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PLAN DE ESTUDIOS COMBINADOS EN MEDICINA

**USO DE BIOMARCADORES EN SANGRE PERIFÉRICA PARA LA PREDICCIÓN DE
MORTALIDAD EN PACIENTES CON COVID-19**

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Prólogo

Quien pensaría que de un día a otro todo iba a cambiar. “Se reportan casos de un virus desconocido en China, pacientes mueren por virus desconocido con síntomas de neumonía”. Esas eran las noticias internacionales en los últimos meses del 2019, en menos de 3 meses se escuchaban reportes de casos en otras partes del mundo, incluyendo países americanos, México no fue la excepción. En febrero del 2020, la sala de urgencias del Hospital General de México tenía pacientes aislados esperando la confirmación del virus por las costosas y escasas pruebas moleculares en el país. Nunca imagine que mi proyecto de doctorado con tema en sepsis que tanto trabajo me había costado planear y presentar al servicio de urgencias para tener su cooperación, de un día a otro ya no se podía desarrollar. De un momento a otro, ni siquiera podíamos entrar al hospital, solamente personal indispensable tenía acceso. Un mes en casa pensando en que iba a ser de mi doctorado ahora sin proyecto, ¿Cuánto tiempo tardaría todo en regresar a la normalidad? Una noche recibí una llamada de mi tutor diciendo que el hospital iniciaría proyectos de investigación sobre COVID-19. Era una difícil decisión. No solo implicaba ir al triaje a captar pacientes y procesar muestras, significaba arriesgarme a mí y a mis seres queridos a un virus desconocido. Pero que oportunidad aquella, contribuir con un poquito de información a un evento histórico. Vestir el traje de astronauta, miles de cambios de ropa en un día, baños llegando a la casa, productos desinfectantes por doquier, “¡viene del hospital, no te le acerques!”. Todo valió la pena.

Resumen

Introducción: Durante la pandemia se presentó una escasez de recursos médicos por lo que identificar a los pacientes con coronavirus del síndrome respiratorio agudo severo tipo 2 (SARS-CoV-2) con mayor mortalidad desde el ingreso hospitalario se volvió primordial. **Objetivo:** Identificar en muestras de sangre periférica los biomarcadores de mayor cambio en pacientes mexicanos con SARS-CoV-2 y combinarlos con datos clínicos y demográficos para generar escalas de predicción de mortalidad. **Materiales y métodos:** Se incluyeron 378 pacientes con diagnóstico de enfermedad por coronavirus 2019 (COVID-19), de los cuales 255 fueron egresados por mejoría y 123 por defunción. Se recabaron los datos demográficos, clínicos y de laboratorio al ingreso, y se tomó una muestra sérica para la determinación de citocinas y quimiocinas. **Resultados:** De los pacientes reclutados, el 63.75% fueron hombres, con una edad media 54 años. Al conjuntar las variables obtenidas, se encontró que el índice linfocitos/neutrófilos (LNR) tenía un área debajo de la curva (AUC) de 0.832, con un punto de corte ≤ 0.008 , con una sensibilidad del 85.00% y una especificidad del 74.19%. En el caso del índice neutrófilos/monocitos (NMR) el AUC fue de 0.890 con un punto de corte ≥ 17.75 con sensibilidad de 89.47% y especificidad de 80.00%. Posteriormente, el inmunoensayo múltiple reveló a la IL-15 como mejor predictor de mortalidad con un AUC de 0.797 y al combinarla con otros parámetros de laboratorio el poder predictivo mejoro significativamente, como en el caso de la albúmina de 0.797 a 0.841 con el índice IL-15/ albúmina. **Discusión y conclusiones:** Los datos anteriores demuestran que el NMR y el LNR pueden ser utilizados en la práctica clínica para identificar pacientes mexicanos con COVID-19 severos con mayor riesgo de mortalidad. Además, parámetros inmunológicos como la cuantificación de citocinas séricas puede mejorar el valor predictivo de los parámetros ya utilizados en la clínica.

Palabras clave: SARS-CoV-2, COVID-19, biomarcadores séricos, mortalidad, curvas ROC.

Abstract

Introduction: During the pandemic there was a shortage of medical resources, so it became essential to identify the patients with severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) with the highest mortality risk at hospital admission.

Objective: To identify the peripheral blood biomarkers with significant differences in Mexican patients with SARS-CoV-2 and combine them with clinical and demographic data to generate mortality prediction scores. **Materials and methods:** We included a

total of 378 patients diagnosed with coronavirus disease 2019 (COVID-19), 255 patients were discharged due to improvement and 123 due to death. We collected all demographic, clinical, and laboratory data at hospital admission, and a serum sample was taken to determine cytokines and chemokines. **Results:** 63.75% of the patients recruited were men, with a mean age of 54 years old. By pooling the variables obtained, it was found that the lymphocyte-to-neutrophil ratio (LNR) had an area under the curve (AUC) of 0.832, with a cut-off point ≤ 0.008 , a sensitivity of 85.00% and a specificity of 74.19%. In the case of the neutrophil-to-monocyte ratio (NMR), the AUC was 0.890 with a cut-off point ≥ 17.75 , a sensitivity of 89.47% and a specificity of 80.00%. Subsequently, the immunoassay revealed IL-15 as the best predictor of mortality with an AUC of 0.797 and when combined with other laboratory parameters the predictive power improved significantly, as in the case of albumin from 0.797 to 0.841 with the IL-15-to-albumin ratio. **Discussion and conclusions:** Our data demonstrates that the NMR and LNR can be used in clinical practice to identify Mexican patients with severe COVID-19 with high mortality risk. In addition, immunological parameters such as the quantification of serum cytokines can improve the predictive value of the parameters already used in the clinic.

Keywords: SARS-CoV-2, COVID-19, serum biomarkers, mortality, ROC curves.

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Listado de abreviaturas y siglas usadas

4C	Coronavirus Clinical Characterization Consortium (consorcio para la caracterización clínica del coronavirus)
ACE2	angiotensin-converting enzyme 2 (enzima convertidora de angiotensina 2)
ALP	alkaline phosphatase (fosfatasa alcalina)
ALT	alanina aminotransferasa
APACHE II	Acute Physiology and Chronic Health Evaluation II (evaluación II de la fisiología aguda y salud crónica)
aPTT	tiempo de protrombina parcial activada
AST	aspartato aminotransferasa
AUC	area under the curve (área debajo de la curva)
BNP	péptido natriurético tipo B
CA	California
CD	cluster of differentiation (grupo de diferenciación)
células/μL	células por microlitro
CIRC	COVID Inpatient Risk Calculator (Calculadora de riesgo de pacientes hospitalizados por COVID)
CK-MB	creatina quinasa fracción cardiaca
CoV	coronavirus
COVID-19	coronavirus disease 2019 (enfermedad por coronavirus 2019)
CPK	creatina-fosfoquinasa
CRP	C reactive protein (proteína C reactiva)

CRT	classification and regression tree (árbol de clasificación y regresión)
CURB-65	confusion, urea, respiratory rate, blood pressure, 65 years old (confusión, urea, frecuencia respiratoria, presión arterial, 65 años)
DNA	desoxyribonucleic acid (ácido desoxirribonucleico)
EndoU	endorribonucleasa específica a uridina
EUA	Estados Unidos de América
ExoN	exorribonucleasa
FiO₂	fracción inspirada de oxígeno
GGT	gamma-glutamyl transferasa
GM-CSF	Granulocyte-macrophage colony-stimulating factor (factor estimulante de colonias de granulocitos y macrófagos)
HBV	hepatitis B virus (virus de la hepatitis B)
HCoV	Human coronavirus (coronavirus humano)
HCV	hepatitis C virus (virus de la hepatitis C)
HDL	lipoproteínas de alta densidad
HGM	Hospital General de México “Dr. Eduardo Liceaga”
IL	interleucina
IMC	índice de masa corporal
IFN	interferón
INR	relación normalizada internacional
IOT	intubación orotraqueal
IP	Interferon-gamma-inducible protein (proteína inducible por interferón gamma)

LDH	deshidrogenasa láctica
LDL	lipoproteínas de baja densidad
lpm	latidos por minuto
MA	Massachussetts
MCP	monocyte chemoattractant protein (proteína quimioatrayente de monocitos)
MERS-CoV	Middle East respiratory syndrome–related coronavirus (coronavirus causante del síndrome respiratorio del Medio Oriente)
mg/dL	miligramos por decilitro
MIG	monokine induced by interferon gamma (monocina inducida por interferón gamma)
MIP	Macrophage inflammatory protein (proteína inflamatoria de macrófagos)
mL	mililitros
mmHg	milímetros de mercurio
N	tamaño de muestra
NEWS2	National Early Warning Score (Puntaje Nacional de Alerta Temprana)
NK	Natural killer cells (células asesinas naturales)
Nsp	No structural protein (proteína no estructural)
NY	Nueva York
OMS	Organización Mundial de la Salud
ORF	open reading frame (marco abierto de lectura)
PAD	presión arterial diastólica

PAM	presión arterial media
PaO₂	presión arterial de oxígeno
PAS	presión arterial sistólica
PCR	polymerase chain reaction (reacción en cadena de la polimerasa)
PSI	pneumonia severity index (índice de gravedad de neumonía)
PT	tiempo de protrombina
qSOFA	quick SOFA (SOFA rápido)
RANTES	regulated on activation, normal T cell expressed and secreted (regulada en la activación, normalmente expresada y secretada por células T)
RdRp	RNA-dependent RNA polymerase (RNA polimerasa dependiente de RNA)
RNA	(ribonucleic acid) ácido ribonucleico
RNasas	ribonucleasas
ROC	receiver operating characteristics (característica operativa del receptor)
rpm	respiraciones por minuto
SARS-CoV	severe acute respiratory syndrome coronavirus (coronavirus causante de síndrome respiratorio agudo severo)
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2 (coronavirus causante de síndrome respiratorio agudo severo 2)
SIRS	Systemic Inflammatory Response Syndrome (Síndrome de Respuesta Inflamatoria Sistémica)
SOFA	sequential organ failure assessment (evaluación secuencial de insuficiencia orgánica)

SpO₂	saturación periférica de oxígeno
TMPRSS2	transmembrane serine protease 2 (serina proteasa transmembranal 2)
TNF	tumour necrosis factor (factor de necrosis tumoral)
TT	tiempo de trombina
UCI	unidad de cuidados intensivos
VACO	veterans health administration COVID-19 (administración de salud de veteranos COVID-19)
VIH	virus de inmunodeficiencia humana
vs	versus

CAPITULO 1: Introducción

Marco Teórico

1. Taxonomía del SARS-CoV-2

Orthocoronavirinae o coronavirus comprende una subfamilia muy diversa de virus de RNA monocatenario positivo que pertenecen a la familia *Coronaviridae*. A su vez, la familia *Coronaviridae* se subdivide en los géneros alfacoronavirus, betacoronavirus, gammacoronavirus y deltacoronavirus.[1] El genoma de estos virus varía entre las 26 y 32 kb y las partículas virales pueden llegar a medir entre 50 y 200 nanómetros de diámetro. Una de las características principales de estos virus son las proyecciones desde la superficie viral que asemejan una corona solar de aproximadamente 20 nanómetros, de las cuales esta familia de virus toma su nombre.[2]

Estos virus son capaces de infectar humanos y otros mamíferos, así como varias especies de aves generando enfermedades del tracto gastrointestinal y/o respiratorio.[1] Los coronavirus capaces de infectar a los humanos son conocidos desde 1966, como es el caso de HCoV-229E y HCoV-OC43 (alfa y betacoronavirus, respectivamente). Estos virus circulan comúnmente entre la población y causan infecciones estacionales leves del tracto respiratorio asociadas a síntomas que conjuntan un cuadro de resfriado común o infecciones gastrointestinales, generando diarreas virales que remiten espontáneamente sin mayor intervención médica. En contraste, históricamente se han detectado en la historia, tres tipos de coronavirus capaces de generar infecciones graves en humanos, el coronavirus del síndrome respiratorio agudo severo (SARS-CoV, 2002-2004), el coronavirus del síndrome respiratorio de oriente medio (MERS-CoV, 2012) y el nuevo coronavirus tipo 2 del síndrome respiratorio agudo severo (SARS-CoV-2).[3,4]

En diciembre del 2019 en la ciudad de Wuhan, China se reportaron los primeros casos de neumonía causada por un virus desconocido. Los análisis bioinformáticos que se realizaron para poder identificar y clasificar al nuevo virus concluyeron una semejanza genética del 79.5% con respecto al ya conocido genoma del SARS-CoV. Además, se encontró una similitud del 96% con el virus del murciélago RaTG13, por lo que se sugirió una infección zoonótica transmitida por el murciélago o algún otro mamífero no identificado.[1] Pese a que su origen sigue siendo un misterio hasta la fecha, se sugiere que el SARS-CoV-2 pudo haber mutado a partir del RaTG13. Por lo tanto, el nuevo virus se clasificó dentro del género betacoronavirus y recibió el nombre de SARS-CoV-2 [5].

2. Estructura del SARS-CoV-2

En cuanto a su estructura, el SARS-CoV-2 posee 4 diferentes proteínas estructurales: pico (S, spike), envoltura (E), membrana (M), nucleocápside (N) y hemaglutinina-esterasa (HE).[6] La proteína S se encuentra en la superficie viral y es una glucoproteína trimérica de fusión clase I. Esta proteína está compuesta por dos subunidades funcionales, la subunidad 1 (S1) que permite la unión al receptor, y la subunidad 2 (S2) que permite la fusión de membranas. La proteína E es un canal iónico transmembranal que facilita el ensamblaje y liberación viral. La proteína M es una proteína transmembranal, es la más abundante en la membrana del virión y provee la curvatura y forma; también esta proteína se encuentra relacionada con el procesamiento, modificación y tráfico de varias proteínas virales, en el ensamblaje y liberación de partículas virales e interfiere en la respuesta inmunológica bloqueando la respuesta antiviral. Por otro lado, la N se encuentra en estrecho contacto con el material genético,

empaquetándolo, además de conferir protección al virión al intervenir con la vía de los interferones.[7,8]

El material genético del SARS-CoV-2 codifica 16 proteínas no estructurales (Nsp1-16) que forman el complejo de replicación y transcripción, en donde 13 proteínas citosólicas están implicadas en la replicación del RNA y 3 proteínas son de tipo transmembranal (Nsp3, 4 y 6).[3,9] Las Nsp3 y Nsp4 modifican las membranas del retículo endoplásmico de la célula huésped formando las vesículas de doble membrana, necesarias para la replicación viral (organelo de replicación), mientras que la Nsp 6 funciona como una proteína de comunicación entre el retículo endoplásmico y este organelo de replicación.[9] La Nsp1 se une específicamente a la subunidad 40 de los ribosomas y promueve la escisión endonucleolítica de RNA mensajeros del huésped, por lo tanto impide la traducción de múltiples genes, incluidos los interferones tipo I, permitiendo evadir la respuesta inmune innata.[8] Nsp2 modula las vías de señalización de supervivencia de la célula hospedera interactuando con varias proteínas de la misma, además se encuentra involucrado en la biogénesis mitocondrial y en el transporte endosomal.[10] La proteína Nsp5 también conocida como proteasa principal hidroliza la poliproteína 1ab, en 11 sitios distintos, produciendo las proteínas no estructurales Nsp4 a Nsp16.[8,11] Las proteínas Nsp7, Nsp8 y Nsp12 son proteínas principales en la maquinaria de replicación viral. La proteína Nsp12 actúa como la RNA polimerasa dependiente de RNA (RdRp). Nsp7 y Nsp8 funcionan como cofactores de la proteína Nsp12 mediando la actividad de la RdRp, estimulando su unión con la plantilla de RNA.[11] La Nsp9 se une a oligonucleótidos de RNA y a las cadenas sencillas de DNA, jugando un papel importante en la formación del complejo de replicación y

transcripción.[12] Las Nsp10, Nsp14 y Nsp16 son proteínas críticas en el taponamiento (*capping*) del RNA mensajero viral vía metilación, lo cual provee estabilidad y asegura la traducción del RNA viral.[11] La Nsp11 es un péptido corto de 13 aminoácidos cuya función permanece poco clara.[13] La Nsp13 tiene actividad de helicasa y RNA 5'-trifosfatasa dependiente de ATP, con esto participa en el desenrollado del DNA o RNA durante la replicación del RNA viral y en el primer paso del taponamiento del RNA mensajero.[14] Al ser una enzima con doble función, la Nsp14 es responsable de realizar la corrección de errores del naciente RNA, con la actividad de 3'-5' exorribonucleasa (ExoN), además de participar durante el taponamiento de RNA mensajero viral con actividad de guanina(-N7-)-metiltransferasa.[15] La Nsp15 es una endorribonucleasa específica a uridina dependiente de magnesio (EndoU), tiene sitios activos y funciones similares a las RNasas eucarióticas, por lo que al unirse a las cadenas negativas de RNA previene la activación de la respuesta inmunológica.[16]

Además de las proteínas anteriormente descritas, el SARS-CoV-2 codifica 9 proteínas accesorias conocidas como proteínas ORF (open reading frame), incluyendo la ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c y ORF10. Estas proteínas juegan un papel importante en la interacción del virus con la respuesta inmunológica del huésped.[11] ORF3b, ORF6, ORF7a y ORF8 se encuentran involucradas en la inmunomodulación de la respuesta del huésped, al igual que impiden la respuesta de los interferones tipo I. ORF3a está implicada en la regulación de las vías de apoptosis de la célula huésped al igual que activa la tormenta de citocinas al estimular vías de señalización como NF- κ B y el inflamosoma NLRP3; además de funcionar como canal iónico involucrado en la liberación de las partículas virales. Por otro lado, las

ORF9b y ORF9c interactúan con los organelos celulares generando la supresión de la respuesta antiviral de las células infectadas.[17]

3. Mecanismos de invasión

El paso inicial de la infección por coronavirus comprende la unión específica de la proteína S viral en su porción S1 con los receptores de entrada celular, en el caso particular del SARS-CoV-2 con la enzima convertidora de angiotensina 2 (ACE2). Además, es necesaria la interacción de la S2 con las proteasas celulares (como la serina proteasa de superficie celular, TMPRSS2 o la cathepsina en los endosomas), que median la adhesión de la proteína S para permitir la posterior unión de las membranas.[18,19]

Existen dos vías en las que el virus puede entrar al citoplasma celular, la primera es mediante la fusión de la membrana viral con la membrana celular, también llamada vía temprana; la segunda vía, o vía tardía, consiste en la fusión de la membrana viral con la membrana endosomal después de haber sido fagocitado.[19] En cualquiera de los dos escenarios, el genoma viral es liberado al citoplasma de la célula huésped para posteriormente utilizar sus ribosomas para comenzar la traducción primaria de dos grandes marcos de lectura abierta, ORF1a y ORF1b. Lo anterior resulta en dos poliproteínas, pp1a y pp1b que son posteriormente fragmentados en los 15 sitios de unión distintos, lo que resulta en las 16 proteínas no estructurales maduras que formarán el complejo de replicación y transcripción, así como las vesículas características de doble membrana u organelos de replicación a partir de la remodelación del retículo endoplásmico.[20] En el interior de estos organelos de replicación ocurre predominantemente la síntesis del RNA viral, conectándose al citosol de la célula huésped para transportar los metabolitos requeridos para la síntesis del RNA.[19]

Después, la polimerasa viral transcribe una serie de RNA mensajeros subgenómicos por transcripción discontinua que son traducidos en proteínas estructurales. La proteína N forma un complejo con el nascente RNA genómico, mientras que las proteínas S, E y M son insertadas en la envoltura viral proveniente del retículo endoplásmico o el aparato de Golgi de la célula huésped.[19] La interacción de estas proteínas con los organelos de la célula huésped genera un cambio en la curvatura de las membranas, permitiendo la formación y separación de los viriones.[18] Los últimos estudios sugieren que el SARS-CoV-2 utiliza la vía de exocitosis lisosomal, lo que resulta en una secreción no lítica de los viriones.[19]

4. Respuesta inmunológica

La respuesta viral innata contra la infección por SARS-CoV-2 incluye varios componentes del sistema humoral, incluyendo los sistemas del complemento y la coagulación, proteínas solubles que reconocen glicanos como las lectinas de unión a manosa, interferones, quimiocinas y anticuerpos naturales.[21] Además, hay varios componentes de la respuesta celular como las células NK o células T gamma delta. Normalmente, estas respuestas son suficientes para evitar la expansión viral, activar la producción de citocinas e inducir el inicio de la respuesta inmune adaptativa.[22]

El virus, es capaz de limitar estas primeras líneas de defensa, particularmente la vía de los interferones, como lo mencionamos anteriormente. El bloqueo de la respuesta temprana de los interferones tipo III y tipo I particularmente, genera una expansión viral rápida y favorece la colonización de las vías respiratorias inferiores, lo cual ha sido correlacionado con la severidad clínica de los pacientes.[23]

Además, el SARS-CoV-2 induce la secreción de citocinas y en algunos casos logra desregular la respuesta inmunológica cambiando el patrón de secreción de citocinas. La hipersecreción de citocinas, también llamada tormenta de citocinas involucra principalmente la IL-1 e IL-6 que inducen inflamación y fiebre.[24] Otras citocinas como la IL-2, IL-7, TNF- α , GM-CSF, IP-10 y MCP-1 también son responsables del estado hiperinflamatorio generando daño en las células del epitelio alveolar, así como en el endotelio vascular con infiltración de neutrófilos y macrófagos en el tejido pulmonar.[25] Este aumento en las poblaciones de fagocitos y cambio en el perfil de citocinas tienen la capacidad de debilitar la respuesta inmunológica enfocada en destruir al virus. Las poblaciones de linfocitos T (tanto CD4⁺ como CD8⁺) se reducen en los pacientes con COVID-19 severa lo que correlaciona con niveles mayores de IL-6 y un mal pronóstico.[26] Finalmente, otras citocinas como el TGF- β , podrían favorecer la remodelación tisular y la fibrosis pulmonar contribuyendo también al compromiso del intercambio gaseoso.[22]

5. Impacto clínico de la infección por SARS-CoV-2

Con respecto a la invasión viral, el receptor ACE2 se encuentra expresado en una gran variedad de tejidos humanos: corazón, riñones, testículos, pulmones (neumocitos tipo I y II), mucosa nasal y oral, incluyendo nasofaringe, enterocitos del intestino delgado, piel, así como endotelios y células de músculo liso de varios órganos incluidos tracto digestivo, cerebro, hígado, riñones y bazo.[27] Particularmente, se ha reportado una mayor expresión en células epiteliales alveolares o neumocitos y en enterocitos del intestino delgado. Lo anterior podría explicar las posibles rutas de entrada del virus, así como los signos y síntomas más comunes en los pacientes.[28]

En diciembre del 2019, la comunidad médica y científica comenzó a reportar casos de neumonía asociada a la infección por SARS-CoV-2; estos pacientes presentaban síntomas clásicos de una neumonía atípica como fiebre, disnea, tos, mialgias, fatiga, entre otros. No obstante, también se reportaron otras manifestaciones clínicas ajenas al tracto respiratorio, tales como diarrea, anorexia, náusea, vómito, dolor abdominal, elevación de enzimas hepáticas, pancreatitis, hematuria, proteinuria, hiperazoemia, confusión, convulsiones, encefalitis, eventos cerebrovasculares, anosmia, ageusia, elevación de biomarcadores de lesión cardíaca, pericarditis, palpitaciones, dolor torácico, arritmia, coagulación intravascular, conjuntivitis, urticaria, erupciones cutáneas, todas estas relacionadas con alteración en el tracto digestivo, el hígado, el páncreas, los riñones, el sistema nervioso central, el sistema cardiovascular, los ojos y la piel.[28] Debido a la gran variedad de síntomas presentes, la infección por SARS-CoV-2 tomó el nombre de COVID-19 (Coronavirus Disease-2019). Desafortunadamente, cuando otros órganos están involucrados en el proceso patológico, se tiene un peor pronóstico que frecuentemente resulta en hospitalización y/o ingreso a terapia intensiva.[27]

El cuadro clínico de los pacientes con COVID-19 es muy heterogéneo, desde infecciones completamente asintomáticas hasta complicaciones mortales. El periodo de incubación en promedio toma 14 días, con un tiempo medio de 4 a 5 días posteriores al contacto.[29] Dependiendo de la severidad, los cuadros de COVID-19 en adultos pueden ser clasificados en 4 grupos. Cabe destacar que los criterios clínicos para cada categoría se pueden traslapar o pueden cambiar durante el curso clínico del paciente. El primer grupo se trata de pacientes asintomáticos, quienes a pesar de haber tenido una prueba positiva para SARS-CoV-2 se encuentran sin síntomas. El segundo grupo son pacientes

con enfermedad leve, estos pueden presentar síntomas comunes como tos, mialgias y cefalea, además de diarrea, odinofagia, astenia, mialgias, alteraciones en el gusto u olfato, congestión nasal, rinorrea o estornudos, mismos que se autolimitan en no más de 10 días.[30] Los cuadros moderados evolucionan generalmente dentro de los primeros 7 días del contacto y se presentan como infección de vías respiratorias inferiores o neumonía, con fiebre, tos, disnea, saturación periférica de oxígeno (SpO_2) mayor o igual a 94% al aire ambiente al nivel del mar y/o infiltrados bilaterales en estudios de imagen.[30] Aquellos pacientes con COVID-19 severa presentan distrés respiratorio: SpO_2 menor de 94% al aire ambiente al nivel del mar, una correlación presión arterial de oxígeno / fracción inspirada de oxígeno (PaO_2/FiO_2) menor a 300 mmHg, una frecuencia respiratoria mayor a 30 respiraciones por minuto o infiltrados pulmonares de más del 50%.[30] La última categoría corresponde a los cuadros críticos, los cuales se definen frecuentemente por presentar síndrome de distrés respiratorio agudo, caracterizado por daño alveolar difuso severo (edema, formación de membranas hialinas, infiltrados inflamatorios, formación de micro-trombos, acumulación de exudados fibromixoides y fibrosis), choque séptico y/o falla orgánica múltiple.[30] Los síntomas y gravedad de la COVID-19 están estrechamente relacionados con los dos principales procesos en la patogénesis viral. La primera etapa corresponde a la replicación viral en el huésped, mientras que la segunda etapa parece estar relacionada a una respuesta inmunológica desregulada que genera daño tisular.

Durante el primer año en el que se detectó la presencia de un nuevo virus no existían pruebas comerciales para detectarlo, la única manera de hacerlo era a través de pruebas de amplificación de ácidos nucleicos, como PCR en laboratorios especializados.

Posteriormente, surgieron varios kits comerciales para la detección de antígenos y anticuerpos que permitieron la detección rápida y económica de la infección.[31] En la actualidad solamente se recomienda realizar pruebas de amplificación de ácidos nucleicos o pruebas rápidas de detección de antígenos en personas sintomáticas donde se sospeche SARS-CoV-2 o en personas en contacto con pacientes positivos 5 días posteriores al contacto.[30] La toma de muestra varía dependiendo de la recomendación de la empresa manufacturera, pero todas consisten en muestras del tracto respiratorio superior (nasofaringe, cornete medio nasal, nasal anterior o saliva).[30]

Debido a la desinformación al inicio de la pandemia, no existían guías de práctica clínica para los pacientes infectados. A lo largo de dos años se propusieron varios tratamientos incluyendo varios grupos de fármacos, no obstante, no se tuvo éxito con ninguno de ellos.[32] Finalmente, se concluyó que para pacientes asintomáticos no es necesario implementar algún tipo de manejo médico. Los pacientes con cuadros leves y moderados deben tener un aislamiento intradomiciliario con un manejo sintomático individualizado (antipiréticos, analgésicos, antihistamínicos, antitusígenos, entre otros).[30] Los pacientes con cuadros moderados, además, deben ser vigilados estrictamente para verificar la evolución del cuadro. En estos grupos de pacientes se deben considerar los tratamientos antivirales por el riesgo de progresión a COVID-19 severa. En orden de preferencia se sugiere: ritonavir-nirmatrelvir (Paxlovid), remdesivir o molnupiravir.[30] En aquellos pacientes severos que requieren hospitalización y oxigenoterapia por COVID-19, se sugiere administrar dexametasona, así como en caso de disponibilidad, se sugiere agregar al manejo baricitinib (inhibidor JAK) o tocilizumab (anti-IL-6), además de heparina en dosis profilácticas a menos de que exista

contraindicación para ello.[30] En cuanto a la oxigenoterapia se deberá ajustar de acuerdo con las necesidades del paciente optando por la vía menos invasiva disponible a la que el paciente responda (cánulas nasales de alto flujo, ventilación no invasiva o intubación y ventilación mecánica).[30] En aquellos pacientes con COVID-19 crítica, se deberá manejar en unidades de terapia intensiva. En relación con la estabilidad hemodinámica se debe optar por soluciones cristaloides balanceadas y norepinefrina como vasopresor de primera línea para conservar la presión arterial media de 60 a 65 mmHg.[30] Sólo se recomienda iniciar antibioticoterapia en aquellos pacientes en donde se sospeche infección bacteriana y hacer las modificaciones pertinentes al recibir los resultados de laboratorio confirmatorios.[30]

6. Epidemiología Mundial y en México

Desde los primeros casos reportados en Wuhan, China, a finales del 2019, millones de casos de COVID-19 han sido reportados en todos los continentes. A nivel mundial, para febrero del 2023, se han registrado más de 600 millones de casos y casi 7 millones de muertes.[33] No obstante, se han publicado diversos estudios donde se evalúa la seroprevalencia en comparación con los casos agudos positivos y se calculan hasta 10 veces más casos en realidad.[34–36] En otro estudio publicado por un grupo de colaboración internacional se reportó que para noviembre del 2021, el 40% de la población mundial ya había contraído SARS-CoV-2 al menos una vez.[37]

Al igual que otros virus el SARS-CoV-2 ha sufrido mutaciones a lo largo del tiempo y ciertas variantes han llamado la atención internacional. Para ser consideradas como variantes de preocupación deben de tener alguna de las siguientes características: aumento en la transmisibilidad o cambios drásticos en la epidemiología, incremento de

la virulencia o cambios en la presentación clínica; o disminución en la efectividad de las medidas de salud pública o de los diagnósticos, medicamentos o vacunas disponibles.[38] La OMS ha designado nombrarlas según el alfabeto griego para su mejor identificación. Las variantes de preocupación reportadas hasta el momento son: alfa con los primeros casos reportados en el reino unido en septiembre del 2020, beta que inicio en mayo del 2020 en Sudáfrica, gamma iniciando en Brasil en noviembre del 2020, delta que surgió en la india en octubre del 2020 y finalmente la variante de preocupación que circula actualmente, ómicron cuyos primeros casos fueron reportados simultáneamente en varios países en noviembre del 2021.[38,39]

En cuanto a la transmisión de este virus, el principal medio de contagio es de persona a persona, que ocurre por contacto estrecho con un caso positivo a través de partículas respiratorias. Los virus son liberados por las personas contagiadas en las secreciones de tos, estornudos o incluso en microgotas de saliva que son capaces de infectar a un nuevo individuo al tener contacto con la mucosa.[40] También el contagio puede ocurrir al tocar superficies contaminadas y posteriormente tocar nariz, ojos o boca.[41–43] El SARS-CoV-2 también ha sido detectado en otras muestras de origen diferente al respiratorio, como heces, sangre, secreciones oculares, leche materna y semen; no obstante, estas secreciones como vías de contagio no han sido demostradas.[44–47] La capacidad de contagio puede iniciar antes del inicio de los síntomas, siendo mayor durante el curso de la enfermedad y disminuyendo durante el transcurso.[48] La transmisión después de los 10 días es poco probable en pacientes inmunocompetentes con infecciones no severas, este tiempo se puede alargar en pacientes inmunosuprimidos o con COVID-19 severa.[39,49,50]

Afortunadamente, gracias a los esfuerzos multinacionales, se lograron diseñar varias vacunas contra el SARS-CoV-2. Estas tienen diversas tecnologías, desde las ya conocidas como fragmentos del virus, hasta nuevas tecnologías como micropartículas con antígenos virales en superficie, virus recombinantes o RNA recombinante.[51] En el mundo se han aplicado más de 13 billones de dosis de vacunas, logrando cubrir alrededor del 70% de la población mundial con al menos una dosis contra el SARS-CoV-2. Desafortunadamente aún existe inequidad en la accesibilidad a las vacunas, por lo que hay países que ya cuentan con el 100% de su población vacunada mientras que países de bajos recursos se encuentran alrededor del 20%.[33]

En México, el primer caso reportado de COVID-19 fue el 27 de febrero del 2020. A partir de esta fecha hasta febrero del 2023, se han reportado un total de 7,450,992 casos confirmados y 332,986 defunciones, con una mortalidad por casos confirmados del 4.5% considerando los datos previamente mencionados. Sin embargo, estos datos oficiales han resultado controversiales y han variado a lo largo del tiempo, calculando hasta 9% de letalidad para pacientes mexicanos infectados por SARS-CoV-2. No obstante, estimando a través de cálculos estadísticos, asumiendo una letalidad real del 1.5%, se calculan un mínimo de casos acumulados de 54,426,833 y un máximo de casos acumulados de 97,968,300 asumiendo una letalidad real del 1.0%. La Secretaría de Salud reportó varios factores de riesgo que aumentan la mortalidad por COVID-19, dentro de los cuales destacan hipertensión, obesidad y diabetes. Además, según los registros de hospitales mexicanos, se reportó mayor incidencia en hombres y en personas alrededor de 40 años. Afortunadamente, para febrero del 2023 se ha logrado una buena cobertura de vacunación en la población mexicana, en total 84% con al menos

una dosis aplicada, impactando claramente en la ocupación hospitalaria, así como en la mortalidad. Considerando las cifras por grupos de edad, en personas mayores de 18 años se cuenta con 91% de cobertura, personas de 12 a 17 años 64% y niños de 5 a 11 años 61%.

7. Biomarcadores para la predicción de mortalidad en COVID-19

Al no contar con tratamientos específicos, la evaluación y predicción de mortalidad fueron herramientas prioritarias para identificar a los pacientes con mayor riesgo de fallecer por COVID-19, así como para administrar de mejor manera los recursos hospitalarios que eran muy limitados. Desafortunadamente, al inicio de la pandemia no se contaba con información para predecir el desenlace de los pacientes infectados por SARS-CoV-2. Los primeros estudios epidemiológicos tanto en México como en el mundo denotaron algunos factores de riesgo que conferían peor pronóstico como la edad (mayores de 65 años), el sexo (hombres) y contar con comorbilidades, como obesidad, diabetes e hipertensión o alguna condición causante de inmunosupresión incluyendo embarazo, enfermedad renal crónica, enfermedad hepática crónica o pacientes bajo medicación inmunosupresora.[30,52,53] No obstante, individuos de todas las edades están en riesgo de infección y enfermedad severa, por lo que identificar biomarcadores que permitieran predecir el curso de la enfermedad se convirtió en prioridad para la comunidad científica y clínica. En este aspecto, muchos biomarcadores utilizados en otras enfermedades se comenzaron a probar en el contexto de COVID-19.[54–59]

Actualmente, después de la publicación de miles de observaciones en distintas partes del mundo, se han realizado metaanálisis y revisiones sistemáticas, sobre el uso de biomarcadores en la predicción de severidad y mortalidad de COVID-19. Los niveles

circulantes de proteína C reactiva han demostrado correlacionar con la severidad de la enfermedad y se han reportado niveles elevados en pacientes no sobrevivientes en comparación con los sobrevivientes.[60–62] De igual manera, diversos estudios han reportado niveles elevados de IL-6, por lo que se han recomendado tratamientos farmacológicos enfocados en este biomarcador.[63–65] Otros biomarcadores típicos de enfermedades crítica como los niveles elevados de protrombina, dímero-D, procalcitonina y deshidrogenasa láctica se han reportado en pacientes críticos infectados por SARS-CoV-2, además de ser útiles en la predicción de mortalidad.[66–72] Otros parámetros comúnmente medidos en un contexto hospitalario, como los aportados por la biometría hemática han sido utilizados para predecir resultados fatales, dentro de los que se encuentran la leucocitosis, linfopenia, neutrofilia o la trombocitopenia.[73–79] Pese a todos los esfuerzos realizados hasta el momento, no existe una recomendación oficial para el uso de biomarcadores en la práctica clínica cotidiana para la predicción de mortalidad.[30]

8. Escalas para la predicción de mortalidad en COVID-19

Además de los factores de riesgo como los demográficos y de biomarcadores identificados para la predicción de mortalidad en pacientes con COVID-19, se comenzaron a evaluar los conjuntos de datos o escalas para predecir mortalidad.

Al inicio se comenzaron a evaluar escalas ya existentes para otras patologías relacionadas, como las escalas utilizadas en pacientes con sepsis o las escalas útiles en el pronóstico de pacientes con neumonía, entre otras. En el caso de la escala SOFA (Sequential Organ Failure Assessment), utilizada para el diagnóstico y pronóstico de mortalidad de pacientes con sepsis o choque séptico, su empleo se ha documentado

para la predicción de mortalidad a 30 o 60 días. En la gran mayoría de ellos se ha encontrado una buena AUC, mayor a 0.80[80–84]; no obstante, existen otros estudios que no recomiendan su uso por tratarse de escalas específicas de una población. [85,86]

La escala qSOFA (quick SOFA), que incluye solamente tres parámetros, estado de alerta, frecuencia respiratoria y presión arterial, en la gran mayoría de estudios ha demostrado de baja a moderada capacidad de predicción de mortalidad, por lo que no se recomienda su uso.[83,84,87–91] Otras escalas utilizadas en pacientes con sepsis fueron probadas en poblaciones diferentes a la mexicana, incluyendo los criterios del síndrome de respuesta inflamatoria sistémica (SIRS), APACHE II y NEWS2, reportando AUC para la predicción de mortalidad de 0.61, 0.73 y 0.85 respectivamente.[82,88,92]

Por otro lado, de las escalas más comúnmente utilizadas en la clínica para la predicción de mortalidad en cuadros de neumonía son las escalas CURB-65 (tomando sus siglas del acrónimo formado por las iniciales de los parámetros que incluye en inglés: confusión, urea >19mg/dL, frecuencia respiratoria $\geq$ 30 rpm, presión sistólica < 90 mmHg o diastólica $\leq$ 60 mmHg, edad $\geq$ 65) y PSI (Pneumonia Sverity Index). En estudios realizados en otros países se reportan AUC entre 0.7 y 0.9, cabe destacar que estos análisis incluyeron no más de 1200 pacientes COVID positivos, ingresados en un solo centro hospitalario.[93–100]

En el caso de un estudio realizado en población mexicana, por Alanís-Naranjo JM y colaboradores, se evaluaron las escalas PSI y SOFA, en 151 pacientes ingresados. En este estudio se reportó un AUC de 0.85 y 0.74 respectivamente. En el caso de la escala PSI, se obtuvo una sensibilidad de 55.8% y una especificidad de 81.6%, mientras que para la escala SOFA, ambas se encuentran alrededor de 78%.[101]

Asimismo, con el paso del tiempo y el aumento de casos, se crearon varias escalas específicas para el pronóstico de mortalidad en pacientes infectados por SARS-CoV-2.[102–105] Dentro de este grupo de escalas, destacan la escala de mortalidad 4C, la calculadora de riesgo para pacientes hospitalizados con COVID-19 (CIRC) y el índice de mortalidad por COVID-19 en la Administración de Salud de Veteranos COVID-19 (VACO). La escala de mortalidad 4C fue originalmente diseñada en población inglesa, y posteriormente validada en distintas poblaciones alrededor del mundo incluyendo Canadá, Japón y EUA.[106–109] Esta escala incluye 8 variables recabadas al ingreso hospitalario: edad, sexo, número de comorbilidades, frecuencia respiratoria, saturación periférica de oxígeno, estado de alerta, niveles séricos de urea y de proteína C reactiva, con un rango de puntaje entre 0 y 21 con un punto de corte de 15, AUC entre 0.61 y 0.76.[106] La escala CIRC fue creada en el centro hospitalario John Hopkins, por el médico Brian T Garibaldi. Se trata de una escala compleja que incluye parámetros demográficos (edad, sexo, raza, vivienda), así como parámetros clínicos como índice de masa corporal, la presencia de síntomas gastrointestinales, respiratorios, constitucionales, ageusia o anosmia, temperatura, tasa de filtración glomerular, frecuencia cardíaca, niveles séricos de troponina, hemoglobina, albumina, dímero-D, ALT, conteo total de leucocitos, conteo absoluto de linfocitos, proteína C reactiva, ferritina y el puntaje de la escala de Charlson (diseñada para evaluar mortalidad a 10 años en pacientes con múltiples comorbilidades).[110,111] Finalmente, la escala VACO, fue diseñada en EUA en pacientes veteranos y posteriormente validada en pacientes no veteranos; esta sólo evalúa las comorbilidades de base del paciente, así como sexo y edad. Se reportó un AUC desde 0.79 hasta 0.84 dependiendo de los puntos de corte en

el pronóstico de mortalidad en caso de contagio.[112,113] Hasta el momento, ninguna de estas escalas ha sido validada en población mexicana.

Por otra parte, en México también se diseñó una escala, tomando en cuenta 400 pacientes en un estudio multicéntrico incluyendo 12 centros hospitalarios. Se trata de la escala LOW-HARM, la cual contiene tanto variables demográficas como parámetros clínicos tales como linfopenia (< 800 células/ μL), saturación de oxígeno ($< 88\%$), glóbulos blancos ($> 10,000$ células/ μL), hipertensión, edad, lesión renal (creatinina sérica > 1.5 mg/dL) y lesión miocárdica (valores anormales de CPK, troponina I o mioglobina). Para esta escala los autores propusieron un punto de corte mayor o igual a 65, con un AUC de 0.80 (IC95% 0.77-0.84), sensibilidad de 63% y especificidad de 97.5%.[114] Sin embargo, para el diseño de esta escala se incluyó una población donde el 50% de los pacientes fallecieron, por lo que esto no es equiparable con la realidad epidemiológica.[115]

Justificación

La infección por SARS-CoV-2 tiene una presentación clínica muy heterogénea. Hasta el momento se han registrado más de 640 millones de casos y más de 6.5 millones de muertes a nivel mundial; México continúa siendo uno de los países con mayores tasas de mortalidad. El sistema inmunológico, con altos niveles de citocinas, juega un papel muy importante en la severidad de la COVID-19. La predicción de mortalidad al ingreso hospitalario permitiría la distribución y optimización de recursos humanos, materiales y de infraestructura, lo cual podría ayudar a disminuir la mortalidad. En México, aún no existe una escala que permita predecir la mortalidad con un área debajo de la curva (AUC) óptima, mayor de 0.90. Además, el uso de biomarcadores en sangre periférica en

conjunto con datos clínicos parece mejorar el rendimiento de las escalas clínicas con AUC mayores o iguales a 0.90, por lo que desarrollar una escala que incluya datos biométricos, clínicos y biomarcadores es fundamental para un mejor manejo de los pacientes con COVID-19 grave.

Pregunta de investigación

¿Cuál es el poder predictivo de mortalidad dada por el AUC, la sensibilidad y la especificidad, de una escala que combine biomarcadores en sangre periférica, datos clínicos y demográficos en pacientes mexicanos con COVID-19 grave?

Hipótesis

Una escala que combine biomarcadores en sangre periférica, datos clínicos y demográficos tendrá un mejor poder predictivo de mortalidad ($AUC > 0.90$), en comparación con escalas preexistentes en pacientes mexicanos con SARS-CoV-2.

Objetivos

OBJETIVO GENERAL

Identificar en muestras de sangre periférica los biomarcadores de mayor cambio en pacientes mexicanos con SARS-CoV-2 y combinarlos con datos clínicos y demográficos para desarrollar una nueva escala de predicción de mortalidad para COVID-19 grave.

OBJETIVOS ESPECÍFICOS

- Describir la población de pacientes con diagnóstico de COVID-19 que ingresen al HGM.

- Conocer la mortalidad de los pacientes con diagnóstico de COVID-19 atendidos en el HGM.
- Caracterizar los subgrupos de sobrevivientes y no sobrevivientes para los pacientes con diagnóstico de COVID-19, a través de variables demográficas, clínicas e inmunológicas.
- Identificar biomarcadores candidatos a través de suero y leucocitos de los subgrupos de pacientes COVID-19 sobrevivientes y no sobrevivientes.
- Proponer una nueva escala que combine valores clínicos, demográficos y biomarcadores para la predicción de mortalidad en los pacientes con infección severa por SARS-CoV-2.

CAPITULO 2: Metodología

Estudio

Se realizó un ensayo prospectivo, longitudinal, observacional y analítico.

Población

Se incluyeron pacientes con diagnóstico de infección por SARS-CoV-2 que acudieron a los Servicios de Urgencias, Neumología, Cardiología y Terapia Médica Intensiva del Hospital General de México “Dr. Eduardo Liceaga”, del 01 de junio del 2020 al 30 de junio de 2021.

Tamaño de la muestra

El tamaño de la muestra se definió a partir de los datos publicados por Alharthy A y colaboradores, quienes realizaron un estudio retrospectivo en pacientes críticos con COVID-19. En este grupo de pacientes se detectaron diferencias significativas en diversos parámetros de laboratorio entre sobrevivientes y no sobrevivientes, tales como el conteo total de leucocitos (20.1 ± 3.6 vs 22.2 ± 2.7 células/mm³ respectivamente) [116].

A través del software G*Power 3.1.9.2, usando la fórmula para comparación de medias para dos grupos independientes a dos colas, con un poder de prueba del 95% y un error $\alpha = 0.05$ predichos, se calculó un tamaño del efecto (d) = 0.66 con base en los valores promedio del conteo total de leucocitos mencionados anteriormente. Con estos datos, se calculó una N = 122 pacientes (61 sobrevivientes y 61 no sobrevivientes). Este valor se ajustó considerando el 20% de pérdidas durante el seguimiento hospitalario, resultando una N ajustada = 146 pacientes (73 pacientes por grupo). (SUPL.1)

Criterios de selección

Criterios de inclusión:

- Pacientes mayores de 18 años.
- Ingreso hospitalario por COVID-19 documentado o clínicamente sospechado, con posterior diagnóstico confirmatorio para SARS-CoV-2 mediante qPCR en hisopado nasofaríngeo.
- Firma del consentimiento informado (Paciente / Familiar / Representante legal).

Criterios de exclusión:

- Imposibilidad de toma de muestra sanguínea.
- Uso de esteroides, quimioterapia, radioterapia, anticuerpo monoclonal o fármacos inmunosupresores en los dos meses previos.
- Embarazo.
- Diagnóstico conocido de inmunodeficiencia primaria o secundaria.
- IMC menor o igual a 18 kg/m².

Criterios de eliminación:

- Pérdida del seguimiento y desconocimiento del desenlace clínico.
- qPCR para SARS-CoV-2 negativa en hisopado nasofaríngeo.
- Documentación de nuevo diagnóstico de infección por VIH, HCV o HBV.
- Datos clínicos incompletos.
- Retiro del consentimiento informado o de su voluntad de seguir participando.

Métodos

1. Reclutamiento de los sujetos

Durante el periodo de la pandemia se estableció un triaje respiratorio en el hospital, en el cual se recibían casos sospechosos de COVID-19. En este servicio se evaluaban las características clínicas del paciente y en caso de tener un cuadro clínico compatible con infección por SARS-CoV-2 se valoraba su requerimiento de ingreso hospitalario. El

paciente debía tener al menos uno de los siguientes criterios de gravedad para ser hospitalizado: saturación de oxígeno periférica (SpO₂) menor de 90% al aire ambiente, taquipnea (más de 30 respiraciones por minuto) o una imagen pulmonar con más del 50% afectación. Al ingreso hospitalario, se tomaba una muestra nasofaríngea para la prueba molecular confirmatoria correspondiente por qPCR. Además, en este momento se solicitaba su consentimiento informado para participar en el presente estudio (SUPL. 2). En caso de aceptar su participación, se tomaba una muestra de sangre periférica, al mismo tiempo que el resto de las muestras para los laboratorios hospitalarios de rutina.

