



FACULTAD DE CIENCIAS DE LA SALUD

CARRERA PROFESIONAL DE MEDICINA HUMANA

**“ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE
CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR
PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19
MODERADOS Y SEVEROS”**

Tesis para optar el grado académico de:
MAESTRO EN EPIDEMIOLOGÍA CLÍNICA Y BIOESTADÍSTICA

Presentado por:

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Lima – Perú

2023

ACTA DE SUSTENTACIÓN

Lima, 05 de abril de 2023.

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Se reúnen para evaluar la tesis titulada:

“ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19 MODERADOS Y SEVEROS”

Presentada por: **Luciano Augusto Palomino Kobayashi**.

Para optar por el Grado de **Maestro en Epidemiología y Bioestadística**

Con la asesoría de la : **Dra. María Jesús Pons Casellas**

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Señor,
Dr. Carlos Zavalaga Reyes
Director General de Investigación, Desarrollo e Innovación
Universidad Científica del Sur
Presente. –

De nuestra consideración,

Yo Luciano Augusto Palomino Kobayashi, estudiante de la maestría en Epidemiología Clínica y Bioestadística de la Universidad Científica del Sur, en conjunto con la asesora María Jesús Pons Casellas, declaramos que este informe final de tesis titulado: "ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19 MODERADOS Y SEVEROS", sustentado para obtener el grado de Maestro en Epidemiología Clínica y Bioestadística es original.

Es decir, no contiene plagio parcial ni total, cuando se utilizó información de fuentes externas se reconoció la autoría mediante la adecuada citación y los resultados obtenidos son producto entero de mi (nuestra) investigación y no han sido falseados ni fabricados. Todo esto en cumplimiento del Código de ética en la investigación, Reglamento del Comité de Integridad Científica, Reglamento de Propiedad Intelectual, Normas y procedimientos de los trabajos de investigación para la obtención de títulos profesionales y grados académicos ¹⁴, que afirmamos conocer en su totalidad.

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¹⁴ Los documentos están en: <https://investigacion.cientifica.edu.pe/reglamentos/>

INFORME DE REVISIÓN DE ORIGINALIDAD

Título del documento evaluado. Estimación de translocación bacteriana mediante cuantificación de zonulina y su potencial como biomarcador pronóstico de muerte en una cohorte de pacientes Covid-19 moderados y severos

Autores. Luciano Augusto Palomino Kobayashi.

Mecanismo de revisión de originalidad. Evaluación con Turnitin (ID 2076469780).

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Estimación de translocación

INFORME DE ORIGINALIDAD



FUENTES PRIMARIAS

- 1** Magnus Lundgren. "Sequential Changes in Serum Cytokines Reflect Viral RNA Kinetics in Target Organs of a Coxsackievirus B Infection in Mice", *Journal of Clinical Immunology*, 05/09/2009
Publicación <1 %
- 2** Soto Vega Elena. "Influencia de los alelos HLA clase I, clase II y el polimorfismo del promotor del TNF- [alfa] en la longevidad de mestizos mexicanos", *TESIUNAM*, 2006
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- 3** hdl.handle.net
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- 4** John Lopera Marín, Amaranto Suárez Mattos, Oscar Gamboa, Martha Piña Quintero, Gretti Terselich. "Sustitución de procarbazona por etopósido en un nuevo esquema de tratamiento OEPA/COPE en los niños y adolescentes con linfoma de Hodgkin: una evaluación de los resultados en el Instituto <1 %

Dedicatoria

Dedico mi tesis de maestría a mi madre Magnolia Azucena Kobayashi Moreno, quien siempre ha dado lo mejor de sí para asegurar alimento, educación y salud en casa; además de darme alientos para alcanzar mis metas profesionales. También dedico este trabajo a mi perrito Scotty, compañero fiel quien contribuía con su compañía mientras redactaba el presente manuscrito.

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A los profesores de la maestría de Epidemiología Clínica y Bioestadística, por los conocimientos brindados empleando el enfoque de Medicina Basada en Evidencia; el cual ha dejado huella en mi formación profesional.

Al personal del Puesto de Salud Punta Hermosa, lugar en donde realicé mi Servicio Rural y Urbano Marginal (SERUMS) al mismo tiempo que realizaba mi tesis, por brindarme su amistad y buen ambiente laboral.

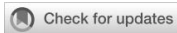
A mis colegas Kevin Reátegui, Orlando Zegarra y José Meléndez, quienes brindan su amistad y buenos consejos profesionales.

Resumen

La zonulina se ha relacionado previamente con la permeabilidad intestinal en diversas enfermedades inflamatorias y, más recientemente, con la fisiopatología de infecciones graves por COVID-19. Nosotros analizamos muestras de suero de un estudio previo de una cohorte peruana de pacientes hospitalizados con COVID-19, para la cuantificación de zonulina por ELISA sándwich. Se realizaron comparaciones con datos clínicos, parámetros hematológicos y bioquímicos y niveles de citoquinas/quimioquinas. Encontramos niveles de zonulina basales más altos en pacientes fallecidos, y la zonulina se asoció con desenlace fatal en análisis multivariable, incluso después de ajustar por presencia de comorbilidades, severidad y sexo. También hubo correlaciones positivas entre los valores de zonulina, creatinina, dímero D y tiempo de protrombina, mientras que se encontraron correlaciones inversas para el cociente Sa/FiO_2 y CCL5 (RANTES). Se recomiendan más estudios longitudinales para analizar la variación de los niveles de zonulina a lo largo del tiempo, así como su relación con COVID persistente.

Abstract

Zonulin has previously been related to intestinal permeability in various inflammatory diseases, and more recently to the physiopathology of severe COVID-19 infections. We analysed serum samples from a previous study of a Peruvian cohort of hospitalised COVID-19 patients, for the quantification of zonulin by sandwich ELISA. Comparisons with clinical data, haematological and biochemical parameters and cytokine/chemokine levels were made. We found higher baseline zonulin levels in deceased patients, and zonulin was associated with fatal outcome in multivariable analysis, even after adjustment for presence of comorbidities, disease severity and sex. There were also positive correlations between zonulin, creatinine, D-dimer values and prothrombin time, while inverse correlations were found for Sa/FiO₂ ratio and CCL5 (RANTES). Further longitudinal studies are recommended to analyse the variation of zonulin levels over time as well as their relationship with long-COVID.



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Zonulin, a marker of gut permeability, is associated with mortality in a cohort of hospitalised peruvian COVID-19 patients

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Zonulin has previously been related to intestinal permeability in various inflammatory diseases, and more recently to the physiopathology of severe COVID-19 infections. We analysed serum samples from a previous study of a Peruvian cohort of hospitalised COVID-19 patients, for the quantification of zonulin by sandwich ELISA. Comparisons with clinical data, haematological and biochemical parameters and cytokine/chemokine levels were made. We found higher baseline zonulin levels in deceased patients, and zonulin was associated with fatal outcome in multivariable analyses, even after adjustment for age, gender, and obesity. There were also positive correlations between zonulin, creatinine, D-dimer values and prothrombin time, while inverse correlations were found for Sa/FiO₂ ratio and CCL5 (RANTES). Further longitudinal studies are recommended to analyse the variation of zonulin levels over time as well as their relationship with long-COVID.

KEYWORDS

microbial translocation, zonulin, COVID-19, biomarker, ELISA, Peru

Introduction

Severe COVID-19 infections are characterised by systemic inflammation, acute respiratory distress syndrome (ARDS) and multi-organ system failure in adults (Kim et al., 2021). Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) binds principally to receptor angiotensin-converting enzyme 2 (ACE2) to enter many different host cells such as type II pneumocytes in the lung, myocardial cells, neurons and glia,

endothelial cells, intestinal enterocytes and others (Lamers et al., 2020; Beyerstedt et al., 2021). Particularly, intestinal enterocytes form a cell layer that is part of the mucosal defence system and are held together by tight junctions. Disruption of this layer (increased gut permeability) translates into translocation of bacterial components from the gut into the bloodstream, contributing to a further increase in systemic inflammation (Dinh et al., 2015).

As reported in other viral infections such as dengue fever (Chanchaoentana et al., 2021) or human immunodeficiency virus (Marchetti et al., 2013), microbial translocation may be caused by direct enterocyte infection from the virus or because of cytokine-mediated alterations and gut dysbiosis. Previous studies (Yonker et al., 2021; Giron et al., 2021; Oliva et al., 2021) have linked higher intestinal translocation with severe cases of COVID-19, describing a higher abundance of bacterial components in the bloodstream compared to milder cases. It has been suggested that microbial translocation, intestinal dysbiosis and underlying inflammatory processes might have a synergistic effect on systemic inflammation and high cytokine levels described in severe COVID-19 infections (Cardinale et al., 2020; Vignesh et al., 2020; Uzzan et al., 2020; Mashaqi et al., 2022).

Zonulin (pre-Haptoglobin 2) is a protein of ~ 47 kDa, which is detectable in its uncleaved form in human serum (Fasano, 2011) and is highly correlated with bacterial DNAemia (Gargari et al., 2021). It is a physiological mediator that reversibly regulates intestinal permeability by modulation of intercellular tight junctions (Tripathi et al., 2009) and is considered a biomarker of impaired gut barrier function (Fasano, 2011). In this sense, it has been hypothesised that the spike protein of SARS-CoV-2 induces overexpression of zonulin, which is related to an increase in intestinal permeability (Llorens et al., 2021). Moreover, zonulin levels have been reported to be higher in patients who died of severe COVID-19 (Giron et al., 2021). These data suggest that SARS-CoV-2 might facilitate passage of intestinal contents into circulation and a higher inflammatory state at intestinal level that could fuel general systemic inflammation. Thus, zonulin might be also an indirect marker of microbial translocation and could be associated with worse outcomes in COVID-19.

Inflammatory responses to viral infection are also influenced by genetic background, for example individual genetic variability of pro-inflammatory cytokines is related to severity in COVID-19 (Vakil et al., 2022). Moreover, population-associated variation of responses to viral infections is differentiated by genetic ancestry (Randolph et al., 2021). The few published studies on serum zonulin levels and COVID-19 come from cohorts/case series of United States of America (Giron et al., 2021; Josyabhatla et al., 2021; Yonker et al., 2022), Italy (Oliva et al., 2021) and Turkey (Kılıçet al., in press) only. Therefore, data from studies with subjects from a different genetic and

geographic background are important for external validity issues.

For the reasons mentioned above, the present study aimed to identify the association between microbial translocation estimated by serum zonulin and patient outcome in a cohort of moderate and severe COVID-19 hospitalised patients from Lima, Peru. Additionally, correlations were established amongst cytokine/chemokine values, routine clinical parameters, and serum zonulin levels.

Materials and methods

Study population and assessment of disease severity

The present study used samples from a cohort of hospitalised COVID-19 patients from *Hospital Nacional Guillermo Almenara Irigoyen* (Pons et al., 2021). A total of 60 patients were classified according to severity: 19 moderate, 26 severe and 15 deaths (initially classified as moderate or severe). All deaths occurred during hospitalisation. The COVID-19 cases were confirmed by quantitative polymerase chain reaction (qPCR) or antibody testing. The follow-up period was from August to October 2020, which was within the context of the first wave of COVID-19 in Peru. Therefore, no patient had received vaccination for COVID-19.

The definition of moderate infection was according to the Berlin 2012 definition of Acute Respiratory Distress Syndrome (ARDS) (ARDS Definition Task Force, 2012), while severe infection was defined whenever one or more of the following states developed: Horowitz index ($\text{PaO}_2/\text{FiO}_2$) < 100, oxygen saturation to fraction of inspired oxygen ratio ($\text{SpO}_2/\text{FiO}_2$, henceforth abbreviated as Sa/FiO_2) < 89, need for mechanical ventilation due to respiratory failure or systemic organ failure requiring admission to the intensive care unit.

Sample conditions

Serum samples from the original study were conserved at -80°C in the laboratories of the *Universidad Científica del Sur*.

Clinical data and cytokine levels

Data pertaining to age, gender, date of hospital admission, date of hospital discharge/death, date of sample according to hospital admission, comorbidities such as obesity, arterial hypertension, diabetes, chronic obstructive pulmonary disease (COPD) and any other underlying conditions, pharmacological treatment received, haemoglobin, platelets, white blood cell

(WBC) counts and Sa/FiO₂ were obtained from the corresponding medical records. All privileged identifiable information of the patients was excluded from the dataset generated and the samples were codified previously to ensure confidentiality.

The cytokine and chemokine concentrations of the serum samples were obtained from the data previously reported in the study by Pons et al. (Pons et al., 2021), in which a commercial kit of Luminox MAGPIX technology (Merck KGaA, Darmstadt, Germany) was used for quantification.

Estimation of microbial translocation by zonulin quantification

Zonulin (ng/mL) was quantified from the serum samples by sandwich ELISA, using the CUSABIO Human Zonulin ELISA Kit (CUSABIO TECHNOLOGY LLC, Wuhan, China) according to the manufacturer's instructions. Briefly, 100 µL of standards and samples were added per well in the coated microplate and incubated for two hours at 37°C. Subsequently, 100 µL of biotin-antibody solution was added and incubated for one hour at 37°C. Afterwards, the liquid was aspirated, and manual washing was done three times with the provided wash buffer. Then 100 µL of horseradish peroxidase-avidin solution was added per well and incubated for one hour at 37°C, and manual washing was done thereafter. Finally, 90 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added per well and incubated for 30 minutes at 37°C. Immediately after, 50 µL of stop solution was added per well and absorbance reading was done at 450 nm (with correction at 540 nm) in a Synergy LX Multi-Mode Reader (Biotek, USA). The samples and standards were run in duplicate.

Statistical analysis

Descriptive analysis was performed using absolute and relative frequencies for summarising categorical variables while median and interquartile range (IQR) were used for numerical variables, unless specified otherwise.

Bivariate analyses were made for comparisons between outcomes (survived vs. deceased) and categorical variables using the Chi-squared or Fisher exact test, while the Mann-Whitney U test was used for comparisons between outcomes and numerical variables. Correlations among WBC counts, biochemical markers, cytokines, chemokines and zonulin levels were normalised using base 10 log transformation and comparisons were made using Spearman tests.

Crude and adjusted multivariable analyses of potential prognostic factors of death were performed using logistic regression with clinical variables, cytokine and zonulin levels at baseline (from first serum sample available since hospital admission) and odds ratios (OR) were obtained. Survival

analyses were done using Cox regression, and crude and adjusted hazard ratios (HR) were estimated accordingly. Significant variables in bivariate analysis were employed for the regression analyses. Epidemiological criteria for adjustment of confounding and the use of variables with less than 20% data loss were employed to decide which adjusted model best fitted the data.

For the interpretation of the zonulin results, samples with a zonulin concentration above the upper limit of the standard (40 ng/mL) were assigned this value. Values below 0.625 ng/mL were used as their actual values for the analyses.

The analyses were conducted using STATA 16.1 SE Edition (StataCorp, TX) and graphics were constructed using R version 4.1.2. 95% Confidence intervals were calculated for OR and HR. Statistical significance was established with $\alpha=0.05$.

Results

Baseline clinical characteristics of the hospitalised cohort

Sixty hospitalised COVID-19 patients were included: 45 patients (75%) survived, and 15 (25%) patients died during the follow-up period. The distribution for case severity was as follows: 39 patients (65%) were classified as severe and 21 (35%) as moderate according to the given case definition and progression during the follow-up period. Severity was associated with outcome ($p=0.042$). The median age was similar in both the survived and deceased groups (55 and 55 years, respectively) and there was no significant difference in age ($p=0.694$) or sex ($p=0.678$) between the two groups. The median (IQR) time from admission to first serum sample were 1(1.5) and 1(5) days in the survived and deceased groups respectively ($p=0.621$). Time from admission to hospital discharge/death (time of hospitalisation) and time from first serum sample obtained to hospital discharge/death were also similar between the aforementioned groups ($p=0.978$ and $p=0.461$, respectively) (Table 1).

Some comorbidities such as obesity, diabetes, high blood pressure and others were present (Table 1). In the deceased group, 12 patients (80%) had comorbidities compared to 22 (48.9%) in the survivor group, and this difference was significant ($p=0.035$). The only comorbidity that was associated with outcome was obesity ($p=0.006$). Corticosteroids were employed as part of the treatment for all the patients in this cohort.

White blood cell counts, biochemical markers, and cytokine/chemokine levels at baseline

Haemoglobin levels at baseline were lower in the deceased, with a mean (standard deviation) of 13.19 (1.93) g/dL

TABLE 1 Characteristics of the study cohort by outcome at baseline.

	Survived	Deceased	p-value
Overall, n=60 (%)	45 (75.00)	15 (25.00)	
Age (years)	55 (19)	55 (25)	0.6943
Sex			
Male (%)	39 (86.67)	12 (80.00)	0.6780
Female (%)	6 (13.33)	3 (20.00)	
Severity			
Moderate (%)	19 (42.22)	2 (13.33)	0.0420
Severe(%)	26 (57.78)	13 (86.67)	
Time from admission to hospital discharge/death (days)	11 (8)	12.5 (12)	0.978
Time from admission to first sample (days)	1 (1.5)	1 (5)	0.621
Time from first sample to hospital discharge/death (days)	9(8.5)	8(14)	0.461
Presence of Comorbidities, n (%)			
None	23 (51.11)	3 (20.00)	0.035
Obesity	9 (20.00)	9 (64.29)	0.006
Diabetes	5 (11.11)	2 (14.29)	0.666
High Blood Pressure	8 (17.78)	5 (35.71)	0.266
CKD	1 (2.22)	0 (0.00)	1.000
COPD	1 (2.22)	2 (14.29)	0.137
CVD	0 (0.00)	1 (7.14)	0.237
Gout	1 (2.22)	0 (0.00)	1.000
Other	3 (6.67)	0 (0.00)	0.566
Haemoglobin, white blood cell count and other biomarkers			
Haemoglobin (g/dL), mean (SD)	14.38 (1.52)	13.19 (1.93)	0.0176
Leucocytes (10 ⁹ /L)	9 (6.87)	12.485 (8.17)	0.2142
Band neutrophils (%)	2 (3)	2 (2)	0.6491
Segmented neutrophils (%)	83 (12)	88 (7)	0.0060
Lymphocytes (%)	10 (10)	6 (4)	0.0045
Total neutrophils (10 ⁹ /L)	7.56 (7.32)	10.82 (8.26)	0.2307
Total lymphocytes (10 ⁹ /L)	0.94 (0.77)	0.59 (0.33)	0.0046
Platelets (count per μ L)	345000 (147000)	274500(45000)	0.1850
CRP (mg/mL)	61.3 (163.8)	168.7 (122.8)	0.1701
D-dimer (μ g/mL)	0.58 (0.51)	0.85 (1.86)	0.1425
Ferritin (μ g/L)	789.7 (679.5)	1261.1 (838)	0.0536
Creatinine (mg/dL)	0.7 (0.2)	0.8 (0.48)	0.4152
PT (s)	11.17 (0.89)	11.57 (1.31)	0.1627
APTT (s)	30.87 (5.15)	32.65 (4.22)	0.1782
Sa/FiO ₂	240 (162.63)	108.73 (35.40)	0.0007

Values of continuous variables are reported as median (interquartile range), unless stated otherwise. CKD, Chronic Kidney Disease; COPD, Chronic Obstructive Pulmonary Disease; CVD, Cardiovascular disease; CRP, CReactive Protein; PT, Prothrombin Time; APTT, Activated Partial Thromboplastin Time; Sa/FiO₂, Oxygen saturation/Fraction of inspired oxygen.

(p=0.018). Regarding WBC counts, segmented neutrophil counts were higher (p=0.006) and lymphocyte counts were lower in the deceased group (p=0.004) with no significant differences being found for the remaining WBC counts in the two groups.

In relation to some biochemical markers such as C-reactive protein, D-dimer and ferritin, there were no significant differences when comparisons were made by outcome (Table 1). However, the Sa/FiO₂ ratio was lower in the

deceased group, with a median (IQR) of 108.73 (35.40) (p=0.0007).

The cytokines interleukin-6 (IL-6) (p=0.029), granulocyte-macrophage colony-stimulating factor (GM-CSF) (p=0.005), IL-15 (p=0.046), IL-2 (p=0.027), IL-8 (p=0.0004), IL-1a (p=0.032), chemokines macrophage inflammatory protein-1 α (MIP α) (p=0.008) and monocyte chemoattractant protein-1 (MCP-1) (p=0.001) showed significant differences according to outcome (Table 2), with all being elevated in the deceased group.

TABLE 2 Zonulin, cytokines and chemokines values in serum at baseline by outcome.

	Survived	Deceased	p-value
Overall, n=60 (%)	45 (75.00)	15 (25.00)	
Zonulin (ng/mL)	1.0029 (0.8887)	3.2644 (4.7096)	0.0002
Overall, n=42 (%)	37 (88.1)	5 (11.90)	
IL-1b (pg/mL)	10 (1.5)	11 (2)	0.1194
GCSF (pg/mL)	11.5 (3)	12 (3)	0.3083
IL-10 (pg/mL)	19.5 (11)	49.5 (54)	0.1246
IL-13 (pg/mL)	8 (2)	8(2)	0.2062
IL-6 (pg/mL)	21 (38)	55 (130)	0.0295
IL-12 (pg/mL)	13 (3)	15 (4)	0.5556
IL-17A (pg/mL)	14 (2)	14 (3)	0.4770
GMCSF (pg/mL)	11 (2.5)	14 (4)	0.0059
IL-15 (pg/mL)	14 (3)	18 (8)	0.0456
EGF (pg/mL)	43 (64)	25 (135)	0.8612
IL-5 (pg/mL)	11 (1)	12 (1.5)	0.3165
VEGF (pg/mL)	20 (15.5)	17 (110)	0.6688
IFN-g (pg/mL)	10 (3)	11 (2)	0.7092
IFN-a (pg/mL)	10 (1)	11 (2)	0.2605
IL1-RA (pg/mL)	12 (4.5)	18 (33.5)	0.0928
TNF-a (pg/mL)	27.5 (10)	50.5 (44)	0.0566
IL-2 (pg/mL)	13 (1)	15 (2)	0.0275
IL-7 (pg/mL)	13 (4)	15 (3)	0.3299
IL-4 (pg/mL)	8 (2)	10 (2)	0.1539
IL-8 (pg/mL)	66 (28.5)	174 (258.5)	0.0004
IL-12p70 (pg/mL)	9 (1)	10 (2)	0.1730
IL-1a (pg/mL)	14 (4)	21 (5)	0.0318
IL-3 (pg/mL)	8 (1)	8 (1)	0.1627
TNF-b (pg/mL)	10 (2)	10 (2)	0.9191
Overall, n=42 (%)	37 (88.1)	5 (11.90)	–
RANTES (pg/mL)	11089 (3360)	10507 (4039.5)	0.6000
Eotaxin (pg/mL)	98 (69)	134 (123.5)	0.8308
MIP-1a (pg/mL)	15.5 (5.5)	22 (23.5)	0.0081
MIP-1b (pg/mL)	118 (99)	173 (589.5)	0.2211
MCP-1 (pg/mL)	1620 (1938)	9113 (1550)	0.0008
IP-10 (pg/mL)	3501.5 (5094)	6720 (4416)	0.1563

Values of continuous variables are reported as median (interquartile range).

Correlations of zonulin versus haematological/biochemical markers and cytokines/chemokines at baseline

Zonulin levels were positively correlated with creatinine ($r=0.288$, $p=0.035$) D-dimer values ($r=0.441$, $p=0.002$), whereas prothrombin time ($r=0.344$, $p=0.011$) and Sa/FiO₂ ratio ($r=-0.4764$, $p=0.0001$) were found to be negatively correlated with zonulin (Table 3, Figure 1).

