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**Multicentric standardized flow cytometry routine assessment of septic patient to predict clinical worsening**

Running head: New tool to predict clinical course during sepsis

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Abbreviation list

AUC: area under the curve

CBC: complete blood count

ED: emergency department

FCGR1: immunoglobulin G Fc-gamma-R1

FCM: flow cytometry

HIV: human immunodeficiency virus

ICU: intensive care unit

IG: immature granulocytes

MAP: mean arterial pressure

NLR: neutrophil/lymphocyte ratio

qSOFA: quick SOFA

ROC: receiver operating characteristics

SAPS: simplified acute physiology score

SIRS: systemic inflammatory response syndrome

SOFA: sequential organ failure assessment

WBC: white blood cell

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Abstract

*Background:* In this study, we primarily sought to assess the ability of flow cytometry to predict early clinical deterioration and overall survival in septic patients admitted in the emergency department and intensive care unit.

*Methods:* Patients admitted for community-acquired acute sepsis from 11 hospital centers were eligible. Early (Day 7) and late (Day 28) deaths were notified. Levels of $CD64^{pos}$ granulocytes, $CD16^{pos}$ monocytes, $CD16^{dim}$ immature granulocytes (IG), T and B lymphocytes were assessed by flow cytometry, using an identical, cross-validated, robust and simple consensus standardized protocol in each center.

*Results:* Among 1062 patients screened, 781 patients with confirmed sepsis were studied (age: 67±48 years, SAPS II: 36±17, SOFA: 5±4). Patients were divided into three groups (sepsis, severe sepsis and septic shock) on Day 0 and on Day 2. On Day 0, septic patients exhibited increased level of $CD64^{pos}$ granulocytes, $CD16^{pos}$ monocytes and IG with T-cell lymphopenia. Clinical severity was associated with higher percentages of IG and deeper T-cell lymphopenia. IG percentages tended to be higher in patients whose clinical status worsened on Day 2 (35.1 ± 35.6 vs 43.5 ± 35.2, $p=0.07$). Increased IG percentages were also related to occurrence of new organ failures on Day 2. Increased IG percentages, especially when associated with T-cell lymphopenia, were independently associated with early ($p<0.01$) and late ($p<0.01$) death.

*Conclusions:* Increased circulating IG at the acute phase of sepsis are linked to clinical worsening, especially when associated with T-cell lymphopenia. Early flow cytometry could help clinicians to target patients at high risk of clinical deterioration.

*Clinical trial registration:* ClinicalTrials.gov: NCT01995448.

Keywords: Sepsis, Inflammation, Immunosuppression, Flow cytometry, Prognosis

**Background**

Sepsis is a major healthcare burden and the leading cause of mortality in critically ill patients. Despite early recognition of septic patients, which is closely related to prognosis [1] and appropriate care, some of them present early clinical deterioration [2-4]. Several clinical trials tried to evaluate the value of various markers such as Presepsin or N-GAL in predicting the clinical course of septic patients admitted to the emergency department (ED) [5-8]. Most of these studies were single-center, small population-based trials and evaluated the ability of a given biomarker to predict late death in septic patients. Nevertheless, no trial has yet assessed the ability of biomarkers to accurately predict early clinical deterioration. Moreover, prior studies showed that patients who are secondarily transferred from a general hospital ward to the intensive care unit (ICU) have a poorer prognosis than patients admitted directly to the ICU from the ED [4,9,10]. Accordingly, a biological marker allowing early prediction of sepsis progression would be highly valuable on clinical grounds to identify the population at high risk of short-term deterioration and to determine the required level of monitoring and hospital admission location.

Sepsis course is not only related to the clinical status and medical history of patients, but also highly influenced by the host's response to infection [11,12] with major changes of circulating white blood cell (WBC) levels. Flow cytometry (FCM) is the reference method to characterize the immunophenotype of WBC. Major improvements have been achieved in FCM harmonization and in quality external controls which allow inter-laboratory comparisons [13,14]. FCM is increasingly used routinely to identify and quantify hematopoietic cells as it is more precise, rapid and reproducible than conventional cytology [15].

In a pilot study [15], we previously showed that the level of immature granulocytes (IG) could be a good candidate to predict early clinical deterioration at the acute phase of sepsis. These preliminary results needed to be validated in a larger population of septic patients based on a multicenter FCM protocol. Accordingly, the primary objective of the present study was to confirm the ability of IG levels to predict early clinical deterioration on Day 2. Secondary objectives were to assess the ability of other leukocyte subsets which are known

to be modified during sepsis, such as inflammatory CD64$^{pos}$ granulocytes, pro inflammatory CD16$^{pos}$ monocytes and CD3$^{pos}$ T-cells and CD24$^{pos}$ B-cells to predict early clinical deterioration (Day 2) and mortality (Day 7 and Day 28). We also evaluated the link between IG levels and mortality (Day 7 and Day 28) of acute septic patients admitted in ICU and ED.

## Material and methods

*Patients and controls*

This prospective, multicenter observational study was accepted by the Ethics Committee of Limoges University Hospital, approval number 119-2013-19. Written information was given to the patients or next-of-kin if patients were unable to approve their participation in the study and informed consent was obtained from all. The trial was registered in ClinicalTrials.gov: NCT01995448.

Patients admitted to one of the participating centers' ED or ICU with at least two criteria in favor of a systemic inflammatory response syndrome (SIRS) and a clinically or microbiologically documented infection evolving for less than 24 hours were eligible. The patients were divided into three groups of severities upon study enrollment, based on previous definitions: (i) sepsis, (ii) severe sepsis and (iii) septic shock [16,17]. Exclusion criteria included pregnancy, solid tumor, HIV infection, history of hematological or inflammatory condition, immunosuppressive treatment and a time to ICU admission exceeding 48 hours. Demographic data and a simplified acute physiology score (SAPS II) were recorded on admission. To evaluate clinical deterioration, organ failure i.e. respiratory (mechanical ventilation 12h/day or PaO2/FiO2 < 300), renal (urine output <0.5 mL/kg/h despite fluid resuscitation), hematological (platelets count < $100x10^9$/L), metabolic (lactatemia > 2 mmol/L) or circulatory failure reflected by hypotension (MAP < 60mmHg or need for vasopressor) and Sequential Organ Failure Assessment (SOFA) were compared between the inclusion visit and that of Day 2 after admission.

Each suspected infection as well as the diagnosis and severity of acute sepsis were validated by an independent adjudication committee including two independent experts based on the initial sepsis definition [16]. In addition, patients were separated according to the initial course of the disease. Sepsis was defined as worsening when deteriorating from sepsis to severe sepsis or septic shock or from severe sepsis to septic shock, or when the patient died by Day 2. In all other cases, sepsis was defined as stable or improved. Vital status was also evaluated on Day 7 and Day 28.

The control group included 20 outpatients without any hematological or infectious disorders who were recruited in all participating centers.

*Flow cytometry*

At the time of patients' enrollment, 8-color immunophenotyping was performed with a FCM protocol close to that currently used in daily clinical practice of participating centers. The two types of flow cytometers used were Navios™ (Beckman Coulter, Miami, FL) or Canto II™ (BD Biosciences, San Jose, CA). The instruments were harmonized as previously described by Solly et al [13]. As training for local laboratories and to insure inter-center reproducibility, FCM results of controls and of the first 20 patients were first analyzed in the local laboratory and then by the coordinating center where results and thresholds proposed by each center were validated before going on with the study. We checked that mean fluorescence intensity and percentage of positive cells for CD16, CD10 and CD64 for these patients were comparable between centers and flow cytometers (e-Figure 1). We also checked that CD3+ T cell absolute counts were in the same range between centers (e-Figure 1).

