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Functional Polymorphisms of Macrophage Migration Inhibitory Factor as Predictors of Morbidity and Mortality of Pneumococcal Meningitis

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Abstract

Pneumococcal meningitis is the most frequent and critical type of bacterial meningitis. As cytokines play an important role in the pathogenesis of bacterial meningitis, we examined whether functional polymorphisms of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) were associated with morbidity and mortality of pneumococcal meningitis. Two functional *MIF* promoter polymorphisms, a microsatellite (-794 $CATT_{5-8}$; rs5844572) and a single nucleotide polymorphism (-173 G/C; rs755622) were genotyped in a prospective, nationwide cohort of 405 patients with pneumococcal meningitis and in 329 controls matched for age, gender and ethnicity. Carriages of the $CATT_7$ and -173 C high expression *MIF* alleles were associated with unfavorable outcome (P=0.005 and P=0.003) and death (P=0.03 and P=0.01). In a multivariate logistic regression model, shock (OR 26.0, P=0.02) and carriage of the $CATT_7$ allele (OR 5.12, P=0.04) were the main predictors of mortality. MIF levels in the cerebrospinal fluid (CSF) were associated with systemic complications and death (P=0.0002). *Streptococcus pneumoniae* strongly up-regulated MIF production in whole blood and transcription activity of high expression MIF promoter *Luciferase* reporter constructs in THP-1 monocytes. Consistent with these findings, treatment with anti-MIF IgG antibodies reduced bacterial loads and improved survival in a mouse model of pneumococcal pneumonia and sepsis. The present study provides strong evidence that carriage of high expression *MIF* alleles is a genetic marker of morbidity and mortality of pneumococcal meningitis and also suggests a potential role for MIF as a target of immune modulating adjunctive therapy.

Significance Statement

Pneumococcal meningitis, the most frequent cause of bacterial meningitis in adults, is associated with substantial morbidity and mortality. In a prospective, nationwide cohort of patients with pneumococcal meningitis, macrophage migration inhibitory factor (MIF), a proinflammatory mediator, was identified as a novel genetic marker of patient's outcome. High expression *MIF* alleles were associated with disease severity and death, a finding consistent with the harmful consequences of robust proinflammatory cytokine responses on brain edema and neuronal damage in the course of bacterial meningitis. These results provide strong evidence that functional *MIF* polymorphisms are genetic predictors of morbidity and mortality of pneumococcal meningitis and suggest that MIF is potential target for immune modulating adjunctive therapies.

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Introduction

Acute community-acquired bacterial meningitis is a life-threatening disease associated with substantial morbidity and mortality and ranks among the top ten infectious causes of death (1). *Streptococcus pneumoniae* (*S. pneumoniae*) is the most common cause of bacterial meningitis in adults of all-age groups, accounting for 50% to 70% of cases in developed countries (2). Pneumococcal meningitis is associated with a mortality ranging from 19% to 37% (3, 4). Neurological sequelae such as hearing loss, focal deficits, motor and cognitive impairments significantly affect the quality of life of survivors (5-7). Predisposing factors for pneumococcal meningitis include pneumonia, otitis, sinusitis, cerebrospinal fluid leaks, splenectomy or asplenic states, debilitating conditions (*i.e.* alcoholism, cirrhosis, diabetes and cancer) and primary or acquired immune deficiencies (*i.e.* multiple myeloma, hypogammaglobulinemia, sickle cell anemia, HIV/AIDS and the use of immunosuppressive agents). Genetic studies of extreme phenotypes have revealed that patients with single-gene inborn errors in *MyD88*, *IRAK4* and *NEMO* affecting the activation of the canonical TLR and IL-1R signaling pathways or in complement factor two are prone to pneumococcal diseases (8, 9). In addition, case-control candidate gene studies identified polymorphisms of genes associated either with increased susceptibility (*MBL2, PTPN22*) to or with protection (*TIRAP*, *NFKBIA, NFKBIE*) from pneumococcal disease (2, 8, 9). The increased susceptibility was related to reduced concentrations of the mannose binding lectin (variants of *MBL2*) or to an increased activity of the PTPN22 phosphatase (variants of *PTPN22*) (10, 11). The protection afforded by the polymorphic *TIRAP* variant is mediated by an attenuation of TLR2 signal transduction due to a defective recruitment of the *TIRAP* variant to TLR2 (12). The functional effects of the polymorphisms of the *NFKBIA* and *NFKBIE* genes coding for the inhibitors of NF-κB (IκB) are unknown (13).

