Can concurrent abnormalities in free light chains and immunoglobulin concentrations identify a target population for immunoglobulin trials in sepsis?

Manu Shankar-Hari[1,2*] PhD MSc FRCA FFICM, Mervyn Singer[3#]MD FRCP FFICM, Jo Spencer[1*#]PhD

[1]Peter Gorer Department of Immunobiology, Programme in Infection & Immunity, King's College London, London, UK; [2]Department of Intensive Care Medicine, Guy's and St Thomas' NHS Foundation Trust, London, UK; [3]Bloomsbury Institute of Intensive Care Medicine, University College London, London, UK

Institutions: This work was performed at the Critical Care Units at Guy's and St Thomas' Hospitals NHS Foundation Trust, London, UK and at The Programme in Infection and Division of Infection and Immunity at King's College London

[#]Equal contributors

***Corresponding author and reprints**

Dr Manu Shankar-Hari MSc PhD FRCA FFICM

1st Floor, East Wing, St Thomas' Hospital, Guy's and St Thomas' NHS Foundation Trust London, UK, SE17EH; [2]Programme in Infection & Immunity, Kings College London, SE1 9RT

email: manu.shankar-hari@kcl.ac.uk

Tel: +44 20 7188 8769; Fax: +44 20 7188 2284

Financial support: Supported in part by the ICU research funds at Guy's and St Thomas' NHS Foundation Trust and by the NIHR Biomedical Research Centre at Kings Health Partners, London, UK

Key words: Sepsis, immunoglobulin kappa light chain, immunoglobulin lambda light chain, prognosis

Word count: Abstract = 260; Main article = 2545

Abstract

Objective: Light chains kappa (κ) and lambda (λ) are immunoglobulin constituents but also circulate independently in blood as free light chains (FLC). We investigated whether a concomitant abnormality in FLC and immunoglobulin levels could identify a high risk of death sepsis subpopulation to inform future IVIg trials. We tested whether light chain allelic inclusion occurs in circulating B cells.

Design: Prospective cohort study

Setting: Adult general intensive care units (ICU)

Patients: Adult sepsis patients without any documented immune comorbidity.

Interventions: None

Measurements and main results: Serum total FLC, IgG, IgA and IgM were measured on ICU Day 1, 3 and 7. Population normal ranges defined normal and abnormal categories. Logistic regression models tested any independent relationship between high-FLC, immunoglobulins and hospital mortality. CD19 B cell subsets expressing cell surface κ and λ were quantified by flow cytometry; their frequencies were compared against healthy subjects and correlation assessed against FLC concentrations. On ICU Day 1, high-FLCλ and high-FLCκ were seen in 46.5% and 75.3% of the study cohort (n=101). Low immunoglobulin levels were commonplace (45.5%) on ICU admission. ICU admission day FLC and immunoglobulin concentrations were significantly correlated. Septic patients had significantly more CD19 B cells expressing both κ and λ compared with healthy controls (median (IQR) 4.1% (2.4, 11.0) vs. 1.3% (1.2, 2.9) respectively; p=0.0001), which correlated with FLC concentrations.

Conclusions: To our knowledge, abnormalities and associations of FLC in critically ill adults with sepsis have not been previously reported. The additional prognostic value of FLCλ and significance of allelic inclusion in B cells in sepsis require further investigation.

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Attempts to favorably modulate a dysregulated immune response with intravenous immunoglobulin therapy (IVIg) failed to improve outcomes, as these trials potentially did not target treatment responsive subpopulations (2-4). Studies that help to enrich sepsis subpopulations based on abnormalities in immunoglobulin biology could inform future IVIg trial design, as new trials are being planned (5). We recently tested whether low immunoglobulin G (IgG) levels on the day of sepsis diagnosis identified a higher risk of death subpopulation (6). Despite a high prevalence of low IgG levels, this abnormality alone did not identify mortality risk.

In health, circulating immunoglobulin consists of heavy chains bound by disulphide bonds to either kappa (κ) or lambda (λ) light chains. These light chains are also measurable as circulating free light chains (FLC), separate from immunoglobulin molecules. Raised FLC levels are associated with sepsis risk factors such as low-grade inflammation, increasing age and renal dysfunction (7-9). Altered FLC concentrations have been reported in a pilot study performed on 21 ICU sepsis patients (10).

Since reduced immunoglobulin and raised FLC concentrations are both associated with sepsis, we investigated whether their concomitant abnormality could enrich a sepsis population for IVIg trials (Table 1) (11, 12). Ideally, enrichment markers should forecast the likely patient outcome irrespective of baseline risk, and should also have biological plausibility (6, 11). Several observations highlight such plausibility for concomitant FLC and immunoglobulin abnormalities in sepsis, in addition to the known associations of FLCs described above (7-10). In health, excess light chains present in the perinuclear space to prevent intracellular heavy chain aggregation prior to immunoglobulin assembly, are secreted as FLC after immunoglobulin assembly (13). Thus, impaired immunoglobulin assembly in sepsis could result in raised FLC and low immunoglobulin levels (2, 3, 14). We also considered whether altered stoichiometry of light chain production could contribute to raised FLC concentrations. Rules of allelic exclusion permit only a single immunoglobulin specificity by each B cell. However, we recently reported that acute inflammation in autoimmune diseases can result in simultaneous expression of both κ and λ light chains by a single B cell.

Thus, light chain allelic inclusion in the context of acute inflammation may result in production of excess FLC (15). Against this background, after describing longitudinal changes in FLC and immunoglobulin levels in sepsis, we tested whether concomitant abnormalities in FLC and immunoglobulin levels on ICU admission day are independently associated with hospital mortality. We also examined surface κ and λ LC expression on B cells.

Materials and Methods

Study design and setting

This prospective observational cohort study was performed in an university hospital general medical-surgical ICU. Ethics committee approval was obtained prior to start of recruitment (10/H0807/81 and 12/LO/0326; Camberwell St Giles Committee, London, UK). Clinical management was at the discretion of the attending physician.