Related to cytokines and chemokines, RANTES was the only chemokine negatively correlated with zonulin levels ($r=-0.5023$,

$p=0.007$). None of the cytokines were significantly correlated with zonulin levels (Table 3, Figure 1).

Zonulin levels at baseline associated with outcome in the bivariate and multivariable analyses

The median (IQR) level of zonulin in the deceased group was 3.264 (4.7096) ng/mL (min 0.7099 ng/mL – max 40 ng/mL, Table 2) showing a significant association with outcome

TABLE 3 Correlations between zonulin levels and haematological / biochemical markers and cytokines/chemokines at baseline.

	Spearman's rho	p-value
Haematological/biochemical marker		
Haemoglobin	-0.2009	0.1305
Leukocytes	0.0027	0.9840
Band neutrophils	0.1993	0.2824
Segmented neutrophils	0.0933	0.4859
Lymphocytes	-0.0374	0.7803
Platelets	-0.0852	0.5287
CRP	0.0114	0.9354
D-dimer	0.4415	0.0024
Ferritin	0.1846	0.1654
Creatinine	0.2880	0.0347
PT	0.3441	0.0108
APTT	0.1311	0.3447
Sa/FiO ₂	-0.4764	0.0001
Cytokines		
IL-1b	-0.1077	0.4971
GCSF	-0.1817	0.2494
IL-10	0.0117	0.9413
IL-13	-0.0113	0.9434
IL-6	-0.0946	0.5514
IL-12	0.9238	
IL-17A	0.1177	0.4579
GMCSF	0.0231	0.8845
IL-15	0.1361	0.3900
EGF	-0.0478	0.7636
IL-5	-0.1591	0.3143
VEGF	0.1512	0.3392
IFN-g	-0.0474	0.7657
IFN-a	-0.0334	0.8336
IL1-RA	-0.0612	0.7002
TNF-a	0.2342	0.1354
IL-2	0.0181	0.9092
IL-7	-0.2031	0.1970
IL-4	-0.1908	0.2262
IL-8	0.1990	0.2065
IL-12p70	0.0738	0.6421
IL-1a	-0.0106	0.9467
IL-3	-0.0859	0.5885
TNF-b	-0.1670	0.2905
Chemokines		
RANTES	-0.5023	0.0007
Eotaxin	0.1928	0.2212
MIP-1a	-0.0400	0.8016
MIP-1b	0.2032	0.1968
MCP-1	0.2268	0.1486
IP-10	0.0891	0.5745

($p=0.0002$, [Figure 2](#)) and obesity ($p=0.017$, [Figure 2](#)) in the bivariate analysis. Notwithstanding, zonulin was not found to be associated with disease severity ($p=0.69$, [Figure 2](#)) in the same analysis.

Several factors in logistic regression, such as obesity (OR 7.200, $p=0.003$), segmented neutrophils (OR 1.145, $p=0.015$), GMCSF (OR 2.220, $p=0.015$), IL-15 (OR 1.332, $p=0.031$), IL-2 (OR 3.089, $p=0.025$), and zonulin levels (OR 2.573, $p=0.003$), presented significant OR values greater than 1 in the crude analysis, which indicate an association with higher odds of mortality. In contrast, haemoglobin (OR 0.624, $p=0.030$), lymphocytes (OR 0.821, $p=0.018$) and Sa/FiO₂ (OR 0.985, $p=0.007$) had significant ORs less than 1. ([Table 4](#)).

Notwithstanding, in the adjusted regression, only obesity (only adjusted for age and gender, OR 8.103 $p=0.003$), segmented neutrophils (OR 1.303, $p=0.010$), lymphocytes (OR 0.726, $p=0.013$), Sa/FiO₂ (OR 0.987, $p=0.025$) and zonulin levels (OR 2.666, $p=0.007$) remained significant after adjustment for age, gender, and obesity ([Table 4](#)).

Cox regression analysis

Cox regression was performed as a survival analysis with the same predictors as logistic regression, considering the period between date of hospital admission to date of hospital discharge/death (days). In the crude Cox regression only obesity (HR 3.923, $p=0.015$), Sa/FiO₂ (HR 0.987, $p=0.032$), GMCSF (HR 1.975, $p=0.034$), IL-15 (HR 1.252, $p=0.023$) and zonulin levels (HR 1.106, $p=0.001$) were found to be significant ([Table 5](#)).

As for the case of the adjusted Cox regression (adjustment for age, gender and obesity, obesity was adjusted for age and gender only), the same variables remained significant except GMCSF and IL-15 ([Table 5](#)). Moreover, segmented neutrophils (HR 1.215, $p=0.023$), lymphocytes (HR 0.764, $p=0.010$) and IL-2 (HR 4.018, $p=0.050$) were not significant in the crude Cox regression analysis but were significant in the adjusted analysis.

Discussion

Even with readily access to vaccines, COVID-19 is still a threat to public health worldwide with many aspects of its pathophysiology, as well as its consequences after clinical improvement of the infection, requiring study. The present study provides knowledge on the detection of zonulin in COVID-19 patients and some risk factors for death by COVID-19. Serum zonulin levels in a Peruvian cohort of hospitalised COVID-19 patients were measured, and an association was found with mortal outcome (both in bivariate

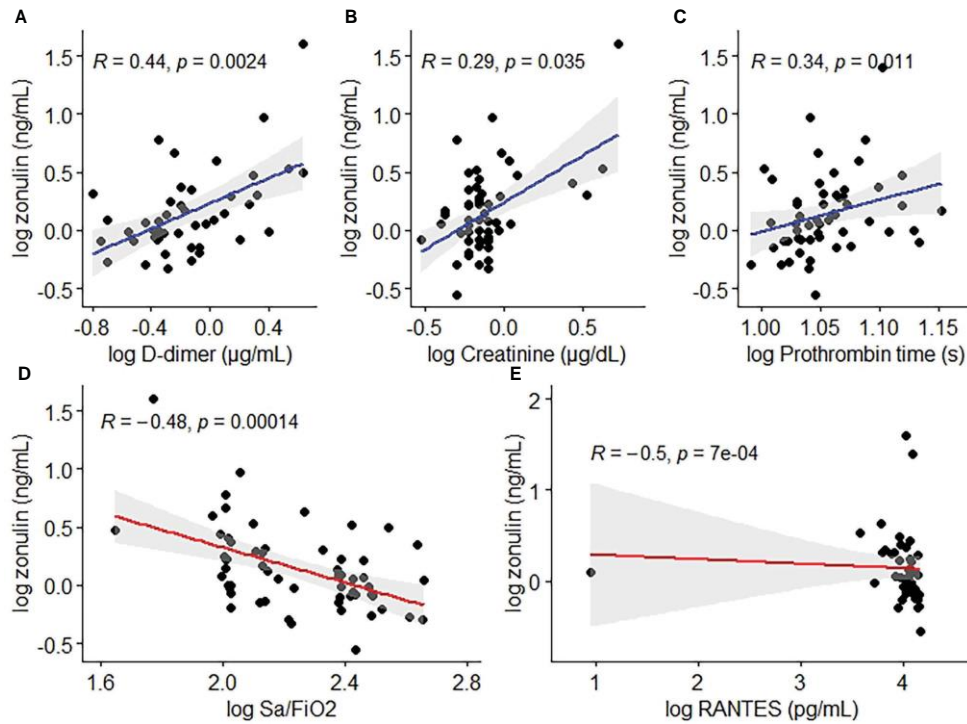


FIGURE 1
Scatterplots depicting significant correlations between zonulin and D-dimer (A), Creatinine (B), Prothrombin Time (C), Sa/FiO₂ (D) and RANTES (E).

TABLE 4 Crude and adjusted* logistic regression analysis for predictability of death according to different factors at baseline.

Variables	Crude OR (95% CI) p value	Adjusted OR (95% CI) p value
Severity		
Severe	4.750 (0.957-23.572) p=0.057	3.518 (0.593-20.874) p=0.166
Moderate*		
Obesity		
Yes	7.200 (1.933-26.812) p=0.003	8.103 (2.067-31.768) p=0.003
No*		
Haemoglobin	0.624 (0.408-0.955) p=0.030	0.703 (0.416-1.186) p=0.187
Seg Neutrophils	1.145 (1.027-1.278) p=0.015	1.303 (1.064-1.596) p=0.010
Lymphocytes	0.821 (0.696-0.967) p=0.018	0.726 (0.563-0.936) p=0.013
SaFiO ₂	0.985 (0.974-0.996) p=0.007	0.987 (0.976-0.998) p=0.025
IL-6	1.012 (0.996-1.028) p=0.157	1.011 (0.994-1.030) p=0.212
GMCSF	2.220 (1.166-4.224) p=0.015	2.222 (0.977-5.050) p=0.057
IL-15	1.332 (1.026-1.730) p=0.031	1.233 (0.939-1.620) p=0.132
IL-2	3.089 (1.153-8.274) p=0.025	2.555 (0.839-7.778) p=0.099
IL-8	1.121 (0.977-1.287) p=0.103	(S)
IL1a	1.056 (0.953-1.169) p=0.297	1.026 (0.920-1.146) p=0.641
MIPa	1.374 (0.933-2.022) p=0.108	1.397 (0.931-2.097) p=0.106
MCP-1	1.001 (1.000-1.001) p=0.010	1.002 (0.999-1.004) p=0.112
Zonulin	2.573 (1.371-4.828) p=0.003	2.666 (1.310-5.426) p=0.007

*Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Cytokines were adjusted for age and obesity only due to collinearity issues with gender. (S): Value could not be computed as log likelihood = 0.

TABLE 5 Crude and adjusted* Cox regression of different factors for survival analysis.

Variables	Crude	Adjusted
	HR (95% CI) p value	HR (95% CI) p value
Severity		
Severe	3.436 (0.759-15.566) p=0.109	4.282 (0.796-23.019) p=0.090
Moderate*		
Obesity		
Yes	3.923 (1.306-11.783) p=0.015	5.888 (1.646-21.068) p=0.006
No*		
Haemoglobin	0.669 (0.444-1.008) p=0.055	0.770 (0.480-1.235) p=0.278
Seg Neutrophils	1.083 (0.980-1.197) p=0.118	1.215 (1.028-1.437) p=0.023
Lymphocytes	0.874 (0.748-1.020) p=0.087	0.764 (0.623-0.938) p=0.010
SaFiO ₂	0.987 (0.975-0.999) p=0.032	0.986 (0.974-0.998) p=0.023
IL-6	1.010 (0.997-1.024) p=0.138	1.013 (0.997-1.029) p=0.122
GMCSF	1.975 (1.051-3.711) p=0.034	2.145 (0.889-5.115) p=0.085
IL-15	1.252 (1.032-1.518) p=0.023	1.218 (0.987-1.502) p=0.066
IL-2	3.080 (0.910-10.427) p=0.070	4.018 (1.001-16.132) p=0.050
IL-8	1.059 (0.997-1.124) p=0.061	1.080 (0.975-1.196) p=0.142
IL1a	1.026 (0.936-1.124) p=0.590	1.011(0.913-1.120) p=0.827
MIPa	1.969 (0.917-4.228) p=0.082	2.671 (0.781-9.129) p=0.117
MCP-1	1.001 (1.000-1.001) p=0.020	1.001 (0.999-1.002) p=0.110
Zonulin	1.106 (1.043-1.173) p=0.001	1.109 (1.038-1.185) p=0.002

*Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Zonulin values were not categorised for the analyses made herein.

and multivariable analyses, even after adjustment for age, gender, and obesity).

Based on the clinical-epidemiological characteristics of this cohort, the relationship of obesity and fatal outcome in COVID-19 patients has previously been described (Pons et al., 2021). This finding is in agreement with other studies reporting obesity as a strong predictor of death in severe COVID-19 infections (Cai et al., 2021; Dessie and Zewotir, 2021; Yang et al., 2021), as it involves both impaired cellular immunity and an amplification of the underlying obesity-related inflammatory status together with the acute inflammation generated by SARS-CoV-2 infection (Mohammad et al., 2021).

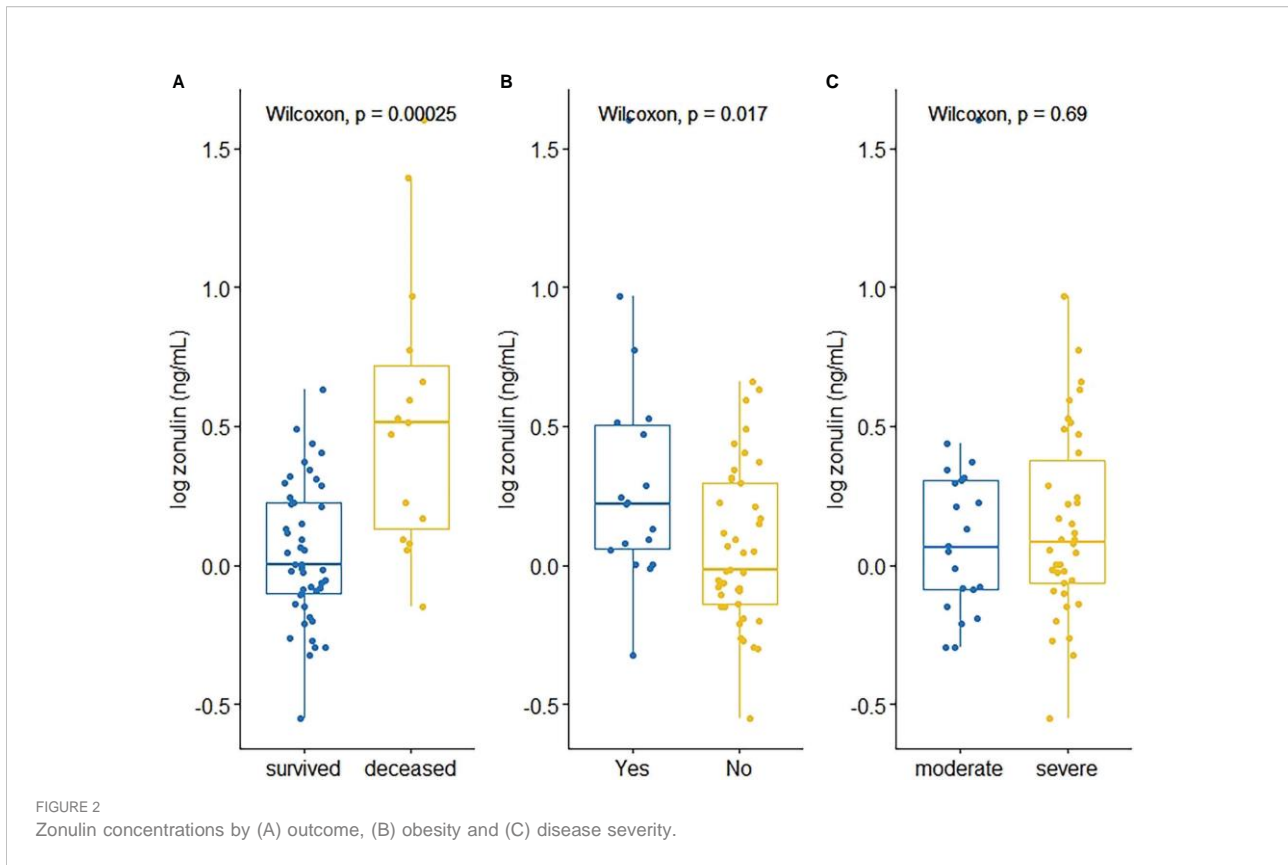
In addition, a lower Sa/FiO₂ ratio and a decreased lymphocyte count were associated with death by COVID-19 (Henry et al., 2020; Huang and Pranata, 2020; Booth et al., 2021; Pons et al., 2021; Gopalan et al., 2022; Soto et al., 2022). It should be noted that lymphopenia might occur due to consequent apoptosis of activated CD4+T cells by SARS-CoV-2 (Shen et al., 2022), thereby inducing immune suppression.

Although there are few reports about the role of zonulin in COVID-19 infections, Giron et al. (Giron et al., 2021) also reported high zonulin levels in the deceased group, similar to our results. In another study, Oliva et al. (Oliva et al., 2021) found median zonulin values similar to those in our study on comparing COVID-19 patients and matched healthy controls. In addition, it is important to highlight the role of zonulin in children with multisystem inflammatory syndrome (MIS), related to the persistence of the virus in the gastrointestinal

tract (Kılıçet al., in press; Josyabhatla et al., 2021). Therefore, zonulin might be associated with a translocation of viral proteins into the bloodstream, contributing to increased inflammation.

Before COVID-19, the zonulin-dependent permeability of intestinal tight junctions had been implicated in chronic inflammatory diseases such as celiac disease, inflammatory bowel disease, type I diabetes (Fasano, 2011), as well as obesity, septicemia, environmental enteropathy, necrotizing enterocolitis, among others (Sturgeon and Fasano, 2016). In these scenarios, the release of zonulin and loss of gut barrier function are followed by microbial translocation, which triggers immune responses and the production of pro-inflammatory cytokines that exacerbate gut permeability in a vicious cycle (Fasano, 2020). Apart from the intestinal system, overexpression of zonulin has also been reported in other organs such as the lungs and brain (Skardelly et al., 2009; Rittirsch et al., 2013).

Related to COVID-19 infection, SARS-CoV-2 affects the gastrointestinal tract as evidenced by gastrointestinal symptoms and detection of the virus in faeces (Groff et al., 2021). Additionally, viral antigens have been found in intestinal enterocytes after resolution of clinical illness in adults (Gaebler et al., 2021) and zonulin hyper permeability of the mucosal barrier coincides with SARS-CoV-2 antigenemia (Yonker et al., 2021). It has previously been speculated that zonulin participates in a complex mechanism linking the wide variety of symptoms associated with SARS-CoV-2 infection, especially involving the digestive system and the nervous system (Llorens et al., 2021). Specifically, disruption of the intestinal barrier with subsequent



microbial translocation could drive inflammatory activation in severe COVID-19 (Giron et al., 2021) with positive feedback of pro-inflammatory cytokines, with the amplified systemic response being stronger in patients with comorbidities (Trottein and Sokol, 2020). Given that our results suggest that zonulin is associated with death, even when adjusted for obesity, this is consistent with the above hypothesis.

Regarding correlations, zonulin was found to be directly correlated with creatinine, D-dimer values, and prothrombin time, all of which have previously been associated with poorer outcome in hospitalised COVID-19 patients (Malik et al., 2021) and inversely correlated with CCL5/RANTES and Sa/FiO₂. As for the chemokine CCL5 (RANTES), elevated values have been found in moderate but not severe COVID-19 cases and is considered as a prognostic factor for survival (Zhao et al., 2020). It should be mentioned that the use of the zonulin inhibitor, larazotide acetate, in a case series of four children with MIS resulted in a faster resolution of gastrointestinal symptoms and faster clearance time of the SARS-CoV-2 spike antigen (Yonker et al., 2022). This inhibitor is also being tested in a clinical trial (ClinicalTrials.gov identifier NCT05022303) in children with long-COVID, for whom a persistent inflammatory effect has been reported (Galán et al., 2022).

It should be noted that zonulin concentrations described in the studies available vary greatly. This variability could be

explained by the shelf life of the samples and differences in the detection methods. Additionally, there are concerns regarding the validity of the ELISAs performed for zonulin quantification, in which the kits may detect not only zonulin but also zonulin-related proteins such as properdin (Scheffler et al., 2018). Another option is quantification of pre-haptoglobin 2 specifically (and no other zonulin-like proteins) with other methods such as western blot and/or mass spectrometry.

As mentioned before, a mounting body of evidence suggests that zonulin is a surrogate marker of tight junction permeability; however, estimation of microbial translocation could be reinforced using other known serum makers such as bacterial DNAemia, b-glucan, LPS-binding protein (LBP), intestinal-fatty acid binding protein (iFABP), soluble CD14 or others (Stehle et al., 2012; Koutsounas et al., 2015; Fukui, 2016), which could be options to consider alongside zonulin in future studies.

The present study has some limitations. The relatively small sample size for zonulin (n=60) and cytokine/chemokine values (n=42) might be responsible for type II errors, therefore some associations expected in previous studies, such as mortality and IL-6 values (Liu et al., in press), were found to be non-significant. This also contributed to the adjusted regressions; because of the small number of events (deaths), the regression models were adjusted for a few key confounders only to avoid model over-fitting. Thus, the findings presented herein are relevant, but a

caveat must be made, as the magnitude of the associations should be interpreted with caution.

In conclusion, zonulin levels (as an indirect measure of microbial translocation levels) were detected in hospitalised patients with COVID-19 and were associated with death even after adjustment for age, gender, and obesity in a peruvian cohort of COVID-19. Future longitudinal studies on zonulin levels are recommended to determine their variation over time, as well as to identify their role in patients presenting systemic inflammatory syndrome/long-COVID.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by CIEI Universidad Científica del Sur-151-2022-POS50. Written informed consent was not provided because the samples were for diagnostic purposes, and, due to the first wave of COVID in Peru, the ethics committee approved the use of diagnostic samples.

Author contributions

MP, LP-K and JR conceived the study. LP-K, MP, BY, AM performed zonulin quantification by ELISA. MU-G obtained the samples and clinical information. LP-K performed the statistical analyses. LP-K and MP wrote the manuscript. All authors made

contributions to the draft and approved the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Zonulin, a marker of gut permeability, and its association with worse outcome in hospitalised Peruvian COVID-19 patients

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13 **Abstract**

14 Zonulin has previously been related to intestinal permeability in various inflammatory diseases, and
15 more recently to the physiopathology of severe COVID-19 infections. We analysed serum samples
16 from a previous study of a Peruvian cohort of hospitalised COVID-19 patients, for the quantification
17 of zonulin by sandwich ELISA. Comparisons with clinical data, haematological and biochemical
18 parameters and cytokine/chemokine levels were made. We found higher baseline zonulin levels in
19 deceased patients, and zonulin was associated with fatal outcome in multivariate analyses, even after
20 adjustment for age, gender, and obesity. There were also positive correlations between zonulin,
21 creatinine, D-dimer values and prothrombin time, while an inverse correlation was found for Sa/FiO₂
22 ratio and CCL5 (RANTES). Further longitudinal studies are recommended to analyse the variation of
23 zonulin levels over time as well as their relationship with long-COVID.

24

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31 1. Introduction

32 Severe COVID-19 infections are characterised by systemic inflammation, acute respiratory distress
 33 syndrome (ARDS) and multi-organic system failure (Kim et al., 2021) in adults. The SARS-CoV-2
 34 virus binds to receptor angiotensin-converting enzyme 2 (ACE2) to enter host cells, mainly type II
 35 pneumocytes in the lung, as well as in the kidney, heart, uterus, brain, vascular walls, and intestine
 36 (Lamers et al., 2020). In particular for the latter, the intestinal enterocytes form a cell layer that is part
 37 of the mucosal defence system and are held together by tight junctions. Disruption of this layer
 38 (increased gut permeability) translates into translocation of bacterial components from the gut into the
 39 bloodstream, contributing to a further increase in systemic inflammation (Dinh et al., 2015).

40 As reported in other viral infections such as dengue fever (Chanchaoenthana et al., 2021) or HIV
 41 (Marchetti et al., 2013), microbial translocation may be caused by direct enterocyte infection from the
 42 virus or because of cytokine alterations and gut dysbiosis. Previous studies (Giron et al., 2021; Oliva
 43 et al., 2021; Yonker et al.) have linked higher intestinal translocation with severe cases of COVID-19,
 44 describing a higher abundance of bacterial components in the bloodstream compared to milder cases.
 45 It has been suggested that microbial translocation, intestinal dysbiosis and underlying inflammatory
 46 processes might have a synergistic effect on systemic inflammation and high cytokine levels described
 47 in severe COVID-19 infections (Cardinale et al., 2020; Uzzan et al., 2020; Vignesh et al., 2020;
 48 Mashaqi et al., 2022).

