Universidad de Oviedo

Programa de Doctorado en Biomedicina y Oncología Molecular

DESCIFRANDO EL PAPEL DE LA SENESCENCIA EN LA PATOLOGÍA CRÍTICA

Doctorando:

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Oviedo, 26 de septiembre 2024

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Departamento de Biología Funcional

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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RESUMEN (en español)

En las últimas décadas, los avances en la medicina intensiva han logrado mejorar significativamente la supervivencia del paciente crítico. Sin embargo, aunque los resultados a corto plazo en estos pacientes han mejorado, los supervivientes hacen frente a una serie de secuelas graves que condicionan su calidad de vida y tienen un gran impacto social y económico. Estas secuelas, que incluyen complicaciones físicas, cognitivas y emocionales, se engloban bajo el término de síndrome post-UCI (PICS, “Post Intensive Care Syndrome”). Esta realidad plantea un nuevo desafío para la medicina intensiva, que ahora busca no solo aumentar la supervivencia de los pacientes en la UCI, si no también reducir la morbimortalidad relacionada con el PICS.

El estudio de los mecanismos patogenéticos responsables de este cuadro es esencial para el desarrollo de nuevos tratamientos que vayan dirigidos al síndrome en su totalidad, en lugar de abordar las complicaciones de manera aislada. Aunque la respuesta inflamatoria es uno de los mecanismos involucrados en el PICS, los tratamientos antiinflamatorios no han demostrado beneficios significativos, dado que este mecanismo desempeña un papel fundamental en la reparación del tejido. La senescencia se define como un estado celular caracterizado por la detención permanente del ciclo celular en respuesta a distintos estímulos, acompañado por un cambio en el secretoma conocido como fenotipo secretor asociado a senescencia (SASP, “Senescence-associated secretory phenotype”). Existen múltiples mecanismos que pueden desencadenar la respuesta senescente en el paciente crítico, ya sea debido a propia enfermedad del paciente o a los tratamientos que estos reciben.

Esta tesis doctoral incluye cuatro trabajos que buscan estudiar el papel de la senescencia en la patología crítica y sus consecuencias a largo plazo. El primer trabajo, consiste en una revisión que tiene como objetivo identificar los mecanismos moleculares que llevan al PICS y explorar nuevos tratamientos para prevenirlos. Este trabajo considera la senescencia como un mecanismo potencial en el desarrollo de este síndrome, proponiéndola como una posible diana terapéutica sobre la que actuar. El segundo trabajo muestra que el daño pulmonar agudo puede activar un programa de senescencia que a corto plazo previene la disfunción del tejido pulmonar. Asimismo, los resultados indican que esta respuesta puede manipularse mediante el uso de lopinavir/ritonavir para incrementar sus beneficios. El tercer trabajo describe cómo el estiramiento mecánico puede activar por si mismo un programa de senescencia y contribuir al desarrollo de una fibrosis mediante la liberación del SASP. Además, señala que el uso de la digoxina, un fármaco con propiedades senolíticas, podría limitar el desarrollo de esta enfermedad. Finalmente, el cuarto y último trabajo explora los mecanismos básicos de la senescencia, sus consecuencias en el paciente crítico, y considera la posibilidad de utilizar fármacos senolíticos y senomórficos en estos pacientes. Esta revisión refuerza los resultados de los artículos científicos de esta tesis doctoral.

En definitiva, estos trabajos revelan la pleiotropía antagónica de la senescencia en la patología crítica y sugieren la posibilidad de implementar una intervención terapéutica personalizada en cada momento de la enfermedad mediante la modulación de la respuesta senescente.

Universidad de Oviedo

RESUMEN (en Inglés)

In recent decades, advances in intensive care medicine have significantly improved the survival rates of critically ill patients. However, although short-term outcomes for these patients have improved, survivors face a range of severe sequelae that affect their quality of life and have a substantial social and economic impact. These sequelae, which include physical, cognitive and emotional complications, are collectively referred to a post-intensive care syndrome (PICS). This reality presents a new challenge for intensive care medicine, which now aims not only to increase patient survival in the ICU but also to reduce the morbidity and mortality associated with PICS.

Studying the pathogenetic mechanisms responsible for this condition is essential for developing new treatments that target the syndrome as a whole, rather than addressing complications individually. While the inflammatory response is one of the mechanisms involved in PICS, anti-inflammatory treatments have shown not significant benefits, as this mechanism plays a crucial role in tissue repair. Senescence is defined as a cellular state characterized by permanent cell cycle arrest in response to various stimuli, accompanied by a change in the secretome known as the senescence-associated secretory phenotype (SASP). Multiple mechanisms can trigger the senescent response in critically ill patients, whether due to the patient´s underlying condition or the treatments they receive.

This doctoral thesis includes four studies that aim to explore the role of senescence in critical illness and its long-term consequences. The first study is a review that aims to identify the molecular mechanisms leading to the development of PICS and explore new treatments to prevent them. This study considers senescence as a potential mechanism in the development of this syndrome, proposing it as a possible therapeutic target. The second study shows that acute lung injury can activate a senescence program that, in the short term, prevents lung tissue dysfunction. The results also indicate that this response can be manipulated using lopinavir/ritonavir to enhance its benefits. The third study describes how mechanical stretching can independently activate a senescence program and contribute to the development of fibrosis through the release of the SASP. Additionally, it reveals that the use of digoxin, a drug with senolytic properties, could limit the development of this disease. Finally, the fourth study explores the basic mechanisms of senescence, its consequences in critically ill patients, and considers the possibility of using senolytic and senomorphic drugs in these patients. This review reinforces the findings of the scientific articles included in this doctoral thesis.

Overall, these studies reveal the antagonistic pleiotropy of senescence in critical illness and suggest the possibility of implementing personalized therapeutic interventions at different stages of the disease through modulation of the senescent response.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOMEDICINA Y ONCOLOGÍA MOLECULAR

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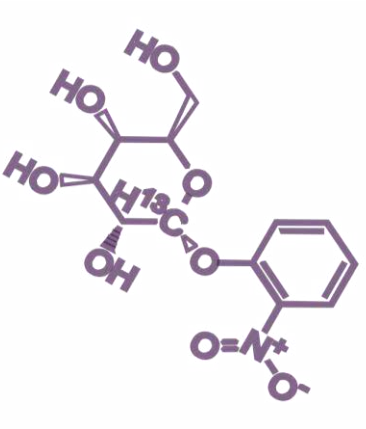

RESUMEN

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development of this syndrome, proposing it as a possible therapeutic target. The second study shows that acute lung injury can activate a senescence program that, in the short term, prevents lung tissue dysfunction. The results also indicate that this response can be manipulated using lopinavir/ritonavir to enhance its benefits. The third study describes how mechanical stretching can independently activate a senescence program and contribute to the development of fibrosis through the release of the SASP. Additionally, it reveals that the use of digoxin, a drug with senolytic properties, could limit the development of this disease. Finally, the fourth study explores the basic mechanisms of senescence, its consequences in critically ill patients, and considers the possibility of using senolytic and senomorphic drugs in these patients. This review reinforces the findings of the scientific articles included in this doctoral thesis.

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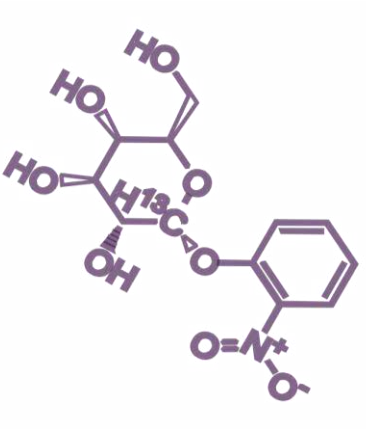
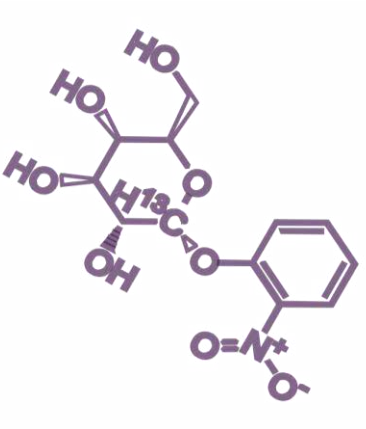


ABREVIATURAS

CARS	*Compensatory Anti-inflammatory Response Syndrome* - Síndrome de respuesta antiinflamatoria compensatoria
DAMPs	*Damage-Associated Molecular Patterns* - Patrones moleculares asociados a daño
DDR	*DNA damage response* - Respuesta al daño en el ADN
EV	*Extracellular vesicles* - Vesículas extracelulares
FPI	Fibrosis Pulmonar Idiopática
HSC	*Hematopoietic Stem Cells* - Células madre hematopoyéticas
ICUAW	*Intensive Care Unit Acquiered weakness* - Debilidad muscular adquirida en la unidad de cuidados intensivos
LADs	*Lamin-Associated Domains* - Dominios asociados a la lamina
LINC	*Linker of Nucleoskeleton and Cytoskeleton* - Complejo de unión del nucleosqueleto y citoesqueleto
MDSCs	*Myeloid-derived suppressor cells* - Células supresoras mieloides
PAMPs	*Pathogen-Associated Molecular Patterns* - Patrones moleculares asociados a patógenos
PARs	*Protease Activated Receptors* - Receptores activados por proteasas
ROS	*Reactive Oxygen Species* - Especies reactivas de oxígeno
SADS	*Senescence associated distension of satellites* - Distensión de satélites asociada a senescencia
SAHF	*Senescence-Associated Secretory Phenotype* – Focos de Heterocromatina Asociados a Senescencia
SASP	*Senescence Associated Secretory Phenotype* - Fenotipo secretor asociado a senescencia
SA-βgal	*Senescence-Associated Beta-Galactosidase* - Beta galactosidasa asociada a senescencia
SDRA	Síndrome de Dificultad Respiratoria Aguda
SIRS	*Systemic Inflammatory Response Syndrome* - Síndrome de respuesta inflamatoria sistémica
SNC	Sistema Nervioso Central
UCI	Unidad de Cuidados Intensivos
VILI	*Ventilator Induced Lung Injury* - Lesión pulmonar inducida por ventilación



INTRODUCCIÓN

SÍNDROME POST-UCI (PICS)

La disminución en las tasas de mortalidad en la unidad de cuidados intensivos (UCI), junto con el aumento de la demanda de atención crítica, ha intensificado la preocupación por el estado de salud a largo plazo de los sobrevivientes de la UCI. Los pacientes que superan la enfermedad crítica se enfrentan a complicaciones a largo plazo englobadas en el síndrome post-cuidados intensivos (PICS, *post-intensive care syndrome*). Este síndrome abarca limitaciones físicas, cognitivas y emocionales que persisten más allá de la hospitalización (1). Los aspectos más estudiados del PICS incluyen la disfunción pulmonar crónica (2), el deterioro cognitivo (3) y la debilidad muscular adquirida (4) en UCI (ICUAW, *Intensive care unit-acquired weakness*). Sin embargo, es fundamental subrayar que el término se encuentra en constante evolución. Nuevas propuestas como la pérdida ósea acelerada, trastornos de deglución y trastornos endocrinos, emergen como componentes adicionales de PICS (5).

En comparación con otras estancias hospitalarias, el espectro de morbimortalidad es más amplio después de una estancia en la UCI (6). Las repercusiones del PICS no solo afectan a la salud y mortalidad del paciente, sino que también generan implicaciones económicas a nivel individual, familiar y social. Este impacto económico intensifica aún más la gran necesidad de investigar y comprender mejor las implicaciones a largo plazo de la enfermedad crítica y el PICS (7).

Disfunción pulmonar crónica

La necesidad de soporte ventilatorio es uno de los principales motivos de ingreso en la UCI, ya sea debido a una lesión pulmonar o a un fallo ventilatorio. Esta estrategia, implica la aplicación de presiones positivas en el pulmón de aquellos pacientes que no pueden mantener de forma adecuada un intercambio gaseoso, siendo por lo tanto esencial para su supervivencia. Sin embargo, estas presiones también pueden ocasionar una lesión en el parénquima pulmonar que, sumada a la enfermedad pulmonar subyacente del paciente puede desencadenar una serie de mecanismos patológicos que conllevan a la transición desde una lesión pulmonar aguda hacia una enfermedad pulmonar crónica. Muchos de los pacientes con lesión pulmonar aguda y necesidad de ventilación mecánica van a desarrollar una función respiratoria anómala, con fibrosis pulmonar cicatricial (2,8).

En este contexto, la inflamación y la remodelación de la matriz, inicialmente encaminados a la resolución de la lesión pulmonar aguda, pueden desempeñar un papel patogenético relevante en enfermedades pulmonares crónicas (9). Durante la lesión pulmonar aguda se liberan un conjunto de moléculas profibróticas que conllevan al remodelado de la matriz extracelular, dando lugar al desarrollo de fibrosis. Paralelamente, existe una respuesta inflamatoria local que desencadena la disfunción de la barrera epitelial y la modificación de la composición celular, lo que, a su vez, contribuye a la activación de mecanismos de transición epitelio-mesénquima. Este proceso, que implica la transformación de células epiteliales en células con características mesenquimales, favorece la

acumulación de tejido fibroso, afectando a la compliancia pulmonar y al intercambio gaseoso (10–12).

Otro mecanismo patogenético activado durante la lesión pulmonar aguda que se ha vinculado con la disfunción pulmonar crónica es la activación de la cascada de la coagulación y el depósito de fibrina. Se ha demostrado que los pacientes con enfermedades pulmonares intersticiales presentan una expresión aumentada de factores procoagulantes dentro del pulmón, entre los que se encuentran algunas proteasas como la trombina, tripsina y catepsinas. Estas activan los receptores activados por proteasas (PARs), desencadenando una serie de eventos de señalización que incluyen la expresión de factores de crecimiento profibróticos y liberación de citocinas proinflamatorias que conducen al desarrollo de la fibrosis (13).

Por último, durante la enfermedad pulmonar crónica, se observa un agotamiento progresivo de las células madre. Este fenómeno se relaciona con un incremento del daño en el ADN y la consecuente activación de la senescencia celular. Las células madre senescentes tienen una capacidad regenerativa reducida y una secreción aumentada de moléculas inflamatorias y remodeladoras de la matriz que perpetúan la enfermedad pulmonar crónica (14).

Deterioro cognitivo

El sistema nervioso central (SNC) recibe señales de aferencias neurales y sustancias circulantes que atraviesan la barrera hematoencefálica. La vía principal para estas señales ascendentes se vehiculiza a través del nervio vago, cuyas fibras aferentes se originan en los

órganos periféricos. Diferentes estructuras sensitivas del nervio vago responden a factores como el estiramiento (15) y la inflamación (16,17), desencadenando complejas vías multisinápticas en el SNC. Por ejemplo, el estiramiento pulmonar activa receptores pulmonares que, a través del nervio vago, influyen en la señalización del cerebro y desencadenan la apoptosis en neuronas del hipocampo.

Durante la enfermedad crítica también existe una traslocación de moléculas y células circulantes al cerebro. Se ha evidenciado que algunas moléculas como los fragmentos de heparán-sulfato (3), liberados durante la sepsis, o citoquinas proinflamatorias como IL-1β e IL-6 (18) pueden llegar al cerebro e intensificar el daño cerebral. Es importante señalar que este proceso se agrava con la respuesta inflamatoria sistémica, la cual reduce la permeabilidad de la barrera hematoencefálica.

Varios estudios preclínicos han identificado algunos de los mecanismos por los cuales la lesión aguda y la ventilación mecánica influyen en el deterioro cognitivo a largo plazo (19,20). En el ámbito clínico, se han constatado mayores marcadores de neuroinflamación y lesión cerebral, así como puntuaciones cognitivas más bajas, en sujetos con duraciones prolongadas de ventilación mecánica. Además, se ha observado que el delirio en pacientes con ventilación mecánica puede asociarse con un deterioro cognitivo a largo plazo. Sin embargo, hacen falta más estudios para establecer el vínculo causal entre la ventilación mecánica, disfunción cognitiva y lesión cerebral, los cuales permitirían desarrollar intervenciones clínicas más efectivas (21).

Debilidad muscular adquirida

La ICUAW es una alteración neuromuscular bilateral y simétrica que afecta a pacientes ingresados en la unidad de cuidados intensivos, siendo muy común en aquellos que reciben ventilación mecánica (22). Estos pacientes experimentan atrofia muscular, que comienza poco después de la admisión en la UCI (23). Factores como la inflamación sistémica, la gravedad de la enfermedad subyacente, el uso de relajantes musculares y la ventilación mecánica contribuyen al desarrollo de la atrofia muscular (24–26). A pesar de que las herramientas de evaluación disponibles no pueden distinguir con precisión entre las causas específicas de la debilidad muscular, el termino de ICUAW se utiliza para describir este síntoma independientemente de su origen (27).

La inflamación sistémica supone uno de los mecanismos moleculares cruciales en el desarrollo de esta secuela. Este proceso se manifiesta a través de la acción de citocinas inflamatorias, siendo TNFα e IL-1β dos de las moléculas implicadas (24). En las fases agudas de la enfermedad crítica, estas citocinas desencadenan una respuesta inflamatoria que va más allá de su función homeostática habitual. El TNFα estimula el catabolismo muscular al interactuar con su receptor y activar el factor nuclear-κB, que induce la expresión de enzimas proteolíticas, resultando en la reducción de proteínas musculares y la pérdida específica de la cadena pesada de miosina (28). Además, la IL-1β, cuyos niveles suelen estar elevados en el suero de los pacientes críticos, se vincula con la perdida proteica y la atrofia muscular, impactando tanto en la síntesis como en la degradación de proteínas musculares (29,30). Este proceso inflamatorio no solo se limita

a la fase aguda, sino que también puede afectar la capacidad de regeneración muscular a largo plazo. Se ha observado en biopsias musculares de pacientes críticos que el fracaso en la regeneración del tejido está asociada con el posible agotamiento de las células madre (4).

Además de la inflamación y el agotamiento de las células madre, existen otros mecanismos que contribuyen a la debilidad muscular persistente en estos pacientes. Se observa que estos presentan daño sensitivomotor distal atribuido a una despolarización de la membrana y una disfunción de los canales iónicos de las neuronas motoras. Asimismo, se evidencia una alteración en la homeostasis del calcio (31). El calcio es un regulador clave en varias funciones metabólicas, incluyendo la síntesis proteica y los procesos mitocondriales. Por lo tanto, cualquier alteración en su metabolismo puede afectar negativamente tanto al balance energético celular como al equilibrio proteico del tejido muscular. La regulación anormal de la autofagia, que desempeña un papel fundamental en la eliminación de componentes celulares dañados y en la preservación de la estructura y función muscular, también puede influir en la debilidad muscular persistente (32).

MECANISMOS MOLECULARES DEL PICS

Las diferentes manifestaciones del PICS comparten una serie de factores predisponentes comunes entre los que se incluye, como ya se ha comentado, la gravedad de la lesión inicial, el tratamiento y soporte orgánico recibido, y la estancia prolongada en la UCI (5). Esta coincidencia sugiere la posibilidad de que la afectación multiorgánica en el PICS responda a una serie de mecanismos sistémicos comunes. Las estrategias actuales abordan las secuelas de manera fragmentada, centrándose en órganos específicos y no considerando el síndrome en su totalidad. Un enfoque más complejo que tome en cuenta la interacción entre los distintos órganos proporcionaría una base más sólida para identificar tratamientos más efectivos en estos pacientes (33).

Hasta la fecha, no se han encontrado mecanismos causales claros sobre los que actuar. La investigación centrada en estos mecanismos biológicos podría ofrecer la posibilidad de prevenir todo el espectro del PICS al intervenir en reguladores ubicados en las etapas iniciales de estos procesos desde el inicio de la fase aguda de la enfermedad. En otras palabras, al comprender y actuar en los reguladores primarios de estos mecanismos, se abriría la puerta a estrategias preventivas más efectivas para mitigar las secuelas a largo plazo en los pacientes críticos (34). En este contexto se exploran tres posibles mecanismos moleculares subyacente al PICS: la inflamación, la mecanotransducción y la senescencia celular. Este último se abordará con más detalle en el siguiente capítulo.

Inflamación sistémica

La inflamación es una reacción normal a una herida, lesión o infección. Esta agresión desencadena la síntesis y liberación de sustancias químicas que producen una respuesta inmunitaria para combatir la infección o sanar el tejido dañado y termina una vez que se cura la herida o la infección (35).

La agresión tisular resulta en la liberación de los denominados patrones moleculares asociados al daño (DAMPs, "Damage-associated molecular patterns") o patrones moleculares asociados a patógenos (PAMPs, "Pathogen-associated molecular patterns"). La interacción de estas familias de moléculas con sus receptores desencadena una respuesta inflamatoria sistémica, denominada síndrome de respuesta inflamatoria sistémica (SIRS, *systemic inflammatory response syndrome*), caracterizada por una liberación de mediadores pro-inflamatorios como factores de crecimiento (G/GM-CSF, FltL) y citocinas (IL-1, IL-6, IL-7), además de células inmunes y mesenquimales (36). Simultáneamente, existe una respuesta anti-inflamatoria compensatoria denominada síndrome de respuesta antiinflamatoria compensatoria (CARS, *compensatory anti-inflammatory response syndrome*), llevada a cabo principalmente por células supresoras mieloides (MDSCs, "Mieloid-derived suppressor cells") que liberan citocinas anti-inflamatorias como IL-10 y TGFβ y antagonistas de citocinas que disminuyen la inflamación como IL-1ra y sTNFRI (37). Estas dos respuestas trabajan en conjunto para mantener el equilibrio homeostático en el sistema inmunológico (38).

Durante las enfermedades graves, como por ejemplo la sepsis, la liberación continua de DAMPs y PAMPs mantiene una respuesta inflamatoria sostenida que supera el equilibrio entre SIRS y CARS. Los granulocitos liberados desde la médula ósea se desplazan a los sitios de la lesión. Una vez allí, no solo participan en la respuesta inmune directa, sino que además liberan factores inflamatorios estimulantes de células madre hematopoyéticas (HSC, "Hematopoietic stem cells"). Las HSC, que normalmente son quiescentes, se activan en respuesta al estrés inducido por la situación crítica. Esto da lugar a la mielopoyesis de emergencia, un proceso en el que las HSC se diferencian preferentemente hacia líneas mieloides. Como resultado, se observa un aumento de granulocitos, macrófagos y células dendríticas, y una supresión de linfopoyesis y eritropoyesis (39).

La activación redundante y sostenida de las HSC resulta en la creación de poblaciones mieloides inmaduras. Entre estas poblaciones, destacan las MDSC, las cuales se caracterizan por infiltrar en tejidos linfoides y reticuloendoteliales y suprimir principalmente la función de células del sistema inmune adaptativo. Tanto la mielopoyesis de emergencia como la formación de MDSC son componentes normales de la respuesta inmune, destinados a controlar la inflamación y prevenir el daño tisular excesivo. Sin embargo, su regulación se vuelve disfuncional en condiciones de inflamación crónica, y pueden contribuir a la coexistencia de una inmunosupresión sostenida y un mantenimiento de este estado inflamatorio. Esta situación crea un entorno propicio para las infecciones nosocomiales, reactivaciones virales y la necesidad continua de intervenciones intensivas, lo que aumenta aún más este estado de

inflamación persistente (40). Los efectos resultantes son numerosos, incluyendo el establecimiento de un entorno inmunológico similar al de una persona de edad avanzada, denominado inmunosenescencia. Se observan cambios en la población de linfocitos, con apoptosis celular de células T y B efectoras, así como cambios en la función de células mieloides y dendríticas (41,42).

Asimismo, la coexistencia de una actividad proinflamatoria y su inmunosupresión acompañante pueden alterar la regulación de los procesos metabólicos, lo que se manifiesta en un aumento del catabolismo y, como consecuencia, a la pérdida de tejido muscular esquelético. La liberación de citocinas proinflamatorias como TNFα, IL-1 e IL-6 está asociada con la activación de la glicólisis, lipolisis y proteólisis. Durante situaciones de estrés, este aumento del catabolismo es necesario para la obtención de energía adicional para hacer frente a las demandas. Las reservas hepáticas de glucógeno se consumen rápidamente para mantener los niveles de glucosa en sangre y suministrar energía a los órganos vitales, especialmente al sistema nervioso central. La glucosa no es suficiente para satisfacer esta alta demanda de energía, por lo que simultáneamente se recurre a la lipolisis y la proteólisis, principalmente en el músculo esquelético, para liberar lípidos y aminoácidos, que actúan como fuentes de energía alternativas. Como consecuencia, estos pacientes experimentan una pérdida de tejido muscular esquelético, como se comentó anteriormente, así como una disminución de peso corporal (43,44).

Todo lo anteriormente expuesto sugiere que es probable que se requiera una combinación de terapias, que incluya agentes antiinflamatorios, adyuvantes inmunológicos, apoyo nutricional y físico en

los pacientes críticos (37), con el fin de bloquear, revertir o atenuar las respuestas no adaptativas a la agresión.

Mecanotransducción

Como se ha mencionado previamente, la ventilación mecánica es una estrategia de soporte vital que, mediante la aplicación de presiones positivas en el pulmón, favorece el intercambio gaseoso. La aplicación de estas presiones en el parénquima pulmonar puede generar un daño conocido como lesión pulmonar inducida por ventilación (VILI, *ventilator-induced lung injury*). La ventilación mecánica puede provocar daño en el parénquima pulmonar a través de tres mecanismos fundamentales (45):

- Barotrauma/Volutrauma: La ventilación con volúmenes o presiones elevadas al final de la inspiración puede provocar rotura alveolar, fugas de aire y edema pulmonar debido a la sobredistensión regional.
- Atelectrauma: La ventilación con volúmenes o presiones bajas al final de la espiración también conlleva la apertura y cierre repetitivos de alveolos y pequeñas vías aéreas, generando hipoxia regional, deterioro de la función del surfactante y formación de membranas hialinas.
- Biotrauma: Las fuerzas físicas aplicadas durante la ventilación mecánica pueden desencadenar, a través de procesos de mecanotransducción, una respuesta bioquímica en el pulmón que puede tener implicaciones tanto a nivel local como a nivel sistémico.

Se entiende por mecanotransducción la capacidad de las células para transformar estímulos mecánicos en señales bioquímicas. El epitelio respiratorio está expuesto a presiones positivas en cada ciclo ventilatorio, por lo que en condiciones basales, este proceso constituye un mecanismo de adaptación celular al entorno, desempeñando un papel clave en la regulación de la estructura, función y metabolismo pulmonar (46). En el caso del pulmón sometido a ventilación mecánica, este mecanismo es particularmente importante, ya que las presiones positivas aplicadas durante la ventilación pueden activar distintas respuestas celulares patológicas, como inflamación (47), apoptosis (48) o remodelado de la matriz extracelular (49).

La detección del estímulo mecánico puede ocurrir a través de distintas vías. Existe una variedad de elementos mecanosensibles en la membrana celular, como receptores de factores de crecimiento, incluyendo receptores tipo tirosina-quinasa y receptores acoplados a proteínas G, así como canales iónicos de Ca2+, incluyendo los canales Piezo (50), que, una vez que detectan el estímulo mecánico, activan cascadas químicas de señalización. Estas señales se transmiten por difusión química o translocación hasta llegar al núcleo.

Adicionalmente, existe otra vía de transducción de señales mucho más rápida, en la cual las fuerzas mecánicas aplicadas en la superficie celular promueven reorganizaciones estructurales en profundidad en el citoplasma y el núcleo (46). Las células están unidas a la matriz extracelular mediante integrinas y cadherinas ancladas a la membrana plasmática, y conectan con el citoplasma a través de toda una red de proteínas filamentosas del citoesqueleto, siendo la actina, los microtúbulos

y filamentos intermedios los más relevantes. Estos se unen al complejo de unión del nucleoesqueleto y citoesqueleto (LINC, "Linker of nucleoskeleton and cytoskeleton"), que representa el enlace del citoesqueleto con el nucleoesqueleto. Este complejo se une a las laminas, filamentos intermedios que forman una red estructural en la cara interna de la membrana nuclear. La lámina nuclear se une a la cromatina a través de interacciones con dominios específicos, llamados dominios asociados a la lamina (LADs, *lamin-associated domains*) (51). Esta unión regula la actividad transcripcional de la célula al influir en la posición y compactación de la cromatina.

Las alteraciones en cualquiera de los componentes de este proceso biológico, que es esencial para la regulación de la expresión génica, podrían contribuir al desarrollo de diversas patologías. Por ejemplo, las integrinas (52) o proteínas encargadas en la regulación del citoesqueleto de actina, como las ROCK quinasas (53), se han visto implicadas en la progresión tumoral. Asimismo, una regulación inadecuada de los factores de transcripción YAP/TAZ (50), también involucrados en esta vía, pueden conducir a una sobreproducción de matriz extracelular y una activación excesiva de fibroblastos, además de interferir en la reparación tisular, contribuyendo todo ello al desarrollo de la fibrosis. Por lo tanto, la comprensión detallada de sus interacciones moleculares y estructurales es fundamental para avanzar en el desarrollo de terapias más precisas.

Los mecanismos de mecanotransducción en el contexto de la ventilación mecánica pueden tener relevancia en diferentes situaciones patológicas. La transmisión de la presión positiva a nivel alveolar aumenta la rigidez nuclear, activando la apoptosis del epitelio. En relación con esta

observación, el bloqueo de la mecanotransducción nuclear interfiriendo con la lámina nuclear logra preservar la integridad celular en modelos de daño pulmonar agudo (54).

También se ha explorado el impacto de la ventilación mecánica en tumores pulmonares. El estrés mecánico durante la ventilación aumenta la invasividad de las células cancerosas al reducir el colesterol intracelular. El colesterol juega un papel crucial aumentando la rigidez celular, lo que limita la capacidad de las células para migrar. Por lo tanto, su disminución bajo condiciones de estrés mecánico promueve la migración celular. En contraste, la intervención terapeútica sobre uno de los componentes del metabolismo del colesterol, PCSK9, revierte el incremento en la invasividad por el estrés mecánico. Esto pone de manifiesto la importancia del metabolismo del colesterol en la respuesta mecanotransductora, señalándolo como una posible opción terapéutica en este contexto (55).

Estos estudios resaltan la importancia de utilizar este mecanismo como una posible diana para el tratamiento de enfermedades asociadas al uso de la ventilación mecánica. En este contexto, varias rutas de señalización relacionadas con la mecanotransducción como Rho/ROCK, TGFβ/Smad, JAK/STAT y Wnt/β, podrían ofrecer múltiples oportunidades terapéuticas (50). Sin embargo, estas rutas están interconectadas y estrechamente reguladas en condiciones normales, manteniendo así la homeostasis celular. Asimismo, al tener múltiples dianas celulares, su manipulación puede ser inapropiada, resultando en respuestas no deseadas y ocasionando efectos sistémicos. Todo esto pone de manifiesto la importancia en el desarrollo de terapias dirigidas a procesos más específicos cuya acción terapéutica se limite a la región de interés. En este

contexto, una alternativa sería utilizar como diana terapéutica las respuestas celulares que se originan como consecuencia de los cambios en la mecanotransducción, las cuales podrían derivar en consecuencias patológicas a largo plazo. Estudios recientes muestran una conexión entre la mecanotransducción y la posterior inducción de la respuesta senescente (56). Esta asociación establece la posibilidad de utilizar este proceso biológico como posible diana terapéutica. Es importante destacar que este mecanismo también puede estar desencadenado por un aumento de la inflamación, que como se mencionó anteriormente es de gran relevancia en el paciente crítico, lo cual hace aún más interesante estudiar la senescencia en este contexto.

SENESCENCIA CELULAR

Se define senescencia como un estado celular en el que existe una detención permanente del ciclo celular acompañado de alteraciones transcriptómicas y cambios morfológicos, metabólicos y epigenéticos, unidos a la secreción de una serie de factores característicos, denominado fenotipo secretor asociado a senescencia (SASP) (57). El concepto de senescencia fue definido inicialmente por Leonard Hayflick y Paul Moorhead (58), a partir de experimentos realizados en condiciones controladas de cultivo utilizando fibroblastos humanos normales. El objetivo de estos estudios era determinar si las células normales eran inmortales bajo las condiciones adecuadas, como se consideraba en esa época. Sin embargo, los resultados de estos experimentos mostraron que los fibroblastos se duplicaban un numero finito de veces antes de dejar de dividirse. Este hallazgo supuso un cambio de paradigma en la comprensión de la replicación celular y dio lugar a la introducción del término "Límite de Hayflick" (59), que establecía que las células normales solo podían dividirse un número limitado de veces.

Después de estos descubrimientos, las investigaciones se centraron en buscar el mecanismo por el cual estas células dejaban de dividirse. Los telómeros son extremos de ADN lineal que no se pueden copiar completamente durante la replicación celular. Esta característica llevó a considerar a los telómeros como elementos cruciales para explicar la limitada capacidad replicativa de las células normales (60). El descubrimiento posterior de la telomerasa, una enzima que alarga los

telómeros, proporcionó una solución a este problema. Mientras que el acortamiento de los telómeros desencadenaba la senescencia celular, la introducción de la telomerasa permitía superar esta limitación (61).

Desde el hallazgo de Leonard Hayflick y Paul Moorhead, son muchos los estudios que han revelado que este proceso celular puede estar desencadenado por una gran variedad de estímulos tanto intrínsecos y extrínsecos y que la senescencia inducida por el acortamiento de los telómeros es solo un subtipo de senescencia. Entre estos estímulos se encuentran la radiación ionizante (62) o el uso de agentes quimioterapéuticos como la doxorrubicina (63), que causa roturas de doble cadena en el ADN, la disfunción mitocondrial (64) y la consiguiente inducción de especies reactivas de oxígeno (ROS, "Reactive oxygen species") o la activación de oncoproteínas (65) que llevan a patrones de replicación aberrantes, inflamación y/o señales de daño tisular. Por otro lado, la senescencia es un componente fundamental para el desarrollo, ya que esta contribuye a la eliminación de estructuras embrionarias transitorias o la sustitución de una población celular por otra (66).

Por lo tanto, podríamos decir que existen tres tipos de senescencia en función del estímulo desencadenante: senescencia replicativa dependiente de telómeros (58), senescencia prematura (67) inducida por estrés no telomérico o senescencia programada (68).

Mecanismos bioquímicos de la senescencia

Como ya se ha definido, las células senescentes se caracterizan por la parada del ciclo celular, la liberación de un secretoma característico y cambios estructurales y metabólicos. Los mecanismos moleculares implicados en cada uno de estos fenómenos son complejos, y en ocasiones se interrelacionan entre sí.

1. Daño en el ADN y parada de ciclo

Uno de los principales desencadenantes de una respuesta senescente es el daño en el ADN y la activación de una respuesta característica (DDR, *DNA damage response*). La rotura de la doble hélice de ADN da lugar al reclutamiento de la quinasa ATM al sitio de daño, impulsando la fosforilación de la histona H2AX y facilitando la formación de complejos de reparación de daño en el ADN. Esta activación de la DDR va acompañada de modificaciones epigenéticas, como la metilación de la histona H3 en su residuo K9, que facilita la unión de la quinasa ATM al sitio de daño y se revierte justo después de que se produzca esta unión. Posteriormente ATM fosforila diferentes sustratos, incluyendo las quinasas CHK1 y CHK2. Estas quinasas son puntos de control del ciclo celular, ya que al activarse lo detienen temporalmente, facilitando la reparación del ADN (69). Si la DDR es persistente, la proteína p53 es fosforilada y como consecuencia las células evolucionan hacia un estado apoptótico o senescente.

El destino celular dependerá de distintos factores como la naturaleza y severidad del daño, la programación específica del tipo celular, el

equilibrio entre las vías pro-senescentes y pro-apoptóticas, y de la señalización de otras vías como PTEN-PI3K-AKT-mTOR. Esta vía desempeña un papel muy importante en el control del crecimiento y la supervivencia celular. Mientras que, en condiciones normales, hay una expresión normal de la fosfatasa PTEN que permite la progresión del ciclo celular, en condiciones de estrés, como puede ser la falta de nutrientes, PTEN es activada para inhibir la actividad de PI3K y por lo tanto reducir la activación de AKT y mTOR. La inhibición de estas proteínas da lugar a una parada del ciclo celular y en algunos casos apoptosis. Sin embargo, en ciertas situaciones de estrés celular donde hay una acumulación excesiva de ROS, se puede producir una desregulación de la vía. Las ROS pueden inactivar PTEN de manera permanente, lo que resulta en una activación persistente de mTOR, desencadenando la senescencia celular (70).

En las células comprometidas con una respuesta senescente, p53 activa el inhibidor de quinasa dependiente de ciclina p21. Esta proteína de 21kDa, codificada por el gen CDKN1A, inhibe la formación de complejos ciclina D-quinasa dependiente de ciclinas 4 y 6. Como resultado, la proteína RB permanece defosforilada, lo que le permite asociarse con el factor E2F y formar el complejo RB-E2F que inhibe la transcripción de los genes del ciclo celular. Asimismo, la activación de p21, inhibe otros complejos ciclina-quinasas dependientes de ciclinas y facilita la formación del complejo DREAM, que reprime los genes del ciclo celular al unirse a su región homologa (71,72). Por lo tanto, ambos son complejos represores de la expresión génica del ciclo celular. Mientras que el complejo RB/E2F regula la entrada y salida de las células en el ciclo celular al suprimir la expresión de genes necesarios para la replicación del ADN y la entrada en

la fase S, el complejo DREAM regula la expresión de genes específicos durante varias fases del ciclo, incluyendo fase G1, S y G2 (73,74). Por lo tanto, p21, a través de la regulación de estos complejos, es capaz de inducir un arresto del ciclo celular en cualquier etapa del ciclo. Además, la expresión de p21 no persiste en las células senescentes ya que simplemente se requiere para la inducción de la senescencia (75).

Cabe destacar también que el control transcripcional de p21 puede darse por mecanismos independientes de p53 y además puede estar regulada al igual que p53 a nivel postraduccional. La fosforilación de p21 en residuos diferentes a los regulados por p53 puede modular su interacción con otras proteínas y cambiar su ubicación subcelular, alterando su función como regulador del ciclo celular. Cuando p21 está presente en el núcleo, inhibe la progresión del ciclo celular, mientras que al ser fosforilado en residuos diferentes a los que lo hace p53, hace que esta proteína se transporte al citoplasma donde funciona como una proteína antiapoptótica (76).

2. Parada de ciclo independiente de DDR

La senescencia puede estar inducida por señales independientes a la DDR mediante la activación de la vía p16/pRB. Esta vía, se activa fundamentalmente por alteraciones epigenéticas y, a diferencia de p21, precisa de una activación sostenida para mantener el estado senescente. P16 es una proteína de 16kDa, codificado por el gen CDKN2A, que interactúa e inactiva complejos ciclina/CDK, evitando la fosforilación de pRB y promoviendo por lo tanto la formación del complejo represor

RB/E2F. Cabe destacar que esta proteína solo se une y desactiva específicamente a CDK4 y CDK6, induciendo un arresto del ciclo celular específico en fase G0/G1(77–79).

Los mecanismos bioquímicos de senescencia celular inducidos por la activación de la DDR y por señales independientes a la DDR se muestran en la Figura 1.

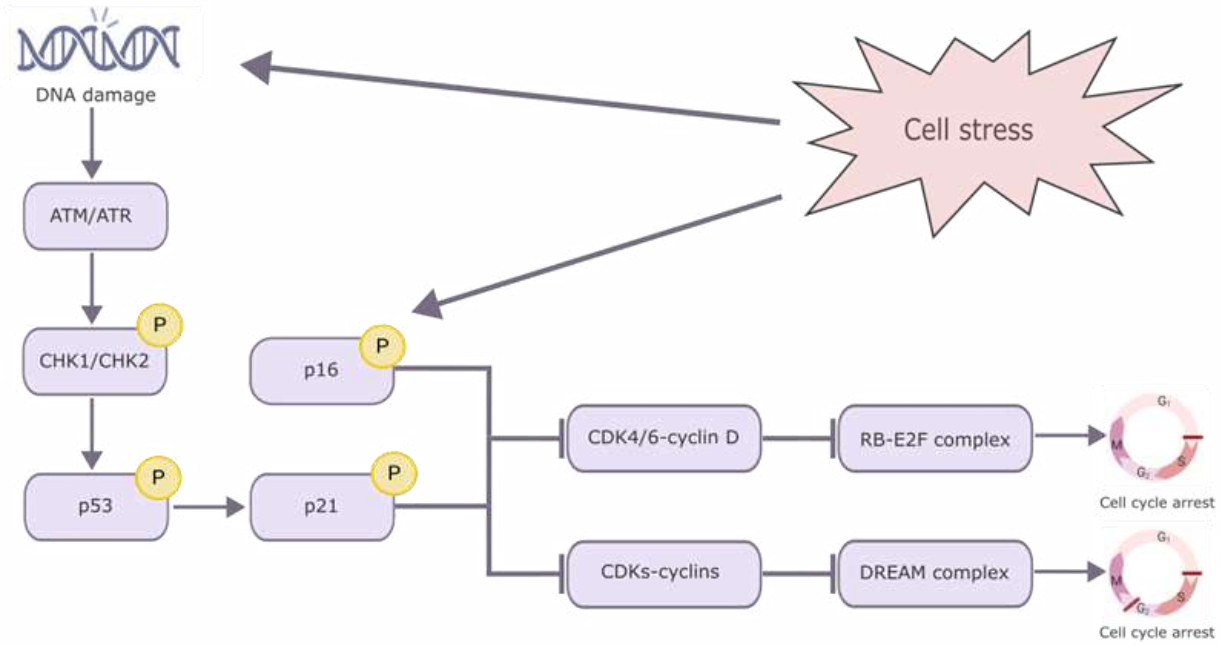


Figura 1. Mecanismos bioquímicos de senescencia celular.