Study population

Consecutive patients with sepsis within the first 12 hours following ICU admission between 05/2011 and 01/2015 were included. Sepsis was defined using the old definition as evidence of two or more systemic inflammatory response syndrome (SIRS) criteria, with proven or suspected infection and at least one organ system dysfunction (cardiovascular, respiratory, renal, haematological or metabolic) (16). Exclusion criteria included patients <18 years, those with congenital hypo-gammaglobulinemia, known protein-losing enteropathies, nephrotic syndrome, neoplastic or proliferative hematological diseases; those having received IVIg therapy in the last 3 months; those receiving high-dose corticosteroid therapy; ongoing blood loss (defined by blood transfusion requirement > 2 units/24 hour period); retroviral disease; and immune dysfunction as defined by Acute Physiology and Chronic Health Evaluation (APACHE) II score co-morbidities (17). We have recently published further details on the B cell changes in sepsis from this population, using the same cohort (14).

Clinical data, blood sampling and flow cytometry

Baseline demographic data, infection details, and variables to record SIRS, APACHE II (17) and Sepsis-related Organ Failure Assessment (SOFA) scores (18) were collected. Blood samples were collected within 12 hours of ICU admission on admission day (D1), on day 3 (D3), and on day 7 (or on the day of demise or discharge if these events occurred earlier).

After clot separation and centrifugation (3000 rpm; 10 minutes; 4°C), serum samples were stored at -70°C. Blinded batch analysis was performed for immunoglobulin and FLC levels to reduce inter-assay variation. Immunoglobulin molecules (total IgG, IgA and IgM) (Dade Behring Nephelometer, Germany) and serum FLC using polyclonal sheep antisera (Freelite™, Binding Site, Birmingham, UK) were analysed on a SPA plus analyser (Binding Site). The free light chain ratio (FLCR) was derived as the ratio of FLCκ to FLCλ.
Flow cytometry was used to ascertain light chain allelic inclusion in circulating B cells using a Canto-A flow cytometer (Becton Dickinson (BD), Franklin Lakes, New Jersey). PBMCs were isolated within 4 hours of blood sampling using Ficoll density centrifugation. Anti-human antibodies, clone and company are reported in parenthesis. Anti-CD19 (PerCP Cy5.5; HB19; BD); anti-κ LC (ACP-H7; G20-193; BD); anti-λ LC (Pacific Blue; MHL-38; BioLegend) and Live/Dead Fixable Aqua (Life Technologies) antibodies were used for cell surface expression of CD19, LC-κ and LC-λ, respectively. Gating during analysis of flow cytometry standard (FCS) files were achieved using isotype controls, fluorescence minus-one controls, and/or single stain controls.

Derivation of abnormal categories

The reference normal ranges for an adult general population in the UK are IgG (6.1 – 16.2) g/l, IgA (0.8 – 5.0) g/l, IgM (0.4 – 2.4) g/l, FLCλ (5.7 – 26.3) mg/l, FLCκ (3.3 – 19.4) mg/l and FLCR (0.26 – 1.65). The upper and lower limits of these normal ranges were used to define normal and abnormal categories. These categorizations allowed generation of prevalence data, and comparison of the proportions of survivor and non-survivor sepsis patients with each abnormality.

Sample size estimation

Sample size was estimated using precision estimates for IgG distribution (19), as IgG is the most often altered immunoglobulin in sepsis (6). One hundred patients were required to achieve a margin of error of 0.74 g/L of IgG measurement based on the population standard deviation of 3.72 g/L observed in the first 30 patients enrolled.

Statistics

Continuous data are presented as mean and standard deviation (SD) when normally distributed, and as median and interquartile range (IQR) when not. Frequency and percentages are presented for categorical data.

Two sets of comparisons described longitudinal changes in FLC. First, we compared the proportions of high FLCs and the concentrations of FLCs between survivors and non-survivors on days 1, 3 and 7. Second, we compared how the proportions of high FLCs and the temporal change in concentration varied between in survivors and non-survivors using paired tests. A similar approach was used to describe changes in immunoglobulins.

To report univariate associations and to inform logistic regression models, a pairwise correlation matrix was generated for admission day FLC, FLCR, IgG, IgA, IgM, white cell count, C-reactive protein (CRP), total SOFA and APACHE II score. Bonferroni corrections were performed for multiple comparisons. Two analyses were performed to assess the association between a concomitant admission day immunoglobulin and FLC abnormality and mortality. First, stratified analyses were performed as shown in Table 1. Second, two logistic regression models were used with acute hospital mortality as the outcome: model 1 had high-FLCλ, total APACHE II score, hypo-IgG, and hypo-IgM as binary covariates, while model 2 had high-FLCκ, total APACHE II score, hypo-IgG, and hypo-IgM as binary covariates.

B cell subsets are reported as proportion of the live CD19 positive B cell population. Proportions were compared between health and sepsis. All analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, www.graphpad.com) and/or Stata/SE Version 13.0 (StataCorp LP, College Station, TX). Reported p values are two sided with p values <0.05 representing a statistically significant result.

Results

Study cohort

The cohort characteristics by hospital survival status is summarized in Table 2. Acute hospital mortality was 26.7%. Non-survivors were older and had greater illness severity as evidenced by greater APACHE II and SOFA scores. The respiratory tract was the most common infection site in both survivors and non-survivors. In two patients, the site of infection could

not be confirmed. On ICU Day 1, the combination of high FLC and low immunoglobulins was more prevalent in non-survivors (Table 2).