49 Zonulin (pre-Haptoglobin 2) is a protein of ~ 47 kDa, which is detectable in its uncleaved form in
 50 human serum (Fasano, 2011) and is highly correlated with bacterial DNAemia (Gargari et al., 2021).
 51 It is a physiological mediator that reversibly regulates intestinal permeability by modulation of
 52 intercellular tight junctions (Tripathi et al., 2009) and is considered a biomarker of impaired gut barrier
 53 function (Fasano, 2011). In this sense, it has been hypothesised that the spike protein of SARS-CoV-2
 54 produces overexpression of zonulin, which is related to an increase in intestinal permeability (Llorens
 55 et al., 2021). Moreover, zonulin levels have been reported to be higher in patients who died of severe
 56 COVID-19 (Giron et al., 2021).

57 For the reasons mentioned above, the present study aimed to identify the association between microbial
 58 translocation estimated by serum zonulin and patient outcome in a cohort of moderate and severe
 59 COVID-19 hospitalised patients from Lima, Peru. Additionally, correlations were established amongst
 60 cytokine/chemokine values, routine clinical parameters, and serum zonulin levels.

61

62 2. Materials and Methods

63 2.1 Study population and assessment of disease severity

64 The present study used samples from a cohort of hospitalised COVID-19 patients from *Hospital*
 65 *Nacional Guillermo Almenara Irigoyen* (Pons et al., 2021). A total of 60 patients were classified
 66 according to severity: 19 moderate, 26 severe and 15 deaths (initially classified as moderate or severe).
 67 The COVID-19 cases were confirmed by quantitative polymerase chain reaction (qPCR) or antibody
 68 testing. The follow-up period was from August to October 2020, which was within the context of the
 69 first wave of COVID-19 in Peru. Therefore, no patient had received vaccination for COVID-19.

70 The definition of moderate infection was according to the Berlin 2012 definition of Acute Respiratory
 71 Distress Syndrome (ARDS) (ARDS Definition Task Force et al., 2012), while severe infection was

72 defined whenever one or more of the following states developed: Horowitz index ($\text{PaO}_2/\text{FiO}_2$) < 100,
 73 oxygen saturation to fraction of inspired oxygen ratio ($\text{SpO}_2/\text{FiO}_2$, henceforth abbreviated as Sa/FiO_2)
 74 < 89, need for mechanical ventilation due to respiratory failure or systemic organ failure requiring
 75 admission to the intensive care unit.

76 2.2 Sample conditions

77 Serum samples from the original study were conserved at $-80\text{ }^\circ\text{C}$ in the laboratories of the *Universidad*
 78 *Científica del Sur*.

79 2.3 Clinical Data and cytokine levels

80 Data pertaining to age, gender, dates of hospitalisation and discharge/death, comorbidities such as
 81 obesity, arterial hypertension, diabetes, chronic obstructive pulmonary disease (COPD) and any other
 82 underlying conditions, pharmacological treatment received, haemoglobin, platelets, white blood cell
 83 (WBC) counts and Sa/FiO_2 were obtained from the corresponding medical records. All privileged
 84 identifiable information of the patients was excluded from the dataset generated and the samples were
 85 codified previously to ensure confidentiality.

86 The cytokine and chemokine concentrations of the serum samples were obtained from the data
 87 previously reported in the study by Pons *et al.* (Pons *et al.*, 2021), in which a commercial kit of Luminex
 88 MAGPIX technology (Merck KGaA, Darmstadt, Germany) was used for quantification.

89 2.4 Estimation of microbial translocation by zonulin quantification

90 Zonulin (ng/mL) was quantified from the serum samples by sandwich ELISA, using the CUSABIO
 91 Human Zonulin ELISA Kit (CUSABIO TECHNOLOGY LLC, Wuhan, China) according to the
 92 manufacturer's instructions. Briefly, 100 μL of standards and samples were added per well in the
 93 coated microplate and incubated for two hours at $37\text{ }^\circ\text{C}$. Subsequently, 100 μL of biotin-antibody
 94 solution was added and incubated for one hour at $37\text{ }^\circ\text{C}$. Afterwards, the liquid was aspirated, and
 95 manual washing was done three times with the provided wash buffer. Then 100 μL of horseradish
 96 peroxidase-avidin solution was added per well and incubated for one hour at $37\text{ }^\circ\text{C}$, and manual
 97 washing was done thereafter. Finally, 90 μL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was
 98 added per well and incubated for 30 minutes at $37\text{ }^\circ\text{C}$. Immediately after, 50 μL of stop solution was
 99 added per well and absorbance reading was done at 450 nm (with correction at 540 nm) in a Synergy
 100 LX Multi-Mode Reader (Biotek, USA). The samples and standards were run in duplicate.

101 2.5 Statistical analysis

102 Descriptive analysis was performed using absolute and relative frequencies for summarising
 103 categorical variables while median and interquartile range (IQR) were used for numerical variables,
 104 unless specified otherwise.

105 Bivariate analyses were made for comparisons between outcomes (alive vs. deceased) and categorical
 106 variables using the Chi-squared or Fisher exact test, while the Mann-Whitney U test was used for
 107 comparisons between outcomes and numerical variables. Correlations among WBC counts,
 108 biochemical markers, cytokines, chemokines and zonulin levels were normalised using base 10 log
 109 transformation and comparisons were made using Spearman tests.

110 Crude and adjusted multivariate analyses of potential prognostic factors of death were performed using
 111 logistic regression with clinical variables, cytokine and zonulin levels at baseline (from first serum
 112 samples at admission) and odds ratios (OR) were obtained. Survival analyses were done using Cox
 113 regression, and crude and adjusted hazard ratios (HR) were estimated accordingly. Significant variables
 114 in bivariate analysis were employed for the regression analyses. Epidemiological criteria for
 115 adjustment of confounding and the use of variables with less than 20% data loss were employed to
 116 decide which adjusted model best fitted the data.

117 For the interpretation of the zonulin results, samples with a zonulin concentration above the upper limit
 118 of the standard (40 ng/mL) were assigned this value. Values below 0.625 ng/mL were used as their
 119 actual values for the analyses.

120 The analyses were conducted using STATA 16.1 SE Edition (StataCorp, TX) and graphics were
 121 constructed using RStudio v. 4.02. 95% Confidence intervals were calculated for OR and HR.
 122 Statistical significance was established with $\alpha=0.05$.

123

124 **3. Results**

125 **3.1 Baseline clinical characteristics of the hospitalised cohort**

126 Sixty hospitalised COVID-19 patients were included: 45 patients (75%) survived, and 15 (25%)
 127 patients died during the follow-up period. The distribution for case severity was as follows: 39 patients
 128 (65%) were classified as severe and 21 (35%) as moderate according to the given case definition and
 129 progression during the follow-up period. Severity was associated with outcome ($p=0.042$). The median
 130 age was similar in both the alive and deceased groups (55 and 55 years, respectively) and there was no
 131 significant difference in age ($p=0.694$) or sex ($p=0.678$) between the two groups.

132 Some comorbidities such as obesity, diabetes, high blood pressure and others were present (Table 1).
 133 In the deceased group, 12 patients (80%) had comorbidities compared to 22 (48.9%) in the alive group,
 134 and this difference was significant ($p=0.035$). The only comorbidity that was associated with outcome
 135 was obesity ($p=0.006$). Corticosteroids were employed as part of the treatment for all the patients in
 136 this cohort.

137 **3.2 White blood cell counts, biochemical markers, and cytokine/chemokine levels at baseline**

138 Haemoglobin levels at baseline were lower in the deceased, with a mean (standard deviation) of 13.19
 139 (1.93) g/dL ($p=0.018$). Regarding WBC counts, segmented neutrophil counts were higher ($p=0.006$)
 140 and lymphocyte counts were lower in the deceased group ($p=0.004$) with no significant differences
 141 being found for the remaining WBC counts in the two groups.

142 In relation to some biochemical markers such as C-reactive protein, D-dimer and ferritin, there were
 143 no significant differences when comparisons were made by outcome (Table 1). However, the Sa/FiO₂
 144 ratio was lower in the deceased group, with a median (IQR) of 108.73 (35.40) ($p=0.0007$).

145 The cytokines interleukin-6 (IL-6) ($p=0.029$), granulocyte-macrophage colony-stimulating factor
 146 (GM-CSF) ($p=0.005$), IL-15 ($p=0.046$), IL-2 ($p=0.027$), IL-8 ($p=0.0004$), IL-1a ($p=0.032$), and
 147 chemokines macrophage inflammatory protein- α (MIP α) ($p=0.008$) and monocyte chemoattractant

148 protein-1 (MCP-1) ($p=0.001$) showed significant differences according to outcome (Table 2), with all
 149 being elevated in the deceased group.

150 **3.3 Correlations of zonulin versus haematological / biochemical markers and** 151 **cytokines/chemokines at baseline**

152 Zonulin levels were correlated with creatinine ($\rho=0.288$, $p=0.035$) D-dimer values ($\rho=0.441$, $p=0.002$),
 153 as well as prothrombin time ($\rho=0.344$, $p=0.011$) and Sa/FiO₂ ratio ($\rho=-0.4764$, $p=0.0001$).

154 Related to cytokines and chemokines, RANTES was the only chemokine inversely correlated with
 155 zonulin levels ($\rho=-0.5023$, $p=0.007$). None of the cytokines were significantly correlated with zonulin
 156 levels.

157 **3.4 Zonulin levels at baseline associated with outcome in the bivariate and multivariate analyses**

158 The median (IQR) level of zonulin in the deceased group was 3.264 (4.7096) ng/mL (min 0.7099
 159 ng/mL – max 40 ng/mL) (Figure 1) showing a significant association with outcome ($p=0.0002$) in the
 160 bivariate analysis (Table 2).

161 Several factors in logistic regression, such as obesity (OR 7.200, $p=0.003$), segmented neutrophils (OR
 162 1.145, $p=0.015$), GMCSF (OR 2.220, $p=0.015$), IL-15 (OR 1.332, $p=0.031$), IL-2 (OR 3.089, $p=0.025$),
 163 and zonulin levels (OR 2.573, $p=0.003$), presented significant OR values greater than 1 in the crude
 164 analysis. In contrast, haemoglobin (OR 0.624, $p=0.030$), lymphocytes (OR 0.821, $p=0.018$) and
 165 Sa/FiO₂ (OR 0.985, $p=0.007$) had significant ORs less than 1 (Table 3).

166 Notwithstanding, in the adjusted regression, only obesity (only adjusted for age and gender, OR 8.103
 167 $p=0.003$), segmented neutrophils (OR 1.303, $p=0.010$), lymphocytes (OR 0.726, $p=0.013$), Sa/FiO₂
 168 (OR 0.987, $p=0.025$) and zonulin levels (OR 2.666, $p=0.007$) remained significant after adjustment for
 169 age, gender, and obesity (Table 3).

170 **3.5 Cox regression analysis**

171 Cox regression was performed as a survival analysis with the same predictors as logistic regression,
 172 considering the variable time to death/hospital discharge (days). In the crude Cox regression only
 173 obesity (HR 3.923, $p=0.015$), Sa/FiO₂ (HR 0.987, $p=0.032$), GMCSF (HR 1.975, $p=0.034$), IL-15 (HR
 174 1.252, $p=0.023$) and zonulin levels (HR 1.106, $p=0.001$) were found to be significant (Table 4).

175 As for the case of the adjusted Cox regression (adjustment for age, gender and obesity, obesity was
 176 adjusted for age and gender only), the same variables remained significant except GMCSF and IL-15
 177 (Table 4). Moreover, segmented neutrophils (HR 1.215, $p=0.023$), lymphocytes (HR 0.764, $p=0.010$)
 178 and IL-2 (HR 4.018, $p=0.050$) were not significant in the crude Cox regression analysis but were
 179 significant in the adjusted analysis.

180

181 **4. Discussion**

182 Even with ready access to vaccines, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)
 183 infection is still a threat to public health worldwide with many aspects of its pathophysiology, as well

184 as its consequences after clinical improvement of the infection, requiring study. The present study
 185 provides knowledge on the detection of zonulin in COVID-19 patients and some risk factors for death
 186 by COVID-19. Serum zonulin levels in a Peruvian cohort of hospitalised COVID-19 patients were
 187 measured, and an association was found with mortal outcome (both in bivariate and multivariate
 188 analyses, even after adjustment for age, gender, and obesity).

189 Based on the clinical-epidemiological characteristics of this cohort, the relationship of obesity and fatal
 190 outcome in COVID-19 patients has previously been described (Pons et al., 2021). This finding is in
 191 agreement with other studies reporting obesity as a strong predictor of death in severe COVID-19
 192 infections (Cai et al., 2021; Dessie and Zewotir, 2021; Yang et al., 2021), as it involves both impaired
 193 cellular immunity and an amplification of the underlying obesity-related inflammatory status together
 194 with the acute inflammation generated by SARS-CoV-2 infection (Mohammad et al., 2021).

195 In addition, a lower Sa/FiO₂ ratio and a decreased lymphocyte count were associated with death by
 196 COVID-19 (Henry et al., 2020; Huang and Pranata, 2020; Booth et al., 2021; Pons et al., 2021; Gopalan
 197 et al., 2022; Soto et al., 2022). It should be noted that lymphopenia might occur due to consequent
 198 apoptosis of activated CD4+T cells by the SARS-CoV-2 virus (Shen et al., 2022), thereby inducing
 199 immune suppression.

200 Although there are few reports about the role of zonulin in COVID-19 infections, Giron *et al.* (Giron
 201 et al., 2021) also reported high zonulin levels in the deceased group, similar to our results. In another
 202 study, Oliva *et al.* (Oliva et al., 2021) found median zonulin values similar to those in our study on
 203 comparing COVID-19 patients and matched healthy controls. In addition, it is important to highlight
 204 the role of zonulin in children with multisystem inflammatory syndrome (MIS), related to the
 205 persistence of the virus in the gastrointestinal tract (Josyabhatla et al., 2021; Kılıç et al.). Therefore,
 206 zonulin might be associated with a translocation of viral proteins into the bloodstream, contributing to
 207 increased inflammation.

208 Before COVID-19, the zonulin-dependent permeability of intestinal tight junctions had been
 209 implicated in chronic inflammatory diseases such as celiac disease, inflammatory bowel disease, type
 210 I diabetes (Fasano, 2011), as well as obesity, septicaemia, environmental enteropathy, necrotizing
 211 enterocolitis, among others (Sturgeon and Fasano, 2016). In these scenarios, the release of zonulin and
 212 loss of gut barrier function are followed by microbial translocation, which triggers immune responses
 213 and the production of pro-inflammatory cytokines that exacerbate gut permeability in a vicious cycle
 214 (Fasano, 2020). Apart from the intestinal system, overexpression of zonulin has also been reported in
 215 other organs such as the lungs and brain (Rittirsch et al., 2013; Skardelly et al., 2009).

216 Related to COVID-19 infection, the SARS-CoV-2 virus affects the gastrointestinal tract as evidenced
 217 by gastrointestinal symptoms and detection of the virus in faeces (Groff et al., 2021). Additionally,
 218 viral antigens have been found in intestinal enterocytes after resolution of clinical illness in adults
 219 (Gaebler et al., 2021) and zonulin hyper permeability of the mucosal barrier coincides with SARS-
 220 CoV-2 antigenemia (Yonker et al.). It has previously been speculated that zonulin participates in a
 221 complex mechanism linking the wide variety of symptoms associated with SARS-CoV-2 infection,
 222 especially involving the digestive system and the nervous system (Llorens et al., 2021). Specifically,
 223 disruption of the intestinal barrier with subsequent microbial translocation could drive inflammatory
 224 activation in severe COVID-19 (Giron et al., 2021) with positive feedback of pro-inflammatory
 225 cytokines, with the amplified systemic response being stronger in patients with comorbidities (Trottein
 226 and Sokol, 2020). Given that our results suggest that zonulin is associated with death, even when
 227 adjusted for obesity, this is consistent with the above hypothesis.

228 Regarding correlations, zonulin was found to be directly correlated with creatinine, D-dimer values,
 229 and prothrombin time, all of which have previously been associated with poorer outcome in
 230 hospitalised COVID-19 patients (Malik et al., 2021) and inversely correlated with CCL5/RANTES and
 231 Sa/FiO₂. As for the chemokine CCL5 (RANTES), elevated values have been found in moderate but
 232 not severe COVID-19 cases and is considered as a prognostic factor for survival (Zhao et al.). It should
 233 be mentioned that the use of the zonulin inhibitor, larazotide acetate, in a case series of four children
 234 with MIS resulted in a faster resolution of gastrointestinal symptoms and faster clearance time of the
 235 SARS-CoV-2 spike antigen (Yonker et al., 2022). This inhibitor is also being tested in a clinical trial
 236 (ClinicalTrials.gov identifier NCT05022303) in children with long-COVID, for whom a persistent
 237 inflammatory effect has been reported (Galán et al., 2022).

238 The present study has some limitations. The relatively small sample size for zonulin (n=60) and
 239 cytokine/chemokine values (n=42), might be responsible for type II errors, and therefore, some
 240 associations expected in previous studies, such as mortality and IL-6 values (Liu et al., 2021), were
 241 found to be non-significant. This also contributed to the adjusted regressions; because of the small
 242 number of events (deaths), the regression models were adjusted for a few key confounders only to
 243 avoid model over-fitting. Thus, the findings presented herein are relevant, but a caveat must be made,
 244 as the magnitude of the associations should be interpreted with caution.

245 It should be noted that zonulin concentrations described in the studies available vary greatly. This
 246 variability could be explained by the shelf life of the samples and differences in the detection methods.
 247 Additionally, there are concerns regarding the validity of the ELISAs performed for zonulin
 248 quantification, in which the kits may detect not only zonulin but also zonulin-related proteins such as
 249 properdin (Scheffler et al., 2018).

250 In conclusion, zonulin levels (as an indirect measure of microbial translocation levels) were detected
 251 in hospitalised patients with COVID-19 and were associated with death even after adjustment for age,
 252 gender, and obesity in a Peruvian cohort of COVID-19. Future longitudinal studies on zonulin levels
 253 are recommended to determine their variation over time, as well as to identify their role in patients
 254 presenting systemic inflammatory syndrome/ long-COVID.

255 **5. Data Availability Statement**

256 The raw data supporting the conclusions of this article will be made available by the authors, without
 257 undue reservation.

258 **6. Ethics Statement**

259 Approbation for this project was given by the Institutional Ethics Committee of the *Universidad*
 260 *Científica del Sur*. Additionally, an addendum to the original study was approved by the Research
 261 Ethics Committee of *IETSI-EsSalud* for COVID-19 for the studies on microbial translocation.

262 **7. Author Contributions**

263 MP, LPK and JR conceived the study. LPK, MP, BY, AMH performed zonulin quantification by
 264 ELISA. MU obtained the samples and clinical information. LPK performed the statistical analyses.
 265 LPK and MP wrote the manuscript. All authors made contributions to the draft and approved the final
 266 version.

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269 9. Conflict of Interest

270 The authors declare that the research was conducted in the absence of any commercial or financial
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275 11. References

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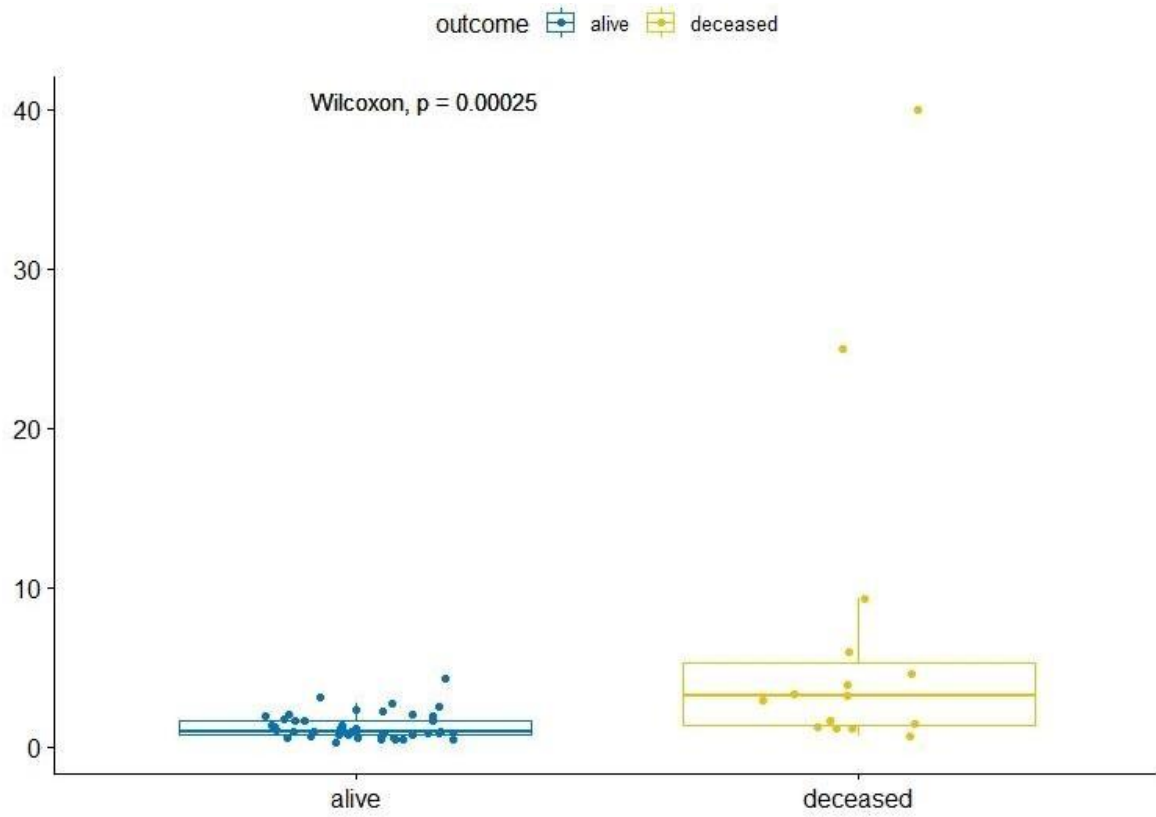
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411 **Figure 1. Zonulin concentrations by outcome**

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438 Table 1. Characteristics of the study cohort by outcome at baseline. Values of continuous variables are
 439 reported as median (interquartile range), unless stated otherwise. CKD: Chronic Kidney Disease,
 440 COPD: Chronic Obstructive Pulmonary Disease, CVD: Cardiovascular disease, CRP: C-Reactive
 441 Protein, PT: Prothrombin Time, APTT: Activated Partial Thromboplastin Time, SaFiO₂: Oxygen
 442 saturation/Fraction of inspired oxygen.

