Innate immunity in critical illness - genetic variations and early inflammatory response

PhD thesis

by

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The Present thesis is based on the following three papers

I. Submitted

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Pre-existing low-grade inflammation affects the inflammatory response to low-dose endotoxemia in healthy volunteers.

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CONTENTS

PREFACE

The clinical part of this study was carried out at the Department of Anaesthesiology, Herlev University Hospital, in the period from 2002-2005. DNA extraction and typing of MBL2 SNPs were analyzed at the Department of Clinical Immunology, Rigshospitalet. Typing of the TNF and IL-18 SNPs and cytokine analyzes at the Center of Inflammation and Metabolism, Department of Infectious Diseases, Rigshospitalet.

I wish to thank my supervisors for their support throughout the years. My main supervisor Jan Bonde for providing the basis of the project, practical guidance as leader of the ICU and being encouraging and supporting. I thank my project supervisor Anders Larsson for his fruitful contribution to the 'form' of the project and never failing support and academic guidance even at a long-distance. I thank Jørn Wetterslev for his valuable methodological contributions, providing statistical analyses and always being encouraging and supporting, Peter Garred for his academic guidance in introducing me into the field of genetic markers, providing statistical analyzes and being encouraging and supporting, Bente Klarlund Pedersen for her fruitful academic contribution to the 'form' of the project, Jens Otto Jarløv for his support and contribution concerning microbiological aspects of the project.

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Without their help this work could not have been accomplished.

Finally I wish to thank the patients and their families who, in a time of great anxiety, gave consent to participation and the young healthy men, who have shown interest and participated in the experimental endotoxin study.

I am grateful to the Department of Anaesthesiology, Herlev University Hospital for providing the financial funding for this work. Furthermore I am very grateful that I was supported by a PhD grant from the County of Copenhagen and by grants from Consultant Johan Boserup and Lise Boserups Foundation; The Foundation of the Danish Society of Anaesthesiology and Intensive Care; The Danish Hospital Foundation for Medical research in Copenhagen, The Faroe Islands and Greenland; Professor Sophus H Johansens Foundation of the 23th of august 1981 and The Foundation of Director Jacob Madsen and wife Olga Madsen.

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ABBREVIATONS

AUC	The area under the curve, as calculated from T=0 to T=480 min
Cmax	The peak response, i.e. the maximum between T=0 and T=480 min.
CRF	Case record form
DNA	Deoxyribonucleic acid
HR	Hazard ratio
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharid, endotoxin
MAP	Mean arterial pressure
MBL	Mannose (or Mannan) - binding lectin
NI	Nosocomial infection
PAMP	Pathogen-associated molecular patterns
SNP	Single nucleotid polymorphisms
SOFA	Sequential organ failure assessment
TLR	Toll-like receptors
TNF	Tumour necrosis factor

SUMMARY

Innate immunity in critical illness - genetic variations and early inflammatory response

The mortality rate of the critically ill septic patients in intensive care units remains to be high despite advances in the treatment regimes during recent years. It has been recognized for a long time that inherited traits influence the individual's ability to respond and resist appropriately to uncontrolled inflammation and infection. With the sequencing of the human genome and the recognition of the degree of genetic variation that exists within the human population, it has become clear, that an individual's genetic constitution probably has an impact on clinical presentation, treatment response and outcome of disease. This is a major research field as to septic patients.

The aim of this Ph.D. thesis was to investigate whether *MBL2* alleles, the single nucleotide polymorphisms (SNP)s; tumour necrosis factor-α (TNFα)-308, TNFα-238 and interleukin (IL)-18-137 were associated with outcome (sepsis susceptibility, severity of sepsis and mortality) in prospectively enrolled patients admitted to intensive care unit (Paper I and II). For the cytokine polymorphisms the association with nosocomial infection and pneumonia was also investigated. We also investigated the effect of low-grade inflammation on a subsequent inflammatory stimulus using the human endotoxemia model (Paper III).

In the study conducted in the intensive care unit at Herlev University Hospital, 547 patients were included corresponding to an inclusion fraction of 96.0% of the patients who were ready for inclusion in the 18 month study period. Homozygosity for *MBL2* variant alleles (*O/O*) was associated with the highest adjusted mortality rate followed by homozygosity for the normal *MBL2* allele (*A/A*), while heterozygous *A/O* patients had the most favourable outcome. *MBL2* alleles were not associated with mortality in the ICU, but the association appeared soon after discharge from the ICU. No difference in *MBL2* frequency was observed between patients and controls at baseline, and between patients classified as having sepsis or not. There was no statistically significant association between the TNFα-308, TNFα-338 and IL-18 SNPs and the risk of sepsis, nosocomial infection or mortality. However, the presence of the TNFα –238 minority allele actually showed a non-significant 42% increase in mortality after an observation period of 28 days. In the endotoxin study we found that young healthy individuals that had been exposed *in vivo* to a low-dose bolus of

E.coli endotoxin and to a second low-dose endotoxin bolus administered 90 minutes later exhibited an altered inflammatory response compared to a single endotoxin bolus.

These studies suggest that the importance of TNFα-308 and IL-18 SNP should not be overestimated, however they support the hypothesis that MBL and TNFα-238 has a role in the critically ill intensive care unit patients. As the *MBL2* effect appears to be independent of the SOFA score and sepsis diagnosis, which both are important prognostic markers of outcome in ICU patients, the MBL-alleles may have the potential to become a prognostic marker in ICU patients.
Moreover in healthy humans, induction of a low-grade inflammatory response by a previous intravenous bolus of LPS affects several aspects of the clinical as well as the biochemical response to a subsequent LPS bolus.

Future studies concerning MBL should include even larger prospective studies in ICU patients with at least one year of follow-up.
Concerning the examined cytokine polymorphisms TNFα-308, TNFα-238 and IL-18-137 larger prospective studies in critically ill patients in intensive care or meta-analysis of already performed studies are warranted.
The 'low-grade' inflammation endotoxin study should be performed with different time intervals between the two endotoxin boluses to examine the influence of the time interval on the inflammatory response.

DANSK RESUMÉ

Medfødt immunitet hos kritisk syge patienter – genetiske variationer og tidligt inflammatorisk respons

Mortaliteten hos kritisk syge patienter med sepsis på intensiv afdelinger er, på trods af nye behandlingstiltag de senere år, fortsat høj. Man har længe vidst, at nedarvede træk har betydning for den enkeltes evne til af få og respondere passende på ukontrolleret inflammation eller infektion. Efter kodningen af det humane genom og erkendelsen af graden af genetisk variation der eksisterer hos mennesker, er det blevet klart, at menneskers genetiske sammensætning formentlig har indflydelse på det kliniske billede, behandling og outcome og dette er med hensyn til septiske patienter et stort forskningsfelt.

Formålet med afhandlingen var at undersøge, om mannose-bindende lektin (*MBL) 2* alleler og enkelt nukleotid polymorfierne; tumor necrose factor-α (TNFα)-308, TNFα-238 og interleukin (IL)-18-137 er associeret med outcome (modtagelighed for sepsis, sværhedsgraden af sepsis og dødelighed) i prospektivt inkluderede patienter indlagt på intensiv afdeling (Manuskript I og II). For cytokin polymorfierne blev endvidere undersøgt, om de var associeret med nosokomiel infektion og pneumoni. Endvidere undersøgte vi effekten af 'low-grade inflammation' på et efterfølgende inflammatorisk stimulus ved hjælp af den humane endotoxin model (Manuskript III).

I de to første studier på patienter indlagt på intensiv afdeling på Herlev Hospital blev inkluderet 547 patienter svarende til 96 % af de inkluderbare i den 18 måneders lange inklusionsperiode. Homozygoti for *MBL2* variant alleller (*O/O*) var associeret med den højeste korrigerede mortalitetsrate efterfulgt af homozygoti for den normale *MBL2* allel (*A/A*), mens heterozygote *A/O* patienter havde det bedste outcome. *MBL2* alleller var ikke signifikant associeret med død på intensiv afdeling, men associationen viste sig hurtigt efter udskrivelse fra intensiv afdeling. Der var ingen forskel i *MBL2* frekvens mellem patienter og kontroller ved inklusionen og mellem patienter klassificeret som havende sepsis eller ikke sepsis. Der blev ikke fundet nogen signifikant association mellem TNFα –308, TNFα -238, and IL-18-137 og risiko for sepsis, nosokomiel infektion og dødelighed, men der var dog en non-signifikant 42% stigning i mortalitets rate for TNFα –238 minoritets allel efter en observations periode på 28 dage. I endotoxinforsøget fandt vi, at unge raske mænd, der har været eksponeret *in vivo* til en lav-dosis bolus af *E-coli* endotoxin og til en anden bolus

90 min. senere udviser et andet inflammatorisk respons sammenlignet med en enkelt endotoxin bolus.

Det konkluderes, at vigtigheden af polymorfierne TNFα-308 og IL-18-137 ikke skal overvurderes hos kritisk syge patienter men støtter, at MBL og TNFα-238 spiller en rolle. Da det ser ud til, at MBL2 effekten er uafhængig af SOFA score og sepsis diagnose, som i sig selv er vigtige prognostiske markører for outcome hos patienter på intensiv afdeling, kan MBL-alleler have potentialet til at blive en prognostisk markør hos intensiv patienter. Desuden konkluderedes, at det inflammatoriske respons på et senere infektiøst stimuli er ændret, hvis patienten i forvejen har low-grade inflammation.

Fremtidige studier med MBL bør være større prospektive studier med patienter indlagt på intensiv afdeling og follow-up tid bør være mindst et år. Med hensyn til. de tre cytokinpolymorfier er der behov for studier med flere inkluderede intensiv patienter eller metaanalyser af allerede udførte studier. Modelstudiet med low-grade inflammation bør udføres med forskellige tidsintervaller mellem de to endotoxin injektioner for at undersøge tidsintervallets betydning for det inflammatoriske respons.

INTRODUCTION

Epidemiology of sepsis

Sepsis is the leading cause of death in the intensive Care Unit (ICU), and is being diagnosed with increasing frequency probably because of an increase in the proportion of older age in the population [1-3]. The SOAP study from European ICUs showed that > 35% of the patients were classified as having sepsis at some point during their ICU stay with a mortality rate of 27%, rising to >50% in patients with septic shock [4]. Infections in the lung, abdomen, the blood and the urinary tract are the most common sites of infection in sepsis. Cultures are positive in about 50-70% of cases [4,5]. The incidence of different causative organisms is different among studies. In the SOAP study Gram-positive organisms were isolated from 40% of patients, Gram-negative from 38%, Fungi from 17% and 18% of all the infections were mixed with several microorganisms [4].

Definition

The definition of clinical criteria developed 15 years ago by Bone and colleagues [6] to identify critically ill patients with systemic inflammation and sepsis has been used ever since as inclusion criteria in a large number of epidemiologic and therapeutic trials.

Clinical presentation of sepsis

Sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated [7]. Initially, sepsis may be characterized by an increased in inflammatory mediators; but as sepsis persists, there is a shift toward an anti-inflammatory immunosuppressive state [8]. Clinical features may include fever, mental confusion, transient hypotension, diminished urine output or unexplained thrombocytopenia. If untreated the patient may develop respiratory or renal failure, abnormalities of coagulation, and profound and unresponsive hypotension [7].

Innate immunity and inflammation in early sepsis

Host defences can be categorized according to innate and adaptive immune system responses. As an example of two important arms of the innate immune system are the Toll-like receptors (TLRs), which are cell-bound and the complement system. Cells of the innate immune system respond rapidly by means of recognizing pathogen-associated mo-

lecular patterns (PAMPs) on different microorganisms e.g. endotoxin (LPS) from Gram-negative bacteria and peptidoglycan from Gram positive bacteria and initiate responses through pattern recognition receptors called TLRs (Figure 1).

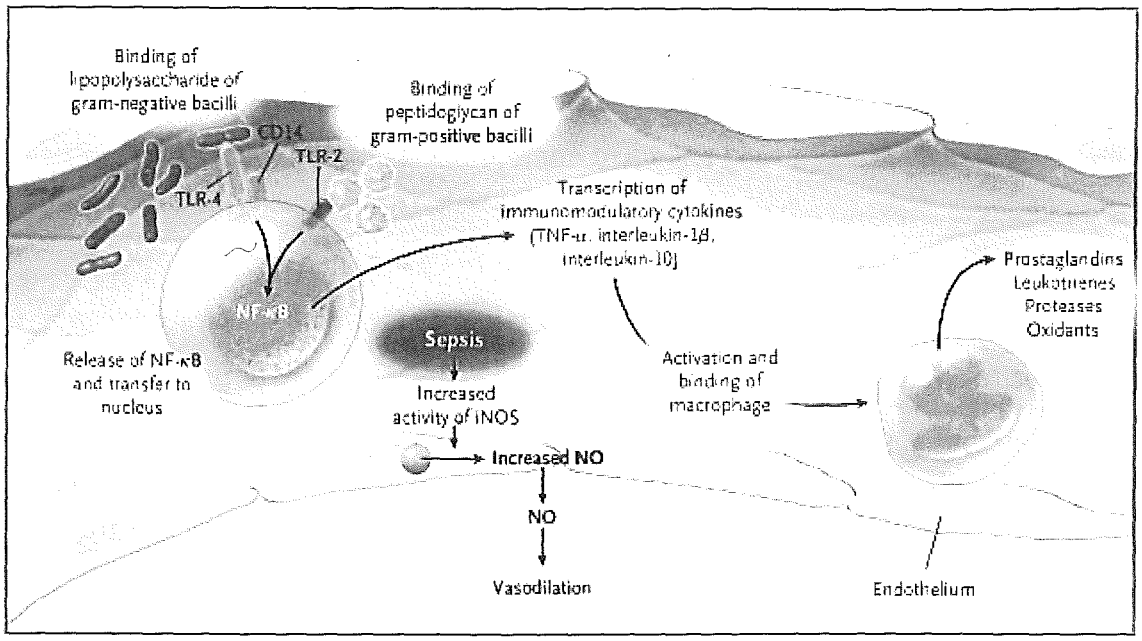

Figure 1 Inflammatory Responses to Sepsis [9]

The binding of PAMPs on TLRs stimulates an intracellular signalling cascade leading to the nuclear translocation of the nuclear factor-κB and ultimately to the activation of cytokine gene promoters. This leads to the transcription of proinflammatory cytokines such as TNFα, IL-1β, IL-6 as well as anti-inflammatory cytokines such as IL-10 [9]. It has also been demonstrated that TLR function plays a major role in the regulation of apoptosis responding to PAMPs [10]. There are more than 10 different TLRs with a wide range of ligand specificity, including bactericidal, fungal and yeast proteins. The one for LPS, a structural component of the outer wall of Gram negative bacteria, is called TLR4 and TLR2 is predominantly responsible for recognizing Gram-positive cell wall structures.

In animal models, administration of TNF induces a syndrome very similar to septic shock, while prior administration of anti-TNF improves the outcome of lethal sepsis in such models [11]. Administration TNF antagonist treatment increased the mortality in septic patients [12] but the fact that anti-TNF strategies failed to prevent death as expected could be related to the difficulty of designing clinical trials in these patients. One practical difficulty encountered is that patients often come to medical attention relatively late in the disease

progression and thereby the blockage of this early cytokine may be too late [7]. Recently the role of TNF-α in combating infection has been underscored by the finding that sepsis and other infectious complications developed in patients with rheumatoid arthritis who were treated with TNF antagonist [13], why treatment in septic patients may be a difficult and like a double-edged sword.

Like the TLRs **the complement system** can be activated by pattern-recognition receptors that have evolved to recognize PAMPs. Most recognition structures involved in complement activation are serum proteins that, in addition to specific antibody, include pattern recognition receptors such as the mannan-binding lectin (MBL), ficolins, C-reactive protein, C1q and natural immunoglobulin M (IgM). There are three different pathways to activate the complement system: classical, lectin and alternative (Figure 2).

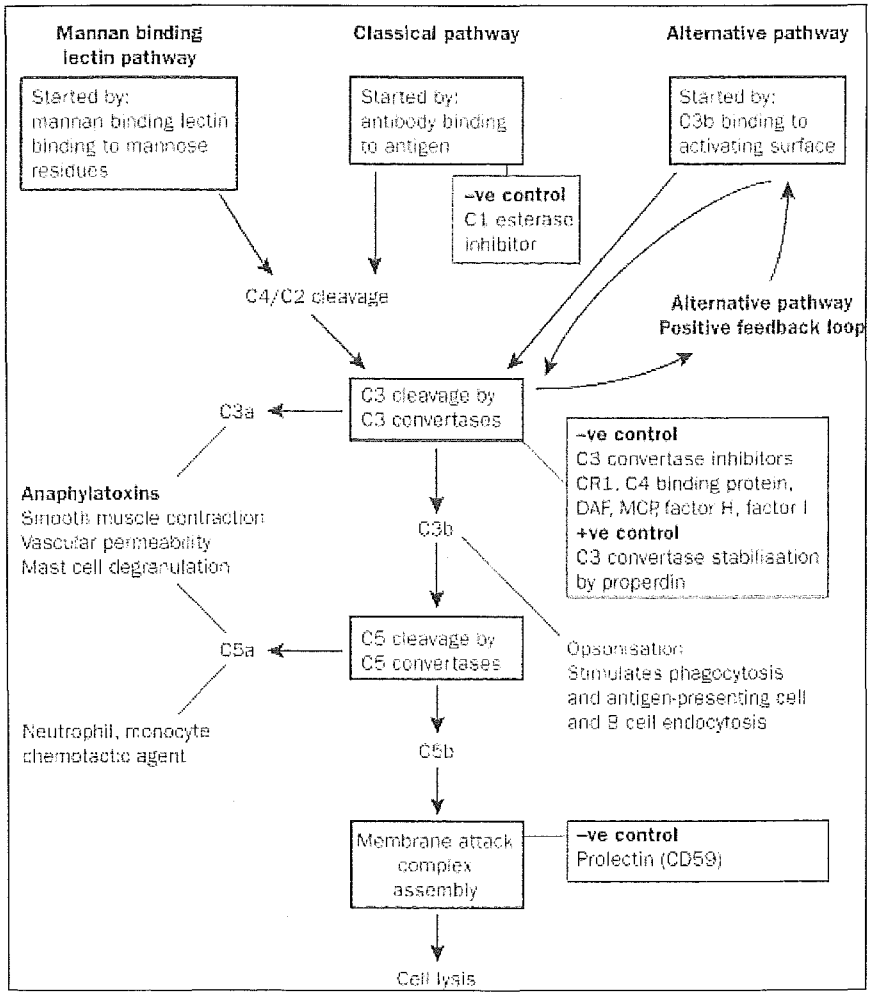

Figure 2 Complement pathways [14]

They differ according to the nature of recognition, but all three pathways share the common step of activating the central component C3. This leads to the final pathway, with the assembly of C5-C9 forming a transmembrane pore (membrane attack complex) in the cell surface and death by osmotic lysis. The classical pathway is activated by antigen-antibody reactions, whereas the two other pathways function in innate immunity. The alternative pathway is continuously turned on because of the spontaneous activation of C3 as it indescriminatly binds a wide range of suitable acceptor sites. The lectin pathway is activated after the recognition and binding of PAMPs by lectin proteins. Until now three members of this pathway have been identified; MBL, ficolin H and ficolin L. The MBL-mediated complement attack is described in Figure 3. Activation of the complement system promotes three main biological activities: opsonisation of pathogens; chemotaxis and activation of leukocytes and direct killing of pathogens. Recently there is evidence that it can function in the disposal of apoptotic cells and also act as an adjuvant by enhancing and directing the adaptive immune system [15,16].

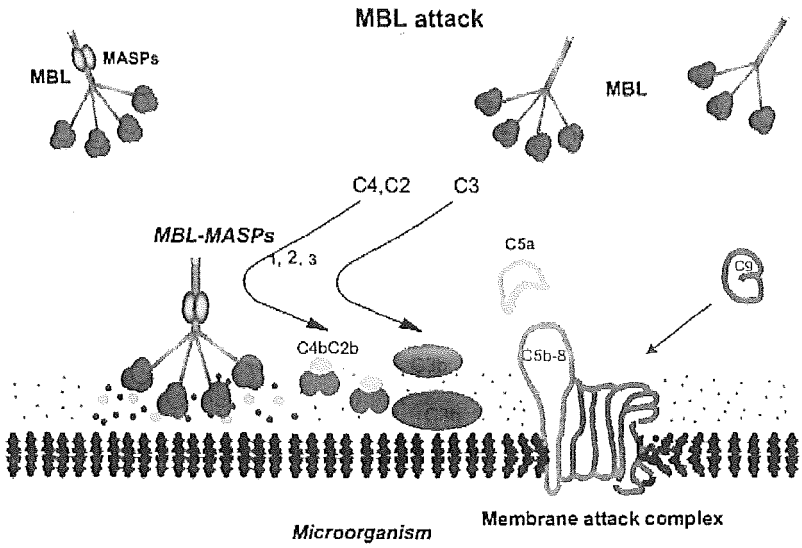

Figure 3 MBL-mediated complement attack. MBL complexed with the MASPs binds to sugar arrays on mikroorganism and mediates a complement attack through MASP2. MASP denote MBL-associated serine proteases [17].

Human MBL is derived from a single gene located on chromosome 10 (mbl2) [18,19]. Inter-individual differences in MBL serum concentration are mainly caused by the structural variant alleles (*B, C,* and *D,* at codons 54, 57, and 52, respectively) in the *MBL2* gene which compromises the assembly of MBL oligomers leading to decreases in the absolute

serum concentration as well as in the functional activity of the protein [17]. Over the last 15 years MBL has been the focus of interest of several research groups owing to its putative clinical role, especially based on epidemiological studies that have suggested that genetically determined variations in MBL serum concentration influences the susceptibility to and course of different types of infections as well as autoimmune, metabolic and cardiovascular diseases (for recent review see [20]).

Secondary infection in the critically ill (The human endotoxinmodel)

Critically ill patients in ICU have an increased risk of secondary infections (nosocomial infections) caused by changes in the environmental load, the resistance pattern of microbes and due to interventions that interfere with natural barriers, such as endotracheal intubation [21]. A change in the inflammatory response to infection may also be implicated [22]. Most critically ill patients have increased plasma levels of pro-or anti-inflammatory cytokines [23,24], the profile of which may have a profound effect on the response to a subsequent inflammatory stimulus.

In the human endotoxemia model, healthy volunteers that received an intravenous bolus injection of endotoxin exhibited a highly uniform and reproducible inflammatory response [25]. This response is characterized by an increase in pro-inflammatory cytokines such as tumor necrosis factor-α (TNF) and interleukin (IL)-1β, followed by an increase in IL-6 and anti-inflammatory mediators such as IL-1 receptor antagonist and IL-10. With large doses of endotoxin (2 to 4 ng per kg), flu-like symptoms peak at 90 minutes, coinciding with the peak in TNF. With lower doses (0.1 to 0.2 ng per kg), the time profile in plasma cytokines is retained albeit at a lower level, whereas none or only minor symptoms are observed [26]. Therefore it is possible that this model could be used to mimic low-grade inflammation.

Genetic aspects of sepsis

On the basis of studies in adoptees and identical twins, genetic factors are known to be major determinants of susceptibility to death from infectious disease [27]. Some persons have single base-pair variations (SNPs) in genes controlling the host response to microbes. Identified alterations include polymorphisms in cytokine genes, TLR, TNF receptors, IL-1 receptors, MBL and Fcγ [20,28-30]. Polymorphisms in cytokine genes may de-

termine the concentration of inflammatory and anti-inflammatory cytokines produced and may influence whether persons have marked hyper-inflammatory or hypo-inflammatory response to infection.

The risk of developing sepsis and mortality from sepsis has been linked to genetic polymorphisms for TNFα and MBL [20,28-30]. Such polymorphisms may ultimately be used to identify patients at high risk for development of sepsis and with high mortality risk. Physicians may in the future be able to use genetic information to dictate immune-based therapy to modulate the response in a given patient.