Cytokines are critical effector molecules of the immune system and play a central role in the orchestration of host defenses against infection. Up until now no polymorphism of cytokine genes (including *TNF, IL6*, *IL10* and *LTA*) has been associated with susceptibility to and outcome of invasive pneumococcal infection (2). Within this large family of mediators, macrophage migration inhibitory factor (MIF) occupies a special place (14, 15). Unrelated to classical cytokine families (tumor necrosis factor, chemokines, interleukins or interferons), MIF is a constitutively expressed pro-inflammatory cytokine acting at the interface of the immune and endocrine systems. Within the innate immune system, MIF positively regulates TLR4 expression, inhibits activation-induced and p53-dependent apoptosis of macrophages and counter-regulates the anti-inflammatory and immunosuppressive effects of glucocorticoids in part by a down-regulation of mitogen-activated protein kinase phosphatase-1 (MKP-1) (16-19). MIF is up-regulated in inflammatory, infectious and auto-immune diseases functioning as a modulator of innate and adaptive immunity (20-22).

Functional polymorphisms of the *MIF* gene locus include a microsatellite repeat of five to eight CATT tetranucleotide ($CATT_{5-8}$) at position -794 (rs5844572) and a single nucleotide polymorphism (SNP) of a G to C transition at position -173 (-173G/C) (rs755622) (23, 24). Genetic studies have revealed a complex picture of the role of polymorphic *MIF* alleles in the pathogenesis of auto-immune diseases (20, 25). Few studies have been performed in patients with infectious diseases, especially in patients with bacterial sepsis (20, 25). We therefore examined the impact of the *MIF* gene locus on the susceptibility to, severity and outcome of pneumococcal meningitis in a large, nationwide cohort of patients. Functional studies of polymorphic *MIF* promoters were conducted in human monocytic cells stimulated with *S. pneumoniae* and analyzed by

Luciferase reporter assays. Lastly, the effect of an anti-MIF treatment strategy was evaluated in a mouse model of *S. pneumoniae* pneumonia and sepsis.

Results

Pneumococcal meningitis cohort. A total of 461 patients with culture-proven, community-acquired *S. pneumoniae* meningitis and 343 controls matched for age (median, 59.4 years *vs*. 60.1 years), gender (female: 53% *vs*. 50.8%) and ethnicity (Caucasian: 94.2 *vs*. 96.0%) were enrolled in a prospective, nationwide cohort study. DNA samples were available from 434 patients and 329 controls who were all Caucasians. The baseline characteristics of patients are shown in Table 1. Briefly, 80.4% of the patients were bacteremic and 43.7% required ICU admission for shock or respiratory failure. During hospitalization, 79.6% developed neurological complications and 38.1% systemic complications. Outcome was unfavorable (defined as a Glasgow Outcome Score of 1 to 4) in 133 patients (32.8%). Thirty patients (7.5%) died (Glasgow Outcome Score of 1).

Association between MIF polymorphisms and susceptibility to, severity and outcome of pneumococcal meningitis. Allelic frequencies and genotypes for the -173 G/C (rs755622) and $CATT_{5\text{-}8}$ (rs5844572) polymorphisms of patients and controls are presented in Supplementary Table 1. No deviation from the Hardy-Weinberg equilibrium was observed for the -173 G/C SNP and $CATT_{5\text{-}8}$ microsatellite. The allele frequencies, genotypes and haplotypes of the -173 G/C and of the $CATT_{5\text{-}8}$ polymorphisms were similar in patients and controls (P>0.5) indicating that there was no association between *MIF* polymorphisms and susceptibility to pneumococcal meningitis. Carriage of the -173 C or of the $CATT_7$ high MIF expression alleles were associated with unfavorable outcome (-173 C: OR 1.9, P=0.003; $CATT_{7:}$ OR 1.89, P=0.005), respiratory failure (-173 C: OR 1.71, P=0.03) and death (-173 C: OR: 2.6, P=0.01; $CATT_7$: OR 2.27, P=0.03) (Table 2). The association between the -173 C or $CATT_7$ high *MIF* expression allelic variants and death was also significant using an additive mode of inheritance (Figure 1). Carriage of the $CATT_7$ allele was associated with markers of inflammation such as C-reactive protein (P=0.02) and

erythrocyte sedimentation rate (P=0.02) and with indicator of disease's severity (i.e. a CSF leukocyte count below 1000 cells per mm^3) (P=0.05) (22). The association between the $CATT_7$ and death remained significant in a multivariate logistic regression model (OR 5.12, P=0.04) and tended to be associated with unfavorable outcome (OR 2.61, P=0.07) after adjustment for relevant co-variables (Table 3).

MIF levels in the CSF and morbidity and mortality of pneumococcal meningitis. MIF CSF levels were measured in 242 patients (52% of the patients included in the study). The median concentration was 2.65 ng/ml (range: 0.025 to 323 ng/ml). The MIF CSF levels were similar in patients in whom antibiotic treatment was started before or after the lumbar puncture (median [range]: 2.49 [0.02-323.22] versus 3.49 [0.02-68.34] ng/ml, P=0.22). MIF CSF levels were markedly higher in non-survivors than in survivors (10.80 [3.29-89.11] versus 3.19 [0.02-323.22] ng/ml, P=0.0002) and also higher in patients with than in patients without unfavorable outcome (P=0.0001), respiratory failure (P=0.006) or systemic complications (P=0.0004) (Supplementary Figure 1).

