



REETTA HUTTUNEN

Factors Associated with Susceptibility to and
Outcome of Bacteraemia with Reference to
Staphylococcus aureus, *Streptococcus pneumoniae*,
 β -haemolytic streptococcus and
Escherichia coli Bacteraemias



ACADEMIC DISSERTATION

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CONTENTS

CONTENTS	5
LIST OF ORIGINAL PUBLICATIONS	8
ABBREVIATIONS	9
ABSTRACT	11
TIIVISTELMÄ	13
1. INTRODUCTION	15
2. REVIEW OF THE LITERATURE	18
2.1. Overview of bacteraemia and sepsis	18
2.1.1. Definitions.....	18
2.1.2. Incidence	19
2.1.3. Prognosis.....	19
2.2. Pathophysiology of bacteraemia and sepsis	20
2.2.1. Inter-bacterial differences	22
2.2.2. Nitric oxide (NO)	24
2.2.3. Endothelium and coagulation.....	25
2.2.4. Immune system	26
2.2.4.1. Apoptosis and immunosuppression.....	28
2.2.4.2. Collectins and mannose-binding lectin (MBL).....	29
2.3. Genetic factors affecting the risk and outcome of bacteraemia and sepsis	31
2.3.1. MBL2 polymorphisms	31
2.3.2. eNOS gene polymorphisms.....	35

2.3.3. Cytokine and innate immune receptor polymorphisms	36
2.4. Host characteristics and underlying diseases affecting the risk and outcome of bacteraemia and sepsis.....	38
2.4.1. Body mass index (BMI).....	39
2.4.2. Smoking	40
2.4.3. Alcohol abuse.....	41
2.5. Indoleamine 2,3 dioxygenase enzyme (IDO).....	42
3. AIMS OF THE STUDY	45
4. SUBJECTS AND METHODS	46
4.1. Subjects (studies I-IV).....	46
4.1.1. Patients	46
4.1.2. Host predisposing factors.....	47
4.1.3. Patient monitoring and laboratory tests.....	47
4.1.4. Controls (Study I).....	49
4.2. Methods	49
4.2.1. MBL2 genotyping	49
4.2.2. eNOS genotyping.....	49
4.2.3. Tryptophan and kynurenine determinations.....	50
4.2.4. Main endpoints.....	51
4.2.5. Statistical methods	51
4.2.6. Ethical considerations	52
5. RESULTS	53
5.1. Characteristics of study material	53
5.1.1. Bacterial aetiology	53
5.1.2. Characteristics and underlying diseases of patients.....	53
5.1.3. Sources of bacteraemia	54
5.1.4. Outcome of bacteraemia	54
5.2. Genotype frequencies in patients and in controls (Study I and II).....	55
5.3. Genetic factors and the risk and outcome of bacteraemia	56
5.3.1. MBL2 genotype (Study I).....	56
5.3.2. eNOS G894T polymorphism (Glu298Asp) (Study II).....	58
5.4. Patient characteristics and underlying diseases and outcome (Study III)	61
5.4.1. BMI	61
5.4.2. Smoking	62

5.4.3. Alcohol abuse.....	63
5.4.4. Gender and age.....	64
5.5. Kynurenine to tryptophan ratio reflecting IDO activity (Study IV).....	64
6. DISCUSSION	68
6.1. Definitions, study population and the outcome of bacteraemia	68
6.2. Genetic variation and the risk and outcome of bacteraemia.....	71
6.2.1. MBL2 genotype	71
6.2.2. eNOS G894T polymorphism (Glu298Asp).....	73
6.3. Host characteristics and underlying diseases and bacteraemia outcome.....	76
6.3.1. Obesity	76
6.3.2. Smoking	78
6.3.3. Other underlying conditions, age and sex	79
6.4. IDO activity as a predictor of outcome in bacteraemia	80
6.5. Future considerations.....	83
7. SUMMARY AND CONCLUSIONS	86
ACKNOWLEDGEMENTS	88
REFERENCES.....	90
ORIGINAL COMMUNICATIONS.....	109

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following four original studies, which are referred to in the text by their Roman numerals I-IV.

I Huttunen R, Aittoniemi J, Laine J, Vuento R, Karjalainen J, Rovio AT, Eklund C, Hurme M, Huhtala H, Syrjänen J (2008): Gene-environment interaction between *MBL2* genotype and smoking, and the risk of gram-positive bacteraemia. *Scand J Immunol* 68:438-44.

II Huttunen R, Hurme M, Laine J, Eklund C, Vuento R, Aittoniemi J, Huhtala H, Syrjänen J (2009): Endothelial nitric oxide synthase G894T (GLU298ASP) polymorphism is associated with hypotension in patients with *E.coli* bacteremia but not in bacteremia caused by a gram-positive organism. *Shock* 31:448-453

III Huttunen R, Laine J, Lumio J, Vuento R, Syrjänen J (2007): Obesity and smoking are factors associated with poor prognosis in patients with bacteraemia. *BMC Infect Dis* 7:13.

IV Huttunen R, Syrjänen J, Aittoniemi J, Oja SS, Raitala A, Laine J, Pertovaara M, Vuento R, Huhtala H, Hurme M (2010): High activity of indoleamine 2,3 dioxygenase enzyme predicts disease severity and case fatality in bacteraemic patients. *Shock* 33:149-54.

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ABBREVIATIONS

ACCP	American College of Chest Physicians
AIDS	acquired immune deficiency syndrome
Allo-SCT	allogeneic stem cell transplantation
AM	alveolar macrophages
APC	activated protein C
AUC ^{ROC}	area under receiver operating curve
BMI	body mass index
BSI	bloodstream infection
C2	complement component 2
C4	complement component 4
C3	complement component 3
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CHD	coronary heart disease
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CTLA4	cytotoxic T-lymphocyte antigen 4
CVVHD	continuous veno-venous haemodialysis
DAMP	danger-associated molecular pattern
DC	dendritic cell
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
EMA	European Medicine Agency
eNOS	endothelial nitric oxide synthase
GAS	group A streptococcus
GBS	group B streptococcus
GCS	Glasgow coma scale
GGS	group G streptococcus
HD	hospital district
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high-performance-liquid chromatography
ICU	intensive care unit
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
kyn	kynurenine
kyn/trp	kynurenine to tryptophan ratio
LPS	lipopolysaccharide
MAP	mean arterial pressure

MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MHC	major-histocompatibility complex
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	methicillin-susceptible <i>Staphylococcus aureus</i>
NF- κ B	nuclear factor kappa B
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthase
O ₂	dioxygen
PAI-1	plasminogen activator inhibitor type 1
PAMP	pathogen-associated molecular pattern
PIRO	predisposition/infection/response/organ dysfunction
PROWESS	Protein C Worldwide Evaluation in Severe Sepsis
PRR	pattern recognition receptor
PVL	Panton-Valentine leukocidin
rhAPC	recombinant human activated protein C
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCCM	Society of Critical Care Medicine
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphism
SOFA	sequential organ failure assessment
SP-A	surfactant protein A
SP-D	surfactant protein D
SSC	surviving sepsis campaign
TDO	tryptophan 2,3 dioxygenase
TFPI	tissue-factor pathway inhibitor
TGFB1	transforming growth factor beta 1
THL	Terveyden ja hyvinvoinnin laitos (The National Institute for Health and Welfare)
TLR	toll-like receptor
TNF- α	tumour necrosis factor alfa
TNM	tumours/nodes/metastases
trp	tryptophan
TSST	toxic shock syndrome toxin

ABSTRACT

Bacteraemia and sepsis are major causes of mortality worldwide. Individual subjects seem to have different risks of developing bacteraemia and sepsis, individual organ failures and death. Despite novel options in the treatment of sepsis, the course of disease in individuals remains unpredicted and the mortality rate high. The lack of knowledge of sepsis pathogenesis and the heterogeneity of patients with bacteraemia and sepsis make risk stratification difficult. The main purpose of this study was to elucidate the role of host genetic factors, living habits, and underlying diseases in bacteraemia outcome, to study the effect of genetic factors on susceptibility to bacteraemia, and to find new approaches in understanding the pathogenesis and prognostication in bacteraemia.

This prospective cohort study involved 149 in-hospital patients (79 male and 70 female) with bacteraemia caused by *Staphylococcus aureus* (*S. aureus*) (41 patients), *Streptococcus pneumoniae* (*Str. pneumoniae*) (42 patients), β -haemolytic streptococcus (β -hml str.) (23 patients) and *Escherichia coli* (*E. coli*) (43 patients) recruited in Tampere University Hospital during the years 1999-2004. Nineteen (12.8%) bacteraemic patients died.

Mannose-binding lectin (MBL) insufficiency caused by point mutations in the *MBL2* gene has been associated with increased susceptibility to infections, but the data are controversial. To study the effect of *MBL2* polymorphisms on susceptibility to and the clinical course of bacteraemia, 145 patients with bacteraemia and 400 controls were examined. **In study I**, *MBL2* structural polymorphisms at codons 52, 54 and 57, and promoter region polymorphisms at position -221 were determined. No difference in *MBL2* genotype frequencies was detected between bacteraemic patients and controls, and *MBL2* genotype had no independent effect on mortality. However, smoking proved a significant risk factor for gram-positive (*S. aureus*, *Str. pneumoniae* or β -hml. str.) bacteraemia among carriers of the variant O allele, while it had no effect on those homozygous for the A allele.

Nitric oxide (NO) is a crucial element in the pathogenesis of sepsis. Endothelial nitric oxide synthase (eNOS) is a key regulator of vascular NO production. The *eNOS* gene polymorphism at position 894 (G>T, Glu298Asp), resulting in the T allele, has been studied in the context of vascular diseases, but its role in sepsis is unclear. **In study II**, the polymorphism of the *eNOS* gene, G894T, was genotyped in patients with bacteraemia. Carriage of the T allele was associated with hypotension and severe disease in patients suffering from *E. coli* bacteraemia, but not in bacteraemia caused by a gram-positive organism. The Glu298Asp polymorphism had no effect on case fatality.

In study III, the major underlying conditions associated with case fatality in bacteraemia were studied. Obesity (body mass index (BMI ≥ 30)) and smoking were independently associated with case fatality in a multivariate model adjusted for the effect of potential confounders. The median BMI was significantly higher among those who died compared to survivors (33 vs. 26, $p=0.003$)

Indoleamine 2,3-dioxygenase (IDO), which is the rate-limiting enzyme for tryptophan catabolism, may play a critical role in various inflammatory disorders. IDO degrades tryptophan (trp) to its metabolite kynurenine (kyn), constituting a suppressor of T-cells. However, the precise role of IDO in different disease processes is largely unknown. **In study IV**, serum tryptophan and kynurenine concentrations were determined by high-performance-liquid chromatography (HPLC) in bacteraemic patients. The kyn/trp ratio, reflecting the activity of the IDO enzyme, was calculated. Maximum IDO activity 1-4 days after blood culture was significantly higher in nonsurvivors

compared to survivors. High IDO activity remained an independent predictor of case fatality in the multivariate model.

In summary, this study showed that obese patients and smokers had significantly higher case fatality rates in bacteraemia compared to their normal-weight and non-smoking counterparts. Smoking also increased the risk of gram-positive bacteraemia in patients carrying the *MBL2* gene structural variant O allele, this constituting a novel example of gene-environment interaction. Carriage of the *eNOS* gene T allele at nucleotide position 894 was associated with hypotension in patients with *E. coli* bacteraemia. A high kyn/trp ratio, reflecting IDO activity was strongly associated with case fatality in patients with bacteraemia. The present findings provide evidence of fundamental differences between gram-negative and gram-positive sepsis and interindividual differences in predisposition to bacteraemia relating to genetic and smoking interactions, and of lifestyle effects on outcome. The contribution of the *eNOS* gene, a gene regulating vascular tone, on disease severity in bacteraemia is shown. IDO may constitute a novel key to the understanding of sepsis pathogenesis.

TIIVISTELMÄ

Bakteremia ja sepsis ovat maailmanlaajuisesti merkittäviä kuolleisuuden aiheuttajia. Yksilöiden herkkyydessä sairastua bakteremiaan, saada siihen liittyvä elinhäiriö tai kuolla siihen on merkittäviä eroja. Vaikka bakteremian ja sepsiksen hoitomenetelmät ovat kehittyneet viime vuosina, taudinkuvat ovat edelleen huonosti ennustettavia ja kuolleisuus tautiin on korkea. Sepsiksen patogeneesiä ei tunneta riittävän hyvin ja potilaiden kuolemanriskiä ei osata arvioida. Tämän tutkimuksen tärkein tavoite on selvittää geneettisten tekijöiden, elintapojen ja yleissairauksien merkitystä bakteremiapotilaiden ennusteeseen, sekä selvittää bakteremiariskiä liittyviä geneettisiä tekijöitä. Tutkimuksessa etsitään entistä parempia keinoja bakteremian ja sepsiksen patogeneesin ymmärtämiseksi, jotta näiden potilaiden hoito voidaan jatkossa suunnata tehokkaammin.

Prospektiivinen tutkimusaineisto koostui 149 bakteremiapotilaasta (79 miestä ja 70 naista), joita hoidettiin vuosina 1999-2004 Tampereen yliopistollisessa sairaalassa. Bakteremian aiheutti 41 potilaalla *Staphylococcus aureus* (*S. aureus*), 42 potilaalla *Streptococcus pneumoniae* (*Str. pneumoniae*), 23 potilaalla β -hemolyyttinen streptokokki (β -hml str.) ja 43 potilaalla *Escherichia coli* (*E. coli*). Potilaista kuoli 19 (12.8%).

Mannoosia sitovan lektiinin (MBL) puute, jonka aiheuttaa pistemutaatiot *MBL2*-geenissä, on yhdistetty riskiin sairastua infektioihin, vaikka ristiriitaisiakin tutkimustuloksia on esitetty. **Osatyössä I** tutkittiin *MBL2*-polymorfoiden vaikutusta riskiin sairastua bakteremiaan ja toisaalta polymorfoiden vaikutusta ennusteeseen 145:n bakteremiapotilaan ja 400:n kontrolliväestöön kuuluvan ihmisen aineistossa. Tutkimuksessa määritettiin *MBL2*-rakennealueen polymorfiat codoneissa 52, 54 ja 57, ja promoottorialueen polymorfiat nukleotidikohdassa -221. *MBL2*-geenin genotyypifrekvenssit eivät eronneet potilaiden ja kontrolliryhmän välillä, eikä *MBL2*-genotyypillä ollut itsenäistä vaikutusta bakteremian ennusteeseen. Tupakointi oli merkitsevä grampositiivisen bakteerin aiheuttaman bakteremian riskitekijä strukturaalialueen O-alleelin kantajilla, mutta ei niillä, jotka olivat homotsygotteja A-alleelin suhteen.

Typpioksidei (NO) on keskeisessä roolissa bakteremian ja sepsiksen patogeneesissä. Endoteelin typpioksidei syntaasi (eNOS) on olennainen verisuonen NO-synteesin säätelijä. *eNOS*-geenin polymorfia nukleotidikohdassa 894 (G>T, Glu298Asp), joka johtaa T-alleeliin, on liitetty verisuonisairauksien patogeneesiin, mutta tämän polymorfian merkitystä sepsiksen patogeneesissä ei tiedetä. **Osatyössä II** bakteremiapotilaiden *eNOS*-geenin polymorfinen nukleotidikohta 894 genotyypattiin. T-alleeli liittyi *E. coli*-bakteremiaa sairastavien merkitsevään verenpaineen laskuun ja vaikeaan taudinkuvaan. T-alleelilla ei ollut vaikutusta grampositiivisen bakteerin aiheuttamissa bakteremioissa. Glu298Asp polymorfialla ei ollut vaikutusta kuolleisuuteen.

Osatyössä III tutkittiin elintapojen ja yleissairauksien merkitystä bakteremiapotilaiden ennusteeseen. Lihavuus (body mass index (BMI ≥ 30)) ja tupakointi säilyivät bakteremiakuolleisuuteen liittyvinä itsenäisinä ennustetekijöinä, vaikka niiden vaikutusta tutkittiin monimuuttujamallissa potentiaalisten sekoittavien tekijöiden kanssa. Bakteremiaan kuolleiden BMI oli tilastollisesti merkitsevästi korkeampi verrattuna niihin, jotka säilyivät hengissä (mediaanit 33 vs 26, $p=0.003$).

Indoleamine 2,3-dioxygenase (IDO) hajottaa tryptofaania (trp) sen metaboliitiksi, kynureniiniksi (kyn). Aiemmat tutkimukset viittavat siihen, että IDO:lla voi olla merkittävä rooli erilaisissa tulehduksellisissa tiloissa. IDO lamaa T-solujen toimintaa, mutta sen kliininen merkitys eri sairauksissa on pitkälti tuntematon. **Osatyössä IV** seerumin tryptofaani- ja kynureniini-pitoisuudet

määritettiin high-performance-liquid chromatography (HPLC)-menetelmällä. IDO-aktiivisuutta kuvastava kyn/trp-suhde laskettiin. Kuolleilla bakteremiapotilailla IDO-aktiivisuus (maksimipitoisuus 1-4 päivää veriviljelystä) oli tilastollisesti merkitsevästi korkeampi verrattuna henkiin jääneisiin. Korkea IDO-pitoisuus säilyi monimuuttujamallissa itsenäisenä kuoleman ennustajana.

Yhteenvedona todetaan, että lihavuus ja tupakointi olivat bakteremiapotilaiden itsenäisiä kuoleman vaaratekijöitä. Tupakointi myös lisäsi grampositiivisen bakteremian riskiä potilailla, jotka kantoivat *MBL2*-geenin strukturaalialueella O-alleelia. Tämä on uusi löydös geenien ja ympäristön välisistä vuorovaikutussuhteista. *eNOS*-geenin T-alleelin kantajuus nukleotidikohdassa 894 liittyi hypotensioon *E. coli* bakteremiassa. Korkea kyn/trp-suhde IDO-aktiivisuuden kuvastajana liittyi voimakkaasti bakteremiapotilaiden kuolemanriskiin. Esitetyt havainnot antavat lisää todisteita siitä, että gramnegatiivisten ja grampositiivisten bakteerien aiheuttamat bakteremiat ovat patogeneettisesti erilaisia. Yksilöiden väliset erot sairastumisherkkyudessa bakteremiaan selittynevät osittain geeni-ympäristöinteraktioilla ja elintavoilla on merkitystä bakteremiapotilaiden ennusteen kannalta. Väitöstyössä osoitettiin, että verisuonitonusta säätelevällä *eNOS*-geenillä voi olla merkitystä sepsispotilaan hemodynamiikan säätelyssä. IDO-välitteisten mekanismien selvittäminen jatkossa tuonee lisää työkaluja sepsiksen patogeneesin ymmärrykseen.

1. INTRODUCTION

Bacteraemia accompanied by a systemic inflammatory response to infection, defined as sepsis, is a heterogeneous class of syndromes and the host response to the disorder involves many concomitant integrated and antagonistic processes involving both exaggerated inflammation and immune suppression (van der Poll and Opal 2008). Septic shock, a severe form of sepsis, is associated with the development of progressive damage in multiple organs and is a leading cause of patient mortality in intensive care units (ICUs) (Feihl et al. 2001). Despite important advances in understanding its pathophysiology, therapy remains largely symptomatic and supportive, as the syndromes of sepsis are defined by nonspecific alterations in physiology rather than by specific cellular processes which would represent potential therapeutic targets. In Finland, the incidence of severe sepsis is 0.38/1000 adults/year (Karlsson et al. 2007).

The successful translation of novel research findings from basic research laboratories into useful clinical strategies for the management of severely ill septic patients has proved to be a difficult challenge (Opal and Patrozou 2009). Despite almost half a century of clinical trials and more than two decades of extensive research, only two pharmaceutical strategies have survived and reached the septic patient: recombinant human activated protein C (rhAPC) and low-dose corticosteroids (Riedemann et al. 2003, Cauwels 2007). The Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study (Bernard et al. 2001), a phase III trial which led to the licensing of rhAPC, suggested that a nonantimicrobial life-saving drug had finally been found for sepsis (Poole et al. 2009). Nonetheless, the beneficial effects of even this agent in terms of survival seem to depend on the severity of the illness (Bernard et al. 2001) and it may even be harmful in patients with a lower risk of death (Riedemann et al. 2003). Recently, the European Medicine Agency (EMA) has required a confirmatory trial of rhAPC without however recalling the drug from the market (Poole et al. 2009). This reflects the despair encountered in attempts to find a resolution to the clinical challenge called sepsis. Studies of the benefits of low-dose corticosteroids have also yielded controversial results (Sprung et al. 2008, Annane et al. 2009). However, some therapeutic interventions have been acknowledged as life-saving in sepsis; there is evidence that mechanical ventilation with low tidal volumes, and prompt and adequate fluid resuscitation may save a significant number of lives (Rivers et al. 2001, Dellinger et al. 2004).

The currently available biomarkers or nonspecific physiologic criteria for the sepsis syndrome or the systemic inflammatory response syndrome (SIRS) do not adequately identify patients who might benefit either from conventional antimicrobial therapies or from therapies targeting specific mediators of inflammation (Marshall and Reinhart 2009). We currently lack the capacity to delineate distinct groups of patients with a discrete disease - a prerequisite in developing specific biologically rational sepsis therapies (Marshall and Reinhart 2009). The significance of underlying diseases and modes of living on bacteraemia outcome have as yet been insufficiently elucidated. Currently, we do not in fact know what causes the death of patients with bacteraemia and sepsis.

A number of factors, for example certain microorganisms, primary focus of infection, blood pressure, body temperature, focus of infection, advanced age, severe underlying diseases and inappropriate initial antimicrobial therapy, have been identified as being significantly associated with the outcome in bacteraemic patients (Weinstein et al. 1983, Pittet et al. 1993, Weinstein et al. 1997). There is evidence that host genetic factors may be involved in the clinical course (Arcaroli et al. 2005). Functional and association studies involving genetic polymorphisms in essential genes, including toll-like receptors (TLRs), cytokines and coagulation factors, have provided important insights into the mechanisms involved in the pathogenesis of sepsis-induced organ dysfunction and failure (Arcaroli et al. 2005).

Mannose-binding lectin (MBL) is a serum acute-phase reactant secreted by the liver. It activates the complement system by binding to carbohydrate structures presented by microorganisms, and is thus considered an important component in the innate immune defence system (Turner and Hamvas 2000, Turner 2003). MBL insufficiency caused by *MBL2* gene polymorphisms in the structural region (codons 52, 54 and 57) and the promoter region (at position -221) of the gene have been shown to increase the risk of infections and worsen outcomes especially in children, in patients with cancer, and in ICU patients (Summerfield et al. 1997, Koch et al. 2001, Gordon et al. 2006, Vekemans et al. 2007). MBL is present in the upper airways and buccal cavity and may thus protect against respiratory infections as a part of innate immunity (Hickling et al. 2004). Smoking, in turn, has substantial detrimental effects on the lung immune system (Sopori 2002). However, the possible interplay of these two, *MBL2* genotypes representing MBL insufficiency and smoking, has not been previously studied.

Nitric oxide (NO) is one of many vasoactive substances released under conditions of endotoxaemia and sepsis. Endothelial nitric oxide synthase (eNOS) is, in turn, a key regulator of vascular NO production (Nathan 1992, Parratt 1998). The *NOS3* gene is highly polymorphic (Jones and Hingorani 2005). Of *eNOS* polymorphisms, the *eNOS* gene polymorphism at position 894

(G>T, Glu298Asp), resulting in the T allele, has also been studied in the context of vascular diseases (Miyamoto et al. 1998), but its role in sepsis has not yet been explored.

Obesity and smoking are major health concerns in Western countries. The consequences of obesity for critical illness are of great public health importance. In the general population, obesity is associated with an increased risk of mortality and excess costs of care (Thompson et al. 1999). Although frequently studied as a chronic disease, the influence of obesity on acute illnesses is poorly understood and the data on the outcome of specific infections, such as bacteraemia in obese people are so far limited (Falagas and Kompoti 2006). Given the high prevalence of obesity in the Finnish population (24% in men, and 28% in women) (Saaristo et al. 2008), this issue is of particular importance. Smoking has substantial effects on the immune system, affecting both innate and adaptive immunity (Sopori 2002). Although the predisposition to invasive bacterial infections has been clearly shown (Fischer et al. 1997, Nuorti et al. 2000), data regarding the outcome of infections in smokers remain limited.

Indoleamine 2,3-dioxygenase (IDO), which is the rate-limiting enzyme for tryptophan catabolism, suppresses T-cells and may play a critical role in various inflammatory disorders. A decreased tryptophan concentration and increased concentrations of kynurenine have been described in various clinical conditions, for example infection, autoimmune syndromes, malignancies, neurodegenerative disorders and pregnancy (Schrocksadel et al. 2006). IDO activity in human serum is commonly measured by determining the ratio of kynurenine to tryptophan (kyn/trp). It is not clear whether IDO activity is beneficial or detrimental to the host (Mellor and Munn 2004). Recent studies on trauma patients have suggested that the degradation of tryptophan may be associated with the development of sepsis (Pellegrin et al. 2005, Logters et al. 2009). However, the role of IDO activity in bacteraemic patients is unclear and its potential prognostic value warrants investigations. This would be essential in order to further define the role of IDO in the pathogenesis of sepsis and to investigate the possibility of scavenging IDO as a part of sepsis therapy.

In this series, the influence of host characteristics, underlying diseases and host genetic factors on the highly variable course of bacteraemia were studied. The risk of bacteraemia in relation to the *MBL2* genotype was investigated and the prognostic value of IDO activity in bacteraemic patients was studied. The data presented might be of value in assessing the prognosis in different individuals in order to target preventive efforts such as immunomodulative therapies on individuals likely to derive the greatest benefit.

2. REVIEW OF THE LITERATURE

2.1. Overview of bacteraemia and sepsis

2.1.1. Definitions

A consensus conference between the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) has defined bacteraemia as the presence of viable bacteria in the blood (Bone et al. 1992). Sepsis was defined as a SIRS in the presence of infection. The SIRS criteria include two or more of the following: temperature >38 or $<36^{\circ}\text{C}$, heart rate $>90/\text{min}$, respiratory rate >20 or $\text{PaCO}_2 <32\text{mmHg}$, white blood cell count >12 or $<4 \times 10^9/\text{l}$ or presence of immature forms $>10\%$. Severe sepsis is sepsis associated with organ dysfunction, hypoperfusion or hypotension. Septic shock is sepsis-induced hypotension despite adequate fluid resuscitation (Bone et al. 1992). In 2001, an International Sepsis Definitions Conference concluded that the diagnostic criteria for SIRS published in 1992 were overly sensitive and non-specific, and thus published an expanded list of signs and symptoms of sepsis which might better reflect the clinical response to infection. The sepsis definition, however, was left practically unchanged (Levy et al. 2003).

One of the most widely used scores for the assessment of organ dysfunction in sepsis is the sequential organ failure assessment (SOFA) score (Vincent et al. 1996). Organ function is graded from 0-4 points (0= no dysfunction, 4= severe dysfunction) in six different organ systems (Vincent et al. 1996). Increasing or constantly high SOFA scores correlate with mortality in patients with severe sepsis (Levy et al. 2005). Organ failure has been defined as a SOFA score ≥ 3 (Brun-Buisson et al. 2004).

Despite the definitions for sepsis, severe sepsis and septic shock outlined above, these terms do not allow of a precise characterization and staging of patients with this condition. To be clinically useful, such a system would stratify patients by both their baseline risk of an adverse outcome and their potential to respond to therapy. Intensive care scoring systems predict the outcome of populations, but they may not be sufficient for the evaluation of individual patients (Skrobik and Kavanagh 2006). Using a variation of the tumours/nodes/metastasis (TNM) classification of

malignant tumours in oncology (Harmer et al. 1970), the International Sepsis Definitions Conference in 2001 developed a classification scheme called PIRO, for sepsis, which stratifies patients on the basis of their *Predisposing conditions*, the nature and extent of the *insult* (in the case of sepsis, infection), the nature and magnitude of the host *response*, and the degree of concomitant *organ dysfunction* (Levy et al. 2003). The PIRO approach is a step towards the concept of “personalized medicine”. Eight years after the introduction of the concept, a few studies have tested and further refined it (Moreno et al. 2008, Rubulotta et al. 2009).

2.1.2. Incidence

During the period 1995-2002, over 50 000 cases of bloodstream infections (BSI) were reported in Finland and the annual incidence increased from 104 to 145 cases/100,000 (40%) (Skogberg et al. 2008). Elderly persons (aged ≥ 75 years) accounted for 28% of cases and showed the largest rate increase. *E. coli*, coagulase-negative staphylococci, *S. aureus* and *Str. pneumoniae* accounted for over half of the obtained isolates and their relative proportions remained unchanged during the period (Skogberg et al. 2008). The increasing incidence was partly explained by increasing blood-culturing activity (Skogberg et al. 2008). According to the national annual infectious diseases report published by the National Institute of Health and Welfare (THL), *E. coli* was noted as the most common organism isolated in blood-culture positive infections in Finland in 2007 (THL 2008).

The annual incidence of pneumococcal bacteraemia in Finland is 9.9/100 000 (Klemets et al. 2008a). The incidence of *S. aureus* bloodstream infections rose from 11/100 000 population in 1995 to 17/100 000 in 2001. Although the increase in incidence was seen in all adult age groups, it was most striking in patients >74 years of age (Lyytikäinen et al. 2005). The incidence of β -haemolytic streptococcus bacteraemia varies between Lancefield groups; in the Pirkanmaa Hospital District (HD), the incidence of Lancefield group G streptococci (GGS) was the highest in 2004 (4.3/100 000 population) and that of group C lowest ($<1/100 000$ population in 2004) (Rantala et al. 2009a).

2.1.3. Prognosis

The prognosis of bacteraemia varies between causative organisms and according to whether sepsis, severe sepsis or septic shock is present. Increasing severity correlates with increasing mortality, which rises from 25-30% for severe sepsis up to 40-70% for septic shock (Lever and Mackenzie 2007). The case fatality rate in methicillin-susceptible *S. aureus* (MSSA) bacteraemia has been shown to vary between 13-24% (Lyytikäinen et al. 2005, Ruotsalainen et al. 2006, Laupland et al.

2008b). The presence of infective endocarditis and a nosocomial origin of bacteraemia increase the case fatality rate in *S. aureus* bacteraemia (Lyytikäinen et al. 2005). The rate has been reported to be 11-17% in *E. coli* bacteraemia (Kuikka et al. 1997, Laupland et al. 2008a), 10-30% in *Str. pneumoniae* bacteraemia (Watanakunakorn et al. 1993, Lujan et al. 2004, Lynch and Zhanel 2009) and 12-14% in β -haemolytic streptococcal bacteraemia (O'Loughlin et al. 2007, Broyles et al. 2009, Rantala et al. 2009a). The presence of toxic shock syndrome or necrotizing fasciitis in streptococcal bacteraemia increases the case fatality rates to 36% and 24%, respectively (O'Loughlin et al. 2007).

2.2. Pathophysiology of bacteraemia and sepsis

Figure 1 represents a summary of the key events in the pathogenesis of bacteraemia/sepsis. Upon inflammatory stimulus, tissue macrophages, monocytes, other myeloid cells, and to some extent endothelial cells contribute to the cellular response seen in sepsis, responding as a first-line defence (Aird 2003). These cells recognize pathogens via pattern recognition receptors (PRRs) which have the ability to interact with the structures of microbes (Janeway and Medzhitov 2002). The interactions between pathogens and host cells induce multiple changes, leading to activation of inflammatory, coagulation and complement cascades (Aird 2003, Opal and Esmon 2003, Ward 2008). This leads to the release of soluble mediators, reactive oxygen and/or reactive nitrogen species (ROS/RNS), cytokines such as tumour necrosis factor alpha (TNF- α), IL-1, and IL-6, arachidonic acid metabolites and NO, which amplify the inflammatory response, affecting the endothelium and activating inflammatory cells (Marsh and Wewers 1996). These changes are manifested as the SIRS. Sepsis perturbs immune homeostasis by inducing an initially unbridled systemic inflammation, which is accompanied by an anti-inflammatory reaction acting as negative feedback. This compensatory inhibitory response, secondly, becomes deleterious, as nearly all immune system functions are compromised (Monneret et al. 2008). However, the timing of the first occurrence of immunosuppression in sepsis is a matter of debate and some investigators suggest that it constitutes a primary rather than a compensatory response to sepsis (Hotchkiss and Nicholson 2006).

Several overlapping mechanisms have been proposed to explain the development of the multiple organ dysfunction syndrome. These may be divided into groups; circulatory failure, leading to an inadequate supply of oxygen to tissues, direct cytotoxic effects of various mediators released in the

course of generalized inflammation, and subsequent immunoparalysis (Parrillo 1993, Feihl et al. 2001, Hotchkiss and Karl 2003).

It has not been completely established what causes the death of sepsis patients. Although the prevailing conception has been that mortality in sepsis results from a tremendous hyper-inflammatory cytokine reaction, novel studies suggest that most deaths from sepsis are actually the result of a substantially impaired immune response due to extensive apoptosis and subsequent death of immune effector cells (Hotchkiss and Nicholson 2006). Table 1 summarizes the factors associated with susceptibility to or outcome of bacteraemia/sepsis.

Figure 1. Schematic presentation on key events in the pathogenesis of sepsis. The inflammatory response may lead to a balanced response, subsequent pathogen elimination and recovery, or to various clinical presentations of sepsis (lower part of the figure). PAMP= pathogen-associated molecular pattern, TLR= toll-like receptor, PRR= pattern recognition receptor, CNS= central nervous system, O₂= dioxygen, TNF= tumour necrosis factor, IL= interleukin, DIC= disseminated intravascular coagulation.

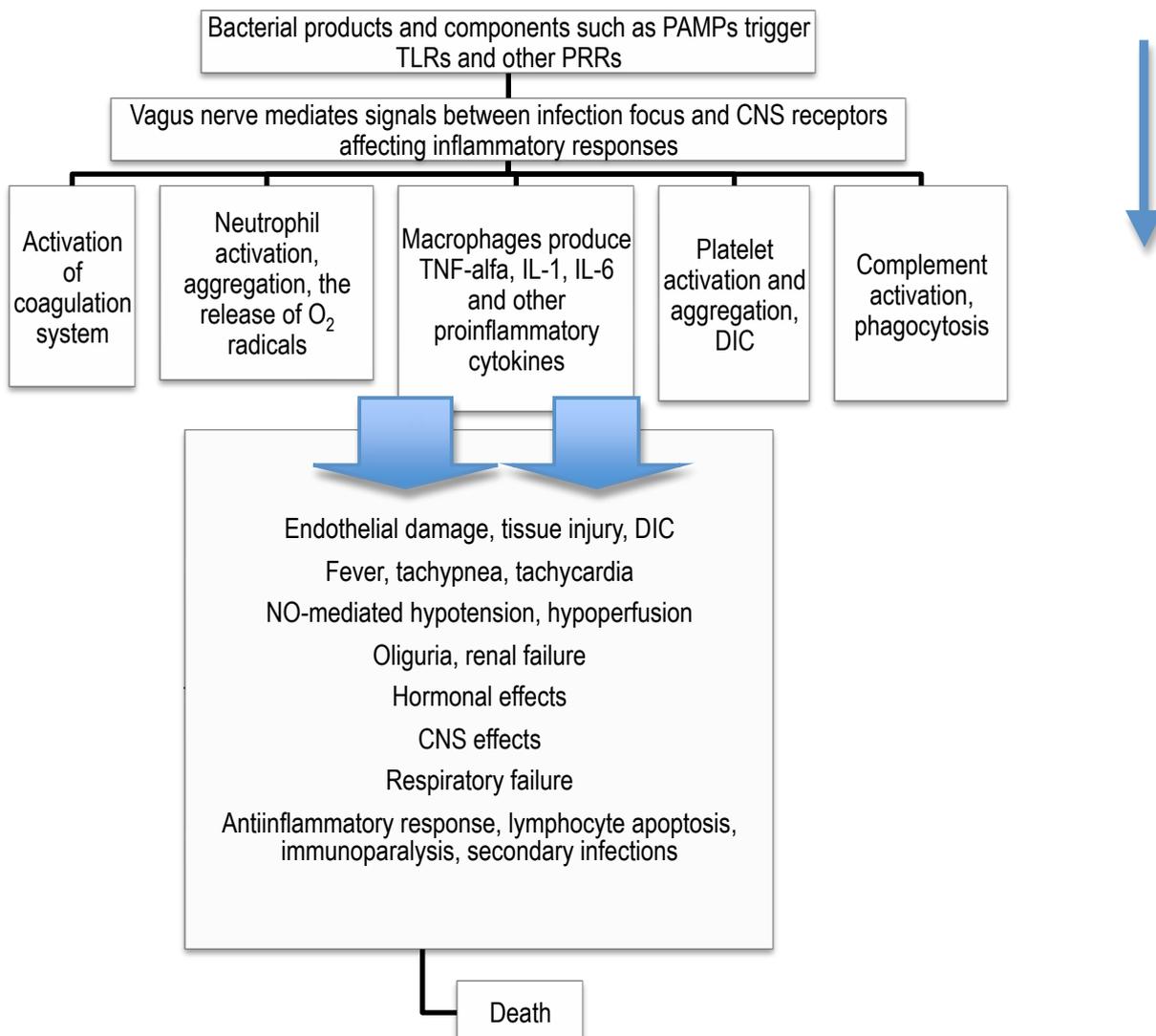


Table 1. Summary of the factors associated with susceptibility to and outcome of bacteraemia/sepsis. TLR= toll-like receptor, MBL= mannose-binding lectin, rhAPC= recombinant human activated protein C.

Factors associated with susceptibility to or outcome of bacteraemia/sepsis
Organism-related factors
Virulence, toxins, antimicrobial resistance, bacterial load
Different organisms signal via disparate mechanisms (e.g. TLRs)
Primary focus of infection
Genetic polymorphisms
Innate receptor and innate system molecule polymorphisms (e.g. MBL)
Cytokine polymorphisms
Host characteristics
Demographic factors (gender, age, race)
Mode of living (BMI, smoking, alcohol abuse)
Underlying diseases
Immunodeficiencies
Malignancies
Ultimately or rapidly fatal diseases
Diabetes
Medical intervention-related causes
Haemodialysis, catheter-related causes
Surgery
Immunosuppressive therapy
Quality of care
Appropriateness and timing of first selected antimicrobial agent
Properties and dosing of antimicrobial treatment
Surgical source control
The adoption of early goal-directed strategies, the use of rhAPC

2.2.1. Inter-bacterial differences

The mechanisms leading to organ failure and death differ between different microorganisms (Opal and Cohen 1999). Much of the damage inflicted on the septic patient is attributable to microbial toxins and the host's response to them (van der Poll and Opal 2008). Endotoxin and superantigens are implicated in the pathogenesis of gram-negative and gram-positive sepsis, respectively, but signal via widely disparate cellular mechanisms and induce very different inflammatory patterns (Hotchkiss and Karl 2003, Carlet et al. 2008). The endotoxin of gram-negative organisms is sufficient to induce shock when given experimentally to laboratory animals or human volunteers (Natanson et al. 1989, van der Poll and Opal 2008). The cell wall of gram-positive bacteria, in turn, contains peptidoglycan and lipoteichoic acid (Opal and Cohen 1999). Both substances can bind to

cell surface receptors and are proinflammatory (Wang et al. 2000), although they are less active on a weight-on-weight basis than lipopolysaccharide (LPS) (Cohen 2002). The production of potent exotoxins like toxic shock syndrome toxin-1 (TSST) by *S. aureus* and the pyrogenic exotoxins by *S. pyogenes* constitute important features of gram-positive organisms.