The gating strategy, performed with the Kaluza™ (Beckman Coulter) software, is presented in supplementary material (e-Table 1 and e-Figure 2). In each laboratory, fluorescence thresholds to assess the percentages of $CD16^{dim}$ and $CD10^{dim}$ granulocytes, $CD64^{pos}$ granulocytes and $CD16^{pos}$ monocytes were set-up by comparison with healthy controls so that more than 95% of controls exhibited less than 5% of each population of interest. Expression stability of the markers was checked up to 12 h after sampling (data not shown).

FCM analysis was performed on leftovers of complete blood count (CBC) within 12 hours after sample collection at ICU or ED admission (e-Table 2). In one single tube, we evaluated B-lymphocytes, defined by the expression of CD24 ($CD24^{pos}$), global T-lymphocytes subsets defined by the expression of CD3 ($CD3^{pos}$). For monocytes subsets we evaluated proinflammatory monocytes which are characterized by the expression of CD16 ($CD16^{pos}$). For granulocytes we evaluated the expression of CD64 which is known to be high during sepsis (proinflammatory granulocytes - $CD64^{pos}$) and IG defined by the low expression of CD16. IG, $CD64^{pos}$ granulocytes, T and B lymphocytes and proinflammatory $CD16^{pos}$ monocytes were quantified as percentages. Absolute counts were then calculated from CBC. For each patient we recorded neutrophil and lymphocyte counts from the CBC performed on the same blood sample as FCM analysis and evaluated the neutrophil/lymphocyte ratio (NLR).

*Statistical analysis*

Analyses were performed on the whole study population and on the 3 distinct groups of septic patients (sepsis, severe sepsis, septic shock). Qualitative variables were expressed as numbers and percentages and quantitative variables were expressed as means ± standard deviations. Population characteristics were compared between the 3 groups. Quantitative variables were compared using Anova tests, qualitative variables were compared using $Chi^2$ tests, or exact Fisher tests when appropriate. Since our study was explanatory, we did not perform alpha adjustment to take into account multiplicity of tests. To assess the ability of all leukocyte subsets analyzed by FCM and NLR ratio in predicting the sepsis course on Day 2, we performed univariate logistic regressions, receiver operating characteristics (ROC) curves and calculated areas under the curve (AUC). Survival curves were estimated using the Kaplan-Meier method, and differences between groups were tested for significance with the log-rank test. Quantitative variables were compared with Student's T tests. Analyses were performed using R software version 3.1.0 and Medcalc 11.1.1 (Ostend, Belgium).

## Results

*Study population*

Between January 2013 and June 2015, 1062 patients were screened (530 from ICU and 532 from ED) in 11 French hospital centers, and 281 of them were secondarily excluded for unconfirmed sepsis (n=242) or lack of FCM data (n=39). Finally, 781 consecutive patients with confirmed sepsis (sepsis: n=343; severe sepsis: n=192; septic shock: n=246) were analyzed (age: 67±48 years; SAPS II: 36±17; SOFA: 5±4) and 769 completed the study (Figure 1, Table 1). Among the 781 consecutive patients, 732 patients had a clinically documented infection – 327 of them with a microbiological confirmation – and the 49 remaining patients had only a microbiologically confirmed infection. Pulmonary and urinary infections were the main causes of sepsis (Table 1). Clinical deterioration on Day 2 was observed in 25 (8%), 21 (12%) and 21 (9%) patients with sepsis, severe sepsis and septic shock, respectively. Forty-eight patients (6%) were deceased on Day 7 and 79 patients (10%) on Day 28 (Figure 1, Table 1).

*Leukocyte subsets on Day 0*

To enumerate precisely IGs with $CD64^{pos}$ neutrophils, monocytes and $CD3^{pos}$ T-cells, we set up a robust standardized 8-color FCM protocol identical in the 11 university hospital centers. Upon admission, the three groups of septic patients, when compared to healthy controls, exhibited significant increased leukocyte counts (14.55 $10^9$/L ± 6.13 in sepsis vs 6.59 $10^9$/L ± 1.65 in controls, Table 2), due to elevated percentages of granulocytes (79.3% ± 10.94 in sepsis vs 57.47% ± 9.24 in controls, Table 2). Conversely, the levels of $CD3^{pos}$ T-cells were significantly lower (0.84 $10^9$/L ± 0.58 in sepsis vs 1.53 $10^9$/L ± 0.58 in controls, Table 2). T-cell lymphopenia was even more profound in severe sepsis and septic shock (Table 2 and Figure 2). The global percentage of monocytes was mildly decreased, but the proportion of $CD16^{pos}$ monocytes was significantly higher (12.37% ± 4.32 of total monocytes in controls vs 20.39% ± 11.51 in sepsis), with values increasing with the severity of sepsis (Table 2 and Figure 2). Among granulocytes, percentages of both $CD64^{pos}$ and $CD16^{dim}$ IGs were

significantly increased (Table 2). Again, these percentages increased with the severity of sepsis, especially for IG (Table 2 and Figures 2 and 3).

NLR was significantly higher in patients with severe sepsis when compared to those with sepsis (21.30 ± 23.5 vs 15.97 ± 19.14: $p<0.05$), but not between patients with septic shock and those with severe sepsis (Table 1).

*Clinical worsening on Day 2*

Percentages of monocytes tended to be higher in patients with early clinical deterioration compared to others (Table 3, 6.7% ± 4.1 vs 5.3% ± 3.1, $p<0.01$) with a proportion of $CD16^{pos}$ pro inflammatory monocytes unchanged when compared to improved/stable patients.

Whole lymphocyte counts were not related to clinical deterioration on Day 2, but $CD3^{pos}$ T-lymphocytes counts tended to be lower in patients with early clinical deterioration (Table 3, 0.5 $10^9$/L ± 0.5 vs 0.7 $10^9$/L ± 0.5, $p = 0.06$). Increased percentages of IG tended to be higher in patients whose clinical status worsened on Day 2 (Table 3, 35.1% ± 35.6 vs 43.5% ± 35.2, $p=0.07$). IG increase on Day 0 was also statistically significantly associated with the occurrence of new organ failures on Day 2 and especially thrombocytopenia on Day 2 (33.4 ± 34.4 vs 67.2 ± 31.8, $p<0.01$) (Table 4). NLR was not associated with clinical deterioration on Day 2 (OR=1.21; 95% CI [0.77; 2.23]: $p = 0.54$; AUC = 0.52].

CD64 expression was statistically significantly higher in patients who presented new renal failure on Day 2 ($p<0.001$) but was not associated with a worsening of sepsis on Day 2 (Table 3 and e-Table 3).

*Early and late death*

We did not find any significant association between NLR and early death (OR = 0.63; 95% CI [0.32; 1.28]; $p = 0.2$; AUC = 0.56) nor between NLR and late death (OR = 1.18, 95% CI [0.69; 2.07]; $p = 0.55$; AUC = 0.52).

As presented in Figure 4A, 77% of the patients who died on Day 7 had a percentage of IG over 30% on Day 0. In contrast, only 42% of patients still alive on Day 7 had IG above this threshold ($p<0.001$).

