This electronic thesis or dissertation has been downloaded from the King's Research Portal at https://kclpure.kcl.ac.uk/portal/

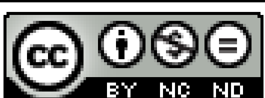
Carbon-13 Breath Delta Value as a Biomarker of infection following Liver Transplantation and Hepatopancreatobiliary Surgery

Jerome, Ellen

Awarding institution:
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

END USER LICENCE AGREEMENT



Unless another licence is stated on the immediately following page this work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International licence. https://creativecommons.org/licenses/by-nc-nd/4.0/

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 13. Jan. 2025

.

Carbon-13 Breath Delta Value as a Biomarker of Infection following Liver Transplantation and Hepatopancreatobiliary Surgery

A thesis submitted for the degree of

Medicine Doctorate (Research)

Ellen Jerome

Institute of Liver Studies

School of Immunology and Microbial Sciences

Faculty of Life Sciences and Medicine

King's College London

London SE1 9NH, UK

February 2024

Word Count 38,089

.

Abstract

Background

Sepsis and infection are the leading cause of morbidity and mortality after surgery, but the inflammatory response to the trauma of surgery can make diagnosis challenging, and no biomarker with high diagnostic accuracy has been discovered or implemented in clinical practice. Carbon-13 breath delta value (BDV) is the ratio of the Carbon isotopes ^{12}C and ^{13}C in breath, where approximately 99% of universal carbon is ^{12}C and 1% is ^{13}C. Data suggests a shift in BDV during high metabolic states, for example sepsis, measured using infrared spectroscopy. ^{13}C BDV has been shown to be discriminant as a novel diagnostic marker for sepsis and infection. This thesis assesses present putative biomarkers and novel breath and circulating biomarkers following major hepatopancreatobiliary (HPB) surgery and Liver Transplantation (LT) to diagnose postoperative infection/sepsis.

Methods

A meta-analysis of Procalcitonin (PCT) and Interleukin-6 (IL-6) was performed. A prospective cohort of 20 participants undergoing HPB surgery and 20 participants undergoing LT were recruited. Breath samples were collected from baseline preoperatively, and on postoperative days (POD) 1-9, with plasma and peripheral blood mononuclear cell (PBMC) samples preoperatively, POD1, 4 and 8 in the HPB group, and 2,5 and 8 in the LT group. Breath samples were analysed using infrared laser spectroscopy to generate BDV (per mil). Plasma was analysed for 9 proinflammatory cytokines using MesoScale Discovery (MSD) immunoassay, PCT using ELISA, and cell surface marker expression on monocytes was phenotyped using flow cytometry in the HPB group. Differences between groups who did and did not develop infective complications was analysed using two-way Analysis of Variance (ANOVA), Mann Whitney U test, and diagnostic accuracy.

.

Results

5/20 HPB participants developed infective complications, mean day of diagnosis POD5. There was no difference between BDV in patients with or without infection. Monocyte count was increased in infected participants at all timepoints, and monocyte expression of programme death-ligand 1 (PD-L1) on POD1, Cluster of differentiation 155 (CD155) and Human-leucocyte antigen - DR isotype (HLA-DR) on POD4 were upregulated, +13.09% (95%CI 1.59 to 24.61, $p<0.05$), +422.5 mean fluorescence index (MFI) (95% CI -770.9 to -73.68, $p<0.05$), and +19.02% (95% CI 37.58 to 0.45, $p<0.05$) respectively. 4/20 LT participants developed infective complications, mean day of diagnosis POD5. There was no difference in BDV, CRP or sequential organ failure (SOFA) score in patients with or without infection. Interleukin-12p70 (IL-12p70), Interleukin-2 (IL-2), Interleukin-4 (IL-4) and tumour necrosis factor α (TNFα) were upregulated and Interferon-γ (IFN-γ), Interleukin-6 (IL-6) and Interleukin-8 (IL-8) were downregulated on POD2 and 5 following surgery. Cytokines were not significantly different between participants with and without infection.

Discussion

In this prospective cohort, BDV was not significantly different between groups following HPB surgery or LT. Monocyte expression of PD-L1, HLA-DR and CD155 is associated with infection, +13.09% ($p<0.05$), +422.5 MFI ($p<0.05$), and +19.02% ($p<0.05$) respectively following HPB surgery. Proinflammatory cytokines were not significantly different between groups following HPB surgery or LT. All markers performed poorly as diagnostic markers.

The innate immune system is dysregulated in infection following major HPB surgery. In this cohort BDV did not predict infection. Further investigation of novel biomarkers including BDV and soluble monocyte markers in a higher-powered study is required.

.

Acknowledgments

This project was the greatest challenge in my career starting at the height of the COVID-19 pandemic, and I had the great opportunity to learn from so many people. I would like to thank my supervisors, to Mr Krishna Menon for taking a chance on me, for enduring support and encouragement, and inspiring me to develop me as a clinician and bringing academia into the challenging world of surgery. Thanks to Dr Mark McPhail for your patience and encouragement and benevolent leadership in the group. Both of your kindness and support gave me the confidence to get through and the clinical and academic work you both do is laudable.

Thanks to my colleagues and friends in the lab group. To Salma Mujib for giving her time and expertise and training me up at the bench; to Dr Francesca Trovato for your passion for data analysis, the freezer and everything else; to Dr Anna Cavazza for great peer support navigating the postgraduate clinician whirlwind; to Salvatore Napoli for bringing so much fun to the lab; to Dr Florent Artu for your Gallic humour and wisdom; to Tengfei, Zhelin, Xiaohong for your great company and kindness; and to Maura, Marc and everyone else who joined the lab.

Thanks to the Spectroscopy group at the Rutherford Appleton Space Laboratory, to Dr Damien Weidmann for your collaboration and expertise, to Tomas Pinto for developing the instrument, and to Dr Thomas Wall for all of the teaching, analysis, Wordle-ing and great company getting through 400 samples.

To the Institute of Liver Studies for bringing me into the team and showing me so much love and support. Thank you to the fellows, for all you taught me on retrieval and in transplant, and for all the breath sampling you did for me, It was a transformative experience and I feel very lucky to have had you as colleagues and friends..

.

I would like to thank my parents Sally and Mike for their ongoing support throughout my career, I'll get there one day. Thanks to Ed, my fiancé, who I was lucky enough to meet when I started this MD(Res) and coming to King's. For every Saturday or Sunday morning spent processing in the lab, or 3am call out for retrieval or transplant, it was so much better to have gone through together.

.

Publications

Jerome E, McPhail MJ, Menon K. Diagnostic accuracy of procalcitonin and interleukin-6 for postoperative infection in major gastrointestinal surgery: a systematic review and meta-analysis. Ann R Coll Surg Engl. 2022 Sep;104(8):561-570. doi: 10.1308/rcsann.2022.0053. PMID: 36044921.

Jerome E, Cavazza A, Menon K, McPhail MJ. Systematic review and meta-analysis of the diagnostic accuracy of procalcitonin for post-operative sepsis/infection in liver transplantation. Transpl Immunol. 2022 Oct;74:101675. doi: 10.1016/j.trim.2022.101675. Epub 2022 Jul 22. PMID: 35878844.

.

List of Abbreviations

^{12}C Carbon 12 isotope

^{13}C Carbon-13 isotope

AA Amino Acid

ACLF Acute-on-chronic liver failure

ACR Acute cellular rejection

ADP Adenosine diphosphate

AIH Autoimmune hepatitis

AKI Acute kidney injury

ALF Acute liver failure

ANOVA Analysis of variance

API All postoperative infection

APR Acute phase response

AR Anatomical liver resection

ArLD Alcohol-related liver disease

ASA American Society of Anaesthesiologist physical status classification

AST Aspartate transaminase

ATP Adenosine triphosphate

AUC Area under receiver operator curve

Aux-OLT Auxiliary orthotopic liver transplant

BDV Carbon-13 breath delta value

BL Biochemical leak – postoperative pancreatic fistula classification

BMI Body mass index

$C_6H_{12}O_2$ Glucose

CAP Community-acquired pneumonia

CCA Cholangiocarcinoma

CCR2 C-C chemokine receptor type 2, CD192

.

CD	Cluster of differentiation
CI	Confidence interval
CIT	Cold ischaemic time
CLP	Caecal ligation and puncture
CO_2	Carbon dioxide
CoV	Coefficient of variance
COVID-19	Coronavirus Disease 2019
CRF	Case report form
CRLM	Colorectal liver metastasis
CRP	C-reactive protein
CT	Computed tomography
CVP	Central venous pressure
CVS	Cardiovascular
DAMP	Damage-associated molecular pattern
DMSO	Dimethyl sulfoxide
DOR	Diagnostic odds ratio
DP	Distal pancreatectomy
EAD	Early allograft dysfunction
EBL	Estimated blood loss
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ESS	Effective sample size
FBS	Foetal bovine serum
FLR	Future liver remnant
FSC	Forward scatter
GB	Gallbladder

.

GI	Gastrointestinal
GJ	Gastrojejunostomy
H_20	Water
HC	Healthy Control
HCC	Hepatocellular carcinoma
HJ	Hepaticojejunostomy
HLA-DR	Human-leucocyte antigen - DR isotype
HAP	Hospital-acquired pneumonia
HPB	Hepatopancreatobiliary surgery
HRP	Horseradish peroxidase
IAI	Postoperative intrabdominal infection
ICU	Intensive care unit
IFNγ	Interferon gamma
IL-1b	Interleukin-1b
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-12p70	Interleukin-12p70
I-MET	Immuno-metabolism in Sepsis, Inflammation and Liver Failure Syndromes
IPMN	Intraductal papillary mucinous neoplasm
IR	Infrared
ISGLS	International Study Group of Liver Surgery
ITS	Infection, trauma or surgery
JJ	Jejunojejunostomy
KDIGO	Kidney Disease: Improving Global Outcomes
LH	Left hepatectomy
LIR	Laser isotope ratio-meters

.

LMR	Lymphocyte monocyte ratio
LPS	Lipopolysaccharide
LT	Liver transplantation
MAP	Mean arterial pressure
MCN	Pancreatic mucinous cystic neoplasm
MEMS	Micro-electro-mechanical
MerTK	Proto-oncogene tyrosine-protein kinase MER
MeSH	Medical subject headings
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOF	Multiorgan failure
MOOSE	Meta-analysis of observational studies in epidemiology reporting
MSD	MesoScale Discovery assay
MWU	Mann Witney U test
NAFLD	Non-alcoholic fatty liver disease
NAR	non-anatomical liver resection
NASH	Non-alcoholic steatohepatitis
NET	Neuroendocrine tumour
NLR	Neutrophil: lymphocyte ratio
NMR	Neutrophil monocyte ratio
NPV	Negative predictive value
ns	Nonsignificant
O_2	Oxygen
OLT	Orthotopic liver transplant
OR	Odds ratio
PA	Pancreatic adenocarcinoma
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline

.

PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cell
PCT	Procalcitonin
PD	Pancreaticoduodenectomy
PD-1	Programmed cell death protein 1, cluster of differentiation 279 (CD279)
PD-L1	Programmed death-ligand 1, cluster of differentiation 274 (CD274)
PDB	PeeDee Belemnite
PHH	Post hepatectomy haemorrhage
PIAI	Postoperative intrabdominal infection
PLR	Platelet monocyte ratio
POD	Postoperative day
POPF	Postoperative pancreatic fistula
PP	Plasma proteome
PPPD	Pylorus preserving pancreaticoduodenectomy
PPV	Positive predictive value
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
PRBC	Packed red blood cell transfusion
PSC	Primary sclerosing cholangitis
QADAS-2	Quality assessment of diagnostic accuracy studies-2 tool
RH	Right hepatectomy
RNA	Ribonucleic acid
ROC	Receiver operator curve
ROLT	Right lobe orthotopic liver transplant
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute 1640 medium
RRT	Renal replacement therapy
RTT	Return to theatre
$Ru(bpy)_3^{2+}$	Cation of Tris(bipyridine)ruthenium(II) chloride

.

SC	Serum Calprotectin
sCD14	Presepsin, soluble CD14
SCr	Serum creatinine
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
SROC	Summary receiver operator curve
SSC	Side scatter
sSDC-1	Syndecan-1
SSI	Surgical site infection
TEG	Thromboelastography
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TMB	Tetramethylbenzidine substrate
TNFα	Tumour necrosis factor alpha
TPRa	Tripropylamine
VOC	Volatile organic compounds
UTI	Urinary tract infection
UW	University of Wisconsin solution
WCC	Leucocyte count/white cell count
WIT	Warm ischaemic time of donor liver graft
Δ	Delta, change in value
δ	delta, change in value

.

Table of Contents

Abstract ... 2

Acknowledgments ... 4

Publications ... 6

List of Abbreviations ... 7

Table of Contents ... 13

Table of Tables ... 16

Table of Figures ... 20

1 Introduction ... 25

1.1 Overview ... 25

1.2 Sepsis, Infection and Surgery ... 26

1.3 Immunity ... 28

1.4 Biomarkers ... 32

1.5 Qualitative Synthesis: Observational Studies examining the Diagnostic Accuracy of Biomarkers of Sepsis and Infection following Major Abdominal Surgery ...

1.6 ^{13}C Breath Delta Value ... 53

1.7 Systematic Review of ^{13}C BDV as a Biomarker of Sepsis and Inflammation ... 55

1.8 Surgery and the Immune System ... 62

1.9 Hepatopancreatobiliary Surgery ... 63

1.10 Liver Transplantation ... 70

1.11 Biomarkers for investigation ... 76

1.12 Hypothesis and aims of the thesis ... 79

2 Methods ... 80

2.1 Participant Recruitment ... 80

.

2.2 Blood Sampling 82

2.3 Breath Sampling 85

2.4 Clinical Data Collection 86

2.5 Blood processing 100

2.6 Breath Processing 101

2.7 Statistical Analysis 113

2.8 Mesoscale Discovery Assay 114

2.9 Procalcitonin ELISA 119

2.10 Monocyte Phenotyping 121

3 Characteristics of Included Participants 125

4 Procalcitonin Meta-analysis 130

4.1 Diagnostic Accuracy of Procalcitonin and Interleukin-6 for Postoperative Infection in Major Abdominal Surgery: Meta-analysis 130

4.2 Diagnostic Accuracy of Procalcitonin for Postoperative Infection in Liver Transplantation: Meta-analysis 146

5 Carbon-13 Breath Delta Value 161

5.1 Introduction 161

5.2 Results 163

5.3 Summary and Conclusions 183

6 Serum Biomarkers of Infection and Inflammation 187

6.1 Introduction 187

6.2 Results 187

6.3 Summary and Conclusions 213

7 Monocyte Phenotyping 214

7.1 Introduction 214

7.2 Results 214

7.3 Summary and Conclusions 223

.

8 General Discussion 224

8.1 Summary and significance of work 224

8.2 Limitations of the study 226

8.3 Methodological flaws 227

8.4 Attempted but as yet unaddressed questions 229

8.5 Future research directions 230

8.6 Personal Reflection 230

9 References 232

10 Appendix 1 252

11 Appendix 2 253

.

Table of Tables

Table 1-1 Summary of studies included in the systematic review examining the diagnostic accuracy of biomarkers of sepsis and infection following major abdominal surgery..............48

Table 1-2 Indications for HPB Surgery...66

Table 1-3 UK Adult Liver Transplant Indications(103) 72

Table 1-4 Biomarkers analysed in the thesis 77

Table 2-1 Sampling Schedule 83

Table 2-2 Blood Samples 84

Table 2-3. Sequential (Sepsis-Related) Organ Failure Score 89

Table 2-4 Center for Disease Control definitions of infection (116, 117)................................90

Table 2-5 ISGLS Postoperative Pancreatic Fistula Criteria(118) 95

Table 2-6 ISGLS Grading of Bile Leakage(119) 95

Table 2-7 ILSGS Grading of Post Hepatectomy Haemorrhage(120) 95

Table 2-8. KDIGO Criteria for Staging AKI...97

Table 2-9 Clavien-Dindo classification of surgical complications(124)...................................99

Table 2-10 Breath sample post storage data collection...108

Table 2-11 Cytokines included in MSD V-PLEX Proinflammatory multiassay 116

Table 2-12. Antibodies used for Flow Cytometry 123

Table 3-1 Characteristics of Included HPB Participants 126

.

Table 3-2 Descriptive statistics: differences between infected and non-infected HPB participants 127

Table 3-3 Characteristics of Included LT Participants 128

Table 3-4 Descriptive Statistics, differences between infected and non-infected LT participants 129

Table 4-1 Characteristics of Included Studies..135

Table 4-2. Subgroup Analysis for Procalcitonin 143

Table 4-3 Characteristics of Included Studies for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis 153

Table 4-4 Subgroup Analysis for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation - Meta Analysis 156

Table 5-1 Direction of vibration of bonds during absorption of IR at their given frequency(158) 162

Table 5-2 Mean and median $^{12}CO_2$ concentration, BDV and number of samples with unrecordable BDV, $^{12}CO_2$ concentration >0.01 and >0.02 163

Table 5-3 Mann-Witney U test for day-to-day difference in BDV between infected and non-infected HPB groups 171

Table 5-4 Mann-Witney U test for day-to-day difference in BDV, WCC, CRP and SOFA between infected and non-infected HPB groups 172

Table 5-5 Univariate logistic regression of BDV for infection following HPB surgery 175

Table 5-6 Mann-Witney U test for day-to-day difference in BDV between infected and non-infected LT groups 177

.

Table 5-7 Mann-Witney U test for day-to-day difference in BDV, WCC, CRP and SOFA between infected and non-infected LT groups 178

Table 5-8 Univariate logistic regression of BDV for infection following LT 180

Table 5-9 Diagnostic accuracy of BDV for infection at baseline and POD1-9 181

Table 6-1 Number and percentage of samples in range for MSD V-PLEX Proinflammatory panel..188

Table 6-2 Coefficient of Variation of repeated samples on MSD plate 1 and 2 188

Table 6-3 Mann-Witney U test for differences in biomarkers of infection following HPB surgery in infected and non-infected groups at baseline, on POD1, POD4 and POD8. 191

Table 6-4 Univariate Logistic Regression for infection at baseline sample in HPB group ...194

Table 6-5 Univariate Logistic Regression for infection on POD1 sample in HPB group......195

Table 6-6 Univariate Logistic Regression for infection on POD4 sample in HPB group......196

Table 6-7 Univariate Logistic Regression for infection on POD8 sample in HPB group......197

Table 6-8 Diagnostic accuracy for MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC,..198

Table 6-9 Mann-Witney U test for differences in biomarkers of infection following LT in infected and non-infected groups at baseline, on POD2, POD5 and POD8 203

Table 6-10 Univariate Logistic Regression for infection at baseline sample in LT group 206

Table 6-11 Univariate Logistic Regression for infection on POD2 sample in LT group 207

Table 6-12 Univariate Logistic Regression for infection on POD5 sample in LT group 208

.

Table 6-13 Univariate Logistic Regression for infection on POD8 sample in LT group 209

Table 6-14 Diagnostic accuracy of ROC curves for POD2 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC, ...210

Table 7-1 Mann-Witney U Test of cell surface markers for infection following HPB surgery. 216

Table 7-2 Univariate Logistic regression for infection for cell surface markers following HPB surgery at baseline...219

Table 7-3 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD1. 220

Table 7-4 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD4. 221

Table 7-5 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD8 222

.

Table of Figures

Figure 1-1 Principle mechanisms of the innate and adaptive immune system[16] 29

Figure 1-2 The Acute Phase Response[19] 31

Figure 1-3 PRISMA flow chart of included studies in the systematic review examining the diagnostic accuracy of biomarkers of sepsis and infection following major abdominal surgery 37

Figure 1-4 Fractionation of Carbon during the Acute Phase Response[72] 54

Figure 1-5 PRISMA diagram of studies included in the systematic review of 13C BDV as a biomarker of sepsis and inflammation 57

Figure 1-6 Anatomy of the Liver, demonstrating the Couinaud classification(79) 63

Figure 1-7 Anatomy of the Pancreas (79) 64

Figure 1-8 Liver sinusoid(87) 65

Figure 1-9 Right Hemi-Hepatectomy and Extended Right Hemi-Hepatectomy(79) 67

Figure 1-10 Left Lateral Segment Resection and Left Hemi-Hepatectomy(79) 68

Figure 1-11 Pancreaticoduodenectomy(79) 69

Figure 1-12 Distal Pancreatectomy and Splenectomy 70

Figure 1-13 Orthotopic Whole Liver Transplantation 74

Figure 2-1 Supel™-Inert Multi-Layer Foil gas sampling bags 85

Figure 2-2. Ventilator Circuit for Breath Sampling 86

Figure 2-3 IR radio spectrometer 101

.

Figure 2-4 MEMS mirror 103

Figure 2-5 Infrared Spectrometer. 104

Figure 2-6 Infrared Spectrometer and Spectra 105

Figure 2-7 Spectra for sample (channel A) and calibrants 1 and 2 (channel B and C). The first large trough represents absorption of ^{12}C and the second ^{13}C.....................................106

Figure 2-8 SULFO-TAG labelled detection antibody bound to analyte 115

Figure 2-9 Electrochemiluminescence at the electrode..115

Figure 2-10 96-well multispot MSD plate 116

Figure 2-11 4-parameter logistic calibration curve..117

Figure 2-12 Gating strategy for Monocyte Phenotyping. 124

Figure 4-1 PRISMA Flow Chart..134

Figure 4-2 Methodological Quality Summary...137

Figure 4-3. Methodological Quality Graph 138

Figure 4-4. SROC, Sensitivity, Specificity and DOR of Procalcitonin for infection following HPB 139

Figure 4-5. SROC, Sensitivity, Specificity and DOR of Interleukin-6 for Infection following LT 141

Figure 4-6. Deek's Test for Publication Bias 142

Figure 4-7 PRISMA Diagram for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis 151

.

Figure 4-8 Methodological Quality Summary Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis 152

Figure 4-9 Methodological Quality Graph Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis 152

Figure 4-10 SROC showing (A)sensitivity, (B) specificity and (C) diagnostic odds ratio (DOR) for Procalcitonin for the diagnosis of post-operative infection/sepsis in Liver Transplantation 155

Figure 4-11 Deek's Test for Publication Bias 157

Figure 5-1 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration (fraction of gas) in a) HPB group and b) LT group 164

Figure 5-2 Spearman r correlation of $^{12}CO_2$ concentration against BDV for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil 164

Figure 5-3 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration (fraction of gas) 165

Figure 5-4 Spearman r correlation of length of time samples stored against $^{12}CO_2$ for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil..........................165

Figure 5-5 Graph to demonstrate the relationship between age of sample (days) and BDV (per mil) in a) HPB group and b) LT group, excluding values where BDV is > -8 permil c) HPB group and d) LT group with the red dotted line showing the mean $^{12}CO_2$ for healthy control samples. 166

Figure 5-6 Spearman r correlation of duration of sample storage (days) and BDV for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil..........................166

.

Figure 5-7 Graph to demonstrate the relationship between $^{12}CO_2$ concentration of sample (fraction) and BDV (permil) 167

Figure 5-8 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration of sample (fraction) and BDV (permil) 168

Figure 5-9 Difference between breath samples taken at the patient end and ventilator end of the ventilator circuit 169

Figure 5-10 ANOVA for BDV in infected and non-infected HPB participant 173

Figure 5-11 ANOVA for BDV in infected and non-infected LT participants 179

Figure 5-12 ROC curves for BDV for infection at baseline and POD1-9.............................182

Figure 6-1 Grouped 2-way ANOVA for HPB showing difference between groups at each POD for MSD V PLEX Proinflammatory Cytokine panel. 192

Figure 6-2 Grouped 2-way ANOVA for HPB infected and non-infected participants for PCT, CRP, Full Blood Count, NLR, LMR, NMR and PLR. 193

Figure 6-4 ROC curves for HPB POD1 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC...199

Figure 6-5 ROC curves for HPB POD4 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC...200

Figure 6-6 Liver Transplantation Grouped 2-way ANOVA showing difference between groups at each POD for MSD V PLEX Proinflammatory Cytokine panel. 204

Figure 6-7 Liver Transplantation Grouped 2-way ANOVA..205

Figure 6-8 ROC curves for Liver Transplantation POD2 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC 211

.

Figure 6-9 ROC curves for Liver Transplantation POD5 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC 212

Figure 7-1 Grouped 2-way ANOVA for HPB cell surface markers, Percentage (%) showing difference between groups at each POD. 217

Figure 7-2 Grouped 2-way ANOVA for HPB cell surface markers, MFI showing difference between groups at each POD. 218

.

1 Introduction

1.1 Overview

Sepsis is a leading cause of critical care admission and mortality, and is a major cause of postoperative morbidity and mortality.(1-3) Surgical site infections are estimated to double postoperative length of stay, and significantly increase the cost of care.(4-7) There is growing interest and research into biomarkers as early predictors of disease, including postoperative infection, to allow for informed decision making before clinical deterioration. As we learn more about host signalling in the immune response to disease and injury – there is potential to utilise use these signalling biomarkers to predict deterioration before traditional clinical and biochemical signs. Early antimicrobial therapy improves morbidity and mortality,(8) although clinical and biochemical signs are shown or may be tested several hours of even days after infection develops. Conversely, non-targeted antimicrobial use risks generating antimicrobial resistance which can contribute to morbidity.(9-11)

The physical injury of an operation, particularly major cavity surgery involving disruption of the gastrointestinal or biliary tract, initiates physiological systemic inflammatory responses. Invasive procedures create potential sources of microbial contamination in sterile spaces and sites for infection. Traditional clinical signs and biochemical investigations are not specific and attributable to disease processes. Biomarkers commonly used to guide clinical decision-making including leucocyte count (WCC) and C reactive protein (CRP) are acute phase markers that can increase in response to major but sterile surgery, limiting their specificity for infection in the postoperative phase. No new biomarkers have been implemented in clinical practice, and there is a need in the post-surgical setting to diagnose postoperative infection before sepsis develops to guide antimicrobial decision making.

.

The aim of this thesis is to examine Carbon-13 breath delta value, a potential novel non-invasive diagnostic biomarker of post-surgical infection/sepsis, along with the cytokine and monocyte response to hepatopancreatobiliary surgery and liver transplantation.

1.2 Sepsis, Infection and Surgery

Sepsis is a clinical syndrome describing the dysregulated immune response to microbial infection, and can cause organ damage, shock, and death.(12, 13) With increasing incidence, 123,000 cases in England in 2015 and a sepsis-associated mortality of 29.9%, there has been a National Health Service England campaign to improve diagnosis and management of sepsis, and reduce avoidable deaths.(13) Surgery – particularly major abdominal surgery with disruption of the gastrointestinal tract containing gut microbial flora – creates potential sources for infection at the surgical site, and risks associated infections due to procedures such as urinary catheterisation, central venous catheterisation, lung atelectasis (following ventilation and due to postoperative hypoventilation), all of which are potential sites for infection to develop. Sepsis is the leading cause of mortality in critical care and is the leading cause of postoperative morbidity and mortality. Surgical site infections are estimated to double postoperative length of stay and significantly increase the cost of healthcare.(2-7)

Early antimicrobial therapy has been shown to improve morbidity and mortality,(8) The Sepsis Six care bundle was shown to reduce the relative risk of death by 46.6%, including administration of broad spectrum antibiotics, and was implemented nationally as part of the sepsis strategy.(14, 15) Onset of change in clinical observations, or change in haematological/biochemical markers of sepsis may be tested or observed several hours or even days following the onset of an infective process. Therefore, there is significant clinical need for a diagnostic marker during the preclinical phase of infection were this possible

.

Sepsis-6 care bundle(1)

1. Give high-flow oxygen via non-rebreathe bag
2. Take blood cultures and consider source control
3. Give intravenous (IV) antibiotics according to local protocol
4. Check lactate
5. Start intravenous fluid resuscitation e.g., Hartmann's or equivalent
6. Monitor hourly urine output and consider catheterisation

Inappropriate antimicrobial use increases both morbidity and mortality as non-targeted use risks antimicrobial resistance.(10, 11, 16) There is a weight of evidence for antimicrobial stewardship, as unnecessary exposure to broad spectrum antibiotics in the lifetime of an individual and the cumulative effect on a population could lead to antimicrobial resistance and increased incidence of multi-drug resistant organisms.

There is growing interest and research in the use of biomarkers as early predictors of disease - including postoperative sepsis – to inform clinical decision making before clinical deterioration. As we learn more about the signalling in the human immune response to disease and injury – there is potential to utilise use these signalling biomarkers to predict clinical outcome or deterioration.

In the context of surgery, the physical injury of an operation – particularly major cavity surgery –initiates a systemic reaction, the 'Acute Phase Response' (APR). This means that traditional clinical and biochemical signs are not always specific and attributable to disease processes. The issue is clouded further in transplant surgery, where immunosuppression, steroid administration, and organ support during recovery from major surgery modulates normal clinical and biochemical responses – making diagnosing postoperative sepsis a greater challenge. Typically, a surgical patient may have perioperative prophylactic antibiotic to control bacterial contamination at the surgical site and limit the development of surgical site infection, with a postoperative course depending on the level of contamination.

.

Prophylaxis can be discontinued in patients making a good recovery without signs and symptoms of infection, minimising use and limiting antimicrobial resistance, but can be continued or escalated in patients with persistently raise inflammatory markers or features of sepsis with therapeutic intent to control infection. There is a role for biomarkers specific to sepsis in the context of surgery, which may quickly and reliably diagnose postoperative sepsis to guide clinical management and antimicrobial use and improve clinical outcome.

1.3 Immunity

The immune system is a network of cells, tissues and molecules that protect the host – in this case human patients – from harmful pathogens and works to prevent or eradicate infections, and stimulates a response to repair damaged tissues.(17) It is divided into the innate and adaptive immune systems.

The innate immune system, or natural immunity, acts in response to pathogens to induce inflammation by accumulating and activating leucocytes and plasma proteins at the infection site. Monocytes are a type of peripheral blood mononuclear cell (PBMC), which can differentiate to macrophages, which perform phagocytosis of pathogens, and dendritic cells, which are antigen presenting cells, presenting antigen on their cell surface to T cells in the adaptive immune system. Chemical mediators called cytokines to recruit immune cells to the infection site, activate the complement cascade , remove pathogen via phagocytosis, and activate the adaptive immune system through antigen presentation.

The adaptive immune system is slower and more specialised, compromising of humoral immunity by B lymphocytes, which recognise antigen and produce antibodies to block and eliminate extracellular microbes, and cell mediated immunity by helper T lymphocytes, which eliminate phagocytosed microbes, and cytotoxic T lymphocytes which kill infected cells and clear reservoirs of infection. The adaptive immune system will not be examined in this thesis.

.

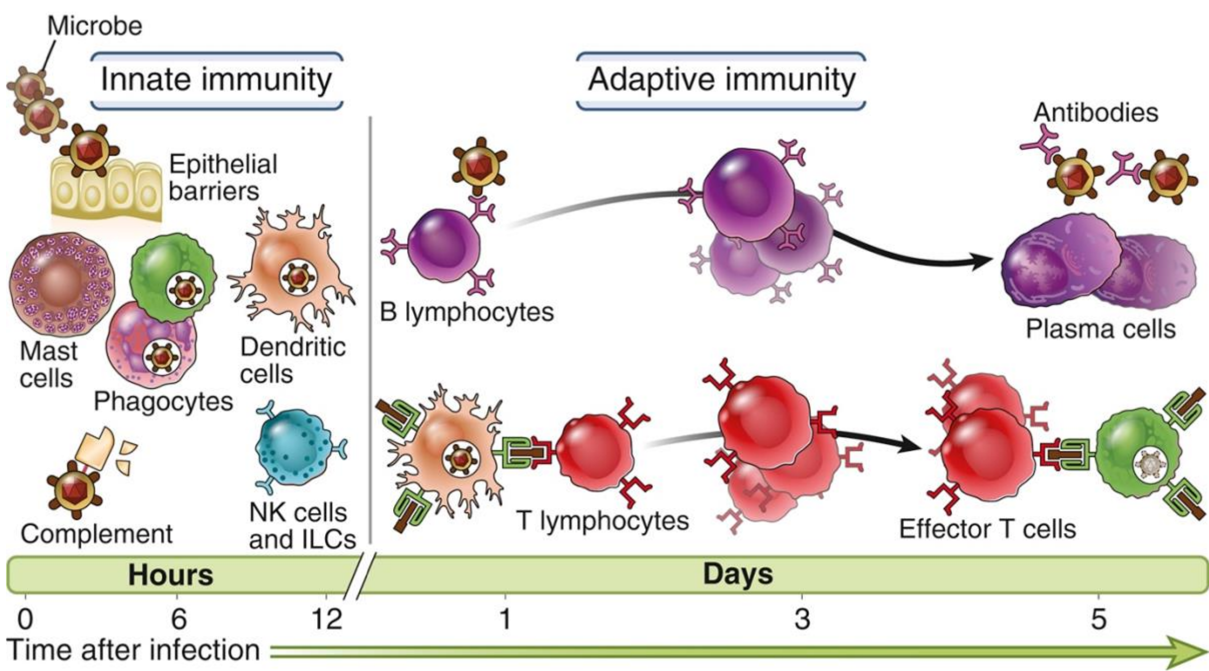


Figure 1-1 Principle mechanisms of the innate and adaptive immune system[16]

Acute Phase Response

The acute phase response (APR) is initiated by the innate immune system in response to a pathogen or injury, such as bacterial lipopolysaccharide (LPS). Monocytes and macrophages recognise the injury or pathogen and produce inflammatory cytokines. These cytokines act on liver hepatocytes which produce acute phase response up to 1000-fold, including production of antimicrobial proteins e.g. CRP, LPS-binding protein, and coagulation proteins e.g. fibrinogen, downregulating others e.g. albumin, and when combined effect may induce leucocytosis, fever, thrombocytosis etc.(18) The clinical syndrome used to describe the effects of acute phase response is the systemic immune response syndrome (SIRS).

.

Monocytes

Monocytes are a large mononuclear white blood cell, whose cytoplasm is equipped to synthesis secretory and membrane proteins. Produced in bone marrow, they circulate in blood in a relatively low proportion, and settle in tissues, differentiating to macrophages and dendritic cells. Monocytes are attracted to sites of infection or injury by endothelial adhesion molecules. Monocytes express cell surface molecules called the cluster of differentiation (CD). CD molecules often act as receptors or ligand that change the behaviour of that cell. Hundreds of CD molecules have been discovered, and there is ongoing research into how their expression related to disease, with potential diagnostic and therapeutic utility.

Cytokines

Cytokines are intercellular signalling polypeptides, produces by many cells and with usually many roles. The main pro inflammatory cytokines are including interleukin-6 (IL-6), interleukin-1β (IL-1β),tumour necrosis factor alpha (TNFα), interferon-γ (IFN-γ)and interleukin-8 (Il-8). These cytokines can be produced by many cell types, but at the induction of APR it is predominantly by macrophages and monocytes at the site of inflammation.(19)

.

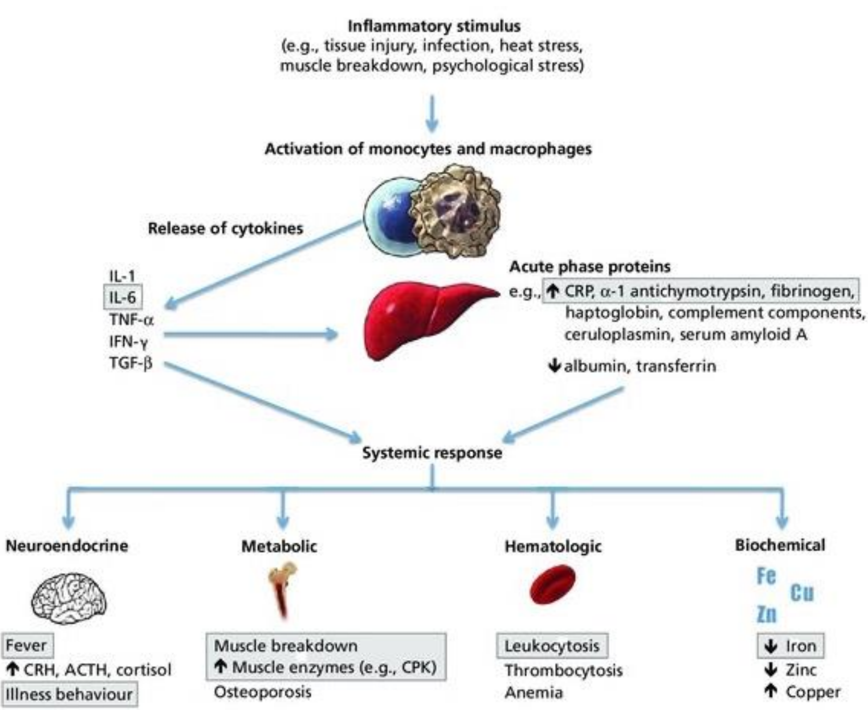


Figure 1-2 The Acute Phase Response[19]

Immunity and Liver Disease

The immune system is dysregulated in liver disease. In cirrhosis, after longstanding injury (from alcohol, infection, autoimmunity etc), healthy liver cells are replaced by fibrotic tissue, causing cirrhosis. This results in immunodeficiency, as the liver loses its local immune surveillance capability, and its ability to produce acute phase proteins in the acute phase response. Simultaneously, immune cell stimulation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattens (DAMPs) from the gut lead to systemic inflammation and upregulation of proinflammatory cytokines.(20) This is known as cirrhosis associated immune dysfunction. Acute liver failure (ALF) is a syndrome of acute onset liver failure due to overwhelming hepatocyte death and multiorgan failure, and is associated with a systemic inflammation and functional immunoparesis, also presenting with immune dysregulation.(21)

.

Immunity in Cancer

Many cancers disrupt. the immune system by causing systemic inflammation.(22) Many cancers disrupt haematopoiesis, leading to leucopenia, often with expansion of immature monocytes and neutrophils which amass at the tumour site, and dysfunctional antigen presentation by dendritic cells.(23) This immature cell population gathering at the tumour site leads to systemic immunosuppression.

1.4 Biomarkers

C-Reactive Protein

C-reactive protein (CRP) is an acute phase protein upregulated in APR with broad functions. CRP binds phosphocholine on dead or dying cells and initiate their phagocytosis, as well as activating the complement system.(19) CRP further induces monocytes to produce further pro-inflammatory cytokines, and PBMCs to produce tissue factor, a procoagulant(24, 25) CRP is one of the routinely used biomarkers in hospital, as quick and relatively inexpensive assay in plasma, clinicians use it to support the diagnosis of an inflammatory or infective process, an monitor for improvement or progression of inflammatory or infective processes, and to guide clinical decisions. In colorectal surgery following resection and anastomosis, many colorectal surgeons use CRP at postoperative day (POD) 3-5 as a screening biomarker of anastomotic dehiscence, with one meta-analysis giving a pooled area under receiver operator curve (AUC) of 0·81 (95% CI 0·75 to 0·86) and a negative predictive value (NPV) of 97% on POD3 with a cutoff of 142ng/L.(26, 27)

.

Leucocyte Count

Similarly, to CRP, the leucocyte count (or white cell count, WCC) as part of the full blood count has long been used as a marker of sepsis or infection. It measures all white blood cells in each sample, and their subgroup counts are presented in the full blood count. WCC $>12 \times 10^9$/L or $<4 \times 10^9$/L is part of SIRS and Sepsis-6 criteria. Clinicians use WCC to support the diagnosis of an inflammatory or infective process, a monitor for improvement or progression of inflammatory or infective processes, and to guide clinical decisions. Leucocytosis is part of the APR, which is a response to both infection/sepsis and tissue injury – as happens in surgery particularly major cavity surgery. The leucocytosis of APR to surgery cannot reliably be differentiated to a response to sepsis/infection, limiting WCC utility following surgery.

Clinical observations

Monitoring of clinical observations or vital signs has long been a key part of hospital care and nursing. Measurement of heart rate, blood pressure, respiratory rate, oxygen saturation and temperature help clinicians assess a patient's clinical condition and monitor for improvement and deterioration. These have been used in the National Health Service as 'medical early warning score' to trigger clinical assessment and intervention, and improve patient outcomes, with a 54% reduction in the number of cardiac arrest calls in one study.(28) These clinical signs are deranged in sepsis representing a physiological response after an infective insult has occurred, which is a limitation.

Each of these markers is used to raise suspicion of an infective process to sepsis, but all can be triggered by the tissue injury response in the early postoperative phase. There is a role for a novel biomarker to diagnose sepsis/infection in the early postoperative phase.

.

Novel Biomarkers

To understand the existing evidence for biomarkers of postoperative sepsis and infection, I conducted a systematic review of available literature on the perioperative use of novel biomarkers in diagnosing postoperative sepsis in patients undergoing major abdominal surgery. The strength of evidence is examined to assess whether novel biomarkers have a role in current practice, or whether further research should be done to discover or validate other biomarkers.

.

1.5 Qualitative Synthesis: Observational Studies examining the Diagnostic Accuracy of Biomarkers of Sepsis and Infection following Major Abdominal Surgery

Methods

Search Strategy

An electronic search of MEDLINE, EMBASE and Cochrane Library was conducted using the terms 'sepsis' (Medical subject heading (MeSH) Major Topic and keyword) or infection (keyword), 'biomarker' (MeSH term and keyword), and 'surgery' (MeSH term or keyword) or from 1996 to June 2020 (searches in supplementary materials). Only human studies and English language studies were considered for inclusion. Bibliographies of relevant studies and the 'related articles' link in PubMed were used to identify additional studies. Any study published only in abstract format or unpublished reports were excluded from the analysis. All citations and abstracts identified were thoroughly reviewed, and secondary references were obtained from the key articles. Studies were reviewed for relevance to diagnostic biomarkers for sepsis in the early postoperative period in patients undergoing major abdominal surgery. Study design and technique were reviewed. Studies were screened by title, abstract, and full text articles were assessed for eligibility with relevant studies included in the synthesis. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidance was utilised.(29)

Inclusion Criteria

Included studies analysed the diagnostic performance of biomarkers for sepsis or infection in patients undergoing major abdominal gastrointestinal (GI) and Hepato-Pancreato-Biliary (HPB) surgery, including transplantation with cutoff values. Abdominal aortic surgery was

.

excluded. Studies were evaluated for duplication or overlapping. Adult and paediatric patients were included, but studies on neonatal patients were excluded.

Exclusion Criteria

Studies were excluded if they reported data from small patient cohorts (<10 patients), or there was overlap with institutions or patient cohorts already published in better quality studies.

Outcome Measures

The primary outcome of interest was the performance (sensitivity and specificity, AUC) of biomarkers in detecting postoperative sepsis. Secondary outcomes were to look at time advantage of the novel biomarker over traditional markers (e.g. Leucocyte count, C-Reactive Protein), and clinical diagnosis of sepsis. Confidence intervals (CI) are reported where given.

Study Selection

Abstracts identified by the search were reviewed to exclude those that did not meet the inclusion criteria. When no abstract was available or the abstract details were inadequate, the full text article was reviewed. Full text articles unavailable online were retrieved using library services.

Results

24 Observational studies and 3 systematic reviews were found that examined the diagnostic performance of biomarkers in postoperative sepsis or infection. 161 were excluded because incomplete results were presented (Figure 1-3). Results are summarised in

.

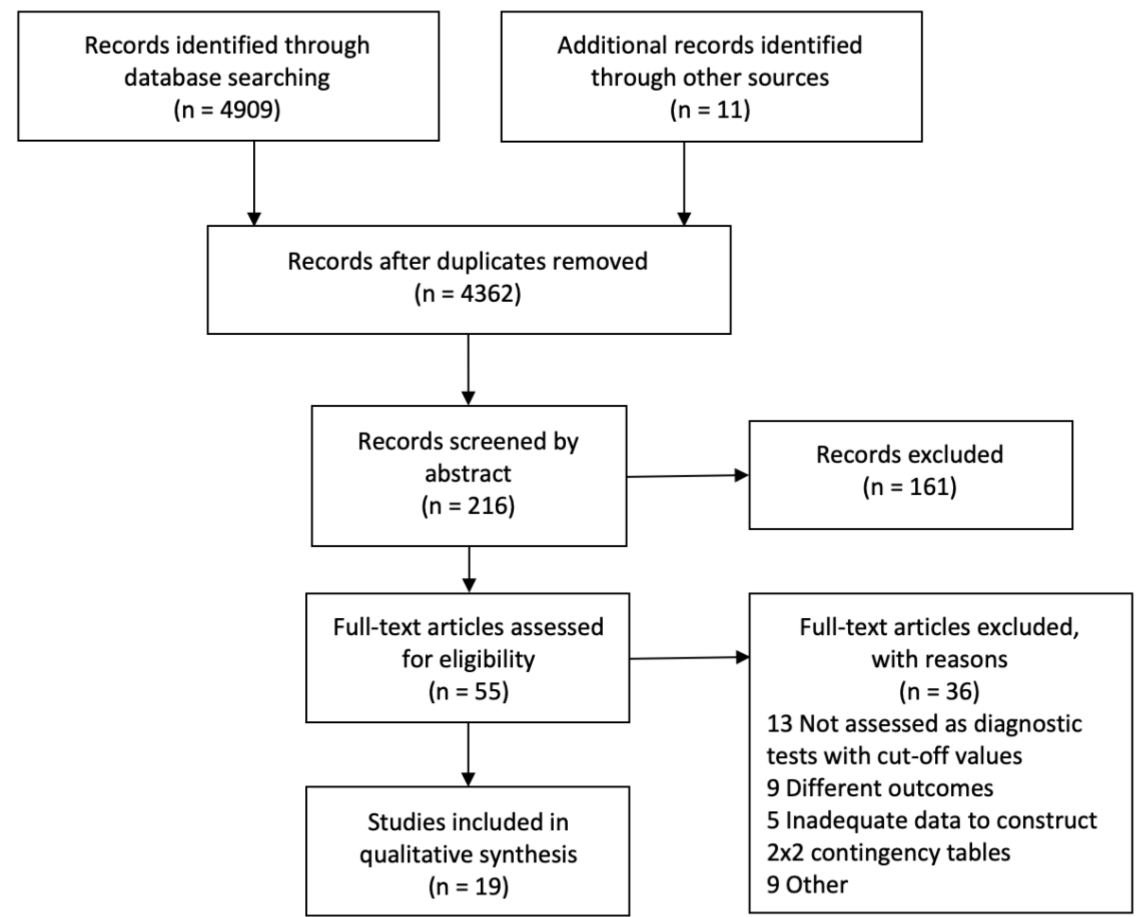


Figure 1-3 PRISMA flow chart of included studies in the systematic review examining the diagnostic accuracy of biomarkers of sepsis and infection following major abdominal surgery

Cytokines

Interleukin-6

A prospective cohort study by Boersama et al examined TNFα, IL1β and IL-6 in 47 adult patients who underwent oncological colorectal resection.(30) TNFα and IL1β were not detectable in all patients, however IL-6 ratio >1.21 on POD1 gave AUC, sensitivity and specificity of 0.825, 0.76 and 0.86 respectively (95% CI 0.69-0.96), compared with AUC of 0.73 for CRP (cut-off value not given). AUC for IL-6 ratio and CRP on POD3 were 0.8 and 0.73 respectively, however timing of infectious complications was not given. These results

.

suggest IL-6 ratio on POD1 performs well to predict postoperative infection, however the paper does not describe the timing of onset of postoperative infection, and incidence of postoperative infection was high (46.8%). The main aim of this study was to examine the relationship between systemic inflammatory cytokines and post-operative ileus, however results did not achieve significance.

Grammatikopoulos et al examined the utility of IL-6, PCT and CRP in differentiating infections and rejection in paediatric liver transplant recipients in a prospective cohort study of 58 patient.(31) PCT and IL-6 performed well as biomarkers of postoperative infection, with AUC, sensitivity and specificity in bacterial infection vs rejection were 0.842, 0.96 and 0.73 for IL-6 ($p<0.0001$ compared to 0.5 area), 1.0, 1.0 and 0.91 for PCT ($p<0.0001$) and 0.739, 0.96 and 0.36 for CRP ($p=0.0046$). Samples were taken at different time points for different patient groups – day 1, day 7 and at the point of a febrile episode and combined in the analysis. The inconsistency in the timing of biomarker sampling limits the ability to draw conclusions on their diagnostic accuracy.

Mokart et al assessed the diagnostic performance of IL-6 and PCT on POD1 in 50 consecutive patients undergoing major elective GI or gynaecological resection.(32) IL-6 yielded the highest diagnostic accuracy, with sensitivity, specificity and AUC were 0.9, 0.58 and 0.821 (95% CI 66.1-98.2) for IL-6, 0.81, 0.72 and 0.749 (95% CI 60.2-89.6) for PCT, and 0.63, 0.72 and 0.664 (95% CI 49.3-83.5) for CRP respectively. All septic events occurred after POD2, with authors concluding that IL-6 and PCT are early markers of postoperative infection.

Zant et al conducted a prospective cohort study of 25 consecutive paediatric patients undergoing liver transplantation examined IL-6, PCT and CRP preoperatively and for 7 consecutive POD.(33) Median age for the population was 2 years, with a range from 19 days to 16 years. IL-6 performed well with AUC, sensitivity and specificity of 0.95, 1.0 and 0.89

.

compared to PCT 0.52, 0.82 and 0.34 respectively. Authors observed that in non-septic patients IL-6 peaked on POD0 then dramatically decreased from POD1, compared with persistently raised values on POD1-7 in septic patients, and significantly correlated with sepsis ($p<0.001$). PCT peaked at POD1 in non-septic patients, followed by as steady decline on POD2-7 contributing to poor specificity (0.34), while PCT in septic patients was persistently raised, but no significant association between PCT and sepsis was found ($p=0.81$). The study is limited by a small patient cohort where sepsis was diagnosed in just 4 patients. IL-6 and PCT values were analysed cumulatively over POD1-7 limiting use as a preclinical predictor of sepsis.

4 studies examining the diagnostic accuracy of IL-6 for sepsis/infection following major abdominal surgery provided adequate data to conduct meta-analysis, which is presented in Chapter 4.

Acute Phase Proteins

Calprotectin

Huang et al reports a prospective case-control study of 163 adults of whom cases were admitted to intensive care following any major operation examined serial changes in serum calprotectin (SC) – a damage-associated membrane protein found in neutrophil cytosol and expressed on monocytes and immature macrophages with inflammation inducing, antimicrobial and apoptosis inducing properties(34) - and PCT to identify cutoff values as a diagnostic screening test for sepsis.(35) The change in SC between POD1 and POD3 (ΔSC_{1-3}) and ΔPCT_{1-3} at 3 days postoperatively showed AUC, sensitivity and specificity of 0.86, 0.87, 0.89 ($p<0.01$) and 0.88, 0.89, 0.9 ($p<0.01$) respectively. ΔSC_{1-3} and ΔPCT_{1-3} The study is limited by heterogeneity in operation types (and ‘major’ operation, elective and

.

emergency), and the 72-hour biomarker assessment limits clinical application as an early predictor of sepsis.

Procalcitonin

Procalcitonin (PCT) has been investigated as a biomarker for postoperative infection/sepsis showing higher diagnostic accuracy than traditional markers such as CRP or WCC,(36-38) which together are often examined as para to index tests for novel biomarkers. PCT is a precursor of calcitonin produced by C cells (parafollicular cells) of the thyroid gland and neuroendocrine cells of the intestine and lung and has been proposed as being a specific marker to bacterial infection and has subsequently been implemented in clinical practice particularly in critical care gaining widespread acceptance during the COVID pandemic when assessing the relative safety of high dose corticosteroid therapy.(36, 37)

In a study of PCT and CRP as markers of postoperative intrabdominal infection (PIAI) following colorectal surgery, Dominguez-Comesana et al measured PCT and CRP in 120 patients on POD0-3.(39) Diagnostic accuracy of PCT was highest on POD1, with sensitivity, specificity of 1.0 (0.48-1.0), 0.69 (0.57-0.78) and 1.0 (0.94-1.0) respectively. Performance of PCT was similar to CRP on POD3 (sensitivity, specificity and AUC of 1.0 (0.4.8-1.0), 0.74 (0.63-0.83) and 0.865 and 1.0 (0.48-1.0), 0.63 (0.52) and 0.864 respectively).
Durila et al examined AST, PCT, IL-6, coagulation profile and thromboelastography (TEG) as biomarkers for postoperative sepsis in this prospective cohort study in 38 patients who underwent oesophagectomy.(40) Researchers tested several cutoff levels on POD1-3. Median time to sepsis diagnosis was 3 days. Coagulation parameters and TEG did not offer significant diagnostic value. AUC values were not given, but for AST, PCT and IL-6 on POD2, sensitivity and specificity were 0.78 and 0.76, 0.78 and 0.76 and 0.78 and 0.83 respectively. The study was limited by small patient numbers, and these results conferred a median diagnostic time benefit of 24h.

.

The IMACORS study by Facy et al examined PCT as a marker of intraabdominal infection (IAI) and all postoperative infection (API) in 501 patients undergoing colorectal surgery, to assess diagnostic performance in 'fast-track' recovery.(41) Sensitivity, specificity and AUC of PCT for IAI on POD4 was 0.82, 0.40 and 0.689 (0.616-0.762) respectively, compared to 0.81, 0.64 and 0.775 for CRP (0.706-0.843). CRP outperformed PCT for API on all days, with PCT AUC on POD4 of 0.671 (0.617-0.724), compared to 0.783 (0.735-0.830) for CRP. Authors concluded that PCT demonstrated lower discriminant ability compared with CRP and in combination with CRP and was not a preclinical biomarker of postoperative sepsis/infection.

A prospective cohort study Figiel et al of 60 adult patients undergoing liver transplantation (LT) examined PCT, neutrophil-lymphocyte ratio (NLR) and CRP and postoperative sepsis.(42) PCT ≥42.8ug/L over 6h postoperatively gave AUC, sensitivity and specificity of 0.64 (0.59-0.89), 0.47 and 0.84 respectively, compared with CRP>142.7mg/L within 48h of 0.728 (0.54-0.92), 0.43 and 0.92. No significant relationship with NLR was observed. The authors concluded that none of these markers were reliable in the diagnosis of post LT sepsis.

Ghiasvand et al conducted a prospective cohort study examining PCT in the diagnosis or infectious disease after adult orthoptic LT.(43) PCT >2ng/L and >5ng/L at POD6-7 performed moderately (AUC 0.7 and 0.67 respectively) with poor sensitivity (0.44 and 0.33), and for PCT>5ng/L on POD1-2 AUC, sensitivity and specificity were 0.78, 0.78 and 0.79 (p values not given). The study was limited by small case numbers (9 LT within cohort) and patients with infections confirmed preoperatively and intraoperatively.

Kaido et al examined PCT preoperatively and in POD2, 3, 5, 7, 14, 21 and 28 in a prospective cohort study of 104 consecutive adult patients undergoing LT. (44) 43 patients

.

(43.3%) developed bacteraemia. PCT ≥2.0ug/L reported positive predictive value (PPV) 0.39 with sensitivity 0.97 and specificity 0.83, and PCT ≥0.5ug/L NPV 0.96, sensitivity 0.57 and specificity 0.97, however p values were not given. Authors found that PCT until day 7 was not significant between groups and so reported on bacteraemia developing on POD8-30 therefore these results cannot be applied as early marker of postoperative sepsis.

Kuse et al conducted a prospective cohort study of 40 adult patients post LT, examining PCT perioperatively and day 0-13.(45) PCT ≥0.8ug/L gave AUC 0.93 – however sensitivity and specificity were not given and authors have not described at what POD this result was available. Optimal cutoff for sensitivity and specificity for PCT (day of diagnosis value – preoperative diagnosis) was given as 5.9ug/L - 1.0 and 0.75 respectively. While clinically significant, these results are at the time of diagnosis – helpful in differentiating sepsis from acute rejection - rather than detecting a preclinical sepsis phase.

Munoz et al examined PCT on POD1 and POD2 in a cohort of 115 patients undergoing laparoscopic sleeve gastrectomy.(46) PCT performed well, but did not outperform CRP for surgical site infection (SSI), with sensitivity, specificity and AUC of 0.7, 0.9 and 0.876 respectively for PCT, and 0.85, 0.9 and 0.923 for CRP.

In a study of 79 patients undergoing elective colorectal surgery, Oberhofer et al measured PCT at baseline and POD1,2,3 and 5(47) On POD2, sensitivity and specificity and AUC were 0.69, 0.79 and 0.75 respectively, comparable to CRP on POD3, 0.76, 0.68 and 0.746 respectively. PCT offers a time advantage with median clinical diagnosis of infection on POD7.

Saeed et al examined PCT on POD1,3 and 6 in 50 patients following cytoreductive surgery for peritoneal malignancy.(48) PCT on POD1 had a sensitivity, specificity and AUC of 0.69.

.

0.62 and 0.689 respectively, outperforming WCC and CRP, but PCT results between infected and non-infected groups converged on POD3.

In a study of 114 consecutive patients undergoing colorectal surgery for cancer by Takakura et al, PCT was measured on POD 1 and POD3.(49) Diagnostic accuracy of PCT for diagnosing SSI was highest on POD1, with sensitivity, specificity and of AUC 0.83, 0.64, and 0.76 respectively, and a higher diagnostic accuracy that CRP on POD3, with DOR 9.79 and 1.74 respectively.

Takeuchi et al conducted a prospective observational study of 30 patients undergoing oesophagectomy, measuring PCT alongside Presepsin (sCD14), WCC and CRP on POD1, 2, 3, 5 and 7.(50) Median day of postoperative complication was POD6. Presepsin performed better than PCT, (and WCC and CRP) with sensitivity, specificity and AUC of 0.6, 0.9 and 0.675 respectively for Presepsin, and 0.5, 0.75 and 0.583 respectively for PCT. Values were highly on POD7, but this is after the median time to clinical diagnosis.

Xiao et al examined PCT in 552 consecutive patients undergoing radical gastrectomy for gastric cancer on POD3 and 5.(51) PCT was more discriminant for post operative infection than WCC on POD3, with sensitivity, specificity and AUC of 0.65, 0.66 and 0.678 respectively for PCT, and 0.51, 0.73 and 0.6 for WCC. The difference between markers was significant (p=0.028), however the diagnostic accuracy of PCT was not particularly high.

Zant et al conducted a prospective cohort study of 25 consecutive paediatric patients undergoing LT and examined IL-6, PCT and CRP preoperatively and for 7 consecutive POD.(33) Median age for the population was 2 years, with a range from 19 days to 16 years. IL-6 performed well with AUC, sensitivity and specificity of 0.95, 1.0 and 0.89 compared to PCT 0.52, 0.82 and 0.34 respectively. Authors observed that in non-septic patients IL-6 peaked on POD0 then dramatically decreased from POD1, compared with persistently

.

raised values on POD1-7 in septic patients, and significantly correlated with sepsis ($p<0.001$). PCT peaked at POD1 in non-septic patients, followed by a steady decline on POD2-7 contributing to poor specificity (0.34), while PCT in septic patients was persistently raised, but no significant association between PCT and sepsis was found ($p=0.81$). The study is limited by a small patient cohort where sepsis was diagnosed in just 4 patients. IL-6 and PCT values were analysed cumulatively over POD1-7 limiting use as a preclinical predictor of sepsis.

10 studies in major abdominal surgery and 6 studies in liver transplantation examining the diagnostic accuracy of PCT in postoperative sepsis/infection provided adequate data to conduct meta-analysis, which is presented in Chapter 4.

Cell Surface Markers

CD64

Jukic et al conducted an observational study examining the change in Cluster of differentiation 64 (CD64) – a cell surface receptor for Immunoglobulin G on neutrophils, monocytes and macrophages – following surgery including colorectal resection, open heart surgery and maxillofacial surgery.(52) CD64 at POD1 and POD2 had a high diagnostic accuracy, sensitivity, specificity and AUC of 0.91, 0.79 and 0.891 on POD1, and 0.82, 0.78 and 0.823 on POD2, and was highly statistically significant in prediction of infection using Pearson Chi-square test on all days $p<0.001$. CD64 outperformed CRP and WCC on POD2 (sensitivity, specificity and AUC of 0.65, 0.72 and 0.68 and 0.36, 0.70 and 0.556 respectively).