	Alive	Deceased	p-value	
443				
444				
445				
446				
447	Overall, n=60 (%)	45 (75.00)	15 (25.00)	-
448	Age (years)	55 (19)	55 (25)	0.6943
449	Sex			
450	Male (%)	39 (86.67)	12 (80.00)	0.6780
451	Female (%)	6 (13.33)	3 (20.00)	
452	Severity			
453	Moderate (%)	19 (42.22)	2 (13.33)	0.0420
454	Severe (%)	26 (57.78)	13 (86.67)	
455	Hospital discharge time (days)	11 (8)	12.5 (12)	0.9786
456				
457	Presence of Comorbidities, n (%)			
458	None	23 (51.11)	3 (20.00)	0.035
459	Obesity	9 (20.00)	9 (64.29)	0.006
460	Diabetes	5 (11.11)	2 (14.29)	0.666
461	High Blood Pressure	8 (17.78)	5 (35.71)	0.266
462	CKD	1 (2.22)	0 (0.00)	1.000
463	COPD	1 (2.22)	2 (14.29)	0.137
464	CVD	0 (0.00)	1 (7.14)	0.237
465	Gout	1 (2.22)	0 (0.00)	1.000
466	Other	3 (6.67)	0 (0.00)	0.566
467				
468	Haemoglobin, white blood cell count and other biomarkers			
469	Haemoglobin (g/dL), mean (SD)	14.38 (1.52)	13.19 (1.93)	0.0176
470	Leucocytes (10 ⁹ /L)	9 (6.87)	12.485 (8.17)	0.2142
471	Band neutrophils (%)	2 (3)	2 (2)	0.6491
472	Segmented neutrophils (%)	83 (12)	88 (7)	0.0060
473	Lymphocytes (%)	10 (10)	6 (4)	0.0045
474	Total neutrophils (10 ⁹ /L)	7.56 (7.32)	10.82 (8.26)	0.2307
475	Total lymphocytes (10 ⁹ /L)	0.94 (0.77)	0.59 (0.33)	0.0046
476	Platelets (count per µL)	345000 (147000)	274500 (45000)	0.1850
477	CRP (mg/mL)	61.3 (163.8)	168.7 (122.8)	0.1701
478	D-dimer (µg/mL)	0.58 (0.51)	0.85 (1.86)	0.1425
479	Ferritin (µg/L)	789.7 (679.5)	1261.1 (838)	0.0536
480	Creatinine (mg/dL)	0.7 (0.2)	0.8 (0.48)	0.4152
481	PT (s)	11.17 (0.89)	11.57 (1.31)	0.1627
482	APTT (s)	30.87 (5.15)	32.65 (4.22)	0.1782
483	Sa/FiO ₂	240 (162.63)	108.73 (35.40)	0.0007

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488 Table 2. Zonulin, cytokines and chemokines values in serum at baseline by outcome. Values of
 489 continuous variables are reported as median (interquartile range).

490

	Alive	Deceased	p-value	
491				
492				
493	Overall, n=60 (%)	45 (75.00)	15 (25.00)	-
494	Zonulin (ng/mL)	1.0029 (0.8887)	3.2644 (4.7096)	0.0002

495

	Alive	Deceased	p-value	
496				
497				
498	Overall, n=42 (%)	37 (88.1)	5 (11.90)	-
499	IL-1 β (pg/mL)	10 (1.5)	11 (2)	0.1194
500	GCSF (pg/mL)	11.5 (3)	12 (3)	0.3083
501	IL-10 (pg/mL)	19.5 (11)	49.5 (54)	0.1246
502	IL-13 (pg/mL)	8 (2)	8(2)	0.2062
503	IL-6 (pg/mL)	21 (38)	55 (130)	0.0295
504	IL-12 (pg/mL)	13 (3)	15 (4)	0.5556
505	IL-17A (pg/mL)	14 (2)	14 (3)	0.4770
506	GMCSF (pg/mL)	11 (2.5)	14 (4)	0.0059
507	IL-15 (pg/mL)	14 (3)	18 (8)	0.0456
508	EGF (pg/mL)	43 (64)	25 (135)	0.8612
509	IL-5 (pg/mL)	11 (1)	12 (1.5)	0.3165
510	VEGF (pg/mL)	20 (15.5)	17 (110)	0.6688
511	IFN- γ (pg/mL)	10 (3)	11 (2)	0.7092
512	IFN- α (pg/mL)	10 (1)	11 (2)	0.2605
513	IL1-RA (pg/mL)	12 (4.5)	18 (33.5)	0.0928
514	TNF- α (pg/mL)	27.5 (10)	50.5 (44)	0.0566
515	IL-2 (pg/mL)	13 (1)	15 (2)	0.0275
516	IL-7 (pg/mL)	13 (4)	15 (3)	0.3299
517	IL-4 (pg/mL)	8 (2)	10 (2)	0.1539
518	IL-8 (pg/mL)	66 (28.5)	174 (258.5)	0.0004
519	IL-12p70 (pg/mL)	9 (1)	10 (2)	0.1730
520	IL-1a (pg/mL)	14 (4)	21 (5)	0.0318
521	IL-3 (pg/mL)	8 (1)	8 (1)	0.1627
522	TNF- β (pg/mL)	10 (2)	10 (2)	0.9191

523

	Alive	Deceased	p-value	
524				
525				
526	Overall, n=42 (%)	37 (88.1)	5 (11.90)	-
527	RANTES (pg/mL)	11089 (3360)	10507 (4039.5)	0.6000
528	Eotaxin (pg/mL)	98 (69)	134 (123.5)	0.8308
529	MIP-1 α (pg/mL)	15.5 (5.5)	22 (23.5)	0.0081
530	MIP-1 β (pg/mL)	118 (99)	173 (589.5)	0.2211
531	MCP-1 (pg/mL)	1620 (1938)	9113 (1550)	0.0008
532	IP-10 (pg/mL)	3501.5 (5094)	6720 (4416)	0.1563

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536 Table 3. Crude and adjusted* logistic regression analysis for predictability of death according to
 537 different factors at baseline.

538

539 Variables	Crude	Adjusted
540	OR (95% CI) p value	OR (95% CI) p value
541		
542 Severity		
543 Severe	4.750 (0.957-23.572) p=0.057	3.518 (0.593-20.874) p=0.166
544 Moderate*		
545		
546 Obesity		
547 Yes	7.200 (1.933-26.812) p=0.003	8.103 (2.067-31.768) p=0.003
548 No*		
549		
550 Haemoglobin	0.624 (0.408-0.955) p=0.030	0.703 (0.416-1.186) p=0.187
551 Seg Neutrophils	1.145 (1.027-1.278) p=0.015	1.303 (1.064-1.596) p=0.010
552 Lymphocytes	0.821 (0.696-0.967) p=0.018	0.726 (0.563-0.936) p=0.013
553 SaFiO₂	0.985 (0.974-0.996) p=0.007	0.987 (0.976-0.998) p=0.025
554		
555 IL-6	1.012 (0.996-1.028) p=0.157	1.011 (0.994-1.030) p=0.212
556 GMCSF	2.220 (1.166-4.224) p=0.015	2.222 (0.977-5.050) p=0.057
557 IL-15	1.332 (1.026-1.730) p=0.031	1.233 (0.939-1.620) p=0.132
558 IL-2	3.089 (1.153-8.274) p=0.025	2.555 (0.839-7.778) p=0.099
559 IL-8	1.121 (0.977-1.287) p=0.103	(\$)
560 IL1a	1.056 (0.953-1.169) p=0.297	1.026 (0.920-1.146) p=0.641
561		
562 MIPα	1.374 (0.933-2.022) p=0.108	1.397 (0.931-2.097) p=0.106
563 MCP-1	1.001 (1.000-1.001) p=0.010	1.002 (0.999-1.004) p=0.112
564		
565 Zonulin	2.573 (1.371-4.828) p=0.003	2.666 (1.310-5.426) p=0.007

566

567
 568 *Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Cytokines were adjusted for age
 569 and obesity only due to collinearity issues with gender. (\$): Value could not be computed as log likelihood = 0.

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578 Table 4. Crude and adjusted* Cox regression of different factors for survival analysis.

579

Variables	Crude HR (95% CI) p value	Adjusted HR (95% CI) p value
Severity		
Severe	3.436 (0.759-15.566) p=0.109	4.282 (0.796-23.019) p=0.090
Moderate*		
Obesity		
Yes	3.923 (1.306-11.783) p=0.015	5.888 (1.646-21.068) p=0.006
No*		
Haemoglobin	0.669 (0.444-1.008) p=0.055	0.770 (0.480-1.235) p=0.278
Seg Neutrophils	1.083 (0.980-1.197) p=0.118	1.215 (1.028-1.437) p=0.023
Lymphocytes	0.874 (0.748-1.020) p=0.087	0.764 (0.623-0.938) p=0.010
SaFiO₂	0.987 (0.975-0.999) p=0.032	0.986 (0.974-0.998) p=0.023
IL-6	1.010 (0.997-1.024) p=0.138	1.013 (0.997-1.029) p=0.122
GMCSF	1.975 (1.051-3.711) p=0.034	2.145 (0.889-5.115) p=0.085
IL-15	1.252 (1.032-1.518) p=0.023	1.218 (0.987-1.502) p=0.066
IL-2	3.080 (0.910-10.427) p=0.070	4.018 (1.001-16.132) p=0.050
IL-8	1.059 (0.997-1.124) p=0.061	1.080 (0.975-1.196) p=0.142
IL1a	1.026 (0.936-1.124) p=0.590	1.011(0.913-1.120) p=0.827
MIPα	1.969 (0.917-4.228) p=0.082	2.671 (0.781-9.129) p=0.117
MCP-1	1.001 (1.000-1.001) p=0.020	1.001 (0.999-1.002) p=0.110
Zonulin	1.106 (1.043-1.173) p=0.001	1.109 (1.038-1.185) p=0.002

609 *Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Zonulin values were not
 610 categorised for the analyses made herein.

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Copias de cartas de respuesta a las observaciones de los revisores

Reviewer 1

Independent review report submitted: 30 Jul 2022

Interactive review activated: 05 Aug 2022

Review finalized: 08 Aug 2022

Initial recommendation to the Editor: Substantial revision is required

Please list your revision requests for the authors and provide your detailed comments, including highlighting limitations and strengths of the study and evaluating the validity of the methods, results, and data interpretation. If you have additional comments based on Q2 and Q3 you can add them as well.

Reviewer 1 | 30 Jul 2022 | 04:06

In this manuscript, Palomino-Kobayashi and colleagues sought to investigate the possible associations between the serum levels of Zonulin (a protein known to mediate intestinal permeability) and COVID-19 outcomes in a cohort of patients admitted to a Peruvian hospital. Notably, the authors explored the associations between this protein with several hematological, inflammatory, and biochemical parameters. Additionally, authors employed robust multivariate analysis revealing that baseline levels of zonulin are heightened among non-survivors, which also correlated with several biochemical and clinical variables. Moreover, regression analysis shows that zonulin associated with a higher risk of death.

In a broad sense, I believe the manuscript is comprehensively written and allows the reader to follow the information adequately. Special attention should be given to reference style (please check Frontier's reference style guide). Even though I believe this study contributes substantially to an enhanced understanding of the pathological nuances surrounding COVID-19, I found several limitations in the manuscript.

A mounting body of evidence suggests that Zonulin is a surrogate marker of tight junction permeability; however estimating microbial translocation based solely on zonulin levels is rather simplistic. Could the authors explain why other makers of microbial translocation (such as bacterial DNAemia, plasma levels of LSP-binding protein [LBP], B-glucan, intestinal-fatty acid binding protein [IFABP], soluble CD14) were not taken into account in the current investigation?

How can one rule out the possibility of systemic inflammation seen in the settings of this investigation were not merely caused by intestinal integrity disruption itself?

Please notice the detailed review report below:

The title is slightly vague; therefore author should rewrite it more descriptively.

Introduction: Overall, the introduction is neatly written, but key pieces of information that support why your study is relevant should be inserted. How does your study differ from previous reports on the associations of zonulin levels and worse outcomes in COVID-19? What is the novelty of your study? what are links between zonulin amounts and systemic inflammation? (as this could be a clear justification for further investigation of inflammatory markers in the this study).

Perhaps the introduction would benefit significantly if the paragraphs were reordered (ie. the 3rd paragraph describes zonulin [the pivotal element in gut permeability, which in turn is described in the first paragraph]). This would allow readers to follow the information more adequately and in a more logical manner.

Line 33- 34: Please state what SARV-COV2 stands for. Plus, the word "virus" after SARV-COV2 seems a little redundant and should be removed.

Line 34: This sentence should be rewritten to avoid ambiguity. The current sentence conveys the idea that type II pneumocytes are the main cell host in all the mentioned organs (eg. lungs, kidney, heart, etc) and this should be corrected.

Line 40: Please avoid acronyms. As it is the first mention of the term " HIV" in the manuscript, such term should be written as "human immunodeficiency virus (HIV)".

Line 42: Please clarify the meaning of "cytokine alterations" in the context of this sentence. Do the authors refer to a switch in intestinal cytokine profile or cytokine-mediated alterations(i.e. functional or morphological) in enterocytes?

Line 54: Please change "produces" to "induces".

Line 105: Please consider changing "alive vs deceased" to "survivors vs nonsurvivors" or "Survived vs Deceased" as all patients were "alive" when admitted to the hospital. If you decide to change the nomenclature, please stick to it throughout the manuscript.

Line 150: There is no reference to tables or figures associated with these results. Authors should provide a descriptive table depicting all the correlations between Zonulin and all the the investigated variables. I believe that authors should provide scatterplots depicting significant correlations.

Line 152: Highlight that zonulin positively correlated with creatine, D-dimer and prothrombine time, whereas negatively correlated with Sa/FiO2 ratio.

Line 163: I believe that authors should provide in this sentence an interpretation for the meaning of OR values higher/less than 1 (ie. these factors are associated with higher odds of fatal outcome) in order to digest the results for the readers.

Figure 1 - Provide the label for the y-axis, making clear that this graph depicts the measurement of zonulin levels and the unit of measure.

Could the authors provide a graph depicting the levels of Zonulin comparing obese patients versus the non-obese ones?

Lines 198 and 216 : Change " The SARS-CoV2 virus" to "SARS-CoV2".

Line 238 : Could the authors elaborate on sample size calculations they might had performed?

Line 245: Regarding the variation of zonulin concentrations among studies, it should be emphasized that which methods may circumvent the cons of ELISA.

Corresponding Author: Maria J. Pons | 08 Aug 2022 | 20:41

1- Could the authors explain why other makers of microbial translocation (such as bacterial DNAemia, plasma levels of LSP-binding protein [LBP], B-glucan, intestinal-fatty acid binding protein [IFABP], soluble CD14) were not taken into account in the current investigation?

1(RESPONSE)-

Good appreciation of the reviewer. At the beginning of the study, we wanted to establish also the DNA load (specifically the 16SrRNA gene) by qPCR quantification. Our surprise was that, after many tests, the qPCR kits themselves gave a signal (due to contamination) and we could not perform the approximation by this method and only measured zonulin. It should be mentioned that the other markers could not be evaluated for economic reasons (the small grant only

allowed us to buy zonulin kits). Although it would be much more interesting to put more markers, we think that zonulin by itself and the correlations and analyses are robust enough to highlight the importance of this marker. It is also in line with other research previously reported by Guiron et al. and COVID-19.

Line 33- 34- The MS has been changed to : "Severe acute respiratory syndrome coronavirus (SARS-CoV-2) binds..."

Line 34: the text has been modified "binds principally to receptor angiotensin-converting enzyme 2 (ACE2) to enter many different host cells such as type II pneumocytes in the lung, myocardial cells, neurons and glia, endothelial cells, intestinal enterocytes and others"

Line 40: The use of acronyms has been avoided.

Line 42: "cytokine-mediated alterations" is more appropriate, thanks

Line 54: "induces" has been added in the text

Line 105: Nomenclature has been changed throughout the manuscript.

Line 152: "Zonulin levels were positively correlated with creatinine ($p=0.288$, $p=0.035$) D-dimer values ($p=0.441$, $p=0.002$), whereas prothrombin time ($p=0.344$, $p=0.011$) and Sa/FiO₂ ratio ($p=-0.4764$, $p=0.0001$) were found to be negatively correlated with zonulin (Table 3)" The above correlations have been highlighted.

Line 163: "zonulin levels (OR 2.573, $p=0.003$), presented significant OR values greater than 1 in the crude analysis, which indicate an association with higher odds of mortality" has been explained better.

Figure 1 -Figure 1 has been improved for better understanding.

A graph of zonulin levels in obese and non-obese individuals has been plotted, with obese individuals having higher levels.

Lines 198 and 216 : Change "SARS-CoV2" has been done.

Line 238 : Could the authors elaborate on sample size calculations they might had performed? Sample size was calculated according to previous literature by Girón et al. The mean zonulin concentration was 110 in the dead group versus 70 in the living. The size calculation was 12 individuals in group 1 vs 36 in group 2 (95% confidence interval and 80% power). So our size complies with the size calculation.

Line 245: Variability in methodology has been better highlighted. "It should be noted that zonulin concentrations described in the studies available vary greatly. This variability could be explained by the shelf life of the samples and differences in the detection methods. Additionally, there are concerns regarding the validity of the ELISAs performed for zonulin quantification, in which the kits may detect not only zonulin but also zonulin-related proteins such as properdin (Scheffler et al., 2018). Another option is the quantification of pre-haptoglobin 2 specifically (and no other zonulin-like proteins) with other methods such as western blot and/or mass spectrometry. "

Reviewer 1 | 08 Aug 2022 | 23:05

I believe the manuscript is well-suitable for publication.

Please check for possible duplication of figure 1.

Reviewer 2

Independent review report submitted: 04 Aug 2022

Interactive review activated: 05 Aug 2022

Review finalized: 15 Aug 2022

Initial recommendation to the Editor: Substantial revision is required

Please list your revision requests for the authors and provide your detailed comments, including highlighting limitations and strengths of the study and evaluating the validity of the methods, results, and data interpretation. If you have additional comments based on Q2 and Q3 you can add them as well.

Reviewer 2 | 04 Aug 2022 | 08:26

This manuscript suggests that increased gut permeability is associated with death due to COVID-19 independently of age and gender in a Peruvian hospitalized cohort. The current study is a follow-up study to an earlier publication analyzing the essentially the same cohort for disease severity associations by the authors. The current study analyzes serum levels of zonulin, which is a widely used marker for increased gut permeability, by a commercial ELISA and compares its levels between deceased and survived patients. As reviewed by the authors, higher zonulin levels and increased gut permeability has already been associated with increased COVID-19 severity by several previous studies, which highlights the importance of gut homeostasis during COVID-19 but also negatively impacts the novelty of the current work.

Major issues that need addressing before the current work can be considered publishable:

- 1) As the authors also rightly point out, the specificity of commercial zonulin ELISAs have been recently questioned. There are other well-established serum markers used to assess gut permeability such as LPS-binding protein and intestinal FABP to name a few. To reliably assess gut homeostasis using serum markers, a combination of markers should be used.
- 2) The serum samples used in the current study are not well-defined. At least, days after symptoms onset and days before death at the time of sampling should be provided so that the results can be adequately assessed. Would be important also to compare severe and moderate cases to each other in terms of gut permeability markers and not only deceased and survived.
- 3) There is no legend or y-axis title provided for Figure 1.

Corresponding Author: Maria J. Pons | 08 Aug 2022 | 20:41

1)

As discussed in the manuscript, it would be ideal to test more markers, but the project, funded by internal university funds, did not allow us to do so. We tried to quantify DNA (16S rRNA) by qPCR, but the signal was obtained with our own Taq and reagents, so it was discarded. The reviewer may question zonulin, but the correlations and few previously reported studies validate and reinforce the need to highlight the results of this article. Because of its association with poor prognosis and its correlations with markers of poor prognosis. There is still much to be known about zonulin, and there is a need for articles that reinforce its role in COVID-19 and its evolution.

As the reviewer comments, it would be ideal to have the date of symptom onset. Still, unfortunately, because the samples were collected during the first wave of COVID-19 in Peru, with the health system collapsed, patient information was only obtained from the time they were admitted to the hospital. From there, samples could be accessed during hospitalisation, until they were discharged or died (a sentence has been included in the text).

The study compared zonulin levels in moderate and severe patients, but no differences were observed. This has been written in the manuscript.

Figure 1 has been improved for better understanding.

Reviewer 2 | 10 Aug 2022 | 10:15

Thank you for the reply. In order to validate the comparison between survived and deceased (or moderate and severe), it would be important to know the time of sampling in relation to disease course. If the date of symptom onset is not available, would you be able to provide the sampling date as adjusted by the time of hospital admission. Another required parameter is the time of death. Did all deaths take place in hospital and if yes, how many days after sampling and admission?

Corresponding Author: Maria J. Pons | 15 Aug 2022 | 08:29

Dear reviewer, thanks for your question.

Yes, all deaths occurred in the hospital. We have information about the admission date, sample date, date of discharge or death. This information has been introduced in the manuscript's material and methods (line 95).

Regarding "" If the date of symptom onset is not available, would you be able to provide the sampling date as adjusted by the time of hospital admission."For this purpose, the median (iqr) time from admission to the first sample (in days) has been placed in table 1.

"if yes, how many days after sampling and admission"- In order to respond, the time (in days) from sample collection to discharge/death is placed in table 1.

Moreover, in Line 147 we added, "The median (IQR) time from admission to first serum sample were 1(1.5) and 1(5) days in the survived and deceased groups respectively ($p=0.621$). Time from admission to hospital discharge/death (time of hospitalisation) and time from first serum sample obtained to hospital discharge/death were also similar between the aforementioned groups ($p=0.978$ and $p=0.461$, respectively) (Table 1)" to clarify the message. No significant differences have been found in both comparisons.

Regarding Cox analysis: The analysis was repeated from sample time to death or discharge and the HR for zonulin increased, remaining significant. We added this information in the manuscript "Cox regression was performed as a survival analysis with the same predictors as logistic regression, considering the period between the date of hospital admission to date of hospital discharge/death (days)." Line 194.

1 **Informe de tesis basado en el artículo**

2 <https://doi.org/10.3389%2Ffcimb.2022.1000291>

3

4 **Zonulina, un marcador de permeabilidad intestinal, está**
 5 **asociada con mortalidad en una cohorte de pacientes**
 6 **COVID-19 hospitalizados de Perú**

7

8 **INTRODUCCIÓN**

9

10 Las infecciones graves por COVID-19 se caracterizan por inflamación sistémica,
 11 síndrome de dificultad respiratoria aguda (SDRA) y falla multiorgánica del sistema
 12 (Kim et al., 2021). El virus del Síndrome Respiratorio Agudo Severo Coronavirus
 13 2 (SARS-CoV-2) se une principalmente al receptor de la enzima convertidora de
 14 angiotensina 2 (ACE2) para entrar en una diversidad de células del huésped tales
 15 como neumocitos tipo II en el pulmón, células miocárdicas, neuronas y glía,
 16 células endoteliales, enterocitos intestinales y otros (Lamers et al.,2020;
 17 Beyerstedt et al., 2021). En particular, los enterocitos intestinales forman una
 18 capa celular que forma parte del sistema de defensa de la mucosa y se mantienen
 19 unidas por uniones estrechas. La alteración de esta capa, aumentando la
 20 permeabilidad intestinal, se traduce en la translocación de componentes
 21 bacterianos del intestino al torrente sanguíneo, contribuyendo a un aumento de
 22 la inflamación sistémica (Dinh et al., 2015).

23

24 Previamente en infecciones virales, como en la fiebre del dengue
 25 (Chanchaoenthana et al., 2021) o la infección por virus de inmunodeficiencia
 26 humana (Marchetti et al., 2013), se ha reportado la presencia de translocación
 27 microbiana, causada por infección directa de enterocitos por el virus o por
 28 alteraciones mediadas por citoquinas y disbiosis intestinal. Estas alteraciones
 29 también pueden asociarse a algunas comorbilidades como obesidad o diabetes
 30 (Verdam et al., 2011; Almugadam et al., 2020). Estudios anteriores (Yonker et al.,
 31 2021; Giron et al., 2021; Oliva et al., 2021) han relacionado una mayor
 32 translocación intestinal con casos más graves de COVID-19, que describen una
 33 mayor carga de componentes bacterianos en el torrente sanguíneo en
 34 comparación con los casos más leves. Se ha sugerido que la translocación
 35 bacteriana, la disbiosis intestinal y los procesos inflamatorios subyacentes
 36 podrían tener una sinergia sobre la inflamación sistémica y los niveles altos de
 37 citoquinas descritos en infecciones graves por COVID-19 (Cardinale et al., 2020;
 38 Vignesh et al., 2020; Uzzan et al., 2020; Mashaqi et al., 2022).