Pathogenic strains of bacterial species differ from commensal strains by the acquisition and expression of specific clusters of virulence genes. In the future, the regulators of microbial virulence genes may also constitute potential therapeutic targets in the care of septic patients (Merrell and Falkow 2004, van der Poll and Opal 2008). Bacterial virulence factors may determine the mode of presentation of infection and many have been implicated in invasiveness and disease severity (van der Poll and Opal 2008). Quorum sensing (the ability of bacteria to assess their population density) is now recognised as a major virulence property (van der Poll and Opal 2008). Within bacterial species there may be differences between different strains in virulence properties, leading to altered infection presentation and mortality. Examples of such strains are the hypervirulent *Clostridium Difficile* strains capable of increased toxin production (Warny et al. 2005) and the community-acquired methicillin-resistant *S. aureus* (MRSA) strains, with increased virulence due to Pantone-Valentine leucocidin (PVL) production (Deresinski 2005).

Infections caused by antibiotic-resistant organisms may involve higher morbidity and mortality rates than similar infections with antibiotic-susceptible strains. However, the extent to which this is encountered may vary according to the causative organism, the infection site and the individual characteristics of the patient (Acar 1997, Cosgrove et al. 2003).

One major difference between organisms in the pathogenesis of sepsis is in binding to TLRs. TLR4 is the LPS receptor and TLR2 is predominantly responsible for recognising gram-positive cell wall structures (Takeuchi et al. 1999). TLR-agonistic molecules such as LPS can regulate NO generation through upregulation of the expression of nitric oxide synthase (NOS) isoforms through nuclear factor kappa B (NF- κ B) activation (Forstermann et al. 1998, Marshall et al. 2004). TLR2 and TLR4 have been shown to induce NOS (inducible NOS, i.e. iNOS) and TNF- α by different signalling pathways (Paul-Clark et al. 2006).

Different organisms cause infections in different loci. Defence mechanisms in the lung and in the peritoneum are dissimilar, leading to differential effects of compounds aimed at manipulating the same inflammatory system (Bagby et al. 1991).

In summary, infections caused by gram-negative and gram-positive organisms differ from each other in several ways. Differences in signalling pathways, virulence factors, patterns of TLR binding and the locations where the infection takes place result in altered clinical presentations of infectious diseases.

2.2.2. Nitric oxide (NO)

Refractory hypotension with inadequate end-organ perfusion is an ominous feature of septic shock, resulting in the dysfunction of one or more vital organs (Cauwels 2007). In 1986 NO was found to be the agent which relaxed blood vessels (Palmer et al. 1987), and NO has since been shown to be important for the physiological and pathological control of vascular tone and a central vasoactive substance released under conditions of sepsis and endotoxaemia (Cauwels 2007). The NO metabolites nitrite and nitrate, indicators of NO production, rise progressively in various experimental models of sepsis (Feihl et al. 2001).

Under physiological conditions NO is produced by two constitutive calcium-dependent enzymes involving NOS in neurones (nNOS) and endothelial cells (eNOS) (Nathan 1992, Parratt 1998). These enzymes produce small amounts of NO in response to increases in intracellular calcium (Nathan 1992). eNOS is ubiquitous in the vascular endothelium, but may also be found in kidney tubular epithelial cells and in the placenta (Forstermann et al. 1995). NO produced by eNOS plays an important homeostatic role in maintaining an appropriate blood flow to vital organs such as the lungs, kidney and liver, and exhibits cytoprotective effects, in part by preventing platelet and neutrophil adhesion to the blood vessel wall (Moncada and Higgs 1993, Forstermann et al. 1998). In bacterial infection, NO formation from L-arginine is enhanced due to the cytokine-mediated induction of an iNOS enzyme in cells (e.g. cardiac myocytes, vascular smooth muscle), which do not normally have the ability to synthesize NO. The result of this excessive iNOS-mediated increased NO production is enhanced bacterial lysis by activated macrophages and profound hypotension (Parratt 1998). NO is considered to be an active player in defence against invasion by micro-organisms, being an effector tool used by activated phagocytic cells (Shiloh et al. 1999). The effects of NO depend on the rate, timing and spatial distribution of its production, as well as the chemical microenvironment (Fang 1997, Feihl et al. 2001). Depending on its concentration and the particular circumstances, NO may exert either pro- or anti-inflammatory effects (Feihl et al. 2001).

While a key event believed to be responsible for hypotension and shock in sepsis is iNOS-derived overproduction of NO, a pivotal role has also been indicated for eNOS (Vo et al. 2005, Doursout et al. 2008). Although the expression of eNOS is constitutive, the level of transcripts for eNOS in vascular endothelial cells is increased by shear stress (Uematsu et al. 1995), variably affected by hypoxia (Le Cras et al. 1998) and reduced by proinflammatory stimuli such as TNF- α (Nathan and Xie 1994, Feihl et al. 2001). NO has major interactions with the pathways of gene expression controlled by NF- κ B, which, in turn, induces the transcription of numerous genes coding for proteins involved in inflammation, for example cytokines and iNOS. Both activation and

inhibition of NF- κ B by NO have been described (Janssen-Heininger et al. 2000). There have been attempts to improve the prognosis of sepsis by NOS inhibitors, but results have been inconsistent (Cobb et al. 1992, Szabo et al. 1994, MacMicking et al. 1995, Assreuy 2006, Harbrecht 2006). This implies that NO acts as a double-edged sword during septic shock (Cauwels 2007), and the focus has consequently shifted towards selective iNOS-inhibitors (Feihl et al. 2001).

Sepsis causes perturbations in vascular responses to vasoactive agents (Julou-Schaeffer et al. 1990). Differential effects are seen in various vascular systems; vasoconstriction may predominate in certain organs, e.g. the kidney and the intestines (Spain et al. 1994a, Spain et al. 1994b), while profound vasodilatation occurs in others, e.g. skeletal muscle (Hershey and Bond 1993). These haemodynamic changes are difficult to predict, being dependent on the timing and experimental conditions (Feihl et al. 2001). In spite of elevated cardiac output, myocardial depression is a common finding in sepsis and septic shock, referred to as septic cardiomyopathy (Flierl et al. 2008). Its mechanisms are poorly understood, although circulating and locally produced myocardial depressant substances may be involved (Parrillo 1993, Brady 1995). The role and impact of NO on septic cardiomyopathy remain a matter of debate (Flierl et al. 2008).

In summary, NO synthases are responsible for the production of NO. A key event implicated in the context of hypotension in sepsis is iNOS-derived overproduction of NO, whereas eNOS has a homeostatic role in maintaining an appropriate blood flow to vital organs. The regulation of both of these synthases is complex, and various inflammatory stimuli play a central role.

2.2.3. *Endothelium and coagulation*

The vascular endothelium plays an important role in regulating immune and inflammatory responses to pathogens. The endothelial dysfunction and impaired microvascular function seen in sepsis are increasingly widely recognized as key characteristics contributing to multiorgan failure and death (Cinel and Dellinger 2007). Endothelial dysfunction is caused by reduced NO bioavailability, which in turn is, regulated by genes such as *NOS* (Horstman et al. 2004). On the cellular level, endothelial dysfunction derives from a progressive loss of endothelial cells determined by the degree of apoptosis in them (Horstman et al. 2004). Altered leukocyte recruitment and impaired perfusion are hallmarks of the microvascular defect (Azevedo et al. 2006). Early manifestation of a microcirculatory defect has been shown to predict mortality (Trzeciak et al. 2007).

Patients with sepsis almost invariably show evidence of activation of the coagulation system, and disseminated intravascular coagulation (DIC) is a central hallmark of severe sepsis.

Inflammation triggers clotting, dampens the activity of natural anticoagulant mechanisms and impairs the function of fibrinolytic system. Inflammatory cytokines are the major mediators involved in coagulation activation, and tissue factor is regarded as the primary initiator of coagulation in sepsis (Esmon 2005). Haemostasis is controlled by three major anticoagulant proteins: the tissue-factor pathway inhibitor (TFPI), antithrombin, and activated protein C (APC) (Esmon 2005). Furthermore, plasminogen activator inhibitor type 1 (PAI-1) is a major inhibitor of haemostasis. During severe sepsis, the activation of TFPI, antithrombin, the protein C-APC-system and fibrinolysis are impaired, resulting in a procoagulant state (Levi and Ten Cate 1999).

2.2.4. Immune system

Sepsis is a result of a complex interaction between the infecting microorganism and the host's reaction to it (van der Poll and Opal 2008). The vertebrates are under constant threat of invasion by microorganisms and have evolved systems of immune defence to eliminate infective pathogens in the body. The innate immune system is an evolutionally conserved host defence mechanism against pathogens (Creagh and O'Neill 2006). The host possesses an array of constitutive protective measures to resist pathogens at the body surface, including physical barriers such as epithelial tight junctions and the mucociliary ladder present on the respiratory epithelium (Sriskandan and Altmann 2008). Surfactants containing antibacterial substances such as defensins are capable of destroying both gram-positive and gram-negative pathogens via pore-forming activity (Selsted and Ouellette 2005).

Innate immune responses are initiated by pattern recognition receptors (PRRs), transmembrane proteins present on the surface of immune cells, which recognize specific structures of microorganisms and play a crucial role in innate immunity. Of the PRRs, TLRs are capable of sensing organisms ranging from bacteria to fungi, viruses and protozoa (Uematsu and Akira 2006). In mammals, thirteen different TLRs have been found. The entire TLR family signals via four adaptor proteins and binding of TLRs also activates intracellular signal-transduction pathways responsible for the activation of transcriptional activators such as cytosolic NF- κ B (Lotz et al. 2004). Activated NF- κ B moves from the cytoplasm to the nucleus, binds to transcription sites and induces the activation of an array of genes for acute-phase proteins, coagulation factors, proinflammatory cytokines, iNOS, and the enzymatic activation of cellular proteases (Uematsu and Akira 2006, Cinel and Opal 2009). The activation of iNOS thus results in the production of NO. Dendritic cell maturation and cytokine production are also induced, resulting in the development of the more specific adaptive immunity (Akira et al. 2006). Dendritic cells, migrating to the lymph

nodes, overexpress antigen-major-histocompatibility complexes (antigen-MHCs) and costimulatory molecules. TLRs, on the one hand, are essential for the early detection of infectious agents, but on the other hand cause excessive inflammation after uncontrolled stimulation, and a sustained inflammatory response can result in tissue damage (van der Poll and Opal 2008). In addition, a reduction in their antimicrobial capacity may predispose to opportunistic infections such as nosocomial infections (Cinel and Dellinger 2007).

Bacteria, viruses, fungi and parasites all possess a limited number of unique cellular constituents not found in vertebrates (Cinel and Opal 2009). These structures are referred to as pathogen-associated molecular patterns (PAMPs), or more precisely, microbial-associated molecular patterns, as these molecules are also present in non-pathogenic bacteria (Granucci et al. 2005, Cinel and Opal 2009). PAMPs bind to PRRs such as TLRs, expressed on the surface of host cells. Previous studies suggest that the specific host immune response to each pathogen is mediated by various sets of PRRs and PAMPs (Cinel and Dellinger 2007, Cinel and Opal 2009). Ischaemia, trauma and tissue necrosis are able to generate danger-associated molecular patterns (DAMPs) (i.e., high mobility group box-1, heat shock proteins, hyaluran etc.) which augment TLR expression like PAMPs (Cinel and Opal 2009). Multiple positive feedback loops between DAMPs and PAMPs and their overlapping receptors represent the molecular basis for the syndrome known as SIRS (Cinel and Opal 2009).

Sepsis is characterized by exacerbated coagulation and impaired anticoagulation. An exaggerated procoagulant state may lead to ischaemic cell injury, an effect which further amplifies the damage caused by excessive inflammation (Cinel and Opal 2009). A microvasculature injured by inflammation and ischaemia further perturbs the host immune response by altering leukocyte trafficking, generating apoptotic microparticles and increasing cellular hypoxia (Cinel and Dellinger 2007, Cinel and Opal 2009). Mitochondrial dysfunction, an acquired intrinsic defect in cellular respiration termed “cytopathic hypoxia,” also has an important role in that it reduces cellular oxygen consumption in sepsis, and growing evidence suggests that perturbations of key mitochondrial functions play a critical role in sepsis related organ failure (Larche et al. 2006, Cinel and Opal 2009).

In summary, sepsis is a result of an interaction between the infecting microorganism and the host's reaction to it. TLR-mediated signalling plays a pivotal role in this network. Like many other genes involved in the pathogenesis of sepsis, the expression of those regulating vascular tone are induced by TLR-mediated signalling pathways.

2.2.4.1. Apoptosis and immunosuppression

It is now widely accepted that the host response to sepsis involves many concomitant, integrated, and antagonistic processes which involve both intensive inflammation and immunosuppression (van der Poll and Opal 2008). The past ten years of sepsis research have revealed that increased apoptosis and resultant immunosuppression of immune effector cells may be a hallmark of the condition (Hotchkiss and Nicholson 2006). In clinical practice, most patients with sepsis survive the initial few days but develop a protracted immunosuppressive state which is manifested by an inability to eradicate the primary infection and the development of secondary infections (Hotchkiss and Nicholson 2006).

Initially, sepsis may be characterized by increases in inflammatory mediators, but as the condition persists there is a shift toward an anti-inflammatory immunosuppressive state (Oberholzer et al. 2001, Hotchkiss and Karl 2003). The mechanisms of immune suppression in sepsis include a shift from inflammatory Th1-type cytokines to Th2-type anti-inflammatory cytokines (Abbas et al. 1996, Opal and DePalo 2000), T cell anergy (Heidecke et al. 1999), apoptotic cell death (Hotchkiss et al. 1999) and death of lymphocytes (Hotchkiss et al. 1999, Hotchkiss and Nicholson 2006). The decrease in the numbers of T and B cells impairs the adaptive immune response, and the cross-talk between the innate and adaptive immune systems is compromised (Hotchkiss et al. 1999). A unique study in which patients who died of sepsis were autopsied within 30-90 minutes of demise revealed extensive apoptosis of lymphocytes and gastrointestinal epithelial cells compared to patients who died of non-septic aetiologies (Hotchkiss et al. 1999). The degree of apoptosis of circulating lymphocytes has been shown to predict outcome in patients with sepsis (Guisset et al. 2007). Another major characteristic of monocytes from septic patients is the decreased surface expression of human leukocyte antigen-DR (HLA-DR) (Lin et al. 1993), and low HLA-DR expression has also been associated with poor sepsis outcome (Fumeaux and Pugin 2006). Although cytokines are considered to be the culprits, they also have beneficial effects in infection and sepsis (Hotchkiss and Karl 2003). Highlighting the aspect that the sepsis syndrome is not purely a syndrome of extensive inflammatory reaction, studies in animal models and in humans have shown that blocking TNF- α may further jeopardize survival (Eskandari et al. 1992, Fisher et al. 1996). Recent data indicate that the proinflammatory cytokine IL-17 increases neutrophil killing capacity and plays a critical role in host protection against sepsis (Freitas et al. 2009).

In summary, recent sepsis research has revealed that increased apoptosis and resultant immunosuppression of immune effector cells may constitute the hallmark of sepsis. The contributors to this phenomenon have not been established.

2.2.4.2. Collectins and mannose-binding lectin (MBL)

The collectins are a small family of glycoproteins (Hickling et al. 2004). They play an important role in innate immune system, recognizing and binding to microorganisms via complex sugar arrays on the microbial surface. Their function is to enhance adhesion and phagocytosis of microorganisms by agglutination and opsonization (Turner 1996, Hickling et al. 2004).

In the lung, two members of the collectin family, the surfactant proteins A and D (SP-A and SP-D), are major protein constituents of surfactant. Another collectin, MBL, is also present in the upper respiratory tract and buccal cavity and may protect against infections (Hickling et al. 2004). The collectins involved in respiratory defence are MBL, SP-A and SP-D. The lectin domain of each collectin binds, with different affinities, to a range of monosaccharides (Hoppe and Reid 1994).

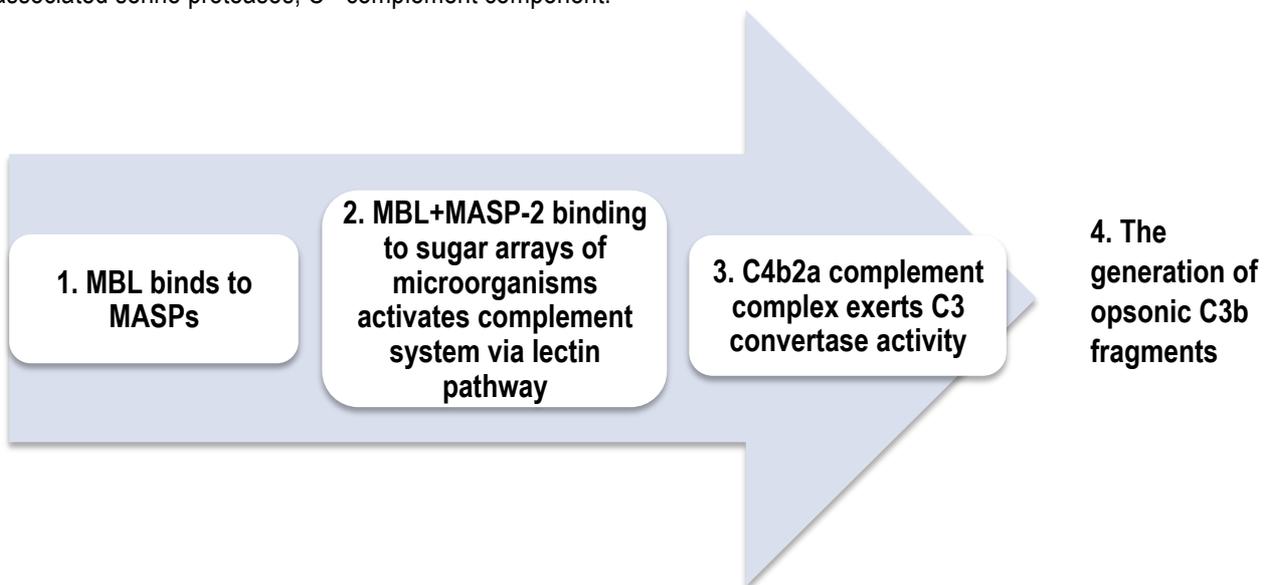
MBL is produced in the liver and is most abundant in the blood but is present in most body fluids, e.g., in the buccal cavity and upper airway secretions, the saliva (Presanis et al. 2003). MBL serum levels can be extremely varied, ranging from 0 to over 3 µg/ml in humans (Hickling et al. 2004). MBL deficiency is common, affecting 5% or more of the population (Super et al. 1989). Debate prevails as to whether MBL acts like an acute-phase protein responding to inflammation (Perez-Castellano et al. 2006, Herpers et al. 2009). A recent study has shown that the acute-phase responsiveness of MBL is highly dependent upon the *MBL2* genotype (Herpers et al. 2009). Serum levels of MBL have been shown to be independent of age and gender (Ytting et al. 2007). In healthy individuals, circulating levels of MBL have been found to be stable over time, not being affected by physical exercise (Ytting et al. 2007). As far back as 1977, a group of infants with recurrent pyogenic infections was described, whose serum failed to opsonize *Saccharomyces cerevisiae* due to a defect in the complement system (Soothill and Harvey 1977).

The complement system is an important mediator of immune responses and contributes to many innate-immune system functions including inflammation, opsonization, and pathogen lysis (Hickling et al. 2004). The system consists of more than 30 proteins, soluble in serum and bound to cell membranes, and can be activated via three pathways, (the classical complement pathway, the lectin pathway, or the alternative pathway) upon recognition of PAMPs (Gasque 2004). MBL mediates activation of the complement system via the lectin pathway in association with ficolins, and mediates opsonophagocytosis directly, thereby constituting an important element in innate immunity (Turner and Hamvas 2000, Turner 2003). MBL binds to a large variety of sugar moieties expressed by many different microorganisms, bacteria, viruses, protozoa and helminths through a pattern-recognition mode of detection and then initiates a range of host responses (Jack and Turner 2003). Complement activation by MBL requires MBL-associated serine proteases (MASPs, Figure

2). Upon MBL binding to carbohydrate-bearing pathogens, MASP-2 is activated and cleaves the C4 and C2 components of complement. The C4b2a complex, in turn, exerts C3 convertase activity, generating opsonic C3b fragments (Casanova and Abel 2004).

The capacity of MBL to bind to different organisms has been shown to vary (Neth et al. 2000). *S. aureus* and some β -haemolytic streptococci exhibit strong binding of MBL, while its pattern of binding to *E.coli* is intermediate (van Emmerik et al. 1994, Neth et al. 2000), while *Str. pneumoniae* binds MBL rather weakly (Neth et al. 2000).

Figure 2. Mannose-binding lectin (MBL)-mediated generation of opsonic complement C3b fragments. MASPs=MBL-associated serine proteases, C= complement component.



2.3. Genetic factors affecting the risk and outcome of bacteraemia and sepsis

The human genome is composed of approximately 3 billion base pairs, encompassing about 30 000 genes. There are approximately 10 million common variations known as single nucleotide polymorphisms (SNPs) (Collins et al. 2003). Recent evidence has revealed that the vast majority of SNPs are not in coding regions of proteins (McVean et al. 2004). SNP is the most common type of stable genetic variation in the population (Brookes 1999). An SNP occurs in approximately 1 out of 1000 base pairs, the most frequent being a C to T substitution (Brookes 1999). It has been estimated that 10% of all SNPs in the genome are functional, possessing thus the potential to alter some biological process (Wjst 2004).

Given the link with pathogenesis, polymorphisms in genes involved in infection, inflammation, and coagulation may influence the risk of bacteraemia and the outcome of patients suffering from sepsis and septic shock (Lin and Albertson 2004). SNPs are thought to explain interindividual differences in susceptibility to infectious diseases as well as the clinical outcomes of infections (Arcaroli et al. 2005). New technologies in molecular biology provide the opportunity to study the genetic basis of susceptibility to these diseases.

2.3.1. *MBL2* polymorphisms

MBL is encoded by the *MBL2* gene in chromosome 10 (10q11.2-q21). MBL insufficiency is caused by SNPs in codons 52 (CGT-TGT, designated D, traditionally any of the variants have also been given the generic designation O allele), 54 (GGC-GAC; B or O), and 57 (GGA-GAA, C or O) in exon 1 of that gene, leading to amino acid substitutions Arg-Cys, Gly-Asp and Gly-Glu in the peptide, which interfere with the encoded protein, thereby lowering the blood levels of MBL, compromising ligand binding, and reducing complement activation (Larsen et al. 2004). Thus, the O allele denotes either a D, B or C variant allele, and the A allele denotes a wild-type allele. The designation AO denotes a variant allele (i.e. either D, B or C) in one chromosome pair, and a wild-type allele in the other), AA denotes homozygosity for a wild-type allele (wild-type allele in both chromosome pairs), and OO genotype denotes homozygosity for the variant allele (i.e. D, B or C in one chromosome pair and D, B or C in the other). Furthermore, the MBL concentration is highly dependent upon promoter region polymorphisms, of which that at position -221 (nucleotide change

G-C; alleles Y-X, respectively) are functionally the most important. Two other polymorphisms of the *MBL2* gene are located in the promoter 1 (position -550, H/L variant) and in the 5'-untranslated region (position +4, P/Q variant) of the gene. Common variant alleles situated in both the promoter and the structural region of the human *MBL2* gene influence the stability and the serum concentration of the protein. There is considerable diversity in the definition of MBL deficiency in different studies, and currently there is no standard for the condition based on a direct functional assessment of a human cohort or an experimental animal model. Various investigators have therefore used either *MBL2* genotypes which typically produce low MBL levels and/or measured low serum MBL levels to define a deficiency state. Recently, *MBL2* genotypes XA/O and O/O have been characterized as being the lowest MBL-producing genotypes in a reanalysis of a large cohort applying individual data from 4 studies (Eisen et al. 2008). There is marked diversity in *MBL2* genotype frequencies between ethnic groups; Asian (Japanese), Caucasian, Hispanic and African-Americans differ from each other in terms of allele, genotype and haplotype distribution (Ivanova et al. 2008).

Several studies have suggested that a genetically determined variation in MBL serum concentration influences the susceptibility to and the course of different types of infections, but the issue is still a subject of debate (Table 2). Some authors even state that human MBL is largely redundant for protective immunity, as approximately 5% of individuals lack functional serum MBL and in prospective studies have not proved prone to severe infections (Casanova and Abel 2004). One experimental study in MBL-knockout mice (i.e. in mice devoid of all MBL activity) showed that all such mice die after exposure to an intravenous inoculation of *S. aureus*, whereas mice with normal MBL production have a mortality of 45% following similar exposure (Shi et al. 2004). Some evidence supports the conception of a dual role for MBL in some infections. There are data indicating that *MBL2* mutations may have a beneficial impact on host defence in leprosy (Garred et al. 1994) and one paper indicates that heterozygosity of *MBL2* genotypes predicts an advantage (heterosis) in relation to fatal outcome in intensive care patients (Helleman et al. 2007).

Table 2. Studies on the *MBL2* genotype and susceptibility to and outcome of infections. MBL= mannose-binding lectin, HIV= human immunodeficiency virus, SIRS= systemic inflammatory response syndrome.

Author/Country	Patients, N/Controls, N	Design	MBL genotypes studied	Endpoint	Significant association between <i>MBL2</i> variant alleles and the endpoint (Yes/No)
Garred 1995, Denmark (Garred et al. 1995)	228 patients suspected of various non-HIV-related immunodeficiencies, 123 controls	case-control	codons 52, 54 and 57	recurrent infections	yes, but only homozygous genotype predisposed
Summerfield 1997, UK (Summerfield et al. 1997)	617 children	observational cohort	codons 52, 54 and 57	infection as a cause of admission to hospital	yes, both heterozygous and homozygous genotype predisposed
Garred 1999, Denmark (Garred et al. 1999)	149 patients with cystic fibrosis, 250 healthy Danes	case-control	promoter -221, codons 52, 54 and 57	the risk of infections and infection-related deaths	yes, associated with both endpoints
Hibberd 1999, UK (Hibberd et al. 1999)	children with meningococcal disease, hospital study (194 patients, 110 controls) and community study (272 patients, 72 controls)	case-control	codons 52, 54 and 57	the risk of meningococcal disease	yes, homozygous genotype was associated with the endpoint
Koch 2001, Greenland (Koch et al. 2001)	252 children <2 years	prospective, population-based cohort, a 2 year morbidity surveillance	promoter -221, codons 52, 54 and 57	the risk of acute respiratory tract infections	yes, the risk association was restricted to children aged 6 to 17 months (RR 2.92) while less effect (RR 1.47) and no effect (RR 1.0) was shown among children aged 0 to 5 months and 18 to 23 months, respectively
Neth 2001, UK (Neth et al. 2001)	100 children with malignancy	prospective observational cohort study	codons 52, 54 and 57	the duration of febrile neutropenic episodes	yes, association with prolonged duration of febrile episodes
Kronborg 2002, Denmark (Kronborg et al. 2002)	141 patients with <i>Str. pneumoniae</i> bacteraemia, 250 controls	prospective case-control	promoter -221, codons 52, 54 and 57	the risk of <i>Str. pneumoniae</i> bacteraemia and outcome	no association with the endpoint
Mullighan 2002, Australia (Mullighan et al. 2002)	97 related allogeneic donor-recipient pairs	retrospective cohort	promoter -221 and -551, codons 52, 54 and 57	the risk of major infection	yes, increased risk of major infections after transplantation
Roy 2002, UK (Roy et al. 2002)	337 patients with invasive pneumococcal disease, 1032 controls	case-control	promoter -221, codons 52, 54 and 57	the risk of invasive pneumococcal disease	yes, but only homozygous MBL codon genotype predisposed
Garred 2003, Denmark (Garred et al. 2003)	272 patients with SIRS	prospective cohort study	promoter -221 and -551, codons 52, 54 and 57	the risk of sepsis, severe sepsis, septic shock and death	yes, associated with all endpoint variables
Dahl 2004, Denmark (Dahl et al. 2004)	9245 individuals from the adult Danish population	a 24 year prospective population-based follow-up	codons 52, 54 and 57	the risk of infections	no association with infection susceptibility or mortality

Table 2. continues from the previous page. ICU= intensive care unit, Allo-SCT= allogeneic stem cell transplantation, BSI= bloodstream infection.

Author/Country	Patients, N/Controls, N	Design	MBL genotypes studied	Endpoint	Significant association between <i>MBL2</i> variant allele and the endpoint (Yes/No)
Fidler 2004, UK (Fidler et al. 2004)	100 consecutive admissions to a pediatric ICU	prospective observational cohort	promoter -221, codons 52, 54 and 57	the risk of SIRS, sepsis and septic shock	yes, codon variants were associated with all endpoint variables
Horiuchi 2005, Japan (Horiuchi et al. 2005)	113 children with cancer receiving chemotherapy	retrospective	promoter -221 and -551, codons 52, 54 and 57	the risk of major bacterial infections	yes, OR 7.9
Sutherland 2005, Canada (Sutherland et al. 2005)	252 critically ill Caucasians with SIRS	cohort study	promoter -221, codons 52, 54 and 57	the risk of positive bacterial cultures, sepsis and death	yes, associated with increased risk of positive bacterial cultures. No association with sepsis, septic shock or death
Eisen 2006, Australia (Eisen et al. 2006)	195 patients (166 patients with BSI:s, 35 with pneumonia), 236 controls	case-control	promoter -221 and -551, codons 52, 54 and 57	the risk of BSI and septic shock	yes, associated with both endpoints
Gordon 2006, UK (Gordon et al. 2006)	172 patients with severe sepsis or septic shock, 353 controls	prospective case-control	promoter -221, codons 52, 54 and 57	the risk of severe sepsis and septic shock	yes, codon variants associated with the endpoints. No effect on survival
Smithson 2007, Spain (Smithson et al. 2007)	62 female patients with acute <i>E. coli</i> pyelonephritis, 133 healthy controls.	case-control study	promoter -550, -221 and +4, codons 52, 54, and 57	the risk of <i>E. coli</i> bacteraemia and septic shock due to <i>E. coli</i> pyelonephritis	predisposition to septic shock but not to bacteraemia
Vekemans 2007, Belgium (Vekemans et al. 2007)	255 adult patients with cancer receiving chemotherapy	prospective observational	promoter -221, codons 52, 54 and 57	the risk of severe infections	yes, associated with severe infections. No predisposition to more-frequent or more-prolonged febrile episodes.
Eisen 2008 (Eisen et al. 2008)	675 patients	reanalysis of 6 previous studies (Kronborg et al. 2002, Fidler et al. 2004, Sutherland et al. 2005, Eisen et al. 2006, Gordon et al. 2006, Perez-Castellano et al. 2006, van Till et al. 2008)	promoter -221, codons 52, 54 and 57	the risk of death due to <i>Streptococcus pneumoniae</i> infection	yes note: XA/O and O/O <i>MBL2</i> genotypes had the lowest median MBL concentrations
Rantala 2008, Finland (Rantala et al. 2008)	111 young men with asthma, 362 without asthma	prospective observational	promoter -221, +4 and -551, codons 52, 54 and 57	the risk of respiratory tract infections	yes, association not dependent on asthma status
Neth 2010, UK (Neth et al. 2010)	131 donor-recipient Allo-SCT-pairs	prospective cohort study	promoter -221, codons 52, 54 and 57	the risk of infections related to Allo-SCT	yes, low MBL levels pre-transplant predisposed to sepsis, fungal and viral infection. Donors' MBL genotypes had no influence

Taken together, previous data indicate that *MBL2* genotypes representing MBL deficiency increase the risk of infections and the risk of poor outcome mainly in specified patient groups, for example in children (Summerfield et al. 1997, Hibberd et al. 1999, Koch et al. 2001, Fidler et al. 2004), in the presence of comorbid conditions (malignancies) (Mullighan et al. 2002, Horiuchi et al. 2005, Vekemans et al. 2007), and in ICU patients (Garred et al. 2003, Fidler et al. 2004, Gordon et al. 2006). Studies showing an effect on susceptibility or outcomes of infections in healthy adults are few in number (Roy et al. 2002, Rantala et al. 2008).

2.3.2. *eNOS* gene polymorphisms

Endothelium-derived NO is formed from L-arginine by eNOS encoded by the *NOS3* gene in chromosome 7q35-36 (Marsden et al. 1993). The *NOS3* gene is highly polymorphic (Jones and Hingorani 2005). Of polymorphisms within the coding region, the *eNOS* gene polymorphism in exon 7 at position 894 (Glu298Asp; glutamic acid substituted by aspartic acid), also defined as G894T, resulting in the T allele, has been linked to the pathogenesis of hypertension (Miyamoto et al. 1998), coronary artery spasms (Yoshimura et al. 1998), acute myocardial infarction (Hibi et al. 1998) and coronary heart disease (CHD) (Casas et al. 2006). However, the data are inconsistent regarding the association with hypertension (Kato et al. 1999, Karvonen et al. 2002, Pereira et al. 2007) and CHD (Cai et al. 1999). In a Finnish cross-sectional case-control study the *eNOS* genotype was not associated with hypertension nor with hypertension-related cardiovascular complications (Karvonen et al. 2002). Recent data, again, indicate a role for the *eNOS* Glu298Asp polymorphism in the pathogenesis of erectile dysfunction (Lee et al. 2009) and ischaemic stroke (Tao and Chen 2009).

There has been debate as to whether the *eNOS* Glu298Asp polymorphism is functional (Jones and Hingorani 2005). However, evidence shows that this polymorphism generates protein products with differing susceptibility to cleavage, suggesting that it has a functional effect on the eNOS protein (Tesauro et al. 2000).

Of other *eNOS* polymorphisms, the *eNOS* 4a/b (intron 4) polymorphism and a T-786C (promoter region) polymorphism in the 5'-flanking region of the *eNOS* gene have been associated with CHD (Casas et al. 2006). The data regarding the role of *eNOS* 4a/b (intron 4) polymorphism and a T-786C (promoter region) polymorphisms in the pathogenesis of ischaemic stroke remain controversial (Casas et al. 2006). According to one meta-analysis studying *eNOS* gene

polymorphisms in the context of vascular diseases, there would appear to be a need for large-scale genetic association studies to confirm or refute the claim of a role of the *eNOS* gene in CHD and in other vascular diseases (Casas et al. 2006).

In non-septic state carriage of the T allele of *eNOS* gene has been linked to reduced basal NO production (Veldman et al. 2002) and blunted endothelial-dependent vasodilatation in healthy volunteers (Godfrey et al. 2007). In addition, mice lacking the *eNOS* gene have been shown to become hypertensive (Huang et al. 1995). Although NO plays a key role in the pathogenesis of sepsis and an essential role for eNOS has also been indicated, there are no data regarding the role of the *eNOS* G894T polymorphism in either bacteraemia or sepsis.

2.3.3. Cytokine and innate immune receptor polymorphisms

Several studies have investigated the role of cytokine, innate receptor and related molecule SNPs in infectious diseases. A summary of relevant studies is given in Table 3.

Table 3. Summary of the most common single nucleotide polymorphisms (SNPs) involving cytokines, innate immune receptors and related molecules in relation to susceptibility to and outcome of sepsis. TLR= Toll-like receptor, CD= cluster of differentiation, IRAK=interleukin-1 receptor-associated kinase, NOD=nucleotide-binding oligodimerization domain, MASP= mannose-binding lectin-associated serine protease, ICU= intensive care unit, TNF= tumour necrosis factor, IL= interleukin, PAI=plasminogen activator inhibitor.

Gene	Polymorphism	Results in different studies reporting the effect of given polymorphism
TLR2	Arg753Gln	Increased risk of life-threatening infections caused by gram-positive organism (Lorenz et al. 2000)
	T-16933A	Increased risk of sepsis caused by gram-positive organism, no effect on outcome (Sutherland et al. 2005)
TLR4	Asp299Gly	Increased risk of infections (Agnese et al. 2002) and septic shock (Lorenz et al. 2002) caused by gram-negative bacteria
	Thr399Ile	Increased risk of infections (Agnese et al. 2002) and septic shock caused by gram-negative bacteria (Lorenz et al. 2002)
CD14	C-159T	No association with the risk of sepsis or mortality (Hubacek et al. 2000)
		Increased risk of septic shock and mortality (Gibot et al. 2002)
		No association with the risk of infections caused by gram-negative organism or outcome (Agnese et al. 2002)
		Increased prevalence of positive bacterial cultures and sepsis but no effect on outcome (Sutherland et al. 2005)
IRAK4	821delT Q293X	Increased risk of infections caused by pyogenic bacteria in children (Picard et al. 2003)
NOD2	NOD2 variant	Increased risk of bacteraemia (Henckaerts et al. 2009)
MASP2	D120G/V377A	Increased risk of death in ICU patients (Henckaerts et al. 2009)
TNF-alpha	G308A	No association with incidence or outcome of severe sepsis (Stuber et al. 1995)
		Increased risk of septic shock and death (Mira et al. 1999)
		Increased risk of surgical infection with sepsis and increased mortality rate due to septic shock in these patients (Tang et al. 2000)
		No association with susceptibility to sepsis, disease severity or outcome (Gordon et al. 2004).
		Increased risk of severe sepsis after burn injury (Barber et al. 2006)
IL-6	G174C	No association with incidence, but GG genotype protective of death in severe sepsis (Schluter et al. 2002)
		Increased risk of severe sepsis after burn injury (Barber et al. 2006)
	G572C	Increased risk of sepsis and increased severity of sepsis in children (Michalek et al. 2007).
		G allele associated with increased risk of sepsis in children, no association with severity of disease (Michalek et al. 2007)
C	C allele associated with increased risk of sepsis in trauma patients (Gu et al. 2008)	
IL-1β	-511	Increased risk of death in meningococcal disease (Read et al. 2000)
		No association with susceptibility to sepsis. Increased mortality in septic patients (Ma et al. 2002)
		No association with outcome of gram-negative sepsis (Jessen et al. 2007)
IL-1α	46 bp VNTR (intron 6)	No association with susceptibility to sepsis. Increased mortality in septic patients (Ma et al. 2002)
IL-1ra	86 bp VNTR (intron 2)	Increased risk of sepsis and mortality (Fang et al. 1999, Ma et al. 2002)
		Increased mortality in sepsis (Arnalich et al. 2002)
PAI-1	4G/5G	No association with the risk of meningococcal infection, but increases the risk of septic shock (Westendorp et al. 1999)
		Increased risk of death in meningococcal disease (Hermans et al. 1999)
		No association with outcome of sepsis (Jessen et al. 2007)
		Increased risk of death in septic shock (Garcia-Segarra et al. 2007)
IL-10	C592A -1082	Increased mortality in sepsis (Lowe et al. 2003)
		No association with sepsis or mortality (Lowe et al. 2003)
		G allele associated with increased risk of septic shock in pneumococcal disease (Schaaf et al. 2003)

2.4. Host characteristics and underlying diseases affecting the risk and outcome of bacteraemia and sepsis

The incidence of infectious episodes is clearly increased in many underlying chronic disorders leading to immunosuppression, including haematologic malignancies (Bodey et al. 1966), neoplasms (Angus et al. 2001, Anatoliotaki et al. 2004), and diabetes mellitus-related organ damage (Dhainaut et al. 2005). Immunosuppressive therapy has been clearly associated with increased infection rates, and the risk of developing infections increases with the degree and duration of granulocytopenia (Bodey et al. 1966).