.

Toll-Like Receptor 4 and 5

Lahiri et al examined early innate immune dysfunction in 69 adult patients undergoing complex major HPB surgery on POD 1 and 2 in a prospective cohort study.(53) Toll like receptors (TLRs) act as pattern recognition receptors in the initiation of SIRS, where TLR4 and 5 interact with LPS and flagellin components of bacteria respectively to activate intracellular signalling pathways. The study found that TLR4, TLR5 and IL-6 were all highly sensitive and specific in predicting SIRS at median 5 days before clinical signs (9/12 sepsis, 3/12 other SIRS response), and were upregulated in all patients who developed SIRS ($p<0.0001$). AUC for intermediate TLR5 ($CD14^{++}CD16^{+}$) on POD 1 and 2 was 0.89 and 1.0. SIRS was associated with increased length of stay and increased mortality. Quantifying cell surface markers is useful to explore signalling pathways, but utility as a diagnostic marker is limited as measurement using flow cytometry is slow and resource heavy.

Coagulation Markers

Durila et al examined AST, PCT, IL-6, coagulation profile and TEG as biomarkers for postoperative sepsis in this prospective cohort study in 38 patients who underwent oesophagectomy. Researchers tested several cutoff levels on POD1-3. Median time to sepsis diagnosis was 3 days. Coagulation parameters and TEG did not offer significant diagnostic value. AUC values were not given, but for AST, PCT and IL-6 on POD2, sensitivity and specificity were 0.78 and 0.76, 0.78 and 0.76 and 0.78 and 0.83 respectively, all were statistically significant. The study was limited by small patient numbers, and these results conferred a median diagnostic time benefit of 24h.

.

Markers of Organ Dysfunction

Syndecan-1

Holzmann et al examined syndecan-1 (sSDC1) – a product of endothelial glycocalyx shedding in sepsis - in 55 patients undergoing elective major abdominal surgery (major abdominal oncological resection or pancreatic duodenectomy) in a prospective single centre cohort study.(54) AUC, Sensitivity and specificity for sSDC1 in sepsis compared with operated controls was 0.84, 0.87 and 0.72 respectively – however these results did not achieve statistical significance. sSDC-1 at POD1 is suggested as an early predictor of sepsis however the time to sepsis diagnosis was reported only as 'within 25 days' so conclusions about the time benefit of sSDC-1 cannot be drawn

Paugam-Burtz et al conducted a preliminary prospective cohort study of 122 adult patients undergoing liver transplantation examining significant plasma proteins - plasma proteome set (PP) - with PCT and CRP, using a derivation and validation set.(55) AUC was not significantly greater in the plasma proteome set compared with PCT and CRP (0.74, 0.73 and 0.73 respectively) and sensitivity was poor in all tests with high specificity (0.99, 0.97 and 0.97) in all tests. Measurement at POD5 limits the clinical value in application preclinical detection and early intervention.

Review Articles

A 2015 scoping review by Xiao et al examined inflammatory mediators in intra-abdominal sepsis or injury. 182 original studies were included in the synthesis, preclinical, clinical and human and animal studies.(56) The focus of these studies was to predict complications and mortality, not specifically related to surgical intervention. While many studies suggested an association with PCT or IL-6 and morbidity or mortality, the authors concluded that the role

.

of mediators remains unclear, with limitations in all studies on small sample sizes, lack of uniformity in design and outcome measures.

Uzzan et al conducted a systematic review and meta-analysis of the diagnostic performance of procalcitonin for sepsis in critically ill adults and those after surgery and trauma.(57) Of 25 eligible studies, 15 were included in the meta-analysis and only 1 of these related directly to a surgical cohort, (Rothenberger et al[i]) following cardiac surgery. Global odds ration (OR) for the diagnosis of systemic infection was15.7 for PCT and 5.4 for CRP, and overall PCT significantly outperformed CRP. Conclusions form this meta-analysis are not applicable to the surgical cohort as they are underrepresented in the synthesis.

Yu et al conducted a systematic review of the diagnostic performance of PCT in solid organ recipients, with subgroup analysis of 4 studies with 175 participant who underwent Liver Transplantation. Pooled sensitivity was 90% (95% CI 75-97), specificity 81% (95% CI 72-88) and AUC 0.90 (95% CI 0.87-0.92). PCT performed well but one of the four included studies reported outcomes that were not infection. This meta-analysis does not contain all of the most current data in the literature.

.

Table 1-1 Summary of studies included in the systematic review examining the diagnostic accuracy of biomarkers of sepsis and infection following major abdominal surgery

Authors (year)	Location	Surgery Group Mean age	No. of patients	Rate of infection	Biomarker	Timing of Sample	Sensitivity %	Specificity %	AUC
Boersama et al**(30)** 2018	Rotterdam, Netherlands	Colorectal resection 68.6 years	47	46.8% (22)	Interleukin-6	POD1	76	86	0.825
Dominguez-Comesana et al**(39)** 2012	Pontevedra, Spain	Colorectal resection 69.9 years	120	13.3% (16)	Procalcitonin	POD1	100	80	-
Durila et al**(40)** 2017	Prague, Czech Republic	Oesophagectomy Not given	38	23.7% (9)	Procalcitonin Interleukin-6 Aspartate Transaminase Thromboelastography	POD2	78 78	79 83	-
Facy et al**(41)** 2016	Dijon, France	Colorectal resection 65.4 years	463	12.1% (56)	Procalcitonin	POD2	82	40	0.648
Holzmann**(54)** 2018	Hamburg, Germany	Gastrointestinal cancer resection	55	21.8% (12)	Syndecan-1	POD1	87	72	84
Huang**(35)** 2016	Beijing, Chine	Major surgery 53.2 years	163	31.3% (51)	Procalcitonin Calprotectin	POD3	89 87	90 89	0.88 0.86

.

Jukic**(52)** 2015	Ljubljana, Slovenia	Major surgery 65.2 years	229	29.3% (67)	CD64	POD2	82.1	78.0	0.823
Lahiri et al**(53)** 2016	London, UK	HPB resection 63.2 years	69	13.0% (9)	Interleukin-6 TLR4 TLR5	POD2	100	83	0.98
Mokart et al**(32)** 2005	Marseille, France	Gastrectomy 50.7 years	50	32.0% (16)	Procalcitonin Interleukin-6	POD1	81 90	72 58	0.749 0.821
Munoz et al**(46)** 2016	Alicante, Spain	Bariatric 44.8 years	115	11.3% (13)	Procalcitonin	POD2	69	78	0.876
Oberhofer et al**(47)** 2012	Zagreb, Croatia	Colorectal resection 64.9 years	79	36.7% (29)	Procalcitonin	POD2	69	78	0.75
Saeed et al**(48)** 2016	Basingstoke, UK	Cytoreductive surgery 54.7 years	50	28.0% (14)	Procalcitonin	POD1	69	62	0.690
Takakura et al**(49)** 2013	Hiroshima, Japan	Colorectal resection 64.4 years	114	15.8% (18)	Procalcitonin	POD1	83	64	0.76
Takeuchi et al**(50)** 2020	Tokyo, Japan	Oesophagectomy 72 years	30	33.3% (10)	Procalcitonin	POD5	50 -	75 -	0.582 -
Xiao et al**(51)** 2020	Changsha, China	Gastrectomy 56.5 years	552	6.7% (37)	Procalcitonin	POD3	65 -	66 -	0.678 -

.

Chen et al **(58)** (2011)	Tianjin, China	Liver Transplantation 53 years	55	25 (46%)	Procalcitonin	On day of suspicion of sepsis	71 (51-88)	87 (69-96)	0.84 (0.73-0.95)
Figiel et al **(42)** (2020)	Warsaw, Poland	Liver Transplantation 50 years	60	9 (15%)	Procalcitonin	POD3	47 (14-79)	84 (71-93)	0.64 (0.54-0.92)
Ghiasvand et al **(43)** (2019)	Tehran, Iran	Liver Transplantation 47 years	28	9 (32%)	Procalcitonin	POD1-2	78 (40-97)	79 (54-94)	0.78 (0.59-0.92)
Grammatikopoulos et al**(31)** (2012)	London, UK	Paediatric Liver Transplant 2 years	58	23 (40%)	Procalcitonin	POD1/7/at febrile episode	100 (85-100)	91 (77-98)	0.97 -
Kaido et al**(44)** (2014)	Kyoto, Japan	Liver Transplantation 52 years	91	26 (29%)	Procalcitonin	POD8	97 (80-99)	38 (44-69)	- -
Kuse et al**(45)** (2000)	Hannover, Germany	Liver Transplantation Unknown age	40	11 (28%)	Procalcitonin	Infection day-1	67 (31-89)	100 (88-100)	0.93 -
Paugam-Burtz et al**(55)** (2009)	Paris, France	Liver Transplantation Unknown age	61	31 (51%)	Procalcitonin	POD5	32 (17-51)	97 (83-100)	0.73 (0.59-0.87)
Zant et al**(33)** (2014)	Regensberg, Germany	Paediatric Liver Transplant 2	25	4 (16%)	Procalcitonin	POD0-7	82 (19-99)	34 (14-57)	0.52 -

.

Discussion

The review presented here shows that PCT performs moderately well as a diagnostic markers of post operative sepsis, with only moderate diagnostic performance seen in the biggest cohorts reported by Facy et al and Xiao et al. Meta-analysis of PCT in LT, and PCT and IL-6 in major GI surgery is conducted in Chapter 6 to pool results. Calprotectin showed promise with sensitivity, specificity and AUC of 0.86, 0.87, 0.89 ($p<0.01$) in a study by Huang et al, as did sSDC1, with sensitivity, specificity and AUC of 0.84, 0.87 and 0.72 respectively in a paper by Holzmann et al.

For PCT there is inconsistency in when it is tested and what the cut off is. This inconsistency is significant as there are large numbers of small studies, so it is difficult to draw conclusions between them.. A large observational study with consistent sampling and cut-off points for PCT may demonstrate more clearly it's diagnostic benefit. There was further heterogeneity between studies from different countries and hospitals with different practices, different surgical groups. While other markers, including Calprotectin, sSDC-1 and sCD-14, showed promise, the study groups were small and not validated, and warrant further investigation before considering using them in clinical practice. PCT has been available to use for quite some time and has not been adopted into routine practice following surgery. The number of novel biomarkers other than PCT being investigated is low.

The review is limited by including publications in English only, and by excluding papers not reporting outcomes of diagnostic accuracy (sensitivity, specificity, AUC). The search terms may have limited the results, where the title and abstract did not describe all markers analysed. The findings are consistent with other reviews of PCT by Xiao, Uzzan and Yu, and meta-analysis is conducted in Chapter 6 to further examine this.

.

The review has not demonstrated a novel biomarker significantly outperforming traditional biomarkers including WCC and CRP. PCT has been extensively investigated and should be examined alongside Carbon 13 breath delta value (BDV) in this cohort.

.

1.6 ^{13}C Breath Delta Value

Carbon is a non-metallic element, which - with oxygen and hydrogen - forms organic compounds which act as substrates for cellular respiration in living organisms. Classically, in aerobic respiration, the substrate glucose ($C_6H_{12}O_6$) with oxygen (O_2) is metabolised to carbon dioxide (CO_2) and water (H_2O), and the energy from this reaction is converted to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP) through a process called oxidative phosphorylation. ATP provides energy to drive energy processes in all living cells. In animals – including humans - as a waste product of respiration, CO_2 is expired through the lungs during ventilation.

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O + \mathit{ATP}$$

Equation 1-1. Aerobic Cellular Respiration

Carbon naturally occurs in 3 isotopes, ^{12}C, ^{13}C and ^{14}C.^{12}C the most abundant isotope (98.9% universally), with 6 protons and 6 neutrons forming its nucleus. ^{13}C represents 1.1% of universal carbon and is a naturally occurring stable isotope of Carbon with an additional neutron in its nucleus. ^{14}C represents <0.1% of Carbon, an unstable isotope whose beta decay is used in carbon dating. ^{13}C is well researched, with differential fractionation between C3 and C4 plant groups,(59-62) and ^{13}C delta value ($\delta^{13}C$) is commonly utilised in geochemistry, paleoclimatology, palaeoceanography and archaeology to examine historical vegetation and diet. (63, 64) ^{13}C has been implemented in clinical care, with ^{13}C labelled ingested urea a widely used test to diagnose helicobacter pylori infection,(65) research into other ^{13}C labelled breath markers,(66-68) and the use of ^{13}C in magnetic resonance imaging to assess the metabolic activity of tissues. (69, 70)

During the acute phase response in sepsis, innate immune cells including monocytes and macrophages use amino acids (AA) containing carbon to synthesise cytokines used in

.

signalling and bioenergetics homeostasis. Researchers hypothesise that lighter AA are preferentially metabolised in the high metabolic state, such as sepsis or during exercise, and this changes the isotopic composition of carbon in exhaled CO_2.(71, 72) This changes the exhaled ratio of $^{13}CO_2$ and $^{12}CO_2$ and has the potential to act as a marker of the onset of sepsis.

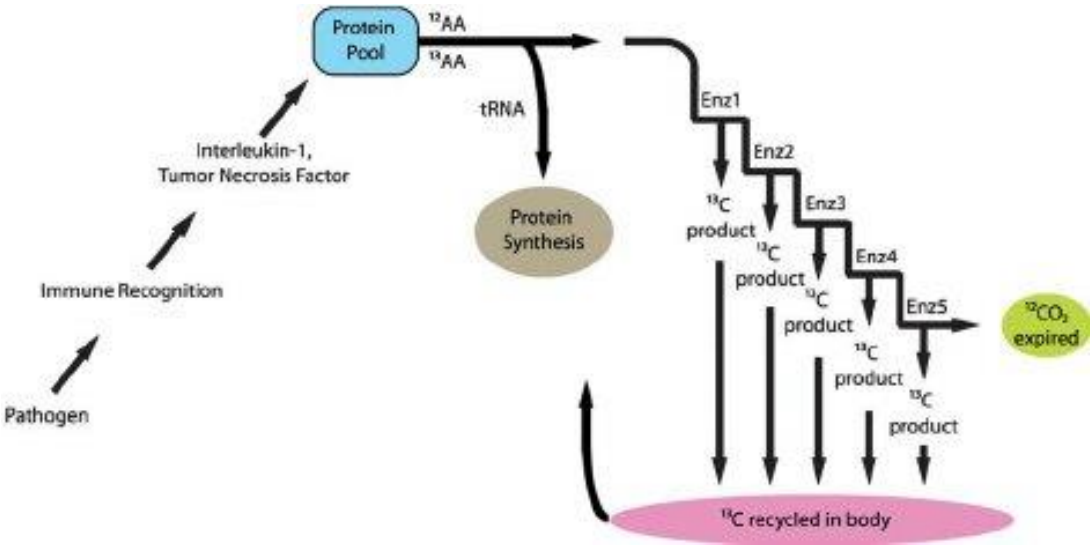


Figure 1-4 Fractionation of Carbon during the Acute Phase Response[72]

Infrared molecular spectroscopy is a well-established method for detecting trace quantities of gases.(73) As infrared electromagnetic radiation passes through a gas, bonds in molecules in the gas absorb the energy and it is converted to kinetic energy in those bonds. Each gas and their isotopes have a characteristic absorption frequency and spectroscopic characteristic, or ‘signature’. The $^{13}CO_2/^{12}CO_2$ breath delta value (BDV) is generated by scanning the absorption frequency of CO_2 by infrared spectroscopy, examining the size of the $^{13}CO_2$ and $^{12}CO_2$ infrared absorption, and comparing to a standard reference material (Pee Dee Belemnite (PDB) where the isotopic ratio is known), as below.

$$\delta = \frac{^{13}CO_2/^{12}CO_2(\text{sample}) - {}^{13}CO_2/^{12}CO_2(\text{PDB})}{^{13}CO_2/^{12}CO_2(\text{PDB})} \times 1000$$

Equation 1-2 Breath Delta Value Calculation(72)

.

Laser Isotope Ratio-meters (LIR) can determine the BDV quickly (<10s), with a precision <0.5‰. Pilot data in a swine model of sepsis have demonstrated that BDV can diagnose the onset of sepsis within 2-4 hours compared to more than 10 hours for physiological parameters.(74) BDV has not been tested in patients hospitalised for sepsis or undergoing major surgery.

1.7 Systematic Review of ^{13}C BDV as a Biomarker of Sepsis and Inflammation

To further assess existing literature on ^{13}C BDV as a clinical marker, I conducted a systematic review of existing literature.

Methods

Search Strategy

An electronic search of Allied and Complementary Medicine Database, British Nursing Index, CINAHL, Embase, Ovid Emcare, Medline, PubMed, and Cochrane Library was conducted from January 1996 to June 2020 using the term ‘breath delta value’. Bibliographies of relevant studies and the ‘related articles’ link in PubMed were used to identify additional studies. All citations and abstracts identified were thoroughly reviewed by the investigators, and secondary references were obtained from the key articles. Studies were screened by title, abstract, and full text articles were assessed for eligibility with relevant studies included in the synthesis. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidance were utilised.

.

Inclusion Criteria

Original studies in humans and animals were considered for inclusion. Adult, paediatric, and neonatal patients were included. Included studies analysed ^{13}C BDV as a marker of infection or inflammation were included. Studies were evaluated for duplication or overlapping.

Exclusion Criteria

Articles which did not report on ^{13}C BDV as a marker of sepsis, infection or inflammation were excluded.

Results

The search returned 6 articles relating to ^{13}C BDV, of which 1 was excluded as it did not relate to ^{13}C BDV as a marker of infection or inflammation, and one was excluded as it was a published tutorial review. Of the 4 remaining papers, 3 were full papers reporting original research, 1 was a published poster abstract excluded due to overlapping data (Figure 1-5). All articles were published by the Butz group, a spectroscopy group in the Department of Animal Studies at the University of Wisconsin – Madison, USA.

.

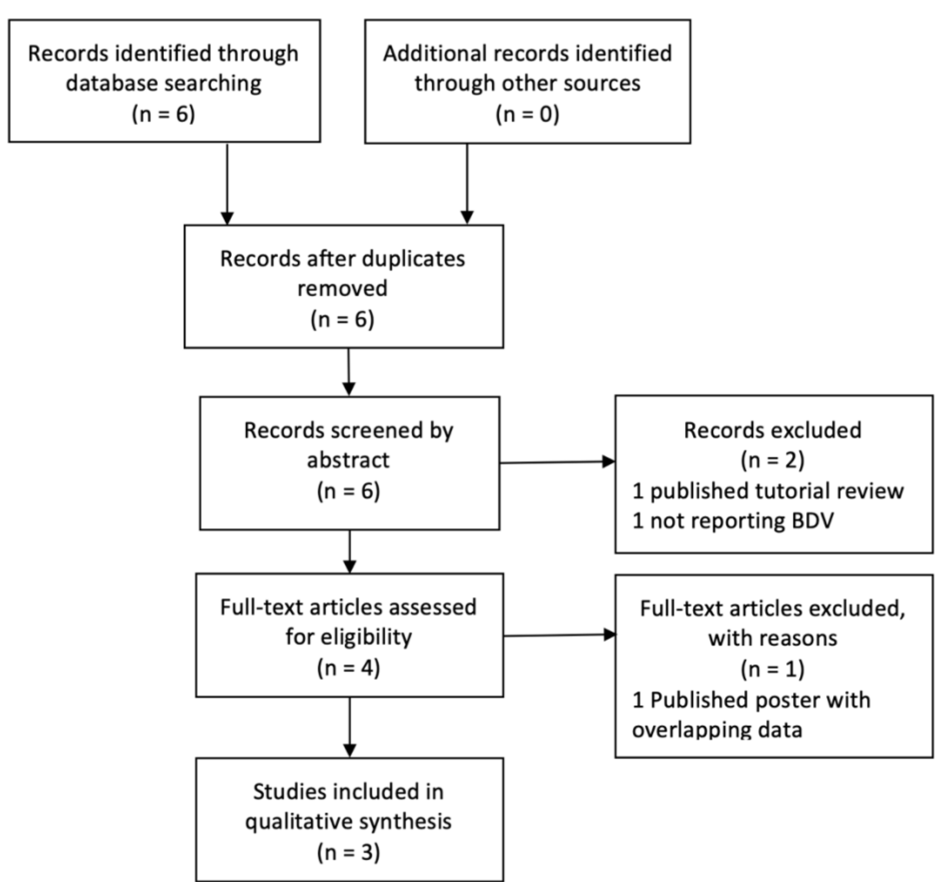


Figure 1-5 PRISMA diagram of studies included in the systematic review of 13C BDV as a biomarker of sepsis and inflammation

Butz et al conducted a case-control study in a swine model, inducing sepsis by caecal ligation and puncture (CLP) in live anaesthetised pigs, and monitoring their ^{13}C BDV, alongside clinical course, physiological parameters, serum markers including CRP and PCT, and serum cytokines.(74) Sepsis was induced CLP in seven pigs, alongside four controls who underwent sham surgery without CLP. Vital signs including mean arterial pressure (MAP) were measured every 15 minutes, central venous pressure and blood glucose was measured every 30 minutes. Where animals central venous pressure (CVP) was <10mmHg or MAP <70mmHg, crystalloid fluid boluses were administered. Blood was collected for plasma isolation at 2, 8 and 15 hours. BDV was monitored continuously via a side stream T-connector. All four control animals survived the 15-hour experiment, 2/7 CLP animals did not respond to fluid boluses and died at 10 and 14 hours respectively, while one CLP animal

.

died from equipment failure. Authors observed a decline in BDV in normotensive BDV CLP animals compared to controls ($p=0.03$) but the slope of the line was not different in hypotensive CLP animals were not different to controls ($p=0.15$). BDV indicated onset of infection at 3.54 hours (standard error of mean (SEM) 0.96), sooner than SIRS criteria – 11.1 hours (SEM 0.94) – ($p<0.001$) in all CLP animals. At 8 hours, BDV was -21.9 (1.5 SEM) in the normotensive CLP group, compared to -19.0 (0.3 SEM) in the control group, and -21.2 (0.7 SEM, $p<0.05$) and -19.2 (0.4 SEM, $p<0.05$) at 15 hours respectively. BDV was not discriminant between the hypotensive CLP group and controls. Cytokines IL-1β, IL-4, IL-12, IFNγ and transforming growth factor-β (TGFβ) did not change during the experiment. TNFα was not detectable in the CLP group (other than a rise at 2 hours only in the hypotensive group), and IL-6 increased in the CLP group at 15 hours. CRP increased in the CLP and control groups and was not discriminant between groups following surgery. PCT increase above baseline by 0.62ng/mL^{-1} (0.12 SEM) at 8 hours and 0.60ng/mL^{-1} (0.08 SEM) at 15 hours in the CLP group. Authors concluded that BDV was the earliest indicator of sepsis, indicating the onset of sepsis before vital signs, including heart rate, MAP and temperature, and plasma cytokines and other markers including CRP and PCT.

This experiment is important as it demonstrates a significant change in the BDV in response to surgical induction of sepsis, despite surgery in all subjects. The change in BDV between control and normotensive CLP group was clear and was significantly different at 8 and 15 hours. The experiment was well controlled, with close homogeneity between animals bred to a similar size (35 kilograms) at the same farm, homogeneity in the surgical procedure and an exact understanding of the time of initiation of sepsis, and homogeneity in their post-surgical management. This was an earlier indicator compared to MAP which was also frequently monitored; however, it is hard to conclude whether this is an earlier indicator than the plasma markers examined as they were only measured at 2, 8 and 15 hours, and may have

.

changed in the interim – particularly between 2 and 8 hours at the time when BDV becomes discriminant.

O'Rourke et al reported the changes in BDV as an early indicator of infection in intensive care patients in 2017.(75) In an observational study, 27 critically ill patients following trauma or acute care surgery were included and breath samples were taken for ^{13}C BDV 4 hourly for 7 days, and correlated with PCT, CRP and clinical diagnosis of infection by a body of independent clinicians taken as the primary endpoint. The mean timing of clinical diagnosis of infection was made on day 3.9 ± 0.63. BDV increased from 1% to 1.7% ($p<0.05$) on day 2. In participants with a clinical diagnosis of infection, with a divergence into significance between the infection and non-infection group on day 1.9, receiver operator curve (ROC) analysis indicated an optimal cutoff value of 1.4%. ^{13}C BDV >1.4% had a sensitivity and specificity of 90.1% and 66.7% respectively, outperforming PCT with sensitivity and sensitivity of 81.8% and 55.6% respectively with a cutoff of 0.05ng/mL, and CRP with sensitivity and sensitivity of 90.9% and 22.2% respectively with a cutoff of 5.0mg/dL.

Boriosi et conducted and observational pilot study on 17 mechanically ventilated paediatric patients without SIRS, with SIRS and with SIRS and shock.(76) BDV was measured in exhaled breath from ventilated participants hourly for 72 hours, and analysed for BDV. BDV was not significantly different in the SIRS group, compared to non-SIRS, and SIRS with shock participants. Participant were further grouped based on infection, trauma or surgery (ITS), to no-ITS, ITS improving, ITS developing infection and ITS septic shock, and participants were included if they provided 9 sequential samples. BDV was significantly lower in ITS developing infection (-23%, SEM 1.3), ITS improving (-23%, SEM 0.5), compared to non-ITS (-19.7%, SEM 0.7) and ITS shock (-19.1%, SEM 1.1)The mean slope of the line was positive for the non-ITS group (0.33, 0.35 SD) and ITS improving groups, and was negative for those developing infection (-0.56, 0.13 SD), and those with septic shock (-0.45, 0.76 SD). BDV was stable in the non-ITS group. Vital signs and WCC were examined

.

at each postoperative day and were statistically different on day 1 for participants developing infection, and on day 1 and 2 for participants in septic shock.

Discussion

These studies from the University of Wisconsin group demonstrate the potential for BDV to be a novel and clinically useful tool in the earlier detection of sepsis in surgical patients. Using a swine model and continuous monitoring, BDV is shown to decrease in response to induction of sepsis, and this is also observed in both adult and paediatric participants with evolving sepsis. In the adult population, BDV is shown to indicate the onset of sepsis before vital signs and other biomarkers and has higher diagnostic accuracy than PCT and CRP.

The studies are limited by their small size, and the number of participants included in the analysis for each group is not well reported. While the swine model showed clear results, animals and the CLP were homogenous, and heterogeneity between the clinical condition of human participants and their interventions may introduce variation and make results difficult to replicate. All studies showed that BDV was not discriminant in hypotensive participants, and authors seem to group participants to exclude those participants from skewing results. The authors may have done this to best demonstrate the difference in BDV in a case without clinical signs and therefore clinical doubt, or to exclude the impact of hypoperfusion on metabolism. They may have done this to create positive results which is likely to introduce bias. The authors cannot conclude that BDV outperforms markers in plasma if they are not measured at the same intervals as BDV.

The review was limited by searching medical journal databases in English only, however all relevant work seems to be produced by one study group at the University of Wisconsin. None of the articles describe the challenges of implementing spectroscopy in a clinical or bedside setting and go into detail about their sampling methodology.

.

Conclusions

BDV is related to developing infection, the authors observe BDV become more negative except for participants with shock and appears to indicate infection before vital signs e.g. MAP, and other commonly used biomarkers including PCT and CRP. ^{13}C BDV should be investigated in the post-surgical setting with frequent observation and several days study, to capture the onset of post-surgical sepsis and infection and examine how this correlates with BDV and other biomarkers of sepsis.

.

1.8 Surgery and the Immune System

Wound creation, tissue resection, reconstruction is recognised as injury by the innate immune system and stimulates APR and the response of monocytes and other white cells to release inflammatory mediators including cytokines. While sterility is maintained where possible, operations on the gastrointestinal tract expose the sterile peritoneal cavity to its colonised gut flora. These operations are described as ‘clean-contaminated’, where contamination is controlled but there is potential for microbial exposure in sterile spaces. The tissue response to injury occurs in 3 phases, the inflammatory stage, the proliferative stage, and the remodelling stage. (77) In the inflammatory stage – in the first 2-3 days – platelets accumulate to achieve haemostasis and build a fibrin clot. Neutrophils act to control microbial invasion, and on day 2-3 are replaced by monocytes and macrophages who produce growth factors. In the proliferative phase, fibroblasts replace neutrophils, monocytes and macrophages and replace the inflammatory matrix with collagen rich granulation tissue, and angiogenesis occurs to deliver blood and nutrients to the healing tissue. In the third stage, remodelling, over weeks and months the number of fibroblasts and macrophages at the injury site reduces and type III collagen is replaced by type I collagen to leave a scar closer to healthy tissue. Gastrointestinal healing at a site of resection and anastomosis happens in a similar fashion, though at a much faster rate and with initial reduced strength at the anastomotic site due to collagenase activity reducing the effect of collagen, stress of transit across the anastomosis, and vulnerability to ischaemia due to hypoperfusion perioperatively.(78)

In this thesis I will be examining patients undergoing Hepatopancreatobiliary (HPB) surgery and Liver transplantation (LT) operated at King’s College Hospital, a large volume tertiary centre in London.

.

1.9 Hepatopancreatobiliary Surgery

Hepatopancreatobiliary (HPB) Surgery is defined as surgery involving the liver, pancreas and biliary system. The liver is a solid organ located in the right upper quadrant of the abdomen, with many functions, and the biliary system is a network of ducts which drain bile from the liver into the digestive tract at the duodenum (first part of the small intestine), and includes the gallbladder, which stores bile and contracts and empties in a hormonal response to the release of cholecystokinin from the duodenum after eating. The liver receives blood from the stomach and intestine via the portal vein (confluence of superior mesenteric vein and splenic vein), and oxygenated blood from the common hepatic artery. Both the artery and vein give branches to the right and left lobes, which further divide/confluence to supply/drain 9 liver segments (Couinaud segment I, II, III, IVa,, IVb, V, VI, VII and VIII) as demonstrated in Figure 1-6.(79) Segments I-IV form the left lobe and segments V-VIII form the right lobe.

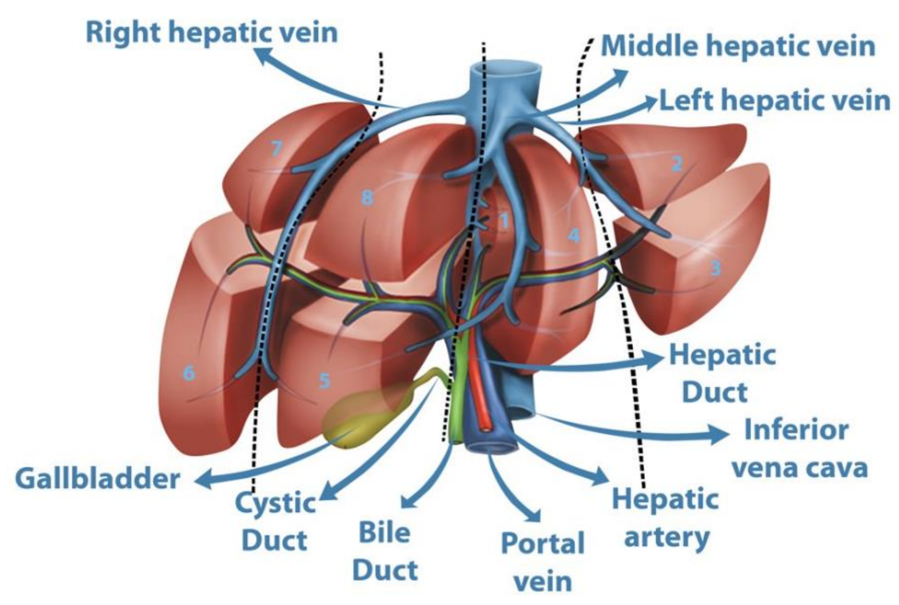


Figure 1-6 Anatomy of the Liver, demonstrating the Couinaud classification(79)

.

The pancreas is a retroperitoneal solid organ which lies behind the stomach in the upper abdomen and drains into the duodenum via the pancreatic duct alongside the common bile duct, with exocrine and endocrine functions, as demonstrated in Figure 1-7.

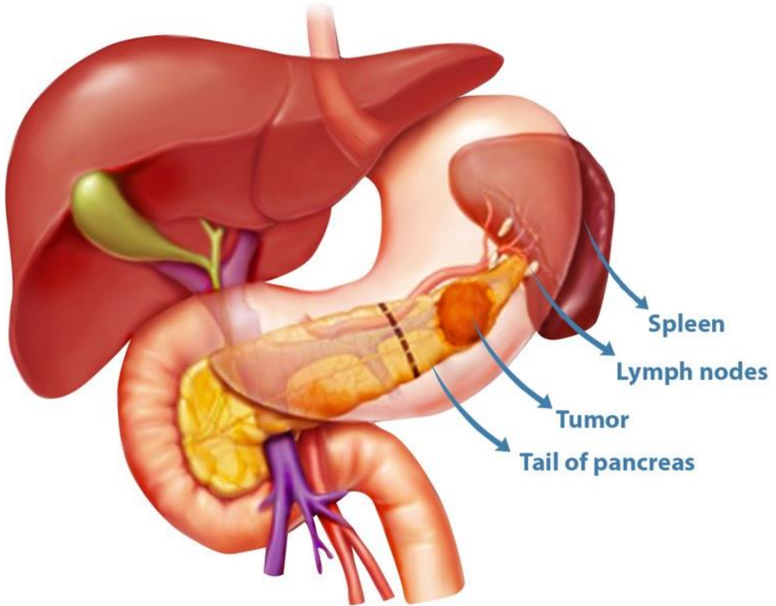


Figure 1-7 Anatomy of the Pancreas (79)

As an endocrine organ, the pancreas produces insulin, glucagon, somatostatin and pancreatic polypeptide, hormones which regulate blood glucose homeostasis. As an exocrine organ, the pancreas produces pancreatic juice, a solution of enzymes including amylase, lipase and trypsinogen, which help break down dietary carbohydrates, fats and proteins. Pancreatic juice drains into the digestive tract alongside bile to digest dietary carbohydrates, lipids and proteins.

Hepatocytes in liver tissue have many functions, glucose homeostasis by gluconeogenesis and glycolysis, protein synthesis and amino acid conversion and storage, lipid regulation by lipogenesis and lipolysis, uptake and storage of vitamins A, B12, D, E, K and minerals such as iron and copper. The liver produces bile – a solution of bile salts, bilirubin, lipids and inorganic salts and water - which helps in the digestion of dietary fats by bile salt anions forming micelles around hydrophobic lipids and dispersing them into droplets. These

.

droplets then increase the surface are for the action of digestive enzymes produced by the pancreas. The liver plays a central role in immunity and the mononuclear phagocyte system. As well as participating in cell-mediated immunity and the complement system, tissue-resident macrophages called Kupffer cells in hepatic sinusoid endothelium encounter and phagocytose gut bacteria, endotoxins and microbial debris from portal venous blood from the GI tract, as demonstrated in Figure 1-8.(80-86)

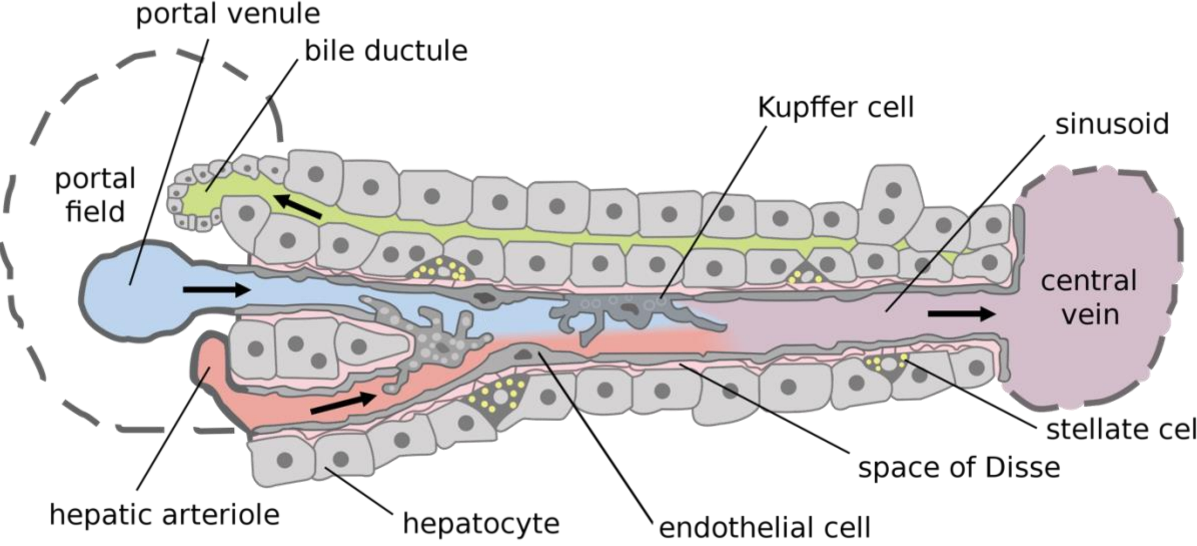


Figure 1-8 Liver sinusoid(87)

.

Indications for HPB Surgery

The most common indication for resection of the liver or pancreas is malignant tumour, leading causes in the liver are hepatocellular carcinoma and colorectal liver metastases, in the pancreas pancreatic adenocarcinoma, and in the biliary system cholangiocarcinoma. Resection is usually planned alongside adjuvant or neoadjuvant chemotherapy, as described in Table 1-2.

Table 1-2 Indications for HPB Surgery

Organ		**Pathology**	**Operation Types**
Liver	Malignant	Hepatocellular Carcinoma (HCC), Colorectal Liver Metastases (CRLM), Gallbladder mass (GB)	Right Hemi-hepatectomy (RH) Extended RH Left hemi-hepatectomy (LH) Extended LH Anatomical liver resection (AR) Non-anatomical liver resection (NAR)
	Benign solid lesions	Haemangioma, focal nodular hyperplasia, hepatocellular adenoma, angiomyolipoma, hepatic lipoma, mesenchymal hamartoma	
	Cystic lesions	Hepatic cyst, hepatobiliary cystadenoma	
Biliary	Malignant	Cholangiocarcinoma	Pancreaticoduodenectomy (PD), Pylorus preserving PD (PPPD), Hepaticojejunostomy (HJ)
	Benign	Biliary stricture	
Pancreas	Malignant	Pancreatic Adenocarcinoma (PA)	Pancreaticoduodenectomy (PD) Distal Pancreatectomy (DP) Hepaticojejunostomy (HJ)
	Benign	Intrapapillary Mucinous Neoplasm (IPMN), Mucinous Cystic Neoplasm (MCN)	

.

HPB Operations

Liver resection is most commonly performed to remove tumours within Liver parenchyma. Lobar and segmental resection follows anatomical principles, resecting affected liver segments/lobes with their arterial and portal venous inflow, and venous and biliary outflow. Surgery is planned to allow for adequate 'future liver remnant' (FLR) – maximising the remnant functional liver tissue post resection to allow for function and regeneration and minimise the risk of post hepatectomy liver failure and its associated morbidity.(88-91)

Right Hemi-Hepatectomy and Extended Right Hemi-Hepatectomy

For tumours involving the right lobe, segments V-VIII are resected with the right portal vein, right hepatic artery, right hepatic duct and right hepatic vein (Figure 1-9). Segments I-IVb are left in situ with perfusion from the left hepatic artery, portal vein, outflow via the middle and left hepatic veins, and biliary drainage through the left hepatic duct. In an extended right hepatectomy, segment IVa and IVb and the middle hepatic vein are resected with segments V-VIII.

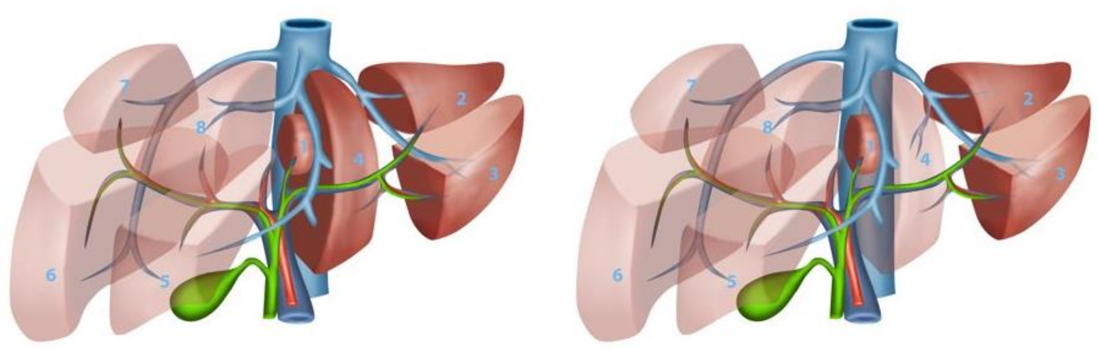


Figure 1-9 Right Hemi-Hepatectomy and Extended Right Hemi-Hepatectomy(79)

.

Left Hemi-Hepatectomy and Left Lateral Segment Resection

For tumours involving the left lobe, segments I, III and IV are resected with the left portal vein, left hepatic artery, left hepatic duct and left hepatic vein (Figure 1-10). Segments I, V-VIII are left in situ with perfusion from the right hepatic artery, portal vein, outflow via the middle and right hepatic veins, and biliary drainage through the right hepatic duct. In a left lateral segment resection, only segments II and III are resected, leaving segment IV in situ.

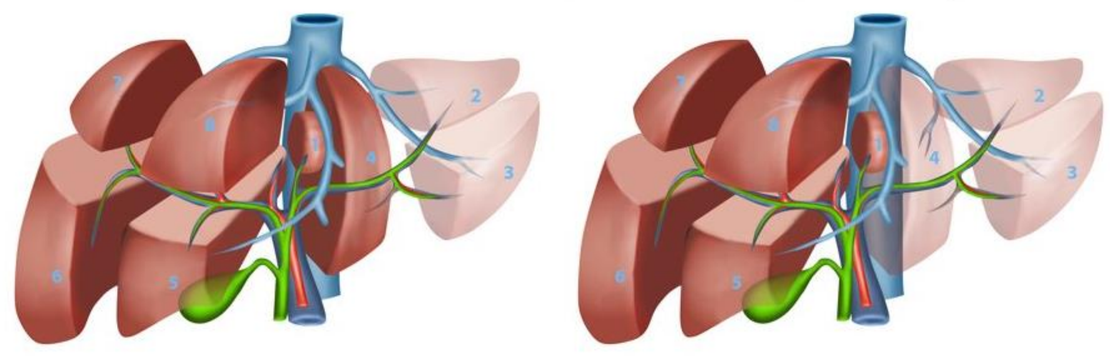


Figure 1-10 Left Lateral Segment Resection and Left Hemi-Hepatectomy(79)

Anatomical/Non-anatomical Liver Resection

Where tumour involves one segment of the liver with a segment dependent tumour margin, that segment can be removed along with potential tumour-bearing portal tributaries. This is called an anatomical resection (AR).(92, 93) The alternative is non-anatomical resection (NAR), where the tumour is resected with grossly macroscopic negative margins (which can be confirmed with frozen section histopathological examination), which clinicians may choose to preserve parenchyma, particularly for HCC which usually develops in cirrhotic livers. The risk and benefit of each remains unclear and operative technique is chosen on an individual case basis.(94-102)

.

Pancreaticoduodenectomy

Pancreaticoduodenectomy (PD) (or Whipple's procedure) is commonly performed to remove tumour in the head of the pancreas or extrahepatic common bile duct (Figure 1-11), as described in Table 1-2. The head of pancreas and distal common bile duct is resected en bloc with the pylorus of the stomach, the duodenum and surrounding lymphatics. The portal vein and superior mesenteric artery lying just posteriorly are left in situ. A Roux-en-Y jejunal limb is formed on which the proximal common bile duct is anastomosed (forming a hepatojejunostomy (HJ)) restoring biliary continuity with the gastrointestinal tract, and the distal pancreatic duct is anastomosed (forming a pancreatojejunostomy (PJ)) restoring pancreatic drainage into the gastrointestinal tract. A gastrojejunostomy is formed to restore continuity from the stomach to the small intestine.

Figure 1-11 Pancreaticoduodenectomy(79)

Hepaticojejunostomy

At the time of PD, some patients are found to have disease progression such that a curative resection is not possible. In such cases, Hepaticojejunostomy (HJ) can be performed, to restore biliary drainage from the liver into the GI tract, which while not curative can decompress post-hepatic biliary obstruction, allowing restoration of liver function and alleviating symptoms of jaundice.

.

Distal Pancreatectomy

Distal Pancreatectomy (DP) is usually performed to remove tumour in the body or tail of the pancreas, as described in Table 1-2 (Figure 1-12). The pancreas is resected proximal to the tumour margin along with the spleen and its associated blood supply (splenic artery and vein) and lymphatic drainage. The spleen is resected due to its shared blood supply with the pancreas and the risk of vascular or lymphatic metastasis.

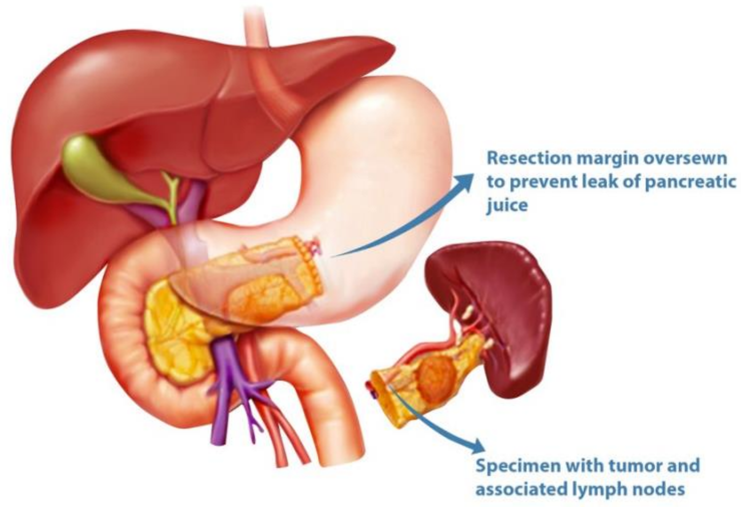


Figure 1-12 Distal Pancreatectomy and Splenectomy

1.10 Liver Transplantation

Liver Transplantation (LT) is a major surgical intervention for people with poor predicted survival or quality of life secondary to acute or chronic liver disease (Immunosuppression is induced using high dose corticosteroids (hydrocortisone or prednisolone), which has a non-specific anti-inflammatory on intracellular receptors which are usually weaned within the first 6-8 postoperative weeks and maintained by a calcineurin inhibitors (usually Tacrolimus or Prograf) which block the T-cell mediated response by inhibiting IL-2 production.

.

Table 1-3). In principle, and following judicious assessment, selection and consent, the diseased recipient liver is surgically removed (explanted) and a non-diseased liver from a cadaveric donor is implanted to restore the function of the liver. Following transplantation, the recipient takes medication to suppress immune-mediated rejection of the donor liver graft. Immunosuppression is induced using high dose corticosteroids (hydrocortisone or prednisolone), which has a non-specific anti-inflammatory on intracellular receptors which are usually weaned within the first 6-8 postoperative weeks and maintained by a calcineurin inhibitors (usually Tacrolimus or Prograf) which block the T-cell mediated response by inhibiting IL-2 production.

.

Table 1-3 UK Adult Liver Transplant Indications(103)

	Aetiology	Brief Pathophysiology
Cirrhosis	Alcohol related liver disease (ArLD), Non-alcoholic fatty liver disease (NAFLD), Non-alcoholic steatohepatitis (NASH), Primary sclerosing cholangitis (PSC), Primary biliary cirrhosis (PBC), Autoimmune Hepatitis (AIH) Viral Hepatitis, Wilson's Disease	Progressive liver injury and fibrosis resulting in portal hypertension and decompensation, including ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, variceal haemorrhage, hepatorenal syndrome, and hepatocellular carcinoma(104, 105)
Tumour	Hepatocellular Carcinoma (HCC) a. Single tumour ≤5cm b. Up to 5 tumours all ≤3cm c. Stable single tumour 5cm-7cm	Usually secondary to cirrhosis (as above), too large or too many to resect
	Unresectable Colorectal Liver Metastases	Metastasis via the inferior portal venous system, unresectable
	Hilar and Intra-hepatic CCA	Adenocarcinoma arising in the biliary system, unresectable
	Unresectable NET liver metastases	Metastasis via the inferior portal venous system, unresectable
Acute Liver Failure (ALF)	Paracetamol overdose Drug induced Viral Hepatitis Idiopathic	Multi-system disorder in which severe acute impairment of liver function with encephalopathy occurs within 8 weeks of the onset of symptoms and no recognised underlying chronic liver disease(106)
Acute-on-Chronic Liver Failure (ACLF)	*(all cirrhosis causes)*	Decompensation of pre-existing liver disease with single or multiorgan failure subsequent to a precipitating event (e.g. infection, GI bleed, alcoholic hepatitis, HBV reactivation) with high mortality risk (107, 108)
Variant	Intractable pruritus; Hepatopulmonary syndrome; Familial amyloidosis; Primary hypercholesterolaemia; Polycystic liver disease; Hepatic epithelioid haemangioendothelioma; Recurrent cholangitis; Nodular regenerative hyperplasia; Hereditary haemorrhagic telangiectasia; Glycogen storage disease; Ornithine transcarbamylase deficiency; Primary hyperoxaluria; Maple syrup urine disease; Porphyria; Amyloidosis-other	

.

Donor Organs

In the UK, most patients receive liver grafts from cadaveric donors, with a small proportion receiving living-related donor liver transplants. After referral for organ donation, and after verification of death - either with brainstem death testing or verification of cardiopulmonary arrest – surgery is performed to retrieve the liver with its biliary drainage, inflow and outflow blood vessels. In brief, the liver undergoes in situ perfusion via the aorta (main artery from the heart) and the portal vein, using an isotonic solution – University of Wisconsin solution (UW) – while draining it of blood via the vena cava (main vein to the heart), flushing out blood and minimising thrombus formation in small and large blood vessels; biliary system flushing to clear as many biliary vessels as possible; and cooled using sterile crushed ice. Dissection is completed and following perfusion, the liver is removed and stored in cold UW surrounded by ice. Cooling minimises ongoing anaerobic cell metabolism after the cessation of perfusion with oxygenated blood, which can lead to the build-up of lactic acid and free radicals which are damaging to tissues. Flushing of vessels and ducts maximises the potential for their patency and function following reimplantation in the recipient, and these techniques are key to optimising liver graft function after implantation.

Liver Transplantation Operations and Principles

Following retrieval and assessment of the liver graft, the recipient patient undergoes orthotopic liver transplantation (OLT) (Figure 1-13). Under general anaesthesia, a laparotomy is performed using a reverse T incision to access the right upper quadrant of the abdomen and remove the recipient's native liver, surgically disconnecting it from its arterial and portal venous inflow, venous outflow and biliary drainage. The donor liver graft is them implanted with using patient and surgeon dependent techniques, but in principle venous outflow is created by anastomosing the confluence of hepatic veins or vena of the donor liver graft to the recipient vena cava, venous inflow by anastomosing the recipient portal vein to

.

the donor liver graft portal vein, arterial inflow by anastomosing the recipient common hepatic artery (or other if variant anatomy) to the liver graft common hepatic artery, and biliary drainage with a ‘duct-to-duct’ recipient to donor choledochal anastomosis or hepaticojejunostomy (donor liver graft bile duct to recipient jejunum roux limb). The liver graft is reperfused in the recipient circulation and the liver graft is rewarmed to core body temperature. Restoration of blood flow homeostatic temperature - with delivery of oxygen and glucose and removal of carbon dioxide and waste – allows cell aerobic respiration to resume and liver graft tissues should begin to function.

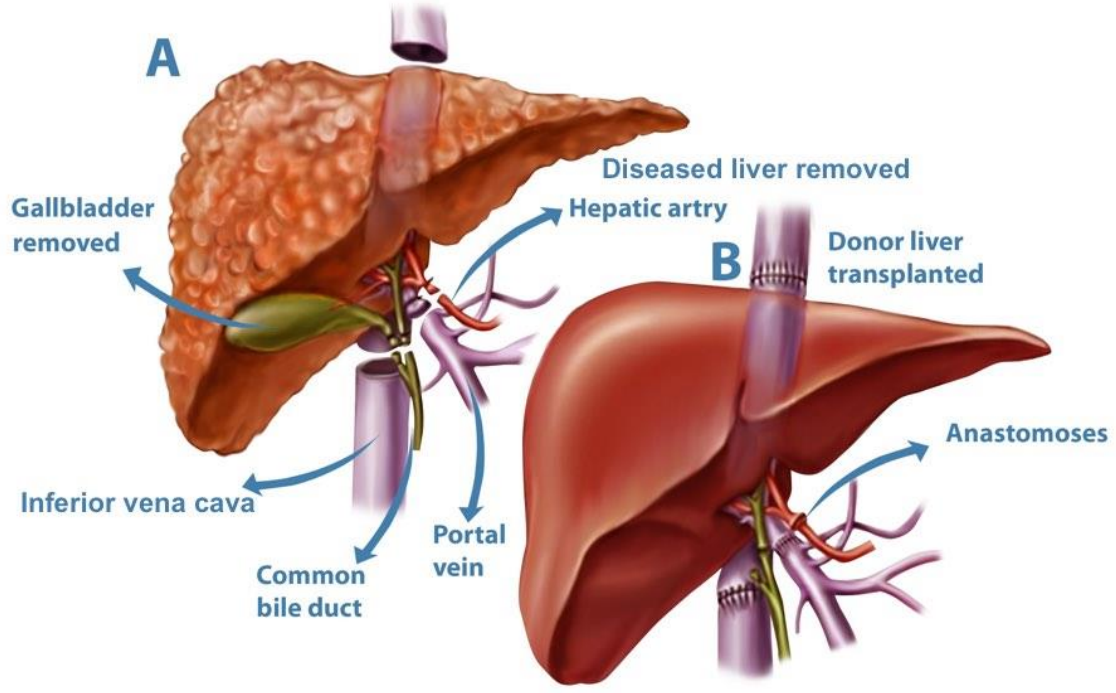


Figure 1-13 Orthotopic Whole Liver Transplantation

Inferior Vena Cava Replacement, Portal Vein Anastomosis, Single Arterial Anastomosis, Primary Choledochal Anastomosis(79)

Orthotopic Right Lobe Liver Transplant (ROLT)

Following the same implantation principles as whole liver transplant however the liver graft has been split with the left lobe or left lateral segment being removed to be transplanted into

.

a second recipient (usually a child) as seen in Figure 1-10. The main donor vessels and ducts stay with the right lobe and are anastomosed as with a whole liver.

Auxiliary Liver Transplantation (Aux-LT)

In cases of ALF where there is potential for recovery of the native liver graft, the surgeon may split the recipient liver leaving the left lateral segment in situ, and removing the recipient right lobe, as seen in Figure 1-9. The right lobe liver graft is then implanted using the same principles alongside the native left, allowing the potential recovery of the native liver. If the native liver recovers, immunosuppression can be weaned over time allowing the native liver to regenerate. If the native liver does not recover, immunosuppression and function of the liver graft is maintained.

.

1.11 Biomarkers for investigation

The cytokines examined in this work were taken from a panel of 10 pro-inflammatory cytokines produced to use in MesoScale Discovery assay, as discussed in Chapter 2-11. The panel has previously been used within the research group with reliable results, and the markers of interest are related to proinflammatory states, in keeping with the aim to investigate alongside the role of BDV in infection and Sepsis. Included cytokines and their actions/features are included in Table 1-4. Cell surface markers examined in the HPB group were selected based on established practice within the group, but particularly their role in inflammation and infection, and inducing the acute phase response, summarised in Table 1-4.

Alongside cytokines and cell surface markers, other biomarkers including CRP, SOFA score, WCC and differential and percentage of WCC, and neutrophil lymphocyte ratio, lymphocyte monocyte ratio, neutrophil monocyte ratio, platelet lymphocyte ratio were analysed from clinical data recorded in the CRF. Recent work has suggested these ratios taken from a simple full blood count may be indicators of infection, bacteraemia, inflammation, and other diseases including cardiovascular disease, and so were included in the analysis to examine their association with BDV and clinical outcomes.(109-113) .

.

Table 1-4 Biomarkers analysed in the thesis

Biomarker	Action	Experiment
BDV	Lighter amino acids used in respiration changes the isotopic ratio of ^{12}C and 13C in exhaled CO_2	LIR
PCT	Precursor of calcitonin which is a specific marker to bacterial infection	ELISA
IFNγ	Pro-inflammatory cytokine, potent activator of macrophages	MSD
IL-1β	Pro-inflammatory cytokine, induces IL-2, B cell maturation and proliferation, fibroblast GF and healing, pyrogenic	MSD
IL-2	T cell growth factors, regulates T cells	MSD
IL-4	B cell stimulatory factor/lymphocyte stimulatory factor, B cell activation and induces DNA synthesis, IgG/IgE expression	MSD
IL-6	Induces acute phase response, differentiates B cells into immunoglobulin secreting cells	MSD
IL-8	Attracts neutrophils, basophils, T cells and neutrophils	MSD
IL-10	Cytokine synthesis inhibitory factor, suppresses pro-inflammatory response	MSD
IL-12p70	Produced by macrophages and T lymphocytes, activates T cells and NK cells to produce IFNγ	MSD
IL-13	Positive regulator of B cell proliferation, macrophage activation, Ig p	MSD
TNF-α	Proinflammatory cytokine produces by macrophages, induces inflammatory response, endogenous pyrogen	MSD
CD14	Receptor for LPS/LBP, LPS recognition	Flow Cytometry
CD16	Component of low affinity Fc receptor, phagocytosis and antibody-dependent cell-mediated cytotoxicity	Flow Cytometry
CD155	Poliovirus receptor participates in a considerable number of immunoregulatory functions through its interactions with activating and inhibitory immune cell receptors	Flow Cytometry
CD163	Monocyte/macrophage-specific membrane marker. Specifically, CD163 is considered a marker of alternatively activated or anti-inflammatory macrophages	Flow Cytometry
CCR2	Mediates monocyte chemotaxis. Monocyte chemoattractant protein-1 is involved in monocyte infiltration in inflammatory	Flow Cytometry
HLA-DR	Present peptide antigens, potentially foreign in origin, to the immune system for the purpose of eliciting or suppressing T-(helper)-cell responses that eventually lead to the production of antibodies against the same peptide antigen	Flow Cytometry
MerTK	Regulation of cytokine secretion and clearance of apoptotic cells	Flow Cytometry

.

PD-L1	Suppressor of the adaptive immune response, by binding the inhibitory checkpoint PD-1, reducing proliferation of antigen specific T cells and apoptosis in regulatory T cells		Flow Cytometry
PD-1	Immune checkpoint which promotes apoptosis(programmed cell death) of antigen-specific T-cells in lymph nodes. Second, it reduces apoptosis in regulatory T cells		Flow Cytometry
CRP	Acute phase protein binds phosphocholine on dead or dying cells and initiate their phagocytosis, induces complement activation, induces monocytes to produce pro-inflammatory cytokines, and PBMCs to produce tissue factor		Clinical Bloods
WCC	Undifferentiated leucocyte count, participating in innate and adaptive immunity to mount inflammatory/cellular response		Clinical Bloods
Leucocyte differential	Neutrophils	Part of the innate immune system, phagocytes at site of infection or inflammation	Clinical Bloods
	Lymphocytes	T and B cells, and innate	
	Monocytes		
Leucocyte ratios	NLR	Neutrophil: lymphocyte ratio, increased value indicates a shift towards neutrophil production,	Clinical Bloods
	LMR	Lymphcyte: monocyte ratio, increased value indicates a shift towards lymphocyte production	
	NMR	Neutrophil: monocyte ratio increased value indicates a shift towards lymphocyte production	
	PLR	Platelet: lymphocyte ratio increased value indicates a shift towards platelet production	

.

