



Faculty of Medicine and Dentistry

Department of Physiology

PhD program: 3102 Biotechnology and
Biomedicine

MOLECULAR MECHANISMS INVOLVED IN THE
INTERACTIONS BETWEEN CHROMATIN-DERIVED
MACROMOLECULES AND ENDOTHELIAL CELLS:
POTENTIAL FOR THE DEVELOPMENT OF SEPSIS
BIOMARKERS

Doctoral Thesis presented by:

Concepción Garcés Díaz

Supervised by:

Dr. Carlos Romá Mateo

Dr. Federico V. Pallardó Calatayud

Valencia, November 2023

D. Federico V. Pallardó Calatayud, Catedrático de Universidad del Dpto. de Fisiología de la Universitat de València

D. Carlos Romá Mateo, Profesor Contratado Doctor del Dpto. de Fisiología de la Universitat de València

D. Juan Viña Ribes, Catedrático de Universidad del Dpto. de Bioquímica de la Universitat de València

CERTIFICAN:

Que la presente memoria, titulada “Molecular mechanisms involved in the interactions between chromatin-derived macromolecules and endothelial cells: potential for the development of sepsis biomarkers”, corresponde al trabajo realizado bajo su dirección por Dña. Concepción Garcés Díaz, para su presentación como Tesis Doctoral en el Programa de Doctorado en Biomedicina y Biotecnología de la Universitat de València.

Y para que conste firman el presente certificado en València a 18 de septiembre de 2023.


CARLOS|ROMA|
MATEO
2023.09.18
13:00:41 +02'00'

Dr. Carlos Romá Mateo

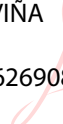
Director

FEDERICO
VICENTE|
PALLARDO|
CALATAYUD

Firmado digitalmente
por FEDERICO VICENTE|
PALLARDO|CALATAYUD
Fecha: 2023.09.18
15:57:34 +02'00'

Dr. Federico V. Pallardó Calatayud

Director

JUAN VIÑA
RIBES -
NIF:22626908
Z

Firmado
digitalmente por
JUAN VIÑA RIBES -
NIF:22626908Z
Fecha: 2023.09.19
10:50:28 +02'00'

Dr. Juan Viña Ribes

Tutor

Esta tesis ha sido financiada por el Ministerio de Universidades mediante la beca de Formación de Profesorado Universitario (FPU18/03969). La estancia internacional ha sido financiada por la organización EMBO mediante la beca *Short-term fellowship* (STF 9849). El trabajo realizado ha sido posible gracias a proyectos financiados por la Generalitat Valenciana (GV/2018/127) y el Ministerio de Ciencia e Innovación (PID2020-119127RA-I00).

AGRADECIMIENTOS

En primer lugar, agradecer al grupo del CIBERER del departamento de Fisiología de la Universidad de Valencia por su acogedor recibimiento y haberme permitido aprender de todos ellos, inmersos siempre en un muy buen ambiente de trabajo. Sobre todo, quiero agradecer la oportunidad de haberme acogido en su laboratorio y formado como científica a mis directores de tesis el Dr. Federico V. Pallardó y el Dr. Carlos Romá. Durante estos casi cinco años me habéis hecho sentir respaldada y guiada, como si fuera una hija más. Gracias por todo lo que me habéis enseñado. En especial quiero agradecer a mi maestro *Jedi* Carlos, por haberme acompañado en los dos lados de la *Fuerza* de la ciencia. Siempre haces todo lo posible por ayudarnos y además de buen humor. Sé que no he podido tener más suerte de poder haber pasado estos años de tesis siendo tu doctoranda ya que han sido productivos y también muy divertidos gracias a ti. Espero haberte hecho sentir orgulloso de tu *padawan*.

En segundo lugar me gustaría agradecer a todos los integrantes del departamento de Fisiología de los cuales he aprendido tanto, a las chicas de secretaría por toda su ayuda siempre tan eficaz y también a mi tutor Juan Viña.

Si algo ha sido importante para la realización de la tesis han sido mis compañeros de laboratorio. Hemos creado una pequeña familia que ha ido aumentando cada año y ellos han sido la razón por la que ir al laboratorio cada día no haya sido duro sino, todo lo contrario, teniendo ganas de ir, ya que se ha creado un ambiente muy bueno. Tamara, Noelia, Elena, Irene, Enric, Víctor, Dani, Miguel, Susana, Irene, Rebeca, Inma...Si nombro a todos, no acabarían nunca estos agradecimientos.

De entre todos mis compañeros quiero agradecer en especial a la actualmente Dra. Laura, te debo mucha parte de la tesis. Muchas gracias por enseñarme todo lo que se del laboratorio, fuiste mi amiga y mi guía durante mi TFM y el principio de la tesis y si esta tesis consiguió salir adelante en los duros comienzos fue gracias a ti.

Por un lado, quiero mencionar a todos los alumnos que he tenido estos años, Marcos, Luis, Alicia y Patri. Una parte de esta tesis también es vuestra. En especial a Patri. Patri

llegaste en un momento de estancamiento y diste un empujón enorme a la tesis. No puedo más que agradecer al laboratorio haberte encontrado y puesto en mi camino porque no solo hemos formado el mejor equipo que pueda haber en ciencia, sino que te has convertido en una persona imprescindible en mi vida. Sabes que, aunque ya no esté en el laboratorio, aquí tienes a tu hermana mayor valenciana siempre.

No cabe olvidar la inmensa ayuda que han supuesto los técnicos de la UCIM para esta tesis. Por un lado, un enorme gracias a Guadalupe Herrera siempre dispuesta a ayudarnos con el citómetro, aunque eso implicase quedarse hasta horas intempestivas. Por otro lado, agradecer la inestimable ayuda de Toni Ibáñez, por haberme enseñado tanto sobre microscopía y haber estado siempre dispuesto a guiarnos con los experimentos que involucraban microscopia, sin tu ayuda no habiéríamos conseguido unas imágenes tan chulas. También agradecer a los profesores José Manuel Morante y Pepe Pertusa por su ayuda y consejos técnicos en la interpretación de resultados.

Por otro lado, también agradecer al laboratorio del Dr. Joseph Penninger en IMBA, Vienna su acogida durante los tres meses que duró mi estancia. En especial a Kirill que me enseñó el apasionante mundo de las iPSC y los organoides con tanta paciencia. Además, también me gustaría destacar a la gente que conocí allí, Victoria, Aranza, Juan Carlos, Miguel y Guisi, que me hicieron sentir como en casa.

Haber llegado hasta aquí, entregando la tesis doctoral, no habría sido posible sin mis chicas PcR, con las que llevo compartiendo el camino desde hace casi diez años y fue gracias a ellas que la universidad fue una etapa increíble. Aunque la ciencia nos ha separado geográficamente siempre estáis presente en mi vida, dándome apoyo y amor a raudales. Os quiero mucho chicas.

Elena, m'has acompanyat en aquesta aventura des del principi. Gràcies per estar sempre al meu costat, inclús acompanyant-me al congrés de Màlaga. He après molt de tu. T'estime molt ♡.

M'agradaria també agrair a la meua família el suport incondicional que m'han donat sempre i tota la confiança que han tingut en mi. En especial al meu cosí Àlvaro, que més que un cosí per a mi és el meu germà.

D'altra banda, a la meua germana, per ser absolutament tot per a mi. Sempre has sigut la meua millor amiga i el meu exemple a seguir, m'has guiat i ajudat en tot, inclús m'has corregit aquesta tesi doctoral. Gràcies també pel millor regal que podies haver-me fet, Adrián. Si sóc qui sóc avui és gràcies a tu i als pares. I love you to the moon and back.

Un enorme gràcies també als meus pares. Gràcies per haver-ho donat tot per mi i per la meua germana. Heu lluitat i esforçat dia a dia perquè nosaltres hàgim pogut fer tot allò que hem volgut. Gràcies per l'educació i els valors que ens heu ensenyat, pel vostre amor, suport i afecte que ens mostreu cada dia. Sense vosaltres, res de tot això hauria estat possible.

Por último, pero no menos importante, a Josevi. Gracias por ser un apoyo incondicional desde antes incluso que empezara la tesis. Gracias por estar siempre dispuesto a escuchar mis frustraciones y alegrías del laboratorio, mis charlas, posters y exposiciones sin importar lo que duraran o que fueran en inglés. Gracias por todas las palabras de ánimo y apoyo y por siempre sacarme una sonrisa. Pero sobre todo gracias por esta pequeña familia de tres que hemos creado junto a Khalifa. Gracias por haber estado y estar siempre a mi lado.

INDEX

INDEX

INTRODUCTION.....	0
1. Sepsis.....	1
1.1. Clinical aspects of the disease	1
1.2. Pathophysiology of sepsis	5
2. Nuclear proteins derived from chromatin	11
2.1. Extracellular histones	11
2.2. HMGB1	16
3. Autophagy	20
3.1 Non-canonical forms of autophagy	25
3.2 Relationship between autophagy, extracellular histones, and sepsis	28
4. Experimental models for studying sepsis and septic shock.....	31
HYPOTHESIS AND OBJECTIVES.....	35
MATERIALS AND METHODS.....	39
1. Cell culture.....	41
1.1. 2D culture.....	41
1.2. Blood vessel organoids.....	41
1.3. Treatments	45
1.4. Transfection.....	46
2. Protein analysis.....	47
2.1. Protein extraction.....	47
2.2. Histone extraction	47
2.3. Western Blot	48
3. Image techniques	50
3.1. Immunofluorescence.....	50
3.2. Proximity Ligation Assay.....	51
3.3. Confocal microscopy	53
3.4. Image analysis	53
4. Flow cytometry.....	60
4.1 Cell cycle analysis	61
4.2 BVOs flow cytometry.....	61
5. RNA extraction and RT/qPCR.....	62
6. Ethics Committee	66
7. Statistical analysis.....	67
8. Facilities	68

RESULTS	69
1. <i>HeLa and Hep3B cell models</i>	71
1.1 Cytotoxic effects of extracellular core histones in HeLa and Hep3B.....	73
1.2 Colocalization studies of core histones and lysosomes in HeLa and Hep3B cells	75
1.3 Effects of autophagy modulation upon core histone treatment in HeLa and Hep3B cells.	79
1.4 Effects of core histone treatment in the cell cycle of HeLa cells.	81
1.5 Changes in miRNA signature upon core histone treatment in HeLa and Hep3B cells.....	82
2. <i>HUVEC cell model</i>	85
2.1 Cytotoxic effects of extracellular core histones in HUVEC cells.	87
2.2 Colocalization studies between histones and autophagy markers LC3B in HUVEC cells.....	88
2.3 Verification of the association between autophagy and core histones.....	92
2.4 Determination of incubation conditions with histone H1-488 and studies of colocalization with LysoTracker Red DND-99.....	95
2.5 Effect of endocytosis blockade on the colocalization between extracellular histones and LC3B protein and its relation to cell viability.....	98
2.6 Effect of Modulation of Autophagy on Histone-Mediated Cytotoxicity and Colocalization with LC3B.....	102
2.7 Changes in release of HMGB1 upon core histone treatment and autophagy modulation.	110
3. <i>Blood Vessel Organoid model.....</i>	111
3.1 Cytotoxic effects of extracellular histones in blood vessel organoids	113
3.2 Localization of extracellular histones and autophagy flux marker LC3B in BVOs	114
3.3 Effect of Modulation of Autophagy on Histone-Mediated changes in BVOs.	118
3.4 Effect of endocytosis blockade on the histone mediated changes in BVOs	123
3.5 Changes in gene expression and miRNA upon chromatin-derived molecules treatments.....	126
3.6 Changes in release of HMGB1 upon core histone treatment and autophagy modulation.	130
4. <i>Potential of HMGB1</i>	133
<i>as sepsis biomarker</i>	133
<i>in human plasma</i>	133
4.1 Evaluation of HMGB1 as a biomarker for sepsis and septic shock	135
4.2 Correlation studies between HMGB1 and sepsis clinical parameters	136

DISCUSSION	139
1. Extracellular histones are cytotoxic for different experimental models	141
2. Extracellular histones are internalized by endocytosis mechanisms and related to autophagy pathway	143
3. Modulation of autophagy influences the alterations driven by extracellular histones	149
4. miRNA signature is altered by extracellular histones and autophagy modulation	153
5. HMGB1 exhibits potential as a biomarker for sepsis progression and severity	157
6. Relevance and limitations of the present work	160
CONCLUSIONS	163
REFERENCES	167
ABBREVIATION LIST	183
INDEX OF FIGURES	186
INDEX OF TABLES	188
ANNEXES	189
ANNEX I: Supplementary figures and tables	191

ABSTRACT

ABSTRACT

Sepsis is a prevalent global syndrome characterized by an exaggerated immune response to infection, resulting in hyperinflammation followed by immunosuppression. Pro-inflammatory molecules, including HMGB1 and histones, are released into the bloodstream during the hyperinflammatory phase, leading to significant physiological changes. Histones, known for their pro-inflammatory and procoagulant effects, particularly impact the endothelium of blood vessels, exacerbating sepsis symptoms. The aim of the study was to elucidate the role of histones in cellular processes and their potential implications in sepsis. The work presents key findings on the cytotoxic effects and cellular localization of extracellular histones, as well as their interactions with autophagy and miRNA expression in various experimental models.

The results demonstrate that high doses of core histones induce cytotoxicity in HeLa, Hep3B, HUVEC cells, and blood vessel organoids. An image analysis tool was developed to quantify the overlap between extracellular histones and fluorescently labeled lysosomes, revealing their predominant localization on lysosomes in HeLa and Hep3B cells. Modulation of autophagy did not significantly affect cell survival or the relative localization of histones and lysosomes in HeLa and Hep3B cells. However, in HUVEC cells and blood vessel organoids, treatment with extracellular histones led to a dose and time-dependent increase in colocalization between histones and the autophagy-associated protein LC3B. Blocking endocytosis reduced the entry of extracellular histones into the cells, resulting in decreased cytotoxicity in HUVEC cells and blood vessel organoids. Inhibition of autophagy in HUVEC cells reduced colocalization between histones and LC3B, while its activation had an impact. In blood vessel organoids, autophagy modulation did not significantly affect histone fluorescence intensity but altered histone localization. Furthermore, core histone treatment influenced miRNA expression, with downregulation of miR-155 observed in HeLa, Hep3B, and HUVEC cells, while its upregulation was found in blood vessel organoids. Additionally, miR-146 was downregulated in HeLa and Hep3B cells, while miR122 was not expressed in any experimental model. Treatment with core histones also induced the secretion of HMGB1 in blood vessel organoids, and plasma levels of HMGB1 were significantly higher in patients with sepsis and septic shock compared to an ICU control.

group. Plasma levels of HMGB1, measured using ELISA, demonstrated strong predictive power for prolonged ICU stays and exhibited significant correlations with clinical indicators in sepsis, such as D-dimer, ICU length of stay, and NtProBNP.

In conclusion, this study provides insights into the cytotoxicity, cellular localization, autophagy interactions, miRNA expression, and HMGB1 secretion induced by core histones in different experimental models. These findings contribute to a better understanding of the role of histones in cellular processes and their potential implications in sepsis.

RESUM

La sepsi és un síndrome global prevalent caracteritzat per una resposta immune exagerada a la infecció, que provoca una hiperinflamació seguida d'immunosupressió. Molècules proinflamatòries, com HMGB1 i histones, es desprenen al torrent sanguini durant la fase hiperinflamatòria, provocant canvis fisiològics significatius. Les histones, conegudes pels seus efectes proinflamatoris i procoagulants, afecten particularment l'endoteli de les parets vasculars, exacerbant els símptomes de la sepsi. L'objectiu de l'estudi era esbrinar el paper de les histones en els processos cel·lulars i les seves possibles implicacions en la sepsi. El treball presenta resultats clau sobre els efectes citotòxics i la localització cel·lular de les histones extracel·lulars, així com les seves interaccions amb l'autofàgia i l'expressió de miRNA en diversos models experimentals.

Els resultats demostren que altes dosis de les histones bàsiques indueixen citotoxicitat en les cèl·lules HeLa, Hep3B, HUVEC i els òrgans vasculars. Es va desenvolupar una eina d'anàlisi d'imatges per quantificar la superposició entre les histones extracel·lulars i lisosomes etiquetats amb fluorescència, revelant la seva localització predominant en lisosomes a les cèl·lules HeLa i Hep3B. La modulació de l'autofàgia no va afectar significativament la supervivència cel·lular ni la localització relativa de les histones i els lisosomes a les cèl·lules HeLa i Hep3B. No obstant això, en les cèl·lules HUVEC i els òrgans vasculars, el tractament amb histones extracel·lulars va provocar un augment dosi i temps dependent en la colocalització entre les histones i la

proteïna associada a l'autofàgia LC3B. El bloqueig de l'endocitosi va reduir l'entrada de les histones extracel·lulars a les cèl·lules, amb una disminució de la citotoxicitat en les cèl·lules HUVEC i els òrgans vasculars. La inhibició de l'autofàgia en les cèl·lules HUVEC va reduir la colocalització entre les histones i LC3B, mentre que la seva activació va tenir un impacte. En els òrgans vasculars, la modulació de l'autofàgia no va afectar significativament la intensitat de fluorescència de les histones, però va alterar la seva localització. A més, el tractament amb histones bàsiques va influir en l'expressió de miRNA, amb una baixada de miR-155 observada a les cèl·lules HeLa, Hep3B i HUVEC, mentre que es va trobar una upregulació en els òrgans vasculars. A més, miR-146 va ser baixament regulat en les cèl·lules HeLa i Hep3B, mentre que miR122 no es va expressar en cap model experimental. El tractament amb histones bàsiques també va induir la secreció de HMGB1 en els òrgans vasculars, i els nivells de HMGB1 en plasma van ser significativament més alts en pacients amb sepsi i xoc sèptic en comparació amb un grup de control de la UCI. Els nivells de HMGB1 en plasma, mesurats mitjançant ELISA, van demostrar un fort poder predictiu per a estades prolongades a la UCI i van mostrar correlacions significatives amb indicadors clínics de la sepsi, com D-dímer, estada a la UCI i NtProBNP.

En conclusió, aquest estudi proporciona informació sobre la citotoxicitat, la localització cel·lular, les interaccions amb l'autofàgia, l'expressió de miRNA i la secreció de HMGB1 induïdes per les histones bàsiques en diferents models experimentals. Aquests resultats contribueixen a una millor comprensió del paper de les histones en els processos cel·lulars i les seves possibles implicacions en la sepsi.

INTRODUCTION

1. Sepsis

1.1. Clinical aspects of the disease

The current definition of sepsis was established at the “Third International Consensus for the definition of Sepsis and Septic Shock (Sepsis-3)” where sepsis was described as a life-threatening medical condition in which the organism's response to an infection is dysregulated and leads to organ and tissue damage. Also, septic shock can be defined as a “subtype of sepsis in which deep cellular, circulatory, and metabolic abnormalities are associated with a major risk of death” (1). Sepsis and septic shock are one of the main causes of death all over the world despite the different treatments and prevention available. Recent studies made in 197 different countries estimated that in 2017 48.9 million cases were diagnosed all over the world. Among these cases, 11 million deaths occurred as a direct consequence of sepsis or septic shock (2).

The first symptoms of sepsis can be very unspecific fever, elevated heart rate, high white blood cell count, and signs of infection. Also, these symptoms could be very difficult to detect in neonates, elderly and immunosuppressed patients, and yet these groups of patients are the ones most likely to develop sepsis after infection (3). Therefore, early diagnosis (preferably within the first six hours) and treatment of sepsis are crucial to increase survival, achieving a 45% relative risk reduction in mortality rates if diagnosed and treated at the early stages of the disease (4). However, the progression from sepsis to septic shock (SS) is currently challenging to predict and depends on the severity of the infection, the patient's pre-existing conditions, the response to the treatment, and the degree of organ dysfunction. Therefore, clinicians face a diagnosis challenge and a prompt, effective intervention becomes critical. So, identifying the early signs of sepsis and being able to predict its progression to septic shock is a major focus and need in critically ill patients. However, it is important to note that in this heterogeneous landscape, no single biomarker can accurately assess the progression of sepsis (5).

Biomarkers and predictive scoring systems

Given the aforementioned reasons, several clinical predictive scoring systems have been developed to facilitate the identification of sepsis and septic shock patients such as Systemic Inflammatory Response Syndrome (SIRS) criteria, the sepsis-related organ failure assessment (SOFA) score, and the quick SOFA (qSOFA) score. They are used to forecast the outcomes, particularly mortality, of patients in intensive care units. These systems aid in standardizing research and evaluating the level of patient care among different ICUs (intensive care units) (6).

Table 1. SOFA Score parameters evaluation. Abbreviations: FIO₂, fraction of inspired oxygen; MAP, mean arterial pressure; PaO₂, partial pressure of oxygen. *With respiratory support. #Catecholamine doses are given as µg/kg/min for at least 1 hour. Modified from (1).

SOFA	Score				
	0	1	2	3	4
PaO ₂ / FIO ₂ (mmHG)	≥ 400	>400	>300	>200*	>100*
Platelets (x10 ³ /µL)	≥ 150	>150	>100	>50	>20
Bilirubin (mg/dL)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12
Cardiovascular parameters	MAP≥70 mmHg	MAP <70 mmHg	Dopamine <5 [#]	Dopamine 5.1-15 or epinephrine/norepinephrine ≤0.1 [#]	Dopamine >15 or epinephrine/norepinephrine ≤0.1 [#]
Glasgow Coma Scale score	15	13-14	10.0-12.0	6.0 - 9.0	<6
Creatinine (mg/dL)	<1.2	1.2-1.9	2.0-3.4	3.5-4.9	>5
Urine output (mL/day)				<500	<200

SIRS criteria consist of several physical and laboratory evidence which indicate a response from the immune system to an infection. To obtain a positive SIRS and therefore a possible sepsis diagnosis, two or more of the following criteria must be met (7):

- Body temperature higher than 38°C or lower than 36°C.
- Heart rate faster than 90 beats/min

- Breathing rate faster than 20 breaths/min or less than 32 mmHg CO₂ partial pressure.
- White blood cell count greater than 12000 or less than 4000 per microliter or containing more than 10% immature cells.

However, the current definition of sepsis (1) is more general than the SIRS criteria, so the SOFA score has become a better scoring system to predict sepsis. This score collects different clinical parameters such as coagulation, CO₂ partial pressure, or platelets, as shown in *Table 1*. In short, the higher the score, the greater the risk of patient mortality and organ dysfunction. Patients could be diagnosed with a SOFA score equal to or higher than two (1). qSOFA was developed to reduce the time of diagnosis as it is the key point, but it has been proved that qSOFA has low sensitivity and moderate specificity for short-term mortality (8).

Other scoring system used nowadays at ICUs is APACHE (Acute Physiology, Age and Chronic Health Evaluation) II and APACHE III. APACHE score evaluates the patient's initial physiological parameters (12 in APACHE II and 17 in APACHE III) (6), age, and pre-existing health status. The final score ranges from 0-71 and the higher the score, the greater the risk of death. It is usually calculated within the first 24 hours at ICUs (9). Most of the studies use the APACHE II score for predicting mortality (9) and it has been proved to be a reliable one for sepsis and septic patients (6).

Non-coding RNA molecules called miRNAs have 19–24 nucleotides in them. They are promising candidates for biomarker applications due to their specificity, selectivity, and great stability. For a quicker and more affordable diagnosis of sepsis, researchers have concentrated on studying circulating miRNAs. Studies have shown that there are quite a few miRNAs whose expression is associated with sepsis (10). Due to their superior stability, selectivity, and specificity when compared to conventional biomarkers, miRNAs (miRNAs) have become promising diagnostic biomarkers for sepsis (11). Recent research has demonstrated that indicators like CRP and PCT are less accurate at diagnosing sepsis than miRNAs, such as miR-25 (12). This shows that miRNAs can help with early identification and treatment by successfully separating sepsis from other illnesses. Additionally, miRNAs can distinguish between various sepsis phases,

offering important prognostic data (12). MiRNAs make it convenient to exploit them as therapeutic targets in the treatment of sepsis. These characteristics make miRNAs ideal biomarkers in various fields, including forensics, clinical diagnostics, and medical research (11). Due to the heterogeneity of the results, more study is required to standardize their usage in the diagnosis of sepsis (12). MiRNAs have considerable promise for enhancing personalized medicine in the treatment of infectious diseases when used as biomarkers in the diagnosis of sepsis (13). In conclusion, because of their distinct characteristics, miRNAs have the potential to be useful biomarkers for sepsis. To overcome the drawbacks and validate their diagnostic and therapeutic potential in sepsis care, more study is required.

Overall, there is not yet a unique biomarker with enough specificity or sensitivity to be used routinely in clinical practice, and the most established have some limitations (14). Therefore, there is still a need for a set of scoring systems which use combined biomarkers which could lead to a precise diagnosis of sepsis and the progression toward septic shock.

Treatment

Before 2001, there were not established protocols for treating sepsis and septic shock that were backed by scientific research. Thus, clinicians aimed for values of cardiac index and oxygen delivery that were above normal levels in critically ill septic patients (15). As it was previously described, the first 6 hours are critical for septic patients, so nowadays sepsis management has adopted early goal-directed therapy (EGDT) which involves early high-risk patient identification, suitable microbiological cultures, and prompt treatment with antibiotics, source control, early hemodynamic optimization, and appropriate supportive care (15,16). This change in sepsis treatment has significantly benefited the outcome of patients (15).

As it has been already stated, one crucial step in the early treatment of sepsis is starting effective antibiotic therapies and addressing the source of infection as soon as possible. In fact, giving antibiotics within the first 60 minutes of identifying sepsis or septic shock can result in a survival rate of up to 80% (17). Other treatments that could provide a benefit in sepsis are resuscitation with balanced crystalloids (18), the use of

vasopressor to maintain optimal blood pressure and cardiac output (19), lactate clearance as a high serum lactate concentration correlates with a high risk of death (20) or glucocorticoids which manage to reduce inflammation by immunosuppression and vascular response to infection (21). However, despite the availability of various treatments and biomarkers for sepsis and septic shock, there is still a lack of specific and effective therapies that can benefit all patients uniformly. Therefore, further investigation is required to advance the development of biomarkers and treatments that can accurately categorize patients and provide tailored interventions. In this regard, advancements in comprehending the underlying molecular mechanisms involved in the onset and progression of sepsis hold great promise for designing targeted therapies and innovative treatments. Additionally, such research efforts have the potential to uncover novel biomarkers that could aid in monitoring treatments and tracking disease progression. In essence, these discoveries could contribute to the field of theragnostics, where both diagnosis and treatment are integrated for optimal patient management.

1.2. Pathophysiology of sepsis

Sepsis is characterized by a systemic exacerbated inflammatory response against infection by microorganisms, due to the dysregulation of intrinsic mechanisms. When an infection takes place, microorganisms express molecules that constitute a so-called PAMP (Pathogen-associated Molecular Pattern) which is detected by the immune system. The immune system also detects other types of molecules called DAMPs (Damage-Associated Molecular Patterns) which are produced by cell death processes in the host. DAMPs and PAMPs are recognized by PRRs (Pattern-Recognition Receptors) (22). The union of DAMP and PAMP to the receptors leads to the activation of diverse signalling pathways which end in the activation of genes related to inflammation, immune response, and cellular metabolism (23). In *Figure 1A* the proinflammatory molecules described are pointed out. At the molecular and cellular level, the pathogenesis of sepsis is very complex, involving processes such as immune dysfunction, imbalance in the inflammatory response, mitochondrial damage, coagulopathy, or autophagy (among others) that ultimately lead to organ dysfunction (24). Based on the chain of events related to the inflammatory response, sepsis physiology could be divided

into two phases: a hyperinflammatory initial phase followed by an immunodepression phase.

