Viral RNA load in plasma is associated with critical illness and a dysregulated host response in COVID-19.

Jesús F Bermejo-Martin1,2,3*, Milagros González-Rivera4,5*, Raquel Almansa1,2,3*, Dariela Micheloud6*, Ana P. Tedim1,2*, Marta Domínguez-Gil7*, Salvador Resino8*, Marta Martín-Fernández1,2, Pablo Ryan Murua9, Felipe Pérez-García10, Luis Tamayo1,11, Raúl Lopez-Izquierdo12, Elena Bustamante13, César Aldecoa1,14,15, José Manuel Gómez16, Jesús Rico-Feijoo1,15, Antonio Orduña17, Raúl Méndez18, Isabel Fernández Natal19, Gregoria Megías20, Montserrat González-Estecha4,5, Demetrio Carriedo21, Cristina Doncel1,2,3, Noelia Jorge1,2,3, Alicia Ortega1,2,3, Amanda de la Fuente1,2,3, Félix del Campo22, José Antonio Fernández-Ratero23, Wysali Trapiello24, Paula González-Jiménez18, Guadalupe Ruiz24, Alyson A. Kelvin25,26, Ali Toloue Ostadgavahi25,26, Ruth Oneizat7, Luz María Ruiz7, Iria Miguéns6, Esther Gargallo6, Ioana Muñoz6, Sara Pelegrín15, Silvia Martín1,15, Pablo García Olivares16, Jamil Antonio Cedeño16, Tomás Ruiz Albi22, Carolina Puertas4, Jose Ángel Berezo1,11, Gloria Renedo13, Rubén Herrán1,11, Juan Bustamante-Munguira27, Pedro Enríquez11, Ramón Cicuendez13, Jesús Blanco11, Jessica Abadia28, Julia Gómez Barquero28, Nuria Mamolar13, Natalia Blanca-López9, Luis Jorge Valdivia21, Belén Fernández Caso19, María Ángeles Mantecón20, Anna Motos3,29, Laia Fernandez-Barat3,29, Ricard Ferrer3,30, Ferrán Barbé3,31, Antoni Torres3,29Δ, Rosario Menéndez3,18Δ, José María Eiros7Δ, David J KelvinΔ25,26.

* These authors contributed equally

Δ These authors contributed equally

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1. Group for Biomedical Research in Sepsis (BioSepsis). Instituto de Investigación Biomédica de Salamanca, (IBSAL), Paseo de San Vicente, 58-182, 37007 Salamanca, Spain

2. Hospital Universitario Río Hortega, Calle Dulzaina, 2, 47012 Valladolid, Spain.

3. Centro de Investigación Biomédica en Red en Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Av. de Monforte de Lemos, 3-5, 28029 Madrid, Spain

4. Department of Laboratory Medicine, Hospital General Universitario Gregorio Marañón, Calle del Dr. Esquerdo, 46, 28007 Madrid, Spain.

5. Department of Medicine, Faculty of Medicine, Universidad Complutense de Madrid, Plaza de Ramón y Cajal, s/n, 28040 Madrid, Spain.

6. Emergency Department, Hospital General Universitario Gregorio Marañón, Calle del Dr. Esquerdo, 46, 28007 Madrid, Spain.

7. Microbiology Service, Hospital Universitario Río Hortega, Calle Dulzaina, 2, 47012 Valladolid, Spain

8. Viral Infection and Immunity Unit, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. de Pozuelo, 28, 28222 Majadahonda, Spain

9. Hospital Universitario Infanta Leonor, Av. Gran Vía del Este, 80, 28031 Madrid, Spain

10. Servicio de Microbiología Clínica, Hospital Universitario Príncipe de Asturias, Carr. de Alcalá, s/n, 28805, Madrid, Spain

11. Intensive Care Unit, Hospital Universitario Río Hortega, Calle Dulzaina, 2, 47012 Valladolid, Spain.
44 12. Emergency Department, Hospital Universitario Rio Hortega, Calle Dulzaina, 2, 47012 Valladolid, Spain.

45 13. Intensive Care Unit, Hospital Clínico Universitario de Valladolid. Av. Ramón y Cajal, 3, 47003 Valladolid, Spain.

46 14. Department of Anesthesiology, Facultad de Medicina de Valladolid, Av. Ramón y Cajal, 7, 47005 Valladolid, Spain.

47 15. Anesthesiology and Reanimation Service, Hospital Universitario Rio Hortega, Calle Dulzaina, 2, 47012 Valladolid, Spain.

48 16. Intensive Care Unit. Hospital General Universitario Gregorio Marañón. Calle del Dr. Esquerdo, 46, 28007 Madrid, Spain.

49 17. Microbiology Service, Hospital Clinico Universitario de Valladolid, Av. Ramón y Cajal, 3, 47003 Valladolid, Spain.

50 18. Pulmonology Service, Hospital Universitario y Politécnico de La Fe, Avinguda de Fernando Abril Martorell, 106, 46026, Valencia Spain.

51 19. Clinical Microbiology Department. Complejo Asistencial Universitario de León. Calle Altos de nava, s/n, 24001 León, Spain.

52 20. Microbiology Service, Hospital Universitario de Burgos, Av. Islas Baleares, 3, 09006 Burgos, Spain.

53 21. Intensive Care Unit. Complejo Asistencial Universitario de León. Calle Altos de nava, s/n, 24001 León, Spain.

54 22. Pneumology Service, Hospital Universitario Rio Hortega / Biomedical Engineering Group, Universidad de Valladolid, Calle Dulzaina, 2, 47012 Valladolid, Spain.
23. Intensive Care Unit. Hospital Universitario de Burgos, Av. Islas Baleares, 3, 09006 Burgos, Spain.

24. Clinical Analysis Service. Hospital Clínico Universitario de Valladolid, Av. Ramón y Cajal, 3, 47003 Valladolid, Spain

25. Department of Microbiology and Immunology, Faculty of Medicine, Canadian Center for Vaccinology CCFV, Dalhousie University, Halifax, Nova Scotia, B3H 4R2, Canada

26. Laboratory of Immunity, Shantou University Medical College, 22 Xinling Rd, Jinping, Shantou, Guangdong, China.

27. Department of Cardiovascular Surgery, Hospital Clínico Universitario de Valladolid. Av. Ramón y Cajal, 3, 47003 Valladolid, Spain

28. Infectious diseases clinic, Internal Medicine Department, Hospital Universitario Río Hortega, Valladolid, Calle Dulzaina, 2, 47012 Valladolid, Spain

29. Department of Pulmonology, Hospital Clinic de Barcelona, Universidad de Barcelona, Institut D investigicions August Pi I Sunyer (IDIBAPS), Carrer del Rosselló, 149, 08036 Barcelona, Spain.

