STUDIES OF UNEXPLAINED INFLAMMATION IN HAEMODIALYSIS AND THE ROLE OF ENDOTOXIN

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School of Life and Medical Sciences

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Index

Acknowledgements iii
Thesis vi
Abstract vii
List of Tables xi
List of Figures xiv
Abbreviations xvii
PhD related Publications xx
Table of Contents xxii
List of Appendices xxvi
Thesis structure and prelude xxvii
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Collaborations

Several studies in this thesis were undertaken in collaboration with other researchers.

The pre-analytical work and methodological studies comparing different endotoxin detection studies described in Chapter 3 of this thesis was carried out with specialist expertise under the supervision of Mr Jim Jeraj (Quality Control – Lister Hospital), Dr Nathan Davies (Royal Free Hospital) and Professor Toru Obata (Shiga University of Medical Science). Studies described in chapter 8, 9, 10 investigating (1-3)-β-D glucan levels in patients was carried out with support from Dr Malcolm Finkelman (Associates of Cape Cod Inc.) who provided expertise and interpretation of (1-3)-β-D glucan measurements. Dr Yonglong Zhang carried out measurements on samples for (1-3)-β-D glucan and Dr Sivaramakrishnan Ramanarayanan helped with data collection for patient demographic and clinical data. Studies described in Chapter 11 measuring intestinal permeability in patients was developed in collaboration with Dr Kaatje Lenaerts and her team at the University of Maastricht. Measurements of blood samples for markers of intestinal permeability was carried out with the help of Dr Dennis Meesters. Cytokine measurements in samples were carried out under the supervision and with help by Dr Ashish Patidar (University of Hertfordshire).

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**Extent of contribution of collaborators for the studies in this thesis**

<table>
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<td>Mr Jim Jeraj</td>
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<td></td>
<td>Professor Toru Obata</td>
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</tr>
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<td></td>
<td>Dr Nathan Davies</td>
<td>Study design, manuscript review and supervision of Jonathan Wong for carrying out kinetic chromogenic assays</td>
</tr>
<tr>
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Endotoxemia and elevated plasma (1-3)-β-D glucan levels are due to increased gastrointestinal permeability and associated with unexplained inflammation in uraemia
Abstract

Clinical outcomes for patients with end-stage kidney disease on haemodialysis remain poor and have similar mortality risk to some cancers. A significant proportion of haemodialysis patients have chronic unexplained inflammation – the pathophysiology of this syndrome is unclear but is associated with poor quality of life and survival. A large number of studies have proposed that circulating endotoxin, fragments of gram negative bacterial cell wall, plays a key role in driving chronic inflammation in dialysis patients in the absence of clinical infection. However, interpretation of published literature is difficult for several reasons. Firstly, reports have mainly been cross-sectional in nature. Secondly, various different types of endotoxin detection assays have been used and reported blood endotoxin levels in clinically stable non-infected patients were exceedingly high which is incompatible with the clinical state. Additionally, nearly all studies used endotoxin detection assays that could be also be activated by (1-3)-β-D glucan, a cell wall constituent of cereals, yeast and fungi. This is a particularly important given that materials used to construct some dialyser membranes are known to leach (1-3)-β-D glucan.

The doctoral research project had two central hypotheses requiring investigation. Firstly, that endotoxin and other exogenous molecules in the gut including (1-3)-β-D glucan are contributors to unexplained inflammation in patients with chronic kidney disease. Second, that their presence in blood is a result of translocation from the gut due to increased gastrointestinal permeability in the uraemic state.

The clinical importance of these hypotheses is that if the relationship between presence of endotoxin and (1-3)-β-D glucan in blood of patients with chronic kidney disease and inflammation were clarified, and a positive association was confirmed, this would justify embarking on endotoxin or (1-3)-β-D glucan lowering interventional studies such as extracorporeal endotoxin adsorption therapy or exploring therapies to modify gut flora.

In order to address these hypotheses systematically, the studies were designed with the following specific aims:
1. To determine the prevalence of chronic unexplained inflammation in patients receiving haemodialysis to determine the scope and extent of the clinical problem.

2. To determine the optimum endotoxin assay, in terms of accuracy and precision, for measurement of endotoxin in uraemic human blood and to limit interference of other factors in the assay such as presence of (1-3)-β-D glucan.

3. To determine the relationship of blood levels of endotoxin measured with an optimised assay and (1-3)-β-D glucan in patients with chronic kidney disease with patient symptoms and inflammation both cross-sectionally and longitudinally.

4. To determine factors influencing the presence of endotoxin and (1-3)-β-D glucan in the blood of patients requiring haemodialysis to determine whether increased gut permeability, potentially induced by intravascular volume depletion during dialysis, is a potential contributor.

To determine the extent of unexplained inflammation, a retrospective study was performed of 444 prevalent haemodialysis patients at the East and North Herts NHS Trusts in which medical records and serial CRP measurements over 3 months were reviewed. Specific potential causes of inflammation were sought for each patient. 64.8% of patients had evidence of elevated CRP >5mg/L on at least two occasions one month apart and 23.4% of the study population had chronic unexplained inflammation.

To determine the optimum blood endotoxin detection assay, a literature review was conducted to identify candidate assays for testing. All published endotoxin studies in dialysis patients used variations of assays based on the Limulus Amoebocyte Lysate (LAL) assay. The kinetic turbidimetric, kinetic chromogenic LAL and Endotoxin Scattering Photometry assay were compared in a direct head-to-head study in terms of accuracy and precision. The kinetic turbidimetric LAL assay was found to have the best performance and used for blood endotoxin detection in clinical studies.

In a study of 40 haemodialysis patients, blood samples were measured simultaneously for endotoxin and (1-3)-β-D glucan. Endotoxin signals correlated significantly with (1-3)-β-D glucan. However, on modifying the kinetic turbidimetric
LAL assay with a blocker which prevents assay activation by (1-3)-β-D glucan, all previously detected signals were extinguished and true endotoxemia in haemodialysis patients in this study was low, contrary to findings from previous studies. ‘Endotoxin’ signals detected using LAL assays suffer from significant false positive interference from elevated (1-3)-β-D glucan in haemodialysis patients.

Given the low prevalence of endotoxemia detected, haemodialysis patients were further studied during clinical states that were hypothesised to increase endotoxin influx including during exercise and after haemodialysis. Blood samples were collected from 10 haemodialysis patients before and after haemodialysis and intra-dialytic exercise. Similarly, after modifying the LAL assay with a blocker to prevent false activation from (1-3)-β-D glucan, only one sample tested positive for endotoxin whereas 43% of samples had elevated (1-3)-β-D glucan.

The relationship between (1-3)-β-D glucan with kidney function, inflammation and endotoxin levels were further studied in a study of 60 haemodialysis patients (30 with high-risk features including chronic unexplained inflammation, hypotension during haemodialysis or high ultrafiltration requirements and 30 low-risk patient [no inflammation, hypotension and low ultrafiltration requirement], 15 peritoneal dialysis patients, 20 patients with CKD 4-5, 20 patients with CKD 1-3 and 20 healthy controls. Similarly, while endotoxin signals were highest in haemodialysis patients, most signals disappeared after modifying the LAL assay with a (1-3)-β-D glucan blocking buffer. Low level endotoxemia was found in only 3 haemodialysis patients. BG levels increased with worsening CKD stage and were highest in haemodialysis patients, 22% having significantly elevated levels. (1-3)-β-D glucan correlated strongly with endotoxin signals (r=0.545, p<0.001) suggesting that previous reports of elevated endotoxin may be artefactual due to raised blood (1-3)-β-D glucan in renal impairment. (1-3)-β-D glucan correlated strongly with markers of inflammation and were associated with higher depression and kidney disease related symptoms scores.

Intestinal barrier dysfunction and increased intestinal permeability has been proposed as a source of inflammation in haemodialysis patients and the haemodialysis procedure has been suggested to exacerbate intestinal permeability due to the large blood volume changes that occur during treatment. Intestinal
permeability has not been studied directly in haemodialysis patients since conventional assays rely on measurement of probes in urine but this method cannot be applied to anuric dialysis patients. Using a novel intestinal permeability assay which measures probes in plasma instead, intestinal permeability was measured in 10 haemodialysis patients before and after haemodialysis treatment and compared with 5 healthy controls. Direct comparison of intestinal permeability between haemodialysis patients and healthy controls was difficult since concentration of probes in plasma was influenced by level of kidney function, although there was a suggestion that small intestinal permeability was increased in haemodialysis patients. However, intestinal permeability was not significantly affected by the haemodialysis procedure.

The main finding of this work is that endotoxemia is relatively uncommon in dialysis patients and hence unlikely to be the sole cause of unexplained chronic inflammation in these patients. Previous reports of apparent high levels of blood endotoxin in dialysis patients were due, in part at least, to false positive interference of LAL assays by elevated (1-3)-β-D glucan present in the blood of patients with kidney disease. The work presented in this thesis supports this. Furthermore, the clinical studies carried out did not demonstrate a convincing association between endotoxin and inflammation. In contrast, this work has demonstrated a progressive increase in levels of (1-3)-β-D glucan with worsening degree of kidney disease and an association of these levels with inflammation, higher depression and kidney-disease related symptom scores. The cause of elevated (1-3)-β-D glucan in dialysis patients is unclear and warrants further study, though an intestinal source is a possibility. The direct intestinal permeability studies carried out in dialysis patients suggest impaired intestinal barrier function, although until an accurate method of assessing intestinal permeability in vivo in uraemic patients is available, this hypothesis remains to be proven.
List of Tables

Table 1-1: US KDIGO stages of kidney disease........................................ Error! Bookmark not defined.

Table 1-2 – Dialysis and non-dialysis related risk factors associated with reduced survival in dialysis patients.................................................................14

Table 2-1: Published levels of endotoxemia detected in dialysis patients.................................30

Table 4-1: Roadmap of studies for thesis ........................................................................47

Table 5-1: Clinical characteristics of patients with and without unexplained inflammation ........64

Table 5-2: Clinical characteristics of inflamed patients with and without potential predisposing risk factors for inflammation........................................................................65

Table 6-1: Coefficient of variation (CV) and reaction times obtained for control standard endotoxin ..77

Table 6-2: Endotoxin content of devices and spike recoveries obtained from water spiked with 1 EU/mL control standard endotoxin solution used to rinse device.........................................................78

Table 6-3: Endotoxin spike recovery from plasma and serum samples spiked with 5 EU/mL of control standard endotoxin..............................................................................................83

Table 6-4: Spike recovery from plasma samples spiked with different concentrations of control standard endotoxin....................................................................................................92

Table 6-5: Spike recoveries for plasma samples with different dilution factors .........................96

Table 6-6: Endotoxin content of detergents ........................................................................102

Table 6-7: Comparison of spike recovery from plasma samples between detergents and water as a diluent................................................................................................................103

Table 6-8: Median endotoxin levels from the kinetic turbidimetric LAL, kinetic chromogenic LAL and ESP assay..................................................................................................................113

Table 6-9: Median spike recovery from kinetic turbidimetric, kinetic chromogenic and ESP assay...114

Table 6-10: Comparison of assay error of turbidimetric and chromogenic LAL assay using linear regression analyses ........................................................................................................116

Table 6-11: Coefficient of variation for all three assays in pooled uraemic and non-uraemic plasma118

Table 6-12: Characteristics of endotoxin assays used in patients with kidney disease .................124

Table 6-13: Endotoxin measurements obtained from the same plasma samples using the kinetic turbidimetric LAL assay (Charles Rivers) and endpoint chromogenic LAL assay (Lonza) ..........127
Table 11-5: AUC for sugar probes in haemodialysis patients with and without residual renal function

<table>
<thead>
<tr>
<th></th>
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<tr>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Test 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

206
List of Figures

Figure 1-1: Schematic diagram to illustrate peritoneal dialysis .......................................................... 9
Figure 1-2: Schematic representation of haemodialysis circuit ............................................................... 10
Figure 1-3: A radio-cephalic fistula ........................................................................................................ 11
Figure 1-4: Diagram of a tunneled dialysis catheter ............................................................................... 12
Figure 1-5: Solute and fluid movement within the dialyser .................................................................... 13
Figure 1-6: Causes and consequences of inflammation in kidney disease ........................................... 16
Figure 2-1: Schematic diagram showing endotoxin embedded in the cell wall of gram negative bacteria ......................................................................................................................... 23
Figure 2-2: Schematic diagram of endotoxin structure .......................................................................... 24
Figure 2-3: Proposed molecular signalling of endotoxin ...................................................................... 25
Figure 3-1: The LAL enzyme cascade .................................................................................................... 34
Figure 3-2: Typical optical density graph obtained from a sample containing endotoxin ..................... 35
Figure 3-3: Images of optical microscopic observations of aggregates formed by the LAL reaction with endotoxin at concentration of 0.05 EU/mL .................................................................................. 40
Figure 3-4: Reaction mechanism for EAA utilising anti-LPS monoclonal antibody and neutrophils from whole blood .................................................................................................................. 43
Figure 5-1: Proportion of haemodialysis patients with and without inflammation ................................. 63
Figure 6-1: Flow chart to illustrate protocol for testing blood collection apparatus for contamination and LAL interfering factors .................................................................................................. 74
Figure 6-2: Optical density graph showing a sample containing 10 EU/mL of endotoxin reaching the onset optical density ............................................................................................................. 75
Figure 6-3: Standard curve from 10 to 0.001 EU/mL (X-axis) against reaction time (Y-axis) ............. 76
Figure 6-4: Optical density graph showing reaction curve ...................................................................... 85
Figure 6-5: Linear portion of reaction curve is used for calculation Vmax .............................................. 86
Figure 6-6: Optical density graph to show reaction time when 50% of the maximum optical density is reached ................................................................. 87
Figure 6-7: Onset time B – reaction time at onset of maximal rate of increase in optical density .......... 88
Figure 6-8: Calculation of endotoxin content using different components of optical density graph ...... 90
Figure 6-9: Schematic diagram of endotoxin structure ................................................................... 99
Figure 6-10: Bubbles in the samples introducing artefact in the optical density graph. ................. 102
Figure 6-11: Mean endotoxin spike recovery from haemodialysis patients .................................. 104
Figure 6-12: Mean endotoxin spike recovery in healthy plasma ................................................. 104
Figure 6-13: Baseline endotoxin content in plasma of haemodialysis patients ............................. 105
Figure 6-14: Gelation process using ESP assay at 10pg/mL ......................................................... 112
Figure 6-15: Bland-Altman analysis of measured versus expected spike recovery ..................... 115
Figure 6-16: Comparison plots showing linear regression lines for measured versus expected endotoxin content in spiked samples for haemodialysis patients and healthy controls with the chromogenic and turbidimetric assay ........................................................................ 116
Figure 7-1: Beta glucan composed of D-glucose monomers linked by (1-3) beta-glycosidic bonds. A linear 1,3 glycosidic chain with a single 1,6 glycosidic chain is shown ................................................................. 131
Figure 7-2: The LAL enzyme cascade .......................................................................................... 132
Figure 8-1: Schematic presentation of spiking experiments .......................................................... 143
Figure 8-2: Endotoxin signal detected using standard LAL without BG blocking buffer displayed by tertiles of (1→3)-β-D glucan. ........................................................................................................ 147
Figure 8-3: Receiver operative curve (ROC) analysis using (1→3)-β-D glucan at cut-off level 62 pg/mL ........................................................................................................................................ 148
Figure 8-4: Comparison of median spike recovery between LAL(-) and LAL(+) in plasma samples spiked with endotoxin ............................................................................................................... 149
Figure 8-5: Comparison of median spike recovery between LAL and LAL reconstituted with different doses of BG blocking buffer (ES buffer) ................................................................. 151
Figure 9-1: Flow diagram of patients recruited .............................................................................. 160
Figure 9-3: Blood sampling time points on exercise and non-exercise day .................................. 161
Figure 9-4: Changes in endotoxin signals detected using LAL(-) across the haemodialysis session an exercise and non-exercise day .................................................................165

Figure 10-1: (1-3)-β-D-glucan in study cohort .................................................................181

Figure 10-2: Proportion of patients with detectable endotoxin using the LAL assay with and without a BG-blocking buffer .................................................................183

Figure 10-3: Endotoxin signal detected using LAL without blocking buffer displayed against serum (1-3)-β-D glucan ...................................................................................183

Figure 10-4: Unadjusted Cox proportional hazard for time to hospitalisation with infection over 18 month period ......................................................................................185

Figure 11-1: Plasma concentrations of lactulose, rhamnose and lactulose: rhamnose ratios on a non-dialysis day and after haemodialysis treatment ...............................................199

Figure 11-2: Plasma concentrations of sucralose, erythritol and sucralose: erythritol ratios on a non-dialysis day and after haemodialysis treatment ......................................................200

Figure 11-3: Plasma concentrations of lactulose, rhamnose and lactulose: rhamnose (L:R) ratio in haemodialysis patients and healthy controls ..............................................................202

Figure 11-4: Plasma levels of sucralose, erythritol and sucralose: erythritol ratios in haemodialysis patients and healthy controls ..............................................................................205
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>(1-3)-β-D glucan</td>
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<td>AVF</td>
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<td>CCI</td>
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<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<tr>
<td>CV</td>
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</tr>
</tbody>
</table>
PhD Related Publications and Presentations


Table of Contents

INDEX ................................................................................................................................. II

ACKNOWLEDGEMENTS ....................................................................................................... III

COLLABORATIONS ............................................................................................................. IV

ABSTRACT ........................................................................................................................ VII

LIST OF TABLES ................................................................................................................ XI

LIST OF FIGURES ............................................................................................................... XIV

ABBREVIATIONS ............................................................................................................... XVII

TABLE OF CONTENTS ........................................................................................................ XX

LIST OF APPENDICES ....................................................................................................... XXVI

THESIS STRUCTURE AND PRELUDE ............................................................................... XXVII

CHAPTER 1 ........................................................................................................................... 1

1. INFLAMMATION IN END-STAGE KIDNEY DISEASE ...................................................... 1
   1.1 The kidneys – anatomy, structure and function ......................................................... 1
   1.2 Measurement of kidney function .............................................................................. 2
   1.3 Classification of chronic kidney disease ................................................................. 4
   1.4 Effect of chronic kidney disease on the body .......................................................... 6
       1.4.1 CKD-associated anaemia ................................................................................... 6
       1.4.2 Bone and mineral disorders ............................................................................. 6
       1.4.3 Cardiovascular disease .................................................................................... 7
   1.5 Options for renal replacement therapy in kidney failure ......................................... 8
       1.5.1 Kidney transplantation ..................................................................................... 8
       1.5.2 Dialysis ............................................................................................................. 8
   1.6 Outcomes for dialysis patients ................................................................................ 14
   1.7 Inflammation in end-stage kidney disease .............................................................. 15
       1.7.1 Other biomarkers of inflammation in kidney disease ......................................... 17
       1.7.2 Causes of inflammation in ESKD ..................................................................... 18
           1.7.2.1 Reduced kidney function and the uraemic milieu ........................................ 18
           1.7.2.2 Patient-related factors .............................................................................. 19
           1.7.2.3 Dialysis-related factors ............................................................................. 20

CHAPTER 2 ........................................................................................................................... 23

2. ENDOXEMIA IN END-STAGE KIDNEY DISEASE ......................................................... 23
   2.1 Endotoxin and inflammation ................................................................................... 24
   2.2 Potential sources of endotoxemia .......................................................................... 26
2.2.1 Contamination of haemodialysis apparatus and devices .................................................. 26
2.2.2 CKD and the leaky gut ........................................................................................................ 27
2.2.3 Periodontal disease and endotoxemia ............................................................................ 28

2.3 Problems with interpreting level of endotoxemia in the dialysis population ................. 29

CHAPTER 3 .................................................................................................................................. 33

3. METHODS OF BLOOD ENDOTOXIN DETECTION .................................................................. 33

3.1 The Limulus Amoebocyte Lysate Assay .............................................................................. 33

3.1.1 End-point LAL assay ........................................................................................................ 34
3.1.2 Kinetic LAL assays ........................................................................................................... 35
3.1.3 Problems with the LAL assay for blood endotoxin detection .......................................... 36
3.1.3.1 Inherent variability of the LAL assay ........................................................................... 36
3.1.3.2 Specific issues with collection and processing of blood samples ................................. 37
3.1.3.3 Specific problems with measuring endotoxin in blood ................................................ 38
3.1.3.4 Interference of LAL by (1→3)-β-D glucan .................................................................. 39

3.2 Alternative direct blood endotoxin detection methods ........................................................ 40

3.2.1 Endotoxin Scattering Photometry (ESP) assay ............................................................... 40
3.2.2 Recombinant factor C assay ............................................................................................ 41
3.2.3 Endotoxin detection methods under development ......................................................... 42

3.3 Surrogate measures of endotoxemia ................................................................................... 42

3.3.1 Endotoxin Activity Assay (EAA) .................................................................................... 42
3.3.2 Lipopolysaccharide binding protein (LBP) .................................................................... 43
3.3.3 Soluble CD14 .................................................................................................................. 44

3.4 Summary ............................................................................................................................... 44

CHAPTER 4 .................................................................................................................................. 45

4. AIMS AND GENERAL METHODOLOGY .............................................................................. 45

4.1 Introduction ........................................................................................................................... 45

4.2 Hypotheses and aims ............................................................................................................ 46

4.3 Study population .................................................................................................................. 47

4.3.1 Retrospective cross-sectional study of the prevalence of unexplained inflammation in the
haemodialysis population .......................................................................................................... 48

4.3.2 Studies to determine the optimum blood endotoxin detection assay in haemodialysis patient .......................... 49
4.3.2.1 Selection of phlebotomy apparatus .......................................................................... 49
4.3.2.2 Optimisation of the LAL assay for blood endotoxin detection .................................. 49
4.3.2.3 Comparison of the turbidimetric and chromogenic LAL assay ................................ 50

4.3.3 Determining the influence of (1→3)-β-D glucan on endotoxin measurements in haemodialysis
patients ...................................................................................................................................... 51

4.3.4 Cross-sectional study to examine the association between blood endotoxin levels and chronic
unexplained inflammation in haemodialysis patients ............................................................. 52

4.3.5 Prospective longitudinal study to determine the effect of haemodialysis on blood endotoxin, (1→3)-β-D glucan and inflammation .................................................................................. 54

4.3.6 Studies of the impact of intra-dialytic exercise and haemodialysis on endotoxemia and
inflammation ................................................................................................................................ 54
4.3.7 Studies of the impact of haemodialysis on gut permeability ........................................ 55
4.4 Research Ethics ................................................................................................................. 56

CHAPTER 5 ............................................................................................................................. 58
5. THE PREVALENCE OF CHRONIC UNEXPLAINED INFLAMMATION IN HAEMODIALYSIS PATIENTS ........................................ 58
5.1 Introduction ....................................................................................................................... 58
5.2 Methods ........................................................................................................................... 58
  5.2.1 Definition of chronic inflammation ........................................................................... 58
  5.2.2 Database search ......................................................................................................... 59
  5.2.3 Dialysis parameters ................................................................................................. 60
  5.2.4 Statistical analysis .................................................................................................... 62
5.3 Results ............................................................................................................................ 62
5.4 Conclusion ....................................................................................................................... 65

CHAPTER 6 ............................................................................................................................. 67
6. STUDIES TO DETERMINE THE OPTIMUM BLOOD ENDOXIN ASSAY IN HAEMODIALYSIS PATIENTS .......... 67
6.1 Introduction ....................................................................................................................... 67
6.2 Study aims ....................................................................................................................... 69
6.3 Testing phlebotomy equipment, blood collection tubes and storage vials for endotoxin contamination and interfering factors ........................................................................... 71
  6.3.1 Method ..................................................................................................................... 71
  6.3.2 Results ..................................................................................................................... 78
  6.3.3 Conclusion ............................................................................................................... 78
6.4 Study population ............................................................................................................. 80
6.5 Determining the difference in spike recovery between plasma and serum ................. 81
  6.5.1 Introduction .............................................................................................................. 81
  6.5.2 Methods .................................................................................................................. 81
  6.5.3 Results .................................................................................................................... 83
  6.5.4 Conclusion ............................................................................................................... 83
6.6 Analysing the optical density graph - use of alternative methods to calculate endotoxin .................................................................................................................. 85
  6.6.1 Introduction .............................................................................................................. 85
  6.6.2 Methods .................................................................................................................. 88
  6.6.3 Results .................................................................................................................... 91
  6.6.4 Conclusion ............................................................................................................... 92
6.7 Studies to determine optimum dilution factor for endotoxin detection using the LAL assay ....................................................................................................................... 94
  6.7.1 Introduction .............................................................................................................. 94
  6.7.2 Method .................................................................................................................... 95
  6.7.3 Results .................................................................................................................... 95
  6.7.4 Conclusion ............................................................................................................... 97
6.8 Studies to investigate the use of surfactants to optimise endotoxin detection using the LAL assay ....................................................................................................................... 99
  6.8.1 Introduction .............................................................................................................. 99
  6.8.2 Methods .................................................................................................................. 100
REFERENCES.................................................................................................................................219

APPENDIX........................................................................................................................................240

12.1 Appendix A ...................................................................................................................................240
12.2 Appendix B – POS-S Renal ........................................................................................................258
12.3 Appendix C – Multidimensional fatigue inventory ....................................................................259
12.4 Appendix D – Patient health questionnaire 9 ..........................................................................260
12.5 Appendix E – Post dialysis recovery time .................................................................................261
List of Appendices

A  Ethical approval of studies
B  POS-S Renal
C  Multidimensional fatigue inventory
D  Patient health questionnaire 9
E  Post dialysis recovery time
Thesis Structure and Prelude

This work is organised into four main sections to defend the thesis offered.

The first section provides an overview of kidney failure and a review of the literature of endotoxemia in patients with kidney disease and methods of endotoxin detection in blood.

The second section describes general methodology relevant to all studies presented in this work.

The third section describes the laboratory and clinical studies used in defence of the hypothesis. This section consists of an initial chapter documenting laboratory experiments conducted to determine the optimum endotoxin detection assay. Subsequent clinical studies using this assay follows which examines endotoxin and (1-3)-β-D glucan levels in haemodialysis and CKD patients.

The fourth section summarises the conclusions derived from the studies described in this thesis and suggests recommendations for future work.
Chapter 1

1. Inflammation in end-stage kidney disease

1.1 The kidneys – anatomy, structure and function

The human body usually contains two kidneys which are bean-shaped organs located in the rear of the abdominal cavity in the retroperitoneal space. They are situated on either side of the spine below the diaphragm. Due to the close proximity of the right kidney to the liver, the right kidney is slightly lower and smaller than the left kidney. The size of the kidneys is dependent on body size and each weighs approximately 150g. The upper parts of both kidneys are protected by the 11th and 12th ribs. The kidneys are surrounded by tough fibrous tissue, the renal capsule which itself is surrounded by perinephric fat, renal fascia and paranephric fat. The kidney parenchyma consists of the outer cortex and the inner medulla. These structures form approximately eight to eighteen cone-shaped renal lobules, each lobule containing renal cortex surrounding a portion of medulla called a renal pyramid. Between each pyramid are projections of cortex called the columns of Bertin. The functional unit of the kidney is the nephron which spans cortex and medulla. There are approximately 1 million nephrons in each kidney. Each nephron is a comprised of a renal corpuscle located in the outer cortex. This drains into the renal tubules consisting of the primary convoluted tubule, loop of Henle, distal convoluted tubule and collecting ducts. The renal corpuscle is the initial blood-filtering component of the kidney and contains the glomerulus and Bowman’s capsule. The glomerulus is a tuft of capillaries which have large fenestrae to allow the passage of filtrate. The Bowman’s capsule consists of an outer parietal layer of simple squamous epithelium and an inner visceral layer of modified squamous epithelial cells called podocytes. These podocytes have long foot processes that wrap around the glomerular capillaries leaving slits in between them. The fenestrated glomerular capillaries, filtration slits formed by the podocytes, together with the fused basal laminar of the glomerular endothelial cells and podocytes form a filtration barrier permitting the passage of water, ions and smaller molecules including low
molecular weight proteins but preventing the passage of red blood cells, leucocytes, platelets and larger molecules. Large volumes of blood are filtered in the kidneys under hydrostatic pressure and approximately 150L of ultrafiltrate are produced daily and enter Bowman’s space. A large proportion of the ultrafiltrate is selectively reabsorbed along the renal tubules to generate approximately 2L of urine per day containing waste products of metabolism which are excreted.

The principal function of the kidneys is to remove the end-products of metabolism such as urea, but they also regulate the volume of fluid in the body together with its osmolarity, acidity, electrolyte content and concentration. The kidneys also have important endocrine functions and are responsible for the production and secretion of a number of important enzymes and hormones including renin, erythropoietin and 1,25-Di-hydroxyvitamin D3. Renin is an enzyme, produced by the granular cells of the juxtaglomerular apparatus, which catalyses the formation of angiotensin from angiotensinogen. Angiotensin is a potent vasoconstrictor peptide and helps to regular salt balance and blood pressure. Erythropoietin is a glycosylated protein produced by the renal cortical interstitial cells and stimulates the maturation of erythrocytes in the bone marrow. 1,25-Di-hydroxyvitamin D3 is the active form of vitamin D3 and is produced by the proximal tubules cells. This steroid hormone promotes intestinal absorption of calcium, increases reabsorption of calcium in the renal tubules, and suppresses the production of parathyroid hormone. 1,25-dihydroxy vitamin D also increases release of calcium from bones via the stimulation of osteoblasts which in turn activate osteoclasts (Briggs et al. 2009). Impairment of kidney function leads to a number of clinically significant problems and the type and severity of problems encountered depends on the severity of impairment.

1.2 Measurement of kidney function

Kidney function is quantified according to the glomerular filtration rate (GFR). GFR is defined as the volume of fluid filtered through the glomerular capillaries into Bowman’s capsule per unit time. The level of GFR reported in healthy individual ranges from 100-130 mL/min/1.73m² in adult men and women (Delanaye et al. 2012). GFR can be measured directly by determining the clearance of an ideal exogenous filtration marker. Inulin, a fructose polymer made from the Jerusalem
artichoke, is considered the gold-standard filtration marker since it is freely filtered through the glomerulus, not secreted, reabsorbed, or metabolized by the kidneys. It is nontoxic, physiologically inert in humans and is exclusively eliminated by glomerular filtration with no apparent extra-renal clearance, making it ideal for measuring GFR. The inulin clearance method first described by Homer Smith and James Shannon (Shannon & Smith 1935) requires the continuous intravenous infusion of inulin and bladder catheterization together with multiple urine and blood collections to measure its renal (urinary) clearance. This method is invasive and impractical and therefore cannot be used routinely in clinical practice. Therefore, GFR is usually estimated using endogenous blood based markers. Creatinine is the most commonly used endogenous marker of renal function. Creatinine is a product of metabolism of creatinine and phosphocreatine in skeletal muscle and levels remain fairly constant throughout the day with a variability of about 8%. Creatinine is freely filtered at the glomerulus and is not reabsorbed but approximately 15% is actively secreted by the tubules (Traynor et al. 2006). However, serum creatinine cannot itself be used to estimate GFR since it can be affected by a number of factors including age, diet, gender, ethnicity and muscle mass (Stevens et al. 2006). However GFR can be estimated by creatinine clearance which also incorporates estimation the volume of, and creatinine concentration in 24 hour urine collection. This has been largely superseded in clinical practice by use of estimated GFR (eGFR). A number of equations based on serum creatinine have been derived which are able to estimate creatinine clearance or GFR with reasonable accuracy, albeit with some limitations. The three most commonly used equations are the Cockroft-Gault equation, the abbreviated MDRD (Modification of Diet in Renal Disease) formula and the most recent CKD-Epidemiology Collaboration (CKD-EPI) formula (Florkowski & Chew-Harris 2011). The Cockroft-Gault equation was originally introduced in 1976 and predicts creatinine clearance based on age, weight, and height and serum creatinine. However, the equation was derived mainly from hospitalised men and the necessity to obtain height and weight for calculation limited its widespread use on a population-wide basis. Subsequently, the MDRD equation was derived from a study cohort of 1628 patients with CKD in 1999. This utilises only four variables (age, gender, ethnicity and serum creatinine) and generates an
estimated GFR corrected for body surface area. The lack of requirement of weight and height made the MDRD equation the preferred formula in clinical practice. However, the MDRD equation tends to underestimate renal function in those with eGFR>90mL/min/1.73m². The CKD-EPI was developed in 2009 from 8254 data points from six studies and four clinical populations and was designed to match the accuracy of MDRD equation at eGFR<60mL/min/1.73m² but to increase the accuracy at higher GFR to avoid inappropriately over diagnosing CKD in the general population. The CKD-EPI equation actually consists of eight different equations, four equations for whites (male, female, above and below specific cut-offs of creatinine) and four equations for blacks with different coefficients. Both US and UK NICE clinical practice guidelines currently recommend the use of CKD-EPI equations for estimating GFR (NICE Clinical Guidelines 2014; KDIGO Workgroup 2013). The CKD-EPI equations are shown below:

\[
\text{Female with creatinine} \leq 62; \text{use } eGFR = 144 \times \left(\frac{Cr}{61.9}\right)^{-0.329} \times 0.993^{Age} \times 1.159 [\text{if black}]
\]

\[
\text{Female with creatinine} > 62; \text{use } eGFR = 144 \times \left(\frac{Cr}{61.9}\right)^{-1.209} \times 0.993^{Age} \times 1.159 [\text{if black}]
\]

\[
\text{Male with creatinine} \leq 80; \text{use } eGFR = 141 \times \left(\frac{Cr}{79.6}\right)^{-0.411} \times 0.993^{Age} \times 1.159 [\text{if black}]
\]

\[
\text{Male with creatinine} > 80; \text{use } eGFR = 141 \times \left(\frac{Cr}{79.6}\right)^{-1.209} \times 0.993^{Age} \times 1.159 [\text{if black}]
\]

where serum creatinine is measured in μmol/L.

### 1.3 Classification of chronic kidney disease

Chronic kidney disease (CKD) is defined as an abnormality in either kidney structure or function persisting for three or more months. The United States National Kidney Foundation have classified CKD into different stages or grades according to level of GFR and the amount of proteinuria present (KDIGO Workgroup 2013):-
Table 1-1: US KDIGO stages of chronic kidney disease

Indicators of kidney damage can be detected by histology, imaging, blood tests (elevated urea and creatinine) or abnormalities in urine dipstick such as the presence of blood and protein. Proteinuria is defined as the presence of significant protein in the urine and is an accurate prognostic indicator of CKD progression and cardiovascular mortality (Jafar et al. 2001; Keane & Eknoyan 1999). Proteinuria is caused by glomerular injury and consist mainly albumin although other proteins such as Tamm-Horsfall protein (uromodulin) are present. Proteinuria was traditionally detected using 24 hour urine collections but due to the inconvenience and unreliability of timed urine collection, these have been largely replaced with the random ‘spot’ urine collections to measure the ratio of urine protein or albumin concentration to urine creatinine concentration (urine PCR or ACR). Thus, although those with stage 1 CKD may have ‘normal’ GFR, these individuals will have other indicators of kidney disease such as proteinuria or structural abnormalities of the kidney. The most severe stage of kidney disease occurs when GFR falls below 15mL/min/1.73m² and uraemic signs and/or symptoms appear. Uraemic signs and symptoms may include volume overload, reduced appetite, confusion, severe hypertension, nausea and vomiting. Once kidney failure or end-stage kidney disease
is established, either transplantation or dialysis treatment is required to reduce the risk of morbidity and mortality.

1.4 Effect of chronic kidney disease on the body

CKD is a complex and prevalent disease with a high economic cost to health systems. Estimated global prevalence of CKD by stages are stage 1: 3.5%; stage 2: 3.9%; stage 3: 7.6%; stage 4: 0.4%; and stage 5: 0.1% (Hill et al. 2016). Progression of CKD itself is associated with a number of serious complications including increased incidence of cardiovascular disease, hyperlipidemia, anaemia, metabolic bone disease and inflammation (Thomas et al. 2008).

1.4.1 CKD-associated anaemia

CKD or renal anaemia is a very common complication and usually occurs in stage 4 and 5 CKD. Whilst CKD-associated anaemia may be due to multiple mechanisms such as iron deficiency, gastrointestinal bleeding, reduced red cell survival and inflammation, the principal cause of CKD-associated anaemia is reduced erythropoietin (EPO) synthesis. EPO is a glycoprotein hormone secreted by kidney interstitial fibroblasts and is essential for the growth and differentiation of red blood cells from the bone marrow. EPO synthesis is impaired with progressive CKD due to tubular atrophy and tubulointerstitial fibrosis. Apart from the symptomatic effect of anaemia such as fatigue, renal anaemia increases morbidity and mortality from cardiovascular complications such as left ventricular hypertrophy and worsening heart failure. Treatment of CKD-associated anaemia is with recombinant human EPO injections. EPO treatment has largely replaced the need for repeated blood transfusions to treat renal anaemia as was practiced historically.

1.4.2 Bone and mineral disorders

CKD leads to defective bone and mineral metabolism and renal osteodystrophy. Renal osteodystrophy encompasses a broad spectrum of histological changes seen in the bony architecture of CKD patients. The kidneys are responsible for phosphate excretion and 1-α hydroxylation of vitamin D, with progressive CKD both of these processes are impaired leading to phosphate retention and hypocalcaemia. As a
consequence, a number of different bone abnormalities may develop including high- and low bone turnover states. A high bone turnover state is due to a compensatory increase in parathyroid hormone that occurs due to hyperphosphatemia, hypocalcemia and low levels of 1,25-dihydroxyvitamin D leading to secondary hyperparathyroidism. Elevated parathyroid levels stimulate osteoclastic activity in the bone leading to weakening and loss of bone mass (osteitis fibrosa cystica). Low bone turnover states such as osteomalacia occur due to inadequate bone mineralisation from insufficient vitamin D synthesis. Another form of low bone turnover disease called adynamic bone disease can also develop predominantly in patients established on dialysis treatment and results from over suppression of parathyroidism and high calcium dialysate concentrations (Epstein et al. 1995). A combination of both high and low bone turnover disease may also co-exist (mixed osteodystrophy). The mainstay of treatment of renal osteodystrophy is reduction of serum phosphate with dietary phosphate restriction and oral phosphate binders. Supplementation with vitamin D and its related compounds is also used to raise serum calcium concentration and to suppress parathyroid hormone secretion. In a proportion of patients with severe hyperparathyroidism, surgical parathyroidectomy may be required. Use of calcimimetics – agents which increase the calcium sensitivity of parathyroid gland, thus down-regulating parathyroid hormone secretion and reducing hyperplasia of the parathyroid gland - may also be useful in those with established hyperparathyroidism.

1.4.3 Cardiovascular disease

CKD is associated with a significant increase in mortality compared to the normal population. There is an increased incidence of cardiovascular disease, myocardial infarction, heart failure, stroke and sudden death (Di Angelantonio et al. 2010). Many traditional Framingham risk factors such as hypertension are more prevalent in CKD patients compared to the general population. Hyperphosphatemia, raised calcium-phosphate ion product and hyperparathyroidism are also independent cardiovascular risk factors, possibly due to its promotion of vascular calcification, atherosclerosis and increased vascular wall stiffness (Thomas et al. 2008). Other non-traditional risk factors such as inflammation are also believed to play a role in increasing
cardiovascular risk in patients with CKD. Inflammation in kidney disease is discussed further in chapter 1.7.

1.5 Options for renal replacement therapy in kidney failure

Once kidney function is unable to meet the metabolic demands of daily life, kidney failure is established and renal replacement therapy (either dialysis or transplantation) is required to prevent death and maintain quality of life. Not all patients may wish to embark on renal replacement therapy and very frail patients may not be suitable for transplantation or benefit from dialysis. This subset of patients may choose a palliative approach to treatment. For those who are able to undergo renal replacement therapy, the aim is to replace the excretory function of the kidney by providing sufficient removal of metabolic waste products, control of fluid and electrolyte balance to maintain health and quality of life.

1.5.1 Kidney transplantation

Patients who receive a kidney transplant benefit from improved clinical outcomes and quality of life compared to well-matched counterparts who remain on dialysis (Wolfe et al. 1999; Rabbat et al. 2000). Kidney transplantation is the preferred option for Renal Replacement Therapy (RRT) due to the superior outcomes. However many patients with kidney failure are elderly and frail and not medically fit enough to receive a kidney transplant. Kidneys are donated from a deceased or living donor and in the past transplantation were only carried out between HLA and ABO compatible individuals although with development of more effective immunosuppressive techniques, HLA- and ABO incompatible transplants are increasing being performed (Barry et al. 2005). Following transplantation, lifelong immunosuppressive medication is necessary to prevent allograft rejection.

1.5.2 Dialysis

For dialysis therapy, two options are available – peritoneal dialysis and haemodialysis. In peritoneal dialysis, the peritoneal membrane in the abdomen is used for the removal of fluid and uraemic solutes. Fresh dialysis fluid containing electrolytes such as sodium, chloride, lactate or bicarbonate and, crucially, a high
concentration of glucose to ensure hypersomolarity, is introduced into the abdominal cavity via a peritoneal dialysis tube or catheter (Figure 1-1). The high concentration of glucose in the dialysate drives the filtration of fluid by osmosis from the peritoneal capillaries into the peritoneal cavity. During this process, the peritoneum acts a semi-permeable membrane and solutes including urea and creatinine are also removed simultaneously. Glucose from the dialysate diffuses rapidly into the blood and after several hours the glucose osmotic gradient is dissipated and the ‘used’ dialysate is drained out and a fresh bag of dialysis fluid is re-instilled. Peritoneal dialysis therapy is a continuous daily treatment and peritoneal dialysate exchanges should be carried out several times each day, either manually in continuous ambulatory peritoneal dialysis (CAPD), or overnight using a mechanical cycler in automated peritoneal dialysis (APD).

Figure 1-1: Schematic diagram to illustrate peritoneal dialysis (reproduced with permission from Blausen Gallery 2014: Medical Gallery of Blausen Medical 2014. Wiki Journal of Medicine)

In haemodialysis, blood from the patient is passed through a synthetic dialyser which removes uraemic waste products before being returned to the patient. The dialyser is a semi-permeable membrane with two separate compartments for blood and dialysis fluid to flow in counter-current directions and uraemic solutes are removed by a combination of diffusion and convection. Systemic anticoagulation with heparin is
necessary during dialysis to prevent blood clotting within the extracorporeal circuit. A schematic representation of haemodialysis is shown in Figure 1-2.

![Schematic representation of haemodialysis circuit](Image)

**Figure 1-2: Schematic representation of haemodialysis circuit (reproduced with permission from Blausen Gallery 2014: Medical Gallery of Blausen Medical 2014. Wiki Journal of Medicine)**

Haemodialysis is the most common form of dialysis used and is usually performed in hospital or a haemodialysis centre three times a week with each treatment session typically lasting 3-4 hours. However, haemodialysis can also be carried out at home by some patients, allowing flexibility, in terms of the timing, duration and frequency of dialysis sessions, which is difficult to offer within the constraints of standard thrice-weekly in-centre haemodialysis. The prospective randomised Frequent Haemodialysis Network trials have found favourable outcomes in terms of mortality, left ventricular mass and physical health scores for patients who dialyse more frequently than patients treated with standard thrice-weekly haemodialysis (Chertow et al. 2010; Hall et al. 2012).

A permanent form of vascular access is required for haemodialysis which enables high blood flow to be delivered to the dialyser to achieve efficient removal of uraemic solutes from the patient. The preferred vascular access is an arterio-venous fistula
(AVF) which is created by surgically joining an artery to a vein, usually between the brachial or radial artery and the cephalic vein. Following the creation of the fistula, the blood flow through the fistula increases to approximately 700-1000mL/min after 1 month. The artery and vein dilates and stretches in response to the high flow and shear stress, eventually the vein is large enough to be cannulated by two large bore needles to allow the efficient delivery and return of blood to the dialyser (Figure 1-3). Occasionally, arterio-venous fistula formation may not be possible and a synthetic graft, usually made of PTFE is used to connect the artery to the vein.

![Diagram of AVF](image)

**Figure 1-3: A radio-cephalic fistula (reproduced with permission from Blausen Gallery 2014: Medical Gallery of Blausen Medical 2014. Wiki Journal of Medicine)**

In some patients, formation of AVF or graft may not be technically possible or the AVF may not have matured rapidly enough for use and in these circumstances a dialysis catheter is inserted into a large vein, usually the internal jugular vein, for haemodialysis (Figure 1-4). Dialysis catheters consist of two lumens – one lumen acts as an arterial limb and diverts blood to the dialyser and the second lumen acts as a venous limb for blood to be returned to the patient. The catheter is tunnelled under the skin and a cuff surrounding the catheter triggers a fibrotic reaction in the surrounding tissue which helps to hold the catheter in position and reduce the risk of
infection. Dialysis catheters are much less preferable than AVFs due to the high risk of infection, malfunction from thrombus formation or kinking. Delivered blood flow rates are usually less than those which can be achieved with AVF leading to reduced dialysis efficiency.

Figure 1-4: Diagram of a tunnelled dialysis catheter (reproduced with permission from Blausen Gallery 2014: Medical Gallery of Blausen Medical 2014. Wiki Journal of Medicine)

Blood is diverted into a dialyser which is usually made from synthetic polymers such as polyamide or polysulfone and is more biocompatible than older dialysers which used to be made of cellulose and caused activation of complement as blood passed through the membrane. Dialyser membranes contain small pores which allow solutes and fluid to be exchanged between the blood and dialyser compartment. Solutes such as urea, creatinine and phosphate diffuse from the patient’s blood into the dialyser, whereas solutes such as bicarbonate and/or calcium move from the dialysis fluid into the blood stream (Figure 1-5).
Modern dialysers have been developed which contain larger pores (high-flux dialysers) which allow larger uraemic solutes such as β2-microglobulin to be removed. β2-microglobulin is a product of normal cell turnover and accumulates in renal failure. It is not removed efficiently by low-flux dialysers and cellulose dialysis membranes. Dialysis-related amyloid – a disabling condition, is caused by the accumulation and polymerisation of β2-microglobulin within tendons, synovium, osteoarticular sites and in other organs. The routine use of high-flux dialysers have been recommended since the publication of two landmark clinical trials (HEMO and MPO study) (Cheung et al. 2003; Locatelli et al. 2009) showed that treatment with high-flux dialyser membranes improved survival in patients with hypoalbuminemia or had been on dialysis for longer than 3.7 years. Removal of larger uraemic solutes (or middle molecules) can be enhanced by the addition of a prescribed convective component to standard high-flux haemodialysis, also known as haemodiafiltration (HDF). In this procedure, between 12-25L of fluid is removed from the patient using a haemodiafiltration pump and replaced with substitution fluid. The development of purification techniques to produce cheap ultrapure dialysis fluid on-line, has allowed HDF to become a viable routine therapy for kidney failure (Canaud & Lertdumrongluk 2012).
1.6 Outcomes for dialysis patients

Despite significant advances in many aspects of dialysis therapy, patient outcomes and survival remain poor. Studies of large US registry data sets show that following dialysis initiation, expected remaining lifespan is approximately 8 years for patients aged 40-44, for those initiating between aged 60-64 years expected remaining lifespan is 4.5 years (Collins et al. 2010), comparable to cancer patients with metastases. Risk factors for poor survival can be separated into dialysis and non-dialysis related (Table 1-2).