S. pneumoniae induces MIF production in whole blood and MIF promoter activity in THP-1 monocytes. Given that *MIF* polymorphisms and MIF levels in the CSF were associated with morbidity and mortality of pneumococcal meningitis, we examined whether *S. pneumoniae* up-regulated blood levels of MIF and whether polymorphic *MIF* alleles affected the transcriptional activity of the MIF promoter. *S. pneumoniae* induced a strong and dose-dependent up-regulation of MIF production in human whole blood (Figure 2A). After 24 hours, the median concentrations of MIF in cell culture supernatants increased 2.4-fold, 3.8-fold and 15.5-fold upon stimulation with 2×10^6, 2×10^7 and 2×10^8 CFU/ml of *S. pneumoniae* (P=0.005, P=0.001 and $P<10^{-8}$ when compared with unstimulated control cells). We then evaluated the transcriptional activity of the four most common polymorphic MIF promoters ($CATT_5$/-173 G, $CATT_6$/-173 G, $CATT_6$/-173 C and

$CATT_7$/-173 C) in human THP-1 monocytes (Figure 2B). The transcriptional activity was up-regulated after stimulation with *S. pneumoniae* and was highest with the $CATT_6$/-173 C and $CATT_7$/-173 C constructs (P=0.03 and P=0.003 when compared with $CATT_5$/-173 G).

Anti-MIF antibody protects against lethal pneumococcal pneumonia and sepsis. Given that high-expression *MIF* alleles and high MIF CSF levels were associated with poor patient's outcome, we tested the impact of anti-MIF treatment strategy in a mouse model of pneumococcal pneumonia and sepsis (Figure 3). When compared with mice treated with control antibody, mice treated with anti-MIF antibody had lower bacterial counts in the lung (median [range] log CFU per gram of tissue: 3.8 [1 – 9.2] versus 1 [1 - 7.9], P=0.008) and higher survival (25% versus 53.6%, P=0.04) indicating that neutralization of MIF was associated with enhanced bacterial clearance and substantial survival benefit in this lethal model of pneumococcal sepsis.

Discussion

In this large, nationwide and well characterized cohort of patients with community-acquired pneumococcal meningitis, high-expression *MIF* alleles and elevated levels of MIF in the CSF were identified as predictors of morbidity and mortality. These results suggest that a vigorous MIF response is detrimental in patients with pneumococcal meningitis, a finding consistent with the harmful consequences of robust proinflammatory cytokine responses on brain edema and neuronal damage in the course of bacterial meningitis (26).

Up to now studies of polymorphisms of cytokine genes have failed to demonstrate association between *TNF*, *IL6*, *IL10* or *LTA* and the susceptibility to or the outcome of bacterial meningitis (2). MIF is therefore the first cytokine whose gene polymorphisms are associated with morbidity and mortality of bacterial meningitis. MIF may exert its effects by promoting the migration, tissue seeding and survival of myeloid cells (such as neutrophils and monocytes) that are important players in the pathogenesis of bacterial meningitis (26, 27). At a cellular level, MIF up-regulates pro-inflammatory responses of immune cells via the activation the MAPK and PI3K/Akt signaling pathways downstream of the CD74-CD44 and CXCR2-CXCR4 MIF receptor complex, which is likely to be detrimental in patients with bacterial meningitis (26, 27).

Few and fairly small studies looked at MIF in patients with pneumococcal diseases (28, 29). Elevated levels of MIF were detected in the CSF of 31 patients with bacterial meningitis (29). Albeit higher in 11 patients with pneumococcal meningitis than in 15 patients with meningococcal meningitis, MIF CSF levels were not associated with patient's outcome. In a study that included 15 patients with pneumococcal meningitis, the frequency of the $CATT_7$ allele, but not that of the -173 C allele, was higher in patients with meningitis than in those with non-CNS pneumococcal infections (28). Associations between *MIF* genotypes and other clinical variables were not reported. Moreover, given the absence of

uninfected control subjects, it was not possible to determine whether the carriage of a $CATT_7$ allele was a risk factor for the development of pneumococcal meningitis. The present study included a large number of patients and had sufficient power to examine these questions. Neither the $CATT_{5\text{-}8}$ nor the -173 G/C *MIF* polymorphisms was a risk factor for susceptibility to pneumococcal meningitis. Yet, high expression *MIF* alleles and high levels of MIF levels in the CSF were associated with markers of inflammation, indicators of disease's severity, complications and mortality.

