and established that severe and mild acute pancreatitis are two different disease groups. While the underlying basic mechanisms of initiation of the disease may stand true for both of them, the progression is governed by different pathways altogether. It begins to explain why the outcomes and disease courses are so different for the same disease. The microarray experiments have identified various genes (98 genes with a fold change of 1.5) that are differentially expressed between mild and severe pancreatitis. Most of these differentially expressed genes are associated with various inflammatory processes and are involved in remodelling of extracellular matrix, recruitment of inflammatory cells, cellular proliferation and angiogenesis. These are all well-known early and key events in the initiation and progression of acute pancreatitis. Results confirm their significance in the disease process. By associating particular genes to these individual processes, it makes a lot of scientific sense to investigate these as potential biomarkers, especially early on in the disease course.

Most interestingly, the functional analysis has shed light on the immune response in the disease and pathways involved. T cell signalling pathways have been found to be among the top most significant canonical pathways. This is a very important finding. This indicates a role of adaptive immunity in pathogenesis that has not been explored and emphasised on before.

Another important canonical pathway that has been diagnosticated to be differentially expressed in SAP is 'role of NFAT in immune response'. Its significance lies in the fact that NFAT has also been noted to be the most significantly implicated gene in our dataset, being a key molecule in at least 12 different pathways (out of the top 25 most differentially expressed pathways). This is of particular importance.

Based on a pilot study of 19 samples including mild and severe patients, a differential miRNA expression between the two disease states was successfully demonstrated. The groups separated out as two different entities, based on the miRNA expression. This was clearly demonstrated on hierarchical clustering. At least 45 miRNA were identified to be differentially expressed between the disease states. This was based on FDR of <0.05 . These miRNA are the point of interest. Looking into their function, specification and interaction with mRNAs and targets will provide invaluable information on the pathogenesis of the disease but also an insight into potential biomarkers. Interpreting canonical pathways for miRNA implicated in this dataset would have to be considered with caution – not much is known about them and therefore, lack of evidence for links to a particular function does not translate to absence of it. In fact, most of the differentially expressed miRNA in this dataset have been found to be of prognostic/predictive value in various malignancies and their role in the setting of inflammation, infection or sepsis is largely unknown.

Of particular note, was the abundance of sno RNAs in our dataset – in fact, almost 50% of our most differentially expressed miRNA were found to be sno RNAs. Small Nucleolar RNA (sno RNA) represent a class of regulatory RNAs responsible for post-transcriptional maturation of ribosomal RNAs (rRNAs). Since defects in ribosome maturation and function can cause disruption of vital processes and lead to diseases and transformation of normal cells into tumour cells [356-358], it is well reasoned to postulate that snoRNA level can affect physiological condition of cells, tissues, and organs, thereby leading to various diseases.

9.1 Biomarker Research in Acute Pancreatitis

Acute pancreatitis continues to be a clinical challenge. While management of the disease is uncertain and unpredictable at best, prognostication poses an even bigger problem. Severe acute pancreatitis, especially, is associated with high morbidity and mortality and therefore,

early identification is the key. The current scoring systems require at least 48 hours to be of full use and even then, suffer from low sensitivity. The crying need of the time is an efficient, robust clinical system, whereby a patient diagnosed to have AP can readily be stratified into a category with good confidence and outcomes predicted. This will help to allocate clinical care in the best suited manner. This will be particularly useful in clinical trials, where categorisation of patients into an appropriate group is of paramount importance. Accurate disease stratification ensures effective assessment of the impact of the treatment. Once a reliable method/molecules is/are identified, this can then be exploited to not only assess severity at the time of admission but also predict different outcomes, i.e., necrosis, organ failure, death etc.

Biomarker discovery has taken an accelerated pace in recent times and is taking advantage of ever growing technologies. The approaches involved are multidisciplinary, from basic to translational and clinical research. As biological processes are complex, multi-technological approaches are crucial in understanding the basic pathophysiology. Prognostication in acute pancreatitis is still evolving. From implementation of Ranson's Score in 1974 [359] to the new genomic markers in the form of miRNA, we have come a long way in unravelling the mysteries of pathophysiology that have led the path for discovering more reliable and clinically useable biomarkers. However, this unfortunately still hasn't translated into ultimate success. We still do not have any biomarker/panel of biomarkers which is efficient enough. More and more clinical parameters and molecular entities are being tested every day.

Many clinical and biochemical markers and scoring systems have been tried in the clinical and experimental arenas – Modified Glasgow scale, APACHE II, organ failure related scoring systems(SOFA, Marshall, MOF/Goris), radiologic scores (Balthazar, CTSI), CRP and procalcitonin are some examples. Cytokines are at the helm of prognostication – there is already sizeable, well established body of data, grounding its claim to be reliable in

predicting severity in AP [360-362]. However, because of their limitations, no cytokines are useful enough to indicate disease progression of AP with simplicity and accuracy for routine clinical use. Recently, Growth differentiation factor-15 (GDF-15) and pentraxin 3 (PTX3) have been shown to have higher discriminatory value than the traditional markers of CRP and APACHE II in AP [363]. Another study found quantitative assessment of raised histone levels in plasma (from early death of immune cells) to be accurate in predicting persistent organ failure and mortality in patients with AP [364].

Results from Early Achievable Severity (EASY) index trial are awaited with great anticipation [365]. This is the first multi-centred, international trial that will endeavour to identify factors, predicting severity of AP by way of easily obtainable parameters which are not only quick to gather but are also representative of the disease course. Along the same lines, is the establishment of Acute Pancreatitis Patient Registry to Examine Novel Therapies in Clinical Experience (APPRENTICE) [366]. It is yet another international, multi-centre consortium that aims to better understand the natural history of AP worldwide and to develop a platform for future randomized clinical trials. This electronic prospective registry will be fed from high-volume international centres with expertise in pancreatic diseases and will describe the current trends of demographics, risk factors, clinical profile, treatment patterns, and outcomes of AP around the world. In addition, the establishment of this unique international collaboration will not only serve as a bank for proteomic and genomic data from the included patients it will also allow for it to be used as a platform for future randomized clinical trials that can translate new therapies in less time and with a diversified patient population.

9.2 T Cell Signalling and Role of Adaptive Immunity In Acute Pancreatitis

T cell depletion (CD4+ and CD8+) in the peripheral blood in the setting of SAP is now a celebrated fact. Many studies have substantiated the claim [254, 367-371]. This begs the question – why? What mechanisms are at play here? It would be reasonable to assume, for starters, that SIRS forces lymphocyte influx into the site/s of inflammation, like pancreas, lungs or kidneys, thereby, depleting the peripheral blood of its presence [372]. It has to be borne in mind that peripheral lymphocytes constitute a very small proportion of the total population as most of it is constantly being redistributed between other lymphoid tissues and organs [373], and therefore, a fall in the peripheral count does not necessarily represent impaired immune response [368]. Be that as it may, recent studies have now implicated role of apoptosis in eliminating excessive T cells which have been found to be primed to apoptosis in AP [369, 374]. Indeed, thymic atrophy as a consequence of apoptosis occurs during SAP in rats [375]. Also backing this piece of evidence is TGF β and intracellular calcium mobilisation induced apoptosis of lymphocytes in rat models of pancreatitis [369, 376]. During the course of the disease, CD4+ are depleted, both in MAP and SAP, whereas CD8+ cell population is relatively unscathed [374] but this does not necessarily translate into impaired host immunity as the CD4+:CD8+ ratio remains unchanged. Among the CD4+ cells themselves, Th1 CD4+ cells have been found to be more suppressed than their Th2 counterparts. This has been eloquently described in many studies and confirmed by a much sharper rise in the Th2 cytokine production as opposed to Th1 [369, 374, 377], prompting a Th1/Th2 cytokine imbalance and a resultant depression of host's immune mechanisms. It is important to briefly mention the role of regulatory T cells (Tregs), whose expression seems to increase through varying stages of SAP. These cells regulate immunosuppression through

their anti-inflammatory and immunomodulatory roles and may provide a compensatory response to the systemic inflammation in SAP [378, 379].

Experimental models of pancreatitis have hailed T cell activation to be at the centre of systemic immune-inflammatory response that is hallmark of AP. T cell deficient and CD4+ depleted mice developed a less severe disease than their healthy counterparts [380], turning the spotlight to the role of the adaptive immune response in this cascade. The very same paper also presented a plausible notion that T cells remain immunocompetent and augment immune responses locally and systemically, much like a burn injury, where the insult primes the immune system for an augmented T cell effect that could potentially precipitate a fatal shock like syndrome [381].

9.3 Nuclear Factor of Activated T Cells (NFAT) as the Key Player in Determining Severity in Acute Pancreatitis

Nuclear Factor of Activated T cells or NFAT was first described as an inducible nuclear factor binding to the Antigen Receptor Response Element – 2 (ARRE-2) of IL-2 promoter in human T cells [382, 383]. At the time of inception, it was considered to be exclusively associated with and expressed by T cells. However, further works have made evident its intimate involvement with various immune and non-immune cells and authenticated its pivotal regulatory role in inflammation, embryonic development, organogenesis, cell development and differentiation, cell motility, angiogenesis, tumorigenesis and cell survival [384-387]. The NFAT family consists of five members, four of which are calcium responsive (NFAT1-4) [388-392] and are activated by Ca²⁺ influx in the cell. This is either mediated through the PLC- γ pathway or store operated Ca²⁺ entry, particularly in lymphoid T cells [393]. Once intracellular levels of calcium increases, Calcineurin (a unique calcium/calmodulin-activated serine/threonine protein phosphatase) dephosphorylates the

isoforms, thereby activating them and leading to nuclear translocation [394-396], where active transcription of downstream gene targets subsequently occurs. This in fact, establishes a direct link between calcium signalling and gene expression [387, 397, 398].

Evidence for role of NFAT signalling in inflammation and sepsis is accumulating. Studies have identified it to be critically important in various inflammatory responses [399] where it increases the expression of proinflammatory cytokines including interleukins (IL 2,3,13), TNF α , GM-CSF and MIP [400, 401]. NFAT is now known to be activated during sepsis and regulates expression of macrophage specific inducible nitric oxide synthase which is crucial for bactericidal activity of macrophages [402]. In the further extension of the same line of work [403], authors demonstrated in a murine model of sepsis induced acute lung injury (ALI) that NFAT regulated inflammatory genes produced by macrophages which seemed to be restricted to macrophages only. They stimulated NFAT deficient macrophages with LPS and discovered resultant attenuated expression of several cytokines, chemokines, and their receptors, thus translating to a profound phenotypic effect of NFAT deletion in sepsis induced ALI. NFAT deletion also showed decreased pulmonary oedema, neutrophilic inflammation and improved arterial oxygenation and survival.

In addition, NFAT activation has been hailed as a momentous step in regulating neutrophil recruitment, systemic inflammation and T-cell dysfunction during abdominal sepsis [404]. Role of NFAT members in different mouse disease models such as spontaneous pulmonary hypertension, diabetic retinopathy and regulation of innate immune responses to fungal pathogens is well documented [405-407].

A study by Awla et al in 2012 avowed for the first time the key role of NFAT in AP [408]. They showed AP was associated with increased NFAT transcriptional activity which was not limited to pancreas only and instead, featured in the lung, aorta and spleen too. This indicated

that AP is associated with local and systemic activation of NFAT signalling. NFAT deficient mice did not exhibit activation of trypsinogen, tissue damage and neutrophil infiltration in the lung or pancreas. They demonstrated and concluded that NFAT signalling is involved in the activation of trypsinogen in the pancreas, co-ordination of neutrophilic infiltration, regulation of systemic pulmonary recruitment of neutrophils and transcriptional regulation of CXCL2 in the pancreas, setting its activity at the heart of the initiation of the disease. Another far reaching notion from this study is the role of NFAT signalling in innate immunity, when it has traditionally been exclusively associated with adaptive immune response. The authors proved through experimental models that initiation of AP which was largely driven by innate immune responses was highly dependent on NFAT signalling, implicating it as a powerful component of the innate inflammatory response. Another study also that Toll-like receptor signalling in bone marrow derived macrophages appears to be dependent on NFAT activity [409].

A fascinating proposition was put forward by in 2010 by da Costa Martins et al. – they believed that a strong and perhaps vital interaction existed between NFAT and miRNA, where both affected and regulated function [410]. This was further authenticated by another study that demonstrated miRNA induced suppression of DYRK1A which reduced nuclear export of NFAT and hence activated it. This may translate into a genetic and an epigenetic role for NFAT, affecting various cellular signalling molecules [411]. It still remains to be seen as to how this works in the setting of inflammatory systemic diseases, such as acute pancreatitis, since most of the evidence is in setting of tumorigenesis. Nonetheless, it stands as a viable, potential area of study.

In addition to its seminal functions in immune surveillance, NFAT has secured a reputation for itself as a key player in diverse pathophysiological states, both inflammation and cancer included. The substratal principle underlying the two main pathologies remains the activation of NFAT proteins in the nucleus and binding to the DNA to cause transcription of its downstream targets. NFAT activity has been shown to be crucial for cell survival and proliferation, invasive migration, and angiogenesis. Up till now only few mediators of the NFAT signalling axis have been identified - several others remain to be discovered. But more important than that, we need to have a clearer idea of the processes that drive NFAT activation. Possible mutations and/or amplifications in NFAT binding partners and export/maintenance kinases may be responsible. – these are frequently seen in several disease processes that are associated with constitutive NFAT nuclear localization. It is perfectly reasonable to opine that there is a lot of cross talk between NFAT and other pro-inflammatory signalling pathways, since NFAT is closely linked to inflammation. Role of calcium signalling in this setting, particularly with reference to Calcineurin/NFAT pathway, cannot be over-emphasised. However, it is still a long way before a case for the use of agents inhibiting release of intracellular calcium release to block NFAT mediated cellular activities can be made. Nonetheless, these findings are encouraging for development of better therapeutics with multi-modal mechanism of action in human diseases.

Another important and extremely significant interaction to consider in our dataset is between calcium/calmodulin dependent phosphatase, Calcineurin and NFAT, both of which were found to be significantly downregulated in SAP. Canonical activation of NFAT family members depends on cytosolic rise in calcium levels, contributed either from the endoplasmic reticulum or from extracellular matrix. This activates calcineurin which leads to the nuclear import of NFAT by dephosphorylating residues and causing exposure of a nuclear

localization sequence [412]. After nuclear import, NFAT reaches out to its nuclear binding partners and activates the expression of NFAT dependent inflammatory and immunomodulatory cytokines [413, 414].

What makes role of NFAT so important, yet complex in immunity? They are the ‘star’ molecules that bridge the innate and adaptive immune responses and also modulate inflammatory responses [399, 415]. We know from evidence that the expression of NFAT family members is cell specific and these have differential binding ability to coactivators. This may explain their distinct roles in the immune system [344]. The calcineurin NFAT axis plays an extremely meaningful role in acquired immunity – in fact, it forms the basis of immunosuppressive treatment in transplant recipients [344, 416]. Per contra, its relevance and contribution in innate immunity is still poorly understood, although there has been a study implicating role of NFAT family members in neutrophil mediated innate immunity during fungal infection with *Candida albicans* [417].

NFAT regulation and NF- κ B signalling were found to be among the most differentially expressed canonical pathways in the current study group. Crosstalk between these two has been identified in previous studies in the context of infection [418]. The two families share Rel homology domain structure, recognise similar DNA binding sequences and work hand in hand to regulate inflammatory cytokines and chemokines [419-421]. Indeed, studies have shown direct interaction between NFAT and NF κ B that effectively integrates 2 disparate signalling pathways in promoting cardiac hypertrophy and ventricular remodelling [422]. While specific mechanisms of these interactions are yet to be determined with certainty, it has been thought to be through their synergistic promotion of gene expression or through a non-coding RNA, which serves as a scaffolding platform for the interaction to occur [423, 424]. Moreover, Calcineurin has also been found to improve NF κ B activity through dissociation and degradation of I κ B (inhibitory molecule) [425].

