UNIVERSITAT DE BARCELONA

Mecanismes implicats en la resposta inflamatòria i noves dianes terapèutiques en la COVID-19

Xavier Solanich Moreno

Tesi doctoral

Programa de Doctorat Medicina i Recerca Translacional
Facultat de Medicina i Ciències de la Salut. Universitat de Barcelona
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MECANISMES IMPLICATS EN LA RESPOSTA INFLAMATÒRIA I NOVES DIANES TERAPÈUTIQUES EN LA COVID-19

Memòria de tesi doctoral presentada per

XAVIER SOLANICH MORENO

per optar al grau de doctor
per la Universitat de Barcelona

Dirigida per

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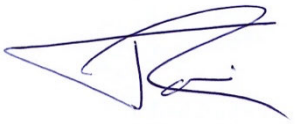
Octubre 2021

Els **Drs. Antoni Riera Mestre** i **Xavier Corbella i Virós**, ambdós doctors en Medicina per la Universitat de Barcelona.

CERTIFIQUEM

Que la memòria titulada "Mecanismes implicats en la resposta inflamatòria i noves dianes terapèutiques en la COVID-19", presentada per **Xavier Solanich Moreno**, ha estat realitzada sota la nostra direcció. Considerem que reuneix les condicions necessàries, i en conseqüència, donem el nostre vist i plau perquè sigui defensada davant el tribunal corresponent, i optar al grau de doctor en Medicina per la Universitat de Barcelona.



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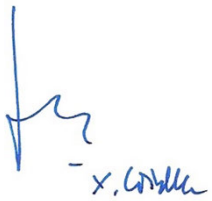

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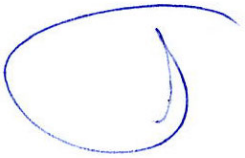

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AGRAÏMENTS

“Tant si penses que pots com si no,

tindràs raó”

Henry Ford

A principis del 2020 la pandèmia per la SARS-CoV-2 va trasbalsar enormement les nostres vides. Com a sanitaris, va suposar un desgast personal, emocional i laboral molt important. I tanmateix, en aquest difícil context, han reeixit els projectes que han acabat conformant la present tesi doctoral. Crec que això ha estat així per un cúmul de circumstàncies. La singular resposta inflamatòria en la COVID-19 va fer que em sentís especialment atret per aquesta malaltia. En aquest sentit, considero que el meu bagatge en l’àrea dels defectes immunitaris em va donar la confiança suficient com per fer recerca en aquest camp. Un cop fet aquest primer pas, els projectes s’han desenvolupat d’una forma fluida, senzilla, natural... gràcies a l’extraordinària implicació, saber fer, i generositat d’un gran nombre de companys. En conseqüència, m’agradaria posar de relleu tota la feina col·lectiva realitzada, i agrair de tot cor cadascuna de les aportacions.

Al **Xavier Corbella** per acompanyar-me i guiar-me en aquest llarg camí. Per saber comprendre i redreçar moments de dificultat. Per la seva exigència que m’ha ajudat a arribar al dia d’avui. Al **Toni Riera** per la seva amistat i els seus consells fets des de l’afecte. Per ser-hi sempre que ho he necessitat, i per la seva enorme generositat amb mi durant tots aquests anys. Al **Gerard Espinosa** per haver-me acompanyat durant els primers anys de la tesi i per l’honradesa de renunciar quan vaig decidir canviar-ne el rumb.

A l’**Antonio Vidaller** per practicar una medicina excel·lent d’una forma tan humana i alegre. Per ser un d’aquells mestres que predica amb l’exemple. Pels

anys que vàrem treballar plegats a Bellvitge i en que va confiar en mi des del primer moment. Per l'amistat i els bons consells. A l'**Arnau Antolí** perquè els treballs d'aquesta tesi han estat possibles en gran part per la seva enorme implicació, i a la **Gemma Rocamora** per fer-ho tot tan fàcil sempre. Tant de bo us pugui ajudar en el futur tant com vosaltres m'heu ajudat a mi. A la **Conxi Lázaro** per la gran predisposició a iniciar nous projectes, per l'enorme perseverança, per obrir-nos les portes del seu laboratori, per pensar en gran. Perquè amb el seu saber fer vàrem establir col·laboració amb el grup d'**Alexander Hoischen** i **Cas van der Made** que està donant ja els seus fruits. A la **Gardenia Vargas** per fer tanta i tan bona feina. Perquè ha estat les mans del nostre estudi de genètica. Per la seva generositat i senzillesa. Al **Joan Sabater** per dinamitzar un dels treballs de la tesi, per la confiança i amistat que m'ha demostrat durant tots aquests anys, per la generositat del seu servei que ha fet una tasca extraordinària en aquesta pandèmia. Al **Raúl Rigo-Bonin** per la seva infinita capacitat de treball, per la iniciativa i enorme capacitat d'anàlisi, i sempre amb bon humor. Al **Fran Morandeira**, i a tot el laboratori d'immunologia, perquè sense la seva implicació i dedicació no haguès estat possible tirar endavant part dels treballs d'aquesta tesi. Al **Roger Colobran** per ser sempre tan accessible i per ajudar-nos a posar a punt algunes tècniques de laboratori. Al **Paul Bastard** i al **Jean-Laurent Casanova** per la seva extraordinària amabilitat, per la facilitat per establir col·laboració, per compartir amb nosaltres els seus coneixements, i per completar part de les nostres investigacions en el seu gran laboratori. Al **Fernando Alcaide**, **Miguel Fernández-Huerta** i **Miguel Santín** per tenir la ment desperta, veure més enllà de les utilitats clàssiques dels IGRA, i compartir amb mi els seus coneixements. Al **Sebastià Videla**, **Pilar Hereu** i **Cristian Tebé**, i als seus respectius equips, per l'extraordinària ajuda oferta mentre dúiem a terme l'assaig clínic, i que encara m'ofereixen sempre que la necessito. A la **Núria Padullés** pels consells i rigorós control dels pacients que varem tractar. A l'**Emili Corbella** per les seves lliçons d'estadística i les converses sobre actualitat. Al **David Chivite** per donar força al català en aquesta tesi. I als **companys de l'Hospital de Bellvitge**, especialment als del servei de medicina interna, per recolzar-me, per les experiències i moments inoblidables que hem viscut i que,

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FINANÇAMENT

Els treballs que conformen aquesta Tesi Doctoral han estat possibles, en part, gràcies al finançament competitiu concedit per la Direcció General de Recerca i Innovació en Salut (DGRI) amb la col·laboració de l'Aquas i el Biocat, que l'any 2020 va atorgar al doctorand Xavier Solanich 108.550,00 €, com a Investigador Principal pel projecte "Pragmatic, controlled, open, single center, randomized, phase II clinical trial to evaluate methylprednisolone pulses and Tacrolimus in hospitalized patients with severe pneumonia secondary to COVID-19".

A més, l'edició 2020 de La Fundació de La Marató de TV3 va concedir finançament competitiu al projecte "Role of Immune System inborn errors as determinants of the COVID-19 severity in hospitalized patients" (202115-30-31). L'equip d'investigadors està format per dos nuclis coordinats de HUB-ICO-IDIBELL, essent la Dra. Conxi Lázaro (ICO-IDIBELL) i el Dr. Xavier Corbella (HUB-IDIBELL) els seus Investigadors Principals, i el Dr. Antoni Riera i el doctorand Xavier Solanich investigadors col·laboradors. El projecte compta amb un ajut de 299.998 € per a tres anys amb data d'inici el Setembre de 2021.

INDEX

GLOSSARI

ACE2 = *angiotensin-converting enzyme 2*
autoAbs = autoanticossos
CD = cèl·lules dendrítiques
CDp = cèl·lules dendrítiques plasmacitoides
CNI = inhibidors de la calcineurina
CoV = Coronavirus
COVID-19 = *Coronavirus disease 2019*
COVIDHGE = *COVID Human Genetic Effort*
EA = esdeveniments adversos
GWAS = *genome-wide association study*
HUB = Hospital Universitari de Bellvitge
IL= interleucina
IFN = interferó
IGRA = assajos d'alliberament d'interferó gamma
IMC = índex de massa corporal
IRA = Insuficiència renal aguda
IRF = *interferon regulatory factor*
ISG = *interferon-stimulated genes*
LDH = lactat deshidrogenasa
LTBI = infecció tuberculosa latent
mTOR = mechanistic target of rapamycin
MyD88 = *myeloid differentiation primary response 88*
NautoAbs = autoanticossos neutralitzants
NF-κB = *nuclear factor-κB*
NK = *natural killer*
NSP = *non-structural proteins* de SARS-CoV-2
ORF = *open reading frames* de SARS-CoV-2
OMS = Organització Mundial de la Salut
PBMC = *peripheral-blood mononuclear cells*

PCR = proteïna C reactiva en plasma

PRRs = *Pattern Recognition Receptors*

QFT-Plus = QuantiFERON-TB Gold Plus

RCT = Assaigs clínics randomitzats

RLR = *RIG-like receptors*

SARS-CoV-2 = *Severe Acute Respiratory Syndrome Coronavirus 2*

SDRA = Síndrome del destret respiratori agut

SoC = *Standard of Care*

ssRNA = àcid ribonucleic de cadena simple

TLR = Toll-like receptor

TNF-α = *tumoral necrosis factor–α*

TRIF = *TIR-domain-containing adapter-inducing interferon-β*

UCI = Unitat de Cures Intensives

INFORME DELS DIRECTORS

Com a directors de la tesi doctoral de **Xavier Solanich Moreno**, titulada "Mecanismes implicats en la resposta inflamatòria i noves dianes terapèutiques en la COVID-19", certifiquem que el doctorand ha participat activament en el disseny, implementació, anàlisi dels resultats i la seva discussió, extracció de conclusions i redacció de cadascuna de les publicacions incloses en aquesta tesi. A continuació s'enumeren cadascun dels treballs, acompanyats pels factors d'impacte de les revistes on es van publicar els resultats. Aquests articles no s'han presentat com a part d'altres tesis doctorals.

TESI EN FORMAT DE COMPENDI D'ARTICLES

La tesi consta de 4 objectius, especificats en l'apartat corresponent, dels quals en deriven les següents publicacions:

1.- **Solanich X**, Vargas-Parra G, van der Made CI, Simons A, Schuurs-Hoeijmakers J, Antolí A, Del Valle J, Rocamora-Blanch G, Setién F, Esteller M, van Reijmersdal SV, Riera-Mestre A, Sabater-Riera J, Capellá G, van de Veerdonk FL, van der Hoven B, Corbella X, Hoischen A, Lázaro C. **Genetic Screening for TLR7 Variants in Young and Previously Healthy Men With Severe COVID-19.** Front Immunol. 2021 Jul 23;12:719115. doi: 10.3389/fimmu.2021.719115.

Factor impacte 7.561 (Q1). Categoria: IMMUNOLOGY - SCIE. Posició 24 de 162 revistes a la categoria. Font JCR 2020.

2.- **Solanich X**, Rigo-Bonnin R, Gumucio VD, Bastard P, Rosain J, Philippot Q, Perez-Fernandez XL, Fuset-Cabanes MP, Gordillo-Benitez MÁ, Suarez-Cuartin G, Boza-Hernandez E, Riera-Mestre A, Parra-Martínez A, Colobran R, Antolí A, Navarro S, Rocamora-Blanch G, Framil M, Calatayud L, Corbella X, Casanova JL, Morandeira F, Sabater-Riera J. **Pre-existing Autoantibodies Neutralizing High Concentrations of Type I Interferons in Almost 10% of COVID-19 Patients Admitted to Intensive Care in Barcelona**. J Clin Immunol. 2021 Sep 27. doi: 10.1007/s10875-021-01136-x.

Factor impacte 8.317 (Q1). Categoria: IMMUNOLOGY - SCIE. Posició 22 de 162 revistes a la categoria. Font JCR 2020.

3.- **Solanich X**, Fernández-Huerta M, Basaez C, Antolí A, Rocamora-Blanch G, Corbella X, Santin M, Alcaide F. **Clinical Significance of Indeterminate QuantiFERON-TB Gold Plus Assay Results in Hospitalized COVID-19 Patients with Severe Hyperinflammatory Syndrome**. J Clin Med. 2021 Feb 26;10(5):918. doi: 10.3390/jcm10050918.

Factor impacte 4.241 (Q1). Categoria: Science Edition - MEDICINE, GENERAL & INTERNAL. Posició 39 de 169 revistes a la categoria. Font JCR 2020.

4.- **Solanich X**, Antolí A, Padullés N, Fanlo-Maresma M, Iriarte A, Mitjavila F, Capdevila O, Molina M, Sabater J, Bas J, Mensa-Vilaró A, Niubó J, Calvo N, Bolivar S, Rigo-Bonnin R, Arregui L, Tebé C, Hereu P, Videla S, Corbella X. **Pragmatic, open-label, single-center, randomized, phase II clinical trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus in patients with severe pneumonia secondary to COVID-19: The TACROVID trial protocol.** Contemp Clin Trials Commun. 2021 Mar;21:100716. doi: 10.1016/j.conctc.2021.100716.

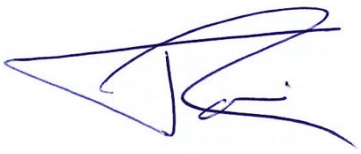
Factor impacte 0.460 (Q3). Categoria: Science Edition - MEDICINE, RESEARCH & EXPERIMENTAL. Posició 133 de 188 revistes a la categoria. Font JCR 2020

5.- **Solanich X**, Antolí A, Rocamora-Blanch G, Padullés N, Fanlo-Maresma M, Iriarte A, Mitjavila F, Capdevila O, Riera-Mestre A, Bas J, Vicens-Zygmunt V, Niubó J, Calvo N, Bolivar S, Rigo-Bonnin R, Mensa-Vilaró A, Arregui L, Tebe C, Videla S, Hereu P, Corbella X. **Methylprednisolone Pulses Plus Tacrolimus in Addition to Standard of Care vs. Standard of Care Alone in Patients With Severe COVID-19. A Randomized Controlled Trial.** Front Med (Lausanne). 2021 Jun 14;8:691712. doi: 10.3389/fmed.2021.691712.

Factor impacte 5.091 (Q1). Categoria: Science Edition - MEDICINE, GENERAL & INTERNAL. Posició 28 de 169 revistes a la categoria. Font JCR 2020.

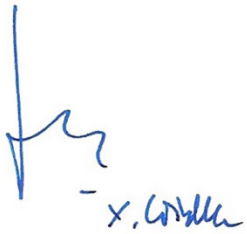
6.- **Solanich X**, Padullés N, Niubó J, Videla S, Antolí A, Rocamora-Blanch G, Corbella X. **Inhibition of SARS-CoV-2 replication using calcineurin inhibitors: are concentrations required clinically achievable?** J Intern Med. 2021 Jun;289(6):926-927. doi: 10.1111/joim.13264.

Factor impacte 8.989 (Q1). Science Edition - MEDICINE, GENERAL & INTERNAL. Posició 12 de 169 revistes a la categoria. Font d'impacte: JCR 2020.



Antoni Riera Mestre

Director I Tutor



Xavier Corbella i Virós

Director

I. Introducció

1. SARS-CoV-2 i la pandèmia per COVID-19

El desembre de 2019 es van detectar els primers casos d'una nova malaltia (COVID-19) causada per un nou coronavirus humà (SARS-CoV-2). Va ser reconeguda per primera vegada a Wuhan, Xina, i es va estendre per tot el món ràpidament [1,2]. L'Organització Mundial de la Salut (OMS) va declarar la COVID-19 com a pandèmia l'11 de març de 2020 [3]. Des d'aleshores aquesta infecció ha continuat propagant-se a nivell mundial causant una morbimortalitat i danys econòmics molt considerables. A data d'1 d'Octubre de 2021, hi ha hagut més de 233.000.000 casos confirmats i més de 4.750.000 morts arreu del mon (https://covid19.who.int). Desafortunadament, la pandèmia resta lluny d'estar controlada, sobretot en els països en vies de desenvolupament on els percentatges de població vacunada són encara baixos.

2. Epidemiologia de la infecció per SARS-CoV-2

SARS-CoV-2 es transmet fonamentalment mitjançant gotes expulsades quan es parla o s'esternuda a una distància curta (<1.8 metres) i durant un temps perllongat (>15 min). Més rarament, el virus es contrau a través del contacte amb fòmites presents a superfícies [4]. El pic de la càrrega viral a la via respiratòria superior sol coincidir amb el debut de la clínica, tot i que l'alliberament de partícules virals començar uns 2-3 dies abans de l'inici dels símptomes [5]. Es creu que més de la meitat de les transmissions es produeixen per l'exposició a persones asimptomàtiques o poc simptomàtiques [6], que constituiria un dels factors que més ha afavorit la disseminació de la SARS-CoV2. Encara que els àcids nucleics del SARS-CoV2 es poden detectar durant vàries setmanes, els cultius virals són generalment negatius passats els 8 dies [7]. Aquest fet seria congruent amb els estudis epidemiològics que han demostrat que rarament hi ha transmissió entre contactes exposats a casos índexs amb més de 5 dies de clínica [4].

L'ús de mascaretes facials ha demostrat ser eficaç per reduir el contagi de la COVID-19 [8]. Les mascaretes quirúrgiques i N95 confereixen una protecció substancial respecte a no utilitzar-les. La distància física i el rentat de mans també son importants per a reduir la transmissió del SARS-CoV-2 [8].

3. Clínica de la infecció per SARS-CoV-2

El període d'incubació de la COVID-19 oscil·la entre 2 i 14 dies, amb una mitjana d'uns 5 dies. La infecció per SARS-CoV-2 presenta un ampli espectre de manifestacions clíniques, des de pacients completament asimptomàtics fins a quadres extremadament crítics. La majoria de pacients es troben asimptomàtics o presenten manifestacions clíniques lleus com una síndrome gripal, febre, tos no productiva o astènia. En aproximadament un 3% dels casos es produeix una pneumònia greu amb síndrome del destret respiratori agut (SDRA) associada. La taxa de mortalitat per infecció (IFR) en poblacions no vacunades és del 1% [1,2]. Des de l'aparició del primer símptoma fins al desenvolupament d'aquestes manifestacions greus sol transcórrer una setmana aproximadament.

La gravetat d'aquesta infecció depèn de factors relatius al virus (p.e noves variants), condicions relatives a l'hoste (p.e. predisposició genètica, immunosupressió, comorbilitats, vacunació, etc), i certs factors ambientals (p.e. accés a sistema sanitari, nivell socioeconòmic, etc) [9].

4. SARS-CoV-2

4.1 Els coronavirus

Els Coronavirus (CoV) són membres de la subfamília *Orthocoronavirinae* dins de la família *Coronaviridae* (ordre *Nidovirales*). Aquesta subfamília comprèn quatre gèneres: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* i *Deltacoronavirus* d'acord a la seva estructura genètica [6]. D'aquests només els alfa i els beta causen patologia en humans. Fins a finals del 2019 només es

coneixien sis CoVs causants de malaltia en humans. Quatre CoV d'ells tenen una baixa patogenicitat (229E, HKU1, OC43 i NL63) i causen un 10-30% dels quadres de refredat comú [10,11]. S'havien descrit també dos CoV altament patogènics causants de la Síndrome Respiratòria Aguda Severa (SARS-CoV) i a la Síndrome Respiratòria d'Orient Mitjà (MERS-CoV), i que eren responsables d'infeccions de les vies respiratòries inferiors d'una elevada mortalitat [11].

4.2 Estructura i genoma de SARS-CoV-2

SARS-CoV-2 és un nou betacoronavirus altament patogènic. Es tracta d'un virus esfèric d'uns 100-160 nm de diàmetre, amb una bicapa lipídica que l'embolcalla i que conté RNA monocatenari (ssRNA) d'aproximadament 30 kilobases de longitud (**Figura 1a**) [12,13]. La regió 5′ terminal del genoma transcriu dos grans *open reading frames* (ORF), ORF1a i ORF1b, que es tradueixen a les proteïnes pp1a i pp1b. La traducció comença a ORF1a i produeix pp1a, que inclou nsp1-11, o bé, pp1ab, un polipèptid més llarg, que inclou nsp12-16. La producció de qualsevol dels dos polipèptids depèn de si el codó stop a ORF1a és reconegut pel ribosoma o és obviat produint-se un canvi en el marc de lectura (*ribosome frameshifting site*). El genoma del virus codifica 2 cisteïnes proteases, una proteasa similar a la papaïna (PLpro o nsp3) i una proteasa similar a la 3C (3CLpro o nsp5). Aquestes proteases divideixen els polipèptids pp1a i pp1b en 16 proteïnes no estructurals (nsp). La RNA polimerasa RNA dependent (RdRp o nsp12) i altres nsp tenen una funció crítica en la replicació / transcripció de SARS-CoV-2. Les proteïnes estructurals i accessòries es sintetitzen mitjançant la traducció dels seus respectius mRNA subgenòmics [10,14]. Hi ha quatre proteïnes estructurals: la proteïna S (*spike*); la proteïna E (*envelope*); la proteïna M (*membrane*); i la proteïna N (*nucleocapsid*); i vuit proteïnes accessòries (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b) [15] (**Figura 1b**). La proteïna N està a l'interior del virió associada al ssRNA viral, i les altres tres proteïnes estructurals es troben en la bicapa lipídica formant l'embolcall viral. La proteïna S forma homotrimers que sobresurten de la superfície viral, donant la característica morfologia que recorda una corona al microscopi electrònic. La proteïna S consta de 2 subunitats funcionals: la subunitat S1, on es troba el *receptor-binding domain* (RBD) que

s'uneix a receptors de les cèl·lules de l'hoste; i la subunitat S2, que intervé en la posterior fusió entre les membranes cel·lulars del virus i de l'hoste [6,16]. Les proteïnes E, M i N faciliten la formació de noves partícules virals [10,17].

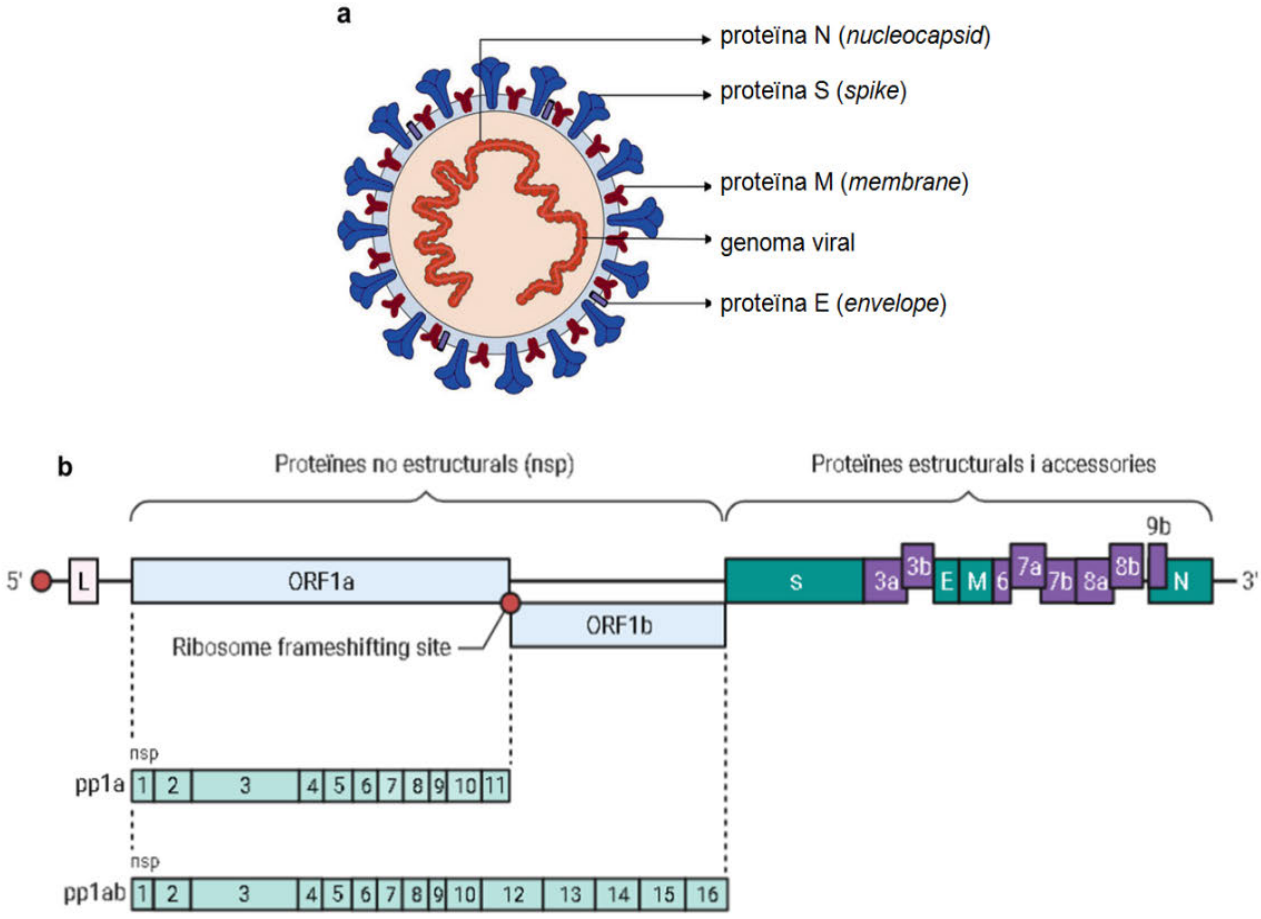


Figura 1. Diagrama esquemàtic de la partícula i el genoma del virus SARS-CoV-2 (Adaptat de Hartemian et al. J Biol Chem. 2020 [10]). A) Estructura de SARS-CoV-2 amb les seves 4 proteïnes estructurals. B) Representació del RNA viral que es tradueix en setze proteïnes no estructurals (blau), 4 proteïnes estructurals (verd) i 8 proteïnes accessòries (lila).

Des de la seva identificació, SARS-CoV-2 ha presentat múltiples mutacions en el seu genoma que han donat lloc a l'aparició de noves variants. De totes elles, unes poques generen un especial interès per la seva importància en termes de salut pública, ja sigui perquè són més transmissibles, causen una malaltia més greu o bé escapen a la immunitat generada per la infecció o la vacunació [18]. Inicialment anomenades en funció del país de procedència, l'OMS les ha

reanomenat utilitzant lletres gregues: alfa (B.1.1.7) [britànica], beta (B.1.351) [sud-africana], delta (B.1.617.2) [índia], etc [19]. Amb la variant alfa (B.1.1.7) va augmentar 2,15 vegades el risc d'ingrés a unitats de cures intensives, i es va incrementar 1,7 vegades el risc de mort en comparació amb les variants prèvies al Regne Unit [20]. Posteriorment, amb la variant delta (B.1.617.2) es va observar el doble de risc d'ingrés hospitalari respecte els infectats amb la variant alfa (B1.1.7) [21]. El risc de patir una COVID-19 crítica amb les variants alfa i delta és aproximadament el doble respecte altres variants [20,21]. El impacte de les variants és evident però els seus efectes no expliquen l'enorme variabilitat clínica interindividual que presenten els pacients infectats per SARS-CoV-2.

4.3 Entrada i replicació viral de SARS-CoV-2 a la cèl·lula de l'hoste

L'enzim convertidor d'angiotensina 2 (ACE-2) i la serin proteasa cel·lular TMPRSS2 permeten el reconeixement i entrada de SARS-CoV-2 a la cèl·lula [22]. ACE-2 s'expressa àmpliament en les cèl·lules epitelials alveolars pulmonars, monòcits i macròfags alveolars, i en les cèl·lules endotelials venoses i arterials [23,24]. S'ha descrit que la Neuropilin1 (NRP1) i la Furina son cofactors importants per a l'entrada del virus, particularment en cèl·lules amb baixa expressió d'ACE2 [25]. Així doncs, SARS-CoV-2 entra a les cèl·lules diana a través de l'endocitosi mediada per receptor i posteriorment es produeix l'alliberament de la nucleocàpsida vírica al citosol de la cèl·lula infectada. Tot seguit, el RNA genòmic del virus es tradueix per produir les 16 nsp que formen el complex de replicació-transcripció (CTR). El CTR participa en la replicació de RNA genòmic i en la transcripció d'un conjunt de mRNA subgenòmic necessari per a que s'expressin els gens de les proteïna estructurals i accessòries. Finalment, els nous virions s'assemblen en el reticle endoplasmàtic - Golgi i s'alliberen per exocitosi [6].

5. Resposta immune de l'hoste enfront a SARS-CoV-2

Per defensar-nos de la majoria d'infeccions víriques és necessària una ràpida resposta de la immunitat innata, seguida d'una resposta adaptativa més sofisticada, que contribuirà a eradicar el virus i que té la capacitat de generar memòria immunològica de llarga durada.

5.1 Immunitat innata

5.1.1 *Pattern recognition receptors*

Cadascun dels *pattern recognition receptors* (PRRs) reconeix uns patrons moleculars específics en els microorganismes coneguts com a *pathogen-associated molecular patterns* (PAMP), i molècules derivades de cèl·lules danyades anomenades *damage-associated molecular patterns* (DAMP). Cada PRR està present en major o menor mesura en els diferents tipus de cèl·lules i condueix a l'activació cel·lular d'una forma diferent, de manera que aquests receptors tenen rols especialitzats no redundants [26]. Els mamífers tenim diverses classes de PRR, inclosos els *Toll-like receptor* (TLR), els *RIG-I-like receptors* (RLR), els *Nodlike receptors* (NLR), els *AIM2-like receptors* (ALR), els *C-type lectin receptors* (CLR) i els sensors de DNA intracel·lular com cGAS.

5.1.2 *Toll-like receptors*

Els TLR es troben altament conservats des la *Drosophila* fins als humans, i comparteixen similituds estructurals i funcionals. Cada TLR està compost per un domini amb repeticions riques en leucina (LRR) que s'encarrega del reconeixement del PAMP, un domini transmembrana, i un domini Toll/IL-1 receptor (TIR) al citoplasma que inicia la senyalització intracel·lular [27]. Els TLR s'expressen en cèl·lules del sistema immune innat (cèl·lules dendrítiques [CD], macròfags, cèl·lules *Natural Killer* [NK]) i de l'adaptatiu (limfòcits T i B), així com en cèl·lules no immunes (fibroblasts, cèl·lules epitelials...) [27,28]. La família TLR comprèn 10 membres (TLR1-TLR10) en humans que es classifiquen en dues subfamílies en funció de si la seva localització és a la superfície cel·lular (TLR1,

TLR2, TLR4, TLR5, TLR6 i TLR10), o intracel·lular (TLR3, TLR7, TLR8 i TLR9). Cada TLR reconeix PAMPs diferents o superposats, com ara lípids, lipoproteïnes, proteïnes i àcids nucleics. Els TLR de la superfície cel·lular reconeixen principalment components de la membrana microbiana com lípids, lipoproteïnes i proteïnes. En canvi, els TLR intracel·lulars reconeixen els àcids nucleics procedents de bacteris i virus, i també els àcids nucleics propis procedent de cèl·lules danyades [28]. TLR3 reconeix RNA viral de doble cadena (dsRNA), RNA petits interferents (siRNA), i RNA propi procedent de cèl·lules danyades. TLR7 i TLR8 provenen de gens duplicats en tàndem al cromosoma X, i reconeixen RNA de cadena simple (ssRNA) viral o endogen, així com oligoribonucleòtids sintètics com ara imidazoquinolina, imiquimod i R-848. Tot i això, aquests dos receptors tenen petites diferències en la seva zona d'unió al lligand i s'expressen en cèl·lules diferents (TLR7 sobretot en CD plasmacitoides [CDp], i TLR8 sobretot en les CD mieloides [CDm]) amb vies de senyalització que difereixen, suggerint que tenen rols especialitzats i no redundants [26,27]. TLR9 reconeix DNA bacterià i viral ric en motius de DNA CpG no metilats, i també reconeix l'hemozoïna generada per Plasmòdium falcíparum [30].

5.1.3 Reconeixement de SARS-CoV-2 pels PPR

SARS-CoV-2 és un virus ssRNA que és reconegut fonamentalment per TLR3 i TLR7 als endosomes, o pels *retinoic acid-inducible gene (RIG)-like receptors* (RLR) al citoplasma de les cèl·lules humanes infectades. Altres TLR podrien reconèixer altres components del virus i estarien també implicats en la patogènia de la COVID-19 [31].

De forma general, quan SARS-CoV-2 és reconegut pels TLR, aquests senyalitzen mitjançant el reclutament de molècules adaptadores específiques. La senyalització es pot dividir en dues grans vies de transducció en funció del tipus d'adaptador: les vies dependents del *TIR-domain-containing adapter-inducing interferon-β* (TRIF), utilitzada per TLR3; i les vies dependents de *Myeloid differentiation primary response* 88 (MyD88), utilitzada per TLR7. Com a resultat, es produeix l'activació de NF-kB i els factors reguladors del interferó (IRF)3 i

IRF7, que es traslladen al nucli i indueixen la producció de citosines proinflamatòries (p.e. interleucina-1 [IL-1], IL-6, factor de necrosi tumoral-α [TNF-α], IL-12, etc) i IFN de tipus I (IFN-I). [32]. Les cèl·lules no hematopoiétiques, com les cèl·lules epitelials respiratòries, expressen TLR3 (però no TLR7) que a través de IRF3 constitutiu produeix IFNs de tipus III [33]. En canvi, les CDp expressen TLR7 (però no TLR3) i tenen nivells constitutius d'IRF7 elevats que produeixen de forma ràpida i potent IFNs de tipus I. Els IFN-I i III secretats s'uneixen als seus receptors d'interferó, activant IRF7 i IRF9 (complex ISGF3), fet que provoca un bucle d'amplificació i la inducció de múltiples gens estimulats per interferó (ISG) [33,34] **(Figura 2)**.

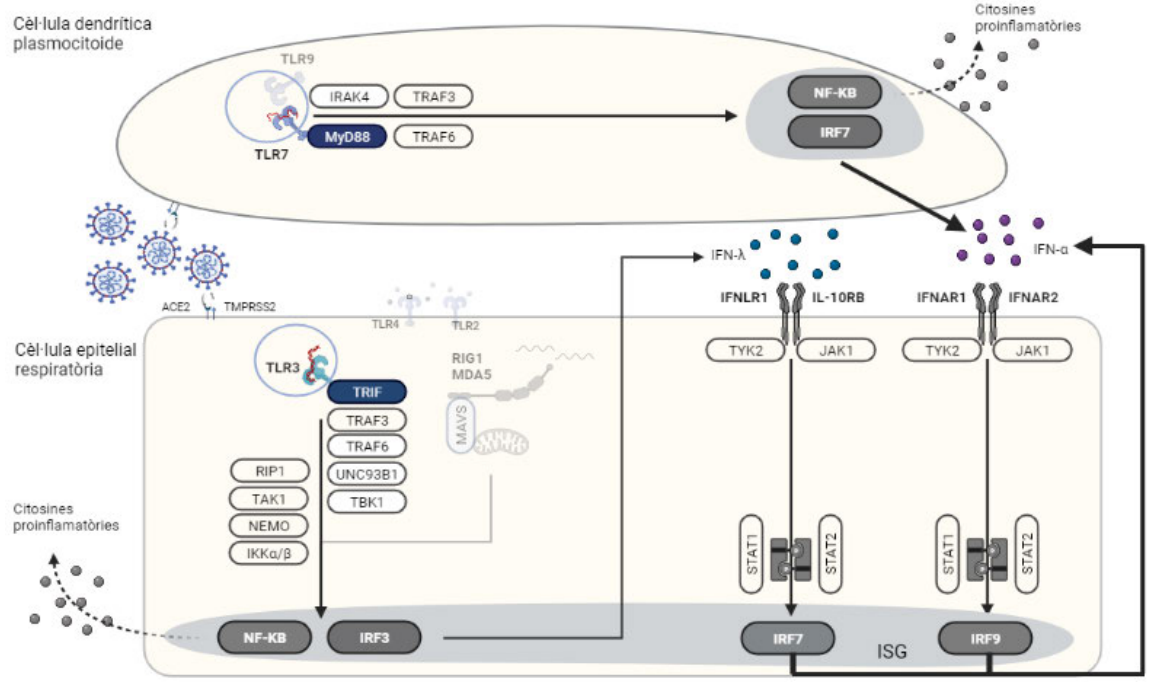


Figura 2. Tropisme i resposta immunitària innata enfront a SARS-CoV-2. (Adaptat de Schultze et al. Cell 2021 [34]) En gris clar s'exposen aquells PRR que no han estat clarament implicats en el reconeixement de SARS-CoV-2. Abreviatures: ACE2, *angiotensin-converting enzyme 2*; IKK, *IκB kinase*; IFN, interferó; IFNAR1/2, *interferon-alpha/beta receptor subunit 1/2*; IRAK, *interleukin-1 receptor-associated kinase*; IRF, *interferon regulatory factor 3/7/9*; ISG, *interferon-stimulated genes*; JAK1, *Janus Kinase 1*; MAVS, *mitochondrial antivirus signaling*; MDA5, *melanoma differentiation-associated protein 5*, MyD88, *myeloid differentiation primary response 88*; NEMO, *Nuclear factor-kappa B essential modulator*; NF-κB, *nuclear factor-κB*; RIG-1, *retinoic acid-inducible gene*; RIP1, *Receptor-interacting protein 1*; STAT, *Signal transducer and activator of transcription*; TAK1, *Transforming growth factor (TGF)-β-activating kinase 1*; TBK1, *TANK-binding kinase 1;* TRIF, *TIR-domain-containing adapter-inducing interferon-β*; TLR, *Toll-like receptor*; TRAFs, *TNF receptor associated factors*; TYK2, *Tyrosine Kinase 2*.

5.1.4 Interferons de tipus I i III en la infecció per SARS-CoV-2

Els IFNs es divideixen en 3 grups: tipus I representat pels IFN-α (-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17, i -α21), IFN-β, IFN-κ, IFN-ε i IFN-ω); tipus II representats per l'IFN-ɣ; i tipus III representat per IFN-ʎ (-ʎ1, -ʎ2, -ʎ3) [35].

Els IFN de tipus I i III tenen una gran quantitat d'efectes antivirals com el bloqueig de l'entrada a les cèl·lules, la interferència amb el trànsit de partícules virals, la inducció de l'expressió de RNAasa i DNAasa per degradar el material genètic del virus, la millora de la presentació d'antígens virals mitjançant MHC-I, la inhibició de la síntesi de proteïnes, la inducció de l'apoptosi de cèl·lules infectades i l'activació de múltiples cèl·lules dels sistema immune (macròfags, cèl·lules NK, limfòcits T citotòxics, i limfòcits T col·laboradors) [35]. El receptors dels IFN de tipus III s'expressen en un nombre limitat de teixits, entre ells els la mucosa respiratoria i gastrointestinal [36], mentre que el receptor del IFNs de tipus I s'expressen d'una forma més ubiqua. Per tant, es creu que la immunitat per IFNs de tipus III regeix la immunitat antiviral local, els defectes de la qual podrien ser menys nocius que els defectes de la immunitat antiviral sistèmica desencadenats pels IFN de tipus I [36].

La resposta immune enfront la infecció per SARS-CoV-2 és una arma de doble tall [37]. Els pacients amb una COVID-19 lleu presenten un pic de IFN-I robust i ràpid amb una disminució posterior, mentre que els pacients amb formes greus tenen uns nivells inicials d'IFN-I escassos i retardats que augmenten progressivament, especialment durant la segona setmana [38,39]. Aquest defecte primerenc d'IFN-I s'associa a una major càrrega viral i a una resposta inflamatòria excessiva, mediada entre d'altres per la via del NFκB, i caracteritzada per un augment de la producció de TNF-α, IL-6, IL8, etc [40].

5.2 Immunitat específica

Tal com s'ha comentat en els apartats anteriors, la resposta immune innata té el propòsit de restringir la replicació viral a les cèl·lules infectades i reclutar cèl·lules efectores del sistema immunitari innat, per crear un estat antiviral a l'entorn dels

teixits afectats. No obstant, la resposta immune innata no només permet defensar-nos ràpidament del patogen, sinó que també determina i orquestra un tipus de resposta immunitària adaptativa específica enfront l'antigen [27]. D'aquesta manera, els IFNs de tipus I i III poden influir positivament en la immunitat cel·lular i humoral.

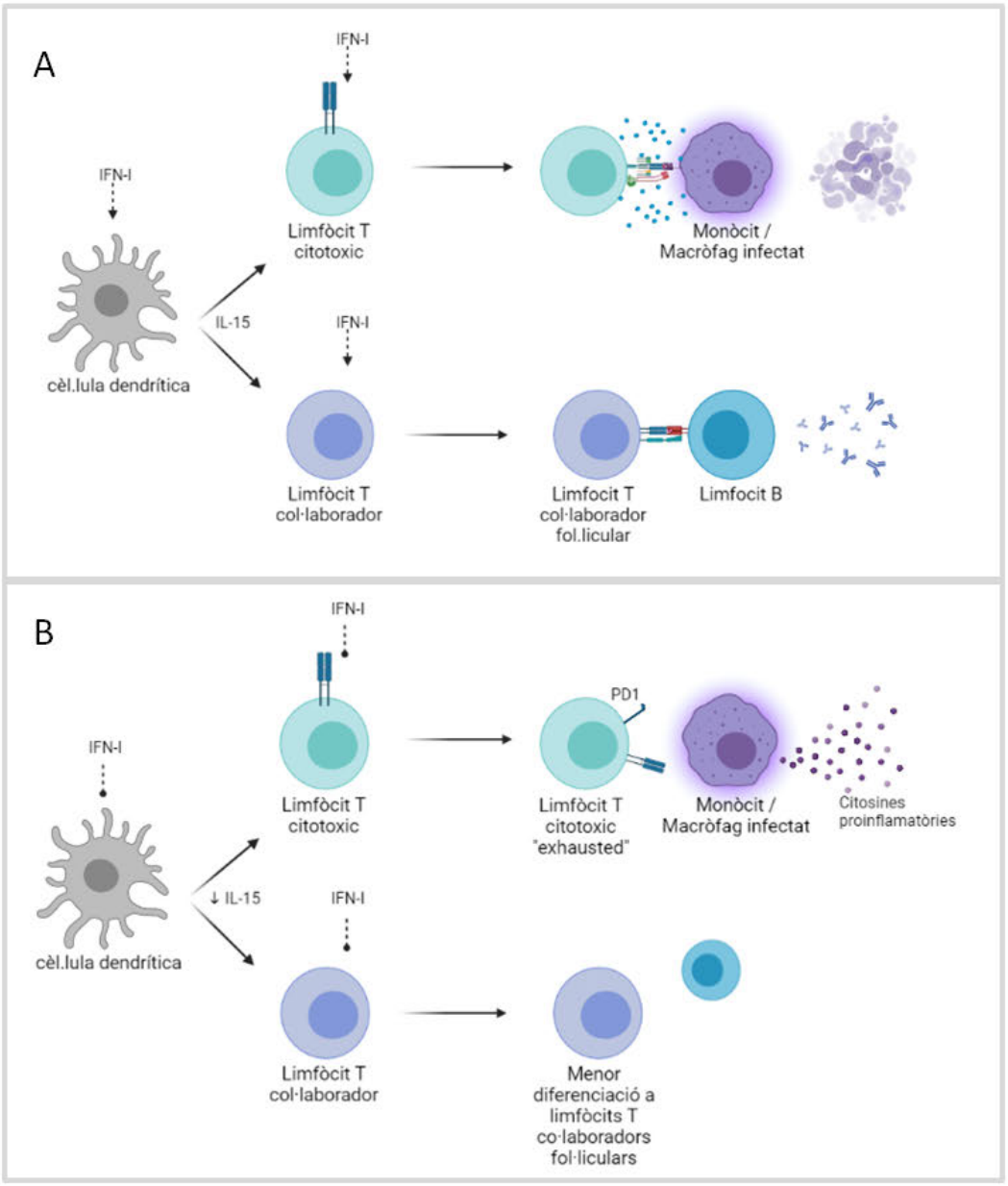


Figura 3. Influencia dels interferons de tipus I en la resposta immune específica (adaptat de King C et al. Trends Immunol, 2021 [32]). A) Diferenciació y proliferació correcta dels limfòcits T i B promoguda per una resposta d'IFN-I adequada. B) Resposta específica T i B defectuosa que és incapaç d'eliminar la infecció

Els IFNs de tipus I i III indueixen l'expressió d'HLA-II i molècules coestimuladores, així com la síntesi d'IL-15 per cèl·lules presentadores d'antígens (CPA). L'activació de les CPA, com ara les CD, indueix la proliferació i diferenciació de cèl·lules T i NK, i en millora la capacitat de secreció d'IFN-γ. Addicionalment, els IFN de tipus I i III actuen directament induint l'expansió i diferenciació dels limfòcits TCD8+ i TCD4+ (inclosos els limfòcits T col·laboradors fol·liculars) **(Figura 3A)**. La resposta immunitària adaptativa és lenta al ser necessari seleccionar i expandir les cèl·lules específiques enfront al virus. Tanmateix, un cop aquestes cèl·lules han proliferat i s'han diferenciades en les poblacions T i B efectores, treballen conjuntament per netejar ràpida i específicament les cèl·lules infectades i virions [32,39].