AIM OF THE STUDY

- We hypothesized that the *MBL2* alleles and the SNPs TNFα-308, TNFα-238 and IL-18-137 in critically ill ICU patients are associated with mortality, the susceptibility to and severity of sepsis and nosocomial infection
- Futhermore we hypothesized that low-grade inflammation, as evoked by an intravenous previous dose of purified *Escherichia coli* endotoxin (lipopolysaccharide, LPS), would enhance the inflammatory response to a subsequent intravenous LPS bolus

The specific aim of the Ph. D. thesis were

- To investigate whether *MBL2* alleles may be associated with outcome (the susceptibility to sepsis and severity of sepsis and mortality) in prospectively enrolled patients admitted to intensive care unit (Paper I).
- To investigate whether the single nucleotide polymorphisms (SNP)s; tumour necrosis factor-α (TNFα)-308, TNFα-238 and interleukin (IL)-18-137 are associated with mortality, nosocomial infections, and nosocomial pneumonia in prospectively enrolled patients admitted to intensive care unit. (Paper II).
- To investigate the effect of low-grade inflammation on a subsequent inflammatory stimulus using the human endotoxemia model. More specifically, we studied the inflammatory response to an intravenous bolus injection of a low dose of purified *Escherichia coli* endotoxin (lipopolysaccharide, LPS) with or without a previous bolus of LPS (Paper III).

METHODS (SHORT)

The ICU study (Paper 1 and 2)

All adult patients (≥ 18 yrs) admitted to the ICU between December 2, 2002 and June 1, 2004 (18 months), were screened for inclusion. Patients readmitted who had been included on their first admission were only registered according to the first admission. Other exclusion criteria were admissions for the sole purpose of fenytoin loading and bronchoalveolar lavage (BAL), constraint commitment because of psychiatric disease, fault referral, and exclusive stay in the post anesthesia care unit due to overbooking of the ICU.

The human endotoxin study (Paper III)

We included 13 male subjects [median age 25 (range, 19-30) yrs; body mass index (BMI), 23.6 (23.2-25.1) kg/ m^2). All subjects were healthy, as defined by an unremarkable medical history, a normal physical examination and normal results on biochemical testing. The subjects had no signs or symptoms of infection in the two weeks preceding either study.

Design

The volunteers underwent three interventions on three separate study days, which were spaced at least one month apart. In a double-blind cross-over design, volunteers were randomized to receive two consecutive intravenous boluses of normal saline or *Escherichia coli* endotoxin (endotoxin, *E. coli*; lot EC-6, United States Pharmacopeia Convention, Rockville, MD, USA), as follows:

Intervention LPS-0.2:	Saline + LPS (0.2 ng/ kg)
Intervention LPS-0.4:	Saline + LPS (0.4 ng/ kg)
Intervention LPS-0.2+0.2:	LPS (0.2 ng/ kg) + LPS (0.2 ng/ kg)

The two bolus injections were spaced 90 minutes apart. Time 0 was defined as the time of the second bolus injection; all times stated below are given in reference to this zero point.

RESULTS

Participants in the ICU study at Herlev University Hospital - Paper I and II

During the 18 months study period 547 ICU patients ≥ 18 years old were included in the study corresponding to an inclusion fraction of 96.0%. More than 99% of the included patients were of Caucasian origin. Of the 547 patients included in the study, 513 (93.8%) met the criteria for SIRS at the first admission date in the ICU and of these 387 (75.4%) met the criteria for sepsis (table 1). Of the 387 patients with sepsis, 199 (51.4%) met the criteria for severe sepsis and 130 (65.3%) of these met the criteria for septic shock.

Mannose-binding lectin (*MBL2*) genotypes in relation to outcome in intensive care patients

The study has shown that ICU patients heterozygous for *MBL2* variant alleles are partly protected from fatal outcome after ICU stay, but more importantly that this effect becomes apparent shortly after discharge from ICU.

We were able to perform complete *MBL2* genotyping in 532 patients accounting for 97.3% of the included patients. At inclusion, genotype frequencies did not differ from those predicted by the Hardy-Weinberg expectations (P>0.55). No significant differences in *MBL2* structural and promoter genotype frequencies were observed between patients and 533 population controls (P>0.8). No significant difference in *MBL2* genotypes between the different SIRS-sepsis groups at admission were observed (P>0.8).
Kaplan-Meier survival curve analysis showed that *O/O* homozygotes were associated with the worst outcome, followed by those with the normal *A/A* genotype, while the heterozygous *A/O* genotype was associated with the most favourable outcome (log rank, P=0.0404) (figure 4 (2a)). It appeared from the survival curve that the *MBL2* association was somewhat delayed in comparison to date of ICU admission, therefore we investigated at what point in time the *MBL2* association appeared. Censoring of patients after ICU discharge revealed that there was no association with the *MBL2* variant alleles during stay at the ICU (log rank, P=0.73) (data not shown)). However, the *MBL2* association appeared immediately after discharge from the ICU and became increasingly apparent during follow-

up when the used baseline was discharge from the ICU (log rank, P=0.0098) (Figure 4 (2b)). In order to pin point in more detail at what time point the *MBL2* association occurred we performed additional Cox regression analyses in a more restricted model after admittance to the ICU corrected for age, gender and the first day SOFA score (Table 4A in Paper I) with censoring at different time points. It was revealed that the *MBL2* alleles overall were independently and significantly associated with survival after 360 days observation period (P=0.023) and after completion of the follow up period (p=0.015). Detailed analysis showed that using the *A/A* genotype as reference, the *A/O* genotype was associated with a significantly better outcome, while the *O/O* genotype was non-significantly associated with the worst outcome compared with the *A/A* genotype (Table 4A in Paper I).

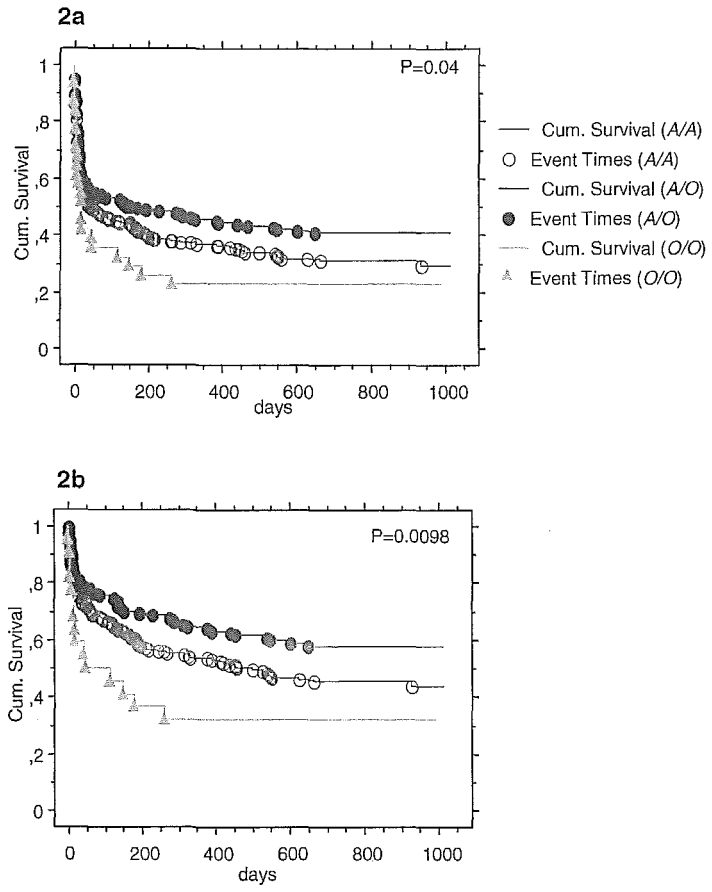

Figure 4

2a Kaplan-Meier survival plot of all 532 included patients during follow-up by the *MBL2* genotype (log rank P=0.0404).

2b Kaplan-Meier survival plot from departure from ICU in 366 patients alive at departure by the *MBL2* genotype (log rank P=0.0098).

The above results indicated the influence of a delayed *MBL2* dependency on mortality. We therefore performed a post hoc analysis in the 366 patients (68.8%) alive at ICU discharge with a determined *MBL2* genotype (Table 4B in Paper I). In this group *MBL2* genotypes were significantly associated with survival already at day 28 after admittance to the ICU with a HR for death for *A/O* of 0.64 (95%CI: 0.4-1.1) and 1.61 (95%CI: 0.8-3.2) for *O/O* (P=0.0477) compared with the *A/A* genotype, while the corresponding figures at completion of follow-up were 0.62 (95%CI: 0.4-0.8) and 1.33 (95%CI: 0.8-2.2), respectively (P=0.0045). Of the individual genotypes only heterozygosity (*A/O*) deviated significantly from the *A/A* genotype, while *O/O* did not. Essentially the same results were observed when we used ICU discharge as baseline (Table 4B in Paper I).
At admission to the ICU 54.7% (n=173) of the patients with the *A/A* genotype had one or more of the following infections; pneumonia, bacteraemia or wound infection, 54.6% with the *A/O* genotype and 51.6% with the O/O genotype. Univariate analysis showed that age, sex, prior disease, immunosuppresion, sepsis, weight, tobacco consumption and the MBL2 genotype were not associated with nosocomial infection (pneumonia, bacteraemia and/or wound infection). Admission type and SOFA score were significantly associated with nosocomial infection both in the univariate and in the multivariate analyses (P=0.003 and P=0.014 respectively).
For patient characteristics, clinical variables and statistical methods see Paper I

The TNF-α SNPs -308 and -238 and the IL-18-137 SNP in relation to outcome in intensive care patients

No statistically significant associations were found between the TNF-α-308, TNF-α-238, and IL-18 -137 SNPs and sepsis, nosocomial infection or mortality. However, it is worth noting that the actually found non-significant 42% increase in early mortality given the presence of TNFα-238 *A* allele cannot be ignored.

We were able to perform a TNFα-238 (rs361525) and a TNFα-308 (rs1800629) genotyping in 535 patients and an IL-18-137 (rs187238) genotyping in 527 patients accounting for 98% and 96% of the included patients, respectively. The SNP genotype frequencies did not differ from those predicted by the Hardy-Weinberg equilibrium at inclusion. Stratifica-

tion of the patients, according to the three examined SNP genotypes, revealed no differences in the rates of SIRS, sepsis, severe sepsis, and septic shock at admission (for all comparisons P>0.7). There was no significant difference in age, sex, type of admission, immunosuppressed, prior disease and admission diagnosis at admission to the ICU stratified according to the different genotypes (for all comparisons P>0.05).

Univariate analyses on all included patients during follow-up until censoring or death revealed that admission type, prior disease, sepsis on admission, immunosuppressed, age, and the worst SOFA score on admission day were significantly associated with mortality (table 4 I Paper II). The TNFα –308, TNFα -238, and IL-18-137 SNPs, sex, pack years of tobacco use, and weight were not significantly associated with mortality. The findings of relations between the 90 days mortality and the examined covariates were similar. However, a non-significant trend for an increased risk of death within 90 days was observed for the presence of the minor *A* TNFα allele in position -238 (P=0.09). Due to the presence of this trend we performed a multivariate analysis for death, adjusted for age and sex at day 28, 90, 360, and in the overall observation period which is shown in table 5 in Paper II. The HR of mortality for the *G/A* genotype compared with the *G/G* genotype was 1.42 (P=0.07) on day 28 and 1.31 for the total observation period (P=0.12).

Correspondingly a similar trend was seen for 28 days survival in a Kaplan-Meier plot (log rank P=0.0546, Figure 5). This trend became less prominent when the whole observation period was taken into consideration (log rank P=0.0945).

For patient characteristics, clinical variables and statistical methods see Paper I

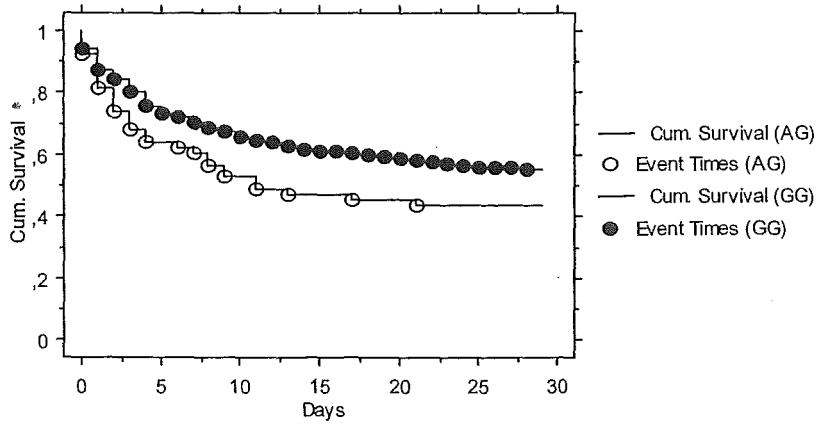

Figure 5. Kaplan-Meier survival plot for 28 days survival of all 535 included patients with a determined TNFα-238 genotype (log rank P=0.0546)

Human experimental endotoxemia

Young healthy humans that have been exposed *in vivo* to a low-dose bolus *E.coli* endotoxin and to a second endotoxin bolus administered 90 minutes later exhibited an altered inflammatory response compared to a single endotoxin bolus. Thus, the temperature increase, lymphopenia and increase in C-reactive peptide are augmented, whereas the TNFα response is attenuated, and the IL-6 response appears not to differ from that observed after a single bolus.

For volunteer characteristics, design, clinical variable and statistical methods see paper III

Physiological variables

Rectal temperature, heart rate and MAP during the three interventions are displayed in Figure 6.

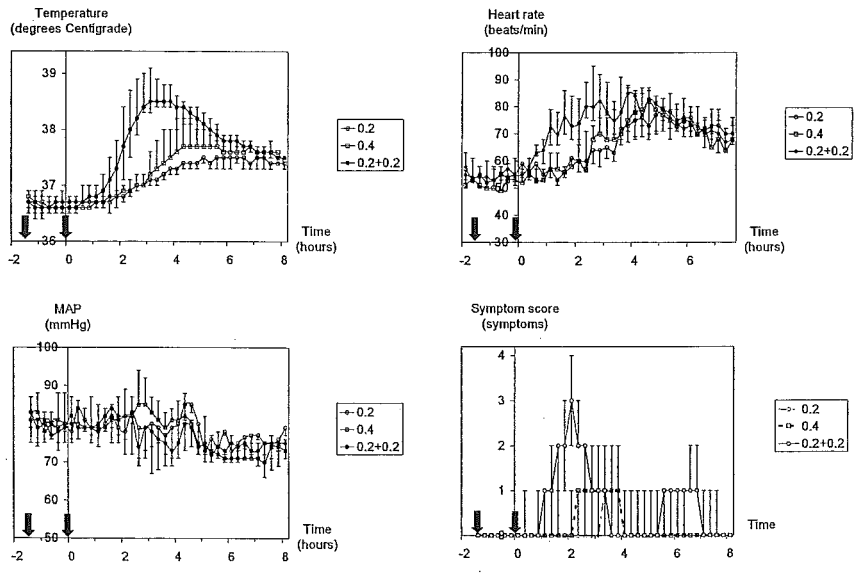

Figure 6 Time course of temperature, heart rate, mean arterial pressure, and symptom score in 13 healthy volunteers after two successive injections of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

Temperature. The temperature increased after all three interventions, to peak at a median temperature of 37.6 (Inter-quartile range 37.4-37.7), 37.7 (37.6-38.3) and 38.5 (38.5-39.2) for LPS-0.2, LPS-0.4, and LPS-0.2+0.2. A significant difference between interven-

tions was found for both the AUC and Cmax (F test for effect of dose, P<0.0005 for both outcome measures; Table 1 and 2 in Paper III). According to the subsequent pair wise comparisons between interventions, both the AUC and Cmax were larger during LPS-0.2+0.2 than during LPS-0.4 and LPS-0.2, respectively. Furthermore, both these outcome measures were higher during LPS-0.4 than during LPS-0.2. According to the definitions given in the Methods section in Paper III, this was interpreted as a supra-additive effect of the split-dose intervention (LPS-0.2+0.2), compared to the single-dose (LPS-0.4 and LPS-0.2) interventions.

Heart rate. An increase in heart rate was observed after all interventions; the peak heart rate was 83 (75-89), 87 (79-89) and 90 (83-99) min^{-1}. The F test revealed a significant difference between interventions for both AUC (P<0.0005) and Cmax (P<0.005). According to the pair wise comparisons, the AUC was larger during LPS-0.2+0.2 than during LPS-0.2 and LPS-0.4. Cmax during LPS-0.2+0.2 was larger than during LPS-0.2, but did not differ from Cmax during LPS-0.4. Furthermore, no difference was found for the outcome measures between LPS-0.4
and LPS-0.2. Altogether, the data were interpreted as "No interpretation possible", i.e., no effect of the split-dose could be inferred when compared to single-dose interventions.

Mean arterial pressure. No change occurred after endotoxin, and no difference was detected between interventions.

Symptom score. Reversible flu-like symptoms were reported during all interventions. During LPS-0.2, mild headache, chills, or myalgia occurred in a total of 10 subjects. During LPS-0.4, chills, headache, shivering (N=4), or myalgias (N=5) were present in a total of 12 subjects. In LPS-0.2+0.2, chills, headache, shivering (N=9), myalgias (N=4), nausea (N=2), and vomiting (N=1) occurred in 12 of 13 subjects. There was a significant difference in symptom scores between the three interventions (Friedman's test, P<0.0005) (Figure 6). The peak symptom score was higher during intervention LPS-0.2+0.2 compared to LPS-0.2 as well as during LPS-0.4 compared to LPS-0.2 (paired Wilcoxons test, P<0.005 for both comparisons), whereas no difference was detected between LPS-0.2+0.2 and LPS-

0.4 (P = 0.29). Together, these results were interpreted as the split-dose having an additive effect on symptom scores, when compared to the single-dose interventions.

Biochemical variables (Table 1 and 2 in paper III, Figs. 7 and 8)

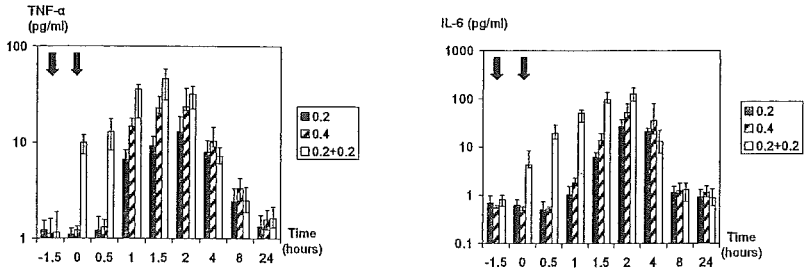

Figure 7

Time course of tumor necrosis factor-α (TNF) and interleukin (IL)-6 in 13 healthy volunteers after two successive injections of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

TNF.

All three interventions were associated with an increase in the arterial concentration of TNF. There was a significant effect of intervention with regard to both AUC and Cmax. Both the AUC and the Cmax were significantly higher during LPS-0.4 than during LPS-0.2. Furthermore, the AUC during LPS-0.2+0.2 was significantly lower than that of LPS-0.4 and did not differ from that of LPS-02. The Cmax did not differ between LPS-0.2+0.2 and LPS-0.2, or between LPS-0.2+0.2 and LPS-0.4. According to the definitions, and since emphasis was put on the effect on the AUC, the results were interpreted as indicating an inhibitory effect of the split-dose endotoxin intervention on the TNF response, compared to the single-dose interventions.

Il-6. The arterial level of this cytokine also increased during interventions. The F test for the difference between interventions yielded a P<0.0005 for both the AUC and Cmax. Both outcome measures were significantly higher during LPS-0.4 than during LPS-0.2, and during LPS-0.2+0.2 than during LPS-0.2, but did not differ during LPS-0.2+0.2 compared to LPS-0.4. This was interpreted as indicating an additive effect of the split-dose intervention on the IL-6 response.

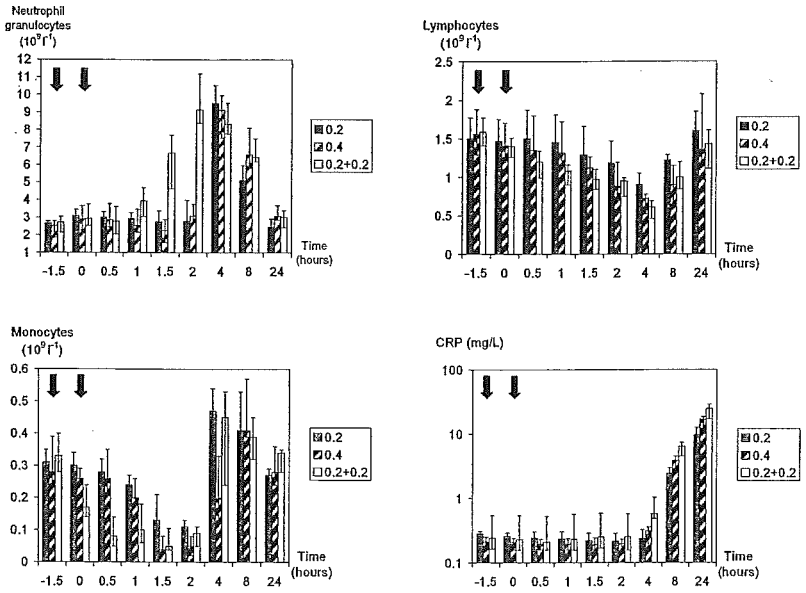

Figure 8
Time course of neutrophils, lymphocytes, monocytes and C-reactive protein in 13 healthy volunteers after two successive injections consisting of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

White blood cell and differential counts. All interventions triggered an increase in total white blood cell and neutrophil counts, and a decrease in lymphocyte and monocyte counts. The F test for the difference between interventions was significant for all these variables with regard to the AUC, and for the lymphocyte count only with regard to the peak response. Furthermore, a dose-response relationship was present only for the lymphocyte count. For this variable, both the AUC and Cmin fulfilled the criteria for a supra-

additive effect of the split-dose intervention. For the remaining cell counts, results were interpreted as indicating no effect of the split-dose compared to the single-dose interventions.

CRP. The CRP, as measured by a high-sensitivity assay, increased after all interventions, and the F test showed a significant difference between interventions with regard to both the AUC and Cmax. These outcome measures showed a dose-effect relationship, i.e. they were higher during LPS-0.4 than during LPS-0.2. Furthermore, they were higher during LPS-0.2+0.2 compared to both LPS-0.4 and LPS-0.2. These results were interpreted as indicating a supra-additive effect on the CRP response of the split-dose compared to the single-dose interventions.

DISCUSSION

METHODS AND ETHICS

The ICU study

The present study provides a number of advantages. Firstly it was a prospective and almost complete study that included 96% of 547 eligible patients in which more than 96% of the patients fulfilling the inclusion criteria over an 18 months period were genotyped, which minimise the risk for selection bias. Secondly it is larger compared with the earlier published MBL and TNFα SNPs studies in ICU populations, which reduces the risk of introducing a type I and a type II error provided that our patients represents the ICU population in general. Herlev Hospital is a general hospital, but has a regional function in nephrology, cancer surgery and oncology. Thirdly the population is an ethnically homogenous caucasian population. Fourthly, the determination of the MBL2 genotype and the three different SNPs TNFα-308, TNFα-238 was done after the data collection was terminated which eliminates the 'confounding by indication' or treatment bias that prior knowledge of the genotypes during treatment at the ICU could have conferred to the results. Fifthly, genotype distribution was in Hardy Weinberger equilibrium [31] and finally, we were able to determine long-term as well as short term outcomes in our patients due to the well established Civil Registration system in Denmark.

