



**UNIVERSIDAD
DE GRANADA**

Tesis Doctoral Internacional / International Doctoral Thesis

**VALORACIÓN DEL ESTATUS DE CINC Y OTROS
PARÁMETROS CLÍNICO-NUTRICIONALES RELACIONADOS,
EN PACIENTE CRÍTICO CON RESPUESTA INFLAMATORIA
SISTÉMICA, CON Y SIN COVID-19**

**ASSESSMENT OF ZINC STATUS AND RELATED CLINICAL-
NUTRITIONAL PARAMETERS IN CRITICAL ILL PATIENT
WITH SYSTEMIC INFLAMMATORY RESPONSE, WITH AND
WITHOUT COVID-19**

Programa de Doctorado en Nutrición y Ciencias de los Alimentos

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Valoración del estatus de Zinc y otros parámetros clínico-nutricionales relacionados, en paciente crítico con respuesta inflamatoria sistémica, con y sin COVID-19

Assessment of Zinc status and related clinical-nutritional parameters in critical ill patient with systemic inflammatory response, with and without COVID-19

Memoria que presenta para aspirar al Grado de Doctor/a con Mención Internacional por la Universidad de Granada D.^a Lourdes Herrera Quintana.

La presente Tesis Doctoral ha sido realizada bajo la dirección de la Dra. D.^a Elena María Planells del Pozo

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Directora de la Memoria de Tesis Doctoral de Título: “Valoración del estatus de Cinc y otros parámetros clínico-nutricionales relacionados, en paciente crítico con respuesta inflamatoria sistémica, con y sin COVID-19”, realizada por D.^a Lourdes Herrera Quintana, autoriza su presentación ante el Tribunal que en su día se designe.

Y para que así conste, y en cumplimiento de las disposiciones vigentes, firma la presente Tesis Doctoral en junio de 2023.

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Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de la directora de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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La doctoranda D.^a Lourdes Herrera Quintana ha realizado la presente Tesis Doctoral como beneficiaria de una beca-contrato con cargo al programa de Formación de Profesorado Universitario (FPU18/03702) del Ministerio de Educación, Cultura y Deporte, por resolución del 12 de junio de 2019, de la Secretaría de Estado de Educación, Formación Profesional y Universidades.

La presente Tesis Doctoral titulada “Valoración del estatus de Zinc y otros parámetros clínico-nutricionales relacionados, en paciente crítico con respuesta inflamatoria sistémica, con y sin COVID-19”, se engloba dentro de la línea de investigación general *Evaluación del estatus nutricional y antioxidante en paciente crítico con Síndrome de Respuesta Inflamatoria Sistémica*, habiendo obtenido financiación a partir de proyectos competitivos nacionales e internacionales, desde el Fondo de Investigación Sanitaria (FIS) del Instituto de Salud Carlos III (Referencias PI07/1228 y PI10/1993), y Proyectos de I+D+i en el marco del Programa Operativo FEDER/ Junta de Andalucía (Referencia: A-CTS-708-UGR20), además de la red Europea Zinc-Net: *The Network for the Biology of Zinc* (FA COST Action TD1304). El trabajo de investigación ha sido realizado en la Universidad de Granada, concretamente en el Instituto de Nutrición y Tecnología de los Alimentos “José Mataix” (Centro de Investigación Biomédica) y en el Departamento de Fisiología de la Facultad de Farmacia. Asimismo, se ha contado con la colaboración de distintos hospitales de la provincia de Granada (Hospital Universitario Virgen de la Nieves, Hospital Clínico San Cecilio, Hospital General de Baza y Hospital Santa Ana de Motril), y con colaboración a nivel internacional, mediante la realización de estancias de investigación financiadas por el Ministerio de Educación, Cultura y Deporte, en la Universidad de Florida (Gainesville, Florida, Estados Unidos) y en la Universidad de Mendel (Brno, República Checa).

“Es mentira que recibes lo que das,
pero das lo que eres,
y eso es lo importante”

Anónimo

A mis padres, por su apoyo y su amor incondicional.

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ABREVIATURAS

ALP: Proteasa Alcalina / Alkaline Phosphatase

APACHE II: Acute Physiology and Chronic Health Evaluation II

ASPEN: Sociedad Americana de Nutrición Enteral y Parenteral /
American Society of Enteral and Parenteral Nutrition

Ca: Calcio / Calcium

CI: Intervalo de Confianza / Confidence Interval

COVID-19: Enfermedad por coronavirus de 2019 / Coronavirus
Disease 2019

CoV-SRAG-2 / SARS-CoV-2: Coronavirus del Síndrome Respiratorio
Agudo Grave de tipo 2 / Severe Acute Respiratory Syndrome-
Coronavirus-2

CRP: Proteína C Reactiva / C-Reactive Protein

Cu: Cobre / Copper

DGEM: German Society for Nutritional Medicine

ECA2 / ACE2: Enzima Convertidora de la Angiotensina 2 /
Angiotensin-Converting Enzyme 2

EPOC / COPD : Enfermedad Pulmonar Obstructiva Crónica / Chronic
Obstructive Pulmonary Disease

ES: Tamaño del efecto / Effect Size

ESPEN: Sociedad Europea de Nutrición Enteral y Parenteral /
European Society of Enteral and Parenteral Nutrition

FAAS: Espectrometría de Absorción Atómica de Llama / Flame
Atomic Absorption Spectroscopy

FC / HR: Frecuencia Cardíaca / Heart Rate

Fe: Hierro / Iron

FiO₂: Fracción inspirada de Oxígeno / Fraction of inspired Oxygen

FR / BR: Frecuencia Respiratoria / Breath Rate

GCP: Good Clinical Practice

GCS: Escala de Coma de Glasgow / Glasgow Coma Scale

GGT: Gamma-Glutamil Transferasa / Gamma-Glutamyl Transferase

GOT (or AST): Transaminasa Glutámico-Oxalacética (o Aspartato
Transaminasa) / Glutamic Oxaloacetic Transaminase (or Aspartate
Transaminase)

GPT (or ALT): Transaminasa Glutámico-Pirúvica (o Alanina
Transaminasa) / Glutamic Pyruvic Transaminase (or Alanine
Transaminase)

GPx: Glutation Peroxidasa / Glutation Peroxidase

Hb: Hemoglobina / Hemoglobin

H₂O: Agua / Water

H₂O₂: Peróxido de hidrógeno / Hydrogen peroxide

ICH: International Conference on Harmonization

ICP-MS: Espectrometría de Masas con Plasma Acoplado
Inductivamente / Inductively Coupled Plasma-Mass Spectrometry

IMC/BMI: Índice de Masa Corporal / Body Mass Index

INR: Índice internacional normalizado / International Normalized Ratio

IQR: Rango Intercuartílico / Interquartile Range

LDH: Lactato Deshidrogenasa / Lactate Dehydrogenase

LC-MS/MS: Cromatografía Líquida con Espectrometría de Masas /
Liquid Chromatography–Tandem Mass Spectrometry

Mg: Magnesio / Magnesium

Mn: Manganeso / Manganese

MPO: Mieloperoxidasa / Myeloperoxidase

MTF-1: Factor de Transcripción regulador de Metales-1 / Metal
regulatory Transcription Factor-1

MTs: Metalotioneínas / Metallothioneins

NADPH: Nicotinamida Adenina Dinucleótido Posfato reducida /
reduced Nicotinamide Adenine Dinucleotide Phosphate

NADP+: Nicotinamida Adenina Dinucleótido Posfato oxidada /
oxidized Nicotinamide Adenine Dinucleotide Phosphate

NOS: óxido nítrico sintasa

O₂: Oxígeno / Oxygen

O₂^{•-}: Radical superóxido / Superoxide radical

•OH: Radical hidroxilo / Hydroxyl radical

OMS / WHO: Organización Mundial de la Salud / World Health Organization

PaO₂/FiO₂: Presión arterial de Oxígeno/Fracción inspirada de Oxígeno / partial Oxygen arterial Pressure/Fraction of inspired Oxygen

qSOFA: quick Sequential Organ Failure Assessment

P: Fósforo / Phosphorus

PAM / MAP: Presión Arterial Media / Mean Arterial Pressure

PCT: Procalcitonina / Procalcitonin

PEEP: Presión positiva al final de la espiración / Positive End-Expiratory Pressure

PTAD: 4-phenyl-3H-1,2,4-triazole- 3,5(4H)-dione

PTH: Parathormona / Parathormone

r: Coeficiente de correlación de Pearson / Pearson's correlation coefficient

RDA: Ingesta Dietética Recomendada / Recommended Daily Allowance

RNS: Especies Reactivas del Nitrógeno / Reactive Nitrogen Species

ROC: Receiver Operating Curve

ROS: Especies Reactivas de Oxígeno / Reactive Oxygen Species

RT-PCR: Reacción en Cadena de la Polimerasa con Transcripción Inversa / Reverse Transcription Polymerase Chain Reaction

R•: Radical lipídico / Lipid radical

SD: Desviación Estándar / Standard Deviation

SDMO / MODS: Síndrome de Disfunción Multiorgánica / Multiple Organ Dysfunction Syndrome

SDRA / ARDS: Síndrome de Dificultad Respiratoria Aguda / Acute Respiratory Distress Syndrome

Se: Selenio / Selenium

SePP: Selenoproteína P / Selenoprotein P

SRIS / SIRS: Síndrome de Respuesta Inflamatoria Sistémica / Systemic Inflammatory Response Syndrome

SOD: Superóxido Dismutasa / Superoxide Dismutase

SOFA: Sequential Organ Failure Assessment

TAC: Capacidad Antioxidante Total / Total Antioxidant Capacity

TNT: Troponina T / Troponin T

TTPA / APTT: Tiempo de Tromboplastina Parcial Activada / Activated Partial Thromboplastin Time

UCI / ICU: Unidad de Cuidados Intensivos / Intensive Care Unit

VM / MV: Ventilación Mecánica / Mechanical Ventilation

VMI / IMV: Ventilación Mecánica Invasiva / Invasive Mechanical Ventilation

ZIP: familia de proteínas importadoras de Zinc / Zinc-Importer family of Proteins

Zn: Cinc / Zinc

ZnT: Familia de Transportadores de Zinc / Zinc Transporter family

β : Standardized Regression Coefficient

1,25-(OH)₂-D₃: 1,25-Dihidroxivitamina D₃ / 1,25-Dihydroxyvitamin D₃

25-OH-D: 25-Hidroxivitamina D / 25-Hidroxyvitamin D

25-OH-D₂: 25-Hidroxivitamina D₂ / 25-Hidroxyvitamin D₂

25-OH-D₃: 25-Hidroxivitamina D₃ / 25-Hidroxyvitamin D₃

RESUMEN

Introducción: El paciente crítico es altamente susceptible de presentar y/o desarrollar una deficiencia de Zinc (Zn) durante su estancia en la Unidad de Cuidados Intensivos (UCI), especialmente en aquellos casos en los que existe una condición inflamatoria generalizada. Sin embargo, la valoración del estatus de Zn sigue siendo un reto en la actualidad debido a la falta de biomarcadores fiables y, por tanto, se desconoce con exactitud su relación con otros parámetros clínico-nutricionales y su efecto sobre la evolución de estos pacientes.

Objetivo: Valorar el estatus de Zn y parámetros clínico-nutricionales asociados, en paciente crítico con Síndrome de Respuesta Inflamatoria Sistémica (SIRS), con y sin COVID-19, a su ingreso en UCI, así como la evolución de dicho estatus durante su estancia.

Metodología: Los pacientes fueron monitorizados desde su admisión en UCI hasta el séptimo día de estancia o hasta el tercer día (en el caso de pacientes con COVID-19). En los pacientes sin COVID-19, los niveles de minerales (Zn y Selenio (Se)) se analizaron en suero y eritrocito mediante Espectrometría de Absorción Atómica de Llama (FAAS), y la actividad de la Glutación Peroxidasa (GPx), la Superóxido Dismutasa (SOD) y la Actividad Antioxidante Total (TAC) se midieron usando métodos colorimétricos. En los pacientes con COVID-19, se utilizaron muestras de suero para medir los niveles de minerales por Espectrometría de Masas con Plasma Acoplado Inductivamente (ICP-MS) y los niveles de Metalotioneínas (MTs) por voltametría diferencial de pulso. Los niveles totales de 25-hidroxivitamina D (25-OH-D) fueron

determinados en ambos casos utilizando muestras de plasma mediante Cromatografía Líquida con Espectrometría de Masas (LC-MS/MS).

Resultados: En pacientes sin COVID-19, los niveles de vitamina D estuvieron directamente asociados con las concentraciones de Zn eritrocitario tras una semana de estancia en UCI. Incrementos en los niveles de Se plasmático, cuyos niveles fueron bajos tanto al inicio y como al final, se asociaron con una mejor evolución en la escala de gravedad SOFA. La actividad de la GPx estuvo inversamente relacionada con la severidad al inicio, mientras que la actividad de la SOD se correlacionó directamente con la TAC. En pacientes con COVID-19, los niveles de 25-OH-D fueron bajos en todos los casos al inicio, disminuyendo durante la estancia en UCI. Los pacientes que no necesitaron ventilación mecánica invasiva presentaron niveles más altos de 25-OH-D₂ al inicio y al final. Además, los cambios en los niveles de Zn tuvieron un efecto positivo sobre parámetros respiratorios (PaO₂/FiO₂ y frecuencia respiratoria). Los niveles de cobre (Cu) y MTs disminuyeron tras 3 días de estancia en UCI, estando inversamente relacionados los cambios en MTs con los cambios en Manganeseo (Mn) y albúmina. Los niveles de hierro (Fe) y Zn mostraron tener un valor predictivo de la severidad y la mortalidad.

Conclusión: En general, los resultados de la presente tesis doctoral indican un estatus deficiente de Zn así como de otros micronutrientes relacionados, tales como vitamina D, Fe, Cu, Se y Mn, los cuales estuvieron relacionados con distintos parámetros antioxidantes, clínicos y bioquímicos, sugiriendo que el restablecimiento de niveles adecuados de Zn y otros micronutrientes podrían tener un efecto beneficioso en estos pacientes.

ABSTRACT

Introduction: Critically ill patients are highly susceptible to present and/or develop Zinc (Zn) deficiency during Intensive Care Unit (ICU) stay, especially in generalized inflammatory conditions. However, the assessment of Zn status continues to be a challenge at the present time due to the lack of a reliable biomarker, and, therefore, its relationship with other clinical-biochemical parameters and its effect on patients' evolution are not completely understood.

Objective: To assess Zn status and related clinical-nutritional parameters in critically ill patients with Systemic Inflammatory Response Syndrome (SIRS), with and without COVID-19, at admission and during ICU stay.

Methodology: Patients were monitored from the first day of ICU admission until the seventh day of stay or the third day (in the case of patients with COVID-19). In patients without COVID-19, mineral levels (Zn and Selenium (Se)) were analyzed in serum and erythrocyte by flame atomic absorption spectrometry, and Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) activity and the Total Antioxidant Capacity (TAC) were measured using kinetic colorimetric methods. In patients with COVID-19, mineral levels were assessed in serum samples by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and Methallothioneins (MTs) levels by differential pulse voltammetric. Total 25-hydroxyvitamin D (25-OH-D) levels were measured in plasma samples by liquid chromatography-tandem mass spectrometry in both cases.

INTRODUCCIÓN

INTRODUCCIÓN

1. El paciente crítico:

1.1 Definición y situación clínica

Aunque no existe consenso acerca de la definición exacta de enfermedad crítica, se puede decir que el término “paciente crítico” (*aquel que padece la enfermedad crítica*) hace referencia a aquellos individuos que presentan un estado de salud deteriorado con disfunción de órganos vitales, un alto riesgo de mortalidad inminente si no reciben tratamiento, y requiriendo, por tanto, de un soporte vital sin el cual su supervivencia no sería posible [1,2]. Hay que tener en cuenta que este concepto se centra en la condición del paciente y su pronóstico (en lugar de en su diagnóstico), pudiendo ser ésta una de las causas de los problemas que aparecen con frecuencia en la práctica clínica. La falta de criterios y conceptos bien definidos en relación con la prognosis podrían derivar en interpretaciones y clasificaciones discordantes de estos pacientes [2].

En la actualidad se han desarrollado distintas guías que promueven la uniformidad de protocolos de actuación y han permitido un mejor abordaje de posibles complicaciones que con frecuencia pueden darse en estos pacientes (por ejemplo, daño renal, síndrome de distrés respiratorio o sepsis). Sin embargo, y a pesar de la sustancial mejora que han supuesto estas herramientas, la mortalidad en el paciente crítico sigue siendo alta [3]. Por lo general, estos pacientes tienen un alto riesgo de

aparición de diversas complicación y progresión de las mismas, lo que lleva a resultados potencialmente mortales [4].

Es importante tener en cuenta que, incluso cuando la condición y características clínicas de varios pacientes son muy similares, la respuesta de cada individuo a una misma intervención puede diferir enormemente. Este hecho, pone de manifiesto la necesidad de una mejor comprensión de las bases en las que se fundamenta esta heterogeneidad entre los pacientes, a fin de conseguir un mejor abordaje en el tratamiento, el cual pasa por hacerlo lo más personalizado posible [3].

1.2 Paciente crítico con SIRS

Debido a la heterogeneidad de las condiciones clínicas de estos pacientes, resulta muy difícil conocer el mecanismo fisiopatológico subyacente exacto de la enfermedad crítica. No obstante, se ha observado que una gran mayoría de los pacientes de la Unidad de Cuidados Intensivos (UCI) sufren o desarrollan lo que se conoce como Síndrome de Respuesta Inflamatoria Sistémica (Systemic Inflammatory Response Syndrome, SIRS) [4,5].

El SIRS consiste en una respuesta defensiva exagerada del organismo ante un factor estresante nocivo no específico (infección, trauma, cirugía, inflamación aguda, isquemia, etc.) que conduce a la desregulación de la homeostasis inflamatoria, estableciéndose una interacción compleja de la respuesta inmune humoral y celular, inclinándose el equilibrio entre las cascadas proinflamatorias y antiinflamatorias hacia la primera.

En la *Figura 1*, elaborada a partir del esquema de Bone et al. [6], se puede ver la relación entre SIRS, infección y sepsis:

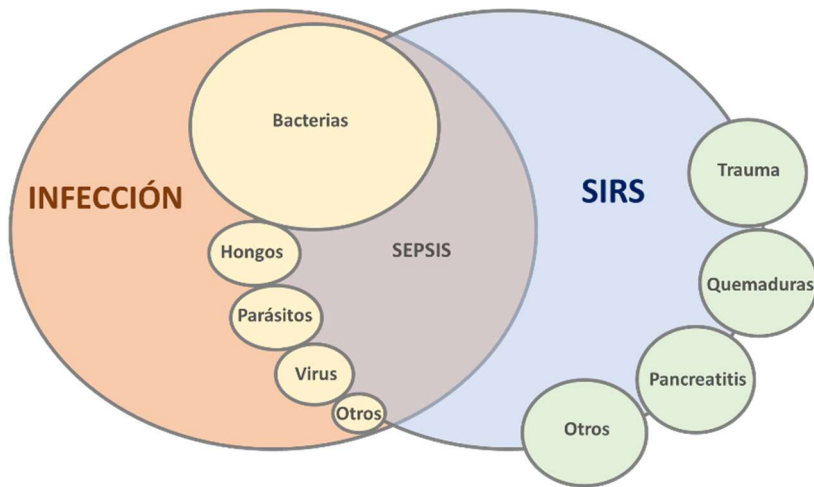


Fig. 1. Relación entre sepsis, SIRS e infección.

Aunque la etiología del SIRS puede ser tanto de naturaleza infecciosa como no infecciosa, una de sus frecuentes complicaciones suele incluir la aparición de sepsis, por tanto, casi todos los pacientes sépticos tienen SIRS, pero no todos los pacientes con SIRS son sépticos [7].

La fisiopatología del SIRS comprende las siguientes fases [8]:

- *Fase I:* en respuesta a un agente nocivo se produce una reacción local y se liberan citoquinas proinflamatorias a fin de promover la reparación del daño, reclutándose células del sistema reticuloendotelial.

- *Fase II:* pequeñas cantidades de citoquinas son liberadas a la circulación sistémica para incrementar la respuesta local. Se produce el reclutamiento de macrófagos y plaquetas, así como la estimulación de la producción del factor de crecimiento. Este proceso de respuesta aguda se mantiene hasta que el daño es reparado, autorregulándose de forma estrecha mediante distintos mediadores y antagonistas del proceso inflamatorio.
- *Fase III:* si la homeostasis no se consigue restablecer, se desarrolla el SIRS. El efecto predominante de las citoquinas pasa a ser dañino en lugar de protector. Los mediadores inflamatorios desencadenan numerosas cascadas humorales y provocan la activación sostenida del sistema endotelial reticular con pérdida de integridad microcirculatoria y daño de distintos órganos.

Una de las secuelas que con frecuencia aparece como resultado del SIRS – en el cual los desórdenes en la coagulación derivan en severas complicaciones que también suelen influir – es lo que se conoce como Síndrome de Disfunción Multiorgánica (Multiple Organ Dysfunction Syndrome, MODS), caracterizado por el desarrollo de disfunción fisiológica progresiva en dos o más órganos, los cuales pierden la capacidad de mantener la homeostasis [9,10].

Finalmente, hay que destacar que la presencia de SIRS se define de forma objetiva como el cumplimiento de, al menos, dos de los siguientes criterios [7]:

- Temperatura corporal $<36^{\circ}\text{C}$ o $>38^{\circ}\text{C}$.
- Frecuencia cardíaca >90 latidos/minuto.

- Frecuencia respiratoria >20 respiraciones/minuto o presión parcial de $\text{CO}_2 <32$ mmHg.
- Recuento leucocitario <4000 o >12000 leucocitos/ μL , o más del 10% de formas inmaduras.

1.3 Paciente crítico con COVID-19

El COVID-19 (Coronavirus Disease 2019) es una enfermedad respiratoria altamente infecciosa causada por la aparición, en el año 2019, de un nuevo coronavirus diferente del resto de coronavirus que producen afecciones leves en el ser humano, el conocido como SARS-CoV-2 (Severe Acute Respiratory Syndrome-Coronavirus-2) [11]. Debido a los alarmantes niveles de propagación y gravedad de esta enfermedad, el 11 de marzo de 2020 la Organización Mundial de la Salud (OMS) declaró el COVID-19 como pandemia, manifestando también una importante preocupación por los alarmantes niveles de inacción e instando a todos los países a tomar medidas para contener el virus [12]. Aunque previamente existían coronavirus que producían, generalmente, afecciones leves a nivel de sistema respiratorio, gastrointestinal y nervioso, tanto en el ser humano como en distintas especies animales [13], el SARS-CoV-2 dio lugar a la aparición de una nueva enfermedad cuya evolución clínica era, y en algunos aspectos todavía sigue siendo, en su mayor parte desconocida [12].

El SARS-CoV-2 es capaz de unirse a un receptor presente en la superficie de ciertas células, el receptor ACE2 (Angiotensin-Converting Enzyme 2), el cual es especialmente abundante en las células epiteliales del tracto respiratorio alveolar, y producir así una enfermedad infecciosa aguda que se propaga principalmente por vía respiratoria. Este receptor

también tiene una presencia destacable en enterocitos del intestino delgado y en las células del endotelio vascular, hecho que supone un primer paso en la comprensión de la patogénesis y de las principales manifestaciones del COVID-19 [14]. La transmisión del SARS-CoV-2 se produce por secreciones respiratorias de un individuo infectado, generalmente en forma de pequeñas gotas. El período de incubación promedio es de 6,4 días, presentándose en la mayoría de los pacientes la enfermedad en su forma leve, con una sintomatología que suele incluir fiebre, tos, disnea, mialgia o fatiga. Mientras que en una minoría de los pacientes se desarrolla una hipoxia severa que requiere hospitalización y ventilación mecánica [15]. Los datos epidemiológicos publicados hasta abril de 2020 mostraron que la media de edad de los pacientes con COVID-19 estuvo alrededor de los 40 años, con una distribución similar entre sexos. No obstante, en los casos graves (los cuales oscilaron en torno al 20% del total, dando lugar a una mortalidad del 6%) la edad media fue de 60 años, afectando mayoritariamente a hombres (61%) [16]. En tales casos, la progresión de la enfermedad puede, eventualmente, dar lugar a neumonía, Síndrome de Dificultad Respiratoria Aguda (Acute Respiratory Distress Syndrome, ARDS), y fallo multiorgánico que, finalmente, podría causar la muerte del paciente [17]. Es por ello por lo que el COVID-19 severo se relaciona con la necesidad de ingreso en la UCI, así como con el aumento de la tasa de infección secundaria y el empeoramiento significativo del pronóstico [18].

Aunque se no se conocen las causas con exactitud, se ha observado que aquellos pacientes con comorbilidades preexistentes como obesidad,

diabetes, malnutrición, inmunosupresión, hipertensión o disfunción renal, eran más propensos a desarrollar COVID-19 severo [16,19]. Por otro lado, la alteración de distintos parámetros clínicos y bioquímicos se ha relacionado con un peor pronóstico en estos pacientes. Algunos ejemplos serían: I) aumento del recuento de glóbulos blancos con disminución de linfocitos, II) niveles incrementados de parámetros como el dímero D, la proteína C reactiva, o el lactato III) disminución del cociente Presión arterial de Oxígeno/Fracción inspirada de Oxígeno ($\text{PaO}_2/\text{FiO}_2$), IV) hipoalbuminemia [19]. Asimismo, se ha observado que la causa más común de muerte en estos pacientes es la insuficiencia respiratoria, existiendo, además, otros procesos patológicos también reseñables, como procesos de coagulación acompañados de reacciones inmunitarias/ inflamatorias excesivas, la activación de la conocida como “tormenta de citoquinas”, o procesos de trombosis y coagulación intravascular diseminada [20]. Aunque el mecanismo fisiopatológico exacto del COVID-19 es desconocido; la evidencia clínica ha revelado que este nivel anormal de citoquinas y la respuesta inflamatoria exacerbada estarían asociados con el grave deterioro de las condiciones de salud en los pacientes infectados [21], procesos a su vez asociados con un incremento de estrés oxidativo [22]. Por tanto, la supresión de esta respuesta inflamatoria excesiva ha sido propuesta como una medida de prevención crucial en la progresión de la enfermedad y la aparición de complicaciones asociadas [23].

1.4 Valoración de la gravedad y situación del paciente

Existen distintos índices que se han desarrollado a fin de valorar la gravedad, pronóstico y diagnóstico del paciente crítico.

Dentro de las puntuaciones que proporcionan un reflejo de la gravedad del paciente se encuentra el sistema de puntuación APACHE II (Acute Physiology and Chronic Health Evaluation II). En la *Tabla 1* se describe el sistema de puntuación, el cual consta de 2 dimensiones (la suma de las puntuaciones de ambas oscila entre 0 y 71, aunque la supervivencia por encima de 55 puntos se ha dado en muy pocos casos) [24,25]:

- 1) Variables fisiológicas: un total de 12 variables fisiológicas que puntúan – a excepción de la Escala de Coma de Glasgow (GCS) – de 0 a 4 en función del grado de desviación respecto al estándar de la normalidad, al cual se le asigna un valor de 0. La GCS se calcula restando el valor GCS del paciente a un valor inicial de 15.

La puntuación de cada una de estas variables se realiza durante las primeras 24 horas de ingreso, escogiéndose el valor más desfavorable para cada una de ellas. Finalmente, se suma el total de cada una de las puntuaciones obtenidas, obteniéndose el primer componente o el “Acute Physiology Score” del APACHE II, considerado una medida de la gravedad de la enfermedad del paciente.

- 2) Edad y el estado de salud previo. Se tienen en cuenta variables como la presencia de enfermedades crónicas del sistema cardiovascular, respiratorio, hepático, inmunológico y renal. Esta segunda escala recibe el nombre de “Chronic Health Evaluation”.

Tabla 1. Sistema de puntuación y dimensiones de la escala APACHE-II

Tabla 11. Sistema de puntuación y dimensiones de la escala APACHE II					
Valoración del “Acute Physiology Score”					
Puntuación	0	1	2	3	4
Temperatura central (°C)	36.0-38.4	34.0-35.9 38.5-38.9	32.0-33.9 -	30.0-31.9 39.0-40.9	< 29.9 > 41.0
Presión arterial media (mm Hg)	70-109	- -	50-69 110-129	- 130-159	< 49 > 160
Ritmo cardíaco (latidos/min)	70 -109	- -	55-69 110-139	40-54 140-179	< 39 > 180
Frecuencia respiratoria (con o sin VM)	12-24	10-11 25-34	6-9 -	- 35-49	<5 >50
Oxigenación* (mm Hg)	>70 <200	61-70 -	- 200-349	55-60 350-499	<55 >500
pH arterial	7.33-7.49	- 7.50-7.59	7.25-7.32 -	7.15-7.24 7.60-7.69	<7.15 >7.70
Sodio (mMol/L)	130-149	- 150-154	120-129 155-159	111-119 160-179	<110 >180
Potasio (mMol/L)	3.5-5.4	3.0-3.4 5.5-5.9	2.5-2.9 -	- 6.0-6.9	<2.5 >7.0
Creatinina** (mg/mL)	0.6-1.4	- -	<0.6 1.5-1-9	- 2.0-3.4	- >3.5
Hematocrito (%)	30.0-45.9	- 46.0-49.9	20.0-29.9 50.0-59.9	- -	<20.0 >60.0
Leucocitos (x10 ⁹ /L)	3.0-14.9	- 15.0-19.9	1.0-2.9 20.0-39.9	- -	<1.0 >40.0
Escala de coma Glasgow (GCS) ***	Puntuación = 15 puntos – puntos de GCS				
Valoración del “Chronic Health Evaluation”					
Edad	≤ 44 años = 0 puntos 45-54 años = 2 puntos 55-64 años = 3 puntos 65-74 años = 5 puntos ≥ 75 años = 6 puntos				
Estado de salud crónico ****	Para pacientes no quirúrgicos o postoperatorio de cirugía urgente = 5 puntos. Para postoperatorio de cirugía electiva = 2 puntos.				

* Si la fracción inspirada de oxígeno (FiO_2) es $> 0,5$, se asignan puntos al gradiente alveolo-arterial ($A-aDO_2$). Si la fracción inspirada de oxígeno es $< 0,5$, se asignarán puntos a la presión parcial de oxígeno arterial (PaO_2).

** La creatinina tendrá doble puntuación en presencia de fracaso renal agudo.

*** La GCS valora tipos de respuesta motora y su puntuación oscila entre 3 (peores resultados) a 15 (mejores resultados) [26].

**** Si el paciente, antes del ingreso en el hospital, tiene historia de insuficiencia severa de órganos o de compromiso inmunitario.

Abreviaturas: VM = Ventilación Mecánica

Sin embargo, en los últimos años se ha resaltado la importancia de utilizar herramientas precisas en el entorno prehospitalario a fin de realizar una buena diferenciación de la situación clínica y comenzar lo antes posible con el tratamiento más adecuado a cada circunstancia, con especial interés en la diferenciación de pacientes que presentan sepsis de aquellos que sólo presentan infección pero no disfunción orgánica [27].

Como se ha mencionado anteriormente, la presencia de SIRS no tiene por qué implicar sepsis, por lo que se han desarrollado distintas herramientas que permitan su identificación, siendo una de ellas la escala SOFA (Sequential Organ Failure Assessment). Dicha escala, la cual se refleja en la *Tabla 2*, fue diseñada para describir una secuencia de complicaciones que con frecuencia aparecen en el paciente crítico, reflejando la situación de disfunción/fallo orgánico [28].

Aunque la valoración de la morbilidad a través de esta escala se pueda relacionar en cierta forma con la mortalidad, no sirve para predecir el resultado final. Su utilidad reside en mejorar la comprensión del proceso de fallo orgánico en relación a su evolución y en valorar los efectos de nuevas terapias en el curso de la disfunción/fallo orgánico, pudiendo ser usado para caracterizar a los pacientes a su ingreso o evaluar los efectos de su tratamiento [27,28].

