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Antiviral resistance and persistent replication of cytomegalovirus and SARS-CoV-2 in immunocompromised patients

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ANTIVIRAL RESISTANCE AND PERSISTENT REPLICATION OF CYTOMEGALOVIRUS AND SARS-COV-2 IN IMMUNOCOMPROMISED PATIENTS

Doctoral thesis report submitted by

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The doctoral thesis titled "*Antiviral resistance and persistent replication of cytomegalovirus and SARS-CoV-2 in immunocompromised patients*" presented by **Marta Santos Bravo** has been conducted in the Department of Microbiology of the Hospital Clinic of Barcelona – ISGlobal and under their supervision and direction, and that accomplishes all the requirements for preparation and defense before the Assessment Tribunal.

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INDEX

GLOSSARY / ABBREVIATURES

Ab: antibody

ACE2: angiotensin-converting enzyme 2

ACV: acyclovir

AIDS: acquired immunodeficiency syndrome

APC: antigen-presenting cells

ATP: adenosine triphosphate

BAC: bacterial artificial chromosome

BDCRB: benzimidazole-D ribonucleosides

cCMV: congenital cytogalovirus

CCR: chemokine receptor

CDC: Centres for Disease Control and Prevention

CDK: cyclin-dependent kinase complexes

CI: confidence interval

CM: central memory

CMV: cytomegalovirus

COVID-19: coronavirus disease – 2019

CPE: cytopathic effect

CYP: cytochrome P450

DC: dendritic cell

DNA: deoxyribonucleic acid

dsDNA: doble strand DNA

E: envelope

EBV: Epstein Barr virus

EC_{50}: effective concentration of the antiviral molecule that reduces 50% of the cytopathic effect.

EM: effector memory

EUA: emergency use authorization

ExoN: exoribonuclease

FDA: food and drug administration

gB/H/L: glycoprotein B, H, L

GCSF-I: granulocyte-colony stimulating factor-I

GCV: ganciclovir

GI: gastrointestinal

gRNA: genomic RNA

GVHD: graft versus host disease

HCMV: Human Cytomegalovirus

HHV-5: human herpesvirus – 5

HIV: human immunodeficiency virus

HSCT: hematopoietic stem cell transplant

HSV-1: herpes simplex virus-1

IC_{50}: concentration of a drug that inhibits 50% of the growth.

IE: immediate early genes

IFN: interferon

IgG: immunoglobulin G

IHQ: immunohistochemistry

IL: interleukin

IR: internal repetition

L: late gene

LZ: leucine zipper

M: membrane

MCH: Major Complex of Histocompatibility

MERS-CoV: Middle East respiratory syndrome coronavirus

MHV: mouse hepatitis virus

MIEP: major Intermediate-Early promoter

mRNA: messenger RNA

mTOR: Mammalian target of rapamycin

N: nucleocapsid

NEC: nuclear egress complex

NGS: Next Generation Sequencing

NiRAN: nidovirus RdRp-associated nucleotidyltransferase

NK: natural killer

NLS: nuclear localization signal

Nps: non-structural proteins

ORF: open reading frame

PCR: polymerase chain reaction

PFU: plaque formed units

PMN: polymorphonuclear

PP1a: popyprotein 1a

PP1ab: popyprotein 1ab

PR: prevalence ratio

PRF: programmed ribosomal frameshifting

PTLD: post-transplantation lymphoproliferative disorder.

Rb: retinoblastoma tumour suppressor protein

RBD: receptor binding domain

RFLP: restriction fragment length polymorphism

RNA: ribonucleic acid

RTC: replicase transcriptase complex

RT-PCR: reverse transcriptase-PCR

S: spike

SARS-CoV-2: severe acute respiratory syndrome coronavirus-2

sgRNA: subgenomic RNA

SNP: Single nucleotide polymorphism

SOT: solid organ transplant

ssRNA: single strand RNA

Teff: effector T cell

TGF: transforming growth factor

TMPRSS2: transmembrane protease serine2

TN: Naïve T cell

TNF: tumour necrosis factor

TR: terminal repetition

Treg: regulatory T cell

TRS: transcriptional regulatory sequences

UL: unique long

US: unique short

VR: variable region

WHO: World Health Organization

ZF: zinc finger

ARTICLES THAT COMPRISE THE THESIS

The present doctoral thesis is submitted as a compendium of research articles.

This thesis comprises 5 objectives, 5 published articles and 2 manuscripts:

1. **Santos Bravo M**, Plault N, Sánchez Palomino S, Mosquera Gutierrez MM, Fernández Avilés F, Suarez Lledo M, Sabé Fernández N, Rovira M, Alain S, Marcos Maeso MÁ. Phenotype and Genotype Study of Novel C480F Maribavir-Ganciclovir Cross-Resistance Mutation Detected in Hematopoietic Stem Cell and Solid Organ Transplant Recipients. Journal of Infectious Diseases. 2021 Sep 17;224(6):1024-1028. IF:7.759. Q1. Microbiology, Infectious Diseases, Immunology.

2. **Santos Bravo M,** Plault N, Sánchez Palomino S, Rodriguez C, Navarro Gabriel N, Mosquera Gutierrez MM, Fernandez Avilés F, Suarez-Lledó M, Rovira M, Bodro M, Moreno A, Linares L, Cofan F, Berengua C, Esteva C, Cordero E, Martin-Davila P, Aranzamendi M, Pérez Jiménez AB, Vidal E, Fernández Sabé N, Len O, Hantz S, Alain S, Marcos MA, REIPI, GESITRA. Genotypic and phenotypic study of antiviral resistance mutations in refractory cytomegalovirus infection. Journal of Infectious Diseases. (Sent for publication).

3. **Santos Bravo M**, Tilloy V, Plault N, Palomino SS, Mosquera MM, Navarro Gabriel M, Fernández Avilés F, Suárez Lledó M, Rovira M, Moreno A, Linares L, Bodro M, Hantz S, Alain S, Marcos MÁ. Assessment of UL56 Mutations before Letermovir Therapy in Refractory Cytomegalovirus Transplant Recipients. Microbiology Spectrum. 2022 Apr 27;10(2):e0019122. IF: 9.043. Q1. Microbiology.

4. **Santos Bravo M,** Nicolás D, Berengua C, Fernandez M, Hurtado JC, Tortajada M, Barroso S, Vilella A, Mosquera MM, Vila J, Marcos MA. Severe Acute Respiratory Syndrome Coronavirus 2 Normalized Viral Loads and Subgenomic RNA Detection as Tools for Improving Clinical Decision Making and Work Reincorporation. Journal of Infectious Diseases. 2021 Oct 28;224(8):1325-1332. IF:7.759. Q1. Microbiology, Infectious Diseases, Immunology.

5. **Santos Bravo M,** Berengua C, Marín P, Esteban M, Rodriguez C, Del Cuerpo M, Miró E, Cuesta G, Mosquera M, Sánchez-Palomino S, Vila J, Rabella N, Marcos MÁ. Viral Culture Confirmed SARS-CoV-2 Subgenomic RNA Value as a Good Surrogate Marker of

Infectivity. Journal of Clinical Microbiology. 2022 Jan 19;60(1):e0160921. IF: 11.677. Q1. Microbiology.

6. Camprubí D, Gaya A, Marcos MA, Martí-Soler H, Soriano A, Mosquera MDM, Oliver A, **Santos M**, Muñoz J, García-Vidal C. Persistent replication of SARS-CoV-2 in a severely immunocompromised patient treated with several courses of remdesivir. International Journal of Infectious Diseases. 2021 Mar;104:379-381. IF: 12.074. Q1. Infectious Diseases.

7. **Santos Bravo M**, Alonso R, Soria D, Sanchez-Palomino S, Sanzo Machuca A, Alcami J, Diez F, Fernandez Avilés F, Bodro M, Rubio E, Villanueva JL, Mosquera MM, Vergara A, Martinez MJ, Soriano A, Marcos MA. Genetic study of SARS-CoV-2 nsp12 in non-responders COVID-19 patients to remdesivir. (Sent for publication).

THESIS SUMMARY

RESISTENCIA A ANTIVIRALES Y REPLICACIÓN PERSISTENTE DEL CYTOMEGALOVIRUS Y EL SARS-COV-2 EN PACIENTES INMUNOCOMPROMETIDOS

INTRODUCCIÓN

1. Paciente inmunocomprometido

El sujeto inmunocomprometido es aquel que tiene un sistema inmune deficitario, lo cual implica una reducida capacidad de la respuesta inmune innata y/o adaptativa ante un patógeno, causando lo que se conoce como infección oportunista. La sospecha de inmunosupresión se basa en la frecuencia, severidad o identificación de ciertos patógenos que indican la presencia de una disfunción inmune subyacente, asociada a graves complicaciones clínicas e incluso la muerte.

El patógeno más prevalente asociado al mayor riesgo de morbi-mortalidad en el paciente inmunodeprimido, especialmente en trasplantados, es el citomegalovirus humano (CMV). El CMV puede producir efectos directos en el organismo, y efectos indirectos, principalmente por la inmunomodulación que provoca en el huésped, provocando el rechazo del órgano/tejido o la enfermedad de injerto contra huésped. Además, incrementa el nivel de inmunodepresión incluso en ausencia de fármacos inmunosupresores, aumentado el riesgo de otras infecciones oportunistas.

Otra infección vírica de gran afectación en el paciente inmunodeprimido ha sido el síndrome respiratorio agudo severo-coronavirus-2 (SARS-CoV-2), causante de la pandemia mundial COVID-19, con un 28-44% de mortalidad en esta población. El SARS-CoV-2 también produce efectos inmunomoduladores, como la disminución de interferón-tipo I (IFN-I) o la tormenta de citoquinas debida a una inflamación exacerbada. El hecho de que un paciente tenga una inmunidad deficiente no está asociado a un factor protector, debido a que la respuesta inmune adaptativa tiene que ser efectiva para limitar la respuesta innata y generar suficientes células efectoras y de memoria para acabar con la infección. De hecho, los centros para el control y la prevención de enfermedades (CDC, por sus siglas en inglés) han incluido ciertos grados de inmunosupresión como un factor de mal pronóstico y riesgo de replicación persistente.

CMV y SARS-CoV-2 pueden convivir en el huésped durante largos periodos de tiempo. El CMV es un herpesvirus que puede permanecer de por vida en estado de latencia dentro de la célula

infectada y reactivarse cuando el individuo sufre alteraciones en el sistema inmune. La infección primaria, reactivación o reinfección por CMV debe ser monitorizada para reducir el tiempo de administración de terapias antivirales, las cuales están asociadas a una alta toxicidad, inmunosupresión y desarrollo de resistencias.

Por otro lado, no se ha descrito hasta el momento que el SARS-CoV-2 pueda establecer latencia, sin embargo, puede permanecer detectable durante meses. En los pacientes inmunodeprimidos, la respuesta mediada por linfocitos B, y la consecuente producción de anticuerpos neutralizantes esta reducida, permitiendo una infección más duradera. La replicación viral prolongada se ha comprobado mediante el aislamiento del SARS-CoV-2 hasta 21 dias después del inicio de síntomas, y se ha rechazado la reinfección mediante secuenciación en diversos estudios. Sin embargo, actualmente no se ha definido el tiempo de replicación activa del SARS-CoV-2 en estado de inmunosupresión.

La replicación persistente es de asunto de gran preocupación para la sanidad pública, ya que favorece la selección de mutaciones que pueden estar asociadas a un fitness viral beneficioso, al escape del sistema inmune o de las vacunas, al desarrollo de nuevas variantes o la aparición de resistencia a antivirales.

La resistencia a los antivirales se define como la falta de respuesta a un tratamiento antiviral, cuya finalidad es tratar o controlar la infección viral inhibiendo algún paso del proceso de replicación del virus. La falta de respuesta al tratamiento puede deberse a que se administren o se alcancen dosis plasmáticas insuficientes, a la toxicidad asociada, a una mala absorción o penetración en el compartimento infectado, a una replicación diseminada mientras se recibe terapia a dosis completas. Esto favorece la selección y desarrollo de mutaciones de resistencia de forma progresiva. Por tanto, su detección temprana es fundamental para el uso de tratamientos adecuados, especialmente en pacientes inmunodeprimidos que sufren infección refractaria por CMV o replicación persistente por SARS-CoV-2.

2. Citomegalovirus humano

La especie CMV-5 pertenece a la familia *Herpesviridae*, y en concreto a la subfamilia *beta-herpesvirinae*. El CMV es especifico de especie, pudiéndose distinguir distintos tipos de citomegalovirus según el mamífero al que infecte. En 1956 se aisló por primera vez y se nombró citomegalovirus debido al aumento de tamaño observado en la célula infectada por la desestructuración de su citoesqueleto.

El CMV es el patógeno humano de mayor tamaño (200nm) descrito hasta el momento. Consiste en una nucleocápside icosaédrica que contiene el genoma viral y está inmersa en el tegumento, compuesto de 27 fosfoproteínas estructurales y moduladoras de la respuesta inmune. El tegumento está envuelto por una membrana fosofolipídica formada por 40 proteínas virales, entre ellas las glicoproteínas fundamentales para la adhesión y la entrada del virus en la célula huésped. La glicoproteína B es el epítopo de reconocimiento de los anticuerpos neutralizantes generados tras la infección y para el cual se están desarrollando las vacunas frente al CMV. Además, sirve para diferenciar los distintos genotipos de cada cepa según su isoforma.

El genoma del CMV consiste en 235000pb de ADN de doble cadena (dsDNA) que codifica para 165 genes. Este genoma se encuentra diferenciado en dos segmentos (UL: unique long; US: unique short) separados por regiones que contienen repeticiones cortas que flanquean cada uno de estos dos segmentos (TR: terminal repetition; IT: internal repetition). Los genes codificados en el genoma del CMV se clasifican en tres grupos según su expresión temporal. Durante el primer periodo de la replicación, el virus entra en la célula huésped y la nucleocápside se transporta hasta el núcleo celular donde los genes inmediatos tempranos (IE) se expresan. Son genes reguladores y funcionan como promotores de la segunda familia de genes: los genes de expresión temprana (E), los cuales son responsables de la replicación del ADN y de activar la transcripción de la familia de genes tardíos (L). Los genes L codifican para proteínas estructurales encargadas de la producción de partículas virales maduras.

El CMV infecta principalmente a los macrófagos, neutrófilos, células dendríticas, células epiteliales y células endoteliales. Es un patógeno de replicación lenta, que aprovecha la maquinaria celular para duplicar su genoma y generar nuevos viriones capaces de infectar a otras células vecinas, causando principalmente una mononucleosis infecciosa cuando afecta a las glándulas salivares. También puede provocar otras afectaciones similares como faringitis, linfoadenopatías, artralgias o hepatitis según el órgano al que infecte. Tras la primoinfección, el CMV incorpora su genoma en el ADN celular y establece latencia de por vida. Cuando el sistema inmunológico del huésped sufre una alteración por estrés endógeno o exógeno, como un tratamiento inmunosupresor, el virus se reactiva. Debido a este estado crónico de latencia, actualmente el CMV es una infección endémica cuya seroprevalencia varía entre un 36-77% en países del hemisferio norte, y alcanza el 100% en países del hemisferio sur.

La infección por CMV en personas inmunocompetentes suele resolverse de forma asintomática o con síntomas leves. Sin embargo, el CMV puede provocar graves complicaciones en pacientes inmunodeprimidos y en el feto cuando la madre se infecta durante el embarazo, conocida como infección congénita. La sintomatología pediátrica consiste principalmente en aborto, problemas

cognitivos o sodera que se desarrolla años más tarde del nacimiento. La sintomatología asociada al paciente adulto consiste en problemas neurológicos, vasculitis, retinitis, mayor riesgo de infecciones oportunistas y rechazo del injerto en el caso de pacientes trasplantados.

Para combatir la replicación del CMV o prevenir su infección en pacientes de alto riesgo, se han desarrollados distintos fármacos antivirales, entre los cuales algunos han sido aprobados para el tratamiento preventivo y/o terapéutico de la infección. Como primera línea de tratamiento se encuentra el ganciclovir (GCV), y su profármaco de administración oral valganciclovir (VGCV), los cuales también están indicados como profilaxis al comienzo del tratamiento inmunosupresor. En segunda línea se encuentran el foscarnet (FOS) y el cidofovir (CDV). Estos cuatro fármacos inhiben con distintos mecanismos de acción a la ADN polimerasa viral. En 2019, letermovir (LMV) fue aprobado como tratamiento profiláctico en receptores adultos de TPH seropositivos para CMV. Este fármaco tiene como diana antiviral la subunidad UL56 del complejo de la terminasa viral, la cual junto con las subunidades UL89 y el UL51 se encargan cortar el ADN concatémero producido durante la síntesis de ADN viral en unidades de un solo genoma. Por otro lado, maribavir (MBV) es un inhibidor de la fosfoquinasa viral UL97, la cual se encarga de fosforilar proteínas virales y del huésped involucradas en la modulación del ciclo celular para ayudar a la replicación del ADN, la regulación de la expresión de los genes víricos, la morfogénesis del virión y la disrupción de la lámina nuclear para facilitar la salida de las nuevas partículas virales. El MBV se encuentra actualmente en ensayos clínicos de fase III, y se está evaluando su indicación para infecciones por CMV refractarias o resistentes principalmente.

El mecanismo de acción del GCV/VGCV necesita de una fosforilación inicial llevada a cabo por la UL97 viral y dos fosforilaciones consecutivas por parte de quinasas celulares para obtener la forma activa del fármaco, el GCV-trifosfato. El GCV-trifosfato compite con la deoxiguanosina trifosfato en el lugar de unión de la ADN polimerasa, y se incorpora en el ADN viral donde ralentiza y eventualmente detiene la cadena de elongación. Las quinasas celulares también transforman al CDV en CDV-difosfato, la forma activa, el cual se incorpora en el ADN viral. Cuando dos CDV-difosfato se incorporan se detiene la cadena de elongación. El FOS se encuentra en forma activa desde la administración intravenosa. Este inhibe la actividad de la ADN polimerasa por competición con el pirofosfato que se debe liberar cada vez que un nuevo nucleótido se une a la nueva cadena de ADN. El LMV interfiere durante el corte y el empaquetamiento del genoma viral mediante la inhibición de la proteína UL56 de la terminasa viral. El MBV altera la morfogénesis y la salida de las nuevas partículas virales mediante la inhibición del UL97. Esta inhibición además impide la fosforilación inicial que realiza sobre el

GCV/VGCV, por lo tanto, provoca un efecto antagonista cuando ambos fármacos se administran conjuntamente.

El uso prolongado o inadecuado de los fármacos antivirales y la incompleta supresión de la replicación viral en presencia de los mismos, ha provocado la aparición de resistencia clínica, que está asociada a un aumento de hospitalización, enfermedad invasiva, disfunción progresiva, rechazo de injerto y alta mortalidad. La resistencia clínica o fallo al tratamiento antiviral puede ser debido a: (I) un sistema inmune deficiente que impide al organismo luchar contra el patógeno a pesar de estar recibiendo tratamiento, (II) a niveles plasmáticos del fármaco sub-terapéuticos debido al sistema metabólico del paciente o, (III) a la aparición de mutaciones en ciertos genes del virus que le permite escapar de la acción del fármaco, confiriendo una resistencia virológica. En ocasiones el fallo al tratamiento se debe una superposición de estos factores.

En el caso de resistencias víricas, más de un 90% de las mutaciones de resistencia a GCV, como primera línea de tratamiento, se encuentran en el gen *UL97*, mientras que las mutaciones en el *UL54* son menos frecuentes y suelen aparecer tras las del *UL97* aumentando así el nivel de resistencia a GCV, o causando resistencias cruzadas a CDV y/o FOS. Además, se ha descrito que el gen *UL54* es muy variable, lo cual dificulta la diferenciación entre mutaciones asociadas a resistencia a los antivirales de polimorfismos naturales sensibles a estos fármacos. El uso compasivo de nuevos antivirales como tratamiento de la infección refractaria o resistente a los antivirales convencionales ha provocado la aparición de resistencia a estas nuevas moléculas. Las mutaciones a LMV aparecen principalmente en el gen *UL56* aunque escasamente se han descrito en el *UL89* y *UL51*. Estas se han descrito en pacientes tras recibir LMV como profilaxis primaria, secundaria, o como tratamiento de la infección. En estudios *in vitro* se ha demostrado que aparecen tras un menor número de pases que para el resto de antivirales, lo cual sugiere una menor barrera genética para la aparición de mutaciones en el *UL56*. Las mutaciones de resistencia a MBV se han detectado principalmente en el gen *UL97* en la región más cercana al extremo amino terminal, respecto a la región carboxilo terminal asociada a resistencias a GCV. También se han descrito mutaciones en el *UL27* que en combinación con mutaciones en el *UL97* aumentan el nivel de resistencia. Sin embargo, hay pocas resistencias cruzadas para GCV y MBV descritas, lo cual respalda el uso de MBV en el tratamiento de CMV resistente a GCV.

La evolución de resistencias virológicas se produce de forma progresiva y escalonada, favorecida por la selección positiva de mutaciones que confieren resistencia o una mayor capacidad replicativa. Por tanto, es esencial la detección precoz de mutaciones emergentes mediante estudios genotípicos.

El estudio genotípico para detectar mutaciones de resistencia está indicado cuando las cargas virales aumentan o se mantienen bajo dosis completas de tratamiento antiviral durante al menos 14 dias. El estudio genotípico consiste en la amplificación y secuenciación de los genes virales que son diana farmacológica. La secuenciación basada en la detección de dideoxy-fluorescencia, conocido como método Sanger, es la más utilizada por su estandarización, rapidez y facilidad para el correspondiente volumen de muestras en los laboratorios de diagnóstico. Su limitación principal es la incapacidad de detectar poblaciones con mutaciones minoritarias (por debajo del 20% de la población vírica total). Otras plataformas de secuenciación masiva (NGS por sus siglas en inglés) superan esta limitación, ya que son capaces de detectar poblaciones cercanas al 1%, permitiendo analizar la diversidad génica completa que hay en la muestra. Sin embargo, para que pueda implementarse se necesita una recalibración y optimización técnica de las plataformas y de la preparación de librerías, refinamiento computacional y adaptación de su excesiva capacidad de secuenciación para un reducido número de muestras.

El estudio genotípico de resistencia a antivirales permite una detección temprana de mutaciones para evitar el fallo al tratamiento y los efectos adversos de un tratamiento infectivo, para así poder mejorar la clínica usando otras alternativas terapéuticas. Sin embargo, en los estudios genotípicos frecuentemente se detectan mutaciones de significado desconocido. Para elucidar dicho significado es necesario realizar ensayos fenotípicos en los que se aísla el virus con la mutación de interés y se enfrenta a los distintos fármacos antivirales para estudiar su respuesta. También se estudia la capacidad de crecimiento del mutante en comparación con la cepa del virus *wild-type* en ausencia de antivirales. En los últimos años en lugar de aislar el virus a partir de una muestra clínica, el cual puede contener otras mutaciones o distintas poblaciones presentes en la misma muestra, se ha optado por producir virus recombinantes por la técnica bacmid. Esta técnica consiste en la introducción de la mutación de interés en un vector (ej. cromosoma artificial bacteriano BAC) que contiene el genoma completo de la cepa de laboratorio AD169 del CMV seguido de un gen reportero fluorescente, mediante mutagénesis dirigida. Una vez introducida la mutación deseada en la posición correcta del genoma del vector, se purifica y se transfecta en la línea celular adecuada para generar nuevas partículas virales con dicha mutación y, además, ser observables mediante el gen fluorescente reportero. Los virus recombinantes se enfrentan a distintas concentraciones del antiviral que se quiera testar para calcular la concentración capaz de inhibir el crecimiento viral al 50% (IC_{50}). El ratio del valor IC_{50} del mutante respecto al hallado en la cepa control AD169 nos permite evaluar el nivel de resistencia a dicho fármaco, considerándose resistente un valor superior o igual a 3. Para evaluar la capacidad replicativa del mutante, se cultiva en paralelo el mutante y la cepa control a

distintas concentraciones en ausencia de fármaco, y se cuentan diariamente las unidades formadoras de placas (PFU) durante al menos 7 días para poder realizar curvas de crecimiento.

Esta correlación entre el significado fenotípico y el genotípico en el momento de sospecha de resistencia es esencial en la práctica clínica para mejorar el manejo farmacológico del paciente y para el diseño de nuevas moléculas antivirales, evitando así la aparición de resistencias cruzadas.

3. Síndrome respiratorio agudo severo por coronavirus 2

El SARS-CoV-2 pertenece a la familia de los *Coronaviridae*, concretamente al género *beta-coronavirus* y subgénero *sarbecovirus*. Su similitud génica (96%) con el virus del SARS procedente de murciélagos y con el procedente del pangolín, sugiere que el origen del SARS-CoV-2 puede haber sido por transmisión zoonótica desde dichos mamíferos al humano. Este salto de barrera de especie data de diciembre del 2019 y cuyo origen fue en Wuhan, China, desde donde se esparció globalmente causando una pandemia mundial, conocida como COVID-19.

El SARS-CoV-2 es un virus envuelto que contiene uno de los genomas más largos de los virus de RNA (32kb). Su genoma de ARN monocatenario (ssRNA) de sentido positivo se encuentra dentro de una nucleocápside (N), y esta a su vez dentro de una envuelta formada por: la proteína de la membrana (M), de la espícula (S) y de la envuelta (E). Su genoma codifica además de para estas 4 proteínas estructurales principales (N, M, S, E), para 16 proteínas no estructurales (nsp) encargadas de los procesos de transcripción, traducción, tráfico de proteínas e inhibición de las defensas del organismo.

Cuando el virus entra en la célula huésped, libera el ARN viral y se traducen las poliproteínas. La replicación y transcripción del ARN genómico viral se lleva a cabo a través de cortes y uniones realizados por el complejo de la replicasa-transcriptasa. Una vez el ARN viral se ha replicado y las proteínas estructurales se han sintetizado, se ensamblan y se empaqueta el genoma viral para formar partículas virales que se liberan e infectan a otras células vecinas. Durante el proceso de replicación del ARN genómico viral se generan ARNs intermediarios de cadena simple negativa que sirven de molde para generar la cadena positiva de ARN genómico, así como otros ARNs subgenómicos (sgRNA) de cadena positiva que codifican para las distintas proteínas que se van a sintetizar. Estos sgRNA poseen una secuencia líder en su extremo 5’ idéntica al ARN genómico seguida por el resto del gen que codifica para las 4 proteínas estructurales y para nsp accesorias (3a, 6, 7a, 7b, 8, 10), mediante saltos durante el proceso de transcripción. Como el sgRNA solo se transcriben durante la replicación viral, se ha propuesto su detección como un indicador de replicación activa del SARS-CoV-2. Algunos estudios han demostrado haber una

asociación significativa entre la detección de sgRNA, altas cargas virales y los primeros 7 dias de síntomas, condiciones asociadas a una mayor transmisión viral. El sgRNA se ha utilizado como marcador subrogado de infectividad en vacunas probadas en modelos primates, en pacientes con ARN genómico persistente, tras el uso de fármacos antivirales y en la monitorización pacientes severos. Sin embargo, otros estudios postulan que no es un buen indicador de infecciosidad debido a que la ausencia de detección de sgRNA precoz se debe a la menor concentración general de transcritos de ARN en comparación con ARN genómico, y no por una falta de replicación.

Actualmente, el cultivo viral sigue siendo el método de referencia para determinar la viabilidad del virus. Estudios de cultivo han aislado SARS-CoV-2 en muestras recogidas tras 1-9 días de síntomas, tras 12 días en pacientes con enfermedad moderada y hasta 20 días en pacientes críticos. Sin embargo, los pocos estudios que han comparado la detección de sgRNA y el cultivo viral, han demostrado una concordancia moderada-alta.

Tanto el ARN genómico como el sgRNA se detecta mediante RT-PCR, técnica altamente sensible y especifica. Esta técnica ofrece un valor umbral (Ct) del ciclo de amplificación al cual se empieza a detectar el ARN viral, que es inversamente proporcional a la carga viral. Sin embargo, este valor es muy variable dependiendo de la técnica de recogida de la muestra, del tipo de muestra, del ensayo y de la plataforma utilizada para la extracción y la ampliación, por lo que limita la utilidad de esta técnica para predecir la carga viral real y la infectividad.

El valor del Ct se ha utilizado como un indicador de pronóstico del paciente en la decisión clínica, sin embargo, la CDC desaconsejó su uso para la toma de decisiones clínicas debido a su gran variabilidad. Esta limitación se puede solventar mediante la estandarización de la técnica, añadiendo curvas de referencia para obtener una cantidad precisa y estándar de copias de ARN viral por PCR, y normalizando dicho valor por el número de células recogidas en la muestra, de forma que se eviten falsos negativos causados por a una recolección inapropiada de la muestra respiratoria.

En la práctica clínica, se ha visto que la PCR puede permanecer positiva durante semanas tras la recuperación clínica del paciente, así como en pacientes asintomáticos. Esto se debe a que la PCR detecta la presencia de ARN viral pero no implica que el virus este activamente replicando, pudiendo estar detectando fragmentos de ARN o detritos virales remanentes una vez superada la infección. De ahí la necesidad de encontrar otras técnicas indicativas de infectividad viral, como la detección de sgRNA.

La duración de la infección depende de la edad, la severidad de la enfermedad y el estado inmune del paciente. En pacientes inmunocompetentes normalmente los síntomas se manifiestan durante 7 días, mientras que en los inmunocomprometidos estos síntomas pueden alargarse en el tiempo debido a una infección persistente. Este hecho se ha descrito en varios pacientes inmunocomprometidos donde la PCR y el cultivo viral fueron positivas hasta 21 después del inicio de síntomas, o incluso más de 100 dias en algunos casos clínicos.

La evolución clínica de pacientes inmunodeprimidos con SARS-CoV-2 suele estar asociada a una alta morbilidad y mortalidad debido a la persistencia de la replicación viral, la cual favorece la aparición de mutaciones asociadas a una ventaja en el *fitness* del virus o resistencia a los antivirales administrados. Debido a la situación de pandemia, ciertos fármacos con capacidad antiviral frente a otros virus se readaptaron para el tratamiento del SARS-CoV-2. Actualmente hay tres antivirales aprobados por la Administración de Alimentos y Medicamentos de los Estados Unidos (FDA) para pacientes con COVID-19: remdesivir (RDV), molnupiravir y paxlovid (nirmatrelvir/ritonavir).

El RDV fue el primer fármaco en ser aprobado en octubre del 2020 indicado para el tratamiento de COVID-19 severo en pacientes que requieren hospitalización. En diciembre del 2021, se autorizó como situación de emergencia el uso de molnupiravir y paxlovid para el tratamiento de COVID-19 leve o moderado en adultos (mulnapiravir) o pediátricos (paxlovid) con alto riesgo de hospitalización o muerte.

El RDV es un profármaco que se metaboliza a un análogo de nucleósido trifosfato cuando entra en las células respiratorias humanas. Una vez en su forma activa, es capaz de inhibir a la ARN polimerasa-dependiente de ARN (RdRp) viral compitiendo por el lugar de unión de la adenosina trifosfato, lo cual impide la corrección correspondiente a la actividad exorribonucleasa de la RdRp y la síntesis de la cadena de ARN mediante la actividad polimerasa de la misma. La RdRp está formada por 4 subunidades: nsp12, nsp7a, nsp7b y nsp8. El RDV se une al nsp12, donde reside la acción catalítica de la polimerasa, interfiriendo con la entrada del próximo nucleótido trifosfato y deteniendo así la elongación de la cadena.

Se ha demostrado en distintos ensayos clínicos que el RDV tiene una buena eficacia y perfil de tolerancia y seguridad. Este fármaco ha sido capaz de mejorar significativamente la clínica, reducir el tiempo de recuperación, de ingresos hospitalarios y de mortalidad en pacientes con COVID-19 severo.

Varios estudios han demostrado la aparición de mutaciones de resistencia a RDV *in vitro*, como F480L, V557L y E802D. Esta última fue detectada posteriormente en un paciente

inmunodeprimido, así como la mutación D484Y en otro caso clínico. Sin embargo, un análisis sobre las variantes circulantes de SARS-CoV-2 ha demostrado no haber una transmisión global de mutaciones de resistencia a RDV. Sin embargo, la identificación y evaluación de la incidencia de mutaciones de resistencia al RDV continua teniendo una fuerte implicación en el manejo clínico, el cual ayudaría a la adecuada elección de estrategias terapéuticas y a la vigilancia evolutiva del virus, altamente evidenciada con la aparición de nuevas variantes de SARS-CoV-2.

HIPOTESIS

Los pacientes inmunocomprometidos son una de las poblaciones más vulnerables a las infecciones oportunistas. La depleción inmune congénita, adquirida o debida a la terapia inmunosupresora impide que el paciente tenga una respuesta defensiva autosuficiente para combatir patógenos, como el CMV y el SARS-CoV-2. Ambos virus son capaces de alterar negativamente el sistema inmune del huésped, exacerbando su estado de inmunosupresión.

La infección por CMV es una importante causa de morbi-mortalidad en el paciente inmunocomprometido debido sus los efectos directos e indirectos. Gracias a la implementación de estrategias preventivas y al desarrollo de fármacos antivirales se ha conseguido reducir la enfermedad por CMV, el riesgo de infección por otros patógenos y la alta mortalidad asociada. Sin embargo, el uso de antivirales ha favorecido el desarrollo de mutaciones de resistencia. Se estima que entre un 5 y un 12% de los pacientes trasplantados experimentan infección refractaria por CMV, incidencia que está infravalorada debido al uso de antivirales de forma empírica sin solicitud sistemática de estudios genotípicos de resistencia.

Según las guías para el manejo del tratamiento antiviral, el estudio de resistencias debe solicitarse lo antes posible para reducir efectos secundarios, la aparición de multi-resistencias y complicaciones clínicas. Las mutaciones de resistencia se detectan usualmente por secuenciación Sanger, ya que es el método más fácil y rápido de secuenciar las dianas farmacológicas. Sin embargo, su limitada sensibilidad puede ser mejorada mediante secuenciación masiva, la cual se permite detectar poblaciones minoritarias y mostrar toda la diversidad génica existente en la muestra.

Determinar la asociación de cada mutación individual con su respuesta al fármaco antiviral, al nivel de resistencia y a la capacidad de replicación viral solo puede llevarse a cabo mediante estudios fenotípicos. El método más fácil y robusto es mediante la generación de virus recombinantes. Esta correlación genotipo-fenotipo es esencial no solo para entender la función biológica de cada residuo proteico, sino también para ampliar las bases de datos públicas de variantes génicas para facilitar la interpretación de los resultados genotípicos, optimizar los tratamientos y mejorar la clínica de los pacientes inmunocomprometidos con infección viral resistente, refractaria o persistente.

Otra infección oportunista de alto impacto en el paciente inmunocomprometido es el SARS-CoV-2. Esta infección está asociada a una alta severidad y mortalidad en este tipo de paciente debido al síndrome de distrés respiratorio, fallo respiratorio e inflamación sistémica que provoca. El material genético de los virus respiratorios se detecta por RT-PCR, el cual provee un valor de Ct

que es muy variable. Las muestras respiratorias además presentan una variabilidad intrínseca que depende de la toma de la muestra. Estas limitaciones se pueden resolver con la estandarización y la normalización de las cargas virales, proporcionando unos valores más precisos que pueden ayudar en la decisión clínica y la monitorización del paciente.

El RNA genómico puede llegar a permanecer detectable prolongadamente después del cese de síntomas, sin implicar necesariamente replicación activa. El cultivo viral es el método de referencia para determinar partículas de virus viables, pero requiere de laboratorios de alta seguridad y el tiempo de respuesta es muy prolongado. Debido a que el sgRNA del SARS-CoV-2 solo se transcribe durante la replicación viral y no se encuentra empaquetado dentro de los viriones maduros, se ha propuesto como indicador subrogado de replicación activa. Conocer la duración de infectividad del SARS-CoV-2 es importante para controlar la transmisión del virus, identificar aquellos individuos asintomáticos/sintomáticos infecciosos, acortar las precauciones de aislamiento y de reincorporación laboral, y para evaluar la eficacia de las vacunas y de los tratamientos antivirales. La selección de mutaciones involucradas en el *fitness* viral, el escape de la inmunidad, la adaptación al huésped o resistencia a antivirales, ocurre preferentemente en pacientes inmunocomprometidos debido a la replicación viral persistente y a la administración prolongada de terapia antivirales.

Dentro de la limitada disponibilidad de tratamientos antivirales, el RDV ha demostrado tener una buena eficacia en el tratamiento del COVID-19 severo. Hasta el momento, no hay evidencias de transmisión de mutaciones de resistencia a RDV extendidas globalmente, sin embargo, la monitorización de mutaciones emergentes es crucial para la vigilancia sanitaria y para guiar en el manejo del paciente.

OBJETIVOS

El objetivo principal de esta tesis es el estudio de las infecciones oportunistas causadas por el citomegalovirus humano y el SARS-CoV-2, profundizando en sus dos mayores implicaciones en el paciente inmunocomprometido: la replicación viral persistente y la resistencia a antivirales. Este objetivo principal se subdivide en:

1. Caracterización de mutaciones asociadas a resistencia a antivirales en la infección por citomegalovirus refractaria en el paciente inmunocomprometido.
    1.1. Estudio genotípico de las dianas farmacológicas de los fármacos antivirales convencionales y nuevos mediante secuenciación Sanger y secuenciación masiva.
    1.2. Estudio fenotípico de mutaciones desconocidas detectadas mediante estudios genotípicos en muestras clínicas por tecnología bacmid para determinar la susceptibilidad a los distintos antivirales y la capacidad replicativa asociada a cada mutación.
    1.3. Búsqueda de mutaciones primarias de resistencia previas a los nuevos tratamientos para la prevención del fallo farmacológico.
    1.4. Determinación de la incidencia de mutaciones de resistencia en el citomegalovirus, de polimorfismos naturales y de mutaciones desconocidas en el paciente inmunocomprometido con resistencia clínica.
2. Cuantificación de las cargas virales normalizadas del SARS-CoV-2 en muestras respiratorias para el estudio de la dinámica del ARN viral.
3. Determinación de la replicación activa del SARS-CoV-2 mediante la detección de ARN subgenómico, y su amplia aplicación en el seguimiento clínico del paciente.
4. Evaluación del paciente con replicación de SARS-CoV-2 persistente y/o COVID-19 severo tratado con remdesivir.
5. Búsqueda de mutaciones asociadas al fallo del tratamiento con remdesivir mediante secuenciación masiva en pacientes con COVID-19 severo.

MÉTODOS Y RESULTADOS

Los 5 objetivos de la tesis se resolvieron mediante 5 artículos publicados y 2 manuscritos:

- **Artículo 1.** Estudio fenotípico y genotípico de la nueva mutación C480F que confiere resistencia cruzada a maribavir y ganciclovir detectada en receptores de trasplante de progenitores hematopoyéticos y de órgano sólido.

- **Artículo 2**. Estudio genotípico y fenotípico de mutaciones de resistencia a antivirales en la infección por citomegalovirus refractaria.

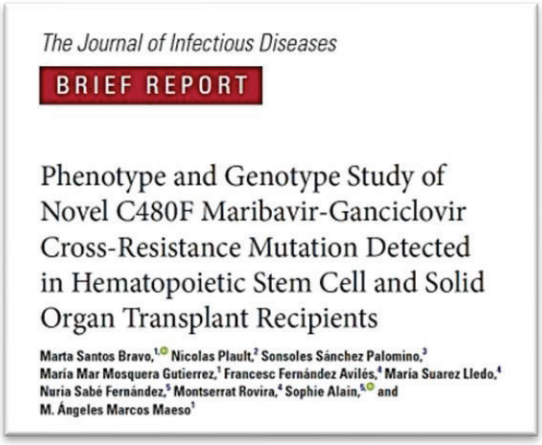
- **Artículo 3.** Evaluación de mutaciones en el gen UL56 previos al tratamiento con letermovir en pacientes trasplantados con citomegalovirus refractario.

- **Artículo 4**. La detección de ARN subgenómico y la normalización de las cargas virales del SARS-CoV-2 como herramientas para mejorar la decisión clínica y la reincorporación laboral.

- **Artículo 5**. El cultivo viral confirma que el ARN subgenómico del SARS-CoV-2 es un buen marcador subrogado de infectividad.

- **Artículo 6.** Replicación persistente del SARS-CoV-2 en un paciente severamente inmunocomprometido tratado con varios cursos de remdesivir

- **Artículo 7**. Mutaciones en el gen nsp12 del SARS-CoV-2 en pacientes con COVID-19 severo no respondedores a remdesivir (Manuscrito pendiente de publicación).



Artículo 1. **Estudio fenotípico y genotípico de la nueva mutación C480F que confiere resistencia cruzada a maribavir y ganciclovir detectada en receptores de trasplante de progenitores hematopoyéticos y de órgano sólido.**

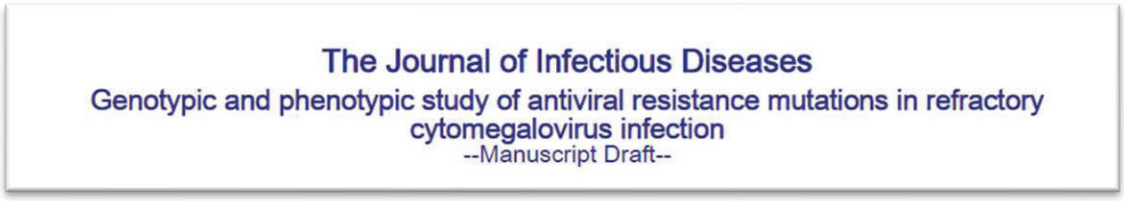
Revista: Journal of Infectious Diseases. 2021 Sep 17;224(6):1024-1028.

Autores: Santos Bravo M, Plault N, Sánchez Palomino S, Mosquera Gutierrez MM, Fernández Avilés F, Suarez Lledo M, Sabé Fernández N, Rovira M, Alain S, Marcos Maeso MÁ.

Resumen:

Dos pacientes trasplantados (1 de riñón y 1 de progenitores hematopoyéticos) recibieron maribavir (MBV) para el tratamiento de la infección por citomegalovirus (CMV) resistente a terapia convencional. Ambos pacientes negativizaron a los 30 y 18 dias, sin embargo, la mutación C480F apareció en el gen *UL97* causando una infección recurrente por CMV después de 2 meses de MBV y 15 o 4 semanas de ganciclovir (GCV), respectivamente. La mutación C480F no fue detectada bajo el tratamiento con GCV previo a la administración de MBV. El ensayo fenotípico recombinante mostró que la mutación C480F confiere un alto nivel de resistencia a MBV y resistencia cruzada a GCV, además de reducir su capacidad de replicación. Este estudio refuerza la importancia del seguimiento clínico del paciente y de los estudios genotípicos y fenotípicos para la evaluación y la optimización del paciente con sospecha de resistencia a MBV.

Respuesta a los objetivos 1.1 y 1.2.



Artículo 2. Estudio genotípico y fenotípico de mutaciones de resistencia a antivirales en la infección por citomegalovirus refractaria.

Revista: Journal of Infectious Diseases. Manuscrito pendiente de publicación.

Autores: Santos Bravo M, Plault N, Sánchez Palomino S, Rodríguez C, Navarro Gabriel N, Mosquera Gutiérrez MM, Fernández Avilés F, Suarez-Lledó M, Rovira M, Bodro M, Moreno A, Linares L, Cofan F, Berengua C, Esteva C, Cordero E, Martin-Davila P, Aranzamendi M, Pérez Jiménez AB, Vidal E, Fernández Sabé N, Len O, Hantz S, Alain S, Marcos MA, REIPI, GESITRA.

Resumen:

Este estudio presenta la caracterización genotípica y fenotípica de nuevas variantes genéticas del citomegalovirus humano (CMV) en una cohorte de 94 pacientes con CMV clínicamente resistente. Se detectaron mutaciones de resistencia en los genes diana *UL97*, *UL54* y *UL56* en 27 de los 94 (28.7%) pacientes incluidos. Mediante el estudio de correlación genotípica-fenotípica se determinó el significado de 5 mutaciones nuevas (G441S, A543V, F460S, R512C, A928T) detectadas en muestras clínicas en el gen *UL54* y 1 (F345L) en el gen *UL56*. Estos resultados demostraron que la mutación A928T confiere una triple resistencia a ganciclovir, foscarnet y cidofovir de alto nivel, y que la mutación A543V tiene una susceptibilidad disminuida a cidofovir con un nivel de resistencia de 10x. El ensayo de crecimiento viral reveló que las mutaciones G441S, A543V y F345L tienen una capacidad replicativa inferior a la cepa control AD169. El estudio computacional mediante modelo de proteínas 3D predijo correctamente el fenotipo de A543V y A928T, pero no de R512C, reforzando la necesidad de caracterizar las mutaciones desconocidas individualmente mediante fenotipo recombinante. Es fundamental ampliar las bases de datos de mutaciones para optimizar los tratamientos y mejorar el manejo de pacientes con infección por CMV refractario o resistente.

Respuesta a los objetivos 1.1, 1.2 y 1.4.

Artículo 3. Evaluación de mutaciones en el gen *UL56* previas al tratamiento con letermovir en pacientes trasplantados con citomegalovirus refractario.

Revista: Microbiology Spectrum. 2022 Apr 27;10(2):e0019122

Autores: Santos Bravo M, Tilloy V, Plault N, Palomino SS, Mosquera MM, Navarro Gabriel M, Fernández Avilés F, Suárez Lledó M, Rovira M, Moreno A, Linares L, Bodro M, Hantz S, Alain S, Marcos MÁ.

Resumen:

Este artículo presenta el estudio de mutaciones *de novo* en la subunidad de la terminasa viral *UL56* y su fenotipo asociado en el contexto de pacientes trasplantados infectados con citomegalovirus (CMV) resistente a los inhibidores de la ADN polimerasa, los cuales nunca han recibido letermovir (LMV). La mutación R246C fue la única variante del gen *UL56* detectada por secuenciación Sanger y por secuenciación masiva, la cual se localiza dentro de la región asociada a resistencias a LMV (residuos 230-370). La mutación R246C fue detectada en 2 de 80 pacientes trasplantados (uno de progenitores hematopoyéticos y otro de corazón) desde el principio de la replicación por CMV, que fueron respondiendo sucesivamente a los distintos antivirales *in vivo*. Los resultados del fenotipo recombinante mostraron que la mutación R246C confiere una capacidad replicativa superior, pero es sensible a todos los antivirales de uso actual (ganciclovir, cidofovir, foscarnet, maribavir y LMV). Estos resultados revelaron un bajo ratio (2.5%) de polimorfismos naturales, dentro de la región asociada a resistencias a LMV, antes de su administración. La identificación de variantes con una capacidad de replicación incrementada en pacientes que no responden al tratamiento o con recidivas puede servir de ayuda para en el ajuste y elección de tratamientos antivirales.

Respuesta al objetivo 1.3.


The Journal of Infectious Diseases

MAJOR ARTICLE

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Severe Acute Respiratory Syndrome Coronavirus 2 Normalized Viral Loads and Subgenomic RNA Detection as Tools for Improving Clinical Decision Making and Work Reincorporation

Marta Santos Bravo,[1,©] David Nicolás,[2] Carla Berengua,[3] Mariana Fernandez,[1] Juan Carlos Hurtado,[1] Marta Tortajada,[4] Sonia Barroso,[4] Anna Vilella,[5] Maria Mar Mosquera,[1] Jordi Vila,[1] and Maria Angeles Marcos[1]

<u>Artículo 4.</u> La detección de ARN subgenómico y la normalización de las cargas virales del SARS-CoV-2 como herramientas para mejorar la decisión clínica y la reincorporación laboral.

<u>Revista</u>: Journal of Infectious Diseases. 2021 Oct 28;224(8):1325-1332.

<u>Autores</u>: Santos Bravo M, Nicolás D, Berengua C, Fernández M, Hurtado JC, Tortajada M, Barroso S, Vilella A, Mosquera MM, Vila J, Marcos MA.

<u>Resumen</u>:

El método de detección de SARS-CoV-2 por PCR provee un valor de Ct poco estandarizado que no permite distinguir la presencia de replicación viral. Por eso, el ARN subgenómico (sgRNA) se ha utilizado para detectar la infecciosidad. Dada la importancia de la detección prolongada de ARN viral por PCR y la necesidad de acortar los periodos de aislamiento y de reincorporación laboral, se ha analizado la utilidad de normalizar las cargas virales (CVN) en las muestras respiratorias para la monitorización del paciente y la detección de sgRNA para indicar infectividad viral.

Para ello, se cuantificó la CVN mediante el gen de la nucleocápside (N) y de la ARN polimerasa dependiente de ARN (RdRp) y el sgRNA en dos frotis nasofaríngeos consecutivos procedentes de 84 trabajadores sanitarios. Las CVN proporcionaron valores precisos y similares para ambos genes en los dos puntos de la infección, superando la variabilidad implícita en la recogida de muestras respiratorias y el hallado en el valor del Ct. Todas las muestras positivas para el sgRNA tenían >4$\log_{10}$RNAcopies/1000cells, mientras que las muestras inferiores a 1$\log_{10}$ eran negativas para el sgRNA. A pesar de que las CVN fueron positivas hasta el día 29 después del inicio de síntomas, el 84.1% de las muestras positivas para sgRNA eran de los 7 primeros dias, lo cual se correlaciona con la viabilidad previamente descrita en cultivo. El análisis multivariado demostró que el sgRNA, las CVN y los dias de síntomas están significativamente asociados ($p<0.001$).

Por tanto, las CVN y la detección de sgRNA son dos técnicas rápidas y accesibles que podrían implementarse fácilmente en la rutina hospitalaria, aportando información esencial sobre la infectividad viral y el seguimiento del paciente.

Respuesta a los objetivos 2 y 3.

Artículo 5. El cultivo viral confirma que el ARN subgenómico del SARS-CoV-2 es un buen marcador subrogado de infectividad.

Revista: Journal of Clinical Microbiology. 2022 Jan 19;60(1):e0160921

Autores: Santos Bravo M, Berengua C, Marín P, Esteban M, Rodríguez C, Del Cuerpo M, Miró E, Cuesta G, Mosquera M, Sánchez-Palomino S, Vila J, Rabella N, Marcos MÁ.

Resumen:

Determinar la infectividad viral del SARS-CoV-2 es crucial para la valoración clínica del paciente y para las medidas de aislamiento. En este estudio se evaluó la funcionalidad del ARN subgenómico (sgRNA) como marcador subrogado de infectividad en muestras respiratorias RT-PCR positivas para SARS-CoV-2 (n=105) por comparación con el cultivo viral, como técnica de referencia de replicación viral. Los resultados del sgRNA y el cultivo fueron coincidentes en 99/105 casos (94%), con un coeficiente de concordancia altamente significativo (Cohen's kappa 0.88, 95% IC 0.75-0.97, p>0.001), una sensibilidad del 97% y un valor predictivo positivo de 94% para detectar virus competentes. Estos resultados apoyan la hipótesis del uso del sgRNA como un marcador subrogado de infectividad del SARS-CoV-2. La PCR del sgRNA es una técnica precisa, rápida y asequible que puede superar las limitaciones del cultivo y del valor del Ct, y que puede ser implementada en la rutina de los laboratorios hospitalarios para detectar infectividad viral, lo cual es esencial para optimizar la monitorización del paciente, la eficacia de los tratamientos y vacunas, las políticas de reincorporación laboral y para reducir de forma segura las precauciones de aislamiento.

Respuesta al objetivo 3.

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Case Report

Persistent replication of SARS-CoV-2 in a severely immunocompromised patient treated with several courses of remdesivir

Daniel Camprubí[a,*], Anna Gaya[b], Maria Angeles Marcos[c], Helena Martí-Soler[a], Alex Soriano[d], Maria del Mar Mosquera[c], Aina Oliver[b], Marta Santos[c], Jose Muñoz[a], Carol García-Vidal[d]

<u>Artículo 6.</u> Replicación persistente del SARS-CoV-2 en un paciente severamente inmunocomprometido tratado con varios cursos de remdesivir.

<u>Revista</u>: International Journal of Infectious Diseases. 2021 Mar; 104:379-381.

<u>Autores</u>: Camprubí D, Gaya A, Marcos MA, Martí-Soler H, Soriano A, Mosquera MDM, Oliver A, Santos M, Muñoz J, García-Vidal C.

<u>Resumen</u>:

La respuesta microbiológica del SARS-CoV-2 al remdesivir en pacientes inmunocomprometidos ha sido escasamente evaluada. Por tanto, este estudio presenta el caso de un paciente severamente inmunocomprometido debido a un linfoma folicular. Este paciente ingresó por una recidiva neoplásica, al cual se diagnosticó además Influenza A, y posteriormente SARS-CoV-2 tratado con lopinavir/ritonavir, hidroxicloroquina, azitromicina y finalmente con remdesivir (RDV) durante 8 días con resolución favorable. A los 3 dias volvió a tener síntomas y tras testar positivo para SARS-CoV-2 se trató con hidroxicloroquina, azitromicina, darunavir/cobicistat, una dosis de RDV e inmunoglobulinas no específicas, y posteriormente con RDV durante 10 dias, el cual terminó resolviendo la infección. Este caso demuestra la eficacia del RDV en pacientes inmunocomprometidos, ya que redujo significativamente las cargas virales y los síntomas del paciente, lo cual no se consiguió con el resto de los tratamientos. Los 60 dias de PCR positiva para SARS-CoV-2 junto con la literatura previa confirman la hipótesis de replicación persistente en pacientes inmunodeprimidos. A pesar de que ciertos ensayos clínicos demostraron que tratar con RDV 10 durante días no aportaba ningún beneficio respecto a 5 días, el aumento de carga viral tras los 8 dias iniciales de RDV, sugieren que una administración corta de RDV puede ser insuficiente en pacientes inmunocomprometidos debido a la replicación prolongada.

Respuesta al <u>objetivo 4.</u>

Artículo 7. Mutaciones en el gen *nsp12* del SARS-CoV-2 en pacientes con COVID-19 severo no respondedores a remdesivir.

Revista: Microbiology Spectrum. Manuscrito pendiente de publicación.

Autores: Santos Bravo M, Alonso R, Soria D, Sánchez-Palomino S, Sanzo Machuca A, Alcamí J, Diez F, Fernández Avilés F, Bodro M, Rubio E, Villanueva JL, Vergara A, Mosquera MM, Puerta P, García C, Martínez MJ, Soriano A, Marcos MA.

Resumen:

El remdesivir (RDV) fue el primer antiviral aprobado por la FDA para tratar pacientes con COVID-19 severo. Inhibe la replicación del SARS-CoV-2 mediante el bloqueo de la proteína no-estructural 12 (nsp12), subunidad de la ARN polimerasa dependiente de ARN (RdRp). Actualmente, no hay evidencias de una diseminación global de mutaciones de resistencia a RDV. Por tanto, el objetivo de este estudio es determinar las mutaciones en *nsp12* antes y después del tratamiento con RDV en pacientes no-respondedores al tratamiento.

Mediante la detección de ARN subgenómico se identificaron 57/149 (38.3%) pacientes que no respondieron a 5-dias (63.2%) o a más de 5 dias (36.2%) de tratamiento con RDV. Variantes del gen *nsp12* fueron detectadas en 17/49 (34.7%) pacientes por secuenciación masiva, entre ellas la mutación *de novo* E83D que apareció en un paciente inmunodeprimido tras 10+8 dias de tratamiento con RDV, así como la mutación L838I detectada antes y después de tratamiento prolongado en 8/49 (16.3%) sujetos. A pesar de que la posición de las mutaciones en modelos de proteína 3D no predijo ninguna una interferencia con RDV, las sustituciones amino acídicas detectadas implicaron cambios en la capa electrostática superficial y en las estructuras secundarias de la proteína, pudiendo estar asociadas a una alteración de la respuesta al antiviral.

La monitorización de aparición de mutaciones de resistencia a los antivirales es esencial para la vigilancia y para mejorar el manejo clínico tras un fallo al tratamiento con RDV, especialmente en pacientes inmunodeprimidos y en aquellos con replicación persistente.

Respuesta a los objetivos 4 y 5.

CONCLUSIONES

1. La incidencia de mutaciones asociadas a resistencia a los antivirales en CMV está infradiagnosticada.
2. El estudio genotípico y fenotípico ha permitido diagnosticar una alta incidencia (≈30%) de mutaciones de resistencia a los tratamientos antivirales convencionales y nuevos en pacientes con infección por CMV refractaria o resistente.
3. Se ha detectado que los polimorfismos naturales en CMV son menos frecuentes en los genes *UL97* y *UL56* que en el *UL54* independientemente del tratamiento antiviral.
4. Las mutaciones desconocidas se han detectado por métodos genotípicos en un 21.3% de los pacientes con infección por CMV refractaria o resistente tras el uso de fármacos antivirales, reforzando la necesidad de ensayos fenotípicos.
5. La técnica de producción de virus recombinantes (bacmid) ha permitido la caracterización de nuevas mutaciones en los genes diana farmacológica *UL97*, *UL54* y *UL56*. Estas mutaciones confieren resistencia triple, cruzada o intermedia a los distintos fármacos antivirales.
6. Mapear las mutaciones del UL97 que confieren resistencia a MBV y/o GCV aporta información útil para el diseño de inhibidores del UL97 alternativos, y así evitar la aparición de resistencias cruzadas.
7. Las mutaciones primarias asociadas a resistencia a LMV son poco frecuentes en pacientes trasplantados con infección por CMV refractaria o resistente que nunca han recibido LMV.
8. El ensayo fenotípico ha permitido identificar alteraciones en la capacidad replicativa de las distintas variantes de CMV, de gran relevancia para el ajuste de tratamientos antivirales y la mejora de la evolución clínica del paciente.
9. No se aconseja estimar el fenotipo de mutaciones desconocidas en el *UL97, UL54* y *UL56* a partir del significado de mutaciones cercanas, ya que no se ha demostrado que no se puede predecir completamente por su posición génica.
10. Los genes dianas a los antivirales deben ser complemente genotipados de forma estándar para reducir resultados falsos negativos y proporcionar una frecuencia real de las mutaciones de resistencia.
11. El modelo de proteínas 3D puede predecir el impacto que las variantes genéticas provocan a través de cambios electrostáticos en las superficies externa de la proteína y en las estructuras secundarias/terciarias de la misma, las cuales pueden modificar

potencialmente la interacción entre la proteína viral y la molécula antiviral. Sin embargo, el nivel de susceptibilidad a los antivirales debe ser confirmado mediante fenotipado.