Por último, hay que mencionar que existen otros inhibidores de quinasas dependientes de ciclinas, como p27 y p15, que también desempeñan funciones críticas en la regulación del ciclo celular y

senescencia. P15 pertenece a la misma familia de CDKIs que p16 e inhibe a CDK4/6, mientras que p27 pertenece a la misma familia p21 y por lo tanto interactúa con varios complejos de CDKs, contribuyendo de esta forma a la detención del ciclo celular. Aunque estas quinasas pueden ser utilizados como marcadores de senescencia, su participación en el programa no está claramente definida ni es tan generalizada (57,80).

3. Fenotipo secretor asociado a senescencia

Las células senescentes secretan un conjunto de moléculas bioactivas que incluye citocinas, quimiocinas, factores de crecimiento, proteasas y lípidos al medio extracelular. Este conjunto de moléculas se conoce como SASP, siendo este el principal causante de los efectos pleiotrópicos de la senescencia. Cuando se produce una lesión, las células del tejido dañado experimentan daño en el ADN y como consecuencia activan la DDR. Como parte de esta respuesta, algunas células se vuelven senescentes y liberan un SASP que permite el reclutamiento del sistema inmune a sitios de lesión, promoviendo así la reparación del tejido. Sin embargo, en un contexto patológico, donde las células no son eliminadas correctamente, se produce un incremento de sus componentes que puede tener consecuencias patogenéticas (80).

Un SASP excesivo está asociado con una inflamación descontrolada que puede contribuir al desarrollo de distintas alteraciones como la fibrosis, al promover una activación de fibroblastos, o la progresión tumoral, al promover un aumento de la angiogénesis. Además, algunos de

sus componentes pueden interferir en la capacidad regenerativa de las células, comprometiendo el mantenimiento de la homeostasis tisular (81).

Asimismo, el SASP puede inducir y reforzar la senescencia mediante señalización autocrina y paracrina. Las señales del SASP confieren resistencia a las células senescentes a ser eliminadas por el sistema inmune. Por ejemplo, las citocinas proinflamatorias del SASP, pueden llevar a un aumento en la expresión de ligandos como el HLA-E, que pueden interferir con la función de las células T CD8+ y las NKs, inhibiendo las respuestas inmunes contra las células senescentes (82). Además, el SASP tiene la capacidad de inducir senescencia en otras células del entorno, lo que se conoce como senescencia paracrina. Como resultado se establece un bucle de retroalimentación positiva que perpetua la senescencia (66).

El SASP se regula a diferentes niveles. A nivel transcripcional, este evento es mediado por distintos factores de transcripción como GATA4, CEBPβ y NF-κB. Este último, cuya activación se desencadena principalmente por el DDR, es de especial importancia al ser necesario para la expresión de numerosos genes proinflamatorios. Además de estos factores de transcripción, p53 también desempeña un papel crucial en el desarrollo del SASP al modular la expresión de muchos de sus genes e interactuar y coordinar otras vías asociadas como NF-κB (83,84).

La transcripción de genes del SASP también pueden depender de cambios epigenéticos. Durante la senescencia se produce una disminución de la marca represiva H3K9me2 en los promotores de los genes *IL-6* e *IL-8*, lo que promueve su expresión (85). Asimismo, existe un aumento de la acetilación en estos mismos promotores, causado por la disminución de la

histona deacetilasa SIRT1, que también estimula la activación de la expresión (86). Otras proteínas como macroH2A1 (87) o la HMGB2 (88) también son importantes en la regulación epigenética puesto que contribuyen al mantenimiento de la estructura tridimensional de la cromatina para la expresión adecuada de los genes del SASP.

A nivel postranscripcional, mTOR juega un papel fundamental al promover la traducción de IL1A, activando así los factores de transcripción NF-κB y CEBPβ, que a su vez estimulan la expresión de genes asociados con el SASP (89). Además, mTOR inhibe indirectamente a la proteína ZFP36L1, encargada de la degradación del ARNm que codifica factores del SASP, prolongando de esta forma la respuesta (90).

Por último, hay que destacar que la composición del SASP es altamente variable, dinámica y depende del tipo de célula, tejido y contexto ambiental en el que se encuentre. Por este motivo, establecer un único SASP como marcador confiable de senescencia supone un gran desafío. Aunque la hipersecreción puede ser un indicador útil de senescencia, las investigaciones actuales se centran en conocer los factores específicos que cada tipo de célula secreta para poder caracterizar los distintos programas de senescencia en función del contexto biológico en el que se encuentre.

4. Aumento del contenido lisosomal

Durante la senescencia, se produce una sobreexpresión de diversas proteínas lisosomales acompañada de un aumento en el contenido lisosomal. Este fenómeno puede atribuirse tanto a la acumulación de lisosomas antiguos por alteraciones en el proceso de eliminación,

evidenciado por la presencia de cuerpos residuales o lipofuscinas, como al aumento en la formación de nuevos lisosomas, aunque los estudios que respalden esta idea aún son contradictorios.

Este incremento del contenido lisosomal puede determinarse mediante la identificación de la enzima lisosomal β-galactosidasa asociada a senescencia (SA-βgal, *Senescence-associated beta-galactosidase*). En condiciones normales, la βgal actúa a un pH más alto que durante la senescencia, donde hay una acidificación de los lisosomas, siendo la SA-βgal activa a pH más bajo. Esta característica es esencial para diferenciar entre células senescentes y no senescentes (91,92).

Aunque esta enzima es ampliamente utilizada para la determinación de la senescencia, no se puede utilizar como único marcador puesto que también puede encontrarse en otros contextos. Además, no se puede utilizar ni en tejidos embebidos en parafina ni en células vivas debido a las restricciones asociadas al pH requerido para la detección de su actividad (93).

5. Remodelación de la cromatina

Las células senescentes experimentan cambios adicionales en su estructura, siendo uno de ellos la remodelación de la cromatina. Estos cambios desempeñan un papel crucial en la configuración del perfil transcriptómico de estas células (80).

Entre estos cambios se destaca la formación de los denominados focos de heterocromatina asociados a senescencia (SAHF, *Senescence-*

associated heterochromatin foci). Estas regiones están enriquecidas con marcas epigenéticas represoras, como H3K9me3 y HP1γ (94,95), que se utilizan comúnmente como marcadores de senescencia. La pérdida de lamina B1 se ha correlacionado con la formación de SAHF (96), al permitir la redistribución y la reubicación de marcas de histonas represoras desde la periferia nuclear hacia estos lugares. Sin embargo, cabe destacar que no es el único determinante para la formación de estas estructuras.

La función de los SAHF es aún algo incierta. Inicialmente se pensaba que estaban involucradas en la represión de genes asociados con la proliferación celular, sin embargo, actualmente se ha indicado que su función puede ser más compleja. Se ha sugerido que los SAHF pueden estar involucrados en la preservación de la estabilidad del genoma de las células senescentes al limitar la señalización del daño en el ADN. Esto podría prevenir que las células senescentes con altos niveles de daño en el ADN entren en apoptosis, preservando así su viabilidad (97).

Otro de los cambios observados en la cromatina que precede a la formación de SAHFs es la distensión de satélites asociada a senescencia (SADS, "*Senescence-associated distension of satellites*"). Este fenómeno implica una descondensación de la heterocromatina constitutiva en las secuencias satélite pericentroméricas. A diferencia de los SAHFs, estas regiones conservan marcas epigenéticas canónicas y no están directamente desencadenadas por vías de señalización molecular asociadas a la senescencia celular, lo que indica que se puede dar en otros contextos (98).

6. Cambios metabólicos

Las células senescentes son metabólicamente muy activas. Este estado está regulado por diversos factores como AMPK, un sensor energético que se activa cuando los niveles de energía de la célula son bajos, desencadenando en las células distintos procesos para generar energía. A su vez, existen otros factores como la disfunción mitocondrial, mTOR y proteínas como RB y p53 que tienen también un papel importante en el mantenimiento del estado metabólico activo de las células senescentes (80).

7. Resistencia a la apoptosis

Otra de las características de las células senescentes es que estas son resistentes a la apoptosis. Estas células aumentan la expresión de proteínas anti-apoptóticas como BCL2 o modulan factores de supervivencia como eNOS o PTEN/PI3/AKT. Otros miembros como FOXO4, p21 y HSP90 también contribuyen a la supervivencia de las células senescentes (70,99).

Además de estos mecanismos, existen multitud de procesos adicionales que varían según el tipo celular y el contexto biológico. A esto hay que añadir que aún no se ha encontrado un marcador universal y específico de senescencia, ya que todos los mecanismos descritos previamente se pueden dar en otros contextos biológicos. Todo ello hace necesario la utilización de un conjunto de marcadores para poder

identificar y cuantificar la senescencia de manera más precisa, lo que supone una limitación para la identificación de tratamientos en base a este proceso.

Senescencia en el paciente crítico

1. Inductores de la senescencia en el paciente crítico

De acuerdo con la definición de la Sociedad Americana de Medicina Intensiva, un paciente crítico es aquel que se encuentra fisiológicamente inestable, y requiere soporte vital avanzado y una evaluación clínica estrecha con ajustes continuos de terapia según evolución (100). Estos pacientes son, por lo tanto, especialmente vulnerables a desarrollar respuestas fisiopatológicas complejas no solo por los mecanismos de daño desencadenados por la enfermedad subyacente, sino también a consecuencia de los tratamientos que reciben.

La respuesta senescente, una de las respuestas fisiopatológicas implicadas, puede estar desencadenada por diversos mecanismos en el paciente crítico, siendo el estrés oxidativo uno de ellos. La generación de ROS puede ocasionar daño en el ADN, activando de esta forma la DDR, que como ya vimos, constituye uno de los mecanismos más relevantes en la inducción de la respuesta senescente. Diversas situaciones contribuyen a la activación del estrés oxidativo, destacando la lesión por isquemia /reperfusión (101), situaciones de hiperoxia (102) o el empleo de la ventilación mecánica (103).

La inflamación es otro de los desencadenantes de la respuesta senescente. En el paciente crítico, ésta toma un papel fundamental puesto que no solo ocurre debido a la condición patológica del paciente, como podría ser una infección (104), sino que también se puede dar como respuesta al tratamiento que este recibe, como es el caso de la inflamación generada por el uso de la ventilación mecánica (47).

2. Dualidad de la senescencia en el paciente crítico

En determinados contextos, la senescencia se considera como una respuesta homeostática esencial tanto para el desarrollo normal de los tejidos como para su reparación tras el daño. Existen tres procesos secuenciales que caracterizan la senescencia programada: primero, la parada del ciclo celular; segundo, una secreción de factores que reclutan células inmunes, como linfocitos T y macrófagos; y tercero, la movilización de células progenitoras cercanas para repoblar el tejido (Figura 2). Este modelo es fundamental para el desarrollo, donde se pueden eliminar estructuras embrionarias transitorias o cambiar una población celular por otra (57).

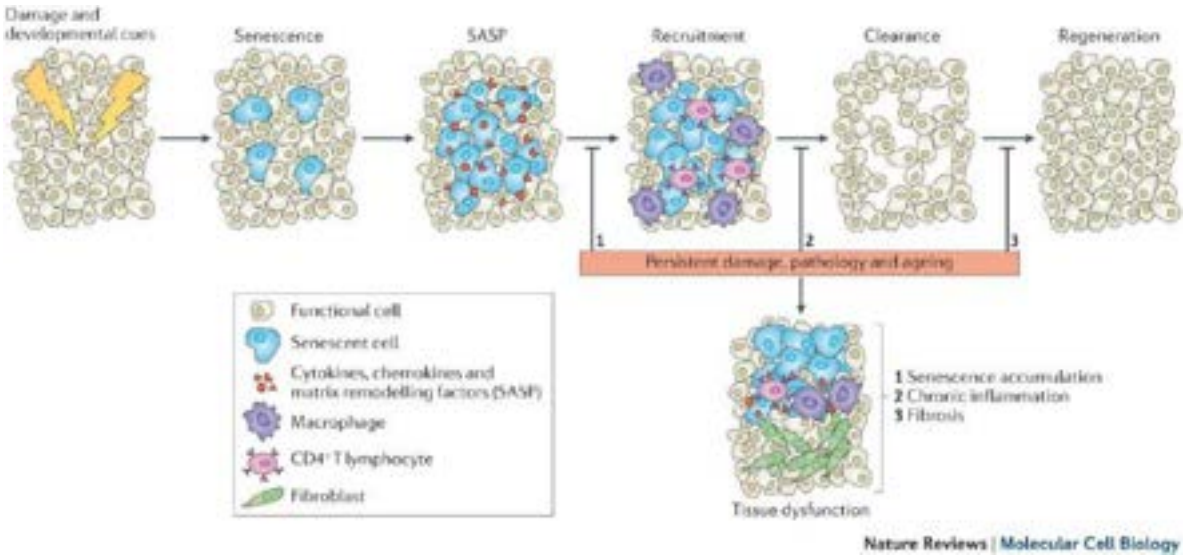


Figura 2. Procesos secuenciales de senescencia celular en la regeneración y disfunción tisular. Tomado de Nat Rev Mol Cell Biol. 2014 Jul;15(7):482-96.

En situaciones de daño tisular en adultos, el modelo de inducción de senescencia, eliminación de células dañadas y regeneración permite la restauración completa del tejido. En el contexto específico del paciente crítico, donde el daño tisular es habitual, ya sea debido a la enfermedad subyacente o a las intervenciones médicas, la senescencia puede desempeñar en algunos casos un papel protector.

La exposición a niveles elevados de oxígeno y la aplicación de ventilación mecánica son prácticas clínicas comunes en pacientes ingresados en la UCI. Se ha demostrado que la hiperoxia conlleva a un aumento de p21 (102) y que este aumento está asociado al mantenimiento de la integridad del ADN durante la fase de reparación celular (105). Asimismo, la modulación de la mecanosensación asociada a la senescencia reduce el daño en el tejido pulmonar tras la ventilación mecánica, además de aumentar los niveles de p21 en la fase de reparación pulmonar (54).

Estos hallazgos sugieren que la activación de la senescencia es un mecanismo protector frente al daño por hipoxia y VILI en el paciente crítico.

En el contexto de la lesión renal, una complicación común en el paciente crítico, se ha observado que la activación de la respuesta senescente limita la proliferación celular excesiva y la inflamación, lo que a su vez ayuda a prevenir el desarrollo de la fibrosis (106). De igual manera, en el ámbito de las infecciones virales agudas, se observa que en algunos casos puede tener un papel beneficioso al mejorar la defensa del huésped mediante el reclutamiento de las células inmunes a través de la liberación del SASP. Estos hallazgos sugieren que la senescencia *a priori* podría desempeñar un papel protector en el contexto de la UCI (107).

Sin embargo, puede ocurrir que los procesos secuenciales que coordinan la senescencia celular no se completen, y que esta, en vez de actuar como un mecanismo homeostático, intensifique aún más la condición patológica del paciente. Este carácter dual de la senescencia se considera un ejemplo de pleiotropía antagónica. El equilibrio entre los efectos beneficiosos y perjudiciales depende de si las células senescentes son de naturaleza transitoria o si se acumulan con el tiempo (57). Distintos factores pueden hacer que la senescencia pase de tener un efecto beneficioso, a contribuir a la cronificación de la enfermedad aguda.

La duración del estímulo es muy importante en este contexto. En condiciones fisiológicas, durante la lesión pulmonar aguda, las células alveolares tipo 2 (ATII) tienen la capacidad de diferenciarse a células alveolares tipo 1 (ATI), lo que favorece la reparación del tejido (108). Sin

embargo, cuando el daño es persistente, estas células pueden evolucionar hacia un estado transicional senescente. Este proceso afecta negativamente a la capacidad de regeneración del tejido y promueve la creación de un entorno profibrótico a través de la liberación de su SASP, lo que impulsa el desarrollo de la fibrosis pulmonar (109).

Otro factor que puede contribuir a la cronificación de la enfermedad es la capacidad del sistema inmune para eliminar las células senescentes. Los pacientes vulnerables como las personas mayores o aquellas con comorbilidades, son más susceptibles a acumular células senescentes debido al deterioro o envejecimiento del sistema inmune, lo que se conoce como "inflammaging" (110). De hecho, en muchas ocasiones este deterioro del sistema inmune puede darse por el efecto de la senescencia *per se*, estableciéndose por lo tanto un bucle de retroalimentación positiva. Por ejemplo, se ha observado que la infección por el virus SARS-CoV-2 favorece, en algunos casos, la acumulación de células senescentes por encima del umbral considerado beneficioso. Esto conlleva a una producción excesiva de factores del SASP, lo que desencadena respuestas inmunológicas dañinas y dificulta la eliminación de células senescentes, contribuyendo a la exacerbación de la patología (42). También puede ocurrir que las células senescentes, mediante la liberación de distintos componentes del SASP, induzcan mutagénesis en el virus SARS-CoV-2, aumentando así su variabilidad genética y confiriéndole capacidad para evadir el sistema inmune del huésped, contribuyendo a la persistencia de la infección (111).

Otro aspecto a tomar en cuenta es la eficiencia en la regeneración celular, que como se ha mencionado anteriormente, es fundamental para

que la senescencia resulte beneficiosa. En algunas ocasiones, como por ejemplo durante el envejecimiento, se observa un agotamiento de las células madre, lo que puede resultar en una regeneración menos efectiva y, en consecuencia, intensificar los efectos perjudiciales de la senescencia (57).

No solo en enfermedades pulmonares, pero también en otras enfermedades como en la lesión renal aguda, se observa qué, aunque *a priori* la senescencia puede tener un papel protector, a largo plazo esta puede ser perjudicial (112).

Senescencia y fibrosis

En los últimos años se ha avanzado de manera significativa en la comprensión de la fibrosis pulmonar, un término que engloba a un grupo de enfermedades pulmonares crónicas, caracterizadas por un depósito progresivo de fibras de colágeno en los pulmones. La fibrosis pulmonar, que puede ser primaria o secundaria a otras patologías, compromete la capacidad de los pulmones para funcionar adecuadamente y en muchos casos, provoca la muerte. La falta de tratamientos efectivos hasta la fecha refleja la necesidad de explorar los mecanismos subyacentes a la enfermedad para de esta forma desarrollar nuevas estrategias de intervención (113).

La senescencia, junto con la secreción del SASP, han emergido recientemente como mecanismos esenciales en el desarrollo de la fibrosis pulmonar. Se ha evidenciado que en enfermedades fibróticas pulmonares crónicas hay una mayor acumulación de células senescentes que contribuyen a la progresión de la fibrosis (114). Varios estudios muestran que pacientes con fibrosis idiopática tienen marcadores aumentados de senescencia celular o mutaciones en genes responsables del mantenimiento de la proliferación celular (115). Además, se ha sugerido que los niveles de biomarcadores senescentes presentes en la circulación sanguínea podrían proporcionar información relevante sobre el estado de la enfermedad (116).

Adicionalmente, el paradigma de la patogénesis de la fibrosis pulmonar idiopática (FPI) ha cambiado, pasando de considerarse una enfermedad causada principalmente por los fibroblastos a una enfermedad originada en el epitelio (117), siendo las células alveolares senescentes, especialmente las ATII, las que desempeñan un papel fundamental en el desarrollo de la misma (118–120). Después de una lesión en el parénquima pulmonar las ATII dañadas pueden adquirir un fenotipo senescente (121,122). En una fase temprana, la senescencia de las ATII, conduce a la secreción de distintos componentes del SASP que pueden participar en la regeneración del tejido (123). Sin embargo, si el daño es persistente, la activación sostenida de la senescencia en el epitelio pulmonar conduce a la secreción de distintos componentes del SASP que promueven una comunicación aberrante epitelio-mesénquima. Esta característica junto con le perdida de la capacidad de regeneración y diferenciación de las ATII senescentes en células ATI, provoca una

tendencia hacia la fibrosis en detrimento de la regeneración del tejido (124).

Las poblaciones celulares senescentes presentes en la FPI liberan distintos componentes del SASP como los factores de crecimiento TGFβ y PDGF, las citocinas TNFα y MIC-1, las metaloproteinasas MMP10 y MMP12, además de otros componentes que promueven la progresión de la fibrosis (125). Un componente interesante en este contexto es el inhibidor del activador del plasminógeno-1 (PAI-1, "*Plasminogen activator inhibitor-1*), que parece estar involucrado en distintos procesos relacionados con la progresión de la fibrosis, como en la síntesis de proteínas de la matriz, la transición epitelio-mesénquima o la activación de macrófagos (126). Asimismo, los distintos factores del SASP también pueden promover senescencia secundaria en otras células ATII y en fibroblastos mediante la generación de estrés oxidativo, inflamación crónica y activación de distintas vías de señalización de manera paracrina (127).

Las células fibrogénicas senescentes, a su vez, mediante la liberación de distintos componentes del SASP, también pueden ser un importante promotor de la fibrosis pulmonar, al inducir la activación de fibroblastos, inflamación y acumulación de la matriz extracelular (128). Asimismo, estas células pueden promover una senescencia celular secundaria en otras poblaciones celulares. Recientemente se ha observado que las vesículas extracelulares (EV, "*Extracellular vesicles*") toman un papel relevante en este contexto. Las EV liberadas por los fibroblastos transportan miRNAs (miR-23n-3p y miR-494-3p) y son capaces de inducir daño mitocondrial, activación de la respuesta al daño en el ADN y la subsiguiente senescencia en células epiteliales (129,130).

Todos estos factores generan un bucle de retroalimentación positiva entre senescencia primaria, senescencia secundaria y fibrosis.

En el caso del paciente crítico, como ya se mencionó previamente, diversos factores como la hiperoxia, la lesión por isquemia/reperfusión o enfermedades respiratorias, como la infección por el SARS-CoV-2, pueden inducir una respuesta senescente. Asimismo, se ha observado que estos factores también pueden contribuir al desarrollo de la fibrosis (101,131,132). Esta coincidencia sugiere la posibilidad de que el desarrollo de la fibrosis pulmonar en el paciente crítico pueda estar asociado con la activación de procesos de senescencia celular.

De hecho, estudios recientes indican que la acumulación de hierro desempeña un papel clave en la activación de la senescencia y el desarrollo de la fibrosis. Esto puede ser muy relevante en el paciente crítico, dado que en ciertas situaciones críticas, como la sepsis o algunas enfermedades respiratorias, se observa un aumento de los niveles de hierro (133).

Senoterapias

Como ya se ha comentado en apartados anteriores, las células senescentes muestran una alta heterogeneidad tanto en su biología molecular como en su función fisiológica. Esta diversidad no solo amplía las posibles dianas terapéuticas, sino que también destaca la necesidad de desarrollar estrategias dirigidas que idealmente preserven las células senescentes en contextos beneficiosos mientras eliminen los efectos perjudiciales.

Los agentes senoterapéuticos pueden estar dirigidos o bien a inducir la apoptosis de las células senescentes (fármacos senolíticos), o bien actuando sobre su fenotipo secretor, modificando el SASP (fármacos senomorfos) (134).

1. Senolíticos de primera generación

Las células senescentes dependen de la activación de vías de supervivencia y antiapoptóticas como mecanismo para evitar la muerte celular. Esta característica ha conllevado a que el desarrollo de senolíticos se enfocara inicialmente en esta estrategia. Entre los senolíticos inductores de apoptosis se encuentran aquellos que tienen como diana BCL-2, una proteína involucrada en la prevención de la muerte celular. Ejemplos de estos fármacos son el navitoclax o ABT-263, venetoclax y flavonoides como la quercetina y la fisetina (135). Algunos de estos fármacos han demostrado ser efectivos en la eliminación de células senescentes, incluidos los fibroblastos activados, en modelos preclínicos de fibrosis pulmonar. Asimismo, se ha observado que ABT-263 tiene un efector protector frente a la infección por SARS-CoV-2, al eliminar parcialmente las células senescentes en hámsteres infectados, disminuyendo la carga viral y la gravedad de la enfermedad pulmonar (136).

La quercetina también ha demostrado tener resultados prometedores. Un estudio realizado en ratones ha determinado que la administración intratraqueal de este fármaco reduce la inflamación, el nivel de citoquinas proinflamatorias y la actividad de la MMP-9, sugiriendo

un potencial terapéutico en condiciones como el síndrome de distrés respiratoria aguda (SDRA) (115).

Algunos inhibidores de tirosinas quinasas, como el dasatinib, tienen un efecto senolítico. En un modelo de SDRA en ratones, se observó que este fármaco disminuía la gravedad de la lesión pulmonar y la respuesta inflamatoria (137). También, se han observado resultados similares en modelos de sepsis. Sin embargo, hay que tomar en cuenta que el control preciso de las dosis de este fármaco es fundamental puesto que la utilización de dosis altas puede generar efectos perjudiciales, al inhibir otras quinasas y proteínas necesarias para la respuesta inmune (138).

Otra diana terapéutica es la HSP-90, una chaperona molecular involucrada en la estabilización y plegamiento de AKT, necesaria para la activación de diversas señales antiapoptóticas. Las células senescentes dependen más de la función de AKT para su supervivencia que las células normales, por eso el impacto de fármacos dirigidos a esta proteína, como la geldanamicina, ansamicina y el resorcinol, promueven la apoptosis celular, mayoritariamente en células senescentes (139,140).

Cabe destacar que algunas vías de supervivencia son redundantes, por lo que en muchas ocasiones son necesarias combinaciones de fármacos senolíticos para conseguir un efecto adecuado (141).

2. Senolíticos de segunda generación

Además de la inhibición de vías moleculares de supervivencia, existen senolíticos que se enfocan en otras características de las células

senescentes. Estas incluyen marcadores de superficie únicos, adaptaciones bioquímicas y cambios estructurales.

Un ejemplo son los glicósidos cardíacos, que han demostrado recientemente su eficacia como senolíticos de amplio espectro. La digoxina, en particular, tiene un efecto senolítico al unirse a la bomba Na+/K+ ATPasa y provocar una despolarización en la membrana celular. Las células senescentes están ligeramente despolarizadas, y la despolarización adicional causada por la digoxina puede superar el umbral crítico para la supervivencia de estas células, pero no para las células normales. Esta característica distintiva hace que este fármaco sea efectivo para la eliminación selectiva de las células senescentes (142). Este fármaco ha resultado ser beneficioso al reducir el crecimiento tumoral y la fibrosis inducida por células senescentes en experimentos realizados en ratones (143) .

Otro ejemplo son aquellos fármacos que se basan en el aumento de la masa lisosomal y la actividad de la SA-βgal. Se están desarrollando nanopartículas unidas a galacto-oligosacáridos que pueden interactuar con la SA-βgal Estas nanopartículas están cargadas con fármacos inactivos que se activan en presencia de la SA-βgal y desencadenan la muerte selectiva de células senescentes (144,145).

Otros fármacos senolíticos se basan en inhibir los sistemas de equilibrio ácido-base, como el metabolismo de la glutamina, necesario para la supervivencia de algunas células senescentes. En algunos casos, estas células pueden tener un pH más ácido debido a la liberación del contenido lisosomal al citoplasma. Para contrarrestar esta acidificación, algunas

células senescentes dependen de estos sistemas de regulación para sobrevivir (146).

También se están desarrollando fármacos que se basan en el uso de células CAR-T conjugadas con anticuerpos dirigidos a proteínas sobreexpresadas en la superficie celular de células senescentes (147).

Es importante señalar que, aunque todas las estrategias senolíticas pueden provocar efectos fuera del objetivo o interferir con poblaciones beneficiosas, estos a menudo pueden limitarse, ya que la mayoría de los tratamientos son susceptibles a estrategias de administración intermitente (148). Estas estrategias son eficaces puesto que las células senescentes tardan alrededor de los siete días en acumularse y desarrollar el SASP.

3. Senomorfos

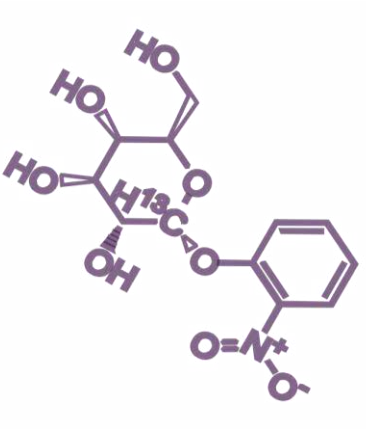
Otro grupo de fármacos que juegan un papel importante en el contexto de la senescencia son los senomorfos. Estos fármacos suprimen el SASP mediante la inhibición del factor de transcripción NF-κβ (149), la vía de transducción de señales JAK-STAT (150), la proteína quinasa mTOR (151), objetivos relacionados con el complejo mitocondrial (152) u otras vías involucradas en la inducción y mantenimiento del SASP.

La metformina, utilizado comúnmente para el tratamiento de la diabetes, también puede tener un efecto senomorfo al modular algunos genes del SASP. Este fármaco ha resultado ser beneficioso en distintos modelos experimentales en animales de lesión pulmonar inducida por ventilación mecánica (153) y de fibrosis pulmonar (154). Además, ha

demostrado reducir la mortalidad en pacientes sépticos con diabetes mellitus (155).

Otros fármacos que podrían tener un efecto senomorfo son las estatinas. En un modelo de fibrosis pulmonar en ratones y cultivos de fibroblastos MRC5, se ha observado que la atorvastatina tiene un efecto beneficioso en la fibrosis pulmonar al inhibir la diferenciación de miofibroblastos, inducir su apoptosis y reducir la acumulación de colágeno (156). Además, la simvastatina se ha propuesto como una posible opción para el tratamiento del SDRA. Un estudio reciente muestra como este fármaco reduce la mortalidad en pacientes con esta enfermedad (157).

Finalmente, cabe destacar que, aunque el desarrollo de la senoterapia haya evolucionado considerablemente en los últimos años, demostrando sus beneficios en algunos contextos, aún no se ha observado que su efectividad este claramente vinculada al efecto de estos sobre la senescencia. Esto se debe a que estos fármacos, participan en la modulación de múltiples vías biológicas que no son específicas de las células senescentes. A esto hay que añadirle que se necesitan herramientas más precisas para detectar la senescencia en pacientes. Estos dos aspectos, resaltan la necesidad de ampliar la investigación en este campo para maximizar la efectividad de las terapias.

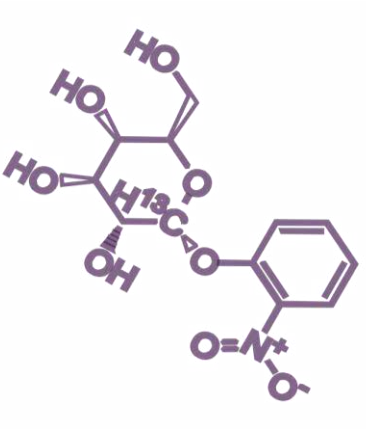


HIPÓTESIS Y OBJETIVOS

La hipótesis de la presente tesis doctoral es que el fallo respiratorio agudo y la ventilación mecánica asociada al mismo activan un programa de senescencia celular en el tejido pulmonar, que puede diseminarse a otros tejidos. Aunque este mecanismo pueda ser beneficioso a corto plazo, limitando la muerte celular, también puede estar asociado a la aparición de las secuelas características del enfermo crítico.

El objetivo general se basa en caracterizar el papel de la senescencia en la patología critica. Para el cumplimiento del objetivo general se plantearon los siguientes objetivos específicos:

- Identificar respuestas senescentes mediante el uso de transcriptomas disponibles de tejido pulmonar en modelos animales de lesión pulmonar aguda y ventilación mecánica, y en muestras de pacientes.
- Caracterizar los mecanismos moleculares responsables de la respuesta senescente en el paciente crítico mediante el empleo de modelos animales de lesión pulmonar y ventilación mecánica, y modelos celulares de estiramiento mecánico.
- Estudiar el potencial de los senolíticos para disminuir las secuelas a largo plazo en el paciente crítico.



RESULTADOS

I. Revisión sobre los mecanismos moleculares del síndrome post-cuidados intensivos.

Los avances en la medicina intensiva han aumentado la tasa de supervivencia de los pacientes críticos en las últimas décadas. Esta situación ha evidenciado la presencia de secuelas físicas, cognitivas y emocionales a las que se enfrentan estos pacientes al salir de la UCI y que se engloban bajo el termino de PICS. Esta realidad, ha supuesto un cambio de paradigma en la medicina intensiva, donde el objetivo no solo se limita a garantizar la supervivencia del paciente, sino también a prevenir que estos desarrollen el PICS, mejorando así su calidad de vida. Hasta ahora los estudios se han centrado en estudiar las secuelas de manera aislada. Sin embargo, las secuelas a largo plazo del PICS podrían derivar de mecanismos sistémicos comunes activados por enfermedades graves, que resultan en una disfunción multiorgánica. El objetivo de esta revisión es, por lo tanto, describir los mecanismos moleculares que se desencadenan al inicio de la enfermedad crítica y que pueden suponer un objetivo terapéutico preventivo para la patología crítica, al abordar el síndrome en su totalidad. Entre otros, esta revisión apunta a que la senescencia podría ser un mecanismo potencial y se podría utilizar como diana terapéutica para el tratamiento de esta enfermedad.

Artículo 1. **Martín-Vicente P**, López-Martínez C, Lopez-Alonso I, López-Aguilar J, Albaiceta GM, Amado-Rodríguez L. Molecular mechanisms of postintensive care syndrome. Intensive Care Med Exp. 2021 Dec 3;9(1):58.

Aportación personal al trabajo.

Fui una de las responsables principales de la elaboración de este manuscrito sobre los mecanismos moleculares involucrados en la patología crítica. La recopilación de la información me sirvió para evaluar los posibles mecanismos moleculares que se manifiestan al principio de la enfermedad y que podrían estar asociados al desarrollo a largo plazo de las secuelas post-UCI. Para ello participé en la búsqueda bibliográfica, organización y escritura del manuscrito.

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REVIEWS Open Access

Molecular mechanisms of postintensive care syndrome

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Effectiveness of intensive care must be evaluated not only by short-term survival after a critical illness, but also by the recovery to an adequate quality of life. The increased evidence of long-term functional disabilities in intensive care survivors led to the definition of post-intensive care syndrome (PICS) [1]. Early diagnosis and effective treatments for these newly recognized conditions are warranted. However, most of the initial efforts to limit long-term sequelae have not yielded satisfactory results [2]. The objective of this review is to identify the molecular mechanisms that lead to organ dysfunction after intensive care and to summarize them into several plausible unifying hypotheses. From these mechanisms, novel therapeutic targets with the potential to prevent PICS may arise [3], allowing for earlier interventions during acute organ failure aimed to improve the quality of life of intensive care unit (ICU) survivors. Cost-effective strategies based on growing pathogenetic evidence on PICS would hence allocate research efforts and funding to implement preventive treatments, to impede pathogenetic mechanisms triggered during ICU stay, rather than exclusively rehabilitate long-term sequelae when they are already established.

The spectrum of postintensive care syndrome

The improvement of mortality rates in the ICU has evidenced that survivors to a critical illness face a number of long-term severe complications and sequelae that can impair their quality of life [4]. Several factors, including the population aging along with the emergence of invasive therapies that may improve the outcomes, have increased the interest in these long-term conditions [5]. An expert panel in 2012 defined PICS as the "new or worsening impairments in physical, cognitive or mental health status arising after critical illness and persisting beyond acute care hospitalization" [1]. It must be noted that this definition provides a framework to improve awareness, research and diagnostic and therapeutic approaches, rather to define a classical syndrome [6].

PICS covers several dimensions, including physical, cognitive and emotional aspects (Fig. 1), for many of which there is no standard definition or diagnostic criteria. Long-term respiratory sequelae include impairments in lung volumes, ventilatory dynamics and diffusion [7]. Although some studies report a mild impairment

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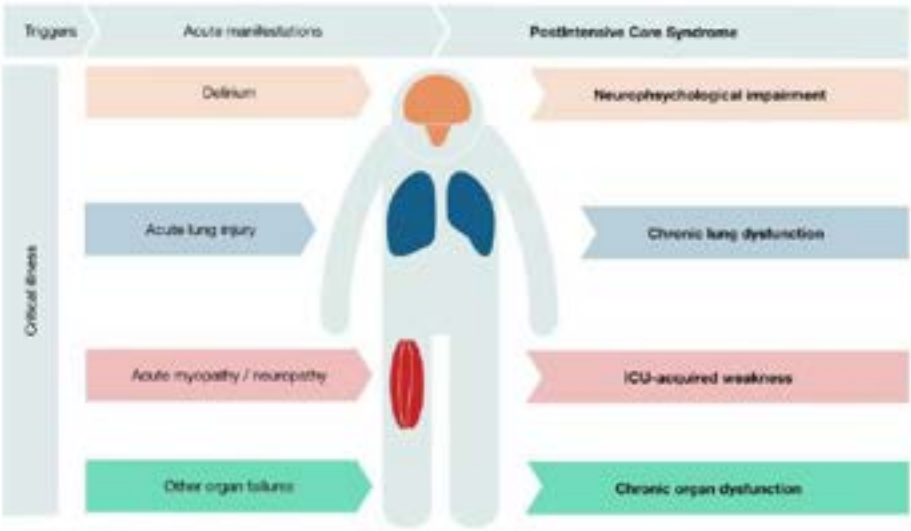

Fig. 1 Spectrum of postintensive care syndrome. Many of the long-term conditions that constitute the postintensive care syndrome have been related to specific syndromes that appear during acute care. However, it is not known if this relationship reflects a common primary cause, a pathogenetic association or simply an association due to underlying confounders

in most of the cases, the recent COVID-19 pandemic has highlighted the relevance of the long-term, post-acute respiratory distress syndrome (ARDS) respiratory sequelae [8]. Musculoskeletal impairments are included under the concept of "ICU-acquired weakness" (ICUAW), defined as a "diffuse, symmetric, generalized muscle weakness, detected by physical examination and meeting specific strength related criteria) that develops after the onset of critical illness without other identifiable cause". ICUAW may result in a severe limitation of daily activities and a significant worsening in quality of life. Although some improvements may occur during the first year after ICU discharge, weakness is persistent in a significant proportion of cases [9]. Finally, neuropsychological alterations regarding cognitive declines in ICU survivors have been also described by several authors. Up to 80% of critically ill patients experience delirium while in the ICU, and a significant number of ICU survivors show signs of moderate cognitive impairment or other neurological, emotional and mental health conditions related to PICS include depression, anxiety, post-traumatic stress disorder and cognitive impairment [10, 11]. Again, these disarrangements may persist well above the first year and cause a severe limitation of patients' activities.

Several ICU-related risk factors and short-term complications have been related to these long-term outcomes (highlighted in Fig. 1). However, as pathogenetic factors are mostly unknown, it is not clear if these short- and long-term symptoms are time-dependent manifestations of a common disease, independent diseases with shared etiologies or independent sequelae caused by the systemic response to a severe injury.

Mechanisms of chronic lung dysfunction

The need of respiratory support is one of the main reasons for ICU admission, either due to lung injury or ventilatory failure. These critically ill patients often require mechanical ventilation. Previous organ damage, along with the use of ventilation, may lead to the development or worsening of lung injury [12], which involves epithelial barrier dysfunction, inflammation and matrix remodelling. In this context, failure to correctly resolve these processes might be involved in the development of long-term sequelae. However, the specific mechanisms by which acute lung damage becomes chronic are yet to be fully elucidated. Most of the knowledge on this topic comes from research on prevalent chronic lung diseases, such as idiopathic pulmonary fibrosis, which may hint to underlying pathogenetic mechanisms. These same processes may play a role in the development of chronic lung dysfunction in the context of post-ICU sequelae (Fig. 2).

Inflammation and matrix remodelling are well described processes involved in the resolution of acute lung injury [13]. However, their perpetuation can be a relevant pathogenetic mechanism of many chronic lung diseases [14]. Persistence of the local inflammatory response has been linked to the development of fibrosis, as released Th2 cytokines (IL-4, -5, -13) have a well-known pro-fibrotic effect and may recruit fibrocytes from the systemic circulation. Moreover, other pro-inflammatory mediators such as IL1b or IL-6 may promote collagen deposition mediated by IL-17 [15].