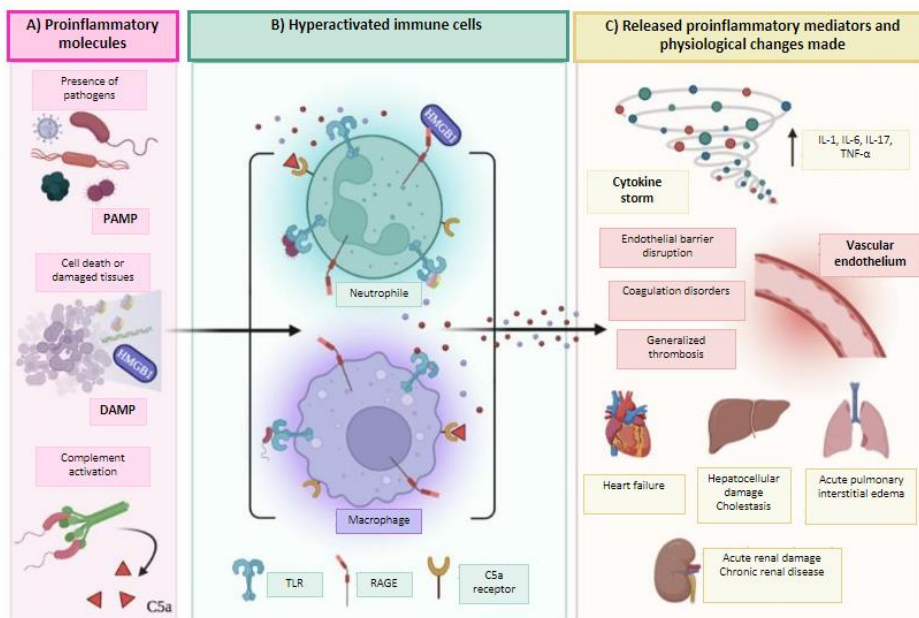


Figure 1. Summary of the pathogenic processes produced in the proinflammatory stage of sepsis. (A) The responsible molecules for triggering the inflammatory response (PAMP and DAMP). (B) Main cells of the immune system which are responsible for detecting PAMP and DAMP through specific receptors, setting in motion the consequent inflammatory response. (C) Different pro-inflammatory mediators whose levels are increased during this phase along with the physiological processes and organs that are affected by the detrimental effects of inflammation dysregulation. Own elaboration figure using BioRender.com. Based on (24).

Inflammatory phase

When a microbial infection or tissue damage is detected, innate immune cells - mostly constituted by macrophages, granulocytes, dendritic cells, monocytes, and natural killer cells- (25), are activated to initiate multiple defence responses (22). This early activation is mediated by PRRs expressed on the surface of immune cells, which interact with exogenous factors derived from pathogens (PAMPs: such as bacterial components, fungal, and viral pathogens or endotoxin and β -glucan (25)) and endogenous factors released by damaged cells (20) (DAMPs: mitochondrial DNA or high mobility group box 1 or HMGB1 (25)). Between all DAMPs released in sepsis, HMGB1 and histones could be highlighted since they act as pro-inflammatory molecules when released into the bloodstream. HMGB1 binds to RAGE (Receptor for Advanced Glycation

End-products), TLR (Toll-Like Receptor), or TREM-1 (Triggering receptor expressed on myeloid cells 1) receptors, activating immune cells and therefore promoting further inflammation and the disturbance of endothelial barrier (22,26). Histones, when released into the bloodstream (from now on called *extracellular histones*) behave equivalently, promoting inflammation, besides having a prothrombotic and procoagulant activity (27).

One of the most common surface receptors for the recognition of DAMPs and PAMPs are TLR, a subfamily of the PRRs, which are responsible for their identification and triggering the inflammatory response (22,28) and promoting complex intracellular signalling pathways with complementary and overlapping effects (27) that include, among other, the activation of inflammation-related genes such as Interleukin (IL)-6, IL-1, type-1 interferons, or tumour necrosis factor- α (TNF- α) (25). However, DAMPs and PAMPs can also be detected at the cytosol of cells by Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLR) and Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (25). In recent years, NOD-like receptors have gained attention in sepsis, specially NLRP3 (NLR-pyrin domain-containing 3). NLRP3 is a component of a multiprotein structure called *inflammasome* (represented in *Figure 2*), which is responsible for activating the pro-inflammatory pro-caspases, resulting in the production and release of very potent pro-inflammatory cytokines such as IL-1 β and IL-18. NLRP3 inflammasome induces the so-called pyroptosis which is a type of programmed cell death (25,29,30). Pyroptosis starts with the recognition of PAMPs by NLRP3 and its binding to pro-caspase 1. This forms a multi-protein complex that activates caspase 1. When caspase-1 is activated, it cleaves pro-IL-1 β and pro-IL-18 to produce active IL-1 β and IL-18, which are then released outside the cell to attract inflammatory cells and increase inflammation. In addition, the N-terminal and C-terminal sequences of Gasdermin D protein are quickly cut, removing the inhibitory effect. This allows it to bind to phosphatidylinositol on the cell membrane and leads to the formation of membrane pores, which disrupt the osmotic balance and cause cell death, and so the release of its content which lead to further exacerbating inflammation (31). Furthermore, these pores have been directly related to the formation and release of NETs (Neutrophil Extracellular Traps) (32). NETosis is a process in which granular

proteins and chromatin are released to form a matrix of extracellular web called NET able to bind to pathogens and eliminate them (33). However, NETs also induce cellular damage, mostly on endothelial cells, contributing to the physiopathology of sepsis (34). The role of pyroptosis and sepsis needs to be further investigated but a link between them has already been proved, showing that moderate pyroptosis has a beneficial effect on pathogen control in sepsis (31,35).

Overall, this process in sepsis ends with the stimulation of inflammation and adaptative immunity (28), leading to the uncontrolled release of proinflammatory cytokines such as IL-6, IL-1 β , or TNF- α (24). These cytokines at the same time can overactivate immune cells driving an increase in proinflammatory molecules and triggering the so-called cytokine storm (24,28), as it is shown in *Figure 1 and Figure 2*.

Within the cytokine storm, IL-17, and MIF (Macrophage migration Inhibitory Factor) stand out. On one hand, IL-17 is produced mainly by the T helper cells and triggers the production of other cytokines such as IL-6, IL-1, and TNF- α , acting as a link between innate and adaptative immune systems (22,28). On the other hand, MIF promotes innate and adaptative immune system response by activating macrophages and T cells, inducing, and amplifying the production of proinflammatory cytokines, regulating the phagocyte TLR-4 production, and blocking p53-mediated apoptosis in macrophages (22).

As we have already seen, a part of the inflammatory response in sepsis is the hyper activation of the immune system to trigger inflammation to fight against infection. In the early stages of sepsis, one of the components of the innate immune system that activates for promoting inflammation is the complement system (36). This system is activated by the presence of DAMPs and PAMPs, generating C5a anaphylatoxin, which behaves like a mediator between proinflammatory and procoagulant responses modulating the coagulation cascade, TLR-4 mediated responses and the release of several cytokines like HMGB1 or MIF (*Figure 1*) (22). Upon C5a generation, it binds to its receptors and generates proinflammatory peptides, reactive oxygen species and the release of enzymes and lipidic mediators (37) triggering extensive cell and tissue damage

which ultimately ends with the disruption of the endothelial barrier that could lead to organ failure and death in acute sepsis (28).

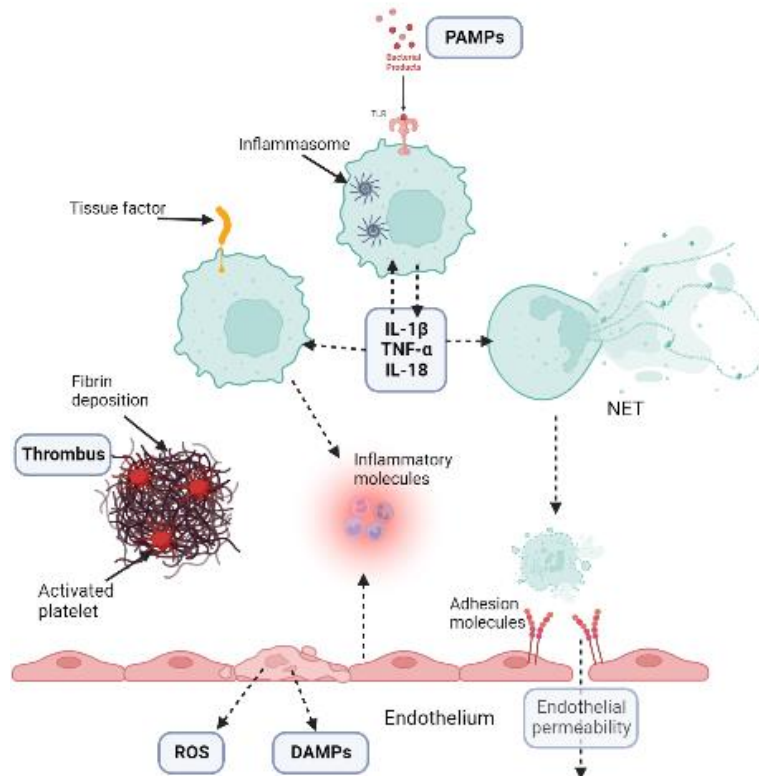


Figure 2. Inflammatory positive feed-back loop in the pathogenesis of sepsis. In sepsis, a series of interconnected physiological changes occur that perpetuate the condition. This includes damage to the endothelial cells, which leads to the activation of certain immune cells, disruption of the barrier function of blood vessels, formation of blood clots within the vessels, and widespread abnormal blood clotting. Own elaboration figure using BioRender.com. Based on (25)

Immunosuppression phase

The immunosuppression phase follows the hyperinflammation phase. Immunosuppression is a direct consequence of the hyperinflammatory environment to which the septic patient is exposed (38). The magnitude of the inflammation is variable and depends on the patient's comorbidities, pathogenic load, nutrition, and severity of the illness. While the proinflammatory response is taking place, an anti-inflammatory response has also been activated to counteract the proinflammatory environment. This anti-inflammatory response is characterized by metabolic dysfunction of immune cells

and changes in gene expression affecting cellular metabolism, cell survival, and immune system activity. Therefore, if the anti-inflammatory response continues over time, it can lead to prolonged immune depression, increasing the risk of secondary infections and consequently, increasing mortality (22,39). Despite that the hyperinflammation phase carries significant damage to the patient, this second phase is also very dangerous because it is related to a major risk of long-term mortality. This phase is associated with the highest amount of mortality, over 80% of the total deaths due to sepsis occurs during this phase (28,38,40).

The effects of the immunosuppression phase include, among others, the loss of function of immune cells leading to the inability of microorganism clearance and therefore, the development of secondary infections caused by opportunistic microorganisms or the reactivation of latent viruses (28,40). The main cells involved in immunity and the changes they undergo during the immunosuppression phase are shown in *Figure 3*. In the context of sepsis, immunosuppression affects innate and adaptative immunity. During this phase, the response is characterized by the production of IL-4, IL-5, IL-10, and IL-12 by TH-2 cells (T-helper 2 cell) which have anti-inflammatory functions and as a consequence, the production of proinflammatory interferon- γ (IFN- γ) and IL-12 by TH-1 cells decreases (22). Therefore, the expression of transcription factors related to antigen-presenting decreases due to the activation of TLR signalling pathways and the transformation of naïve T cells towards regulatory T cells in response to released cytokines (24). The effector lymphocytes of the adaptive immune response (CD4, CD8, and B cells) reach a state of exhaustion, thus limiting their action. Regulatory T lymphocytes do not undergo induction of apoptosis or loss of functionality, so they continue to exert immune suppression functions (38). Therefore, macrophages do not activate correctly during this phase due to the loss of functions of B and T cells, and this leads to a decrease in the ability to eliminate pathogens (28,38). Apoptosis is increased in lymphocytes, dendritic cells, and NK (Natural Killer) cells, triggering a depletion of immune cells and so the immune response is attenuated. This is one of the main reasons underlying the increased risk of septic patients suffering a nosocomial or secondary infection (22).

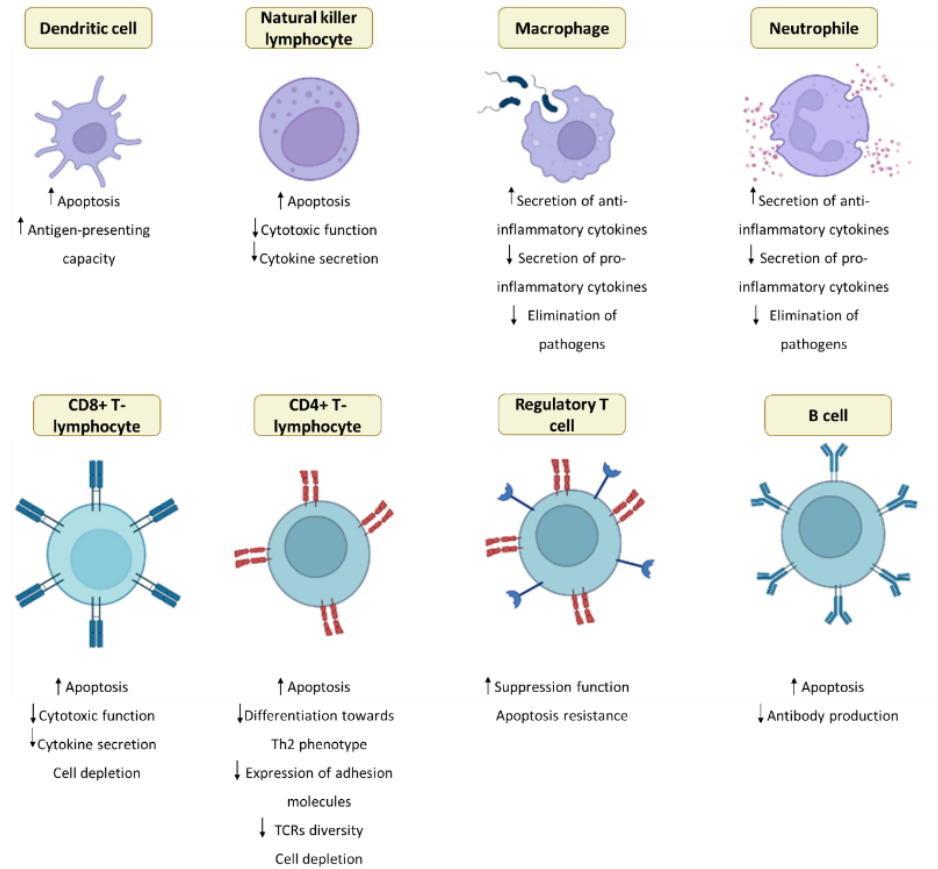


Figure 3. Changes in immune cells during the immunosuppression phase of sepsis. Almost all immune cells show an increase in apoptosis except for neutrophile. They all trigger anti-inflammatory responses. TCR: T-cell receptor, Th2: T-helper 2 cell. Self-elaboration figure using BioRender.com based on (38).

In conclusion, these cellular alterations lead to a state of chronic immunosuppression, defined by persistent and recurrent complications of an infectious nature that lead to reduced long-term survival (40).

2. Nuclear proteins derived from chromatin

2.1. Extracellular histones

Histones are globular proteins with a strong basic character, and they are very conserved in all vertebrates. Histones, together with other nuclear proteins and DNA constitute the chromatin, which is the structural component of chromosomes in the cell

nucleus of all eukaryotes. In humans, the basic structural unit of chromatin is formed by a core of four different histones (H2A, H2B, H3, and H4) in a dimeric way, forming an octamer bound to a strand of DNA of approximately 147 base pairs which wraps this octameric core, leading to the basic structural unit of chromatin, the nucleosome.

Finally, the addition of histone H1 to the nucleosomes triggers a higher level of compaction in which H1 wraps with a short strand of DNA resulting in two turns of DNA covering the octamer. Therefore, the main functions of histones are to provide structural stability, but they also participate in gene expression, by facilitating either the opening or the compaction of chromatin in certain regions (41).

The structure of core histones consists of an interior region, the histone-fold domain, which consists of three alpha-helices and two loops. Core histone structure also contains an extension called the histone tails consisting of amino-terminal extensions of histone proteins. These tails have many different sites where they can suffer post-translational modifications (PTM), such as acetylation, phosphorylation, methylation, ubiquitination, SUMOylation, ADP ribosylation (42) and glutathionylation (43), which could lead to a change of the function. Therefore, if the potential sites of modification are combined with the ability to arrange nucleosomes in the form of tetra or hexamers and the presence of histone variants, the nucleosome can mediate a wide range of regulatory steps to mediate the access of the transcriptional machinery to DNA (44,45). Strong links between alterations of gene expression mediated by histone PTM and several diseases including cancer, infertility, or sepsis have been proved (46). However, when an organism faces stress such as tissue damage or injury that causes cell death, histones, and other nuclear proteins can be released into the extracellular space (47). Once in the extracellular space and into the bloodstream, histones can act as DAMPs, activating the immune system and causing cytotoxicity in the body's cells, which can lead to necrosis and increased release of more DAMPs in a positive feedback process. This release of histones and other nuclear proteins has been observed in patients with different pathologies such as autoimmune diseases, trauma, or sepsis. In this context, systemic immune responses are activated, leading to the release of proinflammatory cytokines and chemokines (48).

In the physiological state, histones are located in the nuclei of cells where they are harmless, so they need to cross the nuclear and plasma membrane to reach the extracellular space and exert pathogenic functions (as shown in *Figure 4*). However, during the progression of sepsis, the release of extracellular histones is exacerbated leading to inflammation, tissue damage, thrombosis, and organ failure between others (26,42). In fact, histones are found in the plasma of sepsis patients in a wide range of concentrations, showing an average of 25 µg/ mL (49,50). On one hand, histones can be released passively due to the death of damaged cells i.e., necrosis, apoptosis, pyroptosis. On the other hand, they can be actively released by NETosis, where the condensed chromatin nets mainly contain histones but also azurophil granule proteins like elastases and myeloproteidases, to kill the microorganisms such as bacteria, fungi, parasites, and viruses (26,42,51).

In the 1950s, Hirsch postulated that histones located outside the nucleus exerted cytotoxic functions on microorganisms (52), but it was not until much later, into the 21st century, that Xu and his team first described the cytotoxic potential of extracellular histones on the organism itself, specifically, on human endothelial cells. They demonstrated that histone administration *in vivo* models caused endothelial damage, thrombosis, and neutrophil margination. In addition, they observed that using different sepsis models in mice, histone plasma levels increased, and if these were administered with APC (activated protein (C) or anti-histone antibody they could improve their survival (50).

Later, Xu and his team defined the proinflammatory role of histones by demonstrating how the administration of sublethal doses of histones causes an increase in the serum levels of proinflammatory cytokines such as IL-6, IL-10, or TNF-α. However, this did not occur when the mouse model used lacked TLR4 receptors. Conversely, if the mice model were deficient in TLR2, the mice could develop a hyperinflammatory response after histone injection. Therefore, they carried out *in vitro* studies on cell cultures demonstrating that histones transduced their signalling through both TLR2 and TLR4 receptors (53).

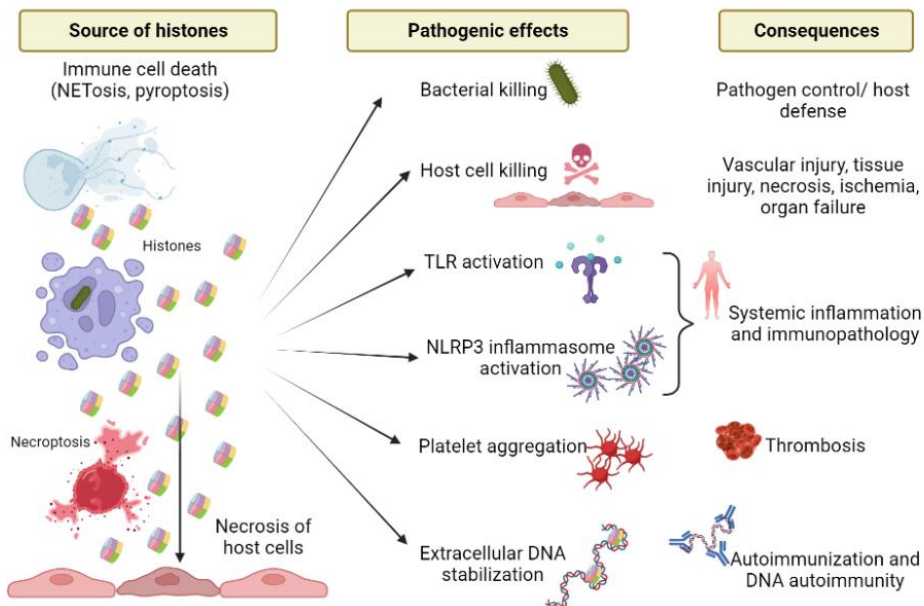


Figure 4. Pathogenic effects of extracellular histones. Different ways of releasing histones into the extracellular space trigger different pathogenic effects on the organism which could finally lead to severe health consequences for disease. Own elaboration figure using BioRender.com. Based on (42)

Thus, there are two mechanisms through which it is postulated that histones exert their extracellular actions. The first one depends on TLRs. TLRs are receptors that are located at the cellular membrane as well as other membranes such as lysosomes, endosomes, or the endoplasmic reticulum (54). TLR-2 and TLR-4 receptors, which are believed to interact with histones, can promote the activation and recruitment of neutrophils, monocytes, and macrophages, as well as triggering the release of proinflammatory cytokines related to sepsis (47). They could exert their functions by forward signalling the MyD88/NF κ B-dependent transcription pathway and via the activation of NOD-like receptors which will ultimately end up activating the NLRP3 inflammasome triggering the release of more cytokines and the recruitment of immune cells causing further inflammation (54). The other mechanism of cytotoxicity of histones is directly driven by the positive charges of arginine and lysine residues present in histone tails. This allows them to preferentially bind to the polar heads of membrane phospholipids through electrostatic interactions, inducing plasma membrane permeabilization and disrupting calcium signalling pathways (55). In fact, histones that underwent post-translational modification to become citrullinated exhibited reduced cytotoxicity towards endothelial cells compared to non-citrullinated histones.

Citrullinated histones did not impact cell viability and did not induce oxidative stress because of their reduced positive charges (56).

However, not all histones have the same cytotoxic potency. Although they all have similar charges, histone H1 is the only one that is neurotoxic, which suggests tissue-specific effects (57). Taken together, these processes lead to cell death by necrosis and trigger the release of intracellular contents, which ultimately drives chronic inflammation and subsequent tissue damage (51,58).

Extracellular histones have also been found in the plasma of septic patients and their impact has also been studied (26). Serum/plasma levels of circulating histones in healthy persons range from 0.8-2.3 µg/mL while the values of patients with sepsis, inflammation, or severe trauma increase drastically (54). Histones are increased in patients with coagulopathy and multiple organ failure (MOF) (59). Another study by Ekaney and collaborators showed that circulating histones were substantially increased and they seemed to cause cellular damage in a TLR4-dependent manner (60) and therefore, histones could be a major mediator of mortality in sepsis by inducing MOF (50). MOF is one of the most desolating consequences of sepsis and its pathogenesis is not fully understood. Some studies highlight the role of circulating histone H3 as the one responsible for initiating MOF, as high concentrations of H3 in the plasma of septic patients it is presume that trigger organ failure (48,59) Jackson and collaborators found higher levels of citrullinated H3 in septic patients than in trauma patients (61).

The evidence of the presence of circulating histones in the serum/plasma of septic patients and their link to the progression to more acute health stage of the patient, underscores the potential of histones as a promising biomarker for the identification and diagnosis of sepsis and septic shock. However, the aforementioned works lack a gold-standard detection method to determine histone concentration and so they could not confirm the power of histone as a diagnostic or prognostic biomarker (62). Nowadays, the most common detection methods of histone levels in plasma are Western Blot and enzyme-linked immunosorbent assay, which has poor reproducibility and variable cross-reactivity (63). Mass spectrometry could be a technique that improves the problems of the previous ones, as well as being more specific and sensitive

(63). In fact, in our group, we have developed and patented a method for quantifying circulating histones in the plasma of septic and septic shock patients by mass spectrometry (MRM-MS). This method uses peptides with specific sequences, isotopically labelled as internal reference (spike-in) (64). We also have developed a preliminary clinical validation of the method (64) and currently, we are performing wider studies with larger samples of patients (65).

All this highlights the fundamental role that histones play in the development of sepsis as one of the most relevant mediators, modulating the intense pro-inflammatory and procoagulant responses characteristic of sepsis (51,66) and their potential as biomarkers (62). Likewise, the different mechanisms through which histones cause cytotoxicity in cells continue to be explored to gain a better understanding of the biological responses that are affected by the action of histones. Among them, as we have already shown, the autophagy pathway must be highlighted (67).

2.2. HMGB1

High Mobility Group Box 1 or HMGB1 protein was first described in the 1970s as a chromatin-binding protein different from histones (68). This protein contains two DNA binding domains called A and B box, and an acidic region located on the C-terminal domain. Inside its sequence, HMGB1 has three cysteines, C23 and C45 located at the A box, and C106 located at the B box. These cysteines can form disulphide bonds, which are essential for the protein to perform its function. Therefore, HMGB1 presents two different nuclear localization domains, a region for binding with TLR4 receptors and another region for binding with RAGE receptor, and a functional domain of proinflammatory cytokine (69). The structure of the protein can be observed in *Figure 5*.

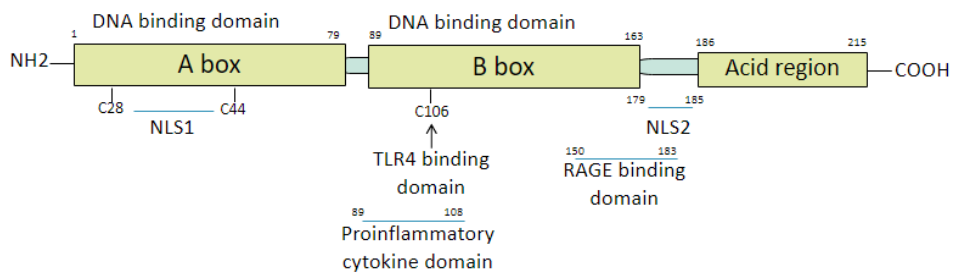


Figure 5. Schematic view of HMGB1 structure. HMGB1 protein contains 215 amino acids divided into three different regions: A and B box and an acidic region. A box that contains a DNA binding domain, nuclear localization site 1 (NLS1), and two of the three cysteines of the sequence. B box contains a DNA binding domain, TLR4 (Tol-like receptor 4), and RAGE (Receptor for advanced glycation end-products) receptors binding domains and a proinflammatory cytokine domain. Between the B box and the acidic region is located the nuclear localization site 2 (NLS2). Own elaboration figure based on (70).

The function of HMGB1 in physiological conditions is to maintain nuclear homeostasis by regulating transcription, DNA damage repair, and the formation of nucleosomes (71). Besides, it can translocate to the cytoplasm under certain circumstances and that's the reason of its name. The nuclear and cytoplasmic forms of the protein are different regarding their oxidative state, the presence of disulphide bonds, and the posttranslational modification pattern (69). Over the past years, HMGB1 has been described as a protein involved in inflammation and signalling cellular stress when it is translocated to the cytoplasm or even released to the extracellular space and delivered into the blood stream. When HMGB1 is located outside of the nucleus it acts as a DAMP molecule and it has been associated with the pathogenesis of different diseases such as cancer, autoimmune diseases, or sepsis (69). The release of HMGB1 into the extracellular space and the bloodstream could be triggered by a passive and active mechanism. Passive release occurs when the cells are under a process of cell death such as necrosis, pyroptosis, or necroptosis in which the cell membrane is disrupted, and the cellular content is released to the extracellular space (69). In this scenario, HGMB1 will act as a DAMP-promoting inflammation by its binding to RAGE and CXCR4 (Chemokine C-X-C type 4 receptor) which will trigger the migration of immune cells (72). On the other hand, the active release of HMGB1 is done mainly by monocytes, enterocytes, and endothelial cells and it occurs when cells are exposed to any stress such as the one caused by a pathological process in which sepsis or trauma are included (73). The active release needs firstly the translocation of HMGB1 from the nucleus to the cytoplasm. Little is known about this type of release, but acetylation and

phosphorylation of the protein are necessary to perform the secretion, which is thought to be driven by secretory exosomes or lysosomes (69). In this case, the released protein could present different states of oxidation which will change its functions. If there is mild oxidative stress in the environment, the cysteines of the A box will form a disulfide bond promoting the acute proinflammatory function of HMGB1. However, if the oxidative stress is continued, the three cysteines are completely oxidized, and this causes the proinflammatory functions of HMGB1 to be neutralized, leading to the resolution of inflammation (72). As mentioned before, HMGB1 has proinflammatory effects, including detrimental effects on the endothelium (69). Circulating HMGB1 can bind endothelial cells via TLR4 and RAGE receptors, and it has been shown to induce increased expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) which are involved in the rolling and adhesion of leukocytes to the endothelium leading to the transmigration of immune cells across the endothelial monolayer, a critical step in the inflammatory process (74).

