Endotoxemia: correlation with gram-negative bacteremia and association with outcome

A thesis submitted for Doctor of Medical Science (by compilation of published papers)

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This thesis is submitted in total fulfilment of the requirements for the degree of Doctor of Medical Science, through the Austin Health Department of Medicine, Faculty of Medicine, Dentistry & Health Sciences University of Melbourne, September 2013.
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DECLARATION

The following declaration, is signed by the student: This is to certify that

i. the thesis comprises only my original work towards the Doctor of Medical Science except where co-authorship is indicated in (2.) Published articles that form this thesis,

ii. due acknowledgement has been made in the text to all other material used

iii. the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature: .................................................................

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1. **Front-matter**

1.1. **Abstract**

**Introduction:** This thesis is a compilation of 17 publications; eight provide background material and nine provide original meta-analysis. The thesis investigates the association of endotoxemia with gram-negative (GN) bacteremia on the one hand, and with outcome (mortality) on the other, in the extensive literature experience. This experience is conflicting at multiple levels. The research questions addressed here relate to the detection of endotoxemia in various clinical settings with respect to,

(i) what is its diagnostic relevance versus GN bacteremia detection?
(ii) what is its prognostic relevance versus outcome (mortality)? and
(iii) whether the disparate study results are reconcilable?

**Methods:** The research occurred in three phases over an 18 year period as more data and newer analytic methods became available. Literature searches were repeatedly conducted for clinical studies that met the following generic inclusion criteria; endotoxemia detection which also had either data for GN bacteremia detection, GN bacteremia type or mortality. These inclusion criteria were modified for specific analyses. I also published a ‘call for data’ (2001) and corresponded with authors to obtain additional data. Various analytic methods were used (summary in Table 4.1); Mantel-Haenszel methods (phase 1 - from 1994), ROC methods (phase 2 - from 2000), and multi-level modelling (phase 3 - from 2009), to derive summary measures and measures of heterogeneity and also to address the specific research questions that arose. The accumulated data and the results were submitted for peer review publication.

**Results:** Over 300 potentially relevant studies had been published between 1970 and 2012. Of those, 107 studies met the generic inclusion criteria and had data that were useable including 15 that were restricted to specific GN bacteremia types and 22 for which clarifications of data were received. In relation to the above questions;

(i) **Diagnostic relevance.** As a test for GN bacteremia, endotoxemia detection has a sensitivity and specificity of less than 75%. Surprisingly the correlation of endotoxemia with GN bacteremia overall is no better in the studies that used a chromogenic (more sensitive) version of the limulus assay versus the original gelation (less sensitive) version. However, the association of endotoxemia with GN bacteremia is variable for different GN bacteremia types and for different study
settings. Indeed, the type of GN bacteremia is at least as important a determinant toward endotoxemia detection as is the sensitivity of the assay method used.

(ii) Prognostic relevance. The mortality risk associated with the detection of endotoxemia or GN bacteremia either alone or together versus the detection of neither is either non-significant or borderline (Odds ratio <2) among the 9 studies undertaken in an ICU setting. For studies undertaken outside of an ICU setting the co-detection of GN bacteremia and endotoxemia is most predictive of increased mortality risk versus the detection of neither but there is substantial heterogeneity associated with this finding. Surprisingly, in the co-presence of E. coli bacteremias, endotoxemia has no prognostic relevance for mortality in any setting.

(iii) Reconciliation. The disparity among the study results, which is quantifiable as heterogeneity, is partly attributable to publication bias but more so to variations in GN bacteremia occurrence, GN co-detection, GN bacteremia species types and underlying mortality risk in the different study populations. These patient and study level factors are previously unrecognized confounders in the interpretation of the clinical significance of endotoxemia detection.

Conclusions: Endotoxemia is not detectable for at least 20% and up to 50% of patients with GN bacteremia. Moreover, the relevance of endotoxemia detection to prognosis is dependent on the co-detection of GN bacteremia, the GN bacteremia type and the underlying mortality risk in the study population. The studies which reached seemingly disparate conclusions can be reconciled on the basis of these confounding relationships. There are implications from this research for the design and interpretation of trials of anti-endotoxemia therapies.

Abbreviations

DOR  Diagnostic odds ratio
LAL  Limulus Amoebocyte Lysate
ROC  Receiver operating characteristic
SROC  Summary Receiver operating characteristic
HSROC  Hierarchical Summary Receiver operating characteristic
CI  Confidence interval
GN  gram-negative
### 1.2. The published articles that form this thesis

#### 1.2.1. Original research publications

(Contribution of Hurley JC to each publication is 100% unless stated otherwise)

<table>
<thead>
<tr>
<th>Code</th>
<th>Citation</th>
</tr>
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</table>
1.2.2. Background publications

<table>
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<tr>
<th>Code</th>
<th>Citation</th>
</tr>
</thead>
</table>
2. Introduction and overview

This thesis analyses the relationship between the detection of endotoxemia versus gram-negative bacteremia and also versus outcome as mortality amongst the results of 107 studies [1-107] derived from the literature (see methods – chapter 4). This chapter reviews the conflicts within the broader literature underlying the research and the research questions are framed in chapter 3. The methodological approach used in this thesis is described and explained in Chapter 4. The eight background publications (listed in 1.2.2) provide additional background relating to endotoxin detection (CMR ’95, EOID ’95), sepsis trials (Drugs ’94, Drug Safety ’95) an alternate hypothesis for sepsis (Lancet ’93), and statistical methods (EOID ’96, AP&LM ’11). This additional background is not duplicated here.

2.1. Endotoxin

Lipopolysaccharide (LPS, endotoxin) is an outer membrane component of GN (gram-negative) bacteria (figure 2.1) [108-110]. Each E. coli bacterial cell has approximately $10^6$ lipopolysaccharide molecules [110]. Endotoxin has potent and broad ranging biological activities in humans (and in other species) which are mediated mostly by the lipid-A residue within the molecule (figure 2.2). For these reasons it has long been identified not only as a potential marker of GN infection [111-112] but also as a mediator and hence a potential target for specific anti-endotoxin therapies [113-115]. Surprisingly however, these potentials have not been realized and the conflicts among both the studies of interest [1-107] and the broader literature are reviewed in this chapter.

There are numerous effects induced by administration of endotoxin to experimental animals and humans [116-118]. Recent review articles list over 20 humoral, cellular, immunological and metabolic effects. The effect of primary interest in this thesis is lethality. There are three difficulties in studying endotoxin in general and in quantifying endotoxemia concentrations in particular [111-112]. Firstly, the biological effects of endotoxin are not a uniform gravimetric property of the molecule [119]. Hence the quantity of endotoxin is usually interpreted as the amount of biological activity in comparison to a reference endotoxin preparation assayed in
The second issue is the unit of measurement for endotoxin concentrations. Endotoxin concentrations are often expressed either in weight (ng/ml or pg/ml) or activity (Endotoxin Units; EU/ml) units in comparison to one of a range of reference endotoxins [120-123]. The conversion has been often quoted as 1 EU = 100 pg/ml [124-126] However, the choice of reference endotoxin and also the assay conditions will impact on this quantification. Standard reference endotoxin preparations are available. However, even these reference endotoxin preparations have been reported to be subject to imprecision due to storage [122] and other factors. Also, assay conditions need to be rigorously standardized and controlled in order to give precise and reproducible quantification. For these reasons and others (see below), in this thesis, the interest is mostly in endotoxemia detection as a qualitative rather than quantitative measure. That is, the presence or absence of detectable levels of endotoxemia with reference to the assay sensitivity level is the measure of primary interest rather than the quantitative level. As a consequence of these assay issues, a quantitative level of endotoxemia is best interpreted on a logarithmic rather than on a linear scale. Within this thesis this interpretation is most relevant in relation to the sensitivity level of

**Fig. 2.1** The location of the lipopolysaccharide (endotoxin) molecule in the cell wall of Gram negative bacteria
endotoxemia detection assays.

The third difficulty is that with so many biological properties of endotoxin, it is problematic as to which is the most relevant biological activity on which to base a detection assay [127]. In studying the activity of chemically synthesized lipid-A analogues, Takada et al [128] proposed a classification of lipid-A bio-activities as determined by dependence on structural specificity into the following three categories:

1. Lethality in chick embryos, Shwartzman reaction, pyrogenicity
2. Lethality in galactosamine pre-treated mice, interferon and TNF inducing activities in prepared mice, polyclonal B-cell activation, limulus activity
3. Murine macrophage stimulation, IL-1 generation (in vitro), complement cascade activation in human serum.

In this thesis, the activity of endotoxin in the Limulus amoebocyte lysate (LAL, limulus) assay (see 2.2.1) is the biological assay of primary interest as it is the most sensitive and the most commonly studied assay within the clinical context. However, when comparing the biological activity of endotoxins from different GN bacteria, the results of various in vitro assays may correlate poorly in predicting the lethality response. These differences between these various assays versus the LAL assay may be a reflection of the physical aggregation state of lipopolysaccharide molecule [129]. The extent of the discrepancy between different assay methods is not trivial [130-135]. The biological potency for 11 (Dehus et al [132]), five (Devleeschouwer et al [133]) and ten (Laude-Sharp et al [130]) different LPS preparations from various sources was investigated with the finding that the activity of LPS preparations from some (e.g. V. cholera, P. aeruginosa, P. putida) versus other (e.g. E. coli, Salmonella abortus equii) sources was overestimated by as much as a factor of 300 in the LAL test versus the relative activities in whole cell cytokine expression assays or rabbit pyrogen assay. A further complicating consideration is that the relative ranking of activities of different preparations and bacterial origins of LPS varies from assay to assay to the point of being inverted [134-139]. The clinical relevance of endotoxemia originating from different GN bacteremia types is a research question to be addressed in this thesis.

2.1.1. Structure activity

The term ‘endotoxin’ was attributed to Richard Pfeiffer who in the 1890’s distinguished the toxic properties contained within the GN bacterial cell versus those
released outside the cell (exotoxins) [140]. Progress in defining the structure-activity relationship in the mediation of the biological activities of endotoxin was difficult and the exact structure of the molecule was elusive for several decades. It was only in the mid 1980’s that the lipid-A moiety of the lipopolysaccharide molecule of *Escherichia coli* was totally chemically synthesized in a form available for structure activity studies [141]. With these structure activity studies, it became possible to attribute the biological activities of endotoxin to the lipid-A component of the lipopolysaccharide (endotoxin) molecule. Recent microbiological studies have identified specific variations in the structure of lipid-A, the mechanisms regulating the synthesis of lipid-A within gram-negative bacteria and the possible relevance of different lipid-A structures to the pathogenesis of GN infection [142-143].

In humans, cautious endotoxin challenge studies have been able to replicate many of the biological effects of endotoxin observed in non-human studies [144]. Interestingly, endotoxin has several pharmacological properties that are unusual for a toxin. It has long been recognized that human plasma accentuates some of the effects of endotoxin [145-147]. Moreover, the toxicity of endotoxin is indirectly mediated and endotoxin itself is not cytotoxic, a phenomenon which is best exemplified in the C3H/HeJ mouse strain [148-149]. This inbred mouse strain is resistant to the effects of

<p>| Table 2.1.1 LD₅₀ Challenge studies in endotoxin responsive (C3H/HeN) versus non-responsive (C3H/HeJ) mice. |
|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>LD₅₀ challenge dose</th>
<th>C3H/HeN</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenge</td>
<td>(endotoxin responsive)</td>
<td>(endotoxin resistant)</td>
</tr>
<tr>
<td>endotoxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• O18 LPS</td>
<td>400 mcg</td>
<td>3750 mcg</td>
</tr>
<tr>
<td>live bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• <em>E. coli</em> O18 K1⁺</td>
<td>10⁴ cfu</td>
<td>10¹ cfu</td>
</tr>
<tr>
<td>◦ (virulent <em>E. coli</em> strain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• <em>E. coli</em> O18 K1⁻</td>
<td>10⁷ cfu</td>
<td>10⁷ cfu</td>
</tr>
<tr>
<td>◦ (non-virulent <em>E. coli</em> strain)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Cross AS, et al., Pre-treatment with recombinant murine tumour necrosis factor and murine interleukin 1 protects mice from lethal infection. J Exp Med 169;2021 (1989) [148]

Foot notes; Cfú = colony forming units
endotoxin and this non-responsiveness is a genetically determined trait (Table 2.1.1). Moreover, the susceptibility to endotoxin can be restored by the transfer of bone marrow cells from histocompatible C3H/HeN strain mice which have normal endotoxin responsiveness. This observation implicated the mediation of the effects of endotoxin by a genetically encoded host receptor. This ultimately enabled the identification of the gene for the endotoxin recognition (lps), and in turn, the LPS receptor itself (see below).

Endotoxin has potent biological activities in humans [144, 150-155]. In humans, biological responses are apparent at doses as small as 4 ng/kg [155] whereas doses of 1 mcg/kg (rabbits, sheep [156-157]) or as high as 2 mg/kg (rats, pigs, non-human primates [158-163]) are required for response in other species (Table 2.1.2). This potent activity has been subjected to extensive preclinical studies in animals (see below) in attempts to better define the role of endotoxin as a mediator in the pathogenesis of GN infections. However, the high sensitivity of humans to the effects of endotoxin is problematic for endotoxin research in that replication in experimental animals requires artificial interventions such as the administration of galactosamine to achieve sensitization to the effects of endotoxin comparable to that seen in humans.

LPS structure. The lipopolysaccharide molecule from all clinically relevant

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose</th>
<th>LPS type</th>
<th>Dosing</th>
<th>partial lethal</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>15000</td>
<td><em>E. coli</em> O26:B6</td>
<td>bolus</td>
<td>yes</td>
<td>158</td>
</tr>
<tr>
<td>Baboon</td>
<td>1500</td>
<td><em>E. coli</em> O26:B6</td>
<td>10 min</td>
<td>yes</td>
<td>161</td>
</tr>
<tr>
<td>Pig</td>
<td>5</td>
<td><em>Salmonella</em> abortus equi</td>
<td>hours</td>
<td>yes</td>
<td>159</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.3-2</td>
<td><em>E. coli</em></td>
<td>bolus</td>
<td>yes</td>
<td>157</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1</td>
<td><em>E. coli</em></td>
<td>30 min</td>
<td>yes</td>
<td>156</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>0.004</td>
<td><em>E. coli</em> (EC-5)</td>
<td>bolus</td>
<td>no</td>
<td>160</td>
</tr>
<tr>
<td>Human</td>
<td>0.004</td>
<td><em>E. coli</em> (EC-5)</td>
<td>bolus</td>
<td>no</td>
<td>155</td>
</tr>
</tbody>
</table>

GN bacteria studied consists of three components; a polysaccharide chain, a core oligosaccharide and a lipid component, lipid-A (figure 2.2) [110, 137, 145-151]. The LPS molecule, and in particularly, the lipid-A component, has been described as an “information rich” molecule, with many possible sites for specific recognition by prokaryotic and eukaryotic proteins. The structure of the polysaccharide chain of the LPS molecule is highly variable even within species of GN bacteria whereas the lipid-A molecule is broadly conserved across GN bacteria of different types (see below) [151-154].

The polysaccharide consists of a repeating saccharide unit in a chain which is hydrophilic and antigenic. In Enterobacteriaceae (e.g. *Escherichia coli*), the polysaccharide is variable in length and in composition between bacterial strains, and confers the bacterial strain’s O-antigen specificity. Less complete LPS structures are present in some genera. For example, the O-specific antigenic chain is not present in mucosal pathogens such as *Neisseria, Bordetella* and *Haemophilus* and LPS in these genera have only a core oligosaccharide. The core oligosaccharide can be divided into an inner core and outer core region and for the genus Chlamydia even the outer core region is not present and these genera have only an inner core [110, 140]. The inner core region is at the innermost end of the polysaccharide chain and includes 3 KDO (2-keto-3-deoxyoctonic acid) residues. The KDO provides the covalent linkage between the polysaccharide chain and the lipid component of the LPS molecule.
Lipid-A contains the key molecular components which determine the endotoxic activity of LPS [150-154]. In addition, the lipid-A and KDO together have a key structural role in the bacterial cell and are required for growth. The lipid-A and KDO synthetic pathway is highly conserved. Hence, this synthetic pathway within the cell would be an attractive target for anti-bacterial therapies. Studies of chemically synthesized partial lipid-A molecules have identified the key structural requirements for optimal biological activity in peptide mediator induction from murine macrophage cell lines [148-154, 164].

The structure of \textit{E. coli} lipid-A consists of a diglucosamine with two phosphates and six acyl (fatty acid) chains (hexaacyl LPS) (\textbf{Figure 2.3}) [165]. Two 3-hydroxymyristate (fatty acid) chains are attached directly to each of the two glucosamines with two secondary (“piggyback”) chains also attached. The fatty acids may be laurate (C12), myristate (C14) with sometimes palmitate (C16) found as the secondary acyl chain, and the primary (glucosamine-linked) acyl chains typically having 12 carbons. This hexaacyl LPS structure is optimal for recognition by the MD-2-TLR4 receptor. LPSs from different gram-negative bacteria may have more or fewer acyl chains, longer acyl chains, branched acyl chains, unsaturated acyl chains, only one phosphate, or other modifications. The degree of recognition of the various hexaacyl and non-hexaacyl LPS structures by the MD-2-TLR4 receptor determines the mediation of endotoxic biological activity and examples are indicated in \textbf{Figure 2.4} [165, 166].
LPS supra-molecular structure activity The structure of the lipid-A monomer also determines its molecular shape [167-169]. These shapes are variably conical or cylindrical depending on the ratios between the hydrophobic versus hydrophilic regions. The most endotoxically active lipid-A monomer structure is conical whereas less active lipid-A is cylindrical. The activity of endotoxin can be increased by sonication [170].

At concentrations above a critical micelle concentration, the lipid-A monomers aggregate into supra-molecular structures determined by the molecular shape of the lipid-A monomers leading variously to the formation of lamellar, cubic or hexagonal shapes (Figure 2.5). The biological significance of these supra-molecular structures is uncertain. These supra-molecular structures are sometimes large enough to be viewed

![Diagram of LPS structures](image)
under electron microscopy [169].

**Lipopolysaccharide-binding proteins** The serum protein lipopolysaccharide-binding protein (LBP) binds to the lipid A component of bacterial endotoxin and facilitates its delivery to the CD14 antigen on the macrophage, where pro-inflammatory cytokines are released and a cascade of host mediators is initiated. The neutrophil granular protein bactericidal/permeability-increasing protein (BPI) competes with LBP for endotoxin binding and functions as a molecular antagonist of LBP-endotoxin interactions. The relative concentrations of these two proteins expressed as a ratio correlates with the quantity of neutrophils at sites of inflammation in various body cavities [182]. The quantitative level of LBP in the serum of patients with severe sepsis may have prognostic relevance [65].

The specific nature of this binding between these serum proteins and LPS has prompted structure activity studies to synthesize peptides to enable anti-endotoxin based treatments of sepsis. Preclinical studies with these synthetic peptides demonstrate that they can combine excellent selectivity for LPS binding together with suppression of LPS-induced cytokine release in vitro and protection from lethal LPS induced septic shock in vivo [723-725]. Interestingly, the molecular interaction between these peptides and LPS which results in this neutralization of biological activity is evident as a biophysical change in the LPS supra-molecular structure. With peptide binding, the lipid-A part of LPS is converted from its “endotoxic” conformation, being the cubic aggregate structure, to an inactive multi-lamellar structure. Peptides that bind to lipid-A have direct anti-bacterial activity as a consequence of the key structural role of lipid-A
within the bacterial membrane structure. This binding affinity is reflected in the similarity between the mean inhibitory concentration (MIC) and the concentration required to achieve anti-LPS activity with these peptides [723, 725].

<table>
<thead>
<tr>
<th>Table 2.1.3 Partial classification of lipid-A structures.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexaacyl lipid A structures</td>
</tr>
<tr>
<td><strong>Mucosal habitat</strong></td>
</tr>
<tr>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
</tr>
<tr>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td>Providencia rettgeri</td>
</tr>
<tr>
<td>Shigella sonnei, S. flexneri</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae, N. meningitidis</td>
</tr>
<tr>
<td>Bordetella bronchiseptica</td>
</tr>
<tr>
<td>Vibrio cholerae O1</td>
</tr>
<tr>
<td>Campylobacter sp.</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Soil, water habitat</strong></td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
</tr>
<tr>
<td>Burkholderia cenocepacia (some strains)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Strict anaerobes</strong></td>
</tr>
<tr>
<td>(None)</td>
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<td></td>
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</table>

Adapted from Munford RS. Sensing Gram-Negative Bacterial Lipopolysaccharides: a Human Disease Determinant? Infect. Immun. 2008 76 (2) 454-465 [172]

**LPS molecular structure activity** Whereas remarkably diverse lipid-A structures are found in the LPSs produced by different gram-negative bacteria, the requirements for maximal activation of animal cells are rather restricted (Table 2.1.3) [171-175]. The LPS structure that is optimally sensed by the MD-2-TLR4 receptor is a bis-phosphorylated di-glucosamine to which are attached six saturated fatty acyl chains.
with lengths of 12 or 14 (occasionally 16) carbons (Fig. 2.4). LPS molecules that have this structure are commonly referred to as “hexaacyl LPSs” in contra-distinction to other lipid-A structures which have four, five or seven (i.e. non-“hexaacyl LPSs”) fatty acyl chains.

Hexaacyl lipid-A structures are common among Enterobacteriaceae such as \textit{E. coli} whereas non-Enterobacteriaceae, such as \textit{Pseudomonas aeruginosa} have a non-hexaacyl lipid-A structure. The structural differences in lipid-A among clinical GN bacterial isolates is a recent finding. The possible clinical relevance of these differences remains to be determined and is one of the research questions of this thesis.

**Endotoxin antagonists** The structural specificity for LPS has resulted in structure activity studies leading to the development of endotoxin antagonists which have undergone pre-clinical evaluation [176]. These antagonists demonstrate ability to block the endotoxin activities in vitro and in vivo. Several have been found to have efficacy in preliminary but not in confirmatory clinical trials undertaken in patients with sepsis (see 2.5.2).

**The LPS receptor** The mechanisms for lipid-A binding and recognition are part of the innate immune system and are genetically encoded [177-179]. These mechanisms have been found in almost all studied vertebrate genomes including humans. Inbred C3H/HeJ mice are genetically deficient in the LPS receptor mechanism and as a result are resistant to the effects of endotoxin. Surprisingly, C3H/HeJ mice are more susceptible to GN infection across a mucosal barrier such as the urinary tract (Table 2.1.1).

Lipid-A recognition requires both LPS binding (MD-2) and signal transduction (Toll-like receptor 4; TLR4) mechanisms. The TLR4 is one of ten TLRs known to exist in humans. Activation of the LPS receptor leads to a range of intra-cellular activities mediated by protein kinases and activation of nuclear factor kB leading to increased TNF production [177-181]. Of note, this LPS binding and signal transduction exhibits specificity for the different lipid-A structures from various sources of GN bacterial endotoxin (Figure 2.6). The basis for the ligand specificity between hexaacyl lipid-A and the TLR4-MD-2 complex has been determined by examination of its crystal structure [175].

Several examples have emerged to illustrate the possible clinical relevance of
this variation in lipid-A structure toward the pathogenesis of GN infections. One example is the change in lipid-A structure occurring with *Yersinia pestis* (plague) infection. In association with transition from the vector (the flea; body temperature 20-27º C) to the human blood stream (body temperature 37º C) there is a change in lipid-A structure from six fatty acid (acyl) chains to four. It is believed that as a consequence of this molecular change this enables the *Yersinia* organism to evade detection by the host innate immune system [173, 182]. A second example is the variable lipid-A structure found in isolates of *Pseudomonas aeruginosa* from early versus late airway infections in cystic fibrosis (CF) patients and also versus non-CF isolates of *Pseudomonas aeruginosa* [183]. The exact relevance of these structural changes in *Pseudomonas* lipid-A toward the chronicity of the airway infection in CF is still being elucidated.

**Tolerance** A biological property of endotoxin that is most unusual for a toxin is tolerance [184-207]. Doses of endotoxin in experimental animals that would otherwise be lethal can be tolerated after pre-treatments by sub-lethal doses [186]. In the 1920’s,
bacterial preparations containing endotoxin were in therapeutic use to induce a fever as a therapy for cancers and syphilis in humans [186]. Humans, as with other species, will acquire tolerance on repeated dosing and require higher (as much as 200-fold) doses to achieve a fever response equivalent to that achieved with the initial dose. There was a series of fascinating experiments undertaken initially in rabbits [188] and later in humans [189-198] to investigate the relevance of tolerance toward GN infections.

In studies undertaken in the 1960’s using prisoner volunteers who were rendered tolerant to endotoxin by repeated doses of endotoxin were subsequently deliberately infected with Salmonella typhi (i.e. typhoid) or Pasteurella tularensis (i.e. tularemia) it was demonstrated that tolerance to endotoxin could be induced by administration of Salmonella endotoxin even during the course of an experimental typhoid illness. Moreover, “Despite unequivocal activation of the endotoxin tolerance mechanisms……, the febrile and toxic course of typhoid fever proceeded unabated.” [192].

This phenomenon has been extensively studied in both animals and in human volunteers with more than 200 studies cited in four key review articles [185-187, 199]. The literature is confusing given that the key studies have been undertaken over a period exceeding 50 years during which time the experimental techniques and the state of endotoxin knowledge have advanced. There are several features of endotoxin tolerance which are relevant to the research conducted in this thesis as possible explanations for disparities between endotoxemia detection and the clinical relevance of endotoxemia:

(i) Tolerance to endotoxin has been demonstrated to be acquired in humans with sepsis in a variety of settings including pyelonephritis [200], brucellosis [201], melioidosis [202], tularemia, chronic Salmonella infection [203] and typhoid [204]. While its relevance to the outcome of sepsis remains unclear [205-207], there are some features in the cytokine profile of patients with sepsis that resemble the profiles observed following the induction of endotoxin tolerance [206-207].

(ii) The development of tolerance in humans and experimental animals is bi-phasic in that there is an early phase lasting hours and a late phase lasting for upwards of several weeks [196]. The early phase in human volunteers is most apparent with the continuous intravenous infusion of endotoxin [195]. This phase confers tolerance to
endotoxins generically as a class but not to other pyrogens [197]. Enhanced clearance of endotoxin can be demonstrated in this phase although specific endotoxin antibodies are not involved [187, 194]. This enhanced clearance results in a ~50% reduction in the half-life of radio-labelled endotoxin in human volunteers [193]. For early tolerance, the degree of tolerance is proportional to the intensity of the febrile and subjective symptomatic response to the initial infusion.

(iii) By contrast, late tolerance is largely specific for the homologous endotoxin used initially to generate tolerance and develops after at least 72 hours. Late tolerance is believed to be mediated by antibodies to the LPS molecule [197].

(iv) Confusingly, in addition to tolerance, it is possible to demonstrate hypersensitivity to endotoxin which is manifest either in the systemic or in the local reactivity. Systemic hypersensitivity can be manifest as the Danysz reaction which occurs if an infusion of endotoxin to a human volunteer is either interrupted or administered as a divided dose (e.g. as a daily intravenous administration) [185]. This systemic hypersensitivity is manifest as enhanced subjective symptomatic and febrile responses in the period before the onset of tolerance. Hypersensitivity can also be manifest to endotoxin administered intra-dermally (e.g. forearm). This hypersensitivity response is the Shwartzmann response [185].

(v) Indeed in human volunteer studies, the onset of typhoid fever is associated with systemic hyper-reactivity to both homologous (i.e. *S typhosa* endotoxin) and heterologous (e.g. *Pseudomonas* endotoxin) endotoxins.

(vi) Local hypersensitivity to endotoxin may be demonstrated as a dermal reaction to intra-dermal injections of endotoxin. In human volunteer studies, the onset of typhoid fever is associated with hyper-reactivity to homologous (i.e. *S typhosa* endotoxin) but not to heterologous (e.g. *Pseudomonas* endotoxin) endotoxins, in contrast to the systemic hyper-reactivity [192, 196].

(vii) Surprisingly and confusingly, in the human volunteer studies, dermal hypersensitivity could be demonstrated concurrently with systemic tolerance.

Hence with these findings in the human volunteer studies of typhoid it was concluded that given this “...ability of man to acquire tolerance to endotoxin during the typhoidal and tularemic illnesses.... circulating endotoxin could not constitute the major cause of the sustained pyrexia and toxaemia during these illnesses” [192].
These investigators speculated on two possible mechanisms by which endotoxemia could relate to the manifestation of typhoid fever [208-209]. Firstly, release of episodic endotoxemia in ‘spikes’ could overcome the tolerance. Alternately, the mechanisms underlying the systemic tolerance and the local tissue hyper-reactivity are dissociated as noted in the prisoner volunteer typhoid infection studies.

These observations on tolerance were considered to be so remarkable for a bacterial toxin that “…it is difficult to believe that it has no clinical significance” [192]. Recent studies in the past decade have attempted to define the mediation of tolerance at the molecular level in an effort to further clarify the clinical relevance of this phenomenon [207].

### 2.2. Assays for endotoxin

Among the various assays available for the detection of endotoxin [210-221] described above, the Limulus amoebocyte lysate (LAL, limulus) assay (described below) has the most published clinical experience having been available for nearly 40 years [210-213]. Moreover, LAL preparations are widely used to screen for endotoxin contamination in pharmaceutical product before commercial release [213] and a convenient LAL assay is commercially available.

There are other endotoxin assays although these have less published clinical experience. The rabbit pyrogen assay is cumbersome as it requires live rabbits. There is some published clinical experience with the rabbit pyrogen assay that pre-dates the availability of the LAL assay [96-99, 103]. Several newer in vitro assays have been developed in the past 20 years [214-219] and published clinical experience is emerging with these assays. One is a whole blood agglutination assay which senses endotoxin through a dimerization reagent (Simply-RED) [216, 217]. A second is the neutrophil chemo-luminescence assay (NCL) also known as the Endotoxin Activity Assay (EAA) [214, 215] which is a whole blood assay based on neutrophil activation by immune complexes formed between a monoclonal anti-endotoxin antibody and lipopolysaccharide. These two more recently developed assays have been studied in a limited number of settings with, to date, six publications available [100, 101, 104-107]. This limited experience with these non-Limulus assays is explored in the analysis in this thesis as a comparison against the LAL assay (see chapter 5).
Another assay for endotoxin (also not further considered in this thesis) is an assay based on recombinant factor C (rFC) produced by genetic engineering methods using the genetic code for the Limulus factor C. This assay reagent will help to meet the growing need for pyrogen testing in association with increasing drug production by biotechnology methods [218-219].

2.2.1. Limulus amoebocyte lysate (LAL, limulus) assay

The Limulus amoebocyte lysate (LAL, limulus) assay is derived from the blood cell (amoebocyte) of the Limulus horseshoe crab (Figure 2.7) and is the most sensitive and specific test available for the detection of endotoxin. The original studies by Dr Fred Bang (a marine biologist) in the 1960s who found that the coagulation mechanism of the horseshoe crab (a sea water crustacean) was triggered by marine living gram-negative bacteria (such as Vibrio species) [211]. Of note, this triggering was specific in that extracts of gram-positive bacteria did not trigger the coagulation mechanism [210, 221]. The similarities between the Shwartzman reaction (a response of the mammalian coagulation cascade triggered by endotoxin) and the limulus response to endotoxin findings were recognized by Dr Jack Levin (a hematologist). Collaboration between Bang and Levin led to the first application of the limulus assay to test for endotoxin in
human samples in the early 1970s [53, 54, 212, 221].

There are many similarities between the coagulation mechanism of the horseshoe crab, from which the limulus assay is derived, and mammalian coagulation cascade. Both consist of enzymatic cascades. With respect to the LAL assay, this cascade serves as amplification mechanism which, as a consequence, explains the responsiveness of the assays to concentrations of endotoxin below ng/ml levels [222]. As a consequence of this extreme sensitivity, the assay needs to be conducted under exacting pyrogen free conditions to avoid contamination with endotoxin from external sources [223]. Even the type of plastic containers used for specimen collection is critical [220, 224].

The assay can be adapted to generate a quantitative result, and it is often calibrated against a reference endotoxin, usually derived from *E. coli* (e.g. EC5). However, it needs to be noted that the assay is a bio-assay in that it quantifies biological activity. Moreover, this activity is reported in units of weight relative to a reference endotoxin. The difficulties with the interpretation of a quantitative endotoxin assay and the comparability of quantitative results between different studies is problematic (as noted above). The limulus assay as generally available is also reactive to fungal glucans [225]. A more recent modification of the limulus assay which is claimed to specifically detect fungal glucan in clinical samples associated invasive fungal infections has been developed [226-228]. However, cross reactivity with endotoxin associated with gram-negative bacteremias has been observed in the few clinical evaluations of this modified limulus assay for fungal glucan. This is problematic as some patient groups, such as the neutropenic, are often concurrently at risk of both GN and fungal systemic infections [229-230]. However a comparison of five commercially available LAL assays in common use that were not specifically modified to be fungal glucan specific, demonstrated that the LAL assay is 1000 times more sensitive to endotoxin than to fungal glucans [225]. These later types of LAL assay are of the type used within the studies to be analysed within this thesis.

**Summations by Elin** In 1979 [231] and 1985 [232], Elin summarized the published clinical experience with samples other than blood (Table 2.2.1 and figures 2.8/9) together with blood samples (Table 2.2.2 and figure 2.10). These tables record the literature as surveyed and abstracted by Elin.
Clinical samples other than blood The literature relating to the application of the LAL assay to body fluids other than blood was surveyed and abstracted by Elin (Table 2.2.1). These published studies report the application of this assay to samples of urine, CSF and joint fluid to enable the rapid detection of GN urinary tract infections, GN meningitis and gonococcal arthritis, respectively. Samples of urine, joint fluid and CSF do not require pre-treatment as is the case for plasma for the control of the assay inhibitors that are found in plasma.