30. Intensive Care Department, Vall d’Hebron Hospital Universitari. SODIR Research Group, Vall d’Hebron Institut de Recerca, Passeig de la Vall d'Hebron, 119, 08035 Barcelona, Spain.

31. Respiratory Department, Institut Ricerca Biomedica de Lleida, Av. Alcalde Rovira Roure, 80, 25198 Lleida, Spain.

**Corresponding author:** David J Kelvin Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, B3H 4R2, Canada. E-mail address: dkelvin@jide.org.
Abstract

Background: COVID-19 can course with respiratory and extrapulmonary disease. SARS-CoV-2 RNA is detected in respiratory samples but also in blood, stool and urine. Severe COVID-19 is characterised by a dysregulated host response to this virus. We studied whether viral RNAemia or viral RNA load in plasma are associated to severe COVID-19 and also to this dysregulated response.

Methods: 250 patients with COVID-19 were recruited (50 outpatients, 100 hospitalised ward patients, and 100 critically ill). Viral RNA detection and quantification in plasma was performed using droplet digital PCR, targeting the N1 and N2 regions of the SARS-CoV-2 nucleoprotein gene. The association between SARS-CoV-2 RNAemia and viral RNA load in plasma with severity was evaluated by multivariate logistic regression. Correlations between viral RNA load and biomarkers evidencing dysregulation of host response were evaluated by calculating the Spearman correlation coefficients.

Results: the frequency of viral RNAemia was higher in the critically ill patients (78%) compared to ward patients (27%) and outpatients (2%) ($p<0.001$). Critical patients had higher viral RNA loads in plasma than non-critically ill patients, with non survivors showing the highest values. When outpatients and ward patients were compared, viral RNAemia did not show significant associations in the multivariate analysis. In contrast, when ward patients were compared with ICU patients, both viral RNAemia and viral RNA load in plasma were associated with critical illness (OR [CI 95%], $p$): RNAemia (3.92 [1.183 - 12.968], 0.025), viral RNA load (N1) (1.962 [1.244 - 3.096], 0.004); viral RNA load (N2) (2.229 [1.382 - 3.595], 0.001). Viral RNA load in plasma correlated with higher levels of chemokines (CXCL10, CCL2), biomarkers indicative of a systemic inflammatory response (IL-6, CRP, Ferritin), activation of NK cells (IL-15), endothelial dysfunction (VCAM-1, angiopoietin-2, ICAM-1), coagulation activation (D-Dimer and INR), tissue...
damage (LDH, GPT), neutrophil response (neutrophils counts, myeloperoxidase, GM-CSF) and immunodepression (PD-L1, IL-10, lymphopenia and monocytopenia).

Conclusions: SARS-CoV-2 RNAemia and viral RNA load in plasma are associated to critical illness in COVID-19. Viral RNA load in plasma correlates with key signatures of dysregulated host responses, suggesting a major role of uncontrolled viral replication in the pathogenesis of this disease.

Key words: SARS-CoV-2, cytokine, sepsis, COVID-19, plasma, RNAemia, viral RNA load, ICU
Background

With well over 43 million cases and 1.56212 deaths globally, Coronavirus disease 2019 (COVID-19) has become the top economic and health priority worldwide [1]. Among hospitalized patients, around 10–20% are admitted to the intensive care unit (ICU), 3–10% require intubation and 2–5% die [2]. SARS-CoV-2 RNA is commonly detected in nasopharyngeal swabs; however viral RNA can be found in sputum, lung samples, peripheral blood, serum, stool samples, and to a limited extent urine [3] [4] [5] [6]. While the lungs are most often affected, severe COVID-19 also induce inflammatory cell infiltration, haemorrhage, and degeneration or necrosis in extra-pulmonary organs (spleen, lymph nodes, kidney, liver, central nervous system) [7] [8]. Patients with severe COVID-19 show signatures of dysregulated response to infection, with immunological alterations involving moderate elevation of some cytokines and chemokines such as IL-6, IL-10 or CXCL10, deep lymphopenia with neutrophilia, systemic inflammation (elevation of C Reactive Protein, ferritin), endothelial dysfunction, coagulation hyper-activation (D-dimers) and tissue damage (LDH) [9] [10] [11] [12] [13] [14].

Our hypothesis is that systemic distribution of the virus or viral components could be associated to the severity of COVID-19, and, in turn to a number of parameters indicating the presence of a dysregulated response to the infection.

While the SARS-CoV-2 virus has been reported to be difficult to culture from blood [4], PCR based methods are able to detect and quantify the presence of genomic material of the virus in serum or plasma, representing an useful approach to evaluate the impact of the extrapulmonary dissemination of viral material on disease severity and also on the host response to the infection [5] [15]. An excellent approach for achieving absolute quantification of viral RNA load is droplet digital PCR (ddPCR). ddPCR is a next-
generation PCR method, which offers absolute quantification with no need of standard curve and greater precision and reproducibility than currently available qRT-PCR methods, as revised elsewhere [16].

We employed here ddPCR to detect and quantify viral RNA in plasma from COVID-19 patients discharged from the emergency room with mild severity, patients admitted to the ward with moderate severity, and critically ill patients. Our objectives in this study were: 1) to evaluate if there is an association between SARS-CoV-2 RNAemia and viral RNA load with moderate disease; 2) to evaluate if there is an association between SARS-CoV-2 RNAemia and viral RNA load with critical illness; 3) to evaluate the correlations between SARS-CoV-2 RNA load in plasma and parameters of dysregulated host responses against SARS-CoV-2.
**Methods**

**Study design:** 250 adult patients with a positive nasopharyngeal swab polymerase chain reaction (PCR) test for SARS-CoV-2 performed at participating hospitals were recruited during the first pandemic wave in Spain from March 16th to the 15th of April 2020. The patients recruited were of three different categories. The first corresponded to patients examined at an emergency room and discharged within the first 24 hours (outpatients group, n=50). The second group were patients hospitalized to pneumology, infectious diseases or internal medicine wards (wards group, n=100). Patients who required critical care or died during hospitalization were excluded from this group, in order to have a group of clear moderate severity. The third group corresponded to patients admitted to the ICU (n=100). Patient’s recruited by participating hospital are detailed in the additional file 1.

20 healthy blood donors were included as controls. These controls were recruited during the pandemics, in parallel to the SARS-CoV-2 infected patients, and were negative for SARS-CoV-2 IgG. This study was registered at Clinicaltrials.gov with the identification NCT04457505.