Several factors (Table 1) have been shown to be associated with an increased risk of bacteraemia and sepsis, for example male sex and an ultimately or rapidly fatal underlying disease (Angus et al. 2001, Martin et al. 2003, Ortega et al. 2007), black race (Martin et al. 2003), and extremes of age (Martin et al. 2003). Liver disease (Foreman et al. 2003), HIV infection (Witt et al. 1987), cancer (Williams et al. 2004) and therapeutic interventions such as in-dwelling catheters (Groeger et al. 1993) and red cell transfusions (Raghavan and Marik 2005) have likewise been associated with increased sepsis rates. Urban residence, haemodialysis, diabetes mellitus, cancer and lung disease have been shown to constitute risk factors for the acquisition of severe bloodstream infection (Laupland et al. 2004).

Regarding various causative agents, the risk of developing invasive pneumococcal disease is higher in persons with certain underlying medical conditions (HIV/AIDS, asplenia, haematological or solid cancer, chronic lung disease, chronic heart disease, diabetes) (Kyaw et al. 2005, Klemets et al. 2008b), who are of low socioeconomic status or who engage in high-risk behaviours such as smoking and alcohol abuse (Pastor et al. 1998, Nuorti et al. 2000, Robinson et al. 2001). In different age groups, the incidence of invasive pneumococcal disease is highest among children younger than 2 years and adults aged 65 years or more (Robinson et al. 2001, Klemets et al. 2008b). The incidence among blacks is higher than among whites (Robinson et al. 2001, Kyaw et al. 2005). Nasal carriage of *S. aureus* is an important risk factor for staphylococcal nosocomial and surgical site infections (Wertheim et al. 2004). Dialysis, organ transplantation, invasive surgery, increased use of intravascular devices, HIV infection, cancer and diabetes are important risk factors for *S. aureus* bacteraemia (Fowler et al. 2005, Laupland et al. 2008b). Invasive group B streptococcal (GBS) disease is a significant cause of death in neonates and a significant cause of morbidity and mortality among pregnant women (Schrag et al. 2000). In nonpregnant adults invasive GBS disease represents a substantial and increasing burden, particularly among older persons, black persons (compared to white), and adults with diabetes (Skoff et al. 2009). The

incidence of invasive group A streptococcal disease (GAS), in turn, is high among elderly persons, infants and black persons (O'Loughlin et al. 2007). Disruption of the cutaneous barrier is a common predisposing factor in GAS and GGS bacteraemias (Rantala et al. 2009a). Risk factors for *E. coli* bacteraemia include low or high age, female sex, dialysis, solid organ transplantation and neoplastic disease (Laupland et al. 2008a).

Ultimately or rapidly fatal diseases (Brun-Buisson et al. 1995), absence of fever at the onset of bacteraemia and incorrect empirical antibiotic therapy have been shown to constitute risk factors for mortality in bacteraemia (Laupland et al. 2004, Ortega et al. 2007). Liver cirrhosis, chronic heart failure and chronic severe renal failure have also been identified as prognostic factors in patients with sepsis (Alberti et al. 2003). Age is a risk factor for severe sepsis; the incidence increases sharply after 60 years (Angus et al. 2001). Several studies have suggested that men are more susceptible to infections (Klein 2000, Laupland et al. 2004), and once infection sets in, they are more likely to die (Schroder et al. 1998, Angus et al. 2001).

Factors associated with poor outcome in bacteraemia vary between different organisms. In *S. aureus* bacteraemia, age > 60 years (Mylotte and Tayara 2000), noneradicable or noneradicated foci, underlying cirrhosis and cancer have been found to be independent predictors of mortality (Kim et al. 2003). Although the mortality rate in MRSA infections has been shown to be greater than in MSSA infections (Cosgrove et al. 2003, Laupland et al. 2008b) and despite the fact that appropriate therapy is essential in treating sepsis, the real impact of resistance on the outcome of patients has remained somewhat controversial (Cosgrove et al. 2003, Kim et al. 2003, Figueiredo Costa 2008). In *E. coli* bacteraemia, increasing age, ciprofloxacin resistance, non-urinary focus and a number of comorbid illnesses are factors independently associated with an increased risk of death (Laupland et al. 2008a). Ultimately or rapidly fatal underlying diseases have been associated with increased case-fatality in β -haemolytic streptococcal bacteraemia (Rantala et al. 2009b).

2.4.1. *Body mass index (BMI)*

Obesity has reached epidemic proportions over the last few decades. The prevalence of obesity in Finland is 24% in men and 28% in women (Saaristo et al. 2008). The Centers for Disease Control and Prevention (CDC) defines a BMI of 25-29 as overweight and a BMI ≥ 30 as obese (CDC 2010). Obesity is associated with increased morbidity and mortality in hypertension, stroke, cardiovascular diseases and cancer and is feared to lower the overall life expectancy over the next decades.

Obesity is a predisposing factor for the acquisition of certain types of infections (Falagas and Kompoti 2006, Falagas et al. 2009), surgical infections in particular (Bamgbade et al. 2007).

Obesity constitutes a risk factor for infection in critically ill patients (Bochicchio et al. 2006), and a risk factor for erysipelas (Karppelin et al. 2009) and community acquired pneumonia (Baik et al. 2000). The underweight state may also in fact constitute a risk factor in this context (Loeb and High 2005), through the alteration of specific immunologic mechanisms such as decreased lymphocyte count and function, decreased macrophage activation, and decreased phagocytosis and cytokine secretion (Solomons 2007, Falagas et al. 2009).

It has not been satisfactorily established whether obesity constitutes a risk factor for adverse outcome in patients with infectious diseases (Falagas and Kompoti 2006, Falagas et al. 2009). Only a few studies have systematically evaluated the impact of obesity on the outcome of infectious diseases, and they have yielded controversial results (Kalfarentzos et al. 1987, Carratala et al. 2003, Bochicchio et al. 2004, Smith et al. 2007). There are also reports to indicate that low BMI is independently associated with higher case fatality (Tremblay and Bandi 2003, Garrouste-Orgeas et al. 2004).

The mechanisms of immune responses to pathogens in different body-weight categories have not been well established in humans (Falagas et al. 2009). Obesity is associated with multiple comorbidities and physiologic derangements (e.g. insulin resistance, pro-inflammatory state), pharmacologic alterations and physical limitations which may potentially complicate acute illness and impede the implementation and efficacy of evidence-based treatment strategies (Ebert et al. 2006, Hogue et al. 2009) in patients with infectious diseases.

In summary, the data regarding the outcome of severe infections in obese patients are sparse. The mechanisms implicated in infection outcome in obese patients have not been adequately established.

2.4.2. Smoking

Cigarette smoking is one of the main preventable causes of death and disability. Although the prevalence of smoking has declined in the past 35 years, nonetheless 30% of Finns smoked in 2007 (26-31% of males and 18-25% of females) (Vartiainen et al. 2009). Smokers are at increased risk of bacterial infections such as invasive pneumococcal disease (Pastor et al. 1998, Nuorti et al. 2000), community-acquired pneumonia (Baik et al. 2000), periodontitis (Obeid and Bercy 2000), meningococcal disease (Fischer et al. 1997) and bacterial meningitis (Gold 1999). The risk of invasive pneumococcal disease has been shown to be increased 2- to 4-fold in smokers compared to non-smokers (Pastor et al. 1998).

Smoking has substantial effects on the immune system, affecting both innate and adaptive immunity (Sopori 2002). Tobacco smoke is a complex mixture of more than 4500 chemicals, many of which have toxic properties (Sopori 2002). It compromises the anti-bacterial function of leukocytes, including neutrophils, monocytes, T cells and B cells (Green and Carolin 1967, Stringer et al. 2007), providing a mechanistic explanation for the increased infection risk. Of specific compounds of smoke, acrolein, a toxic unsaturated aldehyde, affects neutrophil function and reduces the resistance of the lungs to infections (Li and Holian 1998), and nicotine has been shown to induce a significant loss of antibody responses and T-cell proliferation (Sopori 2002). It has been shown that the flora of smokers contains fewer normal flora bacteria and more potential pathogens compared with that of non-smokers (Brook and Gober 2005). Animal models of infection have shown that cigarette smoke affects respiratory immune-inflammatory responses and causes a delayed rate of bacterial clearance (Drannik et al. 2004). Active smoking has been associated with meningococcal carriage (Stuart et al. 1989) and smokers are more densely colonized by a variety of potentially pathogenic bacteria in buccal epithelial cells (El Ahmer et al. 1999). Alveolar macrophages (AMs) from smokers have a reduced ability to phagocytose and/or kill bacteria such as *S. aureus* and *Listeria monocytogenes* (King et al. 1988). Moreover, previous studies indicate that AMs from smokers are functionally impaired and secrete significantly lower levels of proinflammatory cytokines (McCrea et al. 1994). These cytokines are crucial for early responses to potential pathogens and upregulation of local host defence mechanisms (Kishimoto 1989). As regards the adaptive immune system, long-term smoking significantly reduces serum levels of immunoglobulins in humans (Ferson et al. 1979). Data regarding the outcome of infections in smokers are, however, limited (Pittet et al. 1993, Laurichesse et al. 2001, Arvanitidou et al. 2005, Garau et al. 2008, Leithead et al. 2008).

In summary, smoking has been shown to predispose to invasive bacterial infections and to have substantial effects on the immune system, affecting both innate and adaptive immunity. However, the factors contributing to increased infection susceptibility in smokers are not clear and the possible interaction between smoking and genes regulating innate immunity responses has not hitherto been studied.

2.4.3. Alcohol abuse

A recent nationally representative sample of Finns showed that the prevalence of hazardous alcohol drinking (defined as 24 drinks/week in males, and 16 drinks/week in females) was 5.8%. Hazardous drinking was more prevalent among males than females (8.5% vs. 3.1%) (Halme et al. 2008).

Alcohol abuse has been shown to have adverse effects on the immune system, for example alterations in neutrophil and macrophage function and abnormalities in ciliary and surfactant function in the lung (Pavia et al. 2004, Moss 2005). Many of its effects on the innate immune response are dose-dependent, with acute or moderate use associated with attenuated inflammatory responses, and heavy ethanol consumption linked with augmentation of inflammation (Goral et al. 2008). Furthermore, alcohol abuse may be associated with an increased risk of aspiration, poor dental hygiene, malnutrition, suppression of the normal cough reflex, with the physical proximity to other infected people increasing the risk of infectious diseases (Moss 2005).

Alcohol abuse has been shown to be a significant risk factor for invasive pneumococcal disease (Burman et al. 1985), pneumonia (Fernandez-Sola et al. 1995) and severe bloodstream infections (Laupland et al. 2004). Alcoholism has been shown to be associated with the incidence of sepsis in ICU patients (O'Brien et al. 2007).

Several studies suggest that alcohol abuse predisposes to increased severity of infections in subjects with established pneumonia (Fernandez-Sola et al. 1995, Ruiz et al. 1999, Foreman et al. 2003) and sepsis (Moss et al. 2003, O'Brien et al. 2007). Some findings would indicate that alcoholism is a significant risk factor for case fatality in bacteraemia (Ortqvist et al. 1988, Kaech et al. 2006, Rantala et al. 2009b), but not all studies have confirmed this (Pittet et al. 1993, Lääveri et al. 1996, Laupland et al. 2004, Arvanitidou et al. 2005).

2.5. Indoleamine 2,3 dioxygenase enzyme (IDO)

IDO is expressed in a variety of cells, including antigen-presenting cells such as monocyte-derived macrophages and dendritic cells, and is preferentially induced by Th1-type cytokine interferon- γ (IFN- γ) (Mellor and Munn 2004). Other cytokines and LPS are also capable of inducing IDO (Mellor and Munn 2004). IDO catalyzes the degradation of the essential amino acid tryptophan to kynurenine and its derivatives, thereby limiting its availability. Tryptophan in plasma originates both from dietary sources (particularly plentiful in milk, meat, fish, chocolate) (Peters 1991), but is also released by protein turnover (Brown 1996). Tryptophan catabolism has long been known to be operative in antimicrobial defence; since tryptophan is required for protein synthesis, withdrawal of this essential amino acid from the micro-environment arrests this process, with a subsequent growth of pathogens and proliferating cells (Mellor and Munn 2004, Schrocksnadel et al. 2006). However, the biological efficacy of IDO in controlling infections in vivo has remained unclear (Mellor and Munn 2004).

A decreased tryptophan concentration and increased concentrations of kynurenine have been described in various clinical entities, for example infection, autoimmune syndromes, malignancies, neurodegenerative disorders and pregnancy (Schrocksadel et al. 2006). Recently, IDO has been shown to play a role in the process of immune evasion by tumours (Munn and Mellor 2007) by IDO-mediated depletion of tryptophan levels and production of toxic metabolites, resulting in suppression of T cell activation and induction of T cell apoptosis. The overexpression of IDO has been associated with disease progression in cancer (Brody et al. 2009, Curti et al. 2009, Suzuki et al. 2010), and rheumatoid arthritis (Schrocksadel et al. 2006), and in Alzheimer's disease (Widner et al. 2000). The overexpression of IDO by tumour cells, as well as in the dendritic cells that located in the tumour-draining lymph nodes, has been shown to be an independent prognostic variable for reduced survival in patients with melanoma, lung cancer and haematologic malignancies (Brody et al. 2009, Curti et al. 2009, Suzuki et al. 2010). Tryptophan degradation has been shown to be enhanced in patients with Sjögren's syndrome (Pertovaara et al. 2005) and recent data indicate a role for it in the immunoregulation of atherosclerosis (Pertovaara et al. 2007).

In the context of infectious diseases, increased tryptophan degradation has been previously documented in patients with HIV infection (Fuchs et al. 1990) and the overexpression of IDO has also been linked with disease progression and complications such as weight loss and neuropsychiatric disorders (Schrocksadel et al. 2007). Furthermore, antiretroviral therapy significantly reduces tryptophan degradation (Fuchs et al. 1990). Enhanced degradation of tryptophan has been found in patients with neuroborreliosis (Gasse et al. 1994) and in chronic active Epstein-Barr virus infection-associated fatigue syndrome (Bellmann-Weiler et al. 2008). Upregulation of IDO in chronic hepatitis C virus infection has been documented (Larrea et al. 2007). In the case of bacterial infections, recent studies in trauma patients indicate that tryptophan degradation may be associated with the development of sepsis (Pellegrin et al. 2005, Logters et al. 2009, Ploder et al. 2009), and findings in one small study were suggestive of an association between highly increased tryptophan degradation and the severity of *Streptococcus pyogenes* infection (Murr et al. 2001). Recent data indicate that IDO expression during influenza virus infection alters the inflammatory response and facilitates the outgrowth of pneumococci during secondary bacterial pneumonia (van der Sluijs et al. 2006), suggesting that IDO-mediated pathways may contribute to susceptibility to acquire infection.

IDO induces inhibition of T cell proliferation (Hwu et al. 2000) and may thus contribute to the pathophysiology of immunodeficiency. On the other hand, IDO may serve as a natural immunoregulatory mechanism. For example, it prevents rejection of the foetus during pregnancy (Munn et al. 1998). IDO has been shown to be operative in T cell tolerance to tumours (Mellor et al.

2002), and may contribute to the regulation of T cell activity in some autoimmune disorders such as multiple sclerosis (Sakurai et al. 2002). Although the mechanism of T cell suppression has been shown to be mediated by a shortage of tryptophan, toxic catabolites may also play a role (Mellor et al. 2002, Terness et al. 2002). IDO activity in human serum can be measured by determining the ratio of kynurenine to tryptophan (i.e. the first metabolite to substrate). This ratio is regarded as a more reliable marker of IDO-induced tryptophan catabolism than the serum tryptophan concentration alone, which may be influenced by dietary intake of the essential amino acid (Schrocksadel et al. 2006).

Under normal conditions, the kynurenine concentration is related to the tryptophan level. However, reduced dietary intake of tryptophan lowers tryptophan levels without an effect on kynurenine and kyn/trp levels (Schrocksadel et al. 2006). Enhanced tryptophan degradation may result from the activation of either IDO or the hepatic tryptophan 2,3 dioxygenase (TDO) enzyme. TDO is known to regulate homeostatic serum tryptophan concentrations and IDO is up-regulated in response to inflammatory conditions (Schrocksadel et al. 2006).

Recently an increased body of new data has shown that IDO serves more than one function in the immune system (Mellor and Munn 2004, Jung et al. 2009). Its precise role in different disease processes remains nonetheless largely unknown. In particular, it is not clear whether it is beneficial or detrimental to the host. The precise role of IDO activity in bacteraemia patients is likewise unclear, and the utility of IDO measurement as a marker of prognosis in infectious diseases has not previously been studied.

3. AIMS OF THE STUDY

The aims of the present study were to establish:

1. the role of *MBL2* gene polymorphisms in the promoter region (position -221) and in the structural region (codons 52, 54 and 57) in susceptibility to or outcome of bacteraemia
2. the impact of *eNOS* gene polymorphism at position 894 (G>T, Glu298Asp) on the disease severity or case fatality in bacteraemia patients
3. the impact of host characteristics and underlying diseases on the outcome of bacteraemia
4. the role of the kynurenine to tryptophan ratio, reflecting IDO activity, in predicting disease severity and case fatality in bacteraemic patients

4. SUBJECTS AND METHODS

4.1. Subjects (studies I-IV)

4.1.1. Patients

Studies I-IV were carried out in Tampere University Hospital and in the Medical School, University of Tampere. All patients were treated in Tampere University Hospital. The study material comprised 149 Caucasian patients with bacteraemia hospitalized in Tampere University Hospital, Tampere, Finland, from June 1999 to February 2004. The cohort comprised 79 male and 70 female patients with bacteraemia. Their ages ranged from 16 to 93 year (mean 59 years).

In Tampere University Hospital blood cultures are routinely taken from patients with symptoms or signs of systemic infection (fever or hypothermia, tachycardia or tachypnea combined with leukocytosis or leukopenia and/or elevated C-reactive protein (CRP)). Patients were identified according to microbiological blood culture findings and all participants suffered from blood culture-confirmed bacteraemia caused by *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*Str. pneumoniae*), β -haemolytic streptococcae (β -hml.str.), and *Escherichia coli* (*E. coli*). According to the study plan other microbes were excluded beforehand. Blood culture-negative patients with or without sepsis syndrome and those not consenting were not included. Patients at least 16 years of age were enrolled. The clinicians were informed by the clinical microbiologist of a positive blood culture and the patients were thereafter enrolled for the study. The day on which the positive blood culture was drawn was designated as day 0 (diagnosis day). The clinicians (J.S. or J.L.) were informed by the clinical microbiologist (R.V.) of a positive blood culture from Mondays to Thursdays and the patients were enrolled whenever possible to adjust to the daily schedule. Thus, zero to two patients per week during the study period were recruited. Clinicians were not able to obtain details on the patients prior to recruitment. After being informed by the clinical microbiologist the clinician asked patients to participate and interviewed and examined those consenting. Information was also gathered also from hospital records at the time of the hospital visit and hospital records were also reviewed after hospitalization. Patients with polymicrobial bacteraemia were not included in the study. Altogether 149 out of 152 invited patients were deemed

suitable for the study and agreed to participate (i.e. 3 patients did not agree consent). Bacteraemia was defined as hospital-acquired (nosocomial) if blood culture was drawn >48h after admission. During the period in question the BACTEC 9240 (BD Diagnostic Systems, Sparks, MD, USA) blood culture system with standard media was used. All patients were treated with an empiric antibiotic regimen and antimicrobial treatment was changed according to culture results if necessary.

4.1.2. Host predisposing factors

Underlying diseases, social status and alcohol and tobacco consumption were registered. Alcohol abuse was defined as consumption of ≥ 300 g absolute alcohol per week or a known social or medical problem due to alcohol use. Smoking habits were registered and patients were defined as current smokers, ex-smokers, i.e. those who have stopped smoking, and non-smokers, i.e. those who have never smoked. Calculation of BMI (kg/m^2) was based on weight and height as reported by the patient on recruitment. BMI was recorded and patients were defined as obese if their BMI was ≥ 30 . Chronic diseases were registered and the McCabe (and Jackson) classification was used to assess the severity of the underlying medical condition (McCabe 1962). Preceding corticosteroid treatment was registered if corticosteroids (prednisone equivalent) were used in a dose of over 5 mg per day during 1 month before the bacteraemia episode. Neutropenic patients with cancer were not excluded from the study.

4.1.3. Patient monitoring and laboratory tests

Clinical data were registered on the day of blood culture (day 0) and during 6 consecutive days and on day 10-14 after blood culture (Table 4). After patient recruitment, the collection of patient clinical and laboratory data was prospective. Patients were closely monitored during hospitalization and severely ill subjects were transferred to the ICU. The possible need for mechanical ventilation was registered. Body temperature ($^{\circ}\text{C}$) was measured by tympanic thermometer. Alterations in mental status were evaluated on the Glasgow coma scale (GCS). Mean arterial pressure (MAP) ($(\text{systolic} + 2 \times \text{diastolic blood pressure})/3$) was calculated. A patient was classified as hypotensive if the MAP was < 70 mmHg during the bacteraemia episode. In study II, the lowest MAP was calculated for every patient on day 0 (the day of blood culture), during days 1-4 (one to four days after culture), or during days 5-6 (five to six days after culture). The need for vasopressive support was also recorded. A SOFA score (Vincent et al. 1996) was calculated. The SOFA score recorded

1-3 days after blood culture was used in study III. The maximum SOFA score during days 0 to 6 after blood culture was used in analysis in studies I-II and in study IV. Severe organ failure was documented if the score was ≥ 4 . The laboratory tests conducted included plasma levels of C-reactive protein (CRP) (mg/l), bilirubin ($\mu\text{mol/l}$), creatinine ($\mu\text{mol/l}$), albumin (g/l) and alanine aminotransferase (U/l). Other laboratory tests included capillary blood pH, blood white cell count ($\times 10^9/\text{l}$) and blood platelets ($\times 10^9/\text{l}$).

Table 4. Timepoints for the recording of essential clinical and laboratory parameters in 149 patients with bacteraemia. MAP= mean arterial pressure, SOFA= sequential organ failure assessment, ICU= intensive care unit, CRP= C-reactive protein.

Parameter	The timepoint of measurement/evaluation (days after blood culture).
Clinical data	
MAP (mmHg)	On blood culture day, during days 1-6 ^a
SOFA score	During days 0-6 ^b
The need for vasopressive support	During days 0-6
Case fatality	During days 0-14, during days 0-30
The need for mechanical ventilation, the need for ICU treatment	During days 0-14
Laboratory tests	
Plasma CRP (mg/l), creatinine ($\mu\text{mol/l}$), and bilirubin ($\mu\text{mol/l}$). Blood leucocytes ($\times 10^9/\text{l}$) and platelets ($\times 10^9/\text{l}$)	On blood culture day, during days 1-6 ^c
Blood neutrophil count ($\times 10^9/\text{l}$), plasma albumin (g/l) and alanine transferase (U/l), capillary blood pH (c-astrup)	One measurement/patient during days 1-6 ^d

^aMAP available on day 0: 139 patients, day 1: 124 patients, day 2: 120 patients, day 3: 119 patients, day 4: 114 patients, day 5: 87 patients, day 6: 46 patients. Available median 5 blood pressure measurements/patient/separate measurement days during days 0 to 6 after blood culture. In the case of a multiple MAP measurements/day (i.e. ICU patients), the lowest MAP of the single day was used in analysis.

^bAvailable median 3 SOFA measurements/patient/separate measurement days during the period of 0 to 6 days after blood culture.

^cAvailable median 6 CRP measurements/patient, 5 creatinine, leukocyte, and platelet measurements/patient, and 3 bilirubin measurements/patient during the period of 0 to 6 days after blood culture.

^dNeutrophil count available for 124 patients, albumin concentration available for 139, alanine transferase available for 143, and capillary blood pH available for 129 patients

4.1.4. Controls (Study I)

In study I, the control group comprised 400 adult Caucasian persons (151 male and 249 female) aged 60 years (mean, range 31-89), initially recruited from the normal population for an asthma study; only asthma and chronic obstructive pulmonary disease had been excluded (Karjalainen et al. 2002).

4.2. Methods

4.2.1. *MBL2* genotyping

In study I *MBL2* genetic polymorphism testing was carried out in 145/149 of the bacteraemia patients. Genotyping was done after hospitalization, using frozen whole blood samples taken in hospital. Exon 1 of the *MBL2* structural gene was amplified by polymerase chain reaction (PCR) (Madsen et al. 1994). Genotyping of codon 52 (CGT→TGT; designated D, traditionally any of the variants have also been given the generic designation O allele), 54 (GGC→GAC; B or O) and 57 (GGA→GAA; C or O) polymorphisms was performed by sequencing. Structural alleles lacking these SNPs were classified as wild-type (A). For genotyping of promoter region polymorphism at position -221 bp (G→C, designated Y or X alleles, respectively) commercially available fluorogenic allele-specific TaqMan probes and primers were used (rs7096206; Applied Biosystems, Foster City, CA, USA). Genotyping was done by means of the 5' nuclease assay for allelic discrimination using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

4.2.2. *eNOS* genotyping

In study II *eNOS* genetic polymorphism testing was carried out in 147/149 patients with bacteraemia. Genotyping was done after hospitalization, using frozen whole blood samples taken in hospital. Genomic deoxyribonucleic acid (DNA) was extracted from whole blood using a commercially available kit (Qiagen Inc., Hilden, Germany) in accordance with the manufacturer's instructions. DNA samples were genotyped employing the 5' nuclease assay in combination with specific fluorogenic TaqMan MGB probes, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of primers and allele-specific probes, labelled with reporter dyes, were deduced from sequences deposited in the

GenBank database and synthesized in conjugation with Applied Biosystems using the Assays-by-Design tool. PCR reaction tests containing genomic DNA, 1x Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe, were performed in 96-well plates in a total volume of 25 μ L, in accordance with the standard protocol. The endpoint fluorescence reading was measured using the allelic discrimination analysis module, this resulting in clear identification of three genotypes.

4.2.3. Tryptophan and kynurenine determinations

The samples for tryptophan and kynurenine determination were taken during hospitalization. Samples were available for 132 patients on days 1-4 after blood culture. The serum samples were stored at -70° until analyzed. Tryptophan (mmol/l) and kynurenine (μ mol/l) concentrations in peripheral blood were measured by HPLC as previously described (Laich et al. 2002). Tryptophan was separated with a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co, Kyoto, Japan) using a 50-mm BDS Hypersil C18 5 μ m column (Thermo Electron Co, Bellefonte, PA, USA). It was monitored by fluorescence with a Shimadzu RF-10A XL detector at 266 nm excitation and 366 nm emission wavelengths. Kynurenine was separated with a Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA, USA) using a Merck LiChroCart 55–4150 mm cartridge containing a Purospher STAR RP-18 3 μ m column (Merck Co, Darmstadt, Germany). It was determined by ultraviolet absorption at a wavelength of 360 nm with a Hewlett Packard G13144 detector. The kyn/trp (μ mol/mmol) ratio was calculated by relating concentrations of kynurenine (kyn) (μ mol/l) to tryptophan (trp) (mmol/l), this allowing estimation of IDO activity.

Samples for tryptophan and kynurenine determinations were available on day 1-2: (1-2 days after the blood culture was taken): 34 patients, on day 3: 80 patients, on day 4: 104 patients. In addition, 93 patients gave a sample on recovery (>26 days after the blood culture). Since patient recruitment was based on blood culture, which came positive only the day after blood culture was taken, no samples for tryptophan and kynurenine determinations were available on day 0 (blood culture day). There were 1-5 samples (median 4) available per patient, collected on separate days. Multiple samplings in the same patient were always performed on separate days. Patients with no measurements on days 1-4 after blood culture were excluded from the analysis. The maximum value in kynurenine and kyn/trp ratio and the minimum value in tryptophan for every patient measured during days 1-4 after the blood culture were used in analysis.

4.2.4. Main endpoints

The occurrence of bacteraemia was an endpoint in Study I. The case fatality rate was studied within 30 days after the blood culture (d-30 case fatality) (studies I-IV). Also day 14 case fatality was assessed in Study IV. The need for ICU stay, mechanical ventilation, high SOFA score (≥ 4), lowered GCS and hypotension (MAP < 70 mmHg) were the main evaluable endpoints in all studies.

4.2.5. Statistical methods

The SPSS package (version 7.5) was used for statistical analyses and a two-sided p-value < 0.05 was taken as cut-off for statistical significance. Categorical data were analysed by Pearson's chi-square (X^2) test or Fisher's exact test when appropriate. Nonparametric data were analysed by Mann-Whitney U-test.

In study I, binary logistic regression was used to analyse the interaction between *MBL* genotype and current smoking in relation to the risk of bacteraemia in a model adjusted for age and sex, since the patient and control groups were not matched by age and sex. Binary logistic regression was performed, severity of bacteraemia (SOFA score ≥ 4) or need for ICU treatment being dependent factors, and *MBL* genotype, current smoking, interaction between *MBL* genotype and current smoking, age, and sex independent factors. Odds ratios (ORs) were expressed with their 95% confidence intervals (CI). In study I, structural region alleles were designated in statistical analysis as follows: O allele carriage (i.e. AO or OO) =1, no O allele carriage (i.e. AA) =0. Promoter region alleles were designated in statistical analysis as follows: X allele carriage (i.e. YX or XX) =1, no X allele carriage (i.e. YY) =0.

In Study II, the effect of G894T amino acid substitution on hypotension or disease severity was studied in multivariate models adjusting for confounding factors (age, sex and obesity). Binary logistic regression was used to calculate ORs and 95% CI. Alleles were designated in statistical analysis as follows: T allele carriage (i.e. GT or TT genotype) =1, no T allele carriage (i.e. GG genotype) =0.

In Study III, multivariate logistic regression analysis (method enter) was used to assess the effects of independent factors on mortality, controlling for differences in other factors possibly affecting the outcome. Risk ratios (RRs) were expressed with their 95% confidence intervals (CI). The effect of obesity, smoking and alcohol abuse was studied in a multivariate model adjusted for the effect of age (continuous variable), sex and organism.

In Study IV, a logistic regression model was used to study the independent effect of high IDO activity on mortality in models adjusted for potential confounders (age, sex, obesity, alcohol abuse, smoking, McCabe class, high CRP, SOFA score, culprit organism and high creatinine). Odds ratios (ORs) were expressed with their 95% confidence intervals (CI). Survival curves were calculated using the Kaplan-Meier method and differences in survival between groups were compared using the log rank test.

4.2.6. Ethical considerations

The study was approved by the Ethics Committee of Tampere University Hospital. Written informed consent was obtained from patients or first-degree relatives.

5. RESULTS

5.1. Characteristics of study material

5.1.1. Bacterial aetiology

The causative organisms involved were *S. aureus* (41 patients, 27.5%), *Str. pneumoniae* (42 patients 28.2%), *β-hml str.* (23 patients, 15.4%), and *E. coli* (43 patients, 28.9%). All patients were treated with an empiric antibiotic regimen, and when necessary antimicrobial treatment was changed according to culture results. In all patients the causative organism proved susceptible to the first empiric antibiotic treatment selected on the day of blood culture.

5.1.2. Characteristics and underlying diseases of patients

The characteristics and underlying diseases of bacteraemia patients are shown in Table 5. The median BMI of all patients was 26 (range 15-39) and three were underweight (BMI <18.5). The median ages in patients with bacteraemia caused by *S. aureus*, *Str. pneumoniae*, *β-hml. str.* or *E. coli* were 61 years, 58 years, 59 years and 67 years, respectively (p=0.127).

Table 5. Host characteristics and underlying diseases in bacteraemia patients.

Character	All patients N= 149 (%)	<i>S. aureus</i> N= 41 (%)	<i>Str. pneumoniae</i> N= 42 (%)	<i>β-hml. str.</i> N= 23 (%)	<i>E. coli</i> N= 43 (%)	Overall p-value ^e
Obesity (BMI ≥30) ^a	27 (23.7)	9 (25.7)	4 (16.7)	6 (31.6)	8 (22.2)	0.698
Smoking ^b	66 (48.5)	18 (48.6)	26 (65.0)	11 (57.9)	11 (27.5)	0.007
Alcohol abuse	24 (16.1)	5 (12.2)	9 (21.4)	6 (26.1)	4 (9.3)	0.211
Diabetes (type 1 or 2)	34 (22.8)	10 (24.4)	5 (11.9)	4 (17.4)	15 (34.9)	0.077
Malignancy	25 (16.8)	7 (17.1)	6 (14.3)	2 (8.7)	10 (23.3)	0.465
Rheumatoid arthritis	7 (4.7)	5 (12.2)	0	1 (4.3)	1 (2.3)	0.034
Chronic disease ^c	117 (78.5)	33 (80.5)	27 (64.3)	17 (73.9)	40 (93.0)	0.013
Male sex	79 (53.0)	28 (68.3)	24 (57.1)	14 (60.9)	13 (30.2)	0.003
COPD ^d	8 (5.4)	0	5 (11.9)	2 (8.7)	1 (2.3)	0.053
Age >60 years	78 (52.3)	21 (51.2)	20 (47.6)	10 (43.5)	27 (62.8)	0.391

^adata available on 114 patients, ^bcurrent or ex-smoking (data available on 136 patients), ^cat least one chronic disease, ^dchronic obstructive pulmonary disease, ^ep-value indicates the difference between groups of patients with different causative organisms

5.1.3. Sources of bacteraemia

Sources of bacteraemia are presented in Table 6. Bacteraemia was community-acquired in 119 patients (79.9%) and nosocomial in 30 (20.1%).

Table 6. Sources of infection in 149 patients with bacteraemia. One patient may have several focuses.

Source	N=149 (%)	<i>S. aureus</i> n=41	<i>Str. pneumoniae</i> n=42	β -hml. str. n=23	<i>E. coli</i> n=43
Pneumonia	39 (26.2)	2	35	2	0
Skin	37 (24.8)	19	2	16	0
Urinary	30 (20.1)	1	0	1	28
Osteomyelitis/spondylitis	15 (10.1)	12	0	3	0
Gall bladder	7 (4.7)	0	3	0	4
Arthritis	6 (4.0)	5	0	1	0
Endocarditis	6 (4.0)	6	0	0	0
Mediastinitis	4 (2.7)	4	0	0	0
Meningitis	3 (2.0)	1	1	1	0
Gynaecological	3 (2.0)	0	0	2	1
Intravenous/intra-arterial catheter-related	3 (2.0)	3	0	0	0
Focus unknown	17 (11.4)	3	4	3	7

5.1.4. Outcome of bacteraemia

The clinical course in patients with bacteraemia stratified by causative organism is shown in Table 7. Forty-seven per cent of patients had organ failure (assessed by SOFA score ≥ 3), and 77.7% had at least mild organ dysfunction (assessed by SOFA score >0).

Table 7. Clinical data on 149 patients with bacteraemia.

Endpoint	All n=149 (%)	<i>S. aureus</i> n=41 (%)	<i>Str. pneumoniae</i> n=42 (%)	β -hml. str. n=23 (%)	<i>E. coli</i> n=43 (%)	p-value ^g
Died ^a	19 (12.8)	8 (19.5)	8 (19.0)	2 (8.7)	1 (2.3)	0.031
ICU ^b stay	47 (31.5)	15 (36.6)	16 (38.1)	10 (43.5)	6 (14.0)	0.029
Maximum SOFA score ≥ 4 ^c	58 (39.2)	13 (31.7)	17 (40.5)	13 (56.5)	15 (35.7)	0.250
Mechanical ventilation	22 (14.8)	7 (17.1)	10 (23.8)	3 (13.0)	2 (4.7)	0.092
CVVHD ^d	7 (4.7)	5 (12.2)	0 (0)	2 (8.7)	0 (0)	0.006
Hypotension ^e	56 (37.6)	13 (31.7)	19 (45.2)	11 (47.8)	13 (30.2)	0.299
Lowered GCS ^f	60 (40.3)	19 (46.3)	18 (42.9)	10 (43.9)	13 (30.2)	0.45

^adeath due to bacteraemia episode occurred within 30 days from the day of blood culture.

^bintensive care unit, ^csequential organ failure assessment 0 to 6 days after blood culture, ^dneeded continuous veno-venous haemodialysis, ^emean arterial pressure <70 mmHg, ^fGlasgow coma scale lowered (<15), ^gindicates the difference between groups of patients with bacteraemia caused by different organisms

5.2. Genotype frequencies in patients and in controls (Study I and II)

The distributions of *MBL2* genotypes in patients with bacteraemia and in controls, and *eNOS* genotypes at nucleotide position 894 in patients with bacteraemia are shown in Table 8. The genotype frequencies in *MBL2* genotypes in both bacteraemia and controls, and the *eNOS* genotypes at nucleotide position 894 did not deviate from the Hardy-Weinberg equation.

Table 8. Genotype and allele frequencies of polymorphisms studied in patients and controls (Studies I and II).