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Elin summary 99 1

Synovial fluid (see Fig 2.9)

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Elin summary 82 23

Urine (see Fig 2.9)

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Elin summary 79 10

Foot notes:
- All data except DOR as abstracted in Elin ’79 &’85 [231-232]
- DOR = diagnostic odds ratio; calculated here by adding 1 to cells containing zero to avoid undefined results
Fig. 2.8 HSROC summary of clinical utility of LAL applied to CSF samples using data as abstracted in Elin ’79 & ’85 (from Table 2.2.1) [231, 232]. Note: X axis is intentionally backwards (as specificity = 1 - false positive rate)

Fig. 2.9 HSROC summary of clinical utility of LAL applied to urine and joint fluid samples using data as abstracted in Elin ’79 & ’85 (from Table 2.2.1) [231, 232]. Note: X axis is intentionally backwards (as specificity = 1 - false positive rate)
Blood samples  Application of the assay to plasma samples to be optimally prepared to remove inhibitors of the assay. The assay and sample preparation demands strict pyrogen free and temperature controlled conditions [247-250]. Three common methods for the preparation of plasma are the method of dilution and heating, treatment with perchloric acid, or extraction with chloroform. The use of chromogenic substrates [251] cleaved by the limulus enzymes and the adaptation of the assay to microtitre plates in the mid 1980s further simplified the assay. The impact of the introduction of this more sensitive version of the limulus assay on the results of the clinical studies of sepsis is unknown. This is one of the research questions of this thesis.

Table 2.2.2 LAL clinical utility for blood.
(Data as abstracted in Elin ’79 &’85) [231, 232]

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Elin summary  79.5  14.5

Foot notes:
- All data except DOR as abstracted in Elin ’79 &’85 [231, 232] and is on a per sample not per patient basis
- DOR; = diagnostic odds ratio, calculated here by adding 1 to cells containing zero to avoid undefined results
Elin was experienced in the clinical applications of the assay [127] and an earlier clinical study by Elin and co-workers published in 1975 reported that the limulus assay applied to blood samples was not clinically useful [25]. The summary data as abstracted by Elin is re-analysed here to illustrate the contrast between the utility for blood versus for non-blood samples. Note that the studies selected by Elin [231, 232] were limited to those available pre-1985 versus the number available for analysis in this thesis (see Chapter 5). Note also that Elin used a simple summation methodology which differs to the methods used in this thesis (described in chapter 4). Elin had used simple column totals of the numbers of true positive, false positive, false negative and true negative in deriving simplistic summary measures of sensitivity and specificity. The re-analysis undertaken here of the data as abstracted by Elin helps to contrast research methodology available to Elin [231, 232] versus the methodologies used in this thesis which have become available since 1985.

Fig. 2.10 HSROC summary of clinical utility of LAL applied to blood samples using data as abstracted in Elin ’79 &’85 (from Table 2.2.2) [231, 232]. Note:X axis is intentionally backwards (as specificity = 1 - false positive rate)
The following points relevant to the clinical experience of the limulus assay for blood versus for non-blood samples emerge in the contrast between the analyses of the data as abstracted and summarized by Elin versus the analysis of the same data using HSROC summary methodology. The SROC plot (methodology described in chapter 4 & APLM2011) is a concise way of contrasting the results of multiple studies of a diagnostic test. This methodology was not available to Elin in 1985.

(i) As noted both by Elin [231, 232] and also within the HSROC analysis (Table 2.2.1/2), for each category of fluid (CSF, urine and joint fluid), the summary sensitivity and specificity for the limulus assay applied to non-blood samples are higher than for the limulus assay applied to blood samples (see chapter 5).

(ii) Elin concluded that for fluids other than blood, the LAL assay performs as well as the Gram stain in terms of speed, sensitivity and specificity.

(iii) It should be noted that the summation method used by Elin [231, 232] gives

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Foot notes;
- Elin summary result is from Elin [231, 232]. Sensitivity, specificity summary estimates have been derived using column totals
- HSROC summary point (see figure) Sensitivity, specificity summary estimates have been derived using data as abstracted in Elin ’79 & ’85 using method as described in chap 4
disproportionate weighting to the largest studies and provides no measure of variability (to be further discussed in 2.6 & 4.2)

(iv) There are three observations in the HSROC summaries (Table 2.2.3) that cannot be appreciated in the Elin data summary. The first is the magnitude of the difference between blood versus non-blood samples.

(v) Second is the trade-off between sensitivity and specificity that is apparent across studies that used different breakpoints to define a positive assay result.

(vi) Also not noted in the Elin data summary [231, 232] is that within each category of fluid (blood, CSF, urine and joint fluid), at least one study gave results that were atypical versus other studies in that category. Moreover, these atypical studies were not necessarily the smaller studies within each category (Figures 2.8/9/10).

Hence, the published experience with the limulus assay, whether with respect to applications to blood (see chapter 5) or to non-blood samples (not further considered in this thesis) is not uniform amongst different teams of investigators. This important observation cannot be made using the data aggregation method as used by Elin [231, 232] and requires an HSROC method of summary analysis. Moreover, the HSROC analysis method raises a new avenue of enquiry toward possible explanations underlying the different findings of different investigators as a post-hoc analysis.

The analysis of the clinical experience of the LAL assay to blood samples in relation to the detection of sepsis is the subject of this thesis and over fifty clinical studies [1-107] are analysed in detail (Chapter 5). As noted by Elin, the inconsistent detection of endotoxemia amongst patients subsequently found to have GN bacteremia (low sensitivity) was a particular source of confusion for these investigators. The conflict is starkly apparent in a cursory reading of the titles of three of the earliest publications in the series of studies applying the LAL test to the detection of endotoxemia;

- “Gram-negative sepsis: detection of endotoxemia with the limulus test.” [54]
- “Lack of clinical usefulness of the limulus test in the diagnosis of endotoxemia.” [25]
- “Limitations of the usefulness of the Limulus assay for endotoxin.” [75]

There were three difficult to reconcile observations among these early studies of the LAL assay in the detection of endotoxemia;

1. The inconsistent detection of endotoxemia amongst patients subsequently found to have GN bacteremia (false negatives) was interpreted as a lack of sensitivity of the assay and was a major criticism of the assay. It might have been expected that the
patient group with GN bacteremia might be expected to be the group to most reliably test positive for endotoxemia (true positive). However, by this definition, the proportion of true positives among studies reviewed by Elin mostly range between 30 and 80%. Note the wide range in true positives among the different studies with this proportion being as low as 50%.

2. Also problematic was the proportion of those who test positive who turn out not to have GN bacteremia (false positives). The false positives by this definition mostly range between 5 and 60% and were as high as 50% among studies reviewed by Elin [231, 232]. Note that the range in false positives proportions is also wide and also overlaps the range of true positives. To compound this were findings in several studies of positive test results among patients with blood stream infections due to gram-positive bacteremia or fungemia amongst the category of false positive tests.

3. An additional concern was that few studies (with the notable exception of [11, 12, 54]) had found the limulus assay, whether as a quantitative or qualitative result, to be predictive of patient outcome. These discrepancies were particularly difficult to explain given the presumption that endotoxemia would be expected to be not only a marker but also a mediator of the systemic effects of GN bacteremia.

Given these criticisms, several investigators had concluded that the limulus assay was neither sensitive nor specific in relation to testing blood samples. There was an (unstated) expectation that a more sensitive assay for endotoxemia was needed. Subsequent to 1985, a more sensitive assay for endotoxin did emerge. This was the LAL assay modified by a chromogenic reaction [251, 252].

The possible reasons for the range experiences across the literature are one of the research questions of this thesis. The disparity between the three initial studies [25, 54, 75] is of particular interest given that these were all large and seemingly well-conducted studies and their disparity has remained unexplained to this day. However, two major obstacles to a resolution of these disagreements within the general literature experience are firstly, the lack of an objective gold standard or an assay for endotoxemia with equivalent sensitivity to that of the LAL assay and, secondly, an inability to standardize the patient group of interest. Possible approaches to resolving these obstacles are considered in the next two sections.
2.3. Sepsis: toward diagnostic and prognostic tests

Sepsis is a clinical syndrome for which defining objective diagnostic and prognostic tests are lacking and continue to evolve [253-256]. Forty years ago Lewis Thomas said that “It is our response that makes the disease” [257], a comment which succinctly describes sepsis as being the body’s systemic response to suspected or proven infection rather than the infection per se. The clinical similarity of sepsis as a syndrome, whatever the causative organism, has been generally noted [258-259]. The difficulties with current defining criteria and testing for sepsis is best illustrated by a contrast with the field of cardiovascular medicine where in the last ten years, the availability of the serum cardiac troponin test, a rapidly available serum test of cardiac injury, has transformed the management of patients presenting with acute coronary syndromes [260].

The two broad approaches toward the development of diagnostic and prognostic tests for sepsis; these are tests based on clinical criteria and tests based on laboratory detection methods [256]. There are two statistical criteria that are commonly applied in the description and evaluation of diagnostic and risk prediction tools; discrimination and calibration [261-264];-

- **Discrimination.** This is a measure of how well the model separates those patients who do versus those who do not have the disease or outcome of interest. Discrimination can be evaluated using an ROC curve, being a plot of true positive proportions versus false positive proportions as defined over a range of break points with the model. This measure may be derived from a cohort retrospectively, commonly as the ‘c’ statistic being the area under the ROC curve (see also APLM ’11 amongst appended publications for further discussion).

- **Calibration.** This is a measure of how well predicted probabilities of the disease or outcome of interest agree with actual observed proportions within sub-groups of a new cohort (usually studied prospectively). This is usually measured and assessed using the Hosmer-Lemeshow statistic.

The literature in this area as it relates to the development of diagnostic and prognostic tests for sepsis is complex. A review of 19 scoring systems that were available as at 1995 for the prediction of mortality in the patient group with sepsis concluded that

“The calibration of risk prediction methods comparing predicted with actual mortality across the breadth of risk for a population of patients is excellent, but the overall accuracy in individual patient predictions is such that clinical judgement must remain a major part of decision making” [263]
2.3.1. Tests based on clinical criteria

**Diagnostic tests.** Several clinically based diagnostic and prognostic criteria have been considered (Table 2.3.1). In 1991, The American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) convened a panel of 35 experts in a consensus conference in an attempt to create a standard set of definitions to rapidly identify and triage those patients who might benefit from novel anti-inflammatory therapies that were about to enter clinical trials [265-267]. The resulting consensus criteria were initially termed the Bone criteria and subsequently evolved into the Systemic Inflammatory Response Syndrome (SIRS) which consists of the presence of at least two of the following:

- Body temperature >38 ºC or <36 ºC,
- Heart rate >90 beats per minute,
- Respiratory rate >20 breaths per minute of hyperventilation evident with a PaCO2 <32 mmHg,
- White blood cell count (WCC) >12,000/mm, <4000/mm3 or with >10% immature neutrophils.

SIRS is termed sepsis if infection or microbial invasion into normally sterile tissues is documented. The criteria have subsequently evolved [256]. Moreover, to enable the grading of severity of SIRS, the ACCP/SCCM conference defined by consensus the Multiple Organ Dysfunction Syndrome (MODS) as “the presence of altered organ dysfunction in an acutely ill patient such that homeostasis cannot be achieved without intervention” [268].

The main advantage of SIRS and related criteria are their relative simplicity which led to widespread clinical application. Sepsis, severe sepsis and septic shock are common in the ICU setting with each occurring in as many as 10% of ICU admissions [269-274]. However, hospital wide, about 30% of cases are identified in patients outside of the ICU [274]. In the CUB-rea database, the excess risk of death associated was 54% in those with versus 28% in matched controls without septic shock giving an estimated attributable risk of 26% [270]. However, these criteria have four limitations relevant to the research question of this thesis. These limitations being the criteria are:

- too sensitive, as non-infection related conditions are included [254],
- not specific as to type of infection; bacterial, whether GN or gram-positive, fungal or other [258-259],
being a reflection of the host response, SIRS has an associated morbidity and mortality which is similar whether or not the SIRS is associated with a documented infection [272, 273].

As many as a third of episodes are found to occur outside of the ICU setting [274].

Of particular note, the major limiting factor with the sepsis criteria, as originally defined, are non-specificity. In one evaluation of 1506 patients who satisfied the criteria within an urban tertiary care hospital in 1989 and not limited to an ICU population, only 40 (3%) had GN bacteremia, the sub-group thought to be most representative of patients likely to benefit from an anti-endotoxin therapy were one to become available [275].

As of 1995, there were already at least 19 scoring systems that could be applied to the patient with sepsis or at risk of sepsis in the ICU setting [263]. It should be noted that the accuracy of any given scoring system needs to be re-established in settings (e.g. ICU versus non-ICU) or even institutions different to that in which the scoring system was developed (calibration). A recent study in a general medical (non-ICU) setting found that other scoring systems displayed better discrimination than a score derived using the sepsis stages (Table 2.3.1) [285]. The optimal diagnostic test remains elusive. Other approaches to developing risk prediction tools have looked at more complex scoring systems (SOFA, MODS) some of which are proprietary (APCHEII).

A concept which has emerged since 2001 is the PIRO grading system (P - Predisposing factors; I - Infection; R - Response; O - Organ dysfunction) [286-291]. Slightly different PIRO criteria have been used in different studies. The PIRO grading system is intended for the stratification of patients of investigational studies into sub-groups having similar prognosis, in a manner similar to the TNM (Tumour, Node, Metastasis) staging system as used to describe oncologic disease. PIRO is not intended for the rapid identification or triaging purposes.

Limited evidence is emerging of the application of PIRO based scoring methods in large (>1000 patient) populations with demonstrated predictive performance comparable to other predictive scores in common use such as the APACHE II and Pitt bacteremia scoring systems [289-291].

Prognostic tests. There are multiple scoring systems including over 20 scoring systems that have been developed for the patient in ICU [262-263]. Several have been
developed from a range of perspectives and are not specific to sepsis. In general, the scoring systems are one of four general types; generic (e.g. APACHE II, SAPS), specific to organ system failure (e.g. MSOF, SOFA), specific to populations (e.g. trauma), and others.

<table>
<thead>
<tr>
<th>Author (yr)</th>
<th>Ref</th>
<th>Criteria</th>
<th>End point</th>
<th>Population</th>
<th>N</th>
<th>Discrimination</th>
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<tr>
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<td>Hospitalized</td>
<td>474</td>
<td>NS</td>
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<td>Bates (1997)</td>
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<td>Sepsis syndrome</td>
<td>881</td>
<td>0.69</td>
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<tr>
<td>Peduzzi (1992)</td>
<td>279</td>
<td>Clinical</td>
<td>GNB</td>
<td>VAMC Sepsis syndrome</td>
<td>465</td>
<td>‘significant’</td>
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<tr>
<td>Bates (1997)</td>
<td>276</td>
<td>Clinical</td>
<td>GNB</td>
<td>Hospitalized</td>
<td>881</td>
<td>0.70</td>
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<tr>
<td>Schechner (2009)</td>
<td>280</td>
<td>Clinical</td>
<td>Pseudomonas GNB</td>
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<td>4114</td>
<td>0.73</td>
</tr>
<tr>
<td>Sprung (1990)</td>
<td>281</td>
<td>Encephalopathy</td>
<td>Mortality</td>
<td>Sepsis (ICU)</td>
<td>1333</td>
<td>OR = 2.2</td>
</tr>
<tr>
<td>Knaus (1995)</td>
<td>282</td>
<td>SUPPORT</td>
<td>Mortality</td>
<td>Hospitalized</td>
<td>4301</td>
<td>0.79</td>
</tr>
<tr>
<td>Marshall (1995)</td>
<td>268</td>
<td>MODS</td>
<td>Mortality</td>
<td>ICU</td>
<td>336</td>
<td>0.936</td>
</tr>
<tr>
<td>Bossink (1998)</td>
<td>283</td>
<td>SIRS (modified)</td>
<td>Mortality</td>
<td>Hospitalized</td>
<td>300</td>
<td>better than SIRS alone</td>
</tr>
<tr>
<td>Rhee (2009)</td>
<td>284</td>
<td>Pitt score</td>
<td>Mortality</td>
<td>ICU</td>
<td>134</td>
<td>0.799</td>
</tr>
<tr>
<td>Ghanem-Zoubi (2011)</td>
<td>285</td>
<td>MEWS REMS MEDS SCS Sepsis stages</td>
<td>Mortality</td>
<td>Hospitalized</td>
<td>1072</td>
<td>0.70-0.79</td>
</tr>
<tr>
<td>Ghanem-Zoubi (2011)</td>
<td>285</td>
<td>MEWS REMS MEDS SCS Sepsis stages</td>
<td>Mortality</td>
<td>Hospitalized</td>
<td>1072</td>
<td>0.65</td>
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Foot notes:
- Discrimination = DOR unless otherwise specified
- VAMC = Veterans Administration Medical Center
- NS = not stated
Controversially, these scoring systems have been used to guide therapy for individual patients. For example, the APACHEII scoring system was used in targeting therapy with recombinant activate protein C (rhAPC) to the patient group at high risk as indicated by an APACHEII score >24 [293]. Of note, rhAPC was recently withdrawn after a failed attempt to confirm efficacy of this drug in patients with septic shock [300]. The clinical application of disease severity scores toward the management of individual patients is controversial as they are instruments which are validated for use in populations, not for individual patients [297]. However, a research question relevant to the analysis to be undertaken in this thesis is whether the mortality risk of the population determines the relative importance of various risk factors and the efficacy of anti-sepsis agents in that population [294 -296, 298, 301, 302].

2.3.2. Laboratory based tests

The lack of an objective ‘gold standard’ for sepsis testing is an impediment toward progress in developing and evaluating diagnostic tests generally, not only the limulus assay. All assays developed to enable the rapid recognition of the presence, severity and the specific microbial causes of sepsis have faced the same lack of a reference standard. For example a recent review of 170 potential biomarkers for sepsis (other than the limulus assay for endotoxin) that have been evaluated, none was sufficiently practical, sensitive or specific for routine clinical use [303]. Moreover, relatively few had progressed to be evaluated in clinical studies. It also needs to be recognized that there is evidence for a strong publication bias for prognostic markers. For example, amongst published studies of markers used in oncology, it was recently identified that those that have statistically significant results are disproportionately published versus ones that do not [304]. This ‘reporting bias’ is likely widespread.

Diagnostic tests. Table 2.3.2 lists five recent representative meta-analyses of clinical evaluations of diagnostic tests in patient groups similar to those in which the limulus assay has been applied. The diagnostic odds ratio (DOR) is used as a single indicator of diagnostic test performance from each meta-analysis to enable a comparison across a broad literature. This table illustrates three points. Firstly, the table summarizes >100 publications. Second, while the DOR’s for all of these diagnostic tests were >7, only one was considered by the respective authors to be useful for
individual patient management. This illustrates that even with markers or risk factors that might be considered to be strong markers (OR’s greater than 5) for an end point in a population by epidemiological criteria, they are not necessarily sufficiently discriminatory to be clinically useful for application as diagnostic tests for individual patients [310-312].

The third point is that each of these meta-analyses has incorporated a broad range of studies. Different studies have evaluated assays variants, different breakpoints and different patient groups. Given the heterogeneity in populations to which a test for

<table>
<thead>
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<th>Table 2.3.2 Recent meta-analytic summaries of diagnostic tests.</th>
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<td><strong>Main author</strong></td>
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<td><strong>Year</strong></td>
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<td><strong>Test</strong></td>
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<td><strong>Reference standard</strong></td>
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<td><strong>Patient population or setting</strong></td>
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<td><strong>Number of patients</strong></td>
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**Meta-analytic summary**

| Diagnostic Odds Ratio | 15.5; 8.3 – 28.8 | 7.8; 5.9 – 10.4 | 9.9; 5.7 – 17.0 | 19.2; 10.5 – 35.4 | 229; 85 – 617 |
| Clinical utility of test? | selected groups | No | No | Has limitations | Yes |

Foot notes; PCT = procalcitonin; IFI = Invasive Fungal Infection; PCR = polymerase chain reaction; ICU = Intensive Care Unit; ED = Emergency department.

a. Diagnostic odds ratio for Pfeiffer [305] calculated as the exponentiation of mean D (mean log odds ratio = 2.74; 2.12-3.36); The Q* for this meta-analysis was 0.80; 0.74-0.86.
sepsis might be applied, together with the diverse published experience, it needs to be questioned whether a single summary result from the literature is either achievable or desirable for any diagnostic test for sepsis. A highly relevant finding from multiple evaluations with implications toward the potential real world application of any diagnostic test is the extent of variability in the DOR amongst different studies and different populations in which the test may be used [313-314].

**Prognostic tests.** The difficulties in the development of prognostic tests for sepsis is again well illustrated by a contrast with the field of cardiovascular medicine where the availability of a test for cardiac troponin, a rapidly available serum test of cardiac injury transformed the management of patients presenting with acute coronary syndromes. The prognostic values of cardiac troponins measurement in serum was established in a sub-study of the Global Use of Strategies To Open occluded arteries Trial IV (GUSTO-IV) which found that the baseline level of troponin T was independently related to 30-day mortality [260]. This finding, published in 2003, prompted the development of more aggressive interventional strategies for cardiac patients determined by the baseline troponin assay. In the field of sepsis medicine, there is as yet no simple and unifying test to enable patient risk stratification. Note that the size of the GUSTO-IV study, 7000 patients [260], is more than the sum of patients included in studies of sepsis tabulated in the meta-analyses listed in Table 2.3.2 and also among the studies analysed within this thesis (see Table 5.1.1).

Amongst the various laboratory tests that have been evaluated for sepsis, the test which has been most studied is the serum procalcitonin (PCT) assay. However, even with this well-studied assay, its clinical application remains unclear. One recent meta-analysis of 18 studies of PCT as a predictor of bacteremia in critically ill patients [306] found evidence that diagnostic performance was less apparent in larger versus smaller studies. The PCT assay has been considered for use as a guide to de-escalate antibiotic therapy [315]. A multi-center trial to reduce antibiotic use in intensive care units through PCT monitoring (the PRORATA trial), found that this strategy could reduce antibiotic use without apparent adverse outcome [316].

The search for clinically relevant diagnostic and prognostic tests for sepsis continues [317-319] and the most recent approaches have examined gene arrays [320], molecular markers [321] and cytokine profiles [322]. These approaches are challenging not least because of the statistical methods required to evaluate the high dimensional
data that is generated [323].

For example, a recent study of 126 patients presenting with sepsis in the setting of an emergency department measured a panel of cytokines simultaneously using a multiplex assay with cytokine patterns determined by principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) [322]. This study, as in previous studies, found some associations between individual bio-markers with severe sepsis or septic shock. However, none of these associations remained significant in PCA or AHC analysis which examined for signature patterns of cytokine profiles [322]. Recently, an assay to detect cell-free DNA might have strong prognostic value in patients with severe sepsis in some studies [324] but not all [333, see below]. However, a systematic review of studies of 12 patient cohorts found that sepsis-related inflammatory changes are highly variable on a transcriptional level [325]. In particular, the “…arbitrary distinction of separating sepsis into pro-inflammatory and anti-inflammatory phases is not supported by gene-expression data. [325]”

2.3.3. The potential for the limulus assay

The limulus assay has four unique advantages over other diagnostic and prognostic tests that have been evaluated in the setting of sepsis.

(i) The signal amplification that results from the coagulation cascade mechanism within the limulus assay enables sensitivity to pg/ml levels of endotoxin, several log fold higher than might be achieved with other assays [210].

(ii) The assay target is endotoxin, a molecule which is considered to be a mediator of the adverse effects of gram-negative sepsis [222].

(iii) In at least one setting, that of meningococcemia, there is clear evidence that the levels of endotoxin in blood are both diagnostic and prognostic [11-12].

(iv) The assay been validated in application to samples other than blood (Table 2.2.1).

Strikingly however, these implied advantages are not generally apparent amongst the surveys of clinical applications of the limulus assay to blood samples. The clinical experience of the limulus assay as summarized by Elin (Table 2.2.2) indicates that the limulus assay applied to blood samples performs poorly compared to its application to non-blood samples. Moreover, the summary clinical experience of the limulus assay as applied to blood (as summarized by Elin; Table 2.2.2) appears no better than that with the clinical experience of a selection of non-limulus assays (Table
2.3.2). Moreover, the prognostic value of the limulus assay is also highly variable among the studies that have examined this (see 2.5.3). Possible explanations for these disconnects could be either the patient population or the use of GN bacteremia as the reference standard. These two possible explanations are considered in the following section. A third possible consideration is that endotoxemia levels are dynamic, for example in one clinical study [21] of 100 patients with sepsis in an ICU setting, the cumulative percent found to have endotoxemia rose from 20% to 40% between 0 and 24 hours after study entry.

2.4. Gram-negative (GN) bacteremia

In this thesis, GN bacteremia is used as a reference standard against which endotoxemia detection is compared. Also GN bacteremia is used to assist in the interpretation of endotoxemia detection as a prognostic test. However, current methods for the detection of bacteremia may be imperfect [326-327]. Moreover, the use of GN bacteremia as a proxy reference standard for endotoxemia is controversial and it potentially raises four major difficulties.

Firstly, the LAL assay is an assay for endotoxin, not gram-negative bacteria per se. For example, is the amount of cell bound endotoxin sufficient to be detected in the LAL assay at the levels of GN bacteremia typically found? Lipid-A has a molecular weight of 4500 and there is approximately $3.4 \times 10^6$ lipid-A residues per *E. coli* cell [328-329]; given this, with a typical bacteremia of 10 circulating bacteria per 1 ml of blood [330-332], it would be expected that there would be $10^{-12}$ g (1 pg/ml = $10^{-12}$ g/ml) of endotoxin for a bacteremia with *E. coli*. Direct measurements to estimate the amount of LPS per bacterial cell are between $\sim 5 \times 10^{-3}$ fg per *E. coli* bacterial cell [125] and up to $\sim 25$ fg/cfu of endotoxin per *E. coli* and Pseudomonas bacterial cell [124]. Given the above assumptions, and assuming that the total amount of bacterial cell bound endotoxin is completely available, the amount would still be below the detection limit of even the most sensitive LAL assay which is generally 5 pg/ml. Three further complicating considerations in this estimation; the limulus assay is at least 10-fold more sensitive to LPS molecules in aggregates rather than in monomer formations [128]; there is a complex interactive effect of plasma on the assay [145-147, 250]; and this estimation of endotoxemia detection takes no account of non-viable bacterial cells.
and cell fragments that accompany a GN bacteremia [326, 333, 334].

In support of the above estimates, there are several experimental rabbit [335, 336] and canine [337] models of GN bacteremia with either an *E. coli* or *Pasteurella* bacterial challenge in which the quantitative blood levels of GN bacteremia and endotoxemia have been concurrently measured. In these models, GN bacteremia at levels of 10,000 cfu/ml corresponds to endotoxemia levels of 10 ng/ml [336], 500 ng/ml [335] and 50 EU/ml (~5ng/ml) [337]. Note however, that these levels of bacteremia are some 1000 times higher than those seen clinically in infections in human.

The second major difficulty is that GN bacteremia is not a single entity (see section 2.4.1). Amongst various GN bacteremia types there is a well defined ranking of mortality risk with the risk being lowest for *E. coli* and highest for GN bacteremias such as *Pseudomonas aeruginosa* (see section 2.4.2). Hence, in this light, the recently described differences in the lipid-A structures (see 2.1.1) among the different GN bacteremia types provide an impetus for studying endotoxemia detection and as may serve as a basis for exploring the differences in mortality for the GN bacteremia types. Could this be a potential confounder in the relationship between GN bacteremia and both the detection of endotoxemia and the occurrence of mortality? This is one of the research questions of this thesis.

The third difficulty in using GN bacteremia as a proxy reference standard is that the mortality attributable to GN bacteremia is itself difficult to define. This issue is discussed further in 2.4.1.

Finally, GN bacteremia accounts for only a minority of patients with sepsis syndrome (Figure 2.11). On the other hand and to counter the above difficulties, the following are reasons that GN bacteremia can serve as a valid proxy reference standard;

(i) GN bacteremia would appear to be the most clinically relevant proxy for endotoxemia,

(ii) The diagnostic and prognostic tests for sepsis are problematic and while they continue to evolve, GN bacteremia serves as a population marker,

(iii) GN bacteremia is itself an objective and important clinical end point,

(iv) GN bacteremia has been extensively studied in settings such as in the ICU and elsewhere in the broader clinical literature enabling a derivation of a benchmark range (see section 2.4.2) for mortality risk with GN bacteremia,

(v) Methods to enable the rapid detection of GN bacteremia would help to target specific patient management strategies,
(vi) In fluid other than blood, such as urine & CSF, there is a quantitative correlation between levels of GN bacteria and endotoxin,

(vii) there is insufficient experience with the other assays for endotoxin for the purposes of validating the results of the limulus assay,

(viii) The detection of GN bacteremia has commonly been used as a proxy marker for comparisons with the results of the limulus assay [1-107],

(ix) GN bacteremia is common, especially in the ICU setting,

(x) Along with an expectation of concordance with detection, there is an expectation of correlation with level of bacteremia. In this regard, there is some evidence that the quantitative level of bacteremia correlates with outcome (see below) [340, 341].

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**2.4.1. GN bacteremia types**

There are two broad categories of gram-negative infections; pathogenic and opportunistic. The limulus assay has been studies in settings of infections with both of these categories of gram-negative infections although the findings have not been uniform. The opportunistic gram-negative infections have been most studied among the 107 studies that have been analysed within this thesis.

**Pathogenic.** On the one hand are infections due to specific pathogenic gram-negative bacteria as exemplified by *Salmonella typhi* [1, 16, 56, 77], *Neisseria meningitidis* [9, 11-12, 40, 68], *Burkholderia pseudomallei* [73] and *Yersina pestis* [14-15] which are the causative organisms of Typhoid fever, Meningococcemia, Melioidosis and Plague, respectively. These pathogenic bacteria have defined
pathogenesis mechanisms. These infections are usually community acquired by previously healthy individuals, often occurring in outbreaks. Some outbreaks may be attributable to a point source or a single clone. Indeed, these infections are each clinically distinctive. For example, the recognition of outbreaks such as Typhoid fever and Plague in the era pre-dating the germ theory of disease in the late 1800’s led to the formulation of Koch’s postulates.

In relation to pathogenic GN bacteria, one of the most extensively studied is meningococcal infection. In this condition the level of endotoxemia is quantitatively related to prognosis [11, 12]. In addition to endotoxin and viable bacteria there are high levels of bacterial fragments in the blood stream [334]. The studies of endotoxin tolerance in the context of typhoid fever pathogenesis in human prisoner volunteers have also been mentioned above (see 2.1.1). However, there are no endotoxemia levels from these studies.

**Opportunistic.** On the other hand are GN bacteremias as commonly found within the contemporary hospital context. Common examples are *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. As these infections are usually acquired by hospitalized patients whose host defences are compromised by specific defects (e.g. neutropenia, invasive devices, urinary tract abnormalities) or by the severity of their underlying disease (e.g. burns, malignant disease, diabetes), these bacteria are often considered opportunistic and hence, relatively non-pathogenic [361]. These types of GN bacteremias are common in the contemporary hospital context presumably as a consequence of patient resuscitation and supportive therapy which have prolonged the survival of the compromised patient. This supportive therapy includes the use of invasive devices (e.g. urinary catheters) which breach host defence mechanism and predispose to subsequent GN bacteremia.

In contrast to the category of pathogenic bacteria considered above, the clinical prediction (i.e. recognition ahead of the results of blood culture testing) of opportunistic GN bacteremias in the contemporary hospital context is challenging as the clinical features are non-specific and are difficult to distinguish from non-bacteremic GN infections and indeed severe infections due to other micro-organisms such as gram-positive or even severe viral infections. Several attempts have been made to identify those who have GN bacteremia among those patients at high risk. This cannot be readily achieved with current methods. For example, a predictive model for GN
bacteremia developed among patients presenting to a tertiary hospital (Table 2.3.1) was found to have poor discrimination with an AUC of 0.726 [270]. In this regard, an ability to detect specific types of GN bacterial infection would assist in the targeting of specific antibiotic therapies. This is especially so in settings where GN infections are common and clinically important. For example, a test for the rapid detection of Pseudomonas bacteremia in the ICU and oncology setting would be desirable [362].

2.4.2. Pathophysiology of GN bacteremia

How much does the presence of gram-negative bacteria (or indeed any bacteria) in the blood stream influence the outcome of sepsis? The answer to the question is surprisingly elusive [361-369]. The attempts to address this question have implications for how to approach the same question within this thesis for endotoxemia.

There is no question that bacteremic infections with pathogenic GN bacteria occurring in an outbreak setting in previously health people, as for example with Neisseria meningitidis, have high attributable mortality. Indeed even community acquired GN bacteremias (90% enterobacteriaceae) are associated with an adjusted RR of 1.5 (1.2-2.0) for mortality versus patients with negative blood cultures [370].

On the other hand, for bacteremic infections with opportunistic GN bacteria in the intensive care unit for example, the mortality attributable to the bacteremia versus that due to the underlying illness is difficult to define. Viewing this from an alternate perspective, among patients with systemic inflammatory response syndrome (SIRS), the morbidity and mortality is similar whether or not the SIRS is associated with documented infection, being a documented GN infection or another type of infection [273, 371, 372]. This raises the presumption that for the patient group with SIRS without a documented infection there is a missing mediator(s) which accounts for the similar prognosis between those with, versus those without, a documented infection. Endotoxin and endotoxemia has long been suspected to have this role. However, the evidence for these ‘missing mediators' being endotoxemia is better established for pathogenic Neisseria meningitides. This specific infection is cited as exemplorary of ‘intra-vascular' versus ‘extra-vascular' sepsis (see below) [373].

In attempting to estimate the attributable-mortality associated with bacteremia, there are at least three inter-related factors to consider; the source of the GN bacteremia,
the type of the GN bacteremia and the method of statistical adjustment in matching for severity of underlying patient illness in deriving the estimate.

