Interferon-gamma driven elevation of CXCL9: a new sepsis endotype independently associated with mortality

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Summary

Background Endotype classification becomes the cornerstone of understanding sepsis pathogenesis. Macrophage activation-like syndrome (MALS) and immunoparalysis are the best recognized major endotypes, so far. Interferon-gamma (IFNγ) action on tissue macrophages stimulates the release of the cytotoxic chemokine CXCL9. It was investigated if this mechanism may be an independent sepsis endotype.

Methods In this cohort study, 14 patient cohorts from Greece, Germany and Italy were studied. The cohorts were 2:1 randomly split into discovery and validation sets. Sepsis was defined by the Sepsis-3 definitions and blood was sampled the first 24 h from meeting the Sepsis-3 definitions. Concentrations of IFNγ, CXCL9, IP-10 (IFNγ induced protein-10), soluble CD163 and ferritin were measured. The endotype of IFNγ-driven sepsis (IDS) was defined in the discovery set as the combination of a) blood IFNγ above a specified cut-off associated with the minimal risk for immunoparalysis (defined as ≥8000 HLA-DR receptors on CD45/CD14-monoytes); and b) increase of CXCL9. Results were compared to the validation set.

Findings 5503 patients were studied; 3670 in the discovery set and 1833 in the validation set. IDS was defined as IFNγ more than 3 pg/ml and CXCL9 more than 2200 pg/ml. The frequency of IDS in the discovery set was 19.9% (732 patients; 95% confidence intervals-CIs 18.7–21.3%) and in the validation set 20.0% (366 patients; 95% CIs 18.2–21.9%). Soluble CD163, a marker of macrophage activation, was greater in IDS and IDS had features distinct from MALS. The mortality in IDS patients was 43.0% (315 patients; 95% CIs 39.5–46.6%) in the discovery set and 40.4% in the validation set (148 patients; 95% CIs 35.5–45.5%) (p = 0.44 compared to patients of the discovery set). IDS was an independent risk factor for death in the presence of other endotypes, severity

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scores and organ dysfunctions of the multivariate model [hazard ratio 1.71 (95% CIs 1.45–2.01) in the discovery set and 1.70 (95% CIs 1.34–2.16) in the validation set]. Decreases of IFNγ and CXCL9 blood levels within the first 72 h were associated with better outcome.

Interpretation IDS is a new sepsis endotype independently associated with unfavorable outcome.

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Keywords: Sepsis; Interferon-gamma; CXCL9; Macrophages; Outcome

Research in context

Evidence before this study
Failure of randomized controlled trials to show some efficacy of immunotherapies in sepsis led to the understanding that patients need to be classified by endotype and administered treatment tailored to their needs. Two major sepsis endotypes have been established so far: macrophage activation-like syndrome (MALS) and sepsis-induced immunoparalysis. Many more sepsis patients remain unclassified with respect to other endotypes.

Added value of this study
The present study in large cohorts of patients coming from three European countries split into one discovery set and one validation set introduces IDS (Interferon-gamma-driven sepsis) as a novel independent endotype in sepsis pathogenesis. IDS is driven by IFNγ and leads to the production of the cytotoxic CXCL9. The blood biomarkers which define IDS are IFNγ more than 3 pg/ml and CXCL9 more than 2200 pg/ml. IDS is found in almost 20% of patients meeting the Sepsis-3 definitions and it is an independent predictor for 28-day mortality in all patient subgroups irrespective of country of origin, setting of hospitalization, type of infection, underlying comorbidity and implicated pathogens. Decreases of circulating IFNγ and CXCL9 within the first 72 h are linked with favorable outcomes.

Implications of all the available evidence
IDS should be included in all sepsis classification studies as it is universally present regardless of comorbidities, type of infection (lung, abdominal, urinary tract and bloodstream infections) and implicated pathogen (bacterial or viral sepsis). IDS may guide future precision strategies of sepsis adjunctive immunotherapy.

Introduction

Interferon-gamma (IFNγ) is released by cells participating in both the innate and adaptive immune responses like neutrophils, tissue macrophages, natural-killer cells and Th1 lymphocytes. IFNγ primes chemotaxis of neutrophils at the site of inflammation and phagocytosis by neutrophils. Once produced, it binds to type 1 and 2 receptors of tissue macrophages.[1] In response to IFNγ stimulation, tissue macrophages produce and secrete two types of chemokines; IFNγ-induced protein-10 (IP-10) which further primes chemotaxis and neutrophil phagocytosis; and CXC-ligand-9 (CXCL9) which is cytotoxic and pro-apoptotic.[2] Biosynthesis of IP-10 by tissue macrophages is also stimulated by other cytokines like IFNα and tumour necrosis factor-alpha. In contrast, IFNγ is the only known stimulant for CXCL9 production.[3] IP-10 and CXCL9 act on the CXCL3A tissue receptors, where they stimulate tumor growth like in lung and colon cancer. CXCL9 also binds to the CXCL3B receptors where it blocks interference with the vascular endothelial growth factor and limits angiogenesis.[4]

Recent data suggest that CXCL9 is increased in the bloodstream of patients with critical infection by SARS-CoV-2 (COVID-19)[5] and of patients with Chagas disease,[6] an entity where tissue macrophages are infected by *Trypanosoma cruzi*. Since IFNγ is the only known stimulant for CXCL9 production,[3] it is questioned whether excess production of CXCL9 through IFNγ activation may constitute an independent mechanism of sepsis pathogenesis. So far, only two endotypes have been well-characterized in sepsis which represent the extremes of the spectrum of inflammation: macrophage activation-like syndrome (MALS)–a highly pro-inflammatory endotype[7,8]—versus sepsis-induced immunoparalysis–an anti-inflammatory endotype.[9] The present study investigated the existence of an independent sepsis endotype driven by IFNγ (IFNγ-driven sepsis, IDS) and if this endotype is significant for the patient's outcome.

Methods

In this cohort study, samples coming from 14 independent cohorts from patients with sepsis over the years

2016–2023 were studied; 10 cohorts were coming from Greece; one cohort from Italy; and three cohorts from Germany. Patients' sampling was done according to research protocols approved by the Institutional Review Boards of participating hospitals. Approval and informed consent allowed the use of samples for broad proteomic analyses. Patients were enrolled after written informed consent provided by themselves or legal representatives (Supplementary Table S1).

All studied cohorts had common inclusion and exclusion criteria. Inclusion criteria were: a) adults (≥18 years) of either sex; b) presence of infection defined by clinical or microbiological criteria (see Supplement); c) sepsis defined by the Sepsis-3 definitions as any at least 2-point increase of the baseline SOFA (sequential organ failure assessment) score[10]; and d) blood sampling within the first 24 h from meeting the Sepsis-3 definitions. This time frame was the first 24 h from hospital admission for patients admitted with sepsis from the emergencies or the first 24 h from increase of the baseline SOFA score for patients developing sepsis during hospitalization. Patients living with the human immunodeficiency virus, with neutropenia (less than 1000 neutrophils/mm^3 of whole blood), and end-stage malignancy were excluded.