Patients with less than 30% circulating IG had a 95% overall survival rate at Day 28. As shown in Figure 4B, associated deep T-cell lymphopenia (< 0.25 x $10^9$/l) did not significantly modify the prognosis of these patients. As for early clinical deterioration and Day-7 death, percentages of circulating IG at the early phase of sepsis over 30% were significantly linked with the probability of Day-28 death (19.6 ± 17 vs 56.5 ± 42.4 <0.01, Figure 4B). Association with deep T-cell lymphopenia deteriorated the prognosis with approximately 80% overall survival rate on Day 28 (Figure 4B). Of note, the 95% CI of the survival curve of patients with both IG > 30% and T-cell lymphopenia < 0.25 G/L did not overlap that of patients with IG < 30%, irrespective of lymphocytosis (e-Figure 3). When considering the latter group as reference, patients with both increased IG and decreased lymphocytosis exhibited an OR of 3.88 (95% CI [2.14; 7.04]) for D7 death.

**Discussion**

In this multicenter study, we showed the feasibility and the reproducibility of a leukocyte immunophenotyping by FCM in septic patients and its clinical value using a robust FCM protocol close to that used in daily clinical practice. Increased percentages of circulating $CD16^{dim}$ IG at hospital admission were significantly associated with the severity of sepsis. High IG levels also appeared to be associated with a clinical deterioration on Day 2, and with early (Day 7) and late (Day 28) death. This confirms our previous results and the prognostic value of this leukocyte subset in septic patients [15]. We obtained these data using the samples collected for classical CBC, within a few hours after the diagnosis of sepsis either in the ED or in the ICU. Early FCM could thus be informative to help front-line clinicians in the ED or ICU determining the appropriate level of monitoring and the need for ICU admission. Specifically, prognostic scores commonly used in septic patients (e.g., SOFA score and

APACHE II) [18,19] are difficult to implement routinely in the ED. Despite a good prognostic value on a global population, these scores could fail individually in certain patients. In addition, leukocyte immunophenotyping and especially IG level and depth of lymphopenia evaluated on leftovers of a blood sample could provide clinicians with early information on patients' immune status and could be of prognostic value.

This multicenter study was based on the former definition of sepsis [16]. Nevertheless, the prediction of early clinical deterioration of septic patients remains challenging, irrespective of the definition used. Traditional definition of sepsis was based on the association of SIRS and infection [16], whereas the use of quick SOFA (qSOFA) has recently been proposed to better identify high-risk patients with suspected infection outside the ICU [20]. Both qSOFA and SIRS criteria lack specificity in the ED setting since some patients without confirmed sepsis present with a qSOFA or SIRS criteria ≥ 2 [21].

Our study confirmed that early increased percentages of $CD64^{pos}$ granulocytes and, to a lesser extent $CD16^{pos}$ monocytes, were strongly associated with the diagnosis of sepsis [15]. CD64 expression, the high-affinity receptor for immunoglobulin G Fc-gamma-RI (FCGR1), on granulocytes has been reported to have a relevant specific diagnostic value to early identify sepsis in adult patients [22-24]. Although they increased with the severity of sepsis, the levels of either $CD64^{pos}$ granulocytes or $CD16^{pos}$ monocytes did not influence the survival rate of patients with sepsis, suggesting that changes in these leukocyte subsets probably reflect the infectious status but are not linked to clinical severity.

Identification of IG using microscopic blood smear examination by a human being, with the problem of separating band cells from mature neutrophils, is poorly reproducible [11,12,15]. This problem is even more pronounced during sepsis, because granulocytes suffer various morphological changes related to their bactericidal activity, such as degranulation, vacuolization of the cytoplasm or alterations of nucleus lobulation. Automated cytological recognition of cells would be theoretically feasible. Yet, comparison between counting of granulocytes on blood smears with one of the most popular apparatus (DM9600 from

Cellavision®, Lunt, Sweden) and FCM was performed by two of us (EG and JF), and the correlation was very poor (data not shown). In contrast, NLR is an easily obtained hematological parameter [25]. To our knowledge, no trial has yet evaluated the link between NLR and clinical deterioration. Moreover, cutoff values are not well defined [26]. In the present study we did not find any value of NLR to predict a clinical deterioration on day 2 or both early and late death. Some cell counters propose to count IG but these counting methods are based on proprietary methods that preclude any universal solution. Conversely, FCM allows counting cells in a manner that is independent of any industrial supplier. Here, IG were defined by decreased CD16 expression, which spans almost all stages of immature granulocytes [27]. To reduce center-related bias the FCM protocol was standardized and implemented in each routine laboratory of the 12 participating centers, based on a preliminary consensus and on a training step in order to obtain homogeneous results. In including more than 700 patients in the acute phase of sepsis in a multicenter trial, we demonstrate the feasibility of incorporating a simple, robust and standardized FCM protocol in routine care not only in ICU [23], but also in the ED.

Based on the inclusion of a large and heterogeneous population hospitalized in EDs and ICUs for proven sepsis, IG levels measured using FCM appeared as a powerful predictor of the clinical course of sepsis, both in terms of impending organ failure and survival. This is in agreement with the known major role played by granulocyte subsets in the immune response at the acute phase of sepsis [15]. Dysregulation of systemic inflammation, which may be reflected by high percentages of IG [28], is a common trigger of clinical deterioration and worsening organ failures [29-31]. Indeed, as previously shown in a surgical population, IG percentages reflect the intensity of the inflammatory insult [28]. Increased percentages of IG during sepsis are related to the expression of proinflammatory cytokines such as IL-6 and IL-8 [32]. This association between circulating cytokine levels and percentages of IG could explain the significant link between clinical severity and IG levels. On the other hand, IG could directly contribute to the SIRS. Indeed, having some bacterial

phagocytosis and killing properties, IGs express increased levels of tumor necrosis factor-α when compared to IL-10, suggesting a proinflammatory phenotype [33].

Drewry et al. have reported that persistent lymphopenia after a diagnosis of sepsis is associated with mortality [34]. In the present study, global lymphopenia was predominantly related to T-cell lymphopenia. Profound T-cell lymphopenia, a marker of immunosuppression, also appeared to be prognostic, but only when associated with high percentages of circulating IG [31,34]. In that case, the IG subset harbors a myeloid derived suppressor cell (MDSC) fraction able to kill T-cells [15]. The prognostic value of T-cell lymphopenia could be explained by a higher risk of secondary opportunistic infection leading to prolonged hospitalization [31].

The present study has several limitations. First, we enrolled a large cohort of septic patients with various origins and severity coming from the ICU and ED and interpretation of FCM results could somehow vary between centers. This variability could explain why the monocyte count by FCM was not robust enough to be validated, in a multicenter manner, as a predictor of sepsis deterioration. In contrast, by overcoming these sources of variability, this multicenter study allowed the clear demonstration that granulocyte immunophenotyping, and especially IG level, is a robust predictor of clinical course and short and long-term mortality. Other cell markers could have been tested, such as expression of HLA-DR on monocytes[35]. We chose not to assess this parameter because published pre-analytical conditions are rather strict [36], and thus incompatible with the daily practice of the centers participating in this study. To confirm that IG level is a predictor of the sepsis evolution raises the question of immunosuppression in sepsis, since this compartment encompasses MDSCs. In that view, future studies focusing on the PD-1/PD-L1 axis could be of interest [37]. However, expression of these markers would certainly have to be studied not only on granulocytes (including IG and MDSCs) but also on all other circulating leukocyte subsets among them monocytes, dendritic cells or lymphocyte subpopulations.