1.12 Hypothesis and aims of the thesis

The immune system is dysregulated in liver disease and cancer, and no reliable biomarker for diagnosing postoperative infections or sepsis. Given the previous findings on the diagnostic utility of ^{13}C BDV in sepsis and infection, I hypothesise that is that ^{13}C BDV is associated with postoperative sepsis and is a potential biomarker of sepsis following HPB surgery and Liver transplantation. I aim to:

a. Characterise the BDV response to HPB surgery and LT using infrared laser spectroscopy
b. Characterise the cytokine response to HPB surgery and LT using MesoScale Discovery Assay
c. Characterise the monocyte phenotype following HPB surgery
d. Examine differences in these markers between groups who do and do not develop infective complications/sepsis, and compare these to traditional biomarkers of infection

.

2 Methods

2.1 Participant Recruitment

Participants were recruited under the Immuno-metabolism in Sepsis, Inflammation and Liver Failure Syndromes (I-MET) protocol at King's College Hospital London, between June 2020 and March 2021 (Appendix 2). Research ethics was granted with Research Ethics Committee Number 19/NW/0750, Integrated Research Application System number 244089 within 24 hours of admission to hospital.

Screening

Consecutive patients admitted for major HPB surgery or liver transplantation at King's College Hospital London were screened for eligibility. Eligible patients were approached and given verbal and written information before deciding whether to give informed consent. Patients who lacked capacity due to critical illness or pre-existing cognitive impairment were consented by their next of kin consultee where available, or by an independent treating Consultant clinician as professional consultee, and per I-MET protocol. Retrospective patient consent was sought if a patient regained capacity. Healthy controls, and patients with sepsis and liver disease were recruited and sampled to be included as control group.

.

Inclusion Criteria

Adult participants

- Healthy subjects as control, recruited from staff at the Institute of Liver Studies at King's College Hospital who did not meet exclusion criteria.
- Patients undergoing major surgery - Laparotomy/Laparoscopic-assisted surgery requiring post-operative admission to the critical care unit
- Patients with sepsis or suspected sepsis
- Patients with acute hepatic failure or chronic liver disease

Exclusion Criteria

- Age <16
- Evidence of disseminated malignancy (isolated hepatocellular carcinoma without evidence of secondary spread is not an exclusion criteria)
- Pre-existing immunosuppressive states including HIV infection and chronic granulomatous diseases.
- Immunosuppression other than low dose steroids (defined as >40mg prednisolone or equivalent)
- Pregnancy

.

2.2 Blood Sampling

Venous or arterial blood was taken preoperatively (baseline), day 1/2 postoperatively (+- 1 day), day 4/5 postoperatively (+- 1 day) and day 8/9 postoperatively (+-1day)(**Error! Reference source not found.**). Approximately 45ml of blood was taken from central access where available, or peripherally if patients had no central access. Bloods were taken in 3 x 10mL Lithium Heparin tubes, 2 x 5mL Ethylenediaminetetraacetic acid (EDTA), 1 x 5mL Citrate and 1 x Serum tubes (Table 2-1 Sampling Schedule

	Day 1 Baseline	Day 2 POD1	Day 3 POD2	Day 4 POD3	Day 5 POD4	Day 6 POD5	Day 7 POD6	Day 8 POD7	Day 9 POD8	Day 10 POD9
Consent										
CRF	CRF 1		CRF 2 +-1 day			CRF 3 +-1 day			CRF 4 +-1 day	
Clinical Data										
Breath	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Blood	Sample 1		Sample 2 +-1 day			Sample 3 +-1 day			Sample 4 +-1 day	

.

). If patients were anaemic (Haemoglobin <70 or <80 with ischaemic heart disease, symptomatic) or actively bleeding blood sampling did not proceed. If a patient declined blood sampling, bloods were not taken. In patients who were unable to be sampled, plasma and serum samples were requested from remaining routinely collected clinical samples via Viapath (King's College Hospital Central haematology and biochemistry laboratory) and stored at -80°C.

.

Table 2-1 Sampling Schedule

	Day 1 Baseline	Day 2 POD1	Day 3 POD2	Day 4 POD3	Day 5 POD4	Day 6 POD5	Day 7 POD6	Day 8 POD7	Day 9 POD8	Day 10 POD9
Consent										
CRF	CRF 1		CRF 2 +-1 day			CRF 3 +-1 day			CRF 4 +-1 day	
Clinical Data										
Breath	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Blood	Sample 1		Sample 2 +-1 day			Sample 3 +-1 day			Sample 4 +-1 day	

.

Table 2-2 Blood Samples

Specimen Tube	Additive	Volume	Number	Sample Type	Aliquots for Frozen Storage
	Lithium Heparin	9mL	3	PBMCs Lithium Heparin plasma	1-2 x Cryovials PBMCs 2-3 x 500µL Lithium Heparin plasma 2-3 x 130µL Lithium Heparin plasma
	EDTA	4mL	2	Whole blood EDTA plasma	1 x Whole blood EDTA tube 1 x 500µL EDTA plasma 8 x 130µL EDTA plasma
	Citrate	3.2mL	1	Citrate plasma	1 x 500µL Citrate plasma 8 x 130µL Citrate plasma
	Serum	6mL	1	Serum	1 x 500µL Serum 8 x 130µL Serum
	Pagane	2.5mL	1	Intracellular ribonucleic acid (RNA)	1 x PaxGene tube

.

2.3 Breath Sampling

Breath samples were taken daily between 06:00 and 12:00 using Supel™-Inert Multi-Layer Foil gas sampling bags with Thermogreen LB-2 septa, with Screw Cap Valve (SCV) and Push/Pull Lock Valve (PLV) (**Error! Reference source not found.**). These bags are designed for sampling low molecular weight compounds such as carbon dioxide (CO_2). The multi-layer foil is composed of two outer layers of aluminium film providing a barrier to gases permeating through walls of the bag. Thermogreen LB-2 septa installed in the valve fitting minimises the risk of gas leak, and they are chemically inert with moisture and light protection. This means that samples can be gathered and stored for simultaneous processing.

Figure 2-1 Supel™-Inert Multi-Layer Foil gas sampling bags

After a demonstration, participants were asked to inflate the sampling bag as full as they could manage by blowing into the valve, which was then locked and sealed until processing. Where patients were ventilated, samples were taken from the expiratory circuit of the ventilator tubing using Intersurgical Connector 22M (22 French gauge + 6mm stem), at the patient end and ventilator end for later comparative analysis (Figure 2-2).

.

Samples were taken in the morning between 6am and 12pm as close to 8am as possible as schedules would allow. Samples were stored locked and sealed at room temperature until sample analysis.

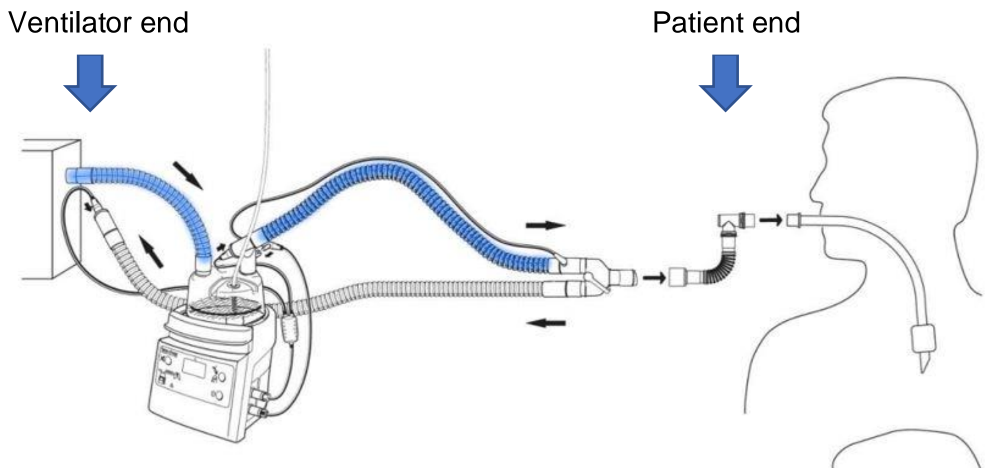


Figure 2-2. Ventilator Circuit for Breath Sampling

The BDV analysis from the sample bags was carried out at the Laser Spectroscopy Laboratory of the Space Science and Technology department (aka RAL Space) of the Rutherford Appleton Laboratory. Samples were stored at room temperature and analysed as discussed in Chapter 4

2.4 Clinical Data Collection

Baseline clinical data were collected on the day of recruitment, including the participant's baseline characteristics, indication for surgery, past medical history, medications, anthropometrics, vital signs and baseline blood results, using a case report form (CRF) and data were stored on a central database. Data on the operative procedures performed, duration of surgery, estimated blood loss (EBL), donor graft factors (for LT participants) and intraoperative complications were collected. Where EBL was documented as <500ml, a

.

value of 500ml was recorded. CRFs were completed at each sampling day including the patients' vital signs, blood results and any clinical issues and interventions including episodes of sepsis, infection, procedures and other complications at the time of breath and blood sampling. Routinely collected vital signs and blood results data (e.g. WCC, CRP) were collected on every day at the time of breath sampling.

Definitions of sepsis, infection, and complications are defined below and recorded depending on vital signs, blood results, microbial cultures, radiological findings and clinician reported diagnoses. Diagnoses were confirmed by 2 independent clinicians.

Sepsis definitions

Sepsis

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, and is associated with in hospital mortality of 10%.(12) Sepsis has previously been defined as a patient having two or more systemic inflammatory response syndrome (SIRS) criteria in the presence of an infective source -temperature < 36°C or > 38°C, heart rate > 90 beats per minute, respiratory rate > 20 breaths per minute, and WCC < 4 x 10^9/Litre (L) and > 12 x 10^9/L - as is referenced in much of the reviewed literature.(114) This was redefined in 2016 by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine, as it was felt to be too sensitive and not representative of a dysregulate host response to infection, not specific to infection particularly in critically ill patients, and not discriminant between infective sources and sterile inflammation.(115) Through a series of meetings and Delphi processes, sepsis was re-defined by the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) as organ dysfunction identified an acute change in total sequential organ failure (SOFA) score ≥2 points consequent to the infection (Table 2-3).

.

Septic shock

Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. Patients with septic shock can be identified as a clinical construct of sepsis with persisting hypotension requiring vasopressors to maintain MAP ≥65 mm Hg and having a serum lactate level >2 mmol/L (18mg/dL) despite adequate volume resuscitation. In patients meeting these parameters, hospital mortality is more than 40%.

Organ dysfunction

Organ dysfunction can be identified as an acute change in total SOFA score ≥2 points consequent to the infection.(12, 114) The baseline SOFA score can be assumed to be zero in patients not known to have pre-existing organ dysfunction. A SOFA score ≥2 reflects an overall mortality risk of approximately 10% in a general hospital population with suspected infection. Even patients presenting with modest dysfunction can deteriorate further, emphasizing the seriousness of this condition and the need for prompt and appropriate intervention, if not already being instituted.

Definitions of Infection

Definitions of infection were taken from the Center for Disease Control criteria, as outlined in Table 2-4 .(116)

.

Table 2-3. Sequential (Sepsis-Related) Organ Failure Score

System	System	Score				
		0	1	2	3	4
Respiration	PaO_2/FiO_2, mmHg (kPa)	>400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7)	<100 (13.3)
Coagulation	Platelet count x $10^3/\mu L$	≥150	<150	<100	<50	<20
Liver	Bilirubin mg/dL (μmol/L)	≤1.2 (≤20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12 (>204)
Cardiovascular	Dose in μg/kg/h for ≥1h	MAP ≥70mmHg	MAP <70mmHg	Dopamine <5 or dobutamine (any dose)	Dopamine 1.5-15 or adrenaline ≤0.1 or noradrenaline ≤0.1	Dopamine >15 or Adrenaline >0.1 or Adrenaline >0.1
Central Nervous System	Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Renal	Creatinine mg/dL (umol/L)	<1.2 (<110)	1.2-1.9 (110-190)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5 (>440)
	Urine Output	-	-	-	<500ml/25h	<200ml/24h

FiO_2, fraction of inspired oxygen; MAP, mean arterial pressure; PaO2, partial pressure of oxygen

.

Table 2-4 Center for Disease Control definitions of infection (116, 117)

Site	Subgroup	Criteria 1	Criteria 2	Criteria 3
Surgical Site Infection (SSI)	Superficial	Within 30 days of surgery	Involves only skin and subcutaneous tissue of the incision	Purulent drainage from the superficial incision *or* organisms isolated from an aseptically obtained culture *or* at least 1 of the following signs or symptoms of: infection: - pain or tenderness - localized swelling - redness - heat *or* diagnosis of superficial incisional SSI by the surgeon or attending physician
Surgical Site Infection (SSI)	Deep	Within 30 days of surgery	Involves deep soft tissues (e.g., fascial and muscle layers) of the incision	Purulent drainage from the deep incision but not from the organ/space component of the surgical site, *or* deep incision spontaneously dehisces/is deliberately opened by a surgeon *and* at least 1 of the following signs or symptoms: - fever (>38°C) - localized pain or tenderness

.

				abscess or other evidence of infection involving the deep incision is found on direct examination/reoperation, or by histopathologic or radiological examination
Surgical Site Infection (SSI)	Organ space	Within 30 days of surgery	Any part of the body, excluding the skin incision, fascia, or muscle layers, that is opened or manipulated during the operative procedure	Purulent drainage from a drain placed into the organ/space *or* Organisms isolated from a culture of fluid or tissue in the organ/space *or* Abscess/infection on direct examination, during reoperation, or by histopathologic or radiologic examination *or* Diagnosis of an organ/space SSI by a surgeon or attending physician.
Pneumonia	Community Acquired Pneumonia (CAP)	Acquired out of hospital	Rales or dullness to percussion on examination *or* Chest Xray showing progressive infiltrate or consolidation	New onset purulent sputum *or* organism isolated from blood culture *or* pathogen isolated from tracheal or bronchial sample *or* virus detected in respiratory secretions *or* diagnostic antibody titre *or* histopathologic evidence of pneumonia
	Hospital Acquired Pneumonia (HAP)	Acquired during hospital admission		
	Aspiration Pneumonia	Following aspiration event		

.

Urinary Tract infection (UTI)	Cystitis Urosepsis Pyelonephritis	Positive urine culture that is, 10^5 microorganisms/c^3 of urine with no more than 2 species of microorganisms	at least 1 of the following signs or symptoms with no other recognized cause: • fever (>38°C) • urgency • frequency • dysuria • suprapubic tenderness	at least 1 of the following - positive dipstick for leukocyte esterase/nitrate - pyuria (urine specimen with <10 WCC/mm^3 - organisms seen on Gram's stain of urine - at least 2 urine cultures with repeated isolation of the same uropathogen (gram- negative bacteria *or* Staphylococcus saprophyticus) with >10^2 colonies/mL in non- voided specimens - 10^5 colonies/mL of a single uropathogen (gram-negative bacteria or S saprophyticus) in a patient being treated with an effective antimicrobial agent for a urinary tract infection - physician diagnosis of a urinary tract infection - physician institutes appropriate therapy for a urinary tract infection.
Bloodstream infection		Recognised pathogen cultured from 1 or more blood cultures	1 of the following signs or symptoms: - fever (>38°C) - chills - hypotension	Unrelated to an infection at another site and common skin contaminant

.

Surgical Complications

All surgery carries risk to patients, related to hospitalisation, anaesthesia, and the procedure performed at the surgical site. These are some of the common early complications seen following surgery.

Anastomotic failure

An anastomosis is a new connection surgically formed between two luminal structures – in this case the GI tract. Though uncommon, an anastomosis can fail (due to healing or technical factors) and the luminal contents to collect out around the anastomosis and in the peritoneal cavity.

Post-operative Pancreatic Fistula

Post-operative Pancreatic Fistula (POPF) is a clinical syndrome caused by leakage of pancreatic fluid at the operated pancreas surgical site, with high morbidity and mortality. Previously defined by the International Study Group of Liver Surgery (ISGLS) as drain output with fluid amylase level > 3 times the upper limit of normal serum amylase,(118) that now defines a biochemical leak. Clinically relevant POPF is defined as a drain output of any measurable volume of fluid with amylase level greater than 3 times the upper Institutional normal serum amylase level, associated with a clinically relevant development/condition related directly to the POPF. This suggests prolongation of hospital or critical care admission, includes use of therapeutic agents specifically employed for fistula management or its consequences (e.g. somatostatin analogues, parenteral nutrition, blood product transfusion or other medications). Postoperative organ failure is defined as the need for re-intubation, haemodialysis, and/or inotropic agents > 24 hours for respiratory, renal, or

.

cardiac insufficiency. Biochemical leak (BL), POPF B and POPF C depending on severity Table 2-5

Bile Leak

Similarly, to POPF, bile leak is the leakage of bile from the operated liver or biliary site (cut surface of biliary anastomosis) and is associated with morbidity and longer hospital admission. It has been defined by the ISGLS as bilirubin concentration in the drain fluid at least 3 times the serum bilirubin concentration on or after postoperative day 3 or as the need for radiologic or operative intervention resulting from biliary collections or bile peritonitis.(119) Severity of bile leakage was classified according to its impact on patients' clinical management.

Post-operative bleeding

Post-hepatectomy haemorrhage (PHH) definition is taken from International Study Group of Liver Surgery (ISGLS). PHH is defined as a drop of haemoglobin level >3 g/dl after the end of surgery compared to postoperative baseline level and/or any postoperative transfusion of PRBCs for a falling haemoglobin and/or the need for invasive re-intervention (e.g. embolization or re-laparotomy) to stop bleeding.(120) To diagnose PHH (and to exclude other sources of haemorrhage) evidence of intraabdominal bleeding should be obtained such as frank blood loss via the abdominal drains if present (e.g. haemoglobin level in drain fluid >3 g/dl) or detection of an intra-abdominal haematoma or active haemorrhage by abdominal imaging (ultrasound, CT, angiography). Patients who are transfused immediately postoperatively for intra-operative blood loss by a maximum of two units of packed red blood cells (PRBC) (i.e. who do not have evidence of active haemorrhage) are *not* diagnosed with PHH.

.

Table 2-5 ISGLS Postoperative Pancreatic Fistula Criteria(118)

Grade	Criteria
Biochemical leak (BL)	Fluid amylase level > 3 times the upper limit of normal serum amylase
B	BL in association with a clinically relevant condition, with deviation from normal postoperative management, e.g. prolonged drains, endoscopic intervention, nil by mouth etc
C	BL with organ failure, e.g. need for re-intubation, haemodialysis, and/or inotropic agents > 24 hours for respiratory, renal, or cardiac insufficiency

Table 2-6 ISGLS Grading of Bile Leakage(119)

Grade	Criteria
A	Fluid bilirubin level > 3 times the upper limit of normal serum bilirubin, with no change in patients' clinical management.
B	Requires active therapeutic intervention but is manageable without relaparotomy
C	Relaparotomy is required

Table 2-7 ILSGS Grading of Post Hepatectomy Haemorrhage(120)

Grade	Criteria
A	PHH requiring transfusion of up to 2 units of PRBCs
B	PHH requiring transfusion of >2 units of PRBCs but manageable without invasive intervention
C	PHH requiring radiological interventional treatment (e.g. embolization) or re-laparotomy

.

Liver Transplant Complications

Hepatic artery thrombosis

Evidence of liver graft hypoperfusion with radiological/surgical evidence of thrombus in the hepatic artery. This leads to graft hypoperfusion and ischaemia, and eventually dysfunction. Management options include early re-exploration of the arterial anastomosis or urgent re-transplantation.

Portal vein thrombosis

Evidence of liver graft hypoperfusion with radiological/surgical evidence of thrombus in the portal vein. Management option include surgical re-exploration, anticoagulation, radiological intervention and re-transplantation where the graft fails.

Early allograft rejection

Early allograft dysfunction (EAD) is a poor allograft function in the early postoperative phase – the first 7 days following transplantation. EAD is associated with allograft quality, donor and retrieval factors. It is associated with poorer long term recipient outcomes.

Olthoff criteria using one or more of,

1. Bilirubin ≥10 mg/dL on postoperative day 7
2. INR ≥1.6 on postoperative day 7
3. aminotransferase level (alanine aminotransferase [ALT] or AST) >2000 IU/mL within the first 7 postoperative days(121)

.

Acute Cellular Rejection

Acute Cellular Rejection (ACR) occurs when recipient T cells responds to alloantigens from the donor liver. Allograft major histocompatibility complex (MHC) molecules initiate a T cell mediated immune response against the liver graft. ACR manifests as allograft dysfunction (usually transaminitis) and can usually be managed by optimising immunosuppression and with a short course of high dose steroids. Diagnosis is made histologically with a liver biopsy.

Medical Complications

Acute Kidney Injury

Acute Kidney Injury (AKI) indicated by rise in serum creatinine (SCr), reduction in urine output, or requirement for renal replacement therapy (RRT). Stage 1, 2 and 3, as defined by Kidney Disease: Improving Global Outcomes (KDIGO) criteria (Table 2-8) (122, 123).

Table 2-8. KDIGO Criteria for Staging AKI

AKI Stage	Serum creatinine (SCr)	Urine Output
1	1.5-1.9 times baseline *or* ≥0.3mg/dL increase	<0.5mL/kg/h for 6-12h
2	2.0-2.9 times baseline	<0.5mL/kg/h for ≥ 12h
3	3.0 times baseline *or* increase to ≥4.0mg/dL *or* Initiation of renal replacement therapy (RRT) *or* decrease in estimated glomerular filtration rate (eGFR) to <35mL/min/1.73m in patients <18years	<0.3mL/kg/h for ≥24h *or* Anuria for ≥12h

.

Cardiovascular event

Acute coronary syndrome including ST-elevation myocardial infarction (STEMI), non-ST elevation MI (NSTEMI), unstable angina, and cardiac arrest.

Cerebrovascular event

Ischaemic injury to brain parenchyma due to thrombus or haemorrhage, including transient ischaemic attack. Diagnosis is confirmed radiologically.

Deep vein thrombosis

Thrombus in the deep veins – typically the lower limb, or lungs (pulmonary embolism (PE)), associated with surgery, critical care, immobilisation and comorbidity. The diagnosis is confirmed radiologically, treated with anticoagulation

Clavien-Dindo Classification

Post-surgery morbidity and mortality outcomes were classified in 1992 by Clavien et al, and modified in 2004 by Dindo et al, to give the widely used Clavien-Dindo score for reporting surgical complications.(124, 125) The classification grades complications based on the level of treatment intervention required and organ system failure, as summarised in Table 2-9.

.

Table 2-9 Clavien-Dindo classification of surgical complications(124)

Grade	Complication
I	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic, and radiological interventions
II	Requiring pharmacological treatment with drugs other than such allowed for grade I complications. Blood transfusions and total parenteral nutrition are also included
III	Requiring surgical, endoscopic or radiological intervention
- IIIb	Intervention not under general anaesthesia
- IIIb	Intervention under general anaesthesia
IV	Life-threatening complication requiring critical care
- IVa	Single organ dysfunction (including dialysis)
- IVb	Multiorgan dysfunction
V	Death of a patient

.

2.5 Blood processing

Plasma Isolation

Lithium heparin, EDTA, Citrate and Serum tubes were centrifuged at 1800 rotations per minute (rpm) for 10 minutes at room temperature (20°C) with the brake on. Supernatants were transferred in 2 mL aliquots and 8 x 130μL and frozen and stored at -80°C.

Peripheral Blood Mononuclear Cell Isolation

Whole blood in lithium heparin was further diluted at 1:1 with phosphate buffer solution (PBS) in 50mL tube(s) and used to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, UK) at 2:1 with Ficoll-Paque Plus. Samples were centrifuged at 2000 rpm for 20 minutes at room temperature (20°C) with the brake off. Peripheral blood mononuclear cells (PBMCs) were collected and resuspended with PBS and then centrifuged twice at 1800 rpm for 10 minutes at room temperature with brake on. The cell pellet was re-suspended in the desired volume according to the pellet size (indicating number of cells), with 500μL of Roswell Park Memorial Institute 1640 Medium (RPMI) and transferred into 1ml cryovials containing 500μL of freezing mix (20% Dimethylsulfoxide (DMSO) + 80% FBS to obtain a final concentration of 10% DMSO, 40% FBS, 50% medium). PBMC Cryovials were frozen in a Nalgenee 'Mr Frosty' container, a polycarbonate container with blue high-density polyethylene closure, white high-density polyethylene vial holder and foam insert surrounded by isopropyl alcohol. This provides controlled 1°C/min cooling rate required for successful cryopreservation of cells. PBMC cryovials were then freeze/stored at -80°C for future phenotyping.

.

2.6 Breath Processing

The BDV analysis from the sample bags was carried out at the Laser Spectroscopy Laboratory of the Space Science and Technology department of the Rutherford Appleton Laboratory (aka RAL Space). Samples were stored at room temperature and analysed as discussed in Chapter 4.

Infrared Spectroscopy

BDV value is generated using the concentrations of the isotopes ^{12}C and ^{13}C in a sample containing CO_2 gas in this experiment expired breath. Concentrations are generated, and used to calculate the relative difference of the $^{13}CO_2/^{12}CO_2$ isotopic ratio of a sample with that of an international reference (Pee Dee Belemnite (PDB)).(138) Breath samples were analysed by and in collaboration with the Spectroscopy Group at the Rutherford Appleton Laboratory Space Science and Technology Department, Harwell Campus, Didcot, UK, with funding from the Science and Technology and Facilities Council., who developed the technique and built the spectrometer.

a.

b.

Figure 2-3 IR radio spectrometer

a. Bench setup, b. sample attached to cell A via silicone tubing

.

The spectrometer is composed of an IR light source – a quantum cascade laser that emits radiation with wavenumber around 2296 cm^{-1}, corresponding to a wavelength of 4.36 μm. The output frequency of the laser is ramped rapidly in time in a saw-tooth pattern. The laser radiation is directed towards an optical beam-steering mirror (MEMs mirror). The MEMs mirror cycles between three positions, each of which directs the laser beam along one of three measurement paths (‘channels’) as shown in Figure 2-4. In the first position the mirror directs the laser beam through the sample cell, through which breath samples are flowed. In the second and third mirror positions the laser beam is directed through the two reference gas cells, respectively. These hermetically sealed glass cells contain reference gas mixtures of 5% (CHECK) CO_2, each with a known $^{13}\partial$ value (-20.x ‰ and -37.x ‰). Each channel has a photodetector used to measure the transmission of the channel’s cell as a function of laser frequency. The mirror has a dwell time of 2 s at each position, the first 0.6 s of which is ‘dead time’ during which mirror vibrations damp down, and the remaining 1.4 s are used for spectroscopic measurements. The laser current is ramp lasts 10 ms, and so during the 1.4 s of measurement time, 140 laser scans are performed, producing 140 spectral traces. The values are used to calculate a mean, and the mean trace is saved.

.

Mirror position	**Detector**	**Gas**	**Current (mA)**
-1	A	Sample	-12.5
0	B	Calibrant 1	0
+1	C	Calibrant 2	+12.5

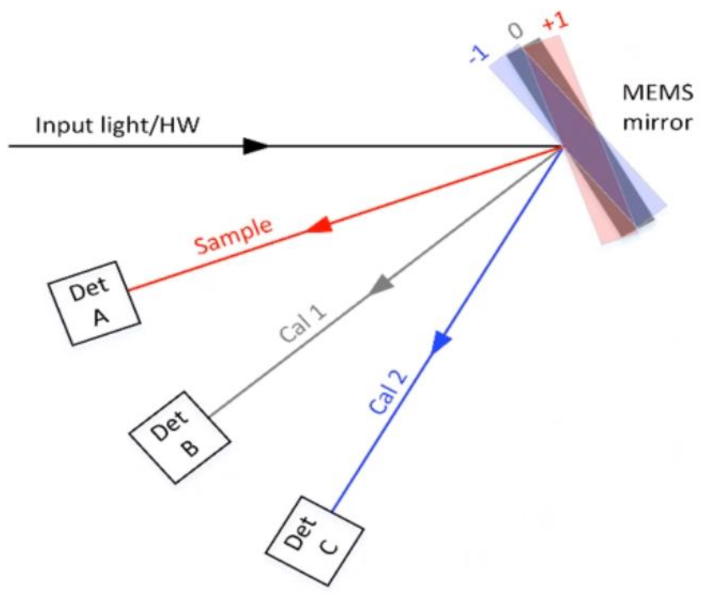


Figure 2-4 MEMS mirror

Rapidly switching the direction of the laser through cells from +1 to -1 to the photodetectors A (sample), B (calibrant 1) and C (calibrant 2)

2 cells are reference cells containing a known concentration of $^{12}CO_2$ and $^{13}CO_2$. The third cell contains a sample – in this experiment breath. The IR laser passes through each cell several times per second, and the amount of IR is measured on a photodetector on the other side of the cell (Figure 2-5).

.

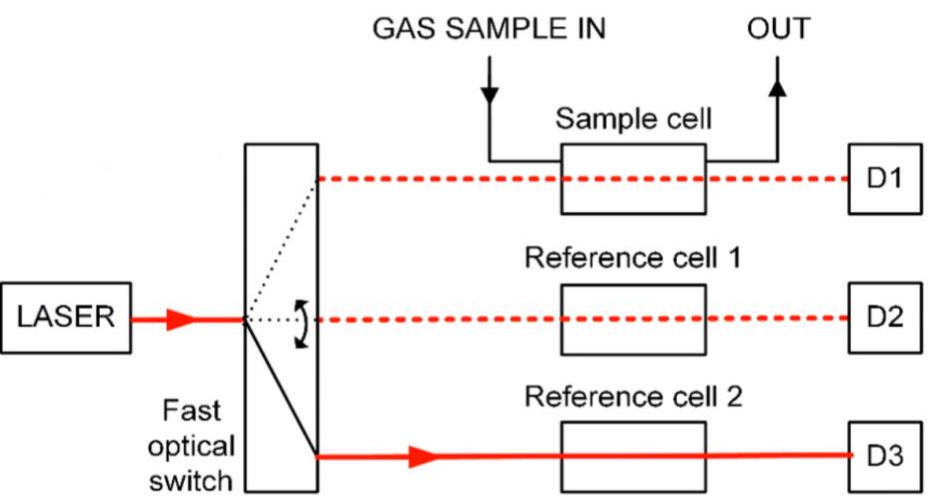


Figure 2-5 Infrared Spectrometer.

The infrared laser is directed through each cell using a MEMS mirror and fast optical switch, and the light power transmitted by that cell is detected by a photodetector (D1-3)

Prior to measurements with a given breath sample, breath from the sample bag is drawn through the sample cell for 1 minute to flush the system of any prior sample/room air during sample changeover, after which the measurement starts. During the measurements, the laser current is periodically linearly ramped, which ramps the laser frequency and output power. As the laser frequency is scanned, the photodetector signal for a given channel includes information about the varying laser power and the absorption by CO_2 in the channel's cell. For most of a laser scan the gas cell transmits almost all the laser radiation. However, when the laser frequency is close to a molecular vibrational frequency, the CO_2 molecules absorb the radiation, reducing the photodetector signal. This absorption is maximal when the laser is 'on resonance': the laser frequency matches the molecular vibrational frequency. This absorption leads to 'dips' in the photodetector signal, centred on laser frequency characteristic for a particular vibration of a particular molecules isotopologue Figure 2-6. These absorption dips can be used to calculate the populations of $^{12}CO_2$ and $^{13}CO_2$ in the sample, from which the isotope ratio can be calculated.

.

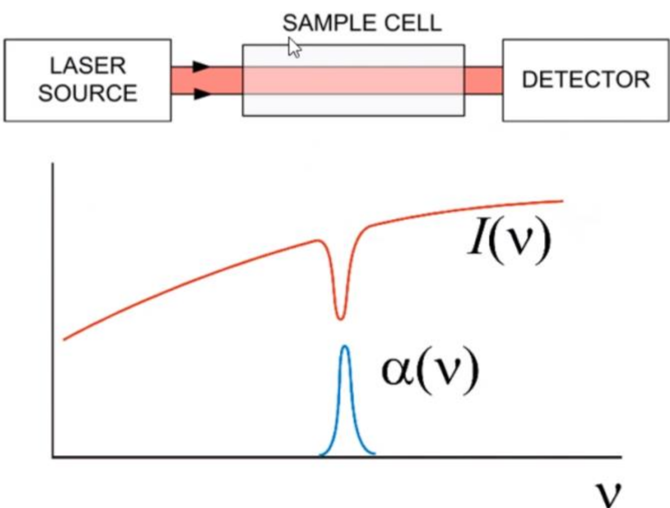


Figure 2-6 Infrared Spectrometer and Spectra

Generating BDV

Figure 2-7 shows examples of measured photodetector signals for each of the three channels. Every laser scan starts with the laser below threshold, meaning that it is emitting no laser radiation. This explains the 'flat line' signal at the start of the signals shown in Figure 2-7. Once the threshold passes threshold and starts to emit radiation, the photodetector signal increases. This increase continues throughout the scan, increasing the detected laser power. The photodetector signals show clear absorption dips from the two isotopologues of CO_2 present in the samples. The two strong absorption features in the middle of each spectrum are used for the isotope ratio measurements: one of the absorption features is from $^{12}CO_2$, and the other from $^{13}CO_2$. By measuring the relative amplitude of these two features, the isotope ratio is calculated. The gas samples in the two calibrant cells have known isotope ratios and are used to calibrate the isotope ratio of the breath sample. The absolute amplitude of the absorption features in each of the channels is a measure of the concentration of the CO_2 in that sample, which varies between the three samples. However, it is the relative amplitude of the two strong absorption features in each gas cell that is measured, and so the results are in principle immune to variations in the overall CO_2 concentration.

.

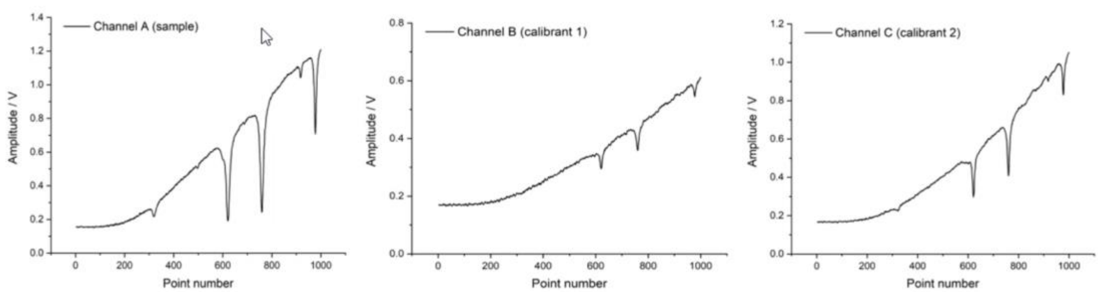


Figure 2-7 Spectra for sample (channel A) and calibrants 1 and 2 (channel B and C). The first large trough represents absorption of ^{12}C and the second ^{13}C

In order to measure the isotope ratio for a given gas cell, the varying baseline of the measured photodetector spectra (which results from the increasing laser power during a scan) needs to be removed. This is done in several sets of analysis, but briefly: (i) first the sub-threshold photodetector is subtracted. This dark signal results from the photodetectors responding to sources of mid-IR radiation other than the laser (any warm body emits mid-IR radiation). The dark signal needs to be removed as it can bias the laser measurements. (ii) Once this background signal has been removed, a polynomial function is fitted to the signal baseline (i.e. all the data points in the scan that do not correspond to molecular absorption). (iii) This fitted function is then used to remove the varying baseline, producing a transmittance spectrum that has a value of 1 when there is no molecular absorption and 0 when the molecules absorb all of the laser radiation. For molecular absorption dips with a minimum value between 0 and 1 it is possible to calculate the molecular concentration.

In order to generate isotope ratio measurements from the transmittance spectra, a model that describes the light-matter interaction is used. This model uses known information about the absorbing strength and frequency response of the $^{12}CO_2$ and $^{13}CO_2$ isotopologue vibrations to produce a calculated transmittance spectrum. For each experimental transmittance spectrum, a numerical fitting routine is employed that varies the $^{12}CO_2$ and $^{13}CO_2$ concentrations in the model in order to find the calculated model spectrum that best agrees with the experimental spectrum. The ‘best-fit’ concentrations are then used to

.

calculate the isotope ratio for the sample. Uncertainties that arise at all stages of the analysis are propagated and combine to provide an overall uncertainty in the isotope ratio.

For a given breath sample, every 6 seconds a new breath sample spectral measurement is recorded, with corresponding measurements for the two calibrant channels. The analysis routine produces an isotope ratio (and uncertainty) for each of these measurements. For each breath isotope ratio, the corresponding isotope ratios measured for the two calibrant samples was used to provide a linear, real-time calibration. Each breath sample was analysed by the instrument for at least 3 minutes, resulting in at least 30 calibrated isotope ratio measurements per breath sample. Each of these isotope ratio measurements has an uncertainty, resulting from the analysis and fitting routine. For each set of measurements for a given breath sample, the isotope ratio uncertainties were used to calculate the weighted mean isotope ratio with an associated mean uncertainty generated from the fitting uncertainties. In addition to this ‘fitting’ uncertainty, the standard deviation of the isotope ratio measurements was calculated, and this measure of the spread of the individual measurements was used as another uncertainty on the mean isotope ratio measurement. In this way, for each breath sample there was a mean isotope ratio measurement, with two accompanying uncertainties. Typically, the uncertainty from the spread of values was greater than the mean fitting uncertainty, indicating that the uncertainty calculated from the analysis and fitting routines does not describe all sources of error. The uncertainty arising from the spread of measurements was used as the measurement uncertainty, except in those rare cases where the ‘fitting’ uncertainty was greater than the ‘measurement spread’ uncertainty.

Sample assessment

Breath samples were stored and sealed at room temperature. Samples were transported to RAL by courier service and further stored. Before each sample was analysed, it was

.

assessed by batch number, valve type (push/pull closure/screw closure) integrity of valve seal (sealed, leaking, left open etc), and filling of sample (well filled to empty). These data were recorded as below, to correlate with results. All samples went through analysis regardless of filling.

Table 2-10 Breath sample post storage data collection

	Measure of data
Batch number	As per label
Valve type	1 - push/pull valve, 2 – screw valve
Valve Integrity	1 – open, 2 - leaking, 3 - good seal
Quality of filling	1 - empty bag, 2 – poorly filled, 3 – moderately filled, 4 – well filled, tense bag

Breath Sampling

Samples were connected to the sample cell A by connecting the valve tube silicone tubing. Sample was drawn into the cell for one minute to flush the chamber of any room air/previous sample. Measurements were taken for four minutes, after which the sample was disconnected and resealed, in case a repeat analysis were required. BDV, error 1, error 2 and $^{12}CO_2$ concentration were generated, as described. Where there was insufficient CO_2 to generate BDV, the value 0 is given for $^{12}CO_2$ and -0.01 for BDV.

Statistical analysis

BDV, error 1, error 2 and $^{12}CO_2$ concentration were analysed. Where there was insufficient CO_2 to generate BDV and the sample will be excluded from the clinical analysis. Samples are presented as baseline and POD1-9. Statistical Analysis was performed using GraphPad Prism 9.2 Statistical software (Graph Pad software, Inc.). Values were tested for normality of

.

distribution using Kolmogorov-Smrivnov test. Parametric data is reported as mean ± SEM, and nonparametric data as median ± interquartile range. Two tailed P value<0.05 was considered to be significant.

Data Quality

$^{12}CO_2$ concentration and BDV were correlated using Spearman r nonparametric XY correlation. This was then repeated after using excluding outliers/non-physiological values, to assess whether BDV at varying $^{12}CO_2$ concentrations were reliable.

Cutoffs

Samples with non-physiological results (samples with BDV more positive than room air, -8 per mil) were excluded. Samples with $^{12}CO_2$ concentration <0.02 - approaching atmospheric $^{12}CO_2$ concentration - were excluded from clinical analysis.

Outliers

Outliers were identified using the ROUT method (GraphPad PRISM). Based on the False Discovery rate, ROUT fits a model to the data where outliers have little impact, then uses a new outlier detection method based on FDR to decide which points are far enough from the prediction of the model to be called outlier. The user can set Q, the maximum desired False Discovery Rate.

Missing Values

Patients with 50% of more BDV values missing or excluded as outliers were excluded from the analysis. Missing data were imputed using interpolation between available data during

.

the sampling period. When data were missing after patients were discharged (without ongoing clinical infection) the last available value was imputed as BDV on subsequent days.

Sample Quality

In LT patients who were ventilated perioperatively, samples were taken at the patient and of the circuit and the ventilator end of the expiratory tubing, as discussed in **Error! Reference source not found. Error! Reference source not found.**. To examine for a difference in BDV and $^{12}CO_2$ in the sampling point (Patient end or Ventilator end) a paired t test using nonparametric Wilcoxon matched pairs signed rank was conducted. There were no ventilated samples in HPB Group.

Bag types

To assess the integrity of storage/seal different between valve types for a difference in BDV and $^{12}CO_2$ between bag types, BDV was correlated with valve type (push/screw) using student's t test.

Clinical Data

Statistical Analysis was performed using GraphPad Prism 9.2 Statistical software (Graph Pad software, Inc.). Two tailed P value<0.05 was considered to be significant. HC samples were compared to infected and non-infected patients using a nonparametric Kruskal-Wallis one-way ANOVA, comparing the mean rank of each column with the mean rank of every other column to assess for a difference between HC and patient sampled.

.

Baseline Characteristics

Baseline Characteristics between groups using Fisher’s exact test for nominal data, and Wilcoxon Rank summary for continuous data.

Infection/Sepsis

To examine for a difference in BDV between patients who do and do not develop a postoperative infection, values were tested for normality of distribution using Kolmogorov-Smirnov test. Parametric data is reported as mean ± standard error of mean (SEM), and nonparametric data as median ± interquartile range. Samples are grouped by whether participants developed postoperative infection/sepsis. Exploratory analysis was performed to determine if BDV was different in infected and non-infected patients using the Mann-Witney U (MWU) test at individual days. To assess the effect of repeated measurements data is also analysed using a two-way ANOVA mixed effects analysis to allow for missing values. Fisher’s LSD test was used, not correcting for multiple comparisons.

Diagnostic Accuracy

The initial statistic plan was to assess diagnostic accuracy of BDV using receiver operating characteristic, and sensitivity, specificity, DOR, and using Youden J statistic to give an optimal cut-off based upon sensitivity and specificity. The cutoff is chosen from the value highest Youden index (sensitivity + specificity – 1). This was to be compared to other clinical markers including WCC, CRP and SOFA, and against experimental markers from MSD and Monocyte Phenotyping.

.

Clinical Condition

To assess whether BDV is associated with continuous biomarkers including SOFA score, WCC, CRP, data were analysed using nonparametric Spearmann R XY Correlation. The relationship between BDV and other parameters including feeding and haemodynamic instability was examined using the MWU test at individual days. The relationship between BDV and clinical outcomes including infection, sepsis, and other inflammatory processes including bile leak, pancreatic leak, was examined using day by day logistic regression.

.

2.7 Statistical Analysis

Statistical Analysis was performed using GraphPad Prism 9.2 Statistical software (Graph Pad software, Inc.). Two tailed P value <0.05 was considered to be significant.
Healthy control samples were compared to infected and non-infected patients using a nonparametric Kruskal-Wallis one-way ANOVA, comparing the mean rank of each column with the mean rank of every other column. Baseline Characteristics between groups using Fisher's exact test for nominal data, and Wilcoxon Rank summary for continuous data. Values were tested for normality of distribution using Kolmogorov-Smrivnov test. Parametric data is reported as mean ± SEM, and nonparametric data as median ± interquartile range. Samples are grouped by whether participants developed postoperative infection/sepsis. Exploratory analysis was performed to determine if BDV was different in infected v non infected patients using the MWU test at individual days. To assess the effect of repeated measurements data is also analysed using a two-way ANOVA mixed effects analysis to allow for missing values. Fisher's LSD test was used, not correcting for multiple comparisons.

.

2.8 Mesoscale Discovery Assay

Cytokines are chemical mediators released by cells and that act on different cell receptors and change physiology, as discussed in Chapter 1.3, and are soluble within serum/plasma. They can be quantified using enzyme-linked immunosorbent assay (ELISA) techniques, but a newer technology called Mesoscale Discovery Cytokine Assay (MSD) uses an electrochemiluminescent multi-array allows several cytokines to be quantified in one sample and experiment, with high sensitivity and low background signal interference.(139)

Electrochemiluminescence (ECL) uses electrical stimulation by labelled analytes to generate light.(139) MSD uses the coreactant pathway, using $Ru(bpy)_3^{2+}$ as a luminophore – a functional group responsible for luminescent properties of a compound – with triprophylamine (TPrA) amine based coreactant. Similarly to ELISA techniques, samples are mixed with SULFO-TAG detection antibody, which bind to analytes – cytokines included in the immunoarray – and allows them to bind to capture antibody (Figure 2-8). SULFO-TAG (is an N-hydroxysuccinimide which couples to the primary amine groups of proteins, and the conjugated proteins are used as detection reagents. The working electrode is connected to an electrical source which controls the electrical potential, and at a particular potential ECL is induced dependent upon the concentration of the lumiphore and coreactant. The coreactant TPrA is oxidised at the electrode to create the reactants TPrA•+ and TPrA•. These radicals react with the luminophore Ru(bpy)32+ to generate an excited state and photon emission, giving luminescence (Figure 2-9). Luminescence is measured on an electromagnetic charge-coupled device. ECL readers can spatially resolve luminescence from the regions of interest (spots) at the electrode, allowing for simultaneous imaging of multiple assays (10 spots for MSD V-PLEX), and microwells within a sample well are precoated with capture antibody (**Error! Reference source not found.**).

.

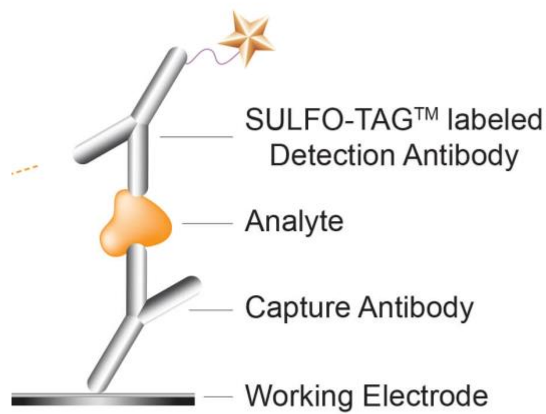


Figure 2-8 SULFO-TAG labelled detection antibody bound to analyte

SULFO-TAG binding to the capture antibody within an electrode microwell or 'spot' to give a reading

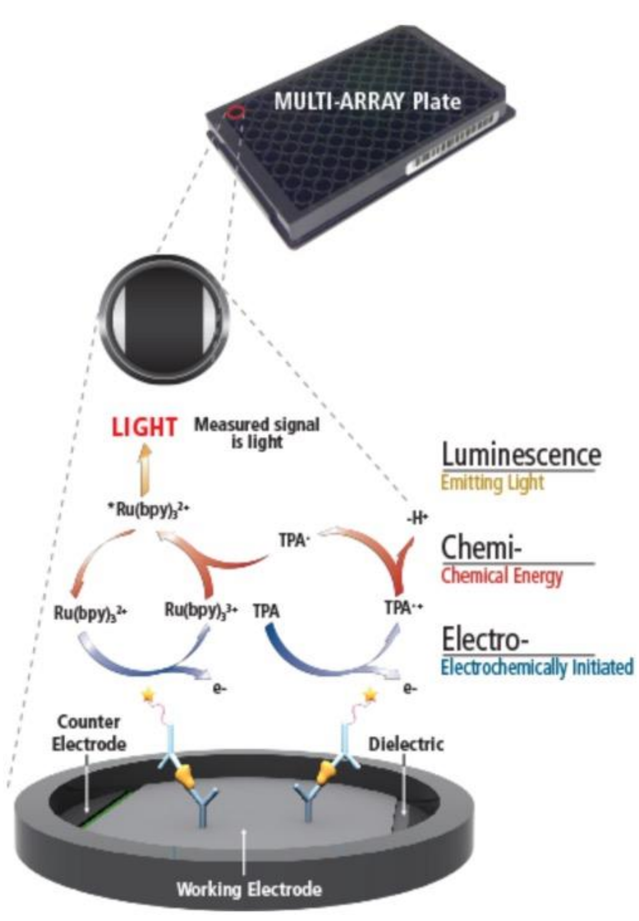


Figure 2-9 Electrochemiluminescence at the electrode

Coreactant TPrA is oxidised at the electrode to create the reactants TPrA•+ and TPrA•. These radicals react with the luminophore Ru(bpy)32+ to generate light

.

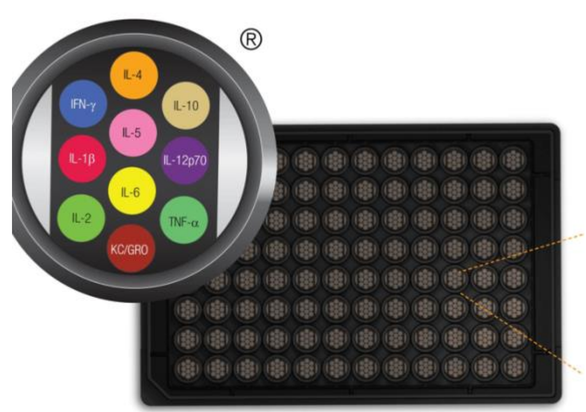


Figure 2-10 96-well multispot MSD plate

Each well contains 10 'spots' or microwells for ECL reading. Experiments were conducted using a MESO Quickplex SQ 12.

Table 2-11 Cytokines included in MSD V-PLEX Proinflammatory multiassay

Cytokine	Action
IFNγ	Pro-inflammatory cytokine, potent activator of macrophages
IL-1β	Pro-inflammatory cytokine, induces IL-2, B cell maturation and proliferation, fibroblast GF and healing, pyrogenic
IL-2	T cell growth factors, regulates T cells
IL-4	B cell stimulatory factor/lymphocyte stimulatory factor, B cell activation and induces DNA synthesis, induces IgG/IgE expression
IL-6	Induces acute phase response, differentiates B cells into immunoglobulin secreting cells
IL-8	Attracts neutrophils, basophils, T cells and neutrophils
IL-10	Cytokine synthesis inhibitory factor, suppresses pro-inflammatory response
IL-12p70	Produced by macrophages and T lymphocytes, activates T cells and NK cells to produce IFNγ
IL-13	Positive regulator of B cell proliferation, macrophage activation, Ig p
TNF-α	Proinflammatory cytokine produces by macrophages, induces inflammatory response, endogenous pyrogen

.

Mesoscale discovery assay was conducted using V-PLEX Proinflammatory Panel 1 Human Kit (IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, as described in Chapter 1.11 Biomarkers for Investigation), from MesoScale Diagnostics, LLC (Rockville, MD, USA) on 2 plates on 9th July 2021. EDTA samples were thawed at room temperature and 2-fold diluted. 50 μL of calibrators or sample (in singlicate) were added to each well of a V-PLEX plate and incubated for 2 hours on a horizontal orbital microplate shaker at room temperature. Plates were then washed three times (20-fold wash buffer concentrate reconstituted). 25 μL of detection antibody solution were added to each well and incubated for 2 hours on a horizontal orbital microplate shaker at room temperature. 150 μL of a 2X Read Buffer T were added to each well and the plate was read using an MESO QuickPlex SQ 120 from MesoScale Diagnostics, LLC (Rockville, MD, USA). Mean of the duplicate readings for each standard and sample were analysed, and analyte concentrations were established fitting a 4-parameter logistic calibration curve on Discovery Workbench U-PLEX software from MesoScale Diagnostics, LLC (Rockville, MD, USA).

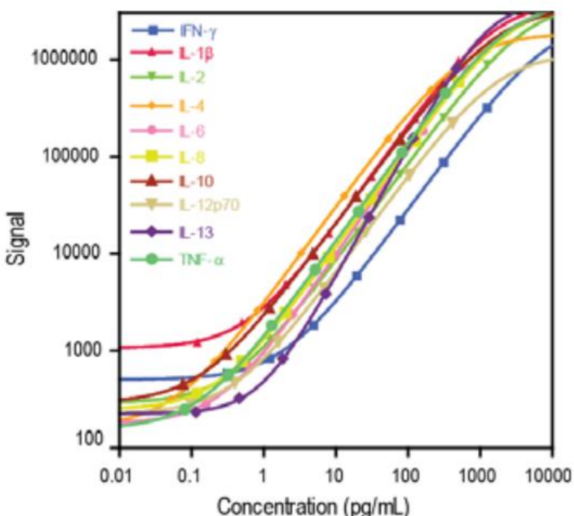


Figure 2-11 4-parameter logistic calibration curve

.

160 samples were included, 3 HCs, 6 septic controls, 73 HPB samples, 75 LT samples and 3 repeated samples. Three samples were repeated on both plates to assess for plate-to-plate variation using coefficient of variance. Coefficient of Variance (CoV) was calculated for each cytokine measured in Microsoft ExCel using the equation below.

CoV= (STDEV(Plate1:Plate5))/(AVERAGE(Plate1:Plate5))*100)

Equation 2-1 Coefficient of Variance using the standard deviation (STDEV) and mean (AVERAGE) between MSD plates in Microsoft Excel

Data were analysed Statistical Analysis was performed using GraphPad Prism 9.2 Statistical software (Graph Pad software, Inc.). Two tailed P value<0.05 was considered to be significant. Mann-Witney U test for sequential samples, one-way ANOVA were carried out to assess differences between groups, and logistic regression for HPB outcomes of infection (no cases of sepsis), pancreatobiliary leak, inflammation (infection or pancreatobiliary leak), and LT outcomes of sepsis, infection, bile leak, inflammation (infection or pancreatobiliary leak), and postoperative bleeding.

Alongside cytokines, other biomarkers including CRP, SOFA score, WCC and differential and percentage of WCC, and neutrophil lymphocyte ratio, lymphocyte monocyte ratio, neutrophil monocyte ratio, platelet lymphocyte ratio were analysed from clinical data. Where CRP values were missing PCT results from EDTA plasma PCT ELISA (Chapter 6) was analysed alongside other markers.

.

2.9 Procalcitonin ELISA

As explored in Chapter 1 and evidenced in the PCT meta-analyses (Chapter 4), PCT is the most used novel biomarkers of bacterial sepsis and is often used to compare the diagnostic performance of other new biomarkers, including BDV. PCT levels in plasma samples from baseline and postoperative days were analysed using ELISA techniques. ELISA – enzyme linked immunosorbent assay – is a common experiment which uses to detect an antigen (PCT) in a biological sample (EDTA plasma) using antigen-antibody interactions. Antigens are added to the ELISA plate and are immobilised on capture antibody. The antigen is then complexed using a capture antibody, which in turn oxidises 3,3′,5,5′-Tetramethylbenzidine, a chromogenic substrate, added to samples. HRP with hydrogen peroxide oxidises TMB to give a blue colour which fluoresces and is read by a microplate reader.

Baseline and sequential EDTA samples from all 20 HPB and 20 LT participants (where available) were included in the experiment, with 13 healthy controls, 13 septic controls and 5 ALF controls (total 200 samples). 1 HC (H27), 1 non-infected HPB participant (S64 d3) and 1 infected LT participant (T165 d7) were repeated on each plate to examine variation between plates.

Human PCT ELISA Kit (Invitrogen, Thermo Fisher Scientific, UK) was used to measure PCT levels in participant EDTA plasma as per manufacturer instructions. ELISA plates were 96-well precoated in human PCT antibody, and the 5-plate sample layout was planned in advance. Plate 1 experiment was conducted on June 4th 2021, and plates 2-5 on June 15th, 2021. EDTA samples were thawed to room temperature and diluted 2-fold in assay diluent and analysed in duplicate. Reconstituted human PCT standard A was diluted 2.75-fold in assay diluent A to give standard 1, which was sequentially diluted 2-fold using diluent A to give standards 2-7, and a blank standard 8 using diluent A.

.

100μL of standards and 100μL of diluted samples were added to plate wells in duplicate. Samples were incubated for 2.5 hours with gentle shaking. Samples were discarded and washed 4 times with wash buffer (20X wash buffer concentrate diluted 20-fold in DD water). 100μL of biotin conjugate (Biotin conjugate diluted 80-fold in diluent B) was added to each well and incubated for 1 hour at room temperature with gentle shaking. Samples were discarded and washed 4 times with wash buffer.100μL of streptavidin horseradish peroxidase (HRP) (700X Streptavidin-HRP diluted 700-fold in diluent B) was added to each well and incubated for 45 minutes at room temperature with gentle shaking. Samples were discarded and washed 4 times with wash buffer. 100μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. 50μL of stop solution was added to each well and plates were analysed.

Optical density was assessed soon after, using a FLUOstar® Omega microplate reader (BMG Labtech Ltd, UK) set to 490 nm. The mean of the duplicate readings for each standard, control, and sample were analysed after subtraction of the mean zero standard optical density. A 4-parameter fit curve was used to extrapolate the data, as recommended by the manufacturer.

Coefficient of variance (CoV) was used to examine plate to plate variation using the equation CoV=(STDEV(Plate1:Plate5))/(AVERAGE(Plate1:Plate5))*100 (Excel, Microsoft, USA). Mann-Witney U test and one-way ANOVA were used to analyse differences between groups using GraphPad Prism 9.4 software (GraphPad Software, La Jolla California, USA). Statistical significance was assessed with non-parametric analysis for data not normally distributed, unless otherwise specified in figure legends. Results are presented as median with interquartile range (IQR), unless otherwise specified in figure legends.

.

2.10 Monocyte Phenotyping

An integral part of the innate immune system, as explored in chapter 1, monocytes are cells within the immune system, circulating in blood in small numbers, which respond to tissue injury and express cell surface markers to initiate tissue response. Monocytes are a type of peripheral blood mononuclear cell – PBMC – along with lymphocytes (T cells, B cells, NK cells). Granulocytes (neutrophils, basophils, and eosinophils) have multi-lobed nuclei, and erythrocytes and platelets have no nuclei. PBMCs can be isolated in blood using a technique called density gradient centrifugation. Diluted whole blood is floated over Ficoll-Paque Plus and centrifuged (as per Methods described in chapter 2). The Ficoll-Paque Plus has a lower density than red blood cells, but a higher density that PBMCs and plasma. The sample is centrifuged into layers by density, with erythrocytes setting at the bottom, a layer of Ficoll, a layer of PBMCs and a layer of plasma and suspension fluid (PBS). This allows for the isolation of these cells for further experiments. Ficoll Paque Plus has low levels of endotoxin (< 0.12 EU/mL) to minimise stimulation of immune cells.

Cell surface markers expressed on monocytes can be read and measured using a technique called flow cytometry. Similarly to ELISA, these markers are labelled with antibody which interacts with the antigen (cell surface marker) which absorbs light at a particular wavelength on the light spectrum. Cells are then run through an instrument called a flow cytometer, which measures cell surface marker expression by generating fluorescence from labelled cell surface markers. The sample is introduced into the cytometry instrument's flow chamber. Hydrodynamic focusing on a fluidics system is used to separate the sample into its single-cell components, and a controlled flow around the sample, forces it into a narrow diameter causing the cells to separate. Separated cells pass through a laser that records each individual cell as an event. Forward scatter (FSC) and side scatter (SSC) are produced by each cell on passing through the laser. The fluorophore becomes excited as it passes through the laser and emits this light which it reaches the appropriate detector for the

.

targeted wavelength and is recorded by the instrument. These detectors recognise the fluorescence of a certain, predetermined wavelength, and the level of fluorescence measured marks the level of CD marker expression on that cell.

Methods

Monocyte phenotype was determined by flow cytometry on thawed PBMCs using Table 2-11 Cytokines included in MSD V-PLEX Proinflammatory multiassay monoclonal antibodies against CD (Cluster of differentiation)14, CD16, CD163, chemokine receptor (CCR) 2 (Biolegend, USA), Human Leukocyte Antigen - DR isotype (HLA-DR) (eBioscience), Mer-Tyrosine Kinase (TK) (R&D Systems, USA), Programmed death-ligand 1 (PD-L1) (Biolegend, USA), Programmed cell death protein-1 (PD-1) (BD) and Fixable Viability Dye eFluor 506 (Invitrogen) (Table 2-12).