Studies exploring the relationship between functional polymorphisms of the *MIF* gene locus and susceptibility to, severity and outcome of infectious and auto-immune diseases have yielded a complex global picture (20, 25). In clinical studies of infectious diseases, carriage of high-expression *MIF* alleles ($CATT_{7/8}$) or haplotypes ($CATT_{6/7}$/-173 C) was associated with severe complications of malaria (30), with mortality in patients with severe sepsis (31) and with survival in patients with community-acquired pneumonia (32). Carriage of the low-expression *MIF* allele ($CATT_5$) protected children from meningococcemia (33), but it predisposed older adults to Gram-negative bacteremia (34). Notwithstanding the possibility that the impact of *MIF* polymorphisms may vary according to the age of the host, the site of infections and the type of microorganism, it also is possible that confounding factors (such as selection biases, ambiguous phenotypes, patient and pathogen heterogeneity and lack of power) account for these discrepant findings (2, 35, 36). To minimize the influence of potential confounders, we elected to conduct our study in a large and homogeneous cohort of patients with community-acquired bacterial meningitis caused by a single pathogen (*S. pneumoniae*). One of the strengths of the study was the use of a well-defined phenotype. Working with a nationwide cohort also protected against the potential risk of single center biases. The choice of controls (patient's partners or proxies) limited the risk of socio-economic and environmental mismatching.

Power calculations indicated that the study had 80% power to detect odds ratios in the range of 1.5 to 3.0 for the main study endpoints.

The study has also some limitations. It included only Caucasians. Given that *MIF* polymorphisms are unequally distributed in different populations (20), our findings might therefore not be extrapolated to other races or ethnic groups. Blood samples were not available to measure MIF in the systemic circulation. Yet, one study looking at MIF levels in patients with CNS infections did not find an association between MIF levels in the CSF and inflammation biomarkers in the systemic circulation (37-40). Attempts to correlate *MIF* polymorphisms with MIF CSF levels were unsuccessful. This may be due to the relatively limited number of CSF and DNA specimen pairs. Additionally, the range of MIF CSF levels was rather broad, likely because lumbar punctures were performed in patients at different stages of the disease. It is also conceivable that carriage of a high expression *MIF* allele plays a detrimental role at the very early stage of disease, promoting inflammation, blood brain barrier disruption and bacterial neuro-invasion, as shown both for MIF or interferon lambda in West Nile encephalitis (41, 42). MIF-driven early pathogenic effects may indeed not be correlated with CSF parameters measured just once at a later stage of the disease. In that scenario, both *MIF* polymorphisms and MIF expression levels might be independent markers of severity at different stages of the disease. Finally, given that MIF is constitutively expressed by innate immune cells and markedly up-regulated upon infection, the release of intracellular MIF pools from dying cells may have increased MIF levels in the CSF compromising pair wise analyses of *MIF* gene polymorphisms and MIF protein levels.

A large body of experimental and clinical evidence indicates that MIF is an upstream mediator of the host antimicrobial defense response and a powerful immunomodulating cytokine. It has therefore been investigated as a target for therapeutic interventions in infectious, inflammatory and autoimmune diseases (19, 32-34, 37-40, 43-45). The

observation that *MIF* is a genetic marker of morbidity and mortality of pneumococcal meningitis is in line with the results obtained in animal models of sepsis and in clinical studies that have linked high levels of MIF with life-threatening bacterial infections (21, 22, 46-49). In a murine model of pneumococcal colonization, the clearance of pneumococci from the nasopharynx, a critical step in the pathogenesis of invasive pneumococcal diseases, was more rapid in wild-type mice than in MIF-deficient mice (45). This finding suggested that MIF might be a protective factor against pneumococcal diseases. However, in the present study there was no relationship between *MIF* expression and susceptibility to pneumococcal meningitis. In a model of pneumococcal pneumonia, wild-type mice had higher bacterial load, higher innate immune cell counts in the lung parenchyma, more severe lung pathology and a higher mortality rate than MIF-deficient mice (50), indicating that MIF played a detrimental role in severe pneumococcal infection. Consistent with this observation, high *MIF* expression alleles and MIF CSF levels were predictors of disease severity and poor outcome in patients with pneumococcal meningitis. Furthermore, anti-MIF therapy using either neutralizing antibodies (present study) or a small-molecule inhibitor of MIF (50) promoted bacterial clearance and increased survival in a lethal pneumococcal sepsis. Likewise, humanized anti-MIF antibodies were shown to be protective in a lethal mouse *E. coli* peritonitis model (51). A first-in-class anti-MIF monoclonal antibody (BAX69/imalumab) is currently under evaluation in phase 1/2a clinical trials in patients with metastatic colorectal cancer or with ovarian cancer and malignant ascites.