2. Captura de datos clínicos y parámetros de laboratorio

Por otro lado, al ingreso hospitalario también se completaba una historia clínica con los datos biométricos y antecedentes médicos de relevancia para el estudio. Se incluyeron como antecedentes personales patológicos de relevancia los siguientes: el diagnóstico previo de obesidad (IMC mayor de 30 kg/m²) o sobrepeso (IMC mayor de 25 kg/m²) por criterios de la OMS, hipertensión arterial sistémica (presión arterial promedio mayor de 135/85 mmHg al ingreso hospitalario en tres mediciones consecutivas con 20 a 30 minutos de diferencia o tratamiento farmacológico correspondiente), Diabetes Mellitus tipo 2 (hemoglobina glicosilada mayor a 6.5% al ingreso o tratamiento farmacológico conocido), antecedente de síndrome coronario agudo conocido, neoplasia conocida, enfermedad autoinmune o inmunosupresora conocida, infecciones latentes por virus o bacterias conocidas, y toxicomanías (alcoholismo, consumo regular promedio de más de 40 g/día (3 bebidas/día) en mujeres, mientras que en hombres más de 60 g/día (4 bebidas/día); tabaquismo: consumo activo de productos con tabaco al menos 1 cigarrillo en los últimos 6 meses).

En cuanto a las pruebas de laboratorio, se incluyeron biometría hemática y parámetros bioquímicos, pruebas de función hepática, pancreática y renal tales como glucosa sérica, triglicéridos, colesterol total, lipoproteínas de baja densidad (LDL), lipoproteínas de alta densidad (HDL), alanino aminotransferasa (ALT), aspartato aminotransferasa (AST), gamma glutamil-transferasa (GGT), fosfatasa alcalina (ALP), bilirrubinas totales, bilirrubina directa, bilirrubina indirecta, amilasa, lipasa, creatina-fosfoquinasa (CPK), creatina quinasa fracción cardíaca (CK-MB), proteínas totales, albumina, deshidrogenasa láctica (LDH), creatinina sérica, ácido úrico, calcio, fosforo, cloro, potasio, sodio y magnesio. Además, se incluyeron los siguientes parámetros de la respuesta inmunológica y pruebas de coagulación: proteína C reactiva (CRP), troponina I, ferritina, procalcitonina, mioglobina, dímero-D, tiempo de protrombina (PT), relación normalizada internacional (INR), tiempo de tromboplastina parcial activada (aPTT) y tiempo de trombina (TT). Todos los parámetros de laboratorio fueron medidos utilizando el analizador de química clínica completamente automatizado Beckman Coulter DxC 700 AU (Beckman Coulter Inc., Brea, CA, EUA), el analizador de hematología automatizado Coulter LH 780 (Beckman Coulter Inc., Brea, CA, EUA) y el sistema BCS® XP (Siemens Healthcare GmbH, Erlangen, Alemania), siguiendo estrictamente los protocolos estándares de operación.

Posteriormente, todos los datos recabados en la historia clínica, así como la exploración física al ingreso y los resultados de las pruebas de laboratorio, imagen y prueba molecular confirmatoria para infección por SARS-CoV-2 al ingreso fueron registrados en una base de datos para su posterior procesamiento y análisis.

3. Inmunoensayo múltiple

Siguiendo estrictas medidas de seguridad, las muestras de sangre periférica fueron tomadas con técnica aséptica en tubos de 5 mL con gel de polímero y activador de coagulación para la separación de suero (Vacutainer® SST™, BD Diagnostics, NJ, EUA). Las muestras fueron centrifugadas a 1500 revoluciones por minuto por 10 minutos para la separación del suero dentro de la primera hora después la toma. Se realizaron alícuotas para su almacén a -80°C y el material restante fue desechado de acuerdo con las normas de bioseguridad correspondientes.

Las muestras se analizaron utilizando el inmunoensayo múltiple Human Cytokine Magnetic 25-Plex Panel (Life Technologies, Carlsbad, CA, EUA). Se prepararon los reactivos y se realizó el ensayo siguiendo el protocolo del producto. Este panel permite la cuantificación de las siguientes citocinas, quimiocinas y factores de crecimiento: IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , GM-CSF, MIP-1 α , MIP-1 β , IP-10, MIG, Eotaxina, RANTES, and MCP-1 en suero humano. Los resultados fueron leídos utilizando el LuminexMagPix (Applied Biosystems, Foster City, CA, EUA).

4. Seguimiento hospitalario y análisis de predicción de mortalidad

Asimismo, se realizó un seguimiento durante toda la estancia hospitalaria de los pacientes para conocer su manejo farmacológico, requerimiento de ventilación mecánica invasiva o cuidados intensivos, además del desenlace clínico, ya fuera pérdida del seguimiento (principalmente por traslado a otro centro médico), alta hospitalaria o fallecimiento. Esto nos permitió agrupar a los pacientes en dos grupos de manera retrospectiva para su posterior análisis: sobrevivientes y no sobrevivientes.

Se realizaron los análisis y se compararon todas las variables demográficas, clínicas y parámetros de laboratorio al ingreso hospitalario, incluyendo las mediciones obtenidas a través del inmunoensayo múltiple. Utilizamos la prueba de Shapiro-Wilk para estimar el tipo de distribución de los datos. También, comparamos las variables cuantitativas entre los grupos de sobrevivientes y no sobrevivientes utilizando la prueba T de Student. Se utilizó la prueba chi-cuadrada para el análisis de variables categóricas. Se detectaron y removieron los valores atípicos utilizando el método de Grubbs, estableciendo un intervalo de confianza del 90%.

Para el análisis de predicción de mortalidad se utilizaron distintos enfoques. El primero fue a través de curvas ROC para variables individuales. Este análisis permite cuantificar la precisión con la que las pruebas de diagnóstico son capaces de discriminar entre dos estados clínicos (por ejemplo, enfermo *versus* no enfermo, sobreviviente *versus* no sobreviviente) [117]. Para estos análisis se obtuvo el área debajo de la curva (AUC), el intervalo de confianza del 95% (IC95%), sensibilidad, especificidad y la razón de momios (OR) para cada variable con diferencias significativas entre grupos en los análisis descriptivos. Además, utilizamos el índice de Youden para calcular los puntos de corte para las variables de interés.

Posteriormente, se analizó la dependencia lineal entre las variables cuantitativas de relevancia, obteniendo las correlaciones de Pearson. Se eligieron las correlaciones con coeficiente de correlación (p) mayor o igual a 0.6, y posteriormente se seleccionaron las correlaciones con significancia clínica para realizar índices entre dichas variables. Estos índices se calcularon dividiendo los valores de las variables de interés. Después, estos

valores agrupados se analizaron a través de curvas ROC para conocer la capacidad de predicción de mortalidad.

Igualmente, se realizaron análisis de supervivencia por curvas de Kaplan-Meier considerando todos los parámetros con significancia clínica para la predicción de mortalidad.

Para todos los análisis se consideró una p igual o menor a 0.05 como significativa. Los análisis se realizaron utilizando los siguientes softwares especializados: GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA 92037, EUA), MDCalc versión 20.112 (New York, NY 10003, EUA), IBM SPSS Statistics versión 26.0 (IBM, Armonk, NY, EUA), y R i386 3.5.2 terminal (Microsoft Corp., Boston, MA, EUA).

CAPITULO 3: Resultados

Inicialmente, evaluamos 545 pacientes que cumplían con los criterios de elegibilidad, de los cuales incluimos 378 pacientes, 255 fueron egresados por mejoría y 123 por defunción. (FIG. 1) Con respecto al sexo de los pacientes, no se encontraron diferencias estadísticamente significativas con respecto a la proporción entre ambos grupos, teniendo una mayor cantidad de hombres con respecto a la de mujeres; mientras que, en la edad, los pacientes no sobrevivientes tuvieron una media de casi 10 años por arriba del grupo de sobrevivientes ($p < 0.001$). En cuanto a la prevalencia de comorbilidades en ambos grupos, no existieron diferencias significativas en diabetes mellitus, hipertensión arterial, cardiopatía isquémica o cáncer. De igual manera, la prevalencia de tabaquismo y alcoholismo en ambos grupos fue similar. En cuanto a la composición corporal, los grupos de pacientes sobrevivientes y no sobrevivientes tuvieron una media de IMC correspondiente a sobrepeso, según las recomendaciones de la OMS (25.8 ± 6.7 vs 27.5 ± 4.4 , respectivamente) sin diferencias significativas entre grupos (TABLA 1).

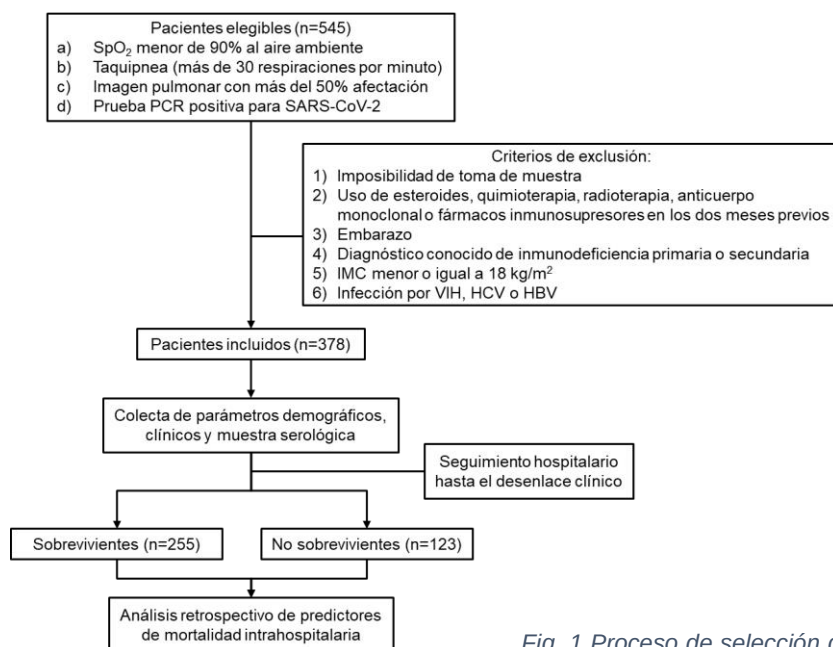


Fig. 1 Proceso de selección de pacientes incluidos en el estudio según los criterios de inclusión y exclusión.

	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	p
Edad (Años)	54.06 ± 13.79	51.47 ± 13.25 (49.83-53.11)	58.96 ± 13.72 (56.61-61.41)	.000*
Sexo (M/H)	137/241	97/158	40/83	.307
DM (%)	148 (39.2%)	92 (36.3%)	56 (45.3%)	.119
HTA (%)	116 (30.7%)	81 (31.9%)	35(28.3%)	.527
Cardiopatía (%)	12 (3.2%)	7 (2.6%)	5 (4.6%)	.333
EPOC (%)	11 (2.9%)	4 (1.7%)	7 (5.5%)	.054
Cáncer (%)	22 (5.9%)	12 (4.7%)	10 (8.3%)	.198
Tabaquismo* (%)	65 (17.3%)	43 (17.0%)	22 (17.9%)	.841
Alcoholismo° (%)	35 (9.4%)	21 (8.2%)	14 (11.9%)	.270
IMC (kg/m ²)	26.73 ± 4.86	25.82 ± 6.72 (23.51-28.14)	27.58 ± 4.34 (24.24-30.91)	.353

*Tabla 1 Variables demográficas de los pacientes incluidos en el estudio. *Tabaquismo: Consumo activo de productos con tabaco al menos 1 cigarrillo en los últimos 6 meses. °Alcoholismo: Consumo regular promedio de: Mujeres > 40 g/día (3 bebidas/día.), Hombres >60 g/día (4 bebidas/día). Datos presentados en medias y DE (IC95%), diferencia de medias por T-student. Frecuencias (%), diferencia de proporciones por X². Las diferencias se consideraron significativas cuando p < 0.05.*

Al ingreso se recabaron además los signos vitales de los pacientes, encontrando diferencias significativas en la frecuencia cardiaca, la frecuencia respiratoria y la saturación periférica de oxígeno. Dentro de otras variables clínicas, los pacientes que a la postre no sobrevivieron tuvieron en promedio estancias hospitalarias más cortas con respecto a los sobrevivientes (7.3±5.2 vs 14.7±9.9 días, respectivamente, $p = 0.014$), mientras que el requerimiento de ingreso a las unidades de cuidados intensivos fue mayor en el grupo de no sobrevivientes en comparación con los sobrevivientes (68.3% vs 23.5%, respectivamente, $p < 0.001$). (TABLA 2)

	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	p
FC (lpm)	89 (80-98)	88.5 (77.75-95.25)	90 (86-99)	.096
FR (rpm)	24 (22-26)	23 (22-25.5)	24 (22-28)	.002*
Temperatura (°C)	37.0 (36.5-37.2)	37.0 (36.5-37.2)	37.0 (36.5-37.0)	.527

PAS (mmHg)	120 (110-130)	120 (110-126.5)	130 (104.5-144)	.201
PAD (mmHg)	76.0 (67.25-80)	76.0 (67.25-80)	74.50 (62.25-92.50)	.981
PAM (mmHg)	90.33 (82.33-96.66)	90.33 (82.33-93.75)	92.83 (77.16-109.16)	.755
SpO ₂ (%)	85 (80-88)	85.5 (82-89)	81 (76-88)	.099
Días de hospitalización	11 (7-16)	12 (9-16)	9 (3.5-14)	.043*
UCI (%)	143(37.8%)	60 (23.5%)	84 (68.3%)	.000*
Días de IOT	7 (4.25-9.00)	8 (6.25-10.25)	5.5(2.5-7.5)	.035*

Tabla 2 Variables clínicas al ingreso hospitalario. Datos presentados en medianas y rangos intercuartílicos, diferencia de medianas por U Mann-Whitney. Frecuencias (%), diferencia de proporciones por χ^2 . Las diferencias se consideraron significativas cuando $p < 0.05$.

En los valores de laboratorio medidos en los pacientes al ingreso hospitalario, los valores de biometría hemática que mostraron diferencias significativas fueron: leucocitos totales, conteo total de neutrófilos, eosinófilos, linfocitos, monocitos y neutrófilos, así como sus respectivos porcentajes ($p < 0.05$) (TABLA 3). Los parámetros bioquímicos que mostraron diferencias significativas fueron: urea, colesterol total y ácido úrico ($p < 0.05$). Con respecto a las pruebas de función hepática, albúmina, AST, ALT y fosfatasa alcalina exhibieron resultados significativos ($p < 0.05$). Por otro lado, la deshidrogenasa láctica y la proteína C reactiva también tuvieron diferencias significativas entre sobrevivientes y no sobrevivientes ($p < 0.05$) (TABLA 4). El dímero D y el tiempo de protrombina fueron los únicos parámetros de la coagulación con diferencias significativas (TABLA 5), mientras que los biomarcadores cardíacos CPK, troponina I y mioglobina mostraron también cambios significativos, específicamente una elevación clara en no sobrevivientes. (TABLA 4) Finalmente, de las gasometrías arteriales, la presión parcial de oxígeno y la saturación de oxígeno fueron significativamente menores en pacientes no sobrevivientes que en sobrevivientes al ingreso hospitalario (TABLA 5).

	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	P
Leucocitos($\times 10^3/\mu\text{L}$)	8.2 (6.3-11.3)	7.6 (6.0-10.1)	9.25 (7.3-12.7)	.000*
Neutrófilos ($\times 10^3/\mu\text{L}$)	6.6 (4.6-9.8)	6.0 (4.2-8.4)	7.8 (5.9-11.7)	.000*
Eosinófilos ($\times 10^3/\mu\text{L}$)	.000 (.00-.00)	.000 (.00-.00)	.000 (.00-.00)	.005*
Linfocitos ($\times 10^3/\mu\text{L}$)	.90 (.60 -1.27)	1.0 (.70 -1.3)	.70 (.50 -1.0)	.000*
Monocitos ($\times 10^3/\mu\text{L}$)	.40 (.30-.60)	.42 (.30-.70)	.40 (.20-.50)	.001*
Neutrófilos (%)	82.8 (74.5-88.9)	78.7 (70.8-86.4)	88.5 (82.5-91.3)	.000*
Linfocitos (%)	10.6 (7.0-17.3)	12.5 (8.7-20.1)	7.8 (4.6-11.1)	.000*
Eosinófilos (%)	.000 (.000-.3)	.1 (.000-.6)	.000 (.000-.1)	.000*
Monocitos (%)	5.1 (3.2-7.1)	6.0 (4.1-8.0)	3.8 (2.5-4.9)	.000*
Hemoglobina (mg/dL)	14.6 (11.57-17.63)	14.5 (11.42-17.58)	14.6 (11.72-17.48)	.922
Hematocrito (%)	43.8 (34.7-52.9)	43.35 (34.15-52.55)	43.8 (33.8-53.8)	.966
Plaquetas ($\times 10^3/\mu\text{L}$)	222 (106-338)	222.5 (107.7-337.3)	220 (100-440)	.564

Tabla 3 Parámetros de la biometría hemática al ingreso. Datos presentados en medianas y rangos intercuartílicos, diferencia de medianas por U Mann-Whitney. Las diferencias se consideraron significativas cuando $p < 0.05$.

	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	P
Glucosa (mg/dL)	148.18 (71.74-224.62)	148.19 (56.06-240.32)	148.16 (87.4-208.92)	.999
Urea (mg/dL)	38.9 (27.9-65.5)	36.5 (26.6-55.3)	50.1 (33.0-93.2)	.000*
Creatinina (mg/dL)	.91 (.71-1.2)	.89 (.70-1.1)	1.04 (.77-1.8)	.005*
Colesterol (mg/dL)	124 (73-175)	128 (74-182)	119 (82-156)	.066
Triglicéridos (mg/dL)	150 (65-235)	150 (64-236)	155 (67-243)	.844
Ácido úrico (mg/dL)	5.2 (8.3- 2.1)	5.1 (2.1-8.1)	5.9 (1.9-9.9)	.035*
Bilirrubina Directa (mg/dL)	.19 (.13-.32)	.16 (.12-.27)	.25 (.41-.16)	.000*
Bilirrubina Indirecta (mg/dL)	.44 (.19-.69)	.43 (.19-.67)	.45 (.14-.76)	.424
Bilirrubinas totales (mg/dL)	.62 (.26-.98)	.59 (.25-.93)	.70 (.16-1.24)	.019*
Albúmina (g/dL)	3.3 (2.9-3.7)	3.5 (3.1-3.8)	3.1 (2.8-3.4)	.004*
ALT(IU/L)	30.0 (18.0-53.0)	31.0 (18.0-56.0)	27.5 (18.0-40.0)	.050*
AST (IU/L)	37 (4-70)	34 (32)	40 (48.35-65.76)	.048*

ALP (IU/L)	89.0 (72.0-119.7)	83.0 (68.0-110.0)	97.5 (78.8-135.0)	.001*
GGT (IU/L)	129.8 (25.8-233.8)	120.29 (104)	149.07 (112)	.938
DHL (IU/L)	400.0 (284.0-513.3)	351.0 (252.8-451.3)	494.0 (367.0-726.7)	.000*
CRP (mg/L)	115.0 (55.6-202.0)	89.1 (37.7-179.0)	162.0 (105.0-285.0)	.000*
Procalcitonina (mg/L)	1.85 (1.07-2.63)	1.6 (1.22-1.98)	2.35 (1.03-3.67)	.000*
Ferritina (mg/L)	758.49 (696.93-820.04)	729.93 (652.35-807.51)	815.32 (714.25-916.39)	.169
CPK (IU/L)	20.0 (15.0-27.0)	19.0 (14.0-25.0)	23.0 (18.0-28.0)	.013*
CK MB (IU/L)	20 (8-32)	19 (8-30)	22 (12-32)	.001*
Troponina I (ng/mL)	5.5 (2.2-19.1)	3.5 (1.7-9.8)	12.5 (5.1-123.5)	.001*
BNP (ng/mL)	39.5 (16.4-113.9)	31.2 (12.6-80.2)	80.7 (29.8-285.4)	.000*
Mioglobina (ng/mL)	66.4 (40.3-150.3)	56.0 (34.8-107.5)	94.9 (58.5-189.6)	.000*

Tabla 4 Parámetros bioquímicos séricos al ingreso hospitalario. Datos presentados en medianas y rangos intercuartílicos, diferencia de medianas por U Mann-Whitney. Las diferencias se consideraron significativas cuando $p < 0.05$.

	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	P
TP (s)	11.7 (10.5-12.9)	11.7 (10.6-12.8)	12.1 (10.5-13.7)	.195
TT (s)	16.7 (15.7-17.9)	16.6 (15.5-17.6)	17.0 (16.1-18.1)	.022*
TTPa (s)	25.9 (19.8-32)	25.65 (20.25-31.05)	26.8 (20.4-33.2)	.209
Fibrinógeno (µg/L)	587 (379-795)	567 (400-730)	609 (208-1010)	.167
INR	1 (.9-1.1)	1 (.9-1.1)	1 (.8-1.2)	.018*
Dímero D (µg/L)	1101.0 (659.8-2540.3)	934.0 (590.0-1871.8)	1913.0 (997.7-3696.3)	.003*
pH	7.41 (7.33-7.49)	7.40 (7.34-7.46)	7.42 (7.41-7.43)	.822
pCO ₂ (%)	32.8 (25.8-39.8)	33.1 (26-40.2)	32.1 (24.2-40)	.146
pO ₂ (%)	65.9 (53.7-79.9)	69.9 (56.0-83.8)	58.0 (46.5-73.3)	.047*
HCO ₃ (mg/dL)	21 (16.3-25.7)	21.15 (16.85-25.45)	20.8 (15.2-26.4)	.695
Lactato (mg/mL)	2.1 (1.5-2.5)	1.93 (1.5-2.4)	2.3 (1.9-2.9)	.002*
SpO ₂ (%)	91.1 (86.2-94.8)	92.3 (87.8-95.4)	87.6 (80.7-93.3)	.001*

Tabla 5 Tiempos de coagulación y parámetros de la gasometría arterial al ingreso. Datos presentados en medianas y rangos intercuartílicos, diferencia de medianas por U Mann-Whitney. Las diferencias se consideraron significativas cuando $p < 0.05$.

Además, se realizó el inmunoensayo múltiple identificando diferencias significativas entre sobrevivientes y no sobrevivientes en las siguientes citocinas y quimiocinas: interleucina (IL)-1beta, IL-2, IL-4, IL-6, IL-10, IL-15 y factor estimulante de colonias de granulocitos y macrófagos (GMCSF) (TABLA 6).

(pg/mL)	Total (n=378)	Sobrevivientes (n=255)	No sobrevivientes (n=123)	P
IL-1 β	14.0 (9.8-22.4)	12.7 (9.3-18.7)	18.9 (13.5-34.3)	.032*
IL-1RA	1896.70 (788.49-3004.90)	1303.05 (146.18-2459.92)	972.51 (195.39-1749.63)	.587
IL-2	6.5 (3.7-10.9)	4.9 (8.6-2.9)	9.3 (7.1-23.1)	.001*
IL-2R	764.86 (559.58-970.15)	538.22 (469.36-907.08)	577.74 (333.09-1031.42)	.124
IL-4	23.6 (14.6-36.37)	19.5 (13.8-26.1)	34.6 (21.5-63.9)	.019*
IL-6	83.5 (28.2-149.6)	60.9 (22.7-120.3)	103.7 (60.4-295.6)	.034*
IL-7	63.40 (23.09-103.71)	56.60 (18.68-94.58)	58.71 (16.63-100.79)	.063
IL-8	116.58 (1.48-6840.14)	58.73 (1.48-2416.36)	71.99 (17.95-2202.53)	.219
IL-10	73.6 (37.7-175.7)	56.5 (26.1-131.5)	110.1 (61.4-257.8)	.023*
IL-12	1610.46 (740.79-2480.13)	1749.91 (922.44-2577.38)	1315.30 (2142.77-1950.03)	.504
IL-13	7.7 (4.2-11.2)	8.54 (6.32-10.76)	9.41 (5.43-13.39)	.522
IL-15	279.7 (180.8-440.2)	230.2 (164.3-312.8)	422.3 (313.3-585.8)	.000*
IL-17a	14.44 (5.92-22.96)	13.03 (8.97- 17.09)	11.63 (4.28-18.98)	.253
IFN- α	750.23 (630.88-869.58)	592.36 (177.64-1007.08)	586.35 (295.62-877.08)	.066
IFN- γ	154.31 (86.3-222.32)	161.02 (105.8-216.24)	131.49 (76.13-186.85)	.450
IP-10	4776.68 (3303.89-6249.46)	3843.13 (2277.22-5409.04)	7302.09 (76.76-14527.43)	.322
MCP-1	1015.83 (881.14-1195.31)	1050.01 (156.75-1943.27)	1203.91 (201.35-2206.47)	.364
MIG	1079.40 (823.17-1335.63)	111.33 (12.85-209.81)	463.56 (291.59-635.53)	.063

MIP-1 α	97.79 (37.7-157.88)	94.91 (59.82-130.00)	90.50 (51.58-129.42)	.208
MIP-1 β	42.46 (34.87-50.05)	41.05 (34.24-47.86)	41.55 (30.39-52.71)	.208
TNF- α	24.08 (2.44-45.72)	16.00 (7.81-24.19)	16.00 (0.83-31.17)	.108
Eotaxina	10792.62 (10192.45-11392.79)	699.43 (246.54-1152.32)	493.79 (335.57-998.69)	.808
GMCSF	6.4 (5.8-8.5)	6.4 (5.6-7.0)	7.8 (6.0-9.8)	.010*

Tabla 6 Inmunoensayo múltiple, cuantificación de citocinas y quimiocinas al ingreso hospitalario. Datos presentados en medianas y rangos intercuartílicos, diferencia de medianas por U Mann-Whitney. Las diferencias se consideraron significativas cuando $p < 0.05$.

En un primer análisis retrospectivo, se formaron dos grupos, uno con 34 pacientes sobrevivientes y otro con 20 pacientes no sobrevivientes, en el caso de la biometría hemática, los parámetros con diferencias significativas entre sobrevivientes y no sobrevivientes fueron el número total de neutrófilos, siendo más elevado en pacientes no sobrevivientes ($p = 0.003$) al igual que el porcentaje de neutrófilos ($p = 0.041$), mientras que los porcentajes de linfocitos, monocitos y eosinófilos resultaron más bajos en este mismo grupo con respecto a los sobrevivientes ($p = 0.001$, $p = 0.001$, $p = 0.007$, respectivamente). Al correlacionar las variables a través del análisis de Pearson para la población total, se obtuvieron correlaciones significativas para las variables en números totales: leucocitos/neutrófilos (0.970, $p < 0.001$) y para las variables en porcentajes se obtuvieron las correlaciones significativas para: neutrófilos/linfocitos (-0.762, $p < 0.001$) y monocitos neutrófilos (-0.728, $p < 0.001$). No obstante, al hacer las mismas correlaciones por grupos, solo se conservaron las correlaciones para neutrófilos/linfocitos y monocitos/neutrófilos. Por esta razón, se decidió analizar dichos cocientes para la predicción de mortalidad.

Al realizar un cociente entre el número total de linfocitos y neutrófilos (LNR), observamos que los no sobrevivientes mostraron un LNR cuatro veces mayor con respecto a los sobrevivientes al ingreso hospitalario ($p = 0.003$) (FIG. 2A), mientras que el cociente del

número total de neutrófilos entre el número total de monocitos (NMR) fue dos veces menor en no sobrevivientes en comparación con sobrevivientes al ingreso hospitalario ($p = 0.001$) (FIG. 2B). Al realizar curvas ROC, para el LNR el área debajo de la curva (AUC) fue de 0.832 (IC95%, 0.701–0.922), con un punto de corte obtenido por índice de Youden de ≤ 0.008 , con una sensibilidad del 85.00%, una especificidad del 74.19% y un riesgo relativo (RR) de 5.8933 (IC95%, 1.9661–17.6652) (FIG. 3A). En el caso del NMR el AUC fue de 0.890 (IC95%, 0.768–0.962) con un punto de corte ≥ 17.75 obtenido por índice de Youden, con sensibilidad de 89.47%, especificidad de 80.00% y RR 8.8542 (IC95%, 2.2864–34.2878) (FIG. 3B).

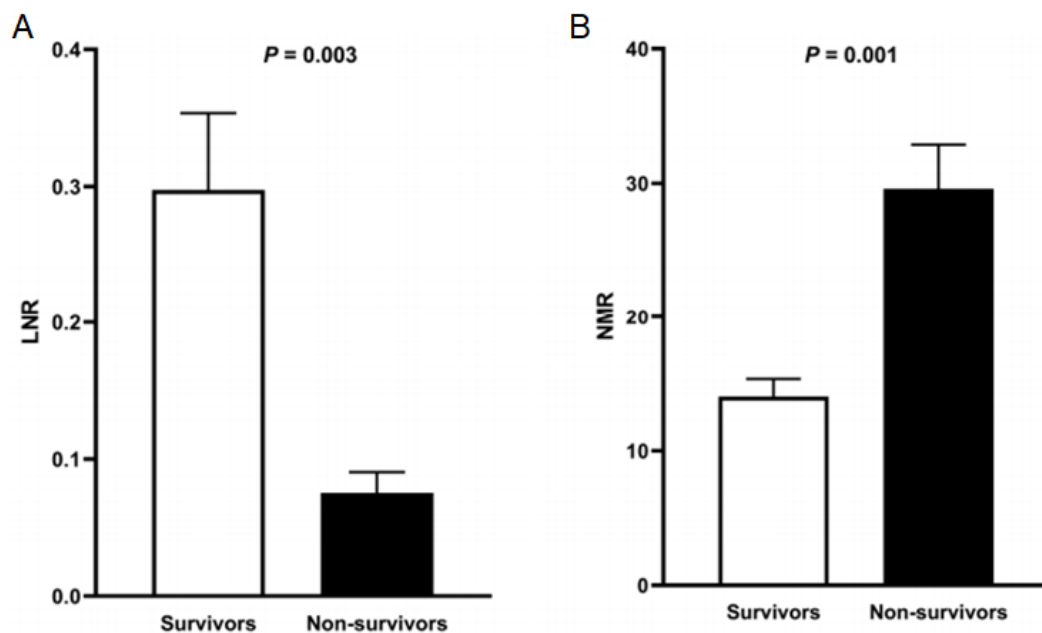


Fig.2 LNR y NMR calculados en la admisión hospitalaria. (A) LNR resultó de la división de la cuenta total de linfocitos entre la cuenta total de neutrófilos en la biometría hemática. (B) NMR resultó de la división de la cuenta total de neutrófilos entre la cuenta total de monocitos en la biometría hemática. Distribución normal estimada por la prueba de Shapiro-Wilk. Se ocupó la prueba T de Student para muestras no pareadas para comparar ambos cocientes entre sobrevivientes y no sobrevivientes. Los datos se representan como medias $\pm$ desviación estándar. LNR, Lymphocyte-to-neutrophil ratio; NMR, neutrophil-to-monocyte ratio.

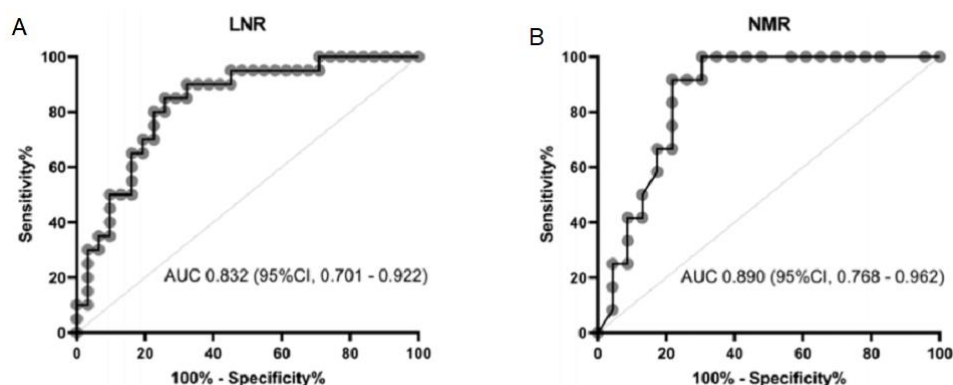


Fig. 3 Curvas ROC de LNR y NMR para la predicción de mortalidad de pacientes con COVID-19 severa, al ingreso hospitalario. (A) Para el LNR el mejor punto de corte resultó ≤ 0.088 , con sensibilidad del 85% y una especificidad del 74.19%; con un índice de Youden de 0.5919 (IC95% 0.3380–0.7387), RR de 5.8933 (IC95%, 1.9661–17.6652) y un OR de 16.2917 (IC95%, 3.7550–70.6837). (B) Para el NMR el mejor punto de corte resultó ≥ 17.75 , con sensibilidad del 89.47% y una especificidad del 80.00%; con un índice de Youden de 0.6947 (IC95%, 0.4349–0.8333), RR de 8.8542 (IC95%, 2.2864–34.2878) y un OR de 27.9286 (IC95%, 5.1435–151.6497). LNR, Lymphocyte-to-neutrophil ratio; NMR, neutrophil-to-monocyte ratio; ROC, Receiver Operating Characteristic curves; AUC, area under the ROC curve; IC, intervalo de confianza; RR, riesgo relativo; OR, Odd ratio.

Al analizar otros parámetros usados en la literatura para la predicción de mortalidad en pacientes con COVID-19 como leucocitos totales, dímero-D, proteína C reactiva o procalcitonina, éstos presentaron un AUC menor al observado en los cocientes LNR y NMR (TABLA 7). Los análisis de supervivencia por curvas de Kaplan-Meier mostraron que los pacientes con $\text{NMR} \geq 17.75$ al ingreso tienen una probabilidad de supervivencia del 50% a los 9 días (FIG. 4A), mientras que los pacientes con $\text{LNR} \leq 0.008$ tienen una probabilidad de supervivencia del 50% a los 12 días (FIG. 4B).

Parámetros	AUC	IC95%
Leucocitos totales	0.702	0.557–0.822
Neutrófilos totales	0.746	0.605–0.857
Linfocitos totales	0.735	0.593–0.849
Monocitos totales	0.605	0.458–0.739
Dímero D	0.730	0.548–0.869
Ferritina	0.777	0.601–0.901
Proteína-C reactiva	0.750	0.569–0.884
Troponina I	0.656	0.464–0.816
Deshidrogenasa láctica	0.758	0.618–0.867
Procalcitonina	0.826	0.682–0.924

Tabla 7 Áreas debajo de la curva para parámetros hematológicos y bioquímicos para predecir mortalidad en pacientes con COVID-19 severa en la admisión hospitalaria. AUC area under the ROC curve; IC, intervalo de confianza.

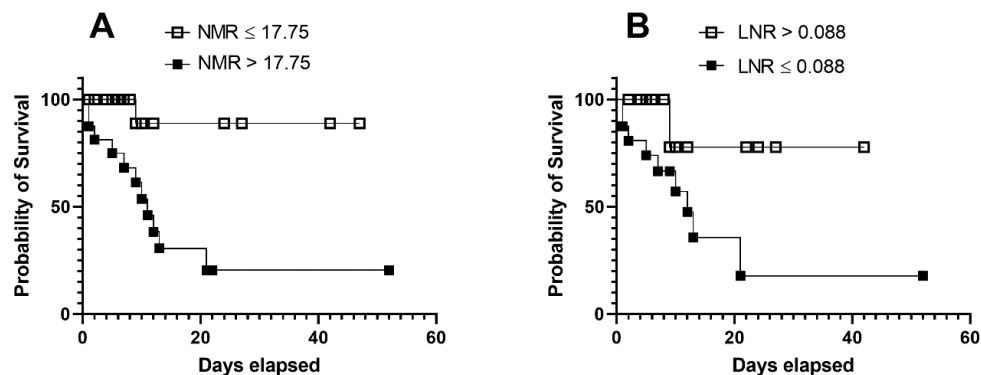


Fig. 4 Curvas Kaplan-Meier comparando la probabilidad de supervivencia a partir del NMR y el LNR al ingreso hospitalario en pacientes con COVID-19 severa. (A) Pacientes con un NMR > 17.75 a la admisión hospitalaria, muestran una probabilidad de supervivencia menor del 50% a partir del día 9 de estancia hospitalaria. (B) Pacientes con un LNR < 0.088 al ingreso hospitalario muestran una probabilidad de supervivencia menor del 50% a partir del día 12 de estancia hospitalaria. NMR, neutrophil-to-monocyte ratio; LNR, Lymphocyte-to-neutrophil ratio.

En este aspecto, propusimos un triaje basado en el NMR y LNR al ingreso, en pacientes mexicanos con COVID-19 severa, lo cual podría priorizar la atención de pacientes con mayor riesgo de fallecer, además de optimizar recursos hospitalarios y de cuidados intensivos (FIG. 5).

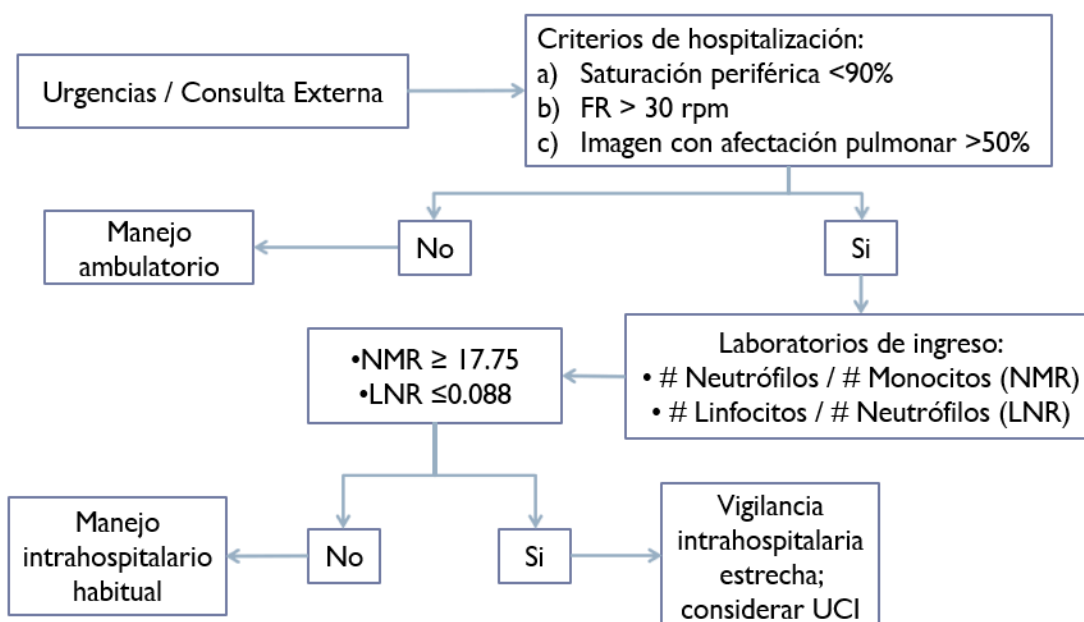


Fig. 5 Triage al ingreso hospitalario propuesto para pacientes con COVID-19 severa usando el NMR y el LNR. Con la biometría hemática de ingreso, se deberá dividir el número total de neutrófilos entre el número total de monocitos (NMR) y el número total de linfocitos entre el número total de neutrófilos (LNR). En caso de que el paciente tenga un NMR ≥ 17.75 o un LNR ≤ 0.088 se recomienda una estrecha vigilancia hospitalaria y se deberá considerar la posibilidad de ingreso a la unidad de cuidados intensivos. FR, frecuencia respiratoria; rpm, respiraciones por minuto; NMR, neutrophil-to-monocyte ratio; LNR, lymphocyte-to-neutrophil ratio.

Al comprobar ambos índices en la población total captada (N=378, 255 sobrevivientes y 123 no sobrevivientes), se calculó un AUC para el NMR de 0.741 (IC95%, 0.687–0.796) y para el LNR de 0.733 (IC95%, 0.679–0.787) (SUPL. 3).

Posteriormente, se analizó la capacidad de predicción de los biomarcadores inmunológicos obtenidos a través de inmunoensayo múltiple, y sólo la IL-15 fue capaz de pronosticar mortalidad con un AUC mayor a 0.700 (TABLA 8). Para los demás datos de laboratorio, sólo se tomaron en cuenta aquellas variables con diferencias significativas entre grupos; siendo la albúmina la única variable individual capaz de predecir mortalidad al ingreso hospitalario con una AUC mayor a 0.700 (TABLA 9). Por esta razón se decidió explorar la posibilidad de conjuntar los valores de albúmina e IL-15 mediante el cociente IL-15/albumina como opción para mejorar la predicción de ambos valores por separado.

Parámetros	AUC	IC95%
Eotaxina	0.606	0.402-0.810
GMCSF	0.688	0.492-0.883
IL-1 RA	0.595	0.398-0.792
IL-10	0.631	0.437-0.834
IL-12	0.682	0.480-0.883
IL-13	0.557	0.331-0.782
IL-15	0.797	0.658-0.915
IL-17a	0.606	0.379-0.833
IL-1 β	0.640	0.442-0.839
IL-2	0.652	0.454-0.850
IL-2R	0.530	0.299-0.761
IL-4	0.680	0.461-0.899
IL-6	0.614	0.418-0.809
IL-7	0.534	0.323-0.745
IL-8	0.627	0.432-0.821
INF- α	0.557	0.349-0.765
INF- γ	0.633	0.416-0.849
IP-10	0.561	0.335-0.786
MCP-1	0.561	0.347-0.774
MIG	0.659	0.465-0.853
MIP-1 α	0.561	0.347-0.774
MIP-1 β	0.540	0.323-0.757
TNF- α	0.509	0.286-0.733

Tabla 8. Áreas debajo de la curva para citocinas y quimiocinas al ingreso para predecir mortalidad en pacientes con COVID-19 severa. AUC area under the ROC curve; GMCSF, granulocyte and monocyte colony stimulating factor; IL, interleucina; R, receptor; INF, interferón; IP, interferon-inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferón; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; IC, intervalo de confianza.

Parámetros	AUC	IC95%
Leucocitos totales	0.597	0.508-0.686
Neutrófilos totales	0.641	0.555-0.728
Linfocitos totales	0.645	0.556-0.735
Eosinófilos totales	0.526	0.434-0.619
Monocitos totales	0.564	0.471-0.657
Urea	0.569	0.473-0.664
Creatinina	0.562	0.467-0.658
Ácido úrico	0.542	0.443-0.642
Bilirrubina directa	0.670	0.578-0.761
Bilirrubinas totales	0.627	0.535-0.720
Albúmina	0.797	0.657-0.889
ALT	0.605	0.516-0.694
AST	0.586	0.492-0.680
ALP	0.635	0.542-0.727
DHL	0.679	0.589-0.768
CRP	0.695	0.615-0.775
Procalcitonina	0.635	0.549-0.722
Ferritina	0.519	0.427-0.610
Dímero D	0.657	0.572-0.741
CPK	0.561	0.470-0.652
CK-MB	0.615	0.527-0.702
Troponina I	0.251	0.176-0.327
BNP	0.672	0.585-0.759
Mioglobina	0.663	0.580-0.747
Lactato	0.662	0.575-0.749

Tabla 9 Áreas debajo de la curva para parámetros de laboratorio al ingreso para predecir mortalidad en pacientes con COVID-19 severa. AUC area under the ROC curve; IC, intervalo de confianza; ALT, alanino aminotransferasa; AST, aspartato aminotransferasa; ALP, fosfatasa alcalina, DHL, deshidrogenasa láctica; CRP, proteína C reactiva; CPK, creatinina fosfoquinasa, CK-MB, creatina quinasa fracción cardíaca; BNP, péptido natriurético tipo B.

Los pacientes no sobrevivientes mostraron valores significativamente más bajos de albumina sérica en comparación con aquellos que sobrevivieron (3.0 ± 0.5 vs. 3.6 ± 0.6 g/dL, $p = 0.004$) (FIGURA 6A). Los niveles de IL-15 fueron casi dos veces mayores en el caso de los pacientes no sobrevivientes en comparación con los sobrevivientes (488.7 ± 242.8 vs. 261.7 ± 137.6 pg/mL, $p < 0.001$) (FIGURA 6B). No obstante, las diferencias entre sobrevivientes y no sobrevivientes fueron más evidentes al comparar el índice IL-15/albúmina; siendo hasta 2.2 veces mayor en no sobrevivientes (167.3 ± 63.8 vs. 74.2 ± 28.5 , $p < 0.001$) (FIGURA 6C).

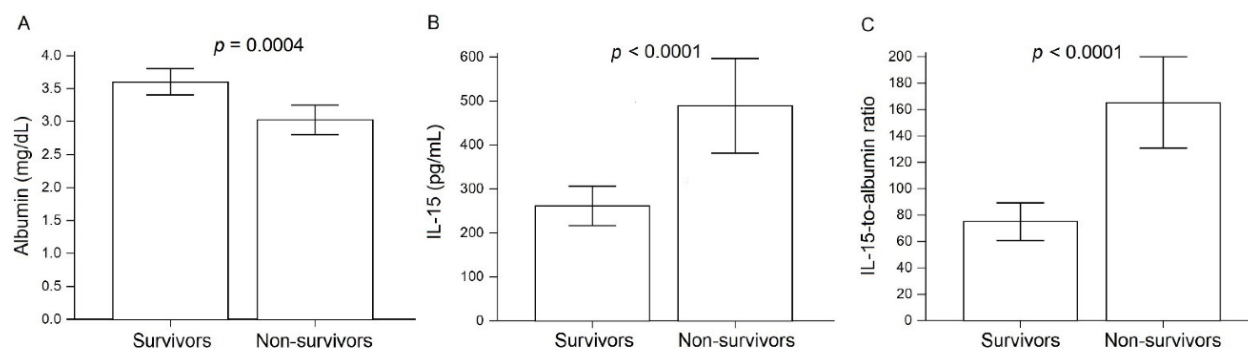


Fig. 6 Niveles séricos al ingreso de albumina (A) e IL-15 (B) en pacientes con COVID-19 severa. (C) El índice IL-15/albúmina resultó de la división del valor sérico de IL-15 entre el valor de albumina al ingreso. Los datos son presentados como medias $\pm$ desviación estándar.

Se hizo el análisis por curvas ROC para los tres parámetros anteriores, el AUC de las variables individuales aumentó significativamente de 0.797 (IC95%,0.657-0.889) para la albúmina (FIGURA 7A) y 0.797 (IC95%,0.658-0.915) para la IL-15 (FIGURA 7B) a 0.841 (IC95%, 0.725-0.922) con el índice IL-15/albúmina (FIGURA 7C). Obtuvimos un punto de corte para este índice de más de 105.4, con una sensibilidad del 72.73% y una especificidad del 87.18% para la predicción de mortalidad en pacientes con COVID-19 severa y un OR de 18.13 (IC95%, 4.81–68.37).

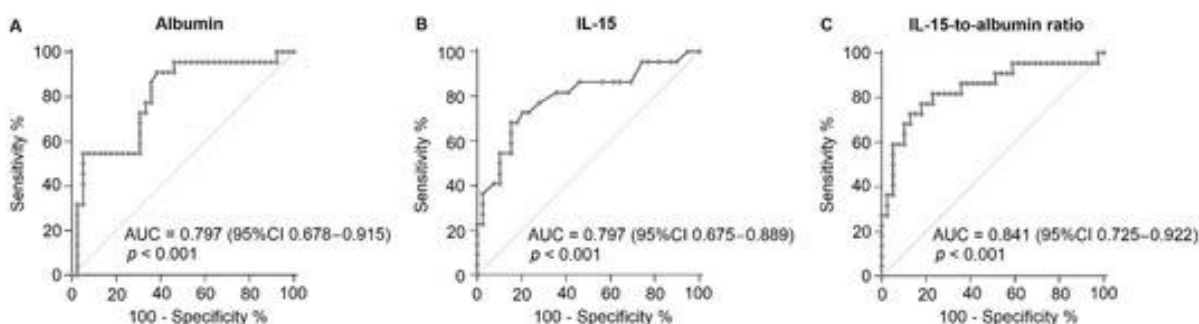


Fig. 7 Análisis por curvas ROC para albúmina (A), IL-15 (B) e índice IL-15/albúmina (C) medidas al ingreso hospitalario como predictores de mortalidad.

Estos datos confirman que la combinación de biomarcadores sanguíneos con parámetros clínicos y de laboratorio aumenta el poder predictivo de mortalidad en pacientes con COVID-19.

CAPITULO 4: Discusión

Durante la pandemia de COVID-19 se vivió una crisis sanitaria alrededor del mundo, que requería una rápida distribución de los recursos tanto materiales como humanos para evitar las elevadas cifras de mortalidad.[118] México se encontró dentro de los países con mayor mortalidad debidas a la infección por SARS-CoV-2[119]; por lo que identificar biomarcadores pronósticos, que fueran población específicos, fue una de las prioridades en el campo de la investigación.