The registration of data was thorough and validated in several steps, the latter performed by the coordinating investigator. All data were initially recorded in case record forms (CRF) by the ICU physicians on rounds, a physician from the project group, or by the coordinating investigator. Data from the surveillance files were entered by two secretaries into a new Access database (Microsoft) designed for this project. Validation was performed in several steps by the coordinating investigator. Control of all the CRF and if data were missing, completion from source data was performed by the coordinating investigator. Information was obtained partly from patient files, the Department of Clinical Biochemistry database, the Department of Radiobiology database, and charts from the Department of Clinical Microbiology. The next step of validation was a visual check of the database with CRF. Later series of both systematic and random data 'extracts' were processed. If inconsistencies where discovered, they were corrected according to the source data.

The primary limitation of the study was the 15 included patients with an unknown *MBL2* genotype (2.7%), and the 20 included patients, which we were unable to get both TNFα-308, TNFα-238 and IL-18-137 genotype from (3.7%). However, these patients were only few and the mortality for the two groups were not different from the other 532 with a determined *MBL2* genotype and the 527 with determined SNPs TNF-α -308, -238 and IL-18-137 respectively. Secondly a registration of causes of dead stratified according to the different genotypes would have been appropriate, but the Danish registry of causes of mortality has not yet been entered for our follow-up period.

We chose to use the *MBL2* genotypes instead of measuring the actual concentration of MBL serum on day one because of the very good correlation between the *MBL2* genotypes and the concentration and function of MBL. Previous studies have showed the strength using *MBL2* genotypes in disease association studies [32].

The determination of infection at admission to ICU or nosocomial infection (NI) (here ICU acquired)

The diagnosis of nosocomial infection (bloodstream infection, respiratory tract infection and wound infection) was based on Centre for Disease Control and Prevention (CDC) criteria [33] with the following modifications: If there was any doubt about the diagnosis of whether the patient suffered from nosocomial pneumonia two physicians from a committee of three intensive care physicians, employed in other ICU's, reviewed the CRF and decided whether the patient had a nosocomial pneumonia or not. If they disagreed the third intensive care physician reviewed the CRFs and made the final decision. All the cases of doubt or disagreement (No=15) were reviewed after the inclusion was closed and in accordance with the new CDC criteria and extra criteria [34]. The use of the CDC criteria for NI makes it possible to compare our study with other studies using similar definitions.

However there are several limitations present. Firstly we chose only to register the three most common nosocomial infections, therefore it is possible that some of the patients indeed had another NI, which was not registered. Due to the difficulties in determining the clinical signs of a urinary tract infection in ICU patients with indwelling bladder catheters and differentiating these infections from colonization of the urinary tract, infections and colonization were not counted as a nosocomial infection.

Secondly, not all the patients had the microbial specimens taken on ICU admission; 98.2% of the included had blood specimen taken, 68.0% of the included had tracheal or sputum sample taken and 87.9% of the patients had a urine specimen taken. The diagnose of pneumonia on admission or the possibility to distinguish between a pneumonia diagnosis at admission or day 3 (NI) was compromised as daily chest-X-ray was not performed routinely.

Cohort size calculation

This was initially calculated on the condition to have enough data material, where it was possible to determine possible associations with nosocomial pneumonia and bacteraemia. However, before the data collection started an association between the examined genetic polymorphism and survival were chosen as the main endpoint in the same time frame for the study as well as data collection in only one centre was maintained why the two studies are slightly underpowered. The study still remains to the largest performed to this date.

The study in human experimental endotoxemia

The human endotoxemia model has been used in several studies to address certain aspects of sepsis [35]. This model is only to a limited extent representative of sepsis [36]. Nonetheless, the early cytokine response after endotoxin injection closely resembles that observed in the early phase of severe infection such as severe septic shock [37]. Thus, for the purpose of investigating the early cytokine response we believe that the model serves to provide an adequate mimicry of early sepsis in humans.

To describe the effect of the split-dose intervention as compared to the single-dose interventions on the outcome measures (AUC and Cmax or Cmin), we defined the effect as supra-additive, additive, inhibitory or no interpretation possible (see Paper III). For the variables for which a dose-response relationship was not detected, even though a paired design was used, the ability of this study to detect such a relationship may have been limited by the relatively small number of subjects. This had a direct bearing on the ability to detect a supra-additive effect as well in these variables, since the dose-response effect was required to be present for a supra-additive (or additive) effect to be inferred. On the other hand, we believe that this very conservative approach, as well as the use of a statistical method that enabled us to control for the level of the outcome measures at T=0 in the split-

dose compared to single-dose interventions, serve to underline the robust nature of the supra-additive effect noted for the rectal temperature, the lymphocyte count and the C-reactive peptide. It is also reassuring that analysis of both the AUC and the peak response in these variables indicated a supra-additive effect, even though the AUC was defined as the most important outcome measure in this regard. The present results were probably critically dependent on the dosage, the nature of the endotoxin and especially the timing of the two separate bolus injections. By spacing the bolus injections by 90 minutes, the second bolus was administered at the time when the plasma concentration of TNF was peaking after the first bolus, i.e., during a phase when the circulating levels of pro-inflammatory cytokines were high and virtually unopposed by anti-inflammatory cytokines.
The design was a randomized double-blind cross-over study. The volunteers underwent three interventions on three separate study days, which were spaced by at least one month apart. In this way, we aimed to avoid any putative residual effect of the endotoxin administered on a previous study day. Moreover, the sequence of the three different interventions was randomised; this further minimised the risk that a systematic effect caused by a previous endotoxin dose would influence data interpretation. Finally, the cross-over design, i.e. the fact that the same volunteer acted as his own control, increased the power of the study to detect a systematic difference between interventions despite a relatively low number of participants.

Ethical issues

The regional ethics committee of Copenhagen County and the Danish Data Protection Agency approved the study protocols and informed written consents were obtained from the patients/volunteers or the next of kin prior to inclusion. With regard to the ICU study, we were obligated to ask the patient and relatives as soon as possible after admission, but we were allowed to take admission blood and microbiological specimens within the first 24 hours after admittance to the ICU, even if the patient or relatives had not yet been asked about study inclusion. If they did not consent, the specimens were destructed. If possible, the patients were informed about the study when regaining a sufficient level of consciousness and were given the option of withdrawing the consent.

Concerning the low-dose endotoxin study, high-dose (2 ng/kg) and low-dose protocols had already been ethically approved and performed in Denmark before our study was per-

formed. It is well-known that high-dose endotoxin induces illness for approximately 5 hours with fever (up to 39°C) and chills, but in young volunteers no haemodynamic effects were observed. No or minimal symptoms were ensued after low-dose endotoxin.

We elected to conduct the endotoxin study despite the risk of inducing discomfort in healthy volunteers in order to obtain further knowledge about the early pathophysiological events in sepsis. Septic patients are notoriously difficult to study, and the results obtained in these patients are hard to interpret because of pre-existing co-morbidities, uncertainty about the time of onset of sepsis, and ensuing organ failure. Moreover, animal studies can not always replace studies in humans. However, an improved understanding of the early inflammatory response might potentially lead to improvements in management and therapy of this life-threatening condition. Apart from the discomfort described above, the human endotoxemia model is generally accepted as safe, and serious adverse events, to our knowledge, have never been reported. As the split-dose intervention had never previously been performed, we elected to administer LPS in low doses (0.2 ng/kg+0.2 ng/kg). The study was performed in the ICU with continuous surveillance by an intensive care physician and the volunteers were not allowed to leave the unit before they were without fever and feeling well. The studies were performed in accordance with the Helsinki Declaration II and the study on human endotoxemia was registered in an international clinical trials database (ClinicalTrials.gov ID NCT00197899).

RESULTS

The ICU study

Heterozygosity for the *MBL2* structural genotypes (*A/O*) was significantly associated with improved survival compared with the normal *A/A* genotype and the homozygous variant *O/O* genotype after follow-up (censuring) period following admittance to the ICU. This association was independent of the SOFA score, age, gender and the SIRS-sepsis consensus criteria on the first day spent in the ICU. These findings indicate that low and intermediate serum levels of MBL may confer a survival advantage in response to both severe infections and acute inflammation. A mechanistic explanation to this observation could be that low and intermediate levels of functional MBL (*A/O*) would confer a relative advantage both in terms in of optimal antimicrobial activity and less pro-inflammatory effect. On the other hand deficiency of functional MBL (*O/O*) will make the patients more prone to become severely infected, while high levels of functional MBL (*A/A*) could directly be associated with the proinflammatory adverse effect following uncontrolled complement activation.

From a clinical perspective was the main and most important finding that may be derived from this study the observation that the *MBL2* alleles almost immediately after discharge from the ICU appeared to be associated with either increased or decreased protection against mortality, while no such effect was observed during ICU stay. Particularly those homozygous for the variant alleles (*O/O*) may have the highest risk. Our interpretation of this finding is that the combination of optimal surveillance and treatment combined with the fact that in a number of patients admitted to intensive care withdrawal of active life support to terminal patients is allowed might mask the *MBL2* effect in relation to survival. Thus, we hypothesize that after intensive surveillance and treatment are halted and the patients are referred to their respective departments with less intensive treatment the *MBL2* effect may become apparent. In addition, as the *MBL2* effect appears to be independent of the SOFA score and sepsis diagnosis, which by themselves are important prognostic markers, it may be helpful to add *MBL2* genotyping to the prognostic staging parameters of ICU patients. In a previous smaller study including 272 ICU patients [38] it was observed that the *MBL2* *O/O* genotype also had a negative impact on survival after ICU stay and that this was independent of the so-called simplified acute physiology score II (SAPSII) and sepsis diagnosis, but no *A/O* advantage could be detected. When MBL serum concentration was

measured in a prospective insulin treatment study in patients that survived at least 5 days in the ICU it was observed that those functionally deficient of MBL, who did not receive insulin had an increased mortality rate consistent with the present findings [39].

We found that the frequency of the *MBL2* alleles at inclusion was similar as the one in the Danish population controls and these findings were consistent with a previous study [38]. The frequency of *MBL2* variant alleles was not significantly different between ICU patients classified as having sepsis or not or the severity of sepsis, which is in variance with a former study [38]. We found that the MBL2 genotype was not associated with nosocomial infection (pneumonia, bacteraemia and/or wound infection).

Our design of this observational study was robust particulaly concerning the analysis of the association with survival and it is the largest study compared with the previous published MBL studies in ICU populations. Concerning nosocomial infections, methodological problems encounterred with the determination of especially nosocomial pneumonia may have lead to an underestimation of the actual number and thereby hamper the validity of the results.

The TNF-α SNPs -308 and -238 and the IL-18-137 SNP in relation to outcome in intensive care patients

We investigated the possible association between each of two TNFα SNPs in position -308 and -238 and one IL-18 in position –137, and sepsis and acquisition of nosocomial infection as well as fatal outcome. Overall no statistically significant associations were observed between the outcome measures and the investigated covariates. The examined TNFα and IL-18 SNPs overall did not show any statistically significant associations between the investigated covariates and the outcome measures. However a non-significant trend that carriers of TNFα -238 G/A genotype had a higher mortality than G/G carriers was found in both univariate analysis and Cox regression analysis after correction for age and gender. The non-significant HR was most pronounced early during follow-up since the HR after 28 days was 1.42 while it was 1.31 at completion of the study.

In a study including 213 caucasian ICU patients, the genotype frequencies for the TNFα-238 SNP were not significantly different in ICU survivors and non-survivors, but 16.3% of the non-survivors had the *G/A* genotype compared with 12.8% of the survivors giving a

relative risk of 1.27 [40], which is within the confidence limits observed in this study. Furthermore in accordance with the above mentioned studies we did not find an association between the TNFα-238 SNP and susceptibility to sepsis and nosocomial infections [40,41]. The possible relationship between the TNFα-238 SNP and an increased mortality should be examined in further larger studies.

We did not detect an association between the TNFα-308 SNP and susceptibility to sepsis and/or mortality. Data from other studies yield conflicting results. Some studies find an association [41-45], while others do not [40,46]. Possible explanations for these conflicting observations are the much smaller size of these studies (maximum 152 included), lack of completeness of the cohort admitted to the ICU, ethnically mixed populations and lack of healthy control groups.

The present finding of no association between the IL-18-137 SNP and the susceptibility to the development of nosocomial infection, or mortality concurs with a study of 69 post-injury patients [47].

We find the design of our study robust and it is the largest performed to date, however it may have been underpowered to detect a relevant difference and indicate that even larger studies than ours must be performed to detect genetic effects. Another possibility would be to include all studies conducted so far into a meta-analysis.

The study in human experimental endotoxemia

The main finding of this study was that in young healthy individuals, *in vivo* induction of a low-grade inflammatory response by a previous bolus injection of *E-coli* endotoxin affects parts of the inflammatory response to a subsequent *in vivo* endotoxin challenge. Thus, a split dose compared to the administration of the same total dose as a single intravenous bolus dose, had supra-additive effects on rectal temperature, lymphocyte count (lymphopenia) and C-reactive peptide. In contrast, the IL-6 response appeared not to be affected by the split-dose when compared to the single-dose interventions (additive effect), whereas the TNF response appeared to be attenuated (inhibitory effect).

In the present study, we found indications of co-existing "priming" [48,49] and "tolerance" [50] phenomena. Thus, the inflammatory response was amplified with regards to tempera-

ture as well as CRP and the lymphocyte count. This amplification was not found with regard to the two cytokines measured; in particular, the TNF response appeared to be significantly lower. This suggests, firstly, that the down-regulation of TNF alone may be a necessary, but not a sufficient stimulus to induce clinical "tolerance" in humans as TNF down-regulation was observed despite of an amplification of clinical symptoms. Secondly, the observations suggest that "tolerance" and "priming" phenomena may not be mutually exclusive phenomena.

A number of studies have demonstrated an attenuated *ex vivo* production of chemo- and cytokines after a single *in vivo* endotoxin challenge in healthy volunteers [51,52]. This attenuated *in vitro* response was demonstrated as early as one hour after endotoxin injection (4 ng/kg), i.e. at a dose that was approximately 10 times as large as the largest dose used in the present study, and at a time that is associated with significant clinical symptoms. These findings also suggest that an attenuated *ex vivo* response may not correlate with an attenuation *in vivo*, and that up- and down-regulation of different aspects of the inflammatory response may coincide.

Healthy elderly volunteers exhibit a more pronounced and protracted inflammatory response to endotoxin [53] and to bacterial infections [54] than their younger counterparts. Therefore, we suggest that the present results would be similar or even more enhanced, had we used healthy older volunteers.

Our first motive to perform this study was to examine if the 'low-grade' inflammation patients in intensive care often have, are what makes them more prone to nosocomial infections. It is not possible to answer this question after this experimental study, however we find that it made a small contribution to the examination of when the immune system goes from amplification of the pro-inflammatory response to an endotoxin tolerance.

CONCLUSIONS

We found that

- ICU patients heterozygous for *MBL2* variant alleles are partly protected from fatal outcome after ICU discharge, but more importantly this effect becomes apparent shortly after discharge from ICU. No association was found with the susceptibility to sepsis and the severity of sepsis, nosocomial infection and nosocomial pneumonia.

- There were no statistically significant associations observed between the selected TNFα-308, TNFα-238 and IL-18 polymorphisms with sepsis susceptibility and severity of sepsis, nosocomial infection, nosocomial pneumonia and mortality in ICU patients. However, the presence of the TNFα –238 minority allele actually showed a non-significant hazard ratio for early mortality of 1.42

- Young healthy individuals that have been exposed *in vivo* to a low-dose bolus *E.coli* endotoxin and to a second endotoxin bolus administered 90 minutes later exhibited an altered inflammatory response compared to a single endotoxin bolus. Thus, the temperature increase, lymphopenia and increase in C-reactive peptide are augmented, whereas the TNF response is attenuated, and the IL-6 response appears not to differ from that observed after a single bolus.

PERSPECTIVES

As the *MBL2* effect appears to be independent of the SOFA score and sepsis diagnosis, which both are important prognostic markers of outcome in ICU patients, the MBL-alleles may have the potential to become a prognostic marker in ICU patients.

Future studies concerning MBL should include even larger prospective studies in ICU patients with at least one year of follow-up.
Concerning the examined cytokine polymorphisms TNFα-308, TNFα-238 and IL-18-137 larger prospective studies in critically ill patients in intensive care or meta-analysis of already performed studies are warranted.
The endotoxin study with 'low-grade' inflammation should be repeated with different time intervals between the two endotoxin boluses to examine the influence of the time interval on the inflammatory response.

In the ICU study data collection a Bio Bank with DNA and daily plasma samples have been collected from the included patients. After the completion of this thesis we have planed to performe a new study where we want to examine a new panel of genetic polymorphisms concerning the innate immune system of significance for infection and inflammation in ICU patients

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Heterozygosity of mannose-binding lectin (*MBL2*) genotypes predict an advantage (heterosis) in relation to fatal outcome in intensive care patients

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Running head: *MBL2* and fatal outcome

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ABSTRACT

Background: Polymorphisms in the *MBL2* gene, which determine function and serum concentration of mannose-binding lectin (MBL), are associated with inflammatory and infectious conditions. The importance of *MBL2* polymorphisms on outcome in critical ill patients is unclear.

Patients and Methods: 532 consecutive critically ill patients admitted to a multidisciplinary intensive care unit (ICU) were included over a period of 18 months. 533 randomly selected individuals served as controls. Vital status for all patients was obtained 15.5 months after the last patient was included. *MBL2* polymorphisms were determined with a PCR-based assay.

Results: Homozygosity for *MBL2* variant alleles (*O/O*) was associated with the highest adjusted mortality rate followed by homozygosity for the normal *MBL2* allele (*A/A*), while heterozygous *A/O* patients had the most favourable outcome (P=0.015). *MBL2* alleles were not associated with death in ICU (n= 166, P=0.7), but the association appeared soon after in patients that were alive at discharge from ICU (n=366): at day 28 after inclusion: hazard ratio (HR) for *O/O* using *A/A* as reference was 1.61 (95%CI: 0.8-3.2) and for *A/O* it was 0.64 (95%CI: 0.4-1.1) (P= 0.0477), while the corresponding figure at completion of the study was: HR 1.33 (95%CI: 0.8-2.2) and 0.62 (95%CI: 0.4-0.8), respectively (P=0.0045). No difference in *MBL2* genotype frequency was observed between patients and controls, and between patients classified as having sepsis or not. However, patients with the *MBL2 O/O* genotype had an increased frequency of *Gram*-positive bacterial infection (P=0.01).

Conclusions: Heterozygosity for *MBL2* variant alleles appeared to confer a protective effect on fatal outcome while homozygosity for *MBL2* alleles was associated with the worst outcome soon after discharge from ICU. Thus, this may be an example of heterosis.

INTRODUCTION

Systemic inflammation and sepsis are the primary causes of death in intensive care units (ICU) despite advances in the treatment regimes over the recent years [1-4], latest with the guidelines of "The Surviving Sepsis Campaign" from 2004 [5]. The SOAP study from European ICUs showed that > 35% of the patients were classified as having sepsis at some point during their ICU stay with a mortality rate of 27%, rising to >50% in patients with septic shock [6]. Even though the pathophysiology of systemic inflammation and sepsis is complex it has long been recognized that inherited traits influence the individual ability to respond and resist appropriately to uncontrolled inflammation and infection [7].

One of the genetically determined factors that have been suggested to be involved in systemic inflammation and sepsis is mannose-binding lectin (MBL) [8]. MBL is a liver derived serum protein, which acts as a pattern-recognition molecule by binding to mannose and N-acetyl glucosamine containing molecular patterns present on various microorganisms (for review see [9]). Moreover, MBL is also involved in sequestration of endogenous waste material and plays an important role in tissue homeostasis [10,11]. In serum MBL is associated with the so-called MBL-associated serine proteases (MASPs) enabling activation of the complement system [12]. Human MBL is derived from a single gene located on chromosome 10 (*MBL2)*. Inter-individual differences in MBL serum concentration are mainly caused by structural variant alleles (*B, C,* and *D,* at codons 54, 57, and 52, respectively) in the *MBL2* gene which compromise assembly of MBL oligomers leading to a decrease in the absolute serum concentration as well as in the functional activity of the protein (for review see [13]). Of particular interest is the fact that heterozygosity for *MBL2* variant alleles causes in average a 85- 90 % drop in the serum concentration of functional MBL compared with the normal genotype while homozygous for the structural variant alleles are devoid of functional MBL [14]. The normal *MBL2* allele is named *A,* and the common designation for the variant alleles is *O.* In addition to the effect

of the structural alleles variants differences in MBL serum levels are determined by polymorphic sites in the promoter region of the *MBL2* gene [15,16]. In particular, a base substitution at codon -221 (G→C; promoter allele *X*) is associated with a lower MBL serum concentration. Presence of *MBL2* polymorphisms is associated with increased risk of respiratory infections during early childhood, especially during the first 6-18 months of life [17], and in patients with a concomitant immunodeficiency or severe disease [18-21]. However, the *MBL2* variant alleles are very frequent in different population around the world [13]. Therefore, we an others have speculated whether the high frequency of these alleles are promoted by selective advantages of being heterozygous in analogy with the sickle cell hemoglobin S allele and malaria [14,22,23].

Mice devoid of MBL have been generated by knock out technology [24]. However, in contrast to the human situation mice have two functional MBL genes. In a sepsis model in which both MBL genes were deleted it was shown that MBL offered protection against *S. aurus* when bacteria were inoculated directly into the blood stream [24]. By contrast in a more physiological model using the ceceal ligation and puncture procedure to induce sepsis in which only one of the mice MBL genes were deleted paradoxically the gene targeted mice where more protected against death than the wild type mice [25]. In another partial MBL knock out mice model blood borne nematode *Brugeria malayi* microfilaria survived significantly longer than their wild type counterparts [26]. However, no differences in cytokine responses were observed. These results clearly indicate a dual and complex role of MBL in relation to systemic inflammation and infections. The importance of MBL as a susceptibility and modifying factor in humans for the development of sepsis and later mortality has been examined in the last years [27,28]. These results indicate that the presence of *MBL2* variant alleles and low serum levels of MBL have been associated with the development of sepsis, and weakly associated to fatal outcome in adult patients admitted to intensive care and in children with the systemic inflammatory response syndrome (SIRS). In a recent British study it was observed that *MBL2* variant alleles are more common in adults with severe sepsis and septic shock than in normal population controls but

no demonstrable influence on outcome was seen [29]. Previously it has been shown that *MBL2* variant alleles are associated with susceptibility for meningococcal disease, but again no significant effect on outcome was observed [30].

The conflicting results and the fact that MBL appears to play a complex role in SIRS and sepsis led us to investigate whether *MBL2* alleles may be associated with outcome in prospectively enrolled patients admitted to intensive care.

MATERIALS AND METHODS

Setting

Herlev Hospital in Copenhagen, Denmark is a university hospital with 568 beds. The central mixed surgical-medical ICU serves the medical and surgical blocks of the hospital. The ICU is a six-bed unit with exclusively single-bed rooms.

Study population and design

A schematic summary of the study design, inclusion, and exclusion criteria is presented in Figure 1. All adult patients (≥ 18 yrs) admitted to the ICU between December 2, 2002 and June 1, 2004 (18 months), were screened for inclusion. Patients readmitted who had been included on their first admission were only registered according to the first admission. Other exclusion criteria were admissions for the sole purpose of fenytoin loading and broncho-alveolar lavage (BAL), constraint commitment because of psychiatric disease, fault referral, and exclusive stay in the post anesthesia care unit due to overbooking of the ICU. The Institutional Review Boards of the County of Copenhagen approved the protocol (file number KA 02071). Informed consent was obtained from the patients or from their relatives.