**Conclusion**

This multicenter study shows that IG is a biological marker of severity at the early phase of sepsis since (i) it accurately identifies patients at high risk of early clinical deterioration who require advanced monitoring, and (ii) it is linked with early and late death, especially when associated with T-cell lymphopenia. To quantify IG by standardized FCM based on decreased CD16 expression was technically simple. In the near future, routine quantification of circulating IG using FCM could help front-line clinicians in predicting the outcome of patients with acute sepsis both in the ED and in the ICU.

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Dr Bruno François takes responsibility for the content of the manuscript, including the data and analysis.

Authors' contributions: TD, EG, PV, JF, BF: designed the study, enrolled patients or performed flow cytometry on samples, analyzed the final data and drafted the manuscript
EM, VG, OH, JPM, FD, NC, CG, MCB, JPQ, CT, JG, GP, AR, GM, EL, NP, MK, OWB, RC, ED, FU, M Roussel, TL, RJ, FV, CF, M Roux, KA: enrolled patients or performed flow cytometry on samples, critically reviewed the manuscript.
All authors read and approved the final version of the manuscript.

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Table 1: Population characteristics

| Criteria | Sepsis (n=343) | Severe Sepsis (n=192) | Septic Shock (n=246) | p |
|---|---|---|---|---|
| Age* | 67 ± 75 | 67 ± 67 | 65 ± 15 | 0.91 |
| Gender, n (%): | | | | |
| - Male | 176 (51%) | 117 (61%) | 152 (62%) | 0.02 |
| BMI* | 27 ± 7 | 27 ± 7 | 29 ± 9 | 0.14 |
| Status, n (%): | | | | |
| - Surgical | 25 (7%) | 16 (8%) | 41 (17%) | <0.01 |
| - Medical | 318 (93%) | 176 (92%) | 205 (83%) | |
| Site of infection, n (%): | | | | |
| - Pulmonary | 109 (32%) | 81 (42%) | 80 (32%) | 0.04 |
| - Urinary | 123 (36%) | 49 (25%) | 55 (22%) | <0.01 |
| - Abdominal | 43 (12%) | 20 (10%) | 53 (21%) | <0.01 |
| - Skin and soft tissue | 39 (11%) | 13 (7%) | 21 (8%) | 0.19 |
| - Other | 29 (8%) | 29 (15%) | 37 (15%) | 0.75 |
| SOFA score* | 2 ± 2 | 5 ± 3 | 9 ± 3 | <0.01 |
| SAPS II score* | 25 ± 11 | 37 ± 13 | 51 ± 17 | <0.01 |
| Organ failures, n (%): | | | | |
| - Respiratory | - | 74 (38%) | 146 (59%) | <0.01 |
| - Kidney | - | 42 (22%) | 86 (35%) | <0.01 |
| - Blood | - | 52 (27%) | 51 (21%) | 0.14 |
| - Metabolic | - | 81 (42%) | 145 (59%) | <0.01 |
| - Hypotension | - | 31 (16%) | 43 (17%) | 0.81 |
| - Vasopressor support | - | - | 246 (100%) | - |
| Clinical worsening at Day-2, n (%) | 25 (7.2%) | 21 (10.9%) | 21 (9.5%) | |
| Dead at Day 7, n (%) | 2 (0.6%) | 8 (4.2%) | 38 (15.0%) | <0.01 |
| Dead at Day 28, n (%) | 10 (2.9%) | 14 (7.3%) | 55 (22.3%) | <0.01 |

Abbreviations: BMI: Body Mass Index; SOFA: Sequential Organ Failure Assessment; SAPS II: Simplified Acute Physiology Score II

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Table 2: Leukocyte subset counts regarding sepsis severity on D0.

| Leukocytes subsets | Control | Sepsis | Severe sepsis | Septic shock |
|---|---|---|---|---|
| Leukocytes, $10^9$/L | 6.59 ± 1.65 | 14.55 ± 6.13* | 13.82 ± 7.04 | 15.87 ± 9.44* |
| Lymphocytes, $10^9$/L | 2.16 ± 0.78 | 1.35 ± 1.43* | 0.89 ± 0.66* | 0.93 ± 0.73 |
| B-Lymphocytes, $10^9$/L | 0.2 ± 0.12 | 0.24 ± 1.19* | 0.16 ± 0.32 | 0.18 ± 0.25 |
| $CD3^{pos}$ Lymphocytes, $10^9$/L | 1.53 ± 0.58 | 0.84 ± 0.58* | 0.54 ± 0.41* | 0.54 ± 0.5 |
| Monocytes, % | 8.29 ± 2.28 | 7.72 ± 3.89* | 6.02 ± 3.73* | 5.3 ± 4.01* |
| $CD16^{pos}$ Monocytes, % | 12.37 ± 4.32 | 20.39 ± 11.51* | 22.54 ± 14.23 | 24.36 ± 14.37 |
| Granulocytes, % | 57.47 ± 9.24 | 79.3 ± 10.94* | 83.98 ± 8.27* | 84.06 ± 11.86 |
| $64^{pos}$ Granulocytes, % | 1.49 ± 5.03 | 47.43 ± 36.23* | 54.18 ± 37.41* | 63.5 ± 33.95* |
| $CD16^{dim}$ Granulocytes, % | 2.97 ± 5.61 | 19.67 ± 26.53* | 34.95 ± 34.04* | 59.27 ± 34.68* |

*$p < 0.05$ (control vs sepsis, sepsis vs severe sepsis, severe sepsis vs septic shock)

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Table 3: Leukocyte subsets evaluated by flow cytometry and clinical deterioration on Day 2, odds ratio its 95% CI and p value associated to the univariate logistic regression, area under the curve and its 95% CI.

| Leukocytes subsets | Improved / Stable | Deteriorated | OR | 95% CI | *p* | AUC | 95% CI |
|---|---|---|---|---|---|---|---|
| Leukocytes, $10^9$/L | 14.9 ± 7.4 | 14 ± 9.4 | 0.98 | [0.95; 1.02] | 0.37 | 0.55 | [0.47;0.63] |
| Lymphocytes, $10^9$/L | 1.1 ± 1.1 | 0.9 ± 0.8 | 0.76 | [0.51; 1.05] | 0.15 | 0.57 | [0.49;0.64] |
| B-Lymphocytes, $10^9$/L | 0.2 ± 0.9 | 0.1 ± 0.1 | 0.33 | [0.04; 1.07] | 0.25 | 0.57 | [0.49;0.65] |
| $CD3^{pos}$ Lymphocytes, $10^9$/L | 0.7 ± 0.5 | 0.5 ± 0.5 | 0.58 | [0.32; 0.98] | 0.06 | 0.59 | [0.52;0.66] |
| Monocytes, % | 6.7 ± 4.1 | 5.3 ± 3.1 | 0.91 | [0.84; 0.97] | <0.01 | 0.59 | [0.52;0.66] |
| $CD16^{pos}$ Monocytes, % | 22.3 ± 13.2 | 22.3 ± 14.7 | 1.00 | [0.98; 1.02] | 0.99 | 0.52 | [0.44;0.59] |
| Granulocytes, % | 81.9 ± 10.8 | 82.1 ± 13.2 | 1.00 | [0.98; 1.03] | 0.89 | 0.53 | [0.46;0.61] |
| $CD64^{pos}$ Granulocytes, % | 53.6 ± 36.3 | 58.9 ± 36.6 | 1.00 | [1.00; 1.01] | 0.25 | 0.53 | [0.46;0.61] |
| $CD16^{dim}$ Granulocytes, % | 35.1 ± 35.6 | 43.5 ± 35.2 | 1.01 | [1.00; 1.01] | 0.07 | 0.59 | [0.53;0.66] |