**Blood samples:** Plasma from blood collected in EDTA tubes samples was obtained from the three groups of patients in the first 24 hours following admission to the emergency room, to the ward, or to the ICU, at a median collection day since disease onset of 7, 8 and 10 respectively, and also from 20 blood donors (10 men and 10 women).

**Biomarker profiling:** a panel of biomarkers was profiled by using the Ella-SimplePlex™ immunoassay (San Jose, California, U.S.A), informing of the following biological functions potentially altered in severe COVID-19, based in the available evidence on COVID-19 physiopathology [13] [17] and also in our previous experience on emerging infections and sepsis [18] [19] [20] [21]: neutrophil degranulation: Lipocalin-2/NGAL, myeloperoxidase; endothelial dysfunction: ICAM-1, VCAM-1/CD106, Angiopoietin 2; T
cell survival and function: IL-7, Granzyme B; immunosuppression: IL-1ra, B7-H1/PD-L1,
chemotaxis: CXCL10/IP10, CCL2; Th1 response: Interleukin 1 beta, IFN-γ, IL-12p70, IL-15, TNF-α, IL-2; Th2 response: IL-4, IL-10; Th17 response: IL-6, IL-17A;
granulocyte mobilization / activation: G-CSF, GM-CSF; coagulation activation: D-Dimer;
acute phase reactants: Ferritin (C Reactive protein and LDH were profiled in each
participant hospital by their central laboratories).

Detection and quantification of SARS-CoV-2 RNA in plasma: RNA was extracted from
100 µl of plasma using an automated system, eMAG® from bioMérieux® (Marcy l'Etoile,
France). Detection and quantification of SARS-CoV-2 RNA was performed in five µl of
the eluted solution using the Bio-Rad SARS-CoV-2 ddPCR kit according to
manufacturer’s specifications on a QX-200 droplet digital PCR platform from the same
provider. This PCR targets the N1 and N2 regions of the viral nucleoprotein gene and also
the human ribonuclease (RNase) P gene using the primers and probes sets detailed in the
CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel [22].
Samples were considered positive for SARS-CoV-2 when N1 and/or N2 presented values
≥ 0.1 copies/µL in a given reaction. RNase P gene was considered positive when it
presented values ≥ 0.2 copies/µL, following manufacturer’s indications. The test was only
considered valid when RNase P gene was positive. Final results were given in copies of
cDNA / mL of plasma. IgG specific for the Nucleocapsid Protein of SARS–CoV-2 was
detected in 150 µl of plasma using the Abbott Architect SARS-CoV-2 IgG Assay (Illinois,
U.S.A). Viral RNA and SARS-CoV-2 IgG were profiled in the same plasma sample.

Statistical analysis: For the demographic and clinical characteristics of the patients, the
differences between groups were assessed using the Chi-square test / Fisher's Exact Test
where appropriated for categorical variables. Differences for continuous variables were
assessed by using the Kruskal-Wallis test with post hoc tests adjusting for multiple
comparisons. Multivariate logistic regression analysis was employed to evaluate the
association between viral RNAemia and viral RNA load in plasma with severity, in the
comparisons [outpatients vs ward patients] and [ward patients vs ICU patients]. Variables
showing significant differences between groups in each comparison in the Kruskal-Wallis
test were further introduced in the multivariate analysis as adjusting variables. The list of
variables considered as potential adjusting variables were [Age (years)], [Sex (male)],
[Alcoholism], [Smoker], [Drug abuse], [Cardiac disease], [Chronic vascular disease],
[COPD], [Asthma], [Obesity], [Hypertension], [Dyslipidemia], [Chronic renal disease],
[Chronic hepatic disease], [Neurological disease], [HIV], [Autoimmune disease], [Chronic
inflammatory bowel disease], [Type 1 diabetes], [Type 2 diabetes], [Cancer], [Invasive
mechanical ventilation], [Non-invasive mechanical ventilation], [SARS-CoV-2 IgG],
[Temperature (ºC)], [Systolic Pressure (mmHg)], [Oxygen saturation (%)], [Bilaterial
pulmonary infiltrate], [Glucose (mg/dl)], [Creatinine (mg/dl)], [Na (mEq/L)], [K
(mEq/L)], [Platelets (cell x 10^3 / µl)], [INR], [D Dimer (pg/ml)], [LDH (UI/L)], [GPT
(UI/L)], [Ferritin (pg/ml)], [CRP (mg/dl)], [Haematocrit (%)], [Lymphocytes (cells/mm3)],
[Neutrophils (cells/mm3)], [Monocytes (cells/mm3)]. Multivariate logistic regression
analysis was performed using the “Enter” method, but also the backward stepwise
selection method (Likelihood Ratio) was employed in each case to confirm the association
between viral RNAemia and viral RNA load in plasma with disease severity (pin < 0.05,
pout < 0.10), not forcing entry of these variables in the model. Correlation analysis were
performed using the Spearman test applying the Bonferroni correction of the p value.
Variables evaluated for correlation with viral RNA load were: [Temperature (ºC)],
[Systolic Pressure (mmHg)], [Oxygen saturation (%)], [Lymphocytes (cells/mm3)],
[Neutrophils (cells/mm3)], [Monocytes (cells/mm3)], [Creatinine (mg/dl)], [LDH (UI/L)],
[GPT (UI/L)], [Platelets (cell x 10^3 / µl)], [INR], [CRP (mg/dl)], and all the biomarkers
analyzed by Ella- SimplePlex. Statistical analysis was performed with IBM SPSS® version 20 (IBM, Armonk, New York, USA).

261 Results

262 Clinical characteristics of the patients (Table 1): Patients requiring hospitalization (either general ward or ICU) were older than those patients discharged to their home from the ER. Critically ill patients were more frequently male than those in the other groups. Comorbidities of obesity, hypertension, dyslipidemia and type 2 diabetes were more commonly found in patients requiring hospitalization, with no significant differences found in the comorbidities profile between critically ill and non-critically ill hospitalized patients.

14% of the patients in clinical wards required non-invasive mechanical ventilation, while 96% of the patients admitted to the ICU required invasive mechanical ventilation.

Critically ill patients had increased glucose levels, along with higher concentration of neutrophils in blood, increased levels of ferritin and C-reactive protein (denoting activation of the systemic inflammatory response). Increased levels of INR and D-dimers (reflecting activation of the coagulation system), as well as LDH and GPT, which levels raise as consequence of tissue and liver damage, were also observed in critically ill patients.