It is now well established that NFAT plays a crucial role not only in activation of T cells, but also in regulation of many aspects of T cell function. Therefore, it is no surprise that it has been considered as a potential target for immune regulatory therapeutics. Calcineurin inhibitors, such as cyclosporine A and FK506, renowned for their use as immunosuppressors to treat graft rejection and autoimmune diseases [426-429], block calcineurin enzymatic activity and therefore, cannot be classified as true NFAT inhibitors [430]. These will block any other target that is dephosphorylated by calcineurin, possibly causing various toxicities associated with their use [431, 432]. The solution to this quandary was identified in the form of VIVIT peptide, a selective inhibitor of calcineurin mediated dephosphorylation of NFAT, which was found to prolong graft survival in experimental model of islet cell transplantation [433]. While far more promising than the earlier alternatives, these peptides still have the potential of blocking calcineurin interactions with other substrates like calcineurin inhibitors (CABIN1/AKAP79) [434]. Answer to this predicament appeared in the form of mutations in the two different regions of calcineurin that were pivotal to calcineurin and NFAT interactions [434, 435]. These newly identified mutations not only selectively impaired calcineurin binding to NFAT but also did not seem to affect any interactions between calcineurin and its other substrates [435]. This, unquestionably, constitutes a reasonably attractive target for design of even more precise inhibitors. As fascinating as the use of these peptide inhibitors appears, there are some plausible issues with their delivery, which could, in principle, be overcome by making use of smaller organic molecules by incorporating structural changes that consequently improves specificity, stability, delivery and distribution. Roehrl et al. [436] came up with a list of compounds in 2004, using Fluorescence Polarization Assay, which specifically inhibited calcineurin and NFAT interactions, culminating in impairment of NFAT dependent cytokine production by T cells. This was closely followed by Venkatesh et al [437] who used a slightly different cell based method to

identify inhibitors of nuclear translocation of NFAT which acted by blocking store-operated calcium channels and hindering calcium mobilisation. These two approaches have provided immensely useful molecules that act upstream of calcineurin, aptly block NFAT dependent transcription and also consolidate cyclosporine effects [437]. These avenues can also be exploited to identify small molecules that will be selective and specific enough to modulate specific NFAT regulated functions. Also to ponder over is the matter of interactions between NFAT and its specific transcriptional partners which may be at the heart of complex signalling pathways that NFAT integrates into, to control various and numerous cellular programmes. The key would be to identify the protein-protein contact surfaces that are specific for these interactions. Molecules could then be designed and modified accordingly to regulate specific functions. These therapeutic molecules could revolutionise the treatment, improving the toxicity profile.

9.4 MicroRNA and SnoRNA as potential Biomarkers in Acute Pancreatitis

MiRNA have emerged as fantastic, novel biomarkers with many advantages to their credentials. Unlike mRNA, they are incredibly stable in circulation (blood, urine and tears) as they are protected from RNases and endure long-term storage well. A single miRNA regulates expression of multiple mRNA and therefore, offers complexity to the process and has rightly been implicated in the pathogenesis of many a disease processes. Recent studies have corroborated that miRNAs are not just the by-products of cell lysis. They possibly enter circulation by being packaged into endosomes or simply avoid degradation by associating with argonaute or nucleophosmin [299, 301, 302]. Another neoteric role for miRNA is as signalling molecules, for they affect functioning of distant cells once out in the circulation, perhaps by influencing the gene expression of the target cells [299]. Their ability to control

gene expression without affecting DNA sequence makes them one of the most powerful epigenetic regulators.

Most of the published work on miRNA biomarkers to date has been for cancer diagnosis.

Many serum/plasma studies have profiled the most abundant miRNAs in the tissue of interest and then assayed these in the circulation on the assumption that miRNAs that are high in the tissue will be secreted and readily detectable in blood. This is truly applicable in case of acute pancreatitis. It will not be clinically and technically feasible to sample pancreatic tissue in patients who are acutely unwell. If a peripheral blood sample, which is much easier to obtain, can provide the same information, such as miRNA profiling reflective of the pancreatic tissue, this could revolutionise the prognostication in this disease state.

To the best of our knowledge, this is the first study that has demonstrated the involvement of snoRNA in acute pancreatitis. The specific role of snoRNA in many disease processes remains a bit of an enigma. While their functions largely remain unknown, there is emerging evidence of their involvement in malignant, neurodegenerative and viral diseases. In fact, they are slowly being recognised as fantastic biomarkers – a new study from China has implicated snoRNA in pathogenesis of colorectal cancer (CRC) and progression from ulcerative colitis to adenocarcinoma, linking it to the ‘inflammation to malignancy’ pathway [438]. They showed that snoRA15 and 41 were upregulated in CRC and as such, behaved as ‘oncogenes’, due to their location at the genomic amplification regions that are characteristically associated with oncogenic function [439]. It was also noted that snoRD33 is downregulated in CRC and may well be a ‘tumour suppressor’, as it suppresses cancer cell growth and metastasis to distant organs by blocking antioxidant pathways [440]. Following the pattern, they identified the same snoRNA to be upregulated in UC patient samples as well. However, snoRD33 expression was found to be highest in CRC, lowest in controls and in between in UC, creating a place for this particular snoRNA as a mediator to promote colon

inflammation. This study very sensibly provides a link between inflammation and carcinogenesis. Expression of these snoRNAs was also found to be associated with disease severity, rendering them particularly useful as biomarkers for CRC prognosis and also for differentiating UC from CRC. This is extremely significant in terms of disease management. snoRNA are very stable in plasma and serum and lend themselves to be excellent biomarkers that can be easily employed in the clinical setting [441]. In fact, use of a snoRNA based biomarker panel in setting of non-small cell lung carcinoma has been very encouraging with 81.1% sensitivity and 95.8% specificity [442].

There is no direct evidence of involvement of snoRNAs in inflammation or sepsis and perhaps, this represents an area of unmet need. They categorically have proved themselves to be incredible biomarkers and their role in the setting of sepsis and acute pancreatitis, where there is a critical need for specific and sensitive biomarkers, is undoubtedly an area for future research. It is hoped that the next step would be quantitative assessment and then validation in an independent cohort.

This study is not without its fair share of limitations, which must be acknowledged and taken into account while interpreting the results. Sample size, while representative of the population, is smaller than anticipated. This was partly due to the fact that the process of recruitment was prospective and severe patients were difficult to recruit as opposed to mild acute pancreatitis. Sometimes, these patients with SAP were too unwell to consent to participate in the study and therefore, couldn't be included. Also, these patients were not as frequent to present as the mild acute pancreatitis, following the natural distribution of the disease severity. Being the tertiary referral centre for SAP, many patients were transferred from other hospitals later than 24 hours of presentation and hence were not eligible for inclusion.

While transcriptomic analysis is a very powerful tool for investigating molecular changes in different biological states, caution has to be exercised in interpreting the data. RNA profiling methods measure the concentration of RNA at a specific state and therefore, it is a plausible speculation that dynamically modulated processes and transcripts could be missed.

Additionally, it does not inform of the regulatory changes associated with translation or of the effects of modulation by other proteins. Sample heterogeneity and preparation can introduce some serious bias in the results. While all care was taken to exclude batch effects, it is impossible to exclude it completely. Finally, both the RNA and miRNA profiling methods can only observe the average effect of multiple cell types and numbers of cells in different states in the sample which can add uncertainty to the data.

Further works would almost certainly involve characterising the mRNA and miRNA better and understanding and identifying their targets. The list generated from this study of potential candidate genes and miRNA is not exhaustive but most definitively a step in the right direction. Next step should be quantification of these in AP patients and assess their value as prognostic markers.

It is anticipated that the next five years will see a rapid expansion in the use of molecular diagnostic tests. This is likely to be particularly true for the introduction of mRNA and miRNA-based tests. Several companies are developing tests that utilize these for determining the diagnosis or prognosis of a variety of diseases. However, the increasing use of high content platforms, such as microarrays, is leading to identification of marker panels, therefore driving us towards the adoption of multiplex detection platforms and multiple marker tests. Given the fact that acute pancreatitis is a complex disease with multiple pathways involving intricately linked innate and acquired immune mechanisms, a panel of biomarkers is likely to be more suited than a single agent. This requires validation studies and testing in clinical trials settings.

CONCLUDING REMARKS

This observational, pilot study has successfully demonstrated that mild and severe acute pancreatitis are two distinct groups and exhibit different courses of disease, evident at the molecular and genomic levels. Microarray based experiments have clearly delineated the two groups and have also provided useful insights into the pathophysiology, highlighting the key pathways and molecules involved. This is of immense value, hinting at potential lines of enquiry for ascertaining prognostication and treatment in AP.

NFAT emerged as the most significantly differentially expressed molecule, playing a pivotal role in many dynamic processes found to be associated with the disease. The relevance of the calcium-regulated NFAT transcription factors to the regulation of many aspects of T-cell function is well stated in the more recent literature. In fact, during T-cell activation, most cytokine genes are regulated by NFAT proteins. As in line with the recent studies, our study has also identified role of NFAT in targeting many other genes that influence the activated T cells. The answer perhaps lies, in part, with the molecular structure of NFAT DNA-binding domain – this has various surfaces, allowing for interactions with different transcriptional factors that result in integration of NFAT into many signalling pathways.

Future studies should focus on better characterisation of regulatory mechanisms surrounding NFAT activation, in the hope that newly identified transcriptional factors and target genes will give a better overview of the involvement of NFAT in various processes, not only including T cells but also other cells of the immune systems and spot the mechanisms that regulate them too.

Another area of future endeavours, which already has attracted a lot of interest, is that of NFAT inhibitors and their development. While these have been tried in various trials, results have been far from encouraging. Derivatives of cyclosporine, although having been evaluated in phase III clinical trials, have not resulted in any commercial products, likely due to their

potential toxicity. Natural compounds possessing NFAT inhibition activities are unfortunately less potent. Peptide inhibitors of NFAT appear more lucrative as these are less toxic and have appeared to hold good potential as drug candidates in recent experimental models.

Micro RNA hold a great deal of promise as biomarkers. The last ten years have seen a rapid rise in recognition of their ability to serve as potential biomarkers in diagnostic, prognostic and predictive arenas. This study has identified miRNA that are differentially expressed between MAP and SAP and their use and development in the field of severity stratification is within realm of possibility.

In short, Genomics has presented us with, in the recent times, an extremely useful and powerful tool to investigate how molecular networks change between biological states. There are however, limitations to their application. It is possible to miss dynamically modulated processes and transcripts with most RNA experiments that measure the concentrations of RNA (for both mRNA and miRNA) at a specific state. Also, one can only contemplate the average effect of various cell types and numbers of cells in different states in the sample. It is also almost impossible to detect regulatory changes with the processes of translation. With regards to miRNA, their levels may not always correspond to their regulatory activities which in turn could have been modulated by other factors/molecules.

One of the most confounding factors in RNA based experiments is the sample preparation and ensuring uniformity in sample handling and taking into account the heterogeneity of the disease within the sample, different cell types in the sample and genetic and environmental factors on individuals.

At any rate, Genomic and transcriptomic analyses, as these stand, are the most powerful tool for interrogating molecular networks associated with disease and disease forms and are likely

to revolutionise the biomarker discovery, with particular emphasis on developing molecular signatures leading to molecular tests, in the coming times.

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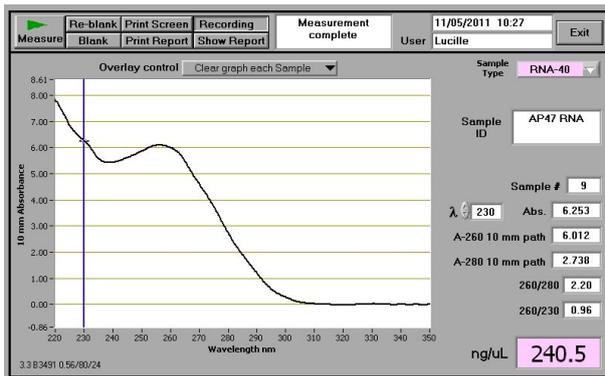
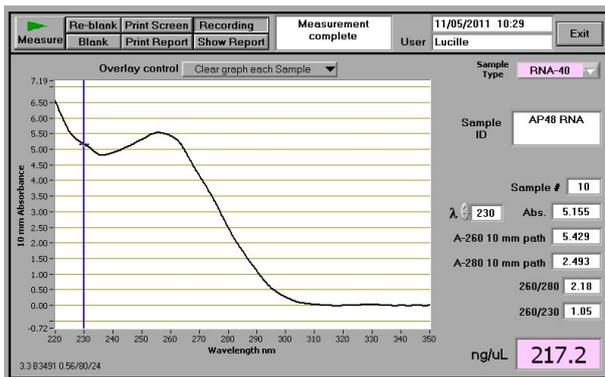
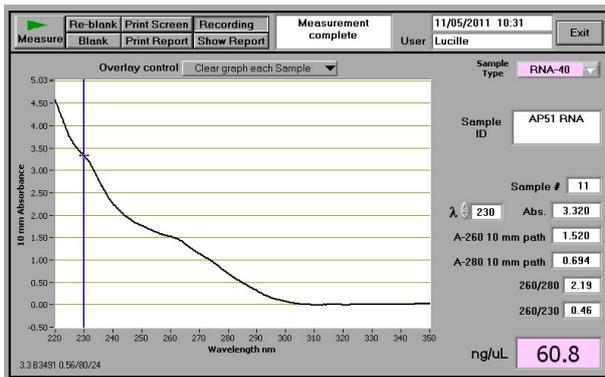
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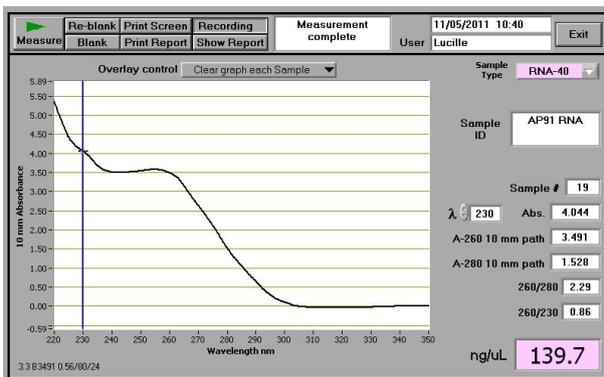
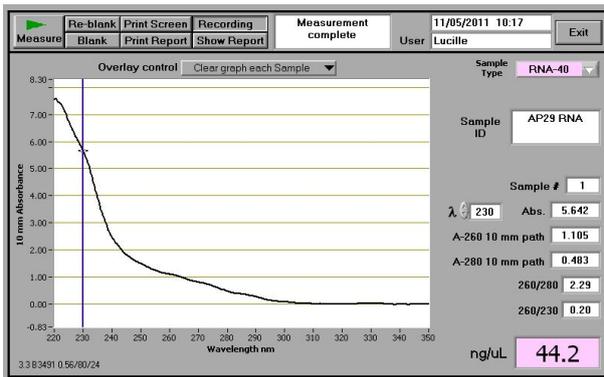
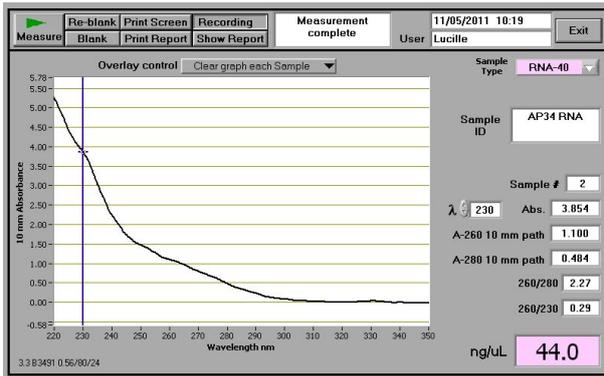
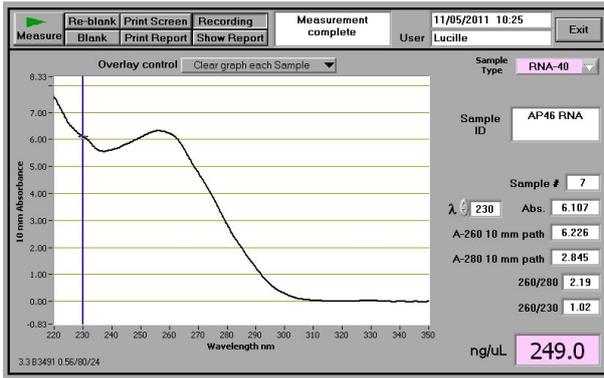
APPENDICES