Altogether, the present study provides strong evidence that carriage of high expression *MIF* alleles is a genetic marker of morbidity and mortality of pneumococcal meningitis and also suggests a potential role for MIF as a target of immune modulating adjunctive therapies for bacterial meningitis and sepsis.

Materials and Methods

Bacteria preparation and whole-blood stimulation assay. A serotype 19 *S. pneumoniae* strain (kind gift of Prof Gilbert Greub, Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne) isolated from the CSF of a patient with acute community-acquired meningitis was used in all stimulation experiments. Bacteria were grown to log phase in brain-heart infusion medium, washed and resuspended in PBS at an OD_{620} of 1.0. Whole blood from five healthy Caucasian subjects was drawn into S-monovette-tubes (Sarstedt, Nümbrecht, Germany) containing 16 U heparin/ml, diluted 5-fold in phenol red-free RPMI 1640 medium containing 2 mM L-glutamine (Life Technologies, Grand Island, NY) (52). Blood was incubated at 37°C with *S. pneumoniae* (53). Penicillin and streptomycin (100 µg/ml) were added to the cultures one hour after stimulation. Cells were pelleted (500 × *g* for 5 min at room temperature) 24 hours after stimulation and cell-free supernatants were used to measure MIF concentrations by ELISA (R&D Systems, Abingdon, UK). The lower limit of detection of the assay was 15.6 pg/ml. LDH was measured using the International Federation of Clinical Chemistry (IFCC) method to evaluate cell death. LDH concentration in samples (9.5-66 units/l) remained in the normal range (*i.e.* <250 units/l).

Patient cohort and controls. After identification by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM, Academic Medical Center, Amsterdam) patients older than 16 years of age and with a positive CSF culture were enrolled between March 2006 and June 2011 in a nationwide prospective cohort study (54). The study was approved by the medical ethics committee of the University of Amsterdam, the Netherlands. Treating physicians obtained informed written consent from either the patient or their legal representatives. Patients with culture-negative community-acquired or with hospital-acquired bacterial meningitis were excluded. Controls consisted of co-dwelling non-related

patients proxies who shared environment and socio-economic milieu and provided a written informed consent (55). Demographic data including age, gender and ethnicity for both controls and patients were collected. A detailed medical history and extensive clinical and laboratory data were obtained from all patients. Outcome was evaluated at discharge according to the Glasgow Outcome Scale (GOS) with the following scoring system: score of 1 for death, score of 2 for persistent vegetative state, score of 3 for severe disability defined as a conscious patient who is dependent for daily activities, score of 4 for moderate disability defined as a patient with some deficits, capable of living independently but unable to return to work, score of 5 for a good recovery (56). A favorable outcome was defined as a score of 5 and an unfavorable outcome as a score of 1 to 4. A CSF leukocyte count below 1000 per mm^3 was used as an indicator of disease's severity (57).

Blood collection and genotyping. Blood from patients and controls was collected in sodium EDTA. DNA extraction was performed by the Gentra Puregene isolation kit (Qiagen, Hilden, Germany) with quality control evaluation for DNA yield and purity. The -794 $CATT_{5-8}$ microsatellite (rs5844572) and the -173 G/C SNP (rs755622) were genotyped as reported previously (33). Given the frequencies of the -173 C minor allele and of the $CATT_7$ allele in the Caucasian population, the study had 80% power to detect odds ratios (OR) of at least 1.5 for the susceptibility to infection, 1.9 for an unfavorable outcome and 3.0 for mortality for these two alleles. These numbers were calculated using the Quanto 1.2.3 software (http://biostats.usc.edu/software).

CSF collection and MIF measurement. CSF was obtained from the first lumbar puncture, centrifuged and supernatant was aliquoted and stored at −80°C until analysis. Human MIF levels in the CSF were measured by the Luminex® technology using a Milliplex assay (Millipore, Billerica, MA, USA). The lower limit of detection of the assay was 12 pg/ml.

***MIF* promoter activity.** A fragment ranging from -1073 to -129 bp of the *MIF* gene of either the -794 CATT5/-173 G, -794 CATT6/-173 G, -794 CATT6/-173 C, or -794 CATT7/-173 C genotype was cloned in the pGL3-basic luciferase vector (Promega) (58). The human monocytic THP-1 (TIB-202, American Type Culture collection, Manassas, VA, USA) cell line was cultured in RPMI 1640 medium containing 2 mM L-glutamine, 50 µM 2-mercaptoethanol and 10% heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO). Forty thousand THP-1 cells in 96-well plates were transfected with 350 ng of the luciferase reporter vector together with 50 ng of the *Renilla* pRL-TK vector (Promega) (59). After 7 hours, cells were incubated with *S. pneumoniae* (MOI 10). Penicillin and streptomycin (100 µg/ml) were added to the cultures one hour after stimulation and incubation was continued for an additional 23 hours. Luciferase and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Results were expressed as the ratio of luciferase activity to *Renilla* luciferase activity. An empty vector served as a negative control (18).