40 La zonulina (pre-Haptoglobina 2) es una proteína de ~47 kDa, detectable en su
 41 forma no escindida en suero humano (Fasano, 2011) y altamente correlacionada
 42 con la DNAemia bacteriana (Gargari et al., 2021). Es un mediador fisiológico que,
 43 de forma reversible, regula la permeabilidad intestinal mediante la modulación de
 44 las uniones estrechas (Tripathi et al., 2009) y se considera un biomarcador de
 45 alteración de la función de barrera intestinal (Fasano, 2011). En este sentido, se
 46 ha planteado la hipótesis de que la proteína espiga del SARS-CoV-2 induce la
 47 sobreexpresión de zonulina, que está relacionada a un aumento de la
 48 permeabilidad intestinal (Llorens et al., 2021). Además, se ha reportado que los
 49 niveles de zonulina son más altos en pacientes que fallecieron por COVID-19
 50 grave (Giron et al., 2021). Estos datos sugieren que el SARS-CoV-2 podría
 51 facilitar el paso de contenidos intestinales a la circulación y un mayor índice
 52 inflamatorio a nivel intestinal que podría alimentar la inflamación sistémica.

53

54 La respuesta inflamatoria a la infección viral también está influenciada por
 55 antecedentes genéticos, por ejemplo, la variabilidad en la respuesta de citoquinas
 56 proinflamatorias, que también se ha relacionado con peor pronóstico de la
 57 COVID19 (Vakil et al., 2022). Además, la variación asociada a poblaciones de
 58 respuestas a infecciones virales se diferencia por ascendencia genética
 59 (Randolph et al., 2021). Los estudios sobre la relación de los niveles de zonulina
 60 sérica y COVID-19 són escasos y provienen de cohortes/series de casos de
 61 Estados Unidos de América (Giron et al., 2021; Josyabhatla et al., 2021; Yonker
 62 et al., 2022), Italia (Oliva et al., 2021) y Turquía (Kılıç et al., en prensa). Por lo
 63 tanto, es importante realizar estudios para ver la asociación de zonulina con la
 64 gravedad de COVID-19 para corroborar que se produce en la población peruana.
 65 Los datos de estudios con sujetos de diferentes antecedentes genéticos y
 66 geográficos son importantes por un tema de validez externa.

67

68 Así, el presente estudio tuvo como objetivo identificar la asociación entre las
 69 concentraciones de zonulina y el desenlace en una cohorte de pacientes
 70 hospitalizados con COVID-19 moderado y severo de Lima, Perú. Además, se
 71 establecieron correlaciones entre los valores de zonulina y los niveles de
 72 citoquinas/quimioquinas y los parámetros clínicos.

73

74 **MATERIALES Y MÉTODOS**

75

76 **Estudio de población y evaluación de gravedad de la enfermedad**

77 El presente estudio utilizó muestras de una cohorte de pacientes hospitalizados
 78 con COVID-19 del Hospital Nacional Guillermo Almenara Irigoyen (Pons et al.,
 79 2021). Un total de 60 pacientes se clasificaron según la gravedad: 19 moderada,
 80 26 severa y 15 muertes (inicialmente clasificadas como moderadas o
 81 severas). Todas las muertes ocurrieron durante la hospitalización. Los casos de

82 COVID-19 fueron confirmados por reacción en cadena de polimerasa cuantitativa
 83 (qPCR) o pruebas de anticuerpos. El período de seguimiento fue de agosto a
 84 octubre de 2020, que estuvo en el contexto de la primera ola de COVID-19 en
 85 Perú. Por lo tanto, ningún paciente había recibido vacunación para COVID-19.

86
 87 La definición de infección moderada fue según la Definición de Berlín 2012 del
 88 Síndrome de Dificultad Respiratoria Aguda (SDRA) (Grupo de trabajo de
 89 definición de ARDS, 2012), mientras que infección severa se definió siempre que
 90 uno o más de los siguientes estados desarrollados: índice de Horowitz
 91 ($\text{PaO}_2/\text{FiO}_2$) < 100, la relación de saturación de oxígeno entre fracción de
 92 oxígeno inspirado ($\text{SpO}_2/\text{FiO}_2$, en lo sucesivo abreviado como Sa/FiO_2) < 89,
 93 necesidad de mecánica ventilación debido a insuficiencia respiratoria o
 94 insuficiencia orgánica sistémica que requieren ingreso en la unidad de cuidados
 95 intensivos.

96

97 **Condiciones de la muestra**

98 Las muestras de suero del estudio original se conservaron a $-80\text{ }^\circ\text{C}$ en los
 99 laboratorios de la Universidad Científica del Sur.

100

101 **Datos clínicos y niveles de citoquinas**

102 Los datos de edad, sexo, fecha de ingreso hospitalario, fecha de alta
 103 hospitalaria/muerte, fecha de toma de muestra según ingreso hospitalario,
 104 comorbilidades como obesidad, hipertensión, diabetes, enfermedad pulmonar
 105 obstructiva crónica (EPOC) y cualquier otra condición subyacente, tratamiento
 106 farmacológico recibido, hemoglobina, plaquetas, glóbulos blancos (WBC,
 107 recuento de glóbulos blancos por sus siglas en inglés) y Sa/FiO_2 se obtuvieron
 108 de los registros médicos correspondientes. Toda la información identificable
 109 privilegiada de los pacientes fue excluida del conjunto de datos generado y las
 110 muestras fueron codificadas previamente para garantizar la confidencialidad.

111

112 Las concentraciones de citoquinas y quimioquinas de las muestras de suero se
 113 obtuvieron de los datos previamente reportados en el estudio de Pons et al. (Pons
 114 et al., 2021), en el que un kit comercial de la tecnología Luminex MAGPIX (Merck
 115 KGaA, Darmstadt, Alemania) se utilizó para la cuantificación.

116

117 **Estimación de la translocación microbiana por cuantificación de zonulina**

118 La zonulina (ng/ml) se cuantificó a partir de las muestras de suero mediante
 119 sándwich ELISA, usando el CUSABIO Human Zonulin ELISA Kit (CUSABIO
 120 TECHNOLOGY LLC, Wuhan, China) según a las instrucciones del fabricante.
 121 Brevemente, 100 μL de estándares y muestras se agregaron por pozo en la
 122 microplaca recubierta, e incubados durante dos horas a $37\text{ }^\circ\text{C}$. Posteriormente,
 123 se añadieron 100 μL de solución de biotina-anticuerpo y se incubó durante una

124 hora a 37°C. Posteriormente se aspiró el líquido y se procedió al lavado manual.
 125 hecho tres veces con el tampón de lavado proporcionado. Luego 100 µL de
 126 solución peroxidasa de rábano-avidina se añadió por pocillo, con incubación
 127 durante una hora a 37°C, con posterior lavado manual. Finalmente, 90 µL de
 128 sustrato 3,3',5,5'-Tetrametilbencidina (TMB) se añadió por pocillo y se incubó
 129 durante 30 minutos a 37°C. Inmediatamente después, se añadió 50 µL de
 130 solución de parada por pocillo y la lectura de absorbancia se realizó a 450 nm
 131 (con corrección a 540 nm) en un lector multimodo Synergy LX (Biotek, EE. UU.).
 132 Las muestras y los estándares se corrieron por duplicado.

133

134 **Análisis estadístico**

135 El análisis descriptivo se realizó mediante frecuencias absolutas y relativas para
 136 resumir variables categóricas mientras se utilizaron la mediana y el rango
 137 intercuartílico (RIC) para variables, a menos que se especifique lo contrario.

138

139 Se realizaron análisis bivariados para las comparaciones entre desenlaces
 140 (sobrevivientes vs. fallecidos) y variables categóricas utilizando la prueba exacta
 141 de Fisher o Chi-cuadrado, mientras que la prueba U de Mann Whitney se utilizó
 142 para las comparaciones entre los desenlaces y variables numéricas.
 143 Correlaciones entre recuentos de leucocitos, marcadores bioquímicos,
 144 citoquinas, quimiocinas y niveles de zonulina se normalizaron utilizando la
 145 transformación logarítmica en base 10 y las comparaciones se realizaron
 146 mediante pruebas de Spearman.

147

148 Se realizó un modelo de regresión de Cox para evaluar el riesgo instantáneo de
 149 muerte considerando zonulina y otras variables mediante criterio epidemiológico,
 150 obteniéndose Hazard Ratios (HR) crudos y ajustados. Para ello, se definió como
 151 tiempo cero la fecha de hospitalización y el tiempo final de seguimiento, la fecha
 152 de alta o muerte en hospital en sobrevivientes y fallecidos respectivamente. Para
 153 determinar cuáles variables de ajuste entraban al modelo de regresión de Cox se
 154 empleó criterio epidemiológico de acuerdo con el modelo conceptual establecido
 155 plasmado en un gráfico acíclico dirigido (DAG) (véase anexo 1). Adicionalmente
 156 para la construcción del modelo se empleó el criterio de selección de akaike
 157 (AIC) para comparar modelos parciales y determinar el modelo ajustado final.
 158 Luego, se realizaron los siguientes análisis post-regresión: Test para probar
 159 supuesto de proporcionalidad de Hazards, interacción entre variables y análisis
 160 de sensibilidad eliminando el valor de zonulina más alto (40 ng/mL), ya que este
 161 se puede considerar como *outlier*.

162

163

164 Para la interpretación de los resultados de zonulina, las muestras con una
 165 concentración de zonulina por encima del límite superior del estándar (40 ng/mL)

166 se les asignó este valor. Los valores por debajo de 0,625 ng/mL fueron utilizados
167 como sus mismos valores para los análisis.

168

169

170 Los análisis se realizaron utilizando STATA 16.1 SE Edition (StataCorp, TX) y los
171 gráficos se construyeron usando R versión 4.1.2. Se calcularon intervalos de
172 confianza del 95% para HR. La significación estadística se estableció con $\alpha=0,05$.

173

174

175 **RESULTADOS**

176

177 **Características clínicas basales de la cohorte hospitalizada**

178 Sesenta pacientes hospitalizados con COVID-19 fueron incluidos: 45 pacientes
179 (75%) sobrevivieron y 15 (25%) pacientes fallecieron durante el período de
180 seguimiento. La distribución por gravedad de los casos fue la siguiente: 39
181 pacientes (65%) fueron clasificados como graves y 21 (35%) como moderado de
182 acuerdo con la definición de caso dada y la progresión durante el período de
183 seguimiento. La gravedad se asoció con el resultado ($p=0,042$). La mediana de
184 edad fue similar tanto en los sobrevivientes como en los grupos de fallecidos (55
185 y 55 años, respectivamente) y no hubo diferencia significativa en edad ($p=0,694$)
186 o sexo ($p=0,678$) entre los dos grupos. El tiempo medio (RIC) desde el ingreso
187 hasta la primera muestra de suero fueron 1 (1,5) y 1 (5) días en los sobrevivientes
188 y grupos de fallecidos respectivamente ($p=0,621$). El tiempo desde el ingreso
189 hasta alta hospitalaria/muerte (tiempo de hospitalización) y tiempo desde primera
190 muestra de suero obtenida al alta hospitalaria/muerte también fueron similares
191 entre los grupos mencionados ($p=0,978$ y $p=0,461$, respectivamente) (Tabla 1).

192

193 Algunas comorbilidades como obesidad, diabetes, presión arterial elevada y
194 otros estaban presentes (Tabla 1). En el grupo de los fallecidos 12 pacientes
195 (80%) tenían comorbilidades en comparación con 22 (48,9%) en el grupo de
196 sobrevivientes, y esta diferencia fue significativa ($p=0,035$). La única comorbilidad
197 que se asoció con desenlace fue la obesidad ($p=0,006$). Se emplearon
198 corticoesteroides como parte del tratamiento para todos los pacientes de esta
199 cohorte.

200

201 **Recuentos de glóbulos blancos, marcadores bioquímicos y niveles de** 202 **citoquinas/quimioquinas al inicio**

203 Los niveles de hemoglobina al inicio del estudio fueron más bajos en los
204 fallecidos, con una media (desviación estándar) de 13,19 (1,93) g/dL ($p=0,018$).
205 Con respecto a los recuentos de glóbulos blancos, los recuentos de neutrófilos
206 segmentados fueron más altos ($p = 0,006$) y los recuentos de linfocitos fueron

207 menores en el grupo de fallecidos ($p=0,004$), no encontrándose diferencias
 208 significativas para los recuentos de leucocitos restantes en los dos grupos.

209

210 En relación con algunos marcadores bioquímicos como la proteína C reactiva, el
 211 dímero D y la ferritina, no hubo diferencias significativas cuando se compararon
 212 por desenlace (tabla 1). Sin embargo, el cociente Sa/FiO₂ fue menor en el grupo
 213 de fallecidos, con una mediana (RIC) de 108,73 (35,40) ($p=0,0007$). Las
 214 citoquinas inteleuquina-6 (IL6) ($p=0,029$), factor estimulante de colonias de
 215 granulocitos y macrófagos (GMCSF) ($p=0,005$), IL15 ($p=0,046$), IL-2 ($p=0,027$),
 216 IL-8 ($p= 0,0004$), IL-1a ($p=0,032$), quimiocinas proteína alfa inflamatoria de
 217 macrófagos (MIPa) ($p=0,008$) y proteína quimioatrayente de monocitos-1 (MCP-
 218 1) ($p=0,001$) mostraron diferencias significativas según el resultado (Tabla 2),
 219 estando todos elevados en el grupo de los fallecidos.

220

221 **Correlaciones de zonulina versus marcadores hematológicos/bioquímicos y** 222 **citoquinas/quimiocinas al inicio**

223 Los niveles de zonulina se correlacionaron positivamente con los valores de
 224 creatinina ($r=0,288$, $p=0,035$) y dímero D ($r=0,441$, $p=0,002$), mientras que se
 225 encontró que tiempo de protrombina ($r=0,344$, $p=0,011$) y la relación Sa/FiO₂ ($r=$
 226 $-0,4764$, $p=0,0001$) se correlacionaron negativamente con la zonulina (Tabla 3,
 227 Figura 1). En relación con las citoquinas y las quimiocinas, RANTES fue la única
 228 quimiocina que se correlacionó negativamente con los niveles de zonulina ($r=$
 229 $0,5023$, $p=0,007$). Ninguna de las citoquinas se correlacionó significativamente
 230 con los niveles de zonulina (Tabla 3, Figura 1).

231

232 **Niveles de zonulina basales asociados con desenlace en análisis bivariado**

233 La mediana (RIC) del nivel de zonulina en el grupo de fallecidos fue de 3,264
 234 (4,7096) ng/mL (mín 0,7099 ng/mL – máx 40 ng/mL, Tabla 2) mostrando una
 235 asociación significativa con el resultado ($p=0,0002$, Figura 2) y obesidad
 236 ($p=0,017$, Figura 2) en el análisis bivariado. No obstante, no se encontró que la
 237 zonulina estuviera asociada con severidad de la enfermedad ($p = 0,69$, Figura 2)
 238 en el mismo análisis.

239

240

241 **Análisis de regresión de Cox**

242 Se encontró que la zonulina se asociaba con tiempo a evento (muerte) tanto en
 243 el análisis crudo (HR 1.11, IC 95% 1.05-1.17) como en el modelo final ajustado
 244 (HR 1.19, IC 95% 1.09-1.29c), el cual incluyó las variables presencia de
 245 comorbilidades, severidad y sexo (Tabla 4). En el modelo conceptual se
 246 consideró la variable edad; no obstante, al aplicar el test de proporcionalidad de
 247 Hazards, dicha variable no cumplía dicho supuesto y se retiró del modelo.
 248 Adicionalmente, si bien es cierto no fue significativo ($p=0,09$, Tabla 4), se

249 encontró evidencia de una posible modificación de efecto de la asociación
 250 mencionada por severidad, y considerando el ajuste por dicha interacción,
 251 cambiaría el *hazard ratio* a 1.27 (0.96-1.69).

252

253 Debido a que el valor de zonulina en un paciente (40 ng/mL) fue mucho más
 254 elevado que los valores promedio, se ejecutó un análisis de sensibilidad al retirar
 255 dicho paciente y volviendo a realizar los cálculos en el análisis crudo y en el
 256 modelo final ajustado. Se encontró un aumento en la magnitud de efecto tanto
 257 en el análisis crudo (HR 1.50, IC 95% 1.18-1.90) y en modelo final ajustado (HR
 258 1.47, IC 95 % 1.10-1.96)

259

260 **DISCUSIÓN**

261

262 La pandemia de SARS-CoV-2 ha causado efectos devastadores, que ha
 263 resultado en más de 6.8 millones de muertes a nivel mundial, de ellas alrededor
 264 de 220.000 en Perú (WHO-2023). Incluso con acceso a la vacunación, la COVID-
 265 19 sigue siendo una amenaza para la salud pública en todo el mundo, con
 266 muchos aspectos de su fisiopatología y consecuencias aún por entender. El
 267 presente estudio aporta conocimiento sobre la asociación de los valores de
 268 zonulina en pacientes con COVID-19 y muerte, describiéndose una asociación
 269 significativa entre concentración de zonulina en suero y hazard de morir por
 270 COVID-19, aún después de ajuste por presencia de comorbilidades (entre las
 271 principales siendo obesidad. En el modelo conceptual se consideró la variable
 272 edad, no obstante al aplicar el test de proporcionalidad de Hazards, dicha variable
 273 no cumplía dicho supuesto y se retiró del modelo.

274

275 Los resultados del presente estudio están en la misma línea, con los escasos
 276 reportes previos, que reportaron el papel de la zonulina en pacientes COVID-19.
 277 De esta manera, Giron et al. (Giron et al. 2021) reportaron niveles más elevados
 278 de zonulina en el grupo de fallecidos, similar a nuestros resultados. En otro
 279 estudio, Oliva et al. (Oliva et al., 2021) encontraron valores de zonulina similares
 280 a los de nuestro estudio en COVID-19 al compararlos con controles sanos
 281 emparejados.

282

283 Aunque se ha informado sobreexpresión de zonulina en órganos como los
 284 pulmones y el cerebro (Skardelly et al., 2009; Rittirsch et al., 2013), la zonulina ha
 285 sido vinculada con alteraciones de la pared intestinal (Fasano et al., 2011). Así,
 286 antes de la COVID-19, la permeabilidad de las uniones estrechas intestinales
 287 dependiente de la zonulina se había implicado en enfermedades inflamatorias
 288 crónicas como la enfermedad celíaca, la enfermedad inflamatoria intestinal, la
 289 diabetes tipo I (Fasano, 2011), así como la obesidad, la septicemia, la enteropatía
 290 ambiental, enterocolitis necrotizante, entre otros (Sturgeon y Fasano, 2016). En

291 estos escenarios, la liberación de zonulina y la pérdida de la función de barrera
 292 intestinal son seguidas por la translocación microbiana, que desencadena
 293 respuestas inmunitarias y la producción de citoquinas proinflamatorias que
 294 exacerban la permeabilidad intestinal en un círculo vicioso (Fasano, 2020). En el
 295 presente estudio se observó un incremento en los niveles de zonulina asociado
 296 con la presencia de obesidad. Además, se ha reportado que la presencia de
 297 algunas comorbilidades están relacionadas con un peor desenlace de los
 298 pacientes COVID-19, en especial la obesidad.(Cai et al., 2021; Dessie y Zewotir,
 299 2021; Yang et al., 2021 En esta cohorte se ha reportado previamente la relación
 300 de la obesidad con el grupo de pacientes COVID-19 que tuvieron un desenlace
 301 fatal (Pons et al., 2021). Este hecho se relaciona con un deterioro de la inmunidad
 302 y una amplificación del estado inflamatorio subyacente relacionado con la
 303 obesidad junto con la inflamación aguda generada por la infección por SARS-
 304 CoV-2 (Mohammad et al., 2021).

305

306 Está demostrada la relación de SARS-CoV-2 y el sistema digestivo, como
 307 demuestra la presencia de los síntomas gastrointestinales y la detección del virus
 308 en las heces en infectados (Groff et al., 2021). Además, se han encontrado
 309 antígenos virales en enterocitos intestinales aun después de la resolución de la
 310 enfermedad clínica en adultos (Gaebler et al., 2021), coincidiendo la
 311 hiperpermeabilidad de la barrera mucosa a la zonulina con la antigenemia del
 312 SARS-CoV-2 (Yonker et al., 2021). Anteriormente se ha especulado que la
 313 zonulina participa en un mecanismo complejo que vincula la amplia variedad de
 314 síntomas asociados con la infección por SARS-CoV-2, especialmente en el
 315 sistema digestivo y el sistema nervioso (Llorens et al., 2021). Específicamente, la
 316 interrupción de la barrera intestinal con la posterior translocación microbiana
 317 podría impulsar la activación inflamatoria en casos graves de COVID-19 (Giron et
 318 al., 2021) con retroalimentación positiva de citoquinas proinflamatorias, siendo la
 319 respuesta sistémica amplificada más fuerte en pacientes con comorbilidades
 320 (Trottein y Sokol, 2020). Dado que nuestros resultados sugieren que la zonulina
 321 está asociada con la muerte, incluso cuando se ajusta por presencia de
 322 comorbilidades, severidad y sexo, esto es consistente con la mencionada
 323 exacerbación de la inflamación y una respuesta mayor de biomarcadores
 324 inflamatorios descrita.

325

326 Es importante destacar el papel de la zonulina también en niños con síndrome
 327 inflamatorio multisistémico (MIS), relacionado con la persistencia de SARS-CoV-
 328 2 en el tracto gastrointestinal (Kılıç et al., en prensa; Josyabhatla et al., 2021).
 329 Estos estudios sugieren que la zonulina podría estar asociada con una
 330 translocación de proteínas virales al torrente sanguíneo, lo que contribuiría al
 331 aumento de la inflamación y a mayores niveles de afectación. Relacionado con
 332 estos estudios, cabe mencionar que el uso compasivo del inhibidor de zonulina,

333 acetato de larazotido, en una serie de casos de cuatro niños con MIS dio como
 334 resultado una resolución más rápida de los síntomas gastrointestinales y un
 335 tiempo de eliminación más rápido del antígeno de pico del SARS-CoV-2 (Yonker
 336 et al. , 2022). Esto ha llevado al desarrollo de un ensayo clínico con este inhibidor
 337 (ClinicalTrials.gov identificador NCT05022303) en niños con COVID-19
 338 prolongado con inflamación persistente (Galán et al., 2022).

339

340 Con respecto a las correlaciones, se encontró que la zonulina está directamente
 341 relacionada con la creatinina, los valores de dímero D y el tiempo de protrombina,
 342 todos los cuales se han asociado previamente con un peor resultado en pacientes
 343 hospitalizados con COVID-19 (Malik et al., 2021) e inversamente correlacionados
 344 con CCL5/RANTES y Sa/FiO₂. En cuanto a la quimiocina CCL5 (RANTES), se
 345 han encontrado valores elevados en casos moderados pero no graves de COVID-
 346 19 y se considera un factor asociado con supervivencia (Zhao et al., 2020). En
 347 tanto, una relación Sa/FiO₂ más baja y un recuento de linfocitos disminuido se
 348 han asociado con la muerte por COVID-19 (Henry et al., 2020; Huang y Pranata,
 349 2020; Booth et al., 2021; Pons et al., 2021; Gopalan et al., 2022; Soto et al., 2022).
 350 Cabe señalar que la linfopenia podría ocurrir debido a la consiguiente apoptosis
 351 de las células T CD4+ activadas por el SARS-CoV-2 (Shen et al., 2022),
 352 induciendo así la inmunosupresión.