<table>
<thead>
<tr>
<th>Non-dialysis related</th>
<th>Dialysis related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased co-morbidity</td>
<td>Inadequate delivered dialysis dose</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Excess ultrafiltration rates (&gt;10mL/kg/hour)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Inadequate ultrafiltration and volume overload</td>
</tr>
<tr>
<td>Elevated calcium-phosphate product</td>
<td>Use of bio-incompatible haemodialysis membranes</td>
</tr>
<tr>
<td>Underlying renal disease</td>
<td>Use of tunnelled dialysis catheters</td>
</tr>
<tr>
<td>(reduced survival with diabetic nephropathy)</td>
<td></td>
</tr>
<tr>
<td>Elderly</td>
<td></td>
</tr>
<tr>
<td>Caucasian ethnicity</td>
<td></td>
</tr>
<tr>
<td>(lower mortality rates observed in Asian/Blacks compared to Caucasian)</td>
<td></td>
</tr>
<tr>
<td>Poor nutrition</td>
<td></td>
</tr>
<tr>
<td>Lack of residual renal function</td>
<td></td>
</tr>
<tr>
<td>Presence of inflammation</td>
<td></td>
</tr>
<tr>
<td>Poor compliance with dialysis</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-2 – Dialysis and non-dialysis related risk factors associated with reduced survival in dialysis patients
The most common cause of death in the dialysis population is cardiovascular disease (Kalantar-Zadeh et al. 2006) despite adjustments for age, gender, race and diabetes suggesting that dialysis patients are more susceptible to accelerated atherosclerosis (Lindner et al. 1974). Traditional Framingham cardiovascular risk factors are more common in patients with end-stage kidney disease (ESKD), however they do not completely account for the high cardiovascular morbidity (Cheung et al. 2000). Furthermore, there is an inverse relationship between some conventional cardiovascular risk factors such as cholesterol and obesity with mortality (Liu et al. 2004; Kalantar-Zadeh et al. 2005). Non-traditional factors such as inflammation are increasingly recognised to be additional important factors. The prevalence of elevated inflammatory biomarkers and the realisation of their close association with poor outcomes in 1990s established inflammation as a non-traditional risk factor for patients with ESKD (Carrero & Stenvinkel 2010).

1.7 Inflammation in end-stage kidney disease

Chronic inflammation is a highly prevalent condition in patients treated with haemodialysis and peritoneal dialysis. Between 30-50% of dialysis patients have evidence of elevated levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and C-reactive protein (CRP) which are not related to systemic infection (Yeun et al. 2000; Zimmermann et al. 1999; Herbelin et al. 1991; Stenvinkel & Alvestrand 2002). Inflammation in ESKD has been linked with a number of problems including increased cardiovascular mortality and protein energy wasting. The term Malnutrition-Inflammation Complex Syndrome (MICS) has been used to describe patients who exhibit this complicated syndrome and exhibit poor prognostic outcomes (Kalantar-Zadeh et al. 2003). Pro-inflammatory cytokines may directly cause anorexia via its influence on the brain leading to reduced oral intake and malnutrition. Inflammation can also lead to increased resting energy expenditure via the suppression of anabolic hormones including insulin growth factor-1 and testosterone (Akchurin & Kaskel 2015).
Other adverse consequences of inflammation includes anaemia and resistance to erythropoietin treatment via multiple mechanisms including suppression of production and stimulatory activity by erythropoietin and disruption of iron metabolism by increasing production of hepcidin. TNF-α is a potent inducer of NF-kB ligand (RANKL), which triggers osteoclast activation and bone resorption and is associated with incident fractures in dialysis patients. IL-6 and IL-1 may also suppress PTH production and it has been suggested in some patients, low PTH may reflect MICS rather than low bone turnover disease (Akchurin & Kaskel 2015).

Figure 1-6: Causes and consequences of inflammation in kidney disease (adapted from Akchurin et al, 2015)

Despite the prevalence of inflammation in patients with kidney disease and the large body of evidence which describes this phenomenon, chronic inflammation remains a poorly understood syndrome. This issue is further complicated due to the lack of consensus on how to define inflammation. CRP is the most commonly reported inflammatory biomarker in studies due to its widespread availability and is produced by hepatocytes in response to elevated IL-6 levels in the setting of acute inflammation. It is considered a prominent marker of cardiovascular mortality in the general population and in CKD patients. However the optimal CRP ‘cut-off’ point to diagnose inflammation remains unclear (KDOQI Workgroup 2005).
**1.7.1 Other biomarkers of inflammation in kidney disease**

IL-6 is a pro-inflammatory cytokine secreted by numerous cell types during an acute inflammatory response. It stimulates the production of CRP from hepatocytes and other acute-phase reactants. Studies in haemodialysis patients show that IL-6 is the most accurate prognostic marker compared to CRP, tumour necrosis factor alpha (TNF-α) and vascular cell adhesion molecules (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (Tripepi et al. 2005; Pachaly et al. 2008).

TNF-α is a traditional pro-inflammatory cytokine originally associated with killing of tumour cells. Together with IL-6 it is elevated in most, if not all inflammatory states and has been recognised as a target of therapeutic intervention (Scheller et al. 2011). TNF-α regulates both pro- and anti-inflammatory cytokine and provides rapid host defence against infection but can be fatal in excess (Panichi et al. 2012). TNF-α levels are linked with increased risk of coronary artery disease (Mendall et al. 1997) and are well reported to be consistently up-regulated in ESKD patients (Panichi et al. 2012).

Recently discovered biomarkers such as pentraxin 3 (PTX-3) and soluble tumor necrosis factor-like weak inducer of apoptosis (TWEAK) have attracted interest in uraemic inflammation (Alles et al. 1994). PTX-3 is an acute phase protein produced in the vasculature by numerous cell types including macrophages, fibroblasts, endothelial cells, neutrophils and dendritic cells and is considered to be a promising inflammatory biomarker. It is released rapidly from neutrophil granules in response to microbial challenge or an inflammatory stimulus making it a more sensitive marker of early inflammation than CRP. PTX-3 levels increased significantly post haemodialysis compared to CRP and IL-6 suggesting that the haemodialysis procedure itself may play a significant role in inducing a chronic inflammatory state (Sjöberg et al. 2012). PTX levels are increased in CKD and haemodialysis patients and strongly predict cardiovascular and all-cause mortality independent of CRP levels (Tong et al. 2007).

TWEAK is a member of the TNF superfamily which mediates its effect via the Fn14 receptor (fibroblast growth factor inducible-14) by inducing an inflammatory and
muscle wasting response (Rietschel et al. 1994). TWEAK participates in inflammatory reactions with other cytokines in the renal tubules. IL-6 and TWEAK levels are associated with increased risk of mortality and muscle wasting in HD patients (Carrero et al. 2009).

Overall, the definition of chronic inflammation in ESKD patients requires clarification although IL-6 and CRP appear to be the most useful biomarkers. It remains unclear whether inflammation causes vascular injury or is a consequence of vascular injury itself, although there is evidence to suggest that inflammatory biomarkers IL-6 and CRP are not simply markers of inflammation but also mediators of atherosclerosis (Paffen & deMaat 2006). High-sensitivity CRP in the general population is able to accurately predict future cardiovascular risk independent of cholesterol, Framingham coronary risk score, blood pressure and in individuals with and without subclinical atherosclerosis (Bassuk et al. 2004).

Chronic inflammation in the ESKD population is a significant clinical problem and targeted anti-inflammatory interventions are currently not available, increasing knowledge and understanding of the pathophysiology could potentially facilitate development of anti-inflammatory strategies to improve quality of life and survival in dialysis patients.

1.7.2 Causes of inflammation in ESKD

The underlying mechanism of chronic inflammation remains to be elucidated but several patient and dialysis-related factors have been proposed.

1.7.2.1 Reduced kidney function and the uraemic milieu

Deteriorating kidney function per se may contribute to increased inflammation due to reduced clearance of pro-inflammatory cytokines. Although the relationship may be bi-directional as inflammation itself may drive loss of GFR (Guessous et al. 2014). Renal failure in animal models induced by nephrectomy leads to a rapid rise in TNF-α due to decreased clearance (Bemelmans et al. 1993). Studies in humans show that urinary excretion of soluble IL-6 receptor is reduced in CKD patients compared to healthy controls, yet the ability to release soluble IL-6 receptor from monocytes
are similar in both CKD patients and controls suggesting that elevated plasma levels of soluble IL-6 receptor is due to reduced renal excretion rather than increased generation (Memoli et al. 2000). Bolton et al found that in multiple regression analysis serum creatinine is the sole determinant of plasma IL-6 level in pre-dialysis and dialysis patients (Bolton et al. 2001). In patients with ESKD treated with peritoneal dialysis, inflammatory markers such as CRP are closely associated with the level of residual renal function (RRF) (Chung et al. 2001). Reduction of kidney function also leads to reduced clearance and subsequent retention of advanced glycosylation end (AGE) products and pro-oxidants contributing to a pro-inflammatory milieu. Uraemia itself also leads to increased carbamylation of proteins such as LDL and carbamylated proteins have greater capacity to inflict endothelial damage and initiate atherosclerotic plaques contributing to the pathogenesis of atherosclerosis (Ok et al. 2005). The retention of uraemic solutes in kidney failure increases the availability of substrates for oxidative injury. For example, the accumulation and increased availability of β2-microglobulin in kidney disease, leads to progressive oxidation and glycosylation by AGE products contributing to the acidification of β2-microglobulin. Isoforms of β2-microglobulin with a more acidic isoelectric point are recognised as a component of amyloid deposits in dialysis patients. In addition, AGE-modified β2-microglobulin binds to AGE receptors on monocytes triggering their activation, cytokine production and further reactive oxygen species formation (Himmelfarb et al. 2002). The pro-oxidative environment in dialysis patients is further compounded by the reduced oral intake of some types of antioxidants such as vitamin C (Kalantar-Zadeh et al. 2002).

1.7.2.2 Patient-related factors

A number of patient-related factors may contribute to the persistent inflamed stated in ESKD patients. Certain underlying renal conditions which may have been the original cause of kidney disease such as systemic lupus erythematosus or amyloid disease may act as a persistent driver of inflammation. Development and progression of other conditions such as cardiac ischemia, peripheral vascular disease and chronic diabetic ulcers may also have a similar impact.
Genetic factors are also likely to play a role due to the observation that the mortality risk is higher in Caucasian ethnic groups than Asian groups amongst US-based dialysis patients (Wong et al. 1999). Black ethnicity also confers a survival advantage in UK dialysis patients despite greater deprivation and co-morbidity scores (Cole et al. 2014). Frequency of single nucleotide polymorphisms in the CRP gene that are associated with increased plasma level of CRP are two-fold higher in Caucasians than Blacks (Szalai et al. 2002). Whereas single nuclear polymorphisms in the gene for the anti-inflammatory cytokine IL-10 that are associated with decreased production for IL-10 are found in those with increased inflammation and cardiovascular morbidity (Girndt et al. 2002).

Periodontitis has also been proposed as an occult source of inflammation in the haemodialysis population (Kadiroglu et al. 2006). Periodontal disease is a spectrum of inflammatory disease affecting the tooth-supporting structures. The disease begins with the adherence of a bacterial biofilm on the surfaces of the teeth resulting in gingivitis which can be reversible if the biofilm is removed (Schaudinn et al. 2009). The disease can progress leading to destructive periodontitis and tooth loss. Periodontal disease is more prevalent in CKD population (Dye et al. 2007) and a recent review has found an association between periodontitis in haemodialysis patients with inflammation and malnutrition (Akar et al. 2011). Moderate to severe periodontitis results in systemic inflammation with elevated levels of pro-inflammatory cytokines and CRP (Loos 2005; Pradeep et al. 2011). Treatment of periodontal disease reduces the levels of inflammatory biomarkers in dialysis patients (Siribamrungwong & Puangpanngam 2012). However it remains unclear if periodontitis causes inflammation or whether chronic inflammation predisposes patients to periodontitis (Kadiroglu et al. 2006). The underlying mechanism is unclear but maybe mediated via endotoxemia (Geerts et al. 2002) (see).

1.7.2.3 Dialysis-related factors

The dialysis procedure itself can contribute to the chronic inflammatory state seen in ESKD patients. The use of dialysis catheters in the haemodialysis population is associated with an elevated inflammatory response even in the absence of infection (Sabry et al. 2014). Clotted arterio-venous grafts and catheters may harbour chronic
or subclinical infection due to biofilm formation. Conversion of haemodialysis catheters to fistulas lead to significant reduction in CRP level in incident patients compared to those who continue to rely on catheters for haemodialysis (Goldstein et al. 2009).

Contaminated dialysis fluid may be a potential source of inflammation. Although dialysis membranes are impermeable to bacteria, soluble pyrogenic bacterial products such as endotoxins may penetrate the dialysis membrane and enter the blood stream activating monocytes and inducing cytokine production. Endotoxins are fragments of bacterial cell wall which are thought to be able to cross the dialysis membrane via back-filtration (Panichi et al. 1998). This hypothesis is further supported by the observation that using ultrapure dialysis fluid significantly reduce IL-6 and CRP levels (Schiffl et al. 2001). UK and European guidelines have advocated the routine use of ultrapure dialysis fluid which may reduce the prevalence and impact of dialysis fluid contamination (European Best Practice Guidelines 2002). Ultrapure dialysis fluid is produced by treating water with reverse osmosis and passing the treated water through a series of filters, the ultrapure water is then mixed with the desired acid and bicarbonate concentrate to obtain ultrapure dialysis fluid that can be safely infused directly into the patient in high volumes. Water quality meets strict criteria as set by the European Best Practice Guidelines (EBPG) and Association for Advancement of Medical Instrumentation (AAMI) to avoid delivering any pyrogenic or inflammatory stimulus to the patient. Ultrapure dialysis fluid must contain <0.1 colony-forming units (CFU)/ml and <0.03 endotoxin units (EU)/ml (Canaud & Lertdumrongluk 2012).

Repeated exposure of blood to artificial membranes, in particular to bio-incompatible membranes made by cuprophane, may activate neutrophils and complements and maintain a chronic inflammatory state. Some studies suggest use of biocompatible membranes help to decrease inflammatory status (Schindler et al. 2000). Similarly for peritoneal dialysis patients, the use of bio-incompatible dialysis fluid may also contribute to systemic inflammation, in particular use of very high glucose concentration dialysis fluid may increase oxidative stress due to the presence of glucose degradation products (Yao et al. 2004).
For haemodialysis patients, the gastrointestinal tract has been proposed as another source of endotoxins or bacterial products that may lead to chronic inflammation. Haemodialysis patients are subject to recurrent episodes of volume overload due to oral fluid intake and accumulation during the inter-dialytic period, during haemodialysis, excessive ultrafiltration may be utilised to remove the accumulated fluid. Gut oedema and ischemia induced by this cycle of volume accumulation and depletion may lead to bacterial endotoxin and fragment translocation through the gut barrier activating signals to release pro-inflammatory cytokines (Hauser et al. 2011). Endotoxemia represents an attractive therapeutic target, recently endotoxin extracorporeal methods have been developed for use in the intensive care setting to treat severe sepsis with some success reported in terms of improved surrogate markers such as inotrope use and haemodynamic stability (Klein et al. 2014). It is possible that such methods could be applied during haemodialysis to reduce endotoxin burden to improve clinical outcomes. However the association between endotoxemia and inflammation requires confirmation before embarking on interventional trials since there are significant limitations with current blood endotoxin detection methods and interpretation of conclusions drawn from endotoxin literature in the dialysis population requires caution (see Chapter 2 – Endotoxemia in end-stage kidney disease).
Endotoxins are complex lipopolysaccharides that are found in the outer cell wall of all gram negative bacteria (Figure 2-1). The terms lipopolysaccharide (LPS) and endotoxin are often used interchangeably.

LPS refers to the purified form used as standards in commercial endotoxin detection assays (Williams 2007). Endotoxins are large molecular structures varying from 10 to 1000 kDa consisting of a lipid A antigen attached to a carbohydrate core and polysaccharide O antigen (Figure 2-2).
Endotoxins are implicated in the pathogenesis of sepsis, especially with gram negative infection. They can activate a systemic inflammatory reaction by triggering complement, coagulation, fibrinolytic, and kinin pathways to release vasoactive peptides and cytokines from monocytes, neutrophils and vascular endothelial cells (Ostro 2013; Hurley 1995). Nearly all these effects are mediated through the lipid A region.

2.1 Endotoxin and inflammation

Endotoxemia was initially proposed as a cause of chronic inflammation in patients with congestive heart failure (Anker et al. 1997), as treatment of this condition with diuretics resulted in significant reduction in levels of inflammatory mediators and blood endotoxin concentration (Niebauer et al. 1999). Proposed molecular mechanisms for the endotoxin-activated inflammation include the production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin (IL-6) following the activation of the CD14/Toll-like receptor 4 (TLR4)
complex found on monocytes and macrophages by the lipid A region of endotoxin (Heumann & Roger 2002; Genth-Zotz et al. 2002).

Activated TLR4 complex stimulates the translation and production of cytokines via transcriptional nuclear factor k-light chain enhancer of activated B cells (NF-κB) (Figure 2-3) (Hauser et al. 2011). The extent of TLR4 activation is dependent on the chemical configuration of the lipid A moieties such as the number of secondary acyl chains and the number and position of phosphate groups. The chemical configuration varies in different species of bacteria, hence the immunogenic potential may differ depending on the bacterial origin of the endotoxin (Gnauck et al. 2016).

Figure 2-3: Proposed molecular signalling of endotoxin (reproduced with permission Wong et al 2014, Seminars in Dialysis)

LPS, lipopolysaccharide; LPS/LBP, lipopolysaccharide binding protein complex; TNF, tumour necrosis factor; PAF, platelet-activating factor; IL, interleukin; TLR, toll-like receptor; NF-κβ, nuclear factor-κβ

Experimentally-induced uraemia in the rat model is associated with elevations in blood endotoxin concentration and inflammatory cytokines such as TNF-α (Hauser et al. 2010). The association of endotoxemia with inflammatory biomarkers has been described in both haemodialysis (El-Koraie & Naga 2013; Christopher McIntyre 2011; Terawaki et al. 2010) and peritoneal dialysis patients (Szeto et al. 2010; Kwan,
Chow, Leung, et al. 2013). Other circulating bacterial products such as bacterial DNA fragments have also been associated with inflammation (Bossola et al. 2009; Kwan, Chow & Leung 2013; Wang, Jiang, et al. 2012).

2.2 Potential sources of endotoxemia

2.2.1: Contamination of haemodialysis apparatus and devices

Patients with chronic kidney disease and especially those on haemodialysis have multiple potential sources for endotoxemia. It is well known that patients who dialyse with tunnelled dialysis catheters have higher rates of infection-related morbidity than patients with arteriovenous fistulas and grafts, due to the formation of bacterial biofilm which are potential sources of persistent bacteraemia and endotoxin (Dasgupta 2002). Biofilm formation may also occur in fluid pathways of dialysis machines and sloughing of bacterial biofilm resulting in bacteraemia and endotoxemia has been described (Dasgupta 2002). With the advent of high flux dialysis, haemodiafiltration and the increasing use of bicarbonate containing dialysates which can support rapid bacterial growth (Oliver et al. 1992), there is a concern that cytokine-inducing substances, such as endotoxin may transfer across the dialysis membrane by convective transfer (back-filtration) or down a concentration gradient (back diffusion) (Glorieux et al. 2012). In-vitro and in-vivo studies have demonstrated that endotoxin transfer into blood compartments is much higher with high-flux than low-flux membranes (Henrie et al. 2008; Vanholder et al. 1992). Although a significant proportion of endotoxins are adsorbed onto the dialysis membrane, there is evidence to suggest that they are still capable of activating monocytes and macrophages (Tsuchida et al. 2004). There have been no studies on the differential impact of standard haemodialysis versus haemodiafiltration on endotoxin influx or clearance. Due to the added convective element of haemodiafiltration, endotoxin influx could be increased due to the increased volumes of substitution/dialysis fluid infused into patients, conversely haemodiafiltration could have a beneficial effect of increasing clearance of endotoxins and other relevant molecules such as pro-inflammatory cytokines from the blood circulation.
More rigorous guidelines for dialysate water purity have been implemented over the last 2 decades. Ultrapure dialysis fluid must have <0.1 colony forming units per mL (CFU/mL) and <0.03 endotoxin units per mL (EU/mL) according to the international guidelines (International Organization for Standardization 2014). However, patients on haemodiafiltration can receive convective volumes of up to 25L per session. If substitution fluid was contaminated with 0.029 EU/ml, this would meet the requirement for microbiological purity but patients could theoretically still receive a significant dose of endotoxin over 700 EU with each dialysis session or approximately 180 EU/hour. It has been established that 5 EU/kg/hour or 350 EU/hour in a healthy 70kg adult is sufficient to induce pyrogenic symptoms such as rigors, fever and nausea (Greisman & Hornick 1969). Recent studies have found that switching patients to ultrapure dialysate resulted in reduction of plasma endotoxin levels (Kwan & Szeto 2013). In a separate study, the proportion of patients with significant endotoxemia reduced from 17% to 9% after switching from standard to ultrapure dialysate. Efforts to reduce patient exposure to bacterial contamination and exogenous endotoxin should be focused on increasing the use of arteriovenous fistulas and increasing microbiological quality of dialysis fluid with regular testing, disinfection of haemodialysis systems to detach biofilms (Ledebo & Nystrand 1999; Man et al. 1998) and installation of ultra-filters in the dialysis circuit (Lonnemann 2004). Given the increase in use of haemodiafiltration which increases water exposure to patients, it may be necessary to consider reducing the threshold tolerated endotoxin contamination from <0.03 EU/ml to <0.001 EU/ml as currently practiced in Japan (Kawanishi et al. 2009).

2.2.2: CKD and the leaky gut

The gastrointestinal tract has a huge reservoir of bacteria which may be a significant source of endotoxemia in patients with CKD. The gut flora is altered in uraemia with evidence of heavy bacterial colonisation of the duodenum and jejunum which are normally not colonised (Simenhoff et al. 1978). This is of particular importance given that there are numerous animal and in-vitro studies showing increased intestinal permeability documented by the penetration of polyethylene glycols of different molecular weights across the intestinal wall in uraemic rats. Bacterial translocation
from the gut into mesenteric lymph nodes, liver and spleen in experimental uraemia has also been shown (Wang, Zhang, et al. 2012). This suggests that translocation of bacterial products or endotoxins may occur in uraemia. Studies from the cardiology literature have demonstrated elevated levels of endotoxins and markers of inflammation in patients with congestive heart failure (Niebauer et al. 1999) possibly due to the effect of increased gut permeability caused by an oedematous bowel wall (Sandek & Bauditz 2007). Invasive assessment of patients with acute heart failure have documented higher concentrations of active lipopolysaccharide in the hepatic vein than the left ventricle, suggesting intestinal origin of endotoxemia (Peschel et al. 2003). In addition to gut oedema, intestinal ischemia may play a significant role given the vulnerability of villous tips to ischemia and hypoxia (Riddington et al. 1996; Takala 1996) that occurs when blood flow is shunted away from the splanchnic region in heart failure (Parks & Jacobson 1985). Mucosal ischemia has been reported in patients on haemodialysis (Diebel et al. 1993) and ultrafiltration also reduces splanchnic blood volume (Yu et al. 1997). It is hypothesised that bacterial translocation across the gut wall could also be occurring in haemodialysis patients who frequently experience large blood volume changes during dialysis. McIntyre et al found that circulating levels of blood endotoxins rose markedly on initiation of haemodialysis (Christopher McIntyre 2011). But currently, there is lack of direct evidence of intestinal bacterial translocation in dialysis patients (Ritz 2011).

2.2.3: Periodontal disease and endotoxemia

Periodontal disease embraces a spectrum of inflammatory disease affecting the tooth-supporting structures. The disease begins with the adherence of a bacterial biofilm on the surfaces of the teeth resulting in gingivitis which is potentially reversible on removal of the biofilm (Schaudinn et al. 2009). The disease can progress leading to destructive periodontitis and tooth loss. Periodontal disease is prevalent in CKD population (Dye et al. 2007) and a recent review found an association between periodontitis in haemodialysis patients with inflammation and malnutrition (Akar et al. 2011). Plaque samples from healthy individuals consist of mainly gram positive aerobic bacteria. With worsening severity of periodontal disease, there is a shift towards increasing numbers of gram negative anaerobic
species such as *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus* and *Porphyromonas gingivalis*. In the non-CKD population, high antibody titres to periodontal pathogens was associated with high endotoxin levels and increased hazard risk for cardiovascular disease (Pussinen et al. 2007). Endotoxin levels increase after gentle mastication in patients with periodontitis (Geerts et al. 2002). It is likely that chronic periodontal disease is a significant contributor to endotoxemia and inflammation in dialysis patients. Aggressive dental treatment of periodontitis reduces levels of high-sensitivity C-reactive protein in peritoneal dialysis patients (Srirbamrungwong et al. 2013). Chronic periodontitis is associated with increased mortality in haemodialysis patients (de Souza et al. 2013), although whether aggressive treatment would improve long term outcome requires investigation.

### 2.3 Problems with interpreting level of endotoxemia in the dialysis population

Reported concentrations of blood endotoxin in both healthy and ESKD populations vary significantly with some studies detecting significant endotoxemia in only a small number of patients (Gnauck et al. 2016). In the CKD population, although most studies report a high prevalence of endotoxemia in dialysis population, there is inconsistency in the literature with some studies detecting endotoxemia in only a small proportion of patients (Markum 2004; Taniguchi et al. 1990), (Table 2-1). This is further complicated by the expression of endotoxin concentration in different units – usually in either weight or endotoxin units (EU). Both of these issues make clinical interpretation of endotoxin levels difficult (Table 2-1).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Method of endotoxin detection used</th>
<th>Reported mean or median endotoxin concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Suzuki &amp; Honda 2011)</td>
<td>HD</td>
<td>LAL turbidimetric</td>
<td>Detectable endotoxaemia in 8/58 patients (0.9-1.0 pg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endotoxin Activity Assay (EAA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.17+/-0.11 to 0.45+/-0.16 EAA units</td>
</tr>
<tr>
<td>(Markum 2004)</td>
<td>HD</td>
<td>LAL chromogenic</td>
<td>4.63 pg/dL to 5.4 pg/dL</td>
</tr>
<tr>
<td>(Christopher McIntyre 2011)</td>
<td>HD, PD, CKD</td>
<td>LAL chromogenic</td>
<td>PD - 0.56 EU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HD - 0.64 EU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CKD v - 0.11+/-0.68 EU/ml</td>
</tr>
<tr>
<td>(El-Koraie &amp; Naga 2013)</td>
<td>HD</td>
<td>LAL gel-clot assay</td>
<td>76.3+/-42.09 pg/ml</td>
</tr>
<tr>
<td>(Szeto et al. 2010)</td>
<td>PD</td>
<td>LAL chromogenic</td>
<td>0.7+/-0.3 EU/ml</td>
</tr>
<tr>
<td>(Kwan, Chow, Leung, et al. 2013)</td>
<td>PD</td>
<td>LAL chromogenic</td>
<td>1.95+/-0.63 EU/ml</td>
</tr>
<tr>
<td>(D. Raj et al. 2009)</td>
<td>HD</td>
<td>LAL chromogenic</td>
<td>0.65 EU/ml</td>
</tr>
<tr>
<td>(Terawaki et al. 2010)</td>
<td>HD</td>
<td>Endotoxin Scattering Photometry</td>
<td>0.23-0.37 pg/ml</td>
</tr>
<tr>
<td>(Taniguchi et al. 1990)</td>
<td>HD</td>
<td>LAL chromogenic</td>
<td>Significant endotoxemia detected in 6/87 patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean 27.6pg/ml</td>
</tr>
<tr>
<td>(Kwan &amp; Szeto 2013)</td>
<td>HD</td>
<td>LAL chromogenic</td>
<td>0.209+/-0.044 EU/ml</td>
</tr>
<tr>
<td>(Szeto et al. 2008)</td>
<td>PD, CKD, controls</td>
<td>LAL chromogenic</td>
<td>PD – 0.44+/-0.18 EU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CKD – 0.035+/-0.009 EU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control – 0.013+/-0.00 EU/ml</td>
</tr>
</tbody>
</table>

Table 2-1: Published levels of endotoxemia detected in dialysis patients. 1EU equates approximately to 100-120pg/ml depending on bacterial source (Stadlbauer et al. 2007)

It is recommended that endotoxin concentration should be reported in endotoxin units (EU) rather than expressing the weight of endotoxin as a unit of measurement (Outschoorn 1982). This is because the potency of control standard endotoxins used in the LAL assay to generate the standard curve differs between manufacturers therefore expressing blood endotoxin levels in terms of weight makes it difficult to interpret. The potency of standard endotoxins are expressed in relation to a single
historical reference standard endotoxin derived from *E.coli* (EC-2) established by the United States Pharmacopoeia (Williams 2007). Earlier studies demonstrated that injecting 1ng/kg of EC-2 could trigger a pyrogenic response in man (Greisman & Hornick 1969). Therefore, one EU was defined as one-fifth of the amount of *Escherichia coli* (EC-2) endotoxin required to bring about a pyrogenic reaction in humans and reporting in EU is recommended since it reflects endotoxic activity of a sample relative to a reference standard endotoxin which would allow comparison of assay results obtained from different manufacturers.

Although the vast majority of endotoxin studies in the dialysis population report detectable endotoxemia in apparently clinically stable non-infected dialysis patients, the levels reported appear unusually high, ranging from 0.209 to 1.95 EU/ml, which exceeds the pyrogenic threshold since it is well established that as little as 4.1-5 EU/kg/hr is sufficient to trigger symptoms such as fever and hypotension in man (Greisman & Hornick 1969) (equivalent to approximately 0.12 EU/mL in an average man weighing 70kg with an average plasma volume of 3L).

Several issues remain unclear:

- Why do levels of endotoxemia in the dialysis population vary so widely?
- Why do studies report such apparently high levels of blood endotoxin in stable dialysis patients that exceed the pyrogenic threshold?
- Why are some studies unable to detect endotoxemia in a significant proportion of their study cohort?

If elevated blood endotoxin levels were a true and consistent phenomenon in the dialysis population it is unclear whether blood endotoxin level is persistently or only transiently elevated and the primary source of endotoxemia is unclear. In addition, it is unknown whether circulating endotoxemia directly contributes to unexplained inflammation that are commonly seen in dialysis patients since not all studies report an association between endotoxemia and inflammatory biomarkers (Gonçalves et al. 2006). The conflicting data and literature reported may partly be due to methodological issues with the detection assays used to measure blood endotoxins – specifically the Limulus Amoebocyte Lysate (LAL) assay (see section 3.1). Before
embarking on interventional trials to examine the efficacy of endotoxin-lowering therapies it is important to clarify the phenomenon of endotoxemia in haemodialysis patients and to examine the reported associations with inflammation
Chapter 3

3. Methods of blood endotoxin detection

The first method approved by the US Food and Drug Administration for endotoxin detection was the rabbit pyrogen test. This method was developed in the 1920s, and its basis was intravenous injection of a test solution into rabbits which were monitored for a rise in body temperature. Rabbits have similar endotoxin tolerance to humans and a rise in body temperature is used to indicate the presence of significant amounts of pyrogens or endotoxin in the injected test solution. This method was expensive and time consuming and was gradually replaced by the Limulus Amoebocyte Lysate (LAL) assay. Due to its high sensitivity and ability to quantify endotoxins, the LAL assay has been the most extensively used and researched method in blood endotoxin detection (Novitsky 1994).

3.1 The Limulus Amoebocyte Lysate Assay

During the 1960s, Levin and Bang observed that Limulus Polyphemus, the horseshoe crab, suffered from disseminated intravascular coagulation when infected with gram negative bacteria. In-vitro work demonstrated that this coagulation reaction was due to endotoxin-initiated activation of a cascade of enzymes present in primitive cells, called amoebocytes found in the haemolymph of the horseshoe crab (Young et al. 1972). The diagnostic potential of this reagent was realised and a purified enzyme system was produced that could be activated by picograms of endotoxin. The principle of the LAL assay is based on a gelation reaction resulting in the formation of a clot, following activation of a cascade of enzymes by endotoxin (Figure 3-1).
Early attempts to detect endotoxins in substances using the gel clot assay involved incubating a test sample with LAL in an assay tube and then inverting the tube to see if the solution had clotted to form a firm gel. This method could only quantify endotoxin semi-quantitatively and is rarely used in practice, photometric methods such as the end-point and kinetic LAL assays are more commonly used.

### 3.1.1 End-point LAL assay

The end-point technique is an earlier photometric method used to measure and quantify endotoxin. The LAL is incubated with the test sample and observed for a period of time (usually around 10 minutes) using a spectrophotometer with a 405-410 nm filter, during this time the LAL reacts with endotoxin present in the sample activating the coagulation cascade and increasing the optical density of the mixture. The reaction is then stopped with stop reagents such as acetic acid and the optical density of the mixture is recorded. Samples with highest concentration of endotoxin react quickly and achieving the highest optical density by the end of the incubation period. A standard curve is constructed based on the optical density recorded at the end of the incubation period for dilutions of control standard endotoxin. The main
advantage of end-point techniques is that results can be obtained relatively quickly, however the disadvantages are that it relies on the user being present to stop the reaction of all the samples at the same time before reading. This may introduce some variability in results. Since the optical density is used to construct the standard curve, the end-point method has a narrow range of sensitivity, this range is generally about a factor of 10, commonly referred to as 1 log (e.g. 0.1 to 1 EU/mL) (Nema & Ludwig 2010). This is because for samples with high endotoxin content, the solution would have fully coagulated already by the end of the incubation, making it difficult to accurately distinguish between samples that have similarly high levels of endotoxin. In samples with low endotoxin, the reaction would not have started after such a short incubation period (usually around 10 minutes), therefore the end-point method is not sufficiently sensitive to detect very low concentration endotoxin.

3.1.2 Kinetic LAL assays

The kinetic LAL assay is a more recent photometric method. In this assay, the reaction between LAL and the test sample is observed with a spectrophotometer using an incubating microplate reader. As the reaction progresses, the mixture starts to coagulate and the turbidity and optical density of the solution progressively increases. The change in optical density over time is used to quantify the concentration of endotoxin present in the sample (Figure 3-2).

![Figure 3-2: Typical optical density graph obtained from a sample containing endotoxin](image)

An alternative method using the same LAL cascade is the kinetic chromogenic assay, which replaces the coagulogen component of the cascade with a synthetic
chromogenic substrate that is linked to p-nitroaniline. Activation of the cascade cleaves p-nitroaniline from the colourless substrate to release a yellow colour (Cohen 2000). Both turbidimetric and chromogenic LAL assays are routinely used for endotoxin detection. The rate of increase in turbidity or intensity of the yellow colour is directly proportional to the concentration of endotoxin in the sample.

Both kinetic methods quantify the endotoxin content of samples either by using the ‘onset reaction time’ (time taken for the optical density of a mixture to reach a critical optical density) or Vmax (the maximum velocity of the reaction calculated using the linear component of the optical density graph). Different parameters derived from the optical density graph have also been used to calculate the endotoxin content previously (Gnauck et al. 2016) (see next Chapter: Validation of endotoxin detection methods). The kinetic LAL assays have significant advantages over the end-point methods including the benefit of automaticity, which reduces the variation introduced by operator technique and it also has a greater range of sensitivity, being able to quantify results over several logs compared to one log with the end-point method.

3.1.3: Problems with the LAL assay for blood endotoxin detection

The LAL assay is licensed for the testing of pharmaceutical products and dialysis fluid for contamination (Glorieux et al. 2012). Testing non-biological samples with the LAL assay is sensitive and specific for endotoxin detection but testing biological samples such as blood is much more difficult. Several inherent problems with the LAL assay and some specific issues relating to its use in blood testing exist therefore testing for endotoxin in blood with the LAL assay is not validated. Despite this, the LAL assay is the most commonly used blood endotoxin detection method.

3.1.3.1 Inherent variability of the LAL assay

The LAL test is a biological assay and is a lysate that is extracted from the osmotic lysis of amoebocytes found in the horseshoe crab. The extract is a crude mixture and not a single purified enzyme and consequently, significant batch-to-batch variation exists in the potency of lysates. Lysate potency is determined by testing each batch against reference standard endotoxin (RSE) usually in serial two-fold dilutions, therefore potency is not determined precisely and may lead to some
variation between different batches. However, RSE is expensive and is an exhaustible supply therefore manufacturers use control standard endotoxin (CSE), whose potency is determined against RSE for every lot of lysate which introduces another potential source of variation (Kumar 2006).

3.1.3.2 Specific issues with collection and processing of blood samples

Meticulous attention to specimen collection, handling and storage is important when obtaining blood samples for endotoxin testing with the LAL assay since multiple factors can influence the accuracy of the result (Hurley 1995). Blood samples should be taken under sterile conditions and collected in endotoxin-free tubes. The blood collection apparatus should not contain polypropylene which can adsorb endotoxins and significantly reduce detection. In haemodialysis patients, blood collection from central venous catheters should be avoided due to the high likelihood of introducing endotoxin contamination into the sample from bacterial biofilms. If plasma is used for testing, the choice of anticoagulant used to obtain plasma is important. Heparin is commonly used but doses as low as 10 IU/ml can be inhibitory to the LAL assay (Sturk et al. 1985). In addition, since heparin is derived from animal products, there is a risk of introducing endotoxin contamination into test samples. If EDTA is used as an anticoagulant this may cause sequestration of divalent cations which are necessary for optimal LAL enzyme activity. Cations may need to be added to compensate for the inhibition by EDTA (Dawson 2005). Processing time is also important since endotoxin is rapidly inactivated in untreated plasma. Samples should be collected and processed rapidly on ice, then frozen to preserve the stability of endotoxin (Hurley 1995). Additionally, a low centrifugal force should be used to obtain platelet-rich plasma since endotoxin can be bound to platelets (Peek et al. 2004; Salden & Bas 1994). Thus, it is clear that the method of blood collection for endotoxin measurement and sample storage requires due consideration to avoid interference with the LAL assay, for many published studies of endotoxemia in the dialysis population it is unclear whether these pre-analytical factors have been considered.
3.1.3.3 Specific problems with measuring endotoxin in blood

Components found in serum or plasma are known to affect the sensitivity of the LAL assay. The identity of many of these factors remain unknown but studies suggest that proteins in blood such as serine proteases (e.g. thrombin, plasmin) and serine protease inhibitors (e.g. anti-plasmin and anti-thrombin III) are components within blood that may non-specifically activate or inhibit the LAL assay (Fields 2006; Levin et al. 1970). The interaction between endotoxin and components of plasma and serum is complex. Components of plasma such as bile salts, proteins and lipoproteins can lead to binding and the formation of complexes or micelles resulting in the hydrophobic lipid A being ‘hidden’ from the aqueous environment, preventing its detection (Hurley 1995; Wang & Quinn 2010; Sandek et al. 2007). Endotoxin may also be removed from the blood by a number of different immune mechanisms including sequestration by immunoglobulins, antibacterial peptides such as bacterial permeability increasing protein, detoxification by macrophages via deacylation and binding by LPS-binding protein (LBP) leading to its transfer to HDL and other systemic lipoproteins (Gnauck et al. 2016). For dialysis patients, the effect of high level of uraemic solutes on the activity of LAL assay is also unclear and there is no published data which investigates this issue. The summation of these processes may lead to variability and difficulties in detecting endotoxin in blood especially in the dialysis population, for which the effect of uraemic solutes on LAL performance has never been investigated.

Additionally, the LAL assay can be subject to interference by multiple substances that may be present in blood caused by medical treatment. These include antibiotics such as penicillin (McCullough & Scolnick 1976), aminoglycosides (Artenstein & Cross 1989) and blood from patients dialysing with cellulose membranes, the materials of which may contain traces of (1-3)-β-D-glucan which can activate the LAL assay by an alternate enzymatic pathway (Taniguchi et al. 1990). It is therefore important to avoid sampling from patients who are treated with antibiotics or dialysis membranes with cellulose constituents.

Historically, multiple methods have been devised to remove interfering factors present in blood and has been reviewed extensively (Hurley 1995), but the most
effective and simple method involves heating and dilution of the sample. However, diluting the sample significantly reduces the threshold of detection. The current limit of detection using kinetic techniques is 0.001EU/ml (Brade 1999), but blood samples are usually diluted by a ten-fold dilution, this reduces the limit of detection to 0.01EU/ml. It is unclear if heating and dilution of the sample is sufficient to maximise recovery of endotoxin from blood samples, since recent work performed in healthy patients suggests that recovery of control standard endotoxin that has been spiked into raw plasma was poor despite dilution and heat treatment suggesting that any LPS sequestration process that occurs in plasma may not be easily removed (Gnauck et al. 2015).

3.1.3.4 Interference of LAL by (1-3)-β-D glucan

The LAL assay is not specific to endotoxin activation, it can also be activated by (1-3)-β-D glucan (BG) via an alternative enzymatic pathway by factor G activation (Morita et al. 1981). BG are major carbohydrate constituents of cereal, yeast and fungal cell walls (Young & Vincent 2005) with a variable molecular weight ranging from thousands to millions of daltons depending on origin. Elevated blood BG has been used a serum marker for invasive fungal infection (Young & Vincent 2005; Rop et al. 2009).

As discussed earlier, treatment with dialysis membranes composed of cellulose material are known to elevate blood BG levels in patients in the absence of invasive fungal infection. This could potentially lead to false activation of the LAL assay via factor G and artefactual elevation of blood endotoxin levels. A review of the reliability of BG detection assay in patients on renal replacement therapy concluded that treatment with modern non-cellulose haemodialysis membranes does not significantly alter serum BG levels (Prattes et al. 2015). However it was commented that in two studies that measured BG blood levels in lung transplant recipients to pre-emptively diagnose invasive fungal infections found that treatment with haemodialysis was a significant predictor of false positive elevation in blood BG (i.e. raised BG but no evidence of systemic fungal infection). Both studies exclusively used polysulfone membranes which are not known to alter blood BG levels and the
authors were unable to explain the relationship between exposure to haemodialysis and raised BG in their cohort (Hanson et al. 2012; Alexander et al. 2010).

Due to the significant methodological issues with the LAL assay for blood endotoxin detection assays, a number of alternative blood endotoxin detection assays have been developed.

3.2 Alternative direct blood endotoxin detection methods

3.2.1 Endotoxin Scattering Photometry (ESP) assay

The ESP assay is a relatively novel endotoxin detection assay with a reported lower limit of detection of 0.01pg/mL (Terawaki et al. 2010), which is approximately ten times more sensitive than current kinetic LAL techniques. The assay also requires the use of LAL reagents, however the technique differs from the conventional kinetic LAL technique by using a light-scattering photometer to detect the formation of coagulin particles which are precursor gel clots formed during the early stages of the LAL coagulation cascade (Figure 3-3).

Figure 3-3: Images of optical microscopic observations of aggregates formed by the LAL reaction with endotoxin at concentration of 0.05 EU/mL. (A) No aggregates at 8 minutes (B) Obscure aggregates at 12 minutes (C) Recognisable aggregates at 14 minutes (D & E) Snow-like crystal aggregates at 20 and 30 minutes respectively. Scale bar denotes 100µm. Reproduced from (Mitsumoto et al. 2009) with permission from John Wiley and Sons publishers.
The onset time of the rapid appearance of these coagulin particles is used to quantify endotoxin content. Since this method is based on the measurement of precursor gel clots from the early phase of the LAL reaction or coagulation cascade, it is able to detect small amounts of endotoxin more rapidly and with greater sensitivity. Use of the ESP assay in the clinical setting has been reported with some success. Endotoxin levels obtained from the ESP assay are higher in patients with septic shock (Kase & Obata 2010). The ESP assay was compared with the kinetic turbidimetric assay for discriminating patients who developed septic shock after emergency gastrointestinal surgery (Shimizu et al. 2013). In stable haemodialysis patients, blood endotoxin level quantified using the ESP assay correlated significantly with CRP (Terawaki et al. 2010).

3.2.2 Recombinant factor C assay

The zymogen factor C is the primer of the LAL coagulation cascade (see Figure 3-1). In vivo, factor C is a biosensor in the horseshoe crab and is activated by the presence of endotoxin. Efforts to conserve the population numbers of the horseshoe crab led to efforts to develop recombinant factor C as an endotoxin detection tool. This also eliminates the problem of batch-to-batch variation in sensitivity and specificity of the LAL assay (Ding & Ho 2010). Several commercial preparations of recombinant factor C are now available including Pyrogene™ Recombinant Factor C assay (Lonza) and the EndoLISA® assay (Hyglos), although currently both assays are slightly less sensitive than the kinetic LAL assays (lower limit of detection for both is 0.005 EU/mL). The recombinant factor C assay is recognised as an alternative method of endotoxin detection to the LAL assay in both the United States Pharmacopoeia (USP) (USP 28 NF 23) and European Pharmacopoeia (European Pharmacopoeia Chapter 5.1.10), however similar to the LAL assay, the recombinant factor C assay has not been validated for blood endotoxin detection. To date, no studies have been published on the use of the recombinant factor C assay for blood endotoxin detection.
3.2.3 Endotoxin detection methods under development

There are a large number of direct endotoxin detection methods under development. Methods based on optical, electrochemical and mass based biosensors using sensing elements such as aptamers, synthetic peptides and antibodies and artificial materials such as gold nanoparticles are currently being explored as potential endotoxin detection methods (Su & Ding 2015).

3.3 Surrogate measures of endotoxemia

Due to the difficulties in measuring endotoxin in blood matrices directly, surrogate biomarkers have been used to estimate endotoxin activity in the blood.

3.3.1 Endotoxin Activity Assay (EAA)

The EAA is a bioassay which measures neutrophil activation by complement opsonised immune complexes of endotoxin as surrogate measure of endotoxemia. The assay is based on the binding of the lipid A region of endotoxin using a specific IgM antibody. This LPS-antibody complex is opsonised with complement and interacts with complement receptors (CR1 and CR3) on neutrophils priming the NADPH oxidase complex. Following priming, neutrophil respiratory burst is evoked using zymosan and detected as light release from the lumiphor, luminol, using a chemiluminometer. The magnitude of respiratory burst is proportional to the logarithmic concentration of endotoxin in the sample and is used to indicate the quantity of blood endotoxin present (Figure 3-4) (Romaschin et al. 2012).
The EAA is a semi-quantitative test that will give an endotoxin activity (EA) result. The EA result will stratify patients into low (EA<0.4), intermediate (0.4-0.59) and high (EA>0.6) risk of developing septic shock. This assay has been approved by the US FDA and European regulatory agency in 2004 as a diagnostic tool to assess the risk of septic shock in human (Romaschin et al. 2012). The EAA can only be used as a semi quantitative indicator of endotoxemia because variations in chain length and the lipid structure of LPS can affect the relationship between EAA levels and the mass of LPS (Marshall et al. 2004). A previous study found a poor dose–response between blood spiked with control standard endotoxin and EA activity (Matsumoto et al. 2013).