In this setting, alveolar macrophages, inflammatory cells and fibroblasts release profibrotic molecules during acute injury, such as transforming growth factor-β (TGFβ), Insulin-like growth factor (IGF-1), platelet derived growth factor (PDGF) or connective tissue growth factor (CTGF) among others [16–19]. TGFβ activates intracellular SMAD complexes via binding to serine/threonine kinase heterodimers in the cell surface. Activated SMAD complexes enter the nucleus and act as

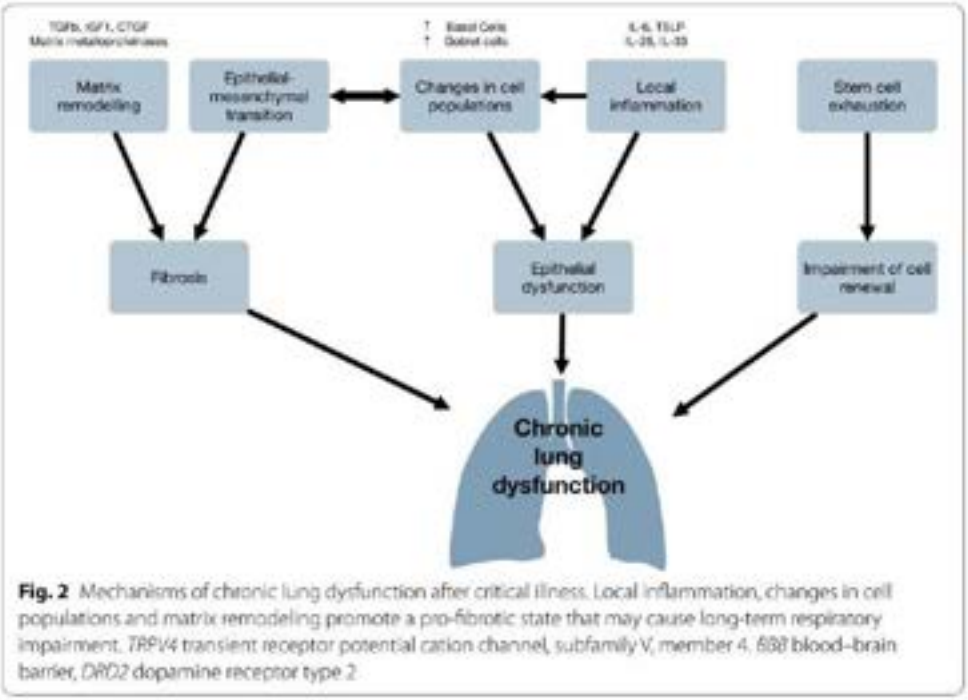

Fig. 2 Mechanisms of chronic lung dysfunction after critical illness. Local inflammation, changes in cell populations and matrix remodeling promote a pro-fibrotic state that may cause long-term respiratory impairment. *TRPV4* transient receptor potential cation channel, subfamily V, member 4, *BBB* blood–brain barrier, *DRD2* dopamine receptor type 2

transcription factors regulating a wide range of cellular processes. In fibrosis, these pathways include extracellular matrix deposition and fibroblast division and differentiation into myofibroblasts [20]. These cells, characterized by an increase in the intracellular content of α-smooth muscle actin (α-SMA), modify the matrix composition by increasing deposition of collagen and disorganizing elastin, generating scar-like lesions [21].

Activation of coagulation and fibrin deposition is another pathogenetic mechanism activated during acute lung injury that has been linked to long-term sequels. It has been shown that patients with lung interstitial diseases have an increased expression of procoagulant factors within the lung, including tissue factor or thrombin, or a decrease in protein C. Activation of proteinase-activated receptors (PARs) by the proteinases from the coagulation cascade (thrombin, trypsin, cathepsins…) provides the mechanistic link between coagulation and fibrosis. Downstream signaling following PAR1 activation results in the expression of several profibrotic growth factors (CTFG, PDGF) and perpetuates the local release of proinflammatory cytokines and TGFβ [22].

Epithelial–Mesenchymal Transition (EMT) has also gained relevance in this scenario, where massive tissue remodeling takes place. EMT is a process in which a polarized epithelial cell acquires a mesenchymal phenotype, that includes synthesis of extracellular matrix components [23]. During EMT, epithelial cells lose part of the epithelial characteristics, such as expression of E-cadherin and cytokeratin, and gain mesenchymal markers including N-cadherin, vimentin or α-smooth muscle actin [24]. At a physiological level, all these processes favour the accumulation of excessive fibrous tissue, decreasing lung compliance and impairing ventilatory dynamics and diffusion. In vivo models of lung injury and mechanical ventilation have shown the activation of EMT, possibly by a *Wnt*-dependent mechanism [25], suggesting a relationship between EMT-like processes and the later development of pulmonary fibrosis [26].

Another common feature in these chronic conditions is epithelial barrier dysfunction, characterized by altered cell composition of the pseudostratified respiratory epithelium with basal and goblet cell hyperplasia and metaplasia [27, 28]. In addition, after the initial lung insult, persistence of epithelial dysfunction is associated with a proinflammatory secretory phenotype due to the activation of airway epithelial cells, dendritic cells and type 2 Innate Lymphoid Cells, and release of epithelial derived cytokines, including thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL- 33 [29]. The resulting sustained inflammation and shift in cellular composition could play an important role in post-ICU lung dysfunction.

Finally, cell renewal is a key feature of chronic lung diseases. Excessive stem cell activation leads to accumulation of DNA damage and cell senescence [30]. In patients with idiopathic pulmonary fibrosis, epithelial cells show an increase expression of senescence markers, such as P16 or P21 and a proinflammatory phenotype [31]. Recently, we have shown the activation of this pathway in response to acute lung injury [32]. These senescent cells have been related to stem cell exhaustion with an impaired regenerative capacity [33] and an increased secretion of inflammatory and matrix remodeling molecules, which in turn may perpetuate fibrosis.

Molecular mechanisms of cognitive impairment

There are several mechanisms that may lead to brain injury in critically ill patients [34]. The central nervous system (CNS) receives signals from neural afferences and circulating factors and cells. Regarding the former, the vagus nerve constitutes the main ascendent pathway from peripheral organs. Distal vagal sensors are responsive to a variety of stimuli, including stretch (via transient receptor potential cation channel, subfamily V, member 4 [TRPV4] and Piezo receptors) [35], or inflammation (via toll-like receptor (TLR)-4, IL1R or tumor necrosis factor [TNF]-receptor present in vagal sensory neurons) [36, 37]. Once these signals reach the brain stem, multisynaptic pathways along the CNS are activated [38, 39]. For instance, lung stretch activates alveolar TRPV4 and purinergic receptors, that, in a vagus-dependent manner, increase dopaminergic signaling and triggers hippocampal apoptosis [40]. Blockade of triggering receptors in distal organs or circulating mediators could decrease the risk of long-term impairment. In animal models, inhibition of peripheral mechanosensation with TRPV4 antagonists, unspecific blockade of nerve conduction with lidocaine or inhibition of type 2 dopamine receptors have decreased hippocampal apoptosis [35].

Circulating molecules and cells may also reach the brain during critical illness. The systemic inflammatory response decreases blood–brain barrier permeability [41] and facilitates the translocation of circulating mediators and/or cells that further promote brain injury. Heparan sulfate fragments released from the endothelial glicocalix during sepsis may translocate to the hippocampus and inhibit brain-derived neurotrophic factor signaling, that results in memory impairment in mice [42]. Circulating IL-6 may also play a role in this setting, as peripheral blockade of IL-6 with a monoclonal antibody prevented ventilator-induced brain injury [43]. In line with these findings, intratracheal instillation of lipopolysaccharide increases the expression of proinflammatory cytokines *Il1b* and *Il6* in the brain stem [44]. Interestingly, only the increase in *Il1b* expression was abolished after vagotomy, suggesting the simultaneous activation of different mechanisms.

The link between these brain responses and functional outcomes has also been assessed. In a large animal model of prolonged protective mechanical ventilation, hippocampal damage was demonstrated [45]. Acute lung damage and mechanical ventilation in mice caused brain inflammation, hippocampal injury and memory impairments, in an steroid-preventable manner [41]. Similarly, conditioning responses, a surrogate marker of memory in mice, were absent 3 days after mechanical ventilation, but not in anesthetized, non-ventilated controls [46]. Although translation of these experimental results into clinical evidence is challenging and remains elusive, this model of brain injury in response to systemic insults (summarized in Fig. 3) provides a framework for prevention, diagnosis and treatment of long-term cognitive dysfunction in critically ill patients.

Mechanisms of ICU-acquired weakness

ICUAW is a bilateral and symmetrical neuromuscular involvement, common in critically ill mechanically ventilated patients. Clinical studies in critical care settings involving electrophysiological tests and muscle histopathology suggest that both polyneuropathy and myopathy may coexist in ICU patients, being myopathy more

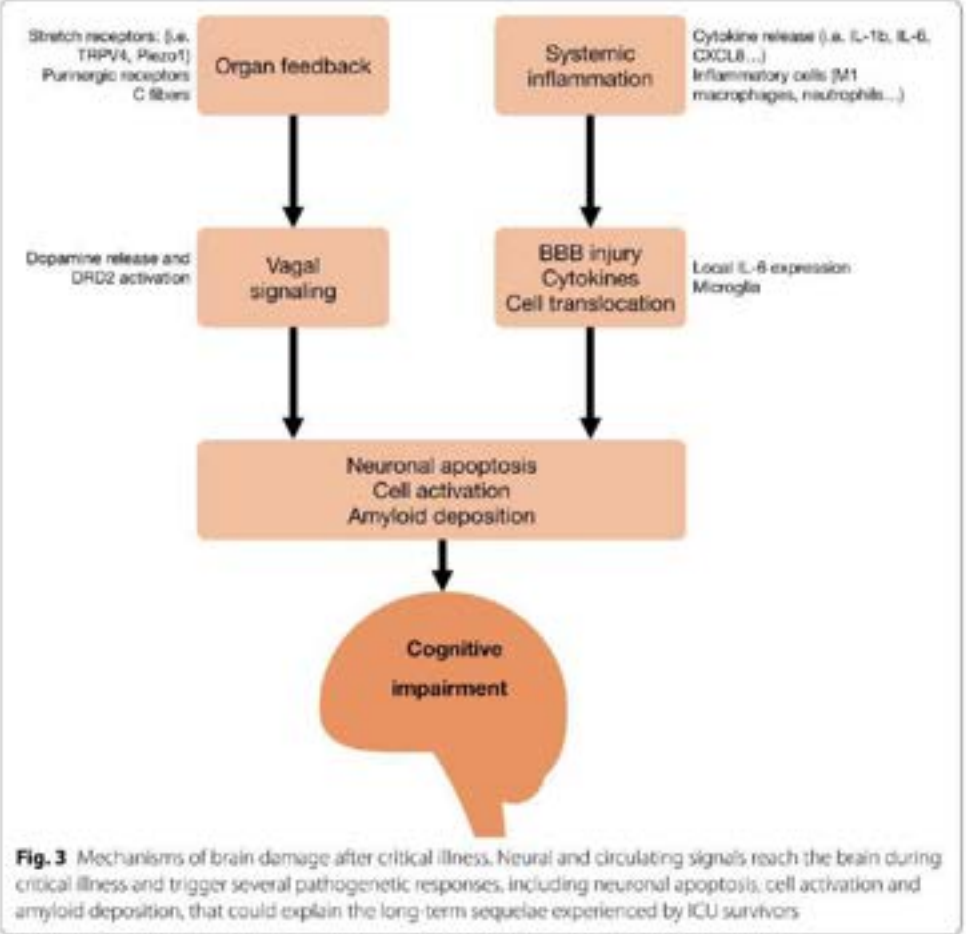

Fig. 3 Mechanisms of brain damage after critical illness. Neural and circulating signals reach the brain during critical illness and trigger several pathogenetic responses, including neuronal apoptosis, cell activation and amyloid deposition, that could explain the long-term sequelae experienced by ICU survivors

frequently identified as the cause of weakness [47]. Critical illness neuropathy has been described as a distal axonal sensory-motor polyneuropathy affecting limb and respiratory muscles. Some evidence suggests that weakness recovery could be worsened and/or delayed when neuropathy accompanies myopathy, being persisting disability associated with polyneuropathy and myopathy coexistence [48, 49]. Because nerve conduction studies and needle electromyography do not accurately discern between both entities, and given the sufficiently relevant clinical problem of muscle weakness in these patients [50], the term ICUAW emerged regardless of its causative nature. Although physical disability related to ICUAW is highly prevalent among ICU survivors, its clinical spectrum varies not only in severity but also in recovery trajectories [51]. Muscle atrophy in the critically-ill has been demonstrated to begin within the first hours after ICU admission in mechanically ventilated patients [52] and its development has been related to several factors, such as systemic inflammation, severity of the underlying disease, use of neuromuscular blockers or mechanical ventilation itself [49, 53, 54].

Multiple molecular mechanisms, either independent or interacting, are involved in muscle wasting and evolve over time, from the onset of critical illness till the long-term recovery phase around 6 months after ICU discharge [55] (Fig. 4). Muscle

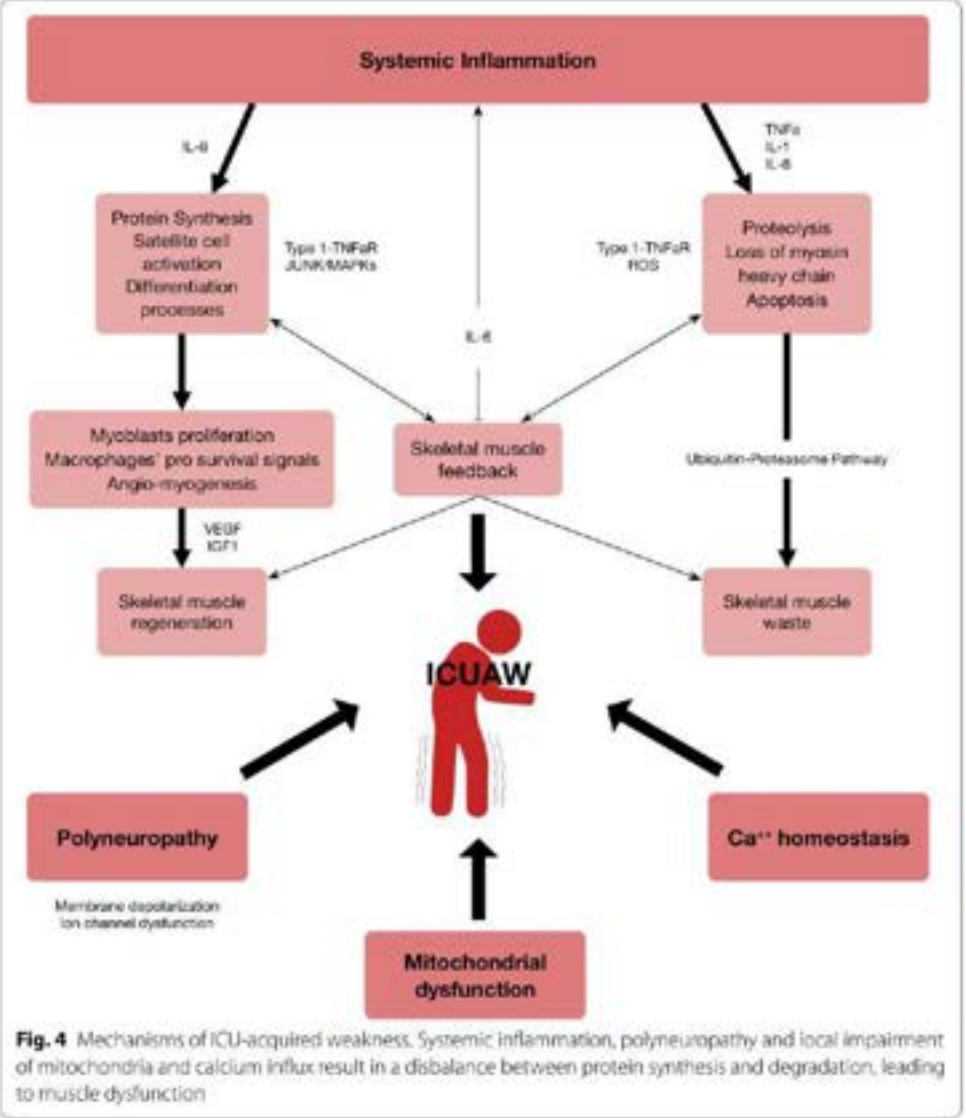

Fig. 4 Mechanisms of ICU-acquired weakness. Systemic inflammation, polyneuropathy and local impairment of mitochondria and calcium influx result in a disbalance between protein synthesis and degradation, leading to muscle dysfunction

wasting results from an increased proteolysis triggered in the acute phase, overwhelming the regenerative capacity of the injured tissue [52, 56]. Early activation of proteolytic pathways, however, is not sustained over time, but instead it may alter muscle biology resulting in an impaired muscle regrowth [57].

Inflammatory cytokines have been suggested to play a relevant role in the development of ICUAW. TNFα has been widely studied in this setting. In differentiated myotubes, TNFα stimulates catabolism by binding to TNF receptor subtype 1 and activating nuclear factor-kB. This transcriptional factor is essential for TNFα-induced reduction in muscle protein and loss of adult myosin heavy chain content [58], which is specifically found in critically ill patients [59]. This pathway is also sensitive to reactive oxygen species, which appear to function as second messengers for TNFα in skeletal muscle [58]. TNFα/nuclear factor-kB signaling is also involved in the differentiation process, representing a mechanism that could be responsible for satellite cells activation and skeletal

muscle recovery following the acute phase [60–62]. TNFα binding to its receptor also stimulates apoptosis and Jun-N-terminal kinases and mitogen-activated protein kinases (MAPKs) in differentiated myotubes. In muscle cells, these signaling events stimulate the expression of genes related to the ubiquitin–proteasome pathway [63, 64], triggering massive intracellular proteolysis [65]. Finally, TNFα is also known to affect the force of muscle contraction even in the absence of atrophy [66] via TNFR1 and mediated by an increased cytosolic oxidant activity [67, 68].

Elevated levels of IL-1 are commonly found in critically ill patients' serum and represent a potential stimulus for protein loss and muscle atrophy. The suggested underlying mechanisms are related to both protein synthesis and degradation [69, 70]. Interestingly, IL-6 has been proven to drive the systemic compensatory anti-inflammatory response syndrome, by inhibiting TNFα release and stimulating IL-10 [71]. In skeletal muscle, IL-6 is involved in myogenesis, lipid metabolism, glucose uptake and both protein synthesis and degradation [72–74]. Skeletal muscle cellular niche has been recognized itself as a myokine secretor organ and even a potential regulator of immune system [75]. In mechanically ventilated patients who developed myopathy, the inflammation-induced acute phase response resulted in a marked increase in IL-6 production in skeletal muscle [76].

Histologic and molecular analyses performed in skeletal muscle biopsies of critically ill patients suggest that recovery failure may be associated with satellite/progenitor cells loss and fibrosis [57], but it is unclear which are the underlying mechanisms leading to the satellite cell depletion or what is the role of the whole skeletal muscle cell niche. In other scenarios, where muscle injury may occur, muscle tissue repair is a complex biological process that necessarily involves activation of stem cells. Myogenic stem cells, so-called satellite cells, reside beneath the basal lamina of muscle fibers [77] and express both NCAM/CD56 and early myogenic cell markers, such as M-cadherin, PAX7, and MYF5 [78]. Satellite cells remain quiescent in skeletal muscle, but they can proliferate and further differentiate into myoblasts in response to activating signals, achieving muscle regeneration [79]. Activated satellite cells may interact with macrophages recruited at the site of muscle regeneration and receive mitogenic signals from these immune cells, mediated by the release of different soluble factors [80]. Myoblasts, and to a higher extent myotubes, also receive cell-contact-mediated pro-survival signals from macrophages [81].

Similarly, the microvascular niche seems to be another partner of satellite cells. Christov et al. have suggested quiescent satellite cells to easily interplay with endothelial cells upon activation to set up coordinated angio-myogenesis in a functional manner [82]. Indeed, angiogenesis and myogenesis could share regulatory factors such as vascular endothelial growth factor (VEGF) [83] or insulin-like growth factor type 1 (IGF1) [84] that reciprocally signal both processes pivotally involved in muscle regeneration.

Apart from inflammation and stem cell depletion, several other mechanisms may be involved in the development of persistent muscle weakness in survivors of critical illness. Distal axonal sensory-motor damage/dysfunction has also been described in ICUAW, being attributable to a reduced membrane excitability resulting from membrane depolarization and ion channel dysfunction [85, 86], together with an altered calcium homeostasis [87]. In critically-ill patients with demonstrated polyneuropathy, motor axons are

depolarized. Chronic membrane depolarization could be related to tissue hypoperfusion or to increased extracellular potassium in patients with kidney failure, and may lead to muscle atrophy [88]. Reduced compound motor action potentials are present in neuropathy and myopathy. In addition, there have been described fibrillation potentials or positive sharp waves that could be explained by denervation or by muscle sodium channel dysfunction [89]. Mitochondrial dysfunction could be a contributing defect involved in energy-dependent processes [90], and a dysregulation in autophagy [91] has also been described to play a role in muscle repair.

In critically ill patients, the limited sample size of the muscle specimens precludes the identification of different mechanism-specific subphenotypes of muscle weakness, with different histopathological findings and driven by different mechanisms, though leading to the wide clinical spectrum of long-term functional disability. These different pathogenetic subphenotypes could explain the heterogeneous recovery observed among survivors of critical illness. For instance, the activated pathways could be promoting proliferation of satellite cells in some patients while related to fibrotic repair and satellite cell depletion in others. Understanding these mechanisms is crucial to identify therapeutic targets that, interfered at the beginning of the process, could modify the clinical course of ICUAW before the 6-month plateau has been achieved, either at the acute phase or during recovery.

Unifying hypotheses

Long-term sequelae included in the PICS framework may be the consequence of organ-specific mechanisms, as described in the previous sections. However, the variety of symptoms included in PICS in response to common triggers (the critical disease and its managements) and the observed links and correlations amongst the different dimensions of the syndrome [92] raise the hypothesis that PICS is the long-term result of underlying mechanisms activated by severe diseases, that become systemic and lead to different degrees of multi-organ dysfunction. There are several stereotypical biological responses and mechanisms that could be involved in the development of PICS. Identification of these shared mechanisms could help to identify patients at risk of developing sequelae. Moreover, this knowledge could lead to novel therapeutic interventions that might prevent the whole spectrum of PICS by interfering with upstream regulators.

Systemic inflammation

The most characterized and studied mechanism in this setting is the inflammatory response. Inflammation is a physiological response necessary to fight against infections or injury and, therefore, restore homeostasis. During the acute phase of the inflammatory response, the presence of damage or pathogen-associated molecular patterns (DAMPs and PAMPs respectively) induces an initial systemic inflammatory response, mediated by the release of pro-inflammatory mediators, such as growth factors (i.e., G/GM-CSF, FltL) and cytokines (i.e., IL-1, IL-6, IL-17) as well as through mesenchymal or immune cells [93]. This coexists with a compensatory anti-inflammatory response syndrome, which is mainly carried out by myeloid suppressor cells (MDSCs) that secrete anti-inflammatory cytokines (e.g., IL-10 and TGFβ) and cytokine antagonists (e.g.,

IL-1ra and sTNFRI) and decrease inflammation without eliminating all protective innate immunity [94].

During chronic inflammation, an unbalance between pro- and anti-inflammatory mediators makes homeostasis impossible to restore. The persistence of stimuli that modify the inflammatory response, either directly or indirectly, may perpetuate the release of inflammatory mediators not only to the lung but also to the systemic compartment, as the increased alveolo-capillary permeability facilitates their translocation. This persistent inflammation has been demonstrated in ARDS survivors even after clinical improvement or recover and related to worse physical recovery [95].

Senescence

Other potential mechanism which might be involved in PICS is related to the activation and spread of senescent responses. Cell senescence is defined as the cell cycle arrest in response to a stimulus, and a shift towards a specific phenotype characterized by the loss of several cell functions and the paracrine release of a variety of molecules (termed senescence-associated secretory phenotype—SASP), including proinflammatory mediators and senescence inductors (thus activating a positive feedback) [96]. There are several molecular pathways that lead to senescence in response to injury (including oxidative stress, release of DAMPs, proinflammatory cytokines, such as TNFα or IL-1α), most of which depend on the activation of P53, P21 and their downstream factors [97]. In a model of acute lung injury, we demonstrated that activation of these pathways results in short-term protection against apoptosis (as senescent cells have an inherent resistance to programmed cell death) [32, 98]. However, the resulting senescent state could lead to long-term sequelae, both locally and in distant organs (in response to SASP). Several of the previously described mechanisms of organ-specific PICS could be manifestation of these senescent responses. Senescence is an emerging pathogenetic pathway in idiopathic lung fibrosis [31], but their involvement in secondary fibrosis is unknown. Several forms of acute lung injury can trigger senescence in response to DNA damage. The previously described satellite cell exhaustion in ICUAW could be also a manifestation of the systemic release of pro-senescent factors. Recently, it has been described the deposition of brain amyloid fibers, a known trigger of neuronal senescence, in response to critical illness [99].

Of note, there is a positive feedback between inflammation and senescence. Inflammation causes activation of senescence through several mediators, such as IL-6. In turn, SASP includes the release of proinflammatory molecules, thus promoting a sustained response [100].

Integrated stress response

A third mechanism potentially involved in the development of PICS is the so-called integrated stress response (ISR). ISR is a conserved cell response to different pathological conditions that results in a general decrease in protein synthesis and expression of a specific gene signature. Its activation is necessary to maintain cell homeostasis in the presence of different stress signals [101]. This response may be activated by four stress-sensing kinases (protein kinase R [PKR], Eukaryotic translation initiation factor 2-alpha kinase 4 [EIF2AK4], heme-regulated inhibitor [HRI] and PKR-like endoplasmic

reticulum kinase [PERK]) that phosphorylate the eukaryotic initiation factor eIF2α, which ultimately leads to a decrease in protein synthesis and the induction of selected genes (such as ATF4 and CHOP) that eventually take part in the cellular response to stress.

ISR plays an important role during acute lung injury or mechanical ventilation. Alveolar overdistension induced by mechanical ventilation results in PERK phosphorylation and subsequent phosphorylation of the factor eIF2α. This alters epithelial permeability, induces proinflammatory cytokine release and cell death [102]. ISR may have a dual role deciding cell fate. Although its main function is maintaining cell survival, exposure to a continuous stress could lead to cell death [103]. In this context, the stress-inducible phosphatase GADD34 dephosphorylates eIF2α and induces a negative feedback mechanism, ceasing the activation of ISR [104]. However, in pathological conditions, ISR is activated but GADD34 expression is attenuated, thus preserving phosphorylated eIF2α [105] and perpetuating this response [106] due to the lack of negative feedback.

From common triggers to cellular and tissue dysfunction

Finally, all these pathways converge in a reduced number of cell responses that mediate tissue dysfunction. The most studied response is apoptosis. There is substantial evidence showing disseminated apoptosis during the acute response to critical illness [107]. This programmed cell death is activated by binding of extracellular signaling molecules (i.e., TNFα) to membrane receptors or intracellular release of cytochrome c from injured mitochondria (i.e., after oxidative stress). These pathways converge in the activation of caspases, that lead to DNA fragmentation and cell death. Although apoptosis is a major pathogenetic mechanism in acute organ failure, and anti-apoptotic drugs may prevent organ damage in this setting, the relationship of acute apoptosis and development of PICS remains to be fully elucidated. Animal models have shown a correlation between apoptosis and later development of pulmonary fibrosis [108] and neurological deficits [46], but human data is scarce. Patients with ICUAW show activation of proapoptotic pathways in peripheral muscles [109].

Activation of senescence, that render cells resistant to apoptosis [110], may be a compensatory mechanism in this setting, but at the price of the promoting persistence of senescent, dysfunctional cells. Senolytics, a heterogeneous family of compounds that inhibit pro-survival kinases in senescent cells (such as *BCL2* or tyrosine-kinases), may promote the selective death of these cells, facilitating a delayed repair [111]. Although promising, these pathways have not been explored in critically ill patients.

Emerging PICS domains

Rather than a settled syndromic condition, PICS is an evolving concept that covers a large variety of symptoms and conditions experienced by ICU survivors. Clinical research in this topic may help to better identify, characterize and manage the long-term consequences of critical illness from the early onset of acute illnesses. The main components of PICS have been covered in the previous sections, but the systemic nature of acute severe diseases may cause other organ injuries.

Critical illness may increase the risk of cardiovascular events after ICU and hospital discharge. Greater rates of atrial fibrillation, heart failure, and myocardial infarction

have been described after sepsis [112]. However, no clear organ-specific mechanisms have been identified to date [113]. Systemic persistent inflammation is a major driver of cardiovascular disease, but a clear causative link is missing [114]. Recently, the role of stress-triggered senescence has been highlighted in this setting [115].

Similarly, there is increasing evidence of long-term impaired kidney function after critical care, even in patients without acute renal failure [116]. The development of kidney fibrosis during the repair phase has been proposed as a major pathogenetic mechanism [117]. In addition, cell cycle arrest, a hallmark of senescence, promotes fibrosis during kidney repair [118]. The previously described cardiovascular impairment could contribute to further deteriorate kidney function.

Finally, transgenerational effects of critical illness, requiring modification of the genome or epigenome of germline cells, have barely been explored. It has been shown that experimental sepsis changes the sperm methylome, mainly in intergenic regions or development-related genes [119]. Both systemic and local inflammation can modify the expression of methyltransferases and thus facilitate cell reprogramming. The consequences of these changes in offspring, however, are controversial [120]. Besides, maternal prenatal exposures in human studies have focused on pregnancy, rarely assessing long term effects of exposures of maternal non-pregnant progenitor in later offspring [121].

Conclusions

The objectives of intensive care must go well beyond ICU survival and aim to provide critically ill patients with the best achievable quality of life. This includes prevention, treatment and/or palliation of the long-term sequels derived from their ICU stay. The complex organ crosstalk and the pleiotropic effects of most of the responses triggered by critical illness make difficult to find treatments that translate into a clinical benefit. In this difficult scenario, knowledge of the underlying biological mechanisms may allow clinicians and researchers to identify novel biomarkers, therapeutic targets and strategies that ultimately will facilitate the identification and treatment of these long-term sequelae even at early and acute stages, thus contributing to improve long-term outcomes.

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Declarations

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Not required.

Consent for publication
Not applicable.

Competing interests
The authors declare that they do not have any competing interests.

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II. La activación de p21 limita la lesión pulmonar aguda e induce senescencia temprana tras la aspiración de ácido clorhídrico y la ventilación mecánica.

La lesión pulmonar aguda y su forma más grave, el SDRA, son condiciones comunes en el paciente crítico que se caracterizan por estar asociadas a una inflamación severa y a una acumulación de líquido en los alveolos, lo que compromete la capacidad de los pulmones para oxigenar la sangre. Como resultado, estos pacientes necesitan de ventilación mecánica, lo cual puede agravar más su situación. La vía p53/p21 se ha visto implicada en la enfermedad pulmonar crónica. Sin embargo, su papel en la lesión pulmonar aguda no se ha explorado de manera exhaustiva. El análisis sistemático de datos transcriptómicos de modelos animales de lesión pulmonar mostraron un enriquecimiento de firmas génicas específicas dependientes de p53 y p21 y un perfil validado de senescencia. Es más, el modelo de ratón de aspiración de ácido clorhídrico y ventilación mecánica utilizado en el estudio, mostró cambios en la envoltura nuclear, la cromatina subyacente, daño en el ADN y activación de la vía p53/p21. Además, la ausencia de Cdkn1a se asoció con una disminución de los marcadores asociados a senescencia y un empeoramiento de la lesión pulmonar debido a un aumento de la apoptosis. En cambio, el tratamiento con lopinavir/ritonavir condujo a la sobrexpresión de Cdkn1a, disminuyendo la apoptosis celular y mejorando la lesión pulmonar. Finalmente, las muestras de autopsias de pulmones de pacientes con SDRA utilizadas en este estudio revelaron un aumento en los focos de heterocromatina asociados a senescencia. En conjunto, estos resultados sugieren que la activación de la vía p53/p21 tiene un papel antiapoptótico

en la lesión pulmonar aguda, estando a su vez implicado en la activación de un programa de senescencia, lo que podría tener consecuencias perjudiciales en el desarrollo de la enfermedad a largo plazo.

Artículo 2. Blázquez-Prieto J, Huidobro C, López-Alonso I, Amado-Rodriguez L, **Martín-Vicente P**, López-Martínez C, Crespo I, Pantoja C, Fernandez-Marcos PJ, Serrano M, Sznajder JI, Albaiceta GM. Activation of p21 limits acute lung injury and induces early senescence after acid aspiration and mechanical ventilation. Transl Res. 2021 Jul;233:104-116.

Aportación personal al trabajo.

Me incorpore a este proyecto desde mi llegada al laboratorio, en el cual dedique la mayor parte de mi tiempo, hasta su publicación. Realicé la mayoría de los ensayos bioquímicos, adquisición de imágenes, estudios histológicos y análisis de los resultados correspondientes. También contribuí en la elaboración del manuscrito, elaboración de figuras, y en el análisis estadístico.

Activation of p21 limits acute lung injury and induces early senescence after acid aspiration and mechanical ventilation

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The p53/p21 pathway is activated in response to cell stress. However, its role in acute lung injury has not been elucidated. Acute lung injury is associated with disruption of the alveolo-capillary barrier leading to acute respiratory distress syndrome (ARDS). Mechanical ventilation may be necessary to support gas exchange in patients with ARDS, however, high positive airway pressures can cause regional overdistension of alveolar units and aggravate lung injury. Here, we report that acute lung injury and alveolar overstretching activate the p53/p21 pathway to maintain homeostasis and avoid massive cell apoptosis. A systematic pooling of transcriptomic data from animal models of lung injury demonstrates the enrichment of specific p53- and p21-dependent gene signatures and a validated senescence profile. In a clinically relevant, murine model of acid aspiration and mechanical ventilation, we observed changes in the nuclear envelope and the underlying chromatin, DNA damage and activation of the Tp53/p21 pathway. Absence of Cdkn1a decreased the senescent response, but worsened lung injury due to increased cell apoptosis. Conversely, treatment with lopinavir and/or ritonavir led to Cdkn1a overexpression and ameliorated cell apoptosis and lung injury. The activation of these mechanisms was associated with early markers of senescence, including expression of senescence-related genes and increases in senescence-associated heterochromatin foci in alveolar cells. Autopsy samples from lungs of patients with ARDS revealed increased senescence-associated heterochromatin foci. Collectively, these results suggest that acute lung injury activates p53/p21 as an antiapoptotic mechanism to ameliorate damage, but with the side effect of induction of senescence. (Translational Research 2021; 233:104–116)

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Abbreviations: ANOVA = analysis of the variance; ARDS = acute respiratory distress syndrome; SAHF = senescence associated heterochromatin foci; SASP = senescence-associated secretory phenotype; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; VILI = ventilator-induced lung injury

AT A GLANCE COMMENTARY

Jorge Blázquez-Prieto et al.

Background

P53 and its downstream factor p21 are involved in the cell response to stress. In addition, they are drivers of senescence. Although these pathways have been studied in chronic lung diseases, their role in acute lung injury has not been systematically explored.

Translational Significance

Our findings show that the antiapoptotic effects of p21 counteract the proapoptotic response triggered by acute lung injury and mechanical ventilation. Drug-induced overexpression of p21 decreases lung injury in this setting. However, activation of p21 also leads to an early senescent response within the lung that may favor long-term side effects.

INTRODUCTION

The lungs have a stereotypic response to acute injury, which is preserved among species and many etiological agents. Once damage is inflicted, lung cells trigger a host response which can include inflammation, matrix remodeling and different forms of cell death, including apoptosis.[1] Although a limited host response may help to clear the injurious agent and promote lung tissue repair,[2] an overexuberant host response can lead to severe injury and gas exchange worsening. Therefore, therapeutic strategies aimed to limit lung damage and interference with lung repair are important.

Lungs are exposed to mechanical load during every breath. In pathologic conditions, generation of higher-pressure gradients necessary for adequate ventilation may cause excessive cell stretch.[3] This is especially relevant during mechanical ventilation with high pressures, which can lead to the so-called ventilator-induced lung injury (VILI).[4,5] In mechanically ventilated patients, a strategy aimed to limit VILI decreased mortality in patients with the acute respiratory distress syndrome (ARDS).[6]

Mechanotransduction is thought to regulate the molecular steps in VILI pathogenesis.[7] The nuclear envelope has been reported as an important cell mechanosensor and signal transducer.[8] Mechanical stretch appears to increase Lamin-A in the nuclear envelope, leading to nuclear stiffening. These changes in the nuclear envelope can also activate p53-dependent pathways. Wildtype p53 is a master regulator of cell homeostasis and fate, and its activation may lead to a variety of responses, ranging from apoptosis to cell cycle arrest. Inhibition of this response has been shown to increase p21 (Cdkn1a) expression and decrease VILI in an experimental model.[9] p53 and its downstream factor p21 are triggers of senescence,[10] a cell response characterized by an stable arrest of the cell cycle and a switch towards a senescence-associated secretory phenotype (SASP). It has been proposed that senescence facilitates the clearance of damaged cells and is required for tissue repair.[11] Interestingly, some of the molecules have a significant overlap with the proinflammatory response associated with VILI.

We hypothesized that p53-dependent pathways play a role in the maintenance of lung homeostasis during acute injury, and that senescence could be a side effect of their activation. To test this hypothesis, we developed a clinically relevant model of lung injury caused by acid aspiration and VILI to assess the activation of p53 and its downstream factors.

MATERIAL AND METHODS

Meta-analysis of transcriptomic data. To explore the main hypothesis, a pooled analysis of published transcriptomic data was performed, using a previously validated 55-gene expression signature of senescence[12] as main endpoint. Datasets reporting lung gene expression in mouse models of acute lung injury and mechanical ventilation were obtained from public repositories (Gene Omnibus Expression -https://www.ncbi.nlm.nih.gov/geo/- and ArrayExpress - https://www.ebi.ac.uk/arrayexpress/-) using the following terms: "Stretch," "Cyclic strain," "Mechanical Ventilation," "Lung," and "Alveolar." Fifty-one datasets were manually reviewed. Studies lacking a control group with intact, spontaneously breathing animals and those reporting less than 40 genes from the endpoint signature were excluded, so 9 datasets were finally used (Supplementary Table 1). When available, raw data was downloaded and normalized using the Robust Multiarray

Average method (for Affymetrix microarrays) or normal-exponential background correction followed by quantile normalization (all the other platforms).

Normalized datasets were pooled using the Combat-Co-normalization using controls (COCONUT) algorithm.[13] This method normalizes gene expression of the different datasets using an empirical Bayes fitting, but applied only to control samples (in this case, spontaneously breathing animals with intact lungs). Then the obtained normalization parameters are applied to the cases (ie, those with lung injury) (Supplementary Fig 1). Three different signatures were studied, corresponding to 116 genes upregulated by p53, 14 genes downregulated by p21[14] and a set of 50 genes consistently up- and down-regulated in senescence.[12] A meta-score was computed for each sample as the geometric mean of the upregulated genes minus the geometric mean of the downregulated genes in the signature.[13] Meta-scores were finally compared among controls and animals with lung injury and/or mechanical ventilation.

Animal models. Male, 12-week-old C57Bl/6 mice, kept under pathogen-free conditions with free access to food and water, were used in all experiments. The Animal Research Committee of the Universidad de Oviedo evaluated and approved the study.

A 2-hit lung injury model, based on chlorhydric acid instillation and mechanical ventilation, was studied. Animals were anesthetized with intraperitoneal ketamine and xylazine and orotracheally intubated using a 20G catheter, through which 50 μL of chlorhydric acid (0.1N, pH = 1.5) were instilled. Two hours after instillation, mice were randomly assigned to receive mechanical ventilation or not. Mice were ventilated with a pressure-controlled mode (peak inspiratory pressure 17 cm H_2O, PEEP 2 cm H_2O, respiratory rate 100 breaths/min) for 120 minutes.

Three additional series of experiments were performed. Mice lacking Tp53 or Cdkn1a (p21, an endogenous inhibitor of cyclin-dependent kinases involved in the senescent response triggered by Tp53) and their wildtype littermates were subjected to the same model of injury, including acid instillation and mechanical ventilation. Genotypes were confirmed by PCR. In separate experiments, wildtype animals were treated with a single dose (200/50 mg/Kg) of lopinavir/ritonavir (a protease inhibitor that inhibits Zmpste24 and disrupts Lamin-A nuclear scaffolding, activating senescence pathways) or saline, administered intraperitoneally immediately after acid instillation, and then ventilated with the parameters described above.

Tissue harvest. Mice were studied in 3 different conditions: baseline, 4 hours after chlorhydric acid instillation without mechanical ventilation and 4 hours after acid instillation including 2 hours of mechanical ventilation. Lungs were removed after exsanguination of anesthetized animals. A laparotomy was performed, the renal artery sectioned, the thorax opened and the heart-lungs removed in bloc. The left lung was instilled with 250 microliters of 4% phosphate-buffered paraformaldehyde, immersed in the same fixative for 24 hours, and then stored in 50% ethanol. The right lung was immediately frozen at −80°C for biochemical analyses.

Patient samples. Paraffin-embedded lung tissue from autopsies of patients were obtained from the tissue bank at Hospital Universitario Central de Asturias, after signed consent from patients' next of kin. ARDS was defined using the Kigali modification of the Berlin definition,[15] to include patients with lung injury but without mechanical ventilation and those without an arterial line. Thirteen samples were recovered (Supplementary Table 2).

Histological studies. After fixation, tissues were embedded in paraffin and 3 slices with at least 1mm of separation between them were cut and stained with hematoxylin and eosin. A pathologist blinded to the experimental settings evaluated the degree and extension of lung damage using a predefined histological score.[16]

Additional lung sections were processed as previously described[17] for detection of myeloperoxidase- and Ki-67-positive cells, using specific antibodies (See Supplementary Table 3 for references). Images from 3 random fields (x200) were taken and then number of positive cells averaged.

For immunofluorescence studies, slides were deparaffinated and antigens retrieved in citrate buffer 0.1M (pH = 9). The autofluorescence of the tissue was diminished using a Sudan black B solution and sections were permeabilized (0.1% Triton X-100 in PBS for 15 minutes), blocked (1% BSA in PBS) and incubated overnight at 4°C with the primary antibody (Supplementary Table 3). After 24 hours, the slices were incubated with the corresponding secondary fluorescent antibody at room temperature for 1 hour. Images were taken using a confocal microscopy (Leica SP8) at 400x and 630x. The number of positive and negative nuclei were automatically quantified using ImageJ software (NIH, Bethesda, Maryland, USA).