En este caso, tras realizar los análisis de la población recibida con COVID-19 severa en un hospital de referencia de la Ciudad de México, pudimos notar que los biomarcadores inmunológicos permiten predecir mortalidad intrahospitalaria con una gran precisión en población mexicana. En primer lugar, el análisis de los parámetros de laboratorio tomados a los pacientes de rutina al ingreso hospitalario reveló una estrecha relación entre las cuentas totales de neutrófilos, linfocitos y monocitos, ligado al pronóstico de los pacientes. Los índices que relacionan estos parámetros, LNR y NMR en particular, han sido ampliamente utilizados como predictores de los desenlaces en otras patologías que involucran un desbalance en la respuesta inmunológica, como sepsis, cáncer o enfermedades autoinmunes[120–125]. En el contexto de COVID-19, se han publicado varios artículos utilizando el LNR como predictor de mortalidad, sin embargo, ninguno en población mexicana[126–132]. En todos estos artículos se concluye que este índice o su inverso NLR, son predictores independientes de mortalidad en pacientes infectados con SARS-CoV-2. En este estudio, encontramos en varios casos un conteo total de linfocitos igual a cero, por lo que se optó por elegir el número total de neutrófilos como dividendo y como divisor el número total de linfocitos. Todos estos datos coinciden con nuestros

hallazgos en la población mexicana, donde el LNR menor a 0.088 es capaz de predecir mortalidad intrahospitalaria (OR = 16.2917, 95% CI 3.7550–70.6837).

En paralelo, el NMR resultó ser mejor predictor de mortalidad en nuestro grupo de pacientes, al tener una mejor sensibilidad y especificidad que el LNR. Además, el índice NMR ha sido utilizado por otros autores para la predicción de mortalidad y de severidad en pacientes con COVID-19, no obstante, la utilidad y puntos de corte han resultado controversiales [128,130]. Por esta razón, consideramos que la búsqueda de biomarcadores y/o escalas de mortalidad debe ser población-específica y adaptada a las particularidades de un grupo de sujetos que comparten un fondo genético, alimentación, exposición a factores ambientales, entre otros, como es el caso de la población mexicana. En estudios elaborados previamente en otros países, biomarcadores como ferritina, proteína C reactiva, dímero-D, entre otros resultaron buenos predictores de mortalidad en pacientes infectados por SARS-CoV-2 [61,72,133]. Sin embargo, nuestro estudio reveló que estos biomarcadores no son tan efectivos en población mexicana como el LNR y el NMR.

La justificación detrás del uso de LNR y NMR para identificar pacientes con COVID-19 severa con mayor riesgo de mortalidad se relaciona a los diferentes roles que desempeñan los linfocitos, monocitos y neutrófilos durante la respuesta inmune contra el virus. En los seres humanos, los neutrófilos son el tipo más abundante de leucocitos con funciones destacadas como primeros respondedores en la inflamación aguda al migrar hacia los tejidos lesionados en respuesta a señales quimioatrayentes como la IL-8 [134]. Los neutrófilos ahora se consideran una de las células inmunitarias más importantes en la defensa del epitelio de las vías respiratorias contra la infección por SARS-CoV-2 al

estimular localmente la producción de IL-1 beta, IL-6, factor de necrosis tumoral alfa (TNF-alfa), y especies reactivas de oxígeno (ROS). Paradójicamente, la activación aberrante y el reclutamiento de neutrófilos intensifican la respuesta inflamatoria aguda, generando aún más daño del tejido epitelial, lo que lleva a la progresión de la enfermedad.[135] Por el contrario, de manera muy general, los linfocitos son encargados de mediar la tolerancia inmunológica a los autoantígenos, la activación de la inmunidad adaptativa específica de patógenos y, por último, la orquestación de los mecanismos inmunomoduladores [76]. Los linfocitos, incluidas las células T y B, pueden dirigir mecanismos inmunomoduladores a través de la producción de citocinas antiinflamatorias como la IL-10 y el factor de crecimiento transformante beta 1 (TGF- β 1) [136]. Paralelamente, los monocitos son leucocitos que se encuentran en circulación, en el caso de los humanos se pueden clasificar en tres subpoblaciones según la expresión de marcadores de superficie celular (CD) 14 y CD16 [137]. La subpoblación clásica de monocitos expresa altos niveles de CD14 y carecen de CD16. Los monocitos intermedios muestran expresión de CD14 y CD16; por el contrario, los monocitos no clásicos expresan niveles muy bajos de CD14 acompañados de una alta expresión de CD16[138]. Curiosamente, la reducción de las subpoblaciones de monocitos intermedios y no clásicos se ha asociado recientemente con una mayor gravedad de la infección por SARS-CoV-2[139]. Además, los monocitos también pueden diferenciarse en macrófagos activados y resolver las respuestas inflamatorias promoviendo la reparación de tejidos a través de la producción de mediadores de la resolución como IL-10, TGF- β 1, lipoxinas y resolvinas[140,141]. En otras palabras, los neutrófilos y las poblaciones celulares de linfocitos y monocitos parecen tener roles antagónicos típicos en múltiples escenarios

inflamatorios, incluido el provocado por la infección por SARS-CoV-2. Por lo tanto, es factible suponer que LNR y NMR podrían reflejar un desequilibrio entre estas células inmunitarias que, a su vez, podría estar relacionado con una inflamación excesiva y una pobre supervivencia en pacientes con COVID-19 severa. Estos datos, no solo ofrecen una opción para predecir mortalidad intrahospitalaria en pacientes con SARS-CoV-2, sino que permiten vislumbrar posibles líneas de tratamiento dirigidas a regular la respuesta inmunológica y a promover la resolución de la inflamación de las vías aéreas[142,143].

Pese a que biomarcadores provenientes de la biometría hemática nos permiten identificar con eficacia que pacientes requieren ser atendidos con prioridad, es importante evaluar nuevas herramientas que nos ayudan a mejorar el valor predictivo de las escalas clínicas utilizadas actualmente, tomando en cuenta el desarrollo tecnológico y el avance en el conocimiento de la inmunología detrás de las enfermedades. Por ejemplo, nosotros propusimos evaluar las citocinas séricas para su uso como biomarcadores de mortalidad en combinación con los parámetros de laboratorio ya analizados de rutina, en particular la IL-15 y la albumina sérica.

La combinación de valores séricos de citocinas con parámetros de laboratorio surgió recientemente como un enfoque prometedor para estimar el pronóstico en pacientes con infección por SARS-CoV-2. Un estudio realizado en China demostró que el uso de IL-2R mejora la precisión del recuento de linfocitos para predecir el riesgo de desarrollar COVID-19 severa a crítica[144]. Asimismo, la combinación entre IL-6 y el conteo de células T CD8+ mejora la predicción de mortalidad en pacientes con COVID-19, con un mejor desempeño que otras herramientas de predicción clínica, como la puntuación

CURB-65 [145]. De acuerdo con la evidencia anterior, nosotros demostramos que la combinación de IL-15 con albúmina a través de una simple división mejoró considerablemente la capacidad para identificar a los pacientes con mayor riesgo de mortalidad. Además, la IL-15 no ha sido el único biomarcador combinado con la albumina para mejorar su poder predictivo de mortalidad en COVID-19, otras combinaciones se han reportado en la literatura, como el índice neutrófilos/albumina (AUC 0.736), BUN/albumina (AUC 0.809) y CRP/albumina (AUC 0.807) [146–148].

Dado que el uso del índice IL-15-albúmina actúa como un predictor preciso de mortalidad, creemos que es de gran importancia discutir los posibles mecanismos a través de los cuales estas moléculas pueden contribuir a la progresión y gravedad de la COVID-19. La disminución de la albúmina sérica es una de las alteraciones de laboratorio más comunes en pacientes con COVID-19 que requieren hospitalización [149]. La hipoalbuminemia también es un componente central de múltiples afecciones, como el cáncer, la cirrosis, los traumatismos y la sepsis [150–153]. De hecho, la albúmina sérica baja es un predictor de mortalidad en pacientes críticos con sepsis y shock séptico [152]. Por lo tanto, creemos que es crucial comprender los posibles mecanismos por los cuales algunos elementos fisiopatológicos de COVID-19 contribuyen a la disminución de los niveles de albúmina sérica. En primer lugar, la disminución de la producción de albúmina hepática se asocia con una mayor liberación de mediadores proinflamatorios pertenecientes a la tormenta de citocinas relacionada con la COVID-19, como proteína C-reactiva, la IL-6 y el TNF- α [150]. En la infección por SARS-CoV-2, Huang y colaboradores informaron una correlación inversa entre la IL-6 y la albúmina sérica en pacientes con menor probabilidad de supervivencia [149]. Además, la IL-6 recombinante

humana induce una disminución dependiente de la dosis y el tiempo en los niveles de ARNm de albúmina en células HepG2 cultivadas in vitro [154]. Esta información se vuelve más significativa en pacientes con COVID-19, en los que los niveles elevados de TNF-alfa e IL-6 pueden disminuir directamente la producción de albúmina y provocar hipoalbuminemia. En segundo lugar, varias enfermedades inflamatorias agudas muestran que el contenido sérico de albúmina puede redistribuirse al espacio intersticial debido al aumento de la permeabilidad vascular y la fuga capilar, lo que lleva a una disminución de los valores de albúmina sérica [150,155]. Esta información concuerda con el hecho de que la COVID-19 se caracteriza por la liberación de potentes mediadores de la permeabilidad vascular, como los metabolitos del ácido araquidónico, la IL-8 y la quimiocina MCP-1, los cuales podrían contribuir a la fuga capilar de albúmina [156]. De esta manera, ambos mecanismos, tanto una disminución en la producción, como una fuga capilar pueden actuar en sinergia disminuyendo los niveles séricos de albumina detectados en pacientes con COVID-19, ambos mecanismos siendo mediados principalmente por la tormenta de citocinas generada por la infección viral. Especulamos que estos mecanismos fisiopatológicos podrían estar detrás de la correlación entre la hipoalbuminemia y la severidad y mortalidad en COVID-19.

En paralelo, existe poca evidencia que respalde el posible papel de la IL-15 en la progresión y mortalidad de COVID-19. La IL-15 es una citocina inmunorreguladora conocida por sus propiedades antivirales y es expresada por células mieloides para contribuir en la respuesta de las células T, activar las células NK y modular la inflamación [140,157]. En los linfocitos, la unión de la IL-15 con su receptor heterodímero IL-2/15R $\beta\gamma$ induce su activación, resultando en el aumento en la proliferación, así como en el

aumento de su supervivencia [158,159]. En estudios previos sobre el papel de la IL-15 en patologías pulmonares, se ha demostrado que la IL-15 inhibe la producción de citocinas proinflamatorias, reduce la hiperplasia de las células caliciformes y regula la obstrucción de las vías respiratorias inducida por alérgenos en ratones mediante la inducción de células T reguladoras productoras de IFN- γ e IL-10 [160]. En otro estudio en un modelo murino de fibroinflamación crónica pulmonar, se demostró que el tratamiento con IL-15 mejora el desenlace a través de la inducción de células NK [161]. Además, se demostró que la IL-15 mejora la actividad de las células NK, mientras que el bloqueo de la IL-15 endógena retrasa la entrada de las mismas células en las vías respiratorias en ratones infectados con influenza, dando como resultado una replicación viral incontrolada [162]. La IL-15 regula tanto la respuesta innata como la adaptativa contra la infección por influenza controlando la replicación viral temprana [157]. De igual manera, en humanos se demostró que los niveles plasmáticos de IL-15 aumentan en pacientes infectados con MERS-CoV, lo que además se asocia a un aumento en el conteo de células NK y T CD8+ [163]. En monocitos humanos, la unión de la IL-15 con su receptor de alta afinidad IL-2R β /IL-15R α induce la liberación de IL-8 y MCP-1, reclutando neutrófilos y monocitos en el espacio bronqueo alveolar contribuyendo al daño tisular y por lo tanto a la insuficiencia respiratoria [164]. También, la actividad autocrina de la IL-15 en macrófagos promueve la liberación de TNF- α , citocina que se ha visto relacionada con la activación de vías apoptóticas en células endoteliales aumentando las lesiones endoteliales y la permeabilidad vascular [165,166]. Todos estos mecanismos fisiopatológicos mediados por la IL-15 podrían estar relacionados con la severidad y/o progresión de la COVID-19. De acuerdo con esta idea, en un estudio se realizó el análisis

de biomarcadores solubles en 175 pacientes con infección grave por SARS-CoV-2; en este, se reveló que la IL-15 aumenta en la misma proporción que la mortalidad [167]. Un estudio transversal mostró que los pacientes con COVID-19 con niveles séricos elevados de IL-15 al ingreso hospitalario, experimentan una estancia hospitalaria más larga [168]. En conjunto, esta información revela un papel fundamental de la IL-15 en la progresión de la infección por SARS-CoV-2 y respalda el uso de esta citocina como predictor de mortalidad intrahospitalaria en pacientes con COVID-19. Sin embargo, se requieren más estudios para develar los mecanismos moleculares subyacentes específicos que correlacionen la albumina, la IL-15 y la supervivencia en pacientes infectados por SARS-CoV-2.

Otro fenómeno captado en el desarrollo de la tesis fue el hallazgo inesperado de que no hubo diferencia entre los sobrevivientes y los no sobrevivientes por género e IMC. Los primeros estudios documentaron que la tasa de letalidad de los casos de COVID-19 era mayor en los hombres que en las mujeres. Sin embargo, algunos estudios han encontrado que una vez que se desarrolla una COVID-19 severa, el riesgo de morir es similar en mujeres y hombres [169]. De esta manera, todos los pacientes con criterios de hospitalización que incluimos en el estudio, no encontramos diferencias significativas en la mortalidad entre hombres y mujeres, lo que concuerda con los hallazgos más recientes. Los hombres tienen más probabilidades de ser hospitalizados que las mujeres; sin embargo, la mortalidad es similar en ambos sexos una vez que se presenta la enfermedad grave. Del mismo modo, aunque notamos que el IMC y la prevalencia de la obesidad tendían a ser más altos en los no sobrevivientes que en los sobrevivientes; no obstante, no encontramos diferencias significativas en la mortalidad por COVID-19. De

acuerdo con nuestros hallazgos, varios estudios han informado que el IMC o la obesidad no son necesariamente predictores independientes de mortalidad hospitalaria en COVID-19 [170–172]. Podemos tratar de explicar esta aparente controversia considerando que, en el momento de la hospitalización, todos los pacientes incluidos en nuestro estudio habían desarrollado la forma severa de la COVID-19, incluyendo dificultad respiratoria y neumonía. Sin embargo, debemos considerar estos hallazgos con precaución, pues se necesita más investigación para comprender los roles del sexo y la obesidad en la mortalidad por COVID-19 dentro de la población mexicana.

CAPITULO 5: Limitaciones y perspectivas

Dentro de las limitaciones en la elaboración de esta tesis se encuentra el tamaño de la muestra, ya que para validar los resultados obtenidos se requiere evaluar los índices encontrados en una n mayor. De la misma forma, para su aplicación en el campo clínico real, se deberán incluir en el análisis pacientes inmunológicamente comprometidos, como mujeres embarazadas o con enfermedades autoinmunes, cáncer, infección por VIH, VHB o VHC, que en este caso fueron excluidos ya que al ser pacientes vulnerables tienden a ser hospitalizados con mayor frecuencia por COVID-19. Finalmente, los primeros índices, LNR y NMR, obtenidos a través de parámetros simples como la biometría hemática son de fácil acceso en contextos de recursos limitados. Para el caso del índice IL-15/albumina, se deben desarrollar tecnologías para su cuantificación rápida, eficaz y a bajo costo en un escenario clínico como el que ofrecen los hospitales del tercer nivel de atención.

Durante la pandemia nos enfrentamos al desconocimiento completo del manejo de los pacientes infectados; la inexistencia de tratamiento específico llevó a muchos países, incluido el nuestro, a una crisis sanitaria. Los biomarcadores son herramientas eficaces que nos permiten identificar a los pacientes con peor pronóstico para dirigir y distribuir los recursos disponibles de la mejor manera posible. En este contexto y para futuros eventos epidemiológicos, el presente trabajo de tesis demuestra que conjuntar biomarcadores comúnmente utilizados en la clínica puede ser un excelente primer recurso para identificar pacientes en riesgo. Sin embargo, para lograr un mayor poder predictivo se debe considerar el uso de nuevas estrategias y tecnologías diferentes a la práctica clínica habitual, como en este caso el uso de un inmunoensayo múltiple.

Además, la cooperación interdisciplinaria es clave para el rápido avance del conocimiento. Por esta razón, es importante sensibilizar al personal clínico sobre la importancia de la investigación traslacional. Así como facilitar el acceso a los datos clínicos y muestras biológicas al personal de investigación (biólogos, químicos, bioinformáticos, etc.) desde el inicio es clave para agilizar la producción del conocimiento que permita mejorar el manejo clínico y desenlace de los pacientes.

CAPITULO 6: Conclusiones

En conclusión, este estudio apoya la idea de estudiar biomarcadores en poblaciones específicas para una mejor predicción de mortalidad hospitalaria, particularmente en pacientes con COVID-19 severo. Esto permitirá la distribución adecuada de recursos en caso de encontrarnos con otra crisis sanitaria como la que se vivió durante los primeros años de la pandemia. La presente tesis muestra dos índices que correlacionan tres parámetros obtenidos de una biometría hemática rutinaria al ingreso hospitalario para la predicción de mortalidad en pacientes infectados por COVID-19, el LNR con un punto de corte inferior a 0.088 y el NMR con un punto de corte superior a 17.75. Asimismo, se muestra como parámetros inmunológicos no medidos rutinariamente son capaces de mejorar la predicción de mortalidad de biomarcadores ya utilizados en la clínica. El índice IL-15/albúmina fue capaz de mejorar el AUC = 0.797 de la albumina individualmente a 0.841 conjuntando ambos valores. Los mecanismos moleculares que correlacionan todos estos biomarcadores con la mortalidad y severidad de la COVID-19 permanecen aún sin ser comprendidos por completo, por lo que estudios que revelen su relación aún son necesarios ya que podrían convertirse en potenciales blancos terapéuticos si se conocen los mecanismos subyacentes.

CAPITULO 7: Manuscritos relevantes

En este capítulo se encuentran las publicaciones en revistas científicas internacionales indexadas directamente relacionadas con la elaboración de la presente tesis.

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CAPITULO 8: Otros Manuscritos

En la presente sección se encuentra un listado de los artículos publicados durante el desarrollo de proyecto doctoral. Estos trabajos complementaron algunos elementos dentro del proyecto. Además, la elaboración de estos permitió explorar diversas técnicas tanto de laboratorio como de análisis que contribuyeron al resultado final de la presente tesis.

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Article

The Neutrophil-to-Monocyte Ratio and Lymphocyte-to-Neutrophil Ratio at Admission Predict In-Hospital Mortality in Mexican Patients with Severe SARS-CoV-2 Infection (Covid-19)

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Abstract: There is a deep need for mortality predictors that allow clinicians to quickly triage patients with severe coronavirus disease 2019 (Covid-19) into intensive care units at the time of hospital admission. Thus, we examined the efficacy of the lymphocyte-to-neutrophil ratio (LNR) and neutrophil-to-monocyte ratio (NMR) as predictors of in-hospital death at admission in patients with severe Covid-19. A total of 54 Mexican adult patients with Covid-19 that met hospitalization criteria were retrospectively enrolled, followed-up daily until hospital discharge or death, and then assigned to survival or non-survival groups. Clinical, demographic, and laboratory parameters were recorded at admission. A total of 20 patients with severe Covid-19 died, and 75% of them were men older than 62.90 ± 14.18 years on average. Type 2 diabetes, hypertension, and coronary heart disease were more prevalent in non-survivors. As compared to survivors, LNR was significantly fourfold decreased while NMR was twofold increased. $LNR \leq 0.088$ predicted in-hospital mortality with a sensitivity of 85.00% and a specificity of 74.19%. $NMR \geq 17.75$ was a better independent risk factor for mortality with a sensitivity of 89.47% and a specificity of 80.00%. This study demonstrates for the first time

that NMR and LNR are accurate predictors of in-hospital mortality at admission in patients with severe Covid-19.

Keywords: SARS-Cov-2; Covid-19; mortality predictor; in-hospital death risk; lymphocyte; monocyte; neutrophil; intensive care unit

1. Introduction

The outbreak of coronavirus disease 2019 (Covid-19) is an ongoing global pandemic caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) that has affected the lives of millions of people worldwide [1]. Although more than 85% of patients with Covid-19 show a self-limiting illness with symptoms such as mild fever, dry cough, and fatigue, some patients develop severe pneumonia that can progress to acute respiratory distress syndrome, multiple organ failure, and death [2]. Mortality rates directly attributed to severe Covid-19 ranges from 3% in countries like China to 10% in countries like Spain [3,4]. However, mortality rates above 10% have been alarmingly reported in other countries like Italy, UK, and Mexico [4–6]. In particular, a 145% excess mortality associated with severe Covid-19 has been recently estimated for some Mexico's cities, which is presumably associated with higher prevalence of comorbidities such as obesity, type 2 diabetes (T2D), and hypertension in our population [7]. Thus, it is of great importance to develop novel tools that help us to estimate the mortality risk at admission in patients with severe Covid-19 that need hospitalization, with the aim of quickly triaging them into intensive care units.

Clinical features of patients with Covid-19 have revealed a number of potential biochemical markers associated with in-hospital mortality. In particular, the blood levels of D-dimer, ferritin, C-reactive protein (CRP), troponin I, lactate dehydrogenase (LDH), and procalcitonin have been extensively studied due to their apparently good accuracy to discriminate patients with the most severe courses of the disease [8–13]. In parallel, numerous studies have also proposed the use of hematological markers such as lymphocyte, monocyte, and neutrophil counts that seem to associate with increased severity and mortality in patients with Covid-19 [14,15]. Actually, it has been consistently demonstrated that, in seriously ill patients with Covid-19, the blood count of lymphocyte and monocyte populations decreases whereas neutrophil counts increase [15]. However, total counts of these white blood cells (WBC) are merely associated with severity and mortality of Covid-19 but fails to predict death risk in clinical practice [14]. For this reason, a growing body of evidence has now proposed that ratios among lymphocytes, monocytes, and neutrophils are more accurate to predict mortality than cell count of lymphocytes, monocytes, and neutrophils by itself [16,17]. As a matter of fact, numerous reports have previously shown that the lymphocyte-to-neutrophil ratio (LNR) and the neutrophil-to-monocyte ratio (NMR) are markers associated with poorer survival in acute lung bacterial infection, sepsis, and many solid tumors including gastroesophageal, colorectal, pancreatic, prostate, and breast carcinoma [18–20]. Nevertheless, NLR and NMR have been scarcely explored as mortality predictors in patients with severe Covid-19, especially in countries with a disproportionate number of deaths related to this disease, such as Mexico.

For this reason, our main goal was to assess the efficacy of LNR and NMR as predictors of mortality at admission in adult patients with severe SARS-CoV-2 infection that met hospitalization criteria.

2. Material and Methods

2.1. Subjects

A total of 54 Mexican patients of both sexes aged 18 years or older, with severe Covid-19, admitted to the Department of Intensive Medical Therapy of the General Hospital of Mexico, were retrospectively enrolled in the study from 23 March 2020 to 26 June 2020. The diagnosis

of Covid-19 was confirmed by specific detection of the SARS-CoV-2 in nasopharyngeal swabs using quantitative polymerase chain reaction (qPCR) according to the World Health Organization (WHO) technical guidance [21]. Patients seriously ill with Covid-19 that needed hospitalization were enrolled in the study if they met at least one of the following criteria: oxygen saturation level (SpO_2) < 93% on room air, respiratory distress > 30 breaths/min, and/or > 50% lung involvement on imaging. Patients were excluded from the study if they had previous diagnosis of cancer, end-stage kidney and/or liver failure, endocrine disorders, infectious diseases, and/or autoimmune disease. We also excluded from the study human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV)-seropositive individuals, patients under long-term immunomodulatory medication including non-steroidal anti-inflammatory drugs, unconscious patients that had lost ability to respond, and pregnant or lactating women. All participants provided written informed consent, previously approved by the institutional ethical committee of the General Hospital of Mexico (registration number of the ethical code approval: DI/20/501/03/17), which guaranteed that the study was conducted in rigorous adherence to the principles that were described in the 1964 Declaration of Helsinki and its posterior amendment in 2013.

2.2. Data Collection

Laboratory and clinical data were collected at hospital admission from all patients enrolled in the study using the digital version of the electronic health record of the General Hospital of Mexico. Demographic data and medical history including previous diagnosis of obesity (body mass index (BMI) > 30 kg/m²), T2D (glycated hemoglobin (HbA1c) > 6.5% or medication to treat T2D), high blood pressure (>130/80 mm Hg or medication to treat hypertension), and coronary heart disease (CHD) were personally recorded at the Department of Intensive Medical Therapy. Furthermore, the need for invasive mechanical ventilation (IMV) with an oral endotracheal tube attached to a mechanical ventilator, time to extubation, inpatient days up to the main outcome, and drug regimen to treat Covid-19 were personally recorded at the Department of Intensive Medical Therapy. All patients were followed-up with daily, until hospital discharge or death, and then were assigned to survival or non-survival groups, respectively.

2.3. Laboratory Measures

Laboratory measures included biochemical parameters, liver function tests, and kidney function test as follows: blood glucose, triglycerides, total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, indirect bilirubin, amylase, lipase, creatinine phosphokinase (CPK), creatinine kinase myocardial band (CK-MB), total protein, albumin, LDH, serum creatinine, uric acid, calcium, phosphorus, chloride, potassium, sodium, and magnesium. Additional measures also included hematological and immune parameters, and coagulation tests as follows: WBC count, red blood cell (RBC) count, lymphocytes, monocytes, eosinophils, basophils, band cells, neutrophils, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW), platelet count, mean platelet volume, CRP, troponin I, ferritin, procalcitonin, myoglobine, D-dimer, fibrinogen, prothrombin time (PT), international normalized ratio (INR), activated partial thromboplastin time (aPTT), and thrombin time (TT). LNR resulted of dividing the total lymphocyte count by the total neutrophil count. NMR resulted of dividing the total neutrophil count by the total monocyte count. All laboratory parameters were measured using the Beckman Coulter DxC 700 AU Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA), the Coulter LH 780 Hematology Analyzer (Beckman Coulter Inc., Brea, CA, USA), and the BCS[®] XP System (Siemens Healthcare GmbH, Erlangen, Germany) following strict adherence to the standard operating procedures.

2.4. Statistics

All laboratory parameters were recorded at hospital admission and then analyzed retrospectively. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student's T-test was used to compare age, BMI, blood glucose, triglycerides, total cholesterol, LDL, HDL, ALT, AST, GGT, alkaline phosphatase, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, LDH, serum creatinine, uric acid, calcium, phosphorus, chloride, potassium, sodium, magnesium, WBC count, RBC count, lymphocytes, monocytes, eosinophils, basophils, band cells, neutrophils, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW, platelet count, mean platelet volume, CRP, troponin, ferritin, procalcitonin, D-dimer, fibrinogen, PT, INR, aPTT, TT, time to extubation, inpatient days, LNR, and NMR between survival and non-survival groups, and data are expressed as mean $\pm$ standard deviation. The chi-squared test was used to compare the prevalence of obesity, T2D, hypertension, coronary heart disease, the number of patients that required IMV, and the sex ratio in survival and non-survival groups, and data are expressed as absolute values or percentages. Receiver operating characteristic (ROC) curves were conducted to calculate area under the curve (AUC) and 95% confidence interval (95% CI) for LNR and NMR. ROC curves together with the Youden index was used to calculate optimal cut-off points, sensitivity, specificity, Relative Risk (RR), Odd Ratio (OR), and 95% CI for LNR and NMR. The sensitivity, specificity, and OR were also calculated for LNR and NMR together by means of the chi-squared test. Multiple regression analysis was performed to estimate the effect of potential confounding variables including obesity, T2D, hypertension, CHD, age, and gender on the accuracy of LNR and NMR as predictors of in-hospital death. Survival analyses were performed for LNR, NMR, and other potential mortality predictors by the Kaplan–Meier method. Differences were considered significant when $p < 0.05$. Statistical analyses were performed by means of the GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA 92037, USA), the MDCalc Software (New York, NY 10003, USA), the IBM SPSS Statistics version 26.0 (IBM, Armonk, NY, USA), and the R i386 3.5.2 terminal (Microsoft Corp., Boston, MA, USA).

3. Results

Figure 1 shows the selection process of patients enrolled in the study based on the inclusion and exclusion criteria.

After hospital admission, 63% ($n = 34$) seriously ill patients with Covid-19 survived, whereas nearly 37% ($n = 20$) patients with a severe course of this disease died (Table 1). Among non-survivors, 75% ($n = 15$) of patients were men ($p = 0.002$). Patients in the non-survival group were, on average, about 9 years older than individuals in the survival group (62.90 ± 14.18 versus 54.06 ± 12.43 years, respectively) (Table 1). In contrast, there were no significant differences between survivors and non-survivors for BMI (28.24 ± 4.60 versus 27.88 ± 4.05 kg/m², respectively). Furthermore, there were no significant differences between survivors and non-survivors with respect to the prevalence of obesity ($p = 0.277$). On the contrary, the prevalence of T2D ($p = 0.037$), hypertension ($p = 0.006$), and CHD ($p = 0.033$) was significantly increased in the non-survival group as compared to the survival group (Table 1). The need for intubation was significantly greater in the non-survival group than in the survival group (83.33 versus 30.43%, respectively) with no significant differences with respect to the time to extubation, number of days that patients spent in hospital, and the five-drug regimen used for treating patients that included aziythromycin, ceftriaxone, enoxaparin sodium, dexamethasone, and acetaminophen (Table 1).

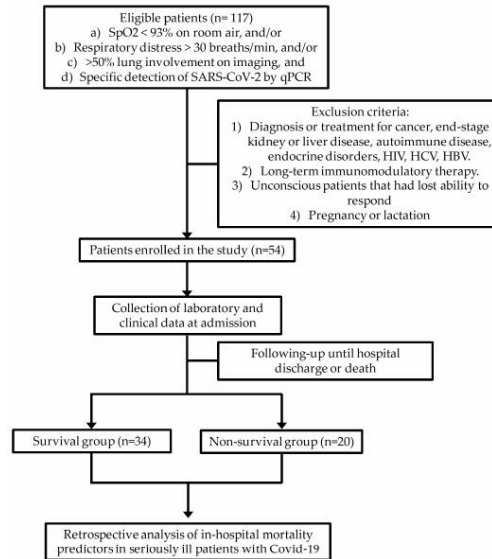


Figure 1. Schematic flow chart showing the selection process of patients enrolled in the study based on the inclusion and exclusion criteria. SpO₂, oxygen saturation level; SARS-CoV-2, novel severe acute respiratory syndrome coronavirus-2; qPCR, quantitative polymerase chain reaction; HIV, human immunodeficiency virus; HCV, hepatitis C virus; HBV, hepatitis B virus; Covid-19, coronavirus disease 2019.

Table 1. Demographic and clinical characteristics in hospitalized patients with severe Covid-19. Abbreviations: W, women; M, men; BMI, body mass index; T2D, type 2 diabetes; IMV, invasive mechanical ventilation. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student’s T-test was used to compare numerical variables and data are presented as mean $\pm$ standard deviation. The chi-squared test was used to compare categorical variables and data are expressed as absolute values or percentages. * Differences were considered significant when $p < 0.05$.

Parameters	Survivors (n = 34)	Non-Survivors (n = 20)	p Value
Gender (W/M)	21/13	5/15	0.002 *
Age (years)	54.06 $\pm$ 12.43	62.9 $\pm$ 14.18	0.020 *
BMI (kg/m ²)	28.24 $\pm$ 4.60	27.88 $\pm$ 4.05	0.903
Obesity prevalence (%)	52.17	41.66	0.277
T2D prevalence (%)	43.47	75.00	0.037 *
Hypertension prevalence (%)	17.39	58.33	0.006 *
Coronary heart disease (%)	8.69	33.33	0.033 *
IMV (%)	30.43	83.33	0.001 *
Time to extubation (days)	2.43 $\pm$ 0.79	3.66 $\pm$ 0.82	0.167
Inpatient days (days)	15.65 $\pm$ 3.13	8.41 $\pm$ 1.66	0.060
Drug regimen	Aziythromycin, ceftriaxone, enoxaparin sodium, dexamethasone, and acetaminophen		-

At hospital admission, there were no significant differences between survivors and non-survivors with respect to serum glucose, creatinine, uric acid, triglycerides, total bilirubin, indirect bilirubin, ALT, ALP, total protein, amylase, lipase, CPK, CK-MB, phosphorus, magnesium, sodium, potassium, chlorine, and calcium (Table 2). On the contrary, blood levels of urea ($p = 0.004$), total cholesterol ($p = 0.010$), HDL ($p = 0.015$), LDL ($p = 0.012$), direct bilirubin ($p = 0.012$), AST ($p = 0.021$), GGT ($p = 0.015$), albumin ($p = 0.001$), and LDH ($p = 0.001$) showed statistically significant differences between survivors and non-survivors at hospital admission (Table 2).

Table 2. Biochemical parameters, kidney function tests, and liver function tests at admission in hospitalized patients with severe Covid-19. Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; CPK, creatinine phosphokinase; CK-MB, creatinine kinase myocardial band. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student’s *t*-test was used to compare numerical variables and data are presented as mean $\pm$ standard deviation. * Differences were considered significant when $p < 0.05$.

Parameters	Survivors (<i>n</i> = 34)	Non-Survivors (<i>n</i> = 20)	<i>p</i> Value
Glucose (mg/dL)	148.19 $\pm$ 92.13	148.16 $\pm$ 60.76	0.999
Urea (mg/dL)	42.05 $\pm$ 37.06	91.07 $\pm$ 77.16	0.004 *
Creatinine (mg/dL)	0.995 $\pm$ 1.18	1.85 $\pm$ 2.79	0.133
Uric Acid (mg/dL)	5.65 $\pm$ 2.81	7.39 $\pm$ 4.09	0.097
Total Cholesterol (mg/dL)	151.77 $\pm$ 34.98	126.06 $\pm$ 20.85	0.010 *
Triglycerides (mg/dL)	166.04 $\pm$ 67.93	169.75 $\pm$ 54.89	0.853
HDL (mg/dL)	34.30 $\pm$ 10.88	23.92 $\pm$ 12.09	0.015 *
LDL (mg/dL)	95.30 $\pm$ 30.14	70.38 $\pm$ 18.69	0.012 *
Total bilirubin (mg/dL)	0.684 $\pm$ 0.401	0.804 $\pm$ 0.315	0.349
Direct bilirubin (mg/dL)	0.185 $\pm$ 0.133	0.323 $\pm$ 0.215	0.012 *
Indirect bilirubin (mg/dL)	0.492 $\pm$ 0.254	0.498 $\pm$ 0.153	0.939
ALT (IU/L)	35.42 $\pm$ 26.29	41.47 $\pm$ 28.92	0.465
AST (IU/L)	34.74 $\pm$ 22.86	59 $\pm$ 47.74	0.021 *
ALP (IU/L)	91.00 $\pm$ 27.22	125.29 $\pm$ 120.86	0.134
GGT (IU/L)	69.54 $\pm$ 43.07	124.33 $\pm$ 101.26	0.015 *
Total Protein (mg/dL)	6.59 $\pm$ 0.525	6.31 $\pm$ 0.627	0.099
Albumin (mg/dL)	3.59 $\pm$ 0.479	2.94 $\pm$ 0.409	0.001 *
LDH (IU/L)	320.63 $\pm$ 132.11	475.63 $\pm$ 195.83	0.001 *
Amylase (IU/L)	46.1 $\pm$ 35.39	56.83 $\pm$ 26.93	0.429
Lipase (IU/L)	116.00 $\pm$ 304.00	52.36 $\pm$ 52.27	0.502
CPK (IU/L)	101.52 $\pm$ 97.49	699.57 $\pm$ 1937.82	0.114
CK-MB (IU/L)	23.80 $\pm$ 11.90	44.43 $\pm$ 59.39	0.099
Phosphorus (mg/dL)	3.89 $\pm$ 2.01	4.25 $\pm$ 1.67	0.516
Magnesium (mg/dL)	2.74 $\pm$ 3.53	2.42 $\pm$ 0.629	0.698
Sodium (mEq/L)	136.00 $\pm$ 6.56	138.53 $\pm$ 6.31	0.182
Potassium (mEq/L)	5.56 $\pm$ 6.67	4.47 $\pm$ 0.719	0.484
Chlorine (mEq/L)	100.28 $\pm$ 7.19	101.84 $\pm$ 6.49	0.441
Calcium (mg/dL)	8.63 $\pm$ 0.686	8.14 $\pm$ 1.20	0.076

There were no significant differences between survivors and non-survivors for prothrombin time, INR, thrombin time, aPTT, fibrinogen, troponin I, and myoglobine (Table 3). On the other hand, non-survivors showed significantly higher values of D-dimer ($p = 0.017$), ferritin ($p = 0.012$), CRP ($p = 0.014$), and procalcitonin ($p = 0.037$) than survivors at the time of admission (Table 3).

Table 3. Coagulation and immune parameters at admission in hospitalized patients with severe Covid-19. Abbreviations: INR, international normalized ratio; aPTT, activated partial thromboplastin time; CRP, C-reactive protein. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student’s *t*-test was used to compare numerical variables and data are presented as mean $\pm$ standard deviation. * Differences were considered significant when $p < 0.05$.

Parameters	Survivors (<i>n</i> = 34)	Non-Survivors (<i>n</i> = 20)	<i>p</i> Value
Prothrombine time (s)	11.97 $\pm$ 2.49	12.96 $\pm$ 1.55	0.237
INR	0.995 $\pm$ 0.246	1.10 $\pm$ 0.167	0.216
Thrombin time (s)	16.76 $\pm$ 1.61	17.78 $\pm$ 1.42	0.085
aPTT (s)	26.18 $\pm$ 6.99	26.36 $\pm$ 5.68	0.941
Fibrinogen (mg/dL)	637.23 $\pm$ 222.29	703.75 $\pm$ 206.92	0.400
D-dimer (Ug/L)	975.33 $\pm$ 477.81	8260.33 $\pm$ 13354.39	0.017 *
Ferritin (ng/mL)	522.62 $\pm$ 451.93	937.00 $\pm$ 415.09	0.012 *
CRP (mg/L)	129.43 $\pm$ 90.10	221.07 $\pm$ 108.32	0.014 *
Troponin I (ng/mL)	37.68 $\pm$ 63.57	68.458 $\pm$ 112.73	0.338
Myoglobine (ng/L)	94.49 $\pm$ 107.76	254.38 $\pm$ 353.69	0.089
Procalcitonin (ng/mL)	0.220 $\pm$ 0.256	2.01 $\pm$ 4.07	0.037 *

No significant differences between survivors and non-survivors were found for most of the hematological parameters including total cell counts of leukocytes, lymphocytes, monocytes, eosinophils, basophils, and RBC (Table 4). However, there were significant differences between survivors and non-survivors with respect to the percentage of neutrophils ($p = 0.041$), lymphocytes ($p = 0.001$) monocytes ($p = 0.001$), and eosinophils ($p = 0.007$), and the neutrophil count ($p = 0.003$) (Table 4).

Table 4. Hematological parameters at admission in hospitalized patients with severe Covid-19. Abbreviations: MCV, mean corpuscular volume; MHC, mean corpuscular hemoglobin; RDW, red blood cell distribution. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student’s *t*-test was used to compare numerical variables and data are presented as mean $\pm$ standard deviation. * Differences were considered significant when $p < 0.05$.

Parameters	Survivors (<i>n</i> = 34)	Non-Survivors (<i>n</i> = 20)	<i>p</i> Value
Leukocytes ($\times 10^3/\mu\text{L}$)	13.48 $\pm$ 25.44	13.19 $\pm$ 6.34	0.960
Neutrophil percentage (%)	73.95 $\pm$ 17.19	84.72 $\pm$ 18.89	0.041 *
Lymphocyte percentage (%)	16.79 $\pm$ 11.35	7.48 $\pm$ 5.65	0.001 *
Monocyte percentage (%)	5.96 $\pm$ 2.54	3.35 $\pm$ 1.42	0.001 *
Band cells (%)	0.000 $\pm$ 0.000	0.08 $\pm$ 0.358	0.217
Eosinophil percentage (%)	0.739 $\pm$ 0.974	0.110 $\pm$ 0.281	0.007 *
Basophil percentage (%)	0.429 $\pm$ 1.23	0.085 $\pm$ 0.123	0.221
Neutrophils ($\times 10^3/\mu\text{L}$)	7.39 $\pm$ 4.44	11.93 $\pm$ 5.99	0.003 *
Lymphocytes ($\times 10^3/\mu\text{L}$)	2.09 $\pm$ 4.40	0.750 $\pm$ 0.426	0.181
Monocytes ($\times 10^3/\mu\text{L}$)	0.519 $\pm$ 0.256	0.450 $\pm$ 0.276	0.364
Eosinophils ($\times 10^3/\mu\text{L}$)	0.096 $\pm$ 0.272	0.015 $\pm$ 0.049	0.193
Basophils ($\times 10^3/\mu\text{L}$)	0.339 $\pm$ 1.87	0.000 $\pm$ 0.000	0.423
Erythrocyte ($\times 10^6/\mu\text{L}$)	4.71 $\pm$ 0.893	4.75 $\pm$ 1.09	0.884
Hemoglobin (g/dL)	14.17 $\pm$ 2.56	14.42 $\pm$ 3.11	0.756
Hematocrit (%)	42.40 $\pm$ 7.58	42.82 $\pm$ 9.44	0.863
MCV (fL)	90.54 $\pm$ 5.88	91.35 $\pm$ 4.15	0.597
MCH (pg)	30.34 $\pm$ 2.73	30.42 $\pm$ 1.64	0.905
RDW (%)	15.07 $\pm$ 3.38	14.72 $\pm$ 2.07	0.685
Platelets ($\times 10^3/\mu\text{L}$)	266.61 $\pm$ 111.11	240.25 $\pm$ 113.83	0.416

On average, LNR showed a significant fourfold decrease in non-survivors as compared to survivors ($p = 0.003$) (Figure 2A). In parallel, NMR exhibited a significant twofold increase in non-survivors with respect to survivors ($p = 0.001$) (Figure 2B).

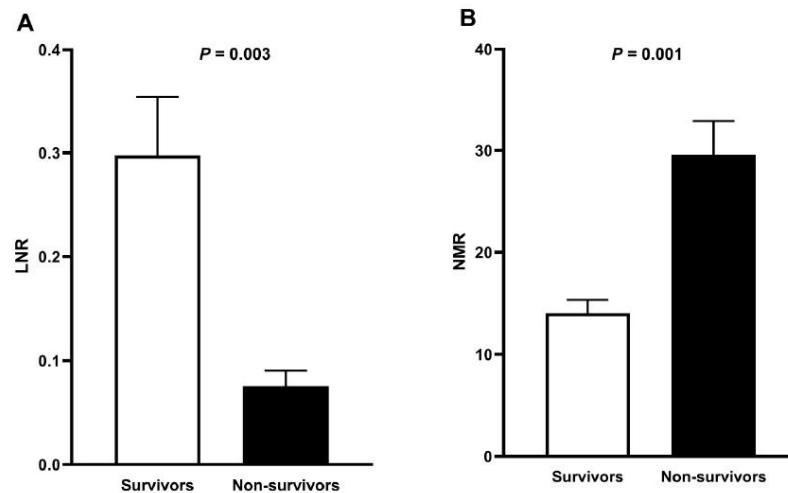


Figure 2. LNR and NMR at admission in hospitalized patients with severe Covid-19. (A) LNR showed a significant fourfold decrease in non-survivors as compared to survivors. LNR resulted of dividing the total lymphocyte count by the total neutrophil count. (B) NMR exhibited a significant twofold increase in non-survivors with respect to survivors. NMR resulted of dividing the total neutrophil count by the total monocyte count. Normality of data distribution was estimated by the Shapiro–Wilk test. The unpaired Student’s *t*-test was used to compare LNR and MNR between survivors and non-survivors, and data are presented as mean $\pm$ standard deviation. Differences were considered significant when $p < 0.05$. LNR, Lymphocyte-to-neutrophil ratio; NMR, neutrophil-to-monocyte ratio.

The area under the ROC curve of LNR was 0.832 (95% CI, 0.701–0.922, $p < 0.001$) (Figure 3A). At hospital admission, the best cut-off point for LNR was ≤ 0.088 with a sensitivity of 85.00% and a specificity of 74.19% (Youden index = 0.5919, 95% CI 0.3380–0.7387). The RR and OR for LNR was 5.8933 (95% CI, 1.9661–17.6652) and 16.2917 (95% CI, 3.7550–70.6837), respectively (Figure 3A). LNR was less than 0.088 in 91% of patients with severe Covid-19 that died, whereas only 20% of patients with severe Covid-19 that survived showed LNR ≤ 0.088 . Simultaneously, the area under the ROC curve of NMR was 0.890 (95% CI, 0.768–0.962, $p < 0.001$) (Figure 3B). The best cut-off value for NMR was ≥ 17.75 with a sensitivity of 89.47%, specificity of 80.00%, and Youden index of 0.6947 (95% CI, 0.4349–0.8333). The RR and OR for NMR was 8.8542 (95% CI, 2.2864–34.2878) and 27.9286 (95% CI, 5.1435–151.6497), respectively (Figure 3B). NMR was greater than 17.75 in 95% of patients with severe Covid-19 that died, whereas only 10% of patients with severe Covid-19 that survived had NMR ≥ 17.75 . Additionally, LNR ≤ 0.088 together with NMR ≥ 17.75 predicted in-hospital mortality in seriously ill patients with Covid-19 with a sensitivity of 81.48%, specificity of 83.33%, and OR of 22.00 (95% CI, 4.556–106.238). The accuracy of LNR and MNR as independent risk factors for in-hospital death was not modified after adjusting by comorbidities, gender, or age in multiple regression analysis.

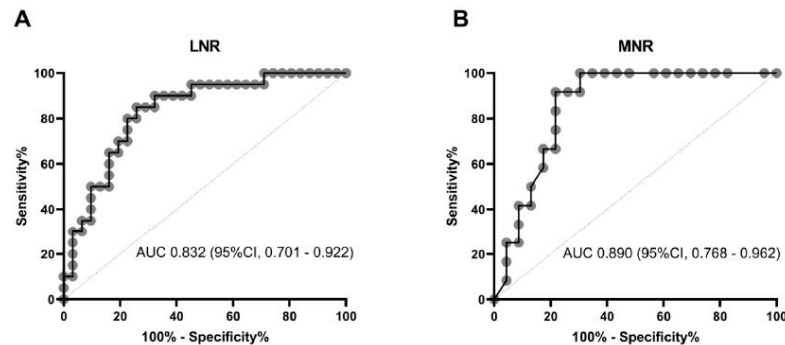


Figure 3. ROC curves for LNR and MNR to predict in-hospital mortality at admission in patients with severe Covid-19. **(A)** For LNR, the best cut-off point to predict in-hospital death was ≤ 0.088 with AUC of 0.832 (95% CI, 0.701–0.922), sensitivity of 85.00%, specificity of 74.19%, Youden index of 0.5919 (95% CI 0.3380–0.7387), RR of 5.8933 (95% CI, 1.9661–17.6652), and OR of 16.2917 (95% CI, 3.7550–70.6837). **(B)** For MNR, the best cut-off point to predict in-hospital death was ≥ 17.75 with AUC of 0.890 (95% CI, 0.768–0.962), sensitivity of 89.47%, specificity of 80.00%, Youden index of 0.6947 (95% CI, 0.4349–0.8333), RR of 8.8542 (95% CI, 2.2864–34.2878), and OR of 27.9286 (95% CI, 5.1435–151.6497). LNR resulted of dividing the total lymphocyte count by the total neutrophil count. MNR resulted of dividing the total neutrophil count by the total monocyte count. Statistical analyses were performed by means of the MDCalc Software (New York, NY 10003, USA) and the IBM SPSS Statistics version 26.0 (IBM, Armonk, NY, USA). Differences were considered significant when $p < 0.05$. LNR, Lymphocyte-to-neutrophil ratio; MNR, neutrophil-to-monocyte ratio; ROC, Receiver Operating Characteristic curves; AUC, area under the ROC curve; CI, confidence interval; RR, relative risk; OR, Odd ratio.