Patients admitted to the ICU also showed a lower haematocrit, pronounced lymphopenia and lower monocyte counts at admission. ICU patients stayed longer in the hospital than ward patients, with 49% having a fatal outcome.

Viral RNAemia, viral RNA load in plasma and specific SARS-CoV-2 IgG in the three groups of patients. As depicted in table 1, the frequency of the detection of SARS-CoV-2 viral RNA (RNAemia) was significantly higher in the critically ill patients (78%) compared to ward patients (27%) and outpatients (2%) (p<0.001). Similarly, the group of critically patients showed higher viral RNA loads in plasma than either ward or outpatients (p<0.001) (Table 1 and Figure 1). Non survivors showed the highest concentrations of viral
RNA in plasma: viral RNA load (N1 region) in ICU non survivors: 1587 copies / ml [10248]; viral RNA load (N1 region) in ICU survivors 574 copies / mL [1872] (results expressed as median [interquartile rank]); viral RNA load (N2 region) in ICU non survivors: 2798 copies / ml [12012]; viral RNA load (N2 region) in ICU survivors 523 copies / mL [1478]. Patients admitted to the wards showed a significant higher frequency of viral RNAemia than outpatients, but viral RNA loads were not significantly different with the latter group (Table 1 and Figure 1). Critically ill patients also had a higher frequency of specific SARS-CoV-2 IgG responses than the other groups (70% in ICU compared to 52% and 49% in the outpatients and ward groups, $p < 0.05$, table 1). No significant differences were found between the group of outpatients and those admitted to the ward. The prevalence of viral RNAemia did not differ between those patients testing positive and those testing negative for SARS-CoV-2 IgG (43.8 % and 40.4 % respectively, $p =0.586$), who in addition showed no differences in viral RNA load (data not shown).

Patients with viral RNAemia showed no differences in the days since onset of symptoms compared to those with no viral RNAemia (8.0 days [6.0]; 8.0 days [7.2], $p = 0.965$). In contrast, samples from patients with SARS-CoV-2 IgG were collected later since disease onset that those without SARS-CoV-2 IgG (10.0 days [7]; 7.0 days [6.0], $p = 0.003$)

**Multivariate analysis to evaluate the association between viral RNAemia and viral RNA load in plasma with moderate disease and critical illness.** While the proportion of patients with viral RNAemia was higher in the wards group compared to the outpatients’ group (table 1), the multivariate analysis did not show a significant association between the presence of viral RNAemia and being hospitalised at the ward, with none of the both methods employed (Additional files 2 and 3). In contrast, when the ward group was compared with critically ill patients, a significant direct association was found between
viral RNAemia and viral RNA load in critically ill patients, using the “Enter” method (Table 2) but also the backward stepwise selection method (additional file 4).

Correlations between viral RNA load in plasma and biological responses to SARS-CoV-2 infection: viral RNA load in plasma (targeting either the N1 and the N2 regions) showed the strongest direct correlations with plasma levels of CXCL10, LDH, IL-10, IL-6, IL-15, myeloperoxidase and CCL-2 (MCP-1) and inverse correlations with lymphocytes, monocytes and O2 saturation (Figure 2). These were the parameters whose levels varied the most in critically patients compared with ward and outpatient groups (Figures 3 and 4 and additional file 5). CXCL10 was the most accurate identifier of viral RNAemia in plasma (area under the curve (AUC), [CI95%], p) = 0.85 [0.80 – 0.89], <0.001), and IL-15 was the cytokine which most accurately differentiated clinical ward patients from ICU patients (AUC: 0.82 [0.76 – 0.88], <0.001). Plasma viral RNA load also showed significant direct correlations with levels of VCAM-1, PDL-1, GM-CSF, G-CSF, neutrophil counts, IL-1ra, CRP, INR, D-dimer, TFNα, Angiopoietin-2, GPT, ICAM-1, IL-7 and Ferritin (Figure 2), with most of these mediators showing the highest variations in the critically ill patients (Figures 3 and 4 and additional file 5).

Discussion

Our study demonstrates that the presence of SARS-CoV-2-RNA in plasma is associated to critical illness in COVID-19 patients, with the strength of association being the highest in those patients with the highest viral RNA loads. This association was independent of other factors also related to disease severity. Moreover, those critically ill patients who died presented with higher viral RNA loads in plasma than those who survived. SARS-CoV-2 viral RNA was detected in the plasma of the vast majority of those COVID-19 patients admitted to the ICU (78%). As far as we know, our study is the largest one to date using ddPCR to quantify SARS-CoV-2 RNA load in plasma from COVID-19 patients, and the
only one with a multicentric design. Our results are in consonance with those from Veyer et al, who, in a pilot study using this technology, found higher viral RNA loads and a prevalence of RNA viremia of 88% in twenty six COVID-19 patients who were critically ill [16]. The results are also in agreement with those of Hagman et al, who, using standard RT-PCR technology, found that the presence of SARS-CoV-2 RNA in serum at hospital admission was associated with a seven-fold increased risk of critical disease and an eight-fold increased risk of death in a cohort of 167 patients hospitalised for COVID-19 [23].

Although our study did not determine if the presence of viral RNA in plasma reflects the presence of live virus in peripheral blood, the association found between the presence and concentration of viral RNA in plasma and critical illness suggests that viral replication is more robust in severe COVID-19, and/or that critically ill patients with this disease are not able to control viral replication. This notion is further supported by the correlations found in our study between viral RNA load in plasma and hypercytokinemia involving CXCL10, IL-10, CCL2, IL-6 and IL-15, where the levels of these cytokines were the highest in patients with critical illness. The correlation between viral RNA load and higher levels of cytokines has also been described in the severe infections caused by H5N1 and pandemic H1N1 influenza strains [24] [25]. Active viral replication stimulates the secretion of cytokines by the recognition of viral RNA by endosomal receptors such as toll like receptor 7 (TLR7) in human plasmacytoid dendritic cells and B cells, or TLR8 in myeloid cells [26]. While the elevation of CXCL10, IL-10, CCL2, IL-6 has been extensively documented in severe COVID-19 [9] [10], our work demonstrates a clear correlation between these cytokines and plasma viral load. Furthermore, we report for the first time a major role of IL-15 in severe COVID-19. High levels of IL-15 in critically ill patients with high SARS-CoV-2 RNA load in plasma could be an attempt to stimulate Natural Killer cells to fight the virus [27]. We previously demonstrated that high levels of IL-15, along
with IL-6, constituted a signature of critical illness in H1N1 pandemic influenza infection [20].