Therefore, it has been proved a link between HMGB1 and the regulation of vascular permeability (75). The endothelial disruption induced by HMGB1 is shown to be time and dose-dependent and it is due to the trans-endothelial electrical resistance in vascular cells by RAGE and p38 MAPK (mitogen-activated protein kinase) signalling pathway (76,77). A study performed by Ito and collaborators showed that the detrimental effects of HMGB1 on the endothelium could be limited by proteolytic cleavage of the protein by thrombin-thrombomodulin complexes (78), pointing to regulatory signalling which reduces the action of HMGB1 on the endothelium, probably by its oxidative state (69). Since HMGB1 is known to be released into the extracellular space and subsequently into the bloodstream, affecting endothelial homeostasis (*Figure 6*), its use as a possible biomarker molecule in diseases that occur with inflammation such as sepsis could be a good approximation. In mice sepsis models, HMGB1 plasma levels are found to be elevated (79), and interestingly, these higher levels arise later than other potent inflammatory cytokines such as IL-6 or TNF- α (79,80). In the case of sepsis in patients, plasma levels of HMGB1 remained high over time and had their maximum after the onset of sepsis, which contrasts with other cytokines (80–82). Also, the high serum levels of HMGB1 in septic patients have been linked to an induction of

the disruption of the endothelial barrier, forming endothelial F-actin stress fibres and starting the redistribution of VE-cadherin (83).

Due to the kinetics of HMGB1, targeting the protein as a therapeutic agent prompt it as a more effective treatment than other proinflammatory cytokines as the time of the onset of septic patients is usually undetermined. An effective treatment is to inhibit the action of HMGB1 using HMGB1-antagonist interacting with RAGE which will bind to the receptor but without activating the proinflammatory signaling pathway (84–86). Another way of inhibiting the action of HMGB1 is the use of antibodies against the protein which block its function and they have been demonstrated to increase survival (26,87). Many different treatments targeting HMGB1 have been tested, mainly in mice but also in humans, such as Zingerone, small molecules inhibitors of HMGB1, peptide inhibitors, herbal extracts, and herbal compounds which decrease the release of HMGB1 or anticoagulant agents. All these treatments aimed to increase survival, reduce the secretion of HMGB1, inhibit its proinflammatory effects, or ameliorate vascular permeability (26,86–89).

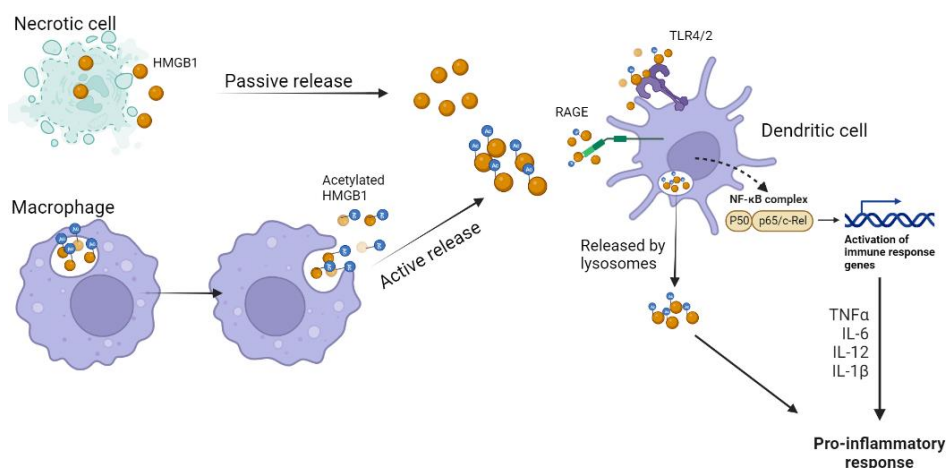


Figure 6. Releasing mechanism of HMGB1. HMGB1 can be actively secreted by immune cells in response to stress such as infection or an inflammatory stimulus, or passively released by damaged cells. When HMGB1 is in the extracellular space can bind to TLR4, TLR2, and RAGE receptors triggering the translocation of HGMB1 from the nucleus to cytoplasm and outside of the cell through lysosomes and the binding of HMGB1 to its receptors will also activate NF-κB complex leading to the release of more proinflammatory molecules. RAGE: Receptor for Advanced Glycated End-products. TLR4/2: Toll-like receptor 4/2. IL: Interleukin. TNF-α: Tumour Necrosis Factor α. Own elaboration figure using BioRender.com based on (89)

Therefore, the presence of HMGB1 is indicative of inflammatory and necrotic processes and it could be postulated as a late predictor of developing a septic shock and

mortality (80,82). The usefulness of HMGB1 as a potential biomarker in sepsis and septic shock depends on the ability to clearly define the effect that it has on endothelial cells and other cell types frequently affected in sepsis as hepatocytes since treatments aimed at eliminating its presence improve the prognosis of sepsis patients (86,90).

3. Autophagy

Autophagy constitutes a relevant highly conserved catabolic process in eukaryotic cells, linked to lysosomes, in which misfolded proteins, and damaged organelles, among others, are degraded. It plays an essential role in energy balance, homeostasis, and cell turnover (91,92). Similarly, it represents a mechanism of cell survival through the suppression of apoptosis and plays a key role in signalling cell stress, leading cells to survival or cell death (67). In addition, there is consistent evidence of the role of autophagy in regulating inflammation and the immune system (93–96). Therefore, it is possible to think that autophagy must be closely related to the pathophysiological processes of sepsis and in fact, our group has recently demonstrated it (67).

As previously highlighted, autophagy plays a crucial role in maintaining cellular homeostasis. For instance, when a cell is confronted with a state of nutrient deprivation, it can activate the autophagy pathway, enabling the transduction of this stimulus into catabolic and anabolic responses. In this scenario, the cell can degrade proteins and cellular organelles to generate ATP (adenosine triphosphate) as a source of energy (91). This process, known as *autophagy by starvation*, involves the formation of vesicles that sequester proteins or organelles and deliver them to lysosomes for degradation. The specific uptake pathways employed for lysosomal delivery vary and include microautophagy, macroautophagy, and chaperone-mediated autophagy, depending on the mode of cargo sequestration (92).

On the one hand, chaperone-mediated autophagy is a type of autophagy for degrading proteins which are marked with KFERQ sequence and therefore they are recognized by the chaperone HSC70 which leads them to the lysosomes for being degraded (97). On the other hand, microautophagy consists of the direct engulfment of the cellular content by the lysosomes (91). However, macroautophagy, hereinafter

called autophagy, is a multistep process in which proteins are entrapped inside a structure called *autophagosome* (which must be previously formed) and then this autophagosome containing the proteins is fused with a lysosome and within the lysosome, the protein is finally degraded (91,98). Macroautophagy is the preferent pathway used for the degradation of damaged organelles (92) and the inhibition of this pathway drives the cell to apoptosis (99).

Therefore, autophagy is involved in various functions and processes of the immune system, through protection against external pathogens and different danger signals that can alter cellular homeostasis, playing a fundamental role in the promotion and regulation of inflammatory responses in the cells of the innate immune system (92).

Mechanism of the autophagic machinery

The mechanism of autophagy is divided into different steps which are represented in *Figure 7*. Autophagy is activated when there are damaged proteins or organelles that must be degraded, so the first step in this process is the selection of the cargo. The cargo proteins are ubiquitinated and this ubiquitination functions as a mark so that they are recognized by the p62/SQSTM1 (sequestosome 1) protein, which contains a ubiquitin-binding domain. Subsequently, p62 interacts with LC3B-II, which is part of the autophagosome, thus releasing the cargo to the autophagosome (100). Other proteins like ALFY (Autophagy-linked FYVE (Fab 1 (yeast orthologue of PIKfyvE), YOTB, Vac 1 (vesicle transport protein), and EEA1) protein) or NBR1 (neighbour of BRCA1 gene 1) can also bind to ubiquitinated proteins and interact with the autophagic machinery to deliver cargos (101,102). However, there is evidence that cargo selection in autophagy may also be a non-specific mechanism (91). The next step is the formation of the autophagosome which will be responsible for receiving the cargo and delivering to the lysosome for degradation. The process is divided into three different stages: initiation, nucleation, and elongation (see *Figure 7*) (91).

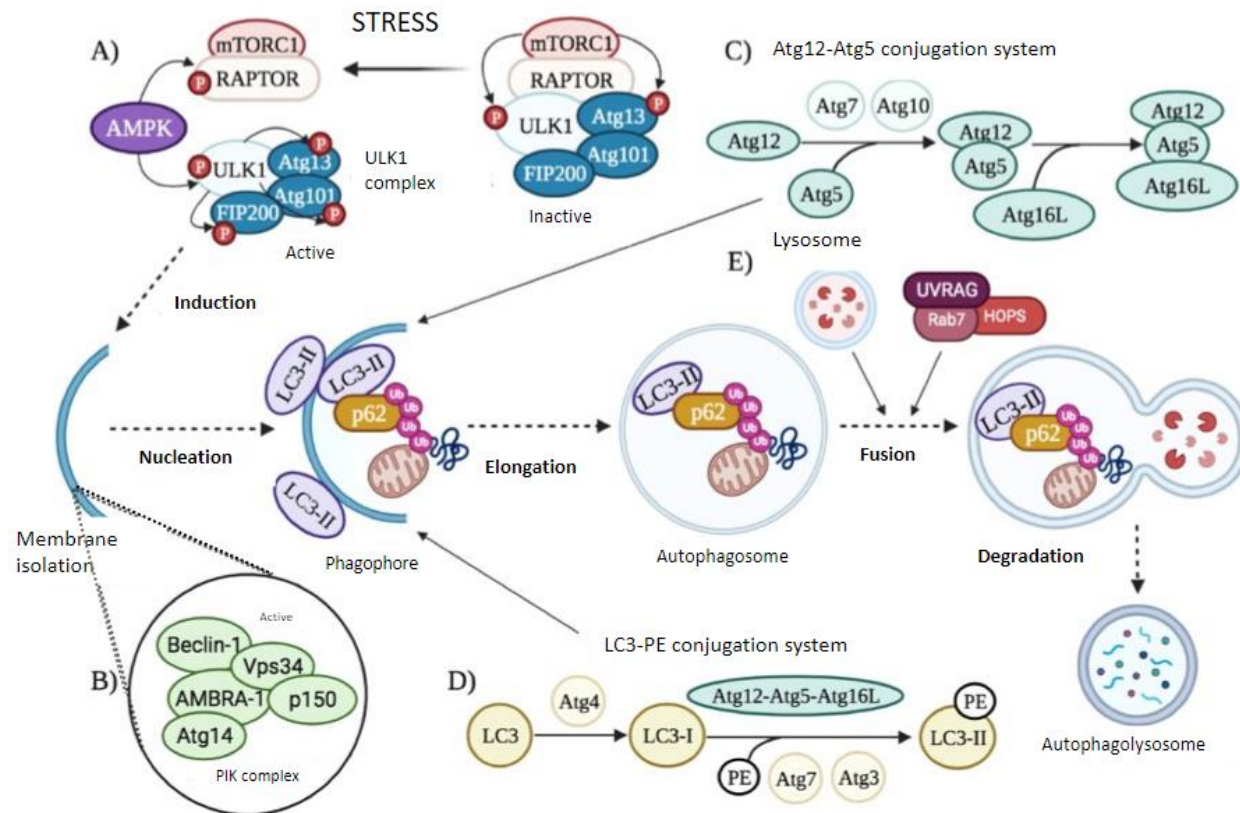


Figure 7. Diagram of the different stages of autophagy. The different complexes that participate in the process are detailed: in the induction phase ULK complex is involved (A), while the PI3K complex participates in nucleation (B). The ubiquitin-type conjugation systems Atg12-Atg5 (C) and LC3-PE (D) are involved in the elongation phase of the phagophore. Finally, the interaction of UVRAG with HOPS stimulates Rab7 to promote the fusion of autophagosomes with lysosomes (E). Own elaboration figure created with BiorRender.com, based on (91,103).

The initiation process is regulated by the Atg1/ULK complex which regulates autophagy induction. In most cases, stress signals are transduced in the cell by activating autophagy in a mTORC1-dependent manner. In these cases of cellular stress, such as under nutrient deficit situations, mTORC1 is inactivated and this leads to the activation of the ULK1/Atg1 complex. The ULK complex dissociates from mTORC1, and it is then when phosphorylation by AMPK activates the complex and with it the formation of a phagosome (91,104–106). But for the formation of the phagophore, a membrane source is also needed. It has been shown that different compartments of the cell can be the source of the membrane used for the formation of the phagophore, among them the mitochondria (107), the endoplasmic reticulum (108) and the Golgi apparatus (109,110). It has also been proven that the plasma membrane can be used for the formation of the phagophore (111). The nucleation process (*Figure 7B*) is the step by which different complexes and the membrane source unite so that the phagophore grows and can house the protein to be degraded. Different complexes such as PtdIns 3-kinase (Vps34, Vps15, Vps30/Atg6, and Atg 14) are involved in this process (104). VPS34 is the enzyme responsible for phosphorylating phosphatidyl inositol and this leads to the formation of phosphatidyl inositol triphosphate, essential for phagophore growth. Another protein that binds to the VPS34 and Beclin-1 complex is AMBRA1 after being phosphorylated by ULK1, for phagophore growth (91).

Elongation is the step in which the phagophore bends and grows to create a double-membrane structure called an autophagosome where it retains the cargo to be degraded. The elongation process is regulated by two ubiquitin-like conjugation mechanisms. The first is the Atg5-Atg12 conjugation mechanism (*Figure 7C*) which, when bound to the Atg16L protein, forms a trimeric complex that can bend the previously formed phagophore. The other conjugation system is the LC3B-PE (*Figure 7D*). In this step, LC3B becomes LC3B-I through the intervention of Atg4 and later, thanks to the Atg12-Atg5-Atg16L trimeric complex and other proteins such as Atg7 and Atg3, convert LC3B-I into LC3B-II and conjugate it with PE. The combination of both conjugation systems manages to elongate and seal the phagophore in a vesicle shape so that the autophagosome is formed. At this point, LC3B-II is found both on the inside and outside of the autophagosome. Therefore, the LC3B-II that is located in the internal part,

when bound to the cargo will be degraded together with it, while the LC3B-II of the external part will dissociate from the autophagosome when the union with the lysosome occurs (91). The last step is the formation of the *autophagolysosome* (Figure 7E). In this part, the autophagosome fuses with a lysosome but it is also thought that first, the autophagosome fuses with endosomes, and then this structure fuses with a lysosome (91,112). The fusion is regulated by many different proteins among which, the HOPS complex stands out. Other important regulatory proteins are SNAREs which are proteins mediating the transport of vesicles (91). Once the autophagolysosome is formed, the cargo protein will be in contact with the digestive enzymes of the lysosome and so it will be degraded.

As we have previously mentioned, autophagy is an essential cellular process that maintains cellular homeostasis by removing misfolded or unfolded proteins and damaged organelles, so when autophagy is not well balanced could be pathogenic. Mutations in autophagy-related genes have been observed in inflammatory diseases, indicating the significance of autophagy in immune responses (95). When the organism faces oxidative stress, autophagy is also induced and, in this scenario, p62 is dependent on NF- κ B, which is negatively regulated by mTOR (113). NF- κ B triggers the production of pro-inflammatory cytokines upon rapamycin-induced mTOR inhibition. However, autophagy is inhibited upon TNF α -mediated activation of NF- κ B, suggesting the complex interplay between autophagy and inflammation (114,115).

Endoplasmic reticulum stress is another form of cellular stress that triggers the unfolded protein response (UPR) to remove misfolded or unfolded proteins by activating stress transducers like PERK, IRE1, and ATF6. However, when the UPR clearance capacity is exceeded, autophagy serves as a pro-survival mechanism to degrade these accumulated proteins. Bcl-2, a key player in the interplay between autophagy and ER stress, localizes both at mitochondria and the endoplasmic reticulum (116,117). However, the anti-apoptotic function of Bcl-2 is lost when it gets phosphorylated by JNK. Phosphorylated Bcl-2 fails to bind to the pro-apoptotic members and also increases the endoplasmic reticulum release of Ca²⁺, resulting in the mitochondrial uptake of Ca²⁺ and leading to apoptosis (91,118).

3.1 Non-canonical forms of autophagy

Canonical autophagy involves the formation of double-membrane-bound autophagosomes that engulf cytoplasmic components, which subsequently fuse with lysosomes to degrade their contents. However, recent studies have described new functions of the autophagic machinery, known as non-canonical autophagy, which link it to the processes of phagocytosis and endocytosis (119).

One key feature of non-canonical autophagy is that it does not require the hierarchical intervention of all the ATG proteins involved in canonical autophagosome formation. Instead, various components of the autophagic machinery, including LC3B, are involved in the conjugation of LC3B to the phagosome and endosome membranes. This suggests that non-canonical autophagy may be able to bypass some of the steps involved in canonical autophagy, such as initiation and nucleation (120,121). Furthermore, during non-canonical autophagy, the double-membrane-bound autophagosome may not necessarily elongate from a single source (120). This contrasts with canonical autophagy, where each of the four steps in autophagosome formation involves the hierarchical activity of ATG proteins, as well as non-ATG proteins such as PtdIns3KC3, p150, AMBRA1, and DFCP1 (121).

Despite these differences, the precise mechanisms underlying non-canonical autophagy are still under debate. One proposed mechanism suggests that non-canonical autophagy bypasses the proteins involved in elongation and closure (ATG7, ATG5, and LC3B), while another suggests that it bypasses proteins that are important for initiation (ULK1) and nucleation (BECLIN1) (122).

Beclin-2 independent autophagy

Canonical autophagy is regulated by the Beclin 1-PI3KC3 complex, which plays a crucial role in the induction and maturation of autophagosomes. However, Non-canonical beclin-1-independent autophagy can bypass some of the hierarchical activity of ATG proteins and the Beclin-1 complex. This type of autophagy occurs in response to specific stimuli, such as pro-apoptotic compounds (120,123). This type of autophagy has also been observed in settings unrelated to cell death, including viral infection (124), differentiation (125) and bacterial toxin uptake (126). These findings suggest that pro-

death and noncanonical autophagic inducers may have therapeutic potential in treating cancer when the function of canonical autophagy proteins is compromised (120,122). Recent research has shown that different membrane sites may be involved in non-canonical autophagosome formation (120,123). For example, resveratrol has been found to promote WIPI-1-dependent LC3B lipidation in the absence of induced phagophore formation (127). This type of autophagy has also been related to the endocytic entry of pathogens that links different ATG proteins at the plasma membrane(123).

These findings suggest that there may be different pathways for autophagic degradation that are independent of the Beclin 1-PI3KC3 complex. However, the mechanisms of non-canonical autophagy are not yet fully understood, and future studies are needed.

ULK-1 independent autophagy

Recent studies have revealed the existence of a non-canonical form of autophagy that bypasses the ULK1 initiation step. This form of autophagy has been observed in response to ammonia or glucose deprivation, and it employs other routes of entry in addition to the evolutionarily conserved ULK1-dependent pathway (123). This finding has been supported by various studies that have shown that autophagy does not always require AMPK activity and therefore the inhibition of autophagy mediated by mTORC1 is not always possible (128,129).

One example of non-canonical autophagy induction is the induction of autophagosome formation by cytosolic DNA that binds and activates cGAMP. This process occurs in an ULK1/2-independent manner, and its mechanism remains an open and interesting question (130). Another example is the induction of non-canonical autophagy by LPS in mouse embryonic fibroblasts, which involves the proteins Atg5 and Atg7 (131).

Overall, the discovery of non-canonical autophagy has broad implications for our understanding of autophagy and its role in various physiological and pathological conditions (120–122). These findings suggest that the regulation of autophagy is more

complex than initially thought and that multiple pathways may contribute to autophagy initiation. Future research may elucidate the precise mechanisms underlying non-canonical autophagy and its potential therapeutic applications.

Non-canonical autophagy and xenophagy

Cells have a mechanism known as xenophagy, which is a form of selective autophagy that helps prevent the invasion of pathogens in the intracellular environment (132). This mechanism involves the formation of phagosomes, which can be recruited to single-membraned vacuoles originating from the plasma membrane (123). Recent studies suggest that there is a connection between autophagy and phagocytosis and that the core autophagy machinery is required for phagosome maturation (133). Toll-like receptors play a role in the recruitment of LC3BII to the phagosomal membrane, which is necessary for phagosome maturation and its fusion with lysosomes (134). The recruitment of autophagy proteins to single-membrane vacuoles is not limited to the entry of pathogenic organisms, as it has been observed in entotic vacuoles and macropinosomes (135). An example could be the endocytosis of TREM-1 and TLR-4 for their recycling in microglia (119). This targeting is dependent on the PtdIns3KC3 complex and the ATG12-ATG5 and LC3BII conjugation systems, but not on upstream mTORC1 elements or the ULK1 and ULK2 complexes (136). The implications of the recruitment of autophagy proteins to these single-membraned vacuoles which are being endocytosed are still not completely understood and require further research.

In conclusion, by removing damaged organelles and improperly folded proteins, autophagy acts as a crucial mechanism for preserving cellular balance. Its complex interaction with inflammation is mediated by complex signaling pathways and key players like NF κ -B and Bcl-2. Thus, autophagy plays a protective role against foreign pathogens and danger signals that upset cellular homeostasis, and it is tightly related to several immune system processes. This engagement becomes essential for organizing and controlling inflammatory reactions inside innate immune cells, which is a crucial step in the development of sepsis. Understanding how autophagy is regulated and how it affects various cellular functions could lead to the discovery of novel therapeutic targets and open up new treatment options for a variety of conditions, including sepsis.

This knowledge has recently been complicated by the discovery of non-canonical autophagy, which highlights the need for more research into its mechanics and potential uses.

3.2 Relationship between autophagy, extracellular histones, and sepsis

Autophagy has been implicated in the regulation of sepsis and pyroptosis; however, the role of autophagy in these processes remains controversial (137). Autophagy is a pro-survival pathway that can be upregulated in response to hypoxia, starvation, and cellular damage. During an infection like in sepsis, autophagic vesicles help to pathogens clearance by engulfing them and driving them to lysosomal degradation (133). In sepsis, autophagy regulates among other functions, the energy metabolism of diverse cell types such as immune cells (137). Autophagy can also act as a safeguarding mechanism against the occurrence of multiple organ dysfunction during sepsis (138). Autophagy has a modulatory effect on pyroptosis, playing an important role in the host's defense against pathogens, but excessive or dysregulated pyroptosis can lead to tissue damage and exacerbate inflammatory responses. Autophagy can negatively regulate pyroptosis by degrading DAMPs and PAMPs (137) and also through the increased generation of reactive oxygen species, which occurs before inflammasome activation. A study made by Kim and collaborators proved that in the early phase of *Streptococcus pneumoniae* infection, ROS can stimulate cell autophagy and in turn inhibit pyroptosis, thus having a pro-survival function (139).

The mTOR signalling pathway, which is a key regulator of cell growth and metabolism, when blocked leads to an increase in autophagy and thus the inhibition of pyroptosis (137). Rapamycin, a specific antagonist of mTOR, has been shown to inhibit the pyroptosis of macrophages and reduce the levels of inflammatory cytokines in a caecal ligation puncture-induced sepsis mouse model. In this work, they showed that the activation of autophagy by rapamycin lead to a decrease in inflammation having a protective effect on the spleen, lungs, and liver (140). Another drug used for increasing autophagy is α -mangostin and it has been proved that upon its treatment in macrophages stimulated with LPS, it prevented the activation of the NLRP3 inflammasome. This, in turn, resulted in a decrease in the release of IL-1 β and in

rebalance in the phagocytic activity of macrophages (141). Studies have demonstrated that metformin, a well-known drug used to treat diabetes, has a protective effect on patients with sepsis. Those who used metformin before hospital admission had a significantly lower mortality rate compared to those who did not (142). Metformin has also been shown to alleviate pyroptosis in various pathological conditions, such as ischemia-reperfusion injury, inflammatory bowel disease, and diabetic cardiomyopathy. This protective effect may be due to metformin's ability to enhance autophagy by inhibiting the inflammasome NLRP3, with the AMPK-AKT-mTOR signaling pathway playing a crucial role in this process (142,143).

However, autophagy may also promote pyroptosis under certain circumstances. Some studies made in a mouse model of sepsis have suggested that the loss of Atg16L1 or Atg7 can lead to the overactivation of the canonical pyroptosis pathway, accompanied by the abundant release of inflammatory cytokines such as IL18 or IL-1 β . In this exacerbated inflammatory scenario, the survival rate of the mice was diminished (144,145). However, whether autophagy acts as a negative modulator of pyroptosis under any circumstances is still controversial. More research is needed to elucidate the complex interplay between autophagy and pyroptosis and to identify the specific conditions and mechanisms under which autophagy exerts its regulatory effects.

On the other hand, a relationship between HMGB1 and autophagy has been shown. Studies have demonstrated that HMGB1 can safeguard against oxidative stress by promoting the process of autophagy and impeding apoptosis (69). HMGB1 in the cytoplasm can bind to beclin1 to generate autophagy initiation complexes that eliminate detrimental oxidative stressors, including those stemming from mitochondria. This binding between HMGB1 and beclin1 happens in the C23 and C45 domains, and alterations to these cysteines hinder the formation of disulfide bridges, thereby reducing autophagy (146). HMGB1 also protects beclin1 and ATG5 from calpain-mediated cleavage, ensuring that autophagy continues in response to redox stress. However, intracellular HMGB1 can be degraded through proteolytic cleavage by caspase-1 during sepsis, suggesting the existence of regulatory pathways to control intracellular HMGB1's effects (69).

The role of HMGB1 in the autophagic pathway in endothelial cells needs to be further defined, especially concerning its interaction with the RAGE receptor. This is important because the RAGE receptor has been linked to autophagy activation in pancreatic cancer and the formation of NETs. Understanding the relationship between HMGB1 and the RAGE receptor can help to identify potential therapeutic agents for sepsis treatment that activate autophagy. Additionally, it is necessary to compare the effects of HMGB1 to those of circulating histones in this context (69). Studies have shown that autophagy plays an important role in the formation of NETs and can be activated through the RAGE receptor in pancreatic cancer (147,148). This highlights the potential of autophagy activators as therapeutic agents for sepsis (149). Previous research by the same group has demonstrated that the addition of histone extracts to human umbilical cord cultures can cause endothelial cell dysfunction, affecting nitric oxide production and superoxide generation (150). Additionally, it has been observed that histones can activate the apoptotic pathway and autophagy in a dose-dependent manner, with TLRs involved in the apoptotic response but not autophagy (67).

The vascular endothelium is a critical regulator of many physiological processes, including inflammation, blood clotting, immune response, and control of vascular tone and blood pressure. These functions are governed by the activation of TLR receptors, which are present on the surface of endothelial cells. However, during the course of sepsis, deregulation of these responses can lead to serious alterations in the endothelium, ultimately leading to the deterioration of the endothelial barrier, alterations in blood flow dynamics, and even multi-organ failure (62). Studies suggest that the release of histones during sepsis significantly influences the endothelial cells that line the blood vessels, reducing their viability and leading to apoptosis. Once these cells enter apoptosis, they acquire procoagulant and pro-adhesive characteristics, which further contribute to the pathogenesis of the disease (67). However, it is currently unknown whether the alteration of autophagy could occur as a cellular response to try to eliminate histones, which would require their internalization first (62,67). It is crucial to reveal how cells internalize histones and/or survive contact with them harbours the potential to design new therapeutic strategies aimed at reducing nuclear protein-related cytotoxicity. Understanding the underlying mechanisms and signaling pathways

that mediate opposing responses in the context of sepsis can aid in the development of new therapeutic strategies aimed at reducing nuclear protein-related cytotoxicity. Therefore, further research is needed to elucidate the signalling pathways that mediate opposing responses, such as survival or cell death, in the context of sepsis.

4. Experimental models for studying sepsis and septic shock

Animal models

In recent years, significant resources have been allocated to study the pathophysiology and treatment of sepsis from basic science to clinical trials with extremely high costs (151). Despite promising findings from preclinical studies involving animal models, most clinical trials testing novel sepsis drugs have been unsuccessful in demonstrating effectiveness (152).

Various animal models have been utilized to replicate sepsis in humans; among others, the animal model that has been more used is the mouse due to its similarities in the development of the pathology (153). Sepsis can be modelled in mice using lipopolysaccharide (LPS) which is a type of endotoxin. This molecule is the primary component of the gram-negative bacterial cell wall and can trigger the release of inflammatory mediators from different cell types (152). The procedure involves injecting LPS into the body to trigger systemic inflammation without establishing an active infection. The host immune system is stimulated by pattern recognition receptors on immune sentinel cells that identify foreign bacterial structures, and triggers immune cell activation, release of inflammatory mediators, and vascular/hemodynamic changes. This immune activation resembles the outcome of human sepsis such as endothelial cell damage, resulting in microcirculatory dysfunction, and eventually, multi-organ failure (153). However, the response to endotoxins can vary considerably among different species. If animals that are naturally resistant to endotoxin are exposed to high doses of it, they may experience toxic effects that are not observed in endotoxin-sensitive species like humans (152).