12. La replicación viral prolongada ha sido frecuentemente detectada en pacientes con COVID-19 severo, especialmente aquellos con previas enfermedades hematológicas. El diagnostico personalizado y las estrategias terapéuticas específicas son necesarias para abordar este reto clínico.
13. Las cargas virales normalizadas del SARS-CoV-2 proporcionan cantidades de RNAg viral similares y robustas, mejorando la variabilidad intrínseca de la toma de muestras respiratorias y el uso inadecuado del valor del Ct.
14. El cultivo viral ha permitido estudiar la cinética de replicación y la citopatología del SARS-CoV-2, así como la validación del sgRNA para detectar infectividad.
15. El sgRNA del gen de la *envuelta* es un buen marcador subrogado de replicación activa del SARS-CoV-2 que supera las limitaciones de largo tiempo de respuesta, de coste de laboratorios de alta seguridad y la necesidad de muestras frescas propias del cultivo viral.
16. El sgRNA aporta información esencial para la reincorporación laboral, el cese de aislamiento, la determinación de replicación persistente del SARS-CoV-2 y la eficacia de las distintas vacunas y tratamientos.
17. La detección de sgRNA y las cargas virales normalizadas del SARS-CoV-2 son dos técnicas rápidas y accesibles fácilmente implementables en la rutina hospitalaria, que ayudan a la determinación de infectividad y el seguimiento del paciente.
18. La detección de sgRNA ha permitido hallar que más del 60% de pacientes con hospitalizados por COVID-19 severo no respondieron a 5 días de tratamiento con remdesivir y que más del 35% no respondieron ante una mayor duración del tratamiento (>5 días).
19. Ninguna resistencia virológica significativa fue identificada por secuenciación masiva después de varios cursos de remdesivir en pacientes no-respondedores hospitalizados por COVID-19 severo, ya que las mutaciones fueron detectadas independientemente del tratamiento con remdesivir, excepto la mutación E83D en el *nsp12* que apareció únicamente después del tratamiento.
20. La duración del tratamiento con remdesivir parece no ser un factor de riesgo de desarrollar mutaciones de resistencia. Sin embargo, se necesitan estudios más extensos para incluir esta práctica en las guías terapéuticas actuales.
21. La ampliación de bases de datos de mutaciones es fundamental para interpretar los resultados genotípicos y para optimizar las guías clínicas de manejo de pacientes que no

responden al tratamiento antiviral o que sufren replicación viral persistente. Sin embargo, se deben excluir mutaciones compensatorias antes de cambiar el tratamiento.

22. La correlación genotipo-fenotipo no es solo importante para definir el nivel de resistencia a los antivirales y la cinética viral, sino para entender la función biológica de cada residuo proteico y así construir mapas mutacionales fiables y comprensibles que faciliten el desarrollo de nuevas moléculas antivirales.
23. La resistencia virológica se desarrolla de forma escalonada y progresiva, por tanto, el seguimiento clínico del paciente y la monitorización viral son esenciales para la detección precoz de mutaciones de resistencia y para evitar su aparición o la de nuevas variantes víricas.

INTRODUCTION

1. IMMUNOCOMPROMISED PATIENTS

Immunocompromised patients are defined by having a deficient immune system, implicating a reduced ability to respond against pathogens. This immunodeficiency status can be caused by certain diseases and conditions, differentiating them in three types according to their origin: primary, secondary or acquired, and iatrogenic immunodeficiency. Primary immunodeficiencies are caused by genetic disorders congenitally inherited that affect different components of the innate and adaptive immune systems leading to serious complications [1,2]. Secondary or acquired immunodeficiencies appear after birth and are not linked to a genetic deficiency. They are caused by a virus infection, such as human immunodeficiency virus (HIV) that infects lymphocytes T CD4+ and causes acquired immunodeficiency syndrome (AIDS)[3]. Hematopoietic and lymphoid malignancies are also acquired immunodeficiencies as they reduce the number of immune effector cells and antibodies (Abs) synthesis[4]. The most common cause of immunodeficiencies is iatrogenic, and resulted from the use of immunosuppressive therapies against cancer, inflammatory diseases, autoimmune diseases or after receiving hematopoietic stem cell (HSCT) or solid organ transplants (SOT)[5].

The immunodeficiency status comprises a heterogenous group of diseases, all affecting the immune system. Suspicion of immunodeficiency is usually raised based on the frequency, severity or identification of pathogens at levels that indicate an underlying immune dysfunction[6]. As they cannot set a robust immune response, infection is the major cause of morbidity and mortality in immunodeficient patients.

1.1. Differences in the immune response of immunocompromised and immunocompetent patients

The immune system is a complex net of cells and tissues that work together to protect the organism against different pathogens. The immune response is coordinated by immune cells that recognize pathogens or part of them (antigens) through receptors in the cell membrane. When a pathogen enters, the innate response starts (figure 1). This is a nonspecific response against any pathogen and does not leave memory cells. It is mediated by Natural Killers (NK) and the phagocytic system composed by macrophages, neutrophils, and dendritic cells (DCs). DCs are able to activate the adaptive response by phagocyting the pathogen, migrating to the closer lymphoid node and presenting the antigen. There are soluble molecules involved in the innate response which are produced by macrophages and activated NK. They have different functions:

antiviral (Interferon (IFN), interleukin (IL) 15 or IL12), proinflammatory (tumour necrosis factor (TNF), IL1) and regulatory function (transforming growth factor (TGF), IL10).

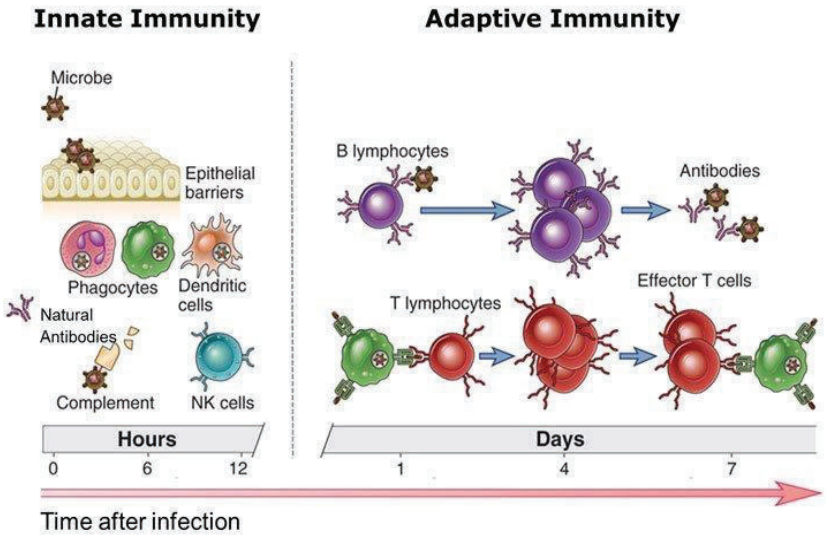

Figure 1. Cells and molecules involved in the innate and adaptive immune responses according to the time after infection. From Abbas et al [7].

The adaptive response is antigen-specific, because immune cells differentiate to be specific for that antigen and proliferate, generating a pull of specialized effector and memory cells that efficiently respond in the case of reinfection with the same pathogen (figure 1). These cells are lymphocytes T and B and the cytokines implicated are IL2, IL4, IL5 and IFN. Lymphocytes T CD8+ have a cytotoxic function, are in charge of controlling the replication of viral infections and clear the virus from the organism by releasing INF-ϒ killing the infected cell. Lymphocytes B are antigen-presenting cells (APC) that disrupt the pathogen and present the antigen in the Major Complex of Histocompatibility class II (MCH-II) to the T cell and produce Abs that recognize the specific antigen.

The persistence of the pathogen in the organisms induce very diverse functional responses. T cells maturate progressively during the immune response presenting different molecules in their surface. They can be differentiated in: naïve, effector, effect memory and central memory cells (figure 2). When naïve T cells (CD27+ CD28+) are stimulated by the recognition of the CMV antigen, they differentiate into effector T cells (CD27+ CD28-) and proliferate generating a pull of specific T cells. Memory T cells are also generated during the infection and depending on the expression of chemokine receptor of signalling of the lymph node CCR7 which helps them to migrate to the lymph node or circulate bloodstream, thus, they can be differentiated in central memory (CCR7+) and effector memory (CCR7-).

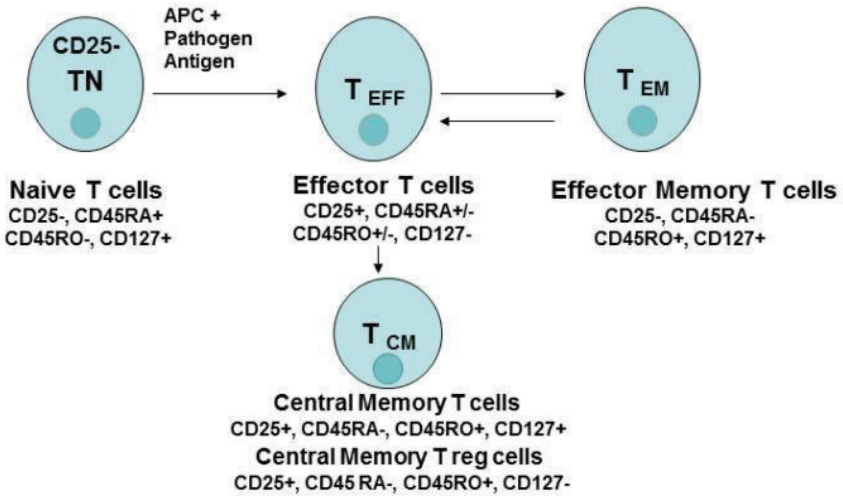

Figure 2. Maturation and differentiation of T cells after antigen recognition and phenotypes of each T cell subset. From Golubovskaya et al [8].

In the case of immunosuppression, the effector T cell gets exhausted and memory cells are produced in less amounts. Immunosuppression involves a deficient innate response and a lower capacity of clonal expansion of adaptive immune cells (CD8+, CD4+ and B) after an antigen restimulation as there is a decreased quantity of cells.

Patients with primary immunodeficiencies are well-controlled thanks to immunomodulator molecules, immunoglobulin G (IgG) infusion, HSCT and gene therapy. On the other hand, antiretroviral drugs have improved CD4+ T cells count in AIDS/HIV patients in the last decade, reconstituting the patients' immune system. However, patients requiring lifelong immunosuppression, such as hematologic malignancies or transplant recipients, are the most affected by opportunistic infection.

The complete reconstitution of the immune system after HSCT or ceasing of immunosuppressive therapy takes years or could not even be completed. It started two to three months after transplant or therapy cessation, with the rise of CD8+ T cells, followed by CD4+ T cells between the three to six months after transplant. B cells are the last to start proliferating from the sixth month until two years post-therapy/post-HSCT. The effect of lymphocyte depletion depending on the degree of immunosuppression renders patients vulnerable to infectious processes. Therefore, the major difficulty clinicians must face is to balance between the right level of immunosuppression required to prevent transplant rejection or malignant lymphoid cells replication, and the level of antiviral treatment to prevent infection.

1.2. Opportunistic infections

The most prevalent pathogen and the major cause of morbidity and mortality in immunodeficient patients, especially in transplant recipients, is human cytomegalovirus (CMV). Immunosuppressive treatment is necessary in the early post-transplant period, however it favours opportunistic infection by CMV. CMV causes mononucleosis-like syndrome as direct effects, but it can also alter the innate and adaptive host immunity to escape from it. Indirect effects involve inflammatory, autoimmune and vascular diseases among many others. The clinical outcome of the immunomodulation caused by CMV is evidenced by graft rejection and graft versus host disease (GVHD) in SOT and HSCT recipients, respectively. CMV also causes immunosuppression even in the absence of immunosuppressive therapy[9], increasing the risk for other opportunistic infections[10–13].

Changing immunosuppressive practices, including the increasing use of T-cell depleting induction Abs, have the potential to affect the risk for CMV infection and disease, even in the face of good prophylactic and preemptive therapy[14] .Prophylactic approaches and treatment with antiviral drugs have significantly reduce the development of CMV infection as well as the risk of other viral and bacterial infections and their associated mortality rates[15,16]. However, prolonged use of antiviral drugs has led to pancytopenia, several toxicities and has favour the emergence of drug resistance.

In transplant settings, the incidence of drug resistance mutations is 5-12% depending on the transplantation. Clinical outcome of patients with resistant infection is associated with a higher morbidity, longer hospitalisation, invasive disease, progressive dysfunction, graft rejection and higher mortality[17]. Thus, the need for early detection of the appearance of resistance mutations is essential in order to implement adequate treatment as described by consensus documents on the management of CMV infection[18].

Immunocompromised hosts, especially patients with hematologic malignancies, have suffered greatly due to the pandemic coronavirus disease (COVID-19), with the highest reported mortality rates (28-44%)[19–21]. SARS-CoV-2 is also associated with immunomodulatory functions, although it is not well-characterized yet. This infection downregulates IFN-I but enhances a chemokine storm causing an exacerbated inflammation. In the context of immunosuppression, it is not a protective factor as an adaptive immune response is needed to limit innate response and to generate efficient effector and memory cells to fight the infection[22].

The Centres for Disease Control and Prevention (CDC) and other international agencies have included as poor prognostic factors patients with some degree of immunosuppression and has associated it as a risk factor of viral persistence[23–26]. Viral persistence is a growing health concern as it can favour the selection of mutants that can confer an advanced viral fitness, immune escape or antiviral resistance. Although no evidence of widespread transmission of resistance mutations to antivirals has been reported until the present study, it is crucial for health surveillance to closely monitor emergent mutations, especially in patients with persistent viral infection.

1.3. Persistence viral replication

CMV and SARS-CoV-2 establish long cohabitation in immunocompromised hosts. It has been demonstrated that CMV can stay in a non-replicative latency livelong and reactivate under alterations in the immune system (section 2.7), whereas SARS-CoV-2 can persistently replicate for months (section 3.9). CMV primary infection, reactivation or reinfection needs to be monitored as it can lead to longer antiviral therapies linked to toxicities, immunosuppression and emergence of resistance, all three worsening patient's outcomes.

On the other hand, it has not been demonstrated that SARS-CoV-2 can stay in latency, but several studies have shown that patients with immunosuppression are at risk for prolonged infection and reinfection with SARS-CoV-2[24]. During immunosuppression, B cell response and the production of neutralizing Abs are deeply impaired leading to persistent replication[27]. Few clinical cases showed successful viral culture in samples from >21 days after symptoms onset[28], and a couple of studies proved prolonged viral replication by the same virus strain instead of reinfection by sequencing[24,29]. However, larger studies to determine time of infection in immunocompromised patients, re-positivity and reinfection should be pursued. Persistent replication is a major concern in this setting as it increases duration of infectivity, it can exhaust the already depleted immune system, delay treatment for hematologic malignancies, and facilitates the progressive emergence of multi-mutational SARS-CoV-2 variants and mutations conferring antiviral resistance[24,29].

1.4. Antiviral drug resistance

Antiviral agents are drugs for the treatment or control of viral infections. They target stages in the viral replication cycle, such as, viral attachment to host cells, uncoating, synthesis of viral messenger RNA (mRNA), translation of mRNA, replication of viral genome, maturation of new viral proteins, budding, release of newly synthesized virus and free virus particles in extracellular medium. For this reason, most antiviral agents are effective only against actively replicating

viruses and not when they are in a latent stage. Antiviral agents must be approved by the Food and Drug Administration (FDA) and are designed to treat or prevent viral diseases and could be repurposed for the treatment of other pathogens.

The objectives for treating acute viral infections are to reduce the severity of the illness and its complications, and to decrease the rate of transmissions of the virus, to prevent viral damage to visceral organs (in the case of chronic infections), to control the viral replication and dissemination in the organism. The therapeutic index (ratio of efficacy to toxicity) must be high for the therapy to be accepted. Antiviral drugs are developed to be used for prophylaxis, preemptive therapy or treatment of the infection. Their utility is limited by three main factors: toxicity, side effects and the development of antiviral resistance by the virus.

Antiviral resistance is understood as the lack of response to an antiviral molecule. The non-response can be due to inadequate dosage, dose-limiting toxicities, poor absorption, poor penetration into the body compartment where replication is occurring, or overwhelming replication, while the antiviral treatment allows for ongoing replication. This eventually selects drug resistance mutations. Mutations mostly appeared on pharmacologic target genes, however, with continued administration of the antiviral drug, the mutations in alternate genes increased the magnitude of antiviral resistance due to cross-resistance to antiviral agents that have yet to be administered. The staggered and progressive evolution of resistance strengthens the importance of early virological studies[30]. Therefore, the need for prompt detection of the appearance of resistance mutations is essential in order to implement adequate treatment, especially in patients with refractory CMV infection or SARS-CoV-2 persistent replication.

2. HUMAN CYTOMEGALOVIRUS

Human CMV or human herpesvirus-5 (HHV-5) belongs to the family *Herpesviridae* and subfamily *Betaherpesvirinae*. CMV is a human-infecting ubiquitous host–restricted virus whose seroprevalence is 45-100% worldwide in the adult population[31]. Its name was derived from the etymology “cyto” meaning cell and “megalo” big, because of the appearance of the cytoskeleton disruption in the infected cell caused by this virus.

2.1. Discovery of cytomegalovirus

In 1881, the first evidence of cytomegalies and body inclusions in kidney and parathyroid gland cells were found by Hugo Ribbert[32], but until 1904 it was not evidenced properly in parallel with Jesionek and Kiolemenoglu[32,33]. The first isolation of the virus, known thereafter “cytomegalovirus”, was achieved in 1956-57 by Smith, Rowe and Weller[34]. In 1984, the human

CMV (strain AD169) was sequenced for the first time and published[35], and only 6 years after, the draft of an annotated CMV genome was reported[36], which at that time was the biggest contiguous genome sequenced (GenBank: BK000394.5).

Since 1990, 406 full-length distinct CMV complete genomes have been published, including lab-attenuated strains, low and high passaged strains or artificial genomes in the Virus Pathogen Database and Analysis Resource[37].

2.2. Epidemiology and prevalence of human cytomegalovirus

CMV infection is an endemic infection. The CMV seroprevalence of northern countries varies between 36% and 77%, while in southern countries this percentage often reaches 100%[38] . CMV seroprevalence has been reported to be highest in Africa, Asia and South America and lowest in Western Europe and United States (figure 3).

According to races, statistical data has shown worldwide seroprevalence among non-whites was 20-30% higher than that of whites (summary prevalence ratio (PR) = 1.59, 95% confidence interval (CI) = 1.57-1.61)[38]. Males generally had lower seroprevalences than females, although these differences were small (summary PR = 1.13, 95% CI = 1.11-1.14). The incidence of CMV seropositivity is higher in persons of lower socioeconomic status (summary PR = 1.33, 95% CI = 1.32-1.35). In countries with low to moderate seroprevalence, age is a predictor of seropositivity since the chance of exposure to CMV continues throughout life. The socioeconomic status may be a reflection of factors that contribute to the exposure to CMV, such as crowded living conditions or numerous infants. However, the severe morbidity caused by CMV in immunocompromised individuals and neonates, and the following long-term disability in children, accounts for the true burden of disease of CMV infections.

CMV infection was frequent (45-100%) among women of reproductive age, who can transmit the virus transplacental to the fetus causing congenital CMV (cCMV) infection. In spite of this high seroprevalence, a significant percentage of women of reproductive age are CMV seronegative, raising the risk of primary CMV infection during pregnancy.

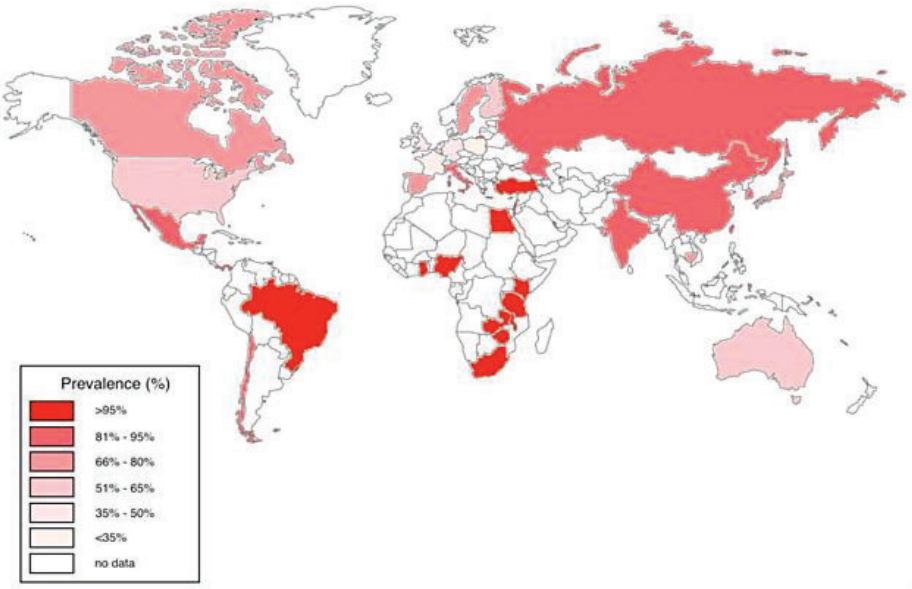

Figure 3. Worldwide CMV seroprevalence percentages in adults aged 16–50 years from 2005 to 2015. From Nevels et al [38].

2.3. Cytomegalovirus structure

CMV is a 200nm of diameter virus and has 4 distinct morphologic units: the core which contains the viral genome; the icosahedral nucleocapsid of 162 capsomers[39]; the tegument which contains 27 structural phosphoproteins, proteins that modulate the immune system and RNA transcripts[40]; and the envelope, a double-stranded lipidic layer with viral glycoproteins and host proteins embedded (figure 4). The tegument is the most heterogeneous structure in CMV, and exerts several functions for viral replication, such as, blocking the cellular response that degrades entering DNA, enhancing IE (immediate early) gene transcription[41–43], and contains the immunodominant target of T cells and Abs, pp65 [44–47].

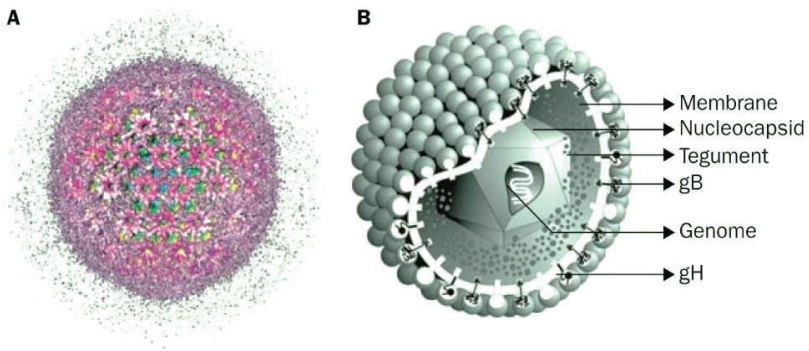

Figure 4. Structure of human cytomegalovirus particles. (A) Particle of CMV viewed along a 3-fold symmetry axis. (B) Virtual 3D model with the different components of the particle indicated. From Gandhi et al [54].

Several glycoproteins have been described, and the pentameric complex gH/gL/gUL128-131 has been shown to elicit neutralizing Abs against several CMV strains[48–50]. The initial binding between CMV and the target cell occurs via viral glycoproteins and cellular proteoglycans that are located in the surface of the CMV particle, followed by binding with more specific receptors and fusion/endocytosis[51–53].

2.4. Cytomegalovirus genome

The CMV genome consists of a double-stranded DNA (dsDNA) packaged in the icosahedral capsid[55]. Its longitude is approximately 235000 bp, the largest human-infecting virus genome, with a 57.8% of GC content [56]. CMV genome has 208 Open Reading Frames (ORFs) encoding over 165 genes, 4 non-coding RNAs and 14 microRNAs. Of the protein-coding genes, 43-44 are core replication genes common to all herpesviruses, while approximately 30 are unique to beta-herpesviruses.

CMV has a type E genome architecture[57], composed of 2 central inverted domains: unique long (UL) and unique short (US) (figure 5). The ORFs are named by the region and the numerical order in which they occur. They are flanked by two repeated regions, one at the terminal end and the other at the intersection with the other unique domain (TR_L-IR_L and TR_S-IR_S). Repetitive regions can recombine, changing the orientation of the UL or US domain, resulting in 4 possible genetic isomers that are found in any infective viral population in equimolar proportion [58,59]. There has been partial or complete deletion of a block of ORFs (UL133-UL148) due to many passages over years of culture of well-characterized laboratory strains CMV AD169 and Towne [60,61]. None of the deleted ORFs has been linked to the anabolism of antiviral agents, but has been associated with latency, reactivation, and the expression of specific genes.

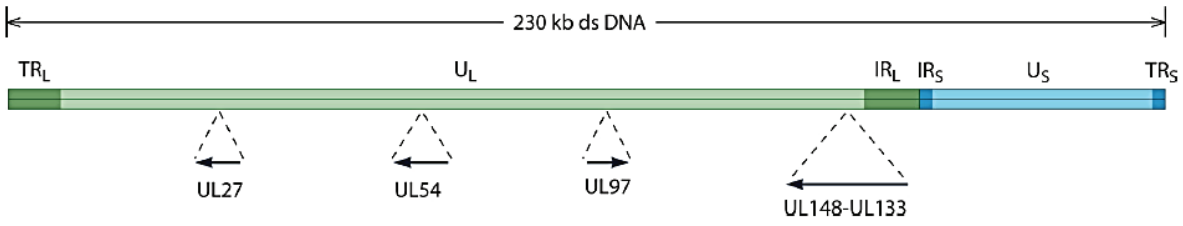

Figure 5. Genetic map of the human CMV genome. Positions of genes of interest are indicated by dotted lines. The direction of the ORF for each gene is indicated with arrows. From Lurain et al[62].

CMV has three gene families temporally regulated and expressed at the immediate early (IE), delayed early (E) and late (L) phases of the replication cycle in infected cells. The *IE* and *E* family of genes regulate the activation of the subsequently expressed genes.

The first period of transcription starts after viral entry. The nucleocapsid is transported to the nucleus where the *IE* genes (*IE-1, IE-2*) are expressed under the control of the major IE promoter (MIEP) and independently of *de novo* viral protein synthesis, thus, *IE-1* is used as a target for detection of CMV infected cells. *IE* genes play regulatory function and *IE-2* transactivated *E* gene promoters in cooperation with DNA replication promoters (*UL54* DNA polymerase, *UL44* polymerase accessory protein, *UL57* ssDNA binding protein, *UL70* primase, *UL102* helicase/primase protein and *UL105* DNA helicase)[63]. *E* genes are mostly involved in DNA replication and are responsible for the activation of *L* gene promoters. Lastly, L phase starts during DNA replication, transcribing *L* genes that mainly encode structural proteins involved in formation of mature virus particles.

2.5. Cytomegalovirus replicative cycle

CMV replication starts with the initial binding between CMV glycoproteins with the cellular proteoglycans of the target cells, followed by the interaction with specific receptors (figure 6). Then, the CMV particle enters through the endocytic pathway or through direct fusion of the envelope with the cellular membrane to release nucleocapsids into the cytoplasm[64,65]. The nucleocapsid is transported to the nucleus where the *IE* genes are expressed and after the *E* genes, which contain the necessary proteins for viral genome synthesis[66]. Although the CMV genome is linear inside the nucleocapsid, it is circularized during replication; first through theta-like replication and subsequently by rolling circle amplification, generating multiple linked copies in tandem[55]. Thereafter, the concatemer is cleaved by the terminase complex, linearized and introduced inside the nucleocapsid formed with the structural proteins encoded by *L* genes [57]. The nucleocapsid leaves the nucleus and reaches the cytoplasm as a partially tegumented viral particle. Secondary envelopment occurs in the cytoplasm at the endoplasmic reticulum (ER)-Golgi intermediate compartment[66]. The virus egression is believed to occur via cell lysis in particular cell subtypes (e.g. fibroblasts), or via exocytosis at the plasma membrane.

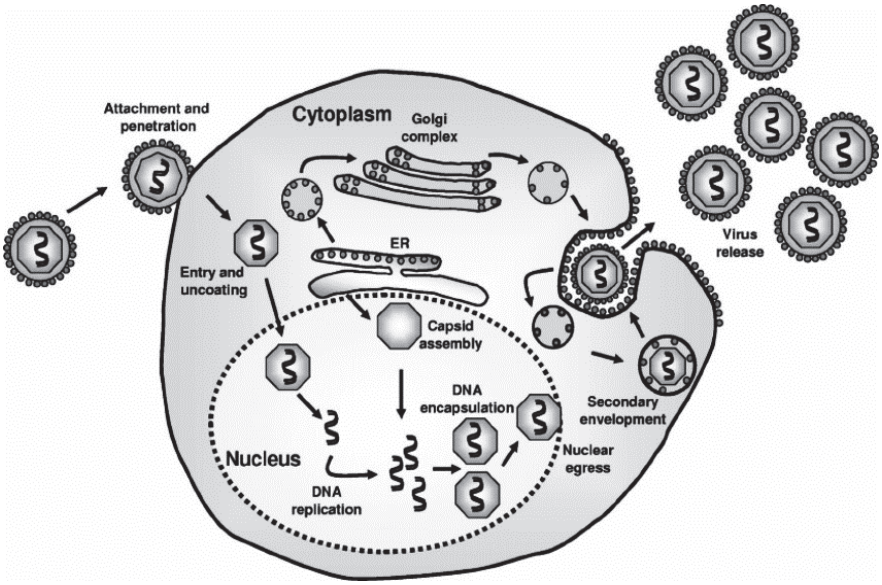

Figure 6. CMV replicative cycle in infected human cells. From Crough et al [67].

2.6. Cytomegalovirus genotypes

The CMV glycoproteins expressed on the viral envelope has been used to genotype CMV. Most glycoproteins are involved in the first step of the replicative cycle in which the virus enters the host cell. Glycoprotein gB (UL55) is in charge of the attachment and the penetration into the host cell, and it is the major target of neutralizing Abs. Glycoprotein H (UL75) and gL (UL115) are covalently linked and is involved in the fusion of the envelope with the cell membrane[68–72].

There are 4 genotypes of CMV gB according to their characteristic nucleotide sequence [73–75]. After the gB is expressed, it experiences glycosylation and proteolytic cleavage between residues 460-461 during transport through the exocytic pathway, but the amino- and carboxy-terminal fragments remain linked by disulphide bonds. This gene is highly variable at the region recognized by the Abs (epitope), the N-terminus and the cleavage site but less variable at the C-terminus. Sequencing highly variable fragments allows its classification.

Many studies have attempted to link gB genotypes and CMV pathogenesis. It has been reported that gB1 genotype is related to a lower mortality compared to other genotypes in HSCT recipients and HIV-infected patients[76]. A possible explanation is that gB1 did not infect T cells while gB2 and gB3 did, as they have different abilities in cell attachment that has been reflected in growth variations in endothelial cells [77]. Nowadays, how each gB is involved in the virulence remains uncertain and no consensus on the classification of CMV strains based on genotype, evolutionary relationship, or clinical relevance has been achieved.

CMV genotyping is most frequently performed by sequencing and restriction fragment length polymorphism (RFLP) of products of polymerase chain reaction (PCR)[78,79]. Methods based on restriction enzymes (enzyme digestion, Southern blot, or RFLPs) only analyse sites sensible to restriction enzymes, failing to detect sequence variability. On the contrary, real-time PCR misses less sequence variability, only those outside the amplified region, but a poor primer design can reduce the sensibility to detect new variations [74]. Nevertheless, the method to detect rapidly and quantify CMV gB and gH genotypes remains real-time PCR[63,80–82]. Multiplex real-time PCR has been shown to be effective in detecting mixed infections and suitable for direct usage on plasma samples, dried blood spots and urine samples of cCMV infected newborn babies[36,83]. Methods based on next generation sequencing (NGS) are more sensitive to the detection of mixed genotypes at very low ratios, but require a large input of samples[84]. In spite of the limited based accuracy and read length, NGS provides an extensive sequencing depth per sample suitable for variant calling. Therefore, NGS is more informative thanks to the increased read yield and sensibility to detect multiple variants.

2.7. Cytomegalovirus infection and latency

Primary infections of children and immunocompetent adults are frequently asymptomatic and are controlled by the host immune response, resulting in clearance of cells that are lytically infected and virus-producing. The viral infection can be acute or chronic. Acute infection is characterized by an intense viral replication and shedding that lasts months, while the chronic infection is characterized by alternating replications and concomitant shedding, with a persistent infection producing undetectable levels of virus.

CMV replicates systemically and has adapted to different biological host niches. Biological niches might place competing demands on the virus depending on the favourable genes in that context. According to the cell type, the infection's outcome is different. For instance, in fibroblasts, the virus replicates lytically to produce infectious virus; in myeloid progenitor cells, an initial burst of lytic gene expression is followed by silence through epigenetic repression, leading to latency establishment.

CMV can establish lifelong latency. Latency is defined as a state in which viral DNA is present inside host cells, but infectious viral particles are not produced. Latency is accomplished by the CMV through viral genome circularization in an episome form in the cellular nucleus of CD34+ hematopoietic progenitors and monocytes that can disseminate the virus to various organs. Although neither cell type is permissive for viral replication, monocytes-derived macrophages are. Latency is established through cell type specific mechanisms of transcriptional silencing. It

appears that there are multiple sites for latency to occur, being blood cells and endothelial cells the major sites of latency[85].

CMV can reactivate from this status of latency and disseminate new infectious viral particles[60]. Reactivation is triggered by the expression of lytic genes through pathways activated during cell stress, such as injury, infection or inflammation, infection, and injury, and during the differentiation of myeloid cells to DCs. CMV exploits cell type specific mechanisms of gene regulation to establish latency and to disseminate infection systemically, evolving a complex relationship with the host immune response. CMV takes advantage of the inflammatory response to infection as an early warning system that allows viral escape, either by cellular damage or infection of the host with another pathogen. Harnessing the immune response to cause cellular injury induced by organ transplantation has been proposed to prevent reactivation of CMV and its clinical outcomes[61]. Reactivation and reinfections have been frequently reported in susceptible populations such as immunocompromised patients (transplanted recipients, HIV-infected, with autoimmune, immunodeficiencies diseases), pregnant women, new-borns, and elderly[86].

2.7.1. Transmission and cell tropism

CMV is species specific and has a particular cell and tissue tropism. It has a slow growth and causes a characteristic cytopathology [83]. The ubiquity of CMV and the rest of herpesviruses could be attributed to their efficient transmission between hosts because of life-long persistence with recurrent shedding. Transmission of CMV occurs through direct exposure to infected body secretions, including blood, saliva, urine, milk and genital secretions, all requiring close contact[87]. Salivary glands secrete higher levels of virus than other organs showing different types of damage. It can also be transmitted transplancentally to the foetus during pregnancy, sexually, by blood transfusion and by transplantation.

CMV has a broad tropism as it can be detected in epithelial cells, endothelial cells, fibroblasts, monocytes/macrophages, neuronal cells, neutrophils, smooth muscle cells, hepatocytes and stromal cells[88–90]. This broad tropism suggests there are multiple cellular receptors that attach to CMV, however, the primary targets are epithelial cells and monocytes, where the initial replication mainly occurs after the virus entry (figure 7). This replication leads to production of both cell-associated and cell-free virus within 24 hours of infection and the virus is spread within polymorphonuclear (PMN) phagocytes via bloodstream to various organs including salivary glands, kidney, lungs, liver, brain, spleen, oesophagus, heart, retina, inner ear and colon[80].

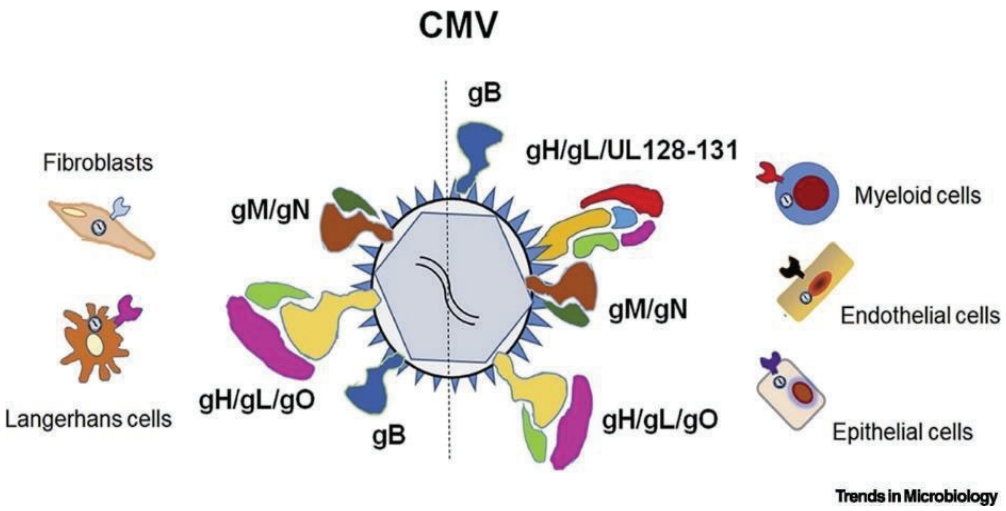

Figure 7. CMV glycoproteins involved in cell tropism. From Sandonís et al[91].

CMV spreading is not prevented by immune evasion mechanisms as monocytes and macrophages are able to infect fibroblast and endothelial cells. This endothelial cell infection can infect more phagocytes[92], and neutrophils as they migrate across the endothelial layer and such PMNs can transmit virus to fibroblasts[93], initiating an inflammatory response. Therefore, CMV DNA is detectable in monocytes, lymphocytes and neutrophils[94], being PMN phagocytes more frequently positive for viral DNA than mononuclear cells[95]. Although the virus does not replicate within leucocytes, they play an important role in virus dissemination throughout the organism. Infection is abortive in PMNs and monocytes but becomes non-lytic cell-associated productive in monocyte-derived macrophages[89,95,96]. There is a large production of intracellular virus, but no release of extracellular virus is detected, as the virus accumulates in large cytoplasmic vacuoles that fail to fuse with the cellular membrane. The virus disrupts microtubules and does not enter the endosomal-lysosomal pathway[97]. In summary, the host response, which normally represses virus spread, enhances viral dissemination because instead of being killed by, CMV replicates in leucocytes.

2.7.2. Pathogenesis and clinical manifestations

The effects of CMV infection in transplant patients can be divided into direct effects of the infection that consists of mononucleosis-like syndrome or tissue-invasive disease, and indirect effects caused by immunomodulation[15,98,99], as represented in figure 8.

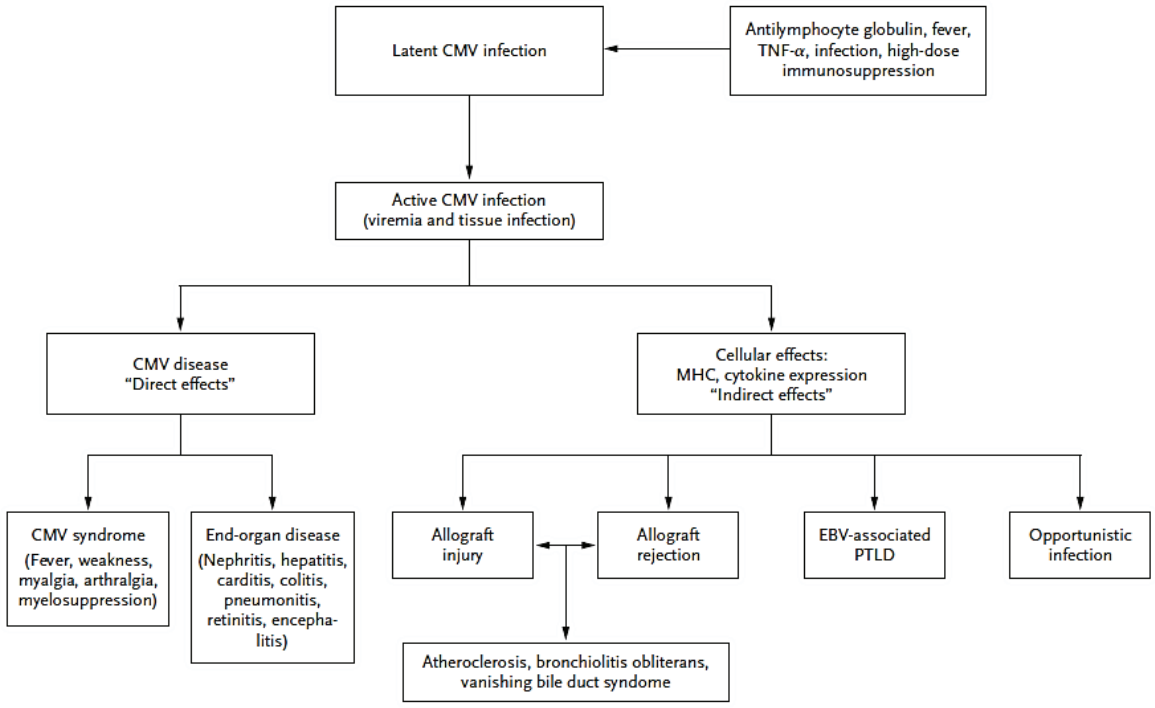

Figure 8. Direct and indirect effects of cytomegalovirus infection. Abbreviatures: MHC major histocompatibility complex, EBV Epstein–Barr virus, PTLD post-transplantation lymphoproliferative disorder. From Fishman et al[15].

2.7.2.1. Direct effects

Clinical manifestations are highly variable among which the mononucleosis-like syndrome is the most common one[100].The characteristic mononucleosis caused by CMV consists of malaise, headache and high fever, which is similar to the one caused by EBV. Other clinical abnormalities include Guillain-Barré syndrome, meningoencephalitis, haemolytic anaemia and thrombocytopenia.

CMV tissue-invasive disease is suspected when high levels of CMV DNAmia are found and is confirmed by detection of the virus in the affected tissue by immunohistochemistry (IHQ). The transplanted organ is the principal target of CMV infection in SOT recipients[15]. CMV can be detected in different organs, but damage occurs only in some of them[101]. CMV damage is found in kidney or salivary glands in transplant recipients, cCMV children and AIDS patients. Mild damage is frequently observed in the retina, liver and gut of adult patients due to direct viral cytopathogenicity, as antiviral agents significantly reduce these effects [101]. Pneumonitis is an often sequelae caused by CMV primary infection or reactivation in kidney, heart, liver or HSCT recipients [102]. However, the incidence of pneumonitis is low in syngeneic HSCT recipients, despite a similar incidence of CMV infection. It could be explained by the different degree of

immunosuppressive therapy each recipient receives, although CMV pneumonitis in AIDS patients where the immunosuppression is severe is infrequent[101]. In the case of cCMV infected children, permanent hearing loss, neurological impairment or miscarriage are the main outcomes[103–105].

CMV cytopathology is low during the acute infection, because of the viral evolved strategies to evade or delay programmed apoptosis. A premature death of infected cells severely limits virus survival and spread of progeny virus within the host.

2.7.2.2. Indirect effects: immunomodulation

CMV is capable of modulating the immune system causing indirect effects on the host, such as graft rejection and immunosuppression in transplant settings (figure 9). These effects are also linked to longer periods of low viral replication caused by the immune response. CMV has also been suggested as a cofactor in the pathogenesis of inflammatory, autoimmune, and vascular disease, such as atherosclerotic coronary artery disease[103–105], as well as a risk-factor for all-cause mortality in large population-based cohorts in the USA and Europe[106].

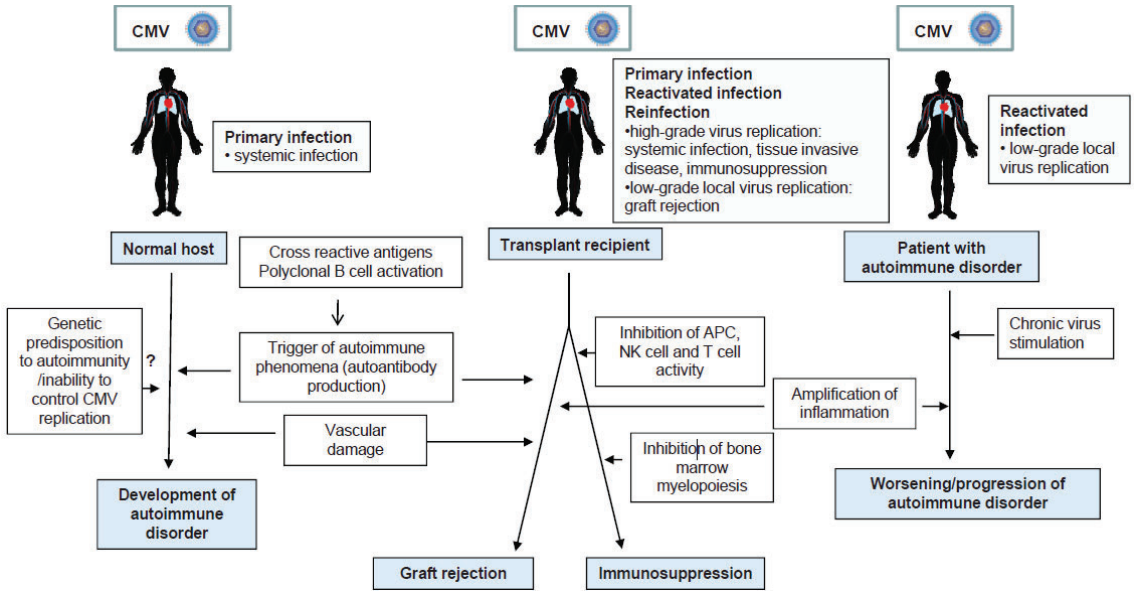

Figure 9. Immunopathology induced by CMV in healthy individuals, in transplant recipients and in patients with autoimmune disorders. From Varani et al[9].

Diverse Abs have been detected in patients with systemic CMV infection[9]. When the humoral response is non-specific and is hyperactivated, the development of specific B cell responses is impeded as a mechanism of CMV immune evasion. This phenomenon has clinical implications

mediated by the production of auto-Abs that cause GVHD in HSCT recipients and graft rejection in SOT recipients [9].

CMV can induce autoimmunity in predisposed individuals as it has been evidenced by concurrent active CMV infection and the development of autoimmunity in many cases. CMV can accelerate the progression of autoimmune disorders by mimicking autoimmune mediated tissue destruction and aggravating inflammation. Paradoxically, CMV infection induces transient but significant immunosuppression in transplant recipients, increasing the risk for opportunistic infections that can be significantly reduced by antiviral prophylaxis[10,13].

The biological function of most CMV genes is to alter innate and adaptive host immunity[107], and a significant fraction of the T cell repertoire in CMV hosts is directed against this virus[100]. Due to CMV persistence in the host, it could be erroneously associated with the pathogenesis of different diseases even if it is not involved. However, evidence of immunopathology attributed to CMV continues to grow, supporting CMV immunomodulatory function.

2.8. Immunotherapy strategies

Viral infections dawn an immune response based on IFN-I and NK cells inhibition of infected cells, as innate response, the blockage of viral epitopes by Abs to avoid the union and entry of the virus, and the cytotoxic effect of T cells to kill infected cells, as adaptive response [108]. The immune response against CMV can be altered by different immunotherapy strategies.

Depending on the immunosuppressive agent, the risk of developing CMV infection is different. For instance, lower rates of CMV infection are found when using mammalian targets of rapamycin (mTOR) (e.g. sirolimus, everolimus). A series of kidney transplant patients were randomized to everolimus with reduced-exposure calcineurin inhibitors or mycophenolic acid with standard-exposure calcineurin inhibitors in the international TRANSFORM trial. Results showed a significant decrease for both CMV infections and syndrome in the everolimus arm[109]. Use of potent induction agents, such as anti-thymocyte globulin, showed higher rates of CMV infection but lower rates of rejection. Although leflunomide, approved for rheumatoid arthritis, was reported to have anti-CMV activity, it was not recommended in the routine practice, together with the antimalarial artesunate, as there is scarce significant clinical data and both present potential toxicities [110,111].

2.9. Antiviral treatment

2.9.1. Antiviral targets

2.9.1.1. UL97 phosphokinase

UL97 gene encodes for a 707 amino acid serine/threonine protein kinase that phosphorylates itself and multiple viral and host proteins[112]. It is conserved among all members of the herpesvirus family. The catalytic domain of protein kinases consists of eleven major conserved regions numbered I to XI, with region I having the highest level of homology[113]. The adenosine triphosphate (ATP)-binding site, the phosphate transfer domain (P-loop) and the substrate-recognition site (catalytic loop) correspond to codon ranges located at positions 337–345 (region I), 481–483 (region VII) and 553–557 (region IX), respectively (figure 10).

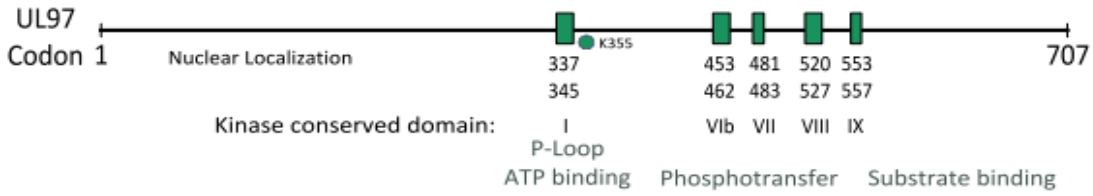

Figure 10. Map of the *UL97* gene. From Lurain et al[62].

UL97 is expressed early during infection and localizes predominantly in the nucleus whereas it is observed later in the cytoplasm[114]. It phosphorylates several proteins involved in many steps of the viral replication cycle, such as, the major IE promoter, the viral UL44 DNA polymerase processivity factor, the nuclear mRNA export factor UL69, and the viral pp65 tegument protein at which is associated in the CMV virion [115-118] .This kinase is a functional orthologue of cellular cyclin-dependent kinase complexes (CDKs) and phosphorylates the retinoblastoma tumour suppressor protein (Rb) on sites normally phosphorylated by CDKs. Once phosphorylated, Rb is unable to repress host genes required for cell-cycle progression to S phase that are indispensable for CMV DNA synthesis[119]. During CMV replication, UL27 delays cell-cycle progression towards the G1/S phase. The mechanism involves the promotion of proteasome-dependent degradation of the cellular histone acetyltransferase, Tip60, which results in an increased expression of the cellular CDK inhibitor, p21[120]. In contrast, UL97 inactivates Rb to promote cell-cycle progression towards the S phase and to allow viral DNA synthesis. When UL97 is inhibited by MBV, Rb remains active and S phase genes required for viral DNA synthesis are silent. Thus, the loss of UL27 could compensate for the loss of UL97 function explaining the mechanism of MBV resistance[121].

On the other hand, the nuclear egress complex (NEC), composed of the two subunits UL50 and UL53, recruits UL97 kinase to the nuclear rim. UL97 then phosphorylates laminin A/C[122] and the NEC leading to disruption of the nuclear lamina and translocation of viral particles into the cytoplasm[123]. Autophosphorylation of UL97 is presumed to be involved in the regulation of its enzymatic activities[124]. UL97 has a wide spectrum of both cellular and viral substrates, which may explain its importance for normal viral replication.

In summary, UL97 plays critical roles in cell-cycle modulation to support viral DNA synthesis, in the regulation of viral gene expression, in the promotion of virion morphogenesis, maturation and encapsidation, in the induction of nuclear lamina disassembly to facilitate the nuclear egress of nascent viral particles and in the tegument proteins disposition, which results in an atypical cytopathic effect (CPE) in cell culture[262].

The essential role of UL97 in the viral replicative cycle has been highlighted by laboratory-engineered UL97-deficient CMV mutant that exhibits a severe replicative defect compared to the wild-type parental strain[223]. Amino acid deletion or changes at the critical well-conserved lysine K355 (K355del, K355M, K355Q) abolish kinase activity[124,125]. At the same time, the enzyme is fortuitously active in the anabolism of antiviral nucleoside analogues. UL97 phosphorylates ganciclovir (GCV) and acyclovir (ACV)[126,127] but not natural nucleosides such as guanosine[114].

2.9.1.2. UL54 DNA polymerase

The virus-specific DNA polymerase encoded by the *UL54* ORF has conserved functional regions shared with other herpesviruses and a wide range of organisms[128,129]. Conserved regions of homology are named Exo I-III (3'-5' exonuclease) and I-VII conserve functional domains of polymerization, along with delta-C region, with an additional homology with some mammalian and yeast delta DNA polymerases[130] (figure 11).

CMV DNA polymerase is the enzyme responsible for viral DNA replication, acting in conjunction with several other essential components of the viral replication complex, including the ssDNA binding protein UL57, the accessory protein UL44, and the primase-helicase complex (UL70, UL102, UL105), which all are highly conserved among CMV strains[131,132]. UL44 forms homodimers that interact through N-terminal residues with the C-terminus of the polymerase, and the specific amino acids involved in the interaction are completely conserved[132].

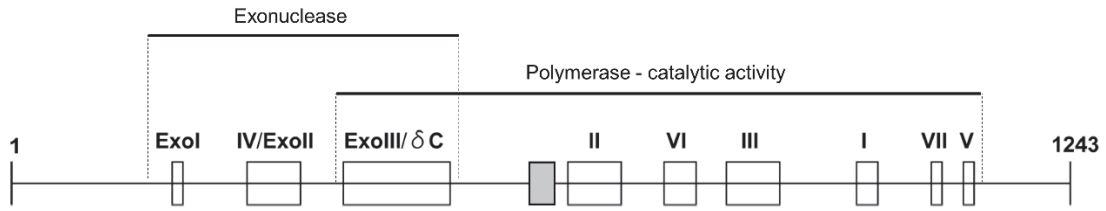

Figure 11. Map of the UL54 gene. Regions associated to the exonuclease and polymerase function of the DNA polymerase are indicated. Conserved domains are shown in white boxes. Adapted from Fillet et al[233].

The main functions associated with DNA polymerase are: DNA synthesis which consists on the binding of the incoming nucleotide triphosphate and release of pyrophosphate; and 3'-5'-exonuclease activity (proofreading) responsible for the high fidelity of replication, which results in a low mutation rate. Figure 11 shows there is overlap of Exo II with region IV and of Exo III with δ-C.

Drug resistance is caused by amino acid substitutions that either inhibit the binding of the active drug to the enzyme or alter the balance of exonuclease and polymerase activities to favour the removal of the incorporated drug[117,133–135]. No drug resistance mutations map to the polymerase region that interacts with UL44[136]; however, the accessory protein homodimer binding is essential for long-chain DNA synthesis. Therefore, there are two potential interactions (Polymerase-UL44 and UL54 homodimerization) that are attractive targets for future antivirals, which can be easily disrupted by a single amino acid change in UL44[137,138].

2.9.1.3. Terminase complex

The viral terminase complex is involved in the cleavage of concatemeric viral DNA and the packaging of unit length linear genomes into preformed capsids. The viral DNA replicates by the amplification of circular templates (figure 12). This step is followed by a rolling circle-based mode of replication that produces concatemers of the genome in head-to-tail fashion; these further act as substrates for the DNA-packaging process[139]. The terminase complex recognizes packaging signal called pac motifs (cis-acting packaging signal) and cleaves concatemeric CMV DNA into unit-length genomes at adenine or thymine (AT)-rich core sequences within these pac motifs located in the TRS and IRS.

The CMV terminase is a heterotrimeric complex formed by UL56, UL89 and UL51 subunits, each carrying a specific function required for the packaging process[140]. These proteins make an initial

free-end specific cut at which packaging starts. The complex formed by the DNA and terminase binds to an empty preformed procapsid at its unique portal vertex, across which the DNA is translocated. A second site-specific cleavage step terminates packaging when a unit length genome has been translocated. DNA packaging is followed by cleavage and expulsion of the scaffold protein and angularisation of the capsid.

The CMV terminase complex is a transient component of the viral particle and does not remain after replication. The small subunit, UL89, seems to early dissociate after DNA cleavage, proved by the absence of UL89-UL56 heterodimers afterwards. It is probably recycled for further cleavage and packaging[141].