Abbreviations: OR: Odds Ratio; AUC: Area Under the Curve; CI: Confidence Interval

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Table 4: Percentage of IG subsets in relation to the occurrence of new organ failure on Day 2, odds ratio its 95% CI and p value associated to the logistic regression, area under the curve and its 95% CI

| Organ failure | Improvement/ Stabilization | Occurrence | OR | 95% CI | p | AUC | 95% CI |
|---|---|---|---|---|---|---|---|
| Respiratory failure | 35.7 ± 35.4 | 48.6 ± 37.2 | 1.01 | [1.00; 1.02] | 0.01 | 0.6 | [0.52;0.68] |
| Kidney failure | 35.1 ± 35 | 61.7 ± 35.7 | 1.02 | [1.01; 1.03] | <0.01 | 0.72 | [0.64;0.79] |
| Thrombocytopenia | 33.4 ± 34.4 | 67.2 ± 31.8 | 1.03 | [1.02; 1.04] | <0.01 | 0.77 | [0.72;0.82] |
| Hyperlactatemia | 35.7 ± 35.2 | 59.8 ± 36.5 | 1.02 | [1.01; 1.03] | <0.01 | 0.69 | [0.6;0.78] |
| Hypotension | 36.1 ± 35.5 | 57.9 ± 36 | 1.02 | [1.01; 1.03] | <0.01 | 0.69 | [0.6;0.77] |
| Vasopressor support | 35.6 ± 35.4 | 53.6 ± 34.9 | 1.01 | [1.00; 1.02] | <0.01 | 0.67 | [0.6;0.73] |

Abbreviations: IG: Immature Granulocytes; OR: Odds Ratio; AUC: Area Under the Curve; CI: Confidence Interval

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Figure legends

Figure 1: Flow chart of the Septiflux2 trial

Figure 2: Box and whisker plot of leukocyte subsets in the control group compared to patients with confirmed sepsis, severe sepsis and septic shock

A: Comparison of $CD16^{pos}$ monocytes, $CD64^{pos}$ granulocytes, $CD16^{dim}$ immature granulocytes percentages

B: Comparison of $CD3^{pos}$ lymphocyte and monocyte counts

Figure 3: Examples of biparametric CD10/CD16 histograms in a typical out patient and patients with different sepsis severity. The red box corresponds to the gate in which $CD16^{dim}$ IG were counted. The detailed gating strategy is given in the e-Figure 2.

Figure 4: Relationship between sepsis evolution and $CD16^{dim}$ IG and CD3 lymphopenia

A: Percentage of patients with the parameter over the 30% threshold according to the vital status on Day 7.

B: Kaplan-Meier overall survival curves for patients according to the proportion of $CD16^{dim}$ IG and $CD3^{pos}$ lymphocyte counts.

