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Mecanismos de transmisión del SARS-CoV-2 e implementación de medidas para reducir su propagación por aerosoles

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Servicio de Publicaciones

ISSN 2254-7606

Tesis Doctoral

**MECANISMOS DE TRANSMISIÓN DEL SARS-COV
-2 E IMPLEMENTACIÓN DE MEDIDAS PARA
REDUCIR SU PROPAGACIÓN POR AEROSOL**

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UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Ingeniería Biomédica

2022



Universidad
Zaragoza



Instituto Universitario de Investigación
de Ingeniería de Aragón
Universidad Zaragoza



UNIVERSITAT POLITÈCNICA
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Tesis Doctoral

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Programa de Doctorado en Ingeniería Biomédica

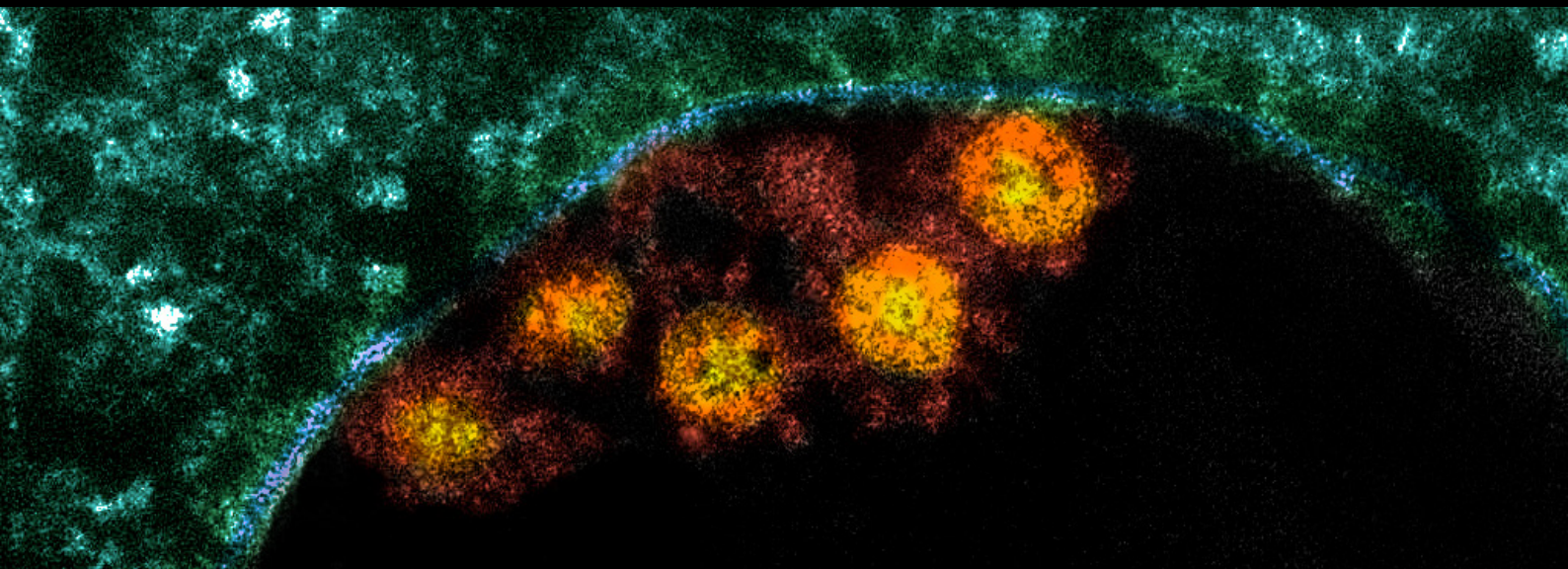
Tesis presentada como requisito para la obtención del título de
Doctora en Ingeniería Biomédica por la Universidad de Zaragoza
y por la Universidad Politècnica de Catalunya



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Zaragoza, 2022



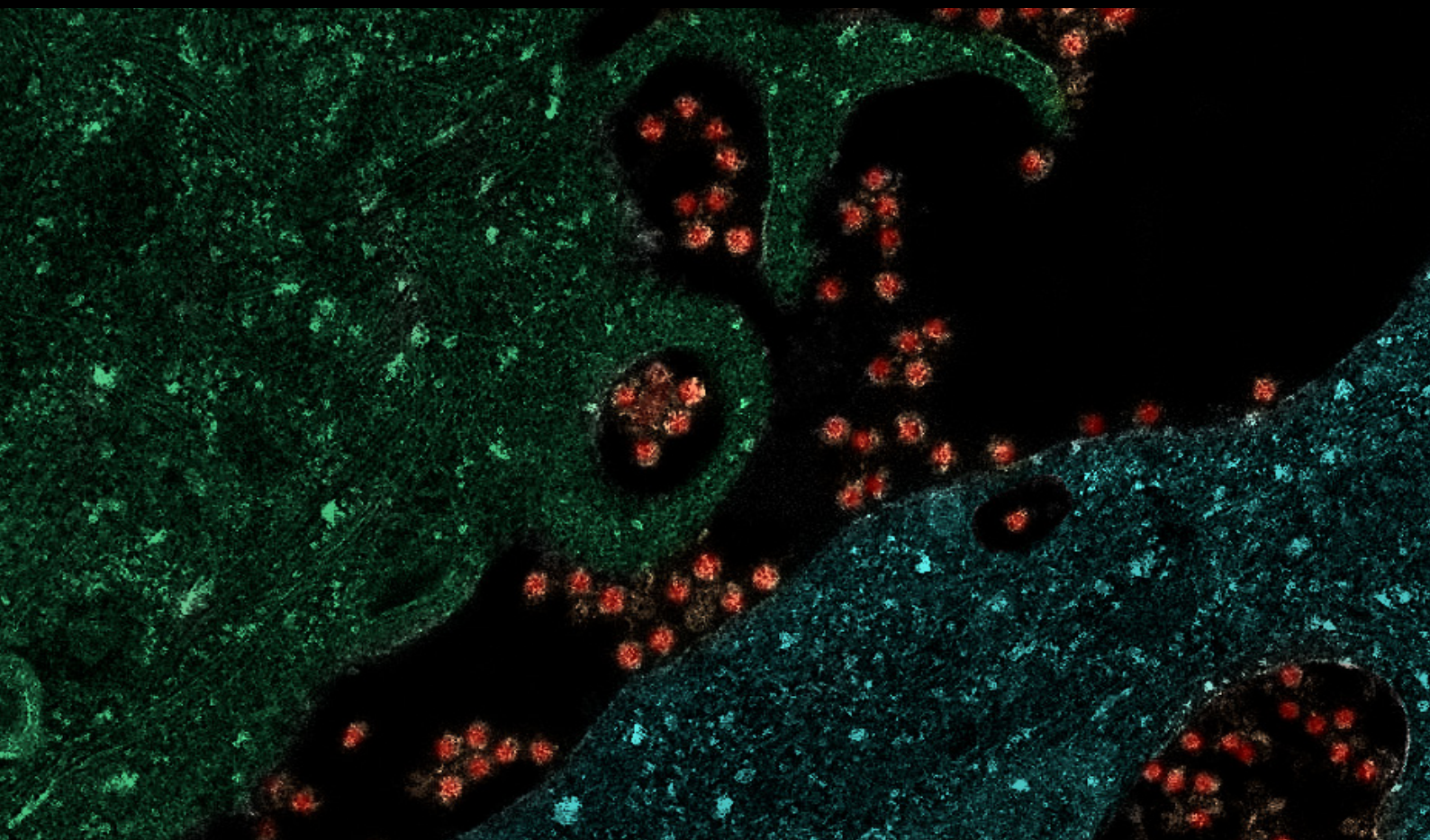
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TESIS DOCTORAL

Programa de Doctorado en Ingeniería Biomédica

Marta Baselga Lahoz

Zaragoza, 2019-2022



Fotografías de portada realizadas por Marta Baselga y Concepción Junquera

Arriba: Virus SARS-CoV-2 adheridos a la membrana plasmática de una célula Vero E6.
Abajo: Dos células infectadas con SARS-CoV-2, mostrando extensiones filopodiales, vesículas con virus internalizados y elevadas cargas virales en la membrana plasmática.

D. ALBERTO JIMÉNEZ SCHUHMACHER, Investigador ARAID, y **D. JUAN JOSÉ ALBA LÓPEZ**, Profesor Titular del Departamento de Ingeniería Mecánica de la Universidad de Zaragoza,

INFORMAN

Que Dña. **MARTA BASELGA LAHOZ** ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada «**Mecanismos de transmisión del SARS-CoV-2 e implementación de medidas para reducir su propagación por aerosoles**» que se corresponde con el plan de investigación aprobado por la Comisión de Doctorado y cumple con los requisitos exigidos por la legislación vigente para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizamos su presentación para que pueda ser juzgado por el Tribunal correspondiente.

En Zaragoza, septiembre de 2022.

Fdo: Alberto Jiménez Schuhmacher

Fdo: Juan José Alba López

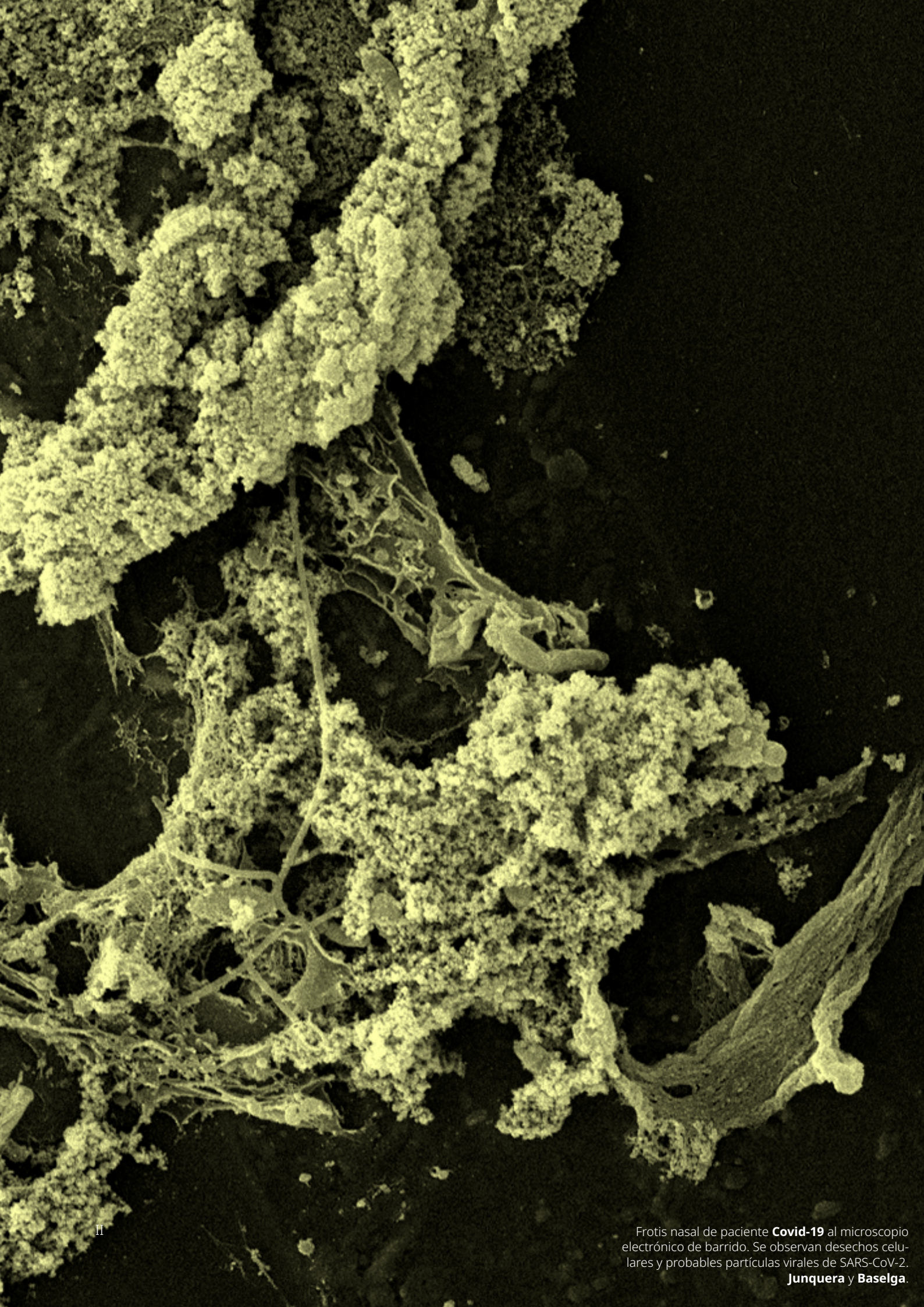


El desarrollo de esta Tesis Doctoral ha sido posible gracias al apoyo y financiación del Instituto de Investigación Sanitaria Aragón. Especialmente, a través la colaboración ciudadana en la campaña «Investiga Covid-19», que tuvo por objeto la recogida de fondos para la financiación de proyectos de investigación sobre el SARS-CoV-2 en el contexto de la pandemia.

A mi querido tío Pepe.

Porque todo lo que me has
enseñado no cabe ni en mil Tesis.





Agradecimientos

En estas páginas se recogen tres años de investigación a ritmo de pandemia. Los aquí citados me han acompañado, motivado y enseñado a lo largo de este periodo. Gracias a ellos esta Tesis ha sido posible; así que, ¿qué menos que dedicarles unas palabras?

A mis directores, el **Dr. Alberto J. Schuhmacher** y el **Dr. Juan J. Alba**, sin los cuales esta Tesis no habría tenido lugar. Gracias por acompañarme en cada paso, por vuestra paciencia y por vuestro compromiso.

A **Alberto**, por siempre estar al pie del cañón. Gracias por inculcarme tu pasión por la ciencia, por sacar mi mejor versión y por confiar en mí. Seguro que el futuro no nos deja indiferentes.

A **Juanjo**, por su sabiduría de la vida, y del lenguaje. Gracias por tu honestidad y ética frente a cualquier situación, por motivarme y apoyarme.

Al **Dr. Antonio Güemes**, porque si no hubieras confiado en mí desde el primer momento, no me dedicaría a la investigación. No tengo palabras para agradecerte lo que me enseñas día a día. Du bist der beste!

A la **Dra. Concepción Junquera**, por su entusiasmo por la ciencia y por hacer investigación honesta. Has convertido tu pasión en mi vocación. Es más fácil encontrar el cilio al TEM que a alguien como tú, así que no puedo estar más agradecida de haberte encontrado.

A la **Dra. Eva Monleón**, gracias por siempre estar dispuesta a ayudarme y a compartir todo lo que sabes. Es un placer trabajar contigo.

Al **Dr. Manuel Arruebo**, **Dr. Víctor Sebastián** y **Dra. Cristina Yus**, por introducirme y formarme en el campo de la nanociencia. Gracias por ayudarme a revisar una y otra vez los trabajos. He aprendido de todos y cada uno de vuestros comentarios.

Al **Dr. Santiago Jiménez**, por su paciencia y su sabiduría. Gracias por poner todos tus recursos al servicio de la sociedad en tiempos de pandemia, y por ofrecerte a ayudar sin buscar nada a cambio.

Al Servicio de Microscopía Electrónica de Sistemas Biológicos, a **M.^a José Marín** y a **M.^a Rosa Bueno** por su disposición y paciencia, y por enseñarme a manejar el microscopio.

Al Laboratorio de Microscopías Avanzadas, a **Gala Simón** y a **Marta Navarro** por introducirme al microscopio electrónico de barrido y por acompañarme durante estos años.

Me gustaría agradecer a los pacientes anónimos que han participado y a los trabajadores del Hospital Clínico Universitario Lozano Blesa y de Exopol que han hecho posible nuestra investigación.

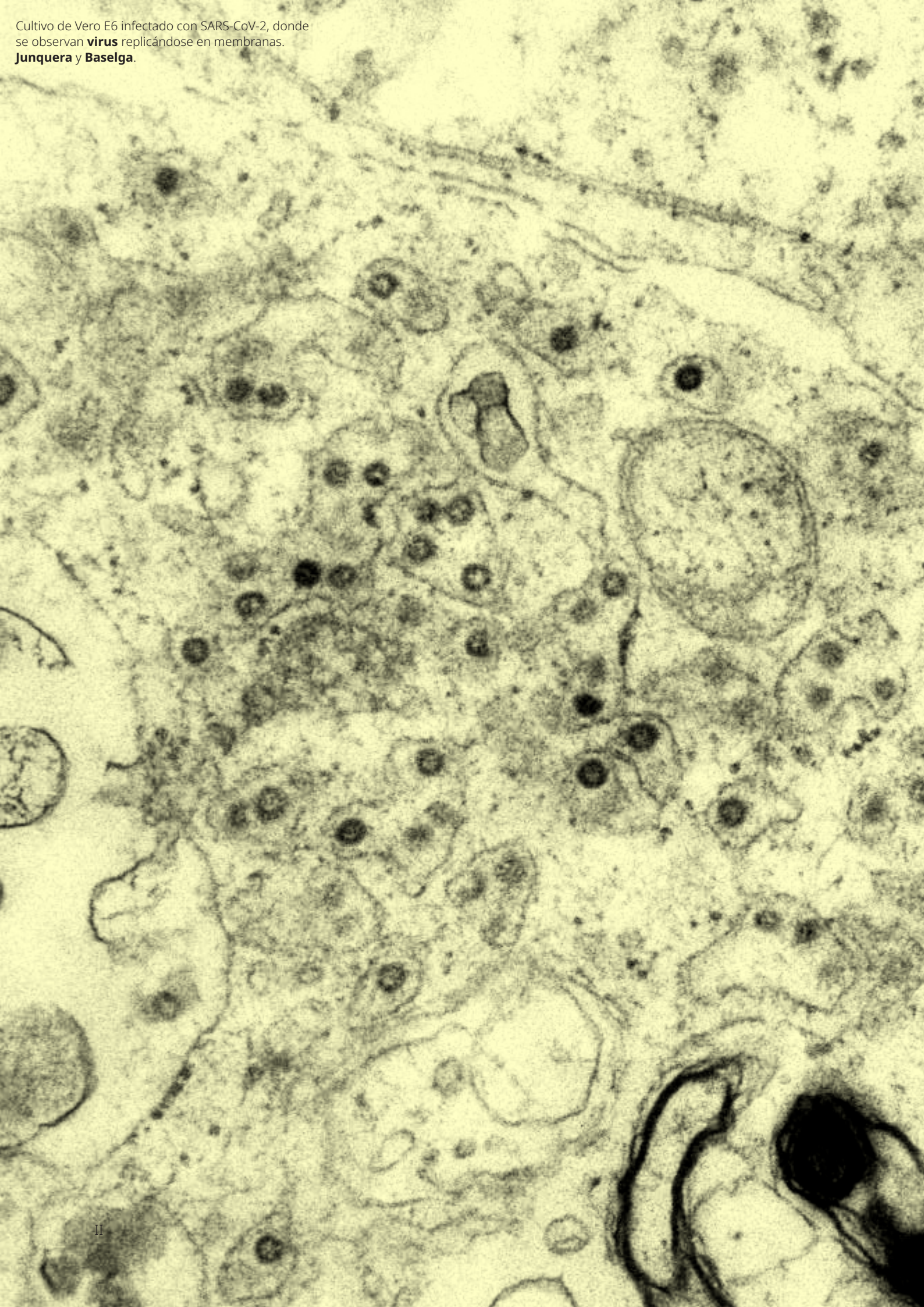
Cuando termina la jornada investigadora, la vida del doctorando no se pone en pausa. Es por esto que, además de agradecer a todos los científicos que me han acompañado este periodo, hay que agradecer a todos aquellos que están día a día en la sombra.

A mi **familia y amigos**, por su apoyo y su comprensión constantes durante esta etapa. Sobre todo a ti, mamá, por tu apoyo incondicional y por inculcarme que con esfuerzo todo es posible. Hacerte sentir orgullosa es la mayor de mis recompensas.

A Javi, por ser mi *Native English Speaking Editor* y por haber recorrido este camino a mi lado. A mis chicas, Bea, Marta, Silvia, Sara y Aza, gracias por darme luz y siempre estar ahí. A mi hermano, por compartir conmigo las alegrías, a pesar de sus «así va la ciencia en España». A mi padre, por siempre estar dispuesto a ayudarme y apoyarme. Y, por último, a mi Abuela que, tal y como le dije en su momento, siempre estará presente en mi camino.

Marta Baselga

Cultivo de Vero E6 infectado con SARS-CoV-2, donde se observan **virus** replicándose en membranas.
Junquera y Baselga.

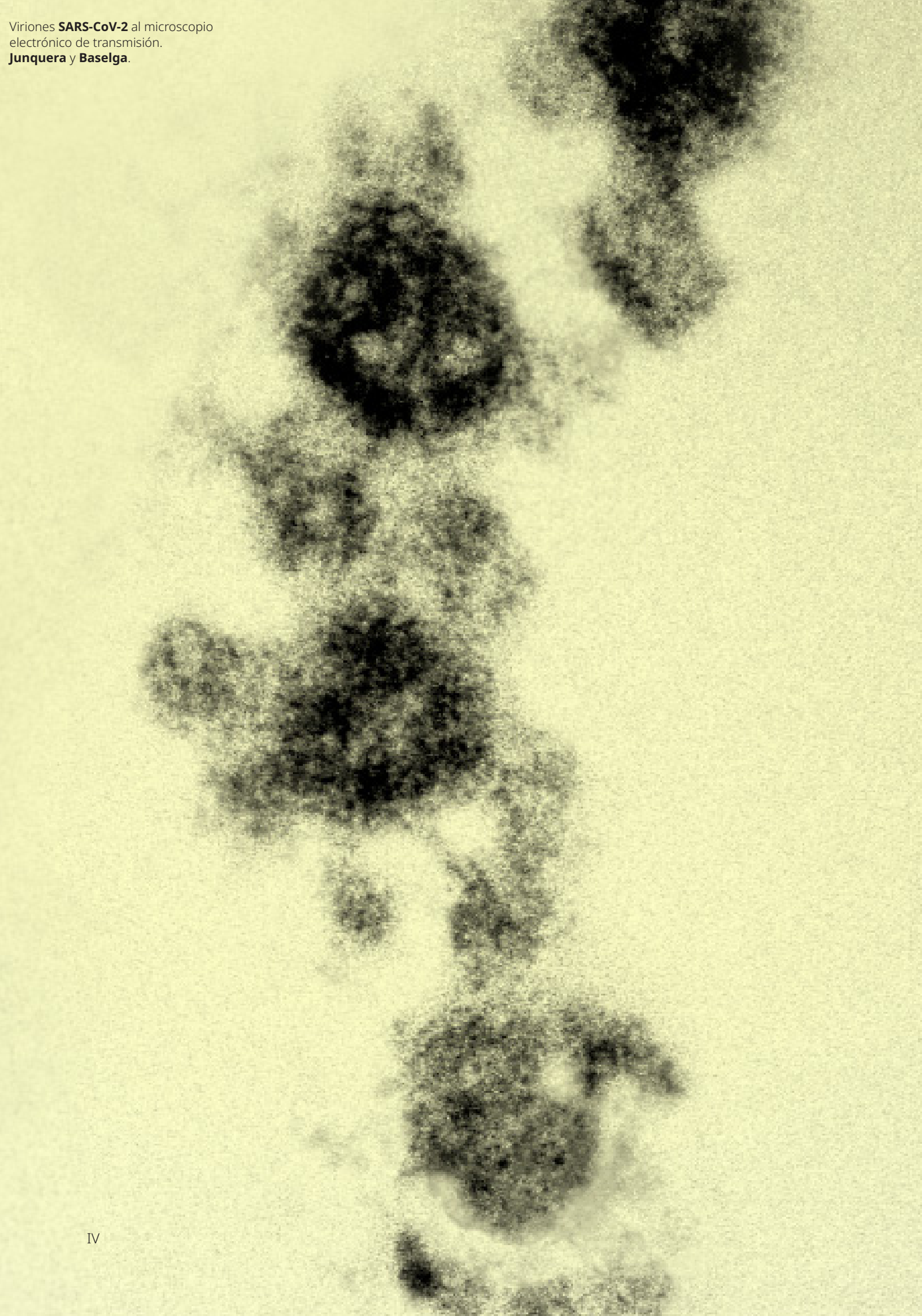


Presentación

La presente Tesis Doctoral, titulada «**Mecanismos de transmisión del SARS-CoV-2 e implementación de estrategias para reducir su propagación por aerosoles**» se presenta como un compendio de publicaciones científicas. En cumplimiento de los requisitos exigidos para esta modalidad según el Programa del Doctorado en Ingeniería Biomédica y la Escuela de Doctorado de la Universidad de Zaragoza, se enumera a continuación la referencia completa de los seis artículos publicados durante la realización de esta Tesis. Además de un séptimo (artículo 5) en fase de revisión (*preprint* en medXriv), que no se incluye en el compendio pero es considerado en esta Tesis.

1. **Baselga, M.**; Uranga, I.; Moreo, E.; Arias, M.; Junquera, C. Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread. *Histochemistry and Cell Biology*. 2022. <https://doi.org/10.1007/s00418-022-02152-7>.
2. **Baselga, M.**; Güemes, A.; Alba, J.J.; Schuhmacher, A.J. 2022. SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity. *Journal of Clinical Medicine*. 2022. 11 (9), 2607. <https://doi.org/10.3390/jcm11092607>.
3. **Baselga, M.**; Uranga, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates. *Materials*. 2022. 15 (14), 4742; <https://doi.org/10.3390/ma15144742>
4. **Baselga, M.**; Yus, C.; Arruebo, M.; Sebastián, V.; Irusta, S.; Jiménez, S. 2022. Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks. *Nanomaterials*. 2022. 12 (6), 925. <https://doi.org/10.3390/nano12060925>
5. **Baselga, M.**; Alba, J.J.; Schuhmacher, A.J. Development and validation of a methodology to measure exhaled carbon dioxide (CO₂) and control indoor air renewal. *Preprint medXriv*. 2022. <https://doi.org/10.1101/2022.09.22.22280262>.
6. **Baselga, M.**; Alba, J.J.; Schuhmacher, A.J. The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases. *International Journal of Environmental Research and Public Health*. 2022. 19: 6605. <https://doi.org/10.3390/ijerph19116605>
7. **Baselga, M.**; Alba, J.J.; Schuhmacher, A.J. Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport. *Science of the Total Environment*. 2023. 855, 158965. <https://doi.org/10.1016/j.scitotenv.2022.158965>.

Considerando los trabajos publicados, se solicita la presentación de esta Tesis Doctoral como compendio de publicaciones por la Universidad de Zaragoza y la Universidad Politécnica de Barcelona.



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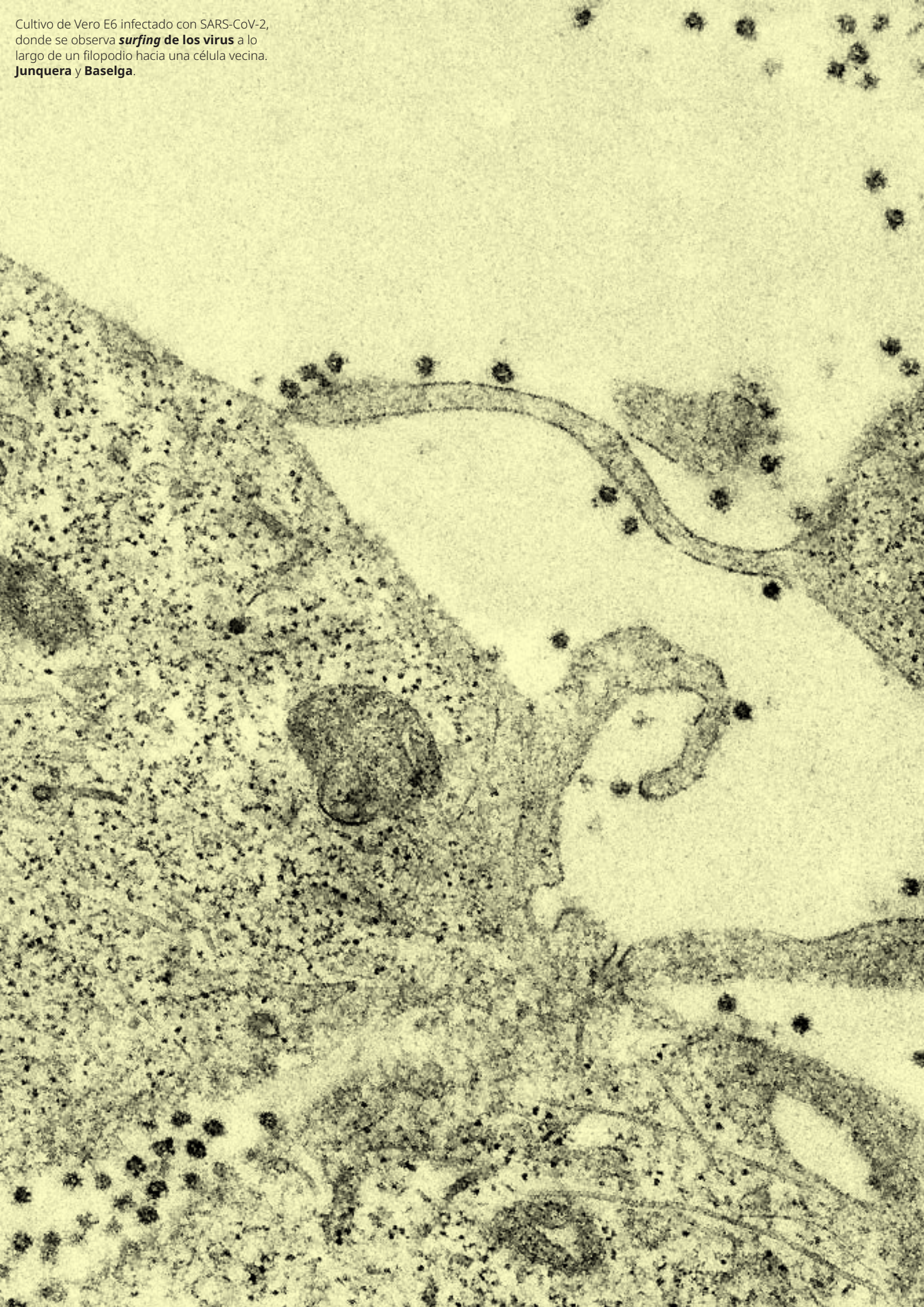
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Cultivo de Vero E6 infectado con SARS-CoV-2, donde se observa **surfing de los virus** a lo largo de un filopodio hacia una célula vecina. **Junquera y Baselga.**



Resumen

El virus SARS-CoV-2 ha supuesto un reto científico y sanitario sin precedentes. Tras más de dos años en pandemia, todavía tenemos un entendimiento parcial sobre cómo afecta este virus al organismo o sobre cómo se transmite entre humanos, entre otras cuestiones. Sin embargo, la inversión investigadora plasmada en este periodo no ha sido en vano, sino que gracias a los avances acometidos ha sido posible paliar las consecuencias del virus: desde la evolución en antivirales hasta las vacunas basadas en mRNA, pasando por los progresos en el conocimiento sobre las rutas de transmisión o los diagnósticos ultrarrápidos. Esta Tesis se presenta como un compendio de seis publicaciones y un artículo en revisión (*preprint* en medXriv) que surgen con el objetivo de dar respuesta a las necesidades sociosanitarias detectadas en diferentes momentos de la pandemia, abordando los retos con un marcado carácter multidisciplinar. En concreto, es posible desglosar el trabajo contenido en esta Tesis en tres áreas temáticas, diferenciadas pero complementarias, relacionadas bajo el marco general de los mecanismos de transmisión del SARS-CoV-2.

En primer lugar, correspondiente a la primera publicación (Baselga *et al.* **Histochemistry and Cell Biology**, 2022), se pone el foco en los **mecanismos de transmisión célula-célula del SARS-CoV-2**. La interpretación de los eventos celulares inducidos por el virus resulta fundamental para la comprensión de las manifestaciones clínicas de la enfermedad. Es por esto que, utilizando microscopía electrónica de transmisión, se analiza el efecto del virus sobre la remodelación del citoesqueleto celular y sus estructuras dependientes. Como conclusión de este primer trabajo, se describen varias rutas subcelulares que optimizan la propagación del virus entre células.

En segundo lugar, se analizan los **mecanismos de transmisión humano-humano del SARS-CoV-2**. Con el objetivo de comprender la heterogeneidad en la transmisión, en un segundo artículo (Baselga *et al.* **Journal of Clinical Medicine**, 2022), se analiza la presencia de RNA viral en bioaerosoles y microgotas de individuos infectados con la variante Ómicron BA.1. Se modela el riesgo de contagio asociado a las diferentes rutas de transmisión directa (microgotas y aerosoles) y se describe la emisión sobredimensionada de un reducido porcentaje de individuos. Finalmente, se discute el papel del umbral de ciclos (Ct) en RT-qPCR como indicador de la infectiosidad del paciente.

En tercer lugar, se realizan cinco trabajos relacionados con **medidas para la prevención de la transmisión del SARS-CoV-2 por aerosoles**. Los dos artículos asociados a los métodos de prevención individual se abordan desde el campo de la nanotecnología. En el tercer artículo (Baselga *et al.* **Materials**, 2022), frente a la creciente expansión de la variante Alfa, se desarrolló un método para recubrir materiales filtrantes basado en nanopartículas de plata y polietilenoimina. Como resultado, se aumentó la comprometida eficiencia de las mascarillas quirúrgicas frente al SARS-CoV-2, alcanzando tasas de inactivación viral del 99.9 %. En el cuarto artículo (Baselga *et al.* **Nanomaterials**, 2022), se describe un método para la producción de materiales filtrantes de alta eficiencia frente a partículas submicrónicas, a partir de desechos plásticos (tereftalato de polietileno) utilizando técnicas electrohidrodinámicas. Además de una posible reducción notoria del impacto ambiental, este trabajo surge durante el azote de la segunda ola de Covid-19 en España (septiembre a diciembre de 2020), donde el desabastecimiento de mascarillas y de medios filtrantes fue notorio.

En relación a los métodos para la prevención colectiva del contagio, se realizan tres artículos de investigación. La popularización de la medición de dióxido de carbono (CO₂) metabólico para el control de la calidad del aire hace necesaria una estandarización el método. En un quinto artículo todavía en revisión (Baselga *et al.* **medXRiv**, 2022) se desarrolla una metodología para el control de la calidad del aire, basada en tres fases: diagnóstico, corrección y monitorización, control y seguimiento (MCS, por sus siglas en inglés *Monitoring, Control, and Surveillance*), que es validada en 40 comercios locales de Zaragoza (España). En el sexto artículo (Baselga *et al.* **International Journal of Environmental Research and Public Health**, 2022) se evalúa la calidad del aire en el tranvía de Zaragoza (España) en funcionamiento y se analizan las variables que afectan a la renovación del aire interior. Además, se modela la probabilidad de riesgo de contagio que, sobre todo, gracias al uso obligatorio de equipos de protección respiratoria resulta disminuida. Sin embargo, dado que el uso de mascarillas y otras medidas basadas en la responsabilidad individual no son opciones a largo plazo, es necesario buscar estrategias para mejorar la calidad del aire de manera definitiva. Con base en lo anterior, en el séptimo artículo (Baselga *et al.* **Science of the Total Environment**, 2023) se evalúa el rendimiento de un equipo de ionización bipolar por punta de aguja implementado en el tranvía de Zaragoza frente a la inactivación de bioaerosoles humanos. Como conclusión, en esta parte de la Tesis se identifican métodos para reducir la transmisión aérea de enfermedades infecciosas.

Las estrategias de salud pública se modulan dependiendo del protagonismo de los mecanismos de propagación, por lo que no es trivial equiparar la vía «airborne» a sus análogas «foodborne» o «waterborne». Como ha quedado demostrado en esta pandemia, la ruta aérea ocupa un puesto predominante en la transmisión de las enfermedades infecciosas respiratorias, de manera que las estrategias de control y prevención deberían establecerse en concordancia.

Palabras clave

Mecanismos de transmisión célula-célula: citoesqueleto, filopodios, SARS-CoV-2, MET, ultraestructura, patogénesis, actina, microtúbulos, nanotubos de efecto túnel, transmisión célula-célula.

Mecanismos de transmisión humano-humano: bioaerosoles, microgotas, transmisión heterogénea, tos, superpropagadores, RNA viral, indicador Ct, Ómicron, Covid-19, infecciosidad.

Métodos para la prevención de la transmisión por aerosoles: mascarillas, filtración, nanopartículas de plata, polietilenimina, nanofibras, PET, CO₂ metabólico, ionización bipolar, renovaciones de aire por hora, control, calidad del aire, ventilación, purificación del aire.

Abstract

The SARS-CoV-2 virus has posed an unprecedented scientific and health challenge. After more than two years of the pandemic, is still partially understood how this virus affects the organism or how it is transmitted between humans, among other issues. However, the research investment made during this period has not been in vain, but thanks to the advances made, it has been possible to mitigate the consequences of the virus: from advances in antivirals to mRNA-based vaccines, through progress in knowledge about transmission routes or ultra-rapid diagnostics. This Doctoral Thesis is presented as a compendium of six publications and an article under review (medRxiv preprint) that aim to respond to the social and health needs detected at different times of the pandemic, addressing the challenges with a marked multidisciplinary nature. Specifically, it is possible to break down this Thesis into three thematic areas, differentiated but complementary, related under the general framework of the SARS-CoV-2 transmission mechanisms.

First, corresponding to the first publication (Baselga *et al.* **Histochemistry and Cell Biology**, 2022), the focus is on the **cell-cell spread mechanisms** of SARS-CoV-2. The interpretation of the cellular events induced by the virus is fundamental for understanding the disease's clinical manifestations. This is why, using transmission electron microscopy, the effect of the virus on cell cytoskeleton remodeling and its dependent structures is analyzed. In the conclusion of this first work, several subcellular routes that optimize the spread of the virus between cells are described.

Second, **SARS-CoV-2 human-human spread mechanisms** are analyzed. To understand the transmission's heterogeneity, in the second article (Baselga *et al.* **Journal of Clinical Medicine**, 2022), viral RNA in bioaerosols and droplets from individuals infected with the Omicron BA.1 variant is analyzed to model the risk of infection associated with the different direct transmission routes. The oversized emission of a small percentage of individuals is described, and the role of cycle threshold (Ct) in RT-qPCR as an indicator of patient infectiousness is evaluated.

Thirdly, five works related to **measures to reduce SARS-CoV-2 airborne transmission** are carried out. The two articles associated with individual prevention methods are approached from the field of nanotechnology. In the third article (Baselga *et al.* **Materials**, 2022), faced with the growing expansion of the Alpha variant, a method was developed to coat filter materials based on silver nanoparticles and polyethyleneimine. As a result, the compromised efficiency of surgical masks against SARS-CoV-2 was increased, reaching viral inactivation rates of 99.9%. In the fourth article (Baselga *et al.* **Nanomaterials**, 2022), a method for producing high-efficiency filter materials against submicron particles is described from plastic waste (polyethylene terephthalate) using electrohydrodynamic techniques. In addition to a possible noticeable reduction in environmental impact, this work arises during the scourge of the second wave of Covid-19 in Spain (September to December 2020), where the shortage of masks and filter media was notorious.

Three research articles are carried out in relation to the methods for the collective prevention of contagion. In this context, the popularization of the measurement of metabolic carbon dioxide (CO₂) to control air quality makes it necessary to standardize the method. Given the above, in the fifth article still under review (Baselga *et al.* **medXriv**, 2022) a methodology for air quality control is developed based on three phases: diagnosis, correction and monitoring, control and surveillance (MCS), which is validated in 40 local businesses in Zaragoza (Spain). In the sixth article (Baselga *et al.* **International Journal of Environmental Research and Public Health**, 2022), air quality in the Zaragoza (Spain) tram is evaluated, and the variables that affect the renewal of the interior air are analyzed. In addition, the risk of contagion probability is modeled, which, thanks to the mandatory use of respiratory protection equipment, is reduced. However, since using masks and measures based on individual responsibility are not long-term options, it is necessary to seek strategies to improve air quality definitively. Based on the preceding, in the seventh article (Baselga *et al.* **Science of the Total Environment**, 2023) the performance of needle-tip bipolar ionization system implemented in the tram against the inactivation of human bioaerosols is evaluated. In conclusion, this part of the Thesis identifies methods to reduce airborne transmission of infectious diseases.

Public health strategies are modulated depending on the prevalence of propagation mechanisms, so it would not be trivial to equate the 'airborne' route to its 'foodborne' or 'waterborne' analogs. Since, as has been demonstrated in this pandemic, airborne route occupies a predominant position in the transmission of respiratory infectious diseases, so control and prevention strategies must be established accordingly.

Keywords

SARS-CoV-2 cell-cell spread mechanisms: cytoskeleton, filopodia, SARS-CoV-2, TEM, ultraestructure, pathogenesis, actin, microtubules, tunneling nanotubes, cell-cell spread.

SARS-CoV-2 human-human spread mechanisms: bioaerosols, microdroplets, heterogeneous transmission, cough, superspreaders, viral RNA, Ct value, Omicron, Covid-19, infectiosity.

Measures to reduce airborne transmission: facemasks, filtration, silver nanoparticles, polyethyleneimine, nanofibres, PET, metabolic CO₂, bipolar ionization, air changes per hour, control, air quality, ventilation, air purification.

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Biología del virus SARS-CoV-2



PREÁMBULO

Este apartado titulado «**Biología del virus SARS-CoV-2**» recoge una introducción a la biología celular y clínica del Covid-19 necesaria para la comprensión de la primera temática de la tesis «**Mecanismos de transmisión célula-célula del SARS-CoV-2**».

1.1. Filogenia y estructura del SARS-CoV-2

Los coronavirus (CoV) son agentes infecciosos que pueden afectar tanto a humanos como a otros mamíferos y aves causando enfermedades respiratorias, entéricas, hepáticas y neurológicas. Se trata de una subfamilia de virus de ácido ribonucleico (RNA, por sus siglas en inglés *Ribonucleic Acid*) monocatenario, con genomas comprendidos entre las 27-32 Kpb, perteneciente a la familia *Coronaviridae* (orden *Nidovirales*).¹ De las 39 especies de coronavirus conocidas, las más relevantes por su prevalencia en el humano son dos cepas de tipo alfa-CoV (alfacoronavirus): HCov-229E y HCov-NL63, que afectan a individuos inmunocompetentes desencadenando enfermedad respiratoria leve.² Las otras cuatro especies destacables, de tipo beta-CoV (betacoronavirus) son: HCov-OC43, HCov-HKU1 de linaje viral A, SARS-CoV, de linaje B y MERS-CoV, de linaje C. Estas cepas de origen zoonótico son causantes de síndrome respiratorio leve en el caso de las de linaje A, Síndrome Agudo Respiratorio Severo (SARS, por sus siglas en inglés *Severe Acute Respiratory Syndrome*) y Síndrome Respiratorio de Oriente Medio (MERS, por sus siglas en inglés *Middle East Respiratory Syndrome*), respectivamente.³

En 2002, se identificó por primera vez el virus SARS-CoV, un beta-CoV de linaje B de etiología difusa, perteneciente a la subfamilia *Orthocoronavirinae* y subgénero *Sarbecovirus*, que inicialmente infectan células epiteliales del tracto respiratorio. En el curso de esta enfermedad, diferentes tipos de células pueden ser infectadas, incluyendo células intestinales, epiteliales tubulares de los riñones, neuro-

nas y células del sistema inmunitario, pudiendo darse, también, lesión indirecta en diferentes órganos.⁴ Este virus se expandió a varios países del sudeste asiático, Europa y América del norte desde Guangdong (China).⁵ Según datos de la Organización Mundial de la Salud (OMS), esta pandemia afectó a 26 países, contagiando a más de 8400 individuos y alcanzando la letalidad en 774 casos. Si bien la tasa de mortalidad media registrada fue del ~9.6 %, en pacientes <24 años se registró una incidencia <1 %, mientras que en casos >65 años, la tasa superó el 50 %.⁶ La neumonía viral asociada al SARS-CoV-1 fue descrita de rápido progreso, con un cuadro inicial inespecífico, aunque los pacientes presentaban picos de fiebre (>38 °C) y sintomatología asociada a enfermedad del tracto respiratorio inferior con evidencia radiográfica de infiltrados pulmonares habituales de neumonía o síndrome de distrés respiratorio.⁷

En 2012, el MERS-CoV fue encontrado en Arabia Saudí, tratándose de un beta-CoV de linaje C perteneciente al subgénero *Merbecovirus* encontrado en camellos y murciélagos de la península. Se conocen dos clados de esta especie: los primeros aislamientos del MERS-CoV pertenecen al clado A y algunas nuevas secuencias nucleótidas aisladas de dromedarios se agrupan en el clado B.⁸ Desde 2012 hasta 2018 se notificaron 2468 casos de MERS, alcanzando una tasa de letalidad del ~34.5 % ($n = 851$). El espectro clínico de neumonía variable, desde la infección asintomática o leve hasta una enfermedad respiratoria aguda severa, con fallo renal en numerosos casos.^{9,10}

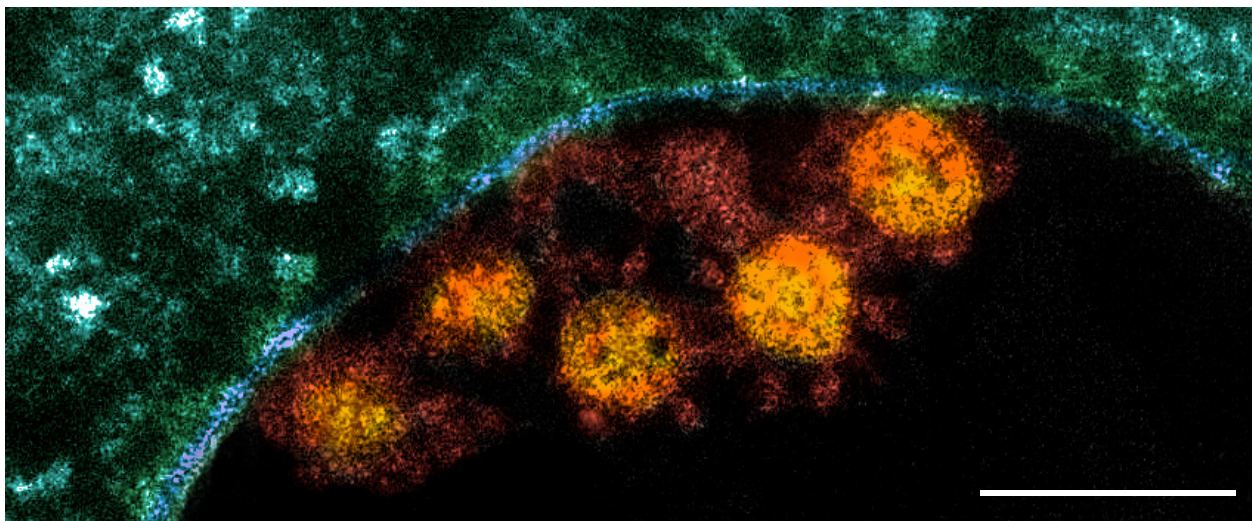


Fig. 1. Micrografía detalle del SARS-CoV-2 obtenida por microscopía electrónica de transmisión. Se observan varias partículas virales de SARS-CoV-2 adheridas a la superficie de una membrana plasmática en un cultivo de células Vero E6 (naranja: SARS-CoV-2, azul: membrana plasmática, verde: citoplasma). La barra de escala representa 50 nm.

A principios de diciembre de 2019, se produjo un brote de la enfermedad por Covid-19, causado por un nuevo síndrome respiratorio agudo severo en la ciudad de Wuhan, provincia de Hubei (China). El 30 de enero de 2020, la OMS declaró el brote del SARS-CoV-2 como una emergencia de salud pública de interés internacional y el 11 de marzo de 2020 fue declarado como pandemia mundial. El SARS-CoV-2 (Fig.1) es un virus encapsulado perteneciente a la subfamilia *Orthocoronavirinae* de orden *Nidovirales* y al linaje B subgénero *Sarbecovirus*. Actualmente, se acepta que el origen zoonótico del SARS-CoV-2 está asociado con los murciélagos, aunque continúa siendo un misterio la presencia de un huésped intermedio para los primeros contagios. Este virus zoonótico, inicialmente denominado nCov-2019, ha sido reconocido como agente etiológico de la enfermedad por Covid-19.^{12,13}

Desde sus inicios, el genoma del SARS-CoV-2 ha sufrido periodos de pequeños cambios genéticos (estasis evolutivas) que se alternan con periodos donde se producen numerosas mutaciones o «variantes» que afectan tanto a las propiedades virales, capacidad infectiva y, en consecuencia, sintomatología.¹⁴ Se han descrito miles de nuevas variantes, organizadas en linajes o clados que surgen del un genoma inicial. La aparición de nuevas variantes representa mayor riesgo para la seguridad pública. Con el objetivo de priorizar el seguimiento e investigación de las variantes se propuso la terminología variante de interés (VOI, del inglés *Variant Of Interest*) y variante de preocupación (VOC, del inglés *Variant Of Concern*). Dentro del

grupo de las VOI pueden ser clasificadas aquellas que presentan cambios en el genoma que afectan a la transmisibilidad del virus, a la gravedad de la enfermedad que causa, o a su capaz de evadir la respuesta del sistema inmunitario, o a la reducción de sensibilidad por métodos diagnósticos. Por su parte, las VOC, además de cumplir con los mismos criterios que las VOI, deben presentar un aumento notable de la transmisibilidad, de su virulencia, un cambio del curso clínico de la enfermedad o una disminución de la eficacia de las medidas sociales, sanitarias y preventivas.^{15,16}

Entre las VOC actualmente se encuentran las variantes Ómicron (B.1.1.529, noviembre de 2021) y Delta (B.1.617, octubre de 2020) detectadas por primera vez en, Botsuana e India, respectivamente, y destacables por el aumento de la transmisibilidad y la susceptibilidad en gente joven sin patologías previas.¹⁹⁻²⁰ Les acompañaron anteriormente la variante Beta (B.1.351, mayo de 2020), detectada por primera vez en Sudáfrica, la variante Alfa (B.1.1.7, septiembre de 2020), descubierta en Inglaterra y considerada como preocupante por un aumento de alrededor de un 60 % de la transmisibilidad respecto al virus inicial y la variante Gamma (P.1, noviembre de 2020), encontrada en Tokio y ampliamente extendida en Brasil con un patrón de rápida transmisibilidad.¹⁵

Entre las VOI, es posible encontrar la variante Epsilon (B.1.427/1.429, Estados Unidos, marzo de 2020), Dseta (P.2, Brasil, abril de 2020), Eta (B.1.525, varios países, diciembre de 2020), Zeta (P.3, Filipi-

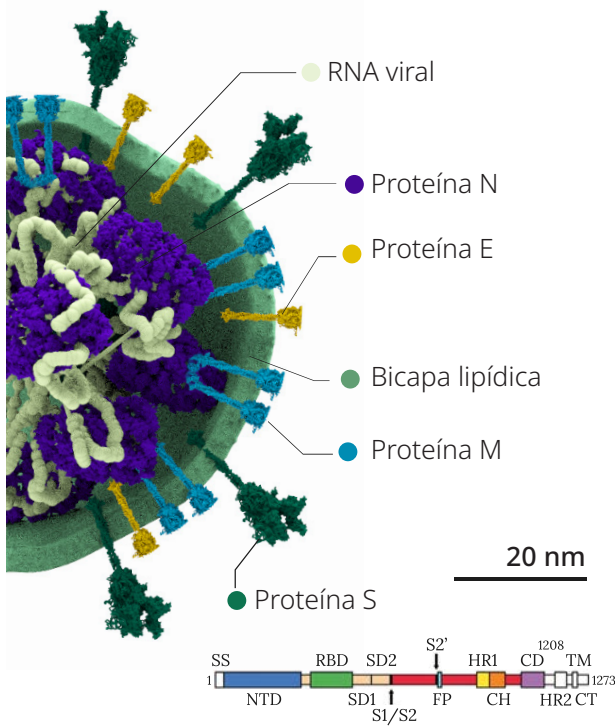
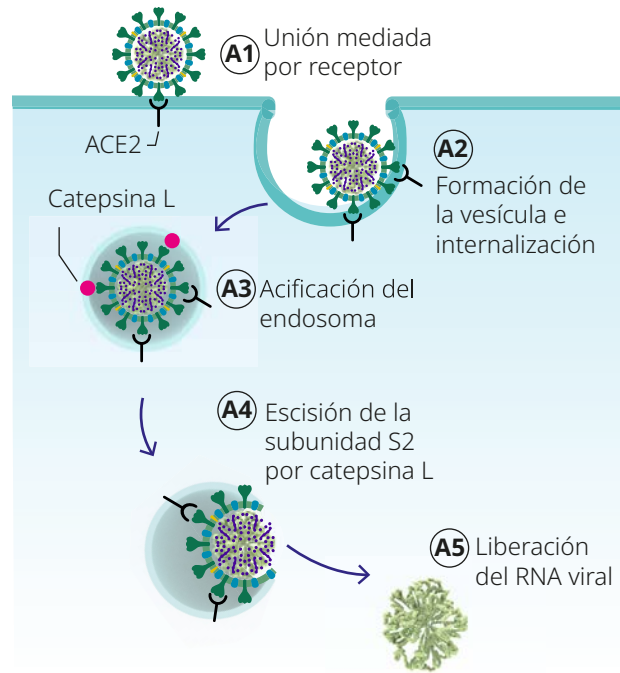


Fig. 2. Representación esquemática de la estructura básica del SARS-CoV-2. Se observan representadas las tres proteínas asociadas a la envuelta viral : E (amarillo), M (azul) y S (verde oscuro), el RNA viral (línea amarilla) y su proteína asociada: N (morado). En la imagen de detalle se observa la bicapa lipídica que conforma la membrana (verde) y la proteína S, cuya estructura describe Wrapp *et al.*¹¹. Los modelos tridimensionales utilizados para la elaboración de la figura han sido recuperados de NIH 3D Print Exchange con licencia CC-BY, y posteriormente ensamblados y renderizados.

nas, enero de 2021), Iota (B.1.526, Estados Unidos, noviembre de 2021), Kappa (B.1.617.1, India, octubre de 2020), Lambda (C.37, Perú, diciembre de 2020) y Mu (B.1.621, Colombia, enero de 2021).¹⁵

El SARS-CoV-2 fue rápidamente secuenciado por Wu *et al.*¹⁸ a través de muestras aisladas de las células epiteliales de las vías respiratorias humanas. Contempla una única cadena de RNA formada por ~30 Kpb que presentan una capucha metilada en el extremo 5' y una cola poliadenilada en el extremo 3'. El genoma viral del SARS-CoV-2 codifica proteínas estructurales y no estructurales, donde se encuentran la proteína S (conocida como *Spike*), la proteína de membrana (M), la proteína de la envoltura (E) y la proteína de la nucleocápside (N), como se observa en la Fig.2. La proteína S se proyecta a través de la envoltura viral. Esta molécula es la encargada de mediar la unión al receptor celular, de manera que, junto con la proteína M que tiene un papel crucial en el ensamblaje del virus, promueve la vía de entrada del virus al huésped. La proteína E es un canal iónico con funciones durante el ensamblaje y salida de los viriones de la célula

A) Entrada endosomal



B) Entrada por fusión de membranas

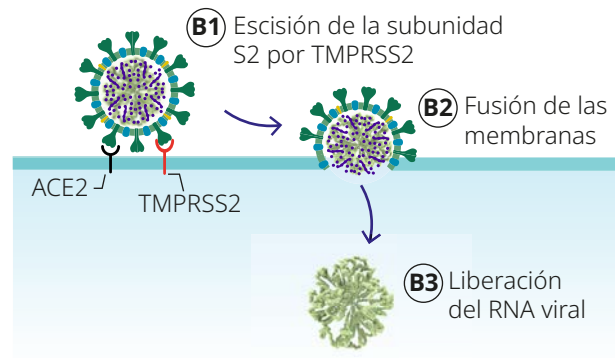


Fig. 3. Representación esquemática de la entrada celular del SARS-CoV-2. La unión mediada por el receptor ACE2 induce a cambios conformacionales de la subunidad S1 exponiendo el sitio S2' de la subunidad S2. En caso de que el complejo virus-ACE2 no encuentre TMPRSS2 o no sea expresado por la célula diana, el virus es internalizado por endocitosis mediada por clatrina (pasos A1 y A2). En presencia de un ambiente ácido en los endosomas, la catepsina L escinde la subunidad S2 (paso A3) liberando péptidos específicos que generan poros de fusión entre membranas (paso A4) para la liberación del genoma viral (paso A5). Cuando ACE2 coexiste con TMPRSS2 en la membrana plasmática, induciendo la escisión de S2 en la superficie celular (paso B1). De nuevo, a consecuencia de la liberación de péptidos específicos de fusión, las membranas se fusionan (paso B2) y el genoma viral es liberado en el espacio intracelular (paso B3). Esquema adaptado de Jackson *et al.*²².

huésped. Finalmente, la proteína N posee un papel protector del genoma, además de un rol en la síntesis de RNA viral.¹⁹⁻²¹

1.2. Ciclo viral

1.2.1. Entrada celular

La comprensión de la ruta celular del virus SARS-CoV-2 permite describir los procesos patogénicos y encontrar dianas terapéuticas efectivas para frenar los efectos de la infección. Hasta el momento se han descrito varias vías de entrada del SARS-CoV-2 al interior celular, entre las que destacan la endocitosis mediada por clatrina (CME, por sus siglas en inglés *Clathrine-Mediated Endocytosis*) (Fig.3A) y la entrada por fusión de membranas (Fig.3B).²² El proceso de CME típicamente comienza el reconocimiento del receptor y la agregación de clatrina en la cara citosólica de la membrana, estableciendo la capa que genera la curvatura inicial y reclutando la carga que va a ser internalizada (Fig.4A).^{23,24} La glicoproteína S del virus consta de dos subunidades (S1 y S2) asociadas de forma no covalente y actúa como mediadora en el proceso de entrada viral. Brevemente, la subunidad S1 se une al receptor Enzima Convertidora de la Angiotensina 2 (ACE2, por sus siglas en inglés *Angiotensin Converting Enzyme 2*), mientras que la subunidad S2 ancla la proteína S a la membrana celular.²⁵ En CME, por debajo de la membrana los promotores de la nucleación activan el complejo Proteínas Relacionadas con la Actina 2/3 (ARP2/3, por sus siglas en inglés *Actin Related Proteins 2/3*), polimerizando nuevos filamentos de actina anexos a los filamentos existentes creando un entramado ramificado cuya estructura geométrica dependerá de la exigencia biomecánica del proceso.^{23,26} Este entramado, unido a la actividad motora de las miosinas de tipo I, genera la fuerza necesaria para la invaginación de la membrana y la extensión de un túbulo hacia el citoplasma. Posteriormente, el cuello endocítico se contrae y la vesícula se genera por fusión de la membrana.²³⁻²⁷ La escisión del sitio S2 de la subunidad homóloga mediado por la proteasa TMPRSS2 (Proteína Transmembrana Serina 2, por sus siglas en inglés *Transmembrane Protease Serine 2*) en la superficie celular o por cathepsina L en el endosoma tras CME libera péptidos específicos que inducen a la formación de poros de fusión para liberar el genoma viral al citosol.²⁸⁻³⁰

1.2.2. Replicación y brotación viral

Tras su entrada en la célula, los filamentos de actina y los microtúbulos junto a proteínas motoras se reorganizan y permiten el tráfico de las vesículas endocíticas con partículas virales hacia los sitios de replicación (Fig.4B-C).³¹ El mecanismo que da lugar

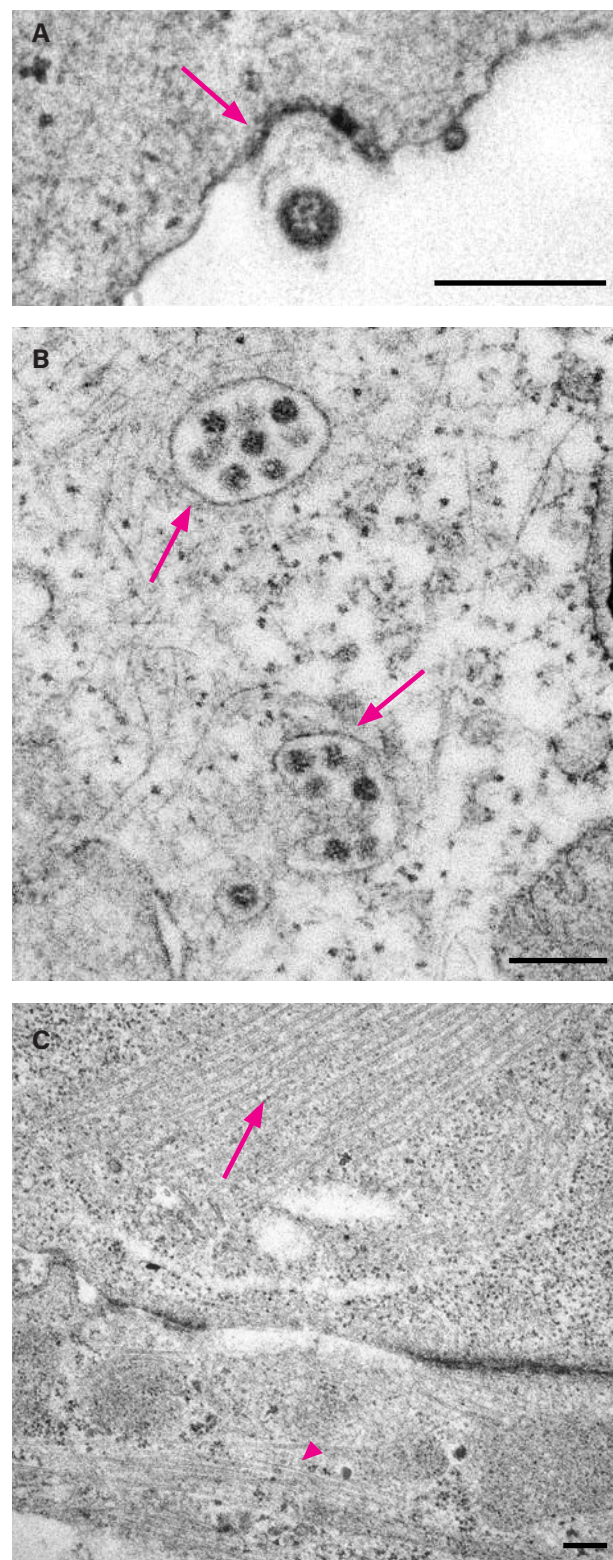
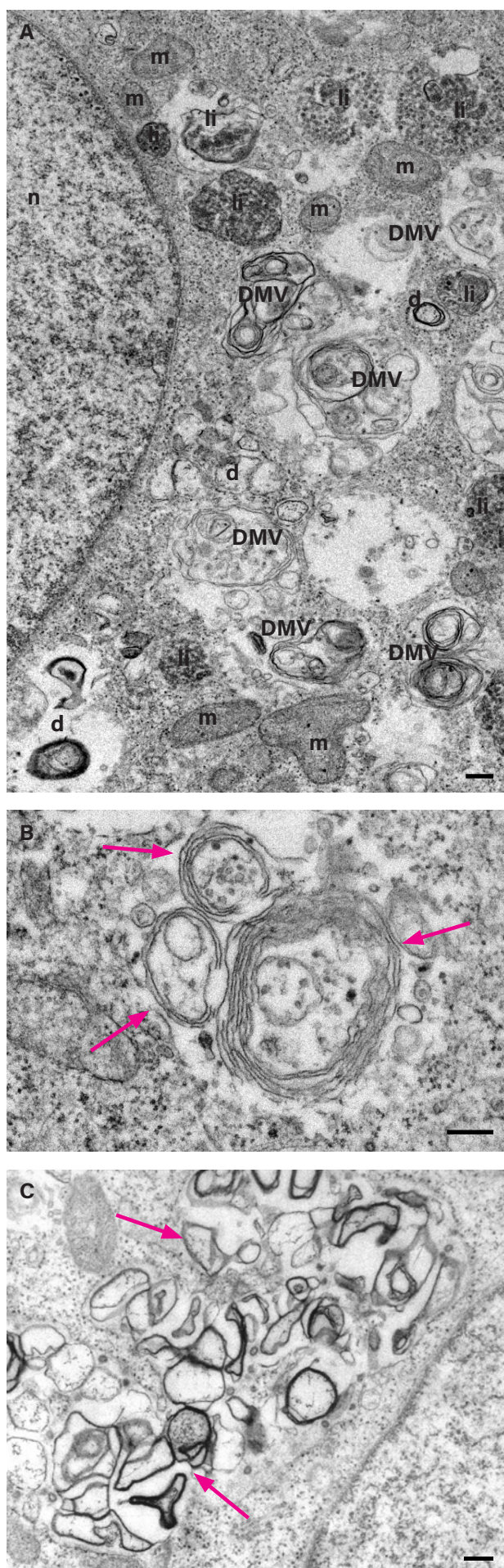


Fig. 4. Micrografías electrónicas de diversos eventos celulares. A-C) Imágenes obtenidas por Microscopía Electrónica de Transmisión (TEM, por sus siglas en inglés *Transmission Electron Microscopy*) de un cultivo infectado de células Vero E6 tras 48 horas. A) Entrada del virus SARS-CoV-2 a la célula por CME: se observa un hoyo recubierto de clatrina (flecha) en la membrana plasmática y una partícula viral en proceso endocítico. B) Partículas virales en vesículas probablemente endocíticas de membrana simple (flechas). C) Citosol de células infectadas donde se observa un citoesqueleto exacerbado. Se muestran microtúbulos (flecha) y filamentos intermedios o de actina (cabeza de flecha). La barra de escala representa 0.2 μm .



a la alteración del citoesqueleto no ha sido descrito todavía,³² razón por la que realizamos la investigación contenida en **el primer artículo de la Tesis**.

En virus de RNA+ se forman nuevas estructuras membranosas de tamaño y morfología variable en el área perinuclear denominadas como «órganulos de replicación», que además protegen al virus del reconocimiento inmunitario (**Fig.5A**).^{33,34} En SARS-CoV-2, estas vesículas de doble membrana (DMV, por sus siglas en inglés *Double Membrane Vesicles*) están típicamente organizadas en membranas dobles y, probablemente, provengan de redes reticulo-lovesiculares. En etapas tempranas de la infección se han identificado vesículas de una sola membrana que han sido asociadas a precursores de DMV, si bien la biogénesis de las estructuras de doble membrana resulta todavía desconocida.³⁵ Dada la necesidad de redes de membranas para la producción de orgánulos de replicación, el engrosamiento del retículo endoplasmático rugoso (RER, por sus siglas en inglés *Rough Endoplasmic Reticulum*) y del Golgi resulta evidente incluso a las pocas horas de la infección.³⁶ Es posible encontrar DMV fusinadas formando paquetes vesiculares de hasta 5 μm (**Fig.5B**).³³ Cuando la replicación viral termina, la célula procesa las DMV formando subproductos membranosos (**Fig.5C**).

La salida de las proteínas estructurales y del genoma replicado hacia los sitios de gemación del complejo intermedio Retículo Endoplasmático-Golgi (ERGIC, por sus siglas en inglés *Endoplasmic Reticulum Golgi Intermediate Compartment*) y Golgi sería posible gracias a la formación de poros moleculares en la doble membrana, según un trabajo reciente de Wolff *et al.*³⁷. En el complejo ERGIC y Golgi se ubican las glicoproteínas virales para la gemación y brotación de nuevas partículas de virus.³⁸ Se ha propuesto que la actina también podría tener un papel regulador en la replicación y transcripción del SARS-CoV-2 a través de la formación de anillos juxtannucleares.^{32,39}

Fig. 5. Micrografías electrónicas de vesículas de doble membrana (órganulos de replicación) en distintas fases del ciclo viral. A-C) Imágenes obtenidas de un cultivo infectado de células Vero E6 tras 48 h. A) Citosol de una célula afectada por la infección con SARS-CoV-2, donde se observa lisosomas digiriendo partículas virales (li), mitocondrias alteradas (m), DMV (DMV), detritus de DMV (d) y el núcleo (n). B) Detalle de varias DMV (flechas) con partículas virales en formación en el interior de las membranas. C) Una vez los DMV han sido utilizados como orgánulos de replicación, se generan subproductos celulares en forma de detritus membranosos (flecha) y pierden su función en el ciclo viral.

En etapas posteriores de la infección, se han observado grandes vacuolas en el espacio intracelular con partículas virales desprovistas de proteínas de membrana y vesículas más pequeñas de origen ERGIC-Golgi conteniendo partículas virales totalmente ensambladas. Las partículas virales desprovistas de proteína S pueden aparecer a consecuencia de un proceso de degradación lisosomal o por defectos en el ensamblaje del virión.³⁶ Estas observaciones podrían estar respaldadas por hallazgos que sugieren la proteína *Spike* no es imprescindible para la gemación y ensamblaje de los CoV, contrariamente a la proteína N.^{40,41} Algunas alteraciones celulares encontradas durante los estudios ultraestructurales han permitido identificar otros agentes relacionados con los procesos de replicación viral. En concreto, se ha observado mayor acumulación de gotas lipídicas en monocitos de pacientes infectados por SARS-CoV-2 en comparación con donantes sanos.⁴² Diferentes autores han demostrado que el reclutamiento de proteínas no estructurales y proteína N de la nucleocápside viral en las gotas lipídicas, lo que podría resultar clave durante el proceso de replicación.^{43,64} Un estudio reciente donde se ha detectado dsRNA como producto intermedio de la replicación viral del SARS-CoV-2 aparece internalizado en mitocondrias celulares, lo que podría representar papel clave de este orgánulo en la replicación viral.^{44,45} Hasta el momento, algunos trabajos describen alteraciones mitocondriales en estudios ultraestructurales,^{46,47} pero no mencionan los posibles eventos causales.

Aunque todavía se desconoce, se ha sugerido que la actina también podría tener un papel regulador en la replicación y transcripción del SARS-CoV-2, aunque sí se ha descrito en estudios anteriores utilizando modelos de virus parainfluenza.³⁹ En otros modelos de coronavirus se ha informado que la reorganización de filamentos de actina cercanos a la membrana para formar anillos podría apoyar la replicación del genoma viral y la síntesis de proteínas virales.⁴⁸

1.2.3. Mecanismos de defensa celular

La autofagia representa un mecanismo clave para la eliminación de patógenos, por lo que numerosos microorganismos han desarrollado estrategias para interferir con la formación y/o maduración de los fagosomas. Los genes *PLAC8* (Gen Asociado a la Placenta 8, por sus siglas en inglés *Placenta Associated gene 8*) y *SPNS1* (Gen Codificante de la Proteína Transportadora de Esfingolípidos 1, del inglés *Sphingolipid Transporter Protein Coding*

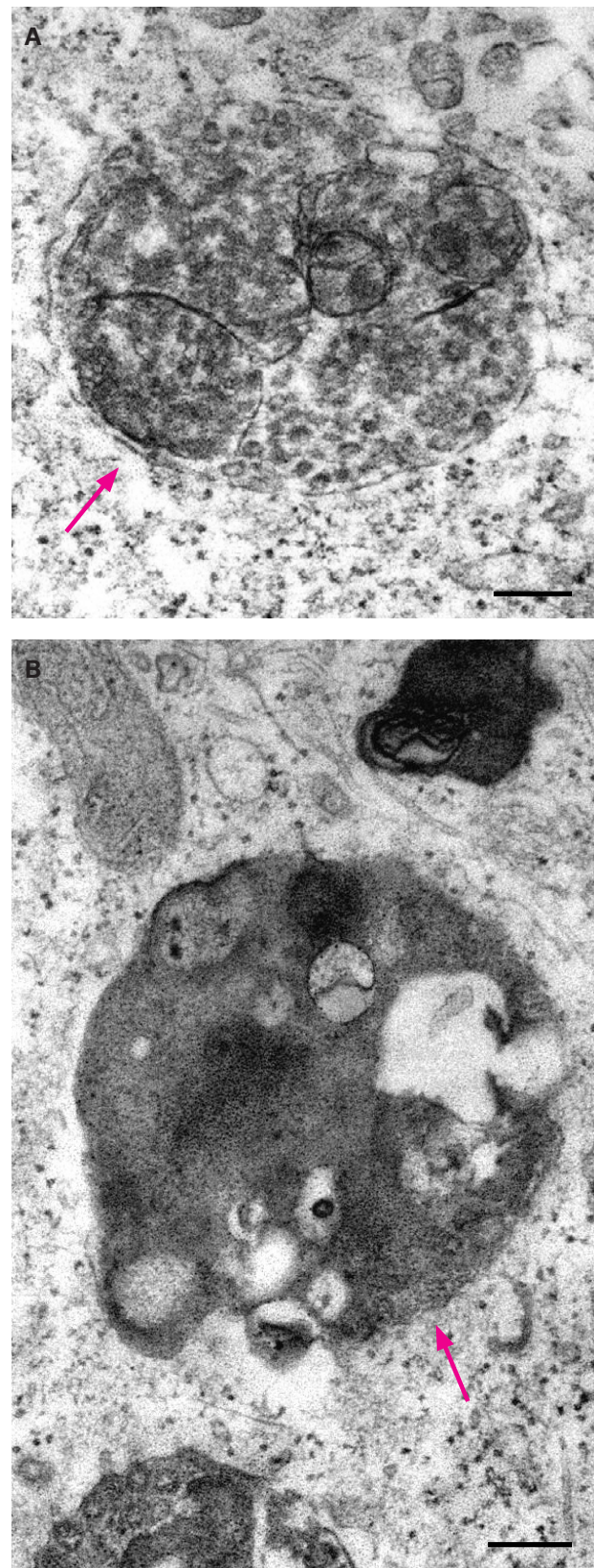


Fig. 6. Micrografías electrónicas de lisosomas digiriendo detritus membranosos y partículas virales. A-B) Imágenes obtenidas de un cultivo infectado de células Vero E6 tras 48 h. A) Lisosoma en fase temprana de degradación (flecha), donde es posible distinguir numerosas partículas virales internalizadas y restos de membrana. B) Lisosoma en fase tardía (flecha), donde se observa un contenido digerido y todavía es posible distinguir detritus membranosos y partículas virales. La barra de escala representa 0.2 μm .

gene) regulan el compartimento autofagolisosomal y afectan el destino intracelular de los viriones SARS-CoV-2 endocitados.⁴⁹ Se ha sugerido que las DMV podrían corresponder a una alteración de fagosomas dado que son positivos para la LC3-II (Cadena Ligera de Proteínas Asociadas a Microtúbulos 3-II, por sus siglas en inglés, *Microtubule-Associated Protein Light Chain 3-II*),⁵⁰ a pesar de que las DMV son de menor tamaño y de mayor persistencia.^{51,52} Eskelinen *et al.*⁵³ propusieron algunas claves para diferenciar la ultraestructura de procesos autofágicos respecto a los procesos de la infección viral asociados a dobles membranas. Brevemente, es posible diferenciar entre el tránsito de los patógenos en estructuras intermedias de tipo autofágico durante su ciclo celular y la captura de los patógenos por vía autofágica para su degradación lisosomal (**Fig.6A-B**). La principal diferencia entre ambos eventos es que la vesícula autofágica siempre se fusiona con un lisosoma. Esta distinción resulta clave para interpretar los eventos del ciclo celular dado que representan procesos contrarios: mientras que el proceso autofágico actúa contra la infección, las DMVs permiten la replicación viral. En línea con lo anterior, se ha informado que el SARS-CoV-2 modula el metabolismo celular para inducir la ubiquitinación y limitar la autofagia.⁵⁴

1.2.4. Extrusión viral

En etapas finales de la infección, el virus ensamblado recurre a la vía de exocitosis para su extrusión.⁵⁵ Al alcanzar la región perimembranosa, la reestructuración de la actina proporciona fuerza para la flección de la membrana durante la exocitosis.^{48,56}

A su paso por la célula, los eventos inducidos por el ciclo viral se presentan como un deterioro celular de grado variable. El estudio de la morfogénesis celular asociada a la infección viral resulta clave para comprender los mecanismos precursores de la manifestación clínica.

1.3. Alteraciones celulares tras la infección por SARS-CoV-2

Hasta la fecha, varios estudios han caracterizado a nivel ultraestructural la morfogénesis de la célula una vez iniciada la infección por coronavirus.^{21,33,46,52,57-59} A grandes rasgos, se han observado varios cambios ultraestructurales, incluyendo la formación de sincitios, la acumulación de gotas li-

pídicas, la reorganización de membranas (descrito en el apartado anterior), alteraciones mitocondriales, el engrosamiento del retículo endoplasmático rugoso (RER, por sus siglas en inglés *Rough Endoplasmic Reticulum*) y la formación de filopodios.

La propagación viral, la evasión inmune y la patogenicidad del SARS-CoV-2 puede ser dependiente de las estructuras que comuniquen de manera directa dos o más células, como es la formación de sincitios y las proyecciones de membrana. Por un lado, los sincitios son células multinucleadas resultantes de la fusión celular. En la infección celular con SARS-CoV-2, este fenómeno está vinculado con una alteración de la subunidad S2 de la proteína *Spike* que conduce a la fusión de la membrana.⁶⁰ Por otro lado, los filopodios son proyecciones finas de membrana que proliferan por prolongación de la actina cortical. Los virus con envoltura pueden propagarse por dos rutas: en el ambiente acuoso intercelular o por contacto célula-célula. A pesar de que la transmisión por el ambiente acuoso requiere de fenómenos de difusión para su transporte y depende de altas cargas virales, es imprescindible para la propagación de humano-humano. La propagación directa célula-célula es más eficiente, por su rapidez y por su evasión inmunitaria.⁶¹ La rápida navegación de virus a través filopodios resulta mediada por el citoesqueleto de actina subyacente y por la miosina II.⁶² Se han descrito tres mecanismos diferentes de los filopodios asociados a la infección viral:⁶³ *surfing* de los virus, retracción filopodial y formación de nanotubos de efecto túnel. Primero, el surf viral consiste en el movimiento ordenado de las partículas virales hacia el cuerpo celular a través de las proyecciones de membrana.⁶² Segundo, la retracción consiste en la atracción del virus hacia el interior celular a través de un impulso mecánico de retracción del filopodio.⁶³ Por último, los nanotubos de efecto túnel (TNT, por sus siglas en inglés *Tunneling Nanotubes*) corresponden a conductos intercelulares que conectan células vecinas y distantes para la transferencia de moléculas, orgánulos y pequeños cuerpos de diversa estirpe.⁶⁵⁻⁶⁷

En etapas finales de la infección pueden aparecer mecanismos de muerte celular para preservar el entorno celular que conlleven a la ruptura de la membrana plasmática y, finalmente, a piroptosis.⁶⁸

Una vez iniciada la infección en el tejido, se induce una respuesta inmunológica que se manifiesta clínicamente con síntomas y signos variables.

1.4. Fisiopatología, clínica y diagnóstico de Covid-19

1.4.1. Fisiopatología de la infección

La respuesta inmunitaria del huésped está sujeta al tropismo celular del virus y a su capacidad para eludir los mecanismos de defensa.⁶⁹ A pesar de que la enfermedad Covid-19 es predominantemente respiratoria, el virus afecta a nivel multiorgánico. Si bien el SARS-CoV-2 presenta tropismo células epiteliales y vasculares del tracto respiratorio, las células renales, cardíacas, biliares, testiculares y gastrointestinales también expresan el receptor ACE2, de manera que son susceptibles a la infección y a un posible efecto citopático.⁷⁰ También se ha informado de neuroinvasión del SARS-CoV-2 desarrollando isquemia localizada y muerte celular.⁷¹ El receptor ACE2 podría no ser exclusivo para permitir la entrada del virus a la célula, sino que coexistiría con receptores alternativos que podrían explicar el amplio tropismo viral y la variabilidad entre manifestaciones clínicas,⁷² aunque el pulmón es el principal órgano afectado en la enfermedad Covid-19.⁷³ Normalmente la infección cursa en las vías respiratorias superiores, aunque un reducido porcentaje de pacientes se progresa hacia una neumonía de intensidad variable.⁷⁴

1.4.2. Sintomatología y curso clínico

La enfermedad Covid-19 abarca un espectro clínico amplio. Dependiendo de la extensión y de la afectación del tejido, la sintomatología de la enfermedad Covid-19 aparece en grado variable, comprendiendo desde una infección asintomática hasta una potencialmente mortal.⁷⁵ Típicamente se manifiesta con fiebre (83-99 %), tos (59-82 %), fatiga (44-70 %), disnea (31-40 %), mialgias (11-35 %), aunque se han descrito otros síntomas inespecíficos como dolor de garganta, congestión nasal, cefaleas, diarrea, náuseas y vómitos. Además de anosmia y ageusia de manera previa al inicio de la sintomatología respiratoria.⁷⁶⁻⁷⁸

En un estudio donde se reclutaron más de 72314 pacientes no vacunados detectaron un 81 % de casos de carácter leve (con neumonía leve, o sin ella), un 14 % de enfermedad moderada (neumonía con hipoxemia) y un 5 % de cursos graves (insuficiencia respiratoria que requiere de ventilación mecánica, shock o fallo multiorgánico).⁷⁹ Algunos pacientes cursan la enfermedad de manera leve-moderada, y evolucionan hacia la neumonía bilateral con in-

filtración pulmonar en imágenes radiológicas y alteración del sistema inmune (manifestada con linfopenia severa, elevada concentración de citocinas proinflamatorias o alteración en linfocitos T). Otro signo frecuente es la disnea, como consecuencia de hipoxemia, que cursa con una progresión de insuficiencia respiratoria. Los hallazgos en estos pacientes (hipoxemia severa e infiltrados pulmonares bilaterales) son compatibles con el Síndrome Distrés Respiratorio Agudo (SDRA).^{80,81} Los pacientes con insuficiencia respiratoria hipóxica presentan inflamación sistémica, incluyendo la liberación de citocinas proinflamatorias, y altas concentraciones de otros marcadores inflamatorios.⁸² Las altas tasas de replicación pueden conducir a daños en células epiteliales y endoteliales provocando fugas vasculares y eventos trombóticos. La inmunotrombosis puede desencadenarse por la respuesta inflamatoria del sistema inmune y se presenta con hipercoagulabilidad y endotelopatía.⁸³ Esto, unido al proceso inflamatorio, puede dar como resultado fibrinólisis, vasodilatación, antiagregación e impermeabilidad vascular en fases terminales de la enfermedad Covid-19 grave.⁸⁴ Los microtrombos, a pesar de no ser patognomónicos en Covid-19, se han encontrado con mayor frecuencia que en otras neumonías de etiología infecciosa.⁸⁵

El periodo de incubación de la enfermedad causada por la variante original del SARS-CoV-2 se estableció entre 5.6 (IC95, 5.2-6.0) y 6.7 (IC95, 6.0-7.4) días, aunque en pacientes con una edad media de 60 años, el percentil 95 fue de 12.5 días, aumentando en torno a un día de incubación por cada 10 años de edad.⁸⁶ En pacientes con comorbilidades como diabetes, hipertensión, enfermedades cardiovasculares o respiratorias, el riesgo del curso grave de la enfermedad aumenta notablemente, así como la tasa de letalidad de la enfermedad.⁸⁷ No obstante, los periodos de incubación dependen de la mutación sobre el virus original. Por ejemplo, en la variante Delta se estimó de 4 días y en Ómicron en 3 días.⁸⁸⁻⁹⁰

La cinética de la carga viral es dependiente del tiempo. Se han reportado tasas máximas de carga viral dos días antes de la aparición de los síntomas,⁹¹ lo cual sugiere una elevada rapidez en la transmisión, incluso en pacientes con síntomas relativamente leves.⁹² Tras el inicio de los síntomas, la carga viral permanece estable durante 5 días y decrece progresivamente durante los 7-8 posteriores,⁹³ aunque se ha detectado RNA viral tras más de 20 días tras el inicio de los síntomas.⁹⁴ Se ha establecido una fase de contagiosidad hasta 8 días

1.4.3. Diagnóstico

después, que cae por debajo del 5 % tras 15.2 días (IC95, 13.4-17.2).⁹⁴ Sin embargo, las nuevas variantes trajeron consigo indicadores diferentes. Por ejemplo, las variantes Delta y Ómicron estuvieron caracterizadas por cargas virales más altas y tiempos más cortos para alcanzar la carga viral máxima y periodos de incubación más reducidos.^{85,95,96} Hay *et al.*⁹⁷ informaron de picos de carga viral inferiores en Ómicron respecto a Delta (Ct 23.3, IC95 22.4-24.3 frente a Ct 20.5, IC95 19.2-21.8, respectivamente) y ratios de eliminación viral similares (3.13 Ct/día, IC95 2.75-3.5 y 3.2 Ct/día, IC95 2.6-3.6, respectivamente). Es preciso tomar en cuenta que en individuos vacunados las cargas virales resultan disminuidas y la eliminación del virus es más rápida,⁹⁴ de manera que la curva de la carga viral está sujeta a un amplio rango de variables.

En función de la gravedad de la enfermedad, la OMS ha establecido cinco categorías que estadifican el curso patológico en:⁹⁹

- 1.** Infección asintomática, donde el paciente no presenta signos clínicos y las imágenes radiológicas no muestran alteraciones detectables.
- 2.** Enfermedad leve, donde el paciente sintomático se ajusta a la definición, sin neumonía ni hipoxia.
- 3.** Enfermedad moderada, donde el paciente presenta signos clínicos de neumonía pero no de carácter grave.
- 4.** Enfermedad grave, donde el paciente presenta signos clínicos de neumonía grave además de alguno de los siguientes indicadores: frecuencia respiratoria >30 inspiraciones/min, dificultad respiratoria grave o SpO_2 <90 %.
- 5.** Enfermedad crítica, que se subdivide en:
 - 5a.** SDRA, donde el paciente puede presentar opacidades bilaterales que no se explican totalmente por sobrecarga de volumen, colapso lobar o pulmonar ni nódulos o insuficiencia respiratoria que no se atribuye a insuficiencia cardíaca o sobrecarga de líquidos. La SDRA puede ser leve ($200 \text{ mmHg} < \text{PaO}_2/\text{FiO}_2 \leq 300 \text{ mmHg}$, con $\text{PEEP} \geq 5 \text{ cm H}_2\text{O}$), moderada ($100 \text{ mmHg} < \text{PaO}_2/\text{FiO}_2 \leq 200 \text{ mmHg}$, con $\text{PEEP} \geq 5 \text{ cm H}_2\text{O}$) o grave ($\text{PaO}_2/\text{FiO}_2 \leq 100 \text{ mmHg}$, con $\text{PEEP} \geq 5 \text{ cm H}_2\text{O}$).
 - 5b.** Septicemia, donde presenta disfunción orgánica aguda y potencialmente mortal secundaria a la disregulación asociada a la infección.
 - 5c.** Choque séptico, donde presenta lactato sérico >2 mmol/l e hipotensión persistente.

El *gold standard* para el diagnóstico de infección por SARS-CoV-2 es la detección de RNA a través de Reacción en Cadena de la Polimerasa Cuantitativa con Transcriptasa Inversa (RT-qPCR, por sus siglas en inglés *Reverse Transcriptase Quantitative Polymerase Chain Reaction*). Sin embargo, la sensibilidad de las pruebas varía en función del momento de la enfermedad en la que se encuentre el paciente. Habitualmente, la carga viral del paciente puede ser cuantificada por el número de ciclos necesarios para la amplificación mínima necesaria para su detección. Se ha estimado la sensibilidad de la prueba en el 33 % cuatro días después del contacto con el infectado, en el 62 % el día de inicio de los síntomas y en el 80 % a los tres días tras el inicio de los síntomas.^{100,101} La tasa de falsos negativos resulta principalmente afectada por la técnica de recolección de la muestra, el tiempo desde la exposición y la localización anatómica. Los lavados broncoalveolares presentan mayor sensibilidad (93 %) frente a las muestras tomadas de las vías respiratorias superiores, seguidas de muestras de esputo (72 %), hisopos nasales (63 %) y frotis faríngeos (32 %) en la variante original del SARS-CoV-2.¹⁰⁰

Existen otras pruebas diagnósticas que, por su bajo coste y fácil manejo, han adquirido popularidad en los últimos tiempos. Entre ellas, destacan los test de detección anticuerpos (normalmente, inmunoglobulinas G y M, IgG e IgM) y de antígenos. Las pruebas basadas en detección de anticuerpos son serologías que identifican la respuesta inmune de los pacientes. Para su detección, existen dos métodos diferenciados: la inmunocromatografía lateral (kits rápidos) y el Ensayo por Inmunoabsorción Ligado a Enzimas (ELISA, por sus siglas en inglés *Enzyme-Linked ImmunoSorbent Assay*). La inmunocromatografía lateral puede detectar anticuerpos totales o específicos (IgG e IgM). Como respuesta inmunitaria, la primera inmunoglobulina en aparecer en sangre es la IgM que, normalmente, brota a los 5-7 tras el comienzo de los síntomas y alcanza valores máximos a las 3-4 semanas tras la infección.^{102,103} Posteriormente, la IgG suele aparecer dos semanas después de la infección para la neutralización viral, y su presencia puede indicar si el individuo se encuentra en fase infecciosa de la enfermedad.⁸⁹ Por su parte, los antígenos del SARS-CoV-2 son los antígenos N, S y el dominio RBD, siendo el antígeno N el más abundante en el virus.¹⁰⁴ Los test rápidos de antígenos típicamente identifican la presencia de proteína N, que es detectable infecciones activas y especialmente

en pacientes con alta carga viral. Se han reportado elevadas tasas de sensibilidad y especificidad, alcanzando el 93.9 % (IC95, 86.5-97.4 y el 100 % (IC95, 92.1-100 %), respectivamente.¹⁰⁵

Además de las pruebas diagnósticas basadas en la detección de estructuras virales y de la respuesta inmune, las técnicas de imagen resultan especialmente útiles para la identificación y caracterización de la neumonía asociada a la Covid-19, especialmente las radiografías y tomografías computarizadas torácicas.^{106,107}

1.5. Epidemiología del virus SARS-CoV-2

1.5.1. Datos globales

El 29 de diciembre de 2019 se notificaron los cuatro primeros casos de Covid-19, vinculados en su totalidad al mercado mayorista de animales vivos de Wuhan (China).¹⁰⁸ La identificación de los casos fue posible debido a que los hospitales en China cuentan con protocolo de vigilancia de «neumonía de etiología desconocida» que fue establecido a raíz del brote del SARS-CoV.¹⁰⁹ Li *et al.*¹¹⁰ recopiló la información de los 425 primeros pacientes confirmados, estudiando la tasa de crecimiento de la pandemia, el tiempo de duplicación de la pandemia y el número de reproducción básico (R_0) desde el 10 de diciembre hasta el 4 de enero de 2020. Entre sus resultados, destacaron una tasa de crecimiento epidémico de 0.1 por día (IC95; 4.2-14) y un tiempo de duplicación de 7.4 días. El valor estimado de R_0 fue de 2.2 (IC95; 1.4-3.9). No obstante, un trabajo de revisión que recopila 41 publicaciones, previas a marzo de 2020, sugiere que el tiempo de duplicación de la pandemia ocurría entre 3 y 7 días y que el R_0 variaba entre 1.9 y 6.5.¹¹¹

En marzo de 2020, al menos dos terceras partes de los primeros contagios en 99 países a nivel internacional correspondían a individuos que habían viajado a Italia, China o Irán.¹¹² El SARS-CoV-2 fue rápidamente propagado a nivel mundial. Desde entonces y hasta la actualidad (junio de 2022), se han reportado un más de 534 millones de casos diagnosticados (**Fig.7**) y más de 6.3 millones de fallecidos como consecuencia de la infección por SARS-CoV-2.^{113,114} Entre los países con mayor número de casos reportados hasta el momento, destacan por orden de infectados: Estados Unidos, India, Brasil y Francia. Hasta junio de 2022, el 85.5 % de la población está vacunada con pauta completa (> 40.5 millones de individuos). La tasa de contagiados pudo ser variable en el tiempo, a su vez, por el cambio en la transmisibilidad de las nuevas variantes. La variante Delta (B.1.617) presentó un índice R_0 de 3.28 (IC95; 3.01-3.58) mayor que la variante ancestral, mientras que se estima que la variante Ómicron (B.1.1.529) es 3.15 (IC95; 2.83-3.50) más infecciosa que la Delta.^{115,116}

1.5.2. Datos en España y en Aragón

El primer caso confirmado de Covid-19 en España fue registrado el 31 de enero de 2020 en Tenerife. Desde entonces, y hasta junio de 2022 se han notificado más de 12.4 millones de casos y más de 100 mil fallecimientos como consecuencia de la pandemia.¹¹³ Actualmente (junio de 2022), España se sitúa en la undécima posición a nivel mundial y la quinta a nivel europeo en número de casos. Si se considera el número de casos relativo, denominado como número de casos por cada millón de personas, España se sitúa en la posición 35. En concreto, en Aragón se han registrado alrededor de 431 mil casos con alrededor de 5 mil fallecimientos.¹¹³ A lo largo de estos últimos dos años, se han considerado 7 «olas» o repuntes asociados a diferentes eventos (**Fig.8**).