3.3.2 Lipopolysaccharide binding protein (LBP)

LBP is a soluble acute phase protein synthesised and released by the liver in the presence of endotoxin. LBP binds and presents endotoxin to important cell surface pattern recognition receptors such as CD14 and TLR4 eliciting the release of pro-inflammatory mediators. The level of LBP has been suggested to reflect the biological activity of circulating endotoxin in patient blood (Sun et al. 2010; Guerra Ruiz et al. 2007). LBP levels are elevated in those with non-alcoholic fatty liver disease (Guerra Ruiz et al. 2007), coronary artery disease (Lepper et al. 2007) and
sepsis (Opal et al. 1999). However, in a large cross-sectional study of 235 patients with septicaemia, the authors found no correlation between endotoxin measured using the LAL assay and LBP levels (Opal et al. 1999).

### 3.3.3 Soluble CD14

Soluble CD14 is a molecule which may also reflect endotoxin bioactivity. In blood, LBP protein forms a complex with endotoxin which is transferred to membrane-bound CD14 (mCD14) resulting in cell activation. This LBP–LPS complex can also bind with soluble CD14 (sCD14), which can facilitate neutralization of endotoxin via HDL pathways or antagonize mCD14-positive cell activation by competitive binding (Seabra et al. 2009). Levels of soluble CD14 increase significantly post-dialysis (Mitzner et al. 1995; Nockher & Scherberich 1995). Two prospective observational cohort studies in haemodialysis patients have shown that blood concentrations of sCD14 correlated positively with inflammation and independently predicted mortality (D. Raj et al. 2009; D. S. C. Raj et al. 2009). One of these studies found that blood sCD14 concentration was significantly associated with endotoxemia (D. Raj et al. 2009).

### 3.4 Summary

In summary, it is possible that endotoxemia is a major contributor to chronic inflammation in dialysis patients. However, the detection of blood endotoxin is complex with a multitude of assays available. To confirm or refute an association between endotoxemia and unexplained inflammation in dialysis patients, validation of an accurate and precise endotoxin detection assay for use in uraemic blood is required.
Chapter 4

4. Aims and General Methodology

This chapter details the hypotheses and aims of this PhD thesis. Rationale for the design and general methodology for all studies in this thesis are summarised.

4.1 Introduction

Chapter 2 summarised the data that suggests endotoxemia as a potentially important mediator of inflammation in the dialysis population (Jefferies et al. 2014; Szeto et al. 2008; Terawaki et al. 2010). There may be multiple sources for endotoxemia but it has been suggested by others that the haemodialysis technique itself and the gastrointestinal tract are major contributory factors (Hauser et al. 2011; Christopher McIntyre 2011). Notably, increased gut permeability and endotoxemia has also been reported in healthy individuals undergoing intensive exercise who experience gastrointestinal complaints (van Wijck & Lenaerts 2011a). This occurs as a result of dehydration and reduced mesenteric blood flow during exercise (de Oliveira et al. 2014). A similar process may occur with patients during haemodialysis treatment and many often report nausea, vomiting, fatigue and a ‘washed-out’ feeling that can last hours to days post-dialysis. Recovery time after haemodialysis treatment can be variable and prolonged (Sklar et al. 1996). The cause of this phenomenon is unclear and contributes to poor quality of life. It may be possible that endotoxins are present in the blood of haemodialysis patients due to increased gut permeability, exacerbated by the haemodialysis procedure contributing to chronic unexplained inflammation and dialysis-related symptoms including prolonged recovery time.

However as discussed in Chapter 2, there are conflicting data with regards to the level of endotoxemia in the dialysis population with some studies reporting a low prevalence of endotoxemia and others reporting unusually high blood endotoxin levels that is likely to exceed the pyrogenic threshold. There are also significant methodological issues with current endotoxin detection assays, none of which have been validated in uraemic blood. It is unclear whether current endotoxin detection
assays are able to accurately measure blood endotoxin in patients with kidney disease.

4.2 Hypotheses and aims

I. Endotoxins are present in the blood of haemodialysis patients.
II. Blood endotoxin can be accurately measured using current available detection assays (such as the LAL assay – the most commonly used endotoxin detection assay).
III. Endotoxemia is associated with chronic unexplained inflammation in haemodialysis patients.
IV. The haemodialysis procedure itself increases gut permeability and blood endotoxin concentration.
V. Gut permeability is increased in patients with end-stage kidney disease.
VI. Endotoxemia is associated with haemodialysis-related symptoms.

The overarching aim of this thesis is to study the role of endotoxin in chronic unexplained inflammation in haemodialysis patients. The studies reported in this thesis have been designed to meet the following objectives:

- To determine an optimum method of measuring blood endotoxin in ESKD and explore the accuracy of current available endotoxin detection assays.
- To explore the association between endotoxemia and unexplained chronic inflammation in haemodialysis patients.
- To determine the effect of haemodialysis on endotoxemia and inflammation.
- To determine the impact of a single haemodialysis procedure on endotoxin levels and gut permeability.

A roadmap of research studies conducted in this thesis is detailed in the Table 4-1.
### Table 4-1: Roadmap of studies for thesis

<table>
<thead>
<tr>
<th>Aims and objectives</th>
<th>Study description</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scoping work to determine the prevalence of unexplained inflammation</td>
<td>Cross-sectional survey to determine the prevalence of unexplained inflammation in haemodialysis patients</td>
<td>4.3.1</td>
</tr>
<tr>
<td>To determine the optimum method of measuring blood endotoxin in ESKD</td>
<td>Comparative laboratory work to determine optimum LAL assay for endotoxin detection</td>
<td>4.3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3.3</td>
</tr>
<tr>
<td>To explore the association between endotoxemia and unexplained chronic inflammation in haemodialysis patients</td>
<td>Cross-sectional study in haemodialysis patients to determine the association between inflammation and blood endotoxin levels. Blood endotoxin levels were also compared with peritoneal dialysis patients and non-dialysed CKD patients</td>
<td>4.3.4</td>
</tr>
<tr>
<td>To determine the effect of haemodialysis on endotoxemia and inflammation</td>
<td>Prospective study – CKD V patients not yet on dialysis were prospectively studied and sampled to measure the change in blood endotoxin levels with haemodialysis treatment over time.</td>
<td>4.3.5</td>
</tr>
<tr>
<td>To determine the impact of a single haemodialysis procedure on endotoxin levels and gut permeability</td>
<td>Cross-sectional study to explore the acute effect of a single haemodialysis session on blood endotoxin and inflammatory biomarkers.</td>
<td>4.3.6</td>
</tr>
<tr>
<td></td>
<td>Cross-sectional study to explore the impact of a single dialysis session on gut permeability and the relationship between increased gut permeability and endotoxemia</td>
<td>4.3.7</td>
</tr>
</tbody>
</table>

If a firm association between endotoxemia and chronic unexplained inflammation in haemodialysis patients was demonstrated and the source of endotoxin established this would justify embarking on interventional trials to examine the efficacy of endotoxin-lowering strategies.

A general overview of each individual study conducted as part of this PhD is described below. A detailed explanation of the methodologies is provided in the relevant chapters.

#### 4.3 Study population

All participants were recruited from patients who were under the care of the Lister Renal Unit at the East and North Herts NHS Trust. Healthy controls for the study were recruited from volunteers and healthcare professionals working at the trust.
The haemodialysis programme at the East and North Herts NHS Trust covers a large number of patients in a wide geographical area covering Stevenage, Luton, Bedford, St. Albans and Harlow. There were approximately 500 prevalent haemodialysis patients at the time of the PhD studies. Patients at our centre are routinely treated with predominantly polysulphone membranes using either high-flux haemodialysis or haemodiafiltration. Water quality is regularly monitored to meet ultrapure standards as recommended in national and international guidelines. Dialysis is prescribed and monitored to achieve minimum target equilibrated Kt/V of 1.2 per dialysis session. Blood tests for purposes of measurement of dialysis adequacy including C-reactive protein are monitored monthly. Residual renal function is monitored monthly with the use of inter-dialytic urine collections to allow estimation of renal urea clearance.

4.3.1: Retrospective cross-sectional study of the prevalence of unexplained inflammation in the haemodialysis population (see Chapter 5)

Although the prevalence of general inflammation in haemodialysis population is well known (Owen & Lowrie 1998), the prevalence of chronic and unexplained inflammation is less clear and there are no published studies which documents this. To determine the extent of chronic unexplained inflammation in the haemodialysis population, a cross-sectional analysis of all prevalent haemodialysis population treated at the East and North Hertfordshire NHS Trust was carried out. Medical records and CRP levels of all haemodialysis patients were reviewed and categorised into those with chronic inflammation and those without inflammation. Patients with transient elevations in CRP were excluded from further analysis. For patients with chronic inflammation, medical notes were reviewed to identify patients with an obvious cause of chronic elevated CRP (e.g. chronic leg ulcers, active vasculitis). If an identifiable cause could not be determined, patients were classified as having chronic unexplained inflammation.
4.3.2: Studies to determine the optimum blood endotoxin detection assay in haemodialysis patients (see Chapter 6)

To determine the optimum blood endotoxin detection assay, studies were carried over several phases as described below.

4.3.2.1 Selection of phlebotomy apparatus

Endotoxins are ubiquitous molecules, therefore precautions are necessary to avoid contaminating blood specimens during the sampling procedure to prevent reporting artificially high results of blood endotoxin levels (Redl et al. 1992). Blood collection tubes may introduce endotoxin contamination due to the additives used (Newhall et al. 2010). To reduce the risk of environmental contamination of blood samples, phlebotomy equipment and blood collection tubes were examined for endotoxin contamination using the standard method defined by the Association for Advancement of Medical Instrumentation (Bryans & Braithwaite 2004). Blood collection and storage tubes were additionally tested to ensure that the materials or additives within the tubes did not contain interfering factors that could significantly activate or inhibit the activity of the LAL assay. This study was conducted to facilitate the selection of phlebotomy equipment and blood collection for use in subsequent clinical studies.

4.3.2.2 Optimisation of the LAL assay for blood endotoxin detection

A review of endotoxemia studies in the dialysis literature showed that the LAL assay was the most commonly used method for blood endotoxin detection. The LAL assay is also frequently used for studies of populations with other disease states (Ronco et al. 2010; Brade 1999). The kinetic LAL assays have a much wider detection range and a lower limit of detection compared to end-point LAL assays. However, as discussed in Chapter 3, use of the LAL assay for blood endotoxin detection can be complex as samples require pre-treatment prior to analysis due to the presence of natural inhibitors and activating factors in blood. Dilution and heat treatment is the simplest and most commonly used pre-treatment method (Hurley 1995).

The purpose of this phase was to determine the blood sample processing requirements to optimise LAL performance in uraemic blood. Once the optimum
A methodology was established this was used to compare the LAL assay with alternative methods of blood endotoxin detection.

Blood samples from 7 healthy controls and 7 haemodialysis patients were collected. Samples were spiked with different standards of control standard endotoxins (CSE) and then subjected to measurement to determine the percentage of the spiked endotoxin that could be recovered from the sample or “spike recovery”. On this basis, experiments were carried to determine the following:

- Is endotoxin spike recovery better in plasma or serum?
- What is the optimum sample dilution factor?
- Can spike recovery be improved with the use of detergents as a sample diluent?

After the optimum blood sample processing conditions were determined. Optical density graphs were analysed to see if calculating the endotoxin content in samples using other parameters other than the onset reaction time such as slope of the optical density curve yielded more accurate spike recoveries (see chapter 6– Kinetic LAL assays).

4.3.2.3 Comparison of the turbidimetric and chromogenic LAL assay (section 6.9)

Using the optimum sample processing conditions and analytical methodology determined from the experiments carried out in section 4.3.2.2, studies were conducted to compare the performance of two commercially available variations of the kinetic LAL assay – the turbidimetric and the chromogenic assay. Both the kinetic turbidimetric and chromogenic methods are regularly used, but it is unclear which assay has better performance in uraemic blood. The best kinetic LAL assay was compared with an endpoint chromogenic LAL assay which has been commonly cited in the dialysis literature and also compared with an alternative novel endotoxin detection method - the Endotoxin Scattering Photometry (ESP) assay, due to its report of increased sensitivity compared to the traditional LAL assays and its success in diagnosing sepsis and correlation with inflammatory markers in haemodialysis patients (see chapter 3).
Blood samples from haemodialysis patients were spiked with different concentrations of CSE and measured to determine spike recovery for each assay. This was used to determine the accuracy and precision of all three assays to determine the best technique to use for blood endotoxin detection in subsequent clinical studies. The study was also repeated in blood samples collected from healthy controls to explore whether uraemia could affect the activity of the LAL assay.

Other promising novel direct endotoxin measurement assays were available but have not been explored in this thesis. The recombinant factor C assay is not as sensitive as the kinetic LAL assays, and there are no reports of its use in blood. The Endotoxin Activity Assay (EAA) is also a promising assay since its use as a diagnostic aid in predicting severe sepsis in patients has received FDA-clearance. However it is semi-quantitative, and only provides surrogate measures of endotoxemia rather than direct measurement. Hence comparison of its performance with other direct endotoxin detection assays would be difficult, so the performance of this assay was not investigated.

4.3.3 Determining the influence of (1→3)-β-D glucan on endotoxin measurements in haemodialysis patients (Chapter 8)

Experiments carried out as described in section 4.3.2.3 showed that out of all three studied assays, the kinetic turbidimetric LAL assay was the most accurate and precise method. This assay was selected for subsequent clinical studies. However, the LAL assay can also be activated by (1→3)-β-D glucan (BG) via the factor G pathway, although this pathway can be blocked with the use of BG blocking buffers (Kambayashi et al. 1991; Tsuchiya et al. 1990). A review of endotoxin studies carried out in the dialysis population listed in Table 2-1, showed that with the exception of one study (Taniguchi et al. 1990), all studies were carried out using LAL assays that were not rendered insensitive to BG stimulation for measurement of endotoxin. This issue is significant due to the reported high levels of BG in the dialysis population (Prattes et al. 2015). Blocking the factor G pathway of LAL assay is associated with significant cost, therefore it is important to confirm the association of high BG levels in haemodialysis patients. A positive finding would suggest that steps should be
taken to remove BG interference when the LAL assay is used for blood endotoxin detection in haemodialysis patients to prevent false positive signals.

Blood samples were collected from 50 stable, non-infected haemodialysis patients. All samples were measured with the LAL assay, with and without the use BG blocking buffers. The same samples were also measured for levels of BG using the Fungitell® assay, an FDA-cleared blood BG detection assay. The association between blood BG and blood endotoxin measurements derived from the LAL assay which did not block account for factor G activation was then determined.

**4.3.4 Cross-sectional study to examine the association between blood endotoxin levels and chronic unexplained inflammation in haemodialysis patients (see Chapter 5)**

A cross-sectional study was carried out to explore the association between endotoxemia and chronic unexplained inflammation. Sixty patients in-centre haemodialysis were recruited, consisting of 30 patients had chronic unexplained inflammation and 30 patients without inflammation (CRP consistently below the detection limit) were studied. Comparisons were made with healthy individuals and patients with less advanced stages of kidney diseases. The rest of the study cohort included 20 healthy controls, 20 patients with CKD stages 1-3, 20 patients with CKD stages 4-5 and 15 peritoneal dialysis patients. All participants were clinically stable and non-infected at the time of study. Blood samples were collected from all study participants and measured for:

1. Endotoxin
2. (1→3)-β-D glucan
3. IL-6
4. TNF-α
5. High sensitivity CRP

The association between blood levels of endotoxin, (1→3)-β-D glucan and inflammatory biomarkers were explored by regression analysis. These parameters were also correlated with a number of quality of life outcome measures including
uraemia related symptoms, depression symptoms, level of fatigue and post-dialysis recovery time.

**Quality of life outcome measures**

**Uraemia related symptoms**: Symptoms related to kidney disease was assessed using Palliative Care Outcome Scale modified for renal disease (POS-S renal) (Murphy et al. 2009). This scale has been validated for the assessment of symptoms in populations with advanced kidney disease (Davison et al. 2015) [Appendix B – POS-S Renal]

**Fatigue**: level of fatigue was captured using the Multidimensional Fatigue Inventory (MFI) (Smets et al. 1995), a 20 item self-report instrument designed to measure fatigue in five dimensions including general fatigue, physical fatigue, mental fatigue, reduced motivation and reduced activity. A global score combining results from each dimension ranges from 20 to 100. Higher scores demonstrate a higher level of fatigue. The MFI is a useful tool for the assessment of fatigue in chronically unwell patients (Lin et al. 2009). [Appendix C – Multidimensional fatigue inventory]

**Depression**: patients were assessed for depressive symptoms using the Patient Health Questionnaire (PHQ-9). This questionnaire used for screening, diagnosing, monitoring and measuring the severity of depression. Depression severity is classified according to the score obtained: - 5-9, 10-14, 15-19 and >20 representing minimal, minor, moderately severe and severe depressive symptoms respectively. The PHQ-9 has been validated for use in dialysis patients (Watnick et al. 2005). [Appendix D – Patient health questionnaire 9]

**Post-dialysis recovery time**: This was assessed by asking participants how long it takes to feel back to normal after a typical dialysis session (Lindsay et al. 2006). Prolonged recovery times are associated with worse quality of life, hospitalisation and mortality (Rayner et al. 2014). [Appendix E – Post dialysis recovery time]
4.3.5 Prospective longitudinal study to determine the effect of haemodialysis on blood endotoxin, \((1\rightarrow3)\)-\(\beta\)-D glucan and inflammation (Chapter 10)

The effects of haemodialysis initiation on blood endotoxin, BG and markers of inflammation were studied. Thirty patients with CKD stage 5 within six months of starting haemodialysis were recruited. Blood samples and quality of life outcome measures as described above were collected up to 6 months prior to starting haemodialysis treatment. Blood samples were similarly analysed for endotoxin, BG, IL-6, TNF-\(\alpha\) and hs-CRP. Participants were prospectively followed and repeat blood sampling and quality of life assessments were repeated within 1-3 months of starting haemodialysis to determine the effect of the haemodialysis process itself on blood levels of endotoxin and BG and on the markers of inflammation.

4.3.6 Studies of the impact of intra-dialytic exercise and haemodialysis on endotoxemia and inflammation (Chapter 9)

There are numerous reports of healthy individuals developing endotoxemia after intense exercise (Bosenberg et al. 1988; Ashton et al. 2003; Lambert 2008) possibly due to gut ischemia (Marshall 1998; ter Steege et al. 2012; van Wijck & Lenaerts 2011a). Haemodialysis may also induce additional circulatory stress on the gastrointestinal tract since some studies suggest that the splanchnic and splenic vascular beds may contract during haemodialysis (Yu et al. 1997). It is possible that patients who undertake intra-dialytic exercise may risk exerting additional circulatory stress to gastrointestinal tract during haemodialysis sessions leading to acute endotoxemia and pro-inflammatory responses. To test this hypothesis, 10 haemodialysis patients who regularly participate in intra-dialytic exercise were studied. Blood was collected from participants before and after haemodialysis on two separate sessions. Patients were asked to carry out their normal intra-dialytic exercise routine on one study day (exercise day) and to abstain from exercise on the second study day (non-exercise day). Differences in blood endotoxin, BG and inflammation levels were analysed before and after haemodialysis on an exercise and non-exercise day to ascertain the acute effect of haemodialysis and intra-dialytic exercise on inflammation and endotoxemia.
4.3.7 Studies of the impact of haemodialysis on gut permeability (Chapter 11)

The gut may be a major source of endotoxin and other bacterial products such as bacterial DNA (Bossola et al. 2009). As discussed in chapter 2, gut permeability may be altered in uraemia and has been reported in animal and non-dialysed CKD patients (Magnusson et al. 1991). However, gut permeability has never been measured in haemodialysis patients.

The conventional method of measuring gut permeability involves the oral administration of non-metabolised sugar probes and measuring their recovery in urine (Bjarnason et al. 1995). In normal circumstances most of the sugar probes are excreted in the stool, however if intestinal permeability is increased, these sugar probes are absorbed into the blood stream and excreted in the urine. The amount of sugars recovered in the urine can be used to assess the degree of gut permeability. Since most haemodialysis patients pass little or no urine, this technique cannot be used. Recently, a novel method of measuring gut permeability assay by measuring the recovery of sugar probes in plasma has been developed at the University of Maastricht (van Wijck & Lenaerts 2011a; van Wijck et al. 2014; van Wijck et al. 2013).

The conventional assays for gut permeability measurement are usually based on a difference in gut absorption of two orally-administered sugars. Under normal conditions, one of these sugars is unable to cross the intestinal barrier easily whereas the second sugar is able to do so freely. However, if gut permeability is increased both sugars are able to cross the intestinal barrier. Changes in the difference in absorption of these sugars can be used to assess changes in gastrointestinal permeability. However, a two sugar-probe test provides only limited information, whereas a multi-sugar probe could provide a more accurate picture. This technique utilises liquid chromatography mass spectrometry (LC-MS) which allows for sensitive measurement of sugars in plasma. As sensitivity is increased, more sugars at a lower dose can be administered so that total sugar load is not higher compared with the classical dual sugar assay. The sugar mixture contains lactulose, sucralose, rhamnose and erythritol. Lactulose and rhamnose are often used as a marker for small intestinal permeability since they are degraded by the
microbiota in the colon (Fink 2002; Hietbrink et al. 2007). Sucralose and erythritol can be used as a marker for whole gut permeability since they resist colonic bacterial fermentation (Farhadi et al. 2003). The calculated plasma lactulose:rhamnose ratios (L/R) is used to assess small intestinal permeability and plasma sucralose:erythritol ratios (S/E) for whole gut permeability. Area under curve was determined for L/R and S/E ratios and comparisons made using the Kruskal-Wallis test to ascertain differences in sugar ratios between dialysis and non-dialysis days.

This novel gut permeability measurement technique was applied in a cross-sectional study of 10 haemodialysis patients. The effect of haemodialysis on gut permeability was determined by measurement of gut permeability on a dialysis and non-dialysis day. The study was also carried out in 5 healthy controls for comparison of gut permeability with non-uraemic subjects.

4.4 Research Ethics

All studies were reviewed and approved by the NHS Research Ethics committee and local Research and Development office. All study participants were given an information sheet to explain the study and informed written consents was obtained from all participants. Letters of approval are shown in Appendix A.

The NHS Research Ethics committee for the studies are given below:-

Studies to determine the optimum endotoxin detection assay in haemodialysis patients – 14/SC/0067

Determining the influence of (1→3)-β-D glucan on endotoxin measurements in haemodialysis patients - 14/EM/1266

Cross-sectional study to examine the association between blood endotoxin levels and chronic unexplained inflammation in haemodialysis patients – 15/EE/0019

Prospective longitudinal study to determine the effect of haemodialysis on blood endotoxin, (1→3)-β-D glucan and inflammation – 15/NW/0192

Studies of the impact of intra-dialytic exercise and haemodialysis on endotoxemia and inflammation – 15/EE/0267
Studies of the impact of haemodialysis on gut permeability – 15/EE/0379
Chapter 5

5. The prevalence of chronic unexplained inflammation in haemodialysis patients

5.1 Introduction

Chronic unexplained inflammation in the dialysis population is a well-recognised phenomenon (Kocyigit et al. 2014; Achinger & Ayus 2013). The prevalence of inflammation in the haemodialysis population is estimated to be 35% (Owen & Lowrie 1998), however the proportions of those with unexplained inflammation and those with obvious clinical causes of inflammation (e.g. chronic infected leg ulcers) is unclear. The prevalence of unexplained inflammation has not been reported.

A cross-sectional study of all prevalent haemodialysis patients treated at the East and North Herts NHS Trust was carried out to determine the extent of unexplained inflammation in the haemodialysis population. Differences in demographic, clinical and dialysis-related parameters between patients with and without inflammation was studied to gain insight into potential implicating factors that may be associated with chronic unexplained inflammation in haemodialysis patients.

5.2 Methods

5.2.1 Definition of chronic inflammation

One of the difficulties in studying inflammation is the lack of consensus on how to define inflammation in the dialysis population (KDOQI Workgroup 2005), despite the recognition that inflammatory biomarkers are persistently elevated in a significant proportion of patients (Santoro & Mancini 2002). Most studies use C-reactive protein (CRP) to identify inflamed individuals but the optimal cut-off CRP to diagnose inflammation is unclear (KDOQI Workgroup 2005). Haemodialysis patients at the East and North Herts NHS Trust have monthly blood tests to measure C-reactive protein (CRP) as part of routine clinical care, therefore CRP was the biomarker used to identify patients with inflammation in this study. Some authors have suggested that a CRP>10 mg/L indicates inflammation caused mainly by infection (Tsirpanlis et al. 2004), whilst others have set this cut-off at >15 mg/L (Stenvinkel et al. 2002).
Some studies have found that a cut-off level of 6 mg/L can accurately predict constant inflammatory state on follow-up (Ortega et al. 2002). In general, a CRP value between 0.1 to 15 mg/L has been suggested to reflect microinflammation (Tsirpanlis et al. 2005). CRP at the East and North Herts NHS was measured using Olympus Beckman-Coulter AU analyser and reagents and healthy individuals are expected have a CRP of <5mg/L using this assay. Therefore values >5mg/L are considered elevated.

To define those with chronic inflammation in this study, patients with two measured levels of CRP >5mg/L at least one month apart within the last 3 months were considered as having chronic inflammation. CRP measurements have to be at least one month apart to avoid classifying patients with only transient rises in CRP as having chronic inflammation.

5.2.2 Database search

A database of all patients receiving haemodialysis treatment at the East and North Herts NHS Trust was generated in October 2013. Medical records and serial CRP measurements in the previous 3 months were reviewed for all patients. Patients with obvious causes for an elevated CRP such as active infection, recent hospitalisation or surgery were excluded from further analysis.

Patients with persistently elevated CRP measurements using the criteria stated in section 5.2.1 but with no obvious clinical cause were classified as having chronic unexplained inflammation. The medical notes of this group were reviewed further to look for potential risk factors which may predispose to the development of inflammation including:

- Use of tunnelled dialysis catheters
- Failed transplant allograft in-situ
- History of gastrointestinal disease such as diverticulitis or colitis
- History of foot or leg ulcers
- History of recurrent urinary tract infections
- Polycystic kidney disease
- History of arthropathy
- History of bronchiectasis or tuberculosis
- Presence of eosinophilia
- Positive HIV
- Long term urinary catheter in-situ
- Active malignancy
- Active hepatitis

Patients were then categorised into three groups:

1. No inflammation (CRP <5 mg/L)
2. Chronic inflammation with potential underlying risk factors
3. Chronic inflammation without potential underlying risk factors

Demographic data (age, gender, weight), co-morbidity (underlying cause of kidney disease and Charlson Co-morbidity Index) and other relevant dialysis parameters were collected for all patients and compared between inflamed (Group 2 and 3) and non-inflamed patients (Group 1). Demographic data, co-morbidity, residual renal function and dialysis parameters were also compared between Groups 2 and 3.

5.2.3 Dialysis parameters

The following dialysis parameters were collected on all patients:

Dialysis time ($T_d$): the duration of the most recent dialysis session in minutes

Interdialytic weight gain as a percentage of dry weight (IDWG%): IDWG as a percentage of dry weight (kg).

Access blood flow ($Q_b$): vascular access blood flow (mL/min).

Renal urea clearance (KRU): renal urea clearance was used to indicate the level of residual renal function present. To calculate KRU, blood was sampled for urea measurement at the end of the first dialysis session of the week and immediately before the next session. Between these samples, urine was collected over the whole inter-dialytic period. KRU was calculated using the formula:
\[ Clearance \ (mL/min) = \frac{2 \cdot (U_{ID} \cdot V_{ID})}{t_{ID} \cdot (C_{post\ HD1} + C_{pre\ HD2})} \]

Where \( U_{ID} \) = urinary urea concentration, \( V_{ID} \) = urine volume, \( t_{ID} \) = collection duration, \( C_{post\ HD1} \) = serum urea concentration at the end of the first dialysis session, and \( C_{pre\ HD2} \) = serum urea concentration immediately before the start of the second dialysis session.

**Dialyser Kt/V**: Dialysis adequacy was measured by calculating the single pool Kt/V urea (spKt/V) using the second generation Daugirdas equation (Daugirdas 1993) and then converted to equilibrated Kt/V using the Tattersall equation (Tattersall et al. 1996).

\[ sp\ Kt/V = -\ln \left( \frac{C_{post\ HD}}{C_{pre\ HD} - 0.008t} \right) + \left( 4 - 3.5 \frac{C_{post\ HD}}{C_{pre\ HD}} \right) \times \frac{UF}{Post\ HD\ weight} \]

Where \( C_{post\ HD} \) and \( C_{pre\ HD} \) = post-dialysis and pre-dialysis serum urea concentration respectively, \( t \) = dialysis time, \( UF \) = ultrafiltration volume and Post HD weight = weight after dialysis (kg)

\[ Equilibrated \ Kt/V = sp\ Kt/V \times \frac{t}{t + 35} \]

Where \( t \) = dialysis time (minutes)

**Renal Kt/V**: KRU from residual renal function contributes to significant urea removal and can be combined with dialyser Kt/V to obtain total Kt/V provided by renal and dialyser urea clearance. KRU is converted to its equivalent intermittent clearance, renal Kt/V, using the equation:

\[ Renal \ Kt/V = KRU \times \frac{f}{V} \]

Where \( f \) is an inflation factor = 5500 for thrice weekly schedules or 9500 for twice weekly (Depner 2005) and \( V \) = volume of distribution of urea calculated using the Watson equation (Watson et al. 1980).
Total Kt/V is calculated by adding renal Kt/V to dialyser Kt/V.

5.2.4 Statistical analysis

Normally distributed data are presented as means ± standard deviation and non-normally distributed data as medians and interquartile ranges. Differences between groups were compared using Student’s T-test or Mann-Whitney U test as appropriate. Categorical data between groups were compared using chi-square. A p value <0.05 was used to indicated statistical significance.

5.3 Results

There were 444 prevalent haemodialysis patients at the time of data extraction. 64.8% (287/444) of patients had chronic inflammation as defined using the criteria stated in section 5.2.1. In this group, 183 patients had obvious clinical reasons (e.g. recent hospitalisation or active infection) for inflammation and were excluded from further analysis representing 41.2% of the study population. 104 patients (23.4% of the study population) had chronic unexplained inflammation and 157 patients (35.4%) did not have systemic inflammation. In patients with chronic unexplained inflammation, 62.5% (65/104) had potential risk factors that may predispose to inflammation and 37.5% (39/104) had no identifiable predisposing risk factor represent 9% and 15% of the whole study population respectively (Figure 5-1).
Clinical characteristics of patients with and without unexplained inflammation are shown in Table 5-1. Patients with inflammation were more likely to be older (67 vs. 63), heavier (80kg vs. 71kg) and have lower dialysis adequacy (Kt/V 1.32 vs. 1.41) despite having a longer dialysis sessions (Td 202 minutes vs. 192 minutes). Both groups were similar with respect to residual renal function, co-morbidity, IDWG and access blood flow and dialysis catheter use. Predisposing risk factors for inflammation were also similar between both groups.

Figure 5-1: Proportion of haemodialysis patients with and without inflammation
**Table 5-1: Clinical characteristics of patients with and without unexplained inflammation**

<table>
<thead>
<tr>
<th></th>
<th>Unexplained inflammation</th>
<th>No inflammation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>67 ± 15</td>
<td>63 ± 16</td>
<td>0.045*</td>
</tr>
<tr>
<td>Male (%)</td>
<td>64</td>
<td>57</td>
<td>0.367</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80 ± 22</td>
<td>71 ± 16</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>12.4</td>
<td>1.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>KRU (mL/min)</td>
<td>0 [IQR 0-1.6]</td>
<td>0.49 [IQR 0-2]</td>
<td>0.171</td>
</tr>
<tr>
<td>Total Kt/V</td>
<td>1.32 ± 0.28</td>
<td>1.41 ± 0.32</td>
<td>0.017*</td>
</tr>
<tr>
<td>Td (min)</td>
<td>202 ± 27</td>
<td>192 ± 45</td>
<td>0.038*</td>
</tr>
<tr>
<td>Qb (mL/min)</td>
<td>325 ± 60</td>
<td>327 ± 56</td>
<td>0.793</td>
</tr>
<tr>
<td>IDWG (%)</td>
<td>1.9 ± 1.1</td>
<td>1.9 ± 1.3</td>
<td>0.819</td>
</tr>
<tr>
<td>Dialysis catheter use (%)</td>
<td>31.7</td>
<td>24.2</td>
<td>0.131</td>
</tr>
<tr>
<td>HDF (%)</td>
<td>70.2</td>
<td>79.6</td>
<td>0.104</td>
</tr>
<tr>
<td>CCI</td>
<td>4 [IQR 2-6]</td>
<td>4 [IQR 2-5]</td>
<td>0.138</td>
</tr>
</tbody>
</table>

**Cause of kidney disease**  
(% affected)

<table>
<thead>
<tr>
<th>Cause of kidney disease</th>
<th>Unexplained inflammation</th>
<th>No inflammation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>23.1</td>
<td>27.4</td>
<td>0.435</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>8.7</td>
<td>19.1</td>
<td>0.02*</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>9.6</td>
<td>7.6</td>
<td>0.575</td>
</tr>
<tr>
<td>Tubulo-interstitial disease</td>
<td>8.7</td>
<td>5.7</td>
<td>0.362</td>
</tr>
<tr>
<td>Hypertension or RVD</td>
<td>16.3</td>
<td>13.4</td>
<td>0.505</td>
</tr>
<tr>
<td>Other</td>
<td>12.5</td>
<td>10.2</td>
<td>0.561</td>
</tr>
<tr>
<td>Unknown aetiology</td>
<td>21.2</td>
<td>15.9</td>
<td>0.282</td>
</tr>
</tbody>
</table>

**Predisposing risk factors**  
for inflammation  
(% affected)

<table>
<thead>
<tr>
<th>Predisposing risk factors for inflammation</th>
<th>Unexplained inflammation</th>
<th>No inflammation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant graft in-situ</td>
<td>8.7</td>
<td>6.4</td>
<td>0.487</td>
</tr>
<tr>
<td>GI pathology</td>
<td>6.7</td>
<td>6.4</td>
<td>0.908</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>2.9</td>
<td>0.6</td>
<td>0.148</td>
</tr>
<tr>
<td>Leg ulcers</td>
<td>6.7</td>
<td>5.1</td>
<td>0.578</td>
</tr>
<tr>
<td>Recurrent urinary tract infections</td>
<td>5.8</td>
<td>2.5</td>
<td>0.184</td>
</tr>
<tr>
<td>Malignancy</td>
<td>6.7</td>
<td>3.8</td>
<td>0.29</td>
</tr>
<tr>
<td>Arthropathy</td>
<td>9.6</td>
<td>9.6</td>
<td>0.987</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>1.0</td>
<td>0</td>
<td>0.218</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>1.0</td>
<td>0.6</td>
<td>0.768</td>
</tr>
<tr>
<td>Eosinophilia</td>
<td>1.0</td>
<td>0</td>
<td>0.218</td>
</tr>
<tr>
<td>Long term catheter in-situ</td>
<td>0</td>
<td>1.9</td>
<td>0.156</td>
</tr>
<tr>
<td>HIV</td>
<td>0</td>
<td>0.6</td>
<td>0.415</td>
</tr>
</tbody>
</table>

KRU, renal urea clearance; Td, dialysis session time; Qb, access blood flow; IDWG, inter-dialytic weight gain; HDF, haemodiafiltration; CCI, Charlson Co-morbidity index; RVD, renovascular disease; GI, gastrointestinal disease; HIV, human immunodeficiency virus; CRP < 5 was designated as ‘0’ for the analysis.

Comparison of clinical characteristics of patients with unexplained inflammation with and without predisposing risk factors for inflammation are shown in Table 5-2. Sub-
group analysis of the cohort of patients with unexplained inflammation did not reveal any differences between those with and without predisposing risk factors in terms of demographic or clinical parameters.

Table 5-2: Clinical characteristics of inflamed patients with and without potential predisposing risk factors for inflammation

<table>
<thead>
<tr>
<th></th>
<th>Unexplained inflammation with risk factors</th>
<th>Unexplained inflammation without risk factors</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>67 ± 15</td>
<td>67 ± 14</td>
<td>0.97</td>
</tr>
<tr>
<td>Male (%)</td>
<td>63.1</td>
<td>64.1</td>
<td>0.916</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77 ± 20</td>
<td>84 ± 24</td>
<td>0.119</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>19.4 [IQR 8.7-19.4]</td>
<td>13 [IQR 10-20]</td>
<td>0.456</td>
</tr>
<tr>
<td>KRU (mL/min)</td>
<td>0.21 [0-1.9]</td>
<td>0 [0-1.2]</td>
<td>0.505</td>
</tr>
<tr>
<td>Total Kt/V</td>
<td>1.34 ± 0.04</td>
<td>1.28 ± 0.04</td>
<td>0.262</td>
</tr>
<tr>
<td>Td (min)</td>
<td>201 ± 28</td>
<td>204 ± 27</td>
<td>0.671</td>
</tr>
<tr>
<td>Qb (mL/min)</td>
<td>322 ± 64</td>
<td>330 ± 52</td>
<td>0.485</td>
</tr>
<tr>
<td>IDWG (%)</td>
<td>1.8 ± 1.2</td>
<td>1.9 ± 0.9</td>
<td>0.668</td>
</tr>
<tr>
<td>HDF (%)</td>
<td>66.2</td>
<td>76.9</td>
<td>0.245</td>
</tr>
<tr>
<td>CCI</td>
<td>4 [IQR 2-6]</td>
<td>4 [IQR 3-6]</td>
<td>0.818</td>
</tr>
</tbody>
</table>

Cause of kidney disease (% affected)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Unexplained inflammation with risk factors</th>
<th>Unexplained inflammation without risk factors</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>16.9</td>
<td>33.3</td>
<td>0.054</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>12.3</td>
<td>2.6</td>
<td>0.087</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>13.8</td>
<td>2.6</td>
<td>0.059</td>
</tr>
<tr>
<td>Tubulo-interstitial disease</td>
<td>9.2</td>
<td>7.7</td>
<td>0.787</td>
</tr>
<tr>
<td>Hypertension or RVD</td>
<td>13.8</td>
<td>20.5</td>
<td>0.373</td>
</tr>
<tr>
<td>Other</td>
<td>15.4</td>
<td>7.7</td>
<td>0.251</td>
</tr>
<tr>
<td>Unknown aetiology</td>
<td>18.5</td>
<td>25.6</td>
<td>0.385</td>
</tr>
</tbody>
</table>

5.4 Conclusion

This cross-sectional survey found that nearly a quarter of the prevalent haemodialysis population has chronic and unexplained inflammation. Inflamed patients were associated with older age, increased weight and lower dialysis adequacy despite having slightly longer session duration. There were no significant
differences in terms of vascular access type, flow, use of haemodiafiltration or residual renal function which could explain this observation. The association between older age and inflammation may be due to heavier co-morbidity, although the CCI scores were similar to those without inflammation. Inflamed patients tended to have lower Kt\V values, although these values still exceed dialysis adequacy targets set by national and international guidelines. These observations are similar to study in PD patients which found an inverse relationship between the pro-inflammatory cytokine IL-6 and Kt/V that was independent of residual renal function (Milan Manani et al. 2016). A lower dialysis dose may result in reduced clearance of cytokines since dialysis membranes are capable of removing some pro-inflammatory cytokines (Tarakcioğlu et al. 2003). However it is difficult to establish direct causal relationship between reduced dialysis dose and inflammation based on observational studies only. A significant proportion of patients had unexplained inflammation despite having no obvious predisposing risk factor which would drive a continuous inflammatory process.

In summary, the prevalence of inflammation is high in the dialysis population and in many instances without an obvious clinical cause. Further studies are needed to investigate the underlying cause of inflammation to help mitigate the poor prognostic outcomes in dialysis patients.
6. Studies to determine the optimum blood endotoxin assay in haemodialysis patients

6.1 Introduction

Determining the optimum blood endotoxin detection assay in haemodialysis patients is necessary before studies can be carried out to determine the association between blood endotoxin and inflammation in haemodialysis patients. As discussed in Chapter 2, blood endotoxin detection is difficult and no assays have been validated for use in blood, although many of these assays are frequently utilised in the research setting. A major problem with the methodology of blood endotoxin measurement may occur during blood sampling phase. Since endotoxin is ubiquitous, contamination of phlebotomy equipment, blood tubes and storage vials may lead to contamination of blood samples and falsely raised blood endotoxin levels. Most blood collection tubes are sterile but are not certified to be endotoxin-free and additives in blood collection tubes may also be a source of contamination (Redl et al. 1992). Conversely, certain additives used to obtain plasma such as ethylenediaminetetraacetic acid (EDTA) and citrate can bind calcium by chelation and inhibit LAL reactivity (Cooper 1990).

Most published endotoxin studies carried out in dialysis patients do not report the methodology used for blood sampling in sufficient detail therefore it is unclear whether steps have been taken by authors to minimise risk of introducing contamination or exogenous factors into blood samples which may affect the accuracy of reported results. Compounding this methodological issue, different LAL techniques and novel assays have been used to measure endotoxin in the dialysis population with a wide range of blood endotoxin level results, some of these are in different units, making it difficult to interpret the results (Table 2-1). No studies that compare the performance of these assays in the uraemic setting have been published.
The principle of the LAL assay is to observe the test sample after the addition of LAL to determine the rate at which the mixture coagulates. This is achieved by observing the increase in optical density of the sample mixture over time. As reviewed recently (Gnauck et al. 2016) and discussed in Chapter 3.1, there are several methods of calculating endotoxin from the generated optical density graphs but it is unclear which method is better for calculating of endotoxin content in uraemic blood samples.

Endotoxin release into the blood may interact with plasma components including bile salts, proteins, lipoproteins leading to binding and the formation of complexes or micelles resulting in the hydrophobic lipid A being hidden from the aqueous environment. These complex interactions may alter the sensitivity of the LAL assay (Hurley 1995; Wang & Quinn 2010; Sandek et al. 2007). Blood contains poorly understood factors that can activate or inhibit the LAL assay such as serine proteases (e.g. thrombin, plasmin) (Fields 2006) and serine protease inhibitors (e.g. anti-plasmin and anti-thrombin III) (Levin et al. 1970). Additionally, blood samples from haemodialysis patients contain high levels of different uraemic solutes, it is unclear whether these solutes would exert effects on LAL activity. Historically, multiple methods have been devised to remove interfering factors present in blood, but the most effective and simple methods involve heating and dilution of the sample and most authors typically use a 1:10 dilution followed by heating for 70°C for 10 minutes prior to testing (Hurley 1995). However, diluting the sample significantly reduces the threshold of detection, therefore a minimum dilution factor that would sufficiently remove interfering factors without compromising sensitivity would be desirable. There have also been reports in studies of patients with liver disease that utilise detergents such as Tween 80 as a diluent have improved recovery of endotoxin (Bode et al. 1993; Fukui et al. 1993). Exploration of such methods may be useful in improving the sensitivity of the LAL assay.

There are a number of different commercial variations of the LAL assay. Kinetic LAL assays are the most recent developments which have the advantages of automaticity, increased sensitivity and a wider detection range compared to the older end-point assays. LAL detection can utilise either turbidimetric or chromogenic
methods (see Chapter 3). The chromogenic LAL assay has been the most commonly used endotoxin detection method in previous human studies and dialysis literature (Ronco et al. 2010). However, in Japan, the turbidimetric LAL assay is routinely used and is covered by the Japanese national health insurance program (Matsumoto et al. 2013). The majority of endotoxin studies carried out in dialysis patients was carried out using an endpoint chromogenic LAL assay (see Table 6-12). No direct head to head comparison studies of chromogenic versus turbidimetric LAL have been published.

There are also a number of emerging novel assays which may have better accuracy and sensitivity than the traditional LAL assays. From a review of current novel assays (see Chapter 3), the Endotoxin Scattering Photometry assay (ESP) appeared to be the most promising due to its reported higher sensitivity and its reports of an association between endotoxemia and inflammation in haemodialysis patients, although this requires further examination (Terawaki et al. 2010).

### 6.2 Study aims

The aims of these studies were to:-

1. Identify phlebotomy equipment and blood storage devices that do not have detectable endotoxin or LAL interfering factors for use in blood collection. [Chapter 6.3]
2. Determine the optimum parameters derived from optical density graph to calculate endotoxin content of blood samples using the LAL assay. [Chapter 6.5 and 6.6]
3. Investigate plasma pre-treatment methods to improve the recovery of endotoxin from blood samples from haemodialysis patients. [Chapter 6.7 and 6.8]
4. Compare the performance of kinetic chromogenic and kinetic turbidimetric LAL assays with the novel ESP assay [Chapter 6.9] and the commonly used endpoint chromogenic LAL assay for endotoxin detection in haemodialysis patients [Chapter 6.10]
This chapter consists of seven sub-sections, each sub-section describes the experiments carried to meet the objectives described above.
6.3 Testing phlebotomy equipment, blood collection tubes and storage vials for endotoxin contamination and interfering factors

6.3.1 Method

All components and apparatus used for blood collection was examined for endotoxin contamination using the method described by the Association for Advancement of Medical Instrumentation (AAMI) (Bryans & Braithwaite 2004). Medical devices are tested for endotoxin by soaking in pyrogen free water for 1 hour at 37°C followed by analysis of the solution for endotoxin contamination. For these studies, endotoxin extracting solution (Wako Chemicals, USA) was allowed to dwell in the lumen of needles, syringes and blood collection tubes rather than soaking the whole device since collected blood will only be exposed to the inner lumen of the device. Endotoxin extracting solution containing of 0.1% human serum albumin (Wako Chemicals, USA) was used in replacement of pyrogen free water as a rinsing solution because endotoxin recovery from devices using water may be less efficient (Tsuchiya et al. 1996). After the dwell period, the rinse solutions were tested for endotoxin using the kinetic turbidimetric LAL assay (Charles Rivers, Ecully, France). This assay is used by the Quality Control department at the Lister Hospital for testing endotoxin contamination in dialysis water at the East and North Herts NHS Trust. The Quality Control department is also contracted to test dialysis water for endotoxin contamination for renal services from other NHS trusts and is subject to regular audits and checks by the Medicines and Healthcare Products Regulatory Agency.

Devices were randomly selected to test for endotoxin contamination. Any devices that tested positive for endotoxin contamination were no longer considered suitable for use in subsequent studies and no further testing was performed on devices with evidence of endotoxin contamination. For devices that passed the initial test for endotoxin contamination (i.e. endotoxin content in rinse solution below the detection limit), a second device (of the same type) was randomly selected from another batch and subjected to repeat measurement for endotoxin contamination.

To ensure that blood collection and storage tubes do not contain any LAL interfering factors, devices that tested negative for endotoxin contamination were instilled with
pyrogen free water spiked with 1 EU/mL of control standard endotoxin (CSE) and allowed to dwell for 1 hour at 37°C. Following the dwell period the solution was tested for endotoxin to measure % spike recovery:

\[
\% \text{ spike recovery} = \frac{\text{Measured endotoxin content in sample}}{\text{Amount of endotoxin added to sample}} \times 100%
\]

The spike recovery is a common method of determining the ability of an assay to measure an analyte of interest. In brief, a known quantity of analyte is added (or used to spike) a sample which is subjected to analysis by the assay. An ideal assay is able to measure the exact quantity of analyte that is added to the sample, thus an ideal 'spike recovery' for an assay would be 100%.