Apoptotic cells in lung slices were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as previously described.[9] Images from 3 random fields were acquired in a Leica SP8 confocal microscope and the positive nuclei were counted and expressed as percentage of the total nuclei count.

Western blot. Nuclei were extracted from fresh lung tissues and subsequently homogenized as described

before.[9] The total amount of protein from nuclear extracts was quantified (BCA Protein Assay Kit, Pierce) and 15μg of each sample was loaded in SDS-polyacrylamide gels, electrophoresed at 120mV and electrotransferred onto PVDF membranes. After blockade with 5% non-fat dry milk, the membranes were incubated with primary antibodies against Caspase-9, Lamin-A/C, Lamin-B1, γH2AX, HP1α or H3 (Supplementary Table 3) in 3% nonfat dry milk overnight at 4°C. After 24 hours, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibodies in 2.5% non-fat dry milk. Proteins were detected by chemiluminescence in a LAS-4000 Imaging system. The intensity of each protein band was quantified using ImageJ software (NIH).

Quantitative PCR. Lung fragments (2 mm x 2 mm) were homogenized with TRIZOL (Sigma, Poole, UK) and RNA precipitated by overnight incubation in isopropanol at −20°C. After 24 hours, samples were washed with ethanol and the RNA resuspended in RNAse-free water and quantified. One μg of total RNA was retrotranscribed into complementary cDNA using an RT-PCR kit (High-capacity cDNA rt Kit, Applied Biosystems). Quantitative PCRs were carried out in triplicate of each sample using 40 ng of cDNA per well. Expression of Plk3, Gdnf, Meis1, Il6, Tp53, Cdkn1a (p21), Cdkn2a (p16), Rb, and Gapdh was quantified using Sybr-green Power up, (Fisher Scientific) and 10uM of the corresponding primers (Supplementary Table 4). The relative expression of each gene was calculated as $2^{-\Delta CT(\text{gene of interest})-\Delta CT(\text{GAPDH})}$.

Statistical analysis. Data are shown as mean ± standard error of the mean. Differences between 2 groups were studied using a T test. Differences among more than 2 groups were assessed using an analysis of the variance (ANOVA). For SAHF counts, 3 slides per animal were counted (considered as technical replicates) and analyzed using a mixed-effects ANOVA. When significant, pairwise comparisons were done using the Tukey's Honest Significant Difference test. A P value lower than 0.05 was considered significant.

RESULTS

Transcriptomic signatures of p53/p21 activation and senescence in lung injury. To test the hypothesis that the p53/p21 pathway is activated during acute lung injury and to identify early markers of a switch towards a senescent phenotype, data from 9 datasets of mouse lung injury and mechanical ventilation (Supplementary Table 1) were pooled and gene expression analyzed (Fig 1, A). Three different transcriptomic signatures related to p53-dependent upregulation (116 genes, 85 available in the pooled data), p21-dependent downregulation (14 genes, 12 available)[14] and senescence[12] (55 genes, 44 available) were analyzed. A meta-score of expression of these genes was computed for each sample and compared to assess the effect of lung injury and mechanical ventilation.

Animals subjected to acid aspiration lung injury and mechanical ventilation showed higher expression of p53-dependent genes (ANOVA p-value<0.001, Fig 1, B), lower expression of p21-downregulated genes (ANOVA P value <0.001, Fig 1, C) and a higher meta-score (Fig 1, D) in the senescence signature than spontaneously breathing controls (ANOVA P value <0.001, Fig 1, D). The expression of each gene of these signatures is shown in Supplementary Fig 2. These results support the notion that lung injury and mechanical ventilation activate p53/p21 pathways and the molecular mechanisms of senescence in acutely injured lungs.

Activation of the p53/p21 pathway in a clinically relevant model. To explore the mechanisms involved in the activation of p53-dependent signals, an experimental model of acid aspiration- and mechanical ventilation induced lung injury was tested. Chlorhydric acid instillation and mechanical ventilation induced a significant increase in lung damage and inflammation, assessed by histological scores (Fig 2, A), neutrophilic infiltrates (Fig 2, B) and Il6 expression (Fig 2, C). Lung damage increased the proportion of both proliferating and apoptotic cells in lung parenchyma (Fig 2, D and E and Supplementary Fig 3). Immunohistochemical studies revealed that TUNEL-positive staining was localized in all the explored cell types, including type I and II pneumocytes, fibroblasts and endothelial cells (Supplementary Fig 4). In line with these findings, the abundance of cleaved caspase-9 in lung tissue was increased with lung injury (Fig 2, F)

We then explored the putative activators of this response to acute injury. Lamins in the nuclear envelope act as cell mechanosensors, regulating chromatin organization in response to mechanical stress. We observed that Lamin-A/Lamin-B ratio increased after mechanical stretch (Fig 2, G). Immunofluorescence studies confirmed the increase in Lamin-A in the nuclear envelope after mechanical stretch, but not after hydrochloric acid instillation alone (Fig 2, H). These changes in the nuclear envelope coexisted with an increase in γH2AX (Fig 2, I) and HP1α (Fig 2, J), markers of DNA damage and chromatin remodeling respectively, in nuclear extracts from ventilated animals. Panel 2K shows representative Western blots of these parameters.

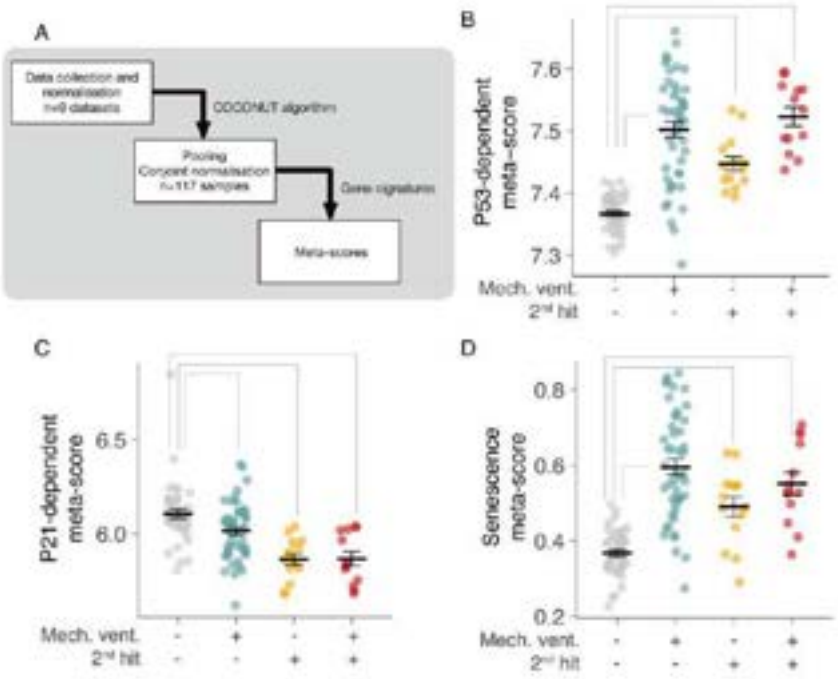

Fig 1. Expression of gene signatures. **A**, Overview of the analysis. Eleven datasets (128 samples) reporting gene expression in animal models of lung injury were pooled and analyzed to calculate different Meta-scores summarizing the expression of genes included in specific signatures. **B**, Meta-score of a p53-dependent signature for each experimental group (second hit refers to any model of lung injury other than mechanical ventilation). **C**, Meta-score of a transcriptomic signature including genes downregulated by p21. **D**, Meta-score of a senescence-specific signature. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc tests).

The expression of the canonical responders to DNA damage Cdkn2a (p16) and Tp53 (p53) and their corresponding downstream factors Rb and Cdkn1a (p21) was also assessed. There were no differences in the levels of Cdkn2a (Fig 2, L), whereas expression of Rb was significantly decreased (Fig 2, M). However, we observed significant increases in Tp53 (Fig 2, N) and Cdkn1a (Fig 2, O) expression with lung injury. The increase in P21 protein was observed mainly in type-I and type-II alveolar epithelial cells (positive for Aquaporin-5 or Surfactant-protein C respectively), and not in fibroblast or endothelial cells (positive for vimentin or Von-Willebrand factor respectively) (Supplementary Fig 5).

Increased lung damage in mice lacking p21. To address the role of p53 and p21 in acute lung damage, $Tp53^{-/-}$, $Cdkn1a^{-/-}$ mice and their wildtype counterparts were subjected to acid instillation followed by mechanical ventilation. In preliminary experiments, absence of Tp53 did not modify lung injury (histological score 2.4 ± 1.6 vs 2.3 ± 1.2, n = 4/group, $P = 0.91$, Supplementary Fig 6), so we focused on the downstream factor p21. In absence of p21, the mice had worse lung injury (Fig 3, A) and higher counts of apoptotic cells (Fig 3, B) and cleaved caspase-9 abundance (Fig 3, C) compared to their wildtype counterparts. There were no differences in Il6 or Tp53 expression nor in abundance of γH2AX or HP1α between genotypes (Fig 3, D–G respectively).

Lopinavir increases p21 and decreases lung damage. We have previously shown that HIV-protease inhibitors modify the nuclear response to mechanical stretch and protect against VILI,[9] an effect that could be due to the inhibition of the Lamin-A protease ZMPSTE24.[18] In our double-hit model, treatment with lopinavir and/or ritonavir impaired the structure of the nuclear lamina, decreasing the abundance of Lamin-A (Fig 4, A), and decreased lung injury (Fig 4, B), apoptotic cell count (Fig 4, C) and cleaved caspase-9 (Fig 4, D). Although abundance of γH2Ax was not modified by this treatment (Fig 4, E), there was a marked decrease in HP1α (Fig 4, F). Panel 4G shows representative blots of these measurements. Finally, treatment with lopinavir and/or ritonavir caused an increase in Il6 expression (Fig 4, H), with no changes in Tp53 expression (Fig 4, I) but an increase in Cdkn1a (p21, Fig 4, J).

Early markers of senescence in acute lung injury. Then, we tried to identify early markers of senescence in our acute model. Acid aspiration and mechanical ventilation-induced lung injury was associated with an increase in the number of nuclei positive for Macro-

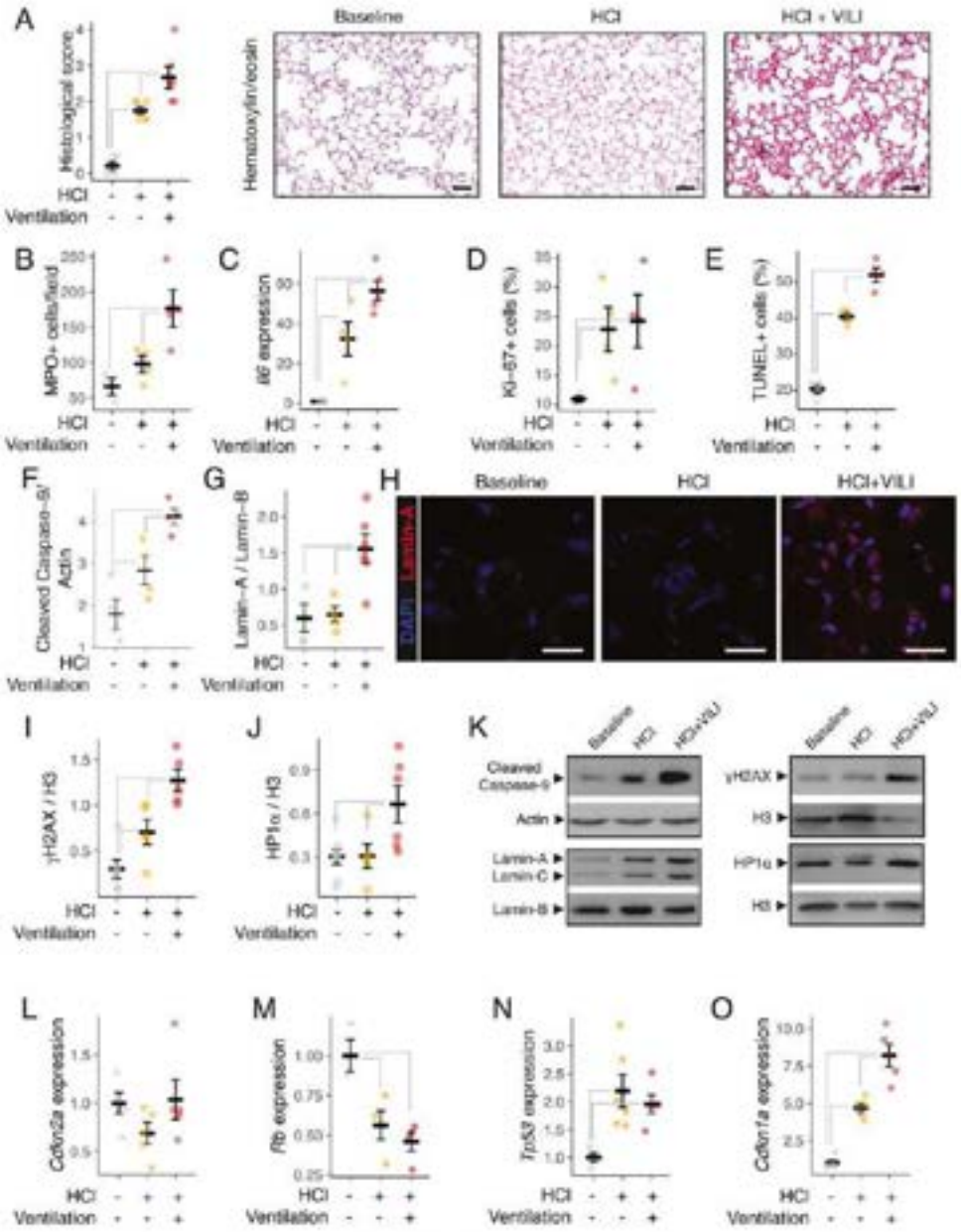

Fig 2. Characterization of lung injury. **A**, Acid instillation and mechanical ventilation caused lung damage assessed using a histological score (scale bar:100 μ). **B**, Myeloperoxidase-positive cell counts in histological sections, showing an increase of neutrophils in the injured lung. **C**, Expression of Il6 in lung tissue. **D**, Quantification of Ki-67 positive cells in histological sections, as a marker of proliferation. **E**, TUNEL-positive cells in histological sections. **F**, Abundance of cleaved Caspase-9 in lung homogenates. **G**–**H**, Changes in Lamin-A/Lamin-B1 ratio in nuclei from lung tissue (**G**) and representative immunohistochemical sections (**H**, scale bar: 25 μ). (**I**–**J**) Abundance of γH2AX (**I**) and HP1α (**J**), markers of DNA damage and heterochromatin respectively, in nuclei from lung tissue. **K**, Representative western blots of the previous quantifications. **L**–**O**, Changes in expression of the canonical senescence inducers Cdkn2a (p16, **L**), Rb (**M**), Tp53 (**N**) and Cdkn1a (p21, **O**). N = 4–6 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc tests).

H2A, a marker of senescence-associated heterochromatin foci (SAHF, Fig 5, A), and changes in Plk3 (Fig 5, B), Gdnf (Fig 5, C), and Meis1 (Fig 5, D), the genes from the senescence signature with the highest differential expression in the previous pooled analysis.

These markers of senescence were modified by manipulation of the p21 pathway. Mutant animals lacking Cdkn1a exhibited a decreased number of SAHF after acid instillation and mechanical ventilation (Fig 5, E) and in expression of the senescence-related

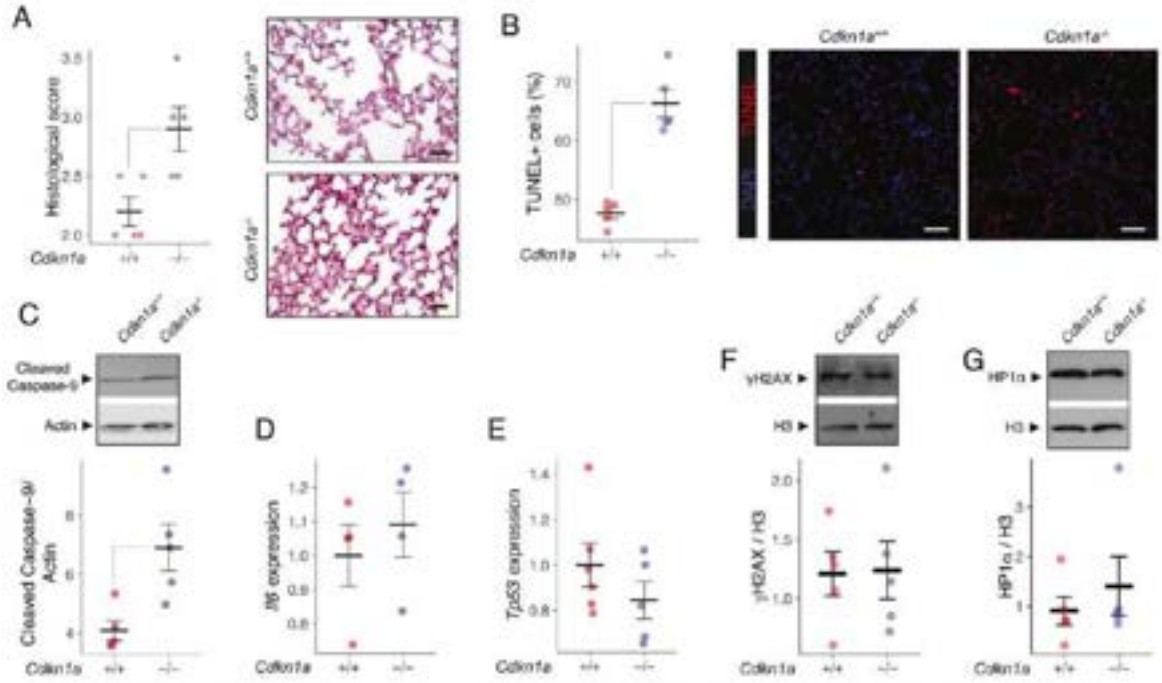

Fig 3. Lung injury in wildtype and Cdkn1a$^{-/-}$ animals. **A**, Histological score of lung damage in both genotypes (scale bar: 100 μ). **B**, Percentage of apoptotic (TUNEL+) cells (scale bar: 50 μ). **C**, Abundance of cleaved caspase-9 in lung homogenates from both genotypes. **D**–**E**, Expression of Il6 (**D**) and Tp53 (**E**) in wildtype and mutant mice. **F**–**G**, Abundance of γH2AX (**F**) and HP1α (**G**), with representative western blots, in lung homogenates. N = 4–6 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in T tests).

gene Plk3 (Fig 5, F). In opposite, treatment with lopinavir and/or ritonavir (that increased Cdkn1a expression, Fig 4, J) was related to lower counts of SAHF (Fig 5, G), but increased Plk3 expression (Fig 5, H).

Finally, to confirm the incidence of SAHF in patients, lung tissue from autopsies of critically-ill patients with and without lung injury and mechanical ventilation (Supplementary Table 2) were stained with antibodies against Macro-H2A. There were no differences in age between the 3 groups of patients (62 ± 6, 61 ± 11, and 54 ± 10 years for patients without ARDS or mechanical ventilation, with ARDS but without mechanical ventilation and ARDS and ventilation respectively, $P = 0.17$ in ANOVA). Similarly, to the animal model, nuclear Macro-H2A increased in those with severe lung injury and mechanical ventilation (Fig 5, I).

Collectively, these findings suggest that lung injury and mechanical stretch trigger the appearance of early markers of senescence. The severity of lung injury and the abundance of senescence markers showed an inverse correlation after manipulation of p21 levels.

DISCUSSION

We provide evidence that acute lung injury and its treatment with mechanical ventilation alters the nuclear envelope and causes DNA damage, activating the p53/p21 pathway. Activation of p21 plays a homeostatic role, limiting the extent of apoptosis in response to injury. Moreover, this effect can be pharmacologically activated to ameliorate lung injury in a clinical setting. In spite of this beneficial effect, this pathway also leads to the appearance of early markers of senescence in lung tissue. Fig 6 summarizes the findings of this work.

The p53/p21 axis in acute injury. P53 and its downstream transcription factor p21 are major regulators of cell homeostasis. It has been shown that p53 regulates permeability in lung endothelial cells after an inflammatory insult.[19] Similarly, activation of this pathway in response to hypertonic saline decreased lung injury and inflammation in human airway epithelial cells.[20] However, our observations in Tp53$^{-/-}$ mice showed no differences in lung injury. Given the pleiotropic effects of p53 in cell homeostasis, this could be due to the existence of both protective and pathogenetic mechanisms.

One of the main effects of the cyclin kinase inhibitor p21 is the blockade of apoptosis.[21] Several

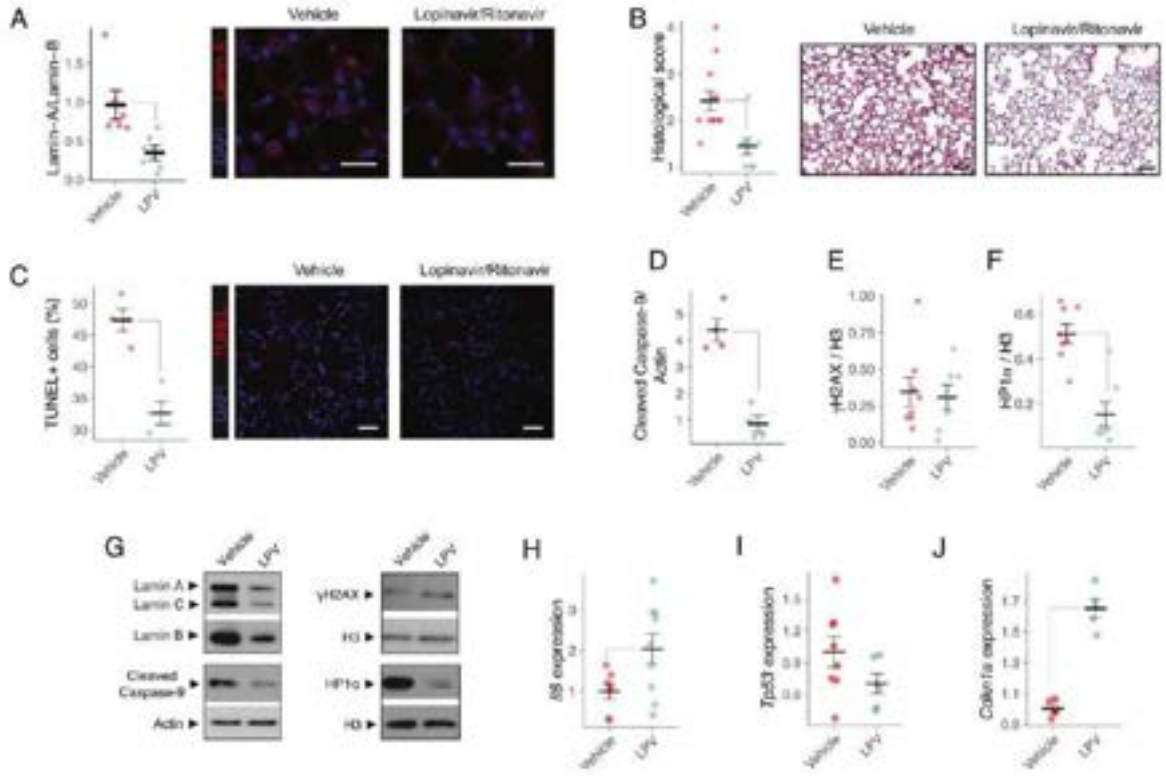

Fig 4. Effects of Lopinavir/Ritonavir on lung injury. **A**, Lamin-A abundance and staining in vehicle- and lopinavir/ritonavir treated animals (scale bar: 25 μ). **B**, Histological score of lung damage (scale bar: 100 μ). **C**, Apoptotic (TUNEL+) cell counts in both groups (scale bar: 50μ). **D-F**, Abundance of Caspase-9 in tissue homogenates (**D**), γH2AX (**E**) and HP1α (**F**), with representative western blots (**G**) in lung homogenates. **H−J**, Expression of Il6 (**H**), Tp53 (**I**) and Cdkn1a (p21, **J**). N = 7−10 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in T tests).

proapoptotic pathways are activated during acute lung injury,[22] as shown in our model. In this setting, p21 may have a compensatory role by avoiding the loss of a large amount of epithelial cells. Our observation of massive cell death in Cdkn1a$^{-/-}$ mice suggests this homeostatic role. It has been proposed that caspase-9 is the downstream target responsible for the antiapoptotic effects of p21.[23] Overexpression of p21 increases the resistance to apoptosis of alveolar epithelial cells,[24] and the beneficial effects of lopinavir and/or ritonavir in VILI could represent the effects of the overexpression of this gene and the observed decrease in caspase-9. In contrast, absence of p21 was associated with more severe lung injury and increased numbers of apoptotic cells, as previously suggested.[25]

The role of mechanical stretch. Mechanical overstretch could be an important pathogenic factor involved in p53/p21 activation. The experimental model using a ventilatory strategy within the limits of protective ventilation (driving pressure 15 cm H_2O, avoidance of zero end-expiratory pressure) was chosen to increase the translational significance of our work. Although ventilation with very high inspiratory pressures and allowing expiratory collapse induces severe lung damage in less than 1 hour, even in healthy lungs, the clinical translation of these findings is less straightforward.

Several mechanisms have linked the mechanical load to a lung biological response, including oxidative stress and MAPK activation.[26] We focused on the role of the nuclear envelope as a critical structure regulating both mechanosensing and senescence. The mechanical load is transmitted from the extracellular matrix to the cytoskeleton and then to the nuclear membrane.[27] This causes a change in the nuclear lamina, reorganization of the underlying chromatin and DNA damage,[28] either mediated by MAPK activation[29] or by a direct mechanical effect.[30] DNA damage is one of the triggers of the p53 pathway. In smooth muscle cells, stretch leads to p53 activation and upregulation of senescence markers,[31] resembling our findings. Similarly, HIV protease inhibitors, such as lopinavir and/or ritonavir, inhibit ZMPSTE-24, a protease responsible for Lamin-A maturation,[18] preserving nuclear compliance, increasing p21 expression and decreasing stretch induced apoptosis and VILI.[9]

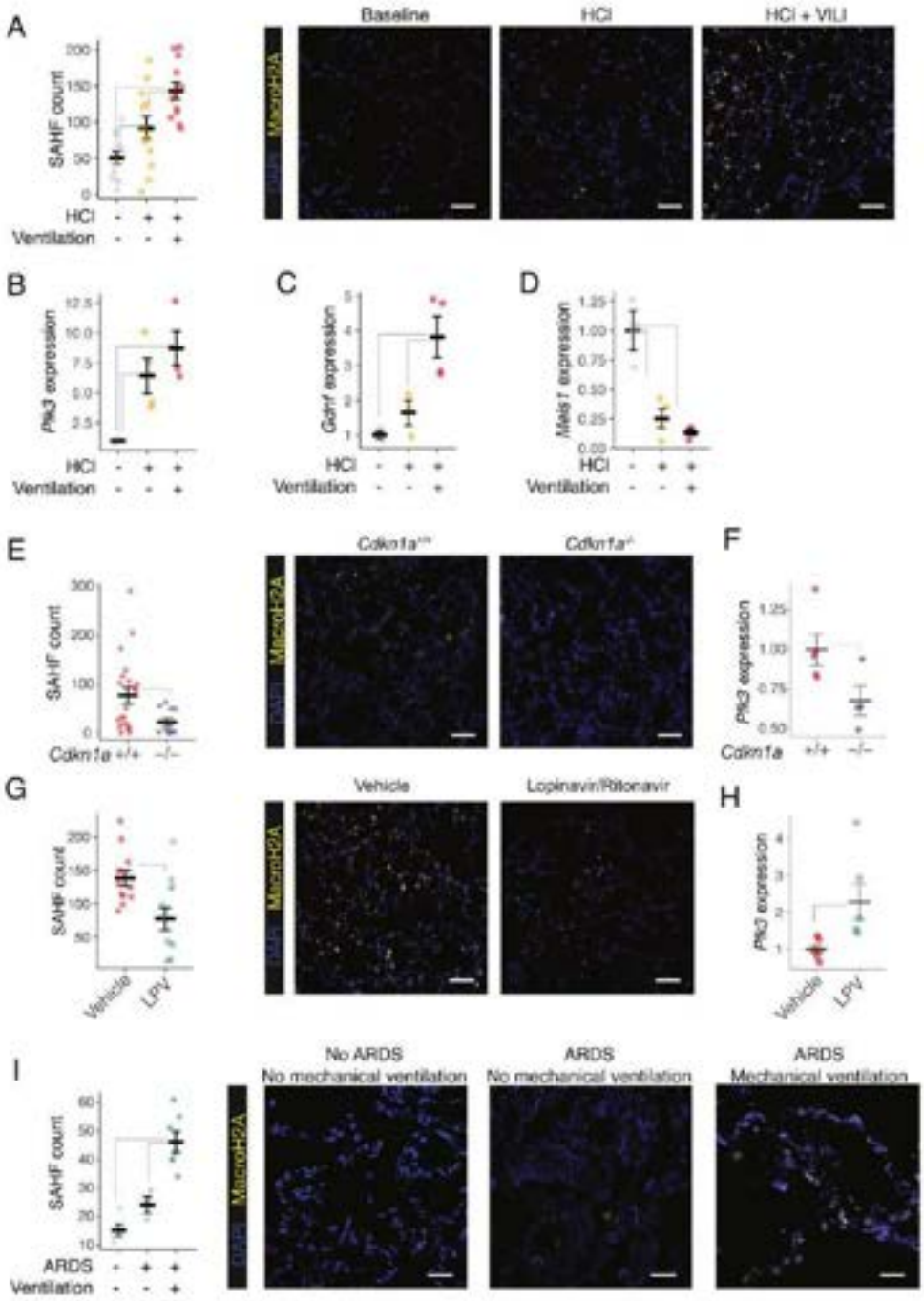

Fig 5. Identification of early senescence markers in experimental models and patients. **A**, Counts of Senescence-associated heterochromatin foci (SAHF) in the experimental model of lung injury of acid instillation and mechanical ventilation (scale bar: 50μ). **B**–**D**, Expression of Plk3, Gdnf, and Meis1 in lung tissue. These senescence-associated genes were identified in the genomic analysis as those with the largest differences between control and injured samples. **E**–**F**, SAHF counts (**E**) and Plk3 expression (**F**) in wildtype and Cdkn1a$^{-/-}$ mice after lung injury. **G**–**H**, SAHF counts (**G**, scale bar: 25μ) and Plk3 expression (**H**) in vehicle and lopinavir/ritonavir (LPV)-treated mice after lung. **I**, Appearance of SAHF in autopsy samples from critically ill patients who died in the Intensive Care Unit with or without mechanical ventilation and acute respiratory distress syndrome (ARDS) (scale bar: 50μ). N = 4–7 animals per group, with 3 slides per animal as technical replicates in SAHF counts. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc or in T tests).

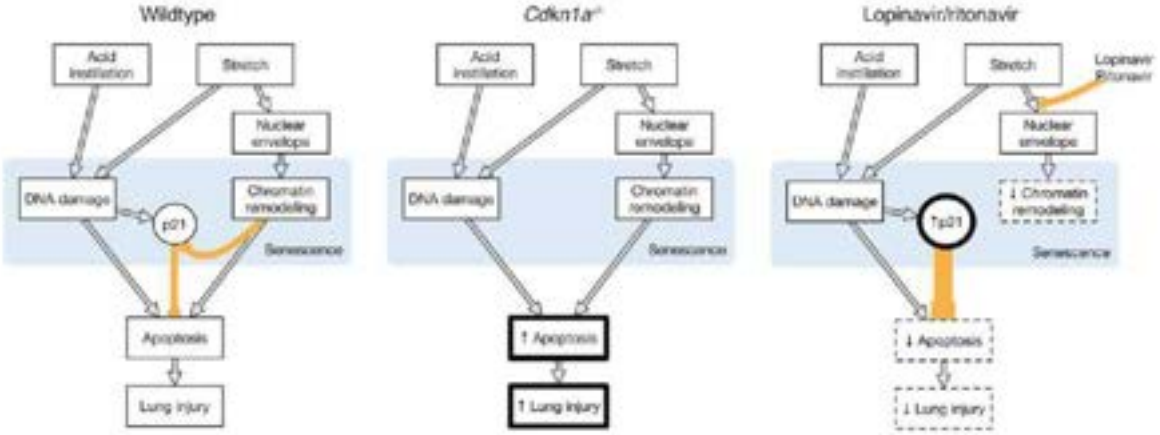

Fig 6. The role of p21 pathway on apoptosis and senescence after acute lung injury. **A**, In control mice, lung injury and mechanical stretch cause DNA damage and changes in the nuclear envelope, activating the cell senescence program. The amount of apoptotic cells depends on the equilibrium between the activation of proapoptotic responses triggered by injury itself and the antiapoptotic effects of the senescence inducer Cdkn1a (p21). **B**, In mice lacking Cdkn1a, absence of this antiapoptotic factor leads to an increase in apoptosis and a more severe lung injury. **C**, Treatment with Lopinavir/ritonavir blocks the Lamin-A mediated chromatin remodeling, triggering a senescence-like response that increases p21 expression, thus decreasing apoptosis and lung damage.

Our immunohistochemical studies suggest that although apoptosis occurs in all the explored cell lines (alveolar epithelium, endothelium and fibroblasts), activation of p21 pathway take place in the alveolar epithelium. Preexisting epithelial damage caused by acid instillation could amplify the mechanical load over the epithelium by increasing heterogeneity of the lung parenchyma. Moreover, previous results in subacute models of lung damage have shown the need for 2 synergic hits to induce lung senescence.[32]

Senescence in lung diseases. One of the known consequences of p53 activation is the cell switch towards a senescent phenotype. Lipopolysaccharide or bleomycin-induced lung injury increases the number of SA-β-galactosidase-positive cells and leads to cell cycle arrest.[33] It has been shown that this activation has no detrimental effects in acute inflammation. However, blockade of the cell cycle has been associated with increased collagen deposition[34] and SASP may perpetuate lung inflammation.[35] Therefore, main features of abnormal lung repair after acute injury (limited cell proliferation, chronic inflammation and fibrosis) could be explained by a persistent senescent response.[36] Inhibition of this response by selective deletion of Tp53 in Club cells ameliorated lung damage related to chronic inflammation,[33] suggesting a novel mechanism amenable to treatment of lung diseases.

The acute nature of our model and its short-term lethality does not allow the identification of canonical senescence markers such as Senescence-associated β-galactosidase, as these require from days to weeks to be positive.[37] However, we identified a set of early markers including changes in chromatin structure and gene expression. In a model of repair after VILI, lung Cdkn1a expression remained elevated up to 2 days of spontaneous breathing after injury.[9] Although it is unclear if these mechanisms may precipitate a full-blown senescent response in the long term, our results highlight the involvement of this molecular machinery in the early phase, and could be a therapeutic target to avoid late consequences.

Clinical implications. Our findings have several implications regarding the pathogenesis of lung injury and its long-term consequences. First, the described p21 response may be beneficial in the acute phase, and could be pharmacologically manipulated using lopinavir. In a recent clinical trial in patients with lung disease caused by the SARS-CoV-2 coronavirus, lopinavir did not reduce mortality, but decreased the risk of ARDS development.[38] However, the associated senescent response could worsen lung repair and long-term outcomes. Survivors after a prolonged ICU stay may have deleterious and prolonged sequels, including respiratory impairment,[39] neuropsychological disturbances[40] and muscle atrophy,[41] particularly the elderly.[42] The mechanisms responsible for these sequels are largely unknown and no effective therapies are currently available. Local activation of senescence and its paracrine and/or systemic spread could contribute to the pathogenesis of these sequels.[43] As previously discussed, senescence may contribute to disordered lung repair. The confirmation of this framework could lead to the use of senolytics[44(p)] in critically ill patients. However, due to the protective nature of

senescence in the early phase,[45] these treatments should be time-coordinated and modulated to optimize their effectiveness.

CONCLUSIONS

We provide new evidence suggesting that acute lung damage activates p21 to limit apoptosis. This response appears to be trigged by the induction of DNA damage and linked to chromatin changes caused by mechanical overstretch. Interaction with the nuclear lamina may enhance this antiapoptotic response. Although p21 activation may be beneficial in the acute phase of lung injury, the long-term effects must be taken into consideration as they could explain some of the long-term sequels of critically ill patients.

DATA STATEMENT

Raw data and R code used in this work are available from the corresponding author (GMA) upon reasonable request.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Authors' contributions: J.B.P., C.H.F., I.L.A., and G.M.A. designed the study. J.B.P., C.H.F., I.L.A., L.A.R., P.M.V., C.L.M., I.C., C.P. and P.J.F.M. performed the experiments. G.M.A. did the bioinformatic analysis. J.B.P., I.L.A., C.H.F., L.A.R., P.J.F.M., and G.M.A. analyzed the results. C.H.F., I.L.A., L.A.R., P.J.F.M., M.S., J.I.S., and G.M.A. discussed the significance of the results. J.I.S., M.S., and G.M.A. wrote the article. G.M.A. is the responsible of the integrity of the whole work.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.01.008.

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Activation of p21 limits acute lung injury and induces early senescence after acid aspiration and mechanical ventilation

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Data supplement

Supplementary table 1. Datasets measuring gene expression in animal models of lung injury and mechanical ventilation. MV: Mechanical ventilation. DP: Driving pressure. VT: Tidal volume. LPS: Lipopolysaccharide.

Dataset	Ref	Platform	Animal	Intervention	n
GSE121550	(López-Alonso et al. 2019)	GPL16570	Mouse	Spontaneous breathing	6
				MV (DP 15 cmH_2O), 2.5h	6
GSE85269	(López-Alonso et al. 2018)	GPL16750	Mouse	Spontaneous breathing	3
				MV (DP 15 cmH_2O), 2.5h	3
GSE18341	(Smith et al. 2010)	GPL1261	Mouse	Spontaneous breathing	8
				MV (VT15 ml/Kg), 2h	7
				Inhaled LPS	8
				MV (VT 15 ml/kg , 2h) + Inhaled LPS	7
GSE11434	(Wray et al. 2009)	GPL1261	Mouse	Spontaneous breathing	5
				MV (DP 20 cmH_2O), 3h	5
GSE9368	(Hong et al. 2008)	GPL1261	Mouse	Spontaneous breathing	3
				MV (VT 30 ml/kg), 4h	3
GSE9314	(Hong et al. 2008)	GPL1261	Mouse	Spontaneous breathing	4
				MV (VT 30 ml/kg), 4h	4
GSE86229	(Otulakowski et al. 2017)	GPL6246	Mouse	Spontaneous breathing	5
				MV (VT 35 ml/kg), 3h	10
GSE9208	(Papasahgari et al. 2007)	GPL8321	Mouse	Spontaneous breathing	3
				MV (VT 30 ml/kg), 2h	3
GSE2411	(Altemeier et al. 2005)	GPL339	Mouse	Spontaneous breathing	6
				MV (VT 10 ml/kg), 4h	6
				Inhaled LPS	6
				MV (VT 10 ml/kg), 4h + Inhaled LPS	6

Supplementary Table 2. Patients' characteristics. ARDS: Acute respiratory distress syndrome diagnosed according to the Kigali modification of the Berlin definition. MV: Mechanical ventilation.

Age	Sex	ARDS	MV	Diagnoses
55	F	No	No	Brain lymphoma
57	M	No	No	Leriche syndrome. Cardiac arrest
67	M	No	No	Cardiac arrest
68	M	No	No	Pneumoconiosis
64	F	No	No	Infective endocarditis
67	F	Yes	No	Bone marrow aplasia. Nosocomial pneumonia
68	F	Yes	No	Community-acquired pneumonia. Septic shock
48	M	Yes	No	Liver cirrhosis. H1N1 influenza. Airway obstruction.
59	M	Yes	Yes	H1N1 influenza
49	M	Yes	Yes	H1N1 influenza
53	M	Yes	Yes	Nosocomial pneumonia
42	M	Yes	Yes	Community-acquired pneumonia
68	M	Yes	Yes	Community-acquired pneumonia

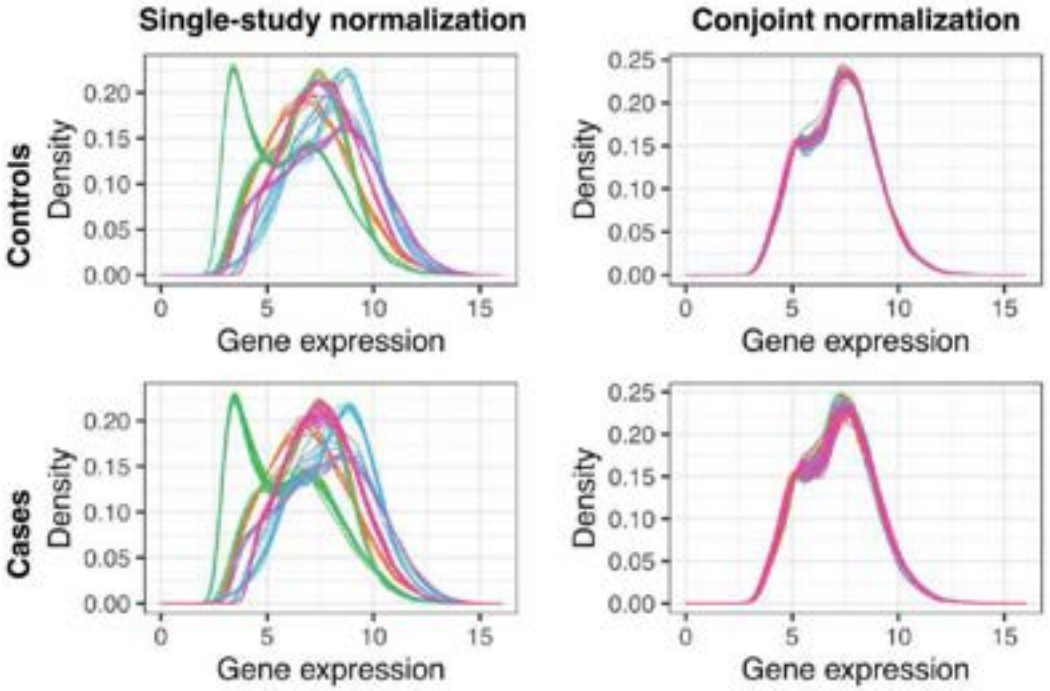
Supplementary Table 3. Antibodies used in the study.