All data were initially recorded in case record forms by the ICU physicians on rounds, physicians from the project group or by the coordinating investigator. At admission to ICU baseline clinical information concerning underlying disease, cause of admission, tobacco consumption, immunosuppressive factors, infection on admission and admission diagnosis where recorded in the CRF. Chest radiography was usually performed at admittance. Bacterial cultures were taken on admission from blood, nasopharynx, urine and tracheal aspirate or expectorate. Cultures were processed according to standardized methods on the Department of Clinical Microbiology. Organ failure at admission

was defined according to the 'worst value' of the first day sequential organ failure assessment (SOFA) score [31]. The SIRS-Sepsis criteria were recorded [32] in the five classes; None, SIRS, sepsis, severe sepsis and septic shock. From the Central Office of Civil Registration in Denmark we requested and received a vital status for all included patients at the 14th of September 2005; if death had occurred the date of death was registered.

Controls

As control subjects served 533 randomly selected individuals (mean age 50 years, range 18-67 from the Danish population by means of the Civil Registration System [33].

Previous disease (Underlying disease): One or more of the following conditions were recorded: acute myocardial infarction (AMI), congestive heart disease, insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus, NIDDM, Acute pancreatitis, chronic pancreatiti hepatic disease, renal disease, chronic obstructive pulmonary disease (COPD), active malignant disease, recent trauma.

Immunosuppressive factors: one or more of the following conditions: diabetes mellitus, cirrhosi of the liver, immunosuppressive agents such as use of high doses of corticosteroids, alcohol or dru abuse, active malignant disease, or renal failure (Se-creatinine ≥200 mol/l) were registered.

Tobacco consumption: Divided in current smoking, ex-smokers, possible ex-smokers, never smoked, not known. Number of pack years registered. One pack year is equal to smoking of 20 cigarettes daily in one year.

Summary of classification criteria (SIRS, sepsis, severe sepsis, and septic shock)

SIRS, sepsis, severe sepsis, and septic shock and none were defined and registered in accordance with the recommendations of the American College of Chest Physicians/Society of Critical Care

Medicine Consensus Conference [32]

Genotyping methods

Blood collected in 10 ml EDTA containing vacutainers was collected for DNA extraction. Anticoagulated blood was frozen at -80°C before processing. *MBL2* single nucleotide polymorphisms (SNPs) in form of the structural variants named *B* (codon 54, rs1800450, *C* (codon 57, rs1800451, and *D* (codon 52, rs5030737) as well as the regulatory variants named *H/L* (-550, rs11003125), *X/Y* (-221, rs7096206), and *P/Q* (+4, rs7095891) were typed by PCR using sequence specific priming (PCR-SSP) as previously described [28]. Although, the typing was performed as SNP-typing the results were combined in haplotypes, based on strong linkage disequilibrium between the SNPs that gives the seven known major *MBL2* haplotypes: Four functional haplotypes *LXPA*, *LYPA*, *LYQA*, and *HYPA* (the normal allele is designated "*A*"), and three defective haplotypes; *LYPB*, *LYQC*, and *HYPD*. All three structural variant alleles (*B*, *C*, and *D*) have a considerable effect on MBL concentrations and to avoid small groups, the three alleles were grouped in one category called allele "*O*" for statistical analyses. Likewise, for statistical analyses we only included the *X/Y* promoter variation at position –221. The *X* variant is always found on a functional haplotype (*LXPA*) and has been shown to have a down regulating effect on MBL expression. Thus, the following six MBL genotypes/haplotypes were defined: the *A/A* group: two normal structural alleles with high-expression promoter activity in position -221 (*YA/YA*) or one high-expression promoter and one low-expression promoter (*YA/XA*) or two low-expression promoters (*XA/XA*); the *A/O* group: one variant structural allele (i.e. defective allele) and one normal structural allele regulated by a high-expression promoter (*YA/O*) or a low-expression promoter (*XA/O)* and the *O/O* group with two defective structural alleles.

Statistical analyses

Contingency table analyses and were used to compare frequencies. Deviation from the Hardy

Weinberg expectations was tested by simple gene counting using the chi-square test for comparing observed and expected values. Kruskall-Wallis or Mann-Whitney tests were used to compare continuous data. Log rank test and Kaplan-Meier curves were used to estimate survival. When appropriate, logistic regression and Cox regression analyses were performed. Only two-sided tests were used.

RESULTS

During the 18 months study period, 680 patients were admitted to the ICU and of these109 were excluded because of exclusion criteria (Figure 1). Of the resulting 571 ready for inclusion a total of 547 were included in the study corresponding to an inclusion fraction of 96.0%. We were able to perform complete *MBL2* genotyping in 532 patients accounting for 97.25% of the included patients. More than 99% of the included patients were of Caucasian origin. At inclusion, genotype frequencies did not differ from those predicted by the Hardy-Weinberg expectations (P>0.55). No significant difference in *MBL2* structural and promoter alleles frequency was observed between patients and 533 population controls (P>0.8) (Table 1).

Of the 547 patients included in the study, 513 (93.8%) met the criteria for SIRS at the first date in the ICU and of these 75.4% met the criteria for sepsis (Figure 1). Of the 387 patients with sepsis, 199 (51.4%) met the criteria for severe sepsis and 130 (65.3%) of these met the criteria for septic shock. Stratification of the patients according to *MBL2* structural variant genotypes and the promoter alleles revealed the same pattern among the 532 genotyped patients: No significant difference in *MBL2* genotypes in the different SIRS-sepsis groups at admission was observed (P>0.8) (Table 1). Because no significant difference was observed in *MBL2* genotype frequencies between patients classified as having SIRS or Non-SIRS we combined these groups in the subsequent analyzes.

Baseline characteristics and admission diagnosis are outlined in table 2. There was no significant difference in age, type of admission, earlier disease or not, diagnosis and smoking habits at admission to the ICU stratified to the *MBL2* genotypes. However, the distribution of sex, patients classified as being immunosuppressed or not and active cancer (subgroup of earlier disease) were significantly different stratified according to the *MBL2* genotypes. A logistic regression model using sep-

sis as a dependent parameter and taking into account the skewed parameters (gender, immunosuppression and active cancer) the *MBL2* structural alleles were still not associated with the sepsis diagnosis (P>0.47).

Univariate analysis on all included patients during total follow-up until death or censuring showed that *MBL2* structural genotype, admission type, previous disease, immunosuppression, sepsis at first date, age and first day SOFA score (Table 3) were significantly related to mortality. Gender, tobacco usage as pack years and weight were not independently related to mortality. The association with *MBL2* genotypes was independent of cofactors that showed a significant association with fatal outcome in the univariate analysis when they were in an expanded Cox regression survival model including the parameters that were predictive in the univariate analysis (Table 3). SOFA score and sepsis at first date were not included in the same model because of the close correlation with each other. However, replacing SOFA score with sepsis at first date in this model revealed that the *MBL2* results were independent of the sepsis diagnosis (data not shown).

Kaplan-Meier survival curve analysis showed that *O/O* homozygotes were associated with the worst outcome, followed by those with the normal *A/A* genotype, while the heterozygous *A/O* genotype was associated with the best outcome (log rank, P=0.0404) (figure 2a). It appeared from the survival curve that the *MBL2* association was somewhat delayed compared to admission to ICU, thus we investigated at what time point the *MBL2* association appeared. Censoring patients after discharge from ICU revealed that there was no association with the *MBL2* variant alleles during stay at ICU (log rank, P=0.73) (figure 2b). However, the *MBL2* association appeared immediately after discharge from ICU and became increasingly more apparent during follow-up when the baseline used was discharge from ICU (log rank, P=0.0098) (figure2c).

In order to pin point in more detail at what time point the *MBL2* association occurred we performed additional Cox regression analyses in a more restricted model after admittance to the ICU corrected for age, gender (included because of the *MBL2*/gender skewing in the demographic data in table 1) and the first day SOFA score (table 4A) with censoring at different time points. It was revealed that the *MBL2* alleles overall were independently and significantly associated with survival after 360 days observation period (P=0.023) and after completion of the follow up period (p=0.015). Detailed analysis showed that using the *A/A* genotype as reference the *A/O* genotype was associated with a significantly better outcome, while the *O/O* genotype was non-significantly associated with the worst outcome compared with the *A/A* genotype (Table 4A). The hazard ratio (HR) for the *A/O* genotype compared with the *A/A* genotype was 0.72 (95% CI: 0.6-0.9) (P=0.0059) for the total observation period, but the tendency was already seen from day 28. Essentially the same results were seen if sepsis/no sepsis was used in the model instead of the first day SOFA score (data not shown).

Based on the observation from the Kaplan-Meier plot we stratified the patients whether they survived stay at intensive care or not. As expected *MBL2* alleles were not independently associated with outcome during stay at ICU when adjusted for the parameters used in table 4A (data not shown).

The above results indicated influence of a delayed *MBL2* dependency on mortality and we made a post hoc analysis in the 366 patients (68.8%) alive at departure from the ICU with a determined *MBL2* genotype (table 4B). In this group *MBL2* genotypes were significantly associated with survival already at day 28 after admittance to the ICU with a HR for death for *A/O* of 0.64 (95%CI: 0.4-1.1) and 1.61 (95%CI: 0.8-3.2) for *O/O* (P=0.0477) compared with the *A/A* genotype, while the corresponding figures at completion of follow-up were 0.62 (95%CI: 0.4-0.8) and 1.33 (95%CI: 0.8-2.2), respectively (P=0.0045). Of the individual genotypes only heterozygosity (*A/O*) deviated

significantly from the *A/A* genotype, while *O/O* did not (table 4B). Essentially the same results were observed when we used departure from ICU as baseline (Table 4B). Further analysis using the same Cox regression model, but including the *MBL2* promoter alleles with the highest expressing *MBL2* genotype *YAYA* as reference showed that this model also was significant regarding *MBL2* alleles (P=0.0392) and that heterozygosity for the structural alleles were associated with a significant better outcome independently of the promoter alleles: *XA/O,* HR 0.44 (95%CI: 0.22-0.86), P=0.0163 and *YA/O*, HR 0.661 (95%CI: 0.44-0.98), P=0.0426. None of the other genotypes *YA/XA*, *XA/XA* and *O/O*, respectively were significantly associated with either reduced or increased survival compared with the *YA/YA* reference genotype (P>0.37).

Microbial specimens obtained at admission to the ICU were culture positive for 242 (44.2%) of the patients (Table 5). 98.2% of the included had blood specimen taken and of these were15.1% culture positive. 68.0% of the included had tracheal or expectorate taken and of these were 40.3% culture positive. 87.9% of the patients had urine specimen taken and of these were 16% culture positive. A admission to the ICU 54.7% (n=173) of the patients with the *A/A* genotype had one or more of the following infections; pneumonia, bacteremia or wound infection, 54.6% with the *A/O* genotype and 51.6% with the O/O genotype. The results positive for c*oagulase negative Staphylococci* were not displayed in the table with admissions cultures since it is usually an insignificant result, if not re-cultured in a later culture from the same place. The *MBL2* homozygous *O/O* defect genotype was associated primarily with *Gram*-positive bacteria since one or more of the *O/O* individual patient cultures were *Gram*-positive in 72.2% (8/11) of the positive cultures compared with 28% (21/75) i the *A/O* genotype and 37.4% (52/139) in the *A/A* genotype (P=0.01), while no significant differenc was observed for *Gram-negative* and anaerobe bacteria or fungi.

DISCUSSION

This study shows that heterozygosity for the *MBL2* structural genotypes (*A/O*) was significantly associated with improved survival compared with the normal *A/A* genotype and the homozygous variant *O/O* genotype after follow-up (censuring) period following admittance to the ICU. This association was independent of SOFA score, age and gender and the SIRS-sepsis consensus criteria on the first day in the ICU indicating that low and intermediate serum levels of MBL may confer a survival advantage in response both to severe infections and acute inflammation. Heterozygous advantage (heterosis) of *MBL2* variant alleles was originally suggested 15 years ago when we and other researchers proposed that the high frequency of these alleles occurs due to a selective pressure promoting heterozygosity [14,22,23,34]. Several studies have attempted to find molecular signatures whether the *MBL2* gene could be subject to evolutionary selection with varying and inconsistent results [35-38]. However neither of these studies did test such a hypothesis in a disease setting, which might simulate an epidemic situation. Although far from ideal ICU does at least to some extent provide such a situation and may give some conceptual clues. Nevertheless, at inclusion of the study the *MBL2* genotypes adhered closely to the Hardy-Weinberg expectations. However, during the observation period an increasingly deviation towards an excess of *A/O* heterozygotes (present both in the *YA/O* and *XA/O* genotype situation) with a depletion of both homozygous *A/A* and *O/O* was observed supporting the notion that such events could take place in an epidemic situation. A mechanistic explanation to this observation could be that low and intermediate levels of functional MBL (*A/O*) would confer a relative advantage both in terms in of optimal antimicrobial activity and less proinflammatory effect. On the other hand deficiency of functional MBL (*O/O*) will make the patients more prone to become severely infected, while high levels of functional MBL (*A/A*) could directly be associated with the proinflammatory adverse effect following uncontrolled complement activation that particularly has been shown for down stream components such as C5a [39].

Nevertheless, from a clinical perspective the main and most important finding that may be derived from this study was the observation that the *MBL2* alleles almost immediately after discharge from ICU appeared to be associated with either increased or decreased protection against death, while no such effect was seen during stay at the ICU. Our interpretation of this finding is that the combination of optimal surveillance and treatment combined with the fact that in a number of patients admitted to intensive care withdrawal of active life support to terminal patients is allowed might mask the *MBL2* effect in relation to survival. Thus, we hypothesize that after intensive surveillance and treatment are halted and the patients are referred to their respective departments with less intensive treatment the *MBL2* effect may become apparent. The Kaplan-Meier curves show that particularly those homozygous for the variant alleles (*O/O*) might have the highest risk and may thus be monitored carefully. Another important lesson from this study is that the *MBL2* effect appears to be independent of SOFA score and sepsis diagnosis, which by themselves are important prognostic markers and may make it even more important to add *MBL2* genotyping to the armament of different parameters that may help in prognostic staging of ICU patients. In a previous smaller study including 272 ICU patients [28] we observed that the *MBL2 O/O* genotype also had a negative impact on survival after ICU stay and that this was independent of the so-called simplified acute physiology score II (SAPSII) and sepsis diagnosis, but no *A/O* advantage could be detected. When MBL serum concentration was measured in a prospective insulin treatment study in patients that survived at least 5 days in ICU it was observed that those functionally deficient of MBL, which did not receive insulin had an increased mortality rate consistent with the present findings [40]. In other studies addressing MBL and ICU survival only mortality related to ICU stay have been reported [29,41]. In these studies *MBL2* polymorphisms were related to sepsis and/or infections but not related to increased mortality during ICU stay in agreement with the present findings. However, in a recent American study patients homozygous for the *B* variant had greater likelihood of septic shock and

development of acute respiratory distress syndrome (ARDS) [42]. In the subgroup of patients with ARDS an increased risk of death was observed after 60 days for *B/B* homozygotes.

We found that the frequency of the *MBL2* alleles at inclusion was similar as the one in Danish population controls consistent with our previous study [28] and the frequency of *MBL2* variant alleles was not significantly different between ICU patients classified having sepsis or not or which developed severe sepsis and septic shock which is in variance with our former study. Nearly the same prevalence of positive bacterial cultures at admission to the ICU was observed between the different *MBL2* genotypes. However, further analysis revealed that the *MBL2 O/O* genotype indeed was associated with increased incidence of *Gram*-positive bacteria. The possible association between *Gram*-positive bacteria and the *MBL2 O/O* genotype has also been observed in other studies [43,44]. Nevertheless, *MBL2* variant alleles have also repeatedly been shown to be associated with *Gram*-negative infections [28,30,45]. The reason why some studies find an association between MBL deficiency and different bacterial isolates probably derives from the fact that many clinically isolates do not bind MBL and differences in the percentage of positive isolates that may be obtained. Another reason for the diversity between the different studies may rely on the fact that MBL also function as a scavenger molecule in maintenance of internal tissue homeostasis and that apparent MBL associations may be due to disturbances in this scavenger system rather than a direct anti-infectious association [10,11]. Moreover the different patient populations are probably very heterogenous even though we try to use objective SIRS/sepsis criteria in order to compare.

It would be tempting to supplement ICU patients with purified or recombinant MBL, which have been used in pilot studies both to patients with infections and healthy volunteers with no adverse effects [46,47]. Recombinant MBL are now in phase 2 trials in order to explore the putative antiinfectious effect of MBL in patients with chemotherapy related neutropenia (www.enzon.com). How-

ever, the results from the present study cast doubts of the use of MBL in ICU patients with systen inflammation and sepsis, but if used only through a narrow therapeutical window.

Although, the general relevance of cohorts studies can always be discussed the present study provide a number of advantages. Firstly it is a prospective and almost complete study in, which more than 97% of the patients fulfilling the inclusion criteria over at 18 months period were genotyped, which minimise the risk for selection bias. Secondly it is larger compared with the earlier publish MBL studies in ICU populations, which reduces the chance for a type I error and thirdly the popu tion is an ethnically homogenous Caucasian population. The primary limitation is the 15 includec patients with an unknown *MBL2* genotype (2.7%), but this is a small number and the mortality fc this group was not different from the other 532 with a determined *MBL2* genotype. We chose to u the *MBL2* genotypes instead of measuring the concentration of MBL serum on day one because (the very good correlation between the *MBL2* genotypes and the concentration and function of M and our previous experience showing the strength using *MBL2* genotypes in disease association studies [48].

In conclusion, the present study has shown that ICU patients heterozygous for *MBL2* variant alle are partly protected from fatal outcome after ICU stay, but more importantly that this effect becomes apparent shortly after discharge from ICU and may thus go unnoticed by the caring physi cians.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1

Flow diagram of study design and patient selection for case control study.

Figure 2

2a. Kaplan-Meier survival plot of all 532 included patients during follow-up by the *MBL2* genotype (log rank P=0.0404).

2b. Kaplan-Meier survival plot, (166 fatal cases) censored at discharge from ICU, by the *MBL2* genotype (log rank P=0.73).

2c. Kaplan-Meier survival plot from departure from ICU in 366 patients alive at departure by the *MBL2* genotype (log rank P=0.0098).

TABLES

Table 1. ***MBL2*** **genotypes, structural alleles, comparisons of patients without sepsis and those with sepsis, severe sepsis and septic shock**

	No (%)						
MBL2 **genotype**	**Controls**	**Patients (Total)**	**No SIRS**	**SIRS without infection**	**Sepsis**	**Severe sepsis**	**Septic shock**
Sum A/A	309 (58.0)	316 (59.4)	22 (68.8)	78 (62.9)	104 (56.2)	38 (57.6)	74 (59.2)
A/B	119 (22.3)	121 (22.7)	5 (15.6)	27 (21.8)	49 (26.4)	15 (22.7)	25 (20.0)
A/C	12 (2.3)	14 (2.6)	1 (3.1)	5 (4.0)	4 (2.2)	0 (0.0)	4 (3.2)
A/D	62 (11.6)	50 (9.4)	3 (9.4)	6 (4.8)	18 (9.7)	9 (13.6)	14 (11.2)
Sum *A/O*	193 (36.2)	185 (34.8)	9 (28.1)	38 (30.6)	71 (38.4)	24 (36.4)	43 (34.4)
B/B	16 (3.0)	19 (3.6)	1 (3.1)	4 (3.2)	8 (4.3)	2 (3.0)	4 (3.2)
B/C	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
B/D	8 (1.5)	9 (1.7)	0 (0.0)	4 (3.2)	2 (1.1)	0 (0.0)	3 (2.4)
C/D	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)
D/D	4 (0.8)	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.0)	0 (0.0)
Sum *O/O*	31 (5.8)	31 (5.8)	1 (3.1)	8 (6.4)	10 (5.4)	4 (6.0)	8 (6.4)
Total	533 (100.0)	532 (100.0)	32 (100.0)	124 (100.0)	185 (100.0)	66 (100.0)	125 (100.0)
Promoter alleles included							
YA/YA	150 (28.1)	157 (29.5)	11 (34.4)	45 (36.3)	48 (25.9)	21 (31.8)	32 (25.6)
YA/XA	135 (25.3)	132 (24.8)	10 (31.3)	28 (22.6)	46 (24.9)	17 (25.8)	31 (24.8)
XA/XA	24 (4.5)	27 (5.1)	1 (3.1)	5 (4.0)	10 (5.4)	0 (0.0)	11 (8.8)
YA/O	130 (24.4)	132 (24.8)	6 (18.8)	24 (19.4)	53 (28.6)	19 (28.8)	30 (24.0)
XA/O	63 (11.8)	53 (10.0)	3 (9.4)	14 (11.3)	18 (9.7)	5 (7.6)	13 (10.4)
O/O	31 (5.8)	31 (5.8)	1 (3.1)	8 (6.5)	10 (5.4)	4 (6.1)	8 (6.4)

A indicates normal structural allele. *O* is the common designation for variant alleles (*B,* codon 54, *C,* codon 57 and *D,* codon 52). *Y* and *X* indicate base exchanges in codon –221which profoundly influence the expression of MBL. *X* is present only on *A* chromosomes. No difference between patients and controls were observed for the total group with respect to structural polymorphisms (chi-square: 0.247, 2 DF, P=0.884) or when the promoter alleles were included (chi-square: 1.246, 5DF, P= 0.94). Nor was any difference observed within the different patient groups (structural polymorphisms: chi-square 3.45, 8DF, P=0.9, including promoter polymorphisms: chi square 16.6, 20DF, P=0.67).