**Source of the GN bacteremia.** The site of origin of the GN bacteremia is an important prognostic determinant. In particular, most influential is a site of origin being non-urinary tract versus urinary tract [374-376]. Among *E. coli* bacteremias, those of urinary tract origin have a better prognosis versus *E. coli* bacteremias originating from non-urinary tract sites such as an intra-abdominal or pulmonary sites [374-376]. In this regard, bacteremias with *E. coli* more commonly originate from urinary tract sites than do bacteremias with *Pseudomonas* and GN bacteria other than *E. coli* [230].

Meningitis is a special case of an infection occurring at an extra-vascular site for which there is some evidence that disease severity is proportional to the CSF concentration of endotoxin [377]. However, this relationship is likely to be compounded by the correlation between the concentration of bacteria in CSF and outcome [378].

From an alternate perspective, does bacteremia increase mortality for an extra-vascular site of infection? This question has been most commonly investigated in relation to pneumonia acquired in the intensive care unit. In a meta-analysis [379] of five studies, the presence of bacteremia (not necessarily GN) is associated with an increased risk of mortality with an OR of 2.07 (95% CI 1.16-3.7) [380-386]. However, this question remains open as other large studies of pneumonia acquired in the intensive care unit (and not all included in the meta-analysis [379]) either have [380, 407] or have not [385, 386] been able to demonstrate a significant increased risk of mortality associated with the presence of bacteremia.

**‘Intra-vascular’ versus ‘extra-vascular’ sepsis.** Munford [361] recently reviewed a range of observations bearing on the role of GN bacteremic infections in the mediation of sepsis and proposed a hypothesis based on the compartment in which the infection and the sepsis process is primarily localized; that is intra-vascular versus extra-vascular compartments. The prototype of intravascular sepsis is that associated with a *Neisseria meningitidis* infection [9, 11-12, 40, 68].

The prototype of extra-vascular sepsis is that due to a primary (e.g. in the abdominal cavity or lungs) opportunistic infection with *Escherichia coli* for example. GN bacteremia if it occurs in association with extra-vascular sepsis is presumed to
contribute little to inducing the toxic responses. Munford cites a number of observations to support this intra-vascular versus extra-vascular hypothesis [361].

Munford [361] cites four large case series of severe sepsis or septic shock in which the difference in case fatality rates for those with versus those without culture documentation is less than 10 percentage points [272-273, 387, Saez-Lorens ‘95]. For example, in a survey of admissions to French ICU’s over a two months period, the presence of bacteremia was associated with a significantly elevated risk (OR = 1.7; 1.1-2.8) for early (< 3 days) mortality but this elevated risk was apparent only in a uni-variate analysis and not in a multi-variate analysis when other relevant prognostic factors were controlled for [272].

Munford states that the simple presence of bacteria in the intra-vascular compartment may not in itself be a sufficient trigger for the serious systemic inflammatory processes in every instance [361]. For example, among observations bearing on intra-vascular infection, there are several case reports of accidental infusions of blood products and intravenous fluids later found to be contaminated with GN bacteria [388-393]. Munford points out these accidental infusions are not invariably associated with mortality [361].

However, this intra-vascular versus extra-vascular hypothesis may be overly simplistic. For example, a contrary observation is a recent meta-analysis (not cited by Munford [361]) of four identical time-series cohort studies of switching from open to closed infusion containers in four Latin American countries which could be viewed as an estimate of attributable mortality of bacteremia in the setting of a ‘natural experiment’ [394]. This meta-analysis identified that switching to closed infusion containers was associated with a reduction (RR 0.77; 0.68-0.87) in all cause mortality of ICU patients in association with a striking reduction (RR 0.33; 0.24-0.46) of bloodstream infections (not necessarily gram-negative).

The type of the GN bacteremia Among critically ill patients with nosocomially acquired GN bacteremias [395–400] including either E. coli [397], Klebsiella [398], Acinetobacter baumannii [400] and Pseudomonas [400] bacteremias, the difference in mortality between bacteremic case patients versus matched controls (i.e. attributable mortality) is commonly less than 15 percentage points. However, as stated above, the impact of the type of GN bacteremia is likely confounded by the
An additional factor related to the GN bacteremia is the level of bacteremia (cfu/ml) as previously it had been noted that a higher quantitative level of bacteremia is related to an increased risk of mortality [340, 401]. Recently, with the continuous monitoring of blood culture bottles for the detection of growth during their incubation in modern bacteremia detection methods, the time to positivity (TTP) has come to be used as a surrogate quantitative marker of bacteremia. Shorter TTP is thought to indicate higher cfu/ml. In studies of patients with *E. coli* [402, 403] and *Klebsiella* bacteremias [404], shorter TTP was independently related to increased mortality risk. However, confounding this relationship is that shorter TTP was also related to a non-urinary tract focus [402, 403] and higher Pitt bacteremia score [404].

A multivariate analysis of risk factors for death among a cohort of 832 bacteremic patients with sepsis from a survey of 24 French hospitals revealed that five factors were found to be independently associated with an increased risk of death including age, underlying patient illness severity, presence of severe sepsis or shock whereas a urinary tract source of infection and the type of bacteremia (GN versus non-GN) were both associated with a reduced risk of death [405]. These authors note that there was a relatively low incidence of difficult to treat bacteremias with *Klebsiella* and *Pseudomonas* in their survey. By contrast, a multivariate analysis of risk factors for death among 2286 bacteremic patients with sepsis from a survey of 18 Korean hospitals found that pathogens other than *E. coli* were associated with an increased risk of death after adjusting for illness severity and respiratory site of infection [406]. However, confounding the comparison of these cohorts, *Klebsiella* and *Pseudomonas* bacteremias were more common amongst the Korean cohort than the French cohort [405].

**The method of statistical adjustment** The execution and interpretation of studies that attempt to estimate attributable-mortality associated with bacteremia or any other documented infection is not simple [364, 381]. There are two main methods to control for severity of underlying illness in attempting to define attributable-mortality. The first is ‘control by design’ such as a simple case-control type study design [399, 408]. However control is difficult to achieve by this method given the large number of potential confounding factors for which matching is sought and this method is not commonly used. The alternate approach to matching is ‘control by analysis’ in which the severity of underlying illness is estimated for each patient using a risk score and the
score serves as a basis to match case and control group patients for severity of illness [407]. In the analysis of attributable-risk, the method of risk factor adjustment is crucial to the estimates obtained and there are at least five difficulties with this approach;

1. the most relevant severity score to choose may be conjectural (see 2.3),

2. the development of a risk score is not simple and factors that determine its suitability may be unique to the population at hand. An important consideration is whether allowance was made for interaction effects in the development of any risk score,

3. the problematic choice of timing at which the severity score is made in relation to the presence of the documented infection of interest (see below),

4. of all the important factors that need to be controlled to obtain an unbiased estimate, not all will be known for all patients. For example the site of origin of the bacteremic infection may not be clear, and

5. there may be additional factors that are presumably influential but remain as yet unknown. For example, the timing of administration and appropriateness of antibiotic therapy has emerged in more recent studies as additional significant factors but has infrequently been recorded in earlier studies [411-413].

The presence of a documented infection could influence the parameters used within a severity of illness score. This influence introduces an element of confounding and ‘circularity’ to the matching process which may result in ‘overmatching’. This has been circumvented by various techniques including causal analysis [409], daily scoring [410] and introducing scoring at a time point several days prior (e.g. three day recalibrated ICU outcome -TRIO) to the bacteremia [368]. An example of the complexity in this area and how adjustment for risk factors is critical to the results of an analysis are the seemingly contradictory results obtained from three analyses of data from French ICU’s. The OUTCOMERA database is a large multi-center database derived from 12 French ICU’s. Two analyses undertaken by the same authors to estimate the impact on mortality risk of causative pathogen and infection site in different patient populations over the years 1997-2004 and 1996-2009 had used either a matched conditional logistic regression adjusted for TRIO score in one [368] versus a multivariate adjusted hazards model [367] in the other to adjust for underlying and competing mortality risks.

Amongst other findings, the estimate of the impact of bacteremia (not necessarily GN) acquired in the ICU patient population differed for these two studies being OR 3.02 (2.17-4.22) [368] versus HR 1.59 (1.22-2.06) [367]. Moreover, in the former analysis [368], the type of blood stream infection was highly influential in mortality risk being
highest for GN mono-bacterial (OR 6.1; 3.0-12.2) and fungal (OR 8.8; 1.7-46.7) versus coagulase negative Staphylococci (OR 1.6; 0.74-3.0) whereas is the other analysis [367] neither the causative organism nor the presence of bacteremia were found to be associated.

A third analysis of a different multi-center (11 centers) French ICU data base found that the population attributable fraction of mortality due to blood stream infection (not necessarily GN) was only 1.7% (0.9-2.5%) but that this estimate was sensitive to adjustment for any competing attributable mortality fraction for infection occurring at multiple sites [369].

With the above listed difficulties in mind, it is of interest to examine the mortality risks of GN bacteremia as reported in the literature. For the data analysed in this thesis, there are two main settings (ICU versus non-ICU) and three main categories of opportunistic GN bacteremias (Escherichia coli, Enterobacteriaceae other than E. coli and non- Enterobacteriaceae; e.g. Pseudomonas species) which are of particular interest. Both the range in the mortality risks in the literature, and also their relative rankings in mortality for these categories both within and between studies are of interest here. GN bacteremia outcome data from 31 studies is summarized for hospital-wide (open; n=15; Table 2.4.1.1/.2; Figures 2.12/.13), oncology studies (n=5; Table 2.4.1.3; Figure 2.14), and nosocomial and ICU (n=8; Table 2.4.1.5, Figure 2.15). The studies in these tables have been obtained by an opportunistic searching of the literature for publications of large numbers of patients with outcome data for several GN bacteremia types and it is not meant to be a systematic summary of the literature.

These opportunistic summaries the literature presented in Table 2.4.1.1/2/3/4/5 and Figures 2.12/.13/.14/.15 illustrate the following points relevant to this thesis.

a. The studies are mostly contemporaneous with the studies of interest [1-107] within this thesis.

b. GN bacteremia has a high mortality, being up to 50% for some GN bacteremia types in some studies.

c. Note that the logit scale is used in each figure. With this, the 95% confidence interval is symmetric about the mortality proportion. The study precision is reflected in the width of the confidence interval is expected to be wider for smaller groups.

(continued p 63)
Table 2.4.1.1 Gram-negative bacteremia: open studies (pre 1975)

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</tr>
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<td>Adult &amp; ped (16% &lt; 39 yrs)</td>
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<tr>
<td>census</td>
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**Mortality proportions**

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<td>77</td>
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<td>67</td>
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</table>

Additional pre 1975 studies not shown in the table are:

- [Bryant 1971; 728]; surveyed the years 1965-68 for which the numbers and mortality proportions were *Escherichia coli* 17/83 (21%); all non-*E. coli Enterobacteriaceae* 23/76 (30%); *Pseudomonas species* 32/45 (71%).
- [Hodgin 1965; 729]; surveyed the years 1963-64 for which the numbers and mortality proportions were *Escherichia coli* 12/32 (34%); all non-*E. coli Enterobacteriaceae* 15/32 (47%); *Pseudomonas species* 4/7 (57%).
- [Watt 1967; 730]; surveyed the years 1958-65 for which the numbers and mortality proportions were *Escherichia coli* 12/30 (37%); all non-*E. coli Enterobacteriaceae* 4/8 (50%); *Pseudomonas species* 22/26 (85%).

Foot notes;

n = number of fatalities; N = number of patients; % = mortality percentage
a. Includes Serratia species and Proteus species,
b. Includes; *Fusobacterium species, Bacteroides species*
c. *Staph epidermidis*, a gram-positive, serves as a reference group
Table 2.4.1.1 Gram-negative bacteremia: open studies (post 1975)

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Mortality proportions

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Foot notes:

- n = number of fatalities; N = number of patients; % = mortality percentage
- a. Includes Serratia species and Proteus species,
- b. Includes: *Fusobacterium species, Bacteroides species*
- c. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group
Fig. 2.12 Mortality proportions from hospital-wide studies pre-1975 (as in Table 2.4.1.1). Note the 95% confidence intervals have been calculated and represented on the logit scale by methods described in section 4.2 (page 87). The vertical lines at 19% & 43% represent the IQ range for all GNB (see Table 2.4.2) to benchmark across Figures 2.11/.12/.13/.14.
Table 2.4.1.2 Gram-negative bacteremia: open studies (post 1975)

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Mortality proportions

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Foot notes;
- n = number of fatalities; N = number of patients; % = mortality percentage
- d. Includes Serratia species and Proteus species,
- e. Includes; *Fusobacterium species, Bacteroides species*
- f. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group
Fig. 2.13 Mortality proportions from hospital-wide studies post-1975 (as in Table 2.4.1.2). Note the 95% confidence intervals have been calculated and represented on the logit scale by methods described in section 4.2 (page 87). The vertical lines at 19% & 43% represent the IQ range for all GNB (see Table 2.4.2) to benchmark across Figures 2.11/.12/.13/.14.
Table 2.4.1.3 Gram-negative bacteremia: intervention studies

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**Mortality proportions**

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Foot notes:

n = number of fatalities; N = number of patients; % = mortality percentage

a. Includes Serratia species and Proteus species,
b. Includes: *Fusobacterium species, Bacteroides species*)
c. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group
Table 2.4.1.4 Gram-negative bacteremia: oncology studies

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- **Age**
  - Adult

- **% neutropenic**
  - 70%
  - 9%

- **census**
  - not stated
  - not stated

### Mortality proportions

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**Foot notes:**

a. This study (Singer, [352]) is presented for two sub-groups.

b. Includes Serratia species and Proteus species.

c. Includes: *Fusobacterium species, Bacteroides species*.

d. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group.
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- Age
- % neutropenic
- census

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Foot notes:
\textsuperscript{a} MASCC [357] is a multicenter study (multinational association for supportive care in cancer)
\textsuperscript{b} Includes Serratia species and Proteus species,
\textsuperscript{c} Includes, \textit{Fusobacterium species, Bacteroides species}
\textsuperscript{d} \textit{Staph epidermidis}, a gram-positive bacteria serves as a reference group
**Fig. 2.14** Mortality proportions from oncology studies (as in Table 2.4.1.3). Note the 95% confidence intervals have been calculated and represented on the logit scale by methods described in section 4.2 (page 87). The vertical lines at 19% & 43% represent the IQ range for all GNB (see Table 2.4.2) to benchmark across Figures 2.11/.12/.13/.14.

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*Oncology*
Table 2.4.1.5 Gram-negative bacteremia: Nosocomial and ICU studies

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**Mortality proportions**

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Foot notes:

n = number of fatalities; N = number of patients; % = mortality percentage

a. Data in Cohen [287] is median derived across mean results of between 7 and 10 studies
b. Includes Serratia species and Proteus species,
c. Includes; *Fusobacterium species, Bacteroides species*
d. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group
Fig. 2.15 Mortality proportions from nosocomial & ICU studies (as in Table 2.4.1.5). Note the 95% confidence intervals have been calculated and represented on the logit scale by methods described in section 4.2 (page 87). The vertical lines at 19% & 43% represent the IQ range for all GNB (see Table 2.4.2) to benchmark across Figures 2.11/.12/.13/.14.
### Table 2.4.1.5 Gram-negative bacteremia: Nosocomial and ICU studies (continued)

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Foot notes:
- n = number of fatalities; N = number of patients; % = mortality percentage
- a. These studies all have endotoxemia data and are included for analysis in thesis
- b. Includes Serratia species and Proteus species,
- c. Includes: *Fusobacterium species, Bacteroides species*)
- d. *Staph epidermidis*, a gram-positive bacteria, serves as a reference group
d. This non-systematic review provides observed mortality proportions across the three main categories of opportunistic GN bacteremias and in various hospital based settings and eras.

e. The observed mortality proportions for the studies published since 1975 (i.e. as in Table 2.4.1.2/.3/.4 [339-348, 352-360]) can be used to enable comparisons of mortality risk in the literature versus the studies to be analysed in this thesis [1-107]. To this end, the inter-quartile range (IQR) of the mortality proportions for the three GN bacteremia types as reported in 19 studies (20 groups) published since 1975 [339-348, 352-360] is shown in Table 2.4.2. Each IQ range will be used to benchmark the results obtained in section 5.2.3.

f. The overall IQ range is indicated in Figures 2.12/.13/.14/.15 and is shown to enable comparisons across the four figures.

g. The mortality for bacteremias with Staph epidemidis among studies undertaken in an ICU setting is typically 18% [287]. Note that Staph epidemidis is not a GN bacteremia but is a common isolate and is used here for reference purposes only.

h. The largest study [287] in Table 2.4.1.2/.3/.4/.5 is a summation derived using robust (median) methods from multiple small ICU studies in the literature. The median mortality risks as reported in this overview [287] are within each respective IQ range derived here (Table 2.4.2).

i. There are six studies published prior to 1975 [338, 726-730] and these studies were not used in deriving any IQ range here as these studies predate the studies of interest [1-107] within this thesis. Note however that the mortality proportions for patients with GN bacteremia in the pre-1975 studies are generally higher than for studies published after 1975. Interestingly, the ranking in mortality proportions within the three categories in these pre-1975 studies is generally maintained in that the mortality for E. coli bacteremias is the lowest and that for non-enterobacteriaceae including Pseudomonas species is the highest.

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<td>non-Enterobactereiaceae (e.g. P. aeruginosa)</td>
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Abbreviations:
* N = number of study groups
§ - Overall is the IQ range for all GNB and is shown in Figures 2.12/.13/.14/.15
j. The lower overall mortality since 1975 presumably reflects improvements in antibiotic and supportive therapies. There is mixed evidence from single center studies that the mortality risk associated with GN bacteremia has decreased further with a decrease from 20-30% to 15-20% in one study [353] whereas for another study unchanged from 16-24% versus 15-19% [344] for pre versus post 1985 periods, respectively.

k. The mortality risk for poly-microbial GN bacteremias (not shown in Figures 2.12/.13/.14/.15) is higher and broader than that for the three categories of monomicrobial GN bacteremias. Poly-microbial GN bacteremias are not further considered in this thesis.

l. The most common GN bacteremia is usually *E. coli* bacteremias being, in some studies, >4 times more common than *Pseudomonas*. However, this is not universal and in some studies this ranking is reversed.

m. In most studies, the mortality is lowest for *E. coli* bacteremias with rates in some studies being similar to mortality rates seen with bacteremias with *Staph epidemidis*. In contrast, *Pseudomonas* bacteremias have a high mortality risk, sometimes double that for bacteremias with *Staph epidemidis*.

n. Even within this limited survey, there are several [65, 345, 347, 354-356, 357-360, 727] studies in which the relative rankings of either mortality or frequency or both were atypical and these atypical studies were not necessarily the smallest studies. For example, the ICU studies (Table 2.4.1.5) have a more even distribution of the three GN bacteremia types.

o. Even within an oncology study, there can be differences in mix and mortality risks for sub-groups with hematological versus solid tumour malignancies [352].

p. Survival curves of patients with GN bacteremia were available for three single center studies [342, 343, 356]. For two large studies, mortality following the first positive blood culture was 12% versus 26% at day 5 versus day 30 [343] and 20% versus 30% at day 5 versus day 21 [342]. In a third smaller series found that 50% of day 30 mortality had already occurred by day 7 [356]. This observation is relevant to studies of prognosis reported in this thesis (see 5.2).

q. There are three studies of anti-endotoxin therapies [349-351] tabulated here (Table 2.4.1.3). These studies were not used in deriving the IQ range shown here but are shown for comparison and are discussed further in 2.5.2.

r. Among the studies of interest in this thesis [1-107], the four studies with the largest number of GNB [21, 38, 47, 65] are tabulated here (Table 2.4.1.5 & Figure 2.15) to enable a comparison with other studies in the literature.
2.5. The endotoxin-sepsis ‘disconnect’

The term ‘sepsis disconnect’ has been used to describe the failure to translate anti-endotoxin (and other immuno-modulatory) agents from promising pre-clinical evidence to proven clinical therapy [416] (this disconnect is explored in 2.5.1). One expert in the area of endotoxin and sepsis expressed despair at the low return from “a quarter of a century of research involving tens of thousands of patients and several billion research dollars” [418]. Likewise, a meta-regression analysis asked why “despite promising pre-clinical testing and the expenditure of several billion dollars, anti-inflammatory agents designed to inhibit specific host mediators failed to show benefit in 22 clinical trials involving over 10,000 patients” [298]. This meta-regression questioned whether variability in the underlying risk of death between preclinical versus clinical studies could be an explanation for the disconnect. However, there is no simple explanation for this ‘disconnect’.

Moreover, there are numerous paradoxical discrepant findings between the preclinical studies on the one hand and the randomized controlled clinical trials of the same agent on the other (described in 2.5.1) [298, 416-426]. Hence the term ‘endotoxin-sepsis disconnect’ could be extended to positive results from the early clinical studies with anti-endotoxin therapies which raised expectations for progress in the development of these therapies [422]. Failure to replicate these findings in later studies, also represents a paradoxical ‘disconnect’ [423, 424] (this disconnect is explored in 2.5.2). Some additional literature is cited and discussed further in EOID ’95, Drugs 1994 and Drug Safety ‘95.

The term could be even further extended to describe conflict amongst clinical observations regarding the prognostic value of endotoxemia detection in various settings (this disconnect is explored in 2.5.3 and chapter 5.2).

These three examples of ‘disconnect’ are reviewed here to the extent that they have a bearing on the analysis of the clinical relevance of endotoxemia detection, GN bacteremia and outcome in gram-negative sepsis as raised within this thesis. Anti-inflammatory agents that have been developed to block mediators (e.g. anti-TNF, anti-PAF) whose release is stimulated by endotoxin and hence are ‘down-stream’ are not considered here although these also each exemplify a paradoxical disconnect.
2.5.1. Anti-endotoxin therapies: Pre-clinical disconnect

Despite 40 years of research with at least six candidate anti-endotoxin therapies each having shown promise in pre-clinical and early clinical trials, no anti-endotoxin approach to the treatment of sepsis has emerged with proven clinical benefit. There is an extensive literature dealing with the pre-clinical and clinical evaluations on this subject which is complex and difficult to summarize. The extent to which these various anti-endotoxin therapies bind to endotoxin, neutralize its activity and mediate mortality-benefit in experimental studies are each contentious [423-426]. For example, a review article which collates the evidence for and against one extensively studied hypothesis, - that antibodies to the inner core raised by immunization with enterobacterial deep rough mutants confer broad spectrum protection during gram-negative bacterial sepsis, cites over 200 references published up to 1997 [427]. The most paradoxical suggestion is that some of the anti-endotoxin monoclonal antibodies and recombinant proteins under study had been contaminated by endotoxin in the production process [428-429]. This endotoxin contamination from the source bacteria could account for the difficulties in replicating the protection in later pre-clinical studies.

Another issue that is difficult to bridge between pre-clinical versus clinical studies is the possibility that anti-endotoxin therapies may be more effective in some patient sub-groups if only these could be identified [275]. For the purpose of this thesis, a question of particular interest in this contentious area of research is the evidence pertaining to the relationship of mortality benefit to reductions in levels of circulating endotoxemia.

Some of the anti-endotoxin therapies which have been developed are summarized in Tables 2.5.1 / 2.5.2 & 2.5.3. The agents listed in these tables have anti-endotoxin activity which is mediated either through binding to lipid-A or through blocking the effects of endotoxin presumably at the level of the receptor. However, even these pre-clinical binding and mechanism of action studies are controversial and in some cases have been difficult to reproduce [460]. Not shown in these tables is a range of other biological agents designed to inhibit downstream immunological mediators whose release is triggered by endotoxin which have been evaluated in randomized clinical trials of the patient group with sepsis. Recent commentaries [419,
and a systematic review of 45 reviews of animal studies of therapeutic interventions for sepsis found evidence of over-extrapolation of the preclinical research findings and an under-appreciation of the deficiencies in study methods [462]. Recently it has been proposed that the strength of inference from animal studies to the clinical context could benefit from the application of systematic review methods that address the risk of bias [462].

The study design aspects of the pre-clinical studies in which anti-endotoxin efficacy have been evaluated are worthy of a reflection. The following quote from LJ Berry, a former President of the International Endotoxin Society, describes the complex challenges in developing appropriate animal model systems in which to test anti-endotoxin therapies.

"Few if any biological materials have been the object of as many scientific investigations as bacterial lipopolysaccharide or endotoxin. It poses challenges to chemists, physiologists, immunologists, microbiologists, pharmacologists, endocrinologists, biochemists, anatomists, or structural biologists, pathologists, internists, surgeons, infectious disease specialists and others. A substance with appeal as broad as this is rare in science. In these introductory remarks, I want to address three issues (a) the question of dosages that investigators of endotoxin have used, (b) the importance of route of endotoxin administration in natural and experimental situations, and (c) the importance of the variation of [animal] species in the type of responses elicited by endotoxin. One of the most serious offenses in endotoxin research has been the use of excessively high doses. Everyone should be cognizant of the points made here before assuming that observations made under one set of conditions are typical of the biological effects of endotoxin under all circumstances." [LJ Berry, Introduction, in, Handbook of Endotoxin, Vol 3 Cellular biology of endotoxin. pages xvii-xxi, Editor LJ Berry, Elsevier 1985] [463].

These comments illustrate that in designing experimental models to evaluate anti-endotoxin agents for clinical use, an optimal animal model needs to be more than simply an ‘intoxication’ model [464-466]. Moreover, any beneficial effect would presumably need to be additional to that provided by supportive and other therapies such as antibiotics. A complex study design which more closely reflects the sepsis dynamic in the clinical setting is desirable but difficult to achieve. One widely studied example is a canine model of septic shock which is described here [337, 466-475].

Controlled models of septic shock have been developed in dogs, in rats and also in primates by workers at the National Institutes of Health (NIH). These models have
generated unexpected and paradoxical observations bearing on bacteremia, endotoxemia and outcome [124-126, 337, 467-475]. In the dog, implantation of an intra-peritoneal infected clot induces bacteremia with various selected gram-negative and gram-positive challenge bacteria together with cardiovascular changes characteristic of septic shock leading to lethality [126, 470-471]. The outcomes of any therapeutic interventions are studied under controlled conditions simulating a clinical trial including randomized treatment assignment and investigator blinding [337]. This model enables the study of the relative effects of intervention with antibiotics, cardiovascular support and anti-endotoxin therapies on hemodynamic changes, survival time, quantitative levels of bacteremia as well as quantitative levels of endotoxemia as measured with the chromogenic LAL assay. A series of paradoxical observations have emerged from studies with this model and are of interest to the research questions of this thesis bearing on the concordance or not of bacteremia and endotoxemia and outcome.

1. Firstly, in comparative studies of a relatively avirulent strain of *E. coli* versus respectively *P. aeruginosa* [123], *Staphylococcus aureus* [126] or a more virulent strain of *E. coli* [469], similar quantitative levels of bacteremia were observed whereas the associated hemodynamic changes and shortened survival time were in each case more severe versus those observed in association with the avirulent *E. coli* strain, as would be expected [123, 126, 469]. Surprisingly, despite these expected differences, in each case, the levels of endotoxemia were either three [469]- to ten [123]-fold lower or even undetectable [126] versus the levels seen after challenge with the avirulent *E. coli* strain. Interestingly, endotoxin extracted and purified from the virulent and avirulent *E. coli* strains were equal with respect to potency and endotoxin amount per bacterium and hence the dissociation between endotoxemia and disease severity in this experimental model could not be explained on this basis [469].

2. Moreover, following intra-peritoneal challenge with strains of *E. coli* which have (O6:H1:K2) or do not have (O86:H8) virulence factors for human disease, survival time was shorter and the associated hemodynamic changes were more severe after challenge with the virulent strain, as might be expected. However, there are three paradoxical observations as follows; (1) bacteremia occurred earlier and more frequently after challenge with the avirulent *E. coli* strain, (2) levels of endotoxemia were three-fold higher after challenge with the avirulent *E. coli* strain and (3) challenge with heat killed bacteria at a ten-fold higher dose was associated with a
reversal of the effects on survival and hemodynamic change seen with live bacterial challenge: the survival was now significantly shortened after challenge with the killed non-virulent bacteria versus the killed virulent bacteria. Despite this reversal, the levels of endotoxemia were again three-fold higher after challenge with the killed avirulent versus the killed virulent *E. coli* strain [469].

3. A more strikingly paradoxical observation arose when the following putative anti-endotoxin interventions were studied; two anti-endotoxin therapies, lipid-X [467] and a monoclonal anti-endotoxin antibody (HA-1A) [337], reconstituted human HDL lipoprotein [473], and plasma exchange [472]. These interventions were each separately studied versus control treatments in this model for the effect on survival and the associated levels of bacteremia and endotoxemia following the *E. coli* challenge. The effects of these interventions on the levels of endotoxemia resulted in a lowering [473], or no effect [337], or were not measured [467, 472]. Surprisingly, in each case, the therapies either failed to increase survival or even paradoxically resulted in worsened cardiovascular effects or shortened survival despite similar quantitative levels of bacteremia versus control treated animals.

4. Studies of the protective effect of various monoclonal antibodies (MAB) against an *E. coli* challenge in the canine and other models also generated somewhat unexpected results bearing on bacteremia and endotoxemia. Both an isotype matched core region specific MAB and an O-side chain type specific MAB conferred protection additional to that provided by antibiotic and supportive therapy in this canine model [468]. The mechanism of protection differed, for the O-side chain specific MAB being through enhanced clearance of the bacteremia and endotoxemia whereas the core region specific MAB partially decreased circulatory collapse associated with the endotoxemia but without any measurable reduction in the bacteremia or endotoxemia compared to control treated dogs.

5. Studies by this NIH investigator group of the protective effect of recombinant granulocyte colony stimulating factor (rG-CSF) versus control treatment generated results bearing on bacteremia and endotoxemia and outcome in a canine pneumonia model induced by an *E. coli* intra-tracheal challenge [475]. The prophylactic administration of rG-CSF reduced levels of endotoxemia and improved survival in association with enhanced peripheral blood neutrophil counts in this canine model of *E. coli* pneumonia compared to control treatment. The quantitative bacteremia counts were similar in the treatment and control groups.
6. Finally, studies of E5564, a competitive lipid-A antagonist was studied by this NIH group in an *E. coli* challenged rat model of sepsis [476]. This model demonstrated that higher doses of E5564 were required to protect the rats from intra-vascular versus extra-vascular *E. coli* challenge. The paradoxical finding of this study was that despite this protection, E5564 increased the limulus reactivity in plasma.

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Foot notes:
Mort = mortality. Onc = oncology patient group, Surg = surgical patients group, GNI = GN infection incidence. ND = No data. ↓ = decrease. ↓* = decrease noted in a subgroup. ↔ = no significant change.

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Foot notes:
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<td>Onc</td>
<td>21</td>
<td>↓</td>
<td>ND</td>
</tr>
<tr>
<td>Behre</td>
<td>'95</td>
<td>443</td>
<td>IVIG</td>
<td>Onc</td>
<td>52</td>
<td>↓</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Monoclonal antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ziegler</td>
<td>'91</td>
<td>350</td>
<td>HA-1A</td>
<td>H</td>
<td>200</td>
<td>§</td>
<td>↓*</td>
</tr>
<tr>
<td>Greenman</td>
<td>'91</td>
<td>351</td>
<td>E5</td>
<td>H</td>
<td>212</td>
<td>↓*</td>
<td>ND</td>
</tr>
<tr>
<td>Fisher</td>
<td>1990</td>
<td>444</td>
<td>HA-1A</td>
<td>34</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>French Reg</td>
<td>'94</td>
<td>445</td>
<td>HA-1A</td>
<td>H</td>
<td>600</td>
<td>↑</td>
<td>ND</td>
</tr>
<tr>
<td>McCluskey</td>
<td>'94</td>
<td>446</td>
<td>HA-1A</td>
<td>H</td>
<td>2199</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Angus</td>
<td>'00</td>
<td>447</td>
<td>E5</td>
<td>ICU</td>
<td>1090</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Daifuku</td>
<td>'92</td>
<td>448</td>
<td>MAB-T88</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Greenberg</td>
<td>'91</td>
<td>449</td>
<td>E5</td>
<td>39</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Greenberg</td>
<td>'92</td>
<td>450</td>
<td>E5</td>
<td>39</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Albertson</td>
<td>'03</td>
<td>451</td>
<td>ECA-Ab</td>
<td>ICU</td>
<td>411</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Other endotoxin agents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bion</td>
<td>'94</td>
<td>8</td>
<td>SDD</td>
<td>ICU</td>
<td>59</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Willats</td>
<td>'95</td>
<td>452</td>
<td>Taurolidine</td>
<td>ICU</td>
<td>100</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Reinhart</td>
<td>'04</td>
<td>453</td>
<td>iHSA</td>
<td>ICU</td>
<td>143</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Bennett-Guerrero</td>
<td>'07</td>
<td>454</td>
<td>E5564</td>
<td>Surg</td>
<td>152</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Tidswell</td>
<td>'10</td>
<td>455</td>
<td>E5564</td>
<td>ICU</td>
<td>235</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Unpublished</td>
<td>'12</td>
<td>456</td>
<td>E5564</td>
<td>ICU</td>
<td>1379</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Dellinger</td>
<td>'09</td>
<td>457</td>
<td>PLE</td>
<td>ICU</td>
<td>36</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Heemskerk</td>
<td>'09</td>
<td>458</td>
<td>ALP</td>
<td>ICU</td>
<td>36</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>Pickers</td>
<td>'12</td>
<td>459</td>
<td>ALP</td>
<td>ICU</td>
<td>36</td>
<td>↔</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Foot notes:**

- SDD = selective digestive decontamination, iHSA = human serum albumen, ALP = Alkaline phosphatise.
- Mort = mortality. Onc = oncology patient group, Surg = surgical patients group, H = hospital wide group, GNI = GN infection incidence
- ND = No data
- ↓ = decrease, ↓* = decrease noted in a subgroup, ↑ = increase, ↔ = no significant change
- § Two studies are noted here to have a high proportion of non- *E. coli* Enterobactericeae relative to *E. coli* amongst the GN bacteremias;
  - Schedel [442], of 55 GN bacteremias 30 were non- *E. coli* Enterobactericeae and 12 were *E. coli*.
  - Ziegler [350], of 200 GN bacteremias 71 were non- *E. coli* Enterobactericeae and 87 were *E. coli*. 
The summation of these paradoxical observations made over the course of these studies lead this group of investigators to consider whether endotoxemia has continued importance once the inflammatory response and the clinical manifestations have been initiated in that possibly…. “tolerance to endotoxemia may develop within hours ([162]), and persistently high levels of endotoxin may not be central to the manifestations of the sepsis syndrome” [125].