Frozen PBMCs were thawed at room temperature and resuspended in 1ml sterile PBS. Cells were centrifuged at 1800 rpm for 5 minutes at room temperature with brake on. The cell pellet was resuspended in 100uL sterile PBS. Cells were counted using Tryphan blue stain on a slide under the microscope, and samples were diluted in PBS to give 500uL of 500,000 cells/500uL sample. Samples were stained using 100uL of live/dead working solution, then a master mix of antibody as above (Table 2-12). Samples were incubated for 30minutes in the dark in the fridge. Fluorescence minus one (FMO) samples were prepared using H27 to prepare the flow cytometry gating, with each FMO containing all but one of the antibodies (one per antibody). Following incubation, 1ml of FACS buffer was added to samples, and were centrifuged at 1800 rpm for 5 minutes at room temperature with brake on. Samples were resuspended in 300uL of FACS buffer, ready for acquisition.

Cell acquisition was performed on a BD LSR Fortessa™ cell analyzer (BD Biosciences, UK). Flow cytometry data analysis was performed in FlowJo™ v10 software (Becton Dickinson &

.

Company). Results are expressed as percentage (%) and/or mean fluorescence intensity (MFI).

Table 2-12. Antibodies used for Flow Cytometry

Primary antibody	**Host**	**Anti**	**Clone**	**Conjugated fluorophore**	**Catalogue number**	**Producer**	**Application**
CD14	Mouse	Human	M5E2	PeCy7	557742	BD	Flowcytometry
CD16	Mouse	Human	3G8	APC-H7	560195	BD	Flowcytometry
CD155	Mouse	Human	SKII.4	BV421	337632	Biolegend	Flowcytometry
CD163	Mouse	Human	GHI/61	PE	556018	Invitrogen	Flowcytometry
CCR2	Mouse	Human	K03602	AlexaFluor488	357226	Biolegend	Flowcytometry
HLA-DR	Mouse	Human	LN3	PerCp-Cy 5.5	45-9956-42	Invitrogen	Flowcytometry
MerTK	Mouse	Human	125518	APC	FAB8912A	R&D	Flowcytometry
PD-L1	Mouse	Human	29E.2A3	BV 605	329724	Biolegend	Flowcytometry
PD-1	Mouse	Human	EH12.1	BV786	563789	BD	Flowcytometry

Gating

Monocytes were identified based upon positive selection CD14, CD16 and HLA-DR expression, using an established gating strategy (Figure 2-12).(140) Surface marker expression was determined in the total monocyte population and the “classical” (CD14+CD16-), “intermediate” (CD14++CD16+) and “non-classical” ($CD14_{low}$CD16+) monocyte subsets.(141)

.

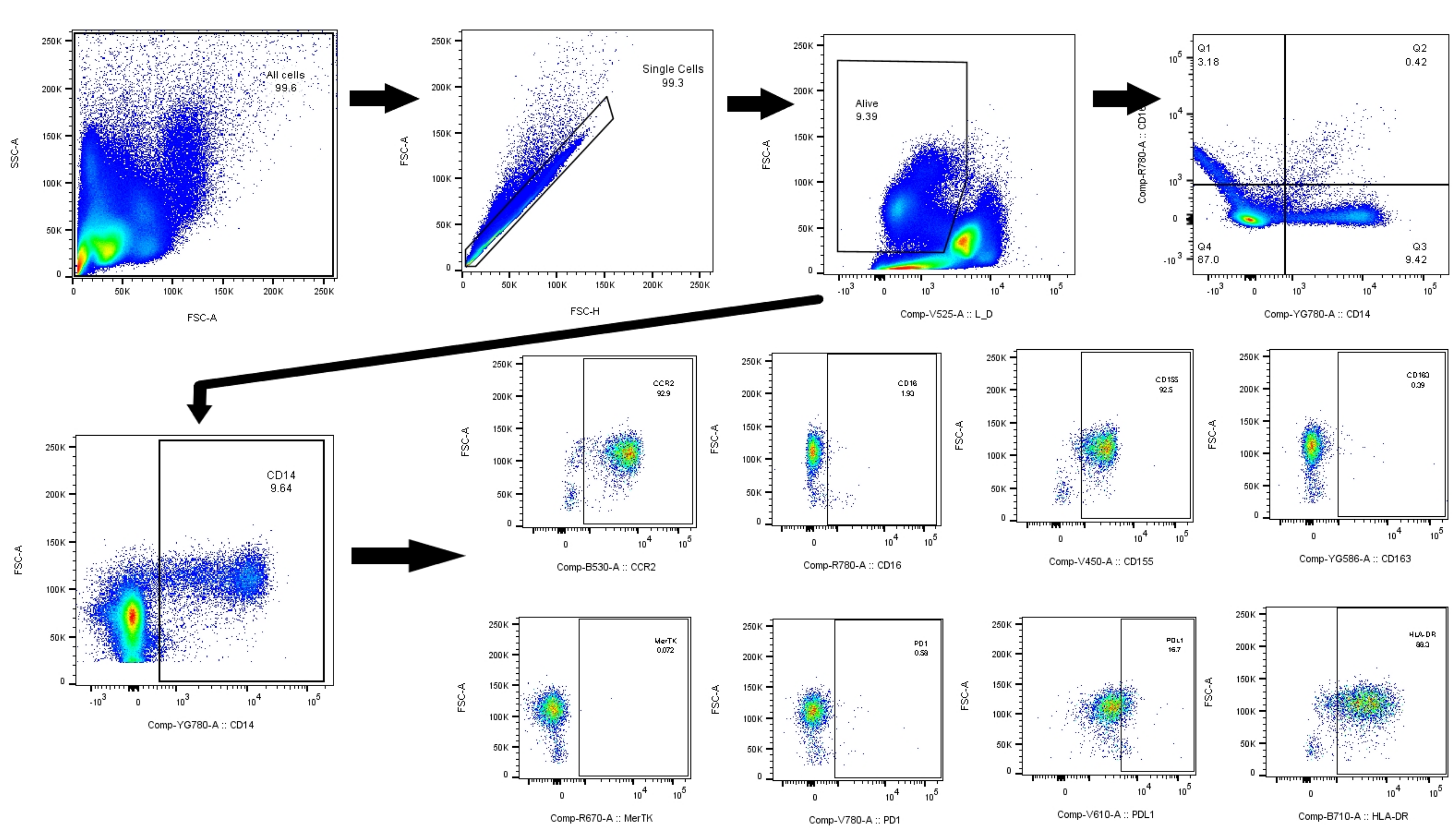


Figure 2-12 Gating strategy for Monocyte Phenotyping.

Gates set using healthy control H27 with FSC-1 and SSC-A. Gating of All cells > Single cells > Alive cells > CD14 (monocytes); and Q1-4, Q1, Q2, Q3

.

3 Characteristics of Included Participants

20 patients undergoing HPB surgery and 20 patients undergoing Liver Transplantation were recruited to I-MET as described Table 3-1(HPB) and Table 3-3 (LT). Participants were grouped by whether they did or did not develop an infective complication in the study period. Differences between groups was analysed using ANOVA for continuous data and Kruskal-Wallis test for categorical data (SPSS). Results are described in Table 3-2 (HPB) and Table 3-4 (LT). Averages of continuous variables are presented as median and interquartile range.

.

Table 3-1 Characteristics of Included HPB Participants

ID	Age (years)	Indication	Operation	Surgery date	LOS (days)	Surgical Complication	Medical Complication	Sepsis	Infection	Clavien Dindo
S044	64	CRLM	Laparoscopic NAR	08/06/2020	7	-	-	-	-	-
S048	67	CCA	PD	15/06/2020	38	POPF (B) POD3	POD3 HAP	POD3	POD3 HAP	II
S064	41	MCN	Laparoscopic DP	03/07/2020	8	-	-	-	-	-
S067	77	IPMN	PD	13/07/2020	25	-	-	-	-	-
S069	72	CLRM	Laparoscopic NAR	17/07/2020	5	-	-	-	-	-
S070	53	CCA	PD	20/07/2020	22	-	-	-	-	-
S074	47	CCA	HJ/GJ*/JJ**	17/08/2020	12	-	-	-	-	-
S078	63	CRLM	Open RH	07/09/2020	9	-	-	-	-	-
S079	71	GB tumour	Laparoscopic NAR	08/09/2020	16	POD4 Bile leak	-	-	POD4 Bile leak	IIIa
S080	71	NET	Laparoscopic DP	10/09/2020	10	POD4 POPF	-	-	-	I
S082	75	CCA	PD	14/09/2020	9	-	-	-	-	-
S084	73	CCA	PD	18/09/2020	35	POD7 SSI	-	-	POD7 SSI	II
S085	79	CCA	PD	21/09/2020	23	-	AKI	-	-	!
S091	48	NET	Laparoscopic DP	05/10/2020	9	POD4 POPF	-	-	-	I
S108	68	CCA	PD	06/11/2020	11	POD5 POPF	-	-	-	I
S123	64	CCA	PPPD	27/11/2020	18	POD3 POPF	AKI	-	-	I
S124	73	CCA	PPPD	27/11/2020	8	POD5 Chyle leak	-	-	-	II
S128	52	PA	Open DP	30/11/2020	7	-	-	-	-	-
S132	65	CCA	Open RH	01/12/2020	9	POD4 Bile Leak	-	-	POD4 Bile Leak	IIIa
S172	41	CRLM	Laparoscopic RH	16/03/2021	13	POD5 Bile Leak	-	-	POD5 Bile Leak	IIIa

*GJ, gastrojejunostomy; **JJ, jejunojejunostomy

.

Table 3-2 Descriptive statistics: differences between infected and non-infected HPB participants Averages are expressed as median and interquartile range. Differences between groups was analysed using ANOVA for continuous data and Kruskal-Wallis test for categorical data (SPSS)

	All	**Non infected**	**Infected**	**p value**
Age	65.9 (52.4-72.8)	63.6 (52.3-73.1)	67 (52.8-71.3)	0.9
Sex (n=)				
Male (M)	12	8	4	0.3
Female (F)	8	7	1	
BMI	24.3 (22.4-27.0)	24.5 (22.3-28.2)	24.2 (24.1-25.9)	0.6
ASA score (n=)				
1	1	1	0	0.7
2	13	10	3	
3	6	4	2	
Frailty Score(142)	3 (2-3)	3 (2-3)	2 (1.5-3)	0.44
WHO PS(143)	1 (0.2-1)	1 (0-1)	1 (0.5-1.5)	0.69
Comorbidity (n=)				
Cardiovascular	2	2	0	0.39
Respiratory	3	3	0	0.29
Diabetes	3	2	1	0.71
Renal	1	1	0	0.55
Obesity	2	2	0	0.39
Neoadjuvant Chemotherapy	9	8	1	0.32
Operation				
Duration (hours)	6.25 (5.1-7.5)	6.75 (5.25-7.5)	5.5 (4.4-6.8)	0.5
EBL (ml)	500 (500-500)	500 (500-500)	500 (500-650)	0.08
LOS (days)	10.5 (8.25-21)	9 (8-18)	16 (11-37)	0.03

BMI, Body mass index; ASA, American Society of Anaesthesiologists Score; WHO PS, World Health Organisation performance status; EBL, estimated blood loss; LOS, length of stay.

.

Table 3-3 Characteristics of Included LT Participants

ID	Age (years)	Sex	Indication	Operation and date of surgery	LOS (days)	Surgical Complication	Medical Complication	Sepsis	Infection	Clavien Dindo
T060	30	M	ALF Seronegative	OLT 24/06/2020	47	-	AKI 3, POD5 EAD	-	-	IVa
T072	47	F	ACLF Wilson's	OLT 15/08/2020	90	POD6 RTT	AKI 3	-	-	IVa
T075	63	M	SC - HCC	OLT 18/08/2020	90	POD1 PHH+RTT	AKI 3, Cardiac arrest	-	-	IVb
T077	62	M	SC ArLD	OLT 23/08/2020	16	-	-	-	-	-
T086	57	M	SCPSC	OLT 28/09/2020	10	POD7 SSI	-	-	POD7 SSI	II
T087	40	F	ACLF Wilson's	OLT 29/09/2020	22	POD2 bacteraemia	AKI 3	POD2	POD2 bacteraemia	II
T090	55	M	SC ArLD	OLT 03/10/2020	14	POD9 HAP	AKI 3	-	POD9 HAP	II
T105	64	F	SC NASH	OLT 10/11/2020	15	-	-	-	-	-
T106	58	M	SC ArLD	OLT 05/11/2020	13	-	AKI	-	-	I
T107	27	F	SC Wilson's	OLT 06/11/2020	14	-	-	-	-	-
T111	66	F	SC ArLD	OLT 19/11/2020	9	-	-	-	-	-
T113	37	M	ALF Seronegative	Aux-LT 20/11/2020	37	-	AKI 3	-	-	IVa
T114	58	M	SC ArLD	OLT 18/11/2020	14	-	-	-	-	-
T119	52	M	SC ArLD	OLT 23/11/2020	11	-	AKI	-	-	IVa
T126	70	M	SC ArLD	OLT 29/11/2020	16	-		-	-	-
T138	39	M	SC ArLD	OLT 30/12/2021	72	POD2 PHH+RTT	AKI 3	-	-	IVa
T139	65	M	ACLF ArLD	OLT 31/12/2021	19	POD2 PHH+RTT	MOF, AKI 3	-	-	IVb
T165	30	F	SC PBC	RL LT 02/02/2021	1	POD2 Bile leak	-	POD9	POD2 Bile leak	IIIa
T166	45	M	SC PSC	OLT 03/02/2021	13	-	-	-	-	-
T175	60	M	SC ArLD	OLT 26/03/2021	12	-	-	-	-	-

RTT, return to theatre; MOF, multiorgan failure.

.

Table 3-4 Descriptive Statistics, differences between infected and non-infected LT participants Averages are expressed as median and interquartile range. Differences between groups was analysed using ANOVA for continuous data and Kruskal-Wallis test for categorical data (SPSS)

	All	**Non infected**	**Infected**	**p value**
Age (years)	56.1 (39.6-62.3)	57.6 (39.4-62.5)	55.3 (35.1-61.0)	0.8
Sex				
Male (M)	12	10	2	0.3
Female (F)	8	5	3	
BMI	27.3 (22.7-33.4)	26.7 (22.8-32.4)	33.7 (21.3-47.4)	0.1
ASA score (n=)				
1	1	1	0	0.2
2	14	9	5	
3	5	5	0	
Frailty Score(142)	3 (2-4)	3 (1-4)	3 (2.5-4.5)	0.4
WHO PS(143)	1 (1-2)	1 (1-2)	1 (1-1.5)	0.83
Comorbidity				
CVS	1	0	1	0.08
Respiratory	3	3	0	0.28
Diabetes	4	4	0	0.2
Renal	1	1	0	0.55
Obesity	7	6	1	0.42
Operation				
Duration (hours)	5.5 (5-7.25)	6 (5-7.5)	5.5 (4.25-6.75)	0.37
EBL (ml)	4985 (3325-7750)	5770 (3100-8000)	4000 (3500-8100)	0.65
P**RBC T**ransfusion (ml)	665 (75-1217)	557 (0-1464)	860 (281-1194)	0.53
CIT (hours)	9.75 (7.94-10.69)	10.5 (8.5-11)	7.0 (6.5-10.25)	0.06
LOS (days)	14.5 (12.25-33.25)	15 (13-47)	14 (5.5-20.5)	0.20

BMI, Body mass index; ASA, American Society of Anaesthesiologists Score; WHO PS, World Health Organisation performance status; CVS, Cardiovascular disease; EBL, estimated blood loss; PRBC, packed red blood cells transfused; CIT, cold ischaemic time of liver allograft; LOS, length of stay.

.

4 Procalcitonin Meta-analysis

Procalcitonin (PCT) and Interleukin-6 (IL-6) have been investigated as biomarkers for postoperative sepsis, infection or other complications, and are often examined as comparator index tests for novel biomarkers. PCT is a precursor of calcitonin produced by C cells (parafollicular cells) of the thyroid gland and by neuroendocrine cells of the intestine and lung, involved in calcium homeostasis. It has been shown to be increased in bacterial infection/sepsis, and high serum concentrations have been associated with mortality.(36, 38, 126) PCT has been implemented as a diagnostic test in clinical practice particularly in critical care to differentiate systemic inflammatory response syndrome (SIRS) from sepsis – SIRS with an infective source. IL-6 is a pro-inflammatory cytokine produced by macrophages, endothelial cell and T cells. It acts on the liver to induce acute phase protein synthesis, induces proliferation of antibody producing B cells and activates T lymphocytes differentiation.(127, 128) It has been shown to be a moderately performing diagnostic test for sepsis, and is often used as a comparator for other biomarkers.(126, 129)

4.1 Diagnostic Accuracy of Procalcitonin and Interleukin-6 for Postoperative Infection in Major Abdominal Surgery: Meta-analysis

The aim is to undertake a systematic review and meta-analysis of available literature on the use of PCT and IL-6 in diagnosing postoperative sepsis/infection in patients undergoing major Gastrointestinal (GI) and hepato-pancreato-biliary surgery (HPB). The evidence is examined to assess whether PCT and IL-6 have a role as sepsis biomarkers in current practice, or whether further research should be done to discover or validate other novel biomarkers to diagnose post-operative infection or sepsis.

.

Methods

Search Strategy

An electronic search of Medline, Embase and Cochrane Library was conducted from 1996 to June 2020 using the terms ‘sepsis’ (MeSH Major Topic and keyword) or ‘infection’ (keyword), ‘biomarker’ (MeSH term and keyword, including Procalcitonin and IL-6), and ‘surgery’ (MeSH term or keyword). Bibliographies of relevant studies and the ‘related articles’ link were used to identify additional studies. Studies published only in abstract format or unpublished reports were excluded from the analysis. Citations and abstracts identified were thoroughly reviewed by investigators, and secondary references were obtained from the key articles. Studies were screened by title, abstract, and full text articles were assessed for eligibility with relevant studies included in the synthesis. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidance and Meta-analysis of observational studies in epidemiology (MOOSE) checklist were utilised (Figure 1).(29, 130) Studies were reviewed for assessment of diagnostic accuracy of PCT and IL-6 for sepsis in the early postoperative period (up to 2 weeks) in patients undergoing major abdominal surgery. Study design and technique were reviewed.

Inclusion Criteria

Only original studies in humans published in English were considered for inclusion. Adult and paediatric patients were included. Studies that analysed the diagnostic performance of Procalcitonin (PCT) and/or Interleukin-6 (IL-6) for sepsis or infection in patients undergoing major abdominal gastrointestinal and hepatopancreatobiliary surgery with cut-off values were included. Studies were evaluated for duplication or overlapping.

.

Exclusion Criteria

Studies were excluded if they reported data from small patient cohorts (<10 patients), or there was overlap with institutions or patient cohorts already published in higher quality studies. Studies on neonatal patients were excluded. Studies were excluded if they included data from patients undergoing other types of operation such as abdominal aortic surgery or cardiac surgery, without reporting a subgroup analysis in major gastrointestinal or hepatopancreatobiliary surgery.

Outcome Measures

The primary outcome of interest was diagnostic performance (sensitivity, specificity and diagnostic odds ratio (DOR)) of Procalcitonin and Interleukin-6 in detecting postoperative sepsis or infection.

Study Selection

Abstracts were reviewed to exclude those that did not meet the inclusion criteria. When no abstract was available or the abstract details were inadequate, the full text article was reviewed. Full text articles unavailable online were retrieved using library services.

Data Extraction

Data were extracted using standardised pro-forma. Data on demographic and clinical parameters were recorded: study characteristics (first author, year of publication, study design, study period, country where the study was performed), population characteristics (number of patients studied, patient demographics), data quality, reported biomarkers with timing of samples, cut-off values and test performance. Test performance variables were

.

extracted as a 2x2 contingency table of true positive, true negative, false positive and false negative from results. If not immediately available they were calculated from published performance results including sensitivity, specificity, negative or positive predictive value. Where insufficient results to construct 2x2 contingency tables were presented, authors were contacted to contribute data.

Quality Assessment

Study quality was assessed by using the QUADAS-2 criteria, examining patient selection, index test, reference standard and flow and timing of testing.(131, 132)

Data Analysis

Data analysis including heterogeneity assessment was performed using the freeware Meta-DiSc version 1.4 (Universidad Complutense, Madrid) (133) DerSimonian–Laird random effects model with over-dispersion corrections was used to calculate pooled sensitivity, specificity and diagnostic odds ratio including heterogeneity using inconsistency assessment (I^2)(134, 135). Summary receiver operator curves (SROC) were generated using Moses-Shapiro-Littenberg model.(136) Studies were examined for publication bias using Deek's test – a funnel plot and effective samples size (ESS) regression analysis. Logarithm of DOR is plotted against 1/√ESS, where ESS=(1/diseased + 1/not diseased).(137) Subgroup analysis using sample size (≤100 vs >100), location (European v non-European) and operation type (Upper gastrointestinal v Colorectal) was performed to investigate heterogeneity using diagnostic accuracy (I^2 DOR).

.

Results

The search returned 4353 results after duplicates were removed, of which 166 were screened by abstract. 32 full text articles were reviewed of which 20 were excluded for reasons given, leaving 12 articles examining the diagnostic accuracy of PCT and/or Il-6 for postoperative sepsis/infection following major abdominal surgery.(144)

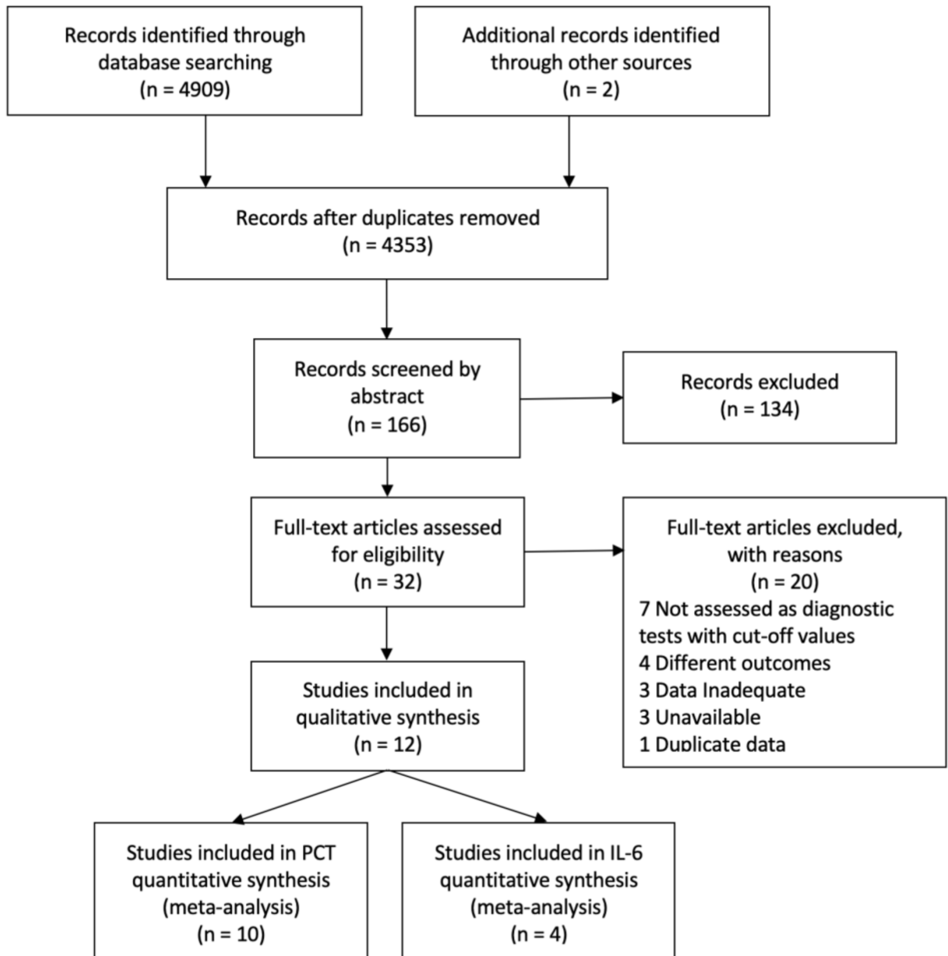


Figure 4-1 PRISMA Flow Chart

.

Table 4-1 Characteristics of Included Studies

Authors (year)	Location	Surgery Group Mean age	No. of patients	Rate of infection	Timing of Sample	Cut-off PCT (ng/mL) IL-6 (pg/mL)	Predetermined cutoff?	Sensitivity % (95% CI)	Specificity % (95% CI)	AUC (SE AUC)
Boersama et al**(30)** 2018	Rotterdam, Netherlands	Colorectal resection 68.6 years	47	46.8% (22)	POD1	- n/a*	No	- 76	- 86	- 0.825
Dominguez-Comesana et al**(39)** 2012	Pontevedra, Spain	Colorectal resection 69.9 years	120	13.3% (16)	POD1	0.5 -	No	100 -	80 -	- -
Durila et al**(40)** 2017	Prague, Czech Republic	Oesophagectomy Not given	38	23.7% (9)	POD2	2.0 273.8	No	78 78	79 83	- -
Facy et al**(41)** 2016	Dijon, France	Colorectal resection 65.4 years	463	12.1% (56)	POD2	0.25 -	No	82 -	40 -	0.648 -
Lahiri et al**(53)** 2016	London, UK	Hepatopancreatobiliary 63.2 years	69	13.0% (9)	POD2	- 97.7	No	- 100	- 83	- 0.98
Mokart et al**(32)** 2005	Marseille, France	Gastrectomy 50.7 years	50	32.0% (16)	POD1	1.1 310	Yes	81 90	72 58	0.749 0.821
Munoz et al**(46)** 2016	Alicante, Spain	Bariatric 44.8 years	115	11.3% (13)	POD2	0.2 -	No	69 -	78 -	0.876 -

.

Oberhofer et	Zagreb,	Colorectal resection	79	36.7% (29)	POD2	1.34	No	69	78	0.75
al**(47)** 2012	Croatia	64.9 years				-		-	-	-
Saeed et al**(48)**	Basingstoke,	Cytoreductive surgery**	50	28.0% (14)	POD1	1.5	No	69	62	0.690
2016	UK	54.7 years				-		-	-	-
Takakura et	Hiroshima,	Colorectal resection	114	15.8% (18)	POD1	0.77	No	83	64	0.76
al**(49)** 2013	Japan	64.4 years				-		-	-	-
Takeuchi et	Tokyo,	Oesophagectomy	30	33.3% (10)	POD5	0.2	No	50	75	0.582
al**(50)** 2020	Japan	72 years				-		-	-	-
Xiao et al**(51)**	Changsha,	Gastrectomy	552	6.7% (37)	POD3	0.695	No	65	66	0.678
2020	China	56.5 years				-		-	-	-
Pooled Result								**72 (66-78)**	**62 (59-64)**	**0.766**
								84 (72-92)	**76 (68-84)**	**(0.032)**
										0.878
										(0.037)

*IL-6 ratio >1.21 (preoperative sample/POD1 sample) rather than cutoff value used

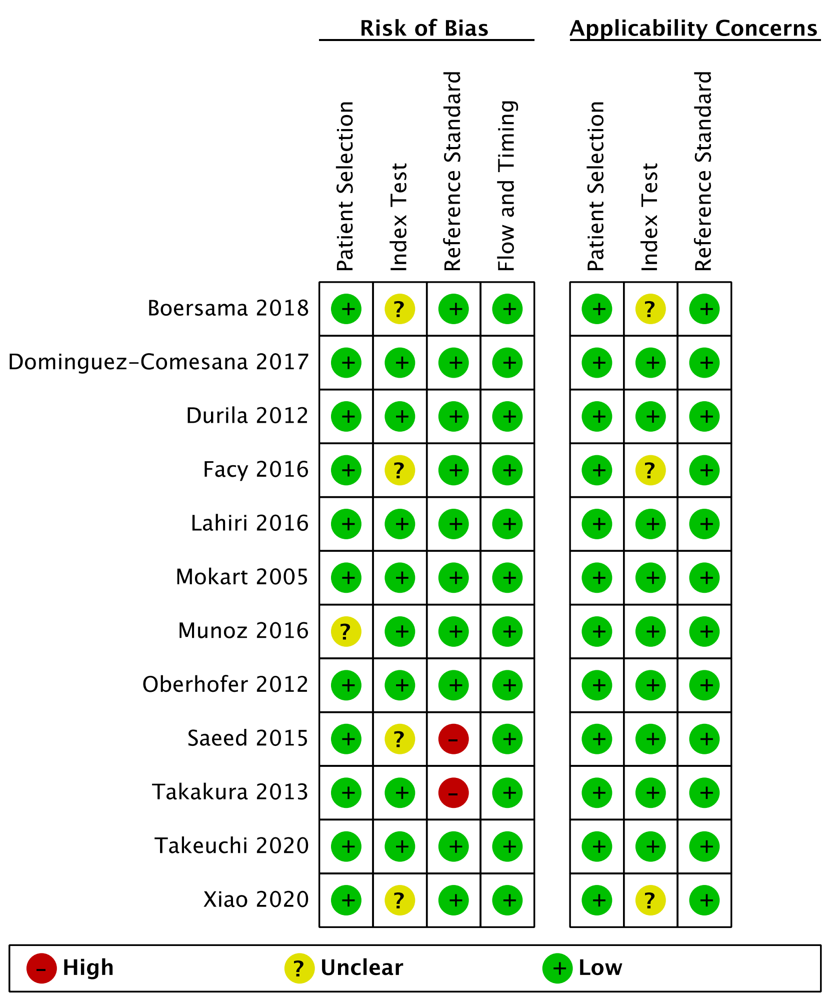
** Cytoreductive surgery including splenectomy for peritoneal malignancy

.

Quality

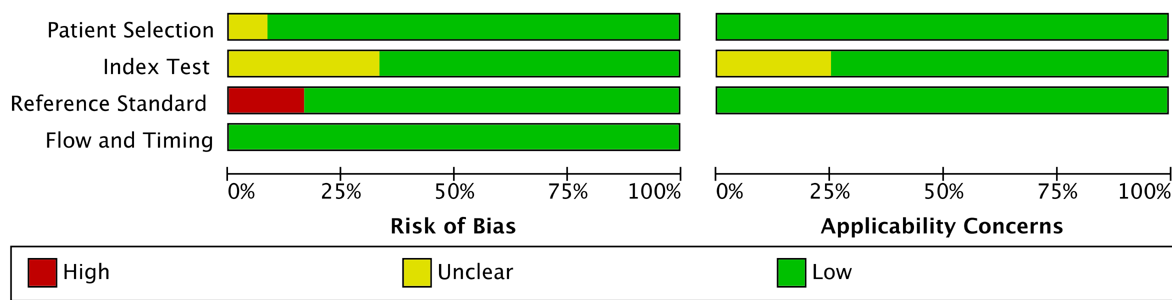
Using QADAS-2 criteria(132) studies showed minimal bias in patient selection and flow and timing of sampling (Figure 4-2, Figure 4-3). There was heterogeneity in the interpretation of index test (PCT/IL-6) with some studies using predetermined cut-off levels, and others using Youden index to calculate cut-off from their ROC curve, sometimes not corresponding with clinically accepted levels. Most studies used international consensus criteria to define clinical diagnosis as the reference standard, however 2 studies used diagnosis from attending clinicians as reference standard. There was low concern about the applicability of tests across most studies.

Figure 4-2 Methodological Quality Summary



.

Figure 4-3. Methodological Quality Graph



Procalcitonin

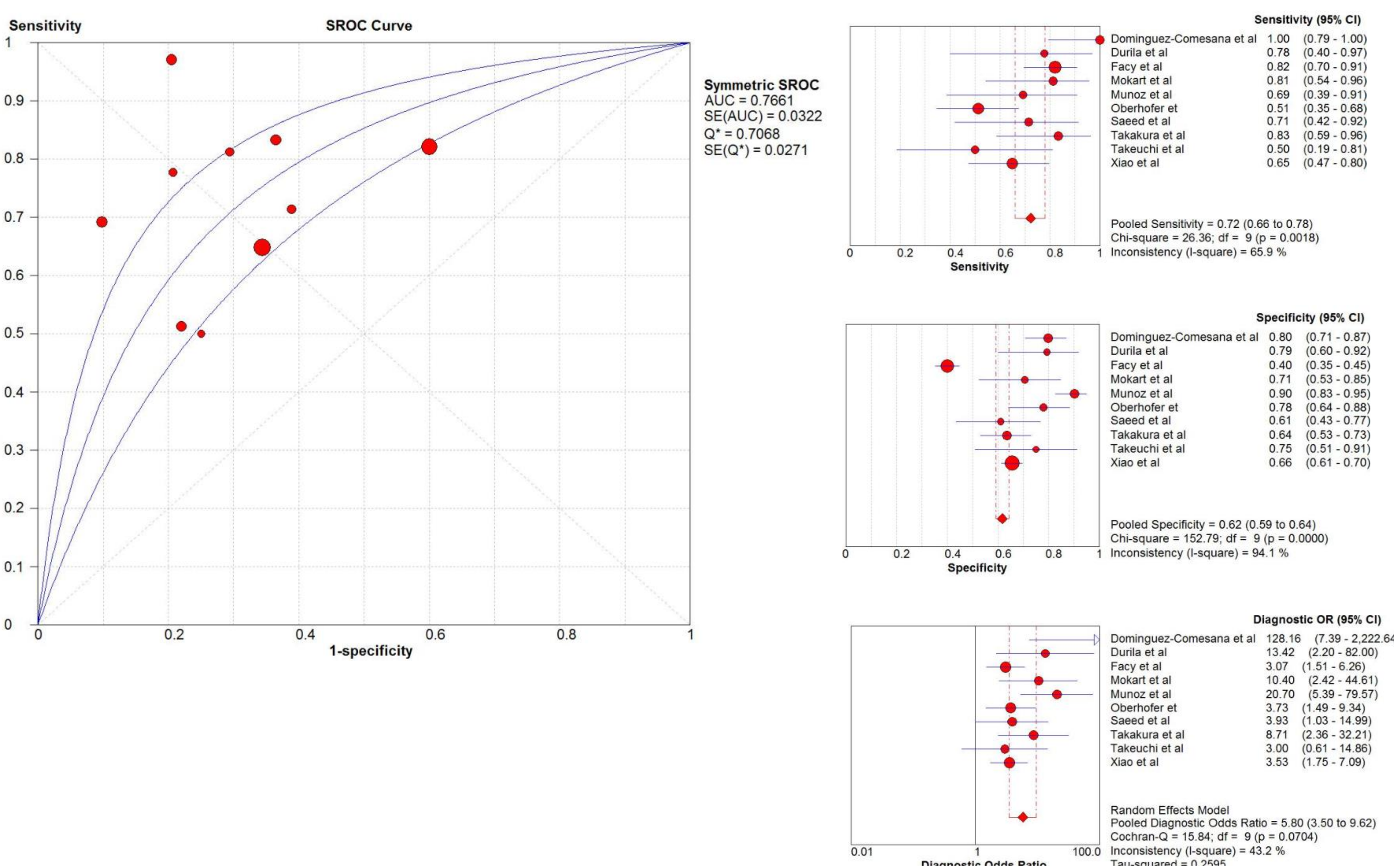
The search returned 13 studies of potential relevance, of which 9 were found to have enough information to go forward into a meta-analysis for PCT. 3 centres were contacted to request data to produce 2x2 contingency tables of which 1 returned appropriate information. In total, data from 10 studies for 1611 patients were included.

Sensitivity, specificity, and SROC

10 studies with 1611 participants reported data on the diagnostic accuracy of Procalcitonin, with pooled sensitivity, specificity and DOR of 72% (95% CI 66–78), 62% (95% CI 59-64) and 5.80 (95% CI 3.50–9.62) respectively (Figure 4-4). Symmetrical SROC was chosen as DOR was constant [b=-0.007 (p=0.976)], giving an area under the receiver operator curve (AUC) (SE) of 0.77 (0.03) with a Q statistic (SE) of 0.71 (0.03).

.

Figure 4-4. SROC, Sensitivity, Specificity and DOR of Procalcitonin for infection following HPB



.

Heterogeneity and subgroup analysis

There was significant heterogeneity between study results, with I^2 of 65.9% for sensitivity, 98.5% for specificity and 43.2% for DOR. There was significant variability in the timing of sampling (POD1 to POD5), a wide range of cut-off values (0.2-2.0ng/mL). Subgroup analysis using sample size (≤100 vs >100), location (European vs non-European) and operation type (Upper gastrointestinal vs Colorectal) was performed to investigate heterogeneity using diagnostic accuracy (I^2 DOR) (Table 4-2).

Interleukin-6

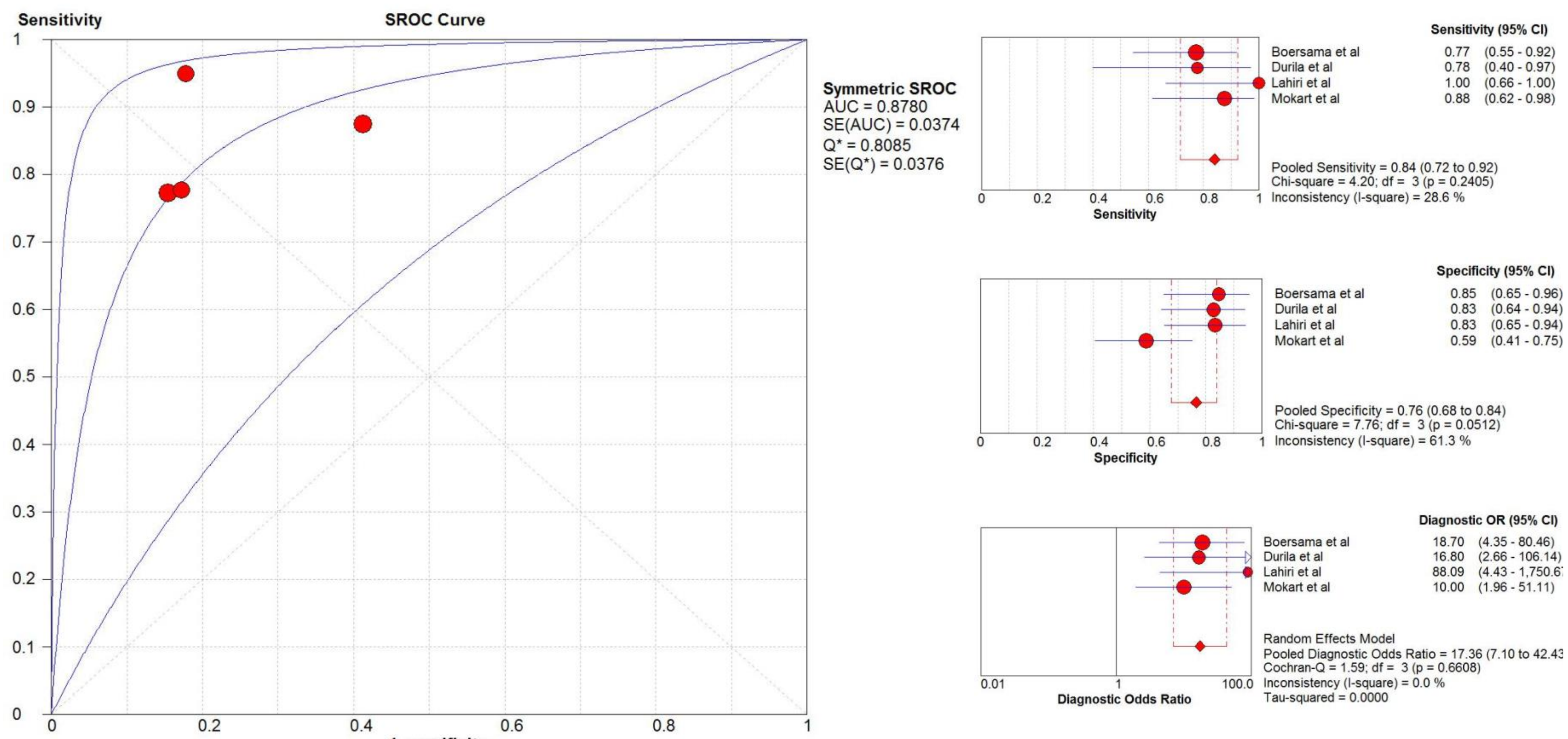
The search identified 4 studies of potential relevance, of which 3 were found to have enough information to go forward into a meta-analysis for IL-6. 1 centre was contacted to request data and, returned appropriate information produce 2x2 contingency tables. In total data from 4 studies for 175 participants were included.

Sensitivity, specificity, and SROC

4 studies with 175 participants reported data on the diagnostic accuracy of IL-6, with pooled sensitivity, specificity, DOR and SROC of 84% (95% CI 72 - 92), 76% (95% CI 68 - 84), 17.36 (95% CI 7.10 - 42.43) and 0.878 respectively (Figure 4-5).

.

Figure 4-5. SROC, Sensitivity, Specificity and DOR of Interleukin-6 for Infection following LT



.

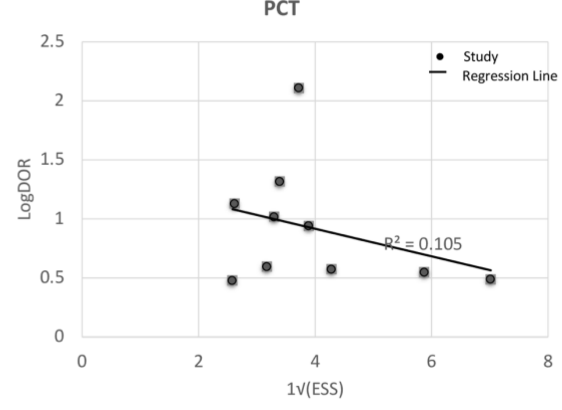
Heterogeneity

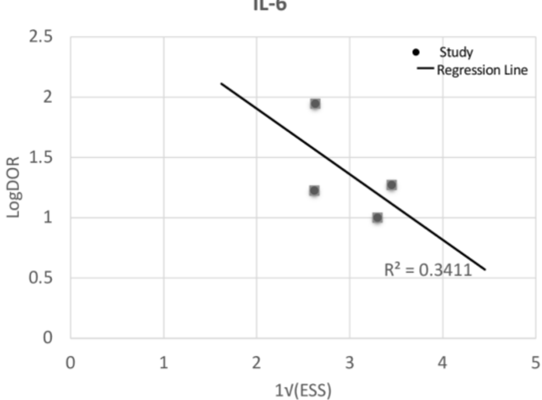
Findings were more consistent between studies, with I^2 of 28.6% for sensitivity, 61.3% for specificity and 0% for DOR. Subgroup analysis was not performed due to the small study number. Samples were consistently sampled on POD1 or POD2, but cut-off values were variable (97.7-310pg/mL), with the cut-off value not reported in one study.(30).

Publication Bias

Linear regression using Deek's test demonstrated no significant publication bias in this study sample for PCT (p=0.2) or IL-6 (p=0.53) (Figure 4-6).

Figure 4-6. Deek's Test for Publication Bias





.

Table 4-2. Subgroup Analysis for Procalcitonin

Subgroup		No. of studies	Pooled sensitivity (%) (95% CI)	Pooled specificity (%) (95% CI)	Pooled LR+ (95% CI)	Pooled LR- (95% CI)	Pooled DOR 95% CI	I^2 DOR %
All	All	10	72.4 (66-78)	61.7 (59-64)	2.57 (1.83-3.61)	0.46 (0.34-0.61)	5.81 (3.50-9.63)	43.2
Operation	Colorectal	5	74.8 (67-82)	53.1 (49-57)	2.28 (1.39-3.74)	0.42 (0.24-0.73)	5.12 (2.43-10.81)	49.8
	Upper GI	5	68.2 (57-78)	70.3 (67-74)	3.00 (1.81-4.98)	0.48 (0.35-0.65)	7.06 (3.19-15.65)	46.5
Sample size	≥100	5	78.9 (70-86)	56.1 (52-60)	2.76 (1.36-5.61)	0.44 (0.27-0.72)	6.96 (2.43-19.9)	65.9
	<100	5	62.6 (52-73)	72.8 (65-79)	2.38 (1.79-3.18)	0.54 (0.39-0.74)	4.94 (2.75-8.88)	0
Region	Europe	7	74.2 (67-81)	58.5 (55-62)	2.89 (1.68-4.97)	0.41 (0.27-0.62)	7.32 (3.50-15.28)	56.1
	Non-Europe	3	67.7 (54-79)	65.6 (62-69)	2.03 (1.66-2.49)	0.52 (2.31-7.31)	4.11 (2.31-7.31)	0

LR+ = positive likelihood ratio, LR- = negative likelihood ratio

.

Discussion

This meta-analysis shows that PCT performs only moderately well as a diagnostic test for post-operative infection/sepsis, comparable to CRP in other studies.(145-147). IL-6 performs better, with a high sensitivity of 0.84 and SROC 0.878, with less heterogeneity between study findings. All studies demonstrated preclinical changes in PCT/IL-6 with median time to diagnosis between POD3-10.

The meta-analysis is limited by number of publications, often with small sample sizes. Some studies were excluded where they presented data that included patient groups not undergoing major gastrointestinal surgery. The search did not return results included in similar meta-analyses where their reported outcome was anastomotic leak/dehiscence, which has been frequently examined in other similar meta-analyses.(57, 148-150) There was heterogeneity between operation types – some for benign disease with others for cancer, however there was little bias observed between studies and the definitions and reporting of outcomes was standardised. Outcome measures were similar between studies, frequently using International Consensus Sepsis definitions or Centre for Disease Control definitions for infection,(12, 114, 116) however some studies used clinician diagnosis which is subjective and a potential source of bias.

Similar meta-analyses have been conducted in colorectal surgery. In a meta-analysis of 11 studies and 2692 patients, Cousin et al tested the diagnostic accuracy of PCT for the early diagnosis of intra-abdominal infection following elective colorectal surgery. Results showed that PCT did not outperform CRP, with pooled sensitivity, specificity and SROC for PCT on POD3 of 0.69, 0.71 and 0.78 respectively, and for CRP 0.75, 0.72 and 0.8 (95% CI 0.76-0.85.(148) although the search included outcomes reported as AL. Tan et al found better results for PCT, with 1629 patients from 8 studies giving pooled sensitivity, specificity and

.

SROC for PCT on POD3 of 0.83, 0.68 and 0.83 respectively.(150) Another meta-analyses of PCT in the diagnosis of AL in colorectal surgery found similar results. Su'a et al reported SROC on POD5 of 0.86 (95% CI 0.79-0.94), and authors concluded that PCT was a useful negative predictor of AL comparable to CRP, but not useful as a diagnostic test.(149) In clinical practice, PCT costs 25 times more than CRP without a significant diagnostic advantage.(148)

Conclusion

There is a role for a biomarker which could quickly and reliably diagnose postoperative sepsis and be used to guide clinical management and antimicrobial use to improve patient outcomes. While a reasonable SROC AUC for PCT has been reported (0.805), sensitivity and specificity are comparable to research traditional markers such as WCC and CRP.(145, 146, 148) Heterogeneity between sampling times and PCT cut-off with significant inconsistency mean that we cannot make accurate conclusions about the accuracy of PCT in diagnosing early postoperative infection. Diagnostic accuracy is better for IL-6, with SROC 0.878, and although the sample size is smaller, studies were more homogenous. A larger, multi-centre study with a fixed sampling time point could provide data which may be comparable to those seen in colorectal surgery.

.

4.2 Diagnostic Accuracy of Procalcitonin for Postoperative Infection in Liver Transplantation: Meta-analysis

Search Strategy

An electronic search of Medline, Embase and Cochrane Library was conducted from January 1996 to June 2020 using the terms ‘sepsis’ (MeSH Major Topic and keyword) or ‘infection’ (keyword), ‘biomarker’ (MeSH term and keyword, including Procalcitonin), and ‘liver transplant/transplantation’ (MeSH term or keyword) (Appendix 1). Bibliographies of relevant studies and the ‘related articles’ link in PubMed were used to identify additional studies. Any study published only in abstract format or unpublished reports were excluded from the analysis. All citations and abstracts identified were thoroughly reviewed by the investigators, and secondary references were obtained from the key articles. Studies were screened by title, abstract, and full text articles were assessed for eligibility with relevant studies included in the synthesis. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidance and Meta-analysis of observational studies in epidemiology (MOOSE) checklist were utilised.(29, 130) Studies were reviewed for relevance to diagnostic accuracy of PCT for sepsis in the early postoperative period (up to 2 weeks) in patients undergoing liver transplantation. Study design and technique were reviewed.

.

Inclusion Criteria

Only original studies in humans published in English were considered for inclusion. Adult and paediatric (<16 years old) patients were included, but studies on neonatal patients were excluded. Included studies analysed the diagnostic performance of PCT for post-operative sepsis or infection in patients undergoing liver transplantation with cut-off values. Studies were evaluated for duplication or overlapping.

Exclusion Criteria

Studies were excluded if they reported data from small patient cohorts (<10 patients), or there was overlap with institutions or patient cohorts already published in better quality studies. Studies which reported other outcomes (e.g. cardiovascular complication) or included other types of operation were excluded.

Outcome Measures

The primary outcome of interest was diagnostic performance (sensitivity, specificity, and Area Under the Receiver Operator Cure (AUC)) of Procalcitonin in detecting postoperative sepsis or infection.

.

Study Selection

Abstracts were reviewed to exclude those that did not meet the inclusion criteria. Where no abstract was available or the abstract details were inadequate, the full text article was reviewed.

Data Extraction

Data were extracted standardised pro-forma. Data on demographic and clinical parameters were recorded: study characteristics (first author, year of publication, study design, study period, country where the study was performed), population characteristics (number of patients studied, patient demographics), data quality, reported biomarkers with cut-off values and test performance. Data were entered into a spreadsheet as binary variables where possible to facilitate further analysis. Test performance variables were extracted as a 2x2 contingency table of true positive, true negative, false positive and false negative from results. If not immediately available values were calculated from published performance results including sensitivity, specificity, negative or positive predictive value. Where insufficient results to construct 2x2 contingency tables were presented, authors were contacted to contribute data. Where samples at several time points were presented, values were taken for the day closest to POD2. Where data were presented for several cut-off levels for PCT, the data presented in the abstract or the highest performing cut-off was included.

.

Quality Assessment

Study quality was assessed by using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) tool, examining patient selection, index test, reference standard and flow and timing of testing, using Revman software.(131, 132, 138)

Analysis

Pooled Sensitivity, Specificity, positive predicative value (PPV), negative predictive value (NPV) and diagnostic odds ratio (DOR) were calculated using the freeware Meta-DiSc version 1.4 (Universidad Complutense, Madrid)(133) using DerSimonian–Laird random effects models with over-dispersion corrections. including heterogeneity using inconsistency assessment (I^2)(135). Subgroup analysis using study population (adult vs paediatric), sample size (≥50 vs <50), location (European v non-European) was performed to investigate heterogeneity using diagnostic accuracy (I^2 DOR). Studies were examined for publication bias using Deek's test – a funnel plot and effective samples size (ESS) regression analysis. Logarithm of DOR is plotted against 1/√ESS, where ESS=(1/diseased + 1/not diseased).(137)

Results

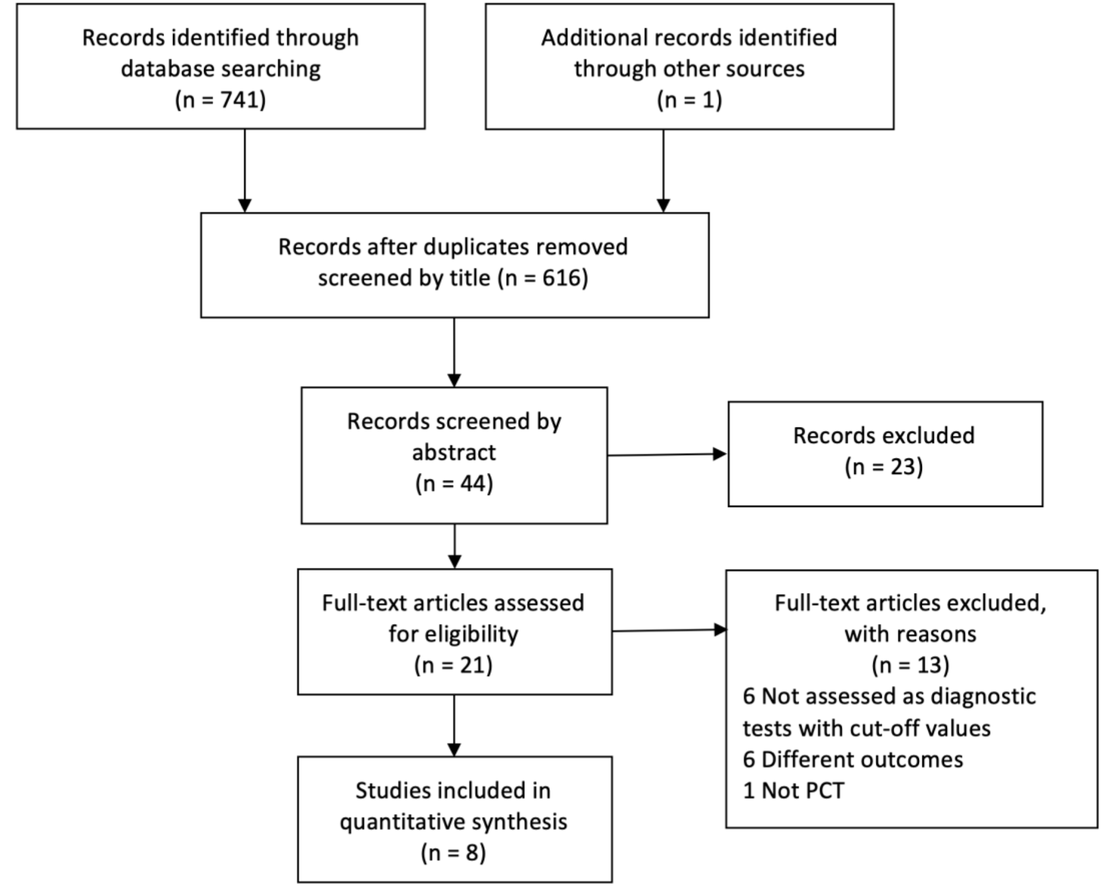
The search identified 616 studies of potential relevance after duplicates were removed, of which 596 were excluded by title or abstract. 13 further studies were excluded at full text review with reasons presented (PRISMA diagram). 8 observational studies including 418

.

patients(31, 33, 42-45, 55, 151) that examined the diagnostic performance of PCT for postoperative sepsis or infection in liver transplantation (Characteristics of Included Studies table).(152) 1 centre was contacted to request data to produce 2x2 contingency tables, and they returned appropriate information. 3 studies presented data exclusively on PCT (43-45), while 5 studies examined PCT alongside other biomarkers, including Interleukin-6, CRP, Plasma Proteome and (1-3)-β-D-glucan.(31, 33, 42, 55, 151). PCT was measured using immunoassay technique (where described) and cut-off values ranged from 0.48ng/mL to 42.8ng/mL. 5 studies generated optimal cut-off points using receiver operator curve analysis, while 2 used pre-determined cut-off values. There was variation in the time points samples were taken, between POD1 and POD8, and some samples examined on the day of or the day before diagnosis of infection.

.

Figure 4-7 PRISMA Diagram for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis



6 studies examined transplantation in adults, with similar median ages ranging from 47-53 years where given. 2 studies examined children, with a median age of 2 years. Most studies did not specify the mode of organ donation (cadaveric vs living donor transplantation), although in Kaido et al, 86.5% (90/104) of patients underwent living donor liver transplantation. Outcomes were comparable, with most studies referenced the Centre for Disease Control criteria to define post-operative infection, and the American College of Chest Physicians/Society of Critical Care Medicine Consensus statement to define sepsis,(114) along with independent clinical assessment considering radiological and microbiological results.

.

Figure 4-8 Methodological Quality Summary Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis

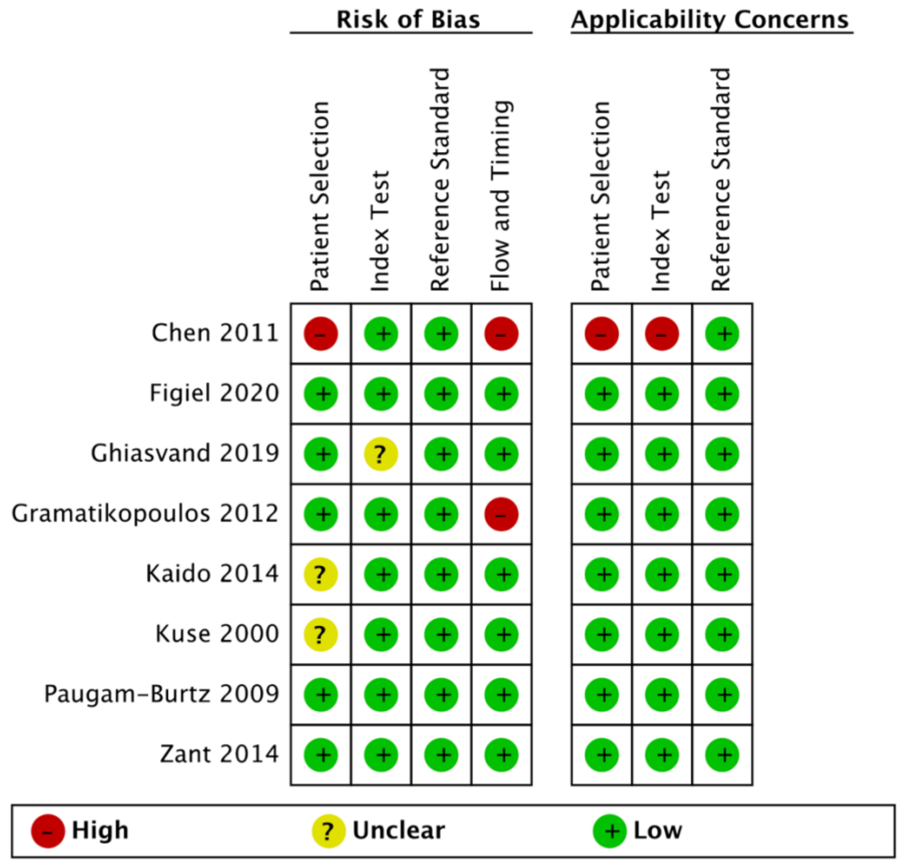
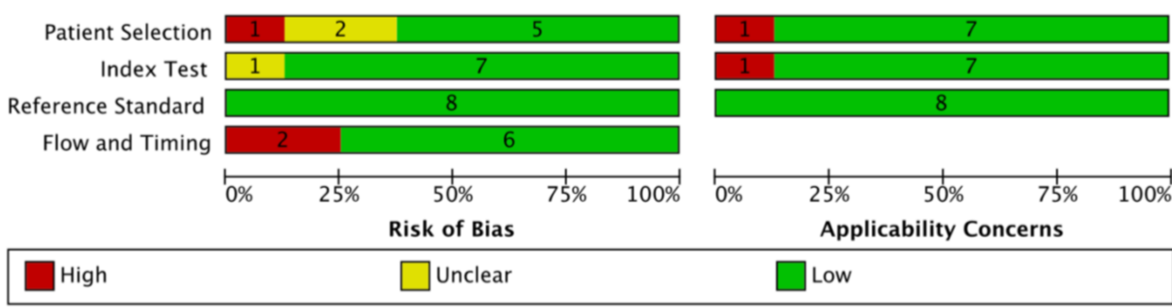


Figure 4-9 Methodological Quality Graph Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis



.