353

354 Cabe señalar que las concentraciones de zonulina descritas en los estudios
 355 disponibles varían mucho. Esta variabilidad podría explicarse por la vida útil de
 356 las muestras y las diferencias en los métodos de detección. Además, existen
 357 controversias con respecto a la validez de los ELISA realizados para la
 358 cuantificación de zonulina, en los que los kits podrían detectar no solo zonulina
 359 sino también proteínas relacionadas con la zonulina, como la properdina
 360 (Scheffler et al., 2018). Otra opción es la cuantificación de pre-haptoglobina 2
 361 específicamente con otros métodos como western blot y/o espectrometría de
 362 masas.

363

364 Como se mencionó antes, un creciente cuerpo de evidencia sugiere que la
 365 zonulina podría ser un marcador de la permeabilidad de las uniones estrechas;
 366 sin embargo, la estimación de la translocación microbiana podría reforzarse
 367 utilizando otros fabricantes de suero conocidos, como la DNAemia bacteriana, el
 368 b-glucano, la proteína de unión a LPS (LBP), la proteína de unión a ácidos grasos
 369 intestinales (iFABP), el CD14 soluble u otros (Stehle et al. , 2012; Koutsounas et
 370 al., 2015; Fukui, 2016), que podrían ser opciones a considerar junto con la
 371 zonulina en futuros estudios.

372

373 El presente estudio tiene algunas limitaciones. El tamaño de muestra
 374 relativamente pequeño para los valores de zonulina (n=60) y

375 citoquinas/quimiocinas (n=42) podría ser responsable de los errores de tipo II.
 376 Como se ha indicado, la producción de zonulina puede deberse a diversos
 377 factores, no tan sólo a translocación bacteriana, lo cual puede constituir una
 378 limitación, no obstante, al existir una asociación entre zonulina y obesidad, y al
 379 haberse descrito que la obesidad favorece la translocación microbiana, el
 380 escenario más probable es que al menos en la mayoría de los casos, el
 381 incremento de zonulina se correlaciona con el incremento de translocación. Otra
 382 limitación fue no disponer de la información de fecha de inicio de síntomas, para
 383 conocer el tiempo de enfermedad, puesto que dicha variable no fue registrada
 384 para dichos pacientes en el momento de ingreso hospitalario debido a la
 385 sobrecarga del sistema hospitalario (primera ola de COVID-19 en Perú). Sobre
 386 el análisis de interacción entre variables, se puede debatir la presencia de una
 387 verdadera interacción entre los niveles de zonulina y la severidad del paciente,
 388 dado que nuestros resultados solo indican un indicio de esta interacción, por lo
 389 que un estudio con mayor tamaño muestral podría evaluar ésta.

390

391 En conclusión, los niveles de zonulina detectados en pacientes hospitalizados
 392 con COVID-19 se asociaron con muerte incluso después de ajustar por
 393 severidad, presencia de comorbilidades y sexo en una cohorte peruana de
 394 COVID-19. Así, un cribado de los niveles de zonulina a ingreso hospitalario podría
 395 ser una herramienta más que permitiese identificar de forma temprana pacientes
 396 con mayor riesgo de muerte. Se recomiendan futuros estudios longitudinales
 397 sobre los niveles de zonulina para determinar su variación en el tiempo, así como
 398 identificar su papel en pacientes que presentan síndrome inflamatorio
 399 sistémico/COVID persistente.

400

401 **ENUNCIADO DE DISPONIBILIDAD DE DATOS**

402 Los datos sin procesar que respaldan las conclusiones de este artículo serán
 403 puestos a disposición por los autores, sin reservas indebidas.

404

405 **ENUNCIADO DE ÉTICA**

406 Los estudios que involucran participantes humanos fueron revisados y aprobados
 407 por CIEI Universidad Científica del Sur-151-2022-POS50. No se proporcionó el
 408 consentimiento informado por escrito porque las muestras tenían fines de
 409 diagnóstico y, debido a la primera ola de COVID en Perú, el comité de ética
 410 aprobó el uso de muestras de diagnóstico.

411

412 **CONTRIBUCIONES DE LOS AUTORES**

413 MP, LP-K y JR concibieron el estudio. LP-K, MP, BY, AM realizaron la
 414 cuantificación de zonulina por ELISA. MU-G obtuvo las muestras y la información
 415 clínica. LP-K realizó los análisis estadísticos. LP-K y MP escribieron el manuscrito.

416 Todos los autores hicieron contribuciones al borrador y aprobaron la versión final.
417 Todos los autores contribuyeron al artículo y aprobaron la versión enviada.

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426

427 **CONFLICTO DE INTERÉS**

428 Los autores declaran que la investigación se realizó en ausencia de cualquier
429 relación comercial o financiera que pudiera interpretarse como un potencial
430 conflicto de interés.

431

432 **NOTA DE LA CASA PUBLICADORA**

433 Todas las afirmaciones expresadas en este artículo pertenecen únicamente a los
434 autores y no representan necesariamente las de sus organizaciones afiliadas, o
435 las del editor, los editores y los revisores. Cualquier producto que pueda ser
436 evaluado en este artículo, o afirmación que pueda hacer su fabricante, no está
437 garantizada ni respaldada por el editor.

438

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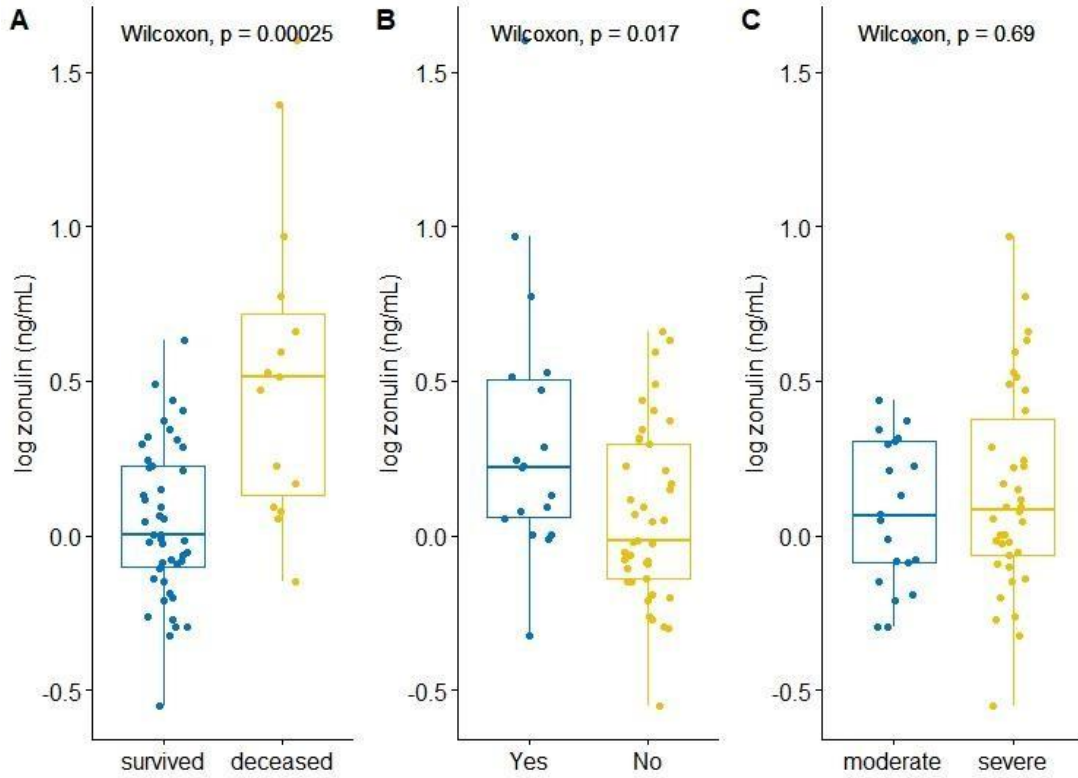
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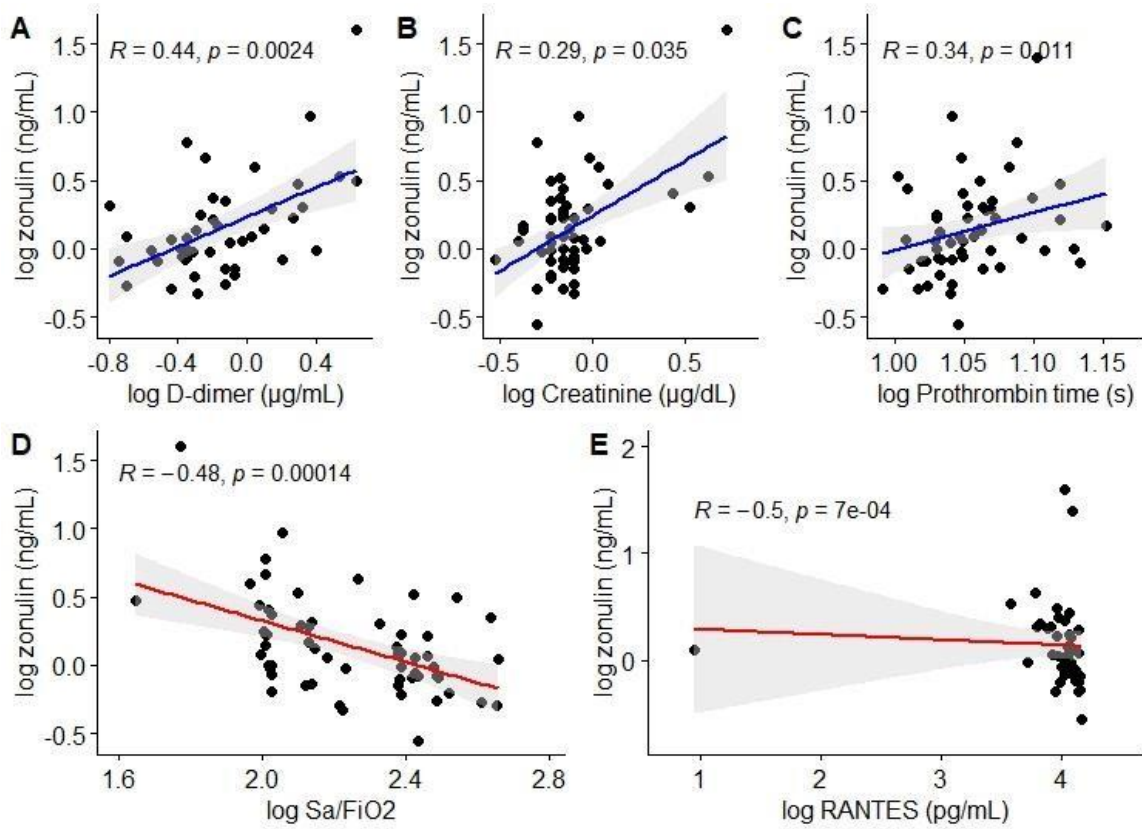
691 Figura 1. Concentraciones de zonulina por desenlace (A), obesidad (B) y severidad (C)



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718 Figura 2. Gráficos de dispersión mostrando correlaciones significativas entre zonulina y
719 Dímero-D (A), creatinine (B), tiempo de protrombina (C), Sa/Fio2 (D) y RANTES €

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741 Tabla 1. Características basales de la cohorte por desenlace (sobrevivientes vs. fallecidos). Los valores de
 742 variables continuas se reportaron como mediana (rango intercuartílico), a menos que se indique lo contrario.
 743 . ERC: Enfermedad crónica renal. EPOC: Enfermedad pulmonar obstructiva crónica. ECV: Enfermedad
 744 cardiovascular. TP: Tiempo de protrombina. TTAP: Tiempo de tromboplastina activada parcial. SaFiO₂:
 745 proporción de saturación de oxígeno/fracción de oxígeno inspirado.

746				
747				
748		Sobrevivientes	Fallecidos	p-valor
749				
750	General, n=60 (%)	45 (75.00)	15 (25.00)	-
751	Edad (años)	55 (19)	55 (25)	0.6943
752	Sexo			
753	Varón (%)	39 (86.67)	12 (80.00)	0.6780
754	Mujer (%)	6 (13.33)	3 (20.00)	
755	Severidad			
756	Moderado (%)	19 (42.22)	2 (13.33)	0.0420
757	Severo (%)	26 (57.78)	13 (86.67)	
758				
759	Tiempo desde admisión hasta			
760	el alta hospitalaria/Muerte (días)	11 (8)	12.5 (12)	0.9786
761				
762	Tiempo desde admisión			
763	hasta primera muestra (días)	(1.5)	1 (5)	0.6208
764				
765	Tiempo desde primera muestra			
766	hasta el alta hospitalaria/muerte (días)	9(8.5)	8(14)	0.4605
767				
768	Presencia de Comorbilidades, n (%)			
769	Ninguna	23 (51.11)	3 (20.00)	0.035
770	Obesidad	9 (20.00)	9 (64.29)	0.006
771	Diabetes	5 (11.11)	2 (14.29)	0.666
772	Hipertensión arterial	8 (17.78)	5 (35.71)	0.266
773	ERC	1 (2.22)	0 (0.00)	1.000
774	COPD	1 (2.22)	2 (14.29)	0.137
775	ECV	0 (0.00)	1 (7.14)	0.237
776	Gota	1 (2.22)	0 (0.00)	1.000
777	Otras	3 (6.67)	0 (0.00)	0.566
778				
779	Hemoglobina, recuento de glóbulos blancos y otros biomarcadores			
780	Hemoglobina (g/dL), media (desv. estándar)	14.38 (1.52)	13.19 (1.93)	0.0176
781	Leucocitos (10 ⁹ /L)	9 (6.87)	12.485 (8.17)	0.2142
782	Abastionados (%)	2 (3)	2 (2)	0.6491
783	Segmentados (%)	83 (12)	88 (7)	0.0060
784	Linfocitos (%)	10 (10)	6 (4)	0.0045
785	Neutrófilos totales (10 ⁹ /L)	7.56 (7.32)	10.82 (8.26)	0.2307
786	Linfocitos totales (10 ⁹ /L)	0.94 (0.77)	0.59 (0.33)	0.0046
787	Plaquetas (recuento por µL)	345000 (147000)	274500 (45000)	0.1850
788	Proteína C-Reactiva (mg/mL)	61.3 (163.8)	168.7 (122.8)	0.1701
789	Dímero D (µg/mL)	0.58 (0.51)	0.85 (1.86)	0.1425
790	Ferritina (µg/L)	789.7 (679.5)	1261.1 (838)	0.0536
791	Creatinina (mg/dL)	0.7 (0.2)	0.8 (0.48)	0.4152
792	TP (s)	11.17 (0.89)	11.57 (1.31)	0.1627
793	TTAP (s)	30.87 (5.15)	32.65 (4.22)	0.1782
794	Sa/FiO ₂	240 (162.63)	108.73 (35.40)	0.0007
795				

796 Tabla 2. Valores basales de zonulina, citoquinas y Quimiocinas en suero por desenlace (sobreviviente vs.
797 Fallecidos). Los valores de variables continuas se reportaron como mediana (rango intercuartílico).

798

	Sobrevivientes	Fallecidos	p-valor	
799				
800				
801	General, n=60 (%)	45 (75.00)	15 (25.00)	-
802	Zonulina (ng/mL)	1.0029 (0.8887)	3.2644 (4.7096)	0.0002

803

	Sobrevivientes	Fallecidos	p-valor
804			
805			

806	General, n=42 (%)	37 (88.1)	5 (11.90)	-
807	IL-1 β (pg/mL)	10 (1.5)	11 (2)	0.1194
808	GCSF (pg/mL)	11.5 (3)	12 (3)	0.3083
809	IL-10 (pg/mL)	19.5 (11)	49.5 (54)	0.1246
810	IL-13 (pg/mL)	8 (2)	8(2)	0.2062
811	IL-6 (pg/mL)	21 (38)	55 (130)	0.0295
812	IL-12 (pg/mL)	13 (3)	15 (4)	0.5556
813	IL-17A (pg/mL)	14 (2)	14 (3)	0.4770
814	GMCSF (pg/mL)	11 (2.5)	14 (4)	0.0059
815	IL-15 (pg/mL)	14 (3)	18 (8)	0.0456
816	EGF (pg/mL)	43 (64)	25 (135)	0.8612
817	IL-5 (pg/mL)	11 (1)	12 (1.5)	0.3165
818	VEGF (pg/mL)	20 (15.5)	17 (110)	0.6688
819	IFN- γ (pg/mL)	10 (3)	11 (2)	0.7092
820	IFN- α (pg/mL)	10 (1)	11 (2)	0.2605
821	IL1-RA (pg/mL)	12 (4.5)	18 (33.5)	0.0928
822	TNF- α (pg/mL)	27.5 (10)	50.5 (44)	0.0566
823	IL-2 (pg/mL)	13 (1)	15 (2)	0.0275
824	IL-7 (pg/mL)	13 (4)	15 (3)	0.3299
825	IL-4 (pg/mL)	8 (2)	10 (2)	0.1539
826	IL-8 (pg/mL)	66 (28.5)	174 (258.5)	0.0004
827	IL-12p70 (pg/mL)	9 (1)	10 (2)	0.1730
828	IL-1a (pg/mL)	14 (4)	21 (5)	0.0318
829	IL-3 (pg/mL)	8 (1)	8 (1)	0.1627
830	TNF- β (pg/mL)	10 (2)	10 (2)	0.9191

831

	Sobrevivientes	Fallecidos	p-valor
832			
833			

834	General, n=42 (%)	37 (88.1)	5 (11.90)	-
835	RANTES (pg/mL)	11089 (3360)	10507 (4039.5)	0.6000
836	Eotaxin (pg/mL)	98 (69)	134 (123.5)	0.8308
837	MIP-1 α (pg/mL)	15.5 (5.5)	22 (23.5)	0.0081
838	MIP-1 β (pg/mL)	118 (99)	173 (589.5)	0.2211
839	MCP-1 (pg/mL)	1620 (1938)	9113 (1550)	0.0008
840	IP-10 (pg/mL)	3501.5 (5094)	6720 (4416)	0.1563

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846 Table 3: Correlaciones entre niveles de zonulina y marcadores hematológicos/bioquímicos y
847 citoquinas/Quimiocinas basales.

848

	Rho de Spearman	p-valor
849		
850	Marcador hematológico/bioquímico	
851	Hemoglobina	-0.2009
852	Leucocitos	0.0027
853	Abastoados	0.1993
854	Segmentados	0.0933
855	Linfocitos	-0.0374
856	Plaquetas	-0.0852
857	Proteína C-Reactiva	0.0114
858	Dímero D	0.4415
859	Ferritina	0.1846
860	Creatinina	0.2880
861	TP	0.3441
862	TTAP	0.1311
863	Sa/FiO ₂	-0.4764
864		
865	Citoquinas	
866	IL-1 β	-0.1077
867	GCSF	-0.1817
868	IL-10	0.0117
869	IL-13	-0.0113
870	IL-6	-0.0946
871	IL-12	-0.0152
872	IL-17A	0.1177
873	GMCSF	0.0231
874	IL-15	0.1361
875	EGF	-0.0478
876	IL-5	-0.1591
877	VEGF	0.1512
878	IFN- γ	-0.0474
879	IFN- α	-0.0334
880	IL1-RA	-0.0612
881	TNF- α	0.2342
882	IL-2	0.0181
883	IL-7	-0.2031
884	IL-4	-0.1908
885	IL-8	0.1990
886	IL-12p70	0.0738
887	IL-1a	-0.0106
888	IL-3	-0.0859
889	TNF- β	-0.1670
890		
891	Quimiocinas	
892	RANTES	-0.5023
893	Eotaxin	0.1928
894	MIP-1 α	-0.0400
895	MIP-1 β	0.2032
896	MCP-1	0.2268
897	IP-10	0.0891

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900 Tabla 4. Modelo multivariable de zonulina y mortalidad por COVID-19. Valores en negrita: p
901 valor <0.05

902

Factores	HR crudo	IC 95%	HR ajustado sin IT	IC 95%	HR ajustado con IT	IC 95%
Zonulina (ng/mL)	1.11	1.05-1.17	1.19	1.09-1.29	1.16	1.06-1.26
Severidad						
Moderado	Referencial					
Severo	3.61	0.80-16.23	15.83	1.31-191.39	4.31	0.39-48.14
Presencia de comorbilidades						
No	Referencial					
Sí	1.67	0.46-6.08	3.30	0.40-27.19	2.14	0.26-17.67
Sexo						
Masculino	Referencial					
Femenino	4.81	1.14-20.23	20.29	2.06-200.31	17.91	1.83-175.30
Zonulin x Severidad						
Zonulina x Severo					1.27	0.96-1.69

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917 Tabla 5. Análisis de sensibilidad al eliminar outlier con concentración de zonulina=40 ng/mL.
 918 Valores en negrita: p valor <0.05

919

Factores	HR crudo	IC 95%	HR ajustado sin IT	IC 95%
Zonulina (ng/mL)	1.50	1.18-1.90	1.47	1.10-1.96
Severidad				
Moderado	Referencial			
Severo	7.23	0.93-56.00	6.04	0.67-54.16
Presencia de comorbilidades				
No	Referencial			
Sí	1.48	0.40-5.47	2.08	0.26-16.70
Sexo				
Masculino	Referencial			
Femenino	5.99	1.33-26.95	17.19	1.82-162.60

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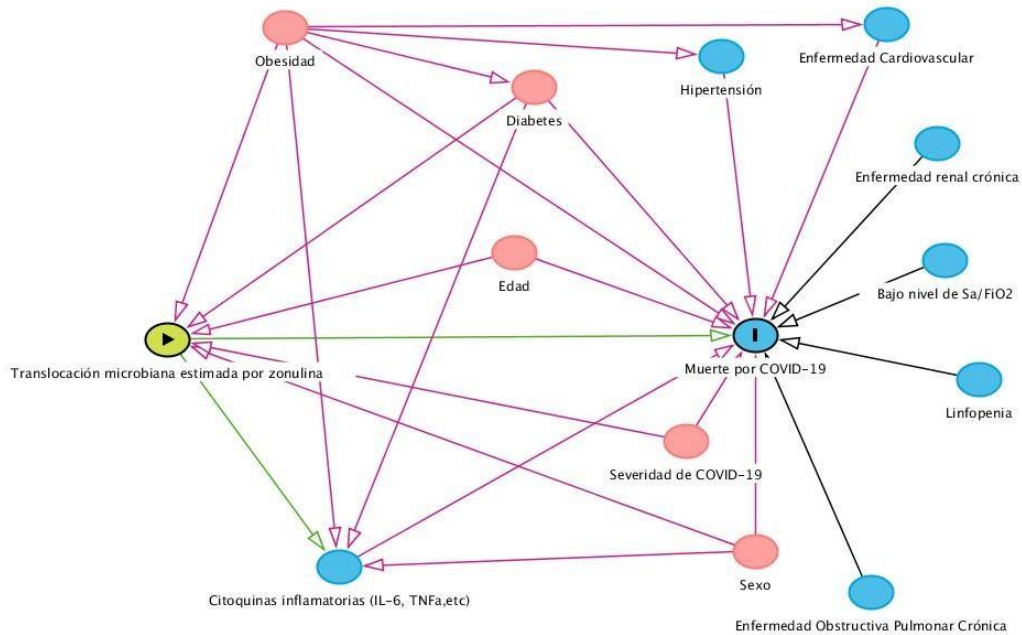
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Anexos

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939 Anexo 1: Gráfico Acíclico Dirigido (DAG) y modelo conceptual



940

941 Gráfico Acíclico Dirigido de la asociación entre translocación microbiana
 942 estimable por zonulina y muerte por COVID-19, construido mediante DAGitty
 943 v.3.0 (<http://www.dagitty.net/dags.html#>)

944

945 Justificación:

946

947 Se construyó el presente DAG para establecer de manera gráfica las posibles variables
 948 confusoras/mediadoras en la relación causal entre la translocación microbiana estimada
 949 por zonulina y muerte en pacientes COVID-19 hospitalizados. Uno de los factores
 950 asociados con mortalidad en COVID-19 es la severidad. La edad es una variable que
 951 tradicionalmente se ha considerado como variable confusora, aunque algunos estudios
 952 proponen que ésta es en realidad una variable mediadora de efecto entre diversas
 953 exposiciones y muerte por COVID-19 (1-2), por lo que es debatible. Se ha establecido
 954 que el riesgo de morir por COVID-19 es mayor en varones que en mujeres en una
 955 revisión sistemática que incluyó 229 estudios (3).