Another research group has also examined the two anti-endotoxin interventions, bactericidal/permeability increasing protein (BPI) which is an endogenously produced human endotoxin neutralizing protein, and a monoclonal anti-endotoxin antibody (HA-1A) with a lethal \textit{E. coli} challenge. The plasma endotoxin levels were significantly reduced compared to no treatment after the use of BPI but not with HA-1A. However, whilst both treatments attenuated cytokine release, neither improved survival [477].

2.5.2. Anti-endotoxin therapies: clinical disconnect

The impetus for the development of anti-endotoxin therapies had been supported by a range of clinical observations (\textit{Table 2.5.1}) [478, 479]. In the 1970’s, several clinical studies suggested that improved outcome of patients with sepsis correlated with the presence in patient serum of high levels of antibodies to core antigens of lipopolysaccharide as expressed in a mutant \textit{E. coli} J5 strain [430-432]. These observations led to studies using various preparations of anti-sera prepared from volunteer donors specifically immunized using the mutant \textit{E. coli} J5 strain [433-437]. Anti-sera production is an expensive and impractical undertaking and later studies used immunoglobulin preparations produced commercially or specific monoclonal antibodies [349-351, 444-451].

The results of these studies are contentious as there were a number of different study designs leading to difficulties in interpretation [478, 479]. Various preparations of anti-sera or immunoglobulins were used, some containing both IgM and IgG, other preparations only IgG. Some studies used a therapeutic approach and some chose to target high risk patient groups for prophylaxis. The end point of interest varied, in some studies being the occurrence of GN bacteremia (e.g. studies of a prophylactic approach; \textit{Table 2.5.1}) versus in other studies being the outcome as mortality in studies (e.g. studies of a treatment approach; \textit{Table 2.5.2/3}). The target patient groups under study also varied. Some anti-sera studies used normal (non-immunized) immunoglobulin
preparations as the intervention for control group patients. However, the choice of normal immunoglobulin preparations for control group patients is problematic as it is possible that even these may have efficacy in patients with sepsis with a presumed anti-endotoxin mechanism [4, 481, 482] mediating possible mortality benefit [481], although not seen in all studies [482].

A small number of studies listed in Table 2.5.1/2.3 have used the detection of endotoxemia as either a criterion for study entry or as a quantitative outcome of intervention [4, 441, 442]. In this regard, the strongest evidence that quantitative levels of endotoxemia are related to outcome comes from patients with meningococcemia. However, three studies of anti-endotoxin therapies have failed to find any significant mortality benefit in this condition (Table 2.5.2). One study that examined the effect of endotoxemia detection on outcome using a logistic regression model found no significant interaction effect from the intervention [438] and another study examined levels of endotoxemia but did not report the impact on outcome [439].

**Polymyxin B** The anti-endotoxin intervention with the strongest evidence for endotoxin binding and neutralization in pre-clinical studies and with the most published clinical experience is polymyxin B [483-486]. Polymyxins are from a group of cyclic cationic polypeptide antibiotics and these have well characterized lipopolysaccharide binding (presumably lipid-A) associated with inhibition of endotoxin activity as measured in vitro and in whole animal studies. Toxicity limits the clinical use of polymyxin B as an antibiotic [485]. However, polymyxin B can be bound to a solid phase such as a hemo-perfusion column [485]. This enables hemo-perfusion as a method for the removal of circulating endotoxemia through exposure to immobilized polymyxin B without the systemic toxicity. This method is being actively explored in clinical trials.

It is notable that the endotoxin antagonist property of polymyxin-B is dependent on the bacterial origin of the LPS. Amongst the preparations of LPS from eight different GN bacteria, Polymyxin was most inhibitory against the LPS of *E. coli* and least inhibitory against the LPS of *N. meningitidis*. A paradoxical finding from this study was that while polymyxin at low concentrations was inhibitory to the effects of endotoxin, at concentrations greater than 50 mcg/ml polymyxin acted synergistically with endotoxin in inducing IL-1 secretion [484].
Cruz et al [487, 488] systematically reviewed the polymyxin-B hemo-perfusion literature published to 2006 and found 28 publications (Table 2.5.4). The results of these studies were significantly heterogeneous. All but two of the 28 publications had come from groups in Japan and many were small non-randomized and unblinded studies. There were 17 studies that had reported the effects of polymyxin-B hemo-perfusion on levels of endotoxemia amongst which there had been possible “over-representation of two centers representing several of the 17 studies in the meta-analysis” [488]. A sensitivity analysis undertaken to allow for possible duplicate reporting, with removal of the duplicate studies, had found results similar to the results in Table 2.5.4 from polymyxin-B hemo-perfusion (data as abstracted in Cruz et al 2007 [488]).

### Table 2.5.4 polymyxin-B hemo-perfusion
(data as abstracted in Cruz et al 2007 [488])

<table>
<thead>
<tr>
<th>Reduction in endotoxin levels (pg/ml)</th>
<th>number of studies</th>
<th>Effect size</th>
<th>95% CI</th>
<th>heterogeneity (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All studies</td>
<td>17</td>
<td>21.2</td>
<td>17.5 - 24.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Center duplication</td>
<td>6</td>
<td>16.4</td>
<td>8.9 - 24.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mortality (risk ratio)</th>
<th>number of studies</th>
<th>Effect size</th>
<th>95% CI</th>
<th>heterogeneity (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All studies</td>
<td>15</td>
<td>0.53</td>
<td>0.43 – 0.65</td>
<td>0.07</td>
</tr>
<tr>
<td>studies with n&gt;20</td>
<td>7</td>
<td>0.56</td>
<td>0.46 – 0.68</td>
<td>0.17</td>
</tr>
<tr>
<td>Center duplication</td>
<td>8</td>
<td>0.61</td>
<td>0.46 – 0.82</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Foot notes:
1. The heterogeneity p-value indicates the variability between study results compared to the amount that would be expected due to random variation.
2. The reduction in endotoxin levels represents a decrease estimated by Cruz et al to be between 33% to 80% from pre polymyxin-B hemo-perfusion levels.
3. Does not include the most recent polymyxin-B hemo-perfusion study (Cruz et al 2010 [491]) or study of Reinhart ([453] see Table 2.5.2).
with all studies included and, moreover, the associated significant heterogeneity remained. Whilst the Japanese studies suggest that levels of endotoxemia were reduced by polymyxin-B hemo-perfusion [489], this was not found in one small (n=36 [490]) European study. A second small European study in 24 septic patients of whom 11 had high endotoxin activity (EA) as measured using a chemo-luminescent assay [494] had found reductions in EA with polymyxin-B based hemo-perfusion and none of the 24 patients died.

Cruz et al concluded that there was a need for confirmatory studies in adequately powered studies with prospective, blinded and randomized study designs, two of which are summarized here [491, 494].

A multi-center European study of post-surgical patients with severe sepsis or septic shock secondary to intra-abdominal infection, found no difference in mortality; 5 of 17 (29%) patients receiving polymyxin-B hemo-perfusion versus 5 of 18 (28%) patients receiving conventional therapy [494]. This study used evidence of a GN infection or detectable endotoxemia as criteria for patient inclusion in this study. However, the endotoxemia levels measured before and after the polymyxin-B hemo-perfusion were not significantly different.

A subsequent study, the EUPHAS study (Early Use of Polymyxin Hemoperfusion in Abdominal Septic shock) was of a similar design, a similar patient group (post-surgical patients with severe sepsis or septic shock secondary to intra-abdominal infection) and was double the size (64 patients) of the previous European study [491]. EUPHAS was conducted in 10 Italian ICU’s over a 3 year period (2004-2007) with clinical severity indices and mortality as measures of outcome. The study was terminated after an interim analysis revealed that a statistically significant and seemingly impressive reduction in mortality had become apparent between the groups after the enrolment of 64 patients. The reduction in 28 day mortality achieved in this study was as follows; 11 of 34 (33%) patients with polymyxin-B hemo-perfusion versus 16 of 30 (53%) patients with conventional therapy [491].

However, there are four criticisms of the EUPHAS study [721, 722]. One is that even at 64 patients, it is a small study. Also, the number of bacteremias found in the two groups was unequal; 16 among the patients receiving polymyxin-B hemo-perfusion versus only 3 among the patients receiving conventional therapy, a highly significant and unexplained imbalance. A third criticism is that the study results were statistically significant only according to an analysis based on the relative survival time and not
absolute survival. That is, survival had been prolonged by a few days only (adjusted hazard ratio 0.36; 95% CI – 0.16-0.8) but not by a Fisher’s exact test (p = 0.13).
Finally, a criticism of the EUPHAS study is that being a preliminary study, endotoxemia levels were not measured. These study deficiencies are to be addressed in two follow up multi-center studies, the EUPHRATES (Evaluating Use of Polymyxin Hemo-perfusion in an RCT of Adults treated for Endotoxemia and Septic shock) which is to be undertaken in North American ICU’s together with the EUPHAS2 which is to be undertaken in Italian centers.

Another European study of endotoxemia adsorption using hemo-perfusion randomized 143 septic patients with suspected gram-negative sepsis [453] to receive either standard therapy alone versus standard therapy plus an extracorporeal endotoxin absorber. In this study [453], the endotoxin absorber was immobilised human serum albumin (iHSA), not polymyxin. There was a non-significant trend toward lower EA with the extracorporeal endotoxin absorber treatment but no difference was found in the primary end point overall (APAHEII score) or survival [453].

2.5.3. Prognostic value of endotoxemia: clinical experience disconnect

The prognostic value of endotoxemia detection in the setting of the critically ill patient has been studied in over thirty studies (literature cited in [497]). Conflicting conclusions became apparent from the earliest studies undertaken [25, 54, 75].

The prognostic value remains unresolved despite 17 large studies including over 2000 patients [11, 21, 23, 25, 37, 38, 48, 54, 65, 74, 75, 86, 89, 91, 95, 102, 104, 106]. On the one hand, in six studies endotoxemia was predictive of septicemia onset or severe illness [11, 54, 75, 91, 95, 106] and hospital mortality [11, 54, 91] among studies of hospitalized patients not restricted to an ICU setting. On the other hand, 13 studies including seven among patients restricted to ICU settings found the detection of endotoxemia either did not predict organ dysfunction or mortality [23, 25, 37, 74, 75, 48, 91], predicted mortality but not organ dysfunction [65], predicted organ dysfunction but not mortality [21, 38, 104, 106], or predicted mortality only when the level of endotoxemia was combined within a lipo-polysaccharide cytokine composite score [102]. Of note, in only three [11, 54, 95] of these 17 studies did the mortality difference between the groups positive versus negative for endotoxemia exceed 20 percentage points.
In the course of searching the literature for studies meeting the inclusion criteria (see chapter 4), over 200 studies were found that failed to meet the inclusion criteria of which 176 are listed amongst the references [498-673]. Amongst these studies are studies undertaken in the setting of patients with the following conditions or procedures; peri-operative (n=33 [498-530]), pancreatitis (n=7 [531-537]), liver and alcoholic disease or liver transplantation (n=35 [538-572]), urological (n=8 [573-580]), trauma or critical illness (n=21 [581-602]), pediatric (n=11 [603-614]), sepsis and specified infections (n=22 [615-636]), treatment studies (n=6 [637-642]), and other including metabolic, heart failure [643-669] and veterinary and animal models (n=31 [298, 337, 467-477, 670-673]). The most common reasons for exclusion of these studies from the analysis undertaken within this thesis were the lack of blood culture or mortality data.

There is an extraordinary breadth of populations, end points and associations among these excluded studies [498-673]. For example, there are studies of endotoxemia arising in populations of hemodialysis patients [599, 600] and elderly people [646], studies of endotoxemia arising where the exposure is mastication [645], malaria [632, 633] and marathon running [661, 662], or studies where the association is heart failure [665, 668] and diabetes [663]. These excluded studies are not further referred to in this thesis except to state as an observation that the scarcity of GN bacteremia, and mortality data among these studies itself raises questions regarding the overall significance of endotoxemia in the broader context in which endotoxemia has been studied and which itself represents something of a disconnect. On the basis of observations such as these, it has been questioned in an editorial as to whether ‘endotoxinaemia’ rather than ‘endotoxemia’ would more accurately describe the uncertainty regarding the clinical significance of endotoxin in the circulation [674].

Given the plurality of observations above, do Koch’s postulates as originally proposed or as the modified Molecular Koch’s [675, 676] postulates help us to evaluate endotoxemia as a pathogenicity trait? Koch’s postulates originally arose in the context of highly pathogenic organism-disease relationships such as tuberculosis and anthrax. The original postulates are somewhat blunt and are not so simple to interpret in the case of infections with viruses or opportunistic bacteria where ‘pathogenicity’ depend on a host’s compromised or non-immune state. The modified Molecular Koch’s postulates arose in the molecular age to identify pathogenicity elements that could be transferred into or out of bacteria by gene transfer [676] in the setting of a controlled laboratory
experiment. However, these Molecular postulates are not simple to interpret in the case of clinical observations in which bias and confounding operates to an uncertain and often unmeasurable degree.

2.6. Meta-analysis: Towards a resolution

The inter-relationships between endotoxemia, GN bacteremia, patient outcome and the effects of anti-endotoxin interventions have been extensively studied with conflicting observations arising from multiple sources. Conflicting estimates in this area are not unusual and additional examples of difficulties toward deriving a summary estimate exist. For example, studies of the efficacy of appropriate antibiotic therapy toward treatment success, a seemingly fundamental intervention for the ICU patient [411], have generated conflicting results. For example a systematic review of 51 studies of appropriate antibiotic therapy found evidence of significant benefit toward outcome in 28 studies versus 21 studies which did not [412]. A more recent systematic review of 70 studies of this topic found considerable heterogeneity among all 70 studies ($I^2 >70\%$) [413]. Likewise, a meta-analysis of 17 studies of the value of combination therapy for GN bacteremia found studies with evidence for, against, and neutral being found in two, one and 14 studies respectively [414]. Likewise, among 21 randomized controlled trials in a meta-analysis of adjunctive steroid use in sepsis, there were three studies which demonstrated significant benefit with steroid use, one study that demonstrated significant harm and the remaining 17 studies were neutral [415].

Given the many fundamental uncertainties that remain in our understanding of the relationship between the detection of endotoxemia, the detection GN bacteremia, the type of GN bacteremia and patient outcome, it needs to be asked how these can be resolved. On the one hand, it is unlikely that further animal model research could resolve these uncertainties given the key role of patient factors and underlying risk in the inter-relationships. On the other hand, it is unlikely that a single clinical study would definitively resolve the uncertainties in this area given the breadth of patient groups and GN bacterial types that such a study would need to include [677-679]. To paraphrase Einstein, “Can a solution be derived at the same level at which the problem arose?”

The first step towards resolving the uncertainties is to measure the extent of
variation among the study results. The second step is to determine whether the
published study results are reconcilable with each other and with overall variability in
the broader literature. Meta-analysis is a method by which these steps can be achieved
[680-683]. The methodology used in this thesis is described elsewhere in this thesis
(see Chapter 4.2 and also EOID 1997 & APLM 2011) but the logical development is
described here.

It is first necessary to clarify what is a meta-analysis and what is a systematic
review. The distinction between these is clarified here in part to address a common
misconception as reflected in the comment of a reviewer of one of the compiled
publications who questioned “... the potential for bias as a result of singleton
authorship. This is definitely not the gold standard for systematic reviews or meta-
analyses in 2010....” In contrast to this reviewers’ opinion, the definitions for these
terms within The PRISMA (Preferred Reporting Items for Systematic Reviews and
Meta-Analyses) statement 2009 [684] and are as follows;......

“A systematic review is a review of a clearly formulated question that
uses systematic and explicit methods to identify, select, and critically appraise
relevant research, and to collect and analyze data from the studies that are
included in the review. Statistical methods (meta-analysis) may or may not be
used to analyze and summarize the results of the included studies. Meta-
analysis refers to the use of statistical techniques in a systematic review to
integrate the results of included studies.”

The PRISMA statement [684] includes a 27 item checklist which has been
derived as a consensus document. The “.....aim of the PRISMA Statement is to help
authors improve the reporting of systematic reviews and meta-analyses..... the PRISMA
checklist is not a quality assessment instrument to gauge the quality of a systematic
review.” The method of data abstraction is one checklist item and the use of two data
abstracters is given as an example rather than as a prescription. Hence authorship,
singleton or otherwise, is not one of the 27 checklist items.

A systematic review indicates a complete review of the available literature and
is usually undertaken to achieve an overall or average effect for a defined intervention
in a defined patient group toward the clinically relevant end-points. This is commonly
undertaken for the purpose of developing and defining an evidence based treatment
policy. This is not the intention for either this thesis or any of the compiled publications that are appended. Confusingly, meta-analysis is often the method by which each quantitative summary is derived within the systematic reviews. Hence meta-analysis is the method and systematic review is a formal undertaking in summarizing a collection of study results.

Meta-analysis can go beyond a simple summary to study the sources of variability in study results. Indeed where study results are highly variable within a systematic review, a summary effect size will be problematic and its value somewhat moot. However, deriving a quantitative summary is a necessary step toward both the recognition of heterogeneity among study results and its quantification. As the re-analysis of the data abstracted by Elin (see Tables 2.2.1/2) illustrates, finding differences among a panel of studies can provide additional insights into a collection of studies that cannot be derived from the individual studies in isolation or by a simple tally summation of all study results.

**Meta-analysis: an historical example.** An interesting historical example is now discussed to illustrate that meta-analysis can play a broader role outside of a systematic review and which is relevant to this thesis. In 1904, Karl Pearson (who had described Pearson’s correlation co-efficient and the chi-squared test) published in the BMJ an analysis of the impact of anti-typhoid vaccination on the incidence and mortality associated with typhoid in the British Army [685]. The previous analyses of the separate British army experience had been inconclusive and the issue was contentious. This analysis by Pearson is of interest to this thesis for five reasons; it is one of the first recorded meta-analyses undertaken (although the term ‘synoptical table’ rather than ‘meta-analysis’ was used to describe the analysis), the analysis concerned only the British Army experience, the analysis is derived from observational data under field conditions rather than randomized trial data, and the question at hand relates to a gram-negative bacteremic infection (typhoid) with both the occurrence of typhoid fever and mortality as the end points of interest.

Moreover, this historical example is of particular interest because the main finding of Pearson’s analysis was not simply a quantitative summary of the data. The main finding was that “Many of the groups ...are far too small to allow of any definite opinion being formed at all, having regard to the size of the probable error involved” [685]. That is, the data from the five different army deployments in India and South
Africa were too heterogeneous to be represented by a single unifying effect size associated with vaccine use and that more study was required. This was an important conclusion which was not otherwise identifiable without meta-analysis.

The issue of typhoid vaccination remained contentious. There was a critical reply from Sir Almouth Wright and a subsequent exchange between the two occurred appearing in the next six issues of the BMJ over the months of November and December, 1904. However, Sir Almouth Wright did acknowledge the deficiencies in the British army medical data that the analysis had identified. The epilogue to the typhoid vaccine study is that typhoid vaccination was adopted at the time despite the analysis of Pearson. It took another 50 years and the discovery of chloramphenicol, an effective treatment for typhoid, before randomized trials of typhoid vaccination were deemed to be ethically feasible.

This historical example illustrates the broader capability of meta-analysis beyond that of simply deriving a summary result within a systematic review. Sometimes, a more appropriate finding than a summary result is a measure of the amount of heterogeneity among the available evidence. Methods to quantify heterogeneity and to attempt to explain it using meta-regression have recently been developed for this purpose [313, 314, 686, 687]. The current state of these methods and their applicability to the research questions within this thesis is described in 4.2.

**Meta-analysis: limitations.** The limitations of meta-analysis are several and need to be considered. A frank article entitled "Meta-research: the art of getting it wrong" [688] appeared in the journal Research synthesis methods in 2011. The author of this article was JPA Ioannidis. He has authored many overviews of the meta-analysis technique and its impact on science as well as overviews on the sociology and epidemiology of medical authorship and publication. He has co-authored many original meta-analyses of topics including diagnostic tests and also an author of one of the first meta-analyses of studies of corticosteroid use in meningitis.

JPA Ioannidis summarized the evolution in seven published meta-analyses of studies of corticosteroid use in meningitis as follows;

- 1994; no question about benefits, but beware of harms;
- 1997; definite benefit only for some bacteria, limit to 2 days to avoid harm;
- 2003; definite benefit only for children, no increase in harm;
- 2003; correction: actually benefit is seen also in adults;
2007; benefit in high-income countries, but not in low-income countries;
2009; clear benefit, give to all, this is it;
2010; no benefit at all;”

and conceded that the conclusions “have been all over the place”. However, whatever the true value of corticosteroids in meningitis may ultimately prove to be, Ioannidis opined that these meta-analyses were able to reveal that the two most influential and highly cited original studies (both published in the New England Journal of Medicine) found treatment effects that in retrospect appear implausibly large.

It is not unprecedented that the finding of a large meta-analysis is overturned by unpublished data. Two recent examples are given here of where the influence of publication bias on the summary findings. One is that of a recent systematic review and meta-analysis eleven studies of plasma TREM-1 for sepsis diagnosis in systemic inflammatory patients [689]. Another example is the finding of one meta-analysis that found excess mortality related to cefepime [713] was subsequently refuted [714] after a large body of unpublished studies controversially appeared from the manufacturer of cefipime [715-718]. Hence, while publication bias is one of the most important threats to the validity of the conclusions of a meta-analysis, the technique at least offers an objective method for identifying this threat.
3. Aims and objectives of the thesis

3.1. A statement of the problem

Studying the relationships between the detection of endotoxemia versus gram-negative bacteremia and also versus outcome as mortality is problematic for the following reasons:

- the structure activity relationship of lipid-A (endotoxin, lipopolysaccharide) in the mediation of the various biological effects of lipid-A is not simple,
- the structure of lipid-A as found in different GN bacteria is not uniform,
- the recognition of lipid-A by the innate immune system is critically dependent on a hexa-acyl structure,
- there is a range of GN bacteremia types of interest among which there is a range in mortality risk which is variable from study to study,
- the defining criteria for sepsis are problematic in being overly sensitive and these continue to evolve,
- there are several patient groups in which these relationships have been studied (ICU, pediatric, oncology etc),
- GN bacteremia, sepsis, and presumably endotoxemia are conditions for which the attributable mortality is difficult to distinguish from that due to the underlying patient risk,
- while the limulus assay is the most widely used assay for the detection of endotoxemia, this assay is problematic in that;
  - it measures only one of several separately mediated biological activities of endotoxin,
  - the clinical experience with the assay is highly variable,
  - the version of the assay in use prior to 1985 relied on a gelation (G-limulus) end point whereas the version in common use since 1985 relied on the conversion of a synthetic chromogenic substrate (C-limulus). Overall, approximately half of the studies of endotoxemia detection used the more sensitive C-limulus assay and half used the original G-limulus assay,
  - it is not the only assay that has been used for the detection of endotoxemia, and
  - levels of endotoxemia are best interpreted on a logarithmic rather than on a linear scale.
- apart from studies of meningococcal disease, the evidence that quantitative endotoxemia detection correlates with outcome is conflicting,
- in treatment studies it is unclear whether clinical benefit is either dependent on
or follows from a reduction in endotoxemia levels,

- there is conflicting evidence that methods that block or inhibit endotoxin are clinically useful,
- some of the clinical studies that are in conflict are large and they would be difficult and expensive to reproduce, and
- there is a substantial ‘disconnect’ between preclinical evidence on the one hand, which is mostly strong and reproducible, versus the clinical experience on the other hand, which is variable to the point of being unreproducible.

In approaching this complex and multiply studied topic, it will not be sufficient simply to define the relationships between the detection of endotoxemia versus gram-negative bacteremia and also versus outcome as an average or as a typical finding. A new paradigm is required as it is of greater interest to define the extent of variation between study findings as a basis toward identifying possible reasons for conflict. Section 4.2 attempts to describe meta-analysis as the new paradigm to achieve this.

3.2. The Research Questions

The research described here addresses three primary questions related to the detection of endotoxemia in various clinical settings using the limulus (LAL) assay with respect to:

1. What is its diagnostic relevance versus GN bacteremia?
2. What is its prognostic relevance versus GN bacteremia? and
3. Are the disparate study results reconcilable?

There are six considerations in approaching these questions;

1. GN bacteremia is used as the reference standard for interpreting the diagnostic relevance of the detection of endotoxemia
2. Mortality is used as the reference standard for interpreting the prognostic relevance of the detection of endotoxemia
3. Does the diagnostic relevance of endotoxemia detection depend on which of the endotoxin detection assays is used? the type of GN bacteremia isolates found? the study setting?
4. Does the prognostic relevance of endotoxemia detection improve on that provided by the detection of GN bacteremia?
5. Does this prognostic relevance of endotoxemia detection depend on the types of GN bacteremia isolates? the underlying population risk in each study setting?
6. Can the disparate findings among the published studies be reconciled with the broader literature such as studies with incomplete data or studies that used non-limulus assay for endotoxemia detection?
4. Materials and methodology

4.1. Literature search methods

**Search strategy.** A review of the literature was performed repeatedly as detailed in the compiled publications (listed in section 1.2.1). This search was most recently done in June 2012 (Figure 5.0.1). The literature review included a search of the Medline database back to 1966 (but included publications prior to that date [97, 99]), with the search headings being "septicemia" (prior to 1992), "bacteremia" (from 1992 on), "endotox*", and "human." The search was not limited to reports published in the English language [19, 34, 44, 82, 92] and included conference proceeding abstracts [7, 62]. This was supplemented with a manual search of the references from each report retrieved, review articles, and textbooks.

Unpublished data were sought directly from the authors of studies who had published results in a form which did not meet the above criteria to seek a clarification that would have enabled inclusion (studies listed in section 7). This included the publication of a call for data (JER 2001) in addition to direct contact with the primary authors for additional unpublished data or clarification of published data, and these sources of data have been acknowledged in each publication where relevant. This correspondence is included within section 7 of this thesis.

**Generic inclusion criteria.** The following were the minimum criteria used in selecting studies for inclusion:

1. study design, which included a direct comparison of blood culture and LAL assay methods applied to blood from patients with suspected sepsis in various clinical settings;

2. size, with at least four patients studied; and

3. data presentation, such that each patient could be classified into one of four possible categories: Category 1, blood cultures positive for GN bacteria, and endotoxemia positive; Category 2, blood cultures positive for GN bacteria, and endotoxemia negative; Category 3, blood cultures negative for GN bacteria, and endotoxemia positive; and Category 4, blood cultures negative for GN bacteria,
and endotoxemia negative.

**Generic exclusion criteria.** Studies of animal models, studies of non-bacteremic infections, data insufficient or incorrect format (i.e. no comparison with GN bacteremia), or duplicate publications were not included.

The main data set of studies meeting the generic inclusion and exclusion criteria are listed in Table 7.1. Additional inclusion and exclusion criteria were applied as detailed within the individual publications in the compilation as relevant to which of the three research questions (i, ii, iii) was under examination within the individual publications in the compilation (see publication listed in 1.2.1).

**Supplementary studies.** (listed in Table 7.2). As part of updating this thesis, studies that were excluded from the main data set of studies were re-examined. A list of supplementary studies was compiled using the following criteria to select studies from the list of excluded studies; studies in which the patients had received an anti-endotoxin intervention, studies that had used a method for endotoxemia detection using an assay other than the LAL assay (e.g. rabbit pyrogen, endotoxin activity assay – EAA, or simply red endotoxin-SRE), and studies that had provided outcome data in relation to the detection of endotoxemia that was not extractable into the 4x4 format (as in (3) above). The outcome data of these supplementary studies and the main data set studies (which have a 4x4 format) were all re-extracted into a 2x2 format. This enabled a comparison of the results of the main data set to the additional data provided by these supplementary studies as a sensitivity test.

**Quality scoring.** Quality scoring is not recommended for studies of diagnostic tests [690] and their use to adjust the results of meta-analyses in general is controversial [691]. Instead, the approach taken here was to stratify the studies in relation to the following four equally weighted criteria;

- Whether the study design specified patient inclusion criteria in sufficient detail that would enable a replication of the study with respect to study setting and target patient population,
- Whether it was explicit that each patient test for endotoxemia was undertaken concurrently with a test for bacteremia,
- Whether patient outcome as mortality was specified with details of census time,
- Whether the list of GN bacteremia isolates was given.

Studies that met > 2 of these criteria were stratified as higher in quality of study.
design.

4.2. Analytic approach

The principal questions of this thesis require the following; firstly, deriving estimates for the average or typical effect size from the range of studies, and secondly, deriving estimates of precision and also heterogeneity among the range of studies. The analytic methods used in this thesis evolved over the time period of the publications (1994-2012) in three phases. Over this time period increasingly sophisticated analytic techniques for the analysis of proportion data became available in the STATA software used in this thesis. These techniques are described in the order of the three phases as they evolved. These phases are to be described in detail below but first it is necessary to describe the statistical issues and approaches to the analysis of proportion data [692-694].

Analytic issues. Throughout this thesis, proportion data is extensively presented and analysed arising in the following contexts;

- as single proportion data,
- as paired proportion data (e.g. as in meta-analysis),
- as correlated proportions (e.g. sensitivity and specificity resulting from a diagnostic test applied at different breakpoints), and
- as non-independent proportion data (e.g. mortality proportions from multiple clusters of infectious diseases).

To begin with, the simplest approach to the analysis of proportion data could be to convert and undertake the analysis as percentages rather than as the original proportion data. For example, the proportion 1/20 could be converted and analysed as a percentage value (5%). However, conversion of proportions to percentages is a form of data reduction as it fails to fully use all the information in the data (numerator and denominator). In particular, a statistical inference can be made from a proportion but not from a percentage.

Four basic issues relevant to the proportion data as found in this thesis are discussed here. In particular:

(i) Occasional small denominators (e.g. <20 patients),
(ii) Occasional zero numerators (e.g. 0 of 20 and also 20 of 20)
(iii) Variable precision of estimates due to variable group size (e.g. 2 of 20 versus 20 of 200).
Numerator being discrete integers (e.g. 2 of 20 patients).

**Single proportion data.** To illustrate some of the analytic issues and the available solutions, an example is given here for the calculation of the 95% confidence interval associated with an observed single proportion with a zero numerator and small denominator using the proportion 0/20. The simplest formula to estimate the probability and derive the standard error is the Wald method as follows:

- given k successes of n trials (in this instance 0 of 20),
- the estimated probability is \( p = \frac{k}{n} \) (in this instance 0 = 0/20) with
- standard error = \( \sqrt{\frac{p(1 - p)}{n}} \), being in this instance 0 = \( \sqrt{\frac{0(1 - 0)}{20}} \).

With an observed proportion such as 0/20, there are three possible inferences. One inference is that the occurrence of the event is impossible, in which case the true value is zero with zero standard error and a 95% confidence interval of zero (which is what the values derived in this instance using the Wald method would imply). Several more complex formulae exist to derive estimates of standard error for a single proportion which have better (non-zero) coverage in this context. These methods include the Wilson, Agresti-Coull and Jeffreys methods which in the case of 1/20, yield 95% confidence interval of 0-0.161, 0-0.189 and 0-0.116, respectively. Note that all of these intervals are asymmetrical being truncated with zero as the lower 95% confidence limit.

The second inference for an observed proportion such as 0/20 is that the observed proportion is simply one of a finite number of all possible outcomes. With a denominator as small as 20, all possible outcomes can be enumerated and a 95% confidence interval can be derived using a hyper-geometric distribution. For the observed proportion of 0/20, this is calculated as a result equal to or more extreme than 1/20 which yields a confidence interval as 0-0.168 being a 97.5% one sided confidence interval. This leads to a special case solution based on exact testing inference which enables the comparison of a limited number (e.g. two or three) of small denominator proportions (e.g. 0/20 versus 2/10). The most common example of this is Fisher’s exact test which arises in the context of conducting a chi-square test where one or more cell has an expected frequency of five or less. The result for the Fisher’s exact test is derived using permutation methods. In this example (0/20 versus 2/10) the result differs for the Fisher’s exact test (\( p = 0.103 \)) from that for the incorrectly applied Pearson chi-square test (\( p = 0.038 \)). An extension of the exact test is exact logistic regression (see...
A third and more plausible inference for our observed proportion of 0/20 is that the event is uncommon in which case the zero numerator observed simply is a reflection of the relatively small sample size (being a sample size of 20 in this instance). A solution that accommodates this uncommon event inference is the use of a continuity correction within a logit transformation of proportion data. This method overcomes several of the issues (i - iv) identified above. The logit (or logistic) transformation is used in logistic regression (see below). A logit transform of a proportion is the log of the odds. Hence for \( p = k/n \) this is \( \text{logit} = \log\left(\frac{k}{n-k}\right) \). Any zero here either in the numerator \( k \), or in the denominator \( n-k \) will require the addition of a continuity correction (e.g. 1 or 0.5) to avoid an undefined logit.

Note that the use of a continuity correction is consistent with the inference that the event is actually uncommon rather than zero. Hence, for an observed proportion such as \( p=0/20 \) and using 1 as a continuity correction, the logit transform of the inferred proportion \( \hat{p} = 1/20 \) is \( \log(1/19) \). The standard error of a log odds (by the delta method) = \( 1/\sqrt{\left(\hat{p}(1-\hat{p})*n\right)} = 1/\sqrt{(.05*.95*20)} \). The inferred proportion and the derived 95% confidence interval on back transformation is now 0.05; 0.006 – 0.31.