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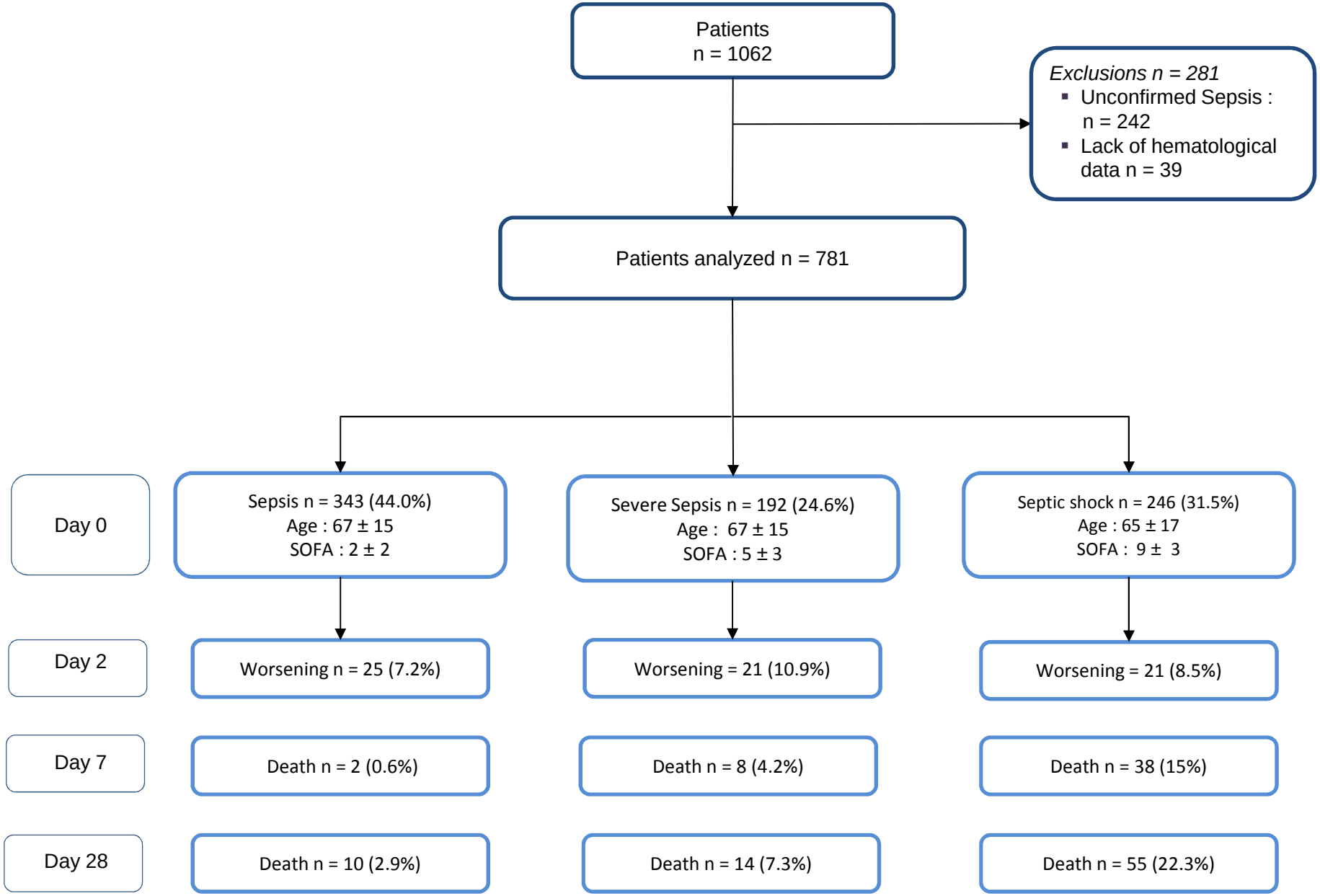
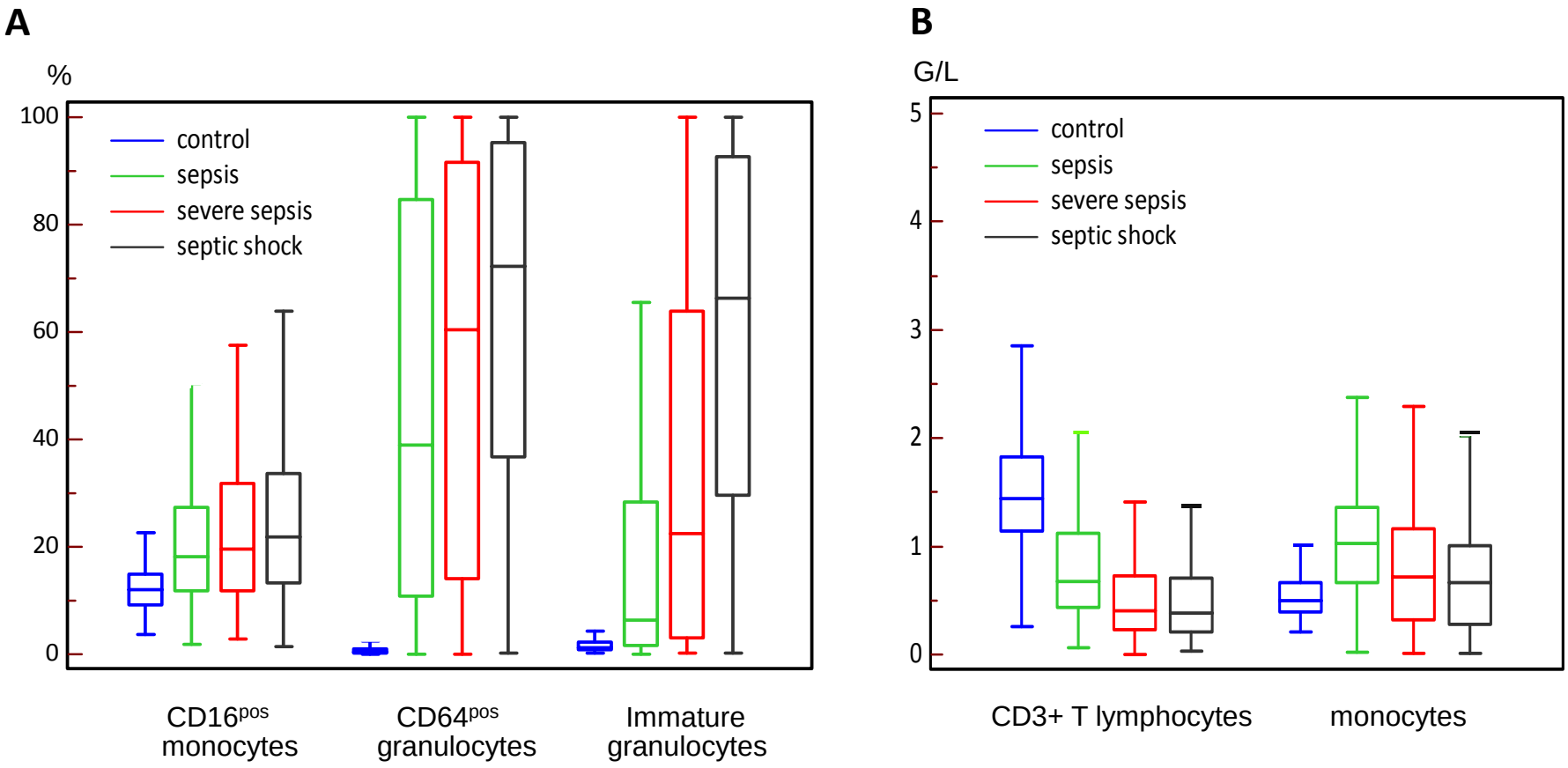
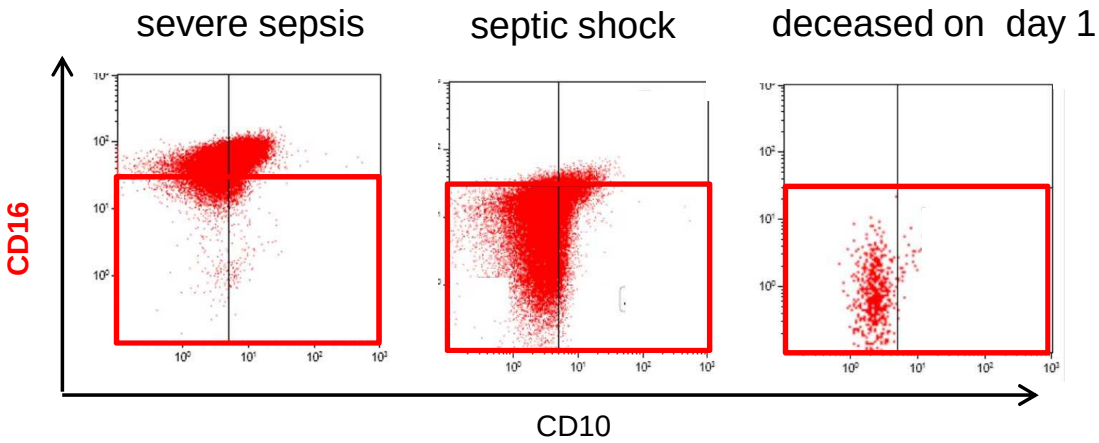
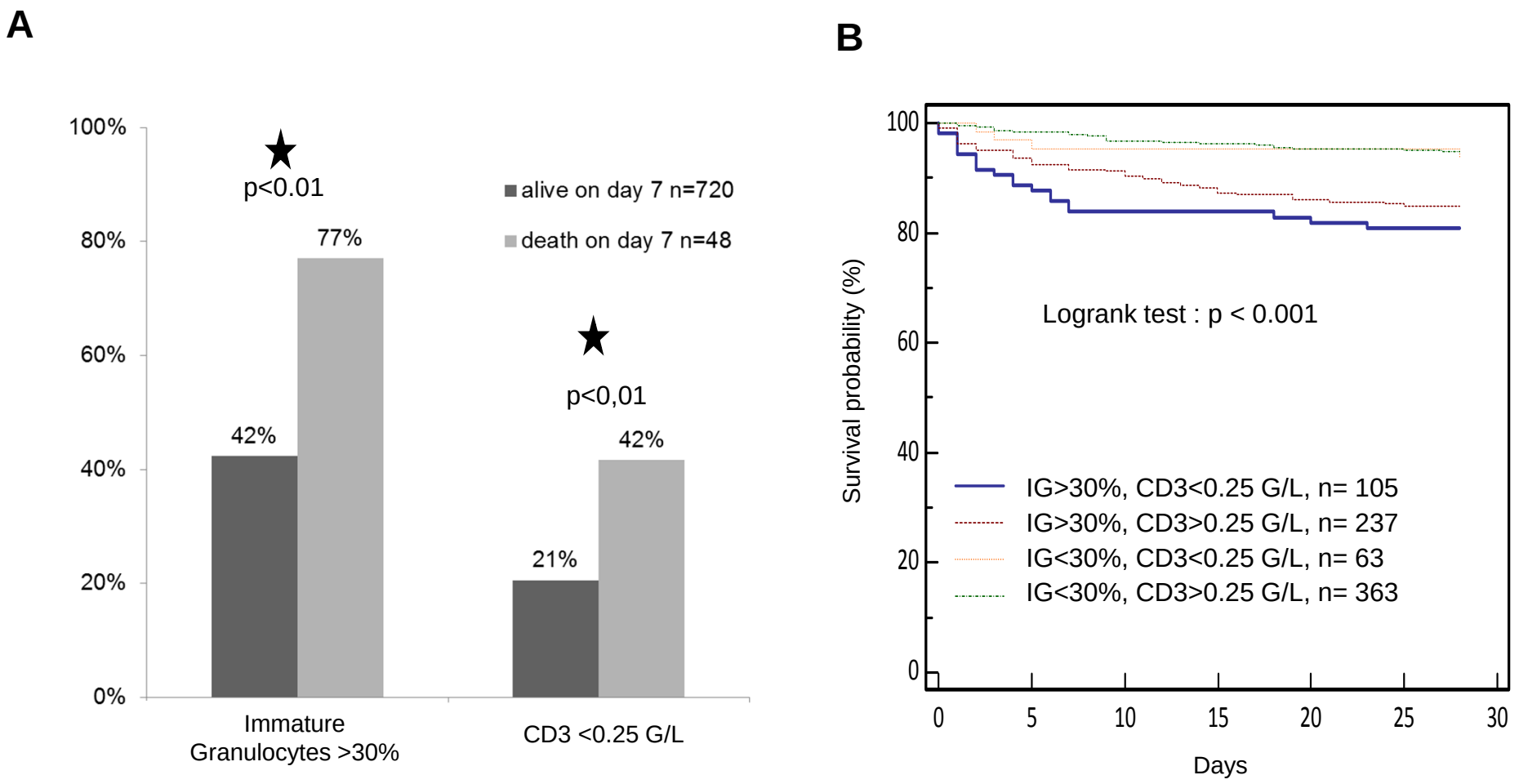