Western blotting	Primary antibodies	Anti-Caspase-9 (#9508, Cell signaling); dilution 1/1000
		Anti-Lamin A/C (sc-20681, Santa Cruz); dilution: 1/2000
		Anti-Lamin B1 (sc-374015, Santa Cruz); dilution: 1/1000
		Anti-YH2AX (sc-517348, Santa Cruz); dilution: 1/500
		Anti-HP1α (sc-130446, Santa Cruz); dilution: 1/500
		Anti-actin (sc-1616, Santa Cruz); dilution: 1/10000
		Anti-H3 (ab1791, Abcam); dilution: 1:10000
	Secondary antibodies	Anti-rabbit IgG-HRP (sc-2004, Santa Cruz); dilution: 1/10000
		Anti-mouse IgG-HRP (sc-2005, Santa Cruz); dilution: 1/10000
Immuno-histo-chemistry	Primary antibodies	Anti-myeloperoxidase (A0398, Dako)
		Anti-Ki67 (GA626, Dako)
	Secondary antibodies	Anti-mouse IgG-HRP (P044701-2, Dako)
		Anti-rabbit IgG-HRP (P044801-2, Dako)
Immuno-fluorescence	Primary antibodies	Anti-macro-H2A (15825913, Fisher); dilution: 1/250
		Anti-Lamin A/C (sc-20681, Santa Cruz); dilution: 1/250
		Anti-p21 (sc-6246, Santa Cruz); dilution 1/250
		Anti-p21 (14-6715-81,eBioscience); dilution 1/250
		Anti-aquaporin 5 (sc514022, Santa Cruz); dilution 1/250
		Anti-surfactant protein C (PA71680, Invitrogen); dilution 1/250
		Anti-VonWillebrand Factor (A0082, Dako), dilution 1/250
	Secondary antibodies	Anti-rabbit IgG-Alexa Fluor 594 (A-21207, Invitrogen); dilution:1/2000
		Anti-rabbit IgG-FITC (sc-2359, Santa Cruz): dilution 1/1000
		Anti-mouse IgG-FITC (sc-2010, Santa Cruz); dilution: 1/500
		Anti-mouse IgG-CFL488 (sc-516176); dilution 1/1000

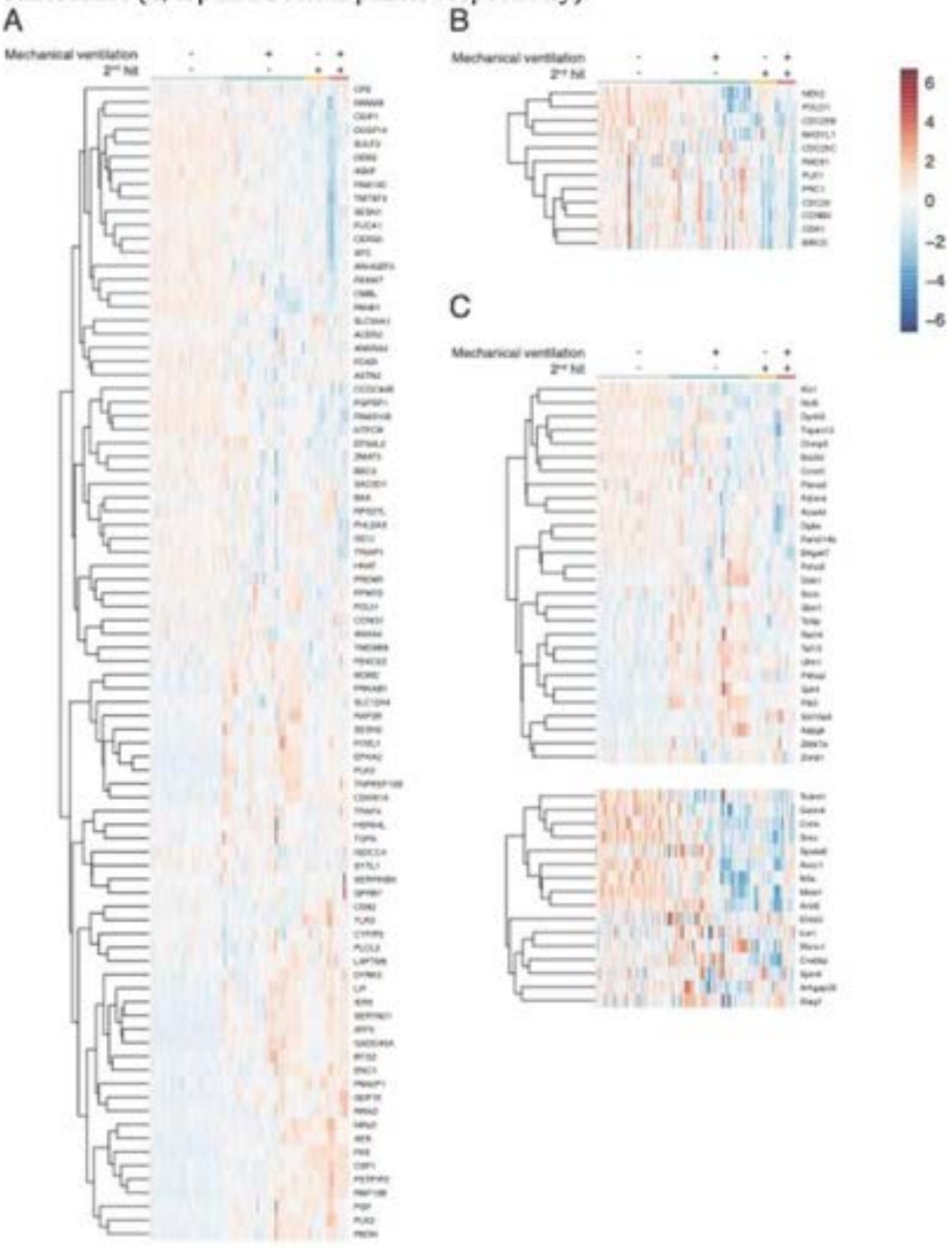
Supplementary Table 4. Primers used for quantitative PCR (FW: Forward; RV: Reverse).

Gene	Species	Direction	Sequence
Tp53	Mouse	FW	5'-CTCTCCCCCGCAAAAGAAAAA-3'
		RV	5'-CGGAACATCTCGAAGCGTTTA-3'
Cdkn1a	Mouse	FW	5'-GGAACATCTCAGGGCCGAAA-3'
		RV	5'-AAGACCAATCTGCGCTTGGA-3'
Cdkn2a	Mouse	FW	5'-CGCAGGTTCTTGGTCACTGT-3'
		RV	5'-TGTTCACGAAAGCCAGAGCG-3'
Il6	Mouse	FW	5'-ACCACTTCACAAGTCGGAGG-3'
		RV	5'-TGCAAGTGCATCATCGTTGT-3'
Plk3	Mouse	FW	5'-GCGCGAGAAGATCCTAAATG-3'
		RV	5'-CTCTGGTTCCAACAGGGTGT-3'
Gdnf	Mouse	FW	5'-GACTTGGGTTTGGGCTATGA-3'
		RV	5'-AACATGCCTGGCCTACTTTG-3'
Meis1	Mouse	FW	5'-AAGGTGATGGCTTGGACAAC-3'
		RV	5'-TGTGCCAACTGCTTTTTCTG-3'
Rb1	Mouse	FW	5'-GCAGTCCAAGGATGGAGAAG-3'
		RV	5'-ACAGGGCAAGGGAGGTAGAT-3'
Gapdh	Mouse	FW	5'-GTGCAGTGCCAGCCTCGTCC-3'
		RV	5'-GCCACTGCAAATGGCAGCCC-3'
TP53	Human	FW	5'-GTGCAGCTGTGGGTTGATTC-3'
		RV	5'-ACCATCGCTATCTGAGCAGC-3'
CDKN1A	Human	FW	5'-TGTCCGTCAGAACCCATGC-3'
		RV	5'-AAAGTCGAAGTTCCATCGCTC-3'
GAPDH	Human	FW	5'-TCGGAGTCAACGGATTTGGTCGT-3'
		RV	5'-TGCCATGGGTGGAATCATATTGGA-3'

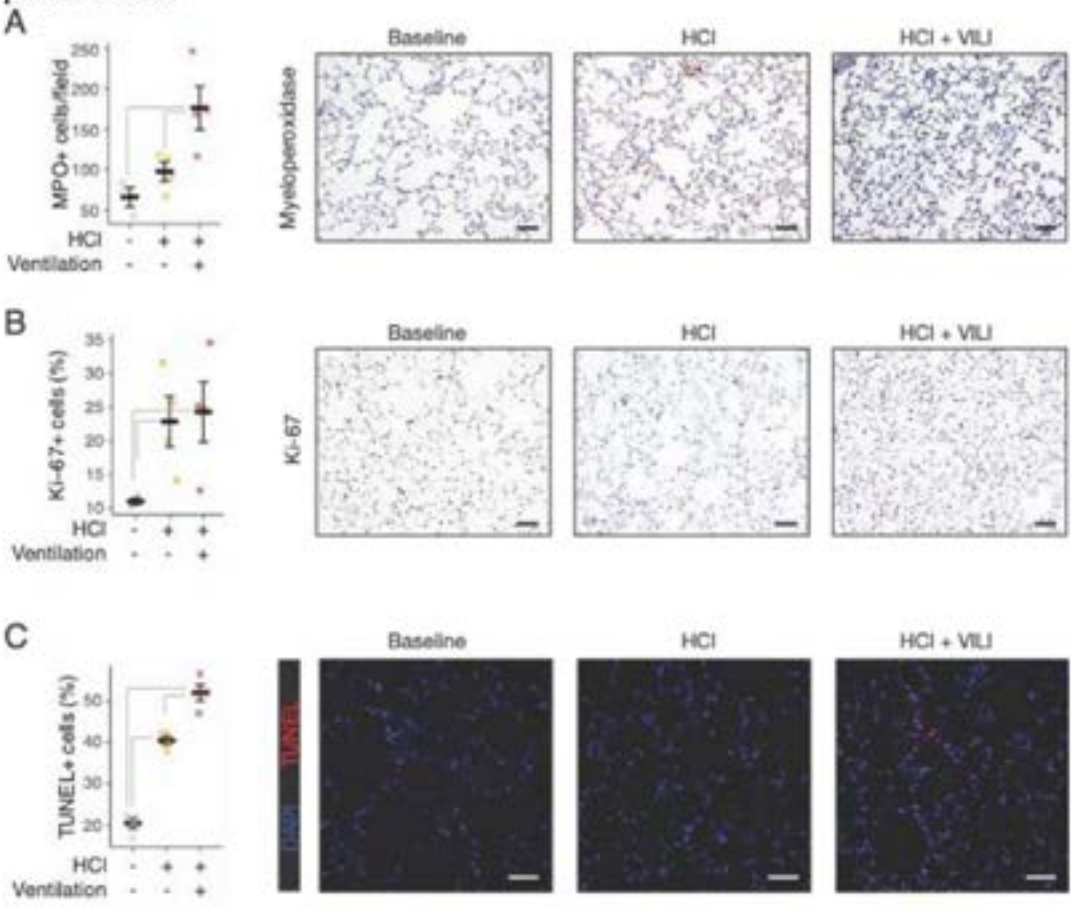
Supplementary figure 1. Gene expression histograms before (single-study normalization) and after normalization with the COCONUT algorithm (conjoint normalization). Controls were spontaneously breathing animals with healthy lungs. Cases were all those with acute lung injury.



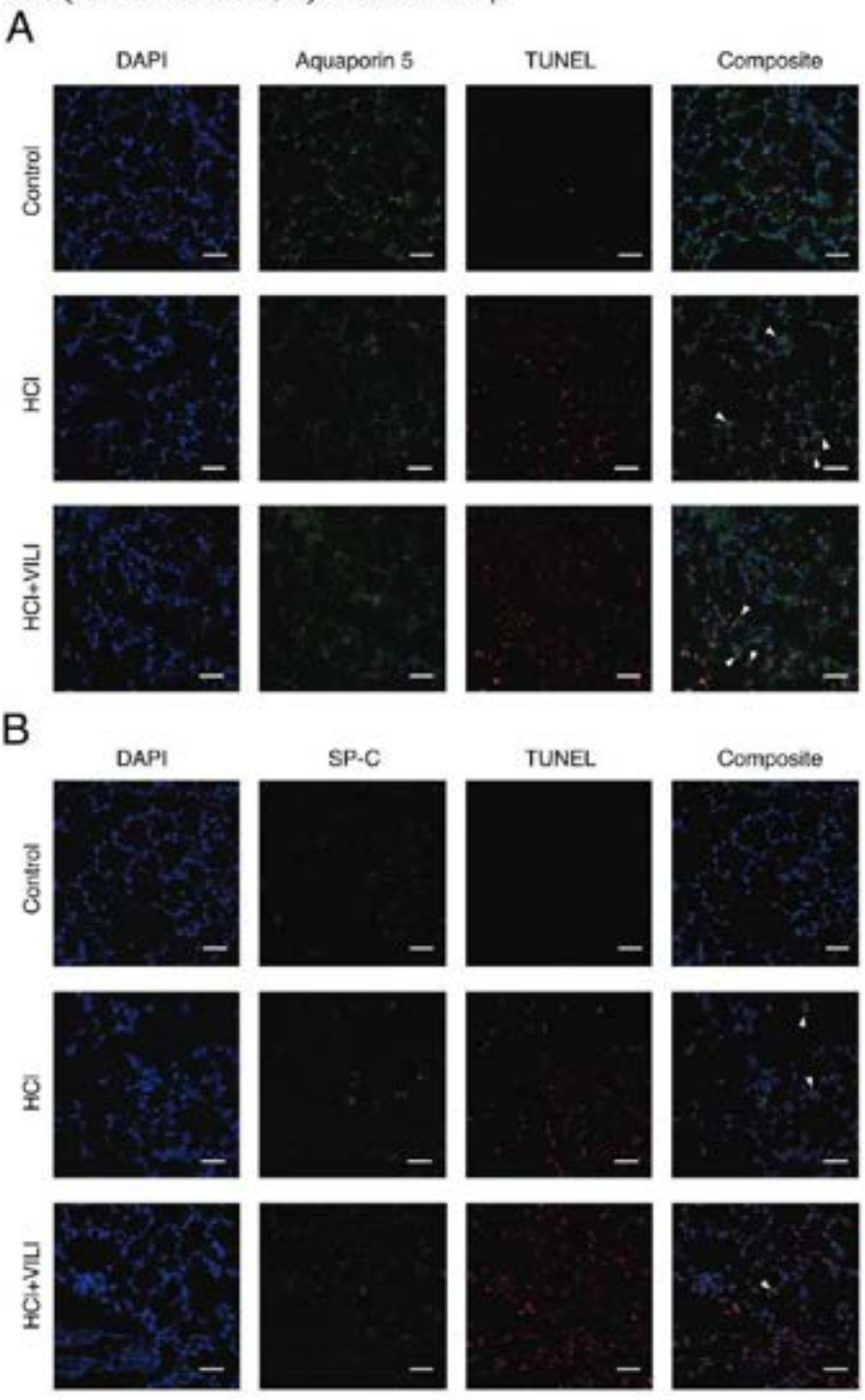
Supplementary figure 2. Heatmaps showing pooled expression of genes included in specific signatures regulated by p53 (A), p21 (B) or up- and down-regulated by senescence (C, top and bottom panels respectively).

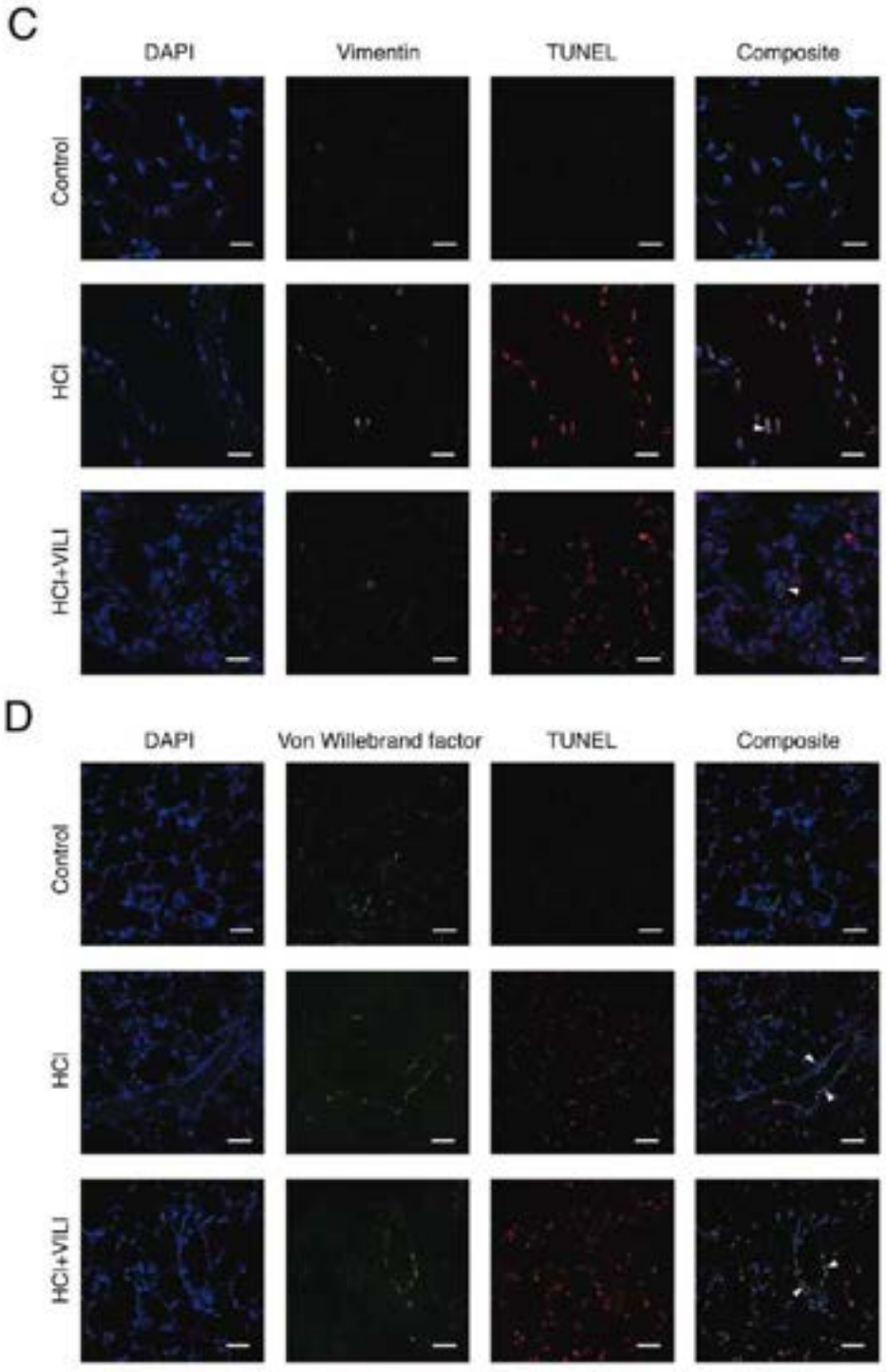


Supplementary figure 3. Representative images of histological slides showing MPO (A, scale bar: 100μ), Ki-67 (B, scale bar: 100μ) and TUNEL (C, scale bar: 50μ) positive cells.

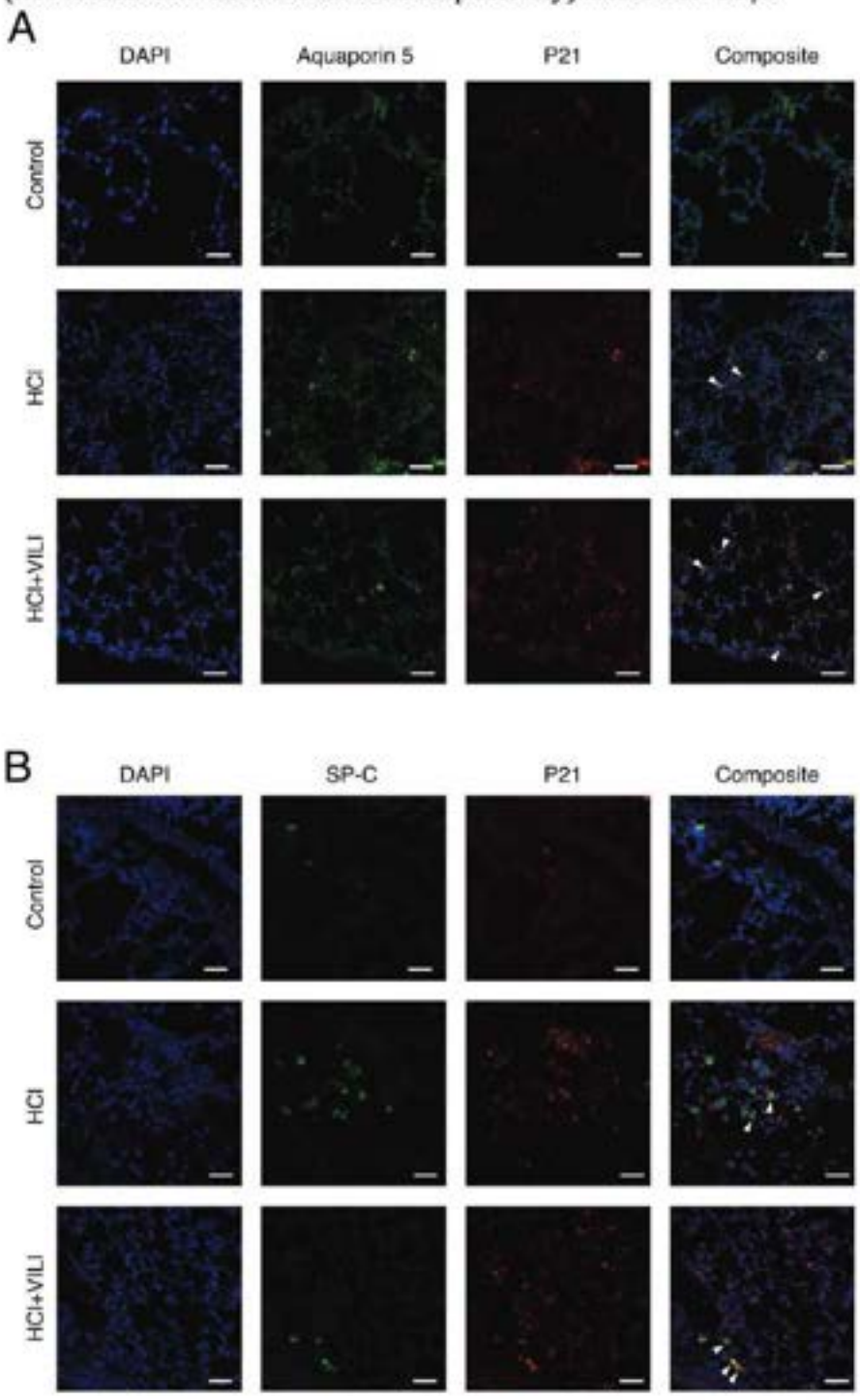


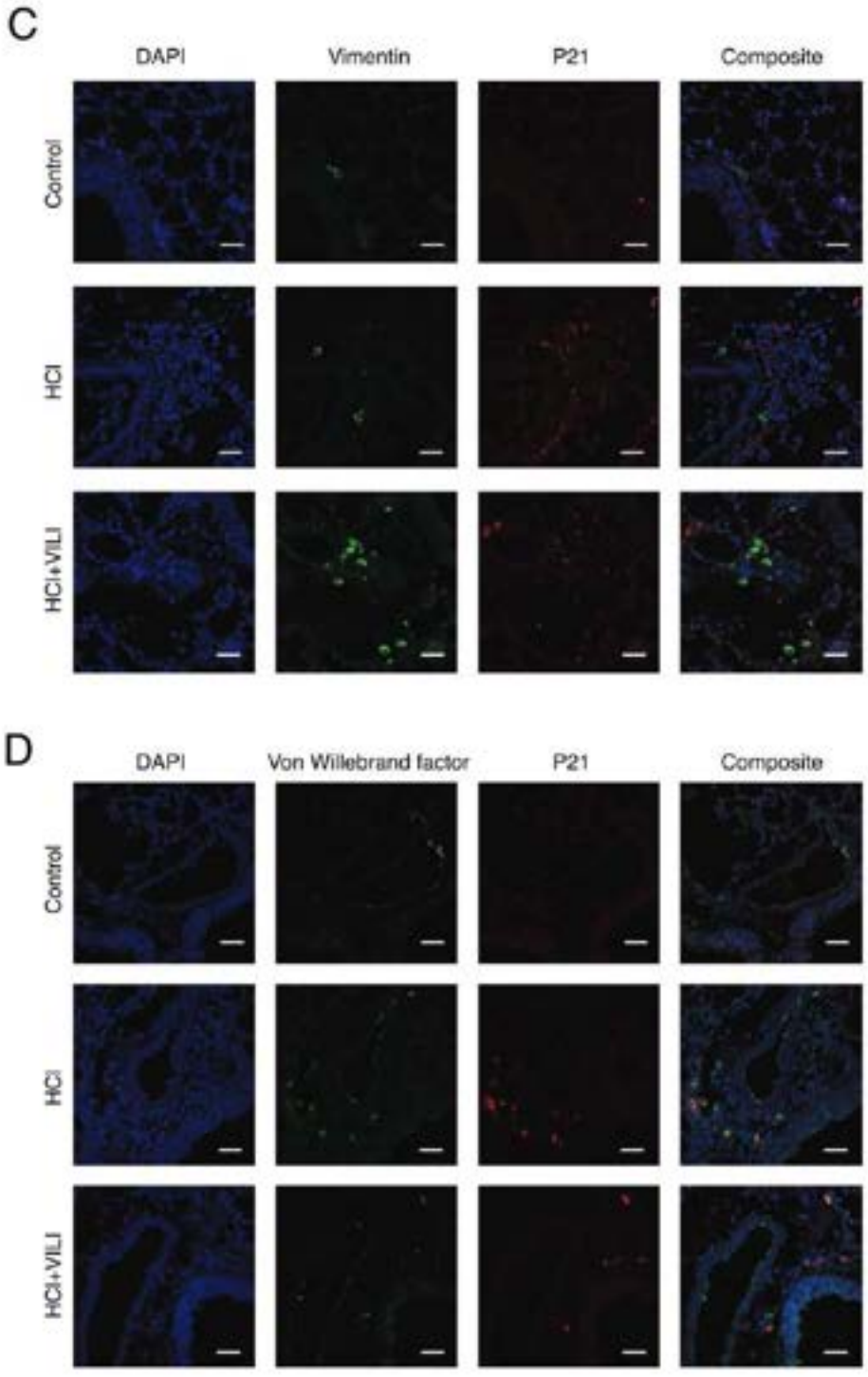
Supplementary figure 4. Immunohistochemical sections showing TUNEL staining in different lung cell lines. Cells positive for TUNEL (arrowheads) were also positive for Aquaporin-5 (marker of alveolar type I cells, A), surfactant protein C (marker of alveolar type II cells, B), vimentin (fibroblasts, C) or Von-Willebrand factor positive cells (endothelial cells, D). Scale bar: 50 µ.



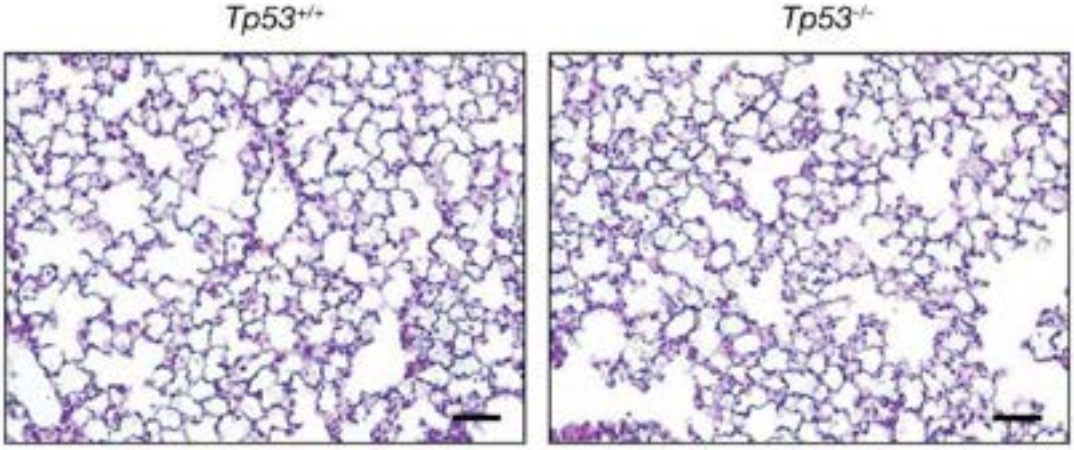

C
DAPI
Vimentin
TUNEL
Composite
Control
HCl
HCl+VILI
D
DAPI
Von Willebrand factor
TUNEL
Composite
Control
HCl
HCl+VILI

Supplementary figure 5. Immunohistochemical sections showing P21 staining in different lung cell lines. Cells positive for P21 were also positive for Aquaporin-5 (marker of alveolar type I cells, A) or surfactant protein C (marker of alveolar type II cells, B), but not in vimentin (C) or Von-Willebrand factor (D) positive cells (fibroblasts and endothelial cells respectively). Scale bar: 50 μ.




C
DAPI
Vimentin
P21
Composite
Control
HCl
HCl+VILI
D
DAPI
Von Willebrand factor
P21
Composite
Control
HCl
HCl+VILI

Supplementary figure 6. Representative histological sections of *Tp53*$^{+/+}$ and *Tp53*$^{-/-}$ mice after acid instillation and mechanical ventilation. There were no significant differences in the severity of lung injury between genotypes. Scale bar: 50μ.



III. El estiramiento mecánico induce la senescencia de las células pulmonares alveolares e impulsa la activación de los fibroblastos por mecanismos paracrinos.

La ventilación mecánica es una terapia de soporte vital necesaria para la supervivencia de pacientes con daño pulmonar severo que no pueden respirar de manera autónoma. Esta, a su vez, puede activar distintos mecanismos patogenéticos que agravan la disfunción pulmonar del paciente. En este estudio se expusieron células epiteliales y fibroblastos a estiramiento mecánico y se evaluó la senescencia celular. Los fibroblastos fueron también expuestos a medios condicionados por células epiteliales senescentes para estudiar su activación. Los resultados mostraron que el estiramiento mecánico induce senescencia en las células pulmonares epiteliales y que los fibroblastos se activan en respuesta al medio condicionado por estas células senescentes. Asimismo, la señalización de Notch fue identificada como una vía clave en la interacción entre el tejido epitelial y mesenquimal. Finalmente, el tratamiento con digoxina redujo la senescencia celular y mejoró la respuesta de los fibroblastos. Estos resultados en conjunto muestran que la senescencia podría ser un mecanismo importante en el desarrollo de la fibrosis secundaria al tratamiento con ventilación mecánica. Además, sugieren que la fibrosis pulmonar puede ser mediada por los efectos paracrinos de las células senescentes, un mecanismo que podría ser manipulado farmacológicamente para mejorar la reparación pulmonar.

Artículo 3. **Martín-Vicente P**, López-Martínez C, López-Alonso I, Exojo-Ramírez SM, Duarte-Herrera ID, Amado-Rodríguez L, Ordoñez I, Cuesta-Llavona E, Gómez J, Campo N, O'Kane CM, McAuley DF, Huidobro C, Albaiceta GM. Mechanical Stretch Induces Senescence of Lung Epithelial Cells and Drives Fibroblast Activation by Paracrine Mechanisms. Am J Respir Cell Mol Biol. 2024 Aug 12.

Aportación personal al trabajo.

Fui la responsable de la mayor parte de este trabajo. Diseñe y desarrolle los distintos modelos celulares. Procedí a la realización de los ensayos bioquímicos, adquisición de imágenes y análisis de resultados. También estuve involucrada en el análisis estadístico, elaboración de figuras y redacción del manuscrito.

Mechanical Stretch Induces Senescence of Lung Epithelial Cells and Drives Fibroblast Activation by Paracrine Mechanisms

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Running title: Stretch-induced epithelial senescence

Authors' contributions: Conception and design: CH, GMA; In vitro-experiments: PMV, CLM, ILA, SMER, LAR, IO, NC. Ex-vivo human lung experiments: CMOK, DFMA; Sequencing: ECL, JG; Bioinformatic analyses: CLM, IDDH, GMA; Analysis and interpretation of results: PMV, GMA; Drafting the manuscript: PMV, GMA; Revision of manuscript for important intellectual content: All authors.

This article has a data supplement, which is accessible at the Supplements tab.

Abstract

Severe lung injury requiring mechanical ventilation may lead to secondary fibrosis. Senescence, a cell response characterized by cell cycle arrest and a shift towards a proinflammatory/profibrotic phenotype, is one of the involved mechanisms. Here, we explore the contribution of mechanical stretch as trigger of senescence of the respiratory epithelium and its link with fibrosis. Human lung epithelial cells and fibroblasts were exposed *in vitro* to mechanical stretch, and senescence assessed. In addition, fibroblasts were exposed to culture media preconditioned by senescent epithelial cells and their activation was studied. Transcriptomic profiles from stretched, senescent epithelial cells and activated fibroblasts were combined to identify potential activated pathways. Finally, the senolytic effects of digoxin were tested in these models. Mechanical stretch induced senescence in lung epithelial cells, but not in fibroblasts. This stretch-induced senescence has specific features compared to senescence induced by doxorubicin. Fibroblasts were activated after exposure to supernatants conditioned by epithelial senescent cells. Transcriptomic analyses revealed notch signaling as a potential responsible for the epithelial-mesenchymal crosstalk, as blockade of this pathway inhibits fibroblast activation. Treatment with

digoxin reduced the percentage of senescent cells after stretch and ameliorated the fibroblast response to preconditioned media. These results suggest that lung fibrosis in response to mechanical stretch may be caused by the paracrine effects of senescent cells. This pathogenetic mechanism can be pharmacologically manipulated to improve lung repair.

Keywords: Senescence; Cell stretch; Secondary lung fibrosis; epithelium-fibroblast crosstalk.

Severe lung damage, often included within the Acute Respiratory Distress Syndrome (ARDS), activates pro-fibrotic programs (1). This secondary fibrosis has major short- and long-term consequences on the outcome by worsening gas exchange and increasing length of mechanical ventilation and mortality (2).

It has been hypothesized that mechanical forces can determine the progression of acute lung injury to fibrosis. By activation of mechanodependent pathways, stretch modulates cell survival, matrix remodeling and local and systemic inflammation (3), all of which can promote collagen deposition (4). Currently, secondary fibrosis is considered the result of the interaction between host factors (i.e. genotype and/or endotype), the disease causing lung damage and the received treatment (5). The specific contribution of this mechanical stress to fibrosis in later stages of the syndrome has not been systematically explored, and no effective drugs have been applied in the clinical practice.

Senescence is a cellular response characterized by cell cycle arrest and a switch towards a proinflammatory/profibrotic phenotype, fueled by the paracrine release of a large number of mediators (known as Senescence-Associated Secretory Phenotype - SASP-) (6). In chronic lung fibrotic conditions, such as idiopathic pulmonary fibrosis,

epithelial senescence is one of the involved pathogenetic mechanisms (7, 8). Moreover, it has been shown that senescence pathways are also activated during experimental acid-induced lung injury (9). However, the consequences of this activation and the specific contribution of mechanical stress in this setting have not been elucidated.

Senescence may constitute a therapeutic target, as several drugs can modulate this response by selective killing of senescent cells (senolytics) or by blocking the acquisition of the characteristic proinflammatory and profibrotic phenotype (senomorphics). Digoxin, an inhibitor of the Na^+-K^+-ATPase, has a senolytic effect by impairing membrane potential and reducing intracellular pH up to levels senescent cells cannot tolerate, selectively triggering apoptosis in this population (10). We have shown that, in the context of acute lung injury, activation of senescence avoids the massive loss of cells due to apoptosis, thus limiting damage (9). Therefore, blockade of senescence at this stage may be detrimental. However, senotherapeutic drugs may have a place to avoid the long-term sequels once the acute phase has been resolved.

The objective of this work is to study the role of mechanical stress as a trigger of senescence in lung cells, and explore the consequences of senescence activation on fibrosis and its potential modulation using digoxin.

Methods

Cell culture experiments. Human lung adenocarcinoma (A549), human bronchial epithelial, non-tumor (BEAS-2b) and human lung fibroblast (MRC5) cell lines were cultured in elastic plates (Bioflex culture plates, Flexcell int, Burlington, NC) in DMEM + 10% FBS (A549 and MRC5 cell lines) or Airway Epithelial Cell Basal Medium (BEAS-2b cells) and standard culture conditions (37°C, 21% O_2, 5% CO_2). Cell lines were authenticated by short tandem repeats profiling. Cells were exposed to cyclic stretch (15% elongation, 15 cycles/min) for 24 hours. Non-stretched cells, cultured in the same conditions or treated with doxorubicin (100 nM) for 24 hours were used as negative and positive controls for senescence respectively. Afterwards, cells were transferred to rigid plates (6 well cell culture plate, Costar, New York, NY) for 6 days. The conditioned media was collected and cells were recovered with trypsin.

Senescence-associated β-Galactosidase measurement. Although there are no universal markers of senescence, we defined senescent cells as those positive for Senescence-Associated β-galactosidase (SA-β-Gal) (11) . Stretched and non-stretched cells were fixed at room temperature in 2% formaldehyde for 10 minutes, washed and incubated with CellEvent Senescence Green Probe (Thermo Fisher Scientific, Waltham, MA), a marker of SA-β-Gal. Data was acquired on a BD FACSAria™ cell sorter. SA-β-Gal positivity was established using predefined gating areas in non-stained cells from the same experiment (Supplementary Figure 1). Cells positive and negative for SA-β-Gal were sorted and RNA extracted (GeneMATRIX Universal RNA Purification Kit, Eurx, Gdańsk, Poland). Stretched and non-stretched cells were also fixed at room temperature in 2% formaldehyde-0,2% glutaraldehyde for 15 minutes, washed and incubated overnight with staining solution containing X-gal substrate for β-Gal activity assays (Agilent, Santa Clara, CA). The next day, slides were washed, and photos were taken with a Motic AE2000 inverted microscope with a 10X objective.

Immunofluorescence studies. Stretched and non-stretched A549 and MRC5 cells were recovered from culture plates, suspended and fixated with 4% paraformaldehyde

on microscope slides (Superfrost Plus Adhesion, Thermo Fisher Scientific). Cells were then incubated with 0.2% Triton X-100 for 10 mins, washed with PBS (phosphate-buffered saline) and, after blockade of non-specific binding sites with albumin, incubated overnight at 4°C with a primary antibody against HP1γ (ab213167, Abcam, Cambridge, UK), a marker of senescence-associated heterochromatin foci. The next day, slides were washed and incubated with the corresponding secondary fluorescent antibody (anti-rabbit AlexaFluor-594, Thermo Fisher Scientific). The slides were mounted with SlowFade Diamond Mountant with DAPI (Invitrogen, Waltham, MA) and scanned in a SP8 confocal microscope. Positive and negative nuclei were quantified automatically using ImageJ software (NIH, Bethesda, Maryland, USA).

Cell cycle analysis. Stretched and non-stretched cells were collected 6 days after stretching and fixed in 70% ethanol. Cells were treated with RNAse-A and stained with propidium iodide. DNA content in these samples was assessed by flow cytometry, and the percentage of cells in the G_1, S, and G_2-M phases of the cell cycle quantified using the *flowPloidy* package for R (12).

RNA sequencing. Stretched A549 cells were sorted according to SA-β-Gal positivity, and RNA extracted and sequenced using the Ion AmpliSeq Transcriptome Human

Gene Expression Kit in a Ion S5 System (Thermo Fisher Scientific). The obtained sequences were pseudoaligned with Salmon v1.10.1, using a GRCh38 human reference transcriptome and imported into R using *tximport* (13). Differential expression analysis was performed using DESeq2 (14). P-values were adjusted using the Benjamin-Hochberg method for a false discovery rate of 5%. A transcriptomic score of senescence was computed as the geometric mean of the expression of genes included in a previously described signature of senescence (15). A Gene Set Enrichment Analysis (GSEA) using all expressed genes ranked using the Wald statistic (16), was performed using ClusterProfiler (17) to identify the gene sets with significant differences in enrichment. The obtained normalized enrichment score reflects the degree to which a gene set is overrepresented at the extremes (top or bottom) of the entire ranked list. Gene ontologies with adjusted p-value lower than 0.05 were clustered according to their core enriched genes using a hierarchical binary algorithm to synthetize redundant categories. All datasets are available in Gene Expression Omnibus (accession number GSE267788, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE267788).