Table 4-3 Characteristics of Included Studies for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation Meta Analysis

Authors (year)	**Location**	**Patient Group Median age (years)**	**n**	**Rate of infection**	**Timing of ample (POD†)**	**Cut-off PCT (ng/mL)**	**Cut-off predeter mined?**	**Sensitivity (%) (95% CI)‡**	**Specificit y (%) (95% CI)**	**AUC§ (95% CI)**
Chen et al **(58)** (2011)	Tianjin, China	Adult 53	55	25 (46%)	On day of suspicion of sepsis	3.1	No	71 (51-88)	87 (69-96)	0.84 (0.73-0.95)
Figiel et al **(42)** (2020)	Warsaw, Poland	Adult 50	60	9 (15%)	POD3	42.8	No	47 (14-79)	84 (71-93)	0.64 (0.54-0.92)
Ghiasvand et al **(43)** (2019)	Tehran, Iran	Adult 47	28	9 (32%)	POD1-2	5.0	Yes	78 (40-97)	79 (54-94)	0.78 (0.59-0.92)
Grammatikopoulos et al**(31)** (2012)	London, UK	Paediatric 2	58	23 (40%)	POD1/7/at febrile episode	0.48	No	100 (85-100)	91 (77-98)	0.97 -
Kaido et al**(44)** (2014)	Kyoto, Japan	Adult 52	91	26 (29%)	POD8	2.0	Yes	97 (80-99)	38 (44-69)	- -
Kuse et al**(45)** (2000)	Hannover, Germany	Adult Unknown age	40	11 (28%)	POD prior to infection diagnosed	1.8	No	67 (31-89)	100 (88-100)	0.93 -
Paugam-Burtz et al**(55)** (2009)	Paris, France	Adult Unknown age	61	31 (51%)	POD5	1.4	No	32 (17-51)	97 (83-100)	0.73 (0.59-0.87)
Zant et al**(33)** (2014)	Regensberg, Germany	Paediatric 2	25	4 (16%)	POD0-7	1.1	No	82 (19-99)	34 (14-57)	0.52 -
Pooled	-	-	-	-	-	-	-	**70 (62-78)**	**78 (73-83)**	**0.87**

†Postoperative day, ‡95% Confidence interval §Area Under Receiver Operator Curve

.

Diagnostic Accuracy

Pooled sensitivity, specificity, diagnostic odd ratio (DOR) and summary receiver operator characteristic (SROC) of PCT for postoperative infection was 70% (95% CI 62 - 78), 78% (95% CI 73 - 83), 15.82 (95% CI 5.81 – 43.12) - and 0.871 respectively (Figure 4-10Figure 4-10 SROC showing (A)sensitivity, (B) specificity and (C) diagnostic odds ratio (DOR) for Procalcitonin for the diagnosis of post-operative infection/sepsis in Liver Transplantation).

Heterogeneity and Subgroup Analysis

Significant heterogeneity was demonstrated between studies using I^2, 86% for sensitivity, 89% for specificity and 47.7% for DOR (Figure 4-10). Subgroup analysis demonstrated high heterogeneity of DOR in paediatric studies compared to adult studies (I^2, 88.2% vs 0%), and in European studies compared with non-European (I^2, 68.1% vs 0%) (

Figure 4-10 SROC showing (A)sensitivity, (B) specificity and (C) diagnostic odds ratio (DOR) for Procalcitonin for the diagnosis of post-operative infection/sepsis in Liver Transplantation

.

Table 4-4). Significant heterogeneity of DOR between study size ≥50 and <50 was not demonstrated (I^2, 53.2% vs 56.6%).

Publication Bias

Linear regression using Deek's test did not show significant publication bias in the sample of studies, p=0.17 (Figure 4-11).

.

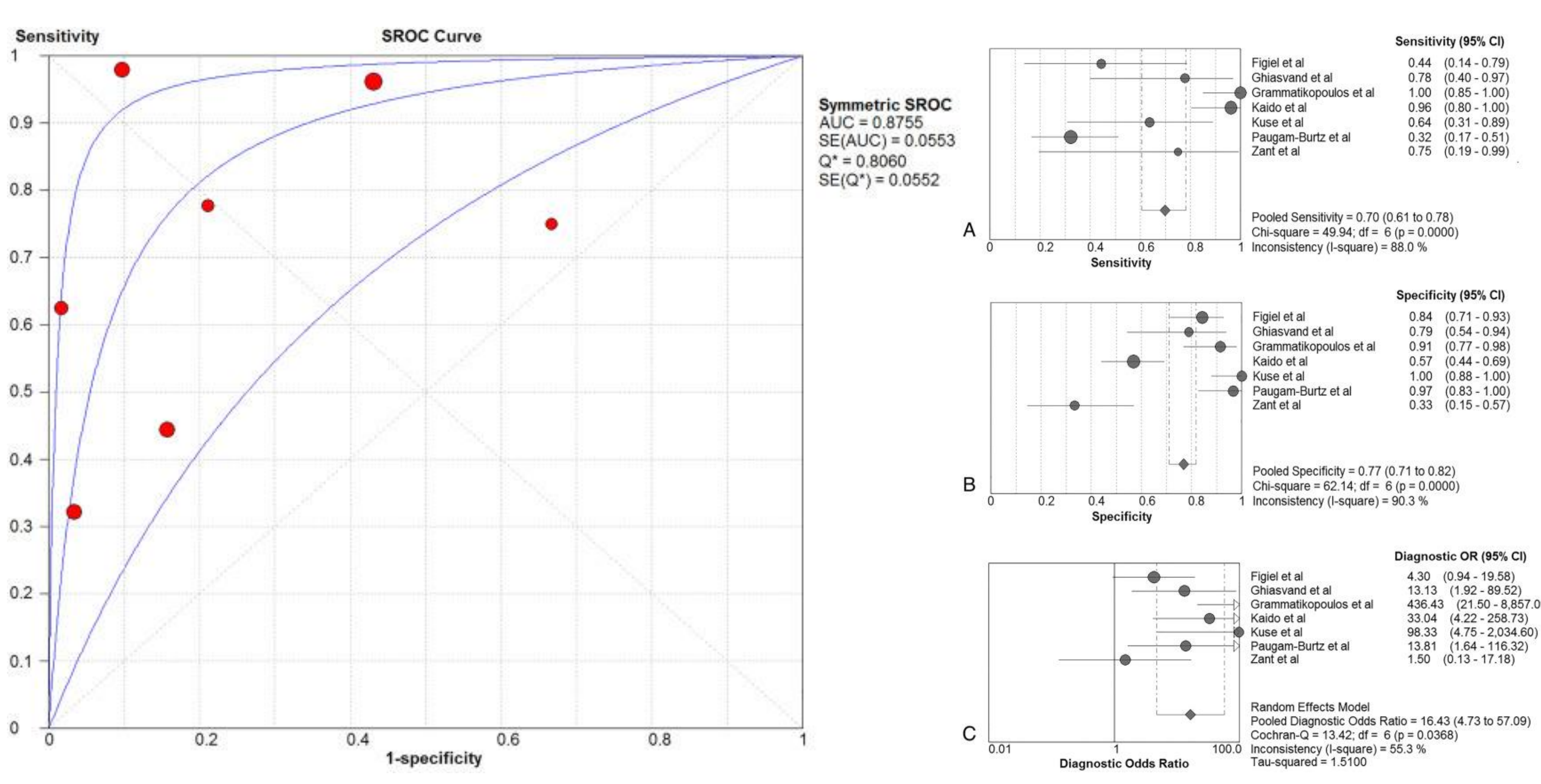


Figure 4-10 SROC showing (A)sensitivity, (B) specificity and (C) diagnostic odds ratio (DOR) for Procalcitonin for the diagnosis of post-operative infection/sepsis in Liver Transplantation

.

Table 4-4 Subgroup Analysis for Diagnostic Accuracy of PCT for postoperative infection following Liver Transplantation - Meta Analysis

Subgroup		**Number of studies**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**	**Pooled LR+ † (95% CI)**	**Pooled LR- ‡ (95% CI)**	**Pooled DOR § (95% CI)**	**I^2 (%) DOR %**
All	All	8	70 (62-78)	78 (73-83)	3.86 (1.95-7.62)	0.36 (0.18-0.71)	15.82 (5.81-43.12)	0.00
	Adult	6	64 (54-73)	80 (74-85)	3.99 (2.01-7.89)	0.41 (0.22-0.74)	13.81 (6.56-29.07)	0.00
	Paediatric	2	96 (81-99)	70 (56-81)	3.29 (0.22-48.95)	0.15 (0.00-10.14)	23.87 (0.09-6731.1)	88.20
Sample size	≥50	5	70 (61-78)	79 (73-84)	4.5 (1.92-10.52)	0.29 (0.10-0.85)	19.33 (5.58-66.91)	53.20
	<50	3	71 (49-87)	74 (62-84)	3.68 (0.63-21.39)	0.38 (0.21-0.70)	10.96 (1.31-91.76)	56.60
Location	Europe	5	60 (49-71)	84 (78-89)	5.19 (1.26-21.29)	0.48(0.22-1.06)	16.41 (2.60-103.43)	68.10
	Non-Europe	3	83 (72-92)	68 (59-77)	3.18 (1.65-6.13)	0.25 (0.11-0.56)	18.31 (6.88-48.78)	0.00

†Positive likelihood ratio, ‡Negative likelihood ratio, §Diagnostic Odds Ratio

.

Figure 4-11 Deek's Test for Publication Bias

LogDOR

3.00
2.50
2.00
1.50
1.00
0.50
0.00

Study
Regression Line

$R^2 = 0.3055$

0.00 1.00 2.00 3.00 4.00 5.00

1√(ESS)

Discussion

This meta-analysis demonstrates good sensitivity and specificity for PCT in the diagnosis of early post-operative infection/sepsis following LT. There is significantly reduced heterogeneity in studies of adults post LT and a strong recommendation to consider the use of PCT in this group can be made.

Immunosuppression is essential post LT despite the presence of infection, in some instances. Discriminating the dominant process has implications for day-to-day decision making, particularly in the early post-transplant phase. Despite reluctance in the adoption of PCT in many non-liver settings, the coronavirus disease 2019 (COVID-19) pandemic has galvanised many centres to rethink its utility. Since immunosuppression is the cornerstone of therapy for COVID-19 pneumonia and the risk of bacterial infection is high, low PCT levels are now widely used to determine the relative safety of augmenting immunosuppression

.

beyond low dose dexamethasone. This is despite the major platform trials not including PCT in exclusion criteria for such agents.

The included studies reported a wide range of cut-off values, many of which were significantly higher than the clinical cut-off of 0.5ng/mL implemented in clinical practice(126, 153), and most cut-off points were generated to optimise performance within their patient group, which could not be generalised between studies. While all studies show a relationship between PCT and diagnosis of postoperative infection/sepsis, variability in time points of PCT sampling should be considered when considering clinical applicability. Some studies have examined PCT at the time of febrile episodes, on the day preceding infection diagnosis or up to POD8. Limited information is presented on the blinding of clinicians to PCT results, which may contribute to bias. Despite patients with severe liver disease already have compromised immune function and receiving immunosuppression in the immediate postoperative phase, PCT remains an option for sepsis monitoring post LT.(154, 155) When investigating their primary outcome of differentiating acute cellular rejection from postoperative infectious episodes in fever of unknown origin, Kuse et al found statistical significance in PCT level on the day of diagnosis with all patients with infectious episodes having elevated PCT.(45) Similarly, Kaido et al found PCT levels in patients bacteraemia diagnosis (5.71 ± 1.27 ng/mL) were significantly higher than those in patients diagnosed with CMV viraemia (0.53 ± 0.08 ng/mL) ($P < 0.001$), and at POD8-30 at the time of diagnosis of acute rejection were 0.42 ± 0.18 ng/mL, significantly lower than those in patients diagnosed with bacteraemia (5.71 ±1.27 ng/mL) ($P < 0.001$).(44)

.

Further limitations in include small sample sizes and exclusion of non-English language publications. Some studies were excluded where they presented data including other complications (cardiovascular etc).(156) Chen et al used a biased cohort already suspected of having central venous catheter related blood stream infections, as reported in the summary of bias.(151) Subgroup analysis did not demonstrate significant heterogeneity in DOR, and significant publication bias was not observed.

While the results of this meta-analysis demonstrate a weaker diagnostic performance of PCT than that of Yu et al 2014(157) (pooled sensitivity and specificity for liver transplant subgroup of 0.9 (95% CI 0.75-0.97) and 0.85 (95% CI 0.77-0.91) respectively), the study by Prieto et al 2009 was excluded in this meta-analysis as it included non-infectious complications in their outcomes.(151, 156) This review does include a larger number of studies and therefore may represent a more realistic interpretation of PCT diagnostic accuracy.

Similar meta-analyses have shown similar diagnostic performance of PCT for infection after Colorectal surgery. A study by Cousin et al(148) (primary outcome intra-abdominal infection including anastomotic leakage) demonstrated a pooled sensitivity, specificity and SROC of PCT on POD3 of 0.69 (95% CI 0.6-0.77), 0.71 (0.69-0.74) and 0.78 respectively (comparable to CRP). Similar studies by Tan et al 2018(150) (primary outcome intra-abdominal infection including anastomotic leakage) showed a pooled sensitivity, specificity and SROC of PCT on POD3 of 0.83 (95% CI 0.73-0.88), 0.71 (0.69-0.74) and 0.83

.

respectively, and Su'a et al 2019(149) (primary outcome anastomotic leakage) reported summary AUC for PCT on POD3 0.86 (95% 0.79-0.94). Despite these findings, it is not common current practice in the United Kingdom to use PCT to guide clinical decision-making following surgery, with most centres still using WCC and CRP, radiological findings and microbiological results to guide treatment decisions. While a reasonable AUC for SROC is found (0.879), sensitivity and specificity are comparable to traditional markers such as WCC and CRP. There is however an ongoing need for an accurate novel biomarker which can rapidly give a diagnosis of post-operative sepsis/infection and its resolution and help guide clinical decisions and the additional requirement of immunosuppressive drug changes makes PCT a more attractive addition to post LT care.

.

5 Carbon-13 Breath Delta Value

5.1 Introduction

Infrared Spectroscopy

Infrared spectroscopy is a technique which measures the interaction between infrared radiation with molecules (in this experiment carbon dioxide, CO_2). As infrared radiation passes through molecules, bonds within functional groups absorb energy which is transferred to vibration or stretching of the bonds. This vibration depends on the length of the bond and the mass of the atoms at in the nuclei, meaning that each different bond will vibrate in a different way. Consequently, a molecule will absorb at characteristic frequencies in the electromagnetic spectrum, depending on the precise nature of the molecule's bonds. Absorption stimulates bonds to vibrate in different directions according to the symmetry and direction of movement as shown in Table 5-1. Because of the vibration frequency's dependence on the mass of the constituent atoms, different isotopes change the absorption frequency, known as the isotope shift. Because of this the characteristic absorption 'fingerprint' of exhaled CO_2 will vary depending on the presence of the ^{12}C and ^{13}C isotopes. A gas sample containing only $^{12}CO_2$ will have a particular infrared absorption spectrum, whereas a gas sample containing only $^{13}CO_2$ will have a similar but different spectrum, with the absorption features shifted to different frequencies. A gas mixture containing both $^{12}CO_2$ and $^{13}CO_2$ will produce infrared spectra containing absorption features from both isotopologues, and the relative strengths of the absorption features can be used to measure the relative concentrations of the two isotopologues in the sample. From studying the infrared spectrum of a gas mixture containing both isotopologues it is therefore possible to measure the delta value of that sample.

.

Table 5-1 Direction of vibration of bonds during absorption of IR at their given frequency(158)

	Symmetric	**Asymmetric**
Radial	Symmetric stretching (v_s)	Antisymmetric stretching (v_{as})
Latitudinal	Scissoring (δ)	Rocking (ρ)
Longitudinal	Wagging (ω)	Twisting (τ)

Atmospheric $\delta^{13}C$ is becoming more negative globally, at -8 to -9 per mil at most recent estimates.(159-161) Existing literature suggests BDV of -21 to -23 permil in healthy human and non-infected subjects, and is always seen to be negative(162, 163) becoming more negative during infective processes.(72, 76) As explored in Chapter 1.6, BDV has been shown to be a potential early biomarker for sepsis. This experiment aims to examine BDV in patients who develop infective complications following HPB surgery and LT.

.

5.2 Results

Sample Quality

Table 5-2 Mean and median $^{12}CO_2$ concentration, BDV and number of samples with unrecordable BDV, $^{12}CO_2$ concentration >0.01 and >0.02

	HC	All	HPB	LT	Mean BDV
n=	8	384	171	213	-22.41
Mean $^{12}CO_2$ (SEM)	0.034 (0.0026)	0.01 (0.00057)	0.013 (0.001)	0.0083 (0.00062)	-
Median $^{12}CO_2$ (IQR)	0.035 (0.032-0.037)	0.0077 (0.0029-0.014)	0.0092 (0.0039-0.02)	0.0064 (0.0023-0.011)	-
Mean BDV (exclusions)	-22.41	--	-20.28	-20.29	-
Mean BDV (SEM)	-22.41 (0.44)	-17 (0.8)	-17 (0.8)	-18 (0.54)	-
Median BDV (IQR)	-23 (-23— -23)	-19 (-21 - -15)	-19 (-21 - -16)	-18 (-21 - -15)	-
Unrecordable	0 (0%)	97 (25.3%)	46 (26.9%)	51 (24.0%)	-
$^{12}CO_2$ >0.01	8 (0%)	107 (27.9%)	57 (33.0%)	50 (23.5%)	-19.6
$^{12}CO_2$ >0.02	8 (0%)	43 (11.2%)	30 (17.5%)	13 (6.1%)	-20.52

.

$^{12}CO_2$ concentration and BDV

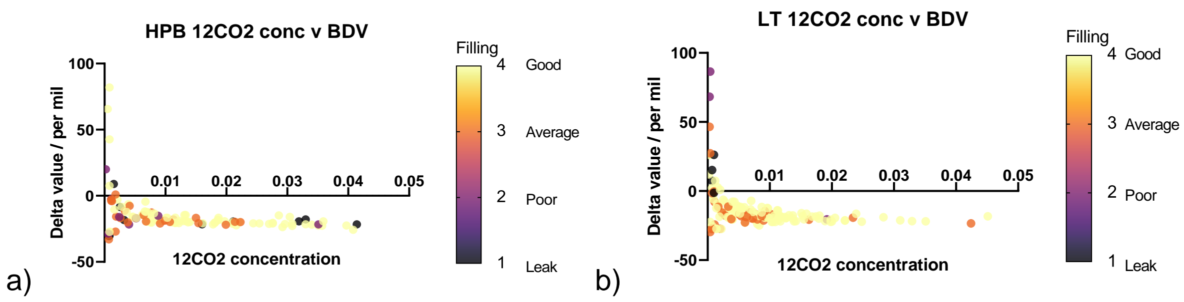


Figure 5-1 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration (fraction of gas) in a) HPB group and b) LT group

The colour bar indicates the adequacy of bag filling, where pale yellow is a well filled sample bag, orange is an average filled sample bag, purple is a poorly filled sample bag and blue is a leaking sample bag. The colours are used to identify trends in $^{12}CO_2$ concentration depending on bag filling and age of sample.

$^{12}CO_2$ concentration and BDV for all samples (HPB and LT, n=384) were plotted to examine whether lower $^{12}CO_2$ is associated with variability in BDV. $^{12}CO_2$ fraction in room air is 0.0005-0.001, and $\delta^{13}C$ in room air is -8 per ml. Spearman r correlation of $^{12}CO_2$ concentration against BDV for all samples showed a negative correlation -0.36 (95% CI -0.46, -0.25) $p<0.0001$.

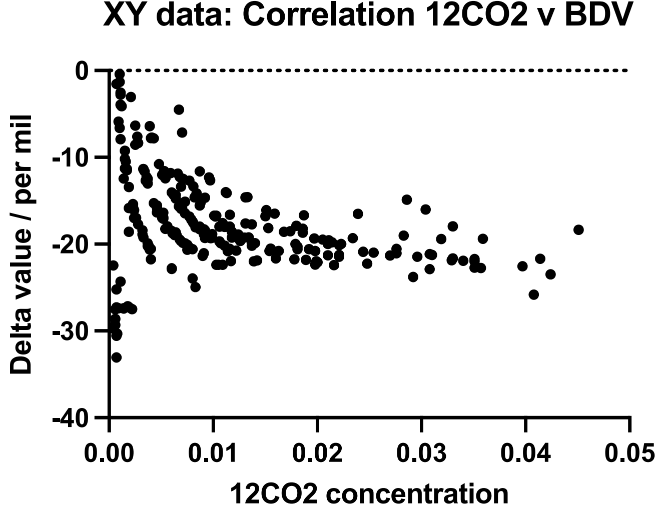


Figure 5-2 Spearman r correlation of $^{12}CO_2$ concentration against BDV for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil

.

Duration of storage and $^{12}CO_2$

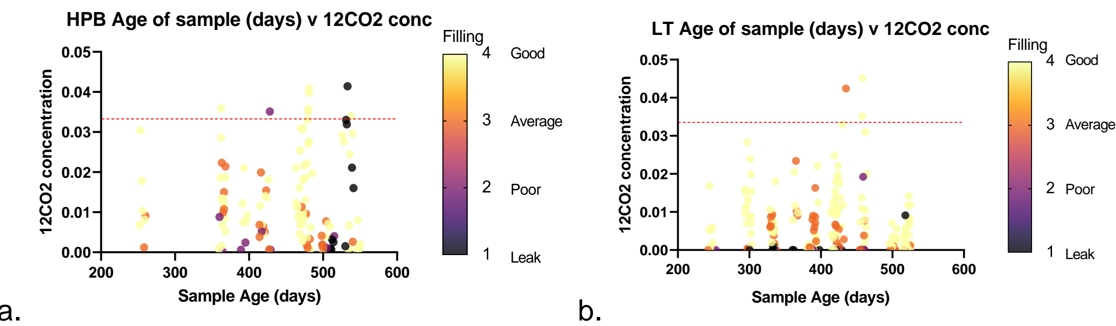


Figure 5-3 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration (fraction of gas)

in a) HPB group and b) LT group, with the red dotted line showing the mean $^{12}CO_2$ for healthy control samples. The colour bar indicates the adequacy of bag filling, where pale yellow is a well filled sample bag, orange is an average filled sample bag, purple is a poorly filled sample bag and blue is a leaking sample bag. The colours are used to identify trends in $^{12}CO_2$ concentration depending on bag filling and age of sample. $^{12}CO_2$ concentration was lower in samples which had undergone prolonged storage

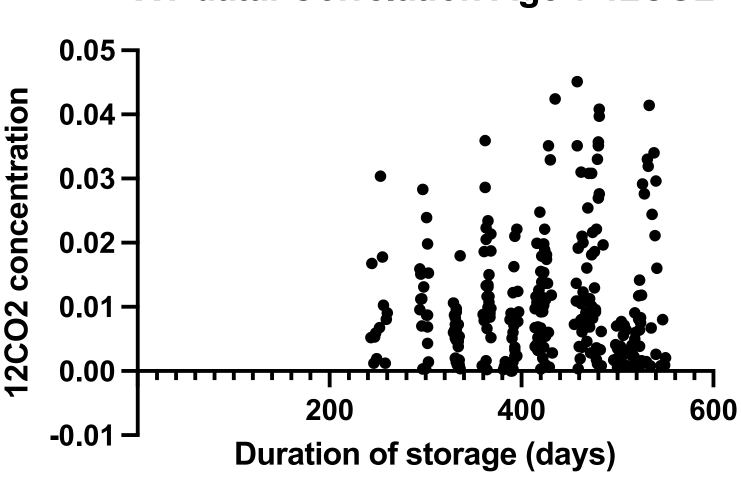


Figure 5-4 Spearman r correlation of length of time samples stored against $^{12}CO_2$ for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil

Duration of storage (days) and $^{12}CO_2$ concentration was analysed using Spearman r correlation for all samples (HPB surgery and LT, n=384). Duration of storage was not correlated with $^{12}CO_2$ concentration, Spearman r -0.00059 (95%CI-0.12,0.1), p=0.99.

.

Duration of storage and BDV

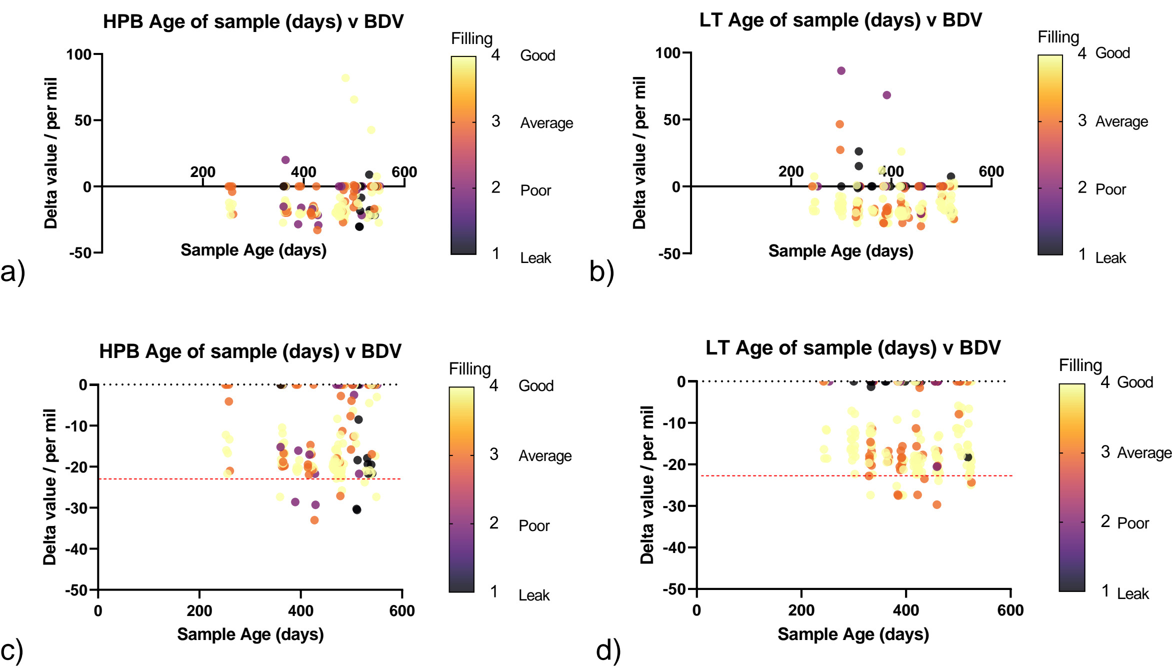


Figure 5-5 Graph to demonstrate the relationship between age of sample (days) and BDV (per mil) in a) HPB group and b) LT group, excluding values where BDV is > -8 permil c) HPB group and d) LT group with the red dotted line showing the mean $^{12}CO_2$ for healthy control samples.

The colour bar indicates the adequacy of bag filling, where pale yellow is a well filled sample bag, orange is an average filled sample bag, purple is a poorly filled sample bag and blue is a leaking sample bag. The colours are used to identify trends in $^{12}CO_2$ concentration depending on bag filling and age of sample.

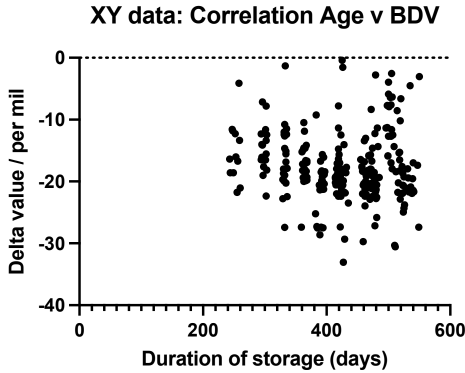


Figure 5-6 Spearman r correlation of duration of sample storage (days) and BDV for all samples (HPB and LT, n=384) excluding values where BDV is >0 permil

Duration of storage (days) and BDV was analysed using Spearman r correlation for all samples (HPB surgery and LT). Duration of storage was negatively correlated with BDV concentration, Spearman r -0.013 (95%CI-0.25, -0.0094), p=0.03

.

Valve type $^{12}CO_2$ v BDV

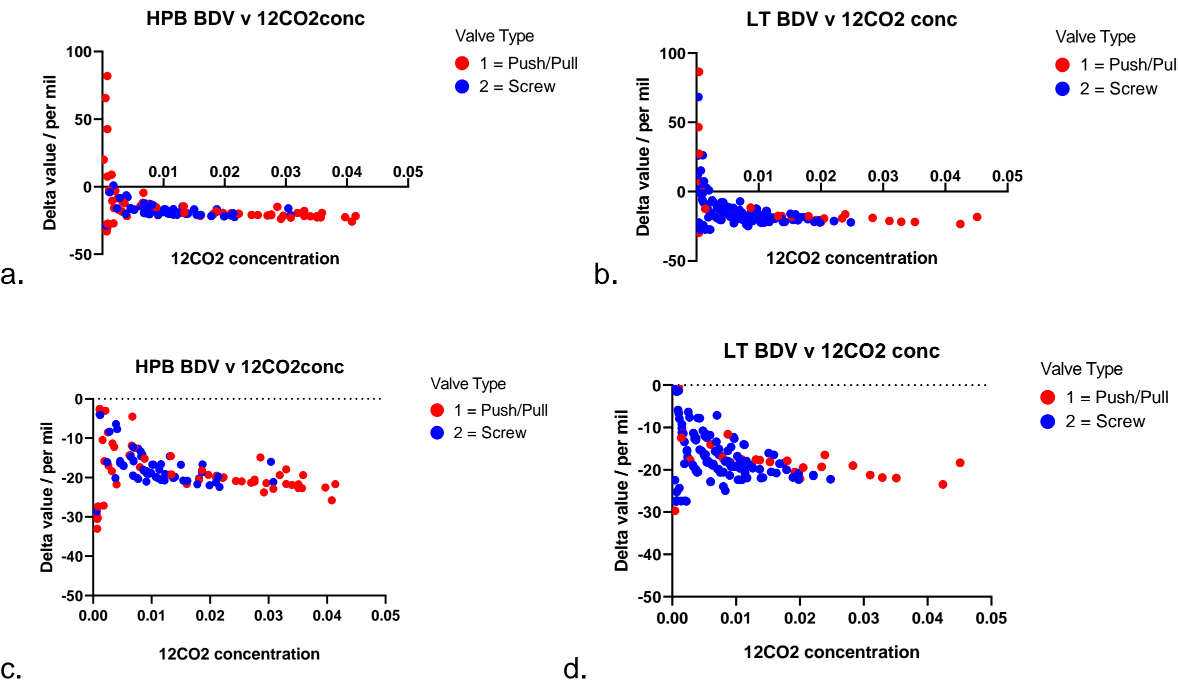


Figure 5-7 Graph to demonstrate the relationship between $^{12}CO_2$ concentration of sample (fraction) and BDV (permil)

in a) HPB group and b) LT group, and excluding values BDV < -8 permil in c) HPB group and d) LT group. The colours indicate the type of valve closing the bag (red, push/pull valve; blue, screw valve) The colours are used to identify trends in BDV and $^{12}CO_2$ concentration, to indicate whether one valve type was more likely to leak sample than the other. These graphs do not demonstrate a different between the valve type and BDV /$^{12}CO_2$ concentration.

.

Duration of sample storage and CO_2 concentration

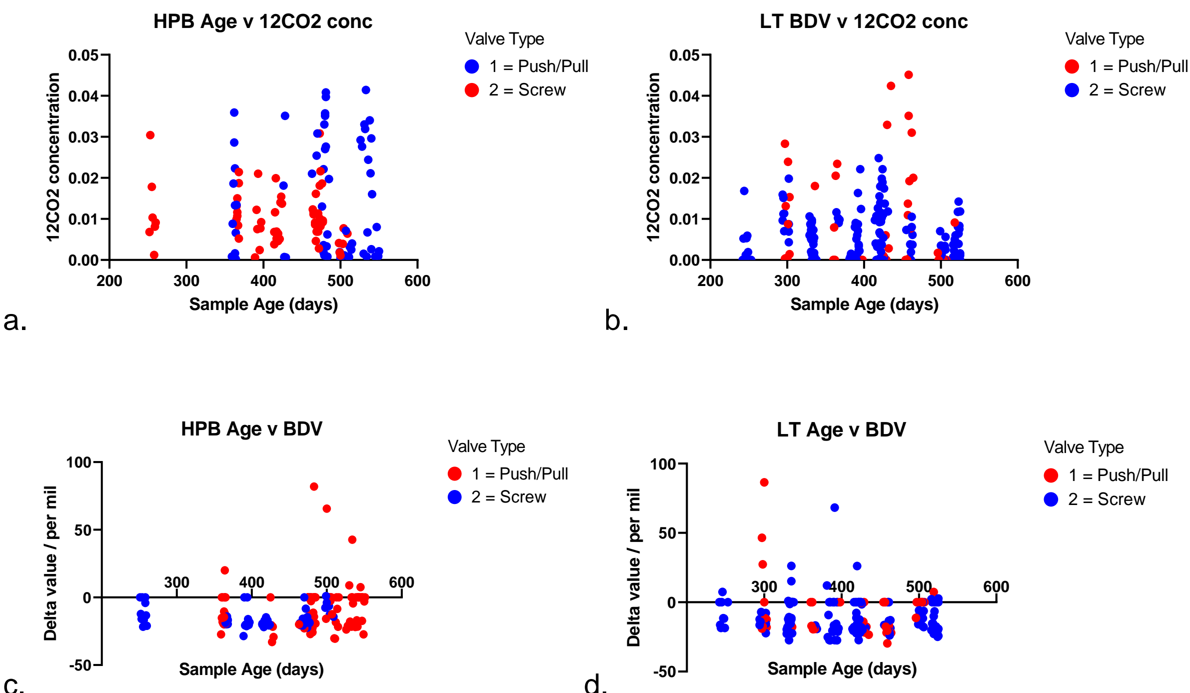


Figure 5-8 Graph to demonstrate the relationship between age of sample (days) and $^{12}CO_2$ concentration of sample (fraction) and BDV (permil)

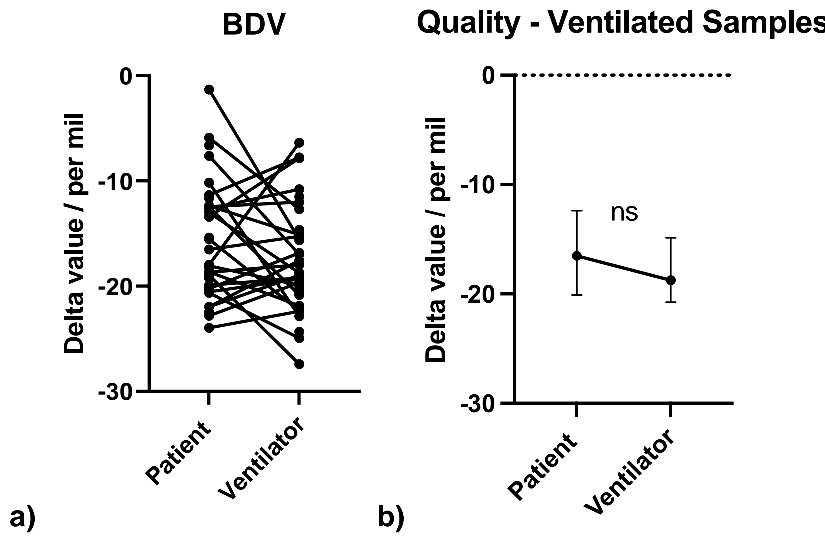
in a) HPB group and b) LT group in different bag types, and excluding values BDV < -8 permil in c) HPB group and d) LT group. The colours indicate the type of valve closing the bag (red, push/pull valve; blue, screw valve) The colours are used to identify trends in BDV and $^{12}CO_2$ concentration, to indicate whether one valve type was more likely to leak sample than the other

.

Ventilated samples in Liver Transplantation

Nonparametric t test Wilcoxon matched pairs signed rank test analysed the difference in $^{12}CO_2$ concentration between paired samples taken from the patient end and ventilator end with no significant difference, p=0.42, and the difference in BDV between paired samples taken from the patient end and ventilator end with no significant difference, p=0.29 as shown in

Figure 5-9. This sample included LT participants only, as no HPB patients were ventilated postoperatively.



.

Figure 5-9 Difference between breath samples taken at the patient end and ventilator end of the ventilator circuit

for a) individual BDV values, b) BDV median and IQR, c) individual $^{12}CO_2$ values, d) $^{12}CO_2$ median and IQR.

.

Mann-Witney U Test between infected and non-infected samples following HPB surgery

MWU test on imputed absolute values for BDV between HPB non-infected and infected groups was significantly different only on POD3, with infected patients' BDV values +3.90 compared to non-infected (p=0.03). The same values were examined, using the change from preoperative baseline, change from POD1 (as baseline), and day-to-day change, but there was no significant difference between groups. MWU test on imputed absolute values for BDV between HPB non-infected and infected groups was significantly different only on POD3, with infected patients' BDV values +3.90 compared to non-infected (p=0.03). MWU for WCC showed, no significant difference, for CRP was significantly higher on POD6 in the infected group (+89.0, p-0.01), and for SOFA score was reached significance on POD6 in the infected (+1, p=0.03).

Analysis of variance between infected and non-infected samples following HPB surgery

Analysis of variance (ANOVA) between HPB infected and non-infected groups showed no significant difference when analysed as imputed absolute BDV values, as a change from preoperative of POD1 baseline, or as a day-to-day change

Univariate Logistic Regression for infection following HPB surgery

Univariate logistic regression for infection was not significant on any postoperative day.

.

Table 5-3 Mann-Witney U test for day-to-day difference in BDV between infected and non-infected HPB groups

	BDV							
Day	Absolute values		∆ from baseline		∆ from POD1		∆ day to day	
	Difference	p	Difference	p	Difference	p	Difference	p
Baseline	-1.24	0.72	0	>0.99	+2.64	0.29	0	>0.99
POD1	+1.44	0.19	+2.64	0.29	0	>0.99	-2.64	0.29
POD2	+4.63	0.10	+5.99	0.28	+0.69	0.93	+0.69	0.94
POD3	**+3.90**	**0.03**	+5.71	0.33	+1.94	0.33	-3.65	0.65
POD4	+1.02	0.72	-1.40	>0.99	+4.18	0.28	+1.23	0.12
POD5	-1.96	0.57	-1.16	0.72	+1.65	0.33	+0.26	0.63
POD6	+0.66	0.80	-0.12	0.96	-0.35	0.72	-0.52	0.31
POD7	-0.43	0.56	-0.32	0.80	-2.82	0.16	+1.37	0.63
POD8	-0.51	0.51	+0.58	0.80	+2.34	0.23	+0.89	0.42
POD9	-1.60	0.44	+1.17	0.80	+2.62	0.12	+1.44	0.19

.

.

Table 5-4 Mann-Witney U test for day-to-day difference in BDV, WCC, CRP and SOFA between infected and non-infected HPB groups

Day	BDV		WCC		CRP		SOFA	
	Difference	p		p		p		p
Baseline	-1.24	0.72	-0.01	>0.99	0	0.39	0	0.93
POD1	+1.44	0.19	-1.72	0.27	-27.0	0.67	+1	0.94
POD2	+4.63	0.10	+2.53	0.93	-15.6	>0.99	+1	0.80
POD3	**+3.90**	**0.03**	+1.70	0.31	-28.0	0.55	0	0.61
POD4	+1.02	0.72	+5.15	0.10	+105.1	0.30	+2	0.13
POD5	-1.96	0.57	+0.27	0.45	+52.6	0.14	0	0.07
POD6	+0.66	0.80	+0.68	0.27	**+89.0**	**0.01**	0	0.89
POD7	-0.43	0.56	+0.05	0.87	+14.5	0.10	**+1**	**0.03**
POD8	-0.51	0.51	+0.83	0.80	+52.0	0.42	**0**	**0.01**
POD9	-1.60	0.44	-0.23	0.55	+28.15	0.91	0	>0.99

.

HPB ANOVA Absolute Values - imputed

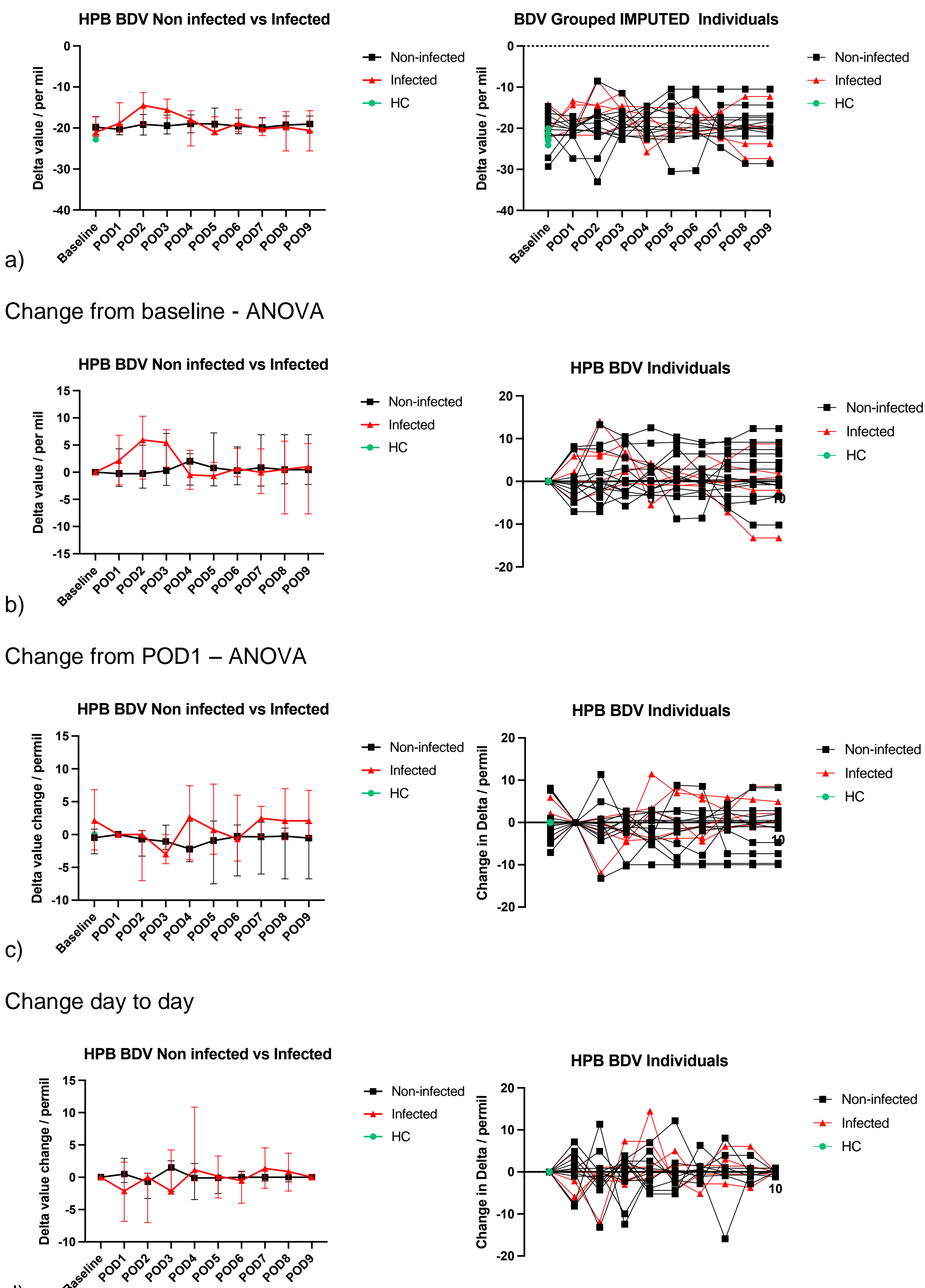


Figure 5-10 ANOVA for BDV in infected and non-infected HPB participant

a) absolute values, b) change from baseline, c) change from POD1, d) change day-to-day

.

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where $p<0.05$, and ** where $p<0.01$. All other points are non-significant.

.

Table 5-5 Univariate logistic regression of BDV for infection following HPB surgery

	OR	95% CI	p=	AUC	95% CI	p=
Baseline	0.99	0.88, 1.04	0.77	0.55	0.18, 0.90	0.79
POD1	1.07	0.99, 1.34	0.31	0.78	0.50, 1.0	0.12
POD2	0.99	0.90, 1.0	0.77	0.65	0.34, 0.95	0.40
POD3	1.08	0.94, 1.29	0.31	0.83	0.59, 1.0	0.04
POD4	0.93	0.63, 1.33	0.68	0.55	0.18, 0.92	0.77
POD5	1.06	0.93, 1.27	0.38	0.60	0.21, 0.99	0.57
POD6	1.18	0.95, 1.69	0.23	0.66	0.32, 1.0	0.37
POD7	0.93	0.61, 1.07	0.53	0.52	0.16, 0.88	0.91
POD8	0.93	0.70, 1.11	0.49	0.62	0.18, 1.0	0.57
POD9	0.74	0.10, 1.23	0.57	0.63	0.16, 1.0	0.64

.

Clinical Results – Liver Transplantation

Mann-Witney U test on imputed absolute values for BDV between LT non-infected and infected groups was not significantly different. The same values were examined, using the change from preoperative baseline, change from POD1 (as baseline), and day-to-day change, but there was no significant difference between groups. MWU test on imputed absolute values for BDV between LT non-infected and infected groups was not significantly different. MWU for WCC was significantly higher (+11.48, p=0.05) in participants who developed infection. CRP and SOFA score showed no significant difference

Analysis of variance between infected and non-infected samples following Liver Transplantation

Analysis of variance (ANOVA) between LT infected and non-infected groups showed no significant difference when analysed as imputed absolute BDV values, as a change from preoperative of POD1 baseline, or as a day-to-day change.

Univariate Logistic Regression for infection following Liver Transplantation

Univariate logistic regression for infection was not significant on any postoperative day.

Diagnostic Accuracy of BDV for infection following Liver Transplantation

BDV did not reach significance on any day.

.

Table 5-6 Mann-Witney U test for day-to-day difference in BDV between infected and non-infected LT groups

Day	Absolute values		Change from baseline		Change from POD1		Change day to day	
	Difference	p	Difference	p	Difference	p	Difference	p
Baseline	+2.14	0.48	0	>0.99	+0.48	0.89	0	>0.99
POD1	+0.01	0.89	-0.48	0.89	0	>0.99	+0.48	0.89
POD2	+1.76	0.89	+0.02	0.89	-2.52	0.50	+2.52	0.50
POD3	+2.53	0.50	+0.83	0.69	+1.68	0.69	+1.30	0.38
POD4	+2.86	0.56	+1.20	>0.99	+0.64	0.75	+1.58	0.46
POD5	-0.57	0.96	+0.41	0.56	-2.65	0.69	-0.14	0.50
POD6	+1.27	0.82	+2.19	0.75	-1.50	0.89	-1.09	0.13
POD7	+0.57	0.69	+1.27	0.96	-0.02	0.82	+0.15	0.23
POD8	+0.43	0.89	-0.69	0.89	-0.04	>0.99	+0.64	0.32
POD9	-0.12	>0.99	+2.88	0.62	+0.54	0.96	0	0.54

.

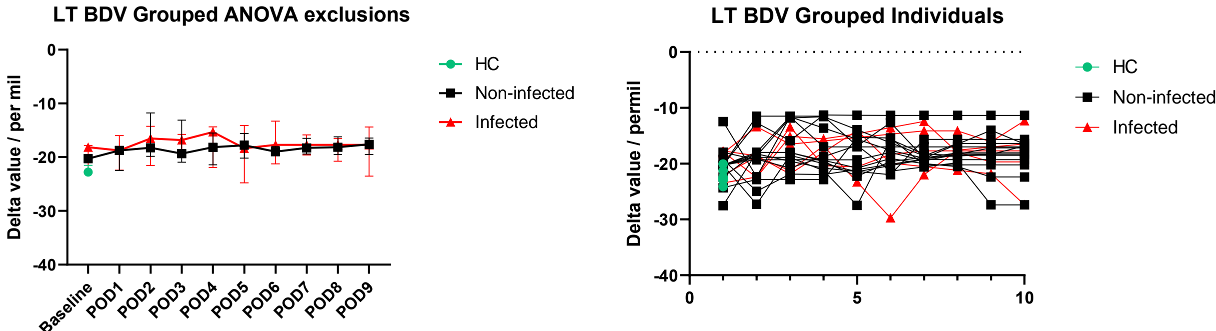
Table 5-7 Mann-Witney U test for day-to-day difference in BDV, WCC, CRP and SOFA between infected and non-infected LT groups

Day	BDV		WCC		CRP		SOFA	
	Difference	p	Difference	p	Difference	p	Difference	p
Baseline	+2.14	0.48	+2.79	0.20	+18.5	0.42	-1	0.14
POD1	+0.01	0.89	+5.2	0.45			+2	0.40
POD2	+1.76	0.89	**+11.48**	**0.05**	+21.5	0.07	+2	0.79
POD3	+2.53	0.50	+11.53	0.10			0	0.71
POD4	+2.86	0.56	+4.26	0.17			+5	0.45
POD5	-0.57	0.96	+2.32	0.31	+10.5	0.57	0	>0.99
POD6	+1.27	0.82	+4.55	0.23			-1	0.94
POD7	+0.57	0.69	+7.56	0.08			-2	0.71
POD8	+0.43	0.89	+4.76	0.17	+51.0	0.81	-2	0.90
POD9	-0.12	>0.99	+6.85	0.05			-6	0.82

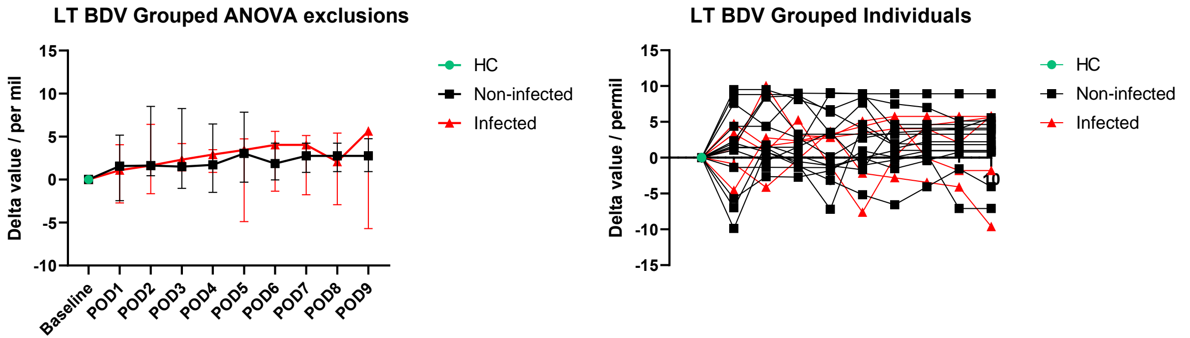
.

.

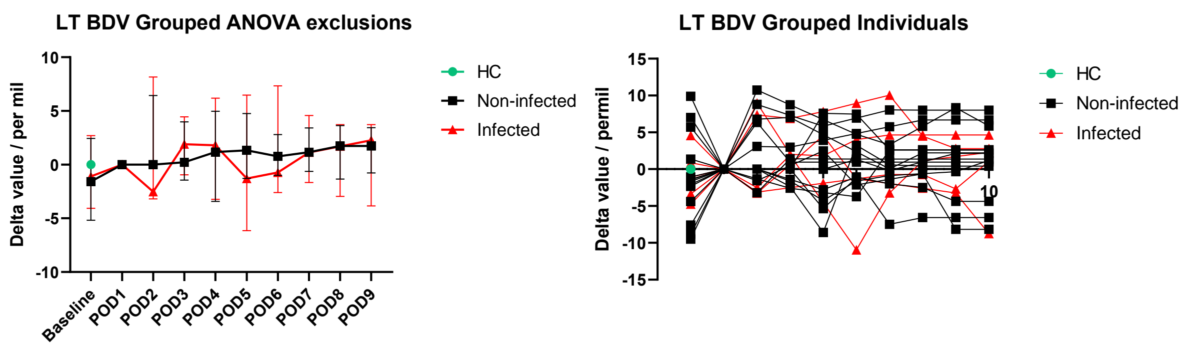
Liver Transplantation Imputed Absolute Values – ANOVA



LT Change from baseline - ANOVA



Change from POD1 baseline – ANOVA



Change day to day

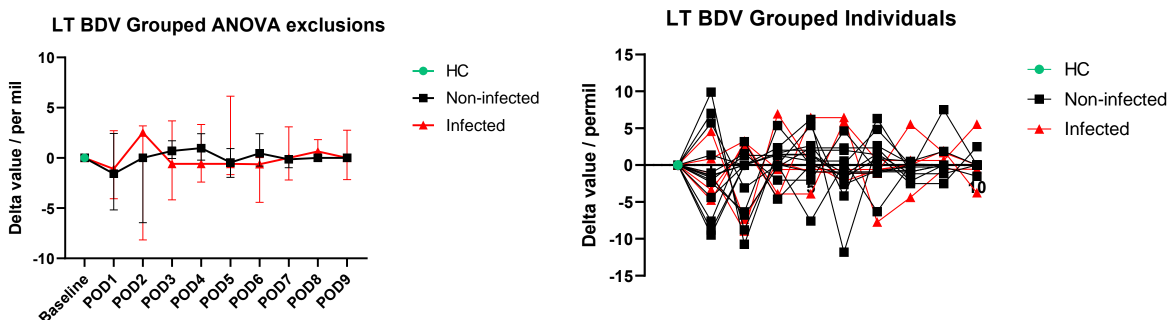


Figure 5-11 ANOVA for BDV in infected and non-infected LT participants

a) absolute values, b) change from baseline, c) change from POD1, d) change day-to-day. Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where $p<0.05$, and ** where $p<0.01$. All other points are non-significant

.

Table 5-8 Univariate logistic regression of BDV for infection following LT

	OR	95% CI	p=	AUC	95% CI	p=
Baseline	0.98	0.67, 1.44	0.93	0.50	0.14, 0.86	>0/99
POD1	1.0	0.74, 1.34	0.99	0.57	0.25, 0.89	0.69
POD2	1.07	0.79, 1.45	0.67	0.58	0.29, 0.88	0.62
POD3	1.05	0.75, 1.44	0.75	0.62	0.33, 0.90	0.48
POD4	0.96	0.69, 1.34	0.80	0.52	0.19, 0.85	0.91
POD5	1.07	0.88, 1.31	0.49	0.58	0.19, 0.98	0.62
POD6	1.26	0.85, 1.91	0.24	0.67	0.27, 1.0	0.32
POD7	1.14	0.64, 1.94	0.61	0.71	0.47, 0.95	0.26
POD8	1.08	0.77, 1.63	0.68	0.58	0.31, 0.86	0.62
POD9	1.20	0.88, 1.88	0.32	0.63	0.33, 0.94	0.42

.

Table 5-9 Diagnostic accuracy of BDV for infection at baseline and POD1-9

Sensitivity, specificity and AUC are given with 95% CI

	Cutoff (per mil)	Sensitivity	Specificity	AUC	p
Baseline	>-19	0.6 (0.23, 0.93)	0.86 (0.60, 0.97)	0.61 (0.27, 0.95)	0.46
POD1	<-18	0.80 (0.38, 0.99)	0.43 (0.21, 0.67)	0.53 (0.24, 0.81)	0.85
POD2	<-21	0.40 (0.07, 0.77)	0.86 (0.60, 0.97)	0.53 (0.23, 0.83)	0.85
POD3	>-19	0.60 (0.23, 0.93)	0.86 (0.60, 0.97)	0.61 (0.27, 0.95)	0.46
POD4	>-21	0.80 (0.38, 0.99)	0.43 (0.21, 0.67)	0.60 (0.29. 0.91)	0.52
POD5	>-20	0.80 (0.38, 0.99)	0.29 (0.12, 0.55)	0.51 (0.17, 0.86)	0.93
POD6	>-18	0.60 (0.23, 0.93)	0.79 (0.52, 0.92)	0.54 (0.16, 0.92)	0.78
POD7	>-18	0.80 (0.38, 0.99)	0.64 (0.39, 0.84)	0.57 (0.25, 0.89)	0.64
POD8	<-17	0.80 (0.38, 0.99)	0.36 (0.16, 0.61)	0.53 (0.24, 0.82)	0.85
POD9	>-18	0.60 (0.23, 0.93)	0.50 (0.27, 0.73)	0.50 (0.18, 0.82)	>0.99

.

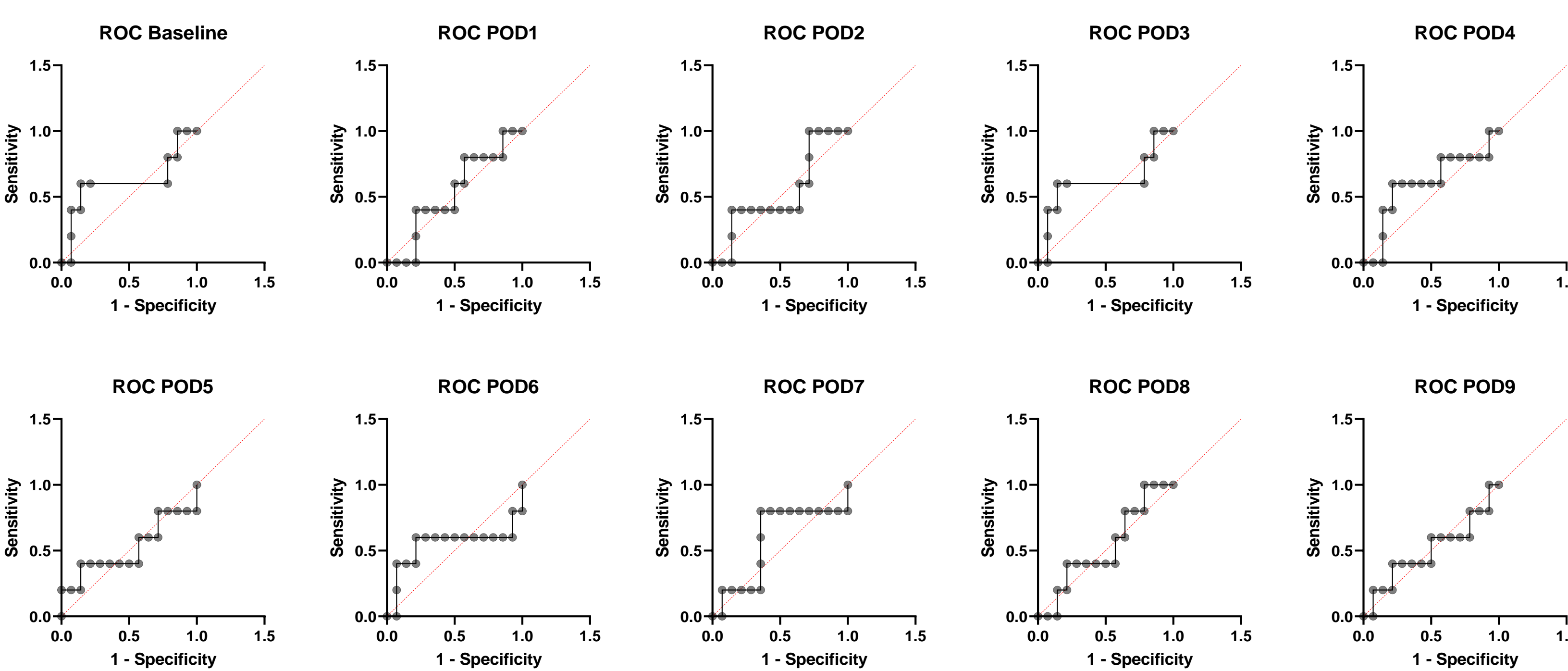


Figure 5-12 ROC curves for BDV for infection at baseline and POD1-9

.

5.3 Summary and Conclusions

Sample Quality

Quality analysis indicates that prolonged storage of breath samples effects results. $^{12}CO_2$ concentration was significantly lower in-patient samples stored for over 200 days compared to HC samples stored for 2-30 days before analysis, although duration of storage for all clinical samples was not correlated with $^{12}CO_2$ concentration. BDV had a weak negative correlation with duration of storage, Spearman r -0.013 p=0.03, suggesting that samples stored for longer had a more negative BDV. $^{12}CO_2$ concentration had a negative correlation with BDV, Spearman r -0.36 (95% CI -0.46, -0.25) p<0.0001, showing that samples with higher concentrations of CO_2 had a more negative BDV. This corresponds with what is known, that the average $^{12}CO_2$ in breath is higher than the atmospheric concentration, and that BDV is more negative in human breath (-21 to -23 per mil) than in the atmosphere (-8 per mil). The low $^{12}CO_2$ concentration among all samples (median = 0.0077, IQR = 0.0029 to 0.045) and high BDV (median =-19 per mil, IQR = -21 to -15) compared to HC $^{12}CO_2$ concentration (median = 0.035, IQR = 0.032 to 0.037) and BDV (median =-23 per mil, IQR = -23 to -22) suggests that the sample bags were not secure over longer duration of storage and leak and diffusion with room air had occurred, lowering the CO_2 concentration and raising BDV closer to atmospheric levels.