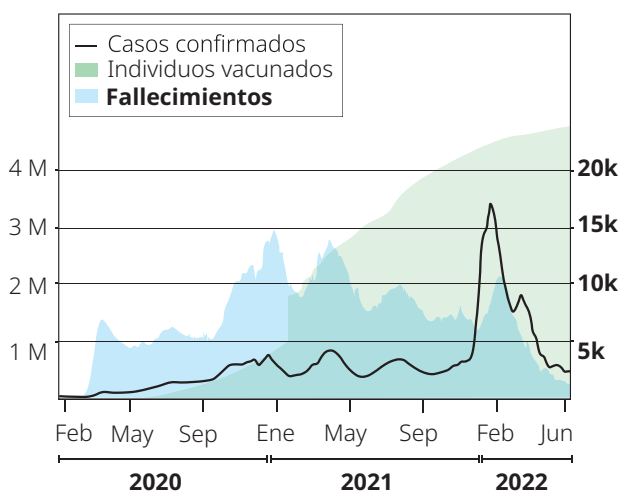


Fig. 7. Media de 7 días de nuevos casos confirmados, fallecimientos y casos diarios de vacunación desde el inicio de la pandemia hasta la actualidad a nivel mundial. Se observa un pico prominente que alcanza 3363784 nuevos casos a finales de enero de 2022, que puede deberse a un aumento de la disponibilidad de autodiagnóstico a nivel global. La curva de fallecimientos resulta reducida respecto al número de casos, especialmente a partir de mediados de 2021, que puede relacionarse con un aumento en el porcentaje de individuos vacunados. Los datos correspondientes al inicio de la pandemia pueden resultar infraestadiados dado el escaso número de pruebas diagnósticas que fueron realizadas a la población. Datos de Our World in Data.¹¹⁴

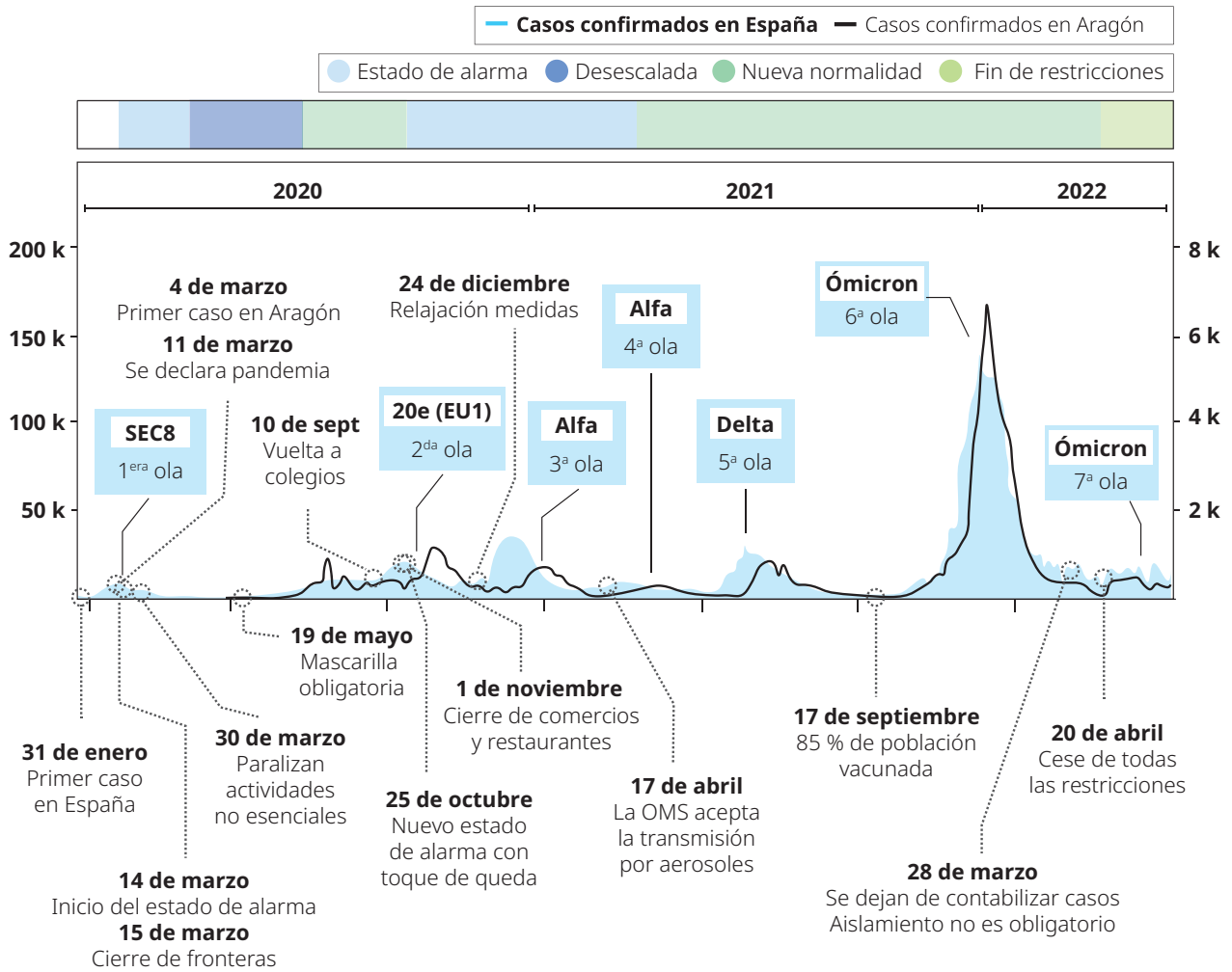


Fig. 8. Media de 7 días de nuevos casos confirmados y fallecimientos por Covid-19 en España y en Aragón desde el inicio de la pandemia. Se observan un resumen cronológico donde se recogen los hitos de interés y las 7 olas registradas a lo largo de dos años de pandemia con sus correspondientes variantes predominantes. Figura diseñada a partir de datos recogidos de Our World in Data.¹¹⁴

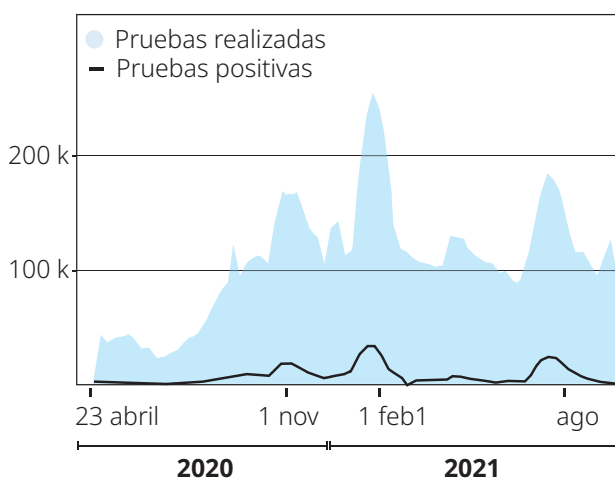


Fig. 9. Relación de pruebas diagnósticas realizadas y pruebas diagnósticas positivas para SARS-CoV-2. Entre abril de 2020 y octubre de 2021. Datos obtenidos de Our World in Data.¹¹⁴

No es desdeñable apuntar a una infravaloración de los contagios, especialmente en la primera y segunda ola, cuando el número de pruebas diagnósticas realizadas resultaba limitado a los recursos materiales y asistenciales disponibles en ese momento (Fig.9). Tras la tercera ola, el muestreo masivo con técnicas diagnósticas ha permitido valorar con mayor precisión la situación epidemiológica. En concreto, según un estudio de seroprevalencia (ENE-COVID) realizado por el Instituto de Salud Carlos III, se estimó que, alrededor de, un 5 % de la población española contrajo la enfermedad durante la primera ola (del 27 de abril al 11 de mayo de 2020).¹¹⁴

Transmisión humano-humano del SARS-CoV-2



PREÁMBULO

Este apartado titulado «Transmisión humano-humano del SARS-CoV-2» recoge un estado del arte actual sobre los mecanismos que rigen la propagación del virus entre humano, en alusión a la segunda temática de la tesis «Mecanismos de transmisión humano-humano del SARS-CoV-2».

2.1. Vías de transmisión del SARS-CoV-2

Tras más de dos años desde el inicio de la pandemia por Covid-19, todavía se desconoce en detalle el papel de las diferentes vías de transmisión en la dinámica de propagación del virus. La transmisión humano-humano ha sido descrita por vía respiratoria directa, donde el paciente sintomático o asintomático expulsa partículas contaminadas en eventos respiratorios y, por otro lado, vía indirecta o fómite, donde la transmisión es por contacto con superficies contaminadas.

En cuanto a la diseminación indirecta, los fómites son objetos que pueden contaminarse con microorganismos patógenos y sirven como vehículos de transmisión.^{117,118} La supervivencia del SARS-CoV-2 es variable depende del material de la superficie; permanece activo hasta 72 horas en plástico y acero inoxidable, hasta 24 horas en cartón y menos de 4 horas en cobre.¹¹⁹ En superficies poliméricas y piel, las variantes Alfa, Beta, Delta y Ómicron exhiben una supervivencia dos veces mayor que la cepa ancestral, alcanzando una persistencia de más de 16 horas en la piel en condiciones experimentales de laboratorio.¹²⁰

En la diseminación indirecta, es posible diferenciar entre el modelo de microgotas (o gotas de *Pflügge*) y el modelo de bioaerosol. Si bien las microgotas predominan en contacto cercano, los bioaerosoles residen suspendidos en el aire con una persistencia variable.¹²¹ A propósito de esta pandemia, la comunidad científica ha redefinido el concepto de

bioaerosol, ampliando su consideración a partículas suspendidas en el aire con diámetros inferiores a 100 μm , atendiendo a los factores comunes relacionados con la aerodinámica de las partículas.¹²²

2.2. Generación y dinámica de la transmisión aérea

2.2.1. Transmisión aérea del SARS-CoV-2

La evidencia científica observada durante la pandemia por Covid-19 ha puesto de relieve la importancia del modelo de propagación aérea, que clásicamente ha sido desestimado como modo principal de transmisión de virus respiratorios entre huéspedes. Tradicionalmente, entre las posibles vías de transmisión predominaban las gotas de *Pflügge* y los fómites. Sin embargo, se ha aceptado globalmente que también se propagan de manera predominante a través de aerosoles respiratorios a corta y larga distancia respecto a la persona infectada que puede ser sintomática o no.¹²³

El término aerosol hace referencia a un coloide de partículas sólidas o líquidas en un medio gaseoso, contemplando tamaños entre 0.002 y 100 μm y ajenas a los compuestos volátiles presentes exclusivamente en fase gaseosa.¹²⁴ Cuando los aerosoles contienen elementos de origen biológico o partículas activas que pueden tener efecto sobre organismos vivos, causándoles algún tipo de alergia, toxicidad o infección,^{125,126} son conocidos como «bioaerosoles» y pueden estar constituidos por virus, bacterias, esporas, polen y, en general, cual-

quier resto de microorganismos.¹²⁷ En el contexto de la transmisión de patógenos aéreos, la generación de bioaerosoles está asociada a las acciones respiratorias típicas.¹²⁸ Las hipótesis mecanicistas tradicionales sobre la transmisión de enfermedades infecciosas apuntaban exclusivamente a la tos y los estornudos como principales eventos respiratorios generadores de aerosoles y gotas. Sin embargo, se ha demostrado que también pueden producirse durante cualquier otro evento respiratorio, como la respiración y el habla. Johnson *et al.*¹²⁹ determinaron la expulsión de bioaerosoles entre ~0.5 y ~10 µm durante el habla, la respiración, la vocalización sostenida y la tos, encontrando menor concentración de aerosoles en la respiración y siendo estos, en promedio, de tamaño más fino. Por el contrario, durante la vocalización sostenida el número de partículas aumentó notablemente, así como el diámetro aerodinámico de la mayor parte de los aerosoles. Durante el habla, otro estudio sugiere la dependencia entre la tasa de emisión de aerosoles y la amplitud de vocalización y, además, apunta a la existencia de eventos de superemisión independientes.¹³⁰

Si bien esta vía de propagación fue descrita con anterioridad en otros virus respiratorios, como el influenza,^{131,133} el SARS-CoV,^{134,135} el MERS-CoV,¹³⁶ el virus sincitial respiratorio,¹³⁷ el virus del sarampión,^{138,139} el rinovirus humano,^{140,141} o el Ébola,^{142,143} la OMS y el Centro de Control y Prevención de Enfermedades (CDC, por sus siglas en inglés *Communicable Disease Center*) no asumieron este método de propagación como una vía de contagio principal a largo alcance hasta 17 (abril, 2021) y 18 (mayo, 2021) meses después, respectivamente, del descubrimiento de la cepa inicial de SARS-CoV-2.

Este modelo de media-larga distancia como vía de contagio de la enfermedad por Covid-19 ha sido demostrada entre pequeños animales, a partir de eventos de superpropagación viral difícilmente demostrables atendiendo a otros modos de transmisión, en escenarios de transmisión a larga distancia donde los individuos infectados no entran en contacto directo, por la transmisión en individuos asintomáticos y por la prevalencia en la propagación en espacios cerrados.¹⁴⁴

Evidencias de RNA viral en el ambiente aéreo

El tamaño del SARS-CoV-2 varía entre 70 y 90 nm,^{145,146} y se ha reportado una concentración promedio del virus en el esputo de 7.0×10^6 copias/ml y máxima de 2.35×10^9 copias/ml.¹⁰³ En consecuen-

cia, en promedio, la carga viral ocuparía un 2.14×10^{-6} % del bioaerosol. Con este valor, Lee¹⁴⁷ estimó un tamaño teórico mínimo e inicial de aerosol de 4.7 µm para contener SARS-CoV-2. Sin embargo, el muestreo experimental ha demostrado la presencia de virus en tamaños de partícula inferiores (incluso <0.25 µm). Liu *et al.*¹⁴⁸ fueron pioneros en investigar la naturaleza aerodinámica del patógeno SARS-CoV-2 cuantificando el RNA viral de aerosoles en diferentes áreas de hospitales de Wuhan (China) durante uno de sus brotes más severos. En su trabajo, determinaron la presencia de virus predominantemente en dos rangos de tamaño: en la región submicrométrica (0.25-1.0 µm) y en la supermicrométrica (>2.5 µm). Trabajos experimentales posteriores han demostrado con mayor o menor éxito la presencia de RNA viral del SARS-CoV-2 en aerosoles utilizando diversos métodos de muestreo, como impactadores sólidos,¹⁴⁹⁻¹⁵¹ ciclones,¹⁵²⁻¹⁶² impigners,^{163,164} filtros de gelatina,^{152,158,165} filtros de partículas¹⁶⁶⁻¹⁶⁹ y sistemas de condensación.^{170,171} En otras investigaciones no se ha logrado recuperar concentraciones detectables de RNA ambiental.¹⁷²⁻¹⁷⁹ De los trabajos previos publicados⁷²⁻¹⁰² es posible deducir una concentración media equivalente de 3.1 ± 2.9 copias/l de aire en un total de 3085 muestreos (313 positivos; que supone un 10.2 % de positividad en muestras).

Evidencias de contagio por aerosoles en modelos animales

La transmisión a larga distancia del SARS-CoV-2 también ha sido demostrada en modelos animales. Diferentes estudios han detallado como algunos animales pueden actuar como reservorio y transmitir la enfermedad, al menos entre individuos de la misma especie.^{180,181} Kutter *et al.*¹⁸² utilizaron la variante BetaCoV/Munich/BavPat1/2020 para infectar *Mustela putorius* (hurones). Colocaron conductos de 118 cm de largo con curvas de 90 ° y un flujo de aire de 100 l/min. El contagio se realizó en 1 a 3 días después de la exposición (dpe) en 4/4 animales con niveles de RNA máximos a las 7 dpe y muestreos positivos hasta 15 dpe (final del experimento). Richard *et al.*¹⁸³ utilizaron la misma variante para infectar *Mustela putorius* utilizando un caudal de 100 l/min para circular el aire. Los animales estuvieron expuestos entre 20 y 21 días y las jaulas estuvieron conectadas a 10 cm para evitar el contacto estrecho. Los niveles de RNA máximo de los *Mustela putorius* donantes fue a los 3 dpe y se detectó hasta 11 dpe en 2 animales y hasta 15 y 19 dpe en otros 2 animales. Se contagiaron 4/4 hurones en contacto directo entre 1 y 3 dpe y el RNA se

detectó hasta 13-15 dpe. En los ensayos con jaulas separadas 10 cm, se transmitió la enfermedad a 3/4 *Mustela putorius* de 3 a 7 dpe y durante los siguientes 13 a 19 dpe. En otro trabajo realizado por Kim *et al.*¹⁸⁴ utilizaron la variante NMC-nCoV02 para infectar *Mustela putorius*. Encontraron RNA máximos 4 días después de la infección (dpi) en animales infectados artificialmente, que persistió hasta 8 dpi. Se evaluó el contacto directo (D) y el contacto indirecto (I). Se expusieron 2 animales no infectados por grupo. Los individuos infectados incubaron 2 dpi antes de la exposición a los sanos. Los *Mustela putorius* D mostraron fiebre y actividad reducida entre 4-6 dpe. El subgrupo I no mostró fiebre ni pérdida de peso tras 12 dpe. Los animales D positizaron a los 2 dpe (primera toma de muestra), mientras que los I (2/6) lo hicieron al 4 dpe (segunda toma de muestra) y la carga viral resultó notablemente inferior en exudados nasales.

Evidencias de contagio por aerosoles en eventos de superpropagación

A lo largo de la pandemia se han reportado algunos eventos de superpropagación típicamente en espacios cerrados que apoyan la teoría de la transmisión predominante por aerosoles.¹⁸⁵⁻²¹⁰ En línea con lo anterior, se ha informado de altas tasas de contagios secundarios en edificios residenciales de gran altura, concepto conocido como «contagio vertical».¹²¹

2.2.2. Generación de bioaerosoles

En individuos infectados, los virus pueden ser transportados de manera primaria a través de bioaerosoles de saliva y mucosa expulsados inadvertidamente por la nariz y la boca. Es posible diferenciar entre bioaerosoles bronquiales, laríngeos y orales, según el mecanismo de generación y la región anatómica donde se originen. De manera orientativa, los aerosoles generados en la zona alvéolo-bronquial son los de tamaño más reducido, encontrándose en el rango de 1 µm, mientras que en la región laríngea aumentan a 5 µm y, en las vías respiratorias superiores, a 50 µm o más.²¹² Las partículas bronquiales se atribuyen predominantemente a la respiración normal y se asocian con la ruptura de la película de líquido en los bronquiolos por fuerzas de cizallamiento.²¹³ La vibración de las cuerdas vocales genera secreciones laríngeas durante el habla y la vocalización.¹²⁹ En cambio, las partículas orales en las que las gotitas superan los 100 µm se producen predominantemente a partir de la saliva en la cavidad oral. Su

tasa de emisión y velocidad dependen del esfuerzo durante el evento de vocalización.¹²⁹ Estos bioaerosoles están predominantemente compuestos por iones (predominantemente Na⁺, Cl⁻, Ca²⁺ y Mg²⁺), partículas orgánicas e inorgánicas y glicoproteínas, principalmente albúmina, mucinas, colesterol y proteínas del surfactante pulmonar, como dipalmitoilfosfatidilglicerol (DPPG) y dipalmitoilfosfatidilcolina (DPPC).²¹⁴ Además de incluir, potencialmente, patógenos virales o bacterianos en individuos infectados. Esto es especialmente relevante durante infecciones que presentan una alta carga viral en el tracto respiratorio superior debido a la proximidad anatómica a las «vías de escape» como es el caso del virus SARS-CoV-2.^{119,215,216}

2.2.3. Dinámica de los aerosoles

Las distribuciones modales de aerosoles adquieren gran relevancia en el contexto de la transmisión de enfermedades debido a que no solo determinan las características aerodinámicas, sino también su dinámica de depósito y la variabilidad en el modelo de colonización viral en función de la profundidad del tracto respiratorio que alcance.²¹⁷

Además de la distribución modal de los aerosoles, la naturaleza de la actividad modificará parámetros asociados a la cinética de las partículas. Para determinar la distribución del tamaño de gotas en función de su posición espacial y del tiempo, Dhawan *et al.*²¹⁸ desarrollaron un modelo matemático considerando un evento donde se analiza un individuo infectado próximo y frente a otro individuo expuesto, sin obstáculos entre ellos y donde los aerosoles solo pueden depositarse en el suelo. El modelo parte de la base de que los aerosoles pueden ser transportados por convección y difusión en el medio aéreo, y que existen fuerzas gravitacionales que provocan su sedimentación. Además, se asume que la evaporación depende de la humedad relativa del ambiente, puesto que tendrá lugar hasta que la presión de vapor de agua en la superficie de los aerosoles expiradas sea igual a la de la humedad relativa ambiental. Este desarrollo parte de la ecuación general de dinámica de los aerosoles formulada por Friedlander²¹⁹ (Ec.1).

$$\begin{aligned} \frac{\partial n(\mathbf{v}, t)}{\partial t} + \nabla \cdot (\bar{\mathbf{v}}_p n(\mathbf{v}, t)) & \quad [1] \\ - \nabla^2 (D n(\mathbf{v}, t)) & \\ = \frac{\partial (G n(\mathbf{v}, t))}{\partial \mathbf{v}} + \mathbf{I}(\mathbf{v} - \mathbf{v}^*) + \frac{1}{2} \int_0^{\mathbf{v}} \beta(\mathbf{v} - \bar{\mathbf{v}}, \bar{\mathbf{v}}) n(\bar{\mathbf{v}}) & \\ - \bar{\mathbf{v}}, t) n(\bar{\mathbf{v}}, t) d\bar{\mathbf{v}} - n(\mathbf{v}, t) \int_0^{\mathbf{v}} \beta(\mathbf{v}, \bar{\mathbf{v}}) n(\bar{\mathbf{v}}, t) d\bar{\mathbf{v}} + \dot{n}(t) & \end{aligned}$$

Donde, el primer término del primer miembro (LHS) constituye la tasa de cambio de la distribu-

ción del tamaño de aerosoles en el intervalo de volumen $V/\partial t (\partial n(v,t)/\partial t)$. El segundo término de ese mismo miembro representa la convección del aerosol $\nabla(\bar{v}_p n(v,t))$. El tercer término constituye la difusión del aerosol $\nabla^2(Dn(v,t))$. En el miembro RHS, el primer término representa la evaporación de los aerosoles $\partial Gn(v,t)/\partial v$, el segundo la formación de nuevas partículas $I(v-v^*)$, el tercero el efecto de coagulación browniana $(\frac{1}{2} \int_0^v \beta(v-\hat{v}, \hat{v}) n(v-\hat{v}, t) n(\hat{v}, t) d\hat{v})$ y el cuarto el término de emisión $(n(v,t) \int_0^v \beta(v, \hat{v}) n(\hat{v}, t) d\hat{v} + \dot{n}(t))$.

Al aplicarlo a la transmisión del SARS-CoV-2 y al no existir el proceso de nucleación, el término segundo del miembro RHS desaparece. De la misma manera, al suponer un número total de concentración de aerosoles emitidos durante actividades respiratorias insignificante, el tercer término del mismo miembro se desprecia. En consecuencia, la **Ec.1** resulta significada según la **Ec.2**.

$$\frac{\partial n}{\partial t} = \nabla^2(Dn) - \nabla(\bar{v}_p n) + \frac{\partial(Gn)}{\partial v} + \dot{n} \quad [2]$$

La convección depende de la velocidad de la gota, y es necesario tomar en consideración la velocidad del aerosol ($\bar{v}_p$), que se describe como $\bar{v}_p = (\bar{u}_{air} - \bar{u}_{ext})$, donde $\bar{u}_{air}$ se refiere a la velocidad del aire y $\bar{u}_{ext}$ a la velocidad del aerosol por fuerzas externas (g), que a su vez viene dada como: $\bar{u}_{ext} = m_p g / 3\pi\mu d_p C$, donde m_p es la masa de la partícula, g la aceleración gravitatoria, μ la viscosidad del aire, d_p el diámetro de la partícula y C el factor de corrección de deslizamiento de Cunningham. Por su parte, la velocidad del aire $\bar{u}_{air}$ viene dada por $\bar{u}_{air} = (\bar{u}_{exp} + \bar{u}_w)$, donde $\bar{u}_{ext}$ se refiere a la velocidad inicial tras la expulsión de los aerosoles y $\bar{u}_w$ a la velocidad del aire ambiental.

Savory *et al.*²²⁰ formularon una expresión simplificada para la determinación de la velocidad de la tos según la **Ec.3**, donde x se refiere a la distancia horizontal desde el individuo infectado en la dirección de la tos.

$$\bar{u}_{tos} = \frac{0.875}{(x + 0.333)^2} \quad (\text{en la dirección de la tos}) \quad [3]$$

Respecto al término de evaporación de aerosoles, se debe considerar que los aerosoles están compuestos por una solución acuosa que contiene, predominantemente, iones orgánicos e inorgánicos, glicoproteínas y, potencialmente, virus. La presencia de iones disueltos afecta a la evaporación de los aerosoles emitidos, mientras que el efecto

de las especies orgánicas sobre la evaporación es despreciable por su baja fracción molar, como se explica en apartados a continuación.

Experimentalmente, Chao *et al.*²²¹ apuntaron a una velocidad media de 11.7 m/s durante la tos y 3.9 m/s al hablar, así como un diámetro promedio de 13.5 μm al toser y 16.0 μm al hablar, con una concentración estimada de 2.4 a 5.2 cm^{-3} en la tos y de 0.0004 a 0.223 cm^{-3} al hablar. Es por esto que la capacidad diseminadora del individuo resulta notoriamente modificada si el individuo está hablando o tosiendo. En concreto, se ha descrito que durante una conversación de un minuto pueden diseminarse más de 1000 aerosoles,²²² y un individuo expele alrededor de 7200 partículas por litro de aire exhalado,^{223,224} mientras que la tos ocurre de manera esporádica, lo que supone una probable mayor liberación de aerosoles durante la respiración y el habla.¹²² Además, en individuos infectados con virus respiratorios se ha descrito un aumento sustancial en la emisión, alcanzando concentraciones del orden de 10^6 aerosoles por tos en pacientes con H1N1.²²⁵ Se han obtenido conclusiones similares utilizando el virus SARS-CoV-2.²²⁶

Es posible definir dos conceptos fundamentales que afectan de manera directa a la propagación de las partículas en el aire: el tiempo de suspensión y la distancia recorrida. El tiempo de suspensión puede determinarse a partir de la Ley de Stokes, donde se describe una relación inversa entre el tamaño de partícula y su velocidad de deposición.¹²² A modo ejemplo, un aerosol de 5 μm de diámetro tarda alrededor de 33 minutos en sedimentar en el suelo, mientras que un aerosol de 1 μm puede permanecer suspendido en el aire durante más de 12 horas.²²⁷ No obstante, otros factores extrínsecos, como los flujos de aire ambientales, afectan tanto al tiempo de suspensión aérea como a la distancia recorrida por el aerosol,²²⁸ por lo que el comportamiento de los aerosoles resulta difícilmente predecible y su estudio requiere de aproximaciones computacionales.^{229,230}

De esta manera, en el modelo «airborne», la eficiencia de la transmisión dependerá del sitio anatómico de excreción del virus, la cantidad y duración de la diseminación del virus infeccioso en el aire, la capacidad del virus para permanecer infeccioso en el aire y la dosis infecciosa requerida para iniciar la infección en el individuo.¹⁸²

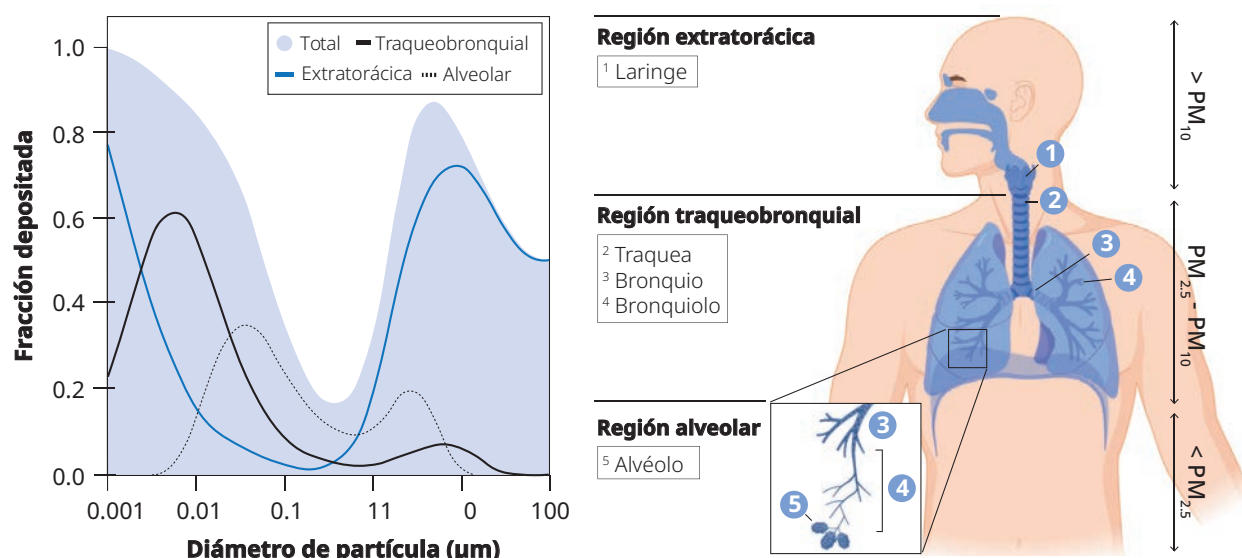


Fig. 10. Deposición de partículas en diferentes regiones del aparato respiratorio según su diámetro aerodinámico. Datos extraídos de Morawaska *et al.*²¹²

2.2.4. Deposición de los aerosoles en el tracto respiratorio

Un individuo sano en reposo consume alrededor de 15 l/min de aire al respirar, mientras que, en ejercicio intenso este consumo puede alcanzar los 75 l/min. El área pulmonar expuesta al aire alcanza los 23 m² en fase de reposo, aumentando hasta 93 m² durante respiraciones profundas. En el aire, existe una suspensión de partículas que pueden ser divididas en fracciones en función de la penetración que alcanzan en: inhalables, torácicas y respirables.²³¹ La inhalable contempla la fracción másica inhalada que es retenida en vías respiratorias superiores, la fracción torácica se refiere a la que penetra hasta la región central (traqueobronquial) y la fracción respirable a la que penetra en vías respiratorias no ciliadas.

Como se observa en la **Fig.10**, los aerosoles se depositan dependiendo de su tamaño en diferentes regiones del tracto respiratorio, según uno de varios mecanismos clave, que incluyen la impactación inercial, la sedimentación gravitacional, la difusión browniana, la precipitación electrostática y la intercepción.²³² Las partículas PM₁₀ se recogen dentro de la fracción inhalable y las PM_{2.5} y PM₁ a la fracción respirable,²³¹ Las partículas menores a 5 µm se depositan predominantemente en la región nasofaríngea (87 a 95 %), principalmente por efectos de impactación inercial y sedimentación gravitacional, aunque también pueden penetrar más profundamente en los pulmones y depositarse en la luz alveolar.²³³ En los aerosoles menores a 0.1 µm gobiernan fenómenos asociados a la difusión browniana, alcanzando las regiones bronquiales

y alveolares.^{227,234} Los factores anatómicos, como el calibre de las vías respiratorias, la distorsión de las vías por fenómenos de inflamación u otros, el patrón ventilatorio, el volumen y el flujo inspiratorio, la humedad y temperatura ambiental o el volumen y frecuencia ventilatoria, son aspectos que influyen en el mecanismo de depósito pulmonar de los aerosoles.²³⁵

Durante la inhalación, algunas partículas, especialmente las mayores a 10 µm, tienden a continuar su trayectoria en lugar de adecuarse al recorrido del tracto respiratorio.²³⁶ Este fenómeno atiende al momento de las partículas, viéndose afectadas por las fuerzas centrífugas de los puntos donde el aire cambie de dirección e impactando con la pared de la vía aérea, principalmente en las diez primeras generaciones bronquiales. Por otro lado, el fenómeno de la intercepción atiende sobre todo a fibras. Éstas se depositan cuando contactan con la pared de la vía aérea. La sedimentación ocurre en partículas de mayor masa, depositándose por la acción gravitatoria.²³⁷ Este fenómeno ocurre cuando la velocidad del aire es baja y aumenta el tiempo de residencia, predominando en las cinco últimas generaciones bronquiales. Finalmente, el fenómeno de la difusión es consecuencia del movimiento browniano de las partículas, por lo que ocurre en partículas con un diámetro inferior a 0.1 µm.²³⁸ Generalmente, las partículas de este tamaño no llegan a depositarse en las zonas distales pulmonares, donde los flujos ventilatorios son muy bajos, y son expulsadas durante la exhalación.²³⁸

Matemáticamente, la fracción de depósito total (DF) en el sistema respiratorio fue desarrollada por la Comisión Internacional de Protección Radiológica como (Ec.4):²¹⁸

$$DF = IF \left(0.0587 + \frac{0.911}{1 + \exp(4.77 + \ln d_{p,i})} + \frac{0.943}{1 + \exp(0.503 + 15.48 \ln d_{p,i})} \right) \quad [4]$$

Donde, $d_{p,i}$ corresponde al diámetro inicial de la partícula e IF a la fracción inhalable definida como (Ec.5):²¹⁸

$$IF = \left(1 - \frac{1}{1 + 4.8 \times 10^{13} d_{p,i}^{2.8}} \right) \quad [5]$$

Para cada moda de partículas, es posible calcular la tasa de deposición en cualquier posición espacial en función del tiempo siguiendo la siguiente formulación (Ec.6). Donde, IR es la tasa de inhalación (aprox, 1.5 m³/h).²¹⁸

$$N_{inh,rate,i,\delta(t)}(x,y,z,t) = N_{i,\delta(t)}(x,y,z,t) IR DF \quad [6]$$

Además de la morfología y tamaño de los aerosoles, su deposición en el tracto respiratorio también dependerá de otros factores, como la fisicoquímica del aerosol (incluyendo, además, densidad, morfología y afinidad al agua), la anatomía de las vías respiratorias (incluyendo diámetro, longitud o ángulos de respiración de los segmentos, entre otros) y la fisiología del tracto respiratorio (como el patrón de flujo de aire).²¹²

2.2.5. Diseminación viral de SARS-CoV-2 en pacientes infectados

La presencia de RNA de SARS-CoV-2 en la exhalación de individuos infectados ha sido ampliamente demostrada.²³⁹ Ma *et al.*¹⁶³ estudiaron a 35 pacientes Covid-19 para cuantificar el RNA SARS-CoV-2 condensando el aliento exhalado. Los ensayos fueron positivos en el aire exhalado de 5 pacientes. Se informó que los sujetos exhalaban una tasa estimada de 10³ – 10⁵ copias/min para un volumen de respiración de 12 l/min. Las tasas más altas (10⁵) se encontraron durante las primeras etapas de la Covid-19. En contraposición, Loconsole *et al.*²⁴⁰ no detectaron carga viral en el aliento exhalado en pacientes con RT-qPCR positiva, de manera que el momento de enfermedad del paciente puede determinar la capacidad emisión de bioaerosoles

cargados de SARS-CoV-2. Malik *et al.*²⁴¹ utilizaron un filtro de aire para cuantificar la carga viral de las gotitas respiratorias del aliento exhalado. De 15 pacientes hospitalizados, se encontraron 70/100 muestras positivas. Los hisopos nasofaríngeos resultaron significativamente más concentrados en carga viral respecto al aliento exhalado. La carga viral media por hisopo fue de 7.97 x 10⁶ (1.65 x 10² - 1.4 x 10⁸ copias) frente a 2.47 x 10³ (7.19 x 10¹ - 2.94 x 10⁴) tras 20 exhalaciones. Viklund *et al.*²⁴² evaluaron 20 respiraciones normales, 10 respiraciones con las vías abiertas y tres toses en una muestra de 25 sujetos. Se encontró RNA viral en 8 muestras de tos (Ct 29.5-36.5), 4 muestras de respiración normal (Ct 33.9-36.8) y 3 con vías respiratorias abiertas (Ct 31.98-33.4) en pacientes con Ct entre 17.2 y 26.4.

La carga viral contenida en las partículas resulta un factor clave para determinar la contribución relativa de la transmisión aérea.¹²² Normalmente, la carga viral contenida en un bioaerosol se asume proporcional a la concentración detectada en el esputo. Sin embargo, varios estudios sugieren que los aerosoles de menor tamaño contienen mayor carga viral relativa, especialmente en los menores a 5 µm, tal y como se ha mencionado en apartados anteriores.^{243,244} En el SARS-CoV-2, un trabajo demostró que un subconjunto de pacientes liberaban de 10⁵ a 10⁷ copias de RNA viral por hora en el aliento exhalado, mientras que otros no diseminaban virus detectables.²⁴⁵ Para la determinación matemática de la carga viral de los aerosoles se asume que la concentración viral de las micro y nanogotas expelidas es equivalente a la presente en el esputo del individuo infectado. En consecuencia, si se conoce la concentración viral en el esputo y el número de aerosoles emitidos, es posible determinar la carga viral emitida, expresada como tasa de emisión de cuantos (ER_q). Definiendo «cuanto» como la dosis de aerosoles necesaria para causar infección en el 63 % de personas susceptibles, puede evaluarse como (Ec.7):²⁴⁶

$$ER_q = c_v c_i V_{br} N_{br} \int_0^{10\mu m} N_d(D) dV_d(D) \quad [7]$$

Donde, c_v corresponde a la carga viral en el esputo (copias de RNA/ml), c_i es un factor de conversión definido como la relación entre un cuanto infeccioso y la dosis infecciosa expresada en copias de RNA viral, V_{br} es el volumen de aire exhalado en la respiración (cm³), N_{br} es la frecuencia respiratoria (respiración/h), N_d es la concentración del número de aerosoles (#/cm³) y $V_d(D)$ es el volumen de un

solo aerosol (ml) en función del diámetro de la gota (D). Para el caso concreto y asumiendo resultados obtenidos por Morawska *et al.* en relación al volumen de los aerosoles, se obtiene la **Ec. 8**, donde j indica las actividades espiratorias consideradas e IR la tasa de inhalación (m^3/h).²⁴⁶

$$ER_{q,j} = c_v c_i IR \sum_{i=1}^4 (N_{i,j} V_i) \quad [8]$$

El análisis por RT-qPCR de mascarillas ha constituido otra estrategia para determinar la emisión de SARS-CoV-2 en pacientes infectados. Williams *et al.*²⁴⁷ utilizaron mascarillas FFP2 durante 30 minutos con tiras de PVA (*strips*) para la recogida de muestras, encontrando cargas virales entre $<10 - 3.3 \times 10^6$ copias/*strip*. Verma *et al.*²⁴⁸ muestrearon el aliento durante 15 minutos. Utilizaron un filtro de gelatina de 4 mm en mascarillas FFP2 de pacientes hablando y en silencio. Se detectó RNA viral en 69/97 participantes. En el grupo A ($n = 53$), donde no se dieron indicaciones, la positividad fue del 79 %. Dentro del grupo B ($n = 44$), donde se habló, se obtuvo un 61 % de positividad y, donde no hablaron, del 43 %. Se correlacionó modestamente con el Ct del paciente, aunque se encontró notoria variabilidad entre muestras.

En animales, Hawks *et al.*²⁴⁹ informó de una tasa de emisión de 700 partículas/min por *Mesocricetus auratus* (hámster sirio), un 99.9 % de ellas de tamaño $<10 \mu m$. Se describió una tasa de emisión de 25 viriones infecciosos/hora en los días 1 y 2 posteriores a la inoculación en partículas de menos de $<5 \mu m$ utilizando la cepa USA-WA1/2020 del SARS-CoV-2. Zhang *et al.*²⁵⁰ utilizaron primates para estudiar la carga viral emitida a lo largo de la enfermedad Covid-19: 2, 4 y 6 días después. A los dos días, se encontraron cargas viral entre 11578 y 28336 copias tras 40 minutos. En promedio, exhalaban 503 virus/min y 209.5 virus/l en el aire. Al cuarto día, se encontraron entre 3369 y 5134 copias tras 40 minutos, lo que supone 106 virus/min y 44 virus/l aire. A los 6 días no se encontró carga viral en el aire exhalado. Se encontraron más partículas entre 2.1-4.7 mm, respecto a 0.65-2.1 y > 4.7 mm, con un recuento de 49.6, 27.4 y 23.0 %, respectivamente. La carga viral resultó más concentrada en las partículas 0.65-4.7 mm (77-79% de la carga viral), mientras que el 21-23% se encontró en partículas >4.7 mm. Sin embargo, hay que considerar que los primates respiraron a una tasa de 2.4 l/min, lo que limita su extrapolación al individuo humano.

2.2.6. Heterogeneidad en la transmisión

La heterogeneidad de la transmisión de enfermedades infecciosas es un concepto bien conocido que ha sido estudiado en varios escenarios epidémicos. Woolhouse *et al.*²⁵¹ identificaron un patrón estadístico conocido como la regla 20/80, que propone que el 80 % de las nuevas infecciones están asociadas a un 20 % infectados, que constituiría el grupo central de alto riesgo causante de la expansión masiva de la enfermedad.²⁵²⁻²⁵⁴ El SARS-CoV-2 está asociado a una dinámica de transmisión no homogénea como el SARS-CoV y el MERS-CoV, donde una cierta cantidad o la mayoría de las transmisiones se pueden rastrear hasta una minoría de portadores reflejada por el parámetro k .²⁵⁵ En el caso del SARS-CoV, el valor del parámetro k fue de 0.06, mientras que el del SARS-CoV-1 fue de 0.20, lo que sugiere que la mayoría de las infecciones se remontan al 6 y 20 %, respectivamente, de los infectados.²⁵⁶ En el caso del SARS-CoV-2, algunos estudios apuntan a un k de entre 0.1 – 0.67, y atribuyen esta heterogeneidad a eventos de superpropagación.^{206,257,258}

La heterogeneidad en la transmisión en la dinámica de los coronavirus no se ha observado en otros virus respiratorios con características virales similares, como el H1N1.²⁵⁹⁻²⁶² Una cantidad sustancial de casos Covid-19 informados proviene de eventos de superpropagación, donde los casos secundarios son desproporcionadamente superiores a los previstos según R_0 .²⁰⁴ A lo largo de la pandemia, se han reportado varios eventos de superpropagación, cuya extensión se apoya exclusivamente en la transmisión predominante por aerosoles.¹⁸⁵⁻²¹¹ Sin embargo, todavía se desconoce qué determina la sobredispersión individual en la transmisibilidad, por lo que no es posible discriminar entre superpropagadores y no propagadores y el manejo de estos individuos resulta limitado a las estrategias preventivas convencionales.^{253,263}

Se ha propuesto la hipótesis de que aparición de superpropagadores podía no ser aleatoria, sino que podía depender de otros superpropagadores.²⁶³ A pesar de que se desconoce en detalle el origen de la superpropagación, principalmente se atribuye a cuestiones fisiológicas del individuo, haciendo difícilmente predecible la infecciosidad del individuo.²⁶⁴ En un análisis sobre las tasas de emisión de aerosoles de 200 individuos sanos, se calculó que las diferencias biológicas podrían afectar la transmisión del virus.²⁶⁵ El trabajo reportó que el 20 % de los participantes del estudio emitieron el

80 % de los aerosoles totales, en línea con la regla 20/80. Aunque también podría estar relacionada con factores ambientales, conductuales y sociales de los individuos que influyen en la dinámica de transmisión del virus, esto solo afectaría a la configuración de los brotes concretos,²⁶⁶ por lo que la superemisión debe estar sujeta a condiciones inherentes al individuo. Para dar luz al conocimiento sobre la heterogeneidad en la propagación surgió la **segunda publicación de esta Tesis**.

2.2.7. Factores ambientales y su influencia en la persistencia viral

La humedad relativa (HR) representa un condicionante para la transmisión por aerosoles de virus. Humedades relativas bajas inducen la evaporación, la disminución del pH y, con ello, cambios conformacionales en las proteínas de su superficie, haciendo del virus un patógeno menos infeccioso.²⁶⁷ Además, a humedades relativas bajas, el tamaño del aerosol disminuye proporcionalmente hasta cristalizar reduciendo notablemente su tamaño en ambientes con una humedad relativa muy baja. En el caso contrario, a humedades relativas altas, el aerosol tiende a adsorber la humedad y aumentar su tamaño.²⁶⁸ Si la humedad relativa se sitúa por debajo del 80 %, los aerosoles respiratorios alcanzan un diámetro final que es del 20 al 40 % su tamaño original.²⁶⁹ En el caso del SARS-CoV-2, se ha reportado menor influencia de la humedad relativa respecto a la humedad o la presencia de radiación solar sobre el deterioro viral.²⁷⁰ Estos condicionantes atmosféricos pueden influir en la transmisión aérea de patógenos: tanto a nivel de dinámica de propagación, como de persistencia. El tamaño de los bioaerosoles exhalados evolucionan en el tiempo como resultado de la evaporación, la coagulación y/o la deposición, afectando de manera directa a su tiempo de suspensión aérea.¹²² En consecuencia, la distribución de tamaños de la concentración aérea de aerosoles variará con el tiempo, ya que las partículas de mayor diámetro sedimentan con mayor velocidad.²⁷²

El método matemático más apropiado para su resolución es un esquema numérico modal por ser fácilmente aplicable a un sistema multicomponente y, además, puede explicar el efecto iónico sobre la evaporación. Se utiliza una forma modal de distribución de tamaños en las que el rango de volumen total para aerosoles se divide en modos discretos. Así, la evolución de la concentración numérica de cada uno con el tiempo y la posición espacial se puede describir según la **Ec.9** como:

$$\frac{\partial N_i(x, y, z, t)}{\partial t} = \nabla^2 (D N_i(x, y, z, t)) - \nabla \cdot (\bar{v}_p N_i(x, y, z, t)) + N_i(x, y, z, t) \quad [9]$$

$N_i(x, y, z, t)$ representa el número de concentración de aerosoles pertenecientes al i -ésimo modo, que resultará modificado a consecuencia de la convección, difusión y emisiones $N_i(x, y, z, t)$. La evaporación resultará en el cambio de diámetro de cada modo. El cambio en el diámetro del i -ésimo modo $d_{p,i}(t)$ debido a la evaporación se puede describir mediante la **Ec.10**.

$$\frac{dd_{p,i}(t)}{dt} = 4 \frac{D_v v_m}{d_{p,i}(t) k_B T} (RH \cdot P_{sat} - d_{p,i}(t)) F(Kn) \quad [10]$$

Donde, d_p corresponde al diámetro de los aerosoles de ese modo, D_v a la difusividad del vapor de agua, v_m al volumen molecular del agua, k_B a la constante de Boltzmann, T a la temperatura, RH a la humedad relativa, P_{sat} a la presión de vapor de saturación del agua, $d_{d,i}$ a la presión de vapor del agua en la superficie de la gota, F al factor de corrección de Fuchs-Sutugin y Kn al número de Knudsen.

Para la resolución del modelo matemático se deben establecer las condiciones de contorno iniciales teniendo en cuenta que el individuo infectado comienza a diseminar aerosoles en $t = 0$ y que las expiraciones duran hasta un cierto $t_{ext'}$, repitiendo un número n de emisiones en intervalos regulares dt , se obtiene que $n = t_{ext'}/dt$. Así, matemáticamente se puede determinar los valores de N_i .

Por otro lado, la presencia de iones afecta a la evaporación de la gota, y por tanto a su dinámica de cambio, mientras que los compuestos orgánicos del esputo resultan insignificantes por la baja fracción molar que representan.²¹⁵ Además, al reducir la componente acuosa del aerosol, los aerosoles están sujetos a cambios morfológicos, de viscosidad y de pH, entre otros.²⁷³

Fears *et al.*²⁷⁴ determinaron una mayor eficiencia dinámica del SARS-CoV-2 respecto a la del SARS-CoV y a la del MERS-CoV, apuntando a una persistencia de la infectividad y de la integridad del virión hasta 16 horas en forma de aerosol de tamaño micrométrico (1.0-3.0 μm). Van Doremalen *et al.*¹¹⁹ sugirieron una vida media similar entre el SARS-CoV-2 (0.6-2.6 horas; 1.1 horas en promedio) y el SARS-CoV (0.8-2.4 horas; 1.2 horas en

promedio), apuntando a que las discrepancias en las características epidemiológicas podrían estar asociadas a altas cargas virales en el tracto respiratorio superior y a la transmisión del virus en pacientes asintomáticos. Smither *et al.*²⁷⁵ reportaron una vida del SARS-CoV-2 entre 30 y 177 min en aerosoles de entre 1.0 y 3.0 μm bajo diferentes condiciones de HR y reportaron una tasa de descomposición de entre el 0.4 y el 2.3 %/min. Schuit *et al.*²⁷⁶ informaron de la independencia entre la tasa de descomposición y la HR del SARS-CoV-2, atribuyendo la pérdida de infectividad al efecto de la luz solar y al medio de suspensión del aerosol. Alineadas con las observaciones de Schuit *et al.*, otros autores reportaron conclusiones similares utilizando otros virus.^{277,278} En contraposición, estudios recientes de Oswin *et al.*²⁷⁹ apuntan a que el 54 % de los bioaerosoles (5-10 μm) cargados de SARS-CoV-2 pierden su capacidad infectiva durante los primeros 5 segundos frente a HR bajas (40 %). En los 5 minutos siguientes, en los virus que persisten, se pierde alrededor del 19 % de la infectividad. En altas HR (90 %), la infectividad cae un 48 % de manera progresiva durante los 5 primeros minutos. A los 20 minutos, se ha encontrado una pérdida del 90 % de la infectividad utilizando diferentes variantes de SARS-CoV-2: SARS-CoV-2/humano/Liverpool/REMRQ0001/2020 (REMRQ0001), B.1.1.7 (la variante Alfa) y un mutante del aislado de SARS-CoV-2 England/2/2020.

El gradiente de temperatura, tanto en las vías respiratorias como a nivel ambiental, afecta la cinética de replicación de numerosos virus respiratorios. La tasa de desintegración aumenta exponencialmente con la temperatura a medida que el calor desnaturaliza las proteínas que componen el virión SARS-CoV-2.²⁸⁰ Un estudio *in vitro* del epitelio respiratorio humano demostró que el SARS-CoV-2 se replicaba de 10 a 100 veces más rápido durante 96 horas a temperaturas superiores (37 °C) respecto a inferiores (33 °C),²⁸¹ pudiendo asociar este fenómeno a eventos asociados al receptor ACE2.²⁸²

Por su parte, la temperatura ambiental y la exposición a radiación ultravioleta ha demostrado influir de manera notable en la transmisión de la enfermedad, esta última daña el material genético y da lugar a la inactivación del virus.²⁸³ Un estudio que evaluó la transmisión del SARS-CoV-2 en países seleccionados por presentar climatología diversa destacó que la relación entre la temperatura y la transmisión de Covid-19 tiene una dirección opuesta, aunque apuntan a que la variación en la

temperatura no fue un parámetro demasiado influyente para la propagación del virus en etapas tempranas de la pandemia.²⁸⁴ Otros trabajos sugieren la presencia de más contagios relativos en países más lejanos del ecuador y presenta resultados consistentes con la hipótesis de que el calor y la luz solar reducen la propagación del SARS-CoV-2 y la prevalencia del Covid-19.^{285,286} Algunos de los grandes eventos de superpropagación se asocia a los mataderos, donde las condiciones de humedad y temperatura aumentan la persistencia del SARS-CoV-2 a mayores periodos de tiempo.²⁸⁷

2.2.8. Dosis-respuesta frente a SARS-CoV-2

El concepto de dosis-respuesta describe la magnitud de la respuesta de un organismo en función de la exposición a un estímulo o factor estresante y resulta un parámetro útil para caracterizar la interacción entre el individuo y los virus.²⁸⁸ El SARS-CoV-2 provoca una variedad de respuestas en quienes contraen el virus, que van desde infecciones asintomáticas hasta insuficiencia respiratoria aguda y muerte.

La información sobre las dosis infectivas suficientes para cursar infecciones con SARS-CoV-2 resulta limitada y sujeta al modelo del estudio. La carga viral posee un papel relevante en la cinética viral en la transmisión de enfermedades infecciosas.²⁸⁹ Fain *et al.*²⁹⁰ encontraron que inóculos iniciales elevados conducen a infecciones breves, pero con picos de títulos virales más altos (10^6), y los inóculos iniciales más pequeños (10^1) reducen el pico del título viral, pero hacen que la infección dure más tiempo. El efecto dosis-infección estimado para humanos ha sido determinado *in vitro*. Zhang *et al.*²⁹¹ determinaron matemáticamente una constante infectiva (k) de 6.4×10^4 a 9.8×10^5 copias para el inicio de la infección, mientras que Therese *et al.*²⁵⁵ reportaron una dosis mínima de 4.0×10^4 copias para una infección *in vitro*. Solo el 21 % de las 10^9 muestras de SARS-CoV-2 aislados de pacientes resultaron lo suficientemente infecciosos como para iniciar una nueva infección. Un estudio reciente realizado por Killingley *et al.*²⁹² ha estudiado la evolución de la infección en humanos voluntarios. Se inocularon 10 TCID₅₀ (SARS-CoV-2/human/GBR/484861/2020) por vía intranasal a 36 voluntarios, alcanzando niveles máximos de RNA 5 dpi (~8,87 log₁₀ copias/ml) e infectando a 18/36 individuos.

Otros estudios con el mismo objetivo han sido llevados a cabo en modelos animales.^{180,184,293-310} Por

aerosoles, la dosis mínima infecciosa probada en pequeños animales es de 630 copias (tasa de infección de 2/2),²⁹⁷ mientras que en grandes animales (monos verdes, *Chlorocebus sabaeus*) es de 2000 copias (tasa de infección 2/2).³⁰⁷ Por vía intranasal se han reportado infecciones desde inóculos de 500 copias (tasa de infección 1/6 en hurones, *Mustela putorius*)²⁹³ hasta 3000000 copias (tasa de infección 8/8 en *Chlorocebus sabaeus*)³⁰⁶. Sin embargo, se han reportado casos donde con cargas virales superiores se ha obtenido menor tasa de ataque; por ejemplo, una inoculación de 70000 copias en ratones hACE2 consiguió infectar solo a 7/19 *Mus musculus* (ratones)²⁹⁵ y 7/9 *Mesocricetus auratus* (hámsters sirios),³¹⁰ mientras que en *Mustela putorius* se reportó un ataque de 6/6 con una dosis de 50000 copias.²⁹³

Métodos para la prevención de la transmisión aérea

3

PREÁMBULO

En la sección denominada «Métodos para la prevención de la transmisión» se recogen los métodos, individuales y colectivos, para la reducción de la propagación aérea del SARS-CoV-2, en relación a la tercera temática «Medidas para la reducción de la transmisión humano-humano».

3.1. Métodos

Los métodos para la prevención de la transmisión aérea pueden ser diferenciados en individuales y en colectivos. Por un lado, la estrategia de los métodos individuales se basa en controlar la emisión de bioaerosoles, ya sea por la limitación física en la emisión (uso de equipos de protección respiratoria) o por la reducción de la emisión (control de aforos). Por otro lado, los métodos colectivos pueden consistir principalmente en: la remoción mecánica (filtración, retención directa) o físico-química (sistemas de inactivación) de los bioaerosoles o la dilución de los bioaerosoles en el aire (ventilación natural o mecánica).

3.2. Remoción de aerosoles

3.2.1. Filtración: mecanismos de filtración de materia submicrónica

La captura de partículas de aerosol mediante filtrado es el método más común para purificar el aire, tanto en sistemas de purificación como en equipos de protección individual. Típicamente, las mascarillas están basadas en filtros fibrosos. Estos filtros constituyen estructuras tridimensionales donde las fibras se disponen de forma aleatoria y los puntos de contacto entre ellas son relativamente infrecuentes, constituyendo filtros de porosidad relativamente alta.³¹¹ La estructura del filtro contempla una posición de las fibras perpendicular al flujo de

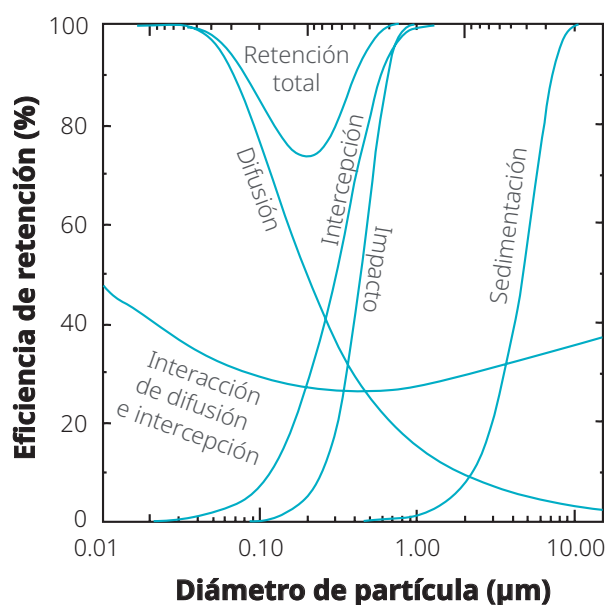


Fig. 11. Eficiencia ideal teórica de los diferentes mecanismos de captura en función del tamaño de la partícula. La predominancia de los diferentes de mecanismos de retención está sujeta al tamaño del aerosol. Yeh y Liu.^{308,316}

aire.³¹² Las fibras que componen estos filtros suelen estar compuestas a partir de celulosa, vidrio, cuarzo y materiales poliméricos y típicamente poseen un tamaño entre 0.5 y 50 μm .³¹² La reducción del diámetro de la fibras mejora de manera notable la filtración, aunque aumenta la pérdida de carga.³¹³⁻³¹⁵ Frente a esto, la tecnología actual permite obtener diámetros de fibra a escala nanométrica, teniendo como objetivo reducir la pérdida de carga y aumentar la eficiencia de filtrado.³¹⁶⁻³¹⁸

La filtración depende, predominantemente, del transporte y la adherencia. Los mecanismos capaces de realizar transporte son el cernido, la sedimentación, la interceptación, la difusión, el impacto inercial y mecanismos de transporte combinados. Por otro lado, los mecanismos de adherencia comprenden las fuerzas de Van der Waals, fuerzas electroquímicas y por enlaces químicos.^{311,319} Como se observa en la **Fig.11**, al diferenciar la materia submicrónica por su tamaño y los mecanismos de retención asociados, podemos distinguir entre tamaños de partículas >600 nm, 300-600 nm y <300 nm. Los más grandes (>600 nm) se retienen predominantemente porque son más grandes que los poros entre fibras (interceptación y sedimentación). Los intermedios (300-600 nm) pueden atravesar los poros del filtro, pero tienen una alta probabilidad de colisionar con las fibras (impacto inercial). Por el contrario, los más finos (<300 nm) siguen una dinámica heterogénea y son capturados principalmente por la difusión browniana.³²⁰⁻³²²

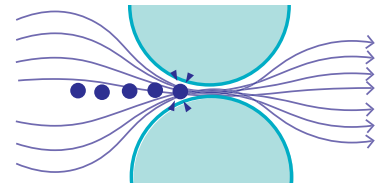
Cernido

El cernido es una operación unitaria que tiene por objetivo separar por tamaños haciendo pasar las partículas a través de una malla de paso definido (**Fig.12A**). Cuando la partícula es de mayor tamaño que los poros del material filtrante, ésta queda atrapada en los intersticios.³²⁴ Este método puede resultar afectado por la adherencia de las partículas, irregularidades del material filtrante, humedad o carga eléctrica de las partículas. Así mismo, las partículas pueden ser interceptadas, adhiriéndose a la fibra. El tamaño de partícula susceptible bajo este fenómeno se encuentra entre 0.1 y 1 mm.³²⁵

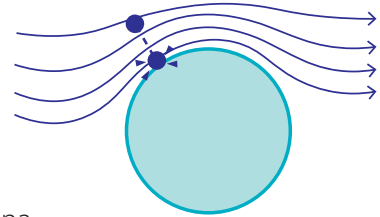
Sedimentación

La sedimentación corresponde al proceso físico mediante el cual las partículas en suspensión son separadas del coloide por efecto gravitatorio (**Fig.12B**). Las partículas capturadas por efecto gravitatorio deben ser de un tamaño del orden de micras y preferiblemente a baja velocidad. Habitualmente, se distinguen tres tipos fundamentales de sedimentación: discreta, difusa y por zonas, pudiendo ser llevados a cabo individualmente o en conjunto. La de tipo discreta se refiere a la sedimentación de partículas como unidades individuales, con velocidad de caída constante y sin interacción entre ellas. La sedimentación difusa corresponde al proceso donde las partículas se agregan, cambian su naturaleza y, al unirse, aumentan su masa y sedimentan a mayor velocidad. La sedimentación por zonas ocurre cuando las fuerzas entre partículas son lo suficientemente elevadas como para

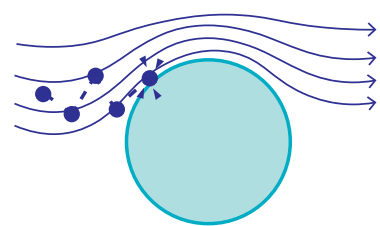
A. Cernido



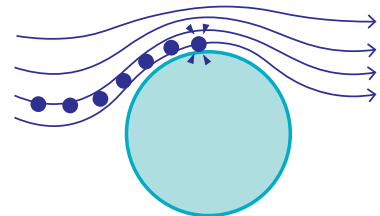
B. Sedimentación



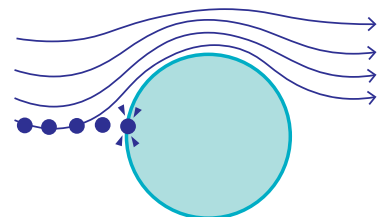
C. Difusión browniana



D. Interceptación



E. Impacto inercial



F. Fuerzas electrostáticas

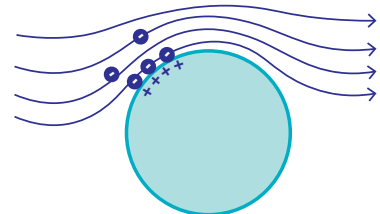


Fig. 12. Representación esquemática de los mecanismos de retención de partículas. Donde se representa A) el cernido, B) la sedimentación, C) la difusión browniana, D) la interceptación, E) el impacto inercial y F) fuerzas electrostáticas.

entorpecer la sedimentación y permanecer en posiciones relativamente fijas. Bajo este supuesto, las partículas forman un aglomerado que precipita como una sola unidad.³²⁶

Difusión browniana

El biólogo y botánico inglés, R. Brown (1773-1857), describió el movimiento browniano como un movimiento aleatorio que se observa en partículas

suspendidas en un medio fluido como resultado de choques contra las moléculas de la propia fase (Fig.12C).³²⁷ Las partículas relativamente pequeñas presentan dicho movimiento errático debido al aumento de la energía termodinámica y a la disminución de la viscosidad del fluido, por lo que la eficiencia del filtrado por difusión es directamente proporcional a la temperatura e inversamente proporcional al diámetro de la partícula. Según la teoría de Einstein, a partir de su ecuación de difusión de partículas brownianas donde el coeficiente de difusión está relacionado con el desplazamiento cuadrático medio, determinó que una partícula browniana sufre del orden de 10^{14} colisiones por minuto.³²⁷ No obstante, existen partículas de tamaño intermedio que permanecen en las líneas de flujo, considerándose «partículas penetrantes». Generalmente, el movimiento browniano aumenta conforme disminuye el tamaño de partícula.³²⁸

Intercepción directa

Habitualmente, el régimen del fluido durante la filtración es laminar y las partículas transcurren a lo largo de las líneas de corriente.³¹¹ El tamaño de las partículas susceptibles de este fenómeno es entre 0.3 y 1 μm y son retenidas cuando las líneas de corriente están a una distancia menor que la mitad del diámetro de las superficies suspendidas, como se representa en la Fig.12D.³²⁵

Impacto inercial

Mediante este mecanismo se describe el comportamiento de las partículas más grandes del aire. En lugar de seguir las líneas del flujo del coloide donde se encuentran dispersas, las partículas con cantidad de movimiento suficiente para mantener su trayectoria colisionan con las fibras del material filtrante como se observa en la Fig.12E. Este evento cobra especial relevancia en partículas entre 0.5 y 1 μm .³²⁹

Atracción por fuerzas combinadas: electrostática y Van der Waals.

La filtración electrostática es consecuencia del campo eléctrico.³³⁰ Las partículas presentan cargas en su superficie debido a la disociación iónica, a cargas no balanceadas, a reacciones químicas con iones de la suspensión y/o a la sustitución isomórfica de su estructura, por lo que el material filtrante atrae y se une a cualquier partícula cargada que pasa a través (Fig.12F). Este mecanismo está vinculado al fenómeno de la adsorción, permitiendo mediante las fuerzas de Van der Waals la retención de partículas.^{126,325}

a. Protección individual: mascarillas y equipos de protección respiratoria

El uso de mascarillas ha sido clave para frenar la transmisión del SARS-CoV-2 en el contexto de la actual pandemia de Covid-19.³³¹ La aceptación global de la propagación de la Covid-19 por aerosoles mejoró el enfoque preventivo, incluyendo el uso obligatorio de mascarillas quirúrgicas en la mayoría de los países. Un meta-análisis comprobó una reducción de hasta el 88 % del riesgo de infección en países donde el uso de mascarilla era obligatorio.³³² Frente a la demanda de equipos de protección respiratoria, son numerosos los modelos de mascarillas que se han dispuesto a la sociedad. No obstante, las mascarillas quirúrgicas, las mascarillas higiénicas y las FFP2 (Pieza Facial Filtrante, por sus siglas en inglés *Filtering Face Piece*) se han posicionado como las opciones más recurrentes. Sin embargo, no todas las mascarillas fueron diseñadas con el mismo objetivo y presentan características diferenciales que modifican sustancialmente su rendimiento frente a partículas submicrónicas. En concreto, la eficiencia de filtración del material y el ajuste de la máscara a la superficie de la cara son las dos características fundamentales.^{333,334}

Las mascarillas quirúrgicas son productos sanitarios que rigen bajo la norma española UNE EN 14683.³³⁵ Es posible diferenciar entre mascarillas de tipo I, tipo II y tipo IIR, cuyas características principales se recogen en la Tabla 1. Los ensayos BFE consisten en la evaluación de una muestra del material de la mascarilla utilizando un impactador en cascada de seis etapas, situado entre una cámara de aerosolización de *Staphylococcus aureus* (1.7×10^3 – 3.0×10^3 CFU/ml), cuyo tamaño oscila entre 0.8 y 1.5 μm de diámetro, y una bomba de vacío. En este sistema, el aerosol bacteriano atraviesa el material de la mascarilla, y se determina la eficacia a partir del recuento de Unidades Formadoras de Colonias (CFU, por sus siglas en inglés *Colony-Forming Units*) que penetran el material. Para determinar la presión diferencial (o respirabilidad) se utilizan muestras que son sometidas a un flujo constante de aire a 8 l/min y evaluadas con manómetros.

Como se observa en la Fig.13, las mascarillas quirúrgicas están típicamente compuestas por tres capas: dos de tejido-no-tejido de polipropileno (spunbond) y una filtrante de meltblown.

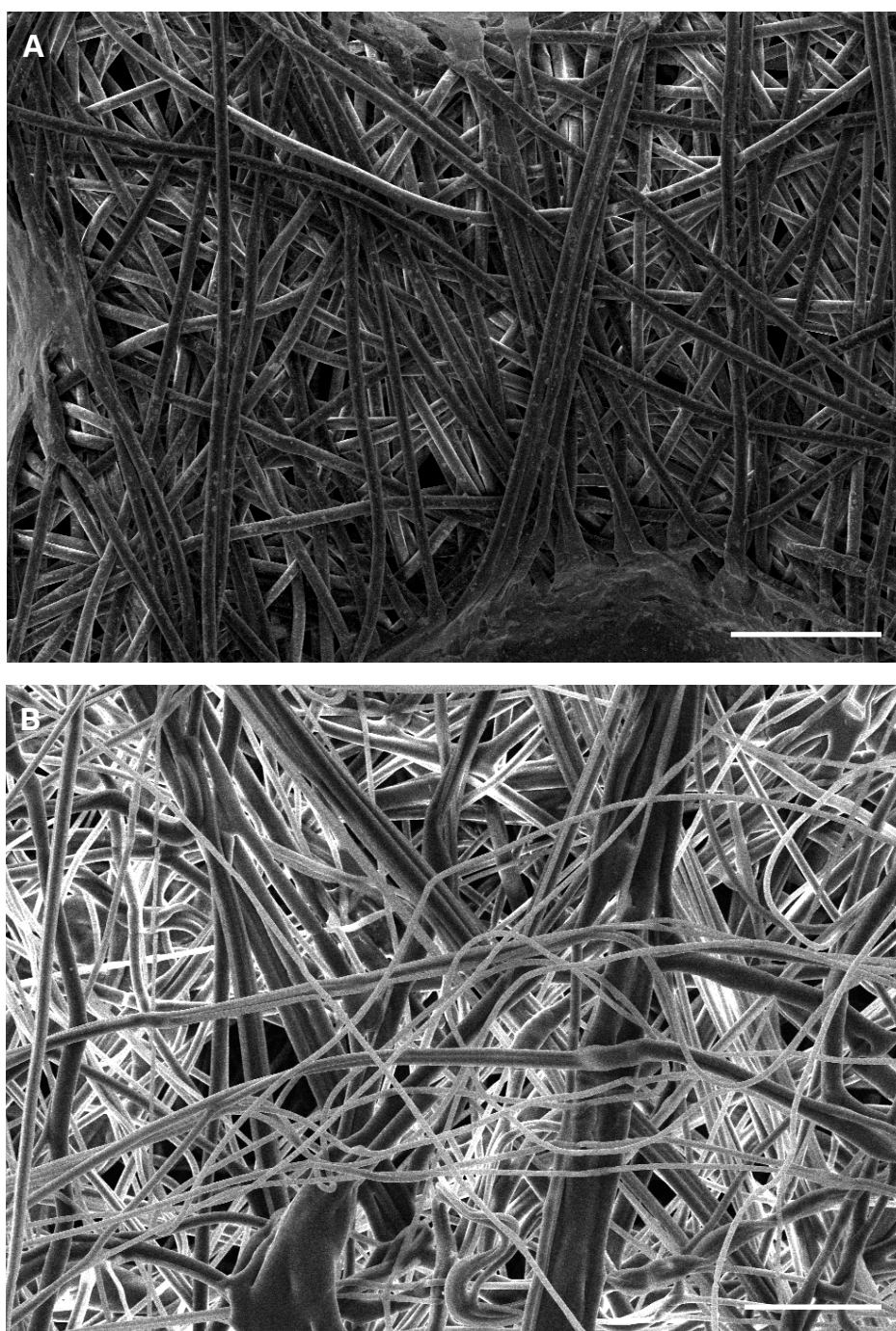


Fig. 13. Micrografías de microscopía electrónica de barrido (SEM, por sus siglas en inglés *Scanning Electron Microscopy*) de las capas de una mascarilla quirúrgica. Donde, se observan A) las capas externa e interna de *spunbond* y B) de *meltblown*. Las imágenes fueron obtenidas de muestras de una mascarilla quirúrgica convencional utilizando el microscopio SEM Inspect F50. Las barras de escala representan 250 μm en A y 50 μm en B.

Tabla 1. Requisitos de las mascarillas quirúrgicas de tipo I, II y IIR según la norma UNE-EN 14683:2019 + AC: 201. Donde, NR = No Requerido. Recuperado de la norma UNE-EN 14683.

Ensayo	Tipo I	Tipo II	Tipo IIR
BFE (UNE-EN 14683:2019)	$\geq 95 \%$	$\geq 98 \%$	$\geq 98 \%$
DP (UNE-EN 14683:2019)	$< 40 \text{ Pa/cm}^2$	$< 40 \text{ Pa/cm}^2$	$< 60 \text{ Pa/cm}^2$
Presión de resistencia a salpicaduras (UNE-EN 14683; ISO 22609/ASTM F1862)	NR	NR	$\geq 16 \text{ KPa}$
Limpieza microbiana (UNE-EN 14683; ISO 11737-1:2018)	$\leq 30 \text{ UFC/g}$	$\leq 30 \text{ UFC/g}$	$\leq 30 \text{ UFC/g}$
Biocompatibilidad (ISO 10993-1)	Aceptable	Aceptable	Aceptable
Citotoxicidad (ISO 10993-5)	Aceptable	Aceptable	Aceptable
Irritación dérmica (ISO 10993-10)	Aceptable	Aceptable	Aceptable

Las mascarillas higiénicas pueden ser de tipo reutilizable (en este caso, recogidas en la norma española UNE 0065) o no reutilizables (UNE 0064). La norma UNE 0065 exige una BFE mínima del 90 % y una respirabilidad inferior a 60 Pa/cm², mientras que en la norma UNE 0064 los valores exigibles son de 90 % en filtración y de 60 Pa/cm² en respirabilidad. Además de presentar requisitos de BFE inferiores a las mascarillas quirúrgicas, su característica diferencial es que éstas no son consideradas producto sanitario, por lo que los ensayos exigibles en la norma UNE EN 14683 no son aplicables en las mascarillas higiénicas. Tanto las mascarillas quirúrgicas como las mascarillas higiénicas presentan un escaso rendimiento frente a la retención de partículas PM₁, incluyendo los bioaerosoles cargados de SARS-CoV-2, motivo por el cual se desarrolló el trabajo contenido en la **tercera publicación de esta Tesis**.

Mientras que las mascarillas higiénicas pueden ser reutilizables, las quirúrgicas tienen una vida útil más reducida. Las mascarillas higiénicas no reutilizables suelen presentar una conformación de capas similar a las mascarillas quirúrgicas, mientras que en las reutilizables es común encontrar máscaras monocapa de fibras poliméricas gruesas y tejidas con recubrimientos para mejorar su hidrofobicidad (Fig.14). Su uso diario y de carácter no reutilizable extendido a gran parte de la población mundial, ha supuesto un impacto ambiental notorio durante el periodo pandémico, motivo por el cual se desarrolló la investigación contenida en la **cuarta publicación de esta Tesis**.

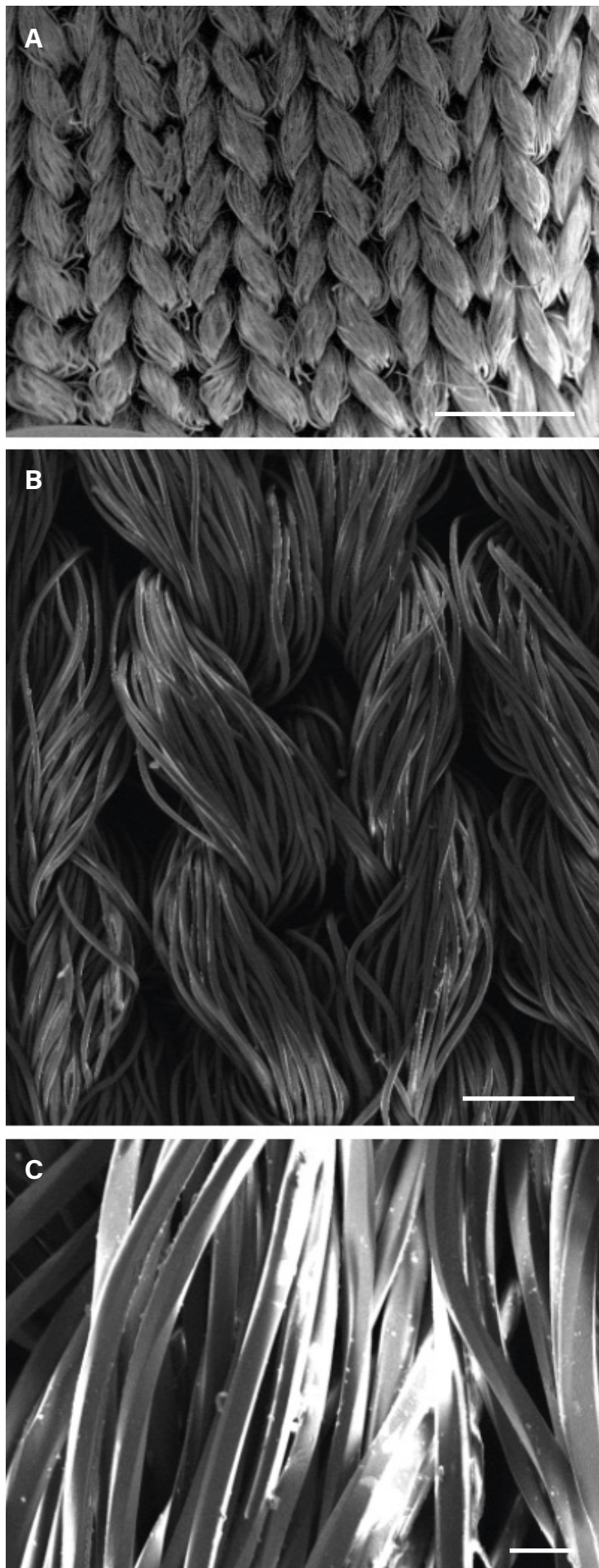


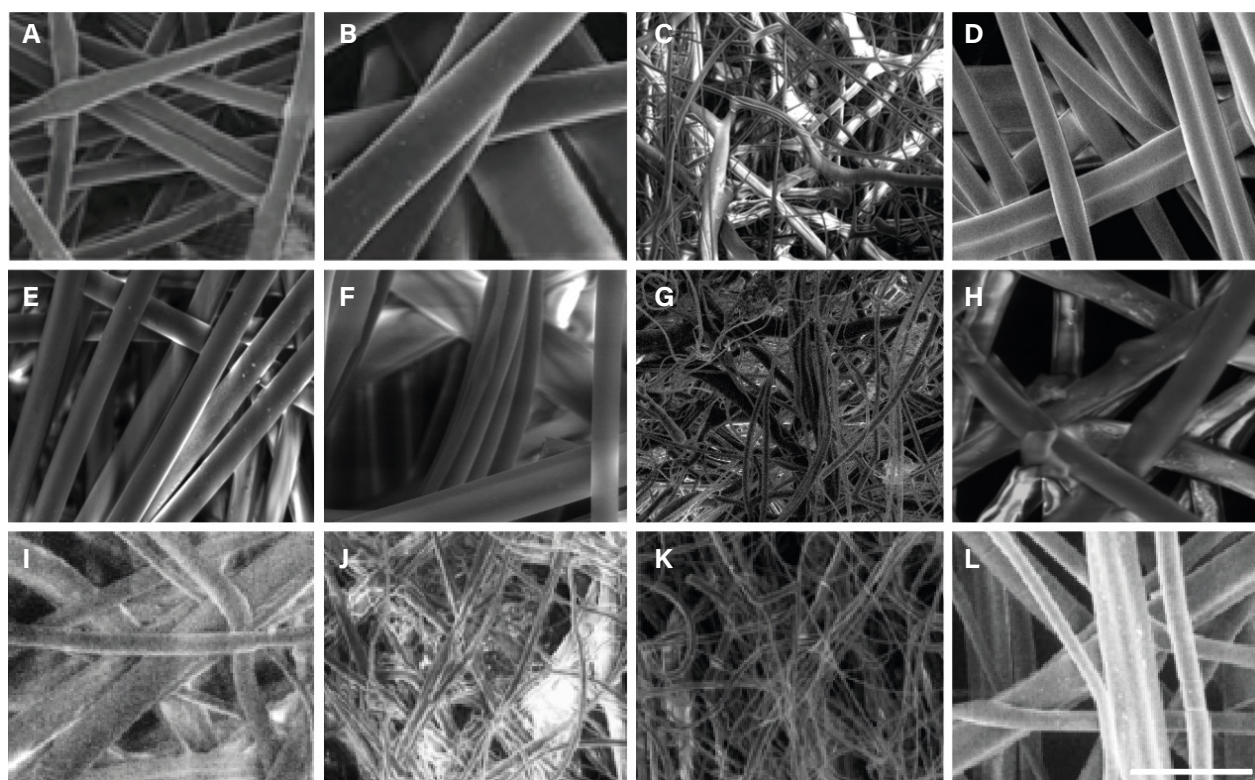
Fig. 14. Micrografías SEM de una mascarilla higiénica reutilizable. Donde, se observa una única capa de fibras poliméricas gruesas tejidas recubiertas con ceras y fotografiada a diferentes aumentos. Las imágenes fueron obtenidas de muestras de una mascarilla higiénica cedida por Ditex SL (Zaragoza, España) utilizando el microscopio SEM Inspect F50. Las barras de escala representan 2 mm en A, 400 μm en B y 50 μm en C.

Las medias máscaras filtrantes según la definición de la norma UNE EN-149:2001+A1:2009 «cubren la nariz, la boca y el mentón y pueden constar de válvulas de inhalación y/o exhalación. La media máscara filtrante consta totalmente, o en su mayor parte, de material filtrante o incluye un adaptador facial en el que el filtro principal constituyen una parte inseparable del equipo». ³³⁶ Estos materiales son considerados Equipos de Protección Individual (EPI), dentro del subtipo de Equipos de Protección Respiratoria (EPR). Según su rendimiento y fuga hacia el interior total, existen tres clases de dispositivos: FFP1, FFP2 y FFP3 (**Tabla 2**). La fuga hacia el interior total refiere a la fuga del aire ambiental hacia el interior del EPR, pudiendo venir a consecuencia de un deficiente ajuste facial.

Tabla 2. Requisitos de penetración del material filtrante según la norma EN 13274. Donde se observan los valores admisibles de fuga hacia el interior total de las máscaras.

	Fuga hacia el interior total		
	FFP1	FFP2	FFP3
Ensayo con aerosoles de NaCl	20 %	6 %	1 %
Ensayo con aerosoles de parafina	20 %	6 %	1 %

Los ensayos que describen el método para la determinación de la fuga hacia el interior total están definidos en la norma UNE-EN 13274-7, donde se recoge la posibilidad de enfrentar el material filtrante a aerosoles de parafina o de cloruro sódico (NaCl). El ensayo de penetración deberá tomarse como una media en el tiempo de 30 ± 3 s, empezando 3 min después del inicio de la aerosolización. En el ensayo con NaCl, se genera un aerosol de partículas mediante atomización de una solución acuosa de NaCl al 1% utilizando un *Collinson* a una presión de 3.45 bar. El líquido pulverizado se introduce en un deflector que elimina las partículas más grandes. Las partículas que persisten son arrastradas por un caudal a 95 l/min y evaporadas en parte al mezclarse con aire caliente, dejando libre el aerosol cristalizado de NaCl. Los aerosoles generados mediante esta técnica presentan polidispersión de tamaños, con un diámetro aerodinámico promedio de 600 nm, según la distribución de tamaños recogida en la propia norma. La concentración del aerosol se mide antes y después del filtro sometido al ensayo utilizando un fotómetro de llama. La resistencia a la respiración (o pérdida de carga) establecida para cada tipología de máscara se recoge en la **Tabla 3**, y es medida a un caudal de 160 l/min, utilizando medidores de presión.



La resistencia a inhalación se ensaya a un caudal continuo de 30 y 95 l/min. Además de los mencionados, en la norma UNE EN-149:2001+A1:2009 se contemplan otros requisitos, como el embalaje, los materiales utilizados, la compatibilidad con la piel, la inflamabilidad, el contenido de dióxido de carbono en el aire de inhalación, el campo de visión, la regulación de válvulas de exhalación, la obstrucción, la regulación de partes desmontables, entre otros. Típicamente, las medias máscaras filtrantes cuentan con, al menos, cuatro capas que incluyen (Fig.15): dos capas de *spunbond* en ambos extremos, una capa prevista para la retención de partículas gruesas y una última capa de *meltblown* para la filtración de las partículas finas.

b. Protección colectiva: equipos de filtración de aire

La limpieza de aire se ha convertido en una intervención crítica en el manejo de la propagación de la Covid-19.

Los filtros de partículas eficientes (EPA, por sus siglas en inglés *Efficiency Particulate Air*), los filtros de partículas de alta eficiencia (HEPA, por sus siglas en inglés *High Efficiency Particulate Air*) y los filtros de aire de penetración ultrabaja (ULPA, por sus siglas en inglés *Ultra-high Efficiency Particulate Air*) se han utilizado ampliamente en diversas industrias y aplicaciones.

Los filtros HEPA (Fig.16), bajo el amparo la norma UNE-EN 1822, se recomiendan para el control de infecciones en entornos sanitarios o de riesgo (p.e., laboratorios biológicos o aviones) por su alta eficiencia y su baja pérdida de carga en relación a los ULPA.²²⁷ En la actual pandemia por SARS-CoV-2, la filtración HEPA ha sido predominantemente implementada a través de equipos portables y se ha demostrado una alta eficiencia para la eliminación de partículas ambientales con tamaños compatibles al del virus.³³⁷

Es común encontrar filtros de menor eficiencia de retención en grandes superficies, transportes colectivos o edificios, acogidos bajo la norma UNE-EN 779, según se enumeran en la Tabla 4. La eficiencia de retención refiere a una eficiencia media (E_m) frente a un diámetro de partícula de 0.4 μm . La clasificación alberga de menor a mayor filtración mínima, abarcando desde el filtro M5 (de menor capacidad filtrante, Fig.17A) hasta el filtro F9 (de mayor capacidad filtrante, Fig.17B).

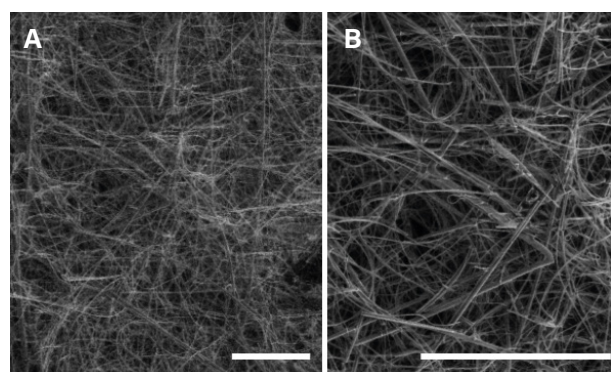


Fig. 16. Micrografías SEM de un filtro HEPA. Las imágenes fueron obtenidas de muestras de una filtro HEPA H13 de un equipo de purificación de aire de Dyson (Malmesbury, Reino Unido) utilizando el microscopio SEM Inspect F50. Las barras de escala representan 100 μm .

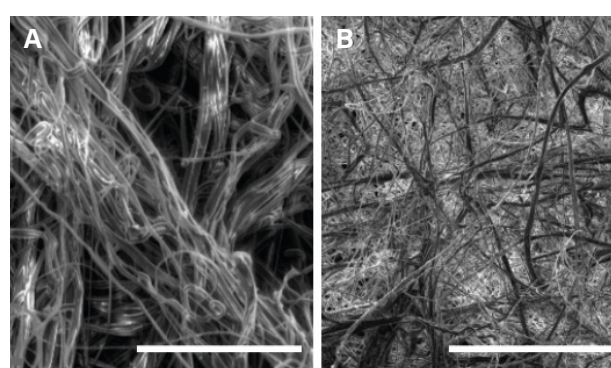


Fig. 17. Micrografías SEM de (a) un filtro M5 y (b) un filtro F9. Las imágenes fueron obtenidas de muestras de un filtro M5 de Camfil (Estocolmo, Suecia) y un filtro F9 de Trox GmbH (Neukirchen, Alemania) utilizando el microscopio SEM Inspect F50. Las barras de escala representan 500 μm .

Tabla 3. Requisitos de resistencia a la respiración según la norma UNE-EN 149. Donde se observan los valores admisibles de pérdida de carga de máscaras FFP1, FFP2 y FFP3.

Tipología de máscara	Resistencia máxima permitida		
	Inhalación		Exhalación
	30 l/min	95 l/min	160 l/min
FFP1	0.6 mbar	2.1 mbar	3.0 mbar
FFP2	0.7 mbar	2.4 mbar	3.0 mbar
FFP3	1.0 mbar	3.0 mbar	3.0 mbar

Tabla 4. Categorías de filtros de ventilación de aire general según la norma UNE-EN 779:2012. Donde, E_m refiere a la eficiencia media en un tamaño de partícula de 0.4 μm .

Clase	Pérdida de carga	Eficiencia de retención
M5	450 Pa	$40 \leq E_m < 60$
M6	450 Pa	$60 \leq E_m < 80$
F7	450 Pa	$80 \leq E_m < 90$
F8	450 Pa	$90 \leq E_m < 95$
F9	450 Pa	$95 \leq E_m < 100$

3.2.2. Inactivación físicoquímica de patógenos

Los sistemas de purificación de aire presentan rendimiento variable. Entre estos sistemas, destacan los basados en ultravioletas (UV-C) y en ionización.