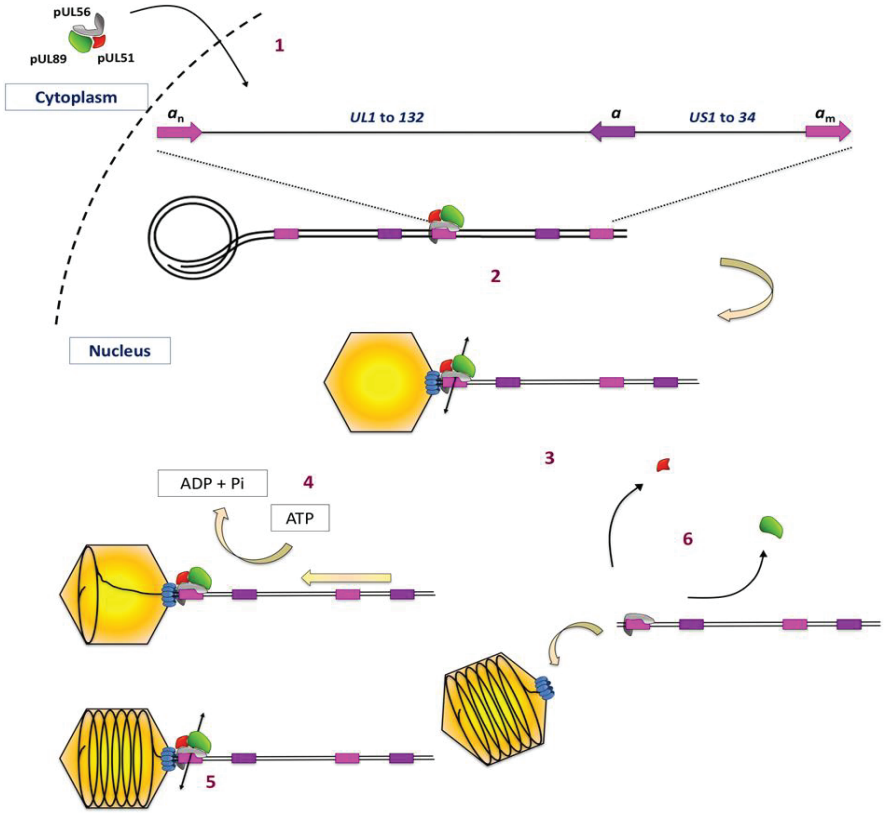

Figure 12. Process of genoma cleaveage and packaging by the CMV terminase complex. (1) Translocation of the terminase complex into the nucleus; (2) CMV terminase specifically binds the pac-site and recruits the empty capsid; (3) cleaves the duplex, (4) exerts its ATPase activity to power translocation of a unit-length DNA genome into the capsid; (5) completes the DNA-packaging process by cutting off excess DNA at the portal region; (6) the DNA-terminase complex dissociates from the filled capsid and is ready for next DNA-packaging step. From Bogner et al[142].

The UL56 is the main subunit of the complex. It is composed of twelve conserved regions (I–XII), as shown in figure 13[143, 146]. The conserved IV region represents the zinc-finger (ZF) domain, and

there are three putative leucine zippers (LZ) and two highly variable regions (VR). When expressed alone, it exists as a dimer formed by two ring-shaped structures connected to each other by a bridge to their base[144]. A short 128 bp sequence containing regulatory *cis* elements in conjunction with *pac* motives is sufficient to mediate efficient CMV genome maturation[145].

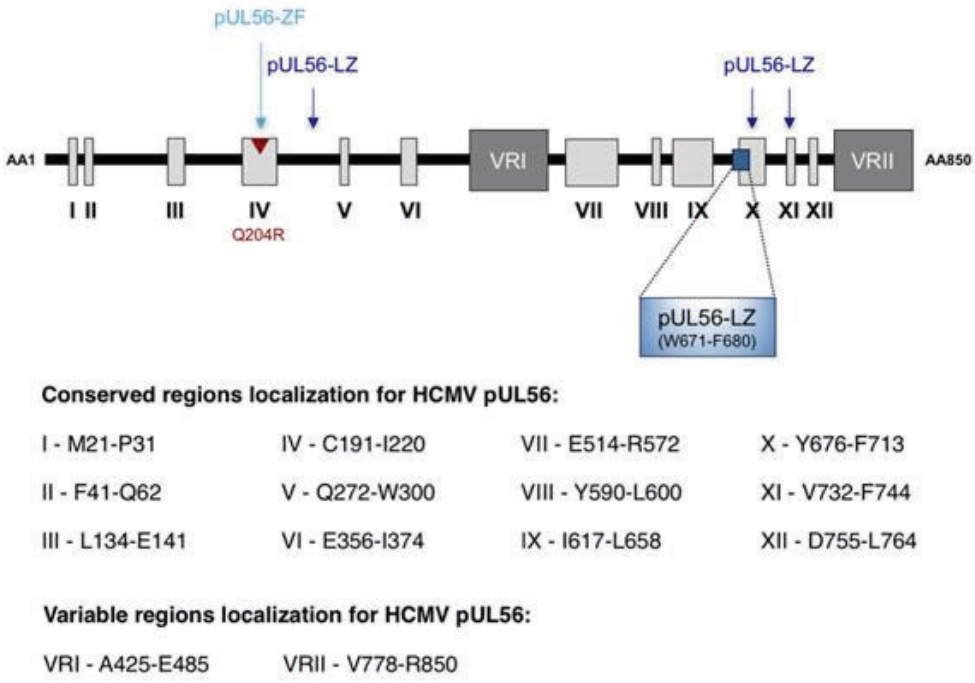

Figure 13. Map of UL56 gene that codifies the catalytic subunit of the terminase complex. Conserved regions I-XII are indicated in light grey, variable regions (VRI-VRII) in dark grey and leucine-zipper (LZ) and zinc-finger (ZF) domains are pointed in blue. The short sequence in the C-terminal region of pUL56 for interaction with pUL89 is indicated. From Ligat et al [146].

Initiation of the DNA-packaging process takes place in nuclear structures known as replication centres, where UL56 accumulates at the end of the infection together with UL112-113 and UL44. Nuclear importation of UL56 alone is mediated by an importin-dependent pathway through the interaction of hSRP1a with the nuclear localization signal (NLS) at the C-terminus of *UL56* (residues 816–827), of which R822 and K823 are essential residues that purpose[147]. Correct nuclear localisation of both UL89 and UL51 requires the concurrent presence and assembly of all three terminase subunits[148]. UL56 interacts with UL89 in the UL56 residues 671-680 and binds to the pac site on the concatemeric viral DNA working as an anchor for UL89[149,150]. Some studies indicate that UL56 has ATP independent endonuclease activity that seems to be *pac* specific[142]. Moreover, UL56 could enhance the endonuclease activity driven by UL89[141].

UL56 also interacts by its C-terminal region with the UL104 during DNA-packaging and forms the portal through which the viral genome enters the capsid[154]. The viral DNA-packaging process requires the terminase ATPase activity, which is only associated to UL56, but enhanced in a 30% when UL89 is bound[151]. The G714 and K715 of the putative ATP binding site (residues 709-715) are essential for ATP hydrolysis[152].

This step of the maturation process is important as it is inhibited by the 2,5,6-trichloro-1-beta-D-ribofuranosyl benzimidazole (TCRB) and the benzimidazole-D ribonucleosides (BDCRB)[153,154]. The *UL56* Q204R mutation is located within a putative ZF, and confers resistance to BDCRB and TCRB, implicating this region in the BDCRB mechanism of action. Although Q204R is located with the region associated to resistance to LMV (residues 180-395), it is sensitive to this drug[143,153].

UL89 is the terminase subunit compound of 674 amino acids conserved among herpesviruses. It has 12 conserved regions (I-XII) as well as its counterpart in other herpesviruses [155]. Conserved regions II, III and V are identified in bacteriophage T4 motifs: adenine binding site, Walker A (γ-phosphate sensor), Walker B (ATPase motor) and motif III (ATPase coupling helicase)[156].

The smallest component of the terminase is UL51, which is composed of 157 amino acids[140]. It has no equivalent in bacteriophages and is homologous to UL33 of herpes simplex virus-1 (HSV-1), only in the C-terminal part [140,157].

It was proposed that the stability of the complex is regulated by the mutual interactions of the three subunits, as well as the subcellular localization and its assembly[148]. Additional proteins, such as, UL52, UL77 and UL93, contribute to the DNA cleavage-packaging process during the CMV replication cycle, but their functions are under study[158,159].

2.9.2. Antiviral molecules

Several antiviral molecules have been approved by the FDA for the treatment of CMV infection. The first line of treatment is GCV, and its oral prodrug valganciclovir (VGCV), which are indicated for prophylaxis after immunosuppression as well. In the second line, there are foscarnet (FOS) and cidofovir (CDV). All four molecules inhibit the CMV DNA polymerase. In 2019, letermovir (LMV) was approved by the FDA for primary prophylaxis of CMV in seropositive HSCT recipients. It has a different pharmacological target, the UL56 terminase subunit. Finally, maribavir (MBV) inhibits the UL97 kinase and is in a phase-III clinical trial. The chemical structure of main CMV antiviral molecules is shown in figure 14.

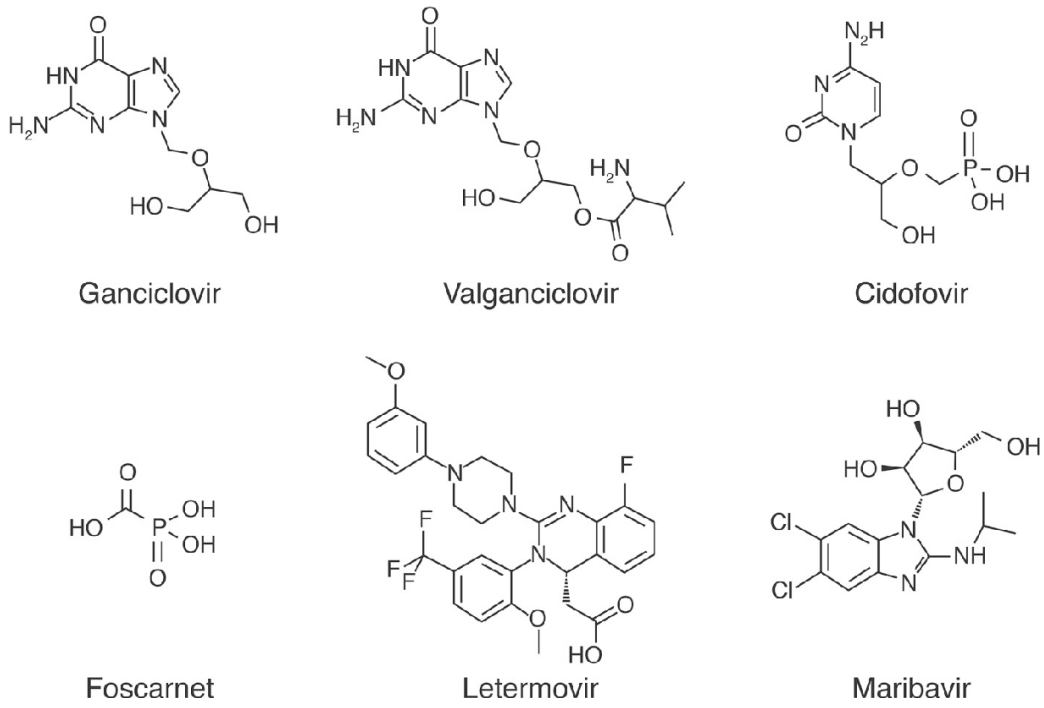

Figure 14. Chemical structures of CMV antiviral agents. From Piret et al [160].

2.9.2.1. DNA polymerase Inhibitors

The main DNA polymerase inhibitors currently in use and following cytomegalovirus guidance management are ganciclovir, valganciclovir, foscarnet and cidofovir. The mechanism of action of these molecules is shown in figure 15. UL97 kinase adds the initial phosphate to GCV. Cellular kinases add two additional phosphates. GCV triphosphate is the active form of the drug incorporated into viral DNA by the viral DNA polymerase. CDV is a monophosphate analog and does not require initial viral kinase activity. Cellular kinases add additional phosphates to produce CDV diphosphate, the triphosphorylated active form of the drug. FOS is a pyrophosphate analog, which does not require activation. The DNA polymerase is the ultimate target of all three drugs.

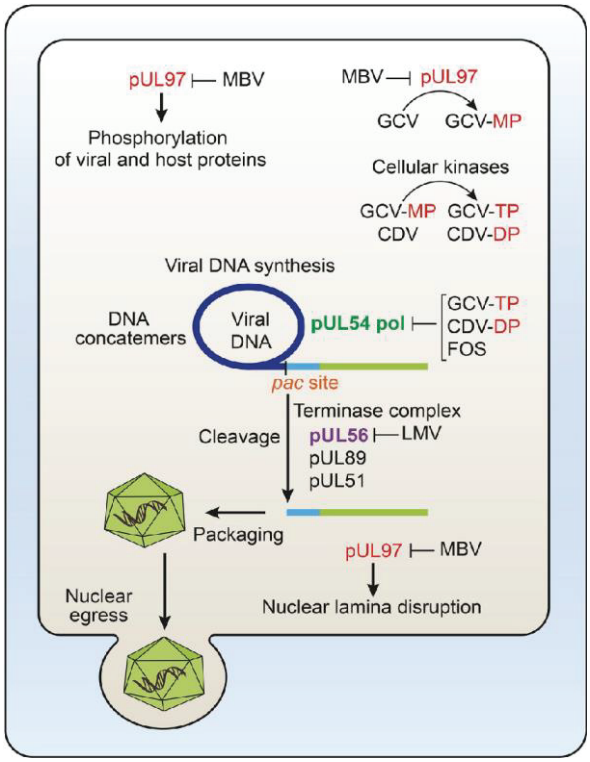

Figure 15. Antiviral agent mechanism of action against CMV infection. From Piret et al [157].

Ganciclovir / Valganciclovir (GCV/VGCV)

The first line of treatment for systemic CMV infections is GCV, a guanosine analog. The drug was initially FDA approved in 1989 for intravenous use. An oral capsule form was released in 1994, but it has poor bioavailability (5.6%) and has been largely superseded by the valyl ester prodrug, VGCV, which was approved by the FDA in 2001. VGCV has much better bioavailability (60%) and provides good tolerability and efficacy[161].

GCV is inactive when administered alone and requires UL97-mediated phosphorylation and two additional phosphate groups added by cellular enzymes for antiviral activity. GCV acts as a potent inhibitor of the UL54 by competing with deoxyguanosine triphosphate on the enzyme binding site. The triphosphate has a free hydroxyl group, which can continue chain elongation; however, incorporation of phosphorylated GCV by the DNA polymerase alters the DNA conformation, which slows and eventually stops chain elongation[160].

The effective concentration of GCV that reduces CPE induced by CMV in fibroblast cells by 50% (EC_{50}) is approximately 3.5 (± 2.3) µM[162]. GCV can be given orally, intravenously or as an ocular implant for the treatment of CMV retinitis. The main toxicity associated with the use of GCV consists of myelosuppression which is an important side effect especially for HSCT recipients.

On the other hand, the major side effect of VGCV is leukopenia, which occurred in 38% of high-risk kidney transplant patients after 200 days of prophylaxis, in 26% after 100 days of prophylaxis, and a similar rate of granulocyte-colony stimulating factor-I (GCSF-I) in both groups (13-14%)[163]. Guidelines recommend using appropriate doses of VGCV based on renal function, and not adjusting to lower doses because of leukopenia, which may produce resistant virus[164].

Several clinical trials demonstrated VGCV 900mg or GCV 5 mg/kg/12h intravenously for 21 days, followed by 900mg/24h VGCV for 28 days, had similar success rates of viremia eradication (21 vs 19 days)[165]. No differences in side effects and long-term outcomes between cohorts were noted. Given this success, international guidelines suggest that VGCV is the prophylaxis and treatment antiviral of choice, with a preference for intravenous therapy noted only for severe or mild disease.

Foscarnet (FOS)

FOS, phosphonoformate sodium, is a pyrophosphate analog which does not require intracellular activation. It binds and blocks the pyrophosphate binding site on the CMV DNA polymerase, thus, preventing incorporation of deoxynucleotide triphosphates into viral DNA[166], halting chain termination. It was approved by the FDA in 1991 for the treatment of CMV infection, as it presented a broad spectrum of antiviral activity against herpesviruses[167]. FOS is used mostly for resistant and refractory CMV disease, although it is sometimes used when SOT recipients have significant leukopenia and are not able to tolerate VGCV/GCV. The EC_{50} value of FOS against CMV ranges from 30 to 90μM and it is administered as large volume intravenous solutions[168].

The most frequent side effects of FOS are renal impairment and electrolyte abnormalities requiring adequate hydration of the patients and monitoring of serum creatinine levels. In a clinical study, virologic failure occurred in 13/39 (33%) cases after 10 years in a cohort of resistant/refractory CMV patients treated with FOS for 32 days on average, and 1% had relapses of viremia[169].

Cidofovir (CDV)

CDV is a non-cyclic cytidine monophosphate analog, which does not require initial phosphorylation by a viral kinase but is dependent on dephosphorylation by cellular kinases for activation[170]. Once in its diphosphate form, CDV inhibits the CMV DNA polymerase by acting as a chain terminator[171]. Two consecutive incorporations of CDV diphosphate are required to efficiently inhibit the incorporation of deoxycytidine triphosphate into viral DNA by viral DNA

polymerase, which disrupts further elongation and terminates DNA chain elongation. CDV has a broad spectrum of antiviral activity against most DNA viruses, and was FDA approved in 1996 for intravenous use for CMV treatment. The EC_{50} value of CDV against CMV ranges from 0.1 to 0.8µM. Nephrotoxicity is the main side effect of CDV, which requires the administration of probenecid and fluid to prevent kidney failure. A multicentre study of CDV for resistant/refractory CMV disease demonstrated high rates of nephrological issues (7/8 patients), with the majority clearing infection, although with a high rate of relapse, as well as significant morbidity and mortality[174]. CDV is also associated with ophthalmologic toxicity, that includes acute iritis and ocular hypotony. Some clinicians use it every 2 weeks for prophylaxis of resistant virus, based on data in the HIV literature[173]

Brincidofovir was developed as an orally bioavailable lipid ester prodrug of CDV and has permitted to avoid the dose-limiting renal toxicity of CDV and led to a safer alternative for the treatment of drug-resistant CMV infections in immunocompromised patients[172].

CDV is rarely used in SOT recipients, except for resistant and refractory CMV disease. However, some of the mutations that convey resistance to GCV, mainly in *UL54*, also convey cross-resistance to CDV.

2.9.2.2. New antiviral therapies

Since DNA polymerase inhibitors are associated with toxicities and the emergence of resistance, efforts were concentrated in the development of new antiviral molecules that target different steps of the viral replicative cycle, such as the terminase complex or the UL97 phosphokinase, to overcome those limitations. Additionally, immunotherapies based on stimulated T-cells or specific CMV-antibodies infusion were considered for the treatment of CMV infection or prophylaxis.

Letermovir (LMV)

LMV was FDA approved for CMV prophylaxis in adults seropositive HSCT recipients in 2019[157]. LMV inhibits the cleavage of viral DNA concatemers and the formation of mature CMV virions by targeting the UL56 subunit of the terminase complex[175]. The antiviral activity of LMV is highly specific to human CMV strains, since it has no significant antiviral activity against other herpesviruses[177]. LMV presents high selectivity and effectivity at low concentrations[176,177], showing a 100-fold more potency than GCV against CMV and effectivity against viral isolates resistant to DNA polymerase inhibitors. Combinations of LMV with GCV and CDV resulted in additive effects, and with FOS resulted in additive/minor antagonistic effects *in vitro*[78].

Therefore, the combinations of LMV with other antiviral agents could be an interesting strategy for the treatment of CMV infections.

LMV is safe, well tolerated and has a good oral bioavailability (35%) and it can be administered orally and intravenously. The intravenous hydroxypropyl β-cyclodextrin formulation of LMV is mainly used immediately after transplantation and in patients presenting gastrointestinal (GI) problems that complicate the ingestion and absorption of oral drugs[179].

LMV can alter biliary excretion of drugs, affecting plasma levels of LMV in patients with renal impairment. LMV shows a weak to moderate inhibitory effect on cytochrome P450 (CYP) activity and was demonstrated to increase exposure to tacrolimus and cyclosporine A, which are usually administered in immunocompromised patients[180]. The most common side effects associated to LMV are nausea, diarrhea, vomiting, peripheral edema, cough, headache, fatigue and abdominal pain.

LMV is not indicated for the treatment of CMV disease as it has shown a low barrier to resistance in vitro and in vivo. It will probably remain administered for primary prophylaxis, but its use might be extended to SOT recipients as there is an ongoing study of LMV prophylaxis in kidney transplant recipients (NCT03443869).

Maribavir (MBV)

MBV is a benzimidazole L-riboside, a competitive inhibitor of the ATP-binding to the UL97 kinase that blocks its kinase activity[181]. The use of MBV leads to defects in the viral replication cycle that correspond to the *UL97*-defective phenotype and interferes with the morphogenesis and nuclear particles in infected cells[182]. However, neither the deletion of *UL97* nor treatment with MBV completely prevents CMV replication. MBV is inhibitory for CMV and EBV but no other human herpesviruses *in vitro*[183].

In contrast to GCV, MBV does not require intracellular phosphorylation to be active[114]. MBV inhibits UL97 kinase activity which is essential to activate GCV, therefore, an antagonist effect is expected if both antivirals are co-administered[187]. Combination therapies of MBV with CDV, FOS and LMV presented synergistic effects against wild-type and drug-resistant CMV strains, and a similar effect was also found in the combination of MBV with rapamycin[188,189]. These data suggest that the benefit of administering MBV with other agents should be further investigated.

The EC_{50} value of MBV is 0.3µM, and is 10-fold more potent than GCV in cell culture. MBV is effective against resistant and refractory CMV disease, across multiple different doses of MBV (400, 800, and 1200mg twice daily)[184]. MBV demonstrated low toxicity against hematopoietic progenitors and different human leukaemia cell lines[181,185]. This lack of toxicity could allow an initiation of drug prophylaxis even before engraftment in HSCT recipients. Nevertheless, in a placebo-controlled phase 3 trial in HSCT, MBV prophylaxis did not prevent CMV disease when started after engraftment[186]. The most common side effect of MBV is dysgeusia.

2.9.3. Immunotherapy strategies for CMV infection.

Intravenous CMV immunoglobulin enriched for higher titter CMV positive donors has shown good results for prophylaxis in thoracic SOT, especially in lung transplant, but limited in the rest of SOT, and for resistant/refractory CMV disease[164]. In this setting, adoptive CMV-specific T cells have been used in a very limited fashion in SOT recipients. Symptomatic improvement, complete resolution or reduction of CMV-associated end-organ disease and DNAemia, the cease of antiviral drugs, has been demonstrated in a 11/13 (84%) SOT recipients given *in vitro* expanded autologous CMV-specific T cells in a phase I clinical trial[190]. Four of these patients displayed reconstitution of CMV-specific T cells in peripheral blood after completion of T-cell therapy and no significant side effects or negative impact on the graft was noted. CMV-specific T cells generated from healthy CMV seropositive subjects in HSCT recipients has the largest experience (n>100)[190]. However, further studies about the efficacy after SOT are needed to integrate into clinical practice autologous or allogeneic adoptive CMV-specific cellular therapy. Moreover, complicated administration, cost, lack of HLA matching (with allogeneic cells) and other factors limit its inclusion.

2.9.4. Clinical use of antiviral drugs

2.9.4.1. Prevention

There are two main strategies to prevent CMV infection: (I) antiviral prophylaxis for three to six months after transplant in risk cohorts, (II) or preemptive therapy that consists of CMV load weekly monitoring for the first twelve to sixteen weeks after transplant with a plan to start antiviral treatment once a certain threshold has been obtained[164]. Although universal prophylaxis for higher risk recipients is more preferred, most experts agree that either approach is appropriate for seropositive recipients. Benefits and contras of both strategies are compared in table 1. The most common prophylaxis agent used after SOT is VGCV, whereas GCV is hardly ever used due to its intravenous administration. Currently, LMV is being implemented in the hospital practice as prophylaxis after HSCT in seropositive recipients. It is important to consider

the cellular immune response by immunodiagnostics for CMV, as it seems very useful in predicting the risk of CMV after transplant in seropositive recipients[191,192].

Table 1. Comparison of preventive strategies for cytomegalovirus infection. From Kotton et al 2018 [164].

	Prophylaxis	**Preemptive therapy**
Early CMV DNAemia / infection	Rare	Common
Prevention of CMV disease	Good efficacy	Good efficacy
Late CMV infection/disease	Common	Rare
Resistance	Uncommon	Uncommon (weekly testing)
Other opportunistic infections	May prevent	Unknown
Safety	Drug side effects	Less drug toxicity
Prevention of rejection	May prevent	Unknown
Graft survival	May improve	May improve

2.9.4.2. Treatment of CMV infection

Treatment of CMV infection is usually with VGCV, although intravenous GCV is recommended for severe disease [164]. In the second line of treatment, FOS and CDV could be administered adjusting the dosage to its kidney function and pancytopenia. An increase antiviral efficacy and decrease development of resistance can be achieved by combination therapy, although it is not yet included in the clinical practice. Useful therapeutic combination profiles were reported *in vitro* for MBV with FOS, CDV, LMV and rapamycin, but not with GCV [188, 189]. Thus, both synergy and antagonism need to be considered cautiously before its clinical use. Additional data is needed, with various other combinations and *in vivo* experiments.

During treatment, CMV load testing should be monitored until negative or very low on one to two tests. After CMV DNA clearance, secondary prophylaxis has not been shown to be effective and is only recommended for high-risk situations. Therefore, CMV load monitoring is recommended weekly with a plan to restart therapy once it reaches a significant level. This level has not been standardized across sample types and assays, although approximately 1500 IU/ml for seronegative recipients and 2000–2500 IU/ml for seropositive recipients may be reasonable thresholds at which to consider restarting therapy. Reduction of immunosuppression should be considered on an individual basis.

2.9.4.3. Management of resistant/refractory disease

Twenty percent of transplant recipients develop CMV infection that is refractory or resistant to anti-CMV treatment[193].These concepts have been defined to guide clinicians on when to suspect resistance, the optimal laboratory testing to diagnose resistance, and how to interpret the results of the assays employed.

- Refractory CMV infection: clinical definition based on criteria for suboptimal response to therapy. It is defined as increased or maintained viral loads after at least 2 weeks of appropriately dosed antiviral therapy.
- CMV antiviral drug resistance: a laboratory definition of a drug-resistant phenotype or the presence of viral genetic alteration that decreases susceptibility to one or more antiviral drugs.

Clinical suspicion of drug-resistant CMV infections is usually based on suboptimal responses to antiviral agents, but confirming virological drug-resistance can only be done by laboratory testing. Treatment failure may also result from other causes, such as adverse host factors or inadequate drug delivery.

Recognizing the risk factors for the development of drug-resistance CMV infection may prompt early diagnosis and management of antiviral resistance. There, we can differentiate between host and virus factors (table 2).

Table 2. Risk factors for cytomegalovirus resistance. Data from Fisher et al[17].

Host factors	**Viral factors**
Prolonged antiviral exposure (>3 months)	CMV viral load rise after >2 weeks of adequate dosing
Previous antiviral exposure	Non CMV load decrease despite appropriate treatment
Recurrent CMV infection	Intermittent low-level CMV viremia
Inadequate antiviral absorption/bioavailability	High CMV viral loads
Inadequate antiviral oral prodrug conversion	
Variation in antiviral clearance	
Subtherapeutic antiviral drug level	
Poor patient compliance with antiviral drug regimen	
T-cell depletion	
Haploidentical /allogenic/cord blood HSCT	
Delayed immune reconstitution	
D^-R^+ serology (HSCT) / D^+R^- serology (SOT)	
Treatment with anti-thymocyte antibodies	
Active GVHD	
Young age	
Congenital immunodeficiency syndromes	

Drug-resistant CMV infections are associated with high morbidity and mortality[161,169,174,194–196]. Resistance mutations may eventually be selected for, if the antiviral treatment allows for ongoing replication either because of inadequate dosage, dose-limiting toxicities, poor absorption, poor penetration into the body compartment where replication is occurring, or overwhelming replication.

CMV drug resistance and symptomatic disease occur in 5-10% of transplant recipients[200], mostly after prolonged drug exposure with incomplete viral suppression, manifesting as increasing viral loads or disease despite therapy[197–199]. The highest-risk group for drug-resistant CMV infection in the SOT setting is donor seropositive receptor seronegative (D^+R^-) combination resulting in a post-transplant primary CMV infection exacerbated by immunosuppression and associated to a 90% of GCV resistance[163,169,199].

Other risk factors for resistant CMV infection include the type of organ transplanted (i.e., lung transplant recipients are at the highest risk[199]), peak CMV viral loads, the intensity of immunosuppression[161,186,201,202] and lower doses or longer duration of VGCV/GCV prophylaxis. Although preemptive treatment might be expected to reduce the incidence of resistance, some studies actually showed a higher incidence of resistance in those on preemptive approaches than those given prophylaxis[169,174]. Prolonged prophylaxis did not appear to increase the incidence of genotypic antiviral resistance significantly[186,201], because of effective suppression of viral replication in most cases.

Various genetic immune factors in both donors and recipients likely contribute to an inability to control CMV, and higher rates of resistance. New data shows that lung transplant recipients with short telomeres and idiopathic pulmonary fibrosis had impaired CMV immunity and more than double the rate of relapsing CMV viremia and higher rates of resistance compared with those with normal telomeres[203].

HSCT recipients suffered many CMV reactivation (29% approximately) due to the T-cell depletion caused by immunosuppressive therapy[204]. Allogenic HSCT recipients with refractory CMV reactivation present a higher incidence of CMV disease and non-relapse mortality than those without refractory CMV. In SOT recipients, several studies on the outcomes of GCV-resistant CMV infections showed an association with longer hospitalization, serious toxicities from alternative therapy, and increased mortality [169,174,190,196,202,205].

Management of resistant/refractory CMV infection involves use of high-dose GCV, rather than VGCV because of preference for intravenous antiviral therapy in this context, or use of FOS,

when life or sight threatening disease occurs[164,206]. Resistance testing data of pharmacologic target genes is crucial to guide further treatment decisions. Additional considerations, as reduction of immunosuppression, switching to mTOR inhibitors, use of CMV immunoglobulin, leflunomide, artesunate or adoptive T-cell therapy may be useful in this setting.

2.9.5. Antiviral resistance in cytomegalovirus

The characterization of CMV antiviral drug resistance mutations has contributed to the advancement of antiviral therapy and to knowledge of the basic biological functions of the viral genes that serve as antiviral drug targets. Viral *UL97* kinase and *UL54* DNA polymerase gene mutations are well-documented mechanisms of resistance to conventional antiviral therapy[62]. Over the 90% of drug-resistant CMV clinical isolates selected from initial treatment with GCV contain one or more mutations in the *UL97* gene, whereas mutations in the *UL54* gene are less frequently encountered[62,160,207]. Clinical use of new antiviral molecules, such as LMV and MBV, has led to the development of resistant mutations found in clinical isolates when used as prophylaxis or salvage therapy after refractory or resistant CMV infection[160].

Table 3. Genetic loci in cytomegalovirus associated with antiviral resistance to various agents. Data from Kotton et al[193].

CMV gene	Associated antiviral resistance	Mechanism of action
UL97	VGCV, GCV, MBV	Kinase
UL54	VGCV, GCV, FOS, CDV, BCDV	Polymerase
UL56 (rarely UL51, UL89)	LMV	Terminase inhibitor
UL27	MBV (low level)	DNA polymerase inhibitor

It is estimated that 5-12% of patients infected with CMV develop resistance mutations, creating the need for antiviral resistance testing. Currently, this is usually based on the detection of mutations in target genes by sequencing and alignment with wild-type viral strains. Accurate interpretation of these genotypic assays depends on an understanding of the levels of resistance and cross-resistance and recognition of sequence variants unrelated to drug resistance which can only be elucidated by phenotypic methods.

2.9.5.1. Antiviral resistance diagnosis by genotypic methods

Genotypic antiviral resistance testing is indicated when there is a rising or maintained viral load while on complete dosage of antiviral therapy during at least fourteen days[164]. Reliable testing requires clinical samples with sufficient viral loads, efficient nucleic acid extraction, enrichment and/or amplification, and appropriate sequencing of the gene regions associated to resistance.

The standard approach consists of PCR amplification of the antiviral target genes where prevalent resistance mutations have been described, followed by fluorescent dideoxy (Sanger) sequencing involving capillary electrophoresis and an automated base calling system for processing the resulting chromatogram files[208]. This method of sequencing is scalable and well suited to the volume of requests for resistance testing in diagnostic laboratories. This technology is widely available and standardized with a short turnaround time. Sanger sequencing presents a limited sensitivity to detect mutant subpopulations under 20% abundance.

There have been many platforms of NGS designed for genotypic antiviral resistance testing, such as pyrosequencing, Illumina and Ion Torrent. Pyrosequencing has been reported to detect mutant subpopulations at an approximately 6% level[210], but the short sequence reads do not let analyse the full range of codons needed for genotypic diagnosis. NGS permits analysis of the whole spectrum of genetic diversity and can identify small subpopulations of roughly 1% of variant sequences in clinical sample[72]. NGS allows the detection of 15% of mutations that are undetectable by Sanger sequencing[209]. One of the benefits of NGS is the earlier indication of impending futility of current therapy, to avoid adverse effects from ineffective treatments, considering better alternatives are available. Nevertheless, its widespread adoption is limited by the development of technical platforms each requiring re-calibration and optimized sample preparation, refinement of computationally intensive algorithms, and sequencing capacity often excessive for the needs of one or a few clinical samples. NGS is compelling in the increased resolution and sensitivity of detection of emerging resistant mutations, for both the study of resistance *in vitro* and for serial sampling during case reports and clinical trials. Its capacity can be used to great advantage for large batches of specimens once it has been optimized. Clinical research can eventually establish the therapeutic impact of detecting resistance mutations earlier and at lower abundance than traditionally.

Simpler methods, as restriction enzyme digestion, are available to detect specific mutations at around 5% abundance[211,212], but specific mutations targeted for analysis limit their usefulness. Therefore, mutation-specific primers and probes and restriction systems still need refinements[210].

Quality control initiatives for genotypic antiviral resistance testing are being developed in order to face their intrinsic limitations. Genotype reports of unusual and mixed sequence populations could appear due to technical artifact from the PCR or the sequencing techniques used, especially when the viral loads are low. Such limitations can be overcome by re-sequencing of additional specimens from the same subject.

Therefore, Sanger sequencing remains the most accessible and scalable solution for routine genotypic diagnosis until issues of accuracy, standardization, practicality, and timely availability of NGS are resolved.

In the future, expanding the scope of standard diagnostic genotyping can provide better insight into the frequency of genetically induced cases of drug resistance with poor response to antiviral treatment and the incidence of each resistance mutation.

Quality control of genotypic sequencing

Quality control is essential to avoid false negative and false positive mutations readouts. Genotypic resistance assays can be falsely negative when resistance mutations are missed due to inadequate coverage of viral genetic loci, insensitive detection, or failure to call out significant mutations in the sequencing data. The risk of false positives has been highlighted during resistance monitoring because of PCR-generated errors, sample misidentification, contaminants, or misinterpretation of sequencing data. False positives provides an erroneous detection of drug resistance, leading to incorrect switching of therapy, or the creation of poorly authenticated entries distracting from the informed interpretation of mutation maps.

Sequencing artefacts favourably appear in low viral copy number clinical samples, leading to an unclear interpretation of mixed peaks on Sanger sequence chromatograms. PCR amplification artefacts can be solved by the use of capture probes for sample pre-processing, but appears useful only for samples with a higher viral load [82,213]. NGS studies, which provides more accurate interpretation of minor subpopulations, require careful replicate testing of controls and samples using the specific technical platform and sample processing parameters.

Distinguishing polymorphisms from resistance mutations

Sequence readouts from genotypic antiviral resistance assays are aligned to a reference virus genome to determine amino acid substitutions, which are checked using databases of sequence polymorphisms and previously characterized resistance mutations. Lists of mutations are summarized periodically, and new mutations continue to be described and published[214]. This information allowed to stablish separated locations in the gene map for antiviral resistance mutations and naturally occurring polymorphisms.

Polymorphisms are not common in the codon ranges listed as hot spots for drug resistance, however, there are many exceptions of polymorphisms confirmed to confer no drug resistance in those hot spots. On the other hand, drug resistance mutations are not expected as dominant sequence variants in specimens without a history of exposure to an associated antiviral drug,

but considerable baseline interstrain sequence variation is known to exist, for instance, in herpesvirus polymerases[62].

Many uncharacterized amino acid sequence variants are found in specimens collected after antiviral drug exposure in clinical practice and during clinical trials, after filtering out sequence variation in baseline isolates. This fact creates uncertainty as to their impact on drug resistance, which can only be elucidated by phenotypic testing[215–218].

2.9.5.2. Phenotypic testing

The significance of unknown mutations can only be determined by phenotypic studies. Conventional phenotypic studies involve the isolation of viruses in cell culture and titration for growth and antiviral susceptibility assays. The long-time consuming, the laboratory infrastructure requirement, the specialisation in cell culture and molecular biology, the inability to study individual mutations and the difficulties to measure growth objectively has made this practice no longer feasible.

In the past years, bacmid technology were developed to generate recombinant viruses with the desired mutation integrated by PCR mutagenesis or homologous recombination (figure 16). The mutation is inserted into the specific position of the viral genome of a baseline laboratory strain cloned into a bacterial artificial chromosome (BAC) and maintained in bacteria [218,219]. The recombinant mutant virus is then reconstituted in cultured cells following transfection of the BAC clone (figure 17).

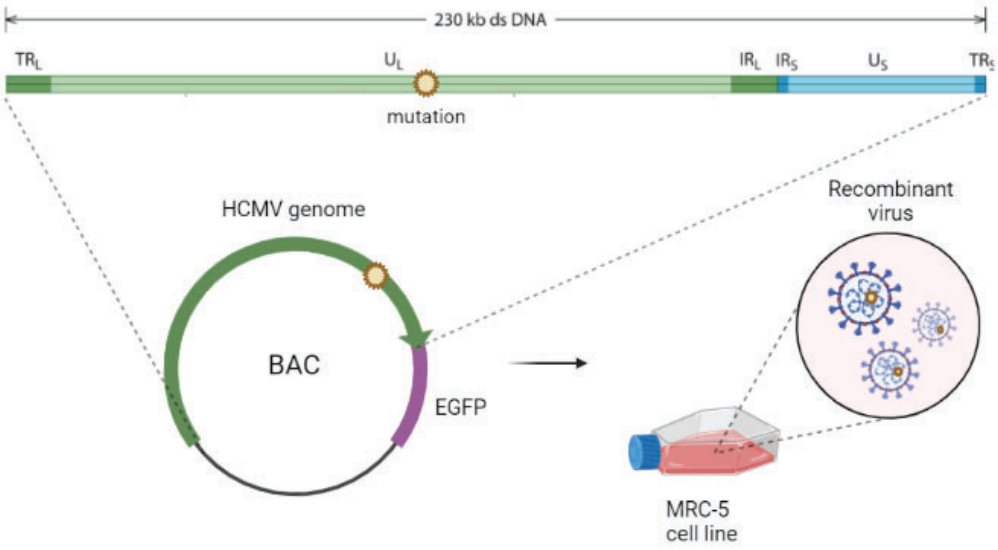

Figure 16. Recombinant virus production by bacmid technology. Original figure.

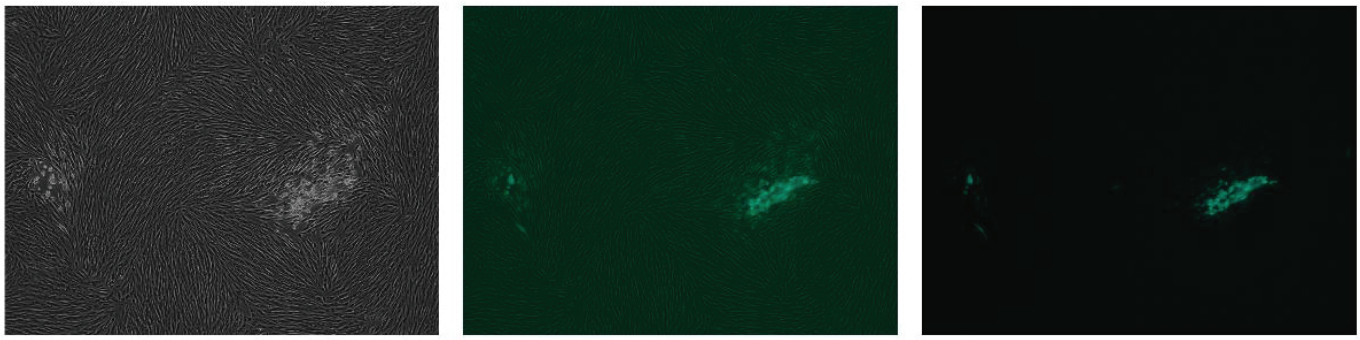

Figure 17. Recombinant CMV generation. The recombinant BAC was transfected into MRC-5 cell culture. Enhanced Green Fluorescence Protein (EGFP) – reporter gene contained in the BAC vector allowed the visualization of recombinant CMV under the fluorescent microscope Nikon Eclipse TS. The cytopathic effect caused by CMV is shown in different fluorescence intensities (0% left to 100% right). Original figure.

Focus reduction assay is used to assess susceptibility of the mutant to serial antiviral concentrations. Viral plaque formed units (PFU) are counted, thanks to a fluorescence reporter gene or bioluminescence (figure 18), to calculate the concentration that inhibits 50% of the viral growth (IC_{50}) of the mutant. The ratio of IC_{50} values of the recombinant mutant virus over the wild-type counterpart is calculated to evaluate the level of drug resistance. Based on recombinant phenotyping, mutations associated with 3-5 fold increase in IC_{50} values over the wild-type strain are considered as low grade drug-resistant, whereas five to ten fold increase in IC_{50} values are considered moderate levels of resistance[164].

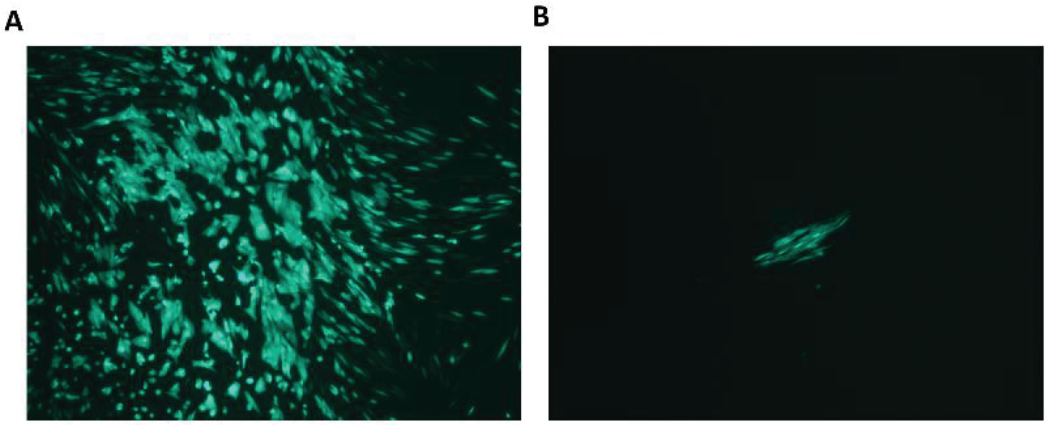

Figure 18. Recombinant CMV with the *UL54* A928T mutation. The mutant CMV strain was generation through the transfection of the A928T-BAC into MRC-5 cell culture. Enhanced Green Fluorescence Protein (EGFP) – reporter gene contained in the BAC vector allowed the visualization of recombinant CMV under the fluorescent microscope Nikon Eclipse TS. (A) Viral

growth of CMV A928T after three passages. (B) Identification of one viral plaque formed unit (PFU) of CMV A928T. Original figure.

Growth assays estimate the impact of the mutation on viral fitness, by counting for one to seven days post-inoculation the recombinant strain and the wild type viruses PFUs thanks to the reporter gene (figure 18).

Mapping resistance-associated mutations and changes in viral kinetics are key to guide physicians in the pharmacology management of the patients and for guiding the designing of alternative inhibitor antivirals to avoid cross-resistance. The presence of small subpopulations with resistance mutations allows antiviral doses to be increased in order to control replication of the virus[220], as well as when a mutation with higher replicative capacity is detected.

The antiviral susceptibility assay is not suitable for routine diagnostic laboratories for technical reasons, as the unavailability of culture isolates in the current molecular diagnostic era. However, the benefits of genotypic testing to detect viral resistance mutations provide timely data to facilitate clinical decisions. It also required consideration of the genetic criteria for diagnosis of resistance, phenotypic correlates of detected mutations, and optimal methodology and therapeutic implications.

2.9.5.3. Cytomegalovirus mutations associated to resistance

Mutations associated to resistance to standard therapy

The UL97 kinase effects the initial phosphorylation of GCV that is required for its antiviral activity. Mutations in the *UL97* that impairs its function are the preferred mechanism of GCV resistance and cross-resistance to any drug that depends on UL97 phosphorylation, such as ACV, or uses UL97 as a viral target, like MBV does. This includes any mutation at the critical lysine K355 or mutations that truncate the expressed UL97 (figure 19). Studies using site-directed mutagenesis of *UL97* functional domains demonstrated UL97 kinase function was impaired by mutations G340V, A442V, L446R, F523C, as loss of autophosphorylation and GCV phosphorylation was found[222]. The severe growth deficiency caused by the loss of normal UL97 function associated to these mutations it not expected to occur in clinical isolates[223].

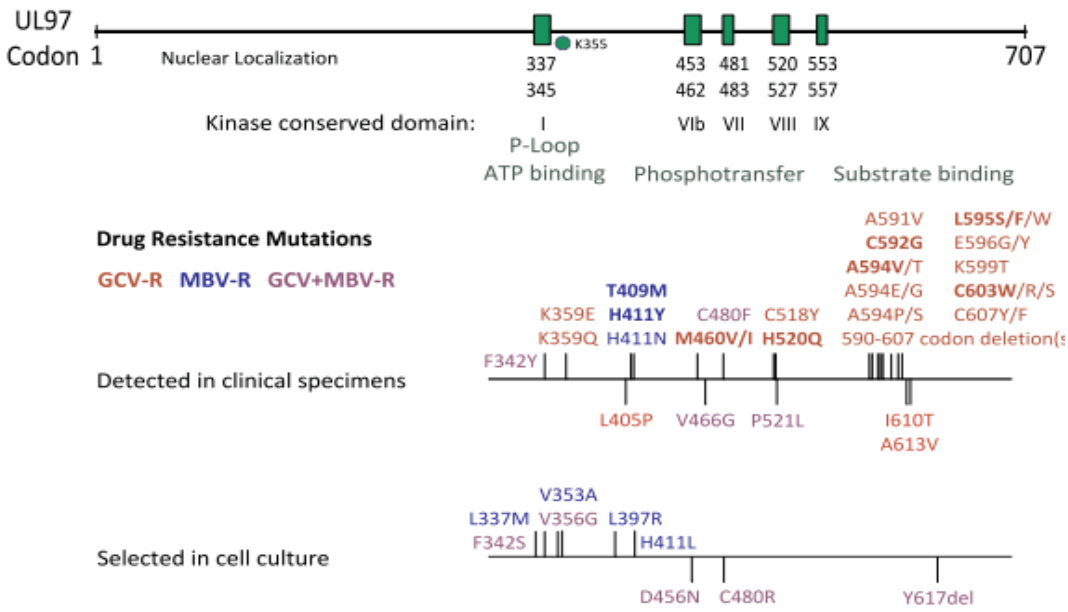

Figure 19. Resistance mutations located in the *UL97* gene map. Green boxes showed conserved kinase domains. Resistance mutations are indicated in colours according to the antiviral they are resistant to. The most frequent mutations are indicated in bold. Mutations underneath the line are less significant due to minor subpopulations, combination of detection in single specimens, severe growth impairment or borderline resistance phenotype. From Lurain et al [62].

Clinical data have established the preferred locus of antiviral resistance mutations at *UL97* codons 405, 460, 466 and 590–607[62,218,224,225]. Mutations in this conserved kinase domain impair the recognition of GCV as a substrate while preserving the UL97 normal biological functions. GCV-resistance mutations in clinical isolates do not substantially impair virus replication in cell culture[226], but may have some subtle loss of viral growth as supported by *in vivo* viral dynamics studies[227].

Seven canonical *UL97* mutations (M460V/I, H520Q, C592G, A594V, L595S and C603W) are found in over 80% of GCV-resistant CMV strains, and do not cause cross-resistance to FOS or CDV[62] (figure 19). Hardly any noncanonical GCV resistance mutations in clinical specimens appear outside the previously defined range that has been reported[218,225].

Since the UL97 kinase also phosphorylates ACV[127], a concern is that prior treatment with ACV elicits GCV resistance *in vivo*. A phenotypic study in patients receiving high-dose ACV did not resulted in GCV resistance[84]. On the other hand, one of the first three reported cases of GCV resistance had evidence of pre-existing resistance before receiving any GCV but after receiving ACV[228].

UL54 DNA polymerase is the pharmacologic target of conventional antiviral therapy (GCV, VGCV, CDV, FOS). Resistance mutations emerge after prolonged GCV therapy and increase the level of resistance conferred by a previous *UL97* mutations[62,229]. The appearance of a *UL54* resistance mutation in the absence of a *UL97* mutation, after initial therapy with GCV, is uncommon but has been reported[230,231]. GCV-resistant CMV clinical isolates with an altered *UL54* DNA polymerase activity result from numerous amino acid changes widely distributed among the different conserved domains of the enzyme, but mostly occur at codons 395–545 and 809–987 (figure 20).

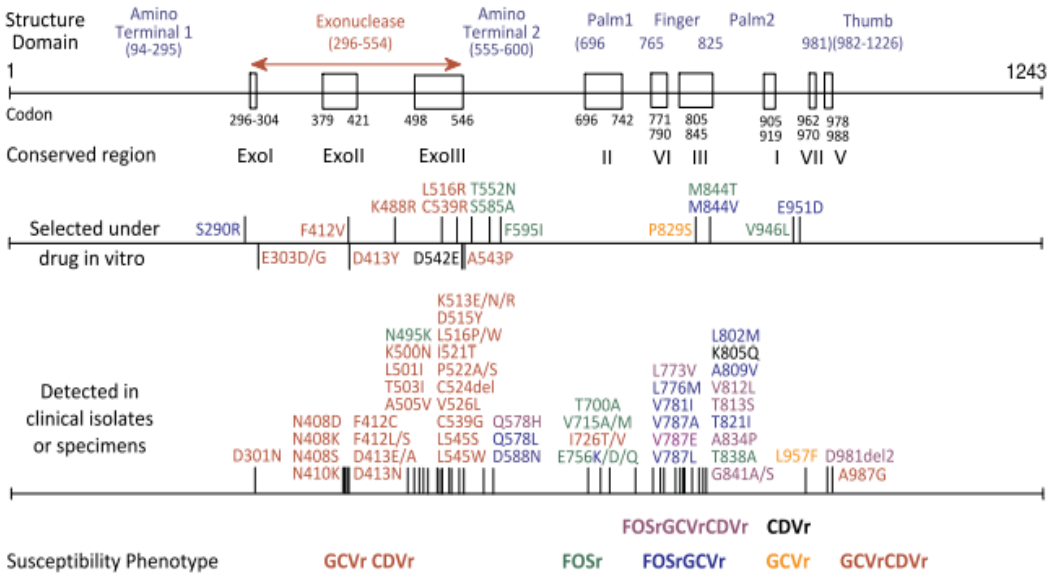

Figure 20. Resistance mutations mapped in the *UL54* gene. Conserved domains of *UL54* are indicated with white boxes. Resistant mutations are indicated with colours according to the antiviral they are resistant to. From Chou et al [238].

Mutations in *UL54* gene that emerge under GCV therapy can confer cross-resistance to CDV and, less frequently, to FOS. Cross-resistance to GCV and CDV is associated with amino acid substitutions located in the exonuclease domains (codons 301, 408–413 and 501–545) and in region V (codons 981–987) of the enzyme (figure 20)[160]. Clusters of amino acid changes found in conserved regions II, VI and III are associated with resistance to FOS alone or to both FOS and GCV. The FOS-resistant mutations V946L and F595I were selected under GCV, and the GCV resistant mutations N408D, K500N and L957F were selected under FOS, yet none of these mutations individually conferred GCV-FOS cross-resistance. Certain combinations, such as N408D+L957F and L545S+P829S showed FOS resistance whereas the single mutations did not. Mutations outside of areas that are directly involved in catalytic activity may result in structural

changes that lead to drug resistance[232]. For instance, A834P and del981-2 confer moderate resistance to all three antivirals (GCV, FOS, CDV). Unlike the situation with *UL97*, there is not a short list of mutations promoting an extensive sequencing that covers the full-length of the gene. Intertrain sequence polymorphisms are relatively common in this gene, which makes it more complicated to distinguish them from true resistance mutations [62,233]. However, the majority of resistance mutations occur within the conserved regions of homology, and many of these mutations are of amino acid residues that are highly conserved among enzymes from herpesviruses[129], as well as enzymes from a wide spectrum of unrelated organisms, such as yeasts, bacteria, and humans[128].

In contrast to *UL97* mutants, recombinant viruses with *UL54* amino acid substitutions conferring drug resistance usually exhibit an attenuated or slow growth phenotype in cell culture compared to their wild-type counterpart[234–236]. This may be a factor in *UL54* mutations not being a preferred genetic pathway of drug resistance. *UL54* G698D and Y818C, yielded either no or poorly viable virus from the corresponding recombinant BACs[219]. These conserved residues probably have a critical function based on the homologous HSV polymerase structure[237], leaving open the question of whether such non-viable mutations represent technical artifacts of genotyping assays or recombinant phenotyping, or a previously unrecognized pathway of drug resistance.

Combinations of mutations in *UL97* and *UL54* genes multiply the effect of each individual mutation and can result in increased GCV IC_{50} values by more than 15-fold and are considered as conferring a high-level of drug resistance[235,239]. Due to the limitations associated with the use of currently approved antiviral agents, the development of new inhibitors of CMV infection that act through different mechanisms of action was thought to be a good solution. However, resistance associated mutations have also been described *in vitro* and in clinical isolates after their administration.

Mutations associated to resistance to new antiviral therapies

LMV resistance mutations map primarily to *UL56* and more rarely to *UL89* and *UL51*, genes encoding the three subunits of the CMV terminase complex. *UL56* exhibits a low natural polymorphism rate among susceptible and drug resistant clinical CMV isolates (intertrain identity >97.7% at both nucleotide and amino acid levels)[240]. LMV-resistant mutations are located at codon 25 and 229-369 of the *UL56* (figure 21)[175,241]. The C325F/Y/R/W mutation is quite prevalent in the clinical practice and confers the highest resistance level (<3000-fold increase) ever described for LMV resistance[242,243]. Mutations in *UL56* conferring resistance to

LMV have minimal to low impact on viral growth fitness compared to their wild-type counterparts. Combinations of two or three mutations in the *UL56* gene result in markedly increased levels of resistance to LMV [242–244]. It was demonstrated that LMV resistance mutations selected *in vitro* appeared much earlier than for currently used antiviral drugs[242], suggesting a lower genetic barrier of resistance to LMV. Two studies before LMV era did not retrieve any resistance mutations in blood of immunocompromised patients naïve of antivirals[143,155] or treated with GCV[245]. Only one study evidenced *de novo* LMV resistance mutation C325Y in two formalin-embedded tissue biopsies from 1/147 patients[246]. Controversially, pre-existing mutations has not been yet found in plasma from transplanted patients, which are the main recipient of LMV.

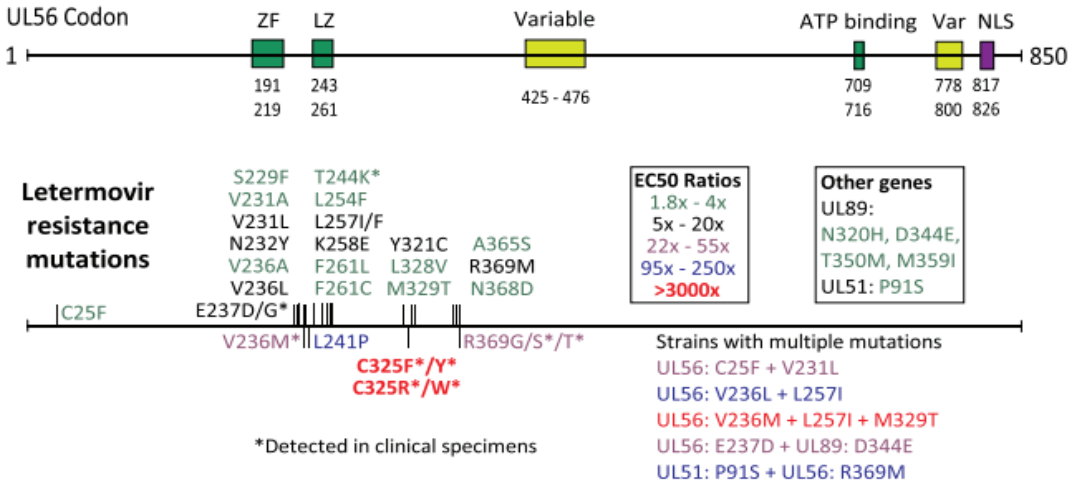

Figure 21. Mutations associated to LMV-resistance mapped in the *UL56* gene. Green boxes indicated the functional domains Zinc Finger (ZF), Leucine Zipper (LZ), ATP- binding site. Yellow boxes showed hyper variable regions. Purple box indicates the Nuclear Localization Signal (NLS). The level of resistance associated to each mutations is indicated with the colour legend. Mutations in *UL89* and *UL51* conferring LMV-resistance are pointed. * Indicates mutations detected in clinical samples. From Chou et al[238].

Resistance mutations to LMV have been described in the clinical practice after LMV administration during phase I-III clinical trials or when used as salvage therapy after refractory/resistance CMV infection[196,249]. The first clinical isolate resistant to LMV (V236M) was detected after suboptimal LMV dosage[250,251]. Those patients achieved virologic suppression after switching to alternative therapy with VGCV or FOS. Even though the indication of LMV is prophylaxis of HSCT recipients, resistance mutations have been clinically described in primary and secondary prophylaxis[195,249,252] and in two cases who had discontinued prophylaxis and received preemptive therapy with GCV[253]. Therefore, the off-label use of LMV for salvage

therapy of refractory or drug-resistant CMV infections should be done with caution and monitoring of LMV resistance should be performed early after viral rebound.

Natural polymorphisms of the *UL89* were shown to be low in susceptible and drug-resistant CMV clinical isolates[240]. Amino acid changes and mutations conferring resistance to LMV in *UL89* were reported by exposure of an error-prone exonuclease CMV mutant to LMV[244,247]. All these amino acid changes (N320H, N329S, D344E and T350M) are located in conserved region V of *UL89* and confer low grade resistance to LMV (figure 21). Recombinant viruses harbouring LMV-resistant mutations in *UL89* exhibit normal cytopathic effects and viral growth in cell culture. The addition of the D344E substitution in *UL89* to E237D, F261L, M329T or Q204R substitution in *UL56* increases the IC_{50} values of the single *UL56* mutants.