Only 27.9% of samples had $^{12}CO_2$ concentration of > 0.01 and 11.2% with $^{12}CO_2$ concentration >0.02, compared to 0% of HC samples with median $^{12}CO_2$ concentration 0.035 (IQR 0.032-0.037). This significant difference suggests that the concentration of CO_2 in the sample did not represent that of human breath, and the majority of samples when analysed did not reliably represent the BDV at the time of sampling.

.

Examining the difference between paired samples taken at the patient end and ventilator end in patients who remained ventilated postoperatively did not demonstrate a statistical difference between samples. Samples taken at the ventilator end which had a higher $^{12}CO_2$ concentration and more negative BDV, though not statistically significant, were included in the clinical analysis. There was no statistical difference in BDV or $^{12}CO_2$ concentration in bags with different valve types (screw valve/push valve).

Hepatopancreatobiliary Surgery Clinical Analysis

The difference in BDV between infected and non-infected HPB groups was significant only on POD3 using MWU, however the results was a greater/more positive BDV (+3.90, p=0.03) contradicting existing literature that BDV becomes more negative during sepsis. Common biomarkers including WCC, CRP and SOFA score did not show higher performance, with CRP significantly higher on POD6, later than the median day of diagnosis of infective complication, limiting its preclinical/diagnostic utility. ANOVA showed no significant difference between groups.

No significant association was found between infection or sepsis outcomes alone and BDV for the HPB group. BDV on POD3 demonstrated a high AUC for patients who developed inflammation (infection, sepsis, or pancreatobiliary leak) AUC 0.83 (p=0.039), and POD1 AUC 0.9 (p=0.0425) for pancreatobiliary leak.

Liver Transplantation Clinical Analysis

The difference in BDV between infected and non-infected LT groups was not significant between groups using MWU. WCC was significantly higher on POD2 (+11.48, p=0.05) in patients who developed infective complications. SOFA score did not show a significant difference on any day. or higher diagnostic performance. CRP was not measured on every

.

sampling day but at baseline, POD2, POD5 and POD8, CRP did not show a significant difference or higher diagnostic performance. ANOVA showed no significant difference between groups.

No significant association was found between infective outcomes in the LT group. BDV demonstrated a high AUC on POD5 for bleeding in LT patients (AUC 0.9, p=0.0425), however both of the two patients who experienced bleeding presented at POD1 with return to theatre on POD1. Both patients remained ventilated throughout the sampling period, with one urgently listed for liver transplantation in critical care with a prolonged period of multi organ failure, and the other patient had right lobe ischaemia, right leg ischaemia and above knee amputation and died after the sampling period, which have introduced confounding bias.

Limitations

There were limitations of the experiment. Missing values due to missing samples or failed IR spectroscopy (e.g. outlying values or empty samples unable to generate BDV), and participants excluded with fewer than 50% of BDV samples, may have missed important changes in BDV. Imputing of data to compensate for missing values using the median between and last values will have diminished the accuracy of results, and may have ‘smoothed out’ trends, and potentially minimising the difference between groups.

There was significant heterogeneity between participants. While age, sex, comorbidity and operative time were not significantly different between groups, there was variation in operative type and post operative management. In the HPB group, patients undergoing GI anastomosis e.g., in pancreaticoduodenectomy, required five postoperative days of fasting. There was variation in operative time and length of stay, with some participants undergoing a relatively small liver resection with minimally invasive approach and discharge after 3-4

.

days, while others underwent more major surgery e.g. hemi-hepatectomy with minimally invasive or open approach and length of stay beyond the sampling period. The indication for some participants was for cancer and others benign with some cancer patients having neoadjuvant treatment that may have contributed to preoperative frailty. In the LT group, five participants were listed for transplantation for acute causes (one seronegative ALF, one paracetamol overdose ALF, two ACLF due to Wilson's disease and one ACLF due to ArLD), and the remaining 15 listed with stable chronic liver disease.

Conclusions

These data suggest that breath samples leaked during prolonged storage, with potential diffusion of room air into the sample, giving a $^{12}CO_2$ concentration lower that that expected of expired human breath and compared to healthy controls (which did not undergo prolonged storage). There was a negative correlation between $^{12}CO_2$ and BDV, Spearman r = -0.36 (95% CI -0.46, -0.25) $p<0.0001$, and a less negative BDV often closer to atmospheric ^{13}C delta value of -8 per mil than. There was no correlation between $^{12}CO_2$ and bag type, duration of storage or patient suggesting the effect was sporadic. All bags were at least moderately well filled by patient breath at the time of sampling, but many were empty/poorly filled at the time of spectroscopy and some bags which looks well filled at the time of spectroscopy had relatively low $^{12}CO_2$ concentration. While many bags showed good integrity of seal and had high $^{12}CO_2$ levels and a reliable BDV, the inconsistency and missing values between samples means that examining trends and differences between groups is unreliable. The manufacturers do not recommend a limit for storage of stable gas samples and were contacted for a recommended time limit which they were unable to give. This should be investigated before further research is conducted.

.

6 Serum Biomarkers of Infection and Inflammation

6.1 Introduction

Results of the V-PLEX Proinflammatory MSD panel are presented alongside cytokines, other biomarkers including CRP, SOFA score, WCC and differential and percentage of WCC, and neutrophil lymphocyte ratio, lymphocyte monocyte ratio, neutrophil lymphocyte ratio, platelets lymphocyte ratio were analysed from clinical data. Where clinical CRP values were missing sampled were analysed from frozen serum using in the same hospital Viapath lab using the same methods. PCT results from EDTA plasma PCT ELISA was analysed alongside other markers.

6.2 Results

Data Quality

A significant proportion of samples were out of range (below the fitting curve or below detection range for IL-12p70 (49.3%), IL-13 (86.3%), IL-1b (91.9%), IL-2 (67.5%), IL-4 (99.4%). This should be considered when interpreting results.

Coefficient of Variance

Plate to plate variation marked by CoV was fairly high (>10%) across most plate. IL-6 and IL-8 had the least plate to plate variation. Low variation was seen in some samples which fell below the fitting curve.

.

Table 6-1 Number and percentage of samples in range for MSD V-PLEX Proinflammatory panel

	In range	Below Fit	Below Detection	Out of range	% in range	% out of range
IFNγ	138	20	6	26	86.25	16.25
IL-10	149	2	9	11	93.125	6.875
IL-12p70	81	62	17	79	50.625	49.375
IL-13	19	85	53	138	11.875	86.25
IL-1b	13	137	10	147	8.125	91.875
IL-2	52	98	10	108	32.5	67.5
IL-4	1	152	7	159	0.625	99.375
IL-6	152	1	7	8	95	5
IL-8	160	0	0	0	100	0
TNFα	102	5	53	58	63.75	36.25

Table 6-2 Coefficient of Variation of repeated samples on MSD plate 1 and 2

IFNγ	Plate 1	Plate 2	CoV
H27	3.82	2.19	27.12
S64 d3	1.84	0.68	**45.79**
T165 d7	5.35	3.48	21.22

IL-2	Plate 1	Plate 2	CoV
H27	0.90	0.65	16.04
S64 d3	0.10	0.11	4.68
T165 d7	6.55	4.30	20.73

IL-10	Plate 1	Plate 2	CoV
H27	1.44	1.16	10.52
S64 d3	0.44	0.33	14.47
T165 d7	26.87	24.76	4.08

IL-4	Plate 1	Plate 2	CoV
H27	0.39	0.64	24.20
S64 d3	0.15	0.09	25.85
T165 d7	0.15	0.01	84.05

IL-12p70	Plate 1	Plate 2	CoV
H27	0.35	0.07	67.74
S64 d3	0.07	0.07	0.31
T165 d7	0.07	0.07	0.00

IL-6	Plate 1	Plate 2	CoV
H27	0.67	0.61	4.70
S64 d3	32.55	31.87	1.06
T165 d7	12.32	11.52	3.36

IL-13	Plate 1	Plate 2	CoV
H27	0.15	0.15	0.00
S64 d3	1.32	0.15	**80.18**
T165 d7	0.15	0.15	0.00

IL-8	Plate 1	Plate 2	CoV
H27	3.07	3.05	0.35
S64 d3	3.05	2.65	7.03
T165 d7	15.84	12.90	10.21

IL-1b	Plate 1	Plate 2	CoV
H27	0.15	0.04	60.95
S64 d3	0.10	0.23	38.75
T165 d7	0.26	0.27	2.96

TNFα	Plate 1	Plate 2	CoV
H27	0.91	0.66	15.39
S64 d3	0.32	0.11	**47.22**
T165 d7	1.89	1.06	28.00

.

Mann-Witney U Test for infection following HPB Surgery

No difference in levels of cytokines was observed using MWU for participants with and without infection. A baseline difference was seen only in IFNγ-5.2 (p=0.04). PCT on POD4 was increased in non-infected participants, +68.8 (p=0.04).

Analysis of Variance for infection following HPB Surgery

There was no statistical difference between infected and non-infected groups for any cytokine at any time point. There was no statistical difference in CRP, WCC, neutrophil count, lymphocyte count, however monocyte level was higher at baseline, on POD4 and POD8 in the infected group, reaching statistical significance. There was no difference in the neutrophil, lymphocyte or monocyte percentage of WCC, and NLR, LMR, NMR and PLR were not significantly different. PCT was significantly higher on POD1 for participants who developed infection.

Univariate Logistic Regression for infection following HPB Surgery

Logistic regression of cytokines for infection significance only for IFNγ at baseline with OR 1.55 (95% CI 1.097, 2.71) p=0.05, however confidence intervals approached 1. SOFA score was not associated with infection.

Diagnostic Accuracy of serum and full blood count markers of infection following HPB Surgery

The diagnostic accuracy for all cytokines was poor, with no AUC achieving statistical significance for cytokines in the MSD V-PLEX panel. PCT on POD4 achieved sensitivity of

.

80% (65% CI 38-99%), specificity of 83% (95% CI 55-95%), and AUC 0.83 (95% CI 0.62-1.0) p=0.04, however this was for values below a cutoff of 126pg/mL. There was no significant difference for CRP, WCC and SOFA scores.

.

.

Table 6-3 Mann-Witney U test for differences in biomarkers of infection following HPB surgery in infected and non-infected groups at baseline, on POD1, POD4 and POD8.

	Baseline	POD1	POD4	POD8
IFNγ	-5.2 (p=0.04)	ns	ns	ns
IL-10	ns	ns	ns	ns
IL-12p70	ns	ns	ns	ns
IL-13	ns	ns	ns	ns
IL-1b	ns	ns	ns	ns
IL-2	ns	ns	ns	ns
IL-4	ns	ns	ns	ns
IL-6	ns	ns	ns	ns
IL-8	ns	ns	ns	ns
TNFα	ns	ns	ns	ns
PCT	ns	ns	+68.8 (p=0.04)	ns
CRP	ns	ns	ns	ns
SOFA	ns	ns	ns	ns
WCC	ns	ns	ns	ns
Neutrophil count	ns	ns	ns	ns
Lymphocyte count	ns	ns	ns	ns
Monocyte count	ns	ns	ns	ns
Neutrophil %	ns	ns	ns	ns
Lymphocyte %	ns	ns	ns	ns
Monocyte %	ns	ns	ns	ns
NLR	ns	ns	ns	ns
LMR	ns	ns	ns	ns
NMR	ns	ns	ns	ns
PLR	ns	ns	ns	ns

Differences in median values between infected and non-infected groups and their p values are presented. Non-significant values are not presented (ns).

.

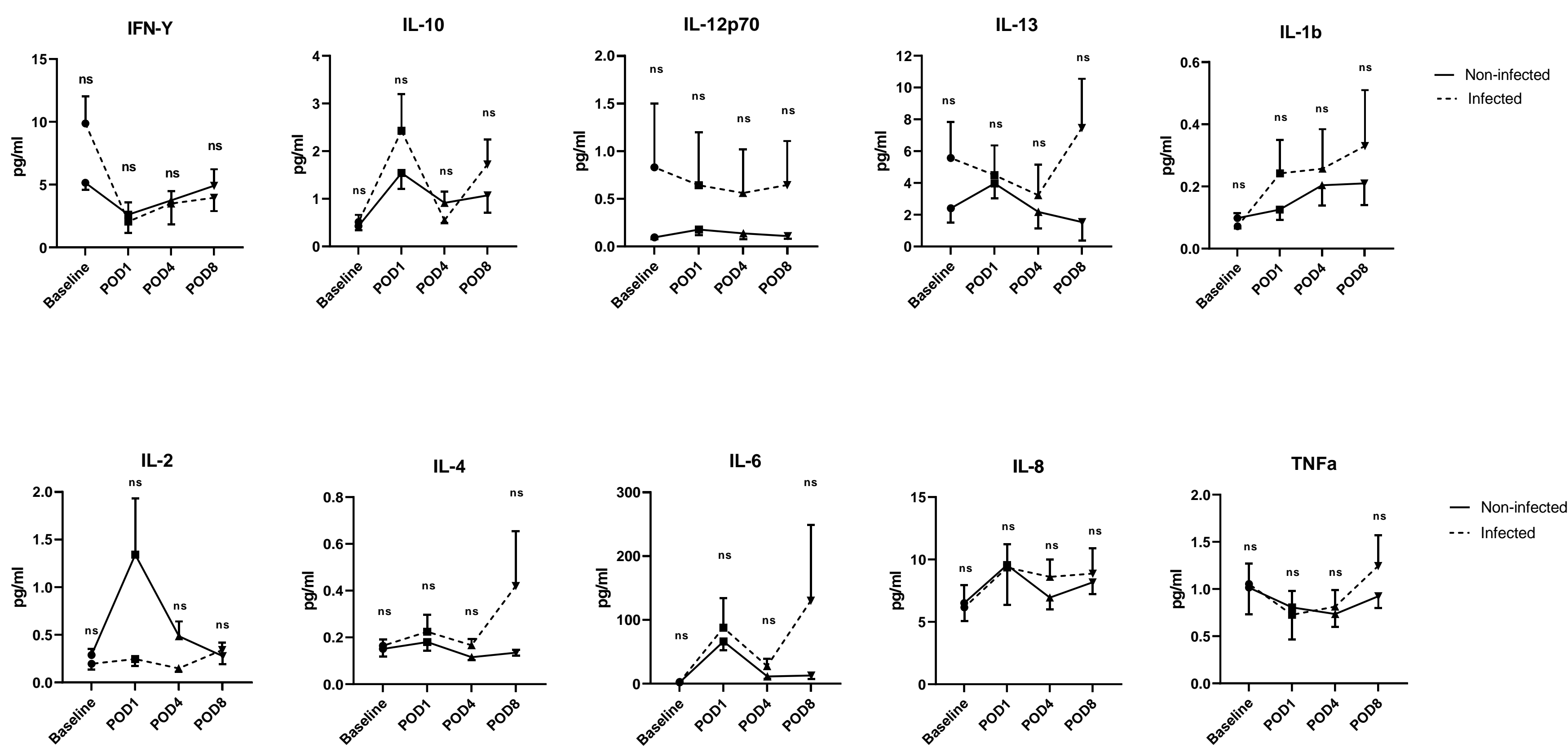


Figure 6-1 Grouped 2-way ANOVA for HPB showing difference between groups at each POD for MSD V PLEX Proinflammatory Cytokine panel.

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where p<0.05, and ** where p<0.01. All other points are non-significant.

.

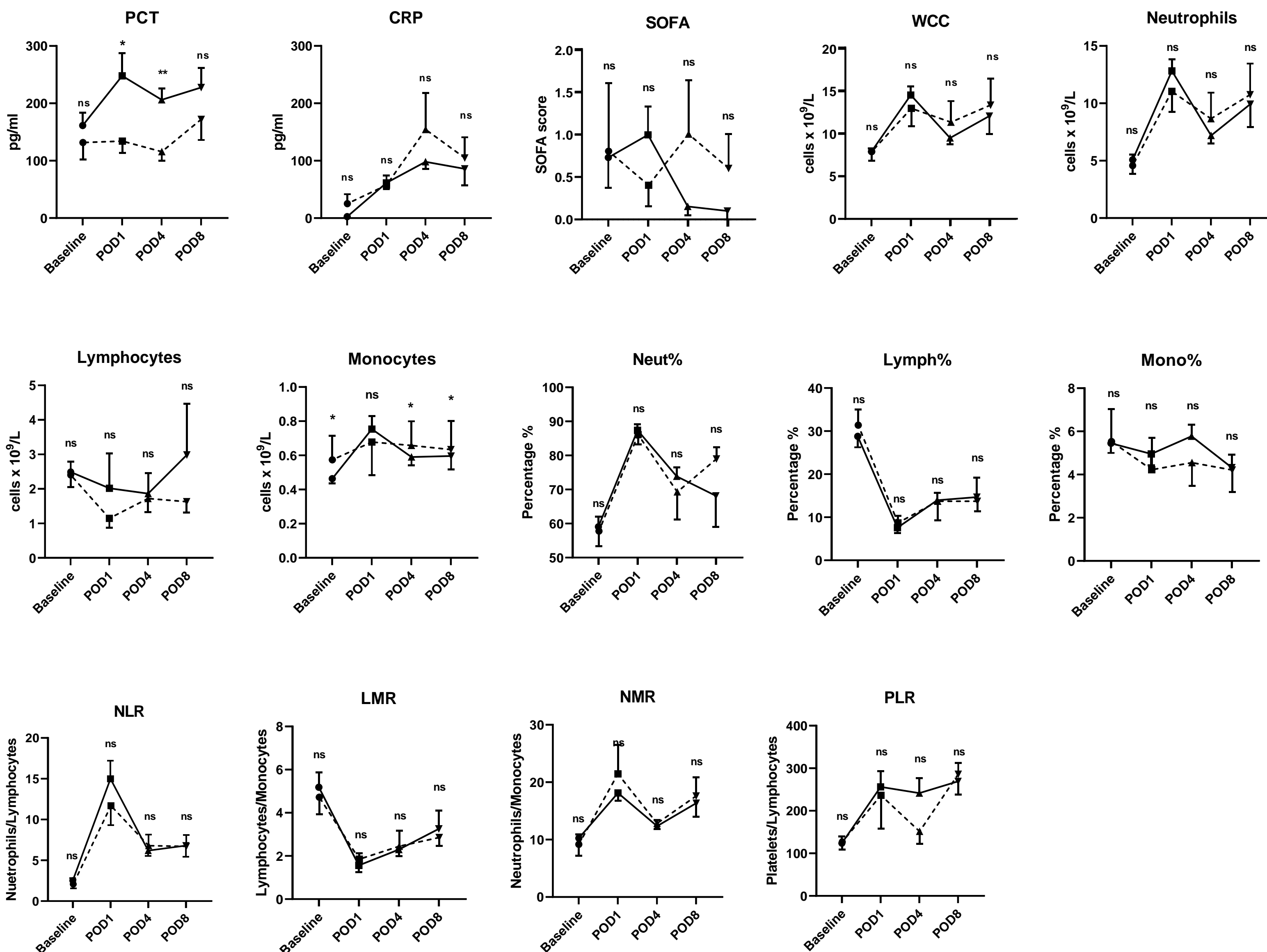


Figure 6-2 Grouped 2-way ANOVA for HPB infected and non-infected participants for PCT, CRP, Full Blood Count, NLR, LMR, NMR and PLR.

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where p<0.05, and ** where p<0.01. All other points are non-significant

.

Table 6-4 Univariate Logistic Regression for infection at baseline sample in HPB group

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	1.55	1.097, 2.71	0.05	0.81	0.58, 1.0	0.04
IL-10	2.10	0.07, 48.5	0.62	0.63	0.36, 0.90	0.41
IL-12p70	7519	1.22,.1372125 6011	0.18	0.76	0.44, 1.0	0.09
IL-13	1.20	0.94, 1.59	0.15	0.70	0.43, 0.97	0.19
IL-1b	2.819 e-006	5.037e-026 to 6951	0.39	0.62	0.35, 0.89	0.43
IL-2	0.06	1.440e-006 to 11.56	0.46	0.68	0.39, 0.97	0.24
IL-4	2.94	2.789e-005 to 34037	0.81	0.61	0.32, 0.89	0.49
IL-6	1.15	0.65,1.93	0.61	0.56	0.26, 0.86	0.69
IL-8	0.98	0.74,1.20	0.89	0.64	0.38, 0.90	0.36
TNFα	1.04	0.24, 2.94	0.94	0.59	0.28, 0.89	0.57
PCT	1.0	0.98, 1.01	0.75	0.57	0.28, 0.87	0.63
CRP	1.18	1.01, 1.77	0.23	0.75	0.46, 1.0	0.11
SOFA	1.03	0.43, 2,02	0.93	0.55	0.24, 0.85	0.76
WCC	0.96	0.50, 1.381	0.91	0.51	0.19, 0.83	0.97
Neutrophil count	0.82	0.38, 1.52	0.56	0.53	0.23, 0.84	0.83
Lymphocyte count	0.92	0.28, 2.37	0.88	0.52	0.21, 0.83	0.90
Monocyte count	28.1	0.10, 17442	0.25	0.5	0.12, 0.88	>0.99
Neutrophil %	0.99	0.89, 1.10	0.81	0.64	0.33, 0.95	0.36
Lymphocyte %	1.03	0.92, 1.16	0.58	0.67	0.38, 0.96	0.28
Monocyte %	1.01	0.62, 1.75	0.94	0.55	0.20, 0.90	0.76
NLR	0.70	0.21, 1.69	0.49	0.65	0.35, 0.96	0.32
LMR	0.91	0.49, 1.40	0.71	0.55	0.25, 0.84	0.76
NMR	0.87	0.56, 1.25	0.49	0.64	0.28, 1.0	0.36
PLR	1.00	0.98, 1.02	0.91	0.61	0.35, 0.88	0.46

.

Table 6-5 Univariate Logistic Regression for infection on POD1 sample in HPB group

OR and AUC are given with 95% CI and p values on POD1

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.94	0.59, 1.28	0.73	0.55	0.21, 0.88	0.76
IL-10	1.56	0.76, 3.35	0.23	0.67	0.38, 0.96	0.28
IL-12p70	2.93	0.61, 64.66	0.26	0.51	0.19, 0.82	0.97
IL-13	1.04	0.77, 1.39	0.77	0.52	0.18, 0.86	0.90
IL-1b	59.79	0.14, 43541	0.18	0.64	0.34, 0.94	0.36
IL-2	0.08	4.620e-005 to 1.059	0.29	0.71	0.43, 0.98	0.18
IL-4	7.18	0.00, 9447	0.56	0.64	0.31, 0.97	0.36
IL-6	1.01	0.99,1.02	0.53	0.53	0.14, 0.93	0.83
IL-8	0.99	0.83,1.17	0.95	0.52	0.22, 0.82	0.90
TNFα	0.80	0.08, 3.95	0.80	0.53	0.17, 0.89	0.83
PCT	0.99	0.97, 1.00	0.22	0.77	0.53, 1.0	0.11
CRP	1.00	0.97, 1.02	0.85	0.52	0.22, 0.82	0.90
SOFA	0.65	0.12, 1.73	0.51	0.56	0.29, 0.83	0.69
WCC	0.89	0.64, 1.16	0.41	0.65	0.34, 0.97	0.32
Neutrophil count	0.96	0.64, 1.34	0.81	0.63	0.16, 1.0	0.49
Lymphocyte count	0.87	0.18, 1.23	0.66	0.51	0.18, 0.84	0.96
Monocyte count	0.46	0.014, 11.97	0.64	0.56	0.19, 0.93	0.69
Neutrophil %	1.0	0.83, 1.24	0.99	0.51	0.17, 0.84	0.97
Lymphocyte %	1.05	0.83, 1.32	0.63	0.65	0.41, 0.90	0.32
Monocyte %	0.74	0.37, 1.25	0.32	0.68	0.34, 1.0	0.24
NLR	0.92	0.77, 1.06	0.31	0.61	0.35, 0.88	0.46
LMR	1.24	0.46, 3.07	0.62	0.76	0.55, 0.97	0.09
NMR	1.08	0.87, 1.29	0.33	0.57	0.24, 0.90	0.63
PLR	1.0	0.99, 1.01	0.99	0.51	0.08, 0.94	0.96

.

Table 6-6 Univariate Logistic Regression for infection on POD4 sample in HPB group

OR and AUC are given with 95% CI and p values on POD4

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.96	0.61, 1.41	0.85	0.58	0.26, 0.91	0.60
IL-10	0.21	0.00, 1.93	0.35	0.57	0.30, 0.83	0.67
IL-12p70	3.95	0.57, 269.80	0.28	0.63	0.28, 0.97	0.43
IL-13	1.08	0.80, 1.44	0.58	0.54	0.23, 0.86	0.79
IL-1b	2.59	0.02, 239.2	0.67	0.50	0.16, 0.84	>0.99
IL-2	0.0002	4.855e-012, 0.7031	0.19	0.78	0.55, 1.0	0.07
IL-4	3.649e+14	3.81,5.401e+62	0.22	0.70	0.43, 0.97	0.21
IL-6	0.16	0.99,1.15	0.13	0.78	0.55, 1.0	0.07
IL-8	1.20	0.85, 1.79	0.32	0.72	0.45, 0.99	0.17
TNFα	1.52	0.12, 19.97	0.73	0.55	0.22, 0.88	0.75
PCT	0.97	0.93, 1.00	0.08	0.83	0.62, 1.0	*0.04*
CRP	1.01	1.00, 1.03	0.20	0.62	0.26, 0.97	0.46
SOFA	3.44	0.95, 26.23	0.11	0.65	0.33, 0.98	0.32
WCC	1.15	0.86, 1.63	0.35	0.52	0.144, 0.90	0.88
Neutrophil count	1.14	0.83, 1.63	0.40	0.51	0.13, 0.88	0.96
Lymphocyte count	0.95	0.35, 1.69	0.87	0.58	0.28, 0.88	0.59
Monocyte count	4.59	0.03, 893.3	0.54	0.57	0.20, 0.94	0.94
Neutrophil %	0.97	0.89, 1.06	0.46	0.52	0.14, 0.90	0.88
Lymphocyte %	1.0	0.83, 1.15	0.95	0.58	0.25, 0.92	0.59
Monocyte %	0.75	0.43, 1.25	0.27	0.68	0.41, 0.94	0.26
NLR	1.10	0.72, 1.77	0.65	0.58	0.26, 0.91	0.59
LMR	1.11	0.42, 2.62	0.81	0.51	0.20, 0.28	0.96
NMR	1.05	0.74, 1.45	0.78	0.63	0.33, 0.93	0.40
PLR	0.98	0.96, 1.00	0.15	0.77	0.51, 1.0	0.08

.

Table 6-7 Univariate Logistic Regression for infection on POD8 sample in HPB group

OR and AUC are given with 95% CI and p values on POD8

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.88	0.55, 1.33	0.56	0.57	0.23, 0.91	0.68
IL-10	1.95	0.60, 9.44	0.30	0.77	0.50, 1.0	0.12
IL-12p70	501020	1.16, 3.348e+15	0.16	0.81	0.55, 1.0	0.07
IL-13	1.27	1.00 1.82	0.10	0.74	0.42, 1.0	0.17
IL-1b	5.01	0.07, 796.5	0.47	0.51	0.12, 0.91	0.94
IL-2	6.00	0.01, 7306	0.57	0.64	0.32, 0.97	0.42
IL-4	1.690e+15	1.63, 4.689e+157	0.51	0.74	0.45, 1.0	0.17
IL-6	1.00	1.00,1.10	0.57	0.60	0.23, 0.97	0.57
IL-8	1.07	0.73, 1.60	0.71	0.54	0.15, 0.93	0.81
TNFα	3.93	0.36, 132.5	0.32	0.60	0.23, 0.97	0.57
PCT	1.00	0.98, 1.01	0.56	0.60	0.26, 0.94	0.57
CRP	1.00	0.99, 1.02	0.65	0.56	0.21, 0.91	0.74
SOFA	4.99	0.69, 114.3	0.18	0.66	0.34, 0.98	0.33
WCC	1.04	0.84, 1.29	0.73	0.51	0.15, 0.87	0.94
Neutrophil count	1.02	0.83, 1.28	0.79	0.55	0.19, 0.91	0.77
Lymphocyte count	0.83	0.26, 1.22	0.55	0.51	0.20, 0.82	0.95
Monocyte count	1.71	0.02, 141.7	0.80	0.54	0.15, 0.93	0.83
Neutrophil %	1.04	0.97, 1.18	0.39	0.58	0.25, 0.90	0.66
Lymphocyte %	0.99	0.86, 1.12	0.86	0.60	0.29, 0.91	0.56
Monocyte %	0.96	0.49, 1.89	0.91	0.55	0.16, 0.94	0.77
NLR	0.99	0.70, 1.40	0.96	0.55	0.23, 0.87	0.77
LMR	0.88	0.36, 1.66	0.70	0.60	0.24, 0.96	0.56
NMR	1.03	0.86, 1.25	0.73	0.50	0.15, 0.85	>0.99
PLR	1.00	0.99, 1.02	0.68	0.60	0.28, 0.92	0.56

.

Table 6-8 Diagnostic accuracy for MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC,

Sensitivity, Specificity and AUC (ROC) with 95% CI on POD1 and POD4 for HPB

		POD1				**POD4**		
	Cutoff	Sensitivity	Specificity	AUC	Cutoff	Sensitivity	Specificity	AUC
IFNγ	<0.16	0.40 (0.07, 0.77)	0.93 (0.70, 1.0)	0.55 (0.21, 0.88) p=0.76	<2.3	0.80 (0.38, 0.99)	0.67 (0.39, 0.86)	0.58 (0.26, 0.91) p=0.60
IL-10	>0.96	0.80 (0.38, 0.99)	0.40 (0.20, 0.64)	0.67 (0.38, 0.96) p=0.28	<0.73	1.0 (0.57, 1.0)	0.42 (0.19, 0.68)	0.57 (0.30, 0.83) p=0.67
IL-12p70	<0.17	0.80 (0.38, 0.99)	0.40 (0.20, 0.64)	0.51 (0.19, 0.82) p=0.97	>0.067	0.60 (0.23, 0.93)	0.75 (0.47, 0.91)	0.63 (0.28, 0.97) p=0.43
IL-13	>5.1	0.60 (00.23, 0.93)	0.73 (0.48, 0.89)	0.52 (0.18, 0.86) p=0.90	>3.8	0.40 (0.07, 0.77)	0.75 (0.47, 0.91)	0.54 (0.23, 0.86) p=0.79
IL-1b	>0.07	1.0 (0.57, 1.0)	0.27 (0.11, 0.52)	0.64 (0.34, 0.94) p=0.36	>0.53	0.40 (0.07, 0.77)	0.92 (0.65, 1.0)	0.50 (0.16, 0.84) p>0.99
IL-2	<0.63	1.0 (0.57, 1.0)	0.40 (0.20, 0.64)	0.71 (0.43, 0.98) p=0.18	<0.18	0.80 (0.38, 0.99)	0.75 (0.47, 0.91)	0.78 (0.56, 1.0) p=0.07
IL-4	>0.24	0.60 (0.23, 0.93)	0.80 (0.55, 0.93)	0.64 (0.31, 0/97) p=0.36	>0.094	1.0 (0.57, 1.0)	0.33 (0.14. 0.61)	0.70 (0.43, 0.97) p=0.21
IL-6	<20	0.60 (0.23, 0.93)	0.87 (0.62, 0.98)	0.53 (0.14,0.93) p=0.83	>17	0.60 (0.23, 0.93)	0.92 (0.65, 1.0)	0.78 (0.55, 1.0_ p=0.07
IL-8	>3.1	1.0 (0.57, 1.0)	0.20 (0.07, 0.45)	0.52 (0.22, 0.82) p=0.90	>5.1	1.0 (0.57, 1.0)	0.50 (0.25,0.75)	0.72 (0.45, 0.99) p=0.17
TNFα	<0.27	0.40 (0.07, 0.77)	0.93 (0.70, 1.0)	0.53(0.17, 0.89) p=0.83	>1.2	0.40 (0.07, 0.77)	0.92 (0.65, 1.0)	0.55 (0.22, 0.88) p=0.75
PCT	<143	0.75 (0.30, 0.99)	0.67 (0.42, 0.85)	0.77 (0.53, 1.0) p=0.11	<126	0.80 (0.38, 0.99)	0.83 (0.55, 0.97)	**0.8 (0.62, 1.0) p=0.04**
CRP	<65	0.80 (0.38, 0.99)	0.53 (0.30, 0.75)	0.52 (0.22, 0.82) p=0.90	>136	0.60 (0.23, 0.93)	0.85 (0.58, 0.97)	0.58 (0.20, 0.96) p=0.59
SOFA	<1.5	1.0 (0.57, 1.0)	0.20 (0.07, 0.45)	0.64 (0.38, 0.90) p=0.36	>1.5	0.40 (0.07, 0.77)	1.0 (0.77, 1.0)	0.65 (0.33,0.98) p=0.32
WCC	<12.0	0.60 (0.23, 0.93)	0.73 (0.48, 0.89)	0.65 (0.34, 0.97) p=0.32	>9.0	0.60 (0.23, 0.93)	0.46 (0.23, 0.71)	0.52(0.14, 0.90) p=0.88

.

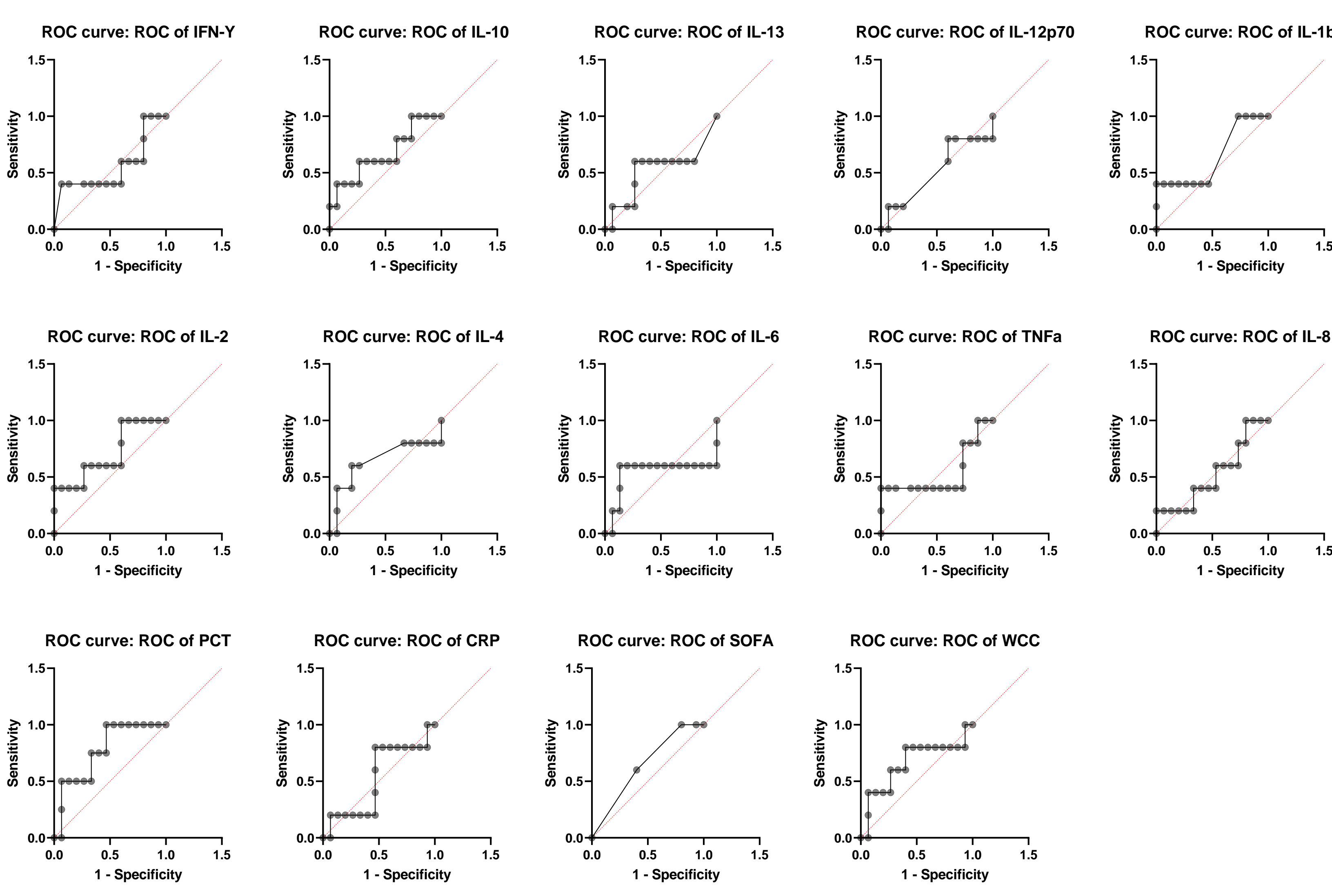


Figure 6-3 ROC curves for HPB POD1 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC

.

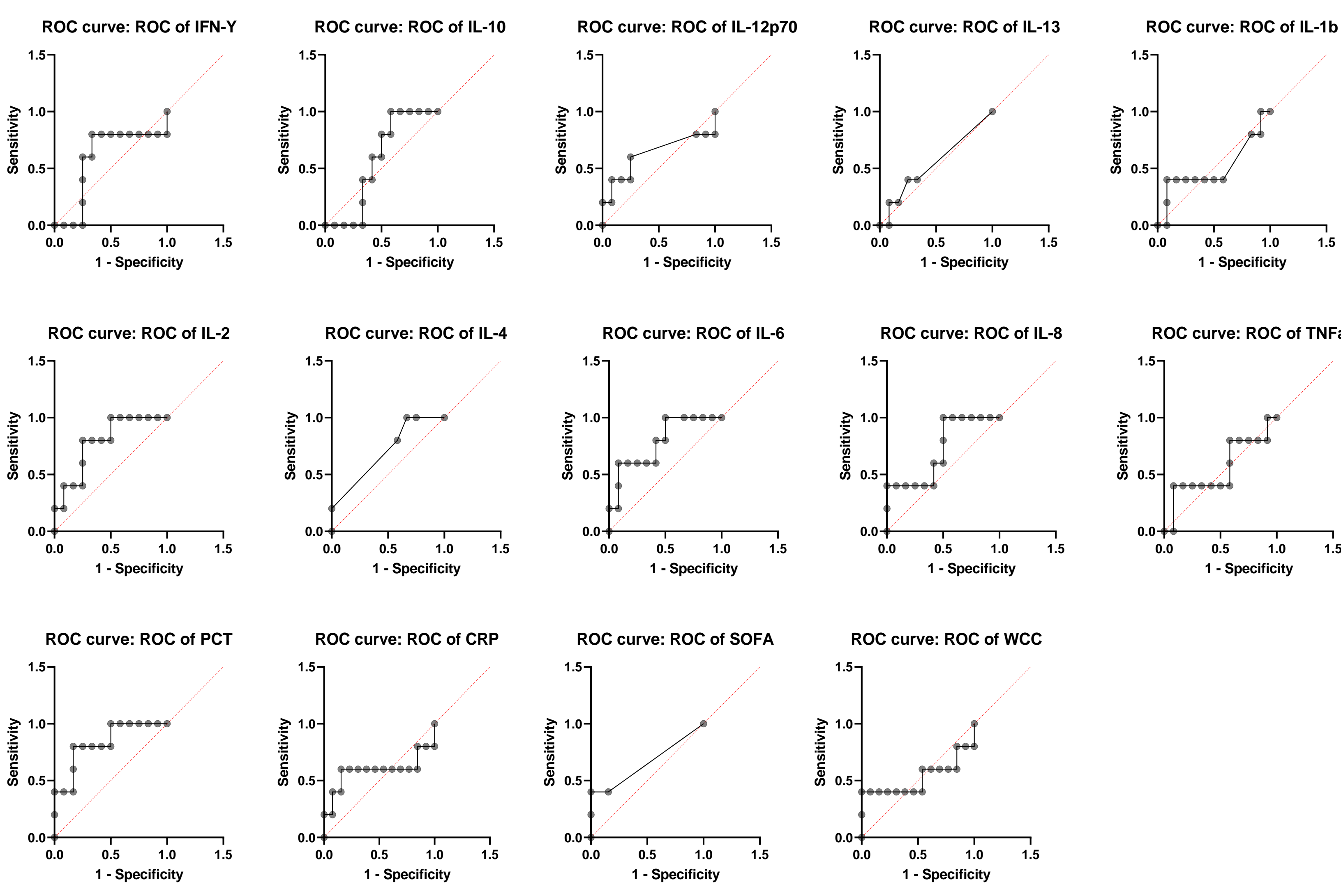


Figure 6-4 ROC curves for HPB POD4 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC

.

Mann Witney U test for infection following Liver Transplantation

Most cytokines were not significantly different between participants with and without infection at baseline and sequential samples. A difference of +0.06 (p=0.03) was seen for IL-1b on POD2. Lymphocytes and monocytes were significantly raised on POD2, +0.6 (p=0.01) and +0.22 (p<0.01) respectively. CRP, PCT, SOFA and WCC did not show a significant difference.

Analysis of Variance for infection following Liver Transplantation

There was no statistical difference between infected and non-infected groups for any cytokine at any time point. WCC were higher in the infected group on POD2. There was no statistical difference in PCT, CRP, neutrophil count, lymphocyte count, or monocyte count. There was no difference in the neutrophil, lymphocyte or monocyte percentage of WCC, and NLR, LMR, NMR and PLR were not significantly different.

Univariate Logistic Regression for infection following Liver Transplantation

Logistic regression of the cytokine V-Plex panel for, infection showed no significant difference between infected and non-infected groups. Increased lymphocyte and monocyte count on POD2 had an increased OR for infection – OR 24.62 (95% CI 2.08, 1016) p=0.03; OR 3706 (95% CI 6.51, 317659159) p=0.004 respectively.

.

Diagnostic accuracy for infection following Liver Transplantation

Sensitivity, specificity and AUC were poor for almost all cytokines, alongside CRP, PCT, SOFA and WCC which did not achieve statistical significance. IL-1b on POD2 was the only marker with a significant result, with sensitivity 100% (95% CI 51-100%), specificity 67% (95% CI 42-85%), and AUC 0.85 (95% CI 67-100%) p=0.04.

.

Table 6-9 Mann-Witney U test for differences in biomarkers of infection following LT in infected and non-infected groups at baseline, on POD2, POD5 and POD8

	Baseline	POD2	POD5	POD8
IFNγ	ns	ns	ns	ns
IL-10	ns	ns	ns	ns
IL-12p70	ns	ns	ns	ns
IL-13	ns	ns	ns	ns
IL-1b	ns	+0.06 (p=0.03)	ns	ns
IL-2	ns	ns	ns	ns
IL-4	ns	ns	ns	ns
IL-6	ns	ns	ns	ns
IL-8	ns	ns	ns	ns
TNFα	ns	ns	ns	ns
PCT	ns	ns	ns	ns
CRP	ns	ns	ns	ns
SOFA	ns	ns	ns	ns
WCC	ns	ns	ns	ns
Neutrophil count	ns	ns	ns	ns
Lymphocyte count	ns	+0.6 (p=0.01)	ns	ns
Monocyte count	ns	+0.22 (p<0.01)	ns	ns
Neutrophil %	ns	ns	ns	ns
Lymphocyte %	ns	ns	ns	ns
Monocyte %	ns	ns	ns	ns
NLR	ns	ns	ns	ns
LMR	ns	ns	ns	ns
NMR	ns	ns	ns	ns
PLR	ns	ns	ns	ns

Differences in median values between infected and non-infected groups and their p values are presented. Non-significant values are not presented (ns).

.

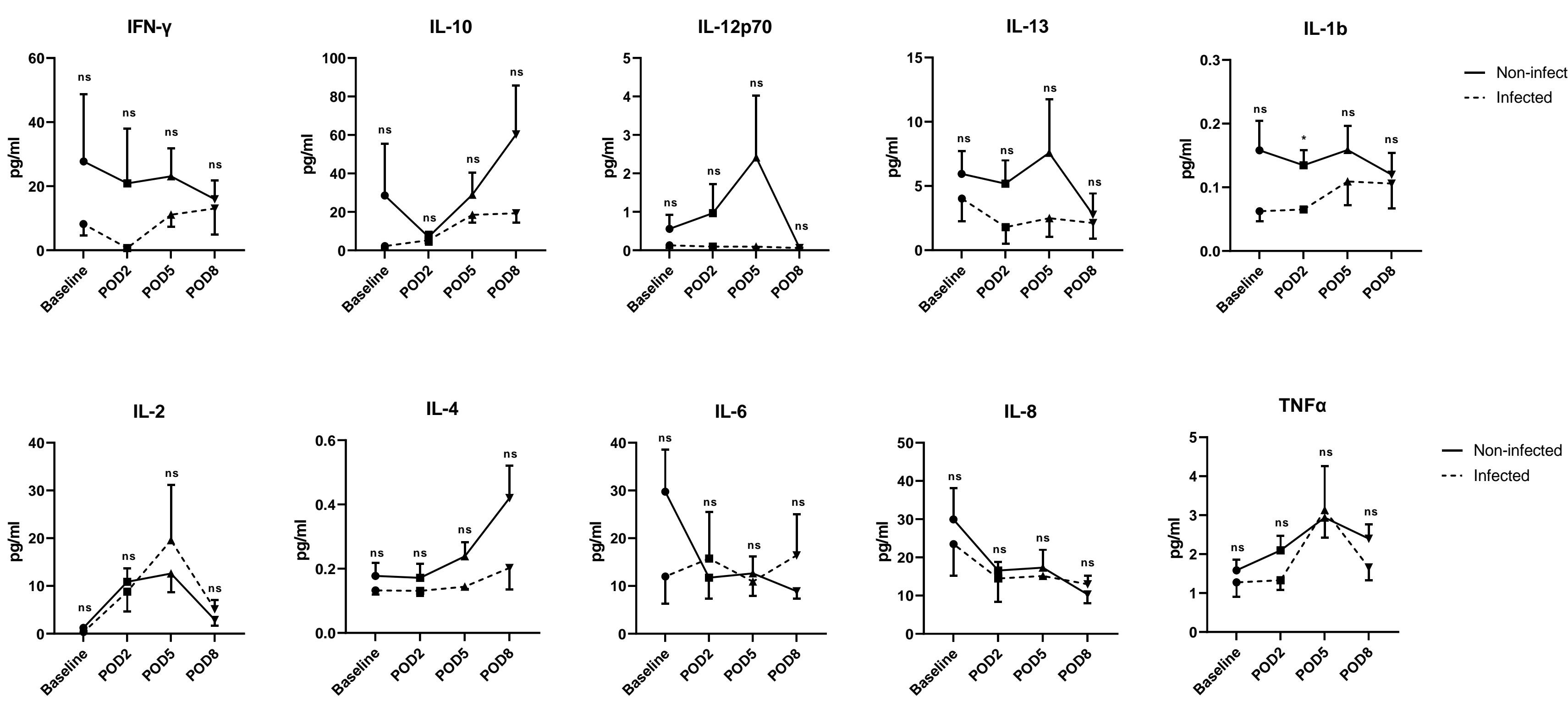


Figure 6-5 Liver Transplantation Grouped 2-way ANOVA showing difference between groups at each POD for MSD V PLEX Proinflammatory Cytokine panel. Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where p<0.05, and ** where p<0.01. All other points are non-significant

.

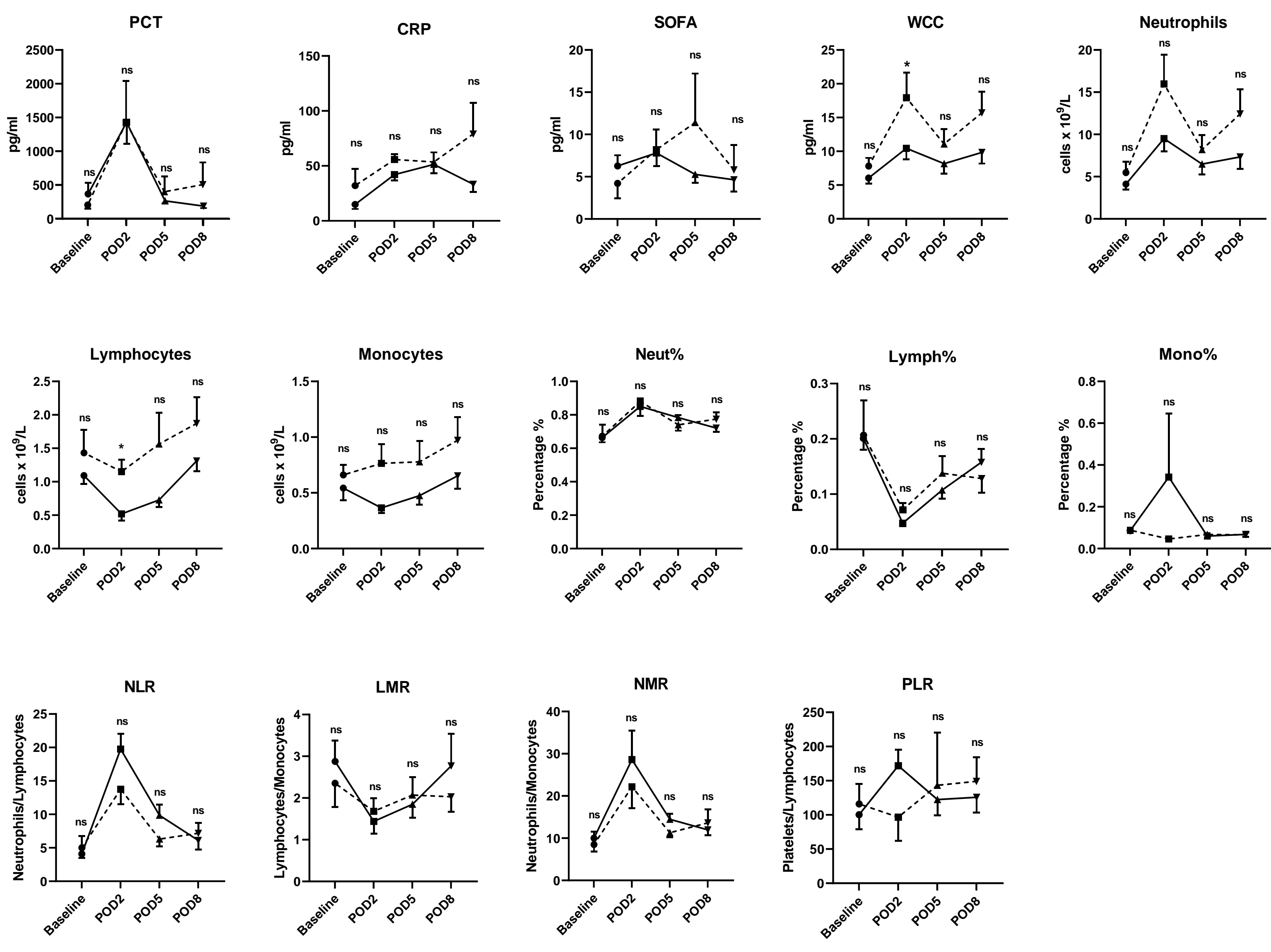


Figure 6-6 Liver Transplantation Grouped 2-way ANOVA

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where $p<0.05$, and ** where $p<0.01$. All other points are non-significant

.

Table 6-10 Univariate Logistic Regression for infection at baseline sample in LT group

OR and AUC are given with 95% CI and p values at baseline

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.99	0.89, 1.01	0.68	0.56	0.27, 0.85	0.69
IL-10	0.99	0.68, 1.01	0.74	0.51	0.17, 0.84	0.97
IL-12p70	0.03	5.22e-7, 1.51	0.40	0.70	0.42, 0.98	0.19
IL-13	0.87	0.52, 1.08	0.39	0.58	0.29, 0.87	0.62
IL-1b	7.57e-12	1.74e-43, 2.36	0.31	0.65	0.37, 0.92	0.34
IL-2	0.32	0.002, 1.24	0.54	0.57	0.28, 0.86	0.66
IL-4	0.03	7.29e-13, 157.7	0.65	0.56	0.25, 0.87	0.73
IL-6	0.97	0.89,1.01	0.31	0.63	0.36, 0.89	0.41
IL-8	1.0	0.97,1.10	0.42	0.61	0.15, 1.0	0.56
TNFα	0.69	0.17, 1.98	0.54	0.55	0.23, 0.86	0.76
PCT	1.0	1.00, 1.00	0.99	0.52	0.16, 0.87	0.92
CRP	1.02	0.96, 1.08	0.50	0.56	0.14, 0.98	0.72
SOFA	0.88	0.56, 1.12	0.41	0.72	0.44, 1.0	0.15
WCC	1.2	0.87, 1.80	0.27	0.71	0.48, 0.93	0.18
Neutrophil count	1.14	0.68, 1.82	0.58	0.59	0.21, 0.96	0.63
Lymphocyte count	2.96	0.48, 23.89	0.25	0.64	0.36, 0.92	0.36
Monocyte count	2.31	0.14, 38.46	0.54	0.69	0.45, 0.92	0.22
Neutrophil %	2.03	0.0002, 36725	0.88	0.60	0.27, 0.93	0.51
Lymphocyte %	2.12	1.031e-5, 145589	0.89	0.56	0.24, 0.88	0.69
Monocyte %	4.48	1.693e-13, 78172256803	0.92	0.53	0.28, 0.79	0.83
NLR	1.12	0.77, 1.63	0.52	0.59	0.26, 0.91	0.57
LMR	0.82	0.33, 1.49	0.57	0.56	0.23, 0.89	0.69
NMR	0.94	0.72, 1.13	0.59	0.55	0.26, 0.83	0.76
PLR	1.00	0.99, 1.02	0.69	0.64	0.38, 0.90	0.36

.

Table 6-11 Univariate Logistic Regression for infection on POD2 sample in LT group

OR and AUC are given with 95% CI and p values on POD2

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.93	0.55, 1.01	0.65	0.60	0.25, 0.96	0.58
IL-10	0.97	0.77, 1.10	0.72	0.53	0.19, 0.87	0.84
IL-12p70	0.004	5.981e-16, 1.223	0.50	0.57	0.29, 0.85	0.69
IL-13	0.94	0.75, 1.12	0.55	0.55	0.27, 0.82	0.76
IL-1b	3.755e-047	6.78e-174, 4.26e-6	0.24	0.85	0.67, 1.0	0.36
IL-2	0.98	0.85, 1.09	0.71	0.58	0.27, 0.89	0.62
IL-4	0.03	7.29e-13, 157.7	0.65	0.01	0.25, 0.87	0.73
IL-6	1.01	0.94,1.08	0.67	0.58	0.25, 0.91	0.62
IL-8	0.97	0.82,1.10	0.67	0.67	0.30, 1.0	0.32
TNFα	0.53	0.12, 1.34	0.27	0.64	0.39, 0.89	0.36
PCT	1.0	1.00, 1.00	0.99	0.52	0.16, 0.87	0.92
CRP	1.04	0.99, 1.12	0.15	0.81	0.62, 1.0	*0.04*
SOFA	1.01	0.83, 1.21	0.89	0.55	0.27, 0.83	0.95
WCC	1.15	1.0, 1.38	0.07	0.79	0.56, 1.0	0.06
Neutrophil count	1.13	0.96, 1.34	0.15	0.67	0.36, 0.99	0.30
Lymphocyte count	24.62	2.08, 1016	*0.03*	0.88	0.73, 1.0	*0.01*
Monocyte count	3706	6.51, 317659159	*0.004*	0.88	0.72, 1.0	*0.01*
Neutrophil %	2.95	0.01, 21844496	0.76	0.69	0.45, 0.93	0.21
Lymphocyte %	4.13e+2	9.35, 1.79e+49	0.08	0.79	0.56, 1.0	0.06
Monocyte %	0.42	9.04e-24, 2.05	0.78	0.66	0.31, 1.0	0.34
NLR	0.86	0.68, 1.01	0.13	0.83	0.65, 1.0	*0.05*
LMR	1.46	0.49, 4.40	0.45	0.77	0.55, 0.98	0.11
NMR	0.99	0.88, 1.0	0.68	0.67	0.33, 1.0	0.30
PLR	0.98	0.94, 1.0	0.08	0.83	0.64, 1.00	*0.05*

.

Table 6-12 Univariate Logistic Regression for infection on POD5 sample in LT group

OR and AUC are given with 95% CI and p values on POD5

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.85	0.84, 1.01	0.43	0.63	0.34, 0.91	0.41
IL-10	0.99	0.72, 1.03	0.71	0.68	0.48, 0.89	0.54
IL-12p70	0.75	1.06e-7, 1.10	0.71	0.61	0.34, 0.8	0.46
IL-13	0.94	0.68, 1.04	0.55	0.55	0.26, 0.83	0.76
IL-1b	0.02	4.51e-9, 99.76	0.48	0.62	0.33, 0.91	0.43
IL-2	1.02	0.96, 1.08	0.45	0.57	0.27, 0.87	0.63
IL-4	3.41e-6	2.04e-26, 5.08	0.40	0.65	0.39, 0.91	0.32
IL-6	0.99	0.86,1.07	0.78	0.60	0.31, 0.89	0.51
IL-8	0.99	0.86, 1.06	0.79	0.73	0.51, 0.96	0.13
TNFα	1.05	0.52, 1.78	0.86	0.50	0.15, 0.85	>0.99
PCT	1.00	1.00, 1.01	0.38	0.57	0.25, 0.90	0.63
CRP	1.00	0.96, 1.05	0.88	0.57	0.30, 0.84	0.66
SOFA	1.01	0.75, 1.30	0.95	0.51	0.21, 0.82	0.93
WCC	1.10	0.91, 1.36	0.31	0.68	0.39, 0.98	0.24
Neutrophil count	1.02	0.77, 1.30	0.85	0.58	0.27, 0.88	0.64
Lymphocyte count	5.55	1.06, 83.02	0.10	0.70	0.34, 1.0	0.22
Monocyte count	2.45	0.09, 51.79	0.55	0.62	0.30, 0.94	0.48
Neutrophil %	9.36e-6	2.81e-14, 296.1	0.20	0.67	0.33, 1.0	0.30
Lymphocyte %	45406	0.001,10172 759715114	0.11	0.77	0.54, 0.99	0.11
Monocyte %	0.0004	2.30e-21. 7950947723	0.27	0.56	0.32, 0.81	0.71
NLR	0.76	0.46, 1.04	0.21	0.77	0.53, 1.0	0.11
LMR	1.41	0.53, 3.61	0.43	0.80	0.62, 0.99	0.07
NMR	0.88	0.61, 1.13	0.38	0.60	0.33, 0.87	0.54
PLR	1.00	0.99, 1.01	0.58	0.59	0.20, 0.99	0.57

.

Table 6-13 Univariate Logistic Regression for infection on POD8 sample in LT group

OR and AUC are given with 95% CI and p values on POD8

	OR	95% CI	p value	AUC	95% CI	p value
IFNγ	0.88	0.55, 1.33	0.56	0.57	0.23, 0.91	0.68
IL-10	1.95	0.60, 9.44	0.30	0.77	0.50, 1.0	0.12
IL-12p70	501020	1.16, 3.348e+15	0.16	0.81	0.55, 1.0	0.07
IL-13	1.27	1.00 1.82	0.10	0.74	0.42, 1.0	0.17
IL-1b	5.01	0.07, 796.5	0.47	0.51	0.12, 0.91	0.94
IL-2	6.00	0.01, 7306	0.57	0.64	0.32, 0.97	0.42
IL-4	1.690e+15	1.63, 4.689e+157	0.51	0.74	0.45, 1.0	0.17
IL-6	1.00	1.00,1.10	0.57	0.60	0.23, 0.97	0.57
IL-8	1.07	0.73, 1.60	0.71	0.54	0.15, 0.93	0.81
TNFα	3.93	0.36, 132.5	0.32	0.60	0.23, 0.97	0.57
PCT	1.00	0.99, 1.01	0.34	0.62	0.32, 0.92	0.46
CRP	1.03	1.0, 1.07	0.11	0.73	0.35, 1.0	0.19
SOFA	1.05	0.83, 1.30	0.67	0.56	0.24, 0.88	0.69
WCC	1.20	0.98, 1.50	0.11	0.78	0.40, 1.0	0.08
Neutrophil count	84207	0.95, 1.50	0.16	0.73	0.41, 1.0	0.18
Lymphocyte count	0.0002	0.47, 21.59	0.29	0.64	0.27, 1.0	0.43
Monocyte count	2.0	0.11, 39.0	0.62	0.57	0.27, 0.88	0.67
Neutrophil %	84207	0.02, 73439563049653	0.19	0.69	0.31, 1.0	0.28
Lymphocyte %	0.99	1.02e-14, 25637	0.51	0.65	0.28, 1.0	0.40
Monocyte %	3.38e+21	1.10e-24, 1.17e+81	0.40	0.69	0.31, 1.0	0.28
NLR	1.09	0.81, 1.47	0.52	0.65	0.28, 1.0	0.40
LMR	0.87	0.26, 1.51	0.70	0.52	0.20, 0.84	0.90
NMR	1.14	0.91, 1.51	0.26	0.60	0.27, 0.94	0.54
PLR	1.00	0.99, 1.02	0.80	0.50	0.15, 0.85	>0.99

.