Por un lado, la tecnología basada en el espectro UV-C (200-280 nm) ha sido ampliamente utilizada en procesos de desinfección y de esterilización. Los componentes intracelulares de los microorganismos (p.e., DNA, RNA y proteínas) presentan una sensibilidad alta y variable para absorber los fotones UV-C, produciendo un daño crítico en su genoma e inhibiendo su correcta replicación.³³⁸ La eficiencia de la desinfección UV varía según la distancia a la superficie y el tiempo de aplicación.³³⁹ Aunque ha demostrado ser efectiva frente a SARS-CoV-2, reduciendo la carga viral entre 0 y 6 log órdenes de magnitud en cultivo celular,³⁴⁰⁻³⁴³ el rendimiento de la tecnología es limitado frente a bioaerosoles cargados de SARS-CoV-2, donde la dosis requerida de UV254 es alta.³⁴³ Escasos estudios avalan su eficacia para la purificación del aire,³⁴⁴ aunque es posible encontrar esta tecnología en combinación con filtración HEPA.³⁴⁵

Por otro lado, la tecnología basada en plasma no térmico se ha posicionado como otra estrategia de purificación de aire en el contexto de la pandemia provocada por el virus SARS-CoV-2. La inactivación plasmática de microorganismos se ha atribuido a la ruptura de la pared celular y al daño del material genético.³⁴⁶ Su mecanismo de acción es múltiple. Destaca la presencia de partículas cargadas, iones, especies reactivas de oxígeno (ROS, por sus siglas en inglés *Reactive-Oxygen Species*) y radicales que contienen oxígeno, UV-C, ultravioleta de vacío y eventos de calentamiento localizado, actuando de forma exclusiva o combinada.³⁴⁷⁻³⁵⁰ El efecto antimicrobiano de esta tecnología ha sido ampliamente explotado en el sector de la salud para esterilizar instrumental quirúrgico.³⁵¹ Ha sido probado en ambientes controlados reportando una excelente eficiencia en la inactivación de especies bacterianas específicas y varios modelos virales con y sin envoltura.^{346,352-354} En la actual pandemia, la tecnología basada en plasma no térmico ha sido probada para otras aplicaciones, obteniendo resultados variables e inferiores a los descritos por los fabricantes.³⁵⁵ Cuestión que llevó a la **séptima publicación de esta Tesis**. Una de las principales preocupaciones y limitaciones de esta tecnología es la generación de subproductos en concentraciones nocivas, donde algunos autores sugieren aumentos en las condiciones reales.^{356,357}

3.2. Dilución de aerosoles**3.2.1. Ventilación**

Otra de las estrategias para reducir la carga viral ambiental a la que se expone un individuo susceptible es la reducción de la concentración de bioaerosoles aéreos. La ventilación de un espacio puede ser definida como el intercambio o renovación de aire interior, permitiendo diluir y evacuar los bioaerosoles en un entorno cerrado.

La incorporación de «nuevo aire» en un espacio, a su vez, puede venir dado por ventilación mecánica, ventilación natural o mixta. Por un lado, la ventilación mecánica viene dada por un sistema de ventilación artificial, típicamente sistemas de climatización o ventiladores. Por otro lado, la ventilación natural se refiere a la recirculación del aire ambiental exterior, exenta del uso de equipos añadidos y normalmente regulada mediante la apertura de puertas y/o ventanas.

En un edificio, la caracterización de la ventilación responde a tres parámetros básicos: la tasa de ventilación (medida como la cantidad de aire exterior que se introduce), dirección del flujo de aire (dirección general del flujo de aire en un edificio) y patrón de flujo del aire.³⁵⁸

En cumplimiento con diversas normativas y estándares (como la UNE 16798 o 171330), los sistemas de climatización recuperan aire exterior y lo filtran y aclimatan para su incorporación al espacio interior. Esto presenta una clara ventaja respecto a la renovación natural de aire, ya que el aire es pretratado (en mayor o menor medida) para limitar la presencia de aerosoles exógenos. Además, la ventilación natural no permite regular el caudal de ventilación ni obtener una calidad de aire interior homogénea. Sin embargo, frente a la carencia de sistemas de ventilación, o la limitación en su regulación, la ventilación natural se posiciona como un elemento imprescindible para la renovación del aire.

Un concepto clave es el de «calidad de aire interior», que puede ser definido «a partir de los niveles de contaminación química y microbiológica así como por los valores de factores físicos» según la norma UNE 171330:2008. No obstante, la medición directa e inmediata de la contaminación microbiológica en el aire todavía es un desafío. Existen herramientas indirectas para su cuantificación en tiempo real, lo que resulta crucial para controlar la renovación de aire.

3.2.2. Control de la renovación del aire

La medición directa de aerosoles es extremadamente compleja y costosa, ya que requiere de equipos altamente especializados para su detección. Frente a esto, se ha sugerido la medición del nivel de dióxido de carbono (CO₂) como un indicador indirecto del riesgo de transmisión aérea de enfermedades infecciosas respiratorias. El CO₂ es coexpirado con bioaerosoles que pueden contener SARS-CoV-2 en personas infectadas,^{359,360} por lo que su cuantificación permite estimar los ratios de renovación de aire, así como la potencial carga viral contenida por volumen de aire.^{359,361,362} A propósito de la actual pandemia, la medición de CO₂ ha sido implementada en diversos contextos, como centros comerciales,³⁶³ transporte público,^{364–369} oficinas³⁶⁴ y centros de estudios.^{364–369} En concreto, un estudio reciente realizado en colegios italianos ha reportado una reducción de la transmisión de la Covid-19 en aulas donde se implementaron protocolos basados en la medición de CO₂.³⁷⁰

Aireamos,³⁷¹ como grupo de trabajo científico independiente a nivel internacional, ha sido pionero en desarrollar las bases para la medición, poniendo a disposición de la población un conjunto de valiosas herramientas y recursos para controlar la renovación del aire interior.³⁶¹ Una de las acciones llevadas a cabo por Aireamos fue la evaluación de diferentes tecnologías en medidores de CO₂, encontrando el ajuste idóneo entre disponibilidad, precio y especificidad en los sensores de tipo N-DIR (por sus siglas en inglés *Non-Dispersive Infrared Detector*).

A través del análisis de la medición de CO₂, es posible determinar qué porcentaje del aire ha sido exhalado por otro individuo y según la **Ec.11**.

$$y = C_e x + C_a(1 - x) \quad [11]$$

Donde, C_e corresponde a la concentración de CO₂ en aire exhalado (estimada en 40000 ppm), C_a a la concentración de CO₂ ambiental y x a la fracción de aire exhalado. De manera que, por ejemplo, si en el valor basal es de 440 ppm, y un grupo de personas consiguen aumentarlo hasta 2300 ppm, el porcentaje aproximado de aire que ya ha sido respirado por esos individuos será el ~4.7 %.

El riesgo de transmisión de infecciones por vía aérea en interiores (para una persona en una hora) P fue descrito a partir de una ecuación alternativa a la de Wells-Riley¹³⁹ (**Ec.12**), enunciada por Rudnick *et al.*³⁷² según se describe en la **Ec.13**.

$$P = 1 - e^{-n} \quad [12]$$

$$P = 1 - \exp\left(\frac{Itqf}{n}\right)R \quad [13]$$

Donde, I es el número de personas infectadas en un espacio, t es el tiempo de exposición medido en horas, q es la cantidad de patógenos diseminados por hora, f es la fracción reinspirada $(C - C_0)/C_a$, n el número de personas expuestas al individuo infeccioso y R la eficiencia de retención de partículas, es decir, la fracción de aerosoles que resultan filtrados por el EPI del individuo expuesto. A su vez, C es concentración de CO₂ en el interior, C_0 la exterior y C_a la concentración exhalada durante la respiración, definida en partes por millón (ppm). El valor n corresponde a la cantidad de dosis infecciosa inhalada por una persona susceptible.

No obstante, Rudnick *et al.*³⁷² asumieron algunas condiciones en el modelo:

1. El aire interior resulta completamente mezclado, de manera que el aerosol infeccioso generado puede encontrarse indistintamente en cualquier lugar del espacio.
2. La concentración externa de CO₂ permanece constante durante el transcurso del evento.
3. La eliminación de aerosoles virales como consecuencia de la supervivencia del virus, la filtración u otros mecanismos resulta insignificante comparada con la ventilación.

Otra alternativa a la formulación de Wells-Riley fue aplicada por Peng y Jiménez³⁶⁰ a propósito de la pandemia de Covid-19. Los autores derivaron expresiones analíticas de la probabilidad de infección en interiores a través de la concentración de CO₂. El valor esperado de $\langle n \rangle$ puede ser calculado para una persona no infectada, asumiendo la probabilidad de que el individuo sea inmune η_{in} , según la **Ec.14**.

$$\langle n \rangle = (1 - \eta_{in})c_p B D (1 - m_{in}) \quad [14]$$

Donde, c_p corresponde al promedio de virus (cuantos.m⁻³), B a la frecuencia respiratoria de la persona susceptible (m³.h⁻¹), D la duración del evento (h) y m_{in} la eficiencia de filtración de la mascarilla durante la inhalación. A consecuencia, suponiendo que no preexistencia de aerosoles virales antes del

evento, la expresión analítica para el valor esperado de c_p puede describirse según la **Ec.15**.³⁶⁰

$$c_p = \frac{\eta_{in}(N-1)E_p(1-m_{ex})}{V} \left(\frac{1}{\lambda} - \frac{1-e^{-\lambda D}}{\lambda^2 D} \right) \quad [15]$$

Donde, N es el número de ocupantes, E_p la tasa de exhalación del SARS-CoV-2 por infectado (cuantos.h⁻¹), m_{ex} es la eficiencia de filtración de la mascarilla durante la exhalación, V el volumen de aire del espacio (m³) y λ la constante de tasa global de pérdida de infectividad del virus (h⁻¹), donde se incluyen todos aquellos mecanismos que puedan afectar a la supervivencia del virus (incluyendo la filtración o la ventilación, entre otros).

Asumiendo que el incremento de CO₂ ($n_{\Delta CO_2}$) del aire interior respecto al del aire exterior es únicamente producido por la actividad humana, se puede describir de la siguiente manera (**Ec.16**).³⁶⁰

$$n_{\Delta CO_2} = \Delta c_{p,CO_2} BD \quad [16]$$

Donde, la relación de mezcla del volumen de incremento CO₂ y la tasa de exhalación de CO₂ por persona $\Delta c_{p,CO_2}$ en m³.h⁻¹, puede describirse como (**Ec.17**), donde λ_0 corresponde a la tasa de ventilación (h⁻¹).³⁶⁰

A consecuencia de este modelo, Peng y Jiménez³⁶⁰ proponen un límite asumible de riesgo de $P = 0.01 \%$, aunque no implica seguridad en cualquier situación, ya que con N y/o D elevadas y/o el evento se produce muchas veces, la probabilidad de infección para la persona susceptible puede resultar infraestimada.

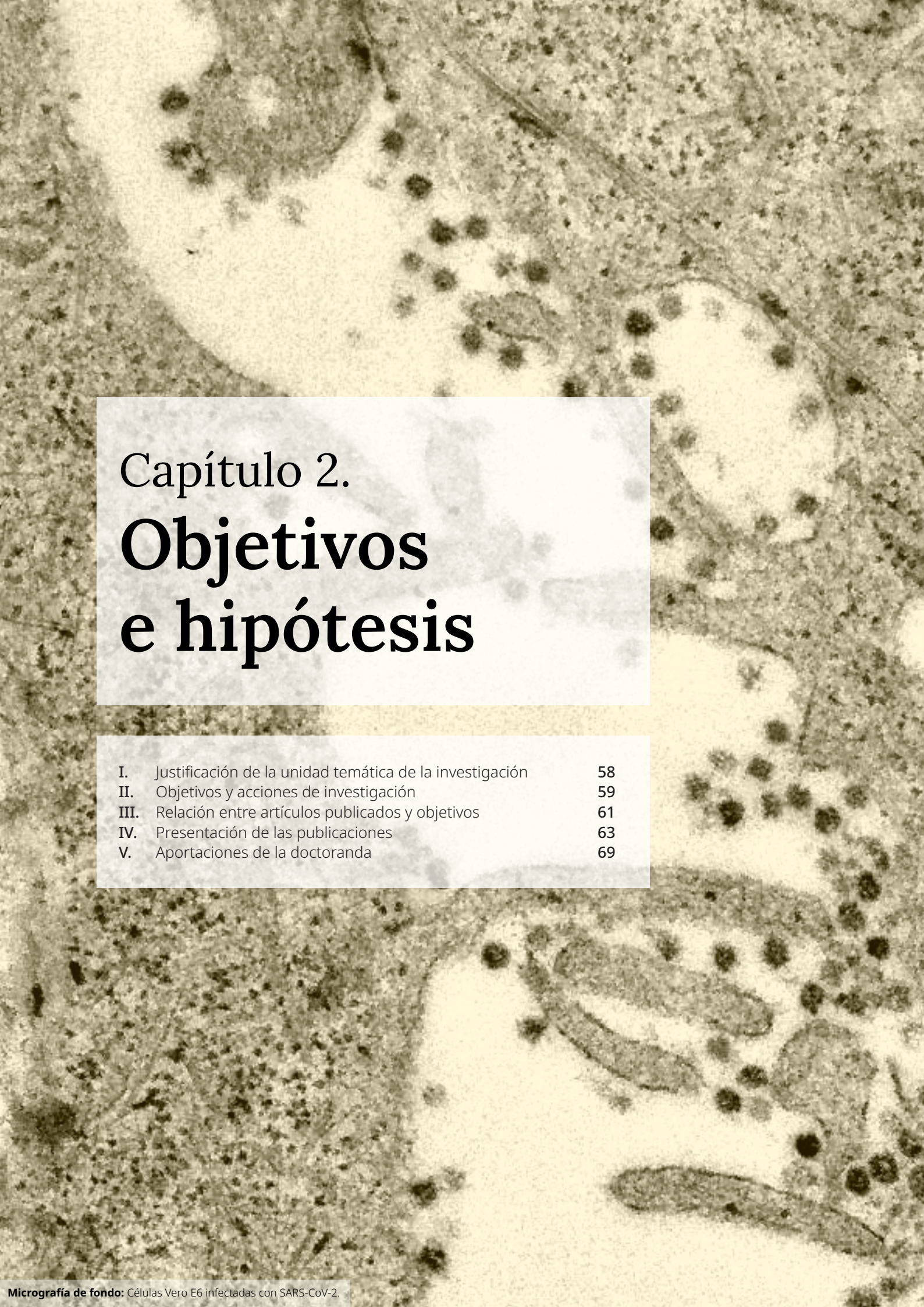
Actualmente, se han propuesto algunos límites de concentración de CO₂ como referencia para la reducción de la transmisión de la Covid-19, que habitualmente se establecen entre 700 y 1000 ppm.^{373,374} No obstante, de carácter previo a la pandemia, existía regulación acerca de la calidad del aire en interiores, que depende de la tipología del edificio. La norma RD 486/1997 aprueba el Reglamento de Lugares de Trabajo, donde se exige una renovación de aire mínima de 30 m³/h en trabajos sedentarios y de 50 m³/h en el resto de casos. Por otro lado, la norma RD 1027/2007 recoge el Reglamento de Instalaciones Térmicas en los Edificios (RITE), que aplica a las instalaciones térmicas de los edificios. Según el RITE, en relación a la categoría de calidad del aire interior (IDA, por sus siglas en inglés *Indoor Air*),³⁷³ en el apartado IT1.1.4.2 y alu-

diendo a la medición de CO₂ como indicador, establece los siguientes límites:

- a) IDA 1** (aire de óptima calidad; hasta 750 ppm): hospitales, clínicas, laboratorios y guarderías. Nota: en situación de pandemia, los mismos autores recomiendan una calidad IDA1 en cualquier tipología de espacio cerrado para prevenir del contagio por Covid-19.
- b) IDA 2** (aire de buena calidad; hasta 900 ppm): oficinas, residencias, salas de lectura, museos, salas de tribunales, aulas de enseñanza y piscinas.
- c) IDA 3** (calidad del aire media; hasta 1200 ppm): edificios comerciales, cines, teatros, salones de actos, habitaciones de hoteles, restaurantes, cafeterías, bares, salas de fiestas, gimnasios, locales de deporte y salas de ordenadores.

Dada la repercusión de la medición de CO₂ en la implementación de medidas eficientes en la renovación del aire, a nivel institucional algunos gobiernos han invertido en este método como recurso para mitigar el riesgo contagio. Ejemplo de ello es el gobierno de Reino Unido, que distribuyó más de 300000 medidores en colegios,³⁷⁵ una inversión de 17 millones de euros realizada por el gobierno alemán³⁷⁶ o de 120 billones de dólares del gobierno estadounidense³⁷⁷ con este mismo objetivo.

Sin embargo, a pesar de los esfuerzos, todavía es necesaria una estrategia global que regule y mejore la calidad del aire de los espacios interiores y compartidos para frenar la expansión de esta y otras enfermedades infecciosas de transmisión aérea, cuestión por la cual surgieron las **publicaciones quinta y sexta de esta Tesis**.

The background of the page is a high-magnification electron micrograph showing SARS-CoV-2 virus particles within Vero E6 cells. The virus particles appear as small, dark, spherical structures with a distinct outer envelope and internal structure. They are distributed throughout the cytoplasm of the cells, which show various organelles like mitochondria and endoplasmic reticulum. The overall image has a grainy, high-contrast appearance typical of electron microscopy.

Capítulo 2.

Objetivos e hipótesis

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I. Justificación de la unidad temática de investigación

La pandemia por Covid-19 ha supuesto un reto a todos los niveles, aunque resultó notorio en el ámbito científico y sanitario. En consecuencia, numerosos investigadores y líneas de investigación se reorientaron para dar respuesta a las necesidades de la crisis sanitaria. Con este mismo objetivo, la presente Tesis Doctoral surge como un compendio de diferentes trabajos realizados con un abordaje notablemente multidisciplinar. Es preciso comprender, en este momento, que las actuaciones y propuestas recogidas en esta Tesis fueron ideadas, desarrolladas o implementadas en diferentes periodos de la pandemia, dando respuesta a los acuciantes requerimientos sanitarios en cada momento.

Los resultados de esta Tesis Doctoral se unen a otros esfuerzos invertidos orientados a la comprensión de este virus de reciente descubrimiento (SARS-CoV-2): desde la patogenia de la enfermedad hasta los mecanismos de transmisión entre humanos, pasando por métodos para la reducción de la transmisión basados en nanotecnología o en el control de la calidad y renovación del aire. Poniendo como epicentro la lucha contra el SARS-CoV-2, se presentan seis trabajos desarrollados desde diferentes áreas de la investigación bajo el abanico general de los mecanismos de transmisión del SARS-CoV-2, recogidos en:

Primera temática

Mecanismos de transmisión célula-célula del SARS-CoV-2

Segunda temática

Mecanismos de transmisión humano-humano del SARS-CoV-2

Tercera temática

Medidas para la reducción de la transmisión del SARS-CoV-2 por aerosoles, haciendo distinción entre las medidas individuales y las colectivas

II. Objetivos y acciones de investigación

A continuación, se exponen los objetivos principales y específicos, vinculados a acciones de investigación.

1^{er} objetivo general: Estudiar la transmisión célula-célula del SARS-CoV-2

Objetivos específicos

- 1a)** Profundizar en el estudio ultraestructural de la morfogénesis celular inducida por la infección por SARS-CoV-2 en cultivo celular.
- 1b)** Estudiar los mecanismos de defensa frente a la infección viral.
- 1c)** Analizar los mecanismos de propagación viral intercelular.

Acciones de investigación

- 1a)** Se realizó un detallado estudio ultraestructural utilizando microscopía electrónica de transmisión en muestras de cultivo de células Vero E6 a las 48 horas tras la infección.
- 1b)** Se reconstruyeron las estructuras subcelulares de interés en tres dimensiones utilizando cortes seriados ultrafinos para mejorar la interpretación de los mecanismos celulares.

2^{do} objetivo general: Analizar la transmisión del SARS-CoV-2 por microgotas y por aerosoles

Objetivos específicos

- 2a)** Constatar la presencia de RNA viral en el entorno aéreo de pacientes Covid-19.
- 2b)** Analizar cuantitativamente el riesgo de contagio tras la exposición a corta y media-larga distancia a pacientes Covid-19.
- 2c)** Evaluar la utilidad del valor Ct en RT-qPCR como indicador de infecciosidad.
- 2d)** Investigar los fenómenos de superpropagación individuales y su papel en la dinámica de la propagación viral.

Acciones de investigación

- 2a)** Se realizaron muestreos ambientales, utilizando ciclones, y de tos en una muestra de 22 pacientes positivos en Covid-19. En las muestras se analizaron las propiedades virales, como la carga viral obtenida a través de la técnica RT-qPCR y la variante por secuenciación genética.
- 2b)** Se optimizó el método de recogida de muestras ambientales, variando parámetros como el medio de transporte viral, el volumen de medio, la técnica de almacenamiento o las condiciones del muestreo.

3er objetivo general: Desarrollar, implementar y validar estrategias preventivas efectivas frente a la transmisión del SARS-CoV-2

Objetivos específicos

3a) Desarrollar y caracterizar herramientas basadas en nanotecnología para optimizar la efectividad de las mascarillas y materiales filtrantes frente a SARS-CoV-2.

3b) Diseñar materiales filtrantes que reduzcan el impacto ambiental que trae consigo el uso de equipos de protección no reutilizables.

3c) Diseñar metodologías preventivas que reduzcan el riesgo de contagio por aerosoles en espacios interiores basadas en la renovación y en la purificación del aire.

3d) Implementar y validar el método preventivo desarrollado en entornos reales.

3e) Validar el rendimiento de sistemas de purificación aérea comerciales frente a microorganismos ambientales.

Acciones de investigación

3a) Se desarrolló y optimizó un nuevo método basado en AgNPs y PEI funcionalizar sustratos poliméricos con un recubrimiento antiviral efectivo contra SARS-CoV-2.

3b) Se desarrolló un nuevo material filtrante basado en fibras electrohiladas de PET obtenido de botellas de plástico y otros materiales desechados para reducir el impacto ambiental de las mascarillas de un solo uso.

3d) Se diseñó una nueva metodología para el control de la renovación de aire interior para reducir el riesgo de contagio de enfermedades infecciosas y fue validada en medios de transporte público, colegios, centros culturales y 40 establecimientos comerciales de Zaragoza.

3e) Se evaluó un sistema de purificación de aire basado en ionización bipolar para la desinfección aérea ambiental en el tranvía de Zaragoza.

III.

Relación entre artículos publicados y objetivos

La presente Tesis se ha realizado como un compendio de publicaciones. En concreto, consta de seis trabajos científicos (y uno más en *preprint*) que han abordado investigaciones relacionadas con los mecanismos de transmisión del virus SARS-CoV-2. Las referencias completas de los artículos de investigación se enumeran a continuación.

La relación entre los objetivos de investigación y los artículos se presenta en la **Tabla 5**.

*Los resultados contenidos en el primer artículo fueron presentados en el IX Congreso Internacional de la Sociedad Española de Histopatología e Ingeniería Tisular (SEHIT) en septiembre de 2022, obteniendo el **Premio al Mejor Póster Científico** en la categoría de Histopatología, tal y como se recoge en el Apéndice 3.

ARTÍCULO 1*

Baselga, M.; Uranga-Murillo, I.; Moreo, E.; Arias, M.; Junquera, C. Ultrastructural characterization of the cellular cytoskeleton morphogenesis after SARS-CoV-2 infection. **Histochemistry and Cell Biology**. 2022. <https://doi.org/10.1007/s00418-022-02152-7>

ARTÍCULO 2

Baselga, M.; Güemes, A.; Alba, J.J.; Schuhmacher, A.J. 2022. SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity. **Journal of Clinical Medicine**. 2022. 11 (9), 2607. <https://doi.org/10.3390/jcm11092607>

ARTÍCULO 3

Baselga, M.; Uranga, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates. **Materials**. 2022. 15 (14), 4742; <https://doi.org/10.3390/ma15144742>

ARTÍCULO 4

Baselga, M.; Yus, C.; Arruebo, M.; Sebastián, V.; Irusta, S.; Jiménez, S. 2022. Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks. **Nanomaterials**. 2022. 12 (6), 925. <https://doi.org/10.3390/nano12060925>

ARTÍCULO 5 (Preprint)

Baselga, M.; Alba, J.J.; Schuhmacher, A.J. Development and validation of a methodology to measure exhaled carbon dioxide (CO₂) and control indoor air renewal. **medRxiv**. 2022. <https://doi.org/10.1101/2022.09.22.22280262>

ARTÍCULO 6

Baselga, M.; Alba, J.J.; Schuhmacher, A.J. The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases. **International Journal of Environmental Research and Public Health**. 2022. 19 (11), 6605. <https://doi.org/10.3390/ijerph19116605>

ARTÍCULO 7

Baselga, M.; Alba, J.J.; Schuhmacher, A.J. Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport. **Science of Total Environment**. 2023. 855, 158965. doi: 10.1016/j.scitotenv.2022.158965.

Tabla 5. Relación entre los objetivos específicos y los artículos de investigación.

	Artículo 1	Artículo 2	Artículo 3	Artículo 4	Artículo 5	Artículo 6	Artículo 7
1. Estudiar la transmisión célula-célula del SARS-CoV-2							
1a) Profundizar en el estudio ultraestructural de la morfogénesis celular inducida por la infección por SARS-CoV-2 en cultivo celular.	●						
1b) Estudiar los mecanismos de defensa frente a la infección viral.	●						
1c) Analizar los mecanismos de propagación viral intercelular, con especial atención al papel del citoesqueleto.	●						
2. Analizar la transmisión del SARS-CoV-2 por microgotas y por aerosoles							
2a) Constatar la presencia de RNA viral en el entorno aéreo de pacientes Covid-19.							
2b) Analizar cuantitativamente el riesgo de contagio tras la exposición a corta y media-larga distancia a pacientes Covid-19.		●					
2c) Evaluar la utilidad del valor Ct en RT-qPCR como indicador de infecciosidad.		●					
2d) Investigar los fenómenos de superpropagación individuales y su papel en la dinámica de la propagación viral.		●					
3. Desarrollar, implementar y validar estrategias preventivas efectivas frente a la transmisión del SARS-CoV-2							
3a) Desarrollar y caracterizar herramientas basadas en nanotecnología para optimizar la efectividad de las mascarillas y materiales filtrantes frente a SARS-CoV-2.			●	●			
3b) Diseñar materiales filtrantes que reduzcan el impacto ambiental que trae consigo el uso de equipos de protección no reutilizables.				●			
3c) Diseñar metodologías preventivas que reduzcan el riesgo de contagio por aerosoles en espacios interiores basadas en la renovación y en la purificación del aire.					●	●	
3d) Implementar y validar el método preventivo desarrollado en entornos reales.					●	●	
3e) Validar el rendimiento de sistemas de purificación aérea comerciales frente a microorganismos ambientales.							●

IV.

Presentación de las publicaciones originales

De acuerdo con los objetivos establecidos en esta Tesis Doctoral, a continuación se describen de manera resumida los hallazgos y los resultados principales de las investigaciones realizadas.

En relación a la primera temática, titulada «Mecanismos de transmisión célula-célula del SARS-CoV-2»

En el primer artículo, titulado **«Ultrastructural characterization of the cellular cytoskeleton morphogenesis after SARS-CoV-2 infection»** (*Histochem Cell Biol*, 2022), se proporciona una caracterización ultraestructural detallada de las principales vías de propagación viral intercelular. En concreto, se describe la formación de puentes filopodiales y nanotubos de efecto túnel como consecuencia de la alteración del citoesqueleto durante el ciclo de infección viral.

Antecedentes: en el momento de la publicación de este artículo, fuimos pioneras en evidenciar estas vías de propagación intercelular en células infectadas por SARS-CoV-2 por TEM. Si bien numerosos artículos se centraron en definir la morfogénesis celular inducida por la infección del virus de la Covid-19, el citoesqueleto y sus estructuras asociadas fueron escasamente descritas, a pesar de su relevancia en la infección celular.

Materiales y métodos: Utilizamos un microscopio electrónico de transmisión JEOL 1010 (Deben UK Ltd; Edmunds, Reino Unido) para analizar un cultivo celular Vero E6 tras 48 horas de la infección con 500 TCID₅₀/ml de la cepa original del SARS-CoV-2 aislado en el Hospital Clínico Universitario Lozano Blesa (Zaragoza, España). Las micrografías fueron tomadas con el software Radius 2.1 con una cámara Gatan Bioscan (Gatan Inc; Pleasanton, EEUU). Las reconstrucciones tridimensionales a partir de cortes seriados ultrafinos se realizaron utilizando el software libre FIJI ImageJ.

Resultados: describimos una intensa remodelación del citoesqueleto caracterizada por la agregación de actina cortical en la región perimembranosa, una estructura de microtúbulos bien definida y la formación de proyecciones de membrana, en forma de filopodios y TNT. Estas estructuras representaron un papel crucial en el ciclo infectivo, ya

que permitieron el transporte de virus replicados desde una célula huésped a una célula vecina a través de dos mecanismos no excluyentes: el *surfing* viral (más frecuente), donde los virus navegan por la superficie de los filopodios mediados por el receptor ACE2, y la ruta intracelular (menos frecuente), donde los virus internalizados en vesículas se transportan por el citoplasma hasta la célula vecina. A través de TEM pudimos evidenciar estos fenómenos de transporte viral, que podrían representar una diana clave en futuras estrategias terapéuticas. Además, proporcionamos las primeras reconstrucciones tridimensionales de estas estructuras utilizando cortes seriados ultrafinos.

Los resultados preliminares de esta investigación fueron presentados en el IX Congreso Internacional de la Sociedad Española de Histología e Ingeniería Tisular, obteniendo el Premio al Mejor Póster en la categoría de Histopatología (Apéndice 3).

En relación a la segunda temática, titulada «Mecanismos de transmisión humano-humano del SARS-CoV-2»

En el segundo artículo, titulado **«SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity»** (*J Clin Med*, 2022), investigamos la transmisión humano-humano del virus SARS-CoV-2 que, tras más de dos años de pandemia, todavía no se ha comprendido completamente.

Antecedentes: la propagación global del virus ha representado un escenario único el avance del conocimiento de enfermedades infecciosas. Las teorías epidemiológicas mecanicistas fueron descartadas rápidamente, como ocurrió con otros coronavirus, y se prestó más atención a otros enfoques que consideraban la heterogeneidad en la propagación. Una de las principales contribuciones a nivel global fue la aceptación general del modelo de transmisión aérea de patógenos que, para el SARS-CoV-2, ha representado una de las vías principales. No obstante, a pesar de los avances, todavía persiste incertidumbre sobre la probabilidad de contagio que representa y su predominancia sobre otros modelos de transmisión.

Materiales y métodos: Con el objetivo de aportar luz a este respecto, analizamos la presencia y

la cantidad de RNA viral en muestras de tos (equivalente al modelo de microgotas) y aire (equivalente al modelo *airborne*) de 22 pacientes infectados con características homogéneas, obteniendo un total de 117 muestras tipificadas. El RNA viral de las muestras fue analizado utilizando RT-qPCR (utilizando las dianas *pan-SARS ESAR* e *IP4*) y secuenciación genética (utilizando los primers F21585 y R22341). Las muestras de aire fueron obtenidas utilizando un Coriolis μ (Bertin Instruments; Rockville, MD, USA) típicamente a 300 l/min en 3 ml de solución salina tamponada con fosfato (PBS, por sus siglas en inglés *Phosphate-Buffered Saline*) durante 10 minutos, mientras que las muestras de tos se recolectaron en un tubo de orina con 1 ml de PBS.

Resultados: encontramos que solo 2/19 muestras de tos presentaron RNA detectable en un subgrupo de pacientes con un Ct promedio de 25.7 ± 7.0 , alcanzando cargas virales de 7.3×10^5 y 8.37×10^8 copias/ml. La significativa diferencia entre la carga viral de ambas muestras puede ser también atribuida a la técnica de recolección, dada la dificultad de estandarizar la tos de un paciente. Sin embargo, este trabajo tuvo por objetivo analizar la transmisión en entornos reales, donde es preciso considerar la variabilidad entre individuos. De los pacientes que fueron sometidos a muestreo aéreo, únicamente detectamos RNA viral en 1/8 de los individuos, en un subgrupo de pacientes con un Ct promedio de 25.0 ± 4.0 . Se encontró una carga viral de entre 1.1 y 4.8 copias/ m^3 , lo cual se ajustó a las cargas virales informadas en estudios anteriores. Un subgrupo de pacientes fue puesto en seguimiento, y no se encontró carga viral ni en tos ni en aire tras el tercer día de síntomas, lo que podría ayudar a ajustar los periodos de aislamiento de los pacientes. Todos los pacientes incluidos en el estudio fueron diagnosticados con Omicron BA.1. Como última conclusión, encontramos que el Ct del paciente no debe ser considerado un indicador de infecciosidad, ya que no pudo ser correlacionado con la carga viral diseminada por los pacientes. Incluso, las muestras ambientales de algunos pacientes con alta carga viral en exudados faríngeos resultaron negativas en RNA viral. Se encontraron discrepancias significativas entre las cargas virales expelidas por diferentes pacientes (en algunos incluso nulas), en línea con las hipótesis de superpropagación atribuidas a la pandemia, donde se atribuye una emisión sobredimensionada a un pequeño porcentaje de personas infectadas.

En relación a la tercera temática, titulada «Medidas para la reducción de la transmisión del SARS-CoV-2 por aerosoles»

El trabajo contenido en el tercer artículo, titulado «**Silver Nanoparticles-Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates**» (*Materials*, 2022), se desarrolló durante los primeros meses de 2021.

Antecedentes: en esta etapa pandémica, el uso de mascarillas como protección individual y de equipos de filtración como protección colectiva permitió controlar gran parte de los contagios entre la población. A pesar de esto, frente a materia submicrónica, como los virus, algunos materiales de filtración y mascarillas presentan una efectividad limitada. Numerosos esfuerzos ejecutados durante la pandemia fueron dedicados a funcionalizar los materiales con nanotecnología para mejorar su rendimiento frente al virus. No obstante, la mayoría de ellos únicamente constituyeron un proyecto investigador pero no alcanzaron una puesta al mercado por su compleja escalabilidad e industrialización. Además, por la rápida propagación del virus a nivel mundial, la dificultad de ensayar los nuevos materiales frente al SARS-CoV-2 limitó su validación como mecanismo antiviral. En este trabajo, planteamos un método de incorporación estable, homogénea, duradera, sencilla y a bajo costo de nanopartículas de plata a sustratos polímeros fibrosos, propios de los materiales filtrantes.

Materiales y métodos: Inicialmente, se propusieron tres estrategias diferenciadas: un método de unión basado en la modificación mediante química tiol, uno por enlace covalente mediado por lisozimas y otro basado en polietilenimina (PEI). En base a los resultados experimentales de control de desprendimiento y homogeneidad del recubrimiento de nanoplatea, se continuó desarrollando esta última alternativa.

Resultados: como conclusión, se obtuvo un nuevo método de bajo costo basado en fuerzas electrostáticas y formación de complejos de coordinación para generar recubrimientos antivirales basados en nanopartículas de plata y polietilenimina. En una primera fase de desarrollo se optimizó la síntesis de nanopartículas de plata variando la concentración molar de los solutos, la proporción de reactivo, la velocidad de agitación y el modo de adición de reactivo. Como resultado, se logró reducir su tamaño hasta un diámetro de 6.2 ± 2.6 nm

para facilitar su oxidación y potenciar su actividad antiviral y mejorar su estabilidad en el tiempo, que fue caracterizada utilizando espectrometría ultravioleta-visible (UV-Vis) diariamente entre el día 0 y el 7 tras la síntesis utilizando el espectrofotómetro Jasco V670 (JASCO International Co Ltd; Tokyo, Japón). La caracterización morfológica de las nanopartículas de plata (AgNPs) se realizó utilizando un microscopio de transmisión electrónica T20-FEI (FEI Company; Hillsboro, EEUU) y el software libre ImageJ. Brevemente, el nitrato de plata (AgNO_3) a una concentración de 1.0 mM y el citrato de sodio tribásico hidratado (TSC) a una concentración de 1.0 mM se disolvieron en una solución acuosa y se mantuvieron bajo agitación (~1250 rpm) durante 5 minutos. Posteriormente, se añadió gota a gota una disolución de borohidruro de sodio (NaBH_4) a concentración 1.2 mM y la mezcla se mantuvo en agitación durante 1 hora. La dispersión coloidal de las AgNP cargadas negativamente se lavó por centrifugación a 13000 rpm durante 15 minutos ya 15 °C. Se eliminó el sobrenadante y el sedimento se redispersó en agua Milli-Q hasta que se logró la concentración coloidal deseada. Tras optimizar la síntesis, se procedió a optimizar el método de recubrimiento. Brevemente, 10 mg/ml de PEI se disolvieron en una solución acuosa. Las muestras de tejido se sumergieron en la solución anterior durante 30 minutos, se secaron al aire libre durante 10 minutos y posteriormente se lavaron con agua desionizada para eliminar el exceso de PEI. Después del lavado, las muestras se suspendieron en una dispersión coloidal de AgNPs a 5 mg/ml en una cantidad no inferior a 0.7 ml/cm² durante 1 hora a temperatura ambiente. Finalmente, las muestras se recuperaron, se secaron a temperatura ambiente durante 10 minutos y se lavaron minuciosamente con agua desionizada para desprender las posibles nanopartículas no adheridas. La carga de plata obtenida fue de 0.5 mg/cm². La homogeneidad del recubrimiento fue caracterizada utilizando SEM y espectroscopia de rayos X de energía dispersiva (EDX, por sus siglas en inglés *Energy Dispersive X-ray Spectroscopy*) con el microscopio CSEM-FEG Inspect 50 (FEI Company, Hillsboro, EEUU), obteniendo los mejores resultados bajo el método de unión descrito. El desprendimiento se determinó utilizando MP-AES (Espectrómetro de emisión atómica de plasma por microondas, por sus siglas en inglés *Microwave Plasma - Atomic Emission Spectrometry*) modelo 4100 (Agilent Technologies; Santa Clara, EEUU) y una balanza de precisión (WLC X2 Radwag; Radom, Poland). Se encontró que durante sonicaciones en ultrasonidos en soluciones de pH alto (~4.5) se perdía alrededor de

un 70 %p/v de plata mientras que, en el mejor de los casos, se perdió un 8 %p/v en un medio salino fosfatado. No obstante, las condiciones utilizadas para cuantificar el desprendimiento resultaron significativamente más agresivas que las condiciones reales de funcionamiento. Realizando únicamente lavados manuales en agua Milli-Q se observaron pérdidas inferiores al ~1 % en todos los materiales ensayados, por lo que se consideró que las AgNPs se incorporaron de manera estable en el contexto en el que iban a ser sometidas en condiciones reales de funcionamiento. Frente a infecciones con 10⁵ Unidades Formadoras de Placas (PFU, por sus siglas en inglés *Plaque-Forming Units*) de SARS-CoV-2 (40 µl), este recubrimiento demostró un alto rendimiento, reduciendo la carga viral en un 98.0 y un 99.9 % a los 10 minutos y a las 2 horas de la infección en cultivos con la línea celular Vero E6.

El cuarto artículo, titulado «**Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks**» (*Nanomaterials*, 2022) fue concebido en un contexto donde el uso de mascarillas de un solo uso supuso un notable impacto ambiental en el contexto de la pandemia por Covid-19.

Antecedentes: La escasa tasa de reciclabilidad de los materiales utilizados para la fabricación de mascarillas unido a la atribución de riesgo biológico supuso una gestión incierta de los residuos secundarios a los equipos de protección. Frente a esta problemática, se planteó el desarrollo de materiales filtrantes a partir de materiales poliméricos desechados.

Materiales y métodos: se utilizaron botellas de PET desechadas para la producción de nano y microfibras utilizando un electrospinner Yflow 2.2 D-500 (Yflow SD; Málaga, España). Los parámetros utilizados durante el electrohilado fueron variados para optimizar el diámetro de las fibras. En concreto, se optimizó la concentración de polímero, la proporción de disolventes DCM (diclorometano): TFA (ácido trifluoroacético), la altura entre aguja y colector, el flujo de salida de la disolución, la diferencia de voltaje para estabilizar el cono de Taylor, la distancia de la aguja al colector y la velocidad de movimiento de la aguja. Se estudió la influencia de estos parámetros del electrohilado en la filtración de los materiales obtenidos, además de la relación entre diámetros de fibra y sus combinaciones y su repercusión en la filtración. Adicionalmente, se optimizó el método para potenciar la homogeneidad a lo largo de todo el tejido electrohilado y se estudió tanto su reproducibilidad bajo diferentes condiciones atmosféricas como su perdurabilidad.

La caracterización morfológica se llevó a cabo utilizando un microscopio electrónico de barrido ambiental (ESEM, por sus siglas en inglés *Environmental Scanning Electron Microscopy*) Quanta-FEG 250 (FEI Company; Hillsboro, EE.UU.). Los ensayos de eficiencia de retención fueron realizados utilizando un generador de aerosoles Topas-ATM226 con una solución salina al 3 %, un Medidor de Ramaño de Partículas por Movilidad (SMPS, por sus siglas en inglés *Scanning Mobility Particle Sizer*) TSI 3936 compuesto por un Analizador de Movilidad Diferencial (DMA, por sus siglas en inglés *Differential Mobility Analyzer*) TSI 3081 y un Contador de Partículas por Condensación (CPC, por sus siglas en inglés *Condensation Particle Counter*) TSI 3782 (para la medición frente a partículas de entre 0.01 y 1.0 μm , con una distribución aerodinámica de tamaños similar a la recogida en la norma UNE EN 13274-7) y por un Medidor de Partículas Óptico (OPS, por sus siglas en inglés *Optical Particle Sizer*) TSI 3330 (para partículas entre 0.5 y 10 μm). En ambos casos se utilizó un flujo de salida de 0.6 l/min y el filtro fue colocado y sellado entre dos discos de bronce con teflón, dejando un diámetro de exposición variable de 4 a 13.3 mm para ajustar al caudal deseado. La pérdida de carga fue medida con una columna de alcohol convencional situada aguas arriba y aguas abajo del filtro dentro del esquema de ensayo.

Resultados: se proporcionó el conocimiento necesario para el desarrollo de materiales filtrantes de eficiencia variable, alcanzando un rendimiento del 99.9 %. Sin embargo, dada la facilidad de fabricación de mascarillas quirúrgicas frente a EPR de tipo FFP2 o FFP3, se decidió desarrollar un método enfocado a la obtención de este tipo de máscaras. Brevemente, se propuso la fabricación de mascarillas quirúrgicas basadas en tres capas: dos de protección y una de filtración colocada entre las capas anteriores. La capa de filtración fue electrohilada bajo las siguientes condiciones: una concentración de 25 %p/v de PET, una diferencia de voltaje de ~ 15 kV, una distancia aguja-colector de 25 cm y una velocidad de flujo de 9.9 ml/h. Para las capas exteriores (de protección) variamos el voltaje hasta ~ 25 kV y la distancia hasta 15 cm para obtener fibras más gruesas que no interfirieran en la pérdida de carga global de la máscara. En el material filtrante se desarrollaron fibras con un diámetro promedio de 1.2 ± 0.7 μm , mientras que en las capas de protección el diámetro aumentó hasta 3.2 ± 2.6 μm . Para una densidad de filtro de ~ 20 mg/cm² (espesor ~ 500 μm), se obtuvo una eficiencia de retención del 98.2 % para partículas de entre 0.5 y 10 μm y del 100 % para partículas superiores a 3 μm , con una pérdida de carga de 0.36 mbar. Adicionalmente, el material electrohilado presentó una hidrofobicidad similar a los materiales comerciales. Y, como valor diferencial, nuestro material demostró la capacidad de ser reciclable bajo la

misma técnica de fabricación inicial, lo que constituiría una estrategia de economía circular que reduciría notablemente el impacto ambiental fruto de esta pandemia.

El trabajo contenido en el quinto artículo (publicado como *preprint* y en estado de revisión), titulado «**Development and validation of a methodology to measure exhaled carbon dioxide (CO₂) and control indoor air renewal**» (*medRxiv*, 2022) surgió como respuesta a la necesidad de establecer estrategias efectivas para el manejo epidemiológico de esta y futuras pandemias.

Antecedentes: Frente a la acuciante necesidad de estimar la carga de aerosoles potencialmente patógenos en entornos cerrados, la interpretación de las mediciones de CO₂ se estableció como un indicador de la ventilación efectivo y eficiente. Por su accesibilidad este método fue rápidamente implementado en numerosos centros e instituciones. Sin embargo, en numerosas ocasiones, las malas prácticas limitan los beneficios de la técnica y no aplican metodologías que garanticen la renovación del aire.

Materiales y métodos: se seleccionaron 40 comercios locales del centro de Zaragoza (España) de diferente tipología y actividad para el estudio, incluyendo restaurantes, floristerías, tiendas de ropa (infantiles y adultas), zapaterías (infantiles y adultas), tiendas de alimentación, ópticas y centros sanitarios, consultorías y tiendas del hogar. Se utilizaron 10 medidores Aranet4 Pro (Aranet; Riga, Latvia) para el estudio de caso en el Centro Ibercaja Actur (Zaragoza, España) y 85 medidores SiO₂ (Signos.io; Zaragoza, España) con tecnologías de Internet de las Cosas (IoT, por sus siglas en inglés *Internet Of Things*) en el estudio de 40 comercios, que monitorizaron el aire de los espacios durante cuatro meses.

Resultados: en este trabajo se propuso una nueva metodología para medir y controlar los niveles de CO₂ interiores en espacios compartidos. En concreto, se formuló una metodología basada en tres etapas: diagnóstico, corrección y monitorización/control/vigilancia (MCS, por sus siglas en inglés *Monitoring, Control, and Surveillance*). En resumen, la primera fase de diagnóstico consistiría en analizar cómo varían los niveles de CO₂ en el espacio dependiendo de las actividades que se desarrollan y tipología del espacio. En una segunda fase de corrección se procedería a diseñar protocolos personalizados para mejorar la renovación del aire según las conclusiones del diagnóstico. Finalmente, en la fase MCS se realizaría una monitorización continua del nivel de CO₂ para garantizar que el espacio se mantenga en niveles de bajo riesgo, según dicte la comunidad científica para cada circunstancia. Si los

niveles de CO₂ sobrepasaran el límite establecido, se alertaría a los responsables del espacio para activar los protocolos definidos en la fase anterior. En el artículo se describe la implementación de cada una de estas fases en un edificio de referencia (que corresponde al Centro Ibercaja Actur, Zaragoza). En este espacio, se realizaron numerosos experimentos para poner a punto la metodología, como la saturación de CO₂ con hielo seco de espacios controlados para analizar el rendimiento de diversas estrategias de ventilación. Una vez descrito el método, fue exitosamente implementado en otros 40 comercios voluntarios durante cuatro meses, demostrando la efectividad de la metodología, que resultó beneficiosa para la renovación del aire en todos los comercios que lo implementaron.

El sexto artículo, titulado **«The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases»** (*IJERPH*, 2022) tuvo por objetivo estimar el riesgo de contagio en el modelo de tranvía Urbos 3 de Zaragoza (CAF, Beasain ES).

Antecedentes: El transporte público fue considerado como uno de los espacios de mayor riesgo de transmisión de la Covid-19, solo por detrás del contexto intrahospitalario. En este trabajo publicamos el primer artículo donde se midió y evaluó CO₂ en un tranvía en funcionamiento.

Materiales y métodos: En concreto, se instalaron 8 medidores Aranet4 Pro (Aranet; Riga, Latvia) a lo largo del tranvía y para analizar la concentración de CO₂ en diferentes eventos (por ejemplo, fin de semana versus entre semana, con y sin recirculación de aire o con diferentes condiciones climáticas) repartidos en 44 trayectos completos. Otro medidor fue ubicado en el exterior de la cubierta superior del tranvía para el registro de valores de referencia. Para valorar el rendimiento del filtro para la retención de partículas submicrónicas en el aire, se utilizó el mismo método descrito en el cuarto artículo. El riesgo de contagio fue determinado utilizando una herramienta de acceso libre desarrollada por Aireamos.

Resultados: en total, se analizaron 79200 registros. Se propuso el indicador ppm/persona para comparar la renovación del aire independientemente del evento, lo que podría resultar muy útil y complementario a la metodología propuesta en el trabajo anterior. Como resultado, se obtuvo un promedio de 835 ±32 ppm y un máximo de 1249 ppm, lo que sugirió una recirculación del aire adecuada a la actividad y ocupación de los coches. Los dos sistemas de Calefacción, Ventilación y Aire Condicionado (HVAC, por sus siglas en inglés *Heating, Ventilation and Air Conditioning*) instalados

en el tranvía proporcionan alrededor de 25 renovaciones de aire por hora (ACH, por sus siglas en inglés *Air Changes per Hour*) y cuentan con equipos de filtración, cuyos filtros (Coarse 75 % de acuerdo con la norma UNE EN ISO 16890, Merak Long Life Filter; Madrid, España) fueron instalados a propósito de esta pandemia. Sin embargo, se observó que la eficiencia de retención de estos filtros frente a partículas submicrónicas (0.1-1 µm) fue reducida, alcanzando el 27.9 % frente a partículas de 300 nm a un flujo de ~2500 m³/h y de 2.4 y 2.3 % frente a flujos de ~162 y ~622 m³/h. Sin embargo, los estudios de filtración pudieron no ser totalmente representativos dadas las condiciones del laboratorio, donde los filtros eran necesariamente apelmazados para su ensayo. Sin tomar en cuenta el porcentaje de aerosoles eliminado por mecanismos de filtrado, considerando una incidencia acumulada a 14 días del momento del ensayo (aproximada a 1150 casos por cada 100000 habitantes), se estimó una tasa de ataque del 0.04 % (para la variante Ómicron, con un nivel de CO₂ promedio de 689 ppm y máximo de 1038 ppm, un tiempo de exposición de 10 minutos, superior al promedio de estancia en el tranvía (estimado en 7 minutos), una ocupación de 60 pasajeros con una inmunidad del 70 % y el uso de mascarilla quirúrgica) que, en el caso más desfavorable (Ómicron, CO₂ promedio de 810 ppm y máximo de 1520 ppm, 10 minutos, 60 pasajeros con una inmunidad del 70 % y el uso de mascarilla quirúrgica) alcanzó el 0.06 %. De manera que, en las condiciones estudiadas, el tranvía de Zaragoza no pareció representar un espacio de alto riesgo de infección por aerosoles de la enfermedad Covid-19.

En línea con lo anterior, en el séptimo artículo, titulado **'Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport'** (*Sci Tot Env*, 2023), se evaluó el rendimiento de eliminación de microorganismos aéreos de un equipo de desinfección ambiental basado en ionización bipolar por punta de aguja PA604 (Tayra SA; Alicante, Spain) en el tranvía de Zaragoza.

Antecedentes: la tecnología basada en plasma no térmico se posicionó como una de las principales estrategias contra la presencia de virus SARS-CoV-2 en el aire. La inactivación plasmática de microorganismos se atribuye a la ruptura de la pared celular y al daño del material genético a partir de diferentes mecanismos, entre otros, la presencia de partículas cargadas, iones o ROS y radicales que contienen oxígeno, actuando de forma exclusiva o combinada. A pesar de representar una tecnología ampliamente utilizada para la esterilización de material quirúrgico, su efectividad para la inactivación de patógenos ambientales resulta incierta. En

concreto, a propósito de esta pandemia, un único trabajo ha evaluado su rendimiento en el espacio interior de un avión *Boeing*, reportando una eficiencia limitada e inferior a la descrita por los fabricantes. En base a lo anterior, este trabajo tuvo por objetivo la determinación de la eficiencia de los sistemas de ionización frente a microorganismos ambientales en el interior del tranvía.

Materiales y métodos: se utilizó un muestreador ambiental Coriolis μ (Bertin Instruments; Rockville, MD, USA) para recoger por triplicado 6000 l de aire a un flujo de 300 l/min en un cono con 5 ml de PBS. En Placas de Recuento de Agar (PCA, por sus siglas en inglés *Plate Count Agar*) se sembraron 5 réplicas de cada muestreo con el equivalente a 450 l de aire y se incubaron durante 72 horas a 30 °C. Se evaluaron cuatro escenarios diferentes: el aire con filtración mecánica del sistema HVAC, el aire con la ionización bipolar activada, el aire con filtración mecánica e ionización bipolar y el aire de control sin filtración ni ionización. Los resultados fueron cuantificados calculando la eficiencia tras recuentos manuales y utilizando el parámetro de la tasa de suministro de aire limpio (CADR, por sus siglas en inglés *Clean Air Delivery Rate*). Las tasas de pérdida de primer orden se caracterizaron utilizando una regresión lineal simple contra los valores de concentración media, tal como se define en la metodología propuesta por la Sociedad Estadounidense de Ingenieros de Calefacción, Refrigeración y Aire Acondicionado (ASHRAE, por sus siglas en inglés *American Society of Heating, Refrigerating and Air-Conditioning Engineers*). Por otro lado, una muestra del filtro dispuesto en el tranvía (Coarse 45 % de acuerdo con la norma UNE EN ISO 16890, Merak Long Life Filter; Madrid, España) fue también analizada en laboratorio para determinar su eficiencia frente a partículas submicrónicas, siguiendo el método descrito en el artículo cuatro. Para evaluar su rendimiento sobre superficies, se tomaron muestras en diferentes objetos (como asientos, barras de agarre, puertas, entre otras). Las 15 CFU más frecuentes en muestras ambientales fueron aisladas e identificadas utilizando espectrometría de masas MALDI Biotyper (Bruker; Massachusetts, EEUU). Estas CFU bacterianas además se visualizaron utilizando el microscopio electrónico de barrido JEOL 6360-LV (Deben UK Ltd; Edmunds, Reino Unido) para determinar su tamaño utilizando el software Image-J (v1.52; National Institutes of Health, 2019).

Resultados: entre los resultados destacables, se observó que las CFU permanecían estables en número durante los 90 minutos de duración de cada ensayo, con una Desviación Estándar Relativa (RSD, por sus siglas en inglés *Relative Standard Deviation*) de 0.04–0.05. Las CFU más frecuentes fueron: *Roseomonas mucosa*, *Micrococcus luteus*, *Tsukamurella paurometabola*, *Dermacoccus nishinomiyaensis*, *Kocuria rhizophila*, *Microbacterium paludicola*, *Staphylococcus haemolyticus*, *Bacillus cereus*, *Dermabacter hominis*, *Deinococcus wulumuqiensis* y *Bacillus sp*, provenientes de mucosas, polvo y dermis, principalmente y con un diámetro inferior a 0.9 μm en todos los casos. La ionización por sí sola (en una concentración de 19.9×10^9 - 31.5×10^9 iones/ m^3) logró reducir las CFU en un 45.9, 61.8 y 69.2 % tras 30, 60 y 90 minutos, lo que resultó consistente con otros estudios anteriores frente a bacterias. Cuando se utilizó filtración de manera exclusiva, las CFU se redujeron en un 73.4, 84.0 y 92.0 % tras 30, 60 y 90 minutos, lo que resultó escasamente eficiente dado que se estiman 25 ACH. Estos resultados estuvieron en línea con los ensayos de filtración realizados en el laboratorio, donde se obtuvo una eficiencia <5 % para partículas de tamaño similar al virus frente a flujos de 2500 m^3/h , en condiciones similares a las del funcionamiento del HVAC. No obstante, estos valores no fueron sorprendentes dado que el filtro, según su norma (UNE-EN 16890), asegura una filtración mínima del 75 % frente a partículas superiores a $\sim 10 \mu\text{m}$, como polvo o polen. Con la acción combinada de la ionización y la filtración no se observó mejoría. En concreto, se detectó un rendimiento del 74.2, 82.8 y 94.1 % tras 30, 60 y 90 minutos. El efecto de la ionización y de la filtración también se caracterizó utilizando el parámetro CADR. En relación con el aire no tratado (CADR = 0,299 m^3/min), la ionización y la filtración reducen las CFU ambientales (CADR = 5.15 y 9.26 m^3/min , respectivamente; y CADR = 13.21 m^3/min , combinados), lo que implica que tienen un impacto sustancial en bioaerosoles de interior. Frente a CFU en superficies la ionización no fue efectiva. Una de las mayores limitaciones de los dispositivos basados en plasma no térmico es la generación de subproductos nocivos para el ser humano. Sin embargo, ese aspecto no fue discutido en este trabajo.

V.

Aportaciones de la doctoranda

La doctoranda ha tenido un papel principal en la concepción, síntesis, elaboración, desarrollo y redacción de todas las publicaciones recogidas en esta Tesis Doctoral. Lo cual ha sido aceptado por el resto de coautores de los artículos, permitiendo su aparición en el primer puesto de autoría como reconocimiento de su protagonismo en los trabajos.

Cabe destacar que la doctoranda ha actuado como investigadora principal (IP o Co-IP) de varios de los trabajos financiados aquí recogidos, en concreto:

a) «**Desarrollo de un recubrimiento basado en nanopartículas de plata como funcionalización viricida para diferentes superficies**» financiado con 20000 € en la convocatoria «Acción de apoyo para proyectos de colaboración entre investigadores del IIS Aragón y del Instituto de Nanociencia y Materiales de Aragón (INMA), relacionados con la Covid-19» resuelta por el Instituto de Investigación Sanitaria Aragón el 28 de mayo de 2021. Proyecto del cual surgió el tercer artículo.

b) «**Adaptación de mascarillas de terapia respiratoria en EPIs de protección: Mascarillas IIS-1 e IIS-2**» financiado con 10000 € en la convocatoria «Acción de apoyo del IIS Aragón Covid-19» resuelta por el Instituto de Investigación Sanitaria Aragón el 22 de marzo de 2020. Proyecto del cual surgió el cuarto artículo.

c) «**CoviBlock. Sistemas flexibles, modulares y de bajo coste para la creación de zonas de bajo riesgo de contagio de la Covid-19**» como una línea de investigación amparada por el IIS Aragón que, a través de contratos con empresas y financiación propia IIS Aragón, ha obtenido una financiación equivalente con un valor aproximado de 60000 €. Proyecto del cual surgieron el segundo, el quinto, el sexto y el séptimo artículo.

Además de lo anterior, CoviBlock obtuvo el *áccesit* en los premios Tercer Milenio a la 'Transferencia de Conocimiento a la Empresa 2021'. En la actualidad, la línea de investigación CoviBlock está migrando hacia una *spin-off*, denominada Great Air, de la que la doctoranda es co-fundadora.

En el mismo evento de los premios Tercer Milenio, la doctoranda obtuvo el *áccesit* '**Joven Talento Investigador 2021**' por sus contribuciones en la pandemia por Covid-19. En paralelo, en ese mismo año, obtuvo el '**Premio Defensa 2021 a la Docencia Universitaria**' del Ministerio de Defensa.

En relación a su aportación en los artículos científicos, la doctoranda ha contribuido de la siguiente manera:

En el **primer artículo**, la doctoranda se encargó de la concepción y desarrollo del trabajo de microscopía electrónica junto a la Dra. Concepción Junquera, Catedrática del área de Histología de la Universidad de Zaragoza. La participación del resto de autores en la publicación consistió en la preparación de los cultivos celulares infectados con SARS-CoV-2. En este trabajo se consideró que la doctoranda también debía aparecer como autora de correspondencia.

En el **segundo artículo**, la doctoranda se encargó del diseño del proyecto junto al Dr. Alberto J. Schuhmacher, fue desarrollado y ejecutado junto al Dr. Antonio Güemes y el Dr. Alberto J. Schuhmacher y validado y supervisado por el Dr. Juan J. Alba, el Dr. Alberto J. Schuhmacher y el Dr. Antonio Güemes. La doctoranda tuvo un papel protagonista durante la concepción del trabajo, la toma de muestras, el procesamiento de las muestras, el análisis de los resultados. De la misma manera, la doctoranda se responsabilizó de la extensa búsqueda bibliográfica presentada en este artículo.

En el **tercer artículo**, la doctoranda fue responsable de la conceptualización del proyecto junto al Dr. Manuel Arruebo. La doctoranda se encargó de la síntesis y optimización de nanopartículas de plata, así como del desarrollo del método de unión a las fibras poliméricas, caracterización de los materiales y trabajo de laboratorio. La metodología y supervisión fue realizada por el Dr. Manuel Arruebo, el Dr. Víctor Sebastián y el Dr. Julián Pardo. Los ensayos sobre el rendimiento de inactivación viral fueron desarrollados por Iratxe Uranga, el Dr. Maykel Arias y el Dr. Diego de Miguel. Por su participación, fue considerada también como autora de correspondencia.

En el **cuarto artículo**, la doctoranda se encargó de la conceptualización del proyecto junto al Dr. Santiago Jiménez y la Dra. M^a Cristina Yus. La doctoranda fue la responsable de la elaboración y caracterización de los materiales filtrantes. Junto al Dr. Santiago Jiménez, la doctoranda realizó los ensayos de filtración desarrollados a lo largo del proyecto. El resto de autores supervisaron el trabajo y aportaron a su metodología. De nuevo, por su participación fue considerada como autora de correspondencia.

En el **quinto artículo**, la doctoranda se responsabilizó de la instalación de los medidores en los comercios, así como de la interpretación de los resultados. La conceptualización y desarrollo de la metodología de control de la calidad del aire fue desarrollada en conjunto al Dr. Alberto J. Schuhmacher y al Dr. Juan J. Alba, que también se encargaron de la supervisión del proyecto y de la búsqueda de financiación.

En el **sexto artículo**, la doctoranda se encargó de la colocación de los medidores, la interpretación de los resultados, los análisis de laboratorio y el estudio estadístico. De nuevo, el Dr. Alberto J. Schuhmacher y al Dr. Juan J. Alba se encargaron de la supervisión del proyecto y de la búsqueda de financiación.

En el **séptimo artículo**, la doctoranda participó en el diseño y concepción del proyecto junto al resto de autores y se encargó de su ejecución, de su análisis y de su trabajo experimental.

La doctoranda se ha encargado de la redacción del manuscrito original, así como de las versiones posteriores y la elaboración de las figuras, de todos los artículos presentados en esta Tesis.

Capítulo 3.

Publicaciones

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Primer artículo

Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread

Histochemistry and Cell Biology,
<https://doi.org/10.1007/s00418-022-02152-7>

Factor de impacto en 2022: 2.531
Factor de impacto a 5 años: 3.617
Cuartil JCR (2021): Q2
Cuartil JCR (2020): D1

Fecha de envío: 21 de junio de 2022
Fecha de publicación online: 29 de septiembre de 2022



Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread

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Accepted: 6 September 2022

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Abstract

The cytoskeleton not only deals with numerous interaction and communication mechanisms at the cellular level but also has a crucial role in the viral infection cycle. Although numerous aspects of SARS-CoV-2 virus interaction at the cellular level have been widely studied, little has been reported about the structural and functional response of the cytoskeleton. This work aims to characterize, at the ultrastructural level, the modifications in the cytoskeleton of infected cells, namely, its participation in filopodia formation, the junction of these nanostructures forming bridges, the viral surfing, and the generation of tunnel effect nanotubes (TNT) as probable structures of intracellular viral dissemination. The three-dimensional reconstruction from the obtained micrographs allowed observing viral propagation events between cells in detail for the first time. More profound knowledge about these cell–cell interaction models in the viral spread mechanisms could lead to a better understanding of the clinical manifestations of COVID-19 disease and to find new therapeutic strategies.

Keywords Cytoskeleton · Actin · Filopodia · Ultrastructure · TNT · SARS-CoV-2

Introduction

SARS-CoV-2 is an enveloped virus belonging to the Orthocoronavirinae subfamily of the order *Nidovirales*. This seventh member of human-infected beta-CoV was rapidly

sequenced by Wu et al. (2020) through samples isolated from human airway epithelial cells. It contemplates a single-stranded RNA of ~29,900 base pairs with a methylated cap at the 5' end and a polyadenylated (poly-A) tail at the 3' end. The viral genome of SARS-CoV-2 encodes structural; namely, the S protein (commonly known as “Spike”), the membrane protein (M), the nucleocapsid proteins (N), and the envelope protein (E), and non-structural proteins (NSP1 to NSP16) encoded by genes located within the 5'-region of viral RNA genome and with different enzymatic activities (Huang et al. 2020; Klein et al. 2020; Wang et al. 2020; Raj 2021).

Several studies have already characterized cell alterations at the ultrastructural level during SARS-CoV-2 infection. The accumulation of cytoplasmic lipid droplets, the thickening of the rough endoplasmic reticulum (RER), and its associated proliferation of double-membrane vesicles (DMV), the mitochondrial alterations, and the formation of filopodia as membrane projections have been well described (Goldsmith et al. 2004; Qinfen et al. 2004; Tseng et al. 2005; Snijder et al. 2006; Knoops et al. 2008; Klein et al. 2020; Barreto-Vieira et al. 2022). Nevertheless, cytoskeleton alterations have been poorly assessed.

In cells, the cytoskeleton plays a crucial role in numerous viral processes, including cell entry, transport to

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cellular regions for viral replication and assembly, and release of new viruses (Foo and Chee 2015; Denes et al. 2018; Bedi and Ono 2019). In the early phases of the infection, various entry routes of coronavirus into the cell have already been described, such as clathrin-mediated endosomal (CME) entry or fusion between viral and cellular membranes (cell surface entry) (Jackson et al. 2022). The contribution of actin polymerization to membrane deformation (i.e., endocytic invagination) is well known. In the CME process, receptor recognition and clathrin aggregation typically begin on the cytosolic side of the membrane, establishing the layer that generates the initial curvature and recruiting cargo to be internalized (Chen et al. 1998; Carlsson and Bayly 2014). Below the membrane, the nucleation promoters activate the Arp2/3 complex, polymerizing new actin filaments attached to the existing filaments, creating a branched framework (Carlsson and Bayly 2014), whose geometric structure will depend on the biomechanical demands of the process (Akamatsu et al. 2020). Together with the type I myosin motor activity, this growth generates the force necessary for the membrane invagination and the tubule extension into the cytoplasm. Subsequently, the endocytic neck contracts, and the vesicle is generated by membrane fusion (Mooren et al. 2012; Carlsson and Bayly 2014). Upon entry into the cell, actin filaments and microtubules with dyneins and kinesins reorganize and allow endocytic vesicles to traffic with viral particles to replication sites (Granger et al. 2014).

Although still unknown, it has been suggested that actin could also have a regulatory role in the replication and transcription of SARS-CoV-2. Nevertheless, it has been described in previous studies using parainfluenza virus models (Bishnu and Banerjee 1999). In other coronaviruses, reorganizing actin filaments close to the membrane to form rings could support the replication of the viral genome and the synthesis of viral proteins (Wen et al. 2020). In the late stages of infection, actin restructuring provides force for membrane bending during exocytosis (Ng et al. 2004; Wen et al. 2020).

The cytoskeleton-dependent structures have a crucial activity in viral infection processes. However, its role in viral propagation has been poorly described, especially for the SARS-CoV-2 virus. At the cellular level, enveloped viruses can spread either through diffusion from the aqueous intercellular environment or by cell–cell interaction. Diffusion-based viral propagation requires high viral loads to be effective, and it is essential for human-to-human spread. Cell-to-cell propagation is more efficient than diffusion due to its speed and immune evasion (Mothes et al. 2010). Lehman et al. (2005) associated filopodia proliferation with an efficient infectious pathway, noting that rapid navigation of viruses into cells through filopodia, which is mediated by the underlying myosin II-dependent actin cytoskeleton. Three mechanisms of filopodia associated with viral infection have been described (Chang et al. 2016): viral surfing, filopodial retraction, and tunneling nanotube formation.

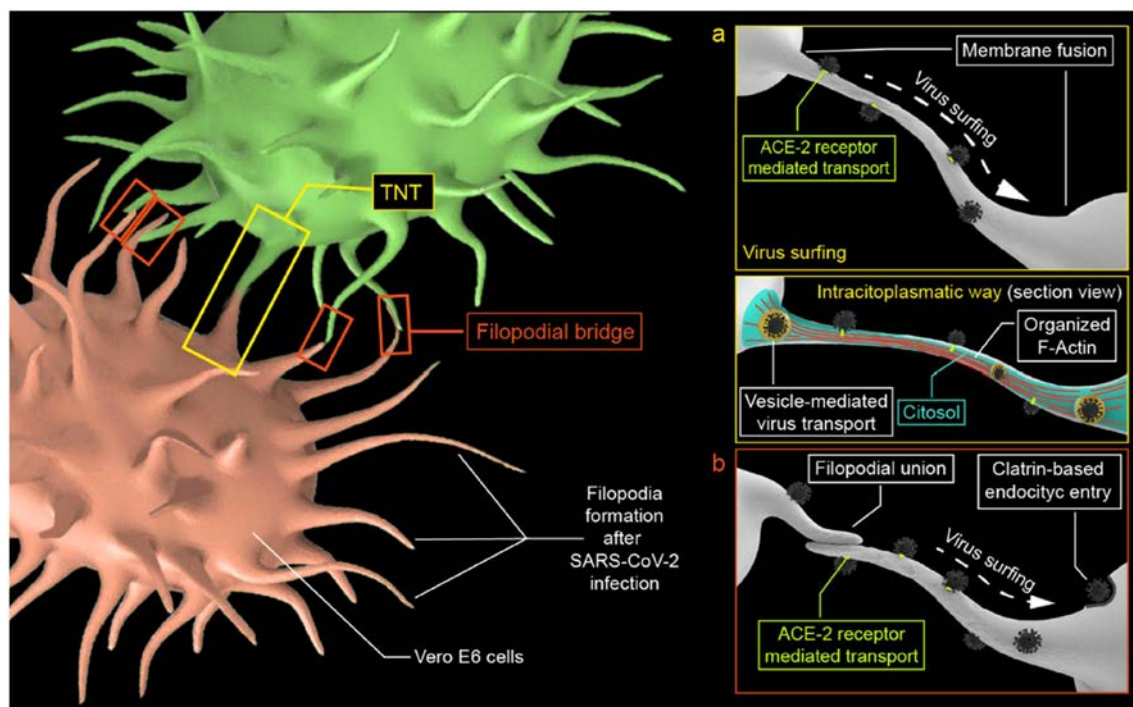


Fig. 1 Conceptual scheme of **a** viral surfing and the intracellular pathway in TNT. **b** Viral surfing along filopodial bridges

Firstly, viral surfing (Fig. 1a) consists of the orderly movement of viral particles toward the cell body through membrane projections before their internalization by fusion with the cell membrane or by receptor-mediated endocytosis (Lehmann et al. 2005). Secondly, retraction consists of the attraction of the virus to the cell through a mechanical impulse of retraction of the filopodium (Bornschrögl 2013). Thirdly, tunneling nanotubes (TNT) (Fig. 1b) correspond to intercellular ducts that connect cells for the intracytoplasmic transfer of molecules, organelles, and small bodies (Jansens et al. 2020). TNTs were originally described by Rustom et al. as "open-ended channels mediating membrane continuity between connected cells" (Rustom et al. 2004). Given the difficulty in interpreting these structures, some essential criteria that define TNTs have been proposed: have a diameter between 50 and 700 nm, have a length up to hundreds of microns, be dynamic and hover on top of the substratum, contain actin, provide cargo transport, have membrane fusion, are open-ended (Zurzolo 2021). Moreover, these structures could contain the three elements of the cytoskeleton: actin filaments, intermediate filaments, and microtubules (Resnik et al. 2019). Its composition may vary depending on the diameter of the TNT (Önfelt et al. 2006).

Ultrastructural analyses of morphogenesis in SARS-CoV-2-infected cells point out filopodia formation as plasma membrane projections (Ng et al. 2004; Caldas et al. 2020; Bideau et al. 2021; Barreto-Vieira et al. 2022). However, uncertainty remains about the involvement of the cellular cytoskeleton in SARS-CoV-2 infection. This article aims to characterize filopodia and TNT formation due to cytoskeleton restructuring in SARS-CoV-2-infected Vero E6 cells utilizing transmission electron microscopy (TEM). Additionally, the role of these subcellular structures in the cell–cell virus spread is discussed.

Materials and methods

Cell culture and SARS-CoV-2 infection

SARS-CoV-2 virus was isolated from a COVID-19 patient at the Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain). Viral stocks were prepared and quantified using the epithelial cell line, Vero E6, (kindly provided by Julia Vergara from Centro de Investigación en Sanidad Animal IRTA-CReSA, Barcelona, Spain) as previously described in Santiago et al. (2021) Vero E6 cells were cultured in Dulbecco's modified Eagle's medium, obtained from Sigma-Aldrich (Darmstadt, Germany) and supplemented with 10% fetal bovine serum (FBS) (Sigma), 2 mM Glutamax (Gibco), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma),

0.25 µg/ml amphotericin B (Sigma), 1% non-essential amino acids (Gibco), and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Biowest), referred as complete medium and used for cell expansion. Vero E6 cells were kept at 37 °C, in a 5% CO₂ humidified incubator and for the antiviral assays, complete medium with 2% FBS was used. Control cells were cultured following the same procedure, excluding SARS-CoV-2 infection.

TEM and 3D reconstruction

For electron microscopy studies, cells were seeded at 100,000 cells/cm² in a Lab-Tek chamber slides of four wells (Nalge Nunc International, Naperville, IL, USA) and infected with 500 TCID₅₀/ml SARS-CoV-2 for 48 h. Then, the cells were washed three times for 2 min with phosphate buffer (PB) fixed in 2.5% glutaraldehyde in 0.1 M PB for 5 min at 37 °C and then 2 h at 4 °C. Afterwards, samples were washed in 0.1 M PB five times and stored at 4 °C. The samples were postfixed in 2% OsO₄ for 1 h at room temperature and stained in 2% uranyl acetate in the dark for 2 h at 4 °C. Then, the samples were rinsed in distilled water, dehydrated in ethanol, and infiltrated overnight in Durcupan resin (Sigma-Aldrich, St. Louis, MO, USA). Following polymerization, embedded cultures were detached from the wells and glued to Durcupan blocks. Finally, ultra-thin sections (0.08 µm) were cut with an Ultracut UC-6 (Leica Microsystems, Wetzlar, Germany), stained with lead citrate (Reynolds solution), and examined under a transmission electron microscope JEOL 1010 (Deben UK Ltd, Edmunds, United Kingdom). Pictures were taken using Radius software (Version 2.1) with a Gatan Bioscan (Gatan Inc, Pleasanton, USA).

Three-dimensional reconstruction from serial electron microscopy sections were performed using ultra-thin sections with 0.05-µm thickness. The subcellular structures were segmented manually using FIJI ImageJ software (Schindelin et al. 2012). Additionally, for the statistical analysis, the length and the diameter were measured using ImageJ software.

Results

SARS-CoV-2 viral particles identification

Virus particles in the extracellular aqueous medium (Fig. 2a), adhered to the cell membrane (Fig. 2b), and grouped inside vesicles have been observed (Fig. 2c), and frequently isolated individually (Fig. 2d). We found an average diameter of 63 ± 11 nm for $n = 50$ viral capsids, although the measurements are underestimated given the poor resolution of the membrane proteins.

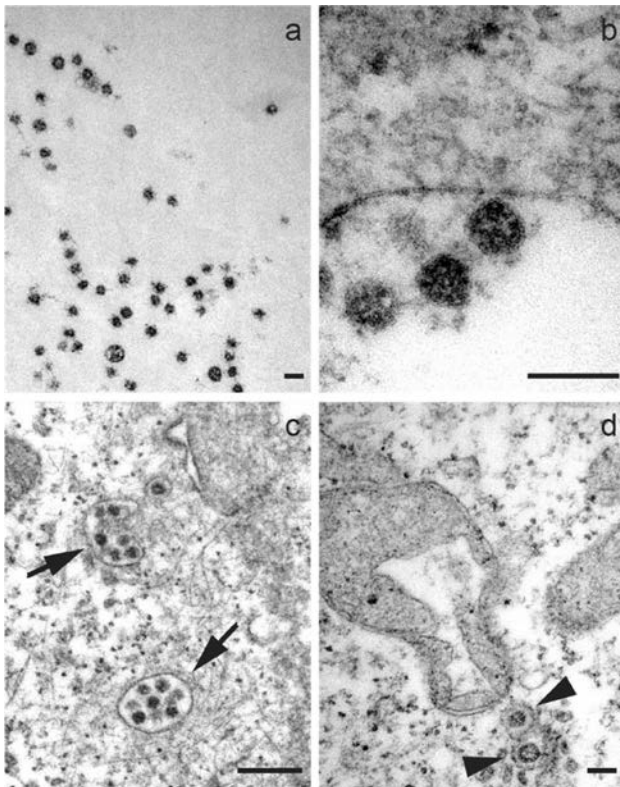


Fig. 2 Micrographs show **a** free viral particles hovering in the extracellular medium. **b** Viruses adhered to the plasma membrane. **c** Clusters of multiple viral particles (*arrows*). **d** Individual viruses within vesicles in the cytosol (*arrowhead*). A mitochondrial alteration induced by SARS-CoV-2 infection is also shown in **d**. Scale bars represent 0.1 μm in **a–d**

Formation of filopodia and cytoskeleton rearrangement

We confirmed the formation of filopodia induced by SARS-CoV-2 infection. In uninfected Vero E6 cells (Fig. 3a), there are tiny membrane projections with no intercellular interaction. In contrast, infected cells showed more developed filopodia, recurrently joined forming between cells (Fig. 3b). In the cytosol of infected cells, DMV structures show a thick membrane which consists of two or more layers closely apposed (Fig. 3c, d).

The infected cells exhibit a higher density of actin/intermediate filaments and microtubules (Fig. 4a) than control cells (Fig. 4b). We found an average length of $2.39 \pm 1.53 \mu\text{m}$ (from 780 nm to 6.58 μm), and a diameter of $120 \pm 47 \text{ nm}$ (from 60 to 245 nm) for $n = 30$ filopodial bridges in infected cells. In control cells, there was a lack of filopodial bridges and entire filopodia. Therefore, it was only possible to determine the average diameter, which was $104 \pm 22 \text{ nm}$ (from 73 to 148 nm) for $n = 30$. The filopodia of control cells were

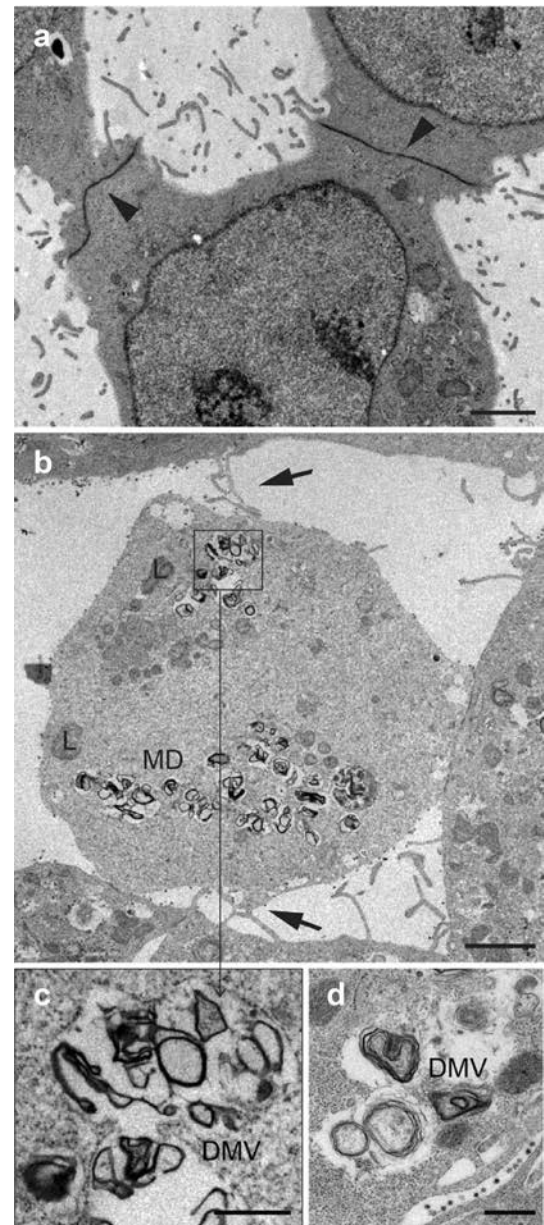


Fig. 3 **a** Uninfected control cells establish extensive junctions between them, with a low number of filopodia and no filopodial bridges. **b** Filopodia formation induced by SARS-CoV-2 is observed in the infected cells, where the formation of filopodium bridges (*arrows*) is usually identified. **c, d** Detail of double membrane vesicles (DMVs). Scale bars represent 2 μm in **a, b**, and 0.5 μm in **c, d**

observed to be slightly finer with a more homogeneous size polydispersity than in infected cells.

Viral particles over filopodial bridges

It was common to find viral particles along the filopodial membranes (Fig. 5a–g). A higher viral load was observed

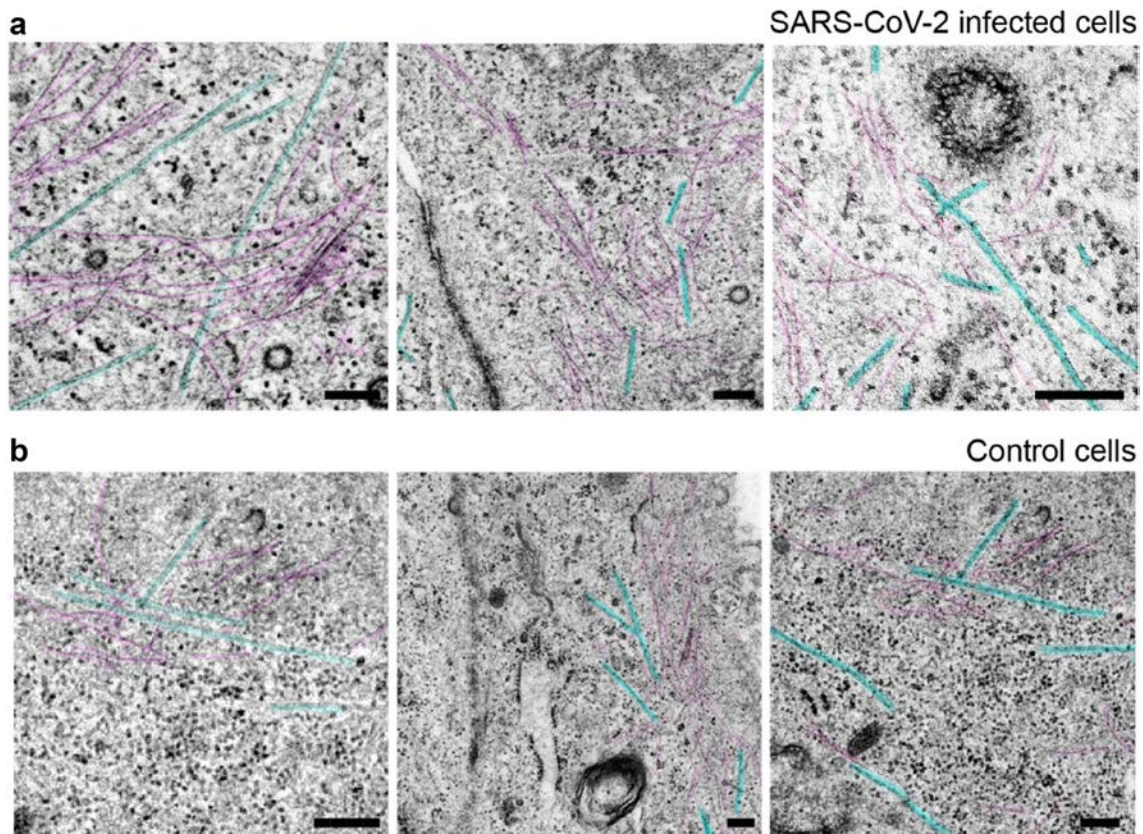


Fig. 4 Comparative cytoskeleton micrographs from infected and control cells. **a** Microtubules (blue) and actin (or intermediate) filaments (purple) in the juxtamembranous region from three SARS-CoV-2-in-

fect cells. **b** Cytoskeleton of three non-infected cells. It can be also appreciated nearby endocytic vesicles probably coated with clathrin and free ribosomal units. Scale bars represent 0.2 μm in **a**, **b**

covering the filopodial surface in comparison with the extracellular medium (Fig. 5b, c).

Even though filopodia came into direct contact with the plasma membrane of one (Fig. 5a) or several (Fig. 5g) cells, most frequent interaction appeared to be the formation of filopodial bridges (Fig. 5b, d–g). These bridges are often built with large overlapping surfaces of filopodia (Fig. 5d), and more regularly with a reduced contact through the distal extreme (Fig. 5e). It was common to find binding points between filopodia with high electron density, associated with protein aggregation (Fig. 5f), as discussed in later sections. The connection between more than two cells has also been observed (Fig. 5g).

Cell projections ultrastructural features

Filopodia present a characteristic cytoskeleton arrangement, being longitudinally traversed by actin filaments (Fig. 6a, b). Additionally, cells emit the thickest cytoplasmic extensions (typically $> 0.25 \mu\text{m}$) that characteristically also have microtubules arranged in parallel with the actin (or intermediate) filaments (Fig. 6c, d).

Cell membranes fusion

In addition to intercellular filopodial bridges, it was possible to identify TNT-like structures mainly characterized by a continuity of the plasma membrane of the connected cells (Fig. 7a, b). These types of structures were less frequently observed, and only once an event similar to a clathrin-mediated endocytic process inside a short nanotube structure was identified (Fig. 7c, d).

Viral extrusion

Extrusion of replicated viruses was identified in the three-dimensional reconstruction (Fig. 8a, b). In our observations, vesicles loaded with multiple viruses (Fig. 8c) fuse with the plasma membrane allowing an outwards virus release (Fig. 8d). At least three neighboring cells filopodia cluster around the extruded viruses. While the majority of these viruses are attached to filopodia, a few are located in the extracellular medium.

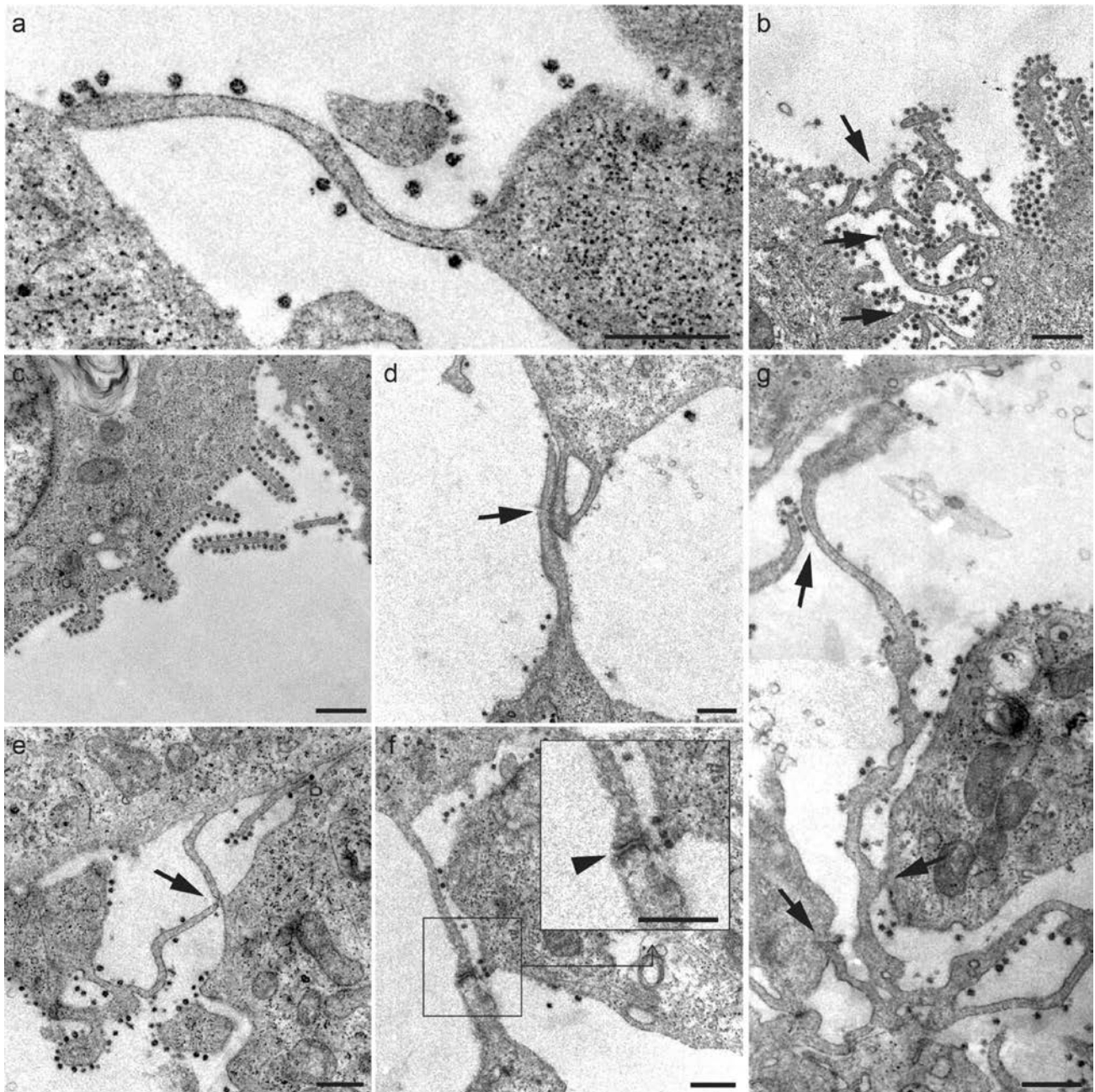


Fig. 5 **a-g** Micrographs of various filopodial structures found in SARS-CoV-2-infected Vero E6 cells. **a** Filopodial extension whose end adheres to the plasma membrane of a neighboring cell. **b-c** The number of viral particles attached to the filopodia was variable, although they were frequently observed in high density surrounding the filopodial surface. **d** Filopodial bridges were constituted through

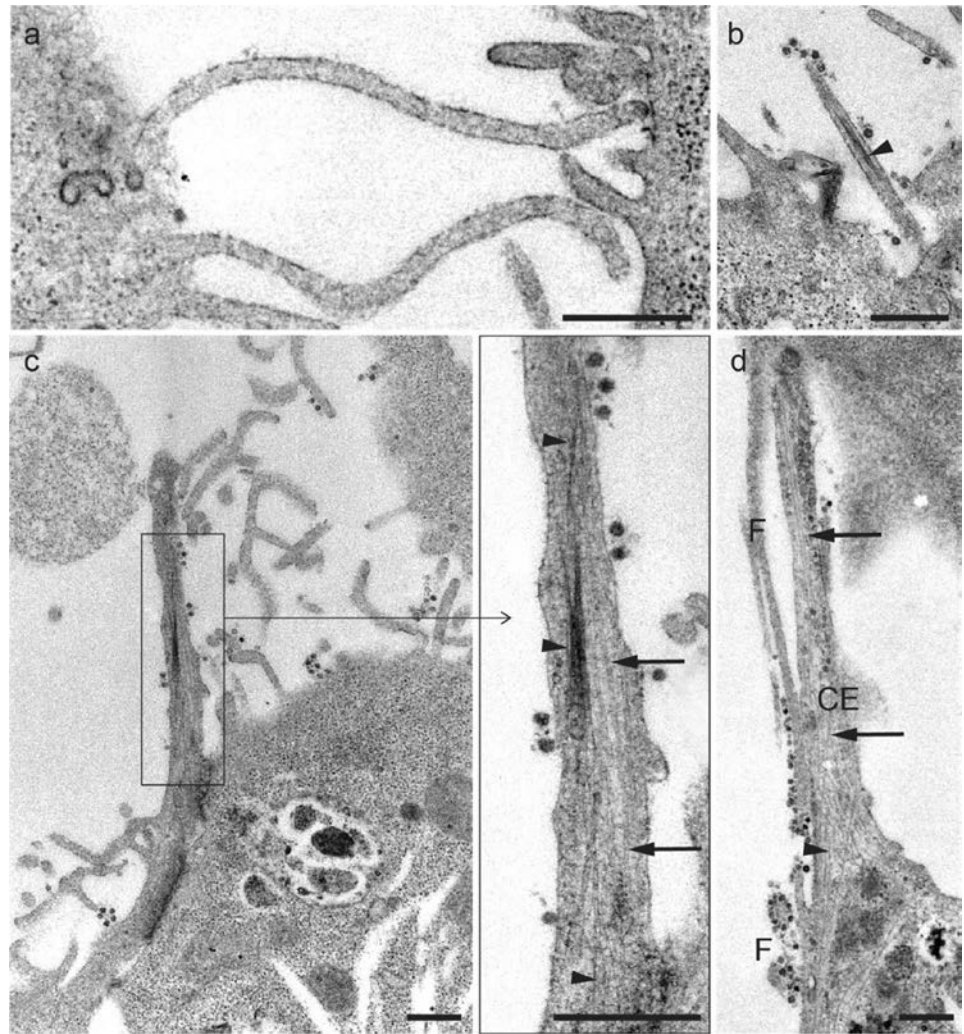
wide intermembrane junctions. **e** Junctions of filopodial bridges were often found in the distal extremes. **f** In many cases, bridge filopodial junctions showed electron-dense reinforcements (*arrowhead*; detail). **g** Commonly, larger filopodia contact more than one neighboring cell. *Scale bars* represent 0.5 μm in **a-g**

Cell membrane fragments in the extracellular space

Cell debris and pyroptotic bodies are visible in the extracellular space (Fig. 9a, b). Membrane fragments are typically surrounded by viruses and it was common to find them in regions close to the cell surface (Fig. 9c, d). So far,

we mainly observed viruses attached to filopodial surfaces (Fig. 5), in the extracellular medium (Fig. 2a), adhered to plasma membranes (Fig. 2b), and inside vesicles (Fig. 2c, d). More rarely, the viral particles were arranged linearly along the membrane debris (Fig. 9b–d).