Longitudinal changes in FLCκ and FLCλ

The prevalence of high FLCκ was similar in survivors and non-survivors on D1 (p=0.45); D3 (p=0.18) and DF (p=0.75). No significant differences were seen in the corresponding FLCκ concentrations at any time point (Figure 1a). The proportion of sepsis patients with abnormal FLCλ was significantly higher in non-survivors only on D1 (74.1% vs. 36.5%; p=0.001) and not on D3 (p=0.24) and DF (p=0.11). There was a statistically significant increase in corresponding FLCλ concentrations over time in both survivors and non-survivors. However, when comparing FLCλ concentrations on D1, 3 and 7 between survivors and non-survivors, there were no differences at any time point (Figure 1b).

Longitudinal changes in FLCR

Despite recruiting septic patients without any documented immune comorbidity, 29.7% of this cohort had a high FLCR. The prevalence was significantly greater in survivors on D1 (36.5% vs 11.1%, p=0.01). The high FLCR decreased significantly on D3 compared to D1 in both survivors and non-survivors (Figure 1c). Analyses restricted to patients with a normal FLCR showed that the proportion of patients with high FLCλ was significantly more common in non-survivors on D1 (75.0% vs. 40.4%; p=0.007) and high-FLCκ similar between survivors and non-survivors (63.8% vs. 83.3%; p=0.11).

Longitudinal changes in immunoglobulin subtypes

Although, hypo-IgG was the most prevalent immunoglobulin abnormality, there was no statistically significant difference in hypo-IgG by hospital survival status D1 (p=0.22); D3 (p=0.92) and DF (p=0.07). The nadir for IgG was on D3 and the non-survivors had a statistically significant drop by D3 compared to D1, which was 8 times that seen in survivors (median (IQR) in survivors was -0.121 (-1.28; 0.87) and in non-survivors was -0.80 (-1.28; -0.3); p= 0.02). Compared to Day 1 IgG concentrations, Day 7 IgG concentrations rose only in survivors (p=0.004; Figure 1d). Similar to IgG concentrations, there were no difference in hypo-IgM by hospital survival status at any time point and the nadir for IgM was on D3, (p=0.02 in non-survivors; Figure 1e). Hypo-IgA was the least prevalent (6.9%) and observed in only 7 survivors (Figure 1f).

Associations of FLC and immunoglobulin

FLCκ was significantly correlated with FLCλ, FLCR, IgG and serum creatinine concentrations. FLCλ was significantly correlated with FLCκ, IgG, IgA and serum creatinine. IgG and IgM were significantly correlated. FLCs were not correlated to markers of illness severity (APACHE II and SOFA) and to acute inflammation (CRP and WBC) (eFigure 1).

High FLCλ is associated with mortality

Using the concept presented in Table 1, the stratified analyses highlighted higher mortality only in patients with high FLCλ, irrespective of hypo-IgG or hypo-IgM status (Figure 2). In our logistic regression models to assess any independent association with mortality and high FLC status, hypo-IgA was not included as it occurred only in survivors on Day 1. APACHE II score accounted for illness severity and differences in age between survivors and non-survivors reported in Table 2. In *model 1*, hospital mortality was significantly associated with the APACHE II score and high FLCλ. The increase in OR (95% CI) for hospital mortality per unit increase in APACHE II score was 1.2 (1.1 – 1.4; p<0.001) and, for high FLCλ, 4.0 (1.3–12.4); p=0.017). In *model 2*, hospital mortality was significantly associated only with the APACHE II score; the OR (95% CI) per unit increase in APACHE II score of 1.2 (1.1–1.4; p<0.001) (eFigure2).

Allelic inclusion in B cells and associations

The gating strategy for identification of light chain allelic inclusion is shown in eFIgure3. The characteristics of the 21 sepsis patients' samples used for flow cytometry are summarized in eTable1. The CD19 B cells expressing cell surface LCκ or LCλ, as a proportion of alive CD19 population and as a ratio of CD19κ:CD19λ were similar in sepsis compared against healthy subjects. The septic patients had a significantly higher proportion of CD19 B cells expressing both LCκ and LCλ ($CD19^{κλ}$) than B cells taken from healthy subjects (median (IQR) 4.1% (2.4, 11.0) vs. 1.3% (1.2, 2.9) respectively; p=0.0001; Figure3a). There was a statistically significant correlation between the frequencies of these $CD19^{κλ}$ cells and the corresponding FLC concentrations (Figure 3b and 3c). Statistically significant correlation was only observed between FLCλ and CD19 B cells expressing cell surface LCλ (Figure 3d and 3e).

Discussion

High FLCκ, high FLCλ and high FLCR are common in patients with sepsis (without known immune comorbidities) admitted to critical care. The presence of a high FLCλ increases the risk of death, even after adjusting for APACHE II score, despite high FLCκ being the more common abnormality. The combination of high FLC and low immunoglobulins on ICU admission day was more prevalent in greater proportion of non-survivors. Consistent with the published literature(6), we too found that hypo-IgG and hypo-IgM were common in sepsis on D1, reaching a nadir on D3, and that more survivors incremented immunoglobulin levels over time. These findings increase the external validity of our results.

The approach presented in Table-1 is discussed in the context of our results to inform trial design. IVIg treatment potentially restores normal serum immunoglobulin concentrations in patients with low IgG levels, in addition to contributing to immunomodulation (3). In clinical trials, the IVIg treatment effect will vary by baseline risk of death, referred to as heterogeneity in treatment effect(20, 21). Thus, enriching sepsis patients with a higher risk of death concomitant with a greater likelihood of treatment response inferred by low immunoglobulins on admission may be more likely to result in successful trials(11, 12). Biomarker based stratification is a valid approach to achieve this. In this study, we position FLCλ as a potential biomarker within the causal pathway from immunoglobulin alterations to death in sepsis patients, after accounting for illness severity. We show that high FLCλ identifies a high risk of death sepsis population. The prevalence of high FLCλ within a sepsis cohort was 46.5%, with 74% of non-survivors having this abnormality. These concepts could be easily validated using data from ongoing trials (5).