Viral RNA load correlated with higher levels of myeloperoxidase in plasma, which were the highest in those patients admitted to the ICU. This is a marker of neutrophil degranulation and a potent tissue damage factor which has been proposed to play a role in the pathogenesis of ARDS secondary to influenza, by mediating claudin alteration on endothelial tight junctions, eventually leading to protein leakage and viral spread [28]. In this regard, the correlation found between viral RNA load in plasma and higher levels of LDH and GPT could suggest a direct or indirect role of viral replication in mediating tissue destruction in COVID-19.

Interesting, but less robust, direct correlations were found between viral RNA load in plasma with GM-CSF and neutrophil counts in blood, further reinforcing the role of neutrophil mediated responses in the pathogenesis of severe COVID-19. The direct correlation with soluble PDL-1 is also relevant, since this the ligand of the inhibitory co-receptor PD1 on T cells, which activation induces anergy of T lymphocytes [29]. This finding reinforces the potential role of immune checkpoint inhibitors in severe COVID-19 [30]. In turn, the association found between viral RNA load in plasma and three mediators of endothelial dysfunction (VCAM-1, angiopoietin 2 and ICAM-1), and with coagulation activation markers (D-dimers and INR prolongation) suggests a potential virally linked mechanism in the pathogenesis of endothelitis and thrombosis in COVID-19 disease [7].

Finally, the correlation with the acute phase reactants CRP and ferritin suggests a connection between shedding of genomic material of the virus to the blood and the induction of a systemic inflammatory response which is observed in those patients needing critical care.
The strongest inverse correlations found in our study was between SARS-CoV-2 RNA load in plasma and lymphocyte and monocyte counts in peripheral blood, for which critically ill patients showed the lowest values. Active viral replication could be a precipitating event in the pathogenesis of lymphopenia and monocytopenia in severe COVID-19 patients [11] [31], by mediating direct cytopathic actions or stimulating the migration of these cells to the extravascular space to reach the infected tissues [32]. A limitation of our work is its observational nature, which precludes to infer causality. Nonetheless, the observed associations could serve as hypothesis generators, leading to the development of animal models to confirm the potential link between SARS-CoV-2 replication and the dysregulated host responses observed in severe COVID-19.

**Conclusion:**

Presence of SARS-CoV-2 RNA in plasma is associated with critical illness in patients with COVID-19. The strength of this association increases with viral RNA load in plasma, which in turn correlates with key signatures of dysregulated host response in COVID-19 (figure 5). Our findings suggest a major role of uncontrolled viral replication in the pathogenesis of this disease. Assessment of viral RNAemia and viral RNA load in plasma could be useful to early detect those patients at risk of clinical deterioration, to assess response to treatment and to predict disease outcome.

**List of abbreviations**

- SARS-CoV-2: Severe acute Respiratory Syndrom-Coronavirus-2
- LDH: Lactate dehydrogenase
- G-CSF: Granulocyte colony-stimulating factor
- TLR: toll like receptor
Declarations

Ethics approval and consent to participate: The study was approved by the Committee for Ethical Research of the coordinating institution, “Comité de Ética de la Investigación con Medicamentos del Área de Salud de Salamanca”, code PI 2020 03 452. Informed consent was obtained orally when clinically possible. In the remaining cases, the informed consent waiver was authorized by the Ethics committee.

Consent for publication: not applicable

Availability of data and materials: the datasets generated and/or analysed during the current study are not publicly available since they are still under elaboration for publication by the authors but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors’ contribution: JFBM, DJK, JB, RF, FB, AT and RM designed the study. JFBM and DJK wrote the manuscript and interpreted the data. RA coordinated the clinical study and drafted the figures. MGR, DM, PR, FPG, LT, RLI, EB, CA, JMG, JR, RM, MIF, GM, MGE, DC, FDC, JFR, WT, PGJ, GR, IM, EG, IM, SP, SM, PGO, JAC, TRA, CP, JAB, GR, RH, JB, PE, RC, JA, JGB, NM, NBL, LJV, BFC, MAM recruited the patients and/or collected the clinical data. MDG, AO, RO, LMR and JME performed the assays for the detection of SARS-CoV-2 IgG and viremia. APT, CD and AO developed the ddPCR works; CD and NJ profiled the immunological mediators. SR, AMF and MMF developed the statistical analysis and drafted the figures. AAK, ATO, AM and LF performed the literature search. All authors read and approved the final manuscript.