Table 2. Baseline characteristics in the 532 included patients classified *MBL2* structural variant alleles					
Variable	**Total** (n=532)	***A/A*** (n=316)	***A/O*** (n=185)	***O/O*** (n=31)	**P-value**
Age (median and range)	67 (18-93)	67 (18-92)	66 (20-93)	70 (46-79)	0.84
Sex female Male	257 (48.3) 275 (51.7)	140 (44.3) 176 (55.7)	105 (56.8) 80 (43.2)	12 (38.7) 19 (61.3)	0.014
Type of admission Acute surgery Elective surgery Medical	 147 27.6) 19 (3.6) 366 (68.8)	 85 (26.9) 11 (3.5) 220 (69.6)	 52 (28.1) 5 (2.7) 128 (69.2)	 10 (32.2) 3 (9.7) 18 (58.1)	0.34
SOFA score first day (median and range)	8 (0-21)	8 (0-21)	8 (0-19)	8 (2-16)	0.84
Immunosuppressant (One and max three)	329 (61.8)	211 (66.8)	97 (52.4)	21 (67.8)	0.0049
Earlier disease (one and max three)	452 (85.0)	272 (86.1)	151 (81.6)	29 (93.5)	0.15
AMI	68 (12.8)	38 (12.0)	27 (14.6)	3 (9.7)	0.61
Congestive heart disease	122 (22.9)	76 (24.1)	42 (22.7)	4 (12.9)	0.36
Diabetes (IDDM and NIDDM)	68 (12.8)	43 (13.6)	21 (11.4)	4 (12.9)	0.76
Pancreatitis (acute and chronic)	10 (1.9)	7 (2.2)	3 (1.6)	0 (0.0)	0.64
Hepatic disease	43 (8.1)	27 (8.5)	11 (5.9)	5 (16.1)	0.14
Renal disease	67 (12.6)	38 (12.0)	26 (14.1)	3 (9.7)	0.7
COPD	146 (27.4)	79 25.0)	58 (31.4)	9 (29.0)	0.28
Active cancer	117 (22.0)	75 (23.7)	31 (16.8)	11 (35.5)	0.033
Other	136 (25.6)	85 (26.9)	43 (23.2)	8 (25.8)	0.6
No previous disease	80 (15.4)	44 (13.9)	34 (18.4)	2 (6.5)	0.15
Tobacco usage as pack years, mean ±SD	31.8 ± 25.6	31.7 ± 25.7	30.7 ± 22.6	38.3 ± 24.2	0.23
Weight kg, mean ±SD	73.1± 16.9	74.2±17.3	72.1±16.9	69.4±14.0	0.11
Primary groups of diagnosis at admittance to the ICU					
Sepsis	137 (25.8)	89 (28.2)	43 (23.2)	5 (16.1)	0.21
Disturbance in liquid/electrolyte balance	18 (3.4)	8 (2.5)	9 (4.9)	1 (5.6)	0.38
Major Intoxication	11 (2.1)	7 (2.2)	4 (2.2)	0 (0.0)	0.7
Neurologic	31 (5.8)	13 (4.1)	16 (8.6)	2 (6.5)	0.11
Respiratory	191 (35.9)	110 (34.8)	67 (36.2)	14 (45.2)	0.5
Cardio-vascular	49 (9.2)	32 (10.1)	16 (8.6)	1 (3.2)	0.42
Gastrointestinal	22 (4.1)	12 (3.8)	8 (4.3)	2 (6.5)	0.76
Renal/Urogenital	43 (8.1)	28 (8.9)	11 (5.9)	4 (12.9)	0.63
Other	30 (5.6)	17 (5.3)	11 (5.9)	2 (6.5)	0.94

Table 3. Univariate analysis of categorical and continuous data for mortality risk in the total observation period			Multivariate analysis of survival following admission	
Quantity/ categorical data	**Mean (SD) of survival time/day**	**P-value**	**Hazard ratio (95% CI)**	**P-value**
Sex				
Females	411.0 (28.7)	=0.276		
Males	358.3 (26.6)			
Admission type				0.17
Medical	407.8 (23.7)		1	
Surgical elective	472.7 (99.6)	=0.040	0.7 (0.42-1.4)	0.41
Surgical acute	308.6 (35.5)		1.2 (0.95-1.5)	0.12
Previous disease				
Present	337.4 (20.4)	<0.0001	1	0.027
Absent	633.1 (50.4)		0.65 (0.44-0.95)	
Sepsis				
Present	505.5 (37.5)	<0.0001		
Absent	332.7 (22.3)			
Immunosuppression				
Present	303.3 (22.9)	<0.0001	1	0.053
Absent	516.7 (33.4)		0.79 (0.62-1.0)	
MBL2 genotypes				0.028
SUM *A/A*	362.5 (25.2)	=0.040	1	
SUM *A/O*	455.7 (34.9)		0.74 (0.59-0.94)	0.013
SUM *O/O*	255.2 (72.3)		1.12 (0.72-1.7)	0.61
Continuous data	**Hazard ratio (95% CI)**			
Weight/kg	0.997 (0.91 -1.0)	=0.283		
Age/year	1.033 (1.02- 1.04)	=0.0001	1.03 (1.02-1.4)	<0.0001
Tobacco/pack years	1.001 (0.97- 1.0)	=0.770		
SOFA score	1.117 (1.09 -1.14)	=0.0001	1.12 (1.1-1.16)	<0.0001

SOFA score and sepsis at first date were not included in the same model because of the close correlation with each other. However, replacing SOFA score with sepsis at first date in the model revealed that the *MBL2* results were independent of the sepsis diagnosis (data not shown).

Table 4A. Mortality risk and hazard ratio with 95% CI in all 532 patients with a determined *MBL2* structural variant genotype (adjusted for *MBL2*, age, gender and SOFA score)

MBL2 genotype	28 days	90 days	360 days	Total
A/A vs A/O vs O/O	(P=0.1949)	(P=0.1210)	(P=0.0243)	(0.0150)
A/A	1	1	1	1
A/O	0.79 (0.6-1.1) (P=0.0996)	0.78 (0.6-1.0) (P=0.0558)	0.74 (0.6-0.9) (P=0.0148)	0.72 (0.6-0.9) (P=0.0059)
O/O	1.11 (0.7-1.8) (P=0.6661)	1.10 (0.7-1.7) (P=0.6953)	1.18 (0.8-1.8) (P=0.4511)	1.09 (0.7-1.7) (P=0.6963)
Age	1.04 (1.0-1.1) (P<0.0001)	1.03 (1.0-1.1) (P<0.0001)	1.03 (1.0-1.1) (P<0.0001)	1.03 (1.0-1.1) (P<0.0001)
Sex (female/male)	1.13 (0.9-1.5) (P=0.3480)	1.06 (0.8-1.3) (P=0.6416)	1.07 (0.9-1.3) (P=0.5661)	1.02 (0.8-1.3) (P=0.8285)
SOFA score 1 day	1.17 (1.1-1.2) (P<0.0001)	1.16 (1.1-1.2) (P<0.0001)	1.14 (1.1-1.2) (P<0.0001)	1.14 (1.1-1.2) (P<0.0001)

Table 4B. Mortality risk and hazard ratio in the 366 patients alive at departure from ICU with a determined *MBL2* genotype (adjusted for *MBL2* genotype, age, sex and 1. day SOFA score) – baseline time was admittance to the ICU except*

MBL2 genotype	28 days	90 days	360 days	Total	*Total (baseline departure from ICU)
A/A vs *A/O* vs *O/O*	(P=0.0477)	(P=0.0353)	(P=0.0049)	(P=0.0045)	(P=0.0048)
A/A	1	1	1	1	1
A/O	0.64 (0.4-1.1) (P=0.0798)	0.65 (0.4-0.99) (P=0.0451)	0.64 (0.5-0.9) (P=0.0139)	0.62 (0.4-0.8) (P=0.0041)	0.62 (0.4-0.8) (P=0.0040)
O/O	1.61 (0.8-3.2) (P=0.1922)	1.47 (0.8-2.8) (P=0.2383)	1.55 (0.9-2.7) (P=0.1127)	1.33 (0.8-2.2) (P=0.3069)	1.30 (0.8-2.2) (P=0.3359)
Age	1.06 (1.0-1.1) (P<0.0001)	1.05 (1.0-1.1) (P<0.0001)	1.04 (1.0-1.1) (P<0.0001)	1.04 (1.0-1.1) (P<0.0001)	1.04 (1.0-1.1) (P<0.0001)
GenderFe-male/male	0.88 (0.6-1.4) (P=0.5862)	0.77 (0.5-1.1) (P=0.1782)	0.85 (0.6-1.2) (P=0.3159)	0.80 (0.6-1.1) (P=0.1495)	0.80 (0.6-1.1) (P=0.1351)
SOFA score day 1	1.11 (1.05-1.2) (P=0.0007)	1.10 (1.04-1.2) (P=0.0004)	1.07 (1.02-1.1) (P=0.0042)	1.06 (1.0-1.1) (P=0.0024)	1.07 (1.0-1.1) (P=0.0016)

Table 5. Microbial species (*-coagulase-negative Staphylococci*) diagnosed in cultures (blood, tracheal and urine) on admission to ICU in 532 *MBL2* genotyped patients					
Patient s	**Total** (n=532)	***A/A*** (n=316)	***A/O*** (n=185)	***O/O*** (n=31)	**P-value**
Cultures					
SUM negative or unknown	307 (57.7)	177 (56.0)	110 (59.5)	20 (64.5)	0.55
SUM positive	225 (42.3)	139 (44.0)	75 (40.5)	11 (35.5)	
Microorganisms					
Gram-positive	**81/225 (36.0)**	**52/139 (37.4)**	**21/75 (28.0)**	**8/11 (72.2)**	**0.01**
Staphylococcus aureus	28 (34.6)	19 (36.5)	8 (38.1)	1 (12.5)	
Streptococcus pneumoniae	26 (32.1)	18 (34.6)	5 (23.8)	3 (37.5)	
Other Streptococci	10 (12.3)	4 (7.7)	4 (19.0)	2 (25.0)	
Enterococcus species	18 (22.2)	10 (19.2)	6 (28.6)	2 (25.0)	
Other Gram- positive	3 (3.7)	2 (3.8)	0 (0.0)	1 (12.5)	
***Gram*-negative**	**136/225 (60.4)**	**85/139 (61.2)**	**46/75 (61.3)**	**5/11 (45.5)**	**0.58**
Escherichia coli	62 (45.6)	37 (43.5)	20 (43.5)	3 (60.0)	
Klebsiella species	22 (16.2)	14 (16.5)	8 (17.4)	0 (0.0)	
Other Enterobactericae	17 (12.5)	12 (14.1)	4 (8.7)	0 (0.0)	
Hæmophilus species	26 (19.1)	17 (20.0)	7 (15.2)	2 (40.0)	
Pseudomonas species	10 (7.4)	4 (4.7)	5 (10.9)	0 (0.0)	
Stenotrophomonas maltophilia	3 (2.2)	3 (3.5)	0 (0.0)	0 (0.0)	
Other Gram-negative	28 (20.6)	16 (18.8)	10 (21.7)	1 (20.0)	
Anaerobe	**2/225 (0.9)**	**0/139 (0.0)**	**1/75 (1.3)**	**1/11 (9.0)**	**0.14***
Fungi	**72/225 (32.0)**	**39/139 (28.1)**	**32/75 (42.7)**	**1/11 (9.0)**	**0.14***
Candida albicans	53 (73.6)	28 (71.8)	24 (75.0)	1 (100.0)	
Candida non albicans	10 (13.9)	5 (12.8)	5 (15.6)	0 (0.0)	
Yeast, not further specified	21 (29.2)	10 (25.6)	11 (34.4)	0 (0.0)	

Chi-square test with 2 DF was used except when indicated* Fisher's were used pooling *A/O* and *O/O* to avoid small numbers. SUM positive: At least one of the admittance cultures (blood, tracheal, expectorate and urine) was positive. A species appearing in > 1 culture /patient was counted only once. Each specimen is shown as part of the main group e.g. gram positive, gram negative and fungi. 98.2% of the included had blood specimen taken, of these 15.1% was positive. 68.0% of the included had tracheal or expectorate taken, of these 40.3% was positive. 87.9% of the included had urine specimen taken, of these 16% was positive. Anaerobes were Clostridium.

FIGURES

Figure 1

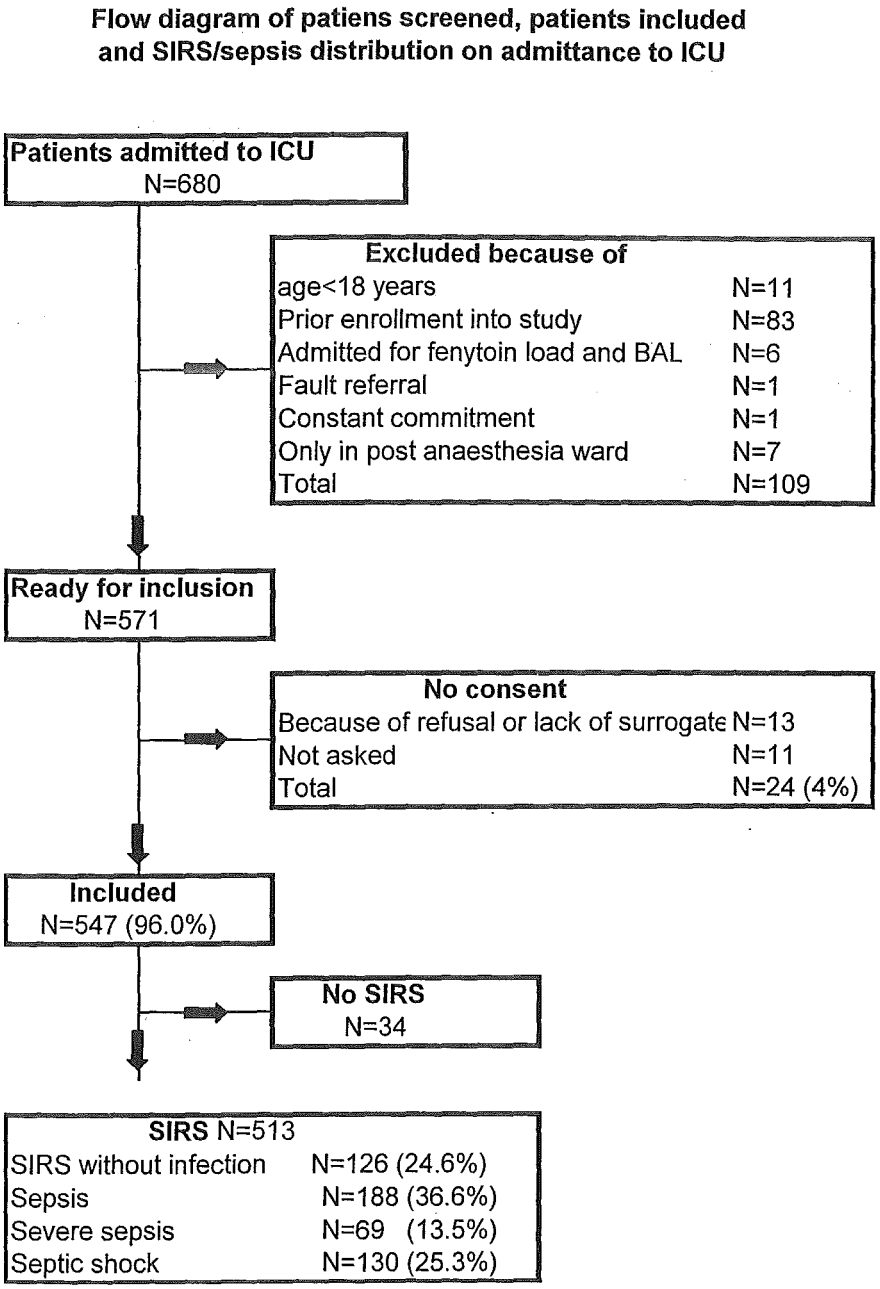
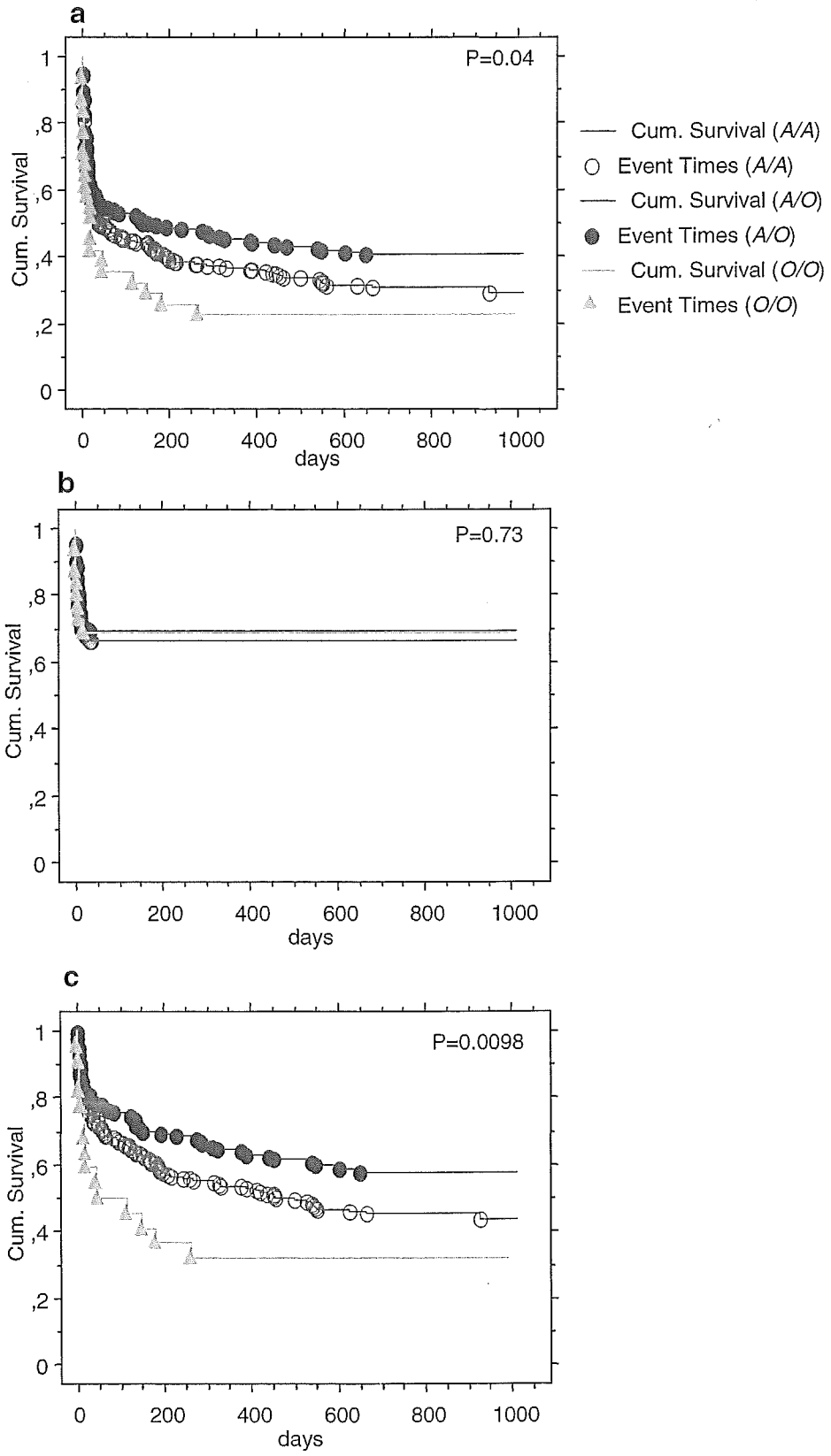


Figure 2


a
P=0.04
Cum. Survival
1
,8
,6
,4
,2
0
0
200
400
600
800
1000
days
Cum. Survival (A/A)
Event Times (A/A)
Cum. Survival (A/O)
Event Times (A/O)
Cum. Survival (O/O)
Event Times (O/O)
b
P=0.73
Cum. Survival
days
c
P=0.0098
Cum. Survival
days

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TNFα and IL-18 promoter polymorphisms in prospectively followed critically ill patients

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ABSTRACT

Objective: To investigate whether the single nucleotide polymorphisms (SNP)s; tumor necrosis factor-α (TNF)-308, TNF-238 and interleukin (IL)-18-137 are associated with nosocomial infections, nosocomial pneumonia and mortality, in critically ill patients.

Design: Observational cohort study of critically ill patients.

Setting: Mixed medical-surgery intensive Care Unit at Herlev Hospital, a University Hospital in Copenhagen.

Patients: A cohort of 547 critically ill patients admitted to the ICU between December 2002 and June 2004 (18 months).

Measurements and main results: DNA was extracted from blood. Clinical data was were collected prospectively and missing data from retrospective chart review. Survival status was determined from the Central Office of Civil Registration in Denmark. The TNFα and IL-18 polymorphisms were genotyped using PCR-based methods. No difference in genotype frequency of the different investigated polymorphisms was observed when the patients were stratified according to diagnosis of sepsis and nosocomial infections (P>0.7). None of the examined polymorphisms; TNFα –308, TNFα -238, and IL-18-137 were significantly related to mortality. However, a non-significant trend towards an increased mortality rate was observed for the TNFα –238 minority allele after an observation period of 28 days (hazard ratio: 1.41, p=0.07).

Conclusions: In this study, there were no statistically significant association between the selected TNFα and IL-18 polymorphisms and risk of sepsis, nosocomial infections and mortality. However, the presence of the TNFα –238 minority allele actually showed a non-significant hazard ratio for death of 1.41, which should be elaborated further in even larger studies.

INTRODUCTION

Sever sepsis is one of the major causes of morbidity and mortality in the Intensive Care Unit (ICU), and is being diagnosed with increasing frequency [1,2]. Genetic variation may partly explain the inter-individual capability to combat infections [3]. Thus the degree of genetic influence on clinical presentation and outcome has become a major research field as to septic patients [4-6].

Sepsis is initiated by a systemic inflammatory response to a microbial challenge, which may subsequently lead to severe sepsis and septic shock with impaired perfusion and multiple organ failure. The pro-inflammatory cytokine tumor necrosis factor TNFα (TNF) is one of the first cytokines released into the circulation during the inflammatory response to infectious stimuli and has been shown to play a central role in the pathogenesis of the inflammatory response and sepsis [7,8]. In the majority of studies, high levels of TNFα correlate with the outcome of patients with severe sepsis in ICUs [9,10]. Thus, any genetic predisposition to increased TNFα production may have profound implications for the risk of developing septic shock and chance of survival.

The gene encoding TNFα is located on chromosome 6 in the region known as the major histocompatibility complex. No mutations have been found in the coding regions of TNFα, but a number of single-nucleotide polymorphisms (SNP) have been detected in the promoter region [11]. Possibly, the changes they introduce, in comparison to the common form, could affect the binding of transcription factors and thus transcriptional regulation of the TNFα gene. The TNFα SNP that has been most thoroughly studied is a bi-allelic variation at position -308 in the promoter region of the TNFα gene causing a G (named allele TNF1) to be substituted with

an A (named allele TNF2) [12]. Although several studies indicate an association between the A-allele and the susceptibility to and/or outcome from septic shock [13-17], the results from other studies cannot support such a notion [9,18]. In addition, results from several in vitro studies examining the effect of the TNFα -308 SNP on TNFα expression [11] and in experimental human endotoxemia are inconsistent [19].

Another TNFα SNP in position –238 not present on the same haplotype as the TNFα -308 SNP also causing a G to A base substitution has been associated with increased basal transcription [11] Although no association between this genotype and TNFα plasma levels has been detected [9] several studies describe an association of the TNFα -238 SNP with infectious and inflammatory diseases such as chronic active hepatitis B and C [20,21], severity of RA [22] and rheumatic heart disease [23]. Only few studies have addressed the TNF-238 SNP in relation to sepsis and outcome. However, thus far no conclusive associations has been reported [15] [9]. Nevertheless, recently the minority allele of the TNFα -238 SNP was shown to be significantly related to adverse outcome in cystic fibrosis [24].

Interleukin (IL)-18, is a pro-inflammatory cytokine, involved in both innate and acquired immune responses. The most prominent function of IL-18 is to induce TNFα, IL-1β, IL-8, GM-CSF secretion and ultimately the induction of IFN-γ production from T, B and NK cells in particular in presence of IL-12 [25]. Interestingly, it has been shown that TNF stimulates the expression of IL-18 in cardiomyocytes [26] and in human skeletal muscle in vivo [27]. Elevated plasma IL-18 concentrations were in a study with 20 patients with severe sepsis associated with increased severity of sepsis as well as adverse outcomes and a significant increase

in IL-18 concentrations was shown to discriminate between Gram-positive and Gram-negative related sepsis [28]. The gene encoding IL-18 is located on chromosome 11. No non-synonymous SNPs have been observed, but a number of SNPs in the promoter region has been verified that potentially interfere with transcription-factor-binding sites [29]. One study found that monocytes with the -137G/G genotype have increased IL-18 production compared with monocytes with the -137 G/C genotype in response to LPS or A23187 + PMA [30].

Previous investigations of the association of the SNPs TNFα-308, TNFα-238, IL-18-137, and the occurrence of sepsis and mortality have been Inconsistent. The aim of this study was prospectively to investigate the association between the TNFα-308 and TNFα-238 and the IL-18 -137 SNP and their possible association with the SIRS-sepsis criteria, acquisition of nosocomial infection as well as fatal outcome, in a large cohort of ICU patients.

MATERIALS AND METHODS

Setting

Herlev Hospital in Copenhagen, Denmark is a university hospital with 568 beds. The central mixed surgical-medical intensive care unit (ICU) serves the medical and surgical blocks of the hospital. The ICU is a six-bed unit with exclusively single-bed rooms.

Study population and design

A schematic summary of the study design, inclusion, and exclusion criteria is presented in Figure 1. All adult patients (≥ 18 yrs) admitted to the ICU between December 2, 2002 to June 1, 2004 (18 months) were screened for inclusion. Patients who were readmitted and had been included on their first admission were only registered according to the first admission. The only other exclusion criteria were admissions for the sole purpose of fenytoin loading and broncho-alveolar lavage (BAL), constraint commitment because of psychiatric disease, fault referral, and an exclusive stay of critically ill patients in the post anaesthesia care unit due to overbooking of the ICU. The Institutional Review Boards of the County of Copenhagen approved the protocol (file number KA 02071). Informed consent was obtained from the patients or from their relatives.