Mouse model of pneumococcal pneumonia and sepsis. Animal experimentations were approved by the Office Vétérinaire du Canton de Vaud (authorization number 877.8) and performed according to the institution and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). Female BALB/cByJ mice (10 weeks-old; Charles River Laboratories, Saint Germain sur l'Arbresle, France) were housed under specific pathogen-free conditions. Mice (8 to 10 per treatment group) were injected intraperitoneally with two mg of rabbit anti-MIF or non-immune (control) IgG (obtained as described in (37)) administered two hours before an intranasal inoculation of 10^4 CFU of *S. pneumoniae* in anesthetized mice. Survival was monitored at least twice daily until the end of the experiment. In selected experiments, mice were sacrificed 48 hours after infection to obtain blood and lung tissue for the measurements of bacterial counts.

Statistical analyses. Graphs were generated with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Data were analyzed using STATA version 11.1 (StataCorp LP, College Station, Texas, USA) and IBM SPSS Statistics 21.0 (IBM, Armonk, NY, USA). Genotype frequencies among patients and controls and Hardy-Weinberg equilibrium (HWE) were calculated using the chi square test. Tests for associations between *MIF* genotypes and susceptibility to and severity of pneumococcal meningitis were performed using logistic regression models. Stepwise multivariate selection was performed using a P value greater than 0.2 for removal of variables in the model. Sex, age and polymorphism carriage were forced into the model. Polymorphisms were analyzed using dominant (comparison of heterozygotes plus homozygotes versus wild-type carriers) and additive modes of inheritance (assessment of the effect of each additional copy of the minor allele). Strength of relationships between CSF levels and continuous variables was evaluated using Spearman's correlation tests. Dichotomous variables were compared with the chi square test. Continuous variable for patient's characteristics and disease parameters were assessed using Mann-Whitney U or Kruskall-Wallis tests. MIF promoter activity and concentrations of MIF released in whole blood after stimulation with *S. pneumoniae* were analyzed using one-way ANOVA for multiple comparisons. The log-rank test was used to compare the Kaplan-Meier survival curves. All tests were two-tailed and P values less than 0.05 were considered to indicate statistical significance.

Author contribution

A.S., M.C.B., T.R., D.v.d.B. and T.C. designed research; A.S., M.C.B., T.R., D.L.R., M.V.S., B.F. and A.v.d.E. performed research; A.S., M.C.B., T.R., P.Y.B., D.v.d.B. and T.C. analyzed data; and A.S., M.C.B., T.R., P.Y.B., D.v.d.B. and T.C. wrote the paper.

Declaration of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Carriage of -173 C or $CATT_7$ high-expression *MIF* allelic variants is associated with death from pneumococcal meningitis. Proportions of survivors (white bars) and non-survivors (black bars) for the -173 (G/G, G/C and C/C) and $CATT_7$ (X/X, 7/X and 7/7) genotypes. Logistic regression analysis was performed using dominant and additive modes of inheritance (P=0.01 and 0.01 for -173 G/C and P=0.03 and 0.01 for $CATT_7$, respectively).

Fig. 2. *S. pneumoniae* induces MIF production in whole blood and polymorphic *MIF* alleles affect the transcriptional activity of the MIF promoter. (**A**) Whole blood from 5 healthy volunteers was stimulated for 24 hours with *S. pneumoniae*. MIF concentrations were measured by ELISA. Bottom, median and top lines of the box mark the 25^{th}, 50^{th} and 75^{th} percentiles. Vertical lines with whiskers show the range of values. P=0.005 (2×10^6), P=0.001 (2×10^7) and $P<10^{-8}$ (2×10^8) when compared to control. (**B**) THP-1 monocytes were transiently transfected with $CATT_5$/-173 G, $CATT_6$/-173 G, $CATT_6$/-173 C and $CATT_7$/-173 C *MIF* promoter pGL3 and *Renilla* luciferase vectors. Cells were stimulated for 24 hours with (solid bars) or without (open bars) *S. pneumoniae* (MOI 10). Data are means + SD of 3 independent experiments performed in triplicates. P=0.05 ($CATT_6$/-173 G), P=0.04 ($CATT_6$/-173 C) and P=0.007 ($CATT_7$/-173 C) for *S. pneumoniae vs.* control. After *S. pneumoniae* stimulation, P=0.03 ($CATT_6$/-173 C) and P=0.003 ($CATT_7$/-173 C) when compared to $CATT_5$/-173 G.