The following data were captured in an electronic database: a) age and sex; b) vital signs; c) comorbidities and Charlson's comorbidity index (CCI); d) type of infection; e) APACHE (acute physiology and chronic health evaluation) II and SOFA (sequential organ failure assessment) severity scores; f) type of organ dysfunction; g) microbiology and antimicrobial susceptibility testing (ANST); h) laboratory data including hemoglobin, total white blood cell counts; total platelet count; international normalized ratio (INR); fibrinogen; D-dimers; C-reactive protein (CRP) and procalcitonin (PCT). Outcome (survival/death) and the exact survival time until day 28 were also captured.

Blood was poured into clean and pyrogen-free tubes (Vacutainer, Becton Dickinson, Cockeysville, USA) and centrifuged following sampling. Serum was kept refrigerated at –80 °C. Samples were transported into a central lab for the measurement of the following proteins: a) IFNγ, IP-10 (Diaclone, Besançon, France), CXCL9, interleukin (IL)-18 and soluble sCD163 (ELK Biotechnology, Denver, USA) with an enzyme immunosorbent assay. The lower limits of detection were 25 pg/ml, 125 pg/ml, 312 pg/ml, 500 pg/ml and 15.6 ng/ml respectively. Samples providing concentrations of IFNγ less than 25 pg/ml, were re-measured using a high-sensitivity enzyme immunosorbent assay with lower detection limit of 0.78 pg/ml (Diaclone); b) ferritin with an immunochemiluminescent assay (CLIA, DiaSorin, Saluggia, Italy) and lower detection limit 0.25 ng/ml; c) triglycerides, aspartate aminotransferase (AST) and bilirubin with an enzymatic assay on Atellica CH 930 analyzer (SIEMENS, Erlangen, Germany) and lower limits of detection 15 mg/dl, 5 IU/l and 0.15 mg/dl respectively. According to the study design of some of the enrolled cohorts, it was provisioned to repeat blood sampling to some patients 72 h after the first sampling; IFNγ and CXCL9 were also measured in these samples.

According to the design of six studies conducted in Greece, it was provisioned to study the rate of immunoparalysis on blood monocytes. Results of this analysis were used to identify a cut-off of blood IFNγ which predicts sepsis-induced immunoparalysis (Supplementary Table S2). Whole blood was poured in tubes covered by ethylene-diamine-tetra acetic acid and incubated for 15 min in the dark with Anti-Human HLA-DR PE/Monocyte PerCP-Cy 5.5 (Quantibrite, BD Biosciences, Franklin Lakes, NJ, USA) which is a CE-marked IVD test. The number of antibodies bound on the surface antigen HLA-DR on CD14-monocytes was counted by the Navios EX (Beckman Coulter, Brea, USA) flow cytometer and was considered an expression of the absolute count of HLA-DR receptors. Sepsis-induced immunoparalysis was diagnosed when the absolute number of HLA-DR receptors was less than 8000 per CD45/CD14-monocyte.[11] This cut-off is derived from the IVD lab test insert.[12]

Study endpoints

The primary study endpoint was the frequency of IDS as an independent sepsis endotype associated with 28-day mortality. The secondary study endpoint was the association of the over-time changes of the IDS endotype with 28-day outcome.

Endotype classification

IDS was classified as a pro-inflammatory sepsis endotype without signs of immunoparalysis, driven by IFNγ and independently associated with 28-day mortality. CXCL9 and/or IP-10 may even be produced when the process of immunoparalysis has started in tissue macrophages and these patients should not be classified with IDS. To achieve accurate classification of IDS, all the following criteria should be present: a) blood IFNγ above the minimum concentration which is associated with the least likelihood for the presence of immunoparalysis; b) increases of CXCL9 and/or IP-10 above specific cut-offs associated with 28-day mortality; and c) independent association of IDS with 28-day mortality in the presence of severity variables and of other sepsis endotypes.

MALS was classified as total hemophagocytosis score (HScore) ≥169[13] and/or the co-presence of hepatobiliary dysfunction (HBD) and disseminated intravascular coagulation (DIC).[14] HScore was calculated by adding the individual points of specific parameters (number of cytopenias, hepato-spleno-megaly, core temperature, AST, fibrinogen, ferritin, triglycerides, immunosuppression) which has already been suggested for patients with secondary MAS.[13] HBD was defined as total bilirubin more than 2.5 mg/dl and serum AST at least 2

times more the upper normal limit. DIC was defined as total ISTH (International Society on Thrombosis and Hemostasis) score ≥5.[15]

Patients with blood IFNγ below the minimum cut-off of inclusion into the IDS endotype are considered having high likelihood for immunoparalysis. These patients are described as "low-IFNγ (immunoparalysis)". We could not classify all these patients as sepsis-induced immunoparalysis since flow cytometry for HLA-DR on CD14-monocytes was only available for a subset and not for all patients studied. Patients who are not classified into one of the above three endotypes have blood IFNγ above the minimum cut-off of inclusion into the IDS endotype but without increases of CXCL9 and/or IP-10. These patients are reported as "High IFNγ without CXCL9 and/or IP-10 increase" and they are considered to represent an adaptive endotype.

Statistical analysis

Patients of each cohort were randomly split 2:1 into discovery and validation populations. Discovery populations of all cohorts were merged into the discovery set and validation populations of all cohorts into the validation set. Quantitative variables were expressed as means and standard deviations for variables following normal distribution and as median and quartiles for variables following non-normal distribution. Measured variables were plotted for groups as medians and interquartile ranges overlying the distribution of individual values. Comparisons between two groups were done by the Student's t-test for normally distributed variables and by the Mann–Whitney U test for non-normally distributed variables. Qualitative variables between two groups were compared by the Fisher's exact test and between more than two groups by the Pearson's Chi-Squared test.

The development of the IDS endotype was performed in the discovery cohort. According to the definition of the IDS classification, patients with IDS should have IFNγ in the bloodstream above a minimal cut-off concentration which is associated with the least likelihood for immunoparalysis. As a first step, a ROC (receiver operator characteristics) curve was plotted to identify the Youden index of IFNγ which may best discriminate patients with less than 8000 HLA-DR receptors on CD45/CD14-monocytes. Then patients were split into those with IFNγ above or below this IFNγ concentration. As a second step, a ROC curve was plotted to identify the Youden index of IP-10 and CXCL9 which may best prognosticate 28-day mortality among patients with IFNγ above the concentration defined at the first step. Then IDS was defined as patients with IFNγ and CXCL9 and/or IP-10 above the estimated cut-offs.

To identify if the presence of IDS was an independent predictor for death, forward step-wise multivariate Cox regression analysis was done in the discovery set with 28-day mortality as the dependent variable and sex, APACHE II, CCI and SOFA scores, organ dysfunctions, and endotypes as independent variables. Age was not included in the equation since it makes part of the APACHE II and CCI scores. The selected independent variables covered for all possible clinical factors which may impact on 28-day outcome. For the purposes of the analyses, quantitative severity scores were transformed into binomial variables using as transformation cut-off the Youden index of the respective ROC curve of each score providing the best prognostication of 28-day mortality; hazard ratios (HR) and 95% confidence intervals (CIs) were calculated.