Currently, there are only two mutations described in UL51 that confer resistance to LMV. P91S substitution was selected from a BAC clone of CMV strain AD169 and was shown to confer low-grade resistance to LMV[247]. The viral growth of the mutant virus was not affected compared to the wild-type strain. Combinations of *UL51* P91S substitution with several *UL56* substitutions (S229F, V236M and/or R329M) multiply the fold-changes of each individual substitution whereas combination with a substitution in *UL89* (D344E) is additive. The second mutation was A95V, which emerged in patients not responding to LMV[248]. Recombinant phenotyping showed A95V conferred 13.8-fold increased LMV resistance itself, but had no impact on viral fitness, like the other 3 LMV-susceptible substitutions described in that report (D12E, 17del, V113L).

On the other hand, MBV resistance mutations primarily map to the *UL97* as well as compensatory mutations in the *UL27* (figure 22). The benzimidazole ring of MBV competes with the ATP-binding site of the *UL97*, specifically, between the P-loop and the catalytic loop of this domain (figure 20)[254]. Most amino acid changes located at the vicinity of the ATP-binding site (residues 337, 353, 397, 409–411) confer resistance to MBV, and did not overlap with those conferring resistance to GCV[255]. Amino acid substitutions or deletion found at distant sites from the ATP-binding loop (D456N, V466G, C480R, P521L Y617del) confer cross-resistance to GCV and MBV; with the exception of F342S, located close to the ATP binding site, which conferred GCV-MBV cross-resistance. Error-prone exonuclease CMV mutants exposed to higher MBV concentrations developed combinations of substitutions (V353A/H411L and V353A/H411Y) that confer high-levels of resistance[255].

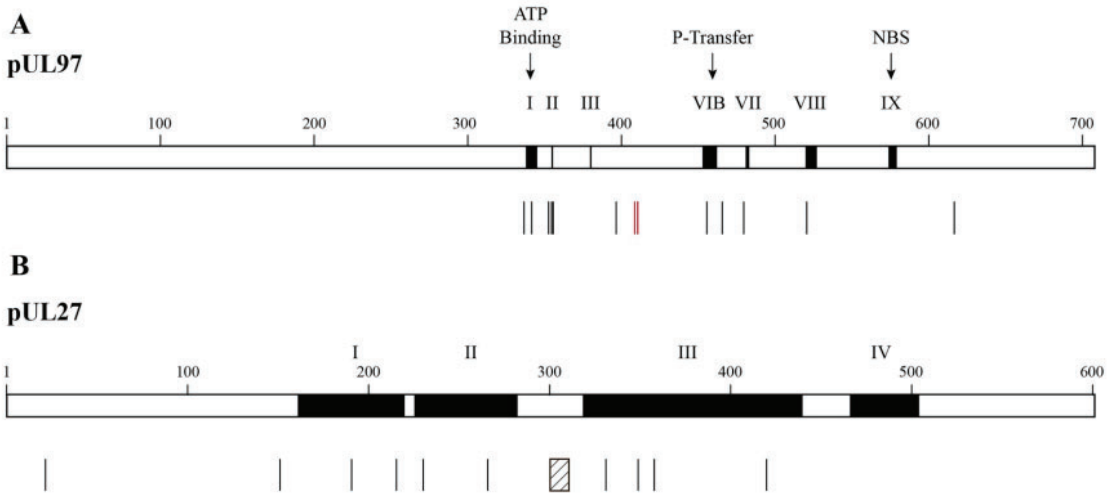

Figure 22. Resistance mutations to MBV in CMV *UL97* and *UL27.* (A) *UL97* conserve regions I-IX are indicated in black boxes and domains implicated in the ATP-binding, phosphate transfer (P-transfer) and nucleoside binding (NBS) are pointed in the map gene. (B) *UL27* conserve regions I-IV are indicated in black boxes. Bars (|) indicate amino acid substitutions and hatched box represents deletion del301–311 associated with MBV resistance. Bars in red correspond to amino acid changes conferring MBV resistance detected in clinical specimens. From Piet and Boivin[160].

Clinical isolate harbouring mutations conferring resistance to MBV were detected in SOT, HSCT recipients as in immunocompromised paediatric patients with resistant CMV infection that received MBV as salvage therapy[202,213,255]. V466G and P521L substitutions identified in clinical specimens isolated from patients treated with GCV confer high-level resistance to GCV, MBV and cyclopropavir (CPV) (figure 22)[224,225,256,257]. Furthermore, these two mutants exhibit a UL97-deficient phenotype with no autophosphorylation and a severe growth defect. D456N and C480R substitutions and del617 selected in CMV strain AD169 exposed to methylenecyclopropane analogue confer cross-resistance to GCV and MBV and mutant strains demonstrate abnormal cytopathic effects and severe growth defects[258].

Recombinant strains resistant to MBV with amino acid changes at codons 337, 353, 397, 409 and 411 exhibit normal cytopathic effects and viral growth[221,255,259]. In contrast, the viral replication of V356G mutant is moderately attenuated[257]. M460I and M460V substitutions that were already known as conferring resistance to GCV are hypersensitive to MBV with 0.14- and 0.17-fold increases in IC_{50} values[221,260]. The M460I mutant was shown to be less efficient to phosphorylate GCV, to autophosphorylate and to utilize ATP than the wild-type strain[260].

Mutations in *UL27* appear spontaneously in genetically pUL97-defective CMV strains in the absence of drugs, suggesting a functional compensation for the loss of pUL97 kinase activity[261]. Substitutions in *UL27* emerged also after exposure of CMV strains to MBV *in vitro* and shown to confer low-level of drug resistance (figure 22). These mutations result in single amino acid substitutions, premature stop codons, or frameshift mutations that lead to truncated proteins. Amino acid changes or deletions were also selected in a CMV clinical isolate[261]. Deletion in *UL27* results in a modest half log reduction in viral titters *in vitro* and no apparent effect on viral growth *in vivo*[262]. Combinations of R233S substitution in *UL27* with S337M or S353A substitution in the *UL97* kinase result in approximately 2-fold increase in MBV IC_{50} values over those of the *UL97* single mutants[259].

3. SEVERE ACUTE RESPIRATORY SYNDROME-COROVAVIRUS-2

The novel coronavirus 2019, formally named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is the pathogen that caused the coronavirus disease 2019 (COVID-19), which has become a global pandemic since December 2019 starting in Wuhan, China, and rapidly spread worldwide[263,264].

The disease has spread across many boundaries, and on March 11, 2020, the World Health Organization (WHO) declared COVID-19 to be a pandemic[265]. The virus initially attacks the respiratory system and causes flu-like symptoms, such as cough and fever and, in severe conditions, leads to difficulty in breathing[266]. According to statistical data, mortality is high in elderly (above 60 years of age) and in individuals with comorbidities, such as previous immunodeficiencies[267]. Apart from SARS and respiratory failure, COVID-19 also manifests as systemic inflammation, acute cardiac injury, leading to sepsis, heart failure, and multiple organ dysfunction in patients at high risk[266].

3.1. Discovery and evolution of SARS-CoV-2

Coronaviruses (CoVs) are the largest group of viruses belonging to the *Coronaviridae* family of order Nidovirales, that consists of two subfamilies: *Coronavirinae* and *Torovirinae* (table 4)[268]. Members of this family are positive-sense single-stranded RNA (ssRNA) viruses and are genetically categorized into the genera Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. There are seven coronaviruses known to infect humans: two belong to the Alphacoronavirus (HCoV-229E and HCoVNL63) and five to the Betacoronavirus (HCoV-OC43, HCoV-HKU1, SARS-CoV-1, MERS-CoV, and SARSCoV-2) genera.

Table 4. Classification of coronaviruses. From Kung et al[269]

Genus	Natural host	Subgenus	Species	Intermediate host	Presence of furin cleave site
Alphacoronavirus	Mammals dominant		HCoV-229E	Camelids	No
			HCoV-NL63	ND	Yes
Betacoronavirus	Mammals dominant	Embecovirus	HCoV-OC43	Bovines	Yes
			HCoV-HKU1	ND	Yes
		Hibecovirus			Yes
		Merbecovirus	MERS-CoV	Dromedary camels	Yes
		Nobecovirus			Yes
		Sarbecovirus	SARS-CoV-1	Palm civets	No
			SARS-CoV-2	Pangolin	Yes
Gammacoronavirus	Bird dominant, mammals				Yes
Deltacoronavirus	Bird dominant, mammals				No

ND: not determined.

SARS-CoV-1 and SARS-CoV-2 belong to Sarbecovirus subgenus and share 79% genetic similarity at the nucleotide level[270]. The overall genetic similarity between SARS-CoV-2 and SARS-related bat coronavirus is 96%, a little higher than the similarity found with the virus infecting Malayan pangolins (92%), indicating SARS-CoV-2 might have originated in bats[269]. Additionally, much higher similarity was found between the S receptor binding domain (RBD) of SARS-CoV-2 and SARS-related bat coronavirus (89.2%) than of the virus infecting Malayan pangolins (97.4%). Moreover, SARS-CoV-2-related coronaviruses have been detected in pangolins in Southeast Asia. Therefore, SARS-CoV-2 could be plausibly originated because of cross-species recombination between bat and pangolin coronavirus.

3.2. SARS-CoV-2 structure

SARS-CoV-2 is an enveloped particle that contains the largest genome among all RNA viruses. The genome is contained inside the nucleocapsid (N), which is further packed by an envelope formed by three structural proteins: membrane protein (M), S protein and envelope protein (E) (figure 23)[271].

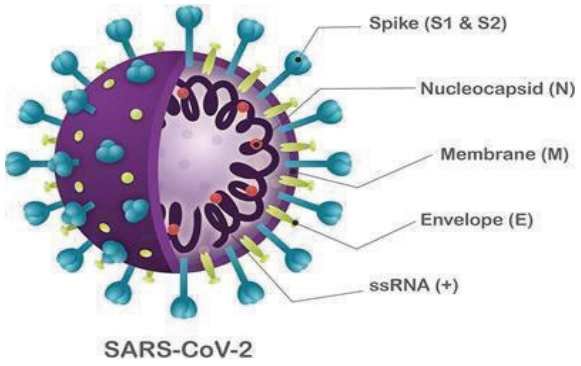

Figure 23. Structure of the SARS-CoV-2. From Santos IA et al[272].

There are sixteen non-structural proteins (nsp1−16)[273] whose functions are shown in table 5. Nsps mediate effects on splicing, translation, and protein trafficking to inhibit host defenses. Nsp and structural proteins are potential targets for antiviral drugs based on their functions and structures which are key for virus survival.

Table 5. Function of SARS-CoV-2 non-structural proteins (nsp). Data from Wang et al[274].

NSP	Function
Nsp1	RNA processing and replication, binds to 18S ribosomal RNA in the mRNA entry channel of the ribosome to interfere with the translation of mRNA.
Nsp2	Modulates the survival signalling pathway of host cell.
Nsp3	Separates the translated protein
Nsp4	Contains transmembrane domain 2 (TM2) and modifies Endoplasmic Reticulum membranes.
Nsp5	Participates in the process of polyprotein during replication.
Nsp6	Presumptive transmembrane domain.
Nsp7	Accessory protein of the RNA-dependent RNA polymerase (RdRp) and helps to adjust the position of the RNA template in the polymerase complex.
Nsp8	Binds to the nsp7 and nsp12 to form the RdRp. Nsp8 disrupts protein trafficking to the cell membrane, together with nsp9.
Nsp9	ssRNA-binding protein.
Nsp10	Cap methylation of viral mRNAs.
Nsp11	Is a 13 amino acid long intrinsically disordered protein with unknown function.
Nsp12	Catalytic subunit of the RdRp involved in virus replication and transcription.
Nsp13	Is a helicase and has nucleoside triphosphatase activity, binds with ATP and the zinc-binding domain (replication and transcription).
Nsp14	Has the proofreading exoribonuclease domain.
Nsp15	Has Mn^{+2}-dependent endoribonuclease activity.
Nsp16	2'-O-ribose methyltransferase and suppresses mRNA splicing.

3.3. SARS-CoV-2 genome

The genome of SARS-CoV-2 is a positive ssRNA of approximately 32000pb and it is decorated with a 5' cap and has a 3' poly(A) tail that functions as translation-ready mRNA[275]. It has a GC content that varies from 32% to 43%, and contains sixteen Open Reading Frames (ORFs) preceded by transcriptional regulatory sequences (TRS). The first two thirds of the whole genome length are occupied by the ORF1a and ORF1ab, which encode replicase polyprotein 1a (PP1a) and polyprotein 1ab (PP1ab), respectively (figure 24). PP1a is proteolytically processed into nsp1 to 11. A structure near the junction of ORF1a and ORF1b causes 21 programmed ribosomal frameshifting (PRF), which in turn leads to the production of another, longer polyprotein, pp1ab, where the short nsp1-11 is replaced by RdRp encoded by the nsp12, followed by nsp13 to 16, which form the replicase–transcriptase complex (RTC). Proteolytic

cleavage of pp1a and pp1ab is executed by the virally proteases encoded by nsp3 and nsp5. Nsp3 and nsp5 cleave the junctions nsp1-nsp4 and nsp4-nsp16, respectively. All the structural and accessory proteins are translated from subgenomic RNA (sgRNA) of coronaviruses. Because these proteases play vital roles in viral protein maturation, previous research has attempted to identify potent, broad-spectrum protease inhibitors.

At the 3' end, SARS-CoV-2 genome contains four major structural proteins from the 5' to 3' order: S, E, M and N[276]. All structural proteins are encoded by the ORF10 and 11 have crucial functions in viral structure and entry into the host. Interspersed among the structural genes, the 3'- end also contains 9 ORFs for structural and accessory proteins (ORF3a, 6, 7a/b, 8, and 10) that are translated from a set of nested sgRNA[263].

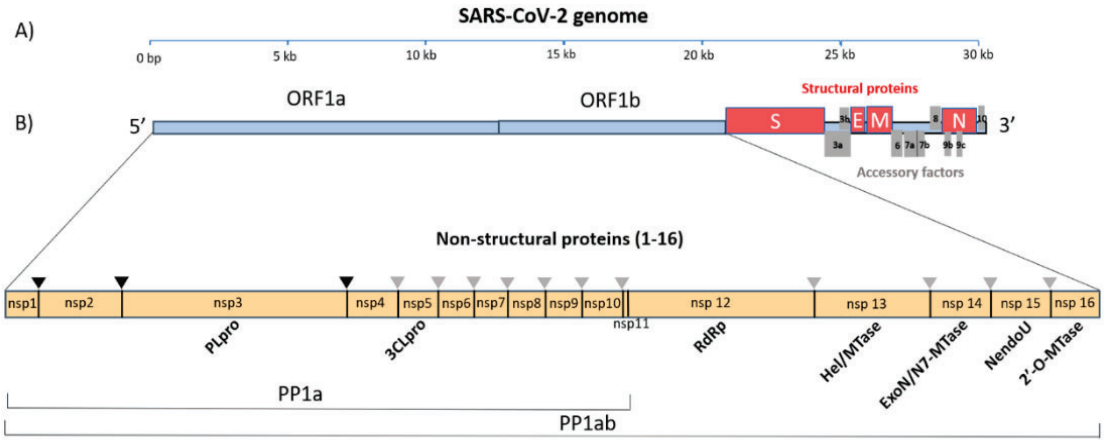

Figure 24. Genome of SARS-CoV-2. (A) Size of the SARS-CoV-2 genome indicated in kilo bases (kb). (B) Genome of SARS-COV-2 organized in individual ORFs. The black and grey triangles indicate the cleavage sites of the protease encoded by nsp3 (PLpro) and nsp5 (3CLpro). Names of functional domains in the nsps are indicated. From Romano et al[277].

3.4. SARS-CoV-2 replicative cycle

Once the virus enters the cell, the viral RNA is released, polyproteins are translated from the RNA genome, and replication and transcription of the viral RNA genome occur via protein cleavage and assembly of the RTC[269]. Viral RNA is replicated, and structural proteins are synthesized, assembled, and packaged in the host cell, after which viral particles are released (figure 25).

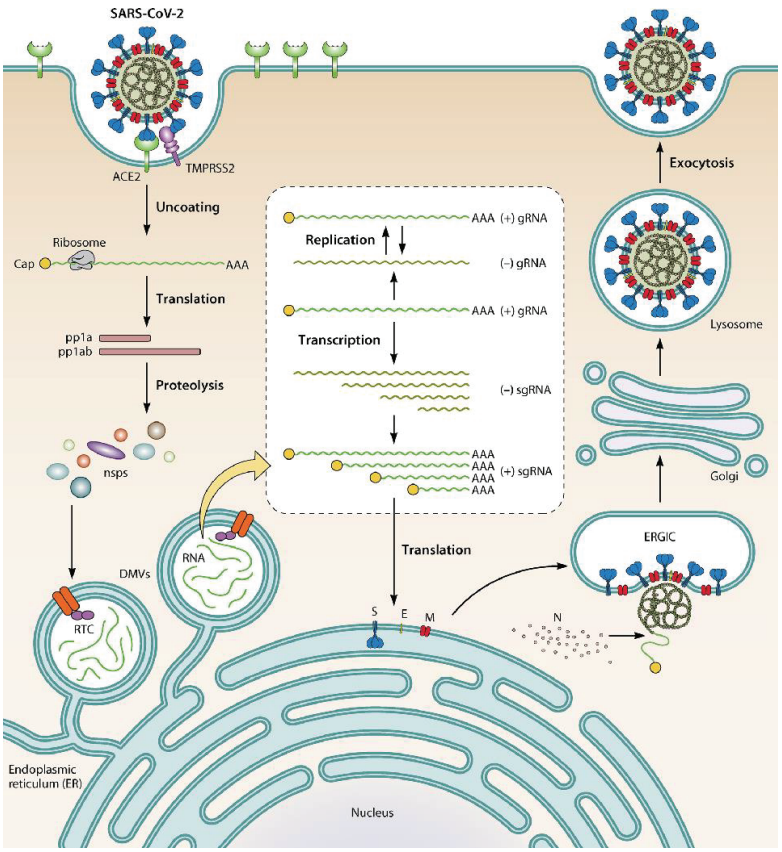

Figure 25. SARS-CoV-2 replicative cycle. From Kung et al[269].

There are three or four viral proteins in the coronavirus membrane[269]. The most abundant structural protein is the M glycoprotein[275]. The S protein as a type I membrane glycoprotein constitutes the peplomers. In fact, the main inducer of neutralizing antibodies is the S protein. However, M plays a predominant role in the intracellular formation of virus particles without requiring S. In the presence of tunicamycin, coronavirus can grow and produce spikeless, non-infectious virions that contain M but devoid of S. Therefore, S protein is essential for binding to the host cell-surface receptor for entry into the host cell.

The spike protein has two subunits S1 and S2[278]. The S1 subunit contains the RBD that binds to the angiotensin-converting enzyme 2 (ACE2) receptor located on the surface of the host cell. When the S protein binds to the receptor, transmembrane protease serine 2 (TMPRSS2), located on the host cell membrane, promotes virus entry into the cell by the fusion of the S2 subunit to the cell membrane. Afterwards, the viral RNA is released into the cytoplasm where translation and genome replication occur.

Upon cell entry, coronavirus utilizes cap-dependent translation. However, coronavirus infection is characterized by global mRNA degradation and robust inhibition of translation[269]. The

mechanism to distinguish between viral and host mRNAs depends on viral nsp1 binding to ribosomal subunits. The nsp liberated by proteolysis participate in the viral RTC within organelles formed by nsp3, nsp4, and nsp6[279–281].The minimal RTC is composed by the nsp12 catalytic subunit of the RdRp, at least one nsp7 subunit and two nsp8 subunits[282–284]. Unlike the replicases of most other RNA viruses, the coronavirus replicase is characterized by proofreading activity that invariably diminishes the efficacy of nucleotide derived analog inhibitors.

The nsp7-nsp8 factor pair binds the finger domain of nsp12 and may serve as a primase[284,285]. The other nsp8 binds the thumb domain of nsp12. Hence, the N terminal extensions of both nsp8 subunits sit in opposite sites and accommodate the exiting RNA. Elongation of the RTC is contributed by two copies of the nsp13 helicase of the 1B helicase superfamily. They also possess RNA 5'- triphosphatase activity and join the RTC. The position of the nsp13 nucleoside triphosphatase domain behind the RTC enables template switching, backtracking, and proofreading[286]. The N-terminal region of nsp9, which is a small RNA binding protein, binds the catalytic pocket of the NiRAN domain. Nsp9 might bring newly synthesized transcripts to the NiRAN domain and generate a GpppA cap. Afterwards, two methylations are performed by nsp14 and nsp16[288,293].

Proofreading is conducted by nsp14, which is a bifunctional protein with exoribonuclease (ExoN) and methyltransferase activities. ExoN activity is performed thanks to the binding with nsp10, forming nsp14-nsp10 active complex with the RNA[288,289]. Nsp14 regulates viral RNA capping via the methyltransferase function and by the junction with the RTC to form a co-transcriptional capping complex[290]. A remarkable feature of SARS-CoV-2 RNA processing is the role of nsp12 in capping. The configuration of this complex permit the design of nucleotide analogues to evade ExoN function[289].

3.5. SARS-CoV-2 subgenomic RNA

The generation of sgRNAs starts from negative-sense RNA intermediates and is regulated by TRSs and mediation of replicase template switching[294–296]. The consensus TRS is 15 nucleotides long and is localized to SL3 of the 5' UTR (leader sequence) and the immediate upstream regions of most accessory and structural genes (body)[297,298]. During minus-strand genomic RNA synthesis, the antisense body sequence in the nascent transcript pairs with the complementary leader sequence of the positive-stranded genome template. This mechanism facilitates the replicase jump from the 3'- to the 5'-end of the template and generates several discontinuous sgRNAs with identical terminal sequences antisense to the leader sequence. These sgRNAs serve as the templates generating protein-coding positive-stranded sgRNAs characterized by 5' leader

sequences identical to genomic RNAs. This template-switching process enables positive-stranded sgRNAs to acquire the 5' leader sequence containing SL1 and SL2 70 nucleotides, preventing nsp1-mediated translational shutoff[299].

These sgRNAs of SARS-CoV-2 are thought to encode the 4 major structural proteins (S, E, M, N), as well as several accessory proteins (3a, 6, 7a, 7b, 8 and 10)(figure 26)[300]. The exact molecular mechanisms that determine either to pause or to continue transcription at the TRSs to generate sgRNAs are yet to be fully clarified, but they likely involve long-range RNA-RNA interactions between complementary sequences. It is thought to be tightly regulated to ensure the optimal ratio of virus proteins.

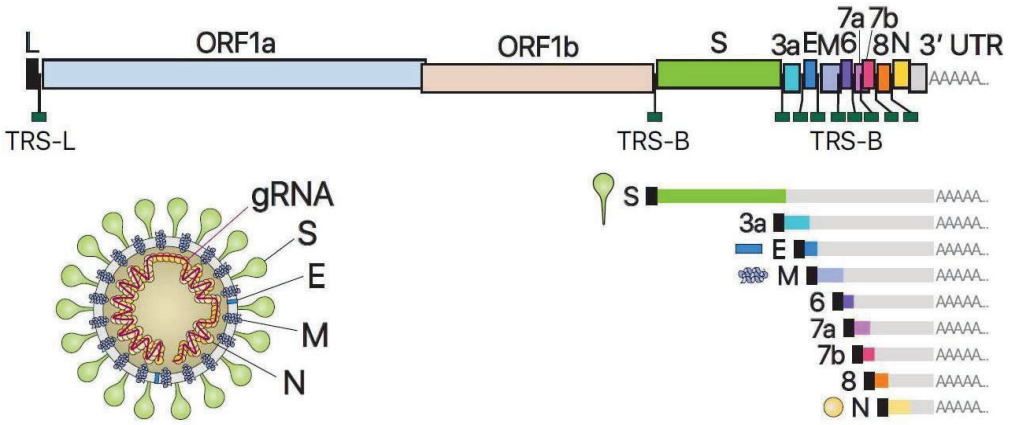

Figure 26. SARS-CoV-2 genome organization, canonical subgenomic RNAs, and the virion structure. The black box shows the leader sequence. From Kim et al[300].

Different amounts of sgRNA species has been detected in Vero-E6 Cells[301]. A study indicated that *S, E, M, N, ORF6, ORF7a* sgRNAs were found at higher levels and *ORF7b* and *ORF10* at low levels in cell culture, data corroborated by NGS in clinical samples. *N* sgRNA was shown to be more abundant than *E* sgRNA and persists for longer periods[301,303]. However, there is no clear data as to whether *E*-gene transcripts are more rapidly degraded by ribonucleases or they better reflect recent transcription. The difference among sgRNA species exist variation could be explained by the enclosure of RNA transcripts in double membrane vesicles and/or extracellular vesicles allowing longer persistence of specific sgRNA species[304–306].

Besides the sgRNA specie, the ratio of gRNA to sgRNA reads varies depending on the RNA sense and number of strands (single or double stranded) is the reverse transcription-PCR (RT-PCR) measuring. Another study detected *E* sgRNA at a level of 0.4% of virus gRNA in sputum samples[302]. Using strand-specific PCRs could cause this variability, for instance, the ratio of the *ORF7a* gRNA-sgRNA was for the positive sense 20-fold and for the negative 150-fold higher for

the gRNA[301]. The overall sgRNA abundance varies among samples and may be related to stage of infection and, importantly, more related to how samples were taken and treated before testing/sequencing [301].

Since sgRNA is only transcribed during viral replication, it has been proposed as an indicator of SARS-CoV-2 active replication. Several reports showed a significant association between sgRNA detection with high viral loads and the first 5-7 days of symptoms, when most viral transmission has been reported. SgRNA has been previously used to test challenged primate models [303,307], to monitor patients persistently positive for SARS-CoV-2 gRNA[28], and in clinical isolates[302,308]. In contrast, some studies have postulated that sgRNA is not a good indicator of infectivity suggesting that the loss of sgRNA detection is due to the lower overall RNA transcript concentration compared with gRNA[309,310].

Nowadays, viral culture is the gold standard for determining virus infectivity (figure 27). Several studies have isolated SARS-CoV-2, showing virus recovery in specimens collected 1-9 days after symptom onset[311], up to 12 days in patients with mild to moderate disease[312], and after 20 days in critically ill patients[313]. Only few reports have compared SARS-CoV-2 culture and sgRNA detection showing a moderate to complete concordance[302,308]. SgRNA detection by RT-PCR presents some benefits compared with culture techniques, which are time consuming, and require BSL-3 facilities and fresh samples.

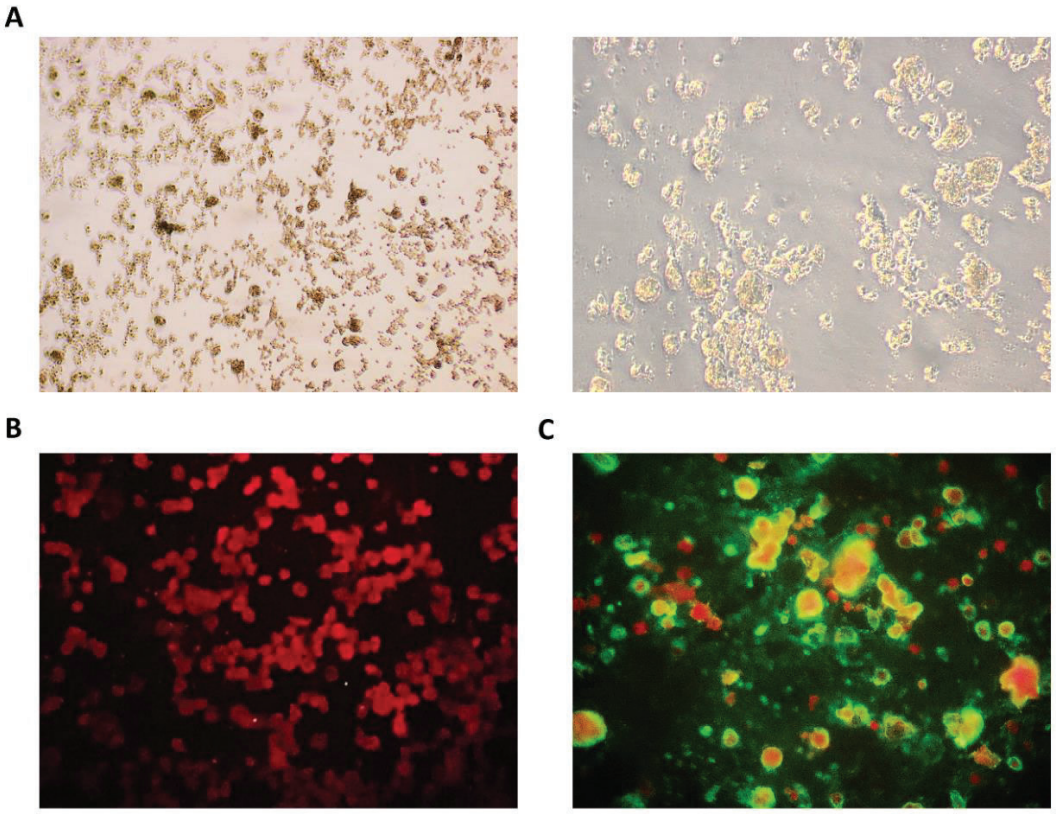

Figure 27. SARS-CoV-2 culture in VERO-E6 cells. (A) Optical microscope visualization of SARS-CoV-2 cytopathic effect on cell culture at day four post-inoculation. Indirect immunofluorescence 10 days after the inoculation of a SARS-CoV-2 negative sample (B) and a SARS-CoV-2 positive sample (C). Red fluorescence shows VERO-E6 cells. Green fluorescence indicates SARS-CoV-2 infected cells. Original figure.

Detection of genomic and subgenomic RNAs

The presence of both continuous gRNA and discontinuous sgRNA during viral replication confounds the interpretation of nucleic acid amplification test results for infectious COVID-19 patients. As real-time RT-PCR has high sensitivity and specificity, it is considered the reference method for SARS-CoV-2 diagnosis. This technique provides a cycle threshold (Ct) value, which is inversely related to viral load, such that every increase in a Ct value of 3.3 corresponds to an approximate 10-fold reduction of RNA amounts. Ct values have been used as a prognostic indicator for clinical decision-making[316], however, Ct values are highly variable depending on the swabbing technique, specimen type, assays and platforms used for RNA extraction and amplification, significantly varying between and within methods[314,315,317].The College of American Pathologists surveyed more than 700 laboratories and demonstrated that different methods using identical control material varied by as much as 14.0 cycles and by up to 12.0 cycle differences within a single gene target using a single method[315]. Therefore, Ct values do not have a linear relation with viral loads, and the dispersion of the measurements is understated. The use of reference standard curves is recommended to calibrate every target and primer/probe design in order to provide accurate viral load determination for patient follow-up.

SARS-CoV-2 RT-PCR can remain positive for several weeks after clinical recovery in sicker patients, as well as in asymptomatic and mild ill individuals, as RT-PCR detects the presence of viral genomic RNA but is not able to distinguish whether an infectious virus is present. To determine whether there is active virus replication and, therefore, potential risk of person-to-person transmission, it is necessary to perform viral culture. However, the correlation between the cycle threshold (Ct) and the culture-based infectivity assay in COVID-19 patients remains controversial. The ranges of Ct ratios obtained for successful SARS-CoV-2 isolation vary among studies [308,318–320]. Various amplicons of *ORF1a/b*, *E, N,* and *S* genes have been widely used. Nevertheless, numerous primer sets recognizing structural genes fail to distinguish gRNAs from sgRNAs. Moreover, low-Ct specimens that failed in virus isolation were found to carry disproportionate numbers of copies of structural and non-structural genes. Hence, disrupted genomes or residual replicating intermediates may have been detected. In contrast, monitoring

the RNA levels of various replicating intermediates and their associations with the culture ability of the clinical specimen revealed that culturable specimens were characterized by the detection of gRNA and sgRNAs soon before the onset of COVID-19 symptoms. Nevertheless, RT-PCR results must be carefully interpreted and correlated with infectiousness.

3.6. Epidemiology of SARS-CoV-2

Since the first reports of cases in the city of Wuhan, the epicentre of the pandemic, in December 2019 through May 2022, over 500 million confirmed cases have been reported on all continents, which have been registered by the WHO and CDC (figure 28)[26,322]. From December 18th to 29th, samples of bronchoalveolar lavage fluid were collected from patients hospitalized for severe pneumonia in Wuhan, and the new coronavirus was isolated. On January 24th, a case series of 41 confirmed SARS-CoV-2 patients treated at a Wuhan hospital was released, and 66% had a history of exposure to the seafood market[266]. In another case series of 138 hospitalized patients in Wuhan, 41% of the infected patients were presumed to have been infected by nosocomial transmission[323]. The infection rapidly escalated and was declared a public health emergency by the WHO on January 30th of 2020.

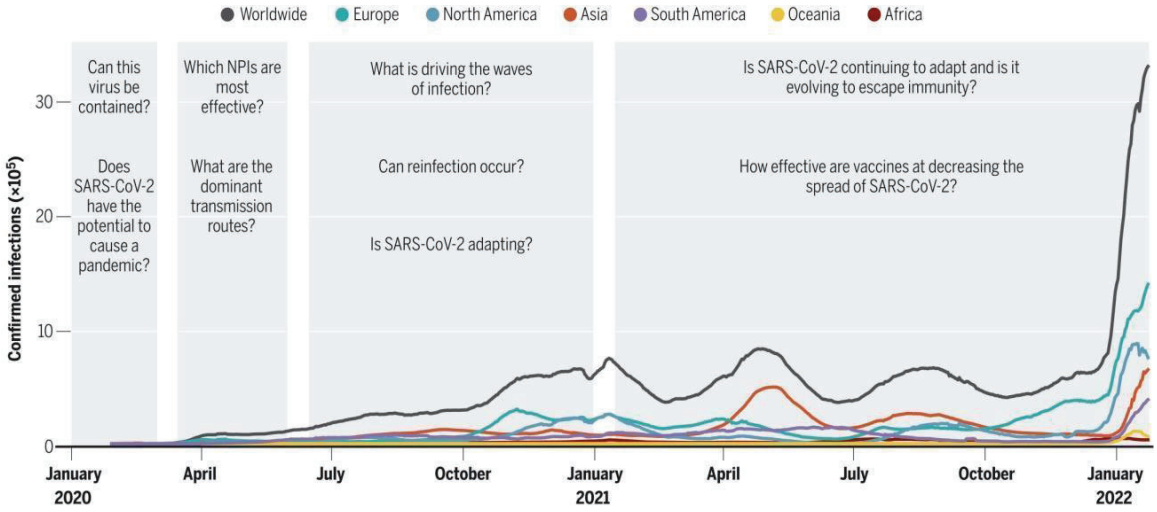

Figure 28. Timeline of confirmed infections of SARS-CoV-2 in a worldwide scale and distributed by continents. From Koelle et al[321].

By the end of June 2020, 214 affected countries have been reported, and the number of confirmed cases worldwide is close to ten million with a total of approximately one half million deaths and over five million cases that have recovered. Measures, such as social distancing, hard-surface disinfection, and face masks, have been implemented in an effort to halt the spread of the disease. Afterwards, vaccine administration decreased the number of hospitalizations,

but not the number of cases due to a lack of health supplies and vaccines which did not reach all continents equally. This fact allowed the evolution of the SARS-CoV-2 to become more transmissible and less pathogenic, which was evidenced by the new circulating variants.

On May 19th 2022, 523 million cases worldwide had been diagnosed and 6.23 millions of COVID-19 related deaths[321]. However, the reported case counts underestimate the overall burden of COVID-19, as only a fraction of acute infections are diagnosed and reported. Seroprevalence surveys in the United States and Europe have suggested that after accounting for potential false positives or negatives exceeds the incidence of reported cases by approximately >10-fold[324].

3.7. Emergent variants of SARS-CoV-2

Advantageous genomic alterations with respect to viral replication, transmission, and immune evasion increase in frequency in a population because of natural selection[325]. Alterations that have effect on viral fitness contribute to the pool of circulating variants[326]. RNA viruses, like SARS-CoV-2, continuously evolve as genetic mutations occur during replication of the genome, generating new variants. The definition of these concepts is the following:

- Mutation: a single variation in the viral genome that confers a change in the characteristics of the virus.
- Variant: a viral genome that may contain one or more mutations that differentiate it from other variants.
- Lineage: a genetically closely related group of virus variants derived from a common ancestor.
- Strain: when variants demonstrably change the virulence and transmission.

SARS-CoV-2 genetic lineages have been routinely monitored since the beginning of the pandemic through epidemiological investigations, virus genetic sequence-based surveillance and laboratory studies. The SARS-CoV-2 Interagency Group (SIG) and the CDC classified variants in four classes[327]:

- Variants of concern (VOC): changes that have a significant impact on transmissibility, increased virulence, resistance to vaccine or acquired immunity from previous infection, and has the ability to elude diagnostic detection.
- Variants being monitoring (VBM): signals through epidemic intelligence, rules-based genomic variant screening, or preliminary scientific evidence. VBM must be present in at least one outbreak, or there must be evidence that there is community transmission

of the variant elsewhere in the world. They have properties similar to those of a VOC, but the evidence is weak or has not yet been assessed by CDC.

- Variants of interest (VOI): genomic properties, epidemiological or in-vitro evidence that these changes could imply a significant impact on transmissibility, severity and/or immunity, realistically having an impact on the epidemiological situation. Evidence is still preliminary or is associated with major uncertainty. In addition, all the criteria for variants being monitored apply.
- Variants of High Consequence (VOHC): has clear evidence that prevention measures or medical countermeasures have significantly reduced effectiveness relative to previously circulating variants.

Variants occur through nucleotide changes that emerge naturally in the viral genome during replication. These changes occur at a higher rate in RNA viruses than in DNA viruses[328] , however, the rate at which these nucleotide changes occur in coronavirus is significantly lower than that of other RNA viruses since they possess a proofreading activity[329].

Apart from replication errors, host-derived pressures, such as RNA editing or RNA modification, can also be essential for the development of SARS-CoV-2 variants. Hosts have evolved RNA editing enzymes as sensory viral innate immune mechanisms[330-332], however, viruses can exploit these mechanisms for their evolutionary potential. The intra-host variability observed in COVID-19 patients has therefore been suggested to be driven by host RNA editing enzymes[333], but how SARS-CoV-2 manipulates these enzymes to introduce nucleotide changes into its viral gRNA remains unclear[330–333]. Intra-host evolution therefore seems to be more pronounced in immunocompromised populations which could serve as a long-term source of new SARS-CoV-2 variants[334,335]. Several studies have revealed strong selection pressure on SARS-CoV-2 during immune-based therapies (convalescent plasma and monoclonal Abs), antiviral therapy and vaccines, or due to environmental factors[334–336].

SARS-CoV-2 variants could have also emerged as a result of recombination[337,338] . Recombination occurs in cells infected with multiple variants, where the genetic material of two variants is packaged into a single virion. This data has been evidenced for SARS-CoV-2 showing different pathogenic properties, especially when the recombinants can escape both natural and vaccine-induced immunity[339,340]. Coronaviruses are known to have relatively high recombination rates, however, the importance of ongoing recombination of SARS-CoV-2 has not yet been resolved[341].

Table 6. SARS-CoV-2 variants. Data from Mistry et al [326].

Variant	Lineage	Type of variant	Time and place of emergence	S protein mutations
Alpha	20I/501Y.V1/B.1.1.7	VOC	UK – September 2020	69/70-, 144Y-, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H
Beta	20H/501Y.V2/B.1.351	VOC	South Africa – October 2020	L18F, D80A, D215G, D242-244del, R246I, K417N, E484K, D614G, A701V
Gamma	P.1/20J/501Y.V3/B.1.1.248	VOC	Brazil – January 2021	K417T, E484K, N501Y, D614G, H655Y, L18F, T20N, P26S, D138Y, R190S, T1027I, V1176F
Delta	21A/B1.617.2	VOC	India – December 2020	L452R, D614G, P681R, T19R, T478K, D950N, R158G, 156-157del
Omicron	21K/ B.1.1.529	VOC	South Africa - November 2021	A67V, D69-70, T95I, G142D/D143-145, D211/L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K

Mutations analysis of SARS-CoV-2 allowed the identification of amino acid changes, that were classified by the identified by the CDC in VOC, VBM, VOI and VOHC (table 6)[336,342]. The original virus was first detected in Wuhan rapidly developed the mutations D614G in the *S* gene that increased it infectiousness, but convalescent serum was effective. Afterwards, four main RBD amino acid substitutions were the focus of studies that assess virulence and immune evasion: N501Y found in the ACE2 binding site of the RBD and is common to Alpha, Beta, Gamma and Omicron variant strains[343,344]; E484K/Q/A and K417T/N that are present in the Beta, Gamma and Omicron strains [343,344]; and L452R that is unique to the Delta variant[343]. In addition, the Omicron variant has between 26-32 amino acid changes in the S protein which are the target investigation[345]. This variant, although having a few substitutions in common with Beta and Delta, has a distinct evolutionary pathway. Omicron variant may affect viral transmissibility, virulence and rate of reinfection by escaping natural and vaccine-induced immunity[346].

SARS-CoV-2 variants were developed progressively and can characterize four distinct waves of the pandemic: the first wave between June-August 2020 was attributed the alpha variant (B.1.1.7), the second wave lasted from November 2020 until February 2021 and was driven by the beta (B.1.351); the third wave was dominated by the delta (B.1.617.2) and occurred from May until October 2021. The most recent fourth wave, beginning in November 2021, was driven by the omicron (B.1.1.529) and has evolved to different subtypes since then to June 2022[347].

3.8. Transmission

Respiratory transmission is the primary means of SARS-CoV-2 infection directly from person to person, especially at close distance (approximately two meters)[348]. The virus is contained in respiratory droplets that are released in secretions when the infected person coughs, sneezes, or talks, involving the infection of surrounding people if virus particles are inhaled or made contact with the mucous membranes (eyes, nose, or mouth). There is also no evidence that SARS-CoV-2 can be transmitted through contact with non-mucous membrane sites, like abraded skin, nor with contaminated surfaces.

SARS-CoV-2 can be airborne transmitted in longer distance through inhalation of particles that remain in the air over time and distance in enclosed poorly ventilated spaces[349], including hospital rooms of patients with mild COVID-19. However, viable virus in air and surface specimens in healthcare settings have rarely been detected[350,351].

SARS-CoV-2 has been detected in non-respiratory specimens, including stool, blood, ocular secretions, saliva and semen, but the role of these sites in transmission is uncertain[350]. Detection of SARS-CoV-2 RNA in blood has also been reported in some but not all studies that have tested for it. Vertical transmission of the virus in pregnancy has not been proven, however it is not known whether there is a risk during delivery through the vaginal canal[351].

The potential to transmit SARS-CoV-2 begins prior to the development of symptoms and is highest early in the course of illness; the risk of transmission decreases thereafter. Initially, it was believed that only people with clinical manifestations could spread the infection. However, it has been shown that asymptomatic carriers also transmit the virus. Although their transmission is less efficient than symptomatic ones[352], they frequently infect more people as asymptomatic patients do not follow isolation precautions until they are aware of their infection.

3.9. Persistent SARS-CoV-2 replication and reinfection

Viral infections are usually a weeklong when symptoms are manifested, therefore, transmission after 7 to 10 days of illness is unlikely in immunocompetent patients with non-severe infection, but it has been frequently reported after 10 days of symptoms onset in immunosuppressed patients, been denominated as persistent infection[335,357].

The duration of viral RNA shedding is variable and may increase with age and the severity of illness[353]. There have been several reports of viral RNA detection in respiratory specimens

months after the initial infection[354–356]. Detectable viral RNA does not necessarily indicate the presence of infectious virus, however, isolation of infectious virus from respiratory specimens has been described over 21 days after symptoms. Prolonged shedding of virus in stools has also been reported, however, further data are needed to understand the frequency and clinical significance of these findings. At this time the RNA dynamics in immunosuppressed patients was unknown[335,357], but a couple of reports proved prolonged infection by the same virus strain instead of reinfection confirmed by sequencing[335,355].

The risk of reinfection in the following 6 to 9 months after infection was 1-20%, but this percentage increases with older age and with Omicron variant in individuals previously infected with other variants[358]. It has been demonstrated that patients with detectable anti-SARS-CoV-2 Abs had lower rates of reinfection[359], and reinfections were milder than initial infections[360], although some studies reported reinfections cases that were more severe than the first infection and some fatal cases[361,362].

3.10. Immunomodulation by SARS-CoV-2

Similar to other RNA viruses, the S protein and dsRNA are recognized by characteristics receptors of the innate immunity (Toll-like receptors and retinoic acid-inducible gene I-like receptors)[363]. Unlike many other respiratory pathogens, SARS-CoV-2 can induce an aggravated immune response. IFN-I and IFN-III responses were only slightly activated, whereas chemokines were highly upregulated in the serum and lungs of severe COVID-19 patients. The consequences of these dysregulated immune responses include a cytokine storm and death.

In the case of immunosuppressed patients, a deficient immune response was first thought to be protective against the cytokine storm and exacerbated inflammation caused during SARS-CoV-2 infection. On the contrary, low lymphocyte counts, specially CD4+ T cells, are considered reliable predictors of disease severity and mortality in COVID-19 patients[364]. This finding highlights the critical role of CD4+ T cells, in orchestrating adaptive immune responses following SARS-CoV-2 infection. Experiments in mice revealed that T cells temper early overactive innate responses and limit the cytokine storm[365-367]. Therefore, having an immunosuppressed adaptive response is prejudicial to fight the infection despite the immunomodulation caused by SARS-CoV-2.

3.11. Immunosuppressive therapy in COVID-19 patients

Immunosuppression is a pathological phenomenon associated with prolonged viral replication and complicated outcomes. The degree of pharmacological immunosuppression is assessed

according to the patient's immunological risk, the type of protocol used, the molecule target and the indication (e.g., neoplasms, transplants, immunological, etc.). Although in all cases it is not easy to directly assess the degree of immunosuppression, some biomarkers reflect the individual's response to therapy (liver function enzymes, genotypes, lymphocytes count, cytokines, leukocyte markers or target enzymes) or is assessed according to the number of immunosuppressant drugs, dose and time employed [368,369].

It is not clear whether transplantation and use of immunosuppressants in this context is a risk or severity factor for SARS-CoV-2 infection. Likewise, in the scenario of SARS-CoV-2 infection, the adjustment or suspension of immunosuppressors should be assessed, and graft function should be protected using glucocorticoid doses and support measures[370].

Multiple recommendations and guidelines have been generated around the use of immunosuppressor or cytostatic drugs, such as discontinuing mycophenolate in critically ill transplant recipients[371], reduce the dosage or the interval of administration of calcineurin inhibitors (tacrolimus, cyclosporine) particularly in kidney recipients[372], and to avoid high doses of corticosteroids, since they could prolong viral replication in patients with COVID-19 [371] . In any case, immunosuppressors must be reserved for specific subgroups of critically ill patients.

Other drugs, not immunosuppressants, but associated with the pyshio-pathogenesis of the disease, such as ACE inhibitors or renin-angiotensin-aldosterone system inhibitors, have not been shown to increase the risk of SARS-CoV-2 infection, and conversely, their withdrawal could be harmful[373].

There is a warning about possible drug interactions between immunosuppressive drugs and those already approved or under investigation for the treatment of COVID-19. Currently, there is no data on possible interactions between RDV and immunosuppressive drugs, unlike chloroquine/hydroxychloroquine and lopinavir/ritonavir [371].

3.12. Antiviral treatment

The global pandemic COVID-19 has created an urgent effort to repurpose antiviral inhibitors to control SARS-CoV-2 replication and improve clinical outcomes[374,375]. To date, three antiviral drugs, created initially against different pathogens, have been approved by the FDA for treatment of COVID-19 patients: RDV, molnupiravir and paxlovid (nirmatrelvir/ritonavir)[376-378].

RDV was the first drug approved by the FDA in October 2020 for the treatment of COVID-19 in hospitalized adults and pediatric two years of age or older requiring supplemental oxygen, invasive mechanical ventilation, or extracorporeal membrane oxygenation[376]. It is an analog of

nucleoside that inhibits the nsp12 subunit of the RdRp. In December 2021, the FDA issued an emergency use authorization (EUA) for molnupiravir and paxlovid for the treatment of mild-to-moderate COVID-19 in adults (molnupiravir) and pediatric patients (paxlovid) at high risk of hospitalization or death. Any nof them is authorized after hospitalization due to COVID-19[377,378]. Molnupiravir is an analog of nucleoside triphosphate that works by introducing errors in the viral genome[377]. Paxlovid consists of nirmatrelvir, which inhibits SARS-CoV-2 protease to stop the virus from replicating, and ritonavir, which slows down nirmatrelvir's breakdown to help it remain in the body for a longer period at higher concentrations[378].

3.12.1. Antiviral target genes: Nsp12 subunit of the RdRp

Coronaviruses use an RdRp complex for the replication of their genome and for the transcription of their genes[379,380]. This RdRp complex is the target of nucleoside analogue inhibitors, such as RDV[381]. The RdRp of SARS-CoV-2 is composed of a catalytic subunit known as nsp12[382] as well as two accessory subunits, nsp8 and nsp7[380,383].

The nsp12 subunit contains the nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain, an interface domain and a C-terminal RdRp domain[283,384]. The RdRp domain resembles a right hand, comprising the fingers, palm and thumb subdomains (figure 29). Subunits nsp7 and nsp8 bind to the thumb, and an additional copy of nsp8 binds to the finger domains. It is known that nsp8 and nsp7 confer processivity to nsp12, and the RNA-dependent RNA extension activity depended on them[383]. RdRp enzyme engaged with over two turns of duplex RNA and reveals a long protruding RNA and extended protein regions in nsp8.

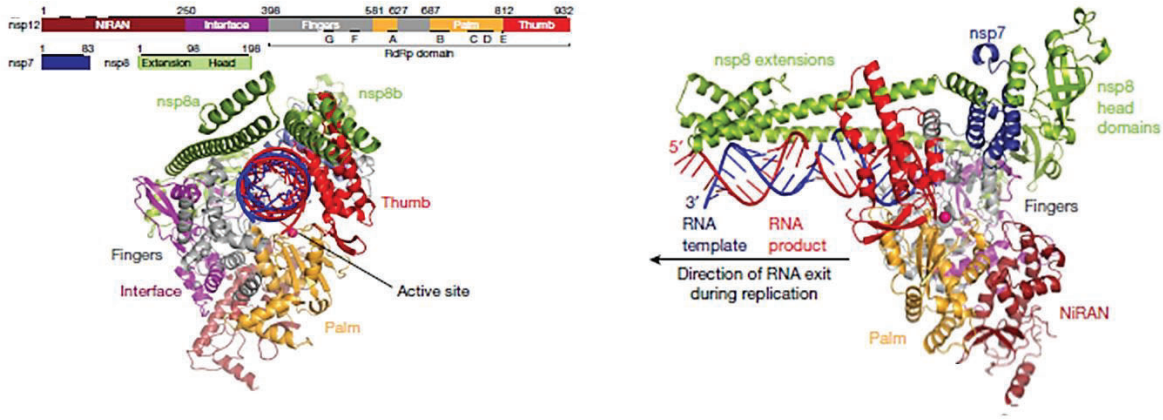

Figure 29. Structure of complex between SARS-CoV-2 RdRp and RNA. a, Domain structure of nsp12, nsp8, and nsp7 subunits of RdRp. In nsp12, the conserved sequence motifs A–G1 6 are depicted. Regions included in the structure are indicated with black bars. b, Three views of the

structure, related by 90° rotations (top, back view; middle, side view; bottom, top view). Colour code for nsp12 (NiRAN, interface, fingers, palm and thumb), nsp8, nsp7, RNA template (blue) and RNA product (red) used throughout. The magenta sphere depicts a modelled21 metal ion in the active site. From Hillen et al [282].

The nsp12 subunit binds to the first turn of RNA between its fingers and thumb subdomains. The active site is located on the palm subdomain, and is formed by five conserved nsp12 elements known as motifs A–E. Motif C binds to the RNA 3'-end and contains the residues D760 and D761, which are required for RNA synthesis[383,385]. The additional nsp12 motifs F and G reside in the fingers subdomain and position the RNA template. The observed contacts of nsp12 with the RNA product strand may retain short RNA during early steps of RNA synthesis.

As the RNA duplex exits from the RdRp cleft, it forms a second helical turn that protrudes from the nsp12 surface[282]. There are no structural elements in the RdRp that restrict the extension of the RNA duplex. These observations are consistent with the production of dsRNA during replication. However, it is unclear whether replication in infected cells results in RNA duplexes or whether RNA strands are separated. It is also unknown when and how RNA strands are separated during the transcription of viral genes, which produces single-stranded product mRNAs that can be translated.

RdRp binds to the incoming nucleoside triphosphate (NTP) substrate in a conserve site, including putative contacts between nsp12 and the NTP. Residues N691, S682 and D623 may recognize the 2'-OH group of the NTP, thereby rendering the RdRp specific for the synthesis of RNA rather than DNA. The NTP is important for the union with the triphosphorylated form of RDV, because there is space to accommodate the additional nitrile group that is present at the 1' position of the ribose ring of RDV. The incorporation of RDV might cause delayed-chain termination, possibly explaining why RDV can escape the surveillance of the coronavirus proofreading activity[389,390].

3.12.2. Antiviral drugs: Remdesivir

RDV was the first antiviral drug approved by the FDA for the treatment of COVID-19 patients in October 2020. RDV was originally developed in response to the 2014-2016 Ebola outbreak in West Africa[391,392] and has shown broad-spectrum activity against pathogenic human and zoonotic coronaviruses, including SARS-CoV, MERS-CoV and the novel SARS-CoV-2[274,391,393,394]. Among the existing broad-spectrum antiviral drugs, RDV was proved to have an improved effective and safe profile against SARS-CoV-2 than other inhibitors.

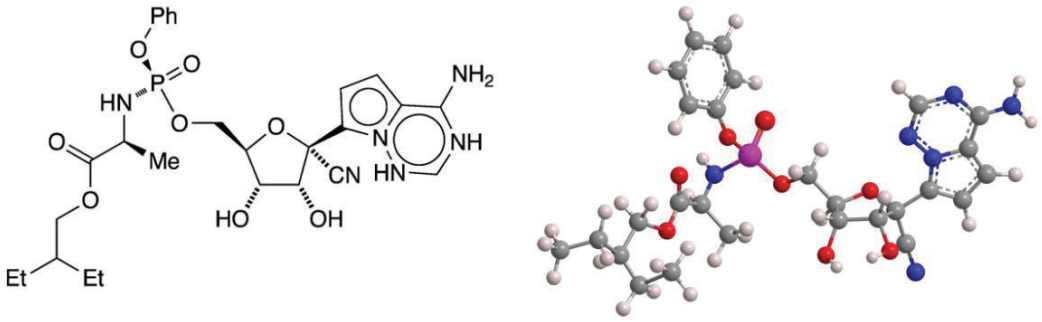

Figure 30. Chemical structure of remdesivir (GS-5734). From the American Chemical Society[395]

RDV, formerly GS-5734, is a phosphoramidate pro-drug that by entrance into respiratory epithelial cells in humans, is metabolized to a nucleoside triphosphate which is the active form (GS-441524) (figure 30)[389]. RDV inhibits the viral RdRp by competing with the usual counterpart adenosine triphosphate, while evading proofreading by viral ExoN. RDV is incorporated by the RdRp into the growing RNA product and allows for addition of three more nucleotides before RNA synthesis stalls. The addition of the fourth nucleotide following RDV incorporation into the RNA product is impaired by a barrier to further RNA translocation. This translocation barrier causes retention of the RNA 3'-nucleotide in the substrate-binding site of the RdRp and interferes with entry of the next nucleoside triphosphate, thereby stalling RdRp. In the structure of the RDV-stalled state, the 3'-nucleotide of the RNA product is matched and located with the template base in the active centre, impairing proofreading and escaping removal from the RNA 3'-end by the viral ExoN nsp14 that binds to the RdRp complex[390].

RDV in its intravenous pro-drug form (GS-5734) has a plasma half-life of one hour approximately[389], and when it is converted to the active triphosphate analogue has a prolonged intracellular half-life (40h). Its dosing regimen positively evaluated in clinical trials is 200mg intravenous on day 1 and 100mg on days 2 through 5. No RDV accumulation was observed following once daily dosing for up to 5 days. However, RDV was expected to have poor oral bioavailaility[392]. RDV is contraindicated in patients with severe hepatic impairment[396], receiving haemodialysis or hemofiltration, but no recommendations for dose adjustments were indicated in patients with mild to moderate renal impairment.

The approval of RDV was supported by the agency's analysis of data from three randomized, controlled clinical trials that included patients hospitalized with mild-to-severe COVID-19. The Adaptive COVID-19 trial (ACTT-1) founded by the National Institute of Allergy and Infectious Diseases showed a significantly clinical improvement and shorter time to recovery from COVID-19 for the group treated with RDV for 10 days compared with the placebo group[397]. A second

trial (SIMPLE) showed significant symptoms improvement in the 5 day-RDV group compared with those receiving only standard of care, and favourable results when comparing the 10-day treatment group with the standard of care ones, but not statistically significantly different[398]. The third clinical trial demonstrated no differences in recovery rates or mortality rates between 5-days and 10-days of RDV treatment [399]. The WHO Solidarity trial showed no improvement in the mortality rate, however, several reports showed a mortality rate reduction, especially after >5 days of RDV treatment[400,401].

Possible side effects include increased levels of liver enzymes, which may be a sign of liver injury, and allergic reactions, that might include changes in blood pressure and heart rate, low blood oxygen level, fever, shortness of breath, wheezing, swelling, rash, nausea, sweating or shivering. Similar safety information was determined when using RDV to treat pediatric patients hospitalized for COVID-19[376].