Tabla 2. Sistema de puntuación y dimensiones de la escala SOFA

SOFA score					
Puntuación	0	1	2	3	4
Respiratorio PaO ₂ /FiO ₂	> 400	≤ 400	≤ 300	≤ 200 Con soporte respiratorio	≤ 100 Con soporte respiratorio
Coagulación Plaquetas (x1000)	> 150	≤ 150	≤ 100	≤ 50	≤ 20
Hepático Bilirrubina (mg/dL)	< 1.2	1.2-1.9	2.0-5.9	6.0-11.9	≥ 12
Cardiovascular	No Hipotensión	PAM < 70 mm Hg	*Dopa ≤ 5 Dobuta (cualquier dosis)	*Dopa > 5 o A ≤ 0.1 o NA ≤ 0.1	*Dopa > 15 o A > 0.1 o NA > 0.1
Neurológico Glasgow	15	13-14	10-12	6-9	<6
Renal Creatinina mg/dl o diuresis/24h	< 1.2	1.2-1.9	2.0-3.4	3.5-4.9 o < 500 ml	≥ 5.0 o < 200 ml
* Inotrópicos administrados durante al menos una hora. PAM: presión arterial media. A: adrenalina. NA: noradrenalina.					

Más recientemente, el consenso de un comité internacional constituido por 55 expertos da lugar a la actualización de las guías del manejo de sepsis (International Guidelines for Management of Sepsis and Septic Shock: 2016) [29]. En este momento aparece también la escala “quick Sequential Organ Failure Assessment” (qSOFA) [30], la cual considera la presencia de sepsis si se cumplen al menos dos de las siguientes condiciones (cada una de las cuales puntuaría como 1):

- Presión sistólica ≤ 100 mm Hg.
- Frecuencia respiratoria ≥ 22 respiraciones/min
- Escala de coma Glasgow ≤ 13.

No obstante, el uso de estas herramientas en la práctica clínica sigue dando lugar a cierta heterogeneidad en los resultados obtenidos. Algunos autores afirman que la escala qSOFA, el criterio de SIRS, o la definición de sepsis, poseen baja sensibilidad a la hora de identificar pacientes con sepsis tanto en el ámbito prehospitalario como a su llegada a urgencias [31]. Otros autores, en cambio, afirman que la escala qSOFA es una herramienta con una buena capacidad predictiva en el diagnóstico de la sepsis a nivel prehospitalario y, por tanto, útil para tal fin [27]. Por otro lado, un estudio retrospectivo que incluyó a más de 180,000 pacientes sugiere que un incremento de 2 o más en la puntuación de la escala SOFA tiene mejor precisión pronóstica para la mortalidad hospitalaria que la escala qSOFA o el criterio de SIRS [32]. En definitiva, podría concluirse que, a día de hoy, todas estas escalas poseen utilidad clínica pero también limitaciones.

1.5 Estrés oxidativo en el paciente crítico

Los procesos biológicos están regulados por reacciones de oxidación-reducción (redox), las cuales son utilizadas por las células en lo que se conoce como ‘señalización redox’ o ‘control redox’, entendiéndose como ‘estrés oxidativo’ al estado de desequilibrio entre oxidantes y antioxidantes a favor de los oxidantes, lo que conduce a una interrupción de la señalización redox y el control y/ o daño molecular [33]. La adopción de este último concepto más mecanicista de la definición de estrés oxidativo, centrándose en esa interrupción de la señalización y del control redox, se considera como una alternativa para

reorientar la investigación y comprensión de los procesos relacionados con el estrés oxidativo [34].

En los organismos aerobios, se produce la formación de radicales libres derivados del oxígeno y de oxidantes relacionados, siendo intermediarios omnipresentes y de corta duración que participan en las reacciones redox [35]. Las especies reactivas de oxígeno (reactive oxygen species, ROS), familia que engloba distintas especies químicas (por ejemplo, el anión radical superóxido ($O_2^{\cdot-}$), el peróxido de hidrógeno (H_2O_2), o el radical hidroxilo ($\cdot OH$) [36]) se generan constantemente en las células como consecuencia del metabolismo endógeno [37], siendo la mitocondria una fuente importante de producción de ROS en las células, ya que los electrones que se escapan de la cadena de transporte de electrones durante la respiración mitocondrial pueden combinarse con oxígeno molecular generando $O_2^{\cdot-}$ [36]. Asimismo, existen otras especies en el organismo que actúan como radicales libres, siendo también comunes las especies reactivas del nitrógeno (reactive nitrogen species, RNS), como por ejemplo el óxido nítrico, el cual puede unirse al $O_2^{\cdot-}$ dando lugar a un nuevo radical reactivo, el peroxinitrito ($OONO^{\cdot}$) [35]. Los principales mecanismos y reacciones involucradas en la formación de estas especies reactivas se pueden ver en el material suplementario, *tabla S1*.

La formación y acumulación de estos radicales, y la subsecuente cascada de reacciones con formación de nuevas especies reactivas, puede conducir a modificaciones oxidativas en biomoléculas, entre las cuales se puede destacar su acción sobre distintas proteínas, la peroxidación lipídica o daños en el ADN [37]. Por tanto, estos fenómenos pueden comprometer la función y la estructura de la célula conduciendo a la degeneración y muerte celular [35], relacionándose los eventos

oxidativos con condiciones patológicas y con el proceso de envejecimiento [36].

A fin de evitar los efectos nocivos que puede causar la acumulación de estas sustancias oxidantes, el organismo cuenta con mecanismos capaces de neutralizarlas; son los conocidos como sistemas antioxidantes, los cuales, en condiciones normales, permiten mantener un equilibrio de los procesos redox. Estos sistemas antioxidantes se suelen clasificar en dos grandes grupos: I) sistemas antioxidantes enzimáticos, que incluyen principalmente superóxido dismutasa (SOD), catalasa y glutathion peroxidasa (GPx), y II) sistemas antioxidantes no enzimáticos, que incluyen vitaminas y otros compuestos [38]. En la *Tabla 3* se pueden ver los principales integrantes de estos sistemas y algunas de sus acciones más relevantes.

Tabla 3. Principales antioxidantes y mecanismos de acción [36,38,39]

Antioxidante	Mecanismo de acción
<u>Enzimáticos</u>	
Superóxido Dismutasa	$O_2^{\cdot-} \rightarrow O_2 + H_2O_2$
Catalasa	$H_2O_2 \rightarrow H_2O + O_2$
Glutathion Peroxidasa	$GSH + H_2O_2 \rightarrow GSSG + H_2O$
<u>No enzimáticos</u>	
Vitamina E	Protección de las membranas celulares frente a la peroxidación lipídica; acción pro- y antioxidante.
Vitamina C	Potente donante de electrones, regenera la vitamina E; disminuye ROS, reacciona con $O_2^{\cdot-}$.
Vitamina A	Reducción de ROS y de la peroxidación lipídica.

N-Acetilcisteína	Incrementa los niveles de glutatión y cede grupos metilo para la conversión de homocisteína a metionina.
Melatonina	Incrementa la actividad de la cadena de transporte de electrones, reduciendo la formación de radicales libres.

Abreviaturas: GSH, glutatión reducido; GSSG, glutatión oxidado; H₂O, agua; H₂O₂, peróxido de hidrógeno; O₂, oxígeno; O₂^{•-}, anión radical superóxido.

Sin embargo, en condiciones patológicas, se produce un incremento en la formación de sustancias oxidantes y, en muchos casos, la acción de los sistemas antioxidantes puede no ser suficiente para mantener la homeostasis, llevando a un incremento del estrés oxidativo. Este efecto se ha observado en patologías de distinta índole, incluyendo cáncer, trastornos neurológicos, aterosclerosis, hipertensión, diabetes, síndrome de dificultad respiratoria aguda, fibrosis pulmonar, enfermedad pulmonar obstructiva crónica, etc. [39]. Además, existe una relación entre las especies reactivas y los procesos inflamatorios, de manera que las ROS y RNS se producen y liberan como consecuencia del proceso inflamatorio, y a su vez promueven una mayor liberación de citoquinas inflamatorias por las células inmunitarias, activando cascadas inflamatorias, y aumentando la expresión de moléculas de la adhesión [40]. Para contrarrestar los efectos de los mecanismos proinflamatorios, en paralelo se activa una respuesta antiinflamatoria compensatoria, existiendo, en circunstancias normales, un equilibrio entre la acción inflamatoria y antiinflamatoria, que promueve el proceso de curación y defiende contra las infecciones secundarias [41].

Durante la enfermedad crítica, los estímulos producidos por las diferentes lesiones que afectan a estos pacientes, activan una respuesta fisiológica mediada por la inflamación y el estrés oxidativo, cuya extensión (dada la gravedad en la mayoría de los casos) sobrepasa y compromete la capacidad antioxidante y antiinflamatoria del organismo

[42]. Se ha observado que la condición crítica de los pacientes ingresados en la UCI se asocia con el empeoramiento del estrés oxidativo, produciendo una alteración en los niveles endógenos de sustancias con capacidad antioxidante y dando lugar a un desequilibrio redox [43]. Asimismo, la respuesta inflamatoria exacerbada y sostenida en el tiempo puede agravar el daño orgánico, determinando un peor pronóstico para el paciente crítico. Además, el incremento del estrés oxidativo se ha relacionado con muchas de las afecciones presentes en pacientes ingresados en la UCI, incluyendo shock cardiogénico, sepsis, síndrome de dificultad respiratoria aguda, o quemaduras [44]. Tanto es así, que algunos autores consideran el estrés oxidativo como el principal determinante de la supervivencia en el paciente crítico, siendo determinante en la inflamación y describiendo la respuesta celular a los estímulos inflamatorios [41].

En base a esto, se ha propuesto como una posible alternativa terapéutica la administración de sustancias antioxidantes conocidas, a fin de contrarrestar la depleción del sistema antioxidante y restablecer el equilibrio redox, reduciendo así la gravedad de la afección en el paciente crítico. Esta posibilidad, pone el foco de atención en el soporte nutricional, ya que muchos de los componentes del sistema antioxidante son micronutrientes, bien de forma directa, como en el caso de las vitaminas, o bien como cofactores enzimáticos, en el caso de algunos minerales (por ejemplo, el Zinc (Zn) y el Manganeseo (Mn) son cofactores necesarios para la actividad de la SOD) [38]. Además, se ha observado que los niveles séricos de estos micronutrientes se ven significativamente

reducidos en los pacientes durante el curso de la enfermedad crítica, pudiendo contribuir al empeoramiento de su situación clínica [42].

1.6 Estatus y soporte nutricional en el paciente crítico

La desnutrición es un fenómeno que se presenta con elevada frecuencia en el paciente crítico, relacionándose con la aparición de complicaciones y un peor pronóstico, como la necesidad de ventilación mecánica, mayor duración de la estancia en UCI o aumento de la mortalidad [45].

Hay que tener en cuenta, que las lesiones sufridas por estos pacientes y la subsecuente liberación de hormonas relacionadas con el estrés (ej.: catecolaminas) y mediadores inflamatorios (ej.: citoquinas), con la posterior aparición de SIRS a la que dan lugar, tiene un gran impacto en todas las reacciones metabólicas del organismo. Este síndrome produce, en primera instancia, un mayor catabolismo de glucógeno, grasas y proteínas, con liberación de glucosa, ácidos grasos libres y aminoácidos a la circulación sistémica. Estos sustratos se desvían de su propósito normal (mantener la proteína periférica, especialmente a nivel muscular) para dirigirse a las tareas de curación y respuesta inmune [46]. Además, el elevado grado de estrés oxidativo, el cual se asocia a un importante daño celular, se suele acompañar de la aparición de estatus carenciales en la mayoría de micronutrientes [47].

El soporte nutricional en el paciente crítico se convierte, por tanto, en una estrategia fundamental para hacer frente a las necesidades metabólicas y mitigar los efectos negativos de las deficiencias de

macronutrientes o micronutrientes que, con alta prevalencia, aparecen en los pacientes a su ingreso en UCI, pudiendo aminorar esos efectos del estado catabólico y de la malnutrición [47]. Sin embargo, y aunque durante esta última década la evidencia acerca de la terapia nutricional ha crecido enormemente y se considera una parte esencial de la atención al paciente crítico, la estrategia para su implantación sigue siendo un desafío en la práctica clínica [46,47].

Uno de los problemas de base a los que se enfrenta la aplicación de la terapia nutricional en UCI es la falta de herramientas validadas para estimar el estado nutricional de estos pacientes [46], lo cual dificulta la identificación de individuos con alto riesgo de malnutrición y la optimización del soporte nutricional administrado [45]. Por otro lado, las recomendaciones sobre la terapia nutricional difieren, en muchos casos, dentro de las distintas guías existentes elaboradas por organismos oficiales, como DGEM (German Society for Nutritional Medicine), ESPEN (European Society of Enteral and Parenteral Nutrition), o ASPEN (American Society of Enteral and Parenteral Nutrition), entre otros [46]. Sin embargo, existen determinados aspectos donde la evidencia actual permite un consenso mayoritario, algunos de ellos serían:

- a) El estado nutricional del paciente crítico debería ser evaluado en el momento del ingreso en UCI y periódicamente durante su estancia [47].
- b) Uso preferente de la nutrición enteral frente a la parental, siempre que no exista ningún tipo de contraindicación. Sin embargo, hay que tener en cuenta que la nutrición enteral por sí sola puede ser

insuficiente para alcanzar los objetivos energéticos y proteicos, especialmente en la fase aguda de la enfermedad crítica, debido a las frecuentes interrupciones de los procedimientos y/o a la intolerancia gastrointestinal [54,55].

- c) En cuanto al mejor momento para iniciar la administración del soporte nutricional, existen pequeñas discrepancias en las distintas guías [48], aunque la tendencia parece abogar por la implementación temprana, incluso durante de las primeras 24-48 h, y de forma gradual, aumentándose paulatinamente el aporte de nutrientes con el transcurso de los días hasta llegar a los valores objetivo [47].

Además, aunque las necesidades de cada paciente dependen de sus circunstancias particulares y el abordaje debe de ser lo más individualizado posible, existen otra serie de consideraciones y recomendaciones generales teniendo en cuenta la etiología de la lesión (*Tabla S2*, material suplementario) [46].

No obstante, a pesar del aparente consenso en determinados aspectos, la nutrición óptima en el paciente crítico sigue siendo un tema de debate en la actualidad [49], incluso en términos de aporte energético y de proteínas [50,51]. Por ejemplo, algunos autores consideran que la recomendación de la ESPEN de un aporte temprano de proteínas de 1.3-1.5 g/kg/día [50] podría ser insuficiente y debería incrementarse por encima de estos valores. Sin embargo, otros autores creen que el aporte temprano alto de proteínas podría ser perjudicial en algunos pacientes, como aquellos que presentan sepsis (por un posible efecto de inhibición de la autofagia) [52]. Se ha observado que, durante las primeras semanas de ingreso en UCI es frecuente la pérdida de masa muscular dado el nivel

de catabolismo en estos pacientes, estimándose un balance de negativo de nitrógeno que equivaldría a la necesidad de un aporte proteico de 2.2-3.5 g/kg/día. Sin embargo, ese estado hipercatabólico, impediría en la mayoría de los casos conseguir un balance de nitrógeno positivo [53].

En cuanto al aporte energético, se ha visto que un aporte temprano de energía (70-80% del gasto energético) se asocia con un mejor pronóstico, datos que parecen diferir en los casos en los que existe sepsis [54]. No obstante, hay que destacar que la mayoría de los estudios utilizan ecuaciones predictivas para calcular los requerimientos energéticos, y ello hace que los resultados estén sujetos a cierta inexactitud, siendo quizá más conveniente el uso de la calorimetría indirecta. En este último caso, los resultados sugieren que tanto la desnutrición como la realimentación parecen ser perjudiciales para el paciente crítico, resaltando la importancia de una estimación del gasto calórico lo más precisa posible [51].

1.7 Micronutrientes en el paciente crítico

Cuando se analizan los niveles circulantes de micronutrientes en el paciente crítico, uno de los hechos más consistentes reportados en la literatura científica es la aparición de bajos niveles plasmáticos en la mayoría de los casos. De tal forma, se ha visto que, al ingreso en UCI, los pacientes presentan niveles bajos de vitaminas y minerales si se compara con individuos sanos [55,56]. Una de las posibles consecuencias de esta aparente deficiencia podría ser el incremento del estrés oxidativo y los efectos negativos asociados al mismo [55]. Sin

embargo, no está claro si estos bajos niveles plasmáticos de antioxidantes son indicadores de deficiencia por sí mismos (pueden producirse procesos de redistribución como consecuencia de la propia condición crítica) o si pueden resultar, incluso, beneficiosos (los pro-oxidantes son un mecanismo esencial de defensa frente a infecciones bacterianas) [56].

Esta frecuente aparición de aparentes estatus carenciales ha llevado a considerar la suplementación con vitaminas (ej.: vitamina A, vitaminas del complejo B, vitamina E, vitamina C) y con elementos traza (ej.: Se, Cu, Zn, Mn, Fe) en dosis farmacológicas como una importante alternativa terapéutica, existiendo fórmulas nutricionales con estos micronutrientes. Sin embargo, existe una gran discrepancia en este sentido, y algunos autores consideran que la administración de micronutrientes y otros antioxidantes no debe hacerse de forma rutinaria [46]. Por su parte, las guías de ESPEN recomiendan el aporte diario de micronutrientes, es decir, de minerales y vitaminas, administrados por vía parenteral, a fin de mantener un adecuado metabolismo (Grado de recomendación: B; consenso fuerte (acuerdo del 100%)); sin embargo, también recomienda la no administración de altas dosis de antioxidantes como monoterapia salvo que haya una deficiencia probada (Grado de recomendación: B; consenso fuerte (acuerdo del 96%)) [57].

2. El Zn y su importancia en el paciente crítico:

2.1 Principales funciones del Zn en el organismo

El Zn es uno de los elementos traza más importantes para la salud humana, caracterizándose por su ubicuidad en el organismo (se encuentra en todos los tejidos del cuerpo humano) [58]. Se trata de un micronutriente esencial que participa en diversas funciones biológicas, como el crecimiento, el metabolismo y la función inmunitaria [59]. Desempeña, por tanto, numerosas funciones en el organismo desde el punto de vista estructural, regulador y metabólico [60].

Este mineral es fundamental para los procesos catalíticos de más de 300 enzimas, las cuales participan en diversos procesos como en el metabolismo de carbohidratos, proteínas y ácidos nucleicos [61]. El Zn puede unirse de forma estable prácticamente al 10% del proteoma humano y mantener su integridad estructural [60]. Algunas de sus funciones más reseñables son: I) importante papel antioxidante, fundamentalmente a través de la acción de la enzima SOD; II) capacidad para formar moléculas conocidas como dedos de Zn, que permiten la unión de distintos factores de transcripción al ADN; III) mediador de procesos de señalización celular; IV) influencia sobre la acción de numerosas hormonas, teniendo un papel esencial, por ejemplo, en la síntesis, almacenamiento y secreción de insulina; V) distintas acciones inmunomoduladoras, siendo esencial para la maduración y la diferenciación de la célula T, entre otras acciones; VI) papel esencial en el buen funcionamiento y desarrollo de sistema esquelético [60,61].

2.2 Distribución corporal y metabolismo del Zn

Después del Fe, el Zn es el segundo ion metálico de transición más abundante en todos los organismos vivos [60]. La cantidad promedio de Zn en el cuerpo humano de un adulto es aproximadamente de 1.5-2.5 g [58], con una distribución desigual en los distintos compartimentos: en torno al 60% se acumula en el músculo, el 20% en los huesos y el 10-15% en la piel e hígado [61]. La concentración tisular de Zn también es muy variable, siendo mayor en tejido muscular esquelético, óseo, prostático y pancreático (unos 200 µg/g), mientras que en otros compartimentos como el corazón, el cerebro y el plasma la concentración es mucho menor (1-23 µg/g); suponiendo el Zn plasmático en torno al 0.1% del Zn corporal y circulando predominantemente unido a albúmina (~80%) y α 2-macroglobulina (~20%) [62].

En cuanto al contenido intracelular, el 50% del Zn se encuentra en el citoplasma, el 30–40% en el núcleo y un 10% en la membrana celular, estando en su gran mayoría unido a proteínas y almacenado en regiones compartimentadas (orgánulos y vesículas), por lo que la concentración de Zn libre citosólico es baja (picomolar y nanomolar) [58,60]. Mediante los procesos de almacenamiento y liberación del Zn unido a proteínas y contenido en los orgánulos, el organismo puede hacer frente a las fluctuaciones de los niveles de Zn ocasionadas por cambios transitorios, manteniendo su concentración prácticamente constante [60]. Los cuatro reservorios celulares de Zn son los siguientes: 1) el Zn estrechamente conjugado con metaloproteínas/metaloenzimas como componente estructural o cofactor; 2) el Zn unido a metalotioneínas (MTs) con una

afinidad más baja (puede suponer un 5–15% del total de Zn celular); 3) el Zn almacenado en orgánulos y vesículas; y 4) el Zn libre citosólico [63].

La homeostasis del Zn está estrechamente regulada, y su distribución y concentración en los distintos compartimentos se controla gracias a la acción de distintas proteínas, incluyendo transportadores de Zn y MTs [60]. El Zn libre no es capaz de atravesar las membranas celulares, y necesita de transportadores para poder movilizarse de un lado a otro [61]. Los transportadores de Zn se clasifican en dos grandes familias: 1) la familia de proteínas importadoras de Zn (Zinc-importer family of proteins; ZIP, o SLC39A) las cuales incrementan las concentraciones citosólicas de Zn; y 2) la familia de transportadores de Zn (Zinc transporter family; ZnT, o SLC30A) que disminuyen la cantidad de Zn del citosol [58]. Asimismo, existe un total de 14 miembros en la familia de transportadores ZIP (ZIP1-ZIP14) y un total de 10 miembros en la familia de transportadores ZnT (ZnT1-ZnT10) [61,64]. Curiosamente, estos transportadores de Zn poseen funciones fisiológicas específicas, aunque tengan en común su capacidad de transportar iones de Zn [64]. Además, cabe destacar la existencia de unas proteínas conocidas como “dedos de Zn” (Zn finger proteins) debido a su estructura. Estas proteínas son las más abundantes en el genoma humano, funcionando muchas de ellas como factores de transcripción mediante la unión a secuencias específicas de ADN [60].

En todas las células eucariotas, las concentraciones intracelulares de iones de Zn se mantienen dentro de límites estrechos gracias a los mecanismos conocidos como “sistemas amortiguadores de Zn” (“Zn buffering”), concepto con cierta analogía a la amortiguación de protones

y control del pH en el organismo [65]. En general, la fluctuación de los niveles de Zn, da lugar a cambios en la transcripción de MTs y de varios transportadores, mediados por la actuación del Factor de Transcripción regulador de Metales-1 (Metal regulatory Transcription Factor-1; MTF-1) [66].

2.3 Fuentes dietéticas, biodisponibilidad y absorción

El Zn es un mineral que se encuentra extensamente distribuido en los alimentos, sin embargo, la cantidad varía ampliamente de unos a otros, siendo los productos de origen animal los alimentos más ricos en Zn, concretamente los mariscos, seguidos de las carnes rojas, derivados lácteos y huevos. Los vegetales no suelen presentar contenidos altos de Zn, con algunas excepciones como las leguminosas y cereales integrales [67]. Debido al estrecho control de los niveles de Zn en el organismo, realmente sólo el 0.1% del contenido de Zn del organismo se repone diariamente a través de la ingesta dietética, gracias a la regulación de los procesos de excreción y absorción [62].

La absorción de Zn se produce por lo enterocitos a nivel de duodeno y yeyuno, mientras que la excreción del exceso de este mineral se logra a través de la secreción gastrointestinal, el desprendimiento de las células de la mucosa y del tegumento, así como por vía renal [62]. El proceso de absorción está determinado fundamentalmente por dos factores: 1) la cinética de saturación del proceso de absorción y 2) la presencia de transportadores de Zn en la membrana del enterocito [68]. Además, existen otros elementos que afectan a la absorción del Zn, como aquellos

que afectan a su biodisponibilidad. En este sentido, el Zn procedente de productos de origen animal se asimila mejor que el Zn que procede de productos de origen vegetal, debido a que factores como la presencia de proteínas animales y de lactosa favorecen la biodisponibilidad de Zn; mientras que otros como la presencia de folatos o de otros minerales como Ca, Fe y Cu afectan negativamente a su absorción a nivel intestinal [61]. Asimismo, otras variables como la edad, la cantidad total de Zn ingerida, condiciones fisiopatológicas previas, etc., también pueden afectar a la absorción de este mineral [68]. En cuanto a la excreción intestinal de Zn endógeno, ésta se regula en respuesta a la absorción reciente y al estatus de Zn previo; considerándose la relación entre la absorción y la excreción de Zn endógeno uno de los mayores determinantes a la hora de conocer los requerimientos de Zn, más que el efecto del fitato sobre las pérdidas intestinales de Zn [68].

2.4 Recomendaciones de ingesta. Deficiencia y toxicidad

La ingesta diaria recomendada de Zn depende, al igual que la de otros nutrientes, de diversos factores como el género, la edad, el sexo, el peso o la dieta. El aporte diario de Zn, según la OMS, debe ser para mujeres y hombres mayores de 19 años de unos 6,5 mg/día y de unos 9,4 mg/día respectivamente [61].

Tanto una deficiencia como un exceso de Zn podrían causar "estrés de Zn", que se asocia con un estatus anormal seguido del deterioro de la señalización celular llevada a cabo por el mismo, induciendo condiciones fisiopatológicas [64]. Múltiples anomalías pueden causar deficiencia de Zn, incluyendo ingesta inadecuada, disminución de la absorción o aumento de las pérdidas [60]. En cuanto a la toxicidad de este mineral,

se produce generalmente por intoxicaciones accidentales causadas por inhalación de vapores de Zn o consumo de alimentos y agua contaminados, afectando a diversos órganos, con especial efecto sobre los sistemas digestivo, respiratorio y nervioso [69].

Dado que la absorción y la excreción de Zn se controlan de forma estrecha a fin de mantener sus niveles constantes, existe una mayor preocupación por los estados carenciales de este mineral desde el punto de vista nutricional, es decir, por aquellos causados por un aporte inadecuado. De hecho, se estima que la deficiencia de Zn afecta a casi 2,000 millones de personas en todo el mundo [60], estando especialmente extendida en los países en vías de desarrollo, donde las dietas a base de cereales son consumidas por la mayoría de la población [70]. Se cree que la deficiencia de Zn es una de las deficiencias de micronutrientes más prevalentes en el mundo, sin embargo, los datos disponibles presentan inconsistencias, fundamentalmente, por la falta de un biomarcador idóneo del estatus de Zn [71].

Teniendo en cuenta la gran variedad y la importancia de las funciones biológicas en las que participa el Zn, incluso una pequeña deficiencia podría tener efectos muy dañinos para la salud, pudiendo ser la causa de una gran variedad de condiciones patológicas, especialmente de aquellas relacionadas con la edad (ej. aterosclerosis); además, en los casos más graves, puede asociarse con fenómenos de morbilidad, observándose una relación con diversas condiciones patológicas, tales como alteraciones a nivel del sistema inmunitario (tanto de la inmunidad innata como de la inmunidad adaptativa) con el subsecuente incremento de riesgo a infecciones, el desarrollo de enfermedades cardiovasculares,

anemia, retraso en el crecimiento, alteración de los procesos de cicatrización de heridas, entre otras [60,62,72].

2.5 Valoración del estatus de Zn: biomarcadores

Uno de los principales problemas existentes en la valoración del estatus de Zn es la falta de un indicador que refleje de forma fiable, sensible y específica dicho estatus. En individuos sanos, las concentraciones de Zn en plasma, pelo y orina pueden utilizarse como biomarcadores relativamente fiables, presentando, no obstante, diversas limitaciones y consideraciones previas a la hora de utilizarlos [73].

Generalmente, la medida más empleada para valorar el estatus de Zn es a nivel sérico o plasmático. Sin embargo, es importante señalar que la concentración plasmática de Zn no puede considerarse un biomarcador completamente fiable ya que no se correlaciona necesariamente con la concentración intracelular de Zn [62]. Aunque bien es cierto que las concentraciones de Zn sérico o plasmático suelen responder a suplementación (especialmente en individuos que inicialmente presentan niveles bajos) así como a situaciones de privación, sus niveles también se ven afectados por otros muchos factores (por ejemplo, condiciones inflamatorias, ayuno o factores relacionados con la alimentación, embarazo, uso de anticonceptivos orales, o ritmo circadiano) [71].

Por tanto, son necesarios biomarcadores adicionales más robustos que permitan la identificación de la deficiencia de Zn en estadios tempranos. En esta búsqueda se han contemplado diversas posibilidades aparte del Zn plasmático, tales como: el contenido de Zn en cabello, orina, eritrocito, leucocito y uñas, las proteínas y enzimas dependientes del Zn,

la expresión génica de transportadores de Zn y MTs, la ingesta dietética de Zn o la función neuroconductual y la agudeza en la percepción gustativa [74,75]. Otros indicadores indirectos de la deficiencia de Zn podrían ser la prevalencia de retraso en el crecimiento, la anemia o deficiencia de hierro (fundamentalmente por similitudes en la etiología de la deficiencia de ambos minerales, como las principales fuentes dietéticas o la absorción limitada por la presencia de fitatos) [71]. Asimismo, parece existir una creciente evidencia que sugiere que el ratio ácido linoleico/ácido dihomo-gamma-linolénico podría utilizarse como un biomarcador adicional y potencialmente más sensible que los contemplados hasta el momento, aunque todavía es necesaria más investigación al respecto [74].

2.6 Aporte y necesidades de Zn en paciente crítico

Como se comentó anteriormente, no existe un consenso sobre la suplementación de micronutrientes en el paciente crítico, de manera que las recomendaciones de las distintas guías relacionadas con el aporte de Zn son cautelosas, consistiendo en recomendaciones como la de evitar suplementación rutinaria o las altas dosis como monoterapia (a menos que haya una eficacia demostrada). No obstante, también reconocen el potencial efecto beneficioso de este mineral y dejan la puerta abierta a la suplementación con dosis seguras en aquellos pacientes que pudieran requerirlo [46]. En general, se recomienda que como mínimo, el aporte de Zn debe ser equivalente al de su Ingesta Dietética Recomendada (Recommended Daily Allowance, RDA) en pacientes hospitalizados, siempre que no exista ninguna contraindicación específica. Estos valores

estarían en torno a los 11 – 19 mg/L por vía enteral y los 2.5 – 5 mg/L por vía parenteral, aunque la condición fisiopatológica y las características de cada paciente deben de ser analizadas de forma individual [76].

A diferencia de lo que ocurre con otros micronutrientes, el organismo no cuenta con un reservorio específico de Zn, o con una proteína análoga a la ferritina (como en el caso del Fe), que lo almacene o lo libere cuando sea necesario. Es por ello por lo que el aporte nutricional resulta fundamental, especialmente en aquellos casos en los que existe riesgo de deficiencia [62]. La deficiencia de Zn se suele asociar a un aumento de la producción de radicales libres. Este efecto podría deberse a la disminución de la actividad de enzimas antioxidantes como la SOD, además de otros efectos como la actividad antioxidante que presenta el complejo Zn-MTs, o la capacidad del Zn de unirse y proteger grupos sulfhidrilo de proteínas [58]. Además, la deficiencia de Zn influye en la generación de citoquinas, las cuales, a su vez, afectan la distribución del Zn. De hecho, se ha observado que la suplementación con Zn puede disminuir los niveles de IL-6, mientras que la IL-6 induce una baja disponibilidad de iones de Zn mediante la modulación de la expresión génica de MTs [60].

Aunque resulta evidente la esencialidad del Zn en los distintos procesos, todavía son necesarios más estudios para determinar las condiciones en las que su suplementación resulta beneficiosa para el paciente crítico. En este sentido, se han encontrado efectos beneficiosos de la suplementación con Zn (50 mg/día) en pacientes con daño renal agudo, asociándose con una menor mortalidad [77]. También se ha observado que la suplementación de Zn, junto con la de Se, en paciente con

COVID-19 se relaciona con una reducción del proceso inflamatorio y mejora de parámetros inmunitarios [78].