Polymorphism	Bacteraemia N=145	Controls N=400	P-value
<i>MBL2</i> gene (Study I)			
Structural region genotype			
A/A	91 (63%)	248 (62%)	
A/O	48 (33%)	136 (34%)	
A/B	29 (20%)	96 (24%)	
A/C	4 (3%)	8 (2%)	
A/D	15 (10%)	32 (8%)	
O/O ^a	6 (4%)	16 (4%)	
			¹ p=0.788
			² p=0.980
A allele frequency	230 (79%)	632 (79%)	
O allele frequency	60 (21%)	168 (21%)	p=0.933
Promoter region genotype			
Y/Y	93 (64%)	264 (66%)	
Y/X	44 (30%)	116 (29%)	
X/X	8 (6%)	20 (5%)	p=0.915
Y allele frequency	230 (79%)	644 (81%)	
X allele frequency	60 (21%)	156 (20%)	p=0.663
<i>eNOS</i> genotype, nucleotide 894 (Study II)			
	Bacteraemia N=147	No controls used	
GG	89 (61%)		
GT	48 (33%)		
TT	10 (7%)		
G allele frequency	226 (77%)		
T allele frequency	68 (23%)		

^avariant in regard to both structural alleles: three bacteraemia patients with B/B, two with B/D and one with D/D genotype, and five controls with B/B, eight with B/D, one with D/D, one with B/C and one with C/D genotype

¹the groups between which the p-value was calculated in bacteraemia patients vs controls (A/A, A/B, A/C, A/D, O/O)

²the groups between which the p-value was calculated in bacteraemia patients vs controls (A/A, A/O, O/O)

5.3. Genetic factors and the risk and outcome of bacteraemia

5.3.1. *MBL2* genotype (Study I)

Susceptibility to bacteraemia in relation to the *MBL2* genotype was assessed in Study I, where the *MBL2* genotype frequencies in patients and controls were compared. No significant difference in *MBL2* genotype frequencies was detected between the patients with bacteraemia and controls (Table 8), between the different organism groups, when gram-positive or gram-negative bacteraemia patients and controls were compared, or between men and women.

Bacteraemic patients who carried the structural O allele were more often current smokers than noncarriers of this allele [20/50 (40%) vs 17/82 (21%), OR 2.5, 95% CI 1.2-5.5; $p=0.017$](Table 9). Patients with gram-positive bacteraemia who were O allele carriers were significantly more often current smokers (53%), compared to controls carrying this allele (21%) (OR 4.2, 95% CI 2.0-9.0; $p<0.001$) (Table 10). The difference between the number of current smokers among O allele carriers was most evident in pneumococcal bacteraemia patients compared to controls (11/18 (61%) vs 32/152 (21%), OR 5.9, 95% CI 2.1-16.4; $p<0.001$). This was not the case in those with bacteraemia caused by a gram-negative rod as compared to controls (*E. coli*, OR 0.3, 95% CI 0-2.3; $p=0.306$). Despite the fact that smoking was more common among men in both bacteraemic and control groups, the interaction between O allele and smoking in relation to acquisition of gram-positive bacteraemia was consistent in both genders (OR 4.6, 95% CI 1.6-13.7 in males and OR 3.6, 95% CI 1.2-10.6 in females). When the interaction between MBL genotype and current smoking in relation to the risk of gram-positive bacteraemia was studied in binary logistic regression adjusted for age and sex, the finding remained significant (OR 3.2, 95% CI 1.1-9.0; $p=0.032$).

MBL2 genotype had no effect on mortality. In a univariate model, carriage of the *MBL2* structural O allele was associated with an increased need for ICU treatment ($p=0.022$), high SOFA score ($p=0.006$), lowered MAP ($p=0.011$), lowered platelet count ($p=0.016$), and elevated creatinine level ($p=0.009$) in males, while this allele had no effect on disease severity in females, nor in the whole study group. The effect of the MBL genotype, current smoking, interaction between MBL genotype and current smoking, age, and sex in relation to severity of disease (SOFA ≥ 4) or need for ICU treatment was analyzed in a binary logistic regression model. O allele or interaction between O allele and smoking did not remain a significant factor associated with severe disease in this model, while smoking did (OR 4.4, 95% CI 1.3-15.1). The O allele, or interaction between this allele and smoking likewise did not remain a significant factor associated with ICU treatment, while smoking

and male sex remained so (OR 3.9, 95% CI 1.0-14.8 and OR 3.5, 95% CI 1.4-8.4, respectively). *MBL2* promoter region polymorphism had no significant effect on the clinical course of the disease.

In summary, *MBL2* genotypes representing MBL insufficiency were not associated with the overall risk of bacteraemia, but smoking in the carriers of structural variant O allele significantly increased the risk of gram-positive bacteraemia. The risk was most prominent in predisposition to pneumococcal bacteraemia. *MBL2* genotypes representing MBL insufficiency were not independently associated with disease severity or case fatality in patients with bacteraemia.

Table 9. Predisposing factors and underlying diseases in bacteraemia stratified by *MBL2* structural genotype.

Character	<i>MBL2</i> structural genotype		OR (95% CI)	p-value
	AA n=91	AO or OO n=54		
Rheumatoid arthritis	7 (8%)	0	*	0.046
Malignancy	11 (12%)	11 (20%)	1.9 (0.7-4.6)	0.179
Alcohol abuse	13 (14%)	11 (20%)	1.5 (0.6-3.7)	0.341
Current smoker ^a	17 (21%)	20 (40%)	2.5 (1.2-5.5)	0.017
Non-smoker ^{a, b}	50 (61%)	17 (34%)	0.3 (0.2-0.7)	0.003
Obesity (BMI ≥30) ^c	20 (30%)	7 (16%)	0.4 (0.2-1.1)	0.075
Chronic disease ^d	69 (76%)	44 (82%)	1.4 (0.6-3.2)	0.427
McCabe II or III ^e	16 (18%)	5 (9%)	0.5 (0.2-1.4)	0.169
Age >60 years	49 (54%)	28 (52%)	0.9 (0.5-1.8)	0.816
Diabetes (type 1 or 2)	21 (23%)	13 (24%)	1.1 (0.5-2.3)	0.891
Male/Female	49 (54%)/42 (46%)	28 (52%)/26 (48%)	0.9 (0.5-1.8)	0.816
COPD	5 (6%)	3 (6%)	1.0 (0.2-4.4)	1.0

^adata available on 132 patients, ^bthose who had never smoked, ^cdata available on 111 patients

^dat least one chronic disease, ^eMcCabe class II or III: ultimately or rapidly fatal disease, *cannot be calculated

Table 10. The number of smokers (%) in gram-positive bacteraemia (including *S. aureus*, *Str. pneumoniae* or β -hml.str.) and in controls in relation to *MBL2* gene structural genotype.

<i>MBL2</i> structural genotype	Bacteraemia n=95	Controls n=400	OR (95% CI)
AA	n=59	n=248	
Current smokers (%)	14 (24%)	45 (18%)	1.4 (0.7-2.8)
AO or OO	n=36	n=152	
Current smokers (%)	19 (53%)	32 (21%)	4.2 (2.0-9.0)

5.3.2. *eNOS* G894T polymorphism (Glu298Asp) (Study II)

The distribution of the *eNOS* genotypes at nucleotide position 894 did not differ statistically significantly between bacteraemias caused by four different organisms ($p=0.354$). Among subjects with *E. coli* bacteraemia, carriage of the T allele was associated with lower MAP ($p=0.004$) and higher SOFA score ($p=0.001$) compared to non-carriers (Table 11). The effect on blood pressure in *E. coli* bacteraemia was most prominent in the early stage of the disease: on blood culture day MAP (median) was 52 mmHg in T allele carriers compared to 91 mmHg in non-carriers ($p<0.001$), as shown in Table 11. This was not the case in bacteraemia caused by a gram-positive organism. In fact patients suffering from gram-positive bacteraemia and having the GG genotype evinced greater severity of disease (assessed by SOFA score) than those carrying the T allele. The effect was most prominent in those suffering from *S. aureus* bacteraemia; the SOFA score was lower in carriers of the T allele compared to non-carriers ($p=0.008$, Mann-Whitney U-test). The number of hypotensive patients was significantly higher among *E. coli* bacteraemia patients carrying the T allele compared to non-carriers during the observation period days 1 to 4 (Table 11), whereas no difference was detected during days 5 to 6. T allele carriage was not associated with increased case fatality in any of the four pathogens studied.

In the whole study population there were more male patients among T allele carriers compared to non-carriers (64% vs 46%, $p=0.035$). T allele carriage remained a significant factor associated with hypotension on day 0 in *E. coli* bacteraemia in a multivariate model adjusted for age (continuous variable) and sex (OR 10.8, 95% CI 2.0-57.7; $p=0.005$). Although the number of obese patients ($BMI\geq 30$) was higher in *E. coli* bacteraemia patients carrying the T allele compared to non-carriers (50% vs 12%, $p=0.027$), obesity was not associated with hypotension in *E. coli* bacteraemia. T allele carriage was significantly associated with hypotension on day 0 in a logistic regression model adjusted for obesity (OR 10.9, 95% CI 1.4-84.0, $p=0.02$).

The effect of *eNOS* T allele carriage on disease severity (assessed by SOFA score) in gram-positive bacteraemia was also studied in multivariate logistic regression analysis. In gram-positive bacteraemia, carriage of the T allele preserved its protective role as regards severe disease (defined as maximum SOFA score ≥ 4) in a model adjusted for the effect of age and sex (OR 0.4, 95% CI 0.2-0.8). All endocarditis patients had GG genotype ($n=6$), none carried the T allele (all cases of endocarditis were caused by a gram-positive organism) (the difference between groups, $p=0.034$). However, if endocarditis patients were excluded from the analysis, carriage of the T allele preserved its protective role in gram-positive bacteraemia as regards severe disease in a model adjusted for the effect of age and sex (OR 0.4, 95% CI 0.2-1.0).

In summary, the results indicate that the T allele at nucleotide position 894 is associated with hypotension in the early stage of disease in patients with *E. coli* bacteraemia but not in bacteraemia caused by a gram-positive organism.

Table 11. Clinical characteristics of patients with *E.coli* or gram-positive bacteraemia in relation to eNOS G894T (Glu298Asp) polymorphism.

Character	<i>E. coli</i>				Gram-positive organism			
	GG, n=30	GT/TT, n=12	OR (95%CI)	p-value	GG, n=59	GT/TT, n=46	OR (95%CI)	p-value
Died (d-30 case fatality)	0	1 (8%)	*	0.286	11 (19%)	7 (15%)	0.8 (0.3-2.2)	0.644
Needed ICU^a stay	2 (7%)	4 (33%)	7.0 (1.1-45.4)	0.046	25 (42%)	16 (35%)	0.7 (0.3-1.6)	0.429
AMI^b	2 (7%)	4 (33%)	7.0 (1.1-45.4)	0.046	4 (7%)	1 (2%)	0.3 (0-2.8)	0.383
Highest SOFA^c score (days 0 to 6), median (quartiles)	1.5 (0-2.3)	5 (3-13)	§	0.001	4 (1-9)	1.5 (1.0-4.3)	§	0.021
Lowest MAP ^d (mmHg), median (quartiles)	80 (75-90)	60 (47-80)	§	0.004	70 (58-84)	77 (63-94)	§	0.071
Lowest GCS ^e , median (quartiles)	15 (15-15)	15 (14-15)	§	0.170	14 (13-15)	15 (14-15)	§	0.052
Needed mechanical ventilation	0	2 (17%)	*	0.077	15 (25%)	5 (11%)	0.4 (0.1-1.1)	0.06
Highest bilirubin value (µmol/l), median (quartiles)	15 (10-29)	20 (16-79)	§	0.085	19.5 (12-50)	17.5 (13-29)	§	0.451
Highest creatinine value (µmol/l), median (quartiles)	98 (72-193)	148 (83-190)	§	0.328	124 (83-202)	91 (71-165)	§	0.098
Lowest platelet count (10 ⁹ /l), median (quartiles)	178 (120-200)	105 (36-163)	§	0.045	128 (62-225)	184 (126-254)	§	0.023
Blood pressure characteristics at different time-points								
Day 0 (blood culture day)								
MAP (mmHg) ^f , median (quartiles)	91 (81-97)	52 (46-81)	§	<0.001	80 (67-97)	87 (76-106)	§	0.08
Hypotension ^g	4 (14%)	7 (58%)	8.8 (1.8-41.6)	0.003	15 (25%)	5 (11%)	0.4 (0.1-1.1)	0.06
Needed vasopressives ^h	1 (3%)	4 (33%)	14 (1.4-144.0)	0.02	6 (10%)	0	*	0.034
During days 1 to 4ⁱ								
MAP (mmHg) ^f , median (quartiles)	94 (88-107)	80 (74-91)	§	0.02	85 (73-100)	92 (80-107)	§	0.236
Hypotension ^g	0	4 (33%)	*	0.004	15 (25%)	7 (15%)	0.5 (0.2-1.4)	0.202
Needed vasopressives ^h	0	4 (33%)	*	0.004	12 (20%)	5 (11%)	0.5 (0.2-1.5)	0.191
During days 5 to 6ⁱ								
MAP (mmHg) ^f , median (quartiles)	99 (90-107)	83 (75-94)	§	0.025	89 (71-100)	97 (81-110)	§	0.045
Hypotension ^g	0	0			7 (18%)	3 (10%)	0.5 (0.1-2.2)	0.358
Needed vasopressives ^h	0	0			5 (11%)	3 (9%)	0.8 (0.2-3.7)	0.796

^aintensive care unit, ^bacute myocardial infarction within 30 days after blood culture, ^csequential organ failure assessment, ^dmean arterial pressure (the lowest value during days 0-6), ^eGlasgow coma scale <15, ^fMAP=mean arterial pressure. The lowest value for each patient during the observation period was recorded, ^gmean arterial pressure <70mmHg at least once during the observation period (on day 0, during days 1 to 4, or during days 5 to 6), ^hneeded vasopressives support at least once during the observation period, ⁱ1 to 4 days after the blood culture, ⁱ5 to 6 days after the blood culture, *cannot be calculated, §continuous variable (OR and CI cannot be calculated)

5.4. Patient characteristics and underlying diseases and outcome (Study III)

The effect of patient characteristics and underlying diseases on case fatality in bacteraemia is shown in Table 12. The case fatality rate was 1/30 (3.3%) in patients with nosocomial bacteraemia compared to 18/119 (15.1%) in community-onset bacteraemia, OR 0.193, p=0.084).

Table 12. Case fatality in 149 bacteraemia patients in relation to host characteristics and underlying diseases.

Character	Nonsurvivors N=19 (%)	Survivors N=130 (%)	RR (95% CI)	p-value
Obesity (BMI≥30) ^a	7 (70.0)	20 (19.2)	9.8 (2.3-41.3)	0.002
Smoking ^b	13 (92.9)	53 (43.4)	16.9 (2.1-133.5)	<0.001
Alcohol abuse	7 (36.8)	17 (13.1)	3.9 (1.3-11.2)	0.008
COPD ^c	4 (21.1)	4 (3.1)	8.4 (1.9-37.1)	0.01
Rheumatoid arthritis	3 (15.8)	4 (3.1)	5.9 (1.2-28.8)	0.045
Diabetes mellitus type 1	0	5 (3.8)	*	1
Diabetes mellitus type 2	6 (31.6)	23 (17.7)	2.1 (0.7-6.2)	0.153
Malignancy	3 (15.8)	22 (16.9)	0.9 (0.2-3.4)	0.902
Liver cirrhosis	1 (5.3)	3 (2.3)	2.4 (0.2-23.8)	0.42
McCabe class II or III ^d	3 (15.8)	21 (16.2)	1.0 (0.3-3.6)	1
Corticosteroid use ^e	2 (10.5)	16 (12.3)	0.8 (0.2-4.0)	0.824
Chronic disease ^f	18 (94.7)	99 (76.2)	5.6 (0.7-43.9)	0.065
Male sex	14 (73.7)	65 (50.0)	2.8 (1.0-8.2)	0.053
Age over 60 years	11 (57.9)	67 (51.5)	1.3 (0.5-3.4)	0.604

^adata available on 114 patients

^bcurrent smoking or ex-smoking, data available on 136 patients

^cchronic obstructive pulmonary disease

^dMcCabe class II or III: ultimately fatal or rapidly fatal disease

^ecorticosteroids (prednisone equivalent) used in a dose of over 5 mg per day during one month prior to the episode of bacteraemia

^fat least one chronic condition

*cannot be calculated

5.4.1. BMI

Obesity was a prominent factor associated with case fatality in bacteraemic patients (Table 12 and 13), the median BMI being significantly higher among those who died compared to survivors (33 vs. 26, p=0.003). When causative organisms were analyzed separately, a statistically significant difference in case fatality between obese compared to non-obese subjects was documented in *Str. pneumoniae* bacteraemia (all who died were obese, p=0.002 indicating the difference between obese and non-obese). Obesity was associated with increased risk of death among those admitted to the ICU (5/7 of obese patients died vs. 3/25 of non-obese; p=0.005, RR 18.3; 95% CI 2.4-140.4). Underweight (BMI <18.5) was not associated with the outcome of bacteraemia. Obesity remained a

significant factor associated with case fatality in bacteraemia in a multivariate model adjusted for the effect of smoking, alcohol abuse, age, sex and causative organism (RR 6.4; 95% CI 1.2-34.4). Obese subjects did not differ significantly from non-obese in the number of current smokers (p=0.608), alcohol abusers (p=0.894), male patients (p=0.372) or the number of patients >60 years of age (p=0.578).

Forty-four per cent of obese bacteraemic patients had previously been diagnosed with type 2 diabetes, as against 12.6% of non-obese patients (p<0.001). Patients with type 2 diabetes died more often than those without this disorder, but the difference was not statistically significant. Obesity remained an independent factor associated with case fatality even after adjustment for type 2 (RR 8.9) or type 1 diabetes mellitus (RR 9.3).

In summary, obesity remained an independent factor associated with case fatality in bacteraemic patients when adjusted for the effect of smoking, alcohol abuse, age, sex and causative organism. The median BMI was significantly higher among deceased patients compared to survivors.

Table 13. Clinical characteristics in obese bacteraemia patients compared to non-obese. BMI data available on 114 patients.

	Obese ^a N=27 (%)	Non-obese ^b N=87 (%)	RR (95% CI)	p-value ^c
Died ^d	7 (25.9)	3 (3.4)	9.8 (2.3-41.3)	0.002
Needed ICU ^e stay	7 (25.9)	25 (28.7)	0.9 (0.3-2.3)	0.78
Needed mechanical ventilation	5 (18.5)	7 (8.0)	2.6 (0.8-9.0)	0.152
Lowered CGS ^f	12 (44.4)	29 (33.3)	1.6 (0.7-3.9)	0.293
Hypotension ^g	10 (37.0)	27 (31.0)	1.3 (0.5-3.2)	0.561
High SOFA score (≥4) ^h	11 (44)	19 (23.8)	2.5 (1.0-6.5)	0.05

^aBMI≥30, ^bBMI<30, ^cp-value indicating the difference between obese and non-obese

^dd-30 case fatality, ^eintensive care unit, ^fGlasgow coma scale <15

^gMAP<70mmHg, ^hsequential organ failure assessment score 1-3 days after blood culture, data available on 105 patients

5.4.2. Smoking

Current or ex-smokers died more often of bacteraemia than non-smokers (Table 12 and 14). When causative organisms were analyzed separately, no statistically significant difference in case fatality was documented among smokers compared to non-smokers. However, in *Str. pneumoniae* bacteraemia, all who needed mechanical ventilation (n=9) were current smokers (p-value <0.001, indicating a difference between smokers and non-smokers).

When current smokers (n=38) were compared to non-smokers (ex-smokers excluded from this analysis) the adverse effect of smoking for prognosis of bacteraemia was emphasized. The day-30 case fatality rate among bacteraemic patients was higher in current smokers than in non-smokers

(21.1% vs. 1.4%, $p < 0.001$, RR 18.4; 95% CI 2.2-153.7). This adverse effect remained even after smoking cessation; 5/28 (17.9%) patients died in the ex-smoker group compared to 1/70 (1.4%) of those who had never smoked ($p = 0.007$, RR 15.0; 95% CI 1.7- 135.1). Fifty-one per cent of males were current or ex-smokers as against 36.9% of females ($p = 0.01$). Smokers (current or ex-smoking) did not differ significantly from non-smokers in the number of obese patients ($p = 0.470$) or in the number of patients with diabetes (type 1 or type 2 diabetes) ($p = 0.213$). Smokers (current or ex-smoking) were younger compared to non-smokers (median age 54 years in smokers and 66 years in non-smokers, $p = 0.001$). Twelve per cent of smokers (current or ex-smoking) had been diagnosed with COPD.

Smoking (current or ex-smoking) remained a significant factor associated with case fatality in a multivariate model adjusted for the effect of obesity, alcohol abuse, age, sex and causative organism (RR 23.0; 95% CI 1.7-321.6).

In summary, smoking was an independent factor associated with case fatality in bacteraemia. The adverse effects of smoking on outcome were seen most strikingly in current smokers compared to non-smokers. The adverse effects of smoking persisted even after smoking cessation.

Table 14. Clinical characteristics in bacteraemia patients stratified by smoking data. Smoking data available on 136 patients.

	Smokers ^a N=66	Non-smokers ^b N=70	RR (95% CI)	p-value ^c
Died ^d	13 (19.7)	1 (1.4)	16.9 (2.1-133.5)	<0.001
Needed ICU ^e stay	26 (39.4)	13 (18.6)	2.9 (1.3-6.2)	0.007
Needed mechanical ventilation	14 (21.2)	4 (5.7)	4.4 (1.4-14.3)	0.008
Lowered CGS ^f	29 (43.9)	23 (32.9)	1.6 (0.8-3.2)	0.184
Hypotension ^g	28 (42.4)	20 (28.6)	1.8 (0.9-3.8)	0.091
High SOFA score (≥ 4) ^h	21 (33.3)	10 (16.4)	2.6 (1.1-6.0)	0.029

^acurrent or ex-smokers, ^bhad never smoked, ^cp-value indicating the difference between smokers and non-smokers

^dd-30 case fatality, ^eintensive care unit, ^fCGS= Glasgow coma scale < 15 , ^gMAP < 70 mmHg

^hsequential organ failure assessment score 1-3 days after blood culture, data available on 124 patients

5.4.3. Alcohol abuse

The case fatality rate was higher among alcohol abusers compared to those not given to alcohol abuse (29.2% vs 9.6%, $p = 0.008$, RR 3.9; 95% CI 1.3-11.2). Eighteen out of 21 (85.7%) alcohol abusers were current smokers or ex-smokers and 4 out of 24 (16.7%) had liver cirrhosis. In *Str. pneumoniae* bacteraemia 56% of alcohol abusers died compared to 9% of those with no alcohol abuse ($p = 0.006$). Eighty-eight percent of alcohol abusers were ≤ 60 years of age and 67% were

males. Thirteen per cent of alcohol abusers had been diagnosed with type 1 or type 2 diabetes compared to 24.8% of those with no alcohol abuse ($p=0.188$).

Alcohol abuse did not remain a significant factor associated with case fatality in a multivariate model adjusted for the effect of obesity and smoking, age, sex and causative organism (RR 1.9; 95% CI 0.1-29.5).

5.4.4. Gender and age

Age was not associated with case fatality in the present study (median age 66 years in nonsurvivors and 61 in survivors, $p=0.390$, Mann-Whitney U-test) even after stratification by causative organism (data not shown). Forty-seven patients needed an ICU stay during the bacteraemia episode; age was not associated with case fatality in those patients, who needed ICU treatment (data not shown).

Males needed the ICU significantly more often compared to females (39% vs. 23%, OR 2.2; 95% CI 1.1-4.5, $p=0.032$), but there was no difference in maximum SOFA scores, in the need for mechanical ventilation or in the occurrence of hypotension (data not shown). In a multivariate model, male sex did not remain an independent factor associated with the need for ICU after adjusting for causative organism (data not shown).

5.5. Kynurenine to tryptophan ratio reflecting IDO activity (Study IV)

The maximum kyn/trp ratios detected 1-4 days after blood culture in 132 patients with bacteraemia, stratified by causative organism are shown in Table 15. The maximum ratios were high in acute illness, and decreased on recovery.

The maximum kyn/trp ratio, detected on days 1-4 after blood culture, was significantly higher in those who died (d-30 case fatality) compared to those who survived bacteraemia (median 193.7 $\mu\text{mol}/\text{mmol}$ (quartiles 124.1-253.3 $\mu\text{mol}/\text{mmol}$) vs. 82.4 $\mu\text{mol}/\text{mmol}$ (quartiles 51.0-138.9 $\mu\text{mol}/\text{mmol}$), respectively; $p=0.001$) (Table 16). Interestingly, the median values of the ratios increased from days 1-2 to day 4 in those who died, whereas in those who survived they decreased (Figure 3). A high maximum kyn/trp ratio was associated with all clinical variables indicative of severe disease and poor outcome (Table 17).

Table 15. Maximum kynurenine to tryptophan (kyn/trp) ratio 1-4 days after blood culture in patients with bacteraemia.

Causative organism	kyn/trp ratio $\mu\text{mol}/\text{mmol}$, median (quartiles) ^b
<i>S. aureus</i>	117.3 (64.4-197.5)
<i>Str. pneumoniae</i>	90.9 (52.5-194.0)
β -haemolytic streptococcus	123.0 (69.6-312.3)
<i>E. coli</i>	67.5 (42.3-113.4)
All patients ^a	89.9 (54.7-167.6)

^aon recovery (>26 days after the blood culture) the median kyn/trp ratio was 36.8 $\mu\text{mol}/\text{mmol}$,

^bp-value 0.006 indicates difference between patient groups stratified by culprit organisms (Kruskal-Wallis test))

Table 16. Maximum kynurenine to tryptophan (kyn/trp) ratio and tryptophan and kynurenine levels in patients with bacteraemia in relation to outcome.

Continuous variables, median (quartiles)	Nonsurvivors n=18	Survivors n=118	p-value
Maximum kyn/trp ratio (days 1-4) ($\mu\text{mol}/\text{mmol}$) ^a	193.7 (124.1-253.3)	82.4 (51.0-138.9)	0.001
Minimum tryptophan concentration $\mu\text{mol}/\text{l}$ (days 1-4)	46.5 (38.0-59.7)	51.1 (38.1-64.4)	0.450
Maximum kynurenine concentration $\mu\text{mol}/\text{l}$ (days 1-4)	8.76 (5.15-12.26)	4.27 (2.92-6.72)	<0.001

^athe day of maximum kyn/trp ratio: median tryptophan concentration 43.2 $\mu\text{mol}/\text{l}$ in nonsurvivors and 51.1 $\mu\text{mol}/\text{l}$ in survivors ($p=0.208$) and kynurenine concentration 7.45 $\mu\text{mol}/\text{l}$ in nonsurvivors and 4.29 $\mu\text{mol}/\text{l}$ in survivors ($p=0.001$)

Figure 3. The medians of kynurenine to tryptophan ratios (kyn/trp) ($\mu\text{mol}/\text{mmol}$) on day 1-2, on day 3 and on day 4 after blood culture in nonsurvivors ($n=18$) and in survivors ($n=118$). The difference between nonsurvivors and survivors statistically significant on day 3 (median in nonsurvivors 164.8 $\mu\text{mol}/\text{mmol}$, quartiles 92.7-227.9 and in survivors 88.7 $\mu\text{mol}/\text{mmol}$, quartiles 54.8-130.8, $p=0.014$) and on day 4 (median in nonsurvivors 193.8 $\mu\text{mol}/\text{mmol}$, quartiles 72.7-242.5 and in survivors 66.9 $\mu\text{mol}/\text{mmol}$, quartiles 43.6-110.3, $p=0.003$).

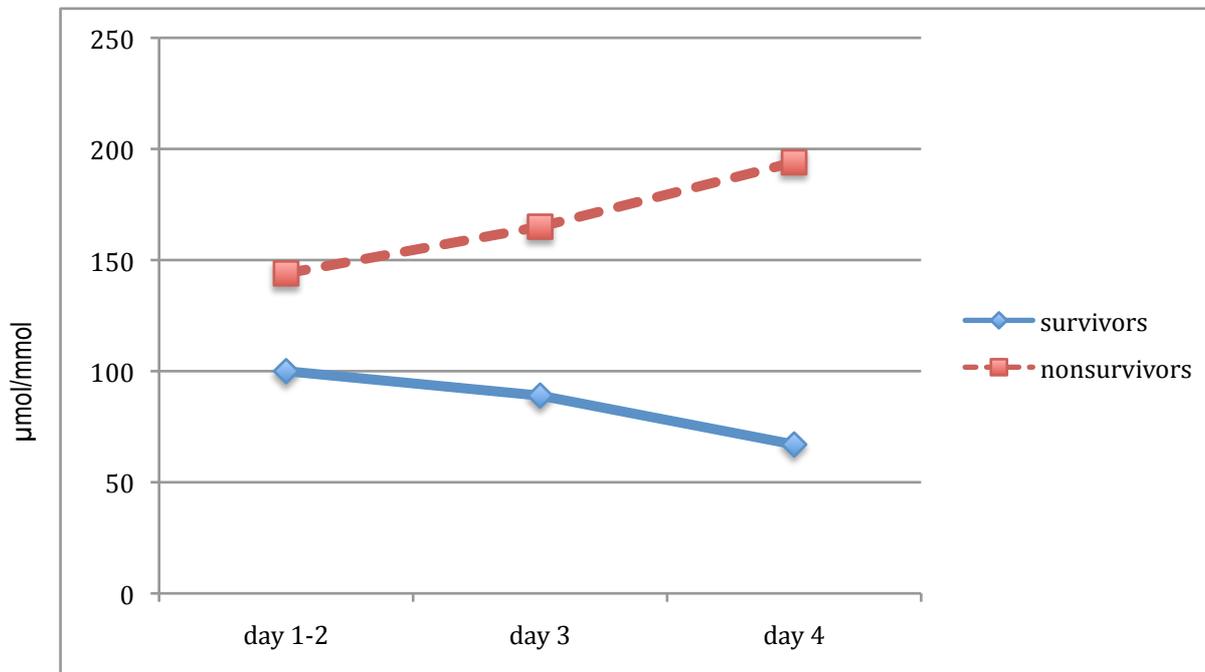


Table 17. Clinical data on 132 bacteremic patients stratified with maximum kyn/trp ratio 1-4 days after blood culture ($\leq 120 \mu\text{mol}/\text{mmol}$ vs. those with $>120 \mu\text{mol}/\text{mmol}$). Categorical data were analysed by chi-square test or Fisher's exact test and continuous data by Mann-Whitney U-test.

Character	maximum kyn/trp ratio $\leq 120 \mu\text{mol}/\text{mmol}$	maximum kyn/trp ratio $>120 \mu\text{mol}/\text{mmol}$	OR (95% CI)	p-value
	n=81	n=51		
Died (d-14 case fatality)	2 (3%)	10 (20%)	9.6 (2.0-46.0)	0.001
Died (d-30 case fatality)	3 (4%)	15 (29%)	10.8 (3.0-39.8)	<0.001
Needed ICU ^a stay	16 (20%)	26 (51%)	4.2 (1.9-9.2)	<0.001
Needed mechanical ventilation	3 (4%)	17 (33%)	13.0 (3.6-47.3)	<0.001
Highest SOFA ^b score ≥ 4	23 (28%)	32 (63%)	4.2 (2.0-8.9)	<0.001
Lowest MAP ^c (mmHg), median (quartiles)	78 (70-93)	63 (54-77)	§	<0.001
Needed vasopressive support	5 (6%)	21 (41%)	10.6 (3.7-30.8)	<0.001
Highest SOFA ^b score, median (quartiles)	1 (0-4)	5 (2-10)	§	<0.001
Highest bilirubin level ($\mu\text{mol}/\text{l}$), median (quartiles)	14 (11-23)	30 (17-60)	§	<0.001
Highest creatinine level ($\mu\text{mol}/\text{l}$), median (quartiles)	90 (71-123)	173 (114-249)	§	<0.001
Lowest platelet count ($\times 10^9/\text{l}$), median (quartiles)	189 (117-243)	108 (65-166)	§	<0.001
Lowest GCS ^d , median (quartiles)	15 (15-15)	14 (13-15)	§	<0.001
Median neutrophil count ($\times 10^9/\text{l}$) (quartiles) ($n=112$)	6.6 (3.8-9.4)	9.3 (6.6-13.3)	§	0.001
Highest CRP (mg/l), median (quartiles)	224 (170-347)	333 (249-405)	§	0.004

^aintensive care unit, ^bsequential organ failure assessment, ^cmean arterial pressure, ^dGCS= Glasgow coma scale, §continuous variable (OR and CI cannot be calculated)

The receiver operating curve (ROC) was used to estimate the optimal cut-off value for maximum kyn/trp ratio in predicting fatal disease. The AUC^{ROC} for maximum kyn/trp ratio was 0.754 (0.639-0.869, p=0.001). The kyn/trp ratio at a cut-off level of 120 µmol/mmol showed a sensitivity of 83% and specificity 69% in detecting fatal disease, and this cut-off point was used to classify patients into those with ≤120 µmol/mmol or >120 µmol/mmol ratio of kyn/trp. The highest CRP level during the bacteraemia episode did not predict case fatality at any cut-off level in the ROC curve (p=0.292). However, the AUC^{ROC} for CRP on the day of blood culture was 0.66 (0.58-0.782, p=0.03). The CRP at a cut-off level of 224 mg/l showed a sensitivity of 72% and specificity 61% in detecting fatal disease, and this cut-off point was used to classify patients into two groups (those with CRP ≤224 mg/l or >224 mg/l in the multivariate model).

Of underlying conditions, alcohol abusers had high kyn/trp ratios (>120 µmol/mmol) more often compared to those not given to alcohol abuse (p=0.017). There was no significant difference in kyn/trp ratios (maximum values ≤120 µmol/mmol or >120 µmol/mmol) between the groups of patients stratified by other underlying conditions or diseases, gender, age, BMI or smoking habits. The association between a high (>120 µmol/mmol) maximum kyn/trp ratio and case fatality in multivariate models adjusted for potential confounders is shown in Table 18. High kyn/trp ratio retained its significance in the multivariate model in all combinations. Obesity and high SOFA score (≥4) also remained independent factors associated with case fatality.

Table 18. Odds ratio for maximum kynurenine to tryptophan (trp/kyn) ratio (>120 µmol/mmol) detected 1-4 days after blood culture in relation to case fatality adjusted for potential confounders in multivariate model.

Variables in model	Odds ratio for high kyn/trp ratio (>120 µmol/mmol)
kyn/trp (>120 µmol/mmol) +	
age and male gender	10.0 (2.7-37.3)
McCabe class II or III	11.3 (3.0-42.0)
CRP >224 mg/l (blood culture day) ^a	9.8 (2.6-37.3)
SOFA score (≥4) ^b	6.6 (1.7-25.6)
obesity (BMI≥30) ^c	7.8 (1.4-42.2)
culprit organism	9.5 (2.5-36.1)
high creatinine (>120 µmol/l)	7.4 (1.8-29.5)
current smoking ^d	10.0 (2.1-48.3)
alcohol abuse	9.7 (2.6-36.1)

^aCRP data available on 124 patients, ^bsequential organ failure assessment score, also remained a significant factor associated with case fatality in multivariate model (p<0.05), ^cBMI data available on 101 patients, also remained a significant factor associated with case fatality in multivariate model, ^dsmoking data available on 120 patients

6. DISCUSSION

6.1. Definitions, study population and the outcome of bacteraemia

The present work was a prospective observational study conducted in Tampere University Hospital in 1999-2004. In study I, a control population was used to determine the risk of bacteraemia related to *MBL2* genotypes.

The overall case fatality (d-30 case fatality) attributable to bacteraemia was 12.8%. In *S. aureus* and *Str. pneumoniae* bacteraemias, case fatality rates (19.5% and 19%, respectively) were in accord with previous studies (Watanakunakorn et al. 1993, Lujan et al. 2004, Lyytikäinen et al. 2005, Laupland et al. 2008b), whereas in bacteraemias caused by *E. coli* and β -haemolytic streptococcae, the case fatality rates (2.3% and 8.7%, respectively) were lower than in other reports (Kuikka et al. 1997, O'Loughlin et al. 2007, Laupland et al. 2008a, Broyles et al. 2009, Rantala et al. 2009a). In this study, the case fatality rate was highest among *S. aureus* bacteraemia patients. *S. aureus* is capable of affecting multiple organs in the body and deep foci due to *S. aureus* bacteraemia are particularly common, resulting in increased case fatality (Mylotte and Tayara 2000). In the present study, all mediastinites and endocardites, infections which carry a marked mortality rate, were caused by *S. aureus*. Since the present study was not population-based, it is possible that some patient groups, e.g. very old patients with multiple comorbidities and severe sepsis had been treated in primary care wards without recruitment to the present study conducted in a university hospital. This may lower the identified case fatality rate in *E. coli* bacteraemia. The number of patients with β -haemolytic streptococcus bacteraemia was low, which may affect the results. Median ages in the respective groups of patients with different culprit organisms did not differ from each other.

Since the patients were recruited for the study according to positive blood culture, which only became positive one day after the blood culture was taken, not all patients were evaluated for respiratory rate on the day of blood culture (on diagnosis day, i.e. day 0). Evaluation of the respiratory rate is one out of the four contributors in the definition of sepsis by the ACCP/SCCM Consensus Conference Committee (Bone et al. 1992). The diagnostic criteria for SIRS are overly sensitive and unspecific (Levy et al. 2003) and in 2001, the International Sepsis Definitions Conference concluded that an expanded list of signs and symptoms of sepsis might better reflect the

clinical response to infection. The definition of SIRS was, however, left unchanged (Levy et al. 2003). According to these guidelines, sepsis is defined as SIRS plus documented infection. The guidelines for the SIRS definition (Bone et al. 1992), strictly taken, do not allow regarding the currently studied population as sepsis patients. However, all patients in the study presented with either fever or hypothermia, elevated CRP and leukocytosis or leukopenia. Given the high prevalence of patients with at least mild organ dysfunction (78%), as assessed by a SOFA score >0 , and with severe organ dysfunction (39%) assessed by SOFA score ≥ 4 , it is highly probable that nearly all patients would eventually fulfil the official definition of sepsis (Bone et al. 1992) at some time-point in their disease. On the other hand, whereas most observational sepsis studies are prone to bias due to the unspecificity of the SIRS criteria in differentiating infectious from non-infectious (e.g. vasculitis) causes of inflammation (Levy et al. 2003), and a definite microbiological diagnosis cannot be made in one third or more of patients with clinical manifestations of sepsis (Sands et al. 1997, Vincent et al. 2006), this problem was overcome in the present cohort by the inclusion of verified bloodstream infections. All patients in the present study had a documented infection, all causative organisms were typical pathogens and in all cases the responsible physician prescribed intravenous antibiotics.