For the LAL assay, spike recoveries between 50–200% are considered acceptable. The wide acceptable range is due to the fact that the LAL test is a biological assay and thus inherent variability in LAL testing is a well-recognised phenomenon due to differences in assay sensitivity, manufacturer reagents and laboratory accessories (Kumar 2006). The sensitivity of each batch of commercial LAL formulations (originally gel clot formulations) is calibrated against Reference Standard Endotoxin (RSE) supplied by the Food and Drug Administration (FDA) or the United States Pharmacopoeia (USP). The concentration of endotoxin at which clot formation occurs is termed lambda. The USP states that the verification of a LAL test’s proper performance (i.e. lack of interference by the sample) is demonstrated by clot formation at \(\frac{1}{2}\) lambda, lambda, or twice lambda. This requirement gave rise to the 50–200% recovery specification used to verify appropriate LAL test performance (Kumar 2006; United States Pharmacopoeia 2013).

The following devices were tested for endotoxin contamination and LAL interfering factors using the method described above and a flow chart is illustrated below (Figure 6-1):

- Terumo 10mL syringe
- Terumo 20mL syringe
- Becton Dickinson serum tube (containing clot activator and gel for serum separation)
- Becton Dickinson tube containing lithium heparin and gel for plasma separation
- Becton Dickinson tube containing liquid EDTA
- Becton Dickinson tube containing buffer sodium citrate (0.109M)
- Terumo Venoject II tubes containing heparin
- Eppendorf Biopur safe-lock tubes
Figure 6-1: Flow chart to illustrate protocol for testing blood collection apparatus for contamination and LAL interfering factors

1. Fill lumen of device with endotoxin extracting solution
2. Allow endotoxin extracting solution to dwell in device for 1hr at 37°C
3. Test solution for endotoxin
4. Does rinse solution contain endotoxin?
   - NO: Repeat testing for endotoxin contamination
   - YES: Discard device
5. Instill device with pyrogen free water containing 1 EU/mL endotoxin
6. Measure rinse solution for endotoxin content to calculate spike recovery
7. Is spike recovery between 50-200%?
   - NO: Repeat testing for endotoxin contamination
   - YES: Use device for blood sampling in subsequent studies
Endotoxin assay

Rinse solutions from devices were tested for endotoxin contamination using a kinetic turbidimetric LAL assay (Endosafe KTA2, Charles Rivers, Ecully). Analysis of samples was carried out using manufacturer supplied equipment including depyrogenated glass tube, pipettes and Eppendorf Endosafe pipette tips. 100μL of sample was added to duplicate wells on 96-well microplates. Endosafe KTA2 reagent was reconstituted with 5.2mL of LAL reagent water (pyrogen-free water). 100μL of this mixture was added to each sample. Analysis of each microplate consisted of duplicate wells containing LAL reagent water to act as a negative control. Plates were analysed using a Biotek ELx808 absorbance microplate reader with Endoscan-V software (version 4.0; Charles River Laboratories, France) and observed at 340 nm. The optical density of each well is observed and the time taken for the optical density to increase by 0.03 units is recorded. This is known as the reaction time (Figure 6-2). The onset reaction time is extrapolated from a standard curve to calculate the endotoxin content of a sample.

![Figure 6-2: Optical density graph showing a sample containing 10 EU/mL of endotoxin reaching the onset optical density of 0.03 at 488.8 seconds](image)

During the initial phase of testing, standard curves were constructed using dilutions of control standard endotoxin ranging from 10 to 0.01 EU/mL as this was the...
detection range used to test for endotoxin contamination in dialysis water. However, the lower limit of detection stated by the manufacturer is 0.001 EU/mL depending on laboratory conditions and equipment. To exclude the possibility of low level endotoxin contamination in devices, the standard curve was extended from 10 to 0.001 EU/mL when examining devices for endotoxin contamination on the repeat test.

The USP regulations stipulate that all constructed standard curves are required to have a correlation coefficient of >0.98 for valid extrapolation of reaction times. Each standard should be analysed in at least two replicates and the coefficient of variation for standards should be <10% or <20% depending on the manufacturer (Richardson & Novitsky 2002). The coefficient of variation (CV) is a measure of precision and is used to assess the degree of agreement among individual test results. The CV is calculated by expressing the standard deviation as a percentage of the mean. The higher the CV the less precise or agreement there are between results. A valid standard curve ranging from 10 to 0.001 EU/mL is illustrated below:

![Standard Curve](image)

**Figure 6-3:** Standard curve from 10 to 0.001 EU/mL (X-axis) against reaction time (Y-axis). Logarithmic scales are used. Correlation coefficient \((r) = 0.992\). Equation for regression line

\[
\text{Log}(RT) = 2.9040 - 0.2184 \cdot \text{Log}(EU)
\]
drawn through data points is shown and is used to calculate endotoxin content of samples using the onset reaction time

However, the reaction times obtained with the lowest standard (0.001 EU/mL) was not precise and occasionally the CVs obtained were as high as 33.57% which invalidated the standard curve (see Table 6-1). Elevating threshold of the lowest standard from 0.001 to 0.0025 EU/mL produced much more precise values for reaction times and multiple runs obtained CV values ranging from 0 to 2.76%. Therefore in subsequent studies to compare the performance of the LAL assay with the ESP assay (Chapter 6.9 and 6.10) the lowest standard used was 0.0025 EU/mL, which represented the lower limit of detection.

<table>
<thead>
<tr>
<th>Concentration of CSE (EU/mL)</th>
<th>Run number</th>
<th>Reaction time (seconds)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>1</td>
<td>4185</td>
<td>7.93</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3195</td>
<td>15.76</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7860</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6465</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5790</td>
<td>13.74</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8535</td>
<td>33.57</td>
</tr>
<tr>
<td>0.0025</td>
<td>1</td>
<td>4380</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4665</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4755</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4320</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4170</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4140</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Table 6-1: Coefficient of variation (CV) and reaction times obtained for control standard endotoxin (CSE) concentrations at 0.001 and 0.0025 EU/mL on multiple runs
6.3.2 Results

Endotoxin content from blood collection tubes are shown in Table 6-2. A number of blood collection tubes were found to have endotoxin contamination or contain factors that may activate the LAL assay. Rinse solution from serum tubes from Becton Dickinson, Terumo Venoject II heparinised tubes and Terumo syringes did not have any detectable endotoxin. Water containing 1 EU/mL of CSE used to rinse these devices were analysed for endotoxin to measure spike recovery. Spike recovery was within the 50-200% suggesting these devices (Becton Dickinson serum tubes, Terumo Venoject II tubes and syringes) do not contain any significant LAL activating or inhibiting factors.

<table>
<thead>
<tr>
<th>Device</th>
<th>Endotoxin content (EU/mL)</th>
<th>Spike recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>Terumo 10mL syringe</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Terumo 20mL syringe</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BD Serum tube</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BD Li Heparin tube</td>
<td>0.352</td>
<td>-</td>
</tr>
<tr>
<td>BD EDTA tube</td>
<td>0.048</td>
<td>-</td>
</tr>
<tr>
<td>BD Sodium citrate tube</td>
<td>0.523</td>
<td>-</td>
</tr>
<tr>
<td>Terumo Venoject II tube</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eppendorf Biopur tubes</td>
<td>&lt;0.001*</td>
<td>86.4</td>
</tr>
</tbody>
</table>

Table 6-2: Endotoxin content of devices and spike recoveries obtained from water spiked with 1 EU/mL control standard endotoxin solution used to rinse device.

* - Eppendorf Biopur tubes are manufacturer certified to contain <0.001 EU/mL.

6.3.3 Conclusion

These series of experiments have determined suitable blood collection tubes and syringes to be used for blood sampling. Examination of a selection of blood collection tubes showed that most tubes contain significant endotoxin contamination.
From the blood collection tubes tested, only Becton Dickinson serum tubes and Terumo Venoject II heparinised tubes tested negative for endotoxin contamination. Becton Dickinson serum tubes contain a clot activating gel for separation of serum from blood and Terumo Venoject II tubes contain heparin as an anticoagulant which allows plasma to be separated from whole blood, these blood collection tubes were used in subsequent studies. A number of syringes (to be used for blood draw) were checked and did not demonstrate significant endotoxin contamination or contain LAL interfering factors. Eppendorf Biopur tubes are manufacturer certified to contain <0.001 EU/mL, spike recovery of 1 EU/mL CSE that were allowed to dwell within the tubes were between 86.4 to 95% suggesting that these storage tubes do not contain any LAL interfering factors.

In conclusion, Becton Dickinson serum tubes, Terumo Venoject II tubes, Terumo syringes and Eppendorf Biopur tubes will be used for blood sampling and storage for subsequent studies.
6.4 Study population

A number of studies were carried out and described in this chapter. The purpose of these studies was to investigate methods to optimise the accuracy of the LAL assay and to compare the performance of different variations of the LAL assays with the ESP assay. These series of experiments were carried out using blood specimens collected from seven haemodialysis patients and seven healthy individuals with no history of renal disease. All participants gave informed written consent.

Inclusion criteria (Haemodialysis patients)

- Adult patients on haemodialysis aged 18 years and above.
- Adult patients dialysing through a functioning arteriovenous fistula.
- Patients with at least two measurements of CRP < 5 mg/L carried at least one month apart within the last 3 months.

Inclusion criteria (Healthy controls)

- Healthy adults aged 18 years and above
- No history of kidney disease

Exclusion criteria

- Anaemia (Hb < 100g/L)
- Ongoing sepsis
- HIV, Hepatitis B or C positive status
- Current pregnancy
- Active vasculitis or connective tissue disease
- Active malignancy
- Liver dysfunction
- Right chamber heart failure
- On oral steroids, NSAIDs or immunosuppressive medications
- Any bowel pathology e.g. diverticulitis or inflammatory bowel disease
- No capacity to consent for themselves
6.5 Determining the difference in spike recovery between plasma and serum

6.5.1 Introduction

Serum and plasma are both components of blood. Serum is the extracellular proportion of blood after coagulation and is devoid of clotting factors such as fibrinogen. Plasma is the liquid, extracellular component of blood that has been treated with anti-coagulants, composing of serum and clotting factors. Both serum and plasma have been used in previous studies for measurement of blood endotoxin. The pros and cons for using serum or plasma for endotoxin measurement has been previously discussed (Hurley 1995). The endotoxin inactivation capacity of serum has been reported to be higher than plasma by some authors, however others have found the opposite (Novitsky & Roslansky 1985). In general, experts consider plasma as the preferred blood component for measurement of endotoxin (Hurley 1995).

A number of studies have also investigated the difference in endotoxin recovery between platelet rich versus platelet poor plasma. Some authors advocate the use of platelet rich plasma due to the observation of endotoxin binding to platelets (Das et al. 1973), although this has been disputed by the lack of difference in the recovery of endotoxin from platelet rich, platelet poor plasma and whole blood (Sakon et al. 1986). Despite the lack of strong evidence, and due to the possibility of platelet binding to endotoxin, many endotoxin studies have been conducted using platelet rich plasma [summarised in (Novitsky 1994)].

The studies carried out in section 6.3 showed that both Becton Dickinson serum tubes and Terumo Venoject II heparinised tubes (to produce plasma) could be used. Although the current literature suggests that plasma is preferred over serum for measuring endotoxin, a brief study was carried out to verify this.

6.5.2 Methods

Venesection and processing of samples

Blood was drawn aseptically from one healthy individual and collected in Becton Dickinson serum tubes and Terumo Venoject II heparinised tubes. Blood collected in
the serum tubes were allowed to rest and fully coagulate prior to centrifuging at 1500g for 10 minutes at 4°C. Serum samples were immediately placed in Eppendorf Biopur safe lock tubes and stored at -80°C. Blood collected using Terumo Venoject II heparinised tubes were centrifuged at 250g for 10 minutes at 4°C to produce platelet rich plasma and stored at -80°C. All blood samples were processed and stored within 30 minutes of collection.

**Endotoxin spiking procedure**

On the day of analysis, plasma and serum samples were thawed to room temperature (20-25°C) and spiked with 5 EU/mL of standard *E.coli* 055:B5 endotoxin. Samples were vortexed and incubated at room temperature for 15 minutes. To remove potential LAL activating or inhibitory factors present in serum/plasma. Samples were diluted 1:10 with LAL reagent water and heated at 70°C for 10 minutes. These heating and dilution conditions were recommended based on an extensive review of studies using different heating and dilution methods (Hurley 1995). Samples were allowed to cool to room temperature prior to measurement for endotoxin.

**Endotoxin measurement**

Samples were measured using the kinetic turbidimetric LAL assay (Endosafe KTA2; Charles River, Ecully) as described above. Samples were analysed in duplicate on sterile 96 well microplates (manufacturer-certified to <0.001 EU/mL) and analysed using a Biotek ELx808 absorbance microplate reader with manufacturer supplied software (Endoscan-V version 4.0, Charles River, Ecully), reaction times were recorded at onset optical density of 0.03. A four point standard curve was constructed using control standard endotoxin with correlation coefficient >0.98 (10-0.01 EU/mL). Coefficient of variation for reaction times for standards and samples was <20%. Spike recovery was calculated for all samples using the method as described in section 6.3.
6.5.3 Results

Endotoxin measurements for plasma and serum samples are shown in Table 6-3. Endotoxin content in both plasma and serum was <0.01 EU/mL. Despite a relatively large spike of endotoxin (5 EU/mL), recovery of endotoxin from serum samples was low at 16.3%, consistent with previous reports of endotoxin neutralising properties of serum (Gnauck et al. 2016; Hurley 1995). Spike recovery from plasma was higher at 58.1% which is within the 50-200% requirement (United States Pharmacopoeia 2013) although considerable amount of spiked endotoxin was not retrieved despite dilution and heat treatment.

<table>
<thead>
<tr>
<th>Blood component</th>
<th>Concentration of endotoxin spike (EU/mL)</th>
<th>Endotoxin measured from sample (EU/mL)</th>
<th>Spike recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.814</td>
<td>16.3</td>
</tr>
<tr>
<td>Plasma</td>
<td>0</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.905</td>
<td>58.1</td>
</tr>
</tbody>
</table>

Table 6-3: Endotoxin spike recovery from plasma and serum samples spiked with 5 EU/mL of control standard endotoxin

6.5.4 Conclusion

The finding from this study suggests that recovery of endotoxin from plasma is better than serum which is in agreement with previous reports (Hurley 1995). Both types of collection tubes were examined and did not contain any endotoxin contamination or LAL interfering factors. The inherent endotoxin neutralising properties of both serum and plasma is well known, although the molecular nature of these inhibitors remain to be fully understood, it appears to be heat-labile and the effects are partially removed by dilution and heat treatment (Novitsky & Roslansky 1985). The spike recovery from plasma was 58.1%, which is just within the 50-200% requirement, although the amount of spiked LPS retrieved is relatively low. Hence it is worthwhile exploring measures to improve the spike recovery such as using different dilutions and use of detergents to improve the recovery (see Chapter 6.7 and 6.8). A considerable large amount of LPS was used to spike the samples (5 EU/mL)
although it is unlikely that samples collected from non-septic haemodialysis patients would have such a high level of endotoxemia. It is unclear, if endotoxin spike recovery would still be in the acceptable range if lower concentrations of endotoxin are used to spike and further studies will be conducted to explore this. Further studies were conducted to determine the spike recovery from samples spiked with endotoxin in the range reported in the dialysis literature (see Table 2-1) (Chapter 6.9). In conclusion, the study findings show that more endotoxin can be detected from plasma compared to serum. The reasons for this are unclear, although it has been reported that endotoxin may be associated with blood components such as platelets that are not found in serum (Das et al. 1973). In subsequent studies described in this thesis to investigate the association between endotoxemia and unexplained inflammation in haemodialysis patients, the plasma component of blood was used for the measurement of endotoxin.
6.6 Analysing the optical density graph - use of alternative methods to calculate endotoxin

6.6.1 Introduction

For kinetic LAL techniques, there are a number of different methods of calculating the endotoxin content using the generated optical density graphs. As discussed in Chapter 3, the most conventional method used by manufacturers is based on the time taken for the optical density to increase to a threshold optical density (typically ranging from 0.03 to 0.2) [Figure 6-4].

![Optical density graph showing reaction curve](image)

Figure 6-4: Optical density graph showing reaction curve

There is no guidance on the threshold optical density and manufacturers may recommend different threshold or onset optical densities, although it is generally recommended that a lower threshold should be used if low concentration standards are used or if a particular batch of LAL reagents reacts more slowly.

The purpose of this study was to determine whether alternative parameters derived from the reaction curve of the optical density graph could better quantify endotoxin content of a sample compared to the conventional method.

The optical density plot is typically a sigmoid curve consisting of a lag phase, followed by a period of rapid increase and ending with a terminal plateau phase. The rate of increase in optical density is a function of the concentration of endotoxin (Hurley et al. 1991). An alternative method of calculation is to calculate the maximum
velocity of the reaction (Vmax) by measuring the slope of the linear part of the reaction curve (Figure 6-5). This method requires the reaction in the sample to reach a plateau phase because most software are only able to determine Vmax from a reaction curve accurately that reaches a plateau (Gnauck et al. 2016).

![Figure 6-5: Linear portion of reaction curve is used for calculation Vmax](image)

Other alternative parameters including using the reaction time taken for the sample to increase by 50% of the maximum optical density (LAL50%) [Figure 6-6].
The disadvantage of using the Vmax and the LAL50% parameters is the requirement for prolongation of the analytical period to allow the reaction curve to reach the plateau phase. Analytical periods may be significantly prolonged for samples with a low amount of endotoxin.

As discussed earlier, the onset optical density threshold set by manufacturers is variable and can range from 0.03 to 0.2. A different onset reaction time was selected based on the point at which the reaction curve starts becomes linear rather than using a ‘fixed’ optical density which is arbitrarily set. This point was chosen since this represents the onset time for which the reaction curve achieves the most rapid rate of increase in optical density (‘onset time B’) [see Figure 6-7].
Figure 6-7: Onset time B – reaction time at onset of maximal rate of increase in optical density

The accuracy of all four parameters including the conventional method, Vmax, LAL50% and onset time B to quantify endotoxin from plasma samples spiked with CSE were compared.

6.6.2 Methods

Blood samples were drawn from two healthy controls using the method already described in section 6.5.2, blood samples were processed to produce plasma and stored at -80°C until analysis.

Plasma samples were thawed on the day of analysis and spiked with CSE ranging from 0.05 to 5 EU/mL and incubated for 15-30 minutes. Samples were diluted 1:10 and heated at 70°C for 10 minutes followed by analysis using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles River, Ecully) as described in section 6.5.2. All samples were incubated at 37°C and analysed using a Biotek ELx808 absorbance microplate reader. Optical density readings were measured every 30 seconds for 9999 seconds which was the analytical time limit of the microplate reader.

Data analysis
Data points from optical density graph for standards and samples were extracted from the microplate reader. An asymmetric sigmoidal 5 parameter logistic regression line was fitted through these data points using Graphpad Prism Software (version 6.01, 2012), the regression line was used for calculation of Vmax, LAL50% and ‘onset time B’.

**Vmax**: was calculated by measuring the slope of the gradient of the linear portion of the sigmoid curve.

**LAL50%**: the reaction time at the point for which the optical density was 50% of the maximum optical density reached by the end of the incubation period was recorded.

**Onset time B**: this is the reaction time at the onset of the maximal rate of increase in the optical density of the sample. The second derivative of the regression line for each sample analysed was plotted and the reaction time for which the maximal rate of increase in optical density was recorded by manual inspection by identifying the first inflection point of the graph of the second derivative of the regression line (see Figure 6-8).
Figure 6-8: (A) Optical density curve of sample, (B) First derivative of curve A, (C) Second derivative of curve A. Onset time B was recorded at the first point of inflection for graph C (red vertical line – represents time of onset of maximal rate of increase in optical density).

Standard curves were constructed using optical density readings from dilutions of CSE ranging from 10 to 0.005 EU/mL. Spike recovery for each spiked sample was recorded and comparisons made between each method.
6.6.3 Results

Spike recovery from plasma samples calculated using all four parameters derived from the optical density graph is shown in Table 6-4. Using the conventional method of calculating endotoxin content (using the reaction time at a onset optical density of 0.03) yielded the most accurate spike recoveries, ranging from 53.9 to 105%, in one sample spiked with 0.05 EU/mL, recovery was only 0%. This may have been due to the low level spike used since 0.05 EU/mL was the lowest limit of detection (the lowest standard used for this study was 0.005 EU/mL, dilution factor was 1:10). Spike recovery using the Vmax was poor ranging from 0 to 27.3%. The onset time B method generated a large variability in spike recovery ranging from -48 to 95.2% respectively. Spike recovery using was LAL50% was similar to that using the conventional method with recovery ranging from 57.9 to 69.8%, although in all plasma samples that were spiked with 0.05 EU/mL, spike recovery was 0%. This suggests that the conventional method of using the reaction time at a fixed optical density was the most accurate method of calculating endotoxin content in comparison to the other alternative methods.
Table 6-4: Spike recovery from plasma samples spiked with different concentrations of control standard endotoxin. Spike recovery derived from using different parameters of optical density graph was compared. Onset OD reaction time, reaction time at onset OD 0.03; Vmax, slope of linear part of reaction curve; LAL 50%, reaction time at 50% of max OD, Onset time B, reaction time at onset of maximal rate of increase in OD

<table>
<thead>
<tr>
<th>Participant</th>
<th>Concentration of endotoxin spike (EU/mL)</th>
<th>Parameter</th>
<th>Measured endotoxin (EU/mL)</th>
<th>Spike recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 (unspiked sample)</td>
<td>Onset OD reaction time</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>0.052</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>0.086</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>Onset OD reaction time</td>
<td>0.053</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>&lt;0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>&lt;0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>0.062</td>
<td>-48</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Onset OD reaction time</td>
<td>0.375</td>
<td>75.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>0.123</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>0.307</td>
<td>61.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>0.437</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Onset OD reaction time</td>
<td>2.697</td>
<td>53.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>0.378</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>2.95</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>4.266</td>
<td>83.6</td>
</tr>
<tr>
<td>2</td>
<td>0 (unspiked sample)</td>
<td>Onset OD reaction time</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>0.164</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>Onset OD reaction time</td>
<td>&lt;0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>&lt;0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>&lt;0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>0.198</td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Onset OD reaction time</td>
<td>3.676</td>
<td>73.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>1.364</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAL50%</td>
<td>3.49</td>
<td>69.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onset time B</td>
<td>4.922</td>
<td>95.2</td>
</tr>
</tbody>
</table>

6.6.4 Conclusion

This study investigated alternative methods of calculating endotoxin content from the optical density graph. Alternative methods using Vmax, the LAL50% and onset time B were not superior to the conventional method of calculating endotoxin content. The Vmax and LAL50% method have the added disadvantage of requiring a prolonged analytical time to allow the reaction curves to reach the plateau phase. Using the
reaction time at the onset of maximal rate of increase in optical density (onset time B) yielded extremely variable spike recoveries and would not be suitable for use.

In summary, using the reaction time at a fixed onset optical density was the optimum parameter for calculating endotoxin content. This method of calculation is built into most manufacturer software which enables automaticity for calculation of endotoxin content and results can be obtained without having to wait for the reaction curves to reach the plateau phase. One limitation of this study was that different threshold levels of optical density was not investigated and it may be possible that having a lower or high threshold optical density value may improve the accuracy of the results although using the standard value recommended by the manufacturer (onset optical density of 0.03), although the majority of spike recoveries was acceptable and within the 50-200% limit.

In summary, using the reaction time at the onset of a fixed optical density provides the most accurate method of quantifying endotoxin content of a sample. This parameter from the optical density graph will be used for calculation of endotoxin in subsequent clinical studies.
6.7 Studies to determine optimum dilution factor for endotoxin detection using the LAL assay

6.7.1 Introduction

Blood consists of complex proteins such as endogenous amidases (e.g. plasmin and thrombin) that can activate the LAL assay and proteins such as anti-thrombin II (a serine protease inhibitor) that may inhibit the activity of the LAL assay (Webster 1980). Inactivation of these plasma components is necessary prior to measurement for endotoxin using the LAL assay. Several historical methods for inactivation of these substances have been previously described including chloroform extraction, gel extraction, solid-phase binding and perchloric acid treatment. These techniques are complex and have largely been superseded by dilution and heat treatment for its simplicity and relative efficacy (Hurley 1995). Although simple, the necessity to dilute samples decreases the sensitivity of the assay. The current limit of detection using kinetic techniques is 0.001 EU/mL (Brade 1999), but blood samples are usually diluted by a ten-fold dilution, this would reduce the limit of detection to 0.01 EU/mL. Thus, a minimum dilution factor should be used to enable sufficient dilution of the interfering factor in the sample without compromising the sensitivity of the assay. The usual dilution factor is 1:10, however more recent endotoxin studies in the dialysis population have utilised a 1:5 dilution (Christopher McIntyre 2011; Jefferies et al. 2014). The studies described in section 0 and 0 were carried using a 1:10 dilution factor for plasma and serum samples. These preliminary studies show that in general, spike recovery from plasma was within the acceptable limit of 50-200%, however there was a general trend for samples spiked with large amounts of endotoxin to achieve lower spike recoveries compared to samples spiked with smaller quantities of endotoxin (see Table 6-4, spike recoveries for samples spiked with 0.05 EU/mL ranged from 75.1 to 105%; spike recoveries for samples spiked with 5 EU/mL ranged from 57.9 to 73.5%). It is possible that for samples with known large quantities of endotoxin, further dilution is necessary to improve recovery. It would also be desirable to investigate if using 1:5 dilution could adequately remove the effects of any activators or inhibitors in plasma since this would the sensitivity of the assay to be increased.
The purpose of this study was to investigate optimal dilution factors for pre-treatment of uraemic plasma for endotoxin detection.

6.7.2 Method

Blood samples were drawn from one healthy control and processed to produce platelet rich plasma using the method as described in section 6.5.2. Samples were stored at -80°C.

On the day of analysis, samples were thawed to room temperature (20-25°C) and divided into aliquots. Samples were spiked with different concentrations of control standard endotoxin (CSE) ranging 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 EU/mL. Samples were incubated for 15 to 30 minutes and diluted 1:5 and 1:10 and subjected to dry heat at 70°C for 10 minutes prior to analysis for endotoxin to calculate spike recovery using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles Rivers). Standard curves were constructed for each plate from 10 to 0.005 EU/mL. Spike recovery for each spiked sample was calculated. Spike recovery was initially compared between 1:5 and 1:10 dilutions. For samples with a spike recovery ≤60%, samples were further diluted to 1:20 to determine if spike recovery could be improved with a greater dilution factor.

6.7.3 Results

Endotoxin spike recovery for samples diluted using different dilution factors are shown in Table 6-5. In general, a 1:5 dilution yielded relatively poor spike recoveries ranging from 0-60%, suggesting that a 1:5 dilution was insufficient to remove the inhibitory effects of plasma on the LAL assay. Using a higher dilution factor of 1:10 revealed similar findings to the results from studies carried out in section 0. Spike recovery was reasonable and within the acceptable limits, ranging from 93.6 to 144%, but recovery progressively reduced with increasing concentration of the endotoxin spike. Samples with 1 to 5 EU/mL of added endotoxin resulted in relatively modest spike recoveries (41.3 to 62.9%). However, diluting the same samples using a 1:20 improved the spike recovery to between 79.3 to 124.9%. 
<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endotoxin (EU/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:5 dilution</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>0.083</td>
<td>0.147</td>
<td>0.245</td>
<td>0.629</td>
<td>1.486</td>
<td>2.063</td>
</tr>
<tr>
<td>Spike recovery (%)</td>
<td>0</td>
<td>55</td>
<td>49</td>
<td>45</td>
<td>60</td>
<td>58</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>1:10 dilution</td>
<td>&lt;0.005</td>
<td>0.072</td>
<td>0.134</td>
<td>0.234</td>
<td>0.47</td>
<td>0.629</td>
<td>1.486</td>
<td>2.063</td>
</tr>
<tr>
<td>Spike recovery (%)</td>
<td>144</td>
<td>134</td>
<td>93.6</td>
<td>94</td>
<td>62.9</td>
<td>59.4</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>1:20 dilution</td>
<td>&lt;0.005</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.249</td>
<td>2.79</td>
<td>3.96</td>
</tr>
<tr>
<td>Spike recovery (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>124.9</td>
<td>111.6</td>
<td>79.2</td>
</tr>
</tbody>
</table>

Table 6-5: Spike recoveries for plasma samples diluted using 1:5, 1:10 and 1:20 dilution factor
6.7.4 Conclusion

This study was in agreement with previous observations that sufficient dilution and heat treatment of plasma is necessary for adequate retrieval of endotoxin. Use of 1:5 dilution factor was borderline sufficient to recover some endotoxin from plasma however in some cases, spike recovery was below the 50% criteria. This is contrary to studies conducted by other investigators (Jefferies et al. 2014; Szeto et al. 2010; Kwan, Chow, Leung, et al. 2013) who used a 1:5 dilution and found elevated blood endotoxin levels (mean endotoxin levels ranged from 0.65-1.95 EU/mL) in dialysis patients. However, these studies were performed using an end-point LAL detection method.

The minimum dilution factor necessary was 1:10 for more accurate endotoxin spike recoveries. However, although spike recovery was accurate for samples containing lower amounts of endotoxin (0.05-0.5 EU/mL), in agreement with the studies described in Chapter 6.5, spike recovery progressively reduced with higher concentrations of endotoxin spike. The reason for this observation is unclear however it is known that the aggregative state of the endotoxin molecule may have differential effects biologically and on the LAL assay itself. Naturally occurring endotoxin are usually in an aggregated state, it has been proposed that endotoxin aggregation is necessary for biological and LAL activity. Ribi et al showed that endotoxin derived from three species of gram negative bacteria was rendered non-pyrogenic using the rabbit pyrogen test when the endotoxin was dissociated using sodium deoxycholate. Removal of sodium deoxycholate with dialysis allowed the subunits to re-aggregate and restored its pyrogenic activity in rabbits (Ribi et al. 1966). Mueller et al examined the biological activity of aggregated and monomeric forms of endotoxin and found that aggregates are more biologically active in terms of cytokine inducing effects on human mononuclear cells and on the LAL assay (Mueller et al. 2004). However, other authors have found the opposite and described increased activity of the LAL assay with monomeric forms LPS (Takayama et al. 1994a; Shnyra et al. 1993). Detergents and surfactants are known to alter the aggregative state of the endotoxin molecule thus altering their biological activity and its subsequent detection by the LAL assay. Increased endotoxin detection from
blood of patients with liver disease using surfactants has been previously reported (Bode et al. 1993), although the mechanism for this have not been elucidated.

This small study suggests that retrieval of endotoxin from plasma in the range 0.05 to 0.5 EU/mL, is possible using current blood sampling methods with a 1:10 dilution and heat treatment. Recovery of endotoxin in samples with higher amounts of endotoxin (>0.5 EU/mL) is not as successful and may need further dilution to obtain a more accurate result. However, this approach would be cumbersome as samples may need to be measured twice although it would be unlikely for many samples from our clinically stable non-infected haemodialysis population to have such high level of endotoxin. Given the success reported by Bode et al, the use of surfactants to improve spike recovery was investigated (see section 6.8).
6.8 Studies to investigate the use of surfactants to optimise endotoxin detection using the LAL assay

6.8.1 Introduction

Endotoxins are lipopolysaccharides comprising of three distinct components (Figure 6-9). The O antigen consists of a polysaccharide region which is variable between species. This is attached to a more conserved core region and lipid A antigen. The lipid A antigen is highly conserved among different species of bacteria and is responsible for the pro-inflammatory and toxic effects of endotoxin. The lipid A region is the component that reacts with biological assays enabling its detection (Williams 2007).

Figure 6-9: Schematic diagram of endotoxin structure

Endotoxin is an amphipathic molecule and can form aggregates and micelles in aqueous form. It has been hypothesised that highly aggregated forms of endotoxin may ‘hide’ the endotoxin from detection (Bode et al. 1993; Fukui et al. 1993). However, most consider the endotoxin aggregates are more active biologically and reactive with detection assays (Tsuchiya 2014; Mueller et al. 2004; Ribi et al. 1966). Although some studies have found the opposite is true and have shown
that monomeric forms of endotoxin are more active (Din et al. 1993a; Takayama et al. 1994b). Thus chemicals that can alter the aggregative state of an endotoxin molecule may be able to improve its reactivity with detection assays.

Some authors have found that use of Tween 80 (polysorbate 80), a non-ionic surfactant, improved the recovery of endotoxin from plasma of patients with liver disease (Fukui et al. 1993; Bode et al. 1993). Use of other surfactants such as Tween 20 (polysorbate 20) and Triton X-100 to improve recovery of endotoxin from plasma has also been reported (Katsuya et al. 1992). Surfactants are known to alter the aggregative state of endotoxin by dissociating them into their monomeric forms (Jang et al. 2009). Due to the reduced spike recovery observed with blood specimens spiked with high amounts of endotoxin (see section 6.8), the use of two surfactants – Triton X-100 and Tween 80, to improve spike recovery from plasma samples was investigated.

6.8.2 Methods

Concentrations of Triton X-100 (Sigma-Aldrich, United Kingdom) and Tween-80 (Merck Chemicals, Germany) similar to those used in previous studies (Bode et al. 1993; Katsuya et al. 1992) were made by dilution with LAL reagent water. Triton X-100 at concentrations of 0.02% and Tween 80 at 0.1% were tested for endotoxin contamination using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles River, France) to ensure that both surfactants were not contaminated with endotoxin. Samples were analysed in duplicate and positive control wells were spiked with 0.1 EU/mL for measurement of spike recovery to ensure there were no significant LAL interfering factors present in the surfactant. LAL reagent water was used as negative control and standard curve ranging from 10-0.005 EU/mL was constructed. Endotoxin results were extrapolated from the standard curve using reaction time at an onset optical density of 0.03.

6.8.2.1 Determining the optimum surfactant

Studies were carried out to determine the optimum surfactant to use as a diluent since both Tween 80 and Triton X-100 have been used previously. Blood samples were collected from one healthy control and processed to produce plasma and
stored as previously described (section 6.5.2). Samples were thawed on the day of analysis, aliquoted and spiked with 0.05, 0.5 and 2.5 EU/mL. Endotoxin spiked samples were diluted in either 0.02% Triton X-100 or 0.1% Tween 80 using a 1:10 dilution factor followed by heat treatment at 70°C for 10 minutes. Samples were analysed using Endosafe KTA2 LAL assay to assess spike recovery.

6.8.2.2 Determining the effect of surfactants on endotoxin spike recovery from plasma

The optimum surfactant determined from the study performed in section 6.8.2.1 was further explored measuring its effect on spike recovery from samples from five healthy controls and five haemodialysis patients to ensure that any potential efficacious effect of surfactants on spike recovery could be replicated in uraemic plasma. Blood samples were collected and processed as described above, aliquots of plasma were spiked with 0.1 and 2.5 EU/mL. Samples were diluted 1:10 using water or the optimum surfactant determined from section 6.8.2.1 and heat treated as described above. All samples were analysed using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles River, France) to compare the difference in spike recovery achieved with using water or detergent as a diluent. Standard curves from 10-0.0025 EU/mL was constructed, endotoxin results were extrapolated from the standard curve using the reaction time at onset optical density of 0.03.

Due to the viscous nature of plasma and surfactants, occasionally bubbles in the samples caused artefacts in the optical density graphs. These artefacts can lead to imprecision in the calculation of endotoxin content in the sample (Figure 6-10). Optical density graphs for all samples were inspected and those containing artefacts were discarded from the analysis and the sample measurement was repeated. Artefacts occurred rarely but was a result of inadequate pipetting which created bubbles in the sample.
Figure 6-10: Bubbles in the samples introducing artefact in the optical density graph.

**Statistical analysis**

Differences in spike recovery between samples diluted in surfactant and LAL reagent water were compared using paired t test. Endotoxin concentration between groups was compared using the Wilcoxon signed-rank test or Mann–Whitney U test as appropriate.

**6.8.3 Results**

Dilutions of Triton X-100 and Tween 80 did not have any detectable endotoxin. Spike recovery for positive product controls for both surfactants was within the 50-200% range (Table 6-6).

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Endotoxin (EU/mL)</th>
<th>Spike recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton 0.02%</td>
<td>&lt;0.005</td>
<td>59</td>
</tr>
<tr>
<td>Tween 80 0.1%</td>
<td>&lt;0.005</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 6-6: Endotoxin content of detergents and respective spike recovery from positive product control

LAL reagent water and both surfactants were used to dilute endotoxin spiked plasma samples (Table 6-7), similar to previous findings, spike recovery using water as a diluent was within the 50-200% valid range, however a progressive reduction in spike recovery was observed in samples with progressively higher concentrations of
endotoxin. Spike recovery was not improved with the use of Triton X-100 with spike recovery ranging from 0 to 67%. Spike recovery using Tween 80 was improved compared to both Triton X-100 and water with recoveries ranging from 92 to 188%.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Concentration of endotoxin spike (EU/mL)</th>
<th>Endotoxin (EU/mL)</th>
<th>Spike recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL reagent water</td>
<td>0</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.054</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.393</td>
<td>78.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.368</td>
<td>54.7</td>
</tr>
<tr>
<td>Triton 0.02%</td>
<td>0</td>
<td>0.089</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.079</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.424</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.67</td>
<td>63.2</td>
</tr>
<tr>
<td>Tween80 0.1%</td>
<td>0</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.094</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.52</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.30</td>
<td>92</td>
</tr>
</tbody>
</table>

Table 6-7: Comparison of spike recovery from plasma samples between detergents and water as a diluent

These initial findings suggest that at the concentrations tested, greater improvement in endotoxin recovery could be achieved with Tween 80 compared to Triton X-100. The efficacy of 0.1% Tween 80 was further examined by repeating in a study comprising of five haemodialysis patients (mean age 58) and five healthy adults (mean age 44).

Endotoxin recovery was similar in both haemodialysis patients and healthy subjects spiked with a low concentration of endotoxin (0.1EU/ml). In plasma spiked with high concentration of endotoxin (2.5 EU/ml), use of Tween 80 significantly improved recovery in both haemodialysis patients (100.1% vs. 70.4%; p < 0.001) [Figure 6-11] and healthy controls (90.4% vs. 59.8%; p = 0.002) [Figure 6-12].

103
Figure 6-11: Mean endotoxin spike recovery from haemodialysis patients. Plasma samples diluted with 0.1% Tween 80 and water. Reproduced from (Wong et al. 2015) BMJ publishing group with permission.

Figure 6-12: Mean endotoxin spike recovery in healthy plasma diluted with 0.1% Tween 80 and water. Reproduced from (Wong et al. 2015) BMJ publishing group with permission.
In unspiked plasma samples, dilution with 0.1% Tween 80 yielded a higher concentration of endotoxin content than in plasma samples diluted with water only (0.04 EU/ml vs. 0.036 EU/ml; p = 0.046) (Figure 6-13).

![Graph showing baseline endotoxin content in plasma of haemodialysis patients diluted with 0.1% Tween 80 and water. Reproduced from (Wong et al. 2015) BMJ publishing group with permission.](image)

Endotoxemia was detected in 4 out of 5 haemodialysis patients and the median endotoxin level was 0.041 EU/ml [95% CI 0 to 0.1 EU/ml]. In healthy controls, low level endotoxemia was detected in only one subject (0.027 EU/ml).

6.8.4 Conclusion

Valid spike recovery of between 50-200% was obtained using both Tween 80 and water. Spike recovery was reduced in samples containing a high quantity of endotoxin when water was used as a diluent. This was improved with the use of 0.1% Tween 80 suggesting it improves detection if samples contain a large quantity of endotoxin. Baseline endotoxin content was also significantly higher in plasma diluted with Tween 80 compared to dilution in water only, further supporting this notion and in agreement with previous studies (Bode et al. 1993; Fukui et al. 1993).
The underlying mechanistic effect which could explain the improved endotoxin detection using detergents is unclear. In general, industry experts consider that the aggregated form of endotoxin molecule is more biologically active (Tsuchiya 2014), although findings from some studies are in conflict with this opinion (Takahama et al. 1994b; Din et al. 1993b). Pharmaceutical products containing surfactants or detergents such as polysorbate have recently been identified as a cause of the phenomenon of ‘low endotoxin recovery’ in products such as vaccines which have been directly spiked with endotoxin (Reich et al. 2016). Dissociation of endotoxin aggregates will only occur in the presence of chelating agent such as sodium citrate. It has been suggested that increasing the concentration of chelating agents disturbs the ionic interactions between endotoxin molecules, reducing the rigidity of aggregates. This allows surfactants such as polysorbate, to intercalate into the destabilized aggregates and forming micelles with endotoxin ‘masking’ the molecule from detection (Reich et al. 2016). Thus, the finding of improved endotoxin detection from blood specimens with surfactants conflicts with the observations noted in the pharmaceutical industry. One hypothesis is that surfactants at a lower threshold concentration causes only partial breakdown of the endotoxin aggregate, allowing the structure to exist as partial aggregates. This better exposes the lipid molecules to Factor C and leads to an increase in the efficiency of endotoxin detection (Wimbish & Reich 2016; Nakamura et al. 1988).

Due to the finding of improved endotoxin detection from plasma samples with the use of 0.1% Tween 80, this was used as a diluent in subsequent studies. The series of studies described in 6.3, 6.5, 6.6 and 6.7 have enabled the optimisation of the kinetic turbidimetric LAL assay with reasonable spike recoveries achieved. However, as discussed in Chapter 3, a number of alternative detection assays are available including the kinetic chromogenic LAL assay and the Endotoxin Scattering Photometry assay. Using the kinetic turbidimetric LAL assay with the methodology defined by the studies so far, comparative studies to evaluate the performance of these assays will be described in the next section.
6.9 Comparative study of blood endotoxin detection methods in haemodialysis patients

6.9.1 Introduction

Endotoxemia is a widely reported phenomenon in haemodialysis patients (Gonçalves et al. 2006), however endotoxin detection in blood is difficult and the optimal assay for use in haemodialysis patients is unknown. Many different endotoxin detection assays have been employed in previous studies in dialysis patients, including the turbidimetric LAL assay, chromogenic LAL assay, Endotoxin Scattering Photometry (ESP) and Endotoxin Activity Assay (EAA). It is important to determine the optimum detection assay for use in patients with end-stage kidney disease since endotoxemia is reported to be associated with chronic inflammation (Hauser et al. 2011) – a poor prognostic marker (Carrero & Stenvinkel 2010). Accurate endotoxin measurements are essential to further understanding of the sequela of endotoxemia in this population and to facilitate the development of potential endotoxin-lowering strategies.

The LAL assay is the most commonly used endotoxin detection assay. LAL is derived from extracts of primitive amoebocytes present in the haemolymph of the horseshoe crab. The lipid A component of endotoxin interacts with pro-clotting enzymes present in LAL activating a coagulation cascade resulting in gelation and eventual clot formation (Williams 2007). High endotoxin content samples lead to rapid gelation. The rate of increase in turbidity can be measured using a spectrophotometer to quantify the endotoxin content of a sample. This technique is known as the turbidimetric LAL assay. A variation of this technique involves the addition of chromogenic substrates to LAL which undergoes a colour change in the presence of endotoxin. The chromogenic LAL assay has been the most commonly used endotoxin detection method in previous human studies and dialysis literature (Ronco et al. 2010). However in Japan, the turbidimetric LAL assay is routinely used and is covered by the Japanese national health insurance program (Matsumoto et al. 2013). It is not know which variation of the LAL assay has better performance since direct comparative studies in haemodialysis patients have not been published.
A series of studies were determined from sections 6.3, 6.5, 6.6, 6.7 and 6.8 to determine appropriate blood sampling methodology and optimising the kinetic LAL assay. Using the methodology determined from these preliminary studies, both variations of the LAL assay was compared with the novel Endotoxin Scattering Photometry assay (ESP). The ESP assay is a novel method of endotoxin detection has been developed capable of detecting very low levels of endotoxins in biological samples. The ESP assay uses an alternative method of detecting LAL activation by using laser photometry to detect the appearance of small particles formed early in the activation of the coagulation cascade. Validation studies have demonstrated its ability to detect levels as a low as 0.01pg/mL or 0.048mEU/ml (Obata et al. 2008). A few clinical studies have demonstrated a superior predictive power of the ESP assay over the LAL assay in the diagnosis of intra-abdominal sepsis (Shimizu et al. 2013) and a small study using the ESP assay detected a strong association between CRP and low level endotoxemia in haemodialysis patients although direct comparison with the LAL assay was not performed (Terawaki et al. 2010).

Endotoxin spike recovery experiments were carried out to directly compare the accuracy and precision of the both variations of the LAL assay with the ESP assay.

6.9.2 Methods

Participant selection

The whole study cohort consisted of 7 clinically stable patients undergoing outpatient haemodialysis (mean age 63) at the Lister Renal Unit (Hertfordshire, UK) and 7 healthy controls (mean age 47) recruited from volunteers and healthcare workers at the Lister Hospital (Hertfordshire, UK). To minimise the possibility of high amounts of native endotoxin present in plasma samples which would interfere with endotoxin spike recovery experiments. Only subjects who were clinically well with no evidence of sepsis were recruited to the study. Additionally, HD patients were required to have a C-reactive protein measurement of <5mg/L within the last month to be eligible for recruitment. Patients with a venous catheter in-situ, liver dysfunction, congestive cardiac failure and active gastrointestinal or inflammatory diseases were not eligible for recruitment.
**Blood collection**

Blood collection was performed as described in section 6.3 and processed to produce plasma, all samples were stored at -80°C prior to analysis.

**Plasma pre-treatment for the LAL assay**

Plasma samples that were used for analysis with the turbidimetric and chromogenic LAL assay was processed identically. Plasma samples were diluted 1:10 in 0.1% Tween 80 (Merck Chemicals, Darmstadt, Germany) and heated at 70°C for 10 minutes to denature plasma proteins and proteases which may interfere with endotoxin detection by the LAL assay (Hurley 1995; Dawson 2005). Batches of 0.1% Tween 80 were consistently found to have no detectable endotoxin and were free of interfering factors (endotoxin spike recoveries ranged from 94% to 106%). Samples were cooled to room temperature (20-25°C) prior to endotoxin measurements.

**Assessment of assay accuracy**

Plasma samples from five haemodialysis patients and five healthy controls were divided into aliquots and directly spiked with five different concentrations of control standard *E.coli* 055:B55 endotoxin (0 [unspiked sample], 0.05, 0.1, 0.5 and 2.5 EU/ml) and then diluted in 0.1% Tween 80 and subjected to heat treatment as described above. Each sample was tested for endotoxin content in sextuplicate using both the kinetic chromogenic and kinetic turbidimetric LAL assay. Samples that contained bubbles introducing artefact into optical density graphs were discarded from the analysis. All samples had between four to six repeated measurements. Percentage spike recovery was calculated for each spiked plasma sample using the formula:

\[
\text{% spike recovery} = \frac{\text{Measured endotoxin content in spiked sample} - \text{Measured endotoxin content in unspiked sample}}{\text{Amount of added endotoxin}} \times 100\%
\]

The optimal spike recovery is 100%, although a spike recovery between 50 and 200% is considered valid (United States Pharmacopoeia 2013). Assay accuracy was
assessed by comparing differences in spike recovery between all three assays. Measured endotoxin content was compared with expected endotoxin content in spiked samples using Bland-Altman analysis (Bland & Altman 1986) and linear regression analysis was used to calculate slope and y-intercept of the line of best fit to estimate magnitude of proportional and constant error (Westgard & Hunt 1973).