Table 5 confirmed that LNR and MNR are more accurate to predict mortality in Mexican patients with severe Covid-19 than WBC count by itself. In this sense, the area under the ROC curve of the total leukocyte count was 0.702 (95% CI, 0.557–0.822) (Table 5). The area under the ROC curves for neutrophil, lymphocyte, and monocyte counts were 0.746 (95% CI, 0.605–0.857), 0.735 (95% CI, 0.593–0.849), and 0.605 (95% CI, 0.458–0.739), respectively (Table 5). LNR and MNR were also better predictors for in-hospital mortality than the serum levels of several biochemical and immune parameters. As a matter of fact, the area under the ROC curves for D-dimer, ferritin, CRP, troponin I, LDH, and procalcitonin were 0.730, (95% CI, 0.548–0.869), 0.777 (95% CI, 0.601–0.901), 0.750 (95% CI, 0.569–0.884), 0.656 (95% CI, 0.464–0.816), 0.758 (95% CI, 0.618–0.867), and 0.826 (95% CI, 0.682–0.924), respectively (Table 5).

Table 5. Area under the ROC curves for hematological, biochemical, and immune parameters to predict in-hospital mortality at admission in patients with severe Covid-19. Statistical analyses were performed by means of the MDCalc Software (New York, NY 10003, USA) and the IBM SPSS Statistics version 26.0 (IBM, Armonk, NY, USA). Differences were considered significant when $p < 0.05$. Abbreviations: CRP, C-reactive protein; LDH, lactate dehydrogenase; ROC, Receiver Operating Characteristic curves; AUC, area under the ROC curve; CI, confidence interval.

Parameters	AUC	CI 95%
Total leukocyte count	0.702	0.557–0.822
Neutrophil count	0.746	0.605–0.857
Lymphocyte count	0.735	0.593–0.849
Monocyte count	0.605	0.458–0.739
D-dimer	0.730	0.548–0.869
Ferritin	0.777	0.601–0.901
CRP	0.750	0.569–0.884
Troponin I	0.656	0.464–0.816
LDH	0.758	0.618–0.867
Procalcitonin	0.826	0.682–0.924

Survival analysis revealed that patients seriously ill with Covid-19 showing NMR greater than 17.75 at admission have 50% survival probability after 9 days of hospitalization (Figure 4A). Patients with LNR less than 0.088 show 50% survival probability after 12 days of hospitalization (Figure 4B). When using the cut-off points for total leukocytes $> 11.4 \times 10^3/\mu\text{L}$, neutrophils $> 6.3 \times 10^3/\mu\text{L}$, lymphocytes $\leq 0.8 \times 10^3/\mu\text{L}$, and monocytes $\leq 0.3 \times 10^3/\mu\text{L}$ separately, the survival probability significantly decrease in patients with severe Covid-19 after 12–20 days of hospitalization (Figure 4C–F, respectively). Despite LDH values significantly differing between survivors and non-survivors (320.63 ± 132.11 versus 475.63 ± 195.83 , $p = 0.001$, respectively), patients with LDH > 362 IU/L show a significant decrease in the probability of survival up to 27 days after hospitalization (Figure 4G). In contrast, patients with severe Covid-19 and procalcitonin values greater than 0.1 ng/mL exhibit a 50% decrease in the survival probability after 12 days of hospitalization (Figure 4H).

Taking these results into account, we propose a new triage based on the use of NMR and LNR to assist clinicians to estimate in-hospital death risk in critically ill patients with Covid-19, with the aim of quickly admitting them into intensive care units and reducing the number of fatalities related to this disease (Figure 5).

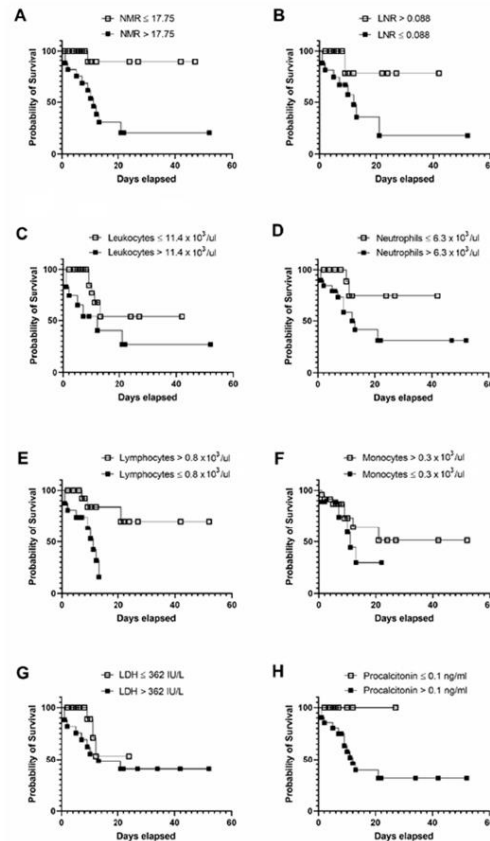


Figure 4. Kaplan–Meier curves for comparing the survival probability of different clinical predictors of in-hospital mortality at admission in patients seriously ill with Covid-19. (A) Patients with severe Covid-19 that show NMR > 17.75 at admission show 50% survival probability after 9 days of hospitalization. (B) Patients with LNR < 0.088 at admission show 50% survival probability after 12 days of hospitalization. (C) Patients with total leukocyte count > $11.4 \times 10^3/\mu\text{L}$ at admission exhibit a significant decrease in the survival probability up to 20 days after hospitalization. (D) Patients with total neutrophil count > $6.3 \times 10^3/\mu\text{L}$ at admission have 50% survival probability up to 13 days after hospitalization. (E) Patients with total lymphocyte count $\leq 0.8 \times 10^3/\mu\text{L}$ at admission show a significant decrease in the survival probability up to 14 days after hospitalization. (F) Patients with total monocyte count $\leq 0.3 \times 10^3/\mu\text{L}$ at admission exhibit a significant decrease in the survival probability up to 23 days after hospitalization. (G) Patients with LDH > 362 IU/L at admission show a significant decrease in the probability of survival up to 27 days after hospitalization. (H) Patients with procalcitonin values > 0.1 ng/mL at admission exhibit a 50% decrease in the survival probability after 12 days of hospitalization. Cut-off points for each marker are described at the top of the corresponding panel. Receiver operating characteristic (ROC) curves together with the Youden index was used to calculate optimal cut-off points for all markers. NMR resulted of dividing the total neutrophil count by the total monocyte count. LNR resulted of dividing the total lymphocyte count by the total neutrophil count. Survival analyses were performed by means of the Kaplan–Meier method using the GraphPad Prism 6.01 software. NMR, neutrophil-to-monocyte ratio; LNR, Lymphocyte-to-neutrophil ratio; LDH, lactate dehydrogenase.

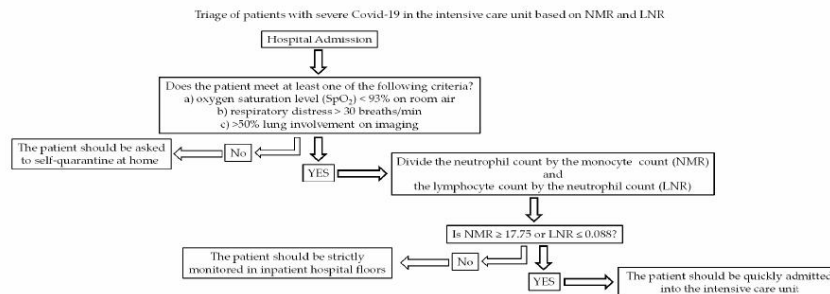


Figure 5. Proposal of triage based on NMR and LNR for patients seriously ill with Covid-19 that meet hospitalization criteria. At hospital admission, the neutrophil count should be divided by the monocyte count (NMR) and the lymphocyte count by the neutrophil count (LNR). If $NMR \geq 17.75$ or $LNR \leq 0.088$, the patient should be quickly admitted into the intensive care unit.

4. Discussion

The search for reliable, sensitive, and specific markers that can help us to discriminate patients with the most severe forms of Covid-19 at the time of hospital admission is still a matter of great urgency. In this sense, the analysis of routine laboratory results has revealed a strongly interdependent relationship among neutrophils, lymphocytes, and monocytes that is linked to the severity of Covid-19 [22].

To date, six retrospective clinical studies examining the potential use of NLR as a prognostic marker for severe Covid-19 have been published; none in Mexican patients until now [22–27]. All these clinical studies consistently conclude that NLR is an independent risk factor of mortality in critically ill patients with Covid-19. Concurring with this information, our results indicate that a LNR less than 0.088 at hospital admission is effective to early predict mortality of patients with severe Covid-19 (OR = 16.2917, 95% CI 3.7550–70.6837).

In parallel, we also wanted to explore if the apparent relationship between neutrophil and monocyte counts might be used to evaluate death risk in seriously ill patients with Covid-19. Unexpectedly, our data demonstrate that NMR is even more sensitive and specific than LNR to predict in-hospital mortality of Mexican patients with Covid-19. Indeed, our results demonstrate that having a NMR greater than 17.75 (OR = 27.9286, 95% CI 5.1435–151.6497) at the time of hospital admission is an independent risk factor for in-hospital mortality in patients with severe Covid-19, even after adjusting for comorbidities, gender, and age. Interestingly, the specificity of NMR slightly increases when using together with LNR, although the sensitivity significantly decreases and its possible clinical implementation should be made taking into account this information. Another important aspect that supports the possible implementation of NMR in clinical practice is the fact that this in-hospital mortality marker may assist in the early identification of patients with Covid-19 that will require a more aggressive clinical management. As a matter of fact, survival analyses revealed that using $NMR > 17.75$ at admission, clinicians might identify patients with severe Covid-19 at higher risk of experiencing 50% reduction in the probability of survival in 9 days. In contrast, identification of patients with poorer survival probability takes longer when using other markers such as lymphocyte count, LDH or procalcitonin. In other words, the use of NMR may help to clinicians to quickly identify patients at higher risk of in-hospital mortality with the aim of giving them priority into intensive care units.

As far as we know, this is the first study showing that NMR can be used as a cheap, fast, and reliable marker of in-hospital mortality in severe Covid-19. Hongmei Zhang and coworkers recently reported that NMR was significantly higher in patients with the most severe forms of Covid-19 as compared to those with mild and moderate courses of the disease [28]. However, these authors also found that NMR

failed to predict which patients had higher risk of developing a more severe form of Covid-19 [28]. This apparent controversy in the accuracy of NMR to discriminate patients at higher risk of death might be explained by understanding the impact of ethnicity on the development and clinical presentation of Covid-19. In this sense, several groups mostly from China have proposed that the serum levels of D-dimer, ferritin, CRP, troponin I, LDH, and procalcitonin are good predictors of mortality in patients with severe Covid-19 [8,9,13]. However, our study shows that in Mexican patients these biochemical and immune parameters exhibit poorer ability to predict mortality than that found for LNR and NMR. Again, these controversial findings may be related to differences in the genetic background and prevalence of comorbidities between Mexican-Mestizo subjects of the south-central region of Mexico and East Asian population. For all these reasons, we firmly believe that searching for markers associated with the most severe courses of Covid-19 should be conducted in a population-specific manner. In other words, LNR and NMR seem to be sensitive and specific markers of in-hospital mortality for severe Covid-19 in Mexican patients but no other populations such as Chinese people. Therefore, the clinical use of these ratios in patients with severe Covid-19 that require hospitalization should be carried out with caution in a population-specific manner.

The rationale behind using LNR and NMR to identify patients with severe Covid-19 at higher risk of death is probably given by the differential roles that lymphocytes, monocytes, and neutrophils play during immune response. In humans, neutrophils are the most abundant type of peripheral WBC with prominent functions in the acute inflammatory response by migrating toward injured tissues in response to chemoattractant signals such as interleukin (IL-) 8 [29]. Neutrophils are now considered one of the most important immune cells in defending the airway epithelium against the SARS-CoV-2 infection by locally stimulating the production of IL-1 beta, IL-6, tumor necrosis factor-alpha (TNF-alpha), and reactive oxygen species (ROS) [30]. Paradoxically, neutrophil hyperactivation and recruitment intensify the acute inflammatory response and worsen epithelial tissue damage thus leading to disease progression [30,31]. On the contrary, lymphocytes are very important leukocytes in charge of mediating immune tolerance to self-antigens, activation of pathogen-specific adaptive immunity, and, last but not less important, orchestration of immunomodulatory mechanisms [32,33]. Lymphocytes, including T and B cells, are able to orchestrate immunomodulatory mechanisms via IL-10 and transforming growth factor-beta 1 (TGF-beta 1) production, a couple of cytokines with key anti-inflammatory and wound-healing actions [34]. In parallel, monocytes are circulating leukocytes that in humans can be sorted into three subpopulations based on the cell surface expression of the cluster of differentiation (CD) 14 and CD16 [35]. The classical monocyte subpopulation expresses high CD14 levels with no CD16 expression. Intermediate monocytes show CD14 and CD16 expression; in contrast, non-classical monocytes express very low CD14 levels accompanied by CD16 expression [36]. Intermediate and non-classical monocytes have been shown to produce high levels of IL-1 beta in response to lipopolysaccharide and in patients with metabolic syndrome [37,38]. Interestingly, reduction in both intermediate and non-classical monocyte subpopulations has been recently associated with increased severity of the SARS-CoV-2 infection [39]. Additionally, monocytes can differentiate into alternatively activated macrophages and prevent inflammatory responses by promoting tissue repair via IL-10 and TGF-beta 1 production, which may also play a pivotal role in regulating hyperactivation of the inflammatory response described in patients with severe Covid-19 [40]. In other words, neutrophils and the cell populations of lymphocytes and monocytes seem to have typical antagonistic roles in multiple inflammatory scenarios including that provoked by the SARS-CoV-2 infection. Thus, it is feasible to assume that LNR and NMR might reflect an imbalance among these immune cells that in turn could be related to excessive inflammation and poorer survival in patients with severe Covid-19. For this reason, present results do not only offer novel mortality markers for severe Covid-19 but also reveal potential lines of treatment aimed to decrease neutrophil hyperactivation and increase lymphocyte and monocyte numbers by means of administering drugs as tofacitinib, reparixin, buspirone, and/or lanimostim. However, the possible efficacy of the aforementioned drugs in patients with severe Covid-19 should still be prospectively examined in randomized, placebo-controlled clinical trials.

Finally, concurring with previous literature [41], our study demonstrates that except for obesity, the prevalence of T2D, hypertension, and CHD was higher in the non-survival group than in patients with severe Covid-19 that survived. Likewise, patients in the non-survival group more frequently required IVM than surviving patients. In this way, our results confirm that the presence of previous non-communicable diseases such as T2D, hypertension, and CHD clearly aggravates disease severity and increases the number of fatalities related to Covid-19. So, the SARS-CoV-2 pandemic remarks again the urgency of more effective politics of public health aimed to control the incidence of noncommunicable diseases in countries like Mexico, which is facing a terrible disproportion in the number of deaths related to Covid-19.

This study has some limitations, including the sample size and exclusion of patients with previous diagnosis of other pathologies such as cancer, autoimmune disease, and/or other viral infections such as HIV and HCV. Improvement of these limitations may help to strengthen our findings and extend the use of LNR and NMR to predict in-hospital mortality in patients with high clinical heterogeneity.

5. Conclusions

This study demonstrates for the first time that an LNR less than 0.088 and an NMR greater than 17.75 at the time of admission can accurately predict in-hospital mortality of patients with severe Covid-19. Indeed, NMR is significantly more sensitive and specific than LNR to estimate the mortality risk in patients with Covid-19 that meet hospitalization criteria. In Mexican adult patients, both NMR and LNR are better predictors for in-hospital death related to severe Covid-19 than other markers such as D-dimer, ferritin, CRP, troponin I, LDH, and procalcitonin. Together with the presence of T2D, hypertension, CHD, male gender, and increased age (>63 years), NMR and LNR should be considered as independent risk factors for mortality in Mexican patients seriously ill with Covid-19. Simply by dividing the neutrophil count by the monocyte count, or the lymphocyte count by the neutrophil count, at the time of hospital admission, clinicians can have cheap, fast, and reliable predictors for in-hospital death in patients with Covid-19. In practical terms, we propose a new triage based on the use of NMR and LNR to assist clinicians to estimate in-hospital death risk in critically ill patients with Covid-19 with the aim of quickly admitting them into intensive care units and reducing the number of fatalities related to this disease (Figure 5). For this reason, we encourage other clinical research groups to assess the efficacy of these mortality predictors not only in Mexicans but also other Latin-American populations.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this article.

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Article

The Combined Use of Cytokine Serum Values with Laboratory Parameters Improves Mortality Prediction of COVID-19 Patients: The Interleukin-15-to-Albumin Ratio

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Abstract: Laboratory parameters display limited accuracy in predicting mortality in coronavirus disease 2019 (COVID-19) patients, as with serum albumin. Emerging evidence suggests that cytokine serum values may enhance the predictive capacity of albumin, especially interleukin (IL)-15. We thus investigated whether the use of the IL-15-to-albumin ratio enables improving mortality prediction at hospital admission in a large group of COVID-19 patients. In this prospective cross-sectional study, we enrolled and followed up three hundred and seventy-eight patients with a COVID-19 diagnosis until hospital discharge or death. Two hundred and fifty-five patients survived, whereas one hundred and twenty-three died. Student’s *T*-test revealed that non-survivors had a significant two-fold increase in the IL-15-to-albumin ratio compared to survivors (167.3 ± 63.8 vs. 74.2 ± 28.5), a difference that was more evident than that found for IL-15 or albumin separately. Likewise, mortality prediction considerably improved when using the IL-15-to-albumin ratio with a cut-off point > 105.4 , exhibiting an area under the receiver operating characteristic curve of 0.841 (95% Confidence Interval, 0.725–0.922, $p < 0.001$). As we outlined here, this is the first study showing that combining IL-15 serum values with albumin improves mortality prediction in COVID-19 patients.

Keywords: IL-15; albumin; COVID-19; SARS-CoV-2; mortality; prognosis

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the causal agent of coronavirus disease 2019 (COVID-19), a global pandemic that has affected more than

two hundred and thirty-five countries with nearly four million deaths up to June 2021 [1]. Clinical manifestations of COVID-19 are highly heterogeneous, ranging from asymptomatic or mild disease to severe or critical illness in patients who develop acute respiratory distress syndrome (ARDS), sepsis, and multiple organ failure [2]. Case fatality rates of COVID-19 vary depending on the geographical area; however, the vast majority of countries report mortality rates of around 2–4% [3]. Conversely, the case fatality rate of COVID-19 in Mexico is as high as 9.2%, revealing the deep need for novel markers to identify patients at higher risk of death in a timely manner [4].

The combined use of laboratory parameters with inflammatory markers increases the ability to identify COVID-19 patients at a higher mortality risk, as with serum albumin, C-reactive protein (CRP), and neutrophilia. In this sense, the CRP-to-albumin ratio better predicts the severity of COVID-19 than CRP or serum albumin separately [5]. Likewise, the combination of the neutrophil count with serum albumin values improves the area under the receiver operating characteristic (ROC) curve for predicting mortality in COVID-19 patients compared to those found when the neutrophil count or serum albumin are used separately [6]. However, emerging evidence suggests that combining laboratory parameters with markers significantly involved in the cytokine storm may also help to predict the mortality risk in COVID-19 patients, mainly albumin and interleukin (IL)-15 [7–9].

Albumin is a plasma protein produced in the liver that exerts multiple physiological functions in blood transport and anticoagulation. Serum albumin is also related to the severity of COVID-19; in fact, patients with the most severe forms of SARS-CoV-2 infection display lower albumin values than patients with the mild-to-moderate disease [10]. Serum albumin is also associated with increased mortality in COVID-19 patients [11]. However, most studies concur that the accuracy of albumin as a mortality predictor in SARS-CoV-2 infection is still limited.

IL-15 is a pleiotropic cytokine expressed by numerous immune and non-immune cells, including monocytes, macrophages, dendritic cells, neurons, epithelial cells, and fibroblasts [12]. IL-15 has a significant role in initiating inflammatory responses against microbial pathogens by modulating innate and adaptive immune cells [13]. A recent study showed that IL-15 serum levels increase in the same proportion as COVID-19 mortality [14]. The use of neutralizing antibodies anti-IL-15 as a potential immunotherapy for patients with severe SARS-CoV-2 infection was recently proposed [15]. Nevertheless, the combined use of IL-15 values with albumin to predict mortality in COVID-19 is unexplored, even though hypoalbuminemia is a common laboratory finding in patients with severe illness, and IL-15 belongs to the cytokine storm that is frequently associated with disease lethality. Thus, the purpose of this study was to examine whether the use of the IL-15-to-albumin ratio allows predicting mortality at hospital admission in a large group of patients with severe SARS-CoV-2 infection.

2. Materials and Methods

2.1. Patients

Three hundred and seventy-eight patients admitted to the Emergency Department of the General Hospital of Mexico from 30 November 2020 to 9 July 2021, were enrolled in this prospective cross-sectional study. Patients of both sexes were enrolled in the study if they met the following inclusion criteria: 18 years and older, COVID-19 diagnosis confirmed by detection of SARS-CoV-2 specific ribonucleic acid (RNA) in nasopharyngeal swabs using quantitative polymerase chain reaction (qPCR), respiratory distress (>30 breaths per minute), hypoxia (peripheral oxygen saturation <92% on room air), or ≥50% lung involvement on imaging. Patients were excluded from the study if they had a previous diagnosis of human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), cancer, endocrine disorders, or autoimmune disease. Pregnant or lactating women and patients under long-term immunomodulatory medication, including non-steroidal anti-inflammatory drugs, were also excluded from the study. All study participants provided written informed consent previously approved by the institutional

ethical committee of the General Hospital of Mexico (registration number of the ethical code approval: DI/20/501/03/17). The study rigorously met the principles described in the 1964 Declaration of Helsinki and its posterior amendment in 2013. This cross-sectional study met the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

2.2. Data Collection

We collected demographic and clinical data from the Emergency Department of the General Hospital of Mexico at admission. We also recorded clinical evolution, drug regimen, and inpatient days up to hospital discharge or death. Demographic and clinical data included sex, age, and previous diagnosis of obesity (body mass index (BMI) > 30 kg/m²), type 2 diabetes (T2D), hypertension, coronary heart disease (CHD), chronic kidney disease (CKD), and chronic liver disease (CLD).

2.3. Laboratory Parameters

We collected laboratory data at admission using the digital version of the electronic health record of the General Hospital of Mexico. Laboratory parameters included albumin, blood glucose, lipid profile, liver function tests, kidney function tests, coagulation markers, hematic biometry, CRP, troponin I, ferritin, procalcitonin, myoglobin, and D-dimer. We measured all laboratory parameters within sixty minutes of the patient's arrival in the hospital using the Beckman Coulter DxC 700 AU Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA), the Coulter LH 780 Hematology Analyzer (Beckman Coulter Inc., Brea, CA, USA), and the BCS[®] XP System (Siemens Healthcare GmbH, Erlangen, Germany), following standard operating procedures.

2.4. IL-15 Serum Levels

At hospital admission, 4 mL blood samples were drawn from all participants and collected in pyrogen-free tubes (Vacutainer[™], BD Diagnostics, NJ, USA) at room temperature. After a centrifugation step at 1000 g/4 °C for 30 min, we obtained serum samples for measuring IL-15 in triplicate by the Enzyme-Linked ImmunoSorbent Assay (ELISA) (PeproTech, Cranbury, NJ, USA). We measured IL-15 serum levels within 180 min of the patient's arrival in the hospital.

2.5. Statistics

We collected all demographic, clinical, laboratory, and immune parameters at hospital admission. We followed up with patients until hospital discharge or death. Then, we formed two groups of patients according to the primary outcome: survival or non-survival. In this way, we analyzed and compared all demographic, clinical, and laboratory parameters retrospectively. We used the Shapiro–Wilk test to estimate the normality of data distribution for numerical variables. We compared numerical variables between survival and non-survival groups using unpaired Student's *T*-tests, showing data as mean ± standard deviation. We used the chi-squared test to analyze categorical variables, showing data as absolute values and percentages. We analyzed ROC curves by obtaining the area under the ROC curve (AUROC) and 95% confidence interval (95% CI), sensitivity, specificity, and odds ratio (OR) for IL-15, serum albumin, and the IL-15-to-albumin ratio as potential mortality predictors. We used the Youden index to calculate optimal cut-off points for IL-15, serum albumin, and the IL-15-to-albumin ratio as potential mortality predictors. The IL-15-to-albumin ratio resulted from dividing the serum levels of IL-15 by albumin. We detected and removed outliers using Grubbs' test. We considered a *p* value < 0.05 as significant. We used the GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA 92037, USA), the MedCalc Software (New York, NY 10003, USA), and the IBM SPSS Statistics version 25.0 (IBM, Armonk, NY, USA) for statistical analyses.

3. Results

Figure 1 shows an overview of the selection process of eligible participants. After applying the inclusion and exclusion criteria, we enrolled three hundred-sixteen patients in the study. After hospital discharge or death, we retrospectively assigned COVID-19 patients to survival ($n = 255$) or non-survival ($n = 123$) groups (Figure 1).

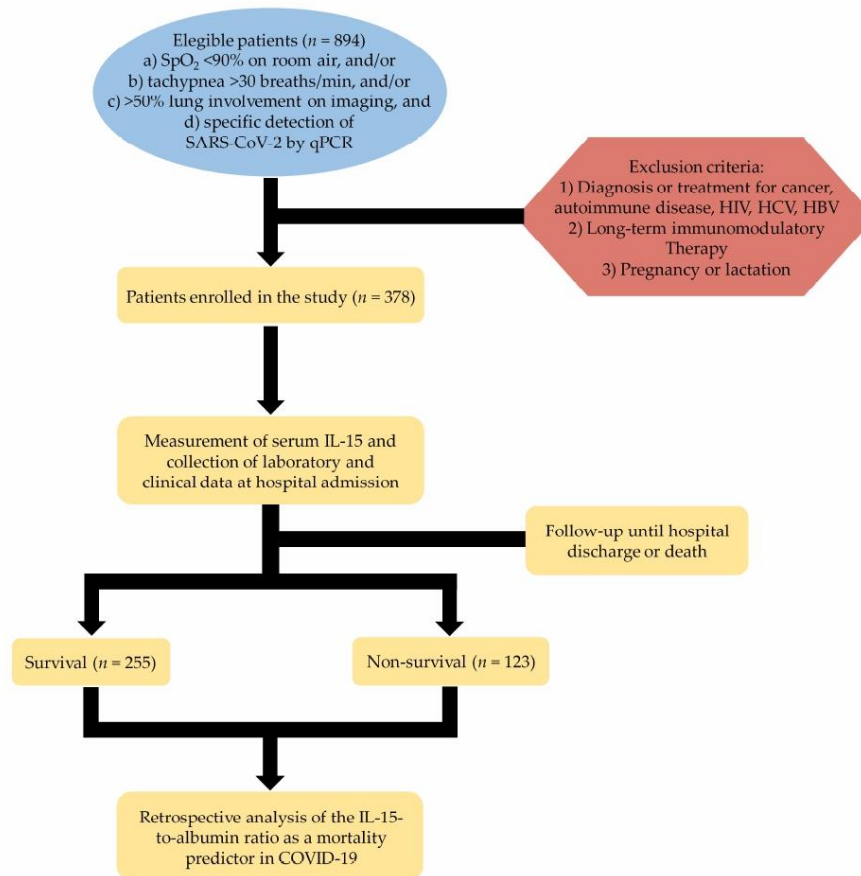


Figure 1. Schematic flow chart illustrating the selection process of eligible patients. SpO₂, peripheral oxygen saturation; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; qPCR, quantitative polymerase chain reaction; HIV, human immunodeficiency virus; HCV, hepatitis C virus; HBV, hepatitis B virus; COVID-19, coronavirus disease 2019.

There were no significant differences between survivors and non-survivors concerning the proportion of women and men ($p = 0.3$) and BMI ($p = 0.35$) (Table 1). Patients in the survival group were, on average, seven years younger than those in the non-survival group (51.4 ± 13.2 versus 58.9 ± 13.7 years, $p < 0.0001$, respectively) (Table 1). There were no significant differences between survivors and non-survivors with respect to the prevalence of obesity, T2D, hypertension, CHD, CKD, and CLD ($p = 0.8$, $p = 0.1$, $p = 0.5$, $p = 0.3$, $p = 0.6$, and $p = 0.3$, respectively). Body temperature and mean arterial pressure showed no changes

between survivors and non-survivors ($p = 0.3$ and $p = 0.1$, respectively). Conversely, there were significant differences between survivors and non-survivors for heart rate, breathing rate, and peripheral oxygen saturation ($p = 0.005$, $p = 0.001$, and $p < 0.001$, respectively) (Table 1). Sixty-eight percent ($n = 84$) of non-survivors required admission to the intensive care unit (ICU) for invasive mechanical ventilation, whereas only 23.5% ($n = 60$) of survivors needed ICU admission ($p < 0.001$) (Table 1). The number of inpatient days was, on average, greater for survivors than non-survivors (14.7 ± 9.9 vs. 7.3 ± 5.2 days, $p = 0.014$, respectively) (Table 1). All patients enrolled in the study received the same drug regimen: azithromycin, ceftriaxone, oseltamivir, enoxaparin sodium, dexamethasone, and acetaminophen.

Table 1. Demographic and clinical characteristics of patients enrolled in the study. Data are expressed as media $\pm$ standard deviation or absolute values and percentages. * Differences were considered significant when $p < 0.05$. We only show clinical data that significantly differed between groups. W, women; M, men; BMI, body mass index; T2D, type 2 diabetes; ICU, intensive care unit.

Parameters	Total ($n = 378$)	Survival ($n = 255$)	Non-Survival ($n = 123$)	p Value
Gender (W/M)	137/241	97/158	40/83	0.3
Age (years)	54 ± 13.7	51.4 ± 13.2	58.9 ± 13.7	$<0.001^*$
BMI (kg/m^2)	26.7 ± 4.8	25.8 ± 6.7	27.5 ± 4.4	0.3
Heart rate (bpm)	89 ± 13.2	87.7 ± 12.9	92 ± 13.5	0.005^*
Breathing rate (bpm)	24.1 ± 4.1	23.6 ± 3.9	25.2 ± 4.5	0.001^*
Oxygen saturation (%)	83 ± 9.5	84.5 ± 8.7	79.8 ± 12.5	$<0.001^*$
ICU needing (%)	143 (37.8)	60 (23.5)	84 (68.3)	$<0.001^*$
Inpatient days	12.1 ± 9.2	14.7 ± 9.9	7.3 ± 5.2	0.014^*

Hematological parameters had no significant differences between survivors and non-survivors, except for the neutrophil count, which was 1.7-fold higher in the non-survival group ($p < 0.001$) (Table 2). Overall, there were no significant differences between survivors and non-survivors for most laboratory parameters, including CRP, procalcitonin, fibrinogen, ferritin, and D-dimer. As compared to survivors, non-survivors showed substantial increases in the values of urea, uric acid, aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatinine kinase-MB (CK-MB), and brain natriuretic peptide (BNP) (Table 2).

Table 2. Laboratory parameters of patients enrolled in the study. Data are expressed as media $\pm$ standard deviation. Differences were considered significant when $p < 0.05$. We only show laboratory parameters that significantly differed between groups. AST, aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK-MB, creatinine kinase myocardial band; BNP, brain natriuretic peptide.

Parameters	Total ($n = 378$)	Survival ($n = 255$)	Non-Survival ($n = 123$)	p Value
Neutrophils ($\times 10^3/\text{mL}$)	8.6 ± 5.6	6.7 ± 3.9	12 ± 6.7	<0.001
Urea (mg/dL)	59.8 ± 54.7	35.1 ± 14.1	96.8 ± 70.8	<0.001
Uric Acid (mg/dL)	6.2 ± 3.1	5.2 ± 1.7	8 ± 4.3	0.001
AST (IU/L)	37.2 ± 19	33 ± 15.3	46 ± 23	0.008
ALP (IU/L)	93.3 ± 33.2	84.8 ± 28.3	109.9 ± 36.4	0.003
LDH (IU/L)	393.3 ± 224.9	311.2 ± 144.4	539 ± 268.2	0.001
CK-MB (IU/L)	22.1 ± 7.8	19.8 ± 5.6	27 ± 9.9	0.002
BNP (pg/mL)	151.2 ± 304.6	19.1 ± 11.5	291.4 ± 396.4	0.004

Non-survivors exhibited significantly lower serum values of albumin than those found in survivors (3 ± 0.5 versus 3.6 ± 0.6 g/dL, respectively, $p = 0.004$) (Figure 2A). IL-15 serum levels showed a significant 1.8-fold increase in non-survivors as compared to survivors (488.7 ± 242.8 versus 261.7 ± 137.6 pg/mL, respectively, $p < 0.001$) (Figure 2B). However, it is worth mentioning that differences between survivors and non-survivors were more evident when circulating values of IL-15 were divided by serum albumin. The

IL-15-to-albumin ratio was 2.2-fold higher in non-survivors than in survivors (167.3 ± 63.8 vs. 74.2 ± 28.5 , respectively, $p < 0.001$) (Figure 2C).

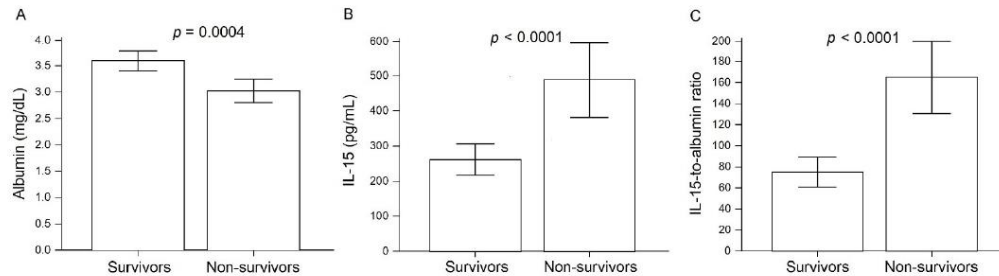


Figure 2. Serum levels of IL-15 and albumin in COVID-19 patients. (A) Serum albumin levels in survivors and non-survivors. (B) Serum IL-15 levels in survivors and non-survivors. (C) The IL-15-to-albumin ratio in survivors and non-survivors. The IL-15-to-albumin ratio resulted from dividing IL-15 serum values by serum albumin. Data are presented as mean $\pm$ standard deviation. We considered a p value < 0.05 as significant.

The AUROC for albumin was 0.797 (95% CI, 0.678–0.915, $p < 0.001$), with a cut-off point ≤ 3.3 g/dL, a sensitivity of 90.91%, a specificity of 61.54%, and an OR of 11.31 (95% IC, 2.84–45.06) (Figure 3A). The AUROC for IL-15 was 0.797 (95% CI, 0.675–0.889, $p < 0.001$), with a cut-off point ≤ 364.6 pg/mL, a sensitivity of 68.18%, a specificity of 84.60%, and an OR of 9.79 (95% IC, 2.91–32.98) (Figure 3B). Conversely, a combination between the values of IL-15 and albumin significantly improved the ability to predict mortality in COVID-19 patients. In fact, the AUROC for the IL-15-to-albumin ratio was 0.841 (95% CI, 0.725–0.922, $p < 0.001$), with a cut-off point > 105.4 , a sensitivity of 72.73%, a specificity of 87.18%, and an OR of 18.13 (95% IC, 4.81–68.37) (Figure 3C).

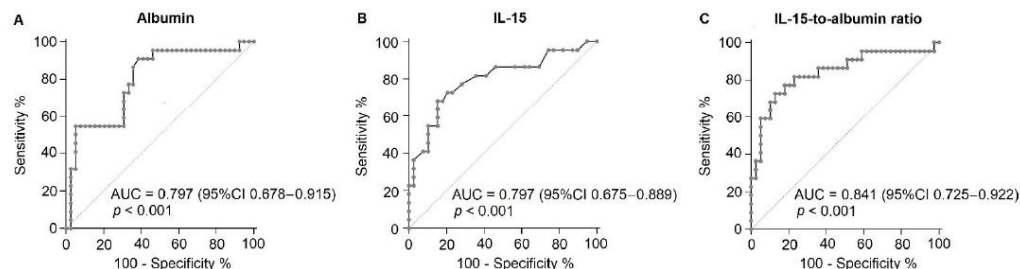


Figure 3. The area under the receiver operating characteristic curves for albumin, IL-15, and the IL-15-to-albumin ratio. (A) AUROC for albumin. (B) AUROC for IL-15. (C) AUROC for the IL-15-to-albumin ratio. We considered a p value < 0.05 as significant. AUROC, area under the receiver operating characteristic curves; AUC, area under the curve; CI, confidence interval.

The use of IL-15 serum values improved the predictive accuracy of albumin and several clinical and laboratory parameters. For instance, oxygen saturation showed an AUROC of 0.795 (95% CI, 0.647–0.902) for mortality prediction (Figure 4A); however, the combined use of IL-15 values with oxygen saturation exhibited an AUROC of 0.823 (95% IC, 0.705–0.909) (Figure 4B). Likewise, the use of IL-15 significantly increased the AUROC values of the neutrophil count, blood urea, AST, and CRP, among others (Figure 4).

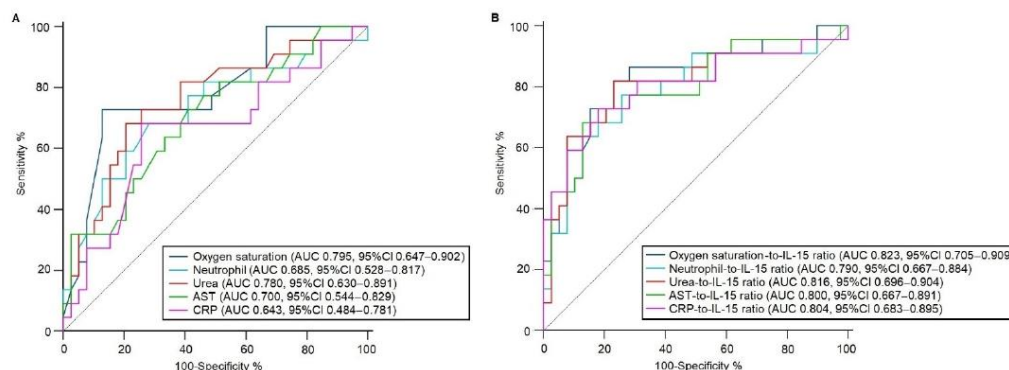


Figure 4. IL-15 serum values improved the area under the receiver operating characteristic curves of clinical and laboratory parameters. **(A)** AUROCs for oxygen saturation, the neutrophil count, blood urea, AST, and CRP. **(B)** AUROCs for the IL-15-to-oxygen saturation ratio, IL-15-to-neutrophil ratio, IL-15-to-urea ratio, IL-15-to-AST ratio, and IL-15-to-CRP ratio. We considered a p value < 0.05 as significant. AUROC, area under the receiver operating characteristic curves; AUC, area under the curve; CI, confidence interval; AST, aspartate aminotransferase; CRP, C-reactive protein.

4. Discussion

The case fatality rate of COVID-19 has continued to increase in the last few months, especially in countries with slow vaccination rates, such as Mexico [1,4]. Thus, there is still a deep sense of urgency to find novel strategies to help improve our ability to identify COVID-19 patients at high mortality risk. Routine laboratory tests can be measured easily, quickly, and at a low cost, making them good candidates to estimate prognosis after hospital admission. However, the accuracy of laboratory parameters to predict mortality in COVID-19 patients remains limited [16,17]. In this sense, we show that combining laboratory markers' values with serum cytokines is an excellent strategy to improve the early recognition of COVID-19 patients with an increased risk of death, mainly albumin and IL-15.

Combining cytokine serum values with laboratory parameters recently emerged as a promising approach to estimate prognosis in patients with SARS-CoV-2 infection. A recent study conducted on COVID-19 patients from China demonstrated that the use of IL-2R enhances the accuracy of the lymphocyte count to predict the risk of developing severe-to-critical illness [18]. Likewise, the combined ratio between IL-6 and the T CD8 cell count improves mortality prediction in COVID-19 patients, performing better than other clinical prediction tools, such as the CURB-65 score [19]. In line with previous evidence, we now report for the first time that combining IL-15 with albumin in a single prognostic ratio considerably improved our ability to identify COVID-19 patients at a much higher mortality risk. This trend was observed not only for albumin but also for other clinical and laboratory parameters. In terms of accuracy, the use of IL-15 seemed to enhance the predictive accuracy of albumin concerning that reported using other clinical and inflammatory parameters. A study conducted in 144 COVID-19 patients showed that the neutrophil count-to-albumin ratio (NAR) is an independent predictor of mortality with an AUROC of 0.736 [6]. In parallel, a study conducted in a large group of COVID-19 patients reported that the blood urea nitrogen-to-albumin ratio (BAR) could predict mortality with an AUROC of 0.809 [20]. Likewise, the CRP-to-albumin ratio (CAR) can predict mortality in COVID-19 patients with an AUROC of 0.807 [5]. As we have shown here, the IL-15-to-albumin ratio can predict mortality in COVID-19 patients with an AUROC of 0.841, which improves the predictive accuracy of NAR, BAR, and CAR. Nevertheless, it is crucial to consider that although the IL-15-to-albumin ratio appears to have higher predictive accuracy than other scales, the

measurement of routine laboratory parameters is more accessible than IL-15 quantification. We will discuss this notion as part of the limitations of this study.

Since the combined use of the IL-15-to-albumin ratio acts as an accurate mortality predictor, we believe that it is of great importance to discuss the possible mechanisms through which these molecules may contribute to the progression and severity of COVID-19. Decreased serum albumin is one of the most common laboratory alterations in COVID-19 patients that require hospitalization [16,17]. Hypoalbuminemia is also a central component of multiple conditions, such as cancer, cirrhosis, trauma, and sepsis [21–24]. As a matter of fact, low serum albumin is a mortality predictor in critically ill patients with sepsis and septic shock [25]. Thus, we believe it is crucial to understand the possible mechanisms by which some pathophysiological elements of COVID-19 may down-regulate serum albumin levels. First, decreased hepatic albumin production is associated with increased release of proinflammatory mediators belonging to the COVID-19-related cytokine storm, such as IL-6 and tumor necrosis factor-alpha (TNF-alpha) [26]. Interestingly, injection of Chinese hamster ovary cells transfected with the human gene encoding TNF-alpha into nude mice decreases albumin synthesis [27]. In SARS-CoV-2 infection, Huang and coworkers reported an inverse correlation between IL-6 and serum albumin in patients with poorer survival probability [16]. Moreover, human recombinant IL-6 induces a dose- and time-dependent decrease in the mRNA levels of albumin in in vitro cultured HepG2 cells [28]. This information becomes more significant in patients with COVID-19, wherein increased levels of TNF-alpha and IL-6 may directly decrease albumin production and lead to hypoalbuminemia. In the second place, several acute inflammatory diseases show that serum content of albumin can be redistributed to the interstitial space due to increased vascular permeability and capillary leakage, leading to decreased serum albumin values [26,29]. This information is in line with the fact that COVID-19 is characterized by the release of potent vascular permeability mediators such as arachidonic acid metabolites, IL-8, and monocyte chemoattractant protein-1 (MCP-1), all of which might contribute to the transvascular leakage of albumin [30]. As we have outlined here, the cytokine storm and vascular permeability may act in synergy with SARS-CoV-2 to decrease serum albumin levels in COVID-19 patients, information that might partially explain why hypoalbuminemia appears to be an excellent contributor to mortality prediction in this disease.

In parallel, there is little evidence supporting the possible role of IL-15 in the progression and mortality of COVID-19. After binding to the high-affinity IL-2R β /IL-15R α receptor, IL-15 induces the release of IL-8 and MCP-1 in human monocytes [31]. In severe SARS-CoV-2 infection, IL-8 and MCP-1 can recruit neutrophils and monocytes to the bronchoalveolar space and contribute to tissue damage and respiratory insufficiency [32,33]. In human macrophages, IL-15 can autocrinally promote the release of TNF-alpha, which in turn can induce apoptosis of human coronary artery endothelial cells and bovine pulmonary artery endothelial cells [34,35]. TNF-alpha-induced endothelial cell apoptosis is also associated with endothelial injury, vascular permeability, and systemic capillary leak syndrome, all of which contribute to the progression of COVID-19 [36–38]. Thus, IL-15 appears to orchestrate a two-hit deleterious action characterized by an exaggerated inflammatory response and increased endothelial cell apoptosis that together may contribute to the severity of COVID-19. Consistent with this idea, the analysis of 66 soluble biomarkers in 175 patients with severe SARS-CoV-2 infection revealed that IL-15 increases in the same proportion as mortality [14]. A recent cross-sectional study showed that COVID-19 patients with increased serum levels of IL-15 at admission experience a longer duration of hospitalization [39]. Altogether, this information reveals a pivotal role of IL-15 in the progression of SARS-CoV-2 infection and supports using this cytokine as a mortality predictor in COVID-19 patients. As we have outlined here, hypoalbuminemia and IL-15 may share a common pathophysiological mechanism mediated by the cytokine storm, which in turn appears to favor vascular permeability and neutrophil infiltration and leads to increased mortality risk. However, we should further explore the exact molecular mechanism through which albumin and IL-15 worsen survival prognosis in COVID-19.

Another phenomenon captured in our study involves the unexpected finding that there was no difference between survivors and non-survivors for gender and BMI. Most studies have documented that the COVID-19 case fatality rate is commonly higher in men than women [40]. However, a few studies have found that once severe COVID-19 occurs, the risk of dying is similar in women and men. We found no significant differences in the case fatality rate between female and male patients in line with this evidence. Men are more likely to be hospitalized than women; however, mortality is similar in both sexes once severe disease occurs. Likewise, although we noticed that BMI and obesity prevalence tended to be higher in non-survivors than survivors, we found no significant effects on the COVID-19 case fatality rate. Concurring with our findings, several studies have reported that BMI or obesity are not necessarily independent predictors of in-hospital mortality in COVID-19 [41–43]. We can explain these controversial findings since, at the time of hospitalization, all patients enrolled in this study had developed the most severe form of COVID-19, including respiratory distress and pneumonia. As mentioned above, most of the studies concur that once severe COVID-19 occurs, the potential contribution of variables such as gender and BMI to mortality rates tends to decrease [40,43]. However, we should consider these findings with caution, and further research is needed to understand the roles of gender and obesity in COVID-19 mortality in a population-specific manner.

Limitations of the study include: (1) the exclusion of HIV, HCV, or HBV seropositive individuals and COVID-19 patients with mild-to-moderate disease, which restricts our findings to a specific group of patients, and (2) the measurement of IL-15 serum levels may not be as easy in primary care. To improve these limitations, we are now conducting additional prospective studies to enroll patients with infectious diseases and other comorbidities. We are also developing rapid IL-15 tests to measure the levels of this cytokine quickly and affordably.

5. Conclusions

As we have outlined here, as far as we know, this is the first study demonstrating that the combined use of the IL-15-to-albumin ratio improves mortality prediction in COVID-19 patients that meet hospitalization criteria. Overall, the combination of IL-15 serum values with other clinical and laboratory parameters significantly increased our ability to identify patients at higher mortality risk. The mechanisms through which IL-15 and albumin contribute to mortality in COVID-19 remain to be elucidated. Although albumin measurement appears to be easier than IL-15 quantification in primary care, IL-15 serum levels can be detected quickly and affordably in tertiary healthcare centers, where most patients with severe COVID-19 are admitted. For this reason, we suggest the use of the IL-15-to-albumin ratio with cut-off point >105.4 to triage COVID-19 patients with increased mortality risk, which becomes more relevant in countries with slow vaccination rates. We encourage other research groups to study the impact of combining cytokine serum values with laboratory parameters in the early identification of COVID-19 patients at a much higher mortality risk.

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Case Report

High Levels of IL-8 and MCP-1 in Cerebrospinal Fluid of COVID-19 Patients with Cerebrovascular Disease

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The coronavirus family has tropism for the Central Nervous System (CNS), however, there is no solid evidence demonstrating that the neurological effects of COVID-19 result from direct viral infection or systemic inflammation. The goals of this study were to examine the cytokine profile and the presence of SARS-CoV-2 messenger ribonucleic acid (mRNA) in cerebrospinal fluids (CSF) from two patients with cerebrovascular disease and COVID-19. Although the SARS-CoV-2 mRNA was not detected in CSF of both patients, we found abnormally high levels of numerous pro-inflammatory cytokines and chemokines, especially IL-8 and MCP-1. Since these chemokines mediate activation and recruitment of neutrophils, monocytes, and macrophages, it is feasible that cerebrovascular disease related-neuroinflammation found in both patients results from an exacerbated inflammatory response instead of SARS-CoV-2 direct invasion to CNS. These results suggest that neuroinflammation plays a key role in cerebrovascular disease and COVID-19.