Another approach to the simulation of sepsis using animal models is the intravascular infusion of living bacteria in mice. The strain of endotoxin that is most used is rarely seen in human bacteraemia, which raises concerns about the significance of the findings (152,154). Furthermore, in most cases, patients are not exposed to an overwhelming bacterial load simultaneously. Instead, they harbour a septic focus that intermittently and continuously disseminates bacteria throughout the body, so this model despite the benefits do not seems to resemble human sepsis (152,154).

Peritonitis models are a valuable tool for sepsis research as they provide a reliable and uncomplicated approach. The most frequently used model is the cecal ligation and puncture (CLP) model, which closely resembles the progression and characteristics of human sepsis (152). The CLP model involves three different types of damage, including tissue trauma from laparotomy, necrosis from cecal ligation, and infection due to peritoneal microbial leakage, leading to peritonitis and translocation of bacteria into the bloodstream. This activates the inflammatory response, ultimately resulting in septic shock. However, the CLP model has limitations, as the onset of peritonitis is uncontrolled and depends on the timing of gastrointestinal perforation (152,155).

Animal models have played a crucial role in understanding the pathophysiology of sepsis, but it is important to carefully evaluate how well they reflect the septic picture in humans. While there are limitations to various models, animal simulations have provided insights into sepsis biomarkers, improved therapy regimens, and shed light on the pharmacokinetics, toxicity, and mechanism of action of drugs. To fully grasp the pathogenesis of sepsis, it may be helpful to use different model variants on different animal species, as this can provide common, objective criteria for evaluating experimental results (152,156).

***In vitro* experimental models**

There are several types of cellular models for studying sepsis, including cell culture models and organoid-like models. Cell culture models involve the isolation and culturing of specific cell types involved in the septic response. These cells can be

stimulated with various agents, such as endotoxins, to mimic the inflammatory response seen in sepsis (157). Organoids and organ-on-a-chip models, on the other hand, involve the creation of miniature organ systems using microfluidic devices. These systems can mimic the function and interactions of multiple cell types in a specific organ (158). Both types of cellular models have advantages and limitations, and their use can provide complementary information to animal models in the study of sepsis. To study the molecular basis of the pathophysiology, a simpler model such a cellular line can be the one of choice. Cellular models have the advantage of being easier to handle, require less space in the lab facilities, are cheaper and have less ethical issues.

The main organs affected during sepsis are liver, circulatory system, kidneys, brain, lungs and heart (159), so a way to study the effect of sepsis in one of these organs is to use a specific cell type model. Sepsis can cause significant damage to the cellular and molecular mechanisms involved in kidney function, which can lead to acute kidney injury (AKI) and even long-term renal dysfunction and is associated with increased mortality (160). To study this molecular mechanism renal tubular epithelial cells are abundantly used (161–164). Cardiac dysfunction is quite common in septic patients, so the use of cardiomyocytes (165–167) or cardiac macrophages (168,169) for understanding the process is a very convenient option.

One of the key factors in the progression of sepsis is the dysfunction of endothelial cells. Endothelial cells play a critical role in maintaining vascular homeostasis, and during sepsis, they become activated and contribute to the immune response and the coagulation system. Because of their central role in sepsis, endothelial cells are a major target for therapy (170,171). For this reason, an important amount of sepsis research uses endothelial cells as experimental model. In our group, HUVEC (Human Umbilical Vein Endothelial Cells) cell cultures treated with extracellular histones to promote inflammation have been widely used for modelling sepsis. With this model our group has shown the role of extracellular histones in activating apoptosis and autophagy (67), disarranging vasoactive mediators release (172), triggering oxidative stress-dependent induction of the NF- κ B/CAM pathway via TLR4 (173), or activating endothelial NLRP3 inflammasome (174), among others. However, these cell cultures do not reflect the complexity of vascular endothelium as they are a monolayer of a unique

cell type. Therefore, several models have been established which employ various types of cells obtained from stem cells or adult tissues *in vivo*, with single or multiple lineages, to construct vascular networks that try to mimic human vascular anatomy and function (175). Previous studies have utilized two-dimensional co-culture of separately differentiated endothelial cells and pericytes, as well as three-dimensional culture of early vascular progenitor cells, to generate networks consisting of both endothelial and pericyte cells (176–178). The development of self-organizing 3D human blood vessel organoids (BVOs) from human pluripotent stem cells (hPSCs) is considered one of the most significant advancements in the field of modeling human blood vessels. These BVOs accurately mimic the functional, morphological, and molecular characteristics of human microvasculature, complete with an endothelium, a continuous basement membrane, and surrounding mural cells (179,180). Furthermore, these BVOs can be transplanted *in vivo* and connected to the endogenous circulation, making them ideal models for studying cardiovascular diseases or tissue tropism in virus infections, such as SARS-CoV-2 (179). In summary, various models have been developed to mimic human vascular anatomy and function using cells obtained from stem cells or adult tissues. The most significant advancement is the development of self-organizing 3D human blood vessel organoids from human pluripotent stem cells, which accurately replicate the characteristics of human microvasculature. These organoids can serve as valuable models for studying the formation and function of blood vessels in health and disease. They can also be used to test the efficacy of new therapies for vascular disorders and to screen for potential drug candidates (181).

In conclusion, the use of these BVO as an experimental model of sepsis stands out as a very interesting approach, since this model provides a three-dimensional structure and several cell types, which make it more similar to human blood vessels and could help to simulate the disease in a more precise way. This model could be suitable for drug screening and to reveal insights into endothelial deregulation which affects many diseases, being sepsis one of them. Therefore, the results obtained with the use of this experimental pluricellular model, in combination with the use of the specific cell line monocultures, could provide a deeper insight on the molecular basis of the pathophysiology of sepsis, bringing us closer to more effective treatments.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

This project is based on the hypothesis that the cytotoxicity exerted by nuclear proteins (histones, HMGB1) when released to the bloodstream is mediated by interactions with the cell membrane that lead to changes in endocytic and autophagic processes. These mechanisms can be specific for either histones or the nuclear protein HMGB1, and importantly, they can be related to changes in the expression pattern of specific miRNAs. Thus, the fine dissection of the molecular pathways triggered by a series of biological models in response to the exposition of histones, under different conditions that modulate endocytosis and autophagic fluxes, can shed light on the potential of histones, HMGB1, and miRNAs to better understand sepsis pathophysiology, as well as provide insights into their potential as disease biomarkers for sepsis patient's stratification, monitoring and prognosis.

OBJECTIVES

This thesis project has been structured around two main objectives: first, to define and characterize the specific interactions between extracellular histones and the cell membrane, as well as the consequences of these interactions regarding the endocytosis / autophagy pathways and the regulation of gene expression by inflammation-related miRNAs; second, to use this knowledge in order to develop specific biomarkers to be tested in a clinical context. To further expand these general objectives, we define a series of specific objectives (SO):

OBJECTIVE 1: Dissection of the molecular mechanisms underlying the effects of extracellular histones.

SO1.1. To analyze the interaction between histones, the cell membrane and endocytic or autophagic machinery using labeled proteins and fluorescence co-localization studies.

SO1.2. To study the alterations in the endocytosis, autophagy, and miRNA-mediated signaling pathways produced after the addition of histones to different cell lines.

SO1.3. To analyse the effect of inhibitors and/or activators of autophagy, on the molecular processes mediated by the addition of purified histones using different cell cultures and 3D vascular organoid models.

OBJECTIVE 2: Study the potential of the nuclear macromolecule HMGB1 as sepsis and/or septic shock biomarker.

SO2.1. To measure the plasma levels of HMGB1 in sepsis and septic shock patients

SO2.2. To correlate plasma levels of HMGB1 with clinical parameters used in the diagnosis, stratification and prognosis of sepsis and septic shock patients.

MATERIALS AND METHODS

1. Cell culture

1.1. 2D culture

HeLa and Hep3B cell lines were purchased from ATCC (American Type Culture Collection). Specifically, HeLa cells were cultured on Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L of glucose and L-glutamine (Thermo Scientific, USA) supplemented with 1% v/v of penicillin/streptomycin (P/S) and 10% v/v of fetal bovine serum (FBS) (Invitrogen, USA.). Hep3B cells were cultured on MEM (Modified Eagle medium) medium (Thermo Scientific, USA) with the same supplementation as HeLa cells but also with 1% v/v of non-essential amino acids and 1% v/v of sodium pyruvate (Thermo Scientific, USA).

HUVEC cells were purchased from Cultek (Lonza, Cultek, Spain). They are a pool of primary cells from several donors to avoid donor variability. The surface of plates and flasks were covered with 1% w/v gelatin (Sigma-Aldrich, United Kingdom). They were cultured on specific growing medium EGM-2 (Endothelial Growth Medium 2) (Lonza, Cultek, Spain) supplemented with 5% v/v of FBS, 0,04% v/v of hydrocortisone, 0,4% v/v of hFG-B (Human Basic Fibroblastic Growth Factor), 0,1% v/v of VEGF (Vascular Endothelial Growth Factor), 0,1 % v/v of R3-IGF-1 (Long Arg3 Insulin-Like Growth Factor-1), 0,1% v/v of ascorbic acid, 0,1% v/v hEGF (Human Epidermal Growth Factor), 0,1% v/v of GA-1000 (Gentamicin sulfate/Amphotericin), 0,1% v/v of heparin (Lonza, Cultek, Spain) and 1% v/v P/S.

All the cell types were incubated at 37°C and 5% v/v CO₂. The passage of cells was done when the cell culture reached an 80% confluency, removing the media, washing cells with phosphate buffer saline (PBS) and detaching them with EDTA (Ethylenediaminetetraacetic acid) trypsin (Sigma-Aldrich, United Kingdom). All cell types were tested for mycoplasma regularly.

1.2. Blood vessel organoids

Blood vessel organoids were obtained through the differentiation of either H9 embryonic stem cells or NC8 human iPSC (induced pluripotent stem cells). The protocol

of BVOs obtention is detailed in (182). Stem cells were grown on Matrigel covered plates with Essential 8 medium (Thermo Scientific, USA). To start the differentiation process and obtention of BVO, iPSC must have been carried out at least two splits to ensure their genetic stability. The differentiation process last fourteen days, following this the process of obtention is explained day by day (Figure A.1 in Annex I section).

Day 0: Suspension of single cell iPSC

iPSC colonies are disrupted and seeded in suspension as single cells to form embryo bodies. The medium of confluent iPSC cells is removed, and they are washed with PBS. After, 1 mL of 0.5 mM EDTA is added, and cells are incubated for 5 min at 37°C. EDTA is removed after the incubation carefully and 1 mL of StemPro Accutase (Thermo Scientific, USA) is added and cells are incubated for 5 min at 37°C. Detached single cell iPSC are collected into a tube with 3 mL of DMEM/F-12 medium (Thermo Scientific, USA) and centrifuged at 300 rpm for 5 min. Then, the supernatant is removed, and cells are resuspended with 1 mL of “Aggregation Medium” (10 mL KnockOut™ DMEM/F-12, 2.5 mL KnockOut™ Serum Replacement, 125 µL GlutaMAX™ Supplement, 125 µL MEM Non-Essential Amino Acids Solution 100X, 125 µL Antibiotic-Antimycotic 100X, 8.75 µL β-mercaptoethanol (Thermo Scientific, USA) and 0.1% of 50 µM “Rock Inhibitor” (Y-27632 DihydrochloridE) (Cayman Chemicals, USA). Finally, cells are seeded into an ultra-low attachment 6 well plate (Corning, USA) in a density between 200000 and 400000 cells/well and incubate for one or two days to form the embryo bodies.

Day 1: Addition of mesoderm induction medium

Once iPSC single cells aggregate and form embryo bodies the medium is changed. Therefore, embryo bodies were collected into a tube and let them precipitate by gravity for 30 minutes. After that, the supernatant is removed and the embryo bodies are firmly resuspended with 3 mL of “day 1 mesoderm medium” which is composed of 10 mL of “N2B27 medium” (25 mL DMEM/F-12, 25 mL Neurobasal Media, 1mL B-27™ Supplement 50X serum free, 0.5 mL N-2 Supplement 100X, 250 µL GlutaMAX™ Supplement, 0.5 mL Antibiotic-Antimycotic 100X and 35 µL β-mercaptoethanol (Thermo Scientific, USA)), 24 µL of ChIR (12uM) stock 5mM used in 1:416.5 dilution (Tocris Bioscience, UK) and 3µL BMP4 (30 ng/mL) stock 100 µg/mL used in 1:3333 dilution (Miltenyi, Biotec, Germany).

Day 2 and Day 3: Resting in mesoderm induction medium

During day two and day three embryo bodies will be left at the incubator with the “day 1 mesoderm medium” added day 1. Big aggregates are broken down by pipetting.

Day 4: Addition of vascular induction medium

Embryo bodies are collected into a tube and let them precipitate by gravity for 10 minutes. After that, the supernatant is removed, and the embryo bodies are firmly resuspended with 3 mL of “vascular induction medium” which is composed of 10mL “N2B27 Medium”, 10 µL of 100 ng/mL VEGF-A used in 1:1000 dilution (Miltenyi Biotec, Germany) and 10 µL of 2µM forskolin used in 1:1000 dilution (Sigma-Aldrich, United Kingdom).

Day 5: Resting in vascular induction medium

During day five embryo bodies are left at the incubator with the “vascular induction medium” added day four. Big aggregates are broken down by pipetting.

Day 6: Embedding organoids into collagen/Matrigel® matrix

In day six, organoids are going to be harvested and placed into a new 12 well plate where they are going to be embedded in a collagen/Matrigel matrix for starting the vessel sprouting.

- Preparation of collagen/Matrigel® matrix

Preparation of “Medium Components Layer1”. For an entire 12 well plate, dispense to each tube the following components:

- 954 µL Ham's F-12 Nutrient Mix (Thermo Scientific, USA)
- 751,2 µL 5x DMEM (Thermo Scientific, USA)
- 73.2 µL MiliQ water
- 38.4 µL GlutaMAX™ Supplement (Thermo Scientific, USA)
- 75.6 µL HEPES 1M (Thermo Scientific, USA)
- 58.8 µL 7.5% sodium bicarbonate (Thermo Scientific, USA)

- Preparation of collagen

4 mL of Collagen solution 3mg/mL is added by reverse pipetting 4mL. Then, the liquid on “medium component layer 1” is transferred to the “collagen layer 1” tube. The mixture will turn yellow because collagen is dissolved on acetic acid,

so 50 μ L of 1M NaOH are added to neutralize it. The color will change to red again.

- Preparation of Matrigel®

1.5mL of Matrigel® are added into a new tube containing 4.5 mL of the collagen previously prepared.

Once the first layer of collagen/Matrigel® is prepared, 500 μ L of it are added to each well of the plate and the plate is incubated 30 min at 37°C. In the meantime, the organoids were collected into a tube and let them precipitate by gravity for 10 minutes. After that, the supernatant is removed, the pellet is washed with 2 mL of DMEM/F12 twice and the pelleted organoids are kept on ice for 10 min. The second layer of collagen/Matrigel® matrix is prepared as previously explained. Pelleted organoids are resuspended with 1.5 mL of collagen/Matrigel® matrix and then 500 μ L of the mixture are added per well. The plate is incubated 5 min at room temperature and then 2 h at 37 °C. After the incubation, 1 mL of “Maturation Media” (17 mL of StemPro 34 medium (50 mL of StempPro34 SFM 1x, 1.3 mL of SP34 and 0.5 mL GlutaMAX™ Supplement (Thermo Scientific, USA), 3mL of fetal calf serum, 20 μ L of 100 ng/mL VEGF-A in a dilution 1:1000 (Miltenyi Biotec, Germany) and 20 μ L of 100 ng/mL FGF-2 in a dilution 1:1000 (Miltenyi Biotec, Germany) is added and the plate is incubated for 3 days without any perturbation.

Day 7 and 8: Resting in maturation medium

During day seven and eight organoids are left at the incubator with the “maturation medium” added day six.

Day 9: Refresh media

Medium must be removed tilting the plate and very carefully without touching the gel. Then, 1 mL of maturation medium is added drop by drop in the middle to each well.

Day 10: Individualization of organoids

When organoids reach the appropriate size, they need to be transfer individually to an ultra-low attachment 96 well plate. The matrix with the organoids embedded are removed from the well with the help of a spatula and a spoon and place it into a Petri

dish. With the help of the microscope and two needles, individual organoids are separated, and the matrix is removed. After, each organoid is placed in one well of a 96 well plate with 170 μL of sprouting medium and incubated for two days.

Day 11 and 12: Resting in sprouting medium

During day eleven and twelve individual organoids are left at the incubator with the “sprouting medium” added day ten.

Day 13: Refresh media

Medium must be removed tilting the plate and very carefully without aspirating the organoid. Then, 150 μL of sprouting medium is added to each well.

Day 14: BVOs ready to use

At day fourteen blood vessel organoids will be ready to use and any treatments (Table 2) can be performed with them.

Table 2. Reagents used for cell culture treatments.

Reagent	Concentration	Techniques
Core histones	10-500 $\mu\text{g}/\text{ml}$	Image techniques, protein analysis, Flow cytometry, RNA analysis
H1-488	10-100 $\mu\text{g}/\text{ml}$	Image techniques and flow cytometry
H3-GFP	10-200 $\mu\text{g}/\text{ml}$	Image techniques
Metformin	2 mM	Image techniques, protein analysis
Krebs-Henseleit medium		Image techniques, protein analysis, Flow cytometry, RNA analysis
NH_4Cl	20 mM	Image techniques, protein analysis, Flow cytometry, RNA analysis
Leupeptin	100 nM	Image techniques, protein analysis, Flow cytometry, RNA analysis

1.3. Treatments

Table 2 shows the reagents used on different cell cultures and their concentrations and the techniques that have been employed.

1.3.1. Histone treatments

Several types of extracellular histones were used:

- Core histones consist of a mixture of H2A, H2B, H3 and H4 histones purified from calf thymus (Sigma-Aldrich, United Kingdom). The concentration used varies from 10 µg/ml to 500 µg/ml.
- Histone H1-488 consists of purified H1 from calf thymus with a 488-fluorescent tag (Thermo Scientific, USA). The concentration used varies from 10 to 100 µg/ml.
- Histone H3-GFP (Histone 3-Green Fluorescent Protein) consists of a home-made purified histone H3 with a GFP tag. The concentration used varies from 10 to 200 µg/ml.

Extracellular histones were added directly to the medium of cultured cells and incubated at 37°C during the specific time depending on the experiment.

1.3.2. Autophagy treatments

For activating autophagy two different treatments were used. For HeLa and Hep3B cells a treatment with metformin (Sigma-Aldrich, United Kingdom) 1mM was added to the cultured cells for 24 hours. For BVOs and HUVEC cells Krebs-Henseleit medium (Thermo Scientific, USA) supplemented with 2.1 g/L of sodium bicarbonate and 0.373 g/L of calcium chloride was used. So firstly, medium was removed, cells were rinsed with PBS and afterwards Krebs-Henseleit medium was added to the cells and incubated for a convenient time (depending on the experiment) at 37°C.

For inhibiting autophagy, 20mM NH₄Cl (Sigma-Aldrich, United Kingdom) and 100 nM Leupeptin (Sigma-Aldrich, United Kingdom) were added to the medium of cultured cells and incubated for different times at 37°C.

1.3.3. Blocking endocytosis treatment

For blocking endocytosis cells were incubated at 4°C for 15 min prior any treatment, and after this 15 min on ice, treatment was accomplished, and the cells returned to the ice for the entire duration of the specific treatment.

1.4. Transfection

HeLa cells were transfected with 2.5 µg of pIRES-Neo-H3.3-GFP plasmid kindly provided by Dr. Sandra B. Hake (Institute for Genetics, University of Giessen, Germany)

using 1 mg/ml polyethyleneimine (PEI) (Polysciences, Germany) in Opti-Mem (Gibco, Thermo Scientific, USA). HeLa cells were incubated for 24h with the mixture of PEI and plasmid and afterwards medium was replaced by DMEM media. Transfected cells were analysed by confocal microscopy (Zeiss, USA) maintaining cells at 37°C and 5 % v/v CO₂, using bright field and argon laser at 488 nm.

2. Protein analysis

2.1. Protein extraction

Hela, Hep3B and HUVEC cells were seeded until they reached 80% confluency, specific treatments were made and afterwards medium was removed, and cells were put on ice immediately. After that, a maximum of 200 µl RIPA buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.5% w/v sodium deoxycholate, 0.1% v/v sodium dodecyl sulphate (SDS), 1% v/v Triton X-100, 0.1% protease and phosphate inhibitors cocktail (Sigma-Aldrich, United Kingdom)) was added to the plate and cells were harvested by scrapping. The cells were collected into cold Eppendorf tubes and incubated in rotation for 10 min at 4°C. Afterwards, tubes were vortexed and centrifuged for 10 min at 15000 g. The supernatant containing total protein extract was collected.

For protein quantification Pierce™ BCA protein assay kit (Thermo Scientific, USA) was employed. It is a colorimetric method by which the formation of Cu⁺¹ is detected, by means of bicinchoninic acid (BC(A), by the reduction of Cu⁺² by the proteins present in an alkaline medium. Briefly, 200 µl of a BCA reagent (a mixture 1:50 of component A and (B), was added to a 96 well plate. After, 25 µl of several concentrations of BSA (bovine serum albumin) for the calibration curve and 25 µl of protein samples (previously diluted if needed). The plate was incubated for 30 min at 37°C and then the absorbance was read at 562 nm and the protein concentrations were calculated.

2.2. Histone extraction

It is a cell fractionation protocol in which the nuclear fraction predominates while histone enrichment is favoured. To do this, we take advantage of the fact that histones

are soluble in sulfuric acid, and that they precipitate in the presence of TCA (trichloroacetic acid).

Hela and Hep3B cells were seeded until they reached 80% confluency, specific treatments were made and afterwards medium was removed, and cells were pelleted. Pelleted cells were resuspended with 1mL hypotonic Lysis Buffer (Tris-HCl 10 mM (pH = 8), KCl 1 mM y MgCl_2 1,5 mM, orthovanadate (10 $\mu\text{L/mL}$) and protease inhibitor cocktail (Sigma-Aldrich, United Kingdom)), and left for 30 min in the rotatory shaker at 4°C. After that, the tubes were centrifuged for 10 min at 10000 rpm at 4°C and the supernatant was discarded, and the pellet was resuspended with 320 μL of H_2SO_4 0,5 N and left overnight in the rotatory shaker at 4°C. After the incubation, cells were centrifuged for 10 min at 13000 rpm at 4°C, the supernatant was collected and transferred to a new tube and 132 μL of TCA were added. Tubes were incubated on ice for 30 min and afterwards they were centrifuged for 10 min at 13000 rpm at 4°C. The supernatant was discarded, and the pellet was resuspended with 0.5 mL of cold acetone and the tubes were centrifuged 10 min at 13000 rpm at 4°C. The last step needs to be repeated twice. Finally, the pellets were dried for 5 min and resuspended with 50 μL of deionized water. Histone quantification was performed using BCA method as previously described.

2.3. Western Blot

Specific protein detection was made by Western Blot technique which allows the detection and semi-quantification of specific proteins thanks to the antigen-antibody reaction. The concentration of total protein or histone-enriched nuclear fractions were calculated and the appropriate quantity of 5x sample buffer (0.25 M Tris-HCl pH 6.8, 0.5 M DTT (Dithiothreitol), 10 % SDS, 50% glycerol and 0.5% bromophenol blue) was added to load 40 μg of protein to polyacrylamide-SDS gel.

In general, proteins were separated in a 15% polyacrylamide-SDS gel at 130V and after proteins were transferred to a nitrocellulose membrane (Whatman GmbH, Germany) at 100V 1h at 4°C. Afterwards, the gel was stained with Coomassie blue solution (Coomassie R-250 1.25 g. ethanol 250 ml, acetic acid 50 ml and 1200 ml of water) to check that the proteins were transferred to the membrane. For reducing non-specific unions of the antibody, the membrane was blocked with 5x BSA-1xTBS (Tris-

Buffered Saline: 200 mM Tris and 150 mM NaCl)-Tween 0.1% at room temperature for at least 1h. Then the membrane was incubated with a primary antibody diluted with 1x BSA-TBS-Tween 0.1% in agitation overnight at 4°C. Specific primary antibodies used can be found on *Table 3*.

Table 3. Primary antibodies used. WB: Western Blotting, IF: Immunofluorescence, PLA: Proximity Ligation Assay, BVOs: Blood Vessel Organoids.

Antibody name's	Reference	Technique and dilution used
β-actin	A1979 Sigma-Aldrich United Kingdom	WB 1:1000
LC3B-II	L7543 Sigma-Aldrich United Kingdom	WB 1:1000 IF 1:1000 or 1:200 (BVOs) PLA 1:1000
p62 SQSTM1	#5114 Cell Signalling	WB 1:5000
GFP	9996 Santa Cruz Biotechnology, USA	IF 1:200 PLA 1:200
H3	06-755 Merck Millipore, Germany	IF 1:1000 or 1:500 (BVOs) PLA 1:1000
CD31 (mouse)	Ab9498 Abcam, UK	IF 1:100 (BVOs)
CD31 (sheep)	# AF806 R&D Systems, USA	IF: 1:200 (BVOs)

The day after, the primary antibody was removed, and the membrane was washed three times for 5 minutes with 1x TBS-T in agitation at room temperature. The corresponding secondary antibody was added (anti-mouse (Sigma-Aldrich, NA931V) diluted 1:5000 in 1x BSA-TBS-Tween 0.1% or anti-rabbit (Sigma-Aldrich, NA934V) diluted 1:10000 in 1x BSA-TBS-Tween 0.1%) and incubated for 1h in agitation at room temperature. Then, the secondary antibody was removed, and the membrane was washed as previously described. For chemiluminescent detection, the membrane was incubated with 1mL of a 1:1 mixture of the reagents from the ECL™ Western Blotting Detection Reagents development kit (Amersham GE Healthcare Bio-Science AB, Sweden) and after this the chemiluminescent signal was detected using the image capture (ImageQuant LAS-4000, General Electrics, USA).

3. Image techniques

3.1. Immunofluorescence

HeLa, Hep3B and HUVEC cells were seeded on 13 mm diameter glass crystals inside twenty-four well plates until they reached the convenient confluency, and the corresponding treatments were made. Afterwards, medium was removed, and cells were rinsed with 1x PBS. For fixation and permeabilization 300 μ L of methanol was added to the cells and they were incubated for 15 minutes at -20°C . Then, methanol was removed, cells were washed three times with PBS-Tween 0.1% for 5 minutes at room temperature in agitation and they were blocked with 10% FBS in PBS-Triton 0.1%. Cells were incubated with blocking solution for at least 1h at room temperature and after that time, blocking solution was removed and primary antibody (see *Table 3*) diluted in blocking solution was added and incubated overnight at 4°C . The next day, primary antibody was removed, the cells were washed three times with PBS for 5 minutes and they were incubated with corresponding secondary antibodies (Alexa-Fluor-488-conjugated goat anti-mouse; A11029, Invitrogen, USA) and Alexa-Fluor-Texas Red-conjugated goat anti-rabbit; T862, Invitrogen USA) diluted 1:2000 in blocking solution for 1h at room temperature. Finally, the crystals were mounted on slides with DAPI-Fluoromount-G[®] mounting medium (Southern Biotech; USA) for nuclei detection and to later be observed under a confocal or fluorescence microscope.

For some experiments, instead of using a primary antibody to mark lysosomes (LC3B-II), a fluorescent probe was used. Briefly, after convenient treatment was made to the cells, generally, the medium was removed and 75nM of LysoTracker Red DND-99 (Invitrogen, USA) was to the cell culture medium and incubated for 30 minutes at 37°C . Afterwards, the medium with LysoTracker was removed, and the immunofluorescence protocol was followed as explained above.