956

957 Otras Variables confusoras serían comorbilidades: Obesidad y diabetes, siendo éstas
 958 condiciones de riesgo de muerte en pacientes con COVID-19 (4,5) y también se asocian
 959 con mayor translocación microbiana debido a que generan estados inflamatorios
 960 crónicos subyacentes en los pacientes, el cual aumenta la translocación microbiana (6–
 961 9). Asimismo, diabetes y obesidad se encuentran relacionadas (10). Otras

962 comorbilidades también se asocian con mortalidad en COVID-19, tales como
 963 hipertensión, enfermedad pulmonar obstructiva crónica, enfermedad cardiovascular,
 964 enfermedad renal crónica, entre otras (11-12) No obstante, su relación con translocación
 965 microbiana no es clara. Además, linfopenia también ha sido asociado con mortalidad
 966 por covid (13). Inicialmente bajos niveles de Sa/FiO₂ se habían asociado con peores
 967 desenlaces en COVID-19 pero una meta-análisis ha puesto en duda dicha asociación
 968 (14).

969

970 Finalmente, un nivel alto de citoquinas proinflamatorias, IL-6 en particular se asocia con
 971 peor pronóstico y muerte por COVID-19 (15). No obstante, varios estudios de revisión
 972 han establecido una hipótesis sobre la fisiopatología del SARS-CoV-2, en la cual una
 973 mayor permeabilidad intestinal con subsiguiente translocación bacteriana junto con
 974 disbiosis intestinal e inflamación subyacente puedan tener un efecto sinérgico en la
 975 inflamación sistémica y mayor nivel de citoquinas en infecciones severas(16–19). Por
 976 ende, las citoquinas inflamatorias en conjunto podrían considerarse como variable
 977 mediadora.

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1069 Anexo 2: Características técnicas del ensayo cuantitativo ELISA Sandwich para
 1070 zonulina humana en suero. Obtenido de la página web del fabricante
 1071 (<https://www.cusabio.com/ELISA-Kit/Human-zonulin-ELISA-kit-154647.html>)

1072

1073 This Human zonulin ELISA Kit was designed for the quantitative measurement of Human zonulin
 1074 protein in serum, plasma, tissue homogenates. It is a Sandwich ELISA kit, its detection range is
 1075 0.625 ng/mL-40 ng/mL and the sensitivity is 0.156 ng/mL .

1076

1077 DETECTION RANGE

1078 0.625 ng/ml-40 ng/ml.

1079

1080 SENSITIVITY

1081 The minimum detectable dose of human zonulin is typically less than 0.156

1082 ng/ml.

1083 The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as

1084 the lowest protein concentration that could be differentiated from zero. It was

1085 determined the mean O.D value of 20 replicates of the zero standard added by

1086 their three standard deviations.

1087

1088 SPECIFICITY

1089 This assay has high sensitivity and excellent specificity for detection of human

1090 zonulin. No significant cross-reactivity or interference between human zonulin

1091 and analogues was observed.

1092 Note: Limited by current skills and knowledge, it is impossible for us to complete

1093 the cross-reactivity detection between human zonulin and all the analogues,

1094 therefore, cross reaction may still exist.

1095

1096 PRECISION

1097 Intra-assay Precision (Precision within an assay): CV%<8%

1098 Three samples of known concentration were tested twenty times on one plate to

1099 assess.

1100

1101 Inter-assay Precision (Precision between assays): CV%<10%

1102 Three samples of known concentration were tested in twenty assays to assess.

1103

1104 The recovery of human zonulin spiked to levels throughout the range of the assay in various

1105 matrices was evaluated. Samples were diluted prior to assay as directed in the Sample

1106 Preparation section.

1107

1108 Sample Type	Average % Recovery	Range
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1109 Serum (n=5)	95	90-101
------------------	----	--------

1110 EDTA plasma (n=4)	97	90-104
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Abreviaturas empleadas:

COVID-19: Enfermedad por coronavirus 2019.

SARS-CoV-2: Coronavirus de tipo 2 causante del síndrome respiratorio agudo severo (*Severe Acute Respiratory Syndrome Coronavirus 2*)

ELISA: Ensayo por inmunosorción ligado a enzimas (*Enzyme-linked Immunosorbent assay*).

CRP: Proteína C-reactiva

PT: Tiempo de protrombina

APTT: Tiempo parcial activado de tromboplastina

Sa/FiO₂: Razón de saturación de oxígeno a la fracción de oxígeno inspirado.

ARDS: Síndrome de distrés respiratorio agudo

ACE2: Receptor de enzima convertidora de angiotensina.

kDa: kilodalton, equivalente a $1,66053886 \times 10^{-24}$ kg

COPD: Enfermedad pulmonar crónica obstructiva.

WBC: Recuento de glóbulos blancos

TMB: 3,3',5,5'-Tetrametilbencidina

IQR: Rango intercuartílico

HR: Razón de riesgos inmediato, *Hazard ratio*

Bacterial DNAemia: Presencia de ácido desoxirribonucleico proveniente de bacterias en suero.

LBP: Proteína de unión a lipopolisacárido

iFABP: Proteína de unión a ácidos grasos intestinales.



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Zonulin, a marker of gut permeability, is associated with mortality in a cohort of hospitalised peruvian COVID-19 patients

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Zonulin has previously been related to intestinal permeability in various inflammatory diseases, and more recently to the physiopathology of severe COVID-19 infections. We analysed serum samples from a previous study of a Peruvian cohort of hospitalised COVID-19 patients, for the quantification of zonulin by sandwich ELISA. Comparisons with clinical data, haematological and biochemical parameters and cytokine/chemokine levels were made. We found higher baseline zonulin levels in deceased patients, and zonulin was associated with fatal outcome in multivariate analyses, even after adjustment for age, gender, and obesity. There were also positive correlations between zonulin, creatinine, D-dimer values and prothrombin time, while an inverse correlation was found for Sa/FiO₂ ratio and CCL5 (RANTES). Further longitudinal studies are recommended to analyse the variation of zonulin levels over time as well as their relationship with long-COVID.

KEYWORDS

microbial translocation, zonulin, COVID-19, biomarker, ELISA, Peru

Introduction

Severe COVID-19 infections are characterised by systemic inflammation, acute respiratory distress syndrome (ARDS) and multi-organic system failure (Kim et al., 2021) in adults. The Severe acute respiratory syndrome coronavirus (SARS-CoV-2) binds principally to receptor angiotensin-converting enzyme 2 (ACE2) to enter many different host cells such as type II pneumocytes in the lung, myocardial cells, neurons and glia,

endothelial cells, intestinal enterocytes and others (Lamers et al., 2020; Beyerstedt et al., 2021). Particularly, intestinal enterocytes form a cell layer that is part of the mucosal defence system and are held together by tight junctions. Disruption of this layer (increased gut permeability) translates into translocation of bacterial components from the gut into the bloodstream, contributing to a further increase in systemic inflammation (Dinh et al., 2015).

As reported in other viral infections such as dengue fever (Chanchaoentana et al., 2021) or human immunodeficiency virus (Marchetti et al., 2013), microbial translocation may be caused by direct enterocyte infection from the virus or because of cytokine-mediated alterations and gut dysbiosis. Previous studies (Yonker et al.; Giron et al., 2021; Oliva et al., 2021) have linked higher intestinal translocation with severe cases of COVID-19, describing a higher abundance of bacterial components in the bloodstream compared to milder cases. It has been suggested that microbial translocation, intestinal dysbiosis and underlying inflammatory processes might have a synergistic effect on systemic inflammation and high cytokine levels described in severe COVID-19 infections (Cardinale et al., 2020; Vignesh et al., 2020; Uzzan et al., 2020; Mashaqi et al., 2022).

Zonulin (pre-Haptoglobin 2) is a protein of ~ 47 kDa, which is detectable in its uncleaved form in human serum (Fasano, 2011) and is highly correlated with bacterial DNAemia (Gargari et al., 2021). It is a physiological mediator that reversibly regulates intestinal permeability by modulation of intercellular tight junctions (Tripathi et al., 2009) and is considered a biomarker of impaired gut barrier function (Fasano, 2011). In this sense, it has been hypothesised that the spike protein of SARS-CoV-2 induces overexpression of zonulin, which is related to an increase in intestinal permeability (Llorens et al., 2021). Moreover, zonulin levels have been reported to be higher in patients who died of severe COVID-19 (Giron et al., 2021). These data suggest that SARS-CoV-2 might facilitate passage of intestinal contents into circulation and a higher inflammatory state at intestinal level that could fuel general systemic inflammation. Thus, zonulin might be also an indirect marker of microbial translocation and could be associated with worse outcomes in COVID-19.

Inflammatory responses to viral infection are also influenced by genetic background, for example individual genetic variability of pro-inflammatory cytokines is related to severity in COVID-19 (Vakil et al., 2022). Moreover, population-associated variation of responses to viral infections is differentiated by genetic ancestry (Randolph et al., 2021). The few published studies on serum zonulin levels and COVID-19 come from cohorts/case series of United States of America (Giron et al., 2021; Josyabhatla et al., 2021; Yonker et al., 2022), Italy (Oliva et al., 2021) and Turkey (Kılıç et al.) only. Therefore, data from studies with subjects from a different genetic and geographic background are important for external validity issues.

For the reasons mentioned above, the present study aimed to identify the association between microbial translocation estimated by serum zonulin and patient outcome in a cohort of moderate and severe COVID-19 hospitalised patients from Lima, Peru. Additionally, correlations were established amongst cytokine/chemokine values, routine clinical parameters, and serum zonulin levels.

Materials and methods

Study population and assessment of disease severity

The present study used samples from a cohort of hospitalised COVID-19 patients from *Hospital Nacional Guillermo Almenara Irigoyen* (Pons et al., 2021). A total of 60 patients were classified according to severity: 19 moderate, 26 severe and 15 deaths (initially classified as moderate or severe). The COVID-19 cases were confirmed by quantitative polymerase chain reaction (qPCR) or antibody testing. The follow-up period was from August to October 2020, which was within the context of the first wave of COVID-19 in Peru. Therefore, no patient had received vaccination for COVID-19.

The definition of moderate infection was according to the Berlin 2012 definition of Acute Respiratory Distress Syndrome (ARDS) (ARDS Definition Task Force, 2012), while severe infection was defined whenever one or more of the following states developed: Horowitz index ($\text{PaO}_2/\text{FiO}_2$) < 100, oxygen saturation to fraction of inspired oxygen ratio ($\text{SpO}_2/\text{FiO}_2$, henceforth abbreviated as Sa/FiO_2) < 89, need for mechanical ventilation due to respiratory failure or systemic organ failure requiring admission to the intensive care unit.

Sample conditions

Serum samples from the original study were conserved at -80°C in the laboratories of the *Universidad Científica del Sur*.

Clinical data and cytokine levels

Data pertaining to age, gender, date of hospital admission, date of hospital discharge/death, date of sample according to hospital admission, comorbidities such as obesity, arterial hypertension, diabetes, chronic obstructive pulmonary disease (COPD) and any other underlying conditions, pharmacological treatment received, haemoglobin, platelets, white blood cell (WBC) counts and Sa/FiO_2 were obtained from the corresponding medical records. All privileged identifiable information of the patients was excluded from the dataset

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generated and the samples were codified previously to ensure confidentiality.

The cytokine and chemokine concentrations of the serum samples were obtained from the data previously reported in the study by Pons et al. (Pons et al., 2021), in which a commercial kit of Luminex MAGPIX technology (Merck KGaA, Darmstadt, Germany) was used for quantification.

Estimation of microbial translocation by zonulin quantification

Zonulin (ng/mL) was quantified from the serum samples by sandwich ELISA, using the CUSABIO Human Zonulin ELISA Kit (CUSABIO TECHNOLOGY LLC, Wuhan, China) according to the manufacturer's instructions. Briefly, 100 µL of standards and samples were added per well in the coated microplate and incubated for two hours at 37°C. Subsequently, 100 µL of biotin-antibody solution was added and incubated for one hour at 37°C. Afterwards, the liquid was aspirated, and manual washing was done three times with the provided wash buffer. Then 100 µL of horseradish peroxidase-avidin solution was added per well and incubated for one hour at 37°C, and manual washing was done thereafter. Finally, 90 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added per well and incubated for 30 minutes at 37°C. Immediately after, 50 µL of stop solution was added per well and absorbance reading was done at 450 nm (with correction at 540 nm) in a Synergy LX Multi-Mode Reader (Biotek, USA). The samples and standards were run in duplicate.

Statistical analysis

Descriptive analysis was performed using absolute and relative frequencies for summarising categorical variables while median and interquartile range (IQR) were used for numerical variables, unless specified otherwise.

Bivariate analyses were made for comparisons between outcomes (survived vs. deceased) and categorical variables using the Chi-squared or Fisher exact test, while the Mann-Whitney U test was used for comparisons between outcomes and numerical variables. Correlations among WBC counts, biochemical markers, cytokines, chemokines and zonulin levels were normalised using base 10 log transformation and comparisons were made using Spearman tests.

Crude and adjusted multivariate analyses of potential prognostic factors of death were performed using logistic regression with clinical variables, cytokine and zonulin levels at baseline (from first serum sample available since hospital admission) and odds ratios (OR) were obtained. Survival analyses were done using Cox regression, and crude and adjusted hazard ratios (HR) were estimated accordingly. Significant variables in bivariate analysis were employed for

the regression analyses. Epidemiological criteria for adjustment of confounding and the use of variables with less than 20% data loss were employed to decide which adjusted model best fitted the data.

For the interpretation of the zonulin results, samples with a zonulin concentration above the upper limit of the standard (40 ng/mL) were assigned this value. Values below 0.625 ng/mL were used as their actual values for the analyses.

The analyses were conducted using STATA 16.1 SE Edition (StataCorp, TX) and graphics were constructed using R version 4.1.2. 95% Confidence intervals were calculated for OR and HR. Statistical significance was established with $\alpha=0.05$.

Results

Baseline clinical characteristics of the hospitalised cohort

Sixty hospitalised COVID-19 patients were included: 45 patients (75%) survived, and 15 (25%) patients died during the follow-up period. The distribution for case severity was as follows: 39 patients (65%) were classified as severe and 21 (35%) as moderate according to the given case definition and progression during the follow-up period. Severity was associated with outcome ($p=0.042$). The median age was similar in both the survived and deceased groups (55 and 55 years, respectively) and there was no significant difference in age ($p=0.694$) or sex ($p=0.678$) between the two groups. The median (IQR) time from admission to first serum sample were 1(1.5) and 1(5) days in the survived and deceased groups respectively ($p=0.621$). Time from admission to hospital discharge/death (time of hospitalisation) and time from first serum sample obtained to hospital discharge/death were also similar between the aforementioned groups ($p=0.978$ and $p=0.461$, respectively) (Table 1).

Some comorbidities such as obesity, diabetes, high blood pressure and others were present (Table 1). In the deceased group, 12 patients (80%) had comorbidities compared to 22 (48.9%) in the survivor group, and this difference was significant ($p=0.035$). The only comorbidity that was associated with outcome was obesity ($p=0.006$). Corticosteroids were employed as part of the treatment for all the patients in this cohort.

White blood cell counts, biochemical markers, and cytokine/chemokine levels at baseline

Haemoglobin levels at baseline were lower in the deceased, with a mean (standard deviation) of 13.19 (1.93) g/dL ($p=0.018$). Regarding WBC counts, segmented neutrophil counts were

Q12 TABLE 1 Characteristics of the study cohort by outcome at baseline.

	Survived	Deceased	p-value
Overall, n=60 (%)	45 (75.00)	15 (25.00)	
Age (years)	55 (19)	55 (25)	0.6943
Sex			
Male (%)	39 (86.67)	12 (80.00)	0.6780
Female (%)	6 (13.33)	3 (20.00)	
Severity			
Moderate (%)	19 (42.22)	2 (13.33)	0.0420
Severe(%)	26 (57.78)	13 (86.67)	
Time from admission to hospital discharge/death (days)	11 (8)	12.5 (12)	0.978
Time from admission to first sample (days)	1 (1.5)	1 (5)	0.621
Time from first sample to hospital discharge/death (days)	9(8.5)	8(14)	0.461
Presence of Comorbidities, n (%)			
None	23 (51.11)	3 (20.00)	0.035
Obesity	9 (20.00)	9 (64.29)	0.006
Diabetes	5 (11.11)	2 (14.29)	0.666
High Blood Pressure	8 (17.78)	5 (35.71)	0.266
CKD	1 (2.22)	0 (0.00)	1.000
COPD	1 (2.22)	2 (14.29)	0.137
CVD	0 (0.00)	1 (7.14)	0.237
Gout	1 (2.22)	0 (0.00)	1.000
Other	3 (6.67)	0 (0.00)	0.566
Haemoglobin, white blood cell count and other biomarkers			
Haemoglobin (g/dL), mean (SD)	14.38 (1.52)	13.19 (1.93)	0.0176
Leucocytes (10 ⁹ /L)	9 (6.87)	12.485 (8.17)	0.2142
Band neutrophils (%)	2 (3)	2 (2)	0.6491
Segmented neutrophils (%)	83 (12)	88 (7)	0.0060
Lymphocytes (%)	10 (10)	6 (4)	0.0045
Total neutrophils (10 ⁹ /L)	7.56 (7.32)	10.82 (8.26)	0.2307
Total lymphocytes (10 ⁹ /L)	0.94 (0.77)	0.59 (0.33)	0.0046
Platelets (count per µL)	345000 (147000)	274500(45000)	0.1850
CRP (mg/mL)	61.3 (163.8)	168.7 (122.8)	0.1701
D-dimer (µg/mL)	0.58 (0.51)	0.85 (1.86)	0.1425
Ferritin (µg/L)	789.7 (679.5)	1261.1 (838)	0.0536
Creatinine (mg/dL)	0.7 (0.2)	0.8 (0.48)	0.4152
PT (s)	11.17 (0.89)	11.57 (1.31)	0.1627
APTT (s)	30.87 (5.15)	32.65 (4.22)	0.1782
Sa/FiO ₂	240 (162.63)	108.73 (35.40)	0.0007

Values of continuous variables are reported as median (interquartile range), unless stated otherwise. CKD, Chronic Kidney Disease; COPD, Chronic Obstructive Pulmonary Disease; CVD, Cardiovascular disease; CRP, CReactive Protein; PT, Prothrombin Time; APTT, Activated Partial Thromboplastin Time; Sa/FiO₂, Oxygen saturation/Fraction of inspired oxygen.

higher (p=0.006) and lymphocyte counts were lower in the deceased group (p=0.004) with no significant differences being found for the remaining WBC counts in the two groups.

In relation to some biochemical markers such as C-reactive protein, D-dimer and ferritin, there were no significant differences when comparisons were made by outcome (Table 1). However, the Sa/FiO₂ ratio was lower in the deceased group, with a median (IQR) of 108.73 (35.40) (p=0.0007).

The cytokines interleukin-6 (IL-6) (p=0.029), granulocyte-macrophage colony-stimulating factor (GM-CSF) (p=0.005), IL-15 (p=0.046), IL-2 (p=0.027), IL-8 (p=0.0004), IL-1a (p=0.032), and chemokines macrophage inflammatory protein-alpha (MIPa) (p=0.008) and monocyte chemoattractant protein-1 (MCP-1) (p=0.001) showed significant differences according to outcome (Table 2), with all being elevated in the deceased group.

TABLE 2 Zonulin, cytokines and chemokines values in serum at baseline by outcome.

	Survived	Deceased	p-value
Overall, n=60 (%)	45 (75.00)	15 (25.00)	
Zonulin (ng/mL)	1.0029 (0.8887)	3.2644 (4.7096)	0.0002
0	Survived	Deceased	p-value
Overall, n=42 (%)	37 (88.1)	5 (11.90)	
IL-1b (pg/mL)	10 (1.5)	11 (2)	0.1194
GCSF (pg/mL)	11.5 (3)	12 (3)	0.3083
IL-10 (pg/mL)	19.5 (11)	49.5 (54)	0.1246
IL-13 (pg/mL)	8 (2)	8(2)	0.2062
IL-6 (pg/mL)	21 (38)	55 (130)	0.0295
IL-12 (pg/mL)	13 (3)	15 (4)	0.5556
IL-17A (pg/mL)	14 (2)	14 (3)	0.4770
GMCSF (pg/mL)	11 (2.5)	14 (4)	0.0059
IL-15 (pg/mL)	14 (3)	18 (8)	0.0456
EGF (pg/mL)	43 (64)	25 (135)	0.8612
IL-5 (pg/mL)	11 (1)	12 (1.5)	0.3165
VEGF (pg/mL)	20 (15.5)	17 (110)	0.6688
IFN-g (pg/mL)	10 (3)	11 (2)	0.7092
IFN-a (pg/mL)	10 (1)	11 (2)	0.2605
IL1-RA (pg/mL)	12 (4.5)	18 (33.5)	0.0928
TNF-a (pg/mL)	27.5 (10)	50.5 (44)	0.0566
IL-2 (pg/mL)	13 (1)	15 (2)	0.0275
IL-7 (pg/mL)	13 (4)	15 (3)	0.3299
IL-4 (pg/mL)	8 (2)	10 (2)	0.1539
IL-8 (pg/mL)	66 (28.5)	174 (258.5)	0.0004
IL-12p70 (pg/mL)	9 (1)	10 (2)	0.1730
IL-1a (pg/mL)	14 (4)	21 (5)	0.0318
IL-3 (pg/mL)	8 (1)	8 (1)	0.1627
TNF-b (pg/mL)	10 (2)	10 (2)	0.9191
	Survived	Deceased	p-value
Overall, n=42 (%)	37 (88.1)	5 (11.90)	–
RANTES (pg/mL)	11089 (3360)	10507 (4039.5)	0.6000
Eotaxin (pg/mL)	98 (69)	134 (123.5)	0.8308
MIP-1a (pg/mL)	15.5 (5.5)	22 (23.5)	0.0081
MIP-1b (pg/mL)	118 (99)	173 (589.5)	0.2211
MCP-1 (pg/mL)	1620 (1938)	9113 (1550)	0.0008
IP-10 (pg/mL)	3501.5 (5094)	6720 (4416)	0.1563

Values of continuous variables are reported as median (interquartile range).

Correlations of zonulin versus haematological/biochemical markers and cytokines/chemokines at baseline

Zonulin levels were positively correlated with creatinine ($r=0.288$, $p=0.035$) D-dimer values ($r=0.441$, $p=0.002$), whereas prothrombin time ($r=0.344$, $p=0.011$) and Sa/FiO₂ ratio ($r=-0.4764$, $p=0.0001$) were found to be negatively correlated with zonulin (Table 3).