Key words: SARS-CoV-2, COVID-19, Cerebrospinal fluid, Cerebrovascular disease, IL-8, MCP-1

INTRODUCTION

The coronavirus disease 2019 (COVID-19) is a respiratory tract infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Besides the lungs, COVID-19 also affects other organs such as kidneys, liver, heart, and brain [1]. Neurological

alterations associated with SARS-CoV-2 infection include anosmia, headache, loss of consciousness, ischemic stroke, meningitis, encephalitis, and microhemorrhage [2]. The coronavirus family has tropism for the Central Nervous System (CNS) [3]. However, there is not yet solid evidence demonstrating that the neurological effects of COVID-19 result from direct viral infection or neuroinflammation associated with systemic inflammation and cytokine storm. Herein, we examine the cytokine profile of cerebrospinal fluids (CSF) from two patients with cerebrovascular disease concurrent with COVID-19, admitted to the Neurosurgery Department of the General Hospital of Mexico. We found high levels of numerous proinflammatory cytokines, especially interleukin (IL-) 8 and monocyte chemoattractant protein-1 (MCP-1). Although the presence of SARS-CoV-2 was confirmed in the airway epithelium by

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real time-polymerase chain reaction (RT-PCR), we did not detect expression of viral particles in CSF of both patients.

CASES REPORT

Case 1

A 54-year old female, with no metabolic disorders or previous surgeries reported, and medical history of chronic obstructive pulmonary disease of 12 years of evolution, treated with nebulized salbutamol as needed. The relatives reported she had mild to moderated headache the last four days, balance impairment, and generalized weakness, although the presence of neurological symptoms associated to COVID-19 such as anosmia and dys-

geusia were denied. She was admitted to emergency room with sudden loss of consciousness with the following vital signs: BP (110/80 mmHg), HR (80 bpm), BF (18 bpm), body temperature (36.1 °C), and a Glasgow Coma Scale (GCS) of 6 points (eye opening: 2 points, verbal response: 2 points, and motor response: 2 points) so we started airway management. The cranial computed tomography (CT) scan revealed cerebrovascular disease (CVD) characterized by acute intra-axial hemorrhage in the left cerebellar hemisphere associated with peripheral edema, which obliterated cortical subarachnoid spaces and cisternae of the posterior fossa and bulges the tentorium (Fig. 1A); hemorrhage extended into the fourth ventricle, which were displaced and compressed to the contralateral direction (Fig. 1A). Likewise, the hemorrhage extended

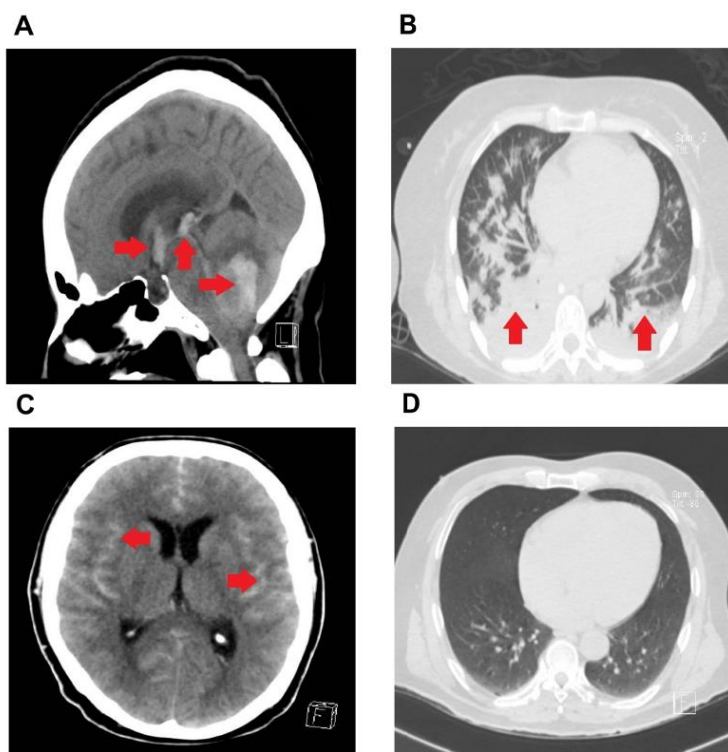


Fig. 1. Cranial and chest CT of patients with cerebrovascular disease and COVID-19. (A) cranial CT in sagittal reconstruction scan showing acute hemorrhage in the left cerebellar hemisphere with fourth ventricle compromise and basal cisternae obliteration, diffuse cerebral edema, obliterated subarachnoid space in posterior fossa, and intraventricular hemorrhage. (B) Chest CT scan in Axial lung window showing several nodules with well-defined and irregular borders. (C) Cranial CT scan, at hospital admission, showing diffuse subarachnoid hemorrhage, spreading through both lateral sulcus predominantly to right side, and the perimesencephalic cisterns; incipient level in hematic range is also observed in the left ventricular inferior horn. (D) Chest CT scan in axial lung window showing homogenous lung parenchyma. Red arrows show sites of hemorrhage (A, C) and pulmonary infiltration (B).

to the choroid plexuses of the supratentorial ventricles and led to obstructive hydrocephalus. The cortical subarachnoid spaces and supratentorial basal cisterns were obliterated (Fig. 1A). Measurement of laboratory parameters showed high leucocyte and neutrophil counts, whereas lymphocyte and monocyte counts decreased. She also presented high serum levels of glucose, total cholesterol, indirect bilirubin, gamma glutamyl transferase (GGT), creatinine kinase myocardial band (CK-MB), fibrinogen, D-dimer, and procalcitonin. There was no concluding evidence of COVID-19 in chest CT scan (CO-RADS III) (Fig. 1B), and then the presence of SARS-CoV-2 mRNA was confirmed by RT-PCR in nasopharyngeal swabs. Urgent right-sided precoronal ventriculostomy was performed. The patient presented clinical findings concurring with brain death immediately after postoperative period, and she died 24 hours later because of cardiopulmonary arrest.

Case 2

A 45-year-old male, with no metabolic disorders or previous surgeries, and medical history of ankylosing spondylitis diagnosed 23 years ago, under treatment with acetaminophen, diclofenac, and methylprednisolone. After reporting an undefined seizure accompanied by psychomotor agitation and disorientation one hour after awakening, he was admitted to the emergency room with vital signs at admission: BP (130/90 mmHg), HR (90 bpm), BF (20 bpm), and body temperature (36.5°C) and GCS of 14 points (eye opening: 4 points, verbal response: 4 points, and motor response: 6 points). The relatives reported that the patient did not present any neurological symptom associated with COVID-19 such as anosmia or dysgeusia. The cranial computed tomography (CT) scan revealed CVD characterized by diffuse subarachnoid hemorrhage (Fisher IV), with WFNS I and HUNT & HESS I (Fig. 1C). The cerebral parenchyma was heterogeneous due to the presence of hyperdense areas in the hematic range, the subarachnoid space, and the grooves of the parietal lobes that extended into the predominantly right Sylvian fissures. The CT scan also showed increased density of interhemispheric fissure and round morphology image. The ventricular system was predominant at the temporal level, wherein incipient periventricular hypodensity and density in the hematic range on the left side were found. In digital cerebral angiography, a saccular aneurysm in the right A1 segment was evidenced. Twelve hours later, he presented sudden neurological impairment, thus we decided to perform a second cranial CT scan in which we found left middle cerebral artery stroke with areas of hemorrhage of acute-subacute stage at the left temporoparietal level and the body of the ipsilateral caudate nucleus. Hematic density inside the left lateral ventricle, at the level of the occipital horn was observed. Laboratory parameters revealed leukocytosis,

lymphopenia, accompanied by high levels of fibrinogen and D-dimer concurring with SARS-CoV-2 infection (Table 1). Chest CT scan showed no typical anomalies (CO-RADS I) (Fig. 1D); however, RT-PCR in nasopharyngeal swabs confirmed the presence of SARS-CoV-2 mRNA. After two weeks of having received conservative treatment, the patient exhibited a negative RT-PCR test result for SARS-CoV-2. Then, left-sided pterional craniotomy

Table 1. Biochemical and immunological parameters of the patients

Parameters	Case 1	Case 2	Reference range in CSF
Glucose (mg/dl)	161.00	122.00	74-106
Total cholesterol (mg/dl)	236	175	<200
Triglycerides (mg/dl)	96	157	<150
Indirect bilirubin (mg/dl)	0.42	0.36	<0.2
GGT (IU/L)	50.00	57.00	M<55 W<38
CK-MB (IU/L)	58.00	9.00	<24
Fibrinogen (mg/dl)	383.00	562.00	200-400
Dimer-D (Ug/L)*	14,760	4,304	0-550
Procalcitonin (ng/ml)	0.06	0.06	<0.01
Leucocytes (×10 ³ /ul)	12.10	15.70	4.5-10
Neutrophils (×10 ³ /ul)	11.30	14.20	3-7
Lymphocytes (×10 ³ /ul)	0.70	0.9	1-3
Monocytes (×10 ³ /ul)	0.100	0.500	0.3-0.8
IL-1β (pg/ml)	18.95	10.00	<4
IL-10 (pg/ml)	32.38	9.79	2.82-5.30
IL-13 (pg/ml)	9.76	6.11	Undetectable
IL-6 (pg/ml)	202.88	24.48	1.11
IL-12 (pg/ml)	322.99	15.87	3.58
RANTES (pg/ml)	261.50	45.15	5.43-226.8
Eotaxin (pg/ml)	75.47	55.06	0.849
IL-17 (pg/ml)	23.29	5.06	1-6
MIP-1α (pg/ml)	317.46	25.00	10.62±6.85
GMC-SF (pg/ml)	6.84	5.66	Undetectable
MIP-1β (pg/ml)	58.06	10.0	4.69/10.3
MCP-1 (pg/ml)*	15,000	337.51	160
IL-15 (pg/ml)	296.44	45.00	3.51
IFN-γ (pg/ml)	11.90	10.0	<0.3
IFN-α (pg/ml)	2,129.62	95.29	2.14
IL-1RA (pg/ml)	382.11	16.71	25.00
TNF-α (pg/ml)	16.00	12.00	<4/1.51-2.11
IL-2 (pg/ml)*	4.51	3.00	70.62±1.1
IL-7 (pg/ml)	89.26	63.40	1.35
IP-10 (pg/ml)	468.07	51.97	2.46-4.07
IL-2R (pg/ml)	56.68	7.11	28.2±3.2
MIG (pg/ml)	20.46	9.99	6
IL-4 (pg/ml)	31.19	3.87	Undetectable
IL-8 (pg/ml)*	62,680.36	377.67	67.5-96.4

*The most apparent differences between cases and reference range are shown.

GGT, gamma-glutamyl transpeptidase; CK-MB, creatine kinase MB; IL, interleukine; MIP 1α/β, Macrophage Inflammatory Protein 1-Alpha/Beta; GMC-SF, Granulocyte Macrophage Colony-Stimulating Factor; MCP-1, monocyte chemoattractant protein-1; IFN-γ/α, interferon gamma/alpha; TNF-α, tumor necrosis factor-alpha; IP-10, interferon gamma-induced protein 10; MIG, monokine induced by interferon-gamma.

and aneurysm clipping surgery was performed. He was hospital discharged due to improvement two weeks after surgery, with normal vital signs as follows: BP (120/70 mmHg), HR (70 bpm), BF (18 bpm), and body temperature (36.5°C).

Both patients were admitted to the emergency department because of neurological symptoms rather than respiratory complications associated with SARS-CoV-2 infection. The relatives of the patients provided written informed consent, previously approved by the Institutional Ethical committee of the General Hospital of Mexico, which guaranteed that the study was conducted in rigorous adherence to the principles described in the 1964 Declaration of Helsinki and its posterior amendment in 2013. Cranial and chest CT scans were obtained from the Carestream Viu Motion software v.12.1.5.7 from the digital electronic file of the General Hospital of Mexico.

The CSF sample was obtained during surgery by ventricular shunt for case 1, whereas the CSF sample was obtained by lumbar puncture at admission for case 2. Viral particles of SARS-CoV-2 including spike S1 protein (S), nucleocapsid (N), envelope (E), and RNA-dependent RNA polymerase (RdRp) were analyzed in CSF by RT-PCR using the next primer sequences: S (forward: GCTGGTGCTGCAGCTTATTA; reverse: AGGGTCAAGTG-CACAGTCTA), N (forward: CAATGCTGCAATCGTGCTAC; reverse: GTTGCGACTACGTGATGAGG), E (forward: TTTCG-GAAGAGACAGGTACGTTA; reverse: AGCAGTACGCACA-CAATCG), RdRp (forward: AGAATAGAGCTCGCACCGTA; reverse: CTCCTCTAGTGGCGCTATT), 18S (forward: CCGC-TACCACATCCAAGGAA; reverse: GCTGGAATTACCGCG-GCT). All tests were performed following the next thermocycling steps: 95°C for 10 minutes, 95°C for 15 seconds, 60–63°C for 30 seconds (depending on the primer sequence), 72°C for 45 seconds, and a final extension step of 72°C for 7 minutes, after reaching 50 cycles.

Although RT-PCR test in nasopharyngeal swabs confirmed the SARS-CoV-2 infection in Case 1 (CT=35.09) and Case 2 (CT=35.17), the expression of S, N, E, and RdRp RNA was not detected in CSF from both patients.

The cytokine analysis was performed using the Human Cytokine Magnetic 25-Plex Panel (Life technologies, Frederick, MD, USA) following the manufacturer's instructions. Results were analyzed in the MILLIPLEXTM Analyst 5.1 Flex software. CSF samples from both patients revealed that levels of IL-6, IL-12, eotaxin, IL-17, MIP-1a, GMC-S, MIP-1b, MCP-1, IL-15, IFN- γ , IFN- α , TNF- α , IL-7, MIG, and IL-8 were dramatically higher than those found in reference values. However, IL-8 and MCP-1 showed the most impressive elevations in Case 1 as compared to Case 2. In fact, the Case 1 exhibited 166- and 44-fold increases in IL-8 and MCP-1,

respectively, with respect to Case 2. IL-2 was the only cytokine below reference values (Table 1).

DISCUSSION

Cases with SARS-CoV-2 infection-related neurological complications are increasing; thus a thorough evaluation of the CNS should be considered in patients with COVID-19. Therefore, patients should provide CSF biopsy, which is a minimally invasive form to obtain a CNS tissue sample. The presence of SARS-CoV-2 in CSF has been documented only in a few COVID-19 patients with neurological symptoms [4]. In this sense, Meinhardt and coworkers proposed that SARS-CoV-2 may enter the CNS via the neural-mucosal interface in olfactory mucosa in the nose, allowing its detection in CSF by means of RT-PCR [5]. In parallel, another study in mice reported that spike S1 protein can cross the blood brain barrier, reaching the CSF of the CNS [6]. However, in this study we were not able to detect the SARS-CoV-2 in CSF by RT-PCR, even though the same molecular test in nasopharyngeal swabs confirmed the presence of SARS-CoV-2 in the airway epithelium of both patients. Nevertheless, the fact that we did not detect SARS-CoV-2 mRNA in CSF samples does not confirm the absence of the virus in the CNS and further studies are needed. On the contrary, the cytokine storm was clearly evidenced in CSF, which presumably associates with the neurological symptoms accompanying COVID-19 [7].

Recently, Mousa-Ibrahim and cols [8] reported intracranial hemorrhage as well as thrombosis within six COVID-19 patients. Moreover, in an exceptional review study, Pramitasuri and cols. show that ischemic stroke is more frequent than intracerebral hemorrhage in SARS-CoV-2 patients. However, ischemic stroke in the context of SARS-CoV-2 infection more frequently evolved to hemorrhage than conventional ischemic stroke, which associates with increased mortality and worst outcome [9].

The relationship between SARS-CoV-2 infection and CVD lies in the ability of the virus to lead to hypercoagulable states that in turn increases fibrin deposition and D-dimer elevation [10]. Upon binding to the angiotensin-converting-enzyme (ACE2) receptor in brain capillary endothelial cells, SARS-CoV-2 causes cerebral endothelial damage that in turn leads to the development of intracranial hemorrhage, release of subintimal collagen, and platelet aggregation associated with clot production [11]. In the present study, we did not evaluate directly the brain capillary endothelium; however, we found no evidence of SARS-CoV-2 mRNA in CSF from both patients by RT-PCR. Therefore, it is feasible that cerebrovascular events observed in these patients were caused by an exacerbated proinflammatory response in the CNS. In this sense,

our data show that both patients had intracranial hemorrhages presumably related to COVID-19, characterized by increased CSF levels of cytokines associated with CNS inflammation such as IL-1 β , IL-6, TNF- α and IFN- γ , and extremely high concentrations of IL-8 and MCP-1.

IL-8 also referred to as CXCL8, is an important chemoattractant of neutrophils with the ability to eliminate cells infected with virus or bacteria, which in turn leads to collateral tissue injury. In a normal physiological state, IL-8 is scantily expressed in the CNS; however, IL-8 increases in several neurological pathologies such as ischemic brain injury, stroke, and traumatic brain injury. After ischemic brain damage, IL-8 increases first in CSF, inducing elevation of this cytokine in plasma, which results in systemic inflammation and persistent damage to CNS [12]. Likewise, MCP-1 also referred to as CCL2, is an important chemokine with the ability to recruit a wide variety of leukocytes such as macrophages and monocytes toward damaged tissues [13]. Similarly to IL-8, increased MCP-1 levels have been involved in neurological pathologies such as multiple sclerosis (MS), cerebral stroke, and traumatic brain injury [12].

Previous studies in Alzheimer Disease (AD) show that brain microvessels can release high levels of thrombin and IL-8, promoting the formation of neutrophil extracellular traps (NETs). In cerebral capillaries of AD patients, thrombin induces the expression of IL-8, thus contributing to the intravascular NETosis. Likewise, the MCP-1/CCR2 axis promotes migration of monocytes to the vascular endothelium of the CNS with the aim of clearing amyloid peptides in AD patients [14]. Despite the function of IL-8 and MCP-1 in the COVID-19-related cytokine storm has been widely studied, the role of these cytokines in the neurological dysfunction associated with COVID-19 is not fully elucidated.

Neutrophilia is a recurrent hallmark of acute infection in COVID-19 patients that leads to the formation of NETs, which are composed by networks of DNA, histones, and proteolytic enzymes that normally function in response to pathogens. Histones of NETs activate the coagulative kallikrein-kinin system and platelets, which present the High Mobility Group protein B1 (HMGB1) to neutrophils, stimulating NET formation. Once NETs bind to the endothelium, they compromise its integrity by the recruitment of more neutrophils, producing endothelial damage [15, 16].

Interestingly, in patients with ischemic strokes due to COVID-19 the role of NETs has a pivotal importance. Additionally, it is believed that neuroinflammation observed in stroke-related COVID-19 associates with the cytokine storm, wherein IL-1 β appears to play a pivotal role. IL-1 β is able to induce the expression of IL-17 and IL-6, which in turn can interact with astrocytes, microglia, and neurons, intensifying the cytokine storm and favoring the recruit-

ment of neutrophils and macrophages to cerebral parenchyma via HMGB1 protein [17]. In this scenario, IL-8 and MCP-1 might also contribute to neuroinflammation by recruiting neutrophils and macrophages to the vascular endothelium of the brain, favoring endothelial damage, NET formation, and the hypercoagulable state.

Despite we cannot precise whether high levels of IL-8 and MCP-1 are a cause or consequence of CVD in COVID-19 patients, this information supports the role of these cytokines in aggravating the severity of the intracranial hemorrhages in patients with SARS-CoV-2 infection.

Identification of the cytokine profile in CSF of patients with CVD concurrent with COVID-19 may allow starting a timely anti-inflammatory pharmacological approach. The use of drugs that inhibit the IL-8 and MCP-1-dependent signaling pathways may help reduce exacerbation of the inflammatory response in patients with COVID-19, thus decreasing the magnitude of the neurological damage. For this reason, we propose to conduct clinical trials aimed to test the efficacy of Tofacitinib and Reparaxyn in hyper-inflamed COVID-19 patients. Tofacitinib is an inhibitor of the JAK-STAT signaling pathway that indirectly obstructs the action of proinflammatory cytokines and chemokines such as IL-6, IL-8, MCP-1, MMP3, and MMP2/9 [18]. In parallel, Reparaxyn is an inhibitor of CXC chemokines receptor types 1 (CXCR1) and 2 (CXCR2), which becomes more relevant in patients with COVID-19 that show marked neutrophilia.

In conclusion, we outlined the cytokine profile in CSF of patients with CVD concurrent with COVID-19. Our findings suggest that CNS damage may result from the excessive inflammatory response mediated by IL-8 and MCP-1, instead of being caused by direct infection of SARS-CoV-2 to the CNS.

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Brief Report

High Incidence Rate of SARS-CoV-2 Infection in Health Care Workers at a Dedicated COVID-19 Hospital: Experiences of the Pandemic from a Large Mexican Hospital

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Abstract: Health care workers (HCW) are at high risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The incidence of SARS-CoV-2 infection in HCW has been examined in cross-sectional studies by quantitative polymerase chain reaction (qPCR) tests, which may lead to underestimating exact incidence rates. We thus investigated the incidence of SARS-CoV-2 infection in a group of HCW at a dedicated coronavirus disease 2019 (COVID-19) hospital in a six-month follow-up period. We conducted a prospective cohort study on 109 participants of both sexes working in areas of high, moderate, and low SARS-CoV-2 exposure. qPCR tests in nasopharyngeal swabs and anti-SARS-CoV-2 IgG serum antibodies were assessed at the beginning and six months later. Demographic, clinical, and laboratory parameters were analyzed according to IgG seropositivity by paired Student's T-test or the chi-square test. The incidence rate of SARS-CoV-2 infection was considerably high in our cohort of HCW (58%), among whom 67% were asymptomatic carriers. No baseline risk factors contributed to the infection rate, including the workplace. It is still necessary to increase hospital safety procedures to prevent virus transmissibility from HCW to relatives and non-COVID-19 patients during the upcoming waves of contagion.

Keywords: health care workers; SARS-CoV-2; COVID-19; incidence of infection; IgG antibody; pandemic

1. Introduction

The global outbreak of coronavirus disease 2019 (COVID-19) was caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. The SARS-CoV-2 is highly transmissible to humans, and health care workers (HCW) attending to COVID-19 patients are at a much higher risk of infection than the general population [2,3]. A study conducted at Rutgers University and two affiliated university hospitals reported that the prevalence of SARS-CoV-2 infection is dramatically higher in HCW than in non-health care professionals (7.3% versus 0.4%, respectively) [4]. A cross-sectional study conducted at the Hospital Universitario of Fuenlabrada in Spain informed a SARS-CoV-2 infection rate of 19.9% in HCW [5]. Kenan Rodriguez and colleagues conducted a cross-sectional descriptive study in two health departments belonging to the Spanish National Public Health System, finding an incidence of SARS-CoV-2 infection of 9.7% in HCW [6]. Likewise, another study reported a SARS-CoV-2 infection prevalence of 14.8% in HCW employed in a large tertiary community hospital in New York City [7]. This information suggests that the SARS-CoV-2 infection rate appears reasonably low in health care personnel who faced at least two COVID-19 waves. However, most reports examining the incidence of SARS-CoV-2 infection in HCW were conducted in cross-sectional studies using quantitative polymerase chain reaction (qPCR) tests to detect positive cases. Since qPCR amplifies the virus's genetic material, this methodology can only identify active infection cases, which may lead to underestimating asymptomatic patients and incidence rates among health care professionals [8]. For this reason, we believe the SARS-CoV-2 infection incidence in HCW should be assessed by conducting prospective, longitudinal cohort studies measuring specific IgG antibodies against the virus to detect all personnel exposed to the infection, irrespective of whether they showed symptoms or not.

At the pandemic's beginning, the Ministry of Health switched the General Hospital of Mexico (GHM) from a tertiary care center to a dedicated COVID-19 hospital. For this reason, we conducted a prospective cohort study to investigate the incidence rate of SARS-CoV-2 infection in HCW of the GHM by examining anti-SARS-CoV-2 IgG antibody serum titers at the beginning of the study and six months later. We also explored risk factors potentially contributing to infection rates in our study cohort, such as comorbidities and working in areas of high, moderate, and low viral exposure.

2. Materials and Methods

2.1. Trial Design and Ethical Considerations

This prospective cohort study consisted of enrolling doctors, nurses, researchers, clinical lab technicians, psychologists, rehabilitators, and administrative personnel from the GHM, one of the largest hospitals of the Ministry of Health, designated for treating COVID-19 patients in Mexico City. The study was approved by the Ethics and Clinical Research committees of the GHM (Approval No: DI/20/501/04/32) and was conducted in strict adherence to the principles of the 1964 Declaration of Helsinki and its posterior amendment in 2013. The enrollment and follow-up of participants took place from August 2020 to January 2021. This study met the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement guidelines for reporting observational studies.

2.2. Selection of Participants

We invited volunteers to participate in the study with no previous diagnosis of SARS-CoV-2, evidenced by a negative quantitative PCR (qPCR) test in oropharyngeal swabs and negative IgG antibodies against the virus, aged 18 years and above, and with no previous COVID-19 vaccination. All HCW that agreed to take part in the study signed an informed consent form and received a full explanation of the purposes and procedures

of the study. Personnel developing administrative tasks were considered to have low SARS-CoV-2 exposure within hospital facilities. Personnel handling and processing blood samples without having contact with COVID-19 patients were believed to have moderate SARS-CoV-2 exposure within the hospital. Personnel working in the emergency room and intensive care units were supposed to have high SARS-CoV-2 exposure within hospital facilities. We chose six months to follow up with all participants based on previous studies examining the COVID-19 incidence in HCW [4,9]. All HCW enrolled in the study agreed to receive weekly monitoring via a phone call interview to register any suggestive COVID-19 symptoms and attend at least two appointments for medical consultation, clinical evaluation, and blood sampling. We provided all HCW enrolled in the protocol with the same N95 respirators, disposable surgical gowns, disposable surgical caps, and face shield masks as personal protection equipment (PPE) throughout the study. We also confirmed that all HCW correctly wore PPE independently of working in low, moderate, or high SARS-CoV-2 exposure areas within the hospital. We labeled a participant as positive for SARS-CoV-2 exposure if the IgG test was positive at the end of the study.

2.3. Sample Size Estimation

The number of workers employed at the General Hospital of Mexico is approximately seven thousand. At the pandemic's beginning, two thousand aged workers were instructed to lock down at home. At this time, we initially estimated a sample size considering that the number of active personnel was five thousand. Based on previous studies [4,5], we expected that 13% of workers were at higher risk of SARS-CoV-2 infection ($n = 650$). Using the equation for population proportion $n = z^2 (p \times q) / e^2 + (z^2 (p \times q)) / N$, with a margin of error of 10% and a confidence level of 99%, we obtained $n = 84$. We considered 25% losses and finally found a sample size of $n = 105$ health care professionals.

2.4. Clinical Evaluation of Volunteers

Demographic, anthropometric, clinical, and laboratory parameters were collected from all study participants. The workplace within the hospital and any current symptoms concurring with SARS-CoV-2 infection were also registered. At the beginning of the study, we took from all participants an initial oropharyngeal swab for the qPCR test and a 4 mL blood sample for specific IgG antibody detection of anti-SARS-CoV-2. According to the World Health Organization (WHO) technical guidance [10], when participants described any symptoms concurring with COVID-19, they were subjected to another qPCR test with nasopharyngeal swab for SARS-CoV-2 detection. At the end of the study, all participants agreed to donate another 4 mL blood sample for specific IgG antibody detection.

2.5. IgG Seroprevalence Analysis

Specific IgG antibody levels against the SARS-CoV-2 nucleocapsid (N) protein were measured in triplicate by the Enzyme Linked-ImmunoSorbent Assay (ELISA) by a standardized kit from Abcam (Abcam, ab274339, Cambridge, UK), using a microplate reader at 450 nm.

2.6. Statistics

We estimated the normality of data by the Shapiro–Wilk test. At the beginning and the end of the study, clinical, anthropometric, and laboratory data were analyzed by paired Student's T-test. We estimated differences in sex proportion by the chi-square test and considered differences significant when $p < 0.05$. We performed statistical analyses using the SPSS version 22 (IBM, Armonk, NY, USA).

3. Results

Figure 1 illustrates the selection process of participants enrolled in the study based on the inclusion and exclusion criteria (Figure 1).

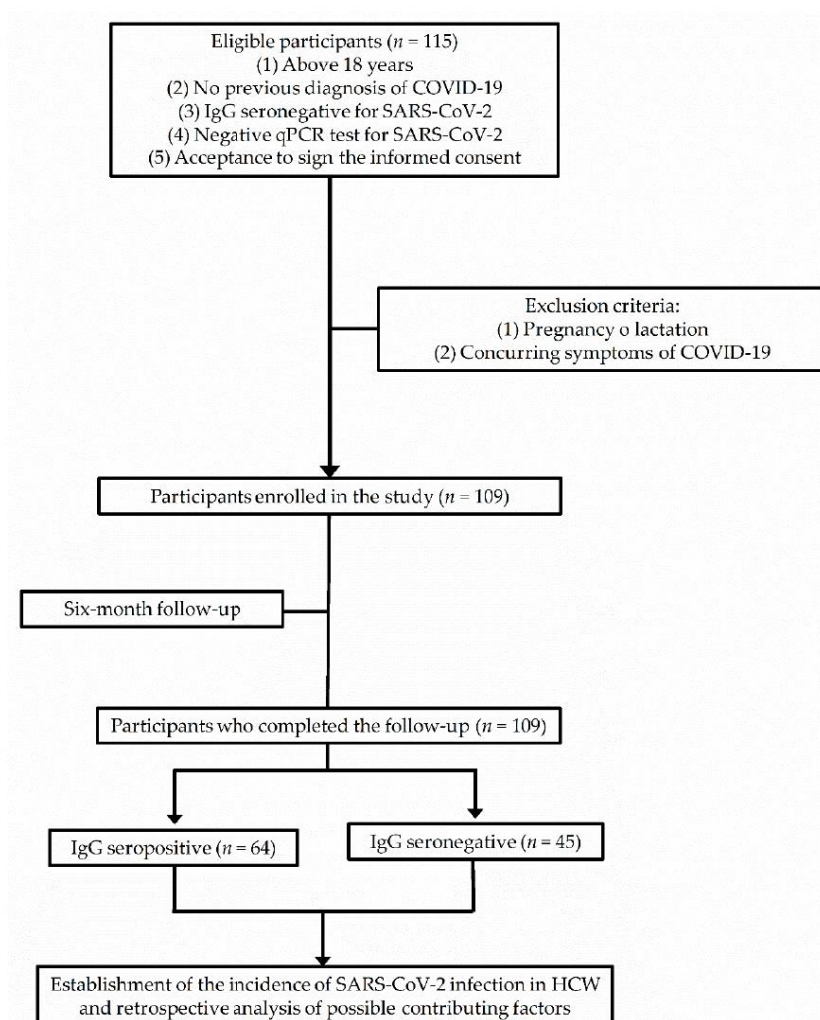


Figure 1. Schematic flow chart showing the selection process of participants enrolled in the study based on the inclusion and exclusion criteria. We conducted the enrollment of participants in strict adherence to the principles of the 1964 Declaration of Helsinki and its posterior amendment in 2013, meeting the STROBE guidelines for reporting observational studies. Abbreviations: IgG, immunoglobulin G; qPCR, quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; HCW, health care workers.

At the end of the study, 64 participants showed specific IgG antibodies against the virus, denoting a 58% incidence of SARS-CoV-2 infection (Table 1). Among IgG seropositive participants, 21 (33%) were symptomatic and presented symptoms concurring with COVID-19, including fever, headache, fatigue, body pain, anosmia, and dysgeusia. Moreover, three (14%) of the symptomatic participants needed non-invasive respiratory support.

In parallel, 67% ($n = 43$) of the IgG seropositive participants were asymptomatic carriers and reported no COVID-19 symptoms during the six-month follow-up. All symptomatic participants were confirmed by a qPCR test, and all of them showed serum titers of IgG antibodies anti-SARS-CoV-2 at the end of the study.

Table 1. Demographic, anthropometric, clinical, laboratory, and working characteristics of HCW.

Parameter	IgG Seropositive <i>n</i> = 64			IgG Seronegative <i>n</i> = 45			<i>P</i> ^A vs. ^B
	Baseline ^A	Final ^C	<i>P</i> ^A vs. ^C	Baseline ^B	Final ^D	<i>P</i> ^B vs. ^D	
Demographic characteristics							
Age, years ($\bar{x} \pm$ SD)	40 $\pm$ 10	-	-	43 $\pm$ 11	-	-	0.21
Men, <i>n</i> (%)	22 (34)	-	-	14 (31)	-	-	0.83
Women, <i>n</i> (%)	42 (66)	-	-	31 (69)	-	-	0.44
Working exposure level							
High, <i>n</i> (%)	37 (64.9)	-	-	20 (44.4)	-	-	0.19
Moderate, <i>n</i> (%)	22 (52.4)	-	-	20 (47.6)	-	-	0.38
Low, <i>n</i> (%)	5 (50)	-	-	5 (50)	-	-	0.39
Use of PPE, <i>n</i> (%)	60 (94)	-	-	39 (87)	-	-	0.28
Prevalence of comorbidities							
Sedentary lifestyle, <i>n</i> (%)	35 (55)	-	-	31 (69)	-	-	0.20
Obesity, <i>n</i> (%)	25 (39)	-	-	21 (47)	-	-	0.57
Diabetes Mellitus, <i>n</i> (%)	3 (5)	-	-	4 (9)	-	-	0.37
Hypertension, <i>n</i> (%)	0	-	-	5 (11)	-	-	0.01
Anthropometric and clinical parameters							
Breathing frequency, ($\bar{x} \pm$ SD)	15 $\pm$ 3	18 $\pm$ 10	0.03	16 $\pm$ 3	20 $\pm$ 13	0.13	0.16
Temperature, °C ($\bar{x} \pm$ SD)	36	36 $\pm$ 0.6	0.20	36	36 $\pm$ 0.1	0.02	0.55
Weight, kg ($\bar{x} \pm$ SD)	69 $\pm$ 12	70 $\pm$ 12	0.02	72 $\pm$ 16	73 $\pm$ 16	0.07	0.37
BMI, kg/m ² ($\bar{x} \pm$ SD)	26 $\pm$ 4.6	27 $\pm$ 4	0.04	28 $\pm$ 5.2	27 $\pm$ 6.7	0.88	0.21
Waist, cm ($\bar{x} \pm$ SD)	88 $\pm$ 10	88 $\pm$ 9	0.10	91 $\pm$ 14	91 $\pm$ 12	0.49	0.23
Hip, cm ($\bar{x} \pm$ SD)	101 $\pm$ 9	102 $\pm$ 8	0.61	103 $\pm$ 11	104 $\pm$ 10	0.06	0.41
Laboratory parameters							
Glucose, mg/dL ($\bar{x} \pm$ SD)	90 $\pm$ 11	87.7 $\pm$ 12	0.09	93 $\pm$ 16	96 $\pm$ 35	0.44	0.19
Urea, mg/dL ($\bar{x} \pm$ SD)	31 $\pm$ 7	31 $\pm$ 7	0.61	33 $\pm$ 11	34 $\pm$ 9	0.62	0.18
Creatinine, mg/dL ($\bar{x} \pm$ SD)	0.8 $\pm$ 0.1	0.7 $\pm$ 1	0.01	0.8 $\pm$ 0.15	0.7 $\pm$ 0.15	0.01	0.77
Uric acid, mg/dL ($\bar{x} \pm$ SD)	5.1 $\pm$ 1.1	5 $\pm$ 1	0.92	5.3 $\pm$ 1.2	5.3 $\pm$ 1.4	0.76	0.35
Cholesterol, mg/dL ($\bar{x} \pm$ SD)	181 $\pm$ 37	186 $\pm$ 36	0.21	183 $\pm$ 30	178 $\pm$ 47	0.35	0.77

At the end of the six-month follow-up, we found that the SARS-CoV-2 infection rate in our cohort of HCW was 58% ($n = 64$), and 67% ($n = 43$) of the IgG seropositive participants were asymptomatic carriers. We used paired Student's T-test to compare numerical variables and presented data as mean $\pm$ standard deviation. We used the chi-squared test to compare categorical variables and expressed data as absolute values or percentages. We considered differences significant when $p < 0.05$. ^A and ^B indicate characteristics of IgG seropositive and seronegative participants, respectively, at the beginning of the study. ^C and ^D indicate characteristics of IgG seropositive and seronegative participants, respectively, at the end of the study. Abbreviations: IgG, immunoglobulin G; PPE, personal protection equipment; BMI, body mass index; HCW, health care workers; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; $\bar{x}$, mean; SD, standard deviation.

At the beginning of the study (baseline), a retrospective analysis revealed no significant differences between participants who further showed positive or negative IgG serum titers for demographic, anthropometric, and laboratory parameters (Table 1). For comorbidities, hypertension was significantly higher in IgG seronegative participants than in IgG seropositive subjects, with no differences in the prevalence of obesity and Diabetes Mellitus (Table 1). Furthermore, there were no differences between IgG seropositive and seronegative participants concerning the correct use of PPE throughout the study (Table 1).

In participants working in hospital areas of low exposure, the incidence of SARS-CoV-2 infection was 50% (Table 1). A similar trend was found in participants working in areas of moderate exposure, wherein the incidence of SARS-CoV-2 infection was 52.5% (Table 1). Conversely, participants working in areas of high viral exposure tended to have the highest incidence of SARS-CoV-2 infection (64.9%) without showing significant

differences (Table 1). We found no differences in the correct use of PPE among participants working in low, moderate, or high SARS-CoV-2 exposure areas within the GHM.

4. Discussion

Herein, we found that the incidence of SARS-CoV-2 infection was as high as 58% in our study sample of HCW. Most studies examining the incidence of SARS-CoV-2 infection in HCW have reported incidence rates of 5–29% [4,5,11,12], which significantly differ from our results. These apparently controversial findings can be explained since most studies assessing the incidence of SARS-CoV-2 infection use the qPCR test in either cross-sectional or prospective studies, which may lead to underestimating actual incidence rates [13,14]. As far as we know, this is one of the first prospective cohort studies reporting a high incidence of SARS-CoV-2 infection in a cohort of HCW by examining titers of anti-SARS-CoV-2 IgG antibodies in a six-month exposure time. A study conducted on a group of HCW from a tertiary care center in Japan showed that almost half of the overlooked infection cases by the qPCR test could be identified when measuring anti-SARS-CoV-2 serum antibodies [15]. Therefore, we think the incidence of SARS-CoV-2 infection in HCW should be assessed by IgG antibody measurement in prospective cohort studies as a strategy that can help identify the personnel exposed to the virus irrespective of having presented COVID-19 symptoms or not. However, it is feasible that converting the GHM into an entirely dedicated COVID-19 care center could have impacted the SARS-CoV-2 infection rate in our study compared to other reports [4,5,11,12]. Therefore, our findings may not necessarily apply to all health care settings admitting COVID-19 patients.

In line with previous studies [16–18], we found that neither demographic and anthropometric characteristics nor laboratory parameters at the beginning of the follow-up were related to the incidence of SARS-CoV-2 infection in HCW of the GHM. However, we observed that hypertension was higher in IgG seronegative participants than in IgG seropositive volunteers. It is feasible that participants recognizing themselves as a vulnerable population with comorbidities such as hypertension had more safety procedures than volunteers without known comorbidities, even though our data show that both groups followed the minimal requirements of PPE.

We found that the workplace within the hospital was not significantly related to the incidence of SARS-CoV-2 infection when all health care professionals were wearing PPE correctly. HCW at the frontline, including emergency room and intensive care units, had similar infection rates to those working in moderate- and low-exposure areas such as hospital administration and medical social work. Our results align with previous studies reporting that the risk of SARS-CoV-2 infection in HCW is not necessarily associated with the workplace but with other factors such as community exposure and contact with family members who are COVID-19 asymptomatic carriers, and the inaccurate use of PPE [17,18].

Finally, our results indicate that only 33% of IgG seropositive participants presented COVID-19 symptoms, which means that two in three subjects were asymptomatic carriers. Abdulla A. Damluji and colleagues found 4.8% of asymptomatic carriers among HCW [19], while another study reported that 40% of HCW with SARS-CoV-2 infection did not develop any symptoms concurring with COVID-19 [20]. Altogether, our protocol shows many SARS-CoV-2 asymptomatic carriers among HCW, which may contribute to increasing transmissibility of the disease within the hospital and in the community.

We are inclined to point out some strengths and limitations to the study. The sample size ($n = 109$) could be a limitation considering that more than seven thousand employees work in the General Hospital of Mexico. However, besides sample size estimation suggesting that 105 participants were enough for statistical analysis, the number of HCW enrolled allowed us to monitor any suggestive COVID-19 symptoms individually for six months, including weekends, strengthening our ability to estimate infection rates and identify asymptomatic carriers. Furthermore, the fact that we conducted this study in one single care center could also be a limitation, considering that a multicenter trial provides an increased number of patients from different geographic places and socioeconomic statuses.

Nevertheless, the General Hospital of Mexico is one of the largest tertiary care centers in the country, attending nearly 200,000 low- and middle-income patients from the south-central region of Mexico per year. Therefore, our findings reflect, to some extent, the exposure levels of HCW working in dedicated COVID-19 hospitals in Mexico and may help consider effective strategies to face emerging health threats like SARS-CoV-2.

5. Conclusions

This prospective study shows, for the first time, in a cohort of HCW followed-up for six months, that the incidence rate of SARS-CoV-2 infection was as high as 58%, among which 67% were asymptomatic carriers. There were no risk factors contributing to the increase in the infection rate, including the workplace within the hospital. There is still a considerable need to increase safety procedures in COVID-19 hospitals, where HCW exposed to SARS-CoV-2 infection can increase virus transmissibility to other health care professionals, relatives, and patients.

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Institutional Review Board Statement: This study was approved by the Ethics and Clinical Research committees of the GHM (Approval No: DI/20/501/04/32) and conducted in strict adherence to the principles of the 1964 Declaration of Helsinki and its posterior amendment in 2013.

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

Data Availability Statement: Data are available upon request.

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Direct bilirubin and the neutrophil-to-monocyte ratio timely predict intensive care unit admission in patients with severe acute respiratory syndrome coronavirus-2 infection (COVID-19)

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Abstract

Objective: Intensive care units (ICUs) collapsed under the global wave of coronavirus disease 2019 (COVID-19). Thus, we designed a clinical decision-making model that can help predict at hospital admission what patients with COVID-19 are at higher risk of requiring critical care. **Methods:** This was a cross-sectional study in 119 patients that met hospitalization criteria for COVID-19 including less than 30 breaths per minute, peripheral oxygen saturation < 93%, and/or $\geq 50\%$ lung involvement on imaging. Depending on the need for critical care, patients were retrospectively assigned to ICU and non-ICU groups. Demographic, clinical, and laboratory parameters were collected at admission and analyzed by classification and regression tree (CRT). **Results:** Forty-five patients were admitted to ICU and 80% of them were men older than 57.13 ± 12.80 years on average. The leading comorbidity in ICU patients was hypertension. The CRT revealed that direct bilirubin (DB) > 0.315 mg/dl together with the neutrophil-to-monocyte ratio (NMR) > 15.90 predicted up to correctly in 92% of the patients the requirement of intensive care management, with sensitivity of 93.2%. Preexisting comorbidities did not influence on the tree growing. **Conclusions:** At hospital admission, DB and NMR can help identify nine in 10 patients with COVID-19 at higher risk of ICU admission.

Keywords: COVID-19. Severe acute respiratory syndrome coronavirus-2. Direct bilirubin. Neutrophil-to-monocyte ratio. Clinical decision-making model. Classification and regression trees.

Introduction

Coronavirus disease 2019 (COVID-19) is an infectious illness caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) that was first identified in Wuhan, China, and has rapidly spread to the rest of the world¹. The vast majority of patients with COVID-19 experience a mild disease that self-limits in < 2 weeks, however, up to 15% of patients can develop severe forms of the disease including respiratory failure, multiple organ failure, and death². Several

studies have consistently reported that near 30% of patients with severe SARS-CoV-2 infection requires critical care, which alarmingly overwhelmed intensive care units (ICUs) during the first global wave of COVID-19 and is expected to happen now during the second wave of this infectious disease³.

Quick admission to ICU clearly decreases mortality rates of patients seriously ill with COVID-19⁴. For this reason, there is a deep need for simple decision-making models that allow clinicians predicting from the very

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first moment of hospital admission what patients with severe SARS-CoV-2 infection might be ICU candidates. Recently, we demonstrated that risk factors such as the neutrophil-to-monocyte ratio (NMR) at admission can accurately predict in-hospital mortality in patients seriously ill with COVID-19 without having yet explored their utility in prioritizing ICU admission⁵.

Classification and regression trees (CRTs) are predictive algorithms that provide decision-making models based on analyzing the influence of several independent numerical variables on a target variable⁶. Moreover, CRT can also model the potential influence of categorical variables on the main clinical outcome, which has recently popularized the use of these decision algorithms in clinical research^{7,8}. In addition, CRT algorithms are able to predict a clinical outcome assuming that the relationship between dependent and independent variables occurs in a non-linear form, as previously reported for the development of complex human diseases⁹. Thus, we hypothesize that CRT might be a powerful tool in developing a clinical decision-making model that can help clinicians predict ICU admission in patients with severe SARS-CoV-2 infection. The purpose of this study was to analyze the most important demographic, clinical, and laboratory risk factors for designing a clinical decision-making model that can predict at hospital admission the need for critical care in patients with severe COVID-19.

Materials and Methods

Patients and study design

It was a cross-sectional, prospective, and analytical study in 119 individuals of both sexes, aged 18 years and older that met at least one of the following criteria of severe COVID-19: oxygen saturation levels (SpO_2) $\leq$ 93% on room air, respiratory distress $\geq$ 30 breaths per minute, and/or $\geq$ 50% lung involvement on imaging. Diagnosis of COVID-19 was confirmed by quantitative polymerase chain reaction following the World Health Organization technical guidance¹⁰. All eligible patients were originally admitted to the Emergency Department from October 5, 2020, to February 26, 2021, and depending on the clinical evolution were transferred to the ICU for ventilatory support. Patients were retrospectively grouped into two different clusters depending on the need for invasive mechanical ventilation in ICU according to the Clinical Care Guidelines for Influenza and SARS-CoV-2 of the Centers for Disease Control and Prevention¹¹. Patients that met criteria of severe

COVID-19 were enrolled in the study if they agreed to provide written informed consent previously approved by the Institutional Ethical Committee with registration number of the ethical code approval: DI/20/501/03/17. The study was conducted in rigorous adherence to the principles described in the 1964 Declaration of Helsinki and its posterior amendment in 2013. This study is reported in compliant with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Patients were excluded from the study if they had previous diagnosis of chronic liver disease, endocrine disorders, and infectious diseases. Patients unable to provide written informed consent and pregnant or lactating women were also excluded from the study.

Demographic, clinical, and laboratory data

Demographic information, clinical data, and laboratory measurements were recorded at hospital admission. Demographic data included gender and age. Clinical data included previous diagnosis of obesity with body mass index $\geq$ 30 kg/m², Type 2 diabetes (T2D), high blood pressure $\geq$ 130/80 mmHg, chronic kidney disease (CKD), coronary heart disease (CHD), autoimmune disease, cancer, smoking, alcoholism, peripheral oxygen saturation, breath rate, heart rate, body temperature, odynophagia, chest pain, diarrhea, inpatient days, date of discharge or death, and drug therapy for COVID-19. Laboratory data were collected using the digital version of the electronic health record of the hospital and included blood glucose, lipid profile, liver function tests, kidney function tests, coagulation markers, hematic biometry, C-reactive protein, troponin I, ferritin, procalcitonin, myoglobin, and D-dimer. NMR resulted of dividing the total neutrophil count by the total monocyte count.