Fig. 6 **a, b** Longitudinal section of filopodia, characterized by the absence of microtubules. **c** Longitudinal section of thick cytoplasmic extensions showing SARS-CoV-2 virus-like particles in the plasma membrane, and microtubules (*arrows*) and filaments (*arrowheads*) arranged parallelly. **d** Comparison between thick cytoplasmic extensions (CE) and filopodium (F) structures. *Scale bars* represent 0.5 μm **a-d**



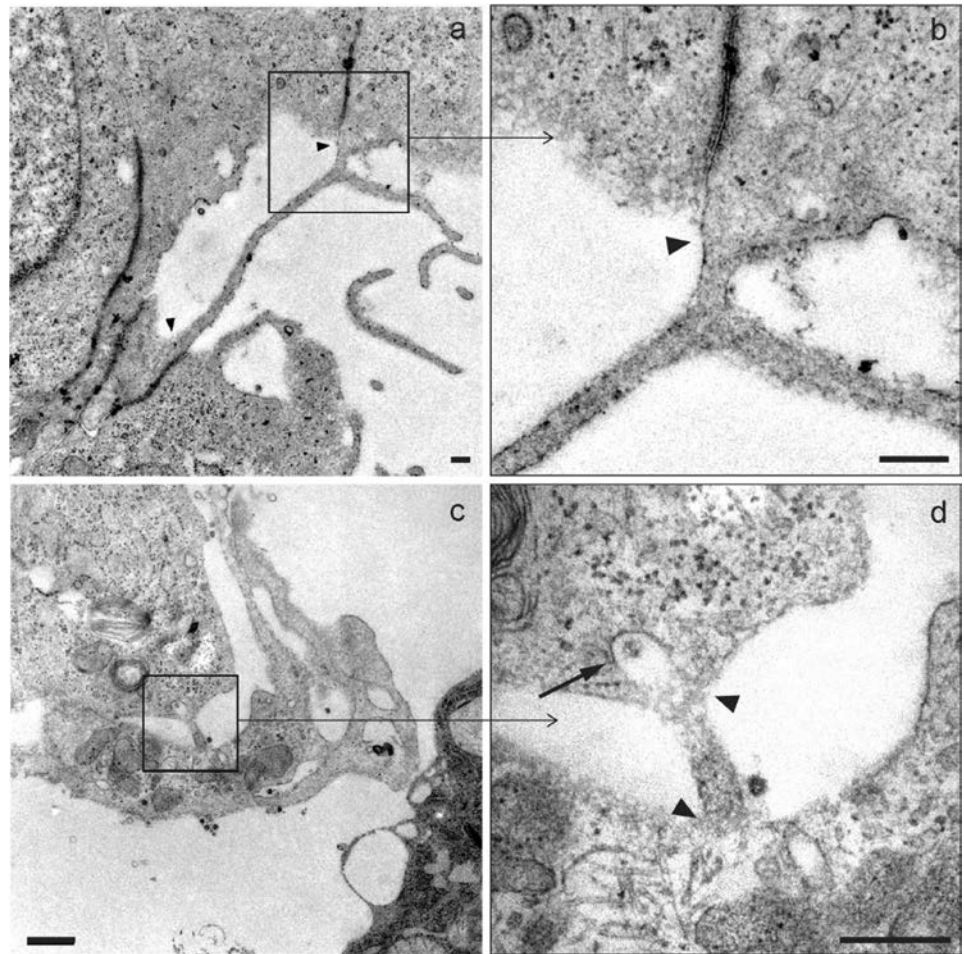
Discussion

Throughout the COVID-19 pandemic, numerous studies focused on the ultrastructural analysis of cell morphogenesis induced by SARS-CoV-2 infection. However, the overwhelming number of articles published in this regard, together with the scant knowledge of the biology of the newly discovered virus, has represented an obstacle when it comes to interpreting electron microscopy studies. As a result, numerous TEM-based reports have continuously confused SARS-CoV-2 viral particles with normal cellular structures. Akilesh et al. (2021) pointed to five cellular structures typically confused with enveloped viruses: clathrin-coated vesicles, secretory vesicles and granules, coatomer- and ER-coated vesicles, multivesicular bodies and exosomes, and microvilli. Expressly, clathrin-coated vesicles represent the structures most frequently misinterpreted as viral particles in TEM (Calomeni et al. 2020; Kniss 2020; Miller and Brealey 2020; Brealey and Miller 2021). Our microscopic observation identified clathrin-containing

vesicles whose sizes ranged from 90 to 115 nm, similar to SARS-CoV-2 (60–140 nm). However, vesicles could be free in the cytoplasm and do not have electron density so they can be quickly differentiated from viral particles. In this work, we found viral particles arranged in three ways: isolated or grouped in the extracellular space, inside vesicles in the intracellular space, and bounded both to the membrane of the cell body and the filopodia projected as a consequence of infection with SARS-CoV-2.

Viral-infected cells rearranged their cytoskeleton. An increase in ribosomal units and polyribosomes was observed in the cytosol for the protein monomers synthesis that make up the cytoskeleton (G-actin and α and β tubulins). Filopodia are cell membrane projections rich in actin filaments arranged in parallel (Mattila and Lappalainen 2008). There are two alternative models that explain filopodia origins. The convergent elongation model postulates that filopodia arise from the filopodium tip complex (FTP) assembly in the plasma membrane or its vicinity (Faix and Rottner 2006), which contain actin polymerases, such as Ena proteins

Fig. 7 Micrographs of structures similar to TNT. **a** A nanotube $> 2.5 \mu\text{m}$ in length and 70–90 nm in diameter promoting direct contact between the cytosols of two adjacent cells is observed. **b** Membrane fusion (arrowhead) detail of (a). **c** A smaller filopodium ($< 0.5 \mu\text{m}$ in length and 90–100 nm in diameter), where a structure similar to a clathrin-coated vesicle (arrow) can be seen endocytosing a viral particle. **d** Detail of the fusion membranes from two neighboring cell bodies (arrowheads) from c. Scale bars represent $0.2 \mu\text{m}$ in a–d



VASP, Dia2, and myosin X that accelerate filament growth (Cheng and Mullins 2020). An alternative model proposes that actin filaments are nucleated at the tips of filopodia by formins (Mattila and Lappalainen 2008).

Filopodia growth induced by SARS-CoV-2 infection in cells has been described in previous studies (Barreto-Vieira et al. 2022). These nanostructures were slightly finer and more homogeneous in the control cells ($104 \pm 22 \text{ nm}$) compared to the infected cells ($120 \pm 47 \text{ nm}$). Similar numbers of filopodia were found in both cultures (around 15–20 filopodia/section/cell). Nonetheless, only in infected cells did filopodia organize in bridges. These membrane projections have played a relevant role in probing the extracellular environment, directed migration, and cell–cell communication processes (Mattila and Lappalainen 2008). Additionally, intercellular communication pathway mediated by filopodia as an effective cell–cell viral propagation model. The role of filopodia was initially described in 2003 as a spread pathway for the human T-cell leukemia virus type 1 (HTLV-1) (Igakura et al. 2003), and was subsequently associated with other enveloped viruses (Lehmann et al. 2005; Sherer et al. 2007; Choudhary et al. 2013). Furthermore, Oh et al.

(2010) found a higher viral load in filopodia than in other regions of the cell membrane. Accordingly, our observations suggest a more significant presence of viral particles adhered to the filopodial surface than in other membrane regions. Viral binding may be mediated by positively charged glycoprotein moieties that recognize negative domains of cellular receptors. Some authors point to a high expression of receptor-negative domains in filopodia, as the heparan sulfate receptors (HS), which could convert them into preferential binding regions (Oh et al. 2010; Tiwari et al. 2012).

Once the viruses bonded in the filopodial surface, they can slide or move laterally along these membrane projections; this phenomenon is known as surfing. Although viruses can enter the cell through other membrane regions, filopodium-dependent viral entry is more efficient. Physical intercellular connection is 2–3 orders of magnitude more efficient than diffusion spread due to the speed of transmission and the evasion of the immune system (Dimitrov et al. 1993; Carr et al. 1999; Miranda-Saksena et al. 2018). This mechanism could also promote the virus propagation between distant cells. In this work, we observed an average length of $2.39 \pm 1.53 \mu\text{m}$ (from 780 nm to

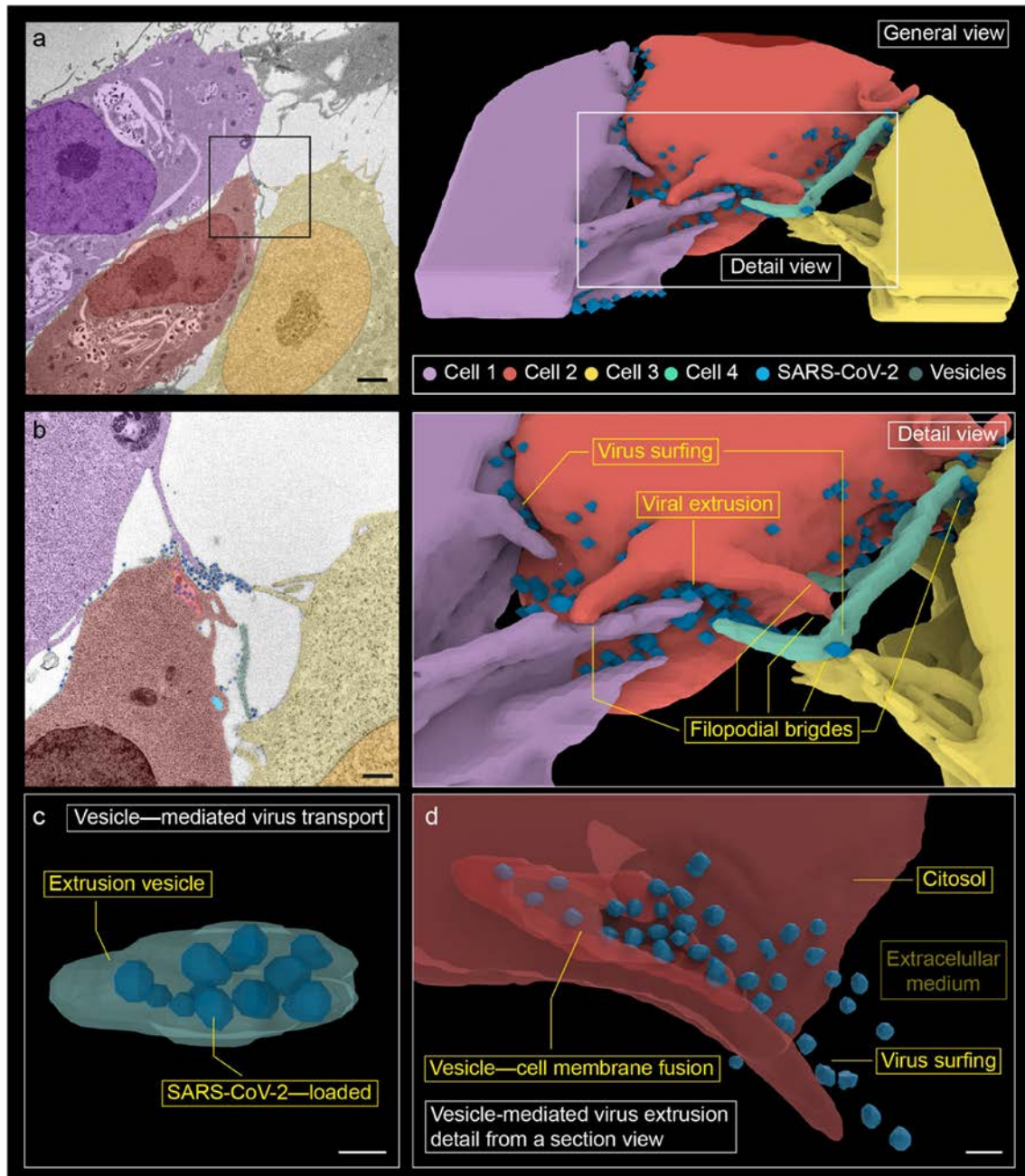


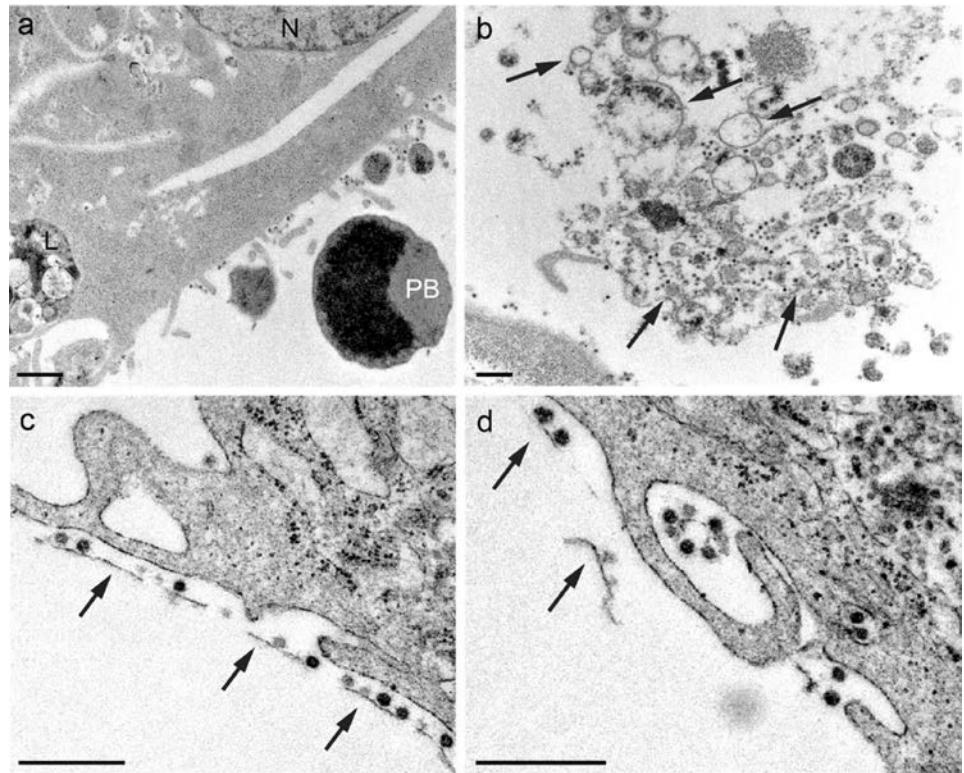
Fig. 8 Three-dimensional reconstruction from 14 serial electron micrographs. **a** Overview of cell–cell interaction in viral propagation. **b** Detail of the membrane projections, where filopodial connections and viral surfing are observed. Numerous membrane projections from (at least) three different cells converge in a region of high viral load. We designate as 'viral extrusion' the region where the membrane of the extrusion vesicle fuses with the plasma membrane. **c** A vesicle with internalized viruses from cell 2 has been reconstructed. It

is observed 12 viral particles that occupy practically the entire vesicle. It is probably an extrusion vesicle externalizing the virus. **d** A detail of viral extrusion has been reconstructed using eight micrographs. The micrographs have been selected to show how a vesicle fuses with the membrane to extrude viruses along a filopodium. This finding supports the hypothesis that filopodia are formed to promote the spread of the virus between cells. Scale bars represent 2 μm in **a**, 0.5 μm in **b**, and $\sim 0.1 \mu\text{m}$ in **c**, **d**

6.58 μm) in filopodial bridges. However, cell culture requires proximity between cells; therefore, longer bridges with more connections could appear *in vivo*. SARS-CoV-2 virus could bind to ACE-2 receptors to navigate through

the filopodial membrane all the way until their eventual entry to the cell (Kloc et al. 2022). Viral envelope proteins have high specificity and affinity to cellular receptors, which are associated with actin filaments. Hence, viral

Fig. 9 Micrographs showing **a** fragments of a pyroptotic body in the extracellular space (*PB* pyroptotic body, *N* nucleus, *L* lysosome). **b, c** Virions adhered to a membrane fragments (arrows). Subcellular fragments in the extracellular space have been observed as a consequence of SARS-CoV-2 infection. In membrane fragments, it has been possible to identify adherent virions. **d** Virus adhered to remains of membranes are occasionally encircled by the filopodia (possible macropinocytosis). Scale bars represent 0.5 μ m in (a–d)



surfing is propelled by microfilament contraction through internal myosin motors (Medeiros et al. 2006) to regions of the membrane with high endocytic activity (Taylor et al. 2011). Furthermore, Seyran et al. (2021) reported that SARS-CoV-2 protein S interacts with the sialic acid layer of the epithelium, which may allow its transport across the epithelial surface until it encounters an ACE-2 receptor.

There is still no consensus on how to refer to the different types of membrane projections. We use the term TNT to refer to filopodial bridges with membrane fusion (Zurzolo 2021; Kloc et al. 2022). Additionally, some authors only mention the intracellular transport through TNT and they do not rule out a dual transfer by surfing (coupling to HS receptors, and entering via the endocytic pathway). In the first reference to a membrane nanotube, membrane fusion was not specified (Ramírez-Weber and Kornberg 1999). The term ‘TNT’ was coined to refer to an intracytoplasmic transport pathway (Önfelt et al. 2004; Rustom et al. 2004). However, as can be seen in some published micrographs (e.g., Figure 2g of (Rustom et al. 2004)), the section of filopodium obtained in the ultrathin section can lead to misinterpretations (e.g., Figure 1g1 from (Rustom et al. 2004)). Therefore, unless the physical separation between filopodia is evident (e.g., Fig. 5d, e of this article), confusion between filopodia bridges and tunneling nanotubes cannot be ruled out (e.g., Figures 5a, b, g of this article). When the ultra-thin section cut longitudinally the entire filopodium (e.g., Fig. 6), the cell structure can be interpreted with a lower uncertainty.

To avoid these limitations, serial ultra-thin sections could represent an appropriate option. Despite TEM limitations for TNT identification, ultrastructural studies using scanning electron microscopy (SEM) entail more difficulty since membrane fusion cannot be clearly identified [as for e.g., in (Caldas et al. 2020), (Lu et al. 2019), or (Pontes et al. 2008)].

Given the high viral load attached to filopodia, we suggest that they could play a crucial role in the SARS-CoV-2 viral cycle. The filopodia formation induced by SARS-CoV-2 was promptly described during the beginning of the COVID-19 pandemic (2020) using Vero E6 cells (Bouhadou et al. 2020). However, no further ultrastructure analyses were performed until two recent studies. In the first report, not yet peer-reviewed, Swain et al. (2022) again suggested that filopodia could contribute to the cell–cell transmission of *Betacoronavirus*. In the second study, Merolli et al. (2022) commented little on its presence in infected cells and described tunneling nanotubes (TNT) as a novel intracytoplasmic route of the virus, despite being previously reported (Kumar et al. 2017; Jansens et al. 2020; Tiwari et al. 2021). Our work found that surfing was the predominant route of viral propagation. Vesicle-mediated cytosolic transport of the virus has also been found less frequently. We have identified the cytosolic pathway in two ways: through TNT and filopodia. Firstly, we have only identified transport through TNT in Fig. 7c. According to this hypothesis, the virus could be internalized in vesicle and transported until being endocytosed by the host cell (Jansens et al. 2020). Note that the

viral transport pathways mentioned are not mutually exclusive but could coexist and increase the yield of intercellular viral propagation.

The findings in Fig. 8 confirm that filopodia act as means of intercellular transport of the virus; herein it is possible to see extrusion of virus replicated from the host cell. Firstly, the large vesicle is loaded with numerous viral particles together with the absence of clathrin accumulation at the vesicular edge (Fig. 8c), makes endocytosis to be unlikely. Secondly, as depicted in Fig. 8a, the host cell (cell 2) is altered by viral infection (numerous replicating DMVs, lysosomes, and autophagic-like events). Given the above, it could be interpreted that viruses have already been replicated in this cell and try to escape to colonize nearby cells. Finally, those viruses are unlikely released from cell 1 and cell 3, since a significant interaction between them coexists in a specific area of cell 2. No nearby exocytic vesicles have been observed in these two neighboring cells. This clustering of filopodia in the high viral load region may suggest underlying molecular mechanisms (Jansens et al. 2020). Additionally, the viral particles are extruded in the direction of the filopodium (see detail in Fig. 8d), which reinforces the hypothesis of cellular transport mediated by filopodia.

Numerous viral particles bound to membranous debris were observed in the extracellular environment (Fig. 9b, c). Pyroptosis occurs in cells infected by SARS-CoV-2, although the mechanisms that trigger it are still unknown in detail (Bittner et al. 2022). One theory is that membranous debris could be associated with the rupture of these cells. The rearrangement of viruses along these structures could have implications for propagation dynamics, so future work should identify its implications in viral processes.

SARS-CoV-2 virus, like other respiratory viruses, targets the ciliated cells of the respiratory epithelium, facilitating dissemination to the lungs. Preferential budding of coronavirus towards membrane projections, as cilia, has also been suggested using *in vivo* samples. For example, Afzelius et al. (Afzelius 1994) observed virions inside and outside hair cells, but not in goblet cells or other human nasal mucosa cells. Ciliary loss has been widely reported both in SARS-CoV-2 and in other coronaviruses that preceded it (such as MERS-CoV or HCoV-229E) (Chilvers et al. 2001; Haverkamp et al. 2018; Schreiner et al. 2022), and it is associated with the internalization of the cilia during the virus entry to the cell. Consequently, deciliation could be related to the recurrent anosmia induced by SARS-CoV-2 infection; correlation between histological damage and olfactory dysfunction is still debated, though (Reyna et al. 2022). This phenomenon of ciliary invagination is similar to the filopodial retraction, where cell filopodia internalize the virus through membrane traction mechanisms (Bornschrögl 2013; Chang et al. 2016).

Given the importance that filopodia and TNT acquires in the viral infection, it is essential to know in greater depth

the interface between viruses and filopodia to understand the viral pathogenesis of SARS-CoV-2, as well as the cellular and molecular mechanisms that govern the course of the disease by COVID-19.

Conclusions

In this work, we have ultrastructurally characterized filopodia formation as a mechanism of cell interconnection in the late stages of SARS-CoV-2 infection (48 hpi) using Vero E6 cells in the primitive variant of the virus. SARS-CoV-2 infected cells shows a more developed cytoskeleton in contrast with control cells, which might be associated with viral cell–cell spread or defense mechanisms linked to virus extrusion. Communication between two or more neighboring cells was evident, finding filopodial bridges (filopodia joined but not fused) and tunneling nanotubes (filopodia with fused membranes).

Viral particles were commonly adhered to the filopodial surface, suggesting a preference of the virus towards these membrane regions.

Viruses surf along membrane projections. Surfing seems to predominate in the viral spread between cells. Evidence of vesicle-mediated intracellular transport was also found via TNT and via filopodia. Vesicular transport by filopodia probably include exocytosis in the host cell and endocytosis in the neighboring cell. Viral spread through TNT occurs intracellularly. Those cytosolic events have been found less frequently. However, it may occur more times since can be hidden in the ultra-thin section.

To the best of our knowledge, no prior studies have reported three-dimensional reconstructions of viral cell–cell propagation. Extrusion of numerous viral particles and the clustering of filopodia from neighboring cells around them was observed. We probably would have not found this event in a single ultrathin section. Therefore, serial sections have been especially useful in this work.

Given the importance of the cytoskeleton for the proper cellular function, it is crucial to characterize in greater depth its alterations to understand the SARS-CoV-2 pathogenesis.

Acknowledgements The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza and to Mario Soriano from Centro de Investigación Príncipe Felipe, Valencia, Spain. This research was funded by the Instituto de Investigación Sanitaria Aragón: Campaña Investiga COVID-19.

Author contributions MB and CJ: Investigation, Original manuscript text preparation, Methodology, Validation. CJ: Project administration, Manuscript review. IU, MA., and EM: Investigation, sample preparation (including cell culture), virus isolation, Manuscript review.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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Segundo artículo

SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity

Journal of Clinical Medicine, 11 (9), 2607

<https://doi.org/10.3390/jcm11092607>

Topic: Fighting against Covid-19: Latest Advances, Challenges and Methodologies

Factor de impacto en 2022: 4.964

Factor de impacto a 5 años: 5.098

Cuartil JCR (2021): Q1

Cuartil JCR (2020): Q1

Fecha de envío: 26 de marzo de 2022

Fecha de publicación: 6 de mayo de 2022

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Article

SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity

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Abstract: The spread dynamics of the SARS-CoV-2 virus have not yet been fully understood after two years of the pandemic. The virus's global spread represented a unique scenario for advancing infectious disease research. Consequently, mechanistic epidemiological theories were quickly dismissed, and more attention was paid to other approaches that considered heterogeneity in the spread. One of the most critical advances in aerial pathogens transmission was the global acceptance of the airborne model, where the airway is presented as the epicenter of the spread of the disease. Although the aerodynamics and persistence of the SARS-CoV-2 virus in the air have been extensively studied, the actual probability of contagion is still unknown. In this work, the individual heterogeneity in the transmission of 22 patients infected with COVID-19 was analyzed by close contact (cough samples) and air (environmental samples). Viral RNA was detected in 2/19 cough samples from patient subgroups, with a mean Ct (Cycle Threshold in Quantitative Polymerase Chain Reaction analysis) of 25.7 ± 7.0 . Nevertheless, viral RNA was only detected in air samples from 1/8 patients, with an average Ct of 25.0 ± 4.0 . Viral load in cough samples ranged from 7.3×10^5 to 8.7×10^8 copies/mL among patients, while concentrations between 1.1–4.8 copies/m³ were found in air, consistent with other reports in the literature. In patients undergoing follow-up, no viral load was found (neither in coughs nor in the air) after the third day of symptoms, which could help define quarantine periods in infected individuals. In addition, it was found that the patient's Ct should not be considered an indicator of infectiousness, since it could not be correlated with the viral load disseminated. The results of this work are in line with proposed hypotheses of superspreaders, which can attribute part of the heterogeneity of the spread to the oversized emission of a small percentage of infected people.

Keywords: COVID-19; bioaerosols; infectious diseases; cough; airborne; superspreaders; transmission heterogeneity

Citation: Baselga, M.; Güemes, A.; Alba, J.J.; Schuhmacher, A.J. SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity. *J. Clin. Med.* **2022**, *11*, 2607. <https://doi.org/10.3390/jcm11092607>

Academic Editor: Yudong Zhang

Received: 26 March 2022

Accepted: 3 May 2022

Published: 6 May 2022

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1. Introduction

After more than two years of the COVID-19 pandemic, the role of the different transmission routes in the dynamics of the spread of the SARS-CoV-2 virus are still unknown. Human–human transmission has been described from direct respiratory spread, where the symptomatic or asymptomatic patient expels contaminated particles in respiratory events, and, on the other hand, indirect spread or via fomites, where the transmission is due to contact with contaminated surfaces. Regarding direct dissemination, fomites are objects that can be contaminated with pathogenic microorganisms and serve as transmission vehicles [1,2]. Variable SARS-CoV-2 survival depends on the surface material; it remains active for up to 72 h on plastic and stainless steel, up to 24 h on cardboard, and less than 4 h on copper [3]. On polymeric surfaces and skin, the Alpha, Beta, Delta, and Omicron variants exhibited survival twice as high as the ancestral strain, reaching a persistence of more than 16 h on the skin [4].

It is possible to differentiate between the droplet model and the bioaerosol model regarding indirect dissemination. Droplets and bioaerosols differ primarily in their aerodynamic diameter and properties. Large droplets predominate in close contact, but they are not capable of infecting individuals at a distance, since they settle rapidly due to gravitational effects. However, the aerodynamic diameter of aerosols allows them to be transmitted over time and distance [5], as represented in Figure 1. This interaction phenomenon between gravity and evaporation, depending on the size of the drop, was described in 1955 by Wells [6]. Suspension time can be determined from Stokes' Law, which describes an inverse relationship between particle size and deposition rate. For example, a 5 μm aerosol takes about 33 min to settle on the ground, while a 1 μm aerosol remains suspended in the air for more than 12 h [7]. Regarding this pandemic, the scientific community has redefined the concept of bioaerosol, extending its consideration to airborne particles smaller than 100 μm , based on evidence and common factors related to the aerodynamics of the particles. Nevertheless, it is necessary to consider that both large droplets and bioaerosols are dynamic, since they can modify their morphology and physicochemical properties, depending on environmental parameters. In this way, as we will discuss later, small droplets can become aerosols and, in turn, larger aerosols can migrate towards smaller aerosols, modifying their aerial dynamics. Fine particles pose greater challenges when it comes to preventing potential contagion: on the one hand, because they correspond to the size range where the retention efficiency of filters is lower (they typically have a minimum efficiency around 300 nm [8,9]); on the other, because they can remain suspended indefinitely and reach greater distances from their emitter.

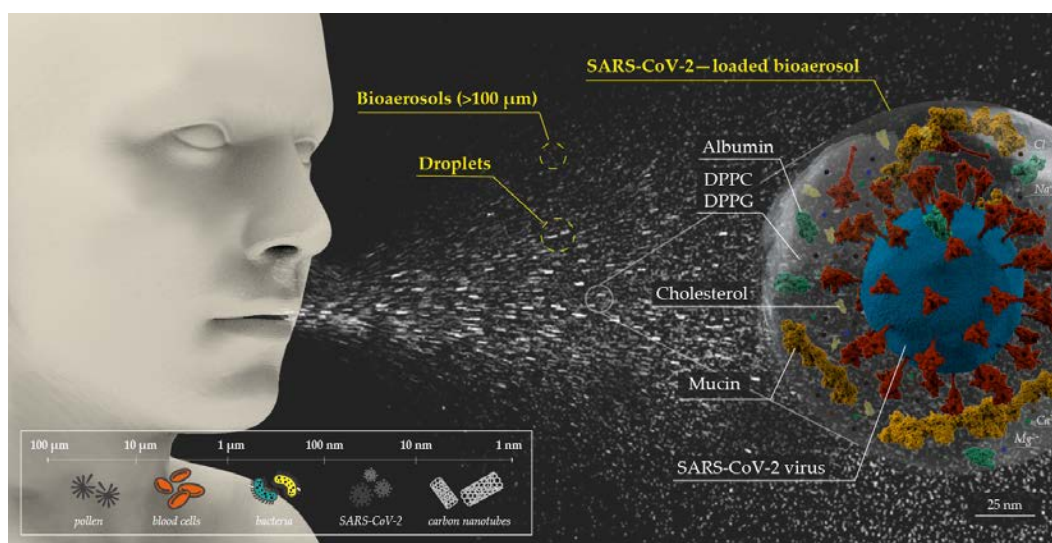


Figure 1. Schematic representation of bioaerosols and droplets emission. Where, DPPC is dipalmitoylphosphatidylcholine protein, and DPPG is dipalmitoylphosphatidylglycerol protein.

The viruses are transported through small secretions of saliva and mucosa in infected people, inadvertently expelled through the nose and mouth. It is possible to differentiate between bronchiolar, laryngeal, and oral bioaerosols, depending on the generation mechanism and anatomical region where they originate. Bronchiolar particles are predominantly attributed to normal breathing and are associated with the rupture of the fluid film in the bronchioles by shear forces [10]. The vibration of the vocal cords generates laryngeal secretions during speech and vocalization [11]. Instead, oral particles where droplets are larger than 100 μm are predominantly produced from saliva in the oral cavity. Their emission rate and velocity depend on the effort during the vocalization event [11]. Other study suggests a dependence between the rate of aerosol emission and the amplitude of vocalization and, furthermore, points to the existence of independent “superspreading” events [12]. These bioaerosols are predominantly composed of ions (predominantly Na^+ , Cl^- , Ca^{2+} ,

and Mg^{2+}), organic and inorganic particles and glycoproteins, mainly albumin, mucins, cholesterol and pulmonary surfactant proteins, such as DPPG and DPPC [13]. They can also include, in infected individuals, viral or bacterial pathogens. This is especially relevant during infections that present a high viral load in the upper respiratory tract due to the anatomical proximity to the 'escape routes', as is the case for COVID-19 [3,14,15].

The airborne model of COVID-19 disease transmission has been demonstrated [16] among small animals [17–19], from viral superspreading events [20], in long-distance transmission scenarios where infected individuals do not come into direct contact [21], by transmission in asymptomatic individuals [22], and by the prevalence of spread in closed spaces [23]. Despite the alerts by scientific groups since April 2020, this route of contagion was dismissed, and greater attention was paid to fomites and contagion by droplets, following the classic models of transmission of respiratory diseases for the COVID-19 pandemic. Consequently, there was controversy about whether asymptomatic infected individuals could be transmitters of SARS-CoV-2, which was an obstacle during epidemiological management. The global acceptance of the spread of COVID-19 by aerosols has modified the preventive approach, including new measures to reduce the risk of contagion. In April 2021, this route of infection was accepted by the WHO as one of the main ones [24].

The size of the SARS-CoV-2 virion varies between 70 and 90 nm [25,26], and a mean virus concentration in sputum of 7.0×10^6 copies/mL and a maximum of 2.4×10^9 copies/mL has been reported [27]. Consequently, the viral load occupies $2.1 \times 10^{-6}\%$ of the bioaerosol on average. With this value, Lee [28] estimated a theoretical minimum and initial aerosol size of 4.7 μm to contain SARS-CoV-2. However, experimental bioaerosol sampling studies suggest the presence of the virus in smaller particle sizes (even <0.25 μm). Liu et al. [29] were pioneers in experimentally investigating the aerodynamic nature of the SARS-CoV-2 pathogen by quantifying viral RNA from aerosols in different hospital areas in Wuhan (China) during one of its most severe outbreaks. In their work, they determined the presence of viruses predominantly in two size ranges: in the submicrometric region (0.25–1.0 μm) as well as in the supermicrometric region (>2.5 μm). Since then, numerous scientific efforts have aimed at characterizing and understanding the dynamics of the spread of COVID-19 associated with aerosols. However, the role of airborne transmission for the SARS-CoV-2 virus and the risk of contagion that they might represent have not yet been well described.

The modal distributions of aerosols acquire great relevance in the context of disease transmission. They determine the aerodynamic characteristics and their deposition dynamics, as well as the variability in the viral colonization model, depending on the depth of the respiratory tract [30]. In addition to influencing the modal distribution of aerosols, the nature of the activity carried out will modify parameters associated with the kinetics of the particles. Chao et al. [31] pointed to a mean velocity of 11.7 m/s during coughing and 3.9 m/s when speaking. Their experimental work described a geometric mean diameter of 13.5 μm when coughing and 16.0 μm when speaking, with an estimated concentration of 2.4–5.2 cm^{-3} in coughs and 0.4×10^{-3} –0.2 cm^{-3} in speaking. The disseminating capacity of the individual is noticeably modified if the individual is speaking or coughing. Specifically, it has been described that during a one-minute conversation, more than 1.0×10^3 aerosols can be disseminated [32], and an individual expels around 7.2×10^3 particles per liter of exhaled air [33,34], while coughing occurs sporadically, implying a likely increased release of aerosols during breathing and speaking [35]. Subsequently, other experimental works have demonstrated, with greater or lesser success, the presence of SARS-CoV-2 viral RNA in aerosols using various sampling methods, such as solid impactors [36–38], cyclones [39–49], impingers [50,51], gelatin filters [39,44,52], particulate filters [53–56], and condensation systems [38,57,58]. However, other investigations have failed to recover detectable RNA concentrations [59–66].

Numerous factors can influence the airborne transmission of pathogens, both at the dynamics of propagation and the virus survival (or persistence). The size of exhaled

bioaerosols evolves due to evaporation, coagulation, and deposition, directly affecting their air suspension time and persistence [35]. Consequently, the size distribution of the airborne concentration of aerosols will vary with time, since larger diameter particles settle faster [67]. However, other extrinsic factors, such as ambient airflows, affect both the airborne suspension time and the distance traveled by the aerosol [68]. At the level of virus survival, the presence of ions affects droplet evaporation. Therefore, the dynamics change, while sputum organic compounds are insignificant due to the low molar fraction they represent [14]. By reducing the aqueous component of the aerosol, aerosols are subject to changes in morphology, viscosity, and pH, among others, modifying the microenvironment of the virus and, therefore, reducing its persistence [69]. Specifically, low relative humidity induces evaporation, a decrease in pH and, with it, conformational changes in the proteins on its surface, making the virus a less infectious pathogen [70]. In addition, the size of the aerosol decreases proportionally until it crystallizes, significantly reducing its size in environments with very low relative humidity. In the opposite case, the aerosol tends to adsorb moisture and increase its size at high relative humidity [71]. If the relative humidity is below 80%, respiratory aerosols reach a final diameter of 20 to 40% of their original size [72]. In the case of SARS-CoV-2, it is suggested that the optimal relative humidity for minimizing the spread of this virus is between 40 and 60% [73]. Initially, Fears et al. [74] determined a greater dynamic efficiency of SARS-CoV-2 than SARS-CoV and MERS-CoV, pointing to the persistence of infectivity and virion integrity up to 16 h in aerosols (1.0–3.0 μm). Van Doremalen et al. [3] suggested a similar half-life between SARS-CoV-2 (0.6–2.6 h; 1.1 h on average) and SARS-CoV (0.8–2.4 h; 1.2 h on average). They pointed out that the discrepancies in the epidemiological characteristics could be associated with high viral loads in the upper respiratory tract and transmission of the virus in asymptomatic patients. Smither et al. [75] reported a lifespan of SARS-CoV-2 between 30 and 177 min in aerosols between 1.0 and 3.0 μm , under different conditions of relative humidity (RH), and reported a decay rate between 0.4 and 2.3%/min. Schuit et al. [76] reported independence between the decay rate and the RH of SARS-CoV-2, attributing the loss of infectivity to the effect of sunlight and the aerosol suspension medium. Aligned with the observations of Schuit et al., other authors [77,78] reported similar conclusions using other viruses. There are considerable discrepancies in the literature about viral persistence in the air. A recent study in a preprint published by Oswin et al. [79] showed that 54% of bioaerosols (5–10 μm) loaded with SARS-CoV-2 lose their infective capacity during the first 5 s at low RH (40%). Within 5 min, persistent viruses lose about 19% of their infectivity. At high RH (90%), infectivity falls by 48% progressively during the first 5 min. At 20 min, a 90% loss of infectivity has been found using different variants of SARS-CoV-2. However, these authors replaced the human mucosal base in the aerosols with a Serum with a different composition, so their direct extrapolation is limited.

To date, most environmental sampling has been carried out in hospital settings, which limits the detection of viral RNA due to constant air renewal (~12 ACH) [60,61], which implies a complete air renewal every 5 min. Few studies have included the carbon dioxide (CO_2) concentration variable as an indicator of space ventilation. In the few reports where CO_2 was measured, the concentration was less than 400 ppm [49,59], suggesting that the presence of RNA in aerosols may be underestimated. A SARS-CoV-2-loaded bioaerosols emission rate reduction has been described a few days after the onset of symptoms, which should be investigated to understand the dynamics of the spread of the disease. In this work, the risk of contagion from aerosols in 8 patients is evaluated and compared with transmission by close contact in 19 patients.

2. Materials and Methods

2.1. Patients Included in the Study

In total, 22 COVID-19-positive patients were included in different study groups. In Group A, 5 volunteer patients were included in environmental sampling (aerosols) and

close contact (coughs). In Group B, 14 volunteer patients were enrolled in close contact sampling. In Group C, 3 volunteers were willing to carry out the aerosol sampling. As shown in Table 1, 2/5 patients in Group A were hospitalized, while the rest were in isolation at home. From Group C, no patient was hospitalized, while from Group B, all were hospitalized. The admitted patients were in the Infectious Diseases area or in the Surgery area of the Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain). Patients presenting severe symptoms were excluded. The mean age was 44.2 ± 20.4 years in Group A, 73.6 ± 13.3 years in Group B, and 28.3 ± 18.3 years in Group C. Vaccination status with COVID-19 mRNA vaccines (Pfizer-BioNTech or Moderna) is indicated in Table 1.

Table 1. Characteristics of the included patients.

Patient	Age	Gender	DASO *	Sampling Period	Initial Ct	Final Ct	Vaccines	Hospitalization	Group
Patient 1	59	Male	3 days	1 day	28.1	N/A	3	Yes	A
Patient 2	45	Female	2 days	1 day	29.8	N/A	3	Yes	A
Patient 3	59	Male	2 days	3 days	19.3	30.4	3 ¹	No	A
Patient 4	22	Male	1 day	4 days	23.7	36.1	2	No	A
Patient 5	26	Male	1 day	3 days	23.2	29.3	2	No	A
Patient 6	89	Male	0 days [†]	1 day	16.7	N/A	3	Yes	B
Patient 7	75	Male	0 days [†]	1 day	16.8	N/A	3	Yes	B
Patient 8	61	Male	0 days [†]	1 day	13.5	N/A	3	Yes	B
Patient 9	59	Female	1 days [†]	1 day	32.7	N/A	No	Yes	B
Patient 10	84	Male	2 days [†]	1 day	32.8	N/A	3	Yes	B
Patient 11	63	Male	0 days [†]	1 day	33.1	N/A	3	Yes	B
Patient 12	89	Male	0 days [†]	1 day	28.8	N/A	3	Yes	B
Patient 13	68	Male	1 day [†]	1 day	24.8	N/A	3	Yes	B
Patient 14	69	Female	6 days [†]	1 day	26.1	N/A	No	Yes	B
Patient 15	88	Female	1 day [†]	1 day	34.8	N/A	3	Yes	B
Patient 16	93	Male	1 day [†]	1 day	28.8	N/A	3	Yes	B
Patient 17	88	Male	1 day [†]	1 day	33.3	N/A	3	Yes	B
Patient 18	54	Female	1 day [†]	1 day	21.8	N/A	3	Yes	B
Patient 19	67	Male	7 days ^{††}	1 day	15.8	N/A	3	Yes	B
Patient 20	22	Female	2 days	2 days	27.1	29.2	2	No	C
Patient 21	49	Female	2 days	1 day	29.3	N/A	2	No	C
Patient 22	14	Female	2 days	1 day	21.2	N/A	2	No	C

* DASO: Days after symptoms onset; ¹ The patient inadvertently received the vaccine while infected with the COVID-19 disease; [†] The period refers from the diagnosis; ^{††} The patients were included because they were not vaccinated, despite being diagnosed more than 48 h earlier.

2.2. Nasopharyngeal Exudate

Nasopharyngeal swab was standardized to obtain comparable results. Conventional swabs with virus transport medium (VTM) were used. The swabs were introduced 3.5–4.5 cm up one nostril of the patient and rotated 180° five times. Immediately afterward, they were placed in the transport medium and stored in a -17°C freezer for less than five days.

2.3. Air Sampling

A Coriolis μ environmental sampler (Bertin Instruments, Rockville, MD, USA) was used for air sampling. The particle collection efficiency is very efficient for sizes greater than 500 nm and 50% for sizes less than 500 nm. As shown in Figure 2, the aspirated flow is collected in a buffer solution inside a sterile sampling cone, forming a vortex. The particles and microorganisms are centrifuged on the cone wall and are separated from the air. Typically, a flow rate of 300 L/min was used for 10 min in 3 mL of PBS (3000 L of air: 1000 L/mL). However, to maximize the detection limits of viral RNA, we decided to sample for 30–110 min in some experiments. These samplings were performed at 300 L/min, maintaining a stable amount of 3 mL of PBS (adding solution as it evaporated between 10-min periods). The volunteers were asked to perform regular breathing and speech actions. This was suggested to collect aerosols from different respiratory activities. All samples

were stored in a freezer at -17°C for later analysis in a period not exceeding 5 days. Patients did not use masks during the sampling period.

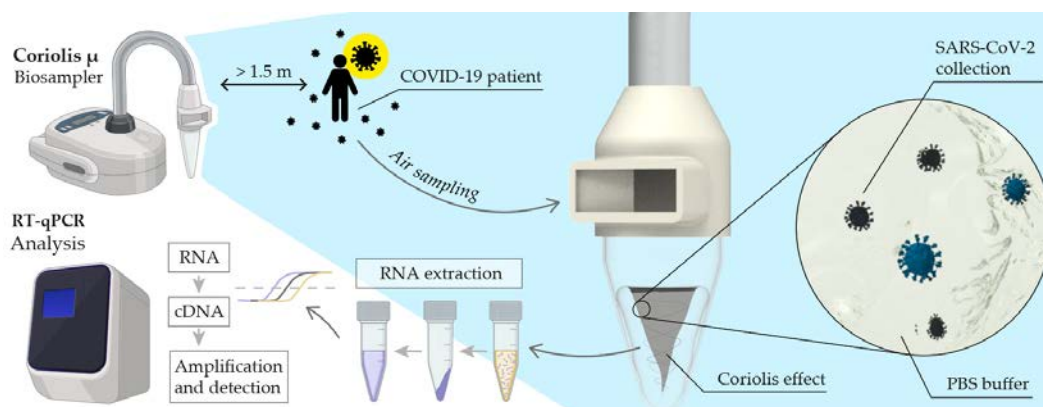


Figure 2. Schematic representation of air sampling and analysis method. Where, PBS is Phosphate Buffered Saline.

Metabolic CO_2 levels were measured using Aranet4 Pro meters (Aranet, Riga, Latvia). These devices are designed with a Non-Dispersive Infrared Detector (N-DIR) meter and record measurements with an accuracy of ± 50 ppm. The meters were placed next to the Coriolis to determine the CO_2 concentration at sampling.

The set-up of the experiments varied depending on whether the patient was hospitalized or quarantined at home. In both cases, the equipment was placed 1.5 m from the patient. It was only placed less than 0.5 m in the case of Patient 3 and Patient 4 to perform additional tests, as detailed in the Results section. The air sampler was located at a distance of at least 1 m from the ground (typically 1.3–1.4 m). As shown in Figure 3, the sampler was placed on mobile tables in the hospital (Figure 3a) and on tables in private homes for quarantined individuals (Figure 3b). This allowed us to position the Coriolis at the desired distance from the patient. The CO_2 m was placed right next to the Coriolis air inlet to measure the CO_2 level of the collected air. At home, patients were placed in small rooms ($>30\text{ m}^2$) and doors and windows were closed to prevent the escape of aerosols. However, they were not completely sealed. Forced ventilation (air conditioning systems) could not be closed in hospitals since it could be dangerous for the rest of the patients and health workers. Patients at home performed the experiments in sitting position, while in the hospital they reclined in bed, positioned with around 135° between legs and abdomen.

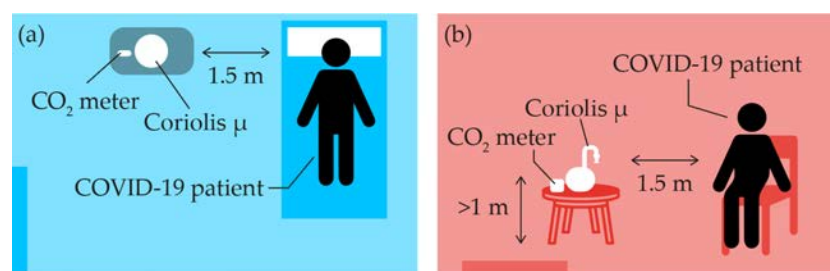


Figure 3. Schematic representation of air sampling set-up at (a) the hospital and (b) in private homes.

2.4. Cough Sampling

In cough samples collection, volunteers were asked to cough three times to clear their throats and, immediately afterward, cough five times into a 5 cm diameter cone containing 1 mL of PBS. The cones were placed 5 cm from the emitter's mouth to simulate close interpersonal distance. To homogenize the samples, they were vortexed for 30 s. Samples were stored in a -17°C freezer and were not kept for more than five days.

2.5. Viral RNA Extraction from Masks

Patients who kept masks from previous days donated them for the study. In some patients, this was not possible since they did not use masks during the sampling period. The masks were chopped and suspended in a 50 mL Falcon tube with 10 mL PBS. The tubes were sonicated in ultrasound for 30 s to favor RNA extraction from the mask's surface. Liquid samples were stored in a -17°C freezer and analyzed within five days.

2.6. RT-qPCR Analysis

All the samples were processed in a biological safety cabinet, complying with the applicable biosafety requirements.

2.6.1. Processing of Environmental Samples

The total sample volume was deposited in Falcon tubes with a 10 kDa Amicon Millipore filter (Millipore, Burlington, MA, USA). They were centrifuged for 15 min at 4000 RPM. Then, 600 μL of the concentrate were transferred to a 600 μL tube of lysis buffer (MagMax Lysis solution, Thermo Fisher, Waltham, MA, USA), homogenized and 200 μL were used for RNA extraction.

2.6.2. Processing of Swabs Samples

The swabs were immersed in 600 μL of lysis buffer (MagMax Lysis solution, Thermo Fisher), and the biological material was detached with rotary movements 3–5 times. They were homogenized and 200 μL was used for RNA extraction.

2.6.3. Nucleic Acid Extraction

According to the manufacturer's instructions, RNA extraction was performed with the MagMax Core RNA/DNA kit (Thermo Fisher) and the KingFisher Flex System automatic extraction kit (Thermo Fisher). The SARS-CoV-2 PCR used 5 μL of extracted RNA and was performed with the validated assay [80] using two key targets (pan-SARS ESAR and SARS-CoV-2 IP4) in a QuantStudio 5 thermocycler (Applied Biosystems, Waltham, MA, USA) [81].

2.7. Identification of SARS-CoV-2 Variants of Concern by Partial Sequencing of the Spike Gene

A pair of primers, F21585 (5'TGCCACTAGTCTCTAGTCAG 3') and R22341.

(5'GCTGTCCAACCTGAAGAA 3'), were designed for the sequencing of the 5' region of the Spike gene that contains the main mutations that characterize the main VOCs of SARS-CoV-2. For this, the reference sequences of the Wuhan-Hu.1 strain (NC_045512), as well as its variants B.1.1.7 (MZ344997), B.1.617.2 (MZ359841), BA.1 (OL672836), and BA.2 (OM296922) were aligned and compared using multiple alignment software MAFFT version 7.

F21585 and R22341 have full homology with all the VOCs studied and generated an amplification product of 756 nucleotides in Wuhan-Hu.1 (NC_045512). The PCR protocol used was 15 min at 45°C , 5 min at 95°C , followed by 40 cycles of 30 s at 95°C , 1 min at 60°C , and 1 min at 72°C , ending with a step of 7 min of extension at 72°C . The PCR product was purified and sequenced using the Sanger technique (StabVida, Caparica, Portugal). The obtained sequences were compared with those cited above as reference sequences of the main VOCs using Clustal Omega v.1.2.2 software (RRID: SCR_001591).

2.8. Determination of the Risk of Infection

Wells and Riley [82] defined the probability of contagion (P) in a susceptible individual according to Equation (1), where n refers to the inhaled amount of the virus infectious doses (expressed in quanta). The SARS-CoV-2 quantum can be described as the probability of infection of $1 - 1/e$ (63%), although it depends on other factors, such as the immunization of the individual, as expressed by Peng and Jiménez [83].

$$P = 1 - e^{-n} \quad (1)$$

Cortellessa et al. [84] adapted this same model to calculate the probability of infection (P) from the RNA dose as expressed in Equation (2). According to this Equation, HID_{63} represents the number of RNA copies needed to initiate the infection with a probability of 63%, estimated in 7×10^2 RNA [85], as discussed in the Results section.

$$P = 1 - e^{-\frac{D_{total}(cv)}{HID_{63}}} \quad (2)$$

Given the limitations in accurately reproducing the conditions in the event of infection, 3 different scenarios will be considered in this work. In the case of determining the risk of infection by large droplets, it will be considered: (1) that the susceptible individual inhales 25% of the droplets, (2) that the susceptible individual inhales 50% of the droplets, and (3) that the susceptible individual inhales 100% of the droplets. Under these scenarios, the interpersonal distance is considered to be narrow and evaporation and gravitational phenomena are neglected to facilitate the estimation. On the other hand, to determine the risk of contagion by aerosols, it will be considered: (1) that the individual breathes contaminated air for 1 min, (2) that the individual breathes contaminated air for 10 min, and (3) that the individual breathes contaminated air for 1 h. In these assumptions, a respiratory flow of 15 L/min will be considered and all the viral RNA detected in the aerosol is infectious.

2.9. Ethical Approval

This study has the approval of the Aragon Community Research Ethics Committee (CEIC Aragón) under references PI20/374 and PI22/130.

3. Results and Discussion

3.1. Ct Value Should Not Be Taken as a Predictor of Infectiousness

Currently, the gold standard for the diagnosis of SARS-CoV-2 infection is the detection of RNA by RT-qPCR, which has the ability to detect target nucleic acids (<100 copies/mL) with remarkable sensitivity [86,87]. The sensitivity varies depending on the stage of the disease the patient is at. The test's sensitivity has been estimated at 33% 4 days after contact with the infected person, 62% on the day of onset of symptoms, and 80% 3 days after onset of symptoms [88,89]. The collection technique, time since exposure, and anatomic location of sampling affect the false-negative rate and efficiency of sampling. Bronchoalveolar lavages have the highest sensitivity (93%) compared to samples taken from the upper respiratory tract, followed by sputum samples (72%), nasal swabs (63%), and throat swabs (32%) [88]. The reference method is nasal exudate due to its accessible collection. Currently, whether the viral load estimate obtained by the Ct value (Cycle Threshold in qPCR analysis) is a determining factor for the outcome of the disease is being discussed [90–93]. However, the quantification of viral RNA is subject to aspects, such as the amount of sample taken, the RT-qPCR kit used, the target used, the thermocycler efficiency, or the storage method [94,95]. For example, RT-qPCR tests that detect more genes have been shown to report lower Ct values [96]. Therefore, individualized interpretation of the test result is necessary. In this work, typified sampling (Section 2.2) has been carried out to reduce the uncertainty between samplings. The mean Ct value was 25.7 ± 7.5 in Group B and 25.7 ± 4.2 in Group C.

A higher concentration of bioaerosols is generated during voluntary coughing, concerning breathing and speech [11]. Cough flows have been observed to be multiphase turbulent clouds with suspended droplets of various sizes [97,98], covering a spectrum from a few nanometers to more than 100 μm [11,99]. Thus, these violent breathing events are critical to infectious diseases spreading [97,100], especially if the pathogens reside predominantly in the upper respiratory tract [101]. Viklund et al. [102] found a higher positivity rate in cough samples (32%) compared to normal breathing (16%). Thus, studying

coughing instead of other respiratory droplets is more efficient for detecting infectious individuals. Viral RNA could only be detected in the coughs of 10.5% (2/19). The two patients who showed detectable viral load in coughing (Patient 4 and Patient 8) presented a Ct of 23.16 and 13.48, respectively. Patient 4 presented a complete vaccination schedule, but without a booster dose (2/3), while Patient 8 was fully vaccinated (3/3). Patient 8's hospitalization made it difficult to follow up due to health problems. However, Patient 4 was able to be sampled on subsequent days, although she only showed positive cough results on the first day of sampling (Table 2). The rest of the patients (17) were negative in coughs, despite presenting lower Ct values.

Table 2. Patient 4 follow-up samples. Where, pan-SARS ESAR refers to Sarbeco E gen detection, and SARS-CoV-2 IP4 refers to RdRp gen detection.

Sample	Amplification	Day 2	Day 3	Day 4	Day 6	Day 7
Nasopharynx	pan-SARS ESAR	25.5 (5.5×10^6 copies/mL)	23.8 (2.0×10^7 copies/mL)	25.9 (4.8×10^6 copies/mL)	33.8 (2.3×10^4 copies/mL)	36.1 (4.9×10^3 copies/mL)
	SARS-CoV-2 IP4	24.6 (3.2×10^6 copies/mL)	23.1 (2.5×10^7 copies/mL)	25.4 (5.4×10^6 copies/mL)	34.4 (1.2×10^4 copies/mL)	Not detected
Oropharyngea	pan-SARS ESAR	29.1 (4.8×10^5 copies/mL)	29.3 (4.8×10^5 copies/mL)	Not detected	Not detected	Not detected
	SARS-CoV-2 IP4	28.9 (3.2×10^5 copies/mL)	29.4 (3.7×10^5 copies/mL)	Not detected	Not detected	Not detected
Coughs	pan-SARS ESAR	28.5 (7.3×10^5 copies/mL)	Not detected	Not detected	Not detected	Not detected
	SARS-CoV-2 IP4	27.4 (9.1×10^5 copies/mL)	Not detected	Not detected	Not detected	Not detected

The viral load found in the cough samples was 7.3×10^5 – 9.1×10^5 copies/mL (Ct 28.5–27.4) and 8.7×10^8 – 6.7×10^8 (Ct 18.2–18.2) in Patients 4 and 8, respectively. These values are higher than Viklund et al. [102] (Ct 29.5–36.5) for patients with Ct between 17.2–26.4. However, Patient 8 showed a higher viral load, although it cannot be compared due to a much lower Ct in the nasopharyngeal exudate.

A correlation between Ct and the infectivity of individuals has been previously reported [103,104]. This indicator has even been taken as an epidemiological prediction tool [105]. However, a clear relationship between patient infectiousness and viral load has not been found in this work. Patients 6, 7, and 19, with Ct values of 16.7, 16.8, and 15.8, were negative for cough samples, while Patient 4 with a Ct of 23.2 emitted SARS-CoV-2-loaded droplets under the same terms. Another case of interest is Patient 5, with characteristics similar to Patient 4, in terms of age and viral load. As seen in Table 3, no viral RNA was detected in either the patient's oropharyngeal exudate or cough samples.

Table 3. Patient 5 follow-up samples. Where, pan-SARS ESAR refers to Sarbeco E gen detection, and SARS-CoV-2 IP4 refers to RdRp gen detection.

Sample	Amplification	Day 2	Day 3	Day 4
Nasopharynx	pan-SARS ESAR	25.1 (7.8×10^6 copies/mL)	23.3 (2.7×10^7 copies/mL)	28.7 (6.7×10^5 copies/mL)
	SARS-CoV-2 IP4	24.6 (8.9×10^6 copies/mL)	22.9 (2.8×10^7 copies/mL)	28.3 (7.4×10^5 copies/mL)
Oropharyngeal	pan-SARS ESAR	Not detected	Not detected	Not detected
	SARS-CoV-2 IP4	Not detected	Not detected	Not detected
Coughs	pan-SARS ESAR	Not detected	Not detected	Not detected
	SARS-CoV-2 IP4	Not detected	Not detected	Not detected

Patients 1, 2, and 5 wore masks the days before sampling. These masks were analyzed by PCR for SARS-CoV-2 detection. The only positive sample was obtained from Patient 1, who presented a Ct of 28.1 at sampling. He was negative on cough and air, although the surgical mask he had worn for the previous three days was positive, with an average viral

load of 29.5 (3.7×10^3 copies/mL). These results indicate that Patient 1 was infectious on previous days.

3.2. Detection of SARS-CoV-2-Laden Bioaerosols

The average Ct value was 24.6 ± 4.3 in Group A and 25.7 ± 4.2 in Group C. A total of 88 air samples from eight different patients from group A and C were collected. All patients included in this subgroup were infected with the variant of concern (VOC), Omicron BA.1 (Supplementary Materials, Table S1). Only seven air samples (8.0%) were detectable for SARS-CoV-2 RNA levels and another eight samples were suspicious (9.0%; Ct > 38). As seen in Table 4, the 15 positive/suspicious samples were collected from the same patient (Patient 20), although on the second day, the samples were taken in the company of two other patients (Patient 21 and Patient 22), since they constituted a coexisting unit.

Table 4. Air samples where viral RNA has been detected. Where, pan-SARS ESAR refers to Sarbeco E gen detection, and SARS-CoV-2 IP4 refers to RdRp gen detection.

Sample	CO ₂ Levels	Amplification	Patient 20
			Day 1
1	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	36.7 (1.1×10^3 copies/mL) Not detected
2	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	36.6 (2.3×10^3 copies/mL) 36.8 (2.0×10^3 copies/mL)
3	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	37.2 (1.6×10^3 copies/mL) Not detected
4	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	38.9 (Suspicious) 38.8 (Suspicious)
5	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	Not detected 38.7 (Suspicious)
6	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	37.9 (9.6×10^2 copies/mL) Not detected
7	1000–1500 ppm	pan-SARS ESAR SARS-CoV-2 IP4	37.2 (1.5×10^3 copies/mL) 39.2 (Suspicious)
8	1000–1500 ppm	pan-SARS ESAR SARS-CoV-2 IP4	38.8 (Suspicious) 38.3 (Suspicious)
9	1000–1500 ppm	pan-SARS ESAR SARS-CoV-2 IP4	39.8 (Suspicious) Not detected
10	1500–2000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	35.5 (4.8×10^3 copies/mL) 36.3 (Suspicious)
11	1500–2000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	37.0 (1.8×10^3 copies/mL) 38.3 (Suspicious)
			Patient 20–22
			Day 3
12	500–1000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	39.0 (Suspicious) Not detected
13	1000–1500 ppm	pan-SARS ESAR SARS-CoV-2 IP4	Not detected 38.9 (Suspicious)
14	1500–2000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	39.0 (Suspicious) Not detected
15	1500–2000 ppm	pan-SARS ESAR SARS-CoV-2 IP4	39.5 (Suspicious) Not detected

In the samples collected on the first day of the trial (2 days after the onset of symptoms), positivity was found in 68.8% (11/16) of the samples, with three suspicious samples. However, on the second day of the trial (4 days after symptom onset), suspicion was only found in 14.8% of samples (4/27). The aerosol viral load results (1.1–4.8 copies/m³) were consistent with those previously reported in the literature [24,31–53]. Given the airborne persistence of SARS-CoV-2, it is important to consider that viral RNA may have been collected from inactivated viruses. Therefore, they would not have the ability to infect humans. Since it is not possible to make precise estimates for the percentage of viable virus, the maximum possible persistent viral load will be considered in this work.

The global acceptance of the COVID-19 airborne spread allowed for an improvement in the preventive strategies, including new techniques for epidemiological management, such as the measurement of exhaled CO₂ as an indicator of the air renewal and, consequently, the risk of contagion [35,83]. The presence of viral aerosols in some infected individuals makes it necessary to implement air control measures in collective environments.

Even under poor ventilation conditions, the remaining patients did not shed detectable viral RNA. Patient 3 (Table 5), with a Ct of 19.1, achieved 2400 ppm metabolic CO₂, implying about ~5% air coming from the individual. However, this same patient tested negative for cough samples on days 2, 4, and 6 after symptom onset (Ct 19.1, Ct 30.6, and Ct 32.6, respectively), despite presenting viral load in the oropharyngeal swab on day 2 (Ct 21.2).

Table 5. Patient 3 follow-up samples. Where, pan-SARS ESAR refers to Sarbeco E gen detection, and SARS-CoV-2 IP4 refers to RdRp gen detection.

Sample	Amplification	Day 2	Day 4	Day 6
Nasopharynx	pan-SARS ESAR	19.9 (1.3×10^7 copies/mL)	30.8 (1.4×10^4 copies/mL)	33.4 (2.5×10^4 copies/mL)
	SARS-CoV-2 IP4	19.1 (2.5×10^7 copies/mL)	30.6 (1.5×10^4 copies/mL)	32.6 (3.7×10^4 copies/mL)
Oropharyngeal	pan-SARS ESAR	21.9 (3.7×10^6 copies/mL)	Not detected	38.9 (Suspicious)
	SARS-CoV-2 IP4	21.2 (5.9×10^6 copies/mL)	Not detected	37.2 (1.6×10^3 copies/mL)
Coughs	pan-SARS ESAR	Not detected	Not detected	Not detected
	SARS-CoV-2 IP4	Not detected	Not detected	Not detected

Patient 4, with a Ct of 24.6 (day 2 after the onset of symptoms), showed positive coughs and positive oropharyngeal exudate (Ct 28.9). However, the aerosol samples were negative, even in a unique sampling where they were concentrated at 33,000 L of air in 2 mL PBS, and the patient was kept talking and breathing at 50 cm distance from the Coriolis. As a control, the collection cone wall and the air inlet were sampled with a swab, which also tested negative for SARS-CoV-2. Similar tests were performed on Patient 3, where he used the Coriolis at less than 20 cm and sang for 10 min, but viral RNA was not detected.

In addition to rapid rates of loss of infectivity [79], the airborne viral load of SARS-CoV-2 is reduced. Although the published studies cannot be directly compared due to the variation in conditions, the reported concentrations point to a viral load between 5.0 and 11.9×10^3 copies/m³ of air (Table S2), and an equivalent mean concentration of 3.1 ± 2.9 copies/L of air is deduced from a total of 3085 samples (313 positives; 10.2%). However, it is necessary to consider that the values should be corrected according to the efficiency of each sampling device. Thus, our results for aerosolized viral load (1.1–4.8 copies/m³) in samples collected from Patient 4 were consistent with those previously reported.

3.3. Do Superspreaders Predominate in the Emission of Bioaerosols with SARS-CoV-2?

Uncertainty persists about the interrelationship between the exposure environment and the transmission networks of the SARS-CoV-2 virus. According to the CDC, droplet transmission (close contact) is predominant, while fomites and aerosols explain special propagation events in punctual events [106]. The role of individuals in the asymptomatic phase has accounted for a substantial portion of infections (Table S3) [107–111]. It has been estimated that 44% (95% CI; 30–57%) of secondary cases became infected during the incubation period [112]. The heterogeneity of infectious disease transmission is a well-known concept that has been studied in various epidemic scenarios. Woolhouse et al. [113] identified a statistical pattern, known as the 20/80 rule, which indicates that 80% of new infections are associated with only 20% of those infected. According to this concept, there is a central high-risk group that may be associated with the massive expansion of the infectious disease [114–116]. In the case of SARS-CoV-2, the dynamics of the spreads pointed to similar conclusions [117,118]. Even the central high-risk group has been reduced to 10–21% of cases [118,119]. Heterogeneity in transmission has already been observed in the dynamics of other coronaviruses, such as SARS-CoV [117,120,121]. Moreover, other respiratory viruses with similar viral characteristics, such as H1N1, do not exhibit these patterns of spread [122].

Sustained superspreading events could explain the massive infection of individuals [123,124]. However, it may also be related to individuals' environmental, behavioral and social factors that influence the dynamics of virus transmission, which could depend on the configuration of specific outbreaks [125]. Beldomenico [126] proposed that the superspreaders might not be random and depend on other superspreaders. Then, superspreaders' secondary infections are more likely to rise to new superspreaders. Although the origin of superspreading is unknown in detail, it is mainly attributed to physiological issues of the individual, making the infectiousness of the individual challenging to predict [127]. A study by Edward et al. [128], on the aerosol emission rate of 200 healthy individuals, emphasized that biological differences could affect virus transmission. The work reported that 20% of the study participants accounted for 80% of the aerosols emitted.

A substantial number of reported COVID-19 cases are from superspreader events, where secondary cases are disproportionately higher than expected based on basic reproduction number (R_0) [129]. In this study, of the four infected individuals who were quarantined, only two had a viral load in coughs and only one in aerosols emitted. The nuclear families of the three individuals who were not infectious through aerosols did not become infected, while Patient 4 infected both her family ($n = 3$) and her partner's family ($n = 4$). Our results point to reduced contagiousness in the incubation period and a predominant role of superspreaders when the disease is already established.

Although SARS-CoV-2 can be explained by the high viral spread of superspreaders [126], it remains unknown what determines individual overdispersion in transmissibility, so it is not possible to discriminate between superspreaders and non-spreaders [115]. Then, a broad understanding of the role of individual variation in the ecology of respiratory viruses in controlling infection transmission is still required.

3.4. Probable Time Required to Inhale SARS-CoV-2 Bioaerosols to Undergo an Infection

The information on the infective doses sufficient to cause infections with SARS-CoV-2 is limited and subject to the study model. It is known that viral load plays a relevant role in viral kinetics in aerial pathogen transmission [130]. Fain et al. [131] found that high initial inocula lead to brief infections but with higher peak viral titers (10^6); smaller initial inocula (10^1) reduce the peak viral titer but make the infection last longer. The estimated dose-infection effect for humans has been determined in vitro. Zhang et al. [132] mathematically determined an infective constant (k) of 6.4×10^4 to 9.8×10^5 copies for the onset of infection. In contrast, Therese et al. [133] reported a minimum dose of 4.0×10^4 copies for an in vitro infection in Vero B4 cells. Only 21% of the 109 SARS-CoV-2 samples isolated

from patients were infectious enough to initiate a new infection. A recent study by Killingley et al. [134] studied the course of infection in human volunteers. Thirty-six volunteers were intranasally infected with 10 TCID₅₀ (SARS-CoV-2/human/GBR/484861/2020), reaching peak RNA levels of 5 dpi (~8.9 log₁₀ copies/mL) and infecting 18/36.

Other studies with the same objective have been carried out in animal models (Table S4). For aerosols, the median aerosol infectious dose tested in small animals (hACE2 mice) is 630 copies (infection rate 2/2) [135], while in large animals (African Green Monkeys), it is 2.0×10^3 copies (infection rate 2/2) [107]. Infections have been reported from 500 copies (infection rate 1/6 in ferrets) [136] to 3×10^6 copies (8/8 African Green Monkeys) [137] by intranasal inoculations. Some cases have been reported where a lower attack rate has been obtained with higher viral loads; for example, inoculations of 7.0×10^4 copies in mice (hACE2) infected only 7/19 mice [138] and 7/9 Syrian hamsters [139], while in ferrets, an attack of 6/6 ferrets with a dose of 5.0×10^4 copies [136].

According to the literature (Table S4), the median infective dose demonstrated was 1.0×10^3 copies to infect 100% of the animals included in the study [135], while Gale et al. [85] estimated 7.0×10^2 copies to infect 63% of human individuals (HID_{63}). However, the infection dynamics are variable from individual to individual, and probabilistic adjustments are limited [140]. By working out the dynamics of the infection in probabilistic terms, it is possible to find infections caused by a single virus, although the probability is minimal [141].

Considering an average persistent viral load of 3.1 ± 2.9 copies/L and a homogeneous concentration of viral particles in the environment and a HID_{63} of 7.0×10^2 RNA copies, if we assume that 100% of the viruses are viable, breathing 15 L of air (1 min), the probability of infection (P) is 0.68, while it reaches 0.99 and 1.0 after inhaling 150 (10 min) and 900 L (1 h). According to this model, a contagion probability of 1.0 would be reached after 15 min of breathing. However, it would be necessary to apply several correction factors that include, for example, the percentage of viruses that do not persist (and, therefore, are not infectious) or the probability of inhalation of these contaminated aerosols.

3.5. Viral Spreading Is Heterogeneous

Assuming an average viral load of 8.2×10^5 copies/mL found in the cough samples of this work, there is a concentration (c_p) of $4.9 \times 10^6 \pm 6.1 \times 10^6$ aerosols per cough in individuals infected with respiratory viruses [142], with an average aerodynamic radius (r) of $0.4 \mu\text{m}$ [11]. Given the variability between individuals [112], the maximum deviation equates the spread by aerosols ($=1.1 \times 10^7$). Under these assumptions, in each cough, a maximum liquid volume of aerosols (v) of 2.95×10^{-4} mL is considered, according to Equation (3).

$$v = \frac{4}{3} \pi r^3 c_p, \quad (3)$$

Note that for this estimate, a perfect sphere has been considered instead of an irregular droplet, an average aerodynamic diameter of $0.8 \mu\text{m}$ (rather than a distribution of particle sizes), and an average amount of aerosols disseminated in coughs in patients infected with H1N1.

Following this approach, Patient 4 was able to shed 242 copies of RNA in his cough, while Patient 8 was able to shed around 115,050 copies. If 100% of cough droplets had been inhaled, the probability of infection would be 0.98 in Patient 4's cough, while in Patient 8, it would be 1.0. In the first case, the viral load resulting from coughing would be equivalent to breathing air for around 10 min at a flow of 15 L/min, with an assumed homogeneous concentration of 3.1 ± 2.9 copies/L. However, in the second case, it would be equivalent to breathing air loaded with SARS-CoV-2 for a minimum of 41 h.

4. Conclusions

The spread patterns of SARS (2003) could not be explained by conventional epidemic models, which assumed homogeneity of transmission. In the same way, SARS-CoV-2 has shown a propagation dynamic that is difficult to parameterize. This variability in the viral spread between people could explain the different growth rates of infected people between populations, although the cause of this variability is still unknown.

In this work, it has been found that the Ct value cannot be considered as an indicator of the individual's infectiousness, since it has not been possible to correlate with the viral load disseminated in aerosols and coughs. Given the limited literature in this regard and the variability in sampling and diagnostic equipment, this parameter should be reconsidered in matters of epidemiological prediction. In our study, patients with a very low Ct (>15) shed less (or no) viral load, while patients with a Ct of up to 27 generated high viral concentrations in the environment.

The viral load in coughs was different between the samples of the two individuals ($n = 19$) that were positive. Concentrations between 7.3×10^5 to 8.7×10^8 copies/mL were found, implying an approximate viral load of between 1.1×10^2 and 1.0×10^5 copies/cough. In this subgroup, the mean Ct was 25.7 ± 7.0 , and the patients who disseminated viral load had a Ct of 23.2 and 13.5. In this case, the patient with the lowest Ct (13.5) had the highest viral load spread in coughs (8.7×10^8 copies/mL). However, not all patients with a positive Ct in oropharyngeal samples coughed up the viral load. In one of the patients with positive sampling, it was possible to carry out a follow-up, where a loss of infectiousness was observed from the third day after the onset of symptoms.

Only one patient shed detectable viral RNA in the air. Additionally, he was the only one who infected seven other individuals. Of the rest of the patients, there is no evidence of secondary infections. The viral load found in the air (1.1–4.8 copies/m³) was consistent with that previously reported in the literature. It was considerably reduced from the first to the third day after symptom onset. In patients with high rates of viral shedding, a cough can be equivalent to 10 min of breathing, making more necessary to improve ventilation and air purification strategies in shared indoor spaces.

It is still necessary to understand the role of the different routes of spread in COVID-19 spreading, considering the influence of parameters, such as the vaccinated population rate and their immunity or the predominant variants. Specifically, knowledge about viral dynamics in aerosols and superspreading events is essential for understanding the spread heterogeneity and better managing future pandemics. Since a low SARS-CoV-2 virus load can initiate an infection, the task of designing epidemiological prediction models is complex, despite being necessary to manage infectious diseases more efficiently in the future.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/jcm11092607/s1, Table S1: Partial sequencing of the viral genome from aerosol-subgroup patients; Table S2: Viral load detected in aerosols per m³ of air using different methods; Table S3: Some superspreading events; Table S4: Dose–response characterization using animal models. References [18,36,38–49,51–53,55,58,129,135–139,143–183] are cited in the supplementary materials.

Author Contributions: Conceptualization, M.B. and A.J.S.; Data curation, J.J.A.; Formal analysis, M.B., A.G., J.J.A. and A.J.S.; Funding acquisition, J.J.A. and A.J.S.; Investigation, M.B., A.G., J.J.A. and A.J.S.; Methodology, M.B.; Resources, A.G.; Supervision, A.G. and A.J.S.; Validation, M.B.; Writing—original draft, M.B. and A.J.S.; Writing—review & editing, A.G. and J.J.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Instituto de Investigación Sanitaria Aragón: Campaña Investiga COVID-19 (CoviBlock).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Aragon Community Research Ethics Committee (CEIC Aragón) under reference PI20/374 and PI22/130.

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

Acknowledgments: We would like to express our gratitude to the Department of Infectious Diseases (Hospital Clínico Universitario Lozano Blesa) for supporting this research. We would also like to thank ‘Exopol SL’ and ‘Institute for Health Research Aragón’ for the support. A.J.S. contribution honors the memory of J.L.R.

Conflicts of Interest: The authors declare no conflict of interest.

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Silver Nanoparticles–Polyethyleneimine–Based Coatings with Antiviral Activity against SARS–CoV–2: a new method to functionalize different substrates

Materials, 15 (14), 4742

<https://doi.org/10.3390/ma15144742>

Factor de impacto en 2022: 3.748

Factor de impacto a 5 años: 4.042

Cuartil JCR (2021): Q1

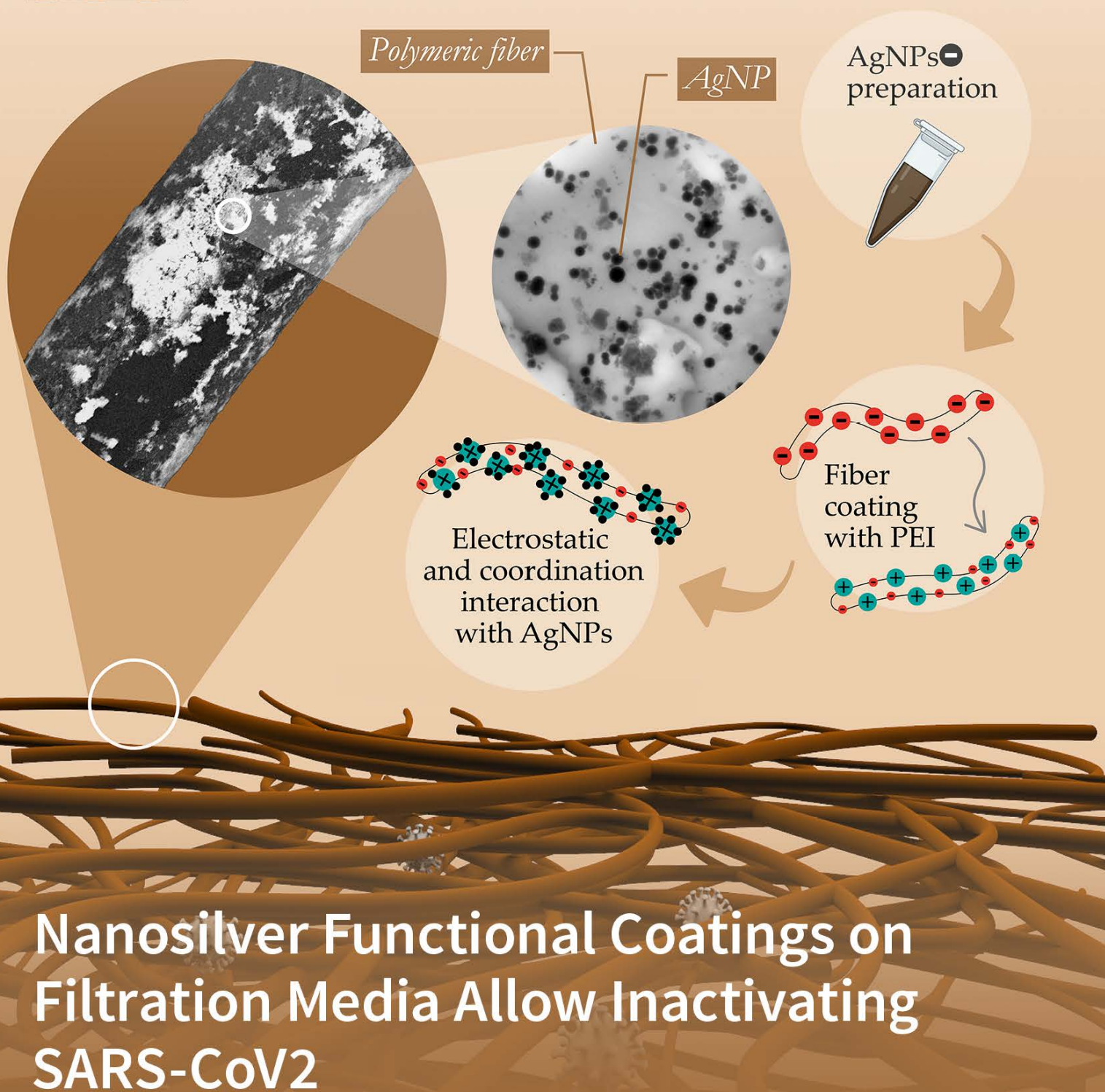
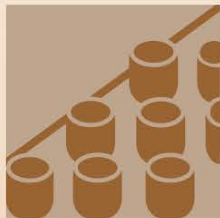
Cuartil JCR (2020): Q1

Fecha de envío: 7 de junio de 2022

Fecha de publicación: 6 de julio de 2022

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Este artículo fue seleccionado como **portada de la Materials** en el volumen 15, de julio de 2022.



Article

Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: A New Method to Functionalize Filtration Media

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Citation: Baselga, M.; Uranga-Murillo, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: A New Method to Functionalize Filtration Media. *Materials* **2022**, *15*, 4742. <https://doi.org/10.3390/ma15144742>

Academic Editor: Alexandru Mihai Grumezescu

Received: 7 June 2022

Accepted: 4 July 2022

Published: 6 July 2022

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Abstract: The use of face masks and air purification systems has been key to curbing the transmission of SARS-CoV-2 aerosols in the context of the current COVID-19 pandemic. However, some masks or air conditioning filtration systems are designed to remove large airborne particles or bacteria from the air, being limited their effectiveness against SARS-CoV-2. Continuous research has been aimed at improving the performance of filter materials through nanotechnology. This article presents a new low-cost method based on electrostatic forces and coordination complex formation to generate antiviral coatings on filter materials using silver nanoparticles and polyethyleneimine. Initially, the AgNPs synthesis procedure was optimized until reaching a particle size of 6.2 ± 2.6 nm, promoting a fast ionic silver release due to its reduced size, obtaining a stable colloid over time and having reduced size polydispersity. The stability of the binding of the AgNPs to the fibers was corroborated using polypropylene, polyester-viscose, and polypropylene-glass spunbond mats as substrates, obtaining very low amounts of detached AgNPs in all cases. Under simulated operational conditions, a material loss less than 1% of nanostructured silver was measured. SEM micrographs demonstrated high silver distribution homogeneity on the polymer fibers. The antiviral coatings were tested against SARS-CoV-2, obtaining inactivation yields greater than 99.9%. We believe our results will be beneficial in the fight against the current COVID-19 pandemic and in controlling other infectious airborne pathogens.

Keywords: silver nanoparticles; polyethyleneimine; SARS-CoV-2; antiviral coating; facemask; filter

1. Introduction

The pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been characterized by a rapid spread between populations due to its high efficiency of viral transmission [1]. The global acceptance of the spread of COVID-19 by aerosols improved a preventive approach, including the mandatory use of filter half masks (KN95) or surgical masks in most countries and the purification of indoor air using filtration systems. It has been reported that the use of masks has reduced by up to 70% the chances of potential infections caused by SARS-CoV-2 [2–4]. However, the efficiency of surgical masks is compromised against submicron particle sizes [5], including SARS-CoV-2-loaded

bioaerosols [6]. Particle leaks between 60% and 80% have been reported with surgical masks as personal protective equipment, which could be explained mainly by the material filtering ability and the poor facial fitting [7–10]. Half-masks are theoretically designed to provide a perfect facial fit, although in practice this is questionable [11–13]. A Centers for Disease Control and Prevention (CDC) report evaluating 21 KN95 masks on 25 volunteers revealed leaks between 6% and 88% of aerosolized particles [14]. In agreement with these results, Park et al. [15] reported leaks up to 21.1% in 3M KN95 masks and up to 73.3% in KF94 masks of Korean origin.

Research efforts towards developing effective filter systems, both as personal protective equipment (PPE) and in air conditioning systems, in the fight against COVID-19 have been directed towards the incorporation of materials with antiviral and antimicrobial properties. Mainly derived from nanotechnology, nanostructured metallic compounds have been the object of special attention in the virucidal functionalization of masks and filters. Examples of those materials include silver, graphene, copper, and zinc [16]. Specifically, silver has been incorporated into various surfaces due to its historical use as an antimicrobial agent [17] and its approval by the FDA in several drugs and devices used in clinical settings, including their use in different antimicrobial medical devices (e.g., catheters), wound dressings, and medical textiles [18]. Specifically, nanoparticulated silver (AgNP) shows an obvious advantage over its analog on a metric scale due to its high surface-to-volume ratio and easy fabrication [19,20]. AgNPs act as a reservoir of ionic silver; the nanoparticles oxidize, releasing the antimicrobial ion while leaving the nanoparticles surface having available chemisorbed ionic silver and being the remaining core prone to successive oxidation cycles. The potential antimicrobial effect depends, among other factors, on the physicochemical parameters that NPs present, including size, shape, surface charge, surface functionalization, specific surface area, concentration, and colloidal oxidation state [21]. Although its ability to destroy microorganisms has been widely demonstrated against gram-negative and gram-positive bacteria [22–25], its virus inactivation ability has been less reported [26,27].

Regarding the COVID-19 pandemic, the interaction of nanosilver with the responsible encapsulated virus has been studied in-depth, proposing two different theories. On the one hand, (1) AgNPs bind to the virus capsid, inhibiting its binding ability to receptor cells. On the other hand, (2) AgNPs bind to viral RNA, inhibiting its replication in host cells. The combined hypothesis of the binding of AgNPs to the spike protein of the SARS-CoV-2 virus at the lipidic envelope and the generation of a hostile environment in the respiratory epithelium, associated with a pH decrease due to the release of silver ions, has also been reported [28]. Although the antiviral mechanism is unknown with precision, colloidal silver has shown high efficiency in the inactivation of the SARS-CoV-2 virus [29,30], presenting better antiviral behavior than other nanostructured metals. Specifically, Merkl et al. [31] reported that silver nanoparticles reduced 98% of the SARS-CoV-2 viral load after 2 h, while copper nanoparticles achieved only 76% reduction and zinc nanoparticles did not show antiviral efficacy. The antimicrobial efficiency of AgNPs has been also reported once incorporated into different filter materials against bacteria and viruses [17,32,33]. Even though several models of masks that incorporate nanosilver are currently marketed, a recent report points out that only ~50% of commercial masks that advertise the incorporation of silver nanoparticles had substantiated antiviral and antibacterial claims [18]. An increase in the antimicrobial effectiveness of nanostructured silver has been demonstrated in numerous research articles when combined with other antimicrobial agents, such as carbon nanotubes [34–36], carbon nitride [37], chitosan [38], or polyethyleneimine (PEI) [39]. Specifically, this last conjugation has shown excellent antimicrobial activity against *S. aureus* and *K. pneumoniae* [39].

This paper reports a new, easy, and affordable method to fabricate a coating based on silver nanoparticles. AgNPs could be toxic to the humans at high doses due to their oxidative stress generation, DNA damage, and cytokine induction production. Consequently, a stable chemical bonding is essential to ensure the stability of the particles on the fibers and

to avoid any potential detachment towards the respiratory tract when incorporated in face masks or in other filtration systems. Polyethyleneimine is a polycationic polymer made from ethyleneimine monomers. By suspending negatively charged polymeric fibers in this polymer, their charge is reversed, becoming positively charged. A strong supramolecular interaction by electrostatic interaction with negatively charged AgNPs is then guaranteed. In addition, the functional amine groups of PEI act as ligands and donate an unshared electron pair to Ag to create a coordinate covalent bond which further improves their strong attachment [40]. As a preventive strategy against the spread of SARS-CoV-2, the method reported herein has five key aspects: (1) industrial viability because of its simplicity, (2) high antiviral efficiency, (3) stable union on different polymeric filtering media, (4) homogeneity in the coating, and (5) preservation of the filtering and breathability properties of the original material. In this work, the coating was applied on different polymeric substrates based on fibers of variable diameters composed of polypropylene, polyester-viscose, and polypropylene-glass, corresponding to surgical masks, hygienic masks, and air conditioning filters of medium and high efficiency of filtration, respectively. The stability of the binding of AgNPs on the polymeric fibers was explored using different techniques. Finally, the inactivation efficiency against the SARS-CoV-2 virus was validated by using a viral isolate obtained from a COVID-19 patient.

2. Materials and Methods

2.1. Synthesis Materials and Procedures

The synthesis of silver nanoparticles was carried out by slightly modifying the method of Goli et al. [41] (Table 1) to obtain the smallest possible particle diameter to facilitate oxidation. Briefly, an aqueous solution of 1.0 mM silver nitrate (AgNO_3) and 1.0 mM tribasic sodium citrate dihydrate (TSC) were dissolved and kept under vigorous stirring (~ 1250 rpm) for 5 min. Subsequently, 1.2 mM sodium borohydride (NaBH_4) was added dropwise, and the mixture was stirred for 1 h. Finally, the colloidal dispersion of negatively charged AgNPs was washed by centrifugation at 13,000 rpm for 15 min and at 15°C . The supernatant was removed, and the pellet redispersed in Milli-Q water until the desired colloidal concentration was achieved (Figure 1). AgNO_3 (CAS 7761-88-8), TSC (CAS 6132-04-3), and NaBH_4 (CAS 16940-66-2) were purchased from Sigma-Aldrich (Darmstadt, Germany) and used as received.

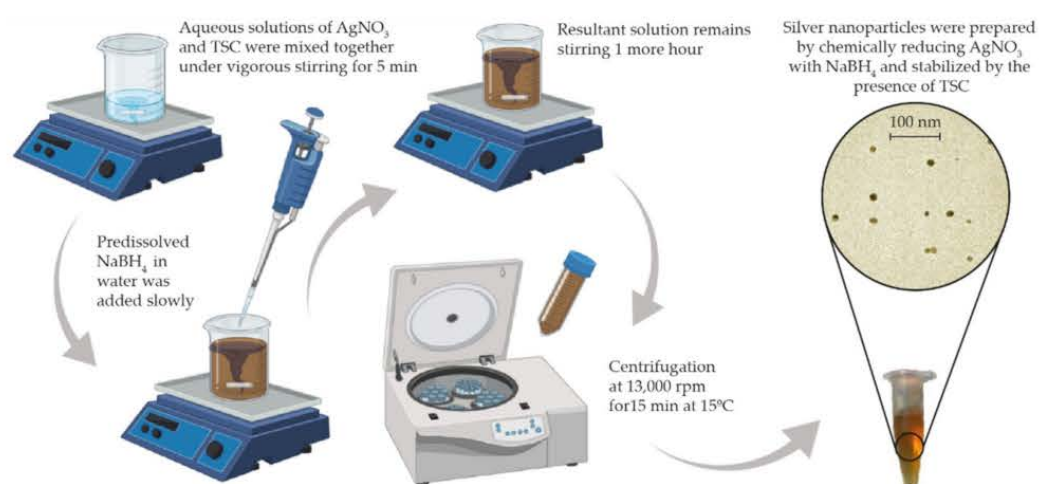


Figure 1. Schematic representation of the silver nanoparticle synthesis method.

During the optimization of the synthesis method, process parameters such as stirring speed, solute concentration, and NaBH_4 reagent mixing mode were varied, obtaining six differentiated syntheses (Table 1). It was decided to select the smallest particle size possible to increase the NPs' virucidal activity, considering that the smaller the size, the faster the oxidation rate and the consequent ionic silver release [22,42].

Table 1. Optimized parameters from the original AgNPs synthesis.