The cut-offs estimated for all variables at the discovery set were applied at the validation set. The frequencies of IDS between the discovery and the validation cohorts were compared by the Fisher' s exact test. The validation of the IDS endotype was further done by one multivariate step-wise Cox regression analysis in the validation set where 28-day mortality was the dependent variable and sex, APACHE II, CCI and SOFA scores, organ dysfunctions, and endotypes were the independent variables. Comparisons of survival between endotypes was done by the log-rank test.

Sensitivity analyses were done to define the impact of the IDS endotype in subgroups by country, type of hospitalization, type of infection, isolated pathogens and comorbidities. HRs and 95% CI were calculated after Cox regression analyses.

In a separate sensitivity analysis, the step-wise Cox regression model was repeated among patients of both cohorts with positive microbiology. In the model, treatment with at least one antibiotic active in vitro against the pathogen according to the ANST was included as an independent variable.

Concentrations of CRP, PCT, D-dimers, ferritin and CXCL9 between patients with different endotypes were compared by the Mann–Whitney U test with Bonferroni corrections for multiple testing. Comparisons of IFNγ and of CXCL9 between baseline and day 4 were done among survivors and non-survivors using the Wilcoxon's signed rank paired test. The relative changes of IFNγ and CXCL9 over the first 72 h of sepsis were calculated. The ROC curves of the relative changes for mortality prediction were plotted and the Youden index was calculated for the estimation of the cut-off which may better predict mortality. Mortalities of patients below and above the cut-off changes of IFNγ and of CXCL9 were compared by the Fisher' s exact test. Odds ratios (ORs) and 95% CIs were calculated by the Mantel and Haenszel test.

Analysis was done using IBM SPSS statistics 26.0. Any p-value less than 0.050 was considered statistically significant. The p-values were corrected by Bonferroni corrections for multiple comparisons where appropriate. In all cases where hazard functions were

evaluated, the Schoenfeld partial residuals were plotted against time.

Role of the funding source

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Results

The total number of participants was 5503; 3670 patients were randomized to the discovery set; and 1833 patients were randomized to the validation set. Patients randomized to each set did not differ by demographics, severity scores, type of infections, comorbidities, laboratory findings, isolated pathogens and 28-day mortality (Table 1).

At the first step of the analysis in the discovery cohort, a ROC curve was plotted to identify the lowest IFNγ concentration with the best prediction for patients to have less than 8000 HLA-DR receptors per CD45/CD14-monocyte. This cut-off was 3 pg/ml (area under the curve 0.65; 95% CIs 0.62–0.69; $p < 0.0001$). The odds ratio for ≥8000 HLA-DR receptors on CD45/CD14-monocytes when IFNγ exceeds 3 pg/ml was 3.97 (95% CIs 2.92–5.41). Since the IDS endotype requires the concentration of IFNγ which is linked with the least risk for sepsis-induced immunoparalysis, the concentration of 3 pg/ml was selected for the rest of the development of IDS.

In the discovery set, high 28-day mortality was found both for patients with IFNγ >3 pg/ml and for patients with IFNγ ≤3 pg/ml. This meant that these patients represented different states of immune activation leading to death (Fig. 1A). Indeed, the absolute count of HLA-DR receptors on CD45/CD14-monocytes of patients with more than 3 pg/ml IFNγ in the blood was much higher than of patients with ≤3 pg/ml IFNγ in the blood (Fig. 1B), signifying that IFNγ ≤3 pg/ml identifies the presence of immunoparalysis. Patients with IFNγ more than 3 pg/ml had significantly greater circulating levels of sCD163 (Fig. 1C), which is a marker of macrophage activation.[16] This further strengthens the evidence that IFNγ more than 3 pg/ml is the appropriate cut-off for IDS.

Then the analysis for the discovery of IDS was focused only on patients with IFNγ more than 3 pg/ml. CXCL9 was higher among non-survivors than among survivors (Fig. 1D). IP-10 levels did not differ among

	Discovery set (n = 3670)	Validation set (n = 1833)	p-value
Age, years, mean (SD)	70.1 (15.9)	69.6 (15.9)	0.252
Male sex, n (%)	1720 (46.9)	842 (45.9)	0.528
APACHE II score, mean (SD)	18.1 (8.7)	18.0 (8.5)	0.760
CCI, mean (SD)	4.11 (2.99)	4.08 (2.96)	0.738
SOFA score, mean (SD)	6.70 (3.95)	6.62 (3.93)	0.480
Main infections, n (%)			
Community-acquired pneumonia	1099 (29.3)	520 (28.4)	0.469
Intrabdominal infection	515 (14.0)	258 (14.1)	0.967
Acute pyelonephritis	522 (14.2)	261 (14.2)	1.00
Pneumonia by SARS-CoV-2	433 (11.8)	216 (11.8)	1.00
Hospital-acquired pneumonia	322 (8.8)	163 (8.9)	0.880
Ventilator-associated pneumonia	339 (9.2)	168 (9.2)	0.961
Primary bacteremia	297 (8.1)	149 (8.1)	0.958
Type of organ dysfunction, n (%)			
ARDS	1601 (43.6)	804 (44.0)	0.818
Septic shock	1660 (45.2)	830 (45.3)	0.977
Acute kidney injury	896 (24.4)	429 (23.4)	0.412
Acute coagulopathy	760 (21.0)	401 (21.9)	0.442
Main comorbidities, n (%)			
Type 2 diabetes mellitus	1051 (28.6)	524 (28.6)	0.975
Chronic obstructive pulmonary disease	522 (14.2)	292 (15.9)	0.099
Chronic heart failure	759 (20.7)	362 (19.7)	0.435
Chronic renal disease	460 (12.5)	225 (12.3)	0.795
Intake of corticosteroids the last 15 days	337 (9.2)	174 (9.5)	0.730
Non-Hodgkin's lymphoma	52 (1.4)	31 (1.7)	0.481
Stage I/II solid tumor malignancy	626 (17.1)	327 (17.8)	0.473
Liver cirrhosis	84 (2.3)	50 (2.7)	0.353
Laboratory findings			
White blood cells,/mm^3, mean (SD)	13,958.8 (7407.8)	13,718.6 (7235.9)	0.266
Procalcitonin, ng/ml, median (Q1-Q3)	0.95 (0.22–6.59)	0.93 (0.21–5.65)	0.707
CRP, mg/l, median (Q1-Q3)	129.2 (44.0–233.0)	113.0 (39.8–227.0)	0.111
Hemoglobin, g/dl, mean (SD)	11.16 (2.29)	11.06 (2.38)	0.357
Platelets, x10^3/mm^3, mean (SD)	225.2 (123.5)	224.4 (120.8)	0.828
Creatinine, mg/dl, median (Q1-Q3)	1.1 (0.8–1.8)	1.2 (0.8–1.9)	0.108
International normalized ratio, median (Q1-Q3)	1.19 (1.05–1.38)	1.18 (1.06–1.39)	0.633
Fibrinogen, mg/l, mean (SD)	539.8 (267.1)	520.2 (203.8)	0.206
D-dimers, μg/l, median (Q1-Q3)	740 (327.8–1657.5)	788 (341.5–1899.0)	0.419
Aspartate aminotransferase, U/l, median (Q1-Q3)	16 (5–37)	16 (5–36)	0.817
Ferritin, ng/ml, median (Q1-Q3)	548.4 (225.7–1229.3)	540.1 (226.7–1125.9)	0.249
Most common isolated pathogens[a], n (%)			
Escherichia coli	365 (9.9)	165 (9.0)	0.286
Klebsiella pneumoniae	259 (7.1)	114 (6.2)	0.255
Staphylococcus aureus	218 (5.9)	116 (6.3)	0.590
Pseudomonas aeruginosa	147 (4.0)	73 (4.0)	1.00
28-day mortality, n (%)	1187 (32.3)	584 (31.9)	0.736