Preconditioning of MRC5 fibroblasts. MRC5 fibroblasts were cultured in rigid plates (24 Well Cell Culture Plate, Costar) in DMEM + 10% FBS and standard culture conditions (37°C, 21% O_2, 5% CO_2) and grown until confluence. Then, a series of concentric scratches were made, floating cells were removed by washing with PBS and conditioned culture medium from stretched and non-stretched A549 epithelial cells was added. Cells were recovered after 6 hours for RNA sequencing (RNeasy Mini Kit, Qiagen, Hilden, Germany) and after 24 hours for protein extraction (18). In additional experiments, either the pan-integrin inhibitor GLPG-0187 (0.5ng/mL), or DAPT (75 μM), a γ-secretase inhibitor that blocks notch signaling, were added and cells incubated for 24 hours for protein extraction. These doses were selected after preliminary experiments as the highest that do not induce cell death.

Wound healing assay. Human lung MRC5 fibroblasts and human lung adenocarcinoma A549 cells were cultured in rigid plates (24 Well Cell Culture Plate, Costar) in DMEM + 10% FBS and standard culture conditions, and grown until confluence. After 24 hours, a scratch was made, floating cells were removed by washing with PBS, and medium from previous experiments (preconditioned by A549 cells cultured after static conditions or stretch) was added. Then, images at regular

time intervals were taken using a Zeiss AxioObserver Z1 microscope with a x10 objective. Wound closure was assessed by repeated quantification of the wounded area using ImageJ (NIH).

Western blot. MRC5 cells were cultured for 24 hours with conditioned medium from stretched and non-stretched A549 cells and with or without inhibitors (see above), and then treated with trypsin to obtain a single cell suspension. Cells were homogenized and proteins extracted. The total amount of protein was measured (BCA Protein Assay Kit, Thermo Fisher Scientific) and 15 µg of total protein for each sample were loaded into SDS-polyacrylamide gels and electrophoresed. Proteins were then transferred to PVDF membranes. After blockade with 5% non-fat dry milk to prevent non-specific binding, these membranes were incubated overnight at 4°C with primary antibodies targeting smooth muscle actin (α-SMA) and β-actin (as loading control). Then, membranes were treated with the corresponding peroxidase-conjugated secondary antibodies, revealed by adding a substrate (Luminata™ Forte HRP Substrate, Merck, Burlington, MA) and bands visualized using chemiluminescence in LAS-4000 Imaging System. The intensity of each protein band was quantified using ImageJ (NIH).

Identification of ligand-receptor networks. Differential expression data from static/stretched A549 and control/preconditioned MRC5 cells were combined to identify potential crosstalk between populations. Using two databases of known ligand-receptor-transcription factor interactions (CellCallEXT (19) and OmniPath(20)) we first selected ligand-receptor pairs with differentially expressed ligands in stretched A549 cells and evidence of receptor expression in MRC5 cells. Transcription factors present downstream the resulting receptors and included in the core enrichment set of a gene ontology category with significant enrichment in preconditioned MRC5 fibroblasts were identified, and the resulting ligand-receptor-transcription factor-ontology networks were constructed.

Digoxin treatment. In separate experiments, A549 cells were stretched, transferred to a rigid culture plate and treated with digoxin in DMEM + 10% FBS for 6 days. Vehicle-treated cells (PBS) were used as control. The medium was collected for preconditioning experiments as previously described.

Proteomic analysis. Proteins from cell culture supernatants were precipitated with trichloroacetic acid, collected by centrifugation at 20000g for 10 mins at 4°C and resuspended in 1 mL of cold acetone. The samples were centrifugated again and the

pellet dried at room temperature. The obtained proteins were separated and quantified using a liquid chromatography system (Evosep One, Evosep, Odense, Denmark) and a high-resolution Q-TOF mass spectrophotometer (ZenoTOF 7600, Sciex, Framingham, MA). Differences in protein abundance between groups were assessed using limma (21).

Human samples. Gene expression from human lungs ventilated ex vivo was recovered from a publicly available dataset (Gene Expression Omnibus, accession number GSE234463). Briefly, human lungs not suitable for transplant were perfused ex vivo and ventilated with continuous positive airway pressure (CPAP 5 cmH_2O) or with a tidal volume of 12 mL per Kg of predicted body weight without positive end-expiratory pressure (to cause ventilator-induced lung injury, VILI). Data were imported to R using *tximport* and normalized using the DESeq2 algorithm as previously described. A transcriptomic score of senescence was calculated for each sample and compared according to the ventilatory strategy.

Statistical analysis. Data are shown as mean and standard deviation. Experimental groups were compared using a Wilcoxon test. In wound closure assays, a repeated-measurements linear model was fitted, including time and experimental group as

within and between-groups factors respectively. Statistical analyses of differential gene expression were previously described. All the calculations were done using R software (22) with the packages *ggplot2* (23), *pheatmap* (24) and *tximport* (13), in addition to those previously cited.

Results

Mechanical stretch triggers epithelial cell senescence

First, we assessed the induction of senescence by mechanical stretch. Compared to cells cultured in static conditions, cyclic stretch increased the percentage of A549 cells positive for SA-β-Gal (Figure 1A-C) and nuclear HP1γ (Figure 1D-E). These senescent cells showed a cell cycle arrest in G1 phase (Figure 1F). Similarly, stretch of BEAS-2b cells led to a significant increase in the proportion of senescent cells and cell cycle arrest (Supplementary Figure S2).

Senescent and non-senescent cells after stretch were sorted according to their SA-β-Gal content, their RNA sequenced and differences in gene expression assessed. The PCA plot including cells cultured in static conditions and stretched cells (senescent and non-senescent) is shown in Supplementary figure S3A. There were 40

differentially expressed genes (Figure 1G, Supplementary Figure S3B and Supplementary table S1). GSEA revealed several functional features of these senescent cells, including downregulation of nucleic acid metabolism and increased inflammation (Figure 1H and Supplementary Table S2). There were no significant differences in the expression of known inducers of senescence, such as *CDKN1A* (p21, Supplementary figure S3C) or *TP53* (Supplementary figure S3D). However, a previously described senescence score (15) calculated using the expression of 9 genes in these cells was higher in the population of senescent cells (Figure 1I), suggesting an established senescent phenotype. Individual expression of the genes included in this signature is shown in Supplementary Figure S3E. Finally, the same score was calculated using publicly available data from *ex vivo* perfused and ventilated human lungs. Mechanical ventilation with large tidal volumes increased the senescence score compared to lungs kept on continuous positive airway pressure (Figure 1J).

Specific features of stretch-induced senescence

To better characterize stretch-induced senescent epithelial cells, their transcriptomic profile was compared to senescent A549 cells obtained after doxorubicin treatment. There were 1307 differentially expressed genes (using an adjusted p-value threshold of 0.05, Figure 2A and Supplementary table S3). There were no significant differences in the calculated transcriptomic senescence score (Figure 2B). A GSEA analysis revealed 555 gene ontologies with an adjusted p-value lower than 0.05 (Supplementary Table S4). These categories were clustered (Figure 2C): compared to doxorubicin-induced senescence, stretch-induced senescent cells showed a lower expression of genes related to inflammation, cell migration, morphogenesis and ion channel activity, and an increased expression of genes involved in RNA processing and ATP metabolism.

Epithelial stretch-induced senescence drives fibroblast activation.

We then studied the senescent response of cultured fibroblasts to stretch. Mechanical stretch did not increase the percentage of SA-β-Gal positive cells (Figure 3A-B). HP1γ had a diffuse pattern in fibroblasts, with no clear foci in either condition (Figure 3C).

There were no differences in cell cycle phases between groups (Supplementary Figure S4).

To further explore the crosstalk between epithelial and mesenchymal cells under stretch conditions, fibroblasts were grown in presence of preconditioned media obtained from A549 cells cultured in static and stretch conditions. Conditioned media from stretched cells increased fibroblast migration, assessed by a wound healing assay (Figure 3D) and abundance of α-SMA (Figure 3E-F). This conditioned media delayed wound closure in A549 cells (Supplementary Figure S5), thus discarding non-specific effects of these media on cell growth.

RNA from these preconditioned fibroblasts was extracted and sequenced. Fibroblasts cultured in presence of media from stretched epithelial cells showed significant differences in the expression of 227 genes (Figure 3G and Supplementary Table S5). Enrichment analysis using GSEA and clustering showed an increase in pathways related with cell adhesion, autophagy, DNA repair and phospholipidic metabolism, and a decrease in those related to cytoskeleton organization, oxidative phosphorylation and peptide transport in this group (Figure 3H and Supplementary Table S6).

Transcriptomic profiles from epithelial cells and fibroblasts were integrated in a network comprising known ligand-receptor-transcription factor relationships, and the downstream gene ontologies. The resulting network is depicted in Figure 4A. Seven ligands overexpressed in epithelial cells interacted with 21 receptors expressed in fibroblasts. We found 13 transcription factors linked to these receptors and to 30 gene ontologies shown in Figure 2C. Collectively, these findings point to integrin signaling activated by collagens and activation of Notch receptors as potential mechanisms that may result in fibroblast reprogramming, triggering changes in proteostasis, autophagy, mitochondrial activity and transmembrane transport mechanisms.

To experimentally test these *in silico* findings, we added GLPG, a pan-integrin inhibitor, or DAPT, a gamma-secretase inhibitor that blocks Notch signaling, to MRC5 fibroblasts cultured with medium preconditioned by stretched A549 cells. Fibroblast activation was assessed by quantifying α-SMA in cell lysates. Inhibition of integrin signaling did not modify fibroblast activation, measured by α-SMA abundance (Supplementary Figure S6). However, in line with the specific enrichment of the Notch signaling pathway in fibroblasts cultured with medium preconditioned by stretched A549 cells (Figure 5A) and the increased expression of *JAG2* in senescent A549 cells

(Figure 5B), blockade of Notch signaling with DAPT decreased the abundance of α-SMA (Figure 5C-D), despite no significant changes in the expression of Notch receptors (Supplementary Figure S7).

Senolytic effects of digoxin

The senolytic effects of digoxin were studied to confirm that the observed pro-fibrotic effects were caused by the senescent response and to identify potential therapeutic strategies. A549 cells were stretched and cultured in the presence of digoxin or their corresponding vehicle. Treatment with digoxin decreased the percentage of senescent cells 1 week after stretch (Figure 6A) without decreasing the abundance of HP1γ-positive foci (Figure 6B). When fibroblasts were exposed to conditioned media from these experiments, we observed that supernatants from digoxin-treated cells inhibited fibroblast growth in wound healing assays (Figure 6C). However, addition of this drug to conditioned media immediately before wounding did not modify closure rates. Finally, we studied cell culture supernatants from vehicle and digoxin-treated cells using proteomics. Among 400 identified proteins, digoxin treatment induced changes in 61 (Figure 6D-E).

Discussion

Our results show that mechanical stretch *per se* can trigger senescence in lung epithelial cells, and that these senescent cells activate fibroblasts by paracrine mechanisms. Removal of epithelial senescent cells using digoxin reversed this SASP-mediated induction of a profibrotic phenotype in fibroblasts. Collectively, these findings link mechanical stretch with the subsequent development of fibrosis and offer novel therapeutic alternatives.

Reduction of lung mechanical load is central in the management of mechanically ventilated patients with acute hypoxemic respiratory failure (25, 26). There is substantial evidence that reduced tidal volumes improve short-term survival (27). However, the relationship between lung overdistension and development of fibrosis is less clear. Some experimental studies have demonstrated that collagen increases early after injurious ventilation (3), and that high tidal volume may activate several profibrotic mechanisms, including epithelial-mesenchymal transition (28) , polarization of macrophages towards a pro-fibrotic M2 phenotype (29) or release of profibrotic factors (30). Furthermore, there is scarce clinical data linking fibrosis directly to

mechanical ventilation and the exact causal relationship is difficult to determine due to the observational nature of the clinical evidence (31). Our model was designed as a proof of concept, aiming to induce a biological response in different cell types, as both distal airways (32) and alveoli (33) can be submitted to significant strain during ventilation. The stretch settings were based on previous results (34). However, the anisotropic nature of bronchial (35) and alveolar (33) expansion during tidal ventilation in lung injury makes difficult to extrapolate our experimental conditions to specific ventilatory settings. Despite this, our results suggest that activation of senescence in response to stretch is not limited to a single epithelial compartment.

Senescence is a cell response to a variety of stimuli. Several studies have identified the activation of senescence in acute lung injury (36). As mechanical ventilation is an essential supportive therapy in patients with acute hypoxemic respiratory failure, we focused on mechanical stress as an activator of senescence. Mechanical forces are transmitted from the extracellular matrix to the cytoplasm and are sensed by the nuclear envelope, which may transmit this force to the underlying chromatin (37). Additionally, these mechanical forces have been shown to induce DNA double-strand breaks (38), oxidative stress (39) and abnormalities in the nuclear envelope (37) , all

of which contribute to the senescence phenotype. The resulting DNA modifications activate the p53-p21 axis, a crucial signaling pathway that block cell cycle progression and induces senescence (6). The transient nature of these triggers (40) explains the lack of significant differences in *TP53* or *CDKN1A* (p21) in our samples, 6 days after the stimuli.

Although senescence may play a homeostatic role in the acute phase by limiting the number of apoptotic cells, thus avoiding massive organ damage (9), it can also facilitate the development of long-term sequelae. Secondary lung fibrosis after acute lung damage is a well-known complication with higher incidence (up to 60%) in severe and prolonged cases (41). Senescence has been identified as a key pathogenetic mechanism in idiopathic lung fibrosis (7, 8). Interestingly, fibroblasts were resistant to stretch-induced senescence. This resistance to mechanical stretch in fibroblasts, which has been described before (42), can be explained by the higher nuclear stiffness in MRC5 (43), that modifies the transmission of mechanical stimuli to the cell nucleus (37). Rather, fibroblast activation and secondary fibrosis after mechanical stretch could be the result of the crosstalk between a senescent epithelium and the underlying fibroblasts, that become activated in a paracrine manner.

Our ligand-receptor analysis revealed signaling through Notch receptors as one of the involved pathways. These receptors may impair alveolar regeneration (44), have known profibrotic effects (45, 46), and are activated during mechanical stretch (47). Senescent cell survival and homeostasis depend on a number of activated pathways that can be pharmacologically manipulated to induce cell death or to limit the development of a senescent phenotype. As previously discussed, digoxin has been proposed as a potential senolytic, by triggering apoptosis of senescent cells. The transcriptomic profile of stretch-induced senescent cells shows differences in the expression of genes related to ion channels, suggesting these cells may be more susceptible to blockers such as digoxin. Moreover, the proteome of culture media of digoxin cells shows significant changes in several senescence-related factors, including an increase in anti-senescent proteins such as S100A6 (48), cathepsins B (49), C (50) and D (51), clusterin (52) or IGFBP6 (53), and a decrease in pro-senescent factors such as Serpin E1 (54), Apolipoprotein E (55) or Insulin-like growth factor-binding protein 3 (IGFBP3) (56).

The major limitation of our study is derived from its in vitro nature, that precludes any direct translation to the clinical practice. The cell lines used are not fully representative

of the complexity of the lung cell composition. Moreover, we focused on the epithelium-fibroblast crosstalk, but stretch of other mechanosensitive cells such as the endothelium (57) or airway smooth muscle (58), or the impact of the secreted SASP on other populations (59), may regulate these interactions. Rather, our results illustrate that stretch is another potential inductor of senescence in different epithelial cell types, opening the door to single cell analyses to characterize its effects in the whole organ. In conclusion, these results suggest that stretch is a direct inducer of senescence in the respiratory epithelium, and that the spread of this response can contribute to the secondary fibrosis observed in ventilated patients. Moreover, this crosstalk between epithelial cells and fibroblasts can be modulated by digoxin, offering novel therapeutic approaches that could help to improve long-term outcomes in critically ill patients.

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Figure legends

Figure 1. Induction of senescence by stretch in A549 lung epithelial cells. A: Senescence-associated-β-galactosidase (SA-β-Gal) positive cells in each condition. B: Representative SA-β-Gal staining. C: Representative flow cytometry plots used to identify SA-β-Gal positive cells. D: Percentage of cells with nuclear HP1γ-positive foci. E: Representative fluorescence images showing HP1γ immunostaining. F: Analysis of cell cycle in static and stretched cells. G: Volcano plot showing changes in gene expression after stretch. H: Enriched Gene Ontology categories after stretch. Each point represents a Gene Ontology category with significant (adjusted p-value lower than 0.05) enrichment, and size proportional to the number of genes included. These categories were grouped using a clustering algorithm (Y-axis). I: Senescence score obtained from gene expression data in A549 cells sorted according to their SA-β-Gal concentration. J: Senescence score obtained from gene expression in human lungs ventilated and perfused *ex vivo*, either with continuous positive airway pressure (CPAP) or with injurious tidal volume (VILI: Ventilator-induced lung injury).

Figure 2. Specific features of stretch-induced senescence in A549 lung epithelial cells. A: Volcano plot showing differences in gene expression in A549 cells after induction

of senescence with stretch or by doxorubicin treatment. B: Senescence score in each condition. C: Enriched Gene Ontology categories in cells with stretch-induced senescence. Each point represents a Gene Ontology category with significant (adjusted p-value lower than 0.05) enrichment, and size proportional to the number of genes included. These categories were grouped using a clustering algorithm (Y-axis).

Figure 3. Induction of senescence in stretched MRC5 lung fibroblasts. A: Senescence-associated-β-galactosidase (SA-β-Gal) positive cells in each condition. B: Representative flow cytometry plots used to identify SA-β-Gal positive cells. C: Immunostaining with antibodies against HP1γ, showing a diffuse pattern in both conditions. D: Results of the wound healing assay of fibroblasts exposed to culture media from stretched and non-stretched epithelial cells. E-F: Quantification of α-smooth muscle actin in each condition and representative western blot. G: Volcano plot assessing differences in gene expression in fibroblasts exposed to preconditioned media from stretched and static epithelial cells. H: Enriched Gene Ontology categories after exposure to preconditioned medium. Each point represents a Gene Ontology category with significant (adjusted p-value lower than 0.05) enrichment, and size

proportional to the number of genes included. These categories were grouped using a clustering algorithm (Y-axis).

Figure 4. Epithelium-fibroblast crosstalk. Network including known ligands with differential expression in senescent A549 cells, receptors with evidence of expression in MRC5 fibroblasts, transcription factors regulated by these receptors with differential expression in MRC5 cells preconditioned by supernatants from stretched A549 cells, and gene ontology categories with significant enrichment in fibroblasts. Position of gene ontology categories in the Y axis corresponds to their enrichment score.

Figure 5. Notch signaling as a mediator of fibroblast activation. A: Gene Set Analysis plot of the Notch signaling pathway in MRC5 fibroblasts preconditioned by supernatants from stretched A549 cells, with the normalized enrichment score (NES). B: Expression of JAG2, an endogenous agonist of Notch receptors in stretched A549 cells. C: Abundance of α-smooth muscle actin (α -SMA) in preconditioned MRC5 cells treated with DAPT, a gamma-secretase inhibitor that blocks Notch signaling, or the corresponding vehicle.

Figure 6. Effects of digoxin as senolytic. A: Percentage of senescent A549 cells after cell stretch in control conditions and after adding digoxin to the culture medium. B:

Percentage of cells with nuclear HP1γ-positive foci after culture with vehicle or digoxin 25 nM. C: Wound closure curves from MRC5 fibroblasts cultured in presence of preconditioned medium from stretched A549 cells in control conditions or after adding digoxin (25 nM, after stretch or immediately before wounding). D: Volcano plot showing differences in protein abundance between culture media from A549 cells with or without digoxin. E: Heatmap showing proteins with significant differences in abundance after adding digoxin to the culture medium.

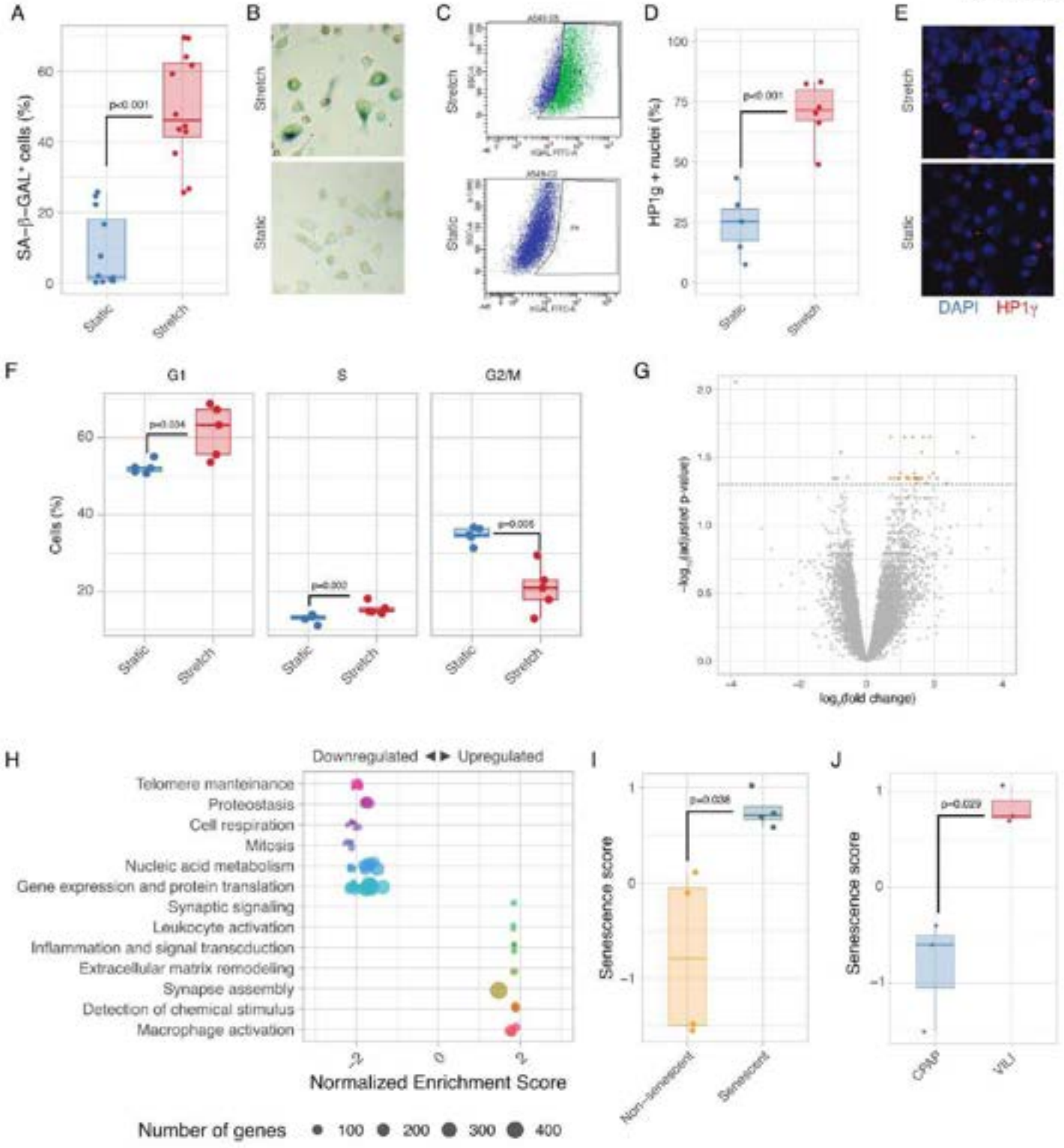
A
SA–β–GAL⁺ cells (%)
p<0.001
Static
Stretch
B
Stretch
Static
C
Stretch
Static
D
HP1g + nuclei (%)
p<0.001
Static
Stretch
E
Stretch
Static
DAPI HP1γ
F
G1
S
G2/M
Cells (%)
p=0.034
p=0.002
p=0.005
Static
Stretch
G
−log10(adjusted p-value)
log2(fold change)
H
Downregulated ◄► Upregulated
Telomere manteinance
Proteostasis
Cell respiration
Mitosis
Nucleic acid metabolism
Gene expression and protein translation
Synaptic signaling
Leukocyte activation
Inflammation and signal transduction
Extracellular matrix remodeling
Synapse assembly
Detection of chemical stimulus
Macrophage activation
Normalized Enrichment Score
Number of genes
100
200
300
400
I
Senescence score
p=0.038
Non-senescent
Senescent
J
Senescence score
p=0.029
CRIP
VIJ

Figure 1

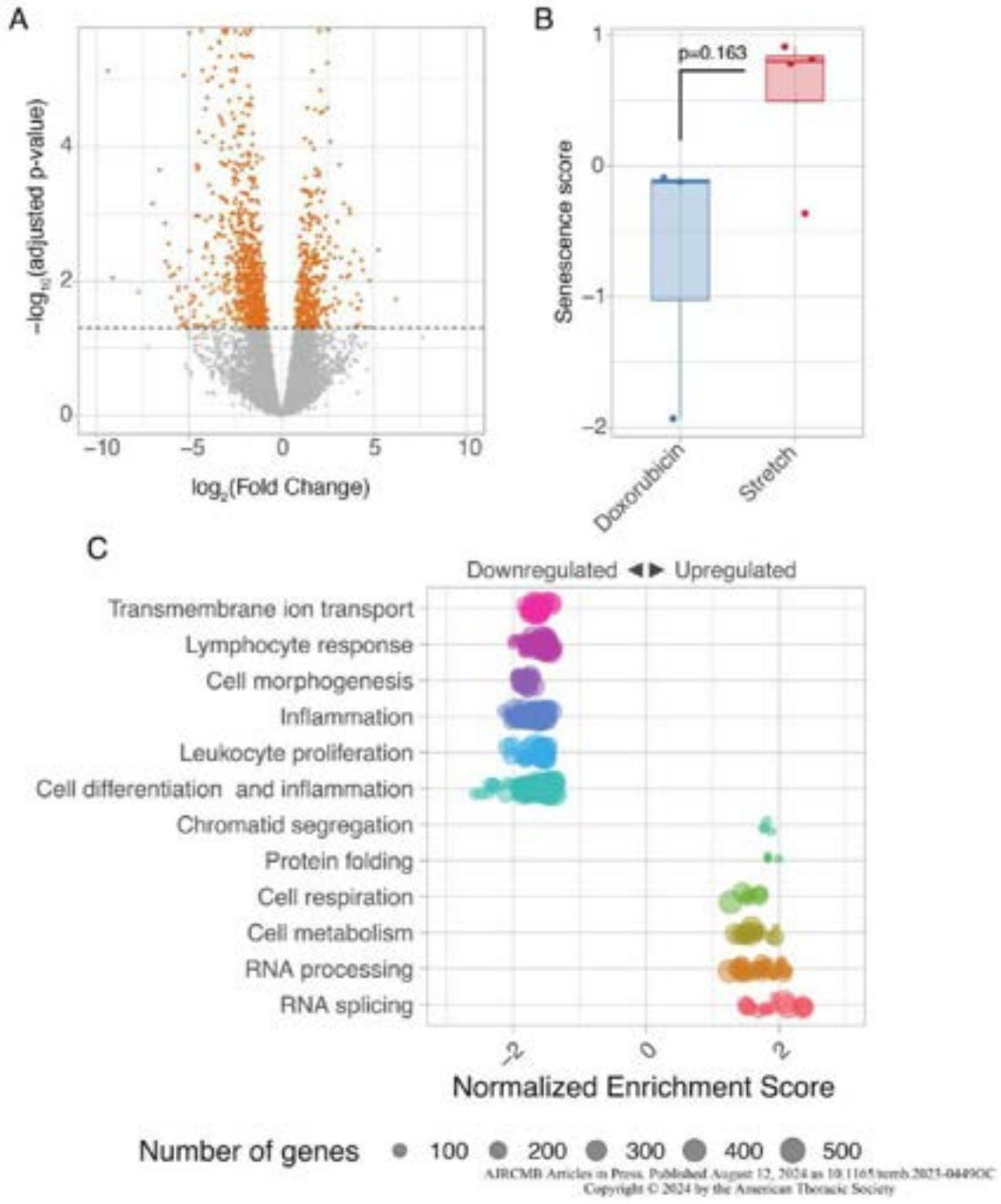

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Figure 2

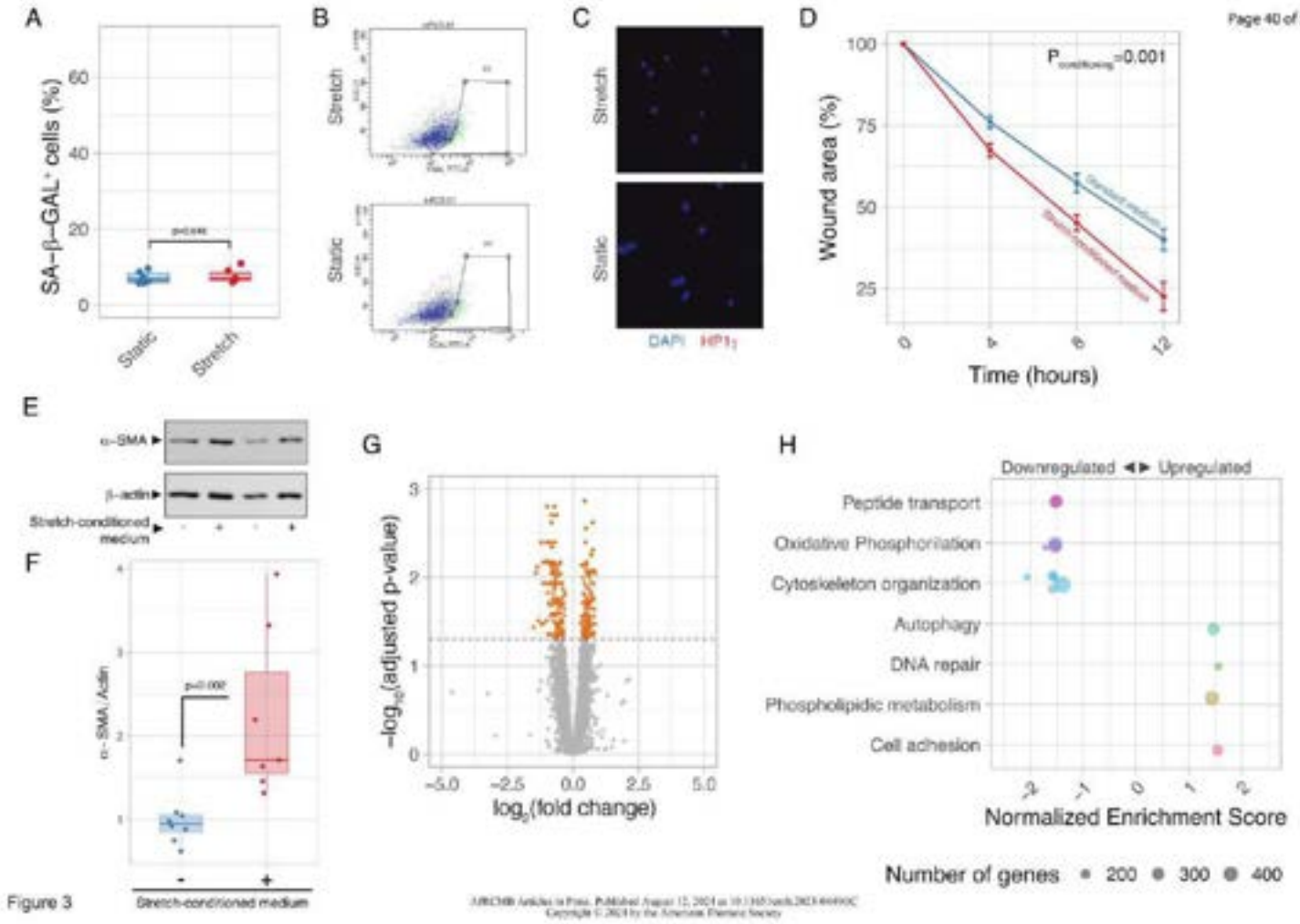
A
SA–β–GAL⁺ cells (%)
Static
Stretch
B
Stretch
Static
C
Stretch
Static
DAPI
HP1γ
D
Wound area (%)
Time (hours)
E
α–SMA
β–actin
Stretch-conditioned medium
F
α–SMA/Actin
Stretch-conditioned medium
G
−log10(adjusted p-value)
log2(fold change)
H
Downregulated ◄► Upregulated
Peptide transport
Oxidative Phosphorilation
Cytoskeleton organization
Autophagy
DNA repair
Phospholipidic metabolism
Cell adhesion
Normalized Enrichment Score
Number of genes 200 300 400

Figure 3

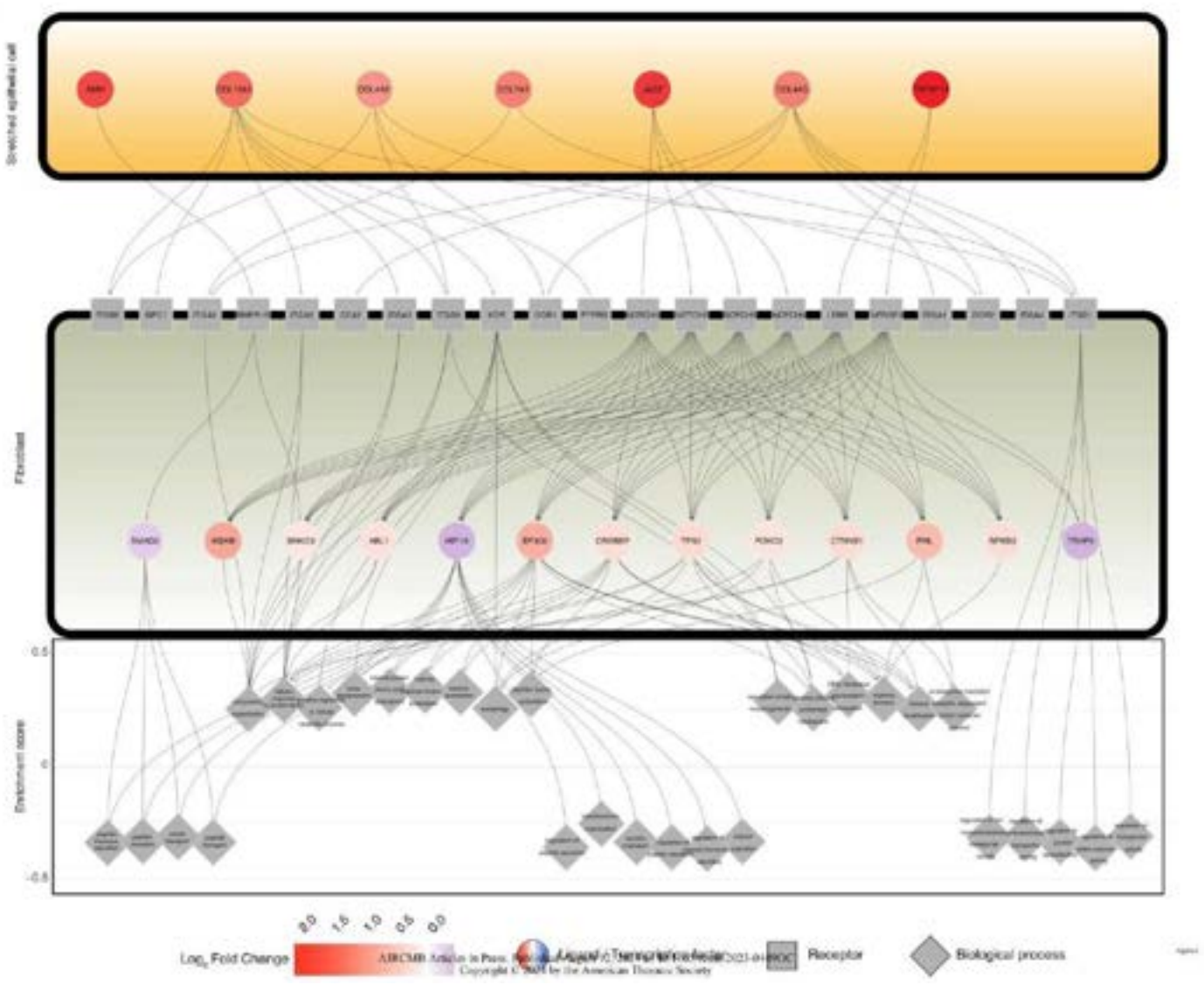
Stretched epithelial cell
Fibroblast
Enrichment score
0.5
0
-0.5
2.0
1.5
1.0
0.5
0.0
Log2 Fold Change
Receptor
Biological process

Figure 4

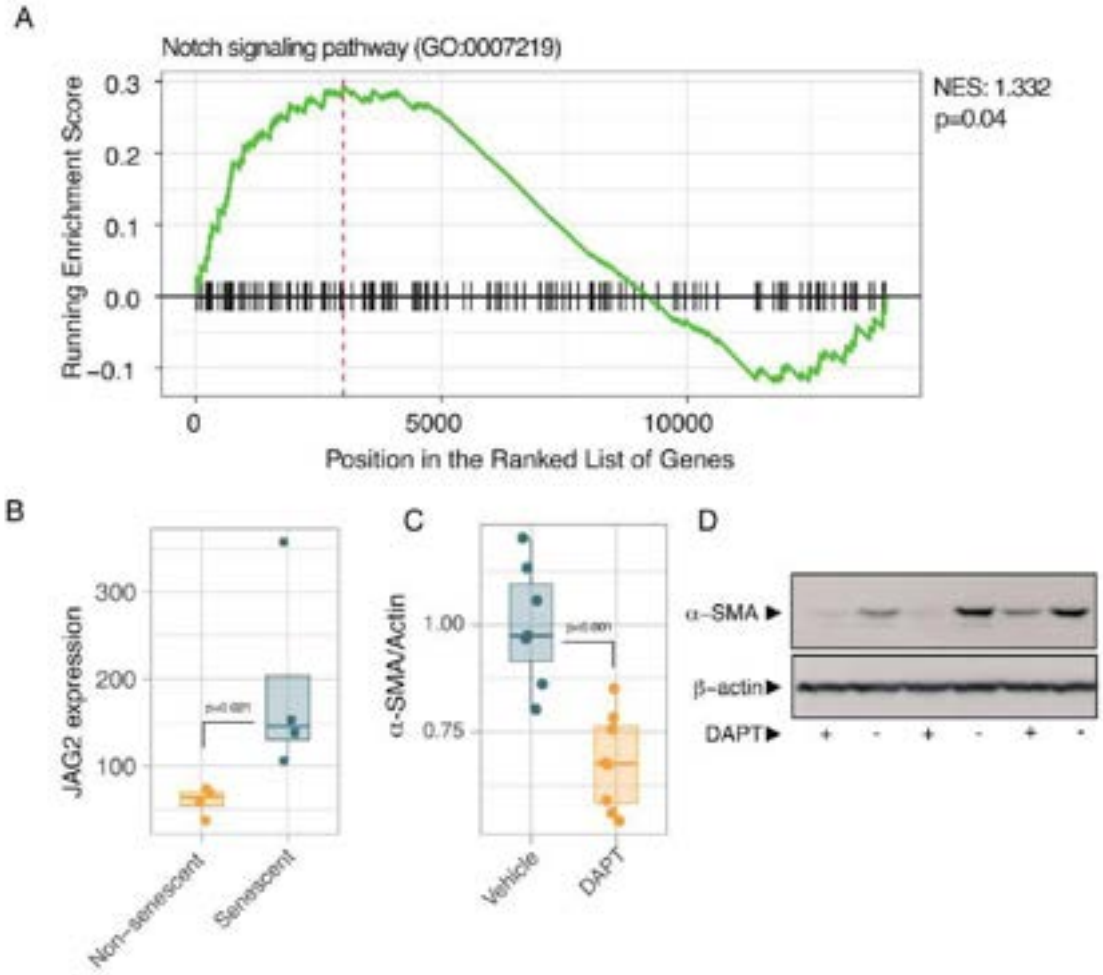

Figure 5

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Figure 5

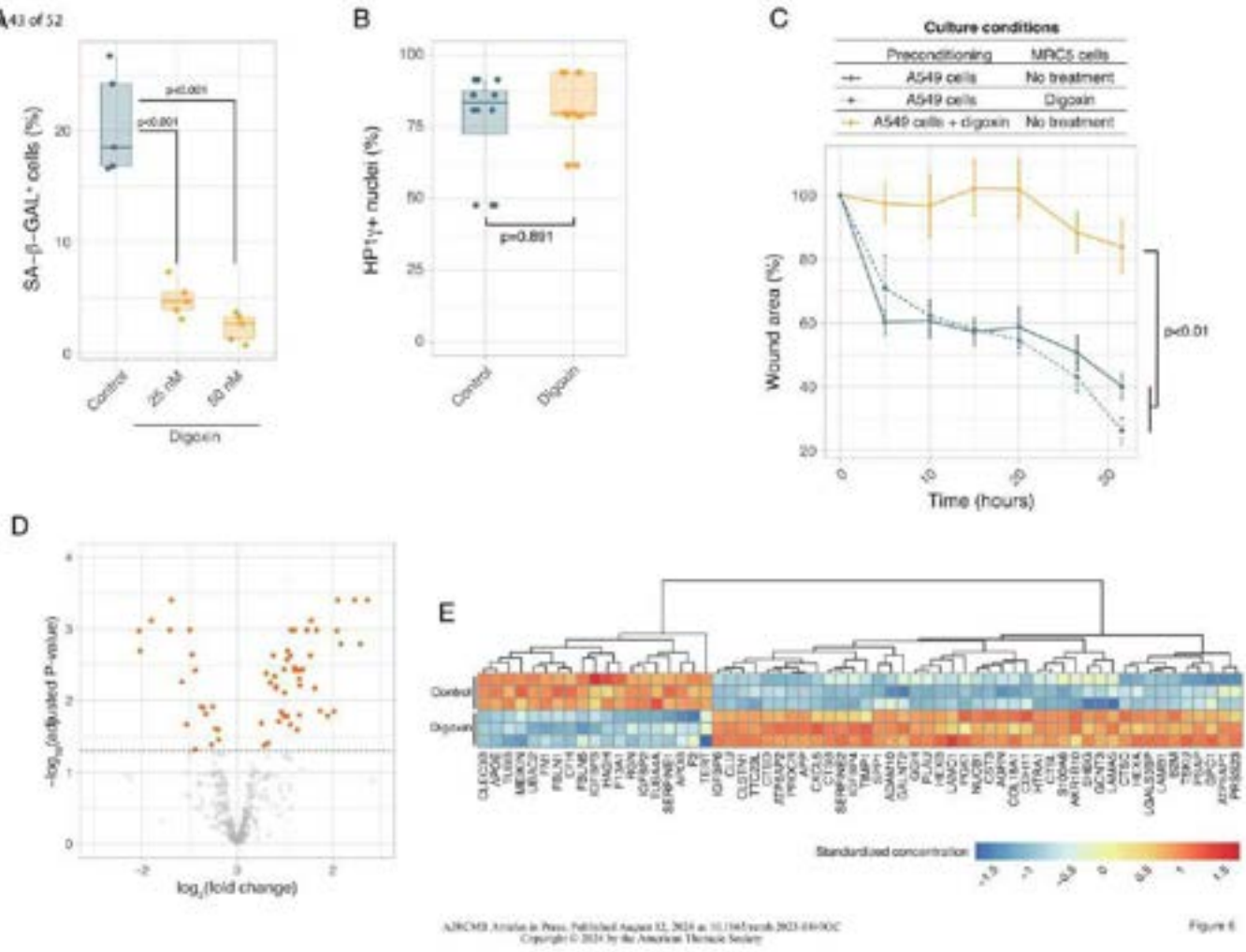

A
SA-β-GAL⁺ cells (%)
p<0.001
p<0.001
Control
25 nM
50 nM
Digoxin
B
HP1γ+ nuclei (%)
p=0.891
Control
Digoxin
C
Culture conditions
Preconditioning
MRC5 cells
A549 cells
No treatment
A549 cells
Digoxin
A549 cells + digoxin
No treatment
Wound area (%)
p<0.01
Time (hours)
D
−log10(adjusted P-value)
log2(fold change)
E
Control
Digoxin
Standardized concentration