3.12.3. SARS-CoV-2 antiviral resistance mutations

Determining emergent mutations prior or subsequent antiviral therapy is a major concern as coronaviruses correct most errors that occur during genome replication. Mutations that arise and are fixed should result in an advantage to the virus, such as antiviral drugs resistance[402-407].

The development of RDV resistance in coronaviruses has been firstly assessed by cell culture in mouse hepatitis virus (MHV), which has similar IC_{50} values to SARS-CoV-1, SARS-CoV-2, and MERS-CoV[408] . F476L and V553L non-synonymous mutations were selected in the nsp12 RdRp after more than 20 passages. Neither of the mutations directly altered the catalytic site or substrate binding pocket of the RdRp, but they did cause minor structural alterations that are thought to impact fidelity checking step of RdRp before catalysis[408,409] . Compared to wild-type virus, these mutations conferred 2.4-fold and 5-fold reduced susceptibility to RDV, respectively, while the double mutant showed 5.6-fold reduced susceptibility *in vitro*[408]. Similar results were found for the substitutions at homologous SARS-CoV-1 residues (F480L and V557L), but not effect was evaluated in SARS-CoV-2 [408]. The EC_{50} values of the mutants (0.057–0.13 μmol/L), however, remained below achievable human drug exposures[408,409]. Furthermore, these mutations appear to confer a fitness cost, with wild-type virus rapidly outcompeting the mutants in the absence of RDV, in accordance with their extremely low frequency of circulation. Of concern, the affected residues are conserved across coronaviruses raising the possibility of a common pathway to resistance.

The E802D was determined to confer 6-fold change in the EC_{50} in two independently generated populations, and it was not present in absence of RDV[410]. E802D mutations did not affect viral replication but did affect virus fitness in a competition assay. Its position is highly conserved between all *beta-coronaviruses* and is located within the palm sub-domains (680-815) and close to the residues implicated in the nsp12-RNA interaction (813-815). This mutation was later found in an immunocompromised patient with acquired B-cell deficiency who developed an indolent protracted course of SARS-CoV-2 infection[411]. This patient experienced a symptoms alleviation and virologic response after RDV treatment, but her course was complicated by recrudescence of high-grade viral shedding. Another report presented the occurrence of the *nsp12* D484Y variant following RDV failure in a patient with a post-rituximab B-cell immunodeficiency and persistent SARS-CoV-2 viremia. Cure was reached after supplementation with convalescent plasma.

Although an analysis of globally circulating SARS-CoV-2 variants (>800000 sequences) showed no evidence of widespread transmission of RDV-resistance mutants [410], the only two clinical cases of RDV-resistance illustrate the importance of monitoring for RDV resistance and the potential benefit of combinatorial therapies in immunocompromised patients with SARS-CoV-2 infection. Identifying and evaluating the incidence of resistance-mutations in the *nsp12* have strong implications for clinical management and virus surveillance.

HYPOTHESIS

Immunocompromised patients are the most vulnerable population for opportunistic infections. The immune depletion congenitally suffered, acquired, or imposed by therapy impairs the self-sufficient defensive response against pathogens, such as CMV and SARS-CoV-2. Both viruses negatively alter the immune system of the host, acerbating their immunosuppressed status.

CMV infection is an important cause of morbidity and mortality in immunocompromised patients due to CMV direct and indirect effects on the host. The development of new antiviral agents and preventive strategies have significantly reduced CMV disease, risk of infection by other pathogens and its associated mortality, but have favoured the emergence of antiviral resistance. It is estimated that 5-12% of transplant recipients experienced CMV resistance infection depending on the transplant type. This incidence is underestimated because clinical decision of antiviral treatment is based on empirical practice and the genotypic antiviral resistance studies are not systematically requested in the hospital routine.

According to the consensus guidelines on the management of antiviral therapies, the study of resistance should be requested as early as possible to reduce side effects of antiviral drugs, the development of multi-resistance and the associated clinical outcomes. Confirming resistant mutations is usually performed by dideoxy sequencing which is the easiest and fastest method to sequence antiviral target genes of the virus. However, its limited sensitivity is overcome by NGS, that can detect minor populations and show all existent genetic diversity.

Determining the association of individual mutation to the antiviral response, the level of resistance and growth capacity can only be carried out by phenotypic studies, and the easiest and most robust way is by generating recombinant viruses. Therefore, genotypic-phenotypic correlation studies are important not only to better understand the biological function of each residue, but also to widen public database of genetic variants to facilitate the interpretation of genotypic testing data, optimize treatment adjustments and improve clinical outcomes of immunocompromised patients with resistant, refractory, or persistent viral infection.

The other opportunistic infection with high impact on immunocompromise patients is SARS-CoV-2. This infection is associated with severe disease and higher mortality in immunosuppressed patients. They suffered respiratory distress syndrome and respiratory failure, but also systemic inflammation. Genetic material of respiratory viruses is detected by RT-PCR, which provides a Ct value that can vary between and within methods. Respiratory samples have intrinsic variability that depends on the operator and the tolerance of the patients.

These limitations can be resolved by standardization and normalization of viral loads, which provide more accurate values that can help clinical decision making and monitoring the patient.

Genomic RNA can remain detectable for long periods after clinical recovery, not necessarily indicating active viral replication. Viral culture is the gold standard to determine viable virus particles, but the requirement of high biosafety laboratory equipment and long-term response limit its usefulness. Since SARS-CoV-2 sgRNA is only transcribed during viral replication and is poorly packaged into virions, it has been proposed as an indicator of active replication. Understanding the duration of SARS-CoV-2 infectivity is crucial to control viral transmission, to identify infectious asymptomatic/symptomatic individuals, to shorten isolation precautions, to reincorporate to work and to evaluate the efficacy of vaccines and antiviral treatments. The emergence of mutations involved in fitness advantage, immune escape, better adaptation to the host or antiviral drug resistance are more prompted to be selected in immunocompromised patients due to persistent viral replication and longer antiviral therapies.

Among the limited antiviral treatments available, RDV showed good effective profiles for the treatment of severe COVID-19. To date, no evidence of widespread transmission of RDV-resistant mutations has been reported, however, it is crucial for health surveillance to closely monitor emergent mutations to guide in the clinical management of patients.

OBJECTIVES

The main objectives of this thesis are to study the opportunistic infection caused by human cytomegalovirus and SARS-CoV-2 inquiring persistent infection and antiviral resistance. These major objectives can be further divided into:

1. Characterization of mutations associated with antiviral resistance in immunocompromised patients with refractory human cytomegalovirus infection.

    1.1. Genotypic antiviral resistance testing of human cytomegalovirus target genes of conventional and new antiviral therapies by Sanger and next generation sequencing.

    1.2. Phenotypic studies of uncharacterized mutations found genotypically in clinical samples by bacmid technologies, to determine the antiviral susceptibility and the replicative capacity of each individual mutation.

    1.3. Searching for baseline resistant mutations before the administration of new therapies to prevent antiviral treatment failure.

    1.4. Determining the incidence of cytomegalovirus antiviral resistance mutations, natural polymorphisms, and uncharacterized genetic variants in immunocompromised patients with clinically resistant cytomegalovirus infection.

2. Quantification of SARS-CoV-2 normalized viral loads in respiratory samples to study the dynamics of total viral RNA.

3. Determination of SARS-CoV-2 replicative capacity during the infection course by the presence of subgenomic RNA, and its broad applicability on the patients' clinical monitoring.

4. Assessment of patients with persistent SARS-CoV-2 replication and/or severe COVID-19 treated with remdesivir.

5. Search of mutations associated with remdesivir failure by next-generation sequencing in severe COVID-19 patients.

MATERIAL AND METHODS AND RESULTS

The materials and methods and results obtained in the thesis are presented as a compendium of research articles:

1. **Santos Bravo M**, Plault N, Sánchez Palomino S, Mosquera Gutierrez MM, Fernández Avilés F, Suarez Lledo M, Sabé Fernández N, Rovira M, Alain S, Marcos Maeso MÁ. Phenotype and Genotype Study of Novel C480F Maribavir-Ganciclovir Cross-Resistance Mutation Detected in Hematopoietic Stem Cell and Solid Organ Transplant Recipients. Journal of Infectious Diseases. 2021 Sep 17;224(6):1024-1028. **Objectives 1.1; 1.2.**

2. **Santos Bravo M,** Plault N, Sánchez Palomino S, Rodriguez C, Navarro Gabriel N, Mosquera Gutierrez MM, Fernandez Avilés F, Suarez-Lledó M, Rovira M, Bodro M, Moreno A, Linares L, Cofan F, Berengua C, Esteva C, Cordero E, Martin-Davila P, Aranzamendi M, Pérez Jiménez AB, Vidal E, Fernández Sabé N, Len O, Hantz S, Alain S, Marcos MA, REIPI, GESITRA. Genotypic and phenotypic study of antiviral resistance mutations in refractory cytomegalovirus infection. (Sent for publication). **Objectives 1.1; 1.2; 1.4.**

3. **Santos Bravo M**, Tilloy V, Plault N, Palomino SS, Mosquera MM, Navarro Gabriel M, Fernández Avilés F, Suárez Lledó M, Rovira M, Moreno A, Linares L, Bodro M, Hantz S, Alain S, Marcos MÁ. Assessment of UL56 Mutations before Letermovir Therapy in Refractory Cytomegalovirus Transplant Recipients. Microbiology Spectrum. 2022 Apr 27;10(2):e0019122.. **Objective 1.3.**

4. **Santos Bravo M,** Nicolás D, Berengua C, Fernandez M, Hurtado JC, Tortajada M, Barroso S, Vilella A, Mosquera MM, Vila J, Marcos MA. Severe Acute Respiratory Syndrome Coronavirus 2 Normalized Viral Loads and Subgenomic RNA Detection as Tools for Improving Clinical Decision Making and Work Reincorporation. Journal of Infectious Diseases. 2021 Oct 28;224(8):1325-1332. **Objectives 2 and 3.**

5. **Santos Bravo M,** Berengua C, Marín P, Esteban M, Rodriguez C, Del Cuerpo M, Miró E, Cuesta G, Mosquera M, Sánchez-Palomino S, Vila J, Rabella N, Marcos MÁ. Viral Culture Confirmed SARS-CoV-2 Subgenomic RNA Value as a Good Surrogate Marker of Infectivity. Journal of Clinical Microbiology. 2022 Jan 19;60(1):e0160921. **Objective 3.**

6. Camprubí D, Gaya A, Marcos MA, Martí-Soler H, Soriano A, Mosquera MDM, Oliver A, **Santos M**, Muñoz J, García-Vidal C. Persistent replication of SARS-CoV-2 in a severely immunocompromised patient treated with several courses of remdesivir. Int Journal of Infectious Diseases. 2021 Mar;104:379-381.**Objective 4.**

7. **Santos Bravo M**, Alonso R, Soria D, Sanchez-Palomino S, Sanzo Machuca A, Alcami J, Diez F, Fernandez Avilés F, Bodro M, Rubio E, Villanueva JL, Mosquera MM, Vergara A, Martinez MJ, Soriano A, Marcos MA. Genetic study of SARS-CoV-2 nsp12 in non-responders COVID-19 patients to remdesivir. (Sent for publication). **Objective 4 and 5.**

The Journal of Infectious Diseases

BRIEF REPORT

Phenotype and Genotype Study of Novel C480F Maribavir-Ganciclovir Cross-Resistance Mutation Detected in Hematopoietic Stem Cell and Solid Organ Transplant Recipients

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Two transplant recipients (1 kidney and 1 hematopoietic stem cell) received maribavir (MBV) after cytomegalovirus (CMV) infection clinically resistant to standard therapy. Both patients achieved CMV DNA clearance within 30 and 18 days; however, the *UL97* C480F variant emerged, causing recurrent CMV infection after a cumulative 2 months of MBV and 15 or 4 weeks of ganciclovir treatment, respectively. C480F was not detected under ganciclovir before MBV treatment. Recombinant phenotyping showed that C480F conferred the highest level of MBV resistance and ganciclovir cross-resistance, with impaired viral growth. Clinical follow-up and genotypic and phenotypic studies are essential for the assessment and optimization of patients with suspected MBV resistance.

Keywords. cytomegalovirus; CMV; antiviral drug resistance; maribavir; ganciclovir; transplantation.

Antiviral therapy for cytomegalovirus (CMV) plays an important role in the clinical management of solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients. However, CMV antiviral therapy can be complicated by drug resistance–associated mutations in the phosphotransferase UL97 and the DNA polymerase UL54 [1]. New therapeutic molecules (letermovir, maribavir [MBV], and brincidofovir) have been developed against different pharmacologic targets; however, resistance mutations have already been described [2].

MBV is a phase 3 antiviral inhibitor of CMV DNA synthesis, viral gene expression, encapsidation, and viral capsid egress by inhibition of pUL97 [3]. Phase 2 trials using high MBV doses for the treatment of CMV infection refractory or resistant to conventional therapy have shown plasma CMV DNA clearance after 6 weeks in 67%–77% of patients. However, about 30% presented CMV recurrent infection with *UL97* mutations conferring MBV resistance [4].

MBV resistance mutations have primarily been mapped to the *UL97* gene and also show compensatory mutations in *UL27* [5]. Only a few mutations in *UL97*, such as the recently described C480F, cause cross-resistance to ganciclovir (GCV) [6].

Our study emphasizes that clinical follow-up, in combination with genotypic and phenotypic studies, is essential for the assessment and optimization of patients with suspected maribavir resistance. In this study we report MBV-GCV resistance mutations and their associated phenotype in the context of 2 transplant recipients.

MATERIALS AND METHODS

Sample Collection

Clinical isolates were obtained from 1 HSCT recipient from the Hospital Clinic of Barcelona (Spain), where MBV was approved for compassionate use, and 1 kidney transplant recipient from the Hospital of Bellvitge (Barcelona, Spain), who was enrolled in the MBV study (Shire, SHP620-303), both with suspected resistance to antiviral agents [7]. Plasma samples and biopsies were sent to the Hospital Clinic of Barcelona to undergo genotypic assays. Plasma CMV monitoring, treatment follow-up, and sample collection and storage were performed as described previously [8].

CMV Viral Load Measurement

Extraction of DNA was performed in MagNA Pure Compact (Roche, Switzerland). The CMV viral load was measured by quantitative real-time polymerase chain reaction (PCR) Cobas CMV (Roche, Switzerland) according to the manufacturer's instructions.

Genotypic Antiviral Resistance Testing

Genotypic testing was based on PCR amplification of CMV *UL97* in the 2 fragments associated with resistance mutations to GCV (residues 400–670) and MBV (270–482) and in 4 fragments of *UL54* (300–1000), followed by Sanger sequencing as described elsewhere [8]. *UL27* was genotyped (284–602) by Sanger sequencing using the primers described by Chou et al [6].

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Phenotypic Assay by Recombinant Bacterial Artificial Chromosome Technology

UL97 C480F mutation, with an unknown phenotype at the time of genotypic detection, was individually tested at the French National Reference Center for Herpesviruses (Limoges) using a phenotypic assay with recombinant bacterial artificial chromosomes (BAC) technology as described previously [9]. C480F single mutation was introduced by "en passant" mutagenesis into a human cytomegalovirus (HCMV) BAC [10] containing an enhanced green fluorescent protein (EGFP) gene in the unique short region derived from the AD169 laboratory strain (provided by M. Messerle). The recombinant BAC was transfected into MRC-5 cells (bioMérieux, Lyon, France) using the liposomal reagent Transfast (Promega, Madison, Wisconsin) following the manufacturer's instructions. The presence of the mutation was confirmed by Sanger sequencing.

A focus reduction assay in 48-well MRC-5 culture plate with a multiplicity of infection (MOI) of 0.01 was used to assess antiviral susceptibility in triplicate to MBV, GCV, and foscarnet (FOS), and 50% inhibitory concentrations (IC_{50}) of the mutant were compared to those of the wild-type control HCMV BAC.

To estimate the impact of the C480F mutation on viral fitness, the recombinant strain and the AD169-EGFP wild-type control were inoculated into 48-well MRC-5 culture with an MOI of 0.01. The number of fluorescent cytopathic foci was counted from days 1 to 8 postinoculation to establish viral growth curves for each recombinant.

Ethical Approval

This study was approved by the Ethical Committee of Hospital Clinic of Barcelona (reference number HCB/2018/0634) as the reference committee for the participating hospitals endorsed by the Grupo de Estudio de la Infección en el Trasplante (GESITRA) according to the Guidance on Good Clinical Practice (CPMP) and the International Conference on Harmonisation (ICH) CPMP/ICH/135/95 regulations. Patient consent was obtained for inclusion in the study.

RESULTS

Clinical Follow-up of Cases With the Novel C480F Mutation

Patient 1 was a 38-year-old CMV-seropositive (R^+) woman who received a myeloablative allogenic HSCT from a CMV-seronegative (D^-) sibling donor due to acute myeloid leukemia (Figure 1A). Prophylaxis for graft-vs-host disease (GVHD) consisted of tacrolimus. At day 41 after transplant, the patient experienced diarrhea and abdominal pain. Lower gastrointestinal (GI) endoscopy revealed diffuse edema and multiple small ulcers in the colon. Gland apoptosis showed histological evidence of acute GI-GVHD. The patient was treated with prednisone 1 mg/kg every 24 hours and beclomethasone 2 mg every 6 hours showing clinical improvement but on day 45, CMV replication was detected in plasma, and valganciclovir (VGCV) 900 mg every 12 hours was started. After 1 week, VGCV was discontinued because of severe cytopenia, and FOS 60 mg/kg every 12 hours was administered for 1 week, resulting in CMV DNA clearance. Two months later, the patient was diagnosed with GI-CMV disease and treated with GCV 5 mg/kg every 12 hours. After 3 weeks, GCV was discontinued due to the development of cytopenia. Since viral loads were not reduced, MBV 400 mg every 12 hours was started and followed for 6 weeks with adequate CMV clearance. One week after stopping MBV, CMV reactivation was detected and MBV was restarted. After 3 weeks of MBV, there was a progressive viral load increase, and resistance mutation genotyping showed the emergence of the *UL97* C480F variant, with a phenotype unknown at that time, in addition to the *UL54* F669L sensitive polymorphism. The patient was then treated with GCV 2.5 mg/kg every 12 hours and again switched to FOS 60 mg/kg every 24 hours for cytopenia until death caused by multiorgan failure related to uncontrolled GI-CMV disease on day 258.

All of the plasma and biopsy samples obtained from the initial CMV replication until patient death were retrospectively genotyped. The naturally occurring polymorphism *UL54* F669L was detected in all of the isolates. Two plasma samples showed previously uncharacterized *UL54* mutations (Y601H, H600P) under GCV treatment on days 136 and 139. Neither mutation was detected in the subsequent esophageal or duodenum biopsies or in plasma samples.

UL97 C480F was first found in a plasma sample (962 IU/mL) 72 days after the first MBV treatment, which is 13 days after the second treatment with MBV. This variant emerged and spread under MBV, GCV, and FOS treatments, being detected in every plasma sample and colon and gastric biopsy from that time until death.

No mutation was detected in *UL27*, which is also related to MBV resistance. No resistance mutation to VGCV-GCV, FOS, or MBV was found at baseline. All fragments were verified with bidirectional sequencing of the PCR product for all target genes with technical and biological repetitions.

Patient 2 was a 44-year-old woman who received a third D^+R^+ kidney transplant due to chronic kidney failure (Figure 1B). Prophylactic treatment during the first 3 months included VGCV (450 mg every 72 hours increased to 900 mg every 24 hours after normalization of kidney function), thymoglobulin, polyclonal antibodies, and corticoids. Maintenance therapy consisted of mycophenolate, tacrolimus, and prednisone. One month after the end of primary prophylaxis, the first CMV viral load (9885 IU/mL) was detected with no associated symptoms, and VGCV 900 mg every 12 hours was started. Clinical symptoms (diarrhea) and an increase in viremia appeared 9 days posttherapy and was treated with GCV 5 mg/kg every 12 hours. Viremia continued to increase (74 687 IU/mL) after 2 weeks on GCV treatment, and GCV was replaced by MBV 400 mg every 12 hours for 2 months until clearance.

Sixteen days after MBV discontinuation, asymptomatic viremia was determined (58652 IU/mL) and a resistance mutation

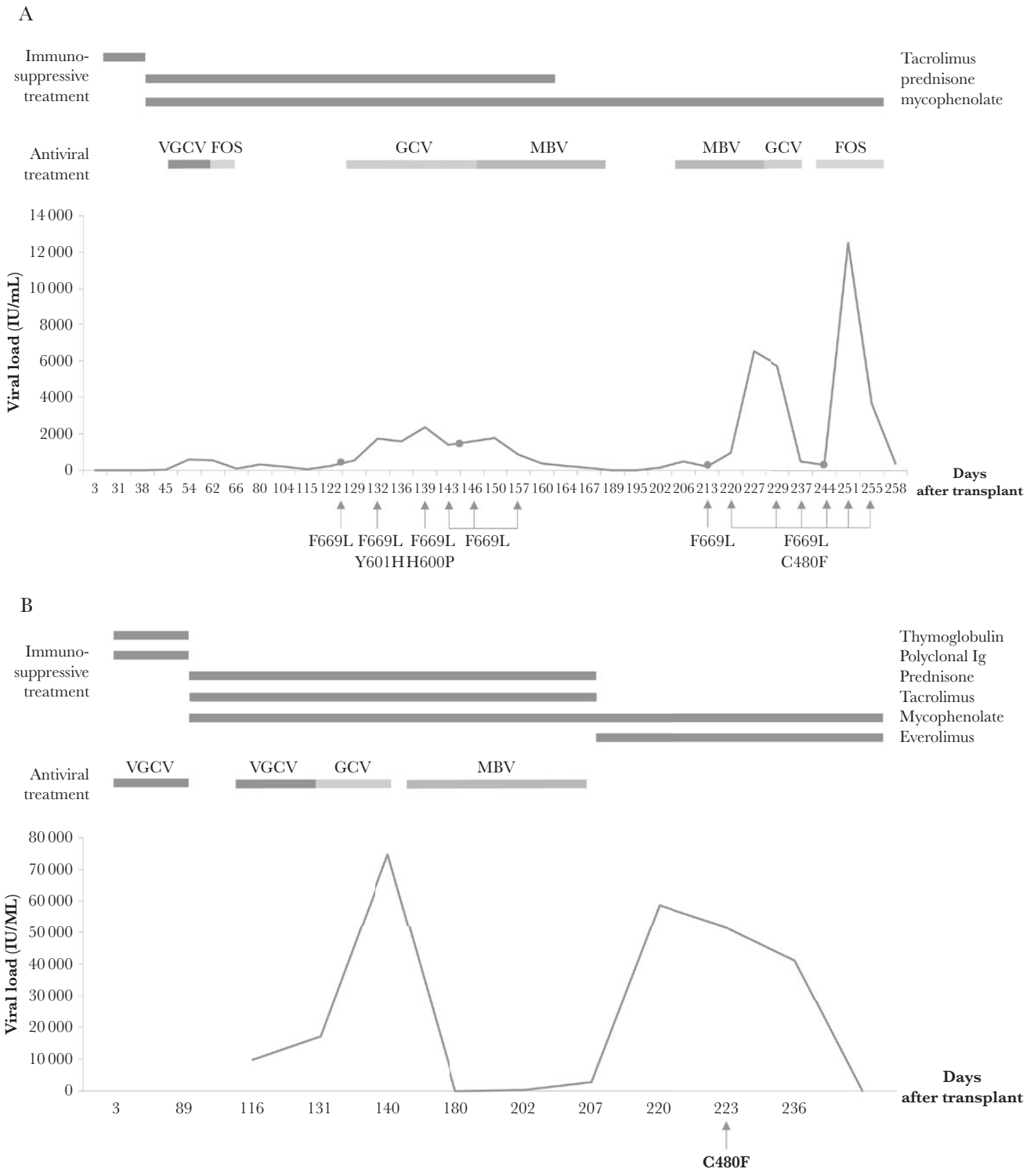

Figure 1. Clinical follow-up of patient 1 (*A*) and patient 2 (*B*). Viral loads were tracked against days after transplantation. Immunosuppressive and antiviral treatment outset and end are indicated. Genotypic assays were performed in the clinical isolates indicated with an arrow. Gastrointestinal biopsies are indicated with a dot; substitutions found in the *UL54* and *UL97* (bold) genes are shown below. Abbreviations: FOS, foscarnet; GCV, ganciclovir; Ig, immunoglobulin; MBV, maribavir; VGCV, valganciclovir.

study was requested. C480F was detected in *UL97*. No amino acid changes were found in *UL54* or *UL27*. However, no specific anti-CMV drug was prescribed owing to the lack of response to previous episodes, and immunosuppression treatment was modified to everolimus and a reduction in mycophenolate until the loss of viremia without CMV-associated clinical outcomes. Unfortunately, clinical isolates from this patient could not be recovered for retrospective genotypic studies.

Phenotypic Analysis of the C480F Mutation

Recombinant virus with the novel C480F mutation was constructed from the standard AD169-BAC with the *EGFP* reporter gene. C480F was verified by sequencing of the *UL97* fragment. The IC_{50} values for the C480F mutant were compared with the AD169 control strain for GCV, FOS, and MBV (Supplementary Table 1). C480F IC_{50} demonstrated resistance to GCV, but for MBV could not be calculated as MBV concentrations did not reduce the foci to 50%, clearly demonstrating a pattern of high-level resistance (Figure 2A). This assay confirmed that C480F conferred high resistance to MBV and GCV cross-resistance.

Growth assay showed a slower replication of the C480F variant compared with AD169, demonstrating deteriorated viral fitness (Figure 2B).

DISCUSSION

This study describes 2 clinical cases in which the recently reported MBV-GCV cross-resistance mutation C480F was found under MBV therapy. Both patients achieved CMV DNA clearance within 18 days (patient 1) and 30 days (patient 2) under MBV treatment; however, the C480F variant emerged, causing recurrent CMV infection detected 13 days after the initiation of a second MBV therapy and 16 days after 2 months of MBV, respectively. Both patients received 400 mg every 12 hours of MBV, a dosage that presented similar efficacy to that of VGCV for clearance CMV viremia. However, a higher dosage related to resistance development has been reported [4, 11].

Previous trials inquired whether C480F was selected under GCV or MBV [6]. In the present study, C480F was not detected under GCV treatment before the initiation of MBV, and it was not present at baseline before GCV or any anti-CMV drug in patient 1. The cumulative duration of treatment before clinical resistance associated with the emergence of this mutant was 2 months of MBV for both patients, 1 month of VGCV/GCV for patient 1, and nearly 4 months of VGCV/GCV for patient 2.

The C480F substitution is located in the catalytic loop of *UL97*, outside the codon range of the ATP-binding site, specified for GCV resistance mutation studies in most laboratories. GCV resistance mutations cluster in the *UL97* domains involved in substrate recognition (residues 460, 520, and 590–607), whereas the majority of mutations located in the vicinity of the ATP-binding site (residues 353–397 and 409–411) confer resistance to MBV but not to GCV [4]. Most commonly selected after exposure, MBV or GCV resistance mutations do not confer cross-resistance to the other [12]. However, only a few studies have reported GCV-MBV cross-resistance mutations in clinical specimens, such as V466G, P521L, or the novel C480F, which were found at distant sites from the ATP-binding loop [13, 14].

The C480F mutation has previously been detected in a patient treated with MBV [14] but was not phenotyped. The same mutation was found in the 2 transplant recipients in this study, and phenotyping of the uncharacterized C480F mutation demonstrated that it confers the highest level of resistance to MBV ever found in addition to GCV cross-resistance, similar to what Chou et al recently described [6].

Genotyping artifacts must be considered when interpreting single-nucleotide mutations. We sequenced the 2 known resistant regions for MBV and GCV of *UL97* to have a widely overviewed genotype. Using bidirectional sequencing, we verified all of the variants found in technical replicates and in different biological samples during the follow-up of the 2 study subjects. Clinical follow-up is highly recommended for routine diagnosis due to the possible sudden emergence of mutants and the limited sensitivity of Sanger sequencing for detecting small populations.

The C480R substitution has also previously been reported in AD169 exposed to the methylenecyclopropane analogue

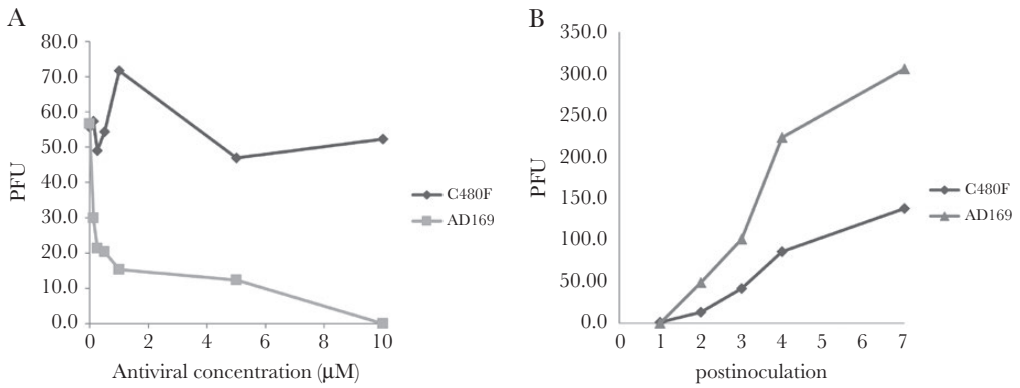

Figure 2. Phenotypic assay of the C480F mutation. The C480F recombinant cytomegalovirus (CMV) strain was compared with an AD169 CMV control strain. *A*, Maribavir plaque reduction curves. Plaque-forming units (PFUs) were counted at day 5 postinoculation. Viral susceptibility was assessed in triplicate. *B*, Comparative growth curves of viral strains. Both strains were inoculated at an equal multiplicity of infection of 0.01. PFUs were counted from days 1 to 7 postinoculation. Data shown are the mean of 3 replicates set up simultaneously.

conferring GCV-MBV cross-resistance and severe growth defects [15], such as those observed with C480F. These data are consistent with the fact that the residue 480 is essential for UL97 phosphotransferase function and HCMV replication.

Mapping of the *UL97* mutation that confers resistance to MBV and GCV may be useful for designing alternative UL97 inhibitor antivirals to avoid cross-resistance. Expanding the scope of standard diagnostic genotyping (residues 355–680) can provide better insight into the frequency of genetically induced cases of drug resistance with poor response to antiviral treatment and the incidence of each resistance mutation. Estimating the phenotypes of new *UL97* mutations from similar or adjacent mutations is inadvisable, and the phenotyping of uncharacterized mutations is essential not only for clinical significance but also for determining levels of resistance.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Running title: Cytomegalovirus antiviral resistance

Title: Genotypic and phenotypic study of antiviral resistance mutations in refractory cytomegalovirus infection

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ABSTRACT

This study describes the genotypic and phenotypic characterisation of novel human cytomegalovirus (HCMV) genetic variants of a cohort of 94 clinically-resistant HCMV patients. Antiviral-resistant mutations were detected in the *UL97*, *UL54* and *UL56* target genes of 27/94 (28.7%) patients. The genotype-phenotype correlation study resolved the status of 5 uncharacterised UL54 DNA polymerase (G441S, A543V, F460S, R512C, A928T) and 1 UL56 terminase (F345L) mutations found in clinical isolates. A928T conferred high triple-resistance to ganciclovir, foscarnet and cidofovir, and A543V had 10-fold reduced susceptibility to cidofovir. Viral growth assays showed G441S, A543V and F345L impaired viral growth capacities compared with wild-type AD169 HCMV. 3D modelling predicted A543V and A928T phenotypes but not R512C, reinforcing the need for individual characterisation of mutations by recombinant phenotyping. Extending mutation databases is crucial to optimize treatments and to improve the assessment of patients with resistant/refractory HCMV infection.

Keywords: cytomegalovirus, antiviral drugs, resistant mutations, phenotype, genotype.

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BACKGROUND

Characterisation of human cytomegalovirus (HCMV) antiviral drug resistance mutations has contributed to improving HCMV therapy and to the knowledge of viral proteins that serve as new antiviral targets. Ganciclovir (GCV), its oral prodrug valganciclovir (VGCV), foscarnet (FOS) and cidofovir (CDV) target the viral UL54 DNA polymerase and are currently licensed for treatment of HCMV infection [1, 2]. Maribavir (MBV) is an inhibitor of UL97 phosphokinase undergoing a phase 3 clinical trial, however, the FDA has not yet approved MBV for patients with post-transplant HCMV infection who do not respond to the antivirals available [3]. Letermovir (LMV) targets the viral terminase complex (*UL51, UL56, UL89*) and has recently been approved for primary prophylaxis of HCMV infections in allogenic haematopoietic stem-cell transplant (HSCT) recipients [4]. HCMV antiviral resistance is an underestimated emergent problem, especially in transplant recipients, presenting an incidence of 5-12% [5].

Resistance to VGCV/GCV, as first-line therapy, is most commonly due to mutations in *UL97* involved in the initial phosphorylation of GCV [6]. MBV resistance mutations have primarily been mapped to this gene and show compensatory mutations in *UL27* [7]; however, only a few mutations in *UL97* cause MBV-GCV cross-resistance [8, 9]. Mutations in *UL54* are associated with resistance to FOS and CDV and can also appear after prolonged GCV therapy, contributing to a high level of resistance to GCV and inducing cross-resistance to FOS or/and CDV [10]. Resistance mutations are mainly located in the conserved domains of the polymerase, whereas natural polymorphisms appear in the highly variable regions located between domains delta-C and II and between domains III and I [11]. Conversely, LMV resistance mutations are mainly described in the *UL56* terminase subunit and rarely in *UL89* and *UL51* [12].

Confirmation of antiviral resistance is based upon the detection of viral mutations that confer drug resistance by genotypic antiviral resistance testing, providing timely data to facilitate clinical decision making. However, the accuracy of genotypic antiviral resistance testing depends on validated databases linking specific mutations with levels of drug resistance. 3D protein modelling provides useful prediction of crucial residues for protein-antiviral molecules interaction [13]. Nevertheless, recombinant phenotyping is the reference method to confirm the level of antiviral resistance and the impact of individual mutations on viral growth.

This study describes the incidence of mutations under GCV, FOS, CDV, LMV and MBV therapy in patients with refractory HCMV infection. We also aimed to phenotype previously uncharacterised HCMV genetic variants detected by genotypic methods by 3D protein modelling and recombinant phenotyping.

MATERIALS AND METHODS

Study specimens and population

Positive samples from patients with refractory HCMV infection (n=94) who fulfilled criteria of suspicion of resistance to antiviral treatment (VGCV/GCV, FOS, CDV, LMV, MBV) were included from April 2012 until September 2021 [1]. Antiviral treatment was administered according to the clinical judgement of the attending physician. The study population has been enlarged from a cohort previously published for a different propose [14]. The patients belonged to the hospitals included in the Spanish Network for Research in Infectious Diseases (REIPI) and the Group for the Study of Infection in Transplantation (GESITRA). All samples were collected on suspicion of antiviral resistance, frozen at -80ºC and sent to the coordinating centre (Hospital Clinic of Barcelona, Spain) for performing genotypic antiviral resistance testing.

HCMV load quantification

The HCMV load was measured in liquid samples by quantitative real time polymerase chain reaction (qPCR) in a Cobas 6800 (Roche, Switzerland) according to the manufacturer's instructions. Viral load of gastrointestinal biopsies was quantified by qPCR (Q-CMV Real Time Complete Kit; Nanogen Advanced Diagnostics, Buttigliera, Italy) using a 7300 Real Time PCR System (Applied Biosystems).

Genotypic antiviral resistance testing

Extraction of total nucleic acids from liquid samples was performed in MagNA Pure Compact (Roche, Switzerland), and with the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) in paraffin-embedded tissue and fresh tissue according to the manufacturer's specifications and using the Bio-Robot EZ1 (Qiagen).

Genotypic testing was done by Sanger sequencing based on PCR amplification of HCMV *UL97* (residues 270–670), *UL54* (300-1000) and *UL56* (180-395) regions. These regions correspond to resistance-associated domains and were sequenced using previously described primers and procedures [14, 15]. Each amplicon was bidirectionally sequenced to avoid artifacts. Sequences were analysed and aligned using the MEGA v.7. software [16] and were compared with the HCMV TB40 strain [GenBank: MF871618.1] using the MRA-Mutation Resistance Analyzer tool provided by the University of Ulm [17].

Prioritization of sequence variants for phenotyping

The gene position of the variants found was used to establish priority for phenotyping. Mutations located at the two previously characterised hyper-variable non-conserved regions

located at residues 614-697 and 874-898 of *UL54* [10] are shown in Table S1 and did not undergo phenotyping.

Phenotypic assay by recombinant bacterial artificial chromosome technology

Mutations with previously uncharacterised phenotypes at the time of genotypic detection were individually tested at the French National Reference Centre for Herpesviruses (Limoges, France) and the Hospital Clinic of Barcelona (Barcelona, Spain) using a phenotypic assay with recombinant bacterial artificial chromosome (BAC) technology as described previously [18]. Each mutation was introduced by "en passant" mutagenesis into a HCMV BAC [19] containing an enhanced green fluorescent protein (EGFP) gene in the unique short region derived from the AD169 laboratory strain (provided by M. Messerle). The recombinant BAC was transfected into MRC-5 cells (bioMérieux, Lyon, France) using the liposomal reagent Transfast (Promega, Madison, Wisconsin) following the manufacturer's instructions. The presence of the desired mutation was confirmed by Sanger sequencing.

A focus reduction assay in a 48-well MRC-5 culture plate with a multiplicity of infection (MOI) of 0.01 was used to assess antiviral susceptibility in triplicate to GCV, FOS, CDV, and/or LMV according to the treatment received by the patient. The half maximal effective concentration (EC_{50}) of the mutant was compared to that obtained for the wild-type control HCMV BAC.

To estimate the impact of each mutation on viral growth, the recombinant strain and the AD169-EGFP control were inoculated into 48-well MRC-5 culture with an MOI of 0.01. The number of fluorescent plaque-forming units (PFU) was counted from days 1 to 4 and on day 7 post-inoculation to establish viral growth curves for each recombinant.

Structure of the protein 3D model

The theoretical structure of the UL54 DNA polymerase was built by homology modelling with the standalone version of MODELLER 9.9 [20]. The UL54 sequence was aligned with three templates as described previously [21]. Sequence alignment included primary structures of the UL52 homolog from herpes simplex virus-1 (HSV-1) (PDB: 2GV9), the C-terminal part of UL54 taken from PDB: 1YYP (i.e., complex of UL44 with fragment 1223-1242 of UL54), and the exonuclease domain (i.e. amino acid sequence 109-342), metal ions and DNA duplex of PDB: 1CLQ. UL54 moieties aligned to non-resolved loops of UL52 were retrieved from sequence alignment for calculations. A fast molecular dynamic optimisation implemented in MODELLER was applied to each 100 calculated structures. Quality structures were assessed by calculating their Q-mean score on a dedicated web server [22]. For *UL56* mutations, the model with an

herpes simplex virus-1 homolog did not allow localising the mutations concerned as there was no correspondence with the amino acid sequence.

Ethical approval

This study was approved by the Ethical Committee of the Hospital Clínic of Barcelona (ref. nº HCB/2018/0634) as the reference committee for all the participating hospitals endorsed by GESITRA according to CPMP/ICH/135/95 regulations. All the patients included in the study provided signed informed consent.

RESULTS

Overview of *UL54*, *UL56* and *UL97* sequence variation under treatment

This study comprised a cohort of 94 patients with refractory HCMV infection who fulfilled the criteria of suspicion of HCMV resistance to standard antiviral treatment (GCV, FOS, CDV). Seven patients additionally received LMV and 4 MBV as salvage therapy. One clinical sample from each patient was collected at the time of suspicion to perform genotypic antiviral resistance testing. Subjects with previously characterised mutations associated with either resistance or sensitive response to antivirals located in the target *UL97, UL54* and *UL56* genes were classified according to their clinical history and sample type (table 1).

Previously described resistant mutations were detected in 24/94 (25.5%) patients, all of whom were transplant recipients (6 HSCT, 18 solid organ transplant (SOT)): 19 patients had resistant mutations in *UL97* (one presenting two mutations), 2 in *UL54* (one presenting two mutations), 1 patient with mutations in *UL54* and *UL56,* and 2 in *UL54* and *UL97*. These mutations conferred resistance to GCV, FOS, CDV, LMV or MBV, as well as multiple- or cross-resistance (Table 2). Data of cumulative treatment, time from transplantation until the detection of the variant, viral loads of the detection sample and level of resistance to each antiviral are shown in Table 2.

The incidence of natural polymorphisms, previously described to be sensitive to antivirals, was 76/94 (80.9%): 5 (5.3%) patients presented them in *UL97*, 67 (71.3%) in *UL54* and 4 (4.3%) in *UL56* (table 1, table S1).

Fourteen previously undescribed genetic variants were found in 16/94 (17.0%) patients, all located in *UL54* but 1 in *UL56* after LMV therapy (table 3, table S1). Each genetic variant was detected in one patient, except for *UL54* A543V and S883I that were detected in 2 different individuals. Five *UL54* and 1 *UL56* variants located outside hypervariable regions were selected for recombinant phenotyping and 3D protein modelling. The *UL56* P800L substitution was previously described by Champier *et al*. in one LMV-naïve patient [23]. However, as it was

detected in association with a LMV resistance mutation in this study, we wanted to measure its potential impact on decreasing sensitivity to LMV and on viral replicative capacity.

Phenotypic results

For antiviral drug susceptibility assays, the resistance index (RI) of each mutant was calculated by dividing the EC_{50} value (µM) of the mutant strain by the EC_{50} of the AD169 HCMV control strain (Table 3). Replicative capacity assays were performed for each mutant and in parallel with the AD169 for each repetition (Table 3; Figure 1).

UL54 A928T presented a triple-resistant pattern to GCV, FOS, CDV at a high level of resistance that any antiviral concentration could inhibit 50% of the replication of the A928T mutant. *UL54* A543V conferred 10-fold decreased susceptibility to CDV, and impaired growth capacity. The three remaining *UL54* (G441S, F460S, R512C) were sensitive to GCV, FOS and CDV, but G441S involved a defective replicative capacity (Figure 1).

None of the phenotypes of the *UL56* mutations (F345L, P800L) conferred reduced response to LMV, or cross-resistance to the DNA polymerase inhibitors tested. However, both mutations involved lower viral kinetics of replication (Figure 1).

Correlation of newly characterised phenotype mutations with clinical outcomes

UL54 A543V was detected in two kidney transplant recipients in combination with the known GCV-resistant mutation *UL97* M460I (figure 2A). In one patient, this mutation emerged after 90 days of GCV and 9 days of FOS treatment, reaching viral loads of 137000 IU/ml. In the other patient, the mutation was detected after 37 days of prophylaxis with VGCV, with a viral load of 3100 IU/ml. The phenotypic results showed A543V conferred a defective replicative capacity and resistance to CDV (table 3), which was not administered to either subject.

UL54 A928T was detected in a plasma sample with a viral load of 21732 IU/ml in a congenital HCMV individual after 2 HSCT (145 days after the first intervention, and 11 after the second) (figure 2B). This mutation was detected after cumulative treatment of 43 days of GCV and 23 days of FOS. CDV was administered afterwards because of adenovirus reactivation and was maintained until limitation of therapeutic efforts; however, HCMV DNA clearance was not achieved until its death. This clinical unresponsiveness correlated with the *in vitro* phenotypic results of GCV, CDV and FOS multi-resistance.

A *UL56* F345L missense mutation was located within the LMV-resistance region (residues 230-370) (Figure S2) in a HSCT recipient after 209 days of VGCV/GCV and 20 days of LMV but not before LMV therapy. Viral loads were 3 $\log_{10}$ IU/ml and were well-controlled until HCMV DNA cleared after 148 days with LMV (figure 2C). This mutation conferred a highly impaired

replicative capacity and was sensitive to GCV, FOS, CDV and LMV in concordance with the clinical antiviral response and clearance after the detection of F345L (Table 3).

P800L was detected in an HSCT recipient at day 163 post-transplant, 7 days before LMV therapy onset (figure 2D). After 28 days receiving LMV, the high level LMV-resistant mutation C325F emerged together with P800L. Despite discontinuation of LMV, both mutations persisted until the patient developed multiorgan failure.

Follow-up of viral loads and antiviral treatment could not be retrieved for the subject with the *UL54* G441S variant. The clinical data of patients infected with wild-type phenotypes characterised in this study are not presented.

Predicting the phenotype of new UL54 variants by 3D protein modelling

UL54 3D protein modelling showed that the amino acids G441S (figure 3A) and F460S (figure 3C) were localised in non-structured regions of UL54 DNA polymerase. These domains are outside conserved regions. This agrees with the absence of impact on antiviral resistance obtained by phenotypic methods. R512C (figure 3D) and A543V (figure 3B) are part of a helix. Removing one amino acid of a helix can change repartition of amino acids exposed to solvent or the interior of the protein. Both are localised in the Exo-III/delta-C region but are associated with a completely different impact on antiviral resistance as shown by recombinant phenotyping. A928T (figure 3E, figure S1) is part of a helix between region I and VII of the palm domain. The change of an alanine to a threonine impacts both the polarity and the size of the amino acid, modifying the potential interaction between *UL54* and antivirals.

DISCUSSION

The results of this study demonstrate a high incidence (n=27/94, 28.7%) of antiviral-resistant mutations in patients with refractory HCMV infection, which is even higher considering all of them emerged in transplant recipients (n=27/83, 32.5%). Recombinant phenotyping of previously uncharacterised mutations showed that the *UL54* A928T mutation conferred high level resistance to GCV, FOS and CDV, and *UL54* A543V conferred intermediate-resistance to CDV. Additionally, *UL54* G441S, A543V and *UL56* F345L, P800L variants showed lower growth capacities than wild-type AD169 HCMV.

Uncharacterised mutations were selected for the correlation of their gene position to antiviral resistance. G441S and F460S were located in the region associated with GCV and CDV cross-resistance (figure S2), but neither conferred resistance to these antivirals, as predicted *in silico.* In agreement, the F460L variant, located in the same residue, was described to be sensitive (1.2-fold shift) to DNA-polymerase inhibitors and did not show a slow-growth phenotype [4].

A543V and R512C were mutations located in the Exo-III/delta-C domain. This domain has been described as being involved in exonuclease and polymerase catalytic function, which are well-conserved among mammals and yeast, and was associated with resistance to GCV, FOS and CDV (figure S1) [24]. Our study showed A543V conferred CDV resistance and impaired its viral growth, whereas a previously reported A543S mutation had a wild-type phenotype [4], suggesting that when alanine is substituted by valine, its bulky lateral aliphatic chain could block its polymerase function, but the small hydroxyl group of the serine does not. Therefore, residue A543 seems to play an important role in viral growth. However, this variant clearly replicated in the two clinical cases presented (Figure 2A), suggesting an A543V growth defect could be compensated by other mutations, such as *UL97* M460I.

The A928T mutation located just outside the catalytic polymerase function, in a region supposedly not associated with a lack of antiviral response, conferred high resistance to three DNA polymerase inhibitors (figure S1). Previous reports showed certain triple-resistance mutations in the surroundings, such as A836P and del981-2, detected in patients receiving prolonged therapy and causing loss of growth fitness [34]. Conversely, many variants in the nearby amino acids (V902G, E903G, K947E, M959T) were susceptible to GCV, FOS and CDV [4]. This results also strengthens the importance of discerning high from intermediate-low levels of resistance, as this influences the dosages and antiviral molecules chosen in clinical practice.

UL56 mutations emerged in the context of infections unresponsive to standard therapy and the use of LMV as salvage therapy. F345L was located inside the LMV resistance region (figure S2) and strongly impaired viral replication, but surprisingly did not involve a loss of response to the different antiviral molecules tested. This suggests that the F345 residue is critical for biological terminase function but not for interaction with LMV, as has been described for R215, since R215C amino acid substitution conferred advanced growth capacity but not loss of response to LMV [14].

The P800L polymorphism emerged before LMV therapy and remained detectable until the patient's death after 28 days of LMV in association with the C325F resistance mutation (figure 1D), suggesting the need to measure its potential impact on decreasing sensitivity to LMV and on viral replicative capacity. Phenotypic assays showed this mutation impaired virus growth and was confirmed as having no effect on virus sensitivity to LMV. As the P800L+C345F variant continued replicating, the defective growth associated with P800L alone seemed to be compensated by the emergence of C325F, the residue of which appeared to be critical for LMV binding but unimportant for HCMV terminase function.

It has been described that *UL54* mutations can confer multi-resistance and may emerge after a previous *UL97* mutation, enhancing the level of resistance [18], as shown in 4 SOT recipients in our cohort. Nevertheless, this study presents the uncommon detection of 2 *UL54* resistance mutations with no previous change in *UL97* and the combination of mutations in *UL54* together with *UL56* after GCV+LMV therapy.

Fewer natural polymorphisms arose in *UL97* than in *UL54* during standard treatment, supporting the existing literature [6, 11], and hardly any were detected in *UL56,* in agreement with the previously described *UL56* genetic conservation among herpesvirus before LMV therapy [14, 35]. However, the prevalence of natural polymorphisms in *UL56* and *UL97* was understated as full-length genes were not amplified and only the regions associated with drug-resistance were sequenced.

This study was limited by the incapacity to recover previous isolates of the patients infected with the newly characterised variants in order to determine the timing of the earliest emergence of the respective mutations. Phenotyping assays involve long-laborious work and results were obtained months after the genotypic resistance testing was requested.

3D protein modelling is a useful and fast tool to predict the impact of genetic variants *in silico*. It correctly predicted the effect of A928T, A543V, G441S and F460S mutations on antiviral susceptibility, but did not with the R512C mutation, highlighting its limits and the need for confirmation by recombinant phenotyping. Therefore, the remaining genetic variants located in the *UL54* highly variable regions detected in this study require future recombinant phenotyping.

This study reinforces the fact that genotype-phenotype correlation studies not only serve to define the level of antiviral resistance and viral kinetics associated with each genetic variant, but also determine the biological role of each residue and allow constructing comprehensive and reliable mutation maps to help develop new anti-HCMV therapies. The high incidence of HCMV resistance mutations in transplant recipients presents a worrisome scenario for their clinical management. However, early genotypic testing and increasing databases of mutations by phenotyping can optimise treatments and improve the assessment of patients with refractory/resistant HCMV infection.

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Potential conflict of interest

None of the authors report any conflict of interest. All authors have submitted the ICMJE Form for Disclosure of Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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FIGURES

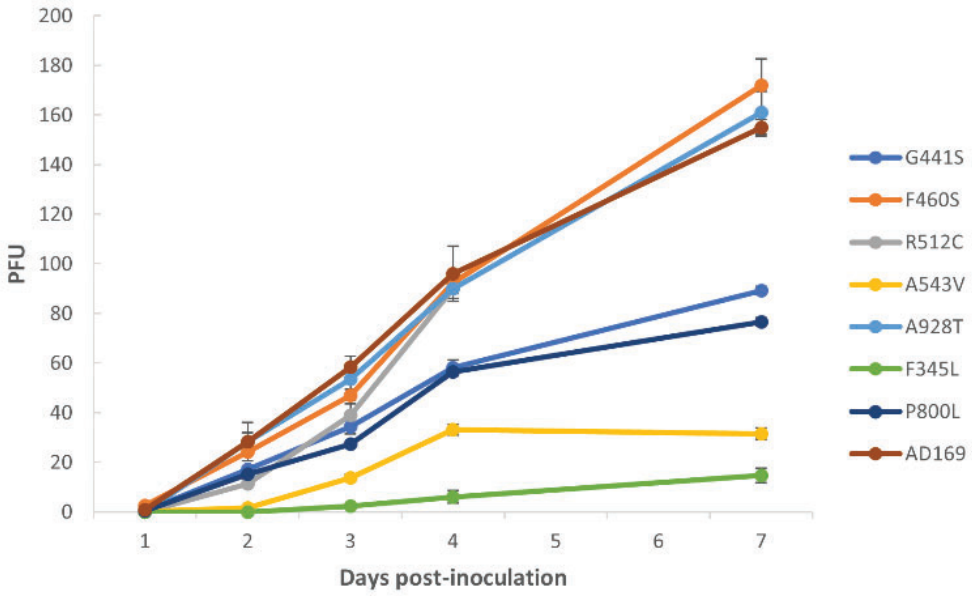

Figure 1. Growth capacity of newly characterized HCMV variants. Each recombinant cytomegalovirus (CMV) strain was compared with an AD169 CMV control strain. Each strain was inoculated at an equal multiplicity of infection of 0.01. Plaque-forming units (PFUs) were counted from days 1 to 4 and on day 7 post-inoculation. Data shown are the mean of 3 replicates set up simultaneously, with the corresponding standard deviation. The AD169 curve is the mean of 7 different experiments (one repetition per variant).

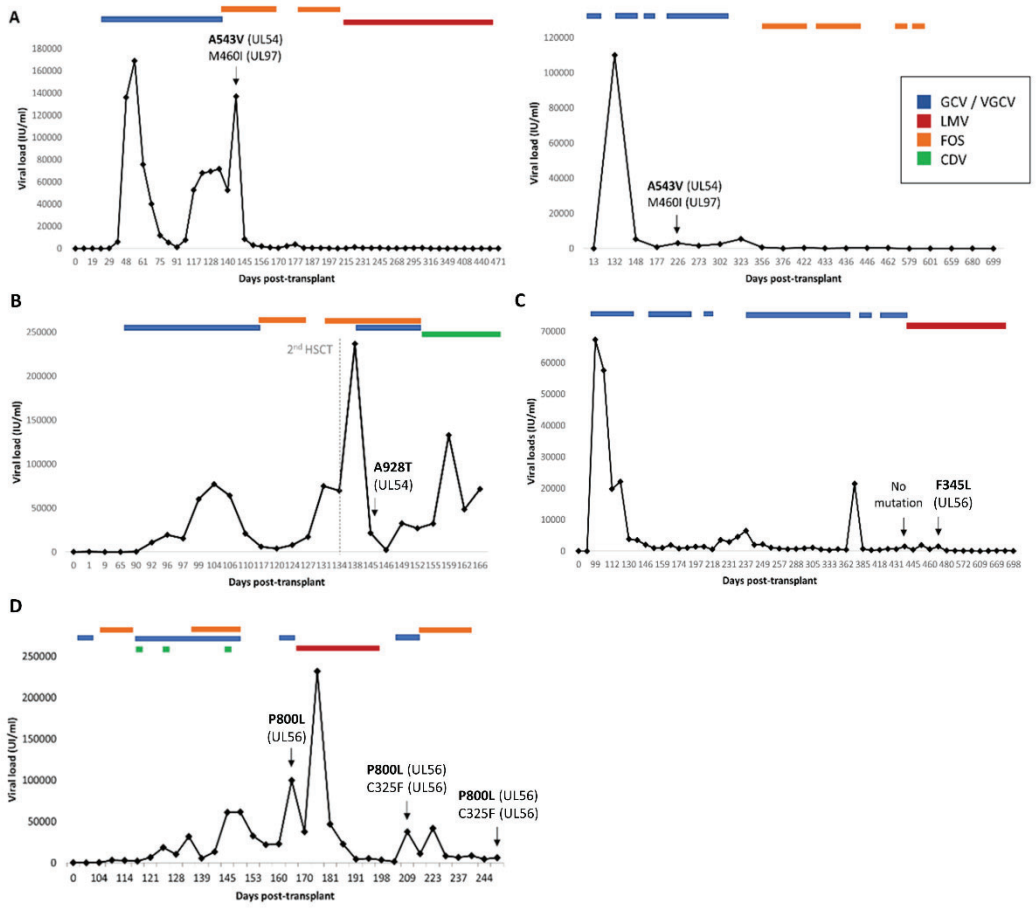

Figure 2. Clinical follow-up of patients with newly characterised phenotype mutations. Viral loads in IU/mL were tracked against days after transplantation. Anti-HCMV treatment outset and end are indicated. Genotypic assays were performed in the clinical samples indicated with an arrow. New phenotype mutations in *UL54* (A-C) and *UL56* (C-D) are indicated in bold, together with previously characterised resistant mutations. Abbreviations: VGCV valganciclovir; GCV ganciclovir; LMV letermovir; FOS foscarnet; CDV cidofovir.

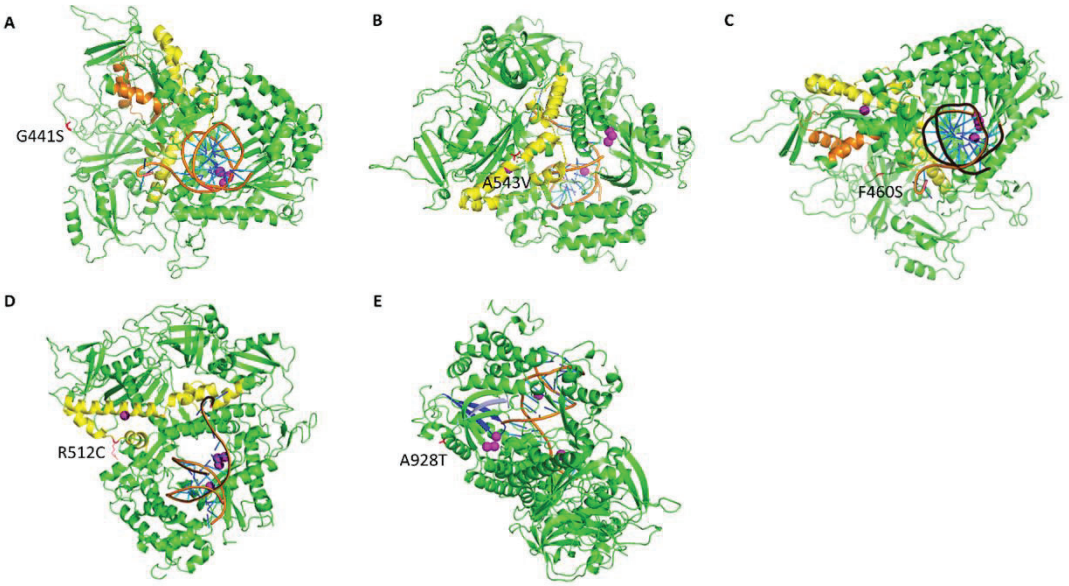

Figure 3. 3D protein models showing the location of newly characterised *UL54* mutations. Theoretical structures of UL54 calculated with MODELLER are represented in cartoon mode. The different domains close to the new mutations are coloured as follows: residues 379 to 421 of region IV/Exo-II in orange, residues 492-588 of region delta-C/Exo-III in yellow, residues 905 to 919 of region I in blue and residues 962 to 970 of region VII in grey. Purple dots in active sites stand represent metal ions. The DNA duplex is coloured in orange and blue. (A) The G441S mutation in red between the Exo-II and Exo-III regions. (B) The A543V mutation in red in the Exo-III region. (C) The F460V mutation in red between the Exo-II and the Exo-III regions. (D) The R512C mutation in red in the Exo-III region. (E) The A928T mutation in red between regions I and VII.