**Assessment of assay precision**

Samples from two haemodialysis patients and two healthy controls were pooled separately. Pooled uraemic and pooled healthy control plasma were spiked with two different concentrations of control standard endotoxin (0.05 and 0.5 EU/ml), diluted and heat treated as described earlier and subjected to ten repeated assay measurements for endotoxin using the kinetic chromogenic and kinetic turbidimetric LAL assay to calculate a coefficient of variation (CV). The CV is used to assess assay precision (Richardson & Novitsky 2002) but there is no industry guidance on a maximum acceptable CV, although most LAL manufacturers impose a CV <10-20% for results to be considered valid (Richardson & Novitsky 2002). CV was calculated using the formula:

\[
\text{Coefficient of variation} = \frac{\text{Standard deviation onset reaction time of samples}}{\text{Mean onset reaction time of samples}} \times 100\%
\]

**Endotoxin assays**

*Kinetic turbidimetric LAL assay:* samples were assayed using Endosafe KTA2 lysate (Charles River Laboratories, France) on sterile 96-well microplates (manufacturer certified to <0.001EU/ml). Assays were carried out using manufacturer supplied equipment including depyrogenated glass tube, pipettes and Eppendorf Endosafe pipette tips (certified <0.005 EU/ml). Analysis of each microplate consisted of duplicate wells containing endotoxin-free water to act as a negative control. Plates were analysed using a Biotek ELx808 absorbance microplate reader with Endoscan-V software (version 4.0; Charles River Laboratories, France) and observed at 340nm with an onset optical density value set at 0.03 as per manufacturer’s recommendations. Six-point standard curves were constructed using onset reaction times from standard dilutions of control standard endotoxin (*E.coli* 055: B5) ranging
from 10-0.0025 EU/ml. All standard curves constructed had a correlation coefficient of >0.98. Due to the ten-fold dilution used for plasma, the lower limit of detection was 0.025 EU/ml.

**Kinetic chromogenic LAL assay:** samples were assayed using Endochrome-K lysate (Charles River Laboratories, France) with manufacturer supplied depyrogenated glass tubes, pipettes and pipette tips as described above. Microplates were analysed using FLUOstar Omega microplate reader with MARS data analysis software (BMG Labtech, Offenburg, Germany) and observed at 405nm with an optical density value of 0.1 as per manufacturer’s recommendations. The same concentrations of control standard endotoxin were used to construct standard curves as described for the kinetic turbidimetric LAL assay.

**Endotoxin Scattering Photometry assay:** assays were carried out on plasma samples using the technique as described by Obata et al (Obata et al. 2008). The principle of the assay is based on the detection of coagulin particles formed by the interaction between endotoxin and LAL reagent early in the coagulation cascade. The appearance of these small particles means that the influence of endotoxins has stimulated activation of the clotting enzyme across the LAL cascade and the timing of their appearance is related to endotoxin concentration. The reaction time of appearance of these coagulin particles are used to calculate the endotoxin content of a sample, rather than the traditional method of using the reaction time at the onset of a set optical density. The ESP assay has a reported lower limit of detection of 0.01 pg/mL (approximately 0.048 milli EU/mL) (Terawaki et al. 2010).

Plasma samples (0.1mL) were diluted in distilled water (0.9mL) containing 0.02% Triton-X and 0.02% denatured human albumin. Samples were vortexed and heated for 10 minutes at 70°C on a dry block heater. After cooling on ice for 5 min, an aliquot (0.62 ml) was mixed with LAL lyophilized powder (Single-Test-Wako ES; Wako Pure Chemical Industries, Tokyo, Japan) in a test tube with a flash mixer for 30 seconds, and 300-μl aliquot was moved to a photometric cuvette for laser scattering photometry. The reaction mixture was observed by laser scattering photometry at 37 °C (model PA-200 or PA-20, analytical software, version 3; Kowa, Nagoya, Japan). A light source is transmitted to the solution and the transparency
and relative particle size count is measured every 5 minutes. At the onset of the coagulation, the formation of coagulin particles is detected by a sharp increase in the particle count of small particles ranging from (1-10um) [Figure 6-14].

Figure 6-14: Gelation process using ESP assay at 10pg/mL. The red arrow indicates time of appearance of small particles in the solution and the reaction time is recorded and used to calculate the endotoxin content. (Obata et al. 2008) [with permission from Analytical Biochemistry]

The time at point of inflection is recorded and extrapolated to a standard curve constructed using dilutions of standard endotoxin (Wako Pure Chemical Industries, Tokyo, Japan) ranging from 0.01 pg/mL to 10000 pg/mL (0.000048 to 48 EU/mL).

6.9.3 Results

Comparison of assay accuracy

This sub-study consisted of five haemodialysis patients and five healthy controls. In unspiked plasma samples, the ESP assay reported the highest level of blood endotoxin out of all three assays (0.272 EU/mL). Endotoxemia was detectable in all subjects using the ESP assay. The kinetic turbidimetric LAL assay detected endotoxemia in four out of five haemodialysis patients whereas the kinetic chromogenic LAL assay detected endotoxemia in one out of five haemodialysis patients (Table 6-8). In healthy controls, the ESP assay detected blood endotoxin in all patients with a median endotoxin level of 0.117 EU/mL (interquartile range 0.068-0.54 EU/mL). The kinetic turbidimetric LAL assay detected endotoxin in one patient
only (0.027 EU/mL). No healthy subjects had detectable endotoxemia using the kinetic chromogenic LAL assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Turbidimetric LAL (A)</th>
<th>Chromogenic LAL (B)</th>
<th>ESP (C)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAEMODIALYSIS patients</td>
<td>0.041 [IQR 0.016-0.081]</td>
<td>0 [0-0.017]</td>
<td>0.272 [0.132-0.321]</td>
<td>A vs. B = 0.068 A vs. C = 0.08 B vs. C = &lt;0.05* A vs. B = &lt;0.05* A vs. C = 0.317 B vs. C = &lt;0.05*</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0 [0-0.013]</td>
<td>0</td>
<td>0.117 [0.068-0.54]</td>
<td></td>
</tr>
</tbody>
</table>

Table 6-8: Median endotoxin levels from the kinetic turbidimetric LAL, kinetic chromogenic LAL and ESP assay

Spike recovery for all three assays is shown in Table 6-9. The kinetic turbidimetric assay was the most accurate assay, spike recovery for each level of endotoxin spike was within the 50-200% acceptable limit for both haemodialysis patients and healthy controls. Spike recovery for the kinetic chromogenic assay was frequently below the 50% suggesting persistent inhibition of the kinetic chromogenic LAL assay by interfering factors present in plasma despite dilution and heat-treatment. The accuracy for the ESP assay was poor with wide ranging spike recoveries obtained. For haemodialysis patients, sample spiked with low concentration endotoxin (0.05 to 0.1 EU/mL), yielded highly variable recoveries with interquartile ranges from -56.9% to 677% and -49.3% to 272% respectively. Samples spiked with larger quantities of endotoxin (0.5 to 2.5 EU/mL) were slightly less variable with less wide interquartile ranges [IQR 97.2% to 219.9% and 47.8% to 206.1% respectively]. These results suggest a high degree of inaccuracy with the ESP assay. For healthy controls, similar observations were found with highly variable and inaccurate spike recoveries obtained (overall spike recovery 147.7% [IQR 35.8-353.9]). Due to the highly variable spike recoveries obtained using the ESP assay, use of linear regression analysis to estimate proportional and constant error was not possible.
### Table 6-9: Median spike recovery from kinetic turbidimetric, kinetic chromogenic and ESP assay. HD, haemodialysis; EU/mL, endotoxin units per millilitre. Numbers in brackets represent interquartile ranges

Comparison of assay error and bias

Bland-Altman analysis was used to assess assay bias (Bland & Altman 1986). Bland-Altman plots for measured and expected endotoxin spike recovery all three assays are shown in Figure 6-15. Overall mean bias was lowest for the turbidimetric assay mean bias at 0.011 EU/ml (95% CI 0.079 to -0.057). For the chromogenic assay was -0.384 EU/ml (95% CI -0.219 to -0.549) and assay bias for the ESP was the highest at -0.689 EU/mL (95% CI -3.84 to 2.47). Sub-analysis of the HD cohort revealed mean bias for the turbidimetric assay was 0.049 EU/mL (95% CI 0.162 to -0.064) and significantly lower than both the chromogenic and ESP assay (both p<0.05).
Similarly, in healthy subjects, bias with the chromogenic and ESP assay were both significantly higher than the turbidimetric assay (-0.478 vs. -0.021 EU/ml and -0.771 vs. 0.021 EU/mL respectively, both p<0.05). [Data points in the Bland-Altman plots align in a straight line because the expected values were ‘fixed’ e.g. 0.05, 0.1, 0.5 etc.]

[Figure 6-15: Bland-Altman analysis of measured versus expected spike recovery]

Linear regression analysis was used to estimate constant and proportional error (Westgard & Hunt 1973). Due to the high inaccuracy of the ESP assay, this method was used to compare the kinetic chromogenic and kinetic turbidimetric assay only.

Comparison plots of measured versus expected endotoxin content in spiked samples were constructed (Figure 6-16). Correlation between measured and expected endotoxin was higher for the turbidimetric assay compared to the chromogenic assay for both haemodialysis patients (r = 0.962 vs. 0.939) and healthy subjects (r = 0.954 vs. 0.922). For haemodialysis patients, estimates of proportional error and constant error were lower for the turbidimetric assay (4.2% and 0.016 EU/ml [-0.13 to 0.162]) compared to the chromogenic assay (32.2% and -0.027 EU/ml [-0.094 to -0.041]). In healthy subjects, overall assay error was similarly lower in the turbidimetric assay compared to the chromogenic assay (proportional error, 11.8% vs. 59.0%; constant error, 0.067 EU/ml [-0.011 to 0.145] vs. -0.011 EU/ml [-0.128 to 0.102]) [Table 6-10 and Figure 6-16].
Figure 6-16: Comparison plots showing linear regression lines (hashed lines) for measured versus expected endotoxin content in spiked samples for haemodialysis patients and healthy controls with the chromogenic and turbidimetric assay. Dotted lines represent 95% confidence intervals for linear regression lines. Each data point represents a mean of 4–6 measurements for one subject). Black reference line indicates perfect agreement between measured and expected endotoxin content.

<table>
<thead>
<tr>
<th>Population</th>
<th>Assay</th>
<th>Slope</th>
<th>Proportional error (%)</th>
<th>Y-intercept (constant error) [EU/mL]</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>Turbidimetric</td>
<td>1.042 [0.927-1.157]</td>
<td>4.2</td>
<td>0.016 [-0.130-0.162]</td>
<td>0.962</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>Chromogenic</td>
<td>0.678 [0.625-0.731]</td>
<td>32.2</td>
<td>-0.027 [-0.094-0.041]</td>
<td>0.939</td>
</tr>
<tr>
<td>Healthy</td>
<td>Turbidimetric</td>
<td>0.882 [0.821-0.943]</td>
<td>11.8</td>
<td>0.067 [-0.011-0.145]</td>
<td>0.954</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>Chromogenic</td>
<td>0.41 [0.320-0.500]</td>
<td>59</td>
<td>-0.011 [-0.128-0.102]</td>
<td>0.922</td>
</tr>
</tbody>
</table>

Table 6-10: Comparison of assay error of turbidimetric and chromogenic LAL assay using linear regression analyses
Comparison of assay precision

The coefficient of variation (CV) calculated using the onset reaction time for both cohorts was <10% for both chromogenic and turbidimetric assays. For the ESP assay, imprecision was high for the ESP assay, with CV for reaction times ranging from 9.5% to 22.6%.

CV calculated using the actual endotoxin content of the sample was <10% for the turbidimetric LAL assay in pooled uraemic plasma samples spiked with high concentration of endotoxin (0.5 EU/ml), however in samples spiked with low concentration of endotoxin (0.05 EU/ml), CV was higher at 24.1% suggesting imprecision of the turbidimetric assay at this low endotoxin concentration in HD patients. With the chromogenic assay, for samples spiked with high concentration of endotoxin (0.5 EU/ml), CV for HD patients and healthy subjects was ranged from 25.8 to 26.5% suggesting high imprecision, CV for samples spiked with low amounts of endotoxin (0.05 EU/ml) could not be defined because spike recovery was 0%. Imprecision was highest for the ESP assay with CV ranging from 33.9 to 66.9% (Table 6-11).
Table 6-11: Coefficient of variation for all three assays in pooled uraemic and non-uraemic plasma

<table>
<thead>
<tr>
<th>Population</th>
<th>Assay</th>
<th>CV - onset reaction time (%)</th>
<th>CV - EU/ml (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Concentration of endotoxin spike (EU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD (n = 2)</td>
<td>Turbidimetric</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Chromogenic</td>
<td>2.4</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>ESP</td>
<td>22.6</td>
<td>14.6</td>
</tr>
<tr>
<td>Healthy (n = 2)</td>
<td>Turbidimetric</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Chromogenic</td>
<td>5.4</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>ESP</td>
<td>20.8</td>
<td>9.5</td>
</tr>
</tbody>
</table>
| Table 6-11: Coefficient of variation for all three assays in pooled uraemic and non-uraemic plasma

6.9.4 Discussion

This is the first comparative study of the ESP assay and two commonly used LAL detection techniques in haemodialysis patients. The accuracy and precision of the ESP assay and two kinetic LAL assays utilising chromogenic and turbidimetric methods were studied.

The ESP assay is a novel method of endotoxin detection assay which relies on the detection of coagulin particles formed earlier in the LAL cascade. Its reported advantages include its increased sensitivity and speed of detection compared to the LAL assay. The LAL reagent used in the ESP assay is also insensitive to (1→3)-β-D glucan (BG) stimulation making it specific to endotoxin activation only. BGs are carbohydrate constituents of cereal, yeast and fungal cell walls (Young & Vincent 2005) and are capable of activating the LAL assay via an alternate enzymatic pathway mediated by factor G activation (Morita et al. 1981) [Figure 3-1]. However,
despite the reported advantages of the ESP assay, recovery experiments from plasma spiked with endotoxin using this assay was inaccurate and imprecision was high. The coefficient of variation obtained from this assay varied from 9.5 to as high as 66.9%. The high degree of inaccuracy and imprecision of the ESP assay suggests that it would not be suitable for blood endotoxin measurement.

Only kinetic LAL assays were compared in this study since kinetic techniques have significant advantages over older LAL techniques including the gel-clot and end-point method. Kinetic assays are more sensitive, able to quantify results over a wider range of endotoxin concentrations and benefits from having an automated process of analysis reducing the variation due to user technique (Williams 2007). However, from the dialysis literature, only one study utilised a kinetic assay (Suzuki & Honda 2011), the remaining studies employed either the end-point techniques (Markum 2004; D. Raj et al. 2009; Szeto et al. 2010; Kwan, Chow & Leung 2013; Christopher McIntyre 2011; Szeto et al. 2008; Jefferies et al. 2014) or the gel-point method (El-Koraie & Naga 2013; Taniguchi et al. 1990). Despite their widespread availability and use, it is not known whether the turbidimetric or the chromogenic assay has superior performance in patients with end-stage kidney disease.

This study, using LAL reagents from the same manufacturer, has shown that the kinetic turbidimetric LAL assay is a more accurate and precise endotoxin detection tool compared to the chromogenic assay in haemodialysis patients and is able to detect endotoxin over a wide range of different endotoxin concentrations (0.05-2.5 EU/ml). Additionally, this investigation showed that uraemic solutes that accumulate in patients with kidney failure do not appear to have any greater significant interference on the LAL assay than non-uraemic plasma.

Endotoxin spike recovery with the chromogenic assay was significantly inhibited by plasma from haemodialysis patients and healthy controls despite attempts to remove interfering factors from samples with dilution and heat treatment. Spike recoveries with the chromogenic were consistently below the minimum requirement of 50% for healthy controls and overall median spike recovery was borderline acceptable at 53.8% for the haemodialysis cohort. In comparison, median spike recovery for the turbidimetric assay was 113.8%, well within the industry specified 50-200%
requirement (United States Pharmacopoeia 2013). The magnitude of assay error and bias was also greater for the chromogenic assay for both haemodialysis and healthy control subjects. Assay precision in this study was assessed using the coefficient of variation calculated from both the onset reaction time and the endotoxin content of the sample. There is a lack of industry guidance on which variable should be used to calculate the CV, depending on the manufacturer either the onset reaction time or the endotoxin read-out measurement can be used (Richardson & Novitsky 2002). It has been suggested that CV calculated using the endotoxin content of samples is a more rigorous measure of assay precision (Richardson & Novitsky 2002). Using the onset reaction time, CV was <10% for both the turbidimetric and chromogenic LAL assay. However, CV calculated using the endotoxin content of the sample showed that the turbidimetric assay was more precise than the chromogenic assay. But in low levels of endotoxemia (≤0.05 EU/ml), assay imprecision was higher (CV - 9.3 to 24.1%) and care should be taken at interpreting samples containing low concentration of endotoxins.

Turbidimetric assays have the advantage of being more economical and a historical comparative study in non-uraemic plasma favoured the turbidimetric over the chromogenic assay because of the interfering effect of chromogenic substrates on the kinetics of the LAL reaction (Hurley et al. 1991). For end-point LAL assays, the chromogenic assay may suffer from interference as plasma and serum samples can absorb light at the measured 405nm wavelength, potentially interfering with the results (Fields 2006).

The LAL assay has been extensively used for the detection of endotoxin in pharmaceutical products, however its use in blood has been a matter of intense debate (Novitsky 1994; Cohen 2000). Currently the LAL assay is not licensed by the Food and Drug Administration for ‘detecting endotoxia in man’. This decision was heavily influenced by studies conducted by Stumacher (Stumacher et al. 1973) in 1973 and Elin in 1975 (Elin et al. 1975) demonstrating lack of concordance between endotoxemia and bacteraemia (Novitsky 1994). However, others have debated the validity of trials which measured the correlation of the LAL assay with positive culture
bacteraemia as a ‘gold-standard’ (Novitsky 1994), which is well known to have a low sensitivity for identifying patients with sepsis (Lee et al. 2007).

Due to current limitations with the LAL assay, a number of different novel detection assays have evolved over the last few decades including the recombinant factor C assay (rFC), the ESP assay and the Endotoxin Activity Assay (EAA) – a bioassay based on neutrophil activation by complement opsonised immune complexes of LPS (Romaschin et al. 2012). No studies on the use of the rFC assay in human blood have been published to date. The EAA is an FDA-approved blood endotoxin detection assay, however the EAA provides a read-out of a patient’s neutrophil oxidative chemi-luminescent response to LPS-antibody complexes rather than the direct endotoxin content present in the sample (Romaschin et al. 2012). A recent investigation found a poor dose-response between blood spiked with control standard endotoxin and EAA activity (Matsumoto et al. 2013). It is noteworthy that most studies in the dialysis literature utilised the end-point chromogenic assay supplied by Cambrex (End-point chromogenic LAL QCL-1000). This assay is now being manufactured by Lonza and further comparison with this specific assay may be useful.

The strengths of this study includes the meticulous attention to pre-analytical factors such as careful selection of blood sampling accessories by testing apparatus for contamination and interfering factors. No blood samples were obtained from tunnelled dialysis catheters to reduce the risk of contamination by bacterial biofilms (Dasgupta 2002). Blood sampling and processing was performed rapidly to reduce the risk of endotoxin inactivation in untreated plasma (Hurley 1995). The United States Pharmacopoeia states that in kinetic LAL testing, test samples need to be verified to be free of assay interfering factors by obtaining a spike recovery between 50-200% from a positive product control (PPC) for an endotoxin measurement from a sample to be considered valid (United States Pharmacopoeia 2013). The PPC is a duplicate of the sample spiked with LPS at a concentration that lies near the mid-point of the standard curve after dilution and heat treatment. In this study, plasma samples were spiked with several different quantities of LPS prior to dilution and heat treatment which allowed examination of the ability of the assays to retrieve LPS
from plasma containing wide range of different concentrations of endotoxin. Spiking samples before dilution and heat treatment to measure spike recovery also more closely resemble the process experienced by naturally occurring endotoxins present in the sample.

The limitations of this study include the relatively small number of participants and the use of control standard endotoxin derived to assess spike recovery. Standard endotoxins are derived from E.coli and are usually stabilised in preservatives such as lactulose and polyethylene glycol which may behave differently in vivo compared to naturally occurring endotoxin. However, natural endotoxins are difficult to standardise and many other published studies also use control standard endotoxin to assess recovery or immune response (Hurley 1995; Sturk et al. 1985; Hurley et al. 1991; Peek et al. 2004; Sharma et al. 2005; Fukui et al. 1993). The incubation times for samples spiked with endotoxin were not controlled due to the large number of samples that were processed, although laboratory analyses of spiked samples was carried out as soon as possible. It is unclear whether different incubation times can affect spike recovery. However since the same spiking procedure was used throughout the whole study, incubation times would have been broadly similar between samples. The LAL assays used in this study have not been modified to be insensitive to BG, thus it unclear whether the endotoxin measured in some of the baseline samples may have been due to BG interference. Further study on this issue is warranted, however, the LAL is reported to be nearly 1000-times more sensitive to endotoxins than BG (Roslansky & Novitsky 1991) and contamination with BG can usually be recognised by significant enhancement of spike recoveries (Cooper et al. 1997), which was not seen in this study. Optimal heat treatment conditions were not investigated in this study, however the plasma pre-treatment conditions selected for this study were based on an extensive review by Hurley (Hurley 1995) and it is likely that the pre-treatment process for removal of interfering factors was sufficient since the overall spike recovery for the turbidimetric assay was >100%. The findings could also be criticised in that, since that comparisons were made between chromogenic and turbidimetric assays using LAL reagent from one manufacturer only (Charles Rivers). The conclusions may not apply with use of reagents from other manufacturers, therefore further comparisons with assays from other manufacturers
may be useful particularly the end-point chromogenic assay by Cambrex and Lonza which has been cited in many dialysis studies (see 6.10).

In summary, this study suggests that the kinetic turbidimetric LAL assay is an accurate blood endotoxin detection method in haemodialysis patients. The kinetic chromogenic assay is prone to significant inhibition by the uraemic plasma despite dilution and heat treatment and the ESP assay has poor accuracy and high imprecision. The kinetic turbidimetric LAL assay could be utilised to determine the relationship between endotoxemia with inflammation in dialysis patients. This assay was used in subsequent studies in this thesis for the measurement of blood endotoxin.
6.10 Measurement of blood endotoxin in haemodialysis patients using the kinetic turbidimetric LAL assay and end-point chromogenic assay

6.10.1 Introduction

The studies described in the previous section suggest superior accuracy of the kinetic turbidimetric LAL assay. However, a review of endotoxin studies carried out in the dialysis population showed that the majority of these studies utilised an end-point chromogenic LAL assay. The end-point techniques have significant disadvantages compared to the kinetic methods due to, 1) a narrower range of detection, 2) the necessity for the user to stop the reaction of all sample wells at the same time, thus introducing the problem of intra- and inter-user variability and 3) end-point chromogenic assays may suffer from interference since plasma and serum samples also absorb light at the measured 405nm wavelength potentially interfering with the results. A summary of endotoxin studies carried out in the dialysis population is summarised in Table 6-12 below:

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Assay method</th>
<th>Manufacturer</th>
<th>(1→3)-β-D glucan insensitive?</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Suzuki &amp; Honda 2011)</td>
<td>Haemodialysis</td>
<td>Kinetic turbidimetric</td>
<td>Pyrostar; Wako</td>
<td>Yes</td>
</tr>
<tr>
<td>(Markum 2004)</td>
<td>Haemodialysis</td>
<td>Endpoint chromogenic</td>
<td>Endospecy; Seikagaku</td>
<td>Yes</td>
</tr>
<tr>
<td>(C McIntyre 2011)</td>
<td>Peritoneal dialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
<tr>
<td>(El-Koraie &amp; Naga 2013)</td>
<td>Haemodialysis</td>
<td>Gel-clot</td>
<td>Pyrotell; Associates of Cape Cod</td>
<td>No</td>
</tr>
<tr>
<td>(Kwan, Chow, Leung, et al. 2013)</td>
<td>Peritoneal dialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
<tr>
<td>(Szeto et al. 2008)</td>
<td>Peritoneal dialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
<tr>
<td>(Szeto et al. 2010)</td>
<td>Peritoneal dialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Lonza</td>
<td>No</td>
</tr>
<tr>
<td>(Wu et al. 2014)</td>
<td>Peritoneal dialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Lonza</td>
<td>No</td>
</tr>
<tr>
<td>(Harrison et al. 2012)</td>
<td>Haemodialysis</td>
<td>Endpoint chromogenic</td>
<td>Endospecy; Seikagaku; Toxicolor Test; Seikagaku</td>
<td>Yes</td>
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<tr>
<td>(Taniguchi et al. 1990)</td>
<td>Haemodialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
<tr>
<td>(Kwan &amp; Szeto 2013)</td>
<td>Haemodialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
<tr>
<td>(Goncalves et al. 2006)</td>
<td>CKD</td>
<td>Endpoint chromogenic</td>
<td>Hycult</td>
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</tr>
<tr>
<td>(D. S. C. Raj et al. 2009)</td>
<td>Haemodialysis</td>
<td>Endpoint chromogenic</td>
<td>QCL1000; Cambrex</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 6-12: Characteristics of endotoxin assays used in patients with kidney disease
The majority of studies utilised an end-point chromogenic assay manufactured by Cambrex. The production of this assay has recently been overtaken by Lonza. As discussed previously, most studies that use the end-point chromogenic assay which report unusually high blood endotoxin levels that exceed the expected pyrogenic threshold of 5 EU/kg (Greisman & Hornick 1969). Endotoxin measurements of blood samples that are known to contain detectable endotoxin using the kinetic turbidimetric assay (Endosafe KTA2, Charles Rivers) were subjected to repeat measurement using the end-point chromogenic LAL assay (QCL1000, Lonza) to determine the correlation between endotoxin measurements obtained from both assays.

6.10.2 Methods

Patient population

Plasma samples from twenty haemodialysis patients that were known to contain endotoxin measured using the kinetic turbidimetric LAL assay were selected for study. Inclusion and exclusion criteria for participants are described in section 6.4. All patients were clinically well and did not have any active infection at the time of the study.

Blood sampling and processing

Blood samples from all participants were collected either through an arterio-venous fistula or from a peripheral vein pre-dialysis and processed to produce plasma. The same blood collection and processing method was used as described in section 6.3. Plasma samples were stored at -80°C until analysis. Each plasma sample was analysed twice – once using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles Rivers) and once using the endpoint chromogenic LAL assay (QCL1000, Lonza).

Endotoxin measurement

*Kinetic turbidimetric LAL assay:* samples were measured for endotoxin using the kinetic turbidimetric LAL assay by Charles Rivers using the same procedure as described in section 6.9.
**Endpoint chromogenic LAL assay:** samples were measured for endotoxin using an endpoint chromogenic LAL assay (QCL1000; Lonza) as per manufacturer’s instructions.

Samples were thawed on the day of analysis and diluted 1:10 in LAL reagent water and heated at 70°C for 10 minutes to denature plasma proteins and proteases that may interfere with the LAL reagent. Samples were cooled to room temperature (20-25°C) and transferred to quadruplicate wells on a 96 well microplate (manufacturer certified <0.001 EU/mL) equilibrated at 37°C on a heating block adapter. Two wells of each sample were directly spiked with 0.4 EU/mL to act as positive product controls. A four point standard curve was constructed using standards control endotoxin from 1 to 0.1 EU/mL with LAL reagent water acting as a blank. Fifty microliters of LAL reagent was added to each well using a multichannel pipette and incubated for 10 minutes at 37°C. After 10 minutes, 100µL of chromogenic substrate was added to each well and samples were incubated for a further 6 minutes. After incubation for a total of 16 minutes, the LAL reaction was stopped using a 10% sodium dodecyl sulfate and the microplate was read at 405nm using a spectrophotometer. Mean change in optical density was determined for each well and endotoxin content of each well was calculated by extrapolation from the standard curve (1-0.1 EU/mL; r >0.98).

**Statistical analysis**

Correlation between endotoxin measurements obtained from the kinetic turbidimetric and end-point chromogenic method was measured using Pearson correlation coefficient. Positive product controls for each sample were required to be within the 50-200% for endotoxin read-out measurements to be valid.

**6.10.3 Results**

Endotoxin measurements for both assays are shown in

Table 6-13. Mean endotoxin measurements obtained using the endpoint chromogenic method was almost one hundred fold higher than the kinetic turbidimetric assay (4.55 EU/mL [95% CI 3.83-5.28] vs 0.051 EU/mL [95% CI 0.037-0.065], p<0.001).
The majority of endotoxin measurements obtained from the end point method were not valid, positive product controls for 13/20 (65%) samples did not have valid spike recovery suggesting that the end-point assay is prone to significant interference from substances present in plasma sample despite dilution and heat treatment. All endotoxin measurements obtained from the kinetic turbidimetric LAL assay were valid, and all samples had valid PPC with spike recoveries within the 50-200% limit. There was no significant correlation between the two assays (r value, 0.196; p=0.408).

**Table 6-13**: Endotoxin measurements obtained from the same plasma samples using the kinetic turbidimetric LAL assay (Charles Rivers) and endpoint chromogenic LAL assay (Lonza)
6.10.4 Conclusion

No correlation was found between both assays with a nearly 100-fold difference in endotoxin levels between the two assays. PPC for all samples measured using the kinetic turbidimetric LAL assay had valid spike recoveries. The majority of endotoxin measurements obtained using the endpoint chromogenic LAL assay was invalid. However, endotoxin measurements from the seven samples with valid positive product controls measurements were still extremely high with a mean endotoxin content of 5.36 EU/mL. These measurements appear high considering all participants were non-infected and clinically stable at the time of blood sampling. Given that 5 EU/kg is sufficient to induce pyrogenic symptoms in humans and assuming an average 70kg human with approximately 3L of circulating plasma volume, it would be expected that as little as 0.12 EU/mL would be sufficient to trigger pyrogenic symptoms. The measurements obtained using the endpoint chromogenic method greatly exceeds this pyrogenic threshold. Notably, the endotoxin measurements obtained using the QCL1000 assay in this study are similar to the values reported in published studies using the same assay (Kang et al. 2013; Lira et al. 2010; Harte et al. 2010).

In conclusion, this specific chromogenic endpoint assay appears to give unreliable results with extremely high endotoxin results. The significant majority of samples despite dilution and heat treatment did not yield valid PPCs. For the kinetic turbidimetric LAL assay, all samples had valid PPC with adequate spike recoveries and yielded lower levels blood endotoxin levels in the range which appear to be more biologically plausible.

The reasons for the lack of correlation between the results obtained from both assays are unclear since all LAL reagents are FDA approved for testing of endotoxin prior to product release. Each batch of LAL is assessed and certified by its manufacturer and should be broadly comparable to similar products from other manufacturers. It is likely that these conditions apply only to testing endotoxin in non-biological pharmaceutical or aqueous products such as dialysis fluid and not to complex substances such as blood and plasma.
6.11 Summary

In summary, the association of endotoxemia with inflammation in dialysis patients needs to be confirmed with an accurate endotoxin detection assay to increase understanding of the potential harmful effects of chronic endotoxemia in this population and to develop endotoxin-lowering interventions.

A series of studies were described in this chapter to determine the optimum method of endotoxin detection specifically for patients with kidney disease. Pre-analytical factors were considered including selection of appropriate blood sampling apparatus, collection and storage tubes (described in section 6.3) to avoid interfering factors from affecting the accuracy of results. The optimum blood component to test for endotoxin was investigated in section 6.5. Current kinetic LAL methods were optimised by exploring alternative means of calculating endotoxin from the optical density graphs (section 6.6), using optimal dilution factors (section 6.7) and alternative diluents such as surfactants (6.8). Optimised variations of the chromogenic and turbidimetric kinetic LAL assays were compared with the novel ESP endotoxin detection assay (section 6.9) and the commonly used endpoint chromogenic assay (section 6.10).

These series of studies found that the kinetic turbidimetric LAL assay was the most accurate and precise assay for endotoxin detection from uraemic blood. This assay will be used in subsequent studies documented in this thesis for the investigation of the association of endotoxemia with inflammation in haemodialysis patients. However, the kinetic LAL reagents used in the studies carried out so far have not been modified to be insensitive to (1→3)-β-D glucan (BG) stimulation. Elevated BG levels have been described in haemodialysis patients particularly in those treated with cellulose dialysis membranes (Kanda et al. 2001). It is important to clarify whether the low level blood endotoxin found in the patients studied described in sections 6.9 and 6.10, is due to BG interference before embarking on larger studies to use this specific assay to determine the association between inflammation and endotoxemia. This issue is further explored in Chapter 7.
Although, a series of experiments have been carried out to determine an optimum method of blood collection and analysis for endotoxin detection. There are several limitations with these studies, firstly the sample size for these studies were small, secondly the endotoxin used to spike samples were derived from control standard endotoxin, ideally the experiments should be repeated using native endotoxin from different bacterial species to ensure that similarly adequate level of spike recoveries are obtained. Finally, although an experiment was carried which found that plasma was the ideal blood component to use for endotoxin detection, these tests were carried out using blood from healthy controls. It is unclear whether observation could be applied to dialysis patients with significant uraemia due to the ‘matrix effect’ – a phenomenon in which undefined plasma proteins, phospholipids and other components interfering with the ability of the LAL assay to detect endotoxin (Rosenberg-Hasson et al. 2014).
Chapter 7

7. (1→3)-β-D glucan in kidney disease

7.1 Introduction

Beta-glucans are polysaccharides that contain D-glucose as a sole monomer unit linked by (1-3)-beta glycosidic bonds. They are structurally diverse and can vary in length and branching of side chains which results in differences in solubility and biological activity (Barton et al. 2016) [Figure 7-1].

![Beta glucan composed of D-glucose monomers linked by (1-3) beta-glycosidic bonds. A linear 1,3 glycosidic chain with a single 1,6 glycosidic chain is shown (reproduced with permission from Barton et al 2016, http://creativecommons.org/licenses/by/4.0/)](image)

(1→3)-β-D glucan (BG) are the most common type of beta-glucans found in fungal cell walls, yeast and seeds of some cereals such as barley and oats (1 (Rop et al. 2009). The molecular weight of beta-glucans can vary from thousands to millions of daltons depending on its origin (Young & Vincent 2005). Cellular components of most pathogenic fungi such as candida and aspergillus contain BG, thus levels of BG in bodily fluids such as serum, broncho-alveolar lavage fluid and cerebrospinal fluid have been used as a pan-fungal cell marker to diagnose invasive fungal infections (Ostrosky-Zeichner et al. 2012).
7.2 Use of blood levels of (1→3)-β-D glucan as a pan-fungal diagnostic marker

In 1968, during the evaluation of a carboxy-methylated beta-glucan as an anti-tumour agent, this was found to induce clotting of the coagulation cascade of amoebocytes of the horseshoe crab despite the absence of endotoxin contamination (Marty & Koo 2009). Further work confirmed that the LAL cascade was activated via the interaction of BG with an alternative serine protease zymogen, factor G (Morita et al. 1981) [see Figure 7-2].

![Figure 7-2: The LAL enzyme cascade (Wong et al 2014, reproduced with permission from John Wiley and Son publisher)](image)

Activation of factor G activates pro-clotting enzymes which cleave a chromogenic substrate, p-nitroanilide from a synthetic peptide in the beta-glucan LAL that replaces the original clot forming coagulogen protein (Wright et al. 2011). Unlike the conventional LAL assays which can range from gel-clot, end-point and kinetic techniques, currently all BG detection assays utilise end-point techniques by measuring the absorbance at 450nm (Novitsky 2009). There are five serum BG detection assays available commercially (Table 7-1): -
<table>
<thead>
<tr>
<th>Assay</th>
<th>Manufacturer</th>
<th>Type of assay and crab species</th>
<th>Cut-off value for positive result (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungitell</td>
<td>Associates of Cape Cod</td>
<td>Limulus Polyphemus (chromogenic)</td>
<td>60-80</td>
</tr>
<tr>
<td>Endosafe-PTS glucan</td>
<td>Charles River Laboratories</td>
<td>Limulus Polyphemus (chromogenic)</td>
<td>10-1000</td>
</tr>
<tr>
<td>Fungitec G-MK</td>
<td>Seikagaku Biobusiness</td>
<td>Tachypleus Tridentalus (chromogenic)</td>
<td>20</td>
</tr>
<tr>
<td>β-glucan test</td>
<td>Wako pure chemical industries</td>
<td>Tachypleus Tridentalus (turbidimetric)</td>
<td>11</td>
</tr>
<tr>
<td>BGSTAR</td>
<td>Maruha Nichiro Corporation</td>
<td>Tachypleus Tridentalus (chromogenic)</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 7-1: Beta glucan assays (adapted from Wright et al 2011)

Evaluation of BG assays as a diagnostic marker of invasive fungal infection was initially carried out in Japan which demonstrated the usefulness of blood BG detection assays to identify patients with invasive fungal infections (Obayashi et al. 2008). Sensitivity and specificity of BG detection assays to detect invasive fungal infections based on autopsy material (using Fungitec-G) was reported to be 90% and 100% respectively, with positive and negative predictive values of 59% and 85% respectively (Obayashi et al. 1995).

Subsequently, a large number of studies have been carried out to examine the diagnostic potential of the BG assay to identify fungal infection. Although cut-off values for a positive result vary depending on the manufacturer and assay used, equivalent diagnostic ability have been reported between assays from different manufacturers (Odabasi et al. 2004). Out of all five assays, the Fungitell assay (Associates of Cape Cod inc.) is the most studied BG detection assay (He et al. 2015). The first multi-centre study to evaluate BG detection assays was carried out
in USA using the Fungitell assay which reported sensitivity and specificity of 69.9% and 87.1% respectively (Ostrosky-Zeichner et al. 2005). A recent meta-analysis involving 4214 patients found that the pooled sensitivity, specificity, diagnostic odds ratio and area under summary receiver operating characteristic for BG detection assays were 0.78 [95% confidence interval (CI), 0.75-0.81], 0.81 (95% CI, 0.80-0.83), 21.88 (95% CI, 12.62-37.93), and 0.8855, respectively, suggesting a high diagnostic accuracy for invasive fungal infections (He et al. 2015). The Fungitell assay has received FDA clearance for use as an adjunct in the diagnosis of invasive fungal infections (Wright et al. 2011) and high plasma or serum levels of BG are now considered as alternative microbiological evidence of fungal infection in the European Organization for Treatment and Research of Cancer/Mycosis Study Group criteria for diagnosis of fungal infection (Ostrosky-Zeichner et al. 2012; De Pauw et al. 2008).

7.3 Causes of false positive and negative results for BG detection assays

False positive (elevated blood BG levels in the absence of fungal infection) and false negative results for BG detection assays may occur due to several reasons (Table 7-2):

<table>
<thead>
<tr>
<th>False positive</th>
<th>False negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemodialysis or haemofiltration</td>
<td>Lipemic blood samples</td>
</tr>
<tr>
<td>Broad spectrum antibiotics</td>
<td>Haemolysed blood samples</td>
</tr>
<tr>
<td>Severe bacterial infections</td>
<td>Infection with fungi lacking in (1→3)-β-D glucan e.g. Cryptococcus, Mucorales, Zygomycetes</td>
</tr>
<tr>
<td>Major surgery</td>
<td></td>
</tr>
<tr>
<td>Administration of blood products</td>
<td></td>
</tr>
</tbody>
</table>

Table 7-2: Causes of false positive and negative results for BG detection assays

The observation that fluid extracts that had been exposed to dialyser membranes used for haemodialysis and haemofiltration are able to activate the LAL assay but without inducing pyrogenicity was reported in the 1980s (Henne et al. 1984; Pearson
et al. 1984). This “LAL-reactive” material was subsequently found to be BG which were derived from cellulose materials used for construction of the dialyser membranes and activates the LAL assay via the alternative zymogen factor G (Yamagami et al. 1986). Venous injection of cellulose dialyser wash solution into healthy subjects increased blood LAL-reactive material and levels of this substance increased ten-fold after dialysis (Kato et al. 2001; Yamagami et al. 1986).

Blood levels of BG increase significantly after dialysis with cellulose membranes, although the phenomenon does not seem to occur if synthetic membranes such as polysulfone are used for dialysis instead (see section 7.4).

Other causes of elevated blood BG in the absence of fungal infection includes treatment with some antibiotics which may contain BG (Marty & Koo 2009). Administration of blood products, albumin and immunoglobulins may also lead to false positive results since BG from cellulose filters may be eluted into blood components by filtration during the manufacturing process (Nagasawa et al. 2003). Major surgery can lead to elevations in blood BG levels due to the absorption of BG released from surgical gauze (Nakao et al. 1997). Severe bacterial infections may also lead to false positive results and some species of streptococcus are known to produce glucan or glucan-like polymers (Pickering et al. 2005; Digby et al. 2003).

False negative results may occur if samples are very haemolysed or lipaemic. BG detection assays are unable to detect fungal infections caused by species such as Cryptococcus, Zygomycetes and Blastomyces dermatidis as these fungi lack or have low amounts of BG in their structure leading to false negative results (Wright et al. 2011).

7.4 Serum (1→3)-β-D glucan levels in kidney disease

7.4.1 Non-dialysed chronic kidney disease patients

No studies published to date looks specifically at the effect of reduced kidney function on levels of serum BG or its effect on BG detection assay. It is unlikely that reduced kidney function or clearance would significantly affect BG levels since animal studies demonstrate that the primary route of removal of BG is by the liver.
and reticulo-endothelial system via oxidative degradative mechanisms (Young & Vincent 2005). Currently, there are no studies that have been published which examines whether uraemic substances may cross-react with BG detection assays (Kato et al. 2001).

### 7.4.2 Haemodialysis and haemofiltration

In the early era of haemodialysis, dialysers were constructed mainly from cellulose-containing products, these have largely been replaced by more biocompatible synthetic membranes such as polysulfone, polycarbonate and polymethyl methacrylate dialysers. Studies of blood BG levels in haemodialysis patients are summarised in Table 7-3:

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Population</th>
<th>Average BG levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified cellulose</td>
<td>Stable haemodialysis patients</td>
<td>330±80 pg/mL increased by 70.6±20.7 pg/mL after dialysis</td>
<td>(Yoshioka et al. 1989)</td>
</tr>
<tr>
<td></td>
<td>Stable haemodialysis patients</td>
<td>9.4 pg/mL (range 1.2-39.1) pre-dialysis; 332 pg/mL (range 108.2-2200) post-dialysis</td>
<td>(Kanda et al. 2001)</td>
</tr>
<tr>
<td>Modified cellulose</td>
<td>Stable haemodialysis patients</td>
<td>7.9 pg/mL (range 1.2-72) pre-dialysis; 9 pg/mL (range 1-29.5) post-dialysis</td>
<td>(Kanda et al. 2001)</td>
</tr>
<tr>
<td>Polysulfone or polymethyl methacrylate (PMMA)</td>
<td>Stable haemodialysis patients</td>
<td>18.8±3.7 pg/mL pre-dialysis; 16.0±3.9 pg/mL post-dialysis</td>
<td>(Kato et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Stable haemodialysis patients</td>
<td>Levels not stated but no significant change in BG level before and after dialysis</td>
<td>(Kanda et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Patients with active Pneumocystis Jiroveci pneumonia</td>
<td>Levels not stated but no significant change in BG level before and after dialysis</td>
<td>(Held &amp; Wagner 2011)</td>
</tr>
<tr>
<td></td>
<td>Lung transplant recipients</td>
<td>383pg/mL dialysed patients; 107.3pg/mL non-dialysed patients</td>
<td>(Alexander et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Critically ill patients on intensive care unit</td>
<td>Levels not stated but exposure to haemodialysis was associated with raised BG levels</td>
<td>(Hanson et al. 2012)</td>
</tr>
</tbody>
</table>

Table 7-3: BG studies in patients treated with haemodialysis or haemofiltration

Haemodialysis using cellulose membranes can raise blood BG levels by 100-200 pg/mL (Yoshioka et al. 1989; Kanda et al. 2001). For modified cellulose membranes,
there are conflicting reports with one study reporting significant elevation of blood BG levels (Kato et al. 2001) and another study finding no effect of modified cellulose membranes on BG levels (Kanda et al. 2001). Most studies have found that synthetic membranes do not significantly influence blood levels of BG. Kanda et al found no difference in BG levels before and after dialysis with polymethyl methacrylate membranes (Kanda et al. 2001). Similarly, Kato et al reported that in patients who had been previously treated with cellulose dialysers, switching to polysulfone membranes led to a sustained reduction in blood levels of BG (Kato et al. 2001). However, an investigation in lung transplant recipients found that blood BG levels were increased for up to 7 days after exposure to haemodialysis despite use of biocompatible polysulfone membranes (Alexander et al. 2010). Another study in critically ill patients found that haemodialysis treatment with polysulfone membranes was a significant determinant of elevated blood BG in the absence of fungal infection (Hanson et al. 2012). Thus, it is unclear whether exposure to haemodialysis membranes regardless of the membrane material, does contribute to elevated blood levels of BG. However, most studies and a recent review on the reliability of blood BG testing in dialysis patients suggest that the impact of dialysis with synthetic membranes on blood BG levels are likely to be negligible (Prattes et al. 2015).

7.4.3 Peritoneal dialysis

There are a very limited number of studies that investigate blood BG levels in peritoneal dialysis patients – all of which are limited to case studies (Ginocchio et al. 2012; Ates et al. 2013). Both studies report elevated blood BG as a useful marker of fungal peritonitis and for monitoring of response to treatment with antifungals. Since there is no exposure of blood to dialyser membranes with peritoneal dialysis, it is anticipated that this modality of dialysis would not significantly raise blood BG levels (Prattes et al. 2015). Currently, there are no published studies that report the level of blood BG in peritoneal dialysis patients without fungal infection.

7.5 Summary

Studies documented in this thesis were carried out on haemodialysis patients treated with biocompatible, synthetic dialyser membranes, although the small number of
published investigations suggest that treatment with synthetic dialyser membranes are unlikely to have any significant effect on blood BG, this finding is not consistent in all studies (Hanson et al. 2012; Alexander et al. 2010). As discussed in Chapter 3, since the LAL assay is not specific for endotoxin and may also be activated by BG via the factor G pathway, it is important to clarify if BG is significantly elevated in the blood of haemodialysis patients and if blood BG present in haemodialysis patients could be responsible for the apparent elevated blood endotoxin levels reported in the literature. This issue is further investigated in chapter 8.