Malauradament, si la producció d'IFN-I s'altera en fases inicials de la infecció genera una activació defectuosa de limfòcits T i B, amb un nombre reduït de limfòcits T citotòxics i una producció reduïda d'anticossos d'alta afinitat, respectivament [32] **(Figura 3B)**. Com a conseqüència, es produeix una eliminació més lenta del virus i de les cèl·lules infectades, que porta a una activació continua del sistema immune que és perjudicial per l'hoste [39].

La reactivitat creuada amb altres betacoronovirus, causants de refredats comuns, podria modular la resposta immunitària de l'hoste [41,42] però no sembla ser suficient per conferir una resposta immunitària protectora. Així doncs, el sistema immunitari innat ha jugat un paper clau per defensar-nos enfront aquesta nova infecció. Amb l'increment del percentatge de població immunitzada, a través de la infecció natural o bé la vacunació, el sistema immune adaptatiu té un rol cada cop més rellevant.

5.3 Tempesta de citosines

La deficient resposta immune innata primerenca, i la subsegüent activació defectuosa de la immunitat adaptativa porta a una eliminació més lenta del virus i de les cèl·lules infectades. És plausible que el sistema immunitari innat intenti superar aquesta situació incrementant la seva funció i produint de forma continuada mediadors inflamatoris amb un desbalanç a favor de vies com NF-kB

(TNF-α, IL-6...) [32]. Com a conseqüència es genera una activació i reclutament excessius de macròfags i neutròfils en els teixits afectats, que es converteixen en una font addicional de citosines proinflamatòries (IL-1β, TNF-α, IL-6, IL-8, IL-12, IL-18, IL-10, MCP-1, IP-10, ROS i diversos DAMPs), la qual cosa autoamplifica la cascada inflamatòria [43]. Aquesta situació es produeix a partir de la segona setmana des de l'inici dels símptomes i condueix a dany tissular, especialment en els pulmons, que pot progressar a SDRA **(Figura 4)** i al fracàs multiorgànic [32,43,44].

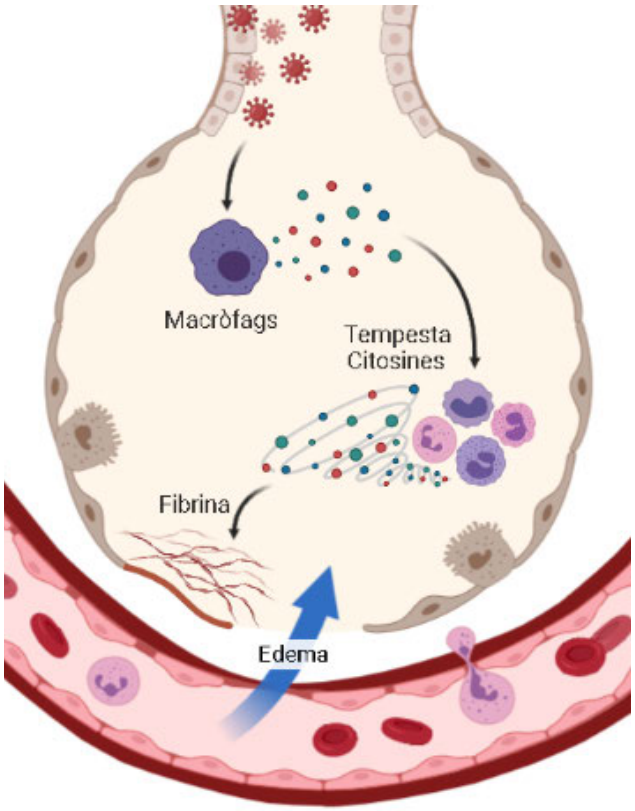


Figura 4. Síndrome de destret respiratori agut en la COVID-19. [Adaptat de Pillalamari et al. Transl Oncol. 2021 [44]). La continua i desbalancejada resposta inflamatòria provoca l'augment de la permeabilitat dels capil·lars (edema) i causa fibrosi a la membrana epitelial danyada.

A la **Figura 5** es pot observar de forma esquemàtica que el moment i la intensitat en que es produeixen els IFNs de tipus I i III és crucial per a que es desenvolupi una resposta immune innata i específica protectora enfront SARS-CoV-2 [39].

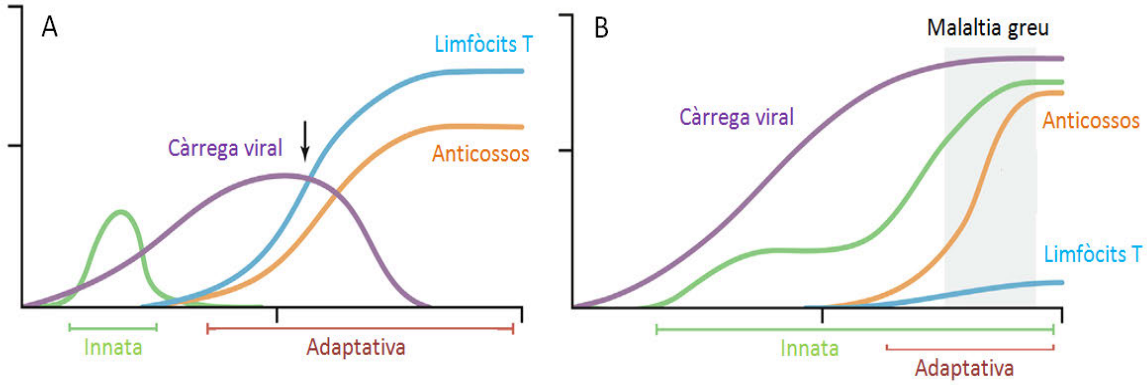


Figura 5. Model que integra la cinètica de la resposta immune amb la gravetat de la infecció per SARS-CoV-2 (adaptat de Sette et al, Cell, 2021 [39]). (A) Infecció per SARS-CoV-2 lleu/moderada. (B) Infecció per SARS-CoV-2 greu. Els efectes positius dels IFN-I/III sobre la resposta immune es produeixen fonamentalment durant les etapes inicials de la resposta immune. Durant aquestes etapes, la producció ràpida i robusta d'IFN-I/III afavoreix la diferenciació eficient del sistema immune adaptatiu, que contribueix a l'eliminació del virus. Tanmateix, quan la síntesi d'IFN-I/III és escassa i/o retardada, la resposta immunitària esdevé proinflamatòria, amb disfunció dels limfòcits T i B.

5.4 Interferència de SARS-CoV-2 en la resposta d'interferons

Els CoV tenen una taxa de mutació elevada que dóna lloc a una considerable diversitat genètica viral, cosa que implica una elevada plasticitat i adaptabilitat per infectar al seu hoste [16]. Després de múltiples replicacions en hostes vius, els CoV han experimentat modificacions que els han permès adaptar-se millor i sobreviure. SARS-CoV-2 té la capacitat d'eludir la inducció de IFNs mitjançant la inhibició de processos cel·lulars generals (p.e. transcripció, traducció). Tanmateix, certs components de SARS-CoV-2 (Nsp1, Nsp5, Nsp6, Nsp13, Nsp14, Nsp15, ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF9b, M i N) **(Figura 1)** actuen específicament sobre proteïnes clau per en la inducció de IFN de tipus I/III (IRF3, TBK1, MAVS, RIG-I i NEMO) o l'autoamplificació (IFNAR1, STAT1, STAT2 i TYK2) **(Figura 6)** [10,14,34,43]. SARS-CoV-2 inhibeix més selectivament la senyalització IFN-I que la senyalització NF-κB, fet que ajuda a entendre els mecanismes patogènics particulars d'aquesta infecció [32].

Tot i no haver-hi tanta evidència, s'ha observat que en la infecció per SARS-CoV-2 es produiria també una supressió de la sintesi d'IFN-ɣ (IFN de tipus II)

pels limfòcits T [45], i que posteriorment, aquesta molècula s'elevaria com a conseqüència de la hiperinflamació i l'intensa activació macrofàgica [43].

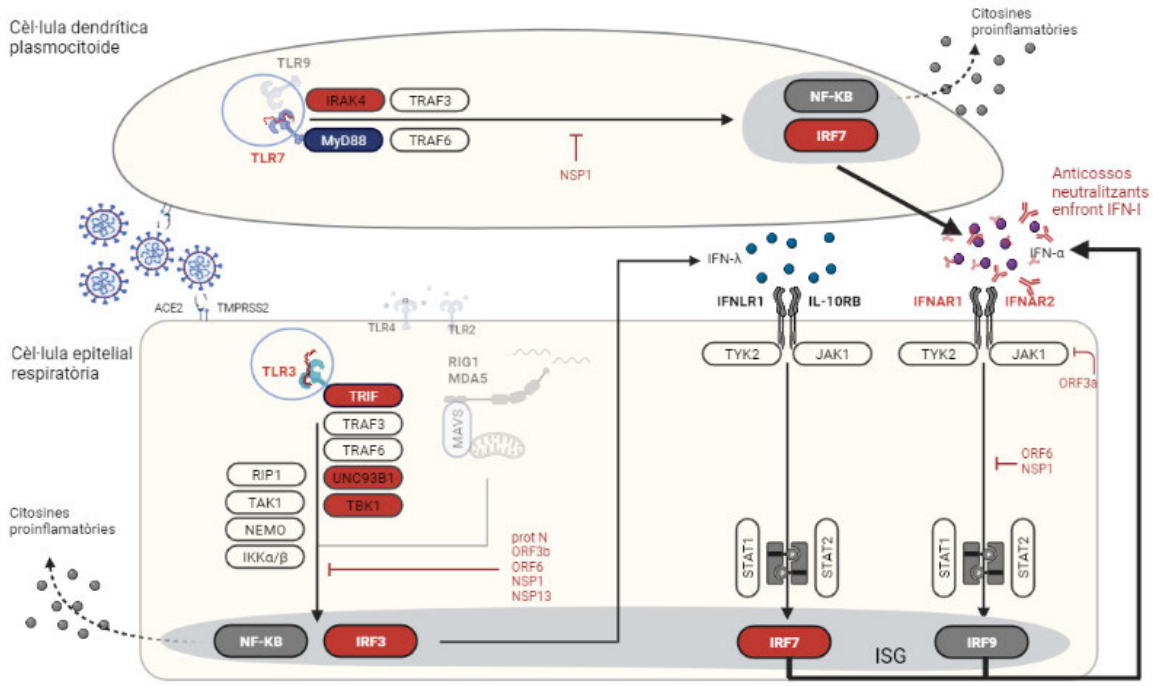


Figura 6. Factors genètics de l'hoste, fenocòpies i molècules virals que interfereixen en la inducció y senyalització de IFNs. Adaptat de Schultze et al. Cell 2021 [34]). S'han identificat diverses variants genètiques de pèrdua de funció o hipomòrfiques (representades en vermell) en components dels sistema immune que reconeixen SARS-CoV-2, així com en components de senyalització importants per a una resposta d'IFNs de tipus I ben orquestrada i robusta. També es representa la fenocòpia (autoanticossos neutralitzants enfront a IFN-I) d'aquestes deficiències innates de la via dels IFN-I. Es pot observar com certs components virals (ORF, NSP) de SARS-CoV-2 poden interferir també la senyalització d'aquesta via. En gris clar s'exposen aquells PRR que no han estat clarament implicats en el reconeixement de SARS-CoV-2. Abreviatures: NSP, non-structural proteins; ORF, open reading frames; la resta d'abreviatures són les mateixes que a la figura 2.

6. Factors de l'hoste relacionats amb una COVID-19 greu

6.1 Demografia i comorbilitats

L'edat avançada és el factor de risc que més intensament es relaciona amb la gravetat de la COVID-19: la mortalitat del 0,002% observada en menors de 10 anys pot arribar a ser de fins al 15% entre els majors de 85 anys [46]. Altres factors demografics i comorbilitats (gènere masculí, obesitat, diabetis, hipertensió...) s'associen a una major gravetat però amb una intensitat més

modesta [47]. Aquestes condicions no permeten predir amb exactitud quins pacients infectats per SARS-CoV-2 tenen un major risc de progressar als estadis més greus de la COVID-19, ni permeten explicar per què la malaltia crítica es desenvolupa en alguns individus joves i aparentment sans.

6.2 Genètica de l'hoste

Tal com succeeix amb altres malalties infeccioses, la genètica de l'hoste pot jugar un paper determinant en la susceptibilitat a patir certes infeccions [48]. Des de l'inici de l'actual pandèmia diversos estudis han identificat variants humanes, haplotips i polimorfismes gènics (ultra rars, rars i comuns) de susceptibilitat a patir una COVID-19 greu, mitjançant aproximacions com *genome-wide association studies* (GWAS), l'anàlisi de genomes, exomes o gens candidats [49]. En general, aquests estudis han evidenciat que la majoria d'aquests al·lels de susceptibilitat condicionen riscos baixos (odds ratio <2), i per tant jugarien un paper limitat com a marcadors genòmics predictius. Tanmateix, al·lels d'alta penetrància en gens que codifiquen per a proteïnes crucials per defensar-nos enfront SARS-CoV-2, podrien ser potencials marcadors pronòstics i servir de guia a l'hora d'administrar certs tractaments farmacològics específics [50]. Les principals variants genètiques i haplotips de susceptibilitat reportats prèviament a la publicació del nostre treball sobre genètica de l'hoste (Juliol de 2021) es troben recollits a l' **annex 1**.

Com a gens candidats cal pensar abans de tot en aquells que codifiquen per a proteïnes implicades en mecanismes de la immunitat innata o adaptativa. En aquest sentit, un consorci internacional anomenat COVID Human Genetic Effort (COVIDHGE), en que investigadors de l'HUB-IDIBELL hi estem col·laborant, va seqüenciar mitjançant exoma complet (WES) o genoma complet (WGS) a 659 pacients amb pneumònia greu per COVID-19 i a 534 individus amb infecció asimptomàtica o lleu. Es va demostrar que fins a un 3,5% dels pacients amb una pneumònia per COVID-19 que havia posat en perill la seva vida eren portadors de defectes genètics en la via d'inducció i senyalització d'IFNs de tipus I depenent de TLR3 i IRF7 [51]. Es varen detectar variants a *IRF7* i *IFNAR1* que

comportaven defectes autosòmics recessius en 4 pacients, i també variants autosòmiques dominants a *TLR3, UNC93B1, TICAM1, TBK1, IRF3, IRF7, IFNAR1* i *IFNAR2* en uns altres 19 pacients [51] **(Figura 6).** Els portadors de les variants tenien una edat compresa entre els 17 i els 77 anys (mitjana 48 anys).

6.2.1 Paper de TLR7 en la COVID-19

TLR7 es un receptor de virus ssRNA, codificat per un gen situat al cromosoma X (Xp22.2). El gen *TLR7* té aproximadament 23 kb i conté 3 exons. Una petita part de la regió codificant es troba a l'exó 2 i tota la resta a l'exó 3 [52]. TLR7 està composat per 1.036 aminoàcids que conformen l'estructura típica d'aquesta família de proteïnes (LRR, domini transmembrana, i un domini TIR) (**Figura 7**) [53]. S'expressa fortament en CDp, monòcits - macròfags i limfòcits B. A nivell de teixits *TLR7* s'ha detectat en pulmons, cervell, melsa, intestí prim i estómac.

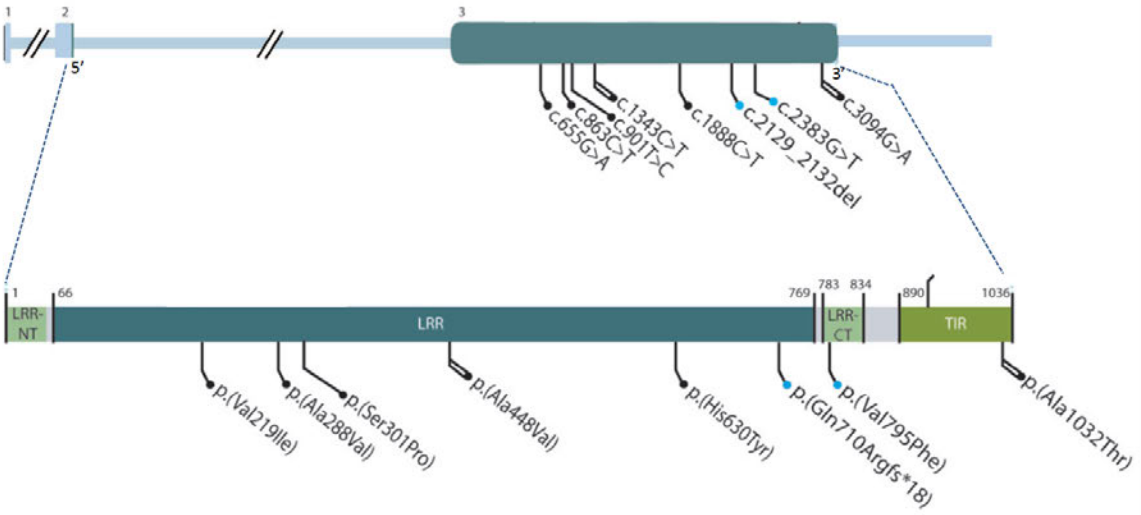


Figura 7. Representació esquemàtica de les variants a *TLR7* (HGNC ID: 15631) en pacients amb COVID-19 greu identificades abans de la publicació dels nostres resultats (Font: van der Made [54] i Fallerini [55]). Situació i nomenclatura de les variants en cDNA (part superior) i en la proteïna (part inferior). Codi de colors: cercles blaus, variants publicades a van der Made [54]; cercles negres, variants publicades a Fallerini [55]. Codi de línia: únic, comunicat en un cas; doble, reportat en 2 casos.

Abans de la pandèmia provocada per SARS-CoV-2 no s'havia descrit la predisposició a infeccions com a conseqüència d'una deficiència de TLR7. L'agost del 2020 es va descriure la relació entre variants de pèrdua de funció al gen *TLR7* i el desenvolupament d'una COVID-19 greu [54], la qual cosa

demostrava per primera vegada la importància que juga TLR7 en la patogènia de la COVID-19. Mitjançant seqüenciació d'exomes complets es va identificar una delecció de 4 nucleòtids (c.2129_2132del; p.(Gln710Argfs*18)) i una variant *missense* (c.2383G>T; p.(Val795Phe)) en dues parelles de germans joves (<35 anys) i aparentment sans procedents de dues famílies diferents que van desenvolupar una COVID-19 crítica. Van analitzar l'expressió de mRNA (IRF3, IRF7, ISG15, i IFNB1) i la producció d'IFN-γ estimulant les cèl·lules mononucleades de sang perifèrica (PBMC) dels casos i de controls sans amb imiquimod (agonista del receptor TLR7); aquesta expressió restava conservada en els controls sans mentre que hi havia una resposta d'IFNs alterada en els portadors de les variants. Aquests resultats es van replicar a Itàlia pel *GEN-COVID Multicenter Study* on es varen analitzar 79 homes amb COVID-19 greu vs. 77 controls amb infecció lleu. En aquest estudi el 2,1% dels homes menors de seixanta anys amb COVID-19 greu eren portadors de variants patogèniques a *TLR7*, mentre que aquestes variants no es trobaven en cap dels casos lleus o asimptomàtics. Es va demostrar que aquestes variants disminuïen la transcripció de gens relacionats amb IFNs de tipus I i II en les PBMC dels casos que van ser tractades amb imiquimod, cosa que suggeria que les variants generaven un efecte de pèrdua de funció o hipomòrfic [55]. En la **Figura 7** es representen esquemàticament les variants a *TLR7* en els casos de COVID-19 greus reportades per aquestes dues sèries.

6.3 Biomarcadors hematològics, serològics i immunològics.

S'han descrit múltiples biomarcadors per predir la gravetat de la COVID-19 mitjançant anàlisi de rutina bàsica d'hemograma, bioquímica i coagulació [43]. Alguns d'ells son un recompte elevat de glòbuls blancs o una disminució del nombre de limfòcits i de plaquetes. Els biomarcadors d'inflamació (proteïna C reactiva, proteïna sèrica amiloide A, ferritina), de lesió cardíaca i muscular, de funció hepàtica i renal, així com algunes proves de coagulació també es troben més elevats en pacients amb COVID-19 greu i fatal. Els biomarcadors immunològics són particularment importants. Diverses citosines i altres

paràmetres immunològics s'han correlacionat amb la gravetat de la COVID-19 **(Taula 1)** [56].

Biomarcador hematològic i serològic	Biomarcador immunològic
Limfopènia	Descens de CD4+, CD8+, NK
Neutrofília	Increment de l'expressió de PD-1 i Tim-3
Elevació del D-dimer	Certs fenotípics monocitaris (CD11b+, CD14+, CD16+, CD68+, CD80+, CD163+, and CD206+
suPAR	Increment de certes citosines (IL-1b, IL1-RA, IL-6, IP-10, MCP-3, IL-8, IL-10, IL-2R, IL-4, IL-18)
Trombocitopènia	Increment de GM-CSF
Elevació de LDH	
Elevació de PCR	
Elevació de SAA	
Elevació de NT-proBNP	

Taula 1. Principals biomarcadors hematològics, serològics i immunològics que s'han associat a una major gravetat de la COVID-19. Abreviatures: CD, *cluster of differentiation*; GM-CSF, *granulocyte-macrophage colony-stimulating factor*; IL, interleusina; IP-10, *Interferon gamma-induced protein 10*; LDH, lactat deshidrogenasa; MCP-3, *monocyte-chemotactic protein 3*; NK, *Natural Killer*; NT-proBNP, propèptid natriurètic cerebral N-terminal; PCR = proteïna C reactiva; SAA, proteïna sèrica amiloide A; PD-1, *programmed cell death protein 1*; suPAR, *soluble urokinase-type plasminogen activator receptor*; Tim-3, *T cell immunoglobulin and mucin domain-containing protein 3*.

6.3.1 Autoanticossos enfront IFNs de tipus I

Hi ha errors de la immunitat innata que alteren específicament la producció de IFNs de tipus II (IFN-γ), IL-17A/F o factor estimulador de colònies de granulòcits-macròfags (GM-CSF), i que predisposen a patir específicament infeccions per micobacteris, candidiasi mucocutània i nocardiosi, respectivament. En els darrers 20 anys s'han descrit diverses fenocòpies autoimmunes d'aquests errors de la immunitat en pacients amb autoanticossos neutralitzants (NautoAbs) enfront IFN-γ, IL-17A/F i GM-CSF [57]. Arrel de la infecció per SARS-CoV-2 s'ha observat que la fenocòpia autoimmune dels errors innats de la via dels IFNs de tipus I podria contribuir al desenvolupament d'una COVID-19 crítica **(Figura 6)**. Aquests NautoAbs es coneixen des de fa uns 40 anys però només s'havia descrit un únic cas d'un pacient a priori sa amb una infecció greu pel virus de la varicel·la zòster [58].

El consorci internacional COVIDHGE ha publicat varies investigacions on es relaciona la gravetat de la COVID-10 amb la presència dels NautoAbs enfront IFN-I. En el seu primer article es va reportar que 101 de 987 pacients (10,2%) amb pneumònia per COVID-19 que posava en perill la vida tenien autoanticossos que neutralitzaven altes concentracions d'IFN-I (10 ng/mL de IFN-α2, IFN-ω o ambdós); en canvi, aquests NautoAbs no es varen detectar en cap dels 663 individus amb infecció lleu o asimptomàtica per SARS-CoV-2. Tots els pacients amb aquests NautoAbs tenien valors d'IFN-α en el límit baix o indetectable durant la malaltia aguda [59]. Aquestes troballes indiquen que aquests NautoAbs podrien permetre identificar a persones amb un risc elevat de desenvolupar COVID-19 greu [51]. Estudis similars han corroborat aquests resultats arreu del mon [60-63]. Posteriorment, la COVIDHGE va mesurar NautoAbs enfront concentracions més fisiològiques d'IFN-I (100pg/ml en plasma diluït 1/10) en 3595 malalts amb COVID-19 crítica i el percentatge de positivitat es va elevar al 13.6%. També s'incrementa la probabilitat de detectar aquests NautoAbs a mesura que augmenta la gravetat (20% en èxitus), i l'edat (20% en majors de 80 anys). A més, el 1,3% dels pacients amb COVID-19 crític i el 0,9% dels pacients èxitus tenen NautoAbs enfront concentracions elevades de IFN-β. Els NautoAbs enfront IFN-β no són més freqüents a major edat [64]. La gran majoria dels pacients amb NautoAbs enfront IFN-I són homes (94%), fet que podria contribuir a explicar la major gravetat de la COVID-19 en aquest col·lectiu [51,59]. Aquests NautoAbs també es troben presents en un alt percentatge de pacients afectes de patologies que alteren la tolerància immunològica com la síndrome poliendocrina autoimmune tipus 1 (APS-1) [65,66], la immunodeficiència combinada per mutacions hipomòrfiques de RAG1 o RAG2 [67], la immunodisregulació, poliendocrinopatia i enteropatia lligada a X (IPEX) [68], o el timoma amb hipogammaglobulinèmia [69]. També estan presents en un percentatge elevat de dones amb *incontinentia pigmenti* [59], en pacients amb lupus eritematós sistèmic [70,71], miastènia gravis [72], i en pacients tractats amb IFN-α o IFN-β [73,74].

En una mostra de 34.159 subjectes no infectats de la població general, es varen detectar NautoAbs enfront de concentracions elevades d'IFN-α2 i/o -ω en el 0,18% dels individus entre 18 i 69 anys, el 1,1% dels de 70 a 79 anys, i en el 3,4% dels de més de 80 anys. En 10.778 d'aquests individus es varen determinar NautoAbs enfront de concentracions més fisiològiques de IFNs, que eren presents en el 1% dels individus de menys de 70 anys, el 2,3% dels de 70 a 80 anys i en el 6,3% dels de més de 80 anys [64]. Aquestes troballes suggereix que els NautoAbs son anteriors a la infecció per SARS-CoV-2 i que podrien contribuir a causar una COVID-19 crítica, més que no pas a ser generats per l'infecció. Addicionalment, aquesta distribució podria contribuir a explicar la major gravetat de la COVID-19 en la població de més edat.

Estudis de *single-cell RNA sequencing* (scRNAseq) de frotis nasofaringis mostren que els pacients amb COVID-19 lleu o moderat tenen marques d'IFNs intenses, mentre que els pacients amb COVID-19 crític tenien respostes més apagades [75]. Els NautoAbs s'han detectat també en frotis nasofaringis i en aspirats traqueals, suggerint que podrien interferir també en la immunitat del tracte respiratori superior. En aquest sentit, s'ha observat que la signatura de IFN-I a nivell nasals es feble en pacients amb NautoAbs enfront IFN-I. Per tant, els NautoAbs enfront a IFN-I podrien comprometre no només la immunitat antiviral sistèmica mediada per IFN-I sinó també la immunitat antiviral local en les primeres etapes de la infecció per SARS-CoV-2 [76].

La detecció d'aquests NautoAbs és tècnicament senzilla i econòmica, així que podria ser un bon marcador de gravetat de la COVID-19 en la pràctica clínica habitual. La determinació d'aquests NautoAbs en col·lectius de risc (ancians o patologies associades), o tan aviat com es fa el diagnòstic d'infecció per SARS-CoV-2 podria permetre identificar una proporció rellevant de persones amb risc de desenvolupar una COVID-19 greu. Aquest fet podria conduir a la instauració de mesures de prevenció i tractaments específics en els pacients amb NautoAbs. En aquest sentit, cal avaluar l'eficàcia de l'IFN-β recombinant en fases primerenques de la infecció, sempre i quan el pacient no tingui NautoAbs enfront a IFN-β [77]. Tanmateix, encara es disposa de poca informació sobre la utilitat de

determinar aquests NautoAbs quan els pacients ja han desenvolupat una forma greu de la malaltia. Tampoc sabem si el tractament de rescat amb recanvis plasmàtics o intensificant els tractaments immunosupressors pot ser una bona opció terapèutica en pacients amb aquests NautoAbs i que es trobin en estadis més avançats de la malaltia [78].

6.3.2 Assaig QuantiFERON-TB Gold Plus

Considerant el paper clau que juga la resposta antiviral mediada pels IFNs, seria molt útil a nivell assistencial disposar d'eines que permetin avaluar si un individu presenta una resposta d'IFNs correcta. L'assaig QuantiFERON-TB Gold Plus (QFT-Plus; Qiagen, Alemanya) [79], es basa en la detecció IFN-γ alliberat per les cèl·lules T després de l'estimulació *in vitro* de sang completa humana amb antígens específics de *Mycobacterium tuberculosis complex* [79]. L'assaig QFT-Plus consta de quatre tubs: 1) un control negatiu (nul), constituït per un tub sense additius i que s'utilitza per determinar si el pacient té un trastorn immunitari preexistent que pugui provocar una lectura falsament positiva; 2) un control positiu basat en mitògens (fitohemaglutinina), dissenyat per provocar una resposta de cèl·lules T inespecífica i així determinar si la resposta immune cel·lular dels individus estudiats és correcta; 3) el tub antigen TB1, que conté els antígens peptídics ESAT-6 i CFP-10 per detectar principalment la resposta de les cèl·lules T CD4+; i 4) el tub antigen TB2, que conté pèptids addicionals més curts d'ESAT-6 i CFP-10 per detectar les respostes de les cèl·lules T CD4+ i CD8+. Com es mostra a la **taula 2**, els resultats de l'assaig QFT-Plus s'informen qualitativament com a positius, negatius o indeterminats.

Les malalties inflamatòries (p.e. malaltia inflamatòria intestinal, artritis reumatoide) en fase activa o l'ús de fàrmacs immunosupressors poden ser la causa de resultats indeterminats del QFT-Plus [79,80]. A més, tal com s'ha comentat prèviament **(Figura 6)**, SARS-CoV-2 ha desenvolupat múltiples mecanismes que interfereixen en la producció de IFNs de l'hoste, i que podrien influir també en els resultats del QFT-Plus

<table>
<tr><th>Nul (UI/ml)</th><th>TB1 menys Nul (UI/ml)</th><th>TB2 menys Nul (UI/ml)</th><th>Mitogen menys Nul (UI/ml)</th><th>QFT-Plus Resultats</th></tr>
<tr><td rowspan="4">≤ 8.0</td><td>≥0.35 i
≥25% del Nul</td><td>Qualsevol</td><td rowspan="2">Qualsevol</td><td rowspan="2">Positiu</td></tr>
<tr><td>Qualsevol</td><td>≥0.35 i
≥25% del Nul</td></tr>
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<25% del Nul</td><td>≥0.50</td><td>Negatiu</td></tr>
<tr><td><0.35 o
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≥0.35 i
<25% del Nul</td><td><0.50</td><td rowspan="2">Indeterminat</td></tr>
<tr><td>>8.0</td><td colspan="3">Qualsevol</td></tr>
</table>

Taula 2. Interpretació dels resultats dels resultats de l'assaig QFT-Plus [79].

Quan s'utilitzen immunosupressors (corticoides, tocilizumab, anakinra...) per a tractar formes greus de COVID-19 es pot produir la reactivació d'infeccions com una infecció tuberculosa latent (LTBI). En aquest sentit, els assajos d'alliberament d'interferó gamma (IGRA), com el QFT-Plus, s'han utilitzat per al cribratge de LTBI en alguns pacients amb COVID-19 greu, abans o durant la teràpia immunosupressora.

Durant els primers mesos de la pandèmia de COVID-19 els microbiòlegs de l'HUB van observar un elevat nombre de resultats indeterminats del QFT-Plus en pacients hospitalitzats per COVID-19. En aquell moment no hi havia informació sobre aquesta troballa i avui en dia encara hi ha poca informació sobre la capacitat del propi COVID-19 o dels fàrmacs utilitzats per a tractar-la per afectar el rendiment del QFT-Plus [81]. Addicionalment, desconeixem si un resultat indeterminat del QFT-Plus pot estar relacionat amb la gravetat i mortalitat de la COVID-19.

7. Tractament

7.1 Tractament actual de la COVID-19

S'han descrit diverses etapes de la COVID-19 en funció de les seves diferents manifestacions clíniques. Les alteracions de les primeres etapes són degudes fonamentalment al propi virus, mentre que en fases més avançades són

secundàries principalment a una resposta inflamatòria inadequada de l'hoste [82]. En funció d'aquesta seqüència clínica temporal s'ha proposat l'administració de tractaments diferenciats **(Figura 8)**.

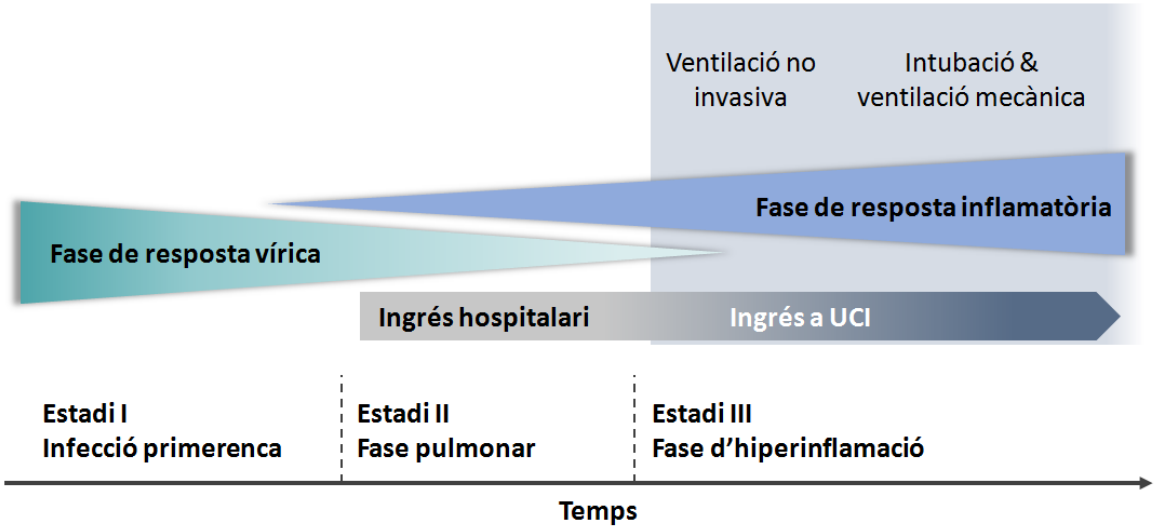

Figura 8. Representació esquemàtica de la història natural de la COVID-19 que s'ha classificat en tres estadis de gravetat creixent (adaptat deSiddiqi, J Heart Lung Transplant. 2020 [82]).

L'estadi I és la fase d'infecció primerenca, on predominen els símptomes de les vies respiratòries superiors. El tractament durant aquesta fase es basa en mesures de suport i el proveïment de tractament simptomàtic d'acord amb les manifestacions clíniques que presenti el pacient [83].

L'estadi II és la fase pulmonar, on el pacient desenvolupa pneumònia amb tots els seus símptomes associats. El remdesivir interfereix en la replicació viral en virtut de la inhibició de la RdRp. Aquest fàrmac ha demostrat reduir el temps fins assolir la millora clínica en el subgrup de pacients hospitalitzats amb hipòxia i amb un màxim de 7 dies des de l'inici dels símptomes [84]. Amb l'objectiu d'evitar l'entrada del virus a les cèl·lules humanes a través del receptor de l'ACE2 s'han desenvolupat anticossos IgG1 humans neutralitzants dirigits enfront la proteïna S del SARS-CoV-2. L'Agència del Medicament Europea (EMA) ha conclòs que la combinació de casirivimab i imdevimab, també coneguda com REGN-COV2, es pot utilitzar per al tractament de pacients amb COVID-19 que no necessiten oxigen suplementari i que tenen un risc elevat de progressar a

formes greus. També s'ha emès una conclusió similar per a la combinació de bamlanivimab amb etesevimab, sotrovimab, i regdanvimab [85].

L'etapa III és la fase d'hiperinflamació, la fase més greu, en que el pacient desenvolupa una SDRA, sèpsia i disfunció multiorgànica. El tractament durant aquesta fase té com a objectiu mitigar la resposta inflamatòria excessiva. Si bé s'ha descrit que alguns medicaments immunosupressors podrien ser nocius, s'ha suggerit paradoxalment que d'altres podrien ser beneficiosos per a tractar aquesta hiperinflamació [86,87]. Actualment sabem que per als pacients amb necessitat d'oxigen, l'ús de 6mg de dexametasona oral durant 10 dies pot reduir la mortalitat [88,89]. L'ús de bloquejadors del receptor de la IL-6 també ha demostrat ser capaç de disminuir la mortalitat en pacients amb hipòxia i inflamació sistèmica, sobretot en ús combinat amb corticosteroides [90,91]. Assajos clínics recents han mostrat resultats exel·lents amb agents antiinflamatoris com el baricitinib (inhibidor selectiu de la Janus kinasa 1/2) [92] o l'anakinra (inhibidor del receptor de la IL-1) [93].

7.2 Assaig clínic TACROVID

El març de 2020 els resultats dels principals assaigs clínics randomitzats (RCT) per a la COVID-19 [87] encara no s'havien publicat, i no es disposava de tractaments per a la COVID-19 més enllà de les teràpies de suport. Degut a la manca de tractaments basats en evidència un gran nombre de pacients van rebre teràpies en ús compassiu i no aprovades, basades en les seves propietats antivirals *in vitro* o immunomoduladores. El reposicionament de medicaments va ser l'estratègia inicial donat el seu perfil de seguretat comprovat [94]. Actualment encara és extremadament necessari realitzar RCT per proporcionar teràpies segures i efectives basades en l'evidència per al maneig de la COVID-19.

Donada la ràpida propagació de la COVID-19 i la manca de teràpies basades en l'evidència, el març del 2020 a l'HUB varem dissenyar un assaig clínic randomitzat, obert i unicèntric per avaluar l'eficàcia i la seguretat dels polsos de metilprednisolona i tacrolimus juntament amb l'*Standard of Care* (SoC), versus

SoC solament, en pacients amb pneumònia per COVID-19 i síndrome hiperinflamatori sistèmic.

El fonament d'aquest RCT es basava en el fet que els corticoides són el pilar del tractament de diversos trastorns immunomediats, amb múltiples mecanismes d'acció que involucren tant la immunitat innata com l'adaptativa. Pel que fa al tacrolimus, el seu ús es basa tant en les propietats antiinflamatòries com antivirals dels inhibidors de la calcineurina (CNI).

7.2.1 Bolus de metilprednisolona

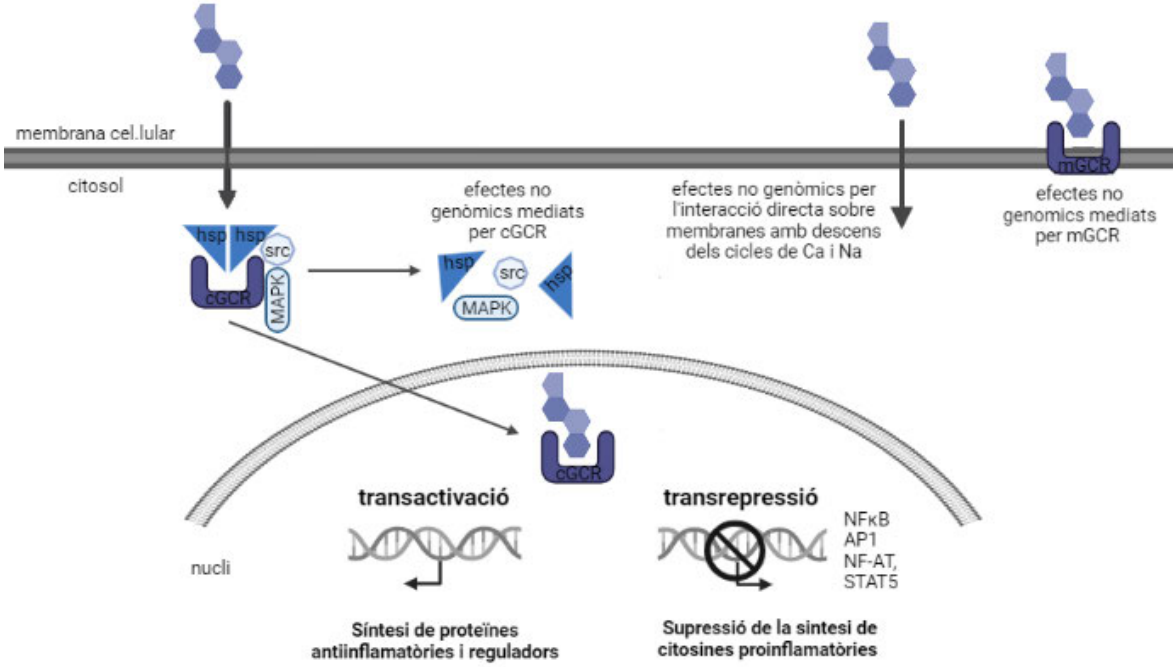

Figura 9. Efectes genòmics i no genòmics dels glucocorticoides (GC). (adaptat de Stahn et al, Nat Clin Pract Rheumatol, 2008 [96]). Els GC passen a través de la membrana citoplasmàtica per unir-se al receptor del GC citosòlic (cGCR). Aquest complex es desplaça cap al nucli on exerceix els seus efectes genòmics: a) transactivació, es a dir, l'expressió de proteïnes antiinflamatòries i reguladores com IL-10, annexin A1 o IκB; b) transrepressió, es a dir, la supressió de la síntesi de proteïnes proinflamatòries (IL-1, IL-2, IL-6, IL-8, VEGF, COX-2, prostaglandines, TNF, IFN-γ). Hi ha 3 grans grups d'efectes no genòmics: a) Per l'acció de les proteïnes que s'alliberen (hsp, MAPK, src) després de la unió dels GC amb el cGCR; b) A través de GCR lligat a la membrana (mGCR); i c) Per la interacció dels GC amb la membrana cel·lular que redueix el cicle de calci i sodi ràpidament. Abreviatures: AP1, *activator protein 1*; cGCR, *cytosolic glucocorticoid receptor*; COX-2, cyclooxygenase 2; hsp, heat shock proteins; IκB, inhibitor of NFκB; IFN-γ, interferon γ; IL, interleukin; MAPK, *mitogen activated protein kinase*; NF-AT, *nuclear factor of activated T cells*; NFκB, *nuclear factor κB*; src, *família de kinases src*; STAT5, *signal transducer and activator of transcription 5*; TNF, *tumor necrosis factor*; VEGF, *vascular endothelial growth factor*.