No obstante, la suplementación con Zn en el paciente crítico, generalmente realizada sin una base empírica sólida (debido a la falta de un biomarcador adecuado del estatus de Zn), sigue siendo un reto. A pesar de ello, se han hecho algunos avances en determinadas áreas, creándose directrices para la suplementación con Zn en ciertas circunstancias (por ejemplo, en pacientes con quemaduras de gran extensión), que podrían servir como base para seguir avanzando en el resto de casos y crear protocolos de intervención adecuados [76].

2.7 Relación del Zn con otros micronutrientes y parámetros de interés en el paciente crítico

Aunque una depleción de Zn tenga un elevado potencial de causar diversas alteraciones por sí misma, es importante tener en cuenta que existen otros factores que pueden tanto afectar, como verse afectados, por esta deficiencia. De tal forma, en el paciente crítico no sólo se ve alterada la homeostasis del Zn, sino también la de otros iones metálicos tales como el Fe, el Cu, el Se y el Mn, que desempeñan un importante papel en el metabolismo [79]. Sin embargo, las concentraciones plasmáticas de muchos micronutrientes tienden a disminuir en estos pacientes debido a la respuesta inflamatoria sistémica, fundamentalmente como respuesta a citoquinas proinflamatorias. Estas citoquinas suprimen la producción hepática de distintas proteínas

transportadoras, aumentan la permeabilidad capilar y promueven que algunos micronutrientes queden retenidos en hígado y otros órganos. Por lo tanto, aunque la disminución en valores plasmáticos de micronutrientes podrían ser indicador de deficiencia, los fenómenos de redistribución impiden confirmar a ciencia cierta este hecho [80].

Además, como se ha mencionado anteriormente, los estados carenciales de Zn se asocian a un mayor estrés oxidativo, los cuales podrían deberse, en parte, a una menor capacidad antioxidante de la SOD. Además, otras enzimas como la GPx (una de las principales enzimas de la defensa endógena antioxidante que trabaja en cooperación con la SOD) también puede ver reducida su actividad durante la enfermedad crítica. En este sentido, se ha observado que el hecho de presentar determinados genotipos para la expresión génica de estas dos enzimas se asocia con la progresión y evolución de la enfermedad, teniendo efecto sinérgico en algunos casos [81].

De tal forma, además de las enzimas con acción antioxidante y de otros minerales, también pueden verse alterados los niveles de vitaminas. En este sentido, la vitamina D tiene papeles importantes como antioxidante natural, algunos de estos efectos están relacionados con sus acciones en los procesos antioxidantes dependientes de iones metálicos, modulando la expresión y actividad de la SOD y MTs, entre otros. Además, también participa en la regulación de mecanismos genómicos y no genómicos relacionados con el transporte de Zn [79].

Por tanto, no sólo resulta de interés el estudio del estatus de Zn y las necesidades del paciente crítico de forma aislada, sino que un mayor conocimiento de la relación existente con otros nutrientes y parámetros

Introducción

clínicos podría proporcionar una visión general de todo el proceso, y, en definitiva, un mejor abordaje del tratamiento de estos pacientes.

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JUSTIFICACIÓN E HIPÓTESIS

JUSTIFICACIÓN

El paciente crítico es altamente susceptible de presentar y/o desarrollar una deficiencia de Zn durante su estancia en UCI, especialmente en aquellos casos en los que se producen procesos inflamatorios generalizados. Además, se ha observado que los estados carenciales de Zn pueden aparecer en fases tempranas de la enfermedad o incluso antes de que el paciente llegue a estar en condición crítica.

El Zn es un mineral esencial que juega un importante papel en múltiples procesos biológicos, incluyendo la respuesta inmunológica, la función antioxidante, la síntesis de proteínas o la cicatrización de heridas. Por tanto, un estatus adecuado es fundamental para un correcto funcionamiento del organismo, y un estado carencial en una situación tan complicada como la que presenta el paciente crítico podría implicar consecuencias nefastas para su evolución.

La monitorización y evaluación del estatus de Zn en estos pacientes, a fin de prevenir o revertir deficiencias, resulta de gran relevancia para un correcto abordaje nutricional. Sin embargo, a día de hoy, no existe un biomarcador de Zn que refleje de forma precisa el estatus real de este mineral. Dentro de las determinaciones más comúnmente utilizadas están los valores circulantes de Zn, concretamente los niveles de Zn sérico. Sin embargo, dichos niveles pueden verse afectados por factores como la inflamación o el estrés, por lo que se recomienda utilizarlos en combinación con otras pruebas de diagnóstico. Este hecho hace que, la

valoración del estatus de Zn sea todavía más compleja en la población de estudio, constituyendo todo un reto en la actualidad.

HIPÓTESIS

En base a lo anteriormente expuesto, se parte de la hipótesis de que el paciente crítico con inflamación sistémica presenta un elevado riesgo de desarrollar deficiencia de diferentes micronutrientes, concretamente de desarrollar estados carenciales de Zn. Dicha deficiencia puede aparecer en fases tempranas de la enfermedad crítica o, incluso, precederla, existiendo una tendencia al empeoramiento del estatus de este mineral durante la estancia en UCI que, en cierta forma, condicionará la evolución clínica, afectando negativamente al pronóstico de estos pacientes.

OBJETIVOS

OBJETIVOS

Objetivo general

Valorar el estatus de Zn y parámetros asociados, en paciente crítico con SIRS, con y sin COVID-19, a su ingreso en UCI, así como la evolución de dicho estatus durante su estancia.

Objetivos específicos

1. Determinar los niveles circulantes de Zn en paciente crítico al ingreso y durante su estancia en UCI.
2. Estudiar parámetros clínico-nutricionales que puedan afectar a los niveles de Zn.
3. Optimizar la valoración del estatus de Zn mediante el estudio de otros biomarcadores relacionados con el mismo, como los niveles de MTs.
4. Analizar la asociación del estatus de Zn con otros parámetros que puedan afectar a la morbilidad y mortalidad en estos pacientes, como los relacionados con el estatus antioxidante.
5. Evaluar el estatus de otros micronutrientes asociados que puedan influir en la evolución de estos pacientes, como la vitamina D y otros minerales clave.

OBJECTIVES

General objective

To assess Zn status and related parameters, in critical ill patients with SIRS, with and without COVID-19, at ICU admission, as well as the evolution of this status during the stay.

Specific objectives

1. To determine Zn circulating levels in critically ill patients at admission and during ICU stay.
2. To study clinical-nutritional parameters which may affect Zn levels.
3. To optimize Zn status assessment through the study of related biomarkers, such as MTs levels.
4. To analyze the association between Zn status and other parameters which could affect patient's mortality and morbidity, as well as antioxidant status' variables.
5. To assess the status of other micronutrients status which may influence patients' evolution, such as vitamin D and other key minerals.

METODOLOGÍA INTEGRADORA

METODOLOGÍA INTEGRADORA

La presente tesis doctoral forma parte de un proyecto abierto más amplio, que aborda el estudio del paciente crítico con SIRS desde su ingreso en la UCI y durante su estancia, evaluando su estatus nutricional y antioxidante; y focalizándose en el estudio del Zn en este trabajo. El diseño del estudio es prospectivo observacional analítico, cuyo plan de trabajo se explica a partir de cuatro estudios. En base a características específicas, los estudios se han clasificado y detallado a continuación dentro de dos capítulos: I) Paciente crítico con SIRS y II) Paciente crítico con SIRS y COVID-19. En la *Figura 2* puede verse un resumen del cronograma del proyecto de tesis.

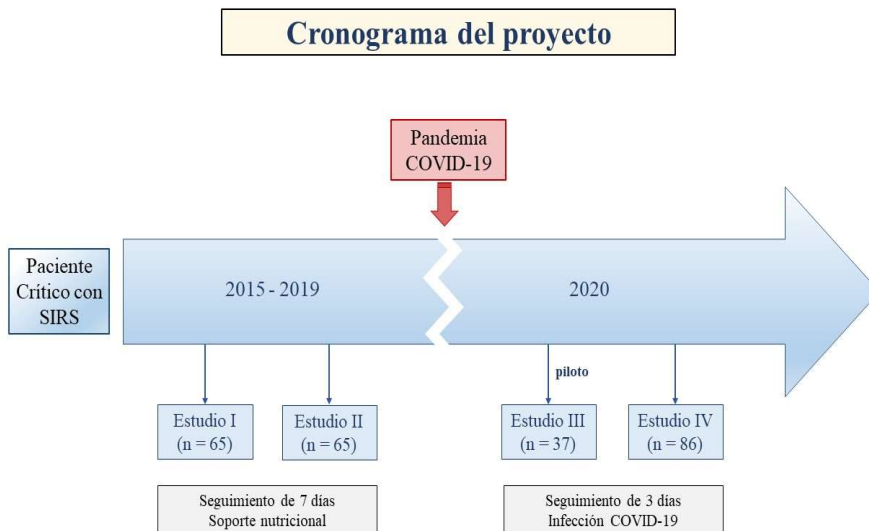


Fig. 2. Cronograma del proyecto de tesis y estudios que lo conforman

En todos los casos, el trabajo realizado se llevó a cabo de acuerdo con los principios de la Declaración de Helsinki (última guía revisada de 2013) [1], siguiendo las normas de Buena Práctica Clínica según los estándares de la Conferencia Internacional de Armonización, y siendo aprobados por el Comité de Ética de la Universidad de Granada (Ref. 149/CEIH/2016).

CAPÍTULO I: Paciente crítico con SIRS

1.1 Sujetos y diseño de los estudios

La muestra de paciente crítico con SIRS estuvo constituida por 65 sujetos (con una edad media de 60 años, un rango de edad comprendido entre los 31 y los 65 años, y un 42% de mujeres) reclutados tras su ingreso en UCI en los siguientes hospitales de la provincia de Granada: Hospital Clínico San Cecilio (Granada), Hospital Virgen de las Nieves (Granada), Hospital General de Baza (Baza) y Hospital Santa Ana (Motril). El periodo de reclutamiento comenzó en marzo de 2015 y finalizó en junio de 2019.

Los pacientes fueron monitorizados al ingreso en UCI (condiciones basales o baseline) y se realizó un seguimiento durante los siguientes 7 días de estancia (seguimiento o follow-up). Durante este periodo la mortalidad fue del 38.5%, siendo 40 el número de pacientes en el “follow-up”. Los criterios de inclusión fueron los siguientes: I) mayoría de edad; II) presencia de SIRS [2]; III) firma de consentimiento informado por parte del paciente o por un familiar. Por otro lado, se establecieron los siguientes criterios de exclusión: I) negativa del paciente o familiar a participar en el estudio; II) embarazo; III) presencia de enfermedad altamente contagiosa; IV) alergias; V) cáncer; VI) ingesta de alimento o administración de soporte nutricional 12h antes de la obtención de muestra.

Por otro lado, y a fin de obtener valores de referencia para poder comparar algunos de los parámetros estudiados, se reclutó una muestra de 56 individuos sanos (55% mujeres) de la misma región, los cuales

constituyeron el grupo control. Para reclutarlos se usó un muestreo consecutivo no probabilístico de conveniencia en función de la edad. Los criterios de inclusión fueron los siguientes: I) mayoría de edad; II) no consumir suplementos nutricionales; III) no presentar ninguna patología que pudiera afectar al estatus nutricional, IV) no estar embarazada, V) ausencia de alergias, VI) ayuno durante las 12h previas a la toma de muestra, VII) firma de consentimiento informado.

1.2 Valoración del perfil nutricional

Los pacientes recibieron soporte nutricional vía enteral, parenteral, o mixta, mediante la administración de fórmulas nutricionales siguiendo los criterios establecidos en el protocolo hospitalario, los cuales estaban basados en los criterios de la ESPEN [3]. Durante los 7 días de seguimiento de estos pacientes, se registró a diario la información de dicho soporte nutricional (es decir, tipo, volumen, composición y tolerancia). La información sobre el aporte de vitaminas y minerales fue calculada en base a la edad, peso y sexo de cada individuo, de acuerdo con las guías ESPEN previamente citadas; y fue registrada por los dietistas-nutricionistas (expresándose como promedio de los 7 días incluidos en el periodo de estudio).

En el grupo control se realizó la valoración cuantitativa de ingesta de nutrientes de manera retrospectiva mediante una entrevista realizada por dietistas-nutricionistas especializados. Para una mayor precisión en la recogida de información, se utilizaron cuestionarios validados (es decir, el recordatorio de 24 h) [4] y libros de fotografías con las comidas y

preparaciones culinarias que frecuentemente se consumen en España [5]. Para el cálculo de la cantidad de macronutrientes y micronutrientes se utilizó la aplicación Dietowin® (version 7.1., Barcelona, Spain).

1.3 Obtención de datos sociodemográficos, clínicos y bioquímicos

Los datos de los pacientes, tanto la información sociodemográfica (es decir, sexo, edad, etc.) como los parámetros resultantes de las analíticas y pruebas realizadas a los pacientes (es decir, todos aquellos parámetros clínicos registrados por los intensivistas y aquellos medidos en el laboratorio del hospital) se obtuvieron de la base de datos electrónica del hospital, registrándose aquellos correspondientes al “baseline” y al “follow-up”.

La valoración y el registro de la gravedad fue realizada por los intensivistas mediante el uso de dos escalas: I) la escala APACHE-II [6]; y II) la escala SOFA [7]. Los parámetros bioquímicos se determinaron en la unidad de laboratorio y análisis del hospital mediante ensayos y metodología usados de forma rutinaria basados en procesos colorimétricos enzimáticos y de inmunoensayo (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).

1.4 Extracción y Procesamiento de muestras

Las muestras se obtuvieron mediante extracción sanguínea a primera hora de la mañana en condiciones de ayuno, tanto en el punto de “baseline” como en el de “follow-up”. La toma de muestras sanguíneas

se realizó en distintos tubos a fin de obtener muestras de suero (BD Vacutainer® SST™ II Advance tubes) y muestras de plasma y eritrocito (BD Vacutainer® K₂EDTA tubes). Todos los tubos fueron procesados de forma inmediata, centrifugándose para separar los distintos componentes (4 °C, 15 min, 3,000 rpm) y alicuotándose en eppendorfs, los cuales fueron almacenados a –80 °C hasta el momento del análisis. En el caso de los eritrocitos, se realizó un lavado previo tras la centrifugación, el cual consistió en 4 lavados con 3 mL de una solución salina al 0.9% de NaCl, mezclando suavemente mediante volteo, centrifugando (4 °C, 15 min, 3,000 rpm), y descartando el sobrenadante.

1.5 Determinación de minerales

La determinación de los niveles de Zn y Se en los estudios realizados en paciente crítico con SIRS se realizó mediante Espectrometría de Absorción Atómica de Llama (Flame Atomic Absorption Spectroscopy; FAAS) (Perkin Elmer A. Analyst'300 Norwalk, Connecticut, United States of America) tras la mineralización previa de las muestras por vía húmeda. Todas las muestras se midieron en un mismo ciclo y dentro del mismo ensayo, a fin de minimizar y controlar los errores de laboratorio en las medidas. Los valores de referencia para el Zn plasmático fueron 10–17 µmol/L (0.65–1.11 mg/L) [8]. Los valores de referencia para el Se fueron establecidos en base al grupo control, tomándose como punto de corte 45.6 µg/L para el plasma y 69.0 µg/L para el eritrocito (valores más bajos del rango en cada caso).

1.6 Determinación de otros parámetros de interés

1.6.1 Determinación de vitamina D

Los niveles de vitamina D se analizaron mediante extracción previa en muestras de plasma, derivatización y posterior medida utilizando Cromatografía Líquida con Espectrometría de Masas (Liquid Chromatography–Tandem Mass Spectrometry, LC-MS/MS), según el protocolo que se describe a continuación.

Para la extracción de vitamina D se utilizaron 200 μL de muestras de plasma, siendo el primer paso la adición de 20 μL de un estándar interno, es decir, de formas deuteradas de la 25-Hidroxivitamina D_3 (25-OH- D_3) y de la 25-Hidroxivitamina D_2 (25-OH- D_2) (Sigma Aldrich, St. Louis, Missouri, United States of America). A continuación, se adicionaron 500 μL acetonitrilo para promover la precipitación proteica. Tras agitación suave y posterior centrifugación (10,000 rpm, 15 min) se recuperó el sobrenadante y se eliminó todo el disolvente mediante el secado con N_2 . Posteriormente, a este residuo seco se le adicionaron 200 μL de acetato de etilo y 100 μL de agua destilada, y tras agitación vigorosa durante 30 s, se centrifugó (3,000 rpm, 5 min) y se recuperó el sobrenadante. Este último paso fue repetido 3 veces, a fin de garantizar una buena extracción, recogiendo los tres sobrenadantes de la misma muestra en un mismo eppendorf, y posteriormente, eliminando el disolvente mediante secado con N_2 . Simultáneamente, se prepararon diluciones de concentraciones crecientes de los estándares, concretamente de 1, 2, 5, 10, 25, 50, y 100 ppb de 25-OH- D_3 y de 25-OH- D_2 (Sigma Aldrich, St. Louis, MO, United States of America), para realizar la línea de

calibración. A estos estándares también se les adicionaron los 20 µL del estándar interno y fueron tratados de la misma forma y a la vez que las muestras de plasma.

Para el proceso de derivatización de las muestras se preparó una solución en acetonitrilo de 4-phenyl-3H-1,2,4-triazole- 3,5(4H)-dione (PTAD) (Sigma Aldrich, St. Louis, MO, United States of America), a una concentración de 0.5 mg/mL. Se añadieron 50 µL de esta solución a cada una de las muestras y a las soluciones estándar, y se dejaron durante 1 h en agitación a temperatura ambiente y cubiertas con papel de aluminio para protegerlas de la luz. Finalmente, las muestras se trasvasaron a viales y se diluyeron con 50 µL de agua destilada, guardándose en el congelador a -20 °C cubiertas con papel de aluminio hasta el momento de inyección en el cromatógrafo.

Las determinaciones se realizaron mediante un cromatógrafo Waters Acquity UHPLC I-Class System chromatograph (Waters, London, United Kingdom), con una columna Acquity UHPLC BEH C18 column 2.1, 50 mm, 1.7 µm, a temperatura ambiente. La fase móvil del canal A fue agua con 50 mM de formiato de amonio, mientras que la del canal B fue metanol. El volumen de inyección de cada muestra fue de 10 µL y la velocidad de flujo de 0.4 mL/min. El detector utilizado fue Waters XEVO-TQ-XS Triple Quadrupole Low Resolution Spectrometer.

Los niveles totales de 25-OH-D fueron calculados como la suma de las formas 25-OH-D₃ y 25-OH-D₂, y fueron clasificados de acuerdo a los criterios de la Sociedad de Endocrinología [9]: niveles superiores a 30 ng/mL fueron considerados como suficiencia, valores comprendidos entre 20–29 ng/mL como insuficiencia, y por debajo de 20 ng/mL como deficiencia.

1.6.2 Determinación de parámetros antioxidantes

Para conocer el estatus antioxidante, se analizaron diversos parámetros relacionados con el mismo. Concretamente, se determinó la actividad de las enzimas GPx y SOD, y se midió la Capacidad Antioxidante Total (Total Antioxidant Capacity, TAC). Para tal fin, se utilizaron diversos kits comerciales basados en cinéticas colorimétricas: I) la actividad GPx se midió en muestras de eritrocito mediante el kit Bioxytech GPx-340™ kit (OxisResearch™); II) la actividad de la SOD mediante el kit Randox Ransod kit (Bioxytech® SOD-525™RANDOX Laboratories Ltd., Antrim, United Kingdom); y III) la TAC se midió en plasma (TAC kit, Jaica, Shizuoka, Japan) [10]. La variabilidad fue testeada y controlada mediante medidas repetidas de la misma muestra, considerando aceptable e incluyendo aquellas medidas cuya variabilidad fue inferior al 5%. Los valores de referencia para cada uno de los parámetros medidos fueron establecidos por el fabricante del kit utilizado.

1.7 Análisis estadístico

Para el análisis estadístico de los datos se utilizó el programa SPSS versión 26.0 (IBM SPSS, Armonk, NY, USA), mientras que para la representación gráfica de los resultados se usó el programa GraphPad Prism 8.0 (GraphPad Software, San Diego, California, United States of America). La normalidad de las variables de los estudios se analizó, en función del tamaño muestral, usando el test de Kolmogorov-Smirnov o

Shapiro–Wilk. Las variables cualitativas de los estudios se encuentran expresadas como frecuencias y porcentajes, mientras que las variables cuantitativas como media y desviación estándar (Standard Deviation, SD). Las comparaciones de variables categóricas entre grupos (“intergroup”) se realizó mediante la prueba de Chi-cuadrado. Las diferencias en la evolución de las variables (“intragroup”) se analizaron mediante la prueba de la *t* de Student para muestras relacionadas, mientras que las diferencias entre grupos (“intergroups”) mediante la prueba de la *t* de Student para muestras independientes. Asimismo, las correlaciones entre variables se analizaron mediante el coeficiente de correlación de Pearson, y en algunos casos se realizaron modelos de regresión linear simple, ajustándose algunos modelos por sexo y edad. En todos los casos, la significación estadística fue establecida para un valor de $p < 0.05$.

CAPÍTULO II: Paciente crítico con COVID-19

2.1 Sujetos y diseño de los estudios

La investigación llevada a cabo en paciente crítico con COVID-19 comenzó en abril de 2020, habiéndose reclutado en junio de este mismo año un total de 37 pacientes (edad media de 60 años, con un rango de edad de 41 y 74 años, y 30% mujeres). Se realizó un estudio piloto cuyo objetivo principal fue valorar el estatus de vitamina D y su relación con otras variables de interés. Posteriormente, se siguió ampliando la muestra hasta diciembre de 2020, consiguiéndose un total de 86 pacientes (edad media de 62 años, con un rango de edad de 36 y 96 años, y 21% mujeres), en los cuales se realizó la evaluación del estatus de Zn y de otros parámetros clínico-nutricionales.

En el caso de estos pacientes, el protocolo consistió en la monitorización desde su ingreso en UCI (condiciones basales o baseline) y seguimiento hasta el tercer día de estancia (seguimiento o follow-up). En este caso, no se recogieron datos del perfil nutricional, y se excluyeron aquellos pacientes que fallecieron antes de superar este periodo. Los criterios de inclusión fueron los siguientes: I) mayoría de edad; II) hospitalización previa de, al menos, 48 horas antes de ingreso en UCI; III) presentar una prueba diagnóstica (Reacción en Cadena de la Polimerasa con Transcripción Inversa; Reverse Transcription Polymerase Chain Reaction, RT-PCR) de infección por SARS-CoV-2 positiva, la cual fue realizada a través de una muestra nasofaríngea [11]; IV) estancia en UCI de al menos 3 días; V) no recibir suplementación con vitamina D. Por otro lado, los criterios de exclusión comprendieron: I) embarazo; II) no

presentar una prueba PCR positiva a pesar de presentar sintomatología compatible con COVID-19.

2.2 Obtención de datos sociodemográficos, clínicos y bioquímicos.

El procedimiento para la obtención de estos datos fue el mismo que el descrito en el apartado 1.3, siendo todos estos parámetros registrados y valorados por los intensivistas y el personal del laboratorio, y recuperándose aquella información relativa al “baseline” y al “follow-up” de la base de datos electrónica del hospital.

2.3 Extracción y Procesamiento de Muestras

Igualmente, el procedimiento de toma y procesamiento de muestra coincide con el descrito anteriormente en el apartado 1.4, obteniéndose muestras de suero, plasma y eritrocito procedentes de la extracción sanguínea tanto en el punto de “baseline” como en el de “follow-up”.

2.4 Determinación de minerales

La valoración de Fe, Zn, Cu y Mn se realizó mediante Espectrometría de Masas con Plasma Acoplado Inductivamente (Inductively Coupled Plasma-Mass Spectrometry, ICP-MS) (Perkin

Elmer® SCIEX Elan-5000 equipment, Markham, Ontario, Canada). Las muestras fueron previamente mineralizadas por vía húmeda y el contenido de los minerales se determinó en el Centro de Instrumentación Científica de la Universidad de Granada, estableciéndose como valores de referencia: Fe = 0.6–1.70 mg/L; Zn = 0.7–1.10 mg/L; Se = 0.6–1.40 µg/L y Mn = 0.4–0.85 µg/L.

2.5 Determinación de otros parámetros de interés

2.5.1 Determinación de vitamina D

Los niveles de vitamina D se analizaron mediante Cromatografía Líquida con Espectrometría de Masas (Liquid Chromatography–Tandem Mass Spectrometry, LC-MS/MS), según el protocolo descrito anteriormente en el apartado 1.6.1 de la metodología.

2.5.2 Determinación de metalotioneínas

La determinación de los niveles de Metalotioneínas (MTs) se realizó mezclando 10 µL de suero con 990 µL de tampón fosfato 0.1 M ($\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$, pH 7.0). A continuación, para eliminar las proteínas y péptidos remanentes (los cuales pueden afectar a la respuesta electroquímica), las muestras fueron sometidas a incubación con agitación a una temperatura de 99 °C durante 20 minutos (Eppendorf 5430, Hamburg, Germany). El tratamiento térmico desnaturaliza y elimina de forma efectiva las proteínas de alto peso molecular no termoestables de las muestras [12]. Los homogeneizados

desnaturalizados fueron centrifugados a 4 °C, 25,000 x g durante 20 min (Eppendorf 5402, Hamburg, Germany), utilizándose el sobrenadante obtenido para el análisis de MTs.

La determinación de MTs se realizó en las muestras procesadas mediante pulso diferencial voltamétrico en medio de reacción Brdicka de acuerdo con el procedimiento modificado [13], utilizando un potenciostato/galvanostato AUTOLAB PGSTAT204 (Methrom, Utrecht, Netherlands) conectado a VA-Stand 663 (Metrohm, Utrecht, Netherlands), y usando una célula estándar de 3 electrodos: (I) un electrodo de gota de mercurio suspendida (Hanging Mercury Drop Electrode, HMDE) con un área de 0.4 mm² como electrodo de trabajo, (II) un electrodo Ag/AgCl/3M KCl como electrodo de referencia, y (III) un electrodo de platino como electrodo auxiliar. Se utilizaron 15 mL de solución Brdicka (1 mM Co(NH₃)₆Cl₃ y 1M tampón amonio (NH₃(aq) + NH₄Cl, pH 9.6)) como electrolito de soporte sin adición de ningún agente tensioactivo, y se añadieron 5 µL de la muestra procesada.

La determinación de MTs se basa en la técnica de separación por adsorción, debido a la alta afinidad de estas moléculas por la superficie del electrodo de trabajo y su fuerte adsorción a la misma en un circuito de electrodos abierto. Tanto la gota de mercurio del electrodo de trabajo como la solución Brdicka se renovaron después de cada determinación. Las condiciones de medida fueron las siguientes: potencial inicial de –0.7 V, potencial final de –1.8 V, tiempo de modulación de 0.05s, tiempo de intervalo de 0.57s, potencial de paso de 3.9 mV y amplitud de modulación de 25 mV. Todos los experimentos se llevaron a cabo a 5 °C usando un termostato Julabo F25 (Labortechnik, Wasserburg, Germany). Las MTs fueron cuantificadas mediante el uso de una línea de calibración

usando una solución comercial de MTs aisladas procedentes de hígado de conejo con una pureza $\geq 95\%$ (Enzo Life Sciences, Inc., New York, United States of America). La línea de calibración obtenida fue la siguiente: $y = 2.017 x - 0.023$; $R^2 = 0.996$. Para el procesamiento de los datos obtenidos en cada medida se utilizó el software NOVA 2.1.4 Metrohm Autolab B.V. (EcoChemie, Utrecht, Netherlands).

2.6 Análisis estadístico

El análisis estadístico de los datos se realizó mediante el programa SPSS versión 26.0 (IBM SPSS, Armonk, NY, United States of America), y la representación gráfica de los resultados mediante el programa GraphPad Prism 9 (GraphPad Software, San Diego, California, United States of America). La normalidad de las variables de los estudios fue testeada en primer lugar, usando el test de Kolmogorov-Smirnov o Shapiro–Wilk, en función del tamaño muestral en cada caso. Las variables cuantitativas con distribución normal están expresadas como media y desviación estándar (Standard Deviation, SD), y las variables con distribución no-normal como mediana y rango intercuartílico. Por otro lado, las variables cualitativas se encuentran expresadas como frecuencias y porcentajes. En el caso de aquellas variables cuantitativas que cumplían con una distribución normal se utilizaron modelos paramétricos para su análisis. Las diferencias en la evolución de las variables (“intragroup”) se analizaron mediante la prueba de la t de Student para muestras relacionadas, mientras que las diferencias entre grupos (“intergroups”) mediante la prueba de la t de Student para muestras independientes. Asimismo, las correlaciones entre

variables se analizaron mediante el coeficiente de correlación de Pearson, y en algunos casos se realizaron modelos de regresión lineal simple. En aquellos casos en los que las variables mostraron una distribución no-normal, se utilizaron pruebas no paramétricas, siendo las diferencias en la evolución evaluadas mediante la prueba de los rangos con signo de Wilcoxon y las correlaciones mediante el coeficiente de correlación de Spearman. El valor predictivo de algunas variables se analizó mediante curvas ROC (Receiver Operating Curve, ROC). Por otro lado, la estimación e interpretación del tamaño del efecto (Effect Size, ES) fue la siguiente: pequeño = 0.01, moderado = 0.06, y grande = 0.14 [14]. En todos los casos, la significación estadística fue establecida para un valor de $p < 0.05$.

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RESULTADOS Y DISCUSIÓN

CAPÍTULO I: PACIENTE CRÍTICO CON SIRS

Estudio I: Vitamin D status in critically ill patients with SIRS and its relationship with circulating Zn and related parameters during ICU stay.

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Abstract: Critically ill patients are exposed to different stressors which may generate Systemic Inflammatory Response Syndrome (SIRS). This situation hinders the assessment of micronutrients status, such as vitamin D or Zinc (Zn), potentially affecting patients' treatment and recovery. The aim of the present study was to assess the evolution of circulating 25-Hydroxyvitamin D (25-OH-D) levels after seven days of Intensive Care Unit (ICU) stay and the influence on changes in plasma and erythrocyte Zn levels, as well as other parameters related to phosphorus–calcium metabolism. A prospective analytical study was conducted on 65 critically ill patients (42% women) aged 31–77 years with SIRS. Total 25-OH-D levels were measured in plasma samples by liquid chromatography-tandem mass spectrometry, and Zn content was analyzed by flame atomic absorption spectrometry. Both 25-OH-D and 25-OH-D₃ levels were directly associated with erythrocyte Zn concentration at follow-up ($p = 0.046$ and $p = 0.011$, respectively). A relationship between erythrocyte and plasma Zn was also found at this follow-up point. No such clear associations were found when considering 25-OH-D₂. Different disturbances in levels of phosphorus–calcium metabolism parameters were found, suggesting a relationship between the changes of 25-OH-D₃ levels and parathormone ($p = 0.019$) and phosphorus ($p = 0.005$). The findings of the present study suggest an interaction between vitamin D and Zn, in which the correct status of these micronutrients could be a potentially modifiable factor and a beneficial approach in the recovery of critically ill patients.

Keywords: vitamin D; Zinc; critically ill patient; Intensive Care Unit; Systemic Inflammatory Response Syndrome.

1. Introduction

Critically ill patients admitted to the Intensive Care Unit (ICU) are exposed to different stressors involved in an acute stress response which may lead to inflammation and metabolic disturbances (e.g., hyperglycemia or mineral imbalances) [1]. An exaggerated host defense response, with the dysregulation of proinflammatory and anti-inflammatory pathway homeostasis, may generate Systemic Inflammatory Response Syndrome (SIRS) [2]. The presence of inflammation in multiple contexts (e.g., surgery, trauma, infection, or many acute or chronic diseases) could influence the assessment of the status of many micronutrients, hence inducing a redistribution from the circulating compartment to other organs [3].