Abbreviations: APACHE, acute physiology and chronic health evaluation; ARDS, adult respiratory distress syndrome; CCI, Charlson's comorbidity index; CRP, C-reactive protein; Q, quartile, SOFA, sequential organ failure assessment; SD, standard deviation. [a]In more than 4% of patients.

Table 1: **Main characteristics of patients of the 14 cohorts split into one discovery set and one validation set.**

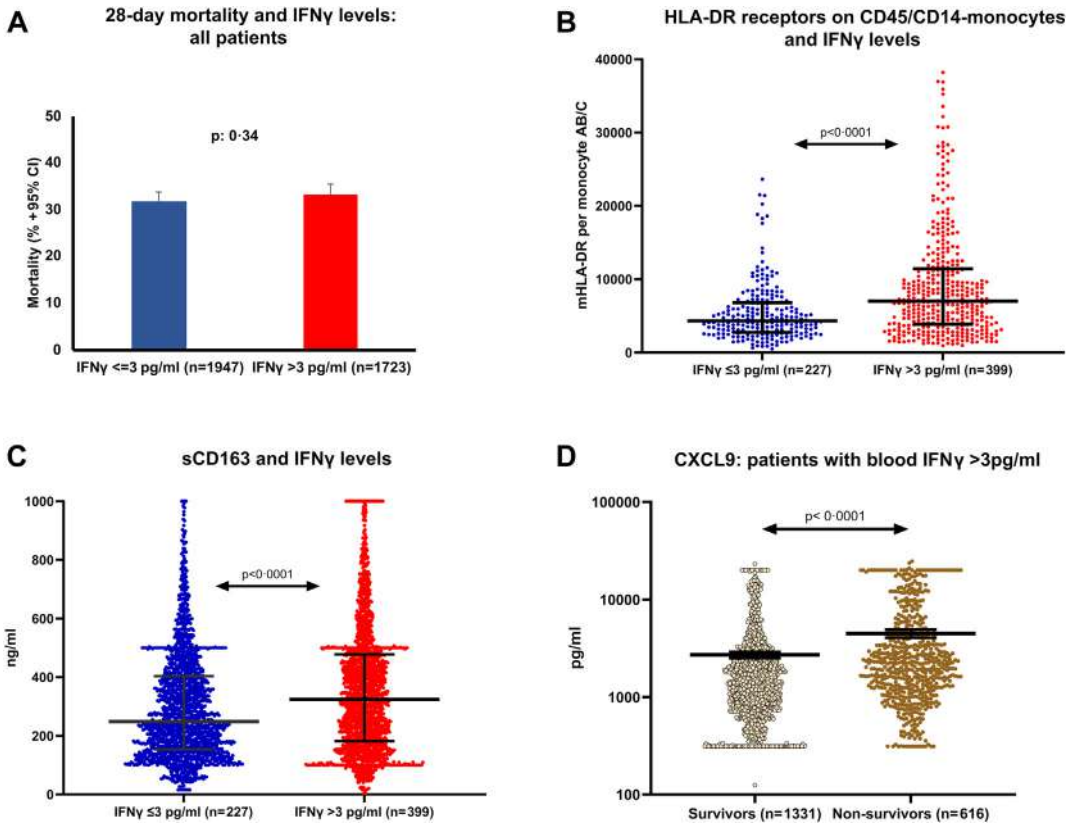

Fig. 1: **Development of the IFNγ-driven sepsis (IDS) endotype in the discovery set**. A) Bar graphs of 28-day mortality in patients with IFNγ more than 3 pg/ml and IFNγ 3 pg/ml or less. The p-value of comparison by the Fisher's exact test is provided. B) Distributions of the absolute count of HLA-DR receptors on CD45/CD14-monocytes in patients with IFNγ more than 3 pg/ml and IFNγ 3 pg/ml or less. Lines overlying the distributions represent medians and interquartile ranges. The p-value of comparison by the Mann–Whitney U test is provided. C) Distributions of sCD163 in patients with IFNγ more than 3 pg/ml and IFNγ 3 pg/ml or less. Lines overlying the distributions represent medians and interquartile ranges. The p-value of comparison by the Mann–Whitney U test is provided. D) Distributions of CXCL9 in survivors and non-survivors. This comparison involves only the patients with IFNγ levels more than 3 pg/ml. Lines overlying the distributions represent medians and interquartile ranges. The p-value of comparison by the Mann–Whitney U test is provided. Abbreviations: CI, confidence interval; IDS, IFNγ-driven sepsis; IFN, interferon; n, number of patients.

survivors and non-survivors (Supplementary Figure S1) so that IP-10 could not be used for the development of the IDS endotype. ROC curve in patients with IFNγ above 3 pg/ml identified a cut-off of CXCL9 of 2200 pg/ml predictable of unfavorable outcome by day 28 (area under the curve 0.63; 95% CIs 0.61–0.66; $p < 0.0001$). IDS was then defined as blood IFNγ more than 3 pg/ml and blood CXCL9 above 2200 pg/ml.

Within the discovery set, patients with MALS presented with all main traits of MALS (co-presence of HBD/DIC; increases of triglycerides, ferritin and HScore) (Supplementary Figures S2–S5). MALS and IDS were distinct endotypes with an overlap of only 0.7% in the discovery set (Fig. 2). Blood CRP, PCT and D-dimers were not different between MALS and IDS (Supplementary Figures S6–S8). Blood ferritin and IL-18 levels were higher in MALS than IDS, and higher in IDS than the other endotypes suggesting that IDS represents a less pro-inflammatory endotype than MALS (Supplementary Figures S9 and S10). CXCL9 blood levels were significantly higher in IDS than any other endotype (Supplementary Figure S11). From the 202 patients classified with MALS at the discovery cohort, 126 patients had IFNγ ≤3 pg/ml in the blood. We consider these patients as having MALS and not immunoparalysis since: a) they meet the independent classification criteria for MALS; and b) it is well described that several patients with MALS have an adaptive decrease of the function of blood monocytes as response to high systemic tissue inflammation. These patients are classified into MALS due to the high mortality of MALS.[9,17]

In both the discovery and the validation sets the blood levels of IFNγ were higher in patients with IDS and in patients with high IFNγ without CXCL9 increase than the other endotypes (Supplementary Figure S12).