Statistics and CRT

Assessment of normality was performed using the Shapiro-Wilk test. Categorical variables were analyzed by the Chi-square test and are expressed as absolute values and percentages. Numerical variables were analyzed by the unpaired Student's t-test and are expressed as mean $\pm$ standard deviation. These statistical analyses were performed using the GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA 92037, USA) and differences were considered significant when $p < 0.05$. Numerical variables were used to build a CRT considering ICU admission as dependent variable. Then, the CRT was explored by categorical variables such as gender, obesity, T2D, hypertension,

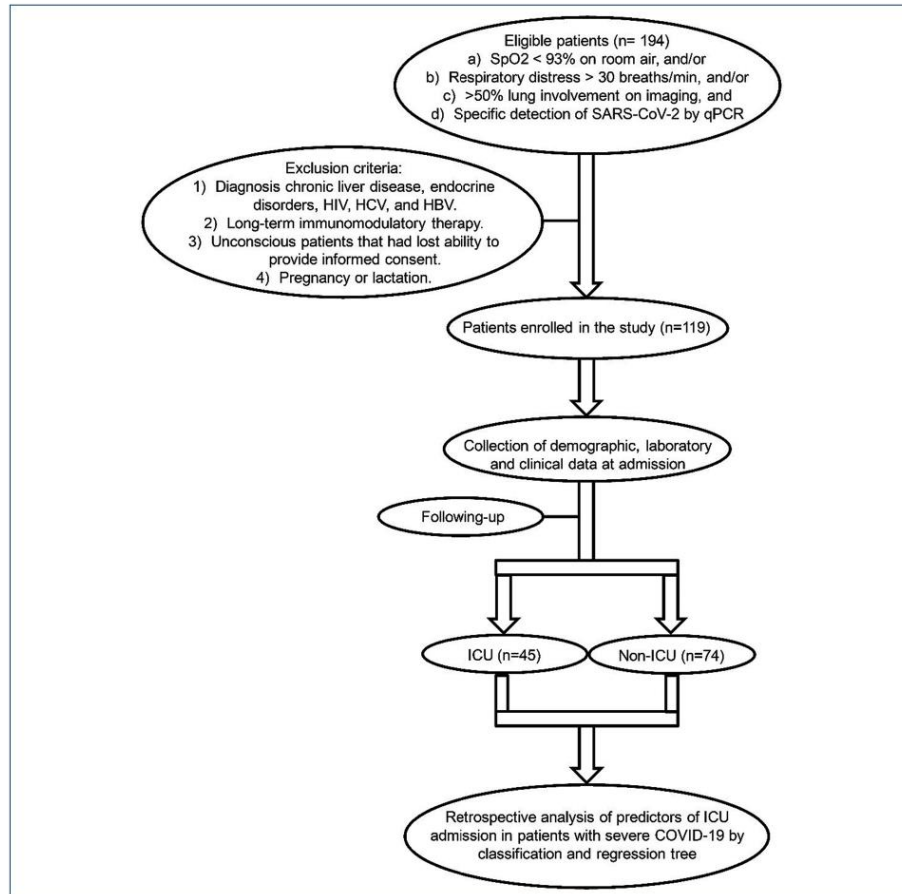


Figure 1. Schematic flowchart showing the selection process of patients enrolled in the study based on the inclusion and exclusion criteria. SpO₂: oxygen saturation level; SARS-CoV-2: novel severe acute respiratory syndrome coronavirus-2; qPCR: quantitative polymerase chain reaction; HIV: human immunodeficiency virus; HCV: hepatitis C virus; HBV: hepatitis B virus; COVID-19: coronavirus disease 2019.

CKD, and CHD that may influence the decision algorithm. The CRT was performed with the minimum number of cases in the parent node of 20% of the sample size, stopping rule for terminal nodes of 10%, 10-fold cross-validation, and tree pruning with a maximum acceptable difference in risk between the pruned tree and the sub-tree of 1 standard error. The CRT contained a root node, four terminal nodes, and three levels. Cutoff point, sensitivity, specificity, odds ratio (OR), and 95%

confidence interval were calculated for each predicting variable of the CRT using the IBM SPSS Statistics Version 26.0 (IBM, Armonk, NY, USA).

Results

The selection process of patients enrolled in the study is shown in figure 1. After being admitted to the Department of Pneumology, 37.8% (n = 45) of patients with

Table 1. Demographic, clinical, and laboratory parameters in patients with severe COVID-19

Parameters	Total (n = 119)	Non-ICU (n = 74)	ICU (n = 45)	p-value
Gender (W/M)	33/86	24/50	9/36	0.142
Age (years)	54.46 ± 11.98	52.84 ± 11.24	57.13 ± 12.80	0.058
Obesity prevalence (%)	60 (50.4)	37 (50)	23 (51.1)	0.920
Comorbidities prevalence (%)	81 (68.1)	53 (71.6)	28 (62.2)	0.286
T2D prevalence (%)	62 (52.1)	40 (54.1)	22 (48.9)	0.584
Hypertension prevalence (%)	36 (30.3)	29 (39.2)	7 (15.6)	0.006*
Chronic kidney disease (%)	5 (4.2)	5 (6.8)	0 (0)	0.075
Coronary heart disease (%)	2 (1.7)	0 (0)	2 (4.4)	0.067
Autoimmune diseases (%)	3 (2.5)	1 (1.4)	2 (4.4)	0.297
Cancer (%)	1 (0.8)	1 (1.4)	0 (0)	0.434
Smoking (%)	27 (22.7)	17 (23.0)	10 (22.2)	0.807
Alcoholism (%)	16 (13.4)	10 (13.5)	6 (13.3)	0.978
Peripheral oxygen saturation (%)	82.61 ± 6.54	84.08 ± 5.62	80.18 ± 7.26	0.001*
Temperature (°C)	36.99 ± 0.562	37.01 ± 0.577	36.94 ± 0.539	0.513
Heart rate (breaths/min)	90.18 ± 12.24	88.30 ± 12.13	93.29 ± 11.91	0.030*
Breath rate (beats/min)	24.50 ± 3.72	23.95 ± 3.97	25.42 ± 3.11	0.035*
Dyspnea	106 (89.1)	64 (86.5)	42 (93.3)	0.246
Cough	102 (85.7)	62 (83.8)	40 (88.9)	0.440
Fever (≥37.3°C)	100 (84.0)	59 (79.7)	41 (91.1)	0.100
Myalgia	81 (68.1)	56 (75.7)	25 (55.6)	0.022*
Headache	48 (40.3)	31 (41.9)	17 (37.8)	0.657
Odynophagia	50 (42.0)	30 (40.5)	20 (44.4)	0.676
Chest pain	23 (19.5)	13 (17.6)	10 (22.7)	0.494
Diarrhea	34 (28.6)	23 (31.1)	11 (24.4)	0.437
Neutrophils (×10 ⁹ /uL)	7.37 ± 3.25	6.87 ± 3.02	8.19 ± 3.47	0.030*
Lymphocytes (×10 ⁹ /uL)	0.913 ± 0.425	0.992 ± 0.439	0.784 ± 0.371	0.009*
Monocytes (×10 ⁹ /uL)	0.439 ± 0.231	0.467 ± 0.238	0.392 ± 0.213	0.086
Neutrophil percentage (%)	81.86 ± 9.29	80.00 ± 9.73	84.93 ± 7.71	0.005*
Lymphocyte percentage (%)	12.27 ± 7.62	13.66 ± 8.25	9.99 ± 5.86	0.010*
Monocyte percentage (%)	5.56 ± 3.39	6.16 ± 3.65	4.57 ± 2.68	0.012*
Total bilirubin (mg/dL)	0.744 ± 0.461	0.641 ± 0.283	0.913 ± 0.625	0.002*
Direct bilirubin (mg/dL)	0.278 ± 0.285	0.212 ± 0.169	0.388 ± 0.388	0.001*
Indirect bilirubin (mg/dL)	0.465 ± 0.214	0.429 ± 0.164	0.525 ± 0.269	0.017*
AST (IU/L)	47.48 ± 30.59	42.87 ± 25.33	54.96 ± 36.69	0.037*
LDH (IU/L)	450.61 ± 185.87	417.43 ± 144.39	505.16 ± 230.59	0.012*
Prothrombin time (s)	11.82 ± 1.29	11.63 ± 1.04	12.12 ± 1.59	0.045*

(Continues)

Table 1. Demographic, clinical, and laboratory parameters in patients with severe COVID-19 (*Continued*)

Parameters	Total (n = 119)	Non-ICU (n = 74)	ICU (n = 45)	p-value
Base excess	-3.18 ± 4.13	-3.86 ± 3.63	-2.07 ± 4.68	0.021*
NMR	23.77 ± 23.06	19.44 ± 14.13	30.88 ± 31.81	0.008*
Inpatient days	14.79 ± 9.23	12.66 ± 5.99	18.29 ± 12.22	0.001*
Mortality	43 (36.1)	12 (16.2)	31 (68.9)	0.000*
Drug regimen	Azithromycin, ceftriaxone, oseltamivir, enoxaparin sodium, dexamethasone, and acetaminophen			

Parameters were recorded at hospital admission. Laboratory parameters with significant differences are shown. Normality of data distribution was estimated by the Shapiro-Wilk test. The unpaired Student's t-test was used to compare numerical variables and data are presented as mean ± standard deviation. The Chi-squared test was used to compare categorical variables and data are expressed as absolute values and percentages.

*Differences were considered significant when $p < 0.05$.

W: women; M: men; T2D: type 2 diabetes; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; NMR: neutrophil-to-monocyte ratio.

severe COVID-19 needed being transferred to ICU (Table 1). Among patients admitted to ICU, 80% ($n = 36$) were men (Table 1). Patients that required critical care were, on average, 5 years older and showed lower hypertension prevalence than patients treated in non-ICU facilities (Table 1). There were no significant differences between non-ICU and ICU patients with respect to dyspnea, cough, headache, chest pain, diarrhea, peripheral oxygen saturation, and body temperature (Table 1). In contrast, heart rate significantly increased in ICU patients as compared to non-ICU patients (Table 1). Function liver tests including conjugated and unconjugated bilirubin and AST were significantly higher in ICU patients than non-ICU patients. Similarly, there were significant differences between non-ICU and ICU patients with respect to LDH, prothrombin time, and cell counts of lymphocytes, monocytes, and neutrophils (Table 1). Notably, NMR was 1.5-fold increase in ICU patients as compared to non-ICU patients (Table 1). Hospitalization days were longer in patients admitted to ICU, who also showed higher in-hospital mortality than patients treated in non-ICU facilities. There were no significant differences between non-ICU and ICU patients with respect to the six-drug regimen used to treat them that included azithromycin, ceftriaxone, oseltamivir, enoxaparin sodium, dexamethasone, and acetaminophen (Table 1).

The CRT decision algorithm for prioritizing ICU admission of patients with severe COVID-19 is shown in figure 2. The root node contains the total of 119 patients, where 37.8% ($n = 45$) required being admitted to the ICU. From the analysis of 59 parameters used to build the CRT, the value of direct bilirubin (DB) at hospital admission was the most important risk factor to allocate patients with severe COVID-19 to the ICU. In

fact, the node 1 shows that DB with cutoff point > 0.315 mg/dl can initially help to triage up to 46% ($n = 21$) of patients with COVID-19 to the ICU with sensitivity of 46.7%, specificity of 89.2%, and OR of 7.219 (95% CI, 2.823-18.458) (Fig. 2). The node 2 indicates that patients who apparently did not meet ICU admission criteria by having $DB \leq 0.315$ mg/dl can be additionally explored using the value of NMR with cutoff point > 15.90 at hospital admission (Fig. 2). The node 3 shows that $NMR > 15.90$ can be used as a complementary risk factor to triage another 46% ($n = 21$) of patients from the total population at risk of requiring ventilatory support (Fig. 2). In this way, the node 4 shows that values of DB and NMR at hospital admission allow predicting up to 92% ($n = 116$) of patients with severe COVID-19 that might require critical care, with sensitivity of 93.2%, specificity of 26.7%, and OR of 5.018 (95% CI, 1.633-15.422) (Fig. 2). Neither gender nor comorbidities such as obesity, T2D, hypertension, CKD, and CHD had an influence on the tree growing (data not shown).

Discussion

The first global wave of COVID-19 reached a peak in the months of March, April, May, and June of this year with thousands of patients admitted to critical care units across Europe, America, and the rest of the world¹²⁻¹⁵. Nowadays, the entire world is experiencing a second wave of COVID-19 that once again is alarmingly overwhelming ICUs¹⁶⁻¹⁸. For this reason, there is a strong sense of urgency to develop simple clinical models that allow predicting what patients with severe COVID-19 are at higher risk of requiring critical care with the aim of increasing patient's survival and optimizing ICU resources.

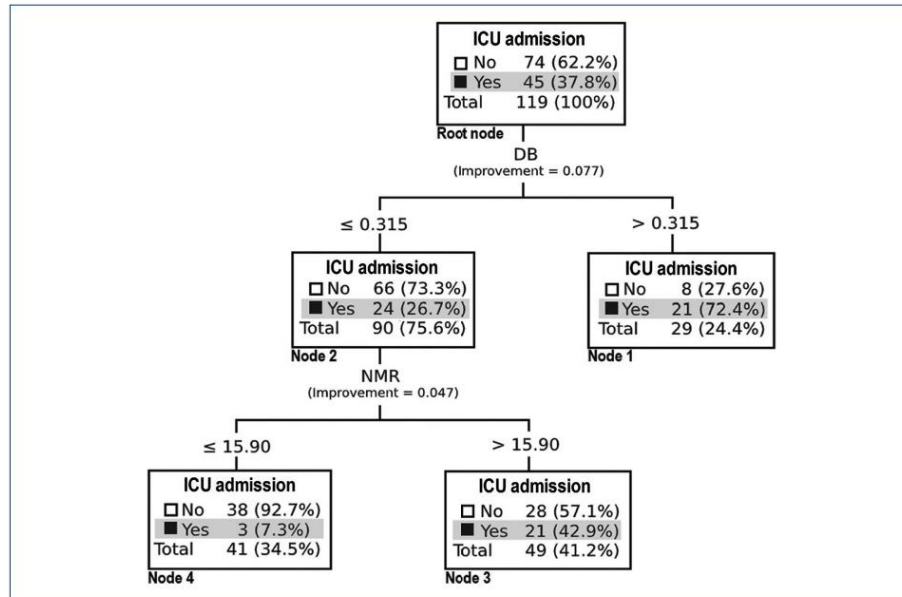


Figure 2. CRT decision algorithm for predicting ICU admission in patients with severe COVID-19. The root node contains the total of 119 patients, where 37.8% ($n = 45$) required being admitted to the ICU. The node 1 shows that DB with cutoff point > 0.315 mg/dl can initially help to triage up to 46% ($n = 21$) of patients with COVID-19 to the ICU. The node 2 indicates that patients who apparently did not meet ICU admission criteria by having $DB \leq 0.315$ mg/dl can be additionally explored using the value of NMR with cutoff point > 15.90 at hospital admission. The node 3 shows that $NMR > 15.90$ can be used as a complementary risk factor to triage another 46% ($n = 21$) of patients from the total population at risk of requiring ICU admission. The node 4 shows that values of DB and NMR at hospital admission allow predicting up to 92% ($n = 116$) of patients with severe COVID-19 that might require critical care with high sensitivity. Neither gender nor comorbidities such as obesity, T2D, hypertension, CKD, and CHD had an influence on the tree growing. The CRT was performed with the minimum number of cases in the parent node of 20% of the sample size, stopping rule for terminal nodes of 10%, 10-fold cross-validation, and tree pruning with a maximum acceptable difference in risk between the pruned tree and the sub-tree of 1 standard error, using the IBM SPSS Statistics Version 26.0 (IBM, Armonk, NY, USA). NMR resulted of dividing the neutrophil count by the monocyte count. ICU: intensive care unit; DB: direct bilirubin; NMR: neutrophil-to-monocyte ratio.

Here, we propose a clinical decision-making model based on the values of DB and NMR at admission that allows predicting up to 92% of the total patient population with severe COVID-19 that might need ICU admission (Fig. 3). Bilirubin is a waste product of hemoglobin catabolism whose water-soluble fraction is secreted into the bile in the form of conjugated bilirubin or DB. Serum levels of DB have been shown to elevate in patients with HBV or HCV and associate with increased liver inflammation and fibrosis^{19,20}. In addition, DB serum values can increase as a result of systemic infection in patients with sepsis-induced cholestasis²¹. This previous information demonstrates that DB is a

biochemical marker that elevates by hepatotropic viral infections and sepsis, both conditions highly frequent in critical patients with the most severe forms of COVID-19^{22,23}. In this study, DB serum levels significantly increased in critical patients with SARS-CoV-2 infection. In parallel, the clinical decision algorithm revealed that DB was the most important risk factor for considering admission of patients with severe COVID-19 to ICU. Cholangiocytes have been shown to highly express the angiotensin-converting enzyme 2 receptor that acts as the SARS-CoV-2 entry point through Spike protein S1 that, in turn, leads to DB accumulation and liver dysfunction in these patients^{24,25}. Considering all

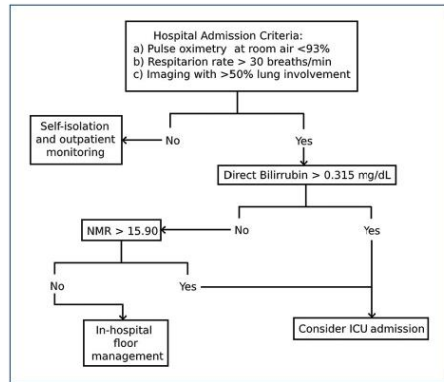


Figure 3. Clinical decision-making model based on the values of DB and NMR at admission for predicting ICU admission in patients with severe COVID-19. At hospital admission, the levels of direct bilirubin and NMR should be measured in patients with severe COVID-19 confirmed by qPCR that also shows pulse oximetry at room air < 93%, respiratory rate > 30 breaths per minute, and/or ≥ 50% lung involvement on imaging. If direct bilirubin > 0.315 mg/dL or NMR ≥ 15.90, the patient should be considered for being admitted to the intensive care unit. NMR resulted of dividing the neutrophil count by the monocyte count.
NMR: neutrophil-to-monocyte ratio.

the above information, it is feasible to assume that BD plays a pivotal role in worsening the severity of COVID-19 and can be used as marker of critical condition in this viral infection. However, we cannot dismiss that some patients with critical SARS-CoV-2 infection might also have an undiagnosed liver condition that, in turn, may increase DB levels and aggravate COVID-19. For this reason, we propose that patients seriously ill with COVID-19 should be fully explored in terms of any hepatic disorder that may increase DB levels and decrease patient's survival probability.

As we previously reported, NMR accurately predicts in-hospital mortality in patients with severe SARS-CoV-2 infection⁵. Now, our clinical decision-making model indicates that NMR with a different cutoff value can be used to complement identification of patients seriously ill with COVID-19 at risk of requiring critical care. Neutrophils play a pivotal role in defending epithelial cells of the lung against the SARS-CoV-2 invasion by secreting pro-inflammatory cytokines and orchestrating immune cell recruiting through chemokine production²⁶. However, neutrophils also appear to be a double-edged sword in

COVID-19-related pneumonia by mediating a pro-inflammatory cytokine storm that intensifies both neutrophilia and lesions in the lungs of patients infected with SARS-CoV-2²⁷. On the other side, monocytes are white blood cells that can be sorted in three different subpopulations according to the cell surface expression of the cluster of differentiation (CD) 14 and CD16. In this way, circulating monocytes expressing high CD14 levels without showing CD16 expression are identified as classical monocytes and comprise the largest monocyte subset in humans (~75-80%)²⁸. In parallel, monocytes that express CD14 and CD16 are called intermediate monocytes, while monocytes that show CD16 expression with low CD14 production are referred to as non-classical monocytes²⁸. Classical monocytes are not only the largest monocyte subpopulation in blood but also the main monocytic source of interleukin (IL-) 10, a cytokine with potent anti-inflammatory actions^{29,30}. Besides playing anti-inflammatory functions by producing IL-10, classical monocytes also exert important roles in wound healing and tissue repair³¹. Altogether, these are the main reasons behind the idea that monocytopenia is associated with the most severe forms of COVID-19³². Thus, increased neutrophilia and monocytopenia reflect the marked imbalance between pro-inflammatory and anti-inflammatory immune responses in patients critically ill with COVID-19³³. Concurring with this idea, our clinical decision algorithm indicates that in patients with severe COVID-19, the ratio between neutrophils and monocytes is a better predictor of ICU needing than the cell count of neutrophils or monocytes separately.

Clinical decision-making models are a useful tool to predict important clinical outcomes based on studying the relationship between dependent and independent variables that behave in a non-linear form as occurs in disease onset and progression³⁴. Here, we offer a clinical decision-making model based on CRT analysis that allows predicting what patients with severe SARS-CoV-2 infection will require ICU admission, as early as they arrive to the hospital. In this sense, Izquierdo et al. recently reported in a large cohort study that age > 40 years, fever > 39°C, tachypnea, and respiratory crackles are accurate predictors of ICU admission in patients with COVID-19³⁵. However, it has been reported that only 43.8% of patients with COVID-19 present fever at hospital admission, whereas 88.7% can develop increased body temperature after several days of hospitalization³⁶. Similarly, tachypnea and dyspnea can appear in a few percent of patients with COVID-19, which may also lead to underestimate the real number of patients at risk of requiring ICU admission in the

upcoming days³⁷. To this respect, our study shows that DB and NMR can predict ICU needing at hospital admission without necessarily waiting a long time to see disease worsening, a notion that might help optimizing ICU resources with the aim of reducing mortality risk. Another work conducted in a large cohort study reported that the National Early Warning Score can predict ICU admission with sensitivity of 71.4%³⁸. In this regard, our clinical decision-making model is able to predict ICU admission of patients with severe COVID-19 with sensitivity of 93.2%, thus increasing the chance of accurately identifying patients at risk of requiring critical care. Although our model presents low specificity, other relevant tools have been historically used with low specificity and high sensitivity, as the Glasgow-Blatchford scale for survival and need for intervention predictions in upper gastrointestinal bleeding, permitting hospital resources administration³⁹. In the same way, in COVID-19 context, chest computed tomography has presented low specificity but a very high sensitivity for diagnosis, allowing to accurately identify infected patients with typical symptoms who had a negative RT-PCR result⁴⁰. Moreover, a recent study proposes the use of the ABC-GOALS score that consists of different variables such as arterial pressure, obesity, glucose, and X-ray image, among others, to predict ICU admission in patients with severe COVID-19⁴¹. Besides this study reports sensitivity values below 90%, we consider that clinical models of critical care triage should be as simple and fast as possible with the aim of timely improving ICU admission of patients with severe SARS-CoV-2 infection. In this sense, DB and NMR can be quickly measured at hospital admission with the intention of timely predicting what patients are at risk of requiring critical care soon. In addition, we encourage the testing of other molecules or methods at admission to predict ICU needing to augment specificity and preserve high sensitivity.

The sample size was a limitation of this study. Despite having enrolled more than 100 patients with severe SARS-CoV-2 infection ($n = 119$), the number of patients requiring ICU admission was, in some sense, small ($n = 45$). A larger sample size may help strengthen our observations with the aim of increasing their accuracy in a clinical scenario.

Conclusions

This study proposes for the 1st time a clinical decision-making model where DB with cutoff point > 0.315 mg/dl and NMR with cutoff point > 15.90 at

admission can quickly predict the need for critical care in patients seriously ill with COVID-19 (Fig. 3). This clinical decision-making model based on CRT analysis allows predicting in an easy manner what patients with severe COVID-19 are at risk of requiring critical care with sensitivity of 93.2%. The search for clinical models to timely predict ICU admission is of great importance during the second global wave of COVID-19 and might allow optimizing ICU resources and decreasing in-hospital mortality rates.

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Conflicts of interest

The authors declare that do not exist conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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In Vitro Exposure of Primary Human T Cells and Monocytes to Polyclonal Stimuli Reveals a Basal Susceptibility to Display an Impaired Cellular Immune Response and Develop Severe COVID-19

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The contribution of the cellular immune response to the severity of coronavirus disease 2019 (COVID-19) is still uncertain because most evidence comes from patients receiving multiple drugs able to change immune function. Herein, we conducted a prospective cohort study and obtained blood samples from 128 unvaccinated healthy volunteers to examine the *in vitro* response pattern of CD4+ and CD8+ T cells and monocyte subsets to polyclonal stimuli, including anti-CD3, anti-CD28, poly I:C, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) recombinant spike S1 protein, and lipopolysaccharide. Then, we started a six-month follow-up and registered 12 participants who got SARS-CoV-2 infection, from whom we retrospectively analyzed the basal immune response pattern of T cells and monocytes. Of the 12 participants infected, six participants developed mild COVID-19 with self-limiting symptoms such as fever, headache, and anosmia. Conversely, six other participants developed severe COVID-19 with pneumonia, respiratory distress, and hypoxia. Two severe COVID-19 cases required invasive mechanical ventilation. There were no differences between mild and severe cases for demographic, clinical, and biochemical baseline characteristics. In response to polyclonal stimuli, basal production of interleukin-2 (IL-2) and interferon (IFN-) gamma significantly decreased, and the programmed cell death protein 1 (PD-1)

increased in CD4+ and CD8+ T cells from participants who posteriorly developed severe COVID-19 compared to mild cases. Likewise, CD14++CD16- classical and CD14+CD16+ non-classical monocytes lost their ability to produce IFN-alpha in response to polyclonal stimuli in participants who developed severe COVID-19 compared to mild cases. Of note, neither the total immunoglobulin G serum titers against the virus nor their neutralizing ability differed between mild and severe cases after a month of clinical recovery. In conclusion, using *in vitro* polyclonal stimuli, we found a basal immune response pattern associated with a predisposition to developing severe COVID-19, where high PD-1 expression and low IL-2 and IFN-gamma production in CD4+ and CD8+ T cells, and poor IFN-alpha expression in classical and non-classical monocytes are linked to disease worsening. Since antibody titers did not differ between mild and severe cases, these findings suggest cellular immunity may play a more crucial role than humoral immunity in preventing COVID-19 progression.

Keywords: T cell, monocyte, SARS-CoV-2, COVID-19, PD-1, IL-2, IFN-gamma, IFN-alpha

INTRODUCTION

The severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) is the causal agent of coronavirus disease 2019 (COVID-19) (1). The clinical presentation of COVID-19 has caught the attention of the scientific community around the globe because of its enormous heterogeneity, ranging from mild and moderate self-limiting viral infection to severe and critical illness (2). However, the mechanisms involved in COVID-19 progression and severity are still a matter of debate (3). A growing body of evidence has pointed out advanced age, male gender, and comorbidities such as hypertension and type 2 diabetes (D) as leading risk factors for developing severe COVID-19 (4–7). Nevertheless, several studies have consistently reported that mild COVID-19 can present even in advanced-age men with comorbidities (2, 6), bringing to light the need to understand additional factors explaining how severe disease develops.

Numerous studies have informed that an exacerbated inflammatory response worsens the clinical course of SARS-CoV-2 infection by increasing the local and systemic levels of tumor necrosis factor-alpha (TNF-alpha), interleukin (IL-) 1 beta, and IL-6 (7–9). However, emerging evidence suggests that a defective cellular immunity may also accelerate COVID-19 progression (10). Compared to patients developing mild symptoms, CD4+ and CD8+ T cell populations from COVID-19 patients admitted to intensive care units (ICU) show reduced production of IL-2, a cytokine with major functions in enhancing T and B cell proliferation (11, 12). Furthermore, CD4+ T cells also express interferon-gamma (IFN-gamma), a cytokine able to inhibit viral replication; however, seriously ill COVID-19 patients show reduced IFN-gamma-producing CD4+ T cells compared to convalescent individuals (10). CD4+ and CD8+ T cell populations from COVID-19 patients in need of hospitalization also show increased expression of cell exhaustion markers, including the programmed cell death protein 1 (PD-1) (13–15). In parallel, the ability of classical and non-classical monocytes to release the antiviral cytokine interferon-alpha (IFN-alpha) decreases as COVID-19 severity increases in patients

critically ill compared to those developing mild-to-moderate disease (16). Of note, even though T cells and monocytes appear to lose the capacity of producing key antiviral cytokines, patients with severe COVID-19 exhibit similar neutralizing antibody titers to those found in subjects with mild symptoms (10). This whole evidence emphasizes the idea that impairment in cellular immunity may play a crucial role in worsening SARS-CoV-2 infection.

Although this information suggests that cellular immunity plays a role in COVID-19 progression by stimulating T cells and monocytes to release crucial antiviral cytokines, most of this evidence comes from cross-sectional clinical studies with polytreated patients, making it challenging to correct data interpretation. We hypothesize that cytokine production mediated by cells such as T lymphocytes and monocytes against the SARS-CoV-2 has basal response patterns with the ability to predispose a patient towards the development of either mild or severe disease. However, this question is hard to respond to in infected patients receiving multiple drug schemes and therapeutic maneuvers able to modify the immune response pattern during the COVID-19 course.

For this reason, we conducted a prospective, longitudinal follow-up for six months in 105 family members of medical staff caring for COVID-19 patients, exploring their immune response pattern to *in vitro* polyclonal stimuli simulating the exposure to SARS-CoV-2. After grading disease severity in the participants resulting infected during that period, we retrospectively examined how CD4+ and CD8+ T cells and monocyte subpopulations responded *in vitro* to anti-CD3, anti-CD28, poly I:C, spike protein, and lipopolysaccharide (LPS) in seeking a basal immune pattern that could associate with the development of mild or severe COVID-19.

MATERIALS AND METHODS

Participants and Ethical Disclosures

We invited 128 healthy adult women and men to participate in the study. We enrolled participants if they met the following

inclusion criteria: family members of health care professionals working at a dedicated COVID-19 hospital in Mexico City, aging 18–65 years old, negative testing for SARS-CoV-2 by quantitative-polymerase chain reaction (qPCR), and seronegative to anti-SARS-CoV-2 IgG antibodies. We excluded subjects from the study if they had the previous diagnosis of the human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), chronic kidney or liver disease, cancer, autoimmune diseases, endocrine disorders, and infectious diseases. We also excluded pregnant or lactating women and patients taking immunomodulatory medication for the last six months. We eliminated participants from the study if they received any vaccine against COVID-19 within six months of enrollment. All study participants provided written informed consent previously approved by the institutional ethical committee of the General Hospital of Mexico (registration number of the ethical code approval: DI/20/501/03/17). The study rigorously met the principles described in the 1964 Declaration of Helsinki and its posterior amendment in 2013.

Study Design

This prospective, longitudinal study with retrospective data analysis took place from December 2020 to September 2021. All family members of health care professionals who agreed to take part in the study signed the informed consent and received a full explanation of the purposes and procedures of the study. We collected demographic, clinical, and biochemical data from all 128 participants at the enrollment. Demographic and clinical data included sex, age, and previous diagnosis of obesity (body mass index (BMI) > 30 kg/m²), type 2 diabetes (D), hypertension, coronary heart disease (CHD), and hypercholesterolemia. Biochemical data included serum albumin, total proteins, blood glucose, lipid profile, liver and kidney function tests, hematic biometry, C-reactive protein (CRP), and lactate dehydrogenase (LDH). We measured all laboratory parameters using the Beckman Coulter Dx² 700 AU Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA), the Coulter LH 780 Hematology Analyzer (Beckman Coulter Inc., Brea, CA, USA), and the BCS[®] XP System (Siemens Healthcare GmbH, Erlangen, Germany). We collected 6 ml venous blood samples from all 128 healthy participants at the enrollment, using tubes containing sodium heparin (VacutainerTM, BD Diagnostics, NJ, USA). After whole blood *in vitro* exposure to polyclonal stimuli, we performed flow cytometry staining for cell surface and intracellular markers of T cells and monocytes, storing flow cytometry data. Polyclonal stimuli were used to simulate the exposure to SARS-CoV-2 in *in vitro* culture settings, using the SARS-CoV-2 recombinant spike S1 protein (the main surface antigen of the virus), Poly I:C (a double-stranded RNA widely used to mimic viral infections *in vitro*), anti-human CD3 and anti-human CD28 (co-stimulatory signals that enhance T cell expansion and activation *in vitro*), and LPS (an endotoxin that enhances monocyte activation). We weekly followed-up to all participants by phone calls for six months, asking for the occurrence of symptoms such as headache, fever (body temperature > 37.5°C), dry cough, tiredness, myalgia,

arthralgia, nasal congestion, runny nose, anosmia, dysgeusia, sore throat, diarrhea, shortness of breath, chest pain, and blue-colored skin or lips. After reporting at least one of the above symptoms, participants attended the General Hospital of Mexico for SARS-CoV-2 infection confirmation by qPCR in nasopharyngeal swabs. Then, we started a daily follow-up on each participant confirmed for SARS-CoV-2 infection, recording relevant clinical and biochemical data at the symptom onset and seven days after, and categorizing the development of COVID-19 as mild-to-moderate or severe-to-critical disease. We classified the level of COVID-19 severity according to the World Health Organization (WHO) criteria as follows: mild COVID-19 cases showed headache, fever, dry cough, tiredness, myalgia, arthralgia, nasal congestion, runny nose, anosmia, dysgeusia, sore throat, and/or diarrhea that participants handled at home without needing of oxygen supply or hospitalization; severe COVID-19 cases presented at least one of the above symptoms plus oxygen saturation level (SpO₂) < 92% on room air, respiratory distress > 30 breath per minute, and/or > 50% lung involvement on imaging that required either hospitalization or mechanical ventilation. Once we confirmed the clinical outcome of COVID-19, we retrospectively analyzed flow cytometry data for mild or severe groups in seeking a basal immune response pattern to *in vitro* polyclonal stimuli that could associate with the disease severity.

Cell Cultures

We collected 6 ml venous blood samples from all participants at the enrollment, using tubes containing sodium heparin (VacutainerTM, BD Diagnostics, NJ, USA). Immediately after, we divided each whole blood sample into 24-well ultra-low attachment surface cell-culture plates (Costar, Kennebunk, ME, USA), adding 200 µl blood plus 400 µl RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 mM HEPES buffer (GibcoTM, Grand Island, NY, USA) per well in triplicate. We designated the first three wells as unstimulated T cell culture control containing 200 µl blood plus 400 µl supplemented RPMI-1640 for 2 hours. The subsequent three wells had 200 µl blood plus 400 µl supplemented RPMI-1640 incubated in the presence of 0.5 µg/ml SARS-CoV-2 recombinant spike S1 protein (Anigo Biolaboratories, Hsinchu City, Taiwan), 100 µg/ml Poly I:C (Sigma Aldrich, St. Louis, MO, USA), and 10 ng/ml anti-human CD3 and anti-human CD28 (BioLegend, Inc., San Diego, CA, USA) for 2 hours. We designated the following three wells as unstimulated monocyte culture control containing 600 µl blood plus 1.2 ml supplemented RPMI-1640 for 2 hours. The last three wells had 600 µl blood plus 1.2 ml supplemented RPMI-1640 incubated in the presence of 0.5 µg/ml SARS-CoV-2 recombinant spike S1 protein (Anigo Biolaboratories, Hsinchu City, Taiwan), 100 µg/ml Poly I:C (Sigma Aldrich, St. Louis, MO, USA), and 10 ng/ml LPS (Sigma Aldrich, St. Louis, MO, USA) for 2 hours. We incubated cell cultures at 37°C in humidified 5% CO₂ atmosphere for 2 hours. We selected a 2-hour stimulation based on time-response curves (Supplementary Figure 1). We treated whole blood cultures for intracellular staining with 1:1000 Brefeldin A (BioLegend, Inc., San Diego, CA, USA) and 1 µg/ml monensin (BioLegend, Inc., San Diego, CA, USA) for 45 min before the culture's ending.

Immunostaining and Flow Cytometry

After incubation, we collected whole blood samples into 5 ml falcon tubes (BD, Bedford, MA, USA), centrifuged tubes at 500 g for 10 min, and washed cell pellets with 200 μ l PBS 1X (Sigma Aldrich, St. Louis, MO, USA) twice. Immediately after, we added 5 ml ammonium-chloride-potassium (ACK) lysing buffer to each cell pellet, mixed gently and incubated for 10 min at room temperature. After centrifuging each tube at 500 g for 10 min, we discarded the supernatant and resuspended the white blood cell (WBC) pellet in 1 ml PBS 1X (Sigma Aldrich, St. Louis, MO, USA). After an extra washing step, we resuspended 5×10^5 WBCs in 50 μ l cell staining buffer (BioLegend, Inc., San Diego, CA, USA). For monocyte cultures, we incubated WBCs with 5 μ l True-Stain Monocyte Blocker™ (BioLegend, Inc., San Diego, CA, USA) for 10 min on ice. Then, we added anti-CD14 APC Fire 750, anti-CD16 PE/Cy5, and anti-HLA-DR PE (BD Biosciences, San Jose, CA, USA) for 20 min in darkness at 4°C. For T cell cultures, we incubated WBCs with anti-CD3 APC Fire 750, anti-CD4 BV 510, anti-CD8 APC, and anti-PD-1 PE for 20 min in darkness at 4°C. Afterward, we incubated WBCs with 100 μ l Fixation Medium A (FIX & PERMTM Cell Permeabilization Kit) (Invitrogen™, Carlsbad, CA, USA) for 20 min at room temperature. After rinsing WBCs with Cell Staining Buffer (BioLegend, Inc., San Diego, CA, USA), we incubated cells with 100 μ l Permeabilization Medium B (FIX & PERM™ Cell Permeabilization Kit, Invitrogen™, Carlsbad, CA, USA) plus anti-IFN-gamma Pacific Blue and anti-IL-2 PE/Cy7 for T cell cultures or IFN-alpha AF 647 (BioLegend, Inc., San Diego, CA, USA) for monocyte cultures during 20 min in darkness at room temperature. After rinsing the cell pellets with cell staining buffer (BioLegend, Inc., San Diego, CA, USA), we acquired cells on a BD FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA), acquiring 10,000 events per test on CD3+ or HLA-DR+ cells, respectively in three different and individual staining.

Gating Strategy

After we confirmed the severity of COVID-19, we retrospectively analyzed flow cytometry data for mild COVID-19 cases or severe COVID-19 participants. For T cells, we first gated single cells on a forward scatter (FSC-H)/side scatter (SSC-A) density plot. Afterward, we gated cells on a time/side scatter density plot to visualize how well the flow of cells was during acquisition. We recognized the lymphocyte population on a side scatter (SSC-A)/forward scatter (FSC-A) plot. Then, we gated lymphocytes using the CD3 expression, acquiring 10,000 events on this gate for posterior analyses. After that, we obtained CD4+ and CD8+ T cells through a rectangular gating strategy using CD4 and CD8 expression. Finally, we analyzed IFN-gamma, IL-2, and PD-1 expression on the CD4+ and CD8+ T cell populations (Supplementary Figures 2A, B, respectively). For monocytes, we first gated single cells on a forward scatter (FSC-H)/side scatter density plot. Then, we gated cells on a time/side scatter density plot. Afterward, we recognized monocytes using HLA-DR expression, acquiring 10,000 events on this gate for posterior analyses. Then, we obtained total monocytes on a CD14/CD16

density plot and subsequently identified gates for classical monocytes (CD14++CD16-), intermediate monocytes (CD14+CD16+), and non-classical monocytes (CD14+CD16+). Finally, we analyzed IFN-alpha expression on each monocyte subset (Supplementary Figure 3). We obtained the Median Fluorescence Intensity (MFI) for IL-2, IFN-gamma, PD-1, and IFN-alpha, considering both positive and negative cell populations for each marker, as shown in Supplementary Figure 4. We obtained the percentage of positive cells for each marker using proper fluorescence minus one (FMO) controls. We performed compensation controls for each fluorochrome by UltraComp eBeads™ (Invitrogen™, Carlsbad, CA, USA). We analyzed data using the FlowJo 10.0.7 software (TreeStar, Inc, Ashland, OR, USA).

Total IgG and Neutralizing Antibodies Anti-SARS-CoV-2

One month after clinical recovery of the twelve patients who developed mild or severe COVID-19, we collected venous blood samples for posterior serum isolation and measurement of anti-SARS-CoV-2 IgG total antibodies and neutralizing antibody percentage in triplicate by the Enzyme Linked-ImmunoSorbent Assay (ELISA). For total antibodies, we measured IgG antibody serum levels against the SARS-CoV-2 nucleocapsid (N) protein using a kit from Abcam (Abcam, ab274339, Cambridge, UK) and a microplate reader at 450 nm. For neutralizing antibody percentage, we used the anti-SARS-CoV-2 Neutralizing Antibody ELISA Kit and a microplate reader at 450 nm (Thermo Fisher Scientific, BMS2326, Vienna, Austria). We calculated the neutralization percentage for unknown samples as follows: neutralization (%) = $1 - (\text{absorbance of unknown sample} / \text{absorbance of negative control}) \times 100$.

Statistics

We evaluated the normality of data by the Shapiro-Wilk test. For *in vitro* assays, we compared the basal expression of IL-2, PD-1, IFN-gamma, and IFN-alpha between mild COVID-19 cases and severe COVID-19 cases by the unpaired Student's T test. We compared the amount of IFN-gamma+IL-2+ double-positive cells in helper and cytotoxic T cells expressing or not PD-1 from mild COVID-19 cases and severe COVID-19 cases by two-way ANOVA. We compared the anti-SARS-CoV-2 IgG total antibodies and neutralizing antibody percentage between mild COVID-19 cases and severe COVID-19 cases by the unpaired Student's T test. We considered differences significant when $P < 0.05$. We performed all statistical analyses using the GraphPad Prism 7 software.

RESULTS

Demographic, Clinical, and Laboratory Parameters in the Study Population

We show the selection process of participants enrolled in the study in Figure 1. After meeting inclusion and exclusion criteria, we eliminated 23 participants from the study because they

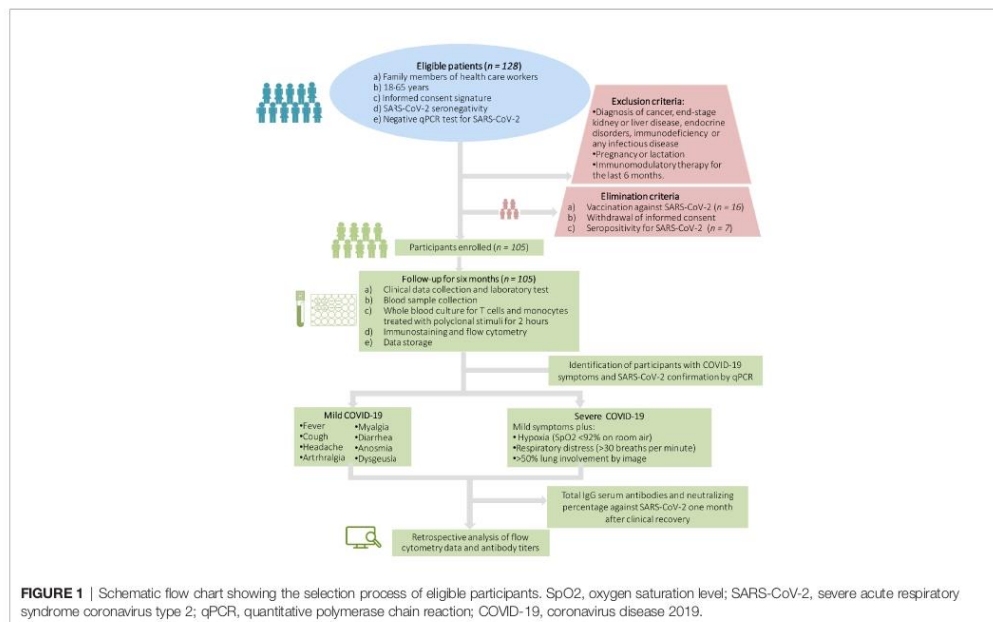


FIGURE 1 | Schematic flow chart showing the selection process of eligible participants. SpO₂, oxygen saturation level; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2; qPCR, quantitative polymerase chain reaction; COVID-19, coronavirus disease 2019.

exhibited specific IgG serum antibody titers against the SARS-CoV-2 N protein ($n = 7$) or got the Pfizer-BioNTech COVID-19 vaccine ($n = 16$). One hundred and five volunteers completed the six-month follow-up without getting vaccinated (**Figure 1**). At the beginning of the follow-up, the whole study population consisted of 41 women and 64 men that were 41.6 ± 11.2 years on average and showed a low prevalence of comorbidities such as obesity, type 2 diabetes, hypertension, coronary heart disease and average values of routine biochemical tests (**Table 1**). After grading COVID-19 severity in participants who got SARS-CoV-2 infection during the six-month follow-up, we registered two women and four men only experiencing a self-limiting disease with mild symptoms such as fever, anosmia, headache, myalgia, and arthralgia, resolved without specific drug treatment after 9–12 days (**Table 2**). Conversely, one woman and five men developed severe COVID-19 characterized by the symptoms mentioned above, plus respiratory distress (36.6 ± 3.1 breaths per minute), hypoxia ($74.0 \pm 7.6\%$), and pneumonia (**Table 2**). Two participants developing severe COVID-19 required ICU admission for invasive ventilatory support (**Table 2**). Of note, the baseline clinical characteristics did not differ between participants who developed mild or severe COVID-19 during the follow-up (**Table 1**). Except for neutrophil and lymphocyte percentages, ALP, and serum albumin, there were no differences between participants who developed mild or severe COVID-19 for the values of hematic biometry, blood glucose, lipid profile, renal parameters, and liver function tests seven days after symptom onset (**Table 2**). As we have outlined here, neither

baseline characteristics nor the clinical presentation of COVID-19 allowed predicting the disease severity in the participants who got SARS-CoV-2 infection during the follow-up.

IL-2, IFN-Gamma, and PD-1 Production in Helper T Cells

Supplementary **Figure 2A** illustrates the gating strategy for analyzing IL-2, IFN-gamma, and PD-1 expression in CD3+CD4+ T cells stimulated with anti-CD3, anti-CD28, poly I:C, and SARS-CoV-2 recombinant spike S1 protein in whole blood *in vitro* cultures at the beginning of the follow-up, when all healthy participants were initially enrolled in the study (**Supplementary Figure 2A**). Representative dot-plots illustrate the comparison of CD3+CD4+ T cells expressing IL-2 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up (**Figure 2A**). In response to polyclonal *in vitro* stimulation, the percentage of CD3+CD4+IL-2+ T cells showed a significant 4-fold decrease in the group that during the follow-up developed severe COVID-19 compared to participants experiencing mild symptoms ($P = 0.0085$) (**Figure 2B**). IL-2 expression behaved in the same way as observed in cell percentage, displaying a significant 2-fold diminution in helper T cells from participants that after SARS-CoV-2 infection developed severe COVID-19 compared to the mild disease group ($P = 0.0075$) (**Figure 2C**). Representative dot-plots exemplify the contrast of CD3+CD4+ T cells expressing IFN-gamma in whole blood samples exposed to polyclonal molecules from participants who experienced mild or

TABLE 1 | Baseline characteristics of the study participants.