3.1.2 BVOs immunofluorescence

Blood vessel organoids were individually seeded in a 96-well plate and the corresponding treatments were made (five organoids were used per condition). Afterwards, organoids were collected into 2 mL tubes and the medium was removed

carefully. For fixation 1 mL of 4% PFA (paraformaldehyde) was added to the organoids, and they were incubated for 2 hours at room temperature. Then, PFA was removed, organoids were washed twice with PBS for 15 minutes at room temperature in agitation and they were simultaneously permeabilized and blocked with 1 ml of blocking solution (3% FBS, 1% BSA, 0.5% Triton X-100 and 0.1% Sodium deoxycholate in PBS) and incubated for 2 hours at room temperature. After that time, blocking solution was removed and primary antibody (see *Table 3*) diluted in blocking solution was added and incubated overnight at 4°C. The next day, primary antibodies were removed, the organoids were washed three times with PBS-Tween 20 0.05% for 15 minutes and they were incubated with DAPI (Invitrogen, USA) diluted 1:750 in blocking solution and the corresponding secondary antibodies (Alexa-Fluor-488-conjugated goat anti-mouse (Invitrogen, USA), Alexa-Fluor-555-conjugated goat anti-sheep (Invitrogen, USA), Alexa-Fluor-647-conjugated goat anti-rabbit (Invitrogen, USA) and Alexa-Fluor-555-conjugated goat anti-mouse diluted 1:250 in blocking solution for 2h at room temperature. Then, the secondary antibodies were removed, and organoids were washed twice with PBS-T and once with PBS. After that organoids were incubated with 300 µL of RapiClear (Sunjin Lab, Taiwan) for 1 h at room temperature for tissue cleaning. After that, the organoids were mounted on a home-made special slide and 80 µL of mounting gel (Sunjin Lab, Taiwan) was added to each sample and another slide was placed on top to seal organoids. Finally, organoids were observed under a confocal microscope. To create visual representations of vascular networks and complete blood vessel organoids, we utilized a confocal microscope equipped with a 10x lens. To ensure optimal z-sectioning when using ZEN software, z-stacks were obtained with a z-resolution.

3.2. Proximity Ligation Assay

Proximity Ligation Assay (PLA) is a technique used to study protein-protein interactions by providing a single fluorescent signal for each interaction event (183). The basis of this technique is represented on *Figure 8*.

HUVEC cells were seeded into eight well plate with glass bottom (IBIDI GmbH, Germany) until they reached 80% confluency, and the appropriate treatments were made. After that, medium was removed, cells were rinsed with 1x PBS, and they were

fixed and permeabilized with methanol for 15 min at -20°C. Then cells were washed three times with 1x PBS for 5 minutes and they were blocked with 100 µL of Duolink® II Blocking Solution (Sigma-Aldrich, United Kingdom) for 1h at 37°C. After the incubation, the blocking solution was removed, and two specific primary antibodies (*Table 3*) diluted with 1x Duolink® II Antibody Diluent (Sigma-Aldrich, United Kingdom) were added and incubated overnight at 4°C. The day after, the antibodies were removed, cells were washed twice with TBST-Tween 0,01% and cells were incubated with the Duolink® in situ PLA® Anti-rabbit PLUS (Sigma-Aldrich, UK, DUO92002) and Anti-mouse MINUS (Sigma-Aldrich, UK, DUO092004) for 1h at 37°C. Then, the probes were removed, and the cells were washed twice with TBS-T 0.01% for 5 min in agitation. Ligation and amplification steps were performed using the in-situ detection reagent kit Duolink® Red (Sigma-Aldrich, UK, DUO92008-100RXN). For ligation, DuoLink® Ligation Stock was diluted 1:5 with ultrapure water and then the ligase was added to the mix in a dilution 1:40. After, 100 µL of the ligation solution were added to the cells, incubated for 30 min at 37°C. Then the ligation solution was removed, and cells were rinsed twice with TBS-T 0.01%. For amplification step reagents and cells must be protected from light. DuoLink® amplification stock was diluted 1:5 with ultrapure water and then the polymerase was added to the mix in a dilution 1:80. After, 100 µL of the amplification solution were added to the cells, incubated for 100 min at 37°C. After, polymerase solution was eliminated and cells were washed once with 1x SSC buffer for 2 min and then with 0,01x SSC buffer for 2 min. Finally, for nuclei detection DAPI Fluoromount-G® (Southern Biotech, USA) was added. All the incubations done at 37°C were made in a humid chamber and all the washing steps were done in agitation. The dots were counted manually, and the results were expressed as the number of interactions (dots) per number of nuclei in each field. The interactions per cell of each condition are expressed as a percentage of the control condition.

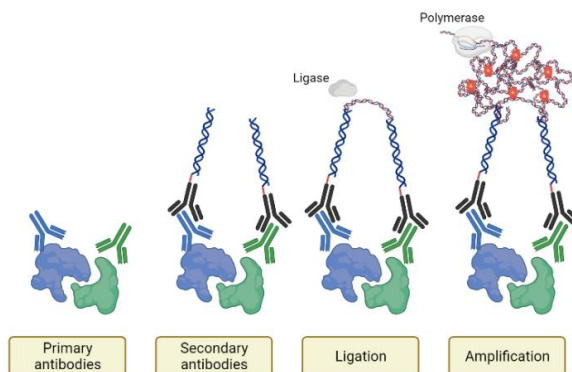


Figure 8. Proximity Ligation Assay molecular basis scheme. PLA is an immunocytochemistry-based technique used for the quantitative analysis of the interaction between two proteins of interest. Firstly, the cells need to be fixed, permeabilized and stained with specific antibodies which must be from distinct species. After, the primary antibodies are detected with secondary antibodies linked to complementary oligonucleotides (MINUS and PLUS probes). If the proteins are close enough (less than 40 nm), the ligase will unite them and afterwards the signal is amplified with a polymerase with fluorescent-labelled nucleotides. Figure of own elaboration using Biorender.

3.3. Confocal microscopy

The slides with the immunofluorescence crystals were placed in a TCS SP8 X confocal microscope equipped with a white laser and 4 spectral detectors, one of them hybrid Leyca HyD (Leica Microsystems, Germany), and observed at 40,000x magnification in immersion oil. A minimum of ten images of each condition were captured when the sample was illuminated with photons of different wavelengths, which indistinctly excited DAPI (365 nm laser), Alexa-488 (488nm laser), Lysotracker™ (576nm laser) and Texas Red (594nm laser), depending on the experimental design.

3.4. Image analysis

The colocalization degree between autophagy, marked by immunofluorescence with LC3B antibody or Lysotracker™ probe and extracellular histones, marked by immunofluorescence with H3 antibody or H1-488 was analysed by different image analysis tools.

3.4.1. Image analysis tool “Image J Macro”

A bank of 200 immunofluorescence images was created as described in the previous section, which included HeLa and Hep3B images treated with histone concentrations of 0, 100, or 200 µg/mL. Using the image bank, we proceeded to verify

the possibility of correctly delimiting cytoplasm and specifically identifying lysosomes and histones in all types of images. Once this possibility was confirmed, the tool was programmed in the specific programming language for ImageJ "ImageJ Macro" (NIH; USA), based on Java.

The tool was created using the ImageJ image analysis software, and it was possible to calculate, automatically and for each cell captured in the images, the percentage of cell area occupied by green fluorescence (theoretically corresponding to histones), the percentage of cell area occupied by red fluorescence (theoretically equivalent to lysosomes), and the percentage of red fluorescence that coincides with green fluorescence (assumed as percentage of lysosome area occupied by histones). *Figure 9* schematically represents the workflow that the tool carries out with each image analysed.

Once the tool is executed from the ImageJ program, the user specifies the cell line to study and selects a folder containing subfolders with images of each experimental condition tested, opening the images one by one and processing them as it is schematized in *Figure 9*. The analysis starts from three images for each visual field, obtained by exciting the preparations with three different lasers (A.1, A.2 and A.3). A.1 shows the cell nuclei and is obtained by illuminating the sample with a 365 nm laser, which excites the DAPI fluorophore. A.2 is obtained by exciting the sample with a 550 nm laser. The LysoTracker Red DND-99, which is a fluorescent probe composed of a fluorophore linked to a weak base, is excited by this laser and emits red fluorescence, and given its basic nature and its ease of penetrating membranes, it allows the identification of acidic organelles such as lysosomes. Image A.3 is obtained by exciting the sample with a 490 nm laser, and theoretically shows the histones present in the visual field, since the cells in the preparations were treated with a rabbit anti-histone H3 antibody and a goat secondary antibody anti-rabbit bound to the FITC fluorophore, which is excited at 490 nm and fluoresces. In *Figure 9*, A represents the fusion of the three channels and corresponds to an unreal image that is never observed under a microscope, generated on the computer by merging the three channels.

Image B is obtained from A.1 using the tool coupled to ImageJ "StarDist" (184), an AI trained with a model that allows to define rounded-shaped nuclei very efficiently. B consists of a binary mask, that is, its pixels can only adopt two values, where the identified nuclei acquire one value and the background a different one. These types of images are useful because they allow individualizing objects to work not with the entire image but with specific parts of it. Once image B is generated, we form image C by processing it with the ImageJ command "Voronoi". This command finds the intermediate distance between the objects of a binary image, which in this case are the nuclei of B, and divides the image into territories defined by the averaged boundaries. The result is an image where each point is included in the territory belonging to the nearest nucleus.

The image E corresponds to a binary image where the objects are the supposed lysosomes, and it is formed by processing A.2 with the "Subtract background" command and then defining an intensity value that will differentiate the object pixels (lysosomes) and bottom. With the "Subtract background" command, we define a maximum size of what will be considered as an object, and we subtract intensity from all those image pixels that apparently form part of larger objects. Thus, it is possible to differentiate the intensity of pixels corresponding to lysosomes from the rest of the cytoplasm, and it is easy to establish an intensity level to form a binary image that contains only lysosomes. Image D is also obtained directly from A.2, but in this case, it is a binary mask of the entire cytoplasm of the cells, and not just the lysosomes. It is possible to define the cytoplasm of the cells from A.2, which in theory should only show lysosomes, thanks to the fact that the cytoplasm of HeLa and Hep3B cells present autofluorescence when excited with the 550 nm laser and make them distinguishable from the background. To form it, A.2 is subjected to a contrast enhancement that seeks to further increase the differences in intensity between pixels with autofluorescence of cytoplasm and pixels corresponding to the background. This was the only differential step between the analysis of HeLa and Hep3B, since in Hep3B a single iteration of this contrast enhancement was sufficient to distinguish background cytoplasm and in HeLa two were necessary. Once the contrast has been increased, A.2 is subjected to a blur filter that works by calculating the median of a 5x5 matrix centred on each pixel and giving the

pixel the result as an intensity value. Applying this filter has a double function: on the one hand it softens the edges of the cells, which after sudden increases in contrast are quite irregular; and on the other, they serve to eliminate pixels outside the cells that have accidentally been highlighted with the contrast increases, since they will be surrounded by pixels with low intensity values (background pixels), and the medians of the matrices centered on them will probably be smaller than those of cytoplasmic pixels that are surrounded by pixels with high intensity values.

Regarding the formation of the F image, we proceeded in a very similar way to how E was formed, although in this case we start from A.3. The image is processed with a "Subtract background" that seeks to increase the differences in histone intensities from those of cytoplasm and background, and then a pixel intensity value is defined from which to differentiate the objects and the background in the binary image F. Image G arises from applying to the binary image F a size filter that eliminates all those objects that are larger than a size defined by the user. We established 15 square pixels as the size, after measuring in the images a multitude of objects that presented green fluorescence and determining a value that differentiated what we consider histones from other bodies that presented fluorescence. Thus, misleading histone precipitates or large auto fluorescent artifacts were excluded from further analysis as they were no longer considered as objects.

H is formed by combining information from B, C and D. For this, the tool coupled to ImageJ "BinaryReconstruct" was used, which reconstructs the objects of a binary image that have some pixel in common with the objects of another binary image. In this case, the tool reconstructs the objects of D from the objects of B, in such a way that all the artifacts that are not part of the cells are not reconstructed as they are clearly differentiated objects from the cytoplasm and do not have pixels in common with objects from B. Next, the area occupied by the nuclei was subtracted from the reconstructed cytoplasm (image B). In this way, a binary image corresponding to the cytoplasm without nuclei was achieved, that is, the regions of the image that contain the data of interest, and then the cells of the cytoplasmic mass are individualized in order to obtain measurements at the single cell level. For this, the territories outlined in

C were used, and each pixel of cytoplasm was considered part of the cell with the nucleus closest to it.

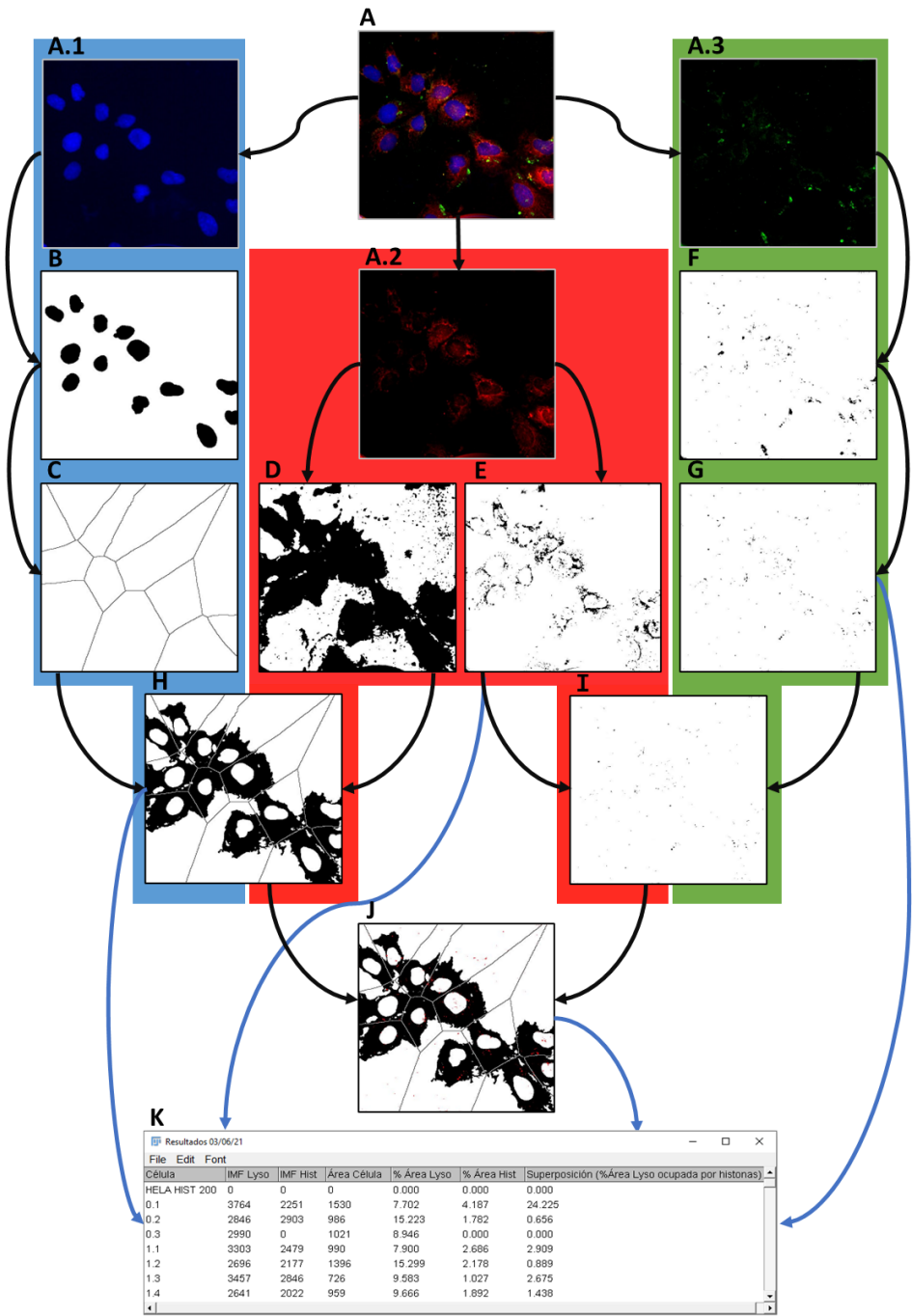


Figure 9. Image analysis tool workflow. The figure shows the process by which the tool isolates specific parts of the cells from the signal of each fluorophore (A.1, A.2, A.3) and creates new images (B-J) on which to obtain

measurements. quantitative variables of interest that are finally collected in a table that includes the results of the entire analysis (K). The black arrows represent image processing that results in new images and in data.

Image I corresponds to the fluorescence corresponding to histones that is superimposed with that corresponding to lysosomes and is formed with a Boolean operation between images: "AND". This function is included in ImageJ's "Image calculator", and it generates an image from two others, with those pixels that in both belong to an object. I is calculated from E and G, by bringing to an image the pixels that are part of objects in both E (lysosomes) and G (histones). Finally, thanks to the measurement zones set to H, it was possible to obtain overlapping data per single cell by working again with the Boolean "AND" operation and the H and I image, so that only the histones overlapping the cells were analysed. cell cytoplasm.

The *Figure 9K* shows the results on each cell analysed were recorded. More data is collected per cell than the overlap data, such as cell area, percentage of cell area occupied by green fluorescence, and percentage of cell area occupied by red fluorescence, which are obtained by measuring areas directly on the binary images that have been generated during the analysis and operating with them. The first column of the table indicates each subfolder that is being analysed and then a two-digit code for each cell analysed, where the first digit indicates the image to which said cell belongs and the second the order of the cell in which it belongs. image in which it appears (from left to right and from top to bottom), and each line of the table contains the data obtained from a single cell. To finish, seeking to avoid losing the results, the tool itself saves the table in txt format (easily exportable to a spreadsheet) in the folder indicated by the user at the beginning.

After completing the development of the image analysis technique for previously optimized culture and treatments conditions with histones, it was used to quantitatively analyse the colocalization between histones and lysosomes from the images obtained previously, during the set-up of the immunofluorescence.

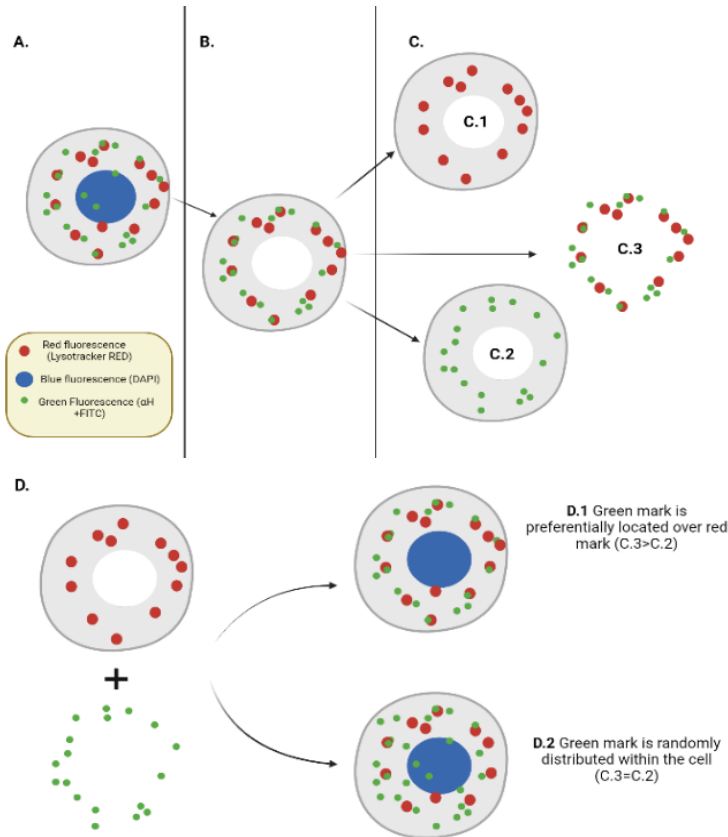


Figure 10. Schematic representation of the analysed images, as well as the quantified variables. (A) Whole cell, with its cytoplasm represented in gray, the DAPI fluorescent label represented in blue, the Lysotracker Red DND-99 fluorescent label in red, and the FITC-linked anti-histone H3 antibody fluorescent label in green. It is appreciated how the fluorescent label from FITC may or may not overlap with the fluorescent label from the Lysotracker. (B) Portion of the cell area that is analysed by the tool. The area corresponding to the nucleus of the cell is eliminated because its histones bind to the anti-H3 antibody linked to FITC, which leads to an overestimation of the number of extracellular histones in the nuclear region. (C) Representation of the variables measured in the cell: C.1, percentage of the cell area occupied by red fluorescent label; C.2, percentage of cell area occupied by green fluorescence label; C.3, percentage of the area occupied by a red fluorescent mark that is also occupied by a green fluorescent mark. (D) Model of possible results for the analysis of the image analysis data: D.1, green fluorescence is located preferentially over red fluorescence, which would be indicative that the histones added as treatment are located preferentially on lysosomes rather than on random points on the cell surface; D.2., Green fluorescence is not preferentially distributed over any region of the cell.

Through the tool, data was obtained at the single cell level on the percentage of the cell area occupied by green mark (histone equivalent), the percentage of cell area occupied by red mark (equivalent to lysosomes) and the percentage of red mark area occupied by green mark (lysosomes colocalizing with histones). In all cases, “cell area” refers to the result of subtracting from the total surface area occupied by a cell in the image, the surface occupied by its nucleus. *Figure 10* shows a schematic representation

of each variable measured by the tool. The regions of the nuclei were excluded from the analysis because they presented green, fluorescent labelling by FITC-conjugated antibodies bound to the cells' own histones, and not to the extracellular histones from the treatments. Prior to analysing the results obtained by the tool, it was theorized that two situations could occur, represented in section D of *Figure 9*. Situation D.1 could suggest that internalization occurs, if the values of C. 3 (percentage of red fluorescent area occupied by green fluorescence) of a cell population were greater than those of C.2 (percentage of cell area occupied by green fluorescence). On the other hand, situation D.2 would indicate that histones do not selectively penetrate lysosomes. This second hypothesis would explain the results in the case that C.3 were equal to C.2.

3.4.2. Leica Software

The quantification of the colocalization analysis was carried out using the colocalization software of the confocal microscope TCS SP8 X (Leica Microsystems; Germany) which takes into account the intensities of the fluorophores in each pixel, giving a percentage of colocalization for each image that reflects the degree of colocalization by the overlap of the green and red fluorescent labels. The software calculates the Person colocalization coefficient and gives as a result the percentage of colocalization between the two marks in the whole image analysed.

4. Flow cytometry

For determining the cytotoxic effect of histone, autophagy and blocking endocytosis treatments in HUVEC, HeLa and Hep3B cells flow cytometry technique was used.

All cell types were seeded in 12-well plates, incubating 24 hours at 37 to 37°C and 5% v/v CO₂, until they achieved around 80% confluency. Subsequently, the cells were subjected to the appropriate treatments indicated previously, to later analyse their impact on the survival capacity by using an Apoptosis detection kit (Inmunostep S.L.; Spain) with Annexin-V labelled with APC (BioLegend, USA) or with FITC (Inmunostep S.L.; Spain) and propidium iodide (Inmunostep S.L.; Spain). To do this, the medium was collected in order to obtain the dead cells which detach from the bottom of the plate,

and then the cells which remained attached to the plate were trypsinized and collected with the medium containing dead cells. After that, cells were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. In this step we had a pellet with dead and alive cells. Then, cells were resuspended in 100µl of labelling buffer (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid/0.1M NaOH (pH 7.4) 1.4M NaCl, CaCl₂ 25mM) and 5 µl of Annexin-V-APC or Annexin-V-FITC and 5 µl of propidium iodide were added and left to incubate for 15 minutes at room temperature. After this time, the samples were analysed using a BD FACSVerse™ Cell Analyzer cytometer (BD Biosciences, USA) and FACSuite software. A 561 nm excitation laser was used for propidium iodide, a 488 nm was used for FITC and 633 nm for the APC. The emission wavelengths used were 614 nm for propidium iodide, 525 nm for FITC and 650 nm for APC.

4.1 Cell cycle analysis

For the determination of cell cycle, a million HeLa cells in exponential growth were used. Both, dead floating cells were reserved and mixed with the attached cells after their trypsinization. Then, both cells were fixed using 70% cold ethanol and incubating them for 15 minutes at -20°C. Afterwards, cells were centrifuged at 2000 rpm for 5 minutes and then they were resuspended in propidium iodide solution (50 µg/mL of propidium iodide and 100 µg/mL of RNase diluted in PBS) and they were incubated overnight at 4°C. Finally, the cells were analysed by flow cytometry using BD FACSVerse™ Cell Analyzer cytometer (BD Biosciences, USA) and FACSuite software. A 561 nm excitation laser was used for propidium iodide, and the emission wavelengths used were 614 nm. The histograms of DNA were analysed using Cylchred software for the analysis of cell cycle (University of Cardiff, UK) and the percentages of cells with different DNA content were determined.

4.2 BVOs flow cytometry

For the flow cytometry analysis of BVOs, first they were exposed to appropriate treatment shown in Table 3. Around 20 organoids per condition will be needed to be tested. After the treatments, the organoids corresponding to each condition were collected into a 15mL tube using a cut P1000 tip. When the organoids were precipitated in the bottom of the tube the supernatant was removed and the pellet was washed

twice with PBS. Then, 5 mL of the pre-filtered enzymatic mix (0.4 mg/mL Liberase TH (Merck, Germany), 3 mg/mL Dispase II (Thermo Scientific, USA) in PBS) were added to the organoids. The organoids were incubated with the enzymatic mix for 25 min at 37°C. After, they were resuspended and incubated for 10 min at 37°C. The last step was repeated twice. Then, the organoids/enzymatic mix were poured to 70 µm cell strainer and 45 mL of inhibition medium (DMEM + 10% FBS) were added onto the strainer to recover any cells. Cells were spined down 10 min 300g 4°C and then, a pellet with single cells was obtained. The supernatant was removed and 100 µl of PBS were added to resuspend the cells and they were transferred to a 96-V bottom well plate. The plate was centrifuged for 5 min at 450 g and cells are resuspend in 50 µl Fixable Viability Dye eFluor™ 780 (Invitrogen, USA) per well (1:1000 dilution in PBS). The plate was incubated at 4°C for 30 min and the samples were transferred into FACS tubes and proceed to analyse them by FORTESA cytometer (BD Biosciences, USA). A 633 nm excitation laser was used for APC and a 488 was used for FITC. The emission wavelengths used were 650 nm for APC and 525 for FITC. The data were analysed using FlowJo software (BD Bioscience, USA).

5. RNA extraction and RT/qPCR

Cell cultures were seeded in 6 well-plate and the appropriate treatments were performed, instead in the case of BVOs, they were individually seeded in a 96-well plate and appropriate histone treatments were done (Table 2). After that, at least 10 BVOs per condition were collected into 2 mL tubes and media was removed carefully. After, in both cases the RNA was extracted using RNAeasy kit (Qiagen, Netherlands) following the manufacturer's instructions. Briefly, cells or organoids were first lysed and homogenized using the buffer provided by the kit. Then, ethanol was added to the lysate to provide ideal binding conditions. After, the lysate was loaded onto the RNeasy silica membrane and in which RNA is bound to the silica membrane while contaminants were washed away. Finally, pure concentrated RNA was eluted in RNase (ribonucleasE) free water and the concentration was measured using Nanodrop spectrophotometer (ThermoFisher, USA).

For analysing gene expression, RT-PCR was performed with random primers to convert RNA into cDNA and amplify it using a commercial master mix (LunaScript® RT SuperMix Kit, New England BioLabs, USA). Then, a qPCR was run using the primers shown at Table 4. A commercial master mix, Luna® Universal qPCR Master Mix (New England BioLabs, USA) was used, and cDNA was amplified following these steps:

- Initial Denaturation: 95°C, 60 seconds
- 40 cycles of:
 - Denaturation: 95°C, 15 seconds
 - Extension: 60°C, 30 seconds and plate read

The relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method (185), using the CT (cycle threshold) value of *EEF1A1* in gene expression analysis, as an internal control for the normalization of the CT of each sample.

Table 4. Primers used in gene expression analyses of human blood vessel organoids.

Transcript	Forward primer	Reverse primer
HMGB1	GCGAAGAACTGGGAGAGATGTG	GCATCAGGCTTTCCTTTAGCTCG
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
IL-1 β	CACAGACCTTCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL-1 α	TGTATGTGACTGCCCAAGATGAAG	AGAGGAGGTTGGTCTCACTACC
ICAM-1	AGCGGCTGACGTGTGCAGTAAT	TCTGAGACCTCTGGCTTCGTCA
VCAM-1	GATTCTGTGCCACAGTAAGGC	GGTCACAGAGCCACCTTCTTG
E-Selectin	TCAAGGGCAGTGGACACAGCAA	GGAAACTGCCAGAAGCACTAGG
<i>EEF1A1</i>	GATGGCAATGCCAGTGGAACCA	GAGAACACCACTCTCCACTCGG

For analysing miRNA expression, RT-PCR was performed with specific primers to convert RNA into cDNA for each miRNA and amplify it using a commercial master mix (TaqMan™ MiRNA Reverse Transcription Kit, ThermoFisher, USA). Then, a qPCR was run using commercial specific primers. A commercial master mix, TaqMan™ Universal Master Mix II, no UNG (ThermoFisher, USA) was used, and cDNA was amplified following these steps:

- Initial Denaturation: 95°C, 10 minutes.