Additionally, there is a significant variation in blood levels of BG in the haemodialysis population (Table 7-3), differences in assay may partly explain this, however other clinical factors may be contributory such as diet, medications, co-existing conditions especially liver and gastrointestinal disease and dialysis factors. The influence of these factors on blood BG in haemodialysis patients (without fungal infection) has not been explored in the current literature and warrants further investigation.
Chapter 8

8. Influence of (1→3)-β-D glucan on endotoxin detection in end-stage kidney disease

8.1 Introduction

As discussed in Chapter 2, endotoxemia is commonly reported in the dialysis population and has been associated with systemic inflammation – a strong prognostic of poor outcome (Carrero & Stenvinkel 2010). Endotoxins are implicated in the pathogenesis of sepsis syndrome and are potent mediators of inflammation. The levels of endotoxemia reported in the dialysis population range from 0.209 to 2.31 endotoxin units/mL (EU/mL) (see Table 2-1). This appears high since it is well established that 4.1-5 EU/kg/hr is sufficient to induce pyrogenic symptoms such as rigor, nausea and hypotension in humans (Greisman & Hornick 1969; Hochstein 1994). Nearly all studies in the dialysis population have employed the use of the LAL assay to detect endotoxin. Due to differences in the types of available LAL assay and sample preparation methods reported in these studies, a number of experiments were carried out to determine the most accurate and precise among LAL assays most commonly used in the dialysis population, further work was carried out to optimise the kinetic turbidimetric LAL assay which showed sufficient accuracy and precision to detect endotoxin from samples that had been spiked with control endotoxin (see section 6.9). However, although LAL is a very sensitive biological assay, capable of detecting sub-picogram/mL levels of endotoxin. LAL is not specific to endotoxin but can also be activated by (1→3)-β-D-glucan (BG) via an alternate enzymatic pathway mediated by factor G activation (see Chapter 3). Given the frequently reported association between elevated BG and exposure to haemodialysis, it is important to ensure that endotoxin ‘signal’ detected using the LAL assay is truly due to endotoxin itself rather than false positive activation by co-existing elevated BG. Taniguchi et al found that endotoxin levels were much lower in hemodialysis patients when blood samples were assayed using LAL devoid of factor G compared with standard unmodified LAL, although they did not measure BG levels
in their study (Taniguchi et al. 1990). It is important to clarify whether blood endotoxin levels are truly raised in dialysis patients since emerging therapies such as extracorporeal endotoxin are in development and their efficacy in the treatment of sepsis syndrome has been reported (Klein et al. 2014).

LAL assay reactivity to BG can be prevented by either using LAL reagent that lacks factor G or by rendering the factor G component of the LAL assay unreactive to BG using BG-blocking buffers. Nearly all endotoxin studies in dialysis population, with one notable exception (Taniguchi et al. 1990), did not use LAL that was rendered insensitive to BG activation. Hence it is unclear whether reported high levels of endotoxemia are truly due to endotoxin or due to false positive interference from BG.

Using the kinetic turbidimetric LAL assay as described in Chapter 8, the influence of BG on endotoxin read-out measurements was investigated by measuring endotoxin in haemodialysis patients with and without the use of a BG-blocking buffer. In a separate experiment, in order to determine whether use of BG-blocking buffers interfered with the detection of true endotoxemia, plasma samples were spiked with known amounts of endotoxin, and measured endotoxin levels with and without the blocking buffer. BG was measured in plasma samples to determine its association with endotoxin.

### 8.2 Methods

**Study design**

This was a single centre cross-sectional study of prevalent haemodialysis patients treated at the East and North Herts NHS Trust. Ethical approval was obtained from the Northampton NHS Research Ethics Committee. Written informed consent was obtained from all participants. All patients dialysed using an arterio-venous fistula and were clinically stable at the time of study. All were treated by on-line hemodiafiltration using ultrapure water. Exclusion criteria were HIV infection, viral hepatitis, abnormal liver function and active gastrointestinal disease.

The study was divided into two phases. The first phase compared the difference in endotoxin read-out measurements between standard LAL (without BG-blocking
buffer [LAL(–)]) and LAL reconstituted with manufacturer supplied BG-blocking buffer (Charles Rivers® ES-buffer [LAL(+)]) to render the LAL insensitive to further stimulation by BG. The second phase of the study was carried out to ensure that BG-blocking buffers themselves do not affect the accuracy or interfere with the sensitivity of LAL to detect endotoxin.

8.2.1 Phase 1: Comparison of endotoxin measurements between LAL(–) and LAL(+)

Fifty patients were recruited. For each subject, plasma samples were assayed for endotoxin using both LAL(–) and LAL(+). Samples were also measured for IL-6, TNF-α and BG. Correlation between BG, markers of inflammation, demographic and clinical factors was explored.

Blood sampling and processing

Blood samples were collected pre-dialysis through the arterio-venous fistula using aseptic technique as described in Chapter 6. Briefly, blood samples for endotoxin and BG measurements were collected in Terumo Venoject II heparinised tubes (Project KBG, Tokyo). Blood samples for cytokine measurements were collected in S-monovette Z-gel tubes (Sarsedt, Germany). Plasma for endotoxin and BG measurements were prepared by centrifugation at 250g for 10min and serum for cytokine measurements were prepared by centrifugation at 1500g for 10min. Plasma and serum samples were immediately frozen and stored at -80°C. Blood collection, processing and storage were completed within 30 minutes for all samples. Phlebotomy equipment including syringes and blood collection tubes were batch checked for endotoxin contamination. Washout from the apparatus were consistently found to have undetectable endotoxin (<0.0025 EU/mL).

Laboratory measurements

Endotoxin assay using standard LAL without BG-blocking buffer [LAL(–)]: Endotoxin measurements were performed using the kinetic turbidimetric LAL assay (Endosafe KTA2, Charles River Laboratories, Ecully). Plasma samples were diluted 1:10 with 0.1% Tween80 (Merck Chemicals, Darmstadt) and heated to 70°C for 10min and cooled to room temperature (20-25°C) prior to analysis. 100µL of diluted
plasma was added to duplicate wells on 96-well microplates. Endosafe KTA2 reagent was reconstituted with 5.2mL LAL reagent water. 100µL of this mixture was added to each sample. The plate was monitored at 340nm using a Biotek ELx808 absorbance microplate reader with Endoscan-V software (version 4.0; Charles River) with an onset optical density set at 0.03. Six-point standard curves were constructed using standard dilutions of control standard endotoxin (E.coli 055:B5) ranging from 10-0.0025 EU/mL. All standard curves had a correlation coefficient >0.98. Coefficient of variation (CV) for onset reaction times for each assay was <20%.

**Endotoxin assay using LAL reconstituted with BG-blocking buffer [LAL(+)]:** To block the factor G pathway, Endosafe KTA2 reagent was reconstituted with 5.2mL BG-blocking buffer (Charles River ES-Buffer) containing 1mg/mL carboxymethylated curdlan(Tsuchiya et al. 1990; Kambayashi et al. 1991). Endotoxin measurements were carried out using the same procedure as described above. For each patient, samples were analysed with LAL(-) and LAL(+) simultaneously on the same microplate. The same batch of LAL reagent was used throughout the study.

**(1-3)-β-D glucan assay:** BG measurements was carried out using the Fungitell® assay (Associates of Cape Cod, Inc.) as per manufacturer’s instructions (Associates of Cape Cod 2011; Petraitiene et al. 2008). In brief, 5µL of sample was mixed with 20µL pre-treatment buffer (0.125M KOH/6M KCl), in microplate wells, and incubated at 37°C for 10min. Fungitell reagent, reconstituted in 0.1M Tris HCl, pH 7.4, was added to sample and standard curve wells (7.8–500pg/mL, Pachyman). The reactions were read kinetically, at 405nm minus 490nm at 37°C, for 40min. Vmean values (milliabsorbance units/min) were calculated for standards and samples and sample titres interpolated from the standard curve. CV for all assays was <20%. Normal human serum contains low levels of BG, typically 10-40pg/mL(Odabasi et al. 2004). Levels <60pg/mL are interpreted as negative and between 60-80pg/mL is interpreted as indeterminate. Levels >80pg/mL is interpreted as positive and in at-risk patients is considered a marker of invasive fungal infection (Associates of Cape Cod 2011; He et al. 2015).

**Cytokine measurements and C-reactive protein measurements:** serum was measured for IL-6 and TNF-α using enzyme-linked immunosorbent assays (Human
Quantikine ELISA, R&D systems). For IL-6, intra- and inter-assay CV was 1.6-4.2% and 2.0-3.7% respectively. For TNF-α, intra- and inter-assay CV was 4.2-5.2% and 4.6-7.4% respectively. All haemodialysis patients at the East and North Herts NHS trust have monthly CRP measurements (Olympus AU2700, Beckman-Coulter) as part of routine clinical care. Monthly CRP measurements for all patients were collected in the preceding 3 months of the study to determine the chronic inflammatory status of subjects.

**Measurement of BG in dialysis fluid:** Dialyzers were investigated for BG contamination. Dialysis priming fluid from 12 randomly selected dialyzers (10 Fresenius FX and 2 Gambro Evodial dialyzers) was measured for BG as described above. The limit of detection of BG in aqueous solutions is 7.8pg/mL.

**8.2.2 Phase 2: Investigating the effect of BG-blocking buffers on endotoxin spike recovery**

To ensure that BG-blocking buffers themselves do not interfere with endotoxin detection, the ability of LAL reconstituted with different doses of BG-blocking buffers to detect endotoxin from plasma samples spiked with control standard endotoxin (lipopolysaccharide; LPS) was explored (Figure 8-1).

![Schematic presentation of spiking experiments](image)

**Figure 8-1:** Schematic presentation of spiking experiments—plasma from each patient was aliquoted and spiked with three different concentrations of control standard endotoxin (LPS) and each spiked sample was assayed using LAL reconstituted with different doses of glucan blocking buffer to calculate % spike recovery. * denotes manufacturer recommended dose of glucan blocking buffer (Charles Rivers ES-buffer®; Endotoxin-Specific buffer) containing 1 mg/mL of carboxymethylated curdlan.
Eight patients with undetectable endotoxemia were recruited for this sub-study. Plasma from each subject was aliquoted and spiked with three different concentrations of LPS (0.05, 0.1 and 1EU/mL). Each spiked sample was diluted, heat-treated as described above and assayed with LAL(-) and LAL reconstituted with different concentrations of BG-blocking buffer. The standard inhibitor concentration of Charles River ES-buffer used to render LAL unreactive to BG stimulation is 1 mg/mL carboxymethylated curdlan (Tsuchiya et al. 1990). Each LPS-spiked plasma sample was assayed with LAL reconstituted with progressively diluted concentrations of carboxymethylated curdlan ranging from 0.01-1mg/mL to calculate percentage spike recovery. Spike recovery was calculated using the following formula: -

\[
\text{% spike recovery} = \frac{\text{Measured endotoxin content in sample}}{\text{Amount of endotoxin added to sample}} \times 100\%
\]

Spike recoveries between 50-200% are considered acceptable as discussed in 6.3.1.

8.2.3 Statistical analysis

Data are presented as means and medians with respective 95% confidence intervals and interquartile ranges. Paired and non-paired data were compared using Wilcoxon-signed rank and Mann-Whitney U test respectively. Ordinal data between multiple groups was compared using Kruskal-Wallis test. Correlation was compared using Spearman’s correlation coefficient. ROC analysis was used to identify optimum cut-off levels of BG to identify patients with detectable endotoxemia using the LAL(-). Level of agreement between patients with detectable endotoxemia and those with elevated BG levels was analysed using the kappa statistic.
8.3 Results

8.3.1 Patient characteristics

Patient characteristics are listed in Table 8-1. Mean age was 64 and median dialysis vintage was 2.8 years. With the exception of one patient who dialyzed using synthetic polycarbonate heparin-grafted dialyzer (Gambro, Evodial), all were treated with synthetic polysulfone dialyzer (Fresenius, FX high-flux). Most had evidence of inflammation, with elevated IL-6 and TNF-α. 58% of patients had evidence of chronic inflammation with median CRP >5mg/L in the three months preceding the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 50</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64 [95% CI, 60-69]</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>74</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.2 [95% CI, 73.1-83.3]</td>
</tr>
<tr>
<td>CCI</td>
<td>3.8 [95% CI, 3.1-4.5] 4 [IQR 2-5]</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>30</td>
</tr>
<tr>
<td>Previous gastrointestinal disease (%)</td>
<td>14</td>
</tr>
<tr>
<td>KRU (ml/min)</td>
<td>0.1 [IQR 0-2.0]</td>
</tr>
<tr>
<td>Kt/V</td>
<td>1.44 [95% CI, 1.36-1.51]</td>
</tr>
<tr>
<td>Dialysis vintage (years)</td>
<td>2.8 [IQR 1.8-7.8]</td>
</tr>
<tr>
<td>Convective volume (L)</td>
<td>18.8 [95% CI, 16.6-23.0]</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>7.0 [IQR 5-11.3]</td>
</tr>
<tr>
<td>High CRP ≥ 5 mg/L (%)</td>
<td>58%</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>38.4 [95% CI, 37.4-39.4]</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>13.6 [IQR 11.6-17.7]</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.7 [IQR 4.6- 14.8]</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>38.6 [IQR 29.0-54.5]</td>
</tr>
<tr>
<td>Dialyzer surface area (m²)</td>
<td>1.8 [IQR 1.8-2.2]</td>
</tr>
</tbody>
</table>

Table 8-1: Patient characteristics

CCI, Charlson co-morbidity index; KRU, residual urea clearance; CRP, median C-reactive protein over the previous 3 months; PTH, parathyroid hormone; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6 (values quoted for healthy controls for IL-6 and TNF-α are <3.1pg/mL and <2.8pg/mL respectively); Kt/V, combined dialyser and renal urea clearance normalised to body volume
**8.3.2 Effect of BG-blocking buffers on plasma endotoxin detection**

Using LAL(-), 50% of patients had detectable endotoxemia with median endotoxin level of 0.038EU/mL, however on repeat measurement with LAL(+), no subjects had detectable endotoxemia (Table 8-2).

<table>
<thead>
<tr>
<th>Population</th>
<th>Plasma endotoxin (EU/mL)</th>
<th>Plasma BG (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAL(-) (No BG blocker)</td>
<td>LAL(+) (with BG blocker)</td>
</tr>
<tr>
<td>Detectable endotoxemia (n = 25)</td>
<td>0.038 [IQR 0.031-0.043]</td>
<td>Undetectable (&lt;0.025)</td>
</tr>
<tr>
<td>No endotoxemia (n = 25)</td>
<td>Undetectable (&lt;0.025)</td>
<td>Undetectable (&lt;0.025)</td>
</tr>
</tbody>
</table>

p-value <0.001

**Table 8-2**: Plasma endotoxin and (1-3)-β-D-glucan levels in patients with and without endotoxemia

**8.3.3 Association between endotoxin signals detected using LAL(-) and (1-3)-β-D-glucan**

Apparent endotoxin signals detected using LAL(-) was higher in patients with BG levels >80pg/mL (positive test) compared to those with BG levels <60pg/mL (negative test) [0.034 vs. 0EU/mL; p=0.02] (Figure 8-2)
Figure 8-2: Endotoxin signal detected using standard LAL without BG blocking buffer displayed by tertiles of (1–3)-β-D glucan.

Endotoxin signals in patients with BG levels >80pg/mL also tended to be higher than those with BG levels between 60-80pg/mL (indeterminate result) although this did not reach statistical significance [0.034 vs. 0.028 EU/mL; p=0.09]. Elevated BG (>60pg/mL) were found in 58% of patients and BG levels was significantly higher in those with detectable endotoxemia compared to those with undetectable endotoxemia (78 vs. 54pg/mL, p<0.001, [Table 8-2]). These findings lend support to the notion that the ‘endotoxin’ signals detected using LAL(-) are false positives due to interference from BG. ROC analyses revealed a strong relationship between high
BG levels and apparent endotoxemia using LAL(-) (AUC=0.79 [0.66, 0.92]; p<0.001) [Figure 8-3].

The optimum cut-off levels for identifying patients with apparent detectable endotoxemia were between 60-70pg/mL. BG levels >62pg/mL identified apparent endotoxemia with sensitivity of 72% and specificity of 76%. Level of agreement between patients with apparent detectable endotoxemia and those with elevated BG levels (>62pg/mL) was moderate (kappa=0.48, p=0.001).

8.3.4 Effect of BG-blocking buffers on endotoxin spike recovery

Endotoxin spike recovery for samples spiked with 0.05 EU/mL and 0.1 EU/ml was elevated at 172% and 130% respectively using LAL(-), suggesting enhancement of the LAL assay. However, this enhancement was abolished on repeat measurement with BG-blocking buffers [LAL(+)], with spike recoveries falling closer to the expected
value of 100% (106% and 97.5% for samples spiked with 0.05 EU/mL and 0.1 EU/ml respectively; p<0.05) [Figure 8-4].

Figure 8-4: Comparison of median spike recovery between LAL(-) and LAL(+) in plasma samples spiked with endotoxin (box plots represent median values with interquartile range, hashed horizontal line denotes the expected optimum spike recovery). LAL(-), standard LAL with no glucan blocking buffer; LAL(+), LAL reconstituted with Charles River® ES-buffer containing 1mg/mL carboxymethylated curdlan.

For samples spiked with high concentrations of endotoxin (1 EU/mL), spike recovery was broadly reduced for both LAL(-) and LAL(+), with no significant differences in spike recovery. Despite this, all spike recoveries were within industry-accepted limits of 50-200% (United States Pharmacopoeia 2013) [Table 8-3].
Table 8-3: Spike recovery from plasma samples with undetectable endotoxin using different concentrations of glucan blocking buffers. Median spike recovery with interquartile ranges presented. Spike recoveries of 50-200% are considered acceptable. Significant P values (*) indicates spike recovery significantly different from expected % spike recovery.

<table>
<thead>
<tr>
<th>Concentration of carboxymethylated curdlan (mg/mL) (n=8)</th>
<th>0</th>
<th>1</th>
<th>0.5</th>
<th>0.1</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard LAL (no glucan blocking buffer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Manufacturer recommended dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of endotoxin spike (Expected endotoxin content) [EU/mL]</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike recovery (%)</td>
<td>172 [108.5-210]</td>
</tr>
<tr>
<td>P</td>
<td>0.028*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of endotoxin spike (Expected endotoxin content) [EU/mL]</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike recovery (%)</td>
<td>130 [102.5-142.5]</td>
</tr>
<tr>
<td>P</td>
<td>0.063</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of endotoxin spike (Expected endotoxin content) [EU/mL]</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike recovery (%)</td>
<td>62.1 [37.6-69.5]</td>
</tr>
<tr>
<td>P</td>
<td>0.012*</td>
</tr>
</tbody>
</table>

Assay measurements using LAL re-constituted with increasingly dilute amounts of carboxymethylated curdlan resulted in progressive enhancement of spike recovery (Table 8-3, Figure 8-5). Lower doses of BG-blocking buffer (0.01-0.1 mg/mL ES buffer) caused activation of the LAL reagent almost immediately upon reconstitution resulting in wide variability of spike recoveries obtained using lower doses of BG-blocking buffer.
Figure 8-5: Comparison of median spike recovery between LAL and LAL reconstituted with different doses of BG blocking buffer (ES buffer). Spike recoveries of 50–200% are considered acceptable. Box plots represent median spike recovery with interquartile range, hashed horizontal line denotes the expected optimum spike recovery.

8.3.5 Measurement of BG in dialysis fluid

High levels of BG were observed in a significant proportion of patients and since BG leaching from cellulose-based HD membranes have been reported (Kanda et al. 2001), dialysers were examined for BG contamination. Pre- and post-dialyser saline washout from the blood compartment of dialysers revealed very low level contamination. Post-dialyser washout tested positive for BG in 6/12 dialysers (range 8-22pg/mL). Median BG levels in post-dialyser washout were 4.8 pg/mL (Table 8-4).
**Pre-dialyzer** | **Post-dialyzer** | **P**
---|---|---
(n =12) | (n=12) | 

| (1-3)-β-D-glucan (pg/mL) | Below detection limit | 4.8 [IQR 0-9.8] | 0.046* |

Table 8-4: BG measurements of saline washout pre- and post-dialyzer. Limit of detection was 7.8pg/mL, values <7.8pg/ml was designated as 0.

### 8.3.6 Clinical correlates of (1-3)-β-D-glucan

Weight and residual urea clearance correlated negatively with plasma BG levels ($r=-0.33$ and -0.31 respectively; $p<0.05$). Plasma BG correlated positively with endotoxin signals detected using LAL[-] ($r=0.55$, $p<0.01$) but did not correlate significantly with markers of inflammation including TNF-α, IL-6, albumin or CRP. There was no relationship between BG and age, co-morbidity, dialysis vintage, dialysis adequacy as determined by Kt/V, convective volume or dialyser surface area (Table 8-5).

<table>
<thead>
<tr>
<th>Variable</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 BG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Endotoxin LAL[-]</td>
<td>0.55**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3 IL-6</td>
<td>-0.11</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 TNF-α</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 CRP</td>
<td>-0.16</td>
<td>0.18</td>
<td>0.33**</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Albumin</td>
<td>-0.01</td>
<td>-0.14</td>
<td>-0.2</td>
<td>-0.33*</td>
<td>-0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Age</td>
<td>0.09</td>
<td>0.14</td>
<td>0.2</td>
<td>0.01</td>
<td>-0.09</td>
<td>-0.32*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Weight</td>
<td>-0.33*</td>
<td>-0.37**</td>
<td>0.21</td>
<td>0.02</td>
<td>0.21</td>
<td>0.05</td>
<td>-0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Kt/V</td>
<td>-0.31*</td>
<td>-0.11</td>
<td>0.06</td>
<td>-0.13</td>
<td>0.13</td>
<td>0.13</td>
<td>0</td>
<td>0.47**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 CCI</td>
<td>-0.12</td>
<td>0</td>
<td>0.30*</td>
<td>0.19</td>
<td>0.01</td>
<td>-0.47**</td>
<td>0.76**</td>
<td>-0.08</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Dialysis vintage</td>
<td>0.14</td>
<td>0.17</td>
<td>0.01</td>
<td>0.32*</td>
<td>-0.03</td>
<td>-0.21</td>
<td>0.15</td>
<td>-0.3*</td>
<td>-0.34**</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Kt/V</td>
<td>0.08</td>
<td>0.2</td>
<td>-0.07</td>
<td>-0.16</td>
<td>-0.07</td>
<td>-0.03</td>
<td>0.33*</td>
<td>-0.15</td>
<td>0.33*</td>
<td>0.23</td>
<td>-0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Convective volume</td>
<td>0.02</td>
<td>-0.22</td>
<td>-0.16</td>
<td>-0.16</td>
<td>0.04</td>
<td>0.15</td>
<td>-0.28</td>
<td>0.26</td>
<td>-0.23</td>
<td>-0.32*</td>
<td>0.12</td>
<td>-0.21</td>
<td></td>
</tr>
<tr>
<td>14 Dialyser surface area</td>
<td>-0.2</td>
<td>-0.24</td>
<td>0.28</td>
<td>-0.01</td>
<td>0.35*</td>
<td>-0.11</td>
<td>-0.07</td>
<td>0.73**</td>
<td>0.19</td>
<td>0.01</td>
<td>-0.06</td>
<td>-0.15</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*p<0.05
**p<0.01

BG, (1-3)-β-D glucan; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, median C-reactive protein over the previous 3 months; Kt/V, residual urea clearance; CCI, Charlson Co-morbidity index; Kt/V, combined dialyser and renal urea clearance normalised to body volume; Endotoxin LAL[-], Endotoxin signals detected using LAL assay without BG-blocking buffers.

Table 8-5: Clinical correlates of (1-3)-β-D glucan, endotoxin signals detected using LAL[-] and markers of inflammation
8.4 Discussion

The levels of endotoxemia reported in the dialysis population are high and often appear close to or exceed the pyrogenic threshold of 4.1-5 EU/kg in humans (Greisman & Hornick 1969; Hochstein 1994). Contrary to the high blood endotoxin levels reported in the literature, the apparent level of endotoxemia detected using LAL(-) in our population was low with a median level of 0.038-0.041 EU/mL. These low levels do not appear to be due to sub-optimal pre-treatment of plasma to remove inhibitory plasma components since spike recovery from all samples was sufficient and well within the 50-200% limit (Figure 8-4). The plasma dilution and heat treatment conditions used in this study was similar to those used by many other authors (Hurley 1995). It is possible that the high endotoxin levels reported in the literature are due to pre-analytical factors such as contamination of phlebotomy and laboratory apparatus. All phlebotomy apparatus used in this study was tested for endotoxin contamination and interfering factors. Only manufacturer-certified endotoxin free laboratory apparatus was used for the analysis.

False positive activation of the LAL from BG may also be partly responsible for the apparently high ‘endotoxin’ levels reported in dialysis patients. The apparent endotoxin signals detected in 50% of the study cohort using LAL(-) became undetectable when the assay was repeated with LAL(+) to block factor G activation. Furthermore, endotoxin signals detected using LAL(-) were significantly higher in those with the highest blood BG levels. These finding suggest that endotoxin signals in this study detected using standard LAL are likely to be false positives due to interference from BG.

The factor G pathway of LAL was inhibited using highly concentrated carboxymethylated curdlan (Kambayashi et al. 1991; Tanaka et al. 1993) – itself a BG. The interaction between BG and factor G is complex (Ohno et al. 1990). Lower molecular weight BG structures such as laminarin and curdlan degradation products have factor G activating properties at low concentrations, but become inhibitory at high concentrations. Consistent with this, there was a progressive increase in endotoxin spike recovery as the concentration of the BG-blocking buffer was progressively reduced and very dilute concentrations of BG-blocking buffer caused
coagulation of the LAL reagent almost immediately upon reconstitution. This peculiar phenomenon is the basis of commercial BG-blocking agents used to eliminate BG interference in LAL endotoxin detection (Young & Vincent 2005) and BG-blocking agents typically consist of solutions containing highly concentrated BG.

Highly concentrated carboxymethylated curdlan used as a BG-blocking buffer does not appear to affect the ability of LAL to detect endotoxin (Kambayashi et al. 1991), although previous studies have tested this in plasma samples spiked with relatively high concentrations of LPS. Since endotoxin content in the current study cohort with detectable endotoxemia was relatively low (median 0.038 EU/ml), and to ensure that the BG-blocking buffer used in this study did not interfere with true endotoxin detection at this level of endotoxemia, the effect of the BG-blocking buffer on the LAL assay’s ability to recover endotoxin from plasma spiked with lower amounts of endotoxin (0.05-0.1EU/mL) was examined.

Spike recovery was enhanced (130-172%) using LAL(-), however on repeat measurement using LAL(+), this enhancement was abolished with spike recoveries falling closer to expected values (97.5-106%). This suggests that within this range of endotoxemia, there could be enhancement of the LAL assay by BG present in the blood, supporting the hypothesis that ‘endotoxemia’ detected in haemodialysis patients may be artefactual due to BG interference. This also demonstrates that BG-blocking buffers used in this study do not interfere with the sensitivity of the LAL assay to detect endotoxin since spike recovery using LAL(+) was close to 100%. All spike recoveries obtained were within the industry-specified limits of 50-200%. As the LAL test is a biological assay and is subject to inherent variability, wide-ranging spike recoveries are permitted (United States Pharmacopoeia 2013), therefore this may lead to LAL users accepting results of endotoxin measurement and not taking steps to rule out BG positive interference.

In samples spiked with a high concentration of endotoxin (1 EU/mL), spike recovery was broadly reduced and there was no significant difference in spike recovery between LAL(-) and LAL(+), possibly reflecting that the contribution of residual BG signal was only a small proportion of the overall signal when higher concentration of
LPS is used. Thus BG blocking buffers are more important and necessary when measuring endotoxin in patients with low level endotoxemia.

Elevated levels of blood BG were found in a large proportion of patients. The clinical significance of high blood BG levels in-vivo is unclear. There was no relationship between BG with markers of inflammation in this study although only two pro-inflammatory cytokines were studied. A broader inflammatory profile may have been useful as BG has been shown to stimulate release of a number of other pro-inflammatory cytokines such as IL-8 (T. Suzuki et al. 2002; Kubala et al. 2003). Different cut-off levels of BG are used as a diagnostic marker in invasive fungal infections and in-vitro evidence suggests that BG may potentiate enhanced Toll-like receptor-induced cytokine production (Kikkert et al. 2007). However, the use of BG as adjunctive therapies for treatment of malignancies has been reported (Young & Vincent 2005; Rop et al. 2009; Yin et al. 2015; Lemieszek & Rzeski 2012). The source of high BG in this study is unknown. Though there was no evidence from single-pass experiments that the dialysis membranes were involved in significant BG generation, it is possible that cumulative treatments over many years may increase BG levels, although there was no correlation between BG levels and dialysis vintage. The gut is a potential source of high BG levels given reports of translocation of intestinal luminal contents due to disturbed gut permeability in uraemia (Wang, Zhang, et al. 2012; Hauser et al. 2011; Vaziri 2012), although there was no relationship between BG levels and history of gastrointestinal disease. There was an inverse relationship between BG with weight and residual kidney function. Animal studies suggest that BG are cleared mainly by the liver (Yoshida et al. 1997; Yoshida et al. 1996) and degraded oxidatively in the reticuloendothelial system(Hong et al. 2004; Young & Vincent 2005), with minimal excretion by the kidney. The role of kidney function in BG excretion is poorly understood (Yoshida et al. 1997) and requires further investigation.

The limitations of this study include the relatively small sample size and the use of reagents from only one manufacturer. It is unclear whether a similar conclusion would be reached if LAL reagents and BG-blocking buffers from other manufacturers were used. However, a previous study similarly found a low prevalence of detectable
endotoxemia in haemodialysis patients when measurements were carried out using LAL devoid of factor G (Taniguchi et al. 1990). As discussed previously, natural endotoxin should ideally additionally be used for assessment of spike recovery although natural endotoxin is difficult to standardise which precluded its use in this study. Furthermore, the efficacy of BG blocking buffers should be confirmed by spiking samples with BG to ensure that BG-blocking buffers are able to prevent any false positive activation of the LAL assay.

In conclusion, BG presence represents a significant limitation of the LAL assay for endotoxin detection in dialysis patients. This data demonstrates that endotoxin measurement, in a setting free of BG contribution from dialyzer equipment or dialysis fluids, suffers from significant, positive BG interference, especially at very low levels of reported endotoxin. Accordingly, endotoxin measurements in dialysis patients should be carried out with LAL rendered insensitive to BG.

This study raises several unexplored issues, firstly although no endotoxin was detected in this unselected group of patients (after accounting for false positive actions of BG), it is unclear whether patients at high risk of death (e.g. those with significant systemic inflammation) may have truly detectable endotoxin since the patients in this study population were relatively well and only mildly inflamed (median CRP 7mg/L). Elevated blood endotoxins may also be transient and detectable only under explicit physiological scenarios such as after exercise or haemodialysis due to the hypothesised ill-effects of exercise and haemodialysis on gastrointestinal vascular system. These issues are further investigated in Chapter 9. Secondly, raised BG levels were found in a significant proportion of haemodialysis patients in this study, the source and clinical significance of raised blood BG is unclear. As discussed in chapter 7, there are a limited number of studies of BG levels in the CKD population and it is difficult to draw any firm conclusions on the consequences of raised BG in the CKD population. Curiously, both potential beneficial and harmful effects of BG have been reported, BG have been suggested to be effective in the treatment of diseases such as cancer, microbial infections, hypercholesterolemia and diabetes (Chen & Seviour 2007), on the other hand, BG can be recognised as non-self-molecules and may activate the immune system via a number of different
mechanisms including the dectin-1, toll-like receptor 2 and complement system leading to the production of cytokines and an inflammatory response (Chen & Seviour 2007). *In-vitro* studies have reported the ability of BG to potentiate the cytokine response of toll-like receptor responses (Kikkert et al. 2007). To further elucidate the effects of endotoxin and BG in the CKD population, blood endotoxin and BG were measured in non-dialysed CKD patients, peritoneal dialysis patients and haemodialysis patients. The long term effects of raised BG and endotoxin in haemodialysis patients were also studied by a prospective, longitudinal follow-up of patients embarking on a haemodialysis treatment (see Chapter 10).
Chapter 9

9. Studies to determine the acute effect of haemodialysis and intra-dialytic exercise on blood endotoxin levels

9.1 Introduction

In the study described in Chapter 8, a sample of an unselected group of prevalent haemodialysis patients with relatively low levels of inflammation did not have any detectable blood endotoxin after accounting for false positive activation by BG. It is possible that blood endotoxin levels were not detected due to the relatively ‘non-inflamed’ status of this study cohort. Blood endotoxin levels may be increased acutely by specific procedures which exert haemodynamic stress such as haemodialysis or exercise (Yu et al. 1997). An acute inflammatory response after intense exercise has been frequently observed in healthy individuals and elite athletes (K. Suzuki et al. 2002). This is purported to be due to the leakage of bacterial endotoxin – potent mediators of inflammation, into the systemic circulation from the gastrointestinal tract due to exercise-induced gut permeability and barrier dysfunction (Lim & Mackinnon 2006). Increased gut permeability related to intense exercise is thought to occur due to a combination of fluid depletion, hyperthermia, ischemia and free radical stress (Pals et al. 1997). Ischaemia occurs due to decreased blood flow to the splanchnic circulation during exercise and many athletes report gastrointestinal symptoms such as abdominal pain, nausea and vomiting during and after exercise (ter Steege & Kolkman 2012). Similarly, many haemodialysis patients experience gastrointestinal complaints after dialysis and also suffer from intestinal ischemia and contraction of the splanchnic circulation during dialysis and ultrafiltration (Yu et al. 1997). In this chapter, studies were carried out to explore blood endotoxin levels in haemodialysis patients during these situations.

However, there is increasing interest to promote the use of intra-dialytic exercise due to a host of possible benefits including improved muscle power, fatigability, cardiac function, blood pressure control, reduction in depression and anxiety and for its anti-inflammatory potential (Parker 2016). Additionally, the haemodialysis procedure itself
can induce an inflammatory response (Bitla et al. 2010) and some studies report increased oxidative stress with a single bout of intra-dialytic exercise (Esgalhado et al. 2015), whereas others have found upregulation of anti-inflammatory cytokines after intra-dialytic exercise (Peres et al. 2015). Given the increasing use of intra-dialytic exercise in haemodialysis units, it is important to determine whether there are any harmful effects from this procedure given that endotoxin levels may be increased after exercise. Although bacterial endotoxin translocation after intense exercise is well reported in healthy individuals (Bosenberg et al. 1988), there is no published data on the effect of intra-dialytic exercise on blood endotoxin levels in haemodialysis patients.

The objective of this study was to determine the acute effect of haemodialysis and intra-dialytic exercise on blood endotoxin levels and markers of inflammation.

9.2 Methods

Study population

This is a single centre study of 10 chronic haemodialysis patients who regularly perform intra-dialytic exercise. Ethical approval was obtained from the NHS Research Ethics Committee and the study adhered to the declaration of Helsinki. Written informed consent was obtained from all participants. All patients were clinically well and non-infected at the time of study. Patients were treated with high flux haemodialysis or on-line haemodiafiltration with ultra-pure water using polysulphone dialysis membranes. The haemodialysis prescription was not altered during the course of the study. Exclusion criteria were those with active infection, HIV, viral hepatitis and active liver or gastrointestinal disease.

Study design

Participants were studied on two separate haemodialysis sessions – in one session patients were asked to abstain from intra-dialytic exercise during haemodialysis treatment (non-exercise day) and in the second session, patients were asked to perform their routine intra-dialytic exercise program (exercise day). These sessions were carried out within one week of each other. Half of the studies were randomly
assigned to be carried with the non-exercise session following the exercise session. The remaining studies were carried out with the sessions completed in reverse order (see Error! Reference source not found.).

Figure 9-1: Flow diagram of patients recruited

Patients exercised using a specially-adapted cycle ergometer (Deluxe Pedal Exerciser, Digiterm Medical Equipment Ltd) during haemodialysis treatment and were advised to perform a 2 minute warm-up period using the lowest resistance setting on the cycle ergometer followed by cycling at their preferred resistance setting aiming for a minimum of 30 minutes at a consistent pace. Patients were allowed to rest or cycle as much as they preferred throughout the exercise session.
Exercise duration and perceived exertion of the exercise session was recorded using the Borg Scale of Perceived Exertion (Borg 1973).

On each study visit, blood samples were collected at three time-points, 1) pre-dialysis [pre-HD], 2) immediately post-dialysis [post-HD 1] and 3) 1-3 hours post-dialysis [post-HD 2], for blood endotoxin, (1→3)-β-D glucan (BG), pro-inflammatory cytokines, high-sensitivity C-reactive protein (hs-CRP) and D-lactate – a sensitive marker of intestinal ischemia (Evennett et al. 2009) (Figure 9-2). Since endotoxins and cytokines can be adsorbed by dialysis membranes (Fujimori et al. 1998; Henrie et al. 2008) and there may be enhanced clearance due to convection with haemodiafiltration (Cole et al. 2004) and smaller endotoxins (10-20 kDa), BG, D-lactate may also be removed during dialysis, additional blood samples were taken 1-3 hours post dialysis to look for a potential rebound rise in any of these parameters caused by either intra-dialytic exercise or the haemodialysis procedure. Such rebound would be anticipated if endotoxin and inflammatory cytokine were generated by the dialysis procedure. For post-HD 1 samples, laboratory measurements were corrected for haemoconcentration by dividing the obtained measurement by the ratio of post-HD albumin to pre-HD albumin.

![Figure 9-2: Blood sampling was performed on an exercise day and non-exercise day. Pre-HD 1, pre-dialysis; Post-HD 1, immediately at the end of dialysis; Post-HD 2, 1-3 hours after completion of dialysis. At each time point, blood was collected for measurement of endotoxin, (1→3)-β-D glucan, IL-6, TNF-α, high-sensitivity CRP and albumin.](image)

Laboratory measurements
**Endotoxin assay:** Endotoxin measurements were carried using a kinetic turbidimetric LAL assay. The procedure is described in detail in Chapter 6. Endotoxin was measured with and without BG-blocking buffer as previously described.

**(1-3)-β-D glucan assay:** BG measurements were carried out using the Fungitell® assay as described in Chapter 8.

**Cytokine, albumin and hs-CRP measurements:** serum was measured for IL-6 and TNF-α using enzyme-linked immunosorbent assays (Human Quantikine ELISA, R&D systems). For IL-6, intra- and inter-assay CV was 1.6-4.2% and 2.0-3.7% respectively. For TNF-α, intra- and inter-assay CV was 4.2-5.2% and 4.6-7.4% respectively. High-sensitivity CRP (hs-CRP) was measured using particle enhanced immunoturbidimetric assay (Roche Diagnostics, cobas c system). Serum albumin was measured using a colorimetric assay (Roche Diagnostics, cobas c system).

**D-lactate measurements:** plasma samples were measured for D-lactate using an endpoint enzymatic spectrophotometric assay on an Olympus AU640 analyser (Megazyme International, Ireland). D-lactate originates from bacteria in the intestinal lumen and is a product of bacterial fermentation. Blood D-lactate levels are usually low in health, but in circumstances of bacterial overgrowth such as short bowel syndrome and acute mesenteric ischemia, large amounts of D-lactate are released into portal and systemic circulation. D-lactate is a marker of intestinal ischemia and a systematic review of biomarkers of intestinal ischemia found that D-lactate had the best overall diagnostic performance (Evennett et al. 2009).

**Statistical analyses**

Parametric and non-parametric data are presented as means and medians with respective 95% confidence intervals and interquartile ranges. Paired differences were evaluated with paired samples t-test and Wilcoxon-signed rank tests as appropriate. Statistical significance of variables were established at the level p<0.05. Data was analysed using statistical software (SPSS version 21.0, IBM, USA).
Results

Patient characteristics

Patient characteristics are shown in Table 9-1, mean age of the study cohort was 62. Most patients were treated with haemodiafiltration using an arterio-venous fistula. Most patients had good dialysis adequacy and retained useful residual kidney function.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>62 [48-76]</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>70</td>
</tr>
<tr>
<td>Vascular access (%)</td>
<td></td>
</tr>
<tr>
<td>Arteriovenous fistula</td>
<td>80</td>
</tr>
<tr>
<td>Dialysis catheter</td>
<td>20</td>
</tr>
<tr>
<td>Charlson Comorbidity Index</td>
<td>4 [3-6]</td>
</tr>
<tr>
<td>Primary renal disease (%)</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>30</td>
</tr>
<tr>
<td>Hypertensive nephropathy</td>
<td>10</td>
</tr>
<tr>
<td>Obstructive nephropathy</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>50</td>
</tr>
<tr>
<td>Dialysis vintage (years)</td>
<td>1.9 [IQR 1.3-3.9]</td>
</tr>
<tr>
<td>Residual Urea Clearance (ml/min)</td>
<td>1.5 [0.5-2.5]</td>
</tr>
<tr>
<td>Kt/V</td>
<td>1.5 [1.2-1.8]</td>
</tr>
<tr>
<td>Haemodialysis modality (%)</td>
<td></td>
</tr>
<tr>
<td>HDF</td>
<td>80</td>
</tr>
<tr>
<td>High-flux HD</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 9-1: Demographic and clinical data

HDF, haemodiafiltration; HD, hemodialysis
Patients exercised for a mean of 100 minutes (95% confidence interval 57.7-142.2) and the mean Borg scale of perceived exertion score was 12 representing “fairly light” exertion for the exercise session. There was no significant difference between exercise and non-exercise days in terms of hemodynamic stability, pre-dialysis weight or ultrafiltration volume (Table 9-2). Post-dialysis systolic BP was lower compared to pre-dialysis systolic BP with intra-dialytic exercise although there was no significant differences in actual change in systolic BP (delta systolic BP). No adverse events were reported.

<table>
<thead>
<tr>
<th>(n=10)</th>
<th>Exercise day</th>
<th>Non-exercise day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise duration (minutes)</td>
<td>100 [57.7-142.2]</td>
<td></td>
</tr>
<tr>
<td>Borg scale of perceived exertion</td>
<td>12 [11-13]</td>
<td></td>
</tr>
<tr>
<td>Pre-HD weight (kg)</td>
<td>69.7 [IQR 66.5-78.4]</td>
<td>69.8 [IQR 66.2-78.8]</td>
</tr>
<tr>
<td>Post-HD weight (kg)</td>
<td>68.5 [IQR 66.7-77.8]</td>
<td>68.7 [IQR 66.1-78.2]</td>
</tr>
<tr>
<td>Ultrafiltration (L)</td>
<td>1.18 [0.36-2.0]</td>
<td>1.26 [0.12-2.39]</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-HD</td>
<td>149 [135-163]</td>
<td>142 [130-155]</td>
</tr>
<tr>
<td>Post-HD</td>
<td>128 [114-142]</td>
<td>132 [114-149]</td>
</tr>
<tr>
<td>p-value (pre-HD vs. post-HD)</td>
<td>0.009*</td>
<td>0.05</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-HD</td>
<td>75 [65-86]</td>
<td>81 [69-93]</td>
</tr>
<tr>
<td>Post-HD</td>
<td>66 [58-75]</td>
<td>68 [56-79]</td>
</tr>
<tr>
<td>p-value (pre-HD vs. post-HD)</td>
<td>0.161</td>
<td>0.1</td>
</tr>
<tr>
<td>Delta systolic BP</td>
<td>21 [6.8-35.2]</td>
<td>10.6 [-5.1-26.3]</td>
</tr>
</tbody>
</table>

Table 9-2: Exercise variables and haemodynamic parameters

HD, haemodialysis; BP, blood pressure; Delta systolic BP = pre-HD systolic BP minus post-HD systolic BP
Effect of intra-dialytic exercise on blood endotoxin and (1→3)-β-D glucan levels

Pre- and post-dialysis blood samples were measured for endotoxin initially using the LAL assay without BG-blocking buffers [LAL(-)]. Repeat measurement was carried out in samples that tested positive using a BG-blocking buffer [LAL(+)]] to render the LAL reagent insensitive to stimulation by BG (Tsuchiya et al. 1990; Kambayashi et al. 1991), to determine if signals detecting using LAL(-) were due to false positive interference by BG. Samples were also separately measured for BG using the Fungitell® assay.

Using the LAL assay without BG-blocking buffers (LAL[-]), only low level endotoxin signals were detected in patients (Table 9-3) with no significant effect of either intra-dialytic exercise or the haemodialysis procedure itself on the level of endotoxin signals (Figure 9-3). Repeat measurement of samples with a BG-blocking buffer extinguished all endotoxin signals with the exception of one sample suggesting that the significant majority of previously detected endotoxin signals using LAL(-) may be due to false positive interference by BG (Table 9-3). BG was elevated (>80pg/mL) in 43% of samples. Mean BG was not significantly altered by the haemodialysis procedure or intradialytic exercise (Table 9-4).

![Exercise day](image1)

![Non-exercise day](image2)

Figure 9-3: Changes in endotoxin signals detected using LAL(-) across the haemodialysis session an exercise and non-exercise day
<table>
<thead>
<tr>
<th>Patient number</th>
<th>Exercise day</th>
<th>Non-exercise day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-HD</td>
<td>Post-HD 1</td>
</tr>
<tr>
<td></td>
<td>Pre-HD</td>
<td>Post-HD 1</td>
</tr>
<tr>
<td>LAL(−) LAL(+) LAL(−) LAL(+) LAL(−) LAL(+) LAL(−) LAL(+) LAL(−) LAL(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>6</td>
<td>0.027</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>7</td>
<td>0.038</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>8</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>9</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.025</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

Table 9-3: Endotoxin signals detected in study population on an exercise and non-exercise day using LAL(−) and LAL(+)  

Pre-HD, pre-dialysis; Post-HD 1, immediately post-dialysis; Post-HD 2, 1-2 hours post-dialysis; LAL(−), LAL assay without BG-blocking buffer; LAL(+), LAL assay with BG-blocking buffer
<table>
<thead>
<tr>
<th></th>
<th>Pre-HD</th>
<th>Post-HD 1</th>
<th>Post-HD 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre-HD vs. Post-HD 1</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise day</td>
<td>8.0 [IQR 6.5-10.1]</td>
<td>9.0 [IQR 6.8-15.5]</td>
<td>11.1 [IQR 8.4-15.7]</td>
<td>0.203</td>
</tr>
<tr>
<td>Non-exercise day</td>
<td>6.3 [IQR 3.9-8.9]</td>
<td>7.5 [IQR 6.6-10.3]</td>
<td>10.9 [IQR 9.9-14.8]</td>
<td>0.445</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise day</td>
<td>2.9 [IQR 2.3-7.3]</td>
<td>2.9 [IQR 2.1-7.4]</td>
<td>3.4 [2.2-8.3]</td>
<td>0.404</td>
</tr>
<tr>
<td>Non-exercise day</td>
<td>2.5 [IQR 1.8-9.6]</td>
<td>2.3 [IQR 1.7-9.3]</td>
<td>3.6 [2.0-10.2]</td>
<td>0.952</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise day</td>
<td>11.7 [IQR 10.8-26.5]</td>
<td>9.6 [4.5-14.4]</td>
<td>8.8 [5.5-15.9]</td>
<td>0.007*</td>
</tr>
<tr>
<td>Non-exercise day</td>
<td>12.2 [IQR 9.4-21.1]</td>
<td>6.3 [4.4-12.0]</td>
<td>12.7 [7.6-18.3]</td>
<td>0.047*</td>
</tr>
<tr>
<td>(1→3)-β-D glucan (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise day</td>
<td>66.8 [39.6-94]</td>
<td>57.2 [46-66.5]</td>
<td>60.8 [53.2-68.4]</td>
<td>0.494</td>
</tr>
<tr>
<td>Non-exercise day</td>
<td>50.1 [34.8-65.4]</td>
<td>53.8 [47-60.5]</td>
<td>62.1 [54.7-69.5]</td>
<td>0.627</td>
</tr>
<tr>
<td>D-lactate μmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise day</td>
<td>0 [IQR 0-6.5]</td>
<td>0 [IQR 0-10.3]</td>
<td>0 [0-12.5]</td>
<td>0.893</td>
</tr>
<tr>
<td>Non-exercise day</td>
<td>0 [IQR 0-12.5]</td>
<td>0 [IQR 0-6.5]</td>
<td>0 [0-10]</td>
<td>0.357</td>
</tr>
</tbody>
</table>

Table 9-4: Effect of haemodialysis and intra-dialytic exercise on markers of inflammation and (1→3)-β-D glucan levels

IL-6, interleukin-6; hs-CRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α
Effect of intra-dialytic exercise on markers of inflammation and D-lactate

The haemodialysis procedure resulted in a rise in circulating levels IL-6 and hs-CRP 1-3 hours after treatment (both p<0.05), intra-dialytic exercise attenuated the rise in IL-6 and hs-CRP with no significant difference between pre- and post-HD levels. For TNF-α, there was no significant difference between pre- and post-HD levels on a non-exercise day. However, intra-dialytic exercise led to a significant fall in levels of TNF-α after HD. Most patients had undetectable D-lactate, the median D-lactate levels pre- and post-HD on both exercise and non-exercise days were below the detection limit (Table 9-4).