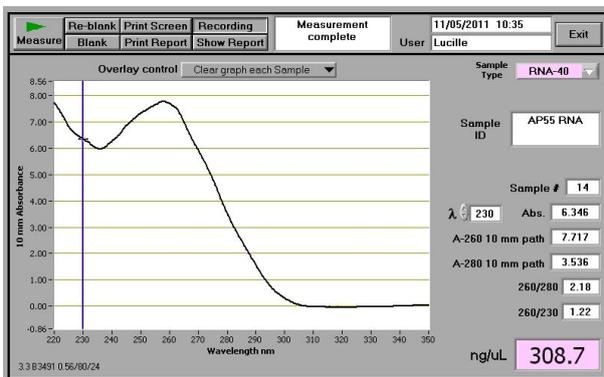
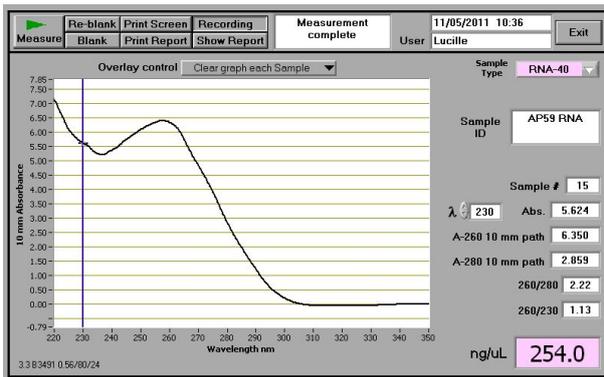
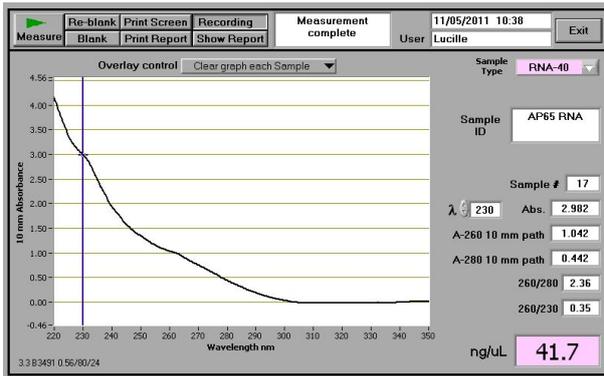
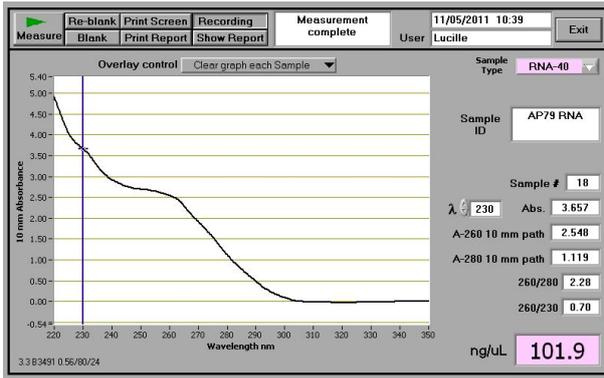
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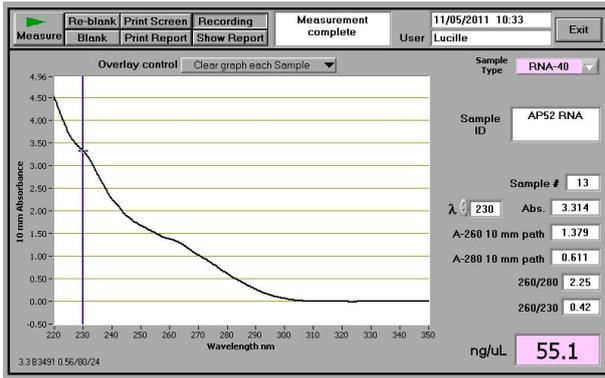
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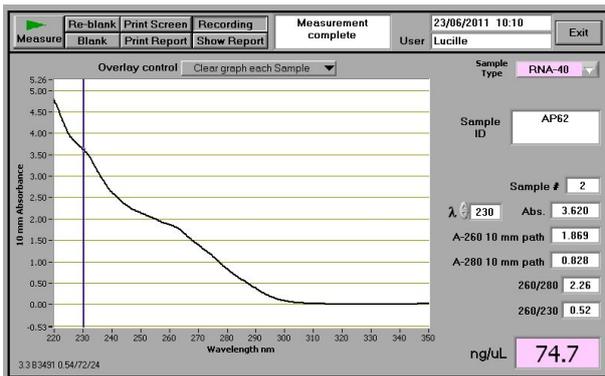
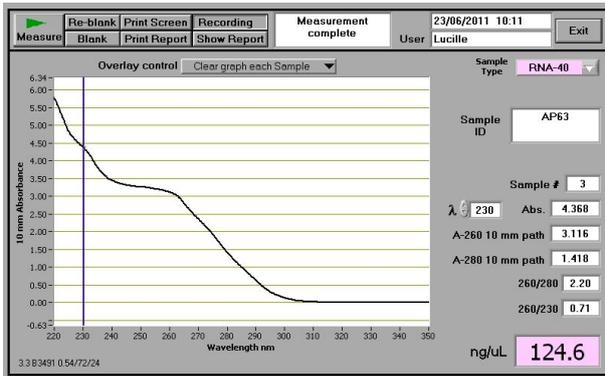


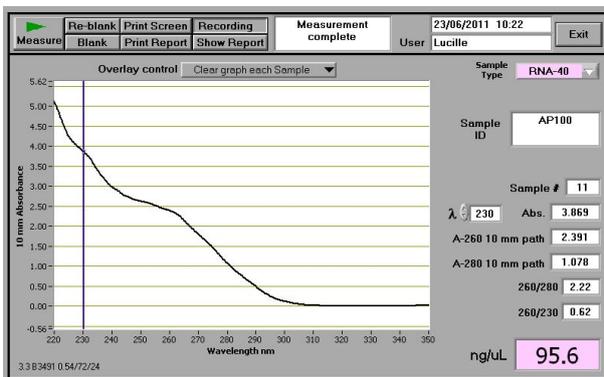
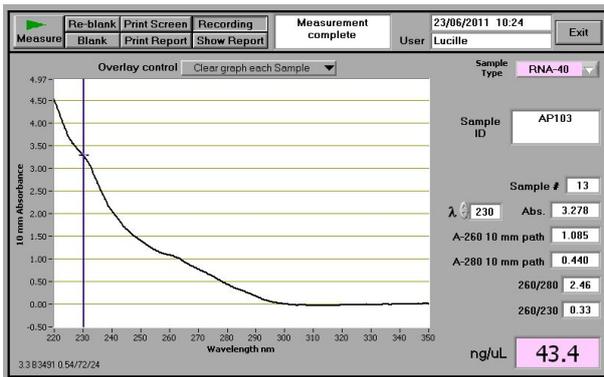
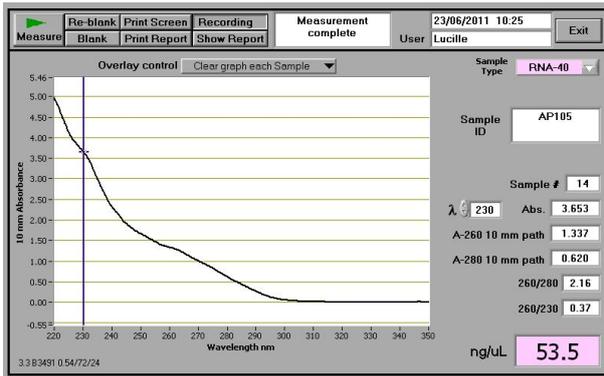
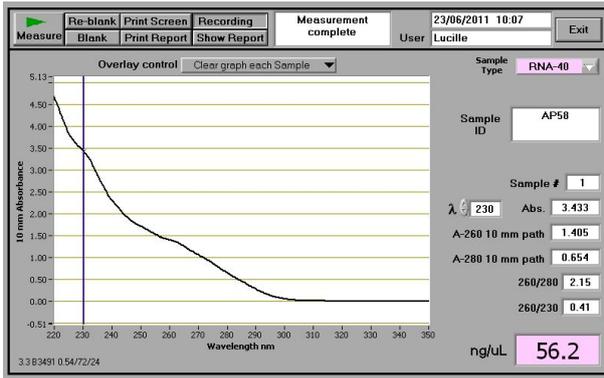


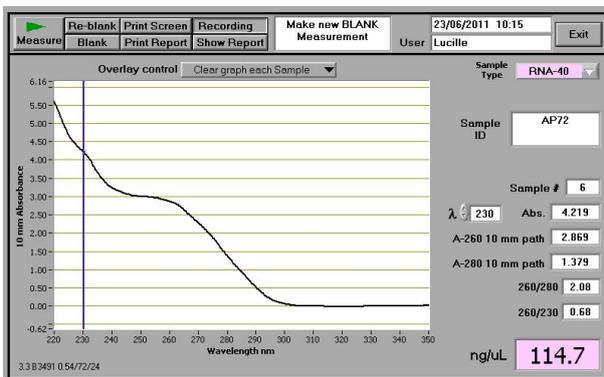
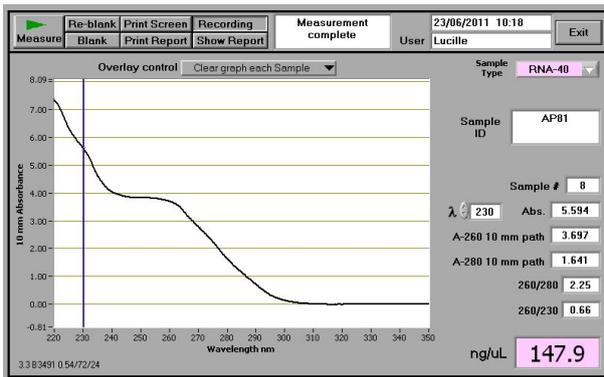
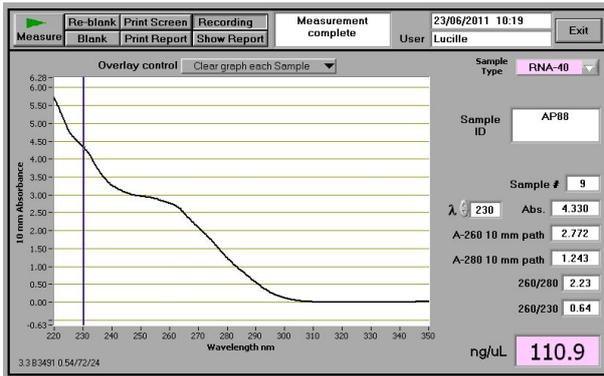
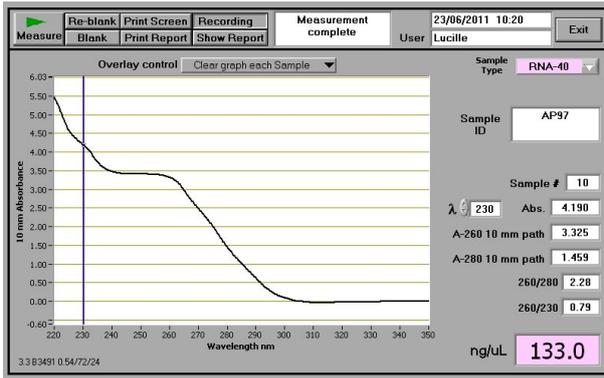


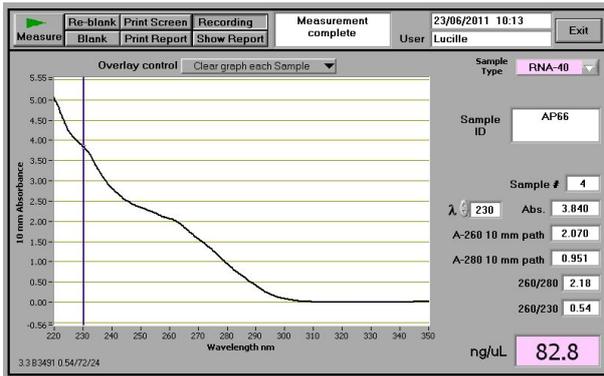
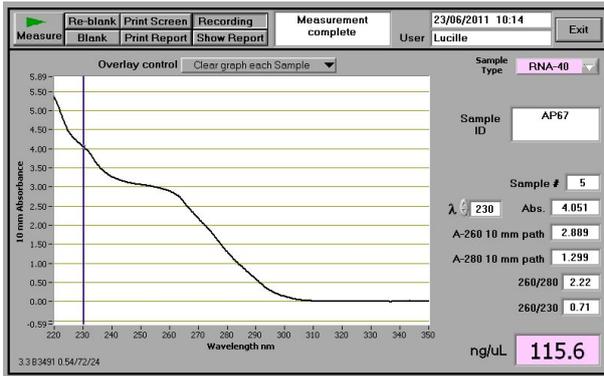


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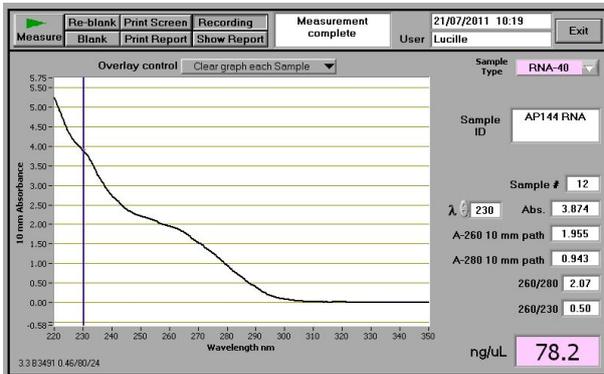