All data were initially recorded in case record forms (CRF) by the ICU physicians on rounds, a physician from the project group, or by the coordinating investigator. At admission to ICU baseline clinical information concerning underlying disease, cause of admission, tobacco consumption, immunosuppressive factors and infection on admission where recorded in the CRF. Chest radiography was usually performed at admittance and later on indication. Bacterial cul-

tures were sampled on admission from blood, nasopharynx, urine and tracheal aspirate or expectorate and later, at the suspicion of an infection, from; blood, tracheal aspirate, urine, wounds and drainages etc. Cultures were processed according to standardized methods on the Department of Clinical Microbiology. Organ failure at admission was defined according to the 'worst value' of the first day sequential organ failure assessment (SOFA) score [31]. The SIRS-Sepsis criteria were registered and defined in accordance with the recommendations of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [32] in the five classes; None, SIRS, sepsis, severe sepsis and septic shock. From the Central Office of Civil Registration in Denmark we requested and received a vital status for all included patients at the 14th of September 2005; if death had occurred the date of death was registered.

Definitions

Definitions of previous diseases, immunosuppressive factors, tobacco consumption and nosocomial infections are given in appendix 1.

SNP analyses

Detailed description of the methodology of the PC-based SNP analyses is given in appendix 2

Statistical analysis

Contingency table analyses and Fisher's exact test were used to compare frequencies. Deviation from the Hardy Weinberg expectations was tested by simple gene counting using the chi-square test for comparing observed and expected values. Kruskall-Wallis or Mann-Whitney tests were used to compare continuous data. Log rank test and Kaplan-Meier curves were

used to estimate survival. When appropriate, logistic regression and Cox regression multivariate survival analyses were performed. Only two-sided tests were used.

RESULTS

During the 18-month study period, 680 patients were admitted to the ICU and of these 109 were excluded due to the fulfilment of one or more exclusion criteria (figure 1). Of the resulting 571 patients ready for inclusion a total of 547 were included in the study corresponding to an inclusion fraction of 96%. We were able to perform a TNFα-238 (rs361525) and a TNFα-308 (rs1800629) genotyping in 535 patients and an IL-18-137 (rs187238) genotyping in 527 patients accounting for 98% and 96% of the included patients, respectively. More than 99% of the included patients were of Caucasian origin. SNP genotype frequencies did not differ from those predicted by the Hardy-Weinberg equilibrium at inclusion.

Of the 547 patients included in the study, 513 (93.8%) met the criteria for SIRS at the first date in the ICU and of these 75.4% met the criteria for sepsis (table 1). Of the 387 patients with sepsis, 199 (51.4%) met the criteria for severe sepsis and 130 (65.3%) of these met the criteria for septic shock. Stratification of the patients, according to the three examined SNP genotypes, revealed no differences of the rates of SIRS, sepsis, severe sepsis, and septic shock at admission (for all comparisons $P>0.7$) (table 1).

Baseline characteristics at admission to the ICU are outlined in table 2. There was no significant difference in age, sex, type of admission, immunosuppressant, earlier disease and admission diagnoses (table is given in appendix 3) at admission to the ICU stratified according to the different genotypes (for all comparisons $P>0.05$). Further, there was no significant difference in smoking habits stratified for genotypes (data not shown).

Univariate analyses on all included patients during follow-up until censoring or death revealed that admission type, previous disease, sepsis at admission, immunosuppression, age, and the worst SOFA score during admission day (table 3) were significantly associated with mortality. The TNFα –308, TNFα -238, and IL-18-137 SNPs, sex, pack years of tobacco use, and weight were not significantly related to mortality. The findings of relations between the 90 days mortality and the examined covariates were similar. However, a non-significant trend for an increased risk of death within 90 days was observed for the presence of the minor *A* TNFα allele in position -238 (P=0.09). Because of this trend we performed a multivariate analysis for death, adjusted for age and sex at day 28, 90, 360, and in the overall observation period which is shown in table 4. The HR for death for the *G/A* genotype compared with the *G/G* genotype was 1.42 (P=0.07) on day 28 and 1.31 for the total observation period (P=0.12). Correspondingly a similar trend was seen for 28 days survival in a Kaplan-Maier plot (log rank P=0.0546, figure 2), while this trend become less prominent when the whole observation period was taken into consideration (log rank P=0.0945). No association with survival was observed for the two SNPs at TNFα-308 and IL-18-137 neither at 28 days nor at completion of the study (log rank P>0.5).

Univariate analysis showed that age, sex, previous disease, immunosuppresion, sepsis, weight, tobacco usage and the TNFα-308 TNFα-238, and IL-18-137 SNPs were not associated with nosocomial infection (pneumonia, bacteriaemia and/or wound infection). Admission type and SOFA score were significantly associated with nosocomial infection both in the univariate and in the multivariate analyses (P=0.003 and P=0.014 respectively). The HR for nosocomial infection pending a surgical acute admission versus a medical admission was 2.3 (95% C.L. 1.2-4.3). The analogous analysis of nosocomial pneumonia revealed that SOFA

score and immunosuppresion were the only significantly associated risk-factors both in the univariate and multivariate analyses (P=0.001 and P=0.007, respectively). The HR for nosocomial infection pending immunosuppression or not was 2.7 (95% C.L. 1.3-5.6).

Microbial specimens obtained at admission to the ICU were culture positive in 232 (42.4%) of all the included patients and with no difference when stratified for genotypes according to the three examined SNPs (see table I appendix 4). Of the included patients 98.2% had a blood specimen and of these 15.1% were culture positive. Of the included patients 68.0% had a tracheal or expectorate specimen and of these 40.3% were culture positive. Of the included patients 87.9% had a urine specimen and of these 16% were culture positive.

The positive results for C*oagulase negative Staphylococci* were not displayed in the table with results from admissions cultures since it is usually regarded as contamination, if it is not re-cultured from the same site at a later episode. There was no statistically significant differences in the distribution of gram positive, gram negative and anaerobe bacteria or fungi when the SNP's where stratified for genotypes (P>0.5). Of the included patients 54.2% had either pneumonia, bacteriaemia and/or wound infection at ICU admission. The investigated SNPs were not associated with the different types of infections (for all comparisons P>0.1).

DISCUSSION

In this study we have investigated the possible association between each of two TNFα SNPs in position -308 and -238 and one IL-18 in position –137, and sepsis, acquisition of nosocomial infection as well as fatal outcome. Overall no statistically significant associations were observed between the outcome measures and the investigated covariates.

Although, the general relevance of observational studies can always be discussed [33] the present study provide a number of strength. First, it is a prospective and almost complete study that included 96% of 547 eligible patients, which reduces the risk of selection bias. Second, the study group is larger than the earlier published TNFα and IL-18 studies in ICU populations, which reduces the risk of both type I and II errors, provided that our patients represents ICUs in general. Third, the population is an ethnically homogenous Caucasian population. Fourth, the determination of the three different SNPs was done after the data collection was terminated which eliminates the 'confounding by indication' or treatment bias that knowledge of the genotypes during treatment at the ICU could have conferred to our results. Fifth, all of the covariates and outcome measures included in the final analyses were predefined prior to the commencement of data collection thereby avoiding data driven analysis bias and outcome measure bias. Sixth, genotype distribution was in Hardy Weinberger equilibrium [34] and finally, we were able to determine long-time as well as short time outcomes in our patients due to the well functioning Civil Registration system in Denmark. The primary limitation is the 20 included patients, which we were unable to get a full genotype from. The mortality in the two groups, however, was not significantly different. Thus this appears to be a minor problem.

Although the presence TNFα and IL-18 SNPs overall did not show any statistically significant associations between the investigated covariates and outcome measures it was a non-significant trend that carriers of TNFα -238 G/A genotype did have a higher mortality than G/G carriers both in univariate analysis and in Cox regression analysis corrected for age and gender. The non-significant HR was most pronounced early during follow-up since the HR after 28 days was 1.42 while it was 1.31 at completion of the study. The increased point estimate of the HR may be a random finding, but may also reflect a type II error. The possible relationship between the TNFα-238 SNP and an increased mortality should be examined in further studies. The TNFα-238 *A/A* genotype was extremely rare in this study since only one patient (0.2%) had this genotype, which is why we chose not to analyze this association. In a study including 213 Caucasian ICU patients, the genotype frequencies for the TNFα-238 SNP were not significantly different in ICU survivors and non-survivors, but 16.3% of the non-survivors had the *G/A* genotype compared with 12.8% of the survivors giving a relative risk of 1.27 [9], which is within the confidence limits observed in this study. Thus, both studies may have been underpowered to detect a relevant difference and indicate that even larger study than ours must be performed to detect genetic effects. Another possibility would be to include all studies conducted so far into a meta-analysis. In this regard it should be noted that in a study of 152 trauma patients of mixed ethnicity [15], all of the 12 TNFα-238 *G/A* heterozygote patients were alive when discharged from hospital. Further in accordance with the above-mentioned studies we did not find an association between the TNFα-238 SNP and susceptibility to sepsis and nosocomial infections [9,15].

We did not detect an association between the TNFα-308 SNP and susceptibility to sepsis and/or mortality. Data from other studies yield conflicting results. Some studies find an asso-

ciation [13-17], while others do not [9,18]. Possible explanations for these conflicting observations are the much smaller size of these studies (maximum 152 included), lack of completeness of the cohort admitted to the ICU, ethnically mixed populations and healthy control groups. Finally functional studies of the TNFα promoter according to the SNP TNFα-308, have displayed conflicting results [11,12,35] and two studies investigating the influence of the TNFα-308 SNP on experimental human endotoxemia with 12 and 87 volunteers respectively showed no significant difference in basal TNFα levels and peak TNF plasma levels after challenge with endotoxin [19,36].

The present finding of no association between the IL-18-137 SNP and the susceptibility to development of nosocomial infection, or mortality concurs with a study of 69 post-injury patients [37]. They found no association between the single SNP and development of infection and sepsis, but when combining the result from the IL-18-137 and IL-18-607 SNPs they demonstrated a significant association between the extended haplotype and development of sepsis. Haplotype analysis was beyond the scope of this project.

In this bias minimised, complete study of critically ill patients which is possibly the largest prospectively planned ICU study performed so far no statistically significant associations were found between the TNFα-308, TNFα-238, and IL-18 -137 SNPs and sepsis, nosocomial infection or mortality. However, it is noteworthy that the actually found non-significant 42% increase in early mortality given the presence of TNFα-238 *A* allele is not ignorable. The true association between TNFα and IL-18 SNPs and outcome in ICU patients may have to await even larger studies and meta-analyses of performed observational studies.

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LEGENDS TO FIGURES

Figure 1

Flow diagram of study design and patient selection for case control study.

Figure 2

Kaplan-Meier survival plot for 28 days survival of all 535 included patients with a determined TNFα-238 genotype (log rank P=0.0546)

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TABLES

Table 1. TNFα-238, TNFα-308 and IL-18-137 genotypes, structural alleles, comparisons of patients without sepsis and those with sepsis, and severe sepsis and septic shock

	No (%)					
Allele genotype	**Patients (Total)**	**No SIRS**	**SIRS without infection**	**Sepsis**	**Severe sepsis**	**Septic shock**
TNFα-238						
GG	482 (90.1)	29 (90.6)	116 (92.8)	165 (89.2)	62 (91.2)	110 (88.8)
GA	52 (9.7)	2 (6.3)	9 (7.2)	20 (10.8)	6 (8.8)	15 (12.0)
AA	1 (0.2)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	535 (100.0)	32 (100.0)	125 (100.0)	185 (100.0)	68 (100.0)	125 (100.0)
TNFα-308						
GG	358 (66.9)	19 (59.4)	82 (65.6)	123 (66.5)	47 (69.1)	87 (69.6)
GA	164 (30.7)	13 (40.6)	41 (32.8)	58 (31.3)	18 (26.5)	34 (27.2)
AA	13 (2.4)	0 (0.0)	2 (1.6)	4 (2.2)	3 (4.4)	4 (3.2)
Total	535 (100.0)	32 (100.0)	125 (100.0)	185 (100.0)	68 (100.0)	125 (100.0)
IL-18-137						
GG	296 (56.2)	21 (65.6)	72 (59.5)	100 (54.9)	38 (55.9)	65 (52.4)
GC	187 (35.5)	9 (28.1)	40 (33.1)	64 (35.2)	23 (33.8)	51 (41.1)
CC	44 (8.3)	2 (6.3)	9 (7.4)	18 (9.9)	7 (10.3)	8 (6.5)
Total	527 (100.0)	32 (100.0)	121 (100.0)	182 (100.0)	68 (100.0)	124 (100.0)

Table 2. Baseline characteristics classified by SNP TNFα-238, TNFα-308 and IL-18-137 respectively.										
	No (%)									
	Total (n=547)	**TNFα-238** (n=535)			**TNFα-308** (n=535)			**IL-18-137** (n=527)		
Variable		*GG* 482 (90.1)	*GA* 52 (9.7)	*AA* 1 (0.2)	*GG* 358 (66.9)	*GA* 164 (30.7)	*AA* 13 (2.4)	*GG* 296 (56.2)	*GC* 187 (35.5)	*CC* 44 (8.3)
Age (median and range)	66 (18-93)	66 (18-93)	68 (28-87)	73	67 (19-91)	66 (18-92)	64 (64-80)	64.5 (19-93)	67 (18-91)	68 (26-85)
Sex female	265 (48.4)	231 (47.9)	24 (46.2)	1 (100.0)	162 (45.3)	87 (53.0)	7 (53.8)	142 (48.0)	91 (48.7)	19 (43.2)
Male	282 (51.6)	251 (52.1)	28 (53.8)	0 (0.0)	196 (54.7)	77 (47.0)	8 (46.2)	154 (52.0)	96 (51.3)	25 (56.8)
Type of admission										
Acute surgery	148 (27.1)	135 (28.0)	10 (19.2)	0 (0.0)	101 (28.2)	43 (26.2)	1 (7.7)	90 (30.4)	45 (24.1)	8 (18.2)
Elective surgery	19 (3.5)	19 (3.9)	0 (0.0)	0 (0.0)	17 (3.9)	2 (1.2)	0 (0.0)	12 (4.1)	6 (3.2)	1 (2.3)
Medical	380 (69.5)	328 (68.0)	42 (80.8)	1 (100.0)	240 (67.0)	119 (72.6)	12 (92.3)	194 (65.5)	136 (72.7)	35 (79.5)
SOFA score first day (median and range)	8 (0-21)	8 (0-21)	8 (0-20)	8	8 (0-21)	8 (0-21)	10 (4-15)	8 (0-21)	8 (0-20)	8.5 (0-16)
Immunosuppressant (One and max three)	341 (62.3)	304 (65.1)	26 (50.0)	1 (100.0)	216 (60.3)	104 (63.4)	11 (84.6)	173 (58.4)	126 (67.4)	29 (65.9)
Earlier disease (one and max three)	464 (84.8)	410 (85.1)	44 (84.6)	1 (100.0)	306 (85.5)	137 (83.5)	12 (92.3)	249 (84.1)	163 (87.2)	36 (81.8)
AMI	69 (12.6)	60 (12.4)	8 (15.3)	1 (100.0)	50 (14.0)	18 (11.0)	1 (7.7)	35 (11.8)	26 (13.9)	6 (13.6)
Congestive heart disease	125 (22.9)	109 (22.6)	13 (25.0)	1 (100.0)	88 (24.6)	32 (19.5)	3 (23.1)	66 (22.3)	46 (24.6)	8 (18.2)
Diabetes (IDDM and NIDDM)	69 (12.6)	63 (13.1)	5 (9.6)	0 (0.0)	46 (12.8)	19 (11.6)	3 (23.1)	28 (9.5)	33 (17.6)	5 (11.4)
Pancreatitis (acute and chronic)	11 (2.0)	8 (1.7)	2 (3.8)	0 (0.0)	6 (1.7)	3 (1.8)	1 (7.7)	6 (2.0)	2 (2.7)	1 (2.3)
Hepatic disease	45 (8.2)	37 (7.7)	7 (13.5)	0 (0.0)	32 (8.9)	9 (5.5)	3 (23.1)	24 (8.1)	18 (9.6)	1 (2.3)
Renal disease	70 (12.8)	9 (1.9)	3 (5.8)	0 (0.0)	48 (13.4)	18 (11.0)	1 (7.7)	39 (13.2)	21 (11.2)	6 13.6)
COPD	149 (27.2)	129 (26.8)	16 (30.8)	0 (0.0)	99 (27.7)	45 (27.4)	1 (7.7)	73 (24.7)	62 (33.2)	9 (20.5)
Active cancer	124 (22.7)	111 (23.0)	8 (15.4)	0 (0.0)	75 (20.9)	40 (24.4)	4 (30.8)	69 (23.3)	40 (21.3)	9 (20.5)
Other	137 (25.0)	123 (25.5)	13 (25.0)	0 (0.0)	92 (25.7)	41 (25.0)	3 (23.1)	75 (25.3)	46 (24.6)	13 (29.5)
No previous disease	83 (15.2)	72 (14.9)	8 (15.4)	0 (0.0)	52 (14.5)	27 (16.5)	1 (7.7)	47 (15.9)	24 (12.8)	8 (18.2)

Table 3. Univariate analysis of categorical and continuous data for mortality risk				
	Mortality in the total observation period		**Mortality in 90 days after ICU admission**	
Quantity/ categorical data	Median observation time (days)	P-value[a]	Median observation time (days)	P-value
Sex				
Females	90	P=0.276	90	P=0.410
Males	37		37	
Admission type				
Surgical elective	260		90	
Surgical acute	19	P=0.040	19	P=0.035
Medical	79		79	
Previous disease				
Present	30	P<0.0001	30	P<0.0001
Absent	582		90	
Sepsis				
Present	28	P<0.0001	28	P=0.001
Absent	387		90	
Immunosuppression				
Present	26	P<0.0001	26	P=0.001
Absent	489		90	
TNFα-238				
GG	67	P=0.119	67	P=0.091
GA	12		12	
AA	(n.a., n = 1)		(n.a., n = 1)	
TNFα-308				
GG	53	P=0.551	53	P=0.887
GA	45		45	
AA	123		90	
IL18-137				
GG	45	P=0.646	45	P=0.592
GC	85		85	
CC	38		38	

a) *p* of difference between Kaplan Meier survival curves (log rank test)

Table 4. Cox regression for TNFα-238 SNP (genotype) and mortality with Hazards ratios (adjusted for age and sex)				
Allele, genotype	**28 days**	**90 days**	**360 days**	**Total**
TNFα-238: GG vs. AG	P=0.0721	P=0.0918	P=0.1226	P=0.1192
TNFα-238: GG	1	1	1	1
TNFα-238: GA	1.42	1.37	1.31	1.31
Age	1.03 (P<0.0001)	1.03 (P<0.0001)	1.03 (P<0.0001)	1.03 (P<0.0001)
Sex	0.91 (P=0.4402)	0.86 (P=0.2129)	0.87 (P=0.1987)	0.87 (P=0.1872)

Figures

Figure 1. Flow diagram of study design, patients included and patients with determined SNPs

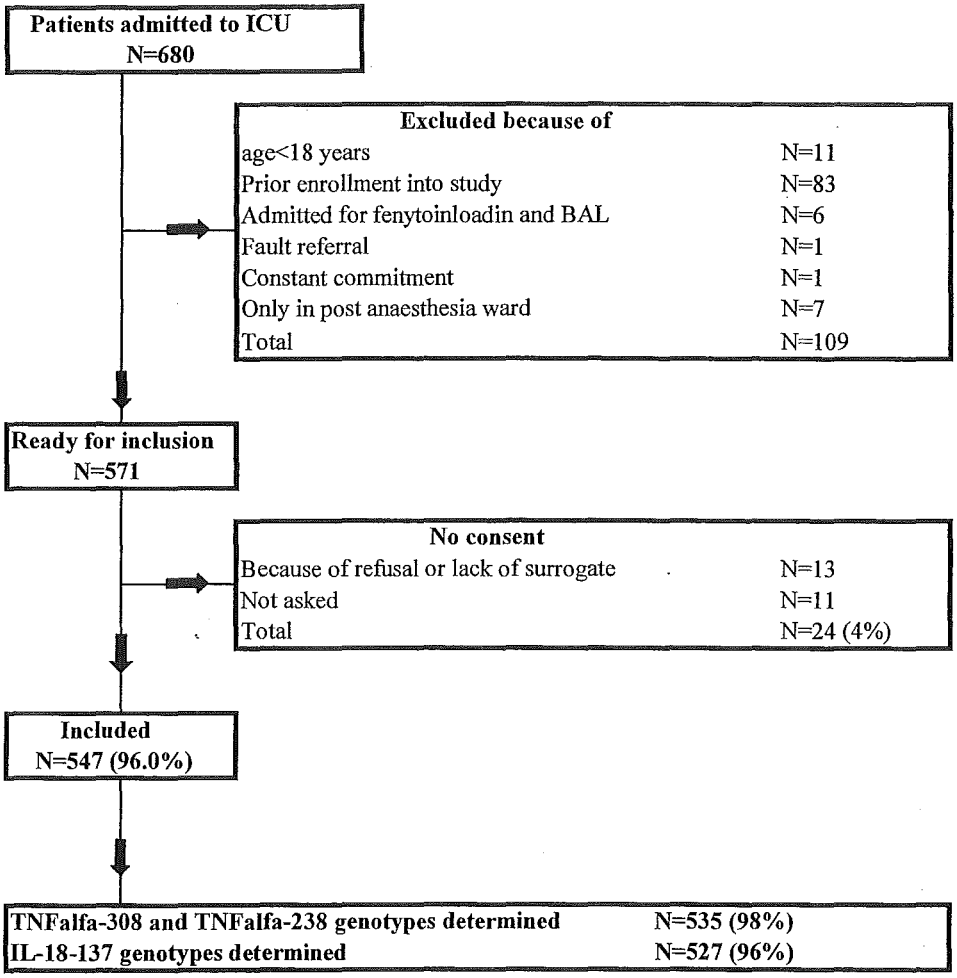
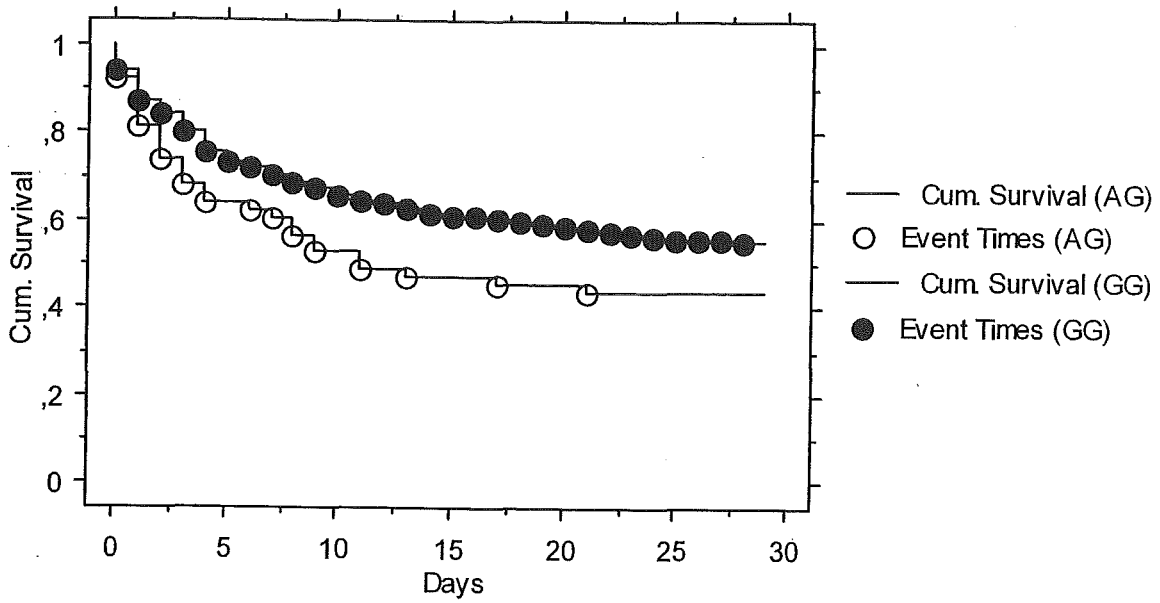


Figure 2


1
,8
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Cum. Survival
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5
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15
20
25
30
Days
Cum. Survival (AG)
Event Times (AG)
Cum. Survival (GG)
Event Times (GG)

Appendix 1

Definitions

Previous disease (Underlying disease): Maximum three of the following conditions were recorded: acute myocardial infarction (AMI), congestive heart disease, insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM), acute pancreatitis, chronic pancreatitis, hepatic disease, renal disease, chronic obstructive pulmonary disease (COPD), active malignant disease, recent trauma.