9.4 Discussion

The changes in levels of blood endotoxin in haemodialysis patients in relation to intra-dialytic exercise were investigated in this study. Due to the lack of endotoxemia detected in haemodialysis patients in the studies carried out in Chapter 8, blood endotoxin levels were measured in haemodialysis patients after haemodialysis treatment and after intra-dialytic exercise – potentially, endotoxemic inducing procedures. Using BG-blocking buffers to render the LAL assay insensitive to false positive activation by BG (Tsuchiya et al. 1990; Kambayashi et al. 1991) – with the exception of one sample, blood endotoxin was not detected in the study population. The majority of signals detected using LAL(-) were extinguished on repeat measurement with BG-blocking buffers (LAL[+]) suggesting that true endotoxemia may be rare in our population. BG was elevated in 43% of samples and given the reports of high blood BG reported in haemodialysis patients (Prattes et al. 2015), this further emphasises the importance of taking measures to prevent activation of factor G using the LAL assay.

However, it may be possible that the LAL assay may not be sensitive enough to detect endotoxin due to its rapid clearance from the circulation by deacylation (Lu & Munford 2011) or due to the binding of endotoxin by proteins in plasma rendering it unavailable for detection by the LAL assay (Munford 2016). The significance of a single sample testing positive for endotoxin with LAL(+) is unclear, this may be a
genuine elevated blood endotoxin result or it could be due to external contamination. Endotoxin is an extremely ubiquitous molecule and there may have been inadvertent contamination during the blood sampling or analytical procedure despite meticulous precautions to minimise this risk. The cause of raised BG levels in our study cohort was unclear since all subjects dialyzed using polysulphone dialysis membrane which is not known to elevate blood BG levels (Prattes et al. 2015). It is unclear whether raised BG is an epiphenomenon of disrupted intestinal barrier due to uraemia or by being on haemodialysis treatment, although its levels were not significantly altered acutely by the haemodialysis procedure or intra-dialytic exercise. This finding will be further explored in Chapter 10.

The study findings suggest that intra-dialytic exercise may attenuate the inflammatory response to the haemodialysis procedure. It has been proposed that the haemodialysis procedure induces an inflammatory response due to activation of peripheral blood mononuclear cells and complement activation in the extracorporeal circuit. IL-6, TNF-α and CRP are prognostically significant pro-inflammatory cytokines in the dialysis population (Carrero & Stenvinkel 2010). IL-6 and hs-CRP increased significantly 1-3 hours post-dialysis on a non-exercise day, however following intra-dialytic exercise, levels of IL-6 and hs-CRP did not increase after HD and remained similar to pre-HD values. TNF-α levels decreased significantly immediately post-dialysis (post-HD 1) but subsequently returned to baseline levels 1-3 hours after completion of dialysis (post-HD 2) on a non-exercise day. An initial reduction in TNF-α with dialysis has also been observed in other studies (Ghysen et al. 1990) and post-HD levels frequently return to levels similar to their pre-HD levels (Ghysen et al. 1990). Given that TNF-α is adsorbed by dialysis membranes (Fujimori et al. 1998) and has a relatively short half-life (5-10 minutes) (Tarakcioğlu et al. 2003), it has been suggested that the HD procedure may still be inducing a net influx or generation of TNF-α despite levels being similar before and after HD (Ghysen et al. 1990; Tarakcioğlu et al. 2003). Intra-dialytic exercise appeared to blunt the rebound rise in TNF-α after dialysis and levels remain significantly lower than pre-HD levels (Table 9-4), overall the observations from this study suggest there may be a potential anti-inflammatory benefit from intra-dialytic exercise.
The anti-inflammatory benefits of exercise have been reported in individuals with and without kidney disease (Parker 2016). The mechanistic reasons for the reported anti-inflammatory effects of exercise are unclear although it appears that the duration and intensity of exercise performed is important. Prolonged and intense exercise increases IL-6 release from muscles significantly and stimulates the release of anti-inflammatory cytokines such as IL-10 and IL-1ra. These anti-inflammatory cytokines inhibit the release of other classical pro-inflammatory cytokines including TNF-α and IL-1β leading to an overall anti-inflammatory effect (Dungey et al. 2013). Whereas short duration and low to moderate intensity activity (similar to the level completed by our study cohort) are still associated with an overall reduction in circulating levels of inflammatory biomarkers but do not stimulate IL-6 release. Dungey et al reviewed the long term effects of exercise on inflammation in dialysis patients and found conflicting evidence (Dungey et al. 2013). Proposed reasons for the conflicting data include lack of a control arms, small sample size, variable exercise protocols used and differences in laboratory assays used to measure different inflammatory biomarkers. The acute effect of intra-dialytic exercise on inflammation is even less clear with only a small number of published studies, one study found an upregulation of anti-inflammatory cytokines such as IL-10 (Peres et al. 2015), another study did not find any effect of exercise on circulating pro-inflammatory cytokines (Dungey et al. 2015), whilst one study found that intra-dialytic exercise reduced antioxidant enzyme activity, suggesting an exacerbation of oxidative stress (Esgalhado et al. 2015). One of the strengths of our study was that samples were collected 1-3 hours after dialysis for better clarification of the effect of dialysis and exercise on inflammation since many molecules of interest such as endotoxin and cytokines are either removed or adsorbed by dialysis membranes.

D-lactate was not a useful marker of intestinal ischemia in stable dialysis patients since most patients had undetectable D-lactate. It is possible that the level of intestinal ischemia experienced by HD patients (due to the low intensity nature of the exercise) was transient and not severe enough to trigger significant elevations in D-lactate, since the diagnostic value of D-lactate is used mainly in patients with severe and extensive mesenteric ischemia.
There were a number of limitations with this study. Firstly, the sample size was small and cross-sectional in nature, these findings would need confirmation in a larger cohort study. Secondly, there may have been selection-bias since the study cohort was a selected group of prevalent patients who regularly participated in intra-dialytic exercise and were relatively well with less comorbidity, stable blood pressures and modest ultrafiltration volumes. The selected study participants may have been more resistant or less susceptible to the potential endotoxemic-inducing effect of intra-dialytic exercise which could explain the lack of detectable endotoxemia observed. Thirdly, other relevant pro-inflammatory and anti-inflammatory cytokines such as IL-1β, IL-10 and IL-1ra which may have better characterised the effect of intra-dialytic exercise on inflammation were not measured. Additionally, the majority of the study cohort was treated with haemodiafiltration with a small proportion on high-flux HD. Dialysis modality may affect cytokine levels since there is enhanced clearance with convection from HDF, future studies examining the acute effect of dialysis on inflammation should ideally control for the different cytokine clearance profiles of HDF vs. standard HD. Fourthly, exercise intensity was not measured in this study and only subjective measures were used. However, given the concerns with exercise-induced translocation of gut bacterial products and endotoxin, the main purpose of the study was to determine the effect of intra-dialytic exercise at the level of intensity and duration typical of that completed by dialysis patients who may have wide varying degrees of fitness. Finally, precise timing of the post-dialysis blood sample (post-HD 2) could not be controlled for all patients since it was impractical to keep patients in the dialysis unit after completion of dialysis treatment and blood sampling was performed after the patient had returned home. However, with the exception of one sample, all samples were taken within 1-2 hours of dialysis.

In conclusion, the findings from this study suggest that acute intra-dialytic exercise does not have any adverse effects on patients. Intra-dialytic exercise does not appear to induce an influx of endotoxin as observed in other trials of healthy athletes during intense exercise. Additionally, intra-dialytic exercise may potentially have beneficial, anti-inflammatory effects. The long term impact of intra-dialytic exercise on inflammation in HD patients requires further investigation. Despite the use of ultra-pure water and biocompatible membranes, the haemodialysis procedure
induces a modest inflammatory effect which is not associated with a measurable increase in plasma endotoxin. This study further highlights the limitations of using the LAL assay to measure blood endotoxin in HD patients which is highly prevalent in the literature partly due to the problem with BG interference. Blood BG was elevated in a significant proportion of patients despite the use of non-cellulosic dialysis membranes, the source and significance of raised blood BG in HD patients warrants investigation. The findings from this study and in Chapter 8 suggest that the emphasis of endotoxemia as a phenomenon and potential trigger of chronic inflammation in stable non-infected HD may not be true and would require re-examination. Elevated BG appears to play a significant role by inducing false positive signals in the LAL assay, potentially being misinterpreted as 'endotoxemia'. However, the studies carried out in this and Chapter 8 are in relatively well, non-inflamed haemodialysis patients. The sample size was also relatively small and without long term follow-up of the study participants. The reasons for elevated BG require further study. It is unclear whether BG is elevated due to renal impairment itself or whether this is due to the effects of being a long term haemodialysis. These issues are further explored in Chapter 10.
Chapter 10

10. Clinical significance of (1-3)-β-D glucan levels in CKD: association with endotoxemia and inflammation

10.1 Introduction

BG are major carbohydrate constituents of yeast, cereal and fungal cell walls which share a diverse common backbone structure consisting of β(1,3)-linked-D-glucopyranosyl units and strongly differ in size and structure depending on the pattern of branching (Camilli et al. 2018). BGs are key pathogen-associated molecular pattern (PAMP) molecules that trigger a number of host immune responses and can stimulate the production of reactive oxygen species and inflammatory cytokines mediated by interaction with dectin-1 and toll-like receptors (Gantner et al. 2003; Brown et al. 2003). The studies carried out in Chapter 8 and Chapter 9 showed that BG was elevated in a significant proportion of haemodialysis patients and may produce false positive test results for blood endotoxin detection with standard methods of detection using the LAL assay. False positive endotoxin signals were detected in 50% of haemodialysis patients in the study described in Chapter 8, which were extinguished on repeat measurement with a BG-blocking buffer. However, these measurements were carried out in a relatively small number of patients without significant inflammation (mean CRP in the population was only 7mg/L). In Chapter 9 patient blood samples were measured for endotoxin whilst in physiological states with the potential to generate endotoxin-influx such as during exercise or immediately after haemodialysis treatment, however there was no significant effect of haemodialysis or intra-dialytic exercise on endotoxin levels. Only low level endotoxin signals detected in the study population and similarly these signals were extinguished on repeat measurement with a BG-blocking buffer, further supporting the notion that BG present in blood samples results in false positive activation of the LAL assay. However, several issues require further exploration – could blood endotoxin be detected in patients with more significant and persistent
inflammation? Why is BG elevated in haemodialysis patients? What is the clinical significance of elevated blood BG in asymptomatic, non-infected individuals?

The reasons for elevated BG in haemodialysis patients are unclear. Animal studies suggest that BG is predominantly degraded oxidatively in the mononuclear phagocytic system (MPS) (Young & Vincent 2005; Yoshida et al. 1997), so one would not expect a direct relationship to renal function. Dialysis membranes have been found to increase BG levels but the effect appears to be confined to now obsolete cellulose membranes and is not seen with modern biocompatible devices. Hence the cause for these elevated levels is not clear. Likewise, there is little understanding of the clinical consequences of high levels of beta-glucan. The prevalence and clinical significance of elevated BG levels in non-infected subjects with different stages of kidney disease has not been studied. Hence a study was carried out to investigate BG levels in patients with various severities of chronic kidney disease and the association of BG with inflammation, endotoxin levels, clinical symptoms and events.

10.2 Methods

Study population

This was a single centre study conducted at the East and North Hertfordshire NHS Trust. Study participants were randomly recruited from patients with CKD, peritoneal dialysis and haemodialysis patients. Inclusion criteria were adults aged 18 years and over. Participants consisted of healthy controls, CKD 1-3, CKD 4-5, peritoneal dialysis (PD) and haemodialysis (HD) patients. Exclusion criteria include participants with active sepsis, positive HIV, hepatitis B or C status, those who were pregnant and those with active vasculitis or connective tissue disease.

Study design

There were two sub-studies.

Sub-study 1
The study aimed to investigate BG levels in healthy controls, CKD patients and dialysis patients. Across this spectrum the associations of BG levels with markers of inflammation, endotoxin levels, clinical symptoms including fatigue and depression and clinical events were explored.

A total of 135 subjects were recruited, 20 healthy controls, 20 patients with CKD 1-3 (eGFR≥30mL/min), 20 patients with CKD 4-5 (eGFR<30mL/min), 15 PD patients and 60 HD patients. The haemodialysis group consisted of two equal subgroups. High-risk patients had chronic unexplained inflammation (CRP>5mg/L) measured on two separate occasions at least one month apart in the three months prior to study recruitment) together with a predilection for intradialytic hypotension (either an ultrafiltration rate >10mL/kg/hr (Flythe et al. 2011) or pre- or post-dialysis systolic blood pressure <100 mmHg within the week prior to study recruitment). Low-risk patients had none of these features. All patients were assessed clinically by to ensure there was no evidence of sepsis, no recent antibiotic treatment or receipt of blood products in the last month since these can elevate blood BG levels (Sulahian et al. 2014).

Blood samples were drawn from subjects and measured for endotoxin, BG, IL-6, TNF-α and high sensitivity CRP. All CKD and dialysis patients were asked to complete several self-report questionnaires to investigate depression symptoms (PHQ-9) (Watnick et al. 2005), fatigue symptoms (MFI-20) (Smets et al. 1995) and kidney disease related symptoms (POS-S renal) (Collins et al. 2015). Subjects were followed up for 18 months from study recruitment for cardiovascular events, infection episodes requiring hospitalisation and mortality.

Sub-study 2

The study aimed to investigate the effect of haemodialysis initiation on blood levels of endotoxin and BG. Thirty patients with CKD stage 5 who were planned for dialysis were recruited for this sub-study. Blood samples were collected up to 6 months prior starting haemodialysis and repeated 1-6 months after initiating haemodialysis.
Blood sampling and processing

Blood samples were collected peripherally using aseptic technique as previously described. For haemodialysis patients, this was done pre-dialysis through the arterio-venous fistula or peripherally if patients did not have a functioning fistula. Blood samples were measured for BG, endotoxin (measured twice – LAL with and without BG blocking buffers) and cytokines (IL-6, CRP and TNF-α) using the same method as described in chapter 6.

Statistical analysis

Non-parametric data were presented as medians with interquartile range. Parametric data were presented as means with 95% confidence intervals. Analysis was performed using IBM SPSS statistics version 21. Comparison were made between groups using the Mann-Whitney U test or T-test as appropriate. Correlation between continuous variables was examined by Spearman rank correlation coefficient. Baseline measures of patients with elevated BG with outcomes was explored using unadjusted and adjusted Cox proportional hazard models. No patients were lost to follow-up during the study period. Co-variates specified in the adjusted models include age, gender, Charlson Co-morbidity index and CKD stage.

10.3 Results

Demographic and clinical characteristics

The study cohort (Table 10-1) consisted of 20 healthy controls, 20 patients with CKD stage 1-3, 20 patients with CKD stage 4-5, 15 PD patients, 60 HD patients (30 high risk and 30 low-risk). Demographic, clinical and biochemical parameters are shown in Table 10-1 and Table 10-2. In general, patients with advanced CKD were older and more highly comorbid than those with lesser degrees of CKD and controls. PD patients had lower dialysis vintage and higher residual kidney function than HD patients. Standard Kt/V between PD and HD patients was not significantly different.
Low- and high-risk HD patient were similar with respect to age, co-morbidity, dialysis catheter use and treatment with haemodiafiltration (HDF) though low-risk patients had lower dialysis vintage, higher pre- and post- dialysis systolic BP, greater residual kidney function, lower body weight, lower sessional duration and higher standard Kt/V. Ultrafiltration rate tended to be higher in high risk patients but this did not reach statistical significance (Table 10-2).

**Table 10-1: Patient characteristics**

* eGFR applies to healthy and non-dialysed CKD patients, KRU applies to dialysis patients. PD, peritoneal dialysis patents; HD, haemodialysis patients; CCI, Charlson Co-morbidity index; eGFR, estimated glomerular filtration rate; KRU, residual urea clearance

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>eGFR/KRU* (mL/min)</th>
<th>Anuric (%)</th>
<th>CCI</th>
<th>Diabetic (%)</th>
<th>Dialysis vintage (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (n=20)</td>
<td>48 [44-52]</td>
<td>73.9 [67.4-80.4]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CKD 1-3 (n=20)</td>
<td>50 [36-64]</td>
<td>79.9 [61.3-98.4]</td>
<td>54.4 [45.7-63]</td>
<td>-</td>
<td>1 [IQR 0-4]</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>CKD 4-5 (n=20)</td>
<td>61 [54-68]</td>
<td>95.9 [86.1-105.5]</td>
<td>14.2 [11.6-16.7]</td>
<td>-</td>
<td>5 [IQR 4-8]</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>PD (n=15)</td>
<td>60 [51-69]</td>
<td>84.8 [77.3-92.4]</td>
<td>4.1 [IQR 1.4-6.3]</td>
<td>6.7</td>
<td>6 [IQR 3-7]</td>
<td>26.7</td>
<td>1 [IQR 0.3-4]</td>
</tr>
<tr>
<td>High-risk HD (n=30)</td>
<td>61 [56-67]</td>
<td>82 [73.3-90.7]</td>
<td>0 [IQR 0-0]</td>
<td>80</td>
<td>5.5 [IQR 3.8-8]</td>
<td>33.3</td>
<td>3 [IQR 1.3-6.1]</td>
</tr>
<tr>
<td>Low-risk HD (n=30)</td>
<td>67 [60-73]</td>
<td>73.7 [66.7-80.7]</td>
<td>1.62 [IQR 0-2.7]</td>
<td>33.3</td>
<td>7 [IQR 4.8-9]</td>
<td>40</td>
<td>1.6 [IQR 0.9-4.3]</td>
</tr>
</tbody>
</table>
Table 10-2: Dialysis clinical parameters

Data represent median with interquartile ranges or mean with 95% confidence interval. Ca, calcium; PO₄, phosphate; PTH, parathyroid hormone; THL, tunneled haemodialysis catheter; Td, session dialysis time; Qb, access blood flow; UFR, ultrafiltration rate; HDF, haemodiafiltration. † – P value for statistical comparison between high risk and low risk HD (haemodialysis) patients, there was no significant difference between PD and low-risk HD patients or high-risk HD. ‧ - standard Kt/V for HD patients represent urea clearance achieved by residual renal function (renal Kt/V) and dialysis Kt/V

<table>
<thead>
<tr>
<th></th>
<th>PD (n=15)</th>
<th>High-risk HD (n=30)</th>
<th>Low-risk HD (n=30)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>2.34 [2.24-2.48]</td>
<td>2.32 [2.23-2.43]</td>
<td>2.37 [2.19-2.42]</td>
<td>0.812</td>
</tr>
<tr>
<td>PO₄</td>
<td>1.61 [1.39-1.82]</td>
<td>1.8 [1.5-2.25]</td>
<td>1.55 [1.42-1.88]</td>
<td>0.128</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>39 [33-31]</td>
<td>41 [38-41.3]</td>
<td>38.5 [36-40.3]</td>
<td>0.058</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>47.5 [27-66.3]</td>
<td>41.9 [22.5-83.8]</td>
<td>43.4 [27.3-63.7]</td>
<td>0.912</td>
</tr>
<tr>
<td>Standard Kt/V ‧</td>
<td>2.2 [95% CI 1.9-2.6]</td>
<td>2.1 [95% CI 2.0-2.2]</td>
<td>2.5 [95% CI 2.3-2.7]</td>
<td>&lt;0.001 †</td>
</tr>
<tr>
<td>Access with THL (%)</td>
<td>-</td>
<td>23.3</td>
<td>16.7</td>
<td>0.519</td>
</tr>
<tr>
<td>Td (min)</td>
<td>-</td>
<td>240 [206-240]</td>
<td>203 [180-240]</td>
<td>0.015</td>
</tr>
<tr>
<td>Qb (mL/min)</td>
<td>-</td>
<td>300 [300-400]</td>
<td>325 [300-363]</td>
<td>0.972</td>
</tr>
<tr>
<td>UFR (mL/kg/hr)</td>
<td>-</td>
<td>5.9 [95% CI 4.6-7.2]</td>
<td>4.4 [95% CI 3.2-5.6]</td>
<td>0.064</td>
</tr>
<tr>
<td>Treatment with HDF (%)</td>
<td>-</td>
<td>73.7</td>
<td>90</td>
<td>0.095</td>
</tr>
<tr>
<td>Pre-HD systolic BP</td>
<td>-</td>
<td>129 [107-139]</td>
<td>152 [138-178]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pre-HD diastolic BP</td>
<td>65 [52-81]</td>
<td>74 [65-86]</td>
<td></td>
<td>0.173</td>
</tr>
<tr>
<td>Post-HD systolic BP</td>
<td>106 [99-127]</td>
<td>135 [120-151]</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Post-HD diastolic BP</td>
<td>58 [49-74]</td>
<td>65 [54-71]</td>
<td></td>
<td>0.504</td>
</tr>
</tbody>
</table>

Blood levels (1→3)-β-D glucan

Blood levels of BG progressively increased with worsening CKD stage. BG levels were highest in high-risk HD patients and were significantly higher than PD, CKD and healthy controls although were not significantly different from low-risk HD patients (Figure 10-1 and Table 10-3). Low-risk HD patients had similar BG levels to PD patients but significantly greater levels than CKD groups and controls. PD

178
patients had significantly greater levels than CKD groups and controls. BG levels in patients with CKD 4-5 were only marginally greater that those with CKD 1-3, but greater than controls. BG levels in patients with CKD 1-3 did not differ from those in controls. In 22% of HD patients (16.7% low-risk and 26.7% high-risk), 13.3% of PD patients, 0% of CKD 4-5 and 10% of CKD 1-3 patients had blood BG levels >80 pg/mL which is considered positive in diagnostic work-up for invasive fungal disease. Across the whole CKD group there was a relationship of BG and Charlson Comorbidity index (rho=0.305, p=0.001) but not with age or gender. In HD patients BG levels were negatively correlated with KRU (rho=-0.414, p=0.001). Patients with minimal kidney function (KRU < 1 ml/min) had higher BG levels than those with more renal function (62 vs 38 pg/ml, p=0.001), and whereas patients receiving HDF had slightly higher levels than those on high-flux HD, these were not significantly different (57 vs. 45 pg/ml, p=0.151). There was no relationship between BG levels and Kt/V.
Table 10-3: Endotoxin, (1-3)-β-D glucan and inflammatory cytokines

BG, (1-3)-β-D glucan; data shown represent median and interquartile ranges. Reference ranges for CRP, IL-6 and TNFα are <5mg/L, <3.1pg/mL and <15.6 pg/mL respectively. BG blocking buffer consists of highly concentrated carboxymethylated curdlan. Applying this buffer to the LAL assay the blocks the factor G pathway of the assay preventing any false activation by any circulating (1-3)-β-D glucan that may be present in the sample

<table>
<thead>
<tr>
<th></th>
<th>High-risk HD (n=30)</th>
<th>Low-risk HD (n=30)</th>
<th>PD (n=15)</th>
<th>CKD 4-5 (n=20)</th>
<th>CKD 1-3 (n=20)</th>
<th>Healthy control (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG (pg/mL)</td>
<td>57 [46.5-81]</td>
<td>55.5 [34.8-73.4]</td>
<td>37 [31-67]</td>
<td>22.5 [16.5-27]</td>
<td>15.5 [10-26]</td>
<td>13.5 [11-17.5]</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>12.9 [7.4-19.3]</td>
<td>2 [0.75-3.8]</td>
<td>4.1 [2.4-11.7]</td>
<td>3.9 [2.3-6.1]</td>
<td>2.9 [1-8.1]</td>
<td>1.5 [0.7-3.5]</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>14.3 [9.6-22.8]</td>
<td>8.6 [6.4-11.6]</td>
<td>6.3 [3.3-11.2]</td>
<td>2.4 [1.4-7.3]</td>
<td>3.1 [0.3-7]</td>
<td>0 [0-0.8]</td>
</tr>
<tr>
<td>Endotoxin level (EU/mL) [without BG blocking buffer]</td>
<td>0.027 [0-0.041]</td>
<td>0 [0-0.036]</td>
<td>0 [0-0.049]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Detectable endotoxin signal (%) [without BG blocking buffer]</td>
<td>53.3 (16/30)</td>
<td>36.7 (11/30)</td>
<td>46.7 (7/15)</td>
<td>15 (3/20)</td>
<td>10 (2/20)</td>
<td>0 (0/20)</td>
</tr>
<tr>
<td>Detectable endotoxin (%) [with BG blocking buffer]</td>
<td>6.7 (2/30)</td>
<td>3.3 (1/30)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 10-1: (1-3)-β-D-glucan in study cohort. Median blood levels of (1-3)-β-D-glucan in study cohort. Error bars represent interquartile range. HD, haemodialysis; PD, peritoneal dialysis; CKD 4-5, chronic kidney disease stage 4-5; CKD 1-3, chronic kidney disease stages 1-3; NS, not significant.
**Associations of blood (1→3)-β-D glucan levels with markers of inflammation**

Levels of inflammatory cytokines interleukin-6 (IL-6), tumour necrosis factor-α (TNFα) and high-sensitivity C-reactive protein (hs-CRP) levels in the different groups are shown in Table 10-3. High-risk HD patients had higher levels of CRP and IL-6 than all other groups. TNF-α was similar in low-and high-risk HD patients but higher than in all other groups. Levels of BG correlated significantly with markers of inflammation. In particular in the whole CKD group there were significant correlations with CRP (ρ=0.271; p=0.003), IL-6 (ρ=0.520; p < 0.001) and TNF-α (ρ = 0.486; p<0.001). On sub-group analysis of the HD group, BG was correlated with CRP only (r=0.277; p=0.032).

**Association of blood (1→3)-β-D glucan levels with endotoxemia**

Endotoxin levels are shown using the standard LAL assay and using the LAL assay combined with a BG blocking buffer which inhibits the factor G pathway rendering the LAL assay insensitive to BG stimulation and endotoxin-specific. Endotoxin measurements obtained using the standard LAL assay revealed low level blood endotoxin in HD, PD and CKD patients (Table 10-3 and Figure 10-2). Both HD and PD patients had significantly higher amounts of endotoxin signal than CKD and healthy controls (p<0.05 for both). However, following repeat measurement with a BG-blocking buffer, nearly all of these signals became negative with only three HD patients in the whole cohort testing positive for blood endotoxin (Figure 10-2). Two patients in the high-risk and one from the low-risk HD group had detectable endotoxin (0.06, 0.071 and 0.037 EU/mL respectively).

Endotoxin signal derived from the LAL assay without blocking buffer correlated strongly with levels of BG (r = 0.545, p<0.001). Endotoxin signal was highest in subjects with high BG (>80 pg/mL) and lowest in patients with low levels (<60 pg/mL) [Figure 10-3]. These collective findings strongly suggest that endotoxin signal detected using the LAL assay in the majority of CKD patients may be artefactual due to elevated BG.
Figure 10-2: Proportion of patients with detectable endotoxin using the LAL assay with and without a BG-blocking buffer.

Figure 10-3: Endotoxin signal detected using LAL without blocking buffer displayed against serum (1-3)-β-D glucan. *Denotes statistical significance p<0.05.

Clinical associations of (1-3)-β-D glucan levels
Table 10-4 shows the results of fatigue (MFI-20), depression scores (PHQ-9) and kidney disease-related symptoms (POS-S renal) across the CKD subject groups. For MFI-20, there were differences in relation to physical fatigue, reduced activity and reduced motivation subscales (only the former shown in Table 10-4) which were highest in the high risk HD group (significant compared to patients with CKD 1-3, but comparable with other groups). All HD patients had higher depression scores than subjects with CKD 1-3 but levels were similar to all other groups. High-risk HD patients reported a higher burden of symptoms (POS-S) than other groups, though this was only significantly higher than CKD 1-3 patients.

Table 10-4: Fatigue, depression and kidney disease related symptom scores across study population

<table>
<thead>
<tr>
<th></th>
<th>CKD 1-3 (A)</th>
<th>CKD 4-5 (B)</th>
<th>PD (C)</th>
<th>Low risk HD (D)</th>
<th>High risk HD (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PHQ-9</strong></td>
<td>2.5 [0.3-5]</td>
<td>3 [2-7]</td>
<td>5.5 [0.8-9.8]</td>
<td>5 [2-9]†</td>
<td>7 [3.5-11.5]†</td>
</tr>
</tbody>
</table>

Across the whole CKD group BG levels correlated with physical fatigue scores (rho=0.269; p=0.005), reduced activity (rho=0.270; p=0.005) and reduced motivation (rho=0.318; p=0.001) There were also correlations between BG and PHQ-9 (rho=0.290; p=0.005) and POS-S scores (0.203; p=0.035). For POS-S, individual symptoms including breathlessness, drowsiness and sleep disturbance correlated significantly with BG levels.
All CKD patients were followed up for 18 months for cardiovascular events, time to hospitalisation with sepsis, and mortality. In univariate Kaplan-Meir analysis, unadjusted hazard ratio (HR) for time to hospitalisation with sepsis was significantly higher in patients with BG>80pg/mL (p=0.007 log rank test) [Figure 10-4]. In a Cox regression model, after adjusting for age, gender, CCI and CKD stage, this association was no longer significant (HR=2.22 [95% CI 0.88 -5.59]; p=0.089). There was no significant relationship of high BG level with mortality or cardiovascular events.

![Figure 10-4: Unadjusted Cox proportional hazard for time to hospitalisation with infection over 18 month period. Population divided based on positive or negative test for BG (cut-off value 80 pg/mL)]

Determining the effect of haemodialysis initiation on serum (1-3)-β-D glucan

The effect of HD initiation on endotoxin and BG levels was explored in a separate cohort of 30 pre-dialysis patients with CKD 5 (Table 10-5). Blood samples were
collected prior to and after starting HD. Serum BG was higher after initiation of HD (26 vs. 41 pg/mL, p=0.002). IL-6 and CRP were not significantly different pre- and after HD initiation but TNF-α levels increased following initiation. All patients had undetectable endotoxin using the LAL modified with a BG-blocking buffer. One patient tested positive for endotoxin after initiation of HD with low level endotoxemia (0.027 EU/mL).

Table 10-5: Patient characteristics with endotoxin, 1-3)-β-D glucan and inflammatory cytokines pre- and after initiation of haemodialysis.

BG blocking buffer consists of highly concentrated carboxymethylated curdlan. Applying this buffer to the LAL assay the blocks the factor G pathway of the assay preventing any false activation by any circulating (1-3)-β-D glucan that may be present in the sample; CCI, Charlson Co-morbidity index

<table>
<thead>
<tr>
<th>Parameter (n=30)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>57 [51-62]</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>70</td>
</tr>
<tr>
<td>CCI</td>
<td>5 [IQR 3-7]</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.7 [80.4-93.1]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Before HD</th>
<th>Post HD initiation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG (pg/mL)</td>
<td>26 [20-38]</td>
<td>41 [24-49]</td>
<td>0.002*</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>5.9 [3.7-13.4]</td>
<td>7.3 [4-11.8]</td>
<td>0.434</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>19.4 [17.5-25]</td>
<td>21.9 [18.5-25.6]</td>
<td>0.005*</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>5 [2-12.6]</td>
<td>5.6 [1.5-11.3]</td>
<td>0.792</td>
</tr>
<tr>
<td>Endotoxin (EU/mL) (without BG blocking buffer)</td>
<td>0 [0-0.032]</td>
<td>0 [0-0.026]</td>
<td>0.552</td>
</tr>
<tr>
<td>Detectable endotoxemia (without BG blocking buffer) [%]</td>
<td>30 (9/30)</td>
<td>26.7 (8/30)</td>
<td>0.774</td>
</tr>
<tr>
<td>Detectable endotoxemia (with BG blocking buffer) [%]</td>
<td>0 (0/30)</td>
<td>3.3 (1/30)</td>
<td>0.313</td>
</tr>
</tbody>
</table>
10.4 Discussion

This study showed that blood BG levels increased progressively with worsening CKD stage. BG levels were highest in HD patients and nearly a quarter of patients had levels above the cut-off value of 80 pg/mL for diagnosis of fungaemia. Elevated BG was associated with increased levels of inflammatory biomarkers, higher fatigue, depression and symptom scores. There was also a possible association with sepsis-related hospitalisation. In addition this study also confirmed the association between high BG levels and false positive endotoxin tests in this setting. The prevalence of true endotoxemia in this cohort was 5% which is much lower than that reported in many other studies (see Chapter 2) but more in keeping with the clinical state of study participants. The source of elevated BG in our population is uncertain as neither kidney failure nor the HD procedure itself are thought to lead to accumulation of BG. BG are large molecules and depending on its branching structure molecular weights vary from tens to thousands kD (Rop et al. 2009). Hence they would not be easily removed by dialysis. Although dialysers constructed with cellulose materials may raise blood BG levels (Kanda et al. 2001; Pearson et al. 1984; Yoshioka et al. 1989), HD patients in this study were treated with modified polysulfone membranes which do not significantly influence blood levels of BG (Prattes et al. 2015; Prattes et al. 2017). BG levels were inversely related to residual kidney function in dialysis patients although there was no relationship to either Kt/V or use of haemodiafiltration. Higher BG levels were also observed after initiation of HD, however we think it is unlikely that loss of residual kidney function or the dialysis membrane itself contributed to high BG levels.

A potential source of BG could be the gut, since translocation of BG from the gut has been described and high blood level of BG has been proposed as a marker of intestinal permeability (Yang et al. 2017; Leelahavanichkul, Worasilchai, et al. 2016). In addition there is a large body of evidence which suggests advanced kidney disease is associated with impaired intestinal barrier function, altered gut microbiome and translocation of gut bacterial products into the circulation (March et al. 2017).
Other factors could be related to the clearance mechanisms of BG. The clearance and metabolism pathways of BG in humans are not well understood, but in animal models BG is primarily removed by the Kupffer cells of the liver. Kupffer cells comprise the major phagocytic activity of the mononuclear phagocyte system (MPS) (Dixon et al. 2013). Following intraperitoneal injection, the majority of BGs are distributed in organs prominent in the MPS such the liver and spleen (Yoshida et al. 1997; Suda et al. 1996) and metabolised by oxidative degradation (Young & Vincent 2005; Suda et al. 1996; Nono et al. 1991). There is little data on Kupffer cell function in CKD, though dysfunction of the MPS has been described (Hörnl 2004). Specific defects of immunity including defective phagocytic function, impaired maturation of monocytes and monocyte-derived dendritic cells have also been reported (Verkade et al. 2006; Lim et al. 2007; Anding et al. 2003; Kato et al. 2008). Elevated blood BG levels detected in our population could therefore relate to a combination of increased intestinal permeability and MPS dysfunction in advanced CKD. In a similar way, the appearance of endotoxin in the systemic circulation in advanced liver disease is thought to result from gut translocation together with MPS dysfunction (Wang et al. 2015).

The immunobiology of BG in humans is complex and not yet fully understood. Interpretation of results from previous studies has been difficult due to the different types of BG used for studies varying from crude extracts from fungal cells to purified BG (Camilli et al. 2018). BG can mediate pro-inflammatory effects via its action on complement receptor 3, dectin-1 and toll-like receptors stimulating phagocytosis and generation of reactive oxygen species. On the other hand, consumption of fungi high in BG have been used for thousands of years in ancient civilisations for health benefits (Rop et al. 2009). BG also appears to have anti-tumour effects (Nono et al. 1991), anti-inflammatory effects and improves survival in animal sepsis models (Camilli et al. 2018). Hence the characteristics of immune responses elicited by BG are variable and depend on cell types, receptors, size and structure of BG.

This is the first study to explore BG levels and their clinical significance in non-infected subjects with different stages of kidney disease. BG and levels were highest
in HD patients and especially in those with increased levels of inflammation, higher UFR and peri-dialytic hypotension and hence a higher likelihood of intradialytic intestinal hypo-perfusion. These findings are consistent with a role for increased intestinal permeability in increasing systemic BG levels. These findings also suggest possible contributions of high BG levels to chronic inflammation, clinical symptoms and susceptibility to sepsis in advanced kidney disease but further studies are required to explore these issues. This study has also confirmed previous observations carried out in chapter 8 and those of others (Taniguchi et al. 1990) that high BG levels cause false positive tests for blood endotoxin, and that the prevalence of endotoxemia in the dialysis population is much lower than widely reported.

There are a number of limitations to this study. Patient sub-groups were not well matched. Residual renal function and dialysis vintage differed between HD and PD groups. Kt/V was higher in low-risk groups compared to high-risk HD patients. However, other parameters such as bone chemistry and use of dialysis catheters were similar. The Fungitell assay is a biological assay and also based on the Limulus assay which has its inherent limitations (Cohen 2000), although it has been well validated in many studies in other patient groups as a pan-fungal marker (Theel & Doern 2013), reports of its accuracy in patients with renal failure are more limited. Furthermore it must be stressed that the relationships between BG levels, inflammation and clinical sequelae are associations only and further work is necessary to establish whether there are any causal relationships.

In conclusion, blood BG levels are elevated in CKD patients, especially dialysis patients, perhaps contributed to by translocation from the gut and MPS dysfunction. High levels appear to be associated with inflammation and adverse clinical sequelae. Increased BG levels can also affect current limulus based endotoxin detection assays leading to apparently high blood endotoxin levels. This study suggests that BGs may play an important clinical role in patients with advanced kidney disease and that further study of BG in this setting is warranted.
Chapter 11

11. Intestinal permeability in haemodialysis patients

11.1 Introduction

Chronic systemic inflammation is highly prevalent in patients with advanced kidney disease. It is a strong cardiovascular risk factor and is associated with other complications including malnutrition, cachexia, anaemia and early mortality (Carrero & Stenvinkel 2010). Previous studies have implicated endotoxemia as a potential driver for sustained chronic inflammation although the studies carried out in chapter 8 to chapter 10 do not support this hypothesis, but ‘endotoxin’ detected in blood samples of dialysis patients appear in part, to be due to false positive activation of detection assays by BG. As mentioned in Chapter 10, serum BG level increases progressively with worsening CKD stage. The source of elevated BG in the absence of invasive fungal infection is unclear. One potential source of elevated BG could be the gastrointestinal tract and it the gut is increasingly being recognised to be a major source of chronic inflammation in the dialysis population (March et al. 2017; Vaziri et al. 2016).

Development of renal impairment leads to alteration in the intestinal microbiome due to changes in the biochemical milieu of the alimentary tract which are known to influence and damage gut barrier function (Kelly et al. 2015; Vaziri 2012). The intestinal barrier acts as a semipermeable membrane for the selective absorption of essential dietary nutrients, electrolytes and water from the intestinal lumen whilst preventing translocation of harmful microbial products and pathogens into circulation (Groschwitz & Hogan 2009). The intestinal barrier consists epithelial cells which regulate transcellular transport of solutes and intercellular junctional complexes or ‘tight junctions’ which seal the spaces between epithelial cells regulating the entry of luminal contents through these paracellular routes (Turner 2009). These tight junctions consists of adhesive transcellular proteins (occludin and claudin families) and actin-binding cytosolic proteins (zonula occludens family) (Vaziri et al. 2016).
A large number of in-vitro and observational studies in animal and humans with chronic kidney disease (CKD) strongly suggest that there is breakdown of intestinal barrier function [reviewed in (Vaziri et al. 2016; Lau & Vaziri 2017; March et al. 2017)]. Studies in non-dialysed CKD patients and uraemic rats demonstrate increased absorption of large size polyethylene glycols indicating increased intestinal permeability or 'leakiness'. In-vivo work in animals has demonstrated marked loss of occludin, claudin and zona occludens from the intestinal tracts of CKD subjects (Vaziri, Yuan, et al. 2012). Breakdown in intestinal barrier function has been hypothesised to contribute to systemic inflammation due to the translocation of large quantities of bacterial components across the ‘leaky’ intestinal wall and entry to the blood circulation (Wang, Jiang, et al. 2012; Ritz 2011; Khoury et al. 2017).

Postulated mechanisms of intestinal barrier damage include direct disruption of tight junctions caused by uremic toxins (Vaziri, Goshtasbi, et al. 2012; Vaziri et al. 2013) and in haemodialysis patients, ultrafiltration during treatment may also cause hypotension leading to bowel ischemia (Rossi et al. 2012; Yu et al. 1997; Jakob et al. 2001), exacerbating uraemia-induced intestinal barrier dysfunction (Vaziri et al. 2016). However, intestinal permeability has not been measured in vivo in haemodialysis patients and direct intestinal permeability changes, induced by the acute effect of haemodialysis, have not been studied. If haemodialysis-induced gut permeability were to be demonstrated, interventions could be targeted at the haemodialysis procedure to minimise this effect.

The lack of studies of intestinal permeability in dialysis patients is likely due to the difficulties in applying the conventional method of measuring intestinal permeability in this population. Intestinal permeability in vivo is measured by determining the urinary excretion of orally administered test substances. These test substances typically consist of a disaccharide and a monosaccharide, the ratio of the urinary concentrations of both providing a specific index of intestinal permeability (Bjarnason et al. 1995). Since a significant proportion of dialysis patients are anuric, measurement of intestinal permeability using this method is not possible. A sensitive assessment of sugars based on liquid chromatography in combination with mass spectrometry (LC-MS) has been developed which allows measurement of sugars in
plasma (van Wijck & Lenaerts 2011b; van Wijck & Lenaerts 2011a; van Wijck et al. 2013).

The purpose of this study was to two-fold, 1) to determine if intestinal permeability is increased in haemodialysis patients and 2) to determine the acute effect of haemodialysis on intestinal permeability.

11.2 Methods

Participant characteristics

Ten patients on maintenance haemodialysis patients and five healthy volunteers were recruited for this study. Haemodialysis patients were medically stable with no active illness at the time of the study. Exclusion criteria were positive HIV or hepatitis B/C status, active gastrointestinal symptoms or disease, liver disease and history of previous bowel surgery.

Gut permeability testing

The classical assays for gut permeability are usually based on a difference in intestinal absorption of two supplied sugars, usually a disaccharide and monosaccharide. A ratio of the concentration of disaccharide over the monosaccharide recovered in the urine or plasma following oral administration is used as an index of intestinal permeability. In states of increased intestinal permeability, there is a relative increase in the absorption of the larger disaccharide molecule due to increased paracellular transport leading to a higher disaccharide to monosaccharide ratio. A multi-sugar solution consisting of lactulose, rhamnose, sucralose and erythritol was used in this study for the assessment of intestinal permeability. Lactulose (disaccharide) and rhamnose (monosaccharide) was used as a marker for small intestinal permeability since they are degraded by the microbiota in the colon (Fink 2002; Hietbrink et al. 2007). Sucralose (disaccharide) and erythritol (monosaccharide) was used as a marker for whole gut permeability since they resist colonic bacterial fermentation (Farhadi et al. 2003). Sugar concentrations in plasma were measured at repetitive time points using liquid chromatography-mass spectrometry as described below. The calculated lactulose: rhamnose ratios (L/R)
were used to assess small intestinal permeability and sucralose: erythritol ratios (S/E) for whole gut permeability. Plasma concentrations of sugars were measured over a 10 hour period. The optimum time period to assess sugar ratios derived from plasma have not yet been established, although for sugar ratios derived from urinary fractions, 0-5 hours and 5-24 hours appear to best reflect small and large intestine permeability respectively (Camilleri et al. 2012). Therefore for comparison of small intestinal permeability between haemodialysis patients and healthy controls, L/R ratios were assessed for 5 hours after sugar ingestion, for whole bowel permeability S/E ratios were assessed for 10 hours. However, since sugar profiles in haemodialysis patients have not been studied previously and may be different from healthy volunteers, comparison of L/R and S/E ratios between dialysis and non-dialysis days were studied during the early period (0-5 hours post sugar ingestion), late period (5-10 hours post ingestion) and over the whole 10 hour study period.

**Study design and sampling**

On the day before and during the test days, all participants were asked to avoid intense physical exercise and consumption of any sweets, confectionary, desserts, sugar-free chewing gum and non-steroidal anti-inflammatory drugs. Products containing erythritol or sucralose were avoided. Participants were tested after an overnight fast, a baseline blood sample was collected from either a cannula inserted in the forearm or from an existing tunnelled dialysis catheter (for haemodialysis patients) using standard aseptic technique. A multi-sugar solution consisting of 1g lactulose (TEVA UK Limited, 3.35g/5mL), 1g rhamnose (Danisco Sweeteners), 1g sucralose (Brenntag, Netherlands), and 1g erythritol (Danisco Sweeteners) dissolved in 100ml water was orally administered. Following ingestion, blood samples were collected hourly for 10 hours. At each sampling point, blood was collected into EDTA tubes and centrifuged at 2300g for 15min to obtain plasma. Plasma samples were aliquoted, frozen and stored at -80°C. Subjects were allowed to eat 2 hours after ingestion of sugars although all sweets, products containing sweeteners were avoided throughout the study period. Participants were not restricted in position or mobility although subjects were not allowed to carry out intense exercise during the study period.
For haemodialysis patients, intestinal permeability was measured on a non-dialysis day and on a haemodialysis day. Both study days were carried out within one week of each other. Testing was randomised such that half of the cohort had initial permeability measurements carried out on a non-dialysis day followed by repeat testing carried out on a dialysis day. The remainder of the cohort were tested in reverse order. For intestinal permeability measurements performed on a haemodialysis day, the study commenced immediately at the end of the dialysis session. The concentration of sugars post dialysis were corrected for dialysis-induced changes in blood volume by multiplying the concentration of the sugar after dialysis with the ratio of serum albumin before and after dialysis at each time point (Korevaar et al. 2004).