Vitamin D is a micronutrient that acts as a hormone, and is involved in a wide range of biological functions through both an endocrine mechanism (e.g., regulation of Calcium (Ca) absorption) and an autocrine pathway (e.g., facilitation of gene expression) [4]. There are roughly 800 human genes for which there is a vitamin D response element; most of them are related to the expression of proteins necessary for the control of cell proliferation, differentiation, and apoptosis [4,5], and other functions related to the immune system [6] or antioxidative actions [7]. Cholecalciferol or vitamin D₃ is the natural form of vitamin D in animals and the form synthesized in human skin on exposure to sunlight, this being the most important source of vitamin D [8]. Typical dietary animal sources of vitamin D₃ are fatty fish, cod liver oil, or egg yolks, and fungal sources, such as mushrooms and yeast exposed to sunlight or UV radiation containing vitamin D₂ (ergocalciferol) [9]. Vitamin D₃ is more potent than vitamin D₂, but is itself not biologically active and needs to

be hydroxylated in the liver, forming, D₃ metabolite, 25-Hydroxyvitamin D₃ (25-OH-D₃) (the major circulating form of vitamin D and reliable biomarker of vitamin D status), and later in the kidney, forming 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂-D₃) [10]. Together, vitamins D₃ and D₂ make up total vitamin D.

Zinc (Zn) is an essential mineral for all organisms because it plays an important role in numerous processes, including protein and nucleic acid synthesis, carbohydrate and lipid metabolism, and both innate and adaptive immunity, and it also acts as a cofactor and component for numerous enzymes and transcription factors, among others [11,12]. Flesh foods are the most important dietary common sources of readily bioavailable Zn, being red meat richer in Zn than fowl and fish [13]. Over 85% of total body Zn is found in skeletal muscle and bone, with only a very small amount (0.1% of total) found in plasma [14]. Plasma Zn concentrations decrease during inflammation, largely because of the redistribution of Zn from albumin to the liver [15], which may further influence or even control the intensity of the acute phase response. In this regard, while acute Zn deficiency causes a decrease in innate and adaptive immunity, its chronic deficiency increases inflammation [16].

The relationship between vitamin D and Zn is not well known. Few lines of evidence have described the administration of vitamin D to effectively reduce the severity of Zn deficiency, suggesting an important effect on Zn absorption [17]. Furthermore, adequate circulating 25-OH-D₃ levels have been associated with improved absorption of essential minerals, including Zn [18]. Zn and vitamin D are important for bone metabolism, their deficiencies being associated with an increased risk of osteoporosis

[19]. Zn has been shown to inhibit osteoclastic bone resorption and stimulate bone formation and mineralization [20,21]. Additionally, the structure of the DNA binding domain of the 1,25-OH₂-D₃ receptor is modulated by Zn, the activity of vitamin D-dependent genes in cells being influenced by intracellular Zn concentrations [22,23]. Moreover, vitamin D has been demonstrated to generate a massive increase in Zn transport in cultured cells [7], and it is also hypothesized that vitamin D may mitigate oxidative damage by improving intracellular Zn concentrations [24]. Hence, based on the above-mentioned aspects, vitamin D and Zn appear to play key roles in many biological processes, which could be crucial for the recovery of critically ill patients. However, little is known about the combined action of these micronutrients.

The present study aimed to assess the evolution of circulating 25-OH-D levels among critically ill patients with SIRS at admission and after one week of ICU stay, and the influence of these levels on changes in plasma and erythrocyte Zn levels and other parameters related to phosphorus–calcium metabolism. We hypothesized that plasma 25-OH-D levels would be decreased at baseline and follow-up, evidencing changes in Zn circulating levels, among others.

2. Materials and Methods

2.1. Subjects and Study Design

The present multicenter observational study was carried out on 65 critical ill patients (aged 31–77 years, 27 women) recruited from Hospital Clínico San Cecilio and Virgen de las Nieves Hospital in Granada (Spain), and Santa Ana Hospital in Motril (Spain), during the

period from March 2015 to June 2019. The participants were monitored from ICU admission (baseline) until day 7 of stay (follow-up), the mortality rate being 38.5% during the study period. All eligible participants enrolled in the study were critically ill patients and fulfilled the following criteria: (i) aged 18 years or older; (ii) with SIRS [2]; and (iii) who agreed to participate in the study, or in which approval of participation was obtained from the family. Exclusion criteria were: (i) refusal to participate in the study as expressed by the patient or his/her legal representatives; (ii) pregnancy; (iii) the presence of highly contagious disease; (iv) allergies; (v) cancer; and (vi) the ingestion of food before obtaining the analytical sample. The present study was conducted in accordance with the principles of the Declaration of Helsinki (last revised guidelines from 2013) [25], following the International Conference on Harmonization (ICH)/Good Clinical Practice (GCP) standards, and was approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016).

2.2. Nutritional Profile

All patients received standard nutritional support via the enteral, parenteral, or combined routes, and were administrated nutritional formulas following the hospital's protocol. A daily nutritional log was kept for each patient (i.e., type, volume, composition of intake and tolerance) from admission to 7 days in the ICU. Mineral and vitamin support was calculated daily based on the age, weight, and sex of each individual, according to ESPEN guidelines [26], registered by the nutritionists and represented as the average of the 7-day period of stay in the ICU. The nutritional support protocol in critically ill patients was

assessed according to the Clinical Nutrition Units of the Hospitals, based on the American Society for Parenteral and Enteral Nutrition and the European Society of Parenteral and Enteral Nutrition guidelines [26].

2.3. Data Collection

The study data, including age, sex, weight, height, Body Mass Index (BMI), calculated as weight (Kg)/height (m²), total proteins, albumin, prealbumin, ferritin, transferrin, triglycerides, total cholesterol, C-Reactive Protein (CRP), Gamma-Glutamyl Transferase (GGT), Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT), Alkaline Phosphatase (ALP), osteocalcin, Parathormone (PTH), and Ca, Magnesium (Mg) and Phosphorus (P) were retrieved from the hospital electronic database system and recorded for each study participant at ICU admission (baseline) and after 7 days (follow-up). Biochemical parameters were determined in the Laboratory Analysis Unit of Virgen de las Nieves Hospital (Granada) (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The Acute Physiology and Chronic Health Evaluation II (APACHE-II) score and Sequential Organ Failure Assessment (SOFA) score were obtained by intensivists in the ICU.

2.4. Blood Sampling and Biochemical Parameters

Two blood extractions were performed in the morning under fasting conditions, at ICU admission (baseline) and after one week of ICU stay (follow-up). Plasma was separated through centrifugation (4 °C for 15 min at 3000 rpm) and the erythrocytes were washed 4 times with 3 mL of 0.9% sodium chloride solution, centrifuging for 15 min at 3000 rpm after each wash and removing the supernatant saline solution from the last wash.

Samples were stored at -80°C until analytical determination of the different parameters. The recorded biochemical parameters were determined using routine hospital analytical assays (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).

2.4.1. Analytical Determination of Zn

As previously described, Zn content was analyzed by Flame atomic Absorption Spectrometry (FAAS) (Perkin Elmer A. Analyst'300 Norwalk, Connecticut, USA) in the previous wet-mineralized way in the Scientific Instrumental Center from the University of Granada [27]. All samples were measured in one run, in the same assay batch and blinded quality control samples were included in the assay batches to assess laboratory error in the measurements. Reference values for plasma Zn concentrations were $10\text{--}17\text{ }\mu\text{mol/L}$ ($0.65\text{--}1.11\text{ mg/L}$) [14].

2.4.2. Analytical Determination of Vitamin D

The extended protocol has been described previously [28]. Briefly: total 25-OH-D levels were measured in plasma samples by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Plasma sample treatment involved protein precipitation, extraction, and derivatization. The Endocrine Society recommends 25-OH-D levels below 20 ng/mL to be termed vitamin D deficiency, concentrations of $21\text{--}29\text{ ng/mL}$ to be termed insufficient, and normal levels should be reserved for serum 25-OH-D values above 30 ng/mL [29].

Total 25-OH-D levels was calculated as the sum of 25-OH-D₃ and 25-OH-D₂ forms.

2.5. Statistical Analysis

Qualitative variables were presented as Frequencies (N) and Percentages (%). Quantitative variables were expressed as the arithmetic mean $\pm$ Standard Deviation (SD). As a previous step to the execution of a parametric model or not, the hypothesis of normal distribution was accepted using the Kolmogorov-Smirnov test. For the comparative analyses at baseline and follow-up, use was made of the paired Student t-test for parametric samples. For the comparative inter-groups analysis, the unpaired Student t-test for parametric samples was applied. Categorical variables were compared inter-groups using Chi-square test. Simple linear regression models were conducted to evaluate the significant associations of 25-OH-D levels with biochemical parameters. β (standardized regression coefficient), R^2 , and p-Values from simple linear regression analyses were obtained. The SPSS version 26.0 statistical package (IBM SPSS, Armonk, NY, USA) was used throughout, and plots were built using GraphPad Prism 8.0 for Mac (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was considered for $p < 0.05$.

3. Results

Table 4 shows the mean changes in the evolution of clinical and biochemical parameters of the study at ICU admission and after 7 days of stay. The mortality rate after 7 days of ICU stay was over one-third of the total study population (38.5%). Only PTH levels were significantly higher in women than in men ($p < 0.05$) at baseline, whereas the rest of the studied parameters showed no differences by sex ($p \geq 0.085$ in all

cases). Similarly, no significant differences were found ($p \geq 0.070$ in all cases) at baseline after categorizing by median age, except for BMI, being higher at greater ages ($p < 0.001$). Most variables were out of the normal reference ranges at the time of ICU admission, and there was an apparent tendency for the values to normalize after one week of stay. In this line of thinking, and regarding phosphorus–calcium metabolism parameters, the mean changes of osteocalcin, PTH, and Ca levels were significant ($p > 0.05$ in all cases), with an increase in osteocalcin and Ca and a decrease in PTH levels after 7 days of ICU stay.

Table 4. Comparative analysis of the studied parameters at baseline and follow-up.

Parameters	Reference	Baseline Mean (SD) N = 65	Follow- Up Mean (SD) N = 40	Δ Change (%)	p-Value Men vs. Women	p-Value < 62 years vs. > 62 years	p-Value Baseline vs. Follow- Up
Age (years)	-	60.1 (11.5)	-	-	0.747	-	-
BMI (kg/m ²)	-	26.7 (4.77)	-	-	0.320	0.001	-
Patient 7-day mortality (n/N, %)	-	25/65 (38.5)	-	-	0.474	0.070	-
APACHE-II score	-	17.2 (4.94)	-	-	0.390	0.895	-
SOFA score	-	9.04 (3.39)	5.12 (3.50)	-43.4	0.230	0.264	< 0.001
Total Proteins (g/dL)	6.60–8.30	5.27 (0.91)	5.53 (0.76)	+4.93	0.138	0.658	0.115
Albumin (g/dL)	3.50–5.20	2.85 (0.58)	2.63 (0.67)	-7.72	0.647	0.649	0.139
Prealbumin (mg/dL)	16.0–42.0	12.5 (4.65)	16.3 (9.80)	+30.3	0.370	0.828	0.143
Ferritin (ng/dL)	20.0– 275.0	401.6 (377.4)	543.7 (482.9)	+35.4	0.861	0.493	0.024
Transferrin (mg/dL)	200.0– 360.0	153.4 (63.1)	146.9 (50.8)	-4.27	0.825	0.868	0.514
Triglycerides (mg/dL)	50.0– 200.0	196.9 (144.3)	197.8 (102.1)	+0.46	0.275	0.366	0.972
Total Cholesterol (mg/dL)	140.0– 200.0	108.5 (38.2)	134.8 (43.7)	+24.2	0.875	0.521	0.002

CRP (mg/L)	0.00–5.00	19.8 (11.8)	10.7 (8.63)	–46.2	0.292	0.164	0.001
GOT (U/L)	< 37.0	104.0 (164.0)	36.2 (23.2)	–65.2	0.290	0.260	0.045
GPT (U/L)	< 41.0	54.9 (78.8)	28.5 (26.0)	–48.0	0.801	0.349	0.082
GGT (U/L)	11.0–50.0	56.6 (60.9)	141.3 (98.4)	+149.7	0.932	0.473	< 0.001
ALP (U/L)	40.0– 130.0	101.5 (76.3)	126.1 (68.8)	+24.3	0.085	0.133	0.062
Osteocalcin (ng/mL)	15.0–46.0	2.97 (1.95)	5.53 (3.98)	+86.2	0.385	0.163	0.002
PTH (pg/mL)	20.0–70.0	248.5 (151.0)	133.5 (97.9)	–46.3	0.013	0.775	< 0.001
Ca (mg/dL)	8.80–10.6	7.53 (0.86)	8.06 (0.71)	+7.04	0.930	0.812	0.001
P (mg/dL)	2.30–4.50	3.91 (1.87)	3.65 (1.47)	–6.65	0.152	0.676	0.452
Mg (mg/dL)	1.60–2.60	2.14 (0.52)	2.31 (0.46)	+7.94	0.564	0.320	0.213

Data are expressed as mean (standard deviation). Abbreviations: ALP = Alkaline Phosphatase; APACHE II = Acute Physiology and Chronic Health Evaluation II; BMI = Body Mass Index; Ca = Calcium; CRP = C-Reactive Protein; GGT = Gamma-Glutamyl Transferase; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamic Pyruvic Transaminase; Mg = Magnesium; P = Phosphorus; PTH = Parathormone; SOFA = Sequential Organ Failure Assessment. D Change (%) = percentage change from baseline to follow-up. Qualitative variables were compared inter-groups using Chi-square test. Quantitative variables were compared inter-groups using the unpaired Student t-test for parametric samples, and intra-groups using the paired Student t-test for parametric samples. Median age (62 years old) was used as cutoff to analyze the differences between lower and higher ages. Significant *p*-Values are shown in boldface. Statistical significance was considered for *p* < 0.05.

The evolution of vitamin D and Zn levels during the ICU stay are represented in *Table 5*. Levels of 25–OH–D were significantly lower than the reference values and remained low after one week, the status being deficient in all cases, at baseline and follow-up. A significant decrease in 25–OH–D₃ levels (*p* = 0.035) was also found. Regarding Zn status, two-fifths of patients presented hypozincemia at baseline and one-third at follow-up. No significant differences were found in the evolution of plasma and erythrocyte Zn levels throughout the time (all *p* > 0.05). Mean daily support of vitamin D and Zn during the studied period were 4.95 µg/day and 7.75 mg/day, respectively, according to ESPEN recommendations.

Figure 3 shows the associations of vitamin D and Zn levels at baseline and follow-up. Non-significant relationships were found for 25-OH-D, 25-OH-D₃, and 25-OH-D₂ with erythrocyte Zn levels and between erythrocyte and plasma Zn at baseline (all $p \geq 0.116$; panels A, C, E, and G), but the latter became significant at follow-up (all $p < 0.05$; model 0; panels B, D, and H) except for 25-OH-D₂ ($p > 0.05$, panel F). The significant direct relationship between erythrocyte and plasma Zn levels at follow-up persisted after including age ($p = 0.022$; panel F; Model 1) and sex ($p = 0.043$; panel F; Model 2) as covariates.

Table 5. Comparative analysis of the studied parameters at baseline and follow-up.

Parameters	Reference	Baseline Mean (SD) N = 65	Follow-Up Mean (SD) N = 40	Δ Change (%)	p-Value Baseline vs. Follow- Up
25-OH-D (ng/mL)	30.0–100.0	6.76 (2.96)	6.51 (3.28)	–3.11	0.506
25-OH-D ₃ (ng/mL)	–	3.90 (2.23)	3.59 (2.10)	–7.18	0.035
25-OH-D ₂ (ng/mL)	–	2.87 (1.60)	2.93 (2.05)	+2.09	0.875
Plasma Zn (mg/L)	0.65–1.11	0.71 (0.51)	0.74 (0.56)	+4.23	0.748
Erythrocyte Zn (mg/L)	–	6.22 (2.81)	6.42 (2.90)	+3.12	0.708
		n/N (%)	n/N (%)		
25-OH-D deficiency	< 20 ng/mL	65/65 (100)	40/40 (100)	0	–
Hypozincemia (plasma Zn)	< 0.7 mg/L	28/65 (43)	13/40 (32.5)	–24.4	0.001

Data are expressed as mean (Standard Deviation). Abbreviations: Zn = Zinc; 25-OH-D = 25-Hydroxyvitamin D; 25-OH-D₃ = 25-Hydroxyvitamin D₃; 25-OH-D₂ = 25-Hydroxyvitamin D₂. Δ Change (%) = percentage change from baseline to follow-up. n = number of participants who presented deficient biochemical values. For the comparative analysis of quantitative variables, the paired Student t-test for parametric samples was used. Qualitative variables were compared inter-groups using Chi-square test. Significant p-Values are shown in boldface. Statistical significance was considered for $p < 0.05$.

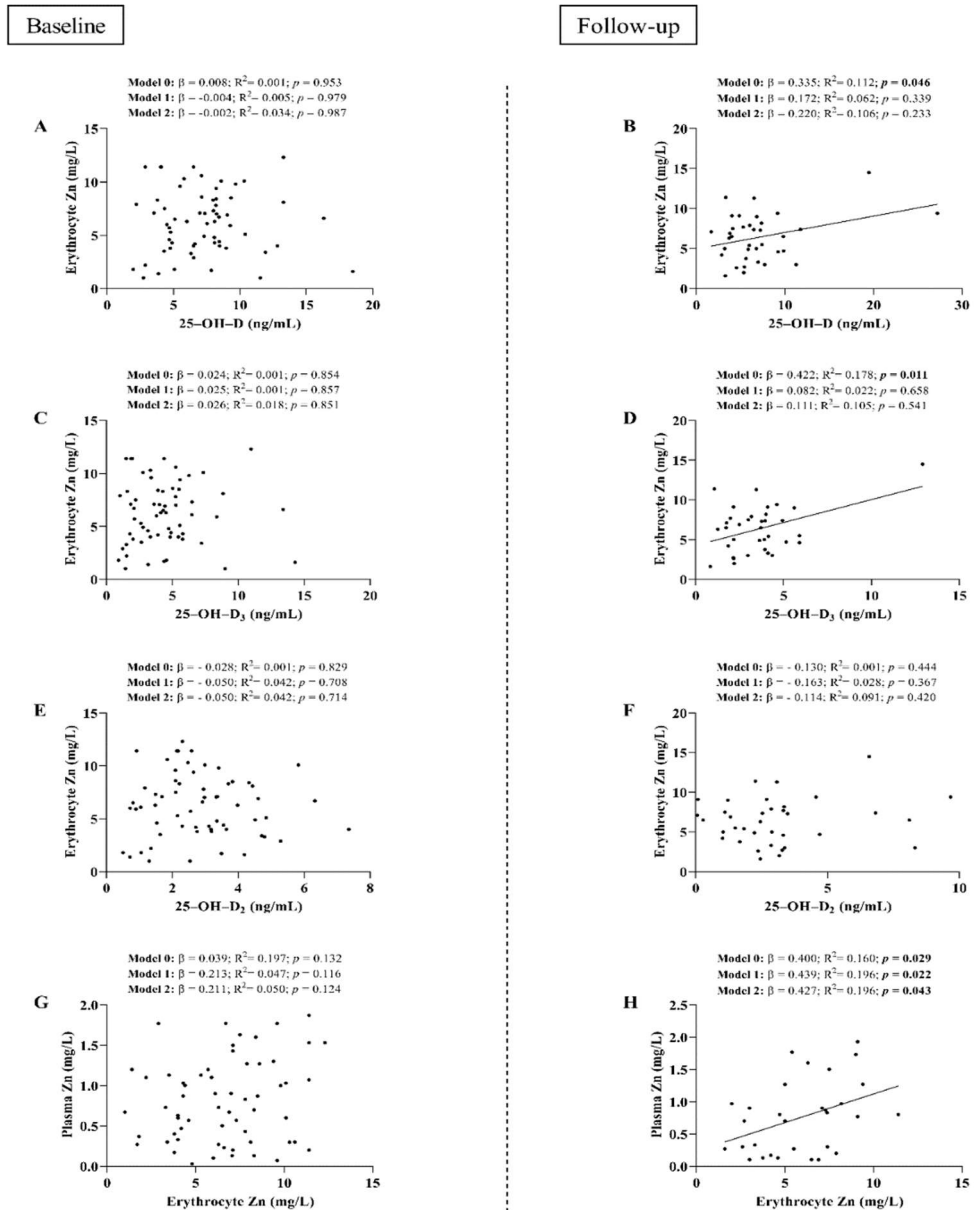


Fig. 3. Associations between vitamin D and Zn levels at baseline and follow-up. (A) 25-OH- D levels with erythrocyte Zn at baseline. (B) 25-OH-D levels with erythrocyte Zn at follow-up. (C) 25-OH-D₃ levels with erythrocyte Zn at baseline. (D) 25-OH-D₃ levels with erythrocyte Zn at follow-up. (E) 25-OH-D₂ levels with

erythrocyte Zn at baseline. (F) 25–OH–D₂ levels with erythrocyte Zn at follow-up. (G) Erythrocyte Zn with plasma Zn at baseline. (H) Erythrocyte Zn with plasma Zn at follow-up. (standardized regression coefficient), R², and p are provided for simple and multiple linear regression analyses. Model 0; unadjusted, Model 1; adjusted for age, Model 2; adjusted for age and sex. Abbreviations: Zn = Zinc; 25–OH–D = 25–Hydroxyvitamin–D; 25–OH–D₃ = 25–Hydroxyvitamin D₃. Significant p-Values are shown in boldface. Statistical significance was considered for $p < 0.05$.

Table 6 shows the significant relationships between changes in 25–OH–D, 25–OH–D₃, and 25–OH–D₂ levels, and changes in phosphorus–calcium metabolism parameters after one week of ICU stay. The changes in 25–OH–D₃ levels were found to be directly related to changes in PTH ($p = 0.019$) and P levels ($p = 0.005$).

Table 6. Associations between changes in vitamin D and changes in phosphorus–calcium metabolism parameters after 7 days of ICU stay.

Characteristic s	Δ 25–OH–D			Δ 25–OH–D ₃			Δ 25–OH–D ₂		
	β	R ²	p-Value	β	R ²	p-Value	β	R ²	p-Value
Δ Osteocalcin (ng/mL)	0.091	0.008	0.672	–0.26 0	0.067	0.221	0.202	0.041	0.343
Δ PTH (pg/mL)	0.211	0.044	0.334	0.484	0.234	0.019	–0.015	0.001	0.945
Δ Ca (mg/dL)	–0.256	0.065	0.218	0.044	0.002	0.833	–0.256	0.065	0.218
Δ P (mg/dL)	0.329	0.108	0.109	0.539	0.291	0.005	0.115	0.013	0.585
Δ Mg (mg/dL)	–0.388	0.150	0.061	0.069	0.005	0.748	–0.402	0.161	0.052

β = standardized regression coefficient. R² and p are from simple regression analysis. Abbreviations: Mg = Magnesium; P = Phosphorus; PTH = Parathormone; 25–OH–D = 25–Hydroxyvitamin D; 25–OH–D₃ = 25–Hydroxyvitamin D₃; 25–OH–D₂ = 25–Hydroxyvitamin D₂. Bold numbers indicate a statistically significant association. Significance was set at $p\text{-Value} < 0.05$.

4. Discussion

The main results of the present study showed that critically ill patients presented deficient levels of 25-OH-D at ICU admission, with a decrease in 7 days, especially in the case of 25-OH-D₃. Both 25-OH-D and 25-OH-D₃ levels were directly associated with erythrocyte Zn concentrations at follow-up, and a relationship also existed between erythrocyte and plasma Zn levels at this time point. No such clear associations were found when considering 25-OH-D₂. The changes in 25-OH-D₃ were positively correlated with the changes in PTH and P levels. These findings shed light on the idea that a relationship exists between vitamin D and circulating Zn, mainly due to 25-OH-D₃ levels.

Patients admitted to the ICU present a critical and complex pathological condition characterized by different clinical and biochemical disturbances, which must be solved for the patient's recovery [30]. After 7 days of ICU stay, we found significant changes in several biochemical parameters, highlighting an increase in osteocalcin and Ca levels, a decrease in PTH and 25-OH-D₃ levels, and a reduction in SOFA score. Although certain parameters remained altered, an apparent tendency to stabilize clinical variables was found at follow-up.

Vitamin D status was deficient in all cases at baseline and follow-up. In critically ill patients, vitamin D deficiency has been associated with increased length of stay, morbidity, and mortality, and it has been demonstrated that significant decrease in vitamin D status occurred throughout the patients' ICU stay [31]. In our study, we observed a significant decrease in 25-OH-D₃ levels after one week of ICU stay, despite better results observed in severity score at this time point, but the

decrease in total 25-OH-D levels was not significant. It should be remembered, however, that without ensuring baseline status and dose adequacy more is not better. A U-shaped curve demonstrated the relationship between 25-OH-D levels (< 30 ng/mL or 60 ng/mL), and 90-day mortality, and inferred that high-dose vitamin D might not be beneficial to patients [32]. Vitamin D metabolomics, transcriptomics, and epigenetics are needed to investigate whether vitamin D deficiency is an independent contributor to morbidity and mortality of critically ill patients with SIRS and whether “body humors” vitamin D correction leads to outcome benefits [33]. Regarding Zn levels, the obtained mean values of plasma Zn were close to hypozincemia and did not change significantly during the ICU stay [34]. The same tendency was found for erythrocyte Zn levels, which did not change after seven days. Although Zn deficiency is difficult to diagnose, due to the lack of a reliable biomarker to assess Zn status, it is known to be a very common and serious nutritional problem because of the widespread variety of clinical manifestations, especially in developing countries, [35]. In the literature, the advantage of measuring serum Zn levels as one of the routine and standard physiological blood tests has been emphasized [34,36]. Ultimately, having a previous correct status of these micronutrients, through diet, and the use of fortified food or supplements in cases of any risk of deficiency, could be a preventive factor [9,13,37]. No relationships were found at baseline when analyzing the associations between vitamin D and Zn levels. On the contrary, erythrocyte Zn levels were positively correlated to 25-OH-D, 25-OH-D₃, and plasma Zn levels at follow-up, this last association persisting between circulating Zn levels after age and sex were included as covariates. The influence of

vitamin D goes far beyond the regulation of Ca and P balance, playing an important pleiotropic role in maintaining global homeostasis [38]. Vitamin D exerts mitochondrial protective effects through the modulation of different signaling pathways and downregulates prooxidative and proinflammatory pathways [39]. Several lines of evidence have described the following different interactions between vitamin D and Zn: (i) vitamin D₃ increases ZnT10 protein expression, (ii) there is a cysteine-rich Zn finger region in the vitamin D receptor, and (iii) vitamin D modulates the expression and activity of Cu/Zn-dependent SOD isoforms and metallothioneins, among others [7,10,19]. Hence, vitamin D could play an important role in Zn-related processes, and it should be investigated more in depth, especially in critically ill patients, where there is a lack of evidence in this line.

Furthermore, the analysis of changes in vitamin D and parameters related to phosphorus–calcium metabolism showed that only the changes in 25–OH–D₃ levels were directly associated with changes in PTH and P levels. In vitro studies indicate that 25–OH–D₃ inhibits the synthesis of PTH, and this negative correlation between PTH and 25–OH–D appears to be supported by most of the published studies. However, some authors have suggested that there is a turning point, or even a plateau, in this correlation [40]. Meanwhile, it has been observed that increased P levels may induce hypocalcemia, which increases PTH secretion, and directly stimulate PTH synthesis and parathyroid cellular proliferation [41]. It must be noted that the PTH levels of our cohort were very high at ICU admission and, although they decreased significantly after one week, remained quite high. Likewise, low Ca levels were found at baseline, significantly increasing during the period but not enough to reach normal values. Extracellular ionized Ca concentrations are tightly controlled,

and PTH and 1,25-OH₂-D₃ are probably the most important factors influencing Ca homeostasis from a clinical point of view [42]. These results are not in line with what would be expected; however, probably these correlations have been affected because of the existence of important disturbances in different studied parameters.

Finally, it should be kept in mind that optimal nutritional status, including healthy levels of vitamin D, is requisite for proper physiological function. Moreover, adequate distribution of metal ions is crucial, and a slight alteration in their levels may induce severe diseases [43]. The interactions between vitamin D and Zn remain incompletely understood. For example, it has been found that Zn supplementation in hemodialysis patients appears to promote bone formation and is not affected by the status of PTH and vitamin D [44]. Vitamin D₃ and Zn are known to shift the immune response towards tolerance individually; however, the evidence suggests that their combination may be a promising option to treat dysregulated immune response in various conditions [45]. Therefore, even though more evidence is needed, the correct status of these micronutrients should be ensured as a potentially beneficial approach for the recovery of critically ill patients.

The findings of the present study should be taken with caution as some limitations arise. Firstly, this study recruited fewer patients than desired due to the difficulty of obtaining the sample and the inherent variable clinical situation and severity of the patients. Secondly, follow-up during the 7-day stay in the ICU did not allow us to establish causal relationships. Thirdly, we had no reliable data on exposure to sunlight, dietary factors, or vitamin D supplementation, all of which affect vitamin

D status. Fourthly, data on 1,25-OH₂-D levels were not available. Fifthly, the overall negative results may be related to the heterogeneity of the subjects and their underlying disease conditions or severity, which may all influence the biochemical profile. Sixthly, we did not find relationships between vitamin D or Zn levels with severity and inflammatory parameters, which could explain the observed differences at baseline and follow-up. As strengths, LC-MS/MS was used to assess the levels of 25-OH-D, which is the gold standard method [46].

5. Conclusions

Critically ill patients with SIRS presented deficient vitamin D status. The deficiency decreased after seven days of ICU stay, mainly due to 25-OH-D₃ levels. The prevalence of hypozincemia decreased at follow-up, but mean plasma Zn levels remained low. A positive association was found between erythrocyte Zn and both 25-OH-D and 25-OH-D₃ levels at follow-up. Different disturbances in levels of the phosphorus–calcium metabolism parameters were found, signifying a relationship between the changes in 25-OH-D₃ levels and changes in both PTH and P. The results of the present study show an interaction between vitamin D and Zn after one week of ICU stay, coinciding with the apparent stabilization of critically ill patients. Thus, these findings suggest that the correct status of these micronutrients is a potentially modifiable factor and beneficial approach in the recovery of critically ill patients.

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Resources: E.P. and J.M.-L.; Writing—original draft preparation, L.H.-Q. and H.V.-L.; Writing—review and editing, L.H.-Q., H.V.-L., Y.G.-M., E.P. and J.M.-L.; Supervision, E.P. and J.M.-L.; Project administration, E.P.; Funding acquisition, E.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016) for studies involving humans.

Informed Consent Statement: An Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analyzed during the current study are not publicly available due the database is very extensive and includes data from other studies complementary to this but are available from the corresponding authors on reasonable request.

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Estudio II: Selenium levels and antioxidant activity in critically ill patients with Systemic Inflammatory Response Syndrome.

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Abstract: The Selenium (Se) status could be an important modifiable factor in critically ill patient outcomes due to the important role of this mineral in several functions. Although there are many clinical trials with Se interventions in the literature, the evidence is not sufficient to establish a common criterion regarding the Se status. Background and aims: An analysis was made of the evolution of selenium (Se) and antioxidant status in critically ill patients with Systemic Inflammatory Response Syndrome (SIRS) over 7 days of staying in the Intensive Care Unit (ICU). Methods: A prospective analytical study was carried out on 65 critically ill patients aged 31–77 years. A healthy control group of 56 volunteers from the same region was recruited to allow comparisons with reference normal values. The selenium levels in both the plasma and erythrocytes were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) activity and the Total Antioxidant Capacity (TAC) were measured using kinetic colorimetric methods. Results: Low erythrocyte and plasma Se levels were found at ICU admission in comparison with the healthy reference group ($p < 0.001$), and the levels further decreased after one week ($p < 0.001$). Smaller changes in the plasma Se levels were associated with greater changes in the Sequential Organ Failure Assessment (SOFA) score ($p < 0.05$). The GPx activity in the critically ill was lower than in the control group ($p < 0.05$), with an inverse correlation to the severity scores at the baseline ($p < 0.05$) and reaching normal values after one week ($p < 0.05$). SOD activity was directly correlated to TAC ($p = 0.03$), with both parameters exhibiting a direct correlation to albumin ($p < 0.05$) after 7 days of ICU stay. Conclusions: A deficient Se status was observed at ICU admission and

worsened further over follow-up regardless of the evolution of the patient severity and the antioxidant parameters. Adequate Se support from the start of admission could preserve and contribute to improve the Se-related outcomes and critical patient recovery during longer periods in the ICU.