One strength of the present study was the enrolment of patients evincing different disease severity; patients with milder symptoms and signs as well as those with septic shock who needed ICU treatment. The study included prospectively gathered detailed clinical data, among them SOFA scores, blood pressure data, Glasgow coma scales and laboratory markers. The inclusion of four different pathogens provided the opportunity to compare outcomes related to genetic and environmental factors in different bacteraemias. On the other hand, it allowed of comparison of these factors also in relation to susceptibility to bacteraemia. However, the inclusion of bacteraemias caused by multiple culprit organisms might also be considered a limitation, as different causative agents increase the infection source- and comorbidity-related heterogeneity of the study population, limiting likewise the sizes of separate subgroups of patients. There were altogether 1756 bacteraemias caused by *S. aureus*, *Str. pneumoniae*, β -haemolytic streptococcus and *E. coli* in the Pirkanmaa HD during the period in question (children and patients treated in other hospitals in the HD are also included in this figure) (unpublished data, oral communication with clinical microbiologist Risto Vuento). Thus, one limitation was that not all patients with bacteraemia in the university hospital district during the study period could be enrolled. Although the incidence of methicillin-resistant *S. aureus* (MRSA) is currently increasing in Finland, during the study period the antimicrobial resistance rates of the organisms in question were low in Pirkanmaa and all pathogens proved susceptible to the first antibiotic selected on admission.

Bacteraemic patients constitute one fraction of all sepsis patients, so that the present material does not represent the whole group of sepsis patients. For example, blood culture-negative sepsis patients were not included in this study. In previous studies, the presence of bacteraemia has been shown to be associated with poor outcome in patients with documented sepsis (Bone et al. 1989, Brun-Buisson et al. 1995). However, patients who had received antibiotics prior to the blood culture may have been missing in the present study since the antimicrobial treatment given may have potentially affected the blood culture findings in some bacteraemic patients. Furthermore, this study represents patients with four specified bacteraemias, and the results may thus not be applicable to all bacteraemias.

The present study was not designed to evaluate the efficacy of different therapeutic strategies in regard to bacteraemia outcome. Many therapeutic interventions have been widely acknowledged as life-saving in sepsis (Rivers et al. 2001, Dellinger et al. 2004). The international Surviving Sepsis Campaign (SSC) published severe sepsis guidelines in 2004 (Dellinger et al. 2004), this comprising an international effort to increase awareness and improve outcome in severe sepsis. These treatment guidelines, based on studies showing that relatively simple therapeutic interventions such as ventilation with low tidal volumes (Dellinger et al. 2004), made it possible to save a significant number of lives. The importance of early appropriate empirical therapy for outcome has been documented in several studies (MacArthur et al. 2004, Ferrer et al. 2008). One recent study showed that the above-mentioned evidence-based sepsis therapies were not used in Finland as often as recommended (Karlsson et al. 2007). The possibility cannot be eliminated that advancing treatment strategies in sepsis (for example rhAPC and the adoption of early goal-directed therapy) would have affected individual outcomes, which would constitute a confounder in the present studies.

Finally, there remain factors impossible to control in clinical studies in general; patients with sepsis typically differ with respect to the inciting organism and its virulence, the focus of infection, and also with respect to optimal approaches in antibiotic selection and surgical source control. Studies have shown that the failure to diagnose and appropriately treat patients with surgical drainage is a common avoidable error encountered in everyday clinical practice (Blosser et al. 1998, Torgersen et al. 2009) and prompt and adequate antibiotic therapy is the cornerstone of survival (Garnacho-Montero et al. 2006).

6.2. Genetic variation and the risk and outcome of bacteraemia

6.2.1. *MBL2* genotype

In Study I, *MBL2* structural O allele carriers who smoked had a strikingly increased risk of gram-positive bacteraemia compared to O allele-carrying controls. The same was not detected in carriers of the AA genotype.

Smoking has previously been shown to predispose to pneumococcal bacteraemia (Nuorti et al. 2000). However, no previous work has studied the possible role of MBL deficiency, a defect in innate immunity, in conjunction with smoking in the context of susceptibility to infection. The effect of the *MBL2* genotype on infection susceptibility and the outcome of infectious diseases, although fairly intensively studied to date, has yielded controversial results in different studies (Summerfield et al. 1997, Hibberd et al. 1999, Kronborg et al. 2002, Roy et al. 2002, Sutherland et al. 2005, Smithson et al. 2007). In several reports, only the homozygous variant genotype has been clearly associated with the risk of bacteraemia (Hibberd et al. 1999, Roy et al. 2002, Sutherland et al. 2005), though totally negative results have also been published (Kronborg et al. 2002, Smithson et al. 2007). In accord with the present findings, abundant data show that MBL deficiency increases the risk of infections in certain specific groups such as in infants (Koch et al. 2001) and in children in general (Summerfield et al. 1997, Hibberd et al. 1999, Fidler et al. 2004), in patients with cancer (Mullighan et al. 2002, Horiuchi et al. 2005, Vekemans et al. 2007), in patients with some other defect in immunity (Garred et al. 1995) and in critically ill patients (Garred et al. 2003, Fidler et al. 2004, Sutherland et al. 2005, Gordon et al. 2006). In non-selected patient populations, the effect is less apparent (Dahl et al. 2004), suggesting that the phenotypic expression of MBL deficiency is favoured by concomitant inherited or acquired risk factors (Casanova and Abel 2004), e.g. smoking in the present study. In contrast to the present findings, a few studies have indicated that *MBL2* variants may also predispose to infections also in healthy young people (Roy et al. 2002, Rantala et al. 2008). In the present study, only a few patients had severe underlying comorbidities, e.g. malignancies, which significantly affect host immunity. This limits conclusions as to the effects of MBL deficiency in cancer patients with bacteraemia in the present cohort.

MBL deficient genotypes did not affect survival here. Previous studies have yielded conflicting results also regarding *MBL2* polymorphisms and case fatality in sepsis/infection (Kronborg et al. 2002, Garred et al. 2003, Gordon et al. 2006). The majority of previous works have focused on the effect of *MBL2* polymorphisms on mortality and outcome in patients with severe septic infection (Garred et al. 2003, Fidler et al. 2004, Sutherland et al. 2005, Gordon et al. 2006). Studies

focusing on the clinical course of bacteraemia in patients with varying disease severity, as the present study, are few in number (Kronborg et al. 2002, Eisen et al. 2006, Smithson et al. 2007). In accord with the present findings, a large population-based prospective study of >9000 participants in an ethnically homogeneous Caucasian population, produced no evidence for significant differences in mortality in MBL-deficient individuals versus controls (Dahl et al. 2004). In contrast, Eisen and associates (2008) reanalyzed individual data from 5 adult studies and 1 paediatric study of MBL and severe bacterial infection, and showed that the risk of death was increased among MBL-deficient patients with severe pneumococcal infection. The genetic heterogeneity of the populations studied, different study protocols, differences in definitions of MBL deficiency, and in the characterization of underlying conditions and way of living constitute a likely explanation for the considerable variability between studies. Current knowledge regarding the relationship between genetic variation and diseases may also be subject to publication bias (Pereira et al. 2007). There is marked diversity in *MBL2* genotype frequencies between different ethnic groups (Ivanova et al. 2008). In the present study, all patients were Caucasians and the genotype frequencies were in Hardy-Weinberg equilibrium. Furthermore, the genotype frequencies in both promoter and exon 1 regions were very similar to those in other studies conducted in the same ethnic group (Rantala et al. 2008).

The risk of bacteraemia in *MBL2* O allele carriers was most prominent in the case of bacteraemia caused by *Str. pneumoniae*, an agent implicated for respiratory tract infections in particular. MBL, a member of the collectin family, is present in the upper airways and buccal cavity secretions, where it may protect against respiratory infections (Hickling et al. 2004). MBL insufficiency causing opsonization deficiency (Super et al. 1989), together with smoking, may have deleterious effects on immunity, thus increasing the risk of gram-positive bacteraemia and the risk of *Str. pneumoniae* bacteraemia in particular. Although *Str. pneumoniae* has been shown to bind MBL rather weakly, in clinical studies (Roy et al. 2002) MBL insufficiency has been associated with the risk of pneumococcal bacteraemia, probably due to its route of infection via the lungs. Interestingly, in most studies showing a positive association between MBL genotypes representing insufficiency and predisposition to bacteraemia caused by a specified agent, the culprit organism in question is encapsulated, which is the case in *Neisseria meningitidis* (Hibberd et al. 1999) and *Str. pneumoniae* (Roy et al. 2002) bacteria. The mechanism underlying this phenomenon is unclear.

The exact mechanism by which smoking contributes to increased infection risk has not been fully established. Smoking has substantial effects on the immune system, affecting both innate and adaptive immunity (Sopori 2002). The findings presented here suggest that the effects of smoking on immunity may, at least in part, be genetically determined. The interplay between genes and

environmental factors, smoking in particular, has been a subject of several other studies. As an example, a gene-smoking interaction on the risk of acquiring rheumatoid arthritis has been found (Pedersen et al. 2007). Gene-environmental interactions between smoking and the predisposition to infectious diseases have previously been described in bacterial vaginosis (Ryckman et al. 2009).

A limitation in the present study was that the control population was not originally chosen for this study. However, smoking habits were very similar in this control group, when compared to corresponding results of the population-based Health 2000 Survey (THL 2007). The lack of MBL serum level measurements in conjunction with MBL genotype analysis could be considered another limitation in the present study. The number of patients homozygous for structural variant allele (OO, n=6) was small. This thus limits conclusions on the effects of structural variant homozygosis on the susceptibility to or outcome of bacteraemia.

In summary, human MBL insufficiency probably does not confer a Mendelian susceptibility to bacteraemia, but it is likely that its phenotypic expression is favoured by concomitant inherited or acquired risk factors such as smoking. Although smoking was more common in males than females here, the finding was consistent in both genders. Furthermore, the effects of smoking on immunity may, at least in part, be genetically determined.

6.2.2. *eNOS G894T polymorphism (Glu298Asp)*

The findings in Study II show that the *eNOS* Glu298Asp polymorphism (T allele) is associated with hypotension in patients with *E. coli* bacteraemia but not in those with bacteraemia caused by a gram-positive organism. The effect on hypotension was most evident in the early stage of the disease. Patients with the variant infected with a gram-positive organism were not at increased risk of hypotension.

In previous studies, the *eNOS* Glu298Asp polymorphism has been suggested to play a role in the development of hypertension, coronary artery spasms and acute myocardial infection (Hibi et al. 1998, Miyamoto et al. 1998, Yoshimura et al. 1998), although controversial data exist (Karvonen et al. 2002, Casas et al. 2006). No previous study has investigated this polymorphism in bacteraemia or sepsis. The phenotypic expression of the T allele in sepsis is unclear, and in non-septic state this polymorphism has been associated with reduced vasodilatation (Godfrey et al. 2007) and NO secretion (Veldman et al. 2002). The *eNOS* Glu298Asp variation has been shown to have biochemical consequences in the human endothelium, for example altered caveolar localization of eNOS and impaired response to shear stress (Joshi et al. 2007).

Previous studies have shown that various ethnic groups differ from each other in terms of the genotype distributions and allele frequencies of the *eNOS* gene. For example, the Finnish population differs from the Japanese, the T allele being less frequent in the latter (Hibi et al. 1998, Karvonen et al. 2002). In the present study, the frequencies of the T and G alleles in the whole cohort were fairly similar as compared to a control population (randomly selected from the national health register) in another Finnish study (23% vs 29% and 77% vs 71%, respectively) (Karvonen et al. 2002).

The balance of eNOS and iNOS during sepsis has not been fully established. Transgenic mice overexpressing eNOS in endothelial cells have been shown to be resistant to LPS-induced hypotension, although iNOS-mediated NO production seemed not to be altered in transgenic compared to control mice (Yamashita et al. 2000). In contrast, recent findings indicate an essential pro-inflammatory role for eNOS; macrophages from eNOS-knockout mice evince reduced NF- κ B activity, iNOS expression and NO production after LPS exposure, compared with wild-type mice (Connelly et al. 2005).

NO may act as a double-edged sword during septic shock. The main contributor implicated for septic shock is cytokine-induced iNOS, whereas eNOS is considered to be physiological, releasing small amounts of NO to maintain tissue flow in vital organs. NO production facilitates host defence by its cytostatic and cytotoxic effects (Nathan 1992, MacMicking et al. 1997) and the nonselectivity of NOS inhibitors may lead to toxic effects of inappropriate eNOS inhibition, as shown in experimental studies (Assreuy 2006). Even though T allele carriage was associated here with hypotension in *E.coli* bacteraemia, T allele carriage did not affect survival. This may also reflect the quality of care and the adoption of evidence-based early goal-directed strategies like fluid resuscitation (Dellinger et al. 2004). On the other hand, all patients with *E.coli* bacteraemia and early hypotension recovered, indicating that pathophysiological mechanisms other than hypotension *per se* may be involved in fatal cases.

The effect of the structural T allele on hypotension was most evident in the early stage of the disease and the effect on blood pressure was no longer documented, starting from day 5 after the blood culture was taken. This makes for a biological rationale, as the effects of NO depend on the rate, timing and spatial distribution of NO production as well as the chemical microenvironment (Fang 1997, Feihl et al. 2001). Furthermore, it has been shown that depending on concentration and particular circumstances, NO may exert either pro- or anti-inflammatory effects (Feihl et al. 2001).

The reason why the T allele was associated with hypotension specifically in *E.coli* bacteraemia in this study remains obscure. Furthermore, the effect of the T allele on disease severity in *E. coli* and in gram-positive bacteraemia here were in contrast to each other. Bacterial toxins such as

superantigens and endotoxin are commonly implicated in the pathogenesis of gram-positive and gram-negative sepsis, respectively, yet they signal via widely disparate cellular mechanisms and induce markedly different inflammatory cascades (Hotchkiss and Karl 2003, Carlet et al. 2008). Gram-negative and gram-positive bacteria differ in binding patterns to TLRs (Uematsu and Akira 2006) and the signalling pathways leading to iNOS induction have been shown to differ in *S. aureus* and *E. coli* infections (Paul-Clark et al. 2006). In experimental models, *E.coli* or LPS have been shown to induce the release of NO more rapidly compared to *S. aureus* (Paul-Clark et al. 2006).

In the present study, carriage of the T allele in patients with *E. coli* bacteraemia was weakly associated with an increased risk of acute myocardial infarction. This might be explained as a consequence of reduced basal eNOS-derived NO secretion as a result of T allele carriage. In accord with this finding, T allele carriage has previously been linked to acute myocardial infarction (Hibi et al. 1998), coronary artery spasms (Yoshimura et al. 1998) and CHD (Casas et al. 2006). It has already been noted that sepsis *per se* leads to endothelial dysfunction, which, in part, may be related to reduced *eNOS* expression and activity. In such conditions, further inhibition/dysfunction of eNOS can exacerbate endothelial dysfunction and further impair microvascular homeostasis (Thiemermann 1997).

Some limitations must be conceded here. The studied subgroups of bacteraemia patients being small, with a limited number of clinical endpoints, no more than a few possible confounders could be added in the multiple regression model as covariates evaluating the independent role of the T allele in relation to hypotension in *E. coli* bacteraemia. Furthermore, there is of course a possibility that the observed associations could be attributable to another co-inherited variant in linkage disequilibrium with the *eNOS* Glu298Asp SNP. The present study, like genetic association studies in general, needs replication (Ioannidis et al. 2001).

In summary, *eNOS* Glu298Asp polymorphism (T allele) was associated with hypotension in patients with *E. coli* bacteraemia but not in bacteraemia caused by a gram-positive organism. The effect on hypotension was most evident in the early stage of the disease. The results may indicate a pivotal role for the *eNOS* gene in the regulation of haemodynamic responses during the course of bacteraemic infection.

6.3. Host characteristics and underlying diseases and bacteraemia outcome

6.3.1. Obesity

Obesity emerged as an independent factor associated with case fatality in bacteraemia patients, BMI being significantly higher in deceased patients compared to survivors.

Only a few studies have systematically evaluated the impact of obesity on the outcome of sepsis or infections in general (Falagas et al. 2009). BMI data are seldom reported in most cohort and population-based studies addressing the outcome of infections, as such studies are usually retrospective by design. In accord with the present findings, there is a study among pneumonia patients (Bochicchio et al. 2004) and one among cellulitis patients (Carratala et al. 2003) showing an association between obesity and increased case fatality in infection. In one prospective study of 182 patients with pneumonia the mortality rate was higher in obese compared to non-obese subjects (Bochicchio et al. 2004) and in a retrospective study of over 300 patients with community-acquired cellulitis, morbid obesity was likewise associated with an increased mortality rate (Carratala et al. 2003). In contrast with the present findings, a prospective study of over 800 ICU patients presenting with different kinds of infections found no significant association between high BMI and increased mortality rate (Smith et al. 2007). Similarly in a small prospective study of patients with intra-abdominal sepsis, obesity was not associated with case fatality (Kalfarentzos et al. 1987).

In contrast with the present findings, the results from two recent meta-analyses show no increased mortality among obese ICU patients and obesity was even found to be protective compared to some other BMI subgroups (Akinnusi et al. 2008, Hogue et al. 2009). However, duration of hospital stay and mechanical ventilation were adversely affected by obesity (Akinnusi et al. 2008, Hogue et al. 2009). Both of these meta-analyses included studies with multiple reasons for ICU admission, and infections or sepsis data were insufficiently reported in most of them. Furthermore, most were retrospective by design.

It remains obscure which factors contribute to the increased case fatality in obese bacteraemia patients in the present study. There are several possible mechanisms. Previous laboratory observations have shown that inflammatory responses are dys-regulated in patients with obesity (Marti et al. 2001). Obesity may also cause increased microvascular injury and activation of prothrombogenic factors upon exposure to infectious stimuli (Scott et al. 2004, Vachharajani et al. 2005, Amar et al. 2007). Moreover, the accumulation of adipose tissue has been related to a higher degree of oxidative stress and platelet activation (Davi et al. 2002). Obesity is known to affect the

respiratory system adversely by reason of mechanical factors as well as the metabolic effects of the excess adipose tissue (McClellan et al. 2008). In fact, the effect of obesity on case fatality was most evident in *Str. pneumoniae* bacteraemia, an agent causing infections involving the upper respiratory tract and lungs in particular. It could thus be assumed that obese patients may have diminished respiratory reserve function to cope with pulmonary infections compared with normal-weight subjects (Falagas et al. 2009). Obesity may adversely affect the outcome of skin and soft tissue infections, since normal adipose tissue has a relatively low blood supply (Rosell and Belfrage 1979) and the blood supply of skin and soft tissues may become impaired in the case of obesity by reason of microvascular changes related to the metabolic effects of obesity (Falagas et al. 2009). Furthermore, an impaired blood supply may be a cause for suboptimal recruitment of host immune mechanisms and consequently, delayed wound healing (Utley et al. 1989, Falagas et al. 2009).

Comorbid conditions related to obesity may be responsible, at least partly, for poorer outcome in patients with bacteraemia. A link to glucose metabolism is one possible mechanism, as type 2 diabetes is a common comorbidity in obese individuals and hyperglycaemia is common in acutely ill patients. Diabetes mellitus is a cause of impaired cellular immune functions, including chemotaxis, phagocytosis and the bactericidal ability of neutrophils and monocytes/macrophages (Geerlings and Hoepelman 1999). Diabetic patients have been shown to have an adverse prognosis in the case of some infectious diseases (Falguera et al. 2005). The occurrence of hyperglycaemia, in particular severe hyperglycaemia, has been shown to be associated with increased morbidity and mortality in a variety of groups of patients (Krinsley 2003, Gale et al. 2007). Over forty per cent of obese patients in the present study had previously been diagnosed with diabetes mellitus. Furthermore, the burden of type 2 diabetes in the general population has been shown to be far more greater than the number of patients with diagnosed diabetes, this applying equally to the present population (Thomas et al. 2005). In a multivariate model, obesity remained an independent predictor of death, even after adjusting for diagnosed type 2 diabetes mellitus. The outcome of obese patients may be adversely influenced because knowledge of the pathophysiology and optimal treatment of obese critically ill patients, especially those with sepsis, is limited. Also limited are relevant data regarding the dosing of most antimicrobials in obesity (Pai and Bearden 2007).

The usefulness of BMI in critically ill patients has been debated in the medical literature, some authors requiring adjustment of patient weights for fluid balances (Goulenok et al. 2004, Schultz and Spronk 2005), since the most severely ill patients frequently need more fluids (especially when saline solutions are used for fluid therapy) which raises the total measured body weight and thus the BMI. However, in the present study the BMIs were based on weight and height as reported by the

patient on admission. Thus, fluid therapy possibly given is an unlikely confounder in the present case.

Low BMI (<18.5) was not associated with the outcome of bacteraemia in the present cohort. However, as there were only 3 underweight patients, no conclusions can be drawn regarding underweight and outcome. It cannot be ruled out that obesity might also have predisposed the present cohort to bacteraemia. However, the proportion of obese patients here (23.7%) was very similar to the prevalence of obesity in the Finnish population (23.5% in men, and 28.0% in women) (Saaristo et al. 2008).

In summary, obesity comprised an independent factor associated with case fatality in bacteraemia in the present study. There are several possible pathophysiological mechanisms underlying the presented findings, which warrant further studies.

6.3.2. Smoking

The findings here showed smoking to be independently associated with case fatality in patients with bacteraemia. The effect on case fatality was most distinct when current smokers were compared to non-smokers, but it also remained significant after smoking cessation.

In previous studies, it has not been adequately evaluated whether the predisposition to an infection in smokers may also translate into a poorer prognosis in patients who become infected. Data on smoking habits are seldom reported in most population-based studies addressing the outcome of infections, and only a few studies have systematically included smoking habits in their multivariate models in seeking factors significantly associated with case fatality in infections.

In accord with the present findings, one retrospective cohort study of 136 patients with liver transplantation (Leithead et al. 2008), and one population-based study of patients with invasive pneumococcal disease (Laurichesse et al. 2001), found smoking to be independently associated with increased infection-related mortality. In contrast with the present findings, a prospective study of nosocomial bacteraemia patients showed smoking not to be associated with case fatality (Arvanitidou et al. 2005) and in a study conducted among septic ICU patients, smoking had no independent effect on mortality (Pittet et al. 1993). In a meta-analysis of 122 studies on factors associated with community-acquired pneumonia outcome, smoking data were available in 8 of the works included (Fine et al. 1996), and smoking was not associated with poor outcome (Fine et al. 1996). One recent retrospective study has even shown reduced mortality among active smokers compared to non-smokers in community-acquired pneumonia (Garau et al. 2008).

The mechanisms whereby smoking affects the outcome in bacteraemic patients remain unclear. It has previously been shown that smoking has substantial effects on the immune system, affecting both innate and adaptive immunity (Sopori 2002). One limitation in the present study, as in others reporting the effects of smoking in infectious diseases (Arvanitidou et al. 2005, Garau et al. 2008, Leithead et al. 2008), was that the quantity of cigarettes smoked per day and smoking duration (years) were not documented. This limits dose-dependent considerations on the effects of smoking. However, an advantage here was that smokers were classified as current smokers, ex-smokers and non-smokers, which enabled evaluation of the significance of smoking cessation on bacteraemia outcome.

In summary, the findings here showed smoking to be independently associated with case fatality in patients with bacteraemia. The effect of smoking on case fatality remained significant in the multivariate model. The present data highlight the significant adverse effects of smoking on bacteraemia outcome.

6.3.3. Other underlying conditions, age and sex

Alcoholism did not remain an independent factor associated with case fatality in the multivariate model where smoking was included. The most likely explanation for this is that most alcohol abusers were also smokers, and the effect on case fatality may thus result from smoking, not alcohol itself.

Alcohol abuse has adverse effects on the immune system (Pavia et al. 2004, Moss 2005). Alcoholism has been shown to constitute a risk factor for sepsis, severe sepsis and hospital mortality (O'Brien et al. 2007), and for increased severity of community-acquired pneumonia (Ruiz et al. 1999, de Roux et al. 2006). There are studies reporting an association between alcoholism and increased case fatality in pneumococcal bacteraemia (Klemets et al. 2008b), *S. aureus* bacteraemia (Kaech et al. 2006) and in β -haemolytic streptococcus bacteraemia (Rantala et al. 2009b). In a study conducted by Moss and associates (2003), chronic alcohol abuse was a risk factor for acute respiratory distress syndrome and increased the severity of nonpulmonary organ dysfunction in patients with septic shock. However, of the above-mentioned studies, multivariate models where smoking was included as a covariate was performed only in the last-mentioned (Moss et al. 2003) and there are also studies where no independent association between alcoholism and case fatality in bacteraemia has been shown (Watakunakorn et al. 1993, Lääveri et al. 1996, Laupland et al. 2004). There is also considerable heterogeneity between different studies in defining alcohol abuse and no uniform approach has been implemented (Aalto et al. 2009). The methods of data collection vary

between studies, i.e. whether patient surveys or questionnaires, register-based data, prospective, or retrospective data registration were used to characterize alcohol dependence. In the present study, alcohol abuse was defined as patient inquiry-based consumption of ≥ 300 g absolute alcohol per week or a known social or medical problem due to alcohol use. This definition excludes the evaluation of patients with moderate consumption; according to the definition used in here, all patients who were defined as alcohol abusers were thus clearly heavy drinkers (Sillanaukee et al. 1992).

Population-based studies constitute a large body of literature on the effect of various underlying diseases on the bacteraemia outcome (Brun-Buisson et al. 1995, Angus et al. 2001, Alberti et al. 2003). In contrast with these, malignancies, ultimately or rapidly fatal underlying diseases or liver cirrhosis did not prove to be significant risk factors associated with outcome in the present study. This may be due to the small numbers of patients with the above-mentioned conditions.

In contrast to several other studies (Angus et al. 2001, Kaech et al. 2006, Klemets et al. 2008b), advanced age was not a risk factor for case fatality here. Since this was not a population-based study, the oldest patients with severe underlying conditions and severe sepsis were probably missed, having been treated in primary health centre wards without admission to the university hospital. Hence the present study was not designed to consider the effect of age on the outcome of patients.

Several studies suggest that men are more susceptible to infections (Klein 2000), and once an infection occurs, are more likely to die (Schroder et al. 1998, Angus et al. 2001). In the present study, the genders did not differ from each other in numbers of deceased patients.

In summary, alcoholism, other underlying conditions, age or sex did not have an independent effect on bacteraemia outcome.

6.4. IDO activity as a predictor of outcome in bacteraemia

The present study showed that the overexpression of IDO activity independently predicted severe disease and case fatality in patients with bacteraemia.

This series was amongst the first to indicate decreased survival in patients with increased IDO activity in bacterial infections or sepsis. In accord with the present findings, recently acquired new data indicate that the degradation of tryptophan may be associated with the development of sepsis and poor outcome after major trauma (Pellegrin et al. 2005, Logters et al. 2009, Ploder et al. 2009). One small study, again, was suggestive of an association between highly increased tryptophan degradation and the severity of *Streptococcus pyogenes* infection (Murr et al. 2001).

Kynurenine to tryptophan ratios reflecting IDO activity in patients with bacteraemia were over three times higher than in healthy Finnish blood donors (Pertovaara et al. 2006), reflecting increased IDO activity in bacteraemic patients. In accord with the present findings, immune cells, including dendritic cells (DCs), macrophages and eosinophils have been shown upon infectious stimuli to induce IDO enzyme expression following Th1-type cytokine interferon- γ (INF- γ)-mediated stimulation (Mellor and Munn 2004). IDO, the rate-limiting enzyme for tryptophan catabolism, has long been known to be operative in antimicrobial defence (Taylor and Feng 1991, Mellor and Munn 1999). Although some microbial organisms have been shown to be sensitive to the tryptophan-depleting activity of IDO in vitro (Pfefferkorn 1984, Gupta et al. 1994), its biological efficacy in controlling infections in vivo has remained unclear (Mellor and Munn 2004). IDO is a natural immunoregulatory mechanism and suppresses T-cell proliferation (Hwu et al. 2000). However, its function in the immune system appears to diverge depending on the type of immune cell and the nature of the stimulus involved (Mellor and Munn 2004) and it is not clear whether IDO is beneficial or detrimental to the host.

IDO activities varied significantly here between different causative organisms and focuses. Although the cellular mechanisms underlying the increased activity of IDO in bacteraemic patients remain unclear, this again reflects the fundamental differences in pathogenesis between the different pathogens, and the particular microorganism undoubtedly plays a particularly important role. Bacterial toxins such as superantigens and endotoxin are commonly implicated in the pathogenesis of gram-positive and gram-negative sepsis, respectively, yet they signal via disparate cellular mechanisms and induce markedly different inflammatory patterns (Hotchkiss and Karl 2003, Bhavsar et al. 2007, Carlet et al. 2008). Furthermore, different organisms cause infections in different loci and the organ in which infection takes place is of paramount importance. For example, it is known that immune mechanisms in the lung and in the peritoneum are markedly dissimilar, leading to differential effects of therapeutic compounds aimed at manipulating the same inflammatory system (Bagby et al. 1991, Carlet et al. 2008). Interestingly, IDO activities here increased markedly in nonsurvivors during the first days after blood culture, whereas in survivors IDO activities decreased, but the significance of and contributors to this finding are unclear.

The findings presented here do not amount to a causal relationship between high IDO activity and case fatality in bacteraemia. One possible mechanism underlying high IDO activity in conjunction with poor outcome could lie in the effects of IDO on T-cell-mediated immunity. Currently, deregulated apoptotic immune-cell death has been suspected to play a major part in immune dysfunction and mortality in sepsis (Hotchkiss et al. 1999, Hotchkiss and Karl 2003, Hotchkiss and Nicholson 2006). Caspase-inhibitors are currently being investigated, as potential

anti-sepsis strategies and their mechanism rely on inhibition of the dysregulated apoptosis of immune cells (Hotchkiss and Nicholson 2006). Interestingly, the IDO enzyme, in addition to its role as a suppressor of T-cell function, induces apoptosis, and is induced by apoptosis (Lee et al. 2002). This latter study showed that T cells became susceptible to death via apoptosis in the absence of free tryptophan, in part through Fas-receptor-mediated signalling, and this was reversed by caspase inhibition. The first evidence supporting the conception of causal effects of IDO in sepsis came from a recent study in mice showing that survival from endotoxin shock was enhanced in IDO-knockout (IDO^{-/-}) and in IDO inhibitor 1-methyl-tryptophan-treated mice compared with wild-type mice (Jung et al. 2009). IDO is encoded by the gene INDO located in chromosome 8. The regulation of INDO transcription is complex and cell-type-specific, and the complete mechanisms are not well established (Mellor and Munn 2004). Recent studies indicate that infection-induced IDO activity may also be genetically determined by the expression of the transforming growth factor beta 1 (TGFB1) gene and cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (Raitala et al. 2007). It remains to be established, whether genetic variation may regulate IDO expression and disease presentation in different clinical entities such as sepsis.

The absence of measurements of IDO on the day of blood culture limits direct comparisons between CRP and IDO. There were generally multiple measurements available for each patient (median 4 per patient) on separate measurement days. This reduces the possibility of bias compared to single measurement protocols and enables evaluation of IDO activity changes during the course of disease. Of separate time-points, on days 3 and 4 IDO activity was significantly higher in non-survivors vs. survivors. IDO activity is commonly measured by determination of the kyn/trp ratio (Schrocksadel et al. 2006) (i.e. by determination of the first metabolite to substrate). Determination of the IDO messenger ribonucleic acid (mRNA) of the cells is another possible method to study IDO activity. However, IDO mRNA determination would have required a rapid and timely analysis of study samples, live cells, and would not have been possible to perform from stored study samples.

In summary, the overexpression of IDO was strongly associated with poor outcome in patients with bacteraemia. When high IDO activity was studied in a multivariate model adjusting for potential confounders, it was found that high IDO activity was an independent predictor of death in bacteraemia.

6.5. Future considerations

The present study showed a novel gene-environment interaction between the *MBL2* structural variant allele and smoking. Future work should be targeted to confirm these findings in population-based studies, and the molecular mechanisms of smoking in the context of immunity should be investigated in detail.

The present findings showed that genes regulating vascular tone may be involved in the pathogenesis of septic hypotension. Large, population-based case-control studies on genes involved in the regulation of vascular tone in relation to infection outcome should be undertaken. A haplotype analysis-based approach and genotyping of multiple SNPs simultaneously may be of value.

The mechanisms underlying the excess case fatality in obese bacteraemia patients reported here remain unclear. Obesity-related comorbidities (e.g., insulin resistance), dys-regulated immunomechanisms, impaired respiratory function, altered antimicrobial pharmacokinetics and pharmacodynamics and obesity-related microvascular changes may be amongst the key elements in the adverse events related to sepsis. Obesity-related factors might complicate acute illness and impede the implementation and/or efficacy of evidence-based interventions. The adoption of evidence-based treatment options for obese individuals may be haphazard, as there is a paucity of information regarding the pathophysiology and treatment of obese critically ill sepsis patients. Thus, the impact of obesity-related mechanisms on infection outcome should be studied in large, population-based prospective studies in different infections.

Cigarette smoking is a worldwide epidemic, and constitutes one of the main preventable causes of death and disability. The present study showed an independent association between smoking and adverse outcome in bacteraemia. The target populations for preventive efforts, for example recommended vaccination populations, should be vigorously studied in order to characterize those likely to derive greatest potential benefit. A recent national population-based study showed that a high proportion (46%) of those who died of invasive pneumococcal disease during the years 1995-2002 did not have a pneumococcal vaccine indication (Klemets et al. 2008b). These findings highlight the need for large, prospective studies investigating the effects of smoking on the outcome of bacteraemia. Studies on the effects of alcohol abuse on infection outcome should be adjusted for smoking habits, and the dose-dependent effects of smoking should be characterized by inclusion of detailed inquiry into smoking habits routinely in study protocols in this area.

The prognostic potential of IDO measurement in sepsis is of paramount interest for the future, as more than 100 distinct molecules have been proposed as useful biological markers of sepsis but

only few are used in routine diagnostics. The utility of a biomarker is a function of the degree to which it adds value to the available clinical information in the domains of screening, diagnosis, risk stratification and monitoring of response to therapeutic strategies (Marshall and Reinhart 2009). The fact that IDO overexpression has been documented in various diseases may limit its clinical applications and specificity in the diagnosis of sepsis. Furthermore, to date measurement of tryptophan and kynurenine concentrations in peripheral blood has been challenging, requiring an HPLC method. The measurement of IDO enzymatic activity needs further studies in sepsis, in other infectious diseases, in critical illness, in all-cause hospital patients and in the healthy population. Assessment of the accuracy of IDO activity in differentiating bacterial infections from noninfective causes of inflammation and differentiating bacterial infections from viral infections calls likewise for further effort.

Manipulating immune responses to improve patient outcomes is an important challenge in a range of inflammatory diseases, including cancer and infectious and autoimmune disorders (Mellor and Munn 2004). In cancer therapy, studies support the suitability of the IDO inhibitor D-1-methyl-tryptophan (1-MT) for human trials aiming to assess the utility of IDO inhibition in blocking host-mediated immunosuppression and enhancing antitumour immunity (Hou et al. 2007). Currently, 1-MT is in phase 1 testing for solid tumours, and a new potent competitive IDO inhibitor has recently been developed (Yue et al. 2009). The potential of IDO inhibition in sepsis therapy warrants further studies. Possible caveats and limitations include its documented beneficial functions as a natural immunoregulatory mechanism. For example, Popov and associates (2006) have shown that in human listeriosis dendritic cells expressing IDO, together with macrophages, are major cellular components of suppurative granulomas *in vivo*, and that repression of IDO might result in exacerbation of granulomatous diseases. Furthermore, IDO is essential to normal, full-term gestation (Munn et al. 1998), and scavenging IDO in these circumstances would be detrimental to the host and the foetus.

For decades, the pathogenesis of sepsis has been viewed as involving excessive inflammation, suggesting that down-regulation of immunity could be beneficial (Thomas 1972, Llewelyn and Cohen 2002, Sriskandan and Altmann 2008). More recently an alternative view is suggested by the finding that impaired monocyte activation and consequent immune deviation is associated with a poor clinical outcome (Hotchkiss and Karl 2003, Remick 2007, Sriskandan and Altmann 2008). Clinical trials on sepsis are indicative of a complex process and lack agreement as to whether we should seek to suppress immunity, boost it, or do both at different time-points (Remick 2007, Sriskandan and Altmann 2008). The studies on cytokine polymorphisms (Table 3) have not firmly established whether these polymorphisms really constitute a risk of susceptibility to or outcome of

infections. To date, the syndromes of sepsis, severe sepsis and septic shock are defined by nonspecific alterations in physiology rather than by specific cellular processes representing potential therapeutic targets. Thus, the question whether IDO constitutes a link between lymphocyte apoptosis, immunosuppression and excess mortality in sepsis, is a matter for further research. The determination of IDO enzymatic activity in peripheral blood mononuclear cells (PBMC) from septic patients would present a valuable continuum to the studies presented here and the signalling pathways leading to IDO induction, IDO-related down-stream mechanisms, and the determination of IDO overexpression-related cytokine profiles in human sepsis urge to further efforts. Defining the possible contribution of IDO in sepsis pathophysiology could enable evaluation of the possible utility of modifying the tryptophan degradation pathway for therapeutic purposes.

7. SUMMARY AND CONCLUSIONS

The influence of host genetic factors on the susceptibility to and clinical picture of bacteraemia, IDO activity as a predictor of case fatality in bacteraemia, and the influence of host underlying factors on the outcome of bacteraemia can be summarized as follows:

I Smoking proved a significant risk factor for gram-positive bacteraemia in carriers of the *MBL2* structural O allele. The same was not detected in carriers of the AA genotype. However, carriage of the *MBL2* variant genotype had no independent effect on disease severity or mortality.

II The findings showed that the *eNOS* Glu298Asp polymorphism (T allele) was associated with hypotension in patients with *E. coli* bacteraemia but not in bacteraemia caused by a gram-positive organism such as *S. aureus*, *Str. pneumoniae*, or β -haemolytic streptococcae. The effect on hypotension was most evident in the early stage of the disease. The T allele did not affect case fatality in any of the four pathogens studied.

III Obesity and smoking proved to be independently associated with case fatality in bacteraemia. The effect of smoking was most distinct when current smokers were compared to non-smokers, but it also remained significant after smoking cessation.

IV The findings showed that a high kynurenine to tryptophan ratio reflecting IDO activity independently predicted case fatality in patients with bacteraemia. High IDO activity was strongly associated with several variables indicative of severe disease.

In conclusion, *MBL2* structural region O allele carriers who smoked were at markedly increased risk of gram-positive bacteraemia as compared to control subjects, this representing a novel interaction between genes and environment. The effects of smoking on immunity may thus, at least in part, be genetically determined. Future population-based studies should be targeted to investigate the mechanisms underlying these findings.