Analysis of sugars

Measurement of sugar probes in plasma was carried out using isocratic ion exchange high performance liquid chromatography (Model PU-1980 pump, Jasco Benelux, Netherlands) and mass spectrometry (Model LTQ-XL, Thermo Electron, Netherlands) (van Wijck & Lenaerts 2011b). 300µL of plasma was transferred into Eppendorf cups containing a 3000 Da cut-off filter (Amicon Ultra 0.5mL 3K, Millipore) to remove plasma proteins. The filter cups were centrifuged for 30 minutes at 11,000g at 4°C to obtain clear plasma filtrate. The plasma filtrate was transferred into 300µL glass insert, spring loaded in a 4mL WISP style vial (Waters, Milford, USA) and placed into a Peltier chilled Gilson 233XL sample processor (Gilson, USA). Chromatographic separation was based on isocratic elution of individual sugars probes on an IOA-1000 9µm cation-exchange column (300mm×7.8mm ID; Illinois), mounted in a Mistral column oven (Separations, Netherlands) at 30°C. An aqueous solution of 20mmol/L formic acid and 10mmol/L trichloroacetic acid was delivered using a Model PU-1580 HPLC pump (Jasco Easton, Maryland) at a flow rate of 0.225mL/min. Samples and standards were injected using a Model 233XL sample processor with Peltier chilled sample storage compartments (10°C), equipped with a 20µL sample loop. After separation, the column effluent was mixed with 30 mmol/L ammonia in 20% methanol/water (v/v) delivered by an additional Model PU 980 pump to allow the formation of ammonium adducts. MS detection was performed
using a model LTQ XL (ThermoFisher Scientific, Massachusetts) equipped with an ion-Max electrospray probe. The mass spectrometer was operated in positive mode. Spray voltage was 4.8kV. Sheath and auxiliary gas were 99 and 30 units respectively with capillary temperature of 220°C. The system was set to a mass range of 125–460Da in full scan enhanced mode.

Statistical analysis

Statistical analysis was performed using Graphpad Prism and SPSS Statistics software. Sugar concentrations were plotted against time for each participant and visually inspected for outliers. Outliers were excluded from the analysis. Area under curve (AUC) was calculated for each sugar, L/R and S/E ratios for all subjects. Derived AUC data for sugars was not normally distributed therefore comparison of AUC between haemodialysis patients and controls were evaluated using Mann-Whitney U test and comparison of sugar profiles between haemodialysis and non-haemodialysis days were evaluated using Wilcoxon signed rank test.

11.3 Results

Patient and healthy control characteristics are displayed in Table 11-1. Haemodialysis patients and controls were similar in terms of age, weight and body mass index. All haemodialysis patients had stable blood pressures before, during and after dialysis, ultrafiltration requirement and rate was moderate. Mean ultrafiltration volume and rate was 1.91L and 6.3mL/kg/hr respectively. 40% of patients were anuric. The median dialysis vintage was 0.85 years. Haemodialysis patients were on several medications as displayed in Table 11-2, no patients were on antibiotics or non-steroidal anti-inflammatory drugs medications at the time of the study. Some patients were on medications that could affect gut motility including opiates (n=3), steroids (n=1). Four patients were on proton pump inhibitors at the time of the study which have been reported to increase upper gastrointestinal tract permeability (MULLIN et al. 2008).
Table 11-1: Subject clinical and demographic data

<table>
<thead>
<tr>
<th>Variable</th>
<th>HD patients (n=10)</th>
<th>Controls (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49 ± 3.7</td>
<td>46.2 ± 4.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>84 ± 7</td>
<td>66 ± 4.2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.72 ± 0.03</td>
<td>1.6 ± 0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>28.1 ± 1.9</td>
<td>25.5 ± 0.98</td>
</tr>
<tr>
<td>Charlson Comorbidity index</td>
<td>3 [IQR 2.5]</td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration volume (L)</td>
<td>1.91 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration rate (mL/kg/hr)</td>
<td>6.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Dialysis session time (min)</td>
<td>230 [IQR 30]</td>
<td></td>
</tr>
<tr>
<td>Pre-dialysis BP (mmHg)</td>
<td>149/76</td>
<td></td>
</tr>
<tr>
<td>Post-dialysis BP (mmHg)</td>
<td>130/78</td>
<td></td>
</tr>
<tr>
<td>Kt/V</td>
<td>1.32 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Residual urea clearance (mL/min)</td>
<td>1.1 [IQR 2.8]</td>
<td></td>
</tr>
<tr>
<td>Proportion with no residual kidney function (%)</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Proportion with tunnelled dialysis catheter (%)</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Dialysis vintage (years)</td>
<td>0.85 [IQR 2.6]</td>
<td></td>
</tr>
</tbody>
</table>

BP, blood pressure; BMI, body mass index; HD, haemodialysis
Table 11-2: Medication use by haemodialysis patients

<table>
<thead>
<tr>
<th>Medication</th>
<th>Number of patients (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitamin D analogues</strong></td>
<td></td>
</tr>
<tr>
<td>Alfacalcidol</td>
<td>7</td>
</tr>
<tr>
<td><strong>Calcimimetic</strong></td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>2</td>
</tr>
<tr>
<td><strong>Antihypertensives</strong></td>
<td></td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>6</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>3</td>
</tr>
<tr>
<td>Diuretics</td>
<td>4</td>
</tr>
<tr>
<td>Alpha blockers</td>
<td>3</td>
</tr>
<tr>
<td>Angiotensin receptor blockers</td>
<td>1</td>
</tr>
<tr>
<td>Vasodilators</td>
<td>1</td>
</tr>
<tr>
<td><strong>Opiates</strong> (codeine phosphate)</td>
<td>3</td>
</tr>
<tr>
<td><strong>Proton pump inhibitors</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Hypoglycaemic agents</strong></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>2</td>
</tr>
<tr>
<td><strong>Phosphate binders</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>Antidepressants</strong></td>
<td></td>
</tr>
<tr>
<td>Selective serotonin reuptake inhibitor</td>
<td>3</td>
</tr>
<tr>
<td><strong>Immunosuppressive medications</strong></td>
<td></td>
</tr>
<tr>
<td>Calcineurin inhibitors</td>
<td>1</td>
</tr>
<tr>
<td>Steroids</td>
<td>1</td>
</tr>
<tr>
<td><strong>Statins</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>Antiplatelets</strong></td>
<td></td>
</tr>
<tr>
<td>Aspirin (omitted 24 hrs prior to study)</td>
<td>3</td>
</tr>
<tr>
<td>Ticagrelor</td>
<td>1</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Gabapentin</td>
<td>5</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>3</td>
</tr>
<tr>
<td>Montelukast</td>
<td>1</td>
</tr>
<tr>
<td>Quinine sulphate</td>
<td>5</td>
</tr>
</tbody>
</table>

The effect of haemodialysis on intestinal permeability

AUC of studied sugar probes were compared in haemodialysis patients on a non-dialysis day and immediately after dialysis to determine the effect of the haemodialysis procedure on intestinal permeability. During the early phase (0-5 hrs) AUC for rhamnose was significantly higher after haemodialysis treatment however, the lactulose levels and L/R ratio were not significantly different (Figure 11-1, Table 11-3). There were no significant differences in lactulose, rhamnose or L/R ratios.
between dialysis and non-dialysis days assessed in the late period (6-10 hrs) or over the whole study period (0-10 hrs). Similarly, there were no significant differences in AUC of sucralose, erythritol and S:E ratio between non-dialysis days and after haemodialysis treatment (Figure 11-2, Table 11-2).

Table 11-3: Area under curve and plasma concentrations of sugar probes on non-dialysis days and after haemodialysis treatment

<table>
<thead>
<tr>
<th></th>
<th>Non-HD day (n=10) †</th>
<th>HD day (n=10) †</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early period (0-5 hrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactulose</td>
<td>5.48 [4.72-8.01]</td>
<td>5.48 [5.01-8.84]</td>
<td>0.72</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>273.1 [234.3-393.5]</td>
<td>308.5 [273.3-464.7]</td>
<td>0.01*</td>
</tr>
<tr>
<td>L:R ratio</td>
<td>0.073 [0.063-0.112]</td>
<td>0.079 [0.064-0.086]</td>
<td>0.72</td>
</tr>
<tr>
<td>Sucralose</td>
<td>26.6 [14.5-29.8]</td>
<td>19.9 [17.1-29.9]</td>
<td>0.95</td>
</tr>
<tr>
<td>Erythritol</td>
<td>1411 [1098-1788]</td>
<td>1635 [1420-1905]</td>
<td>0.26</td>
</tr>
<tr>
<td>S:E ratio</td>
<td>0.055 [0.047-0.09]</td>
<td>0.067 [0.044-0.076]</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Late period (6-10 hrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactulose</td>
<td>6 [5.29-10.88]</td>
<td>8.43 [5.91-10.47]</td>
<td>0.51</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>177.4 [127.7-230.7]</td>
<td>217 [156.6-278.3]</td>
<td>0.17</td>
</tr>
<tr>
<td>L:R ratio</td>
<td>0.146 [0.098-0.19]</td>
<td>0.157 [0.132-0.2]</td>
<td>0.96</td>
</tr>
<tr>
<td>Sucralose</td>
<td>16 [7.4-26.2]</td>
<td>15.3 [10.3-22.5]</td>
<td>0.86</td>
</tr>
<tr>
<td>Erythritol</td>
<td>1021 [779-1277]</td>
<td>1333 [1092-1474]</td>
<td>0.05</td>
</tr>
<tr>
<td>S:E ratio</td>
<td>0.067 [0.029-0.081]</td>
<td>0.051 [0.038-0.062]</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Whole study period (0-10 hrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhamnose</td>
<td>541.2 [428.9-702.7]</td>
<td>634.5 [497.7-805.7]</td>
<td>0.09</td>
</tr>
<tr>
<td>L:R ratio</td>
<td>0.242 [0.188-0.338]</td>
<td>0.274 [0.223-0.314]</td>
<td>0.96</td>
</tr>
<tr>
<td>Sucralose</td>
<td>48.5 [24.6-63.0]</td>
<td>42.8 [30.4-56.7]</td>
<td>0.59</td>
</tr>
<tr>
<td>Erythritol</td>
<td>2800 [2076-3514]</td>
<td>3131 [2943-3743]</td>
<td>0.14</td>
</tr>
<tr>
<td>S:E ratio</td>
<td>0.15 [0.087-0.194]</td>
<td>0.136 [0.094-0.153]</td>
<td>0.31</td>
</tr>
</tbody>
</table>

AUC, area under curve; HD, haemodialysis; L:R ratio, lactulose: rhamnose ratio; S:E, sucralose: erythritol ratio; *denotes statistical significance P<0.05; † one haemodialysis patient with high baseline plasma concentrations of sucralose was excluded and not included in analysis with regards to sucralose, erythritol, and S:E ratios. Data shown are median and interquartile range.
Figure 11-1: Plasma concentrations of lactulose, rhamnose and lactulose: rhamnose ratios on a non-dialysis day and after haemodialysis treatment

L:R, lactulose: rhamnose ratio; Non-HD day, non-dialysis day; HD day: intestinal permeability testing after haemodialysis treatment. Data points and error bars represent median and interquartile range respectively.
Figure 11-2: Plasma concentrations of sucralose, erythritol and sucralose: erythritol ratios on a non-dialysis day and after haemodialysis treatment

S:E ratio, sucralose: erythritol ratio; Non-HD day, non-dialysis day; HD day: intestinal permeability testing after haemodialysis treatment. Data points and error bars represent median and interquartile range respectively.
Comparison of intestinal permeability between haemodialysis patients and healthy controls

Small bowel permeability

Small bowel permeability was assessed by comparing the differential absorption of lactulose and rhamnose. Plasma levels of lactulose increased rapidly after ingestion for both haemodialysis patients and controls (Figure 11-3), for healthy controls lactulose levels peaked after 1 hour, for haemodialysis patients lactulose levels continued to rise throughout the study period and were approximately 3 times greater than peak levels reached by healthy controls (2.05 uM vs. 0.67 uM, p=0.002). AUC of lactulose was significantly higher than healthy controls (5.27 vs 2.66, p=0.001). Plasma levels of rhamnose peaked at 1 hour after ingestion for healthy controls, for haemodialysis patients rhamnose concentration reached peak levels later at approximately 4 hours after ingestion, the peak concentration reached was similar for both groups. There was no significant difference in AUC of rhamnose levels between both groups (283 vs. 307.4, p=0.679). AUC for L/R ratios were significantly higher in haemodialysis patients compared to healthy controls (0.071 vs. 0.034, p=0.001) [Figure 11-3, Table 11-4].
Figure 11-3: Plasma concentrations of lactulose, rhamnose and lactulose: rhamnose (L:R) ratio in haemodialysis patients and healthy controls
Table 11-4: Area under curve and median plasma concentrations of sugar probes in haemodialysis patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n=5)</th>
<th>HD patients (n=10)†</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactulose</td>
<td>2.66 [2.16-3.31]</td>
<td>5.27 [4.43-7.74]</td>
<td>0.001*</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>307.4 [267.4-353.8]</td>
<td>283 [242.2-380.4]</td>
<td>0.679</td>
</tr>
<tr>
<td>L:R ratio</td>
<td>0.034 [0.03-0.048]</td>
<td>0.071 [0.058-0.11]</td>
<td>0.001*</td>
</tr>
<tr>
<td>Sucralose</td>
<td>16.5 [15.2-34.2]</td>
<td>48.5 [24.6-63]</td>
<td>0.019*</td>
</tr>
<tr>
<td>Erythritol</td>
<td>1150 [1111-1732]</td>
<td>2800 [2076-3514]</td>
<td>0.001*</td>
</tr>
<tr>
<td>S:E ratio</td>
<td>0.13 [0.12-0.21]</td>
<td>0.15 [0.09-0.19]</td>
<td>0.797</td>
</tr>
</tbody>
</table>

AUC, area under curve; HD, haemodialysis; L:R ratio, lactulose: rhamnose ratio; S:E, sucralose: erythritol ratio; *denotes statistical significance P<0.05; † one haemodialysis patient with high baseline plasma concentrations of sucralose was excluded and not included in analysis with regards to sucralose, erythritol, and S:E ratios.
Whole bowel permeability

One haemodialysis patient had very high baseline levels of sucralose and was excluded from whole bowel permeability analysis using sucralose and erythritol. Sucralose levels increased post ingestion peaking at 2 hours for healthy controls, for haemodialysis patients sucralose levels peaked at 5 hours and the magnitude of peak levels reached were approximately two-fold greater compared to controls (7.09 uM vs. 3.59 uM, p=0.205). Erythritol levels peaked at 1 hour and reached similar level of magnitude for both haemodialysis patients and healthy controls, however for haemodialysis patients, erythritol levels remained persistently elevated with no discernible reduction in levels for the whole study period, probably reflecting the lack of renal clearance. AUC for S/E ratios were not significantly different between haemodialysis patients and healthy controls (Figure 11-4, Table 11-4).
Figure 11-4: Plasma levels of sucralose, erythritol and sucralose: erythritol ratios in haemodialysis patients and healthy controls

S:E, sucralose: erythritol ratio. Data points and error bars represent median and interquartile range respectively.

The pharmacokinetics of these sugar probes are strongly affected by the lack of renal function as these sugars are predominantly removed by the kidneys. Use of ratios to compare intestinal permeability in haemodialysis patients with healthy controls may not be reliable (see Discussion).
Relationship between level of residual kidney function and concentration of sugar probes

Due to the strong effect of renal function on the clearance of sugar probes. The relationship between of residual renal function in haemodialysis patients with profiles of sugar probes was investigated. There was no significant correlation between residual urea clearance (KRU) and AUC for lactulose, rhamnose, sucralse or lactulose. Differences in AUC for all four sugar probes were compared between anuric HD patients and those with residual kidney function (Table 11-5), there were no significant differences in AUC for all sugar probes between HD patients with and without residual renal function.

Table 11-5: AUC for sugar probes in haemodialysis patients with and without residual renal function

<table>
<thead>
<tr>
<th></th>
<th>No residual renal function (anuric) [n=4]</th>
<th>Residual renal function present (KRU 1.1 to 3.2 mL/min) [n=6]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC Lactulose</td>
<td>16.6 [11.7-30.8]</td>
<td>12.6 [10.7-17.4]</td>
<td>0.394</td>
</tr>
<tr>
<td>AUC Rhamnose</td>
<td>566 [470.5-730.2]</td>
<td>541.2 [375.7-767.8]</td>
<td>0.67</td>
</tr>
<tr>
<td>AUC Sucralose</td>
<td>39.4 [26.7-54.1]</td>
<td>62.5 [21.3-98]</td>
<td>0.624</td>
</tr>
<tr>
<td>AUC Erythritol</td>
<td>2451.5 [2061.8-3202]</td>
<td>3482 [2373-3683]</td>
<td>0.327</td>
</tr>
</tbody>
</table>

AUC, area under curve. Data shown are median and interquartile ranges.
11.4 Discussion

This is the first study to evaluate intestinal permeability in haemodialysis patients and the acute effect of haemodialysis on intestinal permeability in vivo. Breakdown of intestinal barrier function and increased permeability is associated with a number of diseases such as inflammatory bowel disease and is increasingly believed to be implicated in systemic inflammation in uraemic states (Visser et al. 2009; March et al. 2017; Lau & Vaziri 2017). Researchers have attempted to assess gut barrier function using several methods, although each method has its own limitations. Assessment of in vivo gut barrier function with the use of conventional histological methods does not permit measurement of changes in intestinal permeability and data collected from biopsies taken from a small area of the bowel may not be representative for the whole gut.

The classical method to measure intestinal permeability in vivo is with the use of orally administered oligosaccharides first described in 1974 by Menzies (Menzies 1974), initially single test substances of large molecular weights (such as lactulose and polyethylene glycol) were used, however the absorption of these substances could be influenced by pre- and post-mucosal factors other than intestinal permeability such as bacterial degradation, absorptive surface area, gastric dilution and gastrointestinal transit time, differences in systemic distribution of sugars and renal clearance. Thus, the test was modified by introducing a second smaller probe since this is thought to traverse the intestinal barrier freely independent of barrier loss but similarly affected by pre- and post-mucosal factors. A ratio of the urinary concentration of both probes provides a more accurate assessment of para-cellular passage across the gut wall than a single probe (Bischoff et al. 2014). By measuring the appearance of oligosaccharide probes in plasma rather than urine, we attempted to determine intestinal permeability in haemodialysis patients.

The haemodialysis procedure itself did not lead to acute changes in intestinal permeability. There were no significant differences in the L/R and S/E ratios between non-dialysis days and post-dialysis implying that the haemodialysis procedure itself does not appear to induce increased gut permeability. This is in contrary to previous
suggestions that haemodialysis may exacerbate increased gut permeability due to observations of reduced intestinal perfusion from ultrafiltration during haemodialysis (Rossi et al. 2012; Yu et al. 1997; Jakob et al. 2001). Thus, intestinal barrier dysfunction in kidney disease may be due to other causes such as gut oedema (Sandek & Rauchhaus 2008; Sandek & Bauditz 2007; Vaziri et al. 2016) and/or retained uraemic toxins. Previous studies have found that incubating human colonocytes in media containing pre-dialysis serum resulted in a marked drop in transepithelial electrical resistance indicating increased permeability. This was accompanied by loss of transcellular and intracellular protein constituents of the tight junction. The extent of epithelial barrier damage and dysfunction was reduced in cells exposed to serum obtained post-dialysis suggesting that intestinal barrier function is impaired by dialysable retained uraemic toxin(s) (Vaziri, Goshtasbi, et al. 2012). Future studies should be directed at the identification and maximising clearance of these permeability inducing toxins.

Several limitations may account for our findings. Firstly, patients selected for this study were relatively young with little co-morbidity and modest ultrafiltration requirements. Increased intestinal permeability induced by haemodialysis may be detected in patients with greater co-morbidity, high ultrafiltration requirements or haemodynamic instability. Secondly, the study sampling period lasted only 10 hours, previous studies have shown that elimination of some of the sugar probes such as sucralose and erythritol can last up to 48 to 72 hours even in subjects with normal kidney function (Roberts et al. 2000; Bornet et al. 1996; Munro et al. 1998). Intestinal transit time is delayed in uraemia thus 10 hours may have been insufficient to study colonic permeability. Additionally since, sugar probes (sucralose, lactulose) peaked late and did not return to baseline by the end of the study period, AUC for 10 hours could have underestimated intestinal absorption.

Compared to healthy controls, plasma lactulose levels were significantly higher in haemodialysis patients although rhamnose levels were not significantly different. Plasma levels of lactulose progressively increased late into the study period and only started to reduce after 8 hours. The peak levels of lactulose in haemodialysis
patients were significantly higher than controls (approximately 3-fold higher). Whereas for rhamnose, peak levels and AUC for haemodialysis patients were similar to healthy controls, although the high levels of rhamnose were sustained for a longer period in haemodialysis patients (figure 1). This effect is likely to be due to reduced clearance from lack of renal function. Since both lactulose and rhamnose are primarily excreted by the kidney (Maxton et al. 1986; Bjarnason et al. 1994), it would be expected that the magnitude of rise of both sugar molecules in haemodialysis patients relative to healthy controls would be similar. However for lactulose, the degree of absorption by haemodialysis patients is significantly higher than healthy controls whilst there was no significant difference in plasma levels of rhamnose leading to a significantly higher L/R ratio. Although these findings suggest increased small bowel permeability in haemodialysis patients it is difficult to ascertain whether this reflects increased absorption of lactulose due to increased intestinal permeability or accumulation of lactulose due to lack of kidney function. Differences in absorption profiles of these two sugars may also contribute. Studies in humans show that rhamnose is rapidly absorbed after ingestion and further absorption does not occur beyond 1.5 hours post-ingestion despite a significant amount of rhamnose remaining in the gut (McCance & Madders 1930). On the other hand, absorption of lactulose occurs more uniformly in the small intestine and can continue for up to 4 hours after ingestion (Sequeira et al. 2014) which may lead to accumulation and progressively rising plasma lactulose levels in patients who lack kidney function.

Sucralose and erythritol resist bacterial degradation in the colon and were used to evaluate whole intestinal permeability. Both sucralose and erythritol are primarily excreted by the kidney, with the clearance rate of erythritol estimated to be approximately half the rate of creatinine (Meddings & Gibbons 1998; Munro et al. 1998), both are also known to undergo a small but currently unquantified amount of extra-renal metabolism (Bornet et al. 1996; Roberts et al. 2000). In accordance with previous studies, sucralose and erythritol in healthy controls reached peak levels at around 1-2 hours after ingestion (Roberts et al. 2000; Munro et al. 1998; Bornet et al. 1996). Erythritol levels in haemodialysis patients peaked at a similar time scale and magnitude as healthy controls whereas in haemodialysis patients, peak sucralose
levels occurred later in the study period (at around 5 hours post ingestion) and were 2-fold greater than healthy controls. Similar to lactulose, the later peaking of sucralose levels is likely due to accumulation secondary to renal impairment. The S/E ratio in the two groups was not significantly different but the interpretation of this is complicated by the influence of kidney function (Wang et al. 2015). The higher sucralose levels in haemodialysis levels may well reflect increased intestinal permeability but may also indicate the effect of other factors, particularly lack of kidney function.

In addition to the effect of kidney function, other factors may contribute to the differences in plasma oligosaccharide levels between haemodialysis patients and controls. Uraemia may induce delays in gastric emptying and reduce gut motility (Strid et al. 2004). In addition, the haemodialysis patients were on a large number of medications (Table 11-2) including drugs that are known to affect gastrointestinal motility such as proton pump inhibitors and opiates. The effect of medications such as statins and phosphate binders that are frequently used by haemodialysis patients on intestinal permeability has not been studied in detail and it is unclear if medication use may have influenced the plasma levels of sugar probes in this study. Hence, although the differences in renal clearance appear to be the major factor complicating the interpretation of the study findings in relation to differences between haemodialysis patients and controls, there may well be other factors which need to be taken into account.

In summary, an accurate and convenient method of measuring intestinal permeability in haemodialysis patients remains elusive. The study findings suggest that small bowel permeability may be increased in haemodialysis patients although due to the dependence of sugar probes on kidney function for clearance, interpretation and comparison of permeability index between individuals with differing degrees of kidney function is difficult. Use of probes that are not removed by the kidney may overcome this problem but none of the currently available intestinal permeability probes are suitable from this perspective. The intestinal permeability measurement technique described in this study may be used to measure gut
permeability changes in response to an intervention or stimuli, although the need for a prolonged sampling period would make it difficult to apply in the clinical setting. However, it is important to recognise that although intestinal permeability assays have been used frequently in gastroenterology research, the mechanisms that determine oligosaccharide intestinal permeability may be different from that used by bacterial products and intestinal permeability determined by these methods may not correlate with clinically significant bacterial intestinal translocation (Bischoff et al. 2014; Quigley 2016). In conclusion, this is the first study to measure intestinal permeability in dialysis patients in vivo, contrary to previous suggestions this study did not detect any significant acute changes in intestinal permeability in relation to the haemodialysis procedure.
Chapter 12

12. General Discussion

12.1 General overview

Patients with significant reduction in kidney function experience a spectrum of metabolic abnormalities including anaemia, disorders of bone mineral metabolism, acidosis and increased vascular calcification and cardiovascular risk. Chronic and persistent low-grade inflammation is a common feature amongst patients with end-stage kidney disease and those with this syndrome have worse prognosis compared to non-inflamed patients and may exhibit other features such as malnutrition and cachexia. Despite the prevalence and increasing recognition of the significance of this problem (Figure 5-1), chronic inflammation in dialysis patients remains a poorly understood phenomenon and targeted anti-inflammatory interventions are needed.

The underlying cause of chronic inflammation may be multifactorial but endotoxin has been proposed to be a central mediating factor (C McIntyre 2011). Potential sources of endotoxin entering the systemic circulation include biofilm formed in dialysis apparatus or within dialysis catheters, periodontal disease or translocation of endotoxin via the gastrointestinal tract. Thus, endotoxin represents a potential therapeutic target given the advent of extracorporeal filters capable of adsorbing endotoxin and trials have reported short term successes using endotoxin-adsorption as adjunctive-therapy for the treatment of sepsis (Cruz et al. 2009).

However, as discussed in Chapter 2, the published studies reporting endotoxemia in dialysis population are difficult to interpret due to the wide varying degrees of endotoxemia reported, with some studies reporting low level or prevalence of endotoxemia to some studies reporting very high levels of endotoxemia which greatly exceed the pyrogenic threshold in humans and do not appear to correlate with the clinical picture (Table 2-1). Additionally, there are significant methodological issues with endotoxin testing in blood samples and interpretation of studies is complicated by the different types of endotoxin assays used. The overarching aim of this thesis was to explore the association of endotoxemia with chronic inflammation in haemodialysis patients. Confirmation of this association would be of clinical
significance since this would justify embarking on clinical trials of endotoxin lowering interventions to treat chronic inflammation in this population.

In order to achieve this aim, the initial step was to determine an optimum assay that could detect blood endotoxin accurately in dialysis patients. Endotoxin testing in biological samples such as blood is complicated and due to the different types of assays available, it was necessary to carry out some validation work to ensure that assay performance was not affected by uraemic solutes present in the blood of dialysis patients. Chapter 6 describes the pre-analytical work carried out to optimise sample conditions (from blood collection to preparation) for endotoxin detection before direct head-to-head comparative studies between several endotoxin detection assays were carried out. Following this initial phase of validation and laboratory work, the kinetic turbidimetric LAL assay was found to be the most accurate and precise assay for blood endotoxin detection in uraemic blood samples.

However, one of the drawbacks of the LAL assay is that it is not specific for endotoxin and can be activated by (1-3)-β-D glucan (BG), cell wall components of fungi and yeast. Serum levels of BG are also used as a diagnostic adjunct in invasive fungal infections. In a study of non-inflamed haemodialysis patients (Chapter 8), 50% of patients had detectable ‘endotoxin’, although on re-testing samples testing positive for endotoxin with a BG-blocking buffer, these signals were extinguished. Importantly, BG was found to be elevated in a significant proportion of HD patients which could falsely activate endotoxin detection assays therefore for subsequent studies BG-blocking buffers were used to measure blood endotoxin in dialysis patients.

The large body of work carried out to test the accuracy of blood endotoxin detection assays described in chapter 6 and 8 have significantly contributed to our understanding of the limitations of blood endotoxin detection methods and highlights the potential pitfalls of interpreting blood endotoxin levels obtained using the LAL assay – the most commonly used blood endotoxin detection assay in the literature. Our ability to understand the prevalence and effects of endotoxemia in patients will be limited until a more accurate blood endotoxin detection assay is developed.
12.2 The association of endotoxemia with inflammation in dialysis patients

The prevalence of endotoxemia in haemodialysis patients in initial studies (Chapter 8) was lower than previously seen in other studies (C McIntyre 2011; Szeto et al. 2008; Szeto et al. 2010; Terawaki et al. 2010), therefore blood endotoxin was measured in two different states experienced by haemodialysis patients that were hypothesised to increase endotoxin-influx – during exercise and during the haemodialysis procedure. Intense exercise has been frequently reported to contribute to increased blood endotoxin in athletes due damage to the gut wall leading to endotoxin translocation (Pals et al. 1997) and it has been proposed that haemodialysis induces mesenteric ischemia leading to intestinal barrier dysfunction (Rossi et al. 2012; March et al. 2017). In studies described in Chapter 9, neither intra-dialytic exercise nor the haemodialysis procedure itself acutely influenced blood endotoxin or levels of inflammation. In addition, after accounting for BG activation, only one sample tested positive for low level endotoxin but BG was elevated in 43% of samples. Thus, after accounting for important pre-analytical factors such as external contamination of samples and false positive activation of the LAL assay for BG, endotoxemia appears to be a rare phenomenon in haemodialysis patients. Several questions remain unanswered: 1) Why is BG elevated in haemodialysis patients? 2) What is the relationship between BG and kidney function? 3) Is elevated BG (in the absence of fungal infection) clinically significant?

12.3 Clinical significance of (1-3)-β-D glucan in kidney disease

The significance of elevated BG in the context of invasive fungal infection has been well studied (Sulahian et al. 2014), although its significance in non-infected individuals is less clear. BG levels were measured in healthy controls and patients with progressively more severe stages of kidney disease. In a study of 135 clinically stable subjects, levels of BG progressively increased with worsening renal failure (Chapter 10) and highest in patients on dialysis. Elevated BG was also significantly correlated with biomarkers of inflammation, physical fatigue, depression and renal-disease related symptoms scores. On follow-up of subjects with elevated BG (>80pg/mL) after adjusting for important confounding factors such as CKD stage, there was a near significant increased risk of developing sepsis in the 18 month
follow up period \( (p=0.09) \). Importantly, BG was closely correlated with ‘endotoxin’ signal obtained from the LAL assay which highlights the importance of using endotoxin detection assays that are resistant to BG interference when testing for blood endotoxin in dialysis patients. It is possible that previous reports of high levels of endotoxin in dialysis patients may in part be due to BG interference since nearly all published studies do not use endotoxin detection assays which account for BG interference.

The cause of elevated BG in the CKD population is unclear although it appears to be associated with clinical harm. BG is minimally excreted by the kidneys and the dialysis procedure is not known to impact on BG levels (Prattes et al. 2015). A potential source of elevated BG could be the gastrointestinal tract. The harmful effects of uraemia on the intestinal barrier has been investigated in both histological studies and animal models (Vaziri 2012; Lau & Vaziri 2017). Increased translocation of BG has been documented in studies of animals with impaired/altered gut flora (Yang et al. 2017; Leelahavanichkul, Panpetch, et al. 2016) and elevated BG has been reported in Crohn’s disease patients with increased intestinal permeability (Guo et al. 2015). However, increased intestinal permeability has never been proven in dialysis patients since conventional assays currently determine intestinal permeability by measuring the quantity of orally ingested sugar probes that are absorbed into the blood circulation from the gastrointestinal tract and excreted into the urine. This technique would therefore not be possible in many anuric dialysis patients. Utilising a novel technique that measures sugar probes in plasma rather than urine, intestinal permeability was measured in haemodialysis patients and compared with healthy controls (Chapter 11), although there was a suggestion of increased small bowel permeability in the haemodialysis group, valid comparison with healthy controls was difficult due to the strong effect of renal function on the pharmacokinetics of ingested sugar probes.

### 12.4 General limitations

The studies reported in this thesis have certain general limitations. Firstly, to determine an optimum blood endotoxin detection assay, extensive pre-analytical and comparative work was carried out using variations of the LAL assay. The studies
described in this thesis were carried out using a specific type of the LAL assay only. A number of other novel types of blood endotoxin detection methods exist such as the Endotoxin Activity Assay (Romaschin et al. 2012) and cell-based biosensor assays (Hacine-Gherbi et al. 2017) were not studied. Use of these assays may have provided a more accurate means of testing for blood endotoxin and yielded different results. Secondly, although BG appears to play a role in contributing to false positive signals and elevated levels may be of clinical significance, BG was measured using the Fungitell assay, which itself is based on the coagulation cascade derived from LAL extracts. Although this assay is extensively validated as a diagnostic adjunct for fungal infection based on cut-off levels, no validation work has been done on the accuracy of this assay in uraemic blood samples. Hence the possibility that uraemic blood contains an unidentified component that is able to activate the LAL assay, causing false positive signals, cannot be excluded.

12.5 Future directions

There are a number of topics that are discussed in this thesis where further research is needed.

12.5.1 Mediators of chronic inflammation in dialysis patients

The evidence derived from the studies in this thesis do not unequivocally support endotoxin as a mediator of chronic inflammation in dialysis patients. The lack of endotoxemia seen in the studies described in this thesis may be due to endotoxemia being intermittently released or due to is rapid inactivation, several authors have suggested using indirect methods of endotoxemia such as the endotoxin neutralising activity of plasma, anti-endotoxin antibody levels and measuring host response molecules that are released by endotoxin-stimulated cells such as CD14 (D. S. C. Raj et al. 2009). Using of indirect assays in dialysis patients would further increase understanding of the significance of endotoxemia in inflammation.

Other microbial products may be a source of inflammation such as BG. Circulating bacterial DNA and products originating from the gastrointestinal tract has been proposed to be a source of inflammation in dialysis patients (Bossola et al. 2009; Wang, Zhang, et al. 2012). Identification of microbial molecules which mediate
inflammation would be useful to further elucidate understanding of the pathogenesis of chronic inflammation.

12.5.2 The significance of elevated BG in CKD patients

The studies in this thesis have identified BG as a potentially harmful molecule which progressively increases with increasing kidney failure. Several questions remain unanswered:

12.5.2.1 Is elevated BG harmful?

The study carried out in Chapter 10 observed a cross-sectional association between elevated BG and markers of inflammation. On follow-up over 18 months there was no significant association with mortality, cardiovascular outcomes although there was a suggestion of increased risk of developing sepsis. However, the number of patients with a significantly elevated BG (>80pg/mL) was relatively small (around 22% of HD patients), which may have explained the non-significant finding. Therefore, it remains unclear whether BG itself is a harmful molecule. The clinical significance of elevated BG was require exploration in a more long term study and larger cohort of patients with elevated BG.

12.5.2.2 What is the source of elevated BG?

The source of elevated BG in CKD patients is unclear and requires further exploration. Elevated BG in kidney disease may represent either increased influx (e.g. from the gastrointestinal tract) or defective clearance. BG is primarily removed by the reticul-oendothelial system (RES), including Kupffer cells. Specific defects of the immunity including defective phagocytic function, impaired maturation of monocytes and monocyte-derived dendritic cells have been reported in patients with ESKD (Verkade et al. 2006; Lim et al. 2007; Anding et al. 2003; Kato et al. 2008). Abnormalities in immune function are thought to contribute to low-grade chronic inflammation (Kato et al. 2008). Elevated BG detected in our population could be a feature of immune dysfunction in advanced CKD and may explain the association with inflammation. Further work should be aimed at exploring the association between BG and immune function in dialysis patients.
12.5.2.3 BG as a marker of intestinal permeability in CKD patients

As discussed earlier, the gastrointestinal tract could be a potential source of BG originating from intestinal fungi or from dietary sources. Current assays of gut barrier function in dialysis patients are not suitable due to the dependence on renal function for clearance of assay probes. Further work should be directed at identifying means of reliably and easily measuring gut barrier function in dialysis patients to allow its association with BG to be investigated. Given the strong suspicion of the uraemic gut being a significant source inflammation in dialysis patients, it is necessary to determine a reliable test of gut barrier function which can be easily applied in the clinical setting so that the efficacy of interventions (e.g. probiotics) can be assessed. The significance of BG as a marker of intestinal permeability could be tested by measuring BG levels in dialysis patients and controls in response to oral BG loading.

12.6 Summary

In summary, the studies in this thesis have demonstrated that chronic inflammation is a highly prevalent and problematic condition in patients with end-stage kidney disease. Developing our understanding of this condition is important so that interventions can be developed to improve quality of life and survival in dialysis patients. Endotoxin may not be the mediating factor of inflammation as previously proposed. Previous reports of high endotoxin in dialysis patients may have been due to assay interference, in particular by BG – which is, itself, associated with increased inflammation, physical fatigue, depression and kidney disease-related symptoms. Further studies are required to explore the long term significance of elevated BG and the reasons for its increase in patients with kidney disease.
References


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a Randomized controlled trial of Adults Treated for Endotoxemia and Septic shock: study protocol for a randomized controlled trial. *Trials*, 15, p.218.


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Tripepi, G., Mallamaci, F. & Zoccali, C., 2005. Inflammation markers, adhesion molecules, and all-


Appendix

12.1 Appendix A

30 January 2014

Dr Jonathan Wong
Research Fellow
Lister Hospital
Coreys Mill Lane
Stevenage
Hertfordshire
SG1 4AB

Dear Dr Wong

Study title: Establishing a validated method of measuring blood endotoxins in haemodialysis patients
REC reference: 14/SC/0067
IRAS project ID: 147684

Thank you for your letter of 29 January 2014, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Stephanie MacPherson, nrescommittee.south-central-berkshire@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.
Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/R&D office prior to the start of the study (see “Conditions of the favourable opinion” below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblowett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host
organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved by the Committee are:

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<tr>
<td>Investigator CV</td>
<td>(Academic Supervisor - Enric Hall)</td>
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Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known
please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

14/SC/0067 Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee’s best wishes for the success of this project.

Yours sincerely

pp Dr John Sheridan
Chair

Email: nrescommittee.southcentral-berkshireb@nhs.net

Enclosures: “After ethical review – guidance for researchers” [SL-AR2]

Copy to: Dr Shan Gowrie-Mohan, shan.gowrie-mohan@nhs.net
15 January 2015

Dr Jonathan P Wong
Renal Research Fellow
East & North Herts NHS Trust
Lister Hospital, Renal Research Unit
Corey Mills Lane
Stevenage, Hertfordshire
SG1 4AB

Dear Dr Wong

<table>
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The Proportionate Review Sub-committee of the NRES Committee East of England - Cambridge South reviewed the above application on 12 January 2015.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Assistant Nicola Kohut, nrescommittee.eastofengland-cambridgesouth@nhs.net.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the
start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at [http://www.rdforum.nhs.uk](http://www.rdforum.nhs.uk).

Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catheblewwett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Approved documents

The documents reviewed and approved were:

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Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.
User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

With the Committee’s best wishes for the success of this project.

15EE/0019 Please quote this number on all correspondence

Yours sincerely

Dr Leslie Gelling
Chair

Email: nrescommittee.eastofengland-cambridgesouths@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Dr Shan Gowrie-Mohan, East and North Hertfordshire NHS Trust
27 March 2015

Dr Jonathan P Wong
Lister Hospital, Renal Research Unit
Corey Mills Lane
Stevenage, Hertfordshire
SG1 4AB

Dear Dr Wong,

Study title: The longitudinal effects of endotoxemia in haemodialysis patients - pilot study
REC reference: 15/LO/0467
IRAS project ID: 177197

Thank you for your letter of 27th March 2015. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 18 March 2015.

Documents received

The documents received were as follows:

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Approved documents

The final list of approved documentation for the study is therefore as follows:

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<td>Summary CV for supervisor (student research) [Supervisor CV]</td>
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Validated questionnaire [Multi-dimension fatigue inventory]
Validated questionnaire [PHQ.9]
Validated questionnaire [POS-S Renal]

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

15/LO/0467 Please quote this number on all correspondence

Yours sincerely

Amber Ecclestone
REC Assistant

E-mail: nroscommittee.london-hampstead@nhs.net

Copy to: Dr. Shan Gowrie-Mohan, East and North Hertfordshire NHS Trust
Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

23 November 2016

Dr Jonathan Wong
Renal Research Fellow
East and North Herts NHS Trust
Coreys Mill Lane
Stevenage
SG2 0AZ

Dear Dr Wong

Study title: MEASURING GUT PERMEABILITY IN HAEMODIALYSIS PATIENTS; A PILOT STUDY

REC reference: 15/EE/0379
Amendment number: SA4
Amendment date: 29 October 2016
HRA project ID: 190958

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/tra-training/

15/EE/0379: Please quote this number on all correspondence

Yours sincerely

pp. O’Neil

Dr Niki Bannister
Chair
E-mail: NRESCommittee.EastofEngland-CambridgeEast@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Dr Phillip Smith
01 March 2016

Dr Jonathan Wong
Renal Research Lab L94
Coreys Mill Lane
Stevenage
SG14AB

Dear Dr Wong

<table>
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The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Discussion

There were no ethical issues raised.

Approved documents

The documents reviewed and approved at the meeting were:

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Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.
R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

15/EE/0267: Please quote this number on all correspondence

Yours sincerely

[Signature]

Professor Barry Hunt
Chair

E-mail: nescommittee.eastofengland-cambsandherts@nhs.net

Enclosures:

List of names and professions of members who took part in the review

Copy to:

Dr Philip Smith, East and North Hertfordshire NHS Trust
Dr Jonathan P Wong, Lister Hospital

253
02 December 2014

Dr Jonathan P Wong
Lister Hospital, Renal Research Unit
Corey Mills Lane
Stevenage, Hertfordshire
SG1 4AB

Dear Dr Wong,

Study title: Beta-glucans and endotoxins in end-stage kidney disease
REC reference: 14/EM/1266
IRAS project ID: 188251

Thank you for your letter of 01 December 2014, responding to the Proportionate Review Sub-Committee’s request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Rebecca Morledge, NRESCommittee.EastMidlands-Northampton@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.
Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at [http://www.rdforum.nhs.uk](http://www.rdforum.nhs.uk).

Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication tree).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blowett (catherineblowett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).
Approved documents

The documents reviewed and approved by the Committee are:

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Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/
With the Committee’s best wishes for the success of this project.

Yours sincerely,

Mr Ken Willis
Chair

Email: NRESCommittee.EastMidlands-Northampton@nhs.net

Enclosures:  “After ethical review – guidance for researchers”

Copy to:    Dr Shan Gowrie-Mohan
### Appendix B – POS-S Renal

Palliative care outcome scale – Symptoms Renal

**POS-S RENAL – PATIENT COMPLETION**

Below is a list of symptoms, which you may or may not have experienced. Please put a tick in the box to show how you feel each of these symptoms has affected you and how you have been feeling over the past week.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not at all</th>
<th>Slightly</th>
<th>Moderately</th>
<th>Severely</th>
<th>Overwhelmingly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Weakness or lack of energy</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Nausea (feeling like you are going to be sick)</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Vomiting (being sick)</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Poor appetite</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Constipation</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Mouth problems</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Drowsiness</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Poor mobility</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Itching</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Difficulty sleeping</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Restless legs or difficulty keeping legs still</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Feeling anxious</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Feeling depressed</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Changes in skin</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
</tbody>
</table>

Any other symptoms:

<table>
<thead>
<tr>
<th>Not at all</th>
<th>Slightly</th>
<th>Moderately</th>
<th>Severely</th>
<th>Overwhelmingly</th>
</tr>
</thead>
<tbody>
<tr>
<td>□0</td>
<td>□1</td>
<td>□2</td>
<td>□3</td>
<td>□4</td>
</tr>
</tbody>
</table>

Which symptom has affected you the most?

Which symptom has improved the most?

POS-S v1_Renal_P_EN_16052011
12.3 Appendix C – Multidimensional fatigue inventory

**MF® MULTIDIMENSIONAL FATIGUE INVENTORY**

© E. Smets, B. Garsen, B. Bonke (2013)

**Instructions:**

By means of the following statements we would like to get an idea of how you have been feeling lately. There is, for example, the statement:

"I feel relaxed"  
If you think that this is entirely true, that indeed you have been feeling relaxed lately, please, place an X in the extreme left box, like this:

Yes that is true □ □ □ □ □ no, that is not true

The more you disagree with the statement, the more you can place an X in the direction of "no, that is not true". Please do not miss out a statement and place only one X in a box for each statement.

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I feel fit.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>2</td>
<td>Physically, I feel only able to do little.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>3</td>
<td>I feel like doing all sort of nice things.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>4</td>
<td>I feel very active.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>5</td>
<td>I feel tired.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>6</td>
<td>I think I do a lot in a day.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>7</td>
<td>When I am doing something I can keep my thoughts on it.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>8</td>
<td>Physically I can take on a lot.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>9</td>
<td>I dread having to do things.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>10</td>
<td>I think I do very little in a day.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>11</td>
<td>I can concentrate well.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>12</td>
<td>I am rested.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>13</td>
<td>It took a lot of effort to concentrate on things.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>14</td>
<td>Physically I feel I am in a bad condition.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>15</td>
<td>I have a lot of pains.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>16</td>
<td>I tire easily.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>17</td>
<td>I get little done.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>18</td>
<td>I don't feel like doing anything.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>19</td>
<td>My thoughts easily wander.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>20</td>
<td>Physically I feel I am in an excellent condition.</td>
<td>Yes, that is true</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>
12.4 Appendix D – Patient health questionnaire 9

**Patient initials**

**Patient ID**

**PHQ-9 questionnaire**

Over the last 2 weeks, how often have you been bothered by any of the following problems?

(Please circle your answer)

<table>
<thead>
<tr>
<th></th>
<th>Not at all</th>
<th>Several days</th>
<th>More than half the days</th>
<th>Nearly every day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Little interest or pleasure in doing things</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Feeling down, depressed or hopeless</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>Trouble falling or staying asleep, or sleeping too much</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>Feeling tired or having little energy</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>Poor appetite or overeating</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td>Feeling bad about yourself – or that you are a failure or have let yourself or your family down</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7.</td>
<td>Trouble concentrating on things, such as reading the newspaper or watching television</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>8.</td>
<td>Moving or speaking so slowly that other people could have noticed? Or the opposite – being so fidgety or restless that you have been moving around a lot more than usual</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>9.</td>
<td>Thoughts that you would be better off dead or of hurting yourself in some way</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

If you checked off any problems, how difficult have these problems made it for you to do your work, take care of things at home, or get along with other people?

- Not at all
- Somewhat difficult
- Very difficult
- Extremely difficult

Date completed

Score (office only)
12.5 Appendix E – Post dialysis recovery time

Recovery time from haemodialysis

After a dialysis session, how long does it take for you to feel back to normal?

(Please tick one box)

- < 1 hour
- 1-4 hours
- 4-8 hours
- 8-12 hours
- >12 hours