Els glucocorticoides exerceixen una gran varietat d'efectes antiinflamatoris sobre cèl·lules del sistema immune i cèl·lules d'altres òrgans. Els efectes dels glucocorticoides estan mediats per mecanismes genòmics i no genòmics (**Figura 9**). Les dosis baixes exerceixen els seus efectes a través de mecanismes genòmics. Les dosis altes de glucocorticoides (p.e. bolus) permeten obtenir efectes farmacològics addicionals als produïts per les dosis més baixes [95]. Entre aquests efectes afegits hi ha la inhibició de NF-kB per transrepressió o la ràpida interacció amb les membranes cel·lulars [96].

7.2.2 Tacrolimus

En condicions fisiològiques l'activació del receptor de la limfòcits T condueix a la fosforilació de la PLC que tot seguit genera IP3 i DAG. El IP3 permet l'alliberament de calci des del reticle endoplasmàtic cap al citosol. El complex que formen la calcineurina, el calci citosolic i la calmodulina se'n encarrega de la translocació de NFAT al nucli i la transcripció de citosines proinflamatòries (p.e. IL-2, TNF-α, IFN- ɣ...). Posteriorment, citosines com IL-2 permeten l'activació i proliferació de limfòcits T, cèl·lules claus en la generació de la resposta específica **(Figura 10).**

El CNI son un grup de immunosupressors d'ús habitual en el transplantament, i en processos immunològics on hi intervenen les cèl·lules T. Aquest grup farmacològic està format fonamentalment per la ciclosporina A (CsA) i el tacrolimus (TAC, també conegut com FK506). La CsA i el TAC s'uneixen a ciclofil·lina i FKBP12, respectivament, la qual cosa impedeix que aquestes immunofil·lines exerceixin la seva funció **(Figura 10)** [98]. Els complexos resultants de la unió del CNI i la immunofil·lina tenen la capacitat d'inhibir la calcineurina, de manera que no s'indueixen els gens que codifica NFAT, i no es produeixen les citosines necessàries (p.e. IL-2, TNF-α, IFN- ɣ...) per a l'activació i la proliferació dels limfòcits [86,99]. Tanmateix, els CNI poden suprimir també la senyalització de NF-kB, tant en els limfòcits [100] com en cèl·lules no immunes [101]. Les intenses accions antiinflamatòries d'aquests agents es poden atribuir doncs a l'acció binària sobre la funció de les cèl·lules del sistema immune i

també sobre les cèl·lules residents (locals) [101]. Addicionalment, els CoV provoquen una obertura aberrant del porus de transició de permeabilitat mitocondrial (mPTP), que provoca la mort cel·lular. Els complexos CNIs-immunofil·lina podrien evitar l'obertura de mPTP reduint el dany i la mort cel·lular [101,102,103].

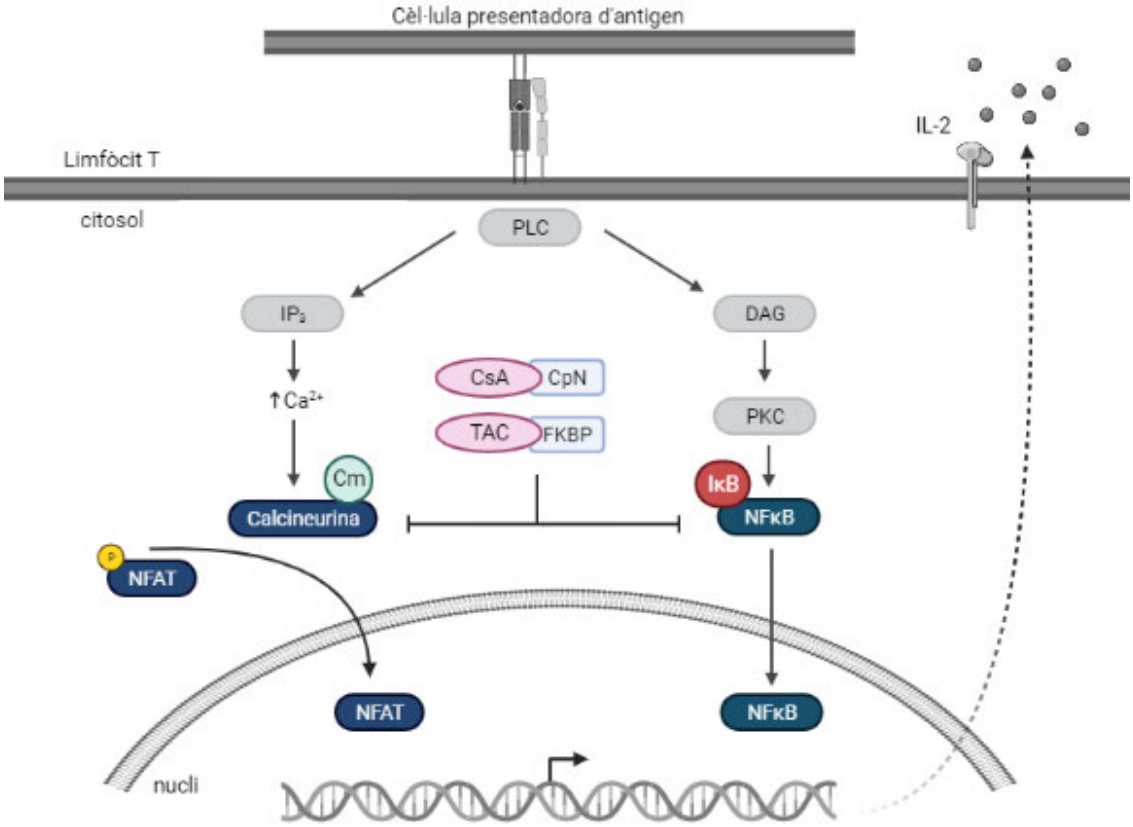

Figura 10. Efectes dels inhibidors de la calcineurina en importants vies de senyalització intracel·lular i sobre les immunofil·lines (Adaptat de Bremer et al [97]). Abreviatures: AP-1, *activator protein-1*; Ca, calci; Cm, calmodulina; CpN, ciclofilina; CsA, ciclosporina A; DAG, diacilglicerol; FKBP, *FK506-binding proteins*; HLA, complex major d'histocompatibilitat; IkB, inhibidor del NF-kB; IL, interleusina; IP_3, inositol trifosfat; JNK, *c-Jun N-terminal kinase*; MAP Kinase, *mitogen-activated protein kinase*; NFAT, *nuclear factor of activated T cells*; NF-kB, factor nuclear kB; PKC, *protein kinase C*; PLC, fosfolipasa C; TAC, tacrolimus; TCR, receptor de cèl·lula T.

Els CNI podrien ser útils per a tractar SARS-CoV-2 inhibint la seva replicació mitjançant un mecanisme que no està relacionat amb les seves propietats antiinflamatòries. Investigacions recents han demostrat que la replicació dels CoV depèn de l'activació de les immunofil·lines. En aquest sentit, concentracions

baixes de CNIs han estat capaces de inhibir *in vitro* la replicació de CoV altament patògens com SARS-CoV i MERS-CoV [98,104,105]. En el cas de la CsA també s'ha observat una millor evolució de la infecció en un model murí *in vivo* [106]. Concretament, es va observar que CsA induïa IRF1 i es produïa una pronunciada resposta de interferons de tipus III (IFNλ). CsA va reduir la replicació de MERS-CoV *in vivo*, correlacionant-se amb nivells elevats d'IFNλ pulmonar i amb una millor evolució clínica [106].

La malaltia greu per la COVID 19 presenta un perfil clínic i de citosines bastant similar al d'altres malalties (p.e. linfohistiocitosis hemofagocítica secundària) [107] on els CNI juguen un paper central en el seu tractament [103,108]. En base a les consideracions exposades prèviament s'han realitzat diversos estudis observacionals per avaluar el paper dels CNI en els pacients hospitalitzats per COVID-19 [109,110].

II. Hipòtesis

- Alguns homes joves i aparentment sans poden ser portadors de variants patogèniques al gen *TLR7* que els predisposa a desenvolupar una COVID-19 greu o crítica.
- Els pacients que requereixen ingrés per COVID-19 a les unitats de cures intensives poden tenir un percentatge elevat d'autoanticossos neutralitzants (NautoAbs) enfront a interferons de tipus I, que determina una evolució de la COVID-19 de pitjor pronòstic que la dels pacients amb NautoAbs negatius.
- Els resultats indeterminats de la prova QuantiFERON-TB Gold Plus poden tenir una prevalença elevada en pacients hospitalitzats per COVID-19, i estar relacionats amb factors clínics i analítics que condicionen una major mortalitat.
- L'ús de polsos de metilprednisolona juntament amb tacrolimus, afegits a l'Standard of Care, pot constituir una combinació farmacològica eficaç i segura per tractar pacients hospitalitzats per una COVID-19 greu.

III. Objectius

Objectiu general:

L'objectiu d'aquesta tesi doctoral es avaluar noves eines que permetin predir quins pacients infectats per SARS-CoV-2 evolucionaran als estadis més greus de la COVID-19. Addicionalment, pretenem avaluar la utilitat de tractaments immunosupressors per als individus que es troben en fases inflamatòries de la malaltia.

Objectius específics:

En base al que hem exposat anteriorment, proposem els següents objectius específics:

- Realitzar l'anàlisi mutacional del gen *TLR7* per identificar variants patogèniques en homes joves i aparentment sans que han desenvolupat una COVID-19 crítica
- Determinar la prevalença d'autoanticossos neutralitzants (NautoAbs) enfront a interferons de tipus I en els pacients amb COVID-19 ingressats a cures intensives, i analitzar si les característiques i evolució dels pacients amb NautoAbs positius és diferent a la dels pacients negatius.
- Determinar la prevalença i els factors associats a un resultat indeterminats de la prova QuantiFERON-TB Gold Plus en pacients hospitalitzats per la COVID-19, i analitzar la relació entre els resultats indeterminats i la mortalitat per COVID-19.
- Determinar l'eficàcia i la seguretat dels polsos de metilprednisolona juntament amb tacrolimus, afegits a l'*Standard of Care*, en pacients hospitalitzats per una COVID-19 greu.

IV. Resultats

ARTICLE 1

Objectiu

Realitzar l'anàlisi mutacional del gen TLR7 per identificar variants patogèniques en homes joves i aparentment sans que han desenvolupat una COVID-19 crítica.

Títol

Genetic Screening for TLR7 Variants in Young and Previously Healthy Men With Severe COVID-19.

Solanich X*,†, Vargas-Parra G*, van der Made CI, Simons A, Schuurs-Hoeijmakers J, Antolí A, Del Valle J, Rocamora-Blanch G, Setién F, Esteller M, van Reijmersdal SV, Riera-Mestre A, Sabater-Riera J, Capellá G, van de Veerdonk FL, van der Hoven B, Corbella X, Hoischen A†, Lázaro C†.

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Contribució del doctorand: revisió de la literatura, disseny, implementació, selecció i preparació de dades, anàlisi i presentació dels resultats, redacció i presentació de l'article.

Material suplementari

Aprovació CEIm

Resum

Introducció: Recentment s'han descrit en un petit nombre d'homes variants de pèrdua de funció a *TLR7* amb una forta predisposició a patir una COVID-19 molt greu. L'objectiu de l'estudi era determinar la presència d'aquestes variants rares en homes joves amb una COVID-19 crítica atesos a l'Hospital Universitari de Bellvitge (Barcelona) i al *Radboud University Medical Center* (Nijmegen).

Mètodes: Es van estudiar prospectivament homes de 18 a 50 anys sense comorbiditats associades a formes greus de COVID-19 i que requerissin almenys de dispositius d'oxigen nasal d'alt flux per a tractar la COVID-19. Es va seqüenciar la regió codificant de *TLR7* per avaluar la presència de variants potencialment patogèniques.

Resultats: Es varen identificar variants *missens* a *TLR7* en dos de 14 pacients (14,3%). L'edat mitjana del total de pacients estudiats era de 38 (IQR 30-45) anys. Ambdues variants no havien estat informades prèviament a les bases de dades de població control, i varen ser reportades com a perjudicials per anàlisi computacionals o *in silico*. En un home de 30 anys es va identificar una variant d'herència materna (c.644A>G; p.(Asn215Ser)), cosegregant al seu germà de 27 anys que també va patir una COVID-19 crítica. Es va trobar una segona variant (c.2797T>C; p.(Trp933Arg)) en un pacient de 28 anys, cosegregant en el seu germà de 24 anys que va desenvolupar COVID-19 lleu. Les proves funcionals d'aquesta última variant van revelar una disminució de les respostes d'interferons de tipus I i II en cèl·lules sanguínies mononuclears perifèriques després de l'estimulació amb un agonista de TLR7 anomenat imiquimod, confirmant un efecte de pèrdua de funció.

Conclusions: Aquest estudi contribueix a justificar el cribratge genètic de variants de *TLR7* en homes joves sense factors de risc associats a formes greus de COVID-19, que paradoxalment desenvolupen una COVID-19 crítica. Diagnosticar una deficiència de TLR7 no només permet tenir en compte noves opcions terapèutiques per al pacient, sinó que també permet determinar la presencia d'aquestes variants a familiars de gènere masculí abans que s'infectin per SARS-CoV-2, i així donar la possibilitat d'iniciar intervencions preventives i terapèutiques precoces.

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ORIGINAL RESEARCH
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Genetic Screening for *TLR7* Variants in Young and Previously Healthy Men With Severe COVID-19

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Introduction: Loss-of-function *TLR7* variants have been recently reported in a small number of males to underlie strong predisposition to severe COVID-19. We aimed to determine the presence of these rare variants in young men with severe COVID-19.

Methods: We prospectively studied males between 18 and 50 years-old without predisposing comorbidities that required at least high-flow nasal oxygen to treat COVID-19. The coding region of *TLR7* was sequenced to assess the presence of potentially deleterious variants.

Results: *TLR7* missense variants were identified in two out of 14 patients (14.3%). Overall, the median age was 38 (IQR 30-45) years. Both variants were not previously reported in population control databases and were predicted to be damaging by *in silico* predictors. In a 30-year-old patient a maternally inherited variant [c.644A>G; p.(Asn215Ser)] was identified, co-segregating in his 27-year-old brother who also contracted severe COVID-19. A second variant [c.2797T>C; p.(Trp933Arg)] was found in a 28-year-old patient, co-segregating in his 24-year-old brother who developed mild COVID-19. Functional testing of this variant revealed decreased type I and II interferon

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responses in peripheral mononuclear blood cells upon stimulation with the TLR7 agonist imiquimod, confirming a loss-of-function effect.

Conclusions: This study supports a rationale for the genetic screening for *TLR7* variants in young men with severe COVID-19 in the absence of other relevant risk factors. A diagnosis of TLR7 deficiency could not only inform on treatment options for the patient, but also enables pre-symptomatic testing of at-risk male relatives with the possibility of instituting early preventive and therapeutic interventions.

Keywords: COVID-19, SARS-CoV-2, host genetics, TLR7, immunodeficiency, genetic screening

INTRODUCTION

A proportion of patients with COVID-19 develop fatal lung injury and multi-organ failure due to systemic host-immune inflammatory processes triggered by the viral infection (1). Advanced age, male sex and chronic disease such as diabetes and obesity are common in patients with more severe forms of COVID-19 (2–4). However, these risk factors cannot explain why critical disease also occurs in young (below 50 years of age) and apparently healthy individuals.

In the past months, several publications have identified loci and genes associated with COVID-19 susceptibility for severe COVID-19 by using comprehensive GWAS studies or genome, exome or candidate gene analyses (5). However, most of these susceptibility alleles showed risk values too low (odds ratio <2) to be regarded as predictive genomic markers. Some of the loci reported include *ABO* blood group (6, 7), *ACE2* (8), *TMPRSS2* (8), several HLA alleles (5, 9), *APOE* (10), and *IFITM3* (11). On the other hand, rare variants in genes encoding for members of the type I/III interferon (IFN) pathway showed a higher estimated risk to severe COVID-19 (12, 13).

In July 2020 rare, deleterious germline variants in the X-chromosomal Toll-like receptor 7 (*TLR7)* gene were reported in young and, otherwise, healthy males with severe COVID-19. In these two brother pairs, rapid whole-exome sequencing identified both a maternally inherited 4-nucleotide deletion [c.2129_2132del; p.(Gln710Argfs*18)] and a missense variant [c.2383G>T; p.(Val795Phe)]. Both variants were associated with impaired type I and II IFN responses upon stimulation with the TLR7 agonist imiquimod, supporting the importance of intact TLR7 signaling in COVID-19 pathogenesis (13). Most recently these findings were replicated in an independent Italian cohort study of males <60 years of age with severe COVID-19 (79 severe cases versus 77 control cases), showing that 2.1% of severely affected males harbored deleterious *TLR7* variants compared to none of the asymptomatic participants. These variants were demonstrated to decrease transcription of type I and II IFN-related genes in patient peripheral blood mononuclear cells (PBMC) treated with imiquimod, supporting a loss of function effect (14).

In follow-up of the recent discovery of TLR7 deficiency in patients with severe COVID-19, we aimed to prospectively determine the presence of *TLR7* variants in young and previously healthy men with severe COVID-19.

METHODS

This is a joint study performed at the Hospital Universitari de Bellvitge – IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain; and the Radboud University Medical Center, Nijmegen, The Netherlands and the Erasmus Medical Center, Rotterdam, The Netherlands.

The Barcelona Cases

From March to July 2020, researchers from Hospital Universitari de Bellvitge - IDIBELL prospectively collected biological samples from young patients without comorbidities related to severe COVID-19. Selection criteria were: 1) patients aged between 18 and 50 years old; 2) absence of known comorbidities associated with most severe forms of COVID-19 (BMI ≥ 30kg/m2, diabetes mellitus, hypertension, chronic heart, pulmonary or kidney disease, or an immunocompromised state); and 3) SARS-CoV-2 related lung injury requiring high flow oxygen devices or mechanical ventilation. Ten male patients fulfilled these selection criteria (**Table 1**). Eight patients (patients 1-8) were also evaluated by the COVID Human Genetic Effort, although no pathogenic variants were identified in any of the 13 type I IFN pathway genes studied (12).

Informed consent was obtained from all patients and relatives, and the IDIBELL Research Ethics Committee approved this study (PR152/20). Demographic, epidemiological, laboratory and clinical data were collected. Treatments specifically used to treat COVID-19 at any time during admission were also documented.

DNA was isolated from total blood either using a Maxwell instrument RSC (Promega, Madison, WI, USA) or QIAGEN Flexigene DNA kit (Qiagen, Germany). Nine PCR primer pairs (Sigma-Aldrich, MO, USA) were designed to cover the whole coding region of *TLR7* (HGNC ID:15631). PCR was performed using DreamTaq MasterMix (ThermoFisher Scientific, Waltham, MA, USA), products were purified using EXO-SAP (New England Biolabs) and sequenced using the BigDye Terminator v.3.1 Sequencing Kit (Applied Biosystems, CA, USA) in an ABI Prism 3730 XL Genetic Analyzer (Applied Biosystems CA, USA). Primers and PCR conditions are available upon request. Mutation Surveyor software was used to detect variants and nomenclature was given according to HGVS guidelines. All variants identified were submitted to Alamut Software Suite v2.15.0 (Interactive Biosoftware) to retrieve population frequency and *in silico* prediction data.

TABLE 1 | Demographic and clinical findings of investigated patients.

Patient	Sequencing	Gender	Age (y)	Ethnicity	Comorbidities	ARDS[a]	ICU	ECMO
1	Sanger	M	31	Caucasian	no	yes	no	no
2	Sanger	M	44	Caucasian	no	yes	yes	no
3	Sanger	M	41	Latin (Venezuela)	no	yes	yes	yes
4	Sanger	M	40	Caucasian	no	yes	yes	no
5	Sanger	M	50	Latin (Peru)	no	yes	yes	no
6	Sanger	M	48	Caucasian	no	yes	yes	no
7	Sanger	M	45	Latin (Peru)	no	yes	yes	no
8	Sanger	M	31	Latin (Honduras)	no	yes	yes	no
9	Sanger	M	47	Latin (Peru)	no	yes	no	no
10[b]	Sanger	M	30	Latin (Dominican Republic)	no	yes	yes	no
11	Sanger	M	21	Caucasian	no	yes	yes	no
12	Sanger	M	36	Caucasian	no	yes	yes	no
13[b]	WES	M	28	Caucasian	no	yes	yes	no
14	WES	M	31	Caucasian	no	yes	no	no

ARDS, acute respiratory distress syndrome; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; F, female; M, male; WES, whole-exome sequencing; Y, years.
[a]*ARDS Definition Task Force. Acute Respiratory Distress Syndrome - The Berlin Definition. JAMA. 2012;307(23):2526-2533. doi:10.1001/jama.2012.5669.*
[b]*Patients 10 and 13 resulted carriers of TLR7 variants and belong to family 1 and 2, respectively.*

The Dutch Cases

At the Radboud University Medical Center in Nijmegen and the Erasmus Medical Center in Rotterdam, the Netherlands, patients were screened prospectively in a clinical setting from December 2020 to February 2021 with the following criteria: 1) males between 18 and 40 years of age; 2) absence of comorbidities known to be associated with severe COVID-19 (BMI ≥ 30kg/m2, diabetes mellitus, hypertension, chronic heart, pulmonary or kidney disease, or an immunocompromised state); and 3) PCR-confirmed SARS-CoV-2 infection requiring high-flow oxygen therapy or ICU admission. A total of 4 patients (patients 11-14) fulfilled these inclusion criteria and underwent clinical Sanger sequencing (patients 11 and 12) or rapid whole-exome sequencing (patient 13 and 14) to specifically assess genetic variants in *TLR7* (**Table 1**). Written informed consent was obtained from patient 13 whose clinical data has been included in this study. Rapid whole-exome sequencing was performed similar to previous reports (13). Sanger sequencing was done according to standard diagnostic procedures at the Department of Human Genetics, Radboud University Medical Center, protocols and primers sequences are available upon request.

For patient 13, in whom a rare *TLR7* missense variant was identified, functional testing was performed to evaluate impaired type I and II IFN responses due to *TLR7* loss-of-function, as described previously (13). In brief, venous blood was drawn and collected in EDTA tubes (Monject). Subsequently, peripheral mononuclear blood cells (PBMC) were isolated by density centrifugation of blood, diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and resuspended in cell culture medium (Roswell Park Memorial Institute [RPMI] 1640, Gibco) supplemented with gentamicin, 10 mg/mL; L-glutamine, 10 mM; and pyruvate, 10 mM. PBMC were then stimulated at 5×10^5 cells/well in round-bottom 96-wells plates (Greiner) for either 4 hours (for transcription of type I IFN genes) and 7 days [for production of the type II IFN, (IFNγ)] in the presence of 10% human pool serum at 37°C and 5% carbon dioxide, in two separate experiments. Apart from a negative medium (RPMI) control, the TLR7 agonist imiquimod (imidazoquinoline compound, Invivogen) was used at a concentration of 5 μg/mL. After the 4 hour incubation period, the supernatants were discarded and the remaining cell pellets were resuspended in RLT buffer (Qiagen) and snap frozen to be stored at −80°C until processing for RNA isolation. In addition, after the 7 day incubation and a centrifugation step, supernatants were collected and stored at −20°C until measured using enzyme-linked immunosorbent assay in case of the 7 day timepoint.

RESULTS

TLR7 Sequencing Results

A total of 14 patients were included, with a median age of 38 (IQR 30-45) years. Putative deleterious *TLR7* missense variants were identified in two patients (patient 10 and 13). Both variants, c.644A>G; p.(Asn215Ser) in patient 10 and c.2797T>C; p.(Trp933Arg) in patient 13, were not previously reported in our in-house databases nor in the population database gnomAD (15). The p.(Asn215Ser) variant affects a highly conserved nucleotide and amino acid in the *TLR7* leucine-rich region domain and is predicted damaging or possibly damaging by *in silico* software (**Table S1**). The p.(Trp933Arg) variant is also located at an evolutionarily highly conserved position within the TIR domain, important for downstream signaling *via* adapter proteins, and is considered deleterious by *in silico* effect predictors (**Table S1**). These *TLR7* variants and other previously reported variants in COVID-19 patients are shown schematically in **Figure 1**.

Patients' Characteristics

Patient 10 (family 1, proband) was a 30-year-old man from Latin origin (Dominican Republic) with no general risk factors predisposing to severe COVID-19. He developed pneumonia

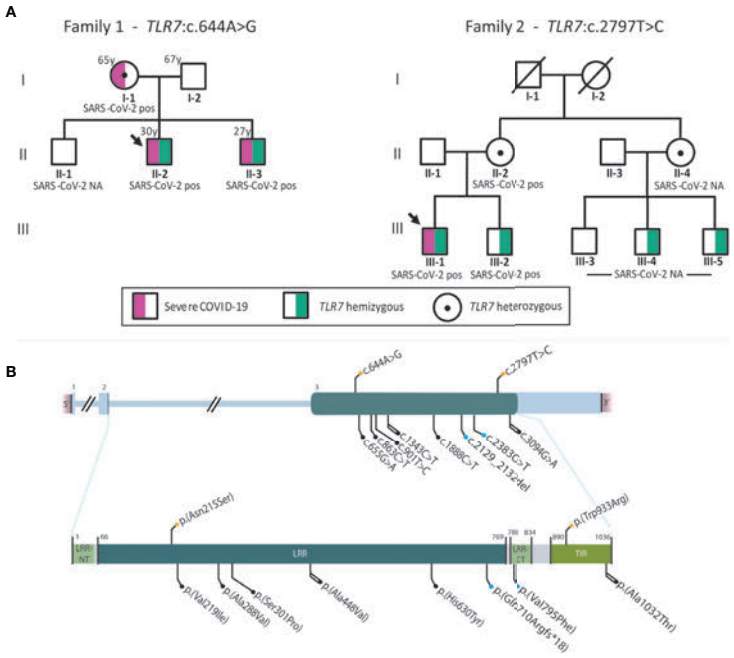

FIGURE 1 | **(A)** displays pedigrees of families 1 (patient 10) and 2 (patient 13) with segregation analysis. **(B)** shows schematic representation of *TLR7* (HGNC ID:15631) variants reported to date in severely affected COVID-19 cases. Variants in cDNA (top) and protein (bottom). Color code: orange, variants found in the present series; blue, previously reported in van der Made (13); black, previously reported in Fallerini (14). Shape code: circle, missense variants; square, frameshift variants. Line code: single, reported in one case; double, reported in 2 cases.

with bilateral consolidations on a computed tomography (CT) scan and fulfilled the criteria of acute respiratory distress syndrome (ARDS) secondary to PCR-proven COVID-19 (**Table 2**). The patient received antiviral treatment with remdesivir and immunosuppressive therapy with dexamethasone. Due to respiratory insufficiency, the patient was intubated and admitted in ICU. The patient was successfully extubated after 4 days of mechanical ventilation and was discharged from ICU after 6 days. After the identification of the *TLR7*:p.(Asn215Ser) variant in the patient, segregation analysis confirmed co-segregation in both his brother and mother, in hemi- and heterozygous state, respectively (**Figure 1**). The eldest brother lives in the Dominican Republic and was therefore not available for testing. According to relatives, there is no evidence that he has been infected with SARS-CoV-2. The youngest brother had no previous medical history but also contracted severe COVID-19, requiring mechanical ventilation and ICU admission at another hospital (**Table 2**). These brothers therefore represent the third brother pair with severe COVID-19, following the initial report (13). The 65 year-old mother suffered from obesity, dyslipidemia, type 2 diabetes and hypertension, and was also admitted in ICU due to critical respiratory failure caused by COVID-19. She was discharged from the ICU 16 days after admission. Main demographic, clinical, laboratory, and radiological findings of the three relatives are summarized in **Table 2**.

Patient 13 (family 2, proband) was a 28-year-old male from Caucasian origin (The Netherlands) without previous medical history or comorbidities. The patient complained of progressive dyspnea and shortness of breath. Following rapid clinical deterioration and respiratory insufficiency, the patient was intubated and hospitalized in ICU at a peripheral hospital. A CT-scan showed multiple diffuse ground-glass opacities and consolidations in all lung segments, meeting the criteria for ARDS. Treatment consisted of mechanical ventilation with prone positioning, intravenous dexamethasone, and the antibiotics ceftriaxone and ciprofloxacin. Despite this therapeutic regiment, the patient's condition further deteriorated and he was referred to the Erasmus Medical Center for possible ECMO treatment. However, with continuing prone positioning he gradually improved before ECMO was required. A repeated CT scan also showed subsegmental pulmonary embolisms for which intravenous heparin was started. In the following weeks, the patient gradually recovered and was successfully extubated and eventually discharged from the hospital. The patients' first-degree family contracted COVID-19 at the time the patient

TABLE 2 | Demographic, clinical, laboratory, and radiological findings of investigated patients.

	Family 1 *TLR7*:c.644A>G			Family 2 *TLR7*:c.2797T>C	Reference ranges
	Proband	**Brother**	**Mother**	**Proband**	
Demographic characteristics					
Date of hospitalization	July/2020	March/2020	August/2020	January/2021	
Age, y	30	27	65	28	
Sex	Male	Male	Female	Male	
Medical history	None	None	Obesity, dyslipidemia, hypertension, type 2 diabetes,	Vasovagal syncope	
Clinical characteristics at presentation					
Time from symptom onset to hospitalization, d	7	6	12	2	
Symptoms at disease onset	Dyspnea, cough, fever, myalgia	Dyspnea, cough, fever, headache	Dyspnea, cough, fever, myalgia	Dyspnea, cough, fever, respiratory arrest	
Imaging features (CT scan)	Bilateral pulmonary consolidations	Bilateral pulmonary consolidations	Bilateral pulmonary consolidations	Multiple ground glass opacities and consolidations in all lung segments	
ICU admission					
Time from symptom onset to ICU admission, d	8	7	12	7	
Medical reason for ICU admission	Respiratory insufficiency	Respiratory insufficiency	Respiratory insufficiency	Respiratory failure, respiratory arrest, resuscitation at home.	
Disease severity status on admission, SOFA score*	3	3	4	6	
Laboratory findings at ICU admission					
Chemistry					
Alanine aminotransferase, U/L	135	14	23.5	41	<40
Albumin, g/L	37	31	28.1	23	35 - 52
Alkaline phosphatase, U/L	131	66	84.0	222	≤ 129
Aspartate aminotransferase, U/L	92	22	73.8	37	≤ 39
Cardiac troponin, high sensitivity, ng/L	NA	7	NA	NA	≤ 13
Creatine kinase, U/L	51	35	180	NA	≤ 189
Creatinine, µmol/L	60	57	57.1	84	44-97
eGFR, mL/min/1.73 m2	>90	>90	>90	>90	>90
γ-Glutamyltransferase, U/L	243	27	34.8	263	≤ 70
Lactate dehydrogenase, U/L	381	432	1088.4	201	<250
Blood count					
Hemoglobin, g/L	112	114	110	121	130 - 165
Lymphocyte count, $\times 10^9$/L	1.8	1.47	2.19	1.64	1.3-3.4
White blood cell count, $\times 10^9$/L	18	10.7	14.74	8.4	3.9-9.5
Platelet count, $\times 10^9$/L	385	408	416	369	149 - 303
Coagulation					
Activated partial thromboplastin time ratio	0.95	0.98	1.00	37	0.8-1.2
D-dimer, ng/mL	<250	463	2400	3660	<250
Fibrinogen, g/L	> 7	> 7	5.5	4,2	2.76-4.71
Prothrombin time ratio	1.38	1.32	1.10	NA	0.8-1.2
Inflammatory markers					
C-reactive protein, mg/L	346.6	267	203.98	196	<3
Ferritin, µg/L	1957.6	920	384.9	845	30 - 400
Procalcitonin, µg/L	0.28	NA	0.1	4.31	<0.5
IL-6, ng/L	48.4	1.5	24	NA	≤ 6.9

(Continued)

TABLE 2 | Continued

	Family 1 *TLR7*:c.644A>G			Family 2 *TLR7*:c.2797T>C	Reference ranges
	Proband	Brother	Mother	Proband	
Secondary complications	None reported	catheter-related bloodstream infection	None reported	Small ventral pneumothorax at admission after resuscitation at home. Bilateral subsegmental pulmonary embolisms.	
Duration of viral shedding after COVID-19 onset (positive SARS-CoV-2 PCR), d	Positive at admission, no follow-up measurement	Positive at admission, no follow-up measurement	Positive at admission, no follow-up measurement	Positive before admission, PCR negative at day 29	
Duration of ventilatory support, d	4	10	7	24 days (ongoing)	
Duration of ICU stay, d	6	12	16	24 days (ongoing)	
Follow-up					
Time from ICU discharge to hospital discharge, d	3	11	5	NA	
Complications during follow-up period	None reported	None reported	None reported	NA	
Treatments	R, D	H, L-R, MP, I, T	R, D, T	D	

COVID-19, coronavirus disease 2019; CT, computed tomography; ICU, intensive care unit; eGFR, estimated glomerular filtration rate; NA, not assessed; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SOFA, Sequential Organ Failure Assessment.

The treatments were administered to the patients as follows: D, Dexamethasone 8 mg daily for 10 consecutive days; R, Remdesivir 200mg intravenously the first day and 100mg daily the next four days; T, Tocilizumab 600 mg single dose intravenously; L-R, Lopinavir 400mg-Ritonavir 100mg orally twice daily for three days; H, Hydroxychloroquine orally 400mg twice daily the first day and 200mg twice daily the next 10 days; I, Interferon β1b 0.25mg every other day subcutaneously for 3 days.

**The SOFA score is calculated using 6 systems: respiratory, coagulation, hepatic, cardiovascular, central nervous, and kidney. Scores range from 0 for normal function to 4 for most abnormal and are summed for a final range of 0 to 24. An initial score of 2 to 3 is associated with 6% mortality; an initial score of 4 to 5 is associated with 20% mortality.*

developed symptoms, including his 24-year-old brother, who had only minor symptoms but was shown to be a carrier of the *TLR7* variant (**Figure 2B**). Information on the exact SARS-CoV-2 viral titers during infections were unavailable. In addition, two male cousins, sons of a maternal aunt, were proven to be carriers of this variant. These individuals had not contracted (symptomatic) COVID-19. Based on the *TLR7* variant carriership, these individuals were fast-tracked for early vaccination.

Functional Testing in Primary Immune Cells

In PBMC isolated from patient 13, the type I and II IFN responses were evaluated upon stimulation with the TLR7 agonist imiquimod, in order to assess the functional impact of the p.(Trp933Arg) variant. It was shown that TLR7 stimulation in the patient resulted in a defective upregulation of *TLR7* mRNA expression, as well as that of the type I IFN-related genes involved in the TLR7 pathway *IRF7, IFNB1* and *ISG15*, as compared to a healthy male control (**Figures 2A, B**). Expression of *IRF3*, a transcription factor that is induced by TLR3 and not TLR7, showed a modest increase of almost 1.7 fold in the patient, but was not induced in the healthy control. Moreover, the production of IFNγ upon exposure with imiquimod was deficient in the patient, compared to two healthy male controls (**Figure 2C**).

DISCUSSION

In this cohort of young males affected with severe COVID-19 rare *TLR7* variants were prospectively identified in 2 out of 14 cases (14.3%). In 10 cases from Spain, one patient was shown to carry a new missense variant [c.644A>G; p.(Asn215Ser)]. The variant was predicted as damaging by *in silico* programs and segregated in a brother who also contracted severe COVID-19. In addition, four Dutch cases were studied, leading to the identification of another patient with a novel, unique missense variant [c.2797T>C; p.(Trp933Arg)] located in the highly conserved TIR domain. Functional analysis of the latter variant demonstrated defective type I and II IFN responses, similar to those documented in the previous reports (13, 14).

A role for the TLR7 receptor has been described in the host defense against single-stranded RNA viruses as well as in the pathogenesis of autoimmune disease such as SLE (16). However, no human mutations in *TLR7* had been reported before the SARS-CoV-2 pandemic. It should be noted that complete TLR7 deficiency is estimated to be extremely rare, because endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) play an essential, non-redundant biological role in host survival (17, 18). Therefore, these rare *TLR7* variants are unlikely to be an explanation for severe COVID-19 in the general population. Distinct from the previously published studies that have identified (enrichment of) rare *TLR7* variants in severe COVID-19 who were 1) males <35 years of age without comorbidities (13), 2) males <60 years of age with or without comorbidities (14) or 3) a group of unselected

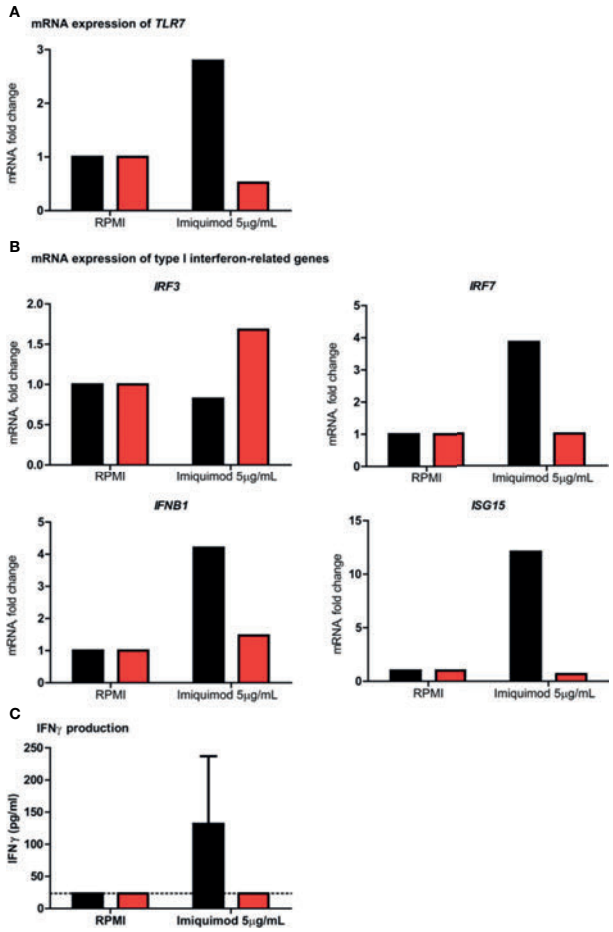

FIGURE 2 | Assessment of *ex vivo/in vitro* type I and II interferon responses in peripheral blood mononuclear cells. In all panels, patient 13 is indicated in red and the healthy controls in black. **(A)** shows the fold change in *TLR7* mRNA expression in patient 13 and a healthy male control after stimulation for 4 hours with the TLR7 agonist imiquimod, compared to the negative medium control RPMI. **(B)** displays the fold change in mRNA expression of type I IFN–related genes *IRF3, IRF7, IFNB1* and *ISG15* induced by imiquimod as compared to RPMI. **(C)** shows the production of IFN-γ production after imiquimod stimulation for 7 days as compared with unstimulated control cells (RPMI) from patient 13 and two healthy male controls. The dotted line indicates the assay's detection limit. Abbreviations: *IRF3*, interferon regulatory factor 3; *IRF7*, interferon regulatory factor 7; *ISG15*, interferon-stimulated gene 15; and *IFNB1*, interferon beta 1.

patients (19), in this study we have selected males <50 years of age without comorbidities predisposing to severe COVID-19. Despite the small cohort, the yield of TLR7 variants was unexpectedly high (14.3%) encouraging that screening for *TLR7* rare variants in severely affected men may be fruitful. Elderly individuals also carry rare, loss of function *TLR7* variants (14), but are more difficult to identify due to other predisposing general risk factors for severe disease in people of advanced age. Therefore, we suggest the following screening criteria: young men (<50 year of age) suffering from severe COVID-19 requiring at least high-flow oxygen therapy, and who were previously healthy. Affected young brother pairs, as well as pedigrees suggestive for X-linked segregation, should be further prioritized.

We furthermore hypothesize that some rare variants compromise essential functional domains in TLR7 and lead to full TLR7 deficiency (20), while other less rare variants might lead only to a partial TLR7 deficiency, and consequently impact a larger group of individuals, but exert a lower relative risk to develop severe COVID-19. Accordingly, these low effect size genetic variants in *TLR7* have been proposed as a possible explanation of the male sex bias in COVID-19 severity because of its localization on the X chromosome and well-established

function in innate immunity (21). Furthermore, it is possible, but only speculative, that the addition of factors that deteriorate *TLR7* function [*e.g.* circulating 25-hydroxy vitamin D [25OHD] levels decline with age (22) and may be accompanied by a lower TLR7 expression and defective function (23)] in patients with these low effect variants may be a common cause of progression to the most severe stages of COVID-19 in male. The same speculative hypothesis could be applicable to other genes involved in immune response regulation after SARS-CoV-2 infection (4, 24).

Strong host genetic factors that confer an increased risk to develop severe COVID-19 might serve as genomic biomarkers, along with other factors, that could be used for early diagnosis and preventative measures, and could allow the identification of possible molecular targets for treatment. In this respect, there would be a strong argument to offer hemizygous *TLR7* deficient males that have not had COVID-19 direct access to early vaccination as an effective preventative measure, similar to other patients with primary immunodeficiencies. This option has indeed been offered to the hemizygous carriers in the family of patient 13. Although no data is yet available to support specific management of *TLR7* deficiency or the at-risk hemizygous carriers, early hospitalization and IFN-based therapies in inborn errors of IFN signaling form rational treatment options (18, 25).

This study has several limitations. First, no biological samples were available to functionally validate the pathogenicity of the p.(Asn215Ser) variant and therefore the functional impact of this variant remains unknown. Nevertheless, the absence of this variant in population databases, the high prediction scores for pathogenicity and the general scarcity of rare variants in *TLR7* (13) suggest this finding is unlikely due to chance. Second, the *TLR7* variant identified in patient 13 segregates in a brother who only experienced mild COVID-19. There are some explanations that may explain why a pathogenic variant in *TLR7* does not become fully penetrant, since protection against SARS-CoV-2 depends on other genetic and environmental factors (*e.g.* exposure to a lower initial viral load, previous exposure to common cold coronaviruses) (26). Moreover, TLR7 function might be specifically influenced by epidemiological factors and certain comorbidities (*e.g.* 25OHD). Thirdly, the size of this study does not permit to draw any firm conclusions on the prevalence of loss of function *TLR7* variants in males with severe COVID-19. Hence, larger cohort studies are required. Most recently, in pan-ancestry whole-exome sequencing data of 586,157 individuals, encompassing 20.592 patients who contracted COVID-19 and 1.266 of those who had severe disease, *TLR7* was the only gene in which the burden of rare variants was significantly increased among patients with severe COVID-19, using a less conservative significance threshold [OR 4.53 (2.64, 7.77)] (19). This is one of the few occasions in which a Mendelian disease gene is replicated through the use of an association study, even without stratifying for male gender or younger age.