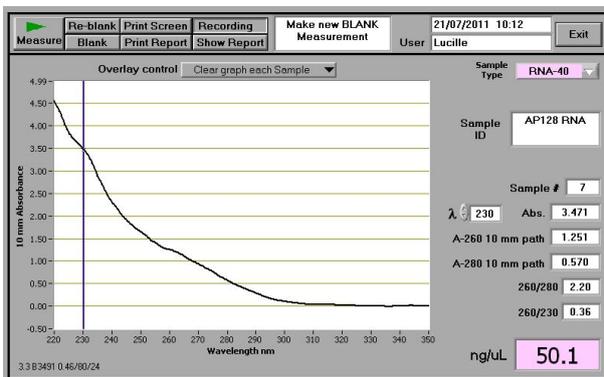
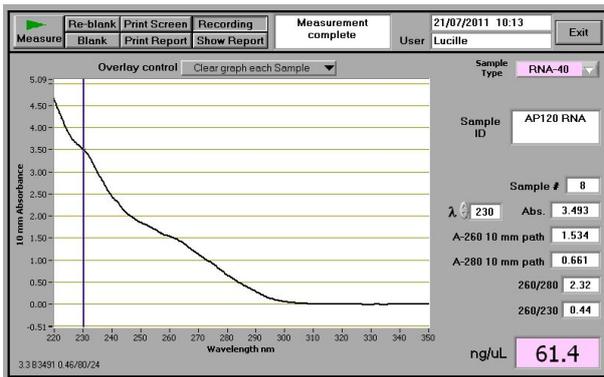
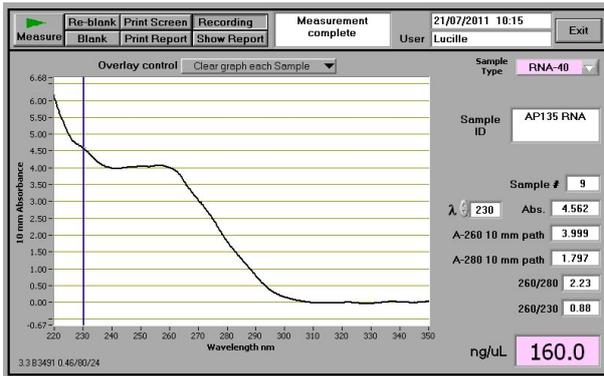
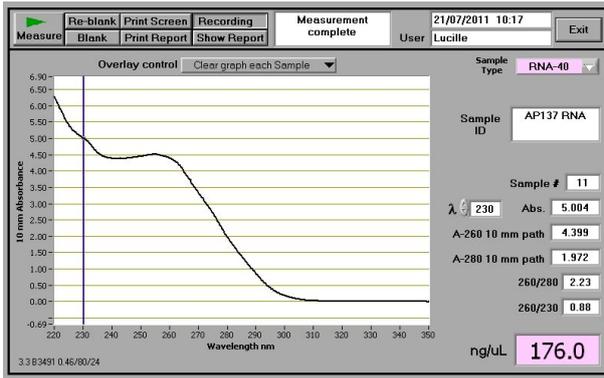


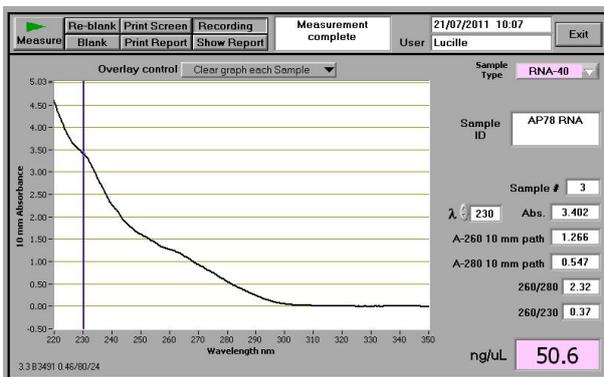
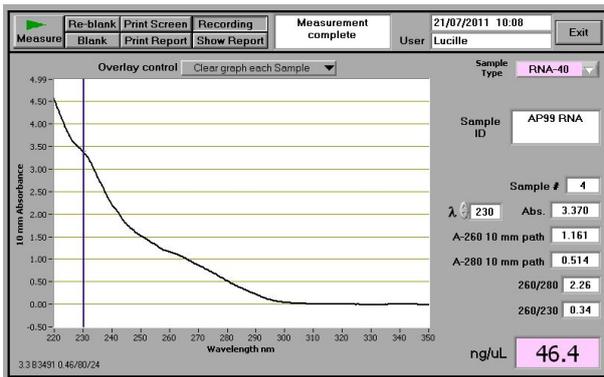
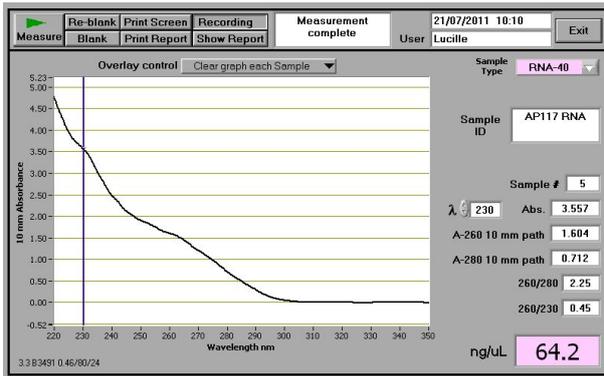
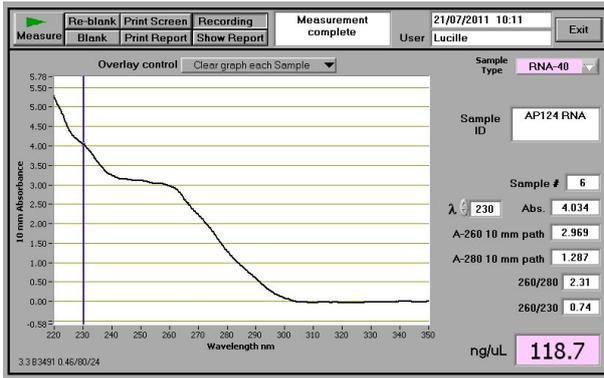


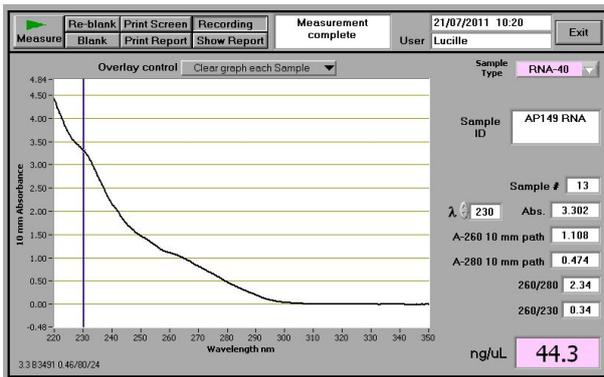
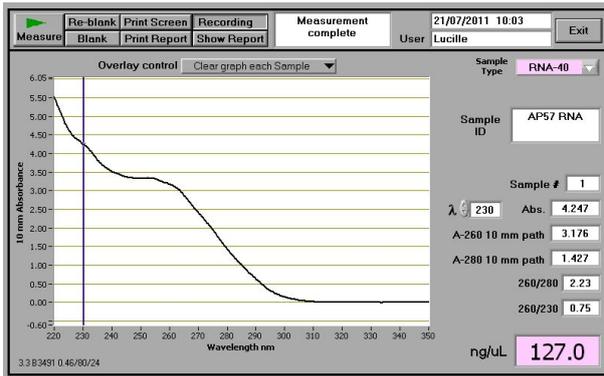
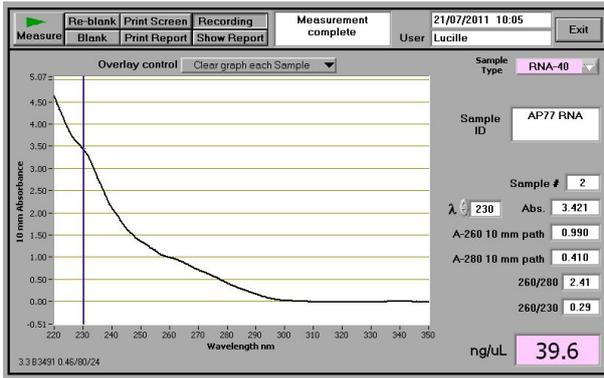


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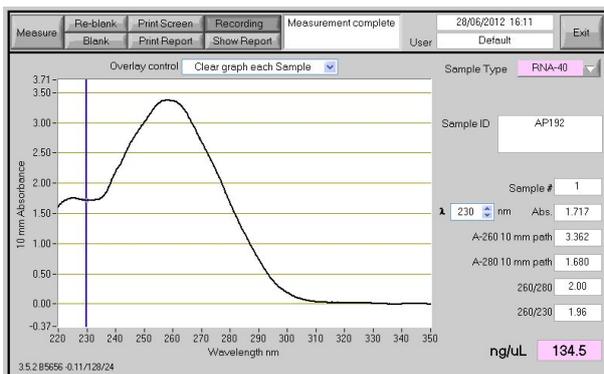


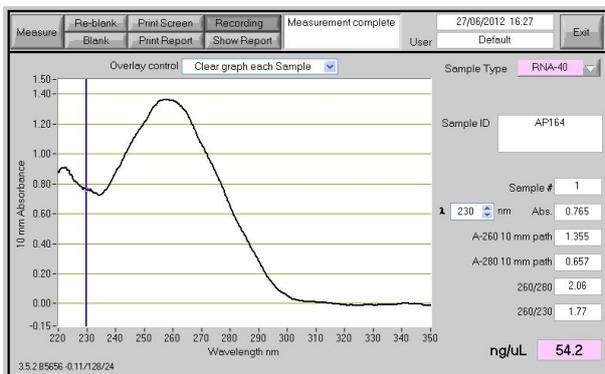
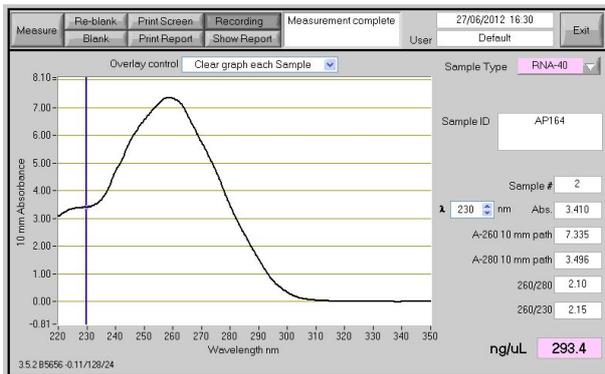
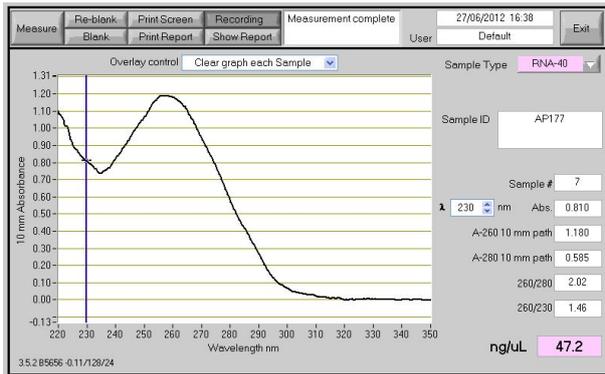
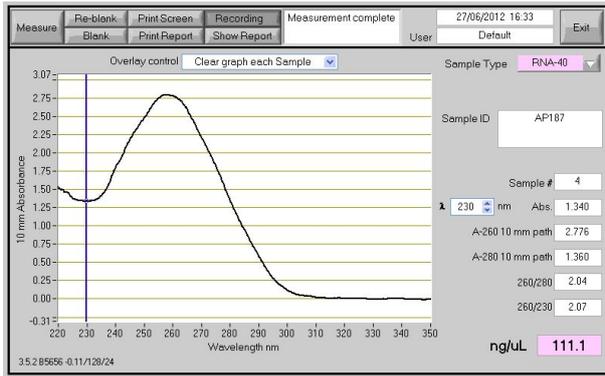


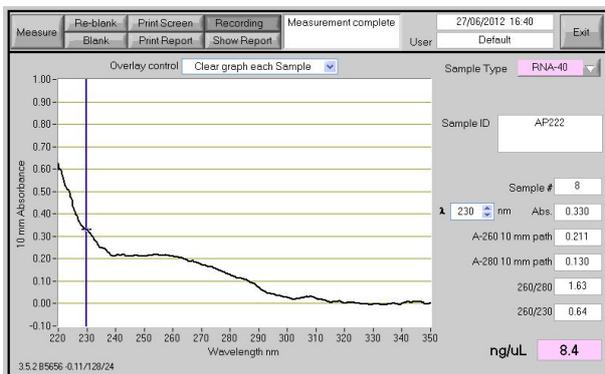
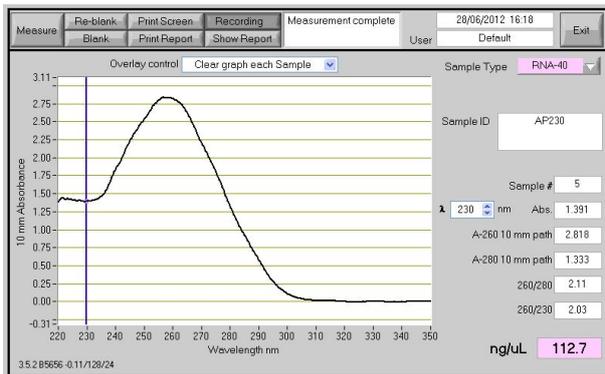
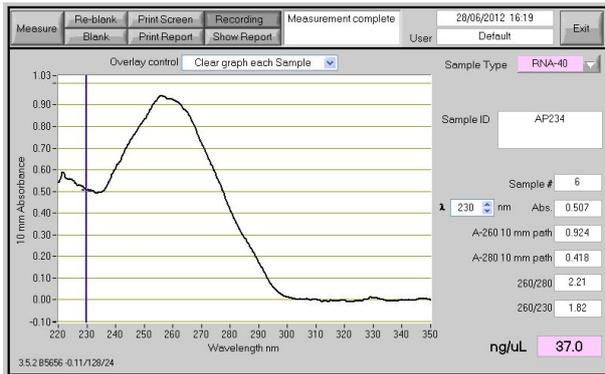
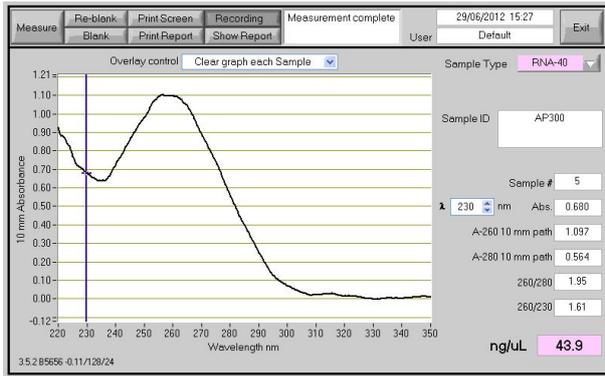


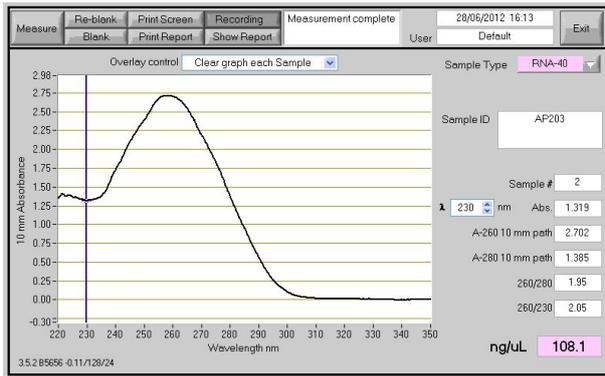
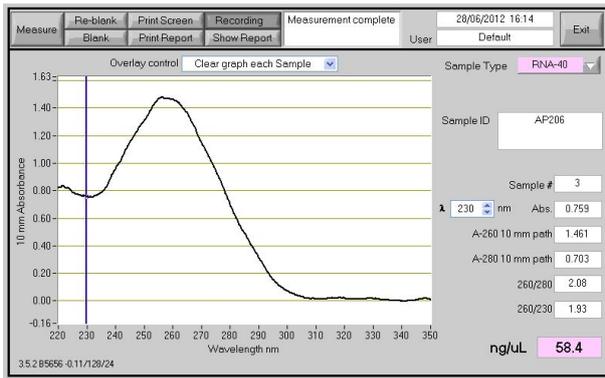


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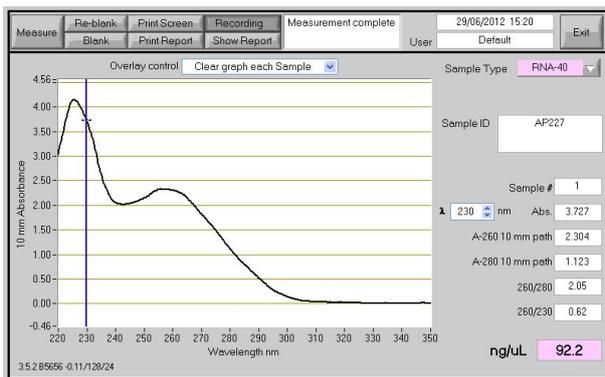