Keywords: selenium; antioxidant; severity; critically ill patient; intensive care unit; systemic inflammatory response syndrome

1. Introduction

Critically ill patients constitute a heterogeneous population characterized by the presence of life-threatening multisystemic disorders [1]. These patients are exposed to different stressors that may lead to acute stress and inflammation, which often are associated with a range of poorly understood metabolic disturbances [2]. When this inflammatory condition is not amended, it may generate Systemic Inflammatory Response Syndrome (SIRS), defined as an exaggerated host defense response to a noxious stressor, with the dysregulation of proinflammatory and anti-inflammatory pathway homeostasis [3].

Decreased concentrations of trace elements, especially in plasma or serum, are a common finding in this population as a result of the inflammatory condition [4]. The micronutrient status plays an important role in maintaining the homeostasis and could prove the determinant in patient recovery. However, it is not clear whether these changes in mineral homeostasis are beneficial or detrimental for critically ill patients [5]. In particular, for Selenium (Se), recent studies have reported decreased Se levels in critically ill patients as early as Intensive Care Unit (ICU) admission compared to patient controls, showing that these values remained stable and were not associated with severity scores or inflammatory parameters such as Sequential Organ Failure Assessment (SOFA) and C-Reactive Protein (CRP) [6]. Contrary, other researchers would have reported the relationship between low Se levels and patient clinical severity according to the simplified acute physiology score and sepsis diagnosis [7], also the strongest predictive factor for ICU mortality [8] Furthermore, the plasma Se status could be affected differently by the patient's inflammation, finding that erythrocyte Se would not be affected

by the inflammatory response [9]. Given the discrepancies in the literature, it is necessary to elucidate the relationship that Se could have with the patient's clinical outcomes and follow-up that may affect their mortality. Moreover, this complex situation in critical illness influences the metabolism and nutritional requirements, with nutrient supply being a possible modifiable risk factor for patient outcomes [10,11].

In this line, trace elements such as Se have been regarded as potential key pharmaconutrients due to their immunological and antioxidant functions [12]. In this regard, Se is incorporated in selenoproteins, with Glutathione Peroxidase (GPx) being the major component of the mammalian antioxidant defense system within the selenoproteome [13]. Se and selenoproteins participate in a wide variety of processes, and their role in maintaining the erythrocyte redox homeostasis is one of their most remarkable functions [14]. In a critical illness, the Se metabolism could be altered by stress and inflammation, suffering redistribution processes. Due to this situation, low Se levels do not necessarily indicate deficiency, making the definition of reliable reference values difficult [15]. In regard to this, it has been carried out in different clinical trials with Se supplementation, even using high doses [16]. However, recent reviews and metaanalyses, which included randomized controlled trials, concluded that the quality of evidence is very low and the risk of bias very high [17,18]. Many factors could influence the potential therapeutic use of Se, such as the previous Se status, which is related to the region [19].

Thus, based on the abovementioned aspects, Se is seen to play a key role in the antioxidant defense, which could be crucial for the recovery of

critically ill patients. However, there is not enough evidence to allow us to establish a common criterion regarding the Se status in this population. The aim of the present study was to assess the evolution of both the Se and antioxidant levels in Spanish critically ill patients with SIRS over 7 days of stay in the ICU, as well as the adequacy of Se support. We hypothesized that Se is decreased both at the baseline and after 7 days of ICU stay, influencing both the severity and antioxidant activity of the studied individuals.

2. Results

The mean differences in the clinical characteristics between the patients upon admission to the ICU and the controls are represented in *Table 7*. The mortality rate after 7 days of ICU stay was over one-third of the total study population. Erythrocyte Se expressed per hemoglobin and SOD activity showed no differences between the groups ($p > 0.05$ in all cases), whereas the rest of the studied parameters changed significantly on comparing the ICU patients versus the healthy controls ($p < 0.001$ in all cases).

Table 8 shows the differences in the main outcomes of the study after 7 days of ICU stay. Regarding the plasma and erythrocyte Se, which were below the reference values at the time of ICU admission, the levels were seen to further decrease after 7 days of ICU stay ($p < 0.001$). In particular, the prevalence of individuals with a high risk of Se deficiency increased after one week, affecting more than a half of the patients in the case of plasma Se. No differences were observed for erythrocyte Se when expressed per hemoglobin ($p > 0.05$). In relation to the antioxidant

activity, the GPx activity increased significantly ($p < 0.05$) after 7 days of ICU stay.

Table 7. Descriptive analysis of the patients at admission to the ICU and the control group, and analysis of the differences between the groups.

Parameters	Healthy Controls	ICU Patients	<i>p</i> -Value
	Mean (SD) <i>N</i> = 56	Mean (SD) <i>N</i> = 65	
BMI (kg/m ²)	25.0 (3.26)	26.7 (4.77)	0.069
Patient 7-day mortality (n/N, %)	-	25/65 (38.5)	-
APACHE-II score	-	17.2 (4.94)	-
SOFA score	-	9.34 (3.90)	-
Total proteins (g/dL)	7.39 (0.37)	5.19 (0.84)	0.001
Albumin (g/dL)	4.41 (0.27)	2.73 (0.62)	0.001
Prealbumin (mg/dL)	27.9 (5.10)	12.7 (6.72)	0.001
Triglycerides (mg/dL)	110.7 (58.1)	186.8 (124.0)	0.001
Total cholesterol (mg/dL)	206.0 (40.8)	108.9 (38.1)	0.001
Hemoglobin (g/dL)	13.4 (1.88)	10.9 (2.70)	0.001
CRP (mg/L)	0.22 (0.25)	19.9 (13.1)	0.001
Se support (µg/day)	69.4 (30.3)	37.8 (23.1)	0.001
Plasma Se (µg/L)	76.4 (18.1)	56.2 (12.9)	0.001
Erythrocyte Se (µg/L)	109.3 (22.5)	79.7 (14.9)	0.001
Erythrocyte Se (µg/g Hb)	0.84 (0.24)	0.76 (0.22)	0.098
GPx activity (U/g Hb)	42.2 (28.7)	29.0 (14.3)	0.004
SOD activity (U/g Hb)	1628.1 (1439.0)	1501.1 (551.1)	0.546
TAC (µmol/L)	801.5 (374.7)	1100.6 (490.5)	0.001

Data are expressed as the mean $\pm$ standard deviation. Abbreviations: SD = Standard Deviation; APACHE-II = Acute Physiology and Chronic Health Evaluation II; BMI = Body Mass Index; CRP = C-Reactive Protein; GPx = Glutathione Peroxidase; Se = Selenium; SOD = Superoxide Dismutase; SOFA = Sequential Organ Failure Assessment; TAC = Total Antioxidant Capacity. For the comparative analysis between groups, the unpaired Student's *t*-test for parametric samples was used. Statistical significance was considered for $p < 0.05$.

Table 8. Comparative analysis of the studied parameters at baseline and follow-up.

Parameters	Baseline Mean (SD) N = 65	Follow-Up Mean (SD) N = 40	ΔChange (%)	p-Value
SOFA score	9.04 (3.39)	5.12 (3.50)	-43.4	0.001
Albumin (g/dL)	2.85 (0.58)	2.63 (0.67)	-7.71	0.139
Prealbumin (mg/dL)	12.5 (4.65)	16.3 (9.80)	30.4	0.143
CRP (mg/L)	19.8 (11.8)	10.7 (8.63)	-46.0	0.001
Plasma Se (μg/L)	57.8 (11.6)	42.6 (10.9)	-26.3	0.001
Erythrocyte Se (μg/L)	84.0 (15.4)	69.2 (14.9)	-17.6	0.001
Erythrocyte Se (μg/g Hb)	0.80 (0.24)	0.76 (0.20)	-5.00	0.343
GPx activity (U/g Hb)	30.6 (18.6)	47.3 (27.9)	54.6	0.016
SOD activity (U/g Hb)	1528.6 (587.2)	1377.2 (652.2)	-9.90	0.265
TAC (μmol/L)	1016.3 (445.1)	849.8 (509.4)	-16.4	0.061
Selenium adequacy				
Plasma Se<45.6 μg/L (n,%)	10 (15.4)	25 (62.5)	150.0	0.033
Erythrocyte Se<69.0 μg/L (n,%)	19 (29.2)	20 (50.0)	5.26	0.167

Data are expressed as the mean ± standard deviation. Abbreviations: SD = Standard Deviation; GPx = Glutathione Peroxidase; Hb = Hemoglobin; Se = Selenium; SOD = Superoxide Dismutase; SOFA = Sequential Organ Failure Assessment; TAC = Total Antioxidant Capacity. Δ Change (%) = Percentage change from baseline to follow-up. For the comparative analysis between groups, the paired Student's t-test for parametric samples was used. Statistical significance was considered for $p < 0.05$.

Figure 4 shows a Pearson's correlation analysis of the antioxidant enzymes with respect to the clinical outcomes, at both the baseline (Figure 5a–c) and follow-up (Figure 5d–f). Regarding ICU admission, GPx activity was inversely correlated to severity scores such as APACHE II ($r = -0.280$, $p < 0.05$; Figure 5a) and SOFA ($r = -0.298$, $p < 0.05$; Figure 5b) and directly correlated with erythrocyte Se (μg/g Hb) ($r = 0.399$, $p < 0.01$; Figure 5c). In the case of the significant correlations found at follow-up, albumin was directly correlated to SOD ($r = 0.501$, $p < 0.01$; Figure 5d) and TAC ($r = 0.404$, $p < 0.05$; Figure 5e), whereas SOD was directly correlated to TAC ($r = 0.444$, $p < 0.05$; Figure 5f).

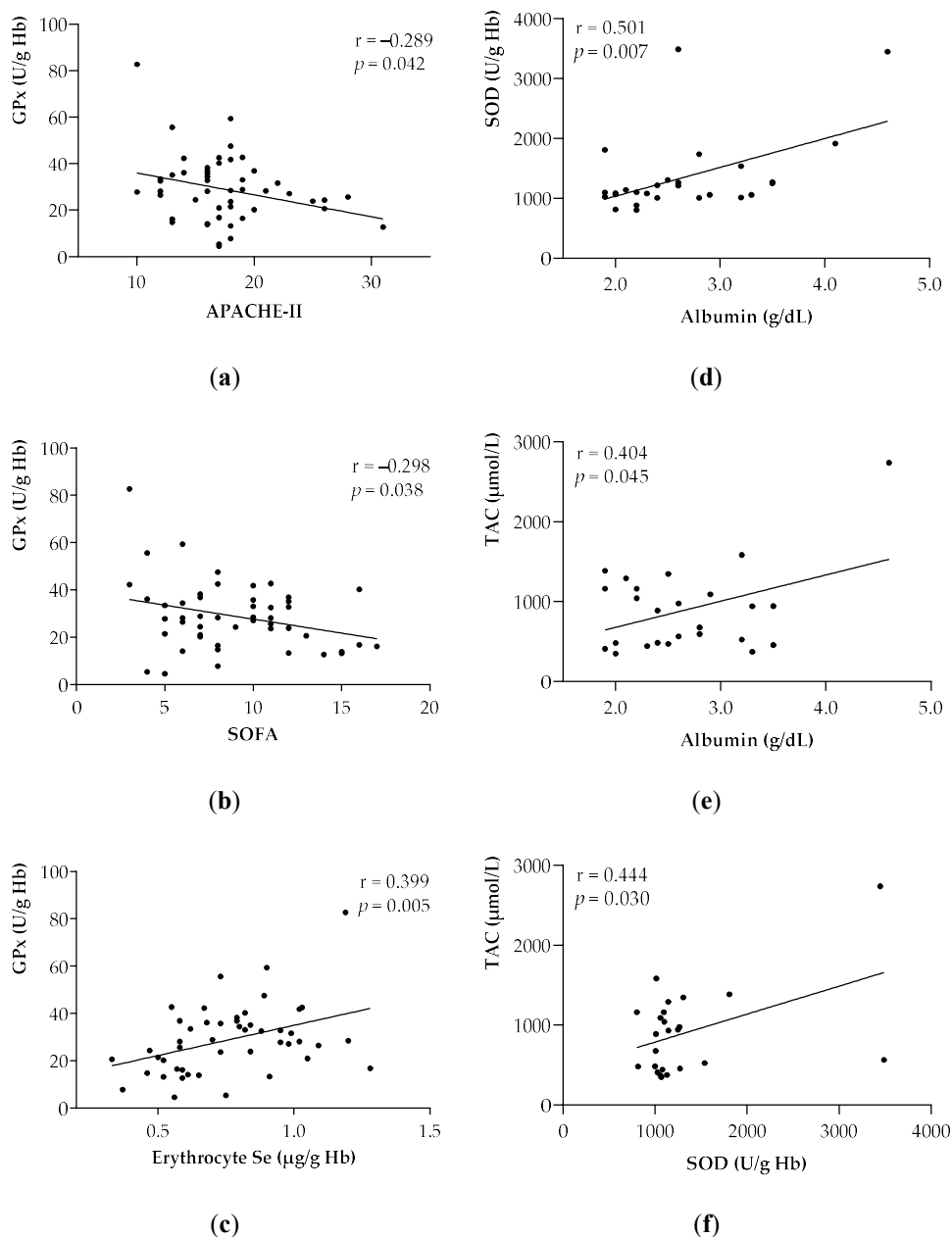


Fig. 4. Associations between the antioxidant enzymes and main parameters of the study at the baseline and follow-up. (a) GPx with APACHE II at the baseline. (b) GPx with SOFA at the base-line. (c) GPx with erythrocyte Se at baseline. (d) SOD with albumin at follow-up. (e) TAC with albumin at follow-up. (f) SOD with TAC at follow-up.

Abbreviations: APACHE-II = Acute Physiology and Chronic Health Evaluation II; GPx = Glutathione Peroxidase; Hb = Hemoglobin; SOD = Superoxide Dismutase; SOFA = Sequential Organ Failure Assessment; TAC = Total Antioxidant Capacity. Pearson's correlation coefficient (r) was used to associate all parameters. Statistical significance was considered for $p < 0.05$.

Figure 5 shows the significant relationships regarding the changes in the Se levels after 7 days of ICU admission. Changes in the plasma Se levels were associated with changes in the erythrocyte Se levels expressed as both $\mu\text{g/L}$ and $\mu\text{g/g Hb}$, respectively ($p < 0.05$ in all cases).

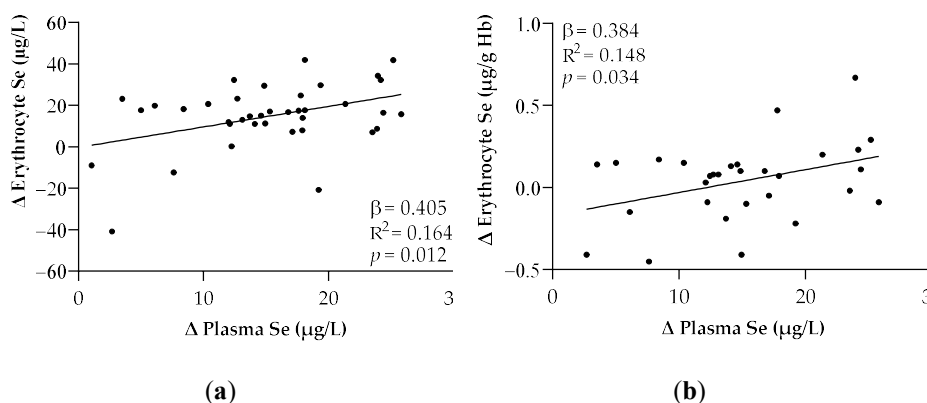


Fig. 5. Relationships between the changes in plasma and erythrocyte Se expressed in $\mu\text{g/L}$ (Figure 6a) and $\mu\text{g/g Hb}$ (Figure 6b) after one week of ICU stay. Abbreviations: Hb = Hemoglobin; Se = Selenium. The β (standardized regression coefficient) and p-values from single linear regression analyses were obtained. Statistical significance was considered for $p < 0.05$.

The relationships between changes in the SOFA score and plasma Se levels after one week of ICU stay are shown in *Figure 6*. Smaller changes in the plasma Se levels were seen to be related to greater increases in the SOFA scores ($p < 0.05$).

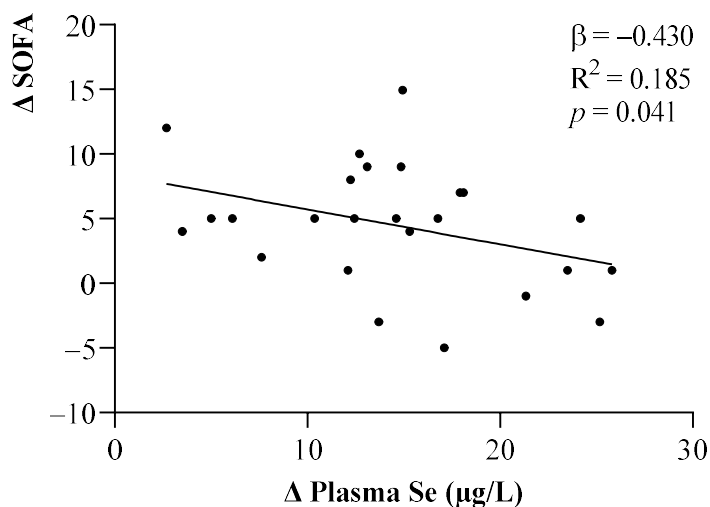


Fig. 6. Linear relationship between changes in the SOFA scores and plasma Se levels. The β (standardized regression coefficient) and p -values from multiple linear regression analyses were obtained. Statistical significance was considered for $p < 0.05$. Abbreviations: Se = Selenium; SOFA = Sequential Organ Failure Assessment.

3. Discussion

The present study was carried out to assess the Se and antioxidant levels and their evolution after one week of ICU stay in critically ill patients. Initially, the prevalence of patients showing Se deficiency was 15.4% for plasma Se and 29.2% for erythrocyte Se. Moreover, plasma and erythrocyte Se were seen to be decreased after 7 days of ICU stay, affecting more than half of the patients in the case of plasma Se, despite the standard support received. Contrary to our primary hypothesis, no significant changes in the SOD activity or TAC were observed, though GPx activity increased significantly in this period. Interestingly, GPx activity was inversely related to both the APACHE and the SOFA scores and to erythrocyte Se at the baseline. Counterintuitively, these associations were not found after one week of ICU stay, though decreases in the plasma Se appeared to reflect increases in severity, as assessed by the SOFA score.

It has been reported that most plasma micronutrients decrease as part of the SIRS response in critical patients. This may occur for several reasons (e.g., redistribution processes, increased catabolism, or urinary excretion), making it necessary to interpret the plasma micronutrient concentrations with caution [20,21]. Additionally, both capillary leakage and endothelial dysfunction caused by sepsis or ischemia–reperfusion injury leads to the additional loss of serum selenoproteins into the interstitium [4] or selenoprotein P (SePP) binding to the endothelium [22]. Regarding the Se levels, profoundly lowered plasma Se concentrations have been widely reported at ICU admission [7], but even significantly lower values have been found in patients with SIRS [23]. In our study, the plasma and erythrocyte Se levels (expressed per

volume) were lower in ICU patients in comparison with the healthy controls at the baseline—the prevalence of individuals with considerably lowered levels being 15%. This percentage increased by up to four times after 7 days in the ICU. In this line, previous studies have shown that patients who, upon admission, present values $< 0.70 \mu\text{mol/L}$, which is consistent with the values recorded in our study ($0.73 \pm 0.14 \mu\text{mol/L}$ upon admission and $0.54 \pm 0.13 \mu\text{mol/L}$ after 7 days in the ICU), have a 3.5-fold higher mortality rate and a three-fold higher organ failure rate [23].

After one week of ICU stay, the Se levels decreased significantly, elevating Se deficiency and affecting more than 50% of the patients. This decrease in erythrocyte Se levels was not significant when expressed per gram of hemoglobin, however. Although hemoglobin is used to adjust for differences in hematocrit [24], it must be noted that other factors could influence these data, such as the role of hemoglobin in exporting Se from erythrocytes [25] or the observed prevalence of anemia caused by Se deficiency—albeit in animal models [26]. The assessment of the real Se status and its evolution in critical patients presents many difficulties. The main drawback of plasma Se is the interpretation of the results in patients with SIRS [7,9]. Erythrocyte Se seems to be unaffected by SIRS, and it may be a more reliable indicator of the Se status in the critically ill [27]. Nevertheless, erythrocyte results may differ depending on how they are expressed. In this regard, when we examined the changes in plasma Se over time, they were related to the changes in erythrocyte Se, both expressed per volume and per gram of hemoglobin,

which corroborates the idea that the risk of Se deficiency in our patients increased during ICU stay.

In reference to Se support, the average amount was less than 40.0 µg of Se per day, which is clearly lower than the Dietary Recommended Values for the healthy adult European population (70.0 µg Se/day) [28] or the suggested Se doses in ICU patients [27]. Our results are consistent with those of other studies, where similar plasma Se levels were found in patients transferred from the ICU to wards [29] and where enteral nutrition did not normalize the plasma levels in the first week of ICU stay [6]. The critically ill patients in our study just received a standard formula with no special supplementation during their ICU stay, and the prevalence of patients with a risk of deficiency increased over time. All this suggests that Se support was insufficient even though the severity scores, and the other clinical parameters improved over time, probably due to the clinical stabilization of the patients. In this regard, Se supplementation remains controversial, mainly due to its narrow therapeutic window [30,31]. Some authors have observed that Se supplementation during 7 days of parenteral nutrition in patients with inflammation increased the Se plasma levels, though not enough to reach or approach the healthy reference values [32]. A recent meta-analysis that evaluated the clinical outcomes of Se supplementation in critically ill patients suggested that it could reduce the overall mortality [33], though the use of high doses could increase the days of ICU stay [34].

Erythrocyte Se has been found to be closely correlated to plasma concentrations and GPx activity in healthy populations [35]. Regarding GPx activity in our critically ill patients, at ICU admission, we observed a direct correlation to the erythrocyte Se levels and an inverse association

to the APACHE-II and SOFA scores. It has been previously reported that both decreased serum Se concentration and GPx activity are inversely correlated to clinical outcomes [27]. This fact is in concordance with the observed correlations between the severity scales and GPx activity at the baseline, and with the changes observed in plasma Se. The GPx activity levels increased over follow-up, and the significant correlation to the SOFA score disappeared. This fact could be caused, on the one hand, by the decrease in sample size due to the high patients' mortality rate during follow-up and, on the other hand, by the decrease in SOFA scores by the hemodynamic stabilization of critical patients, in addition to the underlying processes related to Se metabolism in this deficient situation. It is known that selenomethionine may be incorporated nonspecifically into proteins such as hemoglobin by randomly replacing the (sulfur) methionine [36]. However, knowledge about the redistribution between Se storage and blood is largely lacking, and a decrease in the Se levels does not imply that the selenoenzymes in every compartment are desaturated [19].

On the other hand, in our study, SOD activity and TAC were directly correlated at follow-up and were also associated with low albumin levels. Although we did not clearly observe a significant decrease ($p = 0.061$) in these antioxidant parameters during patient follow-up, other studies have reported decreased antioxidants levels accompanied by lower albumin levels after clinical recovery from severe sepsis [37]. It should be noted that we observed higher TAC in critical patients at the baseline in comparison to healthy controls, maybe due to the initial hypercatabolic state at patients' arrival in the ICU and their acute-phase

response. Nevertheless, plasma TAC must be interpreted with caution due to the heterogeneity of results (i.e., increased plasma TAC has been correlated with worse outcomes after aneurysmal hemorrhage) [38]. In summary, the results of the present study suggest a substantial risk of Se deficiency, with an apparent tendency to further worsen during longer ICU stays, irrespective of the antioxidant and severity parameters, which could affect the overall clinical outcomes in critically ill patients. Furthermore, although Se delivery was ensured in nutritional formulas, it appears insufficient to restore normal values in this population.

Some limitations must be considered in the interpretation of our findings. Follow-up during the 7-days stay in the ICU did not allow us to establish causal relationships, and this study enrolled fewer patients than desired due to the difficulty of obtaining the sample and the inherent variable clinical situation and severity of the patients. On the other hand, there is no information on the other biomarkers of the Se status, such as the levels of the selenoproteins. Therefore, these results should be interpreted with caution, and further studies are needed to assess the impact of long-term Se interventions upon antioxidant activity and the evolution of severity in order to reinforce our findings.

4. Materials and Methods

4.1. Subjects and Study Design

The present study involved a prospective multicenter analytical design and was carried out of patients from different hospitals in Granada (Spain) (Hospital Virgen de las Nieves, Hospital San Cecilio, Hospital General de Baza, and Hospital Santa Ana de Motril) during the period

from March of 2015 to June of 2019. The patients were monitored from admission (baseline) until day 7 of stay (follow-up) in the ICU. Of a total of 65 initially recruited critically ill patients (42% women) by convenience sampling aged 31–77 years (mean age 60 years), 25 (33% women) died during the study period. All eligible participants enrolled in the study were critical patients: (i) aged 18 years or older (ii) with Systemic Inflammatory Response Syndrome (SIRS) following SIRS criteria [3] and (iii) who agreed to participate in the study or in which approval of participation was obtained from the family. Exclusion criteria were: (i) refusal to participate in the study as expressed by the patient or his/her legal representatives, (ii) pregnancy, (iii) the presence of highly contagious disease, (iv) allergies, (v) cancer, and (vi) the ingestion of food before obtaining the analytical sample at the baseline. In order to have reference values for the studied parameters and assess the critical condition, control samples were obtained from 56 healthy controls (55% women) from the same region and matched for age, using non-probabilistic consecutive sampling. The subjects in the control group were: (i) adults over 18 years of age (ii) with no use of nutritional supplements, (iii) no disease conditions that could affect their nutritional status, and (iv) individuals agreeing to participate in the study. The present study was conducted in accordance with the principles of the Declaration of Helsinki following the International Conference on Harmonization (ICH)/Good Clinical Practice (GCP) standards [39] and was approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016).

4.2. Data Collection

The study data, including age, sex, body mass index (BMI), total proteins, albumin, prealbumin, triglycerides, total cholesterol, hemoglobin, and CRP, were retrieved from the hospital electronic database system and recorded for each study participant at ICU admission (baseline) and after 7 days (follow-up). Biochemical parameters were determined in the Laboratory Analysis Unit of Virgen de las Nieves Hospital (Granada) (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The APACHE-II score and SOFA score were obtained by intensivists in the ICU.

4.3. Nutritional Profile

Se intake in the control group was documented through personal interview by staff trained in the use of nutritional techniques, employing questionnaires based on the 24-h recall test [40]. Nutrient intake was then calculated with the Dietowin® application (version 7.1., Barcelona, Spain). The nutritional support protocol (which includes Se support among other micronutrients) in critically ill patients was assessed according to the Clinical Nutrition Units of the hospitals, based on the American Society for Parenteral and Enteral Nutrition and the European Society of Parenteral and Enteral Nutrition guidelines [41]. All patients received standard nutritional support via the enteral, parenteral, or combined routes, administering nutritional formulas elaborated in the pharmacies of the hospitals or in the form of commercial products. A daily nutritional log was kept for each patient (i.e., type, volume, composition of intake, and tolerance) from admission to 7 days in the ICU. Se support was calculated daily and registered by the nutritionists

and was represented as the average of the 7-days period of stay in the ICU.

4.4. Blood Sampling and Biochemical Parameters

Two blood extractions of approximately 10 mL were performed (baseline and followup) in the morning under fasting conditions. The recorded biochemical parameters were determined using routine hospital analytical assays (ECLIA, Elecsys 2010, and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). Plasma and cells were separated using 4 mL of blood through centrifugation (4 °C for 15 min at 3000 rpm), and erythrocytes were washed four times with the physiological saline solution. Samples were stored at –80 °C until analytical determination of the different parameters.

4.5. Measurement of Se, GPx, and TAC

The determination of both the plasma and erythrocyte Se levels was carried out by inductively coupled plasma mass spectrometry (ICP-MS NexION 300D, Perkin Elmer, Waltham MA, USA) using aqueous solutions from wet-mineralized samples diluted with Triton X-100 and nitric acid. In order to evaluate the prevalence of individuals with a high risk of Se deficiency, the lower range values of plasma and erythrocyte Se (45.6 µg/L and 69.0 µg/L, respectively) of 84 healthy subjects from the same region [42] were taken as the cut-off values.

Commercial kinetic colorimetric methods were used to assess the antioxidant parameters. GPx activity was measured in erythrocyte samples using enzymatic immunological methods for GPx with the Bioxytech GPx-340™ kit (OxisResearch™) and SOD activity was

measured using the Randox Ransod kit (Bioxytech® SOD-525™RANDOX Laboratories Ltd., Antrim, UK). The TAC was assessed in the plasma samples (TAC kit, Jaica, Shizuoka, Japan) [43]. The variability was tested repeatedly with 5 samples and considering variability lower than 5% to be included. The reference values for each antioxidant parameter were determined by the manufacturer, and all parameters were analyzed twice. Se, GPx, and SOD in erythrocytes were all calculated as a ratio to hemoglobin concentration in order to correct for hemodilution processes or inaccuracies associated with the pipetting of packed red blood cells [9].

4.6. Statistical Analysis

Qualitative variables were presented as the frequencies (N) and percentages (%). Quantitative variables were expressed as the arithmetic mean $\pm$ standard deviation (SD). For continuous variables, the assumption of normality was tested using the Kolmogorov–Smirnov test as a step prior to the application of a parametric or nonparametric model. For the comparative analyses at the baseline and follow-up, they were made of the paired Student t-test for parametric samples. For the comparative intergroups analysis, the unpaired Student t-test for parametric samples was applied. Correlation analyses and partial correlation coefficients were performed using Pearson's test to study the relationship between the clinical outcomes and antioxidant enzymes. Likewise, simple linear regression models were used to study the associations between the mean differences of the main outcomes. The SPSS version 26.0 statistical package (IBM SPSS, Armonk, NY, USA) was used throughout. Statistical significance was considered for $p < 0.05$.

5. Conclusions

The critically ill patients in our study showed substantially lowered Se levels at ICU admission, and these levels decreased even further after one week of stay in intensive care—a direct correlation observed between the erythrocyte and plasma Se levels, both at the baseline and follow-up. Similarly, GPx activity at the baseline was lower than that observed in healthy controls, being inversely related to the severity scores. In this line, there was an inverse association between the plasma Se levels and the SOFA score, but no relationship between GPx and severity was evidenced after 7 days of ICU stay. Additionally, the standard Se nutritional support in the ICU seemed to be insufficient considering the Dietary Recommended Values and outcomes. Therefore, an adequate increase in the Se supply from the beginning of the ICU stay is advised, aimed at improving both the Se status and the clinical profiles, which could contribute to improving the outcomes and facilitate critical patient recovery.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data available on request due to restrictions on privacy. The data presented in this study are available on request from the corresponding authors [E.P. and J.M.-L.].

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CAPÍTULO II: PACIENTE CRÍTICO CON SIRS Y COVID-19

Estudio III: Bad prognosis in critical ill patients with COVID-19 during short-term ICU Stay regarding vitamin D levels.