Parameter	Baseline characteristics in the entire study population	Baseline characteristics only in participants who developed COVID-19		Reference ranges	P value ^a vs b
		Mild ^a	Severe ^b		
Gender (W/M)	41/64	2/4	1/5	-	0.505
Age (years)	41.6 ± 11.2	47.3 ± 10.3	45 ± 9.6	-	0.343
BMI (kg/m ²)	27.3 ± 4.8	26 ± 1.6	25.5 ± 1.1	<25.0	0.458
Obesity (W/M)	14/10	0/0	0/1	-	1.000
D prevalence (%)	6	0	0	-	1.000
Hypertension prevalence (%)	8.6	0	0	-	1.000
Coronary heart disease (%)	1.7	0	0	-	1.000
Heart rate (beats per minute)	75.4 ± 10.8	72.5 ± 5.9	70.5 ± 5.2	60-100	0.550
Breathing rate (bpm)	15.7 ± 3.3	16.5 ± 2.5	15.6 ± 2.8	15-20	0.609
Body temperature (°C)	36.2 ± 0.2	36.1 ± 0.2	36.1 ± 0.1	≤37.5	>0.999
Systolic blood pressure (mmHg)	128.6 ± 18.3	124.5 ± 11.5	125.8 ± 7.2	<130	0.816
Diastolic blood pressure (mmHg)	82.1 ± 11.3	78.5 ± 9.3	80.1 ± 6.3	<85	0.725
Peripheral oxygen saturation (%)	95.8 ± 2.2	95.6 ± 1.0	96.1 ± 2.7	>91	0.682
Leukocytes (×10 ⁹ /μl)	6.3 ± 1.2	6.1 ± 0.8	6.9 ± 1.8	4.00-11.00	0.213
Neutrophil percentage (%)	58.3 ± 8.6	55.2 ± 10.4	57.2 ± 7.2	37.00-80.00	0.367
Lymphocyte percentage (%)	31.5 ± 7.2	33.8 ± 9.6	27.4 ± 4.1	10.00-50.00	0.106
Monocyte percentage (%)	5.8 ± 0.9	6.3 ± 0.3	7.4 ± 1.6	0.00-8.00	0.084
Band cells (%)	0.04 ± 0.03	0.08 ± 0.08	0.00 ± 0.00	0.00-7.00	0.216
Hemoglobin (g/dl)	16.7 ± 1.0	16.2 ± 1.4	16.9 ± 0.7	13.1-18.00	0.173
Platelets (×10 ⁹ /μl)	219.3 ± 24.6	180 ± 22.9	208.6 ± 26.5	150-400	0.053
Glucose (mg/dl)	96.3 ± 7.4	83.4 ± 6.1	92 ± 9.7	70.0-126.0	0.447
Urea (mg/dl)	30.2 ± 8.6	28.6 ± 4.9	39.2 ± 10.5	20.00-40.00	0.059
Creatinine (mg/dl)	1.1 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.60-1.30	0.180
Total Cholesterol (mg/dl)	207.9 ± 48.0	178.8 ± 18.3	171.4 ± 37.5	50.0-200.0	0.350
Triglycerides (mg/dl)	180.5 ± 82.6	196.8 ± 104	134.8 ± 59.1	30.0-200.0	0.140
HDL (mg/dl)	44.7 ± 8.5	40.6 ± 10.8	41 ± 9.4	≤45.00	0.475
LDL (mg/dl)	110.6 ± 34.2	114.6 ± 33.3	117.8 ± 28.4	<116.00	0.437
Total bilirubin (mg/dl)	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.00-1.00	0.423
Direct bilirubin (mg/dl)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.06	0.00-0.30	0.211
Indirect bilirubin (mg/dl)	0.5 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.20-1.00	0.421
ALT (IU/l)	30.4 ± 8.9	27.6 ± 9.6	24 ± 9.9	30-65	0.288
AST (IU/l)	28.2 ± 5.6	23.6 ± 6.8	19.4 ± 3.6	15-37	0.129
ALP (IU/l)	51.5 ± 14.1	17.6 ± 16.8	59.2 ± 15.7	50-136	0.116
GGT (IU/l)	31.2 ± 19.7	36.4 ± 31.0	35.7 ± 17.4	5-40	0.485
Total Protein (mg/dl)	7.1 ± 0.1	7.2 ± 0.1	7.1 ± 0.2	6.50-8.20	0.275
Albumin (mg/dl)	4.8 ± 0.4	4.29 ± 0.2	4.4 ± 0.3	3.50-5.00	0.267
LDH (IU/l)	194.2 ± 17.0	179.2 ± 4.7	162.6 ± 25.0	100-190	0.118
Amylase (IU/l)	51.3 ± 15.6	45.5 ± 13.2	47.4 ± 17.1	25-115	0.430
Lipase (IU/l)	37.4 ± 14.8	28.5 ± 12.5	24.8 ± 10.8	12-70	0.324

Reference values are shown according to the Clinical Laboratory of the General Hospital of Mexico. We expressed data as mean ± standard deviation. We retrospectively compared baseline characteristics between mild and severe groups using the chi-square test or the unpaired Student's T-test and considered differences significant when $P < 0.05$. COVID-19, coronavirus disease 2019; W, women; M, men; BMI, body mass index; D, type 2 diabetes; bpm, breaths per minute; HDL, high-density lipoproteins; LDL, low-density lipoproteins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase. We show demographic, clinical, and biochemical baseline parameters in all participants and those who developed COVID-19 during the six-month follow-up.

severe COVID-19 throughout the follow-up (**Figure 2D**). The percentage of CD3+CD4+IFN-gamma+ T cells displayed a significant 4-fold reduction in the severe COVID-19 group compared to participants developing a mild disease ($P = 0.0034$) (**Figure 2E**). Likewise, IFN-gamma production significantly decreased in helper T cells from participants experiencing severe COVID-19 compared to those found in individuals with mild symptoms ($P = 0.0369$) (**Figure 2F**). Representative dot-plots illustrate the comparison of CD3+CD4+ T cells expressing PD-1 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up

(**Figure 2G**). The percentage of CD3+CD4+ T cells expressing PD-1 exhibited a significant 2-fold increase in the severe COVID-19 group compared to the mild disease group ($P = 0.0416$) (**Figure 2H**). There were no significant changes between severe and mild COVID-19 participants for PD-1 expression in the population of helper T cells (**Figure 2I**). Representative dot-plots exemplify the contrast of CD3+CD4+ T cells simultaneously expressing IL-2, IFN-gamma, and PD-1 in whole blood samples exposed to polyclonal molecules from participants who experienced mild or severe COVID-19 throughout the follow-up (**Figure 2J**). Interestingly, PD-1 expression was intimately related to IL-2 and

TABLE 2 | Clinical and biochemical characteristics of participants who developed mild or severe COVID-19 during the six-month follow-up.

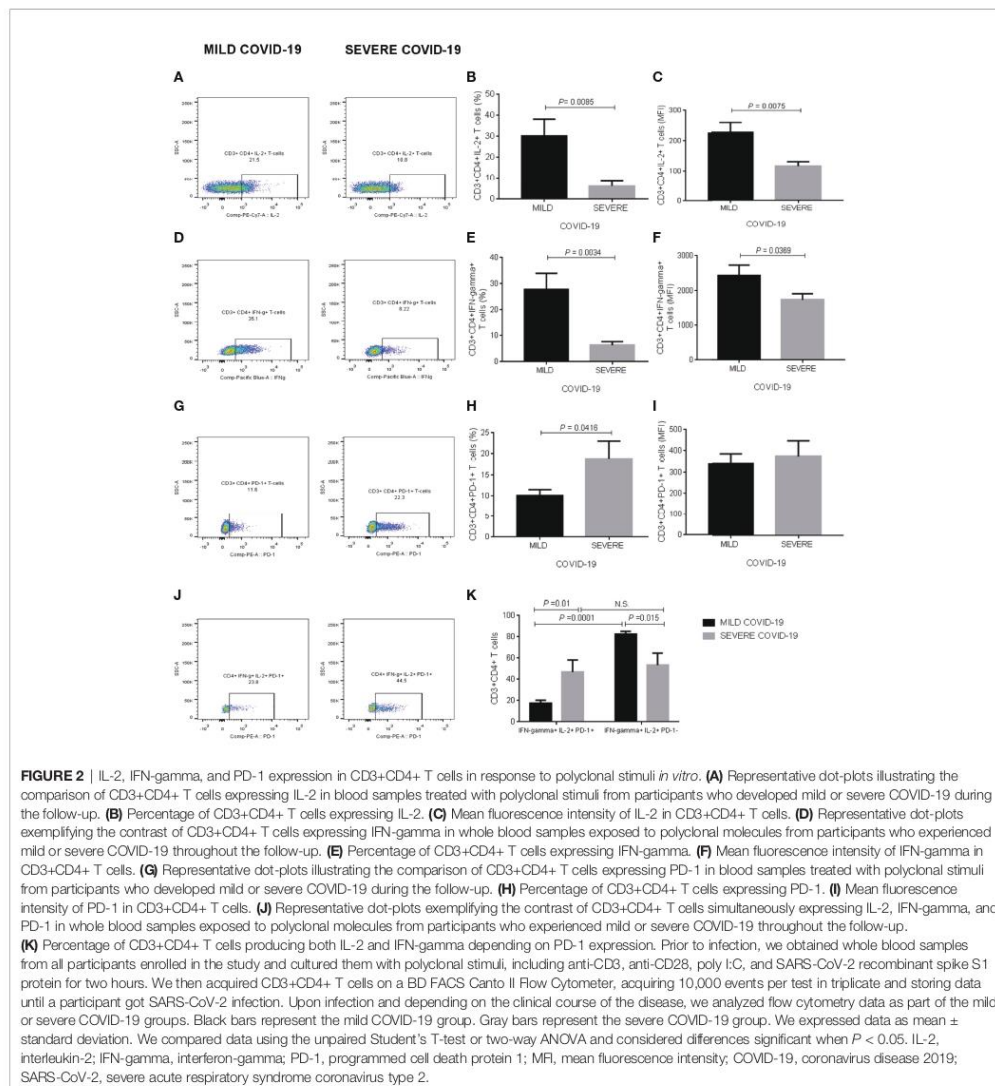
Parameter	Onset of symptoms		Reference ranges	P value ^a vs ^b
	Mild COVID-19 ^a	Severe COVID-19 ^b		
Fever (Yes/No)	4/2	5/1	<37.5°C	1.000
Anosmia (Yes/No)	4/2	3/3	-	1.000
Headache (Yes/No)	6/0	6/0	-	1.000
Myalgia (Yes/No)	4/2	5/1	-	1.000
Arthralgia (Yes/No)	2/4	1/5	-	1.000
Diarrhea (Yes/No)	3/3	2/4	-	1.000
ICU admission (Yes/No)	0/6	2/4	-	0.227
Clinical characteristics seven days after symptom onset				
Heart rate (beats per minute)	69.1 ± 6.3	81.3 ± 8.9	60-100	0.021
Breathing rate (bpm)	19.8 ± 3.0	36.6 ± 3.1	15-20	<0.0001
Peripheral oxygen saturation (%)	95.1 ± 1.7	74.0 ± 7.6	>91	<0.0001
Leukocytes (×10 ⁹ /μl)	5.6 ± 1.4	11.0 ± 5.6	4.00-11.00	0.066
Neutrophil percentage (%)	48.6 ± 14.2	80.1 ± 15.6	37.00-80.00	0.044*
Lymphocyte percentage (%)	40.9 ± 13.0	13.8 ± 14.8	10.00-50.00	0.043*
Monocyte percentage (%)	7.3 ± 0.1	5.8 ± 1.7	0.00-8.00	0.200
Band cells (%)	0.0 ± 0.0	0.0 ± 0.0	0.00-7.00	>0.999
Hemoglobin (g/dl)	16.7 ± 0.7	14.5 ± 1.7	13.1-18.00	0.088
Platelets (×10 ⁹ /μl)	273.5 ± 16.2	283.4 ± 112.5	150-400	0.444
Glucose (mg/dl)	89.0 ± 12.7	208.9 ± 92.1	70.00-100.00	0.088
Urea (mg/dl)	34.8 ± 12.5	58.9 ± 68.6	20.00-40.00	0.355
Creatinine (mg/dl)	0.9 ± 0.0	1.6 ± 2.3	0.80-1.30	0.354
Total Cholesterol (mg/dl)	194.0 ± 42.4	116.7 ± 21.1	50.00-200.00	0.071
Triglycerides (mg/dl)	344.5 ± 86.9	162.8 ± 81.6	30.00-150.00	0.142
HDL (mg/dl)	32.5 ± 7.7	27.0 ± 8.2	≤45.00	0.428
LDL (mg/dl)	118.0 ± 26.8	79.3 ± 27.7	≤115.00	0.142
Total bilirubin (mg/dl)	0.8 ± 0.3	0.5 ± 0.1	0.00-1.00	0.500
Direct bilirubin (mg/dl)	0.1 ± 0.0	0.1 ± 0.0	0.00-0.30	>0.999
Indirect bilirubin (mg/dl)	0.6 ± 0.2	0.4 ± 0.1	0.20-1.00	0.500
ALT (IU/l)	37.5 ± 27.5	49.2 ± 37.8	30-65	0.711
AST (IU/l)	23.5 ± 6.3	48.5 ± 32.5	15-37	0.133
ALP (IU/l)	60.0 ± 2.8	99.3 ± 17.0	50-136	0.044*
GGT (IU/l)	46.0 ± 24.4	56.7 ± 27.3	5-40	0.755
Total Protein (mg/dl)	7.4 ± 0.0	6.7 ± 0.6	6.50-8.20	0.166
Albumin (mg/dl)	4.6 ± 0.0	3.6 ± 0.4	3.50-5.00	0.044*
LDH (IU/l)	162.5 ± 38.8	354.6 ± 146.5	100-190	0.088
Amylase (IU/l)	45.0 ± 9.8	65.0 ± 66.8	25-115	0.700
Lipase (IU/l)	41.0 ± 14.1	45.3 ± 45.6	12-70	0.810

We recorded data from all COVID-19 participants at the onset of symptoms and seven days after. Reference values are shown according to the Clinical Laboratory of the General Hospital of Mexico. We expressed data as mean ± standard deviation. We compared clinical and biochemical data between mild and severe groups using the chi-square test or the unpaired Student's T-test and considered differences significant when $P < 0.05$. The asterisks represent significant differences. COVID-19, coronavirus disease 2019; ICU, intensive care unit; bpm, breaths per minute; HDL, high-density lipoproteins; LDL, low-density lipoproteins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase.

IFN-gamma production in the population of helper T cells. In this sense, participants who developed mild symptoms showed that PD-1+ T cells expressing both IL-2 and IFN-gamma decreased 4-fold compared to PD-1 negative helper T lymphocytes ($P = 0.0001$), indicating that PD-1 expression inversely associated with IL-2 and IFN-gamma production (Figure 2K). Nevertheless, we did not observe this expected behavior in participants developing severe COVID-19 after SARS-CoV-2 infection, whose helper T cells exhibited similar IL-2 and IFN-gamma expression patterns independently of expressing or not PD-1 (Figure 2K). Additionally to cell percentages, we show the corresponding absolute numbers of CD3+CD4+ T cells expressing IL-2, IFN-gamma, and PD-1 in Supplementary Figure 5. We found no detectable IL-2, IFN-gamma, and PD-1 expression in CD3+CD4+ T cells cultured in the absence of anti-CD3, anti-CD28, poly I:C, and SARS-CoV-2 recombinant spike S1 protein (data not shown).

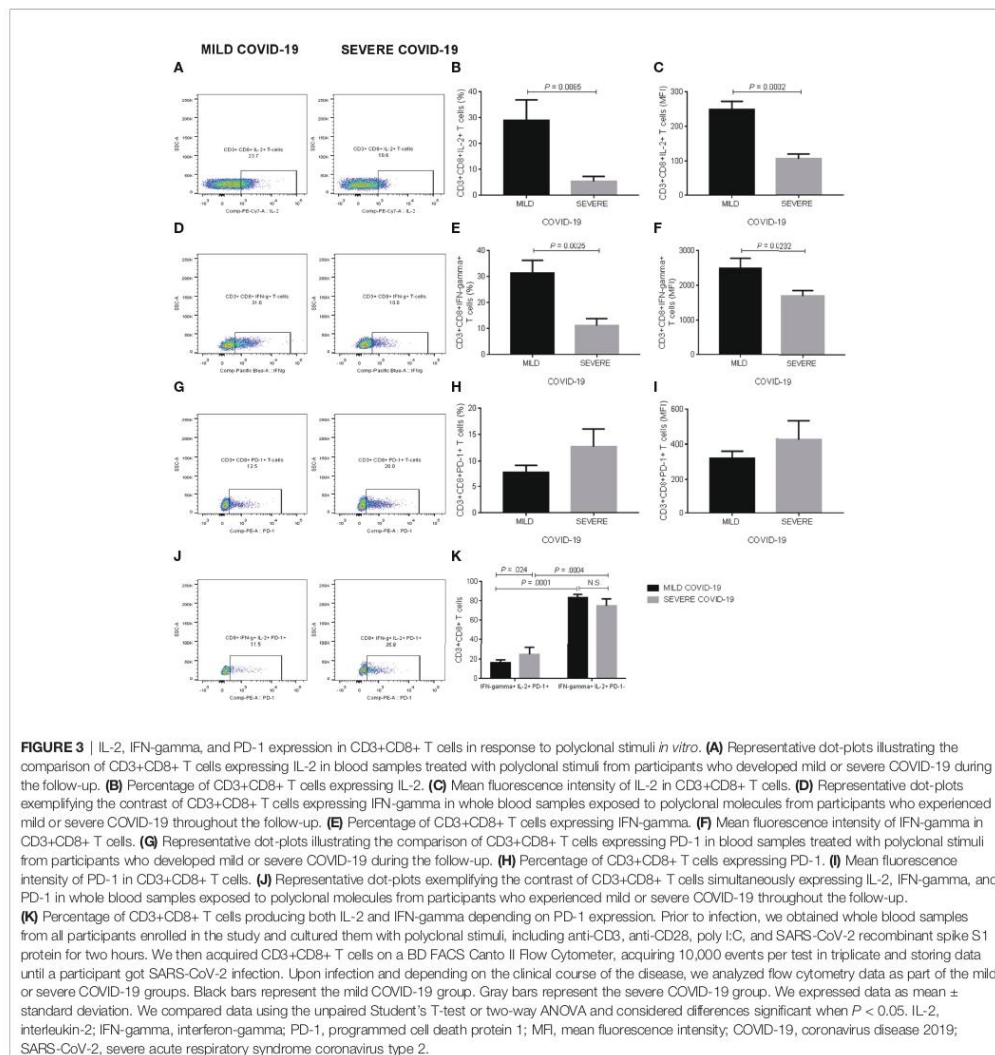
IL-2, IFN-Gamma, and PD-1 Production in Cytotoxic T Cells

Supplementary figure 2B shows the gating strategy for examining IL-2, IFN-gamma, and PD-1 expression in CD3+CD8+ T cells exposed to polyclonal stimuli *in vitro* (Supplementary Figure 2B). Representative dot-plots illustrate the comparison of CD3+CD8+ T cells expressing IL-2 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up (Figure 3A). In response to anti-CD3, anti-CD28, poly I:C, and SARS-CoV-2 recombinant spike S1 protein, whole blood cultures revealed that the percentage of CD3+CD8+IL-2+ T cells exhibited a significant 5-fold decrease in the group that posteriorly developed severe COVID-19 compared to participants with mild symptoms ($P = 0.0085$) (Figure 3B). As expected, IL-2 production significantly reduced in cytotoxic T cells from



participants developing severe COVID-19 compared to those found in subjects experiencing mild disease ($P = 0.0002$) (Figure 3C). Representative dot-plots exemplify the contrast of CD3+CD8+ T cells expressing IFN-gamma in whole blood samples exposed to polyclonal molecules from participants who experienced mild or severe COVID-19 throughout the follow-up (Figure 3D). The percentage of CD3+CD8+IFN-

gamma+ T cells showed a significant 3-fold diminution in the severe COVID-19 group compared to participants experiencing mild symptoms ($P = 0.0025$) (Figure 3E). In parallel, IFN-gamma expression significantly decreased in the cytotoxic T cell population of participants who developed severe COVID-19 compared to that found in subjects with mild symptoms ($P = 0.0232$) (Figure 3F). Representative dot-plots illustrate the



comparison of CD3+CD8+ T cells expressing PD-1 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up (Figure 3G). Contrary to what we expected, neither the percentage of CD3+CD8+PD-1+ T cells nor PD-1 production itself exhibited significant differences between the mild and severe COVID-19 groups (Figures 3H, I, respectively). Representative dot-plots exemplify the contrast of CD3+CD8+

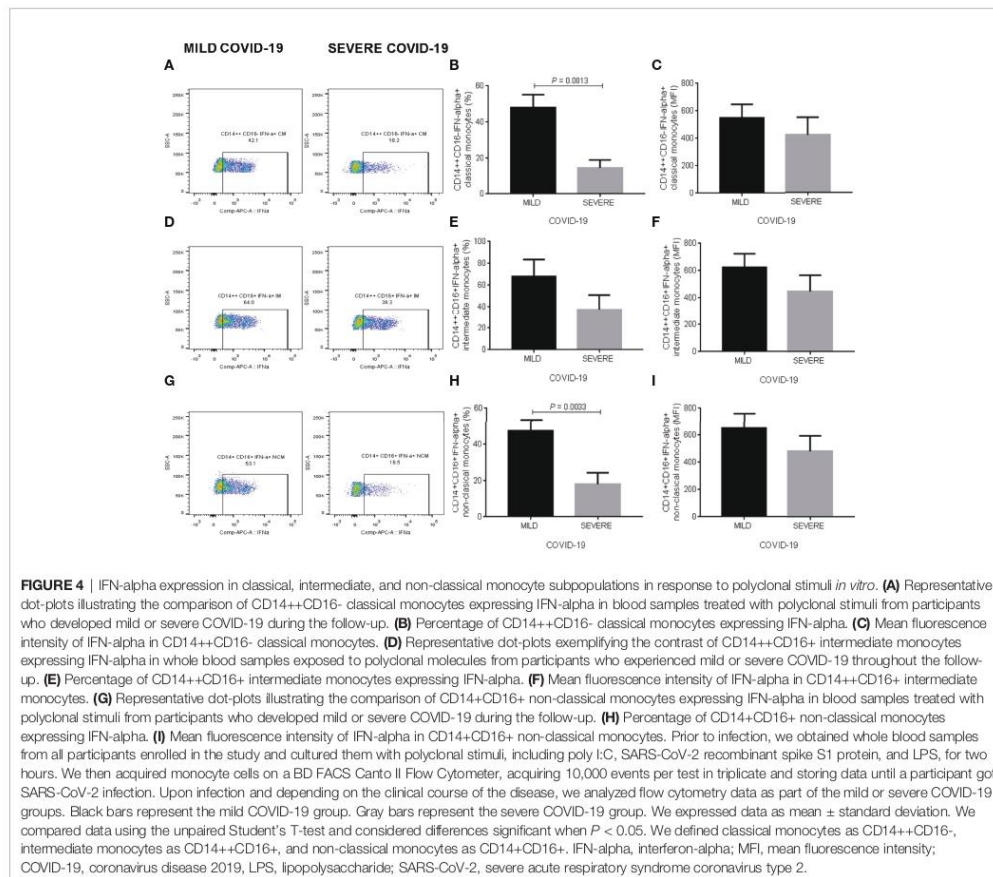
T cells simultaneously expressing IL-2, IFN-gamma, and PD-1 in whole blood samples exposed to polyclonal molecules from participants who experienced mild or severe COVID-19 throughout the follow-up (Figure 3J). However, PD-1 expression conditioned IL-2 and IFN-gamma production in cytotoxic T cells, which expressed higher IL-2 and IFN-gamma levels in PD-1 negative cells than CD3+CD8+PD-1+ T cells independently of having been analyzed in participants that

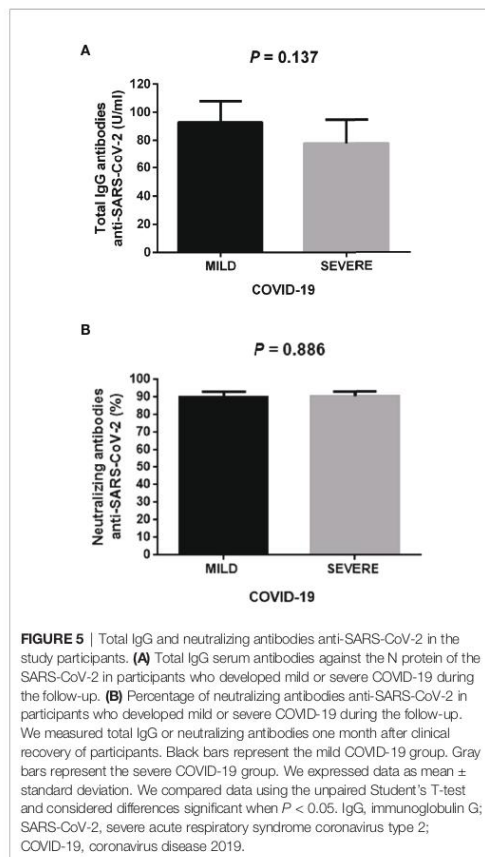
posteriorly developed either mild or severe COVID-19 (Figure 3K). In addition to cell percentages, we provide the corresponding absolute numbers of CD3+CD8+ T cells expressing IL-2, IFN-gamma, and PD-1 in **Supplementary Figure 5**. We observed no detectable IL-2, IFN-gamma, and PD-1 expression levels in CD3+CD8+ T cells cultured without anti-CD3, anti-CD28, poly I:C, and recombinant spike S1 protein (data not shown).

IFN-Alpha Production in Monocyte Subpopulations

Supplementary Figure 3 illustrates the gating strategy for evaluating IFN-alpha expression in classical, intermediate, and non-classical monocytes exposed to SARS-CoV-2 recombinant spike S1 protein, poly I:C and LPS in whole blood *in vitro* cultures (**Supplementary Figure 3**). Representative dot-plots

illustrate the comparison of CD14++CD16- classical monocytes expressing IFN-alpha in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up (Figure 4A). In response to polyclonal *in vitro* stimulation, the percentage of classical monocytes expressing IFN-alpha showed a significant 3-fold decrease in the group who developed severe COVID-19 during the follow-up compared to participants with mild symptoms ($P = 0.0013$) (Figure 4B). There were no significant changes between mild and severe COVID-19 group for IFN-alpha expression in this monocyte subset (Figure 4C). Representative dot-plots exemplify the contrast of CD14++CD16+ intermediate monocytes expressing IFN-alpha in whole blood samples exposed to polyclonal molecules from participants who experienced mild or severe COVID-19 throughout the follow-up (Figure 4D). Neither the percentage





of intermediate monocytes expressing IFN-alpha nor IFN-alpha production itself displayed significant differences between the mild and severe COVID-19 groups (Figures 4E, F, respectively). Representative dot-plots illustrate the comparison of CD14⁺CD16⁺ non-classical monocytes expressing IFN-alpha in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up (Figure 4G). The percentage of non-classical monocytes expressing IFN-alpha exhibited a significant 2.5-fold reduction in participants developing severe COVID-19 compared to that found in individuals experiencing mild symptoms ($P = 0.0033$) (Figure 4H). Conversely, there were no significant changes between mild and severe COVID-19 groups for IFN-alpha expression in this monocyte subset (Figure 4I). Besides showing cell percentages, we present the corresponding absolute numbers of classical, intermediate, and non-classical monocytes expressing IFN-alpha in Supplementary Figure 5. We found no detectable IFN-alpha

expression in classical, intermediate, and non-classical monocytes cultured without recombinant spike S1 protein, poly I:C, and LPS (data not shown).

Total IgG and Neutralizing Antibodies Anti-SARS-CoV-2

The production pattern of IL-2, IFN-gamma, and IFN-alpha suggested that helper and cytotoxic T cells and monocyte subpopulations show a basal defective cellular response against polyclonal stimuli, which is probably associated with predisposing to the development of severe COVID-19 after SARS-CoV-2 infection. To know whether a possible impairment in the cellular immune response led to defective antibody production, we decided to measure the total concentration of IgG serum antibodies and the percentage of neutralizing antibodies against the SARS-CoV-2 in participants who developed either mild or severe COVID-19. Unexpectedly, the total IgG serum titers against the N protein of the SARS-CoV-2 showed no significant changes between participants developing mild or severe COVID-19 ($\bar{x} = 92.8 \pm 15.1$ and $\bar{x} = 77.9 \pm 16.8$, $P = 0.137$, respectively) (Figure 5A). Likewise, the neutralizing antibody percentage against the virus did not differ between participants who developed mild or severe COVID-19 ($\bar{x} = 89.6 \pm 7.1$ and $\bar{x} = 90.2 \pm 6.4$, respectively, $P = 0.886$) (Figure 5B). Thus, defective expression of IL-2, IFN-gamma, and IFN-alpha in T lymphocytes and monocytes did not affect the production of either total IgG or neutralizing antibodies against the SARS-CoV-2.

DISCUSSION

The mechanisms of the cell-mediated immunity contributing to worsening the severity of COVID-19 remain unclear (17, 18). For this reason, we formed a cohort of healthy individuals who were family members of health care professionals working at a dedicated COVID-19 hospital and obtained more than 120 whole blood samples. Then, we exposed T cells and monocytes to polyclonal stimuli *in vitro* to characterize a cytokine response pattern that we could link to the severity of COVID-19 only in those participants resulting infected during a six-month follow-up. We found that participants who got infected and posteriorly experienced mild COVID-19 symptoms exhibit a different immune expression pattern in response to polyclonal stimuli than that observed in subjects that developed severe COVID-19. Participants that responded to polyclonal stimuli by increasing IL-2 and IFN-gamma production and decreasing PD-1 expression in CD4⁺ and CD8⁺ T cells tended to develop mild COVID-19 symptoms. Conversely, subjects with decreased IL-2 and IFN-gamma expression and increased PD-1 production in CD4⁺ and CD8⁺ T cells in response to polyclonal stimuli tended to display the most severe form of COVID-19, including respiratory distress and mechanical ventilatory support needing. These findings reveal a basal immune response pattern to polyclonal stimuli intimately associated with COVID-19 progression, wherein CD4⁺ and CD8⁺ T cells fail

to produce IL-2 and IFN-gamma but show an increased ability to express PD-1.

At the pandemic's beginning, most studies informed that more than 80% of patients seriously ill with COVID-19 tended to exhibit a marked lymphocytopenia at hospital admission (19–21). After that, several lines of evidence confirmed that severe COVID-19 was not only related to a reduced number of circulating lymphocytes but also decreased T cell activity, especially cytokine production such as IL-2 and IFN-gamma (22, 23). IL-2 plays a decisive role in preventing lymphocytopenia by promoting CD4+ T cell proliferation via the Janus kinase 1/Signal transducer and activator of transcription 5 (JAK1/STAT5) (21, 24). In fact, the use of recombinant IL-2 stimulates lymphocyte count recovery and systemic inflammatory response amelioration in patients with severe COVID-19 pneumonia (25). This body of evidence makes it feasible to think that subjects with a low number of IL-2-producing CD4+ T cells in response to *in vitro* polyclonal stimuli display increased susceptibility to severe COVID-19 after SARS-CoV-2 infection. IFN-gamma's primary function in the anti-viral response is acting directly on CD8+ T cells to boost their abundance and reduce viral load (26). IFN-gamma-producing CD8+ T cells considerably decrease in patients with severe COVID-19 compared to patients experiencing mild symptoms (27). What is also relevant is that IFN-gamma is considered an independent risk factor of mortality in COVID-19 patients (28). All this information concurs with our findings and supports the idea that a basal deficiency in IFN-gamma-producing CD8+ T cells, as revealed when we used unspecific polyclonal stimuli, may increase the risk of exacerbating viral load and developing severe COVID-19 after SARS-CoV-2 infection. However, CD8+ T cells are not only able to secrete IFN-gamma but also molecules with potent cytotoxic activity such as granzyme and perforin, which are crucial components in the recognition and lysis of infected cells. Therefore, we still must characterize the production of cytotoxic molecules in CD8+ T cells exposed to polyclonal stimuli to draw significant conclusions regarding the possible role of the cytotoxic activity of CD8+ T lymphocytes in COVID-19 progression.

PD-1 has a pivotal role in preventing exacerbation of immune responses by modulating the activity of T cells via apoptosis promotion, cell proliferation arrest, and cytokine secretion inhibition (29). In COVID-19, the function of PD-1 is still a matter of debate because some research teams have consistently reported that CD4+ and CD8+ T cells from COVID-19 patients express high PD-1 levels and are exhausted (13, 30). In contrast, other working groups have informed that cytotoxic T cells retain their anti-viral functions against the SARS-CoV-2 despite expressing PD-1 (31). We may attribute these controversial findings to the fact that most investigations assessing PD-1 expression in COVID-19 have studied patients treated with several drug cocktails, including cyclooxygenase (COX)-inhibitors, dexamethasone, anticoagulants, among others (32, 33). These drug schemes aim to treat and prevent COVID-19 complications but can also alter the expression of cytokines such as IL-2 and IFN-gamma and immune checkpoints as occurs with

PD-1. For instance, Kailin Xing and colleagues previously demonstrated that dexamethasone increases PD-1 expression and decreases IL-2 and IFN-gamma production in human primary T cells in a dose-dependent fashion (34). Likewise, celecoxib and aspirin, two COX-inhibitors widely used in COVID-19 patients, can increase PD-1 expression in CD4+ and CD8+ chimeric antigen receptor T cells *in vitro* (35). This information illustrates why trying to clarify the contribution of immune cells and mediators such as CD4+ T cells, CD8+ T cells, IL-2, IFN-gamma, and PD-1 to COVID-19 progression is extremely hard in polytreated patients already hospitalized. From a different perspective, our strategy involving unspecific polyclonal stimuli prior to infection allows us to expand on the body of evidence supporting that PD-1 expression increases as IL-2 and IFN-gamma production decreases in severe COVID-19. In other words, our results suggest that a group of individuals may have CD4+ and CD8+ T cells with a basal predisposition to express high PD-1 levels and low IL-2 and IFN-gamma amounts in response to either polyclonal stimuli or SARS-CoV-2. This notion might partially explain why participants that showed helper and cytotoxic T cells with increased PD-1 expression and decreased IL-2 and IFN-gamma production in response to polyclonal stimuli tended to develop severe COVID-19 once infected. Furthermore, these findings support the idea that failure in mounting an adequate T cell-mediated immune response at the beginning of the SARS-CoV-2 infection is associated with increased viral load, systemic inflammatory response occurrence, and death (36, 37). The molecular mechanisms behind this intriguing hypothesis remain to be elucidated, which will positively contribute to expanding our knowledge regarding the very heterogeneous immune responses of humans to pathogens, above all if they are emerging public threats as occurred with SARS-CoV-2.

Besides the response mediated by CD4+ and CD8+ T cells, monocyte subpopulations play a crucial role in the anti-viral immune response by providing the first cell-virus interaction that will lead to antigen presentation and cytokine release (38). Several research teams have shown that monocyte subsets display dynamic changes in COVID-19, including an increase in classical and non-classical monocyte subpopulations and impaired cell ability to express cytokines with anti-viral functions (16, 39, 40). In response to polyclonal stimuli, we did not observe any alteration in the monocyte subset balance; however, we found that classical and non-classical monocytes lost their ability to produce IFN-alpha in subjects that once infected developed severe COVID-19. A study conducted in COVID-19 patients reported that IFN-alpha serum levels considerably decreased as the severity of the disease increased (16). Vanessa Chilunda and coworkers characterized the transcriptional profile of CD16+ monocyte subsets from COVID-19 patients. They informed that intermediate and non-classical monocytes exhibited down-regulation of numerous interferon response-related genes in severe cases compared to subjects that experienced the mild disease (41). In line with these reports, our findings indicate that an apparent susceptibility of classical and non-classical monocytes to express

low IFN- α levels in response to polyclonal stimuli might be associated with a higher risk of developing severe COVID-19 after SARS-CoV-2 exposure.

Cellular immunity mediated by monocytes and T cells provides the first immediate response to pathogens *via* antigen presentation and cytokine release while stimulating B cells to initiate humoral immunity through antibody production. Often, a defective cellular immunity leads to decreased memory B cell expansion and impaired antibody production, as occurs with H1N1/09 influenza vaccine non-responders where failure in CD4+ T cell stimulation and IL-2 secretion concurs with a low percentage of IgG antibody-secreting cells (42). However, the apparent link between cellular and humoral immune responses is still not clear in COVID-19. A recent study reported that PBMCs from severe COVID-19 patients show less CD4+ and CD8+ T cell activation and IFN- γ production than PBMCs from mild cases in response to *in vitro* stimulation with M, N, and S viral proteins (43). Nevertheless, the neutralizing ability of anti-SARS-CoV-2-specific antibodies remained the same between severe and mild COVID-19 patients after a month of having been diagnosed by PCR test (43). Likewise, Irene Cassaniti and colleagues informed that CD4+ and CD8+ T cells from mild COVID-19 patients produce higher IFN- γ concentrations than those found in T cells from severe cases in response to viral peptides (44). However, the authors reported no correlation between the *in vitro* T cell response and anti-SARS-CoV-2 antibody titers (44). In line with this body of evidence, we observed a group of subjects with a robust cellular immunity mediated by T cells and monocytes in response to polyclonal stimuli. This immune response pattern concurred with the development of mild COVID-19 symptoms after SARS-CoV-2 exposure. Conversely, we found another group of individuals that responded to polyclonal stimuli by showing a defective cellular immune activation associated with the development of severe COVID-19 once infection took place. Of note, we detected no changes between mild and severe COVID-19 patients for serum anti-SARS-CoV-2 IgG antibody titers or their neutralizing ability after a month of the symptom onset. Altogether, these findings lead us to suppose that a basal impairment in cellular immunity activation may play a more critical role in preventing COVID-19 worsening than the humoral response mediated by antibodies. We are now working on characterizing the possible mechanisms involved in stimulating PD-1 expression and impairing IL-2 and IFN- γ production in CD4+ and CD8+ T cells and IFN- α secretion in classical and non-classical monocyte subsets, including differential methylation patterns and polymorphic variants.

Finally, we found a SARS-CoV-2 infection rate of around 11% in our study population, among who 50% developed a severe form of COVID-19. The Mexican government officially reported an accumulated number of SARS-CoV-2 positive cases of 725,346 for Mexico City from December 2020 to September 2021 (<https://datos.covid-19.conacyt.mx>). The official number of inhabitants in Mexico City was around 9,209,944 in 2021. These numbers suggest that about 8% of the general population living

in Mexico City got SARS-CoV-2 infection when we conducted the study. Moreover, a recent study indicated that nearly 39% of SARS-CoV-2 positive cases in Mexico were hospitalized due to the COVID-19 severity (45). These data reflect, to some extent, what we found in our study if we consider the limited number of SARS-CoV-2 detection tests available during that period and the remarkable underestimation of the most severe COVID-19 cases in Mexico.

In conclusion, using *in vitro* polyclonal stimuli, we found two basal immune response patterns associated with a predisposition to developing mild or severe COVID-19 once SARS-CoV-2 infection occurs. The pattern linked to severe COVID-19 is characterized by high PD-1 expression, low IL-2 and IFN- γ production in CD4+ and CD8+ T cells, and poor IFN- α expression in classical and non-classical monocytes. Conversely, low PD-1 synthesis and high IL-2 and IFN- γ expression in helper and cytotoxic T cells and an increased IFN- α production in classical and non-classical monocyte subsets are related to a basal predisposition to developing mild COVID-19 symptoms after SARS-CoV-2 exposure. Since the serum anti-SARS-CoV-2 IgG antibody titers or their neutralizing ability did not differ between mild and severe COVID-19 cases, these findings suggest that cellular immunity may play a more crucial function than humoral immunity in preventing COVID-19 progression.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional ethical committee of the General Hospital of Mexico (registration number of the ethical code approval: DI/20/501/03/17). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, GE; Methodology, RV-S, AM-R, HS-V, SR-T, LM-G, VV-S, JG-S, AG-C, JL-P, RF-M, and OR-C; Patient Enrolment, RV-S, SR-T, LM-G, VV-S, JG-S, AA-V, JC-R, AG-C, and JL-P; DATA CURATION, RV-S, AM-R, HS-V, SR-T, LM-G, VV-S, JG-S, AA-V, and JC-R; Formal Analysis, RV-S, AA-V, JC-R, AG-C, JL-P, RF-M, OR-C, and GE; Writing—Original Draft Preparation, RV-S and GE; Writing—Review And Editing, RV-S, AM-R, HS-V, SR-T, LM-G, VV-S, JS-G, AA-V, JC-R, AG-C, JL-P, RF-M, and OR-C; Funding Acquisition, GE. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.897995/full#supplementary-material>

Supplementary Figure 1 | Time-response curves. (A) Based on time-response curves, we selected the earliest *in vitro* culture time to detect IL-2 production in CD3 + T cells. IL-2 production peaked after 2 hours on *in vitro* culture and showed no significant differences at 6, 12, and 24 hours. (B) Based on time-response curves, we selected the earliest *in vitro* culture time to detect IFN-gamma production in CD3 + T cells. IFN-gamma production peaked after 2 hours on *in vitro* culture and showed no significant differences at 6, 12, and 24 hours. (C) Based on time-response curves, we selected the earliest *in vitro* culture time to detect IFN-alpha production in CD14+ monocytes. IFN-alpha production peaked after 2 hours on *in vitro* culture and showed no significant differences at 6, 12, and 24 hours. We expressed data as mean $\pm$ standard deviation. We compared the production of IL-2, IFN-gamma, and IFN-alpha at 0, 1, 2, 6, 12, and 24 hours using one-way ANOVA and considered differences significant if $P < 0.05$. IL-2, interleukin-2; IFN-gamma, interferon-gamma; IFN-alpha, interferon-alpha; MFI, mean fluorescence intensity.

Supplementary Figure 2 | Gating strategy for T cells. (A) Gating strategy for selecting primary human CD3+CD4+ lymphocyte subsets and measuring IL-2, IFN-gamma, and PD-1 precisely. (B) Gating strategy for selecting primary human CD3+CD8+ lymphocyte subsets and measuring IL-2, IFN-gamma, and PD-1 precisely. FSC-H, forward scatter height; FSC-A, forward scatter area; SSC-A, side scatter area; IL-2, interleukin-2; IFN-gamma, interferon-gamma; PD-1, programmed cell death protein 1.

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Supplementary Figure 3 | Gating strategy for monocyte subsets. Gating strategy for selecting primary human HLA-DR+ monocyte subsets and measuring IFN-alpha precisely. FSC-H, forward scatter height; FSC-A, forward scatter area; SSC-A, side scatter area; HLA-DR, human leukocyte antigen-DR isotype; IFN-alpha.

Supplementary Figure 4 | Representative spectra of mean fluorescence intensity for IL-2, IFN-gamma, PD-1, and IFN-alpha in T cells and monocyte subsets. (A) Representative histograms show the comparison of CD3+CD4+ T cells expressing IL-2 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up. (B) Representative histograms show the comparison of CD3+CD8+ T cells expressing IL-2 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 throughout the follow-up. (C) Representative histograms show the comparison of CD3+CD4+ T cells expressing IFN-gamma in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up. (D) Representative histograms show the comparison of CD3+CD8+ T cells expressing IFN-gamma in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 throughout the follow-up. (E) Representative histograms show the comparison of CD3+CD4+ T cells expressing PD-1 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up. (F) Representative histograms show the comparison of CD3+CD8+ T cells expressing PD-1 in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 throughout the follow-up. (G) Representative histograms show the comparison of CD14+CD16- classical monocytes expressing IFN-alpha in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 throughout the follow-up. (H) Representative histograms show the comparison of CD14+CD16+ intermediate monocytes expressing IFN-alpha in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 during the follow-up. (I) Representative histograms show the comparison of CD14+CD16+ non-classical monocytes expressing IFN-alpha in blood samples treated with polyclonal stimuli from participants who developed mild or severe COVID-19 throughout the follow-up. In all cases, control indicates the spectra of MFI from unstimulated cells. IL-2, interleukin-2; IFN-gamma, interferon-gamma; PD-1, programmed cell death protein 1; IFN-alpha, interferon-alpha; MFI, mean fluorescence intensity; COVID-19, coronavirus disease 2019.

Supplementary Figure 5 | Absolute cell numbers for percentages of T cells and monocyte subsets. We show absolute cell numbers for percentages of CD3+CD4+ T cells expressing IL-2, IFN-gamma, and PD-1 on top. We show absolute cell numbers for percentages of CD3+CD8+ T cells expressing IL-2, IFN-gamma, and PD-1 in the middle. We show absolute cell numbers for percentages of classical, intermediate, and non-classical monocytes expressing IFN-alpha on the bottom. We defined classical monocytes as CD14+CD16-, intermediate monocytes as CD14+CD16+, and non-classical monocytes as CD14+CD16+. We expressed data as mean $\pm$ standard deviation. We compared data using the unpaired Student's t-test and considered differences significant when $P < 0.05$. IL-2, interleukin-2; IFN-gamma, interferon-gamma; PD-1, programmed cell death protein 1; IFN-alpha, interferon-alpha; COVID-19, coronavirus disease 2019.

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Blood-brain Barrier Damage is Pivotal for SARS-CoV-2 Infection to the Central Nervous System

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Transsynaptic transport is the most accepted proposal to explain the SARS-CoV-2 infection of the CNS. Nevertheless, emerging evidence shows that neurons do not express the SARS-CoV-2 receptor ACE2, which highlights the importance of the blood-brain barrier (BBB) in preventing virus entry to the brain. In this study, we examine the presence of SARS-CoV-2 messenger ribonucleic acid (mRNA) and the cytokine profile in cerebrospinal fluids (CSF) from two patients with a brain tumor and COVID-19. To determine the BBB damage, we evaluate the Q-albumin index, which is an indirect parameter to assess the permeability of this structure. The Q-albumin index of the patient with an intraventricular brain tumor suggests that the BBB is undamaged, preventing the passage of SARS-CoV-2 and pro-inflammatory molecules. The development of brain tumors that disrupt the BBB (measured by the Q-albumin index), in this case, a petroclival meningioma (Case 1), allows the free passage of the SARS-CoV-2 virus and probably lets the free transit of pro-inflammatory molecules to the CNS, which leads to a possible activation of the microglia (astrogliosis) and an exacerbated immune response represented by IL-13, IFN- γ , and IL-2 trying to inhibit both the infection and the carcinogenic process.

Key words: Blood-brain barrier, SARS-CoV-2, COVID-19, Cerebrospinal fluid, Brain tumor

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) affects the pulmonary apparatus primarily [1]. Nevertheless, increasing evidence has confirmed SARS-CoV-2 infection to the central nervous system (CNS) in patients who also develop severe neurological symptoms with an incidence rate of 36.4% [2]. The most common neurological manifestations of SARS-CoV-2 infection are headache, anosmia, and dizziness; however, some patients can also display febrile seizures, loss of consciousness, convulsions, status epilepticus, and necrotizing encephalopathy that in turn may trigger the onset of multiple sclerosis [3].

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Several studies suggest that neurological symptoms in coronavirus disease 2019 (COVID-19) patients result from systemic inflammatory mediators that do not necessarily involve direct infection of the CNS. However, emerging evidence confirms that SARS-CoV-2 may also enter the brain through different routes [4]. The most accepted way for SARS-CoV-2 entry into brain tissue is via nasal mucosa [5], wherein SARS-CoV-2 binds to motor proteins along olfactory nerves by retrograde axonal transport and is transported to the brain [6,7]. A second possible way is a hematogenous route, wherein after entering the respiratory tract, SARS-CoV-2 may spread to multiple tissues and organs such as the CNS. However, this hypothesis lacks support due to the blood-brain barrier (BBB), which is a semi-permeable barrier that protects the CNS from a wide variety of toxins, molecules, and microorganisms found in blood, making it difficult for the free passaging of pathogens such as virus to the brain [8].

To this respect, several studies have proposed three mechanisms through which the SARS-CoV-2 may enter the CNS via the BBB: transcellular migration, wherein the virus infects endothelial cells of the BBB via the Angiotensin-Converting-Enzyme 2 (ACE2), the primary cell receptor described for SARS-CoV-2; paracellular migration, characterized by viral invasion of tight junctions between endothelial cells of the BBB; and the so-called "Trojan horse strategy", wherein the virus infects macrophages, dendritic cells, and neutrophils that in turn can migrate across the BBB to the CNS [5]. However, all these hypotheses lack support due to the BBB integrity, and we propose that structural damage to the BBB elicits the SARS-CoV-2 infection to the CNS. Herein, we present the case reports of two patients with a brain tumor and COVID-19. Interestingly, we detected viral particles in Cerebrospinal Fluid (CSF) only from the patient with petroclival meningioma and an elevated Q-albumin index (an indirect parameter used to determine the BBB permeability), suggesting a possible BBB damage which highlights the importance of this structure in preventing SARS-CoV-2 infection to the CNS. Moreover, the direct infection of CNS showed an exacerbated cytokine response represented by interleukin (IL)-13, IFN- γ , e IL-2, compared to the CSF sample from the patient with an intraventricular tumor where we did not find viral particles.

CASES PRESENTATION

Case 1

Case 1 was a 43-year-old female patient with no previous chronic or metabolic disorders diagnosis. One month before hospital admission, she presented progressive onset of severe headache, nausea, and drowsiness. She was assessed by neurosurgery service, where a cranial magnetic resonance imaging was performed

and allowed to conclude right petroclival meningioma. She was programmed one week later for a surgical procedure. At hospital admission, she had the following vital signs: blood pressure (BP) (120/80 mmHg), Heart Rate (HR) (78 bpm), Breath Frequency (BF) (17 bpm), body temperature (36.3°C), and Glasgow Coma Scale (GCS) of 13 points eye-opening: 4 points, verbal response: 4 points, and motor response: 5 points. Laboratory parameters at admission suggested total blood cell count abnormalities, including high leucocyte and neutrophil count and low monocyte count. After discarding any probable infection, ceftriaxone was given as antibiotic prophylaxis. We calculated the Q-albumin index by dividing the cerebrospinal fluid albumin by serum albumin and used it as a parameter of BBB damage ($0.0341 \text{ g/dl}/4.62 \text{ g/dl}=7.3 \times 10^{-3}$). Retrosigmoid craniotomy with partial resection of petroclival meningioma (Fig. 1A) and high-pressure valve ventriculoperitoneal shunt system colocation was performed without any complication. After surgery, the patient was transferred to the intensive care unit (ICU) with advanced airway management. A day after, a cranial computer tomography (CT) was performed, revealing the persistence of the tumor accompanied by cerebral edema. The CT scan was extended to chest imaging with consistent data of SARS-CoV-2-related pneumonia (Fig. 1B). The next day, she presented fever (38.6°C) and purulent respiratory secretions; bacterial cultures and SARS-CoV-2 specific detection through bronchoalveolar lavage were performed. Ceftriaxone was suspended, and an empirical antibiotic scheme was started with meropenem and vancomycin; dexamethasone was also added to the treatment. Quantitative polymerase chain reaction (qPCR) confirmed SARS-CoV-2 infection the same day, and *Acinetobacter baumannii* was isolated from cultures one week later, so the antibiotic scheme was changed to colistimethate. She remained with leukocytosis, fever, and neurological impairment during the next days. After 28 days of hospitalization, she died because of cardiopulmonary arrest.