A
%
100
80
60
40
20
0
control
sepsis
severe sepsis
septic shock
CD16pos monocytes
CD64pos granulocytes
Immature granulocytes
B
G/L
5
4
3
2
1
0
control
sepsis
severe sepsis
septic shock
CD3+ T lymphocytes
monocytes

## Outpatient

## Septic patients with low CD16 expression




A
100%
80%
60%
40%
20%
0%
p<0.01
77%
42%
alive on day 7 n=720
death on day 7 n=48
p<0,01
42%
21%
Immature Granulocytes >30%
CD3 <0.25 G/L
B
Survival probability (%)
100
80
60
40
20
0
Logrank test : p < 0.001
IG>30%, CD3<0.25 G/L, n= 105
IG>30%, CD3>0.25 G/L, n= 237
IG<30%, CD3<0.25 G/L, n= 63
IG<30%, CD3>0.25 G/L, n= 363
0
5
10
15
20
25
30
Days

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**e-Table 1:** Presumed deduced cell types from the gating characteristics of each cell studied

| Variable | Gating characteristics |
|---|---|
| Lymphocytes $10^9$/L | SSC $^{low}$, CD45$^{high}$ |
| B-lymphocytes, CD24+ $10^9$/L | SSC $^{low}$, CD45$^{high}$, CD24+ |
| T-lymphocytes, CD3+ $10^9$/L | SSC $^{low}$, CD45$^{high}$, CD3+ |
| Granulocytes $10^9$/L : | SSC $^{high}$, CD45$^{int}$, CD14-, CD3-, CD24- |
| - CD64$^{pos}$ %* | SSC$^{high}$, CD45$^{int}$, CD14-, CD3-, CD24-, CD64+ |
| - CD10$^{dim}$ and not CD16$^{dim}$ % * | SSC$^{high}$, CD45$^{int}$, CD14-, CD3-, CD24-, CD10$^{dim}$-, not CD16$^{dim}$ |
| - not CD10$^{dim}$ and CD16$^{dim}$ % * | SSC$^{high}$, CD45$^{int}$, CD14-, CD3-, CD24-, not CD10$^{dim}$-, CD16$^{dim}$ |
| - CD10$^{dim}$ and CD16$^{dim}$ % * | SSC$^{high}$, CD45$^{int}$, CD14-, CD3-, CD24-, CD10$^{dim}$ |
| Monocytes, $10^9$/L | SSC$^{int}$, CD45$^{high}$, CD14+ |
| - increased CD16 expression, %* | SSC$^{int}$, CD45$^{high}$, CD14+, CD16+ |

*The threshold has been established from a control group

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**e-Table 2:** Antibodies used for flow cytometry

| Antibodies | Manufacturer |
|---|---|
| Fluoresceine Iso Thio Cyanate (FITC) conjugated CD64 mAb (CD64-FITC, clone 22)<br>phycoerythrin (PE) conjugated CD10 mAb (CD10-PE,clone ALB1)<br>PE-cyanine 5,5 (PC5,5) conjugated CD14 mAb (CD14-PC5,5, clone RMO52)<br>PE-cyanine 7 (PC7) conjugated CD3 mAb (CD3-PC7, clone UCHT1)<br>Allophycocyanin (APC) conjugated CD24 mAb (CD24-APC, clone ALB9)<br>APC Alexia Fluor 750 (APC-A750) conjugated CD11b mAb (CD11b-APC-A750, clone Bear 1)<br>Pacific blue (PB) conjugated CD16 mAb (CD16-PB, clone 3G8)<br>Krome Orange (KO) conjugated CD45 mAb (CD45-KO, clone J.33) | Immunotech<br>(Marseille, France). |

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**e-Table 3:** Percentage of CD64$^{pos}$ granulocytes regarding the occurrence of new organ failure on Day 2, odds ratio and p value associated to the logistic regression, area under the curve and its 95% CI

| | Improvement/ Stabilization | New Organ Failure | OR | p | AUC | 95% CI |
|---|---|---|---|---|---|---|
| Respiratory failure | 58.5 | 68 | 1 | 0.43 | 0.53 | [0.44; 0.61] |
| Renal failure | 57.1 | 81.9 | 1.01 | <0.01 | 0.60 | [0.52; 0.68] |
| Thrombopenia | 58.1 | 73.7 | 1.10 | 0.05 | 0.57 | [0.5; 0.64] |
| Hyperlactatemia | 57.2 | 83.8 | 1.02 | <0.01 | 0.63 | [0.54; 0.72] |
| Hypotension | 58.3 | 68 | 1.01 | 0.13 | 0.58 | [0.47; 0.68] |
| Vasopressor support | 57.1 | 81.2 | 1.01 | <0.01 | 0.6 | [0.52; 0.68] |

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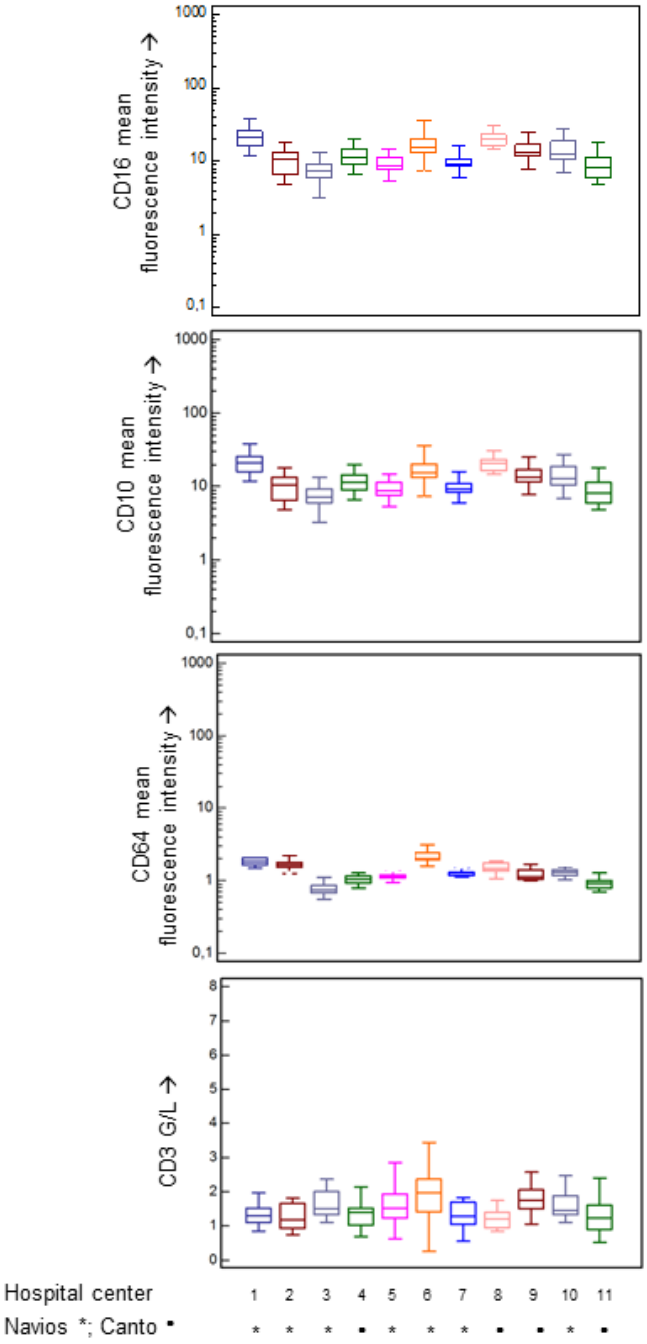
**e-Figure 1:** Box and whisker plot of CD16, CD10, CD64 mean fluorescence intensity and CD3 count subsets in the control group from the eleven centers and depends of flow cytometers used.