Fig. 3. Anti-MIF antibody protects against lethal pneumococcal pneumonia and sepsis. Mice were treated with anti-MIF or non-immune (control) IgG (2 mg injected

intraperitoneally) given two hours prior to an intranasal inoculation of 10^4 CFU of *S. pneumoniae*. (**A**) Box plots of bacterial counts in the lung 48 hours after infection. Data are from five independent experiments with a total of 34 mice per treatment group. Bottom, median and top lines of the box mark the 25^{th}, 50^{th} and 75^{th} percentiles. Vertical lines with whiskers show the range of values. P=0.008 (Mann-Whitney U test). (**B**) Kaplan-Meier survival plot. Data points are from three independent experiments with a total of 28 mice per treatment group. P=0.04 (log-rank test).

Table 1. Characteristics of controls and patients with pneumococcal meningitis.

Characteristics	Controls n=343	Patients n=461
Age (years)	59.4 ± 18.1	60.1 ± 20.8
Female gender	146 (49.2)	220 (47.0)
Caucasian ethnicity	329 (96.0)	434 (94.2)
		Causasian patients with DNA and outcome (n=405)
Predisposing factors (n=405)		
Otitis/sinusitis		191 (47.3)
Immunocompromised status		100 (24.7)
Body temperature in °C (n=401)		39.0 ± 1.5
Glasgow coma scale < 8 (indicating coma) (n=402)		53 (13.2)
Bacteremia (n=357)		287 (80.4)
Neurologic complications (n=404)		257 (79.6)
Systemic complications (n=396)		151 (38.1)
Complications (n=405)		
Shock		25 (6.2)
Respiratory failure		93 (23.0)
ICU admission		177 (43.7)
Mechanical ventilation		140 (34.6)
Outcome		
Glasgow Outcome Scale (n=405)		
1: Death		30 (7.5)
2: Persistent vegetative state		1 (0.2)
3: Severe disability		18 (4.4)
4: Moderate disability		84 (20.7)
5: Good recovery		272 (67.2)
Hearing loss at discharge (n=368)		41 (11.1)

Data are mean ± IQR or number (percent). ICU: intensive care unit.

Table 2. Association between *MIF* gene polymorphisms and patient's outcome.

	Unfavorable outcome (GOS ≤ 4; n=133)		**Respiratory failure (n=93)**		**Death (n=30)**	
Polymorphisms	**OR (95% CI)**	***P value***	**OR (95% CI)**	***P value***	**OR (95% CI)**	***P value***
GC/CC *vs* GG	1.9 (1.24-2.92)	0.003	1.71 (1.06-2.74)	0.03	2.6 (1.01-3.78)	0.01
55/5X *vs* XX	0.88 (0.58-1.33)	0.54	0.87 (0.54-1.39)	0.56	0.66 (0.31-1.43)	0.30
66/6X *vs* XX	0.98 (0.57-1.68)	0.93	1.05 (0.57-1.93)	0.89	1.48 (0.50-4.38)	0.48
77/7X *vs* XX	1.89 (1.21-2.96)	0.005	1.41 (0.98-2.63)	0.06	2.27 (1.07-4.83)	0.03

Logistic regression analysis using a dominant model of inheritance.

GOS: Glasgow outcome scale.

Table 3. Multivariate logistic regression analysis for death and unfavorable outcome.

Prediction	**OR (95% CI)**	***P* value**
Death (n=30/398)		
Shock	26.06 (5.36-48.09)	0.02
Carriage of $CATT_7$ allele	5.12 (1.11-23.67)	0.04
Immunocompromised state	2.85 (1.16-6.98)	0.09
Male gender	2.48 (0.99-6.17)	0.05
Age (per year increase)	1.04 (1.01-1.08)	0.02
Unfavorable outcome (n=133/405)		
Systemic complications on admission	15.8 (1.80-137.97)	0.01
Otitis or sinusitis	3.79 (2.21-6.49)	< 0.001
Carriage of $CATT_7$ allele	2.61 (0.91-7.50)	0.07
Male gender	1.67 (0.96-2.91)	0.07
Age (per year increase)	1.01 (0.99-1.03)	0.18
GCS > 12	0.54 (0.29-1.01)	0.06

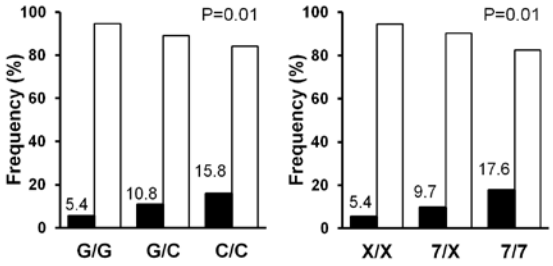
GCS: Glasgow coma score

Supplementary Table 1. *MIF* allelic frequencies and genotypes in patients with pneumococcal meningitis and in controls.