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Abstract: Background and aims: Vitamin D inadequacy may be involved in the mechanisms of SARS-CoV-2 infection and in potential risk factors for disease propagation or control of coronavirus disease 2019 (COVID-19). This study assessed a short-term evolution of vitamin D status and its influence upon different clinical parameters in critically ill patients with COVID-19. Methods: A prospective analytical study in which 37 critically ill volunteers between 41 and 71 years of age with COVID-19 were evaluated at baseline and three days of intensive care unit (ICU) stay. 25-OH-D₃ and 25-OH-D₂ were analyzed by liquid chromatography–tandem mass spectrometry and total 25-OH-D levels were calculated as the sum of both. Results: All patients presented low 25-OH-D levels at baseline, decreasing total 25-OH-D ($p = 0.011$) mainly through 25-OH-D₂ ($p = 0.006$) levels during ICU stay. 25-OH-D₂ levels decreased a mean of $41.6\% \pm 89.6\%$ versus $7.0\% \pm 23.4\%$ for the 25-OH-D₃ form during the ICU stay. Patients who did not need invasive mechanical ventilation presented higher levels of 25-OH-D₂ at baseline and follow-up. Lower 25-OH-D and 25-OH-D₃ levels were associated with higher D-dimer at baseline ($p = 0.003$; $p = 0.001$) and at follow up ($p = 0.029$), higher procalcitonin levels ($p = 0.002$; $p = 0.018$) at follow up, and lower percentage lymphocyte counts ($p = 0.044$; $p = 0.040$) during ICU stay. Conclusions: Deficient vitamin D status in critical patients was established at the admission and further worsened after three days of stay. Lower vitamin D levels were related to key altered clinical and biochemical parameters on patients with SARS-CoV-2 infection. Given the different response of the 25-OH-D₃ and 25-OH-D₂ forms, it would be useful to monitor them on the evolution of the critically ill patient.

Keywords: coronavirus disease 2019; SARS-CoV-2; Vitamin D; critical care; intensive care patient

1. Introduction

The global public health crisis caused by the SARS-CoV-2 virus has created the need for urgent actions in order to reduce the risk of infection, progression, and the severity of coronavirus disease 2019 (COVID-19) [1], which triggers an acute inflammatory process and uncontrolled oxidative stress [2]. This in turn results in severe acute respiratory distress syndrome (ARDS) characterized by a cytokine storm, mainly in critical cases [3], which may lead to multiple organ damage [4] and further complicate the patient's critical condition previously described during their ICU stay [5,6].

There is currently great concern regarding the clinical management and intensive care of patients with critical stages of the disease and who are at a higher risk of death [7]. The implementation of prompt and appropriate nutritional assessment in COVID-19 must be considered, [8,9] because possible modulation of the status of key micronutrients appears to be a relevant factor influencing the development of this disease [10]. No information about nutritional monitoring in critical patients with COVID-19 is available to date [11], and this lack of data precludes the definition of firm micronutrient recommendations in this particular risk population [12].

Certain micronutrients are essential for adequate immunocompetence and antioxidant defense, which are related to inflammatory response, such as vitamin D [13]. 25-Hydroxyvitamin D (25-OH-D) is the metabolite used to assess vitamin D status, due to its long half-life in plasma or serum (one month) [14], and is characterized by synergic action of its two main forms: 25-hydroxyvitamin D₂ (25-OH-D₂), which is obtained from plant sources, and 25-hydroxyvitamin D₃ (25-OH-D₃),

which comes from animal products and endogenous synthesis in skin through exposure to sunlight [15], both of them can be supplemented with commercial products [16]. Recently, vitamin D has generated particular interest because of its role in reducing the risk of pneumonia and viral upper respiratory tract infections at a physical barrier and cellular natural and adaptive immunity level [17,18]. The underlying mechanisms can be grouped into two main actions: anti-inflammatory and anti-infective [19]. Vitamin D is associated with a decrease in proinflammatory cytokines, reducing the cytokine storm induced by the innate immune system, which is exacerbated in COVID-19 [17,20]. Moreover, it must be noted that serum 25-OH-D is considered as a negative acute phase reactant [21] and low vitamin D status in critical ill patients may be related to a decrease of binding protein concentration [22]. On the other hand, vitamin D is able to reduce viral infection and replication rates by inducing transcription of proteins with antimicrobial functions, enhancing autophagic encapsulation of viral particles, favoring lung epithelial cell barrier integrity, and ultimately regulating both innate and adaptive immunity [17–20,23,24].

Thus, vitamin D inadequacy has emerged as a factor that may be involved in the mechanisms of virus infection and in potential risk factors for disease propagation or control [24,25]. In fact, low vitamin D status in patients with COVID-19 has been reported [26–28], being associated with a poorer prognosis, infection risk, or unregulated inflammation. Thus, due to the lack of evidence on the importance of monitoring vitamin D status in critical patients with COVID-19, the present study was designed to assess the short-term evolution of the

status of vitamin D and its influence upon different clinical parameters in critically ill patients with COVID-19 in the province of Granada, Spain.

2. Materials and Methods

2.1. Study Design and Subjects

A prospective analytical study was carried out of patients monitored from the first day of admission to the intensive care unit (ICU) (baseline) until day three of stay (follow-up). Of a total of 43 initially recruited patients, 37 participants from the province of Granada (Spain), aged 41–74 years, were included in the period from 1 April to 1 June 2020, after been informed about the study protocol. Six patients died during the study and were excluded. All eligible participants enrolled in the study were critical patients aged 18 years or older and hospitalized for more than 48 h, who agreed to participate in the study or for whom approval of participation was obtained from the family. All patients had a diagnosis of critical active SARS-CoV-2 infection according to the Chinese Clinical Guideline for the classification of COVID-19 [29] (analyzed by real-time reverse transcriptase–PCR (RT-PCR)) testing of nasal and pharyngeal swab samples) and had an ICU stay of at least three days and did not receive vitamin D support. The present study was conducted in accordance with the principles of the Declaration of Helsinki, following the International Conference on Harmonization/Good Clinical Practice standards, and was approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016).

2.2. Data Collection

Data including age, sex, body mass index (BMI), smoking habits, comorbidities, respiratory and clinical parameters, ICU length of stay, length of hospitalization, and 28-day mortality were retrieved from the hospital electronic database system and recorded for each study participant at ICU admission (baseline) and after three days (follow-up). The Acute Physiology and Chronic Health Evaluation II (APACHE-II) score and Sequential Organ Failure Assessment (SOFA) score were obtained by intensivists at baseline and follow-up.

Patient clinical outcomes were recorded both at admission and during the ICU stay: heart rate (beats per minute); respiratory rate (breaths per minute); mean blood pressure (mmHg); positive end-expiratory pressure (PEEP); fraction of inspired oxygen (FiO_2); partial oxygen arterial pressure/fraction of inspired oxygen ($\text{PaO}_2/\text{FiO}_2$); ARDS; invasive mechanical ventilation (IMV).

2.3. Blood Sampling and Biochemical Parameters

Two measurements were performed (baseline and follow-up). Blood sampling was carried out in the morning under fasting conditions, followed by centrifugation (4 °C for 15 min at 3500 rpm) to separate the plasma. The samples were frozen at –80 °C until analysis of the different parameters. All samples were measured in one run, in the same assay batch, and blinded quality control samples were included in the same assay batches to determine laboratory error in the measurements.

The recorded biochemical parameters were total proteins, albumin, prealbumin, ferritin, transferrin, glucose, total cholesterol, glutamic oxaloacetic transaminase or aspartate transaminase (GOT or AST), glutamic pyruvic transaminase or alanine transaminase (GPT or ALT), lactate dehydrogenase (LDH), C-reactive protein (CRP), procalcitonin (PCT), hemoglobin, leukocytes, neutrophils, lymphocytes, platelets, D-dimer, fibrinogen, calcium (Ca), phosphorous (P), and magnesium (Mg), using routine hospital analytical assays (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).

2.4. Analytical Determination of Vitamin D

Vitamin D was measured in plasma samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Plasma sample treatment involved protein precipitation adding 500 μ L of acetonitrile in an Eppendorf flask with 200 μ L of plasma and 20 μ L of 25-hydroxyvitamin D₃ and 25-hydroxyvitamin D₂ deuterated solutions as Internal Standard (IS) (Sigma Aldrich, St. Louis, MO, USA) (0.5 μ g/mL). The samples were slightly shaken for 1 min on a plate shaker and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected in another Eppendorf flask and dried with N₂. The dry residue was vortexed for 30 s after the addition of 200 μ L of ethyl acetate and 100 μ L of deionized water and centrifuged at 3000 rpm for 5 min. The supernatant was again collected in another Eppendorf flask, and the previous steps were repeated with the remaining liquid phase, subsequently pooling the second supernatant with the first. The total supernatant was dried with N₂.

For samples derivatization, we prepared a solution of 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD) (Sigma Aldrich, St. Louis, MO, USA) in

acetonitrile (0.5 mg/mL), using 50 μ L of this solution in standards and in each sample, with vortexing. All samples were placed on the plate shaker for 1 h at room temperature and covered with aluminum foil. Lastly, the samples were transferred to vials, diluted with 50 μ L of deionized water and stored in freezer at -20°C covered with aluminum foil until injection into the chromatograph. For the calibration line, increasing concentrations of 1, 2, 5, 10, 25, 50, and 100 ppb of the standards 25-OH-D₃ and 25-OH-D₂ (Sigma Aldrich, St. Louis, MO, USA) with 20 μ L of IS were used and dried with N₂ and derivatized at the same time as the samples. For sample measurements, use was made of a Waters Acquity UHPLC I-Class System chromatograph (Waters, London, UK), with the Acquity UHPLC BEH C18 column 2.1, 50 mm, 1.7 m at room temperature. The mobile phase of channel A was water with 50 mM of ammonium formate, while that of channel B was methanol. The injection volume of the sample was 10 μ L and the flow rate was 0.4 mL/min. The detector was a Waters XEVO-TQ-XS Triple Quadrupole Low Resolution Spectrometer. Total 25-OH-D was calculated as the sum of the 25-OH-D₃ and 25-OH-D₂ forms. According to the Endocrine Society Practice Guidelines on Vitamin D, the threshold for biochemical 25-OH-D sufficiency values was considered to be >30 ng/mL, with insufficiency being defined as 20–29 ng/mL and deficiency as <20 ng/mL [28].

2.5. Statistical Analysis

Qualitative variables were presented as frequencies and percentages. Quantitative variables with normal distribution were expressed as the arithmetic mean and standard deviation (SD), and variables with non-

normal distribution were expressed as the median and the interquartile range. Normal data distribution for continuous variables was tested using the Shapiro–Wilk test. The Wilcoxon signed-rank test for nonparametric samples was used for the comparative analyses at baseline and follow-up. The unpaired Student t-test for parametric samples was used for the comparative analysis based on clinical outcomes. The effect size (ES) was estimated and interpreted as follows: small = 0.01, moderate = 0.06, and large = 0.14 [30]. Correlation analyses and partial correlation coefficients were performed using the Spearman test. Statistical significance was considered for $p < 0.05$. The SPSS version 22.0 statistical package (IBM SPSS, Armonk, NY, USA) was used throughout.

3. Results

The demographic and clinical characteristics of the 37 patients enrolled in the study are shown in *Table 9*. The median age of the study population was 60 years, and the gender distribution of the sample was 26 males and 11 females. With regard to the anthropometric parameters, over a third of the patients were overweight, and more than half of them were obese. Most of the patients had one or more underlying diseases. With regard to the severity parameters, the mean APACHE-II and SOFA scores were 12.3 and 6.54, respectively, upon admission. A total of 26 of 37 patients (70.2%) had at least 1 infection, and 5 of them had 3 or more infections during their admission in ICU. The most frequent infection was bacteremia in 25 out of 48 total infections (52%), followed by respiratory infections 15 out of 48 total infections (31%), and finally urinary tract infections associated with urethral catheterization in 7 out of 48 total

infections (14.5%). The most frequent germs causing these infections were Gram-positive (39%), followed by Gram-negative bacteria (33%) and fungi (20%). The mean length of hospitalization was 39.5 days. More than two-thirds of the patients presented ARDS on admission, requiring invasive mechanical ventilation with a mean duration of over 20 days. The mortality rate after 28 days of ICU stay was over two-thirds of the total study population.

Table 9. Demographic and clinical characteristics of the patients.

Baseline characteristics (N = 37)	Mean $\pm$ SD	Min-Max	95% CI
Age (years)	60.0 $\pm$ 10.2	41.0 - 74.0	56.6 - 63.4
Sex (M/F, %)	26/11 (70.3/29.7)	-	-
BMI (kg/m ²)	30.77 $\pm$ 4.17	22.8 - 42.2	29.4 - 32.2
BMI < 25 kg/m ² (n/N, %)	3/37 (8.10)	-	-
BMI 25 - 30 kg/m ² (n/N, %)	14/37 (37.8)	-	-
BMI > 30 kg/m ² (n/N, %)	20/37 (54.1)	-	-
Smoking habit (n/N, %)			
Smokers	3/37 (8.10)	-	-
Ex-smokers	12/37 (32.4)	-	-
Never smokers	22/37 (59.5)	-	-
Patients with comorbidity (n/N, %)	26/37 (70.3)	-	-
Diabetes	13/37 (35.1)	-	-
Hypertension	20/37 (54.1)	-	-
Dyslipemia	11/37 (29.7)	-	-
Chronic kidney disease	2/37 (5.40)	-	-
COPD	10/37 (27.0)	-	-
Cardiovascular disease	6/37 (16.2)	-	-
APACHE-II score	12.3 $\pm$ 3.77	6.00 - 21.0	11.1 - 13.6
SOFA score	6.54 $\pm$ 2.60	2.00 - 13.0	5.67 - 7.41
Bacterial and fungal infection (n/N, %)	26/37 (70.3)	-	-
Sepsis (n/N, %)	7/36 (19.4)	-	-
PaO ₂ /FiO ₂	212.9 $\pm$ 103.8	15.0 - 550.0	169.2 - 248.6
ARDS (PaO ₂ /FiO ₂ < 300) (n/N, %)	26/37 (70.0)	-	-
Mild (300 < PaO ₂ /FiO ₂ $\leq$ 200) (n/N, %)	12/37 (32.4)	-	-
Moderate (200 < PaO ₂ /FiO ₂ $\leq$ 100) (n/N, %)	10/37 (27.0)	-	-
Severe (PaO ₂ /FiO ₂ < 100) (n/N, %)	4/37 (10.8)	-	-
IMV (n/N, %)	30/37 (81.1)	-	-
Duration of IMV (days)	21.7 $\pm$ 14.6	1.00 - 73.0	16.0 - 27.4
ICU length of stay (days)	25.4 $\pm$ 22.6	6.00 - 104.0	17.8 - 32.9
Length of hospitalization (days)	39.5 $\pm$ 27.0	9.00 - 131.0	30.5 - 48.5
Patient 28-day mortality (n/N, %)	26/37 (70.3)	-	-

N = 37. Data expressed as mean standard deviation. Abbreviations: SD = standard deviation; Min–Max = minimum–maximum; CI = confidence interval; M/F = male/female; BMI = body mass index; COPD = chronic obstructive pulmonary disease; APACHEII = Acute Physiology and Chronic Health Evaluation II; SOFA = Sequential Organ Failure Assessment; PaO₂/FiO₂ = partial oxygen arterial pressure/fraction of inspired oxygen; ARDS = acute respiratory distress syndrome; IMV = invasive mechanical ventilation; ICU = intensive care unit.

The clinical and biochemical parameters of the study population at baseline and follow-up are shown in *Table 10*. Respiratory parameters were altered with significant changes in FiO₂ and PEEP after three days. Total proteins ($p = 0.012$), albumin ($p = 0.035$), prealbumin ($p = 0.017$), LDH ($p = 0.002$), CRP ($p = 0.001$), hemoglobin ($p = 0.001$), and fibrinogen ($p = 0.001$) were outside the reference values and decreased significantly after three days of ICU stay. Parameters such as ferritin, transaminases, or D-dimer were also outside the reference values although no changes were observed in their evolution during the ICU stay. The results showed the 25-OH-D, 25-OH-D₃, and 25-OH-D₂ levels to be lower at follow-up versus baseline—with statistical significance being reached for 25-OH-D ($p = 0.011$) and 25-OH-D₂ ($p = 0.006$).

Table 10. Clinical and biochemical parameters of critical patients with COVID-19 at baseline and follow-up.

	Reference	Baseline Median (IQR) N = 37	Follow-up Median (IQR) N = 37	Z	p- Value initial- final	ES
Clinical						
Heart rate (bpm)	60 - 100	79.4 ± 17.6	69.2 ± 19.9	-1.69	0.091	0.411
Respiratory rate (brpm)	15 - 20	28.2 ± 4.42	24.7 ± 10.1	-1.63	0.102	0.582
Mean blood pressure (mmHg)	70 - 105	91.0 ± 11.7	91.8 ± 13.3	-0.31	0.753	0.095
PEEP (cm H ₂ O)	2 - 5	14.0 ± 1.83	12.8 ± 2.21	-2.76	0.006	0.779
FiO ₂	> 68 %	0.72 ± 0.15	0.59 ± 0.16	-3.81	0.001	0.825
PaO ₂ /FiO ₂	200 - 300	213.0 ± 103.8	214.6 ± 76.9	-0.05	0.964	0.010
Biochemical						
Total Proteins (g/dL)	6.60 - 8.30	6.36 ± 0.65	6.09 ± 0.78	-2.51	0.012	0.513

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Albumin (g/dL)	3.50 - 5.20	3.19 ± 0.43	2.99 ± 0.40	-2.11	0.035	0.444
Prealbumin (mg/dL)	16.0 - 42.0	15.5 ± 13.3	28.7 ± 16.2	-2.39	0.017	0.782
Ferritin (ng/mL)	20.0 - 275.0	1,550.0 ± 1,316.0	1,447.0 ± 1,146.0	-0.52	0.603	0.117
Transferrin (mg/dL)	200.0 - 360.0	137.2 ± 29.8	150.8 ± 53.6	-0.82	0.410	0.269
Glucose (mg/dL)	75.0 - 115.0	164.4 ± 54.0	190.2 ± 71.0	-1.62	0.106	0.328
Total Cholesterol (mg/dL)	140.0 - 200.0	137.1 ± 48.6	164.1 ± 63.4	-2.02	0.044	0.574
GOT or AST (U/L)	5.00 - 40.0	50.2 ± 36.8	42.8 ± 32.1	-1.76	0.078	0.351
GPT or ALT (U/L)	0.00 - 55.0	42.8 ± 27.3	50.8 ± 35.3	-1.21	0.228	0.248
LDH (U/L)	0.00 - 248.0	513.5 ± 163.6	446.4 ± 136.7	-3.05	0.002	0.590
CRP (mg/L)	0.00 - 5.00	161.3 ± 105.2	48.9 ± 47.9	-4.66	0.001	0.991
PCT (ng/dL)	0.02 - 0.50	0.32 ± 0.28	0.30 ± 0.46	-1.59	0.112	0.360
Haemoglobin (g/dL)	11.0 - 17.0	12.7 ± 2.09	11.7 ± 2.50	-4.06	0.001	0.789
Leukocytes (*10 ³ /μL)	3.50 - 10.5	11.7 ± 6.70	10.8 ± 5.46	-1.25	0.212	0.240
Neutrophils (%)	42.0 - 77.0	85.9 ± 12.6	86.7 ± 5.45	-0.34	0.737	0.067
Lymphocytes (%)	20.0 - 44.0	7.14 ± 4.33	6.82 ± 3.88	-0.28	0.777	0.056
Platelets (*10 ³ /μL)	120.0 - 450.0	241.3 ± 122.0	275.2 ± 102.0	-2.52	0.012	0.482
D-dimer (ng/dL)	0.00 - 500.0	2,579.0 ± 4,782.0	2,191.0 ± 2,300.0	-1.14	0.254	0.231
Fibrinogen (mg/dL)	200.0 - 350.0	728.7 ± 274.6	571.4 ± 277.2	-3.52	0.001	0.683
Ca (mg/dL)	8.80 - 10.6	8.48 ± 0.61	8.34 ± 0.59	-0.81	0.421	0.190
P (mg/dL)	2.30 - 4.50	3.35 ± 1.25	3.32 ± 1.51	-0.02	0.984	0.005
Mg (mg/dL)	1.60 - 2.60	2.13 ± 0.32	2.23 ± 0.38	-0.80	0.421	0.253
25-OH-D (ng/mL)	20.0 - 100.0	14.4 ± 4.97	12.7 ± 3.59	-2.53	0.011	0.600
25-OH-D ₃ (ng/mL)	-	8.26 ± 4.12	7.78 ± 3.49	-1.35	0.176	0.520
25-OH-D ₂ (ng/mL)	-	6.18 ± 2.18	4.92 ± 1.67	-2.74	0.006	0.278

N = 37. Data expressed as median (interquartile range, IQR). Abbreviations: SD = standard deviation; ES = effect size; bpm = beats per minute; brpm = breaths per minute; PEEP = positive end-expiratory pressure; PaO₂/FiO₂ = partial oxygen arterial pressure/fraction of inspired oxygen; GOT or AST = glutamic oxaloacetic transaminase or aspartate transaminase; GPT or ALT = glutamic pyruvic transaminase or alanine transaminase; LDH = lactate dehydrogenase; CRP = C-reactive protein; PCT = procalcitonin; Ca = calcium, P = phosphorous, Mg = magnesium, *10³ = multiplied by 1000. The sixth column reports statistical significance after the Wilcoxon signed-rank test; evolution is shown after 3 days. ES effect size calculations were also made to determine the effect of ICU stay (ES: small ≤ 0.01, moderate = 0.06, and large ≤ 0.14) [28]. Statistical significance = *p* < 0.05.

Figure 7 shows the distribution of patient vitamin D status upon ICU admission and after three days of stay. In no case were the 25-OH-D levels > 25 ng/mL. Only 16.7% (6/37) of the patients had 25-OH-D > 20 ng/mL at baseline, versus 3.2% (1/37) at follow-up. Furthermore, 22.2% (8/37) presented 25-OH-D < 10 ng/mL—this percentage reaching 25.8% (10/37) after three days of stay.

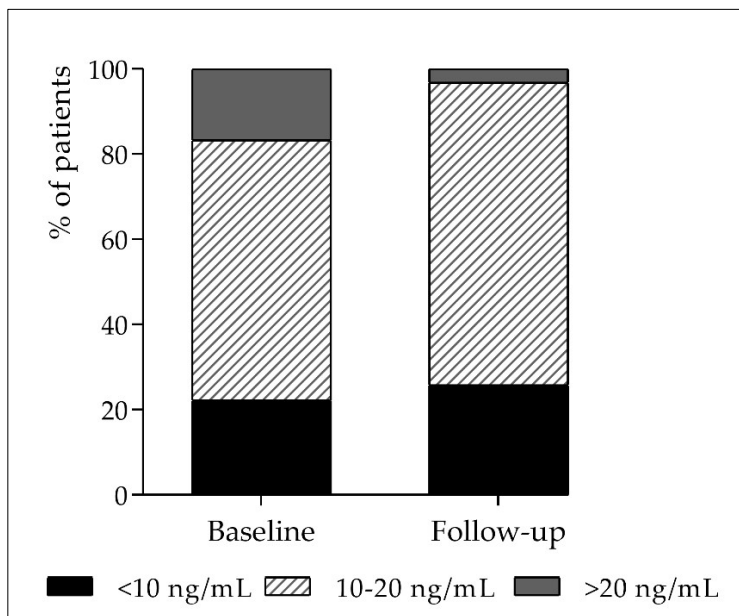


Fig. 7. Vitamin D status in the study population at ICU admission (baseline) and after three days (follow-up).

Figure 8 corresponds to the comparative analysis of clinical parameters in relation to 25-OH-D, 25-OH-D₃, and 25-OH-D₂ levels upon ICU admission and after three days of stay. Based on the sepsis (Figure 8A) and infectious processes (Figure 8B), there was a trend toward statistical significance in the follow-up observing lower levels of 25-OH-D and 25-OH-D₃ for septic patients and lower levels of 25-OH-D₂ for infected

patients. Patients who did not need invasive mechanical ventilation presented higher levels of 25-OH-D₂ at baseline and 25-OH-D and 25-OH-D₂ at follow-up (Figure 8C).

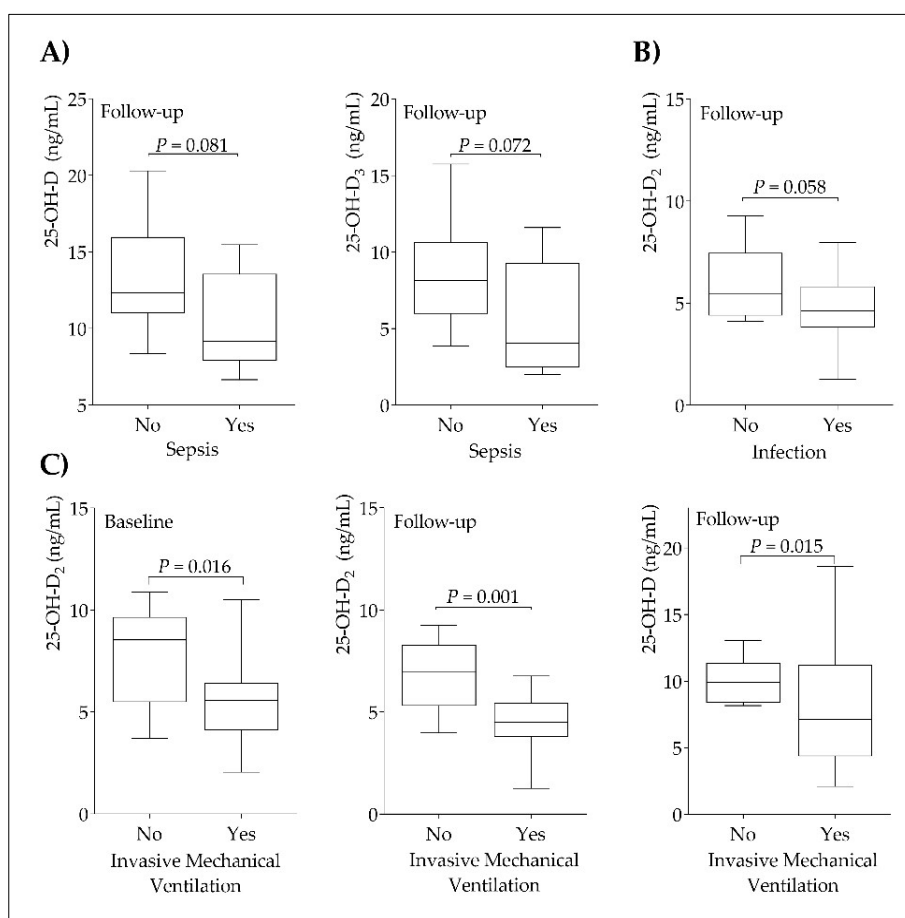


Fig. 8. Comparative analysis of 25-OH-D, 25-OH-D₃ and 25-OH-D₂ levels with sepsis, infection, and invasive mechanical ventilation. Comparative analysis of septic and non-septic patients with 25-OH-D and 25-OH-D₃ levels at follow-up (A); Infection with 25-OH-D₂ levels at follow-up (B); Invasive Mechanical Ventilation with 25-OH-D₂ levels at baseline, and 25-OH-D₂ and 25-OH-D levels at follow-up (C). Statistical significance = $p < 0.05$.

Table 11 shows the Spearman bivariate correlations between the 25-OH-D, 25-OHD₃, and 25-OH-D₂ levels at baseline and follow-up and the clinical and biochemical parameters analyzed in our study. At baseline, the 25-OH-D levels were correlated to albumin ($p = 0.021$), hemoglobin ($p = 0.028$), D-dimer ($p = 0.003$), and fibrinogen ($p = 0.020$)—with albumin also being correlated to 25-OH-D₂ ($p = 0.037$); and D-dimer ($p = 0.001$) and fibrinogen to 25-OH-D₃ ($p = 0.029$). Respiratory rate was negatively correlated to 25-OH-D₂ ($p = 0.025$). At follow-up, 25-OH-D and 25-OH-D₃ were significantly correlated to PCT ($p = 0.002$; $p = 0.018$) and lymphocytes ($p = 0.044$; $p = 0.040$). In the case of D-dimer and Ca, an inverse correlation to 25-OH-D₃ was observed ($p = 0.029$ and $p = 0.006$, respectively). Finally, the 25-OH-D₂ levels showed a significant correlation to both the fibrinogen ($p = 0.003$) and Ca levels ($p = 0.030$). We did not find correlation between mortality rate after 28 days of ICU stay and 25-OH-D₃, 25-OH-D₂, and 25-OH-D levels.

Table 11. Matrix for correlation coefficients (rho) showing the simple linear relationship between clinical and biochemical parameters with 25-OH-D, 25-OH-D₂, and 25-OH-D₃ levels.

	Baseline			Follow-up		
	25-OH-D (ng/mL)	25-OH-D ₃ (ng/mL)	25-OH-D ₂ (ng/mL)	25-OH-D (ng/mL)	25-OH-D ₃ (ng/mL)	25-OH-D ₂ (ng/mL)
Age (years)	-.129	-.132	-.035	-.089	-.132	.065
BMI (kg/m ²)	.111	.165	-.026	.091	.261	-.231
APACHE-II	-.103	-.066	-.040	-	-	-
SOFA	-.154	-.053	-.167	-.133	.008	-.290
Respiratory rate (brpm)	-.176	.053	-.456 ^a	-.277	.047	-.374
Albumin (g/dL)	.403 ^a	.285	.390 ^a	.015	-.097	.234
PCT (ng/dL)	-.270	-.294	-.026	-.587 ^b	-.458 ^a	-.331
Haemoglobin (g/dL)	.387 ^a	.307	.261	.301	.223	.224
Lymphocytes (%)	-.046	-.059	.084	.364 ^a	.371 ^a	.034
D-dimer (ng/dL)	-.521 ^b	-.644 ^b	.148	-.264	-.405 ^a	.302
Fibrinogen (mg/dL)	.350 ^a	.370 ^a	.128	.116	.335	-.521 ^b
Ca (mg/dL)	.285	.180	.306	-.333	-.527 ^b	.426 ^a

Matrix correlations are presented as correlation coefficients (rho). Abbreviations: BMI = body mass index; APACHE-II = acute physiology and chronic health evaluation II; SOFA = sequential organ failure assessment; FiO₂ = fraction of inspired oxygen; PCT = procalcitonin; Ca = calcium. Statistical significance a = $p < 0.05$; b = $p < 0.01$.

4. Discussion

The main finding of the present study was the low 25-OH-D levels in the patients upon admission (baseline), followed by a significant decrease after three days of ICU stay. The entire population was below the sufficiency reference values for 25-OH-D, and most of them presented insufficient 25-OH-D status. We also analyzed the 25-OH-D₃ and 25-OH-D₂ levels, both of which were seen to decrease after three days (though statistical significance was only reached in the case of 25-OH-D₂), thus influencing upon 25-OH-D decreased levels and presenting a worsening during their stay at 3 days. Moreover, vitamin D was associated with clinical parameters such as the need for mechanical ventilation or respiratory frequency and with biochemical parameters also associated with the severity of the critically ill patient such as albumin, hemoglobin, D-dimer, fibrinogen, PCT, and lymphocytes.

Previous evidence points to poorer COVID-19 outcomes associated with factors such as the male gender, older age, BMI > 35 kg/m², and the presence of certain comorbidities [31]. The demographic and clinical characteristics of our patients (Table 9) are consistent with this evidence. In effect, the population was fundamentally elderly, two-thirds were males, and there was a high prevalence of comorbidities and obesity. It should be noted that metabolically ill patients with obesity may have a high risk of suffering inflammatory processes [32], which could contribute to a greater probability of poorer outcomes.

Many of the clinical and biochemical parameters in our patients were altered (Table 10). In the case of PEEP and FiO₂, levels were above reference values and, they even decreased significantly in three days and

remained altered in the most cases. It should be noted that ferritin, CRP, D-dimer, and fibrinogen were well above the reference values. Parameters related to inflammation (such as CRP) or coagulation (such as D-dimer) have been correlated to a poor prognosis and have been described as possible predictive biomarkers of COVID-19 [33].