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| Characteristics                  | Outpatients (1) | Ward (2) | ICU (3) | p value (1 vs 2) | p value (1 vs 3) | p value (2 vs 3) |
|---------------------------------|-----------------|----------|---------|-----------------|-----------------|-----------------|
| **Age [years, median (IQR)]**   | 48.50 [19]      | 64 [20]  | 66 [19] | < 0.001         | < 0.001         | n.s.            |
| **Male [% (n)]**                | 46 (23)         | 50 (50)  | 64 (64) | n.s.            | 0.035           | 0.046           |
| **Alcoholism**                  | 2 (1)           | 0 (0)    | 1 (1)   | n.s.            | n.s.            | n.s.            |
| **Smoking**                     | 4 (2)           | 5 (5)    | 6 (6)   | n.s.            | n.s.            | n.s.            |
| **Drug abuse**                  | 2 (1)           | 0 (0)    | 0 (0)   | n.s.            | n.s.            | n.s.            |
| **Cardiac disease**             | 4 (2)           | 13 (13)  | 9 (9)   | n.s.            | n.s.            | n.s.            |
| **Chronic vascular disease**    | 2 (1)           | 2 (2)    | 5 (5)   | n.s.            | n.s.            | n.s.            |
| **COPD**                        | 2 (1)           | 2 (2)    | 3 (3)   | n.s.            | n.s.            | n.s.            |
| **Asthma**                      | 8 (4)           | 6 (6)    | 2 (2)   | n.s.            | n.s.            | n.s.            |
| **Obesity**                     | 4 (2)           | 26 (26)  | 26 (26) | 0.001           | 0.001           | n.s.            |
| **Hypertension**                | 24 (12)         | 44 (44)  | 45 (45) | 0.017           | 0.012           | n.s.            |
| **Dyslipidemia**                | 16 (8)          | 41 (41)  | 34 (34) | 0.002           | 0.021           | n.s.            |
| **chronic renal disease**       | 2 (1)           | 3 (3)    | 3 (3)   | n.s.            | n.s.            | n.s.            |
| **chronic hepatic disease**     | 2 (1)           | 0 (0)    | 3 (3)   | n.s.            | n.s.            | n.s.            |
| **Neurological disease**        | 0 (0)           | 6 (6)    | 3 (3)   | n.s.            | n.s.            | n.s.            |
| **HIV**                         | 0 (0)           | 0 (0)    | 1 (1)   | n.a.            | n.s.            | n.s.            |
| **Autoimmune disease**          | 2 (1)           | 1 (1)    | 1 (1)   | n.s.            | n.s.            | n.s.            |
| **chronic inflammatory bowel disease** | 0 (0) | 2 (2) | 1 (1) | n.s. | n.s. | n.s. |
| **Type 1 diabetes**             | 0 (0)           | 0 (0)    | 3 (3)   | n.a.            | n.s.            | n.s.            |
| **Type 2 diabetes**             | 0 (0)           | 23 (23)  | 22 (22) | < 0.001         | < 0.001         | n.s.            |
| **Cancer**                      | 6 (3)           | 2 (2)    | 1 (1)   | n.s.            | n.s.            | n.s.            |
| **Invasive mechanical ventilation** | 0 (0) | 0 (0) | 96 (96) | n.a. | < 0.001 | < 0.001 |
| **Non-invasive mechanical ventilation** | 0 (0) | 14 (14) | 34 (34) | 0.004 | < 0.001 | 0.002 |
| **Hydroxychloroquine**          | 77.6 (38)       | 89 (89)  | 99 (99) | n.s.            | < 0.001         | 0.003            |
| **Chloroquine**                 | 4.1 (2)         | 7 (7)    | 0 (0)   | 0.042           | 0.007           | n.s.            |
| **Corticoids**                  | 6.7 (3)         | 29 (29)  | 83 (85) | 0.002           | < 0.001         | < 0.001          |
| **Azithromycin**                | 15.9 (7)        | 84 (84)  | 84 (84) | < 0.001         | < 0.001         | n.s.            |
| **Remdesivir**                  | 0 (0)           | 1 (1)    | 9 (9)   | n.s.            | 0.029           | 0.009           |
| **Tocilizumab**                 | 0 (0)           | 13 (13)  | 33 (33) | 0.008           | < 0.001         | < 0.001          |
| **Lopinavir/ritonavir**          | 74 (37)         | 35 (35)  | 96 (96) | < 0.001         | < 0.001         | < 0.001          |
| **Beta Interferon**             | 0 (0)           | 0 (0)    | 55 (55) | n.a.            | < 0.001         | < 0.001          |
| **Hospital stay [days, median (IQR)]** | -   | 9 (6) | 24 (19) | n.a. | n.a. | < 0.001 |
| **Viral RNAemia [% (n)]**       | 2 (1)           | 27 (27)  | 78 (78) | < 0.001         | < 0.001         | < 0.001          |
| **Viral RNA load in plasma (N1) copies / mL, median (IQR)** | 0 (0) | 0 (91) | 829 (4444) | n.s. | < 0.001 | < 0.001 |
| **Viral RNA load in plasma (N2) copies / mL, median (IQR)** | 0 (0) | 0 (93) | 836 (4939) | n.s. | < 0.001 | < 0.001 |
| **SARS-CoV-2 IgG [% (n)]**      | 52 (26)         | 49 (49)  | 70 (70) | 0.030           | 0.002           | n.s.            |
| **Hospital mortality [% (n)]**   | 0 (0)           | 0 (0)    | 49 (49) | n.a.            | < 0.001         | < 0.001          |
| **Temperature [ºC] median (IQR)** | 36.50 (1.0) | 36.80 (1.4) | 37.00 (1.4) | - | - | - |
| **Systolic Pressure [mmHg] median (IQR)** | 120 (29) | 126 (25) | 120 (26) | n.s. | n.s. | 0.001 |
| **Oxygen saturation [%] median (IQR)** | 96 (3) | 94 (5) | 92 (6) | 0.002 | < 0.001 | 0.002 |
### Table 1: Clinical characteristics of the patients

Continuous variables are represented as [median, (interquartile range, IQR)]; categorical variables are represented as [%, (n)]. INR, International Normalized Ratio; n.s., not significant; n.a., not applicable.

| Measurements at diagnosis | Pulmonary infiltrate [% (n)] | Bilateral pulmonary infiltrate [% (n)] | Glucose (mg/dl) [median (IQR)] | Creatinine (mg/dl) [median (IQR)] | Na (mEq/L) [median (IQR)] | K (mEq/L) [median (IQR)] | Platelets (cell x 10^3 / µl) [median (IQR)] | INR [median (IQR)] | D Dimer (pg/ml) [median (IQR)] | LDH (UI/L) [median (IQR)] | GPT (UI/L) [median (IQR)] | Ferritin (pg/ml) [median (IQR)] | CRP (mg/dl) [median (IQR)] | Haematocrit (%) [median (IQR)] | WBC (cells/mm3) [median (IQR)] | Lymphocytes (cells/mm3) [median (IQR)] | Neutrophils (cells/mm3) [median (IQR)] | Monocytes (cells/mm3) [median (IQR)] |
|---------------------------|-------------------------------|----------------------------------------|--------------------------------|-----------------------------------|---------------------------|---------------------------|---------------------------------|---------------------------|--------------------------------|---------------------------|--------------------------------|--------------------------------|---------------------------|-------------------------|----------------------------|--------------------------------|-----------------------------|--------------------------------|-----------------------------|-------------------------|
| **Measurements at diagnosis** | 72 (36) | 26 (13) | 99.5 (22) | 0.84 (0.18) | 138 (4) | 3.90 (0.50) | 223 [97] | 1.04 (0.10) | 795278 [829234] | 214 (73) | 27 (43) | 359507 [458748] | 1.40 (3.50) | 43.15 (4.72) | 6450 (2815) | 1400 (805) | 4260 (2625) | 500 (300) |
| **Measurements at diagnosis** | 93 (93) | 67 (67) | 112 (31) | 0.91 (0.33) | 138 (5) | 4.10 (0.68) | 207 [113] | 1.11 (0.13) | 1597362 [2024704] | 278 (138) | 29 (29) | 523805 [534757] | 40.90 (89.18) | 42.50 (6.50) | 7005 (4115) | 1000 (433) | 5250 (3918) | 400 (300) |
| **Measurements at diagnosis** | 100 (100) | 93 (93) | 160.50 (83) | 0.88 (0.57) | 138.50 (7) | 3.95 (0.90) | 204 [126] | 1.22 (0.22) | 6182104 [52690022] | 496 (285) | 44 (44.50) | 923687 [1526492] | 91 (182.10) | 38.15 (6.48) | 9145 (6613) | 540 (445) | 8300 (5880) | 300 (280) |
| **Measurements at diagnosis** | < 0.001 | < 0.001 | < 0.001 | - | - | - | - | 0.004 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.021 | 0.002 | n.s. | < 0.001 | < 0.001 | < 0.001 | n.s. |
| **Measurements at diagnosis** | < 0.001 | < 0.001 | < 0.001 | - | - | - | - | - | - | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.002 | 0.031 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| **Measurements at diagnosis** | < 0.001 | < 0.001 | < 0.001 | - | - | - | - | - | - | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.006 | 0.006 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| **Measurements at diagnosis** | < 0.001 | < 0.001 | < 0.001 | - | - | - | - | - | - | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.001 | 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