The T allele at nucleotide position 894, a common variant of the *eNOS* gene, is associated with hypotension in the early stage of disease in patients with *E. coli* bacteraemia but not in bacteraemia caused by a gram-positive organism. The observations in this context emphasize that in devising therapeutic interventions, not only organism-related differences in pathogenesis should be considered but also differences in patients' genetic background of modifying the outcome of bacteraemia and sepsis. The phenotypic expression of T allele carriage in different sepsis models and in population-based approaches should be a subject of future researches.

The results here indicate that obesity and smoking constitute important factors associated with case fatality among bacteraemic patients. With the rising prevalence of obesity in Western countries, future research should focus on the search for mechanisms responsible for the increased mortality in obese bacteraemic patients. The adverse effect of smoking on bacteraemia outcome is an underestimated health risk, and its effects on immunity and infection outcome should be considered in future population-based studies.

Since high IDO activity proved an independent risk factor for case fatality in bacteraemia, measurement of it may provide new insights into sepsis pathophysiology. Further studies are needed to establish whether IDO has an independent role in the pathogenesis of sepsis, and whether interference in tryptophan degradation pathway by specific therapy would prove beneficial to the host. Recent studies indicate that immunosuppression is a major factor implicated in excess mortality in sepsis. Further studies are needed to investigate whether IDO may represent a link between T-cell suppression, immunosuppression and death in bacteraemic and septic patients.

A novel pharmaceutical strategy, rhAPC, raised great hopes at the beginning of the decade, but the beneficial effects of even this agent on survival were not uniform in all subgroups of patients, reflecting the fact that sepsis comprises a heterogeneous group of syndromes. Identification of the risk factors underlying fatal outcome in bacteraemia will allow targeting of preventive and therapeutic efforts on individuals likely to derive greatest potential benefit, and the present findings provide novel tools for the PIRO approach introduced in personalized medicine in the beginning of the ongoing decade.

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Gene–environment Interaction between MBL2 Genotype and Smoking, and the Risk of Gram–positive Bacteraemia

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Abstract

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Mannose-binding lectin (MBL) insufficiency caused by point mutations in the MBL2 gene has been associated with increased susceptibility to bacteraemic infections. We here investigated the effect of MBL2 polymorphisms on the susceptibility and clinical course of bacteraemia. The study cohort comprised 145 patients with bacteraemia and 400 controls. In the case of patients with bacteraemia, laboratory findings and clinical data were registered on admission and during six consecutive days. MBL2 structural polymorphisms at codons 52 (CGT → TGT; designated D or O), 54 (GGC → GAC; B or O) and 57 (GGA → GAA; C or O) in exon 1 of the MBL2 gene and promoter region polymorphisms at position –221 (G → C, designated Y or X alleles) were determined. No difference in MBL2 genotype frequencies between the bacteraemic patients and controls was detected, and MBL2 genotype had no independent effect on mortality, nor disease severity. However, smoking proved a significant risk factor for Gram-positive (*Staphylococcus aureus*, *Streptococcus pneumoniae* or β -haemolytic streptococci) bacteraemia in patients carrying the variant O allele (53% current smokers in Gram-positive bacteraemia patients compared with only 21% in controls, odds ratios 4.2, 95% confidence intervals 2.0–9.0; $P < 0.001$), while it did not have an effect in those homozygous for the A allele. The same effect was not detected in *Escherichia coli* bacteraemia. In conclusion, MBL2 genotypes representing MBL insufficiency were not associated with the overall risk of bacteraemia or disease severity, but smoking in carriers of the structural variant O allele may have a deleterious effect increasing the risk of Gram-positive bacteraemia.

Introduction

Mannose-binding lectin (MBL) is a serum acute-phase reactant secreted by the liver. It activates the complement system by binding to carbohydrate structures presented by micro-organisms and is thus considered an important component in the innate immune defence system [1, 2]. MBL is encoded by the MBL2 gene on chromosome 10. MBL insufficiency is caused by polymorphisms in codons 52 (CGT-TGT, designated D, traditionally any of the variants have also been given the generic designation of O allele), 54 (GGC-GAC; B or O) and 57 (GGA-GAA, C or O) in exon 1 of that gene leading to amino acid substitutions Arg-Cys, Gly-Asp and Gly-Glu in the

peptide, which interfere with the encoded protein, thereby lowering the circulating levels of MBL, compromising ligand binding and reducing complement activation. Furthermore, the MBL concentration is highly dependent upon promoter region polymorphisms, of which that at position –221 (nucleotide change G-C; alleles Y-X respectively) is clinically the most important.

Mannose-binding lectin insufficiency caused by MBL2 gene polymorphisms has been associated with increased susceptibility to infections in both children and adults [3–6]. It has been shown to be particularly important in the presence of another immune deficiency, as in haematological patients [5], while in non-selected patient populations its role in relation to infections has not been fully

established [7–9]. MBL insufficiency is associated with an increased risk of sepsis, severe sepsis and septic shock in critically ill patients [10–13]. The majority of studies have focused on the effect of MBL2 polymorphisms on mortality and outcome in patients with severe septic infection. Studies concerning the clinical course of bacteraemia with varying disease severity are few in number [8, 14, 15].

We sought here to elucidate the role of MBL2 gene polymorphisms together with other clinical factors and comorbidities affecting immunity in the risk of bacteraemia and the outcome of patients with bacteraemia.

Materials and methods

The study material comprised 145 patients with bacteraemia (77 male and 68 female) aged 59 years (mean, range 16–93) hospitalized in Tampere University Hospital, Tampere, Finland, from June 1999 to February 2004. MBL2 genotype frequencies in these patients were compared with those in a control group of 400 adult persons (151 male and 249 female) aged 60 years (mean, range 31–89), initially recruited from the normal population for an asthma study, in whom only asthma and chronic obstructive pulmonary disease had been excluded [16].

In our hospital blood cultures are routinely taken from patients with symptoms or signs of systemic infection [fever or hypothermia, tachycardia or tachypnoea combined with leucocytosis or leucopenia and/or elevated C-reactive protein (CRP)]. During the period in question the BACTEC 9240 blood culture system (BD Diagnostic systems, Sparks, MD, USA) with standard media was used. Patients were identified according to the microbiological blood culture finding and those with bacteraemia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -haemolytic streptococci (β -hml. str.) or *Escherichia coli*, the most common causative organisms among community-acquired bacteraemia, were only included in the study. According to the study plan, other microbes were excluded beforehand. Bacteraemia was defined as community-acquired on admission ($n = 119$, 82%), or hospital-acquired (nosocomial) if a positive blood culture was drawn >48 h after admission ($n = 26$, 18%). After being informed by the clinical microbiologist (R.V.) the clinicians (J.S. and J.L.) asked patients to participate and interviewed and examined those consenting. Information was gathered from hospital records at hospital visits and hospital records were also reviewed after hospitalization (R.H.). Altogether 149 out of 152 patients agreed to participate. MBL2 genetic polymorphism testing was carried out in 97.3% (145/149) of the bacteraemia patients and 400 controls.

The recruitment of the patients and the clinical data collection are described in detail elsewhere [17]. Underlying conditions, alcohol and tobacco consumption were

registered. Alcohol abuse was defined as consumption of 300 g absolute alcohol per week or a known social or medical problem due to alcohol use (data available on 145 patients). Smoking habits were registered, and patients were defined as current smokers, ex-smokers, i.e. those who had stopped smoking and non-smokers, i.e. those who had never smoked (data available on 132 patients). Smoking data were available in 400 controls. Calculation of body mass index (BMI) was based on weight and height as reported by the patient on admission (data available on 111 patients).

The causative Gram-positive organisms were *S. aureus* (40 patients, 27.6%), *Str. pneumoniae* (42 patients, 29%), β -hml. str. (23 patients, 15.9%) and Gram-negative *E. coli* (40 patients, 27.6%). All patients were treated with an empiric antibiotic regimen, and where necessary, antimicrobial treatment was changed according to culture results.

Patients were closely monitored during hospitalization and severely ill subjects were transferred to the ICU. Clinical data were registered on admission and during six consecutive days. The possible need for mechanical ventilation was registered. Alterations in mental status were evaluated on the Glasgow Coma Scale (GCS). A patient was classified hypotensive if the mean arterial pressure (MAP) was <70 mmHg during the bacteraemia episode.

Sequential organ failure assessment (SOFA) score [18] was calculated during the first 6 days after positive blood culture. The highest SOFA score for every patient was used in analysis. Severe organ failure was documented if the highest SOFA score was ≥ 4 . Laboratory findings were registered on admission and during six consecutive days. The laboratory tests included plasma CRP (mg/l), neutrophil count ($10^9/l$), blood platelets ($\times 10^9/l$), plasma bilirubin ($\mu\text{mol/l}$) and plasma creatinine level ($\mu\text{mol/l}$). The case fatality rate was studied within 30 days after the positive culture (day 30 case fatality). The overall case fatality among these patients was 13%.

MBL2 genotyping. Exon 1 of the MBL2 structural gene was amplified by polymerase chain reaction [19]. Genotyping of codon 52 (CGT \rightarrow TGT; designated D, traditionally any of the variants have also been given the generic designation of O allele), 54 (GGC \rightarrow GAC; B or O) and 57 (GGA \rightarrow GAA; C or O) polymorphisms was performed by sequencing. Structural alleles lacking these single nucleotide polymorphisms were classified as wild-type (AA). For genotyping of promoter region polymorphism at position -221 bp (G \rightarrow C, designated Y or X alleles respectively) commercially available fluorogenic allele-specific TaqMan probes and primers were used (rs7096206; Applied Biosystems, Foster City, CA, USA). Genotyping was performed by means of the 5' nuclease assay for allelic discrimination using the ABI Prism 7000 sequence detection system (Applied Biosystems).

Statistical analysis. The SPSS package (version 7.5) was used for statistical analyses and a two-sided *P*-value <0.05 was taken as the level for statistical significance. Categorical data were analysed by chi-squared test or Fisher's exact test when appropriate. Nonparametric data were analysed by Mann–Whitney *U*-test. In a binary logistic regression we analysed the interaction between MBL genotype and current smoking, in relation to the risk of bacteraemia in a model adjusted by age and sex, as the patient and control groups were not matched by age and sex. We also performed binary logistic regression, where severity of bacteraemia (SOFA score ≥ 4) or need for ICU treatment were dependent factors, and MBL genotype, current smoking, interaction between MBL genotype and current smoking, age and sex were independent factors. Odds ratios (ORs) were expressed with their 95% confidence intervals (CI).

Results

The distribution of MBL2 genotypes in patients with bacteraemia and in controls is shown in Table 1. Genotype frequencies in controls were in Hardy–Weinberg equilibrium. The sources of bacteraemia are shown in Table 2. No significant difference in MBL2 genotype frequencies was detected between the patients with bacteraemia and controls, nor between the different bacteria groups, nor when Gram-positive or Gram-negative bacteraemia patients and controls were compared. Furthermore, no difference between genders was detected.

Table 1 MBL2 promoter and structural genotypes in patients with bacteraemia and controls.

Genotype	Bacteraemia (<i>n</i> = 145)	Controls (<i>n</i> = 400)
<i>Structural</i>		
A/A	91 (63)	248 (62)
A/O	48 (33)	136 (34)
A/B	29 (20)	96 (24)
A/C	4 (3)	8 (2)
A/D	15 (10)	32 (8)
O/O ^a	6 (4)	16 (4)
<i>P</i> -value (χ^2 -test)	0.788 ^b and 0.980 ^c	
<i>Promoter</i>		
Y/Y	93 (64)	264 (66)
Y/X	44 (30)	116 (29)
X/X	8 (6)	20 (5)
<i>P</i> -value (χ^2 -test)	0.915	

^aVariant with regard to both structural alleles: three bacteraemia patients with B/B, two with B/D and one with D/D genotype, and five controls with B/B, eight with B/D, one with D/D, one with B/C and one with C/D genotype.

^bThe groups between which the *P*-value was calculated in bacteraemia patients versus controls (A/A, A/B, A/C, A/D, O/O).

^cThe groups between which the *P*-value was calculated in bacteraemia patients versus controls (A/A, A/O, O/O).

Values within parenthesis are expressed in percentage.

Table 2 Source of bacteraemia.

Focus	All, <i>n</i> = 145 ^a (%)	<i>E. coli</i> , <i>n</i> = 40 ^a	<i>S. aureus</i> , <i>n</i> = 40 ^a	<i>Str. pneumoniae</i> , <i>n</i> = 42 ^a	β -hml. str, <i>n</i> = 23 ^a
Pneumonia	39 (27)	0	2	35	2
Skin	37 (26)	0	19	2	16
Urinary	30 (21)	28	1	0	1
Osteomyelitis/ spondylitis	14 (10)	0	11	0	3
Gall bladder	7 (5)	4	0	3	0
Endocarditis	6 (4)	0	6	0	0
Gynaecological	3 (2)	1	0	0	2
Mediastinitis	4 (3)	0	4	0	0
Meningitis	3 (2)	0	1	1	1
Intravenous/ intra-arterial catheter related	3 (2)	0	3	0	0
Arthritis	6 (4)	0	5	0	1
Source unknown	15 (10)	5	3	4	3

^aOne patient may have several focuses.

Values within parenthesis are expressed in percentage.

Predisposing factors and underlying diseases of patients with bacteraemia in relation to MBL2 structural genotype are shown in Table 3. Associations between MBL2 promoter genotype and these predisposing parameters were not detected (data not shown). Bacteraemic patients who carried the structural O allele were more often current smokers than those homozygous for wild-type allele [20/50 (40%) versus 17/82 (21%), OR 2.5, 95% CI 1.2–5.5; *P* = 0.017] (Table 2). The effect was even more pronounced among patients with bacteraemia caused by a Gram-positive organism [19/36 (53%) versus 14/59 (24%), OR 3.6, 95% CI 1.5–8.7; *P* = 0.004] (Table 2), but it was not seen in those with bacteraemia caused by a Gram-negative rod (*E. coli*, *P* = 1.0). Patients with Gram-positive bacteraemia who were O-allele carriers were significantly more often current smokers (53%), compared also to controls carrying the O allele (21%) (Table 4). The risk of Gram-positive bacteraemia in O-allele carriers who smoked was 4.2-fold (Table 4). The risk was most prominent in those suffering from pneumococcal bacteraemia [11/18 (61%) versus 32/152 (21%), OR 5.9, 95% CI 2.1–16.4; *P* < 0.001]. However, no significant difference in smoking was observed between the patients with wild-type genotype (AA) suffering from Gram-positive bacteraemia or pneumococcal bacteraemia, compared with controls who were carrying wild-type (AA) genotype (*P* = 0.328 and 0.119 respectively), nor within the control group between O-allele carriers and non-carriers (*P* = 0.474). Despite the fact that smoking was more common among men in both bacteraemic and control groups, an effect was detected between smoking and Gram-positive bacteremia in O-allele carriers in both genders (Table 4). When the

Table 3 Predisposing factors and underlying diseases of bacteraemia in relation to MBL2 structural genotype.

	Structural genotype, <i>n</i> (%)		<i>P</i> -value	OR (95% CI)
	AA (<i>n</i> = 91)	AO or OO (<i>n</i> = 54)		
Malignancy	11 (12)	11 (20)	0.179	1.9 (0.7–4.6)
Solid malignancy	7 (8)	8 (15)	0.173	2.1 (0.7–6.1)
Haematological malignancy	5 (6)	3 (6)	1.0	1.0 (0.2–4.4)
Obesity (BMI ≥ 30) ^a	20 (30)	7 (16)	0.075	0.4 (0.2–1.1)
Rheumatoid arthritis	7 (8)	0	0.046	– ^f
Alcohol abuse	13 (14)	11 (20)	0.341	1.5 (0.6–3.7)
Current smoker ^b	17 (21)	20 (40)	0.017	2.5 (1.2–5.5)
Non-smoker ^{b,c}	50 (61)	17 (34)	0.003	0.3 (0.2–0.7)
Chronic disease ^d	69 (76)	44 (82)	0.427	1.4 (0.6–3.2)
McCabe II or III ^e	16 (18)	5 (9)	0.169	0.5 (0.2–1.4)
Liver cirrhosis	2 (2)	2 (4)	0.629	1.7 (0.2–12.5)
Age >60 years	49 (54)	28 (52)	0.816	0.9 (0.5–1.8)
Diabetes (type 1 or 2)	21 (23)	13 (24)	0.891	1.1 (0.5–2.3)
COPD	5 (6)	3 (6)	1.0	1.0 (0.2–4.4)
Male/female	49 (54)/42 (46)	28 (52)/26 (48)	0.816	0.9 (0.5–1.8)

BMI, body mass index; COPD, chronic obstructive pulmonary disease; CI, confidence intervals; OR, odds ratios.

^aData available on 111 patients.

^bData available on 132 patients.

^cThose who have never smoked.

^dAt least one chronic disease.

^eMcCabe class II or III: ultimately or rapidly fatal disease.

^fCannot be calculated.

Table 4 Current smokers (%) with Gram-positive bacteraemia (*Staphylococcus aureus*, *Streptococcus pneumoniae* or β -haemolytic streptococci) and controls in relation to MBL2 gene structural genotype.

Structural genotype	All			Male			Female		
	Controls (<i>n</i> = 400)	Bacteraemia (<i>n</i> = 95)	OR (95% CI)	Controls (<i>n</i> = 151)	Bacteraemia (<i>n</i> = 59)	OR (95% CI)	Controls (<i>n</i> = 249)	Bacteraemia (<i>n</i> = 36)	OR (95% CI)
AO or OO	<i>n</i> = 152	<i>n</i> = 36		<i>n</i> = 61	<i>n</i> = 19		<i>n</i> = 91	<i>n</i> = 17	
Current smokers (%)	32 (21)	19 (53)	4.2 (2.0–9.0)	14 (23)	11 (58)	4.6 (1.6–13.7)	18 (20)	8 (47)	3.6 (1.2–10.6)
AA	<i>n</i> = 248	<i>n</i> = 59		<i>n</i> = 90	<i>n</i> = 40		<i>n</i> = 158	<i>n</i> = 19	
Current smokers (%)	45 (18)	14 (24)	1.4 (0.7–2.8)	22 (24)	10 (25)	1.0 (0.4–2.4)	23 (15)	4 (21)	1.6 (0.5–5.1)

CI, confidence intervals; OR, odds ratios.

interaction between MBL genotype and current smoking in relation to the risk of Gram-positive bacteraemia was studied in a binary logistic regression adjusted by age and sex, the finding retained its significance (OR 3.2, 95% CI 1.1–9.0; *P* = 0.032) (Table 4).

Mannose-binding lectin 2 genotype had no effect on mortality. The effect of MBL2 genotype on the clinical course of patients with bacteraemia is shown in Table 5. Carriage of MBL2 structural O allele seemed to be associated with an increased need for ICU treatment (OR 3.0, 95% CI 1.2–7.9; *P* = 0.022), high SOFA score (*P* = 0.006), lowered MAP (*P* = 0.011), lowered platelet count (*P* = 0.016) and elevated creatinine level (*P* = 0.009) in

males only. Univariate model indicated no other significant interactions between O allele and the underlying conditions listed in Table 2 (data not shown) in association with the severity of disease. However, we analysed the effect of MBL genotype, current smoking, interaction between MBL genotype and current smoking, age and sex in relation to the severity of disease (SOFA ≥4) or need for ICU treatment in a binary logistic regression model. O allele or interaction between O allele and smoking did not remain as a significant risk factor for disease severity in this model, while smoking did (OR 4.4, 95% CI 1.3–15.1). O allele, or interaction between O allele and smoking, did not remain as a significant risk

Table 5 Clinical data on patients with bacteraemia in relation to MBL2 structural genotype.

	Structural		OR (95% CI)	P-value
	All (n = 145)			
	AA (n = 91)	AO or OO (n = 54)		
Died ^a (%)	12 (13)	7 (13)	1.0 (0.4–2.7)	0.969
Needed ICU stay (%)	24 (26)	22 (41)	1.9 (0.9–3.9)	0.072*
Highest CRP (mg/l), median (quartiles)	270 (186–352)	285 (188–414)		0.647
Neutrophil count (10 ⁹ /l), median (quartiles) ^b	7.9 (4.4–11.5)	7.4 (4.0–11.5)		0.697
Highest SOFA score, median (quartiles)	2 (0–5)	3 (1–5)		0.082*
Needed mechanical ventilation (%)	12 (13)	10 (19)	1.5 (0.6–3.7)	0.387
Lowest platelet count (10 ⁹ /l), median (quartiles)	168 (104–239)	137 (77–203)		0.159*
Highest bilirubin level (μmol/l), median (quartiles)	18 (12–33)	18 (14–34)		0.587
Lowest GCS, median (quartiles)	15 (14–15)	15 (14–15)		0.537
Lowest MAP (mmHg), median (quartiles) ^c	77 (64–90)	74 (61–83)		0.292*
Highest creatinine level (μmol/l), median (quartiles)	98 (75–161)	139 (79–226)		0.026*

CRP, C-reactive protein; SOFA, sequential organ failure assessment; GCS, Glasgow Coma Scale; MAP, mean arterial pressure.

^aDeath due to bacteraemia episode occurred within 30 days from the day of positive blood culture.

^bData available on 123 patients.

^cThe lowest MAP = [(systolic + 2 × diastolic blood pressure)/3] (median).

*Statistically significant difference in males (χ^2 -test, $P < 0.05$).

Clinical data and laboratory findings were registered on admission and during six consecutive days.

factor for ICU treatment, either, while smoking and male sex remained so (OR 3.9, 95% CI 1.0–14.8 and OR 3.5, 95% CI 1.4–8.4 respectively). MBL2 promoter region polymorphism did not have an effect on the clinical course of the disease.

Discussion

In this study, carriage of MBL2 variant genotype had no independent effect on disease severity or mortality. Furthermore, MBL insufficiency was not connected with the overall risk of bacteraemia. However, MBL2 structural O-allele carriers who smoked had over a fourfold risk of Gram-positive bacteraemia compared with O allele carrying controls. This was not detected in carriers of the AA genotype. O allele carriage in conjunction with smoking was not associated with the risk of *E. coli* bacteraemia.

In several studies, only the homozygous variant genotype has been clearly associated with the risk of bacteraemia [4, 10, 20, 21], although totally negative results have also been published [8, 15]. The association between MBL insufficiency and the risk of bacteraemia is clearer in patients with a more severe course of the disease, e.g. in ICU patients or in patients with comorbidities, e.g. haematological malignancies affecting immunity [5, 22] and in these patients the insufficiency is associated with the development of sepsis, septic shock and severe inflammatory response syndrome [10–13] and even with fatal outcome [12]. In our study, MBL insufficiency was not associated with the overall risk of bacteraemia, which is a somewhat controversial finding as regards some earlier reports [14, 21]. In most of the earlier studies patients

had been recruited from ICU units [10–13], which may induce a selection bias. In our study, in turn, all patients with varying disease severity – patients with milder symptoms and signs, as well as patients who needed ICU treatment – were enrolled. Furthermore, in our study only a few patients had severe underlying comorbidities, e.g. malignancies, affecting significantly on host immunity. Longitudinal follow-up studies will be needed to avoid a possible selection bias when evaluating the effect of MBL insufficiency on the overall risk and outcome of bacteraemia.

There was a significant interaction between MBL2 genotype and current smoking in relation to the risk of Gram-positive bacteraemia, when studied in a binary logistic regression adjusted by age and sex. The finding was consistent in both genders, which is noteworthy because smoking was more common among males than females. The risk was most prominent to bacteraemia caused by *Str. pneumoniae*, an agent causing infections involving the upper respiratory tract and lungs. There are no previous studies in this field reporting similar findings regarding the possible gene–environment interaction between MBL2 genotype and smoking as presented here. MBL, a member of the collectin family, is the most abundant in blood but also present in the upper airways and buccal cavity secretions where it may protect against respiratory infections [23]. Smoking has substantial effects on the immune system, affecting both innate and adaptive immunity [24], and it has been reported as a risk factor for pneumococcal bacteraemia [25]. MBL insufficiency causing opsonization deficiency [26], together with smoking, may have deleterious effects on

immunity, thus increasing the risk of Gram-positive bacteraemia and the risk of *Str. pneumoniae* bacteraemia particularly. Finally, the binding capacity of MBL to different organisms has been shown to be varying [27]. This might cause differences between susceptibilities to infections caused by Gram-positive and Gram-negative bacteria in MBL insufficiency. All bacteria studied bind MBL with various binding capacity [28]. *Staphylococcus aureus*, and some β -hml. str. exhibit strong binding of MBL, while MBL binding pattern to *E. coli* is intermediate [27, 29]. *Streptococcus pneumoniae*, in turn, binds MBL rather weakly, but in clinical studies [21] MBL insufficiency is clearly associated with the risk of pneumococcal bacteraemia, probably due to its infection route via the lungs.

There are certain limitations to our study protocol. The control population was not originally chosen for this study as it was initially recruited from the normal population for an asthma study, in whom only asthma and chronic obstructive pulmonary disease had been excluded [16]. This might mean differences in smoking habits compared with the normal population without such exclusion criteria. However, the prevalence of current smoking was relatively similar in both males and females in this control group, when compared with the respective results of the population-based Health 2000 Survey, a health interview/examination survey of 8028 persons aged 30 or over, carried out in Finland (24% versus 29% in males and 17% versus 18% in females) (<http://www.ktl.fi/terveys2000/index.uk.html>).

There was no demonstrable influence of MBL2 genotype on case fatality or disease severity in this study. Although male patients with bacteraemia carrying the O allele seemed to have a greater severity of disease in univariate analysis, the independent role of O allele carriage was not maintained in multivariate analysis adjusted by age, sex and smoking habits. This might have been due to the fact that smoking was more common in males than in females, and the effect on disease severity might thus have resulted from smoking, not male gender in conjunction with MBL2 genotype itself [17]. Previous studies have produced conflicting results regarding MBL2 polymorphisms and case fatality in sepsis/infection [8, 12, 13]. Indeed, there is a paper indicating that heterozygosity of MBL2 genotypes predicts an advantage (heterosis) in relation to fatal outcome in intensive care patients [30]. It should also be noted that modern intensive care has reduced the mortality rate in sepsis, even if patients are critically ill.

In conclusion, MBL2 genotypes representing MBL insufficiency had no effect on the overall risk of bacteraemia nor on mortality in this study. Thus, the determination of MBL genotypes did not prove to be a useful surrogate marker in assessing the risk or prognosis of bacteraemia in unspecified patient populations. However, MBL2 structural region O-allele carriers who smoked had

over a fourfold risk of Gram-positive bacteraemia compared with control subjects. Our study should be followed up by larger prospective studies in order to confirm these findings and explore the possible gene-environment interactions suggested by our findings.

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Competing interests

The author(s) declare that they have no competing interests.

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Ethics

The study was approved by the Ethics Committee of Tampere University Hospital. Written informed consent was obtained from patients or first-degree relatives.

Authors' contributions

All authors planned and carried out the conception and design of the study. J.L., J.S. and R.H. were involved in patient care and acquisition of data. R.V. was responsible for blood culture interpretation. MBL2 genotyping was carried out by J.A., M.H., C.E., J.K. and A.T.R. All authors were responsible for interpretation of the data. R.H. wrote the first draft of the manuscript and all authors participated in its revision. All authors made an intellectual contribution and all read and approved the final manuscript.

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ENDOTHELIAL NITRIC OXIDE SYNTHASE G894T (GLU298ASP) POLYMORPHISM IS ASSOCIATED WITH HYPOTENSION IN PATIENTS WITH *E. COLI* BACTEREMIA BUT NOT IN BACTEREMIA CAUSED BY A GRAM-POSITIVE ORGANISM

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ABSTRACT—Nitric oxide (NO) as a vasoactive substance is a crucial element in the pathogenesis of sepsis. Endothelial NO synthase (eNOS) is, in turn, a key regulator of vascular NO production. The eNOS gene polymorphism at position 894 (G>T, Glu298Asp) resulting in T allele has been studied in the context of vascular diseases, but its role in sepsis has not yet been explored. We here studied the effect of eNOS Glu298Asp polymorphism on the clinical course of the disease in patients with bacteremia. The study comprised 147 patients with bacteremia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -hemolytic streptococci, or *Escherichia coli*. Laboratory findings and clinical data were registered on admission and during 6 consecutive days. The polymorphism of eNOS gene, G894T, was genotyped. Carriage of the T allele was associated with low MAP ($P = 0.004$) and high Sequential Organ Failure Assessment score ($P = 0.001$) in patients with *E. coli* bacteremia. The effect on blood pressure was most prominent in the early stage of the disease (MAP on admission = 52 mmHg in T-allele carriers vs. 91 mmHg in noncarriers; $P < 0.001$). However, the same was not detected in bacteremia caused by a gram-positive organism (*S. aureus*, *S. pneumoniae*, or β -hemolytic streptococci). The Glu298Asp polymorphism had no effect on case fatality in any pathogen. Carriage of the T allele of the eNOS gene is a risk factor for hypotension in patients with *E. coli* bacteremia but not in bacteremia caused by a gram-positive organism.

KEYWORDS—Bacteremia, NO, endothelial, polymorphism, MAP

INTRODUCTION

Nitric oxide (NO) is one of many vasoactive substances released under conditions of endotoxemia and sepsis. Under physiological conditions, it is produced by two constitutive calcium-dependent enzymes including NO synthase (NOS) in neurons (nNOS) and endothelial cells (eNOS) and has functions ranging from neurotransmission and vasodilatation to inhibition of platelet adhesion and aggregation (1, 2).

After bacterial infection, especially with gram-negative organisms carrying LPS, NO formation from L-arginine is enhanced because of the cytokine-mediated induction of an iNOS enzyme in cells (e.g., cardiac myocytes, vascular smooth muscle), which do not normally have the ability to synthesize NO. The result of this excessive NO production is enhanced bacterial lysis by activated macrophages, hypotension, and myocardial depression. There have therefore been attempts to improve the prognosis of sepsis by NOS inhibitors, but results have been inconsistent (3–7).

Although a key event believed to be responsible for hypotension and shock in sepsis is iNOS-derived overproduction of NO, an essential role for eNOS has also been indicated (8, 9). NO from eNOS plays an important homeostatic role in maintaining appropriate blood flow to vital organs such as the kidney, liver, and lungs and exhibits cytoprotective effects, in part by preventing platelet/neutrophil adhesion to the blood vessel wall (10, 11). Furthermore, studies of animal models of sepsis have shown that the pathogenesis of sepsis is characterized by an initial eNOS activation, with the resultant NO acting as a costimulus for the expression of iNOS, and they therefore highlight a novel proinflammatory role for eNOS (9, 12).

Endothelium-derived NO is formed from L-arginine by eNOS encoded by the *NOS3* gene on chromosome 7q35–36 (13). The eNOS gene polymorphism in exon 7 at position 894 (Glu298Asp; glutamic acid substituted by aspartic acid), also defined as G894T, resulting in T allele has been linked to the pathogenesis of hypertension (14) and coronary artery spasms (15), although negative results have also been published (16–18). In nonseptic state, the carriage of the T allele of eNOS gene has been linked to reduced basal NO production (19). In addition, mice lacking the eNOS gene have been shown to become hypertensive (20).

Although NO plays a key role in the pathogenesis of sepsis and an essential role for eNOS has been indicated, there are

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TABLE 1. eNOS genotype frequencies at nucleotide position 894 in patients with bacteremia

	All n = 147 (%)	<i>E. coli</i> n = 42 (%)	<i>S. aureus</i> n = 40 (%)	<i>S. pneumoniae</i> n = 42 (%)	β -Hemolytic streptococci n = 23 (%)	P
Genotype						
GG 89 (61)	30 (71)	22 (55)	21 (50)	16 (70)	0.354*	
TG 48 (33)	10 (24)	16 (40)	17 (41)	5 (22)		
TT 10 (7)	2 (5)	2 (5)	4 (10)	2 (9)		

*The difference between groups of patients with bacteremia caused by different organisms.

no data regarding the role of eNOS G894T polymorphism in bacteremia/sepsis. We sought here to elucidate the role of this polymorphism, G894T, of the eNOS gene in the clinical course of the disease in patients with bacteremia.

MATERIALS AND METHODS

The study comprised 147 patients with bacteremia (78 men and 69 women) with a mean age of 59 years (range, 16–93 years) hospitalized in Tampere University Hospital, Tampere, Finland, from June 1999 to February 2004.

All blood cultures were taken from patients with symptoms or signs of systemic infection (fever or hypothermia, tachycardia or tachypnea combined with leukocytosis or leukopenia, and/or elevated C-reactive protein). The BACTEC 9240 (BD Diagnostic Systems, Sparks, Md) blood culture system was used with standard media. Patients were identified according to microbiological blood culture findings and those with bacteremia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -hemolytic streptococci, or *Escherichia coli*, the most common causative organisms in community-acquired bacteremia, were included in the study. After being informed by the clinical microbiologists (R.V. and J.A.), the clinicians (J.S. and J.L.) asked the patients to participate and interviewed and examined those who consented. Information was gathered from hospital records at hospital visits, and hospital records were also reviewed after hospitalization (R.H.). Altogether, 149 of 152 patients agreed to participate. eNOS genetic polymorphism testing was carried out in 147 (99%) of 149 patients with bacteremia.

The causative organisms involved were *S. aureus* (40 patients, 27%), *S. pneumoniae* (42 patients, 29%), β -hemolytic streptococci (23 patients,

16%), and *E. coli* (42 patients, 29%). All patients were treated with an empiric antibiotic regimen, and when necessary, antimicrobial treatment was changed according to culture results. In all patients, the causative organism was susceptible to the selected empiric antibiotic treatment. Bacteremia was community acquired in 119 patients (81%) and nosocomial in 28 (19%).

The recruitment of the patients and the clinical data collection are described in detail elsewhere (21). Underlying diseases were registered. Alcohol abuse was defined as consumption of 300 g absolute alcohol per week or a known social or medical problem due to alcohol use. Smoking habits were registered, and patients were defined as current smokers, ex-smokers (those who have stopped smoking), or nonsmokers (those who have never smoked). Calculation of body mass index (BMI) was based on weight and height as reported by the patient on admission. Body mass index was recorded, and patients were defined as obese if their BMI was greater than 30 kg/m².

Patients were closely monitored during hospitalization, and severely ill subjects were transferred to the intensive care unit (ICU). Clinical data were registered on admission and during 6 consecutive days. The possible need for mechanical ventilation was registered. Alterations in mental status were evaluated on the Glasgow Coma Scale. MAP [(systolic + 2 \times diastolic blood pressure) / 3] was calculated. The lowest MAP was calculated for every patient on day 0 (the day of positive blood culture), during days 1 to 4 (1 to 4 days after the positive blood culture), or during days 5 to 6 (5 to 6 days after the positive blood culture). A patient was classified hypotensive if MAP was less than 70 mmHg at least once on day 0, during days 1 to 4, or during days 5 to 6. The need for vasopressive support was also recorded on day 0, during days 1 to 4, or during days 5 to 6.

Sequential Organ Failure Assessment (SOFA) score (22) was calculated during 6 consecutive days after positive blood culture. The highest SOFA score for every patient was used in analysis. Disease severity was assessed by SOFA score, and severe disease was defined if SOFA score was 4 or higher. Laboratory findings were registered on admission and during 6 consecutive days. The laboratory tests included blood platelets ($\times 10^9/L$), plasma bilirubin ($\mu\text{mol/L}$), and plasma creatinine level ($\mu\text{mol/L}$). The whole-blood sample for genomic DNA extraction was taken during days 1 to 4 and stored in -70°C until extraction. The case fatality rate was studied within 30 days after the positive blood culture (day-30 case fatality).

eNOS GENOTYPING

Genotyping was performed afterward from frozen whole-blood sample taken during days 1 to 4. Genomic DNA was extracted from whole blood using a commercially available kit (Qiagen Inc, Hilden, Germany) in accordance with the manufacturer's instructions. DNA samples were genotyped by using the 5' nuclease assay in combination with specific

TABLE 2. Predisposing factors and underlying diseases of bacteremia stratified by eNOS genotype at position 894

Variable	All		<i>E. coli</i>		Gram-positive organism	
	GG n = 89 (%)	TG or TT n = 58 (%)	GG n = 30 (%)	TG or TT n = 12 (%)	GG n = 59 (%)	TG or TT n = 46 (%)
Malignancy*	16 (18)	8 (14)	7 (23)	2 (17)	9 (15)	6 (13)
Obesity (BMI >30 kg/m ²) [†]	17 (25)	10 (23)	3 (12) [¶]	5 (50) [¶]	14 (32)	5 (15)
Cardiac disease [‡]	27 (30)	17 (29)	9 (30)	5 (42)	18 (31)	12 (26)
Essential hypertension	24 (27)	10 (17)	10 (33)	2 (17)	14 (24)	8 (17)
Alcohol abuse	18 (20)	6 (10)	4 (13)	0	14 (24)	6 (13)
Current smoker [§]	25 (31)	12 (22)	4 (14)	0	21 (41)	12 (27)
McCabe II or III	15 (17)	8 (14)	7 (23)	1 (8)	8 (14)	7 (15)
Age >60 y	47 (53)	31 (53)	18 (60)	9 (75)	29 (49)	22 (48)
Diabetes (type 1 or type 2)	18 (20)	16 (28)	8 (27)	7 (58)	10 (17)	9 (20)
Male/female	41 (46)/48 (54) [¶]	37 (64)/21 (36) [¶]	8 (27)/22 (73)	4 (33)/8 (67)	33 (56)/26 (44)	33 (72)/13 (28)

*Solid malignancy or hematologic malignancy.

[†]Data available on 113 patients.

[‡]Coronary artery disease, valvular disease, heart failure, or cardiac myopathy.

[§]Data available on 134 patients.

^{||}McCabe class II or III: ultimately or rapidly fatal disease.

[¶]The difference between groups of patients with GG genotype and TG/TT genotype statistically significant, $P < 0.05$.

TABLE 3. Sources of bacteremia

Focus*	All (n = 147)		<i>E. coli</i> (n = 42)		Gram-positive organism† (n = 105)	
	GG n = 89 (%)	GT or TT n = 58 (%)	GG n = 30 (%)	GT or TT n = 12 (%)	GG n = 59 (%)	GT or TT n = 46 (%)
Pneumonia	19 (21)	20 (35)	0	0	19 (32)	20 (44)
Skin	23 (26)	14 (24)	0	0	23 (39)	14 (30)
Urinary	21 (24)	9 (16)	20 (67)	8 (67)	1 (2)	1 (2)
Osteomyelitis/spondylitis	6 (7)	8 (14)	0	0	6 (10)	8 (17)
Gall bladder	3 (3)	4 (7)	2 (7)	2 (17)	1 (2)	2 (4)
Endocarditis	6 (7)	0	0	0	6 (10)‡	0‡
Gynecological	1 (1)	2 (3)	0	1 (8)	1 (2)	1 (2)
Mediastinitis	2 (2)	2 (3)	0	0	2 (3)	2 (4)
Meningitis	2 (2)	1 (2)	0	0	2 (3)	1 (2)
Intravenous/intra-arterial catheter related	1 (1)	2 (3)	0	0	1 (2)	2 (4)
Arthritis	5 (6)	1 (2)	0	0	5 (9)	1 (2)
Source unknown	11 (12)	5 (9)	6 (20)	0	5 (9)	5 (11)
Nosocomial infection	17 (19)	11 (19)	5 (17)	1 (8)	12 (20)	10 (22)

*One patient may have more than 1 focus.