TABLES

Table 1. Clinical history of the study population. Human cytomegalovirus viral loads (IU/ml) and number of subjects with resistance mutations and polymorphisms in the target genes are indicated according to immunosuppression and the type of sample.

Clinical History	**N[a]**	**Resistance mutation**			**Sensitive polymorphisms**		
		UL97	***UL54***	***UL56***	***UL97***	***UL54***	***UL56***
Congenital HCMV	6	0	0	0	0	5	0
HIV	2	0	0	0	1	2	0
IBD	2	0	0	0	0	1	0
CVID	1	0	0	0	0	1	0
Transplant recipients	**83**	**22**	**5**	**1**	**4**	**58**	**3**
HSCT	30	3	3	1	2	23	2
SOT	53	19	2	0	2	35	1
● Heart	11	4	1	0	0	5	1
● Lung	6	4	1	0	0	5	0
● Liver	8	5	0	0	0	6	0
● Kidney	25	5	0	0	2	17	0
● Liver-kidney	1	0	0	0	0	1	0
● Pancreas-kidney	2	0	0	0	0	1	0
Sample type							
● Plasma	84	21	3	1	5	58	3
● whole blood	4	1	1	0	0	4	0
● GI biopsy	5	0	0	0	0	4	0
● Aqueous humour	1	0	0	0	0	1	0
Total	**94**	**22**	**5**	**1**	**5**	**67**	**3**

N[a]: indicates the number of subjects.

Abbreviations: SNP Single Nucleotide Polymorphism, HCMV Human Cytomegalovirus, HIV Human Immunodeficiency Virus, IBD Inflammatory Bowel Disease, CVID Common Variable Immunodeficiency, HSCT haematopoietic stem cell transplant, SOT solid organ transplant, GI Gastrointestinal.

Table 2. Resistance mutations detected in association with current antiviral drugs in the overall study population.

Gene	Mutation	N[a]	Transplant[a]	Days of cumulative treatment[a,b]	Days until detection[c]	Viral load at the detection (IU/ml)[d]	Resistance level[e]	Reference
UL97	M460V	2	HSCT, Liver	19d VGCV/GCV (n=2)	493	5,82E+03	5-10x GCV	[24]
UL97	M460I	2	Kidney (n=2)	69d VGCV (n=2)	184	7,01E+04	5-10x GCV	[25]
UL97	C480F	2	HSCT, Kidney	60d MBV (n=2), 67 GCV (n =2)	222	9,97E+03	2-5x GCV, 223x MBV	[8, 9]
UL97	C592G	1	Kidney	138d VGCV	235	9,87E+03	2-5x GCV, 2-5x FCV	[26]
UL97	A594V	5	Kidney (n=2), liver (n=2), lung	56d (14; 121) VGCV/GCV (n=5), 52d FOS + 51d IgG (n=1)	137 (100; 156)	2,03E+04 (1,20E+04; 3,68E+04)	5-10x GCV	[27]
UL97	A594P	1	Heart	24d GCV	534	1,84E+04	5-20x GCV	[26]
UL97	L595S	5	HSCT, heart, lung (n=2), kidney	38d (20; 54) VGCV/GCV (n=5), 36d FOS + 16d IgG (n=1)	158 (66; 357)	3,87E+04 (2,29E+04; 5,80E+04)	5x GCV	[28]
UL97	L595W + A594V	1	Heart	14d VGCV	110	4,12E+03	5.1x GCV	[28]
UL54	V781I	1	HSCT	38d GCV + 79d FOS + LT	153	1,63E+03	1-4x GCV, 4-5.2x FOS	[29]
UL54	**A928T**	1	cCMV + 2HSCT	43d GCV + 23d FOS	145	2,17E+04	NP	
UL54	L773V + G841A	1	HSCT	35d VGCV/GCV + 45d FOS + 10d ACV	133	3,55E+03	2x GCV, 5x FOS / 3.2x GCV, 2.6x CDV, 4.3x FOS	[30, 31]
UL54 + UL97	D413N + M460I	1	Lung	24d GCV	668	6,20E+04	6.5x GCV, 11x CDV	[32]
UL54 + UL97	**A543V** + M460I	2	Kidney (n=2)	64d VGCV/GCV + 9d FOS	185	7,01E+04	NP /5-10x GCV	[25]
UL54 + UL97	A987G + C603W	1	Heart	150d VGCV prophylaxis + 84d VGCV	238	1,90E+04	6.8x GCV, 5.3x CDV / 8.3x GCV	[33, 29]
UL54 + UL56	T700A + C325F	1	HSCT	33d VGCV/GCV + 74d FOS + 4d CDV + 27d LMV + LT	198	3,41E+03	4.7x FOS / >3000x LMV	[31, 34]

[a] N: number of patients infected with the HCMV mutant indicated, receiving the transplant type, receiving the treatment indicated.
[b] Days of cumulative treatment until the time of mutation detection are indicated as the median and the (Q1; Q3) when n>2
[c] Days until detection are calculated from the transplant date until the detection of the variant in the clinical sample by sequencing are indicated as the median (Q1; Q3)
[d] Viral loads of the clinical sample in which the variant was detected are indicated as the median (Q1; Q3) in IU/ml.
[e] Level of resistance is indicated as fold-shift reduction of the effective concentration 50% (EC50) of the mutant compared with the CMV control strain.
New phenotype mutation in this study are indicated in bold and by NP (new phenotype) resistance level.

Table 3. Results of antiviral susceptibility and replicative capacity assays of novel HCMV genetic variants.

Gene	Mutation	N[a]	Patient history	Antiviral susceptibility (RI)[b]			
				GCV	CDV	FOS	LMV
UL54	G441S	1	CVID	0.67 (± 0.24)	1 (± 0.75)	1.13 (± 0.8)	
UL54	F460S	1	HSCT	0.86 (± 0.45)	0.88 (± 0.78)	1.11 (± 0.8)	
UL54	A543V	2	Kidney (2)	1.5 (± 0.1)	**10 (± 5.37)**	1.21 (± 0.27)	
UL54	R512C	1	Kidney	1	1	0.74	
UL54	A928T	1	cCMV + 2 HSCT	**>3**	**>3**	**>3**	
UL56	F345L	1	HSCT	1.42 (± 1.07)	1.67 (± 46)	0.98 (± 0.04)	0.99 (± 0.04)
UL56	P800L	1	HSCT	0.23	0.13	1	1.11

N[a]: number of patients in whom the mutation was detected.

RI[b]: The resistance index is the EC50 value (effective concentration 50% (µM)) for the mutant strain divided by the EC50 of the AD169 HCMV control strain. Data are indicated as the mean of 3 biological repetitions of 3 independent experiments and standard deviations (except for R512C, P800L which involved 3 biological repetitions of a single experiment)

RI >3 is considered drug resistant and is indicated in bold.

Abbreviations: HSCT haematopoietic stem cell transplant, CVID common variable immunodeficiency, GCV ganciclovir, CDV cidofovir, FOS foscarnet, LMV letermovir.

SUPPLEMENTARY DATA

TABLES

Table S1. Genetic variants detected by Sanger sequencing.

Sensible polymorphisms			Uncharacterised variants		
Gene	**Mutation**	**N**	**Gene**	**Mutation**	**N**
UL97	N510S	2	UL54	V615S	1
UL97	V758M	1	UL54	Q639H	1
UL54	S612N	1	UL54	G657D	1
UL54	L655S	35	UL54	S682F	1
UL54	S685N	35	UL54	G687S	1
UL54	A688V	1	UL54	S883I	2
UL54	A692V	1	UL54	E888K	1
UL54	F699L	10	UL54	G889E	1
UL54	V759M	1			
UL54	S771P	1			
UL54	I837V	2			
UL54	G841A	1			
UL54	G874R	1			
UL54	T885A	21			
UL54	Ins885S	3			
UL54	P887S	1			
UL54	L890F	1			
UL54	S895N	1			
UL54	S897L	10			
UL54	D898N	28			
UL56	S227R	1			
UL56	R246C	2			

N: number of patients in whom the variant was observed.

FIGURES

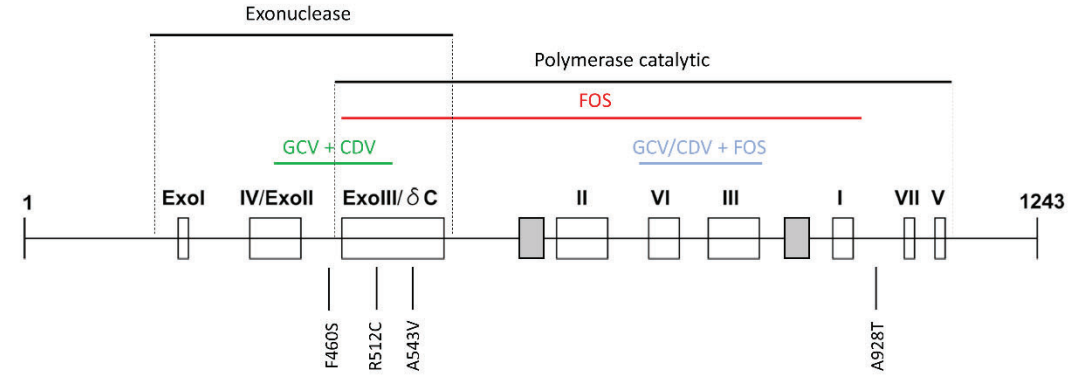

Figure S1. Scheme of the CMV *UL54* gene with novel mutations. Conserved regions are indicated in white boxes (domains I-VII, delta-C); hypervariable regions are indicated in grey boxes. Ranges of codons containing drug resistance mutations to ganciclovir (GCV), foscarnet (FOS) and cidofovir (CDV) are specified. Novel mutations are located between IV-deltaC, within the delta-C domain and between regions I-VII. This gene map is described according to [1].

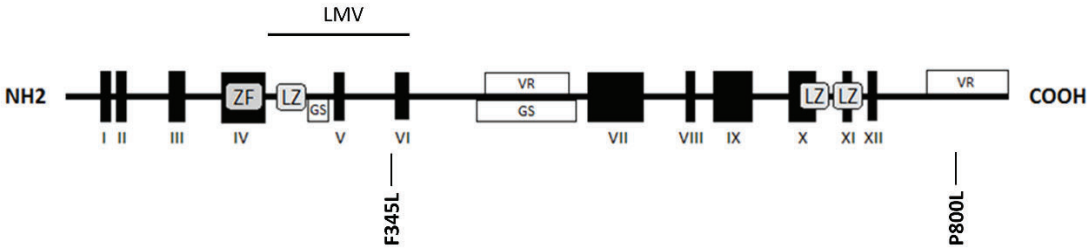

Figure S2. Scheme of the protein UL56 domain organisation with the novel phenotype mutations. Conserved regions are indicated in black boxes (domains I-XII); variable regions (VR) and glycine and serine-rich flexible region (GS) in white boxes; leucine zippers (LZ) and zinc finger domain (ZF), the metal-binding site of which is located in region IV. The region containing mutations associated with resistance to letermovir is indicated [2, 3]. The two novel mutations are located adjacent to domain VI and inside variable region (residues 778-850). The *UL56* genetic structure is based on the previously described scheme [4].

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RESEARCH ARTICLE

Assessment of UL56 Mutations before Letermovir Therapy in Refractory Cytomegalovirus Transplant Recipients

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ABSTRACT *De novo* mutations in the *UL56* terminase subunit and its associated phenotypes were studied in the context of cytomegalovirus (CMV) transplant recipients clinically resistant to DNA-polymerase inhibitors, naive to letermovir. R246C was the only *UL56* variant detected by standard and deep sequencing, located within the letermovir-resistance-associated region (residues 230–370). R246C emerged in 2/80 transplant recipients (1 hematopoietic and 1 heart) since first cytomegalovirus replication and responded transiently to various alternative antiviral treatments *in vivo*. Recombinant phenotyping showed R246C conferred an advanced viral fitness and was sensitive to ganciclovir, cidofovir, foscarnet, maribavir, and letermovir. These results demonstrate a low rate (2.5%) of natural occurring polymorphisms within the letermovir-resistant-associated region before its administration. Identification of high replicative capacity variants in patients not responding to treatment or experiencing relapses could be helpful to guide further therapy and dosing of antiviral molecules.

IMPORTANCE We provide comprehensive data on the clinical correlates of both CMV genotypic follow-up by standard and deep sequencing and the clinical outcomes, as well as recombinant phenotypic results of this novel mutation. Our study emphasizes that the clinical follow-up in combination with genotypic and phenotypic studies is essential for the assessment and optimization of patients experiencing HCMV relapses or not responding to antiviral therapy. This information may be important for other researchers and clinicians working in the field to improve the care of transplant patients since drug-resistant CMV infections are an important emerging problem even with the new antiviral development.

KEYWORDS cytomegalovirus, letermovir, baseline mutations, transplant recipients, phenotype

Human cytomegalovirus (HCMV) is one of the most prevalent infections in solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients. The morbidity and mortality caused by HCMV have been reduced over the last decades thanks to the development of antiviral agents and different strategies to prevent HCMV infection (1). Currently approved DNA polymerase inhibitors (valganciclovir/ganciclovir [VGCV/GCV], cidofovir [CDV], foscarnet [FOS]), are associated with significant toxicities and the emergence of drug resistance (2). The new anti-HCMV drug,

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letermovir (LMV), targets the terminase complex and has not presented cross-resistance with DNA polymerase inhibitors (3).

Mutations conferring resistance to LMV consist of amino acid substitutions located mainly in *UL56*, and rarely in *UL89* and *UL51* terminase subunits (4). They have been described when LMV was administered as salvage therapy of refractory or drug-resistant HCMV infections, and when used as primary or secondary prophylaxis (5, 6). It has been demonstrated that LMV resistance mutations selected *in vitro* appear earlier than for the currently used antiviral drugs (4), suggesting a lower genetic barrier of resistance to LMV. However, there is little information on whether LMV resistance mutations appeared in clinical samples prior or subsequent to LMV administration.

We aimed to determine baseline mutations in *UL56* and its associated phenotypes in the clinical context of HMCV infected SOT and HSCT recipients clinically resistant to the DNA polymerase inhibitors naive of LMV.

RESULTS

Genotypic antiviral resistance testing results. HCMV *UL56* standard sequencing was performed in 80 clinical samples (75 plasma, 2 whole blood, 2 rectal biopsies, 1 aqueous humor) to detect baseline mutations comprising amino acids substitutions before LMV therapy. All samples were from refractory HCMV-infected transplant recipients (30 HSCT; 50 SOT) with resistance suspicion to DNA polymerase inhibitors.

The novel nonsynonymous point mutation R246C was detected in 2 (2.5%) transplant recipients (1 HSCT and 1 heart transplant). R246C is located in the region of *UL56* associated with resistance to LMV (aa 230–370), specifically within the leucine zipper (Fig. S1 in the supplemental material). No nucleotide changes were found in the *UL56* of the remaining patients by standard sequencing.

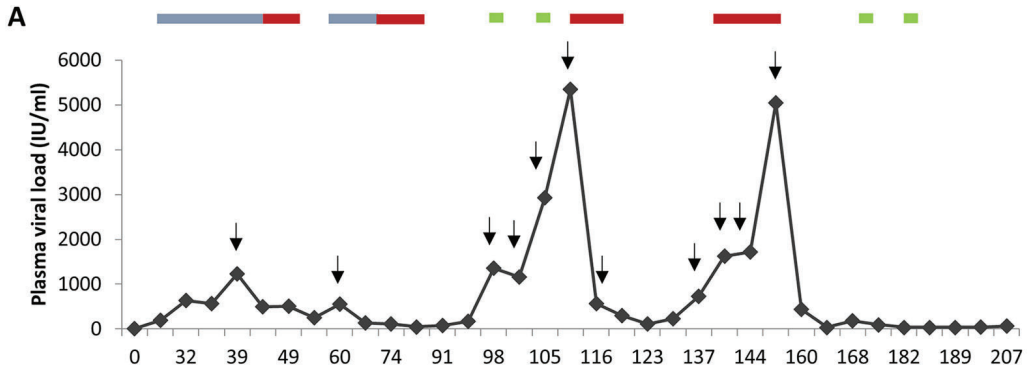
The *UL97* phosphokinase and *UL54* DNA polymerase were sequenced in all samples to test for mutations associated with resistance to the DNA polymerase inhibitors.

Mutations associated with antiviral drug resistance were detected in 21/80 (26.3%) patients: 18 in *UL97* (M460I/V, C480F, C592G, A594P/V, L595S/W) (7, 8), 2 in *UL54* (T700A, V781I) (9, 10), and 1 in *UL97* (M460I) in combination with *UL54* (D413N) (11). None of these subjects coincided with the 2 patients with the *UL56* R246C variant.

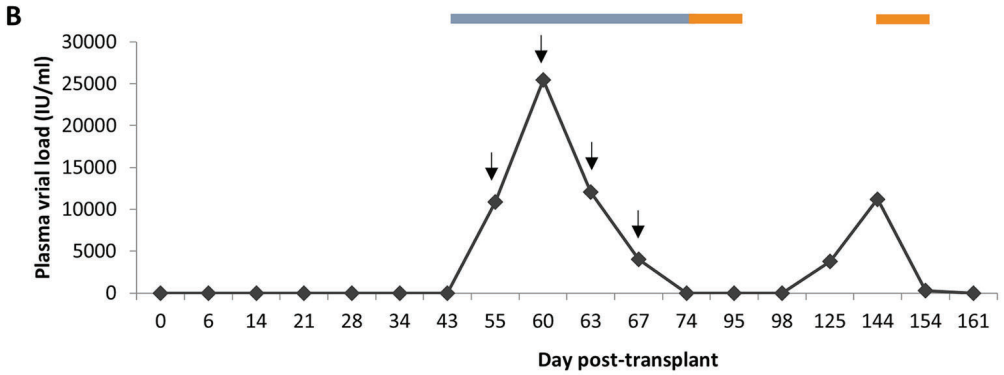
Clinical follow-up of cases with the novel R246C mutation in *UL56*. Patient 1 was a 60-year-old female with chronic myelomonocytic leukemia who underwent D^-R^+ allogeneic HSCT from a mismatch-unrelated donor in the HCB (Fig. 1A). The conditioning regimen was based on fludarabine and busulfan for 3 days, followed by cyclophosphamide and tacrolimus as graft-versus-host-diseases (GVHD) prophylaxis.

The patient presented 4 episodes of HCMV infection. (i) At day 26 post-transplant, HCMV caused primary gastrointestinal (GI) infection which was treated with GCV and then switched to FOS due to myelotoxicity for 10 days each. (ii) 60 days after transplant, HCMV infected mainly the GI tract (biopsy viral load 2437494 copies/10^5 cells) and was treated with GCV for 14 days but changed to FOS during 24 days until CMV-DNA clearance. (iii) 99 days after transplant, HCMV reactivation was treated with CDV every 7 days (day 99 and 106 post-transplant) and was switched to FOS for 13 days due to an increase in CMV replication in plasma (5340 IU/mL) and the GI tract (35152 copies/10^5cells). (iv) At 137 days after transplant, the last HCMV reactivation was treated with FOS. Resistance mutation genotyping was requested and was negative for *UL97* and *UL54*, but the unknown R246C mutation in the *UL56* was detected. FOS therapy was finished when HCMV infection was resolved, and CDV was administered as maintenance treatment (2 doses 14 days apart). Unfortunately, 314 days after receiving the graft the patient died from sepsis and multiorgan failure.

All samples with viral loads >500 IU/mL from patient 1 were retrospectively genotyped for the target genes *UL56*, *UL97*, and *UL54* by standard and NGS methods (Fig. 1A). The allelic frequency of R246C was underestimated by NGS, as the depth per position reached was <1,000 reads; however, it could be clearly confirmed by the



Genotype	Gene	39	60	60*	98	102	105	109	109*	116	137	140	144	154
Standard	UL56	R246C	ND	ND	R246C	-	ND	R246C	**R246C**	ND	R246C	R246C	R246C	R246C
NGS	UL56	R246C (12%)	-	-	-	R246C (13%)	ND	R246C (14%)	-	-	-	R246C (7%)	R246C (7%)	R246C (8%)



Genotype	Gene	55	60	63	67
Standard	UL56	R246C	R246C	R246C	R246C
	UL54	ND	ND	ND	ND
	UL97	ND	ND	ND	ND
NGS	UL56	R246C (50%)	R246C (26%)	-	R246C (9%)
	UL54	ND	D413N[a] (1,5%)	-	ND
	UL97	F342S[b] (3%) A594V[c] (4%)	F342S[b] (3%)	-	F342S[b] (3%)

FIG 1 Clinical follow-up of patient 1 (A) and patient 2 (B). Viral loads (IU/mL) were tracked against days after transplantation. Antiviral treatment during follow-up is indicated in the legend, and arrows indicate the samples that were sequenced. The detection of *UL56* R246C and known antiviral-associated resistance mutations are indicated in the table below, with the allelic frequency percentages in brackets. Gastrointestinal biopsies are indicated with an asterisk. Populations 100% mutant are marked in bold. Hyphen (−) indicates that sequencing over the limit of quality could not be achieved. Abbreviations: GCV, ganciclovir; VGCV, valganciclovir; FOS, foscarnet; CDV, cidofovir; ND: no mutation detected; NGS: next generation sequencing. [a]D413N mutation confers 10-fold-resistance to GCV, 3.8-fold shift to CDV, and sensitive to FOS (7). [b]F342S mutation confers 8-fold resistance to GCV, 2-fold cross-resistance to MBV (26). [c]A594V mutation confers 4.5–10.4-fold resistant to GCV (27).

peaks observed in the Sanger sequencing, except in the GI biopsy collected on day 109 post-transplant (only the R246C variant was presented). Two naturally occurring polymorphisms (F669L, S685N) (12, 13) were detected in the *UL54* by standard sequencing and NGS, but none was identified in the *UL97* during the complete HCMV infection course.

TABLE 1 Antiviral susceptibility assay results of R246C

Mutation	IC_{50}	Ganciclovir	Cidofovir	Foscarnet	Maribavir	Letermovir
R246C	IC_{50}[a]	3 (± 2.0)	1.4 (± 1.8)	211.7 (± 206.4)	0.1	1.2 (± 0.8)
AD169	IC_{50}[a]	5.2 (± 2.4)	0.8 (± 0.47)	238.3 (± 179.7)	0.2	1.5 (± 1.7)
	RI[b]	0.9 (± 0.9)	1.5 (± 0.9)	0.8 (± 0.3)	0.5	1.2 (± 0.5)

[a]IC_{50} value is the inhibitory concentration 50% (μM). Results are shown as the mean of 3 independent experiments when ganciclovir, cidofovir, and foscarnet were tested, 1 for maribavir, and 4 for letermovir and its correspondent standard deviation.

[b]The Resistance Index (RI) is the IC_{50} for the R246C divided by the IC_{50} of the wild-type AD169. Mutants with RI $\geq$3 are considered resistant.

Patient 2 was a 45-year-old man who received a D^+R^- heart transplant in the HCB (Fig. 1B). Maintenance treatment consisted of cyclosporine, mycophenolate, and basiliximab. This patient did not receive prophylactic HCMV treatment, but HCMV viral loads were monitored every 15 days. This patient presented 2 HCMV infection episodes. (i) 55 days post-transplant, HCMV infection appeared and was treated with GCV for 21 days until CMV DNA clearance. During the second week of GCV treatment, viral loads rose (25,448 IU/mL) and genotype testing was requested. Standard sequencing showed the detection of the unknown *UL56* R246C variant, but no resistance mutation was found in either *UL54* or *UL97*. After DNA clearance, VGCV (450 mg/12 h) was administered for 17 days as secondary prophylaxis. (ii) 125 days post-transplant, asymptomatic HCMV reactivation in plasma was treated with VGCV (900 mg/12 h) for 10 days, resulting in CMV DNA clearance and good health controlled annually.

All the clinical isolates from the first episode were retrospectively genotyped. The *de novo UL56* R246C genetic variant was detected by standard sequencing and NGS. GCV-resistant mutations *UL97* F342S (14) and A594V (15), and minor GCV-CDV cross-resistant *UL54* D413N (11) subpopulations, were detected only by NGS. Four naturally occurring drug-susceptible polymorphisms (L655S, S685N, T885A, D898N) (12, 13, 16) were detected in *UL54* by standard sequencing and NGS, but there were no variants in *UL97*. Unfortunately, samples from the second HCMV relapse could not be recovered.

Phenotypic study of the novel R246C mutation. The inhibitory concentration 50% (IC_{50}) value for the recombinant virus with the novel R246C mutation was compared with the AD169 control strain for GCV, CDV, FOS, maribavir, and LMV (Table 1). The resistance index (RI) was calculated as the IC_{50} of the mutant divided by the IC_{50} of the wild type AD169 strain. The mutant is considered resistant to an antiviral when RI $\geq$3. Results showed R246C was sensitive to GCV, CDV, FOS, MBV, and LMV.

The growth assay showed a higher replication of the R246C variant compared with the AD169, demonstrating an advantage in viral fitness with respect to the wild-type HCMV (Fig. 2).

DISCUSSION

This study demonstrates the low frequency of genetic variants found in the *UL56* region associated with LMV resistance before LMV administration in refractory HCMV transplant recipients. The only baseline R246C mutation detected in 2/80 (2.5%) recipients (1 HSCT and 1 heart) was genetic and phenotypic studied resulting in high viral replicative capacity and a sensitive response to antiviral therapies.

Previous reports suggested that natural polymorphisms associated with standard or reduced LMV sensitivity were very unlikely in clinical isolates of patients naive of antiviral treatment (17) or treated with GCV (18), with no cross-resistance being found. Only one study reported the *de novo* LMV-resistant mutation C325Y in 2 formalin-embedded tissue biopsies from 1/147 (0.68%) patient (19).

In our two clinical contexts, the R246C variant emerged at first HCMV replication under GCV and in patient 1 reemerged every time the antiviral therapy was discontinued thanks to its advantaged growth capacity. Follow-up of the antiviral treatment

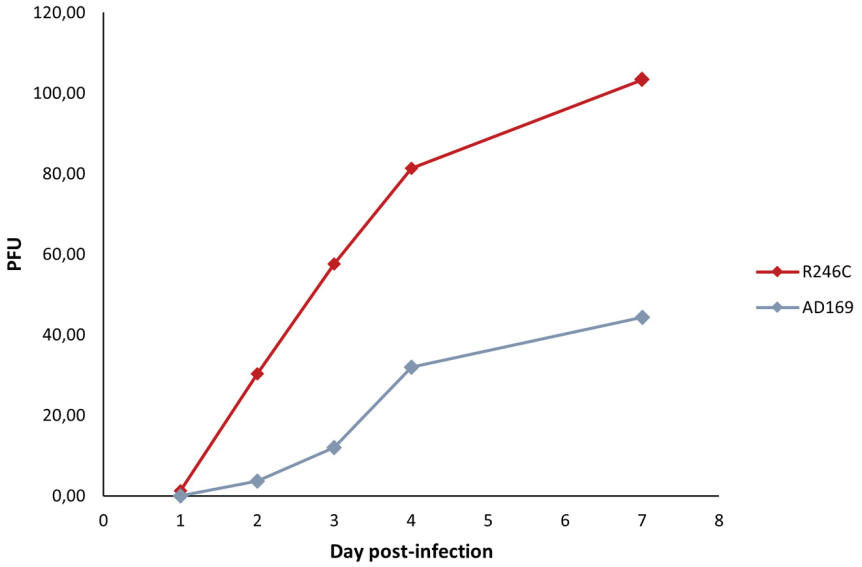


FIG 2 Replicative capacity assay of the R246C recombinant cytomegalovirus (CMV) strain compared with the AD169 CMV control strain. Both strains were inoculated at an equal multiplicity of infection of 0.01. PFU were counted from days 1 to 7 postinoculation. Data shown are the means of 4 independent experiments with 3 replicates per experiment.

and HCMV viral load monitoring of both clinical cases correlated with the antiviral-susceptibility and growth assays results, suggesting a similar impact of this mutation *in vivo*. This information is important as it has an impact in the patient management. In fact, clinical experience has shown LMV was not advisable to treat HCMV infection, due to the rapid and frequent emergence of LMV-resistant mutations once HCMV is replicating (5).

Mutations associated with resistance to LMV were found in the vicinity of R246C (V236M, T244K/R, L257I, C325Y/F/W) (20–22) in clinical isolates from HSCT and SOT recipients, comprising regular or impaired viral fitness. It is unusual to find a susceptible genetic variant within the LMV-resistant-mutation region of *UL56*, which is highly conserved among HCMV isolates, and it is even more infrequent to detect a mutation that entails a superior replicative capacity located within this polemical domain. This fact suggests that the 246 residue enhances *UL56* terminase subunit function but does not interact with LMV itself. Its position also confirms the phenomenon of vicinity of resistance and natural polymorphisms in *UL56*, already described for *UL97* phosphokinase, corroborating the suggestion that mutation phenotypes cannot be fully predicted by their gene position. Phenotyping is critically important not only to determine the resistance level to the different antiviral drugs, but also to understand its impact on the viral kinetic.

NGS is essential for correct follow-up of HCMV-complicated patients, as it enables the detection of small HCMV subpopulations that cannot be detected by standard sequencing. In our study, NGS allowed the detection and determination of the prevalence of minor *UL97* F342S, A594A, and *UL54* D413N variants in patient 2. Although assessment of the significance of minority subpopulations when using NGS in clinical samples should include evidence of their reproducible detection to avoid false positive results (23), NGS could not be repeated in our clinical cases due to the lack of sample volume. Since the evolution of resistance is staggered and progressive, monitorization for an early detection of novel genetic variants is crucial to optimized antiviral and immunosuppressive treatments and avoiding the development of resistance, which are associated with the time of exposure to the antiviral drug (15).

Overall, baseline *UL56* genetic variations before LMV therapy were infrequent in HSCT and SOT recipients. The only *UL56* R246C variant detected in this study by

standard sequencing and NGS emerged in two transplant recipients since first HCMV replication and responded transiently to various alternative DNA polymerase inhibitors. Its increased growth capacity allowed its replication when therapy was discontinued. Identification of high replicative capacity variants in patients not responding to treatment or experiencing relapses could be helpful to guide further therapy and dosing of antiviral molecules.

MATERIALS AND METHODS

Study population. Clinical isolates from HCMV-infected transplant recipients who filled the resistance suspicion criteria to conventional antiviral treatment (VGCV/GCV, CDV, FOS) (2) were included from April 2012 until September 2021. Antiviral treatment was administered according to the clinical judgement of the correspondent physician following the Consensus Guidelines on the Management of Cytomegalovirus in Transplantation (2). None of the patients had received LMV before sample collection. The patients were from the hospitals included in the Spanish Network for Research in Infectious Diseases (REIPI) and the Group for the Study of Infection in Transplantation (GESITRA). All samples were frozen at −80°C and sent to the Hospital Clinic of Barcelona (HCB), Spain, where genotypic antiviral resistance testing was performed.

Genotypic antiviral resistance testing. DNA extraction and HCMV viral load quantification was performed as described elsewhere (24).

(i) Standard sequencing. Genotypic testing was performed by Sanger sequencing based on PCR amplification of the HCMV *UL56* subunit of the terminase complex (residues 180–395), *UL97* phosphotransferase (400–670) and *UL54* DNA polymerase (300–1,000) regions corresponding to resistance-associated domains using the primers and procedure described previously (25). Each isolate was bidirectionally sequenced 3 times to avoid artifacts.

For patients with *UL56* genetic variants, samples with HCMV viral loads >500 IU/mL were retrospectively sequenced by standard and next generation sequencing (NGS) for *UL54*, *UL56*, and *UL97* to determine the timing of the earliest emergence of the respective mutations.

(ii) Next generation sequencing (NGS). A set of 100 strain sequences was provided to the Paragon Genomics teams (CA, USA) for custom panel design of full-length *UL54*, *UL56*, and *UL97* genes in HCMV. A whole-genome alignment of all 100 strains was performed, and this aligned sequence was used to generate a two-pool primer design using the Paragon Genomic's in-house primer design pipeline, ParagonDesigner. The design was able to achieve 100% coverage of all the regions of interest using 267 primers for a total of 57 amplicons. The design incorporated a small number of degenerate primers to account for potential single nucleotide polymorphisms in priming regions. The primers were synthesized with Illumina-specific partial adapters and provided by Paragon Genomics together with Targeted Library Kit Reagents and accessories (CleanPlex Custom Panel, SKU 916028).

Libraries were quantified using the QuantiFlour ONE dsDNA System (Promega, USA) and Bioanalyser (Agilent Technologies) and normalized to 4 nM final concentration. Samples were sequenced using the MiSeq platform (Illumina, USA). Bioinformatics analysis was performed at the French Reference Center for Herpesviruses Genomic Platform (CHU Limoges). Quality check of raw-reads was performed using FastQC v.0.11.5 and MultiQC v.0.9. A trimming step using Trimmomatic-0.39 was done using low stringency parameters (phred33; LEADING:3; TRAILING:3; SLIDINGWINDOW:4:15; MINLEN:36). Trimmed reads were then validated using the previous quality check step. Trimmed reads were aligned against the AD169 reference strain (GenBank: X17403.1) using the BWA-MEM algorithm from Bwa 0.7.17-r1188 (seed 20) and Samtools 1.9 (view, sort, and index options). Genetic variations from alignment were called using Lofreq 2.1.3.1, and variant annotations were made with SnpEff 4.3 software. Mutations were then filtered using Bcftools 1.8 based on Phred score (200) and depth (100). Variant call format (VCF) files were then formatted using SnpSift 4.3.1t (extractFields) and custom sed commands. Consensus files were obtained from VCF files using Bcftools 1.8 (consensus) and formatted using awk custom commands.

Phenotypic assay by recombinant bacterial artificial chromosome (BAC) technology. *UL56* R246C mutation, with an unknown phenotype at the time of genotypic detection, was individually tested at the French National Reference Center for Herpesviruses (Limoges) using a phenotypic assay with recombinant bacterial artificial chromosomes (BAC) technology as described previously (26). R246C single mutation was introduced by "en passant" mutagenesis into a human cytomegalovirus (HCMV) BAC (27) containing an enhanced green fluorescent protein (EGFP) gene in the unique short region derived from the AD169 laboratory strain (provided by M. Messerle). The recombinant BAC was transfected into MRC-5 cells (bioMérieux, Lyon, France) using the liposomal reagent Transfast (Promega, Madison, Wisconsin) following the manufacturer's instructions. The presence of the mutation was confirmed by Sanger sequencing.

A focus reduction assay in a 48-well MRC-5 fibroblast culture plate with a multiplicity of infection (MOI) of 0.01 was used to assess antiviral susceptibility in triplicate to GCV, CDV, FOS, maribavir, and LMV on the basis of fluorescent cytopathic foci counted in an ECLIPSE E200 analyzer (Nikon, New York, USA). Inhibitory concentration 50% (IC_{50}) of the mutant was compared to that of the wild-type HCMV BAC control.

To estimate the impact of the R246C mutation on viral fitness, the recombinant strain and the AD169-EGFP wild-type control were inoculated into 48-well MRC-5 culture plates with a MOI of 0.01. The number of fluorescent cytopathic foci was counted from days 1 to 7 postinoculation to establish viral growth curves for each recombinant.

Ethical approval. This study was approved by the Ethical Committee of the HCB (ref.HCB/2018/0634) as the reference committee for all the participating hospitals endorsed by GESITRA according to CPMP/ICH/135/95 regulations. Signed informed consent was obtained from all the patients included in the study.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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All authors reported no conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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MAJOR ARTICLE

Severe Acute Respiratory Syndrome Coronavirus 2 Normalized Viral Loads and Subgenomic RNA Detection as Tools for Improving Clinical Decision Making and Work Reincorporation

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Background. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) reverse-transcription polymerase chain reaction (RT-PCR) provides a highly variable cycle threshold (Ct) value that cannot distinguish viral infectivity. Subgenomic ribonucleic acid (sgRNA) has been used to monitor active replication. Given the importance of long RT-PCR positivity and the need for work reincorporation and discontinuing isolation, we studied the functionality of normalized viral loads (NVLs) for patient monitoring and sgRNA for viral infectivity detection.

Methods. The NVLs measured through the *Nucleocapsid* and *RNA-dependent-RNA-polymerase* genes and sgRNA RT-PCRs were performed in 2 consecutive swabs from 84 healthcare workers.

Results. The NVLs provided similar and accurate quantities of both genes of SARS-CoV-2 at 2 different timepoints of infection, overcoming Ct-value and swab collection variability. Among SARS-CoV-2-positive samples, 51.19% were sgRNA-positive in the 1st RT-PCR and 5.95% in the 2nd RT-PCR. All sgRNA-positive samples had >4 $\log_{10}$ RNA copies/1000 cells, whereas samples with ≤1 $\log_{10}$ NVLs were sgRNA-negative. Although NVLs were positive until 29 days after symptom onset, 84.1% of sgRNA-positive samples were from the first 7 days, which correlated with viral culture viability. Multivariate analyses showed that sgRNA, NVLs, and days of symptoms were significantly associated ($P < .001$).

Conclusions. The NVLs and sgRNA are 2 rapid accessible techniques that could be easily implemented in routine hospital practice providing a useful proxy for viral infectivity and coronavirus disease 2019 patient follow-up.

Keywords: COVID-19; normalized viral loads; subgenomic RNA; healthcare workers; SARS-CoV-2.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a ribonucleic acid (RNA) virus that emerged in Wuhan, China at the end of 2019 and spread rapidly worldwide with 10%–15% of severe courses and 2.2% of associated mortality [1, 2]. The main method for SARS-CoV-2 diagnosis is reverse transcription-polymerase chain reaction (RT-PCR). This technique provides a cycle threshold (Ct) value, which is inversely related to viral load, such that every increase in a Ct value of 3.3 corresponds to an approximate 10-fold reduction of RNA amounts. This value has been used to aid in interpretation and clinical decision making [3]. However, Ct values are not directly comparable across assays [4] and do not reflect the true viral load, which requires standardization using reference curves. Moreover, further variations may occur due to differences in specimen collection quality and reaction conditions [5]. Sample quality should be verified by cell quantification and normalization of viral loads to complement the RT-PCR results in the diagnosis [6].

Severe acute respiratory syndrome coronavirus 2 RT-PCR can remain positive for several weeks after clinical recovery in sicker patients, as well as in asymptomatic and mild ill individuals, leading to prolonged isolation and long work leave [7]. Higher viral load peaks have been found during the first week of the infectious process generally coinciding with the pre- or asymptomatic period, when most transmissions have been described [8]. However, the duration of infectivity after the onset of clinical symptoms remains uncertain.

Viral culture is the gold standard technique for viral viability. Recent studies have shown efficient transmission of SARS-CoV-2

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to contact-infected hamsters, and the results have been correlated with the detection of infectious virus by culture but not by RT-PCR viral loads alone [9]. Virus culture might be a good surrogate for transmissibility; however, high biosafety laboratory equipment and the long-term response limit the usefulness of this technique.

Viral subgenomic RNAs (sgRNA) in coronaviruses are only transcribed in infected cells and are poorly incorporated into mature virions, indicating the presence of actively infected cells in the samples [10, 11]. Severe acute respiratory syndrome coronavirus 2 sgRNA has been used as a surrogate indicator of active virus replication with a limit of sensitivity of 0.4% of genomic RNA, showing active replication in throat swabs during the first 5 days of symptoms [12], which, however, should be verified with viral culture.

Understanding the duration of SARS-CoV-2 infectivity is crucial for discontinuing isolation, healthcare worker (HCW) reincorporation, particularly in hospitals facing staff shortages, and saving testing supplies of repeated RT-PCRs. Therefore, strategies aiming at an early diagnosis of infected HCWs, preventing nosocomial infections, and deciding work reincorporation policies are still needed.

The main objective of this study was to provide 2 tools for improving clinical decision making and work reincorporation in coronavirus disease 2019 (COVID-19) patients: (1) normalized viral loads (NVLs) for patient monitoring; and (2) sgRNA as a surrogate marker of active replication for discontinuing isolation and work reincorporation in a cohort of HCWs.

MATERIAL AND METHODS

Study Population

According to hospital protocol, RT-PCR screening for SARS-CoV-2 was performed weekly by the Prevention Service on every HCW working in COVID-19 areas in the Hospital Clinic of Barcelona and also in those HCWs with self-reported symptoms suspicious of COVID-19. Every infected HCW was followed by the Home Hospital Unit, with medical and nurse assessment by phone/video calls, home visits, and day-hospital visits if needed for further testing.

This study is based on the prospective cohort of infected HCW from the Hospital Clinic of Barcelona from the first diagnosis of COVID-19 (February 25, 2020) until the of May 25, 2020 [13]. Only patients with 2 consecutive positive SARS-CoV-2 RT-PCR results were included in the analysis. Work reinstatement was decided by the Human Resources and Work Health Department according to governmental protocols.

Laboratory Testing

Inactivation and Extraction of Samples

All samples were inactivated with 1:1 volume of Cobas Omni Lys (Roche, Basilea, Germany), and total nucleic acid was extracted using MagNA Pure Compact (Mannheim, Germany). Throat/nasopharyngeal swabs and elutes were aliquoted and stored at −80°C.

Ribonucleic Acid Reverse-Transcription Polymerase Chain Reaction Quantification and Normalization of Severe Acute Respiratory Syndrome Coronavirus 2

Severe acute respiratory syndrome coronavirus 2 RT-PCR of the *Nucleocapsid* (*N*) and the *RNA-dependent RNA polymerase* (*RdRp*) genes were used to quantify the number of RNA copies per PCR using the SARS-COV-2 R-GENE kit (bioMérieux, Marcy l'Étoile, France) following the manufacturer's instructions. This test has a sensitivity limit of 380 copies/mL. Viral load was quantified in RNA copies per PCR using a standard curve made with AMPLIRUN SARS-CoV-2 RNA CONTROL (Vircell, Granada, Spain). Nasopharyngeal swab quality was checked by the CELL Control r-gene kit (bioMérieux, Marcy l'Étoile, France) that provides a quantified plasmid with the *HPRT1* housekeeping gene for cellular quantification. Samples with <100 cells/PCR were discarded. Viral loads were normalized according to cellular quantification as the number of RNA copies per 1000 cells. Extraction and both amplifications were checked with positive and negative controls for each reaction.

Reverse-Transcription Polymerase Chain Reaction for Subgenomic Ribonucleic Acid Detection for Severe Acute Respiratory Syndrome Coronavirus 2

All samples were tested for the presence of *Envelope* (*E*) sgRNA using the leader-specific primer described by Wölfel et al [12] as well as primers and probes targeting sequences downstream of the start codons of the *E* gene [14]. Reverse-transcription PCR of *E* genomic RNA was performed only in sgRNA-positive samples to estimate the amount of sgRNA through $2^{(\text{Ct sgRNA} - \text{Ct gRNA})}$ [12]. Reverse-transcription PCRs were performed using the SuperScript III Platinum One-Step RT-PCR kit (Invitrogen) with 400 nM primer concentration and 200 nM probe concentration. Cycling involved 15 minutes at 50°C for reverse transcription, 3 minutes at 95°C for Taq activation and 45 cycles of 10 seconds at 95°C, 15 seconds at 60°C (where the fluorescence was quantified), and 5 seconds at 72°C in the thermocycler StepOne (Applied Biosystems).

Severe Acute Respiratory Syndrome Coronavirus 2 Serology

Specific anti-SARS-CoV-2 immunoglobulin G (IgG) was determined using the qualitative Elecsys Anti-SARS-CoV-2 immunoassay (Roche, Basilea, United Kingdom).

Statistical Analysis

We report the number and percentage of patients for categorical variables and the median (first quartile; third quartile) for continuous variables. Categorical variables were compared using the χ^2 or Fisher exact test, whereas 2 groups of continuous variables were compared using the nonparametric Mann-Whitney *U* test. Spearman's correlation coefficient was used to measure the relationship between 2 continuous variables. *RdRp* RNA copies/1000 cells and *N* RNA copies/1000

cells were $\log_{10}$ transformed for normalization before analysis. Linear regression analyses were also used to examine the associations between continuous clinical parameters and *RdRp* RNA copies/1000cells, *N* RNA copies/1000 cells and sgRNA; whereas logistic regression analyses were performed to determine the relationship between dichotomous clinical parameters and *RdRp* RNA copies/1000 cells, N RNA copies/1000 cells and sgRNA. Continuous clinical parameters refers to days of symptoms, age, etc. Dichotomous parameters are gender, comorbidities, treatment, X ray check requirement, etc. The associations were further assessed with regression models adjusting for age, sex, and comorbidities. The effect sizes of association were shown as regression coefficients and 95% confidence intervals (CIs) for continuous parameters and odds ratios and 95% CIs for dichotomous parameters. The level of significance was set at 0.05 (2-tailed). All analyses were performed using IBM SPSS Version 26.0 (IBM Corp., Armonk, NY).

Ethical Approval

The study protocol was evaluated and approved by the Ethical Board of the Hospital Clínic of Barcelona (HCB/2020/0444). The informed consent waiver was provided due to the state of infectious disease emergency. Admission to the Home Hospitalization program was voluntary, as was every medical procedure performed.

RESULTS

Clinical Description

A total of 93 HCWs were selected for having 2 consecutive positive SARS-CoV-2 RT-PCRs with at least a 7-day gap between tests. Assays could not be performed in 9 subjects due to lack of samples, negative RT-PCR repetition, or no clinical data collected. The 84 HCWs analyzed had a median age of 33.5 (interquartile range [IQR], 27–50.5) years and 58 (69%) of them were females. Clinical characteristics are shown in Table 1.

The SARS-CoV-2 infection in this cohort of HCWs was detected by routine hospital screening in 33.8%, due to symptom reporting in 64.6%, and by direct contact in 1.5%. Symptoms were reported at some point during the infection in 91.7% of the total study population, with 89.3% being symptomatic at the time of diagnosis. Only 7 (8.3%) patients remained asymptomatic.

One month after the HCW screening started, specific anti-SARS-CoV-2 IgG serology was performed. All participants presented positive serology, except 1 that remained negative even 43 days after the first detection. Serology was not performed in 3 subjects.

During follow-up, 6 (7%) patients required chest x-ray, 3 presented bilateral infiltrates, and 3 were normal. Five (6%) subjects required treatment according to the local guidelines at that time (azithromycin, hydroxychloroquine, lopinavir/ritonavir, and prednisone). None of the HCWs in the present study required oxygen supplementation or hospital admission.

Table 1. Clinical Description of SARS-CoV-2-Infected Healthcare Workers Cohort

Characteristics	Value
Age, years (median, IQR)	33.5 (27–50.5)
Female, sex, n (%)	58 (69%)
Current smokers, n (%)	3 (3.6%)
Comorbidities[a], n (%)	12 (14.3%)
Clinical Features	
Symptomatic at diagnosis, n (%)	74 (88.1%)
Days from symptoms onset until 1st PCR (median, IQR)	3 (1–6)
Days from symptoms onset until 2nd PCR (median, IQR)	15 (14–19)
Symptoms, n (%)	77 (91.7%)
Cough, n (%)	56 (66.7%)
Fever, n (%)	23 (27.4%)
Dyspnea, n (%)	10 (11.9%)
Hyposmia/anosmia, n (%)	35 (41.7%)
Dysgeusia, n (%)	26 (31%)
Gastrointestinal disorders, n (%)	17 (20.2%)
Asymptomatic, n (%)	7 (8.3%)
Treatment, n (%)	5 (6%)
Serology SARS-CoV-2 IgG	
Positive, n (%)	80 (98.8%)
Negative, n (%)	1 (1.2%)
Days from symptom onset until positive serology (median, IQR)	44 (36–52)

Abbreviations: IgG, immunoglobulin G; IQR, interquartile range; n, number of patients; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a]Comorbidities include dyslipidemia, hypertension, chronic lung disease, ischemic heart disease, chronic kidney disease, and neoplasm.

Microbiologic Results

A total of 168 nasopharyngeal/throat swabs from the 84 patients were selected to quantify viral loads based on the *N* and the *RdRp* genes and normalized per 1000 cells by RT-PCR. All samples were SARS-CoV-2 positive for at least 1 of the target genes and had >100 cells per swab to be considered.

The RT-PCR Ct values and NVLs of the *N* and *RdRp* genes of the 1st and 2nd samples are shown in Table 2. Results demonstrated that Ct values were very variable when testing different genes in the 2nd RT-PCR, whereas NVL results provided similar results for the *RdRp* and *N* genes in the 1st and 2nd detections.

Subgenomic RNA RT-PCR was performed in the 168 samples as an indicator of active replication. The results showed that sgRNA was positive in 43 (51.19%) HCWs in the 1st RT-PCR and in 5 (5.95%) in the 2nd, after a 12-day interval (IQR, 11–16). Subgenomic RNA results were compared with *N* and *RdRp* RT-PCR Ct values and NVL (RNA copies/1000 cells) in linear and logarithmic scales demonstrating a highly significant correlation with both parameters (Table 3). All samples with *N* and *RdRp* RT-PCR Ct values <24.38 were sgRNA positive, whereas samples with Ct values >27.85 were sgRNA negative. In relation to NVL, all samples with >4 $\log_{10}$ RNA copies/1000 cells were sgRNA positive, 1–3 $\log_{10}$ samples were variable, and ≤1 $\log_{10}$ samples were sgRNA negative (Figure 1).

Table 2. RT-PCR Ct Values and Normalized Viral Loads of the SARS-CoV-2 *RdRp* and *N* Genes

	RdRp Gene		N Gene	
Assay	Ct Value[a]	NVL[b]	Ct Value[a]	NVL[b]
1st RT-PCR	26.58 (22.36–28.99)	2.52E + 03 (4.23E + 02; 7.96E + 04)	26.65 (22.07–32.52)	4.00E + 03 (1.38E + 02; 1.24E + 05)
2nd RT-PCR	29.99 (27.63–31.95)	7.55E + 01 (1.85E + 01; 2.45E + 02)	34.25 (30.77–35.56)	1.61E + 01 (7.37E + 00; 4.05E + 01)
Subtraction	3.71 (0.34–7.62)	1.78 $\log_{10}$ (0.59; 3.15)	5.71 (1.32–11.05)	2.1 $\log_{10}$ (0.95; 3.74)

Abbreviations: Ct, cycle threshold; N, nucleocapsid; NVL, normalized viral loads; RdRp, ribonucleic acid (RNA)-dependent RNA polymerase; RT-PCR, reverse-transcription polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a]Median (interquartile range).

[b]Median of RNA copies/1000 cells (interquartile range).

NOTE: Subtraction indicates the difference of the 2nd PCR minus the 1st PCR values.

Microbiologic and Clinical Variables Association

Normalized viral loads of the 74 HCWs symptomatic at diagnosis were compared with days after symptom onset (Figure 2A). Normalized viral loads of the *N* and *RdRp* genes decreased over time. The SARS-CoV-2 samples remained positive until 29 days after symptom onset and only 1 sample 38 days afterwards.

Results showed that sgRNA was positive in 3 asymptomatic HCWs in the 1st RT-PCR and in 1 subject who presented symptoms after the 1st RT-PCR. Of the rest of positive sgRNA samples, 84.1% were from ≤7 days after symptom onset, and this percentage declined over infection-time, as shown in Figure 2B. Dividing the presence of sgRNA by different intervals of days of symptoms, the highest proportion of sgRNA detection was in samples from ≤7 days, being only 7 samples sgRNA-positive from the 7th day onwards (Table 4). Statistical analyses showed that all 3 parameters (sgRNA, *RdRp*, and *N* NVL) were significantly associated with days of symptoms (see Supplementary Table 1).

Univariant and multivariant analyses of microbiological results and the remaining clinical data were carried out to determine potential risk factors (see Supplementary Table 2). Only significant associations ($P < .05$) with a 95% CI were presented (Table 5). The only 2 symptoms that individually correlated with SARS-CoV-2 viral load in the first detection were fever and gastrointestinal disorders (GIDs). Each symptom appeared in patients with viral loads ≈2 $\log_{10}$ higher and positive sgRNA in the case of fever. In contrast, dysgeusia was associated with lower NVL in the 2nd detection.

DISCUSSION

This study presents 2 tools for improving clinical decision making and work reincorporation after SARS-CoV-2 infection: (1) NVLs that provide robust and precise SARS-CoV-2 RNA measurements that overcome Ct values and respiratory sample collection variations; and (2) sgRNA as a possible surrogate

Table 3. Association Between Subgenomic RNA and *N* and *RdRp* RT-PCR Ct Values and Normalized Viral Loads of the 1st and 2nd RT-PCR

Detection	Gene	Variable	Total	Negative sgRNA	Positive sgRNA	P Value
1st RT-PCR	*N*	n	81	38	43	
		Ct value[a]	26.65 (22.07–32.52)	32.66 (30.23–34.53)	22.54 (17.94–25.09)	**<.001**
		NVL[b]	4000 (138.05–124 130.88)	123.28 (39.63– 633.64)	119 915.25 (20 997.88–380 403.46)	**<.001**
		$\log_{10}$ NVL[b]	3.6 (2.14–5.09)	2.09 (1.6–2.8)	5.08 (4.32–5.58)	**<.001**
	RdRp	n	84	41	43	
		Ct value[a]	26.58 (22.36–28.99)	28.97 (27.85–29.95)	22.41 (18.78–24.3)	**<.001**
		NVL[b]	2520.51 (423.06–79 624.99)	396.11 (113.5–1435)	79024 (13 571.43–383 915.02)	**<.001**
		$\log_{10}$ NVL[b]	3.4 (2.63–4.9)	2.6 (2.05–3.16)	4.9 (4.13–5.58)	**<.001**
2nd RT-PCR	*N*	n	79	74	5	
		Ct value[a]	34.25 (30.77–35.56)	34.46 (31.88–35.63)	29.07 (27.5–30.07)	**.010**
		NVL[b]	16.16 (7.37–40.53)	15.36 (7.14–32.29)	1502.86 (477.19–2624.29)	**.001**
		$\log_{10}$ NVL[b]	1.21 (0.87–1.61)	1.19 (0.85–1.51)	3.18 (2.68–3.42)	**.001**
	RdRp	n	84	79	5	
		Ct value[a]	29.99 (27.63–31.95)	30.06 (27.71–31.98)	27.32 (25.68–30.1)	.135
		NVL[b]	75.55 (18.47–245.64)	73 (15.51–236)	1247 (25.7–2914.29)	.123
		$\log_{10}$ NVL[b]	1.88 (1.26–2.39)	1.86 (1.19–2.37)	3.1 (1.41–3.46)	.123

Abbreviations: Ct, cycle threshold; n, number of samples; NVL, normalized viral loads; RdRp, ribonucleic acid (RNA)-dependent RNA polymerase; RT-PCR, reverse-transcription polymerase chain reaction; sgRNA, subgenomic RNA.

[a]Median (interquartile range).

[b]Median (interquartile range) of linear (RNA copies/1000 cells) and logarithmic scales ($\log_{10}$ RNA copies/1000 cells).

NOTE: *P* values obtained from the Mann-Whitney test. $P < .05$ are indicated in bold.

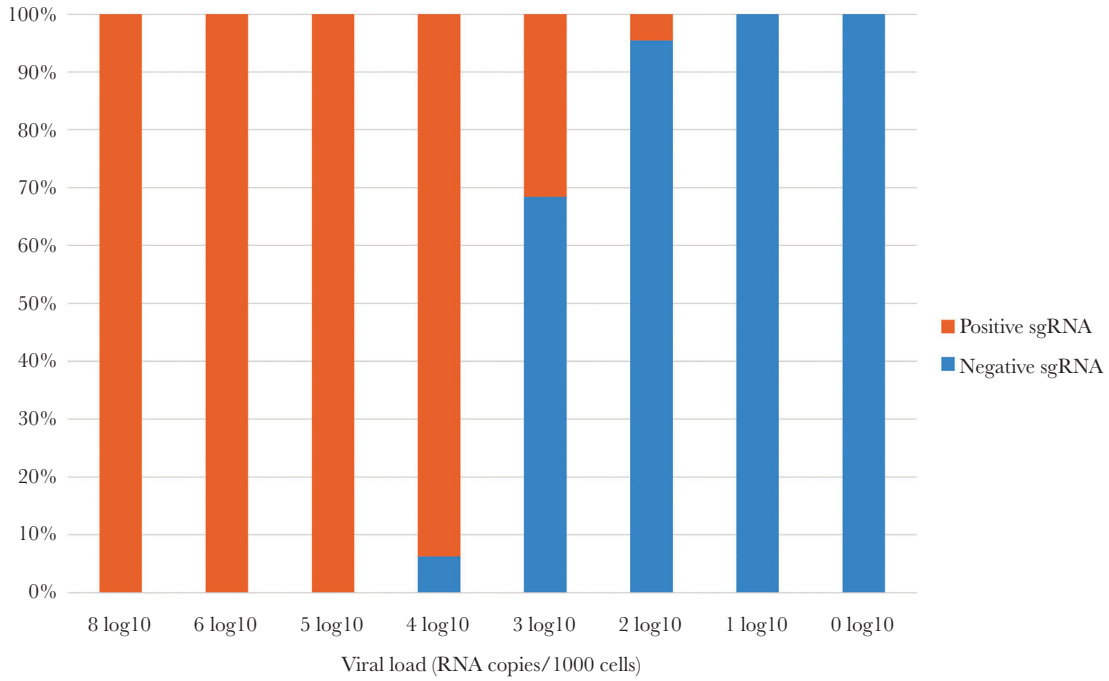

Figure 1. Subgenomic ribonucleic acid (sgRNA) detection compared with normalized viral loads. Normalized viral loads were quantified as $\log_{10}$ RNA copies per 1000 cells. Subgenomic RNA was qualitatively determined as positive or negative by reverse-transcription polymerase chain reaction. The 1st and 2nd sample determinations of each patient are shown.

marker of active viral replication, because it presented a significant association with NVL and days after symptom onset.