Case 2

Case 2 was a 43-year-old male patient with a previous diagnosis of hypertension one year ago and treatment with ACE inhibitors. Three months before hospital admission, he began with progressive, intense headaches in the frontal region without medical treatment. After one month, he developed vomiting and syncope with progressive gait abnormalities and decreased alertness. Then, he was assessed in a primary care center, and cranial magnetic resonance imaging revealed a round and well-defined tumor in the fourth ventricle, causing a cerebrospinal fluid blockage (Fig. 1C). The patient started treatment with prednisone and dexamethasone and was referred to our hospital for neurosurgery assessment. He was admitted with the following vital signs: BP (130/70 mmHg),

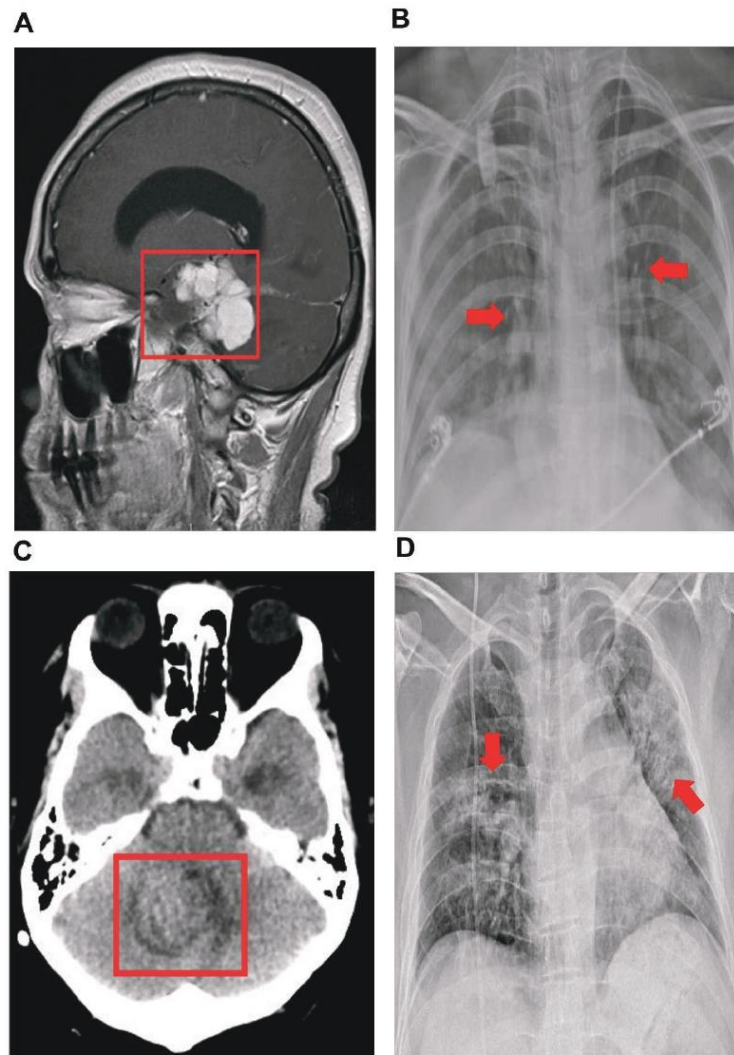


Fig. 1. Cranial and chest CT of patients with a brain tumor and COVID-19. (A) Case 1 magnetic resonance T1 sequence contrasted sagittal section petroclival lesion extending to a protuberance, enhancing the contrast medium compatible with meningioma. (B) Case 1 chest computed tomography (CT) with data suggestive of SARS-CoV-2 pneumonia. (C) Case 2 simple CT scan of the skull in coronal section showing lesion to the cerebellar vermis and right cerebellar hemisphere causing hydrocephalus. (D) Chest CT at admission showing severe pneumonia of Case 2.

HR (75 bpm), BF (18 bpm), body temperature (36.0°C), and GCS of 13 points (eye-opening: 3 points, verbal response: 4 points, and motor response: 6 points). Anosmia and peripheral oxygen satu-

ration of 89% were also documented, and SARS-CoV-2 specific qPCR detection was performed, resulting positive. The relatives reported neurological impairment three days before admission,

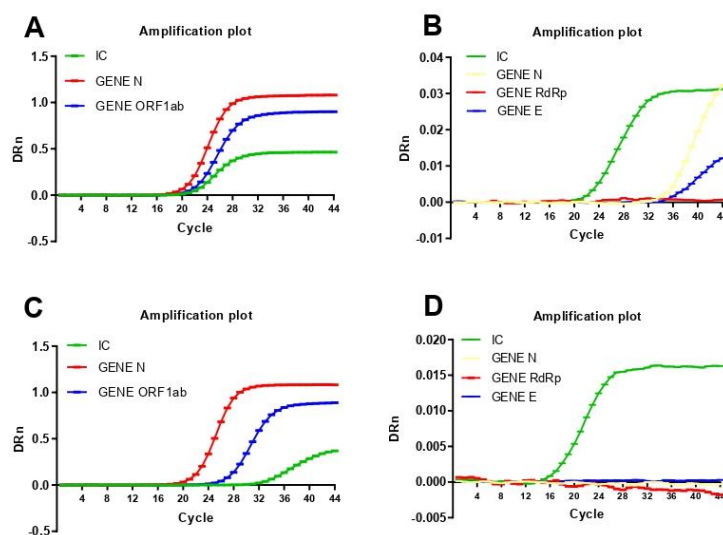


Fig. 2. Analysis of qPCR detection of SARS-CoV-2 viral particles. (A) Determination of viral infection in Case 1 by naso/oropharyngeal swab where N (CT=18.01) and ORF1ab (CT=19.6) SARS-CoV-2 genes were detected as well as the IC gene (CT=20.49). (B) Confirmation of CSF SARS-CoV-2 infection by the amplification of Gene N (CT=37.85) and the IC gene (CT=22.62). (C) Case 2 determination of viral infection by naso/oropharyngeal swab where N (CT=23.21) and ORF1ab (CT=24.86) SARS-CoV-2 genes were detected as well as the IC gene (CT=30.87). (D) Viral infection was not determined in CSF of Case 2; the IC gene amplification was CT=18.05.

without associated respiratory symptoms. Laboratory parameters revealed high leucocyte and neutrophil counts, whereas lymphocyte and monocyte counts decreased. For case 2, we found a Q-albumin index of 0.0016 g/dl/3.01 g/dl= 5.3×10^{-4} . Fig. 1D shows a chest CT at admission revealing severe pneumonia. He was hospitalized in the pneumology department for COVID-19 management with supplemental oxygen therapy at 2 liters per minute using a nasal cannula, and oxygen saturation improved up to 95%. A high-pressure valve ventriculoperitoneal shunt system was placed the next day without any procedural complication. Ten days later, the patient was discharged with neurological improvement, GCS of 14 points (eye-opening: 4 points, verbal response: 4 points, and motor response: 6 points), and no supplemental oxygen needed.

The Institutional Ethical Committee of the General Hospital of Mexico guaranteed that the study was conducted in rigorous adherence to the principles described in the Declaration of Helsinki. Their relatives signed written informed consent. Both patients arrive at the emergency department because of neurological symptoms rather than respiratory complications associated with SARS-CoV-2 infection. The Carestream Viu Motion software v.12.1.5.7 from the digital electronic file of the General Hospital of Mexico

was used to obtain cranial and chest CT scans.

The ORF1ab and N genes of SARS-CoV-2 were detected by the kit for 2019 Novel Coronavirus (DADA0930-DA0932, DAAN Gene Co., Guangzhou, China) in naso/oropharyngeal swabs following the manufacture instructions. SARS-CoV-2 infection was confirmed in both patients by amplification of the N gen in Case 1 (CT=18.01) and Case 2 (CT=23.21), and the Orf1ab gene in Case 1 (CT=19.6) and Case 2 (CT=24.86), respectively. The internal control gene (IC) amplified at CT=20.49 in Case 1 and CT=30.87 in Case 2 (Fig. 2A, 2C respectively).

Both patients gave CSF samples at the beginning of the surgery by ventricular shunt. The serum of the patients was taken at admission for blood biochemistry analysis. The analysis of SARS-CoV-2 detection in CSF was performed following the instructions of the Gene Finder KitTM COVID-19 Plus RealAmp Kit (Cat. Number IFMR-45, Gyeonggi-do, Korea), which can detect the presence of the E, N, and RdRp genes of SARS-CoV-2 and considering amplification of at least one of those genes as a positive result. Amplification of the N gene was detected only in Case 1 (N Gen CT=37.85 and endogenous gene CT=22.62) (Fig. 2B, 2D). CSF and serum were evaluated using the Beckman Coulter DxC 700 AU

Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA) in adherence to the standard operating procedures to determine the values (g/dl) of albumin in both samples. Q-albumin index results from dividing the value of albumin in CSF by the albumin value in serum.

The CSF sample was centrifuged to 13,500 g for 60 minutes at 4°C. The RNA from mononuclear cells was isolated with TRIZOL reagent (Ambion by life technologies, Carlsbad USA Cat number 15596018) following the manufacturer's instructions. Total RNA samples were quantified and synthesized to complementary DNA (cDNA) using the kit M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, USA Cat number: 28025-013). cDNA samples were used for amplification by qPCR (CFX96™ Real-Time System, BIO-RAD) with SYBR Green Master Mix (Jena Bioscience, Jena Germany Cat number PCR-372L) according to the manufacturer's instructions. Specific primers for the cytokines were designed using the Primer-BLAST software from the National Center for Biotechnology Information, U.S. National Library of Medicine, following the thermocycling scheme: denaturation at 95°C for 30 s, annealing at 61°C for 30 s, elongation at 72°C for 45s, 40 thermal cycles. The expression of the cytokines was normalized using the housekeeping gene control 18 s. The expression was reported as $2^{\Delta\Delta CT}$. We used a control CSF sample taken by a ventricular shunt from a patient with non-communicating hydrocephalus and Wallenberg Syndrome. The control case had a negative nasopharyngeal exudate test for SARS-CoV-2 at the time of sample collection. We found an exacerbated cytokine response in Case 1 with petroclival meningioma where we found viral particles. IL-13, IFN- γ , and IL-2 represented overexpressed cytokines. The expression of IL-13 increased by 2-fold in Case 2; meanwhile, in Case 1, this expression raises 19-fold compared to the Control Case. However, the expression of IFN- γ was 14-fold increased in Case 1 and decreased 0.6-fold in Case 2. mRNA levels of IL-2 increased in both patients 13 and 4.7-fold in Case 1 and Case 2, respectively. Similarly, the expression of TGF- β increased to 8 in Case 1 and 2.82 in Case 2. Finally, the IL-4 expression was 7-fold higher in Case 1 and slightly less in Case 2 (0.93-fold) compared to the Control Case. Cytokines such as IL-12, IL-1 β , TNF- α , and IL-6 increased considerably (5-fold approximately) in Case 1, and the expression of these cytokines (exceptionally TNF- α) decreased in Case 2 to 0.3-fold compared to the Control Case (Fig. 3).

DISCUSSION

Several respiratory viruses such as HCoV-OC-43 and H1N1 can infect the CNS via transsynaptic transport. For this reason, a few studies suggested that SARS-CoV-2 could similarly reach the

CNS. However, recent studies reported that ACE2 expresses in endothelial vascular cells, pericytes, and macrophages but not neurons [7], which reduces the chances of the transsynaptic transport as the main route of SARS-CoV-2 entry to the CNS. Moreover, the inability to detect viral particles in CSF from COVID-19 patients despite high titers of anti-SARS-CoV-2 antibodies [9, 10] remarks the possibility that BBB integrity plays a crucial role in SARS-CoV-2 neuroinfection. The BBB is an essential physiologic structure that selectively prevents the passage of pathogens such as viruses to the CNS. Consequently, it is feasible that SARS-CoV-2 can invade the CNS only after structural damage to the BBB.

Brain tumors can disrupt the BBB by releasing proinflammatory mediators, vasoactive cytokines, and growth factors with the ability to affect tight junctions and increase permeability, leading to edema and hemorrhage. Numerous studies have shown down-regulation of tight junction protein expression such as claudin-1 and claudin-3 in specific brain tumors, including astrocytomas and glioblastoma multiforme [11]. Measurement of proteins typically found in serum but not in CSF unless BBB presents damage is an indirect method to estimate BBB integrity. When calculating the Q-albumin index, we obtained 7.3×10^{-3} g/dl for Case 1 and 5.3×10^{-4} g/dl for Case 2. Although reports indicate that a Q-albumin index $> 8 \times 10^{-3}$ g/dl indicates BBB dysfunction [12], no patients exceeded this value. It is worth mentioning that the presence of a brain tumor triggers an inflammatory process where activation of microglia could decrease albumin levels due to albumin uptake from astrocytes [13]. However, the order of magnitude of the Q-al-

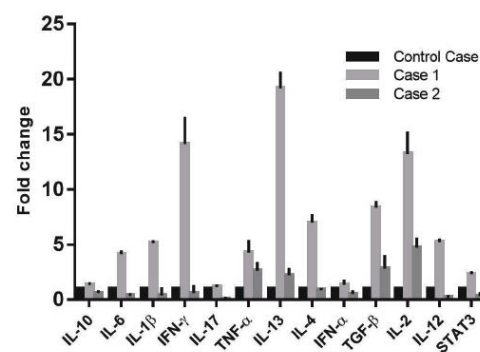


Fig. 3. Cytokine expression in CSF of patients with a brain tumor and COVID-19. IL-13, IFN- γ , and IL-2 represented the most overexpressed cytokines. These cytokines were more expressed in Case 1 (grey bars) compared to Case 2 (dark grey bars) and Control Case (black bars). The expression of cytokines such as TGF- β , IL-4, IL-6, IL-1 β , TNF- α , and IL-12 was overexpressed around 5-fold in Case 1; meanwhile, in Case-2, these cytokines were under-expressed by 0.6-fold, except for TNF- α .

bumin index between patient 1 is greater than patient 2, where we assume BBB integrity. Although Q-albumin is considered a valid parameter to evaluate the BBB permeability, it is indirect evidence which we consider a limitation of this study. Hence, the results presented in this manuscript should be taken with a reserve, and more studies should be conducted to determine the role of BBB in CNS infection by SARS-CoV-2.

In this study, we found viral particles in CSF only in the patient with petroclival meningioma, which causes BBB disruption as suggested by the Q-album index. Meanwhile, we did not find viral particles in the CSF of the patient with an intraventricular tumor, which is in line with our data in two patients with cerebrovascular disease [14]. Several authors, such as Bellon and cols., demonstrated that neurological symptoms in COVID-19 patients were not related to SARS-CoV-2 in CNS [10]. Conversely, they found intrathecal IG synthesis and signals of BBB disruption. These results suggest that virus invasion of the CNS may occur in patients with BBB damage. Moreover, the hypothesis of the neurological manifestations associated with COVID-19 are not necessarily supported by direct invasion of the CNS but through systemic inflammation that precedes the infection [15].

As mentioned earlier, the presence of a brain tumor activates the astrocytes surrounding the tumor-promoting the secretion of growth factors and cytokines trying to repair the damage. Astrocytes can also sense stimuli related to viral infection by expressing a cluster of proinflammatory genes in maladaptive reactive astrogliosis [16]. In this study, it is impossible to determine the observed inflammation source. This response could be due to the tumoral development, the infection by SARS-CoV-2, or by the BBB disruption allowing the free transit of proinflammatory molecules to the CNS. However, the patient with petroclival meningioma, positive for viral particles in CSF who probably had BBB damage, presents a higher expression of proinflammatory cytokines, which can be attributed to local viral infection. It is likely that the CNS infection by SARS-CoV-2 causes a more pronounced local (gliosis) immune response. Cytokine overexpression in these patients was represented by IL-13, IFN- γ , and IL-2, which have been used as immunotherapy to treat brain tumors, mainly in gliomas trying to inhibit tumor proliferation and angiogenesis. High levels of IL-13 were expected since the overexpression of the IL-13 receptor is an essential feature in brain tumors [17].

Moreover, recently IL-13 was recognized as the “driver” of COVID-19 severity, which neutralization in mice reduced both severity and death without affecting viral load [18]. Besides, IFN- γ is a central antiviral immune mediator that is abnormally downregulated in brain tumor cells. So, the overexpression of this cytokine could be exclusively due to viral infection. Initially, it was detected in

respiratory swabs from COVID-19 patients with type I IFN genes (IFN alpha and omega) but not type II IFN genes (IFN- γ) [19]. However, recently was demonstrated that high levels of IFN- γ have a pivotal role in SARS-CoV-2 infection, inducing the expression of ACE2 in epithelial colonic organoid cells and enabling a higher virus replication; therefore, it is proposed that the pharmacological interference of IFN- γ may be a treatment for severe COVID-19 [20]. As well as IFN- γ , IL-2 is downregulated in brain tumors [21], but it is overexpressed in COVID-19 convalescent individuals [22]. Therefore, the overexpression of these cytokines seems to be a set of immune responses due both to tumor development and direct infection of CNS by SARS-CoV-2, specifically in Case 1. Since the CD4+ T lymphocytic origin of IL-2 and IFN- γ (helper and cytotoxic T cells) as a response to viral infection in the pulmonary epithelium, these cytokines or their producing cells may migrate to the CNS, allowed by the BBB damage, however more studies are needed to determine the source of these cytokines in the CNS in patients with a brain tumor, COVID-19, and their relationship with the integrity of BBB.

It is feasible that BBB damage is necessary for virus entry to the CNS, either through binding to ACE2 in endothelial cells or pericytes of the BBB. Due to ACE2 is not expressed in neurons, the integrity of the BBB would play a central role in allowing the passage of SARS-CoV-2 or infected immune cells to the CNS. Further studies are needed to examine ACE2 expression in other brain tissue cells that can host the virus. Accordingly, COVID-19 patients with documented structural disruption or suspected permeability of the BBB due to other diseases such as brain tumors should undergo a specific medical follow-up to monitor possible neurological effects associated with the presence of SARS-CoV-2 in the CNS. In conclusion, this study shows the presence of SARS-CoV-2 particles in the CSF only after damage to the BBB measured indirectly by the Q-albumin index, favoring the inflammatory response regulated by IL-13, IFN- γ , and IL-2, which highlights the possible key role of BBB integrity in SARS-CoV-2 infection.

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Liver biomarkers for prognosis in COVID-19

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Abstract

The novel coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Mortality attributable to COVID-19 remains considerably high, with case fatality rates as high as 8-11%. Early medical intervention in patients who are seriously and critically ill with COVID-19 reduces fatal outcomes. Thus, there is an urgent need to identify biomarkers that could help clinicians determine which patients with SARS-CoV-2 infection are at a higher risk of developing the most adverse outcomes, which include intensive care unit (ICU) admission, invasive ventilation, and death. In COVID-19 patients experiencing the most severe form of the disease, tests of liver function are frequently abnormal and liver enzymes are found to be elevated. For this reason, we examine the most promising liver biomarkers for COVID-19 prognosis in an effort to help clinicians predict the risk of ARDS, ICU admission, and death at hospital admission. In patients meeting hospitalization criteria for COVID-19, serum albumin < 36 g/L is an independent risk factor for ICU admission, with an AUC of 0.989, whereas lactate dehydrogenase (LDH) values > 365 U/L accurately predict death with an AUC of 0.943. The clinical scores COVID-GRAM and SOFA that include measures of liver function such as albumin, LDH, and total bilirubin are also good predictors of pneumonia development, ICU admission, and death, with AUC values ranging from 0.88 to 0.978. Thus, serum albumin and LDH, together with clinical risk scores such as COVID-GRAM and SOFA, are the most accurate biomarkers in the prognosis of COVID-19.

Keywords: Albumin. Lactate dehydrogenase. Bilirubin. Prognosis. COVID-19. SARS-CoV-2.

Introduction

Since the coronavirus disease 2019 (COVID-19) outbreak began in December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has quickly spread worldwide¹. COVID-19 presentation is heterogeneous and ranges from asymptomatic disease to severe illness characterized by pneumonia, acute respiratory distress syndrome (ARDS), and sepsis that may eventually lead to death². Despite the concerted efforts of health systems around the globe, mortality attributable to COVID-19 remains considerably high with case fatality rates as high as 9-11%³. Early medical

intervention in seriously and critically ill COVID-19 patients may reduce fatal outcomes⁴. Therefore, there is an urgent need for novel biomarkers that can help clinicians identify patients with poorer prognosis. Early implementation of more aggressive drug regimens in these patients could not only increase survival rates but also optimize intensive care unit (ICU) resources.

Due to the extensive extrapulmonary manifestations of COVID-19, evaluation of patients typically includes comprehensive blood panels that allow the clinician to simultaneously estimate the effects of the disease on multiple organ systems⁵. Liver function tests (LFT) are commonly included in laboratory analyses and

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measure a variety of molecules such as transaminases, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, and albumin⁶ that indicate the global function and integrity of the liver.

Large-scale descriptive studies have been conducted to examine the relationship between altered liver function and SARS-CoV-2 infection, but only a few of them describe factors that might be used to predict the most adverse outcomes of COVID-19, including ARDS development, ICU admission, and/or death⁷⁻⁹. Herein, we summarize the most promising liver biomarkers for COVID-19 prognosis in an effort to help clinicians rapidly, easily, and inexpensively predict the risk of ARDS, ICU admission, and death at hospital admission.

Serum albumin

Serum albumin (SA) is the most abundant protein in human blood (35 to 50 g/L)¹⁰. SA is synthesized in hepatocytes as prealbumin and then released to the rough endoplasmic reticulum, where an N-terminal peptide is removed to facilitate the conversion of the protein to proalbumin. Proalbumin is in turn processed in the trans-Golgi apparatus to form mature albumin that is subsequently released into circulation¹¹. SA plays multiple physiological roles, including regulation of plasma colloid oncotic pressure, transportation of endogenous and exogenous molecules, anticoagulation, and antioxidation responses¹⁰. In clinical practice, SA level is commonly used as an indicator of mortality risk in severely ill patients such as those experiencing sepsis, trauma, or cancer¹².

Numerous studies support the use of SA as a biomarker of poor prognosis in COVID-19 patients, especially those at much higher risk of ICU admission or death. Three meta-analyses that evaluated several laboratory parameters reported decreased SA at admission in patients with severe COVID-19 (3.50 g/dL, 95% CI 3.26–3.74 g/dL) as compared to non-severe patients (4.05 g/dL, 95% CI 3.82–4.27 g/dL)¹³⁻¹⁶. Hypoalbuminemia (SA < 30 g/L) also correlates with the severity of COVID-19 ($p < 0.0001$) in patients from Italy¹⁷. Two additional meta-analyses showed that SA levels correlate with disease severity but fail to find associations among other liver parameters^{9,16}.

When considering these liver markers in the context of critical care, it is noteworthy that ICU patients with COVID-19 generally have lower SA values at admission than patients admitted to regular hospital floors (31.3 ± 5.2 g/L versus 36.5 ± 5.7 g/L; $p = 0.001$). In fact, SA predicts ICU admission with a HR score of 1.87, 95%

CI 1.05–3.32, $p = 0.034$ ¹⁸. A study conducted in 63 COVID-19 patients from Turkey revealed that SA at admission predicts ICU admission with a cutoff point of ≤ 36 g/L, AUC of 0.989 (95% CI 0.924–1.00; $p < 0.001$), sensitivity of 96.7%, and specificity of 93.9%¹⁹. On the other hand, a study of 128 COVID-19 patients from Dubai showed that SA failed to predict ICU admission (AUC 0.256, 95% CI 0.146–0.366)²⁰. Thus, the use of SA as a predictor of ICU admission in seriously ill patients with COVID-19 should be performed with caution, and clinicians must take into account the genetic background and variability among the patient populations with which they work.

Regarding mortality, a meta-analysis evaluating eleven studies predominantly conducted in China indicated that SA values tend to be lower in non-survivors than survivors (-3.7 g/L, 95% CI $-5.3 - -2.1$; $p < 0.00001$)²¹. A separate study conducted on 207 patients from Italy showed that hypoalbuminemia correlates with death in COVID-19 patients ($p = 0.003$)¹⁷. Another study in 319 patients from Italy concluded that SA < 32 g/L is a risk factor for mortality, with HR of 2.48 (95% CI 1.44–4.26; $p = 0.001$)¹⁸. Concurrently, two additional studies conducted in COVID-19 patients from China revealed that decreased SA at admission was an independent risk factor for mortality (OR = 1.929, 95% CI 1.199–3.104, $p = 0.007$, and OR, 6.394; 95% CI 1.315–31.092, $p = 0.021$, respectively)²². This evidence supports the idea that SA is an independent risk factor for ICU admission and death in patients that meet hospitalization criteria for COVID-19. As we have shown, critical consideration of SA levels may help clinicians identify patients with SARS-CoV-2 infection who are at much higher risk of developing adverse outcomes than other patients. Having a reliable and easy-to-detect biomarker at their disposal would enable clinicians to provide these high-risk patients with more aggressive medical treatments that will increase their probability of survival.

Bilirubin

Bilirubin derives from the breakdown of the heme group in hemoglobin, which occurs in the spleen, bone marrow, and liver²³. In these tissues, heme oxygenase, an enzyme found in macrophages, can catalyze iron release to form carbon monoxide and biliverdin. Biliverdin is in turn reduced to form unconjugated bilirubin, also referred to as indirect bilirubin, which is poorly water soluble²⁴. The 200–300 mg of bilirubin produced by the human body per day is typically excreted.

Excretion of bilirubin occurs when indirect bilirubin (IBIL) is released into the plasma, where it binds albumin that is in turn transported to the liver²⁵. Then, bilirubin is conjugated to uridine diphosphate sugars (UDP) via UDP-glucuronyltransferase. This increases IBIL solubility and allows its excretion through the bile. Conjugated bilirubin, also referred to as direct bilirubin (DBIL), is broken down into urobilinogens by intestinal bacteria. At this point, a small quantity of DBIL can be deconjugated and re-absorbed by intestinal epithelial cells²³. Any increase in the rate of release of the heme group or failure to completely excrete bilirubin leads to a concomitant elevation of total bilirubin, direct bilirubin, or indirect bilirubin depending on the step of bilirubin metabolism that is impaired²⁵.

Common clinical laboratory tests include measures of total bilirubin (TBIL), IBIL, and/or DBIL, which can help establish the cause of hyperbilirubinemia²⁴. Elevation of bilirubin in patients with SARS-CoV-2 infection is not common. However, among COVID-19 patients who do show abnormal liver function test results during hospitalization, TBIL levels have been found to be elevated as high as 18%^{8,26,27}. A large study conducted in 5771 patients from China suggested that TBIL values are independent of the severity of COVID-19²⁸. Indeed, the average values of TBIL, DBIL, and IBIL fell within normal ranges of values in all patients enrolled in this study (10.4 $\mu\text{mol/L}$, IQR 7.9-14.1, 3.0 $\mu\text{mol/L}$, IQR 2.1-4.4, and 7.4 $\mu\text{mol/L}$, IQR 5.2-10.3, respectively)²⁸.

Conversely, emerging evidence now supports the use of bilirubin values at hospital admission to estimate a patient's risk of developing severe COVID-19. Seriously ill patients have higher TBIL and DBIL levels at admission than non-severe patients (for TBIL, 10.6 $\mu\text{mol/L}$, IQR 7.9-15.0 *versus* 10.3 $\mu\text{mol/L}$, IQR 7.9-14.0, $p = 0.053$, respectively; for DBIL, 3.3 $\mu\text{mol/L}$, IQR 2.2-5.2 *versus* 2.9 $\mu\text{mol/L}$, IQR 2.0-4.20, $p < 0.001$, respectively)²⁸. In addition, the peak of TBIL correlates with mortality risk, where TBIL maximum values of 21-63 $\mu\text{mol/L}$ confer a 3-fold increased mortality risk (HR 3.28 95% CI 2.47-4.35; $p < 0.001$), while patients with TBIL greater than 63 $\mu\text{mol/L}$ show 8-fold increased mortality risk (HR 7.98 95% CI 3.88-16.41; $p < 0.001$)²⁸. Higher TBIL levels also confer ~3-fold increased risk of developing severe COVID-19 (OR 2.94, 95% CI 2.18-3.97)²⁹. Likewise, TBIL values are elevated in ICU patients relative to non-ICU patients (15.8 $\pm$ 6.8 $\mu\text{mol/L}$ *versus* 9.1 $\pm$ 3.6 $\mu\text{mol/L}$, $p = 0.0082$, respectively)³⁰. In a population of pediatric COVID-19 patients, Wang and collaborators also reported higher TBIL values in seriously ill patients than in non-severe individuals

(10.13 $\mu\text{mol/L}$ *versus* 6.40 $\mu\text{mol/L}$, $p = 0.05$, respectively) with OR of 1.316, 95% CI 0.575-3.014, $p = 0.516$ ⁴. These studies suggest that bilirubin can be used to estimate the risk of mortality or ICU admission in adult and pediatric patients with COVID-19, especially TBIL and DBIL³¹.

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is a ubiquitous enzyme in human cells. LDH consists of four polypeptide chains that constitute five different isoforms located in the cytosol and mitochondria³². In the cytosol, LDH catalyzes the conversion of lactate to pyruvate by transferring a hydride group from NAD⁺ to NADH³³. In the mitochondria, LDH catalyzes the conversion of lactate to pyruvate by transferring a hydride group from ferri-cytochrome c to ferrocytochrome c. Release of LDH into cells occurs primarily as a result of necrosis^{32,33}. Serum LDH is elevated in a variety of clinical scenarios including hemolysis, infection, sepsis, infarction, liver and kidney diseases, pancreatitis, bone fracture, rhabdomyolysis or myositis, hypoxia, shock, and cancer³⁴.

LDH elevation is one of the most commonly reported laboratory anomalies in COVID-19 patients around the world. In fact, 50-80 percent of patients with SARS-CoV-2 infection show abnormally high LDH levels¹³. Numerous studies report a strong positive correlation between LDH levels and COVID-19 severity, with higher levels present in severe or critical patients. A study conducted in 548 patients reported 250 U/L and > 445 U/L as two cutoff values for LDH that can help distinguish non-severe patients from critically ill patients who are at a much higher risk of death. Patients with LDH > 445 U/L at admission were at a 4.4-fold increased risk of developing severe COVID-19 (OR, 4.4, 95% CI 2.6-7.6) and a 2-fold increased risk of death (HR 2.0, 95% CI, 1.2-3.3; $p = 0.007$)³⁵. In a meta-analysis of 19 papers that gathered clinical information from more than 3000 patients, LDH elevation at admission carried an 8-fold increased risk of developing severe COVID-19 (OR 8.28, 95% CI 4.75 - 14.46). LDH values were higher in patients who required ICU admission, with an OR of 5.78 (95% CI 1.65 - 20.28; $p < 0.001$)³⁶. Numerous studies and meta-analyses report significantly increased LDH levels in non-survivors with respect to survivors ($p < 0.001$)³⁶. This increase in LDH also associates with mortality risk, with OR values ranging from 4.09 to 10.88 depending on the study ($p < 0.001$)³⁷. In line with this, a study conducted in 375 COVID-19 patients from China reported that LDH

Table 1. Liver biomarkers as predictors of pneumonia development, intensive care unit admission, and death in patients with COVID-19

Liver biomarker	Cutoff point	Clinical outcome	AUC (95% CI)	Sensitivity (%), Specificity (%)	Country (Reference)
HSA	< 36 g/L	ICU admission	0.989 (0.924-1.00)	96.7, 93.9	Turkey (Uyar et al.)
	NA	ICU admission	0.256 (0.146-0.366)	NA	United Arab Emirates (Hachim et al.)
LDH	> 365 U/L	Death	0.943 (NA)	NA	China (Yan et al.)
	> 303 U/L	Death	0.829 (0.745-0.895)	NA	China (Feng et al.)
	> 353.5 U/L	Death	0.949 (NA)	94.4, 89.2	China (Dong et al.)
AST/ALT ratio	> 1.38	Death	0.71 (0.67-0.74)	NA	China (Qin et al.)
	> 1.49	Death	0.701 (0.603-0.787)	74, 70	Italy (Zinellu et al.)
	> 1.65	Death	0.713 (0.618-0.807)	57.5, 82.3	Turkey (Medetalibeyoglu et al.)
	> 1.26	ICU	0.636 (0.564-0.709)	64.9, 60.4	
	> 1.55	Pneumonia	0.577 (0.529-0.625)	61.0, 55.6	
Fibrinogen/Albumin Ratio	≤ 0.0883	Pneumonia	0.730 (NA)	NA	China (Bi et al.)
COVID-GRAM		Pneumonia	0.88 (0.85-0.91)	NA	China (Liang et al.)
LDH CRP Lymphocyte	> 365 U/L < 41.2 mg/L > 14.7%	Death	0.978 (NA)	NA	China (Yan et al.)
SOFA score	≥ 3	Death	0.890 (0.826-0.955)	90.00, 83.18	China (Liu et al.)

Summary of the most accurate liver biomarkers for prognosis in COVID-19. Liver biomarkers are accompanied by specific cutoff value, main clinical outcome, AUC, sensitivity, specificity, and country of origin.
 NA: non available; AUC: area under the curve; CI: confidence interval; ICU: intensive care unit admission; AST: aspartate aminotransferase; ALT: alanine aminotransferase; SA: serum albumin; LDH: lactate dehydrogenase; CRP: C reactive protein; SOFA: sequential organ failure assessment.

> 365 U/L predicts mortality with an AUC of 0.943. Similarly, Feng and coworkers found that LDH > 303 U/L predicts death with an AUC of 0.829. Finally, another study conducted in 119 severe-to-critical COVID-19 patients estimated that LDH > 353.5 U/L predicts mortality with an AUC of 0.949, sensitivity of 94.4%, and specificity of 89.2%³⁸. As we have outlined here, LDH appears to be a reliable independent risk factor for mortality in patients who meet hospitalization criteria concurrent with severe-to-critical COVID-19.

Ratios and clinical scores based on measures of liver function

Liang and coworkers proposed the use of the COVID-GRAM score to predict the development of critical COVID-19. The assessment takes into account such factors as lactate dehydrogenase, direct bilirubin, chest radiography, age, hemoptysis, dyspnea,

unconsciousness, cancer history, neutrophil-to-lymphocyte ratio, and number of comorbidities³⁹. This score has an AUC of 0.88 (95% CI 0.85-0.91) for predicting the proportion of patients at higher risk of needing critical care for COVID-19³⁹.

Notably, Yan and collaborators proposed a score based on LDH > 365 U/L, C reactive protein < 41.2 mg/L, and lymphocyte count > 14.7% that was shown to reliably predict mortality in COVID-19 patients, with an AUC of 0.978. In fact, use of this score permitted 100% accurate prediction of a patient's outcome ten days prior its occurrence³⁸.

Another score based on LDH, procalcitonin, smoking history, oxygen saturation, and lymphocyte count predicts ICU admission of COVID-19 patients with an AUC of 0.761 (95% CI 0.71–0.81; $p < 0.001$), sensitivity of 10.5%, and specificity of 99.2%. Likewise, the combined use of LDH, procalcitonin, history of chronic obstructive pulmonary disease (COPD), oxygen saturation, heart rate,

and age predicts mortality in COVID-19 patients with an AUC of 0.87 (95% CI 0.83–0.92; $p < 0.001$), sensitivity of 7.1%, and specificity of 100%³⁶.

Finally, the Sequential Organ Failure Assessment (SOFA) is commonly used to predict mortality in seriously ill patients with sepsis⁴⁰. The SOFA score includes oxygenation index, mean arterial pressure, the Glasgow coma scale, creatinine or urine volume, platelets, and TBIL⁴⁰. Liu and collaborators found that SOFA score at admission predicts mortality of COVID-19 patients with a cutoff point ≥ 3 points, AUC of 0.890 (95% CI 0.826–0.955), sensitivity of 90.00%, and specificity of 83.81%⁴⁰. This information supports the idea that scores that take parameters of liver function into account are better predictors of pneumonia, ICU admission, and death in patients with COVID-19.

Conclusion

As we have outlined here, liver impairment is commonly reported in COVID-19 patients, especially those with severe or critical disease who frequently experience the worst outcomes²⁶. The cause of abnormal liver function in these cases remains unclear, although several hypotheses have been proposed. Firstly, hepatocytes, cholangiocytes, and endothelial cells express the angiotensin-converting enzyme 2 (ACE2) receptor that acts as the main SARS-CoV-2 entry point⁴¹ into cells. Upon SARS-CoV-2 infection, liver cells may undergo necrosis and/or apoptosis. This can, in turn, contribute to inflammation and lead to hepatic damage. It is also believed that ARDS, hypoxia, and coagulation disorders might contribute to liver damage by increasing hepatic ischemia⁴². Further, exacerbation of the inflammatory response directly contributes to the cytokine storm often observed in severe COVID-19 cases, which in turn promotes multiple organ failure, including failure of the liver⁴³. Finally, the use of hepatotoxic drugs such as acetaminophen might also alter liver function tests in patients with COVID-19^{42,44}.

In terms of liver function tests, serum albumin and LDH are better predictors of ICU admission and death than levels of transaminases, ALP, bilirubin, and GGT⁴⁵. Clinical scores that include indicators of liver function such as albumin, LDH, and total bilirubin are also accurate predictors of the most adverse outcomes, including pneumonia, ICU admission, and death, in patients with SARS-CoV-2 infections. Therefore, prognosis in COVID-19 should be assessed using scores that take liver function tests such as albumin and LDH into account, together with other demographic or clinical

parameters. Liver function tests greatly enhance the ability of clinicians to identify patients at higher risk of developing the most severe cases of COVID-19, which could help increase survival rates and optimize hospital resources throughout the current pandemic.

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Conflicts of interest

The authors declare that do not exist conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Suplementos

SUPLEMENTO 1 G*POWER OUTPUT Y GRÁFICA DE DISTRIBUCIÓN

G*Power Version 3.1.9.6

t tests - Means: Difference between two independent means (two groups)

Analysis: A priori: Compute required sample size

Input: Tail(s) = Two

Effect size d = 0.6599663

α err prob = 0.05

Power (1- β err prob) = 0.95

Allocation ratio N2/N1 = 1

Output: Noncentrality parameter δ = 3.6447830

Critical t = 1.9799304

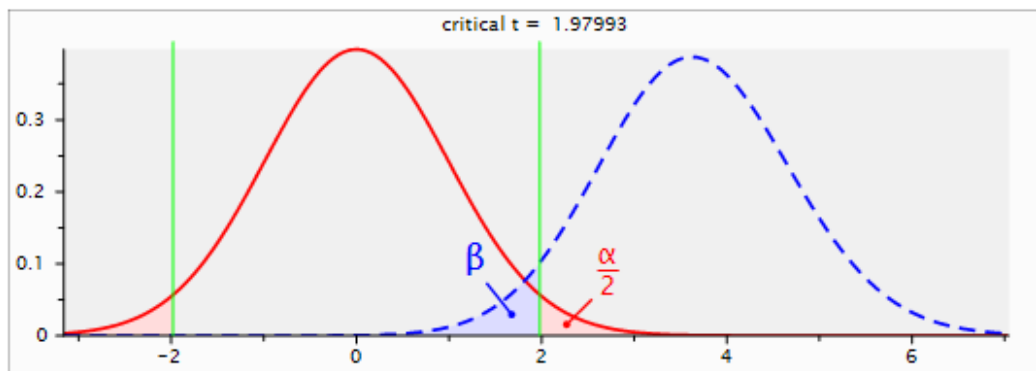
Df = 120

Sample size group 1 = 61

Sample size group 2 = 61

Total sample size = 122

Actual power = 0.9510882



SUPLEMENTO 2 CARTA DE CONSENTIMIENTO INFORMADO



Protocolo de investigación "ESTUDIO DEL PERFIL DE CITOCINAS SÉRICAS EN PACIENTES CON DIAGNÓSTICO DE COVID-19"

CARTA DE CONSENTIMIENTO INFORMADO

El objetivo principal de este protocolo es estudiar un tipo de moléculas en la sangre llamadas citocinas, que pueden desencadenar una inflamación muy fuerte en los pacientes que han sido diagnosticados con Covid-19. Actualmente, se cree que la severidad de esta enfermedad está asociada con un aumento de citocinas inflamatorias en la sangre. Sin embargo, esta información todavía no ha sido demostrada y su participación en este proyecto será de enorme ayuda para entenderla, beneficiando a muchas personas que puedan contraer esta enfermedad a través de un tratamiento personalizado y eficaz.

1. He sido invitada(o) a participar en este protocolo de investigación clínica que lleva por título "Estudio del perfil de citocinas séricas en pacientes con diagnóstico de Covid-19".
2. Me han explicado y entiendo que mi participación en este protocolo consiste en donar 8 ml de sangre, obtenida mediante la inserción de una pequeña aguja en el brazo derecho o izquierdo. He sido enterada(o) que no pagaré el procesamiento de estas muestras y que podré tener acceso a toda la información que se genere con respecto a mi salud.
3. Me han explicado y entiendo que la toma de sangre puede producir un ligero moretón en el brazo, que desaparecerá en uno o dos días sin ninguna consecuencia para mi salud.
4. He sido enterada(o) de que los investigadores y Médicos responsables de este estudio medirán la cantidad de moléculas llamadas citocinas en mi sangre y que esto no tendrá ningún costo para mí, ni repercusiones para mi salud o mi atención futura en este centro hospitalario.
5. He sido enterada(o) que los Investigadores y Médicos responsables de este estudio están dispuestos a responder cualquier pregunta o aclaración acerca del procedimiento, riesgos, beneficios y otros asuntos relacionados con la investigación en cualquier momento que yo lo desee.
6. Me han explicado y entiendo que tengo libertad de no querer participar en el estudio si así lo deseo, o de retirar mi consentimiento en cualquier momento y dejar de participar en el estudio sin que ello afecte por ningún motivo mi salud o atención médica futura en el Hospital General de México "Dr. Eduardo Liceaga".
7. He sido informada(o) que el Dr. Eustacio Galileo Escobedo González estará pendiente de mi salud y seguridad, durante y después de la toma de las muestras de sangre y que en caso de presentarse algún evento adverso durante este procedimiento, él se hará cargo de mi seguridad, así como de mantenerme informada(o) sobre cualquier cosa relacionada con mi salud, aún si eso repercutiera en mi deseo de seguir participando en el estudio.
8. He sido enterada(o) y entiendo que los resultados de este estudio serán totalmente confidenciales y que mi nombre será resguardado en todo momento, mediante el uso de una clave que únicamente el investigador principal y yo conoceremos.
9. Estoy de acuerdo en que los resultados de este estudio sean publicados con fines científicos.
10. He sido informada(o) que este estudio se llevará a cabo en el Laboratorio de Proteómica y Metabolómica del Hospital General de México "Dr. Eduardo Liceaga" y en el Servicio de Infectología del mismo centro hospitalario.
11. Así mismo, he sido informada(o) y entiendo que la realización de este estudio no tendrá ningún beneficio económico directo para los investigadores responsables del mismo, y que mi participación es completamente voluntaria con la finalidad de contribuir con el desarrollo de mejores esquemas de atención médica y hospitalaria.



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Protocolo de Investigación "ESTUDIO DEL PERFIL DE CITOCINAS SÉRICAS EN PACIENTES CON DIAGNÓSTICO DE COVID-19"

12. Me han aconsejado que no debo participar en más de dos proyectos al mismo tiempo, debido a mi condición clínica y en beneficio de mi salud.
13. Manifiesto que mi decisión de participar en este estudio es completamente voluntaria y libre de coerción y cohecho.
14. He sido informada(o) que la Dra. Antonia Cervantes Barrios, Presidenta del Comité de Ética en Investigación, garantizará mi bienestar así como el respeto de mis derechos humanos durante mi participación en el estudio, supervisando que el desarrollo del protocolo esté apegado a la Ley General de Salud, y que mi identidad sea respetada y mantenida de forma confidencial.
15. Los Investigadores y Médicos responsables del proyecto me han informado que si tuviese preguntas sobre este estudio, puedo comunicarme en cualquier momento con el Dr. Galileo Escobedo (Investigador Responsable) al teléfono 5527892000, ext. 5646 o con la Dra. Antonia Cervantes Barrios (Presidenta del Comité de Ética) al teléfono 552789-2000, ext. 1164.

Yo, _____, he leído y comprendido la información anterior y mis preguntas han sido respondidas de manera satisfactoria. He sido informada(o) y entiendo que los datos obtenidos en el estudio pueden ser publicados o difundidos con fines científicos. Por lo tanto, convergo voluntariamente en participar en este estudio de investigación.

Nombre del Paciente

Firma o huella digital

Nombre de Testigo 1 y Parentesco

Nombre de Testigo 2 y Parentesco

Dirección y Teléfono de Testigo 1

Dirección y Teléfono de Testigo 2

Fecha: _____

He explicado a la(a) Sra.(Sr.) _____ la naturaleza y los propósitos de la investigación, así como los riesgos y beneficios que implica su participación. He contestado a las preguntas en la medida de lo posible y he preguntado si tiene alguna duda. Acepto que he leído y conozco la normatividad correspondiente para realizar investigación con seres humanos y me apego a ella.

Dr. Eustacio Galileo Escobedo González

Investigador Responsable

Tel. Of. 5527892000, ext. 5646, Tel. móvil 55-3893-4496, Correo-e: gescobedo@unam.mx



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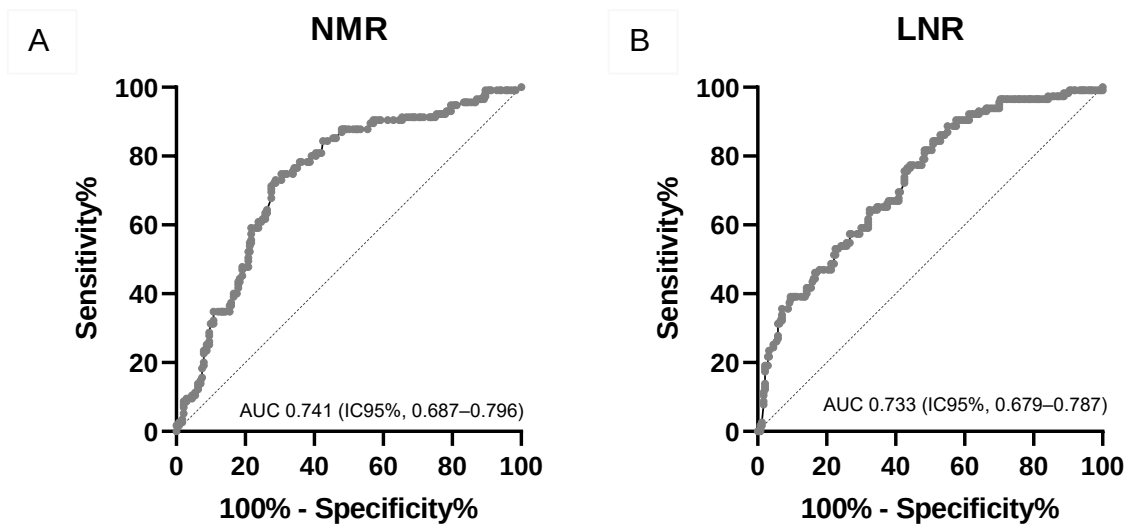
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SUPLEMENTO 3 CURVAS ROC PARA NMR Y LNR AL INGRESO HOSPITALARIO PARA LA PREDICCIÓN DE MORTALIDAD EN PACIENTES CON COVID-19 SEVERA.



LNR, Lymphocyte-to-neutrophil ratio; NMR, neutrophil-to-monocyte ratio; ROC, Receiver Operating Characteristic curves; AUC, area under the ROC curve; IC, intervalo de confianza

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