The frequency of IDS in the discovery set was 19.9% (732 out of 3670 patients; 95% CIs 18.7–21.3%).

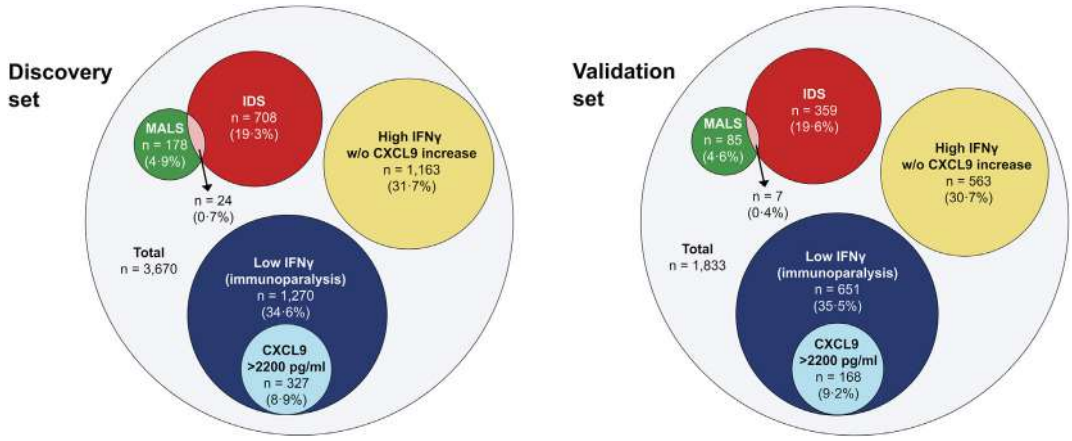

***Fig. 2*: Distribution of endotypes in the discovery and the validation sets of patients**. Patients are classified into four endotypes, MALS, IDS, Low IFNγ and High IFNγ without CXCL9 increase. Patients with low IFNγ (i.e. blood IFNg ≤3 pg/ml) most likely represent patients with sepsis-induced immunoparalysis. The overlaps between MALS and IDS in each set are provided. In the discovery set, 126 patients with MALS have IFNγ ≤3 pg/ml. In the validation set, 65 patients with MALS have IFNγ ≤3 pg/ml. Abbreviations: IDS, IFNγ-driven sepsis; IFN, interferon; MALS, macrophage activation-like syndrome; n, number of patients; w/o, without.

Applying the classification criteria for MALS and IDS endotypes to the validation set showed an overlap between MALS and IDS of 0.4% (Fig. 2). The frequency of IDS in the validation set was 20.0% (366 out of 1833 patients; 95% CIs 18.2–21.9%) (p = 1.00 compared to the frequency of the discovery set). As already described, patients with IFNγ 3 pg/ml or less were classified to the low-IFNγ endotype probably representing patients with sepsis-induced immunoparalysis (Fig. 1B). Some of the patients of the low-IFNγ endotype have increased CXCL9. These patients cannot be part of the pro-inflammatory IDS endotype since they have lab findings compatible with sepsis-induced immunoparalysis (Supplementary Figure S13).

The mortality of patients with MALS was greater than all other patients in both the discovery and the validation sets and this was followed by the mortality of patients with IDS and of patients with low-IFNγ (Fig. 3A and B). The mortality of patients with the IDS endotype was 43.0% (315 out of 732 patients; 95% CIs 39.5–46.6%) in the discovery set and 40.4% in the validation set (148 out of 366 patients; 95% CIs 35.5–45.5%) (p: 0.44 compared to patients of the discovery set). IDS was an independent risk factor for death under the presence of other endotypes, sex, APACHE II, CCI and SOFA scores and of organ dysfunctions in both the discovery and the validation sets of patients (Supplementary Tables S3 and S4). The Schoenfeld partial residuals were plotted against time, showing that they were evenly distributed around zero. This proves that the HRs are time-independent thus verifying the proportional hazards assumption (Supplementary Figure S14). As shown in Fig. 2 for the discovery set, 327 patients with the low-IFNγ endotype had CXCL9 more than 2200 pg/ml and another 1270 patients with the low-IFNγ endotype had CXCL9 ≤2200 pg/ml; 28-day mortality was 39.4% (95% CIs 34.3–44.8%) and 28.5% (26.1–31.0%) respectively (p < 0.0001). As shown in Fig. 2 for the validation set, 168 patients with the low-IFNγ endotype had CXCL9 more than 2200 pg/ml and another 651 patients with the low-IFNγ endotype had CXCL9 ≤2200 pg/ml; 28-day mortality was 43.5% (95% CIs 36.2–51.0%) and 27.5% (24.2–31.1%) respectively (p < 0.0001).

Sensitivity analyses revealed that the presence of IDS was an independent risk factor for death in all studied sub-groups i.e. country of origin, type of hospitalization, type of infection, comorbidities and isolated pathogens (Fig. 4). In total, 649 patients of both cohort sets were hospitalized for pneumonia by SARS-CoV-2 of which 111 patients were classified with IDS and 29 patients were classified with MALS; 51.4% and 79.3% respectively required mechanical ventilation. Among patients of the four endotypes with pneumonia by SARS-CoV-2, blood ferritin was pronounced in MALS (Supplementary Figure S15).

The Cox regression analysis among patients with positive microbiology of both cohorts showed that even when treatment with an active antibiotic was included as an independent variable, the association of the low-IFNγ endotype, IDS and MALS with 28-day mortality remained unaltered (Supplementary Table S5).

In order to further clarify if IFNγ stimulating the production of CXCL9 is an independent driver for death or if increases of CXCL9 are independent from IFNγ three more types of analyses were done including all patients of both cohorts. In the first analysis, a positive association was found between IFNγ and CXCL9 levels