We observed major abnormalities in immunoglobulin levels without an increase in risk of death; this finding is consistent with our previous systematic review (6). Dissecting the relative contributions of altered immunoglobulin production, impaired endothelial recycling, and immunoglobulin consumption for opsonization during sepsis is challenging due to the lack of a well characterized regulatory loop for immunoglobulin homeostasis (22). There are no mechanistic studies explaining immunoglobulin kinetics in sepsis. The first novel finding is that FLCs are raised and high FLCλ was associated with increased risk of hospital mortality in sepsis patients after accounting for illness severity. The kidneys maintain circulating FLC

homeostasis via glomerular clearance, proximal tubule endocytosis and catabolism (23). Impairments in renal function seen in sepsis could increase FLC concentrations, which can be inferred from the positive correlation found between FLC and serum creatinine. The increase in FLC in sepsis could also be due to impaired immunoglobulin assembly. The only other study that reports FLC in sepsis is a pilot of 21 patients study with pneumonia and septic shock, which compared the FLC concentrations between low and normal IgG status to show that the FLC concentrations did not differ by IgG concentrations (10). This study neither reported longitudinal changes in FLC by survival status nor studied mechanisms. The FLCR was similar to our cohort, providing further external validity to our results (10). Thus, important unanswered questions based on our data are the mechanisms by which FLC concentrations increase in sepsis and how this increase affect sepsis biology in terms of illness severity or outcome. The increase proportion of $CD19^{\kappa\lambda}$ dual cells in sepsis and their association with raised FLC concentrations is another novel finding in our study, that requires further validation in sepsis cohorts. In healthy adults, $CD19^{\kappa\lambda}$ dual cells represent only 0.5% of circulating B cells (24); the rise seen in our sepsis cohort could be a random simultaneous rearrangement of both light chain loci, but is most likely to represent allelic inclusion due to impaired kappa deleting element (KDE) activity or a transient phase during light chain revision (24).

There are several limitations to our study. We have not investigated mechanisms behind these novel findings. We cannot ascertain whether the FLC abnormalities were present before the onset of sepsis nor whether FLCs represent a risk factor for sepsis, or mortality irrespective of sepsis (8). We accounted for these challenges by studying patients without immune comorbidity and by showing the independent effect of high FLCλ on hospital mortality after adjusting for APACHE II score. These novel observations should be replicated in other sepsis cohorts.

As FLC concentrations are linked to immunoglobulin assembly in the literature, our findings imply that this FLC-immunoglobulin relationship makes FLC a potential biomarker for simultaneous prognostic and predictive enrichment of sepsis patients for IVIg therapy trials (11). This hypothesis could be confirmed using samples from existing trial data (5). Further questions raised include the contribution of elevated FLC to renal dysfunction in sepsis, as observed in myeloma patients(23). Raised FLC levels could also contribute to the prolonged

neutrophil life span in sepsis (25), acting through enhanced tyrosine phosphorylation (26). The mechanisms involved in B-cell allelic inclusion in sepsis needs exploring.

Conclusions

In a well-characterized cohort of critically ill adults with sepsis without any immune comorbidity, we report a high prevalence of FLC abnormalities, which were associated with immunoglobulin subtype abnormalities and had prognostic utility. We also show evidence of light chain allelic inclusion in B cells in sepsis. Their roles in sepsis pathology and as a putative biomarker for IVIg therapy warrants further study.

Acknowledgements

Manu Shankar-Hari is supported by the UK National Institute for Health Research Clinician Scientist Award (NIHR-CS-2016-16-011). Mervyn Singer is supported by the UK National Institute for Health Research Senior Investigator Award. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the NIHR, or the UK Department of Health.

References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016;315(8):801-810.

2. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol 2013;13(12):862-874.

3. Shankar-Hari M, Spencer J, Sewell WA, et al. Bench-to-bedside review: Immunoglobulin therapy for sepsis - biological plausibility from a critical care perspective. Crit Care 2012;16(2):206.

4. Marshall JC. Why have clinical trials in sepsis failed? Trends Mol Med 2014;20(4):195-203.

5. Welte T, Dellinger RP, Ebelt H, et al. Concept for a study design in patients with severe community-acquired pneumonia: A randomised controlled trial with a novel IGM-enriched immunoglobulin preparation - The CIGMA study. Respir Med 2015;109(6):758-767.

6. Shankar-Hari M, Culshaw N, Post B, et al. Endogenous IgG hypogammaglobulinaemia in critically ill adults with sepsis: systematic review and meta-analysis. Intensive Care Med 2015;41(8):1393-1401.

7. Dispenzieri A, Katzmann JA, Kyle RA, et al. Use of nonclonal serum immunoglobulin free light chains to predict overall survival in the general population. Mayo Clin Proc 2012;87(6):517-523.

8. Anandram S, Assi LK, Lovatt T, et al. Elevated, combined serum free light chain levels and increased mortality: a 5-year follow-up, UK study. J Clin Pathol 2012;65(11):1036-1042.

9. Bradwell AR. Serum free light chain measurements move to center stage. Clin Chem 2005;51(5):805-807.

10. Taccone FS, Stordeur P, De Backer D, et al. Gamma-globulin levels in patients with community-acquired septic shock. Shock 2009;32(4):379-385.

11. Shankar-Hari M, Rubenfeld GD. The use of enrichment to reduce statistically indeterminate or negative trials in critical care. Anaesthesia 2017;72(5):560-565.

12. Prescott HC, Calfee CS, Thompson BT, et al. Toward Smarter Lumping and Smarter Splitting: Rethinking Strategies for Sepsis and Acute Respiratory Distress Syndrome Clinical Trial Design. Am J Respir Crit Care Med 2016;194(2):147-155.

13. Nakano T, Matsui M, Inoue I, et al. Free immunoglobulin light chain: its biology and implications in diseases. Clin Chim Acta 2011;412(11-12):843-849.

14. Shankar-Hari M, Fear D, Lavender P, et al. Activation-Associated Accelerated Apoptosis of Memory B Cells in Critically Ill Patients With Sepsis. Crit Care Med 2017;45(5):875-882.