In summary, we have identified two novel germline variants in the X chromosomal *TLR7* that likely lead to TLR7 deficiency, which is further corroborated by impaired type I and II IFN responses in the patient with the p.(Trp933Arg) variant. These findings reinforce the notion that TLR7 plays a critical role in the recognition of SARS-CoV-2 and the subsequent initiation of an early antiviral immune response that could prevent the development of severe COVID-19. More importantly, a better understanding of strong genetic factors predisposing to severe COVID-19 may help physicians to identify patients at risk. We therefore propose clinical screening criteria for the identification of *TLR7* variants in male patients with severe COVID-19. This could not only enable targeted therapeutic management of the patient, but could also offer relatives the option of pre-symptomatic testing and by extension preventative measures such as early vaccination.

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 IDIBELL Research Ethics Committee (approval number PR152/20). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XS, GV-P, and CM contributed equally to this work. XS, AH, and CL devised the study. GC, CM, AR-M, FS, ME, and XC provided input on the study design. AA, BH, JS-H, FV, and G-RB assisted in patient management. GV-P, AS, JV, and SR designed and performed the sequence and functional analysis. XS, GV-P, CM, AH, and CL had full access to all data and take responsibility for the integrity and the accuracy of the data. XS, GV-P, CM, AH, and CL drafted the manuscript. 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.2021.719115/full#supplementary-material

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ARTICLE 2

Objectiu

Determinar la prevalença d'autoanticossos neutralitzants (NautoAbs) enfront a interferons de tipus I en els pacients amb COVID-19 ingressats a cures intensives, i analitzar si les característiques i evolució dels pacients amb NautoAbs positius és diferent a la dels pacients negatius.

Títol

Pre-existing autoantibodies neutralizing high concentrations of type I interferons in almost 10% of COVID-19 patients admitted to Intensive Care in Barcelona.

Solanich X*,†, Rigo-Bonnin R*, Gumucio VD, Bastard P, Rosain J, Philippot Q, Perez-Fernandez XL, Fuset-Cabanes MP, Gordillo-Benitez MA, Suarez-Cuartin G, Boza-Hernandez E, Riera-Mestre A, Parra-Martínez A, Colobran R, Antolí A, Navarro S, Rocamora-Blanch G, Framil M, Calatayud L, Corbella X, Casanova JL, Morandeira F, Sabater-Riera J.

**co-first author,* †*corresponding author*

J Clin Immunol. 2021 Sep 27. doi: 10.1007/s10875-021-01136-x.

Contribució del doctorand: revisió de la literatura, disseny, implementació, selecció i preparació de dades, redacció i presentació de l'article.

Material suplementari **Aprovació CEIm**

Resum

Antecedents: És important predir quins pacients infectats per SARS-CoV-2 tenen un risc més elevat de patir una COVID-19 que posa en perill la seva vida. Diversos estudis suggereixen que els autoanticossos neutralitzants (NautoAbs) enfront interferons de tipus I podrien predir formes de pneumònia per COVID-19 crítica.

Objectius: Teníem com a objectiu determinar la presencia de NautoAbs enfront IFN-I i descriure les principals característiques dels pacients amb COVID-19 ingressats a cures intensives en funció de si aquests NautoAbs es trobaven o no presents.

Mètodes: Anàlisi retrospectiva de tots els pacients COVID-19 ingressats en una Unitat de Cures Intensives (UCI) i amb disponibilitat de mostra sanguínia des del març del 2020 fins al març del 2021 a Barcelona.

Resultats: Es varen determinar els NautoAbs enfront IFN de tipus I (α2 i / o ω) per ELISA en un total de 275 (70,5%) de 390 pacients ingressats a la UCI, sent positius en 49 (17,8%) d'ells. El plasma diluït 1/10 de 26 (9,5%) d'aquests pacients va mostrar la capacitat de bloquejar concentracions elevades (10 ng / mL) d'IFN α2 i / o ω. Gairebé tots els pacients amb NautoAbs eren homes (92,3%). Els pacients ingressats a la UCI amb NautoAbs positius no varen mostrar diferències rellevants en variables demogràfiques, comorbiditats, variables clíniques i mortalitat, en comparació amb aquells pacients que no tenien aquests NautoAbs. No obstant això, algunes proves de laboratori (leucocitosi, neutrofília, trombocitosi) relacionades amb una major gravetat de la COVID-19, així com la insuficiència renal aguda (17 [65,4%] vs. 100 [40,2%]; p = 0,013) varen presentar-se en un percentatge significativament superior en els pacients amb NautoAbs positius respecte els negatius.

Conclusió: Es van detectar AutoAbs que neutralitzaven concentracions elevades d'IFN de tipus I en un 9,5% dels pacients ingressats per pneumònia per COVID-19 a la UCI d'un hospital de Barcelona. Aquests NautoAbs s'han de determinar precoçment un cop es diagnostica la infecció per SARS-CoV-2, ja que es troben presents en una proporció significativa de casos de COVID-19 que posen en perill la vida.

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ORIGINAL ARTICLE

Pre-existing Autoantibodies Neutralizing High Concentrations of Type I Interferons in Almost 10% of COVID-19 Patients Admitted to Intensive Care in Barcelona

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Abstract

Background It is important to predict which patients infected by SARS-CoV-2 are at higher risk of life-threatening COVID-19. Several studies suggest that neutralizing auto-antibodies (auto-Abs) against type I interferons (IFNs) are predictive of critical COVID-19 pneumonia.

Objectives We aimed to test for auto-Abs to type I IFN and describe the main characteristics of COVID-19 patients admitted to intensive care depending on whether or not these auto-Abs are present.

Methods Retrospective analysis of all COVID-19 patients admitted to an intensive care unit (ICU) in whom samples were available, from March 2020 to March 2021, in Barcelona, Spain.

Results A total of 275 (70.5%) out of 390 patients admitted to ICU were tested for type I IFNs auto-antibodies (α2 and/or ω) by ELISA, being positive in 49 (17.8%) of them. Blocking activity of plasma diluted 1/10 for high concentrations (10 ng/mL) of IFNs was proven in 26 (9.5%) patients. Almost all the patients with neutralizing auto-Abs were men (92.3%). ICU patients with positive results for neutralizing IFNs auto-Abs did not show relevant differences in demographic, comorbidities, clinical features, and mortality, when compared with those with negative results. Nevertheless, some laboratory tests (leukocytosis, neutrophilia, thrombocytosis) related with COVID-19 severity, as well as acute kidney injury (17 [65.4%] vs. 100 [40.2%]; $p = 0.013$) were significantly higher in patients with auto-Abs.

Conclusion Auto-Abs neutralizing high concentrations of type I IFNs were found in 9.5% of patients admitted to the ICU for COVID-19 pneumonia in a hospital in Barcelona. These auto-Abs should be tested early upon diagnosis of SARS-CoV-2 infection, as they account for a significant proportion of life-threatening cases.

Keywords COVID-19 · SARS-CoV-2 · acute kidney injury · type I interferons · auto-antibodies

Abbreviations

8-OS	8-Point ordinal scale
AKI	Acute kidney injury
ALB	Mass concentration of albumin in plasma
ALT	Catalytic concentration of alanine transaminase in plasma
apH	PH in arterial blood
ARDS	Acute respiratory disease syndrome
$aSatO_2$	Substance fraction of oxygen in arterial blood
AST	Catalytic concentration of aspartate transaminase in plasma
auto-Abs	Auto-antibodies
BMI	Body mass index
BIL	Substance concentration of bilirubin in plasma

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CI	Confidence interval
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
CREA	Substance concentration of creatinine in plasma
CRP	Mass concentration of C-reactive protein in plasma
CRRT	Continuous renal replacement therapy
DD	Mass concentration of D-dimer in plasma
DVT	Deep vein thrombosis
ECMO	Extracorporeal membrane oxygenation
FERRI	Mass concentration of ferritin in plasma
FiO_2	Fraction of inspired oxygen
GFR	Glomerular filtration rate
HPE	High-performance Elisa
HRP	Horseradish peroxidase
HUB	Hospital Universitari de Bellvitge
ICU	Intensive care unit
IL	Interleukin
IL6	Mass concentration of interleukin-6 in plasma
IFNs	Interferons
IFN-α2	Interferon-alfa-2
IFN-γ	Interferon-gamma
IFN-ω	Interferon-omega
IgG	Immunoglobulin G
IMV	Invasive mechanical ventilation
IQR	Interquartilic range
IV	Intravenous
KDIGO	Kidney Disease Improving Global Outcomes
LEU	Number concentration of leucocytes in blood
LDH	Catalytic concentration of lactate dehydrogenase in plasma
LYM	Number concentration of lymphocytes in blood
NEU	Number concentration of neutrophils in blood
OR	Odds ratio
$paCO_2$	Partial pressure of carbon dioxide in arterial blood
paO_2	Partial pressure of oxygen in arterial blood
PBS	Phosphate-buffered saline
PLT	Number concentration of platelets in blood
PROCAL	Mass concentration of procalcitonin in plasma
PT	Relative time of prothrombin in plasma
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TMB	3,3',5,5'-Tetramethylbenzidine
TROP-T	Mass concentration of troponin T in plasma
WHO	World Health Organization
UREA	Substance concentration of urea in plasma

Introduction

In December 2019, an emerging disease (COVID-19), caused by a newly identified human coronavirus (SARS-CoV-2), was first recognized in Wuhan, China, and spread worldwide [1, 2]. The WHO declared the COVID-19 epidemic to be a pandemic on March 12, 2020 [3], and it continues to spread globally, causing considerable morbimortality and economic damage.

Age is the greatest risk factor for life-threatening COVID-19 pneumonia [4], and other epidemiological risk factors (men gender, obesity, diabetes, common genetic variants…) can contribute but with a modest effects [5–8]. These conditions do not allow physicians to accurately predict which patients infected by SARS-CoV-2 are at risk to transit into the most severe stages of COVID-19.

Type I interferons (IFNs) are a family of cytokines that mediate the early innate immune response to viral infections limiting viral spread. When SARS-CoV-2 enters human cells, its viral RNA is recognized by endosomal Toll-like receptors such as TLR3 and TLR7, as well as cytosolic MDA-5, which drive a pathway that leads to gene expression of type I IFNs [5, 9].

In the past months, several human genetic variants associated with higher viral binding and entry have been identified, as well as genes related to higher COVID-19 severity [10, 11]. In addition, rare deleterious variants impairing TLR3- and TLR7-driven type I IFNs induction via IRF7 and amplification via IFNAR1 have been identified in about 5% of life-threatening COVID-19 cases younger than 60 years [12, Asano in press].

Recently, an international consortium reported that 101 of 987 patients (10.2%) with life-threatening COVID-19 pneumonia had neutralizing auto-antibodies (auto-Abs) against type I IFNs (IFN-α2, IFN-ω, or both) [13]. All of the patients tested had low or undetectable serum IFN-α values during acute disease. Interestingly, these auto-Abs were present before SARS-CoV-2 pandemic in the patients tested. Nonetheless, these antibodies were absent in 663 individuals with asymptomatic or mild SARSCoV-2 infection. Half of these patients were over 65 years old, and notably, 95 (94%) of the 101 patients with auto-Abs were men. More recently, it was found that auto-abs neutralizing 100-fold lower concentrations of type I IFN were more frequent, found in about 15% of critical cases (Bastard in press).

These findings may provide a first explanation for the excess of older men among patients with life-threatening COVID-19. Furthermore, they might also offer a means in identifying individuals at-risk of evolving into severe or critical stage of COVID-19 [5], as it has been replicated worldwide [14–17]. In addition, the detection of neutralizing auto-Abs against type I IFNs is technically straightforward and not expensive, so that it could be advantageous to apply in routine clinical practice. Finally, these findings might also pave the way for prevention and treatment by using plasmapheresis, plasmablast depletion, or recombinant type I IFNs not targeted by the auto-Abs (e.g., IFN-β) [18–20].

In the present study, we aimed to describe clinical, analytical, and evolutive data of life-threatening COVID-19 patients admitted to the ICU depending on whether or not auto-Abs neutralizing high concentrations of type I IFNs are present.

Methods

Study Design and Patients

This study was conducted at the Hospital Universitari de Bellvitge (HUB), a 750-bed tertiary-care public hospital for adults in Barcelona, Spain. HUB is the referral hospital for 2 million inhabitants with high-complexity diseases from the Southern area of Catalonia. We performed a retrospective study of COVID-19 patients admitted to the ICU during the first year of the pandemic (from March 2020 to March 2021) in whom samples were available. SARS-CoV-2 infection was confirmed by RT-PCR in all patients.

Data were obtained from routine daily practice and anonymized. Personal and clinical data were collected in accordance with the Spanish Data Protection Act (*Ley Orgánica 3/2018 de 5 de diciembre de Protección de Datos Personales*). Informed consent was waived due to the study's retrospective nature, and the mandatory isolation measures applied during in-hospital care. The protocol was approved by the Ethics Committee of the Hospital Universitari de Bellvitge (Barcelona, Spain; approval number PR40/21).

Clinical and Laboratory Variables

Demographic data and main comorbidities were collected from each patient. Laboratory data were registered at admission to the ICU. The WHO 8-point ordinal scale was calculated in each participant (https://www.who.int/blueprint/priority-diseases/key-action/COVID-19_Treatment_Trial_Design_Master_Protocol_synopsis_Final_18022020.pdf). Complications were documented as follows: (1) Thrombotic complications included deep vein thrombosis (DVT), pulmonary embolism (PE), myocardial infarction, mesenteric ischemia, lower limb ischemia, cerebral ischemic attack confirmed by an imaging study; (2) Hemorrhagic complications included major bleeding according to the definition of the International Society on Thrombosis and Haemostasis [21]; (3) Cardiovascular complications included no coronary heart disease (heart failure, arrhythmias, myocarditis); (4) Acute kidney injury (AKI) was defined using the Kidney Disease Improving Global Outcomes (KDIGO) staging. So, patients were classified as stage 1 if they present an increase of concentration of creatinine in plasma (CREA) of 26.5 µmol/L within 48 h, or increase in CREA ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; stage 2 AKI was considered when CREA increase 2.0 to 2.9 times baseline; and stage 3 AKI, when CREA increase ≥ 3.0 times baseline or increase in CREA to ≥ 353.6 µmol/L, or the initiation of renal replacement therapy (RRT), or in patients < 18 years a decrease in eGFR to < 35 mL/min/1.73m^2 [22]; (5) Superinfection included a second infection with a bacterial agent at the time or during ICU admission; (6) Sepsis was defined as an increase in the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score of 2 points or more with respect to baseline SOFA; and (7) Septic shock was identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mmHg or greater and serum lactate level greater than 2 mmol/L (> 18 mg/dL) in the absence of hypovolemia [23]; (8) Multiple organ failure was defined as the SOFA score alteration of two or more organs with a score of ≥ 3 [24]. Treatments specifically used to treat COVID-19, mechanical ventilation duration and other organ support during ICU stay as vasopressors, RRT, nitric oxide, and extracorporeal membrane oxygenation (ECMO) were also analyzed. Length of hospital and ICU stay and death during hospitalization were also recorded. All drugs and procedures were used according to HUB protocol which is detailed in the supplementary materials.

Auto-Abs Against Type I IFNs

Analysis of auto-Abs against type I IFNs (IFN-α2 and IFN-ω) were performed using an ELISA technique according to St. Giles procedure [13]. In brief, NUNC MaxiSorp™ high protein-binding capacity 96 well ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with recombinant human IFN-α2 or IFN-ω by incubation of the diluted cytokine in 100 µL of coating buffer (1 mg/L) overnight at 4° C. Plates were washed three times with PBS, blocked by incubation with PBS supplemented with 5% nonfat milk powder 1 h at room temperature on an agitator, washed again with PBS-Tween 0.005% (v/v), and incubated with 100 µL of 1:50 dilution of serum samples from patients or controls in HPE dilution buffer (Sanquin, Amsterdam, The Netherlands) for 2 h at room temperature

in the agitator. After wash, Fc-specific HRP-conjugated IgG fractions of polyclonal goat antiserum against human IgG (Nordic-MUbio, Susteren, The Netherlands) were added to a final concentration of 2 mg/L. Plates were incubated for 1 h at room temperature and washed. Then, substrate (TMB) was added and incubated 10 min. The reaction was stopped by adding H_2SO_4 0.18 M, and optical density at 450 nm was measured. We considered as positive results of both auto-Abs against type I IFNs any result greater than a cutoff value calculated as the mean value plus two standard deviations of a control group of healthy non-COVID-19 patients with a similar age and gender.

Neutralizing Auto-Abs Against Type I IFNs

The neutralizing ability in vitro of anti-Abs against IFN-α2 and anti-IFN-ω, i.e., their blocking activity, was determined by assessing a reporter luciferase activity [13]. Briefly, HEK293T cells were transfected with the firefly luciferase plasmids under the control of human ISRE promoters in the pGL4.45 backbone, and a constitutively expressing Renilla luciferase plasmid for normalization (pRL-SV40). Next, cells were transfected in the presence of the X-tremeGene 9 transfection reagent (Millipore-Sigma, Burlington, MA, USA) for 36 h. Then, Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) medium supplemented with 10% healthy control or patient serum/plasma and were either left unstimulated or were stimulated with IFN-α2 or IFN-ω (10 ng/mL) for 16 h at 37 °C. Each sample was tested once. Finally, luciferase levels were measured with the Dual-Glo reagent, according to the manufacturer's protocol (Promega Corp., Madison, WI, USA). Firefly luciferase values were normalized against Renilla luciferase values.

Statistical Analysis

Continuous variables were presented as the median and interquartile range (IQR) and categorical data as frequency rates and percentages. Comparisons of the cohorts were made using a chi-square test or Fisher's exact test for categorical variables and a Mann–Whitney *U* test for continuous or ordinal variables. From June 2020, there were significant changes in the treatment of COVID-19 patients, and for this reason, it has been performed a subanalysis of these two periods (first wave vs. second/third wave in Spain). Statistical significance was defined as p-value < 0.05, and we also used odds ratios (OR) and their 95% confidence intervals (CI) for categorical variables. Calculations were performed with the statistical package SPSS version 19 (IBM Corp. Endicott, NY, USA).

Results

From March 10, 2020, to March 6, 2021, 3216 COVID-19 patients were hospitalized at our hospital, and 390 (12.1%) were admitted to the ICU due to respiratory failure. Of them, 275 (70.5%) ICU patients had frozen serum samples stored in the HUB immunology department, and type I IFNs auto-Abs could be tested.

Main characteristics of all included patients are shown in Tables 1, 2, and 3. Patients included belonged to the different epidemic waves (first 125 [45.4%], second 23 [8.4%], and third 127 [46.2%]). Overall, the median age was 64 years old (IQR 55–71), and male gender represented 76.7% of all patients. The most prevalent preexisting comorbidities were hypertension (53.1%), obesity (49.8%), dyslipidemia (49.1%), and diabetes mellitus (28.4%). The median number of days from the appearance of clinical symptoms to admission to the hospital was 8 (IQR 6–11), and later with a median of 2 (IQR 0–6) days, they were admitted to the ICU. The main laboratory parameters at ICU admission showed a median of 0.64 (IQR 0.38–0.96) lymphocytes $\times 10^9$ cells/L, a median LDH of 471.5 (IQR 367.5–610.8) U/L, a median CRP of 136.1 (IQR 52.8–238.3) mg/L, a median ferritin of 1495 (874–2325) mg/L, and a median d-dimer of 879 (454–2862) µg/L. The median paO_2/FiO_2 at ICU admission was 116.5 (IQR 86–166) mmHg/%. Overall, 38 (13.8%) patients belonged to group 5 of the WHO 8-point ordinal scale, 78 (28.4%) to group 6, 16 (5.8%) to group 7, and 143 (52.0%) to group 8 (Table S1). Regarding the drugs administrated during their hospital stay, 92.0% of patients were treated with corticosteroids, 91.2% with enoxaparin, 30.5% with tocilizumab, 19.3% with remdesivir, and 10.5% with interferon beta 1. Most prevalent complications during ICU stay were superinfection 207 (75.3%), sepsis 134 (48.7%), and acute kidney injury 117 (42.5%). In hospital, all-cause mortality was 52.0%.

We found that 49 (17.8%) of these 275 patients were positive for auto-Abs against type I IFNs (IFN-α2 and/or IFN-ω) by ELISA, of which 19 (6.9%) only against IFN-α2, 8 (2.9%) only against IFN-ω, and 22 (8.0%) against both. Next, we aimed to confirm the neutralizing activity of these auto-Abs. A blocking activity of 10 ng/mL was observed in 26 (53.1%) of these 49 patients with positive auto-Abs against IFNs results. Auto-Abs were neutralizing against both IFN-α2 and IFN-ω in 21 (80.8%) of these 26 patients, against only IFN-α2 in four patients (15.4%), and in only one patient (3.8%) for IFN-ω.

We further assessed the clinical, analytical, and evolutive data of life-threatening COVID-19 patients admitted to the ICU depending on whether or not auto-Abs neutralizing high concentrations of type I IFNs are present

Table 1 Main demographic, comorbidities, clinical, and laboratory data of ICU patients with severe COVID-19 infection considering the presence of positive results of auto-Abs IFN-α2 or auto-Abs IFN-ω obtained by ELISA and luciferase activity techniques

Variable	All results for auto-Abs to type I IFNs ($n=275$)	Neutralizing positive results for some or both auto-Abs to type I IFNs ($n=26$)	Neutralizing negative results for both auto-Abs to type I IFNs ($n=249$)	p-value	OR (95% CI)
Pandemic wave					
First; *n* (%)	125 (45.5)	13 (50.0)	112 (45.0)	0.820	n.a
Second; *n* (%)	23 (8.4)	1 (3.8)	22 (8.8)		
Third; *n* (%)	127 (46.2)	12 (46.2)	115 (46.2)		
Demographics					
Age; median (IQC)	64 (55–71)	63 (57–73)	64 (55–71)	0.712	n.a
Sex (male); *n* (%)	211 (76.7)	24 (92.3)	187 (75.1)	**0.048**	3.979 (0.914–17.32)
Comorbidities					
Cancer; *n* (%)	31 (11.3)	2 (7.7)	29 (11.6)	0.750	0.632 (0.142–2.815)
Cardiac disease; *n* (%)	44 (16.0)	4 (15.4)	40 (16.1)	1.000	0.950 (0.311–2.905)
Chronic kidney disease; *n* (%)	38 (13.8)	3 (11.5)	35 (14.1)	1.000	0.798 (0.227–2.798)
Chronic liver disease; *n* (%)	24 (8.7)	3 (11.5)	21 (8.4)	0.484	1.416 (0.392–5.111)
Chronic obstructive pulmonary disease; *n* (%)	45 (16.4)	3 (11.5)	42 (16.9)	0.590	0.643 (0.185–2.239)
Diabetes; *n* (%)	78 (28.4)	7 (26.9)	71 (28.5)	0.864	0.924 (0.372–2.293)
Dyslipidemia; *n* (%)	135 (49.1)	13 (50.0)	122 (49.0)	0.922	1.041 (0.464–2.335)
Hypertension; *n* (%)	146 (53.1)	13 (50.0)	133 (53.4)	0.740	0.872 (0.389–1.957)
Obesity; *n* (%)	137 (49.8)	11 (42.3)	126 (50.6)	0.421	0.716 (0.316–1.620)
Smoking; *n* (%)	20 (7.3)	0 (0.0)	20 (8.0)	0.233	n.a
Symptom onset and admission					
Number of days from the appearance of clinical symptoms to admission to the hospital; median (IQR)	8 (6–11)	7 (6–8)	8 (6–11)	**0.009**	n.a
Number of days from the hospital admission to the ICU; median (IQR)	2 (0–6)	3.5 (1–7)	2 (0–6)	0.352	n.a
Biological quantities at the first day in ICU					
LEU, $\times 10^9$ cells/L; median (IQR)	9.75 (8.59–14.3)	13.7 (9.40–20.0)	9.30 (6.65–13.5)	**0.001**	n.a
NEU, $\times 10^9$ cells/L; median (IQR)	8.41 (5.72–12.7)	12.7 (8.63–19.0)	8.10 (5.65–11.9)	**0.001**	n.a
LYM, $\times 10^9$ cells/L; median (IQR)	0.64 (0.38–0.96)	0.51 (0.41–0.72)	0.66 (0.37–0.98)	0.067	n.a
PLT, $\times 10^9$ cells/L; median (IQR)	232 (173–303)	260.5 (217–325)	230 (168–298)	**0.038**	n.a
apH, 1; median (IQR)	7.35 (7.29–7.43)	7.35 (7.30–7.39)	7.35 (7.29–7.43)	0.800	n.a
$paCO_2$, mmHg; median (IQR)	46 (40–56.5)	47 (40–53)	46 (40–57)	0.856	n.a
paO_2, mmHg; median (IQR)	96.5 (76–125)	90 (73–127)	97 (76–124.5)	0.574	n.a
$aSatO_2$, %; median (IQR)	97.1 (94.5–98.7)	96.7 (94.3–98.4)	97.2 (94.5–98.7)	0.420	n.a
ALB, g/L; median (IQR)	31.6 (27.4–35.0)	32.0 (26.4–35.0)	31.5 (27.7–35.0)	0.741	n.a
LDH, U/L; median (IQR)	471.5 (367.5–610.8)	444.5 (354–538)	474.5 (370–613)	0.395	n.a
ALT, U/L; median (IQR)	34 (23–56.3)	38.5 (28–61)	34 (23–56)	0.421	n.a
AST, U/L; median (IQR)	45 (31–64.8)	41 (27–52)	45 (32–68)	0.165	n.a

Table 1 (continued)

Variable	All results for auto-Abs to type I IFNs ($n=275$)	Neutralizing positive results for some or both auto-Abs to type I IFNs ($n=26$)	Neutralizing negative results for both auto-Abs to type I IFNs ($n=249$)	p-value	OR (95% CI)
BIL, µmol/L; median (IQR)	9.2 (6.5–13.9)	10.4 (6.0–15.0)	9.0 (6.7–13.7)	0.819	n.a
CREA, µmol/L; median (IQR)	81 (61–114)	80 (61–117)	81 (60–111)	0.767	n.a
UREA, mmo/L; median (IQR)	7.9 (5.2–11.5)	8.1 (5.7–11.7)	7.9 (5.2–11.4)	0.588	n.a
TROP-T, ng/L; median (IQR)	14.7 (9.4–28.2)	11.3 (8.4–14.7)	15.8 (9.8–30.9)	0.121	n.a
DD, µg/L; median (IQR)	879 (454–2862)	963 (482–3507)	878 (452–2811)	0.671	n.a
PT, 1; median (IQR)	1.16 (1.08–1.28)	1.23 (1.11–1.25)	1.15 (1.08–1.29)	0.230	n.a
PROCAL, µg/L; median (IQR)	0.26 (0.13–0.68)	0.29 (0.14–0.51)	0.26 (0.13–0.73)	0.875	n.a
CRP, mg/L; median (IQR)	136.1 (52.8–238.3)	212.1 (62.2–366.3)	130.1 (52.7–229.1)	0.055	n.a
FERRI, mg/L; median (IQR)	1495 (874–2325)	1240 (919–2389)	1498 (862–2291)	0.664	n.a
IL6, ng/L; median (IQR)	91.3 (19.5–455.2)	40.4 (30.2–207.9)	95.3 (19.7–474)	0.778	n.a

OR, odds-ratio; *CI*, confidence interval; *ICU*, intensive care unit; *IQR*, interquartilic range; *n.a.*, not applicable; *LEU*, number concentration of leucocytes in blood; *NEU*, number concentration of neutrophils in blood; *LYM*, number concentration of lymphocytes in blood; *PLT*, number concentration of platelets in blood; *apH*, pH in arterial blood; $paCO_2$, partial pressure of carbon dioxide in arterial blood, paO_2, partial pressure of oxygen in arterial blood; $aSatO_2$, substance fraction of oxygen in arterial blood; *ALB*, mass concentration of albumin in plasma; *LDH*, catalytic concentration of lactate dehydrogenase in plasma; *ALT*, catalytic concentration of alanine transaminase in plasma; *AST*, catalytic concentration of aspartate transaminase in plasma; *BIL*, substance concentration of bilirubin in plasma; *CREA*, substance concentration of creatinine in plasma; *UREA*, substance concentration of urea in plasma; *TROP-T*, mass concentration of troponin T in plasma; *DD*, mass concentration of D-dimer in plasma; *PT*, relative time of prothrombin in plasma; *PROCAL*, mass concentration of procalcitonin in plasma; *CRP*, mass concentration of C-reactive protein in plasma; *FERRI*, mass concentration of ferritin in plasma; *IL6*, mass concentration of interleukin-6 in plasma

ALB, LDH, ALT, AST, BIL, CREA, UREA, TROP-T, PROCAL, CRP, FERRI, and IL6 were measured using a Cobas 6000 or Cobas 8000 analyzers (Roche Diagnostics, Risch-Rotkreuz, Switzerland). LEU, NEU, LYM, and PLT were measured using a Sysmex XN-2000 analyzer (Sysmex, Kobe, Japan), and DD, PT from ACL TOP 500 analyzer (Instrumentation Laboratory, Bedford, MA, USA). On the other hand, apH, $paCO_2$, paO_2, and $aSatO_2$ were obtained from GEM Premier 5000 gasometers (Instrumentation Laboratory)

Numbers in bold indicate a p-value < 0.05

(Tables 1, 2, and 3). Table S1 shows the same data but classifies ICU patients following the WHO 8-point ordinal scale. Almost all the patients with positive results of neutralizing auto-Abs were men, being statistically higher than in the group of patients showing negative results (24 [92.3%] vs. 187 [75.1]; $p=0.048$). No relevant differences were observed in the main comorbidities between the two groups.

The median number of days from the onset of symptoms to admission to the hospital was significantly lower in neutralizing auto-Abs group (7 [IQR 6–8] vs. 8 [IQR 6–11]; $p=0.009$), while the number of days from the hospital admission to the ICU (3.5 [IQR 1–7] vs. 2 [IQR 0–6]; $p=0.352$) was not different between the two groups. Overall, the median number of days admitted to the hospital was similar in both groups (30.5 [IQR 14–46] vs. 29 [IQR 16–50]; $p=0.819$). The specific ICU treatment and mechanical ventilation data between both groups were not significantly different.

Regarding analytical variables, those patients with neutralizing auto-Abs showed significantly higher median values of leukocytes (13.710^9 cells/L [IQR 9.40–20.0] vs. 9.30×10^9 cells/L [IQR 6.65–13.5]; $p=0.001$), neutrophils (12.7×10^9 cells/L [IQR 8.63–19.0] vs. 8.10×10^9 cells/L [IQR 5.65–11.9]; $p=0.001$), platelets (260.5×10^9 cells/L [IQR 217–325] vs. 230×10^9 cells/L [IQR 168–298]; $p=0.038$)) than negative neutralizing auto-Abs patients. Furthermore, median CRP values were numerically higher (212.1 mg/L [IQR 62.2–366.3] vs. 130.1 mg/L [IQR 52.7–229.1]; $p=0.055$) in those patients with neutralizing auto-Abs. Drugs specifically used to treat COVID-19 at any time during admission were not different between the two groups.

Table 2 Drugs, mechanical ventilation and other specific ICU treatments of severe COVID-19 patients admitted to ICU considering the presence of positive results of auto-Abs IFN-α2 or auto-Abs IFN-ω obtained by ELISA and luciferase activity techniques

Variable	All results for auto-Abs to type I IFNs ($n=275$)	Neutralizing positive results for some or both auto-Abs to type I IFNs ($n=26$)	Neutralizing negative results for both auto-Abs to type I IFNs ($n=249$)	p-value	OR (95% CI)
Specific ICU treatment and mechanical ventilation data					
Patients with CRRT; *n* (%)	28 (10.2)	3 (11.5)	25 (10.0)	0.736	1.169 (0.328–4.170)
Patients with ECMO; *n* (%)	25 (9.1)	2 (7.7)	23 (9.2)	1.000	0.819 (0.182–3.688)
paO_2/FiO_2, mmHg/%; median (IQR)	116.5 (86–166)	111 (85–153)	120 (86.5–167)	0.313	n.a
Patients treated with IMV; *n* (%)	232 (84.4)	22 (84.6)	210 (84.3)	1.000	1.021 (0.334–3.127)
Patients with nitric oxide administration during IMV; *n* (%)	38 (13.8)	4 (15.4)	34 (13.7)	0.767	1.150 (0.373–3.542)
Patients positioned in prone position during IMV; *n* (%)	205 (74.5)	18 (69.2)	187 (75.1)	0.513	0.746 (0.309–1.800)
Number of days with IMV; median (IQR)	13 (4–27)	11 (3–17)	13 (4–28)	0.291	n.a
Drugs administration					
Patients treated with hydroxychloroquine; *n* (%)	126 (45.8)	13 (50.0)	113 (45.4)	0.653	1.204 (0.536–2.701)
Patients treated with lopinavir/ritonavir; *n* (%)	85 (30.9)	11 (42.3)	74 (29.7)	0.186	1.734 (0.761–3.954)
Patients treated with remdesivir; *n* (%)	53 (19.3)	5 (19.2)	48 (19.3)	0.995	0.997 (0.358–2.778)
Patients treated with azithromycin; *n* (%)	69 (25.1)	5 (19.2)	64 (25.7)	0.469	0.688 (0.249–1.901)
Patients treated with tocilizumab; *n* (%)	84 (30.5)	9 (34.6)	75 (30.1)	0.636	1.228 (0.524–2.880)
Patients treated with corticosteroids; *n* (%)	253 (92.0)	25 (96.2)	228 (91.6)	0.705	2.303 (0.297–17.85)
Patients treated with interferon beta 1; *n* (%)	29 (10.5)	3 (11.5)	26 (10.4)	0.744	1.119 (0.314–3.983)
Patients treated with enoxaparin; *n* (%)	250 (91.2)	26 (100.0)	224 (90.3)	0.144	n.a
Patients treated with anticoagulants with prophylactic or therapeutic goal; *n* (%)	275 (100)	26 (100.0)	249 (100.0)	n.a	n.a

OR, odds-ratio; *CI*, confidence interval; *ICU*, intensive care unit; *IQR*, interquartilic range; *n.a.*, not applicable; *CRRT*, continuous renal replacement therapy; *ECMO*, extracorporeal membrane oxygenation; *IMV*, invasive mechanical ventilation; FiO_2, fraction of inspired oxygen; paO_2, partial pressure of oxygen in arterial blood

Numbers in bold indicate a p-value < 0.05

No significant association between the presence of neutralizing auto-Abs and mortality (12 [46.2%] vs. 131 [52.6%]; $p=0.531$) or other complications was found (Table 3), except for acute kidney injury (AKI) (17 [65.4%] vs. 100 [40.2%]; $p=0.013$). Patients with positive auto-Abs showed approximately three times more probability to present AKI (OR 2.814 [95%CI 1.207–6.563]) than those with negative results. Significant differences were observed in patients at KDIGO-AKI stages 1 ($p<0.001$), 2 ($p<0.001$), and 3 ($p<0.001$) when they were compared with those patients with non AKI. AKI was significantly higher in neutralizing auto-Abs patients who finally died (12 [100%]

Table 3 Length of hospital and ICU stay, and complications of severe COVID-19 patients admitted to ICU considering the presence of positive results of auto-Abs IFN-α2 or auto-Abs IFN-ω obtained by ELISA and Luciferase activity techniques

Variable	All results for auto-Abs to type I IFNs ($n=275$)	Neutralizing positive results for some or both auto-Abs to type I IFNs ($n=26$)	Neutralizing negative results for both auto-Abs to type I IFNs ($n=249$)	*p*-value	OR (95% CI)
Length of hospital and ICU stay					
Number of admitted days to the ICU; median (IQR)	15 (7–31)	13.5 (4–24)	15 (7–31)	0.500	n.a
Number of admitted days to the hospital; median (IQR)	29 (15–49)	30.5 (14–46)	29 (16–50)	0.819	n.a
Complications during ICU stay					
Patients with neurological complications; *n* (%)	77 (28.0)	5 (19.2)	72 (28.9)	0.295	0.585 (0.213–1.612)
Patients with thrombotic complications; *n* (%)	50 (18.2)	5 (19.2)	45 (18.1)	0.795	1.079 (0.389–3.015)
Patients with hemorrhagical complications; *n* (%)	27 (9.8)	4 (15.4)	23 (9.2)	0.301	1.787 (0.567–5.634)
Patients with cardiovascular complications; *n* (%)	56 (20.4)	5 (19.2)	51 (20.5)	0.880	0.924 (0.332–2.570)
Patients with acute kidney injury; *n* (%)	117 (42.5)	17 (65.4)	100 (40.2)	**0.013**	2.814 (1.207–6.563)
Patients with superinfection; *n* (%)	207 (75.3)	19 (73.1)	188 (75.5)	0.785	0.881 (0.353–2.195)
Patients with sepsis; *n* (%)	134 (48.7)	11 (42.3)	123 (49.4)	0.491	0.751 (0.332–1.700)
Patients with septic shock; *n* (%)	70 (25.5)	4 (15.4)	66 (26.5)	0.215	0.504 (0.167–1.517)
Patients with multiple organ failure; *n* (%)	56 (20.4)	5 (19.2)	51 (20.5)	0.880	0.924 (0.332–2.570)
Final status					
Exitus; *n* (%)	143 (52.0)	12 (46.2)	131 (52.6)	0.531	0.772 (0.343–1.736)

OR, odds-ratio; *CI*, confidence interval; *ICU*, intensive care unit; *IQR*, interquartilic range; *n.a.*, not applicable

Numbers in bold indicate a p-value < 0.05

vs. 60 [45.8%]; $p < 0.001$), but not in the rest of the 8-point ordinal scale groups (Table S1). When AKI-related variables were selected and a binary logistic regression analysis was performed, a higher risk of AKI was independently associated with the presence of type I IFNs neutralizing auto-Abs (multivariate OR 7.672 [95% CI 2.286–25.75]), as well as, a glomerular filtrate rate (GFR) < 60 mL/min/1.73m^2 at hospital admission, the need for ECMO, the development of multiple organ failure, the seventh and eighth points of the ordinal scale, and the use of interferon beta 1 during ICU admission (Table S2).

Discussion

From March 2020 to March 2021, a sample of 275 ICU patients could be tested for type I IFNs auto-Abs (α2 and ω), representing 70.5% of all patients admitted to the ICU during the study period. One-fifth (49 (17.8%)) showed positive results, with blocking activity in half of them (26 (9.5%)). There were no relevant differences in the main demographic, comorbidities, and clinical data. Patients with positive neutralizing auto-Abs had a significantly higher leukocytes, neutrophils, and platelet values than negative ones. Interestingly, acute kidney injury was also significantly more frequent in positive patients. Overall, half of these patients (52.0%) died without significant differences between positive and negative neutralizing auto-Abs groups.

A recent study by Koning et al. [14] showed that auto-Abs against IFN-α2 and IFN-ω tested by multiplex particle–based assay and ELISA were found in 35 (16.6%) out of 210 COVID-19 patients, of whom 6 (17.1%) out of 35 had neutralizing auto-Abs using STAT1 phosphorylation assay. Eighty-eight (41.9%) of these 210 COVID-19 patients were admitted to ICU, belonging all 6 patients with neutralizing auto-Abs to this group of greater severity. Accordingly,

Bastard et al. [13] reported that auto-Abs against IFN-α2 and IFN-ω were detected in 135 (13.7%) out of 987 life-threatening COVID-19 patients, showing blocking activity in 101 (74.8%) of these 135 ones. Altogether, these findings suggest that the greater the severity, the higher the proportion of neutralizing antibodies, but even in the critically ill COVID-19 patients, it is important to determine the blocking activity against type I IFNs. In our cohort, half (53.1%) of auto-Abs determined by ELISA showed blocking activity for 10 ng/mL of IFNs using luciferase reporter assays.

According to previous reports [13–17], type I IFN neutralizing auto-Abs may help physicians to identify patients at higher-risk to develop severe COVID-19, at the early stages of the disease. However, there is still limited data on whether characteristics of ICU patients with neutralizing IFN auto-Abs are different from those ICU patients without these auto-Abs. Our results did not show demographic, comorbidity or clinical differences between both groups, except for an excess of men in patients with auto-Abs positive results. It could be explained because an inadequate type I IFN response is a common feature in critical COVID-19 patients [5, 9, 25, 26] regardless of whether this defect is due to auto-Abs against type I IFNs [15, 17], rare inborn errors of immunity, or any other mechanism.

However, some laboratory differences were detected in our COVID-19 patients admitted to ICU considering the presence of neutralizing IFN auto-Abs. Higher CRP values were close to statistical significance in the group of patients with neutralizing auto-Abs, as reported by Troya et al. in a smaller group of ICU patients [16]. In addition, our patients with auto-Abs positive results also showed significantly higher leukocytes, neutrophils, and platelet values. All these blood parameters have been used to stratify patients at higher risk for COVID-19 complications [8, 9] suggesting that positive neutralizing auto-Abs patients may develop more severe forms of COVID-19.

In contrast with previously described in smaller cohorts [14, 16], mortality in our patients was not different between those ICU patients with and without neutralizing type I IFNs auto-Abs. Interestingly, we found a significant association between AKI and neutralizing type I IFNs auto-Abs. AKI can be caused by several mechanisms in critical COVID-19 patients [27], and it should be determined if these auto-Abs play a role in its pathogenesis. It is possible, but only speculative, that type I IFN auto-Abs predisposes to the formation of immune complexes that in turn activate complement. The abnormal presence of plasma-derived complement components in the tubular lumen leads to the assembly of the C5b-9 in the tubular epithelial cells, and it could be involved in the pathogenesis of tubulointerstitial damage. In this regard, a retrospective series of six post-mortem COVID-19 patients showed complement C5b-9 deposition on tubules in all kidneys examined [28]. Although, these findings have to be confirmed, neutralizing IFN auto-Abs might be a biomarker to identify those critical COVID-19 patients with greater risk of developing AKI, helping physicians to make earlier preventive and therapeutic decisions.

Unlike other factors related to increased COVID-19 severity, detection of neutralizing type I IFNs auto-Abs in ICU patients may pave the way for specific therapeutic interventions. In this regard, plasmapheresis was recently reported to decrease the titers of blood auto-Abs in four hospitalized patients with life-threatening COVID-19 pneumonia, even though mortality still was 50% [19]. Little is also known whether the administration of IFN-β, B-cell depletion, or other therapies might be beneficial to treat these patients with auto-Abs against type I IFNs admitted to ICU [20].