- COPD (Chronic obstructive pulmonary disease), HIV (Human Immunodeficiency Virus), INR (International Normalized Ratio), LDH (Lactic Acid Dehydrogenase), GPT (glutamic-pyruvate transaminase); CRP (C-reactive protein), WBC (white blood cell).
Table 2: Multivariate logistic regression analysis comparing wards patients against critically ill patients (Enter method). The association between viral RNAemia or viral RNA load targeting the N1 region, or viral RNA load targeting the N2 region with critical illness was evaluated adjusting by major confounding factors.
Figure 1: Viral RNA load in plasma, targeting the N1 region (left) and the N2 region (right), in the three groups of patients. Results are provided as copies of cDNA per mL of plasma.
Figure 2. Heat map representing the Spearman correlation coefficients between viral RNA load in plasma targeting the N1 and the N2 regions and representative indicators of host dysregulated response.
Figure 3. Levels of laboratory parameters indicating host dysregulated response across groups. † indicates significant difference with the healthy control and the bars significant differences between the other groups.
Figure 4: Levels of laboratory parameters indicating host dysregulated response across groups. † indicates significant difference with the healthy control and the bars significant differences between the other groups.
Figure 5: Integrative model depicting the correlations found between the indicators of host dysregulated responses and SARS-CoV-2 RNA load in plasma.
| Hospital                          | Outpatients | Wards | ICU  |
|----------------------------------|-------------|-------|------|
| Hospital Clínico Universitario de Valladolid | 0           | 0     | 45   |
| Hospital General Universitario Gregorio Marañón (Madrid) | 44          | 4     | 25   |
| Hospital Universitario Infanta Leonor (Madrid) | 0           | 30    | 0    |
| Hospital Universitario y Politécnico de La Fe (Valencia) | 0           | 27    | 0    |
| Hospital Universitario Río Hortega (Valladolid) | 2           | 22    | 23   |
| Hospital Universitario Príncipe de Asturias (Madrid) | 4           | 17    | 1    |
| Hospital Universitario de Burgos | 0           | 0     | 2    |
| Hospital Universitario de León   | 0           | 0     | 4    |
| **Total**                        | **50**      | **100** | **100** |

Additional file 1: recruited patients in each hospital
## Additional file 2. Multivariate logistic regression analysis comparing outpatients against wards patients (Enter method).

The association between viral RNAemia with hospitalization at the wards was evaluated adjusting by major confounding factors.

|                  |          |    |
|------------------|----------|----|
|                  | OR [CI 95%] |  p  |
| Age (years)      | 1.071 [1.024 - 1.121] | 0.003 |
| Obesity          | 5.618 [0.841 - 37.552] | 0.075 |
| Hypertension     | 1.024 [0.279 - 3.756] | 0.972 |
| Dyslipidemia     | 1.521 [0.354 - 6.532] | 0.573 |
| O2 saturation    | 1.077 [0.946 - 1.226] | 0.261 |
| Bilateral infiltrate | 7.652 [2.050 - 28.558] | 0.002 |
| Glucose (mg/dl)  | 1.022 [0.992 - 1.054] | 0.156 |
| INR              | 3.346 [0.121 - 92.829] | 0.476 |
| D Dimer (pg/ml)  | 1.000 [1.000 - 1.000] | 0.111 |
| LDH (UI/L)       | 1.005 [0.998 - 1.012] | 0.176 |
| CRP (mg/dl)      | 1.032 [1.012 - 1.052] | 0.002 |
| Lymphocytes (cells/mm3) | 1.000 [0.998 - 1.001] | 0.440 |
| Monocytes (cells/mm3) | 0.998 [0.996 - 1.001] | 0.223 |
| Neutrophils (cells/mm3) | 1.000 [1.000 - 1.000] | 0.382 |
| Viral RNAemia    | 3.793 [0.344 - 41.845] | 0.277 |
|                  | OR [CI95%]        |   p   |
|------------------|-------------------|-------|
| Age              | 1.057 [1.017 - 1.098] | 0.005 |
| Obesity          | 6.675 [1.129 - 39.472] | 0.036 |
| Bilateral infiltrate | 6.400 [2.153 - 19.024] | 0.001 |
| Glucose (mg/dl)  | 1.027 [0.999 - 1.055] | 0.058 |
| CRP (mg/dl)      | 1.032 [1.013 -1.051] | 0.001 |
| Monocytes (cells/mm3) | 0.997 [0.995 - 1.000] | 0.017 |

Additional file 3. Multivariate logistic regression analysis comparing outpatients against wards patients (backward stepwise selection method / Likelihood Ratio).

The association between viral RNAemia with hospitalization at the wards was evaluated adjusting by major confounding factors, but it was not selected in the final model.
|                          | OR [95% CI]       | p   | OR [95% CI]       | p   | OR [95% CI]       | p   |
|--------------------------|------------------|-----|------------------|-----|------------------|-----|
| Systolic Pressure (mmHg) | 0.976 0.953 1.000 0.049 | 0.974 0.949 0.999 | 0.040 | 0.974 0.950 0.999 | 0.042 |
| Glucose (mg/dl)          | 1.006 1.000 1.012 0.050 | 1.006 1.000 1.012 0.055 | 1.005 0.999 1.012 0.088 |
| D-dimer (pg/mL)          | 1.000 1.000 1.000 0.057 | 1.000 1.000 1.000 0.025 | 1.000 1.000 1.000 0.088 |
| LDH (U/L)                | 1.008 1.004 1.011 0.000 | 1.006 1.003 1.010 0.001 | 1.007 1.004 1.011 0.000 |
| Haematocrit (%)          | 0.798 0.717 0.888 0.000 | 0.789 0.706 0.881 0.000 | 0.775 0.688 0.873 0.000 |
| Lymphocytes (cells/mm³)  | 0.997 0.996 0.999 0.000 | 0.997 0.996 0.999 0.000 | 0.997 0.996 0.999 0.000 |
| Viral RNAemia (Yes)      | 4.270 1.575 11.580 0.064 |       |                  |      |                  |      |
| Viral RNA load (N1) in plasma, log (copies/mL) | 2.005 1.350 2.978 0.001 |       |                  |      |                  |      |
| Viral RNA load (N2) in plasma, log (copies/mL) |       |       |                  |      |                  |      | 2.071 1.419 3.022 0.000 |