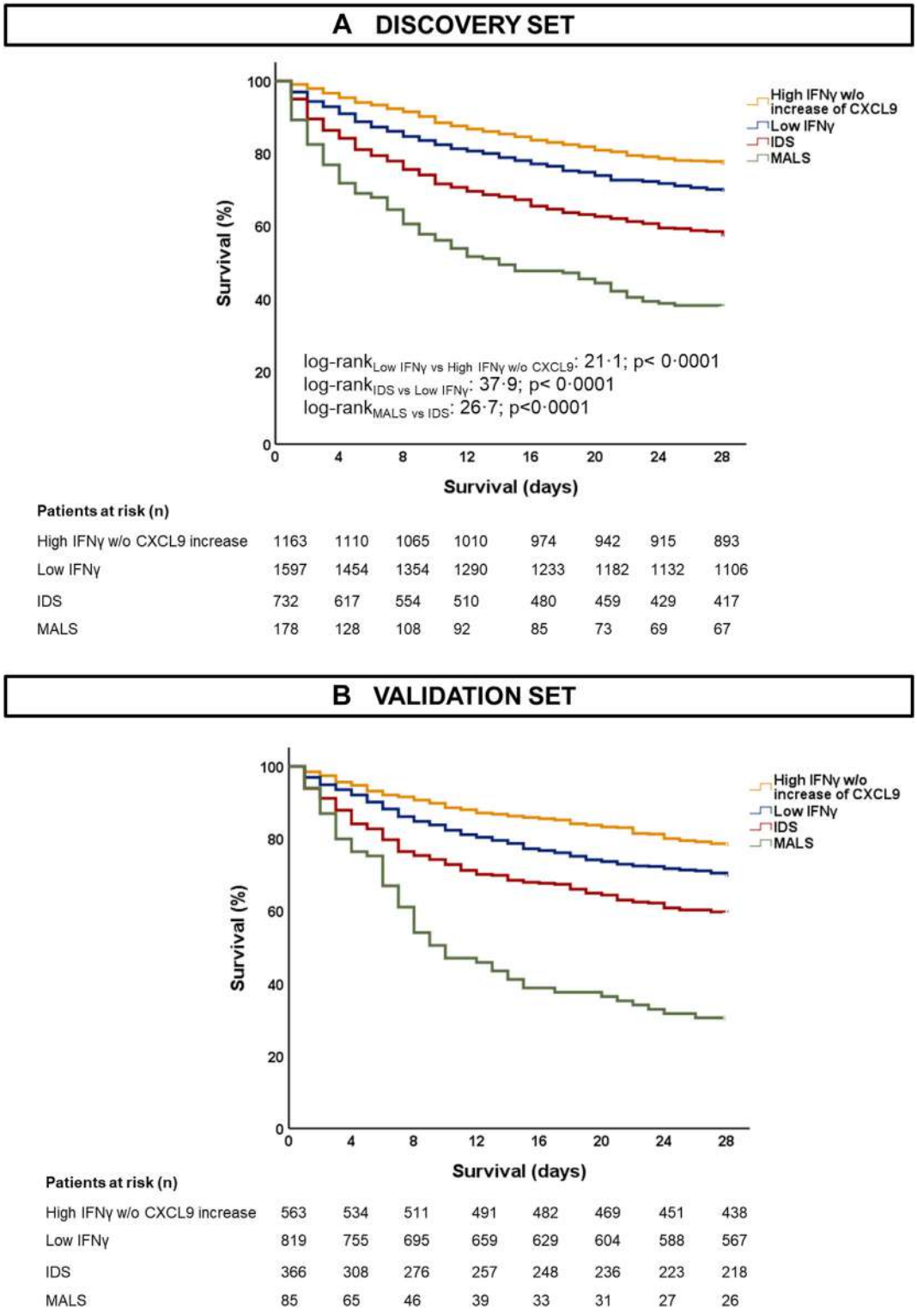

***Fig. 3*: The impact of the endotype of IFNγ-driven sepsis on mortality the first 28 days**. Kaplan–Meier survival curves in the discovery (A) and in the validation (B) sets according to sepsis endotype are shown. The values of the indicated log-rank comparisons and of the respective p-values after Bonferroni corrections for multiple comparisons are provided. In the analysis, patients overlapping in classification with both IDS and MALS are analyzed as IDS. Abbreviations: IDS, IFNγ-driven sepsis; IFN, interferon; MALS, macrophage activation-like syndrome; n, number of patients; w/o, without.

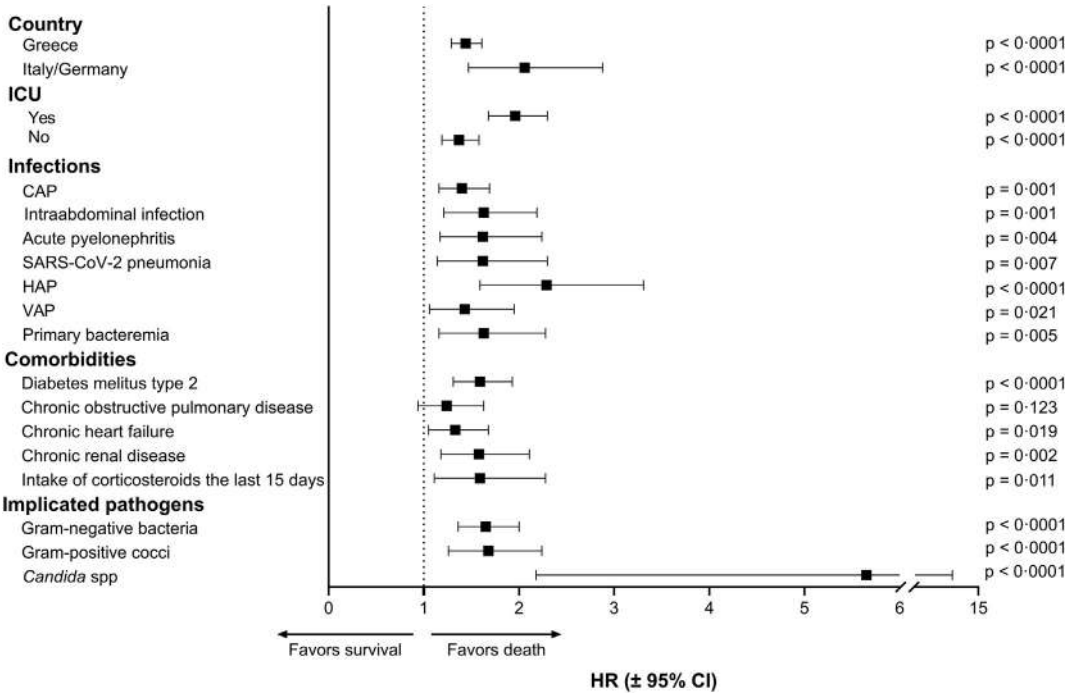

***Fig. 4:* Mortality risk by the presence of the IFNγ-driven sepsis endotype in patient sub-groups**. The HRs of the impact of IDS on 28-day mortality coming from Cox regression analyses within each indicated subgroup are provided. The p-values of significances are shown. Abbreviations: CAP, community-acquired pneumonia; CI, confidence interval; HAP, hospital-acquired pneumonia; HR, hazard ratio; ICU, Intensive Care Unit; VAP, ventilator-associated pneumonia.