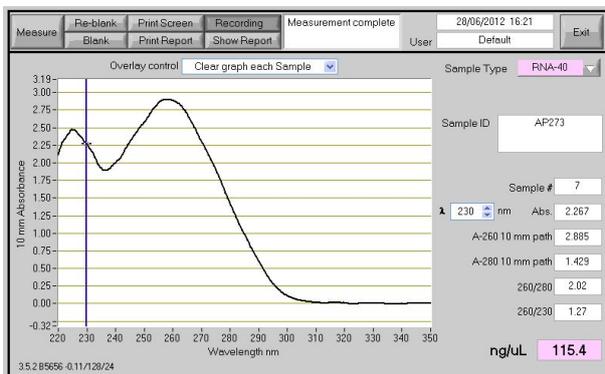
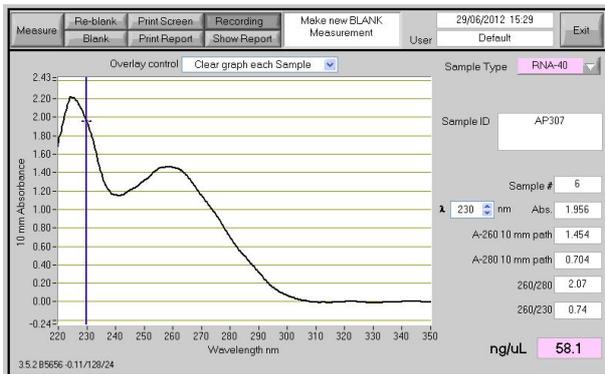
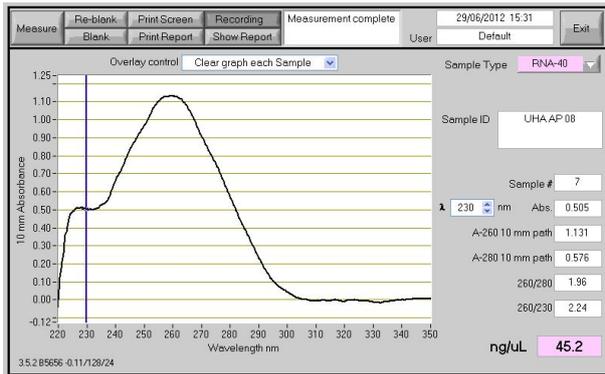
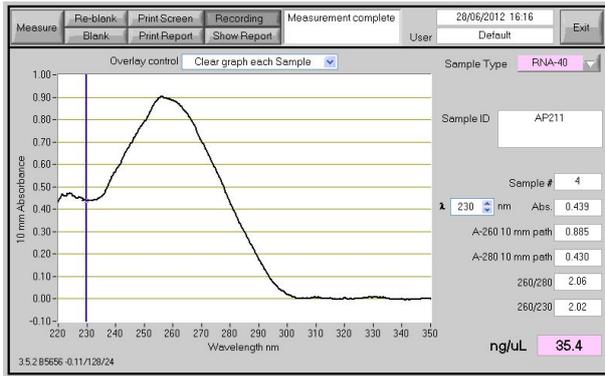


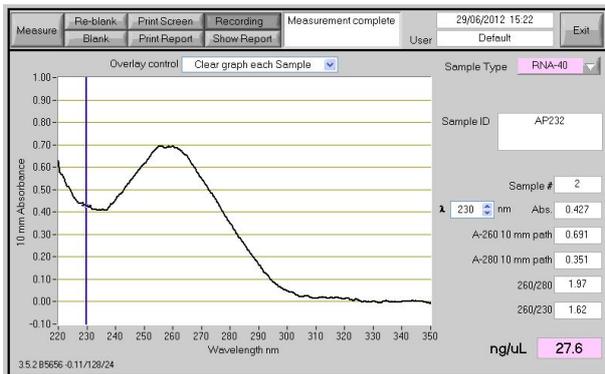
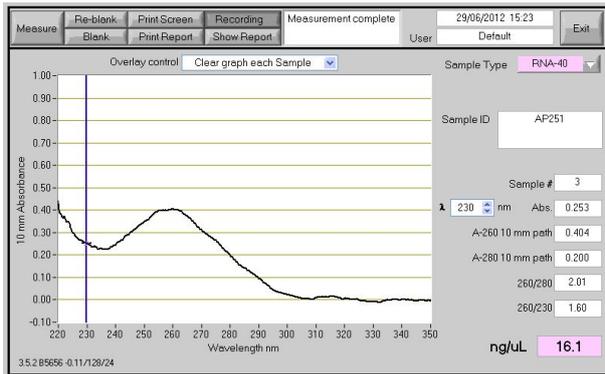
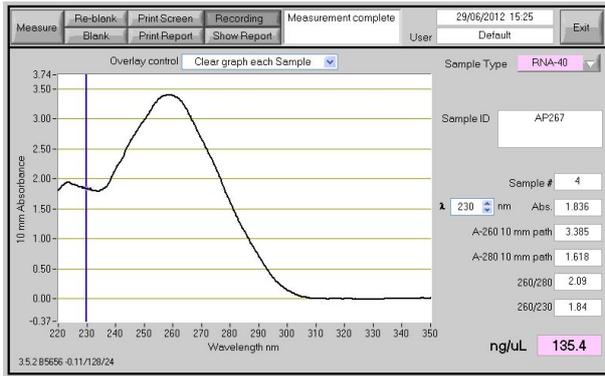




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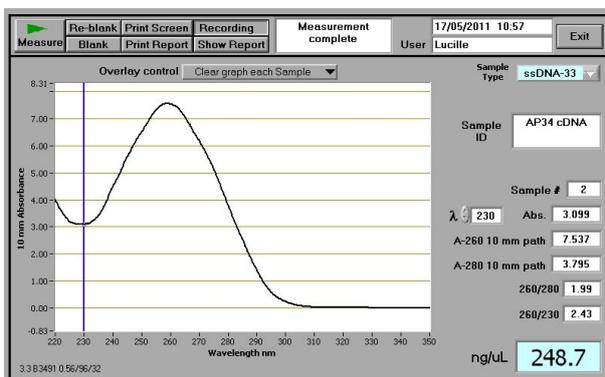
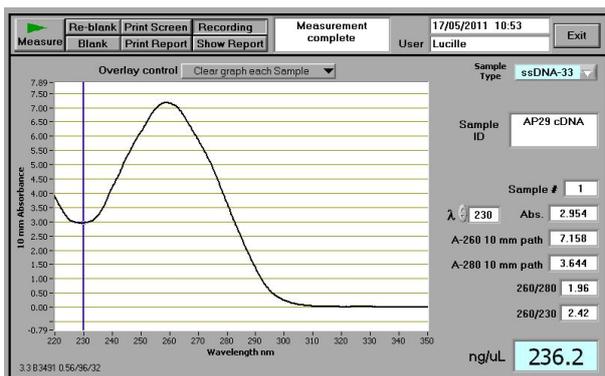
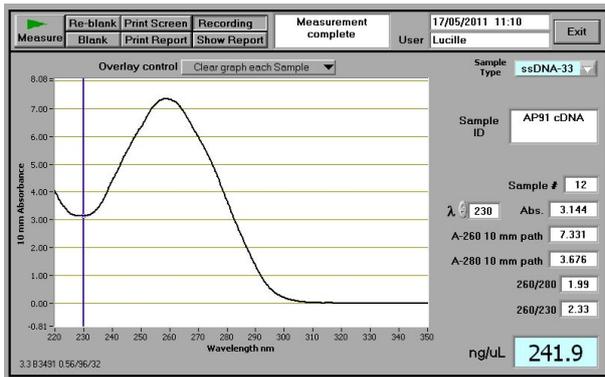


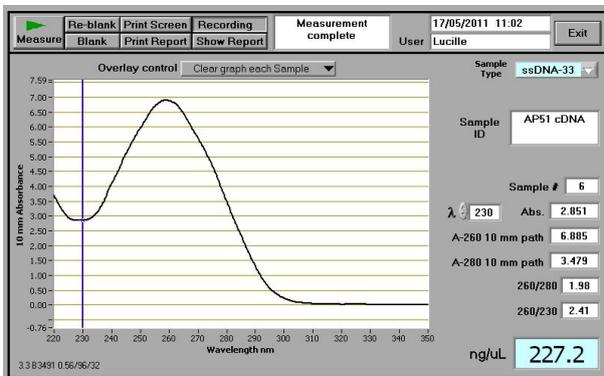
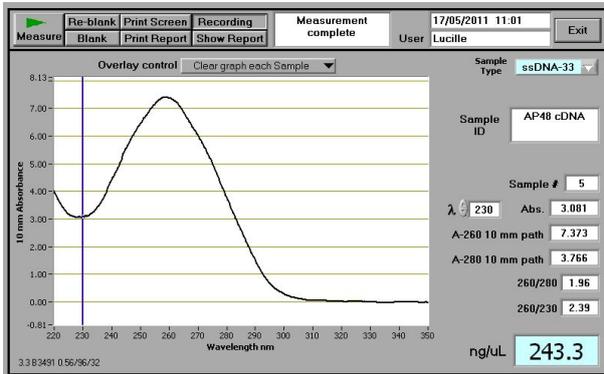
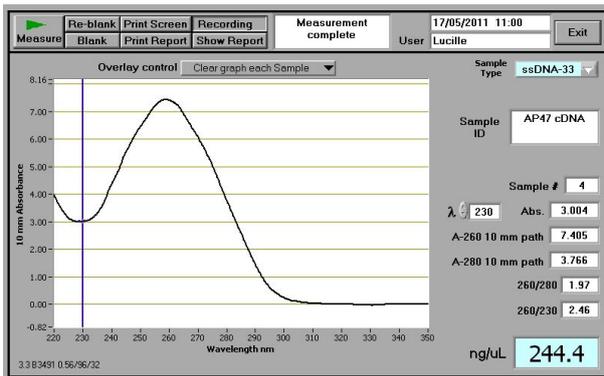
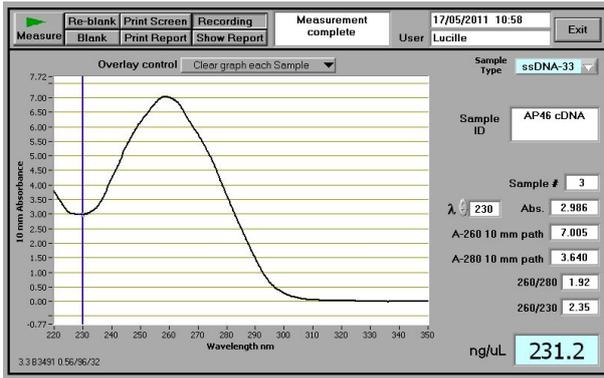


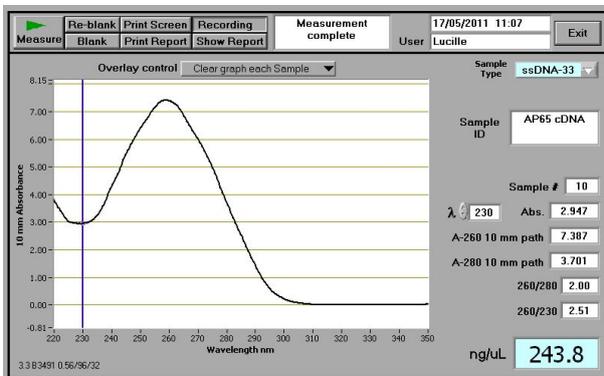
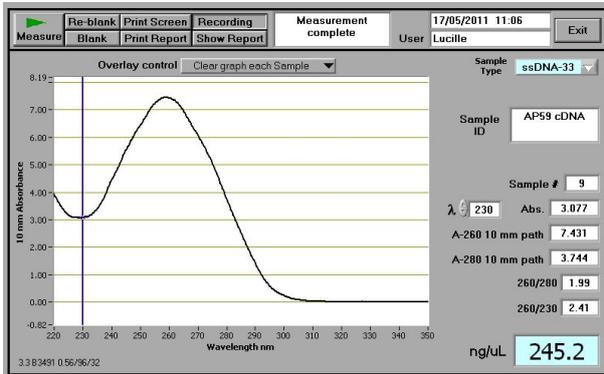
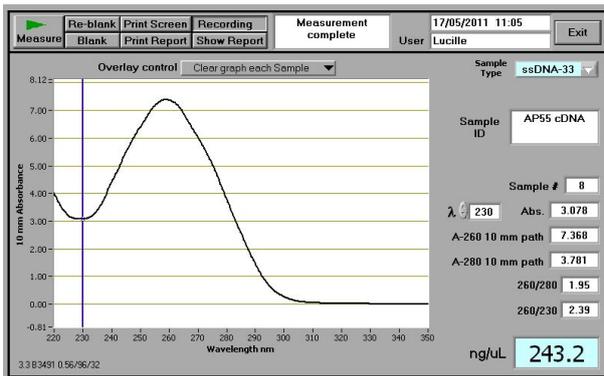
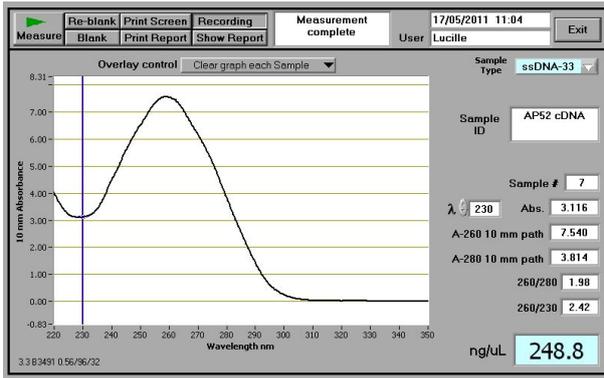


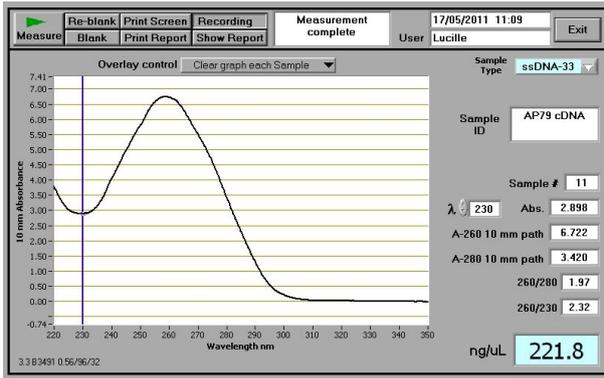
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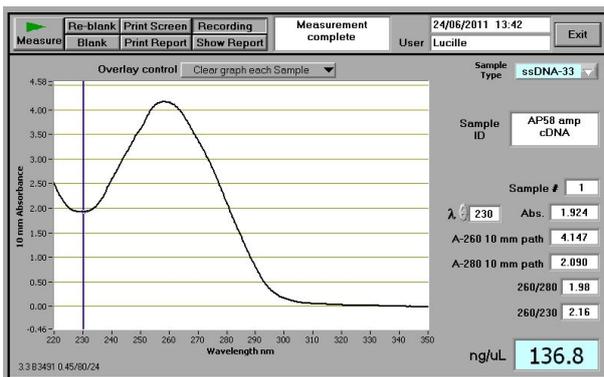
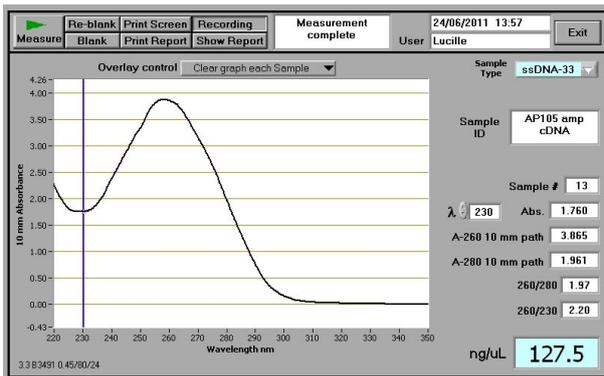