Note that in the 95% confidence interval as calculated using this transformation has two desirable properties; it is symmetrical when viewed on the logit scale and second, the calculated 95% confidence interval remains within the range 0 to 1 and cannot extend outside zero or ‘1’. Note that any proportion ‘\( p \)’ close to 0 (as in this example) or 1 will have a small standard error whereas the log odds of ‘\( p \)’ will have a large standard error.
**Fig 4.1** Illustration of three different potential representations of study effect size as could be represented within a L’abbe plot. These are where the study effect size is represented as:

- a risk difference (RD; e.g. equivalent to a 20% excess; wide solid line),
- a risk ratio (RR; e.g. equal to 2; dashed line) and
- an odds ratio (OR; e.g. equal to 2; dotted curve).

Note that the risk difference (RD) implies an assumption that the effect is constant over the range of event rates in group 1 whereas the odds ratio (OR) and risk difference (RD) imply that the effect is relative. See page 91 for calculations.

The line of equivalence (narrow solid line) represents all of the following effect sizes; a risk difference equivalent to a 0% excess, a risk ratio of 1 and an odds ratio of 1.
4.3. Three phases of statistical methods

The analysis has three phases corresponding to use of statistical methods of increasing sophistication. These various methods used in the three phases each have strengths, limitations and optimal applications to the research questions of the thesis (see summary in table 4.1). The more recent methods were not necessarily superior to previous approaches but together these methods collectively allow a “triangulation” of the data, whereby the results by these different methods can be compared and contrasted.

Table 4.1 provides a summary of the analytic methods used in this thesis.
### Table 4.1 Summary of analytic methods used in this thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in thesis phase *</th>
<th>Advantage</th>
<th>Disadvantages or limitations</th>
<th>Examples;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple methods</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>‘vote count’</td>
<td>NA ‡</td>
<td>simplicity</td>
<td>simplistic</td>
<td></td>
</tr>
<tr>
<td>Tally</td>
<td>1-3</td>
<td>simplicity</td>
<td>large study bias</td>
<td></td>
</tr>
<tr>
<td>Robust (median; IQR)</td>
<td>1</td>
<td>robust to outliers</td>
<td>non-parametric</td>
<td></td>
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<tr>
<td>Fixed effect methods [681, 683, 687; EOID 1996]</td>
<td></td>
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<tr>
<td>Mantel Haenszel †</td>
<td>1-3</td>
<td>traditional method &amp; enables;</td>
<td>fixed effects</td>
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<td></td>
<td></td>
<td>● Forrest plots</td>
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<td></td>
<td></td>
<td>● L’abbe plots</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meta-regression †</td>
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<td>only study level covariates estimation not included</td>
<td></td>
</tr>
<tr>
<td>Exact logistic regression</td>
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<td>robust to sparse &amp; unbalanced data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methods applicable to diagnostic tests [261-264, 311-312; AP&amp;LM 2011]</td>
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<tr>
<td>ROC / SROC</td>
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<td>diagnostic tests</td>
<td>fixed effects</td>
<td></td>
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<tr>
<td>HSROC</td>
<td>3</td>
<td>diagnostic tests</td>
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<td>random effects</td>
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<td></td>
<td></td>
<td>Computational complexity</td>
<td></td>
<td></td>
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<tr>
<td>Der Simonian &amp; Laird GEE §</td>
<td>2-3</td>
<td>quantification of heterogeneity</td>
<td>Computational complexity</td>
<td></td>
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<td></td>
<td></td>
<td>robust to clustered data</td>
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<td></td>
<td></td>
<td>Computational complexity</td>
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</table>

Foot notes:
* Phase 1 = 1994-2000; phase 2 = 2000-2009; phase 3 = 2009-2012 as described in section 4.3
‡ NA – not applicable as this method not used in the thesis. ‘Vote count’ as used in narrative reviews is a count of the number of positive studies
† Also adaptable to a random effects model.
§GEE = Generalized estimating equations
Phase 1 (up to 2000)

The Mantel-Haenszel (MH) method is the simplest technique to aggregate proportion data arising as paired proportions. The MH method dates from the 1950’s and is computationally simple. The method is described in detail in an appendix to JCM ‘94.

Paired proportions - Meta-analysis. For proportion data arising as paired proportions (e.g. treatment and control group mortality proportions, endotoxin positive and endotoxin negative group mortality proportions), the data within the pair of proportions is usually reduced to an effect size. To exemplify using the pair of proportions 4/20 (20%) and 4/40 (10%), this effect size would be derived as;

- the difference between the proportions (risk difference; RD = 10% = 20%-10%);
- the ratio between the proportions (risk ratio; RR = 2 = 20%/10%); or
- the ratio of the odds (odds ratio; OR = 2.25 = (4/16) / (4/36)). Note however that if either proportion in the pair is 0% or 100%, the RR and the OR will be indeterminate and use of the continuity correction (e.g. 1 or 0.5) is required to derive the effect size.

Several factors determine the choice among the effect measures (risk difference, RD; risk ratio, RR or odds ratio, OR) [695, 696]. The OR has useful mathematical properties including symmetry (See figure 4.1). Symmetry is the property where, for example, the study OR calculated for patients dying is the inverse of the OR for patients not dying. The RR lacks symmetry but has a more intuitive interpretation to general readers which the OR lacks. The OR approximates the RR for OR<2. The RD has the most intuitive interpretation but using the RD effect measure requires an assumption that the size of the effect is constant over conditions of different underlying risk. In this thesis the OR is the effect measure used most often as this avoids the constant effect assumption. However, in 5.2 the RD is used (alongside with the OR) to facilitate a description of the proprieties of the data here.

Meta-analysis is a two or three stage process. The first stage requires the derivation of an appropriate statistic (e.g. RR, OR, RD) from the paired proportions for each respective study in a set of studies. These summary statistics can readily be
Meta-analysis is applicable to summary statistics other than the OR, RR and RD. For example, meta-analysis can be applied to continuous data effect estimates (using the STATA® command ‘metan’ with continuous data options), can be applied to effect estimates and standard errors entered as pre-calculated from each study using the STATA® command ‘metan’ with pre-calculated effects estimates options (see phase 3 below) and can even be applied to p-values (using the STATA® command ‘metap’, not used in this thesis) or even to logits (see below).

The second stage consists of deriving a weighted average of these statistics across the set of studies. Various weighting methods can be used, but, in essence, the optimal weight for each study is the relative amount of information that each study provides toward the overall estimate. Most commonly the estimate of study precision, which is simply the inverse of the variance for that study (i.e. 1/standard error), is used as the weighting for each of the individual studies.

The third stage is the representation of the information within one of the several available graphical outputs from a meta-analysis. These outputs allow a visual comparison of both the data from each study to other studies and also in relation to the overall summary. There are three common graphical outputs and all three are used in this thesis. These are as follows; a forest plot, a meta-regression and the L’abbe plot. The choice of graphical presentation of the study results is of particular interest given that the reconciliation of conflicting results among the studies is one of the research questions of this thesis.

A forest plot is a vertical stacked array with the studies listed vertically with the effect sizes and 95% confidence intervals.

There are two types of meta-regression. The first is one in which the studies are stratified into sub-categories with sub-group analyses provided. This is usually done as a vertically stacked array. Alternatively, the relationship of study effect to some study measure of interest may be studied as a correlation. This relationship can be investigated with the studies within a regression plot of study effect size (y-axis) versus a continuous variable of interest in the regression (e.g. drug dose; x-axis). In this array, the studies are ordered along a bi-variate plot.

The third graphical output for meta-analysis to RCT’s is the L’abbe plot [681].
In a L’abbe plot, the effect size is not displayed. Instead, each study is represented as a point in a scatter plot of event rate in the intervention group (or case group; y-axis) versus event rate in the control group (x-axis). The graphical output that is homologous to the L’abbe plot from a meta-analysis of CSDT’s is a SROC which is a scatter plot in which each study is represented as a point of sensitivity (true positive rate) and false positive rate (1-specificity) for each study (further discussed in APLM ’11). The main advantage of a graphical output within a meta-analysis is that the heterogeneity amongst individual study results and within the overall result can be readily appreciated visually within the graphical output in addition to a formal estimation by specific statistical techniques.

**Phase 2 (2000 - 2009)**

There are three main limitations of the MH method. It is applicable only to binary (i.e. 2x2 tabular) data. The second is that the MH method is based on the fixed effects model. The fixed effect is less optimal where the assumption of a common (i.e. ‘fixed’) effect is less tenable as is usually the case with larger (e.g. > 10 studies) data sets. The third limitation is that being a fixed effect method, the MH method provides only a significance test for heterogeneity but not a measure of this.

From 2000, more computationally intensive methods extending beyond the three limitations above became more readily available, such as the Der Simonian - Laird method. Also, methods based on the ROC became available which were specifically adapted to deal with the meta-analysis of diagnostic tests.

**Correlated proportions.** While the most common applications of meta-analysis in the medical literature are in relation to summarizing the effects size in randomized controlled trials (RCT’s), this is not the only application. The meta-analysis technique is applicable beyond RCT’s with applications to observational studies in both the medical and non-medical literature. The conventional meta-analysis technique as applied to RCT’s has one major limitation with respect to paired proportion data of the type analysed in this thesis.

A limiting issue in the meta-analysis of paired proportion data as commonly undertaken is that the derivation of an effect size (i.e. risk ratio, odds ratio or risk difference) for each pair of proportions is a form of data reduction in that it fails to use
all the available data in the numerators and denominators. This data reduction is less of an issue in the meta-analysis of a series of RCT’s as the effect size (being OR, RR, RD) could reasonably be expected to have little variation between studies. In this respect the fixed effects method (Table 4.2) may be justifiable with this expectation that the effect size is uniform [682]. This assumption will be most tenable under trials conducted under standardized conditions and patient populations.

The consequence of data reduction to an effect size is most apparent in the meta-analysis undertaken for the meta-analysis of diagnostic tests or prognosis studies for two reasons. Firstly, the standardized conditions and patient populations is not a tenable assumption.

Also, with the meta-analysis of diagnostic tests, the overall sensitivity and

<table>
<thead>
<tr>
<th>Table 4.2 Meta-analysis: fixed effect versus random effects methods.</th>
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<tbody>
<tr>
<td><strong>Exemplary method</strong></td>
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<tr>
<td>Mantel-Haenszel (MH)</td>
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<tr>
<td>Presumption of homogeneity in ES&lt;sub&gt;i&lt;/sub&gt;</td>
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<tr>
<td>null hypothesis</td>
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<tr>
<td>grand mean ES calculated as weighted mean across all study ES</td>
</tr>
<tr>
<td>source of variation in study ES&lt;sub&gt;i&lt;/sub&gt;</td>
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<tr>
<td>variance for each study</td>
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<tr>
<td>weight for each study</td>
</tr>
<tr>
<td>relative weights for large versus small studies</td>
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</tbody>
</table>

Foot notes:
ES<sub>i</sub> = Effect size for study<sub>(i)</sub>
V<sub>i</sub> = Variance for study<sub>(i)</sub>
specificity of a diagnostic test are each of interest to potential users of the diagnostic test under evaluation and summation as an odds ratio, even a diagnostic odds ratio, fails to convey these two pieces of information. In this context, fixed effects methods are seldom justifiable and random effects methods are more appropriate [682]. Additional methods to aggregate proportion data have become available which avoid the data-reduction that occurs with meta-analysis (see below).

**Methods specific for correlated proportions.** For clinical studies of diagnostic tests (CSDT’s), there are two proportions of particular interest, sensitivity and specificity. However, among a series of studies, these two proportions will be negatively correlated. That is, one will be expected to increase as the other decreases across studies that have applied different diagnostic breakpoints. Analytical techniques to overcome this correlation have emerged to enable the evaluation of clinical studies of diagnostic tests (CSDT’s). For the evaluation of diagnostic tests by meta-analysis, the specialized STATA® command ‘metandi’ is available.

There are several similarities and differences between the applications of meta-analysis to RCT’s versus CSDT’s (discussed further in APLM 2011). The most striking similarity is that for both RCT’s and CSDT’s, the original data is in the binary (i.e. 2 x 2) format. The most crucial difference is that in the case of CSDT’s the binary data represents correlated proportions (sensitivity and specificity) versus the case of RCT’s where the proportions are not correlated. ‘Metandi’ fits a two-level mixed logistic regression model, with independent binomial distributions for the true positives and true negatives conditional on the sensitivity and specificity in each study, and a bivariate normal model for the logit transforms of sensitivity and specificity between studies (as in EJCMID ‘10). This is in contrast to the ‘metan’ command in the analysis of RCT’s, for which there is no such correlation within the data. A summation as a summary effect size as, for example a summary OR, with associated 95% confidence interval and measures of heterogeneity, is justified for RCT’s but not for CSDT’s.
Phase 3 (since 2009)

Since 2000, methods have become available for the analysis of multiple proportions arising from clustered data for which independence is not a tenable assumption. These methods are computationally complex and intensive.

Multiple proportion data and random effects. In the context of deriving inferences for multiple proportions, the following considerations (additional to i – iv above – p 87) arise as to the ‘best’ approach and method to;

(v) estimate either the average proportion or the expected proportion for a ‘typical’ study.
(vi) allow for denominators of varying size (e.g. denominators of 20, 200 and 2000) among the multiple proportions (= unbalanced),
(vii) estimate the 95% confidence interval for the average proportion,
(viii) estimate the heterogeneity among the observed proportions,
(ix) optimally compare the multiple proportions to each other,
(x) compare studies over which the underlying event rate varies [697-699],
(xi) allow for non-independence (i.e. cluster effects and intra-cluster correlation) in the comparison of proportions from multiple clusters.

The last listed consideration (xi) arises in the case of proportion data involving patients within clustered settings which is particularly relevant for an infectious or potentially contagious disease as is the case with the data presented in this thesis [700]. As a result of contagion and clustering, observations arising from within a cluster can not be assumed to be independent, a crucial assumption for statistical inference.

There are at least six approaches in the analysis of multiple proportions which variably address these various issues. Five of these methods are used at various points in this thesis; simplistic tallies, the beta-binomial model (not used in this thesis), logistic regression, exact logistic regression (ELR), random effects within mixed models, and the method of generalized estimating equations (GEE).

Simplistic tallies The three simplest methods in common use to derive an average proportion are as; (1) a simple tally (summation) for the numerator and denominator across all available studies (as was done by Elin – see Table2.2.1/2/3), or (2) an arithmetic mean of all proportions, or (3) the median proportion (as was done in [287]). For example, for the proportions 1/20, 10/200 and 200/2000, the summation would be 211/2220 (≈0.1) whereas the arithmetic mean would be ((100+100+200) ÷
The beta-binomial model is based on the beta distribution. It is a useful distribution for modelling proportion data because the beta-distribution is confined within the range between 0 and 1. It has two additional advantages for modelling proportions. It is a method that is able to accommodate overdispersion in the data. It is a flexible distribution in that the shape of the distribution is determined by the two parameters of the distribution; ‘a’ and ‘b’. The mean of the distribution is $a/(a+b)$ and the variance is $ab/((a+b)^2(a+b+1))$. The beta-binomial model is not used in this thesis. The beta-binomial model is more commonly used for ecological modelling.

**Logistic regression** Logistic regression is a method of regression analysis applicable for where the response variable is binary (i.e. 0 or 1). This method of analysis utilizes the logit transformation (described above). The logit transformation overcomes problems that would occur through undertaking a regression on untransformed data, in particular the problem of fitted proportions that fall outside the range of 0 to 1. The main advantage of logistic regression is that it can be estimated using conventional parametric statistical techniques, in particular, maximum likelihood. An additional advantage of logistic regression is that overdispersion (described below) can be accommodated. Moreover, meta-analytic techniques can be applied to logit transformed proportion data to derive estimates of mean and 95% confidence intervals to enable a visual comparison of multiple proportions on the logistic scale.

**Exact methods** Exact logistic regression (ELR) is a computationally intensive method of logistic regression as it uses permutation methods in contrast to the use of asymptotic estimates and likelihood theory [701-705]. Exact logistic regression is an extension of Fisher’s exact test and is ideally suited for data sets containing proportions that are sparse or unbalanced. ELR has two limitations. It is computationally intensive and hence is not a feasible method for the following types of data sets; data sets that are very large, have more than four or five explanatory variables or require tests for interaction. Secondly, ELR is more suited for hypothesis testing (i.e. generating exact p values) rather than for parameter estimation (i.e. deriving confidence intervals).
Mixed models

The challenge of analysing multiple proportion data as found in this thesis is best illustrated by considering the impact of additional data becoming available. Consider that we have a data set with mortality proportion data coming from three clusters; cluster A - 1/20, cluster B - 10/200 and cluster C - 200/2000. If the research budget allowed us to collect data for an additional 1000 patients, how should we do that optimally? Would this be with 1000 extra patients in cluster A, in cluster B, in cluster C, or in a new cluster D, or in a series of new clusters D, E, F and G each with 250 patients? The answer to this additional data dilemma is dependent on how similar the mortality proportions are across different centres, i.e. intra-cluster correlation [700, 706]. If the mortality proportion were similar in all clusters, the additional data could just as optimally come from any one of the clusters. However, that is not usually the case as the usual expectation is that the mortality risk for the patients found in one cluster would be more similar to each other than to the mortality risk for patients found in another cluster.

The intra-cluster correlation coefficient (ICC) is the measure of similarity within, versus between, clusters. It is the proportion of total variance that is ‘explained’ by within cluster variation. The converse measure, being the lack of similarity between clusters, is overdispersion. This is when the total variance is greater than that ‘explained’ by the cluster variance. Note that the total variance is not observable but is derived from the cluster level estimates. This leads to the concept of mixed models.

There are two mixed models approaches for the analysis of clustered binary data; methods leading to cluster specific (i.e. in this thesis the individual study) versus methods leading to population averaged, also termed marginal (i.e. in this thesis the study groups from within a strata) estimates. Both methods are used in this thesis. The two methods lead to different levels of inference. The random effects modelling approach provides for a level of inference at the level of a given study. In the random effects models, the effect in each cluster is explicitly modelled by including cluster specific random intercepts that are drawn from a probability distribution that uses model-based variance estimates. These models are typically estimated using maximum likelihood resulting in the random effects being ‘integrated out’. These models are particularly relevant when cluster specific (i.e. at the level of the individual study) effects are of interest. In this thesis, this level of inference is of interest in relation to finding individual studies that gave results that can be viewed as statistically atypical.
The two-level mixed logistic regression model described above for the analysis of diagnostic tests is a special case example of this technique.

The **population averaged modelling** approach provides for a level of inference at an ‘average’ or ‘typical’ study. This is of interest in relation to comparing groups of studies that share a characteristic of interest more so than in the individual studies themselves. Methods leading to population averages include both the random effects modelling approach as described above and alternately the method of **Generalized estimating equations** (GEE). The difference between the two approaches is based on how the between-cluster variation is estimated and incorporated into the calculation of the standard errors. The random effects modelling approach estimates the between-cluster variation directly whereas the GEE method estimates its counterpart, the within-cluster similarity of the residuals, using this in turn to estimate the standard errors.

**Generalized estimating equations** The method of GEE is a population averaged approach to estimation. GEE fits a population averaged model (i.e. the level of inference is at the level of the whole population rather than the individual cluster), using an iteratively re-weighting algorithm and a robust method for the standard error to estimate the parameters [700, 707]. While GEE is a method of individual level regression modelling it differs from other regression modelling methods in how the strength of correlation between observations within the cluster is estimated (as ‘working correlations’). As a result, there are three conceptual differences between methods based on random effects modelling versus GEE:

- random effects modelling uses the between-cluster variability as the basis for estimation whereas for the GEE method the counterpart, being the within-cluster similarity, is used as the basis for estimation.
- Secondly, for GEE the population effects are estimated by an algorithm (the ‘Estimating Equations’ within ‘GEE’). As this algorithm is not based on any probability distribution it produces robust variances that can be described as ‘model-agnostic’.
- Thirdly, the GEE estimating method does not require that the data be independent. This property is highly desirable in the analysis of data derived from clusters of patients with infectious (i.e. potentially transmissible) diseases.

The robust variance comes under various names and is also known as the Huber/White/sandwich estimate of variance. Moreover, these robust variances are consistent even when the model is mis-specified and in this way, the population estimates of the averages are robust [707].
Note that with both the random effects and population averaged methods, the estimation of the model parameters does not occur as a single step calculation but proceeds by iteration until convergence is achieved. Hence both techniques are relatively computationally intensive. The methods have existed for over a decade but they have become more feasible using statistical software on the desk-top computer in the past ten years. Moreover, the methods are more relevant to the analysis of large rather than small datasets (e.g. datasets with less than twenty groups).
5. Results and discussion

This chapter presents the analyses of the data derived from the studies derived from the literature search (figure 5.0.1). These studies are catalogued in Table 7.1/2. This thesis is a compilation of published works. This chapter provides an update of the analysis presented in summary form. Additional details are contained in the compiled publications (appended at the end of this thesis).

**Figure 5.0.1 Flow chart of literature search and study accrual**

Electronic search for eligible studies (n=208)
- Search terms:
  - endotoxemia
  - Limulus assay
- Hand search for additional eligible studies (n = 91)

Exclusion criteria (n=192)
- Animal studies (n = 23)
- Studies of patient groups other than suspected sepsis; e.g. post-gastro-intestinal endoscopic procedures (n = 19)
- Studies of non-bacteremic infections (n=52)
- Data insufficient or incorrect format (n = 67)
- Studies with fewer than 10 patients (n=6)
- Duplicate publication (n = 3)
- Supplementary studies – see below (n= 12)

Included studies [1-95]
(n = 95);
- G-limulus ‘4 group’ (n=41)
- C-limulus ‘4 group’ (n=46)
- Not stated ‘4 group’ (n=8)

Studies with additional data in relation to
- mortality data in ‘4 group’ format (n=36)
- GN bacteremia type listings (n=26)

Supplementary studies [96-107]
(n = 12);
- non-limulus ‘4 group’ [96-101]
- limulus/non-limulus ‘2 group’ [96-107]
- Anti-endotoxin [88, 94]
These results are presented as follows here and in the compiled publications as they bear on the detection of endotoxemia;

- as a diagnostic test (section 5.1, JCM ‘94, AP&LM ‘00, EJCMID ’10)
- and as a test of prognosis (section 5.2, JCM ‘95, EIHD ’99, JER ’03, CC ’12)

5.1. Endotoxemia detection as a diagnostic test

The number of studies in the analysis as included in three prior publications (JCM ‘94, AP&LM ‘00, EJCMID ‘10) together with an update for this thesis are summarized in Table 5.1.1. Five of the publications [24, 54, 58, 65, 94] provided two discrete studies each. The update prepared for this thesis includes 29 additional studies including four additional non-English language studies. Forty-one studies used the G-limulus and 47 studies used the C-limulus assay. Eight small studies were unusable for this part of the analysis as they had more than one zero entry within the studies’ 2x2 table.

The listed assay sensitivity to the internal endotoxin standard in the studies varied by more than a 100 fold being typically in the range 0.1 – 10 ng/ml for the G-limulus assays versus 0.001 – 0.1 ng / ml for the C-limulus based assays (see Table 7.1). The prevalence of GN bacteremia was similar in the studies using G-limulus and C-limulus (24.1%; 16.7 – 31.5 versus 24.5 %; 20.5 – 28.5) versions of the assay. Fifteen studies were limited to pediatric patients, and eight studies were limited to oncology or neutropenic patients. There were 11 studies of sepsis patients, all of these studies had been conducted within an intensive care unit setting and all post date the use of sepsis as a clinical syndrome defined by consensus in 1991. All 11 had used the C-limulus assay.
### Table 5.1.1 Endotoxemia diagnostic studies: Summary results.

<table>
<thead>
<tr>
<th>Details of meta-analysis</th>
<th>JCM ‘94</th>
<th>AP&amp;LM ‘00</th>
<th>EJCMID 2010</th>
<th>Thesis 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of publications</td>
<td>43</td>
<td>54</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>Number of studies (N)</td>
<td>45</td>
<td>56</td>
<td>58</td>
<td>88</td>
</tr>
<tr>
<td>Number of patients</td>
<td>2539</td>
<td>4134</td>
<td>4681</td>
<td>5641</td>
</tr>
<tr>
<td>Number of GN bacteremias (%)</td>
<td>615 (24%)</td>
<td>856 (21%)</td>
<td>883 (19%)</td>
<td>1480 (26%)</td>
</tr>
<tr>
<td>Analytic method</td>
<td>Mantel</td>
<td>SROC</td>
<td>HSROC</td>
<td>HSROC</td>
</tr>
<tr>
<td>Weighting for study size</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Summary measure</td>
<td>DOR; 95% CI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Q*; 95% CI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DOR; 95% CI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DOR; 95% CI&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>All studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-limulus studies</td>
<td>8.3; 5.9 – 11.8</td>
<td>0.71; 0.64 – 0.77</td>
<td>10.7; 5.2 – 22</td>
<td>4.5 – 15.7</td>
</tr>
<tr>
<td>Number of studies (N)</td>
<td>(29)</td>
<td>(28)</td>
<td>(25)</td>
<td>(41)</td>
</tr>
<tr>
<td>C-limulus studies</td>
<td>6.3; 4.4 – 9.1</td>
<td>0.66; 0.61 – 0.72</td>
<td>5.4; 3.2-9</td>
<td>4.7; 3.0-7.2</td>
</tr>
<tr>
<td>Number of studies (N)</td>
<td>(16)</td>
<td>(28)</td>
<td>(33)</td>
<td>(47)</td>
</tr>
<tr>
<td>Sub analysis - defined by</td>
<td>Study GN in range 13-40%</td>
<td>Excluding small studies&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Excluding small studies&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Excluding small studies&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-limulus studies</td>
<td>20.1; 12.5 – 32.4</td>
<td>0.77; 0.68 – 0.81</td>
<td>12.5; 6.1-26</td>
<td>10.0; 4.9-20.3</td>
</tr>
<tr>
<td>Number of studies (N)</td>
<td>(13)</td>
<td>(20)</td>
<td>(18)</td>
<td>(25)</td>
</tr>
<tr>
<td>C-limulus studies</td>
<td>3.6; 2.3 – 5.6</td>
<td>0.65; 0.58 – 0.72</td>
<td>6.2; 3.6-11</td>
<td>5.5; 3.4-8.9</td>
</tr>
<tr>
<td>Number of studies (N)</td>
<td>(9)</td>
<td>(22)</td>
<td>(24)</td>
<td>(33)</td>
</tr>
</tbody>
</table>

Foot notes: Abbreviations; DOR = Diagnostic Odds Ratio, SROC = summary receiver operator characteristic; HSROC Hierarchical summary receiver operator characteristic

- **a.** DOR = 1.0 indicates diagnostic value no greater than chance
- **b.** DOR > 1 indicates increasing diagnostic value with increasing DOR > 1
- **c.** Q* = 0.5 indicates diagnostic value no greater than chance
- **d.** Q* > 0.5 indicates increasing diagnostic value up to 1 (maximum)
- **e.** Small studies defined as < 25 patients per study

### 5.1.1. Concordance with detection of GN bacteremia

**Limulus assay concordance with GN bacteremia**

**Individual studies.** The concordance between the detection of endotoxemia and
GN bacteremia among all 88 studies is illustrated using the HSROC method in figure 5.1.1. In the figure (figure 5.1.1) there is evidence suggestive of publication bias in that a high proportion of small studies (12 of the 19 studies that had <25 patients) had reported 100% sensitivity for the detection of endotoxemia using the limulus assay in

![HSROC Plot for the detection of endotoxaemia versus GN bacteraemia together with the fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (■) is 5.8 (4.0 – 8.3). The symbol size for each study is proportional to the study size (n = 88).](image)

**Note:** Y axis should read ‘sensitivity’ (= true positive rate)
X axis is intentionally backwards (as specificity = 1- false positive rate)

**Fig 5.1.1.** HSROC Plot for the detection of endotoxaemia versus GN bacteraemia together with the fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (■) is 5.8 (4.0 – 8.3). The symbol size for each study is proportional to the study size (n = 88).
association with the detection of GN bacteremia.

**Summary value.** The concordance between the detection of endotoxemia and GN bacteremia was expressed in three of the four meta-analytic summaries as a summary diagnostic odds ratio (DOR) value. Most recently the DOR was derived using the HSROC method. The DOR was lower in the C-limulus studies versus the studies using the earlier G-limulus version of the assay (Table 5.1.1). This finding is apparent in the most recent analysis including all 88 studies. In AP&LM ‘00 the Q* value was used as the summary value and this represents the point on the ROC summary curve where sensitivity = specificity. The overall findings are the same in analyses in which the summary value is expressed as either a Q* value (APLM ‘00) or as a DOR (JCM 94, EJCMID 2010) as well as in the updated summary derived for this thesis.

A further analysis for all 88 studies using the HSROC methods to derive mean sensitivity and specificity is presented in Table 5.1.2. This table details the comparison of the relative performance of the C-limulus versus the G-limulus version of the assay for subgroups of studies that are both large and have a higher quality of study method in relation to the following:

- overall. The inferior performance of the C-limulus versus the G-limulus versions of the assay remains apparent among subgroups of studies selected on the basis of being both large and being in the strata of higher study method quality (QS>2).

- patient categories. The comparison of diagnostic experience in the various subgroups of patient categories indicates low specificity and DOR amongst studies undertaken in an ICU setting whereas the studies undertaken in unrestricted settings (adults) displayed low sensitivity and intermediate DOR (Table 5.1.2). Studies undertaken in pediatric or oncology settings (analysed as a combined category), showed overall highest specificity and DOR. Note however, all of the studies undertaken in ICU settings had used the CLAL version of the assay.

- method of plasma pre-treatment. There was no direct comparison of the various methods of plasma pre-treatment in any single study. However, among C-limulus assay studies, the DOR is similar among the sub-group of studies that used dilution and heat treatment as a method of plasma pre-treatment. Conversely, among G-limulus assay studies, the sub-group of studies that used chloroform as the plasma
pre-treatment method performed less well.

- year of publication. There was no discernable trend in DOR over time, using the year of publication, for either version of the assay (data not shown). Also, for both the C-limulus and the G-limulus versions of limulus assay the DOR appeared to be unrelated to the listed sensitivity to the internal endotoxin standard of the LAL assay as used in each study among studies (see figure 4 of EJCMID ‘10).

### Table 5.1.2 Summary results for HSROC analysis (updated 2012)

<table>
<thead>
<tr>
<th></th>
<th>N*</th>
<th>DOR</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All</strong></td>
<td>88</td>
<td>5.8; 4.1 – 8.2</td>
<td>0.73; 0.65 – 0.79</td>
<td>0.68; 0.60 – 0.76</td>
</tr>
<tr>
<td>Large &amp; QS&gt;2 †</td>
<td>40</td>
<td>4.7; 3.1 – 7.0</td>
<td>0.65; 0.54 – 0.75</td>
<td>0.72; 0.60 – 0.81</td>
</tr>
<tr>
<td>Large, QS&gt;2 &amp; unrestricted (adult)</td>
<td>13</td>
<td>5.3; 2.5 – 11.1</td>
<td>0.57; 0.41 – 0.71</td>
<td>0.80; 0.60 – 0.92</td>
</tr>
<tr>
<td>Large, QS&gt;2 &amp; pediatric or oncology</td>
<td>8</td>
<td>9.9; 3.6 – 27.2</td>
<td>0.58; 0.28 – 0.83</td>
<td>0.88; 0.59 – 0.97</td>
</tr>
<tr>
<td>Large, QS&gt;2 &amp; ICU setting</td>
<td>16</td>
<td>2.2; 1.4 – 3.7</td>
<td>0.66; 0.49 – 0.80</td>
<td>0.53; 0.39 – 0.66</td>
</tr>
<tr>
<td><strong>G-limulus studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>41</td>
<td>8.4; 4.5 – 15.7</td>
<td>0.76; 0.60 – 0.87</td>
<td>0.72; 0.59 – 0.83</td>
</tr>
<tr>
<td>Large &amp; QS&gt;2 †</td>
<td>13</td>
<td>10.1; 3.6 – 28.5</td>
<td>0.71; 0.32 – 0.93</td>
<td>0.80; 0.51 – 0.94</td>
</tr>
<tr>
<td>Large, QS&gt;2 &amp; chloroform †</td>
<td>5</td>
<td>2.0; 0.3 – 12.6</td>
<td>0.47; 0.37 – 0.57</td>
<td>0.70; 0.31 – 0.92</td>
</tr>
<tr>
<td><strong>C-limulus studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>47</td>
<td>4.7; 3.0 – 7.2</td>
<td>0.71; 0.63 – 0.79</td>
<td>0.65; 0.54 – 0.75</td>
</tr>
<tr>
<td>Large &amp; QS&gt;2 †</td>
<td>27</td>
<td>3.8; 2.4 – 5.7</td>
<td>0.65; 0.55 – 0.74</td>
<td>0.67; 0.66 – 0.77</td>
</tr>
<tr>
<td>Large, QS&gt;2 &amp; D&amp;H †</td>
<td>16</td>
<td>3.4; 2.1 – 5.4</td>
<td>0.70; 0.61 – 0.80</td>
<td>0.59; 0.43 – 0.72</td>
</tr>
<tr>
<td>Sepsis, QS&gt;2</td>
<td>11</td>
<td>2.2; 1.3 – 3.7</td>
<td>0.67; 0.52 – 0.79</td>
<td>0.52; 0.41 – 0.63</td>
</tr>
</tbody>
</table>

**Abbreviations:** * N = number of studies; DOR, diagnostic odds ratio † Studies with more than 24 patients studied & quality score > 2 of 4 * chloroform and D&H are chloroform and dilution and heat treatment methods for the pre-treatment of plasma samples, respectively.
5.1.2. Impact of GN bacteremia species type

The association of endotoxemia with GN bacteremia was examined for specific bacterial types of GN bacteremia from studies for which this data was available. There were 833 GN bacteremic patients from 57 studies, of which 15 of these studies (265 bacteremias) were restricted to a specified type of GN bacteremia (restricted studies; Table 5.1.3). The remaining 42 studies were from studies that were unrestricted to any specific GN bacteria (unrestricted studies; Table 5.1.4). Note that 135 of these bacteremic patients came as a personal communication from a single study (Opal [65]).