Synthesis	Molar Concentration	Mixing Process	Stirring Speed
Original	2 mM AgNO ₃ ; 2 mM TSC; 2.4 mM NaBH ₄	Not defined	‘Vigorous’
A	2 mM AgNO ₃ ; 2 mM TSC; 2.4 mM NaBH ₄	Drop by drop	~1250 rpm
B	1 mM AgNO ₃ ; 1 mM TSC; 1.2 mM NaBH ₄	Drop by drop	~1250 rpm
C	2 mM AgNO ₃ ; 2 mM TSC; 1.2 mM NaBH ₄	Drop by drop	~1250 rpm
D	1 mM AgNO ₃ ; 1 mM TSC; 2.4 mM NaBH ₄	1 mL in 1 mL	~1250 rpm
E	2 mM AgNO ₃ ; 2 mM TSC; 2.4 mM NaBH ₄	3 mL in 3 mL	~1250 rpm
F	1 mM AgNO ₃ ; 1 mM TSC; 2.4 mM NaBH ₄	Drop by drop	~1500 rpm

2.2. Nanoparticle Attachment to Polymeric Fibers

The coated polymeric substrates were based on polypropylene, polyester-viscose, and polypropylene combined with fiberglass. Specifically, we used spunbond fabric (polypropylene) from a surgical mask, spunlace fabric (polypropylene) from a surgical mask, filter material (polypropylene) from an F9 air conditioning filter, viscose polyester-based fabric from a hygienic mask, and combined polypropylene-glass fibers from a HEPA H13 filter. The hygienic mask fabric was obtained from Ditex SL (Zaragoza, Spain). The H13 and F9 filters were kindly provided by Camfil España SA (Madrid, Spain), and the surgical masks were purchased at a local pharmacy.

Polyethyleneimine (CAS 904759) purchased from Sigma-Aldrich (Darmstadt, Germany) was used for the chemical bonding process. Briefly, PEI was dissolved in an aqueous solution at variable concentrations either 5 or 10 mg/mL. The fabric samples were immersed in the solution for 30 min, dried in the open air for 10 min, and subsequently washed with deionized water to remove all PEI in excess. After washing, the samples were suspended in a colloidal dispersion of AgNPs at 1, 2.5, and 5 mg/mL in an amount not less than 0.7 mL/cm² for 1 h at room temperature. Finally, the samples were retrieved, dried at room temperature for 10 min, and thoroughly washed with deionized water to detach potential non-adhered nanoparticles.

2.3. Particle Size Measurements

Morphologic characterization was carried out using a T20-FEI transmission electron microscope (FEI Company, Hillsboro, OR, USA). TEM samples were prepared by depositing 50 µL of Milli-Q water dispersed colloid on a formvar-coated copper TEM grid and dried for at least 2 h. The nanoparticle size distribution histograms were calculated by measuring the particle diameters from the TEM images with the ImageJ software (National Institutes of Health, Bethesda, MD, USA, v1.53) and using basic statistical analysis ($n = 100$).

2.4. Ultraviolet-Visible Spectroscopy (UV-Vis)

The extinction spectra of the resulting silver colloids were recorded using a Jasco V670 UV-Vis spectrophotometer (JASCO International Co. Ltd., Tokyo, Japan) at the maximum absorbance around 400 nm. Aliquots of the analyzed samples were obtained at different times (1, 3, 5, and 7 days). Monodispersion in the particle sizes was evaluated by direct observation of the amplitude and the full width at half the maximum of the absorbance peak. Over a week, the stability over time was analyzed based on the interposition of the curves of the absorbance peaks obtained.

2.5. Scanning Electron Microscopy with Energy-Dispersive X-ray Analysis (SEM/EDX)

The morphology of the AgNPs-PEI coated fibers was observed using a CSEM-FEG Inspect 50 Field Emission Scanning Electron Microscope (SEM) (FEI Company, Hillsboro, OR, USA) at a high vacuum with an acceleration voltage of 10 kV. For sample preparation, a sample of the corresponding resulting coating was placed on a slide, fixed with carbon tape to a SEM microscope holder, and air-dried.

2.6. Bonding Stability Evaluation Using Microwave Plasma Atomic Emission Spectroscopy

Quantifiable potential particle detachment analyses were performed using MP-AES (4100 MP-AES, Agilent Technologies, Santa Clara, USA). These tests were carried out on the coated polymeric substrates (polypropylene fibers, spunbond, and surgical masks) to test the stability of the coating against different boundary conditions. For the application of mechanical stress, the coated samples were cut out and initially weighed and weighed again after the test to evaluate the mass loss by mass balance. The samples were tested using two complementary techniques: (a) the quantification of the initial amount of AgNPs impregnated in the fabric and (b) the quantification of the AgNPs released after sonication. Those analyses were carried out from digestions with aqua regia (HCl, 37%; HNO₃, 67%, 1:5). In analysis (1), fabric samples having Ag-NPs impregnated were immersed in 1 mL of aqua regia for 30 min. Once digested, they were diluted in 5 mL of Milli-Q water and filtered through 0.2-micron filters. On the other hand, in analysis (2) the supernatant collected after 30 min of sonication (0.5 mL) was digested in 1 mL of aqua regia. Subsequently, it was diluted in 5 mL of Milli-Q and filtered through a 0.2-micron filter. Boundary conditions and the sonication times were varied. Reference sample corresponds to the aqua regia digestion of the fabric samples coated with AgNPs after 20 s of sonication in Milli-Q water used as base value. In order to standardize the results obtained, the samples were measured and weighed after carrying out the tests. The boundary conditions are described in Table 1. Atomic emission measurements were carried out by taking three characteristic peaks of silver (at 328.068, 338.289, and 546.549 nm). The calibration regressions obtained with the standards made were 0.99895, 0.99898, and 0.99907, respectively.

Additionally, the determination of the potential AgNPs release from the modified filter media was evaluated by mass balance using a precision microbalance (WLC X2 Radwag, Radom, Poland) before and after being subjected to 30 successive manual washings at room temperature in Milli-Q water. The cleaning period was, at least, during 10 s each washing using a spunbond surgical mask after its initial AgNPs load being fully characterized using atomic emission spectroscopy.

2.7. Determination of Efficiency against SARS-CoV-2

1×10^5 PFU SARS-CoV-2 virus (40 µL of SARS-CoV-2 virus stock was added) was inoculated on the materials coated with silver nanoparticles and on their respective uncoated materials used as controls. SARS-CoV-2 virus was isolated from a COVID-19 patient at the Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain) [43]. Viral stocks were prepared and quantified using the epithelial cell line, Vero E6 (kindly provided by Julia Vergara from Centro de Investigación en Sanidad Animal IRTA-CReSA, Barcelona, Spain), as previously described in Santiago et al. [43]. Vero E6 cells were cultured in Dulbecco's Modified Eagle Medium, obtained from Sigma-Aldrich (Darmstadt, Germany) and supplemented with 10% fetal bovine serum (FBS) (Sigma), 2 mM Glutamax (Gibco), 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 0.25 µg/mL amphotericin B (Sigma), 1% non-essential amino acids (Gibco), and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Biowest), referred as complete medium and used for cell expansion. Vero E6 cells were kept at 37 °C in a 5% CO₂ humidified incubator and for the antiviral assays complete medium with 2% FBS was used. The materials inoculated with the virus were incubated for 10 min, 1 h, and 2 h at room temperature and subsequently the amount of infective virus was quantified as follows. Each sample was introduced into a 1.5 mL tube with 1 mL of 2% FBS medium and vortexed twice for 30 s. Six serial 1:10 dilutions were made, and 100 µL of each dilution was added in quadruplicate to Vero E6 cells seeded in a 96-well plate (10⁴ cells/well) and incubated for 72 h at 30 °C and 5% CO₂. After the incubation time was completed, plates were fixed with 4% paraformaldehyde for 1 h at room temperature and stained with crystal violet, and the wells with viral cytopathic effect (CPE) were identified. The concentration of viable virus was determined by TCID₅₀/mL. The 50% endpoint titers were calculated according to the Ramakrishnan simple formula

(Equation (1)) [44], where W_{CPE} refers to the total number of wells with CPE and n_W to the number of wells per dilution.

$$\log_{10} \text{ of } 50\% \text{ endpoint dilution} = \left(\frac{W_{CPE}}{n_W} + 0.5 \right) \log_{\text{dilution factor}}, \quad (1)$$

All procedures involving infectious virus handling were conducted in Biosafety Level 3 laboratories (BSL3). The SARS-CoV-2 virus stock was stored in screw cap micro tubes at -80°C . The BSL3 facilities included a controlled air system with HEPA filters, working in under-pressure conditions to prevent air leakages and having an in-place inactivation of contaminated liquid waste. Among the personal safety measures, personal protective equipment (PPE) was worn, comprising disposable full body suits, arm sleeve covers, shoe covers, two pairs of gloves, and face masks. Additionally, samples that needed to be processed outside BSL3 facilities were successfully inactivated beforehand.

3. Results and Discussion

3.1. Silver-Nanoparticles Synthesis and Characterization

Using the original report of Goli et al. [41] as a reference, we studied the different synthesis variables that could have an effect on the resulting particle size distribution (Table 1), namely reagent concentrations, reducing agent concentration, and reagent addition rate. The reference NPs (from the original protocol) showed an average particle diameter of 12.3 ± 2.9 nm (Figure 2a). If the reagent addition was modified by adding them dropwise (Synthesis A-Table 1) to promote a controlled and fast mixing that enables an homogenous nucleation event, a particle size reduction (8.7 ± 2.0 nm) was observed (Figure 2b). In the syntheses where the addition of reagents was added in volumes of 1 mL (Synthesis D-Table 1) and 3 mL (Synthesis E-Table 1), the mean particle size increased by up to 11.2 ± 3.8 nm (Figure 2e) and to 13.4 ± 4.0 nm (Figure 2f), respectively. These results are in agreement with the literature [45] and confirm that the addition of reagents is a key variable in chemical reactions controlled by fast kinetics, such as the nanocrystallization of nanomaterials. On the other hand, if the concentration of reagents was halved to decrease the nanocrystallization rate and to control the growth event (Synthesis B), the mean particle diameter was reduced to only 6.2 ± 2.6 nm (Figure 2c). This phenomenon was also observed for Synthesis C (Table 1), where the concentration of reducing agent was halved and the mean particle size obtained was 8.0 ± 2.4 nm (Figure 2d). The larger mean size of nanoparticles resulted in synthesis C in comparison with B is rationalized by the high silver precursor concentration used in synthesis C. In this case, the nucleation event is similar to the one observed in synthesis B as it is confirmed by a similar size distribution, but the excess of Ag precursors promotes crystal growth and a resulting larger mean size was observed. If the concentration of Ag precursor and TSC was kept constant as in Synthesis B but the concentration of the reducing agent was increased, the particle size distribution was more heterogenous due to the fast and less controlled nucleation event (11.2 ± 3.8 nm, synthesis D, Figure 2e) and (9.8 ± 4.3 nm, Synthesis F, Figure 2g). When comparing Synthesis D and F, is it coherent that the mean size resulted in synthesis F was slightly smaller than that in D since the mixing process was also promoted by the dropwise addition of reagents and the fast stirring rate applied (1500 rpm).

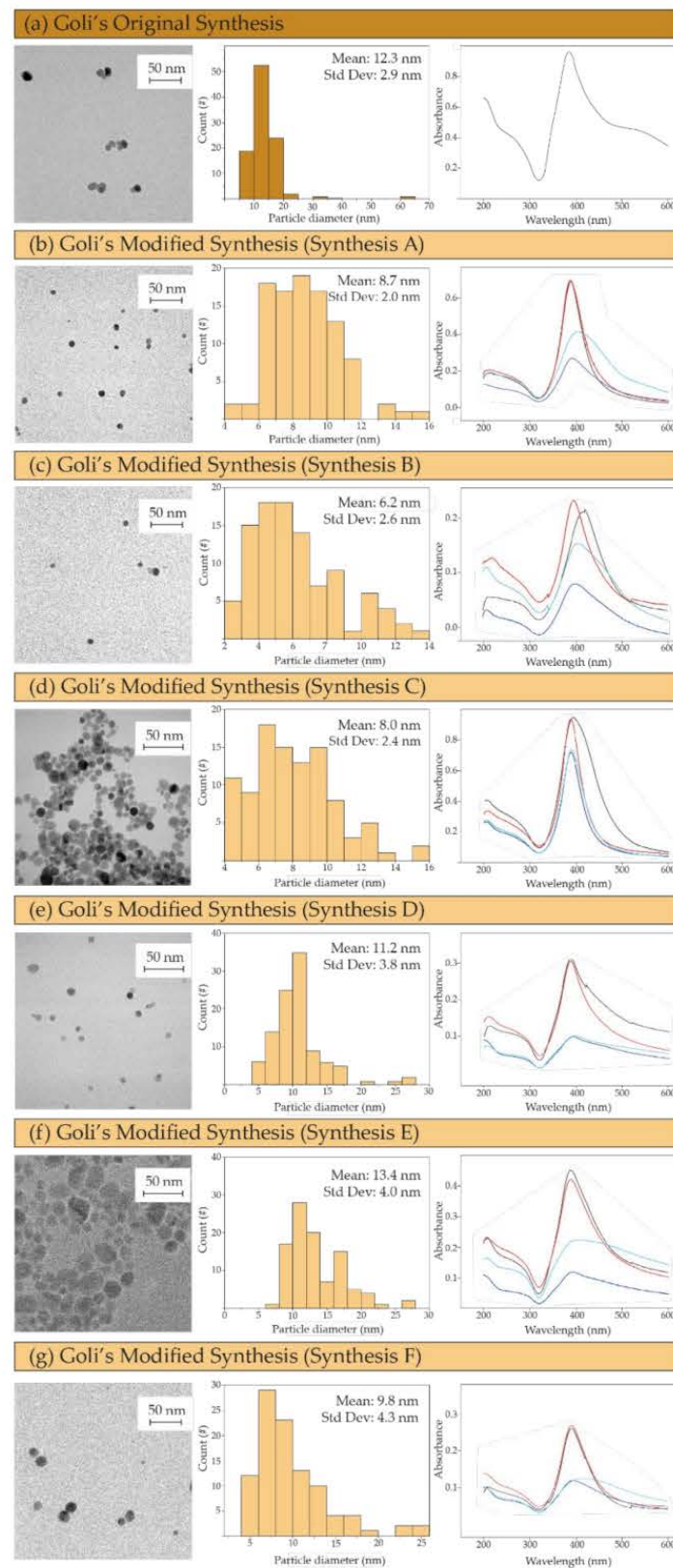


Figure 2. (a–g) TEM images of the silver nanoparticles obtained by following the different chemical syntheses together with their histograms showing the particle size distributions and their UV-Vis spectra on the first day of synthesis (black line) and at 3 (red line), 5 (blue line), and 7 days (green line) after synthesis. Particle size histograms were plotted after considering a population $n = 100$. Where # refers to the number of particles.

As we mentioned before, the antimicrobial potential of silver nanoparticles is closely related to the morphology of the nanoparticles, being this action promoted with a high area per volume ratio, which corresponds with a rapid oxidation and a consequent fast ionic silver release rate [46]. An inverse dependence of the antimicrobial potential of silver nanoparticles has been described as a function of their size [47–49]. In all the selected samples used in this work, the morphology of the particles was typically spherical. Faced with this equality on the morphological conditions, it was decided to choose the synthesis that offered the smallest NP sizes (Synthesis B) for the subsequent studies aiming for a fast dissolution rate, ionic silver release, and a consequent rapid virus inactivation upon contact. In parallel, another desirable aspect of the silver nanoparticles obtained is the long-lasting stability of the resulting colloid. The UV-Vis spectra of the selected sample reported large stability over time when compared with the spectrum of the original synthesis. Specifically, syntheses A, B, and C demonstrated a narrower full width at half maximum of the silver-attributed absorbance peak (around 400 nm) associated with a reduced polydispersity on their composing particle sizes, which is in agreement with the TEM analysis and could benefit when obtaining a homogeneous coating.

3.2. AgNPs-PEI Based Coating Method and Microscopic Characterization

Lee et al. [39] described the combined antimicrobial use of AgNPs and PEI. In their work, the main action attributed to PEI was to stabilize the silver colloid by electrolyte-mediated electrostatic repulsion. They stated that the sustained release of silver ions was prolonged thanks to the protection of PEI on the silver particles surface, acting as a diffusion barrier and prolonging their action.

The high positive surface charge of PEI has been widely used to electrostatically interact with negatively charged surfaces [50,51]. One of the fundamental characteristics of this polymer is its theoretical ratio of primary, secondary, and tertiary amino groups when branched, established as 1:2:1, respectively [51,52]. As we mentioned before, the antibacterial activity of PEI has been widely described in the literature [39,53–55], both in its original form and in conjunction with nanostructured elements. However, to the best of our knowledge, there is still insufficient evidence of its effectiveness against SARS-CoV-2. Theoretically, its antibacterial activity is based on the strong supramolecular electrostatic interactions between this positively charged polymer and the negatively charged bacterial peptidoglycan, which is rich in carboxyl and amino groups. In our work, we took advantage of PEI cationic nature to facilitate electrostatic bonding to the surface of negatively charged polymeric fibers on the one hand and to negatively charged AgNPs on the other side. Specifically, it is presumed that the incorporation of PEI to the polymeric fibers used as substrate generates a highly positive film which remains sandwiched between the polymeric substrate and the AgNPs deposited on top during the coating process, taking advantage of its strong electrostatic and coordinate covalent bonding that amino groups form with metals as demonstrated in the sections below. This strong interaction between amino groups and metals was previously demonstrated by the presence of charge transfer, which led to a change in the protonation state of PEI [56].

During the optimization of the AgNPs-PEI binding method to the polymeric fibers, the concentration of AgNPs in the colloid and the proportion of PEI dissolved in water were varied. The objective of this optimization was to achieve a stable and uniform coating on polypropylene spunbond fibers used as substrates. In preliminary tests, PEI concentrations higher than 15 mg/mL were discarded due to the high solution viscosity, and therefore 5 and 10 mg/mL of PEI aqueous solutions were used in the subsequent studies. In the same way, the concentration of AgNPs in the colloid used in the fabric coating bath ranged from 1 to 5 mg/mL, as depicted in Figure 3. In all cases, even with the minor use of materials (5 mg/mL of PEI and 1 mg/mL of AgNPs), an efficient incorporation of silver on the polymeric fibers was observed. Silver loadings using 10 mg/mL of PEI and 5 mg/mL of AgNPs were used in the subsequent studies because of the even and homogenous coating achieved along the fabric surface and depth. EDX spectra obtained during SEM microscopy

in random areas of the fibers showed the presence of Ag and C, attributable to the silver doping and to the polymer composition of the fibers, respectively.

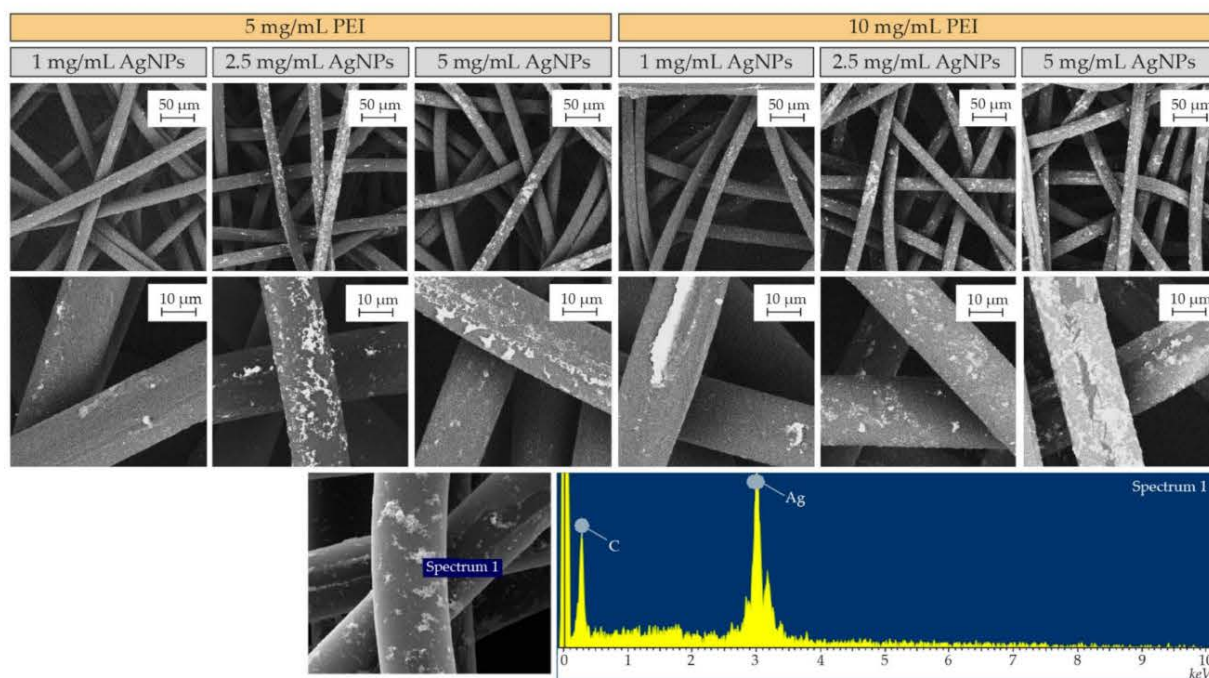


Figure 3. Backscattered electron SEM images of polypropylene spunbond fibers from a surgical mask coated with AgNPs–PEI, using two different PEI concentrations and three AgNPs colloid concentrations. The micrographs of the samples obtained with a higher amount of AgNPs and a higher PEI concentration show a significantly superior silver load. These images demonstrate that the concentration of AgNPs is as important as the amount of PEI to improve the silver incorporation yield. Bottom: EDX analysis on a selected coated area.

With the established method, it was decided to coat other polymeric materials (Figure 4) based on polypropylene fibers, but with different configurations and morphologies, including polyester-viscose fibers in sewn fabrics and polypropylene fibers having glass fibers, used in the manufacturing of filtration media using the same conditions (e.g., 10 mg/mL of PEI and 5 mg/mL of AgNPs).

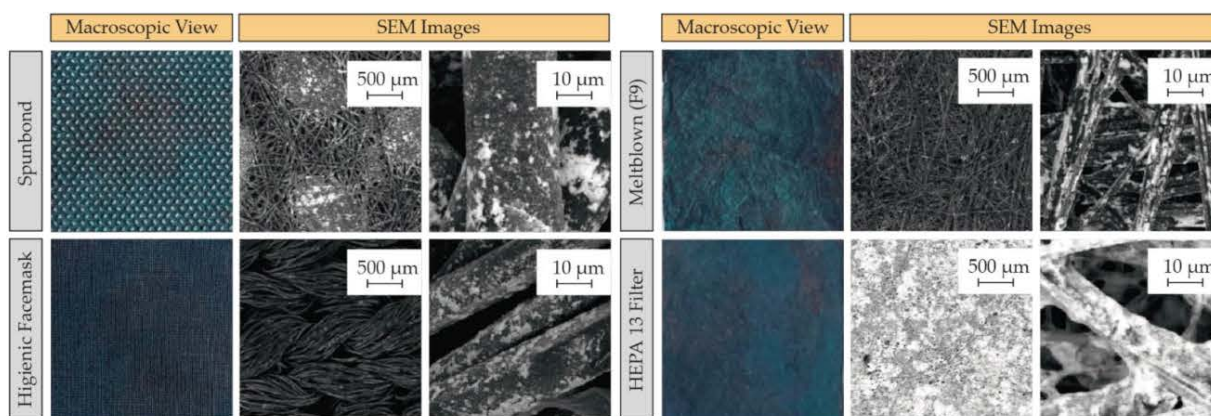


Figure 4. Macroscopic images and backscattered electrons SEM of different fabrics. A superior incorporation of AgNPs was observed in the fibers of smaller diameter (e.g., HEPA H13 filter). These images show that the silver load is homogeneous and high in all the polymeric samples studied (spunbond, hygienic facemask, meltblown, and HEPA filter).

3.3. AgNPs-PEI Based Coating Stability Analysis under Different Boundary Conditions

Atomic emission spectroscopy (MP-AES) revealed a silver loading of $\sim 0.5 \text{ mg/cm}^2$ on spunbond fibers. The results of the stability analysis of the attachment under forced mechanical stress (Table 2) are shown in Figure 5. Samples sonicated in Milli-Q water showed an Ag loss of approximately 13 wt.% at 35 s increasing up to 30 wt.% at 45 s. However, in commercial silver textiles, nanosilver losses of up to 48–75% have been reported after water-based washing. Furthermore, those reports did not use ultrasonic sonication, but only conventional washes, so the presented method for binding silver to polymeric fibers represents a substantial improvement [57,58]. Slightly low binding results were obtained when using water at pH 7 as sonication media, where after 5 s of sonication the loss was 12 wt.% and, after 10 s the measured detachment increased up to 27 wt.%. AgNPs detachment using PBS (10%) were 8 wt.% and 24 wt.% after 5 and 10 s, respectively. Losses obtained after acidic and basic conditions were higher after sonication. At pH 4.5, around 70 wt.% of silver was lost, while at basic pH the loss was 42 wt.% (in the case of using pH 9) and 30 wt.% using pH 14. The acidification of the aqueous medium using HCl led to losses of more than 70 wt.% probably because the dissolution of silver is promoted under acidic conditions and the solubility of PEI increased [59–62]. Under alkaline conditions, the protonation of the amino groups is lost and therefore the interaction with the fabrics as well as with the AgNPs would vanish. However, these mechanical stress tests were much more restrictive than the product's exposure under normal operating conditions. Sonication was used to challenge the strong chemical interaction and to prove that a strong attachment is present.

Table 2. Boundary conditions used for evaluating the release of AgNPs from coated polymeric substrates (polypropylene fibers from spunbond). All samples were initially sonicated in Milli-Q water to remove non-adhering particles. Some were sonicated again in other media, as indicated in the table. The second sonication medium varied from test to test.

Sample	Cleaning Sonication	First Sonication Conditions	Second Sonication Conditions
Reference	20 s in Milli-Q Water	-	-
1	20 s in Milli-Q Water	15 s in Milli-Q Water	-
2	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in Milli-Q Water
3	20 s in Milli-Q Water	15 s in Milli-Q Water	5 s in Tap Water (pH 7)
4	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in Tap Water (pH 7)
5	20 s in Milli-Q Water	15 s in Milli-Q Water	5 s in PBS (10%)
6	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in PBS (10%)
7	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in Tap Water (pH 4.5)
8	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in Tap Water (pH 9)
9	20 s in Milli-Q Water	15 s in Milli-Q Water	10 s in Tap Water (pH 14)

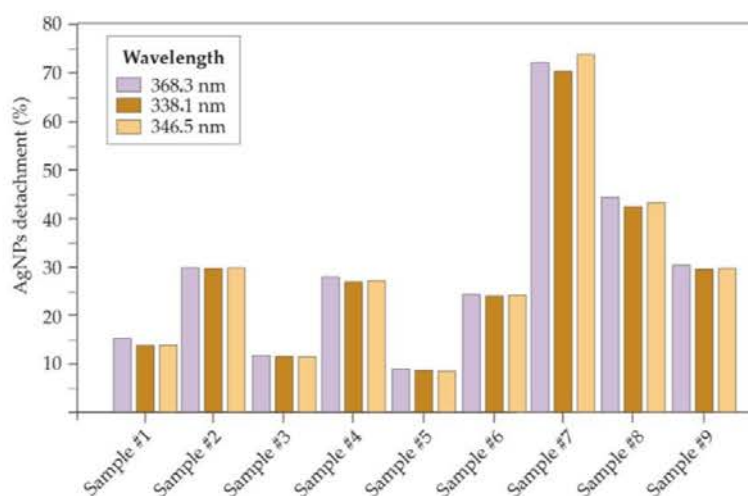


Figure 5. Percentage of AgNPs loss in each of the experiments performed with the AgNPs-PEI coating on polypropylene fibers (spunbond) observing three characteristic absorbance peaks of silver by atomic absorbance spectrometry (MP-AES).

It was possible to quantify the loss of AgNPs from the coated fabrics after 30 manual washes in Milli-Q water thanks to a precision microbalance. Losses of 0.83, 0.44, 0.94, and 1.01 wt.% were measured in the spunbond of a coated surgical mask, a hygienic mask, a meltblown F9 fabric, and a HEPA H13 filter, respectively. Although the washing process is more aggressive than the exposure to air currents, it was decided to recreate this scene as it is foreseen in some standards related to the reuse of hygienic face masks (e.g., EN 0065). In essentially for none of the cases studied a loss greater than 1 wt.% was observed, which shows excellent stability results in Milli-Q water. This study was limited to water used at room temperature (not comparable to the conditions in a washing machine). However, after silver incorporation, fabric washing is required. If a wash is not carried out, the loss of particles can be overestimated since those particles with reduced adherence would be removed. In future works it will be necessary to evaluate the stability of the nanosilver attached on the filtering media against air flows. However, these studies were not considered in this work.

3.4. AgNPs-PEI Based Coating Antiviral Efficiency against SARS-CoV-2

The final goal of this work was the functionalization of common filter materials to provide them with virucidal ability against SARS-CoV-2. As shown in Figure 6a, the silver-coated materials achieve a large reduction in the viral load compared with the control materials (uncoated fabrics). The results indicated that after 2 h of infection, the materials reduced the viral load by more than 99.9%. Remarkably, within the first 10 min after infection, the viral load was reduced by more than 98% when deposited on spunbond fabrics (Figure 6b).

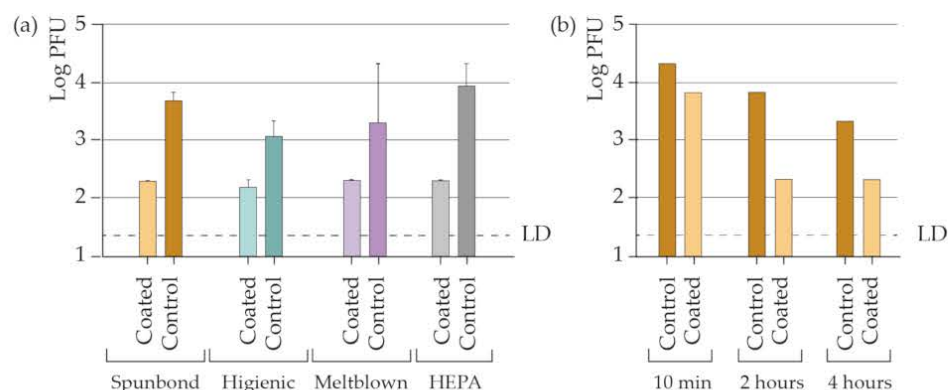


Figure 6. Determination of the infective viral load (a) in materials coated with silver nanoparticles and the control after 2 h and (b) in coated spunbond after 10 min, after 2 h after infection, and after 4 h after infection with the SARS-CoV-2 virus at room temperature.

The role of PEI in the virucidal activity of the coating was also analyzed separately. The successful antiviral activity of this cationic polymer has been reported against several encapsulated viruses, such as influenza virus [63], hepatitis B virus [64], or human cytomegalovirus [65], and against non-encapsulated viruses such as human papillomavirus [65] or herpes virus [66,67]. Its primary mechanism of antiviral action is attributed to the blocking ability of the primary binding of the viral particles to proteoglycans receptors on target cells. However, the antiviral properties of PEI on SARS viruses have not been reported in detail [68].

A 10 mg/mL PEI spunbond coating was taken as a reference for antiviral testing in this work. In addition, 10- and 100-fold more diluted (1 mg/mL and 0.1 mg/mL) samples were prepared to analyze the effect of concentration (Figure 7a). Free PEI (in aqueous solution) was also analyzed to assess the exclusive activity of the polymer (Figure 7b). Thus, the effect of the substrate material (spunbond) was not analyzed during the second phase of the study. No decrease in the viral load was observed, so its virucidal activity was ruled

out at the doses tested. All the antiviral ability of the coating was attributed to the AgNPs single activity. The study suggests that the capacity of PEI to inactivate SARS-CoV-2 is not efficient at the doses tested. In addition, PEI was toxic to Vero cells when incorporated in the coatings as well as in the aqueous solutions where it was used at a concentration of 10 and 1 mg/mL. No cytotoxicity was found at 0.1 mg/mL PEI concentrations and below (Table 3). In the coated samples using AgNPs and PEI, no cellular toxicity was observed at the doses tested.

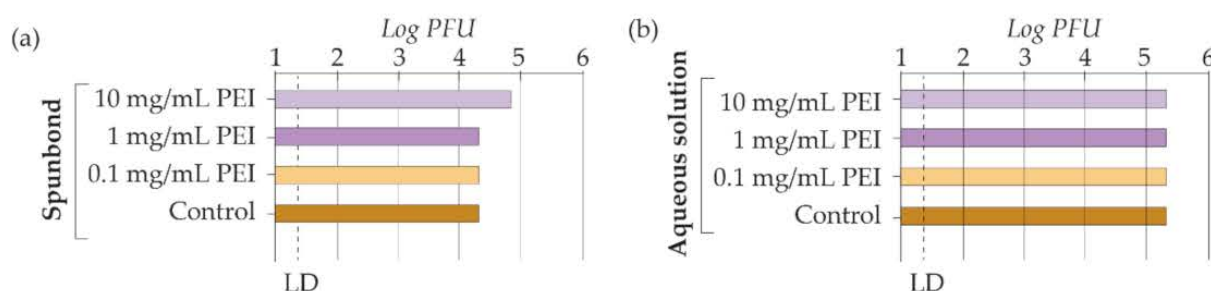


Figure 7. Determination of the infective viral load (a) in spunbond coated with PEI and (b) in PEI dissolved in milli-Q water 2 h after infection with the SARS-CoV-2 virus at room temperature. LD stands for limit of detection.

Table 3. Determination of the PEI toxicity in Vero cells at different dilutions.

	Percent of Toxicity (%)				
	Dilution 0	Dilution 1:10	Dilution 1:100	Dilution 1:1000	Dilution 1:10,000
Spunbond PEI coated (10 mg/mL)	100	100	0	0	0
Spunbond PEI coated (1 mg/mL)	100	0	0	0	0
Spunbond PEI coated (0.1 mg/mL)	0	0	0	0	0
PEI aqueous dissolution (10 mg/mL)	100	100	0	0	0
PEI aqueous dissolution (1 mg/mL)	100	0	0	0	0
PEI aqueous dissolution (0.1 mg/mL)	0	0	0	0	0

4. Conclusions

The widespread use of masks to contain the COVID-19 disease is essential for the epidemiological management of the pandemic, even when individuals are vaccinated. However, the protection offered by surgical masks is compromised compared with the one offered by KN95 (FFP2) or KN99 (FFP3) masks. This paper reported a new antiviral coating that increases the antiviral efficiency of surgical masks and other types of masks (e.g., hygienic) and related materials used as air conditioning filters. The virucidal coating is based on the incorporation of AgNPs in the polymeric fibers. The addition of PEI during the bonding method is key to form a stable bond between the silver and the fibers. This cationic polymer has been reported to exhibit antiviral activity against enveloped and non-enveloped viruses. However, its effect on the SARS-CoV-2 virus was rarely discussed. In this work, we found that PEI did not affect the activity of the SARS-CoV-2 virus at the doses tested. In addition, we found cell toxicity from concentrations of 1 mg/mL onwards. The coatings based on AgNPs and PEI did not show cytotoxicity, although they did show a high viral inactivation efficiency. Two hours after infection, the viral load was reduced by more than 99.9% in all cases. However, part of the effect arises earlier, as at 10 min after infection, a 98% reduction of the viral load was already measured.

This work can be used to improve the epidemiological management of COVID-19. Using this method, the efficiency of respiratory protection equipment and air conditioning filtration systems can be improved. The binding of the silver particles to the fibers is stable. The method we present is easily scalable and with a low cost, which greatly facilitates its large-scale industrialization and production. However, it is still necessary to ensure the bond stability of the particles against air flows since these will be the real operating conditions.

Author Contributions: Conceptualization, M.B. and M.A. (Manuel Arruebo); methodology, V.S., M.A. (Manuel Arruebo) and J.P.; validation, I.U.-M., M.B., D.d.M. and M.A. (Maykel Arias); formal analysis, I.U.-M., M.B., D.d.M., V.S., M.A. (Maykel Arias), J.P. and M.A. (Manuel Arruebo); investigation, I.U.-M., M.B., D.d.M. and M.A. (Maykel Arias); writing—original draft preparation, M.B.; writing—review and editing, I.U.-M., D.d.M., V.S., M.A. (Maykel Arias), J.P. and M.A. (Manuel Arruebo); supervision, V.S., M.A. (Manuel Arruebo), J.P.; project administration, M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Instituto de Investigación Sanitaria Aragón (IIS Aragón) and Instituto de Nanociencia y Materiales de Aragón (INMA).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The microscopy works have been conducted in the “Laboratorio de Microscopias Avanzadas (LMA)” at “INMA–University of Zaragoza”. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011 financed by the Instituto de Salud Carlos III with the assistance of the European Regional Development Fund. Work in the JP laboratory is funded by FEDER (Fondo Europeo de Desarrollo Regional, Gobierno de Aragón (Group B29_17R), Ministerio de Ciencia, Innovación y Universidades (MCNU), Agencia Estatal de Investigación (PID2020-113963RBI00), Fundación Inocente Inocente, ASPANOA and Carrera de la Mujer de Monzón. Postdoctoral Juan de la Cierva Contract (MA) and Sara Borrell contract (DdM) and Predoctoral Grant from Gobierno de Aragón (IUM).

Conflicts of Interest: The authors declare no conflict of interest.

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Cuarto artículo

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***Nanomaterials*, 12 (6), 925**

<https://doi.org/10.3390/nano12060925>

Factor de impacto en 2022: 5.719

Factor de impacto a 5 años: 5.810

Cuartil JCR (2021): Q1

Cuartil JCR (2020): Q1

Fecha de envío: 18 de febrero de 2022

Fecha de publicación: 11 de marzo de 2022

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Article

Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks

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Citation: Baselga-Lahoz, M.; Yus, C.; Arruebo, M.; Sebastián, V.; Irusta, S.; Jiménez, S. Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks. *Nanomaterials* **2022**, *12*, 925. <https://doi.org/10.3390/nano12060925>

Academic Editors: John Vakros, Evroula Hapeshi, Catia Cannilla and Giuseppe Bonura

Received: 18 February 2022

Accepted: 7 March 2022

Published: 11 March 2022

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Abstract: The disposal of single-use personal protective equipment has brought a notable environmental impact in the context of the COVID-19 pandemic. During these last two years, part of the global research efforts has been focused on preventing contagion using nanotechnology. This work explores the production of filter materials with electrohydrodynamic techniques using recycled polyethylene terephthalate (PET). PET was chosen because it is one of the materials most commonly present in everyday waste (such as in food packaging, bags, or bottles), being the most frequently used thermoplastic polymer in the world. The influence of the electrospinning parameters on the filtering capacity of the resulting fabric was analyzed against both aerosolized submicron particles and microparticulated matter. Finally, we present a new scalable and straightforward method for manufacturing surgical masks by electrospinning and we validate their performance by simulating the standard conditions to which they are subjected to during use. The masks were successfully reprocessed to ensure that the proposed method is able to reduce the environmental impact of disposable face masks.

Keywords: PET; fibers; face mask; filtration; electrospinning; nanotechnology; COVID-19; aerosol

1. Introduction

Regarding the COVID-19 pandemic, the European Environment Agency [1] reported in June 2021 a waste of 170,000 tons of face masks and estimated an additional global warming potential of 2.4–5.7 million tons of equivalent carbon dioxide (CO₂). Although the use of protective masks is significantly associated with sociodemographic factors, social behaviors and prevention policies, it has been established in most of the global population [2,3]. The massive use of protective masks will be linked to the epidemiological progress of the pandemic. In the long term, the hypothesis of a procedural change in preventing infectious diseases is considered. The use of a mask will continue to be vital to preventing the spread. Faced with these scenarios, developing new recyclable, and more sustainable materials for application in respiratory protection equipment would be beneficial.

Current technology allows obtaining fiber diameters at the nanometric scale to achieve better filtration performance [4–6]. The diameter of the fibers cannot be reduced indefinitely

due to the increase in pressure drop that this entails [7]. One of the emerging methods that has shown the best performance for the manufacture of filter materials in a submicron scale is electrospinning, which shows improved performance compared to conventional manufacturing techniques (that is, melt-blown) [8]. Nanofibers offer very high surface-to-volume ratio due to their small diameters and reduced fabric thickness, making possible to generate porous membranes that do not present exceptionally high pressure drops [9]. Numerous studies have been aimed at characterizing the effect of electrospinning parameters (such as polymer concentration, polymer type, flow rate or electric field, among others) concerning the behavior of the resulting membrane in the capture of aerosols [5,10–13]. One of the starting parameters during the electrospinning of fibers is the choice of the polymeric base material due to divergence in the resulting morphological and physico-chemical aspects of the resulting electrospun mats [10]. Polyethylene terephthalate (PET) has been the material of choice in research works for its easy processability by electrospinning [14]. The use of PET in the food industry has been extended worldwide due to its chemical characteristics, high recyclability and low cost. The wide availability of disposed PET globally makes it ideal for circular economy strategies. Some works have used electrospun PET recycled from plastic bottles to manufacture membranes for cigarette-derived smoke [15], for fluid microfiltration [16] or air nanofiltration [17]. These publications report particular applications of the material and specific electrospinning conditions without offering a broad point of view on the versatility of PET recycled by electrospinning.

Nonwoven filter media consist of a three-dimensional network of randomly placed fibers where the points of contact between them are relatively infrequent and present a relatively high porosity (~60–90%) [18–20]. Their application in gas filtration has been popularly extended thanks to the balance they offer between filtration and pressure drop [18]. The fibers that make up these filters are usually made from cellulose, glass, quartz, and polymeric materials. Their diameter vary within 0.5 and 50 μm [21]. The capture of aerosol particles by filtering depends on the particle's aerodynamic diameters and is associated with the transport and adherence between the aerosol and the filter medium. The mechanisms capable of carrying out transport are sifting, sedimentation, interception, diffusion, inertial impact, and combined transport mechanisms [22]. When differentiating submicron matter by its size and associated retention mechanisms, we can distinguish between particle sizes >600 nm, 300–600 nm, and <300 nm. The larger ones (>600 nm) are predominantly retained because they are larger than the inter-fiber pores (interception and sedimentation). The intermediate ones (300–600 nm) can pass through the filter pores but have a high probability of colliding with the fibers (inertial impact). In contrast, the finer ones (<300 nm) follow heterogeneous dynamics and are mainly captured by Brownian diffusion [22–24]. On the other hand, adhesion mechanisms include supramolecular interactions such as Van der Waals forces, and electrochemical forces, and chemical bonding, although this is more independent of the particle size and depends on its physical-chemical properties [18,25]. Theoretically, many parameters determine the retention efficiency of the filter material to a greater or lesser extent, which may be associated with both the structural characteristics of the filter and its constitutive fibers and the nature of the retained aerosol. It has been well reported that reducing the diameter of the fibers improves filtration since it increases the probability of impact for the aerosols [7,26,27]. For example, a reduction in fiber diameters from 28.2 to 10.9 μm led to an increased filter efficiency on submicron particles from 12.5% to 84% under the same conditions [7]. This same effect was observed in another study with finer fiber filters, where a filtering efficiency of 38% was obtained for 3.25 μm fibers compared to an efficiency of 98.4% for 1.29 μm fibers on average [17]. Depending on the fiber mesh of the filter, they will have a greater or lesser probability of retention [28,29]. Beyond the fiber diameter itself, there are other morphological aspects, such as the presence of fibers with non-circular cross-sections that increases the surface area and increases the retention rate due to inertial impact [30–32].

In the present work, we explore in-depth the role of PET in the production by electrospinning of materials to filter air with various applications, with special attention to the

production of masks and personal protective equipment against airborne pathogens, such as the SARS-CoV-2. In addition, we present a method for the production of PET-based surgical masks that is easily scalable and transferable to the industry (Figure 1). As face masks are one of the effective measures to slow down the SARS-CoV-2 transmission, the usage of masks has been massively increased. However, the improper disposal of masks is causing a negative impact on the environment and a serious threat to terrestrial and aquatic ecosystems.

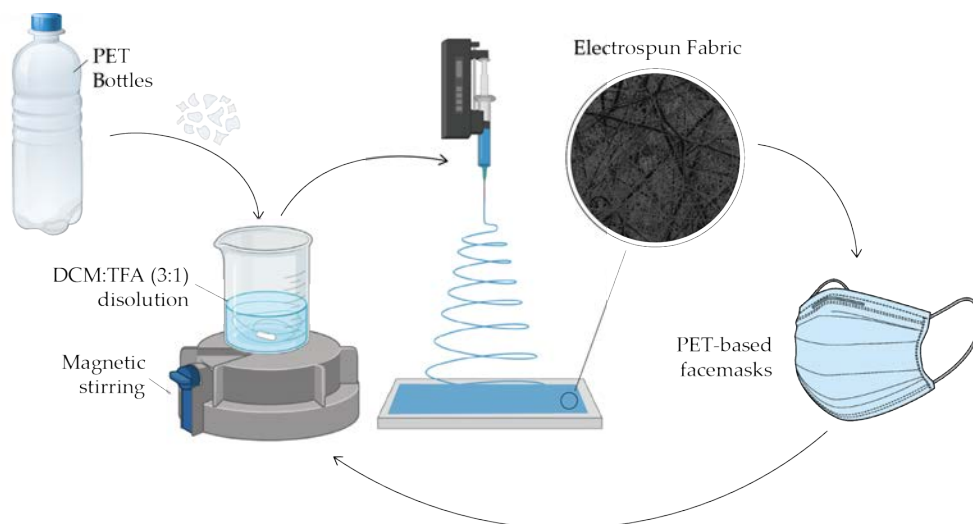


Figure 1. Schematic representation of the PET-based face mask production.

2. Materials and Methods

2.1. Materials

PET nano and microfibers production was carried out using trifluoroacetic acid ($\geq 99.0\%$, TFA) and dichloromethane ($\geq 99.8\%$, DCM) as solvents. Both of them were purchased from Sigma-Aldrich (Darmstadt, Germany). Polyethylene terephthalate was obtained from used water bottles having an intrinsic viscosity of 0.7–0.78 dL/g. The bottles were collected from urban waste. The used PET was dissolved in DCM:TFA (3:1) under continuous stirring for 5 to 10 min at different concentrations: 10, 15, 20, and 25 wt.%. The maximum of 25 wt.% was selected according to the limit of solubility. A minimum of 10 wt.% was selected as the lower limit to maximize electrospinning yield.

2.2. Electrospun PET Fibers

An Electrospinner Yflow 2.2 D-500 (Electrospinning Machines/R&D Microencapsulation, Malaga, Spain) was used for the electrospinning of the PET micro and nanofibers. During the optimization of the synthesis, different electrospinning parameters were varied: flow rate of the polymeric solution (0.5, 2.5, 5, and 9.9 mL/h), the distance from the needle to the collector (15, 20, and 25 cm) and the voltage selected to stabilize the Taylor cone (15 to 25 kV). The fabric samples were obtained using a moving needle at variable speed on the Y-axis (V_y 25 mm/s and 50 mm/s) and constant on the X-axis (V_x 2 mm/s) to obtain an homogeneous mat. Aluminum foil was used on a flat collector having electrical conductivity connected to the negative pole during sample collection.

2.3. Microscopic Characterization

The characterization of the materials was carried out using an environmental scanning electron microscope (ESEM, Quanta-FEG 250, Zaragoza, Spain). Samples were prepared on carbon tape on an aluminum slide and sputtered with palladium to promote electron conduction. The fiber sizes were obtained from manual measurements with the free software Image-J (v1.52; National Institutes of Health, 2019) for $n = 100$, where n is the number of fibers measured.

2.4. Retention Efficiency and Pressure Drop Determination

A saline solution (3 wt.% NaCl in distilled water) was used to produce the aerosols using a Topas-ATM226 generator. The generated microdroplets were dragged with a flow of 3000 NL/h and forced to pass through a tubular air dryer based on silica gel to evaporate the water and obtain solid particles. The particles were lugged into a cabin with controlled extraction where the concentration of particles remained constant. In the submicron range (0.01–0.5 µm), the particle size distribution inside the cabin was measured using a SMPS TSI 3936 composed of an electrostatic classifier (DMA TSI 3081) and a condensation particle counter (CPC TSI 3782). The particles were directed at a flow rate of 0.6 L/min towards a tube with an integrated valve. Depending on the desired measurement, the valve could redirect the flow towards the tested filter holder or towards an exhaust tube without a filter. The filter was placed between bronze discs sealed with Teflon tape, with 30 × 20 mm Teflon washers on each side. The exposed filter area was variable (diameter between 4 and 13.3 mm) to adjust the desired flow rate, but we have typically worked with 13.3 mm to reproduce the expected gas velocities in the mask. The measurements lasted 120 s and were made in duplicate.

The determination of the filtration efficiency on micrometric particles (0.5–10 µm) was carried out following the same scheme and conditions as in the previous section but using an Optical Particle Sizer OPS (TSI 3330) at a flow rate of 1.0 L/min (instead of 0.6 L/min of the DMA). The results retrieved from particles below ~0.5 µm are less reliable in this range using this piece of equipment due to the high ‘dead times’ observed; for this reason SMPS measurements were preferred in this range (Figure 2). Control measurements were carried out between measurements to calculate the relative efficiency (η) according to Equation (1), where C_{up} stands for concentration upstream and C_{down} stands for concentration downstream. The retention efficiency is expressed in global efficiency as number of particles. The distribution of particle concentration depending on their diameter can be seen in Figure S1.

$$\eta = 100 \times \frac{C_{up} - C_{down}}{C_{up}}. \quad (1)$$

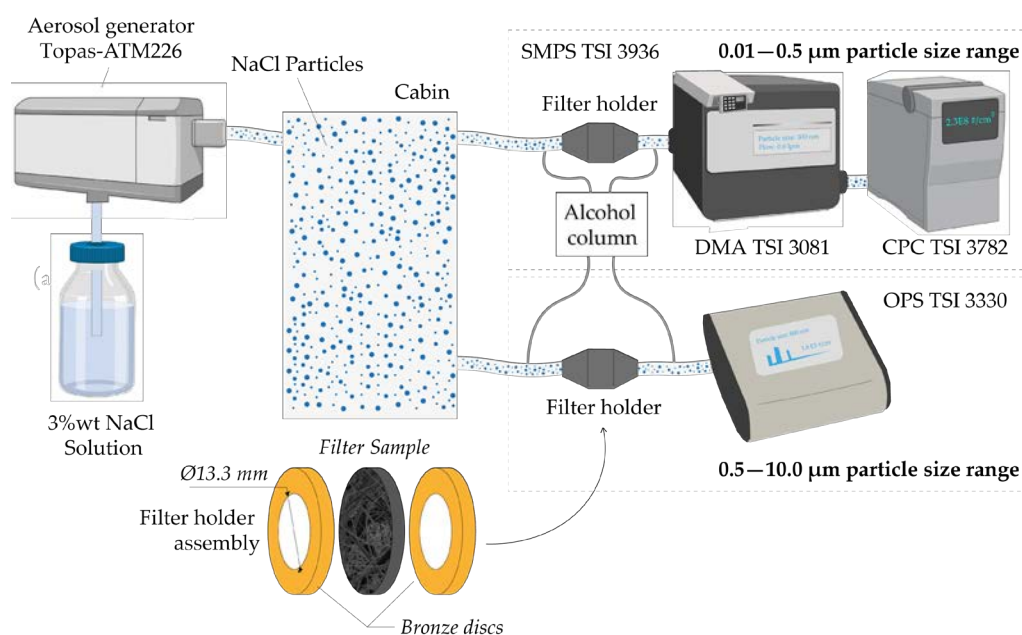


Figure 2. Schematic representation of the equipment used for the particle penetration tests.

The determination of the pressure drop was carried out using alcohol tilted columns connected to both sides of the filter sample holder. Measurements were made with a volumetric flow rate of 0.6 L/min.

3. Results and Discussion

3.1. Fiber Diameter Control

The parameters used during electrospinning were optimized to control the fiber diameters. Firstly, the polymer concentration in the solutions was varied using 10, 15, 20, and 25 wt.%. The viscosity of the solution consisting of PET dissolved in DCM:TFA was varied depending on the polymer concentration. It has been reported that the fiber diameters increase as the electrospun solution viscosity increases [33,34]. The resulting mean diameter was $0.14 \pm 0.04 \mu\text{m}$; $0.29 \pm 0.18 \mu\text{m}$; $0.79 \pm 0.59 \mu\text{m}$ and $1.54 \pm 0.84 \mu\text{m}$ when the polymer concentration was 10, 15, 20 and 25 wt.%, respectively (Figure 3).

3.2. Contact Angle Measurement

The contact angle was measured to evaluate the hydrophobicity of the electrospun materials. For this, a drop of distilled water was placed on the surface of the fibers and contact angle was measured using the Dataphysics OCA equipment (Dataphysics Instruments GmbH, Filderstadt, Germany) at room temperature. Contact angle measurements were performed by triplicate.

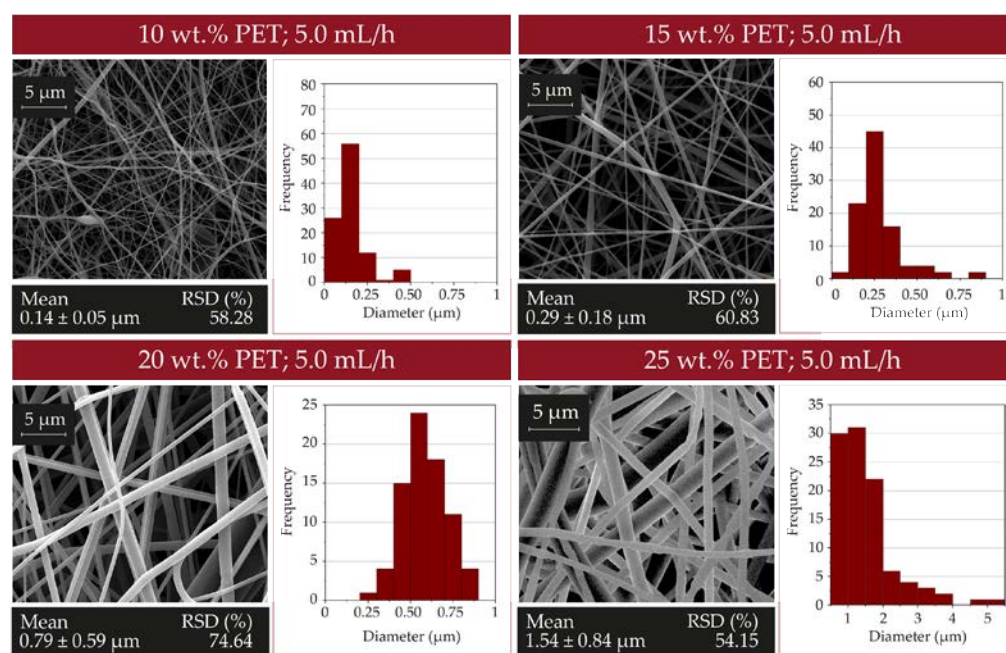


Figure 3. SEM micrographs and fiber size distribution histogram of electrospun fibers ($n = 100$) as a function of the polymer concentration (10, 15, 20 and 25 wt.%) in the solution electrospun at 5.0 mL/h flow rate.

The flow rate of the electrospinning solution is another parameter that directly affects the characteristics of the resulting fibers. High flow rates increase the amount of polymer available for electrospinning, so fibers result in large diameters [35]. Although Zargham et al. [36] reported the influence of flow rates on the distribution of the fiber diameters and they found similar average diameters ($\pm 20 \text{ nm}$) in all cases irrespectively of the flow rates used. In this work, flow rate variations (0.5, 1.0, 2.5, and 5 mL/h) during electrospinning did not appear to significantly alter the resulting fiber diameter or morphology (Figure 4) and the RSD (Relative Standard Deviation) varied from one sample to another without a clear trend. However, a significant presence of beads was observed in the

samples having polymer concentrations of 10 and 15 wt.% as the flow rate of the solution decreased. As the flow rate of the solvent is reduced, the solvents have a longer evaporation time from the tip of the needle to the collector, and the fibers are electrospun with fewer imperfections (i.e., lack of beads). In general, when having high flow rates, the probability of bead formation increases [37]. However, in this work, an increase in bead formation has been observed as the flow rate was reduced. Solutions with low concentrations of PET (10–15 wt.%) presented a reduction in the viscosity of the solution compared to those with high concentrations of PET (20–25 wt.%) and beads are more likely to be formed for the solutions with lower viscosity because under those conditions the elasticity of the electrospun solution shows low relaxation time or low extensional viscosity [38–40]. The presence of these imperfections can directly affect filtration since it alters the morphology and homogeneity of the fibers. However, Zheng et al. [41] reported an increase in the filtration efficiency in electrospun membranes having beads. This increase in performance was attributed to a reduced pore diameter and increased filter's packing density caused by the beads. In this work, the geometry of the fibers was not considered a variable of study. Due to the abundant presence of beads in the fibers produced using a polymer concentration of 10 wt.%, the resulting electrospun mats were discarded for the following filtration tests in order to enhance results reproducibility.

3.3. Influence of Electrospinning Parameters on Filtration

Table 1 shows the variation in filtration efficiency under different electrospinning conditions. 1 mL of 25 wt.% PET solution was electrospun over an area of 125×125 mm, with a distance between the needle and the collector of 20 cm and a voltage difference of $\sim \Delta 15$ kV. Since the liquid flow rate hardly affected the morphology of the fibers, it was decided to maximize this parameter to improve the electrospinning yield, establishing a flow rate of 9.9 mL/h. Initially (Sample 1), the lateral movement was set to 25 mm/s on the Y-axis and 2 mm/s on the X-axis (v_x). In Sample 2, the Y-axis's speed (v_y) was increased to 50 mm/s, maintaining the X-axis speed. The electric field generated between the needle and collector was first set to $\sim \Delta 15$ kV, while Sample 3 was varied to $\sim \Delta 10$ kV. High voltage differences applied between the needle and the collector reduce the diameter of the fibers, although they also generate greater instability during the electrospinning process [42]. In Sample 4, the distance between the collector and the needle was increased, varying from the initial 20 to 25 cm. The distance between the needle and the collector must be large enough to avoid the formation of pores and morphological deformations and small enough to avoid destabilizing the Taylor cone during synthesis [43]. Considering the above, a greater distance is generally associated with reduced diameter fibers [44]. In Sample 5, the flow rate of the solution was reduced from 9.9 to 6 mL/h. Although in this work, it was preferred to work with high flow rates to improve the productivity during the electrospinning process.

Table 1. Variation of parameters used during electrospinning to evaluate their influence on the filtration efficiency of submicron particles.

Sample	PET (wt.%)	Flow (mL/h)	Voltage (kV)	Needle to Collector Distance (cm)	Needle Velocity (mm/s)	Filtration Efficiency (%)	Pressure Drop (mbar)
Sample 1	25	9.9	+10.5/−3.9	20	$25(v_y)/2(v_x)$	91.7	0.24
Sample 2	25	9.9	+10.5/−3.9	20	$50(v_y)/2(v_x)$	95.2	0.39
Sample 3	25	9.9	+5.5/−3.9	20	$25(v_y)/2(v_x)$	77.8	0.23
Sample 4	25	9.9	+10.5/−3.9	25	$25(v_y)/2(v_x)$	95.1	0.19
Sample 5	25	6.0	+10.5/−3.9	20	$25(v_y)/2(v_x)$	98.3	0.25

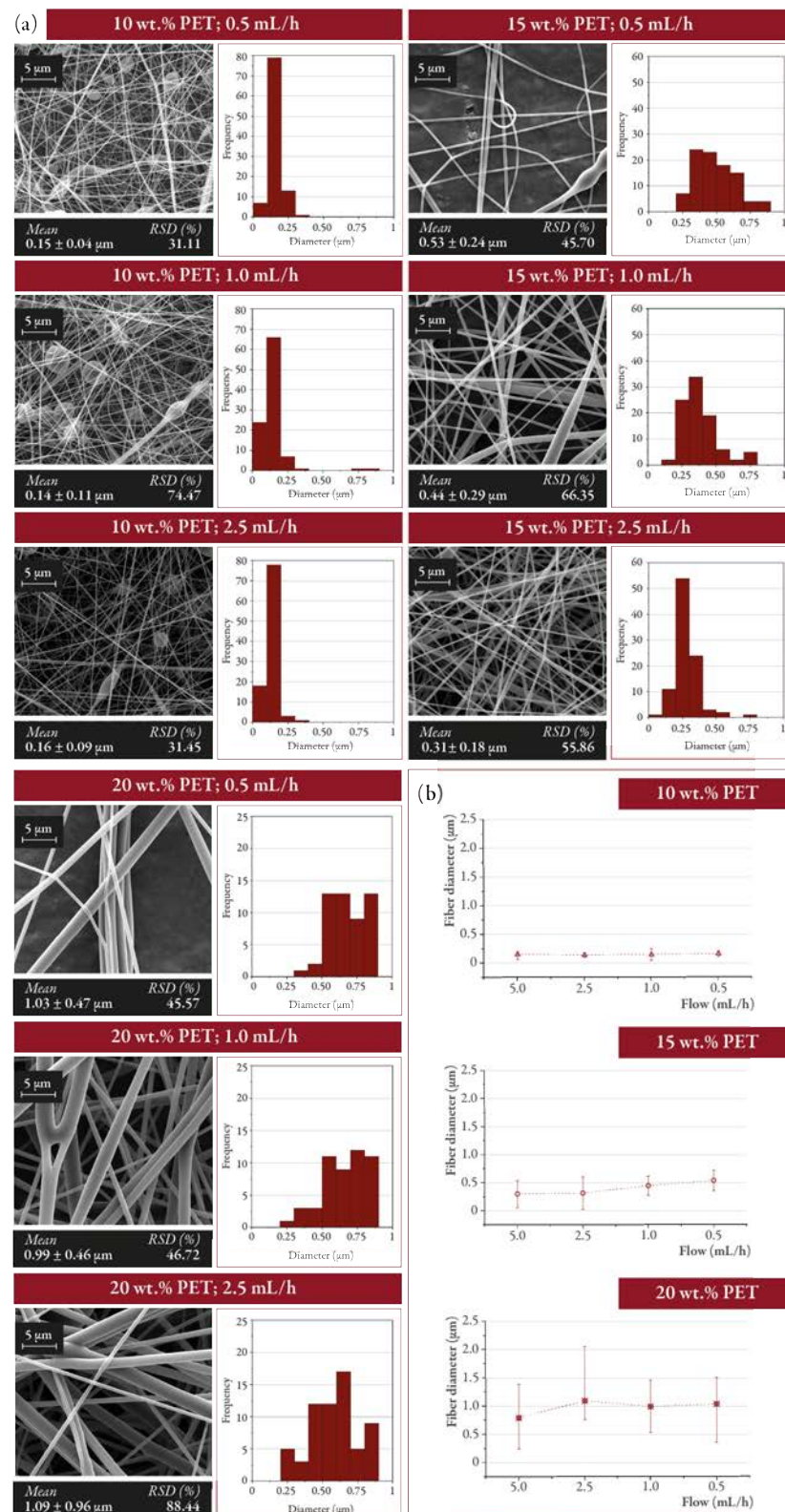


Figure 4. (a) Morphology of the electrospun fibers as a function of the polymer concentration (10, 15, and 20 wt.% PET) and the solution output flow (0.5, 1.0, 2.5, and 5.0 mL/h) and (b) variation of the diameter of electrospun fibers ($n = 100$) depending on the output flow of the ejected solution.

These samples were tested to determine their submicron particle capture performance (Figure 5). The fabric that showed the best performance was Sample 5 (low flow rate:

6.0 mL/h), with an average efficiency of 98.3% and a pressure drop of 0.25 mbar. Next, Sample 4 (produced with an increased needle-collector distance in the needle-collector distance) obtained a yield of 95.1% and 0.19 mbar. Samples 1 and 2 (speed increase in Y-axis) showed similar performances (91.7 and 95.2%, respectively), although sample 1 presented a pressure drop of 0.24 mbar and sample 2 of 0.39 mbar. This could be associated with the fact that Sample 2 presented different thicknesses along its Y-axis due to the long distance covered. The trajectory of the electrospinner needle performs a zigzag movement, where the amplitude of the angles depends on the speed in the Y-axis of the needle. The performance of Sample 3 (Δ kV reduction) was significantly lower than that of the rest of the samples (77.8%; 0.23 mbar). This could be attributed to the fact that the increase in voltage increased the diameter of the fibers and reduced the efficiency in particle retention, as it has been reported in previous works [17,44,45].

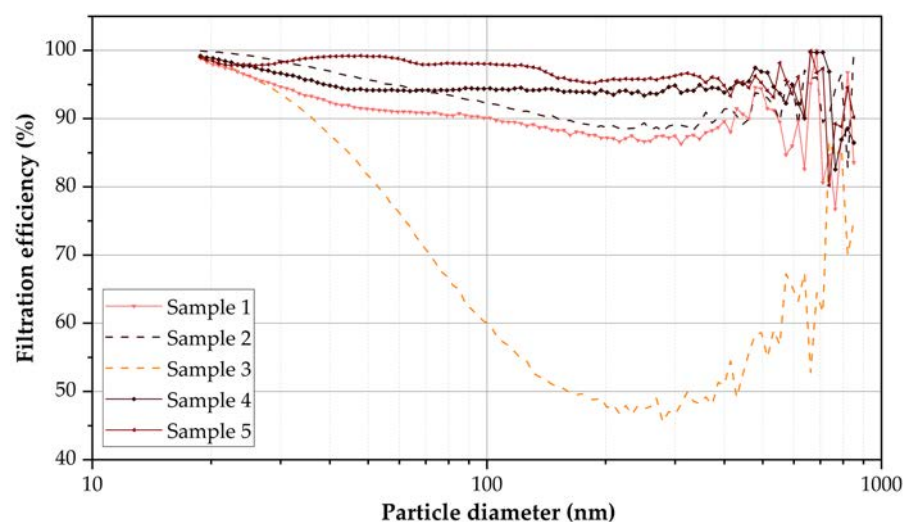


Figure 5. Filtration efficiency using different parameters during electrospinning with a 25 wt.% PET solution. The noise to signal ratio of the curves increases from 0.6 μm because the equipment used is not designed to analyze coarse particles. Particle concentration $>0.6 \mu\text{m}$ ($\sim 1 \times 10^5$ particles/ cm^3) is reduced compared to that of particles between 0.1 and 0.6 μm ($\sim 2.5 \times 10^5$ – 7×10^5 particles/ cm^3).

3.4. Fiber Diameter and Filtration Efficiency

The filtration efficiency is dependent on the fibers diameter. However, the reduction in fiber diameter implies a detrimental increase in pressure drop. In this work, five samples of filter material have been synthesized using variable relative mass quantities of polymer from different concentrations of the parent polymer solutions (15 and 25 wt.% PET) and their combinations, specifically, 1:0, 3:1, and 1:1, and vice versa. The different solutions were electrospun layer by layer one on top of the other. First, the layer of coarse fibers was made. Once the desired amount of polymer had been electrospun, electrospinning continued on top of the previous one using the lowest polymer concentration (i.e., 15 wt.%). The fibers obtained with 15 wt.% showed a fiber diameter of $0.29 \pm 0.18 \mu\text{m}$, as described in section 3.1, while the fibers obtained with 25 wt.% PET reached fiber diameters of $1.54 \pm 0.84 \mu\text{m}$. Experimentally, filtration efficiency increases with the presence of fine fibers (Figure 6a). Superior filtration performance is obtained as the amount of fine fibers increases (15 wt.% PET). In the same way, the pressure drop increases significantly with the reduction of coarse fibers (i.e., the ones obtained using 25 wt.% PET). The filter thickness is also important to achieve the desired filtration performance. Using a composition of 15–25 wt.% PET (3:1), the retention efficiency against submicron particles increases from $\sim 66\%$ (in the case of the thinnest membrane; $\sim 85 \mu\text{m}$) to more than $\sim 99\%$ (in the thickest membrane; $\sim 360 \mu\text{m}$). Figure 6b shows how performance and pressure drop variation depending on the density of the fabric. Therefore, this study shows that it is possible to achieve filters with good filtering capacity—pressure drop performance using combined fiber diameters by

simultaneously electrospinning polymeric solutions of different concentrations. Depending on the pressure drop and gas flow requirements, it is possible to design filters with high filtration performance. For example, to develop a KN95-type mask, the finest fibers should predominate (in this case, 15 wt.% PET) in a high-density fabric (i.e., 48.5 mg/cm²), always controlling to remain within the pressure drop limits established in the standards.

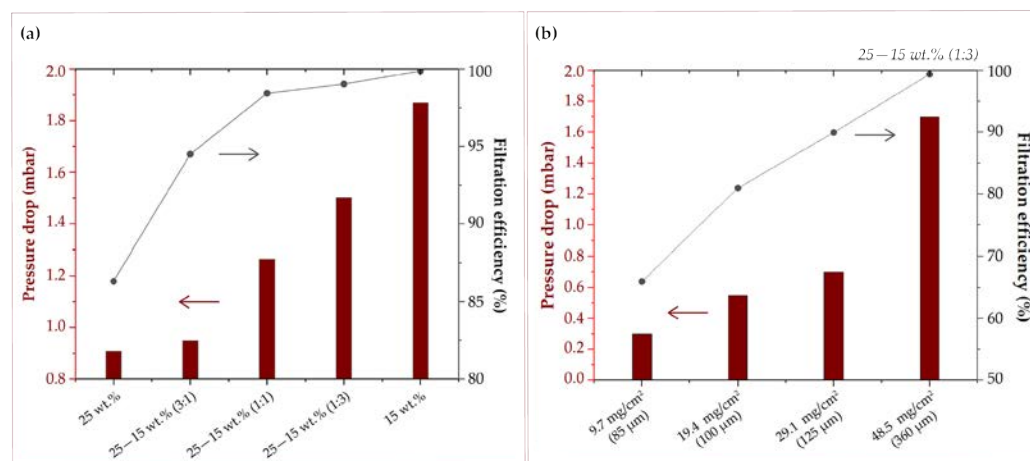


Figure 6. (a) Filtering efficiency against submicronic particles (0.01–1.0 μm) and pressure drop against different combinations of fiber diameters obtained by electrospinning solutions of 15 and 25 wt.% PET at different ratios and (b) solutions of 15–25 wt.% PET (3:1) at different fabric density.

3.5. Homogeneity across the Fabric

One of the problems encountered during the optimization of the fiber production method was the heterogeneity in the filtration efficiency for different samples/regions of a given fabric (Figure S2). This is attributed to the inherent performance of the electrospinning device despite having a moving needle when depositing polymeric mats on large surfaces. To explore the homogeneity of the filter in the same electrospun fabric, samples along one of the diagonals (~17.5 cm) were cut (identified correlatively from A to J from end to end). The sample was electrospun using the same conditions than in Sample 4 for being the sample with the best pressure drop—filtering efficiency ratio and high electrospinning productivity. The filtration and pressure drop tests showed high homogeneity of the central section: samples D, E, F, and G showed filtration efficiencies of 93.6, 94.3, 93.9, and 93.3%, respectively, with a pressure drop of 0.38 mbar for all of them except for sample G (0.34 mbar). However, the homogeneity was lost as the samples approached to the edges of the electrospun mat. The intermediate samples (B, C, H, and I) revealed a filtration efficiency of 91.7, 89.4, 90.9, and 90.4%, respectively, with a pressure drop of 0.36, 0.32, 0.36, and 0.32 mbar, respectively. End samples (A and J, Figure S2) markedly reduced the overall efficiency of the sample having filtration efficiencies of 81.7 and 81.5% and pressure drops of 0.08 and 0.24 mbar, respectively. The results of this experiment presumed the need to process the sample after electrospinning and select the most effective area. However, by using dynamic collectors (i.e., rotating mandrel collectors) this limitation could be easily overcome [46,47]. However, in this work we wanted to show that using a conventional electrospinning system it is possible to fabricate filtering media that fulfills the standards to be used as personal protection equipment in face masks.

3.6. Reproducibility

Atmospheric conditions, such as relative humidity (RH) and ambient temperature (T), may affect fiber formation during electrospinning. The increase in temperature increases the rate of evaporation of the organic solvents used and also decreases the viscosity of the solution. Humidity has a variable effect depending on the physico-chemical properties of the polymer used [48,49]. During one of the syntheses, the measured RH varied from

35 to 50% in a few hours and the temperature, from 20.4 to 23.9 °C. A first sample made during this period synthesis reached 93.1% filtration efficiency, while the final sample obtained showed 74.8%. It is thus necessary to control the environmental conditions at the time of electrospinning in order to ensure the uniformity of the samples generated. The electrospinner used in this work did not allow for a control on the temperature and the relative humidity; however, in other electrospinner models, these parameters can be easily controlled. Again, we wanted to show that with a conventional piece of equipment and without having a climate control unit it is possible to fabricate specific filtering media. Then, an evaluation of the reproducibility of the filter fabrics under similar T and HR was carried out. The particle retention efficiency of the obtained fabrics varied between 90.4 and 94.3% and the pressure drop was maintained (0.38 mbar) in the three samples obtained (Figure 7) in a range of RH of 36–42% and a T of 20.7–22.4 °C.

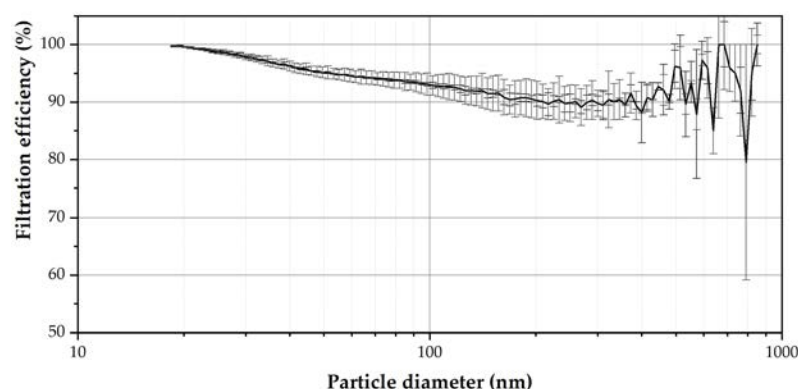


Figure 7. Evaluation of the reproducibility of the electrospinning process at similar atmospheric conditions (RH of 36–42% and a T of 20.7–22.4 °C) in triplicate.

3.7. Evaluation of the Retention Efficiency over Time

The stability of the filters generated with this technique was studied after 2 and 4 months of storage at ambient conditions. Six samples of filter material of different thicknesses synthesized under the same conditions were used (25 wt.% PET; DCM:TFA (3:1); flow rate of the solution 9.9 mL/h; applied voltage $\sim \Delta 15$ kV; distance from the needle to the collector $h \sim 25$ cm; speed on the X-axis: $v_x = 25$ mm/s; speed on the Y-axis $v_y = 2$ mm/s). In the submicron range (Table 2, Figure 8a), losses in retention efficiency between 0.5 and 15.2% were observed after two months, and between 2.9% and 24.8% after four months of storage inside a plastic bag (protected from sunlight) at room conditions. PET degrades under environmental conditions by hydrolysis and photolysis, causing a decrease in crystallinity and mechanical properties, as well as due to an increase in the hydrophilicity of its surface [50,51]. Although PET bottles have a slow rate of degradation depending on temperature and humidity [52], at the nanometric scale, deterioration is accelerated due to the high surface-to-volume ratio of the electrospun nanofibers. Sammon et al. [53] studied 100 nm PET-based films ($M_w = 70,000$ Da), and estimated a period of degradation by hydrolysis between 4 to 8 days in deionized water at 90 °C. In this way, the morphology of the fibers was negatively altered, affecting filtration. In our work, it was observed that, under the appropriate storage conditions (at room temperature and without UV radiation), the filtration was not reduced for particles larger than 400 nm. The results obtained by the OPS (polydisperse size distribution between 0.5 and 10 μm) showed losses in filtration efficiency between ~ 2 and $\sim 13\%$ in all cases. Specifically, the OPS equipment is designed to work with particle sizes greater than 0.5 μm , so the results obtained may not be significant. For aerodynamic diameters between 1 and 10 μm , the efficiency losses were reduced to a range from ~ 0.1 to $\sim 2\%$ (Table 3, Figure 8b). Specifically, considering the EN 14683:2019 [54] standard corresponding to the regulations applicable to surgical masks, the average aerodynamic diameter of the bacteria used in the filtration test is 3 ± 0.3 μm . Against this particle size, the initial tested samples reported a filtration efficiency of 100, 100,

97.0, 100, 95.3 and 97.3%, respectively, while the samples after 4 months storage showed filtration efficiencies as high as 99.8, 98.8, 98.5, 97.8, 98.2 and 98.6%, respectively, which means efficiency losses of less than 2.2% in all cases.

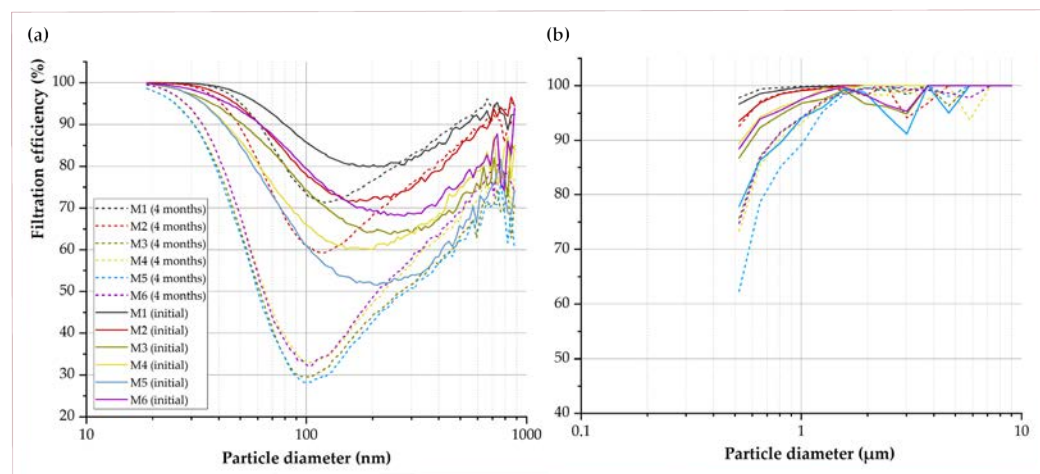


Figure 8. Loss of filtering efficiency after 2 (a) and 4 (b) months of samples stored at room temperature against 0.01–10.0 µm.

Table 2. Loss of filtering efficiency after 4 months of samples stored at room temperature (0.01–1.0 µm particle size range).

Particle Diameter Range 0.01–1.0 µm									
Sample	Thickness (µm)	Filtration Efficiency (%) ¹	Pressure Drop (mbar) ¹	Filtration Efficiency (%) ²	Pressure Drop (mbar) ²	Loss of Efficiency (%) ²	Filtration Efficiency (%) ³	Pressure Drop (mbar) ³	Loss of Efficiency (%) ³
M1	230	89.9	0.80	87.8	0.86	2.4	87.4	0.88	2.9
M2	210	85.5	0.64	85.1	0.76	0.5	80.9	0.68	5.4
M3	110	78.85	0.40	77.1	0.52	2.2	59.3	0.66	24.8
M4	110	76.6	0.42	65.0	0.36	15.0	63.1	0.70	17.5
M5	100	71.2	0.40	67.7	0.42	4.9	58.3	0.64	18.1
M6	190	82.5	0.46	70.0	0.46	15.2	63.5	0.70	23.0

¹ Initial values. ² After 2 months. ³ After 4 months.

Table 3. Loss of filtering efficiency after 4 months of samples stored at room temperature (0.5–1.0 µm and 1.0–10.0 µm).

Particle Diameter Range 0.5–1.0 µm					Particle Diameter Range 1–10 µm		
Sample	Thickness (µm)	Filtration Efficiency (%) ¹	Filtration Efficiency (%) ²	Loss of Efficiency (%) ²	Filtration Efficiency (%) ¹	Filtration Efficiency (%) ²	Loss of Efficiency (%) ²
M1	230	98.1	96.3	1.7	99.9	99.8	0.1
M2	210	96.5	92.5	4.0	99.9	99.8	1.1
M3	110	93.1	86.1	7.0	98.3	98.5	0.0
M4	110	95.2	82.4	12.8	99.7	97.8	1.9
M5	100	89.5	85.5	4.0	97.2	98.2	0.0
M6	190	93.9	82.5	8.5	98.8	98.6	0.2

¹ Initial values. ² After 4 months.

3.8. Development of a Surgical Mask

As a result of this work, the method for manufacturing surgical-type masks has been optimized (Figure 9). Usually, these masks are made up of three layers: an intermediate filter sandwiched between an outer and an inner layer of spun-bond, which protects the

filter placed in the middle [55]. The mask layers were synthesized using the conditions described in Table 4. Despite having better retention efficiency using low flow rates during the electrospinning process, the filtration efficiency required in surgical mask standards was also obtained using high flow rates. That made us to increase manufacturing productivity and increase the flow rate from 6.0 to 9.9 mL/h. The distance from the needle to the collector was kept at 25 cm for the production of the filter, while it was reduced to 15 cm for the production of the outer and inner layers. This allowed the synthesis of larger fibers, which were visible to the naked eye at the outlet of the electrospinner jet. While the sandwiched filter had an average fiber diameter of $1.24 \pm 0.70 \mu\text{m}$, the outer and inner layers were composed of larger fibers ($3.18 \pm 2.63 \mu\text{m}$) that were very heterogeneous. The approximate density of the filter was $\sim 20 \text{ mg/cm}^2$ and the thick middle filtering layer was electrospun to a thickness greater than $500 \mu\text{m}$.

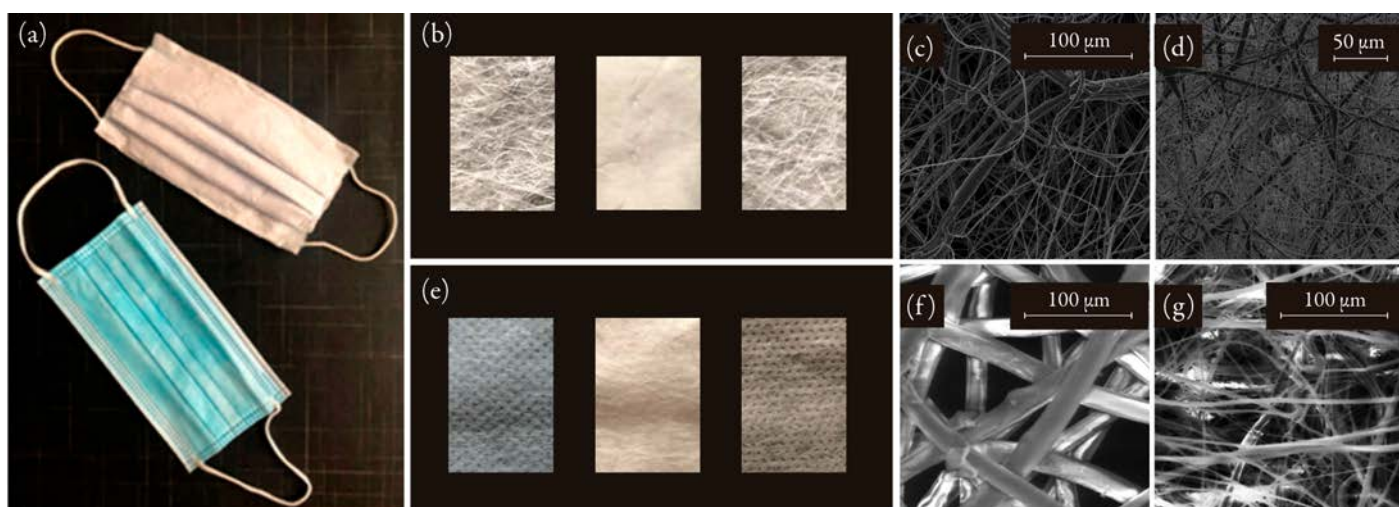


Figure 9. (a) PET-based surgical mask developed in this work (top) and a commercial surgical mask (bottom). (b) Macroscopic detail of the layers that make up the PET-based mask and (c) SEM views of the external layers and (d) the filter. (e) Macroscopic detail of the layers that make up the commercial surgical mask and (f) SEM views of the external layers and (g) the filter placed in the middle.

Table 4. Parameters used for electrospinning a surgical mask.

Parameters	Filter	External Layers
PET concentration	25 wt. %	25 wt. %
Voltage	$\sim \Delta 15 \text{ kV}$	$\sim \Delta 25 \text{ kV}$
Needle distance	25 cm	15 cm
Solution flow rate	9.9 mL/h	9.9 mL/h

The fabric presented a retention efficiency greater than 98.2% against particles between 0.5 and $10 \mu\text{m}$ and 100% against particles of $3 \mu\text{m}$ (Figure 10a), with a pressure drop of 0.36 mbar. Compared to a commercial surgical mask, this PET-based fabric had better retaining ability for fine and coarse particles. Filter samples show high variability when exposed to submicron particles, even when performed under the same conditions. This is not the case when it comes to coarse particles. In this case, there was no significant variability in retention efficiency.

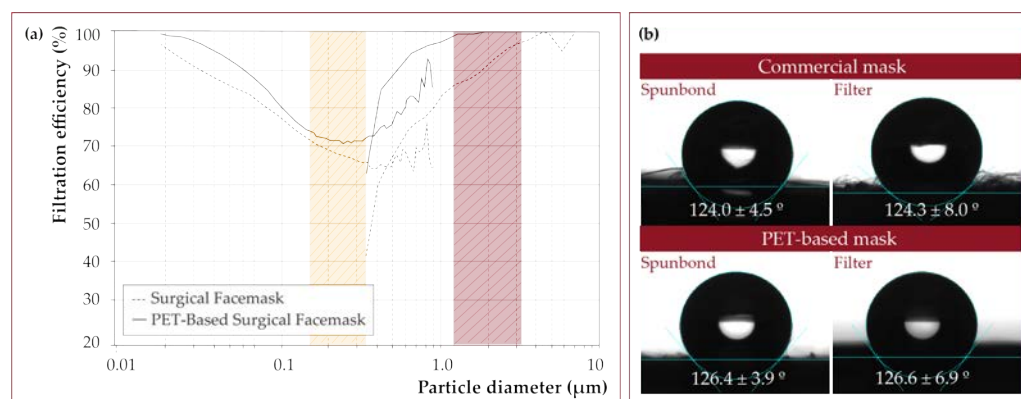


Figure 10. (a) Filtration efficiency of a PET-based surgical mask prepared in this work compared to a commercial surgical mask. The polydispersity range of particles used in the EN 14683:2019 surgical mask test is marked in red, and the range used in the EN 149:2001 standard, applicable to FFP2 (similar to KN95) and FFP3 (similar to KN99) masks, is marked in yellow. Labels for filtration efficiency at specific diameters (100, 300, 500, and 700 nm) can be seen on the graph. (b) The contact angle of the materials obtained compared to a commercial surgical mask.

In the same way, the fabric turned out to be as hydrophobic as the commercial one (Figure 10b). The spun-bond of the commercial surgical masks presented a contact angle of $124.0 \pm 4.5^\circ$, while the outer layers of the PET mask prepared in this work had a contact angle of $126.6 \pm 6.9^\circ$. The sandwiched filter of a commercial mask showed a contact angle of $124.3 \pm 8.0^\circ$, while the PET filter prepared in this work showed a contact angle of $126.4 \pm 3.9^\circ$.

Electrospinning is an easily scalable nanotechnological method, mainly due to its versatility for large-scale production [56,57]. The use of multiple nozzles, as seen in the industrial fiber production, has been investigated for upscaling [58,59]. Electrospun polyacrylonitrile-based masks [13,60] and filter media fabrication [61] are clear examples of large-scale production. Compared to the traditional processes for producing non-woven layers (Table 5), electrospinning offers nowadays lower production capacity. For example, production rates of up to 150 kg/h/m have been reported in meltblown processes (fibers 1–25 μm) using 12,000 nozzles per meter [62]. However, a rate of 30 kg/h/m is obtained in the production of surgical masks by extrapolating the electrospinning conditions used in this work. Additionally, this value might be underestimated because the method must be optimized on an industrial scale. Also, it is necessary to consider that although most of the solvents are evaporated during the electrospinning of the fibers, the fabric samples must be dried to facilitate their extraction from the aluminum foil where they are collected (Figure S3).

3.9. Exploring FFP2/KN95-Type Filters

The method for manufacturing FFP2/KN95-type masks has been explored (Figure 11). However, a fully functional mask model was not made due to the difficulty of obtaining a good facial fit. According to the EN 149:2001+A1:2009 standard [63] (specifically, the test methods section EN 13274-7:2020 [64]), FFP2-type masks must have an efficiency equal to or greater than 95% against NaCl particles between 0.01–1 μm (Stokes mean diameter of ~0.4 μm). In addition, the pressure drop must be less than 2.4 mbar against flows of 95 L/min.

On the one hand, 25–15 wt.% PET (1:1) solutions achieved a global filtration efficiency (0.01–1 μm) of more than 98% in ~10 mg/cm² density filter medium. The pressure drop was 1.26 mbar under similar conditions. Therefore, there is a margin to increase the filtration efficiency and stay within the permitted pressure drop limits. Note that similar filtering performances can be obtained with different parameter combinations. For instance, 15 wt.% PET (~10 mg/cm²) achieved an efficiency higher than 99.9%, but pressure drop increased up to >3 mbar.

Table 5. Advantages and disadvantages of the electrospinning process.

	Electrospinning Process	Traditional Meltblown/Spunbond Process
Advantages	Single production equipment can manufacture the outer layers and the mask's filter. Electrospun fabrics do not require heat-sealing processes to ensure the bonding of the fibers in the outer layers, although they do require fusing one layer with another. Electrospinning parameters can be easily manipulated to obtain different fabrics with variable filtration performance. It offers versatility to functionalize fabrics (e.g., incorporating antimicrobial materials).	Two different types of equipment are required to manufacture the meltblown (filter), and the spunbond (external layers) since the change in fiber diameter requires different techniques. The spunbond material requires a heat-sealing process to bond the fibers obtained and to form the fabric. It is more expensive and complex to manipulate the parameters to obtain different types of filter fabric. The versatility of the process is limited.
Disadvantages	Toxic solvents are used that must be handled in controlled environments. Reduced production rate.	It is not necessary to have controlled environments for handling the materials. High production rate.

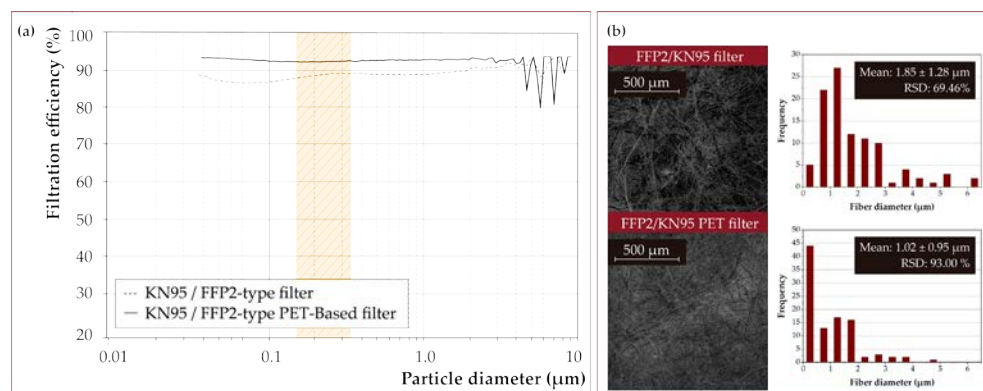


Figure 11. (a) Filtration efficiency of a PET-based FFP2/KN95-type filter prepared in this work compared to a commercial filter mask. The polydispersity range of particles used in the EN 149:2001 standard, applicable to FFP2 (similar to KN95) and FFP3 (similar to KN99) masks, is marked in yellow. (b) SEM micrographs and fiber size distribution histogram of electrospun and commercial fibers ($n = 100$).