Table 6-14 Diagnostic accuracy of ROC curves for POD2 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC,

		POD2				**POD5**		
	Cutoff	Sensitivity	Specificity	AUC	Cutoff	Sensitivity	Specificity	AUC
IFNγ	<1.1	0.75 (0.30, 0.99)	0.67 (0.42, 0.85)	0.77 (0.55, 0.99) p=0.11	<9.9	0.60 (0.23, 0.93)	0.67 (0.42, 0.85)	0.63 (0.34, 0.91) p=0.41
IL-10	<1.6	0.50 (0.9, 0.91)	0.80 (0.55, 0.93)	0.53 (0.19, 0.87) p=0.84	> 11	0.80 (0.38, 0.99)	0.33 (0.15, 0.58)	0.52 (0.26, 0.78) p=0.90
IL-12p70	< 0.09	0.75 (0.30, 0.99)	0.47 (0.25, 0.70)	0.57 (0.29, 0.85) p=0.69	> 0.077	0.40 (0.07, 0.77)	0.80 (0.55, 0.93)	0.61 (0.34, 0.88) p=0.46
IL-13	<2.9	0.75 (0.30, 0.99)	0.47 (0.25, 0.70)	0.58 (0.29, 0.87) p=0.62	<7.2	1.0 (0.57, 1.0)	0.27 (0.11,0.52)	0.55 (0.26,0.83) p=0.76
IL-1b	**<0.08**	**1.0 (0.51, 1.0)**	**0.67 (0.42, 0.85)**	**0.85 (0.67, 1.0) p=0.04**	<0.089	0.80 (0.38, 0.99)	0.53 (0.30, 0.75)	0.62 (0.33, 0.91) p=0.43
IL-2	<2.3	0.50 (0.09, 0.91)	0.73 (0.48, 0.89)	0.58 (0.27, 0.89) p=0.62	>3.5	0.60 (0.23, 0.93)	0.40 (0.20, 0.64)	0.57 (0.27, 0.87) p=0.63
IL-4	<0.088	0.25 (0.01, 0.70)	0.93 (0.70, 1.00)	0.56 (0.25, 0.87) p=0.73	<0.26	1.0 (0.57, 1.0)	0.27 (0.11, 0.52)	0.65 (0.39, 0.91) p=0.32
IL-6	>6.1	0.75 (0.40, 0.99)	0.53 (0.30,0.75)	0.58 (0.25, 0.91) p=0.62	>0.60	(0.23, 0.93)	0.80 (0.55, 0.93)	0.60 (0.31, 0.89) p=0.51
IL-8	<9.5	<0.75 (0.30, 0.99)	0.80 (0.55, 0.93)	0.67 (00.3, 1.0) p=0.32	>14	1.0 (0.57, 1.0)	0.73 (0.48,0.89)	0.73 (0.51, 0.96) p=0.13
TNFα	<2.0	1.0 (0.57, 1.0)	0.47 (0.25,0.70)	0.64 (0.39, 0.89) p=0.36	>2.9	0.50 (0.0.9, 0.91)	0.67 (0.32, 0.85)	0.50 (0.15, 0.85) p>0.99
PCT	<596	0.50 (0.09, 0.91)	0.73 (0.48, 0.89)	0.52 (0.16,0.87) P=0.92	<273	0.80 (0.38, 0.99)	0.40 (0.20, 0.64)	0.57 (0.25, 0.90) p=0.63
CRP	>44	1.0 (0.51, 1.0)	0.80 (0.55, 0.93)	0.90 (0.60, 1.0) p=0.07	>42	0.80 (0.38, 0.99)	0.58 (0.32, 0.81)	0.60 (0.33, 0.87) p=0.53
SOFA	>6	0.60 (0.23, 0.93)	0.60 (0.36, 0.80)	0.55 (0.27, 0.83) p=0.76	>4.5	0.60 (0.23, 0.93)	0.67 (0.42, 0.85)	0.62 (0.29, 0.95) p=0.43
WCC	>18	0.60 (0.23, 0.93)	0.93 (0.70, 1.0)	0.79 (0.56, 1.0) p=0.06	>12	0.60 (0.23, 0.93)	0.87 (0.62, 0.98)	0.68 (0.39, 0.97) p=0.24

Sensitivity, Specificity and AUC with 95% CI and p values on POD2 and POD5 for Liver Transplantation

.

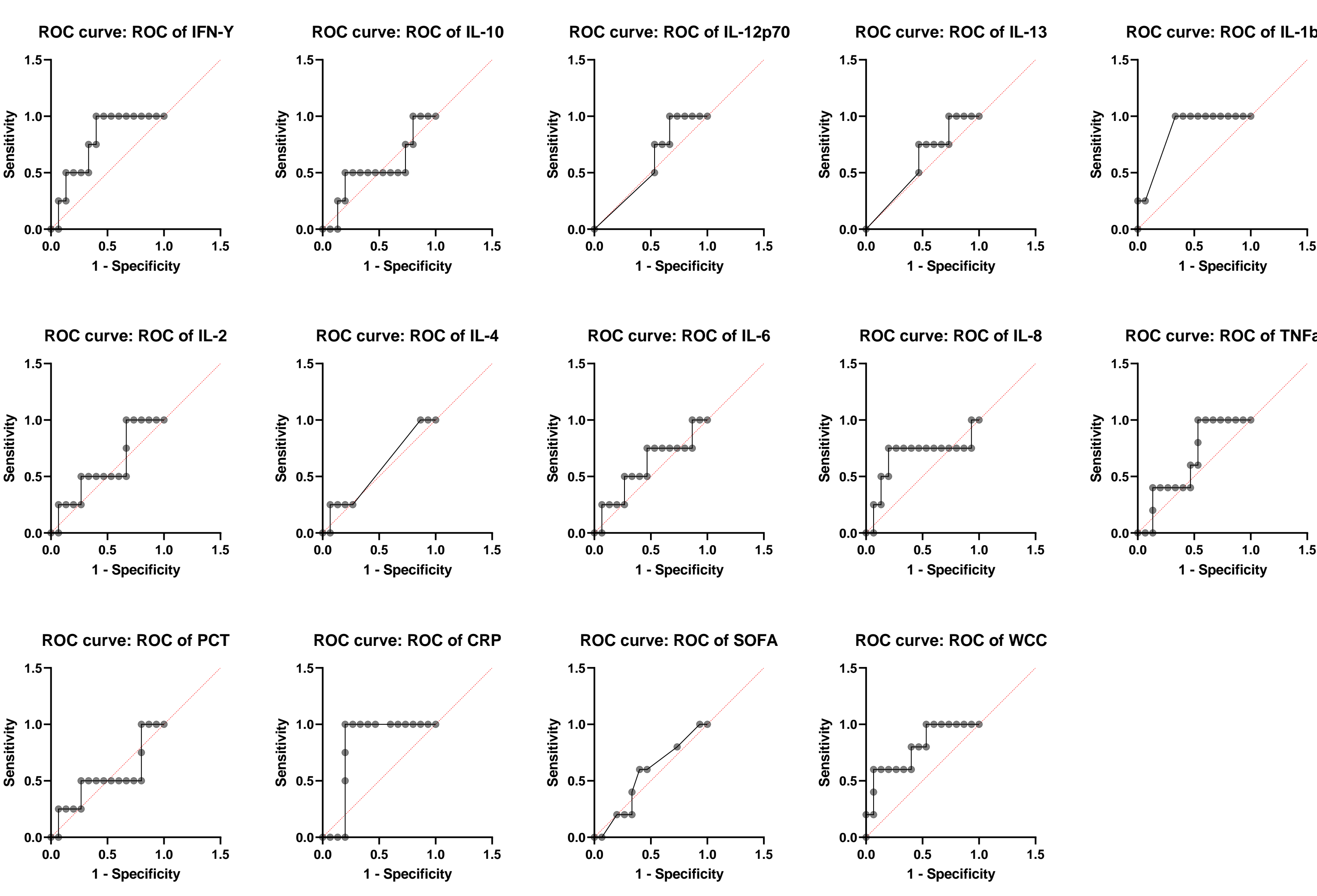


Figure 6-7 ROC curves for Liver Transplantation POD2 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC

.

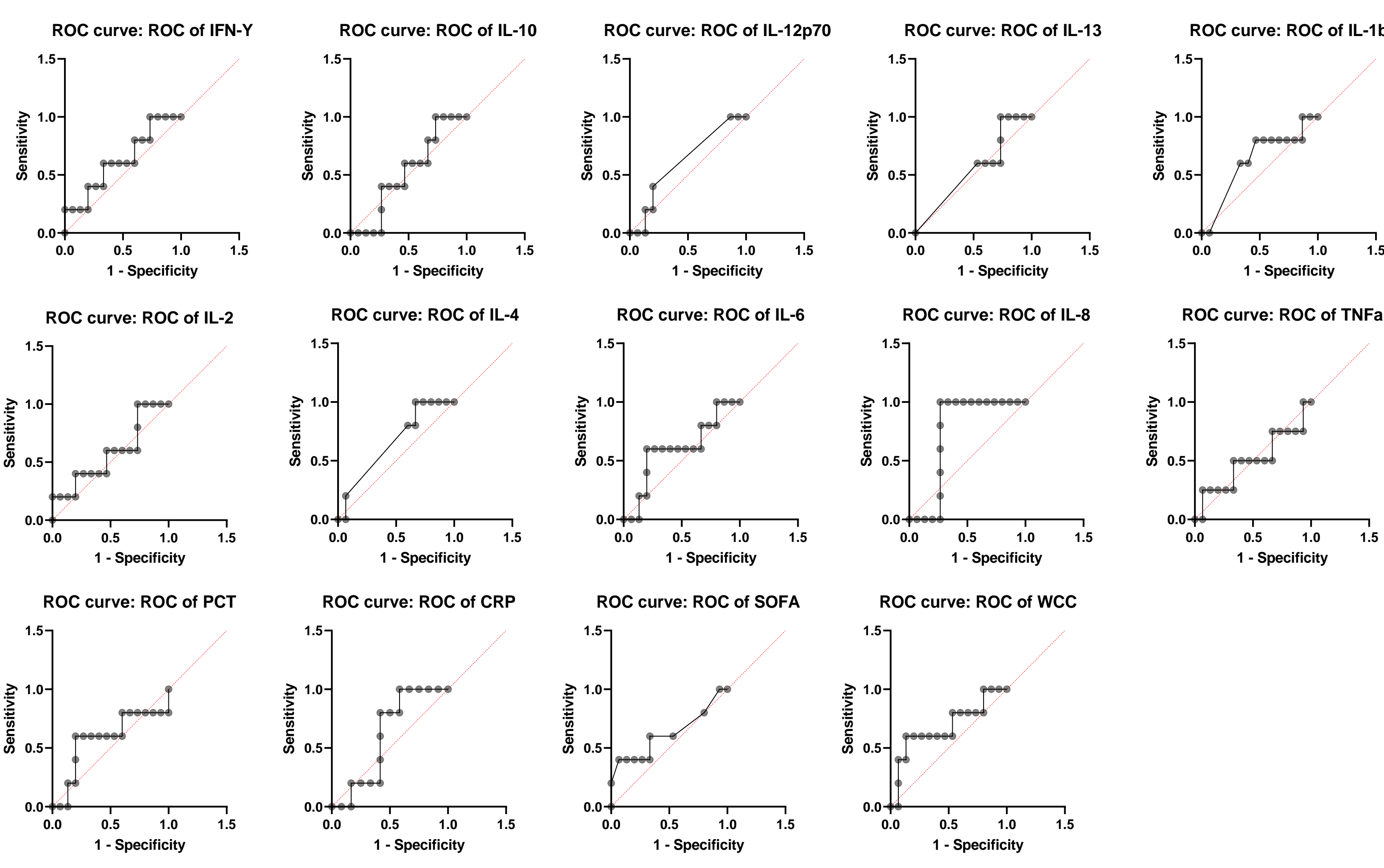


Figure 6-8 ROC curves for Liver Transplantation POD5 MSD V-PLEX Cytokine panel and PCT, CRP, SOFA and WCC

.

6.3 Summary and Conclusions

Detection of a significant number of markers was poor for IL-12p70, IL-13, IL-1b, IL-2 and IL-4. Results for those markers should be interpreted with caution, given the poor rate of detection and measurement above the fit curve,

Analysis using MSD V-PLEX proinflammatory cytokine panel showed no significant difference using ANOVA between groups who did and did not develop infection for any cytokine. The later sequential samples on POD4 and particularly POD8 may be biased as in both cohorts participants had started to be discharged before these time points, meaning that there were missing data (as no samples collected). Samples included were those patients who remained in hospital and be more likely to have a complication.

The diagnostic performance for all markers analysed was poor. The high performance for IL-1b cannot be relied upon, as in the experiment 91.9% of samples were outside of range, and these values were imputed using the lower detection limit which is likely to have biased the analysis.

Standard biomarkers WCC, CRP and SOFA were not discriminant for infection in the LT cohort in all analyses. This supports the suggestion that there is a role for a novel, reliable marker with high diagnostic performance to direct postoperative clinical decision making.

.

7 Monocyte Phenotyping

7.1 Introduction

Flow cytometry was performed on frozen PBMCs for the cell surface markers CD14, CD16, CD155, CD163, CCR2, HLA-DR, MerTK, PD-L1 and PD-1. Monocytes were identified based upon positive selection CD14, CD16 and HLA-DR expression, using an established gating strategy Surface marker expression was determined in the total monocyte population as described in the methods chapter.

.

7.2 Results

67 samples from 20 HPB participants were analysed (baseline and POD1, POD4 and POD8 sequential samples) and 12 HC samples.

MWU for surface markers showed CCR2 MFI to be -1833 (p=0.03) in participants who developed infection. CD14% on POD1 was lower in the infected group, -25.1 (p=0.04). On POD4, CD155 MFI was increased +525.0 (p=0.04), HLA-DR percentage and MFI were increased, +17.05 (p=0.03), +366.5 (p=0.01) respectively in the infected group. CD16. CD163, MerTK, PD-1 and PD-L1 were not statistically different between groups. There was no difference between groups for WCC, CRP, SOFA and PCT.

Grouped 2-way ANOVA for percentage showed a statistically significant decrease in CD14 and PD-L1 expression in the infected group.(Figure 7-1) All other surface markers were not statistically different. Grouped 2-way ANOVA for MFI showed downregulation of CCR2 at baseline and on POD1, and upregulation of CD155 on POD4.(Figure 7-2)

.

Univariate logistic regression for infection did not show a significant increased odds ratio for any cell surface marker examined at baseline, POD1, POD4 or POD8. AUC for HLA-DR % and MFI on POD4 was high – 0.85 (95% CI 0.66, 1.0) p=0.03; and 0.88 (95% CI 0.71, 1.0) p=0.2 respectively – however OR was not significantly increased between groups.

.

Table 7-1 Mann-Witney U Test of cell surface markers for infection following HPB surgery.

	Baseline	POD1	POD4	POD8
CD14 %	ns	-25.1 (p=0.04)	ns	ns
CD14 MFI	ns	ns	ns	ns
CCR2 %	ns	ns	ns	ns
CCR2 MFI	-1833 (p=0.03)	ns	ns	ns
CD16 MFI	ns	ns	ns	ns
CD16 %	ns	ns	ns	ns
CD155 %	ns	ns	ns	ns
CD155 MFI	ns	ns	+525.0 (p=0.04)	ns
CD163 %	ns	ns	ns	ns
CD163 MFI	ns	ns	ns	ns
HLA-DR %	ns	ns	+17.05 (p=0.03)	ns
HLA-DR MFI	ns	ns	+366.5 (p=0.01)	ns
MerTK %	ns	ns	ns	ns
MerTK MFI	ns	ns	ns	ns
PD-1 %	ns	ns	ns	ns
PD-1 MFI	ns	ns	ns	ns
PD-L1%	ns	ns	ns	ns
PD-L1 MFI	ns	ns	ns	ns
PCT	ns	ns	ns	ns
CRP	ns	ns	ns	ns
SOFA	ns	ns	ns	ns
WCC	ns	ns	ns	ns

Values are given as percentage of live cells expressing the surface marker (%), and mean fluorescence intensity (MFI). Non-significant values are presented as ns.

.

Grouped 2-way ANOVA (percentage) between infected and non-infected HPB groups.

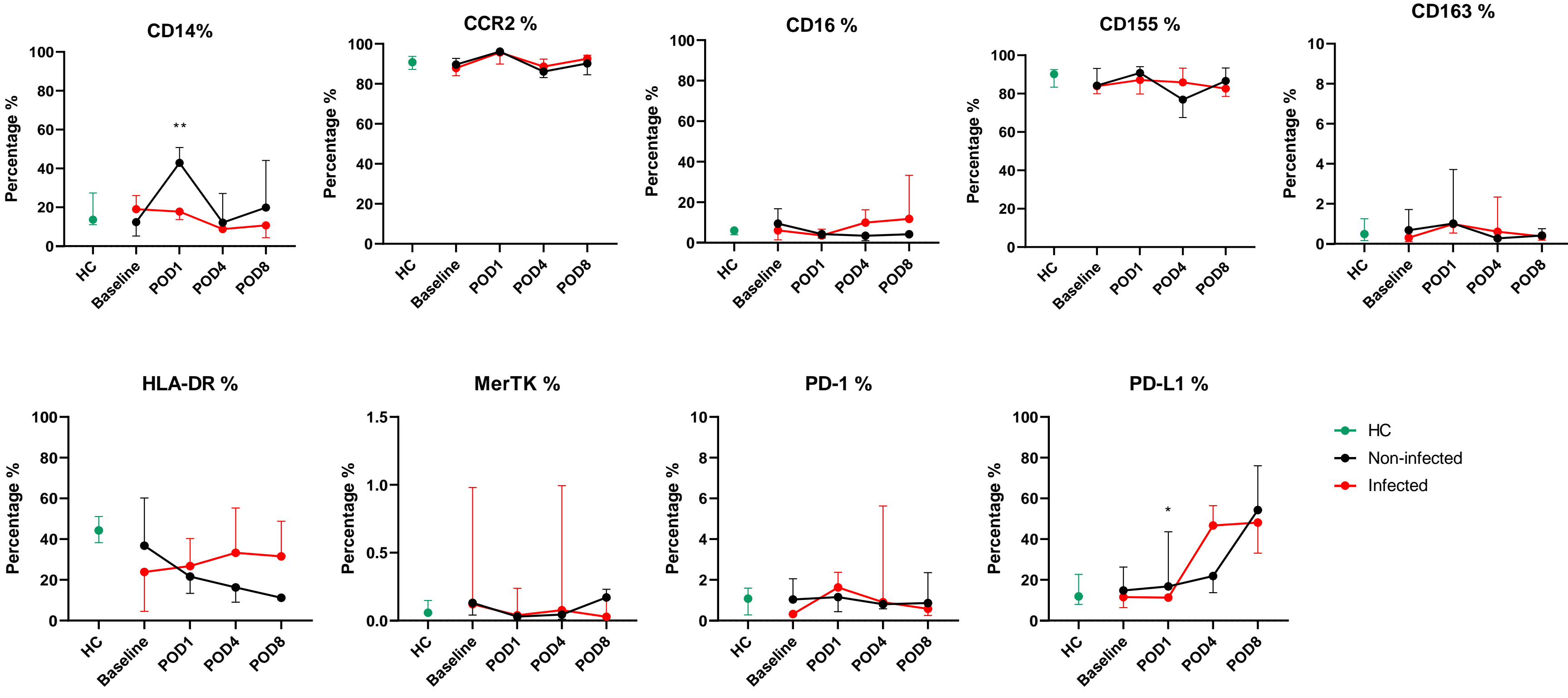


Figure 7-1 Grouped 2-way ANOVA for HPB cell surface markers, Percentage (%) showing difference between groups at each POD.

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where $p<0.05$, and ** where $p<0.01$. All other points are non-significant

.

Grouped 2-way ANOVA (MFI) between infected and non-infected HPB groups

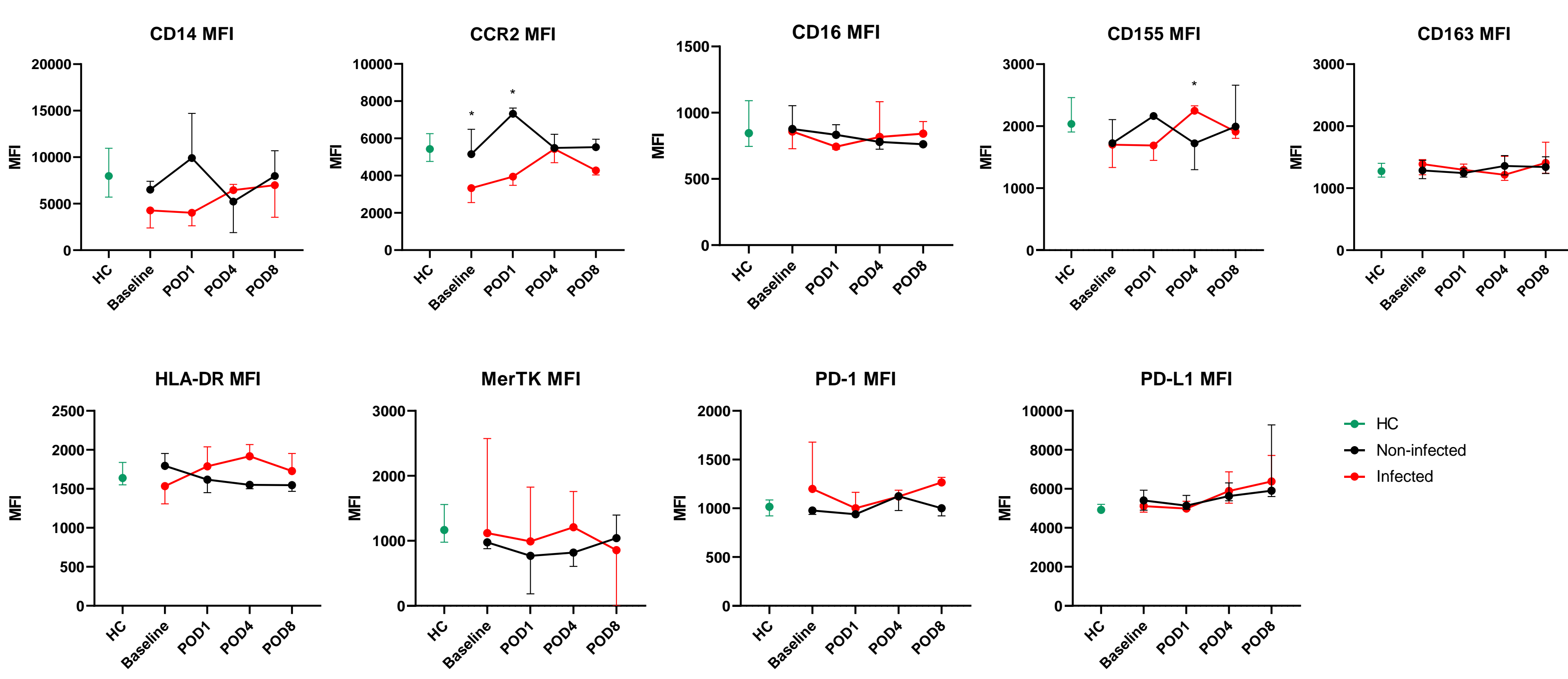


Figure 7-2 Grouped 2-way ANOVA for HPB cell surface markers, MFI showing difference between groups at each POD.

Points represent the mean with error bars denoting standard error of the mean (SEM). Statistical significance is marked using * where $p<0.05$, and ** where $p<0.01$. All other points are non-significant

.

Table 7-2 Univariate Logistic regression for infection for cell surface markers following HPB surgery at baseline.

	OR	95% CI	p value	AUC	95% CI	p value
CD14 %	1.03	094, 1.13	0.53	0.67	0.41, 0.92	0.28
CD14 MFI	1.00	1.0, 1.0	0.69	0.52	0.23, 0.81	0.90
CCR2 %	1.04	0.97, 1.2	0.41	0.53	0.23, 0.83	0.83
CCR2 MFI	1.00	1.00 1.00	*0.04*	0.83	0.57, 1.0	0.03
CD16 %	0.92	0.75, 1.04	0.29	0.64	0.38, 0.91	0.36
CD16 MFI	1.00	0.99, 1.00	0.49	0.57	0.31, 0.84	0.63
CD155 %	1.03	0.97, 1.14	0.42	0.52	0.27, 0.77	0.90
CD155 MFI	1.0	1.0,1.0	0.46	0.59	0.30, 0.87	0.57
CD163 %	0.18	0.003, 1.27	0.25	0.75	0.48, 1.00	0.10
CD163 MFI	1.00	0.97, 1.01	0.43	0.59	0.33, 0.85	0.57
HLA-DR %	0.94	0.87, 1.00	0.10	0.75	0.51, 0.98	0.11
HLA-DR MFI	0.99	0.99, 0.99	0.08	0.80	0.60, 1.0	*0.05*
MerTK %	2.25	0.19, 24.3	0.48	0.51	0.17, 0.86	0.93
MerTK MFI	1.00	1.0, 1.00	0.29	0.59	0.26, 0.91	0.57
PD-1 %	0.66	0.15, 1.41	0.46	0.66	0.33, 0.99	0.29
PD-1 MFI	1.00	1.00, 1.01	0.33	0.57	0.25, 0.90	0.63
PD-L1%	0.97	0.86, 1.05	0.50	0.54	0.26, 0.82	0.79
PD-L1 MFI	1.00	0.99, 1.00	0.22	0.68	0.43, 0.92	0.24

Values are given as percentage of live cells expressing the surface marker (%), and mean fluorescence intensity (MFI), with odds ratio (OR), area under the receiver operator curve (AUC), 95% confidence intervals (95% CI), and p values.

.

Table 7-3 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD1.

	OR	95% CI	p value	AUC	95% CI	p value
CD14 %	0.91	0.79, 0.99	0.09	0.81	0.62, 1.0	0.04
CD14 MFI	1.00	1.0, 1.0	0.14	0.76	0.50, 1.0	0.10
CCR2 %	0.99	0.79, 1.3	0.92	0.50	0.19, 0.81	>0.99
CCR2 MFI	1.0	1.00 1.00	0.29	0.71	0.49, 0.93	0.26
CD16 %	0.85	0.57, 1.19	0.37	0.64	0.40, 0.89	0.35
CD16 MFI	0.99	0.98, 1.00	0.26	0.67	0.39, 0.95	0.27
CD155 %	0.97	0.86, 1.10	0.60	0.68	0.37, 0.99	0.17
CD155 MFI	1.00	0.99,1.00	0.26	0.67	0.35, 0.99	0.27
CD163 %	0.68	0.21, 1.29	0.39	0.54	0.15, 0.93	0.81
CD163 MFI	1.0	1.0, 1.01	0.36	0.69	0.44, 0.94	0.21
HLA-DR %	1.00	0.04, 2.0	0.87	0.53	0.22, 0.84	0.57
HLA-DR MFI	1.01	1.00, 1.02	0.09	0.79	0.57, 1.0	0.06
MerTK %	32.58	0.01, 294291	0.38	0.57	0.23, 0.91	0.64
MerTK MFI	1.00	1.0, 1.00	0.75	0.62	0.27, 0.96	0.50
PD-1 %	0.96	0.40, 1.98	0.91	0.56	0.29, 0.82	0.71
PD-1 MFI	1.0	1.0, 1.01	0.32	0.79	0.58, 1.0	0.06
PD-L1%	0.93	0.76, 1.01	0.25	0.59	0.34, 0.83	0.58
PD-L1 MFI	1.00	0.99, 1.00	0.41	0.61	0.34, 0.89	0.46

Values are given as percentage of live cells expressing the surface marker (%), and mean fluorescence intensity (MFI), with odds ratio (OR), area under the receiver operator curve (AUC), 95% confidence intervals (95% CI), and p values.

.

Table 7-4 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD4.

	OR	95% CI	p value	AUC	95% CI	p value
CD14 %	0.94	0.78, 1.03	0.34	0.60	0.33, 0.87	0.53
CD14 MFI	1.00	1.00, 1.00	0.70	0.62	0.35, 0.88	0.46
CCR2 %	1.0	1.0, 1.0	0.93	0.53	0.25, 0.82	0.83
CCR2 MFI	1.03	0.91 1.20	0.69	0.52	0.23, 0.81	0.92
CD16 MFI	1.09	0.92, 1.31	0.33	0.65	0.37, 0.93	0.34
CD16 %	1.00	1.0, 1.01	0.48	0.63	0.36, 0.90	0.40
CD155 %	1.05	0.96, 1.19	0.32	0.68	0.40, 0.96	0.25
CD155 MFI	1.00	1.00,1.01	0.09	0.83	0.61, 1.0	*0.04*
CD163 %	2.03	0.68, 9.7	0.24	0.72	0.45, 0.98	0.17
CD163 MFI	1.0	0.99, 1.00	0.43	0.65	0.35, 0.95	0.34
HLA-DR %	1.06	1.0, 1.15	0.10	0.85	0.66, 1.0	*0.03*
HLA-DR MFI	1.01	1.0, 1.02	*0.02*	0.88	0.71, 1.0	*0.02*
MerTK %	68.7	0.73, 20799459	0.43	0.68	0.38, 0.99	0.25
MerTK MFI	1.00	1.0, 1.01	0.13	0.77	0.51, 1.0	0.09
PD-1 %	1.73	0.91, 4.56	0.14	0.53	0.16, 0.91	0.83
PD-1 MFI	1.0	0.99, 1.00	0.65	0.55	0.27, 0.83	0.75
PD-L1%	1.03	0.98, 1.09	0.29	0.68	0.41, 0.96	0.25
PD-L1 MFI	1.00	1.0, 1.00	0.54	0.57	0.23, 0.90	0.67

Values are given as percentage of live cells expressing the surface marker (%), and mean fluorescence intensity (MFI), with odds ratio (OR), area under the receiver operator curve (AUC), 95% confidence intervals (95% CI), and p values.

.

Table 7-5 Univariate Logistic regression for infection for cell surface markers following HPB surgery on POD8

	OR	95% CI	p value	AUC	95% CI	p value
CD14 %	0.89	0.71, 1.00	0.18	0.83	0.58, 1.0	0.06
CD14 MFI	1.0	1.00, 1.00	0.35	0.68	0.37, 0.98	0.31
CCR2 %	1.0	0.84, 1.22	0.98	0.51	0.17, 0.86	0.96
CCR2 MFI	1.0	1.0, 1.0	0.96	0.58	0.22, 0.93	0.66
CD16 %	1.01	0.97, 1.33	0.32	0.66	0.32, 1.0	0.37
CD16 MFI	1.00	0.99, 1.01	0.79	0.54	0.16, 0.92	0.83
CD155 %	1.04	0.94, 1.18	0.51	0.60	0.24, 0.96	0.57
CD155 MFI	1.0	1.00,1.00	0.67	0.55	0.22, 0.88	0.77
CD163 %	1.69	0.66, 18.18	0.40	0.51	0.15, 0.87	0.94
CD163 MFI	1.0	1.0, 1.01	0.28	0.69	0.35, 1.0	0.31
HLA-DR %	1.07	0.99, 1.20	0.13	0.74	0.42, 1.0	0.17
HLA-DR MFI	1.01	1.0, 1.01	0.15	0.73	0.39, 1.0	0.19
MerTK %	1.44	0.89, 1410	0.55	0.60	0.23, 0.97	0.57
MerTK MFI	1.00	0.99, 1.00	0.29	0.75	0.38, 1.0	0.20
PD-1 %	1.00	0.53, 1.81	0.99	0.60	0.24, 0.96	0.57
PD-1 MFI	1.00	1.0, 1.01	0.64	0.60	0.25, 0.95	0.57
PD-L1%	1.00	0.94, 1.06	0.97	0.54	0.19, 0.90	0.81
PD-L1 MFI	1.00	1.0, 1.00	0.90	0.65	0.34, 0.96	0.38

Values are given as percentage of live cells expressing the surface marker (%), and mean fluorescence intensity (MFI), with odds ratio (OR), area under the receiver operator curve (AUC), 95% confidence intervals (95% CI), and p values.

.

7.3 Summary and Conclusions

Decreased POD1 CD14 % and increased POD4 CD163 MFI were associated with infection. Increased POD4 HLA-DR % and MFI with associated with infection, Univariate logistic regression did not show significantly increased OR on any day.

POD8 results should be interpreted with caution. Seven participants had been discharged by POD8 and were not included in the analysis due to missing values. Patients discharged earlier were more likely to be well and not suffering a postoperative infection, or more likely to have undergone a less major operation to be safe to discharge. This is likely to have skewed the results and introduced bias. Most cases of infection were diagnosed before POD8, limiting the clinical utility of the test at this late stage. These data suggests that HLA-DR is an important surface expression marker in postoperative infection and inflammation.

.

8 General Discussion

8.1 Summary and significance of work

Evidence of ^{13}C BDV decrease in infection

This experiment has not demonstrated utility for BDV as a clinical biomarker of sepsis or infection. There is good strength of evidence from the research reported in Chapter 1, however study limitations and methodological shortcomings meant that a reliable difference was not observed in this data.

The ideal diagnostic biomarker for postoperative infection should share the characteristics of a screening test, as described in Wilson's criteria.(164)

1. The condition should be an important health problem
2. The natural history of the condition should be understood
3. There should be a recognisable latent or early symptomatic stage
4. There should be a test that is easy to perform and interpret, acceptable, accurate, reliable, sensitive and specific
5. There should be an accepted treatment recognised for the disease
6. Treatment should be more effective if started early
7. There should be a policy on who should be treated
8. Diagnosis and treatment should be cost-effective
9. Case-finding should be a continuous process

Postoperative infection is an important health problem as discussed in Chapter 1, leading to increased morbidity and mortality, increased healthcare cost and length of stay contributing to capacity burden, and increased empirical antimicrobial use with and impact on antimicrobial resistance. Empirical broad spectrum antimicrobial use is

.

common practice in all surgery, and any move toward judicious antimicrobial use would benefit the individual and wider society. The natural history of infection and sepsis is fairly well understood with initiation of the innate immune system and inflammatory response, however this is conflated with the tissue injury response to surgery. A localised response to infection precedes a systemic response leading to organ dysfunction indicated by raised SOFA scores. There is a window of opportunity to screen for and detect postoperative infection and initiate treatment or further investigation or discontinue empirical antimicrobials.

The ideal screening test should be easy to perform and interpret, acceptable, accurate, reliable, sensitive and specific. BDV has great potential as a mode to sample biomarkers as it is non-invasive which is more acceptable to patients than venepuncture which causes pain and a small injury, with limitless continuous sample available, and potential to deliver bedside processing and diagnostics with reduced consumables. The current infrared spectrometer used large, occupying approximately 1x2 metres of floor space, sensitive to surrounding conditions and can only be used for limited periods due to the laser overheating. Data generation goes through a process of fitting to generate a value, requiring the support of collaborators at RAL Spectroscopy group. These factors current model is not appropriate to implement in a clinical setting as a ‘plug and play’ technology, as has been successfully achieved with clinician operated arterial blood gas and thromboelastography machines, however with evidence of clinical utility there is scope to develop and downsize this prototype for bedside diagnostic use with a rapid fitting process and data generation, with limited consumables required adding to its cost-saving and environmental credentials.

While the experiment did not demonstrate diagnostic utility for BDV, likely due to methodological flaws discussed below, the strength of existing evidence and the potential for BDV to act as a biomarker for sepsis warrants further investigation.

.

8.2 Limitations of the study

The study was limited by the sample size, with 2 groups of 20 patients undergoing LT or HPB surgery. While the experimental intention was as a pilot experiment, a group size this small would be unlikely to generate statistically significant data, but no real difference was observed between these small groups. There was significant heterogeneity in participants' clinical condition, operation performed, and post operative management, which was not exclusive to whether they developed infections or not. Participants undergoing LT had a high rate of other organ failures, pre and postoperatively, and haemodynamic instability induced by surgery with significant blood loss. The study using a swine model by Butz et al reported that only swine with sepsis without haemodynamic instability demonstrated a change in BDV, while swine with haemodynamic instability – the most unwell septic subjects - demonstrated little change in BDV.(74) Haemodynamic instability from other causes including bleeding could confound BDV results, affecting many participants in the LT group who left the operating theatre on vasoactive support.

The rate of infection was low, with most patients having an uncomplicated recovery. Defining infective complications was difficult, with a spectrum from severe sepsis with multiorgan failure, to relatively minor infections which did not alter participant's physiological parameters. The differences within this group may confound the data in which patients are grouped. Similarly, there were participants who clinical changes such as tachycardia or hypotension in keeping with sepsis and infection, but which may be related to other factors, for example bleeding, other organ dysfunction, fluid imbalance. Other biomarkers such as WCC, CRP and SOFA scores had poor diagnostic accuracy and were not different between infected and non-infected groups, illustrating the challenge in diagnosing postoperative sepsis/infection. It may be interesting to use the overall impression of the responsible clinician for example with the daily screening question 'do you feel the participant has an infection?', to examine the diagnostic accuracy of clinicians' subjective assessment based

.

on clinical experience, which has been proven to perform as well as the best risk prediction models in general surgery.(165)

8.3 Methodological flaws

The sampling frequency limited the study for both breath and blood. For breath, samples were taken every day for ten days in the morning. A preoperative sample was used as a baseline intended to analyse the response to surgery, and the subsequent 9 days as during which postoperative infections develop. The sampling timeframe was a good duration, however the time between samples was long – 24 hours, alongside WCC CRP and SOFA scores. The potential for inflammatory response to an infective source could easily happen within this period, or other markers could 'catch up' before the period had passed. Other studies examining BDV in sepsis and infection had a higher frequency of sampling within a shorter time frame. In the swine model BDV samples were taken more frequently than blood, so the claim that BDV changes in response to infection before WCC, CRP and PCT may be misleading. More frequent sampling would be better however this was not within the constraints of the IMET study protocol, would significantly add to the sampling and processing workload, and may not be acceptable to participants.

For blood, WCC and CRP was taken daily for HPB participants and was a useful comparator. In the LT groups CRP was not measured daily, and so was not available as a daily comparator to BDV. CRP in the LT group was analysed additionally in the Viapath research lab at King's College Hospital

Analysis suggests that the many of the valves in the breath sampling bags leaked over time in the samples that underwent a long period of storage, as the variation in values out of range from expired CO_2 were not seen in the samples of controls taken 2-4 weeks before analysing them. While the BDV remains stable within a sealed sample, leaking allows

.

diffusion of CO_2 breath out of the sampling bag and diffusion of room air in, lowering the CO_2 concentration and equilibrating with the BDV within the room. The product information does not specify how long samples can be stored and when contacted the manufacturer was unable to specify how long the seal could be used. The Butz group used similar breath sampling bags, but were analysed within 48 hours of sampling.(75, 76) A review of modern breath sampling methodology by Lawal et al found similar methodologies in most studies including polymer bags, aluminium bags, Bio-VOC breath collecting apparatus and glass vials, and subsequently concentrated for analysis in thermal desorption tubes,(166) or in a ReCIVA device collecting breath directly into thermal desorption tubes.(167) Much research is in volatile organic compounds (VOCs) in breath, however the need to stabilise the sample for VOCs and quickly analyse before potential change in the composition of the sample, unlike the stable ^{12}C and ^{13}C isotope measured in BDV in expired CO_2. (168) Another device such as Bio-VOC may provide a longer-term seal to allow for the storage of samples which cannot be processed immediately

The IR spectrometer was based at the Rutherford Appleton Laboratory, Harwell Campus, 70 miles away from King's College Hospital where samples were collected. This created a logistical challenge as samples could not be delivered frequently (e.g. weekly) for measurement. Problems with a pilot run of samples in December 2020 showed an issue with the readings and fitting of the curve (generating non-physiological positive values) and the spectroscopy group asked for more time – eventually 12 months altogether – which meant that samples stayed in sampling bag storage far longer than initially intended. The IR spectroscopy instrument is large, sensitive to surroundings with only limited duration of laser operation, which would make it difficult to implement in its current form in a clinical setting.

The sample size recruited to the study was relatively small, intended as an exploratory pilot study but combined with heterogeneity in participants operations and recoveries it was underpowered to show significant results. Defining infection and sepsis in post-surgical

.

patients is one of the challenges this thesis set out to solve, however it meant that patients with a postoperative complication that looked like an infective process did not always meet diagnostic criteria to group them as such.

Data were analysed using several different methods, Mann Witney U test, 2-way ANOVA, univariate logistic regression and diagnostic accuracy tests – sensitivity, specificity, DOR and AUC. While diagnostic accuracy testing would be the gold standard for a screening test, the participant numbers and infection rates in these groups did not yield any significant results. The other tests were chosen to explore trends in biomarker level over time (2-way ANOVA) particularly for BDV looking for divergence between infected and non-infected groups, association with infection outcome (univariate logistic regression), and whether a preclinical change in a biomarker was predictive on an infective outcome (Mann Witney U). All of these data are presented and discussed. One could be accused of data dredging or p value hacking in testing the values in multiple ways, however these experiments aimed to examine BDV with very little previous clinical research or data, and so it was worth performing multiple analyses to fully explore the results and trends and applying the same principle to the rest of the markers examined.

8.4 Attempted but as yet unaddressed questions

This experiment examining BDV did not answer whether there is a relationship between BDV and postoperative sepsis or infection or whether BDV is a diagnostic test for postoperative sepsis or infection following HPB surgery or LT, during to methodological flaws.

.

8.5 Future research directions

BDV has promise as a diagnostic biomarker for postoperative sepsis or infection, as a non-invasive test with unlimited expiratory sample, acceptable to patients and participants with low use of consumables. The sampling storage and analysis methodology could be improved by earlier analysis e.g. within 48-hours, improved containers/bags with more work to explore and research that methodology, and access to on site IR spectroscopy.

The cohorts examined here are probably too complex with heterogenous diseases, operations and postoperative recoveries unrelated to sepsis to demonstrate difference at this early stage. Hypotension is shown to limit the change in a swine model which affects many patients following liver transplantation.(74) Further investigation could be in participants with more easily defined disease and outcomes, and more heterogenous operations e.g. sampling participants with cholecystitis, cholecystitis with sepsis, pancreatitis, and laparoscopic cholecystectomy to investigate a difference between groups before investigating as a diagnostic marker and proof of concept. There would be larger numbers of participants to recruit with fewer samples to analyse, and if a relationship with BDV and infection/sepsis proven then this group following major surgery could be re-examined.

8.6 Personal Reflection

Undertaking this Medicine Doctorate in Research was probably the biggest challenges of my career to date. I started my studies and clinical role in the Liver Transplant department at King's College at the height of the COVID-19 pandemic on the 1st of April 2020. The country had been in national 'lockdown' for one week, and all clinical roles had been suspended with redeployment to manage COVID-19 patients. With non-COVID-19 research suspended nationally and surgery restricted I could not immediately start my BDV project, which along with further COVID waves delayed my work by several months and limited vists to RAL to

.

analyse breath samples. I was redeployed to critical care and with King's participating in several studies including the RECOVERY trial, Genomicc study along with I-MET research into immunometabolic effects of COVID-19, I was delegated to these studies and recruited many patients admitted to hospital. I learnt quickly how to work within the research team to identify and screen potential participants, how to communicate about clinical research with equipoise, honesty, and uncover and alleviate fears about research participation. It was a swift induction into good medical practice, with several early results published showing clinical benefit for some interventions including Dexamethasone, and no treatment effect of others touted by thought leaders, celebrities and politicians in the media. I saw the benefit of high-quality, simple, innovative and collaborative multicentre research to deliver results which can have a significant impact on patient outcomes.

Undertaking this Medicine Doctorate has given me an understanding of how to design and conduct clinical research, how to perform experiments and conduct statistical analyses, the challenges of resource allocation and of recruitment momentum, and the barriers to conducting research in surgery. I have learnt that careful planning, testing techniques, an enquiring mind and patience and determination are key to achieving results. I hope to continue to engage in clinical academia in the rest of my career by recruiting my patients and site to clinical studies, encouraging the participation of future clinicians in academia, and hopefully developing future surgical trials.

Alongside my research I worked as a Senior Clinical Fellow in Liver Transplantation and Organ Retrieval. I participated in over 35 Adult and Paediatric Liver Transplants and retrieved organs for donation from over 80 donors. I developed a great deal as a surgeon and am grateful for the opportunity to have been a part of the gift of organ donation.

.

9 References

1. Sepsis N. Recognition, diagnosis and early management. London: National Institute for Health and Care Excellence (UK). 2016.

2. Houwink AP, Rijkenberg S, Bosman RJ, van der Voort PH. The association between lactate, mean arterial pressure, central venous oxygen saturation and peripheral temperature and mortality in severe sepsis: a retrospective cohort analysis. Crit Care. 2016;20:56.

3. ICNARC. Case Mix Programme Summary statistic 2018-2019. ICNARC: Intensive Care National Audit and Research Centre.

4. Coello R, Charlett A, Wilson J, Ward V, Pearson A, Borriello P. Adverse impact of surgical site infections in English hospitals. J Hosp Infect. 2005;60(2):93-103.

5. Plowman R, Graves N, Griffin MA, Roberts JA, Swan AV, Cookson B, et al. The rate and cost of hospital-acquired infections occurring in patients admitted to selected specialties of a district general hospital in England and the national burden imposed. J Hosp Infect. 2001;47(3):198-209.

6. General TCaA. The management and control of hospital acquired infection in acute NHS Trusts in England The Stationery Office. London: National Audit Office; 2000.

7. Service SSIS. Protocol for the Surveillance of Surgical Site Infection. Public Health Enlgand; 2013 June 2013.

8. Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, et al. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. Crit Care Med. 2014;42(8):1749-55.

9. Livermore DM. Fourteen years in resistance. Int J Antimicrob Agents. 2012;39(4):283-94.

10. England PH. Surveillance of surgical site infections in NHS hospitals in England April 2018 to March 2019 2019 [updated 2019. Available from:

.

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/854182/SSI_Annual_Report_2018_19.pdf.

11. Agency. HP. HCAI and Antimicrobial Point Prevalence Survey – England 2014 [Available from: Available from: www.hpa.org.uk/Topics/InfectiousDiseases/

InfectionsAZ/HCAI/HCAIPointPrevalenceSurvey/.

12. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). Jama. 2016;315(8):801-10.

13. England N. Improving outcomes for patients with sepsis: a cross-system action plan. 2015. Online information available at https://www england nhs uk/wpcontent/uploads/2015/08/Sepsis-Action-Plan-2312.15-v1.

14. Daniels R, Nutbeam T, McNamara G, Galvin C. The sepsis six and the severe sepsis resuscitation bundle: a prospective observational cohort study. Emerg Med J. 2011;28(6):507-12.

15. Health NIf, Excellence C. Sepsis: recognition, diagnosis and early management: National Institute for Health and Care Excellence (NICE); 2017.

16. Naffaa M, Makhoul BF, Tobia A, Kaplan M, Aronson D, Azzam ZS, et al. Procalcitonin and interleukin 6 for predicting blood culture positivity in sepsis. Am J Emerg Med. 2014;32(5):448-51.

17. Pillai S, Abbas AK, Lichtman AHH. Basic Immunology E-Book: Functions and Disorders of the Immune System: Elsevier; 2019.

18. Parslow TG, Stites DP, Terr AI. Medical Immunology: Lange Medical Books/McGraw-Hill Medical Publishing Division; 2001.

19. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340(6):448-54.

20. Albillos A, Lario M, Álvarez-Mon M. Cirrhosis-associated immune dysfunction: Distinctive features and clinical relevance. Journal of Hepatology. 2014;61(6):1385-96.

.

21. Antoniades CG, Berry PA, Wendon JA, Vergani D. The importance of immune dysfunction in determining outcome in acute liver failure. Journal of Hepatology. 2008;49(5):845-61.

22. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860-7.

23. Hiam-Galvez KJ, Allen BM, Spitzer MH. Systemic immunity in cancer. Nature Reviews Cancer. 2021;21(6):345-59.

24. Ballou SP, Lozanski G. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. Cytokine. 1992;4(5):361-8.

25. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. 1993.

26. Messias BA, Botelho RV, Saad SS, Mocchetti ER, Turke KC, Waisberg J. Serum C-reactive protein is a useful marker to exclude anastomotic leakage after colorectal surgery. Scientific Reports. 2020;10(1):1687.

27. Singh PP, Zeng IS, Srinivasa S, Lemanu DP, Connolly AB, Hill AG. Systematic review and meta-analysis of use of serum C-reactive protein levels to predict anastomotic leak after colorectal surgery. Br J Surg. 2014;101(4):339-46.

28. Patel A, Hassan S, Ullah A, Hamid T, Kirk H. Early triaging using the Modified Early Warning Score (MEWS) and dedicated emergency teams leads to improved clinical outcomes in acute emergencies. Clinical Medicine. 2015;15(Suppl 3):s3.

29. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ. 2009;339:b2535.

30. Boersema GSA, Wu Z, Menon AG, Kleinrensink GJ, Jeekel J, Lange JF. Systemic Inflammatory Cytokines Predict the Infectious Complications but Not Prolonged Postoperative Ileus after Colorectal Surgery. Mediators Inflamm. 2018;2018:7141342.

31. Grammatikopoulos T, Dhawan A, Bansal S, Wade J, Sherwood R, Dew T, et al. Baseline evaluation of serum markers of inflammation and their utility in clinical practice in paediatric liver transplant recipients. Clin Res Hepatol Gastroenterol. 2012;36(4):365-70.

.

32. Mokart D, Merlin M, Sannini A, Brun JP, Delpero JR, Houvenaeghel G, et al. zna. Br J Anaesth. 2005;94(6):767-73.

33. Zant R, Melter M, Knoppke B, Ameres M, Kunkel J. Kinetics of interleukin-6, procalcitonin, and C-reactive protein after pediatric liver transplantation. Transplant Proc. 2014;46(10):3507-10.

34. Stríz I, Trebichavský I. Calprotectin - a pleiotropic molecule in acute and chronic inflammation. Physiol Res. 2004;53(3):245-53.

35. Huang L, Li J, Han Y, Zhao S, Zheng Y, Sui F, et al. Serum Calprotectin Expression as a Diagnostic Marker for Sepsis in Postoperative Intensive Care Unit Patients. J Interferon Cytokine Res. 2016;36(10):607-16.

36. Meisner M, Tschaikowsky K, Palmaers T, Schmidt J. Comparison of procalcitonin (PCT) and C-reactive protein (CRP) plasma concentrations at different SOFA scores during the course of sepsis and MODS. Crit Care. 1999;3(1):45-50.

37. Balc IC, Sungurtekin H, Gürses E, Sungurtekin U, Kaptanoglu B. Usefulness of procalcitonin for diagnosis of sepsis in the intensive care unit. Crit Care. 2003;7(1):85-90.

38. Luzzani A, Polati E, Dorizzi R, Rungatscher A, Pavan R, Merlini A. Comparison of procalcitonin and C-reactive protein as markers of sepsis. Crit Care Med. 2003;31(6):1737-41.

39. Domínguez-Comesaña E, Estevez-Fernández SM, López-Gómez V, Ballinas-Miranda J, Domínguez-Fernández R. Procalcitonin and C-reactive protein as early markers of postoperative intra-abdominal infection in patients operated on colorectal cancer. International Journal of Colorectal Disease. 2017;32(12):1771-4.

40. Durila M BJ, Haruštiak T, Pazdro A, Pechová M, Cvachovec K. . Early diagnostic markers of sepsis after oesophagectomy (including thromboelastography). BMC Anesthesiol. 2012;12:12.

41. Facy O, Paquette B, Orry D, Binquet C, Masson D, Bouvier A, et al. Diagnostic Accuracy of Inflammatory Markers As Early Predictors of Infection After Elective Colorectal Surgery: Results From the IMACORS Study. Ann Surg. 2016;263(5):961-6.

.

42. Figiel W, Grąt, M., Niewiński, G. et al. Applicability of common inflammatory markers in diagnosing infections in early period after liver transplantation in intensive care setting. Sci Rep.10.

43. Ghiasvand F D-KS, Moazzami B, Ebrahimi A , Malekzadeh Z , et al. . Positive Role of Procalcitonin Level in the Diagnosis of Infectious Diseases After Liver Transplantation. Hepatitis Monthly. 2019;19(5):e85668.

44. Kaido T, Ogawa K, Fujimoto Y, Mori A, Hatano E, Okajima H, et al. Perioperative changes of procalcitonin levels in patients undergoing liver transplantation. Transpl Infect Dis. 2014;16(5):790-6.

45. Kuse ER, Langefeld I, Jaeger K, Külpmann WR. Procalcitonin-a new diagnostic tool in complications following liver transplantation. Intensive Care Med. 2000;26 Suppl 2:S187-92.

46. Muñoz JL, Ruiz-Tovar J, Miranda E, Berrio DL, Moya P, Gutiérrez M, et al. C-Reactive Protein and Procalcitonin as Early Markers of Septic Complications after Laparoscopic Sleeve Gastrectomy in Morbidly Obese Patients Within an Enhanced Recovery After Surgery Program. J Am Coll Surg. 2016;222(5):831-7.

47. Oberhofer D, Juras J, Pavicić AM, Rancić Zurić I, Rumenjak V. Comparison of C-reactive protein and procalcitonin as predictors of postoperative infectious complications after elective colorectal surgery. Croat Med J. 2012;53(6):612-9.

48. Saeed K, Dale AP, Leung E, Cusack T, Mohamed F, Lockyer G, et al. Procalcitonin levels predict infectious complications and response to treatment in patients undergoing cytoreductive surgery for peritoneal malignancy. Eur J Surg Oncol. 2016;42(2):234-43.

49. Takakura Y, Hinoi T, Egi H, Shimomura M, Adachi T, Saito Y, et al. Procalcitonin as a predictive marker for surgical site infection in elective colorectal cancer surgery. Langenbecks Arch Surg. 2013;398(6):833-9.

50. Takeuchi M, Yokose T, Kawakubo H, Matsuda S, Mayanagi S, Irino T, et al. The perioperative presepsin as an accurate diagnostic marker of postoperative infectious

.

complications after esophagectomy: a prospective cohort study. Esophagus. 2020;17(4):399-407.

51. Xiao H, Zhang P, Xiao Y, Xiao H, Ma M, Lin C, et al. Diagnostic accuracy of procalcitonin as an early predictor of infection after radical gastrectomy for gastric cancer: A prospective bicenter cohort study. Int J Surg. 2020;75:3-10.

52. Jukic T, Ihan A, Stubljar D. Dynamics of inflammation biomarkers C-reactive protein, leukocytes, neutrophils, and CD64 on neutrophils before and after major surgical procedures to recognize potential postoperative infection. Scand J Clin Lab Invest. 2015;75(6):500-7.

53. Lahiri R, Derwa Y, Bashir Z, Giles E, Torrance HD, Owen HC, et al. Systemic Inflammatory Response Syndrome After Major Abdominal Surgery Predicted by Early Upregulation of TLR4 and TLR5. Ann Surg. 2016;263(5):1028-37.

54. Holzmann MS, Winkler MS, Strunden MS, Izbicki JR, Schoen G, Greiwe G, et al. Syndecan-1 as a biomarker for sepsis survival after major abdominal surgery. Biomark Med. 2018;12(2):119-27.

55. Paugam-Burtz C, Albuquerque M, Baron G, Bert F, Voitot H, Delefosse D, et al. Plasma proteome to look for diagnostic biomarkers of early bacterial sepsis after liver transplantation: a preliminary study. Anesthesiology. 2010;112(4):926-35.

56. Xiao Z, Wilson C, Robertson HL, Roberts DJ, Ball CG, Jenne CN, et al. Inflammatory mediators in intra-abdominal sepsis or injury - a scoping review. Crit Care. 2015;19:373.

57. Uzzan B, Cohen R, Nicolas P, Cucherat M, Perret GY. Procalcitonin as a diagnostic test for sepsis in critically ill adults and after surgery or trauma: a systematic review and meta-analysis. Crit Care Med. 2006;34(7):1996-2003.

58. Abarca-Gómez L, Abdeen ZA, Hamid ZA, Abu-Rmeileh NM, Acosta-Cazares B, Acuin C, et al. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. The Lancet. 2017;390(10113):2627-42.

.

59. Kohn MJ. Carbon isotope compositions of terrestrial C3 plants as indicators of (paleo)ecology and (paleo)climate. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(46):19691-5.

60. Fry B, Sherr EB, editors. δ13C Measurements as Indicators of Carbon Flow in Marine and Freshwater Ecosystems. Stable Isotopes in Ecological Research; 1989 1989//; New York, NY: Springer New York.

61. Retallack GJ. Cenozoic Expansion of Grasslands and Climatic Cooling. The Journal of Geology. 2001;109(4):407-26.

62. O'Leary MH. Carbon Isotopes in Photosynthesis: Fractionation techniques may reveal new aspects of carbon dynamics in plants. BioScience. 1988;38(5):328-36.

63. Schoeninger M. Stable Isotope Analyses and the Evolution of Human Diets. Annual Review of Anthropology. 2014;43:413-30.

64. Watkins J, Blatt S, Bradbury C, Alanko G, Kohn M, Lytle M, et al. Determining the population affinity of an unprovenienced human skull for repatriation. Journal of Archaeological Science: Reports. 2017;12:384-94.

65. Savarino V, Vigneri S, Celle G. The ¹³C urea breath test in the diagnosis ofHelicobacter pylori infection. Gut. 1999;45(suppl 1):I18.

66. Keller J, Layer P, Brückel S, Jahr C, Rosien U. 13C-mixed triglyceride breath test for evaluation of pancreatic exocrine function in diabetes mellitus. Pancreas. 2014;43(6):842-8.

67. Kasicka-Jonderko A, Jonderko K. Phase-1 Evaluation of 13C-Liver Function Breath Tests. Digestive Diseases and Sciences. 2013;58(2):579-81.

68. Lara Baruque S, Razquin M, Jimenez I, Vazquez A, Gisbert JP, Pajares JM. 13C-phenylalanine and 13C-methacetin breath test to evaluate functional capacity of hepatocyte in chronic liver disease. Dig Liver Dis. 2000;32(3):226-32.

69. Kurhanewicz J, Vigneron DB, Ardenkjaer-Larsen JH, Bankson JA, Brindle K, Cunningham CH, et al. Hyperpolarized (13)C MRI: Path to Clinical Translation in Oncology. Neoplasia (New York, NY). 2019;21(1):1-16.

.

70. Woitek R, Gallagher FA. The use of hyperpolarised 13C-MRI in clinical body imaging to probe cancer metabolism. British Journal of Cancer. 2021;124(7):1187-98.

71. Butz DE, Cook ME, Eghbalnia HR, Assadi-Porter F, Porter WP. Changes in the natural abundance of (CO2)-C-13/(CO2)-C-12 in breath due to lipopolysacchride-induced acute phase response. Rapid Communications in Mass Spectrometry. 2009;23(23):3729-35.

72. Butz D, Casperson S, Whigham L. The emerging role of carbon isotope ratio determination in health research and medical diagnostics. Journal of Analytical Atomic Spectrometry. 2014.