Our results with reference to vitamin D status showed all patients to have insufficient levels (<30 ng/mL) both upon admission and during the study period – with the vitamin D status being seen to worsen in only three days. The great majority of patients presented vitamin D deficiency (<20 ng/mL), while extreme deficient values of <10 ng/mL were recorded in a quarter of the study sample. It is known that, compared to the general population, the prevalence of hypovitaminosis D is greater in the critically ill and may constitute a risk factor for adverse outcomes [34]. Moreover, these levels could be influenced by seasonality. In an observational study carried out in critical ill patients from Austria, significant differences were noted in the prevalence of vitamin D deficiency and in the mean 25-OH-D values between the winter and summer months [35]. Our study covered the period from March to June; we, therefore, could not demonstrate the influence of seasonality in our patients.

On the other hand, there is concern about the high prevalence of hypovitaminosis D in the general population—being regarded as a global health issue with important consequences [36]. Moreover, SARS-CoV-2 positivity has been strongly and inversely correlated to circulating 25-OH-D levels – a relationship that persists across latitudes, races/ethnicities, both genders, and age ranges [37], thereby evidencing that the COVID-19 fatality rates parallel the vitamin D deficiency rates

[38]. These negative correlations between vitamin D deficiency and the number of COVID-19 cases and mortality have also been reported in another 20 European countries [39]. It could be expected that countries such as Spain have a better vitamin D status and therefore less severe consequences than other countries in northern Europe. However, our results reinforced the evidence of a possible widespread vitamin D deficiency in the Spanish population. Indeed, vitamin D deficiency in Italy and Spain (the countries presenting the highest age-specific case fatality ratio) [40] is more severe than elsewhere in Europe [41], particularly in the aging population [39].

On the other hand, Maghbooli et al. found 25-OH-D levels >30 ng/mL to reduce the risk of adverse clinical outcomes in patients with COVID-19 [42]. None of our critical patients with COVID-19 presented 25-OH-D levels >25 ng/mL, which is consistent with the findings of Maghbooli. Low 25-OH-D levels have been reported by many authors in hospitalized patients with COVID-19 [43], with such deficiency being associated with a greater mortality risk [26]. In fact, Vassiliou et al. found that low 25-OH-D levels in patients with COVID-19 at ICU admission could predispose to an increased 28-day mortality risk [44]. In the present study, although we did not observe an association between 25-OH-D levels with 28-day mortality risk, we found an association with clinical outcomes, reporting that higher 25-OH-D and 25-OH-D₂ levels were associated with those patients who did not require invasive mechanical ventilation. Moreover, lower 25-OH-D values, as a result of decreased 25-OH-D₃ and 25-OH-D₂ levels were observed with the presence of infection (bacterial/fungal) and sepsis (Figure 8). It should be noted that

25-OH-D₂ levels were in agreement with previous studies in the Spanish population [45], being also similar to those found in other studies performed in Belgian and Chinese populations [46,47]. Likewise, observational studies have also shown that higher 25-OH-D levels would be associated with better clinical outcomes in respiratory diseases [48]. Nevertheless, reference values are needed to have more contrastable evidence on 25-OH-D₂ levels.

In relation to infection, a high percentage of our patients (70.3%) showed bacteriological or fungal infection, which would support the idea that the daily risk rate of infection in COVID-critically-ill patients is increased during ICU stay [49]. Recent studies [50], consistent with the high prevalence of hypovitaminosis D observed in our study, suggest a possible role of low vitamin D in the increased risk of SARS-CoV-2 infection and subsequent hospitalization. Likewise, 25-OH-D levels were inversely associated with coagulation and sepsis, in addition to major comorbidities. Both, 25-OH-D₃ and 25-OH-D₂ forms tended to respond differently in patients with bacteriological or fungal infection and in patients presenting sepsis. On the other hand, it was observed that 25-OH-D₂ levels decreased a mean of $41.6\% \pm 89.6\%$ versus $7.0\% \pm 23.4\%$ for the 25-OH-D₃ form during the ICU stay, which would suggest that the lack of vitamin D support during ICU stay could have allowed this more pronounced decrease in case of the 25-OH-D₂ form, since it would depend on the intake through diet (before the admission) or its supplementation (during ICU stay). A greater decrease in 25-OH-D₂ relative to 25-OH-D₃ could be also related to the lower affinity of 25-OH-D₂ for vitamin D-binding protein, leading to a shorter half-life and a higher rate of clearance from the circulation [51], and in some cases, it even caused a decline, thereby, precipitating in vitamin D deficiency

[52]. This, together with the fact that there is literature that already reports a possible different role of 25-OH-D₃ and 25-OH-D₂ forms [51,53], although not in critical patients, could evidence the different correlations obtained with bacterial/fungal infection or sepsis and invasive mechanical ventilation previously described. Therefore, it would suggest that a lower vitamin D status at admission and worsening during three days of ICU stay may be a modifiable risk factor and an early predictive marker of adverse outcomes in hospitalized patients with COVID-19.

On associating the concentrations of both 25-OH-D and 25-OH-D₃ with other biochemical severity parameters, significantly lower vitamin D levels were correlated to higher D-dimer and PCT levels and a lower percentage of lymphocytes (Table 11). A recent metaanalysis has demonstrated that patients with severe COVID-19 tend to present increased leukocyte and neutrophil counts, neutrophil–lymphocyte ratio, PCT and CRP levels, and a decreased number of total lymphocytes, among parameters, compared to nonsevere individuals [54]. Furthermore, blood hypercoagulability is common among hospitalized patients with COVID-19. Elevated D-dimer levels are consistently reported in this scenario, and a gradual increase of this parameter in the course of the disease is particularly associated with patient worsening. Similarly, lower fibrinogen levels were found in nonsurvivor patients with COVID-19 [55]. In this line, the relationship between fibrinogen and 25-OH-D levels is often reported in the literature in noncritical patients [56,57]. In our study, 25-OH-D and 25-OH-D₃ levels were inversely correlated with D-dimer levels at baseline. Furthermore, 25-

OH-D₃ levels have been correlated with fibrinogen levels at baseline. Our results may reflect a better vitamin D status (mainly due to vitamin D₃) in patients with a more appropriate hematological profile. We observed a positive correlation at baseline between albumin (which was below the reference values) and the levels of both 25-OH-D and 25-OH-D₂. Recently, low albumin levels have been regarded as more of a disease severity marker than as a marker of malnutrition, when such low levels are detected upon admission to hospital [58].

The present study has limitations and strengths. As limitations, the present study enrolled fewer patients than desired due to the difficulty in obtaining the sample and the patient's own clinical situation and severity both at admission and during the ICU stay. Therefore, the data should be treated with caution in order to generalize the findings of the study. Thus, on a comparative level, the effect size was shown for a better understanding. We had no reliable data on exposure to sunlight, dietary factors, or vitamin D supplementation—all of which affect vitamin D status. The overall negative results may be related to the heterogeneity of the subjects and their underlying disease conditions or severity, which may all influence the plasma 25-OH-D levels. Our findings cannot be generalized to other populations or ethnic groups, especially considering the wide range of COVID-19 prevalence. Replicating this study in a larger, prospective, and heterogeneous population and taking into account a control group, would allow for other stratified analyses based on demographic and biochemical characteristics, taking seasonality into account, and could further corroborate our findings. Additional research is therefore needed to validate our findings. As strengths, the present study used LC-MS/MS, which is the gold standard for assessing the levels of 25-OH-D [59], affording greater sensitivity, flexibility, and

specificity than the enzymoimmunoassay techniques commonly used in clinical practice, which tend to overestimate the 25-OH-D values in cases of deficiency [60,61].

Recent studies have reported encouraging results after vitamin D intervention [62,63]. However, further evidence is needed to confirm that improving vitamin D status is of benefit in reducing disease severity and mortality and the probability of developing a critical clinical condition. It is essential to ensure close patient monitoring before establishing intervention guidelines [64]. This study is one of the few that have been conducted in this context, assessing the short-term evolution of the 25-OH-D levels (through 25-OH-D₂ and 25-OH-D₃ levels) and its impact in critical patients with COVID-19.

5. Conclusions

Our data reflect a high prevalence of hypovitaminosis D in all the critical patients at ICU admission, which increased after only three days of ICU stay. On the other hand, the associations observed between 25-OH-D levels, through 25-OH-D₃ and 25-OH-D₂ values and key clinical outcomes and biochemical altered parameters, suggests that it might be helpful to assess vitamin D status in patients with SARS-CoV-2 infection. Given the different response of the 25-OH-D₃ and 25-OH-D₂ forms, it would be useful to analyze them to elucidate the role of each form on the evolution of the critically ill patient. Further investigations are needed to define underlying mechanisms in vitamin D deficiency and useful strategies based on vitamin D interventions aimed at preserving

vitamin D status and enhancing the clinical and biochemical profile of critical patients with COVID-19.

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Data Availability Statement: The datasets generated and analyzed during the current study are not publicly available because the database is very extensive and includes data from other studies complementary to this but are available from the corresponding author on reasonable request.

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Estudio IV: Changes in trace elements and metallothioneins levels and their relationship with clinical, biochemical, and inflammatory parameters in patients with COVID-19 during the early ICU phase.

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(Submitted article)

Abstract: Circulating trace elements (Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn),) and metallothioneins (MTs) could play a key role in modulating the inflammatory reaction and pathogenesis of COVID-19. Thus, the present study aimed to investigate their relationship with clinical, biochemical, and inflammatory parameters in critical care patients with COVID-19 at early intensive care unit (ICU) phase. 86 patients were monitored from the first day of ICU admission until the third day of stay. Mineral levels were assessed in serum samples by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and MTs levels by differential pulse voltammetric. Levels of Cu and MTs decreased (all $p \leq 0.046$) after 3 days, increasing the prevalence of Cu deficient values from 50% to 65.3% ($P=0.015$). Fe and Zn were shown to have a predictive value for mortality and severity. Changes in Fe were directly related to changes in Cu and Mn (all $r \geq 0.266$; $p \leq 0.019$). In contrast, changes in MTs were inversely related to changes in Mn and albumin (all $r \geq -0.255$; $p \leq 0.039$). The present study indicated an apparent risk of trace element deficiency, which were related to different biochemical and clinical parameters, suggesting a possible beneficial effect of restoring proper levels of these micronutrients.

Keywords: COVID-19; Iron; Zinc; Copper; Manganese; Metallothionein; Critical care

1. Introduction

Coronavirus Disease-19 (COVID-19) is a severe acute respiratory syndrome caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which rapidly spread throughout the world and has been declared a pandemic, with a need for hospitalization and intensive therapy in severe cases [1]. Apart from the well-known respiratory symptoms (e.g., respiratory distress, low oxygen saturation, or respiratory failure) – all of them considered to be the main symptoms of COVID-19 –, the appearance of coagulopathy or cytokine storm processes are also clinical signs and symptoms involved in the systemic inflammatory process that occurs during COVID-19 [2,3]. Despite the improvement in disease management, most of the therapies have shown no perceivable impact – no universal cure for COVID-19 disease is available to date – and the rapidly mutating virus has emerged as a matter of great concern – the effort on combating the disease should not be limited to finding drugs and vaccines – [4,5]. In this regard, a proper nutritional status, which has an important role in optimal well-being and immune function, may be an interesting alternative. Thus, some current therapies in clinical settings consist of immunomodulatory agents – including micronutrients to fight against oxidative stress and modulate inflammation – [6].

Trace elements (i.e., Iron (Fe), Zinc (Zn), Copper (Cu), and Manganese (Mn)) are micronutrients mostly responsible for modulating the inflammatory reaction and cytokine production in patients with COVID-19, being their deficiency responsible for the increased risk of morbidity and mortality among these patients [7,8]. It must be noted that excess in circulating mineral levels may also be associated with different

complications: (I) Fe overload, as a consequence of the decomposition of the hemoglobin by SARS-CoV-2, causes oxidative damage, inflammation, and immune dysfunction, which can lead to rapid multi-organ failures [9]; (II) excessive free Zn ions may be toxic by binding at the active site of enzymes or via allosteric inhibition of enzymes [10], and high concentrations of Zn inhibit T cell functions [11]; (III) free Cu may catalyze the formation of highly reactive hydroxyl radicals [12]; and (IV) Mn at elevated levels is neurotoxic [13]. Thus, maintaining proper levels of these trace elements is crucial for a good state of health.

Importantly, metal ion concentrations in the body are controlled by the affinity for their ligands [14]. Metallothioneins (MTs) are a family of ubiquitous small proteins with a high cysteine content, which confer them an optimal capacity for metal ion coordination via detoxification, storage, and delivery, participating in a wide range of stress responses, tumorigenesis, neurodegeneration, and inflammatory processes [15]. MTs bind various divalent trace metal ions, protecting cells and tissues against heavy metal toxicity and playing an essential role in intracellular aspects, especially for Zn and Cu [16]. Additionally, it has been shown that circulating MTs can be increased by different oxidative processes in multiple organ systems, although their production often decreases to protect against oxidative tissue injury [17]. Moreover, overexpression and upregulation of MTs have been associated with carcinogenic processes [16].

To date, the relationship between circulating levels of minerals and MTs and their subsequent effects on clinical and inflammatory parameters have not been investigated, at least in the context of COVID-19 disease.

Therefore, the present study aimed to investigate the association between circulating Fe, Zn, Cu, Mn, and MTs concentrations and their relationship with clinical, biochemical, and inflammatory parameters in critical care patients with COVID-19 at ICU admission and after 3 days of ICU stay. We hypothesized that trace elements could be directly related to MTs levels – their deficiencies worsening during the ICU stay –.

2. Materials and Methods

2.1. Subjects and study design

A prospective observational and analytical study was performed on 86 critically ill patients (18 women) aged 36–96 years with COVID-19 monitored from the first day of admission to the Intensive Care Unit (ICU) (baseline) until the third day of stay (follow-up) at the Hospital Virgen de las Nieves in the province of Granada (Spain). The participants were recruited from 1st April to 1st December 2020 after being informed about the study protocol, which all the patients or their families signed. The diagnosis of COVID-19 was based on a positive Real-Time Reverse Transcriptase–PCR (RT-PCR) test and subsequent RNA sequencing specific for the SARS-CoV-2 testing of nasal and pharyngeal swab samples. The participants were considered critically ill when they presented a respiratory failure requiring mechanical ventilation, needed vasopressor treatment (shock), or had other complications with organ failure requiring monitoring or treatment in the ICU. Inclusion criteria were: (I) 18 years of age or older, (II) to have been previously hospitalized for at least more than 48 hours, (III) to have been admitted to the ICU and to stay for at least 3 days, and (IV) to present a positive

PCR test for SARS-CoV-2 according to the Chinese Clinical Guideline for the classification of COVID-19 [18]. Exclusion criteria were: (I) to be under 18 years, (II) to be pregnant, and (III) to not present a positive PCR test even though they presented symptoms compatible with COVID-19. The present study was conducted in accordance with the principles of the Declaration of Helsinki (last revised guidelines, 2013) [19], following the International Conference on Harmonization/Good Clinical Practice standards, and it was approved by the Ethics Committee of the University of Granada (Ref. 149/CEIH/2016).

2.2. Data collection

Data including positive PCR results against SARS-CoV-2, age, sex, exitus, ICU length of stay, and respiratory and clinical parameters were retrieved from the Hospital electronic database system and recorded for each participant on the first and third days of ICU admission, respectively. The respiratory and clinical parameters collected by the intensivists were: Sequential Organ Failure Assessment (SOFA) scale score [20], Acute Physiology and Chronic Health Evaluation II (APACHE II) scale score [21], days on Mechanical Ventilation (MV), Mean Arterial Pressure (MAP), Heart Rate (HR), Breath Rate (BR), Fraction of Inspired Oxygen (FiO_2); Partial Oxygen arterial pressure/ FiO_2 ($\text{PaO}_2/\text{FiO}_2$), and Positive End-Expiratory Pressure (PEEP).

2.3. Blood sampling and biochemical parameters analysis

Blood samples were collected in the morning under fasting conditions, followed by centrifugation (4 °C for 15 minutes at 3500 rpm) to separate

plasma and serum. The samples were frozen at $-80.0\text{ }^{\circ}\text{C}$ until analysis of the different parameters. All measurements were obtained in triplicate, and blind quality control samples were included in the same assay batches to determine laboratory error in the measurements.

2.3.1. Blood sampling and biochemical parameters analysis

The biochemical and inflammatory variables (i.e., albumin, ferritin, transferrin, Transferrin Saturation Index (TSI), fibrinogen, D-dimer, C-Reactive Protein (CRP), Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT), Gamma-Glutamyl Transferase (GGT), Lactate Dehydrogenase (LDH), Hemoglobin (Hb), leukocytes, percentage lymphocytes and neutrophils, platelets, Troponin T (TNT), Activated Partial Thromboplastin Time (APTT), and International Normalized Ratio (INR)), were analyzed in the Virgen de las Nieves Hospital – which provided the reference values – using “Alinity,” the Abbott Core Laboratory® autoanalyzer for biochemistry and immunochemistry, following enzymatic colorimetry and immunoassay procedures.

The mineral variables (i.e., Fe, Zn, Cu, and Mn) were analyzed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Perkin Elmer® SCIEX Elan-5000 equipment, Markham, Ontario, Canada) following a previous wet-mineralized way in the Scientific Instrumentation Center from the University of Granada – which provided the references values – as previously described [22].

2.3.2. Sample processing and differential pulse voltammetry Brdicka reaction for determination of MTs

A total of 10.0 μl of serum samples were mixed with 990.0 μl of 0.1 M phosphate buffer ($\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$, pH 7.0) and subsequently incubated at 99.0 $^\circ\text{C}$ in a thermomixer (Eppendorf 5430, Hamburg, Germany) for 20 min with shaking to remove ballast proteins and peptides, which could influence the electrochemical response. Heat treatment effectively denatures and removes non-thermostable high molecular weight proteins from samples [23]. The denatured homogenates were centrifuged at 4 $^\circ\text{C}$, 25 000 $\times g$ for 20 min (Eppendorf 5402, Hamburg, Germany), and the obtained supernatant was used for MTs analysis.

Differential pulse voltammetry measurements for the determination of MTs in Brdicka reaction according to the modified Brdicka procedure [16] were performed in the processed samples with a potentiostat/galvanostat AUTOLAB PGSTAT204 (Metrohm, Utrecht, Netherlands) connected to a VA-Stand 663 (Metrohm, Utrecht, Netherlands), using a standard cell and 3 electrodes: (I) hanging mercury drop electrode with a drop area of 0.4 mm^2 was used as the working electrode, (II) $\text{Ag}/\text{AgCl}/3\text{M KCl}$ electrode was the reference, and (III) platinum electrode was the auxiliary electrode. A total of 15 mL of Brdicka supporting electrolyte containing 1 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ and 1 M ammonia buffer ($\text{NH}_3(\text{aq}) + \text{NH}_4\text{Cl}$, pH 9.6) was used without surface-active agent additives and mixed with 5.0 μL of processed sample. Brdicka reaction of MTs follows the principle of the adsorptive stripping technique and is based on the strong adsorption of the target molecule on the surface of the working electrode at an open electrode circuit. After each measurement, the hanging mercury drop electrode and Brdicka

supporting electrolyte are renewed. The measurement parameters were as follows: initial potential of -0.7 V, end potential of -1.8 V, modulation time of 0.05 s, time interval of 0.57 s, step potential of -3.9 mV, and modulation amplitude of 25.0 mV. All experiments were performed at 5 °C using the thermostat Julabo F25 (Labortechnik, Wasserburg, Germany). MTs were quantified from the calibration straight line using a commercially available ≥ 95 % pure solution isolated from rabbit liver (Enzo Life Sciences, Inc., New York, USA). The obtained calibration curve for MTs was as follows $y = 2.017x - 0.023$; $R^2 = 0.996$. NOVA 2.1.4 Metrohm Autolab B.V. (EcoChemie, Utrecht, Netherlands) was used for data processing.

2.4. Statistical analysis

Data normal distribution was examined using the Kolmogorov-Smirnov test prior to further statistical evaluation. Quantitative parameters were represented as the arithmetic mean (Standard Deviation (SD)). The paired Student's t-test for parametric samples was used for the comparative analyses of all the clinical, biochemical, inflammatory, mineral variables, and MTs of the study, and to compare the percentage of deficiency of all minerals between the 1st and 3rd days of ICU admission. The unpaired Student's t-test for parametric samples was used for the comparative inter-group analysis (i.e., changes in clinical, biochemical, and inflammatory parameters of the study by mean changes in mineral and MTs levels) after 3 days of ICU stay. The predictive value of Fe and Zn levels for mortality and severity at baseline and follow-up was evaluated using the Receiver Operating Curve (ROC). Correlation analyses and partial correlation coefficients between mean changes of all parameters of the study after 3 days of ICU stay were performed using

Pearson's correlation. The SPSS version 26.0 statistical package (IBM SPSS, Armonk, New York, USA) was used throughout the study. Statistical significance was considered for $p < 0.05$. GraphPad Prism 9 software (GraphPad Software, San Diego, California, USA) was used for plotting the graphs.

3. Results

The clinical parameters of the study participants are represented in *Table 12 (Supplementary table)*. BR, FiO₂, and PEEP decreased after 3 days of ICU stay (all $p \leq 0.038$), whereas the rest of the parameters showed no mean differences (all $p \geq 0.142$) on the 3rd day of ICU stay.

Table 12 (Supplementary Table) Clinical parameters of critically ill patients with COVID-19 on the 1st and 3rd days of ICU stay.

<i>n</i> =86	References values	1 st day (Mean (SD))	3 rd day (Mean (SD))	Mean differences	<i>p</i> -Value
Clinical					
Age (years)	—	62.2 (13.5)	—	—	—
Exitus	—	—	—	—	—
No/ <i>n</i>	—	59/86	—	—	—
Yes/ <i>n</i>	—	27/86	—	—	—
ICU stay (days)	—	20.5 (19.4)	—	—	—
APACHE II score	—	13.7 (7.7)	—	—	—
SOFA score	—	4.7 (2.5)	5.0 (3.1)	+0.3	0.190
MV (days)	—	15.0 (18.6)	15.0 (18.6)	—	—
MAP (mmHg)	70.0–105.0	95.5 (13.8)	93.2 (10.4)	–2.3	0.560
HR (bpm)	60.0–100.0	76.8 (21.0)	69.6 (18.6)	–7.2	0.142
BR (brpm)	15.0–20.0	26.8 (6.8)	22.1 (6.1)	–4.7	0.001
FiO ₂ (%)	>0.68	0.8 (0.2)	0.6 (0.2)	–0.2	0.001
PaO ₂ /FiO ₂	200.0–300.0	183.4 (111.7)	207.9 (84.5)	+24.5	0.272
PEEP (cm H ₂ O)	2.0–5.0	12.9 (2.3)	12.0 (2.1)	–0.9	0.038

n=86. Data expressed as mean (standard deviation) unless otherwise stated. Paired Student's *t*-test was used to compare the evolution of the variables between the 1st and 3rd days of ICU stay. Mean differences represent the difference between the value of the 3rd day and the value of the 1st day of ICU stay. Significant *p*-Values are represented in bold. Statistical significance = $p < 0.05$. Abbreviations: APACHE-II = Acute Physiology and Chronic Health Evaluation-II; BR = Breathing Rate; bpm = beats per minute; brpm = breaths per minute; HR = Heart Rate; MAP = Mean Arterial Pressure; MV = Mechanical Ventilation; PaO₂/FiO₂ = Partial Oxygen arterial pressure/Fraction

of Inspired Oxygen; PEEP = Positive End-Expiratory Pressure; SD = Standard Deviation; SOFA = Sequential Organ Failure Assessment.

Table 13 represents the biochemical, inflammatory, mineral, and metallothionein values of critically ill patients on the 1st and 3rd days of ICU stay. Fibrinogen, CRP, LDH, hemoglobin, hematocrit, leukocytes, percentage of neutrophils, Cu, and MTs decreased significantly (all $p \leq 0.046$). In contrast, transferrin, D-dimer, GPT, GGT, percentage of lymphocytes, and platelets increased (all $p \leq 0.036$) on the 3rd day of ICU stay. The rest of the parameters showed no mean differences after 3 days of ICU stay (all $p \geq 0.151$).

Figure 9 represents the percentage of patients with deficient status in the studied minerals on the 1st and 3rd days of ICU stay. The percentage of Cu deficiency increased significantly from 50% to 65.3% ($P=0.015$) on the 3rd day of ICU stay. The deficiency prevalence in the case of the remaining minerals showed no significant differences after 3 days of ICU stay (all $p \geq 0.05$).

Table 13. Biochemical, inflammatory, mineral, and metallothionein values of critically ill patients with COVID-19 on the 1st and 3rd days of admission to the ICU.

<i>n</i>=86		References values			<i>n</i>=86			<i>n</i>=86		
Biochemical and inflammatory parameters		parameters			1st day (Mean (SD))		3rd day (Mean (SD))		Mean differences	
Albumin (g/dL)	3.5–5.2				3.2 (0.5)		3.3 (3.0)		+0.1	0.923
Ferritin (ng/mL)	20.0–275.0				1,641.2 (1,130.6)		1,768.7 (1,287.6)		+127.5	0.213
Transferrin (mg/dL)	200.0–360.0				146.1 (25.5)		161.6 (41.5)		+15.5	0.036
TSI (%)	17.1–30.6				46.1 (34.5)		38.3 (26.9)		-7.9	0.183
Fibrinogen (mg/dL)	200.0–350.0				651.1 (211.1)		556.4 (189.9)		-94.6	0.001
D-dimer (ng/mL)	0.0–500.0				1,291.0 (1,246.9)		2,049.6 (1,842.5)		+751.3	0.001
CRP (mg/L)	0.0–5.0				120.6 (86.8)		75.5 (70.6)		-45.2	0.001
GOT (U/L)	5–40				42.2 (29.7)		37.5 (32.7)		-4.7	0.238
GPT (U/L)	0–55				48.5 (46.5)		62.1 (73.7)		+13.6	0.025
GGT (U/L)	1–55				99.8 (108.6)		142.3 (198.3)		+42.5	0.008
LDH (U/L)	0–248				519.6 (19.1)		463.1 (187.8)		-56.5	0.008
Hb (g/dL)	11.0–17.0				13.1 (2.0)		12.4 (2.1)		-0.7	0.001
Hematocrit (%)	30.0–50.0				38.4 (5.7)		36.7 (5.7)		-1.7	0.001
Leukocytes (x10 ³ /μL)	3.5–10.5				11.6 (6.2)		10.5 (5.4)		-1.1	0.046
Lymphocytes (%)	20.0–44.0				7.2 (4.4)		9.7 (8.1)		+2.5	0.005
Neutrophils (%)	42.0–77.0				88.4 (5.6)		82.3 (13.8)		-6.1	0.001
Platelets (x10 ³ /μL)	120.0–450.0				232.5 (89.6)		256.6 (104.5)		+24.1	0.006
TNT (ng/L)	<14.0				19.7 (41.5)		12.9 (28.6)		-6.8	0.151
APTT (s)	26.0–37.0				28.9 (3.9)		29.2 (4.2)		-0.3	0.655
INR	0.80–1.2				1.1 (0.3)		1.0 (0.2)		-0.1	0.367
Minerals										
Iron (mg/L)	0.6–1.70				1.7 (0.9)		1.5 (0.9)		-0.2	0.197
Zinc (mg/L)	0.7–1.10				0.9 (0.3)		0.8 (0.4)		-0.1	0.607
Copper (μg/L)	0.6–1.40				0.6 (0.4)		0.5 (0.3)		-0.1	0.005
Manganese (μg/L)	0.4–0.85				0.5 (0.1)		0.4 (0.1)		-0.1	0.704
Metallothioneins										
MTs (μmol/L)	–				0.3 (0.1)		0.2 (0.1)		-0.1	0.022

n=86. Data expressed as mean (standard deviation) unless otherwise stated. Paired Student's t-test was used to compare the evolution of the variables between 1st and 3rd days of the ICU stay. Mean differences represent the difference between the value of the 3rd and 1st days of ICU stay. Significant P-Values are represented in bold. Statistical significance = P<0.05. Abbreviations: APTT = Activated Partial Thromboplastin Time; CRP = C-Reactive Protein; Cu = Copper; Fe = Iron; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamic Pyruvic Transaminase; GGT = Gamma-Glutamyl Transferase; Hb = Hemoglobin; INR = International Normalized Ratio; LDH = Lactate Dehydrogenase; Mn = Manganese; MTs = Metallothioneins; SD = Standard Deviation; TNT = Troponin T; TSI = Transferrin Saturation Index; Zn = Zinc.

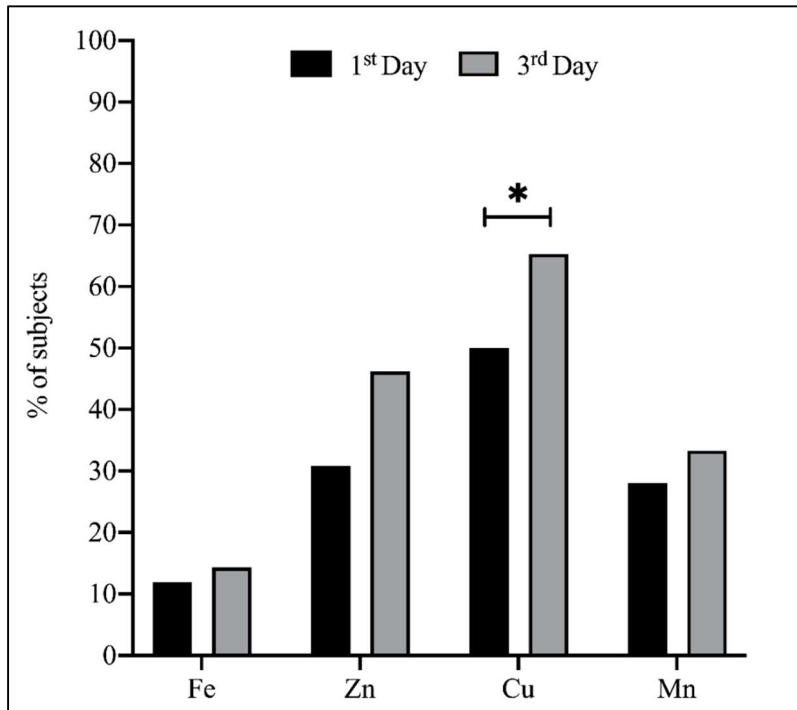


Fig. 9. Percentage of subjects with mineral deficient status on the 1st and 3rd days of ICU stay. Paired Student's t-test was used to compare the evolution of the variables. *Statistical significance = $p < 0.05$. Abbreviations: Cu = Copper; Fe = Iron; Mn = Manganese; Zn = Zinc.

Table 14 shows the differences in changes in the clinical, biochemical, and inflammatory parameters occurring during ICU stay based on the median changes in mineral and MTs levels. Higher changes in Fe levels were found in patients presenting lower changes in HR ($p = 0.005$). Moreover, higher changes in TSI, fibrinogen, CRP, and neutrophils (all $p \leq 0.031$) and lower changes in lymphocyte levels ($p = 0.023$) were found in those presenting higher changes in Cu levels. Furthermore, individuals with higher changes in Mn showed higher changes in albumin ($p = 0.044$) and lowered CRP ($p = 0.038$) levels. The rest of the variables showed no differences based on the median changes in the level of minerals and MTs (all $p \geq 0.073$)

Table 15 represents the relationship between changes in minerals and MTs with clinical, biochemical, and inflammatory parameters after 3 days of evolution in the ICU. Changes in Fe were directly related to changes in Cu and Mn (all $r \geq 0.266$; $p \leq 0.019$). Changes in Zn were positively linked to changes in PaO₂/FiO₂ and platelets (all $r \geq 0.494$; $p \leq 0.010$) and negatively associated with changes in BR and INR (all $r \geq -0.480$; $p \leq 0.026$). Changes in Cu were positively related to changes in fibrinogen and CRP (all $r \geq -0.276$; $p \leq 0.019$). Changes in Mn were directly associated with changes in fibrinogen ($r = 0.250$; $p = 0.039$) and negatively related to changes in GOT ($r = -0.221$; $p = 0.049$). Finally, changes in MTs were inversely associated with changes in Mn and albumin (all $r \geq -0.255$; $p \leq 0.039$).