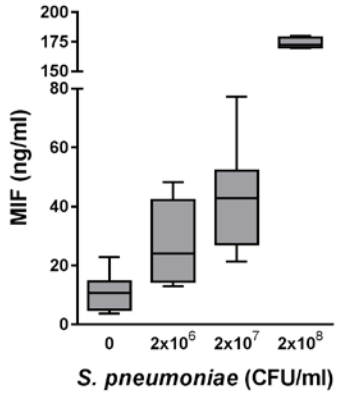
***MIF* gene polymorphism**	**Allele**	**Patients (%)**	**HWE *P* *value***	**Controls (%)**	**HWE *P* *value***	**Genotype**	**Patients (%)**	**Controls (%)**	***P value***
-173 G/C (rs755622)	G	697 (80.3)		542 (82.4)		GG	283 (65.2)	223 (67.8)	
	C	171 (19.7)	0.3	116 (17.6)	0.9	GC	131 (30.2)	96 (29.2)	
						CC	20 (4.6)	10 (3.0)	0.5
$CATT_{5-8}$ (rs5844572)	CATT 5	213 (24.6)		164 (25.0)		CATT 5-5	26 (6.0)	24 (7.3)	
	CATT 6	513 (59.2)		398 (60.7)		CATT 5-6	129 (29.8)	99 (30.2)	
	CATT 7	139 (16.1)		94 (14.3)		CATT 6-6	153 (35.3)	117 (35.7)	
	CATT 8	1 (0.1)	0.9	0 (0)	0.7	CATT 6-7	77 (17.8)	65 (19.8)	
						CATT 5-7	32 (7.4)	17 (5.2)	
						CATT 7-7	15 (3.5)	6 (1.8)	
						CATT 5-8	0 (0)	0 (0)	
						CATT 6-8	1 (0.2)	0 (0)	0.6
CATT/-173 haplotype						5G	212 (24.5)	163 (24.9)	
						6G	479 (55.3)	375 (57.3)	
						6C	36 (4.2)	22 (3.4)	
						7C	132 (15.2)	92 (14.1)	
						8C	1 (0.1)	0 (0)	
						5C	1 (0.1)	0 (0)	
						7G	5 (0.6)	2 (0.3)	0.8

HWE: Hardy-Weinberg equilibrium. Calculation of allele frequencies based on 2xN patients or controls. Genotypes were obtained from 434 (-173 G/C) or 433 ($CATT_{5-8}$) patients and 329 (-173 G/C) or 328 ($CATT_{5-8}$) controls.

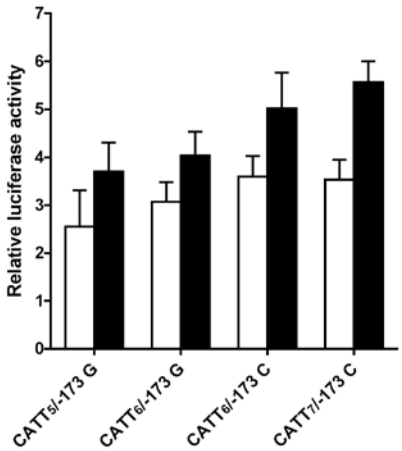
Savva et al., Figure 1



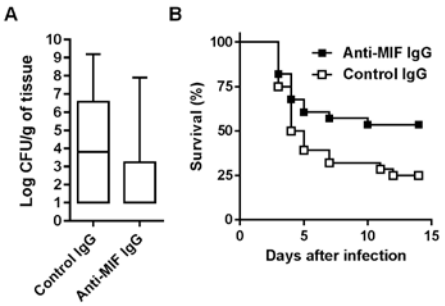
Savva et al., Figure 2



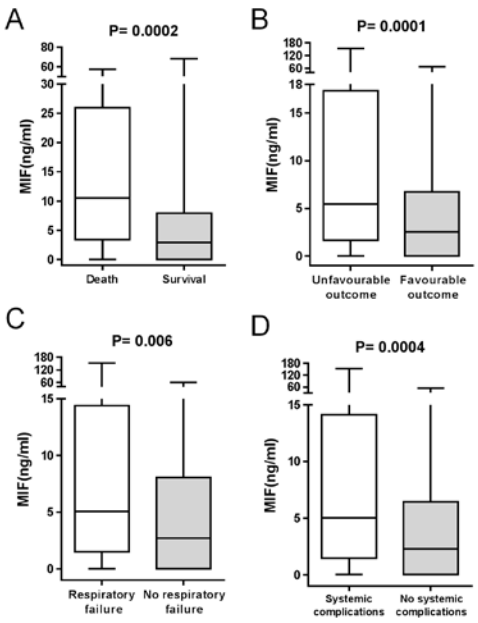
Savva et al., Figure 3



Savva et al., Figure 4



Savva et al., Supplementary Figure 1



Supplementary Figure 1. Associations between CSF MIF levels with death, unfavourable outcome (GOS ≤4), respiratory failure and systemic complications (N=242).