The Ct values have been used as a prognostic indicator for clinical decision making [15]; however, they could vary significantly between and within methods [16]. The College of American Pathologists surveyed more than 700 laboratories and demonstrated that different methods using identical control material varied by as much as 14.0 cycles and by up to 12.0 cycle differences within a single gene target using a single method [17]. Therefore, Ct values do not have a linear relation with viral loads, and the dispersion of the measurements is understated. The use of reference standard curves is recommended to calibrate every target and primer/probe design to provide accurate viral load determination for patient follow-up.

Nasopharyngeal/throat swabs also have an intrinsic variability that depends on the operator and the tolerance of the

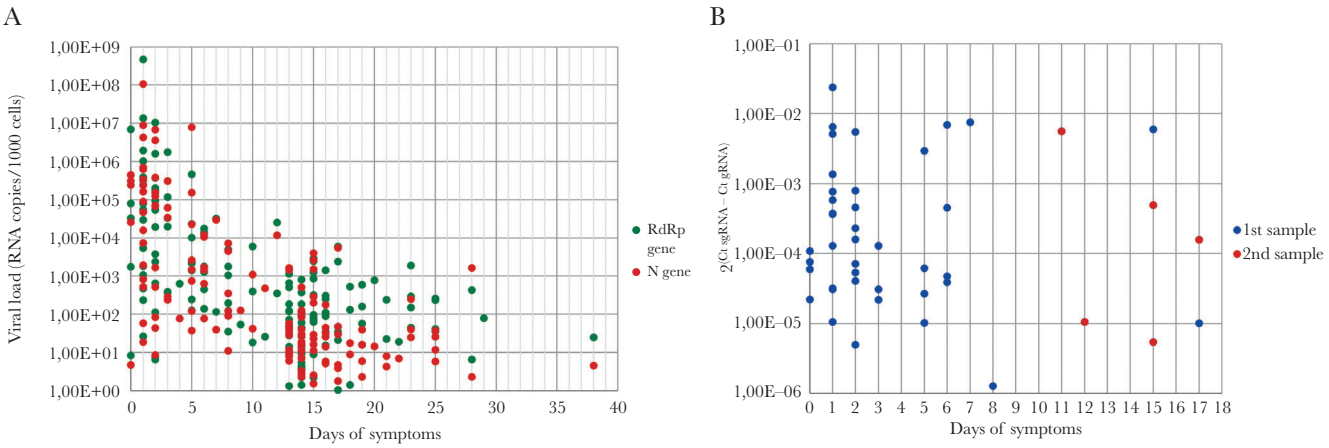

Figure 2. Normalized severe acute respiratory syndrome coronavirus 2 *N* and *RdRp* viral loads and subgenomic ribonucleic acid (sgRNA) of the first and second detection compared with days of symptoms. (A) Viral loads were measured by reverse-transcription polymerase chain reaction and normalized to RNA copies per 1000 cells. Quantification was performed with the *RNA-dependent RNA polymerase* (*RdRp*) and *Nucleocapsid* (*N*) genes in each specimen. (B) Subgenomic RNA transcripts in relation to genomic RNA cycle threshold (Ct) values are expressed as $2^{(Ct\ sgRNA - Ct\ gRNA)}$ and plotted against days of symptoms.

Table 4. Subgenomic RNA of the 1st and 2nd RT-PCR Results Divided by Intervals of Days After Symptom Onset

Days of Symptoms	Positive sgRNA	Negative sgRNA	Total
1–5	34 (68%)	16 (32%)	50
6–7	3 (37.5%)	5 (62.5%)	8
8–10	1 (10%)	9 (90%)	10
11–14	2 (5.9%)	32 (94.1%)	34
>14	4 (8.7%)	42 (91.3%)	46

Abbreviations: RNA, ribonucleic acid; RT-PCR, reverse-transcription polymerase chain reaction; sgRNA, subgenomic RNA.

patients [18]. Therefore, swab collection quality of every sample must be checked to avoid false-negative results and provide reliable values. Our results demonstrate that NVLs showed robust and similar quantities of SARS-CoV-2 of the *N* and *RdRp* genes at 2 different timepoints of the infection in 84 HCWs. Normalization overcame the limitations of Ct value and sample collection variability, providing more accurate values that can guide the monitoring of patients and treatment management.

The gold standard to determine viral infective capacity is viral culture; however, this requires high biosafety laboratories, fresh samples, and long time for response. Severe acute respiratory syndrome coronavirus 2 culture and sgRNA have been used to detect replication-competent virus longitudinally in respiratory samples [12], presenting a high association between the 2 techniques [19].

Our study aimed to determine the potential utility of sgRNA for monitoring actively replicating virus in the context of HCWs to improve work reincorporation policies. Our findings showed sgRNA was mainly detectable in specimens collected ≤7 days after symptom onset. This result is in correlation with several culture studies describing SARS-CoV-2 culture recovery from respiratory specimens obtained 1–9 days after onset in patients with mild to moderate disease [20, 21]. Our results also showed that after 7 days of symptoms only 7 patients presented sgRNA, indicating that a reduction of isolation protocols should be evaluated to ensure adherence and to vacate COVID-19 hospital isolation rooms.

Subgenomic RNA was also compared with Ct values and NVLs obtained by RT-PCR standardized for the *N* and *RdRp* genes. Both genes were tested simultaneously together with the cellular quantification by multiplex RT-PCR always using the same procedure and thermocycler. This explains why sgRNA results significantly correlated with Ct values in this assay and should be standardized in every laboratory before using. To achieve less sample and technique variability, it is more optimal to use NVLs. Considering the significant association between samples with ≥4 $\log_{10}$ RNA copies being sgRNA-positive and <1 $\log_{10}$ being sgRNA-negative, a viral load cut point between 1 and 3 $\log_{10}$ RNA copies/1000 cells could be set to request sgRNA detection.

A correlation between chest x-rays, comorbidities, or serology with NVLs or sgRNA has not previously been reported, but our multivariant analysis showed they are not significantly correlated. Our results confirmed that fever and GID are the only 2 clinical parameters associated with higher viral loads and replication-competent virus (sgRNA positivity) in the case of fever. Furthermore, dysgeusia is likely to be a delayed symptom associated with lower copies of the virus and a tendency to less replicative capacity. No other COVID-19-related symptoms, gender, or age were significantly correlated with SARS-CoV-2 viral load, in accordance with previous reports [15].

In spite of satisfactory sgRNA results, further viral culture studies are required to verify the association of sgRNA with viral replicative capacity and to define the timing of infectivity. In addition, the study population analyzed was not wide

Table 5. Significant Associations Between Normalized SARS-CoV-2 Viral Loads and Subgenomic RNA With Categorical Variables in the Total Study Population

Symptom	Variable	Statistic	Total	No	Yes	P Value
Fever	n	n	81	58	23	**.002**
	$\log_{10}$ NVL[a]—1st RT-PCR	Median (IQR)	3.6 (2.14–5.09)	3.19 (2.08–4.54)	5.18 (2.71–5.8)	
	Positive sgRNA	n (%)	43 (51.2%)	26 (46.6%)	17 (73.9%)	**.011**
	Negative sgRNA	n (%)	41 (48.8%)	35 (57.4%)	6 (26.1%)	
GID	n	n	81	65	17	**.007**
	$\log_{10}$ NVL[a]—1st RT-PCR	Median (IQR)	3.6 (2.14–5.09)	3.2 (2.08–4.79)	5.03 (3.26–5.82)	
	Positive sgRNA	n (%)	43 (51.2%)	31 (46.3%)	12 (70.6%)	.073
	Negative sgRNA	n (%)	41 (48.8%)	36 (53.7%)	5 (29.4%)	
Dysgeusia	n	n	84	58	26	**.006**
	$\log_{10}$ NVL[a]—2nd RT-PCR	Median (IQR)	1.88 (1.26–2.39)	2.04 (1.56–2.51)	1.44 (0.81–1.91)	
	Positive sgRNA	n (%)	5 (6%)	5 (8.6%)	0 (0%)	.318
	Negative sgRNA	N (%)	79 (94%)	53 (91.4%)	26 (100%)	

Abbreviations: GID, gastrointestinal disorders; IQR, interquartile range; n, number of samples; NVL, normalized viral loads; RT-PCR, reverse-transcription polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.

[a]$\log_{10}$ RNA copies/1000 cells.

NOTE: *P* values obtained from the Mann-Whitney test. $P < .05$ are indicated in bold.

enough and did not include immunocompromised or severely ill patients, in whom NVL and sgRNA should be tested to improve patient monitoring and infection control in the hospital setting, and to verify treatment efficacy.

CONCLUSIONS

Normalized viral loads and sgRNA detection are 2 rapid and accessible techniques that can be easily implemented in routine hospital practice, providing a useful proxy for infectivity and patient follow-up. These tools can overcome the obstacles of Ct values, inadequately used in clinical specimens to establish criteria for active infection and transmission, the costs of high biosafety laboratory equipment and long-term of response limitation of viral culture. Subgenomic RNA RT-PCR can provide essential information for safe work reincorporation of infected workers, patient assessment, discontinuing isolation precautions, and optimization of vaccines and treatment approaches.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: no reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Conflicts of Interest.

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VIROLOGY

Viral Culture Confirmed SARS-CoV-2 Subgenomic RNA Value as a Good Surrogate Marker of Infectivity

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ABSTRACT Determining SARS-CoV-2 viral infectivity is crucial for patient clinical assessment and isolation decisions. We assessed subgenomic RNA (sgRNA) as a surrogate marker of SARS-CoV-2 infectivity in SARS-CoV-2-positive reverse transcription PCR (RT-PCR) respiratory samples (n = 105) in comparison with viral culture as the reference standard for virus replication. sgRNA and viral isolation results were concordant in 99/105 cases (94%), indicating highly significant agreement between the two techniques (Cohen's kappa coefficient 0.88, 95% confidence interval [CI] 0.78 to 0.97, $P < 0.001$). sgRNA RT-PCR showed a sensitivity of 97% and a positive predictive value of 94% to detect replication-competent virus, further supporting sgRNA as a surrogate marker of SARS-CoV-2 infectivity. sgRNA RT-PCR is an accurate, rapid, and affordable technique that can overcome culture and cycle threshold (C_T) value limitations and be routinely implemented in hospital laboratories to detect viral infectivity, which is essential for optimizing patient monitoring, the efficacy of treatments/vaccines, and work reincorporation policies, as well as for safely shortening isolation precautions.

KEYWORDS SARS-CoV-2, COVID-19, subgenomic RNA, viral culture, infectivity

Reverse transcription-PCR (RT-PCR) is the most sensitive and widely used technique for the diagnosis of COVID-19. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) RT-PCR can remain positive in respiratory secretions for weeks or months due to the detection of viral particle debris, even in asymptomatic individuals (1). RT-PCR provides a cycle threshold (C_T) value which is inversely related to viral load, with every 3.3 increase in C_T value reflecting a 10-fold reduction of RNA (2). C_T values are highly variable depending on the swabbing technique, specimen types, assays, and platforms used for RNA extraction and amplification, limiting their utility for predicting viral loads or infectivity (3, 4). Determining the duration of active SARS-CoV-2 replication is key for the clinical management of patients, discharge from isolation and work reincorporation.

Viral isolation is the gold standard for determining virus infectivity. Several studies have isolated SARS-CoV-2, showing virus recovery in specimens collected within 1 to 9 days after symptom onset (5), after 12 days in patients with mild to moderate disease (6), and after 20 days in critically ill patients (7).

Subgenomic RNA (sgRNA) is only transcribed in infected cells and is poorly packaged into virions, indicating the presence of active replication (8). Previous studies have shown a significant association between sgRNA detection and high viral loads during the first 5 to 7 days of symptoms, when most viral transmission has been reported (9 to 11).

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sgRNA detection by RT-PCR can overcome the limitations of time-consuming culture techniques which require high-biosafety laboratories (BSL-3) and fresh samples. Only a few reports have compared SARS-CoV-2 culture and sgRNA detection (9, 12); despite using an insufficient sample size to determine agreement, these reports found moderate to complete concordance. Nonetheless, further studies are needed.

This study aimed to determine the correlation of sgRNA with viral culture to verify if sgRNA can be used as a surrogate marker of active SARS-CoV-2 replication.

(Preliminary results from this study were presented at the 31st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID]; 9 to 12 July, Vienna, Austria.)

MATERIALS AND METHODS

Study design and sample collection. The study design consisted of using RNA RT-PCR to select samples that were positive for SARS-CoV-2, which were kept at 4°C for ≤48 h before inoculation for SARS-CoV-2 culture. Aliquots of each sample were stored at −80°C for sgRNA detection by RT-PCR.

A total of 105 samples (88 nasopharyngeal aspirates/swabs, 17 bronchoalveolar lavages) from the Hospital de la Santa Creu i Sant Pau (HSCSP) and the Hospital Clínic de Barcelona (HCB), collected from 6 November 2020 to 25 March 2021, were included for fulfilling the above criteria. They were tested for SARS-CoV-2 RNA using the platforms established in the respective hospitals. Each sample corresponded to an individual subject, except in the case of 1 patient from whom 3 samples were included in the study.

SARS-CoV-2 culture. In the BSL3 lab of the HSCSP, each sample was treated with a mixture of antibiotics (vancomycin and streptomycin) and an antifungal (amphotericin B) for 30 min. After sample treatment, 300 μl was inoculated into the VERO-E6 cells and incubated at 37°C for a maximum of 10 days (see the S1 text at https://doi.org/10.6084/m9.figshare.16802200.v1). The appearance of a cytopathic effect (CPE) was examined daily with an inverted microscope (×40). A culture was considered positive when a characteristic CPE was observed. Each CPE was confirmed as being caused by SARS-CoV-2 either by indirect immunofluorescence using a specific monoclonal antibody, anti-SARS-CoV-2 (CerTest, Spain), and/or by SARS-CoV-2 RT-PCR, where a value of ≥3 cycles lower than the original RT-PCR C_T value confirmed the presence of replicating virus. The viral culture was considered negative if CPE was absent 10 days after inoculation.

SARS-CoV-2 sgRNA RT-PCR. At the HCB, all samples were inactivated with 1:1 volume of Cobas Omni Lys (Roche, Germany), and total nucleic acid was extracted using MagNA Pure Compact (Roche, Switzerland). Respiratory samples and elutes were aliquoted and stored at −80°C.

Extracted RNAs were tested for the presence of *Envelope* (*E*) sgRNA using the leader-specific primer described by Wölfel et al. (9) as well as primers and probes targeting sequences downstream of the start codons of the *E* gene (13) (see Text S2 and Table S1 at https://doi.org/10.6084/m9.figshare.16802200.v1). RT-PCR was performed using the SuperScript III Platinum One-Step RT-PCR kit (Invitrogen) with a primer concentration of 400 nM and a probe concentration of 200 nM. The C_T cutoff for negative samples was >40.

Statistical analysis. We recorded the number and percentage of samples for categorical variables and the median (first quartile [Q1]; third quartile [Q3]) for continuous variables. The assumption of normality was checked using the Kolmogorov-Smirnov test. Categorical variables were compared with a chi-square test. Two groups of continuous variables were compared using a nonparametric Mann-Whitney U test. Cohen's kappa (14) was calculated to measure agreement between the two methods (i.e., culture and sgRNA) for nominal categorical variables. To calculate the yield of the test, we analyzed the area under the receiver operating characteristic (ROC) curve (AUC) (15, 16). We also calculated the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios, along with the 95% confidence intervals (CI). The level of significance was set at 0.05 (2-tailed). All analyses were performed using IBM SPSS Version 26.0 (IBM Corp., Armonk, NY, USA).

Ethical approval. This study protocol was evaluated and approved by the Ethical Board HCB (HCB/2021/0024). Informed consent was waived due to the state of infectious disease emergency.

RESULTS

This study analyzed sgRNA and viral isolation of 105 RT-PCR positive SARS-CoV-2 upper and lower respiratory tract samples (Table 1). The median (Q1; Q3) C_T value of RT-PCR diagnosis was 23 (18; 29), and the median number of days after symptom onset was 4 (1; 15). Eighteen (17.1%) samples were from asymptomatic patients, and 11 (10.5%) were from patients with prolonged viral shedding (RNA positive for >30 days).

sgRNA was detected in 66 (62.9%) specimens: 13 (19.7%) from asymptomatic patients and 38 (57.6%) from patients with 1 to 7 days of symptoms. SARS-CoV-2 isolation was successful in 64 (61%) specimens: 13 (20.3%) from asymptomatic patients and 37 (57.8%) from patients in the first 7 days after symptom onset. The median number of days until characteristic CPE was observed was 3 (2; 3).

Positive and negative sgRNA and viral culture subsets were compared with the C_T value of the diagnostic RT-PCR, the C_T value of the sgRNA RT-PCR, and the number of days after symptom onset stratified by intervals (Table 1). Qualitative sgRNA detection

TABLE 1 Comparative association of subgenomic RNA and viral culture with categorical and continuous variables[a]

		Viral culture			sgRNA		
Parameter	**Total**	**Negative**	**Positive**	***P***	**Negative**	**Positive**	***P***
Total no. (%)	105 (100)	41 (39)	64 (61)		39 (37.1)	66 (62.9)	
Diagnostic C_T	23 (18.0; 29.0)	30.1 (27.3; 34.1)	18.8 (16.5; 22.8)	<0.001	30.8 (27.9; 34.5)	19.1 (16.5; 22.9)	<0.001
sgRNA C_T	29.4 (26.8; 33.8)	37.7 (35.6; 39.0)	28.8 (26.6; 33.1)	=0.003		29.4 (26.8; 33.9)	
Days of symptoms	4 (1; 15)	15 (4; 21)	2 (1; 6.5)	<0.001	14 (4; 22)	2 (1; 7)	<0.001
No. of samples							
Asymptomatic	18 (17.1)	5 (12.2)	13 (20.3)		5 (12.8)	13 (19.7)	
1 day	16 (15.2)	3 (7.3)	13 (20.3)		3 (7.7)	13 (19.7)	
2–7 days	32 (30.5)	8 (19.5)	24 (37.5)		7 (17.9)	25 (37.9)	
8–15 days	15 (14.3)	8 (19.5)	7 (10.9)		8 (20.5)	7 (10.6)	
16–30 days	13 (12.4)	12 (29.3)	1 (1.6)		11 (28.2)	2 (3.0)	
>30 days	11 (10.5)	5 (12.2)	6 (9.4)		5 (12.8)	6 (9.1)	

[a]Data are indicated as number of samples (%) or as the median (first quartile; third quartile). C_T, cycle threshold value of the RT-PCR. *P* values are indicated as <0.05 versus positive group.

was significantly associated with the diagnostic C_T value and days after symptom onset, while viral culture was significantly associated with the diagnostic C_T value, the sgRNA C_T value, and days after symptom onset.

C_T values of diagnostic and sgRNA RT-PCRs were stratified by intervals of days after symptom onset (Table 2). The sgRNA C_T value increased by 1.43 every interval change (approximately 7 days). This ratio could not be appropriately calculated for the diagnostic C_T value due to its high variability over intervals between and within days after symptom onset.

An increase of 11.35 in the diagnostic C_T value between the negative and positive culture samples is shown in Fig. 1A. No virus was isolated when the RT-PCR C_T value was >29, except in one sample from an asymptomatic patient (C_T = 31.4). Only 4 samples presented sgRNA-positive and culture-negative results (Fig. 1B), with sgRNA RT-PCR C_T values of >34 (34.4, 36.8, 38.7, 39.4).

Of the specimens with both positive culture and positive sgRNA, 89% were from <15 days after symptom onset (Fig. 1A). These results were also found in 3 specimens from beyond 240 days after symptom onset, in 3 specimens from 33 to 45 days after symptom onset, and in 1 specimen from 23 days after symptom onset. These last 7 specimens corresponded to immunosuppressed patients with hematological malignancies who had received chemotherapy and required admission to the Critical Care Unit due to severe COVID-19 complications. Seven specimens from 7 to 15 days after symptom onset were positive for both sgRNA and viral culture. These corresponded to patients who presented risk factors (1 with hematological malignancy, 1 with HIV, 3 with severe pneumonia, 1 with morbid obesity, 1 who was 102 years old) for severe COVID-19; however, the early date at which the sample was collected did not allow us to consider them as persistent (>21 days of symptoms) (17).

Cohen's kappa was calculated to measure the agreement between the two methods: culture and sgRNA (Table 3). Over the 105 specimens tested, both methods showed positive results for 62 (59%) specimens, both tests showed negative results for 37 (35.2%) specimens, positive culture and negative sgRNA results in 2 (1.9%) specimens, and positive sgRNA and

TABLE 2 Cycle threshold values of diagnostic and subgenomic RNA RT-PCRs according to days after symptom onset

	C_T value[a]	
Days of symptoms	**Diagnosis**	**sgRNA**
Asymptomatic	20.5 (16.5; 29.9)	28.6 (26.8; 33.1)
1 day	19.3 (17.5; 23.6)	27.9 (25.7; 29.4)
2–7 days	22.3 (17.3; 25.7)	29.4 (27.1; 33.1)
8–15 days	26.1 (19.7; 31.0)	31.1 (27.0; 34.4)
16–30 days	34.1 (26.9; 35.4)	32.5 (25.6; 39.4)
>30 days	23.1 (12.2; 32.8)	33.6 (28.6; 35.4)

[a]Data are indicated as median (first quartile; third quartile).

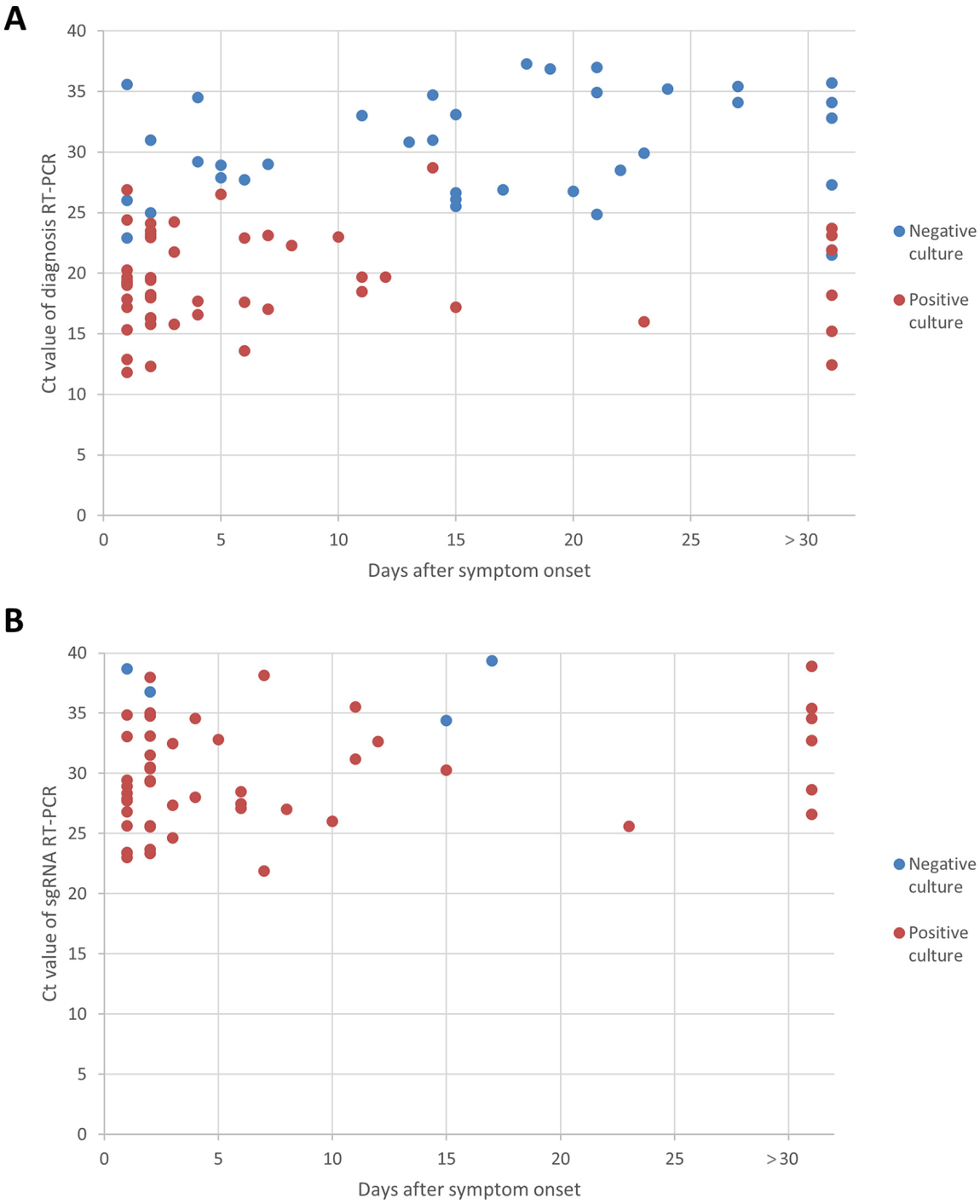

FIG 1 Viral culture results plotted by days after symptom onset and diagnostic C_T value of RT-PCR (A) and sgRNA RT-PCR (B).

negative culture results in 4 (3.8%) specimens (Cohen's kappa 0.88, 95% CI 0.78 to 0.97, $P < 0.001$), indicating a strong significant agreement between viral culture and sgRNA detection.

The predictive performance of sgRNA compared to viral culture is shown in Table 4. The sensitivity, specificity, positive and negative predictive values were 97%, 90%, 94%, and 95% respectively, with positive and negative likelihood ratios of 9.93 and 0.03, respectively. This result indicates that the probability of infection significantly increased with positive sgRNA results and significantly decreased with negative sgRNA results.

TABLE 3 Results of the distribution of samples by sgRNA and viral culture methods

	No. of sgRNA samples		
Culture	Negative	Positive	Total
Negative	37	4	41
Positive	2	62	64
Total	39	66	105

DISCUSSION

This study compared the results of *E* sgRNA with viral culture to evaluate the ability of *E* sgRNA to detect SARS-CoV-2 infectivity. Our findings show that sgRNA was able to detect replication-competent virus with a sensitivity of 97% and a positive predictive value of 94%; with a coefficient of agreement of 0.88 in relation to viral culture as the reference standard. These results suggest that *E* sgRNA could be used as a surrogate marker of active viral replication.

RT-PCR detects the presence of viral genomic RNA but is not able to distinguish whether an infectious virus is present. To determine whether there is active virus replication and, therefore, potential risk of person-to-person transmission, it is necessary to perform a viral culture. However, virus isolation is labor-intensive and has some limitations which make it unsuitable for many laboratories. The growth and identification of virus in culture requires specialized facilities and expertise. Nevertheless, viral cultures should be maintained in experienced laboratories as a reference method to validate new molecular techniques (18).

Several reports support the use of sgRNA as a surrogate marker of infectivity in the context of challenged primate models (19, 20), patients who persistently test positive for SARS-CoV-2 total RNA (17), and clinical isolates with comparative viral culture data (9, 12). In addition, to demonstrate a statistical association between sgRNA and viral culture, our study included a considerable sample size ($n > 100$) and the confirmation of positive culture results with indirect immunofluorescence and/or RT-PCR.

In contrast, some studies have postulated that sgRNA is not a good indicator of infectivity, suggesting that the loss of sgRNA detection is due to the lower overall RNA transcript concentration compared with genomic RNA (11, 21). However, they agree with the previously reported significant association of sgRNA with normalized viral loads and days after symptom onset (10). A significant limitation of these studies was the absence of correlative viral culture data.

There is some controversy regarding the choice of which sgRNA species to measure, as it may have a significant impact on detection and result interpretation. Nucleocapsid sgRNA is more abundant than *E* sgRNA and persists for longer periods (11, 22). This difference between sgRNA species could be explained by the enclosure of RNA transcripts in double-membrane vesicles and/or extracellular vesicles, allowing longer persistence of specific sgRNA species (23); however, there are no clear data as to whether *E* gene transcripts are more rapidly degraded by ribonucleases or whether they better reflect recent transcription. Our study demonstrates that *E* sgRNA has a better coefficient of agreement according to viral culture (Cohen's kappa coefficient 0.88) than that previously obtained using *Nucleocapsid*

TABLE 4 Predictive performance of subgenomic RNA compared to viral culture as the reference standard[a]

Parameter	Value	95% CI
Sensitivity (%)	96.9	91.8–100
Specificity (%)	90.2	79.9–100
Positive predictive value (%)	93.9	87.4–100
Negative predictive value (%)	94.9	86.7–100
Positive likelihood ratio	9.93	3.91–25.22
Negative likelihood ratio	0.03	0.01–0.14
AUC	0.94	0.88–0.99

[a]CI, confidence interval; AUC, area under the ROC curve.

sgRNA (Cohen's kappa coefficient 0.467) (12), suggesting that *E* sgRNA of SARS-CoV-2 is the best option to detect infectivity thus far.

In agreement with the literature, we found that virus isolation was unsuccessful in samples with diagnostic C_T values >29, except in 1 case (24). However, C_T values are no longer recommended as an indicator of infectivity due to their high variability between and within methods and their lack of standardization (4, 24). This study demonstrates that sgRNA detection can overcome the limitations of C_T values, as we compensated for the diagnostic C_T values bias by including diverse platforms from two different hospitals.

The 73% for successful virus isolation was achieved in samples from symptomatic patients within the first 7 days of symptoms, as reported previously (6). These samples were also sgRNA-positive in our study. Prolonged viral replication (>21 days of symptoms) (17), as detected by culture and sgRNA test, was demonstrated in 7 severely immunosuppressed patients who required hospital admission. Virus infectivity was also detected in 13 out of 18 asymptomatic subjects. Therefore, it is key to identify infectious individuals in order to control the spread of infection. sgRNA could be used both to detect asymptomatic infectious individuals and to better characterize the shedding of replicant-competent virus longitudinally from people with moderate to mild disease, as well as severely ill and immunocompromised patients, to define the timing of infectivity.

Our study was limited by the impracticability of repeating viral isolation, since these samples were stored at −20°C, and by the incapacity to quantify sgRNA due to the absence of an approved WHO standard for sgRNA species; therefore, this technique is meant to be qualitatively implemented. Four samples were sgRNA-positive, culture-negative, with sgRNA C_T values close to 40: this suggests that the higher sensitivity of sgRNA RT-PCR found compared to viral culture provides a beneficial overestimation of infectivity, which is crucial for preventing the risk of prematurely releasing patients from isolation or treatment discontinuation.

Overall, sgRNA provides rapid and robust determination of SARS-CoV-2 infectivity in upper and lower respiratory tract samples from patients with different clinical outcomes, including immunosuppressed subjects and individuals with persistent COVID-19 related symptoms. The sgRNA test overcomes both the limitations of C_T values, and the relative insensitivity and need for specialized facilities for viral culture, as it is detected by simple RT-PCR which can be easily implemented in hospital practice. Predicting SARS-CoV-2 infectivity is essential for optimizing patient monitoring, work reincorporation policies, and the efficacy of treatments/vaccines as well as for safely shortening isolation precautions.

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Marta Santos Bravo and Carla Berengua selected the samples included in the study, performed the majority of the experimental techniques, and wrote the manuscript, figures, and tables. Marta Santos Bravo designed and optimized the protocol for the SARS-CoV-2 sgRNA assay and performed it with the help of Genoveva Cuesta. Carla Berengua and Núria Rabella designed and optimized the protocol for SARS-CoV-2 isolation and identification in cell culture. Cell cultures were maintained by Montserrat Esteban with the help of Cristina Rodriguez. Pilar Marín inoculated the clinical samples and performed the immunofluorescence assay. Núria Rabella and María Ángeles Marcos were the promoters of the project and major proofreaders of the manuscript. Sonsoles Sánchez-Palomino, Mar Mosquera, Jordi Vila, Margarita del Cuerpo, and Elisenda Miró reviewed and corrected the manuscript.

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Case Report

Persistent replication of SARS-CoV-2 in a severely immunocompromised patient treated with several courses of remdesivir

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ABSTRACT

Microbiological response of SARS-CoV-2 to remdesivir in immunocompromised patients has not been evaluated. We present the case of a severely immunocompromised patient with persistent replication of SARS-CoV-2, who required different courses of remdesivir. Short courses of remdesivir might be insufficient in immunocompromised patients due to prolonged viral clearance.

More than 37 million people worldwide have been infected and one 1 million have died since the first cases of COVID-19 were described (WHO, 2020). However, data about the efficacy of different treatment agents against SARS-CoV-2 is still scarce (Sanders et al., 2020). Remdesivir is the only antiviral agent that has demonstrated efficacy against COVID-19 in terms of shortening length of hospital stay in a randomized placebo-controlled trial (Beigel et al., 2020). Another randomized clinical trial (RCT) has shown that a 5-day course of remdesivir is as effective as a 10-day regimen in patients with severe COVID-19 not requiring mechanical ventilation (Goldman et al., 2020). However, there is no evidence whether these results can be applied to special populations such as immunocompromised patients. Here we present the case of a severely immunocompromised patient with persistent replication of SARS-CoV-2 who required different courses of remdesivir.

A 37-year-old woman presented to the hospital with fever. During the previous months, the patient had received 3 cycles of R-ESHAP (rituximab, etoposide, cisplatin, cytarabine and methylprednisolone) due to a relapse of a stage IV-A follicular lymphoma. A partial response was observed by PET-CT and a salvage therapy was about to start when the patient was diagnosed with an upper respiratory tract infection by Influenza A virus, requiring treatment with oseltamivir for 10 days two weeks before the present admission.

In March 2020, the patient presented to the hospital with a 3-day history of fever, malaise, anosmia and dysgeusia. At admission, oxygen saturation at room air was 98% and physical exam was unremarkable. Blood tests showed thrombocytopenia and increased D-dimer levels, with no elevation of the other acute-phase reactants and a normal lymphocyte count. Initial chest X-ray did not show any infiltrates. SARS-CoV-2 polymerase-chain reaction (PCR), detecting the envelope (E) and the open reading frame 1a (ORF1a) genes (Roche Diagnostic), performed on a nasopharyngeal swab resulted positive, confirming the diagnosis of COVID-19. Following hospital protocols, treatment with lopinavir/ritonavir, hydroxychloroquine and azithromycin was initiated, but lopinavir/ritonavir was withdrawn after 24 h due to moderate diarrhoea.

The patient showed clinical worsening with persistent fever, diaphoresis, dyspnoea and a slight but painful spleen enlargement. Under the suspicion of neoplastic disease progression, 60 mg

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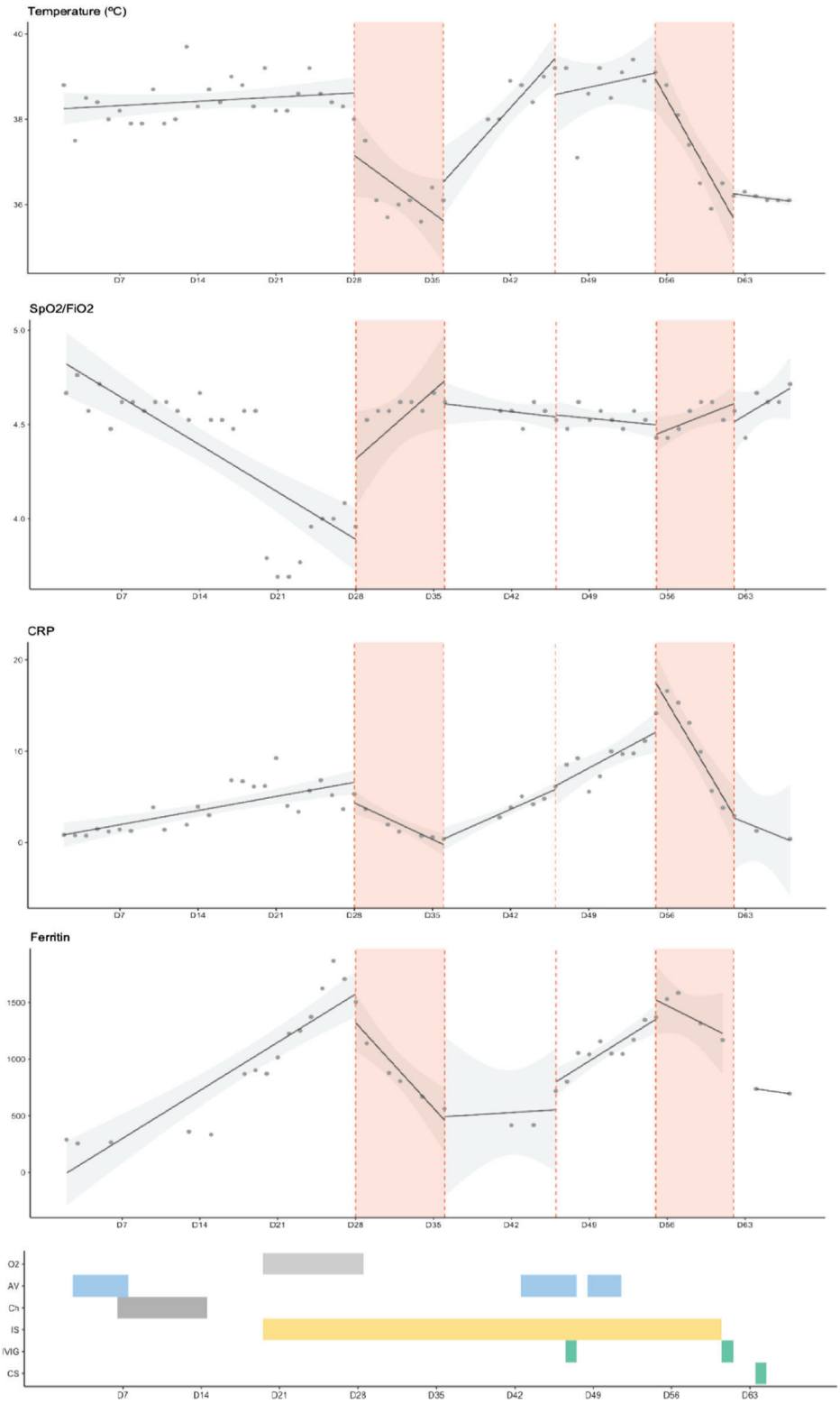

Fig. 1. Microbiological and clinical evolution of SARS-CoV-2 infection.
AV: Antiviral treatment (lopinavir/ritonavir, hydroxychloroquine, azithromycin, darunavir/cobicistat). Ch: chemotherapy (prednisone, cyclophosphamide). CRP: C-reactive protein. CS: convalescent serum. IVIG: intravenous immunoglobulin. IS: immunosupression (steroids, anakinra). O2: oxygen support. SpO2/FiO2: oxygen saturation to fraction of inspired oxygen ratio.
First graph shows changes on SARS-CoV-2 adjusted viral load in serial nasopharyngeal swabs and the different antiviral treatments received. Adjusted viral load was calculated by adjusting cycle threshold (Ct) for the number of cells per sample and is represented as a logarithm of the number of copies per 1000 cells (log_{10} copies/1000 cells). Red areas and dashed lines represent time under remdesivir treatment. Grey areas represent time under other antiviral treatment.
The following graphs show the evolution of temperature (ºC), SpO2/FiO2, CRP and ferritin.
Red areas and dashed lines represent time under remdesivir treatment. The different treatments received are presented in coloured bars.

prednisone and 1000 mg cyclophosphamide, as well as broad-spectrum antibiotics, were administered. In the following days, oxygen needs increased and PET-CT ruled out neoplastic progression and showed signs of organizing pneumonia. Treatment with 250 mg methylprednisolone per day for 3 days followed by 60 mg prednisone daily was then started. However, ferritin levels increased, thus anakinra was also indicated. Bronchoalveolar lavage samples confirmed positive SARS-CoV-2 PCR with a high viral load and discarded other causes of infection. Based on microbiological results and clinical condition, 4 weeks after the onset of symptoms, the patient was enrolled in an RCT being allocated to receive remdesivir (200 mg continued with 100 mg/day). In the following days, the patient presented an excellent clinical evolution, being discharged after 8 days of treatment.

Three days after discharge, the patient started again with fever and cough. Blood tests showing lymphopenia with reduction in all lymphocyte subpopulations and decrease in all immunoglobulin levels confirmed cellular and humoral immunosuppression. A CT-scan showed new bilateral infiltrates and a complete resolution of the previous signs of organizing pneumonia. A new SARS-CoV-2 nasopharyngeal PCR was positive, thus diagnosis of COVID-19 relapse was assumed and treatment with hydroxychloroquine, azithromycin and darunavir/cobicistat was started. Accidentally, a single dose of remdesivir was administered to the patient, becoming afebrile for 24 h afterwards. Concomitantly, a decreasing steroids scheme was continued and, given the severe hypogammaglobulinemia, non-specific intravenous immunoglobulins (IVIG) were administered. Despite the different treatments prescribed, an increase in nasopharyngeal SARS-CoV-2 viral load was observed. Consequently, the patient was included into a second RCT and treated with remdesivir at the same previous doses for 10 additional days. The patient rapidly improved, fever resolved and PCR in nasopharyngeal sample became negative. Given that antibody tests seeking for IgA, IgM, and IgG against SARS-CoV-2 (VITROS® Immunodiagnostic anti-SARS-CoV-2-Total) performed 42 and 64 days after the onset of symptoms resulted negative, another infusion of IVIG and COVID-19 convalescent plasma were also administered.

Fig. 1 shows the evolution of SARS-CoV-2 adjusted viral load in serial nasopharyngeal swabs and the different antiviral treatments received (Lescure et al., 2020). Adjusted SARS-CoV-2 viral load initially increased up to $1{,}1 \times 10^7$copies/1000cells. Coinciding with first remdesivir administration, viral load decreased to 3,1copies/1000cells. However, a new peak on the viral load was observed ($4{,}1 \times 10^3$copies/1000cells) 54 days after symptoms initiation. After a second cycle of remdesivir, a reduction and negativization of viral load was finally achieved (63 days after the onset of symptoms). In this case, remdesivir showed an important antiviral effect in an immunocompromised patient, significantly reducing SARS-CoV-2 viral load, which was not observed with the other antiviral treatments. Remarkably, in our case, the antiviral effect of remdesivir was consistent with different clinical aspects like the resolution of fever, improvement of respiratory insufficiency and decreasing of acute-phase reactants (Fig. 1).

Notably, the patient presented a positive SARS-CoV-2 PCR 60 days after symptoms initiation. Median time of viral RNA clearance in nasopharyngeal samples is 20 days but it has been detected after more than 5 weeks (Zhou et al., 2020). Although some authors suggested possible longer duration of positive PCR in immunocompromised patients, RNA dynamics in this population are unknown (Han et al., 2020; Helleberg et al., 2020; Choi et al., 2020). In this case, the increase in the viral load 54 days after symptoms initiation and the clinical worsening suggested persistent replication of SARS-CoV-2 and supports the hypothesis of persistent replication of SARS-CoV-2 in immunocompromised hosts (Helleberg et al., 2020; Choi et al., 2020).

Finally, although an RCT showed no benefit of 10-day courses of remdesivir compared with 5 days, no specific evaluation of participants with underlying immunosuppression conditions was reported (Goldman et al., 2020). Consequently, these results may not be extrapolated to special high-risk populations like severe immunocompromised patients in whom persistent viral replication can play an essential role on the pathogenesis of the disease (Goldman et al., 2020; Han et al., 2020; Helleberg et al., 2020; Choi et al., 2020). In our patient, a late peak on SARS-CoV-2 viral load was detected despite an 8-day regimen of remdesivir. This suggests that short courses of remdesivir might be insufficient for treating high-risk populations such as severely immunocompromised patients, especially those with humoral immune deficiency (Helleberg et al., 2020).

Declarations

The patient signed an informed consent form and the study was approved the Drug Research Ethics Committee of the Hospital Clinic of Barcelona (CEIm), Barcelona, Spain.

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Genetic study of SARS-CoV-2 nsp12 in non-responder COVID-19 patients to remdesivir

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ABSTRACT

Remdesivir (RDV) was the first antiviral drug approved by the FDA to treat severe COVID-19 patients. RDV inhibits SARS-CoV-2 replication by stalling the non-structural protein 12 (nsp12) subunit of the RNA-dependent RNA polymerase (RdRp). No evidence of global widespread of RDV-resistance mutations has been reported. Determining emergent mutations prior or subsequent antiviral therapy has strong implications for clinical management and virus surveillance.

This study identified 57/149 (38.3%) patients who did not respond to one course (5-days) (63.2%) or prolonged (5-20 days) (36.2%) RDV therapy by subgenomic RNA detection. Genetic variants in the *nsp12* gene were detected in 17/49 (34.7%) non-responder patients by next-generation sequencing, including the *de novo* E83D mutation that emerged in an immunosuppressed patient after receiving 10+8 days of RDV, and the L838I detected at baseline and/or after prolonged RDV treatment in 8/49 (16.3%) non-responder subjects. Although 3D protein modelling predicted no-interference with RDV, the amino acid substitutions detected in the nsp12 involved changes on the electrostatic outer surface and in secondary structures that may alter antiviral response.

It is important for health surveillance to study potential mutations associated to drug resistance and the benefit of RDV retreatment, especially in immunosuppressed patients and in those with persistent replication.

Importance

This study provides clinical and microbiologic data of an extended population of hospitalized patients for COVID-19 pneumonia who experienced treatment failure, detected by the presence of subgenomic RNA. The genetic variants found in the *nsp12* pharmacological target of remdesivir bring into focus the importance of monitoring emergent mutations, one of the objectives of the World Health Organization (WHO) for health surveillance. These mutations become even more crucial as remdesivir keeps being prescribed and new molecules are being repurposed for the treatment of COVID-19.

The present article offers new perspectives for the clinical management of non-responder patients treated and retreated with RDV, and emphasizes the need of further research of the benefit of combinatorial therapies and RDV retreatment, especially in immunosuppressed patients with persistent replication after therapy.

Keywords: COVID-19, remdesivir, resistance mutations, subgenomic RNA, retreatment

BACKGROUND

The global pandemic of novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has created an urgent effort to repurpose antiviral inhibitors to control viral replication and improve clinical outcomes [1]. Remdesivir (RDV) was originally developed in response to the 2014-2016 Ebola outbreak in West Africa [2] and has shown broad-spectrum activity *in vitro* and *in vivo* against pathogenic human coronaviruses, including the novel SARS-CoV-2 [3]. RDV was the first drug to be approved by the FDA in October 2020 and has been extensively used in clinical practice during the COVID-19 pandemic in hospitalized patients [4]. Recently, two oral prodrugs have also been approved for treatment of COVID-19 patients: molnupiravir and nirmatrelvir/ritonavir [5, 6].

RDV, formerly GS-5734, is a nucleoside analogue pro-drug that inhibits the non-structural protein 12 (nsp12) subunit of the RNA-dependent RNA polymerase (RdRp) by competing with its usual natural substrate adenosine triphosphate [7]. The nucleoside analog is incorporated into the generating RNA strand and evades proofreading to successfully inhibit viral RNA synthesis. Several clinical trials and a recent control-case study demonstrated reduced time to recovery, hospitalization time, morbidity and mortality [8-10].

One of the major concerns for health surveillance is determining emergent mutations that could be associated to drug resistance, fitness advantage, immune scape, or better adaption to the host. Only few studies have attempted to characterized amino acid substitutions that could confer resistance to RDV by *in silico* prediction, *in vitro* or in animal models [7, 11-15]. For instance, F480L, V557L and E802D has been described to conferred 2.4x, 5.2x, 6x-fold decrease sensitivity to RDV *in vitro,* respectively [7, 14]. Moreover, E802D was recently detected in an immunocompromised patient after RDV therapy [15], however, no other resistant clinical case or evidence of global widespread transmission of RDV-resistant mutants after treating with RDV for over a year have been described thus far.

This study aimed to identify novel genetic variations in the *nsp12* gene in clinical samples before and after RDV therapy in severe COVID-19 patients who did not respond to therapy with RDV.

MATERIALS

Study population

This was an observational prospective study that included 149 COVID-19 patients admitted in the Hospital Clinic of Barcelona (Spain), from February 2021 until November 2021, who filled the criteria to receive RDV according to the recommendations of the Spanish Medicine Agency. These criteria were the following: (1) SARS-CoV-2 positivity confirmed by RT-PCR, (2) ≤7 days

from symptoms onset, (3) radiological signs of pneumonia, (4) requiring supplemental oxygen support or respiratory rate ≥24 breaths per minute or $Pa0_2$ /FiO_2 <300 mmHg. The RDV dose used was 200 mg as a loading dose the first day and 100 mg/24h for the next 4 consecutive days. Some immunosuppressed patients received prolonged remdesivir therapy (>5d-RDV), decision made by the physician in charge according to the clinical evolution and the immune status of the patient.

Nasopharyngeal/throat swabs were collected before 1st RDV dose and after the last dose for each patient. Both samples were tested for SARS-CoV-2 genomic (gRNA) and subgenomic RNA (sgRNA) by reverse transcriptase-real time polymerase chain reaction (RT-PCR). Although there is some controversy concerning the use of sgRNA to detect active viral replication, we previously validated this technique with viral culture [16]. Non-responder subjects were considered when sgRNA was detected at the end of the RDV treatment. Nucleotide changes were determined by next generation sequencing (NGS) in pre-and post-RDV treatment clinical samples of non-responders. Novel amino acid substitutions found in clinical isolates were evaluated in 3D modelling structure of the protein *in silico.* This study design is showed in figure 1.

Clinical data were collected to study the overall population, considering the following variables: age, significant comorbidities, days of treatment with RDV, Intensive Care Unit (ICU) admission, and all-cause mortality.

RT-PCR for genomic and subgenomic RNA detection for SARS-CoV-2

The presence of SARS-CoV-2 gRNA was determined by real time RT-PCR in the automatic system Cobas 6800 (Roche, Barcelona) according to the manufacturer's instructions.

Inactivation was performed using 1:1 volume of Cobas Omni Lys (Roche, Germany) and total nucleic acid extraction was done using MagNA Pure Compact (Roche, Switzerland). Elutes were used for sgRNA test and NGS. *Envelope (E)* sgRNA was detected by real-time RT-PCR following the procedure previously described [16]. Throat/nasopharyngeal swabs and elutes were aliquoted and stored at -80ºC since their testing. Cycle threshold (Ct) values >40 for gRNA or sgRNA RT-PCRs were considered negative.

Next Generation Sequencing of SARS-CoV-2 complete genome

Retrospectively, the eluted RNA of the SARS-CoV-2 were retrotranscribed into cDNA and SARS-CoV-2 complete genome amplification was conducted following the openly available protocol developed by the ARTIC network [17] using Illumina platform. The sequences obtained went through a bioinformatic pipeline based on a previously described open-source pipeline [18].

Mutations were identified by aligning the consensus sequence with the Wuhan-Hu-1 reference genome [GenBank: MN908947.3] using Nextclade v.1.14.0. Quality of the sequences were determined by the quality control metrics of this tool.

Lineage identity

The lineage of SARS-CoV-2 was identified using Nextclade v.1.14.0 according to the amino acid replacements determinant of each variant as classified by the SARS-CoV-2 Interagency Group (SIG) and the Centres of Diseases Control and Infection (CDC) [19].

Molecular modelling of mutations in the SARS-CoV-2 nsp12 and spike proteins

Mutations found genotypically in the nsp12 subunit of the RdRp were generated in the ribbon structure of the cryo-EM model of the replicating SARS-CoV-2 polymerase complex (PDB 6YY7) using PyMOL Molecular Graphics System (Schrödinger, version 2.5.2). This software was also used for structural visualization. Adaptive Poisson-Boltzmann Solver (APBS) Tool 2.1 Electrostatic Plugin included in PyMOL was used for macromolecular electrostatics calculations and to display the results as an electrostatic potential molecular surface.

Ethical approval

The Ethical Committee of our institution accepted the protocol (HCB/2021/0080) and the included patients signed the informed consent to participate in the study.

RESULTS

In a cohort of 149 patients hospitalized for COVID-19 pneumonia, 111 received a 5-dose course of RDV (5d-RDV) and 38 received prolonged RDV therapy (>5d-RDV) (figure 1). The median age of the 5d-RDV subset was 62.7 (54; 73) years-old, 81 (73%) presented at least one comorbidity, 34 (30.6%) were admitted in the ICU and 7 (6.3%) died by all-cause mortality (table 1). The median age in the >5d-RDV subgroup was 59 (53; 67) years-old and they were treated with RDV for a median of 10 (7; 21) days. Of the 38 patients, 33 (86.8%) had at least one comorbidity, 19 (50%) were admitted in the ICU and 8 (21.1%) died by all-cause mortality, but 3 deaths were not related to COVID-19. Extended clinical data of both subsets is shown in table 1.

SARS-CoV-2 sgRNA identified 57/149 (38.3%) patients with actively replicating virus after treatment, classified as non-responders (figure 1). There were 36/57 (63.2%) non-responders in the 5d-RDV subgroup and 21/57 (36.8%) from the >5d-RDV subgroup. The 17 remaining patients of the >5d-RDV cohort were not tested for sgRNA and were discarded because the pre- or post-treatment swab could not be recovered. Ct values corresponding to the gRNA RT-PCR were maintained or decreased during treatment in non-responder patients. Nucleotide changes in the

nsp12 gene were detected by NGS and were compared before and after RDV therapy in non-responder patients. Sequencing quality was achieved in both samples in 28/34 patients of the 5d-RDV subset, and in all 21 patients of the >5d-RDV subset.

NGS allowed the detection of 18 *nsp12* nucleotide substitutions detected in 17/28 (60.7%) patients from the 5d-RDV subgroup and in 12/21 (57.1%) patients receiving prolonged RDV therapy. Of the 18 nucleotide substitutions identified, 9 conferred an amino acid substitution, pointing genetic evolution after treatment (table 2). Mutations were either detected at baseline (pre-RDV), before and after treatment (pre/post-RDV) or emerged *de novo* after treatment (post-RDV). The only non-synonym mutation only detected after treatment was E83D. E83D emerged in a patient (patient 7, table S1) with diffuse large B-cell lymphoma that was treated with R-CHOP combination chemotherapy and with several antiviral drugs (lopinavir, ritonavir, hydroxychloroquine, azithromycin, RDV) and convalescent plasma. E83D mutation emerged *de novo* after 2 courses (10 + 8 days) of RDV. At the end, this patient was admitted in the ICU and died after 8 months of SARS-CoV-2 infection. The most frequent mutation (18.4%) was L838I, found at baseline in 1 patient and after prolonged treatment in 8 non-responder subjects. Clinical and genetic data of all patients with *de novo* mutations is shown in table S1.

NGS allowed the determination of SARS-CoV-2 lineages. Alpha (B.1.1.7) variant was mainly detected in subjects with 5d-RDV (n=19) compared to >5d-RDV ones (n=3), whereas delta (B.1.167) was more predominant in >5d-RDV subjects (n=12) than in the 5d-RDV subgroup (n=4). The variant B.1.177 emerged as an outbreak in Spain during the summer of 2020, and its incidence was similar in both groups (5d-RDV n=5; >5d-RDV n=6).

Predicting phenotype of novel *nsp12* mutations by 3D protein modelling

The position of all genetic variants detected at any time of the treatment were located in the gene structure of the nsp12 (figure 2) and non-synonym mutations in a 3D protein structure of the RdRp (figure 3A). *In silico* studies postulated that RDV binds strongly to the active pocket of the nsp12 by electrostatic interaction with residues R553, R555, T556, K551, W617, D618, Y619, D623, S682, N691, D760, D761, A762, W800, E811, F812, K813, and S814, and by Van der Waals bonds in the K621, K622, D623, and L758 [11, 12]. Our genotypic results showed the emergence of mutations located next to residues involved in RDV-nsp12 binding (E729D, D738Y, L838I), in the nsp8-nsp12 interaction region (P227L), and inside the RNA-binding domain of the polymerase (T422I). However, RdRp 3D protein modelling shows that all novel amino acid substitutions are distant from the RNA binding domain, where the RDV inhibits the polymerase activity. Replacing one amino acid of a helix for another can change repartition of amino acids

exposed to solvent, as found for the E729D (figure 3B). Slight changes in conformation were detected for the remaining amino acid substitution.

Most mutations changed a negative charged amino acid to aliphatic-chained ones, substituting the highly negative electrostatic outer surface of the nsp12 to a major overall neutral status of the surface (figure 3C). Changes in polarity, charge, and size of the amino acids could potentially modify interactions between nsp12 and antivirals.

DISCUSSION

The study of sgRNA allowed the identification of 57/149 (38.3%) patients who did not respond to 5-days or >5-days of RDV therapy. Eighteen genetic variants in the *nsp12* gene were frequently detected in 17/49 (34.7%) by NGS in non-responder subset. No significant viral mutations were determined to be associated to the failure to RDV treatment, except for the *de novo* E83D mutation that emerged after receiving 18 days of RDV, and the L838I mutation, which was found at baseline in 1 patient and after prolonged treatment in 8 (18.4%) non-responders, and its localized next the previously E802D RDV-resistant mutation.