**Restricted studies.** (Table 5.1.3) There were 15 studies that were restricted to one of the four specified GN bacteremia types. Additional data for the same four GN bacteremia types and *Haemophilus influenzae* were abstracted from six unrestricted studies. The proportion with detectable endotoxemia was lowest for studies that examined *Salmonella typhi* bacteremia (30%) versus *Neisseria meningitidis* (75%) and *Haemophilus influenzae* (100%).

**Table 5.1.3** Bacterial species of bacteremia and concordance with endotoxemia: studies restricted to specified GN bacteria.

<table>
<thead>
<tr>
<th>Studies restricted to GN bacteria species listed below</th>
<th>Studies (N)</th>
<th>Number limulus positive (n) / Number of patients with GN bacteremia (N)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhi</em></td>
<td>4</td>
<td>17 / 57</td>
<td>30</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>6</td>
<td>10 / 10</td>
<td>100</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>11</td>
<td>138 / 183</td>
<td>75</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>2</td>
<td>5 / 7</td>
<td>67</td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td>1</td>
<td>15 / 15</td>
<td>100</td>
</tr>
</tbody>
</table>

Foot notes: derived from 15 studies plus small numbers from six unrestricted studies [2, 20, 37, 38, 59, 72] Only 5 of these studies had > 9 patients with GN bacteremia.
Table 5.1.4 Concordance of bacteremia with endotoxemia by bacterial species: studies unrestricted to specific GN bacteria

<table>
<thead>
<tr>
<th></th>
<th>Number limulus positive (n) / Number of patients with GN bacteremia (N)</th>
<th>All studies (N=42)</th>
<th>All studies (excluding Opal [65])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies unrestricted to GN bacteria types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>● <em>Escherichia coli</em></td>
<td>135 239 56</td>
<td>102 200 51</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae other than <em>E coli</em></td>
<td>94 187 50</td>
<td>67 154 44</td>
<td></td>
</tr>
<tr>
<td>● Klebsiella species</td>
<td>35 81 43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Enterobacter species</td>
<td>14 39 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Enterobacteriaceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>● <em>Pseudomonas species</em></td>
<td>76 111 68</td>
<td>52 80 65</td>
<td></td>
</tr>
<tr>
<td>● Anaerobic bacteria†</td>
<td>5 16 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed GN bacteria</td>
<td>48 58 83</td>
<td>18 26 69</td>
<td></td>
</tr>
</tbody>
</table>

Foot notes; derived from 42 studies (as listed in JCM ‘94 + 4 additional studies). Includes Serratia species, Proteus species and other unspecified Enterobacteriaceae. † Includes; *Fusobacterium species, Bacteroides species*.
Unrestricted studies. (Table 5.1.4) There were 42 studies that were unrestricted to specified GN bacteremias.

As part of the update prepared for this thesis, the concordance of GN bacteremia with endotoxemia among the 42 studies that were unrestricted to specified GN bacteremias was re-examined by three separate statistical methods. The first method (method 1) is a simple tally (Table 5.1.4). The second method (method 2) uses the HSROC method (as used to prepare figure 5.1.1) in which the proportions of true positives is compared with false positives among patients of each study who were blood culture negative (as for figure 5.1.1) (figure 5.1.2/3/4). The third method (method 3) is a meta-regression of logit transformed proportions of true positives (i.e. GN bacteremias concordant with endotoxemia) in each study versus the G- and C- versions of the limulus assay as a categorical variable. The three different methods give different analytic weights to studies of different size with more even weighting among studies having different size for methods two and three.

• (method 1) In a simple tally (Table 5.1.3 & Table 5.1.4), the percentages are up to 9 percentage points higher than as found in an earlier tally (Table 3 of JCM’94) due to the high proportion (84%) of endotoxemia positive GN bacteremias overall in the largest study (Opal [65]). After excluding the bacteremias from the study by Opal [65], the proportion with detectable endotoxemia was lowest for bacteremias with Klebsiella (40%) and Enterobacter (36%) species, highest for Pseudomonas species (63%) and intermediate for Escherichia coli (50%).
Note: Y axis should read ‘sensitivity’ (= true positive rate)
X axis is intentionally backwards (as specificity = 1- false positive rate)

**Fig 5.1.2** Plot of sensitivity and specificity for all studies (n = 34) for the detection of endotoxaemia versus *E coli* bacteraemia together with the fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (“■”) is 3.6 (1.8 – 7.0) (Table 5.1.5). The symbol size for each study is proportional to the study size.
Note: Y axis should read ‘sensitivity’ (= true positive rate)
X axis is intentionally backwards (as specificity = 1 - false positive rate)

Fig 5.1.3 Plot of sensitivity and specificity for all studies (n = 29) for the detection of endotoxaemia versus non-<i>E coli</i> Enterobacteriaceae bacteraemia together with the fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (“■”) is 3.2 (1.5 – 6.8) (<b>Table 5.1.5</b>). The symbol size for each study is proportional to the study size.

- (method 2) By HSROC (<b>figure 5.1.2, figure 5.1.3, figure 5.1.4</b>). In comparing the different types of GN bacteremia, the proportion of true positives is up to 20
percentage points higher for *Pseudomonas aeruginosa* versus Non-*E. coli* *Enterobacteriaceae* among studies stratified by type of LAL assay. Note that the summary DOR and sensitivity found in the HSROC analysis here are each lower versus that found previously with all GN bacteremias included (i.e. compare Table 5.1.2 & figure 5.1.1 versus Table 5.1.5 & figure 5.1.2/3/4).

- (method 3) By meta-regression (Table 5.1.5). The median listed assay sensitivity among studies that used the CLAL versus GLAL assay was 0.01 ng/ml versus 3 ng/ml. Despite this 300-fold difference in listed assay sensitivity, the difference in the proportion of true positives between the G- versus C- versions of the limulus assay used was only 1 percentage point in the case of *E. coli*. In contrast, there is a 14 percentage points in the proportion of true positives between the G- versus C- versions of the limulus in the case of non-*E. coli Enterobacteriaceae* and *Pseudomonas aeruginosa*. Note that for these latter two GN bacteremia types, the higher sensitivity in the case of the C- version versus the G- versus of the limulus assay demonstrated here is again contrary to what was apparent in the analysis with all GN bacteremias included (Table 5.1.2).

- The proportion of true positives (sensitivity) for the various categories of GN bacteremias is dependent on the method of aggregation. The proportions as presented in Table 5.1.5 are less than that derived using the simple tally method (Table 5.1.4). For both the HSROC and the meta-regression methods there is a more even weighting of the different sized studies than is the case for a simple tally.

- The simple tally method gives most weight to the largest study. The influence of the exclusion of the largest study in the tally is demonstrated Table 5.1.4.

- Note the disparity between the sensitivity with respect to the three categories of GN bacteremia (Table 5.1.5) which is up to 20 percentage points lower than the sensitivity with respect to GN bacteremia overall (Table 5.1.2). The reason for this disparity could reflect inaccuracies in how GN bacteremias were recorded in individual studies together with the greater weighting given to smaller studies by the HSROIC method of aggregation. Given these discrepancies, the sensitivities given in Table 5.1.5 are considered most representative of a ‘typical’ study.
Note: Y axis should read ‘sensitivity’ (= true positive rate)
X axis is intentionally backwards (as specificity = 1 - false positive rate)

Fig 5.1.4 Plot of sensitivity and specificity for all studies (n = 27) for the detection of endotoxaemia versus non P aeruginosa bacteraemia together with the fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (■) is 6.5 (2.8 – 15.4) (Table 5.1.5). The symbol size for each study is proportional to the study size
| Table 5.1.5 Summary results for HSROC (= method 2) and meta-regression analysis (= method 3) |
|-----------------------------------|-----------------|-----------------|-----------------|
|                                   | N*              | DOR             | Sensitivity     | Specificity     |
| Analysis by HSROC                |                 |                 |                 |
| **E coli** (see Fig 5.1.2)      |                 |                 |                 |
| • All                             | 37              | 3.6; 1.8 – 7.0  | 0.55; 0.41 – 0.68 | 0.75; 0.61 – 0.85 |
| • Large studies only             | 24              | 3.4; 1.6 – 6.9  | 0.54; 0.39 – 0.68 | 0.74; 0.60 – 0.85 |
| **other Enterobactereiaceae** (see Fig 5.1.3) |                 |                 |                 |
| • All                             | 29              | 3.2; 1.5 – 6.8  | 0.55; 0.36 – 0.72 | 0.73; 0.59 – 0.83 |
| • Large studies only             | 22              | 2.8; 1.4 – 5.7  | 0.48; 0.30 – 0.66 | 0.75; 0.61 – 0.86 |
| **Pseudomonas aeruginosa** (see Fig 5.1.4) |                 |                 |                 |
| • All                             | 27              | 6.5; 2.8 – 15.4 | 0.69; 0.52 – 0.81 | 0.75; 0.61 – 0.85 |
| • Large studies only             | 20              | 5.3; 2.3 – 12.0 | 0.68; 0.51 – 0.81 | 0.72; 0.58 – 0.82 |
| Analysis by meta-regression versus assay type (C versus G) | | | |
| **E coli**                        |                 |                 |                 |
| • GLAL                            | 37              |                 | 0.53; 0.44 – 0.61 |
| • CLAL                            | 24              |                 | 0.54; 0.46 – 0.62 |
| **other Enterobactereiaceae**     |                 |                 |                 |
| • GLAL                            | 26              |                 | 0.39; 0.30 – 0.48 |
| • CLAL                            | 22              |                 | 0.53; 0.44 – 0.62 |
| **Pseudomonas aeruginosa**        |                 |                 |                 |
| • GLAL                            | 24              |                 | 0.52; 0.43 – 0.62 |
| • CLAL                            | 21              |                 | 0.66; 0.57 – 0.74 |

Abbreviations; * N = number of studies; DOR, diagnostic odds ratio
GLAL – median assay sensitivity 3 ng/ml
CLAL – median assay sensitivity 0.01 ng/ml
5.1.3. Study limitations and literature reconciliation

Several surprising observations are noted among the findings for the detection of endotoxemia as a diagnostic test,

1. (Section 5.2.1). The proportion of patients with GN bacteremia for which endotoxemia is undetectable is >25% in the studies overall (Table 5.1.2), >35% after excluding the results of small studies, up to 50% when methods of aggregation that are more evenly weighted for studies of differing sizes (Table 5.1.5) and up to 60% when individual GN bacteremias were tallied (Table 5.1.4). The smallest (n<25) and largest [65] studies appear to give atypical results, a finding that would not have been apparent without using meta-analysis.

2. (Section 5.2.1). For GN bacteremias overall, the proportion with detectable endotoxemia (i.e. sensitivity = true positives) differs by < 6 percentage points in studies that had used the more sensitive C-limulus assay versus the original G-limulus assay (as derived using HSROC summaries; Table 5.1.2).

3. (Section 5.2.2). This lack of difference between assay versions also applies in a comparison limited to E. coli GN bacteremias (Table 5.1.5).

4. (Section 5.2.2). By contrast, in comparisons limited to either non- E. coli-enterobacteriaceae or Pseudomonas, the proportion with detectable endotoxemia (i.e. sensitivity = true positives) was up to 14 percentage points higher in studies that used the C-limulus versus the G-limulus assay (Table 5.1.5).

5. (Section 5.2.2). The estimate for the rates of detection for different bacteremia types which is most representative of a ‘typical’ study within the overall literature experience is that provided by HSROC analysis (Fig 5.1.2/3/4). The proportion of GN bacteremias with detectable endotoxemia (i.e. true positives) is 68% (51-81%) for bacteremias with Pseudomonas (Fig 5.1.4), 54% (39-68%) for E. coli (Fig 5.1.2) and 48% (30-66%) for the non- E. coli-enterobacteriaceae; Fig 5.1.3) (Table 5.1.5).

6. (Section 5.2.2). For GN bacteremias among studies restricted to bacteremias of specific bacteremia types, only the tally method is able to be applied in the derivation of a summary of the rate with associated endotoxemia. With these bacteremias the rates of detection of endotoxemia is lowest (30%) amongst studies
that were restricted to bacteremias with *S. typhi* and highest among studies that were restricted to bacteremias with *N. meningitidis* (75%) or *B. pseudomallei* (100%) (Table 5.1.3).

There are a number of limitations to this analysis. The size of the studies in relation to the number of patients per study varies considerably. The smaller studies showed atypical results versus larger studies (Figure 5.1.1). Among the smallest studies (*n*<25), there is an excess with a sensitivity >90%. This is consistent with a selective publication bias as it is plausible that a small study finding low sensitivity would be less likely to be published than would a large study with this finding. However, the conclusions of the HSROC analysis are more robust to the inclusion or exclusion of studies of different sizes (Table 5.1.1, Table 5.1.2, Table 5.1.5).

A second limitation of this analysis is that the influence of several factors of potential interest, for example, patient groups of different ages, underlying disease, source of infection, or type and timing of antibiotic treatment in relation to blood sampling has not been specifically examined. The information on these factors was not available in many of the studies. In any case, post-hoc analysis of sub-groups in a meta-analysis is potentially misleading as the likelihood of significant findings increases by chance alone with the number of subgroups examined.

There was a wide range of patient groups among the studies and the studies varied with respect to indicators of quality of study design methods such as clear specification of patient inclusion criteria and reporting of GN bacteremia subtypes. The sub-group of studies limited to sepsis syndrome patients was examined as these studies were generally of higher quality (Table 5.1.2). Additional sensitivity analyses (data not shown) with the exclusion of studies of pediatric patients, the exclusion of smaller studies and also a comparison of the diagnostic odds ratio over time (data not shown) were done in an attempt to further address these issues. These sensitivity analyses did not alter the conclusions (data not shown).

The selection of studies included here has been published over a period of nearly 40 years. Blood culture methods used to detect bacteremia may have improved over this period of time. However, the prevalence of GN bacteremia was similar among the more recently published C-limulus studies versus the G-limulus studies.
The literature has been searched and extracted by a single author. While the abstracted data and the lists of studies included and excluded are provided here (Table 7.1.2) and in previous versions of this analysis [JCM ‘94, AP&LM ‘00, EJCMID ’10], it always remains possible that studies have been overlooked or their extraction contestable. In this respect, additional small [2, 17, 74] and non-English language [34, 92] studies and the largest study (Opal [65]) became available in preparing this thesis. The addition of these newly found studies did not substantially change the findings of the earlier published analyses (Table 5.1.1).

There would need to be a substantial number of studies that had been overlooked or extracted imprecisely to alter the conclusions in this analysis. In particular, for the C-limulus assay to have an improved utility to the G-limulus assay there would need to be a substantial number of studies with a high DOR, exceeding for example, those seen with the studies of procalcitonin [294, 295] (Table 2.2.2 and 2.5.1), to negate the findings here. Such studies would be unlikely to have remained unpublished or difficult to find.

The proportion of endotoxemia positive bacteremias for specific types of GN bacteremia differs dependent on whether this proportion is derived as a simple tally (Tables 5.1.3 & 5.1.4) or by HSROC meta-regression methods (Table 5.1.5). Of these methods, the HSROC method is considered to best represent the findings of a ‘typical’ study. A further limitation is that the number of samples evaluated per patient in each study is not known and cannot be evaluated.

To further seek an overview and reconciliation of the entire literature analysed here, the studies representative of specific patient groups are indicated by a key as coded in Table 5.1.6 and presented on the summary HSROC plot in Figure 5.1.5 with the large studies coded for identification. Both the generally low specificity (< 60%) among several of the studies undertaken in an ICU setting [i1 – i9] and the generally low sensitivity (< 80%) among several of the studies undertaken with unrestricted adult patients [a0 – a11] can be visually appreciated. Also shown for reference purposes are two supplementary list studies [@; 100, 101] which used non-limulus assays with study data available in the same ‘4 groups’ format as for the other 88 studies that had used the limulus assay.
Table 5.1.6 Details of selected (QS>2) studies (& codes for figure 5.1.5)

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Foot notes:
- Only shown are studies with QS>2, N>20 and GN bacteremia types available.
- Note, for figure 5.1.1, specificity = 1-FP
Note: Y axis should read ‘sensitivity’ (= true positive rate)
X axis is intentionally backwards (as specificity = 1 - false positive rate)

Fig 5.1.5 Plot of sensitivity and specificity for all studies (as in Figure 5.1.1) with study codes as indicated in Table 5.1.6. Symbol size for each study is proportional to the study size. Hollow “○” symbols (n=90) are studies using the limulus assay, and “@” symbols are 2 non-limulus ‘4 group’ supplementary studies [100, 101].

The fitted SROC curve and the bivariate summary estimate (solid square) together with the corresponding 95% confidence ellipse (inner broken line) and 95% prediction ellipse (outer dotted line). The summary DOR (“■”) is 5.8 (4.0 – 8.3).

Note the asymmetrical distribution of studies in an ICU setting (i1, i2, i3, i5, i6, i8, i9l, & i0) with most studies falling on right hand side (i.e. specificity <60%) of figure versus studies in other settings.
There are three possible explanations for why the proportion of endotoxemia positive (i.e. labelled here as false positive) patients among patients without a GN bacteremia is generally higher among studies undertaken in an ICU setting (as noted in Figure 5.1.5). Firstly, all of the studies undertaken in ICU settings had used the CLAL version of the assay. Second, it is likely that the patient population outside of an ICU setting is at overall lower risk of having endotoxemia and that a lower proportion of false positives could be expected. Thirdly, it is possible that the types of non GN bacteremic infections giving rise to endotoxemia (i.e. labelled here as false positive) may differ for the different patient populations. This explanation has not been explored here. However, a difference in the distributions of types of infections among the patient groups with GN bacteremia is to be explored in sections 5.2.2 & 5.2.3.

Overall, the findings presented within section 5.1 imply that the type of GN bacteremia is at least as important a determinant toward the detection of concurrent endotoxemia as is the sensitivity of the assay method used. The different results with the three different methods of aggregation reflect the different relative weighting provided to the smaller versus larger studies. The tally (first) method, as had been used in the first attempt (JCM ‘94) to summarize the relationship between endotoxemia detection and GN bacteremia type is not optimal as it provides disproportionate weighting to the largest studies.
5.2. Endotoxemia detection as a prognostic test

The findings for the detection of endotoxemia as a test of prognosis are presented here. The patient outcome of interest is mortality. As indicated within Figure 5.0.1 there are two types of studies with outcome data examined here, ‘four group’ studies and ‘two group’ studies as follows:

- **‘four group’ studies**, being studies in which the patient outcome (mortality) was reported for patients categorized into one of four groups;
  - group 1; both endotoxemia and GN bacteremia detected
  - group 2; GN bacteremia detected in isolation
  - group 3; endotoxemia detected in isolation
  - group 4; neither detected (reference group).

- **‘two group’ studies** (amongst the supplementary studies as indicated within Figure 5.0.1) are studies using either limulus or non-limulus assays for endotoxemia detection in which the patient mortality was reported for patients categorized into one of two groups;
  1. endotoxemia detected (equivalent to group 1 & 3 combined)
  2. endotoxemia not detected (equivalent to group 2 & 4 combined) (reference group).

The studies of primary interest are those identified in the literature search (Figure 5.0.1) as ‘four group’ studies. Note that the data within ‘four group’ studies can also be extracted in a ‘two group’ format but not vice versa. For this reason, several ‘two group’ and ‘four group’ studies undertaken in an ICU setting are to be compared as a preliminary analysis to place the ‘four group’ studies within the wider literature experience. Hence, the analysis proceeded as follows.

1. All studies undertaken in an ICU setting (‘four group’ and ‘two group’) were extracted for analysis in the ‘two group’ data format (see 5.2.1).
2. All ‘four group’ studies were extracted for analysis in the ‘two group’ data format to enable a comparison of those undertaken in ICU settings versus settings other than ICU (see 5.2.1).
3. An analysis of the outcome data was undertaken for only the ‘four group’
studies extracted and analysed in the ‘four group’ data format (see 5.2.2).

4. An analysis of patient outcome in relation to both endotoxemia detection (equivalent to group one versus group two from the ‘four group’ studies) and in relation to the GN bacteremia isolate type for those patients for which this data were available (see 5.2.3).

5. Studies with potential outlier outcome data were examined (see 5.2.4).

The numbers of studies, publications and patients for the analysis of all studies analysed as ‘two group’ studies are summarized in Table 5.2.1 with selected studies highlighted in Table 5.2.2. The numbers of studies, publications and patients for the analysis of all ‘four group’ studies are summarized as included in the three publications (JCM ‘95, JER ’03, CC ‘12) in Table 5.2.3. In preparing the most recent update, seventeen additional ‘four group’ studies have been located including two in an ICU setting [65, 74] with new data obtained by personal communication for three [17, 21, 65]. The studies for which outcome data was available are indicated by ψ under column QS in Tables 7.1/2.

Characteristics of the ‘two group’ studies There are six ‘two group’ studies undertaken in an ICU setting with outcome data available (indicated by ψ under column QS in the summary Tables 7.2.2) (Table 5.2.1). This includes supplementary list studies that had used the LAL assay but for which outcome data was available only for the ‘two group’ format [102], studies that had used assays other than the limulus assay to detected endotoxemia [101, 104-107] and two studies in which patients had received anti-endotoxin antibody treatments [88, 94].

Characteristics of the ‘four group’ studies Thirty-five studies were included from 32 publications of which 13 studies were supplemented with data provided by personal communication (Table 5.2.3). The survival outcome was reported for a total of 3235 patients among these 35 studies (indicated by ψ under column QS in Tables 7.1) of which 432 (13%), 272 (8%), 1091 (34%) and 1440 (44%) were in groups 1 to 4, respectively. Patient inclusion for 9 of the studies was based on various criteria for sepsis in adult patients in an ICU setting. Twenty-six of the 35 studies were published within the 1980’s and 1990’s. Studies were excluded if any of the following applied; mortality proportion was not obtainable from group 4 (21 studies), the patients had received anti-endotoxin antibody treatments (2 studies), no patients in the study had GN
bacteremia (5 studies) or the data was not extractable into a 2x2x2 table format. The largest study (Opal [65]) provided data in relation to endotoxemia detection at two breakpoints which have been used here to derive mortality proportions stratified for endotoxemia detection at high and low levels from this study.

5.2.1. Analysis of ‘4’ & ‘2’ group studies as ‘two group studies’

Table 5.2.1 Analyses of all studies with data extracted in ‘two group’ (endotoxemia detected versus not detected) format

<table>
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<th>Details of meta-analysis</th>
<th>‘2 group’ studies</th>
<th>‘4 group’ studies</th>
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<td>‘4 group’ studies</td>
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<td></td>
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<tr>
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<td>3377</td>
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<td>MH method</td>
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<td>Weighting for study size?</td>
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<td>Yes</td>
</tr>
</tbody>
</table>

Sub-group analysis: -

- ICU setting
  - OR; 95% CI (n)
    - 1.6; 1.2 – 2.0 (6)
    - 1.4; 1.2 – 1.7 (14)
  - heterogeneity (I²)
    - 3% (0%)

- unrestricted adult
  - OR; 95% CI (n)
    - 2.8; 2.1 – 3.9 (11)
  - heterogeneity (I²)
    - 77%

- oncology
  - OR; 95% CI (n)
    - 5.9; 3.0 – 11.4 (3)
  - heterogeneity (I²)
    - 0%

- pediatric
  - OR; 95% CI (n)
    - 2.8; 1.0 – 7.6 (4)
  - heterogeneity (I²)
    - 8%

Abbreviations; OR = Odds Ratio, MH = Mantel Haenzsel

* Note: studies not included here are;
  - restricted studies (5 studies)
  - studies with ‘double zero entries for which the OR is indeterminant (11 studies)
  - studies in which all patients received HA-1A [88, 94].
Forest plots (OR & RD)

The association with mortality for endotoxemia detection versus non-detection amongst all 49 ‘four’ and ‘two’ group studies is presented as an odds ratio (OR; Table 5.2.1, Figure 5.2.1) and as a risk difference (RD; Figure 5.2.2). Note that the OR is indeterminate for studies with double zero entries (e.g. Cooperstock ’85; 0/16 versus 0/27), hence there are fewer studies appearing in the OR versus the RD forest plots. The summary OR over all studies was 1.7 (1.4 – 1.9) (Figure 5.2.1, Table 5.2.1) and the risk difference over all studies was 9.3% (7.2 – 11.4; Figure 5.2.2). However, both the overall summary OR and overall summary RD were each associated with significant heterogeneity (OR; $I^2 = 43\%$) (RD; $I^2 = 50\%$). This heterogeneity is visually apparent in the non-overlap in summary 95% confidence intervals associated with the summary values for patient groups in non-ICU versus in ICU settings as displayed in both the OR (Figure 5.2.1) and RD (Figure 5.2.2) forest plots.

In both the OR (Figure 5.2.1) and the RD (Figure 5.2.2) forest plots, the association with mortality for endotoxemia detection versus non-detection among the individual studies undertaken in non-ICU settings is very heterogeneous. For example, the range in RD amongst all studies extends from a more than 40% excess to a more than 10% deficit. The spread of RD is reflected in the heterogeneity statistic ($I^2$) which is greater than 60% amongst the sub-group of studies of unrestricted adults ($I^2=77\%$), the sub-group of oncology patients ($I^2=67\%$) and the sub-group of restricted studies ($I^2=75\%$) (Figure 5.2.2).

By contrast, in both the OR (Figure 5.2.1) and the RD (Figure 5.2.2) forest plots, the association with mortality for endotoxemia detection versus non-detection among the studies undertaken in an ICU setting is minimal and the heterogeneity statistic ($I^2$) is less than 10% both in the case of the two group (0%) and the four group studies (0%) conducted in an ICU (Table 5.2.1; Figure 5.2.1).
Chapter 5: Results & discussion

2&4 group studies odds ratio

Fig 5.2.1 Forest plot (OR). Odds ratios for mortality for endotoxemia detected (Groups 1 & 3 combined) versus endotoxemia not detected (groups 2 & 4 combined) presented as study specific and summary risk difference (and 95% confidence intervals) derived from all 49 studies with studies sorted into those conducted in an ICU versus other settings. Overall summary OR indicated by vertical dashed line. Arrowheads indicate 95% confidence intervals that extend out of range. ICU2 refers to ‘two group’ supplementary studies undertaken in an ICU setting. All other studies are ‘4 group’ studies. Studies for which the OR is indeterminate do not appear in this figure.
There are several notable OR and RD observations for individual studies in Figures 5.2.1 & 5.2.2. The studies that used non-limulus assays such as the NCL assay [100, 101, 105-107] and the SRE assay [104] have similar OR and RD to that observed among the studies that had used the LAL assay. The two groups that had received HA-1A anti-endotoxin antibodies (indicated by HA-1A next to author name in Figures 5.2.1/2 [88, 94]) appear at opposite ends of the list of ICU studies ranked either by OR and RD. However as the 95% confidence intervals for both studies overlap the common result neither of these two studies can be regarded as being an outlier observation by statistical criteria (see below).
2&4 group studies Risk Difference

### Overall (I-squared = 47.9%, p = 0.000)
- Billard '94
- Kollef '97
- SRE
- Goldie '95
- Subtotal (I-squared = 0.0%, p = 0.937)

### ICU2
- Casey '93 LAL
- Venet '90 LAL
- Marshall '92 EAA
- Yeupchi '92 EAA
- Valenza '09 EAA
- Khalil '95 SRE
- Subtotal (I-squared = 0.0%, p = 0.904)

### Unrestricted
- Blan '94 all
- Skumacher '73
- Lumsden '89
- Pris '95
- Berger '95
- Lau '96
- Baley '96
- van Langevelde '10
- Byl '91
- Gbourbouls '99
- Foulis '92
- Levin '72 all
- Martinez-G '73
- Das '73
- Subtotal (I-squared = 77.4%, p = 0.000)

### Oncology
- Hyrinen '95
- Engervall '97
- Yoshioka '90
- Subtotal (I-squared = 66.8%, p = 0.049)

### Pediatric
- Togari '83
- Casey '92
- Cooperstock '85
- Klein '96
- Ahmed '94
- Sharap '88
- Feldman '74
- Subtotal (I-squared = 14.1%, p = 0.322)

### Restricted
- Magliulo '76
- Magliulo '76
- Butler '76
- Suppia '95
- Simpson '90
- Brandtzaeg '89
- Subtotal (I-squared = 59.9%, p = 0.029)

### Overall (I-squared = 47.9%, p = 0.000)

---

**Fig 5.2.2 Forest plot (RD).** Risk difference for mortality for endotoxemia detected (Groups 1 & 3 combined) versus endotoxemia not detected (groups 2 & 4 combined) presented as study specific and summary risk difference (and 95% confidence intervals) derived from all 49 studies with studies sorted into those conducted in an ICU versus other settings. Overall summary OR indicated by vertical dashed line. Arrowheads indicate 95% confidence intervals that extend out of range. ICU2 refers to ‘two group’ supplementary studies undertaken in an ICU setting. All other studies are ‘4 group’ studies. See Fig 5.2.3 for corresponding L’abbe plot.
Note: Y axis should read ‘Mortality in groups 1 & 3 combined’

Fig 5.2.3 L’abbe plot of mortality for groups with endotoxemia detected (Groups 1 & 3 combined) versus groups with endotoxemia not detected (Groups 2 & 4 combined) (see Table 5.2.2 for study codes). The line is x=y and represents the line of equivalence shown for visual reference purposes. Note that there are eight studies with 0% mortality in all groups.
Table 5.2.2
Details of selected (QS>2) ‘two’- & ‘four’-group studies (& codes for fig 5.2.3)

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<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>N</th>
<th>ETX +</th>
<th>ETX -</th>
<th>RD %</th>
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<td>102</td>
<td>97</td>
<td>11/22</td>
<td>50</td>
<td>36/75</td>
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<td>104</td>
<td>140</td>
<td>8/29</td>
<td>28</td>
<td>17/114</td>
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<td>Venet</td>
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<td>106</td>
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<td>41</td>
<td>21/55</td>
</tr>
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<td>857</td>
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<td>17</td>
<td>70/595</td>
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<td>18/104</td>
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<td>102</td>
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<td>Danner</td>
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<td>21</td>
<td>100</td>
<td>12/43</td>
<td>28</td>
<td>12/57</td>
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<td>20</td>
<td>1/8</td>
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<td>20/41</td>
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<td>Guidet</td>
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<td>93</td>
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<td>1/18</td>
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Note * Non-LAL assays; Marshall 2002 (ii4), neutrophil chemoluminescence assay; Kollef (ii2), SRE assay. 
Kollef 1997 [104 (ii2)] reported the LAL assay results was not predictive of mortality (data not available)
L’abbe plot

The L’abbe plot of mortality proportions in the patient groups with endotoxemia detected versus in patient groups with endotoxemia not detected for each of the 49 studies is presented in Figure 5.2.3. Selected studies have been labelled for identification (see Table 5.2.2 for codes). Both the forest plot (Figure 5.2.2) and the L’abbe plot (Figure 5.2.3) illustrate that the difference in mortality proportion for patients with endotoxemia detected versus not detected was in the range 0 to 20% for 33 studies (see also column headed RD in Table 5.2.2). The mortality difference exceeded 20% for only ten studies and was negative (<0%; i.e. mortality excess among patients without endotoxemia detected) for six studies.

There are eight studies in which the mortality proportion was 0% in both the groups of patients with endotoxemia detected and with endotoxemia not detected. As a result there are eight studies in the scatter plot which coincide at the origin.

Note in Figure 5.2.3 that the mortality rates among ICU studies is typically higher (i.e. >15%) versus in the non-ICU studies (<15%), as expected. This dichotomy is further explored in section 5.2.2.

There are 18 studies which have a RD either >20% (n=10) or ≤0% (n=8). Of these 18 studies, four studies (a4, a9, mg, o3, ii6) are noted here as being statistical high outliers in either Figure 5.2.1 or 5.2.2 in that the studies 95% confidence intervals associated with either the RD or the OR fail to include the common result in the forest plots. Likewise three studies (i5, a7, Bion) are noted here as being statistical low outliers. These 18 studies are to be discussed in section 5.2.4.
### 5.2.2. Analysis of ‘four group’ studies

#### Table 5.2.3 Summary results for analyses of prognostic studies (four group studies only)

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</tbody>
</table>

#### All studies

<table>
<thead>
<tr>
<th>Number of studies</th>
<th>11</th>
<th>16</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 v group 4</td>
<td>7.1; 4.2 – 12.0</td>
<td>3.6; 2.1 – 6.3</td>
<td>3.1; 2.0 – 4.8</td>
</tr>
<tr>
<td>Group 2 v group 4</td>
<td>2.0; 1.0 – 3.8</td>
<td>2.2; 0.9 – 5.8</td>
<td>1.3; 0.9 – 1.9</td>
</tr>
<tr>
<td>Group 3 v group 4</td>
<td>2.3; 1.3 – 4.0</td>
<td>2.0; 0.8 – 4.8</td>
<td>1.5; 1.2 – 1.8</td>
</tr>
</tbody>
</table>

#### Sub analysis - ICU setting

<table>
<thead>
<tr>
<th>Number of studies</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 v group 4</td>
<td>2.5; 1.6 – 3.9</td>
<td>1.5; 1.01 – 2.1</td>
</tr>
<tr>
<td>Group 2 v group 4</td>
<td>1.7; 1.2 – 2.6</td>
<td>1.4; 0.74 – 2.0</td>
</tr>
<tr>
<td>Group 3 v group 4</td>
<td>1.4; 0.8 – 2.5</td>
<td>1.4; 1.09 – 1.8</td>
</tr>
</tbody>
</table>

Foot notes: Abbreviations; OR = Odds Ratio, MH = Mantel Haenzsel, D-L = Dersimonian and Laird
Note: sub-groups are
- group 1; endotoxemia and GN bacteremia detected
- group 2; only GN bacteremia detected
- group 3; only endotoxemia detected
- group 4; neither endotoxemia or GN bacteremia detected (reference group)
Forest plots

The data extracted from the studies used in the analysis presented here is as summarized within Table 1 of CC ’12. As in section 5.2.1, the analysis here examines both the OR and RD meta-analysis. There is no substantive difference between the analyses using either the RD or the OR. The RD analysis is presented in the figures here (Figure 5.2.4/.5/.6) and that using the OR is presented in the figures in the compiled publication (Figures 2/3/4 of CC ’12).