3.10. Reprocessing of PET Masks

It is well known that single-use masks substantially impact the environment [65,66]. One of the crucial economical limitations of recycling is the diversity in the materials used in their manufacture. While the mask layers are mainly made of polypropylene, the ear loops are made of polyamide or rubber and the nose adjustment (nose strip) has a metallic component [67]. Hence, any recycling is complex due to the different nature of their components. In addition, there are variations in the use of materials and their final conformation, such as the combination of polypropylene with polyethylene to manufacture mask layers [68]. The separation of the mask components is one of the most significant limitations of recycling. Alternatives have been proposed to alleviate these limitations, including the use of other materials [69,70], sterilization of masks [71,72], and the possibility of recycling mask materials using different technologies [67,73]. However, the cost of masks has fallen as demand has increased [74]. Considering that the price of a surgical face mask is nowadays less than 0.1 USD/unit, its disassembly and pathogen-free guarantee makes its reuse uneconomical to compete in any market. For instance, the cost of sterilization

reprocessing has been estimated at approximately 1.7 USD/unit [75], although it would represent a significant advantage for the environment [76]. In short, the economic cost of recycle them is nowadays prohibitive considering the existing technologies.

Consequently, sustainable alternatives are required to reduce environmental impact. Another advantage that this new strategy offers for manufacturing surgical masks is their ease of reprocessing. Our PET-based fabrics can be re-dissolved in DCM:TFA (3:1), following the steps above, and re-electrospun without affecting the properties of the polymer or the ones of the resulting mat. Figure 12a shows the filtration efficiency for submicron particles of a sample of initial material and a sample obtained by re-electrospinning the same material. There is a small dispersion in the filtering efficiency associated with the climatological variation between the days of the syntheses, but even the recycled sample showed superior efficiencies. As seen in Figure 12b, the morphology of the fibers is not affected by the reprocessing of the PET.

One of the main limitations in reprocessing respiratory protection equipment is the potential contamination with pathogens after use. In this work we chose DCM and TFA not only because they are able to dissolve PET but also because their denaturalizing ability together with the acidic character of TFA [77,78] would contribute to denaturalize potential microorganisms present during reprocessing. For instance it has been reported by Byers et al. [79] that DCM at very low concentrations (78 mM) already inhibits the growth of methanotrophic bacteria. In a future work we will evaluate the inactivation ability of the solvents used (TFA:DCM) on common pathogens (bacteria and viruses) potentially present on used face masks based on the protein denaturizing ability and acidic character of this organic mixture.

The concept of the surgical face mask here proposed would be slightly different from the conventional single-use masks currently existing. In order to improve the life cycle of PET masks (Figure 13) we propose that during the heat-sealing process itself, two holes would be placed in the top and bottom layers of the mask. In this way, reusable and interchangeable rubber bands could be re-used without interfering with the recycling process. In addition, optionally, adhesive nose wires could be used and easily removed, although it is not essential since they could be removed after PET dissolution.

In a potential industrial production those organic solvents evaporated during the electrospinning process would also be condensed and recycled. Both solvents once condensed can be easily separated by distillation due to their different boiling points at 1 atm (72.4 °C and 39.6 °C for TFA and DCM, respectively). Any possible residue would be treated as a hazardous waste according to the legislation.

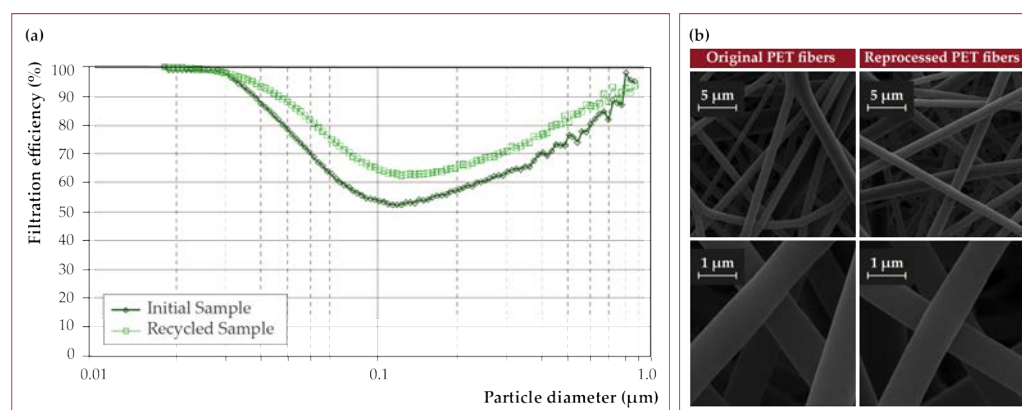


Figure 12. (a) Filtration efficiency of an initial PET fabric sample versus recycled (re-electrospun) PET fabric from an equivalent surgical mask and (b) SEM views of the PET fibers after reprocessing.

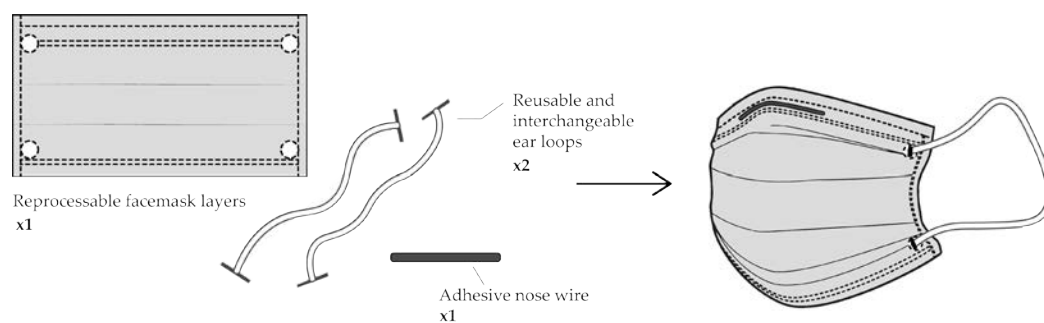


Figure 13. Schematic representation of the PET-based surgical mask concept.

4. Conclusions

In this work, the influence of various electrospinning parameters on the filtration performance of fibers based on recycled PET has been evaluated. The parameters that influenced the filtration efficiency the most were the increase in the distance between the needle and the collector and the reduction in the output flow rate of the polymeric solution, in addition to the polymer concentration influence, which was decisive for the final diameter of the fibers obtained. Using the results reported in this work, it is possible to develop a wide variety of filter media with different applications. For example, one of the strategies used was to combine fibers produced by electrospinning different concentrations of the polymer (i.e., 15 and 25 wt.%), achieving increased efficiency for the samples with a superior concentration of small fibers produced using 15 wt.% polymer concentration. This could be useful for the production of higher efficiency masks (i.e., KN95, KN99 or air filters).

Finally, we report an efficient method for the production of surgical masks based on three layers: two thick fibers ($3.18 \pm 2.63 \mu\text{m}$) and an intermediate one made of finer fibers ($1.24 \pm 0.70 \mu\text{m}$). The two layers of coarse fibers serve as filter protection and have neither pressure drop nor good filtration performance. The intermediate layer acts as a filter and has a filtration capacity greater than 98.2% for particles between 0.5 and $10 \mu\text{m}$ and 100% compared to the $3 \mu\text{m}$ particles used in the standard. The determined pressure drop was 0.36 mbar, within the ranges established in the same standard. The electrospinning process for obtaining this mask was optimized to maximize productivity, using the highest polymer concentration (25 wt.%) and the highest solvent output flow (9.9 mL/h). The resulting material had a hydrophobicity comparable to that of a commercial surgical mask. Surgical masks are usually tested against $3 \pm 0.3 \mu\text{m}$ particles (following EN 14683 protocol), so we demonstrated in this work that the retention capacity of our mats would not be affected significantly over time (up to 4 months) in the capture of particles of those sizes. In addition, our electrospun mats could be re-dissolved and reprocessed without losing properties using the same conditions as in the initial samples, so it could represent a new circular economy method that would reduce the environmental impact caused by the pandemic.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/nano12060925/s1>, Figure S1: Particle concentration distribution depending on their diameter used for the efficiency determination measurements in the range (a) 0.01– $0.5 \mu\text{m}$ (SMPS) and (b) $>0.5 \mu\text{m}$ (OPS); Figure S2: “Evaluation of the homogeneity of a sample of filter fabric”; “Drying of fabric samples” section; Figure S3: SEM images of fibers of the morphological deformation of PET fibers after being dried at 80°C for 30 min.

Author Contributions: Conceptualization, M.B.-L., C.Y. and M.A.; methodology, M.B.-L., C.Y., S.J., S.I. M.A. and V.S.; software, M.B.-L.; validation, S.J., and M.B.-L.; formal analysis, M.B.-L. and M.A.; investigation, M.B.-L., C.Y. and S.J.; writing—original draft preparation, M.B.-L. and M.A.; writing—review and editing, M.A., S.I., S.J., V.S. and C.Y.; supervision, M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Quinto artículo

Development and validation of a methodology to measure exhaled carbon dioxide (CO₂) and control indoor air renewal

[Artículo bajo revisión]

Preprint medRxiv

<https://doi.org/10.1101/2022.09.22.22280262>

Fecha de envío: 19 de junio de 2022

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1 Development and validation of a methodology to 2 measure exhaled carbon dioxide (CO₂) and control 3 indoor air renewal

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8 **Abstract:**

9 The measurement of CO₂ has positioned itself as a low-cost and straightforward
10 technique to indirectly control indoor air quality, allowing the reduction of the
11 concentration of potentially pathogen-loaded aerosols to which we are exposed.
12 However, on numerous occasions, bad practice limits the technique for CO₂ level
13 interpreting and does not apply methodologies that guarantee air renewal. This work
14 proposes a new methodology for measuring and controlling CO₂ levels for indoor air in
15 shared spaces. The proposed methodology is based on three stages: diagnosis,
16 correction protocols, and monitoring/control/surveillance (MCS). The procedure is
17 explained using a cultural center as an actual base case study. Additionally, the
18 procedure was validated by implementing 40 voluntary commercial spaces in Zaragoza
19 (Spain). Standardization of methods is suggested so that the measurement of CO₂
20 becomes an effective strategy to control the airborne transmission of pathogens and
21 thus prevent future Covid-19 outbreaks and novel pandemics.

22 **Keywords**

23 Carbon dioxide; CO₂; infectious diseases; environmental engineering; covid-19; airborne

24 **1. Introduction**

25 Since the global acceptance of the transmission of COVID-19 by aerosols,¹ numerous
26 strategies have been developed to prevent air quality degradation by different means. It is
27 possible to differentiate between air renewal and air purification strategies. On the one
28 hand, renewal techniques evaluate and optimize air renewal in closed spaces. On the other
29 hand, purification methods are varied and struggle to eliminate potential airborne
30 pathogens. Although both strategies share the common goal of reducing the airborne
31 spreading of respiratory diseases, air purification is seriously handicapped because it
32 usually requires higher economic investments (e.g., bipolar ionization systems, UV-C
33 radiation devices), and its efficacy has been poorly tested against SARS-CoV-2. As an
34 exception, high-efficiency portable air cleaners (e.g., HEPA filters) are presented as an
35 affordable, high-performance method that can filter up to 99.9% of submicron particles and
36 reduce SARS-CoV-2 aerial viral viral viral load up to 80%.^{2,3} However, in actual operating
37 conditions, it is necessary to consider the workflow of the equipment and its location to take
38 advantage of its performance.

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.

39 Ideally, measuring aerosols would allow knowing the quality of the air and estimating the
40 risk of contagion. Nevertheless, the direct measurement of aerosols is highly complex and
41 expensive since it requires specialized equipment. To overcome these hurdles, the carbon
42 dioxide (CO₂) measurement has been suggested as an indirect indicator of the risk of
43 respiratory infectious diseases transmission.⁴ The measure of exhaled CO₂ was proposed
44 before the pandemic to control the airborne spread of diseases since it represents a greater
45 probability of breathing air previously inhaled by other people.⁵ Consequently, the indoor
46 CO₂ measurement is suggested as a reasonable ventilation proxy. It is possible to determine
47 what percentage of the exhaled air by another individual (y) according to the expression
48 $y = C_e x + C_a(1 - x)$. Where, C_e corresponds to the concentration of CO₂ in exhaled air
49 (estimated at 40,000 ppm), C_a to the ambient CO₂ concentration and x to the fraction of
50 exhaled air. For instance, assuming a basal value of 440 ppm (fresh air outdoors), which
51 increases indoors up to 2,000 ppm, the approximate percentage of air that those individuals
52 have already breathed will be 3.9 %.

53 One of the significant limitations of CO₂ level measurement is that it cannot be directly
54 related to the concentration of aerosols in the environment since the generation of
55 bioaerosols depends on the type of respiratory activity.⁶ For example, aerosols generated
56 during sustained vocalization (e.g., singing) are not comparable to those generated during
57 silent breathing.⁷⁻¹⁰ Nevertheless, CO₂ concentration limits have been proposed to reduce
58 COVID-19 transmission, usually between 700 and 1,000 ppm, regardless of the event.^{11,12}

59 SARS-CoV-2 is mainly spread over time and distance. An average of 3.1 ± 2.9 copies/L of air
60 viral load average is deduced from a total of 313 samples.¹³⁻³⁵ It has been established that
61 close contact transmission is predominant, while fomites and aerosols explain special
62 propagation events in punctual events, according to CDC guidelines.³⁶ The massive
63 infections associated with superspreading represent a scenario of complex epidemiological
64 management. To date, superspreading events have only been reported indoors.^{28,37-55} Due
65 to the difficulty of discriminating between superspreaders and non-spreaders,^{28,56} it is
66 imperative to improve air quality in public and shared spaces. By implementing the proper
67 measures, it is possible to reduce cases of respiratory diseases in the community and
68 prevent future pandemics with devastating effects.

69 Many countries have implemented CO₂ measurement as a standard in different spaces,
70 such as shopping centers,⁵⁷ collective transport,⁵⁸⁻⁶¹ workspaces,⁶² or university and school
71 classrooms.⁶²⁻⁶⁷ Beyond research projects, some governments have decided to bet on
72 scientific criteria and invest resources to mitigate contagion among the population. For
73 example, the U.K. government distributed more than 300,000 CO₂ meters in its schools to
74 optimize air renewal during classes.⁶⁸ In Germany, a global approach was also carried out,
75 investing more than €17M to guarantee the presence of meters in schools.⁶⁹ An investment
76 of \$350 billion for state and local governments has recently been reported and \$122 billion
77 for schools, which can support making ventilation and air filtration upgrades in the U.S. to
78 reduce the respiratory pathogens spreading.⁷⁰

79 However, there are still no standardized methodologies for measuring and controlling CO₂.
80 "Aireamos" (in Spanish, it refers to "renewing air"),^{71,72} as an international independent
81 scientific working group, has been a pioneer in developing the bases for measurement,

82 making available to the population a set of valuable tools and resources to control the
83 renewal of indoor air. However, there is still no straightforward procedure for intervening
84 in public and shared spaces. Before the pandemic, CO₂ measurement was mainly explored
85 in school settings. Numerous investigations pointed to an improvement in the pupil's
86 concentration in well-ventilated classrooms.⁷³⁻⁷⁵

87 This article proposes a new methodology for effectively measuring and controlling CO₂ in
88 closed and shared spaces. The methodology is based on three stages: diagnosis, correction,
89 and controlled monitoring. The methodology allows us to interpret the air renewal patterns
90 and estimate if the renewal is appropriate to the activity and type of space. Additionally, this
91 method has been validated as a pilot project in a 3-floors building with multiple teaching
92 rooms and cultural activities and a subset of 40 local businesses in Zaragoza (Spain).

93 2. Materials and Methods

94 2.1. General description of the methodology

95 A methodology based on three standard stages with specific goals was developed. They
96 enumerate as follows:

- 97 a) Diagnosis. It is intended to acquire detailed knowledge on how CO₂ concentration
98 levels vary within the physical limits of the shared space and what is its time evolution
99 as a function of specific activities carried out in the shared space. The initial diagnosis
100 includes a description of the space indicating the measures of the shared space (e.g.,
101 size, volume, and architectural geometry), other CO₂ sources (e.g., gas stove), the
102 presence and ubication of windows and doors, as well as the availability of Heating,
103 Ventilation, and Air Conditioning (HVAC) systems and hoods. During this process, it is
104 necessary to identify the "person in charge" and perform minimal instruction about
105 the project and concepts such as air renewal, ventilation, metabolic CO₂, and how to
106 measure it.
- 107 b) Correction. According to the acquired knowledge in the diagnosis stage, it is possible
108 to proceed with the design of customized protocols and procedures to improve the air
109 renovation that will imply variations in the CO₂ concentration. The CO₂ levels will be
110 monitored with a CO₂ meter to avoid trespassing a defined threshold considered as a
111 low-risk level by the scientific community.
- 112 c) Monitoring, control, and surveillance (MCS): Continuous monitoring of CO₂
113 concentration is performed to guarantee that the shared space is always kept at low-
114 risk levels. In case of the CO₂ concentration levels reach the maximum level, an alert is
115 issued so that people in charge of the shared space activate the protocols and
116 procedures defined in the previous stage to fix the situation.

117 For each stage, achieving the primary goals depends on specific characteristics of the
118 shared space, such as main business or social activity, geometrical properties, number and
119 location of windows and doors, or a maximum number of people sharing the room at the
120 same time. A case study and a practical application is presented in this article to explain this
121 methodology.

122 2.2. Measurement of metabolic CO₂

123 Any time measuring metabolic CO₂ levels is required, Aranet CO₂ meters (Aranet Wireless
124 Solutions España SL, Spain) and Signos CO₂ meters (Signos.io, Spain) will be used. The tech-
125 nical characteristics of these measuring devices are shown in Table 1.

126
127 **Table 1.** Technical characteristics of Aranet and Signos SIO₂ CO₂ meters.

Characteristics	Aranet 4 Pro	Signos (SiO ₂)
CO ₂ measurement	< 9,999 ppm	< 5,000 ppm
Temperature measurement	0 – 50 °C	-60 – 80 °C
Humidity measurement	0 – 85 %	0 – 95 %
Atmospheric pressure	0.3 – 1.1 atm	0.3 – 1.1 atm
Sensor	N-DIR	N-DIR
Sampling frequency	1 min	11 min
Transmission	Bluetooth (-12–4 dBm)	LoRa
Precision	± 50 ppm	± 50 ppm
Dimensions / weight	70x70x24 mm / 104 g	100x100x23 mm / 170 g

129 2.3. The methodology explained in a case study

130 As a case study, the methodology for improving air quality was applied in a cultural center.
131 The building has 1,200 m² divided in three floors (Figure 1a). A 132 m² room located in the
132 basement was selected (Classroom 1, see Figure 1b).

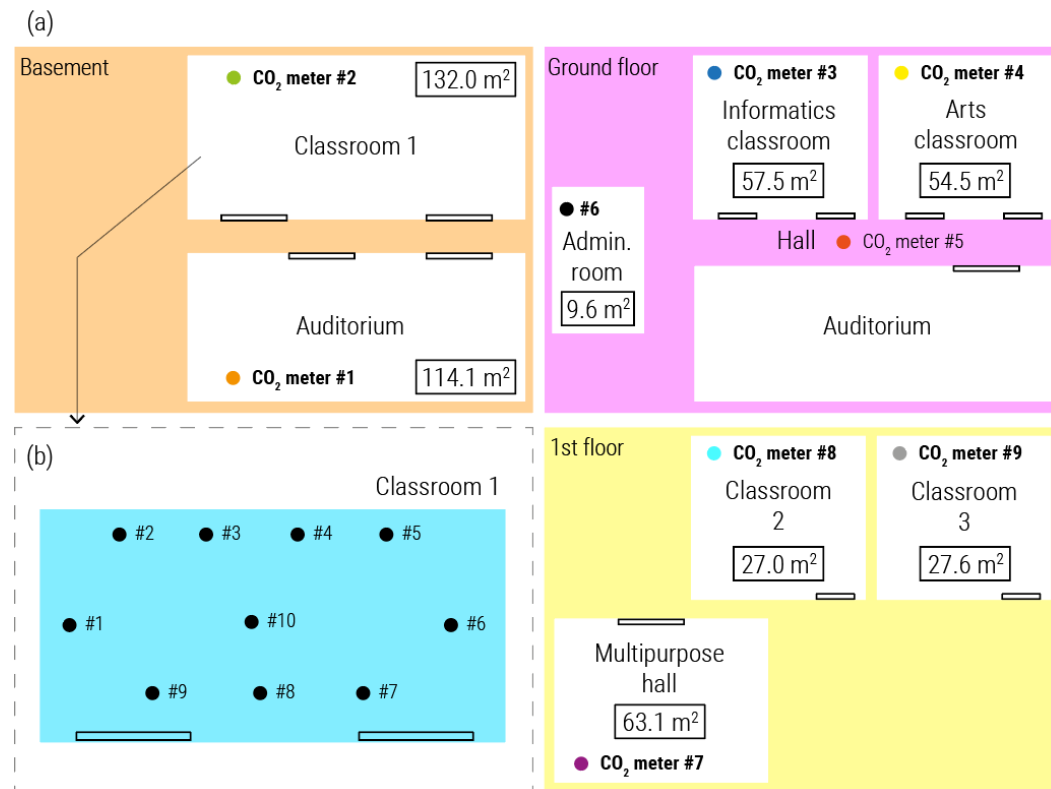
133 a) Diagnosis: The shared spaces were analyzed for four days (February 24th to 27th, 2021)
134 by placing 10 CO₂ meters (Aranet 4) in strategic locations, as shown in Figure 1a. A CO₂
135 measuring device was placed outdoors so that it was possible to calculate the increase
136 in CO₂ (ΔCO_2) associated with human activity according to Equation 1; where (CO₂)_{ext}
137 corresponds to CO₂ outside and (CO₂)_{ind} to CO₂ indoors.

$$138 \Delta CO_2 = (CO_2)_{ind} - (CO_2)_{out} \quad (Equation 1)$$

139 In the first approach, the data showed frequent high CO₂ concentration levels probably
140 linked to specific activities. Subsequently, the shared spaces were carefully monitored
141 for six more days (February 27th to March 5th, 2021) to confirm the hypotheses that
142 could be made after the initial analysis. This process makes it possible to characterize
143 the air renewal patterns in each space based on activity and capacity. At least one CO₂
144 meter was placed every 20 m² at a minimum height of 1.5 m. Ideally, they will be placed
145 near exhaust fans (to measure a significant air sample) and/or in locations where air
146 turnover is poor (e.g. corners), and far from windows and doors.

147 b) Correction: Based on the obtained results, an attempt was made to reduce the level of
148 metabolic CO₂ below 800 ppm. One of the classrooms in the basement of the building
149 (Classroom 1) showed inefficient ventilation patterns. Dry ice was used to saturate the
150 room with CO₂ and find effective strategies to improve air renewal. According to Figure
151 1b, 10 CO₂ meters (Aranet 4) were placed in the classroom to analyze the improvement
152 of changes in the HVAC and natural ventilation (through doors and windows opening
153 or closing).

154 c) MCS: This stage was performed until May 15th, 2022 using 10 CO₂ meters (Signos).
 155 Whenever the cultural center exceeds the 800 ppm level for more than 30 minutes, it
 156 receives a telematic alert or phone call to ask the "person in charge" to correct the
 157 situation.



158
 159 **Figure 1.** (a) General distribution of CO₂ meters in the education building and (b) classroom 1 detail.

160 2.4. Broader validation in commercial spaces

161 Subsequently, the method was applied to 40 businesses / retail stores spaces located in
 162 Zaragoza's city center (Spain). These included: one optician, twelve clothing stores, two
 163 kids' clothing stores, five shoe stores, two jewelry stores, four food stores, two restaurants,
 164 five home stores, two consultancies, two dietary and health centers, and two florists. The
 165 selection of commercial spaces was made to cover different typologies. For example,
 166 customers spend long periods in restaurants, while their exposure to indoor air in flower
 167 retailers is minor. Two CO₂ meters were placed in each establishment, except for a kid's
 168 clothing store, where only one was placed, and another children's clothing store, where
 169 three were placed.

170 Interventions in shared spaces were carried out following the three phases: diagnosis,
 171 correction, and MCS.

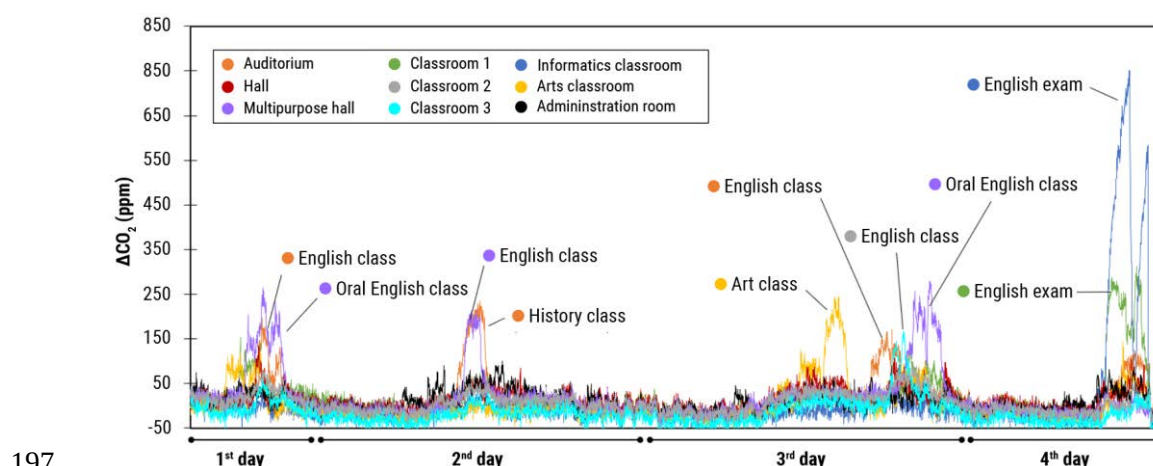
172 a) Diagnostic. Two CO₂ meters (Aranet 4) devices were placed at strategic points for 2
 173 or 3 days in each business. These meters were chosen because they record the CO₂
 174 level every minute. The CO₂ curves were analyzed during the business hours of each
 175 establishment. This phase allowed us to know if the sensors took representative
 176 measurements of the total space and have a global image of the air quality in the
 177 volume area. The diagnostic process might include the use of risk calculators or

178 methods for simulating disease propagation via aerosols based on the standard
 179 Wells-Riley model for infectious respiratory disease transmission by airborne
 180 particles.^{71,72,76}
 181 b) Correction. In spaces where it was necessary (typically presenting CO₂ levels >1,000
 182 ppm for more than 30 minutes), a correction phase was carried out where preventive
 183 measures were implemented to improve indoor air renewal. In most cases, this phase
 184 was superimposed on monitoring to determine the advantage obtained at the time.
 185 c) MCS. The monitoring stage occurred during January and February 2022. For this
 186 stage, CO₂ (Signos) meters were installed in each establishment. These CO₂ meters,
 187 include IoT technology, enabled remote access to streaming data.

188 3. Results

189 3.1. The diagnostic stage to allow detection of inefficient air renewals

190 The diagnosis must be performed on a long enough time interval to cover the specific and
 191 regular casuistry of the establishment. For example, in the base case study (cultural center),
 192 the classrooms usually are occupied for daily classes. Therefore, a selection of days where
 193 the classrooms are occupied is sufficient. In this case, four days were used for the
 194 preliminary analysis of the space. Assuming a proper maximum CO₂ level of 800 ppm, a
 195 maximum increase of 400-450 ppm compared to fresh outdoor air was considered
 196 acceptable.



197
 198 **Figure 2.** Increment of CO₂ values recorded in the diagnostic period.

199 As shown in Figure 2, there are peaks in the increment of CO₂ associated with the activity of
 200 each room. Knowing the activity and the number of occupants is essential to interpret the
 201 CO₂ measurements. Determining the ppm/person ratio can be helpful, as we proposed in
 202 previous work (see ⁶¹). This value incorporates the occupancy variable and allows a more
 203 precise definition of the efficiency of air renewal, although it assumes homogeneity in
 204 exhalation between individuals. In addition, it is crucial to request the usual activity in the
 205 space. Those responsible must not consciously increase ventilation (e.g., opening windows)
 206 to avoid underestimating CO₂ measurements. A typical table with essential information is
 207 shown in Table 2. The method to fill in the table must be explained in the training phase
 208 since it is of utmost importance to interpret the data properly later. In the base case, the
 209 room had no open windows, except for the Hall and the Art Classroom. In contrast, all doors
 210 were open during diagnostic measurements.

211

Table 2. Example of a data sheet for CO₂ measurements interpretation.

Day	Duration	Activity	Occupation	Location	Mean ΔCO ₂	Max ΔCO ₂	Mean ppm/person	Max ppm/per- son
1 st day	1:30 h	English class	8 pax	Auditorium	106 ppm	196 ppm	13.3 ppm	24.5 ppm
2 nd day	2:00 h	History class	15 pax	Auditorium	164 ppm	234 ppm	11.0 ppm	15.6 ppm
3 rd day	2:00 h	English class	9 pax	Auditorium	116 ppm	170 ppm	12.9 ppm	18.9 ppm
1 st day	1:30 h	English class	10 pax	Classroom 1	89 ppm	153 ppm	8.9 ppm	15.3 ppm
3 rd day	2:00 h	English class	8 pax	Classroom 1	76 ppm	140 ppm	9.5 ppm	17.5 ppm
4 th day	2:30 h	English exam	18 pax	Classroom 1	219 ppm	312 ppm	12.2 ppm	17.3 ppm
4 th day	4:00 h	English exam	7 pax	Informatics	328 ppm	751 ppm	46.9 ppm	107.3 ppm
2 nd day	3:00 h	Art class	6 pax	Art classroom	68 ppm	121 ppm	11.3 ppm	20.2 ppm
3 rd day	1:30 h	Art class	6 pax	Art classroom	34 ppm	125 ppm	5.8 ppm	20.8 ppm
3 rd day	3:00 h	Art class	6 pax	Art classroom	79 ppm	243 ppm	13.2 ppm	40.5 ppm
3 rd day	2:30 h	Art class	4 pax	Art classroom	58 ppm	118 ppm	9.7 ppm	19.7 ppm
1 st day	2:30 h	English class	7 pax	Multi. hall	163 ppm	263 ppm	23.3 ppm	37.6 ppm
2 nd day	1:00 h	English class	10 pax	Multi. hall	181 ppm	213 ppm	18.1 ppm	21.3 ppm
3 rd day	2:30 h	English class	8 pax	Multi. hall	177 ppm	279 ppm	22.2 ppm	34.9 ppm

212 Comparing the ppm/person indicator in similar activities in different spaces makes it
 213 possible to determine the relative efficiency of air renewal in each space. For instance, in
 214 English classes (not oral), 9.2 ± 16.4 , 13.1 ± 21.7 , and 18.1 ± 21.3 ppm/person were achieved
 215 in Classroom 1, the Auditorium, and the Multipurpose Room, respectively. It indicates that
 216 air renewal is similar in all rooms, although slightly improved in Classroom 1. On the
 217 contrary, disparate ratios were obtained in the Cambridge exams in Classroom 1 and the
 218 Informatics Classroom. While in Classroom 1, the ratio was 12.2 ± 17.3 ppm/person, in the
 219 Informatics Classroom, a 46.9 ± 107.3 ppm/person was found. Again, this indicates more
 220 efficient air renewal inside the basement room.

221 Some gaps may go unnoticed during the diagnostic phase. Due to the atmospheric
 222 conditions or the activity during the diagnosis, the CO₂ levels may be decreased. Although
 223 we recommend analyzing 3-4 critical days, subsequent days (e.g., 5-6 days) should be closely
 224 monitored to ensure representative results. For example, in the base case, on the
 225 monitoring days after diagnosis, higher CO₂ concentrations were recorded in Classroom 1
 226 than in other areas, even in similar conditions of capacity and activity.

227 **3.2. Interpretation of CO₂ measurements, methods to correct gaps, and** 228 **monitoring**

229 After detecting spaces that can be improved, it is necessary to implement efficient corrective
 230 measures that guarantee adequate air renewal. First, it is crucial to interpret the
 231 measurements and begin to rule out possibilities. In the case of Classroom 1, the following
 232 hypotheses were prepared:

- 233 • Hypothesis 1. The activities studied in the initial diagnosis were not representative,
 234 and the high activity in the classroom, so more CO₂ is exhaled. Then, the following
 235 12 English classes in Classroom 1, comprising the months of April to June, were
 236 analyzed. In the studied activities, no significant increase in CO₂ was observed,

except for the classes held in June, where an increase of 522 ppm was reached. Figure 3 shows the evolution of average CO₂ from April to June, showing an increase in concentration in the latter month. However, CO₂ exhalation in the records studied is similar, so this hypothesis is ruled out.

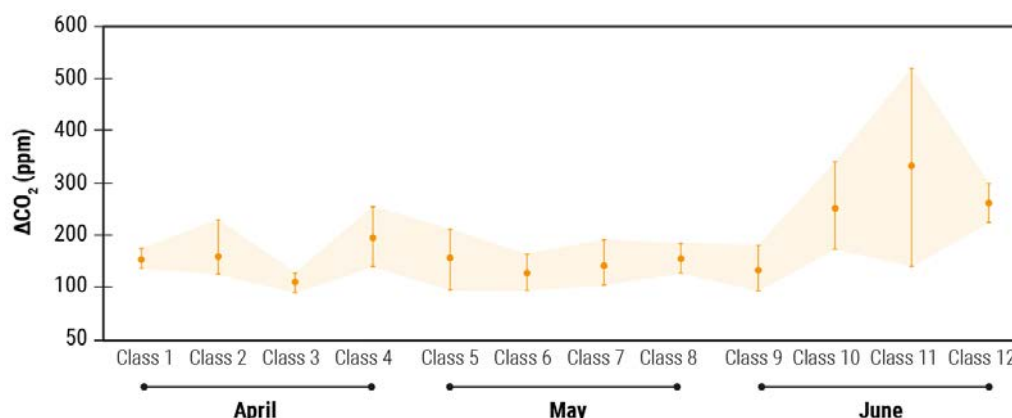


Figure 3. Evolution of average ΔCO₂ in English classes from April to June.

- Hypothesis 2. The weather conditions of the initial diagnosis were favorable for air renewal ratios. The weather influence on the ventilation ratios was evaluated by analyzing the incidences (values above 800 ppm for more than 30 minutes) recorded from April to July (regardless of the activity carried out). Of the 13 incidences found, 46 % (6) came from July, while 31 % (4) came from June, and the remaining 23% (3) from May. The climatic variation has affected the ventilation performance of the space. Working on days with less favorable weather will be necessary to test the corrective measures.
 - Testing corrective measures. An experiment where the space was saturated with CO₂ using dry ice was conducted, stabilized as a function of the amount evaporated. Two scenarios were evaluated with an activated HVAC system: with and without opening doors. Figure 4 shows the results obtained. With doors closed, it takes ~1,600 ppm ~37 minutes to reduce the CO₂ concentration to ~800 ppm, at 22 ppm/minute. While, with doors open, the reduction from ~1,600 ppm to ~800 ppm occurs in ~17 minutes, at 47 ppm/minute. The opening of doors with the HVAC system active supposes a remarkable improvement of the ventilation in this classroom.
- In addition, this method helps calculate actual air changes per hour (ACH) in space. The HVAC flow in Classroom 1 is 750 m³/s. For a volume of 363 m³ corresponding to the space, a theoretical ACH of 2.06 (door closed) is estimated. An ACH of 4.42 (with opened doors) and 2.03 (closed doors) has been determined by dry ice tests. It suggests that the tests have been carried out correctly and that the opening of the doors represents a considerable improvement in air renewal compared to the closed space.

Sometimes, the subsequent monitoring phase can be used to assess the benefit of the corrective actions imposed. In the base case, opening doors in Classroom 1 was enough to achieve adequate air renewal, even in the summer months. Ideally, during monitoring, it would be necessary to implement a system that alerts those

responsible for the space if the established CO₂ limits are exceeded. This strategy allows for preventing possible incidents and correcting CO₂ levels exceeded in streaming, thus, guaranteeing adequate air quality.

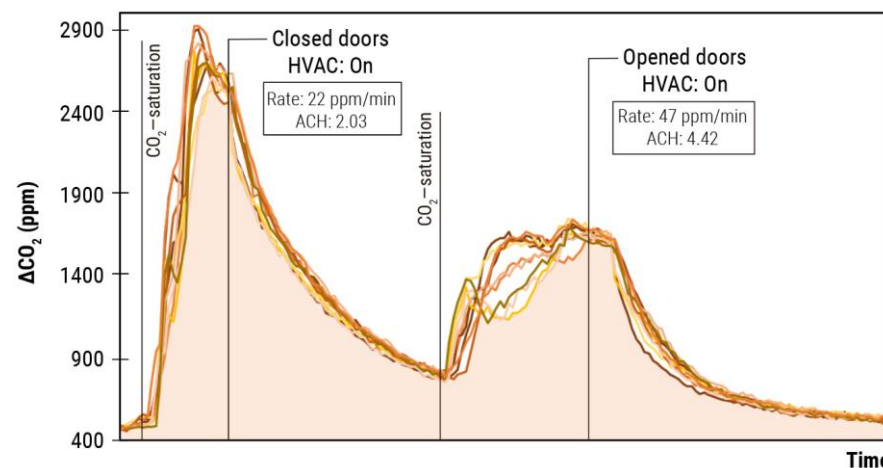


Figure 4. Results of the experiment with dry ice in Classroom 1, with and without open doors.

3.3. Applying and validating the method in local businesses

To perform a broader application of the methodology to diagnose, correct, and monitor CO₂ we performed a study in 40 retail stores. To this end we modified the phases to meet the pre-established requirements, and the absolute CO₂ concentration was measured (instead of the outdoor-indoor CO₂ increase). In addition, the ppm/person indicator could not be used since the businesses could not provide a continuous record of the occupancy. Initially, a preliminary characterization (diagnosis) of the participant businesses was conducted. 23 businesses with CO₂ values above the maximum recommended (800 ppm) were identified. Eight of them did not require specific follow-up interventions as they were minimal and discrete increases. The other 15 participants were subjected to an exhaustive evaluation that served to design and implement a series of prevention measures that had to be implemented to improve air recirculation in the commercial spaces.

The implementation of corrective measures was modified from the original approach. Instead of *in-situ* experimenting, it was decided to assess the improvement of the proposed measures throughout the MCS stages. Thus, we evaluate the air renewal patterns from week to week. In our case, it was enough to agree on the terms with each person in charge through fortnight reports and phone calls. The presented strategy allowed us to reduce the costs of the project and to be able to evaluate several stores at the same time.

In the period studied (January-February 2022), the average CO₂ of all businesses was 547 ± 87 ppm in the first fortnight of January, 559 ± 86 ppm in the second fortnight of January, 531 ± 82 ppm in the first fortnight of February, and 529 ± 89 in the second half of January (Figure 5a). Substantial differences were observed according to the type of store, as shown in Figure 5b. Specifically, the highest CO₂ peaks were observed in Kids' clothing stores (691 ± 209 ppm with maximums of 2,297 ppm). It may be associated with a limited opening of doors or windows to maintain the thermal comfort of the children. In contrast, florists reported the

lowest CO₂ levels (mean of 466 ± 47 ppm with a maximum of 874 ppm), possibly due to customers' reduced time during service.

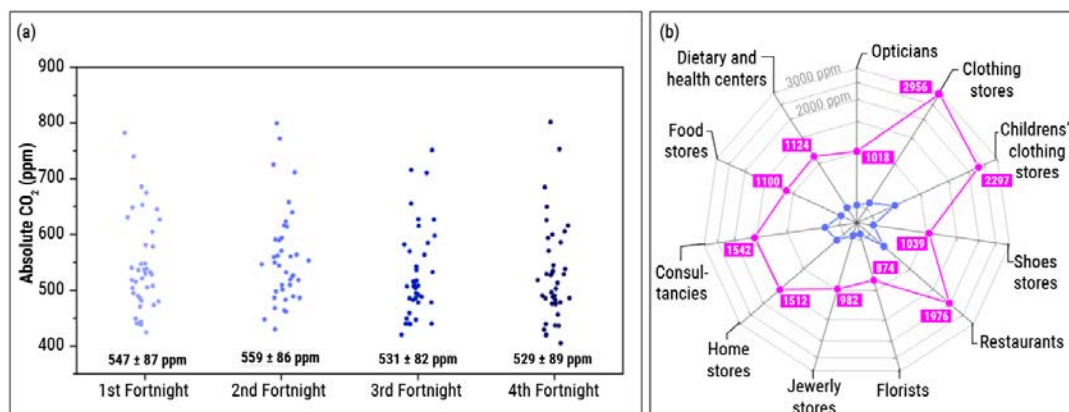


Figure 5. (a) Mean of CO₂ value (inbox) of all businesses based on the fortnight and (b) mean of CO₂ and maximum measurement depending on the type of business where the mean value (blue) and the maximum values (pink label) are shown.

A total of 15 businesses were selected for exhaustive monitoring. Among them, only five agreed to implement the corrective measures. The following sections describe the evolution of 3 establishments under the three typical scenarios:

1. Retail stores with good starting air quality. Initially, 17 shops showed a renewal of the air appropriate to the architecture of the space and the type of activity carried out. Three cases are shown below as an example.

Example 1) In a shoe store, the limit of 800 ppm was not exceeded during the recorded period (Figure 6a). Specifically, it obtained an average CO₂ value of 441 ± 42 ppm, reaching a maximum value of 776 ppm.

Example 2) A food store was kept below 800 ppm (Figure 6b). Despite the large influx of customers, the levels maintained an average of 454 ± 39 ppm, reaching a maximum value of 782 ppm

Example 3) In one of the clothing stores, the maximum established by the scientific community of 800 ppm was not exceeded (Figure 6c). On average, it reached a value of 469 ± 33 ppm, reaching a maximum value of 783 ppm.

2. Businesses with improvable air quality that did not implement corrective actions. Of the 23 stores that showed levels above 800 ppm during the first week, only 15 of them did so continuously and with values above 900 ppm. The remaining eight stores were recommended to maximize the frequency of opening doors and windows. This section shows the evolution of CO₂ in a business that refused to establish the proposed corrective measures.

Example 1) On average, a clothing store reached a value of 709 ± 295 ppm, reaching a maximum of 2956 ppm (Figure 6d).

Example 2) A restaurant establishment reached an average value of 589 ± 139 ppm, reaching a maximum value of 1976 ppm (Figure 6e).

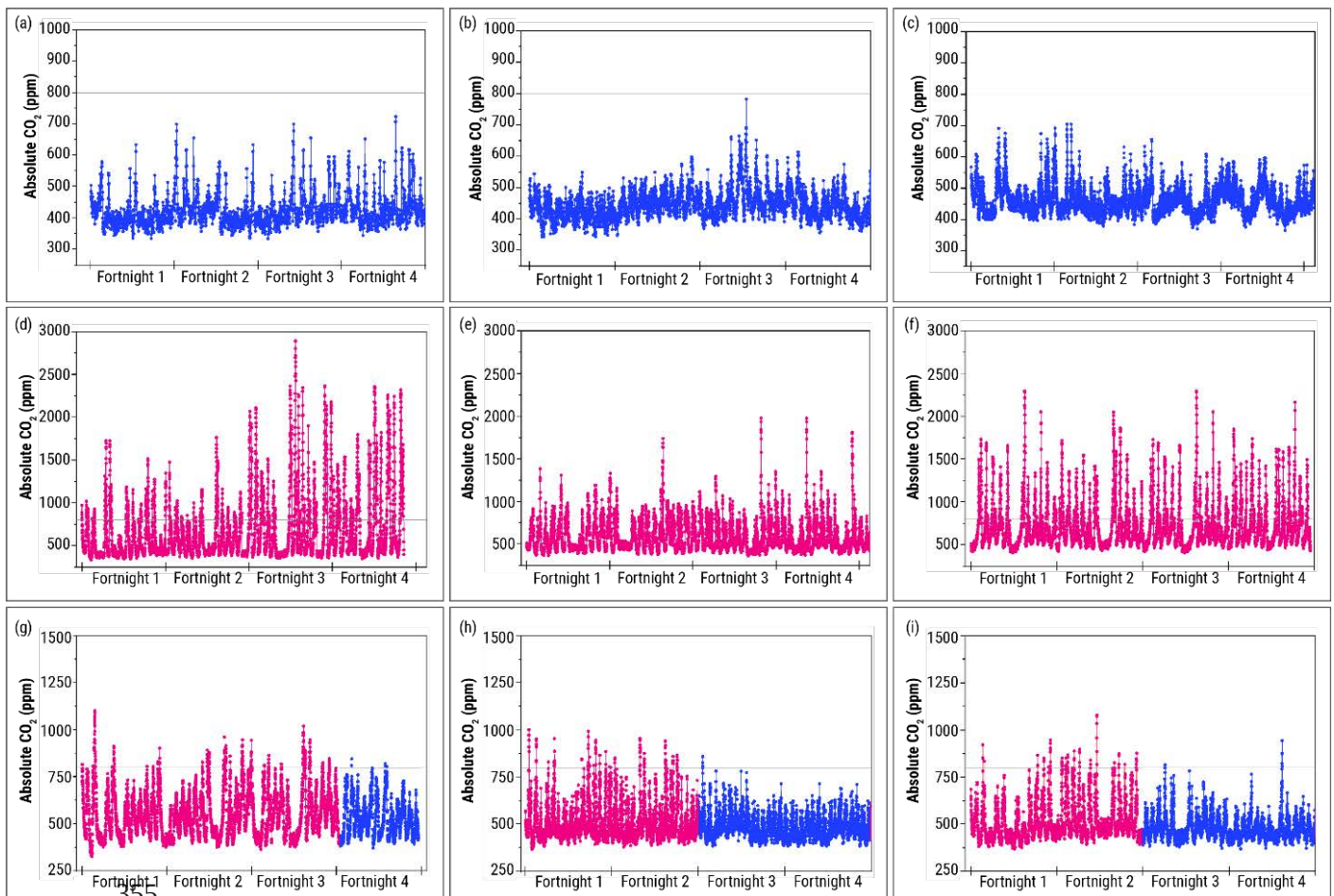
Example 3) The mean of CO₂ in a children's clothing store was 762 ± 229 ppm, reaching a maximum of 2297 ppm (Figure 6f).

339 3. Spaces with improvable air quality that implemented corrective actions. After
340 implementing the prevention measures (especially the reinforcement of natural
341 ventilation), the reduction of CO₂ in the establishments was substantial. Three cases
342 are described below. The values of the first weeks concerning the final weeks are
343 comparable, given that similar trends and averages are observed in the previous
344 section.

345 *Example 1)* In a clothing shop, the mean of CO₂ was reduced from 605 ± 78 ppm
346 (maximum of 1100 ppm) to 529 ± 78 ppm (745 ppm) after trying different measures.
347 The increase in the frequency of door opening (Figure 6g) was more effective in the
348 fourth fortnight. In this period, no values over 800 ppm were registered.

349 *Example 2)* CO₂ levels on average were reduced from 536 ± 89 ppm (maximum of
350 1001 ppm) to 485 ± 51 ppm (714 ppm) from the third fortnight in a food store, as
351 shown in Figure 6h.

352 *Example 3)* In a shoe store, the mean of CO₂ was reduced from 528 ± 67 ppm
353 (maximum of 946 ppm) to 473 ± 45 ppm (764 ppm). Although, at one point, 800 ppm
354 was exceeded (Figure 6i).



356 **Figure 6.** Evolution of CO₂ levels in the monitoring period (January-February 2022) in (a-c) businesses
357 with good starting air quality, (d-f) businesses with improvable air quality that did not implement cor-
358 rective measures, and (g-i) businesses with improvable air quality that implemented corrective actions.
359 The pink dots indicate the CO₂ curves of stores with poor air renewal, while the blue dots represent
360 CO₂ curves of establishments with good air renewal.

4. Conclusions

This work presents and discusses a new methodology for measuring CO₂ in shared spaces based on diagnosis, correction, and MCS (monitoring-control and surveillance phases). This methodology was validated in a subset of 40 local businesses in Zaragoza (Spain). The COVID-19 pandemic has pointed to the need to rethink the epidemiological control techniques. In particular, the increasing knowledge on airborne transmission of diseases makes it imperative to implement new public health strategies that guarantee that air quality follows specific standards. If the incorporation of these strategies proves effective, it will be possible to prevent numerous respiratory affections and potential new pandemics.

Briefly, the methodology can be sequenced in three stages:

1. Diagnosing. After training the responsible person, the diagnostic phase was carried out. A first assessment of the shared space is made by placing CO₂ meters for a time interval long enough to address the maximum possible casuistry. Usually, 3 or 4 business days is enough. Ideally, placing more than 1 CO₂ meter will let us know if the measurement represents the space. Additionally, it will bring preliminary -but no definitive- information about how the air renewal occurs in the shared space. After an in-depth analysis of the evolution of CO₂ levels in the diagnosis phase, it is necessary to follow up in subsequent days (typically 6 or 7 days) to ensure that the diagnosis is representative of the space.
2. Correcting. Poor air renewal must be identified once an exhaustive analysis of the space has been carried out. Based on current scientific knowledge, a criterion of not exceeding 800 ppm for more than 20-30 minutes may be valuable. After detecting these incidences, it is necessary to look for the cause of the problem. It is helpful to raise possible hypotheses and try to rule them out to propose efficient corrective measures. Ideally, these proposed measures can be validated by conducting experiments (e.g., saturating with CO₂ and analyzing the advantage of each one) or carrying out a detailed follow-up in the monitoring phase. Note that this phase can be performed linearly (e.g., after diagnosis), or it can also be implemented during monitoring if necessary.
3. MCS. A monitoring and follow-up phase is crucial to warn of any incident where the CO₂ limit is exceeded. It is the only strategy that guarantees that good indoor air quality. It is possible that, in spaces where natural ventilation influences the space air renewal, the CO₂ concentration fluctuates depending on the weather. In these cases, it may be necessary to re-implement the correction phase to maintain an adequate air renewal. Ideally, an alert system can be valuable for the monitored center, allowing it to prevent the accumulation of CO₂ or modulate the capacity and, ultimately, correct the situation.

The results obtained from the validation in 40 retail stores in the center of Zaragoza (Spain) suggest that it is possible to implement measures that favor the renewal of shared air quickly. Opening doors and windows is one of the most straightforward and immediate methods applied in almost any situation. We have shown that the methodology is practical and can reduce the risk of transmission of respiratory diseases by aerosols by increasing air renovation. We hope this work contributes to laying the methodological foundations for CO₂ measurement to become a valuable, standardized, and guaranteed tool.

405 Acknowledgments

406 We would like to express our gratitude to "Fundación Ibercaja" and "Federación de
407 Empresarios de Comercio y Servicios de Zaragoza (ECOS Zaragoza)" for supporting this
408 research and their invaluable collaboration. We thank Signos.io for help and support with
409 the CO₂ meters. We also would like to thank "Ayuntamiento de Zaragoza" and "Instituto de
410 Investigación Sanitaria Aragón" for the received support.

411 Conflict of Interest

412 The authors declare no competing financial interest.

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Sexto artículo

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***International Journal of Environmental Research
and Public Health, 2022,19 (11), 6605***

<https://doi.org/10.3390/ijerph19116605>

Factor de impacto en 2022: 4.614

Factor de impacto a 5 años: 3.789

Cuartil JCR (2021): Q1

Cuartil JCR (2020): Q1

Fecha de envío: 26 de abril de 2022

Fecha de publicación: 28 de mayo de 2022

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Article

The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases

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Abstract: The global acceptance of the SARS-CoV-2 airborne transmission led to prevention measures based on quality control and air renewal. Among them, carbon dioxide (CO₂) measurement has positioned itself as a cost-efficiency, reliable, and straightforward method to assess indoor air renewal indirectly. Through the control of CO₂, it is possible to implement and validate the effectiveness of prevention measures to reduce the risk of contagion of respiratory diseases by aerosols. Thanks to the method scalability, CO₂ measurement has become the gold standard for diagnosing air quality in shared spaces. Even though collective transport is considered one of the environments with the highest rate of COVID-19 propagation, little research has been done where the air inside vehicles is analyzed. This work explores the generation and accumulation of metabolic CO₂ in a tramway (Zaragoza, Spain) operation. Importantly, we propose to use the indicator ppm/person as a basis for comparing environments under different conditions. Our study concludes with an experimental evaluation of the benefit of modifying some parameters of the Heating–Ventilation–Air conditioning (HVAC) system. The study of the particle retention efficiency of the implemented filters shows a poor air cleaning performance that, at present, can be counteracted by opening windows. Seeking a post-pandemic scenario, it will be crucial to seek strategies to improve air quality in public transport to prevent the transmission of infectious diseases.

Keywords: airborne; CO₂; collective transport; SARS-CoV-2; tramway; filtration; infectious diseases; epidemiology; public health; COVID-19

Citation: Baselga, M.; Alba, J.J.; Schuhmacher, A.J. The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases. *Int. J. Environ. Res. Public Health* **2022**, *19*, 6605. <https://doi.org/10.3390/ijerph19116605>

Academic Editors: Sachiko Kodera and Essam A. Rashed

Received: 26 April 2022

Accepted: 27 May 2022

Published: 28 May 2022

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1. Introduction

Public health strategies are modulated by adjusting to the development of knowledge about the transmission routes of COVID-19. The viral transmission of SARS-CoV-2 human–human has been described from direct respiratory dissemination and indirect dissemination. On the one hand, direct respiratory dissemination, where the symptomatic or asymptomatic patient expels contaminated particles in respiratory events, and, on the other hand, indirect dissemination or via fomites, where transmission is due to contact with contaminated surfaces. On the other hand, it is possible to differentiate between the droplet and bioaerosol models with indirect dissemination. While droplets predominate in close contact, bioaerosols can be transmitted through the air over time and distance [1]. Regarding this pandemic, the scientific community has redefined the concept of bioaerosol, extending its consideration to airborne particles smaller than 100 µm, based on evidence and common factors related to the aerodynamics of the particles [1,2]. The spread patterns of SARS-CoV-2 could not be explained by traditional epidemic models, where homogeneity in the transmission is assumed [3]. As recognized by the WHO in April 2021 [4], a predominance airborne way has been suggested compared to other propagation models [1,5].

The size of the SARS-CoV-2 virion varies between 70 and 90 nanometers [6,7], and an average concentration of the virus in the sputum of 7.0×10^6 copies/mL and a maximum of 2.35×10^9 copies/mL [8]. Consequently, the viral load occupies $2.14 \times 106\%$ del bioaerosol on average. With this value, Lee [9] estimated a theoretical minimum and initial aerosol size of $4.7 \mu\text{m}$ to contain SARS-CoV-2. However, experimental bioaerosol sampling studies suggest the presence of the virus in smaller particle sizes (even $< 0.25 \mu\text{m}$) [10–13]. Despite numerous factors influencing the airborne transmission of pathogens, such as dynamics or their aerial persistence, contagion events can only be explained by a medium and long-distance airborne transmission model [5]—for example, among small animals [14,15], from viral superspreading events [16], in the long-distance transmission where infected individuals do not come into contact direct [17], by asymptomatic individuals transmission rates [18], and by the prevalence of spread in closed spaces [19]. Specifically, a superspreading event affected public transportation. Shen et al. [20] reported a massive infection of 24/68 (35.3%) people from a single infected individual while being transported in a bus with air recirculation and poor ventilation.

At the pandemic's beginning, this route of contagion was dismissed, and more attention was paid to contagion by droplets and fomites. Consequently, there was controversy about whether asymptomatic infected individuals could be transmitters of SARS-CoV-2. However, currently, it has been estimated that 44% (CI95; 30–57%) of secondary cases were infected during the incubation period [21], where the individuals were asymptomatic. The global acceptance of the COVID-19 airborne spread allowed an improvement in the preventive methods, including new techniques for epidemiological management, such as the measurement of exhaled carbon dioxide (CO_2) as an indicator of the risk of contagion [2,22].

Carbon dioxide measurement began to be used in the 19th century to design ventilation systems in architecture [23]. In the pre-pandemic period, CO_2 measurement helped improve academic performance in schools and colleges [24] and, sporadically, control infectious diseases [25]. Due to the COVID-19 pandemic, CO_2 measurement has become one of the preferred preventive strategies to reduce the risk of contagion by aerosols [22,26,27]. The direct measurement of aerosols to determine the risk of contagion by SARS-CoV-2 is highly complex and expensive since it requires highly specialized equipment. While there are handheld instruments or simple sensors to direct measure of aerosol concentrations, these instruments present different limitations such as they can not discriminate human-exhaled versus environmental aerosols; usually, they cover a limited range of particle diameter and hardly measure the submicronic particles. To overcome these hurdles, the CO_2 level has been suggested as an indirect indicator of respiratory infectious diseases' transmission [22]. CO_2 is co-expired with bioaerosols that may contain SARS-CoV-2 in infected people [28–30]. Its quantification provides an idea of indoor air renewal and establishes the risk of infection as it depends on the viral load [31]. Consequently, the measurement of indoor CO_2 is suggested as a reasonable ventilation proxy for respiratory infectious disease. Through its reading, it is possible to determine what percentage of the air has been exhaled by another individual (y) according to the expression $y = C_e x + C_a(1 - x)$, where C_e corresponds to the concentration of CO_2 in exhaled air (estimated at 40,000 ppm), C_a to ambient CO_2 concentration, and x to the fraction of exhaled air. For example, if we assume a basal value is 440 ppm (fresh air outdoors), a group of people manages to increase it to 2300 ppm. In that case, the approximate percentage of air that those individuals have already breathed will be around 4.7%.

Despite the ventilation rates being known to influence the concentration of microorganisms in the environment [32], the increase in the exhalation rate of aerosols depending on CO_2 has been poorly explored [30]. The concentration of airborne particles and the level of CO_2 cannot be directly related due to a disparity between the bioaerosols generated and the respiratory activity [28]. For example, aerosol generation during forced vocalization or coughing is not comparable to emission rates during respiration [33]. Thus, two

different scenarios (for example, a library versus a gym) with similar CO₂ levels have to be interpreted individually.

To date, COVID-19 superspreading events have been reported indoors [34–40]. Thus, the use of air renewal proxy indoors is crucial for maintaining spaces with a low risk of contagion by aerosols. Many countries are making high economic investments to equip schools with CO₂ meters [41,42]. In addition, other isolated initiatives have successfully implemented this methodology in shopping centers [43], collective transport [44–46], offices [47], or university and school classrooms [47–52]. Specifically, a recent study in Italy reported an 82% reduction in secondary COVID-19 infections in schools where they controlled air renewal from CO₂ measurements [53].

Currently, CO₂ concentration limits have been proposed as a reference to minimize COVID-19 spreading. Usually, it is set between 700 and 1000 ppm regardless of the event [54,55]. Urban collective transport is one of the policies designed to promote sustainable cities [56]. To prevent respiratory infectious diseases spread, it is important to analyze the risk involved in every specific means of transport. References on the emission of bioaerosols in collective transport are scarce despite being the environment with the second-highest transmission rate of SARS-CoV-2. Lan et al. [57] point to 18% of cases in the transport sector, only behind the health sector (22%) in the transmission rate of COVID-19 disease. Before the pandemic, some reports pointed to metabolic CO₂ concentrations of up to 3700 ppm in buses [58–61], suggesting poor air renewal. However, due to the pandemic, it has been possible to reduce it to <800 ppm by implementing simple ventilation measures [45]. The operating conditions of the subways require reinforcement of artificial ventilation, for which values close to 1000 ppm have been found [44,62,63].

Trams have similar characteristics to buses since they circulate outside, and the contribution of natural ventilation can substantially favor air renewal. However, no specific information on air quality in trams has been reported. This work evaluates the accumulation of metabolic CO₂ in the Zaragoza Tram (Spain) in circulation under different conditions. On the one hand, the objective was to analyze the concentration of CO₂ in different events (e.g., weekend versus midweek, with and without air recirculation or with different weather conditions). To compare air renewal regardless of the event, the ppm/person indicator was used. However, secondary air purification methods that affect contagion risk, such as added air filtration, must also be considered. Then, the performance of the installed filtration system is analyzed against the concentration of submicron aerosols (such as the airborne virus SARS-CoV-2). The work concludes with suggestions for measuring CO₂ and recommendations to reduce the risk of contagion in collective transport.

2. Materials and Methods

2.1. Measurement of Metabolic CO₂

The metabolic CO₂ level was measured using Aranet 4 Pro meters (Aranet Wireless Solutions España SL, Madrid, Spain), with technical characteristics shown in Table 1. The increase in CO₂ (ΔCO_2) was determined according to Equation (1):

$$\Delta\text{CO}_2 = \text{CO}_{2,\text{indoors}} - \text{CO}_{2,\text{outdoors}} \quad (1)$$

Table 1. Technical characteristics of the Aranet 4 Pro meters.

	CO ₂	<9999 ppm
Parameters measured	Temperature	0–50 °C
	Relative humidity	0–85%
	Atmospheric pressure	0.3–1.1. atm
	Sensor type	N-DIR (Non-Dispersive Infrared)
Communication technology	Bluetooth (–12–4 dBm)	
Sampling frequency	1 min	

Precision	±50 ppm (CO ₂)
Dimensions/Weight	70 × 70 × 24 mm/104 g

The Urbos 3 tram models (CAF, Beasain ES) have a total length of 33 m, a width of 2.65 m, and a height of 3.2 m. They have a capacity of 200 seats, of which 54 are seats. Travelers wore a mask at all times, and the windows remained partially open during all routes. Eight CO₂ meters were installed at a 2.25 m height at different points of the Tram, according to the distribution of Figure 1a. The objective was to obtain realistic and uniform measurements, representative of the level of exposure experienced by an average user without running the risk that the measurement would be altered due to the direct exhalation of the passengers. As shown in Figure 1b, the meters were installed on grab bars, for which it was necessary to manufacture anti-vandal housings with holes to guarantee air transfer.

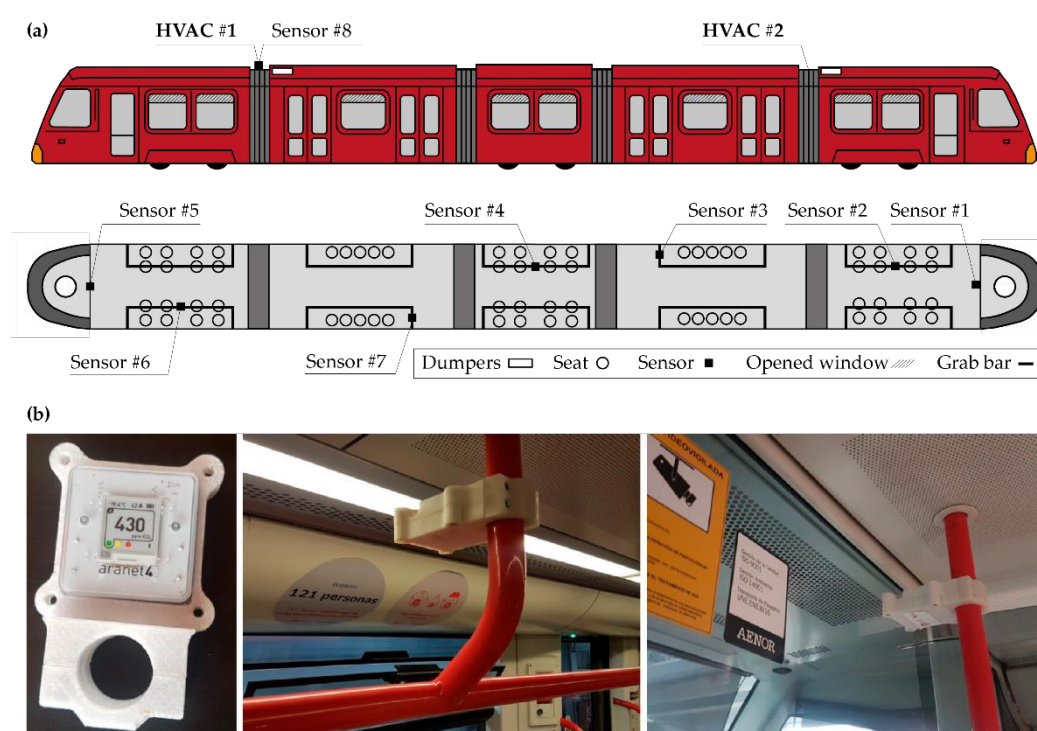


Figure 1. Schematic representation (a) of the meters distribution in the Tram and (b) installed sensors in the Tram. Where, # refers to the meter ID.

2.2. Probability of Contagion Determination by the CO₂ Level

The CO₂ measurement was used as a tool to determine the risk of contagion. This was possible thanks to the theoretical model updated by Peng and Jiménez [22] and the Aireamos consortium [31].

The risk of airborne indoors transmission (for one person in one hour) P was described from an alternative equation to that of Wells–Riley [64] (Equation (2)), enunciated by Rudnick et al. [65] (Equation (3)):

$$P = 1 - e^{-n} \quad (2)$$

$$P = 1 - \exp\left(\frac{Itqf}{n}\right)R \quad (3)$$

where I is the number of infected people in a space, t is the exposure time measured in hours, q is the number of pathogens spread per hour, f is the fraction re-inhaled ($[C - C_0]/C_a$), n is the number of people exposed to the infectious individual, and R is the particle retention efficiency, that is, the fraction of retained aerosols by the PPE from the

exposed individual. In turn, C is the concentration of CO₂ indoors, C_0 outside, and C_a the concentration exhaled during respiration, defined in parts per million (ppm). The value of n corresponds to the infectious dose inhaled by a susceptible person. However, Rudnick et al. [65] assumed some conditions for the model's description: (1) the indoor air is thoroughly mixed, so the infectious aerosol generated can be found anywhere in the space. (2) The external concentration of CO₂ remains constant during the event. (3) Removal of viral aerosols due to virus survival, filtration, or other mechanisms is negligible compared to ventilation.

Peng and Jiménez [22] applied another alternative to the Wells–Riley formulation regarding the COVID-19 pandemic. The authors derived analytical expressions for the probability of infection indoors through the concentration of CO₂. The expected value of $\langle n \rangle$ can be calculated for an uninfected person, assuming the probability that the individual is immune η_{in} according to Equation (4):

$$\langle n \rangle = (1 - \eta_{in})C_p B D (1 - m_{in}) \quad (4)$$

where C_p corresponds to the average number of viruses (quantos.m³), B to the respiratory rate of the susceptible person (m³ h⁻¹) that will vary depending on the activity carried out at that time, D the event duration (h), and m_{in} the filtration efficiency of the mask during inhalation. Consequently, assuming no pre-existence of viral aerosols before the event, the analytical expression for the expected value of C_p can be described by Equation (5):

$$C_p = \frac{\eta_{in}(N - 1)E_p(1 - m_{ex})}{V} \left(\frac{1}{\lambda} - \frac{1 - e^{-\lambda D}}{\lambda^2 D} \right) \quad (5)$$

where N is the number of occupants, E_p is the exhalation rate of SARS-CoV-2 per infected person (quantos.h⁻¹), m_{ex} is the filtration efficiency of the mask during exhalation, V is the volume of air in the space (m³), and λ the global rate constant of virus infectivity loss (h⁻¹), including all those mechanisms that may affect virus survival (filtration, ventilation, etc.). Assuming that the increase in CO₂ (ΔCO_2) of the indoor air concerning that of the outdoor air is only produced by human activity, it can be described as follows (Equation (6)):

$$n_{\Delta\text{CO}_2} = \Delta C_{p,\text{CO}_2} B D \quad (6)$$

where the CO₂ increment volume and the CO₂ exhalation rate per person mixing ratio (ΔCO_2), in m³.h⁻¹, can be described as (Equation (7)), where λ_0 corresponds at ventilation rate (h⁻¹):

$$\Delta C_{p,\text{CO}_2} = \frac{N E_{p,\text{CO}_2}}{V} \left(\frac{1}{\lambda_0} - \frac{1 - e^{-\lambda_0 D}}{\lambda_0^2 D} \right) \quad (7)$$

As a result of this model, Peng and Jiménez [22] propose an acceptable probability of infection limit of $p = 0.01\%$. Although it does not imply safety in any situation, since with high N and/or D and/or the event occurs many times, the probability of infection for the susceptible person is understated.

2.3. Studied Routes of the Zaragoza Tram

Eighty-eight round trips (44 complete trips) with an average of ~40 min each were analyzed. As shown in Figure 2, each complete path stops at 42 stations. The routes included in stations #7–#10/#33–#36, and #10–#16/#27–#33 correspond to the university area and the city center, respectively.

The CO₂ meters were installed for three months in the vehicle. We hypothesized that the variation in the meteorological data obtained during the study days could translate into variations in the Tram's ventilation capacity. The variables of interest for five reference days of December 2020 are shown in Table 2. According to data provided by the Zaragoza weather station, the average wind speed value in December was 3.25 (± 1.65) m/s, so it can be considered that on days B, C, and D, the values of wind speed and maximum gusts were low. Low wind speed was an unfavorable condition for the natural ventilation of the Tram.

Table 2. Meteorological variables of the reference days A, B, C, D, and E. Information prepared by the Agencia Estatal de Meteorología of Spain (data collected at the Valdespartera Station, Zaragoza Spain, 23 December 2020).

Day	T_{aver}	T_{min}	T_{max}	D_w	$W_{s,aver}$	$W_{s,max}$	P_{max}	P_{min}
A	9.6 °C	6.1 °C	13.1 °C	30 °	3.1 m/s	8.9 m/s	996.8 atm	990.0 atm
B	8.2 °C	4.4 °C	11.9 °C	16 °	1.7 m/s	6.1 m/s	996.8 atm	990.0 atm
C	5.4 °C	3.3 °C	7.4 °C	10 °	1.9 m/s	5.0 m/s	998.2 atm	996.0 atm
D	7.9 °C	3.8 °C	12.0 °C	16 °	1.9 m/s	5.6 m/s	994.7 atm	990.8 atm
E	8.9 °C	6.2 °C	11.6 °C	31 °	4.7 m/s	11.1 m/s	994.9 atm	992.4 atm

T_{aver} : temperature (average); T_{min} : temperature (minimum); T_{max} : temperature (maximum); D_w : wind direction; $W_{s,aver}$: wind speed (average); $W_{s,max}$: wind speed (maximum); P_{max} : atmospheric pressure (maximum); and P_{min} : atmospheric pressure (minimum).

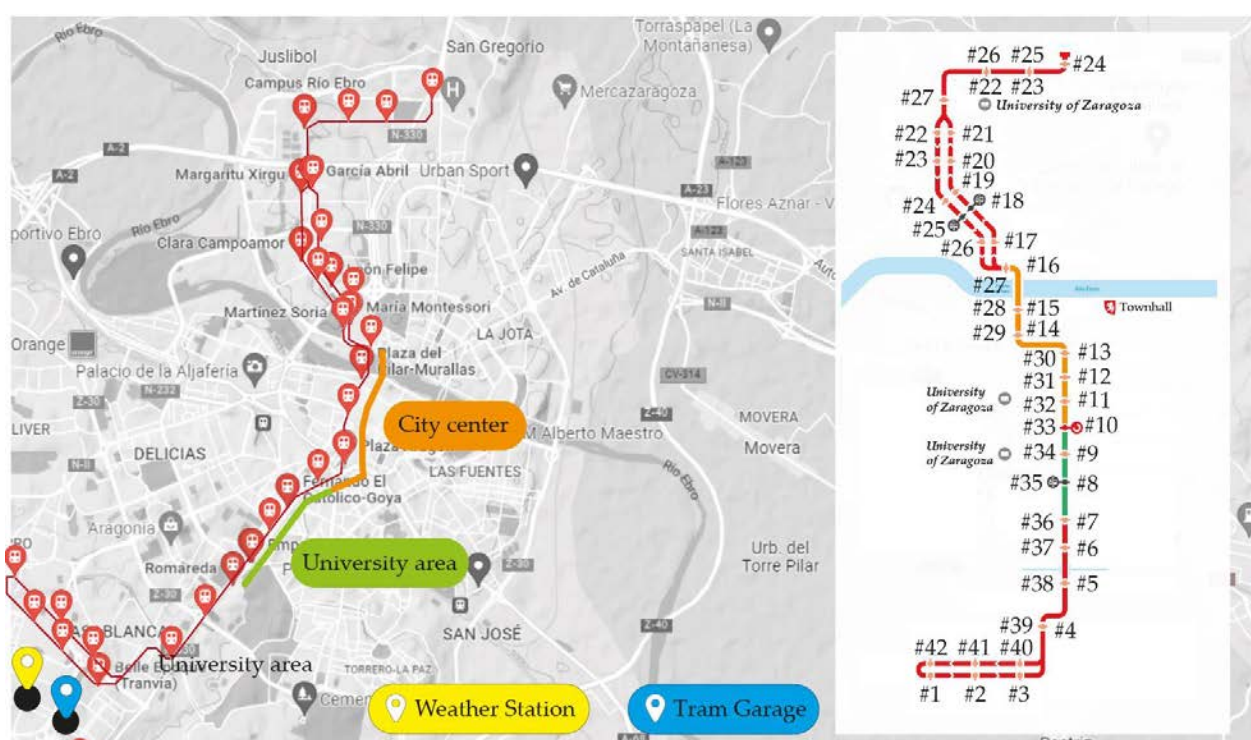


Figure 2. Route of the Zaragoza Tram. Where, # refers to the station ID.

2.4. Determination of Filtration Efficiency against Submicron Particles and Filters' Pressure Drop

The filter's performance was studied in-vitro to assess its effectiveness against submicron particle sizes, as is the case with the SARS-CoV-2 virus and other respiratory viruses. The filter used during the tests was specially implemented due to the current COVID-19 pandemic (Coarse 75% according to UNE-EN ISO 16890, Merak Long Life Filter, Madrid SP). As depicted in Figure 1, two filters were arranged in two HVAC units

installed in the Tram, which drive a total flow of 2800–3300 m³/h, with an air ratio of 1:3 fresh/return air.

As shown in Figure 3a, NaCl aerosols were produced using a Topas-ATM226 generator with a saline solution of sodium chloride (3 wt.% NaCl in distilled water). Microdroplets were evaporated using a tubular silica air dryer to produce solid particles. The particle size distribution (Figure 3b) inside the cabin was measured using an SMPS TSI 3936 composed of an electrostatic classifier (DMA TSI 3081) and a condensation particle counter (CPC TSI 3782). An 0.6 L/min flow rate drags the particles. The filter was placed between bronze discs sealed with Teflon tape, with 30 × 20 mm Teflon washers on each side. The desired flow rate was adjusted varying the exposed filter area (2.05, 4.1, and 8.1 mm). The measurements lasted 120 s and were made in duplicate. Measurements were made passing through a free tube between measurements to calculate relative efficiency according to Equation (8), where C_{up} stands for concentration upstream and C_{down} stands for concentration downstream. The retention efficiency is expressed in global efficiency as ‘global number of particles’:

$$\eta = 100 \times \frac{C_{up} - C_{down}}{C_{up}} \quad (8)$$

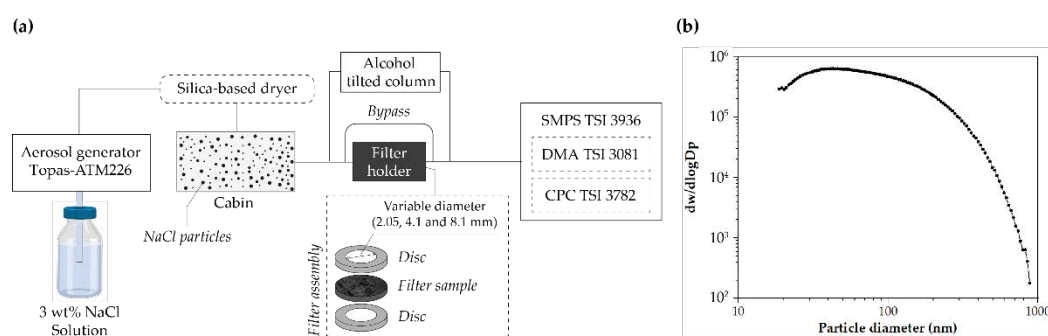


Figure 3. Performance test. (a) Diagram of the equipment used to characterize the filters and (b) particle concentration distribution for efficiency determination measurements in the range 0.1–1.0 µm.

According to Bernoulli’s principle, the pressure drop was carried out using alcohol columns connected to the free ends of the tubes. Measurements were also made with a 0.6 L/min volumetric flow rate.

2.5. Statistic Analysis

The statistical analysis of the data has been carried out using the R-UCA v.4.0.2 software (University of Cadiz Spain, 2017) [66]. Mean comparisons were made with the Student’s *t*-test at a 99% confidence interval (CI99).

3. Results

3.1. CO₂ Levels along the Route Are Closely Related to Occupancy

As shown in Figure 4a,b, the increase in the CO₂ concentration inside the Tram gradually increases as it approaches the city’s downtown area #33–#36 and #27–#33 stations; approximately, at minutes 20 and 70 on the outward and return routes, respectively. The CO₂ increase corresponds to the difference between the Tram indoor values concerning the external reference value (atmospheric) registered with sensor #8. Analyzing the increment makes it possible to determine the global CO₂ concentration corresponding to metabolic CO₂ to rule out possible external contamination. The calculated ppm/person ratio (Figure 4c) suggests a concentration of ΔCO_2 in the final areas of the route associated with an accumulation of CO₂ in the vehicle, which begins to be evident after driving through the city center. It may be explained because the number of travelers increases in the city

center and accumulates CO₂ not recirculated at subsequent stops. On average, the Tram doors open for 16.6 ± 3.6 s at each stop.

ΔCO_2 concentration is closely related to tram occupancy (Figure 5a), although there is some dispersion associated with external variables (Figure 5b). In absolute CO₂ values, the maximum average was 835 ± 232 ppm, reaching a maximum value of 1229 ppm. In contrast, the lowest average was 541 ± 82 ppm. The pattern of ΔCO_2 concentration on weekdays compared to weekend days is different, although it follows similar trends. As shown in Tables S1 and S2, the average of the trips made on weekends in the morning was 565 ± 318 ppm; in the afternoon, it was 580 ± 323 ppm, and, at night, it was 602 ± 330 ppm. On weekdays, an average of 592 ± 319 ppm was obtained in the morning, 595 ± 324 ppm in the afternoon, and 541 ± 292 ppm at night.

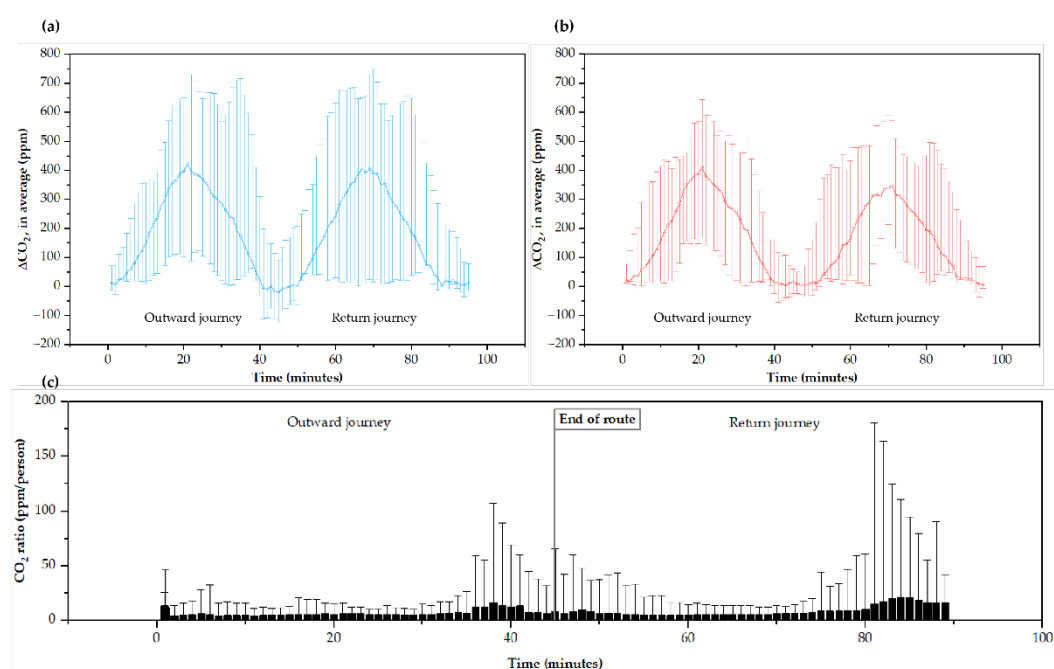


Figure 4. CO₂ increment average levels (a) in all weekday and (b) in all weekend routes, and (c) ppm/person ratio average and maximum gap along routes. The error bars in (a,b) correspond to the difference between the maximum/minimum data and the average data of all studied routes.

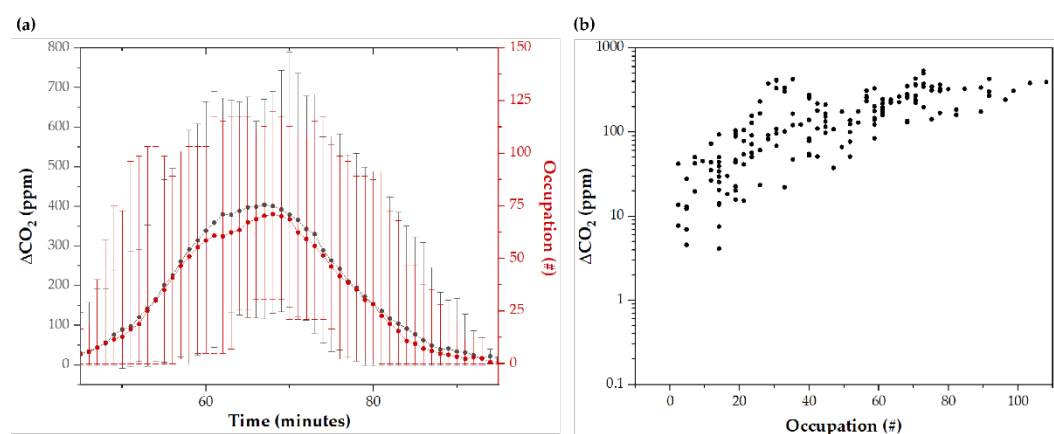


Figure 5. ΔCO_2 increment and tram occupancy as (a) a function of time, and as (b) a function of tram occupancy. The error bars correspond to the difference between the maximum/minimum data and the average data of all the studied routes.

3.2. CO₂ Levels Distribution Is Similar at Different Points Inside the Tram

ΔCO_2 dispersion measurements at the different points of the Tram were assessed using the records from each sensor, as shown in Figure 6. Passenger occupancy is rarely uniform along the Tram, and differences in the capacity distribution can lead to spatially disparate values. The average Relative Standard Deviation (RSD) was determined to determine the homogeneity of the CO₂ distribution in the Tram. The RSD of 0.09 ± 0.02 suggested that the measurements were relatively homogeneous, although accumulation tendencies are typically observed in the central area of the Tram.

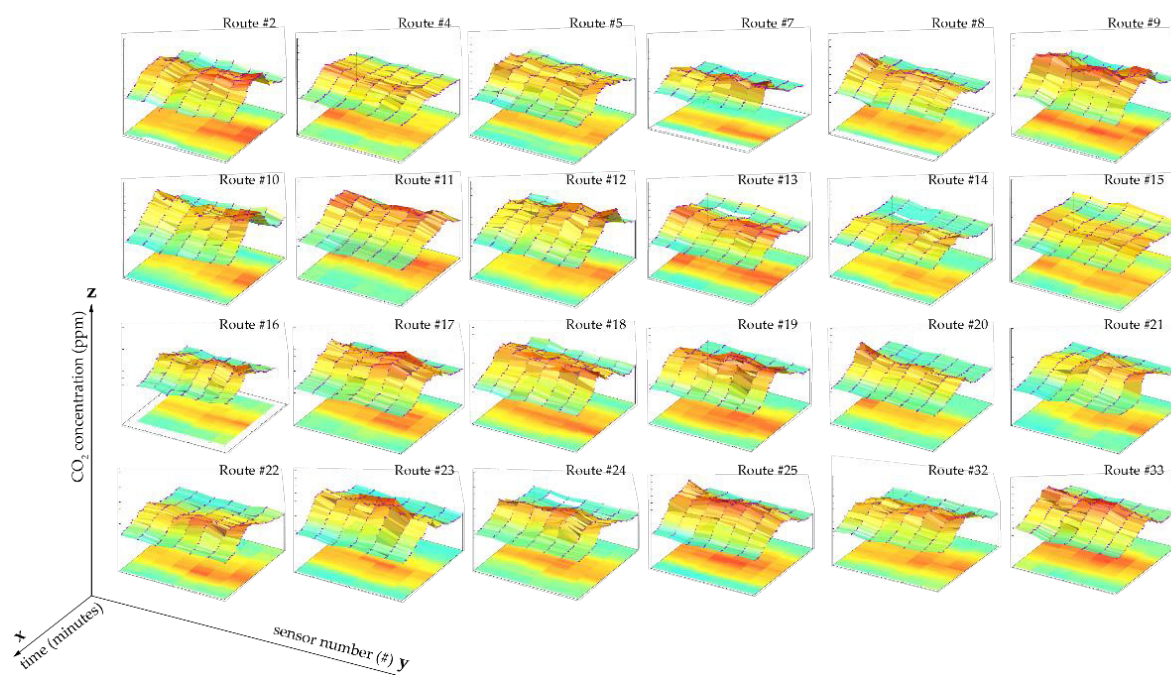


Figure 6. Distribution of ΔCO_2 in the Tram (z-axis) as a function of time (x-axis) and ΔCO_2 measures (y-axis) on routes #2, #4–#25, and #32–#33, where there is homogeneity in the CO₂ measurement along the tram and a strong relationship with occupancy.

3.3. Improving the Air Renewal by the Closing of the Air Return

To study the influence of the return of air from inside the Tram to the air conditioning equipment, we worked with the data obtained through Sensors #3 and #7, located just below the grilles of the air return ducts. Days C and D were selected as a reference for the study due to the similarity between meteorological variables. The ΔCO_2 varies when the air return is closed, as deduced in Figure 7. From the analyses carried out, the extreme values at the beginning and end of the route corresponding to the accumulation of gas in the Tram have been removed, offering a more realistic view of the internal atmosphere during the tour. Under these conditions, the average ppm/person rate without return was 3.5 ± 0.1 (Sensor #3) and 5.1 ± 0.1 ppm (Sensor #7) without air return, and 4.9 ± 0.7 (Sensor #3) and 6.0 ± 0.4 (Sensor #7) with air return. A reduction in ΔCO_2 between 9% and 36% can be seen concerning air return ΔCO_2 levels.

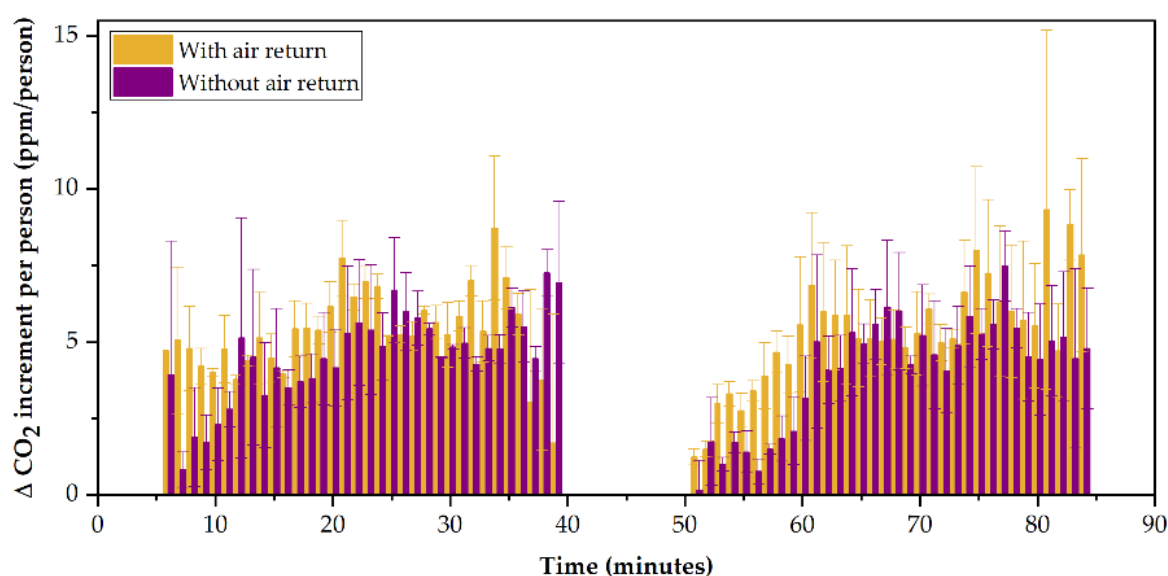


Figure 7. Registered ppm/person values depending on the air return.

3.4. Wind Speed Contributes to Increasing Ventilation Rates

The days were divided depending on the wind speed into Set A (Day A and E) and Set B (Day B to D). Sets were made to assess whether the weather plays a crucial role in the ventilation. The objective of this section is to compare the ventilation pattern on days with different weather, with special attention to the average wind and gusts and the average temperature. The average weather conditions of interest for the days of each Set are shown in Table 3. In Set A, the average wind speed was 3.9 ± 1.1 m/s, while, in Set B, it was 1.8 ± 0.1 m/s, with maximum gusts of 10.0 ± 1.6 m/s and 5.6 ± 0.6 m/s, respectively. Figure 8a,b represent the ppm/person index for Set A and Set B, respectively. In addition, 88.4% of the ppm/person indices was higher in Set B than Set A. It was found that the means of the data from Set B were significantly lower than those from Set A using a hypothesis contrast (CI99; -3.26 – -1.38). Limits are harmful in the CI, confirming that higher data on Set A. A Student's *t*-test shows that the average ΔCO_2 concentration increases on days with lower wind speeds are higher. However, the difference between the ppm/person index in Set B compared to Set A is 2.3 ± 3.3 ppm, compared to averages of 5.2 ± 3.7 ppm (Set A) and 7.5 ± 3.0 ppm (Set B), which represents a reduction of between 31 and 44%. These data suggest that the weather can substantially affect the recirculation of air inside the Tram, as shown in Figure 8.

Table 3. Meteorological variables of the reference Sets A and B. Information prepared by the Agencia Estatal de Meteorología of Spain (data collected at the Valdespartera Station, Zaragoza Spain, 23 December 2020).

Day	T_{aver}	T_{min}	T_{max}	D_w	$W_{s,aver}$	$W_{s,max}$
Set A	9.6 ± 0.5 °C	6.1 °C	13.1 °C	30 ± 0.5 °	3.9 ± 1.1 m/s	11.1 m/s
Set B	7.2 ± 1.5 °C	3.3 °C	12.0 °C	14 ± 3.5 °	1.8 ± 0.1 m/s	6.1 m/s

T_{aver} : temperature (average); T_{min} : temperature (minimum); T_{max} : temperature (maximum); D_w : wind direction; $W_{s,aver}$: wind speed (average) and; $W_{s,max}$: wind speed (maximum).