Our study has several limitations that deserve further comment. First, it was not possible to obtain plasma samples from all the patients admitted to the ICU during the study period, although we were able to analyze more than 70% of them. Nevertheless, this was a representative group with little potential for bias. Second, we exclusively detected the most frequent type I IFNs (α2 and ω) by ELISA, and, therefore, it is possible that some study patients presented other antibodies that were not detected (i.e., auto-Abs against IFN-β). Third, we analyzed blocking activity for 10 ng/mL of IFNs according with previous reports [13–16], but blood IFN-α concentrations of mild/moderate COVID-19 patients typically range from 1 to 100 pg/mL, and they are even lower in severe and critical ones [25], so auto-Abs neutralizing concentrations of type I IFNs below 10 ng/mL may underlie life-threatening COVID-19 pneumonia in more than 9.5% of cases, as suggested by a recent study [Bastard in press]. Fourth, since our study was retrospective, confounders could be overlooked, and missing data might have altered some results. Fifth, the study design does not permit us to establish if the antibodies play a pathogenic role or are simply a biomarker of increased risk for developing renal failure among such patients. Finally, the present study does not allow assessing the usefulness of auto-Abs in those patients at earlier or milder stages of the disease.

In summary, one-fifth of COVID-19 patients admitted to ICU presented auto-Abs against type I IFNs (IFN-α2 and/or IFN-ω), and blocking activity against 10 ng/mL of type I IFNs in half of them. In such life-threatening COVID-19 population, the presence of neutralizing IFNs auto-Abs was remarkably and statistically greater in men, associated with increased inflammatory laboratory parameters related to COVID-19 severity, and also related with a higher risk for developing acute kidney injury. Conversely, mortality between both groups was not different. Therefore, the early identification of these auto-Abs help to identify a significant proportion of patients at higher risk to develop critical COVID-19 pneumonia, its usefulness being more limited when patients are in the ICU. Further

research is needed to assess the clinical and pathogenic role of neutralizing auto-Abs against type I IFNs in order to better select the most appropriate therapies.

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Author Contribution XS and RR-B contributed equally to this work. XS, RR-B, FM, and JS-R devised the study. XS, RR-B, FM, and JS-R had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. XS, RR-B, VDG, PB, AR-M, XC, JLC, FM, and JS-R provided input on the trial design. XS, VDG, XLP-F, MPF-C, MAG-B, GS-C, EB-H, AA, GR-B, and JS-R assisted in patient management. RR-B, PB, JR, QP, RC, JLC, and FM designed and performed the laboratory analysis. XS, RR-B, VDG, FM, and JS-R were responsible for acquiring, analyzing, and interpreting data. XS, RR-B, PB, FM, and JS-R drafted the manuscript. VDG, JR, QP, XLP-F, AR-M, RC, XC, and JLC critically revised the manuscript. XS and RR-B contributed to the statistical analysis. XS, RR-B, FM, and JS-R verified the underlying data. All authors contributed to conducting the trial and read and approved the final manuscript.

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

Code Availability Not applicable.

Declarations

Ethics Approval Ethical approval for the study was obtained from the Hospital Universitari de Bellvitge – IDIBELL (L'Hospitalet de Llobregat, Barcelona, Spain) Research Ethics Committee (approval number PR40/21). Informed consent was waived due to the study's retrospective nature and the mandatory isolation measures applied during in-hospital care.

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ARTICLE 3

Objectiu

Determinar la prevalença i els factors associats a un resultat indeterminats de la prova QuantiFERON-TB Gold Plus en pacients hospitalitzats per la COVID-19, i analitzar la relació entre els resultats indeterminats i la mortalitat per COVID-19.

Títol

Clinical Significance of Indeterminate QuantiFERON-TB Gold Plus Assay Results in Hospitalized COVID-19 Patients with Severe Hyperinflammatory Syndrome.

Solanich X*†, Fernández-Huerta M*, Basaez C, Antolí A, Rocamora-Blanch G, Corbella X, Santin M, Alcaide F.

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Contribució del doctorand: revisió de la literatura, disseny, implementació, selecció i preparació de dades, anàlisi estadístic, presentació dels resultats, redacció i presentació de l'article.

Aprovació CEIm

Resum

Antecedents: El rendiment de l'assaig QuantiFERON-TB Gold Plus (QFT-Plus) es pot veure afectat per condicions de desregulació immune. Se sap poc sobre la fiabilitat del QFT-Plus en pacients amb COVID-19.

Objectius: El nostre objectiu era determinar la prevalença i els factors relacionats amb una prova QFT-Plus indeterminada en pacients hospitalitzats amb COVID-19, i analitzar la seva relació amb la mortalitat hospitalària.

Mètodes: Varem realitzar una anàlisi retrospectiva de tots els pacients amb COVID-19 hospitalitzats als quals es va realitzar l'assaig QTF-Plus en un hospital públic d'atenció terciària durant la primera onada epidèmica a Espanya (març-abril de 2020).

Resultats: D'un total de 96 pacients inclosos, 34 (35,4%) varen presentar un resultat indeterminat, en tots els casos per falta de resposta en el control del mitogen. Factors relacionats amb la gravetat de la COVID-19, com la lactat deshidrogenasa (LDH) (odds ratio [OR] 1,005 [interval de confiança del 95% [IC] 1.002-1.008]) i l'administració prèvia de corticosteroides (OR 4.477 [IC del 95% 1.397-14.345]), varen ser predictors independents d'un resultat indeterminat del QFT-Plus. A més, els resultats indeterminats varen ser més freqüents entre els pacients amb COVID-19 que varen morir durant l'hospitalització (29,1% vs. 64,7%; p = 0,005).

Conclusió: L'assaig QFT-Plus va donar una prevalença inesperadament alta de resultats indeterminats en pacients amb COVID-19 hospitalitzats. Els factors relacionats amb una pitjor evolució de la COVID-19, com la LDH, així com l'ús de corticosteroides abans de realitzar el QFT-Plus, podrien ser predictors d'un resultat indeterminat. S'ha de seguir avaluant el paper que pot tenir un resultat indeterminat del QFT-Plus com a predicció de gravetat i mortalitat en la COVID-19.

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Article

Clinical Significance of Indeterminate QuantiFERON-TB Gold Plus Assay Results in Hospitalized COVID-19 Patients with Severe Hyperinflammatory Syndrome

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Abstract: Performance of the QuantiFERON-TB Gold Plus (QFT-Plus) assay could be affected by conditions of immune dysregulation. Little is known about the reliability of QTF-Plus in COVID-19 patients. Our aim was to determine the prevalence and the factors related to an indeterminate QFT-Plus test in COVID-19 hospitalized patients, and to analyze its relationship with in-hospital mortality. A retrospective analysis of all hospitalized COVID-19 patients on whom a QTF-Plus assay was performed in a tertiary care public hospital during the first epidemic wave in Spain (March–April 2020). Out of a total of 96 patients included, 34 (35.4%) had an indeterminate result, in all cases due to a lack of response in the mitogen control. Factors related to COVID-19 severity, such as higher lactate dehydrogenase (LDH) (odds ratio [OR] 1.005 [95% confidence interval [CI] 1.002–1.008]) and previous administration of corticosteroids (OR 4.477 [95% CI 1.397–14.345]), were independent predictors for indeterminate QFT-Plus assay. Furthermore, indeterminate results were more frequent among COVID-19 patients who died during hospitalization (29.1% vs. 64.7%; $p = 0.005$). We conclude that QFT-Plus assay yielded an unexpected, high prevalence of indeterminate results in severe COVID-19 patients. Factors related to worse COVID-19 outcome, such as LDH, as well as corticosteroid use before the QFT-Plus assay, seem to be predictors for an indeterminate result. The role of an indeterminate QFT-Plus result in predicting COVID-19 severity and mortality should be evaluated.

Keywords: QuantiFERON-TB Gold Plus; indeterminate; COVID-19; SARS-CoV-2; corticosteroids

1. Introduction

In December 2019, an emerging disease (COVID-19), caused by a newly identified human coronavirus, was first recognized in Wuhan, China, and spread worldwide [1,2]. The WHO declared the COVID-19 epidemic to be a pandemic on 12 March 2020 [3], and it continues to spread globally today, causing considerable morbimortality and economic damage.

Multiple drugs have been repurposed to treat severe SARS-CoV-2 infections presenting with systemic hyperinflammatory syndrome, some of them having an immunosuppressive

effect such as corticosteroids, tocilizumab, anakinra, etc. [4–7]. Therefore, reactivation of several dormant infections, including latent tuberculosis infection (LTBI), is of concern among COVID-19-treating physicians. In this regard, interferon-gamma release assays (IGRAs), such as the QuantiFERON-TB Gold Plus (QFT-Plus; Qiagen, Germany) [8] assay, have been unsystematically performed for LTBI screening in some severe COVID-19 patients, before or during immunosuppressive therapy.

QFT-Plus is based on the detection of interferon-gamma (IFN-γ) released by a T-cell-mediated immune response following in vitro stimulation of human whole-blood by antigens specific to the Mycobacterium tuberculosis complex [8]. Additionally, the QFT-Plus assay includes a mitogen-based control designed to nonspecifically elicit a T-cell response and thus infer the immunological fitness of the studied individuals. It is well known that active inflammation or immunosuppressive drug use is associated with QFT-Plus indeterminate results [8,9].

During the first months of the COVID-19 pandemic, microbiologists observed a surprisingly high rate of QFT-Plus indeterminate results in patients hospitalized with COVID-19 at our hospital. At that time, there was no information about this finding and, nowadays, there is still little information about how COVID-19 itself and the immunosuppressive drugs can impact QFT-Plus performance [10]. In addition, it is not known if an indeterminate QFT-Plus result might have a relation to COVID-19 severity and mortality.

Our aim was to determine the prevalence and the factors related to an indeterminate QFT-Plus test in COVID-19 hospitalized patients, and to analyze its relationship with in-hospital mortality.

2. Materials and Methods

2.1. Study Design and Participants

The study was conducted at the Bellvitge University Hospital (BUH), a 750-bed tertiary-care public hospital for adults in Barcelona, Spain. BUH is the reference hospital for 2 million inhabitants with high-complexity diseases from the Southern area of Catalonia.

We performed a retrospective study of all hospitalized patients with COVID-19 from March to April 2020. SARS-CoV-2 infection was confirmed by RT-PCR in all patients on whom the QFT-Plus test was performed. Demographic, epidemiological, laboratory and clinical data were also collected. Laboratory tests were determined within 24 hours before or after the QFT-Plus test. The QFT-Plus indeterminate results were defined according to the manufacturer´s criteria. Nil value comes from the negative control tube that contains no additives, and it is used to determine if the patient has a pre-existing immune disturbance which could cause a false-positive or false-negative reading on the test (that is, nil > 8 UI/mL or nil-corrected mitogen value < 0.5 IU/mL) [8]. Mild hypoxemia was defined as PF (PaO_2/FiO_2) ratio < 300 and/or SF (SpO_2/FiO_2) ratio < 357; moderate hypoxemia was defined as PF ratio < 200 and/or SF ratio < 214; and severe hypoxemia was defined as PF ratio < 100 and/or SF ratio < 89) [11]. In accordance with the WHO 8-point ordinal scale [12], a moderate disease was defined as hospitalized patients with or without supplementary oxygen; and a severe disease was considered in patients with non-invasive ventilation or high-flow oxygen, intubation and mechanical ventilation, additional organ support, renal replacement treatment or extracorporeal membrane oxygenation (ECMO), or death. We calculated the WHO 8-point ordinal scale corresponding to the same day that the QFT-Plus assay was performed for each participant. Treatments specifically used to treat COVID-19 at any time during admission, and immunosuppressive treatments administered before the QFT-Plus test was performed were also analyzed. According to the March to April 2020 BUH guidelines, corticosteroids were administered as methylprednisolone iv 125 mg daily on 3 consecutive days, and iv tocilizumab as a single dose (600 mg if ≥75 kg or 400 mg if <75 kg). Length of stay from QFT-Plus assay until hospital discharge or death was also recorded.

2.2. Statistical Analysis

Results from continuous variables are presented as median and interquartile range (IQR), and categorical data as absolute frequencies and percentages. Comparisons of the cohorts were made using a chi-square test or Fisher's exact test for categorical variables and a Mann–Witney U test for continuous variables. Multivariable logistic regression analysis using the stepwise method was performed to assess factors related to an indeterminate QFT-Plus assay result for not inter-correlated and univariate p-value ≤ 0.1 co-variables, since we do not know which ones may be the most relevant due to the lack of previous studies in patients with COVID-19. Survival analysis was conducted using Cox univariate regression and Kaplan–Meier curves to assess the impact of an indeterminate QFT-Plus assay on the COVID-19 in-hospital mortality. Statistical significance was defined as p-value < 0.05 and we also used odds ratios (OR) or hazard ratios (HR) and their 95% confidence intervals (CI). Calculations were performed with the statistical package SPSS version 19 (IBM Corp., USA).

3. Results

The QFT-Plus test was performed on 96 (6.8%) of a total of 1403 patients admitted due to COVID-19 during the study period. Main comorbidities, clinical status, laboratory tests and treatment characteristics are summarized in Table 1. In all cases, the QFT-Plus assay was indicated for the screening of LTBI in patients receiving or potentially going to receive immunosuppressive therapy to treat severe COVID-19. Finally, of the overall 96 cases, 66 (68.8%) received corticosteroids and/or tocilizumab, and the rest did not. The QFT-Plus test was performed with a median of 12 (IQR 10–16) days from the onset of COVID-19 symptoms. Of the overall 96 patients tested, 54 (56.3%) were negative, 8 (8.3%) were positive, and 34 (35.4%) were indeterminate. Any patient developed tuberculosis after a median follow-up period of 74 days (IQR 61–78). All indeterminate results were due to a lack of response to the mitogen control.

There was a significant association between an indeterminate QFT-Plus result and lymphopenia (0.64 [0.48–1.15] vs. 0.88 [0.64–1.27] $\times$ 109; $p = 0.017$), leukocytosis (8.6 [6.3–13.9] vs. 6.8 [5.6–9.6] $\times$ 109; $p = 0.016$), and neutrophilia (7.3 [5.4–12.4] vs. 5.5 [3.5–8.3] $\times$ 109; $p = 0.002$). Indeterminate QFT-Plus assay results were associated with higher levels of lactate dehydrogenase (LDH) (412 [330–517] vs. 317 [265–424] U/L; $p = 0.003$) and D-dimer (636 [353–2913] vs. 361 [250–946] µg/L; $p = 0.025$), as well as hypoxemia ($p = 0.015$). Additionally, corticosteroid use prior to the QFT-Plus test was significantly higher in the indeterminate subset ($n = 10/34$ [29.4%] vs. $n = 6/62$ [9.7%]; $p = 0.013$). Significantly, five of the 34 (14.7%) patients who did not receive immunosuppressive treatment prior to testing showed indeterminate results. However, no association between indeterminate QFT-Plus and age, gender, comorbidities or previous days of COVID-19 was found. In the adjusted analysis, higher LDH (OR 1.005 [95% CI 1.002–1.008]) and corticosteroid use before performing the QFT-Plus test (OR 4.477 [95% CI 1.397–14.345]) remained independently associated with an indeterminate test (Table 1).

Seventeen (17.7%) patients died during hospitalization. In this respect, a significant association was found between mortality and an indeterminate QFT-Plus assay result ($n = 23/79$ [29.1%] vs. $n = 11/17$ [64.7%]; $p = 0.005$). Patients with an indeterminate QFT-Plus test showed four times more risk for in-hospital mortality (HR 4.025 [95% CI 1.486–10.903]) than those patients with interpretable results (Figure 1).

Table 1. Main characteristics of 96 COVID-19 hospitalized patients according the QuantiFERON-TB Gold Plus (QFT-Plus) test result.

	Indeterminate QTF-Plus (*n* = 34)	Interpretable QTF-Plus (*n* = 62)	*p*-Value	Unadjusted Model		Adjusted Model	
				OR (95%CI)	*p*-Value	OR (95%CI)	*p*-Value
Gender (male)	22 (64.7%)	45 (72.6%)	0.422	0.693 (0.282–1.700)	0.422		
Age (years)	70.5 (56.9–76.2)	63.7 (49.2–74.9)	0.237	1.020 (0.990–1.051)	0.203		
Comorbidities							
Hypertension	16 (47.1%)	34 (54.8%)	0.466	0.732 (0.316–1.694)	0.466		
Dyslipidemia	19 (55.9%)	28 (45.2%)	0.315	1.538 (0.663–3.569)	0.315		
Diabetes	11 (32.4%)	12 (19.4%)	0.154	1.993 (0.766–5.182)	0.154		
Cardiovascular disease	7 (20.6%)	12 (19.4%)	0.885	1.080 (0.381–3.066)	0.885		
Lung disease	6 (17.6%)	9 (14.5%)	0.686	1.262 (0.408–3.906)	0.686		
Immunocompromised patient	4 (11.8%)	3 (4.8%)	0.212	2.622 (0.551–12.480)	0.212		
Candidate for invasive measures (yes)	25 (73.5%)	52 (83.9%)	0.224	0.534 (0.193–1.480)	0.224		
COVID-19 onset to admission (days)	7.5 (5.75–12.25)	8 (7–10.25)	0.401	1.013 (0.928–1.105)	0.778		
Laboratory tests (*)							
Leukocytes ($\times 10^9$/L)	8.6 (6.3–13.9)	6.8 (5.6–9.6)	0.016	1.131 (1.024–1.248)	0.015		
Neutrophils ($\times 10^9$/L)	7.3 (5.4–12.4)	5.5 (3.5–8.3)	0.002	1.147 (1.032–1.274)	0.011		
Lymphocytes ($\times 10^9$/L)	0.64 (0.48–1.15)	0.88 (0.64–1.27)	0.017	0.287 (0.105–0.783)	0.015		
Serum ferritin (µg/L) (*n* = 92)	1756 (1081–2574)	1444 (692–2233)	0.159	1.000 (1.000–1.001)	0.201		
LDH (U/L) (*n* = 95)	412 (330–517)	317 (265–424)	0.003	1.005 (1.001–1.008)	0.005	1.005 (1.002–1.008)	0.003
C-reactive protein (mg/L)	79 (29–197)	87 (27–160)	0.654	1.002 (0.997–1.006)	0.447		
IL-6 (ng/L),(*n* = 52)	100 (20–1044)	99 (55–193)	0.955	1.001 (1.000–1.012)	0.130		
Troponin (ng/L), (*n* = 69)	10.0 (7.0–25.0)	10.5 (6.0–18.2)	0.546	1.000 (0.991–1.008)	0.926		
D-dimer (µg/L), (*n* = 93)	636 (353–2913)	361 (250–946)	0.025	1.000 (1.000–1.001)	0.016		
Hypoxemia			0.015				
None	4 (11.8%)	21 (33.8%)		-			
Mild	6 (17.6%)	20 (32.3%)		1.557 (0.386–6.423)	0.536		
Moderate	22 (64.7%)	20 (32.3%)		5.775 (1.690–19.734)	0.005		
Severe	2 (5.9%)	1 (1.6%)		10.5 (0.758–145.359)	0.079		
COVID-19 onset to QFT-Plus (days)	11 (9–18)	12 (10-16)	0.797	1.005 (0.937–1.077)	0.893		
Severe WHO 8-OS on QFT-time	9 (26.5)	5 (8.1)	0.015	4.104 (1.248–13.491)	0.015		
IS prior to QFT-Plus							
None	5 (14.7%)	28 (45.1%)	0.003	0.209 (0.072–0.612)	0.003		
Corticosteroids	10 (29.4%)	6 (9.7%)	0.013	3.889 (1.270–11.912)	0.013	4.477 (1.397–14.345)	0.012
IL-6 blockage	3 (8.8%)	5 (8.1%)	0.898	1.103 (0.247–4.928)	0.898		
Corticosteroids and IL-6 blockage	16 (47.1%)	23 (37.1%)	0.342	1.507 (0.646–3.519)	0.342		

Qualitative data are expressed as a number (percentage), and quantitative data are expressed as a median (interquartile range). Abbreviations: OR (95%CI), odds ratio (95% confidence interval); IL-6, interleukin-6; QFT, QuantiFERON-TB Gold Plus; LDH, lactate dehydrogenase; IS, immunosuppressive drugs; WHO 8-OS, World Health Organization 8-point Ordinal Scale. (*) Laboratory tests when the QFT-Plus assay was performed.

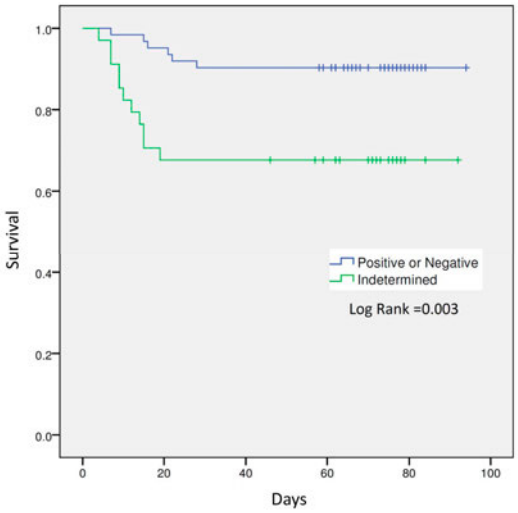

Figure 1. Kaplan–Meier survival curves for patients with interpretable (positive or negative) and indeterminate QFT-Plus assay results.

4. Discussion

The present study shows an unexpected, high prevalence of indeterminate results in hospitalized patients with COVID-19. Factors related to COVID-19 clinical severity such as higher levels of LDH and corticosteroid use before the performance of the QFT-Plus assay seem to be related to an indeterminate result. Furthermore, a significant association between an indeterminate QFT-Plus result and higher in-hospital mortality has also been found.

The sensitivity of IGRAs in the detection of LTBI can be affected by conditions of immune dysregulation. Our research group previously assessed IGRAs in several immune-related disorders, detecting a percentage of indeterminate results of 2.1% in pre-transplant cirrhotic patients [13], 7.7% in patients with corticosteroids who are going to receive anti-TNF alpha treatments [14], or 9.5% in HIV-positive patients without antiretroviral treatment and a CD4 count <100 cells/mm^3 [15]. Of great concern, the present study found that 35.4% of the hospitalized COVID-19 patients studied had an indeterminate QFT-Plus assay result. This proportion is consistent with the 36.4% rate reported by Torre et al. in a recent work from an Italian series [10]. So, the frequency of indeterminate results might be significantly high in COVID-19 patients.

In our study, the QFT-Plus test was mostly performed in severe COVID-19 patients hospitalized after 10 days of symptoms onset, and the results obtained are representative of the COVID-19 inflammatory phase [16]. According to previous reports, COVID-19 patients at this inflammatory stage of the disease show high plasma proinflammatory cytokines levels (IL-1b, IL-2, IL-4, IL-7, IL-10, MCP-1, GCSF, MIP-1A, TNF-α, IFN-γ and IP-10) [17,18]; however, this fact should not alter the accuracy of the QFT-Plus test since indeterminate results were not due to a high IFN-γ concentration in the nil tube (>8 UI/mL) [8].

Therefore, we investigated those potential factors which may cause indeterminate QFT-Plus results due to IFN-γ concentrations in the mitogen-based control below the limit established by the test manufacturer (that is, nil-corrected mitogen value < 0.5 IU/mL). Accordingly, our rationale was that COVID-19 patients at severe stages might suffer for some kind of immunosuppressant condition. According to our data, two groups of factors might be associated with indeterminate results: firstly, some determinants that have been previously related to worse COVID-19 outcome (lymphopenia, leukocytosis, neu-

trophilia, LDH, D-dimer, hypoxemia) [17,19] and, secondly, the use of immunosuppressive treatments administered before the QFT-Plus assay, especially corticosteroids.

Corticosteroids and other immunosuppressive drugs are well known to be the cause of indeterminate QFT-Plus assay results [14]. However, it should be noted that the indeterminate results rate remained high (14.7%) even in the subset of our studied patients who had not received any immunosuppressive treatment prior to test, highlighting COVID-19 itself as a determining factor of an indeterminate result.

SARS-CoV-2 has evolved several mechanisms to impair host immune response, including the inhibition of IFN induction and signaling. In fact, impaired IFN-I/III signatures have been related to a persistent blood viral load and an inflammatory disturbance that leads to a worse COVID-19 prognosis [18,20]. Nevertheless, there are conflicting data regarding the role of type II IFNs such as IFN-γ. In several studies, elevation of multiple inflammatory cytokines, including IFN-γ, has been related to a more severe COVID-19 [18,21]. On the other hand, recent data show that COVID-19 has the ability to induce an early and profound suppression of T-cell IFN-γ production [22]. Possibly, these data suggest that timing is the key, as IFN-γ could be protective early in a disease but later could become pathologic and increase as a consequence of multiple stimuli received by the hyperinflammatory cytokine storm. In clinical practice, there is a lack of diagnostic tools to evaluate the failure of host protective immunity while suffering from COVID-19. In this respect, the QFT-Plus test might be a standardized and accessible functional immunoassay to study the IFN- γ host immunity.

Worryingly, the rate of patients with an indeterminate QFT-Plus assay result was higher in patients who died during admission (64.7% vs. 29.1%). Several factors have been related to in-hospital mortality in COVID-19 [14,16], but there is scarce information regarding the role of an indeterminate QFT-Plus result as a marker of severity or death.

This preliminary investigation of COVID-19 patients with an indeterminate QFT-Plus assay result has several limitations that deserve further comment. We performed a retrospective analysis so confounders could be overlooked and missing data might have altered some results. Only 6.8% of COVID-19 patients admitted to hospital were tested for QFT-Plus assay, and therefore this is a highly selected group with potential for bias. Furthermore, the QFT-Plus assay was unsystematically performed in hospitalized patients with COVID-19, although in most cases was done in those who transited into inflammatory stages receiving or going to receive immunosuppressive agents such as corticosteroids and/or tocilizumab. Therefore, the present study does not allow us to assess the QFT-Plus assay usefulness in patients at earlier stages of the disease. The number of COVID-19 patients on whom a QFT-Plus assay was performed is relatively low to draw solid conclusions about its role in predicting clinical outcomes of the disease.

5. Conclusions

The indeterminate QFT-Plus assay results show an unexpected, high prevalence in hospitalized COVID-19 patients with host-immune hyperinflammatory syndrome, losing reliability in this context. Factors related to worse COVID-19 outcome such as LDH, as well as corticosteroid use before the QFT-Plus assay, seem to be predictors for an indeterminate result. Furthermore, indeterminate QFT-Plus results were found in a higher proportion in those COVID-19 patients who died. Therefore, further and larger studies are needed to assess the real significance of indeterminate QFT-Plus test results on COVID-19 clinical outcomes.

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Data Availability Statement: The data sets generated and analyzed during the current study are available on request to the corresponding author upon reasonable request.

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ARTICLE 4

Objectiu

Determinar l'eficàcia i la seguretat dels polsos de metilprednisolona juntament amb tacrolimus, afegits a l'*Standard of Care*, en pacients hospitalitzats per una COVID-19 greu.

Títol

Pragmatic, open-label, single-center, randomized, phase II clinical trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus in patients with severe pneumonia secondary to COVID-19: The TACROVID trial protocol.

Solanich X*†, Antolí A, Padullés N, Fanlo-Maresma M, Iriarte A, Mitjavila F, Capdevila O, Molina M, Sabater J, Bas J, Mensa-Vilaró A, Niubó J, Calvo N, Bolivar S, Rigo-Bonnin R, Arregui L, Tebé C, Hereu P, Videla S, Corbella X.

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Contribució del doctorand: revisió de la literatura, disseny i promoció de l'assaig clínic, preparació de dades, redacció i presentació de l'article.

Aprovació CEIm **Aprovació AEMPS**

Resum

Introducció: Alguns pacients amb COVID-19 desenvolupen una afectació pulmonar greu amb una síndrome hiperinflamatòria sistèmica desencadenada tant per la infecció per SARS-CoV-2 com per la posterior resposta immune de l'hoste. En aquest sentit, quan vàrem dissenyar l'assaig (març de 2021), s'intuïa que l'ús d'agents immunomoduladors podria ser útils però el seu ús encara era molt controvertit. La nostra hipòtesi de treball era que els polsos de metilprednisolona i el tacrolimus podia ser una combinació de medicaments eficaç i de segura per tractar pacients amb COVID-19 greus.

Mètodes: TACROVID va ser un assaig clínic randomitzat, obert i unicèntric per avaluar l'eficàcia i la seguretat de polsos de metilprednisolona i tacrolimus, afegits a l'*Standard of Care*, respecte l'SoC solament, per tractar a pacients amb COVID-19 que desenvolupaven lesió pulmonar i resposta hiperinflamatòria sistèmica. Els pacients es varen assignar aleatòriament (1:1) a un dels dos braços de tractament (42 pacients de cada grup). L'objectiu principal va ser avaluar el temps fins a l'*estabilitat clínica* des de l'aleatorització. L'*estabilitat clínica* es va definir com a temperatura corporal ≤37,5°C; i PaO_2/FiO_2> 400 i/o $SatO_2/FiO_2$> 300; i freqüència respiratòria ≤24 rpm; durant 48 hores consecutives.

Discussió: Vàrem considerar que la metilprednisolona i el tacrolimus podien ser beneficiosos per tractar als pacients amb COVID-19 que evolucionaven cap a una insuficiència respiratòria greu i una síndrome hiperinflamatòria sistèmica. El fonament del seu ús era el ràpid efecte dels polsos de metilprednisolona i la capacitat del tacrolimus per inhibir tant la replicació del CoV-2, així com la posterior tempesta de citosines. Ambdós medicaments tenen un baix cost i es poden fabricar a gran escala; per tant, si fossin efectius i segurs, un gran nombre de persones podrien ser tractats als països desenvolupats i en desenvolupament.

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Pragmatic, open-label, single-center, randomized, phase II clinical trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus in patients with severe pneumonia secondary to COVID-19: The TACROVID trial protocol

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ABSTRACT

Introduction: Some COVID-19 patients evolve to severe lung injury and systemic hyperinflammatory syndrome triggered by both the coronavirus infection and the subsequent host-immune response. Accordingly, the use of immunomodulatory agents has been suggested but still remains controversial. Our working hypothesis is that methylprednisolone pulses and tacrolimus may be an effective and safety drug combination for treating severe COVID-19 patients.

Methods: and analysis: TACROVID is a randomized, open-label, single-center, phase II trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus plus standard of care (SoC) versus SoC alone, in patients at advanced stage of COVID-19 disease with lung injury and systemic hyperinflammatory response. Patients are randomly assigned (1:1) to one of two arms (42 patients in each group). The primary aim is to assess the time to clinical stability after initiating randomization. Clinical stability is defined as body temperature

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≤37.5 °C, and PaO2/FiO2 > 400 and/or SatO2/FiO2 > 300, and respiratory rate ≤24 rpm; for 48 consecutive hours.
Discussion: Methylprednisolone and tacrolimus might be beneficial to treat those COVID-19 patients progressing into severe pulmonary failure and systemic hyperinflammatory syndrome. The rationale for its use is the fast effect of methylprednisolone pulses and the ability of tacrolimus to inhibit both the CoV-2 replication and the secondary cytokine storm. Interestingly, both drugs are low-cost and can be manufactured on a large scale; thus, if effective and safe, a large number of patients could be treated in developed and developing countries.
Trial registration number: NCT04341038 / EudraCT: 2020-001445-39

Abbreviations

CoV = Coronavirus
SARS-CoV = Severe Acute Respiratory Syndrome Coronavirus
MERS-CoV = Middle East Respiratory Syndrome Coronavirus
SARS-CoV-2 = Severe Acute Respiratory Syndrome Coronavirus 2
COVID-19 = Coronavirus disease 2019
SoC = Standard of Care
PaO2 = arterial oxygen partial pressure
SatO2 = Oxygen saturation
FiO2 = fractional inspired oxygen
PIC = pro-inflammatory cytokines
HLH = hemophagocytic lymphohistiocytosis
CADM = clinically amyopathic dermatomyositis
MDA-5 = anti-melanoma differentiation-associated gene 5
RT-PCR = Reverse transcription polymerase chain reaction
LDH = lactate dehydrogenase
CRP = c-reactive protein
ALT = alanine aminotransferase
ICF = informed consent form
ECMO = extracorporeal membrane oxygenation
GFR = Glomerular filtration rate
BMI = body mass index
PSI = Pneumonia Severity Index
SOFA score = Sequential Organ Failure Assessment Score
6MWT = 6-min walk test
eCRF = electronic case report form
ARDS = acute respiratory disease syndrome
JAK = Janus Kinase
IL = interleukin
BUH = Bellvitge University Hospital

1. Background

In December 2019, an emerging disease (COVID-19), caused by a newly identified human coronavirus (SARS-CoV-2), was first recognized in Wuhan, China and spread worldwide [1,2]. The WHO declared the COVID-19 epidemic to be a pandemic on March 12, 2020 [3].

Unfortunately, there are still no proven effective and safe therapies for treating the COVID-19 illness other than supportive care. Despite the lack of evidence, the urgency of care leads to a large number of severe patients receiving off-label and compassionate use therapies, based on their in vitro antiviral or immunomodulatory properties. Among suggested treatments, the repositioning of older drugs may be a plausible strategy given that their proven safety profile [4,5]. Furthermore, there are several ongoing randomized controlled trials (RCTs) assessing different drug regimens for treating patients with COVID-19 [6].

Of great concern, some COVID-19 patients evolve to fatal lung injury and multi-organ failure due to the serious inflammatory process triggered by the viral infection [7–12]. While the use of some immunosuppressive drugs has been reported to be potentially harmful, other such drugs, paradoxically, have been justified in treating the excessive inflammation triggered by the viral infection [13]. Unfortunately, studies performed following pragmatic randomized controlled trials (pRCT) are still very limited to date and current international recommendations have not taken a position either in favor of or against the use of immunomodulatory therapy in such patients.

Our working hypothesis is that methylprednisolone pulses and tacrolimus might be an effective and safe drug combination strategy for severe COVID-19 patients. Accordingly, given the rapid spread of COVID-19 and the current health emergency worldwide, we performed a proof-of-concept study in a randomized, open-label, single-center, clinical trial to evaluate the efficacy and safety of methylprednisolone and tacrolimus plus standard of care (SoC), versus SoC alone, in severe COVID-19 patients with lung injury and systemic hyperinflammatory syndrome.

The rationale for the present pRCT is based on the fact that glucocorticoids such as methylprednisolone are a mainstay in the treatment of several immune-mediated disorders, with multiple mechanisms of action involving both the humoral and cell-mediated arms of immunity. As for tacrolimus, the rationale for its use is based on its specific mechanism of action leading to an impaired lymphocyte function and a decrease in pro-inflammatory cytokines (PIC) [14]. Interestingly, severe COVID-19 disease presents a fairly similar clinical and cytokine profile to other diseases such as secondary hemophagocytic lymphohistiocytosis (sHLH) [15] or clinically amyopathic dermatomyositis (CADM) associated with anti-melanoma differentiation-associated gene 5 (MDA-5), where calcineurin inhibitors such as tacrolimus or cyclosporine play a central role in its treatment [16]. Furthermore, some human coronavirus (CoV) replication depends on active immunophilin pathways, which can be inhibited by tacrolimus, at low, non-cytotoxic concentrations in cell culture [17]. In this regard, anecdotal case series suggest that tacrolimus may exert a protective effect in solid organ transplanted patients affected by MERS-CoV [13]. Thus, tacrolimus has an immunosuppressive effect but would also block viral replication and may have beneficial impacts on severe COVID-19 lung injury.

2. METHODS: (no word limit)

2.1. Study design

TACROVID is a pragmatical, randomized (1:1), open-label, single-center, phase II clinical trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus plus SoC, versus SoC alone, in severe COVID-19 patients with lung injury and systemic hyperinflammatory syndrome.

2.2. Population

Patients are being prospectively recruited and included in the study for subsequent randomization, if they meet all the inclusion criteria and there is no reason for exclusion.

2.2.1. Inclusion criteria

1) COVID-19 infection confirmed by nasopharyngeal RT-PCR,
2) New pulmonary infiltrates (either by chest X-ray or computerized axial tomography),
3) Respiratory failure (PaO2/FiO2 <300 or satO2/FiO2 <220),
4) High analytical inflammatory parameters (CRP >100 mg/L, and/or D-Dimer >1000 μg/L and/or Ferritin >1000 μg/L).

2.2.2. Exclusion criteria

1) Critically ill patients with life expectancy ≤24h,
2) Glomerular filtration ≤30 mL/min/1.73 m^2,
3) Leukopenia ≤4000 cells/μL or other conditions that cause immunosuppression,
4) Concomitant potentially serious infections,
5) Contraindication for the use of corticosteroids or tacrolimus according to the Summary of Product Characteristics,
6) Known hypersensitivity to any of the study drugs, their metabolites, or formulation excipient,
7) Previous participation in a RCT in the last 3 months.

All patients (or a legal representative) have to provide informed consent (ICF) prior to initiation of the study procedures.

2.3. Setting

The TACROVID trial is being conducted at Bellvitge University Hospital (BUH), a 750-bed tertiary care public hospital in Barcelona (Catalonia, Spain). BUH is the reference hospital for high complexity patients from the Southern Area of Catalonia, a region of approximately 2 million inhabitants.

2.4. Randomization

On day 0, patients are centrally and randomly assigned using the RedCap computer platform that allows data collection and patient randomization. Patients are not stratified by baseline variables. Participants are randomized in a 1:1 ratio into one of two study groups: methylprednisolone pulses, tacrolimus and SoC, or SoC alone.

2.5. Treatment

Patients are being randomly assigned to one of the following arms:

1. Experimental: methylprednisolone pulses of 120 mg/day are administered on 3 consecutive days (if not previously administered) and immediate release tacrolimus is administered twice daily until clinical stability is achieved. Tacrolimus dosing is individualized through therapeutic drug monitoring (TDM) to achieve blood trough levels of 8–10 ng/mL. In addition, patients in the experimental arm can receive standard of care (SoC) for their management in accordance with treating physicians.
2. Control (SoC): SoC includes measures of supplemental oxygen and respiratory support, fluid therapy, antipyretic treatment, postural measures, low molecular weight heparins, and may also include other treatments, whether antiviral or immunosuppressive drugs, or those necessary at the discretion of the treating physician, except for cyclosporine and/or tacrolimus.

The experimental drugs start immediately after having been randomly assigned to this group. The experimental treatment is discontinued after "*clinical stability*", which is achieved after fulfilling all of the following criteria for 48 consecutive hours: body temperature ≤37.5 °C; PaO2/FiO2 > 400 and/or SatO2/FiO2 > 300; and respiratory rate ≤24 rpm.

Experimental treatment is also discontinued if the included patient has a severe or potentially severe infection, required invasive mechanical ventilation, extracorporeal membrane oxygenation (ECMO), or has any serious medication-related adverse event (of special interest refractory HTA, decrease of more than 50% of the GFR compared to the baseline, or ventricular tachycardia).

2.6. Study visits

All patients are followed from day 0 through day +56 or death. The planned visits are: 1) randomization visit (day 0) in which the patient is screened and eventually included in the study; 2) Hospitalization visits that are variable according to the period of admission; 3) Visits on day +28 ± 3 and on day +56 ± 3 from randomization. These visits are face-to-face to evaluate whether patients present a relapse or worsening of the disease, as well as the presence of adverse events or mortality. Specific procedures that are performed on each visit are detailed in Table 1 and the *evaluation methods* section.

2.7. Evaluation methods

2.7.1. Clinical parameters

The randomization visit (day 0) includes the collection of demographic variables [date of birth, sex, ethnicity], weight and height, clinical data [symptoms onset date, hospital admission date, clinical manifestations, radiological characteristics of pulmonary infiltrates], comorbidities [smoking and alcohol consumption, Charlson index],

Table 1
Flow-chart of the trial showing the procedures that will be conducted in each visit.

	Randomization visit (day 0)	Hospitalization visits (days n)	Visit 28 ± 3 days after the start of treatment	Visit 56 ± 3 days after the start of treatment
Inclusion/ exclusion	x			
Informed consent	x			
Randomization	x			
Demographics Comorbidities	x			
COVID-19 clinical data	x			
Vital signs	x	x	x	x
8-point Ordinal scale	x	x	x	x
Analysis (Hematology, Chemistry, Coagulation)	x	x	x	x
Tacrolimus levels		x		
Cytokines	x	x	x	x
Viral load (blood)	x	x	x	x
Nasopharyngeal SARS-CoV-2 PCR	x		x	x
Clinical stability	x	x	x	x
Lung tests (x-ray, CT scan, chest US, 6MWT, functional tests)	x	x (1)	x (1)	x
Study treatment	x	x	x	x
Concomitant medication	x	x	x	x
AE registration	x	x	x	x

(1) according to the attending physician.

functional and cognitive assessment [Barthel and Pfeiffer index respectively], 8-point Ordinal scale (https://clinicaltrials.gov/ct2/show/NCT04280705), vital signs [temperature, blood pressure, heart rate, respiratory rate, oxygen assessment and supply (type of oxygen mask and FiO2, non-invasive or invasive ventilator support devices, or ECMO, PaO2/FiO2 and/or SatO2/FiO2), and conscious level using the Glasgow Coma Scale], as well as concomitant medication. With all these data we automatically calculate body mass index (BMI), the Pneumonia Severity Index (PSI) and CURB-65 index, and determine the extent of organ failure (SOFA score). The hospitalization visits include the collection of vital signs, 8-point Ordinal scale, adverse events and mortality. Visits on day +28 ± 3 and +56 ± 3 include vital signs, ordinal scale, relapse or worsening of the disease, adverse events and mortality.

2.7.2. Tests and other procedures

A blood analysis is performed before starting the study treatment with the following biomarkers: 1) hematology (hemoglobin, hematocrit, platelet count, absolute lymphocyte count, leukocyte count with differential) assessed using Sysmex® XN2000 analyzer (Sysmex Europe GmbH; Norderstedt; Germany); 2) basic chemistry (creatinine, albumin, ALT, total bilirubin) and inflammatory parameters (ferritin, D-dimer, CRP, LDH) measured with Cobas® 6000/8000 analyzer (Roche Diagnostics®, Risch-Rotkreuz, Switzerland), which has spectrophotometry and immunochemistry modules with electrochemiluminescent detection; and 3) coagulation (activated partial thromboplastin time, prothrombin time and fibrinogen) using ACLTOP® 550 analyzer (Werfen®; Barcelona, Spain). Furthermore, all patient samples are collected, processed and stored at randomization, and weekly after randomization by the BUH-IDIBELL Biobank following standard operating procedures. During hospitalization, a blood analysis is performed 3 times a week with the same parameters as used at randomization. Tacrolimus levels are measured using a method based on ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) [18].

The QTc interval is evaluated using an ECG before randomization, and an electrocardiogram 3 times a week is recommended during hospitalizations. All pulmonary tests performed during hospitalization at the discretion of the treating physician are being collected (chest x-Ray CT scan). A chest CT scan, the 6-min walk test (6MWT) and functional respiratory tests on day +56 ± 3 follow-up visit are performed.

In addition, a follow-up oropharyngeal SARS-CoV-2 RT-PCR is performed on day +28 ± 3 and +56 ± 3 visits. Blood viral load will be evaluated using quantitative RT-PCR to assess the impact of immunosuppressive treatment on viral dynamics. Microbiological tests are being carried out by the Microbiology Department at the BUH.

All samples will be sent to the Immunology Laboratory of the Biomedical Diagnostic Center at the Clinic Hospital in Barcelona to perform a multiple cytokines quantification technique by Luminex®. Concentration of the following cytokines will be analysed: IL-6, IL-18, CXCL10, CXCL9, TNF-alpha, IFN-alpha, IFN-beta, IFN-gamma, IL1RA, IL1-beta, IL10 and IL-2R/CD25.