†Bacteremia caused by *S. aureus*, *S. pneumoniae*, or β -hemolytic streptococci.

‡ $P = 0.034$.

fluorogenic TaqMan MGB probes, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif). The nucleotide sequences of primers and allele-specific probes, labeled with reporter dyes, were deduced from sequences deposited in the GenBank database and synthesized in conjugation with Applied Biosystems using the Assays-by-Design tool. Polymerase chain reaction tests containing genomic DNA, 1 \times Universal PCR Master Mix (Applied Biosystems, Foster City, Calif), 900 nM of each

primer and 200 nM of each probe, were performed in 96-well plates in a total volume of 25 μ L, in accordance with the standard protocol. The end-point reading of fluorescence was measured using the allelic discrimination analysis module, this resulting in clear identification of three genotypes.

Statistical analysis

The SPSS package (version 7.5; SPSS Inc Headquarters, Chicago, Ill) was used for statistical analyses, and a two-sided

TABLE 4. Clinical data on patients with *E. coli* or gram-positive bacteremia in relation to *eNOS* G894T (Glu298Asp) polymorphism

	<i>E. coli</i>				Gram-positive organism			
	GG n = 30	GT or TT n = 12	OR (95% CI)	<i>P</i>	GG n = 59	GT or TT n = 46	OR (95% CI)	<i>P</i>
Died*	0	1 (8%)	†	0.286	11 (19%)	7 (15%)	0.8 (0.3–2.2)	0.644
Needed ICU stay	2 (7%)	4 (33%)	7.0 (1.1–45.4)	0.046	25 (42%)	16 (35%)	0.7 (0.3–1.6)	0.429
Acute myocardial infarction‡	2 (7%)	4 (33%)	7.0 (1.1–45.4)	0.046	4 (7%)	1 (2%)	0.3 (0–2.8)	0.383
Highest SOFA score, median (quartiles)	1.5 (0–2.3)	5 (3–13)		0.001	4 (1–9)	1.5 (1.0–4.3)		0.021
Needed mechanical ventilation	0	2 (17%)	†	0.077	15 (25%)	5 (11%)	0.4 (0.1–1.1)	0.06
Lowest platelet count, median (quartiles), $\times 10^9/L$	178 (120–200)	105 (36–163)		0.045	128 (62–225)	184 (126–254)		0.023
Lowered GCS	15 (15–15)	15 (14–15)		0.170	14 (13–15)	15 (14–15)		0.052
Highest bilirubin value, median (quartiles), μ mol/L	15 (10–29)	20 (16–79)		0.085	19.5 (12–50)	17.5 (13–29)		0.451
Lowest MAP, median (quartiles), mmHg	80 (75–90)	60 (47–80)		0.004	70 (58–84)	77 (63–94)		0.071
Highest creatinine value, median (quartiles), μ mol/L	98 (72–193)	148 (83–190)		0.328	124 (83–202)	91 (71–165)		0.098

*Death due to bacteremia episode that occurred within 30 days from the day of positive blood culture.

†Cannot be calculated.

‡Acute myocardial infarction within 30 days from the positive blood culture.

GCS indicates Glasgow Coma Scale.

TABLE 5. The occurrence of hypotension in patients with *E. coli* and gram-positive bacteremia in relation to eNOS G894T (Glu298Asp) polymorphism during observation period of day 0, days 1 to 4, and days 5 to 6

	<i>E. coli</i>				Gram-positive organism			
	GG n = 30	GT or TT n = 12	OR (95% CI)	<i>P</i>	GG n = 59	GT or TT n = 46	OR (95% CI)	<i>P</i>
On day 0*								
Hypotensive [†]	4 (14%)	7 (58%)	8.8 (1.8–41.6)	0.003	15 (25%)	5 (11%)	0.4 (0.1–1.1)	0.06
MAP, [‡] median (quartiles)	91 (81–97)	52 (46–81)		<0.001	80 (67–97)	87 (76–106)		0.08
Needed vasopressives [§]	1 (3%)	4 (33%)	14 (1.4–144.0)	0.02	6 (10%)	0		0.034
During days 1–4 [¶]								
Hypotensive [†]	0	4 (33%)		0.004	15 (25%)	7 (15%)	0.5 (0.2–1.4)	0.202
MAP, [‡] median (quartiles)	94 (88–107)	80 (74–91)		0.02	85 (73–100)	92 (80–107)		0.236
Needed vasopressives [§]	0	4 (33%)		0.004	12 (20%)	5 (11%)	0.5 (0.2–1.5)	0.191
During days 5–6 [#]								
Hypotensive [†]	0	0			7 (18%)	3 (10%)	0.5 (0.1–2.2)	0.358
MAP [‡] , median (quartiles)	99 (90–107)	83 (75–94)		0.025	89 (71–100)	97 (81–110)		0.045
Needed vasopressives [§]	0	0			5 (11%)	3 (9%)	0.8 (0.2–3.7)	0.796

*On the day of positive blood culture.

[†]MAP <70 mmHg at least once during the observation period (on day 0, during days 1–4, or during days 5–6).

[‡]The lowest value for each patient during the observation period was recorded.

[§]Needed vasopressor support at least once during the observation period.

^{||}Cannot be calculated.

[¶]One to 4 days after the positive blood culture.

[#]Five to 6 days after the positive blood culture.

$P < 0.05$ was taken as the level for statistical significance. Categorical data were analyzed by χ^2 test or Fisher exact test when appropriate. Nonparametric data were analyzed by Mann-Whitney U test. Odds ratios (ORs) were expressed with their 95% confidence intervals (CIs). The effect of G894T amino acid substitution on hypotension or disease severity was studied in a multivariate model adjusting for confounding factors. Binary logistic regression was used to calculate ORs and their 95% CIs.

RESULTS

The distribution of the eNOS genotypes at nucleotide position 894 in patients with bacteremia is shown in Table 1. The genotype frequencies followed the Hardy-Weinberg equilibrium. Table 2 shows the most important predisposing factors and underlying diseases; and Table 3, the sources of bacteremia in relation to eNOS G894T polymorphism in all patients and in patients with *E. coli* or gram-positive bacteremia, including *S. aureus*, *S. pneumoniae*, or β -hemolytic streptococci. The effect of the eNOS G894T polymorphism on the clinical characteristics of patients with *E. coli* and gram-positive bacteremia is shown in Table 4. Among those with *E. coli* bacteremia, carriage of the T allele was associated with lower MAP ($P = 0.004$) and higher SOFA score ($P = 0.001$), and there were more hypotensive patients among the T-allele carriers compared with noncarriers (7/12 vs. 6/30; $P = 0.015$). The effect on blood pressure was most prominent in the early stage of the disease: on admission, MAP (median) was 52 mmHg in T-allele carriers compared with 91 mmHg in noncarriers ($P < 0.001$), as shown in Table 5. The same was not detected in bacteremia caused by a gram-positive organism. In fact, the patients with gram-

positive bacteremia and who have GG genotype had greater severity of disease (assessed by SOFA score) than those carrying T allele (Table 4). The effect was most prominent in those with *S. aureus* bacteremia; SOFA score was lower in carriers of the T allele compared with noncarriers ($P = 0.008$). T allele did not have an effect on case fatality in any of the four pathogens studied. The overall case fatality among these patients was 13%.

T-allele carriage remained a significant risk factor (OR, 10.8; 95% CI, 2.0–57.7; $P = 0.005$) for hypotension on day 0 in *E. coli* bacteremia in a model adjusted by age (continuous variable) and sex. The number of obese patients (BMI >30 kg/m²) was higher in patients with *E. coli* bacteremia carrying T allele compared with noncarriers (Table 2). However, obesity was not associated with hypotension in *E. coli* bacteremia (data not shown). T-allele carriage also remained a significant risk factor for hypotension on day 0 in a logistic regression model adjusted by obesity (OR, 10.9; 95% CI, 1.4–84.0; $P = 0.02$).

The effect of eNOS T-allele carriage on disease severity (assessed by SOFA score) in gram-positive bacteremia was also studied in a multivariate logistic regression analysis. In gram-positive bacteremia, carriage of T allele preserved its protective role as regards severe disease (OR, 0.4) in a model adjusted for the effect of age, sex, and endocarditis. Endocarditis was included in the model because there were significantly more patients with endocarditis in patients with GG genotype in gram-positive bacteremia as shown in Table 3.

DISCUSSION

The present findings show the eNOS Glu298Asp polymorphism (T allele) to be associated with hypotension in

patients with *E. coli* bacteremia but not in bacteremia caused by gram-positive organisms such as *S. aureus*, *S. pneumoniae*, or β -hemolytic streptococci. The effect on hypotension was most evident in the early stage of the disease. T allele did not affect case fatality in any of the four pathogens studied.

Cytokine-induced iNOS plays a key role in septic hypotension, whereas eNOS is considered to be physiological, releasing small amounts of NO to maintain tissue flow. The carriage of the T allele of *eNOS* gene has been previously linked to reduced basal NO production in non-septic state (19). The balance of eNOS and iNOS during sepsis has not been fully established. Yamashita and associates (23) have shown that transgenic mice overexpressing eNOS in endothelial cells are resistant to LPS-induced hypotension, although iNOS-mediated NO production does not differ between transgenic and control mice. In contrast, recent studies have indicated an essential proinflammatory role for eNOS (12). Connelly and associates (12) have found that macrophages from eNOS knockout mice demonstrate reduced nuclear factor κ B activity, iNOS expression, and NO production after LPS exposure, compared with wild-type mice. However, no previous study has linked G894T (Glu298Asp) polymorphisms to septic hypotension or studied the functional effects of this polymorphism in sepsis. In contrast, this polymorphism has been linked to hypertension, although the data are still somewhat ambiguous and its functional consequences are not yet well established (17, 24). The reason why T allele was associated with hypotension in *E. coli* bacteremia in this study remains unknown. The carriage of T allele in patients with *E. coli* bacteremia seemed to be weakly associated with an increased risk of acute myocardial infarction. One explanation may be coronary artery spasms, which have been associated with T-allele carriage (15). Although the functional consequences of T-allele carriage in sepsis are not well known, and systemic hypotension might be a plausible explanation responsible for decreased myocardial perfusion, this observation could also be a consequence of reduced basal eNOS-derived NO secretion as a result of T-allele carriage.

In our study, the effects of the T allele on disease severity in *E. coli* and in gram-positive bacteremia were in contrast to each other. One possible explanation is the tie to Toll-like receptors (TLRs); gram-negative bacteria activate TLR4, whereas gram-positive bacteria activate TLR2. Toll-like receptor agonistic molecules such as LPS can regulate NO generation through upregulation of expression of NOS isoforms through nuclear factor κ B activation (11, 25). Furthermore, the signaling pathways leading to iNOS induction have been shown to be different in *S. aureus* and *E. coli* infections, and *E. coli* or LPS has been shown to induce the release of NO at earlier time points compared with *S. aureus* (26).

There are limitations in our study. First, the study group is fairly small. To follow acceptable statistical practices, we cannot include more than a few possible confounders in the multiple regression model as covariates evaluating the independent role of T allele in relation to hypotension in *E. coli* bacteremia. Second, in bacteremia caused by gram-positive bacteremia, there were significantly more patients

with endocarditis in GG genotype compared with T-allele carriers (Table 3). This might cause bias when evaluating the independent role of genotype associated with the severity of disease in gram-positive bacteremia. However, T allele remained a significant factor associated with milder disease severity also in logistic regression model, where endocarditis was included. The strength of our study is that patients with different disease severity were included, those needing ICU treatment and being hypotensive as well as those with milder disease spectrum.

It remains a subject of debate whether NO or NOS is beneficial or detrimental in sepsis (27, 28). Attempts to improve the prognosis of sepsis by introducing NOS inhibitors have yielded inconsistent results (5–7). One explanation here lies in the cytostatic and cytotoxic effects associated with NO production that facilitate host defense (1, 29), and another is the nonselectivity of hitherto introduced NOS inhibitors attributed to the toxic effects of inappropriate eNOS inhibition (3). Although T-allele carriage was associated with hypotension in *E. coli* bacteremia, T-allele carriage did not affect survival. It should be noted that there are also other factors that have been shown to affect the course of sepsis, especially the quality of care (30, 31).

In conclusion, our results indicate that the T allele at nucleotide position 894, a common variant of the *eNOS* gene, is a risk factor for hypotension in the early stage of disease in patients with *E. coli* bacteremia but not in bacteremia caused by a gram-positive organism. Larger studies are needed to confirm these preliminary findings in bacteremia. Our observations emphasize that, in efforts to devise therapeutic interventions, not only organism-related differences in the pathogenesis should be considered, but also differences in the genetic background of patients modifying the outcome of sepsis.

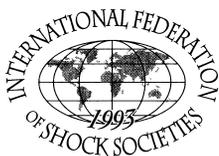
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Research article

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Obesity and smoking are factors associated with poor prognosis in patients with bacteraemia

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Abstract

Background: Bacteraemia is still a major cause of case fatality in all age groups. Our aim was to identify the major underlying conditions constituting risk factors for case fatality in bacteraemia patients.

Methods: The study involved 149 patients (79 male and 70 female) with bacteraemia caused by *Staphylococcus aureus* (*S. aureus*) (41 patients), *Streptococcus pneumoniae* (*Str. pneumoniae*) (42 patients), β -hemolytic streptococcae (β -hml str.) (23 patients) and *Eschericia coli* (*E. coli*) (43 patients). Underlying diseases, alcohol and tobacco consumption and body mass index (BMI) were registered. Laboratory findings and clinical data were registered on admission and 6 consecutive days and on day 10–14. Case fatality was studied within 30 days after positive blood culture. Associations between underlying conditions and case fatality were studied in univariate analysis and in a multivariate model.

Results: Nineteen patients (12.8%) died of bacteraemia. We found obesity ($p = 0.002$, RR 9.8; 95% CI 2.3 to 41.3), smoking ($p < 0.001$, RR 16.9; 95% CI 2.1 to 133.5), alcohol abuse ($p = 0.008$, RR 3.9; 95% CI 1.3 to 11.28), COPD ($p = 0.01$, RR 8.4; 95% CI 1.9 to 37.1) and rheumatoid arthritis ($p = 0.045$, RR 5.9; 95% CI 1.2 to 28.8) to be significantly associated with case fatality in bacteraemia in univariate model. The median BMI was significantly higher among those who died compared to survivors (33 vs. 26, $p = 0.003$). Obesity and smoking also remained independent risk factors for case fatality when their effect was studied together in a multivariate model adjusted with the effect of alcohol abuse, age (continuous variable), sex and causative organism.

Conclusion: Our results indicate that obesity and smoking are prominent risk factors for case fatality in bacteraemic patients. Identification of risk factors underlying fatal outcome in bacteraemia may allow targeting of preventive efforts to individuals likely to derive greatest potential benefit.

Background

Bacteraemia is a common infection with significant morbidity and in most severe instances also mortality. The importance of underlying conditions such as immunosuppression, ultimately and rapidly fatal diseases and chronic organ insufficiency for bacteraemia outcome has been emphasized in a number of studies [1-3].

Obesity is an increasing health concern in Western countries. In the general population, a relationship between obesity and increased mortality has been shown [4-6]. Data on the incidence and outcome of specific infections, especially community-acquired infections, in obese people are so far limited [7]. There are no papers on the effect of obesity on case fatality in bacteraemia patients.

Smokers and alcohol abusers display an increased susceptibility to bacterial infections, especially those involving the lung. In addition, smoking [8] and alcoholism [9] are major risk factors for invasive pneumococcal disease. There are data showing that alcoholism is a risk factor for case fatality in *S. aureus* [10] and *Str. pneumoniae* bacteraemia [11,12]. However, some studies have found no statistically significant association between alcoholism and case fatality [2,3,13]. A recent study of epidemiological and clinical features as potential prognostic factors for outcomes of hospital-acquired bacteraemia found no differences between non-survivors and survivors in sex, age or smoking habit [14]. In bacteraemias other than pneumococcal, the effect of smoking and alcoholism on case fatality has not been widely studied.

Moreover, studies of bacteraemia with varying disease severity are few in number. Most materials in this field consist of patients merely treated with severe infection in ICU; patients originally evincing milder signs and symptoms of infection are not included. The aim of our study was to elucidate the underlying conditions, especially the role of obesity, smoking and alcohol abuse, as risk factor for case fatality in bacteraemia.

Methods

The study material comprised 149 patients (79 male and 70 female) with bacteraemia hospitalized in Tampere University Hospital, Tampere, Finland, from June 1999 to February 2004. Their ages ranged from 16 to 93 years (mean 59 years).

In our hospital blood cultures are routinely taken from patients with symptoms or signs of systemic infection (fever or hypothermia, tachycardia or tachypnea combined with leucocytosis or leucopenia and/or elevated C-reactive protein (CRP)). The study focused on patients with bacteraemia caused by *S. aureus*, *Str. pneumoniae*, β -hml str. or *E. coli*, the most common causative organisms

among community-acquired bacteraemia. Patients were identified according to microbiological culture finding. The clinicians (J.S. or J.La.) were informed by clinical microbiologist (R.V.) of a positive blood culture from Mondays to Thursdays and the patients were enrolled to the study whenever possible to adjust to the daily schedule. We were able to recruit zero to two patients per week during the study period. Since the clinicians were unable to know any details about the patients nor their disease severity before the recruitment, the selection was purely based on the blood culture finding. After being informed by clinical microbiologist the clinician asked patients to participate and interviewed and examined those consenting. Information was gathered from hospital records at the time of hospital visit and hospital records were also reviewed after hospitalization (R.H.). Altogether 149 out of 152 patients agreed to participate. The causative organisms involved were *S. aureus* (41 patients, 27.5%), *Str. pneumoniae* (42 patients, 28.2%), β -hml str. (23 patients, 15.4%), and *E. coli* (43 patients, 28.9%). Patients with two episodes of bacteraemia were not included in the study. During the period in question the BACTEC 9240 (BD Diagnostic Systems, Sparks, MD, USA) blood culture system with standard media was used. Bacteraemia was defined hospital-acquired (nosocomial) if positive blood culture was drawn >48 h after admission.

Underlying conditions, social status and alcohol and tobacco consumption were registered. Alcohol abuse was defined as consumption of 300 g absolute alcohol per week or a known social or medical problem due to alcohol use. Smoking habits were registered and patients were defined as current smokers, ex-smokers i.e. those who have stopped smoking and nonsmokers i.e. those who have never smoked (data available from 136 out of 149 patients). Calculation of BMI was based on weight and length as reported by the patient on admission. BMI was recorded and patients were defined as obese if their BMI was > 30 (data available from 114 out of 149 patients; 10/19 of deceased patients and 104/128 of survivors). Chronic diseases were registered and McCabe (and Jackson) classification was used to assess the severity of the underlying medical condition [15]. Preceding corticosteroid treatment was registered if corticosteroids were used in a dose of over 5 mg per day during 1 month before the bacteraemia episode. All patients were treated with an empiric antibiotic regimen and antimicrobial treatment was changed according to culture results if necessary.

Patients were closely monitored during hospitalization and severely ill subjects were transferred to the ICU. Clinical data were registered on admission and during 6 consecutive days and on day 10–14 after admission. Possible need for mechanical ventilation was registered. Alterations in mental status were evaluated on the Glasgow

Coma Scale (GCS). A patient was classified hypotensive if mean arterial pressure (MAP) was < 70 mmHg during the bacteraemia episode.

A SOFA score (Sequential Organ Failure Assessment) [16] was calculated on 1–3 days after positive blood culture finding (data available from 135/149 patients). Severe organ failure was documented if the SOFA score was ≥ 4 . A patient did not need ICU treatment if the SOFA score was lower than 4. Laboratory findings were registered on admission, during 6 consecutive days and on day 10–14. The laboratory tests included plasma C-reactive protein (mg/l), blood white cell count ($\times 10^9/l$), blood platelets ($\times 10^9/l$), plasma bilirubin ($\mu\text{mol/l}$), plasma creatinine level ($\mu\text{mol/l}$), plasma alanine aminotransferase (U/l) and plasma alkaline phosphatase (U/l). The case fatality rate was studied within 30 days after the positive blood culture (day 30 case fatality).

The study was approved by the Ethics Committee of Tampere University Hospital. Written informed consent was obtained from patients or first degree relatives.

The SPSS package (version 7.5) was used for statistical analyses and a two-sided p-value < 0.05 was taken as the level for statistical significance. Categorical data were analysed by χ^2 test or Fisher's exact test when appropriate. Nonparametric data were analysed by Mann-Whitney U-test. Multivariate logistic regression analysis (method enter) was used to assess the effects of independent factors on mortality, controlling for differences in other factors possibly affecting the outcome. Risk ratios (RRs) were expressed with their 95% confidence intervals (CI). We studied the effect of obesity, smoking and alcohol abuse in a multivariate model adjusted with the effect of age (continuous variable), sex and organism (*S. Aureus*, *Str. Pneumoniae*, β -hl str., or *E. coli*).

Results

Nineteen patients (12.8%) died of bacteraemia. The case fatality rate differed in bacteraemias caused by different organisms: *S. aureus* 8/41 (19.5%), *Str. pneumoniae* 8/42 (19.0%), β -hml str. 2/23 (8.7%), and *E. coli* 1/43 (2.3%). Bacteraemia was community-acquired in 119 patients (79.9%) and nosocomial in 30 (20.1%).

Predisposing factors and underlying diseases of bacteraemia are given in Table 1. Four differed bacteraemias differed statistically significantly from each other in the number of smoking patients (current smokers or ex-smokers, $p = 0.007$). Smoking was common in pneumococcal bacteraemic patients (65.0% of patients current smokers or ex-smokers) whereas 27.5% of *E. coli* bacteraemic patients were current smokers or ex-smokers. *S. aureus* bacteraemia was the most common pathogen in patients

with rheumatoid arthritis. Bacteraemias differed statistically significantly from each other in the number of male patients ($p = 0.003$). Only 30.2% of *E. coli* bacteraemia patients were male whereas 68.3% of *S. aureus* bacteraemic patients were male. *S. aureus* was a common pathogen in nosocomial infection; 39% of *S. aureus* bacteraemias were nosocomial infections.

The sources of the infection were identifiable in 132 patients and are listed in Table 2. Clinical data on all patients and on patients with bacteraemia caused by different organisms are shown in Table 3. The case fatality rate in relation to predisposing factors and underlying diseases is given in Table 4. Obesity, smoking, alcohol abuse, COPD and rheumatoid arthritis proved significant risk factors for case fatality in univariate analysis (table 4).

Myocardial infarction occurred in 11 (7.4%) patients and cerebral infarction in 7 (4.7%) during the month following positive blood culture. Twenty-six per cent of patients older than 60 years were treated in ICU compared to 38 per cent of those aged 60 or younger ($p = 0.104$).

Obesity

Day 30 case fatality was higher in obese bacteraemic patients than in nonobese patients (25.9% vs. 3.4%, $p = 0.002$, RR 9.8; 95% CI 2.3–41.3). The median BMI was significantly higher among those who died compared to survivors (33 vs. 26, $p = 0.003$). Obese and nonobese study groups did not differ statistically significantly from each other in numbers needing ICU treatment (25.9% vs. 28.7%, $p = 0.78$, RR 0.9; 95% CI 0.3–2.3). Obese patients needed mechanical ventilation more often than non-obese, but the difference was not statistically significant (18.5% vs 8.0%, $p = 0.152$, RR 2.6; 95% CI 0.8–9.0). However, more obese than nonobese patients died in ICU treatment (5/7 vs. 3/25; $p = 0.005$, RR 18.3; 95% CI 2.4–140.4). The obese had high SOFA scores (value >4 on day 1–3 after positive blood culture finding) more often than nonobese, but the difference was not statistically significant (44.0% vs 23.8%, $p = 0.05$, RR 2.5; 95% CI 1.0–6.5). The obese and nonobese groups did not differ statistically significantly in the occurrence of hypotension (37.0% vs 31.0%, $p = 0.561$, RR 1.3; 95% CI 0.5–3.2), or in the number of patients with neurological deficit (lowered GCS) (44.4% vs 33.3%, $p = 0.293$, RR 1.6; 95% CI 0.7–3.9)

Forty-four per cent of obese bacteraemic patients had previously been diagnosed with type 2 diabetes as against 12.6% of nonobese patients ($p < 0.001$). Patients with type 2 diabetes died more often than those without type 2 diabetes, but the difference was not statistically significant (Table 4).

Table 1: Predisposing factors and underlying diseases of bacteraemia

Predisposing factor	All patients N = 149 n (%)	<i>S. aureus</i> N = 41 n (%)	<i>Str. pneumoniae</i> N = 42 n (%)	β -haemolytic streptococci N = 23 n (%)	<i>E. coli</i> N = 43 n (%)	p-value
Obesity ^a	27 (23.7)	9 (25.7)	4 (16.7)	6 (31.6)	8 (22.2)	0.698
Current smoker or ex-smoker ^b	66 (48.5)	18 (48.6)	26 (65.0)	11 (57.9)	11 (27.5)	0.007
Alcohol abuse	24 (16.1)	5 (12.2)	9 (21.4)	6 (26.1)	4 (9.3)	0.211
Type 1 diabetes	5 (3.4)	1 (2.4)	-	-	4 (9.3)	0.1
Type 2 diabetes	29 (19.5)	9 (22.0)	5 (11.9)	4 (17.4)	11 (25.6)	0.426
COPD	8 (5.4)	-	5 (11.9)	2 (8.7)	1 (2.3)	0.053
Haematological malignancy	11 (7.4)	2 (4.9)	3 (7.1)	1 (4.3)	5 (11.6)	0.723
Solid malignancy	15 (10.1)	6 (14.6)	3 (7.1)	1 (4.3)	5 (11.6)	0.589
Rheumatoid arthritis	7 (4.7)	5 (12.2)	-	1 (4.3)	1 (2.3)	0.034
Liver cirrhosis	4 (2.7)	2 (4.9)	-	1 (4.3)	1 (2.3)	0.511
Cardiac disease ^c	44 (29.5)	14 (34.1)	10 (23.8)	6 (26.1)	14 (32.6)	0.705
McCabe II or III ^d	24 (16.1)	8 (19.5)	5 (11.9)	2 (8.7)	9 (20.9)	0.462
Corticosteroid treatment ^e	18 (12.1)	6 (14.6)	4 (9.5)	2 (8.7)	6 (14.0)	0.857
Chronic disease ^f	117 (78.5)	33 (80.5)	27 (64.3)	17 (73.9)	40 (93.0)	0.013
Age >60 years	78 (52.3)	21 (51.2)	20 (47.6)	10 (43.5)	27 (62.8)	0.391
Male sex	79 (53.0)	28 (68.3)	24 (57.1)	14 (60.9)	13 (30.2)	0.003
Nosocomial infection	30 (20.1)	16 (39.0)	4 (9.5)	3 (13.0)	7 (16.3)	0.004

^a(BMI >30), data available from 114 patients

^bData available from 136 patients

^cCoronary artery disease, valvular disease or documented heart failure

^dMcCabe class II or III: ultimately fatal or rapidly fatal disease

^eCorticosteroids used in a dose of over 5 mg per day during one month prior to the episode of bacteraemia

^fAt least one chronic disease

Table 2: Source of bacteraemia^a

Focus	All n = 170 ^b (%)	<i>S. aureus</i> n = 56 ^b (%)	<i>Str. pneumoniae</i> n = 45 ^b (%)	β -hml str. n = 29 ^b (%)	<i>E. coli</i> n = 40 ^b (%)
Pneumonia	39 (22.9)	2 (3.6)	35 (77.8)	2 (6.9)	0 (0)
Skin	37 (21.8)	19 (33.9)	2 (4.4)	16 (55.2)	0 (0)
Urinary	30 (17.6)	1 (1.8)	0 (0)	1 (3.4)	28 (70.0)
Source unknown	17 (10.0)	3 (5.4)	4 (8.9)	3 (10.3)	7 (17.5)
Osteomyelitis/spondylitis	15 (8.8)	12 (21.4)	0 (0)	3 (10.3)	0 (0)
Gall bladder	7 (4.1)	0 (0)	3 (6.7)	0 (0)	4 (10.0)
Endocarditis	6 (3.5)	6 (10.7)	0 (0)	0 (0)	0 (0)
Arthritis	6 (3.5)	5 (8.9)	0 (0)	1 (3.4)	0 (0)
Mediastinitis	4 (2.4)	4 (7.1)	0 (0)	0 (0)	0 (0)
Meningitis	3 (1.8)	1 (1.8)	1 (2.2)	1 (3.4)	0 (0)
Gynaecological	3 (1.8)	0 (0)	0 (0)	2 (6.9)	1 (2.5)
Intravenous/intra-arterial catheter-related	3 (1.8)	3 (5.4)	0 (0)	0 (0)	0 (0)

^aOne patient may have several focuses

^bIndicating the number of focuses

Table 3: Clinical data on 149 patients with 4 different bacteraemias

Disease severity	All n = 149 (%)	<i>S. aureus</i> n = 41 (%)	<i>Str. Pneumoniae</i> n = 42 (%)	β -hemolytic streptococci n = 23 (%)	<i>E. coli</i> n = 43 (%)	p-value*
Died ^a	19 (12.8)	8 (19.5)	8 (19.0)	2 (8.7)	1 (2.3)	0.031
Needed ICU stay	47 (31.5)	15 (36.6)	16 (38.1)	10 (43.5)	6 (14.0)	0.029
Needed mechanical ventilation	22 (14.8)	7 (17.1)	10 (23.8)	3 (13.0)	2 (4.7)	0.092
Needed CVVHD	7 (4.7)	5 (12.2)	0 (0)	2 (8.7)	0 (0)	0.006
Needed haemodialysis	2 (1.3)	2 (4.9)	0 (0)	0 (0)	0 (0)	0.097
Sofa Score >4 ^b	39 (28.9)	9 (25.0)	10 (26.3)	11 (50.0)	9 (23.1)	0.122
Low platelet count ^c	21 (14.1)	8 (19.5)	3 (7.1)	4 (17.4)	6 (14.0)	0.411
Elevated bilirubin level ^d	51 (34.2)	14 (34.1)	12 (28.6)	10 (43.5)	15 (34.9)	0.687
Lowered GCS ^e	60 (40.3)	19 (46.3)	18 (42.9)	10 (43.9)	13 (30.2)	0.45
Hypotensive ^f	56 (37.6)	13 (31.7)	19 (45.2)	11 (47.8)	13 (30.2)	0.299

^aDeath due to bacteraemia episode occurred within 30 days from the day of positive blood culture.

^bSOFA score was registered 1–4 days after positive blood culture finding (median 3 days). Data available from 135 patients.

^cPlatelet count at least once lower than 50 10⁹/L 0–8 days after positive blood culture

^dBilirubin level elevated (>25 μ mol/L) at least once 0–8 days after positive blood culture

^eGlasgow coma scale lowered (<15) at least once 0–6 days after positive blood culture

^fHypotensive (MAP <70 mmHg) at least once 0–6 days after positive blood culture

*The difference between groups of patients with bacteraemia caused by different organisms

Table 4: Case fatality in relation to predisposing factors and underlying diseases (univariate analysis)

Predisposing factor	All patients N = 149 n (%)	Deceased N = 19 n (%)	Survivors N = 130 n (%)	p-value	RR (95% CI)
Obesity ^a	27 (23.7)	7 (70.0)	20 (19.2)	0.002	9.8 (2.3–41.3)
Current smoker or ex-smoker ^b	66 (48.5)	13 (92.9)	53 (43.4)	<0.001	16.9 (2.1–133.5)
Alcohol abuse	24 (16.1)	7 (36.8)	17 (13.1)	0.008	3.9 (1.3–11.2)
Type 1 diabetes	5 (3.4)	0 (0)	5 (3.8)	1	1.0 (0.9–1.0)
Type 2 diabetes	29 (19.5)	6 (31.6)	23 (17.7)	0.153	2.1 (0.7–6.2)
COPD	8 (5.4)	4 (21.1)	4 (3.1)	0.01	8.4 (1.9–37.1)
Haematological malignancy	11 (7.4)	0 (0)	11 (8.5)	0.36	0.9 (0.9–1.0)
Solid malignancy	15 (10.1)	3 (15.8)	12 (9.2)	0.41	1.8 (0.5–7.2)
Rheumatoid arthritis	7 (4.7)	3 (15.8)	4 (3.1)	0.045	5.9 (1.2–28.8)
Liver cirrhosis	4 (2.7)	1 (5.3)	3 (2.3)	0.42	2.4 (0.2–23.8)
Cardiac disease ^c	44 (29.5)	6 (31.6)	38 (29.2)	0.834	1.1 (0.4–3.2)
McCabe II or III ^d	24 (16.1)	3 (15.8)	21 (16.2)	1	1.0 (0.3–3.6)
Corticosteroid treatment ^e	18 (12.1)	2 (10.5)	16 (12.3)	0.824	0.8 (0.2–4.0)
Chronic disease ^f	117 (78.5)	18 (94.7)	99 (76.2)	0.065	5.6 (0.7–43.9)
Age over 60 years	78 (52.3)	11 (57.9)	67 (51.5)	0.604	1.3 (0.5–3.4)
Male sex	79 (53.0)	14 (73.7)	65 (50.0)	0.053	2.8 (1.0–8.2)
Nosocomial infection	30 (20.1)	1 (5.3)	29 (22.3)	0.084	0.2 (0.03–1.5)

^a(BMI >30), data available from 114 patients

^bData available from 136 patients

^cCoronary artery disease, valvular disease or documented heart failure

^dMcCabe class II or III: ultimately fatal or rapidly fatal disease

^eCorticosteroids used in a dose of over 5 mg per day during one month prior to the episode of bacteraemia

^fAt least one chronic disease

Smoking

The day 30 case fatality rate in bacteraemic patients was higher in current or ex-smokers than in nonsmokers (19.7% vs. 1.4%, $p < 0.001$, RR 16.9; 95% CI 2.1–133.5); ninety-three per cent of patients who died were current or ex-smokers (Table 4). Current or ex-smokers needed ICU treatment (39.4% vs 18.6%, $p = 0.007$, RR 2.9; 95% CI 1.3–6.2) and mechanical ventilation (21.2% vs 5.7%, $p = 0.008$, RR 4.4; 95% CI 1.4–14.3) more often than nonsmokers during the bacteremia episode. Current or ex-smokers died more often in ICU treatment compared to nonsmokers (10/26 vs 1/13, $p = 0.044$, RR 7.5; 95% CI 0.8–66.9) and had high SOFA scores (value > 4 on day 1–3 after positive blood culture finding) more often than nonsmokers (33.3% vs 16.4%, $p = 0.029$, RR 2.6; 95% CI 1.1–6.0). The current or ex-smoker patient groups did not differ statistically significantly from the nonsmoker group in the occurrence of hypotension (42.4% vs 28.6%, $p = 0.091$, RR 1.8; 95% CI 0.9–3.8) or in numbers with neurological deficit (lowered GCS) (43.9% vs 32.9%, $p = 0.184$, RR 1.6; 95% CI 0.8–3.2).

Thirty-eight patients (35.2%) were current smokers while 70 (64.8%) had never smoked. When current smokers were compared to nonsmokers (ex-smokers excluded from this analysis) the adverse effect of smoking for prognosis of bacteraemia was emphasized. The day 30 case fatality rate in bacteraemic patients were higher in current smokers than in nonsmokers (21.1% vs. 1.4%, $p < 0.001$, RR 18.4; 95% CI 2.2–153.7). Current smokers needed ICU treatment (50.0% vs 18.6%, $p = 0.001$, RR 4.4; 95% CI 1.8–10.5) and mechanical ventilation (31.6% vs 5.7%, $p < 0.001$, RR 7.6; 95% CI 2.3–25.8) more often than nonsmokers during the bacteremia episode. This adverse effect remained even after smoking cessation; 5/28 (17.9%) patients died in the ex-smoker group compared to 1/70 (1.4%) of those who had never smoked ($p = 0.007$, RR 15.0; 95% CI 1.7–135.1). Fifty-nine per cent of males were current or ex-smokers as against 36.9% of females ($p = 0.01$).

Alcohol abuse

The day 30 case fatality rate in bacteraemic patients was higher in alcohol abusers compared to those not given to alcohol abuse (29.2% vs 9.6%, $p = 0.008$, RR 3.9; 95% CI 1.3–11.2). Alcohol abusers needed ICU treatment (66.7% vs 24.8%, $p < 0.001$, RR 6.1; 95% CI 2.4–15.5) and mechanical ventilation (41.7% vs 9.6%, $p < 0.001$, RR 6.7; 95% CI 2.5–18.4) more often than those not abusing. Alcohol abusers died more often in ICU treatment, the difference being, however, not statistically significant (7/16 vs 8/31, $p = 0.211$, RR 2.2, 95% CI 0.6–8.0). Alcohol abusers had high SOFA scores (value > 4 on day 1–3 after positive blood culture finding) more often than those without alcohol abuse (65.2% vs 21.4%, $p < 0.001$, RR

6.9; 95% CI 2.6–18.1). The occurrence of hypotension was more common among abusers than in their counterparts (70.8% vs 31.2%, $p < 0.001$, RR 5.4; 95% CI 2.1–14.0) and were more likely to develop neurological deficit (lowered GSC) (75.0% vs 33.6%, $p < 0.001$, RR 5.9; 95% CI 2.2–16.0). Eighteen out of 21 (85.7%) alcohol abusers were current smokers or ex-smokers and 4 out of 24 (16.7%) had liver cirrhosis.

The effect of obesity, smoking and alcohol abuse on day 30 case fatality were studied together with age, sex and organism in a multivariate model. Obesity remained a significant risk factor associated with case fatality also in this adjusted model ($p = 0.03$, RR 6.4; 95% CI 1.2–34.4), together with smoking ($P = 0.02$, RR 23.0; 95% CI 1.7–321.6).

Discussion

Obesity, smoking, alcohol abuse, COPD and rheumatoid arthritis proved to be significantly associated with case fatality in bacteraemia in univariate model. The effect of obesity and smoking on case fatality also remained significant when studied in a multivariate model together with alcohol abuse, age (continuous variable), sex and causative organism.