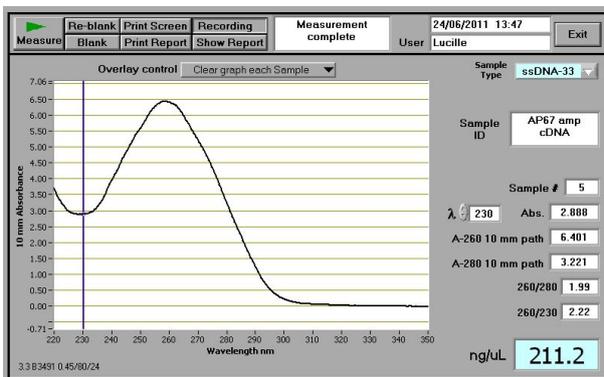
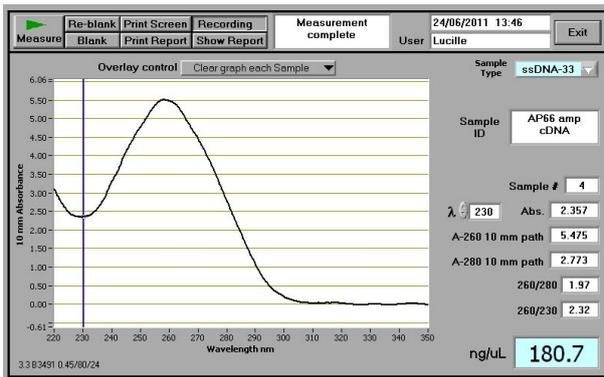
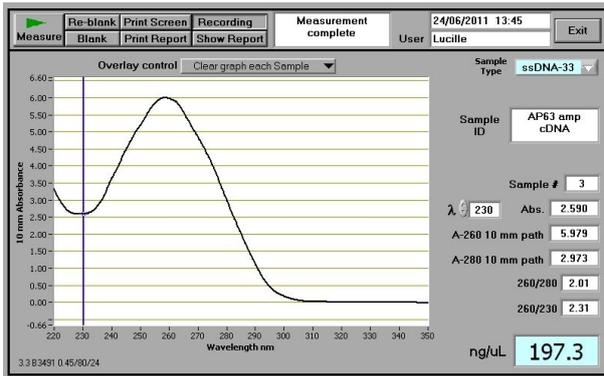
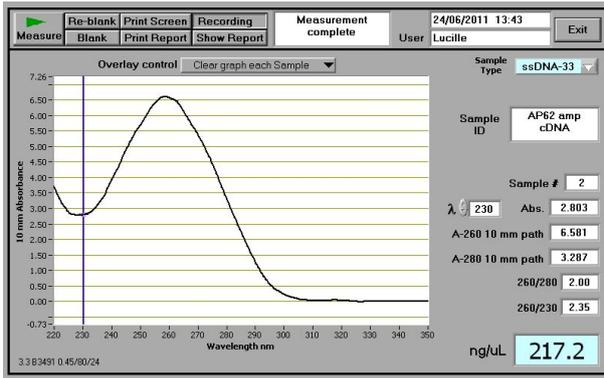


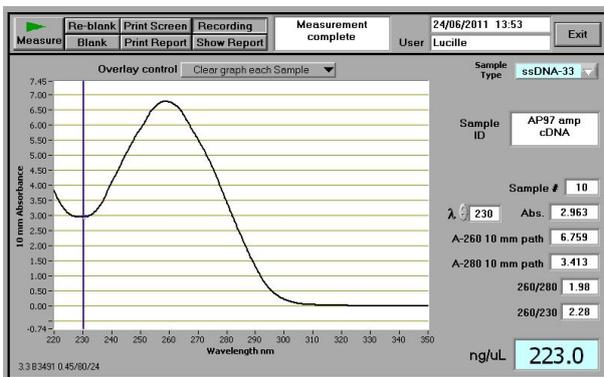
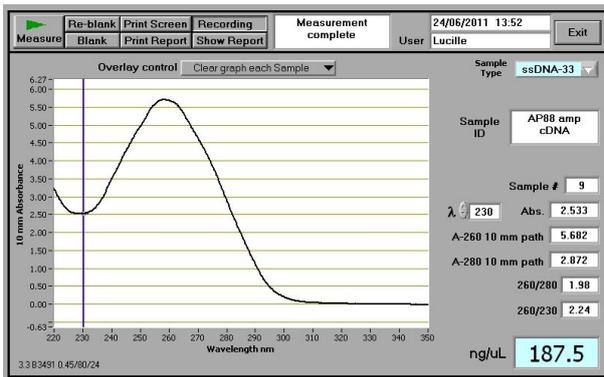
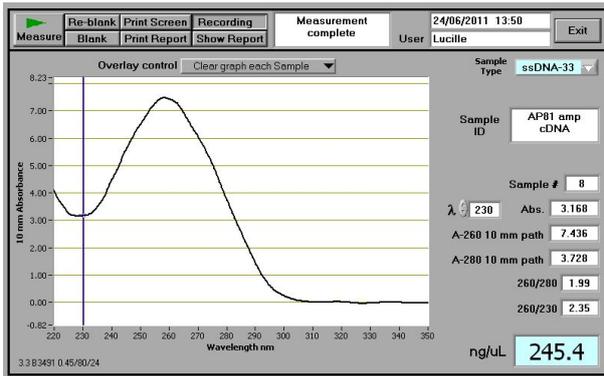
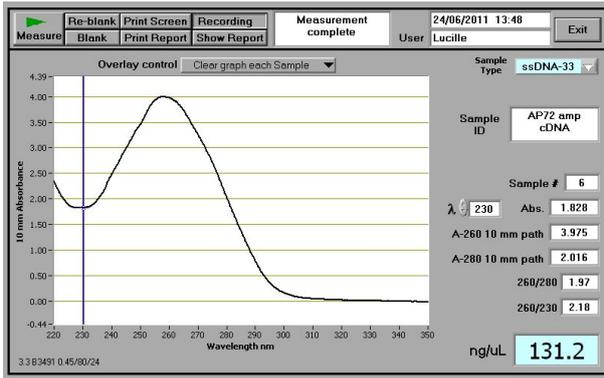


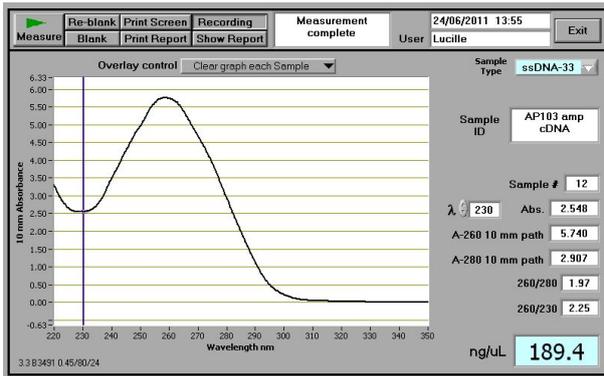
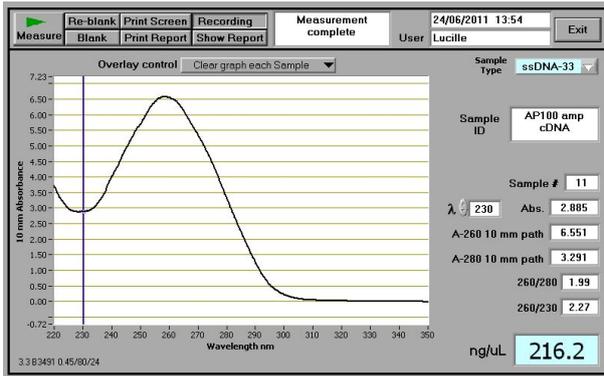


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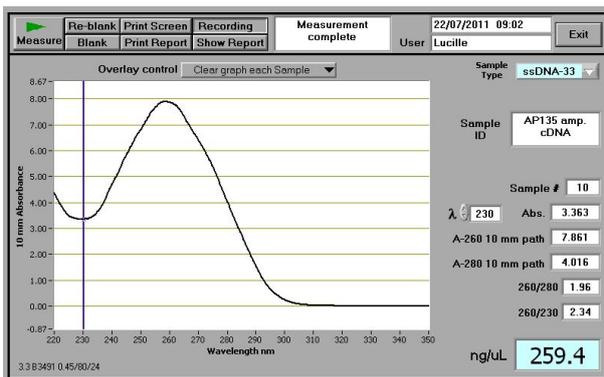


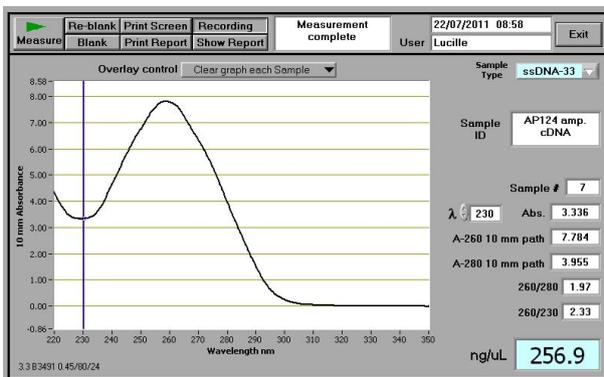
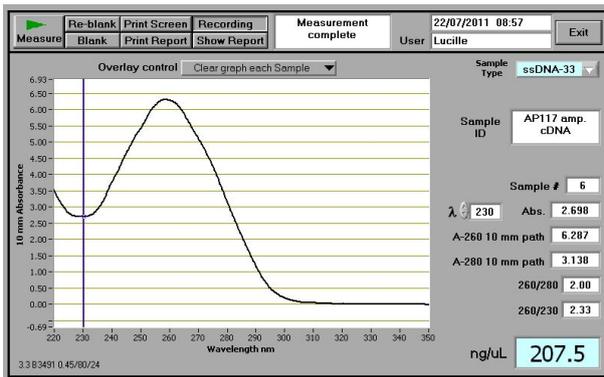
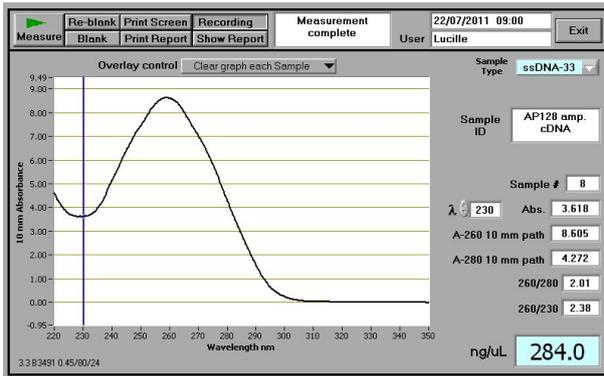
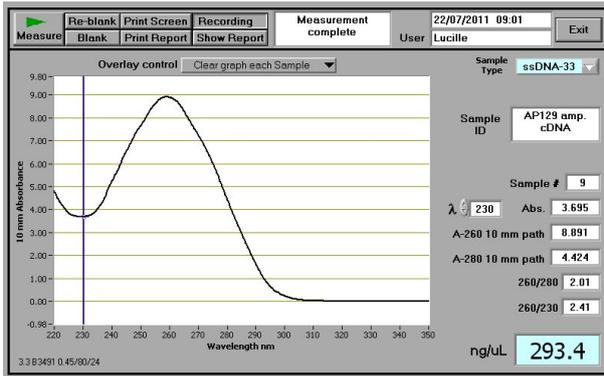


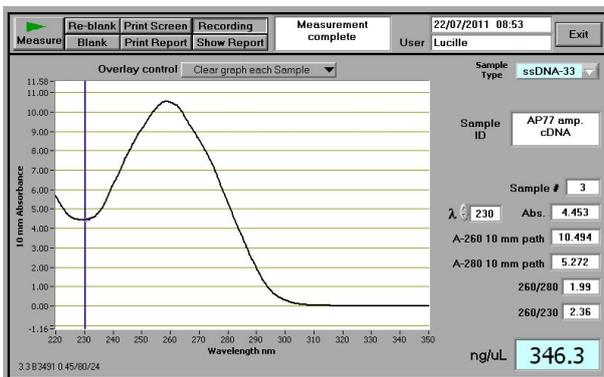
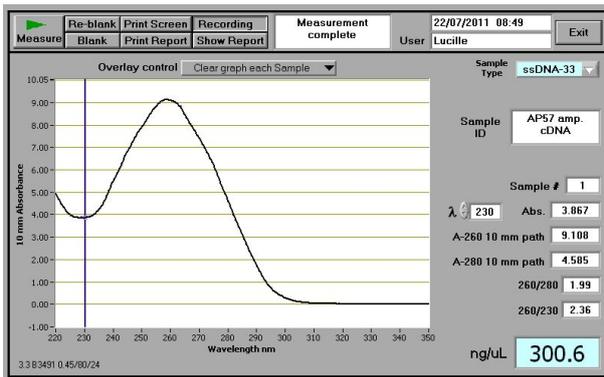
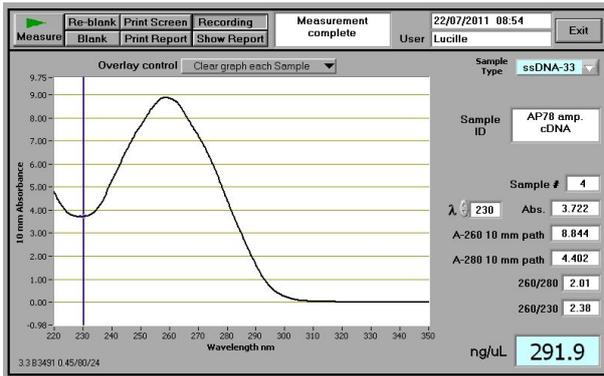
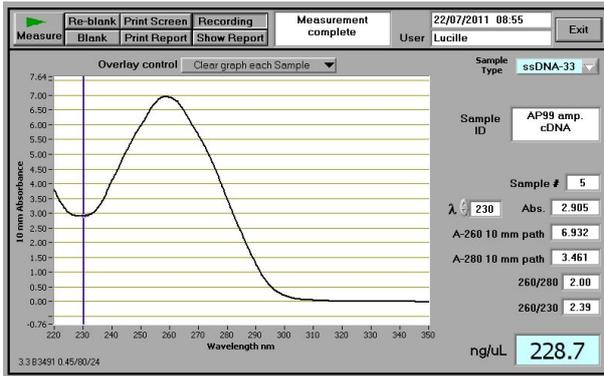


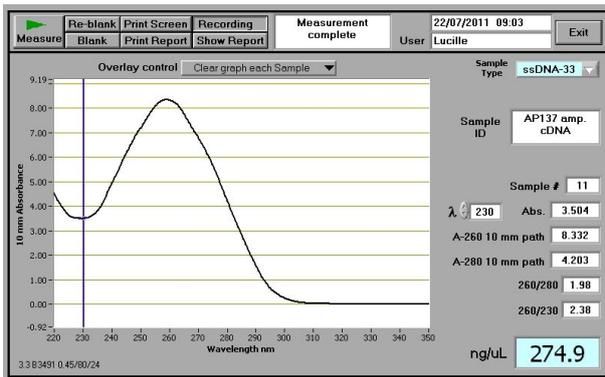
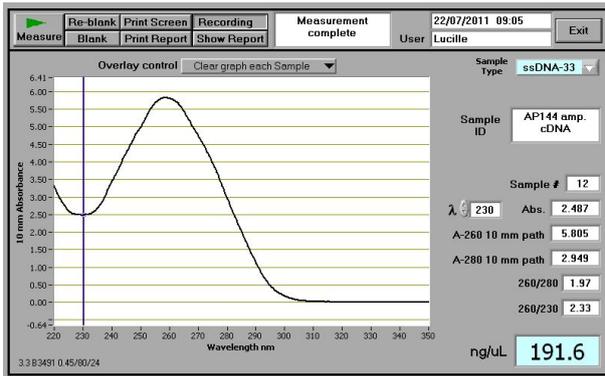
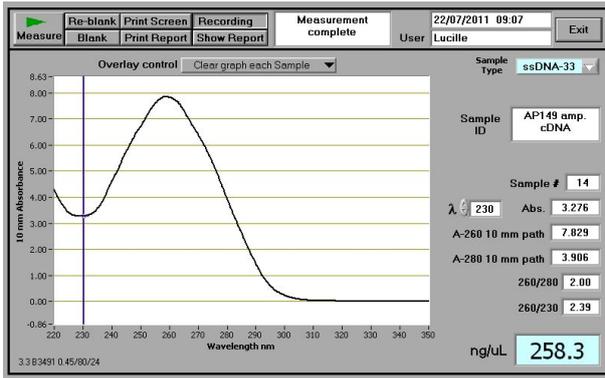