Immunosuppressive factors: one or more of the following conditions: diabetes mellitus, cirrhosis of the liver, immunosuppressive agents such as use of high doses of corticosteroids, alcohol or drug abuse, active malignant disease, or renal failure (se-creatinine $\geq$200 μmol/l) were registered.

Tobacco consumption: Divided in current smoking, ex-smokers, possible ex-smokers, never smoked, not known. Number of pack years registered. One pack year is equal to smoking of 20 cigarettes daily in one year.

Determination of a nosocomial (ICU acquired) infection: A nosocomial infection was defined as an infection that occurred more than 48 h after admission to the ICU. The diagnosis of nosocomial infection (bloodstream infection, respiratory tract infection and wound infection) was based on Centers for Disease Control and Prevention (CDC) criteria [38] with the following modifications: If anyhow at doubt about the diagnosis of a patient having nosocomial pneumonia two physicians from a committee of three intensive care physicians, employed in other ICU's, reviewed the CRF and decided whether the patient had a nosocomial pneumonia or not. If they disagreed the third intensive care physician reviewed the CRFs and made the final decision. All the cases of doubt or

disagreement (No=15) were reviewed after the inclusion was closed and according to the new CDC criteria and extra criteria [39].

The CDC criteria for infections were also used to define whether patients had an infection before 48 h after admission to the ICU.

Due to the difficulties to determine clinical signs of an urinary tract infection in patients in the ICU with indwelling bladder catheter and differentiate it from colonization the urinary tract, infections and colonization was not counted as a nosocomial infection.

Appendix 2.

SNP analyses

DNA was extracted from whole blood that had been stored at −80°C using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany). The following SNPs were determined using fluorescence-based real-time PCR (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, CA, USA): TNFα-308 (rs1800629), TNFα-238 (rs 361525), and IL-18-137 (rs187238). Predeveloped assays (Applied Biosystems) were used for all SNPs as follows: TNFα -308 (C_7514879_10), TNFα-238 (C_2215707_10) and IL-18-137 (C_2408543_10). PCR amplification was performed in a total reaction volume of 5 μl. The reaction mixture consisted of 0.4 μg/μL gDNA, primer and probe mix (20X), nuclease free water and 2X TaqMan Universal MasterMix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase Uracil N-glycosylase, dNTPs with dUTP, ROX as passive reference and

buffer components. Immediately after a regular PCR run (cycle profile: 50°C for 2 min + 95°C for 10 min + [95°C for 15s + 60°C for 1 min] x 50 cycles), an allelic discrimination run was performed.

Appendix 3.

Table. Admission diagnosis at ICU classified by respectively SNP TNF-α-238, TNF-α-308 and IL-18-137

	No (%)									
	Total (n=547)	**TNF-α-238** (n=535)			**TNF-α-308** (n=535)			**IL-18-137** (n=527)		
Variable		*GG* 482 (90.1)	*GA* 52 (9.7)	*AA* 1 (0.2)	*GG* 358 (66.9)	*AG* 164 (30.7)	*AA* 13 (2.4)	*GG* 296 (56.2)	*GC* 187 (35.5)	*CC* 44 (8.3)
Sepsis	142 (26.0)	125 (25.9)	15 (28.8)	0 (0.0)	97 (27.1)	39 (23.8)	4 (30.8)	75 (25.3)	52 (27.8)	13 (29.5)
Disturbance in liq-uid/electrolyte balance	18 (2.7)	18 (3.7)	0 (0.0)	0 (0.0)	11 (3.1)	7 (4.3)	0 (0.0)	11 (3.7)	6 (3.2)	1 (2.3)
Major Intoxication	14 (2.6)	12 (2.5)	0 (0.0)	0 (0.0)	6 (1.7)	6 (3.7)	0 (0.0)	6 (2.0)	6 (3.2)	0 (0.0)
Neurologic	34 (6.2)	28 (5.8)	4 (7.7)	0 (0.0)	19 (5.3)	13 (7.9)	0 (0.0)	22 (7.4)	7 (3.7)	3 (6.8)
Respiratory	197 (36.0)	166 (34.4)	23 (44.2)	1 (100.0)	130 (36.3)	56 (34.1)	3 (23.1)	108 (36.5)	65 (34.8)	12 (27.3)
Cardio-vascular	49 (9.0)	44 (9.1)	5 (9.6)	0 (0.0)	34 (9.5)	14 (8.5)	1 (7.7)	24 (8.1)	17 (9.1)	7 (15.9)
Gastrointestinal	23 (4.2)	21 (4.4)	1 (1.9)	0 (0.0)	16 (4.5)	4 (2.4)	2 (15.4)	10 (3.4)	10 (5.3)	0 (0.0)
Renal/Urogenital	45 (8.2)	38 (7.9)	4 (7.7)	0 (0.0)	29 (8.1)	13 (7.9)	1 (7.7)	22 (7.4)	14 (7.5)	7 (15.9)
Other	30 (5.5)	30 (6.2)	0 (0.0)	0 (0.0)	16 (4.5)	12 (7.3)	2 (15.4)	18 (6.1)	10 (5.3)	1 (2.3)

Appendix 4.

Table.	**Microbial species[a] diagnosed in cultures (blood, tracheal and urine) and infections at admission to the ICU all stratified to the three SNP's TNFα-238, TNFα-308 and IL-18-137**									
	No (%)									
Patient or microorganism		**TNFα-238** (n=535)			**TNFα-308** (n=535)			**IL-18-137** (n=527)		
Variable	**Total** (n=547)	*GG* (n=482)	*AG* (n=52)	*AA* (n=1)	*GG* (n=358)	*GA* (n=164)	*AA* (n=13)	*GG* (n=296)	*GC* (n=187)	*CC* (n=44)
Patient no (%)										
SUM negative or unknown	315 (57.6)	275 (57.1)	30 (57.7)	1 (100.0)	203 (56.7)	96 (58.5)	7 (53.8)	177 (59.8)	100 (53.5)	24 (54.5)
SUM positive	232 (42.4)	207 (42.9)	22 (42.3)	0 (0.0)	155 (43.3)	68 (41.5)	6 (46.2)	119 (40.2)	87 (46.5)	20 (45.5)
Gram positive bacteria	84 (36.2)	76 (36.7)	6 (27.3)	0 (0.0)	51 (32.9)	29 (42.6)	2 (33.3)	42 (35.3)	27 (31.0)	10 (50.0)
Gram negative bacteria	142 (61.2)	127 (61.4)	12 (54.5)	0 (0.0)	95 (61.3)	42 (61.8)	2 (33.3)	73 (61.3)	54 (62.1)	11 (55.0)
Anaerobe bacteria	2 (0.9)	2 (9.1)	0 (0.0)	0 (0.0)	2 (1.3)	0 (0.0)	0 (0.0)	1 (0.8)	1 (1.1)	0 (0.0)
Fungi	73 (31.5)	65 (31.4)	7 (31.8)	0 (0.0)	56 (36.1)	14 (20.6)	2 (33.3)	36 (30.3)	30 (34.5)	6 (30.0)
Pneumonia, bacteriaemia and/or wound infection	299 (54.7)	258 (53.5)	33 (63.5)	0 (0.0)	201 (56.1)	85 (51.8)	5 (38.5)	157 (53.0)	104 (55.6)	25 (56.8)
Pneumonia	253 (46.3)	218 (45.2)	28 (53.8)	0 (0.0)	174 (48.6)	69 (42.1)	3 (23.1)	132 (44.6)	88 (47.1)	22 (50.0)

Pre-existing low-grade inflammation affects the inflammatory response to low-dose endotoxemia in healthy volunteers

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ABSTRACT

Objective: In critically ill patients, it is unknown to which extent the pre-existing cytokine profile affects the response to a subsequent inflammatory stimulus, such as an infection. We hypothesized that low-grade inflammation, as evoked by an intravenous previous dose of purified *Escherichia coli* endotoxin (lipopolysaccharide, LPS), would enhance the inflammatory response to a subsequent intravenous LPS bolus. This hypothesis was tested in healthy volunteers.

Design: Randomized, double-blind, crossover trial.

Setting: Intensive care unit.

Subjects: Thirteen healthy male volunteers.

Interventions: On three separate occasions, volunteers received two intravenous bolus injections spaced 90 minutes apart, of 1) saline + LPS (0.2 ng/kg) (LPS-0.2); 2) saline + LPS (0.4 ng/kg) (LPS-0.4); or 3) LPS (0.2 ng/kg) + LPS (0.2 ng/kg) (LPS-0.2+0.2).

Measurements and main results: Physiological variables as well as plasma concentrations of tumor necrosis factor-alpha (TNF), interleukin (IL)-6, C-reactive protein (CRP), white blood cell (WBC) and differential counts were measured until 8 hours after the second injection. Outcome measures were 1) the area under the curve (AUC), as calculated from T=0 to T=480 min; and 2) the peak response. Analysis of covariance (ANCOVA) was used. Flu-like symptoms occurred during all three interventions; the peak severity was increased during LPS-0.4 and LPS-0.2+0.2 as compared to LPS-0.2. A supra-additive response during LPS-0.2+0.2 compared to LPS-0.4 and LPS-0.2 was found with regard to the rectal temperature, lymphocyte count and CRP, whereas the effect on TNF was inhibitory, and the effect on IL-6 was additive. No conclusions could be made with regard to heart rate, total WBC, the neutrophil count or the monocyte count. The mean arterial pressure remained unchanged throughout.

Conclusion: In healthy humans, induction of a low-grade inflammatory response by a previous intravenous bolus of LPS affects several aspects of the clinical as well as the biochemical response to a subsequent LPS bolus.

Keywords: endotoxins, sepsis, interleukin-6, tumor necrosis factor-alpha, human experimentation, critical illness.

INTRODUCTION

Critically ill patients in the intensive care unit (ICU) are of high risk of dying from sepsis (1). Apart from an increased risk of infection due to changes in the environmental load and resistance pattern of microbes and to interventions that interfere with natural barriers, such as endotracheal intubation (2), a change in the inflammatory response to infection may also be implicated (3). Most critically ill patients have increased plasma levels of pro-or anti-inflammatory cytokines (4,5), the profile of which may have a profound effect on the response to a subsequent inflammatory stimulus.

In the human endotoxemia model, healthy volunteers receiving an intravenous bolus injection of endotoxin exhibit a highly uniform and reproducible inflammatory response (6). This response is characterized by an increase in pro-inflammatory cytokines such as tumor necrosis factor-α (TNF) and interleukin (IL)-1β, followed by an increase in IL-6 and anti-inflammatory mediators such as IL-1 receptor antagonist and IL-10. With large doses of endotoxin (i.e. 2 to 4 ng per kg), flu-like symptoms peak at 90 minutes, coinciding with the peak in TNF. With lower doses (0.1 to 0.2 ng per kg), the time profile in plasma cytokines is retained albeit at a lower level, whereas no or only minor symptoms are observed (7), facilitating repeated studies in the same individual.

In the present study, we aimed to investigate the effect of low-grade inflammation on a subsequent inflammatory stimulus using the human endotoxemia model. More specifically, we studied the inflammatory response to an intravenous bolus injection of a low dose of purified *Escherichia coli* endotoxin (lipopolysaccharide, LPS) with or without a previous bolus of LPS. We hypothesized that low-grade inflammation, as induced by the previous dose of LPS, would enhance the inflammatory response to the subsequent LPS dose, measured by changes in the principal

outcome measures TNF and IL-6 and the white blood cells (WBC), and C-reactive protein (CRP). Therefore, the second dose of LPS was administered at 90 minutes after the previous dose, i.e. at the time of the peak in plasma TNF after the previous dose.

Since the doses of LPS to be administered were rather small, we did not *a priori* expect to find any effect on the clinical response, as measured by symptoms, rectal temperature, heart rate (HR), and mean arterial pressure (MAP).

MATERIALS AND METHODS

Volunteers.

We included 13 male subjects [median age 25 (range, 19-30) yrs; body mass index (BMI), 23.6 (23.2-25.1) kg/ m^2)]. All subjects were healthy, as defined by an unremarkable medical history, a normal physical examination and normal results on biochemical testing. The subjects had no signs or symptoms of infection in the two weeks preceding either study. Written informed consent was obtained from each volunteer.

The Institutional Review Boards of the County of Copenhagen approved the protocol (file number KA 04015). The protocol was registered in an international clinical trials database (ClinicalTrials.gov ID NCT00197899).

Study design

The volunteers underwent three interventions on three separate study days, which were spaced at least one month apart. In a double-blind cross-over design, volunteers were randomized to receive two consecutive intravenous boluses of normal saline or *Escherichia coli* endotoxin (endotoxin, *E. coli*; lot EC-6, United States Pharmacopeia Convention, Rockville, MD, USA), as follows:

Intervention LPS-0.2:	Saline + LPS (0.2 ng/ kg)
Intervention LPS-0.4:	Saline + LPS (0.4 ng/ kg)
Intervention LPS-0.2+0.2:	LPS (0.2 ng/ kg) + LPS (0.2 ng/ kg)

The two bolus injections were spaced 90 minutes apart. Time 0 was defined as the time of the second bolus injection; all times stated below are given in reference to this zero point.

On each study day, the subject reported to the intensive care unit at 07.00 a.m. after an overnight fast. With the subject resting in the supine position, an arterial catheter was inserted into a radial artery for blood sampling and invasive measurement of the mean arterial pressure (MAP). In the contralateral arm, a peripheral intravenous catheter was inserted into a cubital vein for injection of study medications and infusion of saline. Rectal temperature, arterial pressure, heart rate as measured by standard 5-lead electrocardiography, and peripheral oxygen saturation were continuously monitored and recorded every 15 minutes; any symptoms noted by the volunteer were recorded every 15 minutes on the chart as well. Subjects remained in the fasting state until 4 hours after the second bolus, when they were allowed to eat a light meal. Monitoring was maintained until 8 hours after the second bolus. At this time, the volunteers were discharged; they reported back to the department at 24 hours for the last blood sample.

Blood samples were drawn from an arterial catheter at time points -1.5, 0, 0.5, 1, 1.5, 2, 4, 8 and 24 hours for measurement of WBC and differential counts, cytokines, and CRP. Plasma levels of endotoxin were not measured.

Measurements

Cytokines. Blood was drawn into tubes containing EDTA and trasylol and spun immediately at 3500 ***g*** for 10 min, after which plasma was stored at −80°C until arrival at the laboratory for analysis where it was stored at -20°C. Plasma concentrations of TNF and IL-6 were measured by the enzyme-linked immunosorbent assay (ELISA) technique; all samples were analyzed in duplicate, and mean concentration was calculated. TNF: Plasma concentration was measured using a high-sensitivity assay (Quantikine HS, R&D Systems, Minneapolis, MN, U.S.A.;

lower detection limit 0.5 pg/ml). IL-6: Plasma concentration was measured using a high-sensitivity assay (Quantikine HS, R&D Systems, Minneapolis, MN, U.S.A.; lower detection limit 0.156 ng/L).

WBC and differential counts. Standard laboratory procedures were employed.

CRP. Plasma concentrations of CRP were measured using a high-sensitivity assay measured with nephelometry (Dade Behring, Rødovre, Denmark). The lower limit of detection using this assay was 0.158 mg/l; for statistical analysis, this value was applied for samples where CRP was undetectable.

Symptom score. Based on prospectively registered symptoms, a symptom score was constructed retrospectively. According to this score, headache, chills, myalgias and nausea were separately graded at three arbitrary levels, i.e., from 0-2, where 0 refers to no symptoms, 1 to slight symptoms, and 2 to moderate-to-severe symptoms. The sum of scores was the symptom score, with a resulting range of 0-8.

Statistical analyses

For all variables except the symptom score, the primary outcome measures were 1) the area under the curve (AUC), as calculated from T=0 to T=480 min; and 2) the peak response, i.e. the maximum (for all variables, except lymphocyte and monocyte counts) or minimum (for lymphocyte and monocyte counts) value (Cmax or Cmin as appropriate) between T=0 and T=480 min. All AUC and Cmax or Cmin values for each variable were logarithm transformed prior to analysis. The transformed values were then analysed in a linear mixed model, in which dose was incorporated as fixed effect; whereas the effect of patient and the effect of sequence of endotoxin administration were incorporated as random effects. In all analyses of variance, the logarithm-transformed baseline value of the variable was used as a covariate adjusting for differences at T=0 in the model (analysis of covariance (ANCOVA) (8)); this was done in order to compensate for the

possible effect of the first endotoxin dose during intervention LPS-0.2+0.2, leading to different baseline values among groups. SPSS version 14 (SPSS, Chicago, IL) was used for statistical analysis. $P<0.05$ was considered statistically significant.

The primary objective of the study was to explore whether the inflammatory response after the split-dose intervention (LPS-0.2+0.2) would differ from that observed after a single-dose intervention (LPS-0.2 and LPS-0.4). To describe the effect of the split-dose intervention as compared to the single-dose interventions on the outcome measures (AUC and Cmax or Cmin), the following definitions were used:

Supra-additive:

1. The F test is significant for the difference between interventions; and
2. The effect of intervention LPS-0.4 on the outcome measures is larger than that of intervention LPS-0.2 (indicating the presence of a dose-effect relationship); and
3. The effect of intervention LPS-0.2+0.2 is larger than the effect of both intervention LPS-0.4 and intervention LPS-0.2.

Additive:

1. The F test is significant for the difference between interventions; and
2. The effects of both intervention LPS 0.2+0.2 and intervention LPS-0.4 on the outcome measures are larger than that of LPS-0.2; and
3. The effect of intervention LPS-0.2+0.2 does not differ from that of intervention LPS-0.4.

Inhibitory:

1. The F test is significant for the difference between interventions; and
2. The effect of intervention LPS-0.4 on the outcome measures is larger than that of intervention LPS-0.2; and
3. The effect of intervention LPS-0.2+0.2 is smaller than that of intervention LPS-0.4

No interpretation possible:

1. The F test for the difference between interventions yields a P>0.05; or
2. The results of the pairwise comparisons fail to fit into any of the categories listed above.

This interpretation was applied to the analysis of both AUC and Cmax / Cmin. For the final decision whether endotoxin effects differed between interventions, emphasis was put on the effect on AUC, which was interpreted as the impact of the intervention during the initial 8 hour observation period after the two injections.

Since symptom scores were categorical data, and because the peak symptom severity was considered the major outcome measures for this variable, differences between interventions were analyzed by Friedman's test followed by a Bonferroni-corrected Wilcoxon's test for paired data between any two interventions to pinpoint the difference.

RESULTS

No complications occurred during the study; all subjects were well and discharged home after the 8-hour study period.

Physiological variables

Rectal temperature, heart rate and MAP during the three interventions are displayed in Fig. 1.

Temperature. The temperature increased after all three interventions, to peak at a median temperature of 37.6 (Inter-quartile range 37.4-37.7), 37.7 (37.6-38.3) and 38.5 (38.5-39.2) for LPS-0.2, LPS-0.4, and LPS-0.2+0.2, A significant difference between interventions was found for both the AUC and Cmax (F test for effect of dose, $P<0.0005$ for both outcome measures; Table 1 and 2). According to the subsequent pairwise comparisons between interventions, both the AUC and Cmax were larger during LPS-0.2+0.2 than during LPS-0.4 and LPS-0.2, respectively; moreover, both these outcome measures were higher during LPS-0.4 than during LPS-0.2. According to the definitions given in the Methods section, this was interpreted as a supra-additive effect of the split-dose intervention (LPS-0.2+0.2), compared to the single-dose (LPS-0.4 and LPS-0.2) interventions.

Heart rate. An increase in heart rate was observed after all interventions; the peak heart rate was 83 (75-89), 87 (79-89) and 90 (83-99) min^{-1}. The F test revealed a significant difference between interventions for both AUC ($P<0.0005$) and Cmax ($P<0.005$). According to the pairwise comparisons, the AUC was larger during LPS-0.2+0.2 than during LPS-0.2 and LPS-0.4; Cmax during LPS-0.2+0.2 was larger than during LPS-0.2, but did not differ from Cmax during LPS-0.4. Furthermore, no difference was found for the outcome measures between LPS-0.4

and LPS-0.2. Taken together, the data were interpreted as "No interpretation possible", i.e., no effect of the split-dose could be inferred when compared to single-dose interventions.

Mean arterial pressure. No change occurred after endotoxin, and no difference was detected between interventions.

Symptom score (Table 3, Fig. 1)

Symptom scores during the three interventions are displayed in Table 3 and Fig. 1. Reversible flu-like symptoms were reported during all interventions. During LPS-0.2, mild headache, chills, or myalgia occurred in a total of 10 subjects. During LPS-0.4, chills, headache, shivering (N=4), or myalgias (N=5) were present in a total of 12 subjects. In LPS-0.2+0.2, chills, headache, shivering (N=9), myalgias (N=4), nausea (N=2), and vomiting (N=1) occurred in 12 of 13 subjects. There was a significant difference in symptom scores between the three interventions (Friedmans test, $P<0.0005$). The peak symptom score was higher during intervention LPS-0.2+0.2 compared to LPS-0.2 as well as during LPS-0.4 compared to LPS-0.2 (paired Wilcoxons test, $P<0.005$ for both comparisons), whereas no difference was detected between LPS-0.2+0.2 and LPS-0.4 ($P = 0.29$). Together, these results were interpreted as the split-dose having an additive effect on symptom scores, when compared to the single-dose interventions.

Biochemical variables (Table 1 and 2, Figs. 2 and 3)

TNF. All three interventions were associated with an increase in the arterial concentration of TNF. There was a significant effect of intervention with regard to both AUC and Cmax. Both the AUC and the Cmax were significantly higher during LPS-0.4 than during LPS-0.2; furthermore, the AUC during LPS-0.2+0.2 was significantly lower than that of LPS-0.4 and did not differ from that of LPS-02. The Cmax did not differ between LPS-0.2+0.2 and LPS-0.2, or between LPS-0.2+0.2 and LPS-0.4. According to the definitions, and since emphasis was put on the effect

on the AUC, the results were interpreted as indicating an inhibitory effect of the split-dose endotoxin intervention on the TNF response, compared to the single-dose interventions.

Il-6. The arterial level of this cytokine also increased during interventions. The F test for the difference between interventions yielded a $P<0.0005$ for both the AUC and Cmax; both outcome measures were significantly higher during LPS-0.4 than during LPS-0.2, and during LPS-0.2+0.2 than during LPS-0.2, but did not differ during LPS-0.2+0.2 compared to LPS-0.4. This was interpreted as indicating an additive effect of the split-dose intervention on the IL-6 response.

White blood cell and differential counts. All interventions triggered an increase in total white blood cell and neutrophil counts, and a decrease in lymphocyte and monocyte counts. The F test for the difference between interventions was significant for all these variables with regard to the AUC, and for the lymphocyte count only with regard to the peak response. Furthermore, a dose-response relationship was present only for the lymphocyte count. For this variable, both the AUC and Cmin fulfilled the criteria for a supra-additive effect of the split-dose intervention. For the remaining cell counts, results were interpreted as indicating no effect of the split-dose compared to the single-dose interventions.