15. Fraser LD, Zhao Y, Lutalo PM, et al. Immunoglobulin light chain allelic inclusion in systemic lupus erythematosus. Eur J Immunol 2015;45(8):2409-2419.

16. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med 2003;31(4):1250-1256.

17. Knaus WA, Draper EA, Wagner DP, et al. APACHE II: a severity of disease classification system. Crit Care Med 1985;13(10):818-829.
18. Vincent JL, Moreno R, Takala J, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 1996;22(7):707-710.
19. Arnold DM, Burns KE, Adhikari NK, et al. The design and interpretation of pilot trials in clinical research in critical care. Crit Care Med 2009;37(1 Suppl):S69-74.
20. Iwashyna TJ, Burke JF, Sussman JB, et al. Implications of Heterogeneity of Treatment Effect for Reporting and Analysis of Randomized Trials in Critical Care. Am J Respir Crit Care Med 2015;192(9):1045-1051.
21. Welton NJ, Soares MO, Palmer S, et al. Accounting for Heterogeneity in Relative Treatment Effects for Use in Cost-Effectiveness Models and Value-of-Information Analyses. Med Decis Making 2015;35(5):608-621.
22. Junghans RP. IgG Biosynthesis: No “Immunoregulatory Feedback”. Blood 1997;90(10):3815-3818.
23. Basnayake K, Stringer SJ, Hutchison CA, et al. The biology of immunoglobulin free light chains and kidney injury. Kidney Int 2011;79(12):1289-1301.
24. Giachino C, Padovan E, Lanzavecchia A. kappa+lambda+ dual receptor B cells are present in the human peripheral repertoire. J Exp Med 1995;181(3):1245-1250.
25. Taneja R, Parodo J, Jia SH, et al. Delayed neutrophil apoptosis in sepsis is associated with maintenance of mitochondrial transmembrane potential and reduced caspase-9 activity. Crit Care Med 2004;32(7):1460-1469.
26. Cohen G, Rudnicki M, Deicher R, et al. Immunoglobulin light chains modulate polymorphonuclear leucocyte apoptosis. Eur J Clin Invest 2003;33(8):669-676.
27. Lee WL, Slutsky AS. Sepsis and endothelial permeability. N Engl J Med 2010;363(7):689-691.

Figure and Table Legends

Table legends

Table 1: Potential relationships between immunoglobulins and free light chains abnormalities in sepsis patients

We have considered the observable scenarios depicting acute changes in FLC and immunoglobulin concentrations, their possible underlying biological explanations and FLC as a surrogate marker linked to immunoglobulin biology with potential to enrich or stratify sepsis patients (11) in intravenous immunoglobulin trials. The rationale for this approach and the hypothetical scenarios presented here is that FLC levels are considered a valid surrogate marker of immunoglobulin production and potentially related to outcome, which places FLC as a biomarker in the causal chain between sepsis, immunoglobulins and death. We tested four possible combinations of two variables FLC and immunoglobulins, categorised into normal and abnormal based on their normal clinical laboratory range. Low immunoglobulins in sepsis could represent excess immunoglobulin consumption or extravasation of immunoglobulins(27) or impaired IgG recycling(3), or a normal immunoglobulin consumption but with impaired immunoglobulin assembly due to B cell abnormalities in sepsis(14). *High* FLC in sepsis could be related to either excess production related to sepsis related inflammation or due to allelic inclusion of light chains in B cells(13, 15). Enrichment principles are further explored in these reviews (11, 12). Abbreviations: FLC – Free light chains; IVIg – intravenous immunoglobulins

Table 2: Cohort characteristics

Table shows the salient sepsis specific and generic case mix of study cohort. [1]continuous data presented as mean and standard deviation when normally distributed; [2]Categorical data-presented as number (proportion); [3]continuous data presented as median and inter quartile range when not normally distributed. APACHE – Acute physiology and chronic health evaluation; WCC – white cell count; CRP – C-reactive protein; SOFA- Sequential organ failure assessment; LOS – Length of stay; FLC = free light chains; FLCR – Free light chain kappa/lambda ratio. IgG – immunoglobulin G; IgM – immunoglobulin M; IgA – immunoglobulin The admission day concentrations of immunoglobulins, FLC and FLCR are summarized

Table-3: Prevalence of different scenarios by survival status in the overall cohort

Normal/raised FLC and normal/low immunoglobulin categories were derived as described under *Derivation of abnormal categories* section. Then for each of the scenarios described in table-1 the proportion of patients meeting each scenario within survivors and non-survivors were estimated. These analyses were performed as the prevalence of each scenario and their potential association to survival status will inform future trial design. As our study was powered using precision sample size estimation of Immunoglobulin G concentrations, no formal statistical tests were done for comparisons between groups. There were 27 non-survivors in this cohort. Abbreviations: IgG – immunoglobulin G; IgM – immunoglobulin M; FLCκ = free light chain kappa; FLCλ = free light chain lambda