Batch 3

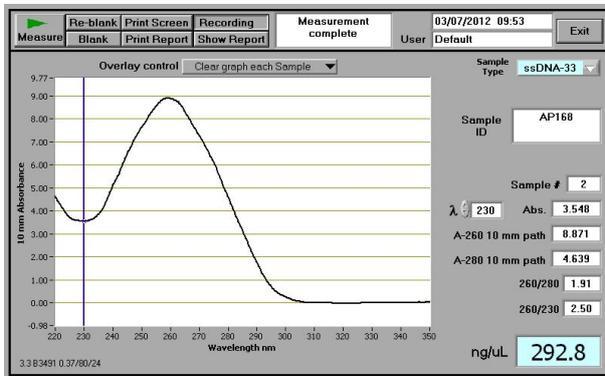
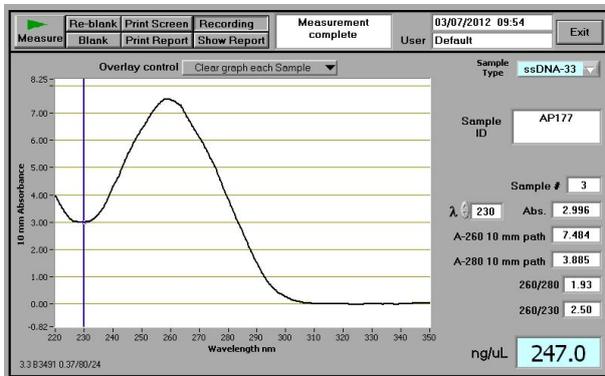
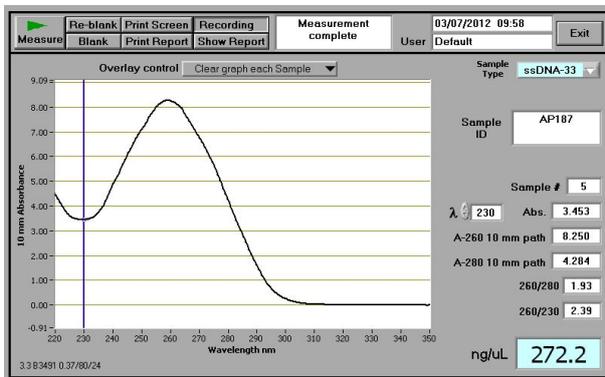


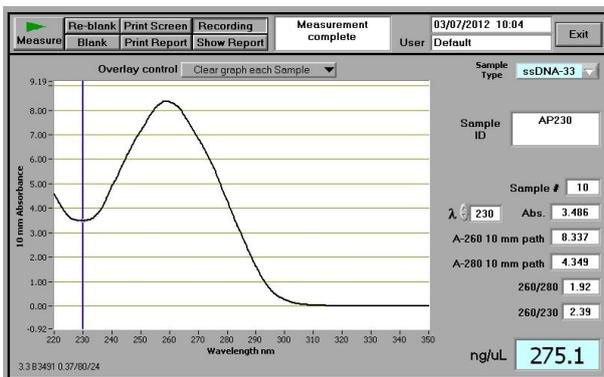
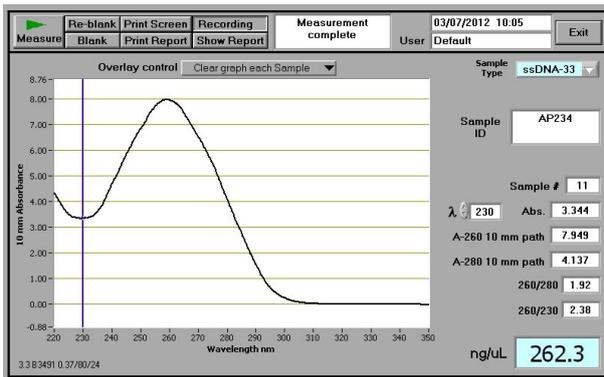
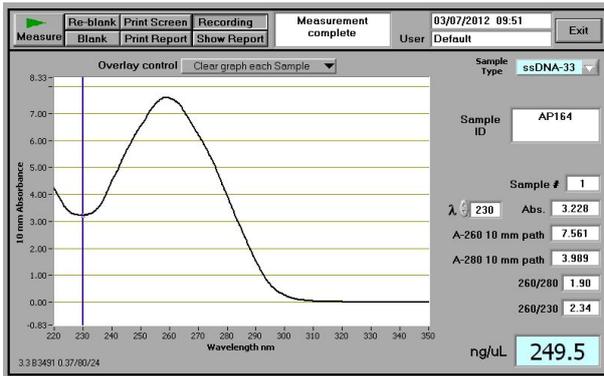
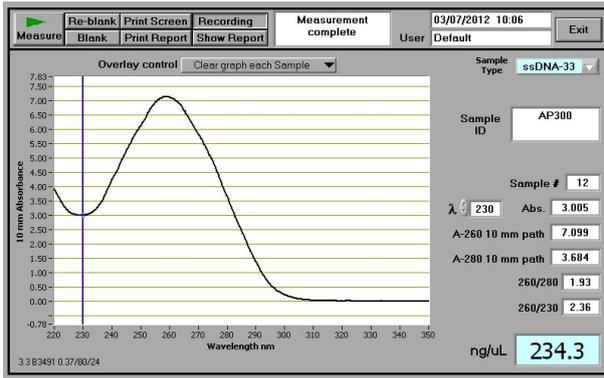


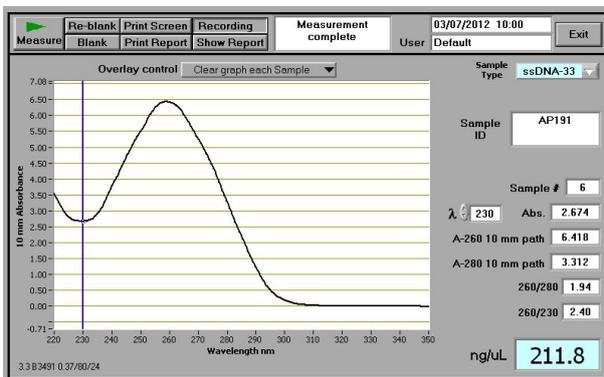
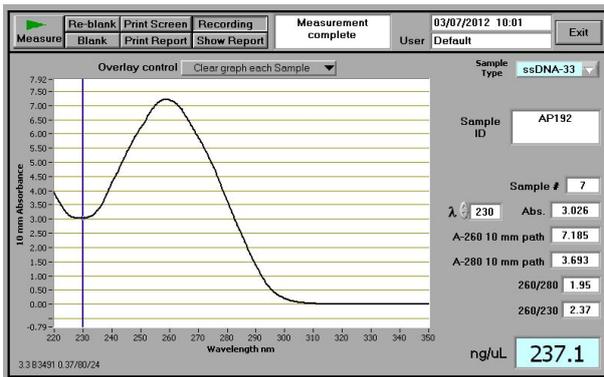
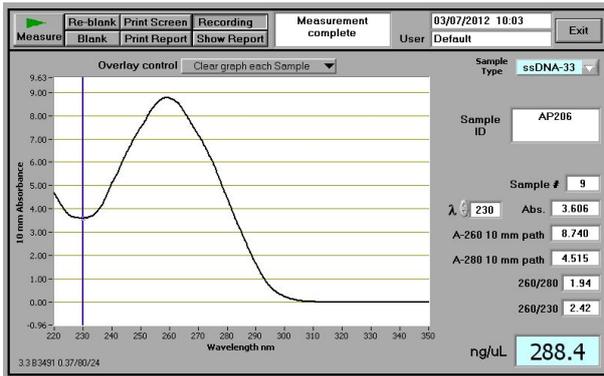
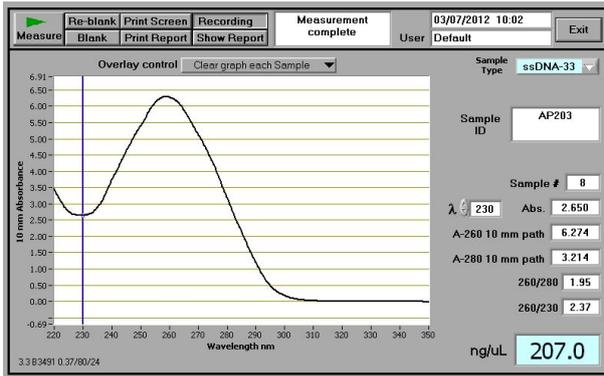




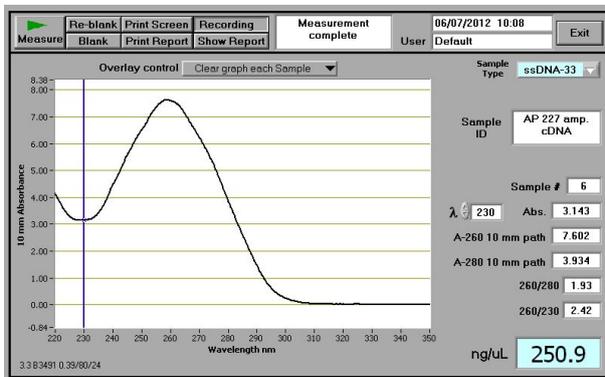
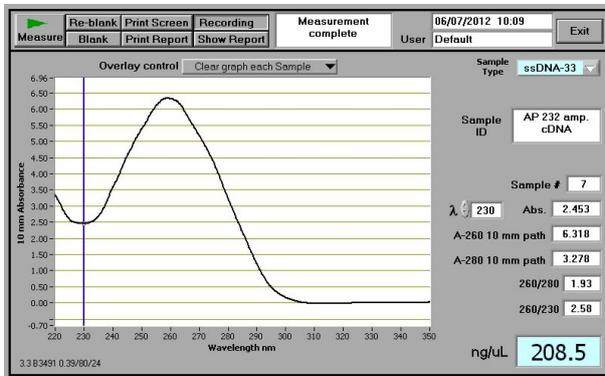
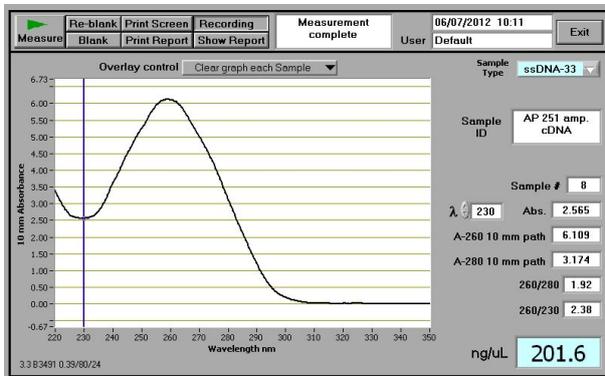
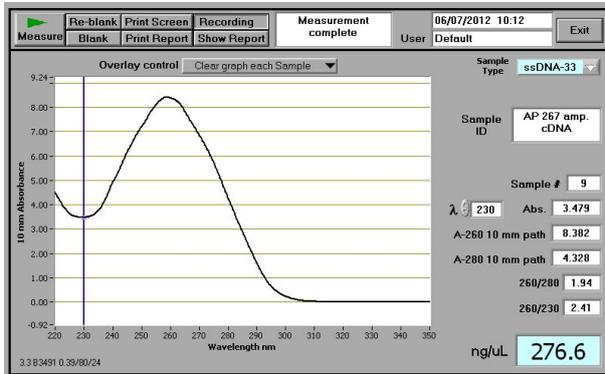
Batch 4

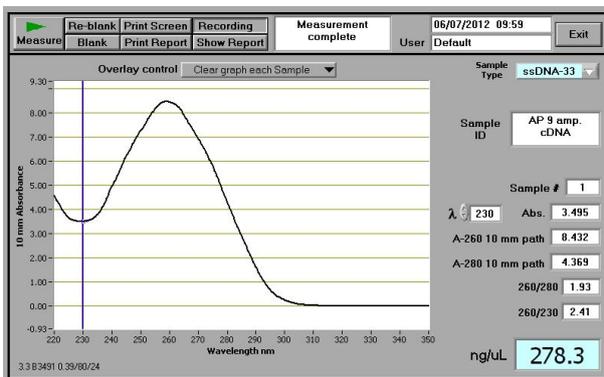
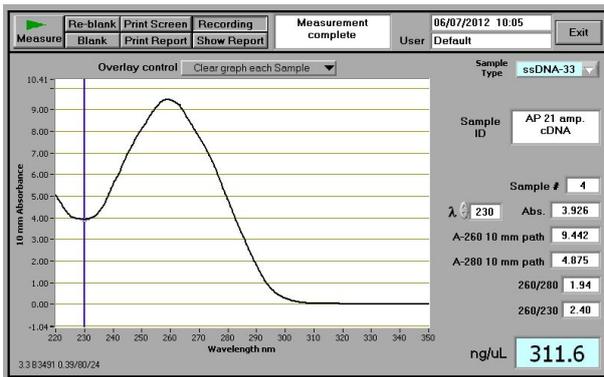
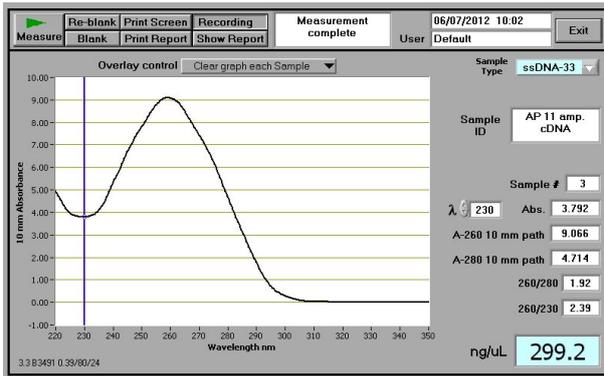
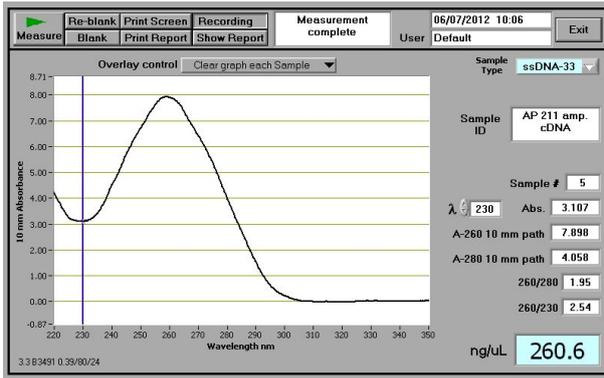