**Additional file 4. Multivariate logistic regression analysis comparing wards patients against ICU patients (backward stepwise selection method / Likelihood Ratio).** The association between viral RNAemia and viral RNA load with critical illness was evaluated adjusting by major confounding factors.
|                    | Healthy Controls (0) | Outpatients (1) | Ward (2) | ICU (3) | p (0 vs 1) | p (0 vs 2) | p (0 vs 3) | p (1 vs 2) | p (1 vs 3) | p (2 vs 3) |
|--------------------|----------------------|-----------------|----------|---------|------------|------------|------------|------------|------------|------------|
| ICAM-1 pg/ml       | 289208 [92857]       | 358431.50 [88702.30] | 377655 [150081.80] | 466818 [202189.80] | 0.036 | < 0.001 | < 0.001 | n.s | < 0.001 | < 0.001 |
| Lipocalin-2 pg/ml  | 82634.50 [29205.30]  | 73134.50 [32007.30] | 94077 [49868] | 100165 [57819.80] | n.s | n.s | n.s | 0.011 | < 0.001 | n.s |
| Myeloperoxidase pg/ml | 27541.50 [12906.80] | 41902 [30413.80] | 88244.50 [78145] | 141971.50 [148623.50] | n.s | < 0.001 | < 0.001 | < 0.001 | < 0.001 | n.s |
| VCAM-1 pg/ml       | 618380 [195510.50]   | 800393 [403378] | 1191387 [618227] | 1248699.50 [603679] | 0.021 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | n.s |
| PD-L1 pg/ml        | 55.85 [33.27]        | 110.50 [120.68] | 178 [114.25] | 228 [137.75] | 0.031 | < 0.001 | < 0.001 | 0.045 | < 0.001 | 0.031 |
| G-CSF pg/ml        | 6.90 [7.67]          | 23.55 [20.48] | 31.95 [42.85] | 36.25 [53.35] | < 0.001 | < 0.001 | < 0.001 | n.s | < 0.001 | n.s |
| IL-1b pg/ml        | 0.44 [1.65]          | 0.16 [0.30] | 0.44 [0.53] | 0.38 [0.51] | n.s | n.s | n.s | 0.005 | < 0.001 | n.s |
| IL-10 pg/ml        | 1.59 [0.48]          | 4.19 [6.68] | 9.11 [10.14] | 17 [22.25] | 0.002 | < 0.001 | < 0.001 | 0.007 | < 0.001 | < 0.001 |
| IL-17A pg/ml       | 1.95 [1.08]          | 2.38 [1.45] | 2.32 [1.44] | 3.06 [2.87] | n.s | 0.047 | 0.002 | n.s | n.s | n.s |
| GM-CSF pg/ml       | 0.66 [0.42]          | 1.07 [0.54] | 1.10 [0.67] | 1.62 [1.01] | n.s | n.s | < 0.001 | n.s | < 0.001 | < 0.001 |
| IL-7 pg/ml         | 1.81 [1.49]          | 2.04 [3.12] | 3.19 [4.19] | 4.84 [5.17] | n.s | 0.009 | < 0.001 | 0.017 | < 0.001 | n.s |
| CXCL10 pg/ml       | 91.9 [71.85]         | 470.50 [731.50] | 977.50 [943] | 1609.50 [1693.75] | 0.003 | < 0.001 | < 0.001 | 0.008 | < 0.001 | 0.001 |
| Angiopoietin-2 pg/ml | 708 [260.75]          | 809 [479.75] | 1128 [779.50] | 1769.50 [2064.25] | n.s | 0.001 | < 0.001 | 0.008 | < 0.001 | 0.001 |
| IL-1ra pg/ml       | 478 [1159.75]        | 653.50 [1039.25] | 1796 [1896] | 1634 [4472.50] | n.s | < 0.001 | < 0.001 | < 0.001 | < 0.001 | n.s |
| IL-6 pg/ml         | 1.27 [0.83]          | 6.34 [12.42] | 33.25 [48.10] | 105.50 [252.23] | 0.027 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| CCL2 pg/ml         | 103.50 [39]          | 189 [157.50] | 283.50 [304] | 490 [881.75] | 0.012 | < 0.001 | < 0.001 | 0.031 | < 0.001 | < 0.001 |
| IL-12p70 pg/ml     | 0.62 [0.71]          | 1.46 [0.67] | 1.51 [0.71] | 1.36 [0.94] | < 0.001 | < 0.001 | < 0.001 | n.s | n.s | n.s |
| IL-2 pg/ml         | 0.02 [0.09]          | 0.53 [0.39] | 0.49 [0.57] | 0.63 [0.83] | < 0.001 | < 0.001 | < 0.001 | n.s | n.s | n.s |
| IL-4 pg/ml         | 0.13 [0.27]          | 0.33 [0.34] | 0.33 [0.39] | 0.29 [0.37] | 0.021 | 0.006 | 0.041 | n.s | n.s | n.s |
| IL-15 pg/ml        | 2.11 [0.54]          | 3.85 [3.7] | 4.89 [2.90] | 8.38 [5.19] | 0.001 | < 0.001 | < 0.001 | n.s | < 0.001 | < 0.001 |
| Granzyme-B pg/ml   | 12 [15.75]           | 33.20 [27.92] | 46.15 [40.03] | 39.25 [35.58] | < 0.001 | < 0.001 | < 0.001 | n.s | n.s | 0.036 |
| INFpg/ml           | 0.68 [0.45]          | 4.35 [14.16] | 7.42 [15.13] | 3.44 [8.50] | < 0.001 | < 0.001 | < 0.001 | n.s | n.s | 0.021 |
### Additional file 5. Laboratory parameters ‘levels across groups.

| TNFα (pg/ml) | 5.11 [1.79] | 10.60 [4.18] | 13 [6.46] | 12.95 [10.34] | < 0.001 | < 0.001 | < 0.001 | 0.021 | 0.006 | n.s. |
|-------------|-------------|-------------|------------|--------------|---------|---------|---------|-------|-------|-----|