Figure legends

Figure 1: Changes in FLC and Immunoglobulins over time by survival status

Scatter plots showing all data points and median (IQR). D1= ICU admission day, D3= third day following admission and DF=Final sampling day. The shaded regions in 1a, Ib and 1c represents high-FLC category and in 1d, 1e and 1f represent hypo-Ig category. Comparisons by survival status were done using t-tests and within survivors and non-survivors were done using paired t-tests or non-parametric equivalent. **[1a]** FLCκ showed no significant differences in high-FLCκ prevalence and in FLCκ concentrations over time. **[1b]** FLCλ showed significant differences in high-FLCλ prevalence between survivors and non-survivors on D1 (p=0.001). The FLCλ concentrations increased significantly over time in survivors and non-survivors. **[1c]** FLCR showed significant differences in high-FLCR prevalence between survivors and non-survivors on D1 (p=0.01); D3 (p=0.51) and DF (p=0.34) and that there was a significant drop in FLCR between D1 and D3 in both survivors and non-survivors. **[1d]** IgG showed no significant differences in hypo-IgG prevalence between survivors and non-survivors on D1 (p=0.22); D3 (p=0.92) and DF (p=0.07). In survivors D1 vs. D3 was not significant (p=0.57). In non-survivors D1 vs. DF (p=0.82) and D3 vs. DF (p=0.44) were not significant. **[1e]** IgM showed no significant differences in hypo-IgM prevalence between survivors and non-survivors on D1 (p=0.63); D3 (p=0.62) and DF (p=0.49). The survivors had a higher IgM by the DF when compared to D1 and D3 levels. The D3 IgM concentrations were significantly lower than D1 only in non-survivors (p=0.02) vs. p=0.43 in survivors. **[1f]** On D1, seven

survivors had hypo-IgA. The IgA concentrations were similar in survivors and non-survivors on D1, D3 and DF. In survivors D1 vs. D3 was not significant p=0.50. In non-survivors D1 vs. D3 (p=0.28), D1 vs. DF (p=0.82) and D3 vs. DF (p=0.57) were not significant.

Figure 2: Stratified analyses of mortality by high-FLC and immunoglobulin status

The prevalence of FLC and immunoglobulin abnormality and their association to hospital mortality are reported. These analyses show outcomes in the scenarios A = Normal FLC + Normal immunoglobulin; B = Normal FLC + Low immunoglobulins; C = High-FLC + Normal immunoglobulin and D = High-FLC + Low immunoglobulin

Figure 3: Allelic inclusion in B cells in sepsis

SS – Sepsis; H – Healthy; κ– Igκ on CD19 cells; λ– Igλ on CD19 cells; $CD19^{κλ}$– CD19 expressing both Igκ and Igλ on cell surface. **[3a]** Scatter plot showing all data points, the mean and 95% confidence intervals. Mann-Whitney U tests were used to generate p values by comparing sepsis (n=21) and healthy samples (n=15). The proportions of $CD19^{κλ}$ expressing B cells were seen significantly more common in sepsis patients. [**3b and 3c]** Statistically significant positive correlation was seen between the proportion of $CD19^{κλ}$ dual B cells in sepsis patients and the corresponding admission day FLCλ and FLCκ concentrations. [**3d]** Statistically significant positive correlation was seen between the proportion of $CD19^{λ}$ B cells in sepsis patients and the corresponding admission day FLCλ concentrations. **[3f]** There was no correlation between the proportion of $CD19^{κ}$ B cells and the corresponding FLCκ concentrations. We used linear regression to derive R^2 when there was significant correlation.

Table-1: Hypothetical relationships between measured concentrations of immunoglobulins and free light chains and enrichment for trial design

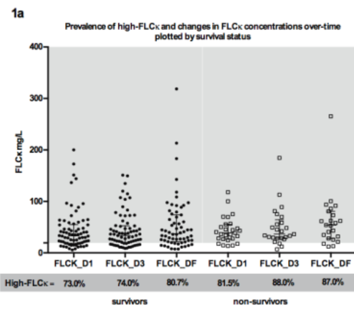
Abnormality	Normal immunoglobulin	Low Immunoglobulin
Normal FLC	***Scenario A*** Production and consumption of immunoglobulin light and heavy chains in balanced equilibrium	***Scenario B*** Either immunoglobulin consumption or loss is higher than assembly
Raised FLC	***Scenario C*** Effective immunoglobulin assembly meeting the consumption and loss demands seen in sepsis but there is excess light chain production n	***Scenario D*** Rapid immunoglobulin consumption or excessive loss with ineffective immunoglobulin assembly and large excess of light chain production

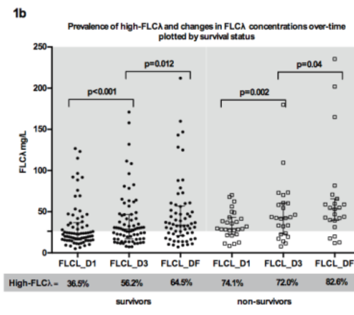
Table-2: Admission day cohort characteristics

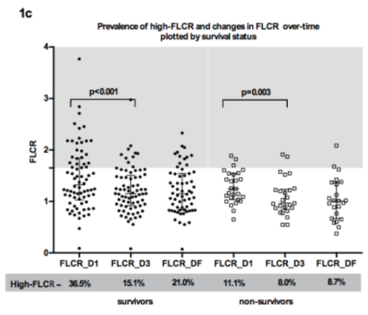
Variable	Survivors (N=74)	Non-survivors (N=27)
[1]Age years	62.3 (12.6)	71.9 (14.1)
[2]Female n (%)	26 (35.1%)	12 (44.4%)
[1]APACHE II Score	18.4 (4.9)	24.0 (5.6)
[2]Infection site n (%) Respiratory Intra-abdominal Wound and Soft tissue Urosepsis Meningitis Osteomyelitis Unknown	 46 (62.2%) 12 (16.2%) 7 (9.5%) 6 (8.1%) 1 1 1	 19 (70.4%) 3 (11.1%) 3 (11.1%) 1 (3.7%) - - 1
[3]WCC count	14.6 (9.9, 19.0)	13.6 (10.3, 21.8)
[3]CRP	148 (74, 278)	131 (93, 248)
[3]Total SOFA score	7 (5, 9)	8 (6, 11)
[3]Immunoglobulin concentrations IgG g/l IgA g/l IgM g/l	 7.2 (4.7 – 10.2) 2.3 (1.5 – 3.0) 0.6 (0.4 – 0.8)	 8.0 (5.4 – 10.5) 2.2 (1.6 – 3.2) 0.7 (0.4 - 1.2)
[3]Free light chain concentrations FLC-Lambda mg/l FLC-Kappa mg/l FLC Kappa/Lambda Ratio	 23.2 (15.8 – 36.3) 33.2 (18.6 – 54.5) 1.3 (1.0 – 1.8)	 29.6 (22.5 – 43.1) 38.8 (26.8 – 55.5) 1.2 (1.0 – 1.5)
[3]ICU length of stay	7 (3,12)	12 (6,18)
[3]Hospital length of stay	23 (14, 41)	13 (7, 18)