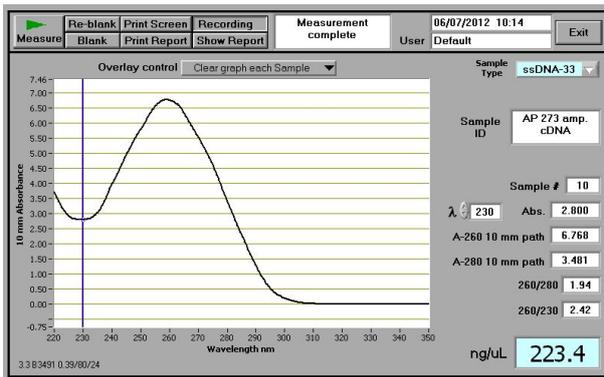
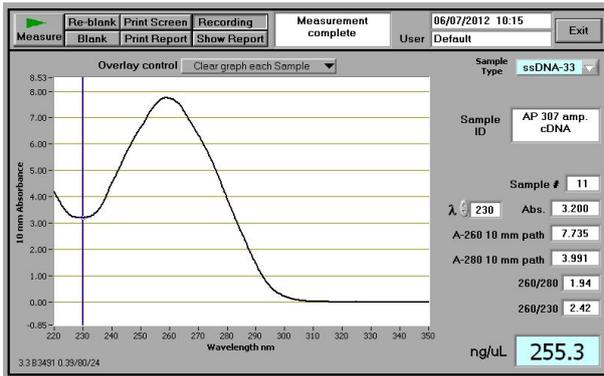
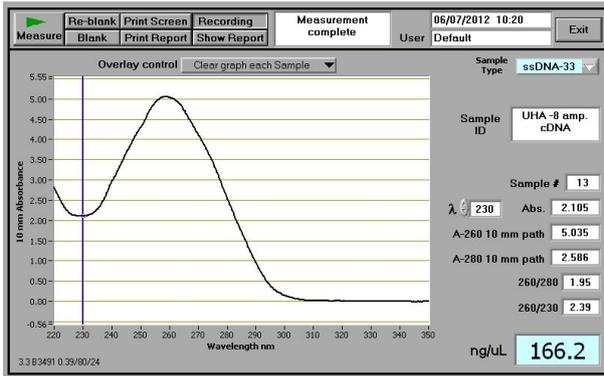




Batch 5







Appendix 2

List of all differentially expressed upregulated genes in severe and mild acute pancreatitis

Expr p-value	Expr Fold Change	Symbol	Entrez Gene Name	Location	Type(s)
0.000108	4.49	MMP8	matrix metalloproteinase 8	Extracellular Space	peptidase
0.0005	3.419	OLAH	oleoyl-ACP hydrolase	Cytoplasm	enzyme
0.0000419	3.095	ADAMTS2	ADAM metalloproteinase with thrombospondin type 1 motif 2	Extracellular Space	peptidase
0.000028	3.085	RETN	resistin	Extracellular Space	other
0.00263	3.077	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	Cytoplasm	enzyme
0.000335	3.072	SLC1A3	solute carrier family 1 member 3	Plasma Membrane	transporter
0.00108	2.918	OLAH	oleoyl-ACP hydrolase	Cytoplasm	enzyme
0.00316	2.915	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	Cytoplasm	enzyme
0.0028	2.87	OLAH	oleoyl-ACP hydrolase	Cytoplasm	enzyme
0.000604	2.847	GPR84	G protein-coupled receptor 84	Plasma Membrane	G-protein receptor
0.000368	2.739	SLC51A	solute carrier family 51 alpha subunit	Plasma Membrane	transporter
0.000132	2.701	CRISP3	cysteine rich secretory protein 3	Extracellular Space	other
0.000838	2.624	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	Cytoplasm	enzyme
0.0018	2.551	DAAM2	dishevelled associated activator of morphogenesis 2	Cytoplasm	other
0.0000173	2.538	CD163	CD163 molecule	Plasma Membrane	transmembrane receptor
0.00332	2.537	FGF13	fibroblast growth factor 13	Extracellular Space	growth factor
0.000477	2.534	ANKRD22	ankyrin repeat domain 22	Nucleus	transcription regulator
0.000163	2.452	VSIG4	V-set and immunoglobulin domain containing 4	Plasma Membrane	other
0.0000131	2.445	BPI	bactericidal/permeability-increasing protein	Plasma Membrane	transporter
0.000208	2.398	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	Cytoplasm	kinase
0.0000661	2.391	TIMP4	TIMP metalloproteinase inhibitor 4	Extracellular Space	other
0.000666	2.335	ARG1	arginase 1	Cytoplasm	enzyme
0.00036	2.315	SH3PXD2B	SH3 and PX domains 2B	Cytoplasm	other
0.000874	2.31	HGF	hepatocyte growth factor	Extracellular Space	growth factor
0.000334	2.302	HGF	hepatocyte growth factor	Extracellular Space	growth factor
0.000518	2.252	PTGES	prostaglandin E synthase	Cytoplasm	enzyme
0.000349	2.238	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	Nucleus	other
0.0000832	2.227	TMEM45A	transmembrane protein 45A	Plasma Membrane	other
0.000094	2.221	CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1	Plasma Membrane	transporter
0.00183	2.21	DLC1	DLC1 Rho GTPase activating protein	Cytoplasm	other
0.0000795	2.196	TCN1	transcobalamin 1	Cytoplasm	transporter
0.000477	2.195	DHRS9	dehydrogenase/reductase 9	Cytoplasm	enzyme
0.000799	2.182	SEMA6B	semaphorin 6B	Plasma Membrane	other
0.00161	2.165	LCN2	lipocalin 2	Extracellular Space	transporter
0.00273	2.157	CYP1B1-AS1	CYP1B1 antisense RNA 1	Other	other
0.0000261	2.151	ADAMTS2	ADAM metalloproteinase with thrombospondin type 1 motif 2	Extracellular Space	peptidase
0.000497	2.149	DHRS9	dehydrogenase/reductase 9	Cytoplasm	enzyme
0.00292	2.134	LTF	lactotransferrin	Extracellular Space	peptidase

0.00104	2.124	CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1	Plasma Membrane	transporter
0.000751	2.111	CRISP2	cysteine rich secretory protein 2	Extracellular Space	other
0.000224	2.103	HGF	hepatocyte growth factor	Extracellular Space	growth factor
0.00269	2.1	C1QC	complement C1q C chain	Extracellular Space	peptidase
0.000875	2.094	THBS1	thrombospondin 1	Extracellular Space	other
0.00129	2.09	CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1	Plasma Membrane	transporter
0.0000365	2.073	SIRT5	sirtuin 5	Cytoplasm	enzyme
0.000357	2.06	DHRS9	dehydrogenase/reductase 9	Cytoplasm	enzyme
0.000345	2.053	FGF13-AS1	FGF13 antisense RNA 1	Other	other
0.0000247	2.048	ERLIN1	ER lipid raft associated 1	Plasma Membrane	other
0.000131	2.027	PDE4D	phosphodiesterase 4D	Cytoplasm	enzyme
0.000595	2.012	KL	klotho	Extracellular Space	enzyme

List of all differentially expressed downregulated genes in severe and mild acute pancreatitis

Expr p-value	Expr Fold Change	Symbol	Entrez Gene Name	Location	Type(s)
0.000152	-1.931	RPS6KA5	ribosomal protein S6 kinase A5	Nucleus	kinase
0.000479	-1.935	TRAC	T-cell receptor alpha constant	Plasma Membrane	other
0.000391	-1.936	GBP5	guanylate binding protein 5	Plasma Membrane	enzyme
0.000664	-1.939	NR3C2	nuclear receptor subfamily 3 group C member 2	Nucleus	ligand-dependent nuclear receptor
0.000941	-1.954	CD40LG	CD40 ligand	Extracellular Space	cytokine
0.000183	-1.954	MYBL1	MYB proto-oncogene like 1	Nucleus	transcription regulator
0.0000454	-1.966	CXCR3	C-X-C motif chemokine receptor 3	Plasma Membrane	G-protein coupled receptor
0.000521	-1.968	YME1L1	YME1 like 1 ATPase	Cytoplasm	peptidase
0.000208	-1.97	BCL11B	B-cell CLL/lymphoma 11B	Nucleus	transcription regulator
0.000231	-1.972	LCK	LCK proto-oncogene, Src family tyrosine kinase	Cytoplasm	kinase
0.000103	-1.975	RORA	RAR related orphan receptor A	Nucleus	ligand-dependent nuclear receptor
0.000401	-1.977	PYHIN1	pyrin and HIN domain family member 1	Nucleus	other
0.000425	-1.982	CD3D	CD3d molecule	Plasma Membrane	transmembrane receptor
0.000189	-1.984	THEMIS	thymocyte selection associated	Cytoplasm	other
0.000522	-2	CAMK4	calcium/calmodulin dependent protein kinase IV	Nucleus	kinase
0.00274	-2.003	LOC105369609	uncharacterized LOC105369609	Other	other
0.00174	-2.007	ISM1	isthmin 1	Other	other
0.00165	-2.012	ETV7	ETS variant 7	Nucleus	transcription regulator
0.0000993	-2.016	ATP8B2	ATPase phospholipid transporting 8B2	Plasma Membrane	transporter
0.000717	-2.02	GRAMD1C	GRAM domain containing 1C	Other	other
0.0000249	-2.022	RPS6KA5	ribosomal protein S6 kinase A5	Nucleus	kinase
0.000147	-2.026	DPP4	dipeptidyl peptidase 4	Plasma Membrane	peptidase
0.00035	-2.03	TRAC	T-cell receptor alpha constant	Plasma Membrane	other
0.000122	-2.068	IFNLR1	interferon lambda receptor 1	Plasma Membrane	transmembrane receptor
0.000382	-2.075	PRKCQ-AS1	PRKCQ antisense RNA 1	Other	other
0.00321	-2.082	BEX2	brain expressed X-linked 2	Nucleus	other
0.000694	-2.09	TRAV24	T cell receptor alpha variable 24	Other	other
0.000213	-2.091	CD160	CD160 molecule	Plasma Membrane	transmembrane receptor
0.0000443	-2.104	TBC1D4	TBC1 domain family member 4	Cytoplasm	other
0.00103	-2.113	CAMK4	calcium/calmodulin dependent protein kinase IV	Nucleus	kinase
0.000194	-2.139	CD28	CD28 molecule	Plasma Membrane	transmembrane receptor
0.0000954	-2.159	GATA3	GATA binding protein 3	Nucleus	transcription regulator
0.000319	-2.165	TRAV9-2	T cell receptor alpha variable 9-2	Other	other
0.000101	-2.178	GBP4	guanylate binding protein 4	Cytoplasm	enzyme
5.51E-06	-2.201	RPS6KA5	ribosomal protein S6 kinase A5	Nucleus	kinase
0.000125	-2.208	BCL11B	B-cell CLL/lymphoma 11B	Nucleus	transcription regulator
0.0000298	-2.214	YME1L1	YME1 like 1 ATPase	Cytoplasm	peptidase
0.0000878	-2.235	GZMK	granzyme K	Cytoplasm	peptidase
0.000529	-2.254	KIAA1671	KIAA1671	Other	other
0.00037	-2.283	TRAT1	T cell receptor associated transmembrane adaptor 1	Plasma Membrane	kinase
0.000166	-2.292	INPP4B	inositol polyphosphate-4-phosphatase type II B	Cytoplasm	phosphatase
0.00018	-2.299	THEMIS	thymocyte selection associated	Cytoplasm	other

0.0000354	-2.321	P2RY10	purinergic receptor P2Y10	Plasma Membrane	G-protein coupled receptor
0.0000236	-2.364	RORA	RAR related orphan receptor A	Nucleus	ligand-dependent nuclear receptor
0.0000358	-2.386	YME1L1	YME1 like 1 ATPase	Cytoplasm	peptidase
0.0000328	-2.398	TRDV3	T cell receptor delta variable 3	Other	other
0.00125	-2.405	YME1L1	YME1 like 1 ATPase	Cytoplasm	peptidase
0.000218	-2.407	YME1L1	YME1 like 1 ATPase	Cytoplasm	peptidase
0.000285	-2.519	NOV	nephroblastoma overexpressed	Extracellular Space	growth factor
0.00045	-2.594	NELL2	neural EGFL like 2	Extracellular Space	other

Appendix 3

List of all differentially expressed miRNA in severe and mild acute pancreatitis

Rank	ID	F p-value	Adjusted p-value	Log Fold Change	Fold Change	Absolute Fold Change
1	MIR631	3.69E-06	0.013909811	-0.89813	-1.86365	1.863649
2	MIR3129	7.20E-06	0.013909811	-0.66696	-1.58772	1.587725
3	snoU13	9.09E-06	0.013909811	-0.37461	-1.29649	1.296491
4	MIR1291	2.04E-05	0.018768512	0.858892	1.813645	1.813645
5	ACA50	1.82E-05	0.018768512	-0.39981	-1.31933	1.319331
6	SNORD30	2.58E-05	0.019781918	-0.34226	-1.26774	1.267737
7	MIR758	4.26E-05	0.021760076	0.828388	1.7757	1.7757
8	ACA9	3.73E-05	0.021760076	-0.637	-1.55509	1.555091
9	snoU13	3.91E-05	0.021760076	-0.53997	-1.45394	1.453938
10	snoU13	4.91E-05	0.022441665	0.59434	1.509782	1.509782
11	MIR3157	5.83E-05	0.022441665	-0.51723	-1.4312	1.431205
12	snoU13	5.86E-05	0.022441665	0.423748	1.341408	1.341408
13	MIR548n	6.94E-05	0.024523767	-0.82394	-1.77023	1.770233
14	MIR302e	8.66E-05	0.026509154	-0.60491	-1.52089	1.520889
15	MIR513c	8.34E-05	0.026509154	0.538149	1.452108	1.452108
16	MIR548j	9.79E-05	0.028097164	-0.54481	-1.45883	1.458825
17	SNORA51	0.000131	0.03542224	-0.32097	-1.24917	1.249168
18	MIR758	0.000148	0.03572517	0.712447	1.638581	1.638581
19	MIR3187	0.000141	0.03572517	-0.66193	-1.58219	1.582195
20	SNORA70	0.000162	0.037128192	-0.16524	-1.12136	1.121356
21	MIR184	0.00019	0.03787902	0.666223	1.586912	1.586912
22	MIR199b	0.000186	0.03787902	-0.62895	-1.54644	1.546441
23	SNORD109A	0.000188	0.03787902	-0.51885	-1.43281	1.432809
24	MIR636	0.000248	0.039727972	0.792679	1.732288	1.732288
25	MIR1976	0.000237	0.039727972	-0.7054	-1.6306	1.630598
26	snoU13	0.000265	0.039727972	-0.65159	-1.5709	1.570901
27	snosnR60_Z15	0.000229	0.039727972	0.624232	1.54139	1.54139
28	14qII-17	0.000268	0.039727972	0.562621	1.47695	1.47695
29	SNORA25	0.000241	0.039727972	-0.54568	-1.4597	1.459705
30	MIR23a	0.000224	0.039727972	-0.52215	-1.43609	1.436094
31	MIR192	0.000253	0.039727972	-0.28773	-1.22071	1.220714
32	SNORA8	0.000312	0.042511604	0.600929	1.516693	1.516693
33	snoU13	0.000298	0.042511604	-0.43351	-1.35051	1.350514
34	SNORA3	0.000315	0.042511604	0.368014	1.290575	1.290575
35	SNORA27	0.000327	0.042862865	0.612952	1.529385	1.529385
36	MIR432*	0.000353	0.044994617	-0.65758	-1.57743	1.57743

37	SNORA8	0.000373	0.045138987	0.768009	1.702918	1.702918
38	snoU13	0.000374	0.045138987	0.447187	1.36338	1.36338
39	U8	0.000406	0.047838964	0.404085	1.32325	1.32325
40	SNORA22	0.000421	0.04784593	0.757156	1.690155	1.690155
41	MIR373	0.000458	0.04784593	0.703811	1.628801	1.628801
42	SNORD56	0.000456	0.04784593	0.441721	1.358223	1.358223
43	MIR589	0.00044	0.04784593	-0.39282	-1.31295	1.312953
44	MIR135b	0.000428	0.04784593	-0.33667	-1.26284	1.262841
45	MIR487b	0.000479	0.048927839	0.349223	1.273875	1.273875