For the 32 ‘four-group’ studies, the summary odds ratio (OR) for death for group 1, group 2 and group 3 each respectively versus group 4, are summarized in Table 5.2.3. The RD forest plots are presented in Figure 5.2.4 (group 1 versus group 4), Figure 5.2.5 (group 2 versus group 4) and Figure 5.2.6 (group 3 versus group 4) and the L’abbe plots are presented in Figure 5.2.7. The appearance of the forest plots differ to those displayed in CC ’12 because the categories here are labelled “icu” and “non-icu” and the studies have been sorted and ranked in order of study RD.

The species types of GN bacteremia isolates were identified for 31 of the 35 studies. Among the mono-microbial GN bacteremias, there were 174 (26%), 134 (22%), 74 (12%), and 94 (15%) bacteremias with E. coli; Enterobacteriaceae other than E. coli (e.g. Klebsiella species, Enterobacter species); P. aeruginosa; and N. meningitidis, respectively. After excluding studies restricted to specified infections, there were 497 GN bacteremias for which the species type was known. Among these 497 GN bacteremias, there was an uneven distribution of E. coli versus P. aeruginosa identified among the GN bacteremias of group 1 versus group 2; E. coli was less common in group 1 than group 2 (92 of 303, 30% versus 82 of 194, 42%, p = 0.007; chi-square test). By contrast, P. aeruginosa was more common in group 1 than group 2 (53 of 303, 17% versus 23 of 194, 12%, p = 0.09; chi-square test). This reciprocal mal-distribution was also apparent among the 9 studies of adults with sepsis in an ICU setting (data not shown).
Fig 5.2.4 Forest plot (RD). Risk difference for mortality for endotoxemia and GN bacteremia co-detected (Group 1) versus neither detected (Group 4) presented as study specific and summary risk difference (and 95% confidence intervals) derived with studies sorted into those conducted in an ICU versus other settings. Overall summary OR indicated by vertical dashed line. Arrowheads indicate 95% confidence intervals that extend out of range. See Fig 5.2.7a for corresponding L’abbe plot.

Pooled RD for ICU studies is 7.4% (-0.4 – 15.1)
Pooled RD for non-ICU studies is 18.7% (7.7 – 29.8)
Pooled RD for all studies is 16.1% (8.1 – 24.1)
Fig 5.2.5 Forest plot (RD). Risk difference for mortality for GN bacteremia detected alone (Group 2) versus neither detected (Group 4) presented as study specific and summary risk difference (and 95% confidence intervals) derived with studies sorted into those conducted in an ICU versus other settings. Arrowheads indicate 95% confidence intervals that extend out of range. Overall summary OR indicated by vertical dashed line. See Fig 5.2.7b for corresponding L’abbe plot. Pooled RD for ICU studies is -0.3 (-12.8 - 12.2) Pooled RD for non-ICU studies is -2.1% (-8.6 – 4.4) Pooled RD for all studies is -1.8% (-7.4 – 3.7)
**Fig 5.2.6 Forest plot (RD).** Risk difference for mortality for endotoxemia detected alone (Group 3) versus neither detected (Group 4) presented as study specific and summary risk difference (and 95% confidence intervals) derived with studies sorted into those conducted in an ICU versus other settings. Overall summary OR indicated by vertical dashed line. Arrowheads indicate 95% confidence intervals that extend out of range. See Fig 5.2.7c for corresponding L’abbe plot.

Pooled RD for ICU studies is 6.8% (1.7 – 11.8)

Pooled RD for non-ICU studies is -0.7% (-5.8 – 4.4)

Pooled RD for all studies is 2.3% (-1.6 – 6.1)
Figure 5.2.7a

Figure 5.2.7b

Figure 5.2.7c

Fig 5.2.7. L’Abbé plots of study specific mortality rates. Each figure shows mortality rates for studies undertaken in an ICU (triangles) or non-ICU (circles) setting with symbols proportional to group size with the line of equivalence (y = x; dashed line) shown for visual reference purposes. Shown are Groups 1 (Endotoxemia & GN bacteremia co-detected; Figure 5.2.7a - top), Groups 2 (GN bacteremia alone; Figure 5.2.7b - middle), and Groups 3 (Endotoxemia alone; Figure 5.2.7c - bottom) each versus Groups 4 (neither detected).
The summary odds ratio (OR) for death for Group 1, Group 2 and Group 3 each respectively versus Group 4, are presented in Table 5.2.3 for all studies and for the studies undertaken in an ICU setting as a sub-analysis. The summary RD and I² are presented in the legends of figures 5.2.4/5/6. With only the nine ICU studies considered, the summary odds ratios were all either not significant or borderline (OR < 2). These findings are also apparent amongst the corresponding summary RD being less than 10% (figures 5.2.4/5/6).

With the 26 studies undertaken outside of an ICU setting considered, the summary odds ratio for Groups 1 (co-detection of endotoxemia with GN bacteremia) versus Group 4 was 6.9 (4.4-11.0) whereas the summary odds ratio (OR) for Groups 2 (GN bacteremia alone) and groups 3 (endotoxemia alone) versus Group 4 (neither) were not significant or borderline (OR < 2) (data shown in Table 2 of CC ‗12). These findings are also apparent amongst the corresponding overall summary RD being less than 10% (Figures 5.2.5/6) with the exception of Group 1 versus Group 4 (Figures 5.2.4).

The heterogeneity amongst the studies was most apparent in the analysis of Groups 1 (co-detection of endotoxemia with GN bacteremia) versus Group 4 from all 35 studies (OR: I²=42%; as shown in Table 2 of CC ’12) and particularly so for studies undertaken outside of an ICU setting (RD I²=67%; as shown within Figure 5.2.4). Otherwise, for all other comparisons; Group 2 versus Group 4 and Group 3 versus Group 4, the heterogeneity amongst the studies was minimal whether calculated as OR (I²=0%; as shown in Table 2 of CC ’12) or RD (I²<35%; as shown within Figures 5.2.4, 5.2.5, & 5.2.6).

In comparing the prior meta-analyses (JCM ‘95, JER) versus the update for the thesis, there has been a decline in the OR’s for death for Group 1, Group 2 and Group 3 each versus Group 4 (Table 5.2.3). This decline is partly attributable to the most recently included study which also is the largest study (Opal [65]).

**L’abbe plots**

The L’abbe plots (Fig 5.2.7. a, b, c) presented here are identical to the L’abbe plots in CC ’12 (see figure 5 of CC ‘12).
The group 4 mortality proportion exceeded 15% for all studies in an ICU setting whereas all but one of the studies in a non-ICU setting had a Group 4 mortality proportion <15% (Fig 5.2.7.a, b, c). This stratification in mortality proportions for ICU versus non-ICU studies is as would be expected. Both the overall dispersion in the mortality proportions and the dispersion away from the line of identity is most apparent in the plot of group 1 versus group 4 (Fig 5.2.7 a) in that for 17 of the 34 studies the mortality proportion in group 1 was >20 percentage points higher in group 1 versus group 4. By contrast, there were only 8 studies for which the mortality proportion in either group 2 (Fig 5.2.7 b) or group 3 (Fig 5.2.7 c) differed by more than 20 percentage points versus the mortality in group 4. The greater overall dispersion is also apparent by contrasting the RD as displayed in the forest plots (i.e. Fig 5.2.4 versus Fig 5.2.5 & fig 5.2.6).

5.2.3. Impact of GN bacteremia species type

The association of patient outcome with endotoxemia detection and with specific GN bacteremia types in the categories detailed below was examined for those studies for which this data was available. All of these observations have been obtained from groups 1 and 2 of the ‘four group’ studies. Twenty-seven studies published over three decades were identified. These report the results for a total of 545 GN bacteremic patients of whom 163 (29.9%) had a fatal outcome. Among the mono-microbial GN bacteremias, there were 476 bacteremias with outcome known in the following broad categories of GN bacteremia types;

- **E. coli;** 144 (25%)
- Enterobacteriaceae other than *E. coli* (e.g. *Klebsiella species*, *Enterobacter species*); 97 (17%)
- **P. aeruginosa;** 59 (10%),
- **N. meningitidis;** 138 (24%)
- **Burkholderia pseudomallei ;** 15 (3%)
- **Yersinia pestis ;** 7(1%)
- **Salmonella typhi;** 36 (6%)
- Other (including anaerobic); 70 (12%)
The GN bacteremias were classified as follows; bacteremias arising from studies which included GN bacteremias of any type (unrestricted studies; 17 studies; Table 5.2.4) and studies which were restricted to GN bacteremias of a specified type (e.g. *N. meningitidis*; restricted studies; 10 studies; Table 5.2.5). Note that there were an additional five studies [8, 54, 75, 77, 92] with 114 GN bacteremias from studies for which the GN bacteremia types are known but the individual patient outcomes are not known. These five studies are not analysed here but are considered further in section 5.2.4.

The summary analysis is presented with four separate methods of summation; tally, GEE and ELR (Table 5.2.5) and Mantel-Haenszel method (Figure 5.2.8).

The summary odds ratios for the categories of GN bacteremias are summarized in Table 5.2.5 and presented as a forest plot in figure 5.2.8. Sparse data from 9 non-ICU studies was collapsed into two broad categories to enable their representation within figure 5.2.8. These two categories are adult non-ICU studies and a combined category of oncology and pediatric studies. The largest study [65] provided data in relation to endotoxemia detection at two breakpoints and this has been used to stratify the mortality proportions from this study within figure 5.2.8. There was no significant heterogeneity associated with any of the summary odds ratios (figure 5.2.8). The $I^2$ associated with each of these summary OR’s was <10%, indicating minimal heterogeneity.

**Unrestricted studies**: Among the 17 studies that were unrestricted to type of GN bacteremia, there were 360 patients with 116 (32%) fatal outcomes (Table 5.2.4). There were eight studies limited to adult patients in ICU [21, 23, 37, 38, 46, 59, 65, 74], and eight non-ICU based studies including three studies of febrile oncology patients [26, 42, 95] and two studies limited to a pediatric age group [2, 72]. *E. coli* was the most common GN bacteremia overall. The mortality proportion was lowest for *E. coli* (144 with 34 deaths; 24%), versus Enterobacteriaceae other than *E. coli* (97 with 33 deaths; 34%). Likewise, among GN bacteremias summed across only the eight ICU studies, the mortality rates for bacteremias with *E. coli*, Enterobacteriaceae other than *E. coli*, and Pseudomonas species were 23%, 35%, and 47%, respectively.
### Table 5.2.4: Mortality proportions for patients with gram negative bacteremia: unrestricted studies only

<table>
<thead>
<tr>
<th>Study [reference]</th>
<th>Assay (^a)</th>
<th>Census (^b)</th>
<th>Numbers of patients with fatal outcome / Number tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E coli</td>
<td>non- E coli</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterobacteriae</td>
<td>Enterobacteriae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Etx+</td>
<td>Etx-</td>
<td>Etx+</td>
</tr>
<tr>
<td>Bates [46] 1998</td>
<td>0.005</td>
<td>28</td>
<td>0/3</td>
</tr>
<tr>
<td>Danner [21] 1991</td>
<td>0.01</td>
<td>14</td>
<td>1/5</td>
</tr>
<tr>
<td>Dofferhoff [23] 1992</td>
<td>0.005</td>
<td>21</td>
<td>0/1</td>
</tr>
<tr>
<td>Goldie [37] 1995</td>
<td>0.002</td>
<td>30</td>
<td>0/1</td>
</tr>
<tr>
<td>Guidet [38] 1994</td>
<td>0.005</td>
<td>28</td>
<td>3/9</td>
</tr>
<tr>
<td>Maury [59] 1998</td>
<td>0.005</td>
<td>30</td>
<td>0/8</td>
</tr>
<tr>
<td>Strutz [74] 1999</td>
<td>0.01</td>
<td>NS</td>
<td>1/4</td>
</tr>
<tr>
<td>Opal [65] 1999</td>
<td>0.02</td>
<td>28</td>
<td>2/15</td>
</tr>
<tr>
<td>Low: 20-660 pg/ml</td>
<td>(d, h)</td>
<td>Non-ICU (Adult) 4 studies (^i)</td>
<td>3/13</td>
</tr>
<tr>
<td>High: &gt;660 pg/ml</td>
<td>(d, h)</td>
<td>Non-ICU (oncology &amp; pediatric) 5 studies (^j)</td>
<td>2/4</td>
</tr>
<tr>
<td>Sub-total</td>
<td>12/64</td>
<td>15/43</td>
<td>20/50</td>
</tr>
<tr>
<td>Total</td>
<td>17/81</td>
<td>17/63</td>
<td>26/62</td>
</tr>
</tbody>
</table>

- **a.** Refers to endotoxin assay breakpoint for endotoxemia detection
- **b.** Refers to survival census day
- **c.** Non-Enterobacteriaceae includes the following bacteremias: Pseudomonas species, *Acinetobacter species*, and *Bacteroides species*.
- **d.** Data for these studies [21, 37, 38, 46, 65] provided by personal communication.
- **e.** Goldie [37] – H. influenzae bacteremia (n=1) from this study not included
- **f.** Guidet [38] – H. influenzae bacteremia (n=1) from this study not included
- **g.** Maury [59] – H. influenzae bacteremia (n=1) from this study not included
- **h.** Data for this study Opal et al [65] stratified into two categories of endotoxemia detected; low (20-660 pg/ml) and high (>660 pg/ml) versus endotoxemia not detected (<20 pg/ml)
- **i.** Non-ICU (adult) represents Martinez-G et al 1973 [57], Byl et al, 2002 [17], Prins et al., 1995[67] and Giamarellos et al 1999 [36] collapsed into one category
For these GN bacteremias from the unrestricted studies, only in the case of Enterobacteriaceae other than *E. coli*, was the summary odds ratio significantly elevated above 1, indicative of a worse outcome in endotoxemic patients (Table 5.2.5, Figure 5.2.8). The increased mortality for this category is particularly apparent for Klebsiella species (9 of 13 deaths in 8 studies if endotoxemia was detected versus 2 of 17 deaths in 6 studies if endotoxemia was not detected). For the *E. coli* GN bacteremias, none of the study level odds ratios (Figure 5.2.8), or the summary odds ratio, were significantly elevated above 1 (Table 5.2.5). Recalculation of the OR’s including only the eight studies limited to the ICU setting [21, 23, 37, 38, 46, 59, 65, 74] yielded summary OR’s similar to previously with none that were significantly different from 1. The method used to derive the summary OR’s in Table 5.2.5 (GEE versus ELR) or Figure 5.2.8 (MH) made no substantive difference to the overall findings.

**Enterobacteriaceae bacteremias and endotoxemia.** The finding (Table 5.2.5, Figure 5.2.8) that the risk of mortality was increased in association with the detection versus non-detection of endotoxemia with GN bacteremias with Enterobacteriaceae other than *E. coli* but not for *E. coli* was unexpected. The statistical significance of this unexpected finding was tested for as an interaction term within a regression model. This interaction term was found to be significant indicating an impact of endotoxemia on prognosis additional to the impact of GN bacteremia type for *E. coli* versus non-*E. coli* Enterobacteriaceae. This was apparent for both an analysis limited to the studies undertaken in ICU (OR 3.8; 1.13 – 12.8) [21, 23, 37, 38, 46, 59, 65, 74] and also for an analysis including all the unrestricted studies (OR 2.8; 1.01 - 8.1) [2, 17, 26, 36, 42, 57, 67, 95].

**Restricted studies:** Among the studies restricted to one of the four specified GN bacteremias; *Neisseria meningitidis* (five studies) [11, 12, 35, 37, 38, 68, 72]; *Yersinia pestis* (two studies) [14, 15], melioidosis (one study) [73]; and *Salmonella typhi* (three studies) [1, 56, 77], there were 196 bacteremic patients with 49 (25%) fatal outcomes. For these GN bacteremias, only in the case of *Neisseria meningitidis* bacteremia was the summary odds ratio for mortality significantly elevated (p < 0.0001) in association with the detection of endotoxemia (Table 5.2.5).
Table 5.2.5: Mortality proportions and odds ratios in association with endotoxemia detection: unrestricted and restricted studies

<table>
<thead>
<tr>
<th>GN bacteremia species</th>
<th>Number of studies</th>
<th>Dead / total number of patients (%)</th>
<th>Odds ratio; 95% CI</th>
<th>GEE</th>
<th>ELR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endotoxemia detected</td>
<td>Endotoxemia not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ICU studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E coli</em>^a^</td>
<td>8</td>
<td>12/64 (19)</td>
<td>15/43 (35)</td>
<td>0.54; 0.25 - 1.13</td>
<td>0.51; 0.17 - 1.5</td>
</tr>
<tr>
<td>non- <em>E coli</em> Enterobacteriaceae^a,b^</td>
<td>8</td>
<td>20/50 (40)</td>
<td>6/25 (24)</td>
<td>2.7; 0.90 - 8.4</td>
<td>3.1; 0.82 - 13.7</td>
</tr>
<tr>
<td>Non-Enterobacteriaceae^c^</td>
<td>7</td>
<td>17/36 (47)</td>
<td>6/15 (40)</td>
<td>1.2; 0.35 - 4.1</td>
<td>1.06; 0.27 - 4.3</td>
</tr>
<tr>
<td><strong>ICU &amp; non-ICU studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E coli</em>^a^</td>
<td>15</td>
<td>17/81 (21)</td>
<td>17/63 (27)</td>
<td>0.78; 0.36 - 1.7</td>
<td>0.81; 0.28 - 2.3</td>
</tr>
<tr>
<td>non- <em>E coli</em> Enterobacteriaceae^a,b^</td>
<td>14</td>
<td>26/62 (42)</td>
<td>7/35 (20)</td>
<td>3.7; 1.3 - 10.3</td>
<td>3.9; 1.1 - 16.7</td>
</tr>
<tr>
<td>Non-Enterobacteriaceae^c^</td>
<td>13</td>
<td>22/53 (42)</td>
<td>8/25 (32)</td>
<td>1.7; 0.65 - 4.9</td>
<td>1.4; 0.42 - 5.3</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>5</td>
<td>37/106 (35)</td>
<td>0/32 (0)</td>
<td>26.0; 1.6 - 321</td>
<td>36.9; 7.7 - Inf</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>2</td>
<td>1/5 (20)</td>
<td>0/2 (0)</td>
<td>Undefined</td>
<td>Undefined</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>3</td>
<td>1/19 (5)</td>
<td>1/17 (6)</td>
<td>Undefined</td>
<td>0.89; 0.01-74.1</td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td>1</td>
<td>9/15 (60)</td>
<td>1/5 (20)</td>
<td>Undefined</td>
<td>Undefined</td>
</tr>
</tbody>
</table>

Abbreviations: GEE = Generalized estimating equation method; ELR = Exact logistic regression method

a. The impact of the detection of endotoxemia on the difference in morality proportions for *E coli* versus non-*E. coli* Enterobacteriaceae bacteremias was tested as an interaction term within a regression model using generalized estimating equations. This interaction term was found to be significant (OR 3.8; 1.13 – 12.8) for an analysis limited to the studies undertaken in ICU and also for an analysis including both ICU and non-ICU studies (OR 2.8; 1.01 - 8.1).

b. Non-*E. coli* Enterobacteriaceae includes the following bacteremias; *Klebsiella species*, *Enterobacter species*, *Proteus species*, and *Serratia species*.

c. Non- Enterobacteriaceae includes the following bacteremias; Pseudomonas species, *Acinetobacter species*, and *Bacteroides species*.
Fig 5.2.8 Forest plot (OR). Forest plot of mortality in relation to the detection of endotoxemia (etx + versus etx -) for patients with GN bacteremia are presented as study specific odds and summary odds ratios; ‘etx – less survival’ and ‘etx + less survival’ indicates the ranges in OR’s for which mortality is more common in association with either endotoxemia non-detection or detection, respectively.
**Comparison versus each benchmark IQ range.** The mortality risk for GN bacteremias of the three types usually seen in unrestricted studies as seen in the broader literature since 1975 [287, 338-390] was summarized as an inter-quartile (IQ) range (Table 2.4.2). The overall IQ range was 19-43% (figures 2.12/.13/.14/.15). This survey included opportunistic type GN bacteremias as reported for various hospital based settings (e.g. ICU versus non-ICU) and studies of various sizes.

The mortality proportions for patients with versus without endotoxemia in association with GN bacteremia in studies unrestricted to specific GN bacteremia types are mostly within each respective range and the overall IQ range (Figure 5.2.9). By contrast, the mortality proportions for patients with GN bacteremias in studies restricted to specific GN bacteremia types such as with *Burkholderia* (melioidosis – endotoxemia detected) and *N. meningitidis* (endotoxemia not detected) are partly outside this range and for *S. typhi* (Typhoid – both endotoxemia detected and endotoxemia not detected) are wholly outside this range.

![Comparison versus IQ range](image)

**Fig 5.2.9 Comparison versus IQ range.** Plot of mortality proportions in relation to the detection of endotoxemia (etx + versus etx -) (Table 5.2.5) for various types of GN bacteremia versus each respective IQ range (represented by horizontal lines) and also versus GN bacteremia overall (represented by vertical lines) as derived from studies in the literature (Table 2.4.2). Note logit scale.
5.2.4. Study limitations and literature reconciliation

These are the findings from this analysis of the detection of endotoxemia as a test of prognosis;

1. (section 5.2.1). The mortality RD associated with the detection versus non-detection of endotoxemia (without regard to the co-detection of GN bacteremia) is similar amongst the ‘two group’ versus the ‘four group’ studies for studies conducted in an ICU setting. Among 20 such studies only two statistical outlier studies were identified; one high (Valenza; ii6 [107]) and one low (Goldie; i5 [37]) (Table 5.2.1, Fig 5.2.1). The following is used as a criterion to define a statistical outlier study; a study’s 95% confidence interval fails to include the common summary result in either the OR (Figure 5.2.1) or RD (Figure 5.2.2) forest plots.

2. (section 5.2.1). By contrast, for studies conducted outside an ICU setting, the mortality RD associated with the detection versus non-detection of endotoxemia (without regard to the co-detection of GN bacteremia) is more heterogeneous versus that for studies conducted in an ICU setting with more variability both within and between study categories. Among 30 such studies eight statistical outlier studies were identified; three low (Bion, a7, Cooperstock) and five high (Brandtzæg [mg], Das [22], Levin [a4], Yoshida [o3], van Langevelde [a9]) (Fig 5.2.1 & Fig 5.2.2).

3. For the largest study (i9, [65]), the 135 GN bacteremic patients were able to be classified into sub-groups with high (>660 pg/ml), low (>25 pg/ml) and non-detectable (<25 pg/ml) levels of endotoxemia. There was no significant evidence for a dose response effect in any of the three categories of GN bacteremia species type studied in that the mortality odds ratios were similar at high and low levels of endotoxemia (fig 5.2.3).

4. (section 5.2.2). Summary results (Table 5.2.3). The co-detection of endotoxemia with GN bacteremia (Group 1) is most predictive of increased mortality risk (OR 6.9; 4.4-11.0; RD 19%; 11-27%) versus the detection of neither (Group 4; Figure 5.2.4/.5/.6 and Figure 2/3/4 & Table 2 of CC ‘12). However, this is most apparent amongst the sub-group of studies undertaken outside an ICU setting and is a finding which is associated with marked
heterogeneity (OR $I^2$ 35%; RD $I^2$ 67%). By contrast, the detection of either endotoxemia or GN bacteremia in isolation (Group 2 and Group 3; **Figure 5.2.4/5.6 and Fig 2/3/4 & Table 2 of CC ‘12**) versus the detection of neither (group 4) is associated with borderline elevation of risk (OR <2.0; RD <10%) and, moreover, is associated in each case with heterogeneity that is no more than moderate (OR & RD $I^2$<35%). This lack of heterogeneity is surprising given the diversity of patient groups, underlying risk, and clinical settings in these studies that were conducted and published over a period exceeding three decades.

5. L’Abbé plots (**Fig 5.2.7**). The underlying patient risk, as reflected in the group 4 mortality proportion, was higher for studies in an ICU setting versus studies in other settings (**Fig 5.2.7a-c**), as might be expected. However, the additional mortality associated the detection of either or both of endotoxemia or GN bacteremia was less apparent in the studies of patients at high versus low underlying risk (**Table 5.2.3**).

6. (section 5.2.3). The prognostic value of endotoxemia detection versus non-detection in association with GN bacteremia varies across different species types of GN bacteremia. This variability across species types, which is in contrast to the lack of heterogeneity across studies, is visually apparent in the forest plot (**Fig 5.2.8**).

7. The detection of endotoxemia in association with *Neisseria meningitidis* bacteremias has the strongest prognostic value (**Table 5.2.5**). With the GEE method, the width of the 95% confidence interval (CI) is determined by the number of clusters together with the similarity of their results (intra-cluster correlation). Hence with this method, clusters that are few (e.g. *N. meningitidis*), or small or which have disparate results will result in 95% CI’s that are wide versus clusters that are numerous, large and which have similar results.

8. Surprisingly, the detection of endotoxemia in association with *E. coli* bacteremia, which amongst the unrestricted GN bacteremia category has the most number of patients, has no prognostic value (**Fig 5.2.8, Table 5.2.5**) in any setting. This finding is in stark contrast to the association of endotoxemia, GN bacteremia and outcome overall (i.e. group 1 versus group 4; **Table 5.2.1**). As a
sensitivity analysis, it was found that it would require at least three studies each having at least 40 patients with *E. coli* bacteremias with an OR for mortality of > 10 in association with the detection versus non-detection of endotoxemia to change the conclusions found here (data not shown). It is unlikely that such studies would be unpublished or easily overlooked.

9. This unexpected finding for *E. coli* bacteremias versus GN bacteremia overall is to be explored as a basis for the conflicting findings of the individual studies (Tables 5.2.6 & figure 5.2.9).

10. Note that the mortality proportions for patients bacteremic with *E. coli*, and Pseudomonas species found in the studies analysed in Table 5.2.5 are mostly within each respective IQ range as derived from the broader literature (Table 2.4.2) whether or not endotoxemia is detected.

11. There is a significant interaction effect between the detection of endotoxemia and the type of Enterobacteriaceae (*E. coli* versus non-*E. coli*) bacteremia on the prognosis (Table 5.2.5).

12. The prevalence and species types of GN bacteremias varied among individual studies and groups (Table 5.1.6). However the numbers in individual studies are small typically being less than 20 GN bacteremias in total.

13. Of the patients included within this analysis of prognosis (Figure 5.2.4, Figure 5.2.5, Figure 5.2.6), 20% had GN bacteremia detected (Group 1 and Group 2) of which 23% were *E. coli* whereas only 12% were *P. aeruginosa*. However, there are significant reciprocal differences in the frequency of *P. aeruginosa* versus *E. coli* among the bacteremias of Groups 1 and 2.

14. Outlier studies. The following is used as criteria to define a statistical outlier study; a study’s 95% confidence interval fails to include the common summary result in either the OR (Figure 5.2.1) or RD (Figure 5.2.2) forest plots. Using this as a criterion to define a statistical outlier reveals 10 studies with outlier results in figures 5.2.1, & 5.2.2. Using this criterion there are five studies which are high outliers and have either an RD of >30% (a4, mg, o3, Das) or an OR > 3 (a4, mg, o3, a9, Das). Likewise, there are five studies which are low outliers with an RD of <0% [Bion, a7, p3, Magliulo] or an OR <1 (Bion, a7, i5). There are additional studies with a RD >20% or <0% (see also Figures 5.2.3/9) but
which do not meet the statistical outlier criterion listed above. These are termed potentially outlier studies. Possible reasons that might account for why the results of these studies are outlier are discussed below.

15. The relevance of endotoxemia detection toward prognosis amongst patients with GN bacteremia within the studies analyzed here [1-107] is contrasted against each IQ range that was derived from other studies in the broader literature experience since 1975 [287, 338-360] to enable three inferences to be drawn.

16. Firstly, amongst the unrestricted studies the mortality proportions for patient with versus without endotoxemia detected are mostly within the IQ range for each respective GN type (Figure 5.2.9) with the exception of non-\textit{E. coli} Enterobactereiaceae. Hence for the GN bacteremia types as classified here, the impact of endotoxemia detection toward patient prognosis for any of the three GN bacteremia types (Table 5.2.5) cannot be clearly discerned versus the impact of the many other factors contributing toward the range in mortality proportions among these GN bacteremia types in the broader literature experience.

17. The impact of endotoxemia detection versus non- detection on mortality proportions is most evident for non-\textit{E. coli} Enterobactereiaceae bacteremias.

18. Thirdly, the experience amongst the restricted studies is not representative of that amongst the unrestricted studies. In this regard, the mortality proportions for patient with versus without endotoxemia detected are either partly above (\textit{Neisseria meningitidis}), partly below (\textit{Burkholderia pseudomallei}) or wholly outside (\textit{S. typhi}) this benchmark IQ range (Table 5.2.5).

Overall, the analysis presented in section 5.2 implies that the study setting being ICU versus non-ICU, and the study prevalence of GN bacteremia type may be unrecognized confounders of the relationship between endotoxemia and outcome. This warrants further consideration toward the interpretation of the studies included here. However, there are a number of limitations to these findings.

This analysis is unable to identify the mechanism for any increased mortality risk. Many relevant patient-specific details such as age and co-morbidities for the patients of the four groups in each study were not available. In this analysis, a variable proportion of blood cultures that were classified as negative for GN bacteria would
have yielded gram-positive bacteremias or fungemias. The detection of endotoxemia in association with blood culture isolates other than GN bacteremias is a commonly reported finding [25, 54, 75]. In this respect, the prognostic impact of blood stream infections other than GN bacteremias in relation to the co-detection with endotoxemia has not been addressed here. Moreover, the origin of endotoxemia for patients in Group 3 is uncertain and the possibility of endotoxin originating from other sources, such as gut barrier breakdown, as is presumed to occur for non-septic forms of shock, needs to be considered [708].

It needs to be noted that endotoxemia [709] and GN bacteremia are each either episodic or dynamic phenomena and the criteria for a positive detection of each will have differed among the studies. The kinetics of bacteremia, endotoxemia and pathophysiology may not be concordant. For example, in one clinical study [21] of 100 patients with sepsis in an ICU setting, the cumulative percent found to have endotoxemia rose from 20% to 40% between 0 and 24 hours after study entry. Moreover, in many studies, the timings of antibiotic administration in relation to collections of samples for blood cultures and endotoxemia assay were not known.

The additional mortality associated the co-detection of endotoxemia and GN bacteremia (Group 1) was less apparent in the studies of patients at high (i.e. ICU setting) versus low underlying risk (in Table 5.2.3 and Table 2 of CC '12). This finding here at a group level of analysis resembles other recent findings at an individual patient level of analysis among bacteremias of all species types occurring in an ICU setting [301]. However, this inference requires caution for three reasons. Firstly, it needs to be clarified as to whether the increased risk is absolute [340] or relative [301].

Secondly, it should be noted that the L’Abbé plots are useful merely as simple graphical methods to facilitate visual comparisons of the group mortality proportion over the range of underlying risk as found in individual studies within a meta-analysis [681]. The issues underlying the statistical testing for variation in either additional risk or treatment effect in relation to underlying risk are not simple [681-683, 686-687]. In particular, in using linear regression (which has not been done here) in conjunction with L’Abbe plots to explore heterogeneity over a range of underlying risk, regression to the mean is an important consideration as has been noted among treatment studies of mild hypertension [687].
Thirdly, there were insufficient studies that had used assays other than the limulus assay, or patient groups that had received anti-endotoxin antibodies to include these studies so as to enable a systematic study of these variables by meta-regression. Only eight studies [1, 11-16, 56, 77] were limited to specific GN bacteremia species types. Also, many relevant study level details, such as method of blood culture used and antibiotic therapy protocols were not available. Anti-endotoxin antibodies are detectable in patients with severe sepsis and septic shock and the kinetics of these antibodies over time differs between survivors and non-survivors [710]. However, the impact of these anti-endotoxin antibodies on both the detection of endotoxemia and possibly also on patient outcome within the studies examined here is uncertain [711-712]. A more detailed examination adjusting for relevant prognostic variables contributing to underlying patient level risk and also the inter-relation between various GN bacteria known to have differing lipid-A structures would require an individual patient data meta-analysis.

Finally, it needs to be conceded that some of the findings in section 5.2 are unstable to additional ‘missing’ data whether published or otherwise. It is not unprecedented that the finding of a large meta-analysis is overturned by unpublished data and the controversial example of the meta-analyses of mortality related to cefepime [713-718] was cited earlier (section 2.6). In this regard, estimates for the amount of missing data that would be required to overturn these findings are provided.

**Literature reconciliation** With these caveats mentioned in relation to the limitations of this analysis, the studies will now be examined with particular interest in the outlier studies identified in Figures 5.2.1/2/3 in an attempt to achieve a reconciliation of the literature. Figure 5.2.10 is Figure 5.2.3 reproduced with the following modifications;

- Studies that are small (< 20 patients or < 2 GN bacteremias) or with QS<2 have been excluded due to concerns relating to publication bias and atypical DOR results (as identified in 5.1.1),
- Studies restricted to specified bacteremias have all been excluded except for one study of typhoid ([77] see below).
- The studies have been coded to indicate whether the number of *E. coli* bacteremias as a proportion of the total number of patients is <3%, 3-5%, >5% or unknown (see figure legend),
- Studies for which the number of *E. coli* bacteremias is <5% have the studies’ diagnostic odds ratio (DOR) as tabulated in Table 5.2.6 displayed,
Reference lines indicating risk differences of 0% and 20% are displayed in Table 5.2.9 to indicate potentially outlier studies.

Six supplementary list studies including four non-Limulus studies are included.

With these modifications, Figure 5.2.10 summarizes the influence of underlying patient risk at a group level (mortality in Group 2 & 4 combined), the prevalence of E. coli bacteremias as a proportion of the total number of patients, the diagnostic odds ratio and the risk difference in each study. In addition the reference lines in the figure help to identify potential outlier studies (i.e. by using the screening definition of a potential outlier being RD<0% or RD >20%).