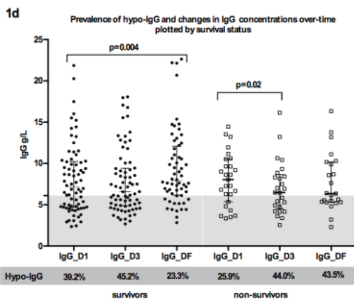
Table-3: Prevalence of different scenarios by survival status in the overall cohort

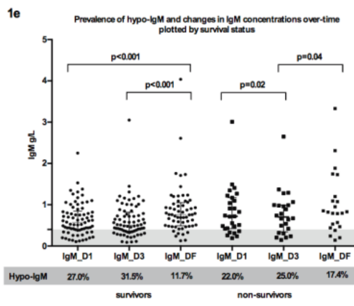
Parameter		Normal IgG (N=65) Survivors vs non-survivors Reported as %	Low IgG (N=36) Survivors vs non-survivors Reported as %	Normal IgM (N=75) Survivors vs non-survivors Reported as %	Low IgM (N=26) Survivors vs non-survivors Reported as %
FLCλ category	Normal FLCλ (N=54)	31.1% vs 14.8%	32.4% vs 11.1%	44.6% vs 18.5%	18.9% vs 7.4%
	High-FLCλ (N=47)	29.7% vs 59.3%	6.8% vs 14.8%	28.4% vs 59.3%	8.1% vs 14.8%
FLCκ category	Normal FLCκ (N=25)	12.2% vs 7.4%	14.9% vs 11.1%	14.8% vs 11.1%	11.1% vs 12.2%
	High-FLCκ (N=76)	48.7% vs 66.7%	24.3% vs 14.8%	58.1% vs 66.7%	14.8% vs 14.9%

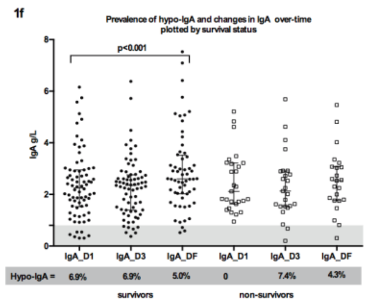

1a
Prevalence of high-FLCκ and changes in FLCκ concentrations over-time
plotted by survival status
FLCκ mg/L
400
300
200
100
0
FLCK_D1
FLCK_D3
FLCK_DF
FLCK_D1
FLCK_D3
FLCK_DF
High-FLCκ = 73.0% 74.0% 80.7% 81.5% 88.0% 87.0%
survivors
non-survivors

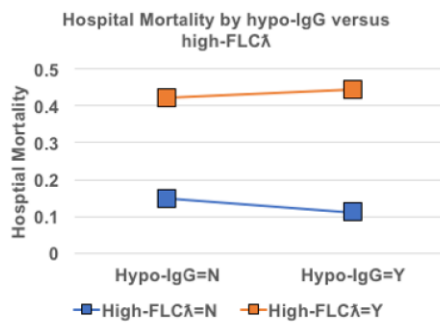

1b
Prevalence of high-FLCλ and changes in FLCλ concentrations over-time
plotted by survival status
FLCλ mg/L
250
200
150
100
50
0
p<0.001
p=0.012
p=0.002
p=0.04
FLCL_D1
FLCL_D3
FLCL_DF
FLCL_D1
FLCL_D3
FLCL_DF
High-FLCλ = 36.5% 56.2% 64.5% 74.1% 72.0% 82.6%
survivors
non-survivors

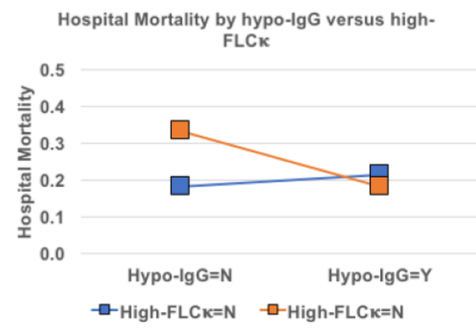

1c
Prevalence of high-FLCR and changes in FLCR over-time
plotted by survival status
FLCR
4
3
2
1
0
p<0.001
p=0.003
FLCR_D1
FLCR_D3
FLCR_DF
FLCR_D1
FLCR_D3
FLCR_DF
High-FLCR = 36.5% 15.1% 21.0% 11.1% 8.0% 8.7%
survivors
non-survivors

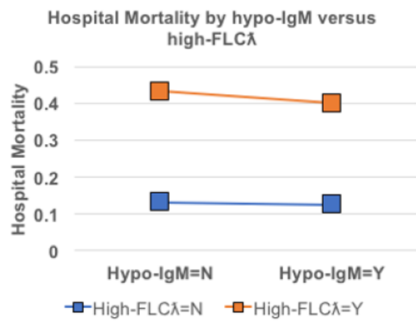

1d
Prevalence of hypo-IgG and changes in IgG concentrations over-time
plotted by survival status
IgG g/L
25
20
15
10
5
0
p=0.004
p=0.02
IgG_D1
IgG_D3
IgG_DF
IgG_D1
IgG_D3
IgG_DF
Hypo-IgG 39.2% 45.2% 23.3% 25.9% 44.0% 43.5%
survivors
non-survivors