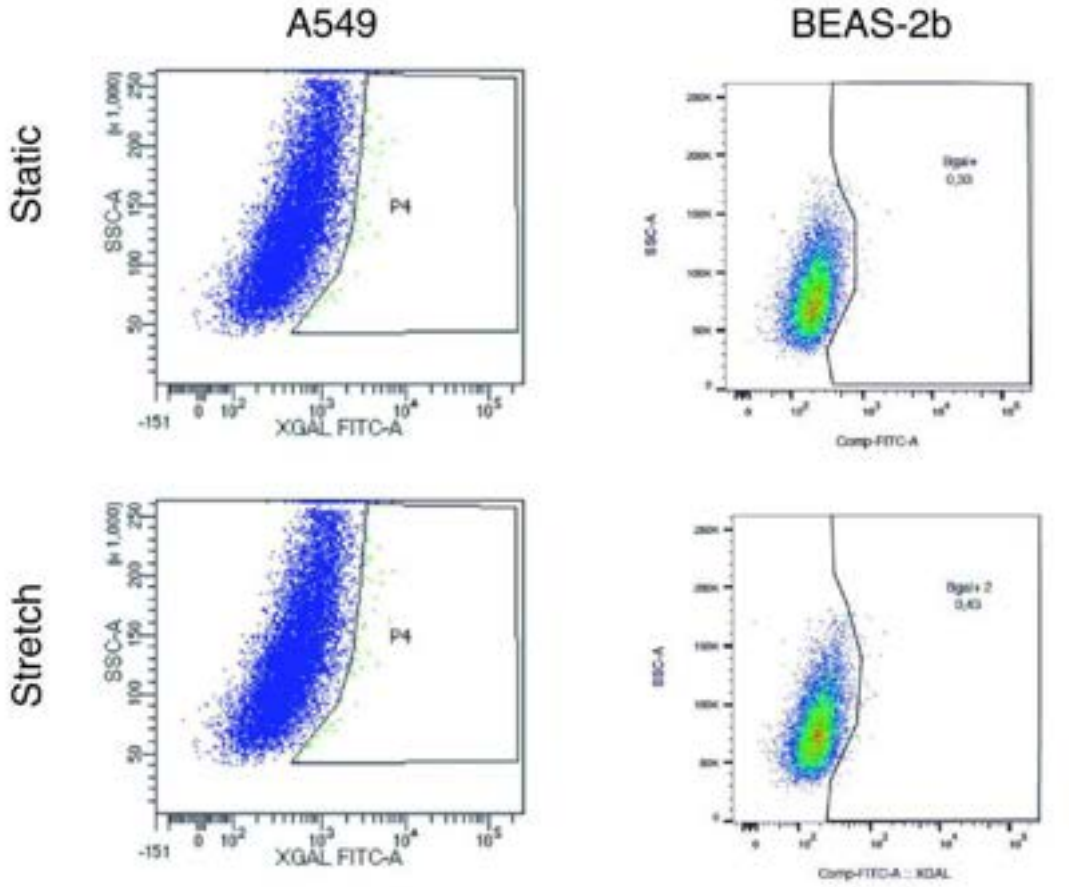
Figure 6

Mechanical stretch induces senescence of lung epithelial cells and paracrine fibroblast activation

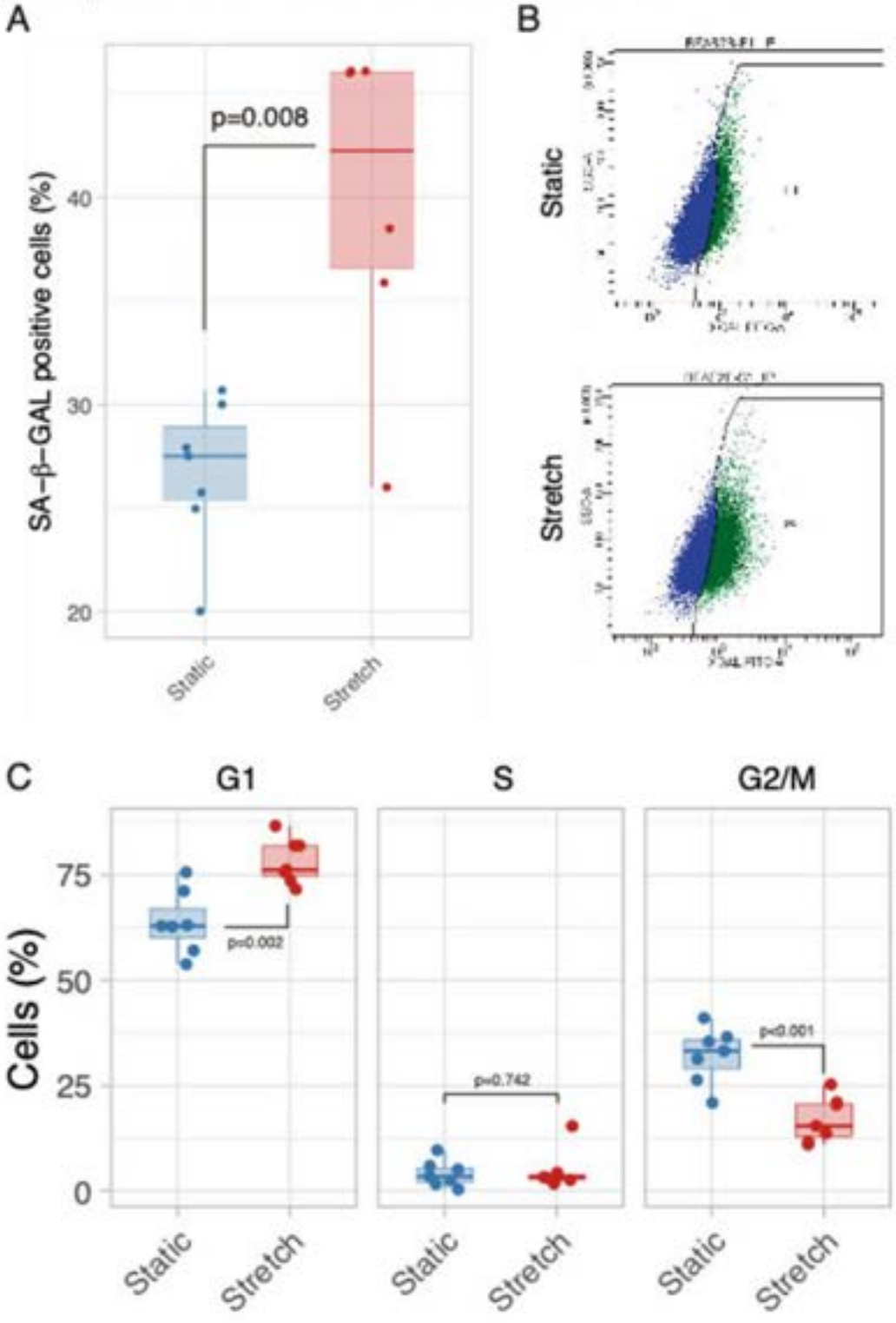
Paula Martín-Vicente, Cecilia López-Martínez, Inés López-Alonso, Sara M Exojo-Ramírez, Israel David Duarte-Herrera, Laura Amado-Rodríguez, Irene Ordoñez, Elias Cuesta-Llavona, Juan Gómez, Natalia Campo, Cecilia M O'Kane, Daniel F McAuley, Covadonga Huidobro, Guillermo M Albaiceta

Supplementary results

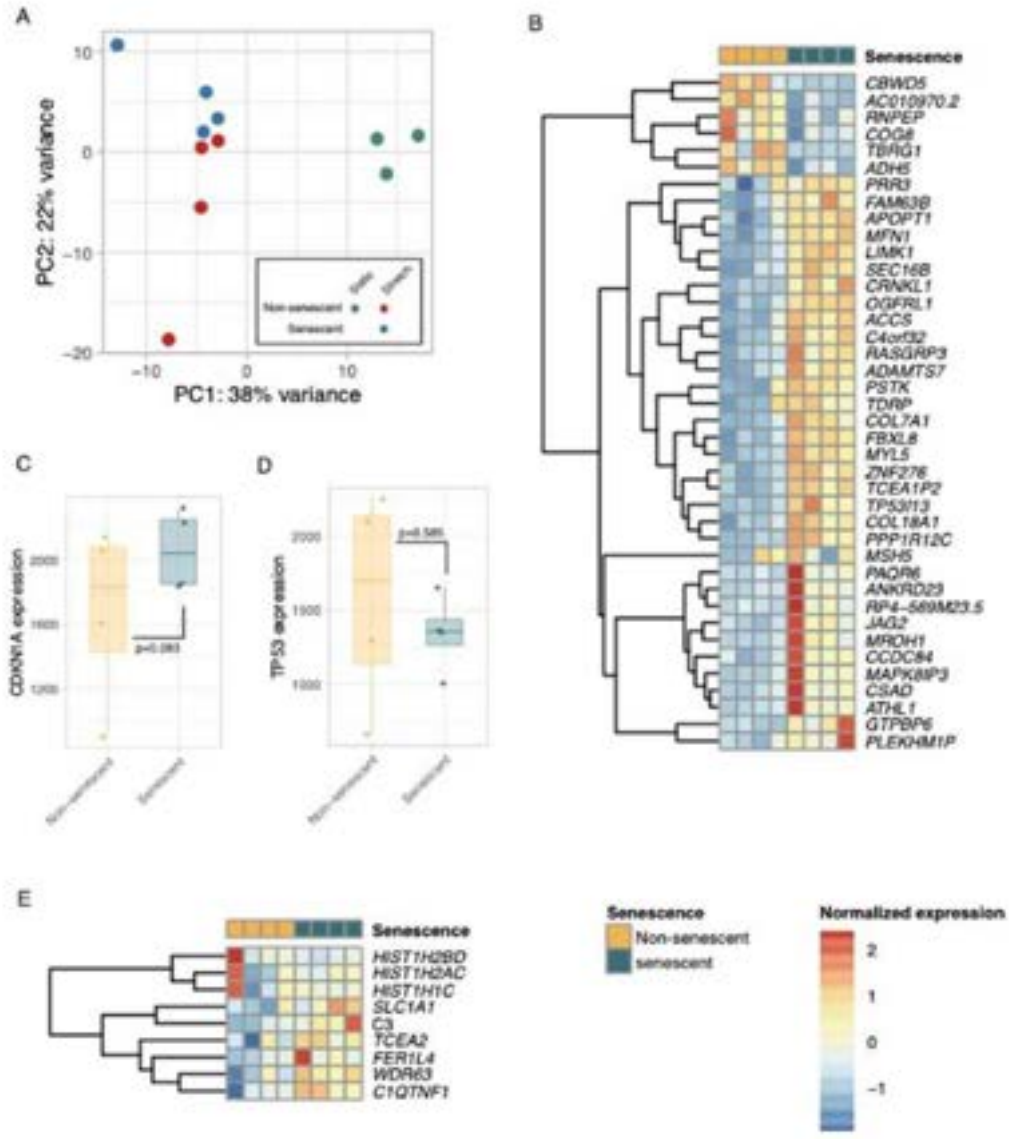
Supplementary Figure S1. Representative examples of flow-cytometry analyses of A549, and BEAS-2b cells acquired without X-gal staining to define positivity thresholds.



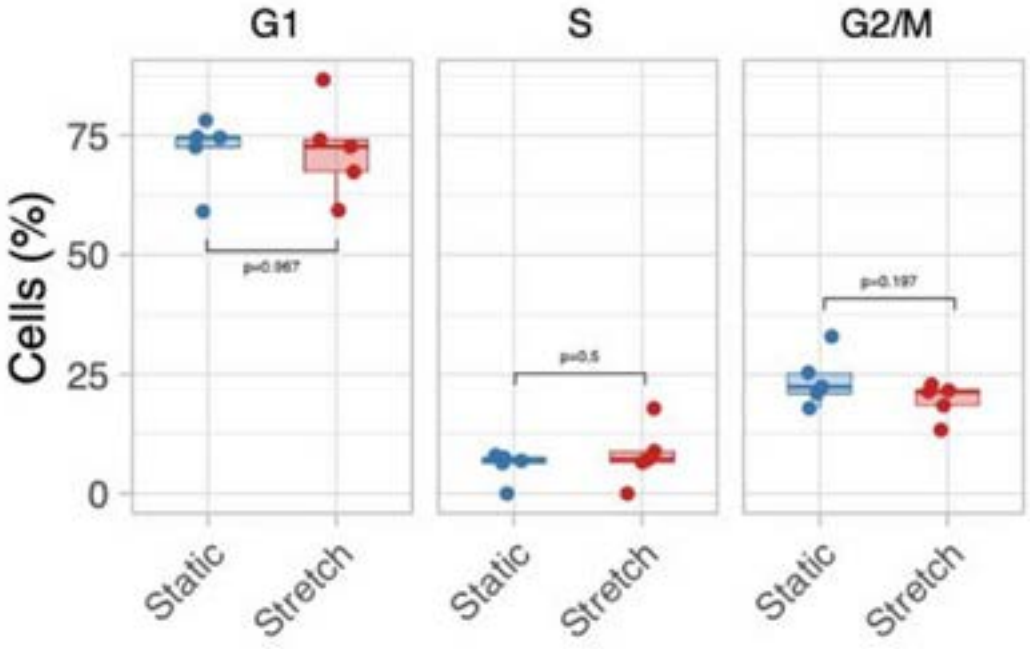
Supplementary figure S2. A: Percentage of BEAS-2b cells positive for senescence-associated beta galactosidase (SA-b-GAL) cultured in static conditions or after mechanical stretch. B: Representative flow cytometry plots from each experimental group. C: Cell cycle analysis of BEAS-2b cells cultured in static conditions or after stretch.



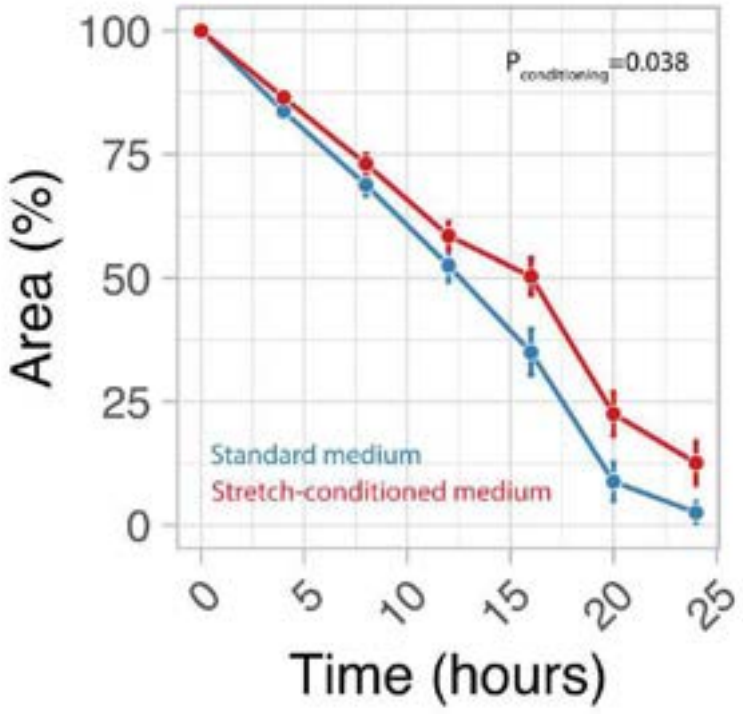
Supplementary figure S3. A: Principal component analysis of transcriptomes from senescent and non-senescent A549 cells after mechanical stretch, and cells cultured in static conditions B: Heatmap showing expression of genes with significant differences in senescent (positive for senescence-associated β-galactosidase) and non-senescent epithelial cells (A549 cell line). C-D: Expression of CDKN1A (p21) and TP53 in senescent and non-senescent cells. E: Heatmap showing individual expression of genes included in a transcriptomic signature of senescence.



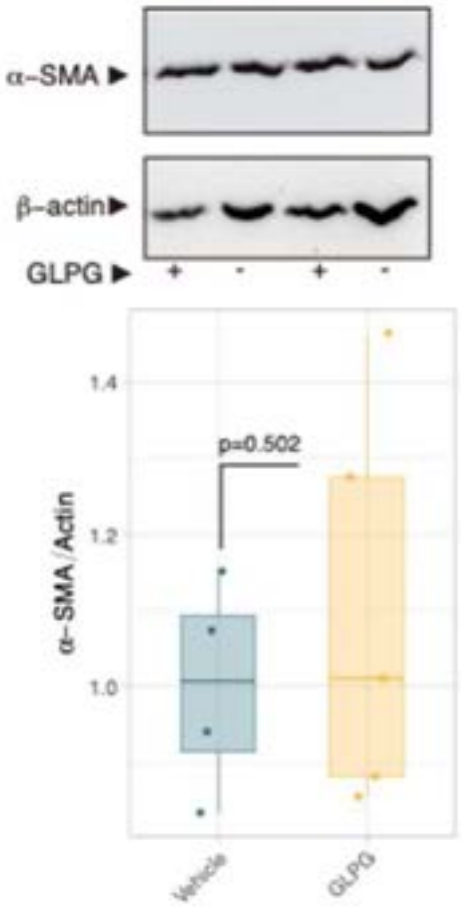
Supplementary figure S4. Cell cycle in MRC5 fibroblasts cultured in static conditions or after mechanical stretch.



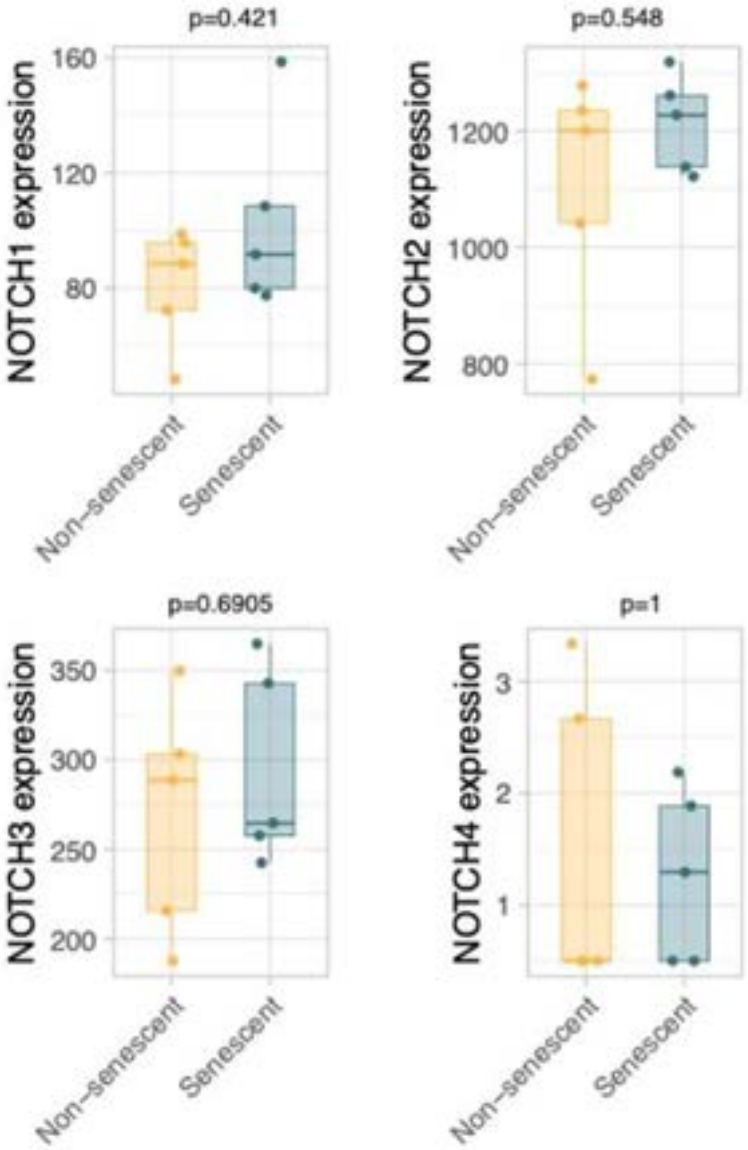
Supplementary figure S5. Wound closure curves in epithelial cells (A549 cell line) cultured with standard medium or preconditioned by stretch-induced epithelial senescent cells. The p-value corresponds to the group factor of the repeated-measurements ANOVA.



Supplementary Figure S6. Inhibition of integrin signaling. MRC5 fibroblasts were exposed to culture medium from stretched A549 cells to induce activation, and treated with GLPG, a pan-integrin inhibitor or the corresponding vehicle. This treatment had no significant effect on α-smooth muscle actin (α-SMA).



Supplementary Figure S7: Expression of the genes of the different Notch receptors in MRC5 fibroblasts preconditioned with medium from senescent A549 cells.



Supplementary tables

Supplementary table S1. Differences in gene expression in senescent and non-senescent epithelial cells after mechanical stretch.

Supplementary table S2. Pathways with differential enrichment (according to GSEA) in senescent and non-senescent epithelial cells after mechanical stretch.

Supplementary table S3. Differences in gene expression in cells with senescence induced by stretch or doxorrubicin.

Supplementary table S4: Pathways with differential enrichment (according to GSEA) in cells with senescence induced by stretch or doxotrubicin.

Supplementary table S5. Differences in gene expression in in fibroblasts cultured in medium preconditioned with stretched and static epithelial cells.

Supplementary table S6: Pathways with differential enrichment (according to GSEA) in fibroblasts cultured in medium preconditioned with stretched and static epithelial cells.

IV. Revisión sobre los mecanismos, consecuencias y oportunidades terapéuticas de la activación de la senescencia en el paciente crítico.

La senescencia celular implica un arresto proliferativo irreversible del ciclo en el cual las células permanecen metabólicamente activas y secretan una serie de factores proinflamatorios y profibróticos como parte de su SASP. Este estado puede darse como consecuencia del envejecimiento fisiológico, pero también puede estar desencadenado por otros estímulos. En esta revisión se resumen los mecanismos principales de la senescencia celular y su papel en la patología crítica. La evidencia científica muestra que distintas situaciones críticas pueden activar un programa de senescencia donde esta puede tener un papel beneficioso a corto plazo, ya que ayuda a limitar la apoptosis celular y está involucrado en la reparación del tejido, pero a largo plazo puede ser un mecanismo patogenético importante en el desarrollo del PICS. Esta revisión resalta la importancia de profundizar en el estudio de la senescencia celular dado que esta podría ser un objetivo terapéutico importante para mejorar la salud a largo plazo del paciente crítico.

Artículo 4. **Martín-Vicente P**, López-Martínez C, Rioseras B, Albaiceta GM. Activation of senescence in critically ill patients: mechanisms, consequences and therapeutic opportunities. Ann Intensive Care. 2024 Jan 5;14(1):2.

Aportación personal al trabajo.

Fui la responsable principal de la elaboración de este manuscrito que resume y destaca el papel de la senescencia en la patología crítica. Para ello participé en la búsqueda bibliográfica, organización y escritura del manuscrito. La recopilación de la información me sirvió para entender como la senescencia pueda ser un objetivo terapéutico potencial para mejorar la salud a largo plazo en el paciente crítico, reforzando los resultados de esta tesis doctoral.

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REVIEW

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Activation of senescence in critically ill patients: mechanisms, consequences and therapeutic opportunities

Paula Martín-Vicente[1,2,3], Cecilia López-Martínez[1,2,3], Beatriz Rioseras[4] and Guillermo M. Albaiceta[1,2,3,5*]

Abstract

Whereas aging is a whole-organism process, senescence is a cell mechanism that can be triggered by several stimuli. There is increasing evidence that critical conditions activate cell senescence programs irrespective of patient's age. In this review, we briefly describe the basic senescence pathways and the consequences of their activation in critically ill patients. The available evidence suggests a paradigm in which activation of senescence can be beneficial in the short term by rendering cells resistant to apoptosis, but also detrimental in a late phase by inducing a pro-inflammatory and pro-fibrotic state. Senescence can be a therapeutic target. The use of drugs that eliminate senescent cells (senolytics) or the senescence-associated phenotype (senomorphics) will require monitoring of these cell responses and identification of therapeutic windows to improve the outcome of critically ill patients.

Keywords Senescence, Senotherapeutics, Apoptosis, DNA damage response, Post-ICU syndrome

Introduction

Critical illness is often characterized by a systemic response beyond the primary site of injury. Severe insults trigger a large variety of responses, evolutionarily optimized and aimed to survive, that regulate essential cell processes such as active cell death (programmed or not), cell division, inflammation or regulation of metabolism [1]. The development of supportive techniques that preserve life despite massive injuries has improved early survival rates, but the persistent activation of those adaptative mechanisms turns them into pathogenetic, contributing to late organ dysfunction and death [2]. This dual nature of the response to aggression (tuned by evolution but also pathogenetic) difficults the identification of therapeutic targets that further improve the outcomes of our severe patients.

Senescence is defined as a cell state characterized by a stable arrest of cell cycle and a phenotypic change that includes the release of paracrine factors that, among other actions, promote inflammation and fibrosis [3]. Although senescence has often been linked to aging, it must be noted that any challenge to cell integrity, including DNA damage or breakdown, results in its activation irrespective of the age of the subject. Senescence is a paradigmatic example of a pro-survival mechanism, deeply rooted in our biochemical machinery, that may also be pathogenetic. In this review, we will discuss how a critical disease can promote accelerated aging by activating senescence mechanisms.

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An overview of senescence mechanisms

The term senescence was first used to refer to the finite proliferative capacity of primary fibroblasts observed in vitro [4]. This cell cycle arrest is now known to be a subtype of cellular senescence, termed replicative senescence, and caused by telomere shortening.

Nowadays, hundreds of different stimuli have been found to trigger a senescent response, and most of them are directly or indirectly related to the development of DNA damage and activation of the DNA damage response (DDR). These include ionizing radiation that cause double strand breaks [3], telomere shortening as consequence of multiple replication cycles [4], mitochondrial dysfunction and the consequent production of reactive oxygen species (ROS) [5], or activation of oncoproteins that lead to aberrant replication patterns [6]. Each one of these stimuli, either by direct activation of the DDR or by action of other cellular stress responses, lead to the activation of cyclin-dependent kinase inhibitors [6]. P53 is a master regulator of diverse cellular processes that becomes activated in response to these damages, and plays a part in deciding the fate of the cell towards apoptosis or senescence [7]. Following the senescence path, P53 will activate the cyclin-dependent kinase inhibitor p21, but other inhibitors such as p16, p27 or p15 can also participate in the cell cycle arrest. Due to this inhibition activity, the RB protein will remain dephosphorylated, allowing its action as inhibitor of the E2F transcription factor family and thus effectively arresting the cell cycle [8].

However, cell cycle arrest is not the only characteristic of senescent cells. The senescent phenotype involves other changes such as secretion of several mediators, increased lysosomal content, nuclear reorganization, apoptosis resistance, endoplasmic reticulum stress, metabolic reprogramming, changes in membrane components, or changes in cell size [9]. It's important to highlight that not all these characteristics are found in every senescent cell, nor these processes are exclusive to senescent cells, which makes identifying and studying this cellular state a real challenge.

The development of a senescence-associated secretory phenotype (SASP) is one of the most studied characteristics of senescent cells because of its consequences [10]. Senescent cells secrete cytokines, chemokines and proteases that in a physiological state are aimed to promote tissue remodeling and attract immune cells that will clear the tissue of unwanted cells, including the senescent ones. However, in a pathological context in which there is no clearance of the senescent cells, the SASP will produce chronic inflammation and fibroblast activation leading to development of fibrosis [3]. This secretory phenotype is orchestrated by the transcription factors NF-κB and CEBPβ, and includes the release of IL-6, IL-8, monocyte chemoattractants, macrophage inflammatory proteins and growth factors such as TGFβ [11].

Another key characteristic of senescent cells is an increase in lysosomal content and proteins, including the lysosomal enzyme β-galactosidase. Increases in the quantity of this enzyme have been used as a surrogate marker for this change in lysosomal activity, and the positive assay of this enzyme at the suboptimal pH of 6.0 is considered a senescence marker, termed senescence-associated β-galactosidase [12, 13].

Changes in chromatin organization can also be observed in senescent phenotypes. Phosphorylation of histone H2AX occurs in sites of DNA breaks and drives the recruitment of DNA repair complexes. Also, heterochromatin foci form as a mechanism to repress proliferation-promoting genes, for which HP1γ is a marker [14, 15].

Of note, no specific marker of senescence has been validated in patients and using routinely available samples. This lack of biomarkers directly implies that identification and quantification of the "senescent state" of a tissue or individual in the clinical practice is an almost impossible task, and will have its consequences to identify therapeutic windows to manipulate senescence.

Activation of senescence in critical illness

Critically ill patients face a series of challenges that arise from both the underlying disease and the necessary medical interventions for their treatment. This interaction between pathology and therapy activates a set of damage mechanisms that can lead to the development of complex pathophysiological responses, including cellular senescence.

There are different critical care scenarios where an increase of the senescent response has been observed. A widely studied trigger is ischemia/reperfusion injury [16], which refers to the damage caused by a temporary lack of blood supply followed by its restoration. This critical situation commonly occurs in emergency surgery, organ transplantation, and cardiovascular events. Hyperoxia per se can also activate senescence mechanisms [17–20]. In both situations, the main underlying mechanism that leads to cellular senescence is oxidative stress. In the case of ischemia, cells face a decrease in the supply of oxygen and essential nutrients. This oxygen deprivation can trigger the production of ROS. When blood is restored during reperfusion, cells may experience a sudden increase in the supply of oxygen and nutrients, that paradoxically, can lead again to an increase in ROS due to the release of stored free radicals during ischemia [21–23]. In the case of hyperoxia, prolonged exposure to elevated oxygen levels can increase ROS [24]. The production of

reactive oxygen and nitrogen species can directly damage nucleic acids, thereby triggering the DDR and the following senescent response [25]. This makes oxidative stress a fundamental mechanism in the critically ill patient to address senescence not only in these two contexts but also across a wide range of scenarios.

Inflammation is another relevant mechanism. Systemic inflammation is not only a common feature of critical illnesses, such as viral infections [26], but can also arise as a consequence of their treatment, including interventions such as mechanical ventilation [27]. Inflammation initiates or intensifies cellular senescence through various mediators, such as IL-6. Additionally, senescent cells can contribute to systemic inflammation by their SASP, which includes proinflammatory cytokines, growth factors and proteolytic enzymes. Consequently, a positive feedback loop is established between inflammation and senescence [28, 29].

Despite its supportive nature, mechanical ventilation induces a large variety of lung responses, including oxidative stress or inflammation that can promote senescence. Moreover, mechanical stress itself can also directly activate cell senescence mechanisms. Transmission of mechanical forces from the extracellular matrix to the cell nucleus can alter the nuclear envelope, leading to changes in the mechanical properties of the nucleus and regulating gene expression [30]. Different studies have shown that abnormalities in the nuclear envelope make cells prone to enter senescence [31]. When exposed to high tidal volumes, alveolar cells change the mechanical properties of the nuclear envelope, activate the P53/P21 axis and show markers of senescence.

Overall, the complex interplay between the underlying pathology, medical interventions, and the mechanisms they trigger in critically ill patients can lead to the activation of the senescent response. This cellular response can have significant implications for the patients' health and contribute to additional complications. Understanding these processes is essential for improving critical care and developing strategies that minimize damage by targeting these mechanisms therapeutically.

The short- and long-term consequences of senescence

Senescence must be viewed as a homeostatic response rather than the sole consequence of aging. In fact, these pathways play key roles in tissue development and repair. Therefore, its activation during critical illness must be expected as part of the normal response to severe injuries. However, a dysregulated or persistent response may have long term, negative consequences. Moreover, the spread of SASP components can explain the systemic involvement seen in the most severe patients. Recent research has contributed to decipher the role of senescence in different syndromes commonly observed in critical care.

Senescent responses during acute lung injury have been widely characterized [32]. An increase in P53-P21 signaling has been described in experimental models of acute inflammation and in humans with ARDS or receiving mechanical ventilation [33]. Recent single-cell sequencing studies in necropsy samples show signs of DNA damage and the overexpression of the main triggers of senescence (*TP53*, *CDKN1A*) [34]. This COVID-induced senescence is seen mainly in endothelial and epithelial lung cells, although most cell types showed an increase in expression of SASP-related genes [34]. Interestingly, there is some evidence that blockade of senescence during the acute phase increases apoptosis and severity of injury [33], illustrating its early beneficial effects. It has been also proposed that the pro-inflammatory environment induced by the SASP reduces viral replication in respiratory infections [35]. However, there is also experimental and clinical evidence showing that senescence is a major player in lung fibrosis. In the context of acute lung injury, persistent activation of senescence creates a pro-fibrotic environment, and acquisition of a senescent phenotype by lung transitional or stem cells can result in impaired repair [36]. Survivors of SARS-CoV-2 infections with severe pulmonary damage show fibrotic changes that are related to telomere length [37].

A similar pattern has been observed during acute kidney injury. Renal ischemia, oxidative stress and systemic inflammation activate senescence programs, mainly in tubular epithelial cells [38]. In the acute phase, these senescent cells may promote wound repair, limit the proliferation of damaged cells and avoid a large cell loss due to apoptosis [39]. It has been shown that mutant mice lacking key senescence triggers show more severe damage in experimental models of kidney injury [40]. However, persistence of senescence results in activation of pro-fibrotic pathways that may lead to chronic kidney disease [41].

Endothelial dysfunction and injury have been described in different critical syndromes, including sepsis or acute lung, kidney or liver failure [42]. Senescent endothelial cells increase their size and number of nuclei, and release SASP-related mediators [43]. These changes promote atherosclerosis, thrombosis and vascular wall inflammation, and could explain the increased rate of cardiovascular events observed in sepsis survivors [44].

All this evidence fits into a model in which senescence is activated early as part of the acute phase response, contributing to organ homeostasis and repair. Prolonged or dysregulated senescent mechanisms can become injurious [45], favoring a prolonged pro-inflammatory and

pro-fibrotic state that can spread beyond the initial site of injury (Fig. 1).

Immunosenescence in critically ill patients

Immunosenescence is characterized by alterations of the innate and adaptative immune system producing an impairment of their normal function (Fig. 2). The main alterations involve the decrease of the repertoire diversity of naïve CD4+and CD8+T lymphocytes in favor to an increment of highly differentiated phenotypes. T lymphocytes with this terminally differentiated phenotype are exhausted memory lymphocytes with impaired proliferative and responsiveness capacities and a more potent proinflammatory activity. One hallmark of immunosenescence is inflammageing, described as a sterile, non-resolving, low-grade, and chronic inflammation that progressively increases with age. Inflammageing and immunosenescence processes show mutual interaction [46]. Inflammageing is caused by the increased production of pro-inflammatory cytokines mainly by innate immune cells [47, 48]. Although the underlying mechanisms of inflammageing are still unclear, one of the most studied causes is the 'Garb-aging' theory. It is based on the evidence that cellular debris accumulated over time

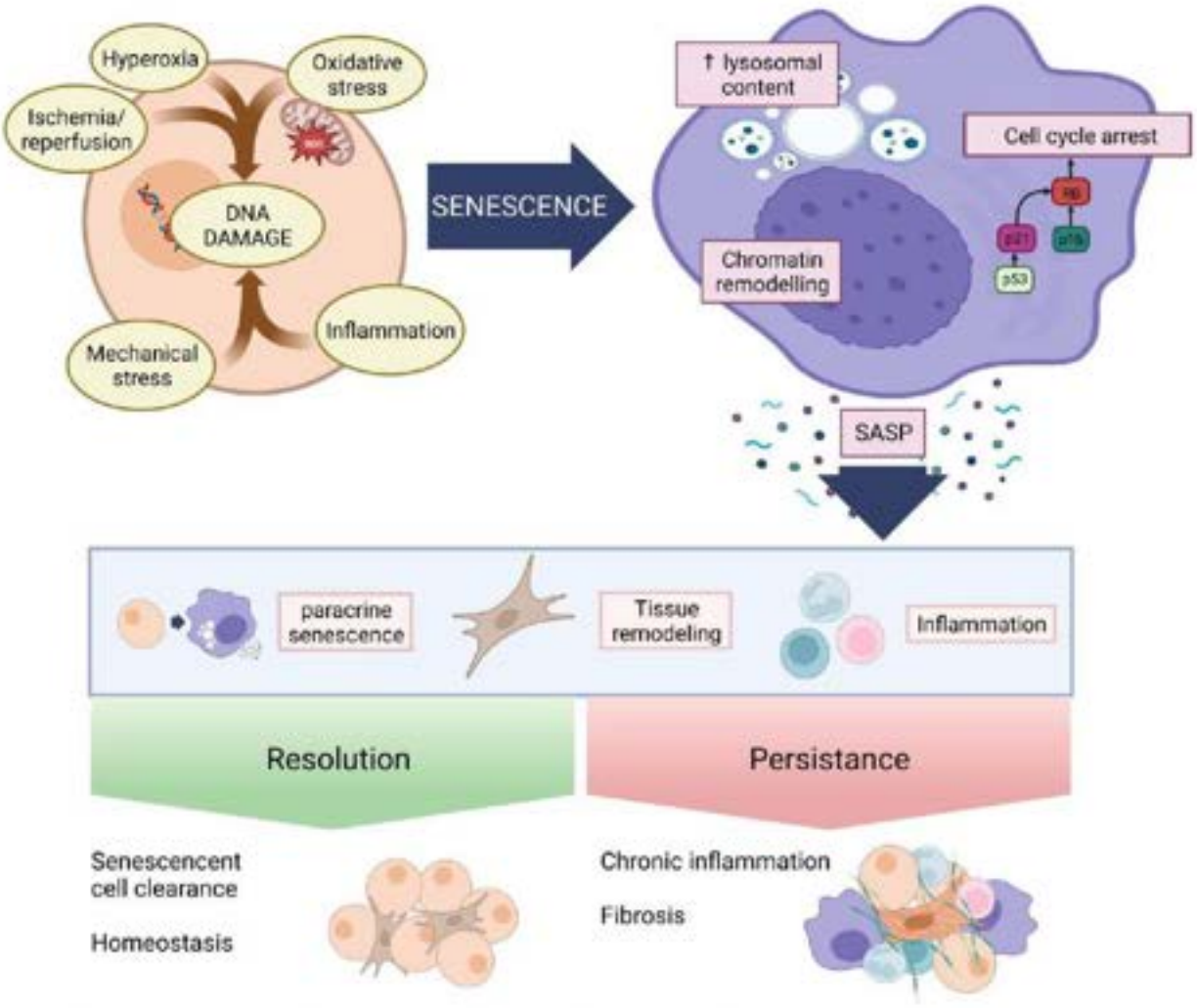

Fig. 1 Activation of senescence and its consequences. Critically ill patients are exposed to a variety of injurious stimuli that may activate senescence mechanisms, in which P53, P21 and P16 play a key role. Senescent cells show several specific features including cell cycle arrest, changes in cell structure and release of a number of factors (known as Senescence associated secretory phenotype -SASP-) that result in systemic spread of the response and modulate inflammation and tissue remodeling. Although these mechanisms are aimed at tissue repair and clearance of damaged and senescent cells, their persistence leads to chronic inflammation and fibrosis. Created with BioRender.com

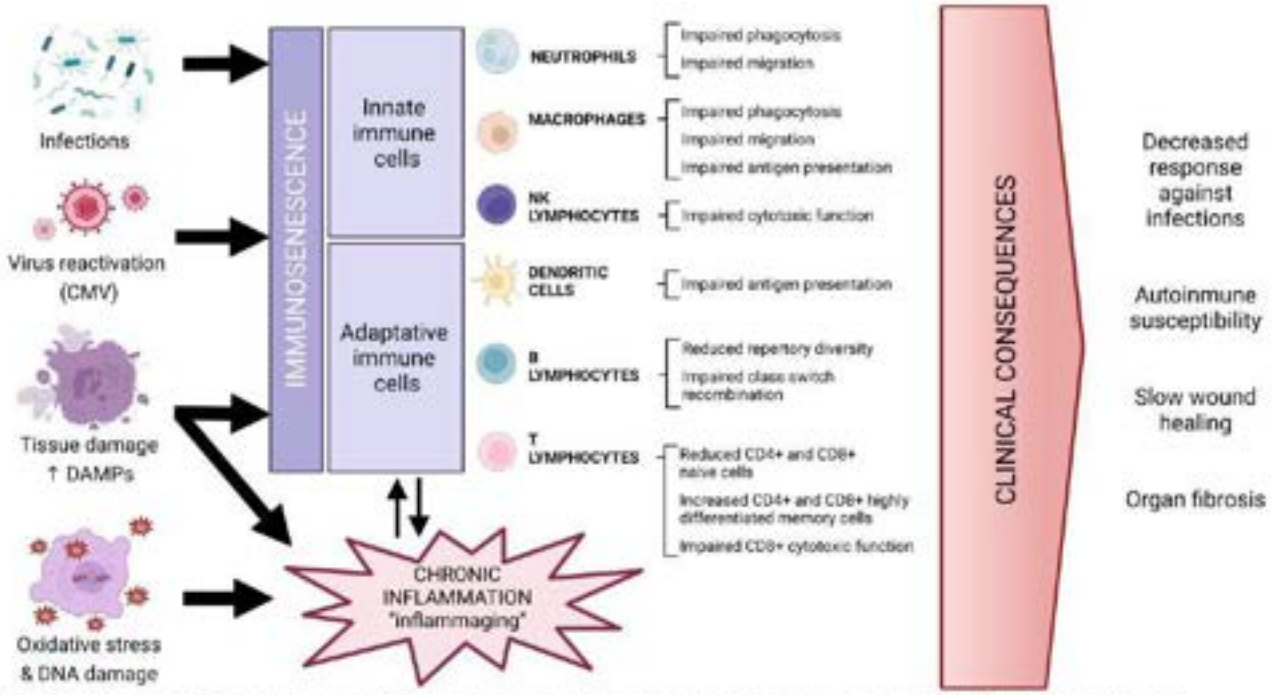

Fig. 2 Mechanisms of immunosenescence. Triggers of senescence induce several changes in immune cell populations, favoring the shift towards a state of impaired immune response (both innate and adaptative). This "inflammaging" includes a low intensity pro-inflammatory response, in part due to the release of immune mediators from senescent cells. All these mechanisms lead to dysregulation of the immune response and establish a positive feedback loop that perpetuates this state. Created with BioRender.com

due to cellular damage increment and progressive failure of reparation mechanisms triggers inflammation by innate immunity activation through known signaling pathways as the NF-kB transcription factor [49]. There are several circulating pro-inflammatory markers that are known to be elevated during inflammageing, contributing to SASP, as proteins associated with macrophages (sCD163, YKL-40, sCD14) and neutrophils (elastase, PR3, IL-8) and other pro-inflammatory molecules as IL-6, TNF-α and C reactive protein. Many of them have been associated to the increase of comorbidities, grater frailty and a worse outcome of some diseases [50].