- 40 cycles of:
 - Denaturation: 95°C, 15 seconds
 - Extension: 60°C, 30 seconds and plate read

The relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method (185), using the CT (cycle threshold) value of U6 in snoRNA expression analysis, as an internal control for the normalization of the CT of each sample. **ELISA**

Patients

We chose a group of people ($n = 53$) and divided them into three different groups to precisely assess and compare the levels of HMGB1 by ELISA kit in categorizing sepsis and septic shock patients. Every patient who took part in this study gave their informed consent. The first group was made up of healthy persons ($n = 17$), with an average age of 49.76 $\pm$ 5.18 years and consisting of 10 men and 7 females. This group was not expected to have any detectable nucleoproteins in their plasma. The second group, which acted as a control, consisted of nine nonseptic patients ($n = 9$) from the ICU of the Clinical University Hospital of Valencia (HCUV), who had instead had a spontaneous intracerebral haemorrhage rather than being afflicted by an infection, autoimmune condition, or polytrauma injury. The remaining two groups were patients with proven bacteraemia (positive blood culture at 48 hours) who had sepsis ($n = 10$) or septic shock ($n = 17$). These patients came from the same ICU as the previously described control group. The clinical features of the patients are described in *Table 5*. The most notable clinical distinctions, as demonstrated in *Table 5*, were observed between the control group, consisting of nonseptic patients, and the septic shock patients across all standard parameters. The measurements that displayed statistically significant differences among the groups were C-reactive protein ($p > 0.0001$), activated partial thromboplastin time (APTT) ($p = 0.014$), as well as the clinical APACHE II and SOFA scores ($p > 0.03$ and $p < 0.001$, respectively). It is important to note that mortality rate, duration of ICU stay, and other traditional biochemical biomarkers such as lactate and PCT did not exhibit statistically significant differences between sepsis patients and those with septic shock.

Using EDTA tubes, peripheral blood samples were taken from both ICU patients and healthy controls. Blood samples were taken from ICU patients within the first six

hours of their admission. To extract the plasma from each sample, it was centrifuged at 2500 rpm for 10 minutes at ambient temperature. The plasma was then kept at -80°C until analysis.

Table 5. Initial clinical characteristics of the patients with sepsis and the control group admitted to the intensive care unit (ICU). ICU: intensive care unit. APACHEII: Acute Physiology and Chronic Health Evaluation score during the 1st day of ICU admittance. SOFA score: Sequential Organ Failure Assessment score during the 1st day of ICU admittance. CRRT: continuous renal replacement therapy. CRP: C-reactive protein. PCT: procalcitonin. APTT: activated partial thromboplastin time. LOS: length of stay. Significant differences (p value < 0.05) between groups.

	Control ICU non septic	Sepsis	Septic shock	p Value
Age (years) (mean $\pm$ SD)	64,38 $\pm$ 8,31	65,1 $\pm$ 13,1	66,18 $\pm$ 11,6	n.s.
Male gender (%)	66,7	70,8	58,8	n.s.
APACHE II score (mean $\pm$ SD)	15,11 $\pm$ 4,8	15,8 $\pm$ 4,9	22,12 $\pm$ 9,07	0.03
SOFA (mean $\pm$ SD)	3,44 $\pm$ 3,09	5 $\pm$ 2,21	9,65 $\pm$ 3,43	<0.0001
Vasopressor therapy (%)	11,1	20	94,1	<0.0001
CRRT (%)	0	0	11,76	n.s.
Mechanical ventilation (%)	33,3	0	21,4	n.s.
White blood cells (mean $\pm$ SD)	10603 $\pm$ 4939	12977 $\pm$ 11589	16395 $\pm$ 9612	n.s.
CRP (mg/L) (mean $\pm$ SD)	25,14 $\pm$ 26,31	250,2 $\pm$ 133,46	339,35 $\pm$ 189,72	<0.0001
PCT (ng/mL) (mean $\pm$ SD)	0,06 $\pm$ 0,07	2,21 $\pm$ 3,08	1,52 $\pm$ 2,29	n.s.
Lactate (first hour) (mmol/L) (mean $\pm$ SD)	1,61 $\pm$ 6,98	1,91 $\pm$ 1,25	1,81 $\pm$ 1,99	n.s.
Platelets count/L (mena $\pm$ SD)	240400 $\pm$ 89300	229600 $\pm$ 147360	169400 $\pm$ 122800	n.s.
APPT (seconds) (mean $\pm$ SD)	29,67 $\pm$ 2,88	99,833 $\pm$ 118,82	117,43 $\pm$ 154,83	0.014
ICU LOS (days) (mean $\pm$ SD)	5,9 $\pm$ 6,6	12,6 $\pm$ 15,62	9,47 $\pm$ 8,61	n.s.
Hospital LOS (days) (mean $\pm$ SD)	15,4 $\pm$ 14,6	20,6 $\pm$ 13,1	19,88 $\pm$ 17,8	n.s.
ICU Mortality (%)	22,2	20	23,5	n.s.

The following were the exclusion criteria for the ICU controls, sepsis patients, and cases of septic shock: Patients outside the age range of 18 to 85 years, patients with an active neoplastic process, patients receiving antioxidant therapy, patients with a life

expectancy of less than 24 hours, patients with a hospital ward stay longer than 24 hours prior to ICU admission, patients transferred from another hospital, and patients undergoing surgery are examples of patients who fall under this category. Patients who have undergone cardiac resuscitation, those who may have viral infections, and expectant mothers, as well as patients who did not give their consent, were omitted. In patients with sepsis and septic shock, cultures for Gram-negative bacteria revealed positive results in 3 sepsis patients and 11 septic shock patients. In 2 patients with sepsis and 2 patients with septic shock, gram-positive bacteria were found. Nine patients with sepsis and six patients with septic shock had negative results from cultures.

HMGB1 ELISA

Samples were diluted with 30µl of the dilution buffer provided by the kit used, HMGB1 ELISA kit (IBL, Germany). The protocol recommended by the manufacturer was then followed using the sensitive method with a standard curve from 0 to 10 ng/ml. The absorbance was measured in the SpectraMax Plus 384 plate reader (Molecular Devices; USA) and the concentration it was calculated by interpolation of the absorbance values with the concentrations of the standard curve.

The same ELISA kit was used to analyse secreted HMGB1 on supernatant from BVOs. Supernatant samples were diluted 1:3 with the dilution buffer provided and the protocol was performed the same way.

6. Ethics Committee

All participants gave their informed consent statement. The local Biomedical Research Ethics Committee of HCUV gave its approval before the experimental protocols and procedures were carried out, and they did so in accordance with the rules and regulations set forth by the World Medical Association Declaration of Helsinki for conducting ethical research involving human subjects. The INCLIVA Biobank provided the samples and data for this investigation, and their provision was accompanied by the required authorisation from the Ethical and Scientific Committees. Following the principles outlined in the World Medical Association Declaration of Helsinki for ethical

conduct in research involving human subjects, all experimental protocols and methods were carried out with the informed consent statement of patients, with the consent of the local Biomedical Research Ethics Committee of HCUV and in compliance with pertinent policies and regulations. The Spanish Ministry of Science and Innovation (PID2020-119127RA-I00) and the GVA Starting Grant (GV/2018/127) both provided funding for the research study. The INCLIVA Biobank (PT20/0029; B.000768 ISCIII), a member of the Valencian Biobanking Network and the Spanish National Biobanks Network, contributed the samples and patient data used in this investigation. The processing of samples and the gathering of data were done in accordance with standard operating proceeding that had been approved by the Ethics and Scientific Committees.

7. Statistical analysis

The statistical analysis of results from HMGB1 ELISA performed with plasma samples were performed calculating the parameters as mean, median, standard deviation, and confidence intervals. One-way ANOVA and post hoc test (Tukey's test) were used for between-group comparisons. ROC curve analyses were performed by calculating AUC (areas under ROC curves), standard error, confidence interval (CI), optimal concentration cut-off value, and percent sensitivity and specificity for diagnostic and stratification purposes. Values of $p < 0.05$ were considered statistically significant. Analyses were performed by SPSS, v.24 (IBM Corporation, USA)

For the rest of the experiments made by other techniques different from that ELISA, a descriptive analysis of the results was carried out using the GraphPad Prism statistical program (GraphPad Software; USA), calculating the parameters as mean and standard deviation. The data has been represented as the average of at least three biological experiments or independent replicates. One-way or two-way ANOVA followed by post hoc test (Tukey's test) were performed for comparisons between groups, considering statistically significant differences from $p < 0.05$. The asterisks that appear in the graphs show the level of significance of the comparisons, representing * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$ and **** when $p < 0.0001$.

8. Facilities

The experiments were conducted at the Laboratory of Physiopathology of Rare Diseases - Department of Physiology - Faculty of Medicine and Dentistry - University of Valencia and at the Human Disease Modelling Laboratory at IMBA – Vienna (Austria). The facilities of both spaces were utilized, particularly the *“Unidad Central de Investigación”* from Faculty of Medicine with special focus in microscopy and cytometry services and their technicians Guadalupe Herrera and Toni Ibáñez, for which the provided support is gratefully acknowledged.

On the other hand, we would like to thank the INCLIVA Biobank, as well as the HCUV ICU team led by Edurne Carbonell for providing the plasma and clinical data of the patients analysed.

RESULTS

1. HeLa and Hep3B cell models

1.1 Cytotoxic effects of extracellular core histones in HeLa and Hep3B.

Given that it has been demonstrated that the addition of extracellular histones affects cell survival in a dose-dependent manner (67), we aimed to determine the percentage of cell survival under different culture conditions after incubation with extracellular histones, using the immortalized human cell lines HeLa and Hep3B. Commercially purified core histones (see *Materials and Methods*) were used at concentrations ranging from 25 to 500 µg/mL and added to either HeLa or Hep3B culture plates grown to 80% confluency for 0.5, 2, and 4 hours. Cell survival was analysed using an apoptosis study kit for flow cytometry measurement, as described in the Materials and Methods section. The results are shown in *Figure 11*. It is observed that the Hep3B line is more sensitive to treatment with extracellular histones than the HeLa cell line, both in terms of incubation time and the concentration to which they are subjected. The mechanisms of apoptosis induced by extracellular histones are significant after 0.5h incubation time in Hep3B but not in HeLa; however, they are significant in both cell lines after 2h of incubation when compared to untreated cells. On the other hand, cell death is induced in both cell lines at short incubation times when exposed to very high concentrations of histones (500 µg/mL).

The minimum concentration of histones that causes significant changes in either of the two cell lines is 200 µg/mL. Therefore, based on these data and in order to optimize treatments that would enable observation of changes occurring in cells that interact with extracellular histones but survive, experimental conditions for subsequent experiments were established as 4-hours incubation time.

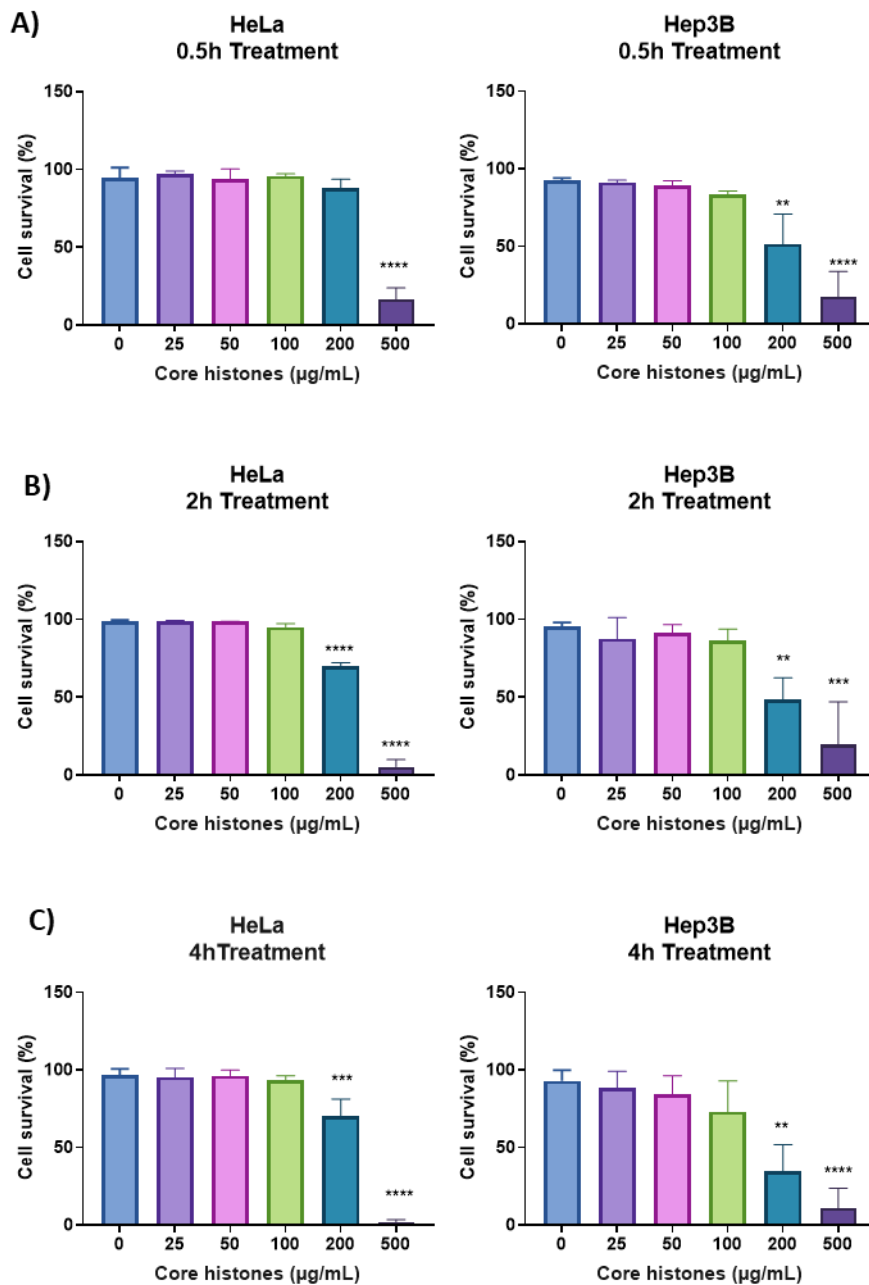


Figure 11. Analysis of histone-mediated cytotoxicity in HeLa and Hep3B cell models. HeLa (left panels) and Hep3B (right panels) cells were incubated in the absence or presence of concentrations of core histones ranging from 25 to 500 μg/mL and maintained for 0.5h (A), 2h (B) or 4h (C). After the indicated incubation times, the cells were harvested and labelled using an Annexin V kit, and subsequently analysed by flow cytometry. The mean values are shown as bars, expressed as a percentage $\pm$ the standard deviation (n=3). Statistical significance was considered at a p-value < 0.05, calculated in all cases using a one-way ANOVA statistical test indicated by ** when $p < 0.01$, *** when $p < 0.001$ and **** when $p < 0.0001$.

1.2 Colocalization studies of core histones and lysosomes in HeLa and Hep3B cells

Once the incubation conditions with extracellular histones were established, immunofluorescence conditions were optimized to study the localization of extracellular histones added to HeLa and Hep3B cell cultures. Both cell lines were treated with histones at different concentrations for 4 hours and then observed and photographed under a fluorescence microscope. Histones, lysosomes, and cell nuclei were labelled using: a rabbit antibody specific for histone H3 and a goat anti-rabbit antibody conjugated to FITC; the acid organelle marker LysoTracker Red DND-99; and DAPI, respectively (see *Materials and Methods* section). *Figure 12* shows examples of images from all experimental conditions studied. It can be observed that in the absence of extracellular histones, all histone labelling locates to the cell nucleus, while in the presence of 100 and 200 $\mu\text{g}/\text{mL}$ of extracellular histones, green fluorescence appears dispersed in perinuclear or cytoplasmic areas. From direct observation of the images, it was not possible to draw conclusions about whether histones were introduced into lysosomes or not, and for this reason, an image analysis routine was developed to provide quantitative data at the single-cell level that could indicate a greater or lesser degree of histone internalization into lysosomes. This analysis was performed automatically from our own image bank created using the aforementioned methodology.

The image analysis tool was successfully developed using the *ImageJ* image analysis software, allowing for the automated calculation of the percentage of cellular area occupied by green fluorescence (theoretically corresponding to histones), the percentage of cellular area occupied by red fluorescence (theoretically equivalent to lysosomes), and the percentage of red fluorescence coinciding with green fluorescence (assumed as the percentage of lysosome area occupied also by histones).

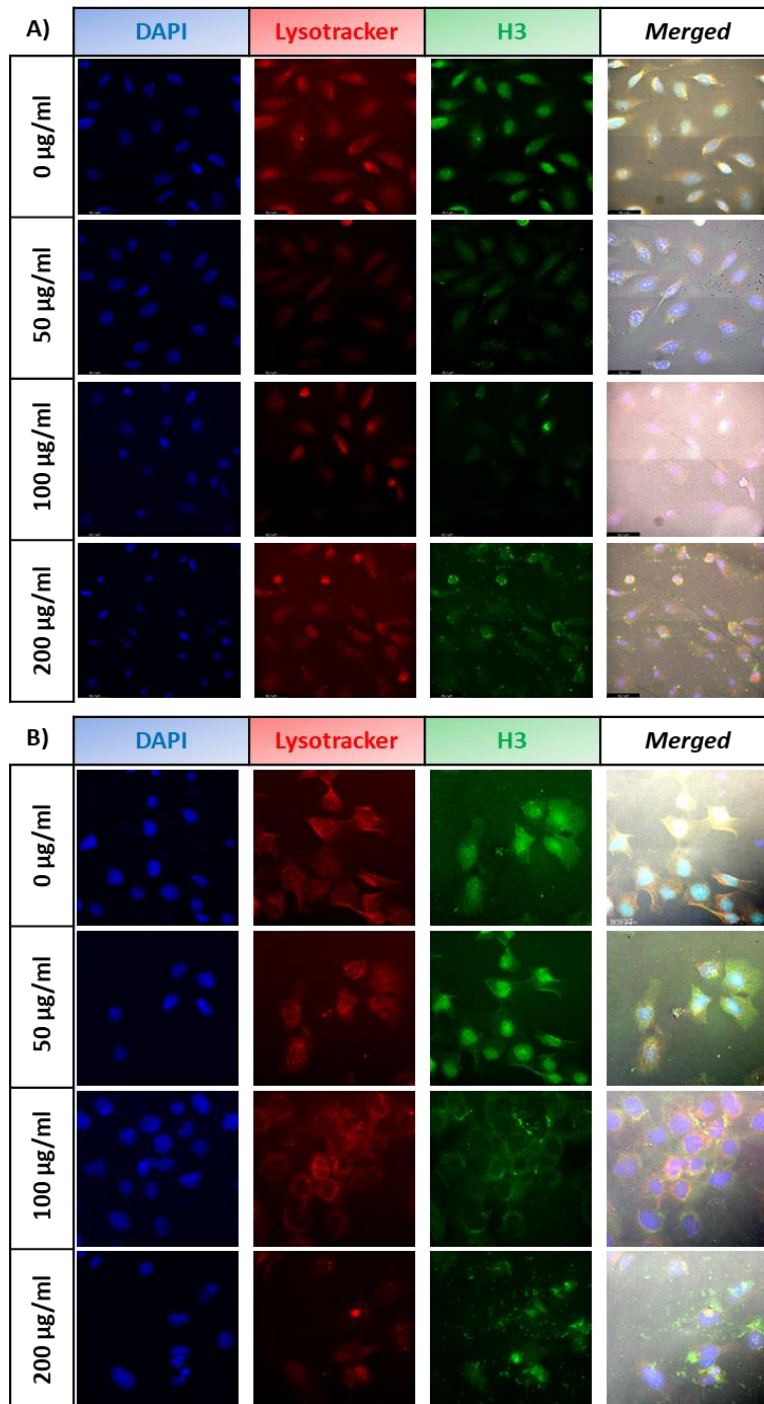


Figure 12. Fluorescence microscopy images of Hep3B and HeLa cells under core histone treatments. Both HeLa (A) and Hep3B (B) were treated with of 0, 50, 100, and 200 $\mu\text{g/ml}$ of core histones for 4 hours and within the last 30 minutes of incubation, 75nM of Lysotracker Red DND-99 were added to the cultures. Afterwards, an immunofluorescence assay was performed using a mouse anti-histone H3 primary antibody and a FITC

labelled goat-anti-mouse secondary antibody to label histones, LysoTracker Red DND-99 to label lysosomes, and DAPI to label nuclei. Preparations were observed under a fluorescence microscope, specifically the DMI-8 model, and photographs were captured using a Leica-DFC 9000 GT camera attached to the microscope (see Materials and Methods). Each row corresponds to images from the same field of view, obtained by exciting the samples with lasers at specific wavelengths of 490 nm, 550 nm, or 365 nm to excite FITC, LysoTracker, or DAPI, respectively. The fourth column shows a fusion of the three corresponding images from a single field of view using a 40x magnification. Scale bar 40 μ m.

After completing the development of the image analysis technique (Figures 9 and 10 in *Materials and Methods* section) using previously optimized culture and histone treatment conditions, it was used for quantitative analysis of histone-lysosome colocalization based on the previously obtained immunofluorescence images. The technique provided single-cell level data regarding the percentage of cellular area occupied by green labelling (representing histones), the percentage of cellular area occupied by red labelling (representing lysosomes), and the percentage of red labelling area occupied also by green labelling (lysosomes colocalizing with histones). The results obtained from analysing a total of 96 images with 484 cells for HeLa and 96 images with 501 cells for Hep3B are depicted in *Figure 13*. As shown in graph 13A, the tool accurately identifies histones, as the percentage of cellular area coinciding with green fluorescence (variable C.2, *Figure 10*) being directly proportional to the added histone concentration. Graph 13B demonstrates that the percentages of red fluorescence area occupied by green fluorescence (variable C.3, *Figure 10*) for both cell lines are significantly higher with extracellular histone treatments at 200 μ g/mL compared to 100 μ g/mL. However, this may be attributed to the fact that by adding more histones to the cells, they cover larger portions of the cell surface and are more likely to overlap with the red labelling. Section C of *Figure 13* compares the data represented in 13A and 13B for HeLa and Hep3B. These graphs illustrate that the percentage of red fluorescence area overlapping with green fluorescence (*Figure 13B*) is significantly higher than the percentage of cellular area occupied solely by green fluorescence (*Figure 13A*) under all the studied conditions, confirming that green labelling is primarily present on areas with fluorescent red labelling rather than on any random point within the cellular area. Additionally, it can be observed that for HepB, the differences between C.3 and C.2 are more pronounced compared to HeLa (*Figure 13C*).

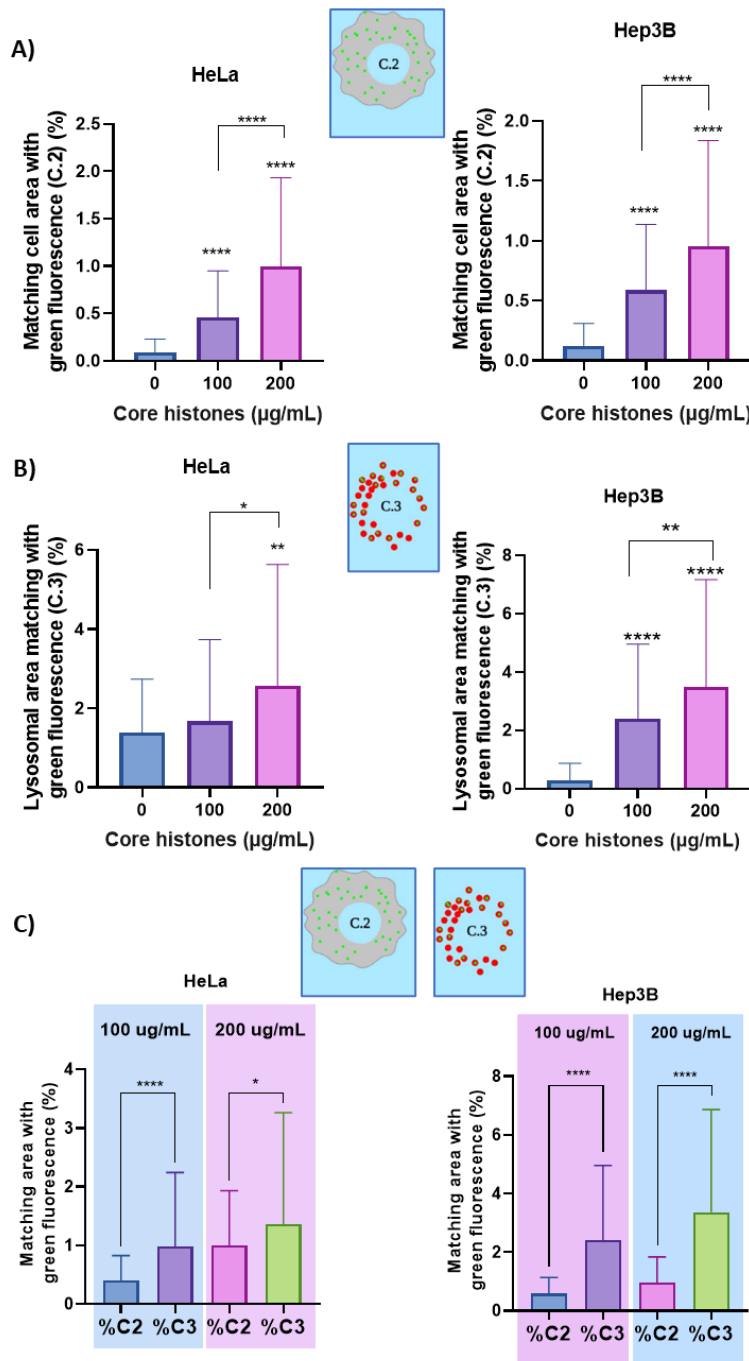


Figure 13. Subcellular co-localization study of histones using the developed image analysis tool. A total of 96 images with 484 cells were analysed for HeLa (215 cells treated with histones at a concentration of 0 µg/mL, 164 cells treated with 100 µg/mL, and 105 cells treated with 200 µg/mL), and 96 images with 501 cells were analysed for Hep3B (239 cells treated with 0 µg/mL, 133 cells treated with 100 µg/mL, 129 cells treated with 200 µg/mL). Graphs on left column represent the results for HeLa images, while graphs on right represent the results for Hep3B images. (A) displays the percentage of cellular area coinciding with green, fluorescent labelling (variable C.2, as presented in Figure 10) for each of the three histone treatments. (B) represents the

*percentage of red fluorescent labelling area overlapping with green, fluorescent labelling (variable C.3, as presented in Figure 10). (C) compares the data from the previous graphs. These graphs compare, for each cell line and for the 100 and 200 µg/mL treatments, the percentage of cellular area occupied by green fluorescence versus the percentage of red fluorescence area occupied by green fluorescence, i.e., C.2 versus C.3. Statistical significance was considered at a p-value < 0.05, calculated by one-way ANOVA, indicated by * when $p < 0.05$, ** when $p < 0.01$ and **** when $p < 0.0001$.*

In conclusion, provided that the identified red fluorescent labelling corresponds to lysosomes and the green labelling corresponds to histones, this analysis supports the hypothesis of colocalization between histones and lysosomes, with a stronger effect observed in Hep3B cells than in HeLa cells.

1.3 Effects of autophagy modulation upon core histone treatment in HeLa and Hep3B cells.

To investigate the relationship between extracellular histone-mediated cytotoxicity, autophagy, and histone internalization, and based on previous data from our group (67), we decided to repeat both the colocalization and cytotoxicity experiments, this time treating the cell cultures with autophagy activators or inhibitors. In order to further explore the relationship between extracellular histones and autophagy, we studied the extent to which autophagy activation or inhibition affected histone-mediated cytotoxicity and histone internalization.

Firstly, we investigated the effect of modulating autophagy on the colocalization between lysosomes and histones. For this purpose, HeLa and Hep3B cells were subjected to treatments with metformin or NH_4Cl and leupeptin (to induce or inhibit autophagy, respectively), followed by treatments with histones at concentrations of 100 µg/mL or 200 µg/mL for 4 hours. The cells were fluorescently labelled with DAPI, LysoTracker Red DND-99, and a primary anti-histone H3 antibody (green). The samples were observed and photographed under a fluorescence microscope (shown in *Figure A 2* in *Annex I* section). The results did not show clear trends regarding changes in the overlap between green and red fluorescent labels, making it necessary to repeat the treatments, fluorescent labelling of cells, and image capture to create a robust and coherent image bank to be able to make the analysis with the image analysis tool.

To assess the potential protective role of autophagy against extracellular histone-mediated cytotoxicity, the cells were treated with core histones and autophagy modulators as mentioned before and then they were labelled with an annexin-V apoptosis kit and analysed by flow cytometry. The results are shown in *Figure 14*.