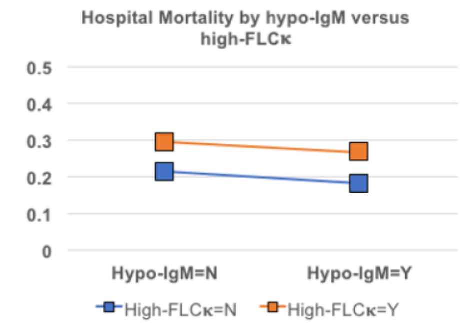

1e
Prevalence of hypo-IgM and changes in IgM concentrations over-time
plotted by survival status
IgM g/L
5
4
3
2
1
0
p<0.001
p<0.001
p=0.02
p=0.04
IgM_D1
IgM_D3
IgM_DF
IgM_D1
IgM_D3
IgM_DF
Hypo-IgM 27.0% 31.5% 11.7% 22.0% 25.0% 17.4%
survivors
non-survivors

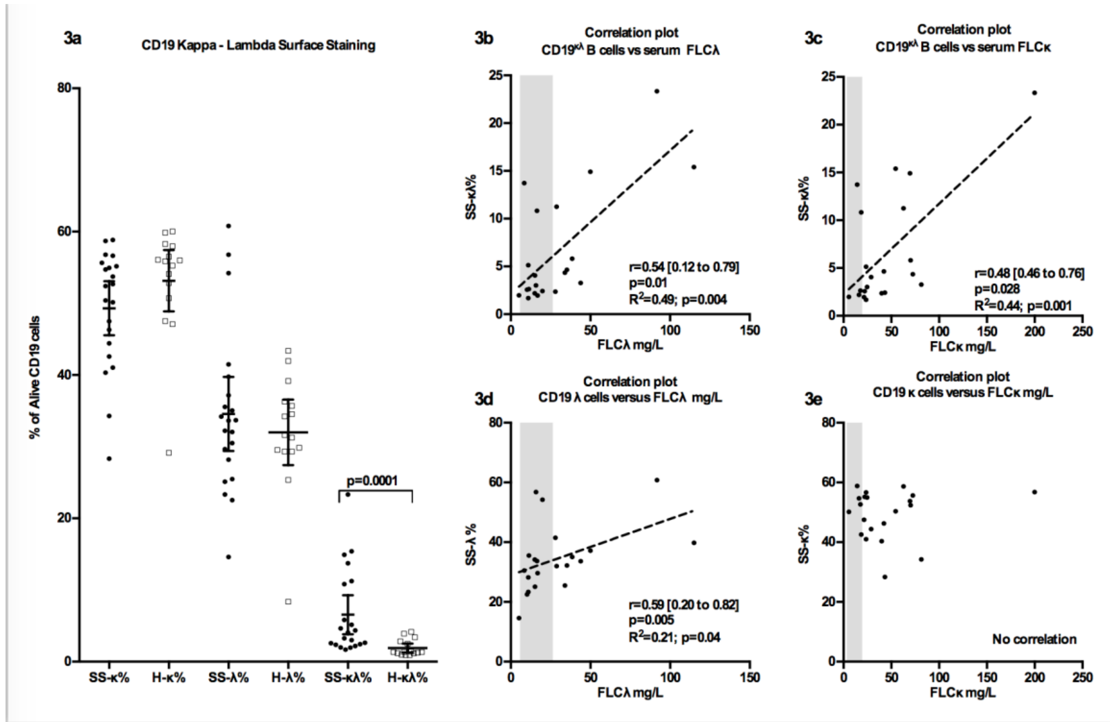

1f
Prevalence of hypo-IgA and changes in IgA over-time
plotted by survival status
IgA g/L
8
6
4
2
0
p<0.001
IgA_D1
IgA_D3
IgA_DF
IgA_D1
IgA_D3
IgA_DF
Hypo-IgA = 6.9% 6.9% 5.0% 0 7.4% 4.3%
survivors
non-survivors


Hospital Mortality by hypo-IgG versus high-FLCλ
Hospital Mortality
0.5
0.4
0.3
0.2
0.1
0
Hypo-IgG=N
Hypo-IgG=Y
High-FLCλ=N
High-FLCλ=Y


Hospital Mortality by hypo-IgG versus high-FLCκ
Hospital Mortality
0.5
0.4
0.3
0.2
0.1
0.0
Hypo-IgG=N
Hypo-IgG=Y
High-FLCκ=N
High-FLCκ=N


Hospital Mortality by hypo-IgM versus high-FLCλ
Hospital Mortality
0.5
0.4
0.3
0.2
0.1
0
Hypo-IgM=N
Hypo-IgM=Y
High-FLCλ=N
High-FLCλ=Y


Hospital Mortality by hypo-IgM versus high-FLCκ
0.5
0.4
0.3
0.2
0.1
0
Hypo-IgM=N
Hypo-IgM=Y
High-FLCκ=N
High-FLCκ=Y


3a
CD19 Kappa - Lambda Surface Staining
% of Alive CD19 cells
80
60
40
20
0
SS-κ%
H-κ%
SS-λ%
H-λ%
SS-κλ%
H-κλ%
p=0.0001
3b
Correlation plot
CD19κλ B cells vs serum FLCλ
SS-κλ%
25
20
15
10
5
0
0
50
100
150
FLCλ mg/L
r=0.54 [0.12 to 0.79]
p=0.01
R2=0.49; p=0.004
3c
Correlation plot
CD19κλ B cells vs serum FLCκ
SS-κλ%
25
20
15
10
5
0
0
50
100
150
200
250
FLCκ mg/L
r=0.48 [0.46 to 0.76]
p=0.028
R2=0.44; p=0.001
3d
Correlation plot
CD19 λ cells versus FLCλ mg/L
SS-λ %
80
60
40
20
0
0
50
100
150
FLCλ mg/L
r=0.59 [0.20 to 0.82]
p=0.005
R2=0.21; p=0.04
3e
Correlation plot
CD19 κ cells versus FLCκ mg/L
SS-κ%
80
60
40
20
0
0
50
100
150
200
250
FLCκ mg/L
No correlation