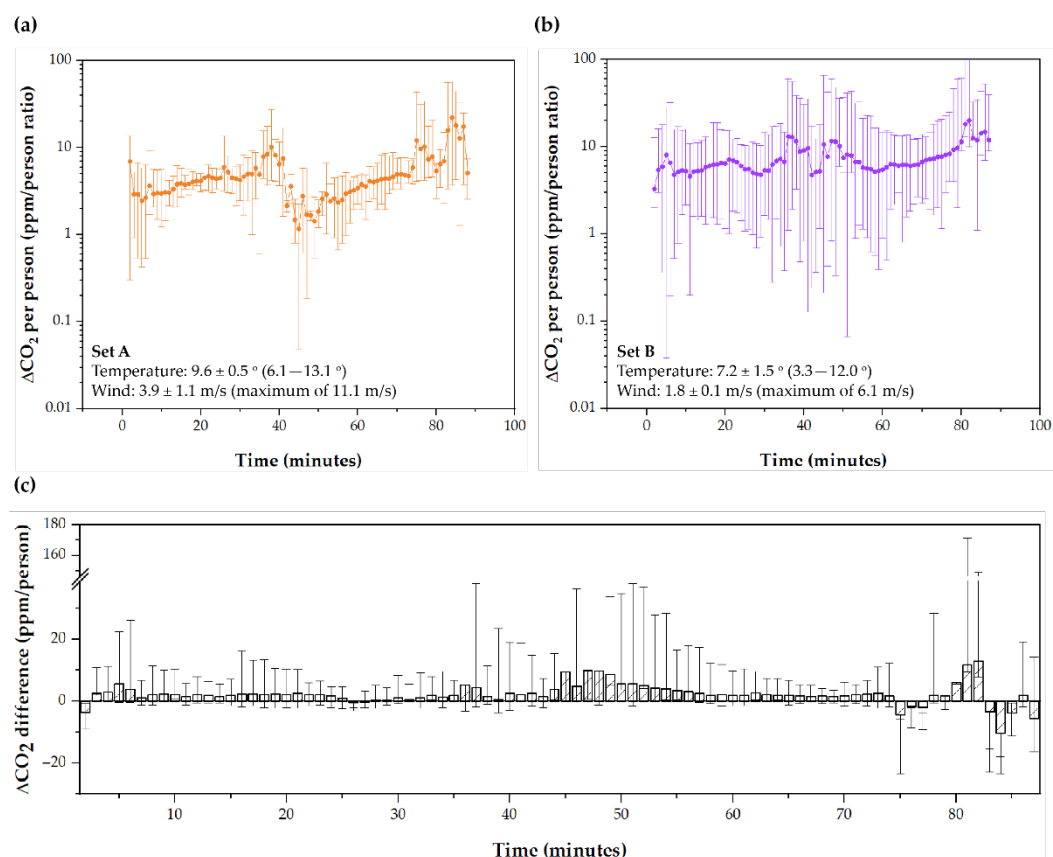


Figure 8. ΔCO_2 per person ratio (ppm/person) in (a) Set A, and in (b) Set B depending on time; (c) difference between Set B and Set A depending on time.

3.5. Tram Speed Does Not Influence the Indoor Ventilation Rate

Tram speed while circulating did not seem to have a substantial effect on the reduction of ΔCO_2 (Figure 9a), nor on the average reduction rate of ΔCO_2 at 3 min (Figure 9b). The Student's *t*-test showed no significant relationship between the rate of reduction of ΔCO_2 compared to two different speed ranges: 1–20 km/h and 21–40 km/h. It may be due to the flow of the HVAC system, which generates internal drafts so that the inflow of air through the window does not alter the ventilation rates substantially.

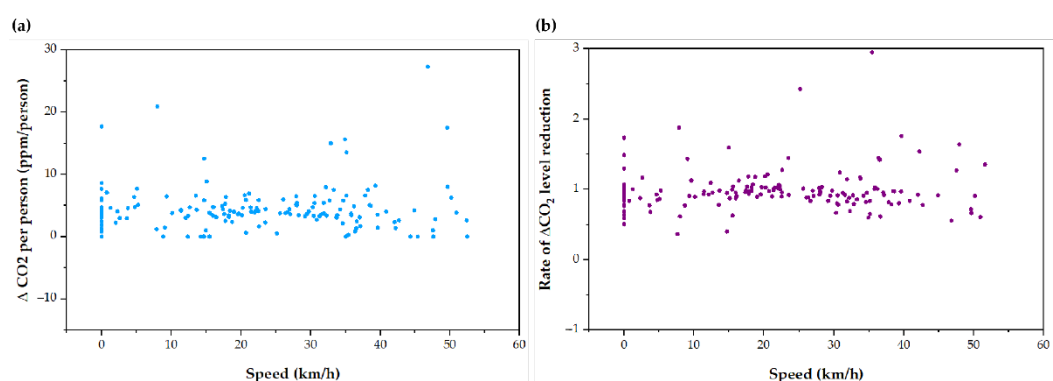


Figure 9. CO_2 measurements depending on Tram speed. (a) ΔCO_2 per person ratio, and (b) average reduction rate of ΔCO_2 depending on the Tram speed.

3.6. The Filtration System Is Not Efficient against Submicron Matter

The tests have been carried out with the filter usually installed on the Tram (Coarse 75% Filter Media). However, the Coarse 75% filter specially implemented due to the

current COVID-19 pandemic has been characterized in the laboratory. The Air Changes per Hour (ACH) of the Zaragoza Tram remained in the unit's regular operation at 25 ACH. As shown in Figure 10, the Coarse 75% filter presented an approximate retention efficiency of 27.9% for 300 nm particles at a flow rate of ~2500 m³/h. The filtering efficiency decreases up to 2.4 and 2.3% using ~162 and ~622 m³/h, respectively. The largest particles present more inertia at high flow rates [67,68], resulting in a higher retention rate in the filter medium. Even though the clogging of NaCl particles observed in the head loss tests may have overestimated these results, which could be seen as an increase up to 440 Pascals of pressure drop (Table 4), this flow would be the most representative of the working conditions in the HVAC of the Tram system.

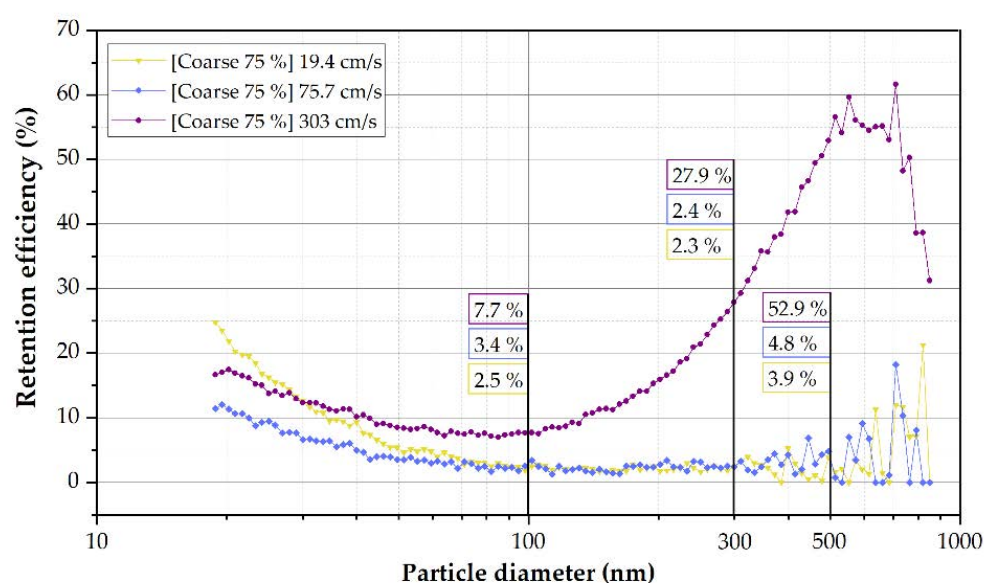


Figure 10. Coarse 75% filter retention efficiency depending on the particle diameter at different speeds (flow rates).

Table 4. Conditions used in the filtration tests and pressure drop determination.

Area	Flow Rate	Velocity in Filter	Pressure Drop
2281.6 cm ²	~161.8 m ³ /h	19.4 cm/s	6 Pa
2281.6 cm ²	~621.8 m ³ /h	75.7 cm/s	34 Pa
2281.6 cm ²	~2488.8 m ³ /h	303.0 cm/s	440 Pa

3.7. Probability of Infection

The probability of infection and the attack rate were calculated following the models proposed by Peng and Jiménez [22] and Aireamos [31]. Based on the average daily CO₂ values collected in Tables S1 and S2, an attack rate of 0.06% was determined in the least favorable case (higher CO₂ values) and an attack rate of 0.04% in the average case (global average CO₂). According to Peng et Jiménez's proposal, the probability of contagion <0.01% is acceptable, so the Tramway did not represent a high risk of contagion under the conditions studied, as shown in Table 5.

Table 5. Determination of the infection probability and attack rate by aerosols in two different scenarios simulates the Tram’s ventilation conditions, using Covid Risk Airborne [31] based on the model Wells–Riley [64].

Scenario	Facemask	Occupation	Exposure Time	CO ₂ Level	Variant	<i>p</i>	Attack Rate
#1 (global average)	Surgical mask	60 pax	10 min	689 ppm (average)/1038 ppm (max)	Omicron	0.01% *	0.04% *
#2 (maximum average)	Surgical mask	60 pax	10 min	810 ppm (average)/1520 ppm (max)	Omicron	0.01% *	0.06% *

* Considering a 78% vaccination with a proportionate immunity of 70%; a cumulative incidence (CI) of 1150 to 14 days/100,000 hab.

4. Discussion

SARS-CoV-2 bioaerosols dissemination in infected individuals’ exhalation is widely demonstrated [69–71]. In addition, the virus’s presence and persistence in the environmental air have also been extensively studied [10,11,72–78]. Given the apparent predominance of the airborne route of transmission of COVID-19, various strategies have been investigated to mitigate the risk of contagion. Public transport environments represent the second sector with the highest transmission of SARS-CoV-2, only behind the health sector [57]. However, computational studies point to a 1.5–1.6% attack rate [79,80]. Even though numerous works have been aimed at evaluating the behavior of bioaerosols in collective transport by computational fluid dynamics [81–85], extrapolation to actual conditions is an enormous limitation. One of the strategies that allow the indoor ventilation rate to be quantified in situ is the measurement of CO₂, which has positioned itself as a standard for air control [22,86,87].

In this work, CO₂ measurements were collected in 88 round trips, which is equivalent to more than 79,200 records obtained from eight sensors strategically distributed in the Tram. The distribution of CO₂ throughout the vehicle follows a similar trend ($RSD\ 0.09 \pm 0.02$), so the location of the HVAC systems and natural air intakes seem to favor all points of the Tram equally. The average absolute CO₂ of all the routes studied was 685 ± 59 ppm (572 ± 75 ppm– 835 ± 232 ppm). This value suggests that the percentage of air already breathed is <0.7%. Considering the virus emission rates in exhaled breath [71,88], average time spent in the Tram (~7 min), and the mandatory use of facemasks, the interior of the vehicle does not represent a risk space of contagion by aerosols (probability of infection [22], $p = 0.01\%$; attack rate < 0.1%) in the most unfavorable scenario (844 ppm average; 1571 ppm maximum). In this sense, Moreno et al. [78] reported attack rates between 0.00–0.72% in buses depending on the respiratory activity, bus air conditions, and the infected individual without a mask. Thus, the environment of the bus at that time was more dangerous.

Before the pandemic, some reports pointed to metabolic CO₂ concentrations of up to 3700 ppm in buses [58–61]. However, a study on the bus in Barcelona (Spain) points to concentrations close to 1000 ppm that can be easily reduced to <800 ppm by implementing simple ventilation measures (i.e., opening windows) [45]. The operating subways conditions require a reinforcement of artificial ventilation, for which values close to 1000 ppm have been found [62,63].

In this paper, we propose using ppm/person indicator as a measure that allows ΔCO_2 levels comparison on different days and circumstances. A key aspect and an obvious one is the increase in ΔCO_2 as the number of passengers increases. Analyzing the ΔCO_2 data measurements, a gradual increase in CO₂ concentration could be misinterpreted as an accumulation. However, looking at the ppm/person ratio, it can be seen that the increase in ΔCO_2 comes from an increase in capacity.

Trams are typically similar to buses since they circulate outside and substantially favor measures to reinforce natural ventilation. In this work, it was found that the speed of the external wind reduced the ppm/person rates to around 2.3 ± 3.3 ppm. Although it seems a slight benefit, it represents a reduction of between 31 and 44% compared to days with less wind. Closing the air return (total external air intake) favored ventilation, reducing the ΔCO_2 level between 9 and 36%. Tram speed did not affect ventilation rate, at least in two data sets with different speed ranges (2–20 km/h versus 20–40 km/h). However, the data could not be compared with the stopped Tram since the conditions were different at that moment. There are no sources of CO_2 generation (there are no passengers), and the doors open entirely, so the air is wholly recirculated in a few minutes.

Favoring natural ventilation (opening windows), the HVAC system, and the use of masks have been shown to significantly reduce the risk of transmission [30,79,84,89,90]. Masks reduce the bioaerosols emission variably, depending on the type of mask and the aerodynamics of the scattered particles [91–94]. In addition, HVAC systems should consider the filter, but it is also possible to optimize it to maintain adverse thermodynamic conditions for the virus [95,96].

One of the most significant limitations of CO_2 measurement is that its interpretation cannot be generalized but must be individualized. Aerosol generation fluctuates substantially depending on the individual's respiratory event [2,33,97–99]. In addition, environmental conditions directly influence the spread and persistence of the virus [2,100,101]. Therefore, it is not easy to define an effective viral load dependent on CO_2 , at least in absolute terms. However, this and other studies demonstrate the effectiveness of CO_2 measurement to implement effective air renewal patterns and reduce the risk of transmission of infectious diseases.

5. Conclusions and Recommendations

This work suggests that the measurement of the ΔCO_2 concentration inside collective transport constitutes a cost-efficiency strategy that can reduce the rates of spread of the respiratory virus by aerosols, as is the case of the SARS-CoV-2 virus. In this work, the interpretation of the exhaled CO_2 levels per person (ppm/person) has made it possible to analyze the behavior of the air inside the Zaragoza Tram. Maintaining the typical parameters of the HVAC units and implementing the partial opening of the windows, the maximum CO_2 level was 1249 ppm. On average, 835 ± 232 ppm have not been exceeded on any of the routes studied, which indicates that air recirculation is adequate for vehicle occupancy. In addition, the absolute CO_2 in all the routes studied was 685 and 690 ± 59 ppm, on average and median, respectively. However, it must be considered that capacity was reduced on the studied days due to the COVID-19 pandemic restrictions. It presents a limitation when extrapolating the data to post-pandemic operating conditions. The passengers' exposure to the Tram air must also be considered since the average route usually lasts around 7 min, and passengers wear a mask and keep their distance when possible. Under the conditions studied, the following recommendations are suggested to reduce the risk of infection by aerosols and/or improve ventilation performance:

- Maximize outside air intake: by opening windows, increasing door openings in stations, and minimizing the rate of return air in HVAC units;
- Completely recirculate the air between outbound and return routes to avoid exposing new passengers to the air breathed by previous passengers;
- Consider implementing efficient filtration systems against particles ($0.1\text{--}100\ \mu\text{m}$) instead of coarse-type filters, efficient against pollen or dust. Additionally to filtration systems, other air purification technologies can be beneficial in improving air quality. Even so, its performance needs to be demonstrated under operating conditions and not just in the laboratory or theoretically;

- Limit the respiratory activity of passengers to calm breathing and speech and the use of masks and other personal protection equipment and promote interpersonal distance.

In addition, from experience gathered during the CO₂ measurement experiments in public transport, the following recommendations can be drawn:

- Initially, characterizing the distribution of CO₂ inside the vehicle is essential so that the location of the sensors allows representative measurements of the space to be taken;
- Analyzing the increase in CO₂ instead of absolute CO₂ allows for quantifying only the CO₂ generated by passengers, discriminating external pollution. Additionally, we propose to use the ppm/person ratio as the main indicator to compare the exhaled CO₂ measurements on different scenarios. This ratio can be easily calculated by dividing the increase by the number of people. For example, if the increase in CO₂ is 500 and there are 50 people, the ratio will be 10 ppm/person. In case of studying two separate days, for example with different weather, we can find that one day the ratio is 10 ppm/person and another day it is 30 ppm/person. With this information, we can determine how the change of variables affects independently of the occupation.
- Place the gauges at a sufficient height to avoid the direct exhalation of the passengers. For example, they were placed 2.25 m above ground level for this work. Moreover, locating meters near doors and windows should avoid underestimating CO₂ levels.
- Evaluate weather conditions, especially airspeed, to interpret the measurement results on measurement days correctly. For example, in our study, the weather substantially affected the ventilation ratio inside the Tram. On the days with the greatest wind, ppm/person rates of up to 44% lower were recorded with respect to the days with the least wind.
- Recording occupancy levels (number of passengers) is essential to estimate the ventilation rate and to be able to compare data in different samples.
- Deduct the minimum number of meters to obtain representative measurements of the space. The heterogeneity in vehicle occupancy requires a consistent distribution of meters. For example, a meter was placed for every 35 m³ of air in this work.
- Considering the respiratory activity of the vehicle occupants is desirable when normalizing the ppm/person rates. In addition, the CO₂ records must be individually interpreted depending on variables such as interpersonal distance, the use of masks or other PPE, and the implemented filtration systems (or other air purification devices).

Under the conditions studied, the Tram does not present itself as a space with a high risk of infection by aerosols (by using Aireamos Covid Risk Airborne tool [31]; see Section 3.7). Air quality monitoring began to gain popularity due to the COVID-19 pandemic. However, once the focus is on the air [2], a post-pandemic scenario presents uncertainty when the windows are completely closed and the capacity increases. Consequently, it will be necessary to implement a standard that allows air quality to be regulated in these post-pandemic conditions. The poor filtration performance against the submicronic matter of the typically implemented filters is a significant limitation. It is necessary to find new air control and purification strategies that reduce the risk of disease transmission in the future.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/ijerph19116605/s1, Table S1: Weekend journeys data analysis (from Friday evening to Sunday), and Table S2: Weekday journeys data analysis (from Monday to Friday evening).

Author Contributions: Conceptualization, M.B., J.J.A. and A.J.S.; Data curation, M.B.; Formal analysis, M.B., J.J.A. and A.J.S.; Investigation, M.B. and J.J.A.; Methodology, M.B. and A.J.S.; Project administration, J.J.A. and A.J.S.; Resources, J.J.A.; Supervision, A.J.S.; Writing – original draft, M.B., J.J.A. and A.J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Instituto de Investigación Sanitaria Aragón: Campaña Investiga COVID-19 (CoviBlock).

Acknowledgments: We would like to express our gratitude to “Los Tranvías de Zaragoza S.E.M.” and “CAF Spain S.A.” for supporting this research and its invaluable collaboration. We also would like to thank “Ayuntamiento de Zaragoza” and “Institute for Health Research Aragón” for the received support.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Séptimo artículo

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Science of Total Environment, 2023, 855, 158965

<https://doi.org/10.1016/j.scitotenv.2022.158965>

Factor de impacto en 2022: 10.753

Factor de impacto a 5 años: 10.237

Cuartil JCR (2021): D1

Cuartil JCR (2020): D1

Fecha de envío: 26 de abril de 2022

Fecha de publicación: 29 de septiembre de 2022



Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

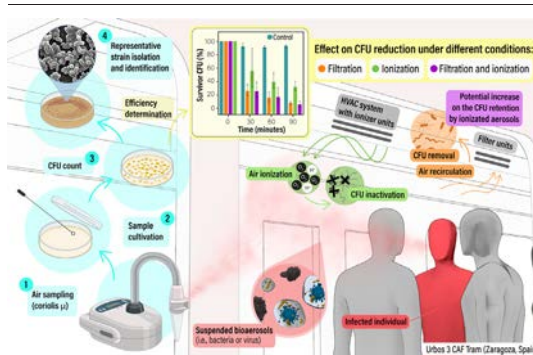
Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport

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HIGHLIGHTS

- Collective transport is considered one of the environments with the highest rate of COVID-19 propagation.
- Air purification can reduce airborne transmitted diseases.
- Standard filters installed in trams present reduced efficiency for submicron particles.
- A bipolar ionization unit in a tram has been evaluated for the first time.
- Bipolar ionization can reduce environmental bioaerosols but not CFUs on surfaces.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Warish Ahmed

Keywords:

Collective transport
Tramway
Bipolar ionization
Bioaerosols
Infectious diseases
SARS-CoV-2

ABSTRACT

The transmission rate of SARS-CoV-2 is higher in collective transport than in other public environments. Transport companies require preventive strategies to mitigate airborne risk of contagion which not imply responsible use at the individual level. Air purification systems, such as UV-C or needle-tip bipolar ionization, are attractive alternatives. However, only a few studies addressing the validation of this technology against bioaerosols in actual operation conditions have been published so far. In this work, the efficiency of a bipolar ionization unit in the Zaragoza Tram has been evaluated. Against environmental bioaerosols, ionization ($\sim 25.7 \cdot 10^9$ ions/m³, on average) reduced the concentration of colony-forming units (CFU) by $\sim 46\%$ and $\sim 69\%$ after 30 and 90 min. No clear benefits were obtained against microorganisms on inner tram surfaces (seats, grab bars, walls, and windows). ‘Pre-pandemic’ filtration equipment located in the HVAC based on a Coarse 45 %-type filter removed $\sim 73\%$ and $\sim 92\%$ of aerial CFU by itself after 30 and 90 min. Microscopic visualization of the CFUs revealed that they were mostly $>1\ \mu\text{m}$, much larger than the SARS-CoV-2 virion ($\sim 100\ \text{nm}$) and SARS-CoV-2-loaded bioaerosols (from $0.25\ \mu\text{m}$). Then, we studied the filter behavior under normalized laboratory methods. The filters efficiency against submicron particles was limited (between 5 and 12 % against 0.1 to $0.3\ \mu\text{m}$ NaCl particles). Another ionization strategy was to generate aerosol agglomerates to enhance filtration performance, but the combined action of ionization and filtration did not improve substantially. The effect of these technologies was also characterized using the clean air delivery rate (CADR). Relative to untreated air (CADR = $0.299\ \text{m}^3/\text{min}$), ionization and filtration reduce ambient CFUs (CADR = 5.153 and $9.261\ \text{m}^3/\text{min}$, respectively; and CADR = $13.208\ \text{m}^3/\text{min}$, combined) which implies that it has a substantial impact on indoor bioaerosols.

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<http://dx.doi.org/10.1016/j.scitotenv.2022.158965>

Received 26 April 2022; Received in revised form 12 September 2022; Accepted 19 September 2022

Available online 23 September 2022

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1. Introduction

The airborne transmission model refers to the spread of a disease through aerosols up to 100 μm , which can be transmitted through the air over time and distance (Siegel et al., 2019). At the beginning of the COVID-19 pandemic, this route of infection was not considered relevant, and more attention was paid to the transmission through droplets and infected surfaces (Wang et al., 2021). The global acceptance of the spread of SARS-CoV-2 by aerosols allowed an improvement in the preventive approach, including new techniques for epidemiological management, such as the measurement of exhaled carbon dioxide (CO_2) as an indicator of the risk of contagion (Wang et al., 2021; Peng and Jimenez, 2021) and air purification technologies.

The term ‘bioaerosol’ refers to those particles of biological origin or active particles that can affect living organisms, causing them some allergy, toxicity, or infection (Rose, 1994; Sánchez-Monedero et al., 2006). Bioaerosols can include viruses, bacteria, spores, pollen, and, in general, any other microorganisms with an aerodynamic diameter between 0.5 and 100 μm (Cox and Wathes, 1995). In the context of airborne pathogen transmission, following mechanistic hypotheses about disease transmission, the spread of bioaerosols is associated with respiratory events (Hsiao et al., 2020), mainly coughs and sneezes (Asadi et al., 2019). However, the generation of bioaerosols has also been demonstrated during breathing and speech. Disseminating aerosols size ranges from a few nanometers to hundreds of micrometers (Asadi et al., 2019). Regarding the COVID-19 pandemic, bioaerosols released by a person infected with SARS-CoV-2 may contain some virus or traces of it, and the viral load contained in the particles is a crucial factor in determining the relative contribution of airborne transmission (Wang et al., 2021; Sattar and Ijaz, 1987). The modal distributions of aerosols acquire great relevance in infection transmission. Particle size determines the aerodynamic characteristics and deposition dynamics and the variability in the viral colonization model depending on the depth of the reached respiratory tract (Wang et al., 2021; Shinya et al., 2006; Guzman, 2021).

A higher transmission rate of SARS-CoV-2 has been observed in means of transport compared to other public and shared spaces. Thus, Lan et al., 2020 analyzed the relationship between the transmission of COVID-19 and the type of work, pointing to an incidence of 18 % of cases in the transport sector, only behind the health sector (22 %). Zhao et al., 2020 determined an association between the number of infected patients and the domestic transportation route of Wuhan by train, private car, and flights, finding a significant relationship between the infection rate and transportation by train, but not in flights and private vehicles. This trend was also studied using other infectious disease models (Horna et al., 2010; Troko et al., 2011; Goscé and Johansson, 2018).

The risk of contagion in public transport is determined by the seat's proximity to the infected person, the number of passengers, the number of interactions with other passengers, the duration of the trip, and the capacity performance of air renewal (Nasir et al., 2016). Generic measures have been adopted and have proven effective to reduce the virus spreading: surfaces sanitation, social responsibility measures (such as the use of a mask, social distancing, and hand hygiene), and ventilation measures (Moreno et al., 2021; Di Carlo et al., 2020). Regarding basic interior sanitation, solutions based on chlorine and isopropyl alcohol effectively disinfect surfaces (European Centre for Disease Prevention and Control (ECDC), 2020). Recommendations from the Centers for Disease Control and Prevention (CDC) and The Transportation Research Board (TRB) include at least two daily cleanings and suggest having particular account high-touch zones (American Public Transportation Association, 2020). The survival of SARS-CoV-2 varies depending on the material on which it is deposited. SARS-CoV-2 remains active for up to 72 h on plastic and stainless steel, up to 24 h on cardboard, and <4 h on copper (Van Doremalen et al., 2020), therefore it would be necessary to consider heterogeneity within the different areas of the same public transport unit.

HEPA filtration is very effective eliminating of microorganisms present in the ambient air in the means of transport since it allows efficient elimination of microorganisms reaching efficiencies close to 99.997 % (Askar et al.,

2012; Leder and Newman, 2005). However, the performance of this technology will depend on the number of air changes and the feasibility of incorporating these filters to the installed air conditioning system. The percentage of recirculated air is another aspect to consider in the air quality in transport (Zitter et al., 2002). Additionally, natural air renovation, such as opening windows, can considerably reduce the concentration of CO_2 inside a vehicle as well as the number of airborne containing microorganisms (Matose et al., 2019). Concerning the COVID-19 pandemic, the preventive strategy to reduce the risk of contagion by aerosols has been managed along two different lines: on the one hand, based on the measurement of CO_2 as an indicator of air renovation. On the other hand, by purifying or cleaning the air by different means including ultraviolet C (UV-C) radiation technology, dry fumigation with hydrogen peroxide (H_2O_2) or technology based on non-thermal plasma, among others.

Technology based on the UV spectrum between 200 and 280 nm (UV-C) has been widely used in disinfection processes. The intracellular components of microorganisms (DNA, RNA, and proteins) present a high and variable sensitivity to absorb UV-C photons, producing critical damage to their genome and inhibiting their correct replication (Bolton and Cotton, 2008). The efficiency of UV disinfection varies depending on the distance from the surface and the application time (Li et al., 2017). This technology has proven helpful against the SARS-CoV-2 virus (Liang et al., 2021; Biasin et al., 2021; Ruetalo et al., 2021), reducing the viral load between 0 and 6 log orders of magnitude in culture media (Raeiszadeh and Adeli, 2020). Although the performance of the technology is limited against bioaerosols loaded with SARS-CoV-2, where the required UV_{254} dose is high (Raeiszadeh and Adeli, 2020), some studies highlight its effectiveness for air purification (Bowen et al., 2021) and especially if combined with HEPA filtration (Barnewall and Bischoff, 2021).

Non-thermal plasma-based technology has positioned itself as one of the leading air purification strategies in the context of the pandemic caused by the SARS-CoV-2 virus. Plasma inactivation of microorganisms has been attributed to cell wall rupture and damage of the genetic material (Liang et al., 2012). Its mechanism of action is multiple. The presence of charged particles, ions, reactive oxygen species (ROS) and oxygen-containing radicals, UV-C, vacuum ultraviolet (VUV), and localized heating events stand out, acting exclusively or in combination (Gallagher et al., 2007; Laroussi, 2007; Sakudo and Shintani, 2010; Nehra et al., 2008). The antimicrobial effect of this technology has been widely exploited in the health sector to sterilize surgical instruments (Klämpfl et al., 2012). Moreover, it has been tested in controlled environments reporting excellent efficiency in the inactivation of specific bacterial species (Liang et al., 2012) and enveloped/non-enveloped viruses (Sakudo et al., 2013; Sakudo et al., 2017; Sakudo et al., 2016). However, a limited number of studies support its efficiency in other settings.

Non-thermal plasma-based technology has been tested for other applications regarding the current pandemic, obtaining variable and lower results than those described by the manufacturers (Licht et al., 2021). One of the major concerns and limitations of this technology is the generation of by-products in harmful concentrations (Sleiman and Fisk, 2009), where some authors suggest increases in actual conditions (Zeng et al., 2021). However, this aspect is not discussed in this article. This work provides information on possible systems to reduce the risk of infectious diseases in public transport. First, the efficacy of needle-point bipolar ionization against eliminating environmental bioaerosols in the Zaragoza Tram (Zaragoza, Spain) is evaluated. Given biosafety and ethical constraints, we decided to perform the assays using environmental bacteria (not artificially dusted). Afterward, the efficiency of the isolated filtration media is tested against non-biological particles. These tests carried out in the laboratory allow the efficiency of the filter to be measured against submicron matter (hardly characterizable using the previous tests). Finally, the combined action of both strategies is studied. In parallel, the antimicrobial performance of ionization for surface disinfection is evaluated. This work evaluates the air purification systems for their implementation in local public transport.

2. Materials and methods

2.1. Needle-tip bipolar ionization system

As shown in Fig. 1, two PA604 ionization units of the 600 Series (Tayra SA, Spain) were installed in the suction vertices of the delivery fan of the two air conditioning units arranged in the Zaragoza Tram Unit (Model Urbos 3 CAF, ES). The two HVAC (Heating, Ventilation, and Air Conditioning) units installed in the Tram drive a total flow of 2800–3300 m³/h, with a fresh air ratio of 1:3 fresh/return air. The selected ionizers were of the needle tip brush type (PA604, Tayra SA Spain). They produce an equal amount of positive and negative ions. They have a maximum treated flow of 4100 m³/h and a <5 kV DC voltage between brushes. In the case of the tests where ionization was evaluated, an Air Ion Counter COM-3200PRO II (Com System INC, Tokyo JA) was used to ensure the correct generation of ions.

2.2. Conditions and preparation of the tram units

The Urbos 3 tram model (CAF, Spain) has a total length of 33 m, a width of 2.65 m, and a height of 3.2 m. It has a capacity of 200 seats, of which 146 are standing seats (3.5 people per m²) and 54 are seats. It operates in Zaragoza, the fifth biggest city in Spain, with a population close to 700 k inhabitants, located midway between Madrid and Barcelona. The tram units included in the study were in operation for a full day, and the usual protocol was not carried out night cleaning. During the morning of the following day, the tram unit provided partial service lasting approximately 4 h. During this time, the hydroalcoholic gel dispensers were removed to avoid disinfection of the tram surfaces included in the study, and the windows were closed during the journeys. Travelers wore masks. Upon arrival at the depot, all the tram doors were opened for 15 min to renew the interior air and replace it with fresh ambient air. Doors opening were performed to maximize the homogeneity and reproducibility of the assays. The conditions used for the preparation of the Tram Units are detailed in Table 1.

The bipolar ionization units were turned on and stabilized for at least 15 min prior to the start of the test. The filter used during the tests was the usual one recommended by the manufacturer (Coarse 45 % according to UNE-EN ISO 16890, Merak Long Life Filter, Madrid SP). They were kept in the same usual conditions and were within the period of useful life determined by the manufacturer. The air conditioning system was kept off for the reference sampling, and the Tram was ventilated between tests, recirculating the depot air for at least 15 min.

2.3. Environmental sampling conditions and cultivation

A total of 6000 l of air was sampled at a flow rate of 300 l/min in an initial 5 ml of phosphate buffered saline (PBS) solution (Sigma-Aldrich, Darmstadt DE) using a Coriolis μ (Bertin Technologies, Montigny-le-Bretonneux FR). Sampling was carried out at three different points of the

Table 1

Set-up conditions established in each air sampling tests. Note that the HVAC A/C ventilation conditions have remained stable in all the samples.

Sampling conditions	Dampers	Filter	HVAC conditions	AFNPBI
Without ionization and filtration (stability test)	Closed	No	22 °C	No
Ionization	Closed	No	22 °C	Yes
Filtration	Closed	Yes	22 °C	No
Ionization and filtration	Closed	Yes	22 °C	Yes

Tram to homogenize the sampling and collect microorganisms at points characterized by different ionic concentrations. The manufacturers initially modeled the ion concentration throughout the Tram Unit, guaranteeing a sufficient concentration for the inactivation of microorganisms in any of their locations. In each test, three samplings were carried out with a frequency of 30 min to evaluate the system's efficiency for 90 min, corresponding to the travel time in each tram line. In addition, an initial sampling was carried out to calculate the relative efficiency. Once the sampling was finished, the volume corresponding to 450 l of air sample aspiration was seeded on a Plate Count Agar (PCA) plate (Scharlab, Spain), for 72 h at 30 °C. The final sampled solution was variable depending on the climatological conditions of the sampling. Thus, the counts were normalized to be comparable, taking the environmental sampling prior to the intervention as a reference. Five replicates of each sample were made. Colony-forming units (CFU) were manually counted after the incubation period (72 h at 30 °C). The CFU counted in each replica were averaged and normalized. Efficiency in CFU inactivation was calculated by comparing the percentage of surviving CFUs with the reference sample.

2.4. Identification and visualization of the bacterial species found

Among the CFUs, the 15 most representative specimens collected during the samplings were analyzed. The identification of the microorganisms has been carried out using MALDI-TOF mass spectrometry technology (MALDI Biotyper, Bruker Massachusetts USA), comparing the results to databases (Bruker, Massachusetts USA). The morphology of the bacterial strains fibers was observed using a Scanning Electron Microscopy (SEM) JEOL 6360-LV (Deben UK Ltd., Edmunds, United Kingdom). For sample preparation, a sample of the corresponding CFU was placed on a slide, fixed with carbon tape to a SEM microscope holder and sputtered with Au/Pd to promote electron conduction. The average of CFUs diameters and standard deviations (SD) were obtained from manual measurements with the free software Image-J (v1.52; Schindelin et al., 2012) for $n = 50$.

2.5. Sampling conditions of surfaces and cultivation

Rodac-PCA plates (Plate Count Agar-Scharlab, Spain) were used for surface sampling. At least 30 surface samples per test were taken at different points of the Tram, at different levels, and considering different types of

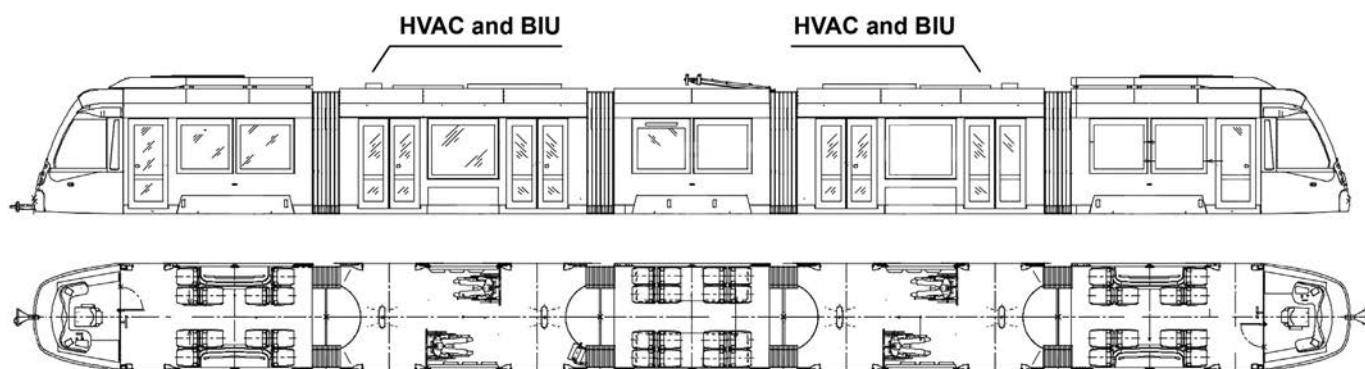


Fig. 1. Schematic representation of the bipolar ionization units and the HVAC systems location in the studied in the Urbos 3 tram model studied. Where the implementation of the BIU systems in the two HVAC is observed.

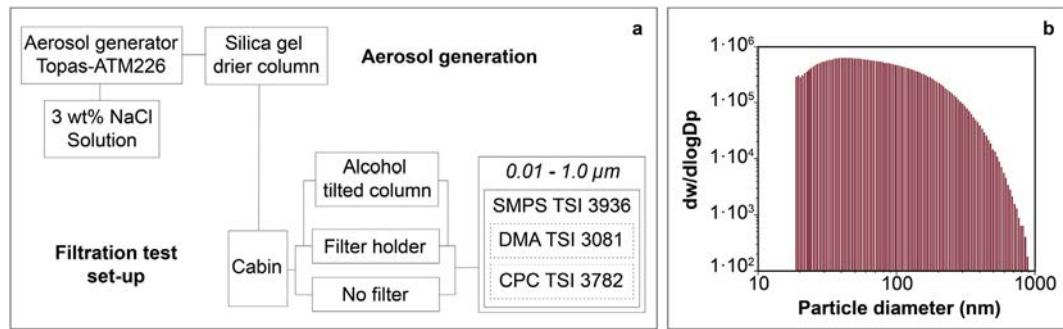


Fig. 2. a) Diagram of the equipment used to characterize the filters. b) Particle concentration distribution for efficiency determination measurements in the range.

surfaces. Seats, backrests, grab bars, intermediate supports, walls, and windows were included. Despite being carried out initially, the soil samples were excluded due to their high variability associated with residual contamination and dirt. The final surface sample was compared with a nearby (5 cm proximity) reference sample, and the ionization efficiency was calculated following the same method as for air sampling. Samples collected by contact in the Rodac-PCA plates were cultivated for 72 h at 30 °C.

2.6. Determination of filtration efficiency against submicron particles

As shown in Fig. 2-a, aerosols were produced using a Topas-ATM226 generator with a saline solution of sodium chloride (3 % NaCl in ddH₂O). The obtained microdroplets pass through a tubular silica gel air dryer to evaporate the water and produce solid particles. The particle size distribution (Fig. 2-b) inside the cabin was measured using an SMPS TSI 3936 composed of an electrostatic classifier (DMA TSI 3081) and a condensation particle counter (CPC TSI 3782). The particles were dragged at a 0.6 l/min flow rate. The filter was placed between bronze discs sealed with Teflon tape, with 30 × 20 mm Teflon washers on each side. The exposed filter area was variable (2.05, 4.1, and 8.1 mm) to adjust the desired flow rate. The measurements lasted 2 min and were made in duplicate. Due to the concentration of particles variation, measurements were made passing through a free tube between measurements to calculate relative efficiency according to Eq. (1). Where C_{up} stands for concentration upstream and C_{down} stands for concentration downstream. The retention efficiency is expressed in global efficiency as ‘number of particles’.

$$n = 100 \times \frac{C_{up} - C_{down}}{C_{up}} \quad (1)$$

Pressure drop testing was carried out using alcohol columns based on Bernoulli's principle. The free ends of the tubes have been inserted into the two quick couplings located on both sides of the filter sample holder. Measurements were made with a volumetric flow rate of 0.6 l/min.

2.7. Determination of air purification efficiency

System performance has been characterized using different approaches. Firstly, the calculation of the relative efficiency in the air and surface samples was carried out according to Eq. (2). The determination of the final CFU (CFU_2) was given as the average between the counts of the five replicates of each sampling. The initial CFU data (CFU_1) was taken from the previous test to assess performance every 30 min. Those cultures plates replicates with CFUs with values higher than three times those of the rest of the plates of the same sample were eliminated as they were considered non-representative extreme values. Secondly, clean air delivery rate (CADR) and first order loss rates using a simple linear regression against $\ln$ -transformed mean concentration values was determined as described by Stephens et al. (2022).

$$n = 100 \times \frac{CFU_1 - CFU_2}{CFU_1} \quad (2)$$

3. Results and discussion

3.1. Effect of filtration and needle-tip brush bipolar ionization on air

3.1.1. CFU stability in the environmental air

Airborne microorganisms are significantly affected by weather and environmental conditions (Fang et al., 2018; Zhang et al., 2017; Ehrlich et al., 1970). To guarantee a stable concentration of environmental bacteria during the tests, air samples were taken every 30 min without filtration and ionization. As depicted in Fig. 3, the results of this test suggest that the environmental microbiota is stable over time during the study hours on the same day. An average of 40.4 ± 1.5 CFU/m³ of air was obtained during the first test and 53.8 ± 3.0 CFU/m³ during the second test. CFU counts were averaged using all five replicates of each sample. In the plates at 30 and 90 min from the first test, 40.0 CFU/m³ were counted in each one

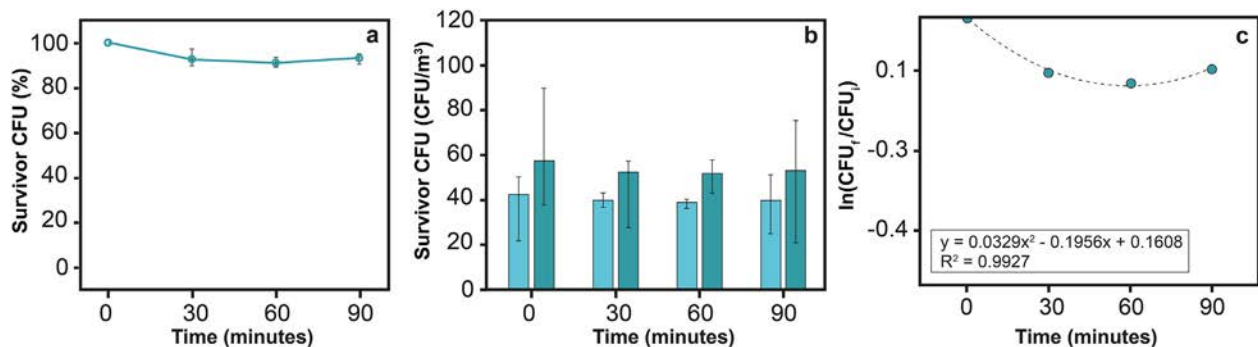


Fig. 3. a) Average percentage of survivor CFU. b) CFU/m³ count of the two samples stability tests from the initial reference value. c) First-order loss rate constant regression in CFU stability tests.

Table 2

Results of the bacterial species identification. Where it is observed that they come mainly from human origin and are Gram positive in nature.

Ref	Strain	Score ^a	Gram	Average $\pm$ SD	Probable origin
S1	<i>Roseomonas mucosa</i>	1.76	Neg	$3.5 \pm 0.7 \mu\text{m}$	Mucous (Romano-bertrand et al., 2016)
S2	<i>Micrococcus luteus</i>	2.13	Pos	$1.2 \pm 0.8 \mu\text{m}$	Soil, dust, cutaneous (Kookan et al., 2014)
S3	<i>Tsukamurella paurometabola</i>	1.71	Pos	$1.0 \pm 0.1 \mu\text{m}$	Pathogen (Munk et al., 2011)
S4	<i>Kocuria rhizophila</i>	1.77	Pos	$0.9 \pm 0.2 \mu\text{m}$	Cutaneous (Takarada et al., 2008)
S5	Not identified	–	–	$3.9 \pm 0.5 \mu\text{m}$	Not defined
S6	<i>Dermacoccus nishinomiyaensis</i>	1.75	Pos	$1.1 \pm 0.1 \mu\text{m}$	Cutaneous (Williams and MacLea, 2019)
S7	<i>Microbacterium paludicola</i>	2.02	Pos	$4.2 \pm 1.3 \mu\text{m}$	Not defined
S8	<i>Dermabacter hominis</i>	1.91	Pos	$1.2 \pm 0.2 \mu\text{m}$	Cutaneous (Jones and Collins, 1988)
S9	<i>Roseomonas mucosa</i>	2.6	–	$1.3 \pm 0.2 \mu\text{m}$	Mucous (Romano-bertrand et al., 2016)
S10	Not identified	–	–	$1.1 \pm 0.1 \mu\text{m}$	Not defined
S11	<i>Staphylococcus haemolyticus</i>	2.1	Pos	$0.9 \pm 0.1 \mu\text{m}$	Cutaneous (Eltwisy et al., 2020)
S12	<i>Deinococcus wulumuqiensis</i>	2.36	Pos	$1.5 \pm 0.2 \mu\text{m}$	Not defined
S13	Not identified	2.0	–	$1.0 \pm 0.1 \mu\text{m}$	Not defined
S14	<i>Bacillus cereus</i>	1.95	Pos	$1.5 \pm 0.2 \mu\text{m}$	Not defined
S15	<i>Bacillus</i> sp.	–	Pos	$3.5 \pm 0.9 \mu\text{m}$	Not defined

^a The score indicates the effectiveness of the identification. Scores >2 indicate an excellent identification of the bacteria. Scores <2 indicate that the spectrum is related to the indicated bacterium but its concordance may not be exactly, so it could not be the indicated strain.

with respect to the initial 42.5 CFU/m^3 . In the plates of the second test, 52.5 and 53.1 CFU/m^3 were found at 30 and 90 min, compared to the initial 57.5 CFU/m^3 . To determine the stability of the CFUs, the Relative Standard Deviation (RSD) of each set of plates was calculated. The obtained RSD of ~ 0.04 in the first test and ~ 0.06 in the second test suggests that the bacterial environmental contamination is stable in air during the 90 min of sampling, so that the rest of the tests presented below are performed under robust and reproducible conditions throughout each test. Loss-rate regression of CFU has been fitted to a polynomial to characterize stability (Fig. 3c). The resulting CADR of $0.299 \text{ m}^3/\text{min}$ and the loss rate constant of 0.0012 per minute during the stability control condition reinforces the previous conclusion about the stability of environmental CFUs.

3.1.2. Bacterial species identification

As enumerated in Table 2, the 15 most frequent colonies were isolated and identified. The bacterial identification is of interest since the efficiency

of the BIU is evaluated predominantly for this subset of strains, despite the existence of others in a smaller proportion. In addition, its visualization allowed to evaluate its morphology and size, which is relevant for filtration studies. As depicted in Fig. 4, most bacterial strain (12/15) have sizes $>1 \mu\text{m}$ in its longest dimension. The measured dimension represents the minimum size in which each strain of CFUs can be found in the air.

3.1.3. Bipolar ionization reduces aerial CFUs

The effect of ionization was considered exclusively to evaluate the single efficiency of the bipolar ionization unit. The objective of this study was to isolate the efficiency of the ion system and quantify the improvement that it supposes by itself in the absence of other perturbations, such as the filtration of the air conditioning system. An increasing efficiency was observed from the initial sampling to the final samplings. Ion concentration varied ($19.9 \cdot 10^9$ – $31.5 \cdot 10^9 \text{ ions/m}^3$) in all the samplings carried out due to the heterogeneity in the air distribution at the different points of

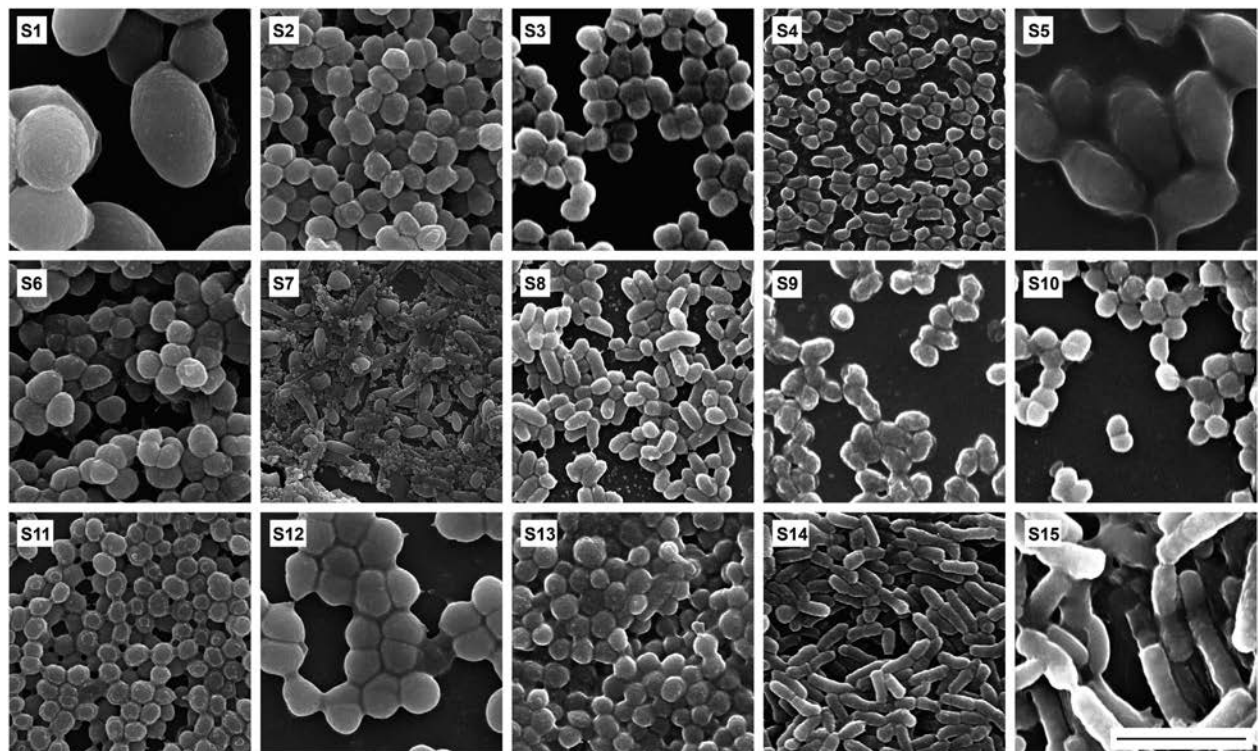


Fig. 4. Electron micrographs of isolated CFUs from air samples. Scale bar represents $5 \mu\text{m}$ for all images.

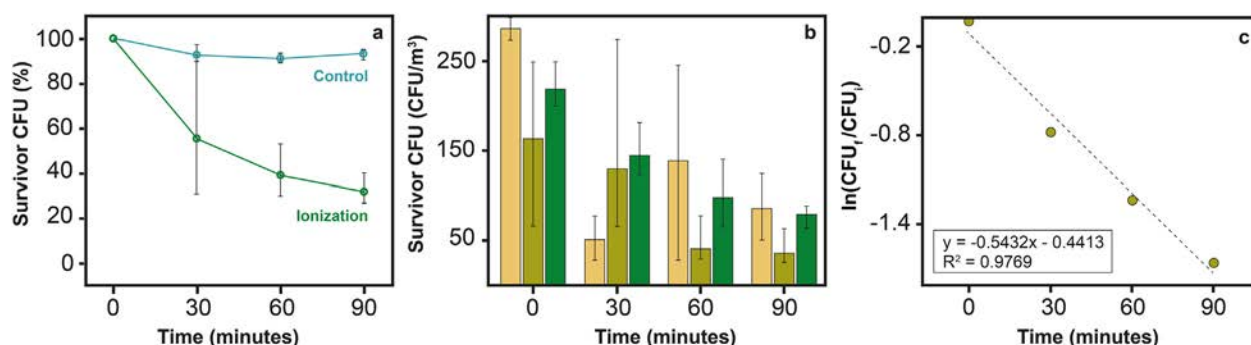


Fig. 5. a) Average percentage of survivor CFU. b) CFU/m³ count of the three samples in ionization tests from the initial reference value. c) First-order loss rate constant regression in ionization inactivation tests.

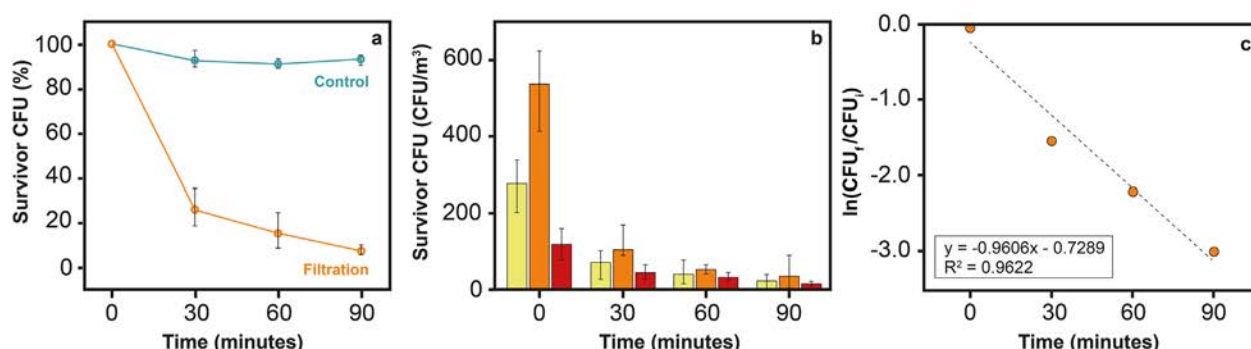


Fig. 6. a) Average percentage of survivor CFU. b) CFU/m³ count of the three samples in filtration tests from the initial reference value. c) First-order loss rate constant regression in filtration inactivation tests.

the Tram. On average, during the first 30 min of the experiment, CFUs were reduced by 45.9 % (54.1 % persisted). As shown in Fig. 5, an efficiency of 61.8 % (38.2 % persisted) was estimated after 60 min. The efficiency increased to 69.2 % (30.8 % persisted) after 90 min. Licht et al., 2021 evaluated the efficiency of needle-point bipolar ionization systems (~10–20 kV) in airplanes, obtaining a reduction of CFU (*Staphylococcus epidermidis*) variable between 20.7 % and 60.0 % after 1 h of ionization, which is consistent with the results obtained in the present study. The resulting loss rate constant is 0.018 per minute under ionization conditions, and the estimated CADR was 5.153 m³/min. Regarding the control values, it is observed that the clean air delivery rate increases, which implies a greater amount of air free of bioaerosols. Specifically, this value increased in >17 times.

3.1.4. Filtration reduces aerial CFUs

The electrostatic potential influence of the surface on the deposition of bacteria from the air has been demonstrated (Meschke et al., 2009). Various studies suggest that bipolar ionization reduces particulate matter in the air due to increased particle deposition and/or filtration efficiency associated with the increase in the aerodynamic diameter of aerosols due to the agglomeration of fine particles (Pushpawela et al., 2017; Wu et al., 2015; Zeng et al., 2021). This phenomenon is mainly associated with electrostatic effects (Mayya et al., 2004; Grabarczyk, 2001) and is dependent on particle size and composition, relative humidity, ionization time, and surface material (Grabarczyk, 2001). Filters can interfere in estimating the efficiency of the ionization system. Then, the effect of the filtration implemented in the air conditioning system of the Tram has been characterized separately. The tests were carried out with the filter usually installed on the Tram (Coarse 45 % filter medium). The Air Changes per Hour (ACH) of the Zaragoza Tram remained as they are in the normal operation of the unit at 25 ACH. This indicates that the air passes through the filter 12.5 times/h on average. As seen in Fig. 6, loss rate constant was 0.033 per minute, and the estimated CADR was 9.261 m³/min. These parameters imply

that filtration is more effective than ionization. CFU amount upon filtration suggests a reduced efficiency considering the number of air changes inside the tram unit. On average, CFUs were reduced by 73.4 % (26.6 % persisted), 84.0 % (16.0 % persisted), and 92.0 % (8.0 % persisted) during the first 30, 60, and 90 min, respectively. In this sense, the CFUs are large enough to favor the filtration mechanisms since they are, for the most part, >1 µm.

Faced with submicron particles, smaller in size than the bacteria tested, the efficiency of the filter is limited according to the conditions studied (Table 3). The filter complies with the UNE-EN 16890 standard and refers to a 75 % filtration for particles greater than ~10 µm. This filter is efficient for the retention of dust or pollen. However, it is inefficient against fine particles and viruses. The tests carried out are limited. Due to the conditions of the equipment used for the determination of filtration efficiency against submicron particles, the filter may have been 'clogged' by the concentration of NaCl particles, assuming a notable increase in pressure drop and an ornament in the efficiency result. The Coarse 45 % filter was evaluated in a more compact format than natural: 2–3 mm thick instead of 20–30 mm. The results suggest that the speed at which the air passes through the filter medium retains the larger particles. However, no notable difference is observed in the flows studied. The curves could not be very representative of this filter's actual efficiency (in working conditions in the air conditioning system of the Tram).

Table 3
Laboratory conditions used in the filtration tests.

	Area (cm ²)	Flow (m ³ /h)	Speed (cm/s)
Coarse 45 % filter	3740	~261.2	19.4
		~1019.2	75.7
		~4079.6	303.0

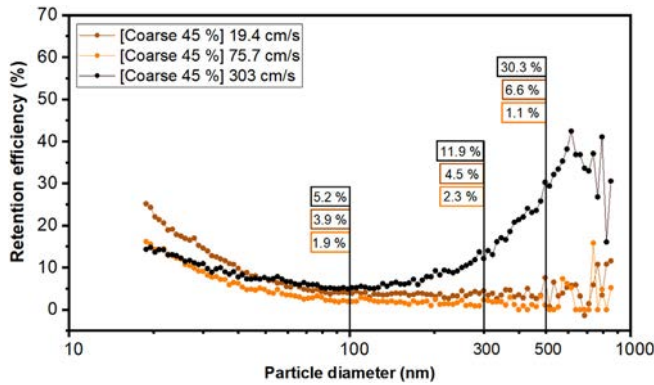


Fig. 7. Coarse 45 % filter retention efficiency depending on the particle diameter at different velocity in filter (flow rates). Where it is observed that the efficiency is dependent on the flow rate and the particle size.

Table 4

Pressure drop obtained from the Coarse 45 % filter. Where it is shown that pressure drop depends on the flow rate (measured as velocity in filter).

Velocity in filter (cm/s)	Pressure drop (Pa)
	Coarse 45 % filter
19.4	~6
75.7	~40
303.0	~380

In experimental sampling tests, SARS-CoV-2 viral RNA has been mainly found in particle sizes from 0.25 μm (Kenarkoochi et al., 2020; Liu et al., 2020). Therefore, determining the efficiency of the filter against submicronic sizes and above is relevant to quantify its performance. As shown in Fig. 7, the Coarse 45 % filter presented an approximate retention efficiency of ~30 % for 0.5 μm particles at a flow rate of ~4079 m^3/h . For flow rates lower than this, the efficiency of the filter decreases to values <7 %. These tests suggest that larger particles have more inertia at high speeds (high flow rates) and, therefore, a higher retention rate in the filter medium (Yeh and Liu, 1974a, 1974b). However, the clogging of NaCl particles observed in the head loss tests may have overestimated these results. In the tests where the highest speed is simulated, clogging is observed, which translates into an increase up to 380 Pa of pressure drop (Table 4).

3.1.5. Effect of combined ionization and filtration efficiency against aerial CFUs

Once the effects of ionization and filtration were evaluated separately, the combination's performance was intended to study. However, no clear advantage was obtained from the combination of mechanisms. The first 30 min reduced the CFU concentration by 74.2 % (25.8 % persisted), while at 60 min, the efficiency increased to 82.8 % (17.2 % persisted), and at 90 min, 94.1 % was eliminated (5.9 % persisted) (Fig. 8). While these data are promising, the

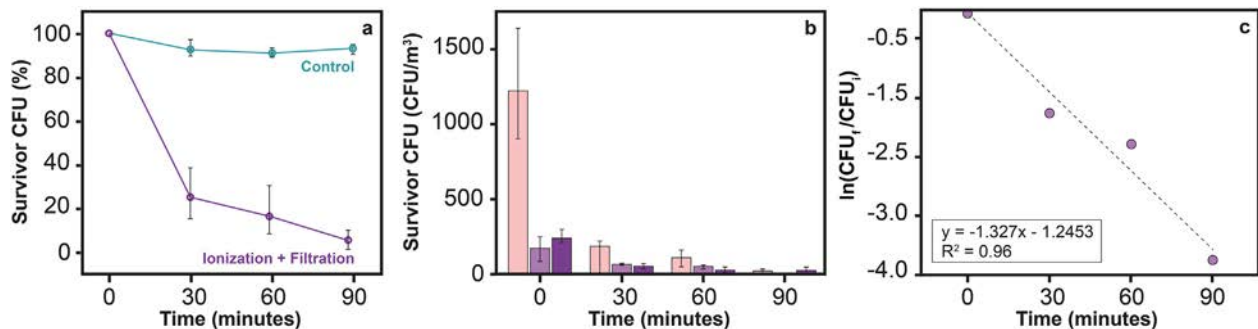


Fig. 8. a) Average percentage of survivor CFU. b) CFU/ m^3 count of the three samples in simultaneous ionization and filtration tests from the initial reference value. c) First-order loss rate constant regression in both ionization and filtration inactivation tests.

Table 5

Results of surface stability tests. Where, CFU_i refers to the CFU count in initial ($t = 0$) samples and CFU_f to final ($t = 2$ h) samples CFU counts.

Test	RSD (average)	$\text{CFU}_i = \text{CFU}_f$	$\text{CFU}_i < \text{CFU}_f$	$\text{CFU}_i > \text{CFU}_f$
Test 1	0.39	39.3 %	21.4 %	39.3 %
Test 2	0.64	17.2 %	58.6 %	24.2 %
Test 3	0.39	23.1 %	30.8 %	46.1 %
Test 4	0.61	13.3 %	33.3 %	53.4 %

CFU_i = Initial CFU; CFU_f = Final CFU.

filtration alone reduced the CFU concentration by 73.4 % and 92.0 %, respectively, after 30 and 90 min. The maintenance of filtration efficiency can be explained because the highest rate of particle agglomeration due to electrostatic phenomena occurs in the finest particles (<0.15 μm). Then, there is a higher concentration of particles >0.3 μm (Zeng et al., 2021), which does not favor the performance of filtration (Yeh and Liu, 1974a, 1974b).

The loss rate constant was 0.047 per minute, and the estimated CADR was 13.208 m^3/min . The value of CADR of ionization and filtration together was higher than filtration and ionization separately by 3.947 and 8.055 m^3/min , respectively.

3.2. Effect of the bipolar ionization in the elimination of CFUs on surfaces

3.2.1. Evaluation of the CFUs stability on surfaces

To guarantee the uniformity of the tests, four surface samples have been carried out, with a total of at least 60 Rodac PCA plates per test: 30 at $t = 0$ (CFU_i) and 30 at $t = 2$ h (CFU_f). Ideally, the CFU_i should be equal to the CFU_f (ie the percentage of $\text{CFU}_i = \text{CFU}_f$ would ideally be 100 %). However, we have considered it relevant to analyze whether there are more or fewer CFU at the end of the experiment to determine if it is a matter of stability or simply variability across the surface (i.e., $\text{CFU}_i < \text{CFU}_f$ or $\text{CFU}_i > \text{CFU}_f$). If the CFUs had deteriorated over time, a trend greater than $\text{CFU}_i > \text{CFU}_f$. This has not been observed, so it is assumed that the CFUs are stable during the time of the experiment (2 h) but there is a non-negligible need between the neighboring test surfaces. To quantify this dispersion, the RSD of each set of plates has been calculated. RSD close to zero suggests high uniformity in the samples. The variability of the results (Table 5) required an increase in the sample to obtain representative conclusions.

3.2.2. Bipolar ionization efficiency on surface samples

On the one hand, “effectiveness” refers to the percentage of PCA plates where final CFU number was lower than the initial ($\text{CFU}_i > \text{CFU}_f$). That is, in this column the percentage of CFU plates that showed a potential action of ionization has been studied. On the other hand, “efficiency” represents the percentage of “removed” bacteria in these plates potentially due to ionization effect, which is calculated according to Eq. (2). It is relevant to differentiate between the efficiency for plates with $\text{CFU} > 50$, $\text{CFU} > 20$, and $\text{CFU} > 10$ to avoid distorting the results obtained. However, the global efficiency does not discriminate between values obtained (Table 6).

Table 6

Effect of bipolar ionization tests on surfaces. Where, CFU_i refers to the CFU count in initial ($t = 0$) samples and CFU_f ($t = 2$ h) to final samples CFU counts.

Test	Effectiveness (CFU _i > CFU _f)	Global efficiency	Efficiency CFU > 50	Efficiency CFU > 20	Efficiency CFU > 10
Test 1	60 %	65.1 %	65.9 %	72.1 %	66.6 %
Test 2	53 %	44.0 %	43.4 %	36.3 %	40.9 %
Test 3	30 %	54.6 %	38.6 %	47.7 %	46.3 %
Test 4	53 %	73.0 %	65.9 %	78.4 %	73.0 %
Test 5	42 %	78.1 %	73.4 %	70.5 %	78.1 %
Test 6	40 %	81.4 %	69.2 %	83.6 %	81.4 %

CFU_i = Initial CFU; CFU_f = Final CFU.

According to our findings, in this experimental set-up, the ionization did not present a major role on the inactivation of microorganisms on surfaces. The average ionization effectiveness was 46 %, while the average CFU_i > CFU_f in the stability study was 36 %. We cannot exclude that the difference between initial and final CFUs could be due to effects other than ionization.

4. Conclusions

The increasing understanding of the mechanisms behind COVID-19 infection transmission should drive a shift in the way we address the prevention of the transmission of this disease and other respiratory infections. A higher transmission rate of SARS-CoV-2 has been observed in transport compared to other public and shared spaces. It is required to implement preventive measures for different transportation settings and countries. In previous studies we found that the Zaragoza Tram did not represent a high risk of contagion (for more information see Baselga et al., 2022). Nevertheless, after the pandemic, preventive strategies are relaxed and the potential risk of contagion increases, so it is necessary to find long-term solutions for the improve the quality of the air we breathe. Although masks are very efficient in expelling fewer potentially pathogenic microorganisms into the air, it is a temporary measure that probably will not be used indefinitely.

Studies on environmental bioaerosols in air samples suggest that BIU systems have a beneficial effect on eliminating CFUs. The main limitation for the implantation of ionization systems is a possible generation of by-products due to the electrostatic interaction between elements, although this aspect is not discussed here, and it is out of the scope of this work. Under the conditions studied, which are favorable (including closed tram unit and long exposure times), efficiencies close to 69.2 % were obtained after 90 min of ionization. The implemented filtration system offers better results in the same period against microorganisms present in the air (92.0 %). The combined action of both systems slightly improved the filtration performance (94.1 %), not representing a major improvement. According to the literature, the predominance of agglomerates is close to 300 nm. However, the Tram filters tested in this study did not perform well in laboratory analyses against those particle sizes (<10 %). We cannot exclude a synergy of bipolar ionization with other filter types that could retain these agglomerates.

The filtration tests assayed in the studied Tram units are not very efficient for submicron particles. The Coarse 45 %-type filters are designed to eliminate dust, pollen, and larger particles. However, considering the number of air changes per hour (~25 ACH, ~1450 m³/h) approximately 6 % of the CFUs are retained each time the air passes through the filter leading to a ~75 % efficiency observed during the first half-hour (note that no CFU sources existed during the tests). In addition, the main bacterial families found almost always presented sizes >1 µm, so the filtration of submicron matter would be more representative in the case of viruses. A limited filtration of fine particles is observed in the tests carried out with submicron particles, where the retention of 0.1 µm particles was <6 %. Faced with these sizes, HEPA filters are an effective strategy. However, it must be considered that the quality of the filter is as important as its performance (measured in m³/h). This is why the implementation of HEPA filters in the HVAC system of the Tram is unthinkable due to its pressure drop. Installation of stand-

alone HEPA purification systems to reduce airborne CFUs and viruses could be considered, although this would require separate studies.

Through the technical feature recently agreed upon by the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE), it was possible to quantify the impact of the technologies (ionization and filtration) on the interior air of the Tram. It was performed using the loss rate value and the CADR parameter. Combined action of ionization and filtration (CADR = 13.208 m³/min) present a substantially advantage over untreated air (CADR = 0.299 m³/min). Even so, the filtration was also effective on its own (CADR = 9.261 m³/min). Exclusive ionization did not have such a notable effect (CADR = 5.153 m³/h), although also reduced the aerial CFU concentration.

All the assays described here were performed under favorable conditions for air purification since there was no external dissemination of bioaerosols and the air was constantly recirculated. Of note, our findings highlight the importance of filtration and air cleaning in public transport. Importantly, the development and validation of new effective air purification systems may be essential for minimizing the spread of infectious diseases in the future and this field merits further research.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to acknowledge to Los Tranvías de Zaragoza S.E.M. and CAF Spain S.A. for requesting this project and their invaluable collaboration throughout it. Authors also thank to the IIS Aragón and Liftec (CSIC) for their support. Authors would also like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

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A scanning electron micrograph (SEM) showing a dense, intricate network of fine, fibrous strands. The fibers are thin and elongated, creating a complex, interwoven mesh structure. The lighting highlights the texture and three-dimensional nature of the fibers, with some appearing more prominent than others.

Capítulo 4.

Discusión general

La enfermedad por Covid-19 fue declarada como emergencia de salud pública a nivel mundial por la OMS en 2020. A pesar de los devastadores perjuicios sociales, económicos y sanitarios de la pandemia, la lucha contra este virus ha traído consigo un escenario científico sin precedentes, ofreciendo los mejores medios al servicio de la investigación e involucrando a decenas de miles de científicos a nivel mundial. No obstante, pese a los esfuerzos invertidos en estos dos años, todavía se desconocen algunos aspectos fundamentales del virus, como los relacionados con su ciclo celular o con sus mecanismos de transmisión entre humanos.

Primera temática:

Mecanismos de transmisión célula-célula

Los tiempos de publicación y las tasas de aceptación de artículos científicos fueron reajustados para hacer frente al ritmo de la pandemia, lo que supuso un arma de doble filo. Si bien esta estrategia resultó clave para compartir los avances con investigadores de todo el mundo, el reducido conocimiento sobre el virus en etapas iniciales fue un obstáculo para el cribado de manuscritos.³⁷⁹ En concreto, las publicaciones relacionadas con la interpretación ultraestructural errónea de las biopsias ha comprometido el conocimiento sobre la repercusión de la infección en el organismo. Por ejemplo, la continua confusión de las partículas virales de SARS-CoV-2 con estructuras celulares normales, ha llevado a la identificación equivocada del virus en diferentes órganos y tejidos y, por tanto, a un mal diagnóstico. En una carta al editor, Akilesh *et al.*³⁸⁰ apuntaron a cinco estructuras celulares que típicamente se confunden con virus envueltos, estos son las vesículas cubiertas de clatrina, las vesículas y gránulos secretores, las vesículas cubiertas de coatómero, los cuerpos multivesiculares y exosomas y las microvellosidades. En concreto, las vesículas recubiertas de clatrina representan las estructuras más frecuentemente malinterpretadas como partículas virales en TEM.³⁸¹⁻³⁸⁴ En las observaciones realizadas a propósito de esta Tesis Doctoral, encontramos que las vesículas recubiertas de clatrina medían entre 90 y 115 nm, mientras que hemos identificado el SARS-CoV-2 en tamaños comprendidos entre 60 y 80 nm, de acuerdo con otros autores que describen el tamaño del virión entre 70 y 90 nm.^{145,146}

El virus afecta predominantemente a células epiteliales y vasculares del tracto respiratorio, aunque también presenta tropismo por otras células

a nivel multiorgánico.^{70,385,386} La entrada a la célula ha sido principalmente descrita a partir de dos mecanismos diferenciados: mediante endocitosis mediada por clatrina y por fusión de membranas, que principalmente depende de la presencia de TMPRSS2.²² A pesar de que la entrada masiva a la célula ocurre en momentos iniciales de la infección, en esta Tesis hemos observado eventos de entrada viral a través de hoyuelos recubiertos de clatrina, pero no de fusión de membranas, lo que podría sugerir que esta vía es predominante, tal y como apuntan otros autores.^{387,388} El mecanismo de entrada a la célula podría ser determinante en la patogénesis viral, dado que la vía CME requiere necesariamente de la implicación del citoesqueleto para el transporte de los endosomas tempranos, lo que podría alterar el tráfico intracelular. En células miocárdicas, se ha sugerido que esta modificación en la distribución dinámica intracelular podría contribuir a la vulnerabilidad de pacientes con cardiopatías,³⁸⁹ aunque todavía se requiere de más investigación para analizar las implicaciones de los diferentes mecanismos de entrada.

Considerando la TEM como una herramienta clave para la interpretación de los mecanismos celulares, los estudios ultraestructurales han permitido identificar en gran medida, pero no en su totalidad, algunos eventos de vital importancia para la búsqueda de dianas terapéuticas y para la comprensión de los eventos sistémicos asociados al curso de la enfermedad por Covid-19. A grandes rasgos, la morfogénesis celular inducida por el virus se ha descrito con la formación de sincitios, la acumulación de gotas lipídicas, la proliferación de DMV, las alteraciones mitocondriales, el engrosamiento del RER y formación de filopodios como proyecciones de membrana.^{15,33,37,46-49} Los cambios inducidos por el virus son ultraestructuralmente detectables a partir de las 6 horas tras la infección, mientras que a las 8 horas se observan cambios masivos.⁵⁸

Encontramos sincitios en el cultivo infectado, como describen otros autores.⁴⁶ La formación de sincitios se ha correlacionado con una mayor gravedad de la enfermedad Covid-19 y con la linfopenia.^{52,390} Otro hallazgo interesante incluye la escasez de sincitios inducidos por la variante Ómicron,³⁹¹ lo que podría contribuir a la explicación de la sintomatología leve que caracteriza la infección con esta variante.³⁹² La reprogramación metabólica de lípidos y la formación de gotas lipídicas está relacionada con la enfermedad infecciosa, incluyendo la regulación metabólica, la respuesta inmunitaria innata o adaptativa y la señalización celular.^{62,393}

La remodelación de las estructuras intracelulares de membrana es inducida por el SARS-CoV-2 y otros virus para generar sitios de replicación viral. En concreto, el rol de las DMVs durante la infección contempla la protección física del RNA viral para su evasión inmunitaria y la posibilidad de replicación viral.^{33,394} En línea con estos autores, en esta Tesis hemos encontrado una marcada presencia de restos membranosos en células Vero E6 48 horas después de la infección. Se ha observado con frecuencia el ciclo de infección viral en diferentes fases, donde se han podido identificar diversos orgánulos membranosos de replicación y ensamblaje viral. En las células más afectadas, hemos encontrado que estos residuos de membrana ocupan una proporción significativa del citosol, lo que necesariamente impide la normal función celular. Otra de las características distintivas de estas células fue la ausencia de viriones de SARS-CoV-2, lo que sugeriría que la célula ya ha sido aprovechada para su replicación y ensamblaje. En ciclos virales fracasados, se han observado lisosomas con partículas virales en diferentes fases de degradación, como mecanismo para la eliminación del virus una vez internalizado en la célula.

Otro hallazgo ha consistido en la observación de alteraciones mitocondriales. Para evadir la respuesta innata del huésped, la alteración de mitocondrias resulta otra estrategia clave.^{394,395} La disfunción mitocondrial está relacionada con la reducción de la detección del oxígeno y, en consecuencia, la desregulación del oxígeno y del suministro de oxígeno sistémico, lo que podría representar un agravante en el déficit respiratorio del paciente.^{395,396} La constricción de la arteria pulmonar aparece como respuesta a la hipoxia patológica en vías respiratorias, lo que conduce a cambios en el flujo hacia los alvéolos.³⁹⁶ En los alveolos, además, se desarrolla una inflamación en grado variable que dificulta el intercambio de oxígeno y progresa hacia una insuficiencia respiratoria. El RNA viral vertido al contenido citosólico desencadena la secreción de interferonas (IFN-I y III) y citocinas proinflamatorias (interleucinas, IL-1 β e IL-18) como mecanismo antiviral que revierte en el reclutamiento de células inmunitarias reguladoras y efectoras. En tejido pulmonar, los macrófagos alveolares responden fagocitando células epiteliales infectadas y apoptóticas, induciendo la eliminación viral y secretando citocinas proinflamatorias y quimiotácticas (p.e., IL-6 e IL-8).³⁹⁷ A consecuencia de la secreción de citocinas y quimiocinas, las células inmunitarias son atraídas hasta el sitio de la infección, desencadenando un aumento de la relación neutrófilos/linfocitos. Los

mecanismos de respuesta de las células epiteliales y macrófagos alveolares inducen, a su vez, a la invasión de neutrófilos y células Natural Killer (NK, por sus siglas en inglés *Natural Killer*) y a la diferenciación de monocitos y células dendríticas en macrófagos que promueven la inflamación.^{398,399}

La formación de filopodios es otro de los signos distintivos de las células infectadas.⁴⁶ Las proyecciones de membrana juegan un papel relevante en el sondeo del entorno extracelular, la migración dirigida y los procesos de comunicación de célula a célula,⁴⁰⁰ lo que puede resultar clave para el desarrollo de la infección en el organismo.

El citoesqueleto celular desempeña un rol crucial en el ciclo de infección viral.⁴⁰¹⁻⁴⁰³ Tanto en la endocitosis como en la exocitosis del virus, la polimerización de actina cortical permite hacer frente a los requerimientos biomecánicos de la membrana celular.^{23,24} Tras su ingreso a la célula, los filamentos de actina y los microtúbulos con dineínas y quinesinas se reorganizan para permitir que los endosomas tempranos trasladen las partículas virales a los sitios de replicación.³¹ En otros coronavirus además, se ha descrito que la reorganización de los filamentos de actina en la región perimembranosa podrían respaldar la replicación del genoma y la síntesis de proteínas virales.⁴³ Sin embargo, como estructuras dependientes del citoesqueleto, la formación de conexiones intercelulares en la transmisión viral célula-célula para la progresión de la infección ha sido escasamente estudiada en general y esquiva en el caso del SARS-CoV-2.⁴⁰⁴⁻⁴⁰⁶

En esta Tesis hemos descrito la ultraestructura asociada a la formación de filopodios y de nanotubos de efecto túnel, así como los mecanismos de transporte viral por *surfing* y a través de la vía intracitoplasmática. Por un lado, los filopodios como vía de comunicación intercelular fueron inicialmente descritos para el virus linfotrópico T humano 1,⁴⁰⁷ y posteriormente ampliada otros modelos de virus envueltos.^{53,408,409} Sin embargo, todavía no habían sido descritos en profundidad para el virus SARS-CoV-2 y rara vez en conjunto en un mismo artículo. Se ha propuesto que las partículas virales podrían tener preferencia por la unión a la membrana filopodial respecto a otras regiones de la membrana por la presencia de receptores específicos que serían mayoritariamente expresados en esta superficie celular.^{410,411} En línea con lo anterior, en nuestro trabajo hemos observado una presencia significativamente mayor de partículas virales adheridas a la superficie filopodial respecto a otras regiones

de la membrana. Una vez los virus se unen a la membrana filopodial, el *surfing* viral entre células a su través representaría una vía de transmisión eficiente, donde las proteínas de la envoltura viral se unirían a receptores ACE2 físicamente conectados a los microtúbulos y transportarían el virus hasta regiones de la membrana huésped con alta capacidad endocítica.⁴¹²⁻⁴¹⁶ Por otro lado, los TNT fusionan las membranas de dos o más células. Estas estructuras nanométricas ofrecerían dos rutas alternativas: por un lado, al igual que en los filopodios, el *surfing* viral y, por otro lado, la ruta intracitoplasmática.⁵⁶ Sin embargo, en nuestro trabajo hemos identificado estas estructuras de manera aislada, lo que podría sugerir que la ruta prioritaria es el *surfing* viral a través de filopodios. Utilizando cortes seriados ultrafinos, realizamos la primera reconstrucción tridimensional de filopodios inducidos por la infección viral. Con este modelo, fue posible confirmar que los filopodios con altas cargas virales están asociados a la extrusión del virus.

Más allá de la formación de nuevas proyecciones de membrana, es probable que la alteración del citoesqueleto también interfiera en estructuras dependientes normales que se traduzcan en una manifestación clínica del paciente Covid-19. La pérdida ciliar ha sido ampliamente reportada tanto en SARS-CoV-2 como en otros CoV que le precedieron (como MERS-CoV o HCoV-229E) y se asocia con la internalización de los cilios.⁴¹⁷⁻⁴¹⁹ En consecuencia, la deciliación podría estar relacionada con la anosmia recurrente inducida por la infección por CoV en el epitelio respiratorio. Todavía existe incertidumbre a este respecto y la correlación entre el daño histológico y la función olfativa es motivo de debate.⁴²⁰ Compartimos la opinión de que este fenómeno de invaginación ciliar es similar al fenómeno de retracción filopodial, donde los filopodios celulares internalizan el virus a través de mecanismos de tracción de la membrana,^{54,55} por lo que no podría descartarse que ambos mecanismos estén relacionados.

Dada la importancia que adquiere el importante desarrollo de los filopodios frente a la infección viral y en la normal función celular, es fundamental conocer con mayor profundidad la interfase entre virus y filopodios para entender la patogenia viral.

Segunda temática:

Mecanismos de transmisión humano-humano

Al igual que ocurre en la propagación viral a nivel celular, la transmisión del SARS-CoV-2 humano-hu-

mano ha sido descrita a partir de diferentes mecanismos. Si bien las posibles rutas de transmisión del virus han sido ampliamente descritas, todavía se desconoce el papel que juega cada una de ellas en la propagación de la enfermedad. Hasta el momento, principalmente se contempla un modelo de diseminación directa, donde el individuo infectado expelle partículas contaminadas por eventos respiratorios, y un modelo de diseminación indirecta, donde los fómites sirven como vehículos de transmisión.^{117,118} Dada la baja probabilidad de que la diseminación indirecta sea una ruta principal en la transmisión del SARS-CoV-2,⁴²¹ esta Tesis se ha centrado en el modelo de propagación directa.

En la propagación directa es posible diferenciar entre las microgotas y los aerosoles, dependiendo de las características aerodinámicas de la partícula «biocargada». Antes de la pandemia la interfase entre ambos modelos de transmisión se estableció en 5 μm aunque, actualmente, a consecuencia de un mayor estudio sobre estos mecanismos de propagación se ha redefinido 100 μm .¹²² La vía aérea de transmisión, bien conocida como «airborne» ya había sido descrita para otros modelos de virus,^{131-133,137-143} incluyendo otros coronavirus.¹³⁴⁻¹³⁶ No obstante, este modelo fue erróneamente desestimado como uno de los principales mecanismos de propagación. En consecuencia, durante el primer año de la pandemia existió controversia sobre el potencial infectivo de la vía airborne a pesar de las crecientes evidencias de influencia en la curva de contagios.¹⁴⁴ En un breve análisis estadístico realizado como parte de esta Tesis concluimos una carga viral promedio de 3.1 ± 2.9 copias/ m^3 de aire en un total de 3085 muestreos (313 positivos; 10.15 %) a partir de trabajos previos publicados.^{149,150,159-168,151,169-171,152-158} En línea con lo anterior, en nuestro estudio hemos detectado alrededor de 1.1–4.8 copias/ m^3 en muestras aéreas del ambiente de pacientes infectados.

En la literatura, la carga viral reportada varía entre 5.0 y 11.9×10^3 copias/ m^3 ,⁷²⁻¹⁰² lo que sugiere que este valor es dependiente de la eficiencia de la técnica de recolección, de las condiciones y eventos fisiológicos del paciente y de las condiciones del muestreo, entre otras. Por tanto, resulta complejo identificar con precisión la carga viral aérea. En esta Tesis muestreamos el ambiente de 8 individuos contagiados con Ómicron BA.1 y vacunados con pauta completa utilizando un muestreador Coriolis μ (Bertin Instruments; Rockville, MD, USA) en condiciones similares, donde los participantes se encontraban en etapas iniciales de la infección,

con cargas promedio en frotis nasal de 24.6 ± 4.3 en el subgrupo A y de 25.7 ± 4.2 en el subgrupo C. Solo detectamos RNA viral por RT-qPCR (dianas *pan-SARS ESAR* e *IP4*) en la muestra de aire de 1 de los 8 individuos. A este individuo se le realizó un seguimiento de 2 días. Durante la primera jornada (3 días tras el inicio de los síntomas) se encontró una positividad del 68.8 % (11/16) con 3 muestras sospechosas ($Ct > 38$), mientras que en la segunda jornada (4 días tras el inicio de los síntomas) solo se detectó sospecha de RNA viral en el 14.8 % de las muestras (4/27). En las muestras ambientales del resto de individuos infectados no fue posible detectar RNA viral, incluso en condiciones de ventilación pobres (hasta 2400 ppm), cortas distancias (> 50 cm) y largos muestreos de aire (hasta 33000 l de aire). En los individuos infectados que se prestaron a realizar estos ensayos extraordinarios, el Ct fue de 19.1 y de 24.6 en el momento del muestreo, lo que supondría mayor carga viral que el paciente que diseminó partículas virales, con un Ct 27.1 el primer día y un Ct 29.2 el segundo. Lo cual sugiere que la capacidad de propagación viral del paciente no es dependiente de la carga viral que presente en las vías respiratorias altas, aunque sí del tiempo, dado que en el segundo día del muestreo los niveles de RNA viral en el aire fueron reducidos respecto al primer día.

Durante la tos se genera mayor concentración de bioaerosoles y de su tamaño aerodinámico respecto a otros eventos respiratorios, como la respiración y el habla,¹²⁹ que pueden estar altamente cargados de patógenos que colonizan en las vías respiratorias superiores, como es el caso del SARS-CoV-2.⁴²²⁻⁴²⁴ Por ejemplo, Viklund *et al.*²³⁹ encontraron el doble de muestras positivas en tos respecto a muestras de respiración en los mismos pacientes Covid-19. De manera que analizar la tos de los individuos es más eficiente que utilizar otras muestras de diseminación respiratoria. En esta Tesis, en los subgrupos B (Ct 25.7 ± 7.5) y C (Ct 25.7 ± 4.2) se tomaron muestras de tos a un total de 19 individuos para simular la capacidad de propagación de los pacientes en el modelo de transmisión por microgotas. Solo fue posible detectar RNA viral en el 10.5 % (2/19) de individuos con Ct 25.5 y 13.5, respectivamente. Se encontró una carga de 7.3×10^5 copias/ml (Ct 28.5) y 8.7×10^8 copias/ml (Ct 18.2), respectivamente. La hospitalización de uno de los pacientes impidió su seguimiento, pero el segundo paciente (Ct 25.5) pudo ceder muestras de tos los días posteriores, aunque solo se detectó carga viral en el primer día de muestreo. La dosis de virus detectada en las muestras fue superior a la detec-

tada en un estudio similar, donde se encontró un Ct 29.5 – 36.5 en pacientes con un Ct 17.2 – 26.4.²⁴²

No hemos encontrado correlación entre el Ct del paciente y su capacidad infectiva. Sin embargo, la correlación entre estas variables ha sido típicamente establecida y escasamente justificada, considerándose como un factor de predicción epidemiológica.^{105,425,426} Tres de nuestros pacientes con valores de Ct de 13.5, 15.8 y 16.7 dieron negativo para muestras de tos, mientras que otro individuo con un Ct de 23.2 emitió gotas cargadas de SARS-CoV-2 en los mismos términos. Incluso, detectamos RNA viral en frotis faríngeos de algunos pacientes que luego no pudimos identificar en sus muestras de tos. En línea con lo anterior, las muestras de aire solo resultaron positivas para un paciente con un Ct de 27.1, mientras que en otros pacientes con Ct 19.3, 21.2 y 23.2 no fue posible detectar RNA viral en el aire. Una explicación inmediata de disociación entre ambas variables podría ser que la cuantificación de la carga de RNA viral por RT-qPCR está sujeta a aspectos como la cantidad de muestra tomada, el kit utilizado, la diana detectada, la eficiencia del termociclador o el método de almacenamiento, entre otros.^{427,428} Sin embargo, en esta Tesis hemos utilizado muestreos normalizados para estandarizar, en la medida de lo posible, la toma de muestras. En cualquier caso, algunos de los kits que detectan más genes han reportado valores de Ct inferiores,⁴²⁹ por lo que creemos se requiere una interpretación individualizada de cada paciente y su implicación pronóstica y epidemiológica resulta cuestionable. No obstante, el estudio resulta limitado por el número de pacientes, por lo que estas conclusiones deben ser contrastadas con una muestra más amplia.

La superpropagación (**Fig.18**) podría explicar la heterogeneidad en la propagación de la enfermedad. Como ya ocurrió con el SARS precedente en 2003, los patrones de propagación no pueden ser explicados por modelos epidémicos convencionales, que asumen la homogeneidad de la transmisión. Una de las teorías relacionada con la heterogeneidad en la dinámica de la propagación es la regla 20/80, que atribuye el 80 % de infecciones secundarias a un 20 % de la población, por lo que la expansión masiva de la enfermedad estaría vinculada a un grupo de superpropagadores.^{251-254,265} En la actual pandemia, ese grupo de individuos representaría un 10–21 % sobre los casos totales.^{430,431} En esta Tesis hemos encontrado que, al menos el 18.2 % (4/22) de los individuos eran infecciosos (incluyendo un paciente en el que se detectó RNA

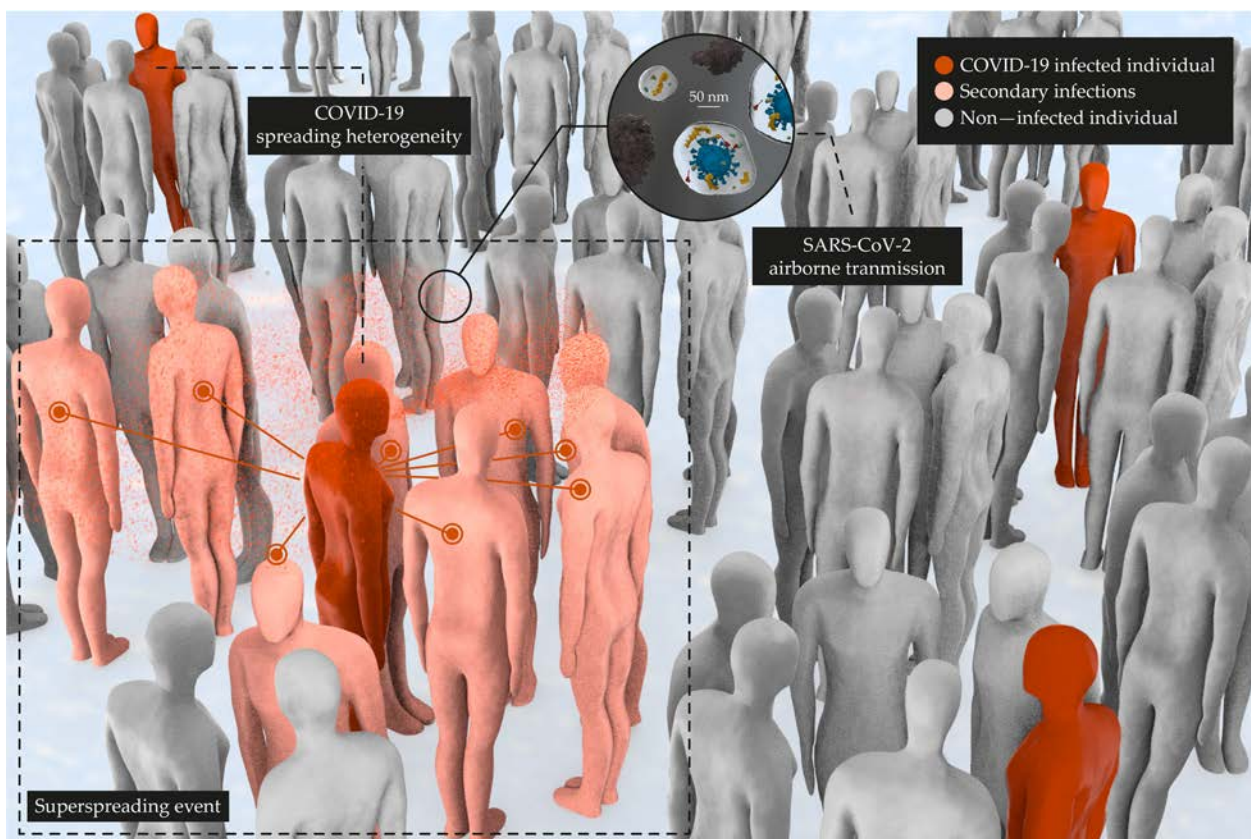


Figura 18. Abstracto gráfico del artículo *SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity*. Se observa esquemáticamente el modelo de superpropagación.

viral en la mascarilla utilizada en días anteriores), aunque no puede descartarse que el resto lo haya sido en otro momento de la enfermedad. Se ha estimado que alrededor de un 44 % de las infecciones secundarias provendrían de individuos en periodo de incubación,⁴³² lo que dificulta la investigación en este subtipo de pacientes al desconocer la presencia de la infección. En línea con lo anterior, no hemos encontrado individuos potencialmente contagiosos más allá de los 3 días tras el inicio de los síntomas, lo que podría contribuir en la definición de los periodos y protocolos de aislamiento, que han resultado claves para aplanar las curvas de contagios.⁴³³

Los individuos con capacidad infectiva identificados en nuestro estudio propagaron la enfermedad en su núcleo de convivencia, mientras que del resto no hemos registrado contagios secundarios. Aunque la superpropagación se ha atribuido a características fisiológicas del individuo, todavía se desconoce la causa de esta diseminación masiva,²⁶⁶ de manera que resultan difícilmente trazables.²⁵³ Por ejemplo, en un estudio se detectó RNA viral de SARS-CoV-2 en el 70 % de las muestras de aliento,²⁴¹ mientras que en otro estudio solo detectaron muestras de aliento positivas en RNA viral en el

14 % de los pacientes ($10^3 - 10^5$ copias/min).¹⁶³ Además, las tasas más altas se asociaron a pacientes en etapas tempranas de la infección por SARS-CoV-2, en línea con lo anterior. En paralelo, una teoría propone que los superpropagadores podrían ser fruto de un contagio secundario de otro propagador.²⁶³ En esta Tesis, encontramos que el individuo que diseminó RNA viral en el aire contagió a su núcleo familiar completo ($n = 3$), incluyendo a su pareja que, a su vez, contagió a otro núcleo familiar completo ($n = 4$), lo que podría respaldar las suposiciones anteriores. En contraposición, no se registró ningún contagio secundario en los pacientes que fueron positivos en muestras de tos, aunque esto pudo ser dependiente de las medidas de prevención utilizadas en los diferentes escenarios, por lo que no podemos sugerir una relación entre los aerosoles y la superpropagación.