Figure 10 shows the ROC curves of Fe and Zn as mortality and severity predictors. The areas under the curve were 0.655 (95% CI 0.533–0.778) for changes in Fe predicting mortality and 0.652 (95% CI 0.531–0.773) for Fe predicting SOFA at ICU admission. In the same way, the areas under the curve were 0.709 (95% CI 0.503–0.914) for Zn predicting APACHE on the 1st day of ICU stay and 0.768 (95% CI 0.586–0.951) for Zn predicting SOFA on the 3rd day of ICU stay.

Table 14. Changes in clinical, biochemical, and inflammatory parameters based on the median changes in minerals and MTs occurring during the ICU stay.

$n=86$ Δ Change	Δ Fe		Δ Zn		Δ Cu		Δ Mn		Δ MTs	
	Δ	P-Value	Δ	P-Value	Δ	P-Value	Δ	P-Value	Δ	P-Value
Clinical parameters										
SOFA	+0.05	0.917	+0.45	0.576	+0.06	0.908	+0.88	0.170	+0.08	0.895
MAP	+5.50	0.506	-2.40	0.893	+16.6	0.095	+12.8	0.182	+3.62	0.658
HR	-25.1	0.006	-6.15	0.592	+2.88	0.807	-11.5	0.295	+3.73	0.677
BR	-0.48	0.862	-1.43	0.329	+3.23	0.253	-2.09	0.521	+2.15	0.509
FiO ₂	+0.01	0.806	-0.03	0.456	-0.04	0.439	+0.05	0.480	-0.05	0.431
PaO ₂ /FiO ₂	+23.1	0.618	-0.12	0.415	+21.6	0.679	+43.1	0.499	+74.1	0.151
PEEP	+0.93	0.266	-1.23	0.146	-1.10	0.242	-0.87	0.396	+0.81	0.322
Biochemical and inflammatory parameters										
Albumin	+0.75	0.325	-2.25	0.363	-0.04	0.617	+1.95	0.044	-0.81	0.359
Ferritin	+177.7	0.392	-71.5	0.884	+15.5	0.948	-92.8	0.720	-117.4	0.613
Transferrin	-23.4	0.091	+26.4	0.214	-1.19	0.944	-5.11	0.764	+1.25	0.943
TSI	+12.1	0.301	-17.5	0.347	+25.5	0.031	+19.0	0.153	-5.02	0.726
Fibrinogen	+27.2	0.527	+58.4	0.427	+148.5	0.001	+54.1	0.319	+15.3	0.737
D-dimer	-1315.0	0.294	+1797.0	0.484	+504.6	0.723	-1526.2	0.340	+265.7	0.855
CRP	-13.8	0.438	+18.3	0.605	+44.0	0.017	-45.4	0.038	+6.62	0.734
GOT	-13.9	0.090	+9.71	0.402	-2.09	0.812	-15.8	0.115	-2.01	0.832
GPT	-22.1	0.073	+11.7	0.544	-19.9	0.121	+2.51	0.866	-8.50	0.522
GGT	+35.2	0.284	+25.4	0.471	+43.4	0.229	+87.1	0.211	+24.6	0.498
LDH	+31.6	0.464	-50.9	0.371	+78.0	0.104	+18.0	0.746	+68.2	0.151
Hb	-0.12	0.615	+0.28	0.524	+0.18	0.505	+0.06	0.843	+0.30	0.270
Haematocrit	-0.61	0.432	+0.90	0.527	+0.46	0.588	+0.34	0.714	+0.62	0.463

Leukocytes	+0.38	0.719	+2.63	0.113	+1.41	0.221	+0.20	0.876	+0.14	0.898
Lymphocytes	-0.85	0.639	-0.67	0.880	-4.57	0.023	+0.95	0.671	+1.74	0.364
Neutrophils	+1.63	0.605	-6.33	0.407	+6.51	0.030	-0.46	0.890	-3.48	0.243
Platelets	-2.33	0.897	+54.9	0.085	-6.65	0.716	+0.53	0.979	-16.1	0.416
TNT	+13.5	0.290	+9.25	0.208	-1.12	0.936	+24.2	0.161	-11.1	0.444
APTT	-1.74	0.098	+1.85	0.192	-0.20	0.849	-1.07	0.401	-0.62	0.585
INR	-0.12	0.075	-0.14	0.258	+0.02	0.811	+0.01	0.942	-0.01	0.841

$n=86$. Data expressed as mean (standard deviation) unless otherwise stated. Δ represents the difference between the value on the 3rd and the 1st days of the ICU stay. Unpaired Student's t-test was used to compare the changes in clinical, biochemical, and inflammatory parameters of the study based on the median changes in the levels of minerals and MTs . Significant P-Values are represented in bold. Statistical significance = $P<0.05$. Abbreviations: APTT = Activated Partial Thromboplastin Time; BR = Breathing Rate; CRP = C-Reactive Protein; Cu = Copper; Fe = Iron; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamic Pyruvic Transaminase; GGT = Gamma-Glutamyl Transferase; Hb = Hemoglobin; HR = Heart Rate; INR = International Normalized Ratio; LDH = Lactate Dehydrogenase; MAP = Mean Arterial Pressure; MTs = Metallothioneins; PaO_2/FiO_2 = Partial Oxygen arterial pressure/Fraction of Inspired Oxygen; PEEP = Positive End-Expiratory Pressure; SOFA = Sequential Organ Failure Assessment. TNT = Troponin T; TSI = Transferrin Saturation Index; Zn = Zinc.

Table 15. Matrix for correlation coefficients (r) showing the simple linear relationship between minerals and MTs with clinical, biochemical, inflammatory, minerals, and MTs parameters of the study.

n = 86 Δ Change	Δ Fe	Δ Zn	Δ Cu	Δ Mn	Δ MTs
Clinical parameters					
SOFA	-0.076	+0.026	-0.127	+0.209	+0.042
MAP	+0.087	-0.377	+0.226	+0.159	+0.300
HR	-0.258	-0.189	-0.079	+0.073	+0.146
BR	-0.229	-0.815*	+0.251	-0.366	-0.133
FiO ₂	0.058	-0.179	-0.220	+0.390	+0.142
PaO ₂ /FiO ₂	-0.166	+0.963*	+0.262	-0.653	+0.113
PEEP	-0.002	-0.295	-0.083	+0.288	-0.095
Biochemical and inflammatory parameters					
Albumin	+0.060	-0.059	-0.068	+0.043	- 0.328*
Ferritin	+0.054	+0.223	+0.069	+0.021	+0.030
Transferrin	-0.163	+0.359	-0.050	+0.029	+0.025
TSI	+0.066	-0.138	+0.404	+0.232	-0.230
Fibrinogen	+0.131	-0.148	+0.447**	+0.250*	+0.077
D-dimer	+0.015	+0.185	+0.081	-0.066	+0.033
CRP	-0.015	-0.264	+0.276*	-0.079	+0.053
GOT	+0.029	-0.114	-0.086	-0.221*	+0.030
GPT	+0.031	+0.115	-0.195	-0.164	-0.054
GGT	+0.114	+0.215	+0.063	+0.113	+0.001
LDH	+0.112	-0.210	+0.205	+0.145	+0.103
Hb	-0.035	+0.219	+0.160	-0.111	+0.083
Hematocrit	-0.064	+0.313	+0.122	-0.151	0.051
Leukocytes	+0.153	+0.070	+0.129	+0.078	-0.194
Lymphocytes	-0.139	-0.031	-0.204	+0.012	+0.060
Neutrophils	-0.003	-0.183	+0.173	+0.011	-0.076
Platelets	+0.117	+0.494*	+0.076	+0.068	-0.193
TNT	+0.001	+0.156	+0.019	+0.298	-0.022
APTT	-0.174	-0.065	+0.006	-0.117	-0.064
INR	-0.147	-0.480*	-0.199	-0.154	+0.040
Minerals					
Fe	-	-	-	-	+0.039
Zn	-0.262	-	-	-	-0.152
Cu	+0.282*	-0.330	-	-	-0.022
Mn	+0.266*	+0.197	+0.059	-	- 0.255*

$n=86$. Pearson's correlation was used to compare the simple linear relationship between changes in minerals and MTs and clinical, biochemical, inflammatory, minerals, and MTs values. Δ represents the difference between the value of the 3rd and the 1st days of the ICU stay. Significant p -Values are represented in bold. Matrix correlations are presented as correlation coefficients (r). Statistical significance = * $p<0.05$ and ** $p<0.001$. Abbreviations: APTT = Activated Partial Thromboplastin

Time; BR = Breathing Rate; CRP = C-Reactive Protein; Cu = Copper; Fe = Iron; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamic Pyruvic Transaminase; GGT = Gamma-Glutamyl Transferase; Hb = Hemoglobin; HR = Heart Rate; INR = International Normalized Ratio; LDH = Lactate Dehydrogenase; MAP = Mean Arterial Pressure; MTs = Metallothioneins; PaO₂/FiO₂ = Partial Oxygen arterial pressure/Fraction of Inspired Oxygen; PEEP = Positive End-Expiratory Pressure; SOFA = Sequential Organ Failure Assessment. TNT = Troponin T; TSI = Transferrin Saturation Index; Zn = Zinc.

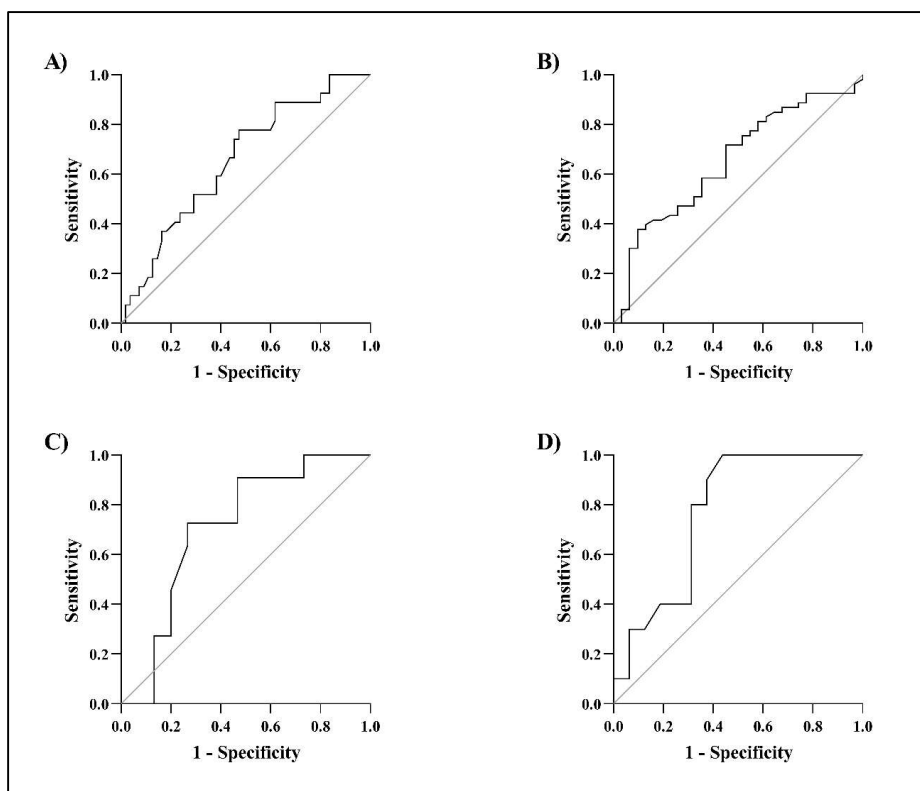


Fig. 10. ROC curves of Fe and Zn as mortality and severity predictors. A) ROC curve of changes in Fe predicting mortality. B) ROC curve of Fe predicting SOFA at ICU admission. C) ROC curve of Zn predicting APACHE at ICU admission. D) ROC curve of Zn predicting SOFA on the 3rd day of ICU stay.

4. Discussion

The main findings of the present study showed a relatively high prevalence of mineral deficiency (i.e., 11.9–50%) at ICU admission, increasing (i.e., 14.3–65.3%) after 3 days – in the case of Cu being significant–. Additionally, MTs levels decreased after 3 days of ICU stay. Changes in Fe levels were directly associated with changes in circulating Mn and Cu –these changes in Fe also being a prognostic biomarker of severity and mortality –. Changes in Zn levels were also predictors of severity. Contrary to our previous hypothesis, no relationship was found between changes in MTs and changes in both Zn and Cu levels, being MTs changes inversely associated with changes in Mn and albumin levels. The present study highlights the potential benefit of assessing the mineral status and MTs at early stages in critical patients with severe COVID-19 infections to characterize the disease, identify and reverse deficiency conditions, and improve the treatment and prognosis of these patients.

SARSCoV-2 infection is a heterogeneous illness because not all patients develop the same symptoms. However, some parameters have been reported as a common finding in cases with worse prognosis (i.e., thrombocytopenia or higher CRP, D-dimer, transferrin, and LDH levels, among others) [24]. In our study, patients showed altered values for most clinical and biochemical parameters at ICU admission, observing changes with an apparent trend towards normalized values (i.e., BR, $\text{PaO}_2/\text{FiO}_2$, transferrin, fibrinogen, leukocytes) after 3 days. However, levels of many variables remained altered, worsening during ICU stay (i.e., D-dimer, GGT, GPT), underscoring the complexity of the clinical situation of these patients. Over-exuberant inflammatory response and

dysregulated host immune system are common findings in severe cases of COVID-19 [25]. Among other effects, inflammatory processes lead to oxidative stress and altered levels of different trace elements in serum [26].

Regarding Fe levels, our results showed normal mean values for this mineral at baseline and follow-up, being the prevalence of deficiency around 10%. Moreover, Fe levels at baseline predicted severity, and changes in its levels during ICU stay predicted mortality. The relationship between Fe levels and susceptibility to infection remains complex because, on the one hand, Fe could reduce the vulnerability to respiratory infections via enhancing natural killer cell activity, T lymphocyte proliferation, or production of T helper 1 cytokines, but on the other, infections caused by organisms that spend part of their life-cycle intracellularly may be enhanced by Fe [27]. Indeed, it has been described that coronavirus can capture Fe and generate reactive oxygen species to damage the human immune system [28]. Additionally, it must be noted that, although Fe levels were within normal values, important disturbances were found in ferritin levels. It has been reported that changes in Fe metabolism can be used to predict death in patients admitted to ICU because serum ferritin has been identified as one of the predictors of death in COVID-19 patients [29,30]. Hyperferritinemia – a key acute-phase reactant – could represent a host defense mechanism depriving bacterial growth of iron and protecting immune cell function. However, there is a need for further investigation of the role of ferritin in uncontrolled inflammatory conditions [29].

Similarly, we found normal mean values of circulating Zn – more than one-third of the patients presenting deficient levels – at ICU admission and after 3 days of stay. Lower Zn levels during the early phase of ICU stay in patients with COVID-19 infection have been reported previously [31,32]. Hypozincemia in these patients is characteristic, and it is possible due to redistribution processes from blood to the liver at the expense of Zn in other tissues [33]. Furthermore, we found that changes in Zn levels were directly associated with changes in $\text{PaO}_2/\text{FiO}_2$ and platelets and inversely related to changes in BR and INR. Likewise, Zn levels presented predictive values for severity scales. These results, altogether, appear to suggest that higher levels of Zn are related to better clinical outcomes. In consistency with that, Maares et al. [34] found an association between serum Zn levels and increased risk of death, suggesting that Zn may serve as a prognostic marker for the severity and course of COVID-19. Additionally, Notz et al. [35] reported that circulating Zn – below the reference range in patients with severe COVID-19 – was restored after supplementation within two weeks of intensive care, supporting the possible beneficial effect of Zn supplementation.

Circulating Cu levels decreased during ICU stay – the mean levels being below normal values – reaching 65% the prevalence of deficiency at the first 3 days of ICU stay. Cu levels were directly related to fibrinogen and CRP levels, and inversely related to INR. Moreover, patients with higher changes in Cu levels presented higher variations in TSI and neutrophils and lower changes in lymphocyte levels. A decrease in lymphocyte levels has been previously linked to a higher mortality risk, and an increase in CRP is linked to severe prognosis in COVID-19 patients [24]. Our study shows that better clinical, biochemical, and inflammatory

parameters are associated with lower concentrations of circulating Cu during this acute phase of infection. Indeed, Cu levels have been previously associated with CRP, suggesting an association between serum Cu levels and COVID-19 severity [31]. Thus, a possible inflammatory and pro-oxidant role of Cu in COVID-19 pathogenesis has been hypothesized [36]. In contrast, Hackler et al. [37] found elevated serum Cu levels at hospital admission in the surviving patients compared with non-survivors and with the reference range – no patient showing Cu levels below the reference range –. These discrepancies could be due to differences in patients' severity and inflammatory conditions. Further research is needed to know the role of circulating Cu on outcomes in critically ill patients with COVID-19.

Concerning Mn levels, our study revealed that the prevalence of deficiency affected one-third of patients at follow-up, being Mn changes inversely related to changes in GOT and MTs levels and directly associated with changes in fibrinogen, Fe, and Cu. No differences in Mn levels were found by Zhou et al. [38] when they compared mild and severe groups of patients with COVID-19. On the other hand, Zeng et al. [39] found higher urinary excretion of Mn and other trace elements in severe patients than in non-severe cases. In general, Mn levels appear to follow the trend of other trace elements, and Mn could play a role in the pathogenesis of this disease. However, the evidence assessing Mn levels and their effects on COVID-19 is scarce and inconclusive to date.

Although MTs play a major role in the homeostasis of Zn and Cu and detoxification processes, their biological function is still debatable [40,41]. In cellular processes, MTs are known to have metallo-regulatory

functions (i.e., growth, differentiation, and protective role in oxidative stress) [16]. Our results showed a significant decrease in circulating MTs concentration during ICU stay, being these changes in MTs levels inversely correlated to changes in albumin levels. Metal-Cys clusters in MTs – together with disulfide moieties in serum albumins – are considered good interceptors of free radicals [42]. Therefore, circulating MTs could be determinant in antioxidant processes; their levels are influenced by oxidative stress and inflammation. Moreover, contrary to our previous hypothesis, no relationship was found between changes in MTs levels and Zn and Cu levels, being only inversely associated with changes in Mn levels. It has been observed that chronic low level exposure to Mn in rats significantly decreases hepatic MTs – the liver being the organ with the highest concentration of MTs – [43]. Furthermore, the effect of inflammation on circulating MTs and mineral levels remains unclear. In patients with chronic exacerbated pancreatitis, a significant increase in circulating MTs concentration was accompanied by a decrease in Zn and an increase in Cu levels [44]. Nevertheless, MTs levels were lower in patients with hepatitis C than in healthy controls. In contrast, in other chronic hepatitis cases, MTs levels increased gradually, followed by the progression of the disease to liver cirrhosis and hepatocellular carcinoma [45]. Further studies are needed to fully understand the relationships between MTs and mineral levels and their implications in clinical outcomes, especially during inflammatory conditions. Moreover, the last revised ESPEN guidelines [46] about early nutritional supply and micronutrient supplements should also be considered in these patients' management.

The findings reported in the present study should be treated with caution as some limitations arise. Firstly, follow-up during the 3-day stay in the

ICU did not allow us to establish causal relationships as no intervention was performed. Secondly, the recruited patients were from a single Hospital, and some potential confounding factors (sociodemographic and socioeconomic status) were not evaluated. Thus, these outcomes cannot be generalized to other populations, especially considering the wide range of COVID-19 prevalence. Finally, the overall negative results may be related to the heterogeneity of the subjects and their underlying disease conditions or severity. Replicating this study in a larger and heterogeneous critically ill population with a control group could further corroborate our findings.

5. Conclusions

In summary, the present study in critically ill patients with COVID-19 indicated an apparent risk of deficiency of trace element status at ICU admission, worsening after 3 days of stay. Cu and MTs levels decreased significantly during this period, whereas Fe and Zn levels were shown as mortality and severity predictors. Different biochemical and clinical parameters were related to the studied variables. Therefore, we suggest monitoring the mineral status and performing nutritional interventions, when appropriate, that could help to improve the altered parameters, such as inflammatory conditions and, thus, the prognosis in critically ill patients with COVID-19.

Informed Consent Statement: Informed consent and consent for publication was obtained from all subjects involved in the study.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Availability of Data and Materials: Data will be shared upon reasonable request by the corresponding authors: Héctor Vázquez-Lorente & Lourdes Herrera-Quintana.

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LIMITACIONES Y PERPESTIVAS FUTURAS

LIMITACIONES Y PERSPECTIVAS FUTURAS

El presente trabajo presenta una serie de limitaciones que cabe mencionar.

En primer lugar, el número de pacientes reclutados fue inferior al deseado debido, entre otros motivos, a la dificultad de obtener la muestra y a la variable situación clínica y gravedad de los pacientes, todo ello inherente a su condición patológica.

En segundo lugar, sería necesaria la realización de un seguimiento durante un periodo más prolongado que permitiese valorar la evolución de las distintas variables a largo plazo.

En tercer lugar, existe la posibilidad de que parte de los resultados negativos obtenidos sean debidos a la gravedad o condición patológica de los pacientes, o a la heterogeneidad existente entre ellos, pudiendo afectar a la mayoría de los parámetros analizados.

En cuarto lugar, los pacientes incluidos en el estudio proceden de la misma región, por lo que algunos potenciales factores de confusión, fundamentalmente de tipo sociodemográfico, no fueron evaluados.

Por último, también existen algunas limitaciones metodológicas en la determinación de algunos parámetros, tales como la valoración del estatus de vitamina D (no se dispuso de datos referentes a la exposición solar, la suplementación con vitamina D o los niveles de la forma 1,25-OH₂-D), la valoración del estatus de Se (falta de datos de otros

biomarcadores como las selenoproteínas), o el análisis de los niveles de Zn en otros compartimentos del organismo.

Dadas las limitaciones mencionadas con anterioridad, los datos aportados en la presente tesis doctoral deben ser tratados con precaución a la hora de generalizar los resultados. A continuación, se realiza una serie de propuestas a fin de complementar y mejorar el estudio realizado:

- Dada la gran heterogeneidad que presentan estos pacientes, sería conveniente hacer un estudio a gran escala, aumentando el tamaño muestral, incluyendo y diferenciando a cada paciente en grupos más homogéneos, y mejorando también la potencia estadística. Además, la inclusión de una población de otras regiones permitiría reducir la variabilidad existente por las diferencias sociodemográficas.
- Sería interesante disponer de datos nutricionales y hábitos dietéticos previos a la condición crítica, lo cual resulta realmente difícil. Se podría realizar una aproximación teniendo en cuenta los hábitos alimenticios del paciente y las características generales de la población según la región, a fin de tener una idea de las posibles carencias nutricionales y la probabilidad de las mismas.
- Sería conveniente mejorar y homologar los protocolos de actuación en el tratamiento y nutrición de los pacientes, sobre todo en aquellos casos en los que los datos vayan a ser utilizados con fines de investigación, y así poder realizar comparaciones entre estudios con menor riesgo de sesgo.

- También sería interesante homologar la metodología empleada en las determinaciones analíticas, a fin de tener datos más fácilmente comparables y de reducir la variabilidad intersujeto.
- La realización de estudios de intervención, mediante la suplementación de Zn y de otros micronutrientes, controlando el aporte y los niveles de todos ellos, así como de otros parámetros relacionados con su estatus, podría ser útil a la hora de comprender todos los fenómenos (y los mecanismos subyacentes) que acontecen durante la enfermedad crítica.
- Una mejor cuantificación de las pérdidas de Zn y de otros micronutrientes sería esencial para realizar una mejor estimación de los requerimientos. La determinación de la excreción urinaria puede ser útil en este sentido, no obstante, cuantificar pérdidas por otras causas resulta muy difícil.
- La búsqueda de biomarcadores del estatus de Zn es indispensable, y se debe seguir trabajando a fin de encontrar la forma más fiable y precisa de estimar el estatus real de Zn, y en base a esto, poder calcular los requerimientos nutricionales para cada sujeto durante las diferentes etapas de la vida, tanto en situación fisiológica como fisiopatológica.

CONCLUSIONES

CONCLUSIONES

Conclusión general

En general, los resultados de la presente tesis doctoral indican un elevado riesgo de deficiencia de Zn, así como de otros micronutrientes relacionados, tales como vitamina D, Fe, Cu, Se y Mn, mostrando asociaciones con un peor perfil clínico-nutricional y, poniendo de manifiesto la necesidad de restablecer niveles adecuados de Zn y de otros micronutrientes en estos pacientes críticos.

Conclusiones específicas

1. La prevalencia de hipozinquemia fue alta tras la admisión en UCI, sin observarse mejoras tras el seguimiento.
2. La mayoría de los parámetros clínicos y bioquímicos se mostraron alterados en los pacientes del estudio, relacionándose en algunos casos con los niveles bajos de Zn. Asimismo, los cambios en los niveles de Zn se asociaron con una mejor evolución de parámetros respiratorios en pacientes con COVID-19.
3. En pacientes sin COVID-19, las concentraciones de Zn eritrocitarias en el seguimiento estuvieron directamente relacionadas con los niveles de vitamina D y vitamina D₃, mientras que en pacientes con

COVID-19, los cambios en los valores de MTs no mostraron relación con los cambios observados en los niveles de Zn.

4. El análisis de las variables relacionadas con la gravedad mostró que el incremento en los niveles de Se plasmático se asoció con una mejor evolución en la escala SOFA en pacientes sin COVID-19, mientras que los niveles de Zn y de Fe fueron predictores de gravedad y mortalidad en pacientes con COVID-19.
5. En cuanto a los parámetros antioxidantes, la actividad de la enzima SOD se asoció con los niveles TAC, mientras que la actividad GPx estuvo inversamente correlacionada con las escalas de severidad al inicio en pacientes sin COVID-19.
6. El estudio de otros micronutrientes mostró bajos niveles de elementos traza (Fe, Cu, Se and Mn) tras la admisión en UCI, con una disminución general durante la estancia, mostrando un elevado riesgo de deficiencia. De forma similar, se observó un estatus deficiente de vitamina D en todos los pacientes, relacionándose los niveles más bajos con peores valores de diferentes parámetros clínicos y bioquímicos.

En resumen, los resultados muestran un elevado riesgo de deficiencia de Zn y de otros micronutrientes, sin embargo, es necesario disponer de biomarcadores fiables del estatus de Zn para poder establecer de forma precisa y fiable los requerimientos reales de cada paciente.

CONCLUSIONS

General conclusion

In general, the overall results of the present doctoral thesis indicate a high risk of Zn deficiency, as well as other micronutrients deficiencies as vitamin D, Fe, Cu, Se, and Mn, showing relationships with a worse clinical-nutritional profile and suggesting a possible beneficial effect of restoring proper levels of Zn and other micronutrients in these patients.

Specific conclusions

1. The prevalence of hypozincemia was high at ICU admission, not improving at follow-up.
2. Most clinical and biochemical parameters were altered among critically ill patients, being related with low Zn levels in some cases. Likewise, in patients with COVID-19, changes in Zn levels were associated with better outcomes in respiratory parameters.
3. In patients without COVID-19, erythrocyte Zn concentrations were directly related to vitamin D and vitamin D₃ levels at follow-up, meanwhile, in patients with COVID-19, changes in MTs were not related to changes in Zn levels.

4. The analysis of severity variables showed that increases in plasma Se levels were associated with better evolution in SOFA score in patients without COVID-19, while Zn and Fe levels were predictors of severity and mortality in patients with COVID-19.
5. Regarding antioxidant parameters, an association between SOD activity and TAC was observed, whereas GPx activity was inversely correlated to severity scores at baseline in patients without COVID-19.
6. After analyzing other micronutrients status, we observed low levels of trace elements (Fe, Cu, Se and Mn) at ICU admission, with a general decrease during the stay, showing a high risk of deficiency. Similarly, deficient vitamin D status was observed in all patients, with lower levels associated with worse values of different clinical and biochemical parameters.

In summary, these results showed a high risk of Zn and related micronutrients deficiency. Nevertheless, a reliable biomarker of Zn status is needed in order to establish, accurately, the real requirements of each patient.

MATERIAL SUPLEMENTARIO

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Tabla S1. Principales mecanismos involucrados en la producción de especies reactivas en el organismo [1,2].

Especie reactiva	Enzima/ión	Mecanismo/Reacción
Anión Superóxido ($O_2^{\bullet-}$)	NADPH oxidasa	$NADPH + 2O_2 \rightarrow NADP^+ + 2 O_2^{\bullet-} + H^+$
Peróxido de Hidrógeno (H_2O_2)	SOD	$2 O_2^{\bullet-} + H^+ \rightarrow H_2O_2 + O_2$
Radical Hidroxilo ($OH^{\bullet}$)	Fe^{2+}	$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$
Ácido Hipocloroso ($HClO$)	MPO	$H_2O_2 + Cl^- + H^+ \rightarrow HClO + H_2O$
Óxido Nítrico ($NO^{\bullet}$)	NOS	$L\text{-Arginina} + O_2 \rightarrow L\text{-Citrulina} + NO^{\bullet}$
Peroxinitrito ($OONO^-$)	$NO^{\bullet}$	$NO^{\bullet} + O_2^{\bullet-} \rightarrow OONO^-$
Radical Hidroperoxil ($HOO^{\bullet}$)	$O_2^{\bullet-}$	$O_2^{\bullet-} + H_2O \rightarrow HOO^{\bullet} + OH^-$
Radicales peroxilo ($ROO^{\bullet}$)	$R^{\bullet}$	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

Abreviaturas: H_2O , agua; MPO, mieloperoxidasa; NADPH, nicotinamida adenina dinucleótido fosfato reducida; $NADP^+$, nicotinamida adenina dinucleótido fosfato oxidada, NOS, óxido nítrico sintasa; O_2 , oxígeno; $R^{\bullet}$, radical lipídico; SOD, superóxido dismutasa.

Tabla S2. Visión general de los aspectos y consideraciones más relevantes a la hora de aplicar la terapia nutricional en el paciente crítico teniendo en cuenta la situación patológica [3].

Grupo de pacientes	Consideraciones
Principios básicos generales	- Todo paciente crítico está en riesgo de malnutrición - Examinar riesgo de malnutrición de forma periódica - Monitorización estrecha y adaptación del soporte nutricional - Preferible la administración de nutrición enteral temprana - Los micronutrientes son necesarios en nutrición parenteral - Necesidad de adaptación individual
Malnutrición Preexistente	- Nutrición temprana - Déficits de micronutrientes - Síndrome de realimentación
Obesidad	- Diagnóstico de malnutrición - Alteraciones de utilización de sustrato - Metabolismo alterado - Síndrome metabólico y comorbilidades
Sepsis/Shock séptico	- Nutrición enteral temprana tras estabilización hemodinámica (catecolaminas y lactato) - Tolerancia gastrointestinal y metabólica - Alteraciones de utilización de sustrato
Edad avanzada	- Malnutrición y deficiencia de micronutrientes - Comorbilidades y síndrome de fragilidad - Rechazo a comer habitualmente o anorexia - Movilidad
Afectación del SNC	- Alto riesgo de estancia prolongada en UCI y de malnutrición iatrogénica - Aspiración y disfagia - Frecuente dismotilidad gastrointestinal en sedación profunda
Afectación del sistema respiratorio	- Restricción de fluidos - Evitar exceso de aporte - Valorar presencia de hipofosfatemia
Afectación del sistema cardiovascular	- Caquexia y malnutrición frecuente - Alto riesgo de estancia prolongada en UCI y de malnutrición iatrogénica - Inestabilidad hemodinámica y cambios en las demandas de volumen

	- Riesgo de sangrado gastrointestinal durante la administración de anticoagulantes
Afectación del tracto gastrointestinal	- Anatomía funcional comprometida con maldigestión y malabsorción - Protección de las anastomosis gastrointestinales - Pérdida de proteínas y micronutrientes
Afectación del sistema renal	- Gasto energético incrementado en fallo renal crónico - Centrarse en los electrolitos y la hidratación - Pérdida de proteínas y micronutrientes - Considerar calorías aportadas por el citrato en el tratamiento renal sustitutivo
Trauma/quemados	- Alta variabilidad en el gasto energético - Pérdida de nutrientes a través de las heridas - Valorar necesidad de nutrientes inmunomoduladores

Abreviaturas: SNC; Sistema Nervioso Central

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