2.8. Outcomes

2.8.1. Primary end point

Average of days until *clinical stability* from treatment randomization.

Treatment failure is defined as: 1) patient who does not meet the criteria for clinical stability 56 days after starting treatment; 2) patient presenting with a grade 3 or 4 adverse event attributed to trial treatment; or 3) patient who dies after being included in the clinical trial.

2.8.2. Secondary endpoints

- Days until normalization of each of the clinical parameters (body temperature, PaO2/FiO2 and/or SatO2/FiO2, respiratory rate) from day +1 through day +56.
- Days until normalization of each of the analytic parameters (D-dimer, CRP, ferritin, LDH, IL-6) from day +1 through day +56.
- Number of patients requiring non-invasive and invasive ventilatory support devices during hospitalization.
- Clinical status according to the 8-point Ordinal scale from day +1 through day +56.
- Number of patients who reach a clinical status ≤2 after 10 days or hospital discharge, whichever is first.
- Number of patients who reach clinical stability after 10 days or hospital discharge, whichever is first.
- Value of each of the analytical values (D-dimer, CRP, ferritin, LDH, IL-6) after 10 days or hospital discharge, whichever is first.
- Days with trial experimental treatment.
- Days until hospital discharge.
- Changes in blood quantitative viral load by RT-PCR before start treatment and weekly during hospitalization.
- Changes in expanded cytokine profile before starting treatment and weekly during hospitalization.
- Long-term efficacy (28 and 56 days after trial treatment initiation) measuring whether clinical stability are maintained and the incidence of relapse of COVID-19 illness.
- Description of the radiological abnormalities (chest x-ray, chest CT scan, functional tests) at day 56.
- Adverse events according to their severity and relationship to trial experimental treatment.
- COVID-19 related mortality at day +28 and + 56 after randomization.
- All-cause mortality at day +28 and + 56 after randomization.

2.8.3. Criteria for withdrawing a patient from the study

Patients must be withdrawn from the study in any of the following situations: 1) any adverse event for which clinicians consider it necessary to withdraw trial experimental therapy; 2) the patient requests to be withdrawn from the study (at any time during the patient's participation); 3) the principal investigator (PI) considers that there has been a serious protocol violation; 4) lost to follow-up; and 5) pregnancy during the study.

When a patient withdraws from the study, the investigator records the reason/s in the clinical chart and the electronic case report form (eCRF). If the reason for withdrawal is a serious adverse event, the patient must be followed-up until resolution or stabilisation of the event. Patients who withdraw will not be replaced.

2.9. Statistical analysis

2.9.1. Determination of sample size

The median time to clinical stability in the control group is expected to be 16 days. If the hazard ratio of clinical stability of control patients in relation to the patients in the experimental group were 0.52, it would be necessary to include 42 patients in each group in order to be able to reject the null equality hypothesis with a power of 80%. The probability of Type I error associated with this hypothesis test is 0.05 and includes 5% of withdrawal.

2.9.2. Statistical considerations

A descriptive analysis of the study variables will be carried out. Continuous variables will be described as mean and Standard Deviation (SD) or as median and range; and categorical variables as absolute frequencies and percentages. The main analysis will be performed when all patients reach clinical stability or failure. If patients are in hospital for 56 days, they will be considered clinical failures. Comparison between average time to achieve clinical stability between the two study groups will be measured using the log-rank test. To quantify the degree of association, hazard ratio will be estimated with a Cox proportional hazards model using a 95% confidence interval.

Efficacy analyses will be performed for the intention to-treat (ITT)

population. If a proportion of subjects >10% is detected to have relevant deviations from the protocol (for example, non-compliance with the selection criteria, non-compliance or inability to receive the trial treatment), the per protocol (PP) analysis will exclude those subjects with such deviations. If this group is defined, all exploratory efficacy analyses will be repeated as a sensitivity test.

As additional analysis, a Cox proportional hazards model, adjusted for clinically relevant confounding factors such as age, sex, comorbidities using the Charlson index, indexes to assess the severity of pneumonia (PSI and CURB-65), index of organic dysfunction (SOFA) and inflammatory parameters will be performed. In addition, prespecified subgroup analyses involving sensitivity analysis will be used to evaluate outcomes in patients not receiving invasive mechanical ventilation.

For evaluation of secondary variables, we will calculate unadjusted and adjusted estimations of effect size and the corresponding 95% confidence intervals using linear, logistic or proportional hazards Cox regression. Study adverse events will be described according to their severity and relationship with other treatments, and will be compared between treatment groups. Midway through recruitment, i.e. 21 patients per arm, an intermediate efficacy and safety analysis will be performed. For this, a correction of the type I error will be applied following LanDeMets (O'Brien – Fleming) in evaluation of efficacy.

The IDIBELL Biostatistical Unit will perform analysis and analysts will be blind to the treatment received by the patients (intervention vs. usual care). R version 3.6.2 or higher for Windows (R Foundation for Statistical Computing, http://www.r-project.org) will be used for data treatment and analysis.

2.10. Monitoring

An electronic case report form (eCRF) has been created using the RedCap computer platform. IDIBELL Clinical Research and Clinical Trials Unit (UICEC-IDIBELL) is carrying out the monitoring of the trial. Regular monitoring is performed by the UICEC IDIBELL according to the ICH GCP. Compliance with the approved protocol and verification of data is evaluated according to applicable regulatory requirements. The key data in the Monitoring Plan are those related to informed consent, primary outcome, mortality and safety data.

2.11. Adverse events reporting and quantification

2.11.1. Definitions

- *Adverse event (AE)*: any injury related to medical management (including all aspects of care) that occurs during the patient's participation in the clinical trial will be considered an adverse event. An adverse event may be related to the study medication or be non-related.
- *Adverse drug reaction (AR): any 'adverse drug event' that occurs when the medication is used as directed and in the usual dosage will be considered an adverse drug reaction.*
- Serious AE (SAE) or serious AR (SAR) are considered to be those that at any dose:
  - provoke death (Note: death is a possible evolution of SARS-CoV-2 infection);
  - put patient's life at risk;
  - require the patient's hospitalization or the extension of an existing hospitalization.
  - cause permanent or significant disability or incapacity;
  - cause a congenital anomaly or malformation;
  - are considered medically relevant.
- *Adverse drug event of particular interest for the study*: The investigator shall record in the eCRF and communicate to the promoter, the AE that are considered of special interest as soon as possible and no later than 15 days after he becomes aware of them. The AEs that are considered of special interest are:
  - refractory hypertension (defined as poor blood pressure control despite 3 antihypertensive drugs including a diuretic);
  - renal impairment (decrease of more than 50% of the GFR compared to the baseline);
  - ventricular tachycardia.

2.11.2. Reporting

The UICEC-IDIBELL is carrying out pharmacovigilance of the trial. All SAEs (including death), regardless of their relationship with the investigational medications, have to be notified by the investigator within 24h. The investigator has to make the notification via the SAE Notification Form, sending it by email to the pharmacovigilance unit that will review it and, if appropriate, will request additional information from the investigator. The investigator will provide information to the promoter or pharmacovigilance unit whenever requested and, in any case, when its initial evaluation changes regarding severity or causation. Likewise, all additional information regarding the AE, until the end of the study or until its definitive outcome, must be communicated without delay, via follow-up reports following the notification procedure previously described.

In case of a suspected unexpected serious adverse reaction (SUSAR), UICEC-IDIBELL will report to the competent authorities. The sponsor will also report the development update safety report (DSUR) once a year to the ethics committee and the Spanish Agency for Drugs and Health Products (AEMPS).

2.12. Ethical considerations

This trial conforms to the Declaration of Helsinki and Good Clinical Practice guidelines, and personal data protection as required by Spanish law (LOPD 3/2018). The protocol and informed consent form (ICF) were approved by the BUH's Ethical Committee for Drug Research (EC) and by the Spanish Agency for Drugs and Health Products (AEMPS) in March 2020, in accordance with current legislation, Royal Decree 1090/2015 of December 4 and European Regulation 536/2014 of April 16, regulating clinical trials with drugs.

The patient, his or her closest legal or family representative (in case of incapacity due to the severity of the clinical situation) has to accept the ICF. As patients with COVID-19 can infect researchers via the ICF document, patients can consent orally with a witness, and this is documented in the patient's medical history. If the legal representative or a close relative is in quarantine due to COVID-19 quarantine, informed consent is provided orally by telephone and documented in the patient's medical history. The written ICF is obtained when the patient or his/her closest legal or family representative are able to give consent, as they are not quarantined.

Methylprednisolone and tacrolimus are commercially available as generic drugs, so no special permissions are required. Both the promoter and the center are respectively responsible for the treatment of patient data and commit to comply with Regulation (EU) 2016/679 of the European Parliament and of the Council of April 27, 2016 on Data Protection (RGPD), as well as with all other laws and regulations in force and applicable (Organic Law on Protection of Personal Data and Guarantee of Digital Rights 3/2018 of December 05). The data collected for the study are identified by a code, so as not to include any information that can identify the patient (name, surname, initials, address, social security number, etc.). Only the study clinician/collaborators are able to relate such data to the patient and his/her medical history. Therefore, a patient's identity is not revealed to any other person except the health authorities when required, or in the case of medical emergency. Access to patient information is restricted to the attending physicians, the health authorities AEMPS, the Clinical Research Ethics Committee, and personnel authorised by the sponsor when they need to check the data and procedures used in the study, always maintaining confidentiality in accordance with current legislation. If participants wish to know more, they can contact the Promoter's Data Protection Officer.

2.12.1. 1 Protocol amendments

The protocol was approved by the EC and the AEMPS on March 31, 2020, in a process of authorization adapted to the pandemic situation and based on a protocol synopsis (March 28, 2020). A substantial amendment to the original protocol was submitted to the EC and the AEMPS in accordance with Spanish legislation, and accepted on April 9, 2020. We decided to reduce tacrolimus plasma levels from 10 to 15 to 8–10 ng/mL and not to maintain corticosteroids by protocol beyond the three pulses of methylprednisolone in the experimental group. In addition, some exclusion criteria were added, such as glomerular filtration ≤30 mL/min/1.73m2, leukopenia ≤4000 cells/μL or other conditions that cause immunosuppression, and concomitant and potentially serious infections. Another additional amendment was accepted by the EC and AEMPS on May 25, 2020, including a complete clinical study protocol with new secondary key outcomes (8-point Ordinal scale and radiological abnormalities) that are being analysed in several COVID-19 clinical trials. These outcomes are being prospectively reported in the trial's eCRF, but were not reported in the protocol synopsis. We do not remove any of the previous key study outcomes.

2.13. Publication plans

The trial is currently actively recruiting patients. Completion of patient recruitment is expected for Q3-Q4 of 2020. The sponsor commits itself to publishing the data within 12 months of completion of the study. Results will be analysed and reported in accordance with CONSORT guidelines.

3. Discussion

Clinically, COVID-19 patients can evolve into 3 stages of progressive severity. Viral incubation and early establishment of the disease are the predominant components in the first week from the onset of symptoms. In the second stage, viral replication and transition into moderate acute lung involvement are the most important components. The third and most advanced stage manifests as severe pulmonary injury and systemic multi-organ failure, resulting from cytokine storm and systemic hyperinflammatory response. Older age, comorbid chronic conditions, elevated body mass index, lymphopenia, and elevated transaminases, LDH, D-dimer, ferritin, and soluble IL-2 receptor (sIL-2R) on admission are some of the reported risk factors associated with higher mortality [7–12]. From a pathological viewpoint, two different pathological mechanisms appear to coexist in the COVID-19 illness; the first, triggered by the virus itself, and the second, by the host-immune response. Accordingly, a two-step sequence of antiviral and anti-inflammatory drug administration has been proposed, regarding the natural 3-stage evolution of COVID-19 disease [19].

Despite randomized clinical trials (RCTs) being the only way to find effective and safe treatments, very limited available RCTs for treating COVID-19 have been reported to date [20]. Given the emergency situation, most patients are receiving compassionate, unproven therapies to avoid clinical progression into severe advanced stages where host-immune inflammatory response is the most important component [21].

Several studies demonstrate that cells infected by coronavirus (CoV) produce elevated levels of PICs in order to tackle the invading virus. The overproduced PICs may cause immuno-mediated damage to the lungs and other organs, resulting in severe lung injury and systemic hyperinflammatory syndrome [22]. Once this excessive inflammatory response has been triggered, it may self-perpetuate in some patients despite a decrease in the CoV viral load. So, it may be necessary to add "anti-inflammatory" treatments such as corticosteroids, anti-IL-6 or anti-IL-1 inhibitors, Janus kinase (Jak) inhibitors, polyclonal immunoglobulins, etc. to reduce the secondary deleterious inflammatory response triggered by the virus [6].

In this regard, glucocorticoids appear as a key first-line option, especially given their worldwide availability and cost. However, their use has been controversial in patients with SARS-CoV, MERS-Cov or influenza infections. On the one hand, intravenous steroid use has been associated with delayed elimination of coronaviruses in the blood and lungs of patients with MERS-CoV [23] and SARS-CoV [24], and steroids associated with an increased risk of mortality and adverse events in influenza patients [25–27]. On the other hand, a Cochrane review of glucocorticoids as adjunctive therapy in influenza found evidence of low quality due to confounding by indication [28], and there are some series which suggest improvement of severe COVID-19 lung injury after administration of steroids [8,10].

Furthermore, a small retrospective observational study conducted in China [29] suggests the efficacy of tocilizumab (an IL-6 inhibitor) in the treatment of 21 COVID-19 patients with severe pneumonia and high IL-6. But tocilizumab can cause even deeper immunosuppression than steroids, increasing the risk of sepsis, bacterial pneumonia, gastrointestinal perforation, and hepatotoxicity [20]. Another drawback of tocilizumab is its shortage in some hospitals worldwide and its high cost to national health systems. Interestingly, IL-1 blockade has shown particular promise in cytokine storm syndrome and high-dose regimens have been shown safe even in the context of overt sepsis [30]. Emapalumab (anti-IFNγ) is FDA-approved for HLH and may be effective in COVID-19 inflammatory phase. JAK inhibition appears promising; however, the safety of this medication in severe viral infection remains unknown. Accordingly, some ongoing RCTs are studying glucocorticoids and blockage of IL-1, IL-6, and IFNγ in COVID-19 [6]. However, at the end of April 2020, no trials assessing calcineurin inhibitors, whether tacrolimus or cyclosporine, had been registered either at www.clinicaltrials.gov nor EU Clinical Trials Register, thus prompting us to carry out the present investigation.

Given the scarcity of medical reports supporting the use of immunosuppressive therapy in severe COVID-19, the TACROVID trial was initiated on March 29, 2020. The study is a pragmatical, randomized, open-label, single-center, phase II clinical trial to evaluate the effectiveness and safety of methylprednisolone and tacrolimus in combination regimen plus SoC, versus SoC alone, in COVID-19 with severe lung injury and systemic hyperinflammatory syndrome. We decided to consider methylprednisolone pulses for their rapid onset of action, combined with the ability of tacrolimus to inhibit both the multiple PICs, and the SARS-CoV replication in cell cultures. Moreover, the rationale of our study is aligned with an Ovid MEDLINE review article [13] searching for current evidence for immune-suppressing or stimulating drugs to treat COVID-19, which concludes that low-dose methylprednisolone and tacrolimus may have a beneficial impact in such COVID-19 populations.

Tacrolimus has been administered for decades to prevent allograft rejection in transplanted patients as well as to treat patients with severe autoimmune diseases, and it has a well-known safety profile. In addition, it is a low-cost drug and can be manufactured on a large scale. Thus, if tacrolimus is effective in treating the inflammatory process triggered by COVID-19, a large number of patients could be treated in developed and developing countries. Despite the fact that tacrolimus has been shown to be safe for other diseases, we [researchers] are particularly aware of the possible occurrence of associated AE throughout the present TACROVID trial. In this respect, most patients in the advanced stage of the COVID-19 illness are currently given on immunosuppressive treatment and it is well-known that this facilitates certain opportunistic infections, making it difficult to attribute them to one particular drug. Moreover, the drawback of tacrolimus is that it may interact with some antiviral treatments used in COVID-19 (especially Lopinavir). Finally, tacrolimus is started when patients transition into the most severe clinical stage which, in turn, facilitates the occurrence of AE.

Interestingly, in addition to its immunomodulatory effect, tacrolimus has been also reported to show certain in vitro activity against different human coronaviruses, inhibiting viral replication through immunophilins, although its efficacy and safety have not been assessed in clinical

practice. For this reason, an interim analysis will be conducted when half of patients (21 per arm) will be recruited in order to measure the basal and weekly viral load, and the potential AE related to immunosuppression.

Regarding the study design, the present trial was not designed as a double-blind trial. This was considered unrealistic given the emergency situation and the extensive workload that it would involve for the hospital pharmacy service. For the above-mentioned reasons, the TACROVID trial was designed as an open study which allows the use of all drugs that treating physicians prescribe, even without clear evidence to support their use. To minimize the impact of an open study, the primary end-point "time to clinical stability", is based on objective, quantitative measures for 48 consecutive hours: body temperature, and PaO2/FiO2 and/or SatO2/FiO2, and respiratory rate. Likewise, the statistician performing analysis is blind to the treatment that patient receives (experimental vs control).

Cytokine measurement throughout the trial can reveal new available information on COVID-19 evolution and potential new treatment targets. IFNγ and IL-1β are very relevant cytokine in MAS, but they are not easily assessed in the peripheral blood. CXCL9, a stable chemokine, is a useful surrogate for IFNγ activity in MAS, as are IL-18 and IL-1RA for IL-1β. Thus, in this trial we will not only analyse how the cytokine profile evolves after treatment with tacrolimus, but we will also be able to detect increases in cytokines that can also be treated by other proven specific inhibitors (e.g. IL-18 with Tadekinig alpha, IFNγ with Emapalumab).

In summary, the TACROVID trial assesses the combined use of tacrolimus and methylprednisolone, in addition to the standard of care in patients with severe COVID-19 illness. The aim is to evaluate both the beneficial effect of tacrolimus on controlling viral replication and, in combination with methylprednisolone pulses, modulating the host-immune inflammatory response triggered by the virus. In addition, potential adverse events related to this immunosuppressive therapy are carefully followed-up throughout the investigation. The results of the present trial might encourage further RCTs to assess the efficacy and safety of new antiviral and anti-inflammatory drugs regimens, in sequential combination, at earlier clinical stages of COVID-19.

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Contributors

XS, AA, CT, SB, PH and XC took part in the study design, review of the protocol and manuscript writing. NP, MFM, AI, FM, OC, MM, JS, JB, AMV, JN, NC, SB, RRB, and LA took part in the review of the protocol. All authors read and approved the final manuscript.

Declaration of competing interest

Authors declare no conflicts of interest.

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ARTICLE 5

Objectiu

Determinar l'eficàcia i la seguretat dels polsos de metilprednisolona juntament amb tacrolimus, afegits a l'*Standard of Care*, en pacients hospitalitzats per una COVID-19 greu.

Títol

Methylprednisolone Pulses Plus Tacrolimus in Addition to Standard of Care vs. Standard of Care Alone in Patients With Severe COVID-19. A Randomized Controlled Trial.

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Contribució del doctorand: revisió de la literatura, disseny, implementació, selecció i preparació de dades, presentació dels resultats, redacció i presentació de l'article.

Material suplementari	**col·laboradors**	**protocol de l'assaig**

Resum

Introducció: En alguns pacients amb COVID-19 es produeix una afectació pulmonar greu desencadenada tant per la infecció per SARS-CoV-2 com per la posterior resposta immunitària de l'hoste.

Mètodes: Varem dur a terme un assaig clínic aleatoritzat, fase II, unicèntric i obert, amb l'objectiu d'avaluar l'eficàcia i la seguretat dels polsos de metilprednisolona i tacrolimus, juntament amb l'*Standard of Care* (SoC), vs l'*SoC* solament, en pacients hospitalitzats amb COVID-19 greu. L'*outcome* principal era el temps fins a l'*estabilitat clínica* dins dels 56 dies posteriors a la randomització.

Resultats: De l'1 d'abril al 2 de maig de 2020 es varen incloure prospectivament 55 pacients; 27 van ser assignats al grup experimental i 28 al grup control. El tractament experimental no es va associar amb un menor temps fins a l'*estabilitat clínica* (*hazard ratio* 0,73 [IC 95% 0,39-1,37]) ni es varen observar diferències en la majoria d'*outcomes* secundaris. Les dosis mitjanes acumulades de metilprednisolona varen ser significativament menors (360 mg [IQR 360-842] vs. 870 mg [IQR 364-1451]; p = 0,007) i es varen administrar durant un temps més curt (mediana de 4,00 dies [3,00-17,5] vs. 18,5 dies [3,00-53,2]; p = 0,011) al grup experimental que al grup control. Tot i no arribar a la significació estadística, el grup experimental va mostrar una mortalitat per totes les causes numèricament inferior al grup d'*SoC*, especialment al dia 10 [2 (7,41%) vs. 5 (17,9%); odds ratio 0,39 (IC del 95%: 0,05-2,1); p = 0,282]. El nombre total d'esdeveniments adversos no greus va ser de 42 en cadascun dels dos grups. Els que varen rebre tractament experimental varen tenir una taxa numèricament superior d'esdeveniments adversos infecciosos no greus [16 (38%) vs. 10 (24%)] i esdeveniments adversos infecciosos greus [7 (35%) vs. 3 (23%)] que els que varen rebre solament l'SoC.

Conclusions: L'ús combinat de polsos de metilprednisolona més tacrolimus, a més de l'*SoC*, no va millorar significativament el temps fins a l'*estabilitat clínica* o altres *outcomes* secundaris en comparació amb l'*SoC* solament en hospitalitzats amb COVID-19 greu. Tot i no assolir la significació estadística, els pacients que rebien la teràpia experimental tenien una mortalitat per totes les causes numèricament inferior als que rebien només *SoC*, corroborant estudis recents no aleatoritzats amb inhibidors de la calcineurina.

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Methylprednisolone Pulses Plus Tacrolimus in Addition to Standard of Care vs. Standard of Care Alone in Patients With Severe COVID-19. A Randomized Controlled Trial

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Introduction: Severe lung injury is triggered by both the SARS-CoV-2 infection and the subsequent host-immune response in some COVID-19 patients.

Methods: We conducted a randomized, single-center, open-label, phase II trial with the aim to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus plus standard of care (SoC) vs. SoC alone, in hospitalized patients with severe COVID-19. The primary outcome was time to clinical stability within 56 days after randomization.

Results: From April 1 to May 2, 2020, 55 patients were prospectively included for subsequent randomization; 27 were assigned to the experimental group and 28 to the control group. The experimental treatment was not associated with a difference in time to clinical stability (hazard ratio 0.73 [95% CI 0.39–1.37]) nor most secondary outcomes. Median methylprednisolone cumulative doses were significantly lower (360 mg [IQR 360–842] vs. 870 mg [IQR 364–1451]; p = 0.007), and administered for a shorter time (median of 4.00 days [3.00–17.5] vs. 18.5 days [3.00–53.2]; p = 0.011) in the experimental group than in the control group. Although not statistically significant, those receiving the experimental therapy showed a numerically lower all-cause mortality than those receiving

SoC, especially at day 10 [2 (7.41%) vs. 5 (17.9%); OR 0.39 (95% CI 0.05–2.1); $p = 0.282$]. The total number of non-serious adverse events was 42 in each the two groups. Those receiving experimental treatment had a numerically higher rate of non-serious infectious adverse events [16 (38%) vs. 10 (24%)] and serious infectious adverse events [7 (35%) vs. 3 (23%)] than those receiving SoC.

Conclusions: The combined use of methylprednisolone pulses plus tacrolimus, in addition to the SoC, did not significantly improve the time to clinical stability or other secondary outcomes compared with the SoC alone in severe COVID-19. Although not statistically significant, patients receiving the experimental therapy had numerically lower all-cause mortality than those receiving SoC, supporting recent non-randomized studies with calcineurin inhibitors. It is noteworthy that the present trial had a limited sample size and several other limitations. Therefore, further RCTs should be done to assess the efficacy and safety of tacrolimus to tackle the inflammatory stages of COVID-19.

Clinical Trial Registration: Identifier [NCT04341038/EudraCT: 2020-001445-39].

Keywords: COVID-19, SARS-CoV-2, methylprednisolone, tacrolimus, inflammation, lung injury

INTRODUCTION

In December 2019, a new type of human coronavirus (SARS-CoV-2), causing an emerging diseases (COVID-19), was first recognized in China and spread globally (1, 2). The COVID-19 was declared a pandemic by the WHO on March 12, 2020 (3), and it continues to spread worldwide, causing considerable morbimortality and economic damage.

SARS-CoV-2 has evolved some mechanisms to disturb host-immune response. In fact, impaired interferon (IFN) signature in early stages leads to a persistent blood viral load and a later hyper-inflammatory response that has been related with a worse COVID-19 outcome (4, 5). Accordingly, antiviral followed by anti-inflammatory drugs have been recommended (6). While some immunosuppressive treatments could be potentially harmful, others have been suggested for treating the disproportionate inflammation triggered by the SARS-CoV-2 infection (7).

When the TACROVID trial was designed in March 2020, data from the main COVID-19 randomized controlled trials (RCT) (8–10) were still not available and there were no therapies for treating the COVID-19 illness other than supportive care. Due to the lack of evidence-based treatments, a large number of patients received off-label and compassionate therapies, based on their *in vitro* antiviral or immunomodulatory properties. The repurposing of older drugs was the initial main strategy given their proven safety profile (11). Today, RCTs are still needed in order to provide evidence-based effective and safe therapies for COVID-19 management (12).

Our hypothesis was that methylprednisolone pulses plus tacrolimus could be an effective and safe drug combination for severe COVID-19 patients. Accordingly, given the health emergency due to the rapid spread of SARS-CoV-2 worldwide, we conducted a proof-of-concept study in a randomized, single-center, open-label clinical trial with the aim to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus plus standard of care (SoC), vs. SoC alone, in severe COVID-19 patients with lung injury and systemic hyperinflammatory syndrome.

The rationale for the current RCT was based on the fact that corticosteroids, such as methylprednisolone, are a pillar in the treatment of multiple inflammatory diseases, with several mechanisms of action impacting both the innate and adaptive arms of immunity. Regarding tacrolimus, the reason for its use was based on both the anti-inflammatory and anti-viral actions of calcineurin inhibitors (CNIs). As an immunomodulatory agent, tacrolimus impairs lymphocyte function and consequently decreases in pro-inflammatory cytokines (7, 13). In this respect, severe COVID-19 disease presents a similar clinical and cytokine profile to other disorders like secondary hemophagocytic lymphohistiocytosis (14), where CNIs play a central role in its treatment (15). Additionally, several human coronavirus replication depends on immunophilin pathways, which can be inhibited by CNIs in cell culture (16, 17). Based on these two mechanisms, it has been suggested that CNIs could be used to treat COVID-19 (18). In fact, recent non-randomized studies suggest that cyclosporine could reduce mortality, mainly in patients with moderate to severe COVID-19 (19, 20). Our study

Abbreviations: AE, adverse event; ALT, alanine aminotransferase; ARDS, acute respiratory disease syndrome; HUB, Hospital Universitari de Bellvitge; CNIs, calcineurin inhibitors; CoV, Coronavirus; COVID-19, Coronavirus disease 2019; CRP, C-reactive protein; CXCL, chemokine (C-X-C motif) ligand; CXR, chest x-ray; ECMO, extracorporeal membrane oxygenation; eCRF, electronic case report form; FiO_2, fractional inspired oxygen; G-CSF, granulocyte colony-stimulating factor; GFR, glomerular filtration rate; ICF, informed consent form; IFN, interferon; IL,interleukin; ITT, intention to treat; LDH, lactate dehydrogenase; MCP, monocyte chemoattractant protein; PaO_2, arterial oxygen partial pressure; RCT, randomized controlled trial; RT-PCR, reverse transcription polymerase chain reaction; SAE, serious adverse event; SARS-CoV, Severe Acute Respiratory Syndrome Coronavirus; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SF,SpO_2/FiO_2 ratio; SoC, Standard of Care; SOFA score, Sequential Organ Failure Assessment Score; SpO_2, oxygen saturation; TNF, tumor necrosis factor.

is the first RCT assessing the effect of corticosteroids plus a CNI (tacrolimus) in hospitalized patients with severe COVID-19. They are low-cost drugs with a well-known safety profile that could be produced on a large scale if they were effective at treating COVID-19.

METHODS

Study Design

TACROVID was a pragmatic, randomized (1:1) with parallel-groups, open-label, single-center, phase II clinical trial to evaluate the efficacy and safety of methylprednisolone pulses and tacrolimus plus SoC, vs. SoC alone, in severe COVID-19 patients with lung injury and systemic hyperinflammatory syndrome.

The TACROVID trial was conducted at Hospital Universitari de Bellvitge (HUB), a 750-bed tertiary care public hospital for adults in Barcelona (Catalonia, Spain). HUB is the reference hospital for high complexity patients from the southern area of Catalonia, a region of ∼2 million inhabitants.

In March 2020, the HUB's Ethical Committee for Drug Research and the Spanish Agency for Drugs and Health Products approved the protocol and informed consent form (ICF). This trial complies with the Declaration of Helsinki and Good Clinical Practice guidelines, and personal data protection as required by Spanish law (LOPD 3/2018). The trial registration numbers are NCT04341038 and EudraCT 2020-001445-39. All patients (or a legal representative if patients were unable) had to provide ICF prior to initiation of the trial procedures. The protocol is available online (21).

Population

Patients were included in the trial if they met all the inclusion criteria and none of the exclusion criteria. *Inclusion criteria:* (1) COVID-19 infection confirmed by nasopharyngeal RT-PCR; (2) New pulmonary infiltrates (either by chest X-ray or computerized axial tomography); (3) Respiratory failure defined by $PaO_2/FiO_2 < 300$ or $SpO_2/FiO_2 < 220$; (4) High analytical inflammatory parameters: CRP > 100 mg/L, and/or D-Dimer > 1,000 μg/L, and/or Ferritin > 1,000 μg/L. *Exclusion criteria:* (1) Critically ill patients with life expectancy ≤ 24 h; (2) Glomerular filtration ≤ 30 ml/min/1.73m^2; (3) Leukopenia ≤ 4,000 cells/μl or other conditions that cause immunosuppression; (4) Concomitant potentially serious infections; (5) Contraindication for the use of corticosteroids or tacrolimus according to the Summary of Product Characteristics; (6) Known hypersensitivity to any of the study drugs, their metabolites, or formulation excipient; (7) Previous participation in a RCT in the last 3 months.

Randomization

After obtaining the ICF, patients were randomized using the RedCap, a secure web application for building and managing electronic case report forms (eCRF). Patients were randomly (1:1) assigned to one of the following arms with no baseline stratification:

1. Experimental arm: methylprednisolone pulses of 120 mg/day had to be administered on 3 consecutive days after randomization (if not previously administered). The administration of higher doses or longer duration of corticosteroids was allowed if their treating physicians considered it appropriate. Tacrolimus starting dose was 0.05 mg/kg (Adoport®) twice daily. Patients using lopinavir-ritonavir received 0.2 mg (Modigraf®) every 48 h. Thereafter, tacrolimus dosing was individualized through therapeutic drug monitoring to achieve blood trough levels of 8–10 ng/ml. In addition, patients in the experimental arm could receive standard of care (SoC) for their management in accordance with treating physicians.
2. Control arm (SoC): SoC included measures of supplemental oxygen and respiratory support, fluid therapy, antipyretic treatment, postural measures, low molecular weight heparins, and could also include treatments with unproved antiviral (lopinavir-ritonavir, hydroxichloroquine, etc.) or immunosuppressive (any regimen of corticosteroids, tocilizumab, anakinra, etc.) drugs, or those necessary at the discretion of the treating physician, except for cyclosporine and/or tacrolimus.

The experimental drugs were started immediately after the participants were randomly assigned to that group. The experimental treatment was discontinued after patients achieved *clinical stability*, which was defined in the *outcomes* section. Experimental treatment was also discontinued if the included patient presented a severe or potentially severe infection, required invasive mechanical ventilation, extracorporeal membrane oxygenation (ECMO), or had any serious medication-related adverse event (of special interest refractory high blood pressure, decrease of more than 50% in the GFR compared with the baseline, or ventricular tachycardia).

Procedures

All patients were followed from day 0 through day 56 or death. The planned visits and procedures are detailed in the TACROVID trial protocol (**Supplementary Table 1**) (21). Follow up visits were face-to-face to evaluate disease outcomes, and data was collected using an eCRF. The Bellvitge Biomedical Research Institute (IDIBELL) Clinical Research and Clinical Trials Unit (UICEC-IDIBELL) carried out the monitoring of the trial. Regular monitoring was performed by the UICEC-IDIBELL according to the International Conference on Harmonization (ICH) good clinical practice (GCP) requirements. The UICEC-IDIBELL carried out pharmacovigilance of the trial.

Outcomes

The primary outcome was time (days) to *clinical stability* within 56 days after randomization. *Clinical stability* was defined as fulfilling all of the following criteria for 48 consecutive hours: body temperature ≤ 37.5°C; $PaO_2/FiO_2 > 400$ and/or $SpO_2/FiO_2 > 300$; and respiratory rate ≤ 24 rpm. Treatment failures were defined as: (1) patients that did not meet criteria for clinical stability 56 days after starting treatment; (2) patients presenting a serious adverse event attributed to the experimental treatment; or (3) patients dying after being included in the clinical trial.

The secondary outcomes included the number of days until normalization of each of the clinical and analytic parameters

from day 0 through day 56; the clinical status according to the eight-point ordinal scale (22) from day 0 through day 56; patients who achieved a clinical status $\leq$ 3 after 10 days or hospital discharge (whichever was first), and on days 28 and 56; patients who achieved *clinical stability* after 10 days or hospital discharge (whichever was first), and on days 28 and 56; value of each of the analytical parameters after 10 days or hospital discharge (whichever was first), and on days 28 and 56; number of days receiving trial experimental treatment; days until hospital discharge; number of patients and days requiring non-invasive and invasive ventilatory support devices during hospitalization; changes in blood quantitative viral load by RT-PCR before start of treatment and during follow up; changes in expanded cytokine profile before starting treatment and on days 14, 28, and 56; pulmonary parenchyma involvement using chest x-ray (CXR) pulmonary severity score (23) at baseline, and at day 56; adverse events according to their seriousness and relationship to trial experimental treatment; COVID-19 related mortality at day 28 and 56 after randomization; all-cause mortality at day 28 and 56 after randomization.

Statistical Analysis

The intention-to-treat (ITT) population consisted of all randomized patients. We estimated that the assignment of 42 patients with 1:1 randomization would provide at least 80% power to reject the null hypothesis that the experimental and control survival curves are equal. The hazard ratio of clinical stability of control patients in relation to the patients in the experimental group was 0.52, and a median survival time in the control group of 16 days was assumed. The probability of Type I error associated with this hypothesis test was 0.05 and 5% withdrawal was anticipated.

A descriptive analysis of the baseline profile of patients included in the ITT population was carried out. The primary efficacy outcome was time (days) to *clinical stability* within 56 days after randomization, and was estimated using the Kaplan–Meier method, and cumulative incidence curves were compared between the two groups with the log-rank test. The stratified Cox proportional-hazards model was used to estimate the hazard ratio (for experimental group as compared with control) and 95% confidence interval. The main analysis was repeated on each *clinical stability* criterion. Moreover, odds of clinical stability were compared at 10, 28, and 56 days using a logistic regression.

Time to WHO eight-point ordinal scale $\leq$ 3 and time to death (secondary outcomes) were compared between the two groups using the Kaplan–Meier approach, and using a logistic regression at 10, 28, and 56 days. A sensitivity analysis of the time to hospital discharge was performed. Safety was assessed in all patients; patients were grouped according to the study group. A safety review was performed by the UICEC-IDIBELL.

The IDIBELL Biostatistical Unit performed the analysis and analysts were blinded to the treatment received by patients (intervention vs. usual care). R version 4.0.3 for Windows (R Foundation for Statistical Computing, http://www.r-project.org) was used for data management and analysis.

RESULTS

Fifty-five patients were prospectively included from April 1, 2020 to May 2, 2020 in the trial for subsequent randomization. Twenty-seven were assigned to the experimental group and 28 to the control group (ITT and safety population) (**Figure 1**). Of those assigned to the experimental group, 24 (88.9%) patients received the treatment as assigned. Three patients discontinued the treatment during the first 5 days and were excluded from the per-protocol analysis population. Of those assigned to the control group, 26 (92.9%) were eligible for the per-protocol analysis. Two deceased patients were excluded owing to a short follow-up (<5 days).

The mean age of the 55 patients included in the ITT analysis was 63.2 (SD 13) years; 44 (80%) were male (**Table 1**). Overall, 39 (70.9%) of the patients were Caucasian and 16 (29.1%) were Latino. The most common pre-existing comorbidities were hypertension (43.6%), obesity (43.6%), and diabetes (27.3%). Thirty-eight (69.1%) patients had no smoking history. The median Charlson index was 3 in both groups. Except for one patient in the experimental group, all patients showed independence in tasks of daily living without cognitive impairment. There were no patients admitted from long-term care facilities or nursing homes.

Some imbalances existed at enrollment between the two groups. The time between symptom onset and randomization was 11 days (IQR 9–17) in the experimental group compared to 14 days (IQR 9.75–19.2) in the control group. A higher proportion of the control group had required high-flow nasal cannula or non-invasive mechanical ventilation and corticosteroids. Conversely, the experimental group showed higher CRP and creatinine kinase. No other major differences in symptoms, signs, laboratory results, disease severity, or treatments were observed between the groups at baseline (**Table 1**).

Patients in the experimental group received a median of 9 (IQR 7–11) days of tacrolimus, with a median time from symptom onset to tacrolimus administration of 11 days (IQR 9–17). Median tacrolimus dose per kg bodyweight was 0.0375 mg/kg twice daily (IQR 0.0276-0.05), and it was 0.0025 mg/kg every other day (IQR 0.0024-0.0029) when receiving concomitant lopinavir-ritonavir. Tacrolimus median trough levels were 8.4 ng/ml (IQR 4.6-15.1). The need for high flow devices and mechanical ventilation (invasive or not) during the follow up was similar in the two arms of the trial (**Table 2**). All patients received corticosteroids, with a median time from symptom onset to corticosteroid therapy of 10 days (IQR 8.00–14.0) in the experimental group and 10 days (IQR 8.75–15.0) in the control group. The dose of any type of corticosteroid received from admission to day 56 of the trial was converted to methylprednisolone, and the cumulative doses were significantly lower in the experimental group than in the control group (median methylprednisolone cumulative doses were 360 mg [IQR 360–842] vs. 870 mg [IQR 364–1451]; $p = 0.007$), as was the duration of corticosteroid treatment (median of 4.00 days [3.00–17.5] vs. 18.5 days [3.00–53.2]; $p = 0.011$). Most of the patients included also received tocilizumab (25 [92.6%]

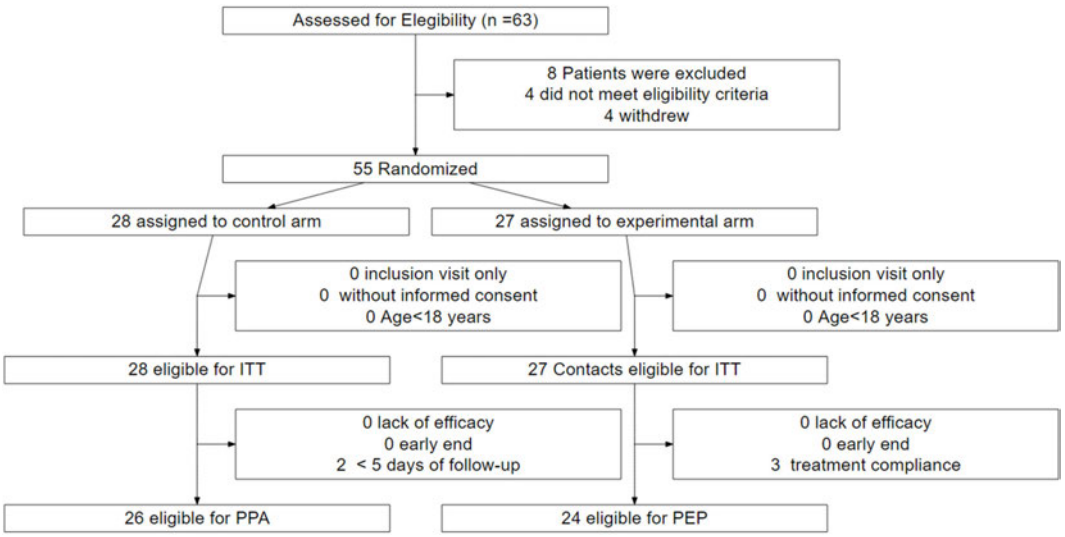

FIGURE 1 | Trial profile.

in the experimental group vs. 24 [85.7%] in the control group); $p = 0.669$). Two patients (7.14%) in the control group received anakinra. After tacrolimus initiation no patients in the experimental group received any additional immunosuppressant drug other than steroids. No significant differences were observed among the two groups in the number of patients who received lopinavir-ritonavir, hydroxychloroquine, or antibiotics. Length of oxygen support, as well as the rate and duration of ventilation support were not significantly different between the two groups (**Table 2**).

The final study follow-up was on June 27, 2020. In the ITT population, no statistically significant differences were observed in time to *clinical stability* within 56 days after randomization between the two groups (median 10.0 days [IQR 7.0–13.0] in the experimental group vs. 11.0 days [8.0–18.0] in the SoC group; HR 0.73 [95% CI 0.39–1.37]; Asymptotic Logrank test *p*-value = 0.325) (**Table 3** and **Figure 2**). The times to normalization of each of the variables that compound *clinical stability* (body temperature, PaO_2/FiO_2 or SpO_2/FiO_2; and respiratory rate) did not differ significantly between arms. Results for time to *clinical stability* were similar in the per-protocol population (median 10.0 days [IQR 7.00–12.5] in the experimental group vs. 11.0 days [IQR 8.0–18.8] in the SoC group; HR 0.77 [95% CI 0.40–1.47]; Asymptotic Logrank test *p*-value = 0.473). At 56 days after randomization, the number of patients who had achieved *clinical stability*, those with an eight-point ordinal scale ≤ 3, and patients discharged did not differ significantly between the groups (**Table 3**).

Although not statistically significant, those receiving the experimental therapy showed a numerically lower all-cause mortality than those receiving SoC, especially at day 10 (2 [7.41%] vs. 5 [17.9%]; OR 0.39 [95% CI 0.05–2.1]; $p = 0.282$) and at day 28 (4 [14.8%] vs. 6 [21.4%]; OR 0.65 [95% CI 0.14–2.67]; $p = 0.551$) (**Figure 3**). Patients in the experimental group died later at a median of 13 days from randomization (IQR 10.0–26.0), while in the control group the median was 7 days (3.25–10.0); but these differences were not statistically significant (logrank test *p*-value 0.710). The number of available events by group (four deaths per study arm) was not enough to get reliable estimators to analyze the effect of experimental therapy on all-cause mortality adjusting by age or sex. Similar results were obtained for COVID-19-related mortality (**Table 3**) (**Supplementary Figure 1**).