El tiempo de exposición es otro factor clave para determinar el riesgo de contagio. La carga viral contenida en los bioaerosoles emitidos por los pacientes es crucial para determinar la contribución relativa de la transmisión aérea.¹²² Sin embargo, la diseminación viral puede también ser variable en el tiempo y estar condicionada a determinados momentos. Estimar la probabilidad de contagio de

un individuo es complejo. Por un lado, el concepto de dosis infectiva mínima puede conducir a malinterpretaciones ya que, aunque con menor probabilidad, potencialmente una sola copia de virus podría iniciar la infección en un individuo expuesto. Por otro lado, la carga viral encontrada en aire o toses no necesariamente implica que el virus sea viable. Es decir, es posible detectar RNA viral por RT-qPCR proveniente de virus inactivos, por lo que no tendrían capacidad infectiva. Incluso, existe la probabilidad de que esos bioaerosoles con carga viral persistente no sean respirados por el individuo. En cualquier caso, asumiendo una carga viral persistente promedio de 3.1 ± 2.9 copias/m³, una concentración homogénea de estas partículas en suspensión y un TCID₅₀ de 7.0×10^2 copias, a un flujo respiratorio de 15 l/min, la total probabilidad de infección se alcanzaría a los 15 minutos de la exposición. En paralelo, atendiendo a las cargas virales encontradas en muestras de tos, sería equivalente a respirar el ambiente contaminado durante 10 minutos.

A pesar de los avances conseguidos hasta el momento, todavía se requiere una comprensión más amplia sobre el papel de la variación individual en la transmisión de los virus respiratorios. Los resultados de esta Tesis sugieren una significativa heterogeneidad entre individuos, aunque todavía no se ha identificado su origen. Hemos tratado de cuantificar matemáticamente el riesgo de contagio del modelo de microgotas frente al modelo airborne, aunque concluimos que es algo difícilmente parametrizable. No obstante, los resultados de esta Tesis están limitados a un bajo número de pacientes, por lo que sería necesario contrastar las conclusiones aquí mostradas en estudios más amplios. Las microgotas predominan en el contacto estrecho, dada su mayor concentración y tamaño, mientras que el riesgo aumenta con la vía aérea por su capacidad de propagación a larga distancia y su difícil manejo. Dado lo expuesto, resulta imperativo establecer medidas de prevención que prevengan de futuras pandemias.

Tercera temática: Medidas para la reducción de la transmisión del SARS-CoV-2 por aerosoles

(Medidas de prevención individual)

El uso de mascarillas a nivel global ha representado una estrategia clave para el control de la pandemia, reduciendo hasta en el 70 % de los potenciales contagios por SARS-CoV-2.⁴³⁴⁻⁴³⁶ Sin embargo, no todas las tipologías de mascarilla disponibles

ofrecen el mismo rendimiento en la captación del virus. La exigencia de las normas UNE:EN 149 (medias máscaras filtrantes) y UNE:EN 14683 (mascarillas quirúrgicas) no es comparable entre sí. Mientras que las medias máscaras exigen rendimientos superiores al 95 % frente a partículas polidispersas con un diámetro promedio de 0.6 µm, las mascarillas quirúrgicas únicamente deben retener al menos el 95% de bacterias con un diámetro promedio superior a 1 µm.

La eficiencia real de las mascarillas y medias máscaras dista enormemente de su valor teórico, dado que un ajuste facial compacto es esencial para preservar la capacidad del tejido. En este sentido, las medias máscaras teóricamente se diseñan para proporcionar un ajuste facial perfecto, aunque en la práctica es cuestionable.⁴³⁷⁻⁴³⁹ Un reporte realizado por el CDC donde se evaluaron 21 medias máscaras filtrantes FFP2 en 25 voluntarios reveló fugas de entre el 6 y el 88 % de partículas.⁴⁴⁰ En la misma línea, Park *et al.*⁴⁴¹ reportaron fugas de hasta el 21 % en mascarillas FFP2 de 3M y de hasta el 73 % en mascarillas FFP2 de origen coreano. En investigaciones que hemos realizado de manera complementaria a esta Tesis, se evaluó el rendimiento global que ofrecen las máscaras, incluyendo la variable del ajuste facial utilizando un maniquí. En el caso de la FFP2, las fugas representaron el 30 % del total de partículas, mientras que en la FFP3 no alcanzaron el 3 %. La elevada fuga hacia el interior que presentaron las máscaras FFP2 estuvo asociada a la forma de «pico de pato», ya que las mascarillas FFP3 utilizadas, donde se incluía una banda de espuma para el ajuste nasal y cintas de sujeción completas, mostraron fugas mínimas y un resultado similar al sellar la superficie. En un trabajo realizado analizaron el *fitting* facial de mascarillas FFP2, encontrando una caída pronunciada de la eficiencia en las mascarillas con forma de «pico de pato», incluyendo notable variabilidad interpersonal y equiparándose a la protección de una mascarilla quirúrgica. En concreto, apuntaron a la forma de «pico de pato» como la tipología de mascarilla con menor ajuste facial independientemente del individuo.³³³ En esta Tesis, la mascarilla FFP2 mostró un rendimiento ligeramente mejorado en el maniquí más pequeño, lo que demuestra que la eficiencia en la retención de partículas depende sustancialmente de las características faciales del individuo. Por el contrario, como era esperable, se observaron fugas cercanas al 80 % en modelos con barba prominente, donde el ajuste resultaba comprometido. Por su parte, las mascarillas quirúrgicas no están diseñadas para adaptarse a la cara y, por tanto,

presentaron pérdidas próximas al 30 %. En condiciones similares a las de nuestros ensayos, Rengasamy *et al.*⁴⁴², Chen *et al.*⁴⁴³, Obeng *et al.*⁴⁴⁴ y Weber *et al.*⁴⁴⁵ encontraron fugas cercanas al 60-80 % en mascarillas quirúrgicas.

A pesar de sus limitaciones, en la práctica el uso de mascarillas quirúrgicas ha ganado popularidad dada su alta respirabilidad (baja pérdida de carga), precio, disponibilidad y escaso ajuste facial, que se traduce en una mayor comodidad para el individuo. Numerosos esfuerzos investigadores se han orientado a aumentar su capacidad protectora frente al SARS-CoV-2. En concreto, la modificación de materiales preexistentes utilizando nanomateriales se ha posicionado como una de las opciones más atractivas para dotarlas de capacidad antiviral.

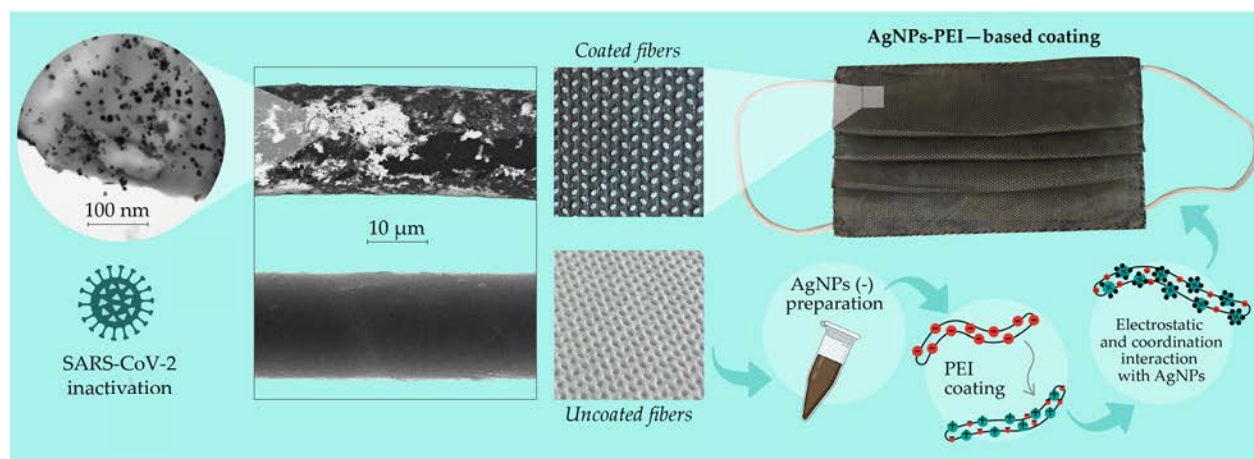
La deposición de nanorecubrimientos sobre fibras poliméricas del material filtrante ha recibido especial atención a lo largo de la pandemia. Por sus propiedades, los óxidos metálicos nanoestructurados se han posicionado históricamente como epicentro de las estrategias antisépticas, incluyendo materiales como el cobre, la plata, el grafeno o el zinc.⁴⁴⁶ Específicamente, la plata se ha incorporado en superficies de diversa tipología por sus marcadas propiedades antimicrobianas y por estar aprobado por la FDA en entornos clínicos.^{447,448} La reducción de su tamaño hasta la escala nanométrica, en forma de nanopartículas de plata (AgNPs), muestra una ventajas evidentes sobre su análogo en una escala métrica por su alta relación superficie-volumen y liberación iónica a largo plazo.^{449,450}

Conforme disminuye el tamaño de nanopartícula, aumenta su potencial antimicrobiano.⁴⁵¹⁻⁴⁵³ En consecuencia, durante la síntesis de nanopartículas en esta Tesis, se optimizó el procedimiento propuesto por Goli *et al.*⁴⁵⁴ hasta reducir el tamaño de las partículas de 12.3 ± 2.9 nm a 6.2 ± 2.6 nm.

El comportamiento antimicrobiano de la plata ha sido ampliamente demostrado frente a diferentes cepas bacterianas,⁴⁵⁵⁻⁴⁵⁸ pero escasamente estudiado frente a virus.^{459,460} Aunque se han propuesto algunos mecanismos que explicarían la inactivación del SARS-CoV-2 inducida por la nanoplate, todavía no existe un consenso.⁴⁶¹ Sin embargo, ese aspecto se escapa del alcance de esta Tesis y pocos trabajos publicados lo discuten. Utilizando este CoV, se han obtenido altas tasas de inactivación^{462,463} y más elevadas en relación a otros metales nanoestructurados,⁴⁶⁴ por lo que su posible explotación en este contexto resulta de interés.

En el planteamiento inicial de este proyecto, el recubrimiento de mascarillas basado en plata resultaba novedoso para el SARS-CoV-2 (Fig.19). Sin embargo, en línea con otras áreas de investigación, fruto de la necesidad han surgido diferentes iniciativas con esta misma finalidad. Una revisión apunta a que solo el ~50 % de las mascarillas comerciales que aseguran contenido en nanoplate presentan una capacidad antimicrobiana eficiente.⁴⁴⁸ En esta Tesis, a partir de AgNPs se ha desarrollado un recubrimiento de material filtrante que presenta un elevado rendimientos de inactivación: del 98.0 al 99.9 % a los 10 y 120 minutos tras la exposición al virus. Otras mascarillas basadas en plata pero for-

Figura 19. Abstracto gráfico del artículo *Silver Nanoparticles-Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: A New Method to Functionalize Filtration Media*. Se observa esquemáticamente representado el método de unión de AgNPs a fibras de PP.



muladas con otros compuestos han demostrado eficiencias cercanas al 100 %. Valdez-Salas *et al.*⁴⁶⁵ conjugaron la nanoplata con surfactantes para su unión a las fibras de polipropileno y reportaron eficiencias superiores al 99 % frente a otros modelos de virus y bacterias. En combinación con óxido de titanio, las máscaras desarrolladas por Li *et al.*⁴⁴⁷ redujeron la concentración de *E. coli* y *S. aureus* en un 100 %. Hamouda *et al.*⁴⁶⁶ unieron la plata a las fibras a partir de enlaces iónicos, aunque el tamaño de sus partículas (de entre 6 y 70 nm, mayoritariamente entre 10 y 20 nm) unido a su escasa concentración en el tejido, logro inactivar el SARS-CoV-2 en un porcentaje inferior al 50 %.

La estabilidad en la unión de las nanopartículas a las fibras es primordial para evitar su desprendimiento hacia la vía respiratoria, aunque no se ha contemplado en otros estudios.^{447,465,466} El desarrollo de esta Tesis consiste en un recubrimiento cargado con ~0,5 mg/cm² de AgNPs en combinación con un polímero catiónico conocido como polietilenimina, cuya unión es dependiente de fuerzas electrostáticas y de complejos de coordinación. De esta manera, ha sido posible generar una unión estable. Frente a condiciones de sonicación agresivas, se han observado pérdidas de nanoplata de hasta el 30 %p/v en baños de ultrasonidos. En este sentido, en la literatura se han identificado pérdidas de entre el 48 y 75 % en lavados convencionales.^{467,468} Frente a 30 lavados manuales, nuestro recubrimiento mostró pérdidas del 1 % e inferiores, por lo que la mejora en la estabilidad de esta nueva formulación resulta notoria. No obstante, dada la toxicidad de las AgNPs para el organismo humano, en un trabajo futuro será preciso evaluar la estabilidad de la unión frente a corrientes de aire que simulen su aplicación real.

Dada la imperativa necesidad del uso de mascarillas para el control de la pandemia, en numerosos países han sido establecidas como obligatorias a lo largo de diferentes periodos de la pandemia. Lo que a mediados de 2021, según estimaciones de la Agencia Europea del Medio Ambiente, ha supuesto un desperdicio de 170000 toneladas de mascarillas faciales de un solo uso que equivaldrían a un potencial de calentamiento global adicional de 2.4 a 5.7 millones de toneladas de CO_{2,eq}.⁴⁶⁹ Bajo este escenario, la búsqueda de alternativas más sostenibles representa una estrategia clave para reducir el impacto ambiental.

Las mascarillas higiénicas reutilizables se presentan como un sustituto atractivo frente a las masca-

rillas quirúrgicas. En concreto, se ha estimado que las mascarillas reutilizables generarían un impacto 3.5 veces menor sobre el cambio climático.⁴⁷⁰ Sin embargo, esta tipología de mascarilla presenta una limitada eficiencia de filtración, lo que compromete su rendimiento frente al SARS-CoV-2. Un estudio apuntó a penetraciones de entre el 9 y 98 % frente a partículas de entre 0.02 y 1.0 µm.⁴⁷¹ A pesar de la variabilidad entre mascarillas dependiendo de su ajuste, materiales y capas, en estudios no publicados de esta Tesis hemos encontrado eficiencias inferiores al 25 % en todos los casos frente a partículas de entre 0.01 y 1.0 µm, de acuerdo con estudios anteriores.

El desarrollo de mascarillas con estrategias de reducción del impacto medioambiental representa una estrategia atractiva para paliar con las limitaciones descritas anteriormente. En consecuencia, en el marco de esta Tesis desarrollamos materiales filtrantes basados en nano y microfibras de tereftalato de polietileno (PET) proveniente de botellas desechadas. La tecnología de electrohilado permite obtener nanofibras, que por su elevado ratio superficie-volumen y su reducido diámetro, permite generar tejidos fibrosos con alta porosidad para reducir la pérdida de carga y aumentar el rendimiento de filtración, características que se han aprovechado para la producción de mascarillas.^{313,314,472} En nuestro trabajo, estudiamos la relación entre los diámetros de fibra y su capacidad de retención de partículas submicrónicas. Tal y como había sido descrito anteriormente,^{313,473,474} observamos una fuerte correlación entre el diámetro de las fibras y su eficiencia del filtrado, siendo ésta una relación inversa. El parámetro que permitió obtener cambios en el diámetro de fibra más reproducible fue la proporción de polímero (de 15 a 25 %p/v) en la disolución, tal y como respaldan otros autores.^{317,321} En las muestras con fibras más pequeñas (0.3 ± 0.2 µm) se obtuvieron eficiencias superiores al 97 % frente a partículas de entre 0.01 y 1.0 µm, mientras que en las fibras de mayor diámetro (1.54 ± 0.84 µm) se redujo hasta el 83 %. En contraposición, la pérdida de carga aumentó conforme se redujo el diámetro de fibra, dado que el tamaño del poro fue menor, desde 1.1 mbar (en las fibras de mayor diámetro) hasta 1.9 mbar (en las de menor tamaño). Para maximizar la retención y la respirabilidad, se consideraron dos estrategias diferenciadas. Por un lado, aumentar la densidad de la membrana y, por otro lado, combinar fibras finas y gruesas en un mismo tejido. Con estos resultados, se obtuvieron gráficas con las que fue posible diseñar materia-

les filtrantes a medida. Hasta la fecha, solo hemos encontrado dos trabajos similares. En el primero, desarrollado por Opálková *et al.*⁴⁷⁵ los autores no investigaron el potencial del PET para el desarrollo de materiales de eficiencia variable y se limitaron a evaluar una sola membrana. En el segundo, Bonfim *et al.*⁴⁷⁶ estudiaron el comportamiento de tres membranas basadas en PET frente a partículas de 3 a 300 nm, lo cual no es representativo de su capacidad filtrante a otros rangos de partícula, por lo que su estudio resulta limitado. Que se sepa, el estudio que nosotros presentamos es el más completo en relación al desarrollo de materiales filtrantes electrohilados basados en PET y el estudio de su comportamiento en la filtración de partículas en la región sub y super-micrométrica.

Para concluir el trabajo, se optimizó la fórmula para desarrollar una mascarilla quirúrgica basada en tres capas similares a las de una máscara convencional. Las dos capas externas estuvieron compuestas por fibras gruesas ($3.2 \pm 2.6 \mu\text{m}$) heterogéneas que fueron diseñadas para reducir la pérdida de carga, aumentar la resistencia de la mascarilla y proteger el medio filtrante. La capa filtrante, por su parte, fue sintetizada con fibras de $1.24 \pm 0.7 \mu\text{m}$ a una densidad de $\sim 20 \text{ mg/cm}^2$, que se traduce en un espesor de $\sim 500 \mu\text{m}$. Frente a partículas de $0.5\text{-}10 \mu\text{m}$, presentó una eficiencia del 98.2 %, mientras que frente a partículas de $3.0 \mu\text{m}$ (amparada bajo la norma UNE EN 14683) la captación fue del 100 %, con una pérdida de carga de 0.36 mbar. En comparación con una mascarilla quirúrgica comercial, este tejido a base de PET mejoró la capacidad de retención de partículas finas y gruesas y presentó una hidrofobicidad similar en todas las capas. En otros trabajos con objetivos similares se ha buscado el uso de materiales biodegradables como sustituto de los materiales bioestables actualmente utilizados para la producción de mascarillas.⁴⁷⁷ Algunos de los materiales propuestos han estado basados en proteínas,^{478,479} celulosa,⁴⁸⁰⁻⁴⁸³ quitosano,⁴⁸⁴ o PLA,⁴⁸⁵ entre otros. Sin embargo, el reaprovechamiento de materiales desechados para la producción de mascarillas ha sido poco explotado. Zhao *et al.*⁴⁸⁶ combinaron el PET con fibras de elastómero de poliéster termoplástico para la producción de mascarillas con objetivos diferentes al reaprovechamiento del material.

La característica diferencial del material desarrollado en esta Tesis es su capacidad de re-electrohilarse indefinidamente sin perder propiedades, lo que podría traer consigo una estrategia de economía circular que redujese el impacto ambiental.

A pesar de que las mascarillas y los equipos de protección respiratoria han sido cruciales para el manejo epidemiológico de la pandemia, su uso viene dado por la responsabilidad individual de los usuarios y, probablemente, tenga un carácter temporal vinculado a la Covid-19. Esto, unido a la creciente necesidad de medidas preventivas para reducir la transmisión aérea de enfermedades, hace necesario implementar estrategias a largo plazo que no dependan del interés individual. Y, para potenciar la bondad de las medidas individuales, realizar campañas de formación, sensibilización e información para el público general.

(Medidas de prevención colectiva)

A pesar del incesante control de la transmisión de enfermedades vehiculizadas a través de la comida («foodborne») y el agua («waterborne») y los exigentes estándares sobre los que se sustenta,⁴⁸⁷ todavía no existe una regulación que vele por la salubridad del aire ni por la reducción de la transmisión de enfermedades «airborne». En esta pandemia, esta ruta de transmisión se ha establecido como una de las predominantes dada la creciente investigación a este respecto.¹²² Por tanto, las estrategias que fomentan el control y la mejora de la calidad del aire deberían permanecer en el tiempo como estrategia preventiva, tratando de ser diseñadas buscando la máxima compatibilidad con las políticas de eficiencia energética definidas en cada momento.

La medición del CO_2 metabólico ha representado una medida eficiente para controlar la calidad del aire interior, dado que la medida de su concentración actúa como indicador indirecto de la presencia de aerosoles potencialmente patógenos.^{360,372} Estableciendo un rango aceptable entre 700 y 1000 ppm,^{373,374} este recurso sencillo y a bajo costo se ha extendido a diferentes entornos y configuraciones, trayendo consigo beneficios y desventajas.³⁵⁸ Por un lado, la instalación de medidores en centros comerciales,³⁶³ en transporte público,⁴⁸⁸⁻⁴⁹⁰ en oficinas³⁶⁴ y en instituciones educativas³⁶⁴⁻³⁶⁹ permite ventilar los espacios con criterio y de manera más eficiente, lo que redundará en una reducción del riesgo de transmisión de la enfermedad infecciosa.⁴⁹¹

En esta línea, algunos gobiernos han intensificado las inversiones en medidores de CO_2 para el control independiente del aire en escuelas y centros educativos.³⁷⁵⁻³⁷⁷ Como contrapunto, frente a una creciente implementación de esta tecnología, re-

sulta imprescindible la unificación y la estandarización de un método para evitar el mal uso o la malinterpretación de los niveles de CO₂ metabólico, que podría traer como consecuencia la infraestimación del aire en los espacios.

A pesar de que existen iniciativas internacionales, como Aireamos,³⁷¹ grupo de científicos que auna su esfuerzo para promover una medición e interpretación eficiente del nivel de CO₂, es necesario establecer un consenso internacional que regule esta práctica. Dado lo anterior, uno de los objetivos de esta Tesis se ha dedicado al desarrollo y validación de un protocolo para el análisis, interpretación y monitorización de CO₂. En concreto, proponemos un método basado en tres fases: diagnóstico, corrección y monitorización, control y seguimiento (MCS, por sus siglas en inglés *Monitoring, Control, and Surveillance*). La primera fase de diagnóstico (2-3 días) permite valorar cómo varían y evolucionan los niveles de concentración de CO₂ en función de las actividades concretas que se desarrollan en el espacio. Considerando los hallazgos en el diagnóstico, se procede a diseñar protocolos y procedimientos personalizados para mejorar la renovación del aire en una fase de corrección. En la implementación de medidas correctoras, la ventilación natural es una de las estrategias prioritarias para mejorar las renovaciones de aire.^{362,492} La metodología finaliza con la fase MCS, donde se propone la monitorización de CO₂ continuada en el espacio con el objetivo de garantizar la calidad del aire a largo plazo. Una de las principales ventajas de este método es que el responsable del establecimiento puede visualizar la medición de CO₂ y actuar en consecuencia. Hasta el momento, no se han publicado metodologías que unifiquen la implementación de la medición de CO₂ en espacios compartidos. Antes de la pandemia, la interpretación del nivel de CO₂ ya era utilizada como un indicador de la calidad del aire interior para el diseño de edificios. Sin embargo, no existe un procedimiento estandarizado que regule esta práctica.⁴⁹³

El método propuesto en esta Tesis fue diseñado en estudios previos en centros de formación, colegios y residencias de la tercera edad, y finalmente validado en 40 comercios locales en Zaragoza (España) utilizando 85 medidores y durante un periodo de 4 meses. De entre ellos, 23 presentaron niveles de CO₂ por encima de los recomendado en tiempos de pandemia (800 ppm) en la fase de diagnóstico. Encontramos que 8 de ellos solo aumentaron este valor puntualmente, mientras que los 15 restantes fueron sujeto de una evaluación exhaustiva en la

fase de corrección para mejorar su renovación del aire interior. En 5 de ellos, gracias a la cooperación de los comercios, fue posible implementar nuestra metodología que redujo la concentración de CO₂ por debajo del límite establecido mediante nuevos protocolos de ventilación natural. Otros 10 comercios no se implicaron lo suficiente en el proyecto y, en consecuencia, no mejoraron la calidad de su aire interior. La tipología y actividad en el comercio afectó a la calidad de aire interior. En este aspecto, los establecimientos estudiados con mejores indicadores de CO₂ fueron las floristerías y las zapaterías. Por otro lado, los de restauración y los comercios de ropa infantil constituyeron los espacios con peores tasas de renovación de aire, pudiendo ser debido a un mayor tiempo de residencia o a una actividad más intensa dentro del establecimiento. Dado lo anterior, se pone de relieve que las pautas para la corrección del aire deben ser individualizadas al espacio concreto.

Dado que el transporte colectivo representa el segundo entorno con mayor tasa de transmisión de la Covid-19,⁴⁹⁴ otra acción enmarcada en esta Tesis estuvo orientada a la evaluación y a la mejora de la calidad del aire interior del tranvía de Zaragoza (Fig.20). En valores absolutos de CO₂ la media máxima fue de 835 ±232 ppm, alcanzando un valor máximo de 1229 ppm. Como era esperable, encontramos que el incremento de CO₂ (Δ CO₂) era dependiente del número de personas en su interior;⁴⁹⁵ observando niveles aumentados en la zona centro de la ciudad. Además, la renovación de aire interior varió sustancialmente dependiendo del clima. En el subconjunto estudiado de días con mayor viento se observaron reducciones de entre el 31 y 44 % en la acumulación de CO₂ metabólico. En otros trabajos no se ha encontrado una influencia notoria,⁴⁹⁶ aunque puede ser debido a cambios menos bruscos en las condiciones atmosféricas. Dado lo anterior, propusimos el uso del indicador ppm/persona para analizar la renovación del aire independientemente de las condiciones externas.

Además de la implementación de la apertura parcial de ventanas, se buscaron medidas secundarias que fomentasen las renovaciones de aire interiores. Entre ellas, el porcentaje aire recirculado en el tranvía es un aspecto crítico para la calidad del aire interior.⁴⁹⁷ En esta Tesis, limitar los retornos de aire al 50 % mostró una reducción de entre el 9 y 36 % del nivel de CO₂ interior. Si bien los sistemas HVAC permiten la limpieza mecánica de grandes partículas (polvo o polen, entre otros) en el aire, los filtros del tranvía no mostraron un comportamiento

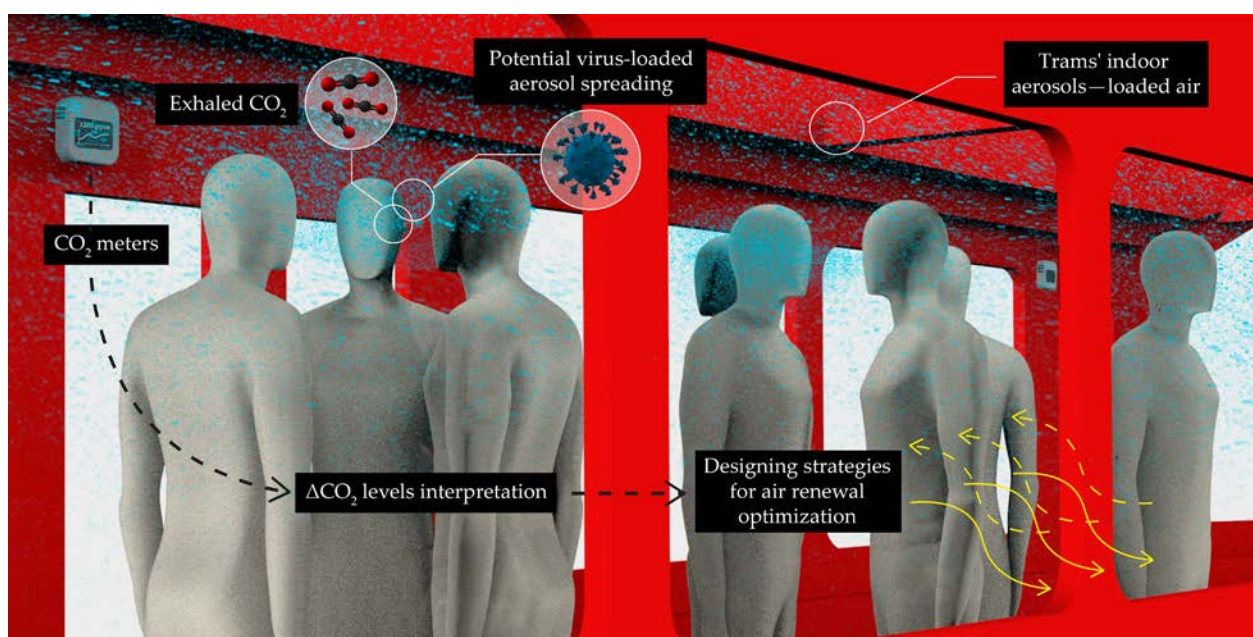


Figura 20. Abstracto gráfico del artículo *The Control of Metabolic CO₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases*. Se observa esquemáticamente representado el concepto de medición de CO₂ en el tranvía de Zaragoza.

aceptable frente a materia submicrónica, como es el caso del SARS-CoV-2. De manera que, el cierre del retorno del aire debería ser más beneficioso que perjudicial bajo las condiciones estudiadas.

La presencia de otros sistemas de limpieza o de purificación del aire, como los equipos de filtración, pueden generar un desajuste en la relación entre la potencial carga viral y el nivel de CO₂ metabólico. Este es un aspecto que todavía no ha sido ampliamente descrito en literatura y representa una casuística que debe ser considerada. En el tranvía de Zaragoza se evaluó el potencial rendimiento de equipos de ionización bipolar PA604 (Tayra SA; Alicante, España) frente a bioaerosoles ambientales, principalmente provenientes de la microbiota humana (p.e. *Roseomonas mucosa*, *Kocura rhizophila*, *Dermaococcus nishinomiyaensis* o *Staphylococcus haemolyticus*, entre otros). Frente a una concentración de 19.9×10^9 - 31.5×10^9 iones/m³, se cuantificó una reducción de Unidades Formadoras de Colonias (CFU, por sus siglas en inglés *Colony-Forming Units*) viables del 45.9, 61.8 y 69.2 % tras 30, 60 y 90 minutos respectivamente (**Fig.21**). El rendimiento encontrado frente a bacterias fue inferior al reportado en condiciones de laboratorio,^{349,351,353} pero fue consistente con los resultados publicados por Licht *et al.*³⁵⁵ utilizando condiciones similares en aviones, con una reducción de entre el 20.7 y el 60.0 % tras 60 minutos de ionización.

Por otro lado se estudió la eficiencia de los filtros implementados en el sistema HVAC en relación a la limpieza mecánica de bioaerosoles, identificando una reducción de CFU en un 73.4, 84.0 y 92.0 % tras 30, 60 y 90 minutos de funcionamiento. Sin embargo se observó que las 15 cepas bacterianas más frecuentes presentaron un tamaño promedio superior a 0.9 μm , por lo que el ensayo podría no ser representativo frente a SARS-CoV-2 y otros virus. Para corroborarlo, el filtro fue ensayado frente a condiciones simuladas en laboratorio, donde se observó una capacidad de retención inferior al 12 % frente a partículas de 300 nm. Otro efecto de la ionización es la promoción de la aglomeración de aerosoles a través de mecanismos electrostáticos,³⁵⁷ por lo que el incremento del tamaño aerodinámico de las partículas debería mejorar el rendimiento de la filtración. Sin embargo, en nuestro trabajo no observamos un rendimiento mejorado de la capacidad filtrante, dado que la reducción de CFU fue del 74.2, 82.8 y 94.1 % tras 30, 60 y 90 minutos, lo que pudo ser debido a un efecto de agregación predominante en partículas ultrafinas.³⁵⁷

No obstante, la purificación del aire a través mecanismos físico-químicos puede no estar exenta de generar subproductos nocivos para el ser humano.³⁵⁶ Aunque es una cuestión que no se discute en profundidad en esta Tesis, creemos que la eliminación de aerosoles por mecanismos de filtración

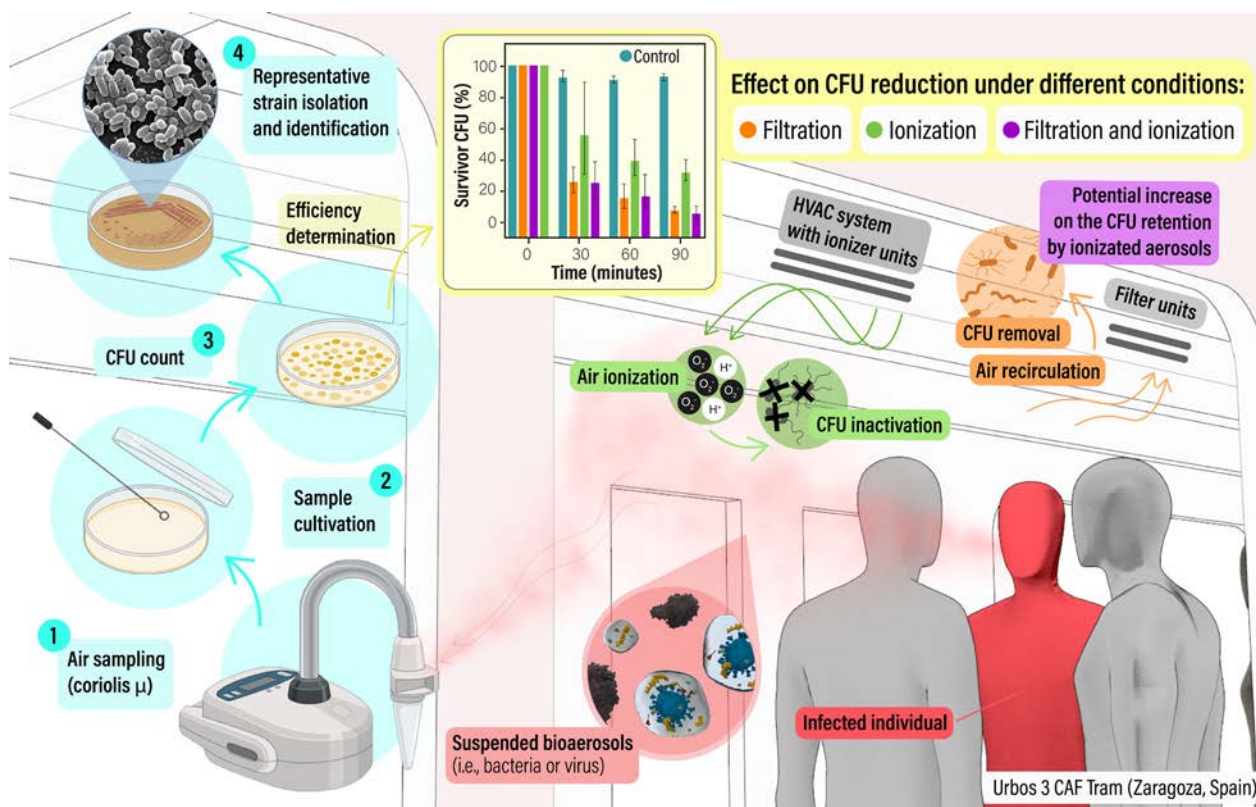


Figura 21. Abstracto gráfico del artículo *Impact of need-le-point bipolar ionization system in the reduction of bioaerosols in collective transport*. Se observa esquemáticamente representado el funcionamiento del sistema de ionización bipolar en el tranvía de Zaragoza y el método utilizado para su validación frente a bioaerosoles ambientales.

se presenta como una alternativa ventajosa frente a la ionización, los sistemas UVC o el ozono. Mientras que el potencial de acción de estos métodos depende de su acción en los componentes intracelulares de los microorganismos (como el DNA, el RNA o las proteínas) y puede no ser igualmente eficiente frente a cualquier especie,^{338,347,348} la retención de los aerosoles en filtros depende mayoritariamente de su tamaño y no es dependiente de la resistencia de los patógenos.^{311,323} Si bien en los equipos de protección respiratoria resulta tan importante la capacidad de filtración inherente al material como su ajuste facial, en los equipos de filtración de aire es igualmente crucial el volumen de aire por hora que son capaces de gestionar. Por ejemplo, el sistema HVAC del tranvía de Zaragoza funciona a un caudal 2800-3300 m³/h, lo que equivale a 25 ACH. Frente a condiciones similares a estas, el filtro convencional (Coarse 45 %) fue capaz de retener <12 % de las partículas de 300 nm, mientras que el filtro instalado a propósito de la pandemia (Coarse 75 %) solo mejoró su capacidad hasta < 28 % frente a partículas del mismo tamaño. Sin embargo, posiblemente aumentar las ACH no sea una opción para mejorar la captación

de aerosoles dado que la velocidad del aire en el filtro es una variable que afecta notoriamente a la capacidad de filtración en sentido inverso.^{311,323} De manera que resulta imprescindible considerar las características del espacio a intervenir para proponer sistemas de purificación del aire efectivas.

No obstante, la tarea de purificación del aire no puede atajarse utilizando dispositivos electrónicos no probados, sino que los sistemas a implementar deberían demostrar su efectividad en el entorno en el que van a ser instalados.⁴⁹⁸

Las estrategias de control de salud pública se han modulado ajustándose a la predominancia y a la prevalencia de los modos de transmisión de enfermedades. Uno de los avances más relevantes que ha traído consigo la pandemia por Covid-19 es la aceptación global de la predominancia de la transmisión aérea de los patógenos respiratorios. Dado lo anterior, cabría esperar que la reconsideración de esta vía de propagación indujese a la reformulación de los estándares exigibles de calidad de aire interior, especialmente en espacios interiores y compartidos.

The background of the slide is a high-magnification electron micrograph showing the cellular ultrastructure of Vero E6 cells infected with SARS-CoV-2. The image displays various organelles, including mitochondria with visible cristae, and numerous small, dark, electron-dense viral particles scattered throughout the cytoplasm. A semi-transparent white rectangular box is overlaid on the left side of the image, containing the chapter title.

Capítulo 5. **Conclusiones**

Conclusiones

Sobre la primera temática:

Mecanismos de transmisión célula-célula del SARS-CoV-2

1. Mediante microscopía electrónica de transmisión se observó que la formación de proyecciones de membrana, en forma de filopodios o de nanotubos de efecto túnel, viene dada por la remodelación del citoesqueleto asociada a la infección por SARS-CoV-2. La transmisión célula-célula del virus está principalmente mediada por el *surfing* y, en menor proporción, por la ruta intracitoplasmática. Se observaron mayoritariamente partículas virales organizadas y adheridas a la superficie de los filopodios, lo que sugiere una preferencia del virus hacia estas regiones de membrana.
2. La reconstrucción tridimensional de eventos de propagación viral célula-célula, demostró la extrusión de numerosas partículas virales y la agrupación filopodios como proyecciones de las células vecinas en forma de mecanismo de defensa.
3. Las alteración estructural subcelular relacionadas con la transmisión del virus célula-célula podrían, por un lado, representar nuevas estrategias terapéuticas y, por otro lado, comprender la manifestación clínica de la enfermedad Covid-19.

Sobre la segunda temática:

Mecanismos de transmisión humano-humano del SARS-CoV-2

4. El patrón de propagación del SARS-CoV-2 muestra una dinámica heterogénea que no puede ser explicada a partir de los modelos epidémicos convencionales. En línea con las teorías de superpropagación, observamos una emisión sobredimensionada de RNA viral en un reducido subgrupo de individuos infectados, mientras que otros subgrupos no diseminaron carga viral ni en tos ni en aerosoles bajo condiciones equivalentes de la enfermedad.
5. Se encontró que el valor de Ct no podía ser considerado como un indicador de la infectividad del individuo al no presentar correlación con la carga viral diseminada en aerosoles y en tos.. A pesar del limitado número de pacientes incluidos en el estudio, otros trabajos similares complementan estas conclusiones. Por tanto, el indicador Ct debería ser reconsiderado en la práctica clínica.
6. Aunque todavía se desconoce la contribución de cada vía de propagación en la dinámica de la transmisión del SARS-CoV-2 entre personas, la ruta de microgotas parece predominar en el contacto estrecho, mientras que los bioaerosoles gobiernan en largas distancias. El manejo preventivo de la transmisión por microgotas es eficiente mediante el uso de mascarillas y distancia interpersonal. Sin embargo, debería prestarse más atención a las estrategias preventivas de la transmisión por aerosoles.

Sobre la tercera temática:
Medidas para la reducción de la transmisión del SARS-CoV-2 por aerosoles

7. El uso de mascarillas y equipos de protección respiratoria ha sido crucial para el manejo epidemiológico de la pandemia por SARS-CoV-2. Las mascarillas quirúrgicas, así como tipologías análogas, presentan un rendimiento comprometido frente a la captación de aerosoles. Dado lo anterior, una de las contribuciones de esta Tesis ha sido el desarrollo de un método para aumentar su eficiencia frente al virus consistente en un recubrimiento basado en nanopartículas de plata y polietilenimina que, en conjunto, logran reducir la carga viral hasta en un 99.9 %. El método propuesto es fácilmente escalable y de bajo coste, lo que facilitaría enormemente su industrialización y producción a gran escala y redundaría en una prevención más eficiente .

8. El uso generalizado de mascarillas y equipos de protección respiratoria ha impactado de manera notoria a nivel medioambiental. El método desarrollado para la producción de medios filtrantes utilizando botellas de PET desechadas a partir de técnicas electrohidrodinámicas puede reducir este impacto. Aunque el trabajo estuvo centrado en el desarrollo de mascarillas de tipo quirúrgico por su prevalencia en la sociedad, con los resultados aquí reportados es posible desarrollar una amplia variedad de medios filtrantes con diferentes aplicaciones. Además, una de las contribuciones de mayor interés en este trabajo fue la propuesta de reprocesar los tejidos filtrantes y re-electrohilarlos sin perder propiedades. De esta manera, se podría establecer una estrategia de economía circular que redundase en una reducción notable del impacto ambiental.

9. El uso de mascarillas y equipos de protección respiratoria depende de la responsabilidad individual, por lo que es probable que se trate de una estrategia temporal. Dada la importancia de la vía de transmisión aérea para la propagación de las enfermedades infecciosas respiratorias, concluimos que deberían establecerse medidas colectivas que garantizaran la calidad del aire de manera definitiva.

10. Las estrategias basadas en la medición de CO₂ metabólico constituyen una alternativa sencilla y efectiva para controlar la calidad y la renovación del aire. Metodología basada en tres fases: diagnóstico, corrección y monitorización, control y seguimiento. Este protocolo fue validado en 40 comercios locales de Zaragoza (España), donde la correcta implementación de cada fase condujo a una mejora significativa en la calidad del aire interior del espacio.

11. El transporte colectivo representa un entorno de alto riesgo para la transmisión de enfermedades infecciosas. En el tranvía de Zaragoza se observó un bajo riesgo de contagio en condiciones pandémicas, donde las mascarillas resultan un elemento clave para el control de la transmisión. Sin embargo, tras la relajación de las medidas y con vistas a un escenario pospandémico, la situación podría no ser tan favorable. Es por esto que implementar medidas efectivas contra la transmisión aérea de patógenos es fundamental para garantizar el bajo riesgo. La filtración mecánica del aire es una de las estrategias más efectivas, aunque su rendimiento dependerá de las propiedades del filtro y de las capacidades del HVAC. Los equipos de filtración implementados en el tranvía de Zaragoza no están diseñados contra bioaerosoles, por lo que su eficiencia resulta comprometida. Dado lo anterior, es preciso buscar alternativas definitivas y efectivas para mejorar la calidad del aire.

Conclusions

On the first theme: SARS-CoV-2 cell-cell spread mechanisms

1. Through transmission electron microscopy, it was observed that membrane projections formation, in the form of filopodia or tunneling nanotubes, is due to the cytoskeleton remodeling induced by SARS-CoV-2 infection. Virus cell-cell spread is mainly mediated by surfing and, to a lesser extent, by the intracytoplasmic route. Organized viral particles adhered to the surface of the filopodia were mainly observed, suggesting a preference of the virus towards these membrane regions.
2. The three-dimensional reconstruction of cell-cell viral spread events demonstrated the extrusion of numerous viral particles and the grouping of filopodia as projections from neighboring cells, as a cellular defense mechanism.
3. Subcellular structural alterations related to cell-cell spread of the virus could, on the one hand, represent new therapeutic strategies and, on the other hand, understand the clinical manifestation of the Covid-19 disease.

On the second theme: SARS-CoV-2 human-human spread mechanisms

4. The spread pattern of SARS-CoV-2 shows heterogeneous dynamics that conventional epidemic models cannot explain. In line with superspreading theories, we observed an outsized release of viral RNA in a small subgroup of infected individuals, while other subgroups did not shed viral load either in coughs or aerosols under equivalent disease conditions.
5. Ct value could not be considered as an indicator of the individual infectivity as it did not present a correlation with the viral load disseminated in aerosols and/or coughs. Other similar works complement these conclusions despite the limited number of patients included in the study. Therefore, the Ct indicator should be reconsidered in clinical practice.
6. Although the contribution of each propagation route in the dynamics of SARS-CoV-2 transmission between people is still unknown, the droplet route seems to predominate in close contact, while bioaerosols rule over long distances. Preventive management of droplet transmission is efficient through the use of masks and interpersonal distance. However, more attention should be paid to preventive strategies for aerosol transmission.

**On the third theme:
Measures to prevent SARS-CoV-2 airborne transmission**

7. The use of masks and respiratory protection equipment has been crucial for the epidemiological management of the SARS-CoV-2 pandemic. Surgical masks, as well as similar types, have a compromised performance against the capture of aerosols. Given the above, one of our contributions has been the development of a method to increase its efficiency against viruses. It consists of a coating based on silver and polyethyleneimine nanoparticles that reduce the viral load by up to 99.9 %. The proposed method is easily scalable and low cost, which would greatly facilitate its large-scale industrialization and production and result in more efficient prevention.

8. The widespread use of masks and respiratory protection equipment has had a notorious environmental impact. The method developed for producing filter media using PET bottles from electrohydrodynamic techniques can reduce this impact. Although the work was focused on the development of surgical-type masks due to their prevalence in society, with the results reported here, it is possible to develop a wide variety of filter media with different applications. Additionally, one of the most interesting contributions in this work was the proposal to reprocess the filter fabrics and re-electrospun them without losing properties. In this way, a circular economy strategy could be established that would significantly reduce environmental impact.

9. Using masks and respiratory protection equipment depends on individual responsibility, so it is likely a temporary strategy. Given the importance of the airborne transmission route for spreading respiratory infectious diseases, we conclude that collective measures should be established to guarantee air quality.

10. Strategies based on measuring metabolic CO₂ constitute a simple and effective alternative to control air quality and renewal. Our methodology is based on three phases: diagnosis, correction, and monitoring, control, and surveillance (MCS). This protocol was validated in 40 local businesses in Zaragoza (Spain), where the correct implementation of each phase led to a significant indoor air quality improvement.

11. Public transportation represents a high-risk environment for the transmission of infectious diseases. In the Zaragoza tram, a low risk of contagion was observed in pandemic conditions, where masks are crucial in controlling transmission. However, the situation may not be so favorable after the measures relaxation in a post-pandemic scenario. This is why implementing effective measures against airborne transmission of pathogens is essential to ensure low risk. Mechanical air filtration is one of the most effective strategies, although its performance will depend on the filter's properties and the HVAC's capabilities. The filtration equipment implemented in the Zaragoza tram is not designed against bioaerosols, so its efficiency is compromised. Given the above, looking for definitive and effective alternatives to improve air quality is necessary.

Glosario y abreviaturas

Glosario
Abreviaturas

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Glosario y abreviaturas

Definiciones extraídas, y en ocasiones modificadas, de la Real Academia Española y de Wikipedia en su versión en inglés.

Glosario

A

Aerosol. Partícula diminuta ($< 100 \mu\text{m}$), sólida o líquida, suspendida en el aire u otro gas.

Ácido desoxirribonucleico. DNA, ácido nucleico que contiene las instrucciones genéticas usadas en el desarrollo y funcionamiento de todos los organismos vivos y algunos virus; también es responsable de la transmisión hereditaria.

Ácido ribonucleico. RNA, ácido nucleico que participa en la síntesis de las proteínas y realiza la función de mensajero de la información genética.

Actina. Familia de proteínas globulares.

Ageusia. Pérdida completa del gusto.

Amina. Compuesto orgánico y grupos funcionales que contienen un átomo de nitrógeno básico con un par solitario.

Aminoácido. Molécula orgánica con un grupo amino ($-\text{NH}_2$) en uno de los extremos de la molécula y un grupo carboxilo ($-\text{COOH}$) en el otro extremo.

Aminoalcohol. Compuesto orgánico con grupos funcionales de amina e hidroxilo.

Anosmia. Pérdida completa del olfato.

Anticuerpo. Glicoproteína del tipo gamma globulina empleada por el sistema inmunitario para identificar y neutralizar cuerpos extraños.

Antígeno. Sustancia que puede ser reconocida por los receptores del sistema inmunitario adaptativo.

Artromialgia. Dolor articular y muscular.

Autofagia. Proceso celular mediante el cual el citoplasma, incluyendo el exceso de orgánulos o aquellos deteriorados o aberrantes, son secuestrados en vesículas de doble membrana y liberados dentro del lisosoma/vacuola para su descomposición y eventual reciclado.

C

Catepsina. Enzima que se encuentra en tejidos animales y cataliza la hidrólisis de proteínas a polipéptidos.

Caspasa. Familia de enzimas perteneciente al grupo de las cisteín-proteasas, caracterizadas por presentar un residuo de cisteína que media la ruptura de otras proteínas. Intervienen en la apoptosis.

Cardiotónico. Sustancia que aumenta la eficiencia de la función cardíaca al disminuir el consumo de oxígeno.

Cepa. Grupo de organismos emparentados, como las bacterias, los hongos o los virus, cuya ascendencia común es conocida.

Ciclo de amplificación. Proceso de qPCR que tiene por objetivo amplificar (o crear copias) de determinados fragmentos de DNA.

Cisteína. Aminoácido que contiene azufre y que se sintetiza a partir de la metionina-homoserina y la posterior disociación de la cistationinal.

Citoesqueleto. Entramado tridimensional de proteínas que provee soporte interno en las células, organiza estructuras internas e interviene en los fenómenos de transporte, tráfico y división celular.

Citopático. Daño celular causado por la infección de un virus.

Citosol. Líquido que se localiza dentro de las células y constituye la mayoría del fluido intracelular.

Citoquina. Molécula de señalización celular.

Choque séptico. Es un estado anormal grave del organismo en el cual existe hipotensión prolongada por cierto período, generalmente dos horas o más, causada por una disminución de la perfusión tisular y el suministro de oxígeno como consecuencia de una infección y la sepsis que de ella resulta.

Clado. Cada una de las ramificaciones del árbol filogenético.

Coagulación. Proceso por el cual la sangre pierde su liquidez, convirtiéndose en un gel.

Coatómero. Complejo proteico que participa en la formación de las cubiertas de vesículas, en el inicio de las vías secretoras.

Coloide. Que, disgregado en un líquido, aparece como disuelto por el tamaño de sus partículas.

Comorbilidad. Presencia de uno o más trastornos; o efecto de estos trastornos en enfermedades adicionales.

Condensación. Cambio de estado de la materia que pasa de forma gaseosa a líquida.

Convección. Propagación de calor u otra magnitud física en un medio fluido por diferencias de densidad. Movimiento vertical del aire.

Covalente. Enlace producido en dos átomos no metálicos cuando se unen y comparten uno o más

electrones del último nivel.

D

Diana terapéutica. Lugar del organismo donde un fármaco ejerce su acción, la mayoría de las dianas terapéuticas son del tipo lípidos, proteínas y ácidos nucleicos.

Difusión. Movimiento térmico de todas las partículas a temperaturas por encima de cero absoluto.

Dineína. Es, junto con la quinesina, la proteína motora más importante asociada a los microtúbulos.

Disnea. Dificultad para respirar.

E

Edema. Acumulación de líquido en el espacio extracelular o intersticial.

Electrospinning. Electrohilado, técnica de nanofabricación basada en electromagnetismo.

ELISA. Técnica de inmunoensayo en la cual un antígeno inmovilizado se detecta mediante un anticuerpo enlazado a una enzima capaz de generar un producto detectable, como cambio de color o de algún otro tipo.

Endemia. Proceso patológico que se mantiene de forma estacionaria en una población o espacio determinado durante periodos prolongados.

Endocitosis. Mecanismo por el cual las células internalizan moléculas grandes, partículas extracelulares e incluso pequeñas células, englobándolas en una invaginación de la membrana plasmática.

Endosoma. Compartimento dentro del citoplasma delimitado por una membrana simple de clatrina con la función de transportar material celular.

Envoltura viral. Membrana lipídica que rodea a la cápside vírica.

Enzima. Molécula orgánica que actúa como catalizadora de reacciones químicas.

Escisión. Fragmentación.

Esfingolípido. Lípido complejo que deriva del aminoalcohol insaturado de dieciocho carbonos esfingosina.

Esfingosina. Aminoalcohol formado por dieciocho carbonos, que forman una cadena hidrocarbonada insaturada.

Especificidad. En qPCR, capacidad de obtención de un solo producto amplificado.

Espujo. Flema que se arroja de una vez en cada expectoración.

Estasis evolutiva. Estabilización evolutiva, en un periodo se muestran pocos cambios evolutivos.

Epidemia. Dícese cuando una enfermedad infec-

ciosa se transmite a un número de individuos superior al esperado en una población durante un tiempo determinado.

Exocitosis. Forma de transporte activo y transporte por arrastre en el que una célula transporta moléculas al exterior celular.

Exosoma. Vesícula extracelular que se produce en los endosomas.

F

Fagocitosis. Proceso por el cual una célula utiliza su membrana plasmática para engullir una partícula grande, típicamente $>0.5 \mu\text{m}$.

Fagosoma. Vacuola formada alrededor de una partícula asimilada por fagocitosis.

Fase. Cada una de las partes homogéneas físicamente separables en un sistema formado por uno o varios componentes.

Fibrilación. Trastorno del ritmo cardíaco.

Fibrinolisis. Degradación de las redes de fibrina formadas en el proceso de coagulación sanguínea, evitando la formación de trombos.

Fibrosis. Desarrollo en exceso de tejido conectivo fibroso en un órgano o tejido como consecuencia de un proceso reparativo o reactivo.

Filamento de actina. Estructura de actina F que forma parte del citoesqueleto celular.

Filopodio. Proyección citoplasmática delgada que está regulada por la actina cortical.

Fómite. Cualquier objeto carente de vida que, si se contamina con algún patógeno viable es capaz de transferirlo de un individuo a otro, por lo que también se les denomina «vector pasivo».

G

Gen. Unidad de información en un locus de DNA que codifica un producto génico: proteínas o RNA.

Genoma. Secuencia de nucleótidos que constituye el DNA de un individuo o de una especie.

Glicoproteína. Proteína conjugada cuyos componentes no proteicos son hidratos de carbono.

Gota de Plügge. Pequeña gota de secreción que se expulsa de forma inadvertida por boca o nariz.

H

Hidrocarbonada. Cadena de átomos formada por una estructura de átomos de carbono unidos entre sí a través de enlaces simples, dobles o triples y estos a su vez unidos a átomos de hidrógeno.

Hidroxilo. Grupo funcional formado por un átomo

de oxígeno y otro de hidrógeno.

Hipoxemia. Disminución anormal de la presión parcial de oxígeno en la sangre arterial.

I

Impactador en cascada. Instrumento para la recolección de muestras aéreas utilizado para fraccionar las partículas por tamaños.

Impigner. Instrumento para la recolección de muestras aéreas en forma de botella.

Infectividad. Capacidad de un agente patógeno para invadir un organismo y provocar infección.

Invaginación. Formación de una bolsa o pliegue en una membrana hacia el interior.

L

Linfocito. Célula del sistema inmunitario; un tipo de leucocito que proviene de la diferenciación linfóide de las células madre hematopoyéticas.

Linfopenia. Número de linfocitos inferior al normal en la sangre.

Lípido. Macromolécula que es soluble en solventes no polares, con funciones de almacenamiento de energía, señalización y estructurales.

Lisosoma. Orgánulo relativamente grande, formado a partir del aparato de Golgi, que contiene hidrolasas ácidas encargadas de degradar el material celular.

M

Macropinocitosis. Proceso de endocitosis inducible y transitorio asociado con la formación de grandes extensiones de la membrana plasmática.

Meltblown. Tipo de tejido cuya fabricación se realiza mediante soplado en fusión.

Metilación. Adición de un grupo metilo ($-\text{CH}_3$).

Mialgia. Dolor de los músculos.

Microorganismo. Sistema biológico de tamaño micrométrico o submicrométrico.

Microtúbulo. Estructura celular formada por polímeros proteicos, parte del citoesqueleto.

Microvellosidad. Prolongación digitiforme rodeada por plasmalema, presente en la superficie luminal de algunas células.

Miosina. Proteína fibrosa que, en conjunción con la actina, permite la contracción de los músculos e interviene en la división celular y el transporte de vesículas.

Momento angular. Equivale al producto de la masa por la velocidad.

Monocatenario. Formado por una sola cadena.

Morfogénesis. Proceso biológico que lleva a que un organismo desarrolle su forma.

Mutación. Alteración en la secuencia del ADN de un organismo, que se transmite por herencia.

N

Nanotubo de efecto túnel. Tubo largo y fino formado a partir de la membrana plasmática que conecta el citoplasma de células alejadas entre sí.

Neumonía. Inflamación del pulmón o de una parte del mismo.

Núcleo de condensación. Partícula que provee de una superficie donde las moléculas de agua pueden agregarse.

P

Pandemia. Epidemia de una enfermedad infecciosa que se ha propagado en un área geográficamente extensa y ha afectado a un número considerable de individuos.

Patógeno. Cualquier microorganismo capaz de producir enfermedad o daño en un huésped.

Patogenicidad. Capacidad de un agente para producir enfermedades.

Patognomónico. Dicho de un signo o de un síntoma que caracteriza y define una determinada enfermedad.

Péptido. Molécula formada por la unión de varios aminoácidos mediante enlaces peptídicos.

Perinuclear. Que rodea el núcleo celular.

Piroptosis. Subrutina de muerte celular dependiente de caspasa-1, asociada con la generación de mediadores pirogénicos como *IL-1 β* e *IL-18*.

Poliadenilación. Adición de una cola de poli(A) a un mRNA.

Polimerización. Proceso químico por el que los monómeros se agrupan químicamente, dando lugar a una molécula de gran peso o bien una cadena lineal o una macromolécula tridimensional.

Prevalencia. Proporción de individuos de un grupo que presentan una misma característica.

Proteína. Macromolécula formada por cadenas de aminoácidos.

Proteína motora. Proteína que convierte energía química en trabajo mecánico, generalmente por medio de una ATPasa.

R

R_0 (número de reproducción básico). Medida promedio de la infecciosidad de un patógeno.

Reacción en Cadena de la Polimerasa. Técnica de biología molecular que tiene por objetivo obtener un gran número de copias de un fragmento de DNA particular.

Receptor. Proteína o glicoproteína que permite la interacción de determinadas sustancias con los mecanismos del metabolismo de la célula.

Régimen del fluido. Laminar o turbulento.

Replicación (viral). Término utilizado para describir el proceso de multiplicación de los virus.

Retículo endoplasmático rugoso. Orgánulo de la célula que se encarga del transporte y síntesis de proteínas de secreción o de membrana.

S

Sensibilidad. En qPCR, cantidad mínima de DNA necesaria para que se produzca la amplificación.

Septicemia. Infección generalizada producida por la presencia en la sangre de microorganismos patógenos o de sus toxinas.

Serología. Estudio químico y bioquímico de los sueros, especialmente del suero sanguíneo.

Seroprevalencia. Prevalencia global de un anticuerpo en la sangre.

Sincitio. Célula con varios núcleos resultante de la fusión de varias células.

Síndrome de Distrés Respiratorio Agudo. Definido como una insuficiencia respiratoria grave, por edema pulmonar, no cardiotónico, causado por aumento de la permeabilidad de la barrera alvéolo-capilar y secundario a daño pulmonar agudo.

Spunbond. Material similar a la tela hecho de fibras discontinuas y fibras largas, unidas entre sí mediante tratamiento químico, mecánico, térmico o con solventes.

Submicrónico. De tamaño inferior a 1 μm .

Surfing viral. Navegación de los virus a lo largo de la membrana plasmática.

T

Transcripción. Primer proceso de la expresión genética, donde se transfiere la información contenida en la secuencia del DNA hacia la secuencia de proteína utilizando diversos RNA como intermediarios.

Transmembrana. Que puede atravesar la membrana celular o se dispone a través de ella.

Tropismo. Movimiento de orientación de un organismo sésil como respuesta a un estímulo.

Tomografía computarizada. Obtención de imágenes con rayos X de cortes o secciones de algún objeto.

Trombo. Formación de un coágulo en el interior de un vaso sanguíneo.

U-V

Ubiquitinación. Proceso de adición de cadenas de ubiquitina a una proteína marcada para la degradación.

Unidad formadora de colonias. Unidad de medida que se emplea para la cuantificación de microorganismos.

Vacuola. Orgánulo celular unido a la membrana que ayuda a retener los productos de desecho.

Vesícula. Orgánulo que forma un compartimento pequeño y cerrado, separado del citoplasma por una bicapa lipídica que almacena, transporta o digiere productos y residuos celulares.

Virión. Partícula vírica morfológicamente completa e infecciosa.

Virus. Microorganismo infeccioso acelular compuesto de material genético (DNA o RNA) protegido por un envoltorio proteico.

Y-Z

Yuxtannuclear. Que está cercano al núcleo.

Zoonótico. Enfermedad o infección que puede transmitirse entre animales y seres humanos.

Abreviaturas

ACE. Del inglés *Angiotensin Converting Enzyme* (Enzima Convertidora de Angiotensina).

ACH. Del inglés *Air Change per Hour* (Renovaciones de Aire por Hora).

Alfa-CoV. Alfacoronavirus.

ARP. Del inglés *Actin-Related Proteins* (Proteínas Relacionadas con Actina).

Beta-CoV. Betacoronavirus.

CDC. Del inglés *Communicable Disease Center* (Centros de Control y Prevención de Enfermedades).

CEIC Aragón. Comité de Ética de la Investigación de la Comunidad Autónoma de Aragón.

CFU. Del inglés *Colony-Forming Unit* (Unidades Formadoras de Colonias).

CME. Del inglés *Clathrine-Mediated Endocytosis* (Endocitosis Mediada por Clatrina).

CoV. Coronavirus.

CPC. Del inglés *Condensation Particle Counter* (Contador de Partículas por Condensación).

Ct. Ciclo de cuantificación en qPCR.

Dpe. Días después de la exposición.

Dpi. Días después de la infección.

DCM. Diclorometano.

DPPC. Dipalmitoilfosfatidilcolina.

DPPG. Dipalmitoilfosfatidilglicerol.

DMA. Del inglés *Differential Mobility Analyzer* (Analizador de Movilidad Diferencial).

DMV. Del inglés *Double Membrane Vesicle* (Vesícula de Doble Membrana).

DNA. Del inglés *Deoxyribonucleic Acid* (Ácido Desoxirribonucleico).

ELISA. Del inglés *Enzyme-Linked ImmunoSorbent Assay* (Ensayo por Inmunoabsorción Ligado a Enzimas).

EN. Del inglés *European Norm* (Norma Europea).

EPI. Equipo de Protección Individual.

EPR. Equipo de Protección Respiratoria.

ERGIC. Complejo intermedio ER (Retículo Endoplasmático)-Golgi.

ESEM. Del inglés *Environmental Scanning Electron Microscope* (Microscopía Electrónica de Barrido Ambiental).

FFP. Del inglés *Filtering Face Piece* (Pieza Facial Filtrante).

Hpi. Horas tras la infección.

H1N1. Influenzavirus A subtipo H1N1.

HVAC. Del inglés *Heating, Ventilation, and Air Conditioning* (Sistema de Calefacción, Ventilación u Aire Acondicionado).

IC. Intervalo de confianza.

IDA. Del inglés *Indoor Air* (Aire Interior).

IFN. Interferon.

IgG. Inmunoglobulina G.

IgM. Inmunoglobulina M

IL. Interleucina.

IoT. Del inglés *Internet Of Things* (Internet de las Cosas).

Kbp. Kilopares de bases.

LC. Del inglés *Microtubule-Associated Protein Light Chain* (Cadena Ligera de Proteínas Asociada a Microtúbulos).

MERS. Del inglés *Middle East Respiratory Syndrome* (Síndrome Respiratorio de Oriente Medio).

MP-AES. Del inglés *Microwave Plasma-Atomic Emission Spectrometry* (Espectrometría de Emisión Atómica de Plasma por Microondas).

N-DIR. Del inglés *Non-Dispersive Infrared Detector* (Detector Infrarrojo No Dispersivo).

NK. Del inglés *Natural Killer* (Célula Asesina, anteriormente conocida como linfocito grande granuloso).

OMS. Organización Mundial de la Salud.

OPS. Del inglés *Optical Particle Sizer* (Medidor de Partículas Óptico).

pan-SARS ESAR. Diana del gen *Sarbeco E* SARS-CoV-2.

PaO₂. Presión parcial de oxígeno.

PBS. Del inglés *Phosphate-Buffered Saline* (Solución Salina Tamponada con Fosfato).

PCA. Del inglés *Plate Count Agar* (Placas de Recuento de Agar).

PEEP. Del inglés *Positive End-Expiratory Pressure* (Presión Positiva al Final de la Espiración).

PET. Del inglés *Polyethylene Terephthalate* (Tereftalato de Polietileno).

PFU. Del inglés *Plaque-Forming Unit* (Unidad Formadora de Placas).

PLAC. Del inglés *Placenta Associated gene* (Gen Asociado a la Placenta).

PM. Del inglés *Particulated Matter* (Materia Particulada).

PVA. Del inglés *Polyvinyl Acetate* (Acetato de Polivinilo).

qPCR. Del inglés *Quantitative Polymerase Chain Reaction* (Reacción en Cadena de la Polimerasa Cuantitativa).

R₀. Número de reproducción básico.

RE. Retículo Endoplasmático.

RER. Retículo Endoplasmático Rugoso.

RITE. Reglamento de Instalaciones Térmicas en los Edificios.

RH. Del inglés *Relative Humidity* (Humedad Relativa).

ROS. Del inglés *Reactive Oxygen Species* (Especies Reactivas de Oxígeno).

RNA. Del inglés *Ribonucleic Acid* (Ác. Ribonucleico).

RSD. Del inglés *Relative Standard Deviation* (Desviación Estándar Relativa).

RT-qPCR. Del inglés *Reverse Transcriptase qPCR* (qPCR con Transcriptasa Inversa).

SARS. Del inglés *Severe Acute Respiratory Syndrome* (Síndrome Respiratorio Agudo Grave).

IP4. Diana del gen *RdRp* en SARS-CoV-2.

SDRA. Síndrome Distrés Respiratorio Agudo.

SEM. Del inglés *Scanning Electron Microscope* (Microscopía Electrónica de Barrido).

SMPS. Del inglés *Scanning Mobility Particle Sizer* (Medidor de Tamaño de Partículas por Movilidad).

SPNS. Del inglés *Sphingolipid Transporter Protein Coding gene* (Gen Codificante de la Proteína Transportadora de Esfingolípidos).

SpO₂. Saturación de Oxígeno en sangre.

TCID50. Del inglés *Median Tissue Culture Infectious Dose* (Dosis Infecciosa del 50 % en Cultivo de Tejido).

TEM. Del inglés *Transmission Electron Microscope* (Microscopía electrónica de transmisión).

TFA. Del inglés *Trifluoroacetic Acid* (Ácido trifluoroacético).

TMPRSS2. Del inglés *Transmembrane protease serine 2* (Proteasa Transmembrana Serina 2).

TNT. Del inglés *Tunneling Nanotube* (Nanotubo de efecto túnel).

TSC. Del inglés *Trisodium Citrate* (Citrato trisódico).

UNE. Una Norma Española.

VOC. Del inglés *Variant of Concern* (Variante de Preocupación).

VOI. Del inglés *Variant of Interest* (Variante de Interés).

VTM. Del inglés *Virus Transport Medium* (Medio de Transporte de Virus).

The background of the slide is a scanning electron micrograph (SEM) showing a dense, chaotic network of thin, light-colored fibers against a darker background. These fibers are identified as PET electrospun microfibers, and the image highlights various defects such as breaks, loops, and irregular thicknesses.

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A scanning electron micrograph (SEM) showing a dense field of Bacillus cereus bacteria. The bacteria are rod-shaped and appear to be arranged in chains or clusters, covering the entire background of the page.

Apéndices

Apéndice 1. Factor de impacto de las revistas y áreas temáticas correspondientes a las publicaciones. 246

Apéndice 2. Renuncia de los coautores. 247

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Apéndice



Factor de impacto de las revistas y áreas temáticas correspondientes a las publicaciones

Tabla 5. Factor de impacto, categoría JCR y cuartil de las revistas donde se han publicado los trabajos contenidos en esta Tesis Doctoral.

Referencia	IF (2021)	IF (5 años)	Categoría JCR	SCIE 2021	JCI 2021	ISCI 2020
Baselga, M.; Uranga, I.; Moreo, E.; Arias, M.; Junquera, C. Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread. <i>Histochemistry and Cell Biology</i> . 2022. https://doi.org/10.1007/s00418-022-02152-7	2.531	3.617	Microscopy	Q2 (4/9)	Q3 (5/9)	D1 (1/9)
			Cell biology	Q4 (162/194)	Q2 (78/203)	Q3 (106/195)
Baselga, M.; Güemes, A.; Alba, J.J.; Schuhmacher, A.J. 2022. SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity. <i>Journal of Clinical Medicine</i> . 2022. 11 (9): 2607. https://doi.org/10.3390/jcm11092607	4.964	5.098	Medicine, General & Internal	Q2 (54/172)	Q1 (50/329)	Q1 (39/167)
Baselga, M.; Uranga, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates. <i>Materials</i> . 2022. 15 (14), 4742; https://doi.org/10.3390/ma15144742	3.748	4.042	Metallurgy & Metallurgical Engineering	Q1 (18/79)	Q1 (21/91)	Q1 (17/80)
			Physics, Applied	Q2 (56/161)	Q2 (67/178)	Q2 (51/160)
Baselga, M.; Yus, C.; Arruebo, M.; Sebastián, V.; Irusta, S.; Jiménez, S. 2022. Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks. <i>Nanomaterials</i> . 2022. 12 (6): 925. https://doi.org/10.3390/nano12060925	5.719	5.810	Physics, Applied	Q1 (37/161)	Q1 (44/178)	Q1 (35/160)
			Nanoscience & Nanotechnology	Q2 (53/109)	Q2 (48/138)	Q2 (51/106)
Baselga, M.; Alba, J.J.; Schuhmacher, A.J. The Control of Metabolic CO ₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases. <i>International Journal of Environmental Research and Public Health</i> . 2022. 19: 6605. https://doi.org/10.3390/ijerph19116605	4.614	4.798	Public, Environmental & Occupational Health	Q1 (45/182)	Q2 (109/392)	Q1 (42/176)
			Environmental Sciences	Q2 (100/279)	Q2 (86/324)	Q2 (118/274)
Baselga, M.; Alba, J.J.; Schuhmacher, A.J. Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport. <i>Science of the Total Environment</i> . 2022. 158965. https://doi.org/10.1016/j.scitotenv.2022.158965	10.753	10.237	Environmental Sciences	D1 (26/279)	D1 (15/324)	D1 (25/274)

Apéndice

2

Renuncia de los coautores

Tabla 6. Relación de renuncias de los coautores a presentar los artículos contenidos en esta Tesis como parte de otra Tesis Doctoral.

Referencia	Coautores no doctores	Página
Baselga, M.; Uranga-Murillo, I.; Moreo, E.; Arias, M.; Junquera, C. Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread. <i>Histochemistry and Cell Biology</i> . 2022. https://doi.org/10.1007/s00418-022-02152-7	Uranga-Murillo, I. Moreo, E.	246 247
Baselga, M.; Güemes, A.; Alba, J.J.; Schuhmacher, A.J. 2022. SARS-CoV-2 Droplet and Airborne Transmission Heterogeneity. <i>Journal of Clinical Medicine</i> . 2022. 11 (9): 2607. https://doi.org/10.3390/jcm11092607	Todos los coautores son doctores.	No aplica.
Baselga, M.; Uranga-Murillo, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates. <i>Materials</i> . 2022. 15 (14), 4742; https://doi.org/10.3390/ma15144742	Uranga-Murillo, I.	246
Baselga, M.; Yus, C.; Arruebo, M.; Sebastián, V.; Irusta, S.; Jiménez, S. 2022. Submicronic Filtering Media Based on Electrospun Recycled PET Nanofibers: Development, Characterization, and Method to Manufacture Surgical Masks. <i>Nanomaterials</i> . 2022. 12 (6): 925. https://doi.org/10.3390/nano12060925	Todos los coautores son doctores.	No aplica.
Baselga, M.; Alba, J.J.; Schuhmacher, A.J. The Control of Metabolic CO ₂ in Public Transport as a Strategy to Reduce the Transmission of Respiratory Infectious Diseases. <i>International Journal of Environmental Research and Public Health</i> . 2022. 19: 6605. https://doi.org/10.3390/ijerph19116605	Todos los coautores son doctores.	No aplica.
Baselga, M.; Alba, J.J.; Schuhmacher, A.J. Impact of needle-point bipolar ionization system in the reduction of bioaerosols in collective transport. <i>Science of the Total Environment</i> . 2022. 158965. https://doi.org/10.1016/j.scitotenv.2022.158965	Todos los coautores son doctores.	No aplica.

A continuación se adjuntan los impresos de Renuncia de Coautores de los trabajos presentados como parte de una Tesis Doctoral en la modalidad de compendio de publicaciones.

RENUNCIA DE LOS COAUTORES DE LOS TRABAJOS PRESENTADOS COMO PARTE DE UNA TESIS DOCTORAL EN LA MODALIDAD DE COMPENDIO DE PUBLICACIONES

1.- Datos personales del coautor		
Apellidos: Uranga Murillo	Nombre: Iratxe	
DNI/Pasaporte/NIE: 17756429S	Teléfono	Correo electrónico iratxe.um@gmail.com

2.- Tesis Doctoral
Título: Mecanismos de transmisión del SARS-CoV-2 e implementación de medidas para reducir su propagación por aerosoles
Autor: Marta Baselga Lahoz
Programa de doctorado: Programa de Doctorado en Ingeniería Biomédica

3.- Publicaciones que formarán parte de la tesis y de las que el firmante es coautor
Artículo publicado: Baselga, M.; Uranga-Murillo, I.; de Miguel, D.; Arias, M.; Sebastián, V.; Pardo, J.; Arruebo, M. Silver Nanoparticles–Polyethyleneimine-Based Coatings with Antiviral Activity against SARS-CoV-2: a new method to functionalize different substrates. Materials. 2022. 15 (14), 4742; https://doi.org/10.3390/ma15144742
Artículo aceptado: Baselga, M; Moreo, E.; Uranga-Murillo, I; Arias, M; Junquera, C. Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread. Histochemistry and Cell Biology. 2022. https://doi.org/10.1007/s00418-022-02152-7

RENUNCIA:
Renuncio a que las publicaciones anteriores puedan ser presentadas como parte de otra tesis doctoral en la modalidad de compendio de publicaciones.
<lugar>, <fecha> Zaragoza, 21/09/22



RENUNCIA DE LOS COAUTORES DE LOS TRABAJOS PRESENTADOS COMO PARTE DE UNA TESIS DOCTORAL EN LA MODALIDAD DE COMPENDIO DE PUBLICACIONES

1.- Datos personales del coautor		
Apellidos: Moreo Lapieza		Nombre: Eduardo Javier
DNI/Pasaporte/NIE: 77135728F	Teléfono 680709224	Correo electrónico ejmoreo@gmail.com

2.- Tesis Doctoral
Título: Mecanismos de transmisión del SARS-CoV-2 e implementación de medidas para reducir su propagación por aerosoles
Autor: Marta Baselga Lahoz
Programa de doctorado: Programa de Doctorado en Ingeniería Biomédica

3.- Publicaciones que formarán parte de la tesis y de las que el firmante es coautor
Artículo aceptado: Baselga, M; Moreo, E.; Uranga-Murillo, I; Arias, M; Junquera, C. Ultrastructural analysis and three-dimensional reconstruction of cellular structures involved in SARS-CoV-2 spread. Histochemistry and Cell Biology. 2022. https://doi.org/10.1007/s00418-022-02152-7

RENUNCIA:
Renuncio a que las publicaciones anteriores puedan ser presentadas como parte de otra tesis doctoral en la modalidad de compendio de publicaciones.
<lugar>,<fecha> Zaragoza, 22 de septiembre de 2022
Firma:

Apéndice

3

Póster científico y certificado de premio

Parte de los resultados del trabajo contenido en el primer artículo de esta Tesis fue presentada en formato póster en el IX Congreso de la Sociedad Española de Histología e Ingeniería Tisular, obteniendo el premio en la categoría de Histopatología.



SEHIT-22

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**XXI CONGRESO DE LA SOCIEDAD ESPAÑOLA DE HISTOLOGÍA E INGENIERÍA TISULAR,
IX INTERNATIONAL CONGRESS OF HISTOLOGY AND TISSUE ENGINEERING y
VIII CONGRESO IBEROAMERICANO DE HISTOLOGÍA**

CERTIFICA que la Comunicación en PÓSTER:

***Ultrastructural Analysis of Filopodium Formation Induced by the
SARS-CoV-2 Virus Infection in Vero E6 cells***

de la que son Autores:

Baselga M., Uranga I., Moreo E., Arias M., Monleón E., Güemes A., Pérez J., Sarriá S.J. and Junquera C.

Ha sido **presentada** en este Congreso celebrado en Granada entre el 6 y el 9 de septiembre de 2022.
Y para que conste, se expide y firma el presente certificado en
Granada, a 9 de septiembre de 2022



Juan Francisco Madrid Cuevas
Presidente de la SEHIT



Pascual Vicente Crespo Ferrer
Presidente del Congreso SEHIT-22



Antonio Campos Muñoz
Presidente del Comité Científico



SEHIT-22

El Comité Organizador del

**XXI CONGRESO DE LA SOCIEDAD ESPAÑOLA DE HISTOLOGÍA E INGENIERÍA TISULAR,
IX INTERNATIONAL CONGRESS OF HISTOLOGY AND TISSUE ENGINEERING y
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Ha recibido el **premio al mejor póster de su categoría** en este Congreso celebrado en Granada entre
el 6 y el 9 de septiembre de 2022.
Y para que conste, se expide y firma el presente certificado en
Granada, a 9 de septiembre de 2022



Juan Francisco Madrid Cuevas
Presidente de la SEHIT



Pascual Vicente Crespo Ferrer
Presidente del Congreso SEHIT-22



Antonio Campos Muñoz
Presidente del Comité Científico

Ultrastructural Analysis of SARS-CoV-2 Cell-Cell Propagation Mechanisms: Filopodial Bridges and Tunneling Nanotubes

Baselga M.¹, Uranga I.^{1,2,3}, Moreo E.², Arias M.^{1,2,3}, Monleón E.^{1,4}, Güemes A.^{1,5}, Pérez J.⁴, Sarriá S.J.⁴, and Junquera C.^{1,4}

¹ Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain; ² Department of Microbiology, Pediatrics, Radiology and Public Health, University of Zaragoza, Zaragoza, Spain; ³ Networking Research Center on Infectious Diseases CIBERINFEC, Madrid, Spain; ⁴ Department of Anatomy and Histology, University of Zaragoza, Zaragoza, Spain; ⁵ Department of Surgery, University of Zaragoza, Zaragoza, Spain.

INTRODUCTION. The cytoskeleton plays a crucial role in numerous viral processes, including cell entry, transportation to viral organelles, and release of new viruses. However, the cytoskeleton alteration after SARS-CoV-2 infection in epithelial cells has been scarcely reported. This work aims to ultrastructurally characterize the cytoskeleton reorganization, its associated filopodia and tunneling nanotubes assembly, and their participation in cell-cell viral propagation.

ABBREVIATIONS. af: actin filaments; cj: cell junction; CME: clathrin-mediated endocytosis; DMV: double-membrane vesicles; ev: endocytic vesicle; f: filopodium; fb: filopodial bridge; fe: filopodial extension; L: lysosome; m: mitochondria; mc: membrane continuity; MT: microtubule; n: nucleus; TNT: tunneling nanotubes; tv: transport vesicle; vp: viral particle.

MATERIALS AND METHODS. Vero E6 cells were infected with the original variant of SARS-CoV-2 virus and cultured for 48 hours [1]. Ultrathins were visualized using conventional Transmission Electron Microscopy. Three-dimensional models were reconstructed from serial ultrathin sections using Fiji Image J software [2].

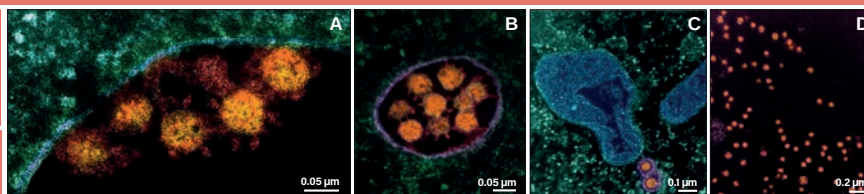


FIG.1. Coloured electron micrographs. **A)** Viruses adhered to the plasma membrane. **B)** Clusters of multiple viral particles. **C)** Individual viruses within vesicles in the cytosol. **D)** Free viral particles in the extracellular space. A mitochondrial alteration induced by SARS-CoV-2 infection is also shown in **C**. SARS-CoV-2: orange; cytosol: blue; vesicle: purple; mitochondria: blue.

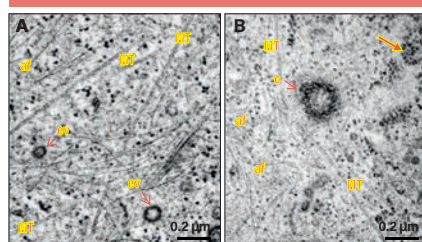


FIG.2. **A)** High density of microtubules (MT) and actin/intermediate filaments (af) in the juxtamembranous region, with endocytic vesicles (ev) probably coated with clathrin and free ribosomal units. **B)** A microtubule-organizing center or centrosome (c: centriole). It is also possible to identify polyribosomes (arrow).

RESULTS AND DISCUSSION. The formation of filopodia induced by SARS-CoV-2 infection was frequently observed (FIG.1). The infected cells showed an evident development of the cytoskeleton, with characteristic formations of filopodial unions between cells (FIG.2). Two different types of filopodial structures were found: inter-filopodial connections without membrane fusion (referred to as 'filopodial bridges') (FIG.3) and with membrane fusion (referred to as 'tunneling nanotubes, TNT') (FIG.4). Filopodial bridges and nanotubes could promote viral cell-cell propagation from two different phenomena: by viral 'surfing' and through the intracytoplasmic pathway. Viral surfing refers to the transport along the filopodial membrane, and it is mediated by specific receptors. In the intracytoplasmic route, virus could travel from cell to cell through the cytosol [3,4]. Even filopodial structures connect more than two cells at a time, as shown in FIG.5. Filopodium structure was characterized by ordered F-actin bundles (and/or intermediate filaments), microtubules, and protein packages of few tens of nm (FIG.6), although its composition may vary depending on its size and morphology. Higher density of viral particles has been identified in filopodial membranes regarding other regions of plasma membrane. It suggests that these membrane regions represent a preferential viral binding area (FIG.7). Viral cell-cell propagation was frequently observed via viral surfing route. In contrast, the intracytoplasmic route was occasionally identified so viral surfing represent a preferred cell-cell spread model.

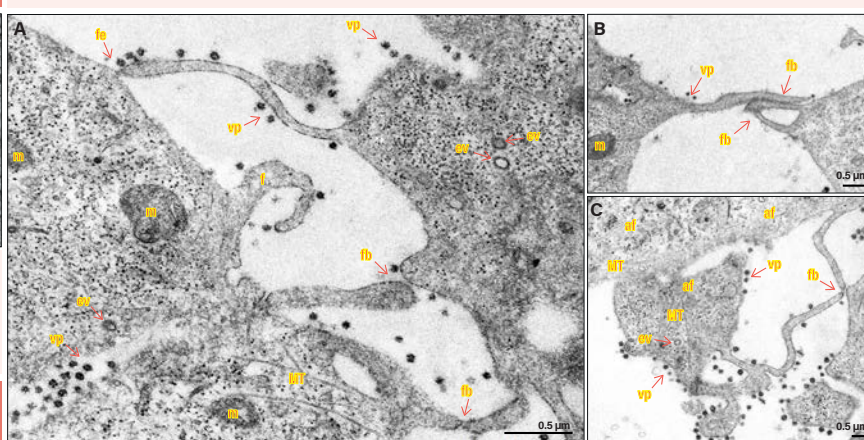


FIG.3. Cell-cell interaction structures found in SARS-CoV-2 infected cells. **A)** Filopodial extensions (fe) whose endings adheres to the plasma membrane of neighboring cell and through which the viral particles (vp) are surfing. **B)** Filopodial bridges (fb) were constituted through wide intermembrane junctions, **C)** or smaller junctions in the apical regions.

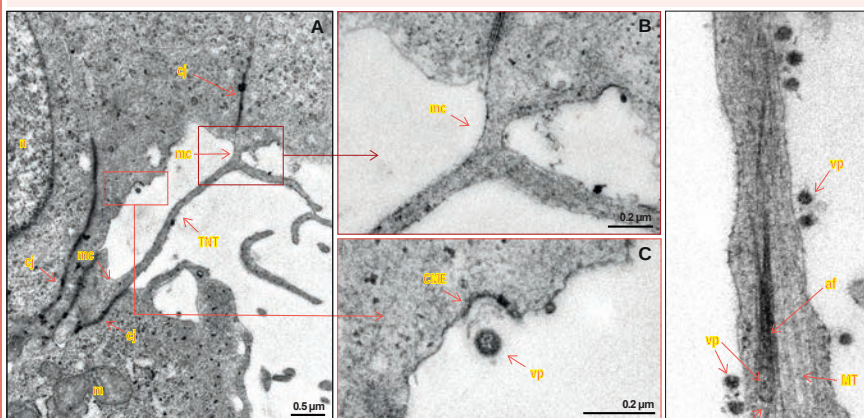


FIG.4. **A)** Tunneling nanotube (TNT) with membrane continuity (mc), where the nanotube is >2.5 µm in length and direct contact the cytosols of two adjacent cells. **B)** Detail of the membrane continuity. **C)** Detail of a clathrin-mediated endocytosis (CME) of a viral particle (vp).

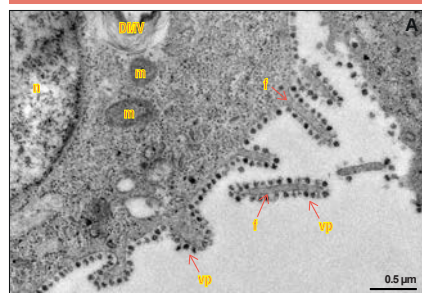


FIG.5. **A-B)** Viral particles (vp) attached to the filopodia (f), which are frequently surrounding plasma membranes of filopodia. This finding suggests the presence of preferential viral binding zones on the filopodial surfaces.

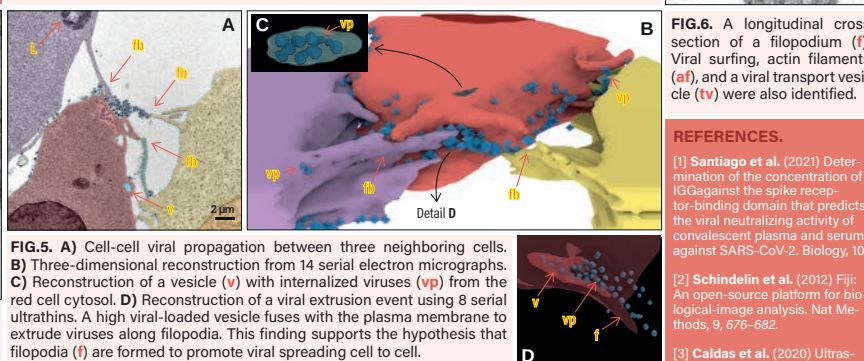


FIG.6. A longitudinal cross section of a filopodium (f). Viral surfing, actin filaments (af), and a viral transport vesicle (tv) were also identified.

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CONCLUSIONS. Extensive filopodia structures appeared in SARS-CoV-2 infected cells. These membrane projections play a relevant role in probing the extracellular environment, directed migration, and cell-cell communication processes. High concentration of viral particles attached to the filopodia observed in the electron micrographs suggests that these membrane projections could be critical during viral infection and could represent strategic and novel therapeutic targets.