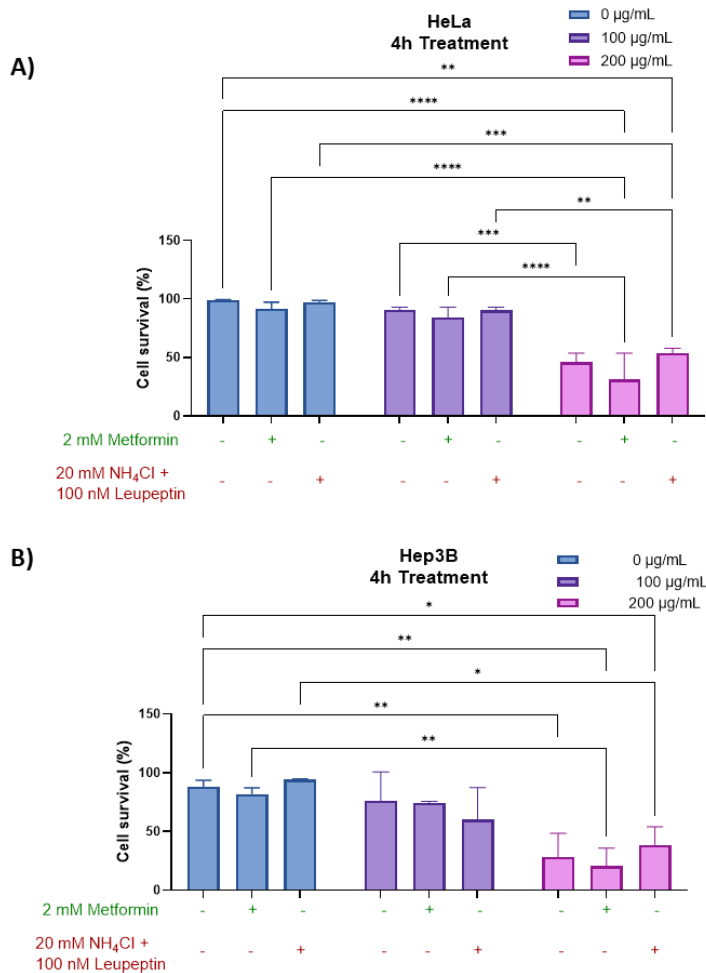


Figure 14. Analysis of histone-mediated cytotoxicity following treatment of cells with autophagy modulators. HeLa (A) and Hep3B (B) cells were subjected to 2 mM Metformin treatment for 24 hours or 20 mM NH₄Cl plus 100 nM leupeptin treatment for 1.5 hours. Subsequently, histones were added to cells treated with either of the two conditions, as well as to untreated cells, at concentrations of 0, 100, and 200 µg/mL. After 4 hours, cells were harvested and labelled using an annexin V kit, followed by flow cytometry analysis. The mean values are shown as bars, expressed as a percentage $\pm$ the standard deviation ($n=3$). Statistical significance was considered at a p -value <0.05 , calculated in all cases using a two-way ANOVA statistical test, indicated by * when $p<0.05$, ** when $p<0.01$, *** when $p<0.001$ and **** when $p<0.0001$.

No significant effects of metformin or NH₄Cl plus leupeptin on cell survival were observed in either of the cell lines tested at any of the histone concentrations examined,

although NH_4Cl plus leupeptin appeared to show a trend towards reducing histone-induced cytotoxicity. Thus, the results obtained so far do not suggest that autophagy exerts a protective effect against histone-mediated cytotoxicity under the studied conditions.

1.4 Effects of core histone treatment in the cell cycle of HeLa cells.

Afterwards, and in order to assess whether histone treatment affected cell proliferation without triggering cell death, cell cycle alterations were studied following exposure to core histones. HeLa cells were treated with various concentrations of core histones (10-400 $\mu\text{g/mL}$) as described previously. Subsequently, cell cycle analysis was performed by flow cytometry to determine any changes in the distribution of the stages of the cell cycle.

As it can be observed in *Figure 15*, HeLa cells exposed to core histones resulted in a significant alteration in the cell cycle profile. Specifically, it can be noticed an increase in the proportion of cells in the G1 phase when cells are treated with high doses of core histones (300 and 400 $\mu\text{g/mL}$). This indicates that cell cycle is arrested at the G1 checkpoint when cells are exposed to high doses of core histones. These findings suggest that exposure to extracellular histones leads to cell cycle alterations, specifically causing G1 phase arrest.

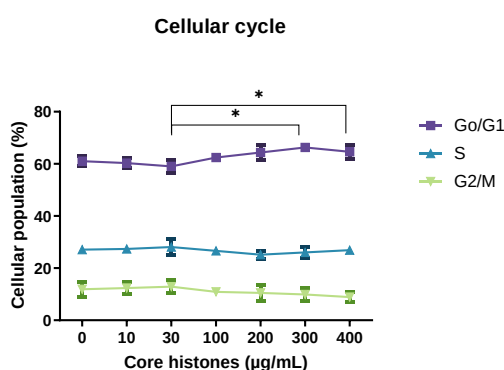


Figure 15. Analysis of HeLa cell cycle upon core histone treatment. HeLa cells were seeded and treated with core histones (0-400 $\mu\text{g/mL}$) for 4h and then 300.000 cells of each condition were collected and stained with propidium iodide. Then, the cell cycle was analysed by flow cytometry. The dots represent individual values expressed as a percentage $\pm$ the standard deviation ($n=3$). Statistical significance was considered at a p-value <0.05 , calculated in all cases using a two-way ANOVA statistical test indicated by * when $p<0.05$.

1.5 Changes in miRNA signature upon core histone treatment in HeLa and Hep3B cells

Once the cytotoxic potential of core histones and their connection to the modulation of autophagy was evaluated, and since the modulation of autophagy did not seemingly affect cell viability, but some changes were observed in terms of cell cycle regulation, we decided to study the possible effect of histone treatments on the signalling pathways related to inflammation controlled by miRNAs. Recently, our group has reported that miRNAs 155 and 146 are altered in a neuroinflammatory context. This study describes their potential as biomarkers for Lafora disease, which is characterized by neuroinflammation (186). Additionally, various miRNAs have been associated with sepsis-associated encephalopathy (187). Hence, it is plausible to consider that these miRNAs may also be involved in the modulation of inflammation in our experimental model.

Consequently, HeLa and Hep3B cell lines were subjected to treatment with autophagy modulators as previously described. Afterwards, they were exposed to various concentrations of core histone (100 and 200 µg/mL). Then, the cells were collected, and the RNA was extracted using affinity columns. According to existing literature (186) (187), several miRNAs of interest for our study were selected and analysed by specific RT-qPCR for each miRNA. The results are depicted in *Figure 16*.

In the case of miR-155 (*Figure16A*), it can be observed that its expression is downregulated in HeLa cells exposed to core histones under control conditions. A similar trend is observed in Hep3B cells although not statistically significance is found. Surprisingly, when autophagy is modulated in HeLa cells, the expression of miR-155 is decreased in all tested the conditions. However, Hep3B cells exhibit contrasting behaviour with an opposite trend.

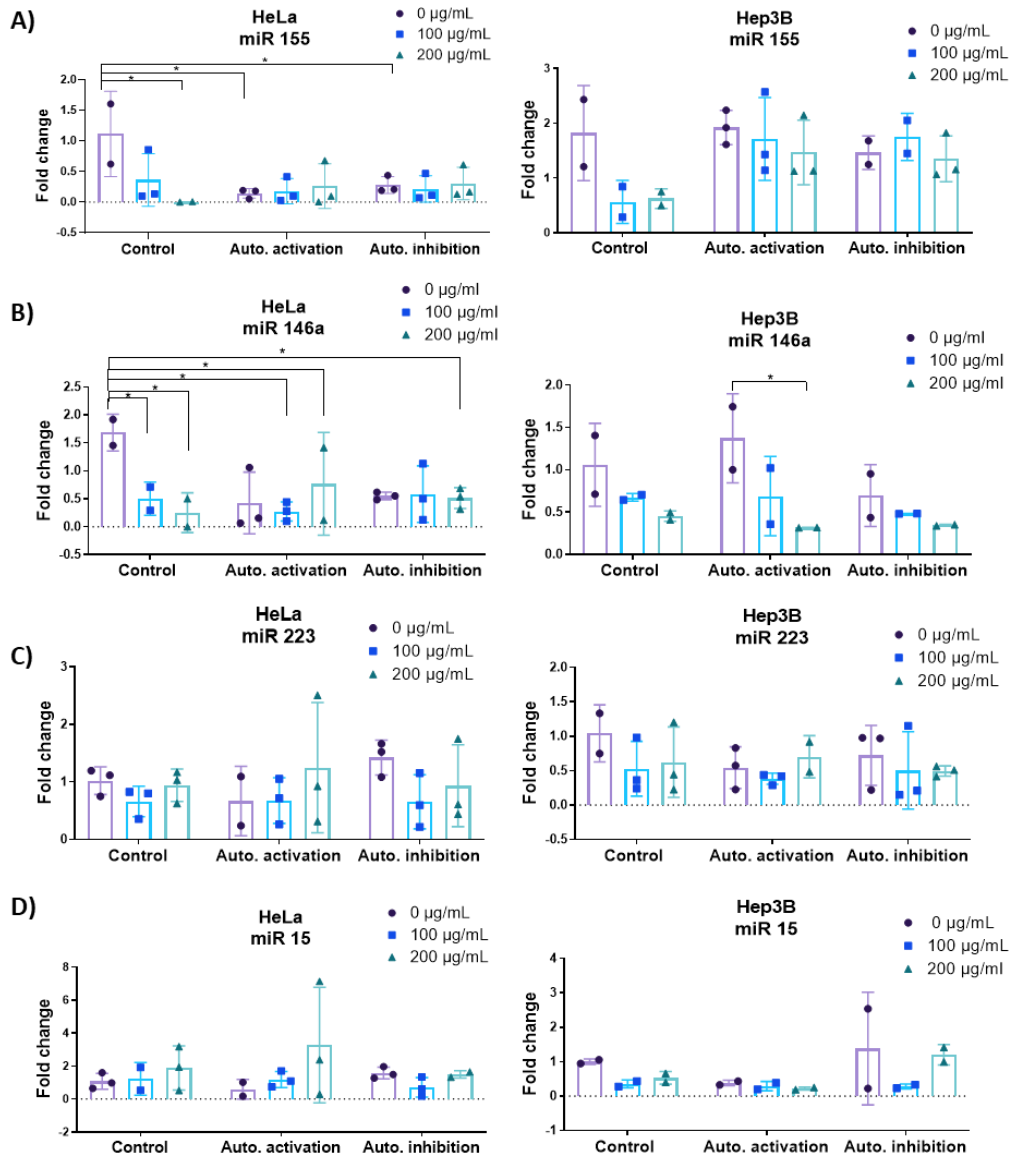


Figure 16. MiRNAs analysis of HeLa and Hep3B cells after modulation of autophagy and core histone treatments. HeLa (left panels) and Hep3B cells (right panels) were treated with 2 mM Metformin treatment for 24 hours (autophagy activation) or 20 mM NH_4Cl plus 100nM leupeptin treatment for 1.5 hours (autophagy inhibition). Subsequently, histones were added to cells treated with either of the two autophagy modulators, as well as untreated cells, at concentrations of 0, 100, and 200 $\mu\text{g/mL}$. After four hours, the cells were harvested, RNA was extracted, and miRNA expression was assessed by specific RT-qPCR. The mean values are shown as bars, expressed as a fold change $\pm$ the standard deviation ($n=3$, except for miR-15 $n=2$). Statistical significance was considered at a p -value <0.05 , calculated in all cases using a two-way ANOVA statistical test indicated by " * " for $p<0.05$. "Auto." Refers to autophagy.

Regarding miR-146a (Figure16B), HeLa cells demonstrate a clear downregulation under control conditions when treated with core histones and a similar trend is observed in Hep3B cells. Interestingly, when autophagy is modulated, this dose-

dependent downregulation is altered in HeLa cells, and there are no differences observed between core histone treatments. In contrast, Hep3B cells do not show any alteration in the core histone-dependent downregulation of miR-146 upon autophagy modulation. No alterations in the expression of miR-223 (*Figure16C*) and miR-15 (*Figure16D*) were observed under any condition tested in both cell models.

In conclusion, miR-155 is consistently downregulated in HeLa cells in the control group when autophagy is modulated, while Hep3B cells show an opposite trend. MiR-146a is downregulated in both cell types under basal conditions, with alterations in HeLa cells when autophagy is altered. These findings suggest that core histones and autophagy may differentially regulate the expression of miRNAs in different cell types, emphasizing the complex and cell-specific nature of their interactions. Further investigation is warranted to fully understand the underlying mechanisms and potential implications of these observed patterns in cellular functions.

2. *HUVEC cell model*

2.1 Cytotoxic effects of extracellular core histones in HUVEC cells.

Once the cytotoxic potential of core histones and their relationship with autophagy had been evaluated, given the observed co-localization between these extracellular histones and lysosomes in experimental models of HeLa and Hep3B cells, it was necessary to confirm these results using a cellular model more suitable to study the basis of the pathophysiology of sepsis. Therefore, the following studies focused on HUVEC cell cultures, since endothelial cells are among the first cells upon which extracellular histones exert a harmful effect in the context of sepsis.

To accomplish this, the first step was to assess the cytotoxic potential of the previously used core histones on HUVEC cells. Cells were treated with increasing concentrations of core histones (10-500 $\mu\text{g/mL}$) and incubated for various durations (4, 6, 8, and 18 hours). Subsequently, the cells were collected, and cell survival was evaluated using flow cytometry. The results presented in *Figure 17* demonstrate dose- and time-dependent cytotoxicity, since longer incubation time with core histones (*Figure 17D*) required a lower concentration (25 $\mu\text{g/mL}$) to cause a decrease in cell survival. Conversely, at shorter incubation times such as 4 hours, significantly higher concentrations (400 $\mu\text{g/mL}$) were necessary to achieve a decrease in cell survival. Therefore, the cytotoxic potential of core histones on HUVEC cells was confirmed, with dose and time dependency. The findings underscore the harmful impact of extracellular histones on endothelial cells, underscoring its importance in the context of sepsis.

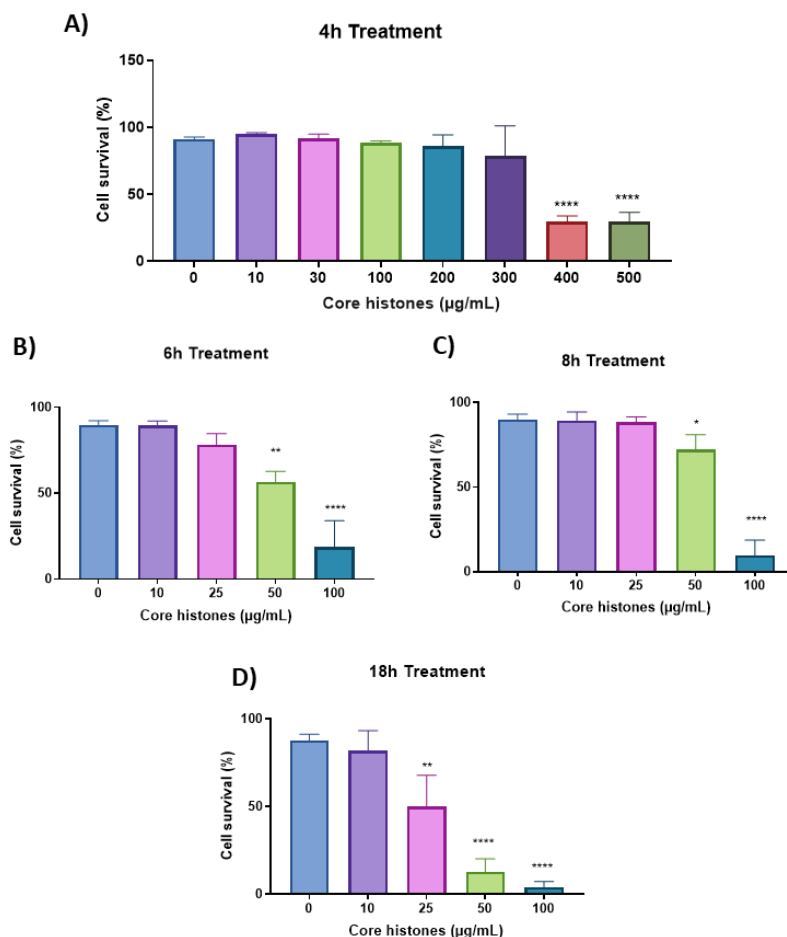


Figure 17. Analysis of histone-mediated cytotoxicity in HUVEC cells. HUVEC cells were incubated in the absence or presence of several concentrations of core histones. After the indicated incubation times, the cells were harvested and labelled using an Annexin V kit, and subsequently analysed by flow cytometry. The mean values are shown as bars, expressed as a percentage $\pm$ the standard deviation ($n=3$). Statistical significance was considered at a p -value < 0.05 , calculated in all cases using a one-way ANOVA statistical test indicated by * when $p < 0.05$, ** when $p < 0.01$ and **** when $p < 0.0001$.

2.2 Colocalization studies between histones and autophagy markers LC3B in HUVEC cells.

Once the cytotoxic potential of core histones in HUVEC cells was evaluated, a 4-hour incubation period was determined as the standard time. Regarding the concentrations, different values were used for the next experiments, depending on the parameter being evaluated, considering that cell viability decreases above 400 µg/mL of core histones. Subsequently, the previously observed relationship between core

histones and autophagy in HeLa and Hep3B cells was investigated. First, subcellular localization studies of core histones were conducted using immunofluorescence. Then, the degree of colocalization between H3 histone and autophagic protein LC3B was quantified. Cells were treated with core histones for 4 hours at various concentrations, followed by immunofluorescence staining of H3 histone and LC3B. Images were acquired using a confocal microscope, capturing at least ten fields per condition. Colocalization analysis was performed as described in section 3.5.2 of *Materials and Methods* section. The results are presented in *Figure 18*.

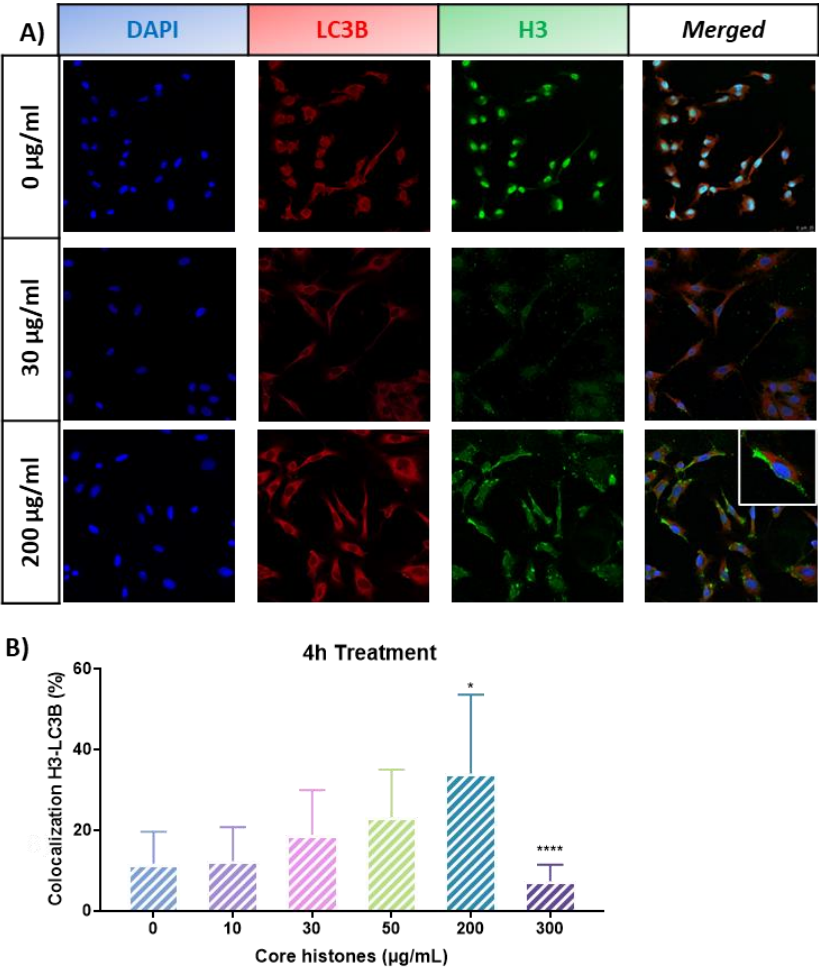


Figure 18. Analysis of colocalization between commercial H3 histone and LC3B. (A). Confocal microscopy images of HUVEC cells treated with core histones at concentrations of 0, 30, and 200 $\mu\text{g/ml}$ are presented. Each row displays images from the same field of view. Blue fluorescence corresponds to cellular nuclei stained with DAPI, red fluorescence corresponds to LC3B protein labelled with a Texas Red-conjugated secondary antibody, and green fluorescence corresponds to H3 histones stained with an Alexa488-conjugated secondary antibody. Furthermore, a fourth column displays the merge of the three channels used. The magnification used was 40X. The insert was obtained using a 4x zoom from the indicated image. Scale bar 25 μm . (B) Quantification

of colocalization was performed using the TCS SP8 X confocal microscope software (Leica Microsystems; Germany) (see section 3.5.2 of Materials and Methods) between commercial H3 histones and LC3B protein under each studied condition. The mean values of colocalization percentage from 10 images per condition are represented as a bar graph, along with the standard deviation ($n=3$). Statistical significance was considered when $p<0.05$ compared to the control ($0 \mu\text{g/ml}$)., calculated using a one-way ANOVA statistical test indicated by * for $p<0.05$ and **** for $p<0.0001$.

Figure 18A displays representative examples of confocal microscopy images for some of the analysed conditions. The histone mark is concentrated in the nucleus in the absence of extracellular histones (control), while increasing concentrations of histones lead to green fluorescence dispersing throughout the cellular space. Examples of images from all studied conditions can be found in Figure A3 in the Annex I section. The colocalization analysis between green and red fluorescence was performed for each condition using the TCS SP8 X confocal microscope software (Leica Microsystems; Germany). This software calculates the percentage of overlap between the two fluorophores per image (see section 3.5.2 of the Materials and Methods section). Quantification revealed a dose-dependent increase in colocalization between the two proteins, with a very high significant increase observed at a core histone concentration of $200 \mu\text{g/mL}$. However, at the dose of $300 \mu\text{g/ml}$ no significant results were obtained with respect to the control ($0 \mu\text{g/ml}$) possibly because at that high concentrations cells are starting to die and then the autophagy is not being activated.

In order to confirm that the previously obtained results were due to colocalization with extracellular histones used in the treatment rather than endogenous histones present in the cells, a study on colocalization was conducted between a recombinant form of histone H3 fused with green fluorescent protein (GFP) (hereafter referred to as H3-GFP) and the autophagic marker LC3B (red fluorescence) through immunofluorescence and confocal microscopy (Figure 19). In this case, cultured HUVEC cells were treated with a histone extract enriched with H3-GFP obtained from HeLa cells transfected with the pIRES-Neo-H3.3-GFP plasmid to express the H3 protein tagged with GFP (section 1.4, Materials and Methods). However, there was fluorescence loss due to the histone extraction procedure. Therefore, GFP was used not as a fluorescent marker itself but as a tag for specific detection of exogenously added histones, using a specific antibody to detect the GFP protein (Figure 19).

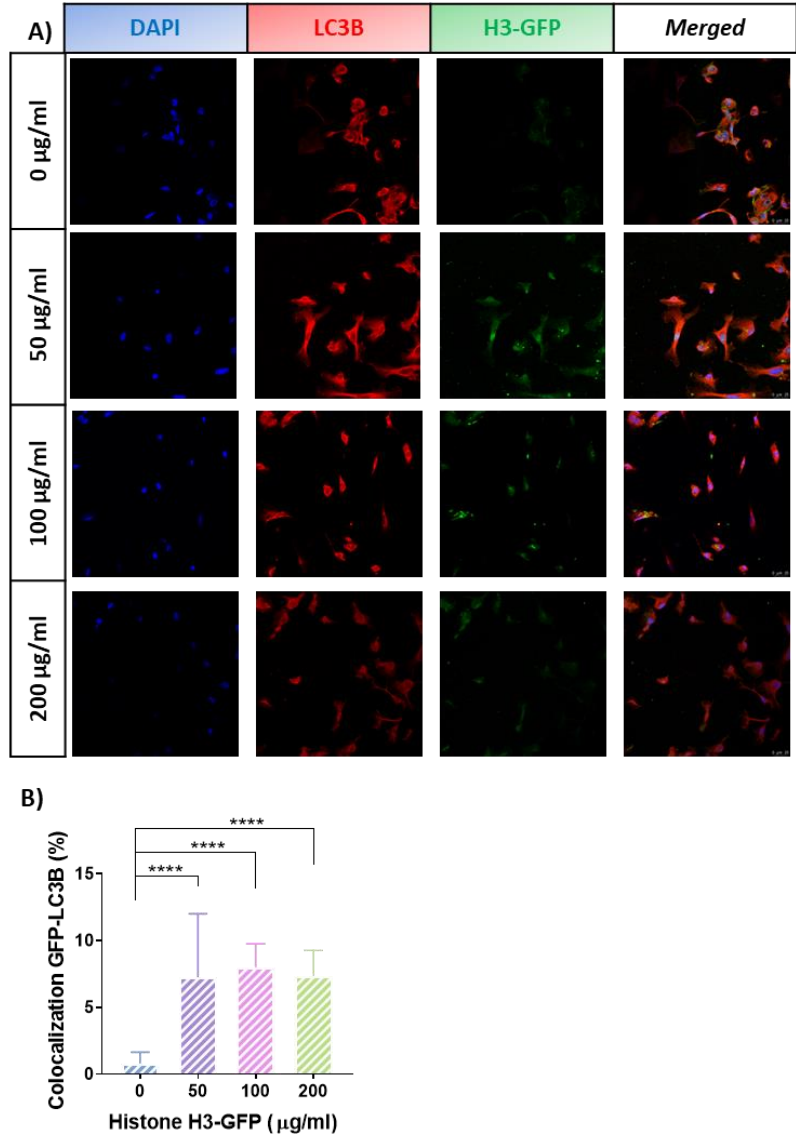


Figure 19 . Study of colocalization between histones H3-GFP and LC3B. (A) Photographs of confocal microscopy of HUVEC cells treated with different concentrations of an extract rich in histones H3-GFP. Each row collects the images of the same visual field. The blue fluorescence corresponds to the cell nuclei stained with DAPI, the red with the LC3B protein and green with histones H3 by binding to an antibody secondary conjugated to the fluorophore Texas Red and Alexa488 respectively. It shows also a fourth column with the fusion of the three channels used. 40X magnification. Scale bar 25 μm . (B) Quantification of colocalization between histone H3-GFP and the LC3B marker by image in percentage form made with TCS SP8 confocal microscope software X (Leica Microsystems; Germany) (see section 3.5.2 Materials and Methods). are represented the mean values in the form of bars $\pm$ the standard deviation of 10 images per condition ($n=3$). Statistically significant differences were considered with respect to the control (0 $\mu\text{g/ml}$) when $p<0.05$, calculated using ANOVA one-way indicated by **** when $p<0.0001$).

The histone extract was administered to cultured HUVEC cells at concentrations ranging from 10 to 200 $\mu\text{g/ml}$ for 4 hours, and LC3B and GFP proteins were labelled with secondary antibodies conjugated to different fluorophores, while cellular nuclei were stained with DAPI as previously described. Confocal microscopy images illustrating examples of the analysed conditions are presented in *Figure 19A*. Once the results were quantified, a significant dose-dependent increase in colocalization between the LC3B marker and the histone- H3-GFP protein was observed again (*Figure 19B*).

2.3 Verification of the association between autophagy and core histones

In order to confirm the previous results obtained in the analysis of colocalization by indirect immunofluorescence and confocal microscopy, the interaction between LC3B protein and both histone H3 (*Figure 20*) and histone H3-GFP (*Figure 21*) was evaluated using the Proximity Ligation Assay (PLA) technique and fluorescence microscopy. In this procedure, if both proteins (labelled with primary antibodies) are in close proximity (at distances <40 nm), the complementary nucleotide tails attached to the secondary antibodies can be linked by ligases. The addition of polymerases and fluorescent nucleotides increases the ligation complex, resulting in the emission of red fluorescence that reflects protein interactions (188).