There were no significant differences in the evolution of analytic parameters (lymphocytes, CRP, ferritin, LDH, IL-6, D-dimer) between the two arms (**Supplementary Table 2**), or in the expanded cytokine profile (**Supplementary Table 3**). Serum cytokine levels at different time points during the trial showed increased levels of pro-inflammatory cytokines like CXCL10 (IP-10), IL-6, IL-18, TNF-alpha, and soluble IL-2 receptor alpha; and regulatory cytokines such as IL-10 and IL-1RA at day 0 and day 14. In both groups, serum cytokine levels tended to have decreased by day 28 and day 56 (**Supplementary Figure 2**). In the same way, there were no significant differences between groups in pulmonary parenchyma involvement according to the CXR pulmonary severity score at inclusion or at day 56 (**Supplementary Table 4**).

All 55 patients were nasopharyngeal and oropharyngeal RT-PCR positive at diagnosis, but viral load data was available in 24 (88.8%) patients in the experimental group and 20 (71.4%) in the control group. The median baseline viral load of upper respiratory tract swabs was 244,954 (IQR 8,566–7,458,132) $\log_{10}$ copies per ml in the experimental group and 388,329 (IQR

TABLE 1 | Baseline characteristics.

	Experimental (*N* = 27)	Control (*N* = 28)
Age (years)	61.5 (13.9)	64.8 (12.1)
Sex (male)	23 (85.2%)	21 (75%)
Race or ethnic group		
Caucasian	20 (74.1%)	7 (25%)
Hispanic or Latino	7 (25.9%)	9 (32.1%)
Other	0 (0%)	1 (3.6%)
Coexisting conditions		
Smoking history	7 (25.9%)	10 (35.7%)
Hypertension	10 (43.5%)	14 (53.8%)
Diabetes mellitus	6 (22.2%)	9 (32.1%)
Obesity	11 (40.7%)	13 (46.4%)
Coronary heart disease	3 (11.1%)	1 (3.6%)
Charlson Index	3 (1–3)	3 (2–4)
Barthel Index	100 (100–100)	100 (100–100)
Time from symptom onset to randomization, days	11 (9–17)	14 (9.75–19.3)
Early (≤10 days from symptom onset)	13 (48.1%)	10 (35.7%)
Late (>10 days from symptom onset)	14 (51.9%)	18 (64.3%)
Body temperature, °C	36.3 (0.5)	36.31 (0.53)
Respiratory rate, breaths per min	25.7 (7.8)	25.0 (4.4)
PaO_2/FiO_2	236.7 (220.6–261.2)	217.9 (124.2–237.5)
SpO_2/FiO_2	178 (160–193.7)	157.5 (106.1–165.4)
FiO_2	0.5 (0.5–0.6)	0.6 (0.6–0.9)
Score on eight-point ordinal scale	5 (5–5)	5 (5–6)
5.Hospitalized, requiring supplemental oxygen	23 (85.2%)	16 (57.1%)
6.Hospitalized, receiving non-invasive ventilation or high-flow oxygen devices	4 (14.8%)	12 (42.9%)
PSI	77.0 (27.3)	79.4 (23.3)
NEWS	7.5 (2.1)	7.71 (1.88)
SOFA score	1 (0–1)	1 (0–1)
Laboratory		
Lymphocyte count, $\times 10^9$/L	0.72 (0.58–0.91)	0.56 (0.44–0.78)
Platelets count, $\times 10^9$/L	311 (115)	288 (123)
Serum creatinine, mg/dl	0.84 (0.23)	0.79 (0.24)
ALT, U/L	47.0 (32.0–70.5)	51.5 (35.8–92.2)
AST, U/L	39.0 (30.5–56.0)	41.0 (24.8–67.0)
LDH, U/L	435 (114)	450 (183)
CRP, mg/L	139.5 (24.1–195.75)	39.2 (17.3–109.9)
Ferritin, μg/L	1735.3 (1420.15–2346.15)	1695.2 (1212.1–1894.6)
IL-6, ng/L	86.1 (37.6–785)	80.4 (41.4–667)
Creatinine kinase, U/L	75.0 (43.5–198)	47.5 (31.5–65.0)
D-dimer, μg/L	612 (250–2672.5)	741 (352–2195.5)
Baseline viral load of nasopharyngeal and oropharyngeal swabs, log_{10} copies per ml	244,954 (8,566–7,458,132)	388,329 (22,551–980,683)
Treatments at baseline		
Lopinavir-ritonavir	23 (85.2%)	22 (78.6%)
Hydroxychloroquine	27 (100%)	28 (100%)
Antibiotic	13 (48.1%)	8 (28.6%)
Corticosteroids	10 (37.0%)	17 (60.7%)
Tocilizumab	24 (88.9%)	22 (78.6%)

Data are median (IQR) or mean (SE), and n (%). ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; FiO_2, fractional inspired oxygen; IL-6, interleukin-6; LDH, lactate dehydrogenase; NEWS, National Early Warning Score; PaO_2, arterial oxygen partial pressure; PSI, Pneumonia Severity Index; SOFA, Sequential Organ Failure Assessment; SpO_2, oxygen saturation.

22,551–980,683) log_{10} copies per ml in the control group. During follow-up, upper respiratory tract viral load decreased over time similarly in both arms, becoming undetectable at day 28 and 56 in most patients. Blood RT-PCR at baseline was available in 24 (88.8%) patients in the experimental group and 21 (75%) in the control group. Almost all of them showed undetectable viral RNA in blood samples at baseline and during follow-up (**Supplementary Table 5** and **Supplementary Figure 3**).

Adverse events (AE) occurred in 46 (83.6%) patients. Twenty-two (44%) patients had one AE, five (9.1%) had two AE, 11 (20%) had three AE, and eight (14.5%) had four or more AE. Sixty-two AE were reported in 23 (85.2%) patients in the experimental group, 20 of them met the seriousness criteria (corresponding to nine patients), and nine were assessed as related to the experimental treatment. In the control group, 55 AE were reported in 23 (82.1%) patients, of which 13 were considered as serious AE (corresponding to 10 patients) (**Supplementary Table 6**).

The total number of non-serious AE was 42 in each of the two groups. Those receiving experimental treatment had a numerically higher rate of non-serious infectious AE (16 [38%] vs. 10 [24%]), and serious infectious AE (7 [35%] vs. 3 [23%]) than those receiving SoC. In contrast, the control group showed poorer glucose metabolism and a higher overall bleeding rate. Five (18.5%) patients in the experimental group developed special interest AE, these being hypertension in three (60%) of them and renal impairment in two (40%). Four deaths in each group were judged by the site investigators to be related to COVID-19 acute respiratory distress syndrome. One death reported in the experimental group was attributed to hemorrhagic stroke, and regarding the two deaths in the control group, one was attributed to *Staphylococcus aureus* septicaemia and the other to hemopericardium (**Supplementary Table 6**).

DISCUSSION

The TACROVID trial found that methylprednisolone bolus plus tacrolimus did not significantly improve the time to *clinical stability* (primary outcome), mortality or other secondary outcomes compared with the SoC in

TABLE 2 | Treatments received during hospitalization and the trial period.

	Experimental (*N* = 27)	Control (*N* = 28)	*P*-value
Duration of oxygen support from randomization, days	11.0 (8.00–19.5)	13.0 (7.75–23.0)	0.953
High-flow or ventilatory support therapies	14 (51.9%)	18 (64.3%)	0.509
HFNC and/or non-invasive mechanical ventilation	13 (48.1%)	18 (64.3%)	0.350
Invasive mechanical ventilation	5 (18.5%)	4 (14.3%)	0.729
ECMO	0 (0%)	1 (%)	..
Duration of high-flow or ventilatory support from randomization, days	8.00 (5.00–27.2)	6.50 (4.25–14.2)	0.303
HFNC and/or non-invasive mechanical ventilation	5.00 (4.00–9.00)	5.00 (3.25–9.00)	0.532
Invasive mechanical ventilation	22.0 (11.0–29.0)	10.00 (4.25–21.5)	0.327
Renal replacement therapy	2 (7.4%)	0 (0%)	..
Corticosteroid therapy	27 (100%)	28 (100%)	..
Duration of corticosteroid therapy, days	4.00 (3.00–17.5)	18.5 (3.00–53.2)	0.011
Methylprednisolone cumulative dose	360 (360–842)	870 (364–1,451)	0.007
Tocilizumab	25 (92.6%)	24 (85.7%)	0.669
Anakinra	0 (0%)	2 (7.14%)	0.491
Lopinavir-ritonavir	23 (85.2%)	22 (78.6%)	0.729
Hydroxychloroquine	27 (100%)	28 (100%)	..
Heparin	27 (100%)	28 (100%)	..
Antibiotics	23 (85.2%)	26 (92.9%)	0.422

Data are n (%) compared using Pearson's chi-squared test, and median (interquartile range) compared using the Wilcoxon test. ECMO, extracorporeal membrane oxygenation; HFNC, high-flow nasal cannula.

TABLE 3 | Effect of allocation to experimental group on key study outcomes.

	Experimental (*N* = 27)	Control (*N* = 28)	HR/OR [CI 95%]	*P*-value
Time to clinical stability, days	10.0 (7.00–13.0)	11.0 (8.00–18.8)	0.73 [0.39–1.37]	0.327^
Time to body temperature normalization, days	1.00 (1.00–2.00)	1.00 (1.00–1.00)	0.8 [0.47–1.36]	0.415^
Time to $PaO_2/FiO_2 > 400$ and/or $SpO_2/FiO_2 > 300$	9.00 (7.00–11.0)	11.0 (8.00–18.8)	0.81 [0.43–1.53]	0.525^
Time to respiratory rate < 24 bpm	5.00 (2.00–9.00)	5.00 (3.00–7.00)	1.03 (0.59–1.81)	0.909^
Patients who achieved clinical stability				
at day 10	11 (40.7%)	11 (39.3%)	1.06 [0.35–3.19]	0.915*
at day 28	18 (66.7%)	21 (75.0%)	0.67 [0.20–2.21]	0.515*
at day 56	21 (77.8%)	22 (78.6%)	0.96 [0.25–3.61]	0.946*
Time to an eight-point ordinal scale ≤ 3	12.5 (8.00–15.2)	15.0 (9.00–24.0)	0.92 [0.49–1.71]	0.787^
Patients who reach an eight-point ordinal scale ≤ 3				
at day 10	9 (33.3%)	7 (25.0%)	1.48 [0.45–5.03]	0.515*
at day 28	18 (66.7%)	20 (71.4%)	0.80 [0.25–2.59]	0.714*
at day 56	20 (74.1%)	21 (75.0%)	0.95 [0.27–3.33]	0.940*
Discharge at day 56	21 (77.8%)	21 (75.0%)	1.16 [0.32–4.28]	0.819*
Duration of hospital stay, days	13.0 (8.50–21.0)	14.0 (9.00–22.5)	..	0.933**
COVID-19-related mortality				
at day 10	2 (7.41%)	3 (10.7%)	0.69 [0.07–4.88]	0.705*
at day 28	3 (11.1%)	4 (14.3%)	0.76 [0.13–4.02]	0.747*
at day 56	4 (14.8%)	4 (14.3%)	1.04 [0.21–5.13]	0.958*
Time from randomization to COVID-19-related death, days	18.0 (9.50–27.8)	7.00 (3.50–12.5)	0.96 [0.24–3.84]	0.953^
All-cause mortality				
at day 10	2 (7.41%)	5 (17.9%)	0.39 [0.05–2.10]	0.282*
at day 28	4 (14.8%)	6 (21.4%)	0.65 [0.14–2.67]	0.551*
at day 56	5 (18.5%)	6 (21.4%)	0.84 [0.21–3.28]	0.800*
Time from randomization to all-cause death, days	13.0 (10.0–26.0)	7.00 (3.25–10.0)	0.80 [0.24–2.61]	0.707^

*Data are median (IQR) or n (%). COVID-19, Coronavirus disease 2019; FiO2, fractional inspired oxygen; OS, ordinal scale; PaO_2, arterial oxygen partial pressure; SpO_2, oxygen saturation. Clinical stability (the event) was defined as fulfilling all of the following criteria for 48 consecutive hours: body temperature ≤ 37.5°C; $PaO_2/FiO_2 > 400$ and/or $SpO_2/FiO_2 > 300$; and respiratory rate ≤ 24 breaths per minute (bpm). Treatment failure was defined as: (1) patient who does not meet the criteria for clinical stability 56 days after starting treatment; (2) patient presenting a serious adverse event attributed to trial treatment; or (3) patient who dies after being included in the clinical trial. Differences are expressed as ^ Hazard Ratio [CI 95%] and p-value; *Odds ratio [CI 95%] and p-value; ** Wilcoxon test and p-value.*

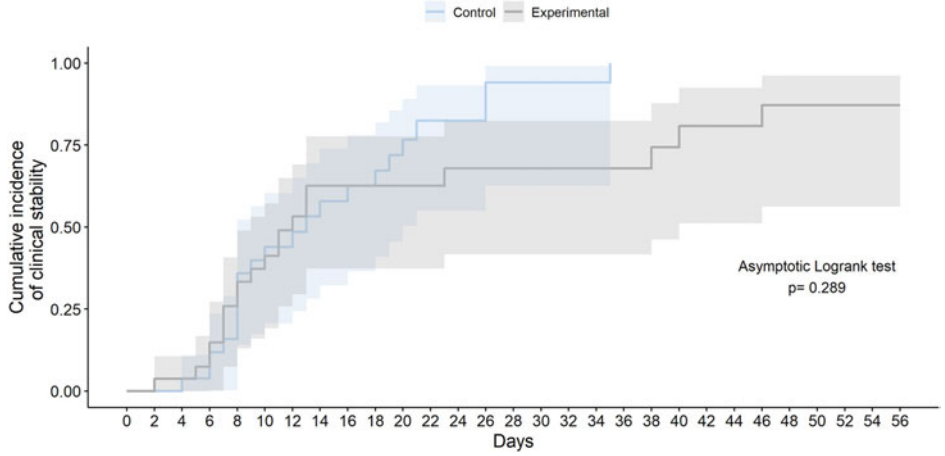

FIGURE 2 | Time to clinical stability in the intention-to-treat population.

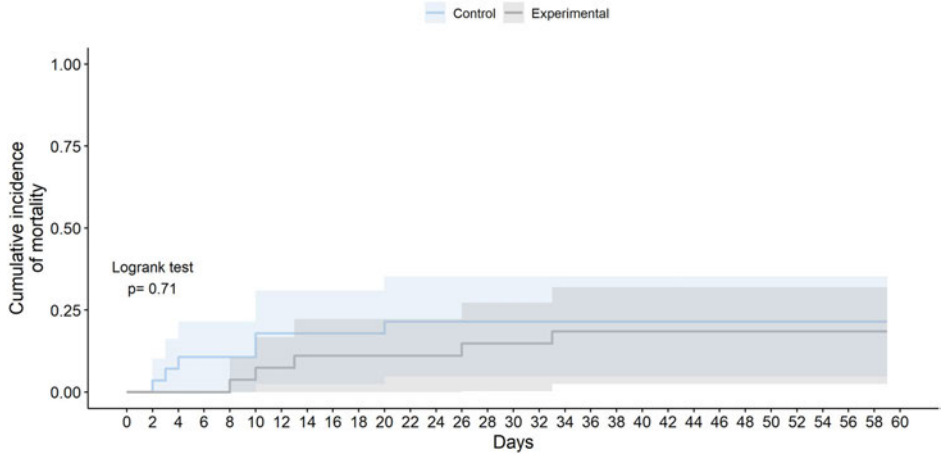

FIGURE 3 | All cause mortality in the ITT population.

hospitalized patients with severe COVID-19. Furthermore, no differences were observed in the clearance of the virus or in the rate of adverse events between the two groups.

The TACROVID trial was initiated in March 29, 2020 when there were no medical reports supporting the use of immunosuppressive therapy in severe COVID-19. Nonetheless, all of the trial's patients received corticosteroids. Methylprednisolone and tacrolimus lead to impaired lymphocyte function (7, 13) and therefore it could facilitate SARS-CoV-2 replication and also promote the development of other infections. On the other hand, CNIs have been shown to inhibit the growth of human coronaviruses at low micromolar, non-cytotoxic concentrations in cell cultures by immunophilin pathway inhibition (16, 17). Based on this finding, it has been suggested that CNIs could be used as an antiviral agent to treat COVID-19. However, we would like to highlight that the concentrations used in cell culture are not clinically achievable, as they correspond to highly toxic blood levels in humans (24). Accordingly, the proposed use of tacrolimus should be restricted to the inflammatory stages of COVID-19. In this trial, tacrolimus had no significant effect on SARS-CoV-2 RNA loads

either in the upper respiratory tract or in blood specimens in our patients.

The ratio of most treatments (antibiotics, lopinavir-ritonavir, hydroxychloquine, heparin, and tocilizumab) used was similar in the two groups. Interestingly, the largest and longest corticosteroids doses were used in the control group, although we do not know the exact reasons. During follow up both groups had similar laboratory test results, needed similar rates of high-flow and ventilation devices, and developed similar CXR parenchymal involvement. Notably, those patients receiving the experimental therapy had a numerically shorter time to achieve an eight-point ordinal scale $\leq$ 3 than those receiving SoC within 10 days of randomization, although this trend was later reversed. Finally, this was an open-label trial and the control group patients who could not receive tacrolimus may have received more corticosteroids or other immunosuppressants (anakinra).

Although not statistically significant, patients receiving the experimental therapy had numerically lower all-cause mortality than those receiving SoC, especially during the first 28 days. This data supports non-randomized studies that showed that cyclosporine could reduce mortality, mainly in patients with moderate to severe COVID-19 (19, 20). Interestingly, tacrolimus use had a positive independent effect on survival vs. all other immunosuppressant (cyclosporine, mycophenolate and/or mTOR inhibitors) according to a multi-center European study carried out on 243 adult liver transplant recipients with symptomatic COVID-19, suggesting that it could be even more beneficial than cyclosporine (25). The mortality data from days 28 to 56 of the trial are less valuable because only five patients were still admitted to the hospital in the experimental group (**Supplementary Table 7**), and experimental therapy was withdrawn previously in all of them due to mechanical invasive ventilation or serious AE.

Likewise, there was no difference in adverse events overall between groups. Patients in the experimental group seemed to have a slightly higher number of non-serious and serious AE infections. We also have to consider that corticosteroids have been associated with gastrointestinal bleeding, hyperglycaemia, and neuromuscular weakness (26). In fact, the group treated with tacrolimus received a significantly lower dose of corticosteroids, having better control of glucose metabolism and a lower rate of bleeding.

However, the trial had some limitations. First, the current trial was not conducted as a double-blind trial. This was considered unrealistic given the intense workload experienced at the beginning of the pandemic in our local setting. To minimize the impact of an open-label design, the statistician performing the analysis was blinded to the trial arm. Second, the TACROVID trial had a limited sample size and clearly was not sufficiently powered to detect a difference in time to *clinical stability* and mortality between the two groups after the early termination that occurred with 29 (34.1%) patients fewer than expected. Furthermore, the limited sample size caused certain imbalances in the baseline characteristics between the two groups after randomization. Third, all included patients received corticosteroids, heparins, and hydroxychloroquine; and most (89.1%) of them also received tocilizumab as part of the SoC. In this respect, the additional use of any other medication regimens (except for cyclosporine) in both arms, as part of the SoC, limits the assessment of which was the real effect of each drug on clinical outcomes, laboratory data and the occurrence of AE. Moreover, tacrolimus strongly interacts with some treatments (especially lopinavir) used at that time in COVID-19. Most (85.2%) patients in the experimental group received concomitant treatment with lopinavir-ritonavir, as it was extremely difficult to achieve the recommended plasma levels of tacrolimus. Finally, the lack of medical evidence supporting immunosuppressive therapies in COVID-19, when the trial was conducted, made us more cautious, withdrawing experimental therapy when mechanical invasive ventilation was implemented. Therefore, its efficacy and safety cannot be assessed by this trial in this subset of patients with life-threatening COVID-19.

In summary, the combined use of methylprednisolone pulses and tacrolimus, in addition to the SoC did not significantly improve the time to *clinical stability* or other secondary outcomes compared with SoC alone in hospitalized patients with severe COVID-19. Although not statistically significant, patients receiving the experimental therapy had numerically lower all-cause mortality than those receiving SoC. No relevant differences were observed in the clearance of the virus or in the rate of adverse events between the two groups. The reason why the largest and longest corticosteroid doses were used in the control group remains unclear. It is noteworthy that the present trial had a limited sample size and several other limitations. Therefore, further RCTs should be done to assess the efficacy and safety of tacrolimus to tackle the inflammatory stages of COVID-19.

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 Bellvitge University Hospital's Ethical Committee for Drug Research. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XS, AA, GR-B, CT, and XC had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. XS, AA, AR-M, CT, SV, PH, and XC provided input on the trial design. XS, AA, GR-B, AR-M, CT, and XC were responsible for the acquisition, analysis, and interpretation of data. XS, AA, GR-B, AR-M, CT, NP, and XC drafted the manuscript. MF-M, AI, FM, OC, JB, AM-V, SV,

and PH critically revised the manuscript. CT contributed to the statistical analysis. XS and XC verified the underlying data. All authors contributed to conducting the trial, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.691712/full#supplementary-material

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

ARTICLE 6

Objectiu

Determinar l'eficàcia i la seguretat dels polsos de metilprednisolona juntament amb tacrolimus, afegits a l'*Standard of Care*, en pacients hospitalitzats per una COVID-19 greu.

Títol

Inhibition of SARS-CoV-2 replication using calcineurin inhibitors: are concentrations required clinically achievable?

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J Intern Med. 2021 Jun;289(6):926-927. doi: 10.1111/joim.13264.

Contribució del doctorand: revisió de la literatura, preparació de dades, anàlisi i presentació dels resultats, redacció i presentació de l'article.

Resum

Es tracta d'una carta a l'editor sobre l'article de Galvez-Romero et al. titulat '*Cyclosporine A plus lowdose steroid treatment in COVID-19 improves clinical outcomes in patients with moderate to severe disease. A pilot study'* i que va ser publicat a Journal of Internal Medicine [109].

L'article original de Galvez-Romero et al. tenia com a objectiu avaluar el benefici de la ciclosporina A (CsA), afegida als esteroides, per al tractament de pacients hospitalitzats per COVID-19 moderada o greu. Els autors suggereixen que l'efecte beneficiós de la CsA podia ser degut tant a les propietats antiinflamatòries com antivirals d'aquest fàrmac.

En aquesta carta volíem posar de manifestat que, tot i que els fàrmacs anticalcineurínics (CNI) inhibeixen la replicació del SARS-CoV en cultius cel·lulars, la concentració efectiva 50 (EC_{50}) en cèl·lules Vero per CsA és de 3,3 μM (corresponent a nivells plasmàtics de 3968 ng / mL), que són 10 vegades superior al rang terapèutic recomanat en humans; i per tacrolimus és 6,9 μM (corresponent a nivells plasmàtics de 5540 ng / mL) que és 1000 vegades superior.

Per tant, vàrem mostrar el nostre acord a Galvez-Romero et al. en que la CsA podria ser una bona alternativa terapèutica per als pacients amb COVID-19 en fases inflamatòries de la malaltia, bloquejant la resposta immune excessiva, però no en les primeres etapes, quan creix i es propaga el virus. Addicionalment, vàrem fer una crida per a que es desenvolupessin assajos clínics per avaluar l'eficàcia i la seguretat dels CNI, per abordar la resposta inflamatòria excessiva present en la COVID-19 greu.

JIM Letter to the Editor

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Inhibition of SARS-CoV-2 replication using calcineurin inhibitors: are concentrations required clinically achievable?

Dear Editor,

We read with great interest the article by Galvez-Romero et al. entitled 'Cyclosporine A plus low-dose steroid treatment in COVID-19 improves clinical outcomes in patients with moderate to severe disease. A pilot study' published in the Journal of Internal Medicine.[1]

This study aimed to assess the added benefit of cyclosporine A (CsA) to steroids in the treatment of life-threatening COVID-19, according to the two pathological mechanisms that appear to coexist in severe SARS-CoV-2 infections, the first triggered by the virus itself and the second by the host-immune response. The study is a nonrandomized observational investigation with 209 hospitalized patients with COVID 19, showing better outcomes in patients treated with CsA plus steroids, compared to patients treated with steroids alone. Furthermore, a significantly lower death rate across all grades of severity was observed, even more marked in those with moderate-to-severe lung injury.

The authors hypothesize that this beneficial effect may be due to both the anti-inflammatory and the antiviral properties of CsA. Accordingly, they report that, in addition to the well-known role as immunomodulatory agent, CsA shows an interesting certain antiviral activity, inhibiting in vitro the replication of several coronavirus, including SARS-CoV and MERS-CoV.[2-4]

In this regard, we would like to remark that, although calcineurin inhibitors have been shown to inhibit SARS-CoV replication in cell cultures, the 50% effective concentrations (EC_{50}) of CsA in Vero cells have been reported to be 3.3 μM (corresponding to plasma levels of 3968 ng/mL),[2] which is 10-fold higher than the recommended therapeutic range in humans, and 6.9 μM (corresponding to plasma levels of 5540 ng/mL)[5] in the case of tacrolimus, which is 1000-fold.

Therefore, we agree that the use of CsA might be justified in those severe COVID-19 patients at risk to transit into the second stage of the disease, by blocking the excessive inflammatory response, but not at the early stages, when viral growth and spread occurs. In this regard, we have recently launched a randomized controlled trial (RCT) using tacrolimus to treat severe COVID-19 patients,[6] and we encourage the development of new other RCTs to assess the efficacy and safety of calcineurin inhibitors, at recommended dose, focused on to tackle the systemic host-immune hyperinflammatory response present in severe COVID-19.

Conflicts of interest

All authors declare no competing interests.

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Author contribution

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V. Discussió

L'edat avançada o altres comorbilitats no permeten predir amb exactitud quins pacients infectats per SARS-CoV-2 progressaran als estadis més greus de la COVID-19. Per tant, calen noves eines que ens permetin entendre millor la gran variabilitat interindividual per emmalaltir d'aquesta infecció. En aquest sentit, els tres primers treballs d'aquesta tesi avaluen la utilitat de diverses tècniques (variants rares a *TLR7*, autoanticossos neutralitzants enfront IFN-I, i QFT-Plus indeterminat) per a predir la gravetat de la COVID-19.

Des d'Abril de 2020, investigadors de l'HUB col·laborem amb el consorci internacional anomenat *COVID-19 Human Genetic Effort* (COVIDHGE) que considera que algunes formes greus de COVID-19 es poden explicar per errors congènits de la immunitat o les seves fenocòpies [47]. Cap dels pacients atesos a l'HUB inclosos al primer estudi genètic de la COVIDHGE tenien variants patogèniques en els 13 gens avaluats de la via d'IFNs de tipus I depenent de TLR3 i IRF7. L'Agost de 2020 van der Made et al. [54] van descriure la relació entre una COVID-19 crítica i variants de pèrdua de funció al gen *TLR7.* Conjuntament amb genetistes del Programa del Càncer Hereditari de l'ICO – IDIBELL, Dra. Conxi Lázaro i Dra. Gardenia Vargas-Parra, vàrem iniciar un estudi per determinar si homes joves i sans amb quadres de COVID-19 greu atesos a l'HUB tenien alguna variant patogènica a *TLR7*. Per a fer-ho, varem col·laborar amb Alexander Hoischen i Cas van der Made del *Department of Human Genetics, Radboud University Medical Center, Nijmegen, Netherlands.*

El **primer estudi** que exposem és fruit de la col·laboració entre HUB – ICO – IDIBELL amb el grup de Nijmegen. Es varen identificar variants rares a *TLR7* en 2 dels 14 casos analitzats. En un dels 10 casos atesos a l'HUB es va identificar una nova variant *missense* [c.644A>G; p.(Asn215Ser)]. La variant va ser predita com a patogènica per anàlisi computacional o *in silico* i segregada en un germà que també va patir una COVID-19 crítica. En un dels quatre casos dels Països Baixos es va identificar una altra nova variant *missense* [c.2797T>C; p.(Trp933Arg)] situada al domini TIR, molt conservat filogenèticament. L'anàlisi funcional d'aquesta segona variant va demostrar respostes d'IFNs de tipus I i II

defectuoses, similars a les documentades en estudis anteriors [54,55]. Així doncs, es confirma la primera hipòtesi de la present tesi doctoral.

Abans de la pandèmia per SARS-CoV-2, no s'havien descrit alteracions a TLR7 associades a una major predisposició a patir infeccions en humans. Es considera que la deficiència completa de TLR7 és extremadament rara, perquè els TLR endosòmics (TLR3, TLR7, TLR8 i TLR9) tenen un paper biològic essencial i no redundant en la supervivència dels individus [111,112]. Per tant, és poc probable que variants de pèrdua de funció de *TLR7* expliquin el desenvolupament d'una COVID-19 greu en una proporció gran de la població. En la nostra cohort, el rendiment del cribatge per detectar variants a *TLR7* va ser bastant elevat (14,3%), pel que la detecció d'aquestes variants podria ser d'interès en pacients joves seleccionats. Estudis recents mostren que les persones de major edat també poden presentar variants rares de pèrdua de funció a *TLR7* [55,113], però són més difícils d'identificar ja que l'edat i altres factors de risc poden justificar les formes greus de COVID-19. Segons la nostra experiència podria ser interessant analitzar variants de *TLR7* si es compleixen els següents criteris: homes joves (<50 anys d'edat) amb una pneumònia per COVID-19 que requereixi almenys de dispositius d'alt flux o ventilació mecànica i que no presentin condicions associades a una COVID-19 greu. Addicionalment, s'hauria de prioritzar l'estudi si hi ha altres germans homes i joves afectes, així com en famílies amb una segregació suggestiva d'estar lligada al cromosoma X.

El consorci internacional COVIDHGE va descriure que fins a un 3,5% dels pacients amb COVID-19 greu eren portadors de defectes genètics en la via els IFNs de tipus I depenent de TLR3 i IRF7 [51]. No obstant, l'associació d'aquests gens amb la gravetat de la COVID-19 no s'ha pogut replicar en alguns estudis posteriors. En un d'aquests estudis es va seqüenciar l'exoma o el genoma complet de 1.864 casos de COVID-19 (713 amb malaltia greu i 1.151 amb malaltia lleu) i 15.033 controls, precedents de 4 biobancs independents, i no es va observar associació entre una COVID-19 greu i les variants rares de pèrdua de funció en els gens de la via dels IFN-I depenents de TLR3 i IRF7 descrites per la COVIDHGE [114]. En el segon estudi es va seqüenciar l'exoma complet de

586.157 individus de diferents ascendències, englobant a 20.592 pacients amb COVID-19 (1.266 dels quals amb quadres greus). Els 13 gens de la via dels IFNs de tipus I depenent de TLR3 i IRF7 tampoc es van associar a formes greus de COVID-19, sent *TLR7* l'únic gen en què la càrrega de variants rares va augmentar significativament entre els pacients amb COVID-19 greu [OR 4,53 (2,64-7,77)] [115]. Aquesta associació es va observar fins i tot sense haver estratificat per sexe o edat.

El nostre estudi reforça la hipòtesi que algunes variants rares en dominis funcionals essencials de TLR7 poden comprometre la inducció de interferons i causar una resposta antiviral defectuosa [116]. Les variants genètiques rares podrien servir de biomarcadors genòmics, juntament amb altres factors de risc, i servirien així per al diagnòstic precoç i per instaurar mesures preventives. En aquest sentit, la detecció d'aquestes variants de *TLR7* permetria recomanar la vacunació enfront a SARS-CoV-2 de forma precoç, una estratègia similar a la seguida amb altres immunodeficiències primàries. Aquesta opció es va oferir als portadors hemizigots de la família del pacient 13 (cas índex de la cohort Neerlandesa). Tot i que encara hi ha poca informació al respecte, els individus infectats per SARS-CoV-2 amb variants deletèries a *TLR7* podrien beneficiar-se d'una hospitalització precoç i de tractaments basats en IFNs com a opcions de teràpia personalitzada [112,117].

A nivell teòric, és possible que altres variants menys rares de *TLR7* puguin condicionar una deficiència parcial de TLR7 i condicionin un risc relatiu baix de desenvolupar COVID-19 greu. *TLR7* té una herència lligada al cromosoma X, i en conseqüència, aquestes variants genètiques de menor efecte s'han proposat com una possible explicació del biaix sexual masculí en la gravetat de la COVID-19 [118]. L'addició de factors que deterioren la funció de TLR7 (p.e. baixos nivells circulants de 25-hidroxi-vitaminaD [25OHD] [119], major edat) [120]) en homes amb aquestes variants de menor efecte, podria ser una causa freqüent de progressió als estadis més greus de la COVID-19. En canvi, en les dones, cada cèl·lula inactiva aleatòriament un dels seus dos cromosomes X per igualar la dosi de gens amb els mascles XY. No obstant això, entre el 15 i el 23% dels gens

humans lligats a X, inclòs *TLR7*, s'escapen de la inactivació del cromosoma X de manera que els dos al·lels es poden expressar simultàniament [121]. En conseqüència, l'expressió bial·lèlica de *TLR7* podria augmentar la susceptibilitat a patir malalties autoimmunes com el lupus eritematós sistèmic [122], però en canvi permetria a les dones desenvolupar respostes immunes més eficients enfront infeccions víriques com la SARS-CoV-2.

El nostre estudi té diverses limitacions. En primer lloc, no disposàvem de mostres biològiques per validar funcionalment la patogenicitat de la variant identificada al pacient del nostre campus, la variant p.(Asn215Ser) i, per tant, no tenim cap mesura objectiva del seu l'impacte funcional. Tot i això, l'absència d'aquesta variant a les bases de dades poblacionals, les altes puntuacions predites de patogenicitat en diferents predictors *in silico*, així com l'escassetat de variants rares al gen de *TLR7* entre la població general, que indiquen que és un gen molt intolerant a les mutacions (pLI = 0,98) [54], reforcen la patogenicitat de la variant. En segon lloc, la variant a *TLR7* identificada en el pacient 13 segrega en un germà que només va experimentar COVID-19 lleu. Que una variant patogènica de *TLR7* no esdevingui completament penetrant podria explicar-se pel fet que la protecció enfront SARS-CoV-2 depèn també d'altres factors (genètics, epidemiològics, comorbilitats, ambientals, etc) [50]. En tercer lloc, la mida mostral del nostre estudi no permet bastir conclusions fermes sobre la prevalença de variants de pèrdua de funció de *TLR7* en homes que desenvolupen una COVID-19 greu, per la qual cosa caldrien estudis de cohorts més grans. En aquest sentit, a l'agost de 2021, després de la la publicació del nostre article, el consorci COVIDHGE va reportar la presència de variants patogèniques a *TLR7* en 16 homes no emparentats d'una cohort de 1202 homes de diferents edats (mediana 36,7 anys [rang 7-71]) que varen patir una COVID-19 greu. Segons aquest estudi el 1,8% dels homes menors de 60 anys són portadors de variants patogèniques a *TLR7* [113], proporció similar a la observada prèviament per Fellarini et al. [55] A la taula de l'**annex 2** es detallen les variants a *TLR7* reportades fins ara a la literatura.

L'Octubre de 2020 el consorci internacional COVIDHGE va reportar que el 10,2% dels pacients amb pneumònia per COVID-19 greu tenien autoanticossos neutralitzants (NautoAbs) enfront IFN-I. Conscients de la rellevància d'aquesta troballa, investigadors de l'HUB vàrem posar-nos en contacte amb el Dr. Roger Colobran de l'Hospital Vall d'Hebrón per determinar a l'HUB els autoanticossos enfront IFN-α2 i -ω mitjançant la tècnica d'ELISA. A més, vàrem contactar amb els Drs. Paul Bastard i Jean-Laurent Casanova del *Laboratory of Human Genetics of Infectious* Diseases, INSERM (Paris, França) i de la *Rockefeller University (New York, USA)* per a determinar l'activitat bloquejant enfront aquests interferons dels nostres pacients. En vàries cohorts [59-63] la determinació d'aquests NautoAbs ha permès identificar una proporció rellevant dels casos que evolucionen a COVID-19 greus o crítics. No obstant, es desconeixia si els pacients ingressats a la UCI per COVID-19 amb els NautoAbs enfront IFN-I tenien característiques clíniques, analítiques i evolutives diferents a les dels pacients sense aquests NautoAbs. La col·laboració entre diferents serveis de l'HUB (UCI, immunologia, laboratori clínic, medicina interna, pneumologia) va permetre dur a terme el **segon estudi** exposat d'aquesta tesi doctoral.

Vàrem analitzar la presència de NautoAbs enfront IFN tipus I (α2 i ω) en mostres sanguínies de 275 pacients ingressat a cures intensives, gairebé tres quartes parts de tots els pacients ingressats a la UCI de l'HUB des del març de 2020 fins al març de 2021. En una cinquena part dels pacients els NautoAbs van resultar positius, amb capacitat neutralitzant en la meitat dels casos. No hi va haver diferències rellevants en les principals dades demogràfiques, comorbiditats, ni característiques clíniques entre els pacients amb NautoAbs positius respecte els pacients negatius. Els pacients amb NautoAbs positius tenien valors significativament més elevats de leucòcits, neutròfils i plaquetes que els negatius. Cal destacar que la insuficiència renal aguda també va ser significativament més freqüent en els pacients amb NautoAbs positius. Globalment una mica més de la meitat del pacients ingressats a la UCI per COVID-19 varen ser èxitus, sense que s'observessin diferències significatives entre la mortalitat del grup de pacients amb NautoAbs positius respecte la dels

negatius. Per tant, es confirma la hipòtesi que un percentage elevat de pacients ingresats a UCI té NautoAbs enfront a IFN-I, però en canvi, tot i observar certes diferències, l'evolució de la COVID-19 no sembla ser pitjor en els pacients amb NautoAbs positius respecte els negatius.

Un estudi recent de Koning et al. [60] va detectar autoAbs enfront IFN-α2 i IFN-ω mitjançant tècniques d'assaig basat en partícules múltiplex i ELISA en 35 (16,6%) de 210 pacients amb la COVID-19, presentant sis (17,1%) d'aquests 35 la capacitat de neutralització d'IFN-I mitjançant l'assaig de fosforilació d'STAT1. Vuitanta-vuit (41,9%) d'aquests 210 pacients amb COVID-19 varen ingressar a la UCI, pertanyent els únics 6 pacients amb NautoAbs a aquest grup de major gravetat. En la mateixa línia, el primer article de Bastard et al. [59] va detectar autoanticossos enfront IFN-α2 i IFN-ω en 135 (13,7%) de 987 pacients amb COVID-19 que posava en perill la vida, mostrant activitat bloquejant 101 (74,8%) d'aquests 135 pacients. En conjunt, aquestes troballes suggereixen que com més greu és la COVID-19, major és la proporció de NautoAbs, però fins i tot en els pacients amb COVID-19 més greu és important determinar l'activitat bloquejant enfront als IFN-I. En la nostra cohort, la meitat (53,1%) dels autoAbs enfront IFN-I determinats per ELISA varen mostrar capacitat per bloquejar 10 ng / mL d'IFN mitjançant l'assaig de la luciferasa.

Segons estudis previs [59-63], els NautoAbs enfront a IFN-I poden ajudar a identificar, en les primeres etapes de la malaltia, a pacients amb un risc elevat de desenvolupar quadres potencialment mortals de COVID-19. Tanmateix, encara hi ha poca informació sobre les característiques diferencials dels pacients de les UCI amb aquests NautoAbs respecte les de la resta pacients crítics que no tenen aquests NautoAbs. Els nostres resultats no van mostrar diferències demogràfiques, de comorbilitats ni clíniques entre ambdós grups, excepte un excés d'homes en els pacients amb NautoAbs positius. Aquesta similitud es podria explicar perquè la resposta d'IFN-I deficient és una característica comuna en pacients crítics amb COVID-19 [40,43,47,123], independentment de si aquest defecte es deu a NautoAbs enfront a IFN-I [61,124], a errors innats d'immunitat [51,54,55,113] o a qualsevol altre mecanisme.

No obstant això, es varen detectar algunes diferències en paràmetres de laboratori en els nostres pacients amb COVID-19 ingressats a la UCI en funció de la presència o absència dels NautoAbs enfront a IFN-I. El grup de pacients amb NautoAbs positius varen mostrar una associació gairebé significativa amb valors més elevats de proteïna C reactiva (PCR), tal com havien informat prèviament *Troya* et al. analitzant un grup més petit de pacients d'UCI [62]. A més, els nostres pacients amb NautoAbs positius també varen presentar valors significativament més elevats de leucòcits, neutròfils i plaquetes. L'elevació de tots aquests paràmetres sanguinis s'ha associat a un major risc de complicacions per la COVID-19 [43,125], cosa que suggereix que els pacients amb NautoAbs podrien desenvolupar formes més greus de COVID-19.

A diferència del que s'ha descrit anteriorment en sèries més petites [60,62], la mortalitat en els nostres pacients no va ser diferent en funció de la presència dels NautoAbs enfront IFN-I. Cal destacar que es va detectar una associació significativa entre la insuficiència renal aguda (IRA) i la positivitat d'aquests NautoAbs. La IRA pot ser deguda a múltiples mecanismes en pacients crítics amb la COVID-19 [126], i caldrà aclarir si aquests NautoAbs juguen un paper en la seva patogènesi. És possible, però només especulatiu, que els NautoAbs enfront IFN-I predisposin a la formació de immunocomplexes que al seu torn podrien activar el complement. La presència anormal de components del complement a la llum tubular renal condueix a la formació del C5b-9 en cèl·lules epitelials tubulars, i podria estar implicat en la patogènesi del dany tubulointersticial. En aquest sentit, una sèrie retrospectiva de sis pacients *post mortem* amb COVID-19 va mostrar el dipòsit dels factors de complement C5b-9 en els túbuls de tots els ronyons que es varen examinar [127]. Tot i que s'han de confirmar aquestes troballes, els NautoAbs podrien esdevenir un biomarcador per identificar aquells pacients afectes de COVID-19 crítica amb un major risc de desenvolupar IRA, ajudant els clínics a prendre decisions preventives i terapèutiques.

A diferència d'altres factors relacionats amb l'augment de la gravetat de la COVID-19, la detecció d'aquests NautoAbs en pacients crítics permet plantejar

l'administració de tractaments específics. En aquest sentit, recentment s'ha reportat que la plasmafèresi disminuïa els títols de NautoAbs en la sang i en el tracte respiratori de quatre pacients hospitalitzats amb pneumònia greu per COVID-19, tot i que la mortalitat va ser del 50% [78]. Es desconeix si l'administració d'IFN-β, la depleció de cèl·lules B o altres teràpies poden ser beneficioses per a tractar a aquest subgrup de pacients [128].