Instead of focusing on the clinical findings associated with poor prognosis in bacteraemia, which have been well studied (such as hypotension, leukopenia or leukocytosis or the number of evolving organ dysfunctions) [17,18], we sought to focus on the underlying conditions and chronic illnesses possibly constituting risk factors for case fatality in bacteraemic patients. Most studies in this field deal with patients with severe bacteraemia requiring ICU treatment [3,18]. One of the major advantages of our study was the enrolment of patients evincing different disease severity; patients with milder symptoms and signs as well as patients with septic shock who needed ICU treatment. One of the limitations was that we could not enrol all patients with bacteraemia in our university hospital district during the study period. This limitation excludes determination of population-based incidence rates. However, the investigators did not select the patients they included in the study, the inclusion being based on the microbiological culture finding, and all patients having the same possibility to be recruited by the investigators from Mondays to Thursdays during the study period. Since the clinicians were unable to know any details about the patients nor their disease severity before the recruitment, the selection was purely based on the blood culture finding. This kind of recruitment of patients probably did not cause any selection bias.

Obesity emerged as an independent risk factor for case fatality and obese patients died more often despite ICU

treatment compared to nonobese patients. The published studies examining the association between obesity and in-hospital case fatality give conflicting results concerning the role of body mass index (BMI) as a risk factor for in-hospital case fatality. We found no studies reporting increased mortality due to bacteraemia among obese patients. There are three papers reporting an increased obesity-related case fatality rate in ICU [19-21], these involving patients with multiple reasons for ICU admission, not only infectious causes. In contrast to our study there are also studies where high BMI is not found to be a predictor of case fatality in ICU patients [22-25], and where a low BMI is independently associated with higher case fatality [22,23].

The physiologic mechanisms prevailing between obesity and mortality are unknown. It remains obscure which factors contribute to the increased case fatality in obese in-hospital patients reported in some series. One study showed that obesity exacerbates sepsis-induced inflammation and microvascular dysfunction in the mouse brain [26]. The investigators in question noted microvascular inflammatory and thrombogenic responses, including activation of endothelial cells with subsequent expression of adhesion molecules such as P-selectin in obese mice [26]. Bornstein and associates found that plasma leptin levels are increased in survivors of acute sepsis [27]. The group found that mean leptin levels were three-fold higher in patients who survived the episode than in non-survivors, and concluded that in addition to its function as an anti-obesity factor, leptin may play a role in a severe stress state such as acute sepsis [27]. Recent findings indicate that obesity is an independent risk factor for lipid peroxidation [28] and impaired endothelial cell function [29]. In addition, there appears to be a chronic low-grade inflammation state with elevated acute-phase mediators, cytokines and soluble adhesion molecules which persists in obese individuals [30,31].

Smoking was an independent risk factor for case fatality due to bacteraemia in our study. The effect was most distinct when current smokers were compared to nonsmokers, but it also remained significant after smoking cessation. Smokers more often needed mechanical ventilation and ICU stay, their SOFA score was more often higher than in nonsmokers and they died more often despite ICU treatment. Although the importance of smoking cessation has been emphasized in the therapeutic plan of patients with serious infections [32], there are only a few other studies of the effect of smoking on case fatality in bacteraemia. Arvanitidou and colleagues studied epidemiological and clinical features as potential prognostic factors for outcomes of hospital-acquired bacteraemia in a tertiary care teaching hospital in Greece [14]. They found no differences between non-survivors and survivors in sex,

age or smoking habit [14]. Pittet and associates concluded that smoking or alcohol abuse did not reach statistical significance as independent risk factors for case fatality in septicaemia. However, the number of co-morbidities, also including smoking and alcohol abuse, predicted mortality [2].

Alcoholism emerged as a risk factor for case fatality in univariate analysis, this effect being however diminished when studied in an adjusted model together with obesity, smoking, sex, age and organism. This might be explained by the fact that most alcohol abusers were also smokers, and the effect on case fatality may thus result from smoking, not alcohol itself. In accordance with our findings, there are studies showing that alcoholism is a risk factor for case fatality in pneumococcal bacteraemia in univariate analysis [11,12]. There are however also studies where such an association is not confirmed. Lääveri and associates found no statistically significant association between alcohol abuse and increased case fatality in bacteraemic pneumococcal disease [13], while a group under Laupland et al conducted a population-based surveillance cohort study of severe bloodstream infections and found that alcoholism increased the case fatality rate in bacteraemic patients although the difference was not statistically significant [3].

We had seven patients with rheumatoid arthritis, three of whom died of bacteraemia. The number of patients with rheumatoid arthritis is small, but the results in univariate model suggest that rheumatoid patients have increased case fatality in bacteraemia. Sihvonen and associates conducted a cross-sectional cohort study of rheumatoid arthritis patients and concluded that they carried an increased risk of death from various causes, and they were at increased risk of dying of infections when compared to the general population or controls [33].

Conclusion

In conclusion, our results indicate that obesity, together with smoking, constitutes an important risk factor for case fatality in bacteraemic patients. With the rising prevalence of obesity in Western countries, future research should focus on finding mechanisms responsible for increased mortality in obese bacteraemic patients. The adverse effect of smoking on bacteraemia outcome is an underestimated health risk. Identification of risk factors underlying fatal outcome in bacteraemia may allow targeting of preventive efforts to individuals likely to derive greatest potential benefit.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

All authors planned and carried out the conception and design of the study. J.La., J.S. and R.H. were involved in patient care, and acquisition of data. R.V. was responsible for the blood culture interpretation. All authors were responsible for interpretation of the data. R.H. wrote the first draft of the manuscript, and all authors participated in its revision. All authors had intellectual contribution, and all read and approved the final manuscript.

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HIGH ACTIVITY OF INDOLEAMINE 2,3 DIOXYGENASE ENZYME PREDICTS DISEASE SEVERITY AND CASE FATALITY IN BACTEREMIC PATIENTS

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ABSTRACT—Indoleamine 2,3-dioxygenase (IDO), which is the rate-limiting enzyme for tryptophan (trp) catabolism, may play a critical role in various inflammatory disorders. Recent studies on trauma patients have suggested that the degradation of trp is associated with the development of sepsis. The role of IDO activity in bacteremic patients is unclear. We studied IDO activity in 132 patients with bacteremia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -hemolytic streptococcae, or *Escherichia coli*. The serum concentrations of trp and its metabolite kynurenine (kyn) were measured by reverse-phase high-performance liquid chromatography 1 to 4 days after the positive blood culture and on recovery. The kyn-to-trp ratio (kyn/trp), reflecting the activity of the IDO enzyme, was calculated. The maximum value in the ratio for every patient during 1 to 4 days after positive blood culture was used in analysis. The maximum kyn/trp ratio was significantly higher in nonsurvivors versus those who survived (193.7 vs. 82.4 $\mu\text{mol}/\text{mmol}$; $P = 0.001$). The AUC^{ROC} of maximal kyn/trp in the prediction of case fatality was 0.75 (95% confidence interval, 0.64–0.87), and the kyn/trp ratio at a cutoff level of 120 $\mu\text{mol}/\text{mmol}$ showed 83% sensitivity and 69% specificity for fatal disease. A kyn/trp ratio greater than 120 $\mu\text{mol}/\text{mmol}$ was associated with increased risk of death versus low (≤ 120 $\mu\text{mol}/\text{mmol}$) ratios (odds ratio, 10.8; confidence interval, 3.0–39.8). High IDO activity also remained an independent risk factor for case fatality in a multivariate model adjusted for potential confounders. The data in this report demonstrate that IDO activity is markedly increased in bacteremia patients, constituting an independent predictor of severe disease and case fatality.

KEYWORDS—Bacteremia, indoleamine 2,3-dioxygenase, kynurenine, tryptophan, case fatality, enzyme, IDO, T cell

INTRODUCTION

Severe sepsis is characterized by systemic release of a number of proinflammatory and anti-inflammatory mediators, and the mortality rate is associated with the magnitude of this inflammatory process (1). However, the significance of the blood concentration of any given individual inflammatory mediator as a predictor of clinical outcome is still largely unknown.

Indoleamine 2,3-dioxygenase (IDO) is expressed in a variety of cells, including antigen-presenting cells such as monocyte-derived macrophages and dendritic cells, and is preferentially induced by Th1-type cytokine INF- γ (2). Other cytokines and LPS are also capable of inducing IDO (2). Indoleamine 2,3-dioxygenase catalyzes the degradation of the essential amino acid tryptophan (trp) to kynurenine (kyn) and its derivatives, thereby limiting the availability of trp. Because trp is required for protein synthesis, withdrawal of this essential amino acid from the microenvironment arrests this

process, and subsequent growth of pathogens and proliferating cells (2, 3). A decreased trp concentration and increased concentrations of kyn have been described in various clinical conditions, for example, infection, autoimmune syndromes, malignancies, neurodegenerative disorders, and pregnancy (3). Indoleamine 2,3-dioxygenase induces inhibition of T-cell proliferation (4) and may thus contribute to the pathophysiology of immunodeficiency. On the other hand, IDO may serve as a natural immunoregulatory mechanism. For example, it prevents rejection of the fetus during pregnancy (5). Physiological IDO activity has been implicated in T-cell tolerance to tumors (6) and as a protective negative regulator in autoimmune disorders (7). Although the mechanism of T-cell suppression has been shown to be mediated by trp shortage, toxic catabolites may also play a role (2, 8). Indoleamine 2,3-dioxygenase activity in human serum can be measured by determining the ratio of kyn to trp (i.e., the first metabolite to substrate).

According to a recently increasing body of new data, it is clear that IDO serves more than one function in the immune system (2, 9). However, its precise role in different disease processes is largely unknown. In particular, it is not clear whether it is beneficial or detrimental to the host (2). Previous studies on trauma patients have suggested that degradation of trp is associated with the development of sepsis and poor outcome after major trauma (10, 11). One recent study in mice has shown that survival from endotoxin shock is increased in

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TABLE 1. Maximum kyn/trp ratio 1 to 4 days after positive blood culture in 132 patients with bacteremia and after stratification by causative organism

	Maximum kyn/trp ratio, $\mu\text{mol}/\text{mmol}$, median (quartiles)	P
All patients*	89.9 (54.7–167.6)	0.006†
<i>Staphylococcus aureus</i>	117.3 (64.4–197.5)	
<i>Streptococcus pneumoniae</i>	90.9 (52.5–194.0)	
β -hemolytic streptococcus	123.0 (69.6–312.3)	
<i>Escherichia coli</i>	67.5 (42.3–113.4)	

*Median trp concentration, 50.7 $\mu\text{mol}/\text{L}$ and median kyn concentration, 4.48 $\mu\text{mol}/\text{L}$ on the day of maximal kyn/trp ratio. On recovery (>26 days after the positive blood culture), the median kyn/trp ratio was 36.8 $\mu\text{mol}/\text{mmol}$ (median of trp concentration, 81.8 $\mu\text{mol}/\text{L}$ and kyn concentration, 3.18 $\mu\text{mol}/\text{L}$).

†P value was calculated between four patient groups classified by causative organisms (Kruskal-Wallis test).

IDO knockout (IDO^{-/-}) and IDO inhibitor 1-methyl-trp-treated mice compared with wild-type mice (9).

We determined here the IDO activity in sera from bacteremia patients to study the possible association of IDO with various clinical parameters and case fatality.

MATERIALS AND METHODS

The study material comprised 132 adult patients with bacteremia (70 men and 62 women) aged 60 years (mean; range, 18–93 y) admitted to Tampere University Hospital, Tampere, Finland, from June 1999 to February 2004. Patient recruitment, clinical data collection, and sample collection were prospective. Samples for kyn and trp measurements were analyzed after hospitalization.

In our hospital, blood cultures are routinely taken from patients with symptoms or signs of systemic infection (fever or hypothermia, tachycardia or tachypnea combined with leukocytosis or leukopenia and/or elevated C-reactive protein [CRP]). The BACTEC 9240 (BD Diagnostic Systems, Sparks, Md) blood culture system was used with standard media. Patients were identified according to the microbiological blood culture finding, and only those with bacteremia caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, β -hemolytic streptococcus, or *Escherichia coli*, the most common causative organisms among community-acquired bacteremia, were included in the study. According to the study plan, other microbes were excluded beforehand. Blood culture-negative patients with or without sepsis

TABLE 2. Maximum kyn/trp ratios 1 to 4 days after positive blood culture in 132 patients with bacteremia in relation to sources of infection

Source of infection†	Maximum kyn/trp ratio $\mu\text{mol}/\text{mmol}$, median (quartiles)		P*
	Source, yes	Source, no	
Pneumonia (n = 33)	124.6 (67.6–246.7)	85.7 (50.7–143.8)	0.025
Urinary (n = 29)	61.3 (41.3–106.3)	100.0 (62.3–193.8)	0.003
Skin (n = 33)	123.5 (66.0–229.7)	89.5 (51.2–146.2)	0.057
Osteomyelitis/ spondylitis (n = 13)	82.5 (56.3–104.8)	90.9 (54.6–176.8)	0.549
Gall bladder (n = 6)	135.0 (84.4–321.2)	89.7 (53.2–165.7)	0.146
Endocarditis (n = 4)	160.3 (138.7–193.3)	89.5 (54.0–163.4)	0.097
Mediastinitis (n = 4)	106.5 (90.5–248.7)	89.5 (54.0–167.6)	0.346
Gynecological (n = 3)	145.0 (66.0–)	89.8 (54.2–166.7)	0.298

*Mann-Whitney U test.

†One patient may have several foci.

syndrome and those not consenting were not included. Only patients at least 16 years old were enrolled. The clinicians (J.S. or J.L.) were informed by the clinical microbiologist (R.V.) of a positive blood culture from Mondays to Thursdays, and the patients were enrolled in the study whenever possible to adjust to the daily schedule. We were able to recruit zero to two patients per week during the study period. Because the clinicians had no knowledge of details regarding the patients or their disease severity before recruitment, the selection was based solely on the blood culture finding. After being informed by the clinical microbiologist, the clinicians (J.L. and J.S.) asked patients to participate and interviewed and examined those consenting. Information was gathered from hospital records at the time of a hospital visit, and hospital records were also reviewed subsequent to hospitalization (R.H.). Altogether, 149 of 152 patients agreed to participate. Indoleamine 2,3-dioxygenase activity during 1 to 4 days after positive blood culture was determined in 132 patients, and these were recruited into the final study population. The study was approved by the ethics committee of Tampere University Hospital. Written informed consent was obtained from patients or first-degree relatives.

Underlying diseases and sources of bacteremia were registered. Alcohol abuse was defined as consumption of 300 g absolute alcohol per week or a known social or medical problem due to alcohol use. Patients were defined as current smokers and nonsmokers, that is, those who had never smoked or had stopped smoking. Calculation of body mass index (BMI) was based on weight and height as reported by the patient on admission. Patients were defined as obese if their BMI was greater than 30 kg/m².

Clinical data and laboratory findings were registered on admission and during 6 consecutive days. Alterations in mental status were evaluated on the Glasgow Coma Scale. MAP ([systolic + 2 \times diastolic blood pressure] / 3) and Sequential Organ Failure Assessment (SOFA) score (12) were calculated. The maximum SOFA score (days 0–6) for every patient was used in analysis. Disease severity was assessed by SOFA score, and severe disease was defined as a SOFA score greater than or equal to 4. Laboratory tests included plasma CRP (milligram per liter), blood platelets ($\times 10^9/\text{L}$), plasma bilirubin (micromole per liter), plasma creatinine level (micromole per liter), and blood leukocyte count ($\times 10^9/\text{L}$). The case fatality rate was studied within 14 and 30 days after a positive blood culture (d-14 and d-30 case fatality).

trp and kyn determinations

The samples for kyn and trp determination were taken during patients' hospitalization. Samples were available for 132 patients altogether on days 1 to 4 after the positive blood culture. The serum samples were stored at -20°C until analyzed. Tryptophan (millimole per liter) and kyn (micromole per liter) concentrations in peripheral blood were measured by reverse-phase high-performance liquid chromatography, as previously described (13). Tryptophan was separated with a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co, Kyoto, Japan) using a 50-mm BDS Hypersil C18 5 μm column (Thermo Electron Co, Bellefonte, Pa). It was monitored by fluorescence with a Shimadzu RF-10A XL detector at 266-nm excitation and 366-nm emission wavelengths. Kynurenine was separated with a Hewlett Packard 1100 liquid chromatograph (Palo Alto, Calif) using a Merck LiChroCart 55–4150 mm cartridge containing a Purospher STAR RP-18 3 μm column (Merck Co, Darmstadt, Germany). It was determined by

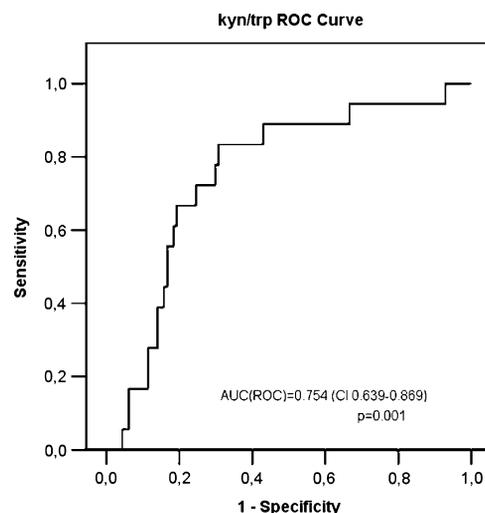


FIG. 1. Receiver operating characteristic curve for maximal kyn/trp ($\mu\text{mol}/\text{mmol}$) ratio detected on days 1 to 4 after positive blood culture in bacteremic patients in relation to case fatality.

ultraviolet absorption at 360-nm wavelength with a Hewlett Packard G13144 detector. The kyn-to-trp ratio (kyn/trp; micromole per millimole) was calculated by relating concentrations of kyn (micromole per liter) to trp (millimole per liter), thus allowing estimation of IDO activity.

Samples for trp and kyn determinations were available on days 1 and 2 (1–2 days after the blood culture was taken), 34 patients; on day 3, 80 patients; on day 4, 104 patients. In addition, 93 patients gave a sample on recovery (>26 days after the positive blood culture). Because patient recruitment was based on blood culture, which only became positive the day after blood culture, no samples for trp and kyn determinations were available on day 0 (blood culture day). There were 1 to 5 samples (median, 4) available per patient collected on separate days. Multiple samplings in the same patient were always performed on separate days. Patients with no measurements on days 1 to 4 after positive blood culture were excluded from the analysis. The maximum value in kyn and kyn/trp ratio and the minimum value in trp for every patient measured during 1 to 4 days after the positive blood culture were used in analysis.

Statistical analysis

An SPSS package (version 7.5) was used for statistical analyses, and a two-sided *P* value less than 0.05 was taken as cutoff for statistical significance. Categorical data were analysed by chi-square test or Fisher exact test when appropriate, nonparametric data by Mann-Whitney *U* test or Kruskal-Wallis test. A logistic regression model was used to study the independent effect of high IDO activity on mortality models adjusted for potential confounders. Odds ratios (ORs) were expressed with their 95% confidence intervals (CIs). The survival curves were calculated using the Kaplan-Meier method, and survival differences between groups were compared using the log-rank test. The accuracy of maximal kyn/trp ratio and maximal CRP and blood culture day CRP in predicting case fatality was evaluated using receiver operating characteristic (ROC) curves (14). By this method, a test that is perfect has 100% sensitivity and no false positives (1-specificity = 0) and will have an

area under the curve (AUC) of 1.0, whereas a test of no diagnostic value would have an AUC of 0.5. The 95% CIs were calculated.

RESULTS

The causative organisms involved here were *S. aureus* (32 patients; 24%), *Str. pneumoniae* (37 patients; 28%), β -hemolytic streptococcus (22 patients; 17%), and *E. coli* (41 patients; 31%). All subjects were treated with an empiric antibiotic regimen, and when necessary, antimicrobial treatment was changed according to culture results. In all patients, the causative organism proved susceptible to the first empiric antibiotic treatment selected on admission. The maximum kyn/trp ratios detected 1 to 4 days after positive blood culture in 132 patients with bacteremia, stratified by causative bacteria, are shown in Table 1 and by the source of infection in Table 2. The maximum kyn/trp ratios were high in acute illness, and ratios decreased on recovery (Table 1). A significant difference in maximal kyn/trp ratios was detected between the causative organisms; ratios were highest in those patients with β -hemolytic streptococcus bacteremia and lowest in those with *E. coli* bacteremia (Table 1). Furthermore, ratios were significantly higher in patients with pneumonia and lower in those with urinary tract infection compared with those without (Table 2).

TABLE 3. Underlying conditions and clinical data in 132 bacteremic patients stratified by maximum kyn/trp ratio 1 to 4 days after positive blood culture ($\leq 120 \mu\text{mol}/\text{mmol}$ vs. those with $>120 \mu\text{mol}/\text{mmol}$)

Character	Maximum kyn/trp ratio $\leq 120 \mu\text{mol}/\text{mmol}$ (n = 81)	Maximum kyn/trp ratio $>120 \mu\text{mol}/\text{mmol}$ (n = 51)	OR (95% CI)	<i>P</i>
Underlying condition				
Obesity (BMI $>30 \text{ kg}/\text{m}^2$)*	14/62 (23%)	12/39 (31%)	1.5 (0.6–3.8)	0.359
Alcohol abuse	8 (10%)	13 (26%)	3.1 (1.2–8.2)	0.017
Current smoking [†]	16/74 (22%)	17/46 (37%)	2.1 (0.9–4.8)	0.067
Cancer (solid or hematological)	17 (21%)	6 (12%)	0.5 (0.2–1.4)	0.174
Diabetes mellitus (type 1 or 2)	22 (27%)	11 (22%)	0.7 (0.3–1.7)	0.470
Rheumatoid arthritis	3 (4%)	4 (8%)	2.2 (0.5–10.3)	0.429
Cardiac disease [‡]	23 (28%)	18 (35%)	1.4 (0.6–2.9)	0.404
Male	39 (48%)	31 (61%)	1.7 (0.8–3.4)	0.157
Age >60 y	41 (51%)	30 (59%)	1.4 (0.7–2.8)	0.357
Clinical data				
Died (d-30 case fatality)	3 (4%)	15 (29%)	10.8 (3.0–39.8)	<0.001
Died (d-14 case fatality)	2 (3%)	10 (20%)	9.6 (2.0–46.0)	0.001
Needed ICU stay	16 (20%)	26 (51%)	4.2 (1.9–9.2)	<0.001
Needed mechanical ventilation	3 (4%)	17 (33%)	13.0 (3.6–47.3)	<0.001
Highest SOFA score ≥ 4	23 (28%)	32 (63%)	4.2 (2.0–8.9)	<0.001
Lowest MAP median (quartiles)	78 (70–93)	63 (54–77)		<0.001
Lowest platelet count ($10^9/\text{L}$), median (quartiles)	189 (117–243)	108 (65–166)		<0.001
Highest SOFA score, median (quartiles)	1 (0–4)	5 (2–10)		<0.001
Highest bilirubin level ($\mu\text{mol}/\text{L}$), median (quartiles)	14 (11–23)	30 (17–60)		<0.001
Highest creatinine level ($\mu\text{mol}/\text{L}$), median (quartiles)	90 (71–123)	173 (114–249)		<0.001
Lowest GCS, median (quartiles)	15 (15–15)	14 (13–15)		<0.001
Highest CRP, median (quartiles)	224 (170–347)	333 (249–405)		0.004
Median neutrophil count ($\times 10^9/\text{L}$) (quartiles) (n = 112)	6.6 (3.8–9.4)	9.3 (6.6–13.3)		0.001

Categorical data were analysed by chi-square test or Fisher exact test and continuous data by Mann-Whitney *U* test.

*Obesity data available on 101 patients.

[†]Smoking data available on 120 patients.

[‡]Valvular, coronary artery disease, heart failure, or cardiac myopathy. GCS indicates Glasgow Coma Scale.

TABLE 4. The effect of predisposing factors and clinical parameters on case fatality in 132 patients with bacteremia in univariate model

	Deceased, n = 18	Survivors, n = 118	OR (95% CI)	P
Grouping variables in univariate model				
Obesity*	7/10 (70%)	19/91 (21%)	8.8 (2.1–37.5)	0.003
Alcohol abuse	6 (33%)	15 (13%)	3.3 (1.1–10.1)	0.041
Current smoking†	7/13 (54%)	26/107 (24%)	3.6 (1.1–11.8)	0.024
McCabe class II or III‡	3 (17%)	19 (17%)	1.0 (0.3–3.8)	1.0
Male	13 (72%)	57 (50%)	2.6 (0.9–7.8)	0.079
kyn/trp >120 µmol/mmol	15 (83%)	36 (32%)	10.8 (3.0–39.8)	<0.001
CRP >224 g/L (blood culture day)§	13/18 (72%)	41/106 (39%)	4.1 (1.4–12.4)	0.008
SOFA score (≥4)	16 (89%)	39 (34%)	15.4 (3.4–70.3)	<0.001
Continuous variables, median (quartiles)				
kyn/trp on days 1 to 2 (n = 34)	143.8 (123.5–312.2)	100.0 (55.3–229.3)		0.166
kyn/trp on day 3 (n = 80)	164.8 (92.7–227.9)	88.7 (54.8–130.8)		0.014
kyn/trp on day 4 (n = 104)	193.8 (72.7–242.5)	66.9 (43.6–110.3)		0.003
Maximum kyn/trp ratio (days 1–4) (µmol/mmol)¶	193.7 (124.1–253.3)	82.4 (51.0–138.9)		0.001
Maximum kyn concentration (days 1–4) (µmol/L)	8.76 (5.15–12.26)	4.27 (2.92–6.72)		<0.001
Minimum trp concentration (days 1–4) (µmol/L)	46.5 (38.0–59.7)	51.1 (38.1–64.4)		0.450
Maximum CRP (mg/L)	304 (209–411)	268 (182–380)		0.292
CRP (mg/L) on the day of positive blood culture§	246 (198–400)	186 (85–303)		0.03

Continuous variables are expressed in medians (Mann-Whitney test).

*Obesity data available on 101 patients.

†Smoking data available on 120 patients.

‡McCabe classes III or III: ultimately fatal or rapidly fatal disease.

§CRP data available on 124 patients.

¶The day of maximum kyn/trp ratio: median trp concentration 43.2 µmol/L in nonsurvivors and 51.1 µmol/L in survivors ($P = 0.208$) and kyn concentration 7.45 µmol/L in nonsurvivors and 4.29 µmol/L in survivors ($P = 0.001$).

Eighteen bacteremic patients died (d-30 case fatality) (8/32 patients in *S. aureus*, 7/37 patients in *Str. pneumoniae*, 2/22 patients in β -hemolytic streptococcus, and 1/41 patients in *E. coli* bacteremia). The maximum kyn/trp ratio, detected on days 1 to 4 after positive blood culture, was significantly higher in those who died (d-30 case fatality) versus those who survived (median, 193.7 µmol/mmol [quartiles 124.1–253.3 µmol/mmol] vs. 82.4 µmol/mmol [quartiles 51.0–138.9 µmol/mmol], respectively; $P = 0.001$). The same was detected in relation to d-14 case fatality (185.3 µmol/mmol [quartiles 123.0–247.8 µmol/mmol] vs. 86.9 µmol/mmol [quartiles 51.2–146.9 µmol/mmol], respectively; $P = 0.005$).

The optimal cutoff value for maximal kyn/trp ratio in predicting fatal disease was estimated using ROC curve, illustrated in Figure 1. The kyn/trp ratio at a cutoff level of 120 µmol/mmol showed a sensitivity of 83% and a specificity of 69% in detecting fatal disease, and this cutoff point was used to classify patients into those with less than or equal to 120 µmol/mmol or greater than 120 µmol/mmol kyn/trp. Underlying conditions and clinical data in cases with kyn/trp greater than 120 µmol/mmol versus less than or equal to 120 µmol/mmol are shown in Table 3. Of underlying conditions, alcohol abusers had somewhat more often high kyn/trp ratios (>120 µmol/mmol) than the patients with no alcohol abuse. High maximum kyn/trp ratio was associated with all clinical parameters indicative of severe disease and poor outcome (Table 3).

The effect of predisposing factors and underlying conditions on case fatality in patients with bacteremia is shown in Table 4. The maximum CRP level did not predict case fatality at any cutoff level in the ROC curve ($P = 0.292$). However,

the AUC^{ROC} for CRP on the day of positive blood culture was 0.66 (0.58–0.782; $P = 0.03$). The CRP at a cutoff level of 224 mg/L showed a sensitivity of 72% and specificity of 61% in detecting fatal disease, and this cutoff point was used to classify patients into those with CRP less than or equal to 224 mg/L or greater than 224 mg/L in the multivariate model. The following grouping variables were associated with case fatality in univariate model: obesity, smoking, alcohol abuse,

TABLE 5. The effect of maximum kyn/trp ratio (>120 µmol/mmol) detected 1 to 4 days after positive blood culture on case fatality in bacteremia adjusted for potential confounders

Variables in multivariate model	OR for high kyn/trp ratio (>120 µmol/mmol)
kyn/trp >120 + age + male sex	10.0 (2.7–37.3)
kyn/trp >120 + CRP >224 g/L (blood culture day)*	9.8 (2.6–37.3)
kyn/trp >120 + McCabe class II or III	11.3 (3.0–42.0)
kyn/trp >120 + causative organism	9.5 (2.5–36.1)
kyn/trp >120 + SOFA score (≥4)†	6.6 (1.7–25.6)
kyn/trp >120 + obesity†,‡	7.8 (1.4–42.2)
kyn/trp >120 + alcohol abuse	9.7 (2.6–36.1)
kyn/trp >120 + current smoking§	10.0 (2.1–48.3)
kyn/trp >120 + high creatinine (>120 µmol/L)	7.4 (1.8–29.5)

*CRP data available on 124 patients.

†Also remained a significant risk factor associated with case fatality in multivariate model ($P < 0.05$).

‡Obesity data available on 101 patients.

§Smoking data available on 120 patients.

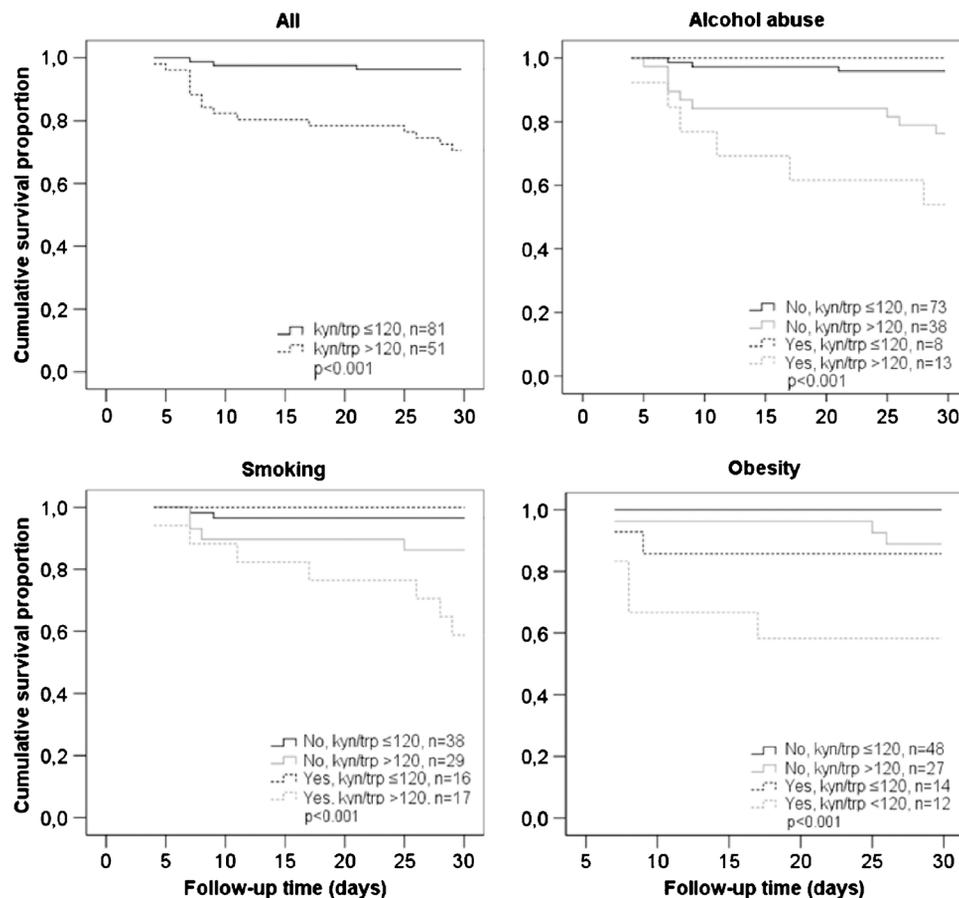


FIG. 2. Cumulative 30-d survival in bacteremia patients with maximum kyn/ntp ratio (days 1 to 4 after positive blood culture) ≤ 120 $\mu\text{mol}/\text{mmol}$ versus those with greater than 120 $\mu\text{mol}/\text{mmol}$ (first inset) and after stratification by underlying conditions proving significant risk factors for case fatality in univariate analysis (alcohol abuse, current smoking, and obesity (BMI >30 kg/m^2). Of underlying conditions, only obesity remained as an independent predictor for case fatality in addition to high kyn/ntp ratio in the multivariate model. Day 0 = the day of positive blood culture (admission). The survival curves were calculated using the Kaplan-Meier method, and survival differences between groups were compared using log-rank test.

high maximum kyn/ntp ratio (>120 $\mu\text{mol}/\text{mmol}$), and high CRP (>224 mg/L) on the day of positive blood culture (Table 3). Interestingly, the median values of kyn/ntp ratios increased from days 1 to 2 to day 4 in those who died, whereas in those who survived, the ratios decreased (Table 4).

The effect of high (>120 $\mu\text{mol}/\text{mmol}$) maximum kyn/ntp ratio on case fatality adjusted for confounders in multivariate models is shown in Table 5. High kyn/ntp ratio retained its significance in the multivariate model in all combinations. Obesity and high SOFA score (≥ 4) also remained independent factors associated with case fatality. Figure 2 shows the cumulative 30-d survival in patients with low and high kyn/ntp ratios and after stratification by underlying conditions, proving significant risk factors for case fatality in univariate analysis.

DISCUSSION

In the present study, the maximum kyn/ntp ratio in patients with bacteremia was more than three times higher than in healthy Finnish blood donors (15), reflecting increased IDO activity in bacteremic patients. Moreover, the present findings show that high IDO activity independently predicts severe disease and case fatality in patients with bacteremia.

Indoleamine 2,3-dioxygenase is an inducible enzyme in immune cells, including dendritic cells, macrophages, and

eosinophils. However, its function in the immune system seems to diverge depending on the type of immune cell and the nature of the stimulus (2, 9). It is not clear whether it is beneficial or detrimental to the host. Indoleamine 2,3-dioxygenase, the rate-limiting enzyme for trp catabolism, has long been known to be operative in antimicrobial defence (16, 17). Some organisms, for example, *Chlamydia pneumoniae*, *Toxoplasma gondii*, and mycobacteria, have been shown to be sensitive to the trp-depleting activity of IDO *in vitro* (18–20), but the biological efficacy of IDO in controlling infections *in vivo* has remained unclear (2). Indoleamine 2,3-dioxygenase-dependent suppression of T-cell responses, in turn, has been shown to function as a natural immunoregulatory mechanism (17). This conception is based mainly on evidence that this mechanism inhibits maternal T-cell immunity against fetal tissues during mammalian gestation (5).

Our findings leave it unclear whether IDO is simply a marker of severe disease or whether it also plays an independent causative role in the pathophysiology of sepsis. The first evidence supporting the latter conception came from a recent study in mice showing that survival from endotoxin shock was increased in IDO^{-/-} and in IDO inhibitor 1-methyl-trp-treated mice compared with wild-type mice (9). Blocking or knocking out IDO reduced IL-12 levels while increasing those of IL-10 in LPS-induced endotoxin shock, resulting in increased survival.

TNF- α and IL-6, which are important proinflammatory cytokines in sepsis pathophysiology and have been shown to lead to septic shock (21), were also markedly lower after LPS challenge in IDO^{-/-} mice (9), indicating that IDO may increase case fatality in sepsis by potentiating the inflammatory reaction. In humans, two previous studies on trauma patients have suggested that the degradation of trp might be associated with the development of sepsis and poor outcome after major trauma (10, 11), and one small study was suggestive of an association between highly increased trp degradation and the severity of *Streptococcus pyogenes* infection (22).

Although high kyn levels and IDO activity were clearly associated with case fatality in this study, trp levels did not predict case fatality. One explanation here could be nutritional status sufficient to maintain endogenous trp levels despite increased degradation. However, nutritional factors are an unlikely explanation for the increased kyn/trp ratios in the present context because kyn and kyn/trp levels are not affected by dietary intake (3). Enhanced trp degradation may result from the activation of either IDO or the hepatic trp 2,3 dioxygenase enzyme. However, IDO seems a more likely activator of the enhanced trp degradation, as trp 2,3 dioxygenase is known to regulate homeostatic serum trp concentrations, and IDO is up-regulated in response to inflammatory conditions. The ratio of kyn/trp is commonly used to reflect IDO activity (3), and it is regarded as a more reliable marker of IDO-induced trp catabolism than serum trp concentration, which may be influenced by dietary intake of the essential amino acid. Alcoholics had high maximum kyn/trp ratios in the present cohort. However, maximum kyn/trp predicted case fatality independently of alcohol abuse in the multivariate model. Although the kyn/trp ratio correlated here with creatinine in bacteremia patients, and hyperkynurinemia has been found in previous studies also in patients with renal failure (23), IDO predicted case fatality also independently of high creatinine.

The study has some limitations. Indoleamine 2,3-dioxygenase activity was not measured on admission, and there were only few patients whose IDO activity was measured 1 to 2 days after the positive blood culture was drawn. However, on days 3 and 4, IDO activity was significantly higher in nonsurvivors versus survivors. The maximum IDO activity in each patient during 1 to 4 days after the positive blood culture was used in analysis. This was considered acceptable because all patients were admitted to the hospital at subjective time points in relation to their disease course, and the duration of symptoms of sepsis could have varied considerably between different individuals on hospital admission. High kyn/trp ratios on days 5 and thereafter are less likely to be bacteremia related than are ratios closer to the time point of positive blood culture.

The current shortage of validated biomarkers of disease severity in sepsis is an important limitation when attempting to stratify patients into homogeneous groups, to study pathogenesis, or develop therapeutic interventions. Further studies are needed to address the specific contribution of IDO to host defence against infection and to establish whether blocking IDO may have effects beneficial to the host.

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