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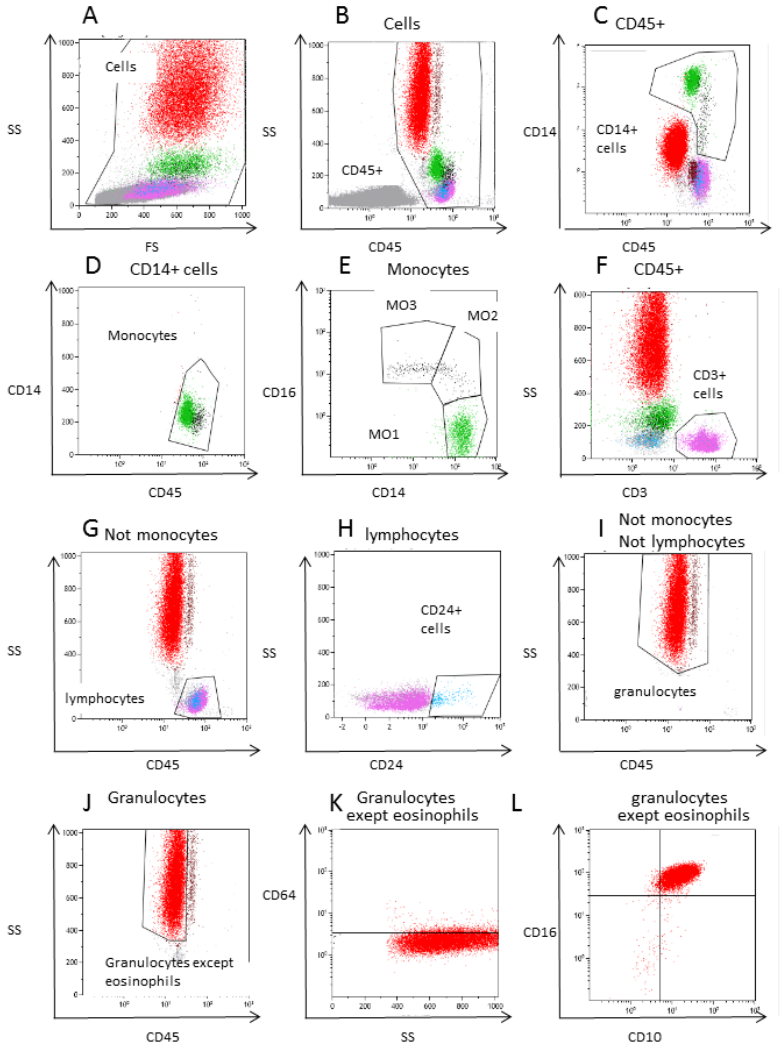
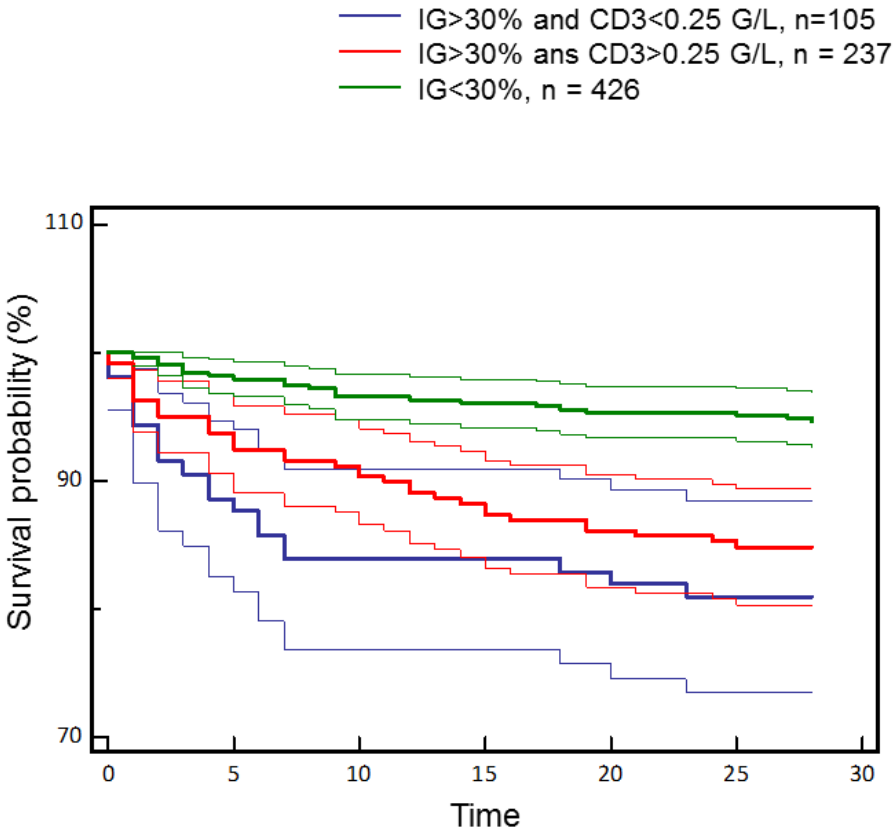
**e-Figure 2:** Gating strategy to identify leukocyte subsets in a representative case of outpatient used as control
A: Cells were selected on morphological parameters (FS versus SS)
B: Hematopoietic cells were defined as CD45$^{pos}$ cells.
C, D: Monocytes were roughly selected as CD14$^{pos}$ cells and then refined as CD45$^{pos}$/SS intermediate cells.
E: Monocytes were separated into two subtypes (i) classical CD14$^{pos}$/CD16$^{neg}$ (classical and MO1), (ii) CD14$^{pos}$/CD16$^{pos}$ (intermediate or MO2) and CD14$^{low}$/CD16$^{pos}$ (non-classical or MO3).
F: CD3$^{pos}$ T-lymphocytes were gated.
G: Lymphocytes CD45$^{high}$/SS$^{low}$ were separated on cells after exclusion of monocytes
H: CD24$^{pos}$ B-lymphocytes were gated.
I: Granulocytes were gated after exclusion of monocytes and lymphocytes.
J: Exclusion of CD45$^{high}$ eosinophils.
K, L: CD10, CD16 and CD64 expression on granulocytes without eosinophils.
Thresholds were determined by comparison with 20 control subjects in each center.



**e-Figure 3:** Kaplan-Meier overall survival curves for patients according to the proportion of CD16$^{dim}$ IG and CD3$^{pos}$ lymphocyte count including 95% IC in graph



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