(Supplementary Table S6). This association was missing among patients with MALS (Supplementary Table S7). In the second analysis, patients of both cohorts were split into four subsets: those with IFNγ >3 pg/ml and CXCL9 ≤2200 pg/ml; those with IFNγ ≤3 pg/ml and CXCL9 ≤2200 pg/ml; those with IFNγ ≤3 pg/ml and CXCL9 >2200 pg/ml; and those with IFNγ >3 pg/ml and CXCL9 >2200 pg/ml. It was found that the last three subsets were independently associated with 28-day mortality proving that IFNγ stimulating the production of CXCL9 is an independent pathway for unfavorable outcome (Supplementary Table S8). In the third analysis, it was found that the expression of HLA-DR on CD45/CD14-monocytes is higher in patients with IFNγ >3 pg/ml and CXCL9 >2200 pg/ml than in patients with IFNγ ≤3 pg/ml and CXCL9 >2200 pg/ml. This indicated the presence of immunoparalysis when IFNγ is ≤ 3 pg/ml and CXCL9 >2200 pg/ml hence probably implying a different state of immune activation. It is concluded that IDS is an endotype which is functionally distinct than the cases of CXCL9 without increase of IFNγ (Supplementary Figure S16).

The frequencies of all organ dysfunctions were higher among patients with MALS than patients with the other endotypes. The frequencies of acute respiratory distress syndrome and septic shock were similar between patients with the low-IFNγ endotype and patients with IDS. However, the frequencies of acute kidney injury and acute coagulopathy were higher in IDS than the low-IFNγ endotype. No major differences in the main comorbidities were found between the four endotypes (Supplementary Table S9).

In a subset of patients with IDS, measurements of circulating IFNγ and CXCL9 were repeated after 72 h. Although blood levels of IFNγ decreased over-time in both survivors and non-survivors, levels of CXCL9 remained stable in non-survivors (Fig. 5A and B). It was found that the 28-day mortality in patients experiencing at least 50% decrease of IFNγ from baseline measurement was significantly lower than in patients with less than 50% decrease of IFNγ (Fig. 5C). Similar differences in 28-day mortality were found for patients experiencing at least 30% decrease of CXCL9 from baseline and patients with less than 30% decrease of CXCL9 from baseline (Fig. 5D).

Discussion

The present study in large cohorts of patients coming from three European countries split into one discovery set and one validation set introduces the novel IDS endotype as an independent mechanism of sepsis pathogenesis. The positive association between blood levels of IFNγ and CXCL9 in the entire patient

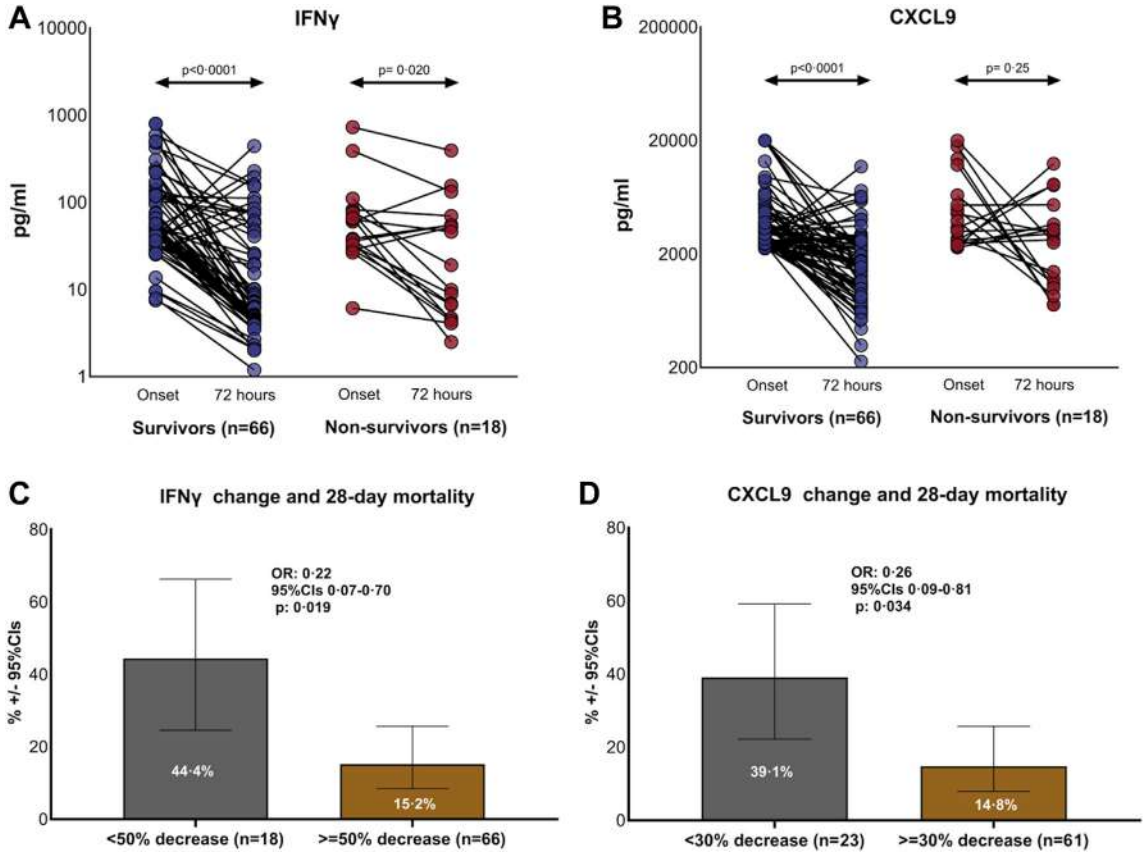

Fig. 5: **Changes of IFNγ and CXCL9 over the first 72 h and 28-day outcome**. Measurements of blood IFNγ and of CXCL9 were repeated for 84 patients after 72 h. A) Paired changes of IFNγ between the two time points, separately for 28-day survivors and 28-day non-survivors. The p-values of paired comparisons by the Wilcoxon's signed rank test are provided. B) Paired changes of CXCL9 between the two time points, separately for 28-day survivors and 28-day non-survivors. The p-values of paired comparisons by the Wilcoxon's signed rank test are provided. C) 28-day mortality for patients with less than 50% decrease of blood IFNγ and for patients with ≥50% decrease of blood IFNγ within the first 72 h. The 50% cut-off is defined as the Youden index following ROC curve analysis of the relative changes of IFNγ to predict 28-day mortality (area under the curve 0.69; 95% confidence intervals 0.55 to 0.82; p = 0.016). D) 28-day mortality for patients with less than 30% decrease of blood CXCL9 and for patients with ≥30% decrease of blood CXCL9 within the first 72 h. The 30% cut-off is defined as the Youden index following ROC curve analysis of the relative changes of CXCL9 to predict 28-day mortality (area under the curve 0.65; 95% confidence intervals 0.525 to 0.82; p = 0.046). Abbreviations: CI: confidence interval; IFN; interferon; OR: odds ratio; n: number of patients; ROC, receiver operator characteristics.

population but not in patients with MALS suggests that IDS is driven by IFNγ which leads to the production of the cytotoxic CXCL9. The biomarkers defining IDS are IFNγ more than 3 pg/ml and CXCL9 more than 2200 pg/ml. IDS is found in almost 20% of patients meeting the Sepsis-3 definitions and it is an independent predictor for 28-day mortality in all patient subgroups irrespective of the country of origin, the setting of hospitalization, the type of infection, the underlying comorbidity and the implicated pathogen. Decreases of circulating concentrations of IFNγ and of CXCL9 over the first 72 h are linked with favorable outcomes.