Genetic variants in *nsp12* arose similarly after 5 days of RDV or longer treatment independently of the duration of therapy (5d-RDV 57.1% vs. >5d-RDV 60.7%) and, surprisingly, 5d-RDV subjects presented more *de novo* mutations (n=6) than >5d-RDV (n=3). Almost all mutations were detected at baseline in at least one subject showing an evolutionary tuning of the viral proteins to a new host, although a response for antiviral selective pressure cannot be excluded in those that persist after treatment. In non-responder patients, viral loads progressively increased during treatment, as Ct values were maintained/decreased and sgRNA remained detectable, which could be due to either slow viral shedding or failure to treatment.

Despite the high frequency of substitutions in *nsp12*, none of them have been previously described to confer resistance to RDV [15]. We detected the L838I mutant, nearby the E802D RDV-resistant mutation, in 8 patients before and after treatment, and in 1 patient only post-therapy. All of them received either 10, 20 or 21 days of RDV therapy, 6 of them were admitted in the ICU and 3 died, thus, it seems to be associated to a worse prognosis, although the number of cases is scarce to confirm it. It suggests that this naturally occurring variant may provide an improved viral scape of the inhibitor, since no evidence that RDV acts as a mutagen driving spontaneous mutations has been reported and the template stalling action of the RDV limits spontaneous mutations emergence [7, 20].

E83D emerged in the SARS-CoV-2 delta variant that infected an immunosuppressed patient, who was admitted in the ICU and died after 9 months of infection. The E83D mutation was detected

after RDV retreatment (10 + 8 days), but not before, making it potential for phenotyping. 3D protein modelling did not predict any interference in the interaction with RDV, however, its implication in fitness advantage, inhibitor scape or adaption to the host is unknown.

E729D and D738Y may also have an implication of antiviral response, as they were located in the palm subunit of the polymerase active site next to the residues involve in the RDV-nsp12 interaction and E729D also modified an alpha-helix structure. T422I is located in the conserve motif G in charge of the RNA template attachment. It is close to the previously described resistant mutations *in vitro* (F480L, V557L) and *in vivo* in an immunocompromised patient with persistent viremia (D484Y) [7, 21]. They did not alter the RdRp catalytic site but are thought to impact RdRp fidelity checking step before catalysis. Molecular surveillance of this region in RDV-treated COVID-19 patients is suggested to be warranted [20]. Even though 3D protein modelling predicted none of the mutations found in this study block the binding pocket of RDV, they involved changes on the electrostatic outer surface and in secondary structures that may alter antiviral response.

Lineages could be a concern of the severity of the disease and the antiviral response. Alpha variant was more frequent in the 5d-RDV and delta in the >5d-RDV, which agrees with its higher pathogenicity. Besides the possible bias caused by the time of inclusion of the patients, delta lineage could influence on a worse response to RDV and the need of a second course of treatment.

Available literature about RDV retreatment only reported few clinical cases [22-23] but no large studies have been carried out, making this practice still unaddressed in current treatment guidelines [24]. However, this study provides further information of the response to RDV treatment and retreatment in patients whose active replication was previously checked by sgRNA and clinical data was exposed.

In conclusion, no significant virological resistance was determined after different courses of RDV in non-responders severe COVID-19 patients and the duration of RDV treatment does not seem to be a risk factor for developing RDV-resistance mutations. However, mutations found in this study, especially E83D, were potential to be further evaluated by recombinant phenotyping. It is crucial to monitor antiviral resistance as one of the objectives of the World Health Organization (WHO) for health surveillance, and to study the potential benefit of combinatorial therapies and RDV retreatment, especially in immunosuppressed patients or with persistent replication.

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FIGURES

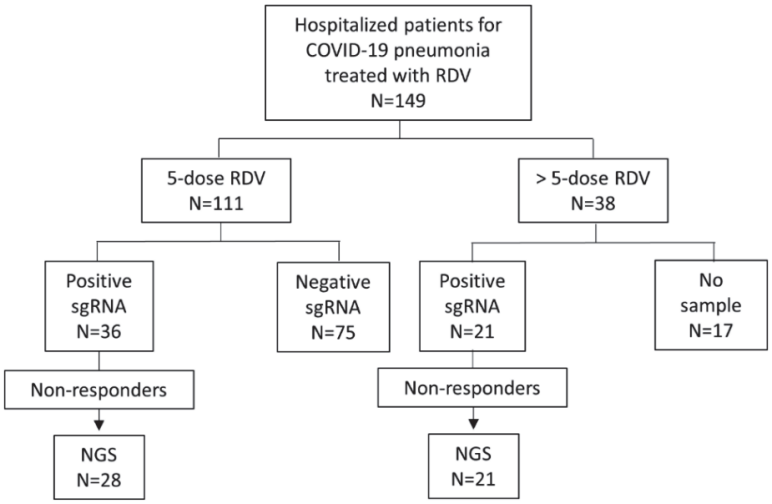

Figure 1. Scheme of the study design. Patients positive for SARS-CoV-2 that were admitted in the Hospital Clinic of Barcelona (Spain) for COVID-19 pneumonia and were treated with remdesivir (RDV) were included in the study. They were treated with 5 doses (1 course) of RDV or with longer treatments (>5 doses). Subgenomic RNA (sgRNA) detection was performed in all samples in order to detect viral replication before and after treatment. Patients with positive sgRNA after treatment were classified as non-responders and were sequenced by next-generation sequencing (NGS). Not all clinical samples could be sequenced with enough quality to be analysed and included in the study due to RNA degradation or low viral load in the sample. Of the samples from the >5-dose subpopulation that could be studied, all were sgRNA RT-PCR positive in the last sample. N indicates the number of subjects included in each cohort.

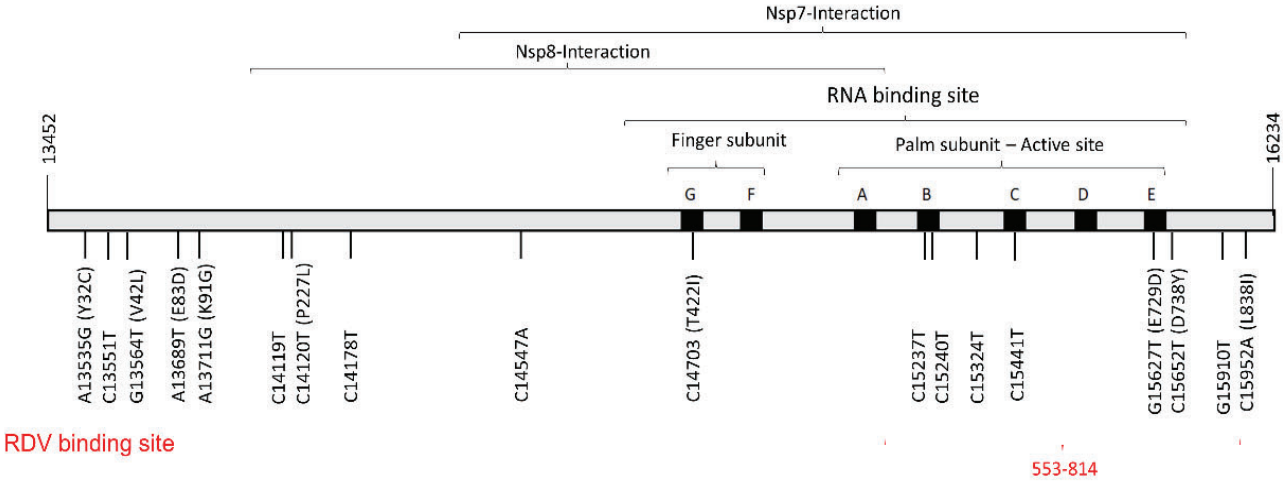

Figure 2. Gene structure of the SARS-CoV-2 non-structural protein 12 with the novel mutations detected. The nucleotide position is indicated respect to Wuhan-Hu-1 reference genome [GenBank: MN908947.3]. Amino acid substitutions are indicated in brackets. Gene structure is based on Gao et al, 2020 [25].

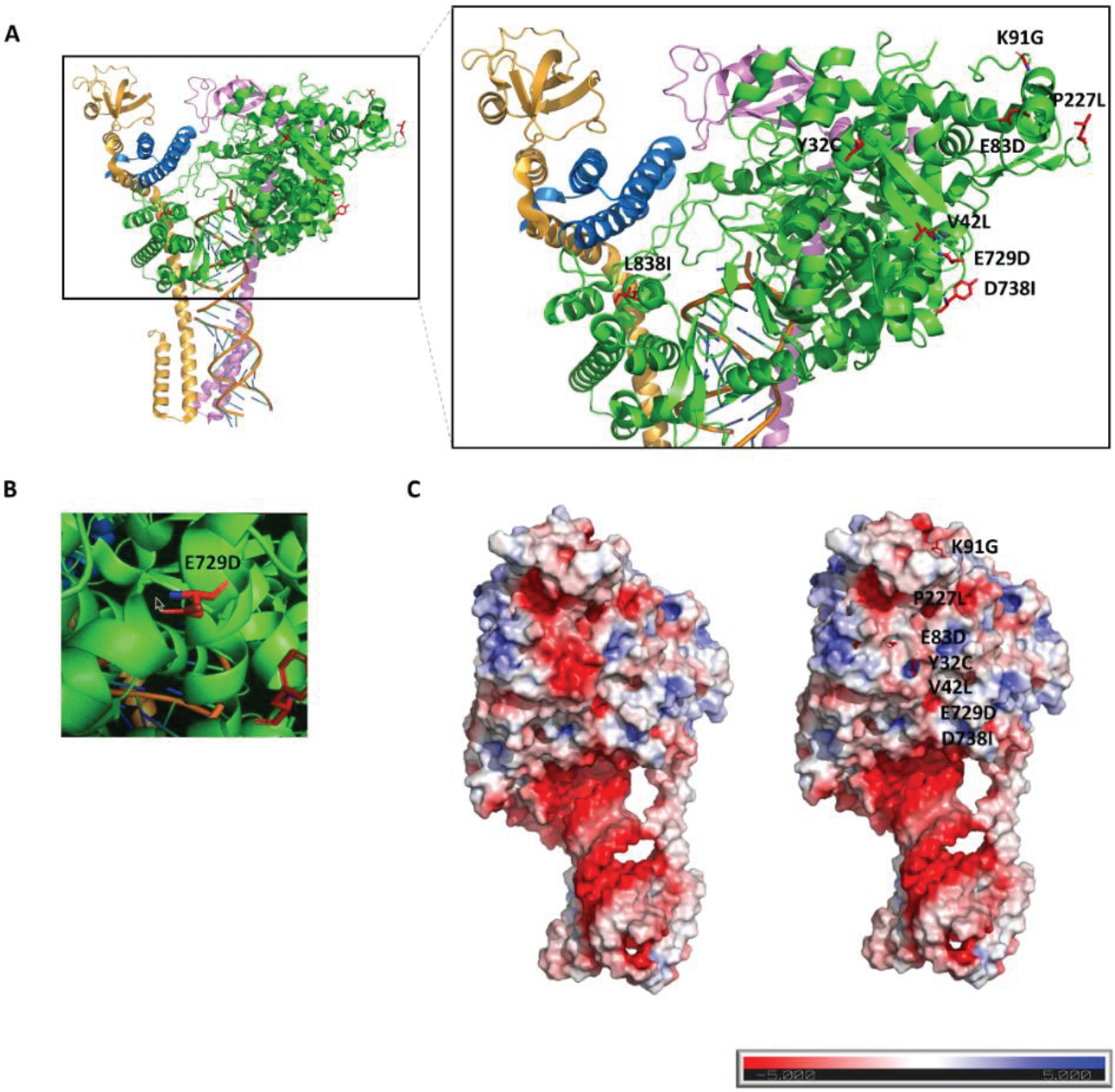

Figure 3. Location of novel RdRp mutations in 3D protein models. (A) Theoretical structure of RdRp is represented in the ribbon structure of the cryo-EM model using PyMOL. The different subunits of the RdRp are colored as follow: nsp7 in blue, nsp8a in yellow, nsp8b in pink, nsp12 in green. The RNA duplex is colored in orange and blue. Novel genotypcally detected mutations indicated in red, in the right figure. (B) E729D mutation breaks the alpha-helix secondary structure of the nsp12 (C) APBS-generated electrostatic surface of the RdRp. Negative charged areas are indicated in red and positive charged areas in blue. Mutations are visualized in red and tagged in the figure.

TABLES

Table 1. Clinical characteristics of the study population according to the treatment of remdesivir received.

Clinical characteristics	5d RDV	>5d RDV
N	111	38
Age (median; IQR)	62.7 (54; 73)	59 (56; 67)
Days of RDV therapy (median; IQR)	5	10 (7; 21)
Comorbidities (n,%)	81 (73%)	33 (86.8%)
• Hypertension (n,%)	51 (45.9%)	8 (21.1%)
• Diabetes mellitus (n,%)	26 (23.4%)	5 (13.2%)
• Obesity (n,%)	18 (16.2%)	3 (7.9%)
• Cardiovascular disease (n,%)	32 (28.8%)	3 (7.9%)
• chronic pulmonary disease[a] (n,%)	24 (21.6%)	6 (15.8%)
• chronic kidney failure (n,%)	9 (8.1%)	1 (2.6%)
• haematological malignancies[b] (n,%)	15 (13.5%)	25 (65.8%)
• Solid malignancy with active chemotherapy (n,%)	4 (3.6%)	1 (2.6%)
• Transplant recipients (n,%)	4 (3.6%)	6 (15.8%)
• Other disorders treated with immunosuppressors (n,%)	7 (6.3%)	1 (2.6%)
ICU admission (n,%)	34 (30.6%)	21 (55.3%)
Mortality (n,%)	7 (6.3%)	8 (21.1%)

N: number of subjects

Age and days of treatment are indicated as the median and the interquartile range Q1; Q3.

[a] Chronic pulmonary disease includes chronic obstructive pulmonary disease and asthma

[b] Haematological malignancies include lymphoma or leukaemia

Abreviatures: RDV remdesivir, ICU Intensive Care Unit

Table 2. *Nsp12* nucleotide substitutions detected at baseline (pre-RDV), at baseline and after treatment with remdesivir (pre/post-RDV) and after (post-RDV) therapy in clinical isolates.

Mutations[a]	Location	Pre-RDV[b]	Pre/post-RDV[b]	Post-RDV[b]	ICU[b]	Mortality[b]
A13535G (Y32C)		2	1 (10d)	1 (5d)	1	0
C13551T				1 (18d)	1	1
G13564T (V42L)			1 (5d)		0	0
A13689T (E83D)				1 (18d)	1	1
A13711G (K91G)			1 (5d)		0	0
C14119T	Nsp8-interaction		1 (5d)		0	0
C14120T (P227L)	Nsp8-interaction		3 (5d n=2, 10d)	1 (5d)	3	1
C14178T	Nsp8-interaction		1 (5d)		0	0
C14547A	Nsp7-8-interaction		2 (5d)	3 (5d)	3	0
C14703T (T422I)	RNA binding site (motif G)		1 (10d)		1	0
C15237T	RNA binding site (motif B)			1 (10d)	0	0
C15240T	RNA binding site (motif B)		1 (5d)		1	1
C15324T	RNA binding site	2	1 (30d)	2 (5d)	3	1
C15441T	RNA binding site (motif C)		1 (5d)		0	0
G15627T (E729D)	RNA binding site (motif E)		1 (5d)		1	0
C15652T (D738Y)	RNA binding site		1 (5d)	1 (5d)	1	0
G15910T			1 (10d)		0	0
C15952A (L838I)			8 (10d n=7; 21d)	1 (20d)	6	3

[a] The nucleotide position is indicated respect to Wuhan-Hu-1 reference genome [GenBank: MN908947.3]. Amino acid substitution is indicated in brackets.

[b] Indicate the number of subjects with the specific substitution, admitted in the ICU or died due to all-cause mortality. Days of treatment received when the mutation was found are indicated in brackets.

Abbreviatures: Nsp non-structural protein, RDV remdesivir, ICU intensive care unit.

Table S1. Clinical characteristics of the patients with SARS-CoV-2 non-structural protein 12 and/or Spike mutations of interest.

ID	Age	Comorbidity	ICU admission	Mortality	Immunosuppressive treatment	Antiviral treatment	Date of infection	Linage	NSP12	
									Before RDV	After RDV
1	60	Hypertension, enolic dilated myocardiopathy	no	no	BNB	RDV 5d	March 2021	Alpha (B.1.1.7)	-	A13535G (Y32C), C14120T (P227L), C15324T
2	68	Hypertension	yes	no	TCZ, CORT	RDV 5d	April 2021	Alpha (B.1.1.7)	C14120T (P227L)	C14120T (P227L), C15324T
3	40	No	no	no	BNB	RDV 5d	March 2021	Alpha (B.1.1.7)	-	G14547A
4	47	No	no	no	TCZ, BNB, CORT	RDV 5d	April 2021	Alpha (B.1.1.7)	A13535G (Y32C), C14120T (P227L), C15324T	G14547A
5	40	No	yes	no	TCZ, BNB, CORT	RDV 5d	April 2021	Delta (B.1.167)	A13535G (Y32C), C14120T (P227L), C15324T	G14547A
6	49	Thalassemia minor	no	no	BNB	RDV 5d	June 2021	Alpha (B.1.1.7)	-	G15652T (D738Y)
7	83	Diffuse large B-cell lymphoma	yes	yes	R-CHOP[a]	LPV + RTV + HCQ 7d, AZM 5d, RDV 10d + 8d, plasma	March – December 2021	Delta (B.1.167)	-	C13551T, A13689T (E83D)
8	67	Kidney transplant, arterial hypertension, hypercholesterolemia	no	no	BNB 10d	RDV 10d	July 2021	Delta (B.1.167)	G15910T	C15237T, G15910T
9	64	Mantle lymphoma in complete remission	yes	No	DEX 10d, TCZ, BNB, anakinra	RDV 20d, TEC, IVM + plasma	August 2021	Delta (B.1.167)	-	C15952A (L838I)

[a] R-CHOP is a chemotherapy composed by the combination of rituximab, cyclophosphamide, hydroxidaunorubicine, oncovin and prednisone

Abbreviations: ICU intensive care unit, LPV lopinavir, RTV ritonavir, RDV remdesivir, HCQ hydroxychloroquine, AZM Azithromycin, TEC teicoplanin, IVM ivermectin, TCZ tocilizumab, BNB baricinitinib, DEX dexamethasone, CTX cyclophosphamide, PDN prednisone, CORT other corticoids.

DISCUSSION

In this thesis we discuss CMV and SARS-CoV-2 opportunistic infections in immunocompromised patients and their two major challenges: antiviral resistance and persistent infection following the order of the objectives.

CMV has an endemic prevalence worldwide and it has a high morbidity and mortality rates when it comes to immunocompromised patients due to its deficient immune system and the virus pathogenesis. Antiviral drugs have reduced these mortality rates but have promoted the emergence of resistance. We hypothesized that the 5-12% of incidence of antiviral resistance previously stated is underestimated due to clinical decision-making and is based on empirical practice and the genotypic studies are not systematically requested. Therefore, the **objective 1** was to characterize genotypically and phenotypically mutations associated with antiviral resistance of the cytomegalovirus (articles 1, 2 and 3).

To accomplish it, we studied a cohort of patients with different immunodeficiencies (HIV, common variable immunodeficiencies, inflammatory bowel disease), congenital CMV infection, but mainly, SOT and HSCT recipients with refractory CMV infection, who fulfilled the criteria of suspicion of resistance to standard antiviral treatment. These patients belonged to the hospitals included in the Spanish Network for Research in Infectious Diseases (REIPI) and the Group for the Study of Infection in Transplantation (GESITRA).

Clinical samples were genotypically analysed at the moment of suspicion of resistance to identify CMV resistance mutations (n=28) in 25.5% of the patients; among them, 79.2% were localized in *UL97*. This supports the existing literature that states that antiviral resistance mutations emerged mainly in the *UL97*[62] and that *UL54* mutations can confer multi-resistance and may emerge after previous *UL97* mutation enhancing the level of resistance[412], as was found in 4 SOT recipients in the present cohort (article 2). Otherwise, 2 *UL54* resistance mutations emerged without any previous change in *UL97*, as well as the uncommon combination of mutations in *UL54* together with *UL56* after GCV+LMV therapy. This evidence reinforces that all target genes should be genotype, and not only *UL97* as occurs in standard diagnostic genotyping in many laboratories.

Genotypic resistance testing allowed the detection of 14 unknown genetic variants in clinical isolates from this cohort; some of them were phenotype. *UL97* C480F mutation was detected in 2 transplant recipients, in who CMV DNA clearance was achieved after 18 and 30 days of MBV treatment, respectively (article 1). MBV was administered as salvage therapy after clinical

resistance or refractory infection, as it still in a phase III clinical trial. C480F variant caused recurrent CMV infection 13 days after the initiation of a second MBV course and after a month of VGCV/GCV treatment in one patient; and after 2 months of MBV and nearly 4 months of VGCV/GCV in the second patient. Previous trials inquired whether C480F was selected under GCV or MBV[413]. In the present study, C480F was not detected under GCV treatment before the initiation of MBV, and it was not present at baseline before GCV or any anti-CMV drug, suggesting MBV was the selective pressure. One of the causes for treatment failure is an inappropriate dosage. They received 400mg every 12 hours of MBV, a dosage that presented similar efficacy to that of VGCV for clearance CMV viremia. However, a higher dosage related to resistance development has been reported[202,414]. In this case, it was due to this mutation and associated to cross-resistance to GCV and MBV, as shown by antiviral susceptibility testing.

The residue C480 is located in the catalytic loop of UL97, outside the codon range of the ATP-binding site, specified for GCV resistance mutation studies in most laboratories. GCV resistance mutations cluster in the *UL97* domains involved in substrate recognition (residues 460, 520, and 590-607), whereas the majority of mutations located in the vicinity of the ATP-binding site (residues 353-397, and 409-411) confer resistance to MBV but not to GCV[202].

Most commonly selected after-exposure MBV or GCV resistance mutations do not confer cross-resistance to the other mutations as they have different mechanisms of action[224]. However, only a few studies have reported GCV-MBV cross-resistance mutations in clinical specimens, such as V466G, P521L[256]and the novel C480F, which were found at distant sites from the ATP-binding loop[213,224,256]. C480F has previously been detected in a patient treated with MBV[213], but was not phenotyped. Different amino acid substitution in the same residue (C480R) has also been reported in AD169 exposed to the methylenecyclopropane analogue conferring GCV-MBV cross-resistance and severe growth defects[258], such as those observed with C480F. This data is consistent with the fact that the residue C480 is essential for GCV and MBV mechanism of action, UL97 phosphotransferase function, and thus, CMV replication.

Novel mutations in the *UL54* and *UL56* detected by genotypic methods in this cohort were phenotyped (article 2). The genotype-phenotype correlation study resolved the status of 5 uncharacterized UL54 (G441S, A543V, F460S, R512C, A928T) and 1 UL56 (F345L) mutation. Antiviral susceptibility assay showed A928T presented high triple-resistance to GCV, FOS and CDV, A543V conferred intermediate resistance to CDV, and the rest had an antiviral-susceptible phenotype. This result also strengthens the importance of discerning high from intermediate-

low levels of resistance, as it influences the dosages and antiviral molecules chosen in the clinical practice.

The location of uncharacterized mutations on the gene map can provide an insight of its impact on antiviral-response, viral kinetics or the biological role of particular residues on the protein's function. However, *UL54* A928T mutation is located just outside the catalytic polymerase function, in a region supposedly not associated with a lack of antiviral response, but conferred triple-resistance. Previous reports showed certain triple-resistance mutations in the surroundings, such as A836P and del981-2, detected in patients receiving prolonged therapy, and causing loss of growth fitness[415]. Conversely, many variants in the nearby amino acids (V902G, E903G, K947E, M959T) were susceptible to GCV, FOS and CDV[195].

UL54 A543V and R512C are mutations located in the Exo-III/delta-C domain. This domain has been described to be involved in exonuclease and polymerase catalytic function, which are well-conserved among mammals and yeast, and was associated with resistance to GCV, FOS and CDV[62]. Our study showed A543V conferred intermediate-CDV resistance, whereas previously reported A543S mutation had a wild-type phenotype[195], suggesting that when alanine is substituted for valine, its bulky lateral aliphatic chain could block its polymerase function, but the small hydroxyl group of the serine does not. Although growth assays showed this mutant had a defective replicative capacity, A543V clearly replicated in the two clinical cases infected with this variant, suggesting A543V growth defect could be compensated by other mutations, such as *UL97* M460I which emerged together with A543V in both cases. Therefore, residue A543 seems to play an important role in viral growth.

UL54 G441S and F460S mutations were located in the region associated with GCV and CDV cross-resistance, but none conferred resistance to these antivirals. In agreement, the F460L variant, located in the same residue, was described to be sensitive to DNA-polymerase inhibitors and did not show slow-growth phenotype[195]. On the contrary, G441S impaired viral replication.

Recombinant phenotyping assays involve long and laborious work, making it infeasible to be implemented in the hospital routine. To overcome this limitation, 3D protein modelling has shown to be a useful and rapid tool to predict the impact of genetic variants *in silico,* however, its predictive efficacy has not been validated. Recombinant phenotyping and 3D modelling of *UL54* mutations were compared (article 2). 3D protein modelling correctly predicted the effect of *UL54* A928T, A543V, G441S and F460S mutations on antiviral susceptibility, but did not with the R512C mutation, highlighting its limitations and the need for confirmation by recombinant

phenotyping. Previous protein models of homolog viruses limit the usefulness of this tool, for instance, novel *UL56* mutations could not be localized in the 3D protein as there was no correspondence with the amino acid sequence in the homolog model of herpes simplex virus-1.

Novel *UL56* mutations were then characterized by bacmid technology. F345L emerged after, but not before, LMV therapy in an HSCT patient (article 2); and R256C was detected and followed-up in two transplant recipients naïve of LMV (article 3). P800L, a polymorphism previously reported[143], was detected 7 days before LMV therapy and remained after 28 days of LMV in association with the *UL56* C325F resistance mutation until the patient's death (article 2). This context made it interesting to measure their potential impact in decreasing sensitivity to LMV and in the virus replicative capacity.

Both, F345L and R246C are located within the LMV-resistant-mutant region of UL56, but none of them confer resistance to LMV or cross-resistance to any DNA polymerase inhibitors. Many LMV-resistant mutations (V236M, T244K/R, L257I, C325Y/F/W) were found in the vicinity in clinical isolates from HSCT and SOT recipients, comprising regular or impaired viral fitness[62,184,416]. It is unusual to find a susceptible genetic variant within this polemical domain, which is highly conserved among CMV isolates, and it is even more infrequent to detect a mutation that entails a superior replicative, as R246C does. Since R246C mutation does not seem to interfere with the mechanism of action of either DNA polymerase and terminase inhibitors, it could be a compensatory mutation developed to replicate more efficiently to counteract the depletion caused by the antiviral molecules, as a viral escape. On the contrary, F345L strongly impaired viral replication, but surprisingly did not involve a loss of response to the different antiviral molecules tested. This fact suggests that the F345 residue impairs and R246 enhances the biological UL56 terminase function, but any of them interact with LMV itself.

In the case of the P800L polymorphism, phenotypic assays showed this mutation did not favour viral growth and was confirmed to have no effect on the response to LMV. As P800L+C345F were detected together after LMV therapy and continued replicating, the defective growth associated to P800L alone seemed to be compensated by the emergence of the C325F, whose residue appeared to be critical for LMV binding but unimportant for CMV terminase function.

Among the large number of variants found after refractory CMV infection, the newly characterized mutations in the presented research articles were located close to previously associated codons or domains, but the borderline between resistant mutations and polymorphisms is not delimited. This confirms the phenomenon of vicinity of resistance and

natural polymorphisms in *UL54* and *UL56*, already described for UL97 phosphokinase [416], corroborating the suggestion that mutation phenotypes cannot be fully predicted by their gene position and strengthening the need for individual characterization of mutations. Therefore, the remaining *UL54* genetic variants located in highly variable regions detected (article 2) required future phenotyping.

After elucidating the phenotypes of unknown *UL97*, *UL54* and *UL56* mutations, the incidence of antiviral resistant mutations (n=27/94, 28.7%) was increased, and was even higher considering all of them emerged in transplant recipients (n=27/83, 32.5%). Natural polymorphisms were detected in an 80.9% of the patients, which arose less in *UL97* than in *UL54* during standard treatment, supporting the existing literature[211,233].

Hardly any natural polymorphisms were detected in *UL56* (article 2), as *UL56* is well-conserved among herpesvirus and in patients naïve of antiviral treatment[143] or treated with GCV[240], with no cross-resistance being found. However, LMV-resistant mutations have been mainly described when it is administered as salvage therapy for refractory/resistant CMV infections, and when used as primary or secondary prophylaxis[195,249]. LMV resistance mutations selected *in vitro* appeared earlier than for the currently used antiviral drugs[242]. This data suggested a lower genetic barrier to mutate after LMV administration. Clinical experience has shown LMV was not advisable to treat CMV infection, due to the rapid and frequent emergence of LMV-resistant mutations once CMV is replicating[249]. However, there was scarce information on whether LMV resistance mutations appeared in clinical samples prior or subsequent to LMV administration. Only one study reported the *de novo* LMV-resistant mutation C325Y in 2 formalin-embedded tissue biopsies from 1/147 (0.68%) patient[246].

For this reason, we aimed to elucidate the presence of baseline mutations in *UL56* and its associated phenotypes in 80 transplant recipients infected with CMV naïve of LMV from the described cohort (article 3). R246C was the only variant detected by standard sequencing and NGS in 2 transplant recipients. This mutation emerged since first CMV replication and responded transiently to various alternative DNA polymerase inhibitors. Recombinant phenotyping showed R246C conferred an increased replicative capacity and was sensitive to GCV, CDV, FOS, MBV and LMV. Follow-up of the antiviral treatment and CMV viral load monitoring of both clinical cases, showed its advanced growth capacity allowed its replication when therapy was discontinued. Clinical information correlates with phenotypic results, suggesting a similar impact of this mutation *in vivo.* Therefore, in response to objective 1.3, these results suggested an absence of primary resistant mutations to LMV and demonstrated a low rate (2.5%) of natural occurring

polymorphisms within LMV-resistant-associated region before its administration, in concordance with what was previously hypothesized[160,243].

NGS provides an increased sensitivity of detection of minor populations of emerging genetic variants. NGS capacity is more useful for large batches or for serial sampling during case reports. NGS allowed the detection and determination of the prevalence of minor *UL97* F342S, A594A and *UL54* D413N variants (article 3). Assessment of the significance of minority subpopulations when using NGS in clinical samples should include evidence of their reproducible detection to avoid false positive results[253], however, NGS could not be repeated in our clinical cases due to the lack of sample volume. Even though NGS was attempted for the two reported clinical cases with UL97 C480F variant, the DNA quantification obtained after preparation of the library was not sufficient to perform it after being firstly checked by Sanger methods (article 1). Sanger sequencing remains the most accessible and scalable solution for routine genotypic diagnosis until issues of practicality and timely availability of NGS are resolved. In our studies, all clinical isolates were bidirectionally sequenced by Sanger method and results were verified in technical replicates and in different biological samples during the clinical follow-up, to avoid sequencing artifacts.

Genotypic antiviral resistance testing usually covers codon ranges *UL97* 440-670, *UL54* 300-1000 and *UL56* 229-369[238], but these are not all-inclusive for known resistance mutations. New antiviral molecules and mutations require expanded analysis of viral gene regions, such as *UL27* and the upstream of *UL97* (codons 335-680) to detect MBV-resistance mutations, or the *UL89* and *UL51* terminase subunits to determine mutations associated with LMV resistance. We sequenced the two known resistant regions for MBV and GCV of *UL97* to have a widely overviewed genotype (article 1), and the three genes encoding CMV terminase complex by NGS (article 3). On the other hand, complete gene sequences need to be amplified to determine outbound resistance mutations and natural polymorphisms as they are usually located far from hot spots for drug resistance. Probably, the prevalence of natural polymorphisms in *UL56* and *UL97* was underestimated as full-length genes were not amplified, and only the regions associated to drug-resistance were sequenced (article 2).

Early detection and viral loads monitoring can improve the assessment of patients and avoid the selection of mutants. Determining the timing of the earliest emergence of resistant mutations is essential to understand the clinical background that favour this selection. A retrospective determination could be achieved in the 4 clinical cases extensively presented (articles 1 and 3). However, in the study where most phenotypes were performed (article 2), results of antiviral

susceptibility and growth capacity assays were obtained months after the genotypic resistance testing was requested, which restricted the capacity to recover previous isolates, especially in patients from different hospitals.

Studies of viral genetic mutations have been bi-directionally informative throughout the development of antiviral drugs, which were not fully understood until their drug resistance mutations were mapped. Therefore, our studies reinforce the proposal that genotype-phenotype correlation serves not only to define the level of antiviral resistance and viral kinetics, but also to widen the biological role of each residue. This aids to define conserved and variable domains and their associated function in order to construct comprehensive and reliable mutation maps.

Due to impaired defenses, immunocompromised patients present higher risk of severe COVID-19 progress and worse clinical outcomes. Moreover, in the case of SARS-CoV-2 we are facing two challenges: the follow-up of viral RNA dynamics and the determination of active viral replication. Both challenges are absent in the case of CMV infection, as CMV DNA detection in plasma samples directly implies active replication and is not continually detectable if the virus is latent or not viable. Oppositely, SARS-CoV-2 gRNA can remain positive in respiratory samples for several weeks after clinical recovery in sicker patients, as well as in asymptomatic and mild ill individuals. Therefore, the CDC stated in September 2020 that identification of SARS-CoV-2 RNA through RT-PCR does not equate to the presence of viable infectious virus within patients[26].

The Ct value provided by the RT-PCR presents high variability between and within methods[317], thus, it cannot be directly comparable across assays[314]as it does not reflect true viral loads. Real time-PCR requires standardization using reference curves and cellular controls in the case of respiratory samples [417]. Nasopharyngeal/throat swabs have an intrinsic variability that depends on the operator and the tolerance of the patients[315]. Swab collection quality of every sample must be checked to avoid false-negative results and provide reliable values. Normalization of viral loads using cellular loads has been demonstrated to complement the validation of real time-PCR results in the diagnosis of respiratory viruses[418]. With that propose, we accomplished the **objective 2** of the thesis that was to demonstrate that NVLs can provide robust results of SARS-CoV-2 loads avoiding the intrinsic variability of respiratory samples (article 4). Data obtained by measuring the *N* and *RdRp* genes at 2 different timepoints of the infection, showed NVLs overcome the limitations of Ct value and sample collection variability, providing more accurate values that can guide the monitoring of patients and treatment management.

Nevertheless, positive gRNA RT-PCR or NVLs does not necessarily implicate viral replication as non-viable virus particles or virus debris can be detected in nasopharyngeal/throat swabs after the infection course. Determining if the virus is still actively replating is important to control the infection and determine persistent viral replication. The gold standard to determine virus viability is viral culture. However, it is labour-intensive and requires BSL-3 facilities that make it unsuitable for many laboratories. Intermediate sgRNA species detection was proposed as a surrogate indicator of infectivity by Wölfel et al [302] at the beginning of the pandemic COVID-19. Therefore, as stated in **objective 3,** we aimed to determine if sgRNA can detect competent viruses by studying the kinetics of sgRNA in two consecutives respiratory samples of 84 healthcare workers (article 4). Due to the impossibility to access to BSL-3 in our hospital at that time, we statistically compared the result of sgRNA detection with NVLs, days of symptoms onset and gRNA Ct values in both samples of each subject to provide two easy and rapid tools for clinical decision making.

SgRNA and NVLs were significantly associated and allowed to define a viral load interval (1-3 log_{10} copies/1000 cell) which sgRNA is recommended to be requested. SgRNA was significantly correlated with days after symptoms onset as well. Our findings showed sgRNA was mainly detectable in specimens collected during the first week of symptoms, generally coinciding when most transmissions have been described[322]. This result agrees with several studies describing SARS-CoV-2 culture recovery from respiratory specimens obtained 1–9 days after infection onset in patients with mild to moderate disease[308,311,419]. However, sgRNA has been detected up to 17 and 21 days after symptoms onset in 12 and 99 SARS-CoV-2-positive swabs, respectively[28,301]. As they were healthcare workers, they were not suspected to be immunocompromised, however, 12/83 presented comorbidities and 7 of them had positive sgRNA after 7 days of symptoms (article 4), supporting a correlation of comorbidities with longer time of infectivity [353,356,420,421]. This fact was reinforced by the results obtained when comparing sgRNA and viral culture, showing 73% of successful virus isolation and sgRNA detection in samples from the first 7 days of symptoms (article 5). Prolonged viral replication (>21 days of symptoms)[315] detected by culture and sgRNA test was demonstrated in 7 severely immunosuppressed patients who required hospital admission. Moreover, virus infectivity was detected in 13 of 18 asymptomatic subjects, therefore, sgRNA test is key to identify infectious individuals in order to control the spread of infection, and to better characterize the shedding of a replicant-competent virus longitudinally from people with moderate to mild disease, as well as severely ill and immunocompromised patients.

Even being extensively reported that Ct values are highly variable[317], sgRNA detection correlated with the Ct values of gRNA RT-PCR (article 4). The explanation is that both genes were tested simultaneously together with the cellular quantification by multiplex RT-PCR always using the same procedure and thermocycler, emphasizing gRNA RT-PCR assays should be standardized in every laboratory before using. Conversely, in the sgRNA and viral culture study, gRNA Ct values bias was considered by including diverse platforms from two different hospitals, and even so, we demonstrated that sgRNA detection can overcome the limitations of Ct values variability (article 4 and 5).

This data suggested sgRNA could be a good surrogate marker of infectivity as it was previously supported by studies in challenged primate models [303,307], in patients persistently positive for SARS-CoV-2 total RNA[28] and comparing different sgRNA species and gRNA[302,308]. At that time, only a couple of reports performed viral culture and sgRNA in a small number of samples. On the contrary, some reports suggested that the loss of sgRNA detection was due to the lower overall RNA transcript concentration compared with gRNA [309,310], but their results similarly showed significant association between sgRNA with viral loads and days after symptom onset. However, these studies were limited by the absence of correlative viral culture data.

Therefore, our objective was to validate sgRNA value as surrogate indicator of infectivity by viral culture, thanks to managing to access to BSL-3, in a considerable sample size (n=105) and confirming positive culture results by RT-PCR and/or using specific monoclonal antibodies (article 5). Our findings showed a significantly high coefficient of agreement between both techniques and that sgRNA detection has a sensitivity of 97% and a positive predictive value of 94% to detect competent-replicative virus. Recently, Phuphuakrat and colleagues[422] performed *N* sgRNA RT-PCR and viral isolation in 260 nasopharyngeal swabs from 36 patients and supported the used of sgRNA to detect infectivity. A 100% of specimens containing culturable SARS-CoV-2 from a set of 123 clinical samples were successfully identified by E sgRNA in another report[423]. Kim et al achieved a 100% of sensitivity when using gRNA and sgRNA of the N and S genes, but the specificity of sgRNA was higher (65-68%) than that of gRNA (23-17%), suggesting a significant advancement on the transmissibility-based approach beyond the detection of gRNA[424]. The small differences found among comparative studies can be explained by the cell type used for viral isolation, as Vero E6 cells expressing TMRPSS2 extended the analytical sensitivity found of viral isolation and resulted in a faster isolation[423]. In our study (article 5) and the mentioned reports[308,422], culture was performed in Vero E6 cells that did not express TMRPSS2, showing a slightly increased sensitivity in sgRNA RT-PCR over viral culture. Similar levels of sensitivity and positive predictive value of sgRNA were shown in another report[425], but were not reached when

using Quidel Sofia SARS Antigen Fluorescent Immunoassay (FIA) (57.7%) or gRNA RT-PCR (59.3%) in positive viral culture samples. Therefore, sgRNA seems a better biomarker of live virus than antigen tests or gRNA RT-PCR, and the slightly increased sensitivity of sgRNA RT-PCR[308,422] provides a beneficial overestimation of infectivity, that prevents the risk of prematurely releasing patients from isolation or treatment discontinuation.

There is some controversy regarding the choice of sgRNA species to measure, as it may have a significant impact on detection and result interpretation. *N* sgRNA is suggested to be more abundant than *E* sgRNA and persists for longer periods[310,426], as reported in a subset of positive *N* sgRNA samples that was subjected to *E* sgRNA detection and 69.0% of these samples were negative for *E* gene sgRNA. Another study on *E* and *N* sgRNAs from nasopharyngeal swabs obtained from 185 SARS-CoV-2-positive patients revealed the median duration of symptoms to negative test of 14 and 25 days for *E* and *N* sgRNAs, respectively [303]. However, a recent study showed the median time to *S* sgRNA and *N* sgRNA negativity were 11 days in both cases[422]. These differences between sgRNA species could be explained by the enclosure of RNA transcripts in double membrane vesicles and/or extracellular vesicles allowing longer persistence of specific sgRNA species[427]. There are no clear data of whether *E* transcripts are more rapidly degraded by ribonucleases or they better reflect recent transcription. Nevertheless, our study demonstrated that *E* sgRNA has a better coefficient of agreement according to viral culture (kappa's coefficient 0.88) than that previously obtained using *N* sgRNA (kappa's coefficient 0.467)[308], suggesting that *E* sgRNA of SARS-CoV-2 is the best option to detect infectivity, as corroborated it by studies published afterwards [428].

An active replication of the virus produces minus-strand RNA intermediates that can be detected by PCR. A research group designed a strand-specific real time RT-PCR assay to detect plus and minus-strand RNA intermediates separately. Minus-strand RNA was detected beyond the 14-day period previously reported for sgRNA, however, whether SARS-CoV-2 was actively replicating could not be confirmed by lack of viral culture data[401]. It is important to comment that sgRNA RT-PCR detected both positive and negative strand *E* sgRNA species, as they are both generated during discontinuous transcription within virus replication cycle (articles 5, 6 and 7).

Our studies presented some limitations as the impracticability of repeating viral isolation since these samples were stored at -20ºC and the incapacity to quantify sgRNA due to the absence of an approved WHO standard for sgRNA species; therefore, this technique is meant to must be qualitatively implemented. However, the significant correlation found between both methods allowed the validation of sgRNA as a good surrogate marker of SARS-CoV-2 infectivity.

Predicting SARS-CoV-2 infectivity is a critical need to safely shorten isolation precautions, to vacate COVID-19 hospital rooms, to reduce work reincorporation policies, and to check the efficacy of treatments/vaccines, especially during the pandemic COVID-19 period. Defining the time of infectivity is especially important in immunosuppressed patients as they have high risk for prolonged SARS-CoV-2 infection, which favours the development of viral mutations and worsen patients' clinical outcomes.

The origins of VOC, such as alpha, beta, gamma, delta and omicron, are not yet understood, but several case reports have demonstrated that multi-mutational variants have arisen during the course of such persistent SARS-CoV-2 infection in immunocompromised individuals receiving cancer chemotherapy, organ transplant recipients, and people with uncontrolled advanced HIV disease [334,355,429]. Failure to clear SARS-CoV-2 due to sub-optimal immune responses results in persistent infections that allow a form of rapid, multistage evolutionary jumps and the accumulation of mutations that can alter Abs recognition and other immune evasive properties[430], as seen in the already emerged variants. However, no evidence that the evolved variants in immunocompromised patients successfully spread into the general population [347,410,431]. Since a large number of persons are living with innate or acquired immunosuppression, the association between immunosuppression and the evolution of VOC requires further delineation and mitigation strategies.

Some factors have been associated with longer duration of viral infectivity, including older age, sex, presence of comorbidity, the receipt of steroid and immunosuppression [353,356,420,421]. Nevertheless, prolonged SARS-CoV-2 replication has been reported in several immunocompromised patients, and reinfection was dismissed as sequencing results showed the same virus strain was presented during the infectious course[355,429]. However, further data was needed to understand the frequency and clinical significance of these findings. The **objective 4** inquired about the evaluation of patients with severe COVID-19 and persistent replication (article 6). This clinical case presented an immunocompromised patient with positive SARS-CoV-2 RT-PCR 60 days after symptoms onset, where NVLs and antiviral and immunosuppressive treatments were followed-up during the infectious course. At this time the RNA dynamics in immunosuppressed patients was unknown and sgRNA was not validated. However, the increase in the viral load 54 days after symptoms and the clinical worsening of this patient, together with previous reports of prolonged viral shedding [357,429], supports the hypothesis of persistent replication of SARS-CoV-2 in immunocompromised patients.

Stopping the replication of SARS-CoV-2 in a compromised host by means of an effective monoclonal antibody or antiviral molecules offers an opportunity to improve clinical outcome

and to halt new threatening mutations. Among the limited antiviral drugs available, RDV has shown good efficacy and safety profiles in a 5-dose regimen for the treatment of severe COVID-19[376]. A report of non-hospitalized patients who were at high risk for COVID-19 progression demonstrated that a 3-day course of RDV had an acceptable safety profile and resulted in an 87% lower risk of hospitalization or death than placebo[432]. On the other hand, clinical cases of RDV retreatment showed an initial response to every course of RDV by experiencing symptoms cease and/or negative PCR[357,429,43], but 7/16 severe retreated patients died for all-causes. Although a clinical trial demonstrated that 10 days of RDV did not provide any profit to 5-days treatment[434], the increase of viral loads after the initial 8 days of RDV in the patient presented (article 6), suggested that short administration of RDV could be insufficient in the context of immunocompromised patients with prolonged replication. However, the infection resolution experienced after a second course of 10 days with RDV confirmed that this antiviral is highly effective in the treatment of immunocompromised patients, since viral loads and clinical symptoms were significantly reduced with RDV but not with the rest of antivirals (article 6).

Nevertheless, available literature about RDV retreatment remains scant and no large studies have been reported, making this practice still unaddressed in current treatment guidelines. SgRNA offers a determination of treatment efficacy. A recent report showed that RDV treated samples had lower sgRNA levels detected by NGS, even when high viral RNA persisted, than samples from untreated patients[435]. We aimed to provide further information of the response to RDV treatment and retreatment by sgRNA detection in article 7 and in the published article[354.]

First of all, sgRNA screening determined persistent viral replication in a cohort of 124 patients with haematological malignancies[354]. Among them, 67 were eligible for viral persistence (positive gRNA RT-PCR at the 3rd week since the diagnosis) but only 17 were confirmed by positive sgRNA RT-PCR. Some of them did not respond to 5-day course of RDV, and convalescent plasma and prolonged antiviral treatment were necessary at times for improvement; others initially responded to RDV but fell ill or had a viral load increase afterwards; and others did not respond to antiviral strategies at all. Each of these situations requires a personalized assessment, therefore, further studies of the potential benefits of RDV retreatment and combinatorial therapies with monoclonal antibodies and/or plasma were needed.

SgRNA detection identified 57/149 (38.3%) patients hospitalized for COVID-19 pneumonia who did not respond to 5-dose or longer RDV treatment (article 7). Failure of RDV treatment could be explained by risk factors of the host, such as age, comorbidities or immunosuppressive treatment, or by the emergence of SARS-CoV-2 antiviral resistance mutations. Strategies to ensure that appropriate selection and monitoring for rapid detection of resistance to antiviral

treatment is a key part of medical and public health management. Only a few studies have attempted to characterize amino acid substitutions that could confer resistance to RDV by *in silico* prediction, *in vitro* or in animal models [408,410,436–439]. To determine if the clinical failure in our patients was due to resistance mutations, we aimed to identify novel genetic variations in the *nsp12* gene in clinical samples of non-responder patients before and after RDV, as outlined in **objective 5.**

Although 18 genetic variants in *nsp12* were detected in 34.7% of non-responders by NGS, no significant viral mutations were determined to be associated with failure of RDV treatment, except for the *de novo* mutations E83D and L838I which were potential to have an impact. E83D was detected in the SARS-CoV-2 delta variant that infected an immunosuppressed patient who was admitted to the ICU and died after 9 months of infection. The mutation E83D was detected after RDV retreatment (10 + 8 days) but not before. 3D protein modelling did not predict any interference in the interaction with RDV, however, its implication in fitness advantage, inhibitor escape or adaptation to the host is unknown.

L838I was located in the nearby of the E802D resistant mutation, which conferred a 6-fold decreased sensitivity to RDV *in vitro* [410] and was reported in an immunocompromised patient after RDV therapy[440]. L838I was detected in 8 patients before and after treatment, and in 1 patient only post-therapy. These patients received 10, 20 or 21 days of RDV therapy, 6 were admitted to the ICU and 3 died, suggesting that this mutation is associated with a worse prognosis, although the number of cases is too scarce to confirm it. This naturally occurring variant may provide an improved viral escape for the inhibitor molecule, since no evidence that RDV acts as a mutagen driving spontaneous mutations has been reported as the template stalling action of RDV limits the emergence of spontaneous mutations[408,441] .

E729D and D738Y may also be involved in antiviral response, as they were located in the palm subunit of the polymerase active site next to the residues involved in the RDV-nsp12 interaction and E729D also modified an alpha-helix structure. T422I is located in the conserve motif G in charge of maintaining the position of the RNA template. It is close to the F480L and V557L mutations that are described to confer 2.4x and 5.2x-fold, respectively, RDV resistance *in vitro* [408] and to the RDV-resistant D484Y mutations detected in an immunocompromised patient with persistent viremia [442]. Any of them altered the RdRp catalytic site but were thought to impact the RdRp fidelity checking step before catalysis. Molecular surveillance of this region in RDV-treated COVID-19 patients is suggested to be warranted[441].

Even though 3D protein modelling predicted that none of the mutations found in this study blocked the binding pocket of RDV, they were involved in changes in the electrostatic outer surface and in secondary structures. Changes in polarity, charge, and size of the amino acids could potentially modify interactions of RdRp with antivirals. However, recombinant phenotyping should be performed to confirm it as we previously stated by the slight discordance found between both methods (article 2).

Almost all mutations were detected at baseline in at least one subject (article 7), showing an evolutionary tuning of the viral proteins to a new host, although a response for antiviral selective pressure cannot be excluded in those that persisted after treatment. Indeed, in non-responder patients, Ct values were maintained/decreased and sgRNA remained detectable during treatment, suggesting viral loads progressively increased due to either slow viral shedding or treatment failure. Interestingly, *nsp12* variants arose equally independently of the time of therapy and 5d-RDV subjects presented even more *de novo* mutations than the >5d-RDV subgroup. Another study deep-sequenced RDV-treated and untreated patients and found many synonym variations in the *nsp12* but did not detect any potential resistance[435]. To date, no other resistant clinical case or evidence of global widespread transmission of RDV-resistant mutants, after treatment with RDV for over a year, have been described.

Differences in pathogenicity and transmissibility have been described for the different VOC, mainly due to mutations in the *S* gene, but their relation to the antiviral response is not clear. The alpha variant was more frequent in the 5d-RDV subgroup and the delta in the >5d-RDV subgroup, which agrees with its higher pathogenicity (article 7). Besides the possible bias caused by the inclusion time of the patients, the delta lineage could influence worse response to RDV and the need for a second course of treatment. Analysis of genetic information currently available for all VOC, including the Omicron variant, showed no prevalence of mutations in the RdRp that hampered the efficacy of RDV[443]. The activity of RDV against SARS-CoV-2 alpha, beta, gamma, delta and epsilon, through *in vitro* antiviral testing have been confirmed [443]. Similarities found in the RdRp suggest that RDV will continue to be active against the Omicron variant.

Presence of mixed viral haplotypes has been reported [436], which was likely to have evolved within each host early in infection, persisting thereafter, in some cases possibly within discrete tissue compartments in the lung. We confirmed that there was no reinfection as the same linage infected the first and the last sample, and NGS showed both sequences had identical patterns (article 7). This agrees with the low risk of reinfection in the following 6-9 months calculated in the general population, prior to the emergence of the Omicron variant[359]. The considerable variations obtained in 4/9 patients in the study of Boshier et al[436] appeared to be unrelated to

RDV treatment, as RDV suppressed viral replication in one patient but did not impact of the viral RNA in another subject with identical strain, therefore RDV resistance was not found to account for difference in response. This suggests heterogeneous response to RDV is not due to resistance but rather is likely to be caused by suboptimal tissue levels, as compartmentalization within lung-tissue occurs in respiratory infections, such as influenza or *M. Tuberculosis.*

Evolution of mutations in the *S* gene identical to mutations of VOC/VOI have also been reported in cell culture after several passages before and after RDV-treatment [410]. This indicated that they can arise *in vitro* in the absence of immune selection (similar environment to immunosuppressed patients), demonstrating that *in vitro* populations and the circulating lineages are evolving under driving evolutionary convergence, and that RDV does not favour their development, but rather prevent its replication[410,436].

Scientific attention to the intersection of CMV and SARS-CoV-2 biologic features, host immunologic characteristics, and the unique concerns of immunocompromised hosts is warranted. Increased vigilance is needed to benefit clinical management of the affected individuals and prevent the emergence of mutations associated to fitness advantage, immune escape, antiviral resistance, increased transmissibility, or better adaption to the host, as proved by the emergence of novel SARS-CoV-2 variants and CMV antiviral resistance strains.

The increasing database of CMV and SARS-CoV-2 mutations facilitate the interpretation of genotypic testing results. Identification of low or high replicative capacity variants in patients not responding to treatment or experiencing prolonged replication could be helpful to guide further immunosuppressive therapy and dosing of antiviral molecules. However, compensatory mutations must be evaluated before changing treatment instructions.

Clinical follow-up is highly recommended due to the possible sudden emergence of mutants and the limited sensitivity of Sanger sequencing for detecting small populations. Since the evolution of resistance is staggered and progressive, monitorization for an early detection of novel mutations is crucial to avoid the development of resistance or new threatening variants.

CONCLUSIONS

1. The incidence of mutations associated to antiviral resistance in CMV is underdiagnosed.

2. The genotypic and phenotypic studies determined the high incidence (≈30%) of CMV resistant mutations to standard and new antiviral therapies in patients with refractory/resistant CMV infection.

3. CMV sequencing determined that natural polymorphisms in CMV are less frequent in *UL97* and *UL56* than in *UL54* regardless of antiviral treatment.

4. Novel uncharacterized mutations in the CMV were detected by genotypic methods in 21.3% of refractory/resistant CMV infection after antiviral therapy, strengthening the need for phenotypic assays.

5. The technique of recombinant virus generation (bacmid) allowed the characterization of new mutations in the *UL97*, *UL54* and *UL56* antiviral target genes of CMV. These mutations conferred triple resistance, cross-resistance or intermediate resistance to different antiviral molecules.

6. Mapping of *UL97* mutations that confer resistance to MBV and/or GCV is useful for designing alternative *UL97* inhibitor antivirals to avoid cross-resistance.

7. Baseline mutations associated with resistance to LMV are infrequent in transplant recipients with refractory/resistant CMV infection naïve of LMV.

8. Phenotypic assay identifies alterations in the replicative capacity of CMV variants, highly relevant to optimize the adjustment of treatments, and thus, improve patients' clinical outcomes.

9. Estimating the phenotypes of new CMV *UL97*, *UL54* and *UL56* mutations from similar or adjacent mutations is inadvisable, as mutation phenotypes cannot be fully predicted by their gene position.

10. Widen the scope of antiviral target genes should be implemented in standard diagnostic genotyping to avoid false negative results and to provide better insight into the actual frequency of resistant mutations.

11. 3D protein modelling can predict the impact of genetic variants through changes in the electrostatic outer surface and in secondary/tertiary structures of the protein that may potentially modify interactions between the viral protein and the antiviral molecule. However, antiviral susceptibility level must be confirmed by phenotyping.

12. Prolonged viral replication has been frequently determined in patients with severe COVID-19, especially in those previously suffering hematologic malignancies. A personalized diagnostic approach and specific therapeutic strategies are necessary to address this clinical threat.

13. SARS-CoV-2 normalized viral loads provide robust and similar quantities of viral genomic RNA, avoiding respiratory sampling collection variability and the inadequate use of Ct values.

14. Viral culture has permitted the study of SARS-CoV-2 replication kinetics and cytopathology, and the validation of sgRNA to detect infectivity.

15. *Envelope* sgRNA is a good surrogate marker of SARS-CoV-2 active replication that overcomes the long-term response, the costs of high biosafety laboratory equipment and fresh samples requirements that limit the practicality of viral culture.

16. SgRNA provides essential information for safe work reincorporation, discontinuing isolation precautions, detect persistent SARS-CoV-2 replication and determine the efficacy of different vaccines/treatment approaches.

17. SARS-CoV-2 sgRNA detection and normalized viral loads of are two rapid and accessible techniques that can be easily implemented in routine hospital practice giving a useful proxy for infectivity and patient follow-up.

18. SgRNA screening identified that >60% of patients did not respond to one course (5 days) of remdesivir treatment and >35% did not respond to longer remdesivir therapy (>5 days).

19. No significant virological resistance was determined by NGS after different courses of remdesivir in hospitalized severe COVID-19 non-responder patients, since most genetic variants were detected irrespectively of remdesivir treatment, except for the *nsp12* E83D mutation that only emerged after therapy.

20. Duration of remdesivir therapy does not seem to be a risk factor for developing resistance mutations. However, larger studies on retreatment are needed to address this practice in current treatment guidelines.

21. Extending mutation databases is crucial to interpretate genotypic testing results and to optimize clinical guidelines in the management of patients not responding to antiviral treatment or with persistent viral replication. However, compensatory mutations must be evaluated before changing treatment instructions.

22. Genotypic-phenotypic correlation studies are important to define the level of antiviral resistance and viral kinetics, to understand the biological role of each residue constructing comprehensive and reliable mutation maps and to widen the landscape for new antiviral molecules.

23. Virologic resistance are gradually and progressively developed, thus, clinical follow-up and virus monitoring are essential for the early detection of resistant mutations and to avoid their appearance or the emergence of new threatening variants.

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