Related to cytokines and chemokines, RANTES was the only chemokine negatively correlated with zonulin levels ($r=-0.5023$,

$p=0.007$). None of the cytokines were significantly correlated with zonulin levels (Table 3).

Zonulin levels at baseline associated with outcome in the bivariate and multivariate analyses

The median (IQR) level of zonulin in the deceased group was 3.264 (4.7096) ng/mL (min 0.7099 ng/mL – max 40 ng/mL, Table 2) showing a significant association with outcome ($p=0.0002$, Figure 1) and obesity ($p=0.017$, Figure 1) in the

TABLE 3 Correlations between zonulin levels and haematological / biochemical markers and cytokines/chemokines at baseline. .

	Spearman's rho	p-value
Haematological/biochemical marker		
Haemoglobin	-0.2009	0.1305
Leukocytes	0.0027	0.9840
Band neutrophils	0.1993	0.2824
Segmented neutrophils	0.0933	0.4859
Lymphocytes	-0.0374	0.7803
Platelets	-0.0852	0.5287
CRP	0.0114	0.9354
D-dimer	0.4415	0.0024
Ferritin	0.1846	0.1654
Creatinine	0.2880	0.0347
PT	0.3441	0.0108
APTT	0.1311	0.3447
Sa/FiO ₂	-0.4764	0.0001
Cytokines		
IL-1b	-0.1077	0.4971
GCSF	-0.1817	0.2494
IL-10	0.0117	0.9413
IL-13	-0.0113	0.9434
IL-6	-0.0946	0.5514
IL-12	0.9238	
IL-17A	0.1177	0.4579
GMCSF	0.0231	0.8845
IL-15	0.1361	0.3900
EGF	-0.0478	0.7636
IL-5	-0.1591	0.3143
VEGF	0.1512	0.3392
IFN-g	-0.0474	0.7657
IFN-a	-0.0334	0.8336
IL1-RA	-0.0612	0.7002
TNF-a	0.2342	0.1354
IL-2	0.0181	0.9092
IL-7	-0.2031	0.1970
IL-4	-0.1908	0.2262
IL-8	0.1990	0.2065
IL-12p70	0.0738	0.6421
IL-1a	-0.0106	0.9467
IL-3	-0.0859	0.5885
TNF-b	-0.1670	0.2905
Chemokines		
RANTES	-0.5023	0.0007
Eotaxin	0.1928	0.2212
MIP-1a	-0.0400	0.8016
MIP-1b	0.2032	0.1968
MCP-1	0.2268	0.1486
IP-10	0.0891	0.5745

bivariate analysis. Notwithstanding, zonulin was not found to be associated with disease severity ($p=0.69$, [Figure 1](#)) in the same analysis.

Several factors in logistic regression, such as obesity (OR 7.200, $p=0.003$), segmented neutrophils (OR 1.145, $p=0.015$), GMCSF (OR 2.220, $p=0.015$), IL-15 (OR 1.332, $p=0.031$), IL-2 (OR 3.089, $p=0.025$), and zonulin levels (OR 2.573, $p=0.003$), presented significant OR values greater than 1 in the crude analysis, which indicate an association with higher odds of mortality. In contrast, haemoglobin (OR 0.624, $p=0.030$), lymphocytes (OR 0.821, $p=0.018$) and Sa/FiO₂ (OR 0.985, $p=0.007$) had significant ORs less than 1. ([Table 4](#)).

Notwithstanding, in the adjusted regression, only obesity (only adjusted for age and gender, OR 8.103 $p=0.003$), segmented neutrophils (OR 1.303, $p=0.010$), lymphocytes (OR 0.726, $p=0.013$), Sa/FiO₂ (OR 0.987, $p=0.025$) and zonulin levels (OR 2.666, $p=0.007$) remained significant after adjustment for age, gender, and obesity ([Table 4](#)).

Cox regression analysis

Cox regression was performed as a survival analysis with the same predictors as logistic regression, considering the period between date of hospital admission to date of hospital discharge/death (days). In the crude Cox regression only obesity (HR 3.923, $p=0.015$), Sa/FiO₂ (HR 0.987, $p=0.032$), GMCSF (HR 1.975, $p=0.034$), IL-15 (HR 1.252, $p=0.023$) and zonulin levels (HR 1.106, $p=0.001$) were found to be significant ([Table 5](#)).

As for the case of the adjusted Cox regression (adjustment for age, gender and obesity, obesity was adjusted for age and gender only), the same variables remained significant except GMCSF and IL-15 ([Table 5](#)). Moreover, segmented neutrophils (HR 1.215, $p=0.023$), lymphocytes (HR 0.764, $p=0.010$) and IL-2 (HR 4.018, $p=0.050$) were not significant in the crude Cox regression analysis but were significant in the adjusted analysis.

Discussion

Even with ready access to vaccines, COVID-19 is still a threat to public health worldwide with many aspects of its pathophysiology, as well as its consequences after clinical improvement of the infection, requiring study. The present study provides knowledge on the detection of zonulin in COVID-19 patients and some risk factors for death by COVID-19. Serum zonulin levels in a Peruvian cohort of hospitalised COVID-19 patients were measured, and an association was found with mortal outcome (both in bivariate and multivariate analyses, even after adjustment for age, gender, and obesity).

Based on the clinical-epidemiological characteristics of this cohort, the relationship of obesity and fatal outcome in COVID-

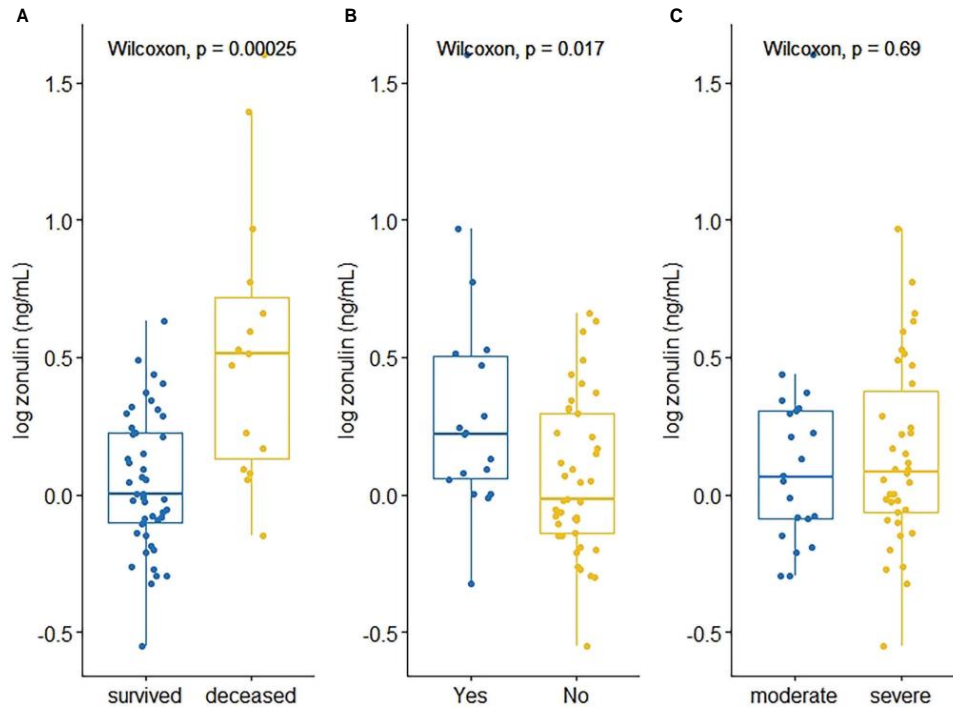


FIGURE 1 Zonulin concentrations by (A) outcome, (B) obesity and (C) disease severity.

TABLE 4 Crude and adjusted* logistic regression analysis for predictability of death according to different factors at baseline.

Variables	Crude	Adjusted
	OR (95% CI) p value	OR (95% CI) p value
Severity		
Severe	4.750 (0.957-23.572) p=0.057	3.518 (0.593-20.874) p=0.166
Moderate*		
Obesity		
Yes	7.200 (1.933-26.812) p=0.003	8.103 (2.067-31.768) p=0.003
No*		
Haemoglobin	0.624 (0.408-0.955) p=0.030	0.703 (0.416-1.186) p=0.187
Seg Neutrophils	1.145 (1.027-1.278) p=0.015	1.303 (1.064-1.596) p=0.010
Lymphocytes	0.821 (0.696-0.967) p=0.018	0.726 (0.563-0.936) p=0.013
SaFiO ₂	0.985 (0.974-0.996) p=0.007	0.987 (0.976-0.998) p=0.025
IL-6	1.012 (0.996-1.028) p=0.157	1.011 (0.994-1.030) p=0.212
GMCSF	2.220 (1.166-4.224) p=0.015	2.222 (0.977-5.050) p=0.057
IL-15	1.332 (1.026-1.730) p=0.031	1.233 (0.939-1.620) p=0.132
IL-2	3.089 (1.153-8.274) p=0.025	2.555 (0.839-7.778) p=0.099
IL-8	1.121 (0.977-1.287) p=0.103	(S)
IL1a	1.056 (0.953-1.169) p=0.297	1.026 (0.920-1.146) p=0.641
MIPa	1.374 (0.933-2.022) p=0.108	1.397 (0.931-2.097) p=0.106
MCP-1	1.001 (1.000-1.001) p=0.010	1.002 (0.999-1.004) p=0.112
Zonulin	2.573 (1.371-4.828) p=0.003	2.666 (1.310-5.426) p=0.007

*Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Cytokines were adjusted for age and obesity only due to collinearity issues with gender. (S): Value could not be computed as log likelihood = 0.

TABLE 5 Crude and adjusted* Cox regression of different factors for survival analysis.

Variables	Crude HR (95% CI) p value	Adjusted HR (95% CI) p value
Severity		
Severe	3.436 (0.759-15.566) p=0.109	4.282 (0.796-23.019) p=0.090
Moderate*		
Obesity		
Yes	3.923 (1.306-11.783) p=0.015	5.888 (1.646-21.068) p=0.006
No*		
Haemoglobin	0.669 (0.444-1.008) p=0.055	0.770 (0.480-1.235) p=0.278
Seg Neutrophils	1.083 (0.980-1.197) p=0.118	1.215 (1.028-1.437) p=0.023
Lymphocytes	0.874 (0.748-1.020) p=0.087	0.764 (0.623-0.938) p=0.010
SaFiO ₂	0.987 (0.975-0.999) p=0.032	0.986 (0.974-0.998) p=0.023
IL-6	1.010 (0.997-1.024) p=0.138	1.013 (0.997-1.029) p=0.122
GMCSF	1.975 (1.051-3.711) p=0.034	2.145 (0.889-5.115) p=0.085
IL-15	1.252 (1.032-1.518) p=0.023	1.218 (0.987-1.502) p=0.066
IL-2	3.080 (0.910-10.427) p=0.070	4.018 (1.001-16.132) p=0.050
IL-8	1.059 (0.997-1.124) p=0.061	1.080 (0.975-1.196) p=0.142
IL1a	1.026 (0.936-1.124) p=0.590	1.011(0.913-1.120) p=0.827
MIPa	1.969 (0.917-4.228) p=0.082	2.671 (0.781-9.129) p=0.117
MCP-1	1.001 (1.000-1.001) p=0.020	1.001 (0.999-1.002) p=0.110
Zonulin	1.106 (1.043-1.173) p=0.001	1.109 (1.038-1.185) p=0.002

*Adjustment for age, gender, and obesity. Obesity was adjusted for age and gender only. Zonulin values were not categorised for the analyses made herein.

19 patients has previously been described (Pons et al., 2021). This finding is in agreement with other studies reporting obesity as a strong predictor of death in severe COVID-19 infections (Cai et al., 2021; Dessie and Zewotir, 2021; Yang et al., 2021), as it involves both impaired cellular immunity and an amplification of the underlying obesity-related inflammatory status together with the acute inflammation generated by SARS-CoV-2 infection (Mohammad et al., 2021).

In addition, a lower Sa/FiO₂ ratio and a decreased lymphocyte count were associated with death by COVID-19 (Henry et al., 2020; Huang and Pranata, 2020; Booth et al., 2021; Pons et al., 2021; Gopalan et al., 2022; Soto et al., 2022). It should be noted that lymphopenia might occur due to consequent apoptosis of activated CD4+T cells by SARS-CoV-2 (Shen et al., 2022), thereby inducing immune suppression.

Although there are few reports about the role of zonulin in COVID-19 infections, Giron et al. (Giron et al., 2021) also reported high zonulin levels in the deceased group, similar to our results. In another study, Oliva et al. (Oliva et al., 2021) found median zonulin values similar to those in our study on comparing COVID-19 patients and matched healthy controls. In addition, it is important to highlight the role of zonulin in children with multisystem inflammatory syndrome (MIS), related to the persistence of the virus in the gastrointestinal tract (Kılıçet al.; Josyabhatla et al., 2021). Therefore, zonulin might be associated with a translocation of viral proteins into the bloodstream, contributing to increased inflammation.

Before COVID-19, the zonulin-dependent permeability of intestinal tight junctions had been implicated in chronic inflammatory diseases such as celiac disease, inflammatory bowel disease, type I diabetes (Fasano, 2011), as well as obesity, septicaemia, environmental enteropathy, necrotizing enterocolitis, among others (Sturgeon and Fasano, 2016). In these scenarios, the release of zonulin and loss of gut barrier function are followed by microbial translocation, which triggers immune responses and the production of pro-inflammatory cytokines that exacerbate gut permeability in a vicious cycle (Fasano, 2020). Apart from the intestinal system, overexpression of zonulin has also been reported in other organs such as the lungs and brain (Skardelly et al., 2009; Rittirsch et al., 2013).

Related to COVID-19 infection, SARS-CoV-2 affects the gastrointestinal tract as evidenced by gastrointestinal symptoms and detection of the virus in faeces (Groff et al., 2021). Additionally, viral antigens have been found in intestinal enterocytes after resolution of clinical illness in adults (Gaebler et al., 2021) and zonulin hyper permeability of the mucosal barrier coincides with SARS-CoV-2 antigenemia (Yonker et al.). It has previously been speculated that zonulin participates in a complex mechanism linking the wide variety of symptoms associated with SARS-CoV-2 infection, especially involving the digestive system and the nervous system (Llorens et al., 2021). Specifically, disruption of the intestinal barrier with subsequent microbial translocation could drive inflammatory activation in severe COVID-19 (Giron et al., 2021) with positive feedback of pro-inflammatory cytokines, with the amplified systemic

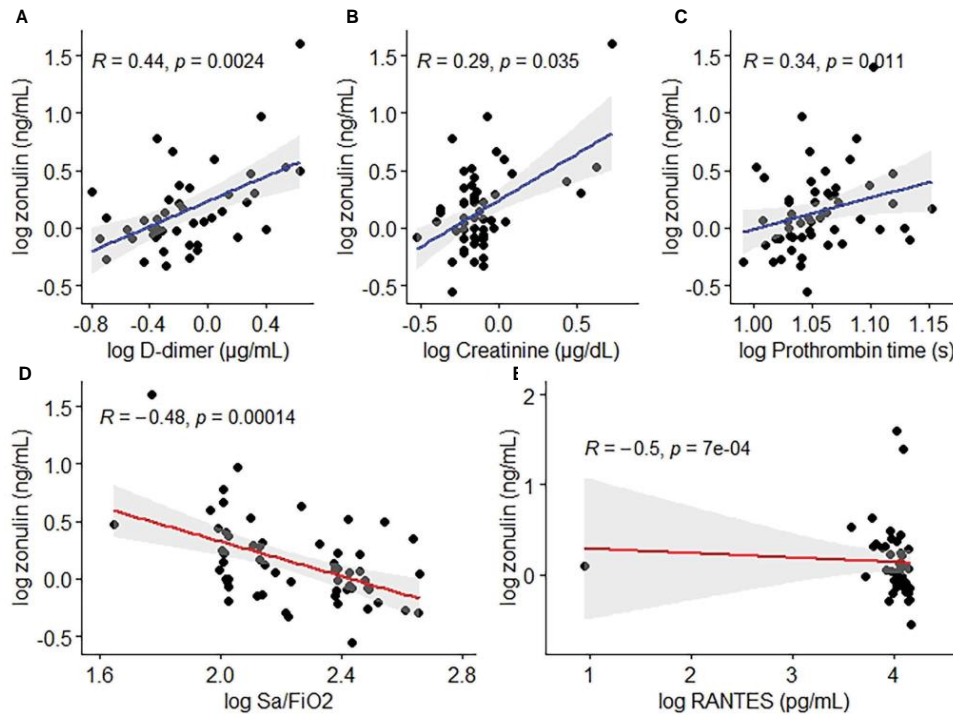


FIGURE 2
Scatterplots depicting significant correlations between zonulin and D-dimer (A) Creatinine (B), Prothrombin Time (C), Sa/FiO₂ (D) and RANTES (E).

response being stronger in patients with comorbidities (Trottein and Sokol, 2020). Given that our results suggest that zonulin is associated with death, even when adjusted for obesity, this is consistent with the above hypothesis.

Regarding correlations, zonulin was found to be directly correlated with creatinine, D-dimer values, and prothrombin time, all of which have previously been associated with poorer outcome in hospitalised COVID-19 patients (Malik et al., 2021) and inversely correlated with CCL5/RANTES and Sa/FiO₂. As for the chemokine CCL5 (RANTES), elevated values have been found in moderate but not severe COVID-19 cases and is considered as a prognostic factor for survival (Zhao et al.). It should be mentioned that the use of the zonulin inhibitor, larazotide acetate, in a case series of four children with MIS resulted in a faster resolution of gastrointestinal symptoms and faster clearance time of the SARS-CoV-2 spike antigen (Yonker et al., 2022). This inhibitor is also being tested in a clinical trial (ClinicalTrials.gov identifier NCT05022303) in children with long-COVID, for whom a persistent inflammatory effect has been reported (Galán et al., 2022).

It should be noted that zonulin concentrations described in the studies available vary greatly. This variability could be explained by the shelf life of the samples and differences in the detection methods. Additionally, there are concerns regarding the validity of the ELISAs performed for zonulin quantification,

in which the kits may detect not only zonulin but also zonulin-related proteins such as properdin (Scheffler et al., 2018). Another option is quantification of pre-haptoglobin 2 specifically (and no other zonulin-like proteins) with other methods such as western blot and/or mass spectrometry.

As mentioned before, a mounting body of evidence suggests that zonulin is a surrogate marker of tight junction permeability; however, estimation of microbial translocation could be reinforced using other known serum makers such as bacterial DNAemia, b-glucan, LPS-binding protein (LBP), intestinal-fatty acid binding protein (iFABP), soluble CD14 or others (Stehle et al., 2012; Koutsounas et al., 2015; Fukui, 2016), which could be options to consider alongside zonulin in future studies.

The present study has some limitations. The relatively small sample size for zonulin (n=60) and cytokine/chemokine values (n=42) might be responsible for type II errors, therefore some associations expected in previous studies, such as mortality and IL-6 values (Liu et al., 2021), were found to be non-significant. This also contributed to the adjusted regressions; because of the small number of events (deaths), the regression models were adjusted for a few key confounders only to avoid model overfitting. Thus, the findings presented herein are relevant, but a caveat must be made, as the magnitude of the associations should be interpreted with caution.

In conclusion, zonulin levels (as an indirect measure of microbial translocation levels) were detected in hospitalised patients with COVID-19 and were associated with death even after adjustment for age, gender, and obesity in a peruvian cohort of COVID-19. Future longitudinal studies on zonulin levels are recommended to determine their variation over time, as well as to identify their role in patients presenting systemic inflammatory syndrome/long-COVID.

authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by CIEI Universidad Científica del Sur-151-2022-POS50. Written informed consent was not provided because the samples were for diagnostic purposes, and, due to the first wave of COVID in Peru, the ethics committee approved the use of diagnostic samples.

Author contributions

MP, LP-K and JR conceived the study. LP-K, MP, BY, AM performed zonulin quantification by ELISA. MU-G obtained the samples and clinical information. LP-K performed the statistical analyses. LP-K and MP wrote the manuscript. All authors made contributions to the draft and approved the final version. All

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CONSTANCIA N° 192-CIEI-CIENTIFICA-2022

El presidente del Comité Institucional de Ética en Investigación de la Universidad Científica del Sur (CIEI-CIENTÍFICA) da constancia que el proyecto de investigación, cuyos datos se registran a continuación, ha sido **aprobado** por el CIEI-CIENTÍFICA.

Código de registro: **151-2022-POS50**

Título: **ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19 MODERADOS Y SEVEROS**

Investigador(a): **Luciano Augusto Palomino Kobayashi**

La aprobación del proyecto de investigación implica que el documento presentado a evaluación cumple con los lineamientos de Universidad en materia de investigación y ética, los cuales se sustentan en la normativa internacional vigente. Adicionalmente, en el contexto de la pandemia de enfermedad por COVID-19, le recomendamos cumplir con las normas publicadas por el estado peruano (accesos, medidas de bioseguridad, etc.) y tener los permisos de las instituciones en las que realizará el estudio antes de ejecutarlo.

En tal sentido, esta aprobación carecerá de valor si es que el proyecto de investigación arriba indicado es modificado de cualquier forma. Toda enmienda, añadido, eliminación o eventualidad (eventos adversos, etc.) posterior a la fecha de aprobación debe ser reportado al CIEI-CIENTÍFICA.

La vigencia de la aprobación de este documento es de dieciocho (18) meses (**hasta el 16 de diciembre del 2023**), periodo en el que puede desarrollarse el proyecto. Cualquier trámite para su renovación deberá ser enviado antes del término de la vigencia de este documento, debiendo suspenderse todo desarrollo hasta que la renovación sea aprobada. La presente aprobación sería ratificada en la sesión del CIEI más próxima.

Villa El Salvador, 17 de junio de 2022



Mg. Fulton Paul Rivera Albinagorta
Presidente

Comité Institucional de Ética en Investigación

RESOLUCIÓN DIRECTORAL ACADÉMICA DE CARRERA No. 431 - DACMH-DAFCS-U. CIENTIFICA-2022

Lima, 22 de agosto del 2022

VISTO:

El informe de revisión independiente académica y la aprobación de un Comité de Ética del proyecto de investigación titulado: **“ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19 MODERADOS Y SEVEROS”**.

CONSIDERANDO:

Que, de acuerdo al Reglamento General de la Universidad Científica del Sur y el reglamento y Procedimientos de trabajos de Investigación para obtener el **Grado de Maestro**.

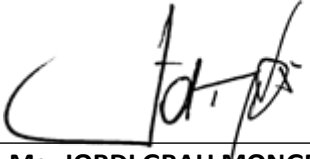
Que, de acuerdo a la normativa vigente de la Universidad Científica del Sur, en uso de las atribuciones conferidas al Director Académico de la Carrera.

SE RESUELVE:

ART. 1° Aprobar, en vías de regularización e inscribir el proyecto de tesis titulado **“ESTIMACIÓN DE TRANSLOCACIÓN BACTERIANA MEDIANTE CUANTIFICACIÓN DE ZONULINA Y SU POTENCIAL COMO BIOMARCADOR PRONÓSTICO DE MUERTE EN UNA COHORTE DE PACIENTES COVID-19 MODERADOS Y SEVEROS”**, presentado por el alumno Luciano Augusto Palomino Kobayashi con N° de registro: **151-2022-POS50**.

ART. 2° Nombrar al Docente Maria Jesús Pons Casellas , como asesor para el desarrollo de la tesis en cuestión.

Regístrese, comuníquese y archívese.



Mg. JORDI GRAU MONGE
Director Académico (e)
Carrera de Medicina Humana