El nostre estudi té diverses limitacions que cal comentar. En primer lloc, no va ser possible obtenir mostres de plasma de tots els pacients ingressats a la UCI durant el període d'estudi, tot i que varem poder analitzar més del 70% d'aquestes. Per tant, considerem que es tracta d'un grup representatiu amb poca probabilitat de biaixos. En segon lloc, hem detectat exclusivament els IFN-I més freqüents (α2 i ω) per ELISA i, per tant, és possible que alguns pacients de l'estudi presentessin altres autoanticossos que no es varen analitzar (p.e, enfront IFN-β). En tercer lloc, hem analitzat l'activitat bloquejant a 10 ng / mL d'IFN basant-nos en el que s'havia publicat prèviament [59-63], però les concentracions sanguínies d'IFN-α en pacients amb COVID-19 lleus / moderats oscil·len entre 1 i 100 pg / mL, i són fins i tot més baixes en els pacients greus / crítics [40], de manera que autoAbs que neutralitzin quantitats d'IFN-I inferiors a 10 ng / mL podrien contribuir al desenvolupament de pneumònies per COVID-19 crítiques en més del 9,5% dels casos, tal com suggereix Bastard et al en un estudi recent [64]. En quart lloc, atès que el nostre estudi és retrospectiu, podem haver passar per alt alguns factors de confusió i haver deixat d'incloure pacients (*missings)* que hagin alterat certs resultats. En cinquè lloc, el disseny de l'estudi no ens permet establir si aquests NautoAbs tenen un paper patogènic o són simplement un biomarcador de major risc per a desenvolupar insuficiència renal aguda en els pacients amb COVID-19 que requereixen ingrés a UCI. Finalment, aquest estudi no permet avaluar la utilitat d'aquests NautoAbs en aquells pacients en fases anteriors o lleus de la malaltia.

Pensem que calen investigacions addicionals per a avaluar el paper de la genètica de l'hoste i dels NautoAbs enfront IFN-I en la patogènia i evolució de la COVID-19 que ajudin a seleccionar teràpies més adequades i personalitzades.

En aquest sentit, a la darrera edició de La Marató de TV3, dedicada a la COVID-19, es va concedir finançament a 36 projectes dels 229 projectes que es varen presentar. Un dels projectes seleccionats és el del nostre grup de l'ICO-HUB-IDIBELL titulat "Role of Immune system inborn errors as determinants of the COVID-19 severity in hospitalized patients" (#202115-30-31). El nostre proper objectiu és avaluar un nombre superior de gens de susceptibilitat mitjançant un panell NGS específic i determinar el significat clínic dels NautoAbs en diferents contexts.

Durant els primers mesos de la pandèmia de COVID-19, els microbiòlegs de l'HUB Fernando Alcaide i Miguel Fernández-Huerta van observar un elevat nombre de resultats indeterminats de l'assaig QuantiFERON-TB Gold Plus (QFT-Plus) en pacients hospitalitzats per COVID-19. Conjuntament amb el Dr. Miguel Santin del servei de malalties infeccioses de l'HUB ens varen proposar de determinar la prevalença i els factors associats a un resultat indeterminat de l'assaig QFT-Plus en aquests pacients i analitzar la relació entre un resultat indeterminat amb la mortalitat per COVID-19. En el **tercer estudi** que exposem vàrem observar una prevalença inesperadament alta de resultats indeterminats en l'assaig QFT-Plus en pacients hospitalitzats per COVID-19. Addicionalment, vàrem observar que factors relacionats amb la gravetat clínica de la COVID-19 (p.e. nivells elevats de LDH) i l'administració de corticosteroides abans de la realització del QFT-Plus estaven associats a aquests resultats indeterminats. Per últim, vàrem detectar una associació estadísticament significativa entre un resultat indeterminat del QFT-Plus i una major mortalitat hospitalària per COVID-19. Per tant, es confirma la tercera hipòtesi d'aquesta tesi doctoral.

La sensibilitat dels IGRA en la detecció de la LTBI es pot veure afectada per condicions que alteren el sistema immune. A l'HUB s'ha avaluat prèviament la precisió dels IGRAs en diversos trastorns relacionats amb la immunitat, amb un percentatge de resultats indeterminats del 2,1% en pacients cirròtics pretransplantament [130], del 7,7% en pacients amb corticosteroides que abans

d'iniciar tractaments amb anti-TNF-α [131], o del 9,5% en pacients VIH positius sense tractament antiretroviral i amb un recompte de CD4 <100 cèl·lules / mm^3 [132]. El nostre estudi va trobar que el 35,4% dels pacients hospitalitzats per COVID-19 tenien un resultat indeterminat de l'assaig QFT-Plus. Aquesta proporció és concordant amb la taxa del 36,4% reportada per Torre et al. en un estudi similar realitzat a Itàlia [81]. Per tant, la freqüència de resultats indeterminats sembla ser bastant elevada en pacients amb COVID-19.

En el nostre estudi, l'assaig QFT-Plus es va realitzar principalment en pacients de COVID-19 hospitalitzats, greus i després dels 10 dies del inici dels símptomes, per la qual cosa els resultats obtinguts són representatius de la fase inflamatòria de la COVID-19 [82]. Segons el que s'ha publicat anteriorment, els pacients amb la COVID-19 en aquesta fase de la malaltia presenten nivells sanguinis elevats de múltiples citosines proinflamatòries (IL-1β, IL-2, IL-4, IL-7, IL-10, MCP-1, GCSF, MIP -1A, TNF-α, IFN-β i IP-10) [43,133]. Tanmateix, aquest fet no va alterar la precisió del QFT-Plus, ja que els resultats indeterminats no van ser deguts a una concentració elevada de IFN-γ al tub nul (> 8 UI / mL) [79].

Tots els resultat indeterminat del QFT-Plus van ser deguts a concentracions d'IFN-γ per sota del límit establert pel fabricant en el control basat en mitògens (és a dir, el valor del mitògen menys el valor del nul va ser < 0,5 UI / ml). Per tant, vàrem considerar que alguns pacients amb una COVID-19 greu podien estar patint algun tipus de deterior de la funció del sistema immune. Segons les nostres dades, dos grups de factors s'associen a resultats indeterminats: en primer lloc, alguns paràmetres analítics que prèviament s'han relacionat amb una pitjor evolució de la COVID-19 (limfopènia, leucocitosi, neutrofília, LDH elevada, dímer D elevat, hipoxèmia) [40,133] i, en segon lloc, l'ús de tractaments immunosupressors administrats prèviament a la realització del QFT-Plus, especialment els corticoides.

Se sap que l'ús de corticosteroides i altres fàrmacs immunosupressors pot contribuir a un major nombre de resultats indeterminats de l'assaig QFT-Plus

[132]. Tot i això, cal remarcar que la taxa de resultats indeterminats es va mantenir molt elevada (14,7%) fins i tot en el subgrup de pacients que no havien rebut cap tractament immunosupressor abans de la prova. Aquest fet ens fa pensar que el propi SARS-CoV-2 podria ser un factor que contribueixi als resultats indeterminats. A més, cal destacar que la taxa de pacients amb un resultat indeterminat de l'assaig QFT-Plus va ser significativament superior en pacients que van morir per la COVID-19 (n = 23/79 [29,1%] vs. n = 11/17 [64,7%]; p = 0,005). Els pacients amb una prova de QFT-Plus indeterminada tenien quatre vegades més mortalitat (HR 4.025 [IC 95% 1.486-10.903]) que aquells pacients amb resultats determinats (positius o negatius).

Com s'ha comentat prèviament, SARS-CoV-2 ha desenvolupat diversos mecanismes per deteriorar la resposta immune de l'hoste, inclosa la inhibició de la inducció i la senyalització dels IFNs. No obstant això, hi ha dades contradictòries sobre el paper dels IFN de tipus II, com l'IFN-γ, en la COVID-19. L'elevació de múltiples citosines inflamatòries, entre elles l'IFN-γ, s'ha observat en fases avançades de la malaltia. D'altra banda, SARS-CoV-2 ha demostrat tenir també la capacitat d'induir una supressió primerenca i profunda de la producció d'IFN-γ per les cèl·lules T [45]. Aquestes dades suggereixen que la fase de la malaltia és la clau, sent l'IFN-γ protector en fases primerenques de la COVID-19, mentre que en fases més avançades podria ser perjudicial, i augmentar com a conseqüència dels múltiples estímuls rebuts per la hipeactivació del sistema immune.

A la pràctica clínica, hi ha una manca d'eines diagnòstiques per avaluar defectes de la immunitat que ens ajudin a predir l'evolució a COVID-19 greu. En aquest sentit, la prova del QFT-Plus podria ser un immunoassaig funcional estandarditzat i accessible per avaluar defectes en la resposta d'IFNs dels pacients infectats per SARS-CoV-2, i podria contribuir a predir quins pacients patiran quadres greus de COVID-19.

Aquesta investigació preliminar de pacients amb COVID-19 amb un assaig QFT-Plus indeterminat té diverses limitacions. Vàrem realitzar una anàlisi

retrospectiva on els factors de confusió i *missings* podrien haver alterat certs resultat. Només es va realitzar el QFT-Plus en el 6,8% dels pacients amb COVID-19 ingressats a l'hospital durant el període d'estudi, per tant, es tracta d'un grup molt seleccionat i amb elevat potencial de biaixos. A més, l'assaig QFT-Plus es va realitzar de manera no sistemàtica en pacients hospitalitzats amb COVID-19, tot i que en la majoria dels casos es va determinar quan es trobaven en fase inflamatòria, i rebent o a punt de rebre immunosupressors com corticosteroides o tocilizumab. Per tant, el nostre estudi no ens permet avaluar la utilitat de l'assaig QFT-Plus en pacients en fases anteriors de la malaltia. Per últim, el nombre de pacients avaluats en aquest estudi és baix i per tant no permet extreure conclusions sòlides sobre el paper d'un QFT-Plus indeterminat com a factor predictor de mala evolució de la COVID-19.

Conscients de les limitacions d'aquest estudi, hem seguit analitzant als pacients ingressats per COVID-19 als que s'ha realitzat QFT-Plus durant les diferents onades pandèmiques. Considerem que les primeres 24-48 hores d'hospitalització son el moment més adient per a realitzar aquest assaig si es pretén detectar pacients amb un major risc de desenvolupar formes greus de la COVID-19. A més, cal considerar que com més aviat es realitzi aquest assaig menor serà l'impacte de fàrmacs immunosupressors en els resultats del QFT-Plus. Per a avaluar si l'assaig QFT-Plus pot contribuir a predir l'evolució a COVID-19 greu, actualment estem analitzant exclusivament a pacients als que s'ha realitzat un QFT-Plus durant les primeres 48 hores del ingrés per COVID-19.

Com a clínics hem tingut un especial interès en poder oferir alternatives terapèutiques eficaces i segures als malalts greus que han hagut de ser hospitalitzats per la COVID-19. Durant els primers mesos de la pandèmia, teníem encara poca informació sobre la patogènia de la malaltia, i no es disposava d'assaigs clínics controlats que permetessin administrar tractaments en base a l'evidència. Les **últimes tres publicacions** que presentem en aquesta tesi s'emmarquen en el context de la urgència sanitària viscuda el març del 2020 arrel de la pandèmia per COVID-19. Vàrem dissenyar un assaig clínic,

aleatoritzat, unicèntric i obert que tenia l'objectiu d'avaluar l'eficàcia i la seguretat dels polsos de metilprednisolona i tacrolimus, juntament amb l'*Standard of Care* (SoC), versus l'SoC solament, en pacients hospitalitzats amb COVID-19 greu (assaig Tacrovid). Aquest assaig, dut a terme en una situació molt excepcional, va ser possible gràcies a la extraordinària implicació de múltiples departaments de l'IDIBELL, així com a la col·laboració desinteressada d'innumerables professionals sanitaris de l'HUB.

En l'assaig Tacrovid, el tractament experimental no va millorar significativament el temps fins a l'*estabilitat clínica* (*outcome* primari), mortalitat o altres variables de validació secundàries en comparació amb l'*SoC* en pacients hospitalitzats amb COVID-19 greu. Tampoc es varen observar diferències en l'eliminació del virus o en la taxa d'esdeveniments adversos entre els dos grups. Així doncs, el nostre assaig clínic no permet afirmar que els polsos de metilprednisolona juntament amb tacrolimus, afegits a l'*Standard of Care*, sigui una combinació eficaç i segura per tractar pacients hospitalitzats per una COVID-19 greu.

Tot i no assolir la significació estadística, els pacients que varen rebre la teràpia experimental varen presentar una mortalitat per totes les causes numèricament inferior a la dels pacients que varen rebre l'*SoC*, sobretot durant els dies 10 i 28. Les nostres dades reforcen els resultats d'estudis no aleatoritzats en que es va observar que la ciclosporina reduïa la mortalitat, principalment en pacients amb COVID-19 moderat a greu [109,110]. Addicionalment, en un estudi europeu multicèntric realitzat a 243 receptors de trasplantament hepàtic adults amb COVID-19 simptomàtic, l'ús de tacrolimus va tenir un efecte beneficiós independent en termes de supervivència, comparant-lo amb la resta d'immunosupressors (ciclosporina, micofenolat o inhibidors de mTOR), suggerint que podria ser fins i tot més beneficiós que la ciclosporina [134]. Considerem que les nostres dades de mortalitat als 56 dies han de ser valorades amb prudència perquè només cinc pacients del grup experimental estaven encara ingressats a l'hospital i la teràpia experimental es va retirar prèviament en tots ells a causa de la necessitat d'administrar ventilació mecànica invasiva o d'algun esdeveniment advers greu.

D’altra banda, s’ha demostrat que els fàrmacs inhibidors de la calcineurina (CNIs), a concentracions micromolars baixes i no citotòxiques, bloquegen el creixement de coronavirus humans en cultius cel·lulars, mitjançant la inhibició de la via d’immunofil·lina [104,105]. Basant-se en aquesta troballa, s’ha suggerit que els CNIs es podrien utilitzar com a agent antiviral per tractar la COVID-19. Desafortunadament, cal destacar que les concentracions utilitzades en cultiu cel·lular no són clínicament assolibles, ja que corresponen a nivells sanguinis altament tòxics en humans **(article 6)**. En conseqüència, la indicació de tacrolimus s’hauria de restringir a les etapes inflamatòries de la COVID-19.

L'assaig TACROVID es va iniciar el 29 de març de 2020, quan no s’havien publicat encara els assajos clínics que donen suport a l'ús de teràpia immunosupressora en els pacients amb una COVID-19 greu. Tot i això, tots els pacients de l’assaig varen rebre corticoides per protocol (en el grup experimental), o bé, com a part del SoC indicat pel metge tractant. Cal destacar que dosis de corticosteroides més elevades i durant un període de temps major es varen utilitzar en el grup control, tot i que desconeixem les raons exactes. Durant el seguiment tots dos grups varen tenir resultats similars en les proves de laboratori, varen necessitar en proporcions semblants dispositius d’alt flux i ventilació, i varen desenvolupar una afectació pulmonar parenquimatosa similar per radiografia toràcica. Cal remarcar que els pacients que rebien la teràpia experimental varen assolir un valor ≤ 3 de l’escala ordinal en un temps numèricament menor que aquells pacients que rebien l’SoC dins dels 10 dies posteriors a la randomització, tot i que aquesta tendència es va revertir posteriorment. Finalment, es tractava d’un assaig obert en què els pacients del grup control no podien rebre tacrolimus ni ciclosporina per protocol, així que és possible que els seus metges tractants pautessin alternativament més corticoides o altres immunosupressors (p.e. anakinra). Tanmateix, els tractaments rebuts per a tractar la COVID-19 o les seves complicacions (antibiòtics, lopinavir-ritonavir, hidroxicloroquina, heparines i tocilizumab) van ser similars en els dos grups.

La metilprednisolona i el tacrolimus suprimeixen intensament el sistema immune [86,99] i, per tant, podrien facilitar la replicació de la SARS-CoV-2 i afavorir el desenvolupament d'altres infeccions. En aquest assaig, el tractament experimental no va comportar cap canvi significatiu sobre la càrrega viral de SARS-CoV-2 a les vies respiratòries superiors ni a les mostres de sang dels nostres pacients. No hi va haver tampoc diferències en els esdeveniments adversos (EA) globals entre els dos grups. Els pacients del grup experimental varen presentar un nombre superior d'EA infecciosos no greus i greus. Cal recordar que els corticosteroides s'associen a sagnat gastrointestinal, hiperglucèmia i debilitat neuromuscular [135]. De fet, el grup tractat amb tacrolimus va rebre una dosi significativament inferior de corticosteroides, amb un millor control del metabolisme de la glucosa.

L'assaig TACROVID tenia vàries limitacions. En primer lloc, l'assaig no es va dur a terme com a doble cec. Això es va considerar poc realista atesa l'elevada càrrega de treball experimentada pel sistema sanitari a l'inici de la pandèmia. Per minimitzar l'impacte d'un disseny obert, l'estadístic que va realitzar les anàlisi era cec respecte al grup al que pertanyien els pacients. En segon lloc, aquest assaig tenia una mida mostral limitada i, que clarament no era suficient per a detectar diferències respecte el temps fins a l'*estabilitat clínica* o la mortalitat entre els dos grups després de la finalització precoç que es va produir amb 29 (34,1%) pacients menys del que s'esperava. A més, la petita mida de la mostra va causar certs desequilibris en les característiques basals entre els dos grups després de l'aleatorització. En tercer lloc, tots els pacients inclosos varen rebre corticoides, heparines i hidroxicloroquina, i la majoria (89,1%) també varen rebre tocilizumab com a *SoC*. Aquest fet dificulta avaluar l'efecte individual de la teràpia experimental i de cadascun dels medicament utilitzats com a *SoC* en els resultats clínics, les dades de laboratori i l'aparició d'EA. Addicionalment, el tacrolimus interactua fortament amb alguns tractaments (especialment amb el lopinavir) utilitzat en aquell moment com a tractament per a la COVID-19. La majoria (85,2%) dels pacients del grup experimental varen rebre tractament concomitant amb lopinavir-ritonavir, fet que va dificultar assolir els nivells

plasmàtics recomanats de tacrolimus. Finalment, la manca d'evidències mèdiques que donessin suport a les teràpies immunosupressores per a la COVID-19, quan es va dur a terme l'assaig ens va fer ser prudents, retirant la teràpia experimental quan era necessària la intubació orotraqueal per iniciar ventilació mecànica invasiva. Per tant, la seva eficàcia i seguretat en aquest subgrup de pacients afectes de COVID-19 no es pot avaluar mitjançant aquest assaig.

Un cop finalitzat l'assaig TACROVID, hem seguit col·laborant en la recerca de noves teràpies per a tractar a pacients hospitalitzats per la COVID-19 **(annex 3)**. Conscients que, per obtenir resultats sòlids, és necessari disposar d'un nombre important de pacients, hem prioritzat l'inclusió dels nostres pacients en assajos clínics multicèntrics. Al igual que l'assaig TACROVID, els assajos en què estem participant avaluen la utilitat i seguretat de fàrmacs amb propietats antiinflamatòries (p.e. Canakinumab, Immunoglobulines policlonals a dosis immunomoduladores, etc).

VI. Conclusions

1.- Hem identificat dues noves variants germinals al gen *TLR7* que probablement causen una funció deficient del receptor i que podrien explicar la COVID-19 greu en els pacients portadors de les mateixes. En el pacient portador de la variant p.(Trp933Arg) s'ha pogut realitzar un estudi funcional que corrobora la seva patogenicitat, atès que en la seva presència s'observen respostes d'IFN de tipus I i II disminuïdes.

2.- Aquestes troballes reforcen la idea que TLR7 juga un paper essencial en el reconeixement de la SARS-CoV-2 i en la posterior inducció d'una resposta antiviral precoç, que podria evitar el desenvolupament d'una COVID-19 greu.

3.- El diagnòstic de deficiència de TLR7 no només obre la porta a nous tractaments personalitzats per als pacients, sinó també a poder fer proves presimptomàtiques als familiars homes en risc i, per extensió, iniciar intervencions preventives i terapèutiques precoces.

4.- Una cinquena part dels pacients amb COVID-19 ingressats a cures intensives de l'HUB varen presentar autoanticossos enfront IFN de tipus I (IFN-α2 i / o IFN-ω), mostrant en la meitat dels casos activitat bloquejant enfront a altes concentracions (10 ng / mL) d'aquests IFNs.

5.- En aquests pacients afectes de pneumònia per COVID-19 crítica, la presència d'aquests autoanticossos neutralitzants enfront IFN-I va ser notable i significativament superior en homes. La seva presència es va associar a valors més elevats de paràmetres de laboratori relacionats amb la gravetat de la COVID-19, i també a un major risc de desenvolupar insuficiència renal aguda. Per contra, la mortalitat va ser similar tant en presència com en absència d'aquests autoanticossos.

6.- Un terç dels pacients hospitalitzats per COVID-19 a qui es va demanar la prova QuantiFERON-TB Gold Plus va presentar resultats indeterminats. Aquesta prevalença notablement alta posa en dubte la fiabilitat d'aquesta prova com a screening d'infecció tuberculosa latent en aquests malalts.

7.- Alguns factors associats a pitjor evolució de la COVID-19, com la concentració plasmàtica de LDH, així com l'ús de corticosteroides abans de la realització de la prova QuantiFERON-TB Gold Plus, podrien ser predictors d'un resultat indeterminat. En aquest sentit, es va documentar una proporció significativament superior de resultats indeterminats en els pacients hospitalitzats per COVID-19 que varen ser èxitus.

8.- La combinació de polsos de metilprednisolona i tacrolimus, a més de l'*Standard of Care*, no va millorar significativament el temps fins a l'estabilitat clínica o altres criteris de validació (*outcomes*) secundaris, en comparació amb l l'*Standard of Care* solament, en pacients hospitalitzats amb COVID-19 greu.

9.- Tot i no assolir la significació estadística, els pacients que varen rebre la teràpia experimental, combinant polsos de metilpredinisolona i tacrolimus, varen presentar una mortalitat per totes les causes numèricament inferior que la del grup que va rebre només l'*Standard of Care*, recolzant els resultats obtinguts prèviament per estudis no aleatoritzats amb inhibidors de la calcineurina.

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VIII. Annexes

ANNEX 1. VARIANTS GENÈTIQUES O HAPLOTIPS DE SUSCEPTIBILITAT A SARS-COV-2 REPORTADES AMB ANTERIORITAT A LA PUBLICACIÓ DELS TREBALLS DE LA TESI

Variant genètica o haplotip	Risc [OR]	Freqüència [MAF]	Referències
TLR3, *UNC93B1*, *TICAM1*, *TBK1*, *IRF3*, *IRF7*, *IFNAR1*, *IFNAR2* (autosomal-dominant model)	9	<0.001	Zhang et al
IRF7, IFNAR1 (autosomal-recessive model)	>50	<0.001	Zhang et al.
rs769208985—missense variant of *FURIN*	N.A	<0.001	Latini et al.
rs150892504—missense variant of *ERAP2*	N.A	0.002	Hu et al.
rs138763430—missense variant of *BRF2*	N.A	0.002	Hu et al.
rs147149459—missense variant of *ALOXE3*	N.A	0.002	Hu et al.
rs117665206—missense variant of *TMEM181*	N.A	0.006	Hu et al.
rs114363287—missense variant of *TMPRSS2*	N.A	0.006	Latini et al.
rs61756766—missense variant of *TNFRSF13C*	12.3	0.008	Russo et al.
rs7626962—missense variant of *SCN5A*	8.7	0.008	SeyedAlinaghi et al.
rs1805128—missense variant of *KCNE1*	9.0	0.009	SeyedAlinaghi et al.
HLA-DRB*27:07	N.A	0.02	*Novelli* et al.
rs72711165—intronic variant of *TMEM65*	1.2	0.02	COVID-19 H.G.I.
rs115492982—intronic variant of *MRPS21*	2.5	0.02	Dite et al.
rs74956615—3'UTR variant of *TYK2*	1.6	0.03	Pairo-Castineira et al.
rs2034831—intronic variant of *ITGA4*	1.2	0.05	Dite et al.
rs76374459—intronic variant of *LZTFL1*	1.2	0.05	Dite et al.
rs35652899—intronic variant of *LZTFL1*	1.2	0.05	Dite et al.
rs10490770—intronic variant of *LZTFL1*	2.0	0.06	COVID-19 H.G.I.
rs333—CCR5-Δ32	0.7	0.07	Cuesta-Llavona et al.
rs73064425—intronic variant of *LZTFL1*	2.1	0.08	Pairo-Castineira et al. Ellinghaus et al.
rs11385942—intronic variant of *LZTFL1*	1.8	0.07	Ellinghaus et al.
rs1886814—intronic variant of *FOXP4*	1.3	0.07	COVID-19 H.G.I.
rs76488148—intronic variant of *GYG1*	1.3	0.07	Dite et al.
rs2271616—5'UTR variant of *SLC6A20*	1.1	0.08	COVID-19 H.G.I.
HLA-DQB1*06:02	N.A	0.08	*Novelli* et al
rs143334143—intronic variant of *CCHCR1*	1.9	0.09	Pairo-Castineira et al.
HLA-DRB1*15:01	N.A	0.10	*Novelli* et al.
rs12252:G allele of *IFITM3*	2.2	0.13	Alghamdi et al.
rs4801778—intronic variant of *PLEKHA4*	1.0	0.16	COVID-19 H.G.I.
rs6598045—5'UTR variant of *IFITM3*	N.A	0.19	Kim et al.
rs429358—missense variant of *APOE*	2.3–2.4	0.20	Kuo et al.
rs12610495—intronic variant of *DPP9*	N.A	0.25	Moon et al.
rs12329760—intronic variant of *TMPRSS2/MX1*	0.9	0.25	Andolfo et al.
rs2298661—missense variant of *TMPRSS2/MX1*	0.9	0.25	Andolfo et al.
rs3787946—intronic variant of *TMPRSS2/MX1*	0.9	0.28	Andolfo et al.
rs9983330—intronic variant of *TMPRSS2/MX1*	0.9	0.28	Andolfo et al.
rs9380142—3'UTR variant of *HLA-G*	13	0.29	Pairo-Castineira et al.
rs2109069—intronic variant of *DPP9*	1.4	0.33	Pairo-Castineira et al., COVID-19 H.G.I.
rs9985159—intronic variant of *TMPRSS2/MX1*	0.9	0.33	Andolfo et al.
rs75603675—missense variant of *TMPRSS2*	N.A	0.36	Latini et al.

rs1405655—intronic variant of *NR1H2*	1.1	0.37	COVID-19 H.G.I.
rs12329760—missense variant of *TMPRSS2*	0.9	0.39	Hou et al.
rs657152—intronic variant of *ABO*	1.3	0.41	Ellinghaus et al.
rs677800—intronic variant of *ABO*	N.A	0.55	Moon et al.
rs6020298—intronic variant of *TMEM189-UBE2V1*	1.2	0.58	Wang et al.
rs10735079—intronic variant of *OAS1/3*	1.3	0.64	Pairo-Castineira et al.
rs8065800—intronic variant of *MAPT*	1.7	0.65	COVID-19 H.G.I.
rs10774671—intronic, splicing variant of *OAS1*	1.1	0.67	COVID-19 H.G.I.
rs13050728—intronic variant of *IFNAR2*	0.9	0.69	COVID-19 H.G.I.
rs2236757—intronic variant of *IFNAR2*	1.3	0.71	Pairo-Castineira et al.
rs3131294—intronic variant of *NOTCH4*	1.5	0.90	Pairo-Castineira et al.
HLA-A*11	N.A	N.A	Fricke-Galindo et al.
HLA-A*11:01:01:01	2.3	N.A	Khor et al.
HLA-A*25:01	N.A	N.A	Fricke-Galindo et al.
HLA-B*46:01	2.1	N.A	Lin et al., Fricke-Galindo et al.
HLA-B*51:01	N.A	N.A	Fricke-Galindo et al.
HLA B*54:01	5.4	N.A	Lin et al.
HLA-C*01	N.A	N.A	Fricke-Galindo et al.
HLA-C*01:02	N.A	N.A	Fricke-Galindo et al.
HLA-C*05	N.A	N.A	Fricke-Galindo et al.
HLA-C*12:02:02:01-HLA*52:01:02:02	2.3	N.A	Khor et al.
HLA-C*14:02	N.A	N.A	Fricke-Galindo et al.
HLA-C*17	N.A	N.A	Bonaccorsi et al.
HLA-DQB1*04	N.A	N.A	Fricke-Galindo et al.
HLA-DQB1*08	N.A	N.A	Fricke-Galindo et al.
HLA-E*0101/0103	2.1– 2.7	N.A	Vietzen et al.
*KLRC2*del	2.6–7.1	N.A	Vietzen et al.
ACE1 I/D genotype	2.5	N.A	Verma et al.
C9orf72 with HREs > 10 units	2.4	N.A	Zanella et al.
c.2129_2132del, p.Gln710Argfs*18—frameshift variant of *TLR7*	N.A	N.A	van der Made et al.
c.2383G>T, p.Val795Phe—missense variant of *TLR7*	N.A	N.A	van der Made et al.
c.901 T>C, p.Ser301Pro—missense variant of *TLR7*	N.A	N.A	Fallerini et al.
c.3094G>A, p.Ala1032Thr— missense variant of *TLR7*	N.A	N.A	Fallerini et al.
c.2759G>A, p.Arg920Lys—missense variant of *TLR7*	N.A	N.A	Fallerini et al.
c.863C>T, p.Ala288Val—missense variant of *TLR7*	N.A	N.A	Fallerini et al.
c.1342C>T, p.Ala448Val—missense variant of *TLR7*	N.A	N.A	Fallerini et al.
c.655G>A, p.Val219Ile—missense variant of *TLR7*	N.A	N.A	Fallerini et al.
rs140312271—missense variant of *ACE2*	N.A	N.A	Novelli et al.

Adaptat de Colona VL, et al. Hum Genomics. 2021

ANNEX 2. VARIANTS DE *TRL7* RELACIONADES AMB UNA COVID-19 GREU PUBLICADES A LA LITERATURA

Autor‡	Variant	aminoàcid	Tipus	Activitat al·lel	Origen ètnic
Asano	c.223A>C	p.(Asn75His)	missense	LOF	Middle East
Asano	c.401T>C	p.(Leu134Pro)	missense	LOF	Admixed American
Asano	c.471delC	p.(Asn158Thrfs*11)	deletion	LOF	European NF
Solanich	c.644A>G	p.(Asn215Ser)	missense	-	Latino
Fallerini	c.655G>A	p.(Val219Ile)	missense	Hypomorphic	European NF
Asano	c.680delT	p.(Leu227fs*)	deletion	LOF	Middle East
Asano	c.730G>T	p.(Asp244Tyr)	missense	LOF	Middle East
Fallerini	c.901T>C	p.(Ser301Pro)	missense	LOF	European NF
Asano	c.928T>C	p.(Phe310Leu)	missense	LOF	Middle east
Asano	c.1114C>T	p.(Leu372Met)	missense	Hypomorphic	Central Asian
Asano	c.1514T>C	p.(Ile505Thr)	missense	LOF	European NF
Asano	c.1888C>T	p.(His630Tyr)	missense	LOF	European NF
Asano	c.1970T>C	p.(Ile657Thr)	missense	Hypomorphic	European NF
Asano	c.(2010_2011del; 2013_2014insC)	p.(Phe670Leu*8)	Deletion /insertion	Hypomorphic	European F
Asano	c.2050A>T	p.(Lys684*)	nonsense	LOF	European NF
Van der Made	c.2129_2132del	p-(Gln710Argfs*18)	deletion	LOF	European NF
Asano	c.2143C>T	p.(Pro715Ser)	missense	Hypomorphic	Latino
Asano	c.2342A>T	p.(His781Leu)	missense	LOF	Middle East
Van der Made	c.2383G>T	p.(Val795Phe)	missense	LOF	African
Asano	c.2455G>A†	p.(Met854Leu)	missense	LOF	European NF
Fallerini	c.2759G>A	p.(Arg920Lys)	missense	-	European NF
Solanich	c.2797T>C	p.(Trp933Arg)	missense	LOF	European NF
Asano	c.2963T>C	p.(Leu988Ser)	missense	LOF	Middle East
Fallerini	c.3094G>A	p.(Ala1032Thr)	missense	LOF	European NF

‡Primer autor del treball on es reporta la variant de *TLR7*.

†La variant c.2445G>A està conjuntament amb la variant c.2963T>C en un mateix pacient.

- Anàlisi funcional no disponible**;** *European NF (Non-Finnish), European F (Finnish)*

Font: van der Made et al. Colona VL, et al. JAMA. 2020; Fallerini C, et al. eLife. 2021; Asano T, et al. Sci Immunol. 2021; Solanich X, et al. Front Immun 2021.

ANNEX 3. PROJECTES D'R+D+I RELACIONATS AMB LA TEMÀTICA DE LA TESI EN ELS QUE EL DOCTORAND HI HA PARTICIPAT O HI PARTICIPA COM A INVESTIGADOR

3.1. Participació en projectes d'R+D+I finançats en convocatòries competitives d'Administracions d'entitats públiques o privades

FUNCIÓ DESENVOLUPADA: Promotor – Investigador Principal**.** ***Pragmatic, open-label, single-center, randomized, phase II clinical trial to evaluate the efficacyand safety of methylprednisolone pulses and tacrolimus in patients with severe pneumonia secondary to COVID-19: the TACROVID trial*** (Nº EudraCT: 2020-001445-39). ENTITAT FINANÇADORA: PERIS - COVID19: Departament de Salut de la Generalitat de Catalunya - CERCA Programme. 108.550,00 euros. INICI/ FINALIZACIÓ: Abril a Desembre de 2020.

FUNCIÓ DESENVOLUPADA: Investigador col·laborador. ***Immune and Omics Cell Atlas of COVID-19 patients with Pre-existing Autoimmunity and Immunodeficiency*****.** INVESTIGADOR PRINCIPAL: Esteban Ballestar i Roser Vento. ENTITAT FINANÇADORA: Chan Zuckerberg Initiative (grants 2017-174169, 2020-216717, 2020-216799, 2020-216949, 2020-216954 and 2020-217820).National Institute of Health, and the American Lung Association COVID-19 Action Initiative grant, and an anonymous gift through the Broad Institute to support the COVID-19 single-cell genomics work. INICI/ FINALITZACIÓ: 2020 – 2023.

FUNCIÓ DESENVOLUPADA: Investigador col·laborador. ***COVID-19, immunological risk profile.*** INVESTIGADOR PRINCIPAL: Ricardo Pujol Borrell. ENTITAT FINANÇADORA: Instituto de Salud Carlos III – Convocatoria de proyectos de investigación sobre el SARS-CoV-2 y la enfermedad COVID-19 – 144.623,56 euros. INICI/ FINALITZACIÓ: Juny 2020 – juny 2023.

FUNCIÓ DESENVOLUPADA: Investigador col·laborador. ***Role of Immune System inborn errors as determinants of the COVID-19 severity in hospitalized patients (202115-30-31).*** INVESTIGADOR PRINCIPAL: Conxi Lázaro i Xavier Corbella. ENTITAT FINANÇADORA: La Marató de TV3 - 299.998 €. INICI/ FINALITZACIÓ: Juliol 2021 – Juliol 2024.

3.2. Contractes, convenis o projectes d'R+D+I, no competitius amb Administracions o entitats públiques o privades

FUNCIÓ DESENVOLUPADA: Investigador principal**.** ***Phase 3 multicenter, randomized, double-blind, placebocontrolled study to assess the efficacy and safety of canakinumab on cytokine release syndrome in patients with COVID-19-induced pneumonia: CAN-COVID-19*** (Nº EudraCT 2020-001370-30). ENTITAT FINANÇADORA: Novartis. INICI/ FINALITZACIÓ: Maig – Juliol 2020.

FUNCIÓ DESENVOLUPADA: Investigador principal. ***A Multicenter, Randomized, Open-label Parallel Group Pilot Study to Evaluate Safety and Efficacy of High Dose Intravenous Immune Globulin (IVIG) plus Standard Medical Treatment (SMT) versus SMT alone in Hospitalized Subjects with COVID-19*** (Nº EudraCT 2020-001696-32). ENTITAT FINANÇADORA: Grifols. INICI/ FINALITZACIÓ: Maig 2020 – Gener 2021.

FUNCIÓ DESENVOLUPADA: Monitor mèdic. ***Phase II, proof-of-concept, randomized, open-label, multicenter clinical trial to evaluate the efficacy and safety of icantibant in patients infected with SARS-COV-2 (COVID-19) and admitted to hospitalization units, without mechanical ventilation invasive, compared to standard of care: ICAT COVID*** (Nº EudraCT 2020-002166-13). ENTITAT FINANÇADORA: Takeda. INICI/ FINALITZACIÓ: Febrer 2021 – actiu.

FUNCIÓ DESENVOLUPADA: Investigador Principal. ***Evaluación de la eficacia y la seguridad de PTC299 en pacientes hospitalizados con COVID-19 (FITE19)*** (Número de protocol: PTC299-VIR-015-COV19). ENTITAT FINANÇADORA: PTC Therapeutics, Inc. INICI/ FINALITZACIÓ: Maig 2021 – actiu.

ANNEX 4. PRESENTACIONS A CONGRESSOS RELACIONADES AMB LA TEMÀTICA DE LA TESI EN QUE HA PARTICIPAT EL DOCTORAND

1.1 PONÈNCIES A CONGRESSOS:

Defectos inmunitarios y COVID-19. XI Reunión virtual de Enfermedades Minoritarias. 28-30 de Abril 2021. On-line. Ponent: Solanich X.

1.2 PÒSTERS O COMUNICACIONS ORALS:

Solanich X, Antolí A, Rocamora-Blanch G, Padullés N, Fanlo-Maresma M, Iriarte A, Mitjavila F, Capdevila O, Molina M, Sabater J, Bas J, Mensa-Vilaró A, Niubó J, Calvo N, Bolivar S, Rigo-Bonnin R, Arregui L, Tebé C, Hereu P, Videla S, Corbella X. **PRAGMATIC, OPEN-LABEL, SINGLE-CENTER, RANDOMIZED, PHASE II CLINICAL TRIAL TO EVALUATE THE EFFICACY AND SAFETY OF METHYLPREDNISOLONE AND TACROLIMUS IN PATIENTS WITH COVID-19 SEVERE PNEUMONIA**. 19th Biennial Meeting of The European Society for Immunodeficiencies. Birmingham (UK) - Online 14-17/10/2020.

Mondou E, Solanich X, Pedro-Botet ML, Domingo P, Carbone J, Estrada V, Almirante B, Calderon E, Ribó N, Navarro-Puerto J. **STUDY DESIGN TO EVALUATE SAFETY AND EFFICACY OF HIGH-DOSE INTRAVENOUS IMMUNOGLOBULIN IN HOSPITALIZED COVID-19 PATIENTS**. 19th Biennial Meeting of The European Society for Immunodeficiencies. Birmingham (UK) - Online 14-17/10/2020.

Solanich X, Pedro-Botet ML, Domingo P, Carbone J, Estrada V, Almirante B, Calderon E, Ribó N, Navarro-Puerto J, Mondou E. **SEGURIDAD Y EFICACIA DEL TRATAMIENTO CON ALTAS DOSIS DE IMMUNOGLUBULINA INTRAVENOSA EN PACIENTES HOSPITALIZADOS POR COVID-19:**

EFECTOS DEL TRATAMIENTO EN LOS PARÁMETROS HEMATOLÓGICOS. DISEÑO DEL ESTUDIO. LXII Concreso nacional de la SEHH y XXXVI congreso nacional de la SETH. Online 26-30/10/2020.

Tiraboschi JM, Scevola S, Blanco J, Calatayud L, Prieto P, Soriano I, Arregui L, Solanich X, Antolí A, Imaz A, Saumoy M, Silva A, Praderas E, Carratalà J, Padzamczer D. **NEUTRALIZING ANTIBODY RESPONSES FOLLOWING SARS-CoV-2 INFECTION** (ID 1502). Congres on Retroviruses and Opportunistic Infections (CROI) 2021. On line. 07-10/03/2021.

Solanich X, Rocamora-Blanch G, Antolí A, Padulles N, Fanlo-Maresma M, Iriarte A, Mitjavila F, Capdevila O, Niubó J, Tebé C, Hereu P, Videla S, Corbella X. **PRAGMATIC, OPEN-LABEL, SINGLE-CENTER, RANDOMIZED, PHASE II CLINICAL TRIAL TO EVALUATE THE EFFICACY AND SAFETY OF METHYLPREDNISOLONE AND TACROLIMUS IN PATIENTS WITH COVID-19 SEVERE PNEUMONIA**. 19th European Congress of Internal Medicine (ECIM). On line – 18-20/03/2021.

Vargas-Parra G, Solanich X, Del Valle J, Rocamora-Blanch G, Setién F, Esteller M, Capellá G, Corbella X, Lazaro C. **VARIANTES DE TLR7 EN PACIENTES JÓVENES APARENTEMENTE SANOS CON COVID-19 GRAVE**. 2º Congreso Nacional Multidisciplinar COVID19 de las Sociedades Científicas de España. On line – 12-16/04/2021.

ANNEX 5. ALTRES PUBLICACIONS RELACIONADES AMB LA TEMÀTICA DE LA TESI EN QUE HA PARTICIPAT EL DOCTORAND

Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann HH, Zhang Y, Dorgham K, Philippot Q, Rosain J, Béziat V, Manry J, Shaw E, Haljasmägi L, Peterson P, Lorenzo L, Bizien L, Trouillet-Assant S, Dobbs K, de Jesus AA, Belot A, Kallaste A, Catherinot E, Tandjaoui-Lambiotte Y, Le Pen J, Kerner G, Bigio B, Seeleuthner Y, Yang R, Bolze A, Spaan AN, Delmonte OM, Abers MS, Aiuti A, Casari G, Lampasona V, Piemonti L, Ciceri F, Bilguvar K, Lifton RP, Vasse M, Smadja DM, Migaud M, Hadjadj J, Terrier B, Duffy D, Quintana-Murci L, van de Beek D, Roussel L, Vinh DC, Tangye SG, Haerynck F, Dalmau D, Martinez-Picado J, Brodin P, Nussenzweig MC, Boisson-Dupuis S, Rodríguez-Gallego C, Vogt G, Mogensen TH, Oler AJ, Gu J, Burbelo PD, Cohen JI, Biondi A, Bettini LR, D'Angio M, Bonfanti P, Rossignol P, Mayaux J, Rieux-Laucat F, Husebye ES, Fusco F, Ursini MV, Imberti L, Sottini A, Paghera S, Quiros-Roldan E, Rossi C, Castagnoli R, Montagna D, Licari A, Marseglia GL, Duval X, Ghosn J; HGID Lab; NIAID-USUHS Immune Response to COVID Group; COVID Clinicians (**Solanich X**); COVID-STORM Clinicians; Imagine COVID Group; French COVID Cohort Study Group; Milieu Intérieur Consortium; CoV-Contact Cohort; Amsterdam UMC Covid-19 Biobank; COVID Human Genetic Effort, Tsang JS, Goldbach-Mansky R, Kisand K, Lionakis MS, Puel A, Zhang SY, Holland SM, Gorochov G, Jouanguy E, Rice CM, Cobat A, Notarangelo LD, Abel L, Su HC, Casanova JL. **Autoantibodies against type I IFNs in patients with life-threatening COVID-19**. Science. 2020 Oct 23;370(6515):eabd4585. doi: 10.1126/science.abd4585. Epub 2020 Sep 24. PMID: 32972996; PMCID: PMC7857397.

Factor impacte 47.728 (Q1). Categoria: Science Edition - MULTIDISCIPLINARY SCIENCES. Posició 2 de 73 revistes a la categoria. Font d'impacte: JCR 2020.

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