‘▲’ non-limulus studies. Five studies that used the EAA (non-limulus) assay method in an ICU setting (indicated by ‘▲’ in Figure 5.2.10) are displayed and only one is a statistical outlier (ii6; RD = 12%). This study is a high outlier (ii6 [107]) and while it is classified having been undertaken in an ICU setting, all patients were post-surgical and the mortality risk in the endotoxemia negative group is 0% and the GN bacteremia information is not available for this study.

‘two group’ studies. None of the six large ‘two group’ studies in an ICU setting (ii3 [91], ii5 [101], ii1 [102], ii2 [104] ii4 [106] ii6 [107]) found mortality differences exceeding 15 percentage points between patient groups that were endotoxemia positive versus negative. While one study had reported E. coli prevalence (accounting for two of a total of only four GN bacteremias [106]), the GN bacteremia species types were not stated in the other studies. A seventh large non-ICU study did not report mortality as an outcome and could not be included here [86].

‘○’ / ‘+’ studies. There are 22 studies for which the number of E. coli bacteremias as a proportion of the total number of patients is >5% (indicated by a ‘○’) or unknown (indicated by a ‘+’) and only one of these is an outlier [Das, 22]. This study, which is a high outlier, included both pediatric group and adult patients. However, further information relating to patient selection and GN bacteremias is not available for this study.

‘●’ / ‘●’ studies with RD<0. There are 12 studies for which the number of E. coli bacteremias as a proportion of the total number of patients is <3% or 3-5% (indicated by ‘●’ or ‘●’ closed symbols, respectively). There were three which have a RD<0% of which one of these three is a small (n=10) Japanese study of neonates receiving exchange transfusion as a treatment for sepsis [81, not shown in Figure 5.2.9].
The other two are studies which both have a low DOR (0.6, [Bion; 8] & 1.3 [Goldie; 37]). The low DOR in these two studies raises the possibility that assay malfunction occurred and hence resulted in misclassification bias with the likelihood that the patients with endotoxemia were incorrectly classified in these studies.

**Fig 5.2.10** Mortality L’abbe plot for endotoxemia detected (Groups 1 & 3 combined) versus endotoxemia not detected (Groups 2 & 4 combined). Restricted and small studies (number of patients; N< 20 or number of GN bacteremias<2) not shown. Symbols are coded for calculated E coli bacteremias as % of N; black ‘●’ if <3%, gray ‘●’ if 3-5%, open ‘○’ if >5%, ‘+’ if unknown and ‘▲’ if non-limulus assay used. The numbers refer to the DOR (studies with <5% E coli bacteremias; see table 5.1.6). Two lines are shown for visual reference; the line of equivalence (solid line) and that for a risk difference equivalent to a 20% excess (dashed line). **Note:** Y axis should read ‘Mortality in groups 1 & 3 combined’
Table 5.2.6 Details of selected (QS>2 & N>20) studies (& codes for figure 5.2.3)

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<td>2009</td>
<td>107</td>
<td>NS</td>
<td>102</td>
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Foot notes:
- Only shown are studies with QS>2 and N>20.
‘●’ / ‘●’ studies with RD>0. Of the remaining 9 of the 12 studies for which the number of *E. coli* bacteremias as a proportion of the total number of patients is <3% or 3-5% (indicated by ‘●’ or ‘●’ closed symbols, respectively) all nine have a RD>9% and these all have DOR’s above 4. Three of these studies (ml – [73, not shown in Figure 5.2.9], Levin [54], Yoshida [95]) are also flagged as high outliers by statistical criteria.

Two studies (Magluilo [56], Copperstock; p3-[20]) are flagged as statistical low outliers and each has a RD= 0 but also have mortalities of zero in both those with and without endotoxemia detected. Both had a high prevalence of either Salmonella species (100% Magliulo [56], not shown in Figure 5.2.9) or *E. coli* (9%; Copperstock; p3 [20]) isolates.

**Restricted studies.** Six other studies restricted to specified bacteremias are not displayed in Figure 5.2.9 but four warrant specific mention. The studies of meningococcemia (mg [11]) and melioidosis (ml [73]) both have RD>20%. One study of typhoid (t [1]) has a RD of 0%. Another study of typhoid is displayed in Figure 5.2.9 (Suyasa, [77]) is classified under the category of restricted studies although 4 of the 21 typhoid patients of this study were found to also have a Pseudomonas bacteremia. This study [77] has a RD of 18%, a DOR of 20.5.

Finally, the three original studies published in 1972-1975 which produced conflicting conclusions and which inspired this thesis can be examined [25, 54, 75]. One does not provide either mortality or GN bacteremia data [Elin et al; 25] and hence cannot be further assessed here. The second [a7; Stumacher; 75] has a high prevalence of *E. coli* (15%) isolates together with a relatively low DOR (1.4) as possible explanatory factors for being a statistical low outlier study. The third [a4; Levin; 54] has a low prevalence of *E. coli* (3%) isolates together with a high DOR (10.4) as possible explanatory factors for being a statistical high outlier study.
6. Summary and conclusions

6.1 Summary of findings

The research described here addresses three questions related to the detection of endotoxemia in various clinical settings using the limulus (LAL) assay with respect to:

1. what is its diagnostic relevance versus GN bacteremia detection,
2. what is its prognostic relevance versus outcome (mortality), and
3. whether the disparate study results are reconcilable?

In the four decades following the inception of the limulus assay for endotoxemia in 1972, the following developments bearing on the above three questions have occurred:

- There are over 300 studies using the limulus assay in various clinical contexts including patients at risk for sepsis [1-107] and various other settings [498-673].
- the chromogenic limulus assay superseded the less sensitive gelation-based limulus assay after 1984,
- the patient population of particular interest within this thesis is the population within the ICU setting with sepsis which has been defined most commonly using the 1991 consensus sepsis criteria (Sepsis Syndrome). However, these definitions will continue to evolve and non-ICU populations are also of interest.
- the structure activity of endotoxin has become better understood with recognition of the importance of a hexaacyl (6 chain) as opposed to non-hexaacyl (4, 5 or 7 chain) structure of the lipid-A component of the lipopolysaccharide molecule (endotoxin) in host recognition by the human immune system,
- the statistical techniques of meta-analysis used in the derivation of summary estimates have evolved. In addition, the derivation of estimates of heterogeneity as a measure of the diversity of experience within the literature is now possible. In the most recent phase of this study, various multi-level methods became available to derive estimates of summary proportions that are optimal for data for which the assumption of independence is not tenable, which is most pertinent to the analysis of data in an infectious diseases setting.

1. What is the diagnostic relevance of the detection of endotoxemia versus GN bacteremia detection?

1.1. Endotoxemia is not detectable in at least 25% and up to 60% of patients with GN bacteremia.

1.2. The ‘typical’ proportion of patients with GN bacteremia in which endotoxemia is not detectable is dependent on the method of data aggregation across the studies. The HSROC method is considered optimal to derive estimates that are most representative of a ‘typical’ study.
1.3. The choice of method of plasma pre-treatment prior to the limulus assay may be important and the optimal method identified here is the method of dilution and heating.

1.4. There is no direct comparison of the gelation versus the more sensitive chromogenic limulus assay methods in the same patient population in the literature. In the analysis here using GN bacteremia overall as the ‘gold standard’, the newer chromogenic assay is not a superior assay.

1.5. Surprisingly, there appears to be no relationship between the diagnostic odds ratio and the listed sensitivity of the limulus assay to the internal endotoxin standard as used within each study over a range of more than 1000 fold (<.01 ng/ml to >10 ng/ml).

1.6. Using HSROC methods of analysis, the proportion of GN bacteremias with detectable endotoxemia is 68% (95% CI: 51-81%) for bacteremias with Pseudomonas, 54% (39-68%) for E. coli and 48% (30-66%) for the non-E. coli-enterobacteriaceae. (Table 5.1.5).

1.7. With the three categories of specified GN bacteremia types there appears to be a differential relationship between the detection of GN bacteremia and the associated endotoxemia as follows; the chromogenic version is more sensitive than the gelation-version for the detection of endotoxemia in association with bacteremias with Pseudomonas and the non-E. coli-enterobacteriaceae. (Table 5.1.5).

1.8. However, the 300 fold increase in sensitivity limit in the limulus assay method (chromogenic- versus gelation-versions) is associated with an increase in the sensitivity toward endotoxemia detection in association with bacteremias with Pseudomonas and the non-E. coli-enterobacteriaceae by no more than 14 percentage points.

1.9. The two versions (chromogenic- versus gelation-versions) are equivalent in sensitivity for E. coli bacteremias (Table 5.1.5).

1.10. The specificity of the assay is dependent on the study setting with less specificity among studies undertaken in an ICU setting (51%; 38-65%) versus a pediatric or oncology setting (88%; 59-97%). The sensitivity is not dependent on study setting. (Table 5.1.5).

1.11. Overall, the findings presented here imply that the type of GN bacteremia is at least as important a determinant toward the detection of concurrent endotoxemia as is the sensitivity of the limulus assay method used.

1.12. Surprisingly, the relationship between endotoxemia detection and the type of GN bacteremia is not simply determined by the type of lipid-A structure as originally predicted at the outset of this thesis.

1.13. For a ‘typical’ study having 100 patients and 24 GN bacteremias, overall the expectation would be that there would be 16 true positives (concurrent endotoxemia and GN bacteremia detected), 8 false negatives (GN bacteremia in isolation), 57 true negatives (neither endotoxemia nor GN bacteremia detected), and 19 false positives (endotoxemia in isolation) (Table 5.1.2).

1.14. The expected number of true positives for a ‘typical’ study
would need to be modified as follows; downward for *E. coli* and non-*E. coli*-enterobacteriaceae GN bacteremias. For 24 *E. coli* bacteremias there would be an expectation of 13 true positives and 11 false negatives and with 24 non-*E. coli*-enterobacteriaceae bacteremias there would be an expectation of 12 true positives and 12 false negatives (Table 5.1.5).

1.15. For 24 non-enterobacteriaceae GN bacteremias the expected number would be modified upwards; there would be 16 true positives and 8 false negatives expected.

2. **What is the prognostic relevance of the detection of endotoxemia versus outcome (mortality)?**

2.1. The prognostic value of endotoxemia detection versus non-detection has been most commonly studied in the ICU setting. In the ICU setting, endotoxemia detection has little (OR = 1.4; 1.2-1.7, RD 7%; 3-10%) prognostic value in studies reporting either as ‘two group’ (Figure 5.2.1) or ‘four group’ studies (Figure 5.2.2).

2.2. Endotoxemia detection has been studied in several non-ICU settings where it has been found to have prognostic value which is generally higher but more variable than in ICU settings (Figure 5.2.1 & fig 5.2.2).

2.3. Amongst the ‘four group’ studies, the co-detection of endotoxemia with GN bacteremia (Group 1) is most predictive of increased mortality risk versus the detection of neither (group 4; Figure 5.2.3). While this is most apparent amongst the sub-group of studies undertaken outside an ICU setting (OR 6.9; 4.4-11.0; RD 19%; 11-27%), this finding is associated with at least moderate heterogeneity (OR I² 35%; RD I² 67%).

2.4. By contrast, the mortality risk in association with the detection of either endotoxemia or GN bacteremia in isolation (Group 2 and Group 3; Figure 5.2.4 & Figure 5.2.5) versus the detection of neither (Group 4) is associated with borderline or non-significant elevation of risk (OR <2.0; RD <10%). Moreover, this finding applies in both ICU and non-ICU studies and the associated heterogeneity is no more than moderate whichever summary effect size is used (OR & RD I²<35%).

2.5. The prognostic value of endotoxemia detection versus non-detection in association with GN bacteremia varies across different species types of GN bacteremia (Figure 5.2.8, Table 5.2.5) as follows;

2.6. The detection of endotoxemia in association with bacteremias with *Neisseria meningitidis* has the strongest prognostic value. Whilst this is the largest category of GN bacteremia, these patients have mostly come from two studies [11, 12] from a single center. The possibility that this infection is epidemic and clonal in the region in which this center is located needs to be considered. The limited number and lack of diversity of these clusters is reflected in the wide 95% CI calculated by the GEE method.

2.7. Surprisingly, the detection of endotoxemia in association with *E. coli* bacteremia, which is the second largest GN bacteremia category, has no prognostic value in any setting. This experience is derived from eight ICU studies. The experience among small numbers from an additional 6 non-ICU
studies is not significantly different. It is notable that this lack of prognostic value with *E. coli* bacteremia is at variance with the experience for GN bacteremia overall as this provides a basis for exploring the conflicting results of individual studies.

2.8. There is a significant interaction effect between the detection of endotoxemia and the type of Enterobacteriaceae (*E. coli* versus non-*E. coli*) bacteremias on the prognosis.

2.9. This last finding is surprising given the expected commonality of the hexa-acyl structure of lipid-A amongst all Enterobactereiaceae whether *E. coli* or non-*E. coli*.

3. *Are the disparate findings among the published studies reconcilable?*

3.1. There is a large range in diagnostic odds ratio among the studies. The results of the three original studies [25, 54, 75] published in 1972-1975 could not be considered have been outlier or atypical in the analysis of the 107 studies overall. (Figure 5.1.5).

3.2. Toward the detection of GN bacteremia, the sensitivity limit of the limulus assay is not the only limiting factor and other factors bearing on the diagnostic utility of the assay such as the setting, the plasma preparation method and the types of GN bacteremias are at least as important.

3.3. For studies conducted in an ICU setting, the mortality RD associated with the detection versus non-detection of endotoxemia (without regard to the co-detection of GN bacteremia) is similar amongst studies whose data is reported in the ‘two group’ versus the ‘four group’ format (Table 5.2.1).

3.4. By contrast, for studies conducted outside an ICU setting whose data is analyzed in the ‘two group’ format, the mortality RD associated with the detection versus non-detection of endotoxemia (without regard to the co-detection of GN bacteremia) is generally higher and more variable versus that amongst studies conducted in an ICU setting (Table 5.2.1).

3.5. In regard to the interpretation of the limulus assay results in relation to prognostic importance, the setting, the mix of GN bacteremias and the DOR obtained all appear to be important determinants towards the findings in each study (Section 5.2.2.3.4).

3.6. The seemingly contrary results reported in the literature could be reconciled by the mix of GN bacteremia seen, with a higher proportion of *E. coli* potentially able to account for the ‘negative’ conclusions of some studies in comparison to studies with a lower proportion of *E. coli*. (Figure 5.2.10).

3.7. The major contribution from this thesis is that the interpretation of the detection of endotoxemia in a given patient in relation to the literature experience as analysed within this thesis [1-107] and possibly even more broadly [498-673] cannot be given without knowledge of three contextual factors; whether the patient in question has a GN bacteremia, if so what type of GN bacteremia and the background mortality risk of the population in which the patient belongs.
6.2 Recapitulation of study limitations and strengths

**Limitations.** The limitations of the research described here have been mentioned in 5.1.3 and 5.2.4. Several additional limitations are listed here;

1. The data is observational; this limits any causal interpretation due to the inherent confounding and bias that exists such as selection, sampling and publication biases.

2. The data has been abstracted for each meta-analysis by a single author. However, this is not unprecedented in this area [231-232, 685]. These have each been submitted for peer review and accepted for publication as meta-analyses and not as systematic reviews.

3. The data was incomplete for the majority of the 107 studies.

4. The definitions of study populations were imprecise for many studies, more so for those undertaken outside of an ICU setting.

5. Some of the sub-groups of studies (e.g. pediatric) were small (< 8 studies) and this necessitated analysis as a composite sub-group (e.g. with oncology studies).

6. The exposure classification (endotoxemia positive versus negative) varies from study to study due to variable endotoxemia detection thresholds.

7. Some important study parameters, and in particular mortality census, were missing. More so for those studies undertaken outside an ICU setting.

8. GN bacteremia has been used as a reference standard. The limitations and strengths relating to this have been discussed in section 2.4

9. With the exception of six ‘two group’ supplementary studies, endotoxemia detection has been defined with respect to activity in the limulus assay and generalization of these findings beyond endotoxemia as defined by detection with the limulus assay is cautioned.

10. The influence of underlying patient risk as presented in this analysis may be misleading. Underlying patient risk has been defined at the group level for each study using the mortality rate of the reference group (Group 4; neither endotoxemia nor GN bacteremia detected) rather than at the patient level (see below).

11. There is considerable variability with respect to study sizes. As a consequence of this there are slight differences in summary effect sizes depending on the method of data aggregation used in the analysis.

12. The data for the 107 studies is somewhat dated having been mostly published more than ten years ago.

13. The contributory effect of other important factors such as appropriate antibiotic therapy could not be examined.

14. That the origin of the endotoxemia is from the concomitant GN bacteremia is a presumption.

15. The most surprising finding here, that endotoxemia has no prognostic relevance for mortality when found in the co-presence of *E. coli* bacteremias, cannot be readily corroborated.
16. There being only 51 non-Enterobactericeae GN bacteremias from seven studies undertaken in an ICU setting (Table 5.2.5), there is insufficient data to derive a definitive answer to the question of the effect of non-hexa-acyl lipid-A structure toward clinical relevance of endotoxemia detection.

17. There are limitations to the interpretations of the findings of a meta-analysis particularly with the cautions required for any post-hoc analysis.

**Strengths.** On the other hand, there are a number of strengths of the research described here.

1. A large number of studies in several categories have been found after repeated searching of the literature.

2. The data has been expanded by a call for data with additional data obtained by personal communication.

3. The abstracted data is appended to this thesis (section 7.1/.2). For each of the eight compiled research publications appended to this thesis, the data as abstracted from the original studies was available for scrutiny during the peer review process prior to publication and remains accessible there. Moreover, the data has been updated as new data has come to hand.

4. Meta-analytic methods have been used to enable the quantitative overview of a complex literature that could not otherwise be achieved. In particular, the techniques used have enabled estimations of measures of variability which are of particular interest.

5. Recently developed meta-analytic methods have been used as these have become available and these are optimally suited for;
   - variable endotoxemia detection thresholds between studies,
   - variable study size,
   - variable underlying patient risk (at the group level),
   - derivation of measures of heterogeneity,
   - graphical presentation of study results to enable a visual overview of the extensive literature and identification of studies with atypical results,
   - enabling estimations of the number of studies that would need to be unpublished or ‘missing’ to overturn the findings,
   - data analysis issues arising from inclusion of study groups that are small, unbalanced and clustered,
   - enabling comparisons with other meta-analytic summaries of diagnostic and prognostic tests applicable to the study populations of interest as reported in the literature (Table 2.3.2).

6. The data as abstracted and summated by Elin has been re-analyzed to enable the findings as reported by Elin to be compared with those obtained with the more robust contemporary methods used here (Figure 2.8/.9/.10).

7. The original conclusions are robust to re-analysis with new data. Namely, the findings as published in JCM '94 and JCM '95 with 45 and 11 studies,
respectively are in each publication broadly the similar, albeit with attenuated OR’s as in the update for this thesis with 88 and 32 studies, respectively (Table 5.1.1 and Table 5.2.3).

8. Two attempts have been made to reconcile the findings here with the broader literature. Firstly, special effort has been made to compare the results of studies with (e.g. the ‘four group’ studies) versus without (e.g. the ‘two group’ studies) complete data (Table 5.1.2) and the findings are consistent. Second, the findings here for mortality rates for the three main GN bacteremia types have been compared for each of these GN bacteremias to the broader literature. These are mostly within the inter-quartile range with the exception of non-\textit{E. coli} Enterobactereiaceae (Figure 5.2.9).

9. The main study questions, the effect of limulus assay version, and the effect of hexa-acyl versus non-nexa-acyl lipid-A structure toward clinical relevance of endotoxemia detection, can each be regarded as pre-specified in that the questions arose independently of the data.

6.3 Future prospects

With the main findings of the research together with the limitations and strengths of the analysis listed above, it is timely to address the implications of the findings.

1. The diverse and conflicting literature can be reconciled if it is accepted that, regardless of the sensitivity level of the endotoxemia detection assays that have been used, not all GN bacteremias have detectable endotoxemia and conversely, not all patients with endotoxemia will have GN bacteremia. The first implication of this is that the detection of endotoxemia will not serve as a practical rapid screening test for GN bacteremia (in contrast to the case for fluids other than blood) (see Figures 2.7 & 2.8).

2. The second implication is that unrecognized confounders, namely underlying patient risk and type of GN bacteremia, could account for the different findings among different studies with respect to the prognostic relevance of endotoxemia detection. Also, study size needs to be recognized as an important confounding factor together with the compounding effect of publication bias. Variable reporting as a result of confounding and study imprecision is not unusual in the literature. However, only through the application of meta-analytic techniques as used in this thesis can these influences be recognized.

3. The influence of underlying patient risk as presented in this analysis may be
misleading. As stated above, underlying patient risk has been defined for each study using the mortality rate of the reference group (Group 4; neither endotoxemia nor GN bacteremia detected, being generally the largest group of each study) rather than specifically at the patient level. The underlying risk of Group 4 is unlikely to be representative of the underlying patient risk for the patients in other groups in each study, especially so in studies that were small or had used imprecise patient inclusion criteria to define the study populations. However, the pertinent inference from this analysis is that in studies with a high background underlying patient risk (i.e. ICU settings) endotoxemia detection has a prognostic value which is weak and adds little to that provided by GN bacteremia detection.

4. As stated above, the data is observational and this limits any causal interpretations, in particular whether endotoxemia has a causal influence in the outcome of patients with suspected GN sepsis. However, the detection of endotoxemia in a study undertaken in the ICU setting is associated with a mortality risk difference of 10% (Figure 5.2.2 & 5.2.3) or an OR of 1.4 to 1.6 (Figure 5.2.1 & Table 5.2.1). Hence, the evidence accumulated here would suggest that the prognostic inference associated with the detection of endotoxemia (without regard to the detection of GN bacteremia) overall is similar to that associated with the detection of GN bacteremia (without regard to the detection of endotoxemia) as discussed in section 2.4.2.

5. The analysis here in relation to the prognostic inference associated with the detection of endotoxemia raises the likelihood that this is unequal for different GN bacteremias. Surprisingly, endotoxemia may have a strong prognostic inference in patients with non-\textit{E. coli}\ Enterobactericeae GN bacteremias versus no prognostic inference in \textit{E. coli} bacteremias (Table 5.2.5).

6. Moreover, the mix of GN bacteremias within individual studies and their distribution between groups 1 and 2 may be an unrecognised confounder leading to the conflicting results of studies of the prognostic value of endotoxemia. In this regard, the relative rankings of the three common GN bacteremia types in relation to both their relative frequency and mortality risk (Table 2.4.1./1./2./3./4./5) overall versus the atypical rankings found amongst the smaller sized studies is of note (as is apparent in Figures 2.12./13./14./15).
7. The effect of endotoxin derived from non-\textit{E. coli} Enterobactericeae warrant greater study in animal models and other pre-clinical studies as it has been relatively under-studied in comparison to the extent of experimental study devoted to the effect of endotoxin derived from \textit{E. coli}.

8. There are too few studies [100-107] of the newer assays (SRE, EAA) for the detection of endotoxemia to determine whether these perform any differently from studies that used the limulus assay. However, the confounding influences mentioned above will need to be considered in any comparison.

9. The results of randomized trials of novel anti-endotoxin therapies warrant a re-examination in the light of the findings here. In particular, the types of GN bacteremia under study needs attention. However, mostly, the types of GN bacteremias were either not stated (\textbf{Tables 2.5.1 & 3}), or the studies were restricted to meningococcemia primarily (\textbf{Table 2.5.2}). Few of the studies found a significant reduction in mortality with the anti-endotoxemia treatment (as discussed in section \textbf{2.5.2}). However there are two studies (as indicated within \textbf{Table 2.5.3}) in which a significant reduction in mortality with the anti-endotoxemia treatment was noted and which also had an unusually high proportion of non- \textit{E. coli} Enterobactericeae versus \textit{E. coli} and versus total numbers of GN bacteremias (71 versus 87 versus 200 [350] and 30 versus 12 versus 49 [442]). Whilst this is a speculative conclusion derived from a post hoc analysis, it is worthy of further examination in future randomized trials.

10. The research questions in this thesis have been limited to endotoxemia detection with the limulus assay and gram-negative bacteremia with particular interest in the patient group with sepsis. Newer techniques are emerging to assist progress toward finding the pathogenesis of sepsis. In particular, newer methods of detection of blood stream infection in the patient group with sepsis are emerging. These techniques, such as MALDI-TOF (Matrix Assisted Laser desorption-ionization Time Of Flight mass spectrometry fingerprinting) offer not only rapid detection but also rapid identification [719]. Also of promise are methods for the molecular profiling of both the bacterial and host molecules in the diagnosis and investigation of sepsis. There are methodological challenges associated with the evaluation of these molecular methods (discussed in section \textbf{2.3.2}). To date, the results of cytokine profiling in sepsis patients have not
always been as expected. For example, evidence supporting the classic two phase (inflammatory/ anti-inflammatory) model of sepsis has not yet been found. One study suggested that the incremental cost in using these PCR methods is justifiable only when the rate of inadequate initial anti-microbial therapy is greater than 25% [720].

11. Finally, two recent studies [333, 720] are of interest in relation to research question two of this thesis (endotoxemia versus outcome -mortality). These studies address the predictive value of microbiological identification made amongst culture negative sepsis patients using polymerase chain reaction (PCR) methods that is additional to that obtained with culture detection. The smaller of the two studies [333] examined blood culture versus PCR methods for 126 critically ill (criteria not specified) adult ICU patients. The finding of this first study [333] was that the SOFA score was similar for the 64 PCR positive (8.2 (SD 3.6)) versus the 134 PCR negative (8.3 (SD 4.0)) patients, respectively.

12. The larger of the two studies [720] investigated the prognostic value of using a broad (not limited to GN bacteremia) multiplex PCR method applied to 221 sepsis patients among which the 30-day non-survival was 59/221 (27%) overall. The patients of this study could be divided into four groups corresponding to those that were both PCR and blood culture positive (not limited to GN bacteremias), those that were only blood culture positive, those that were only PCR positive, and a fourth group that was positive for neither. Note that these four groups are homologous to the four groups as detailed within section 5.2 of this thesis. The finding of this study [720] was that the 30-day mortality was 15/33 (45%), 3/11 (27%), 9/40 (23%), and 10/37 (27%), for groups 1 to 4, respectively. Note the risk differences between the percentages between groups 1 to 3 each versus group 4 (as the reference group) in this study are similar to the risk differences reported in the footnotes to Figures 5.2.4/5.6. In particular, for this study [720] the greatest risk difference (18%) versus group 4 is that observed for group 1 and in this thesis, the risk difference was 16.1% (Figure 5.2.4).
### 7. Catalogue of studies and personal correspondence

Column headings and explanatory notes for **7.1 & 7.2**:

1. First author and reference
2. LAL: G = Gelation version C = chromogenic version; SRE & EAA are non-limulus assays for endotoxemia (see 2.2); R = rabbit assay.
3. ng/ml = sensitivity limit of assay to the internal endotoxin standard
4. Pl = method used to pre-treatment plasma prior to LAL assay
5. Set_ = Setting & _Pop = population;
   - A_ = Adult
     - _fever/shock/sepsis = fever/shock/clinically suspected sepsis
     - ASCCM shock
     - _GI = gastrointestinal conditions (e.g. pancreatitis, GI surgery, cirrhosis)
     - _UTI = documented urinary tract infection
     - _malaria = malaria
   - P_ = pediatric
     - _CVS = cardiovascular surgery
     - _maln = malnutrition
     - _HUS = hemolytic uremic syndrome
     - _NEC = necrotizing entero-colitis
   - O_ = oncology
   - ICU_ = Intensive care unit
     - _2_shock = shock
   - R_ = studies restricted to;
     - _Ty = typhoid
     - _Mg = meningococcal disease
     - _Pg = plague
     - _S = Salmonellosis
     - _MI = melioidosis
6. **N** = Total number of patients (= TP + FN + FP + TN)
7. **TP** = True positives; GN bacteremia with concomitant bacteremia
8. **FN** = False negatives; GN bacteremia without concomitant bacteremia
9. **FP** = False positives; endotoxemia without concomitant GN bacteremia
10. **TN** = True negatives; Neither endotoxemia nor GN bacteremia
11. **GNB%** = Percent of patients with GN bacteremia (= (TP+FN)/T)
12. **QS** = quality score symbols code; \(\bigcirc/\bullet/\psi/\$\) = 1; 0 = 0
    - 1: study inclusion criteria specified
    - 2: sampling strategy; i.e. blood culture with each endotoxemia sampling
    - 3: outcomes detailed as patient survival (‘\(\psi\)’ indicates data used in 5.2)
    - 4: listing of GN bacteremia isolates given (‘\(\$\)’ indicates data used in 5.1.2, 5.2.3 or 5.2.4)
13. code = study code used in figures 5.1.5 & 5.2.3 to assist with literature reconciliation
14. notes:
   - **NE** = non-English language study
   - **PE** = per-episode data
   - **pc** = Information received by personal communication
   - **R** = study restricted to specified GN bacteremias
   - **D** = duplicate study (only one study shown)
   - **HA-1A** = anti-endotoxin monoclonal antibody (HA-1A) given to all
### 7.1 Summary of included studies

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**Legend:**
- **Ref:** Reference number
- **LAL:** Laboratory analysis
- **ng/ml:** Nanograms per milliliter
- **P1:** Parameter 1
- **Pop:** Population
- **Set:** Setting
- **N:** Number
- **TP:** Truth grade
- **FN:** False negative
- **FP:** False positive
- **TN:** True negative
- **GNB %:** Gram-negative bacteria percentage
- **QS:** Quality score
- **Code:** Code for further analysis
- **nb:** Note

- **T - R:** Treatment - Retention
- **P1:** Parameter 1
- **Mg - R:** Magnesium - Retention
- **Mg - R, pc:** Magnesium - Retention, paired comparison
- **RS:** Retention, significance
- **PC:** Paired comparison
- **PE:** Paired evaluation
- **NB:** Not specified
- **DF:** Difference in frequency
- **IF:** Information flow
- **PF:** Parameter flow
- **TF:** Truth flow
- **FN:** False negative
- **FP:** False positive
- **TN:** True negative
- **GNB %:** Gram-negative bacteria percentage
- **QS:** Quality score
- **Code:** Code for further analysis
- **nb:** Note

**Note:**
- **●●●●:** High significance
- **●●●‡:** Medium significance
- **●○○○:** Low significance
- **●○○○:** Highest significance
- **●○○○:** Lowest significance
- **●○○○:** Null significance
- **●○○○:** Significant
- **●○○○:** Not significant
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### 7.2.2 ‘2 group’ studies using LAL or non-LAL assays

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8. References


Chapter 8: References


223. Hwang SH, Park DU, Joo SI, Park HH, Yoon CS: Comparison of endotoxin levels and gram-negative bacteria under different conditions in microbial laboratories and a biowaste site. Chemosphere, 85(1):135-139.


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Surgery


499. Berger D, Bolke E, Seidelmann M, Beger HG: Time-scale of interleukin-6, myeloid related proteins (MRP), C reactive protein (CRP), and endotoxin plasma levels during the postoperative acute phase reaction. Shock 1997, 7(6):422-426.


Pancreatitits


Cirrhosis, obstructive jaundice & liver disease


Liver transplantation: donors and recipients


570. Winchurch RA, Thupari JN, Munster AM: Endotoxemia in burn patients: levels of circulating endotoxins are related to burn size. Surgery 1987, 102(5):808-812.


Urological


Trauma


Critical illness including ARDS


Pediatric and neonate


Sepsis and specific infections


Treatment studies


Other


Metabolic and heart failure


Veterinary and animals models


Statistical methods


Discussion


7.3. Acknowledgement of correspondence with authors

1. Dr D Berger, Klinikum der Universität Ulm, Germany (1 page)
2. Dr P. Brandtzaeg, Ulleval University Hospital, Oslo, Norway (5 pages)
3. Dr B Byl, Erasme University Hospital, Belgium;
4. Dr R Danner, NIH, Bethesda, MD, USA (2 pages)
5. Dr P Engervall, Karolinska Hospital, Stockholm, Sweden (3 pages)
6. Dr KCH Fearon, Royal Infirmary, Edinburgh, UK (3 pages)
7. Dr EJ Giamarellos-Bourboulis, Laiko General Hospital, Athens (3 pages)
8. Dr B. Guidet, Hopital Saint-Antoine, Paris, France (2 pages)
9. Dr P Ketchum (Associates of Cape Cod) and Dr D Bates, Brigham and Women’s Hospital, Boston, MA, USA. (4 pages)
10. Dr J. Levin, VA Medical Center, San Francisco, USA (1 page)
11. Dr D. Massignon, Center Hospitalier Lyon Sud, France (3 pages)
12. Dr E Maury, Hopital Saint Antoine, Paris, France. (1 page)
13. Dr MJ McMahon, Leeds Teaching Hospital, Leeds, UK (1 page).
14. Pr G. Offenstadt, Hopital Saint Antoine, Paris, France. (3 pages)
15. Prof Steven M Opal, MD, Infectious Disease Division, The Alpert Medical School of Brown University, Providence, Rhode Island;
16. Dr. Jan M. Prins, Academic Medical Center, Amsterdam (2 pages)
17. Dr JT van Dissel, Leiden University Medical Center, The Netherlands (6 pages)
18. Dr SM Willatts, Bristol Royal Infirmary, Bristol, UK (1 page)
19. Dr M Yoshida, Jichi Medical School, Japan, (3 pages via Dr J Levin)
# 9. Appendix: the publications

## Original research publications

(Contribution of Hurley JC to each publication is 100% unless stated otherwise)

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## Background publications

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Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Hurley, James Crowther

Title:
Endotoxemia: correlation with gram-negative bacteremia and association with outcome

Date:
2013

Citation:
Hurley, J. C. (2013). Endotoxemia: correlation with gram-negative bacteremia and association with outcome. Doctorate, Department of Medicine (Austin Health), Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne.

Persistent Link:
http://hdl.handle.net/11343/38393

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