73. Butz DE, Casperson SL, Whigham LD. The emerging role of carbon isotope ratio determination in health research and medical diagnostics. J Anal Atom Spectrom. 2014;29(4):594-605.

74. Bütz DE, Morello SL, Sand J, Holland GN, Cook ME. The expired breath carbon delta value is a marker for the onset of sepsis in a swine model. Journal of Analytical Atomic Spectrometry. 2014;29(4):606-13.

75. O'Rourke AP, Buckman SA, Evans DC, Kerwin AJ, Breunig EA, Bütz DE. Changes in exhaled 13CO2/12CO2 breath delta value as an early indicator of infection in intensive care unit patients. Journal of Trauma and Acute Care Surgery. 2019;86(1):71-8.

76. Boriosi J, Maki D, Yngsdal-Krenz R, Wald E, Porter W, Cook M, et al. Changes in breath carbon isotope composition as a potential biomarker of inflammatory acute phase response in mechanically ventilated pediatric patients. Journal of Analytical Atomic Spectrometry. 2014;29:599.

77. Eming SA, Krieg T, Davidson JM. Inflammation in Wound Repair: Molecular and Cellular Mechanisms. Journal of Investigative Dermatology. 2007;127(3):514-25.

78. Thompson SK, Chang EY, Jobe BA. Clinical review: Healing in gastrointestinal anastomoses, part I. Microsurgery. 2006;26(3):131-6.

79. WA TLC. 2022 [Available from: https://www.livercentrewa.com.au/treatments/pancreas-surgery-pancreatitis-specialist-perth.

.

80. Nguyen-Lefebvre AT, Horuzsko A. Kupffer Cell Metabolism and Function. Journal of enzymology and metabolism. 2015;1(1):101.

81. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 2010;10(11):753-66.

82. Li Y, Cao G, Zheng X, Wang J, Wei H, Tian Z, et al. CRACC-CRACC interaction between Kupffer and NK cells contributes to poly I:C/D-GalN induced hepatitis. PLoS One. 2013;8(9):e76681.

83. You Q, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. Hepatology. 2008;48(3):978-90.

84. Pangburn MK, Müller-Eberhard HJ. Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J Exp Med. 1980;152(4):1102-14.

85. Helmy KY, Katschke KJ, Jr., Gorgani NN, Kljavin NM, Elliott JM, Diehl L, et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. Cell. 2006;124(5):915-27.

86. Hinglais N, Kazatchkine MD, Mandet C, Appay MD, Bariety J. Human liver Kupffer cells express CR1, CR3, and CR4 complement receptor antigens. An immunohistochemical study. Lab Invest. 1989;61(5):509-14.

87. Frevert U, Engelmann S, Zougbédé S, Stange J, Ng B, Matuschewski K, et al. Intravital Observation of Plasmodium berghei Sporozoite Infection of the Liver. PLOS Biology. 2005;3(6):e192.

88. Breitenstein S, Apestegui C, Petrowsky H, Clavien PA. "State of the art" in liver resection and living donor liver transplantation: a worldwide survey of 100 liver centers. World J Surg. 2009;33(4):797-803.

89. Vauthey JN, Abdalla EK, Doherty DA, Gertsch P, Fenstermacher MJ, Loyer EM, et al. Body surface area and body weight predict total liver volume in Western adults. Liver Transpl. 2002;8(3):233-40.

.

90. Kishi Y, Abdalla EK, Chun YS, Zorzi D, Madoff DC, Wallace MJ, et al. Three hundred and one consecutive extended right hepatectomies: evaluation of outcome based on systematic liver volumetry. Ann Surg. 2009;250(4):540-8.

91. Kawasaki S, Makuuchi M, Matsunami H, Hashikura Y, Ikegami T, Chisuwa H, et al. Preoperative measurement of segmental liver volume of donors for living related liver transplantation. Hepatology. 1993;18(5):1115-20.

92. Makuuchi M, Hasegawa H, Yamazaki S. Ultrasonically guided subsegmentectomy. Surg Gynecol Obstet. 1985;161(4):346-50.

93. Makuuchi M. Surgical treatment for HCC – Special reference to anatomical resection. International Journal of Surgery. 2013;11:S47-S9.

94. Zhou Y, Xu D, Wu L, Li B. Meta-analysis of anatomic resection versus nonanatomic resection for hepatocellular carcinoma. Langenbecks Arch Surg. 2011;396(7):1109-17.

95. Cucchetti A, Cescon M, Ercolani G, Bigonzi E, Torzilli G, Pinna AD. A comprehensive meta-regression analysis on outcome of anatomic resection versus nonanatomic resection for hepatocellular carcinoma. Ann Surg Oncol. 2012;19(12):3697-705.

96. Hasegawa K, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, et al. Prognostic impact of anatomic resection for hepatocellular carcinoma. Ann Surg. 2005;242(2):252-9.

97. Yamashita Y, Taketomi A, Itoh S, Kitagawa D, Kayashima H, Harimoto N, et al. Longterm favorable results of limited hepatic resections for patients with hepatocellular carcinoma: 20 years of experience. J Am Coll Surg. 2007;205(1):19-26.

98. Regimbeau JM, Kianmanesh R, Farges O, Dondero F, Sauvanet A, Belghiti J. Extent of liver resection influences the outcome in patients with cirrhosis and small hepatocellular carcinoma. Surgery. 2002;131(3):311-7.

99. Ziparo V, Balducci G, Lucandri G, Mercantini P, Di Giacomo G, Fernandes E. Indications and results of resection for hepatocellular carcinoma. Eur J Surg Oncol. 2002;28(7):723-8.

.

100. Capussotti L, Muratore A, Amisano M, Polastri R, Bouzari H, Massucco P. Liver resection for hepatocellular carcinoma on cirrhosis: analysis of mortality, morbidity and survival--a European single center experience. Eur J Surg Oncol. 2005;31(9):986-93.

101. Kaibori M, Matsui Y, Hijikawa T, Uchida Y, Kwon AH, Kamiyama Y. Comparison of limited and anatomic hepatic resection for hepatocellular carcinoma with hepatitis C. Surgery. 2006;139(3):385-94.

102. Chen J, Huang K, Wu J, Zhu H, Shi Y, Wang Y, et al. Survival after anatomic resection versus nonanatomic resection for hepatocellular carcinoma: a meta-analysis. Dig Dis Sci. 2011;56(6):1626-33.

103. Zalewska K. Liver Transplantation: Selection Criteria and Recipient Registration. In: Transplant NBa, editor. 2018.

104. Ge PS, Runyon BA. Treatment of Patients with Cirrhosis. New England Journal of Medicine. 2016;375(8):767-77.

105. Schuppan D, Afdhal NH. Liver cirrhosis. Lancet (London, England). 2008;371(9615):838-51.

106. Bernal W, Wendon J. Acute Liver Failure. New England Journal of Medicine. 2013;369(26):2525-34.

107. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology. 2013;144(7):1426-37, 37.e1-9.

108. Arroyo V, Moreau R, Jalan R. Acute-on-Chronic Liver Failure. N Engl J Med. 2020;382(22):2137-45.

109. Yang Y-T, Jiang J-H, Yang H-J, Wu Z-j, Xiao Z-M, Xiang B-D. The lymphocyte-to-monocyte ratio is a superior predictor of overall survival compared to established biomarkers in HCC patients undergoing liver resection. Scientific Reports. 2018;8(1):2535.

110. Neofytou K, Smyth EC, Giakoustidis A, Khan AZ, Williams R, Cunningham D, et al. The Preoperative Lymphocyte-to-Monocyte Ratio is Prognostic of Clinical Outcomes for

.

Patients with Liver-Only Colorectal Metastases in the Neoadjuvant Setting. Annals of Surgical Oncology. 2015;22(13):4353-62.

111. Cummings M, Merone L, Keeble C, Burland L, Grzelinski M, Sutton K, et al. Preoperative neutrophil:lymphocyte and platelet:lymphocyte ratios predict endometrial cancer survival. British Journal of Cancer. 2015;113(2):311-20.

112. Mano Y, Shirabe K, Yamashita Y-i, Harimoto N, Tsujita E, Takeishi K, et al. Preoperative Neutrophil-to-Lymphocyte Ratio Is a Predictor of Survival After Hepatectomy for Hepatocellular Carcinoma: A Retrospective Analysis. Annals of Surgery. 2013;258(2):301-5.

113. Song M, Graubard BI, Rabkin CS, Engels EA. Neutrophil-to-lymphocyte ratio and mortality in the United States general population. Scientific Reports. 2021;11(1):464.

114. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101(6):1644-55.

115. Vincent J-L, Opal SM, Marshall JC, Tracey KJ. Sepsis definitions: time for change. Lancet (London, England). 2013;381(9868):774-5.

116. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control. 2008;36(5):309-32.

117. Horan TC, Gaynes RP, Martone WJ, Jarvis WR, Emori TG. CDC definitions of nosocomial surgical site infections, 1992: a modification of CDC definitions of surgical wound infections. Infect Control Hosp Epidemiol. 1992;13(10):606-8.

118. Bassi C, Marchegiani G, Dervenis C, Sarr M, Abu Hilal M, Adham M, et al. The 2016 update of the International Study Group (ISGPS) definition and grading of postoperative pancreatic fistula: 11 Years After. Surgery. 2017;161(3):584-91.

.

119. Koch M, Garden OJ, Padbury R, Rahbari NN, Adam R, Capussotti L, et al. Bile leakage after hepatobiliary and pancreatic surgery: a definition and grading of severity by the International Study Group of Liver Surgery. Surgery. 2011;149(5):680-8.

120. Rahbari NN, Garden OJ, Padbury R, Maddern G, Koch M, Hugh TJ, et al. Post-hepatectomy haemorrhage: a definition and grading by the International Study Group of Liver Surgery (ISGLS). HPB (Oxford). 2011;13(8):528-35.

121. Olthoff KM, Kulik L, Samstein B, Kaminski M, Abecassis M, Emond J, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transplantation. 2010;16(8):943-9.

122. Group KDIGOKAKIW. KDIGO Clinical Practice Guideline for Acute Kidney Injury. Kidney inter, Suppl. 2012;2(1-138).

123. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. Nephron Clinical Practice. 2012;120(4):c179-c84.

124. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004;240(2):205-13.

125. Clavien PA, Sanabria JR, Strasberg SM. Proposed classification of complications of surgery with examples of utility in cholecystectomy. Surgery. 1992;111(5):518-26.

126. Harbarth S, Holeckova K, Froidevaux C, Pittet D, Ricou B, Grau GE, et al. Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis. Am J Respir Crit Care Med. 2001;164(3):396-402.

127. Ma L, Zhang H, Yin Y-l, Guo W-z, Ma Y-q, Wang Y-b, et al. Role of interleukin-6 to differentiate sepsis from non-infectious systemic inflammatory response syndrome. Cytokine. 2016;88:126-35.

128. Abbas AK. Basic Immunology: Functions and Disorders of the Immune System. 6th edition.. ed. Lichtman AHH, Pillai S, editors2019.

.

129. Tsalik EL, Jaggers LB, Glickman SW, Langley RJ, van Velkinburgh JC, Park LP, et al. Discriminative value of inflammatory biomarkers for suspected sepsis. J Emerg Med. 2012;43(1):97-106.

130. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. Jama. 2000;283(15):2008-12.

131. Whiting P, Rutjes AW, Reitsma JB, Bossuyt PM, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. BMC Med Res Methodol. 2003;3:25.

132. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8):529-36.

133. Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. BMC medical research methodology. 2006;6(1):1-12.

134. Zamora J, Muriel A, Abraira V. Meta-DiSc statistical methods. 2006.

135. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med. 2002;21(11):1539-58.

136. Moses LE, Shapiro D, Littenberg B. Combining independent studies of a diagnostic test into a summary ROC curve: data-analytic approaches and some additional considerations. Stat Med. 1993;12(14):1293-316.

137. Deeks JJ, Macaskill P, Irwig L. The performance of tests of publication bias and other sample size effects in systematic reviews of diagnostic test accuracy was assessed. J Clin Epidemiol. 2005;58(9):882-93.

138. Robinson I, Butcher HL, Macleod NA, Weidmann D. Hollow waveguide integrated laser spectrometer for 13CO2/12CO2 analysis. Optics Express. 2019;27(24):35670-88.

139. Zhang J, Arbault S, Sojic N, Jiang D. Electrochemiluminescence Imaging for Bioanalysis. Annual Review of Analytical Chemistry. 2019;12(1):275-95.

.

140. Abeles RD, McPhail MJ, Sowter D, Antoniades CG, Vergis N, Vijay GK, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. Cytometry A. 2012;81(10):823-34.

141. Liaskou E, Zimmermann HW, Li KK, Oo YH, Suresh S, Stamataki Z, et al. Monocyte subsets in human liver disease show distinct phenotypic and functional characteristics. Hepatology. 2013;57(1):385-98.

142. Rockwood K, Song X, MacKnight C, Bergman H, Hogan DB, McDowell I, et al. A global clinical measure of fitness and frailty in elderly people. Canadian Medical Association Journal. 2005;173(5):489-95.

143. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. American journal of clinical oncology. 1982;5(6):649-56.

144. Jerome E, McPhail MJ, Menon K. Diagnostic accuracy of procalcitonin and interleukin-6 for postoperative infection in major gastrointestinal surgery: a systematic review and meta-analysis. The Annals of The Royal College of Surgeons of England. 2022;104(8):561-70.

145. Lee Y, McKechnie T, Doumouras AG, Handler C, Eskicioglu C, Gmora S, et al. Diagnostic Value of C-Reactive Protein Levels in Postoperative Infectious Complications After Bariatric Surgery: a Systematic Review and Meta-Analysis. Obes Surg. 2019;29(7):2022-9.

146. Straatman J, Harmsen AMK, Cuesta MA, Berkhof J, Jansma EP, van der Peet DL. Predictive Value of C-Reactive Protein for Major Complications after Major Abdominal Surgery: A Systematic Review and Pooled-Analysis. PLOS ONE. 2015;10(7):e0132995.

147. Welsch T, Muller SA, Ulrich A, Kischlat A, Hinz U, Kienle P, et al. C-reactive protein as early predictor for infectious postoperative complications in rectal surgery. Int J Colorectal Dis. 2007;22(12):1499-507.

.

148. Cousin F, Ortega-Deballon P, Bourredjem A, Doussot A, Giaccaglia V, Fournel I. Diagnostic Accuracy of Procalcitonin and C-reactive Protein for the Early Diagnosis of Intra-abdominal Infection After Elective Colorectal Surgery: A Meta-analysis. Ann Surg. 2016;264(2):252-6.

149. Su'a BU, Mikaere HL, Rahiri JL, Bissett IB, Hill AG. Systematic review of the role of biomarkers in diagnosing anastomotic leakage following colorectal surgery. Br J Surg. 2017;104(5):503-12.

150. Tan WJ, Ng WQ, Sultana R, de Souza NN, Chew MH, Foo FJ, et al. Systematic review and meta-analysis of the use of serum procalcitonin levels to predict intra-abdominal infections after colorectal surgery. International Journal of Colorectal Disease. 2018;33(2):171-80.

151. Chen J, Wang Y, Shen Z, Zhu Z, Song Y, Han R. Early diagnostic value of plasma PCT and BG assay for CRBSI after OLT. Transplant Proc. 2011;43(5):1777-9.

152. Jerome E, Cavazza A, Menon K, McPhail MJ. Systematic review and meta-analysis of the diagnostic accuracy of procalcitonin for post-operative sepsis/infection in liver transplantation. Transpl Immunol. 2022;74:101675.

153. Schuetz P, Beishuizen A, Broyles M, Ferrer R, Gavazzi G, Gluck EH, et al. Procalcitonin (PCT)-guided antibiotic stewardship: an international experts consensus on optimized clinical use. Clin Chem Lab Med. 2019;57(9):1308-18.

154. Eberhard O, Langefeld I, Kuse E, Brunkhorst F, Kliem V, Schlitt H, et al. Procalcitonin in the early phase after renal transplantation-will it add to diagnostic accuracy? Clinical transplantation. 1998;12(3):206-11.

155. Meisner M, Tschaikowsky K, Hutzler A, Schick C, Schmidt J, editors. Postoperative plasma concentrations of procalcitonin after different types of surgery. Critical Care; 1998: Springer.

156. Prieto B, Llorente E, González-Pinto I, Alvarez FV. Plasma procalcitonin measured by time-resolved amplified cryptate emission (TRACE) in liver transplant patients. A

.

prognosis marker of early infectious and non-infectious postoperative complications. Clin Chem Lab Med. 2008;46(5):660-6.

157. Yu XY, Wang Y, Zhong H, Dou QL, Song YL, Wen H. Diagnostic value of serum procalcitonin in solid organ transplant recipients: a systematic review and meta-analysis. Transplant Proc. 2014;46(1):26-32.

158. Molecular Vibration Wikipedia [Available from: https://en.wikipedia.org/wiki/Molecular_vibration.

159. Ohlsson KEA, Yang B, Ekblad A, Boman C, Nyström R, Olofsson T. Stable carbon isotope labelled carbon dioxide as tracer gas for air change rate measurement in a ventilated single zone. Building and Environment. 2017;115:173-81.

160. Ghasemifard H, Yuan Y, Luepke M, Schunk C, Chen J, Ries L, et al. Atmospheric CO2 and δ13C Measurements from 2012 to 2014 at the Environmental Research Station Schneefernerhaus, Germany: Technical Corrections, Temporal Variations and Trajectory Clustering. Aerosol and Air Quality Research. 2019;19(3):657-70.

161. Xu J, Lee X, Xiao W, Cao C, Liu S, Wen X, et al. Interpreting the 13C ⁄12C ratio of carbon dioxide in an urban airshed in the Yangtze River Delta, China. Atmos Chem Phys. 2017;17(5):3385-99.

162. Epstein S, Zeiri L. Oxygen and carbon isotopic compositions of gases respired by humans. Proc Natl Acad Sci U S A. 1988;85(6):1727-31.

163. Guillon S, Agrinier P, Pili É. Monitoring CO2 concentration and δ13C in an underground cavity using a commercial isotope ratio infrared spectrometer. Applied Physics B. 2015;119(1):165-75.

164. Wilson JMG, Jungner G, Organization WH. Principles and practice of screening for disease. 1968.

165. Wong DJN, Harris S, Sahni A, Bedford JR, Cortes L, Shawyer R, et al. Developing and validating subjective and objective risk-assessment measures for predicting mortality after major surgery: An international prospective cohort study. PLOS Medicine. 2020;17(10):e1003253.

.

166. Lawal O, Ahmed WM, Nijsen TME, Goodacre R, Fowler SJ. Exhaled breath analysis: a review of 'breath-taking' methods for off-line analysis. Metabolomics. 2017;13(10):110.

167. Belluomo I, Boshier PR, Myridakis A, Vadhwana B, Markar SR, Spanel P, et al. Selected ion flow tube mass spectrometry for targeted analysis of volatile organic compounds in human breath. Nature Protocols. 2021;16(7):3419-38.

168. Markar SR, Wiggins T, Antonowicz S, Chin S-T, Romano A, Nikolic K, et al. Assessment of a Noninvasive Exhaled Breath Test for the Diagnosis of Oesophagogastric Cancer. JAMA Oncology. 2018;4(7):970-6.

169. Esposito S, De Simone G, Boccia G, De Caro F, Pagliano P. Sepsis and septic shock: New definitions, new diagnostic and therapeutic approaches. J Glob Antimicrob Resist. 2017.

170. Zubert S, Funk DJ, Kumar A. Antibiotics in sepsis and septic shock: like everything else in life, timing is everything. Critical care medicine. 2010;38(4):1211-2.

171. Bhattacharjee P, Edelson DP, Churpek MM. Identifying Patients With Sepsis on the Hospital Wards. Chest. 2017;151(4):898-907.

172. Bernsmeier C, Triantafyllou E, Brenig R, Lebosse FJ, Singanayagam A, Patel VC, et al. CD14+CD15-HLA-DR- myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure. Gut. 2017.

173. Arroyo V, Moreau R, Jalan R, Gines P, Study E-CCC. Acute-on-chronic liver failure: A new syndrome that will re-classify cirrhosis. J Hepatol. 2015;62(1S):S131-S43.

174. Karvellas CJ, Pink F, McPhail M, Austin M, Auzinger G, Bernal W, et al. Bacteremia, acute physiology and chronic health evaluation II and modified end stage liver disease are independent predictors of mortality in critically ill nontransplanted patients with acute on chronic liver failure. Critical care medicine. 2010;38(1):121-6.

175. McPhail MJ, Shawcross DL, Abeles RD, Chang A, Patel V, Lee GH, et al. Increased Survival for Patients With Cirrhosis and Organ Failure in Liver Intensive Care and Validation of the Chronic Liver Failure-Sequential Organ Failure Scoring System. Clin Gastroenterol Hepatol. 2015;13(7):1353-60 e8.

.

176. Bajaj JS, O'Leary JG, Reddy KR, Wong F, Biggins SW, Patton H, et al. Survival in infection-related acute-on-chronic liver failure is defined by extrahepatic organ failures. Hepatology. 2014;60(1):250-6.

177. Carrara E, Pfeffer I, Zusman O, Leibovici L, Paul M. Determinants of inappropriate empirical antibiotic treatment: systematic review and meta-analysis. International journal of antimicrobial agents. 2018;51(4):548-53.

178. Jensen JU, Bouadma L. Why biomarkers failed in sepsis. Intensive Care Med. 2016;42(12):2049-51.

179. Molter G, Seifert H, Mandraka F, Kasper G, Weidmann B, Hornei B, et al. Outbreak of carbapenem-resistant Acinetobacter baumannii in the intensive care unit: a multi-level strategic management approach. Journal of Hospital Infection. 2016;92(2):194-8.

180. Bruns T, Reuken PA, Stengel S, Gerber L, Appenrodt B, Schade JH, et al. The prognostic significance of bacterial DNA in patients with decompensated cirrhosis and suspected infection. Liver Int. 2016;36(8):1133-42.

181. Volk HD, Reinke P, Krausch D, Zuckermann H, Asadullah K, Muller JM, et al. Monocyte deactivation--rationale for a new therapeutic strategy in sepsis. Intensive Care Med. 1996;22 Suppl 4:S474-81.

182. Kox WJ, Volk T, Kox SN, Volk HD. Immunomodulatory therapies in sepsis. Intensive Care Med. 2000;26 Suppl 1:S124-8.

183. Antoniades CG, Berry PA, Davies ET, Hussain M, Bernal W, Vergani D, et al. Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. Hepatology. 2006;44(1):34-43.

184. Wasmuth HE, Kunz D, Yagmur E, Timmer-Stranghoner A, Vidacek D, Siewert E, et al. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. J Hepatol. 2005;42(2):195-201.

185. Metges C, Kempe K, Schmidt HL. Dependence of the Carbon-Isotope Contents of Breath Carbon-Dioxide, Milk, Serum and Rumen Fermentation Products on the Delta-C-13 Value of Food in Dairy-Cows. Brit J Nutr. 1990;63(2):187-96.

.

186. Butz DE, Morello SL, Sand J, Holland GN, Cook ME. The expired breath carbon delta value is a marker for the onset of sepsis in a swine model. J Anal Atom Spectrom. 2014;29(4):606-13.

187. Augustin S, Pons M, Maurice JB, Bureau C, Stefanescu H, Ney M, et al. Expanding the Baveno VI criteria for the screening of varices in patients with compensated advanced chronic liver disease. Hepatology. 2017;66(6):1980-8.

188. Rajajee V, Williamson CA, Fontana RJ, Courey AJ, Patil PG. Noninvasive Intracranial Pressure Assessment in Acute Liver Failure. Neurocrit Care. 2018;29(2):280-90.

189. Feng S, Goodrich NP, Bragg-Gresham JL, Dykstra DM, Punch JD, DebRoy MA, et al. Characteristics associated with liver graft failure: the concept of a donor risk index. Am J Transplant. 2006;6(4):783-90.

.

10 Appendix 1

Systematic Review Searches

EMBASE - ((((biomarker*).ti,ab OR exp "BIOLOGICAL MARKER"/) AND ((surgery).ti,ab OR exp "ABDOMINAL SURGERY"/ OR exp "CARDIOVASCULAR SURGERY"/ OR exp "THORAX SURGERY"/ OR exp "LIVER SURGERY"/ OR exp "LIVER TRANSPLANTATION"/ OR exp "LIVER RESECTION"/)) AND ((infect*).ti,ab OR exp INFECTION/ OR (sepsis).ti,ab OR exp SEPSIS/)) [DT 1996-2020] [English language] [Languages English] [Human age groups Child unspecified age OR Preschool Child 1 to 6 years OR School Child 7 to 12 years OR Adolescent 13 to 17 years OR Adult 18 to 64 years OR Aged 65+ years] [Humans]

Medline - ((((biomarker*).ti,ab OR exp BIOMARKERS/) AND ((infect*).ti,ab OR exp INFECTIONS/ OR (sepsis).ti,ab OR exp SEPSIS/)) AND ((surgery).ti,ab OR exp "GENERAL SURGERY"/ OR exp "THORACIC SURGICAL PROCEDURES"/ OR exp "COLORECTAL SURGERY"/ OR (transplant*).ti,ab OR exp "LIVER TRANSPLANTATION"/)) [DT 1996-2020] [Human age groups Child,preschool OR Child OR Adolescent OR Young adult OR Adult OR Middle Aged OR Aged OR Aged,80 and over] [Languages English] [Humans]

11 Appendix 2

IMET Study Protocol

FULL TITLE OF STUDY: IMMUNO-METABOLISM IN SEPSIS, INFLAMMATION AND LIVER FAILURE SYNDROMES

SHORT TITLE: I-MET

PROTOCOL: Version 3, dated 14 August 2020

RESEARCH REFERENCE NUMBERS:

Research Ethics Committee No.:	19/NW/0750
IRAS No.:	244089

LIST of CONTENTS

GENERAL INFORMATION	Page No.
TITLE PAGE	1
RESEARCH REFERENCE NUMBERS	1
SIGNATURE PAGE	2
LIST OF CONTENTS	3
KEY STUDY CONTACTS	4
STUDY SUMMARY	4
FUNDING	5
ROLE OF SPONSOR AND FUNDER	5
KEY WORDS	5
SECTION	
1. BACKGROUND	6
2. RATIONALE	8
3. RESEARCH QUESTION/AIM(S)	9
4. STUDY SETTING	10
5. SAMPLE AND RECRUITMENT	11
6. ETHICAL AND REGULATORY COMPLIANCE	18
7. REFERENCES	19
8. DISSEMINATION POLICY	19
9. TIMETABLE	19
10. APPENDICES	20

KEY STUDY CONTACTS

Chief Investigator	Dr Mark McPhail mark.mcphail@kcl.ac.uk; 0203 299 9000
Study coordinator	John Smith; john.smith27@nhs.net; 0203 299 9000
Sponsor	Professor Reza Razavi Director of Research Management and Innovation, King's College London, Room 5.23, James Clerk Maxwell Building, Waterloo Campus, 57 Waterloo Road, London SE1 8WA
Funder(s)	Science and Technology Facilities Council Medical Research Council
Key protocol contributors	Mr John Smith, Dr Grace Hatton, Dr Sinead Helyar

STUDY SUMMARY

Study Title	Immuno-metabolism in sepsis, inflammation and liver failure syndromes
Internal ref. no. (or short title)	I-MET
Study Design	Cohort observation; basic science study involving procedures with human participants
Study Participants	(1) Patients with acute hepatic failure and chronic liver disease (2) Patients with sepsis or suspected sepsis (3) Healthy subjects (control) (4) Patients with sterile inflammation (control)
Planned Size of Sample (if applicable)	250
Follow up duration (if applicable)	90 days
Planned Study Period	01/07/2019 to 01/07/2021
Research Question/Aim(s)	To investigate the cross-talk of immunity and metabolism in patients with sterile inflammation, sepsis and liver disease by sequential observation during acute hospital admissions. To develop new biomarkers of sepsis including the use of carbon isotope breath measurement

FUNDING AND SUPPORT IN KIND

FUNDER(S)	FINANCIAL AND NON-FINANCIAL SUPPORT GIVEN
Science and Technology Facilities Council Healthcare Challenge	Financial support for staff and consumable expenditure
MRC Confidence in Concept Award	Financial support for staff and consumable expenditure

ROLE OF STUDY SPONSOR AND FUNDER

The study sponsor is King's College London, who take legal responsibility over the overall setup and management of the study. The study design, conduct, data analysis, interpretation, manuscript writing and dissemination of results are the responsibility of the chief investigator, principal investigators, sub-investigators and researchers within the study group. The funder(s) have no role in these aspects of the study.

KEY WORDS: sepsis, inflammation, biomarkers, breath, cirrhosis, acute liver failure, acute-on-chronic liver failure; metabolism

STUDY PROTOCOL

Immuno-metabolism in sepsis, inflammation and liver failure syndromes

(1) BACKGROUND

(i) Lay Summary

Patients with critical illness are susceptible to infections leading to high mortality rates. When the immune response does not function appropriately, this can lead to severe infection (sepsis). Sepsis can be identified clinically through observations such as change to heart rate, blood pressure, respiratory rate and bodily temperature.

Poorly functioning, circulating white blood cells called monocytes are seen in sepsis. Monocytes normally display a molecule known as human leucocyte antigen (HLA)-DR on their surfaces, but in sepsis, HLA-DR expression is reduced or lost, in addition to other changes in their surface markers. This can mean that the ability of white blood cells to signal one another and defend against infection is impaired. A well-known cause of this HLA-DR dysfunction is liver disease, and here there is a high risk of sepsis from bacterial translocation and immune dysfunction. Abnormal metabolic responses can increase the risk of death, but the link between metabolism and immunity is currently poorly understood. Bodily organs can also become inflamed without the presence of an infection, but which can still lead to similar problems in terms of low blood pressure or confusion as seen in septic infection. It is important to know whether this inflammation is due to sepsis (and needs

appropriate treatment with antibiotics) or is not associated with infection. New blood or breath markers which measure the number of bacteria or how they metabolise energy could mean we can identify and treat true sepsis much earlier than previously possible, and also ensure that we only give antibiotics to patients who need them. This would prevent complications such as bacterial resistance developing through avoiding unnecessary treatment.

(ii) Background

Sepsis is one of the most significant causes of premature death in the world(169) and is implicated in 750,000 deaths annually at a cost of $20billion in the USA alone. NHS data shows that in the UK there are 120,000 critical care unit admissions and 44,000 deaths per annum attributed to sepsis, and 14,000 of those are thought to be preventable with improved diagnosis and reduced treatment delays.

Microbial infection induces a variable host immune response, and when this response is dysregulated and associated with organ dysfunction, is called sepsis.(12) Failure of multiple other organs can ensue, with increased mortality linked to the number of organs in failure; organism; severity of the immune response; and, crucially, the time to diagnose sepsis and start treatment.(170) Diagnostic and therapeutic delay increase the risk of death, and national strategies are in place to improve the time to recognise sepsis and start

antimicrobials. Despite this, up to half of deaths in hospitalised patients can be attributed to sepsis.(171)

Certain patient groups are also at higher risk: For example, patients with liver cirrhosis are prone to infection from immune dysfunction(172), and up to a third of acute hospitalisation episodes due to acute decompensation (AD) or acute-on-chronic liver failure (ACLF)(173, 174) are sepsis-related(175, 176). Conversely, the failure to recognise when systemic responses are not caused by pathogens and do not require antibiotics leads to excess prescribing and the risk of avoidable drug-induced complications; selection of resistant organisms; and excess cost and length of hospital stay(177).

The diagnosis of sepsis is often clinically based on typical history, organ system findings and systemic responses such as fever, tachycardia, tachypnoea and hypotension. When systemic responses are blunted (such as in liver disease or pre-existing critical illness) or temperature homeostasis disrupted (neurological failure), the risk of false positive or false negative diagnosis rises. Highly accurate biomarkers would help address this clinical difficulty, though many plasma-based methodologies have been unsuccessfully proposed(178). These have variable diagnostic performance and are often confounded by sterile inflammatory processes which do not require treatment with antimicrobials.(179) Whole blood bacterial DNA analysis has been proposed but requires translocation of the organism into the bloodstream in sufficient quantities for analysis. While patients with advanced liver disease have higher rates of such translocation, it does not necessarily indicate sepsis.(180)

A reduction on the monocyte white blood cell surface expression of the human leucocyte antigen (HLA) –DR molecule is the hallmark of this anti-inflammatory state. This abnormal monocyte phenotype (that is, the molecules that are displayed on the cell surface) as well as abnormal monocyte function have been described in severe liver disease and critical illness.(181-184). It is not known whether abnormal monocyte phenotype and function is the result of ongoing localised pro-inflammatory process 'spilling over' into the periphery with ensuing compensatory down-regulation, or whether global pro- and anti-inflammatory processes drive these. This is of importance, as pro-inflammatory immune modulating agents have been suggested for patients with monocyte de-activation. However, if the primary problem driving this phenomenon is localised hyper-inflammation, augmenting the immune system and the acute phase response further would not only be counteractive to any treatment interventions, but ultimately would contribute to enhanced morbidity and mortality rates.

Isotope ratio methods in exhaled breath have recently been investigated as a novel metabolic biomarker of the APR to infection. 99% of carbon in the universe is in the form of 12C with 1% present in the isotope 13C which has one neutron more than 12C. Carbon is a ubiquitous atom in biological organisms, and this isotopic ratio is modulated in the long term by diet(185) and in the shorter term by metabolic responses, where the mass of carbon is relevant to chemical reactions. Sepsis mobilises amino acids (AA) for cytokine signalling and bioenergetics homeostasis. Lighter AA are more likely to be metabolised to CO2 and hence the exhaled 13CO2 /12CO2 ratio is altered.(71) Infrared molecular spectroscopy is a well-

established method for detecting trace quantities of gases.(73) Each gas has a characteristic frequency and spectroscopic characteristic. The 13CO2 /12CO2 breath delta value (BDV) is calculated by scanning the absorption frequency of CO2 by infrared spectroscopy and comparing to a standard reference material (Pee Dee Belemnite (PDB) where the isotopic ratio is known), as below.

$$\delta = \frac{^{13}CO_2/^{12}CO_2(\text{sample}) - ^{13}CO_2/^{12}CO_2(\text{PDB})}{^{13}CO_2/^{12}CO_2(\text{PDB})} \times 1000$$

Based on this approach, Laser Isotope Ratio-meters (LIR) can determine the BDV quickly (<10s), with a precision <0.5‰. Pilot data in murine and porcine models of sepsis have demonstrated that BDV can diagnose the onset of sepsis within 2-4 hours compared to more than 10 hours for physiological parameters(71, 186). In ongoing studies in the USA in patients admitted for major trauma, BDV was specific for the onset of late sepsis (unpublished data). This has not been tested in patients hospitalised for sepsis, or in those with a high risk of sepsis (eg liver disease) or where inflammatory processes are sterile (eg post trauma or major surgery).

Sepsis and liver failure syndromes often lead to a multi-organ failure picture. It is already known that liver inflammation can affect its stiffness, but little is known about other abdominal organs failing during critical illness.

Elastography is an ultrasound based non-invasive tool able to estimate the stiffness of several organs (liver, spleen, kidneys).

In cirrhotic patients it is widely used and is predicting of variceal haemorrhage and decompensation, (187) but potentials of this technique in acute setting (ie. acute liver failure syndromes or sepsis) needs to be further explored.

Ultrasound can also be used in estimating intracranial pressure and brain failure non-invasively, monitoring the transcranial doppler signal of the intracranial arteries(188).

(2) RATIONALE

This study proposes to identify at the cellular and molecular level, the changes that occur in the phenotype and function of circulating immune cells in patients with liver failure and sepsis and compares this to the immune-metabolic signatures in health and sterile inflammation.

By studying this, we hope to be able to identify immunotherapeutic targets and understand whether potential immunotherapy could be applied locally or systemically. Our observations in this study could provide the basis for future development of immune-modulating agents, which may reduce susceptibility to infection and could reduce mortality in critically ill patients with sepsis.

(3) OBJECTIVES

This project aims to concurrently characterise the phenotype and function of peripheral immune cells and systemic metabolism (measured by metabonomics and breath carbon dioxide ratio).

This research is designed to answer whether immune changes commonly seen are due to sepsis or sterile inflammation and whether breath can be used to discriminate early sepsis from sterile inflammation.

In addition, this research will investigate whether any associations exist between the appearance and function of innate immune cells, inflammation, metabolism, imaging and clinically relevant outcomes such as survival or length of hospital stay. This will help design new therapies in order to use metabolic pathways to improve immune function in a physiological manner.

As part of the response to the COVID 19 pandemic, this study has been amended to collect data of relevance to that condition. Patients with COVID-19 pneumonia fit the definition of sepsis and so are within the group of patients of interest for this study. However certain COVID specific aspects warrant further exploration.

A specific area of concern is the high number of patients who develop cardiovascular and renal failure. The collection of urine samples from COVID patients with critical illness and additional measurements of proteins and biomarkers relevant to cardiac and renal dysfunction will better stratify the immunometabolic progression to multiple organ failure and hence provide valuable insight into which patients are likely to require early intensive

monitoring or specific therapies or placement (e.g., in intensive care units which can provide renal replacement therapy).

(4) STUDY SETTING

In order to investigate the phenotypic and functional changes in immune cells and metabolism in liver disease, participants with acute or chronic liver diseases across the spectrum of severity will be recruited from appropriate hospital settings: participants with stable liver disease from outpatient clinics; inpatients with acutely decompensated chronic liver disease from general or liver-specific wards; critically ill patients with acute liver failure or acute-on-chronic liver failure in intensive care units. To investigate immune-metabolism in patients with severe inflammatory responses with or without sepsis we will recruit from the emergency department and general critical care units. Abdominal organs (liver, spleen, kidney) stiffness will be assessed non-invasively by ultrasound (elastography) during the hospital admission, together with the splanchnic vasculature and/or transcranial doppler to provide macroscopic evidence of inflammation and organ failure.This is a single centre study at King's Health Partners (Kings College Hospital and Kings College London). Patients will be recruited from the liver, liver intensive care and general critical care units of King's College Hospital. Medical laboratory work will be performed at the Institute of Liver Studies and the James Black Centre, King's College London. The Liver Unit and Liver Intensive Care Unit at King's College London has a world class reputation for the treatment of liver failure syndromes and has the highest throughput of cases in Europe providing an ideal environment where translational therapies can be introduced to clinical care. They are world leaders in the characterisation of immune and metabolic responses during acute liver illnesses.

During the lifetime of the project the new Kings Critical Care Centre will open offering the largest critical care centre in the UK which will act as an important area for recruitment.

The BDV analysis from the sample bags will be carried out at the Laser Spectroscopy Laboratory of the Space Science and Technology department (aka RAL Space) of the Rutherford Appleton Laboratory (RAL).

(5) RECRUITMENT AND SAMPLE COLLECTION

5.1 Eligibility Criteria

5.1.1 Inclusion criteria:

Main disease group inclusion criteria for analysis of blood, urine and exhaled breath

(1) Patients with sepsis or suspected sepsis

(2) Patients with acute hepatic failure or chronic liver disease

Main disease group inclusion criteria for analysis of blood and urine (where the viral content of exhaled breath may be pathological)

(1) Patients with confirmed or suspected COVID 19 infection requiring admission to hospital or critical care

Control Group Inclusion Criteria:

Healthy subjects

Patients with inflammation who are at risk of sepsis

Post major trauma or

Post major elective surgery

5.1.3 Exclusion criteria:

Age <16

Evidence of disseminated malignancy (isolated hepatocellular carcinoma without evidence of secondary spread is not an exclusion criteria)

Pre-existing immunosuppressive states including HIV infection and chronic granulomatous diseases.

Immunosuppression other than low dose steroids (defined as >40mg prednisolone or equivalent)

Pregnancy

Definitions

Sepsis

life-threatening organ dysfunction caused by a dysregulated host response to infection.

Organ dysfunction

an increase in the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score of 2 points or more (assume a baseline of 0 if no baseline available), OR

qSOFA>1 of respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure of 100 mm Hg or less (SEPSIS 3 and quick SOFA).

Liver disease

Acute liver failure – coagulopathy (INR>1.5), jaundice (bilirubin >30umol/l) and any hepatic encephalopathy with previously normal liver function

Chronic liver disease – cirrhosis by clinical, biochemical, radiological or histological criteria, subdivided into

Stable cirrhosis (SC)

Acute decompensation (AD)

Acute on chronic liver failure (ACLF)

Major trauma

Traumatic injury with an injury severity score of >8

Major elective surgery

Laparotomy/Laparoscopic-assisted surgery requiring post-operative admission to the critical care unit

5.2 Consent

Potential subjects will be identified by their clinical team responsible for their care and approached to see whether they would be interested in participating in this research project. If agreeable, they will be given the 'patient information sheet' to read. After an appropriate time period and the opportunity to ask any questions, written consent will be taken with a member of the research team. Ambulant patients who are subsequently re-admitted for liver transplantation will be re-sampled on days 1, 3, 7 and 10 during their admission using the

original consent - but will have a further discussion with an investigator at admission and the opportunity to withdraw their consent to further sampling. Patients awaiting surgery may be contacted to share study information in advance of admission by telephone, post, email or part of their pre-operative assessment/education.

Patients with liver failure, critical illness or sepsis, may have impaired consciousness due to the evolution of their critical illness or encephalopathy, and require sedation and intubation. The objective of this project focuses on the immunological dysfunction of these critically unwell patients. In addition, a large number of patients will be intubated and ventilated at a referring hospital, making it difficult to consent participants agreeing to take part in the study. In these situations, where the potential subject is unable to consent, an appropriate consultee will be sought. The consultee will most often be a close personal contact of the potential participant e.g., the patient's next of kin, a family member, carer or friend. This may also be a clinician caring for the patient but not involved in the study. They will be suitable to act as the consultee by the virtue of their relationship, availability and willingness to do so. In the process of considering inclusion into the study, the patient's wishes and feelings will be assessed, and written information will be provided in the form of the Consultee Information Sheet. After an appropriate time period and the opportunity to ask any questions, the consultee will sign the 'consultee declaration form'. If the patient recovers consent then we will approach them to confirm they wish their data and samples to be kept and ask them to sign the patient consent form.

5.3 Specimen collection

All patients will have sampling of blood, breath and urine on days 1, 3, 7 and 10 following acute admission to King's College Hospital, and at a single visit for healthy controls and ambulant patients in outpatient clinics or who may be discharged from hospital. Patients admitted for surgery will be sampled pre-operatively, on day 1 post-operatively, and days 7 and 10 during their admission. Ambulant patients who are subsequently re-admitted for liver transplantation will be re-sampled on days 1, 3, 7 and 10 during their admission. Patients will be subsequently followed for 90 days remotely. Patients will also have urine collected on these days, and more frequently (up to 12 hourly) for first 48 hours after admission (if practically possible) in order to assess renal biomarkers/inflammation at early stages of acute kidney injury. As breath is more straightforward to sample and can detect changes at an earlier time than blood we will sample breath on each (working) day.

Clinical parameters Demographics, full blood count, C-reactive protein, international normalized ratio, liver and renal function tests, lactate, ammonia, hospital mortality and infection status and clinical variables will be collected prospectively. The following disease severity scores will be calculated: Acute Physiology and Chronic Health Evaluation II (APACHE II), SOFA, and qSOFA (Quick SOFA) scores and SEPSIS-3 criteria(12). In liver patients, additional scores will be determined Child–Pugh, model of end-stage liver disease (MELD), and CLIF-SOFA.(173) In patients who undergo surgery, details of their operation will be recorded, including anaesthetic assessment (ASA (American Society of

Anaesthesiologists) score, performance status), anaesthetic details (medication used, blood products given), operative details (time, intra-operative findings, procedure, blood loss, intra-operative/post-operative complications. In patients undergoing Liver Transplantation, data on the donor organ will be collected, including donor age, donor infection, mode of donation (donation after Brain death/donation after Cardiac death), Donor Risk Index(189), Liver cold ischaemic time).

Blood sampling

A small sample of blood (up to 45mls or 9 teaspoons) will be taken for research purposes within 48 hours of admission to hospital or critical care. The amount of blood taken will not harm the patient in any way. If the patient is in the intensive care, the blood will be taken from tubes already within the blood vessels (intravenous access) and no venepuncture with a needle will be necessary. If the patient is on the ward and does not have intravenous access, a blood sample will be taken by venepuncture. There may be some minor discomfort during the blood sampling as well as a risk of some bruising. To follow the immune system profile during the course of the hospital admission, blood sampling may occur sequentially with the patient's consent on days 1, 3, 7 and 10 after admission. Where possible, this will be taken at the same time as the patient's diagnostic blood tests ordered by the clinical team to avoid repeated venepuncture.

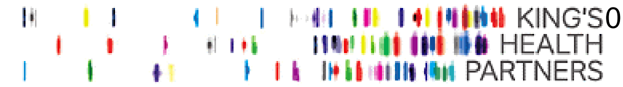
White blood cells will be separated by density gradient centrifugation and either used immediately or stored at -800C. The fluid supernatant (plasma and serum) will also be

collected and stored for further analysis of inflammatory chemicals (cytokines), or other important molecules involved in infection (such as lipids or lipopolysaccharide). Whole blood will be immediately stored at -800C for later quantification of bacterial DNA.

Urine Sampling

10 ml of urine will be drawn from existing urinary catheters within 48 h of admission to hospital or critical care and sequentially, or self-collection in ambulant patients without urinary catheter. Urine samples will be stored at -800C. Renal biomarker assays will subsequently be conducted on these urine samples. This will be by using the Nephrocheck assay. It is a CE marked device and is approved by the FDA in conjunction with clinical evaluation in patients who currently have, or have had within the past 24 hours, acute cardiovascular and / or respiratory compromise and are ICU patients, as an aid in the risk assessment for moderate or severe acute kidney injury (AKI) within 12 hours of patient assessment. This will be of particular importance during the COVID19 pandemic as progression to severe AKI requiring renal replacement therapy is required to delineate where scare renal replacement therapy (RRT) equipment can be deployed and or where patients can be further placed (e.g., in non-hospital derogated sites that do not provide RRT). In the non-pandemic setting these markers will remain of interest to determine which patients with sepsis experience multiorgan failure.

Trial procedures	Pre-screening	Screening	Baseline (Day 1)	Day 2	Day 3 (+/-2)	Day 4, 5 and 6	Day 7 (+/-2)	Day 8, 9	Day 10 (+/-2)	Day 90
Informed consent		X								
Eligibility criteria	X	X								
Demographics*			X							
Medical History*			X							
Targeted physical exam*			X							
Vital signs (BP/HR)*			X	X	X	X	X	X	X	
FBC, INR, Liver, Renal and Bone profile*			X	X	X	X	X	X	X	
Physiological measurements			X	X	X	X	X	X	X	



Prognostic scores* ɣ			X		X		X		X	
Blood sampling			X		X		X		X	
Breath sampling			X	X	X	X	X	X	X	
Urine sampling			X	X	X		X		X	
Sepsis status (Y:N)			X	X	X	X	X	X	X	X
Microbiological results			X		X		X		X	
Ultrasound Imaging			X	X	X	X	X	X	X	
Conmeds*	X	X	X		X		X		X	
Adverse Events			X	X	X	X	X	X	X	X
Mortality										X

Table 2: Schedule of activity. * Standard of care ɣ SOFA, CLIF SOFA, APACHE II, qSOFA scores will be calculated. Ambulant patients sampled once (e.g. patients with stable cirrhosis) who are re-admitted for surgery or due to clinical deterioration will be resampled from baseline day 1 at acute admission.

Breath Testing

Exhaled breath will be captured in five-layer gas sampling bags, discarding the first few hundred millilitres that are diluted in the airways. The samples will then undergo analysis by laser isotope ratio meter measurement of 13CO2 to 12CO2 ratio as a measure of the underlying inflammatory response or sepsis. These bags will be stored at KCH prior to sending to Rutherford Appleton Laboratory, Didcot, Oxfordshire for analysis. As breath is more straightforward to sample and can detect changes at an earlier time than blood we will sample breath on each (working) day.

Imaging

Ultrasound scan will be done during the hospital admission. Splanchnic vasculature flows, solid organs stiffness (measured by elastography) and transcranial doppler will be done regularly during the study period (up to daily for patients in intensive care with rapid change of clinical conditions).

5.4 Laboratory processing

Sample storage

Samples will be stored in the laboratories located in:

the Institute of Liver Studies, KCH

the James Black Centre, KCL

Samples are stored in a pseudo anonymised fashion identified by a code number. The primary researcher will hold the key as participants will need to be identified to link the experimental findings with clinical and physiological data. Breath samples will be transferred to Rutherford Appleton Laboratory, Didcot, Oxfordshire for analysis.

5.5 Experimental techniques

A) Flow Cytometry analysis (FACS): Fluorochrome linked anti-human antibodies will be used for cell surface and intra-cellular molecules and quantification of phagocytosis on whole EDTA blood prior to lysis, as well as separated peripheral blood mononuclear cells (PBMCs) and selected monocytes. Results will be expressed as the number or percentage of a particular cell population expressing that molecule, or the density of that molecule's expression (mean fluorescence intensity).

B) Enzyme-Linked Immunosorbent Assay (ELISA): For analysis of cytokine concentrations in plasma/sera/supernatants from clinical samples.

C) Enzyme-linked immunosorbent spot assay (ELISPOT): Analysis of cytokine production by monocytes/macrophages as well as T-cell stimulation by monocytes/macrophages.

D) Bacterial DNA analysis: Total, 16S and metagenomics analysis of bacterial DNA in blood and/or ascites (in patients with liver disease)

E) Laser isotope ratio meter measurement: Exhaled breath analysis of 13CO2 to 12CO2 ratio as a measure of the underlying inflammatory response or sepsis.

G) Metabonomics of blood plasma using 1H NMR or ultra-performance liquid chromatography mass spectrometry

H) Gene transcriptomic profiling: Evaluation of the protein (messenger or micro RNA) expression of genes in immune cells and plasma to understand the relationship of gene products on function.

Proteomics: Proteins relevant to progression in critical illness relevant to sepsis and cardiovascular compromise (such as ACE) will be measured by untargeted and targeted proteomic analysis

J) NephroCheck™ is a simple point of care test that quantifies cell cycle arrest biomarkers (Tissue Inhibitor of MetalloProteinases-2 (TIMP-2) and Insulin-like Growth Factor Binding Protein-7 (IGFBP-7)) These biomarkers indicate renal stress, before damage or dysfunction has occurred, and have a high predictive performance for kidney injury in many clinical settings, including critical care

As people can vary in the sub-types of immune molecules they produce, we may want to investigate a variety of important molecules for immune function. These include subtypes of HLA-DR, TAM tyrosine kinase receptors (Mer, Axl & Tyro3), migration markers (CX3CR1, CCR2, CCR5 & CCR7) amongst others. Therefore, DNA extraction and polymerase chain reaction-sequence specific polymerisation to identify common immune related polymorphisms will be performed.

5.6 Data analysis

Clinical, biochemical, haematological, physiological, demographic and outcome data for all patients will be collected daily and entered into a secure database. Subsequently, the immunological data will be correlated with these parameters. As this is a pilot study, there is no need for statistical power calculations. Data will be assessed for normal distribution. Paired univariate statistical testing will be used to compare monocyte and macrophage phenotypic and functional differences. Correlation of the immunological data with clinical data will be performed.

Area under the receiver operating curve (AUROC) comparison will be performed by the Hanley-McNeill method. Univariate comparison of plasma levels of markers of infection/immune response will be performed by one-way ANOVA (on log transformed data if

necessary for normalisation) within R(Bioconductor) and MedCalc (MedCalc Software, Mariakerke, Belgium). Repeated measures ANOVA will be used for longitudinal data.

Power calculation: To diagnose bacterial infection using EBA in a group of definite infected patients against a group of patients without infection at an area under the receiver operating curve of 0.85 and compared to a null hypothesis value (0.75), 150 patients would need to be recruited. This assumes a power of 80% and alpha of 0.05. 70 patients with sterile inflammation and 30 healthy controls will also be recruited. Therefore 250 patients are required.

5.7 Data storage

Subject data will be stored in a de-identified fashion and the primary investigator will hold the key. Any hard copies of data, such as investigator files, will be kept in a locked dedicated research office. All data kept on NHS or University computers will be protected by password access. All data kept on laptops or portable storage devices will be encrypted and password protected. If any data is sent outside the above mentioned areas for statistical analysis, it will be fully de-identified and password protected. Following the end of the study, any surplus biological samples will be transferred to the Liver Bio-Bank (REC ref 08/HO704/117). Breath samples will be destroyed following analysis. Ultrasound imaging will be stored anonymously for teaching and research purpose.

6) ETHICAL AND REGULATORY CONSIDERATIONS

6.1 Assessment and Management of Risk

Risks to the patient are minimal. Blood sampling (40ml) is known to be safe, would be checked with the senior clinicians responsible for the patient's care and would not in any case would not be taken in severely anaemic patients requiring blood transfusion. Breath sampling will not be performed on patients with severe respiratory failure on mechanical ventilation where it is not safe to disconnect the ventilator and attach the breath bag.

6.2 Research Ethics Committee (REC) Review and Reports

Before the start of the study, a favourable opinion was sought from the NRES Committee, informed consent and consultee advice forms. Substantial amendments that require review by NHS REC will not be implemented until that review is in place and other mechanisms are in place to implement at site. All correspondence with the REC will be retained. It is the Chief Investigator's responsibility to produce the annual reports as required. The Chief Investigator will notify the REC of the end of the study. If the study is ended prematurely, the Chief Investigator will notify the REC, including the reasons for the premature termination. Within one year after the end of the study, the Chief Investigator will submit a final report with the results, including any publications/abstracts, to the REC.

7) PEER AND REGULATORY REVIEW

The study has been peer reviewed in accordance with the requirements outlined by KCH R&I. The Sponsor considers the procedure for obtaining funding from the Science and Technology Facilities Council to be of sufficient rigour and independence to be considered an adequate peer review.

8) ADVERSE EVENTS AND INCIDENT REPORTING

Definitions of Adverse Events

Term	Definition
Adverse Event (AE)	Any untoward medical occurrence in a patient or study participant, which does not necessarily have a causal relationship with the intervention/treatment/procedure involved.
Serious Adverse Event (SAE).	Any adverse event that: results in death, is life-threatening*, requires hospitalisation or prolongation of existing hospitalisation**, results in persistent or significant disability or incapacity, or consists of a congenital anomaly or birth defect

*A life- threatening event, this refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

** Hospitalisation is defined as an in-patient admission, regardless of length of stay. Hospitalisation for pre-existing conditions, including elective procedures do not constitute an SAE.

Assessments of Adverse Events

Each adverse event will be assessed for severity, causality, seriousness and expectedness as described below.

Severity

Category	Definition
Mild	The adverse event does not interfere with the participant's daily routine, and does not require further procedure; it causes slight discomfort
Moderate	The adverse event interferes with some aspects of the participant's routine, or requires further procedure, but is not damaging to health; it causes moderate discomfort
Severe	The adverse event results in alteration, discomfort or disability which is clearly damaging to health

Causality

The assessment of relationship of adverse events to the procedure is a clinical decision based on all available information at the time of the completion of the case report form.

The following categories will be used to define the causality of the adverse event:

Category	Definition
Definitely:	There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.
Probably:	There is evidence to suggest a causal relationship, and the influence of other factors is unlikely
Possibly	There is some evidence to suggest a causal relationship (e.g. the event occurred within a reasonable time after administration of the study procedure). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant events).
Unlikely	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the study procedure). There is another reasonable explanation for the event (e.g. the participant's clinical condition).
Not related	There is no evidence of any causal relationship.
Not Assessable	Unable to assess on information available.

Expectedness

Category	Definition
Expected	An adverse event which is consistent with the available information about the intervention/treatment/procedure in use in this study.
Unexpected	An adverse event which is not consistent with the available information about the intervention/treatment/procedure in use in this study*

* this includes listed events that are more frequently reported or more severe than previously reported

Procedures for recording adverse events

All adverse events will be recorded in the medical records in the first instance.

All Adverse events will be recorded in the CRF following consent.

All adverse events will be recorded in the CRF until (insert as appropriate e.g. the participant completes the study)

Procedures for recording and reporting Serious Adverse Events

All serious adverse events will be recorded in the medical records and the CRF.

All SAEs (except those specified in section 16.5 as not requiring reporting to the Sponsor) must be recorded on a serious adverse event (SAE) form. The CI/PI or designated individual will complete an SAE form and the form will be emailed to the R&I Office (kch-tr.research@nhs.net) within 1 working day of becoming aware of the event.

Where the event is unexpected and thought to be related to the intervention/treatment/procedure this must be reported by the Investigator to the REC and

Health Research Authority, using the SAE Report form for non-CTIMPs (available from the HRA website) within 15 days.

Serious Adverse Events that do not require reporting

In the patient groups under investigation the nature of their critical illness is such that events such as death, critical illness deterioration is unlikely to be related to the study procedures of blood or breath sampling. Therefore, unless causality is possible these major deteriorations will not be reported as AE but will be captured in the clinical course of the study.

Reporting Urgent Safety Measures

If any urgent safety measures are taken the CI/ PI shall immediately and in any event no later than 3 days from the date the measures are taken, give written notice to the relevant REC, Health Research Authority and R&I office of the measures taken and the circumstances giving rise to those measures.

Protocol deviations and notification of protocol violations

A deviation is usually an unintended departure from the expected conduct of the study protocol/SOPs, which does not need to be reported to the sponsor. The CI will monitor protocol deviations.

A protocol violation is a breach which is likely to effect to a significant degree –

(a) the safety or physical or mental integrity of the participants of the study; or

(b) the scientific value of the study.

The CI and R&I Office should be notified immediately of any case where the above definition applies during the study conduct phase.

Trust incidents and near misses

An incident or near miss is any unintended or unexpected event that could have or did lead to harm, loss or damage that contains one or more of the following components:

a. It is an accident or other incident which results in injury or ill health.

b. It is contrary to specified or expected standard of patient care or service.

c. It places patients, staff members, visitors, contractors or members of the public at unnecessary risk.

d. It puts the Trust in an adverse position with potential loss of reputation.

e. It puts Trust property or assets in an adverse position or at risk.

Incidents and near misses must be reported to the Trust through DATIX as soon as the individual becomes aware of them.

A reportable incident is any unintended or unexpected event that could have or did lead to harm, loss or damage that contains one or more of the following components:

It is an accident or other incident which results in injury or ill health.

It is contrary to specified or expected standard of patient care or service.

It places patients, staff members, visitors, contractors or members of the public at unnecessary risk.

It puts the Trust in an adverse position with potential loss of reputation.

It puts Trust property or assets in an adverse position or at risk of loss or damage.

9) MONITORING AND AUDITING

The Chief Investigator will ensure there are adequate quality and number of monitoring activities conducted by the study team. This will include adherence to the protocol, procedures for consenting and ensure adequate data quality.

The Chief Investigator will inform the sponsor should he/she have concerns which have arisen from monitoring activities, and/or if there are problems with oversight/monitoring procedures.

10) TRAINING

The Chief Investigator will review and provide assurances of the training and experience of all staff working on this study. Appropriate training records will be maintained in the study files

11) INDEMNITY ARRANGEMENTS

King's College London holds insurance against claims from participants for harm caused by their participation in this clinical study. Participants may be able to claim compensation if they can prove that KCL has been negligent. However, if this clinical study is being carried out in a hospital, the hospital continues to have a duty of care to the participant of the clinical study. King's College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. This applies whether the hospital is an NHS Trust or otherwise.

12) DISSEMINATION

The results of this research will go towards publications in major peer reviewed journals and discover novel therapeutic targets on which to base immunomodulatory therapies and novel blood and breath based biomarkers to improve outcome in this condition. Authorship will be decided based on the International Committee of Medical Journal Editors (ICMJE) recommended criteria.

13) TIMETABLE

It is expected that it will take two years to recruit the patients for this study

15) APPENDICES

Appendix 1: PROTOCOL VERSIONS

Versions No	**Version Date**	**Status**
2	07/04/20	Previous
3	14/08/20	Current