Although these senescence processes were described in aging, promoted by the many contacts of the immune system with different antigens throughout life, there were also found in other situations as some chronic diseases [51, 52]. Underlying disease, ICU conditions and invasive treatments that are usually applied in critically ill patients, are known to alter the immune system homeostasis producing an immunosenescence phenotype [53, 54]. More specifically, infections, inflammation status and tissue damage associated to critical illness could be some of the specific triggers of immunosenescence.

Infections and reactivation of chronic viruses are some of the most frequent complications in critically ill patients and are known to be closely related to immunosenescence development. This response is instigated by the recognition of pathogen-associated molecular patterns (PAMPs) by the immune system, which are molecular signals derived from various pathogens. Specifically, cytomegalovirus (CMV) reactivation is the best-known exogen factor inductor of immunosenescence [55]. Up to 36% of immunocompetent patients admitted to the ICU suffer latent CMV reactivation during their stay. This is associated to an adverse outcome [56, 57]. Each viral reactivation cycle generates a subset of CMV specific T lymphocytes, with the consequent decrease of naïve T-lymphocyte repertoire and promoting a terminally differentiated phenotype and the pro-inflammatory chronic state due to the continuous immune stimulation [58, 59].

Tissue damage, also occurring due to underlying pathology and invasive treatments in critically ill patients, promotes the release of danger-associated molecular patterns (DAMPs), consisting in products from extracellular matrix and cytosol of damaged or apoptotic cells. These PAMPs/DAMPs are recognized by intracellular and extracellular toll-like receptors (TLRs) present mainly in innate immune cells generating a pro-inflammatory response. The massive release of DAMPs can also activate adaptative immune responses favoring their immunosenescence phenotype

and at the same time triggering autoimmunity processes [46, 60].

Finally, immunosenescence might also be one of the mechanisms driving the accumulation of senescent cells in tissues both during critical illness and aging. These changes in immune cell function might render them unable to reach the local senescent cells and clear the tissue from them, which will further exacerbate the cycle of systemic inflammation [61, 62]. All these described processes that frequently occur in critically ill patients, would act as a positive feedback loop, perpetuating the systemic inflammation which leads to a worse prognosis of these patients.

Interaction between senescence and aging

Senescence is part of physiological aging, but, as previously discussed, can also be triggered by other stimuli. This implies that, although correlated, senescence and aging can be independent phenomena. The crosstalk between senescence and aging is only partially known.

Activation of senescence molecular mechanisms and accumulation of senescent cells increase with age. Repeated exposure to injurious stimuli and telomer shortening due to cell replication are responsible for this phenomenon along the lifespan. One of the consequences of this activation is the persistence of a low intensity proinflammatory response due to the concurrent SASP [63]. In old critically ill patients, this increased proinflammatory milieu can impair tissue repair and recovery, thus contributing to the poor outcome in this population [64]. In addition, this increased activation of senescence may modify the acute response to new injuries. Aged animals show an attenuated inflammatory response in experimental models of sepsis, with dysfunctional cell populations and only minor changes in the expression of proinflammatory genes [65]. It is unclear how these two counteracting conditions (activation of senescence in baseline conditions and decreased senescent response after a new stimulus) modulate the pathogenesis of critical diseases.

Monitoring senescence in critically ill patients

Given the dual nature, adaptative and pathogenetic, of the senescence response to acute injuries, monitoring becomes a critical issue to identify therapeutic time windows in which pharmacological manipulation achieves the maximal benefit. This task is difficult due to the lack of specific and universal markers. Moreover, all the approaches based on monitoring of SASP components [66] are invalid in critically ill patients, due to the massive release of proinflammatory mediators triggered by the underlying disease.

A more specific approach to systemic monitoring of senescence could be achieved using multiparametric techniques. Sequencing RNA from circulating cells allows the quantification of several proposed transcriptomic signatures in peripheral blood [67, 68]. By quantifying the expression of several genes, these signatures can be synthetized in a single value. Recently, a detailed proteomic analysis has been able to quantify aging (not senescence) at an organ-specific level by measuring tissue specific protein signatures [69]. Finally, flow cytometry allows the identification and quantification of senescence markers at a cell level [70]. However, the validity of these approaches must be confirmed in a complex scenario such as critical care.

Monitoring senescence could help to define optimal windows of opportunity to treat patients with drugs that modify the senescent response (see below). One could speculate that this kind of drugs should be tested in enriched clinical trials, in which only those patients with a clear senescent phenotype should be included and treated. This kind of precision medicine, that stems from the underlying pathogenetic mechanisms and applies phenotype-specific therapies, constitutes the future of critical care [71].

Therapeutic modulation of senescence: senotherapeutics

Senescent cells depend on the activation of pro-survival and anti-apoptotic pathways to avoid cell death. Therefore, any drug that inhibits these biochemical routes will trigger the selective apoptosis of senescent cells. Most of these so-called senolytics target intracellular factors involved in regulation of apoptosis. For instance, BCL-2 inhibitors, such as navitoclax or venetoclax, have been widely used in experimental models to remove senescent cells. This approach has shown beneficial results in models of lung fibrosis [72]. Recently, it has been shown that navitoclax decreases viral load, systemic inflammation and SASP markers in a model of COVID-19 in aged hamsters, in line with its senolytic activity [73]. Other pathways can be blocked using kinase inhibitors. Among these, dasatinib, a non-selective inhibitor of tyrosin-kinases and SRC-kinases, has been used in experimental models of sepsis and acute lung injury with favorable results [74, 75]. However, these drugs have significant toxicities, including peripheral blood cytopenias and cardiovascular events that raise concerns on their use in critically-ill patients. As an alternative, flavonoids may have senolytic properties, at least in part due to their effect as BCL2-inhibitors, and have a better safety profile. Quercetin and fisetin have been used in models of senescence, alone or combined with other drugs. There are several reports on the use of flavonoids in critical

care [76, 77]. Targeting HSP-90, a pro-survival protein involved in sepsis and acute lung injury, also promotes apoptosis by facilitating the elimination of the pro-survival kinase AKT. Several drugs, such as geldanamycin, ansamycin or resorcinol act at this level, and have shown benefits in experimental models of acute lung injury or sepsis [78, 79].

Other widely used drugs have been repurposed as senolytics. Digoxin and other cardiac glucosides promote the death of senescent cells [80, 81], as these cannot cope with the changes in intracellular concentrations of pH and calcium triggered by the drug. This drug decreases lung fibrosis after bleomycin administration by clearance of senescent cells [81]. However, no clinical data are available. It must be taken into account that these findings are limited by the pleiotropic nature of the tested inhibitors and their targets, thus precluding any firm conclusion regarding their specific effects on senescence. In addition, there is no evidence of benefits in critical care settings.

A different approach is the use of drugs to inhibit SASP. These drugs are termed senomorphics. Most of the drugs that block the inflammatory response, such as rapamycin or NF-kB or JAK-STAT inhibitors, may fall in this category. In critically ill patients, where a pro-inflammatory response is usually part of the pathogenesis of the disease, these drugs may have potential benefits. However, as in the case of senolytics, it is not clear that these benefits are linked to a specific effect on senescence.

Again, several drugs have senomorphic effects. Metformin, at least in part by their effects as blocker of the NF-kB and Nrf2 pathways, decreases experimental lung injury caused by endotoxin or alveolar overdistension [82], and the development of fibrosis after bleomycin-induced inflammation [83]. Clinical observational data has shown a reduction of mortality in patients with sepsis and diabetes [84] or pneumonia, but, again, the link with senescence has not been tested. Similarly, statins can modulate SASP by upregulating several sirtuins, a family of proteins that inhibit senescence. Several observational studies and trials have addressed the use of statins in critically ill patients. Although some works report benefits in survival and long-term sequels [85–87], randomized trials have not supported their use in unselected populations in critically ill patients [88–90].

Given the previously proposed model of senescence in critical illness, timing of these treatments is essential. Early blockade of senescence may promote harm, as it blocks a homeostatic mechanism. Most of the potential benefits of senescence-targeting drugs are related the avoidance of late sequels. Preclinical research has also pointed to these benefits, usually in experimental models of chronic diseases such as lung or kidney secondary fibrosis [38]. This highlights the need for animal models of late sequels of critical illness to test the proposed therapeutic approaches. In these experimental models, time windows can be defined a priori. However, clear identification of the transition from early to late phases of the disease in patients may be difficult, and would probably imply the use of systemic or local biomarkers, as previously proposed.

Conclusions

Despite the strong link between aging and senescence, the latter is now understood as a cell mechanism activated in response to a variety of stimuli. Aging implies the continued activation of the senescence machinery, but in critically ill patients, senescence may be activated irrespective of patients' age to play a key role in tissue homeostasis by increasing cell resilience to injury, decreasing apoptosis and promoting tissue remodeling and clearance of injured cells. As virtually all the homeostatic mechanisms during critical illness, the continued, dysregulated activation of senescence favors tissue damage, usually caused by persistent inflammation and fibrosis. This raises the hypothesis that the so-called Post-intensive care syndrome can be, at least in part, the manifestation of accelerated aging [45]. Knowledge of these senescence pathways can allow their monitoring and pharmacological manipulation, probably in specific time windows, to improve the outcome of critical care patients.

Abbreviations

CMV	Cytomegalovirus
DAMPs	Danger-associated molecular patterns
DDR	DNA damage response
PAMPs	Pathogen-associated molecular patterns
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
TLRs	Toll-like receptors

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None.

Author contributions

PMV, CLM, BR and GMA outlined the manuscript and wrote all the sections. CLM made the figures. PMV and GMA wrote the final version that was reviewed by all the authors.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

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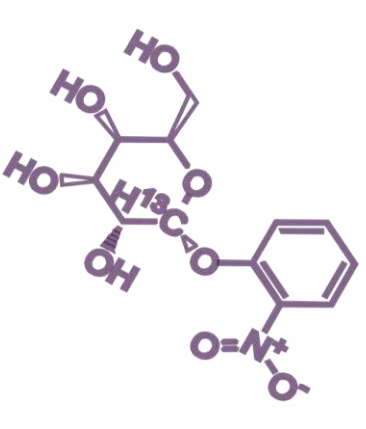


DISCUSIÓN

Desde su definición inicial, el concepto de senescencia ha experimentado una evolución constante no solo en cuanto a la caracterización del fenotipo de las células senescentes, sino también en su función celular, identificándose distintos tipos de senescencia según el contexto biológico en el que se encuentre. Son muchos los estudios que han mostrado el papel dual de la senescencia tanto en la homeostasis, como en el envejecimiento y la disfunción tisular. Los resultados expuestos en esta tesis doctoral revelan el efecto pleiotrópico de la senescencia en el contexto del paciente crítico. Se observa que el daño pulmonar agudo junto con el uso de la ventilación mecánica promueve la activación de una respuesta senescente que puede ser beneficiosa a corto plazo, ya que limita la apoptosis y el daño histológico en el pulmón. Sin embargo, a largo plazo, el estiramiento mecánico puede desencadenar un programa de senescencia que contribuye al desarrollo de una fibrosis secundaria. Esto pone en evidencia la implicación de la senescencia tanto en la reparación del tejido pulmonar como en el desarrollo de las secuelas post-UCI, sugiriendo la posibilidad de utilizar este mecanismo como diana terapéutica para mejorar la salud a largo plazo de los pacientes críticos.

Activación y modulación de la vía p53/p21 en la lesión pulmonar aguda y su papel en la regulación de la apoptosis

La lesión pulmonar aguda es una condición crítica que se presenta con frecuencia en pacientes con enfermedades graves en la UCI. Esta afección puede variar desde formas menos severas hasta el SDRA, asociado con una alta mortalidad. Un mecanismo patogenético muy importante que

contribuye a las formas más severas de la enfermedad es la disrupción de la membrana alveolocapilar a consecuencia de la inflamación. Esta alteración permite la filtración de líquidos desde los capilares a los alveolos, lo que afecta negativamente al intercambio gaseoso. Como resultado, a menudo estos pacientes requieren de ventilación mecánica, lo que, a su vez, puede agravar la situación (158). La vía p53/p21 está implicada en la respuesta celular al estrés, pero su papel en el daño pulmonar agudo aún no ha sido esclarecido.

Los resultados expuestos en el segundo trabajo de esta tesis doctoral muestran una activación de la vía p53/p21 en respuesta a la lesión pulmonar aguda y el uso de la ventilación mecánica en el compartimento alveolar. Además, el análisis sistemático de datos transcriptómicos de modelos animales de lesión pulmonar muestran un enriquecimiento de firmas génicas específicas de p53 y p21 y un perfil validado de senescencia. Esta coincidencia sugiere que la activación de esta vía en el contexto de la lesión pulmonar aguda podría estar relacionada con el inicio de la respuesta senescente.

Asimismo, la inhibición de p21 se correlaciona con un aumento de la apoptosis celular y el daño histológico. Distintos autores han identificado la senescencia de las células alveolares como un mecanismo destinado a evitar la apoptosis celular, previniendo de esta manera la disfunción del tejido (159,160). Las células epiteliales alveolares, además de ser vitales para garantizar la eficiencia en el intercambio gaseoso, constituyen la primera línea de exposición a agentes dañinos en el pulmón y forman una barrera física que protege a los tejidos subyacentes de patógenos y toxinas (161). El control exhaustivo de la apoptosis en estas células es, por lo tanto,

crucial para el mantenimiento de la integridad y funcionalidad del pulmón. Una apoptosis excesiva de las células epiteliales alveolares está relacionada con el desarrollo de algunas enfermedades pulmonares. Estas células pueden liberar un conjunto de factores, como moléculas procoagulantes o agentes vasoconstrictores, que pueden participar en el desarrollo de la fibrosis (162,163).

Para estudiar el papel de la senescencia en la lesión pulmonar aguda se establecieron dos modelos animales de daño. En el primero, el daño se inducía mediante la aspiración de ácido clorhídrico. En el segundo, además de la aspiración de ácido clorhídrico, se utilizó la ventilación mecánica para agravar el daño pulmonar. En ambos modelos se observó una activación de la vía p53/p21, así como un aumento de la apoptosis celular y el daño histológico. Para observar la relevancia de esta activación, se inhibió p21 y p53. La inhibición de p53 no mostró ningún cambio en cuanto a la severidad del daño pulmonar, posiblemente por el efecto pleiotrópico de p53. Sin embargo, la inhibición de p21 resultó en una muerte celular excesiva y un mayor aumento del daño histológico, indicando la importancia de este gen en el mantenimiento de la homeostasis pulmonar.

Se ha descrito que p21 tiene un papel inhibitorio sobre la apoptosis al suprimir la actividad de las CDKs, la cual interfiere con la activación de la caspasa-9, una proteína fundamental en la apoptosis celular (164). Nuestros resultados evidencian que la inhibición de p21 está asociado a un aumento en los niveles de esta proteína, lo que parece indicar que la inhibición de caspasa-9 es el mecanismo por el cual p21 protege a las células frente a la apoptosis excesiva después del daño pulmonar agudo.

Asimismo, nuestro trabajo muestra que la ventilación mecánica, en combinación con el ácido clorhídrico, incrementa significativamente la expresión de p21, así como la apoptosis celular y el daño histológico. Es más, la activación de la vía p53/p21 se correlacionó con cambios en las laminas nucleares, en línea con hallazgos previos (54). Como se detalló en la introducción, estas proteínas no solo proporcionan un soporte estructural a las células, sino que también desempeñan un papel vital durante el estiramiento mecánico y en la regulación de la mecanotransducción (54). Basándonos en estos resultados y el hecho de que el estrés mecánico se haya visto implicado en la activación de la respuesta senescente (165), cabría esperar que en nuestro modelo las señales mecánicas estén activando un proceso de mecanotransducción que module la respuesta celular frente al estrés y, en consecuencia, active la vía p53/p21.

Para explorar el papel del estiramiento mecánico en la regulación de esta vía, se utilizaron los inhibidores de proteasas ritonavir/lopinavir. Estos fármacos, inhiben a la proteína ZMPSTE-24, una proteasa encargada de la maduración de la prelamina A a lamina A (166) y han demostrado mantener la compliancia del núcleo celular en condiciones de estiramiento mecánico, disminuyendo la apoptosis y daño en el parénquima pulmonar (54). El uso de estos fármacos se correlacionó con la sobreexpresión del gen p21, además de cambios en proteína HP1α, implicada en la organización y mantenimiento de la estructura de la cromatina, y una disminución del daño histológico y la apoptosis celular, mostrando un efecto protector del ritonavir/lopinavir frente al VILI.

Además de las alteraciones observadas en la envoltura nuclear y la estructura de la cromatina, el daño pulmonar agudo y el uso de la ventilación mecánica se asociaron con un incremento del daño en el ADN, que no parecía revertirse por el uso de ritonavir/lopinavir. Es más, la coincidencia de este evento con el aumento tanto de p21 como de la caspasa-9, sugirió que el daño en el material genético suponía una vía alternativa por la cual tanto el daño pulmonar agudo como el estiramiento mecánico estaban activando simultáneamente un programa de senescencia y apoptosis celular.

En definitiva, la cantidad de células apoptóticas en nuestro modelo depende del equilibrio entre la activación de la respuesta apoptótica por el daño en sí mismo y los efectos antiapoptóticos del inductor senescente p21, que van a estar regulados tanto por el daño en el ADN como por la remodelación de la cromatina. El tratamiento con lopinavir/ritonavir favorece la sobreexpresión de p21 y contribuye a reducir la lesión pulmonar aguda (Figura 1).

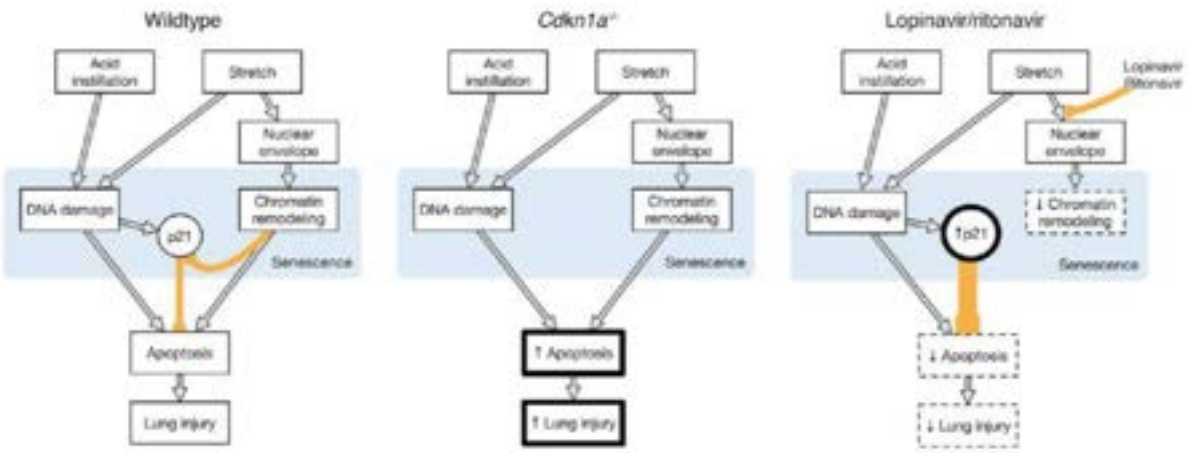


Figura 1. Esquema del papel de la vía p21 en la apoptosis y la senescencia celular tras el daño pulmonar y el estiramiento mecánico en ratones controles (A), ratones deficientes en Cdkn1a (B) y ratones tratados con lopinavir/ritonavir (C). Transl Res. 2021 Jul;233:104-116.

Hacía una respuesta senescente estable en el daño pulmonar agudo

La activación de la vía p53/p21 pueden llevar a las células a iniciar un programa de senescencia celular. Para ello, las células detienen su ciclo celular (167) y experimentan cambios profundos en su funcionamiento y estructura, entre los que se encuentra la remodelación de la cromatina. Como ya se detalló en la introducción, durante la senescencia se forman estructuras conocidas como SAHF (97), que desempeñan un papel muy importante en el establecimiento del perfil transcriptómico de las células senescentes. Además, estas estructuras marcan la transición entre la activación de esta respuesta hacia un estado de senescencia más avanzado y estable (168).

Nuestros resultados mostraron que el daño pulmonar y el uso de la ventilación mecánica estaba asociado a la expresión de algunos genes

asociados con senescencia, además de a la formación de los SAHF. Es más, la inhibición de p21 o el uso de lopinavir/ritonavir estaba asociado con una disminución de estas estructuras nucleares. Asimismo, en un contexto más clínico, se encontró un aumento de este marcador en autopsias de pacientes con SDRA sometidos a ventilación mecánica.

Estos resultados demuestran la activación y desarrollo de una respuesta senescente inducida por el daño pulmonar agudo y el uso de la ventilación mecánica. Sin embargo, la imposibilidad para identificar un programa de senescencia ya establecido impide determinar cómo esta respuesta podría influir a lo largo del tiempo. Para ello, sería necesaria la identificación de otros marcadores canónicos de senescencia como puede ser la acumulación de SA-βgal, el arresto del ciclo celular y el desarrollo de un SASP (168) en un modelo que nos permita monitorizar la senescencia con el tiempo.

Senescencia inducida por estiramiento mecánico y su papel en el desarrollo de la fibrosis pulmonar

Según muestran los resultados del tercer trabajo de esta tesis doctoral, el estiramiento mecánico puede inducir directamente la senescencia en las células epiteliales y estas, mediante la liberación del SASP, activar fibroblastos por mecanismos paracrinos.

Como se detalló en la introducción, las células del parénquima pulmonar están constantemente sometidas a fuerzas mecánicas durante la respiración normal. Estas señales, se transmiten al núcleo y activan diversas vías de señalización intracelular que regulan la proliferación,

diferenciación y supervivencia celular (46). Durante la ventilación mecánica, las células del parénquima pulmonar experimentan fuerzas mecánicas que están por encima de los umbrales observados durante la respiración espontanea. Sin embargo, hay situaciones donde las fuerzas mecánicas aplicadas se mantienen dentro de los umbrales fisiológicos. En estos casos, estas señales permiten a las células adaptarse a cambios en su entorno mecánico y por ejemplo, puede actuar como un estímulo de reparación, como se observó en el segundo trabajo de esta tesis doctoral, contribuyendo a la homeostasis celular.

Sin embargo, cuando el estrés mecánico es excesivo o prolongado, este puede inducir daño pulmonar dando lugar a la activación de distintos mecanismos patogenéticos (2). Es por ello que la reducción de la carga mecánica en pacientes con fallo respiratorio agudo es esencial para disminuir este daño, y de hecho ha demostrado aumentar la supervivencia en estos pacientes (169–171). Sin embargo, las diferentes estrategias preventivas en el manejo del paciente referido al ajuste de los parámetros ventilatorios no son suficientes para evitar el desarrollo subsiguiente de enfermedades a largo plazo de estos pacientes (172). Esto pone de relieve la importancia de conocer los mecanismos moleculares que se activan por el uso de la ventilación mecánica, para evitar el desarrollo de PICS.

La doble naturaleza de la senescencia (57) nos hace plantearnos la posibilidad de que este mecanismo, que como se ha demostrado anteriormente tiene un papel importante en el mantenimiento de la homeostasis pulmonar, también pueda estar asociado al desarrollo de las secuelas a largo plazo en el paciente crítico. Es por ello, que el modelo utilizado en este tercer trabajo, se estableció con el objetivo de identificar

los mecanismos moleculares que se activan a largo plazo en respuesta al estiramiento mecánico.

Para ello, inicialmente el estudio se centró en la búsqueda de marcadores canónicos de senescencia, como la parada de ciclo celular, la acumulación de SA-βgal o la formación de SAHF, los cuales se manifiestan en etapas posteriores a la inducción de la senescencia (168). La identificación de estos marcadores tras el estiramiento mecánico nos permitió confirmar la activación de un programa de senescencia y por lo tanto nos abrió la posibilidad de estudiar cómo este fenómeno celular podría contribuir a las complicaciones a largo plazo que pueden desarrollar los pacientes críticos.

La senescencia se ha identificado con un mecanismo patogenético clave en el desarrollo de la fibrosis pulmonar idiopática (114,118). Además, varios estudios han encontrado correlaciones entre el estiramiento mecánico y la fibrosis (173–175). Aunque en un contexto clínico no se ha podido vincular el uso de la ventilación mecánica con el desarrollo de la fibrosis secundaria debido al lapso temporal entre el tratamiento de soporte vital y el desarrollo de la enfermedad (176), este trabajo plantea la hipótesis de que la senescencia inducida por el estiramiento mecánico podría ser un desencadenante de la fibrosis secundaria.

El SASP representa un mecanismo fundamental por el cual las células senescentes pueden influir en el desarrollo de la fibrosis (133,177). Nuestro trabajo mostró una activación de los fibroblastos por el efecto paracrino de las células senescentes, lo que sugiere que la fibrosis

secundaria después del estiramiento mecánico puede ser el resultado de la comunicación entre el epitelio senescente y el fibroblasto subyacente. Además, se mostró que el estiramiento inducía senescencia en las células epiteliales, pero no en fibroblastos, lo que sugiere que la respuesta es específica de tipo celular. La rigidez de la membrana celular puede afectar a cómo el estiramiento se transduce a una respuesta biológica dentro de la célula (54), por lo que es posible que las variaciones en esta característica entre los diferentes tipos celulares (178) esté afectando a la activación de los mecanismos que conducen a la senescencia.

Asimismo, los resultados mostraron que la vía de los receptores de Notch podría estar implicada en la comunicación epitelio-mesénquima. Se ha descrito, que la activación de estos receptores de superficie celular afecta en la capacidad proliferativa de las células progenitoras alveolares, influyendo así en la capacidad regenerativa del tejido alveolar (179). Además, estos pueden activarse en respuesta al estiramiento mecánico (180) y dar lugar a la formación de tejido fibroso (180–182).

Uso de senolíticos para la prevención de la fibrosis pulmonar secundaria en el paciente crítico

Los resultados expuestos anteriormente sugieren que la eliminación de las células senescentes podría ser un objetivo terapéutico clave para abordar la fibrosis pulmonar secundaria en respuesta al estrés mecánico. Como se detalló en la introducción, los senolíticos son fármacos que promueven la apoptosis de células senescentes (135). En concreto, la

digoxina tiene un efecto senolítico al unirse a la bomba Na+/K+ ATPasa (143).

Las diferencias encontradas en la expresión de genes relacionados con canales iónicos en las células senescentes inducidas por estiramiento sugieren que estas células pueden ser susceptibles a este fármaco. En nuestro modelo, el uso de la digoxina tuvo el potencial de eliminar a las células senescentes y reducir su efecto profibrótico. Asimismo, se observó un aumento en proteínas anti-senescencia como S100A6, catepsinas B, C y D, clusterina o IGFBP6, y una disminución en factores pro-senescenia como Serpina E1, Apolipoproteína E o la proteína transportadora 3 del factor de crecimiento similar a la insulina (IGFBP3) en aquellas células tratadas con digoxina. Estos cambios indican que la eliminación de células senescentes provoca una modificación de su secretoma, lo cual reduce el efecto pro fibrótico observado con el estiramiento.

La digoxina ha demostrado ser efectiva como senolítico en modelos animales de fibrosis pulmonar (143) y se utiliza comúnmente en clínica para tratar afecciones cardíacas (183) por lo que tiene un perfil de seguridad bien establecido en este contexto. Todo esto sugiere que podría ser una opción terapéutica prometedora para el tratamiento de la prevención de la fibrosis pulmonar secundaria.

Limitaciones

Los resultados mostrados en esta tesis doctoral tienen ciertas limitaciones. Aunque los trabajos muestran la activación de la senescencia en respuesta a distintas situaciones críticas y destacan la diferencia en respuesta entre algunos tipos celulares, la naturaleza de los modelos y las herramientas utilizadas para el estudio impiden representar todos los tipos celulares presentes en el pulmón.

Por otro lado, la configuración del estiramiento utilizado en nuestros estudios no se puede trasladar a parámetros ventilatorios determinados en clínica. Los parámetros ventilatorios utilizados en nuestro modelo animales no son directamente extrapolables a la clínica debido a las diferencias en el tamaño y anatomía pulmonar, las diferencias fisiológicas entre especies y la duración de los estudios. Además, en el modelo in vitro utilizado, las células se expusieron a condiciones controladas de estiramiento mecánico que no reflejan la complejidad tridimensional ni la heterogeneidad de la expansión alveolar de los pulmones lesionados. Este modelo solo buscaba provocar una respuesta biológica al estiramiento, pero no replicar las condiciones fisiológicas exactas que ocurren en el pulmón humano durante la ventilación mecánica.

Estos estudios podrían complementarse utilizando diferentes técnicas como la secuenciación de célula única, que permitiría una comprensión más precisa de las diferencias en la activación de la senescencia entre las distintas poblaciones celulares del pulmón. También podrían utilizarse otros modelos, como el modelo ex vivo de perfusión y ventilación pulmonar, que permitiría un control más riguroso de las

condiciones experimentales y una medición más detallada de los parámetros funcionales del pulmón.

El papel de la senescencia y el SASP en las secuelas post-UCI

Como se detalló en la introducción, el paciente crítico se enfrenta a una serie de situaciones durante su estancia en la UCI que conlleva, en muchas ocasiones, al desarrollo de una serie de secuelas graves entre las que se incluye la disfunción pulmonar crónica (2), la debilidad muscular adquirida (4) y el deterioro cognitivo (3). Distintos estudios, revisados en uno de los trabajos que integran la presente tesis doctoral, han identificado varios mecanismos moleculares involucrados en la enfermedad, pero hasta ahora, no se ha encontrado un tratamiento que pueda dirigirse con éxito a esos procesos biológicos para mejorar la salud de los pacientes (37).

Durante esta tesis doctoral se han llevado a cabo distintos trabajos con el objetivo de apoyar la hipótesis de que la senescencia celular supone un mecanismo unificador entre la fase aguda de la lesión pulmonar y el desarrollo de las secuelas a largo plazo experimentadas por los supervivientes, en concreto en el desarrollo de una fibrosis secundaria al uso de la ventilación mecánica. El segundo trabajo de esta tesis doctoral describe que la activación de la respuesta senescente inducida por el daño pulmonar y el uso de la ventilación mecánica podría tener un papel homeostático en la fase aguda de la enfermedad. Es más, nuestros resultados muestran que esta respuesta puede ser manipulada farmacológicamente mediante el uso de lopinavir/ritonavir para incrementar el papel beneficioso de la activación de p21. En cambio, los

resultados del tercer trabajo de esta tesis doctoral sugieren que la activación de la senescencia en el epitelio respiratorio puede contribuir al desarrollo de una fibrosis secundaria en pacientes ventilados mediante la difusión del SASP. Además, propone que el efecto senolítico de la digoxina podría contribuir a limitar el desarrollo de esta enfermedad.

Aunque los resultados de esta tesis doctoral se limitan a estudiar el papel de la senescencia inducida por el estiramiento mecánico en el desarrollo de la fibrosis pulmonar, distintos estudios sugieren que este mecanismo puede ser un factor patogenético clave en el desarrollo de otras secuelas post-UCI en diferentes situaciones críticas. Un estudio publicado recientemente establece que la sepsis es un importante inductor de la senescencia en el tejido muscular, la cual está directamente relacionada con la debilidad muscular resultante (184). La enfermedad renal crónica también parece ser un importante inductor de la senescencia de las células progenitoras musculares, dando como resultado una atrofia muscular. Estas células liberan un SASP que da lugar a la producción de mediadores inflamatorios y la subsiguiente perdida muscular (185). Además, en ambos estudios, el tratamiento con senolíticos demostró reducir la senescencia celular y ayudó a frenar la perdida muscular. La inducción de la senescencia en la lesión cerebral también ha sido objeto de investigación en varios estudios. Se ha encontrado que tanto el traumatismo craneoencefálico como la lesión por isquemia-reperfusión pueden activar un programa de senescencia y el aumento subsiguiente del SASP, el cual desempeña un papel significativo en el deterioro cognitivo (186,187). Además, la eliminación de la carga senescente mediante el uso de

senolíticos también ha demostrado reducir los niveles de senescencia y mejorar la función cerebral(187).

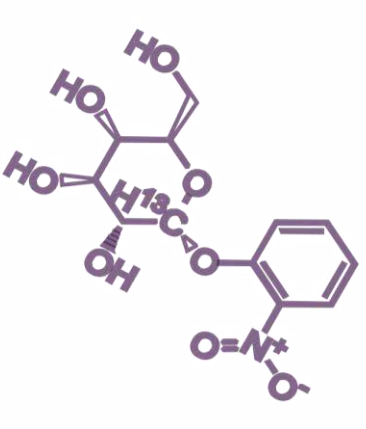
En todos estos estudios, el SASP mostró ser el principal responsable de los efectos paracrinos asociados a la senescencia, tomando un papel muy importante en el desarrollo de la debilidad muscular y el deterioro cognitivo. Además, se ha comprobado que las moléculas secretadas por las células senescentes pueden translocarse y, una vez en la circulación, llegar a tejidos distantes aumentando la inflamación de estos y alterando su microambiente (188). Es decir, el efecto del SASP no se limita únicamente al tejido donde se activa el programa de senescencia, sino que también puede tener implicaciones en tejidos distantes.

Los resultados de esta tesis doctoral vinculan el uso de la ventilación mecánica con la inducción de la respuesta senescente y su implicación en la fibrosis pulmonar a través del SASP. Aunque aún no se haya estudiado si la activación de la senescencia por ventilación mecánica está involucrada también en el desarrollo de otras secuelas, distintos estudios evidencian el uso de la ventilación mecánica con un aumento de la atrofia muscular (22) y el deterioro cognitivo (189) a largo plazo en el paciente crítico, siendo la inflamación sistémica uno de los mecanismos patogenéticos involucrados

La coincidencia de que la inflamación suponga uno de los mecanismos moleculares cruciales en el desarrollo de estas secuelas, junto con el hecho de que el SASP pueda contribuir a una inflamación sistémica, sugiere que la inducción de la senescencia por la ventilación mecánica no solo pueda estar afectando al tejido pulmonar a través de su SASP, sino que también pueda llegar a otros tejidos, como el tejido cerebral y muscular, y

pueda participar en el deterioro cognitivo y la debilidad muscular desarrollada a largo plazo en el paciente crítico.

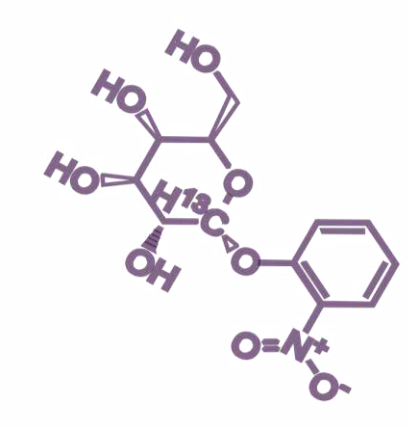
Aunque hagan falta más estudios para comprender todos los efectos de la senescencia y el SASP en el paciente crítico, estos resultados reflejan que la senescencia desempeña un papel crucial en el desarrollo de las secuelas post-UCI. En este contexto, la senescencia podría actuar como un mecanismo patogenético común a las diversas complicaciones observadas en estos pacientes, lo que la convierte en un objetivo terapéutico potencial para la prevención del PICS.



CONCLUSIONES

1. La activación de la senescencia en el daño pulmonar agudo limita la apoptosis y el daño histológico del pulmón. Esta respuesta parece estar causada por la inducción del daño en el ADN y cambios en la cromatina causados por una sobredistensión mecánica. La interferencia con la lamina nuclear puede potenciar esta respuesta antiapoptótica.
2. El estiramiento mecánico es un inductor directo de la senescencia en el epitelio alveolar y la propagación de esta respuesta puede contribuir a la fibrosis a largo plazo observada en los pacientes que reciben ventilación mecánica.
3. La eliminación de las células senescentes mediante el uso de la digoxina modula la comunicación entre células epiteliales y fibroblastos, limitando la fibroproliferación en respuesta al secretoma liberado por las células senescentes. El efecto senolítico de este fármaco ofrece una posibilidad terapéutica para el tratamiento de la fibrosis secundaria en el paciente crítico.
4. La senescencia supone un mecanismo patogenético clave en el desarrollo de las secuelas post-UCI, lo que la convierte en un objetivo terapéutico potencial para la prevención del PICS.

1. The activation of senescence in acute lung injury limits apoptosis and histological lung damage. This response seems to be caused by the induction of DNA damage and chromatin changes due to mechanical overdistension. Interference with the nuclear lamina can enhance this anti-apoptotic response.
2. Mechanical stretch is a direct inducer of senescence in the alveolar epithelium, and the spread of this response may contribute to the long-term fibrosis observed in patients undergoing mechanical ventilation.
3. The elimination of senescent cells using digoxin modulates the communication between epithelial cells and fibroblasts, limiting fibroproliferation in response to the secretome released by senescent cells. The senolytic effect of this drug offers a therapeutic possibility for the treatment of secondary fibrosis in critically ill patients.
4. Senescence represents a key pathogenetic mechanism in the development of post-ICU sequelae, making it a potential therapeutic target for the prevention of PICS.



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