The failure of randomized controlled trials (RCTs) of the past which studied the efficacy of adjuvant immunotherapies in sepsis led to the understanding that patients present different endotypes reflecting different mechanisms of immune activation. RCTs must be re-designed so that patients can receive treatment tailored to their endotypes and respective clinical needs.[18] One recent example is the SAVE-MORE trial for COVID-19 pneumonia[19] in which patients were selected for enrolment using the biomarker suPAR (soluble urokinase plasminogen activator receptor) which surrogated the early activation of the IL-1 pathway and where patients were randomized to adjuvant treatment with either placebo or the IL-1-blocker anakinra. The relative benefit of anakinra treatment was 64%; this was the greatest recorded benefit of any drug used for COVD-19 treatment during the pandemic. The

precision strategy of patient selection may have been crucial for this beneficial outcome.

In the present study three well-characterized endotypes prevailed in both the discovery and the validations sets; MALS, IDS and low-IFNγ, with the latter probably featuring immunoparalysis. MALS is driven by IL-1 activation.[20] In non-sepsis patients with secondary macrophage activation syndrome (MAS) due to solid tumor malignancies, lymphomas, autoimmune disorders and viral infections that affect the lymphoid tissues, e.g. Epstein–Barr virus and cytomegalovirus, circulating IFNγ and CXCL9 are increased.[21] In these patients, CXCL9 is correlated with other features of MAS like increased levels of HScore, triglycerides, ferritin, soluble IL-2 receptor (sIL-2R) and soluble receptor of tumour necrosis factor (sTNFR).[21] Our findings suggest that the markers of the IFNγ pathway in secondary MAS cannot guide the identification of the IDS endotype in sepsis. MALS and IDS are distinct because: a) the applied classification system showed only 0.7% overlap in the discovery set and 0.4% in the validation set; and b) MALS is a pro-inflammatory condition with remarkable hyper-ferritinemia.[22] Although ferritin and IL-18 levels of IDS were greater than the other endotypes, they were significantly lower than in MALS, even in patients with pneumonia by SARS-CoV-2, suggesting that IDS is an endotype with intermediate pro-inflammatory features between MALS and the remaining patients; and c) the cut-off of CXCL9 indicating IFNγ activation in secondary MAS is 517 pg/ml[23] which is much lower than the 2200 pg/ml cut-off of IDS of sepsis. The fourth described endotype encompasses patients with blood IFNγ above 3 pg/ml and it is associated with the least risk for 28-day mortality. The lack of increase of CXCL9 most probably suggests that this is an adaptive endotype not reflecting some specific cell activation.

Of pathomechanistic interest is the question if the deleterious effect of IDS is driven by IFNγ or by CXCL9. Tissue histology of MAS induced in mice following a challenge of the toll-like receptor 9 remained unaltered when mice had simultaneously a homogeneous deficiency for *CXCL9*.[24] In a recent open-label trial, 14 patients with Still's disease refractory to corticosteroids and secondary MAS were treated with emapalumab, a monoclonal antibody which blocks the activity of IFNγ[25]; MAS was improved in 50% of patients in the first 30 days; and in all patients the first 60 days. Emapalumab treatment decreased circulating CXCL9 levels suggesting that targeting IFNγ improves the syndromic entity of such patients.

Three main limitations of the present study should be acknowledged. The first limitation is that the description of IDS is based mainly on protein measurements and does not include the analysis of molecular pathways. However, transcriptomic analysis was probably unnecessary because: a) patients had increases of sCD163 indicating tissue macrophages as the effector cells of IDS[16]; b) the cut-off of blood IFNγ is selected to be associated with the minimal risk of immunoparalysis, making IDS a pro-inflammatory endotype; and c) IDS was identified as an independent predictor for 28-day mortality which is a requirement for any variable of importance in sepsis biology already suggested by the Task Force of the Sepsis-3 definitions.[10] The second limitation is the analysis of patients with bacterial and viral sepsis together. However, analysis showed that IDS brings independent risk for death in either bacterial or viral sepsis consistent with a conserved maladaptive host response. The third limitation is the lack of precision of the time period between the blood sampling and the onset of sepsis. This was dealt by enrolling patients with blood sampled within the first 24 h from meeting the Sepsis-3 definitions.

The presented findings improve substantially our understanding of sepsis pathogenesis and may guide new strategies for precision treatment. We describe for the first time IDS as an entirely new endotype associated with unfavorable outcome irrespective geographic location, type of infection, type of implicated pathogen and comorbidities. IDS should be taken into consideration in all new studies of sepsis classification.

Contributors

EJG-B conceptualized the study, was responsible for the acquisition of funding and study resources, supervised the study, analyzed the data, wrote the original draft, revised the manuscript for intellectual content and approved the final version for submission. EJG-B has accessed and verified the data, and he is responsible for the decision to submit the manuscript.

IK, CP, DT-R, KM, JB, GD, PK, SA, EA, GV, CT, MA, AI, EK, MN, EG, VP, IP, GdP, MK, and EM contributed to data acquisition, investigation and project administration, revised the manuscript for intellectual content and approved the final version for submission.

KD developed the study database, curated the data, analyzed the data, revised the manuscript for intellectual content and approved the final version for submission.

MA, FB, AJR and MB analyzed the data, drafted the manuscript, revised the manuscript for intellectual content and approved the final version for submission.

EJGB and KD have accessed and verified the data, and EJGB is responsible for the decision to submit the manuscript.

Data sharing statement

Data are available with publication by the corresponding author by a signed data access agreement.

Declaration of interests

EJG-B reports honoraria and consultation fees from Abbott Products Operations, bioMérieux, Brahms GmbH, GSK and Sobi (granted to the National and Kapodistrian University of Athens); independent educational grants from AbbVie, InCyte, Novartis and UCB (granted to the National and Kapodistrian University of Athens) and from Abbott Products Operations, bioMérieux Inc, Johnson & Johnson, MSD, and Swedish Orphan Biovitrum AB (granted to the Hellenic Institute for the Study of Sepsis); and funding from the Horizon 2020 European Grants ImmunoSep and RISCinCOVID and the Horizon Health grants EPICCROWN-2, POINT and Homi-Lung (granted to the Hellenic Institute for the Study of Sepsis).

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MK is inventor of a patent covering a method for quantification of C-terminal peptides of AAT (applicant: Jena University Hospital) (JUH); EP4224163A1; status: application), and the inventor of other patents covering C-terminal AAT peptides in inflammation (applicant: Jena University Hospital (JUH): Method for determining the origin of an infection (EP3239712B1 [granted]) and Diagnosis of Sepsis and Systemic Inflammatory Response Syndrome (EP2592421B1, EP2780719B1, CN104204808B, US10712350B2, JP6308946B2 [all granted]).

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The other authors do not declare any conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105414.

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