CRP. The CRP, as measured by a high-sensitivity assay, increased after all interventions, and the F test showed a significant difference between interventions with regard to both the AUC and Cmax. These outcome measures showed a dose-effect relationship, i.e. they were higher during LPS-0.4 than during LPS-0.2; furthermore, they were higher during LPS-0.2+0.2 compared to both LPS-0.4 and LPS-0.2. These results were interpreted as indicating a supra-additive effect on the CRP response of the split-dose compared to the single-dose interventions.

DISCUSSION

The main finding of this study was that in healthy humans, *in vivo* induction of a low-grade inflammatory response by a previous bolus injection of endotoxin affects parts of the inflammatory response to a subsequent *in vivo* endotoxin challenge. Thus, a split dose compared to the administration of the same total dose as a single intravenous bolus dose, had supra-additive effects on rectal temperature, the lymphocyte count and C-reactive peptide. By contrast, the IL-6 response appeared not to be affected by the split-dose as compared to the single-dose interventions (additive effect), whereas the TNF response appeared to be attenuated (inhibitory effect).

The human endotoxemia model has been used in several studies to address certain aspects of sepsis (9). Following an intravenous injection of endotoxin, an inflammatory response ensues, leading to dose-dependent clinical symptoms that occur after 60 to 90 minutes and persist for approximately four to six hours. This model is only to a limited extent representative of sepsis (10). Nonetheless, the early cytokine response after endotoxin injection closely resembles that observed in the early phase of severe infection such as severe septic shock (11). Thus, for the purpose of investigating the early cytokine response, we believe that the model serves to provide an adequate mimicry of early sepsis in humans.

We found a dose-dependent relationship between endotoxin dose and the subsequent inflammatory response for rectal temperature, TNF, IL-6, lymphocytes and CRP, as 0.4 ng/kg triggered a stronger inflammatory response than did 0.2 ng/kg. This is in agreement with previous studies [12,6]. For HR, MAP, WBC, neutrophils and monocytes the difference was not significant. The absence of any effect on MAP of low-dose endotoxin has been repeatedly shown in young and healthy volunteers [13,7,14]. For the remaining variables for which a dose-response relationship was not detected, even though a paired design was used, the ability of this study to detect such a

relationship may have been limited by the relatively small number of subjects. This had a direct bearing on the ability to detect a supra-additive effect as well in these variables, since the dose-response effect was required to be present for a supra-additive (or additive) effect to be inferred. On the other hand, we believe that this very cautious approach, as well as the use of a statistical method that enabled us to control for the level of the outcome measures at T=0 in the split-dose compared to single-dose interventions, serve to underline the robust nature of the supra-additive effect noted for the rectal temperature, the lymphocyte count and C-reactive peptide. It is also reassuring that analysis of both the AUC and the peak response in these variables indicated a supra-additive effect, even though the AUC was defined as the most important outcome measures in this regard.

It would have been interesting to measure circulating endotoxin levels in order to establish whether the observed effects were due to a change in the way, that endotoxin was cleared from the bloodstream. However, even though endotoxin triggers an inflammatory response of several hours' duration, the substance in itself is cleared rapidly in humans. Following an intravenous injection of 2 ng/kg purified *Escherichia coli* endotoxin in healthy volunteers, endotoxin was detectable in the circulation for 15 minutes at a peak concentration of 15 ng/L, as measured by a chromogenic Limulus assay, which had a detection limit of 3 ng/L (15). Given the rapid clearance rate the low dosage used in the present study (0.2 to 0.4 ng/kg), and the relatively high detection limit of the assay, we consider it highly unlikely that we would have been able to detect even a clinically relevant change in endotoxin clearance. Thus, we cannot rule out the possibility that the present observations were caused by, e.g., slower clearance of endotoxin after the split-dose intervention.

The term "priming" alludes to the synergistic action of endo- and exotoxins *ex vivo* (16). It is based on the principle that application of a first stimulus, e.g., endotoxin, does not fully activate target cells, but renders them more susceptible to a second stimulus, e.g., to pore-forming

exotoxins. For example, in an isolated lung preparation from rats, endotoxin as a single stimulus failed to cause significant pulmonary hypertension, lung edema, or mismatch of ventilation and perfusion. However, the response to a subsequent injection of *E. Coli* hemolysin (exotoxin) was dramatically amplified (17). The opposite phenomenon, "tolerance", is used for scenarios in which the pro-inflammatory response to a stimulus is down-regulated during repeated stimulation, diminishing the detrimental clinical effects (e.g., mortality) of endotoxin [18,19]. This phenomenon was demonstrated in humans *in vivo* by Greisman et al. (20), who found that rectal temperature was rapidly downregulated during a continuous infusion of three different types of endotoxin, and that the dosage required to evoke a fever response was increased the day after the initial endotoxin infusion. Tolerance has been directly related to downregulation of TNF. This was shown by Astiz et al. (21), who pretreated healthy volunteers with monophosphoryl lipid A derived from *Salmonella Minnesota R595* endotoxin at 20 units/kg, inducing a moderate increase in temperature, heart rate, TNF, IL-6 and IL-8. After 24 hours, they received either *E. coli* endotoxin 20 units/kg (2 ng/kg) or vehicle control. The febrile response, tachycardia, TNF, IL-6 and IL-8 response were all significantly reduced after pretreatment with monophosphoryl lipid A.

In the present study, we found indications of co-existing "priming" and "tolerance" phenomena. Thus, the inflammatory response was amplified with regard to temperature as well as CRP and the lymphocyte count, but this amplification was not found with regard to the two cytokines measured; in particular, the TNF response appeared to be significantly less. This suggests, first, that downregulation of TNF alone may be a necessary, but not a sufficient stimulus to induce clinical "tolerance" in humans, since TNF downregulation was observed in the face of an amplification of clinical symptoms. Second, the observations suggest that "tolerance" and "priming" phenomena may not be mutually exclusive phenomena.

A number of studies have demonstrated an attenuated *ex vivo* production of chemo- and cytokines after a single *in vivo* endotoxin challenge in healthy volunteers [22,23]. This attenuated *in vitro* response was demonstrated as early as one hour after endotoxin injection at 4 ng/kg, i.e., at a dose that was approximately 10 times as large as the largest dose used in the present study, and at a time that is associated with significant clinical symptoms. These findings also suggest that an attenuated *ex vivo* response may not correlate with an attenuation *in vivo*, and that up- and down-regulation of different aspects of the inflammatory response may coincide.

Several pathways are intricately linked in the inflammatory response, and it is impossible to hypothesize from this study which pathway mediated the supra-additive response for some variables in the face of an unchanged IL-6 response and an apparent downregulation of TNF. However, two potential alternative pathways explaining these findings involve, first, IL-1β; this cytokine is a critical mediator of fever (24) and may be capable of inducing hepatic synthesis of CRP (25), even though the production of this mediator is usually intimately linked to IL-6 (26). Secondly, cortisol downregulates TNF (27) and may be responsible for endotoxin-induced lymphopenia (28). Thus, the present findings that the TNF response was inhibited, the temperature and CRP responses were augmented, and the lymphopenia was more profound during the split-dose intervention, may, in theory, be due to increased levels of IL-1β and cortisol as compared to the single-dose interventions. However, we elected not to measure IL-1 because of concerns about measurement accuracy; also, cortisol was not part of the predefined variables to be measured, although this would, in retrospective, have been highly relevant. Finally, even if additional measurements showed an increase in IL-1β and cortisol after split-dose endotoxin, this would only have indicated an association, but would not have proven causality or assisted in demonstrating the pathways for this potential relationship.

Compared to the study by Astiz et al. (21), the delay between the two endotoxin doses in the present study was smaller, the doses were lower, and the same type of endotoxin was used for both doses. Thus, the response to a repeated challenge with endotoxin may depend on the delay between the two doses, as well as the dosage and nature of the endotoxin. In particular, the present results were probably critically dependent on the timing of the two separate bolus injections. By spacing the bolus injections by 90 minutes, the second bolus was administered at the time when the plasma concentration of TNF was peaking after the first bolus, i.e., during a phase when the circulating levels of pro-inflammatory cytokines were high and virtually unopposed by anti-inflammatory cytokines.

Healthy elderly volunteers exhibit a more pronounced and protracted inflammatory response to endotoxin (29) and to bacterial infections (30) than their younger counterparts. Therefore, we suggest that the present results would be similar or even more enhanced, had we used healthy older volunteers.

In conclusion, young healthy humans that have been exposed *in vivo* to a low-dose bolus E.coli endotoxin and to a second endotoxin bolus administered 90 minutes later exhibit an altered inflammatory response compared to a single endotoxin bolus. Thus, the temperature increase, lymphopenia and increase in C-reactive peptide are augmented, whereas the TNF response is attenuated, and the IL-6 response appears not to differ from that observed after a single bolus.

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FIGURE LEGENDS

Figure 1

Time course of temperature, heart rate, mean arterial pressure, and symptom score in 13 healthy volunteers after two successive injections of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

Figure 2

Time course of tumor necrosis factor-α (TNF) and interleukin (IL)-6 in 13 healthy volunteers after two successive injections of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

Figure 3

Time course of neutrophils, lymphocytes, monocytes and C-reactive protein in 13 healthy volunteers after two successive injections consisting of saline + endotoxin 0.2 ng/kg (0.2), saline + endotoxin 0.4 ng/kg (0.4), and endotoxin 0.2 ng/kg + endotoxin 0.2 ng/kg (0.2+0.2). Times of injection (-1.5 and 0 hrs) are indicated by arrows. Values are medians and the inter-quartile ranges.

TABLES

Outcomes	Variables	F test for the effect of intervention on log(AUC)	P-values for the pairwise comparisons of effects of interventions, if F test reveals significance			Effect of split dose compared to single dose
			LPS-0.2 vs. LPS-0.4	LPS-0.2 vs. LPS-0.2+0.2	LPS-0.4 vs. LPS-0.2+0.2	
Clinical variables	Temperature	< 0.0005	0.01	< 0.0005	< 0.0005	Supra-additive
	Heart rate	< 0.0005	NS	< 0.0005	< 0.001	NA
	MAP	NS	ND	ND	ND	NA
Biochemical	TNF-alpha	< 0.0005	< 0.0005	NS	< 0.05	Inhibitory
variables	IL-6	< 0.0005	< 0.0005	< 0.001	NS	Additive
	WBC	< 0.0005	NS	<0.0005	<0.0005	NA
	Neutrophil count	<0.0005	NS	<0.0005	<0.0005	NA
	Lymphocyte count	< 0.0005	< 0.0005	< 0.0005	< 0.05	Supra-additive
	Monocyte count	< 0.01	NS	NS	<0.005	NA
	CRP	< 0.0005	<0.0005	<0.0005	<0.0005	Supra-additive

AUC: Area under the curve

MAP: mean arterial pressure.

TNF: tumor necrosis factor.

IL: interleukin.

WBC: white blood cell count.

CRP: C-reactive protein.

NS: not significant.

ND: Analysis not done.

NA: Not applicable (no interpretation possible).

NS: Not significant.

ND: Analysis not done.

NA: Not applicable (no interpretation possible).

Table 3. Peak symptoms scores during interventions.

Intervention	Number of persons with a given peak symptom score			
	0	1-2	3-4	5-6
LPS-0.2	3	9	1	0
LPS-0.4	1	8	3	1
LPS-0.2+0.2	1	4	8	0

FIGURES

Figure 1

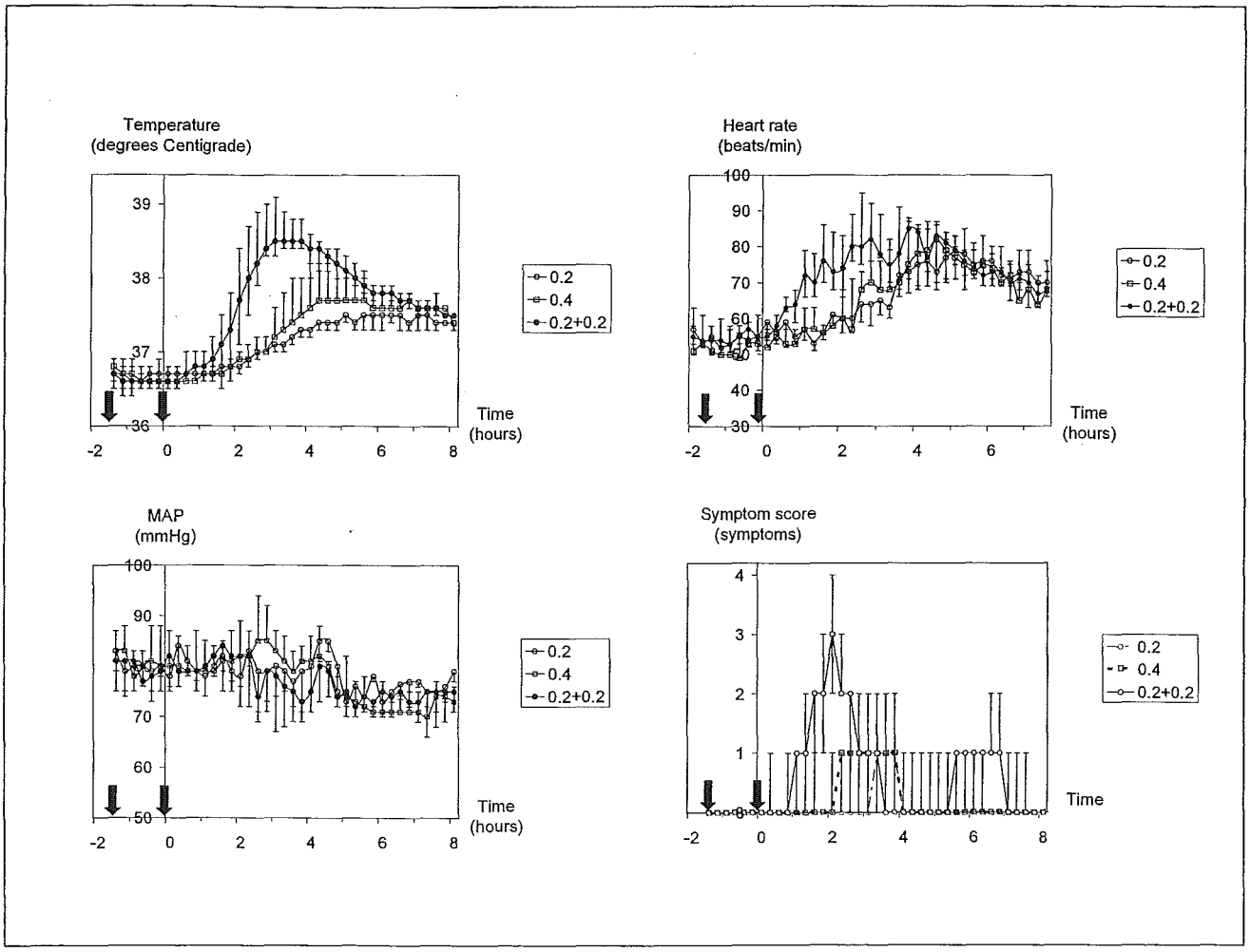


Figure 2

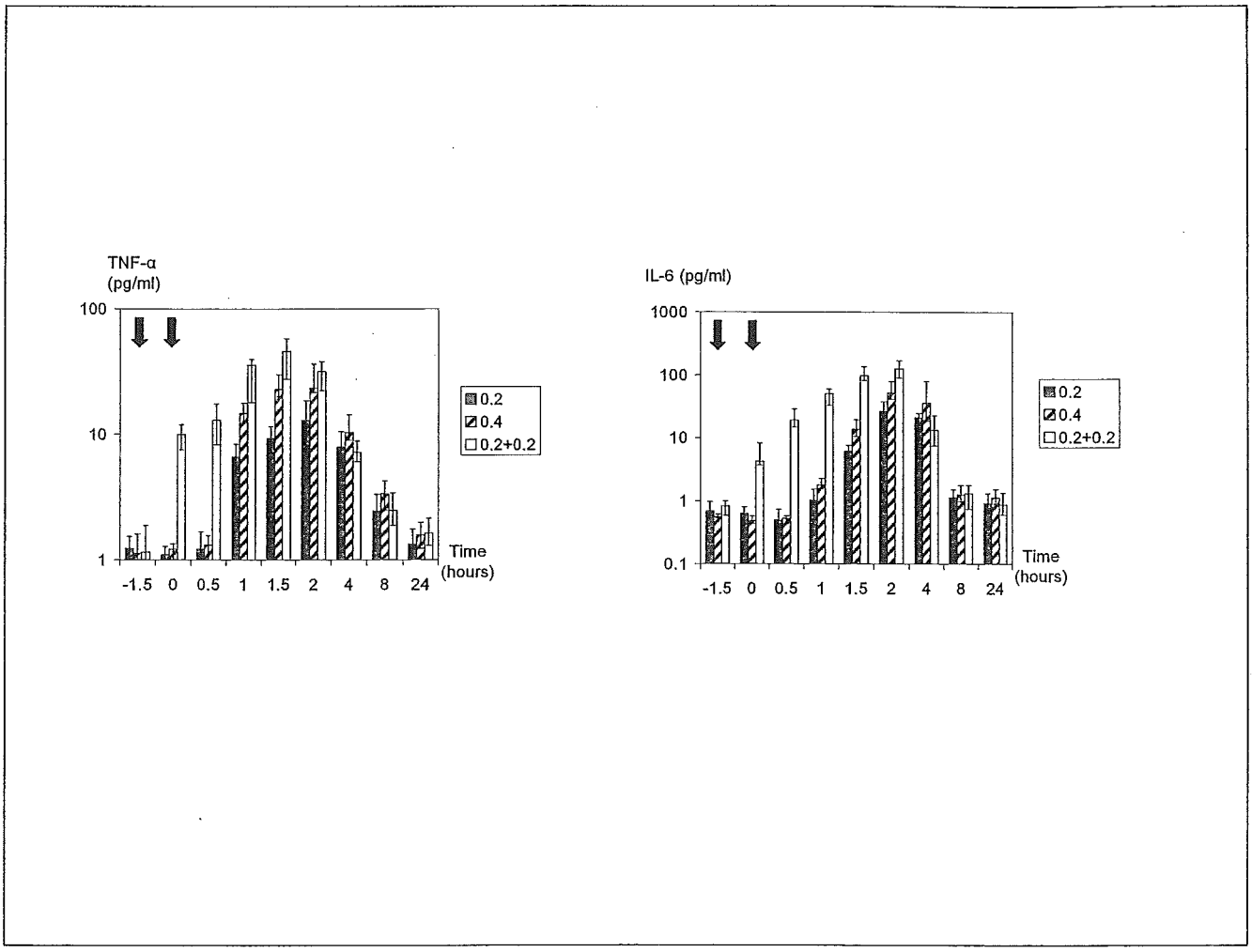
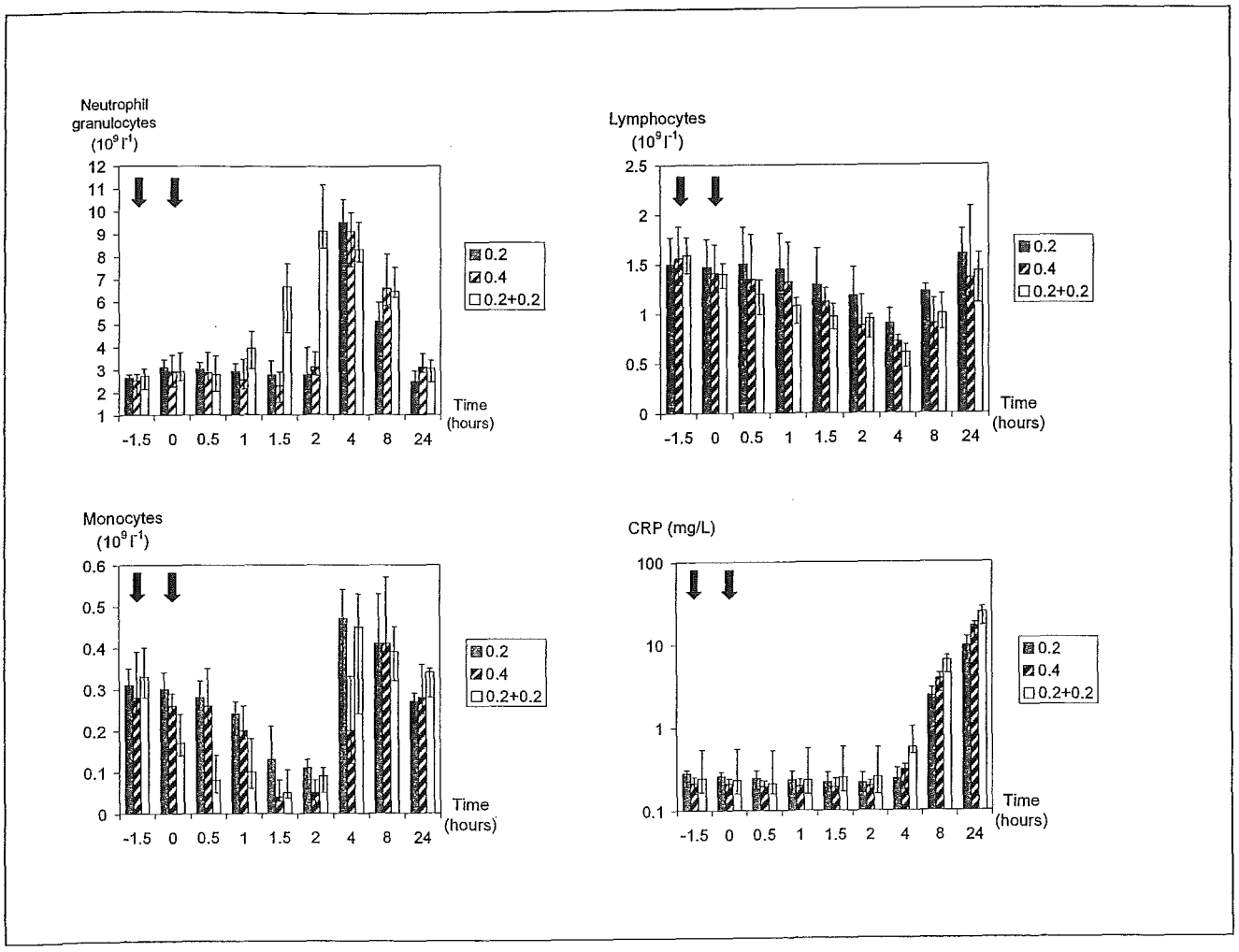

TNF-α
(pg/ml)
100
10
1
-1.5 0 0.5 1 1.5 2 4 8 24
Time
(hours)
0.2
0.4
0.2+0.2
IL-6 (pg/ml)
1000
100
10
1
0.1
-1.5 0 0.5 1 1.5 2 4 8 24
Time
(hours)
0.2
0.4
0.2+0.2

Figure 3


Neutrophil granulocytes ($10^9 l^{-1}$)
12
11
10
9
8
7
6
5
4
3
2
1
-1.5 0 0.5 1 1.5 2 4 8 24
Time (hours)
0.2
0.4
0.2+0.2
Lymphocytes ($10^9 l^{-1}$)
2.5
2
1.5
1
0.5
0
-1.5 0 0.5 1 1.5 2 4 8 24
Time (hours)
0.2
0.4
0.2+0.2
Monocytes ($10^9 l^{-1}$)
0.6
0.5
0.4
0.3
0.2
0.1
0
-1.5 0 0.5 1 1.5 2 4 8 24
Time (hours)
0.2
0.4
0.2+0.2
CRP (mg/L)
100
10
1
0.1
-1.5 0 0.5 1 1.5 2 4 8 24
Time (hours)
0.2
0.4
0.2+0.2