To perform this assay, HUVEC cells in culture were treated with core histones at concentrations between 10 and 200 $\mu\text{g/ml}$, and with H3-GFP histone extract at concentrations between 50 and 200 $\mu\text{g/ml}$ for 4 hours. The protocol established in section 3.2 of *Materials and Methods* section was followed. In at least 80 cells from each condition, quantification of fluorescent red dots derived from the interaction between LC3 and H3 or H3-GFP was performed. Results are shown in *Figure 20 and Figure 21*. Both figures show examples of fluorescence microscopy images corresponding to some of the studied conditions, which are fully presented in *Figures A4 and A5* of the Annex I. In the experiment with core histones (H2AB, H3, and H4), quantification revealed a significant increase in the interaction between histone H3 and LC3B at concentrations of 10 and 100 $\mu\text{g/ml}$ compared to the control (0 $\mu\text{g/ml}$), while at a concentration of 200 $\mu\text{g/ml}$, the number of interactions was significantly reduced (*Figure 20B*).

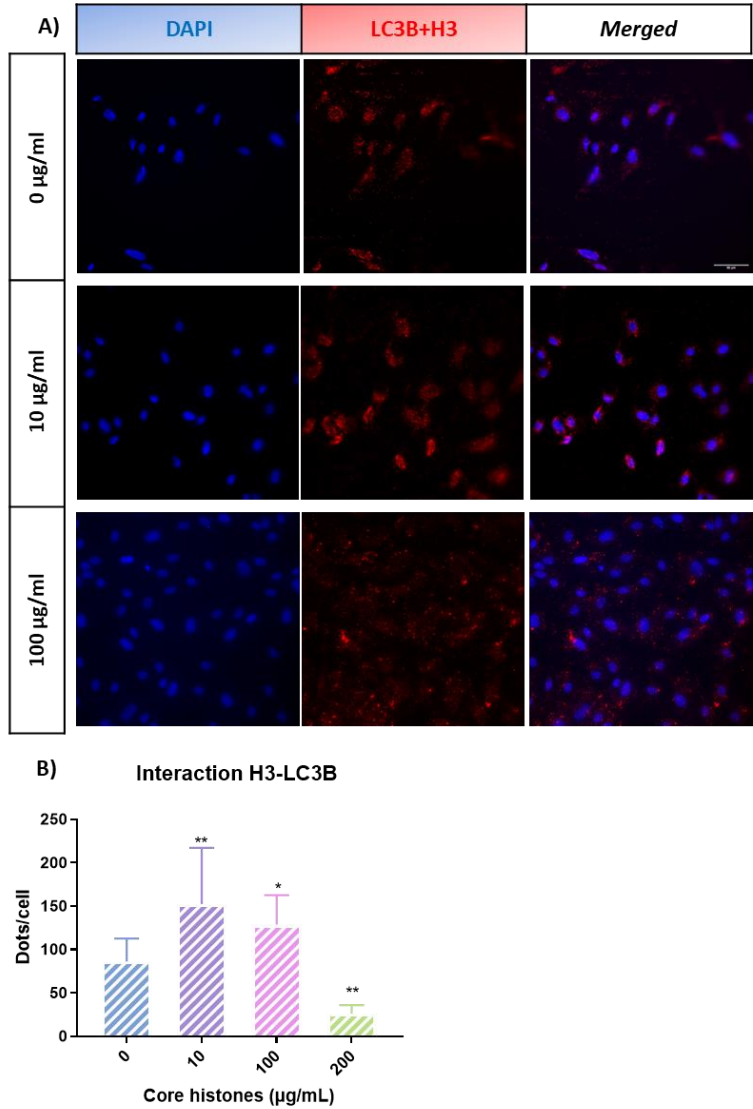


Figure 20. Analysis of interactions between commercial histones H3 and LC3B. (A) Fluorescence microscopy images of HUVEC cells treated with increasing concentrations of purified commercial histones (H2AB, H3, and H4). Blue fluorescence corresponds to cellular nuclei stained with DAPI, while red fluorescence represents the interactions between histone H3 and LC3B (as they are physically close, allowing the binding of nucleotide tails attached to secondary antibodies by ligases and their amplification by polymerases with fluorescent nucleotides). The third column represents the merged image of the two channels used. Magnification 40X. Scale bar 52 μm . (B) Quantification of interactions between LC3B and histone H3 per cell. Mean values of the interactions observed in at least 80 cells from each condition are represented as bars, along with the standard deviation ($n=3$). Significantly different values compared to the control (0 $\mu\text{g/ml}$) are indicated when $p<0.05$, calculated using ANOVA one-way indicated by * for $p<0.05$ and ** for $p<0.01$.

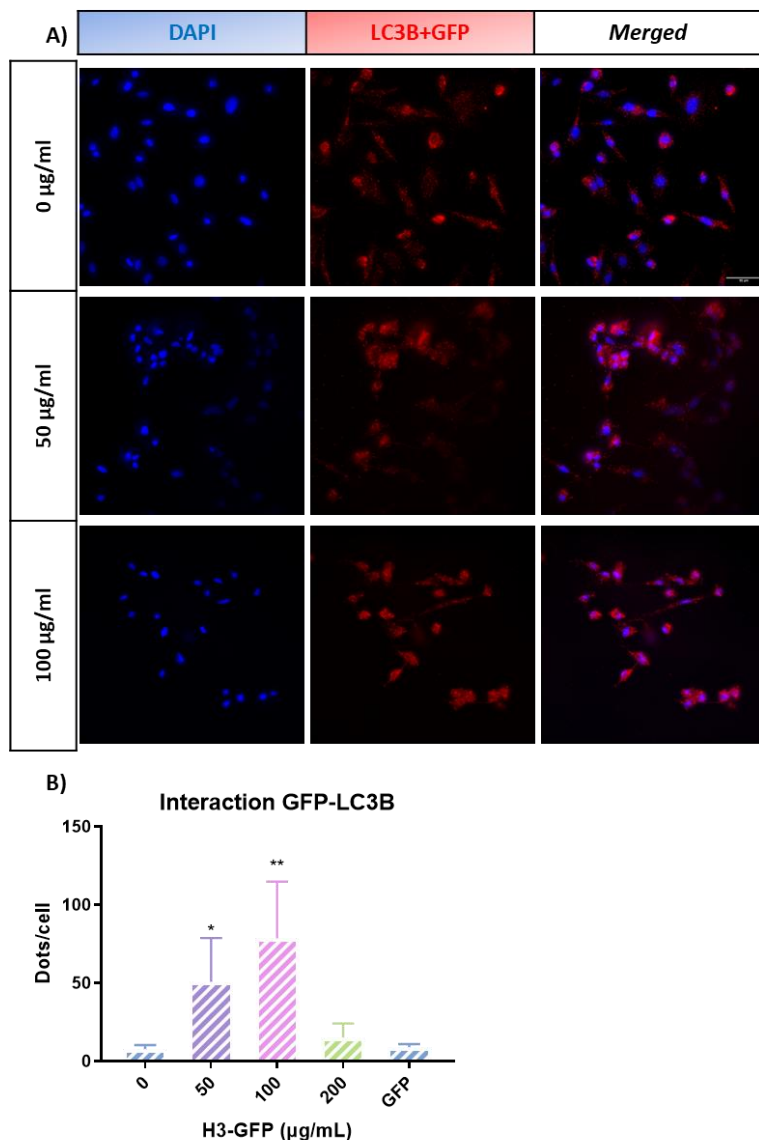


Figure 21. Analysis of interactions between H3-GFP histones and LC3B. (A) Fluorescence microscopy images of HUVEC cells treated with increasing concentrations of H3-GFP histone extract. Blue fluorescence corresponds to cellular nuclei stained with DAPI, while red fluorescence represents the interactions between GFP protein attached to histone H3 and LC3B protein (due to their proximity within the intracellular space, leading to the binding of nucleotide tails conjugated to secondary antibodies by ligases and their amplification by polymerases with fluorescent nucleotides). The third column represents the merged image of the two channels used. Magnification 40X. Scale bar 52 μm . (B) Manual quantification of interactions between LC3B and H3-GFP histones per cell. Mean values of the interactions observed in at least 80 cells from each condition are represented as bars, along with the standard deviation ($n=3$). Statistical significance is indicated when $p<0.05$ calculated using ANOVA one-way indicated by * when $p<0.05$ and ** when $p<0.01$.

Regarding the interactions with H3-GFP histones (Figure 21B), a significant increase was observed at concentrations of 50 and 100 $\mu\text{g/ml}$ compared to the control

(0 µg/ml), while they significantly decreased at a dose of 200 µg/ml compared to 100 µg/ml. In addition, possible interactions with the GFP protein itself were individually analysed to confirm that the observed effects were due to the action of histone and not due to their fusion with the high molecular weight GFP protein. As expected, it was found that treatment with GFP did not interfere with the obtained results.

2.4 Determination of incubation conditions with histone H1-488 and studies of colocalization with LysoTracker Red DND-99.

Although the previous results suggest an interaction between exogenously applied core histones and the autophagy marker LC3 in the different cell cultures, the treatments used required immunofluorescence labelling for the visualization of histones under the microscope, making the obtained results indirect. Therefore, it was decided to employ a treatment with a fluorescent commercial histone H1 (directly conjugated with the Alexa-488 fluorophore (from now on called H1-488) and analyse their subcellular localization in HUVEC cells when applied exogenously to the cultures.

Prior to this, to optimize the experimental conditions for incubation with these histones, the cytotoxicity range was examined on the HUVEC cell line. A curve of increasing concentrations of H1-488 histones ranging from 10 to 100 µg/ml was used, added to the cell culture plates with HUVEC cells for 24 hours. Cell survival was analysed using flow cytometry, employing an apoptosis study kit with Annexin V-APC, as described in section 4 of *Materials and Methods*. The results are presented in *Figure 22*.

No significant changes were observed in terms of cytotoxicity induced by histone H1-488 treatment in endothelial cells. However, a trend towards reduced cell viability can be observed from the concentration of 50 µg/ml onwards, which remains consistent at higher concentrations without reaching significance (*Figure 22*). Therefore, to ensure cell viability while studying changes in cells due to interactions with extracellular histones, a concentration of 50 µg/ml of H1-Alexa488 histones was selected for further experiments.

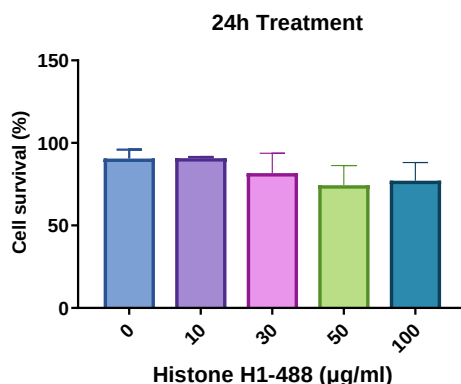


Figure 22. Cytotoxicity analysis of H1-488 histone. HUVEC cells were incubated in the absence or presence of several concentrations of H1-488 for 24h. After incubation, the cells were harvested and labelled using an Annexin V kit, and subsequently analysed by flow cytometry. Mean survival values are presented as bars, expressed as a percentage $\pm$ standard deviation ($n=3$). No statistical difference was found.

Subsequently, a colocalization analysis was performed between H1-Alexa488 histones (green fluorescence) and the acid organelle marker LysoTracker Red DND99 (red fluorescence) at different time points, using indirect immunofluorescence and confocal microscopy (Figure 23). Cultured HUVEC cells were treated with histone H1-488 at a concentration of 50 $\mu\text{g/ml}$ for 2, 4, and 24 hours. Nuclear staining with DAPI was performed. The images shown in Figure 24A represent examples of each studied condition. Quantification of the colocalization between LysoTracker and histone H1-488 marks using the TCS SP8 X confocal microscope software (Leica Microsystems; Germany) revealed a significant increase in colocalization between both proteins when the treatment was extended to 24 hours, compared to both the control (0 hours) and the 2 and 4-hour time periods (Figure 23B).

These findings, together with those obtained in previous experiments, seem to indicate that histones are capable of contacting endothelial cells at short times, and subsequently, entering into them. Furthermore, the cells appear to direct these histones towards autophagosomes (organelles involved in autophagy). To confirm these findings, a series of assays were designed based on the modulation of endocytosis and autophagy processes.

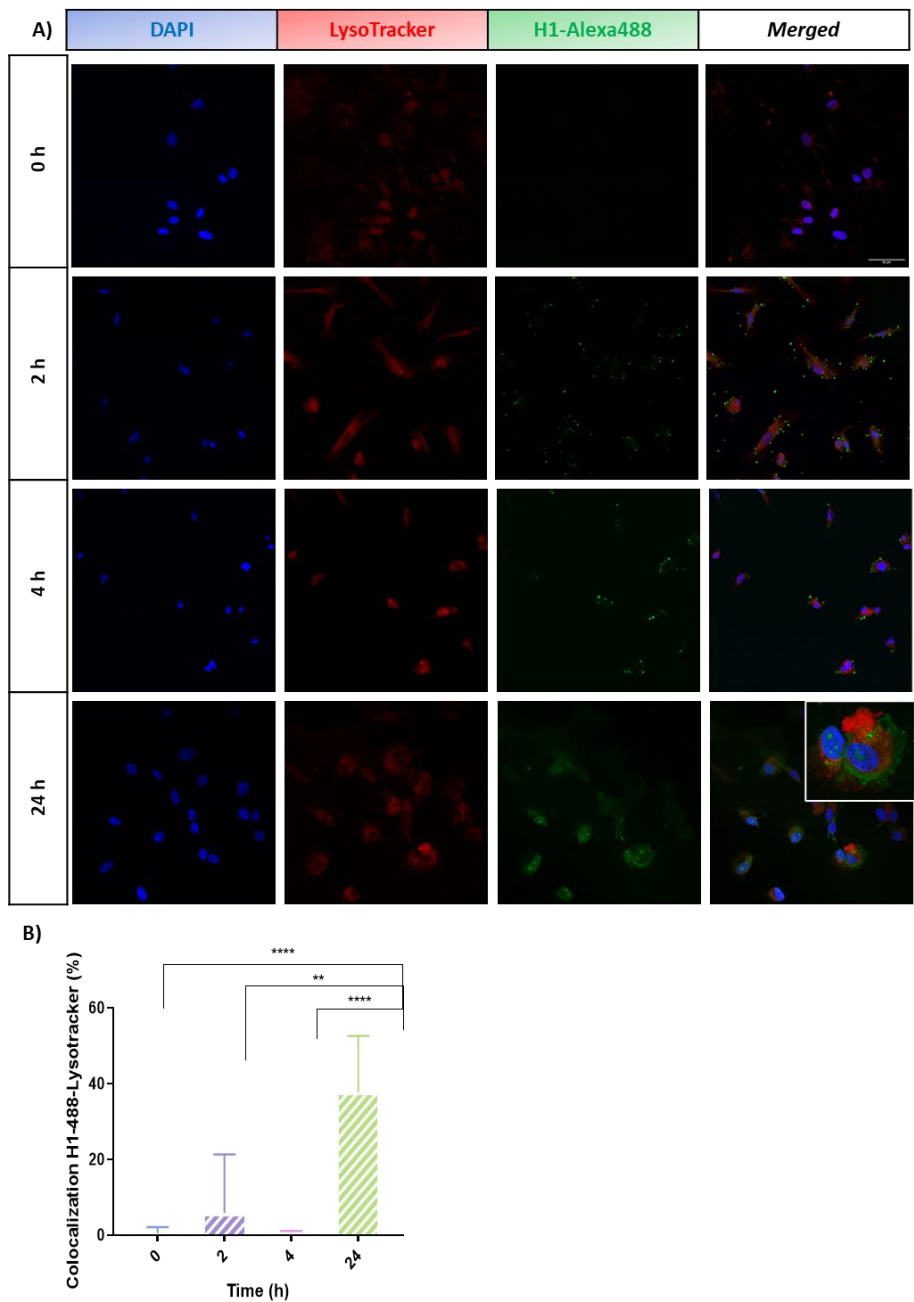


Figure 23 Analysis of colocalization between histones H1-Alexa488 and LysoTracker Red DND-99. (A) Confocal microscopy images of HUVEC cells that were subjected to treatment with histone H1-a488 (50 µg/ml) for 2, 4 and 24 hours. The blue fluorescence of each row shows cell nuclei by DAPI staining, red with the LysoTracker Red DND-99 acid organelle marker and the green one with the autofluorescence of histone H1-Alexa488, corresponding to the same visual field. In addition, a fourth column is shown with the fusion of the three channels used. Magnification, 40X. The insert was obtained using a 4x zoom from the indicated image Scale bar 52 µm. (B) Quantification of colocalization between LysoTracker Red DND 99 and histone H1-Alexa488 at the different incubation times with treatment, using TCS SP8 X confocal microscope software (Leica Microsystems; Germany) (see section 3.5.2 Materials and Methods). The bars represent the mean values ± the

*standard deviation of the percentage of colocalization of each condition (n=3). Statistically significant differences were considered with respect to the control (0 hours), and between the different conditions when $p < 0.05$, calculated using ANOVA one-way indicated by ** from $p < 0.01$, and **** when $p < 0.0001$.*

2.5 Effect of endocytosis blockade on the colocalization between extracellular histones and LC3B protein and its relation to cell viability.

Based on the existing evidence in the literature, which suggests that nucleosome internalization into cells occurs through endocytosis (189), it was decided to repeat the indirect immunofluorescence and confocal microscopy experiments under conditions that would promote blocking of endocytosis in cultured cells. Therefore, if the histones are entering HUVEC cells through endocytosis, a reduction of the colocalization between the autophagy flux marker LC3B and extracellularly added histones would be expected. So, the aim was to observe the effect on the colocalization between histone H3 (green fluorescence) and LC3B protein (red fluorescence) in this context (*Figure 24*).

To achieve this, once the cells reached 80% confluency, they were incubated for 15 minutes on ice, maintaining a temperature of 4°C as indicated in the studies by Wang et al (189). Subsequently, the cells were treated with core histones and incubated on ice for 4 hours. The cell nuclei were stained with DAPI, while histone H3 and LC3B protein were labelled by indirect immunofluorescence staining. *Figure 24A* presents examples of fluorescence microscopy images of the analysed conditions. As shown in *Figure 24B*, there was no significant increase in colocalization between histone H3 and LC3B protein dependent on the dose, as observed in the previous assays. This suggests that histones are internalized into cells through endocytosis and that it is the reason why the blockage with low temperature prevents the increase of colocalization.

The experiment was repeated using a different type of histone, histone H1-488, to confirm that the observations made using the core histone were not artifacts resulting from cold incubation. *Figure A6* in *Annex I* section illustrates how 4-hour and 24-hour incubations on ice do not affect cell viability.

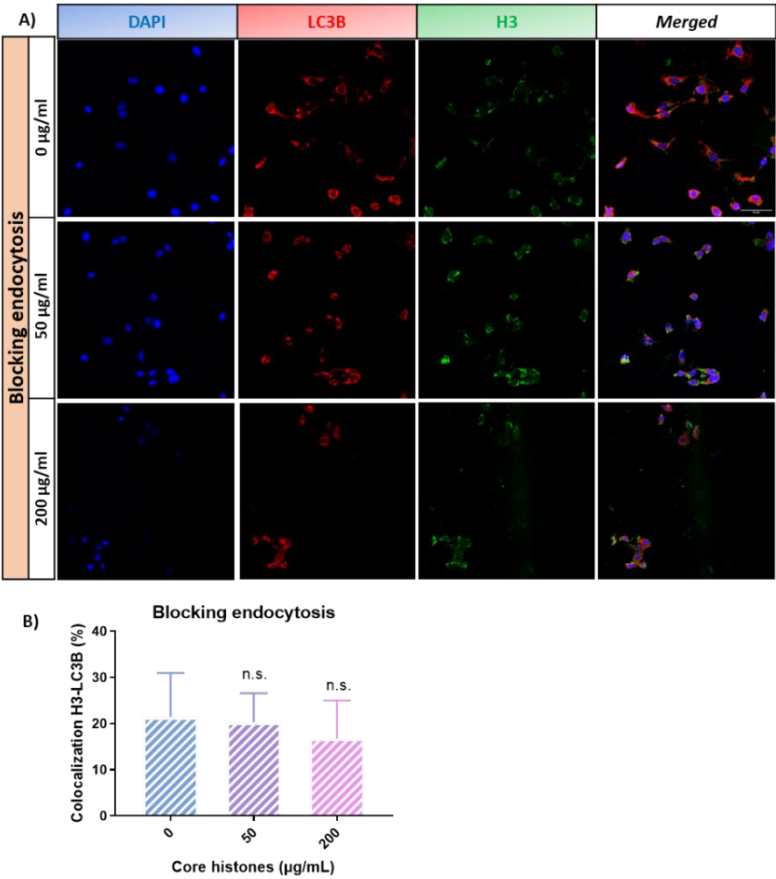


Figure 24. Effect of blocking endocytosis on colocalization between histone H3 and LC3B. (A) Confocal microscopy images of HUVEC cells subjected to endocytosis blockade conditions and treated with core histones at concentrations of 50 and 200 $\mu\text{g}/\text{ml}$. Each row shows images from the same field of view, with blue fluorescence corresponding to cell nuclei stained with DAPI, red fluorescence representing LC3B protein, and green fluorescence indicating histone H3 through binding to a secondary antibody conjugated to Texas Red and Alexa488 fluorophores, respectively. The fourth row shows the merged image of the three previous channels. Magnification 40X. Scale bar 52 μm . (B) Quantification of colocalization between histone H3 and LC3B protein under endocytosis blockade conditions performed using the TCS SP8 X confocal microscope software (Leica Microsystems; Germany). Mean values are represented as bars $\pm$ standard deviation ($n=3$). Significant differences were considered at $p<0.05$ calculated using ANOVA one-way indicated by n.s. as non-significant.

To accomplish this, HUVEC cells were cultured until reaching 80% confluence. Once this point was reached, the cells were incubated on ice for fifteen minutes, followed by treatment with 50 $\mu\text{g}/\text{mL}$ of histone H1-488 for 24 hours at 4°C, in the case of blocking endocytosis, and in the control condition cells were treated with 50 $\mu\text{g}/\text{mL}$ of histone H1-488 for 24 hours at 37°C. The cell nuclei were stained with DAPI, histone H1 was directly observed through its fluorescent tag and the LC3B protein was labelled using indirect immunofluorescence staining. Figure 25A presents examples of

fluorescence microscopy images for the analysed conditions. As depicted in *Figure 25B*, a decrease in colocalization was observed when cells were exposed to blocking endocytosis conditions. These results are consistent with previous findings using the histone core treatment, thereby indicating endocytosis as the pathway for histone entry into HUVEC cells.

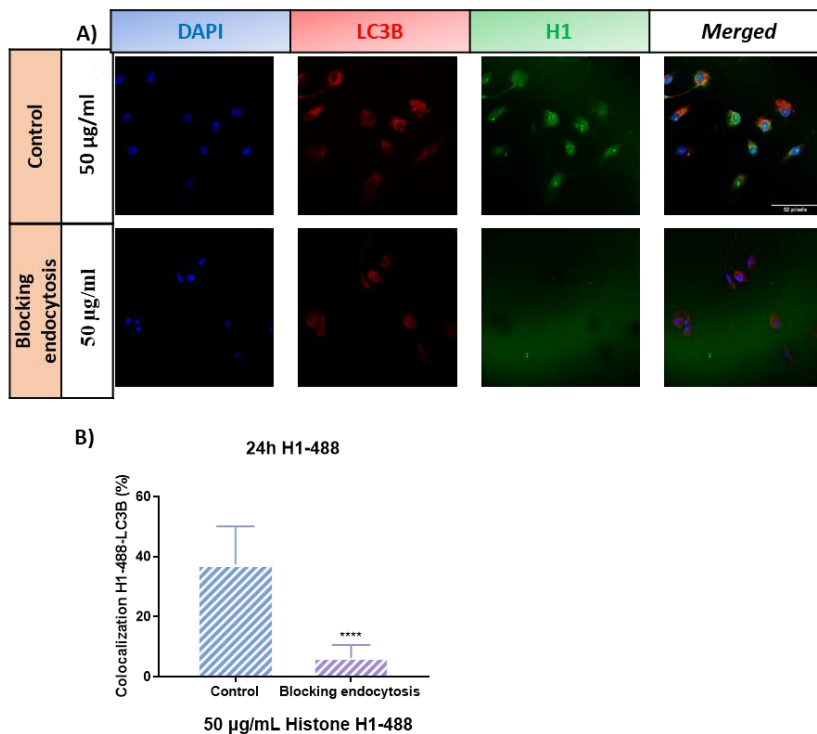


Figure 25 Effect of blocking endocytosis on colocalization between histone H1-488 and LC3B. (A) Confocal microscopy images of HUVEC cells subjected or not to endocytosis blockade conditions and treated with histone H1-488 at 50 µg/ml. Each row shows images from the same field of view, with blue fluorescence corresponding to cell nuclei stained with DAPI, red fluorescence representing LC3B protein, and green fluorescence indicating histone H1 directly observed through its green, fluorescent tag. The fourth row shows the merged image of the three previous channels. Magnification 40X. Scale bar 52µm. (B) Quantification of colocalization between histone H1-488 and LC3B protein under endocytosis blockade conditions performed using the TCS SP8 X confocal microscope software (Leica Microsystems; Germany). Mean values are represented as bars $\pm$ standard deviation ($n=3$). Significant differences were considered at $p<0.05$ calculated using t-test calculated by t-test (**** when $p<0.0001$).

To further confirm the entry of histones into cells in the case of treatments with histone H1-488, if the histone was indeed entering the cells, quantifiable FITC signals (corresponding to the tag of histone H1) should be obtained within the cells, as we have previously observed green dots corresponding to histone H1-488 in the immunofluorescence images. Therefore, cells were treated with different

concentrations of histone H1-488 (0, 10, 25, and 50 $\mu\text{g/mL}$) for 24 hours at 37°C (standard treatment conditions). In parallel, endocytosis was blocked as explained earlier, in addition to treating the cells with 10 $\mu\text{g/mL}$ of H1-488. Subsequently, the cells were collected and the FITC signal from histone treatment was analysed using flow cytometry. In this case, if we obtain FITC signal when cells are treated at 37°C with histone H1-488 this would mean that the histone is being internalized. Results are represented in *Figure 26A*. As it can be observed, even the lowest concentration of histone H1-488 significantly increased the FITC signal within the cells compared to the condition without histone (0 $\mu\text{g/mL}$), and this signal remained consistent at higher treatment doses. This indicates that histone H1-488 is indeed present within the cells. On the other hand, in the condition treated with 10 $\mu\text{g/mL}$ of histone H1-488 but incubated on ice for blocking endocytosis, there is just a residual FITC signal, with no significant differences compared to untreated cells. Additionally, a drastic difference can be observed between the condition of 10 $\mu\text{g/mL}$ of histone H1-488 cultured under normal conditions and the condition of 10 $\mu\text{g/mL}$ of histone H1-488 with blocked endocytosis. All of this suggests histone entry through endocytosis. Lastly, we wanted to determine if blocking histone entry had a beneficial effect on cells, thus confirming that the cytotoxic effects observed with core histone treatments depend on histone entry through endocytosis or just produced after histone contact with the cell membrane. To do this, HUVEC cells were cultured and treated with 400 $\mu\text{g/mL}$ of histone core for 4 hours. In the control condition, this incubation was carried out at 37°C, while in the endocytosis-blocking condition, the cells were incubated at 4°C as explained earlier. After completing the treatments, cell survival was analysed using flow cytometry, employing an apoptosis study kit with Annexin V-FITC, as described in section 4 of the *Materials and Methods* section. The results are presented in *Figure 26B*.

As observed in *Figure 26B*, treatment with 400 $\mu\text{g/mL}$ of histone core for 4 hours is cytotoxic to the cells, however, when cells are incubated at 4°C, cell survival increases compared to normal conditions, indicating a beneficial effect of endocytosis blockade as it prevents histone entry into the cells.

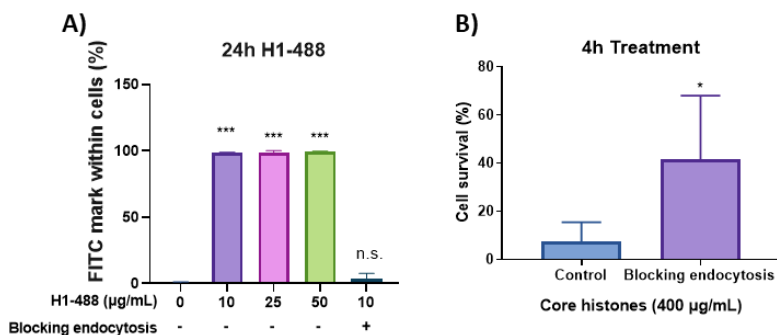


Figure 26. Blocking endocytosis prevents histone entry to HUVEC cells and reduces histone-induced cytotoxicity. (A) HUVEC cells were incubated in the absence or presence of several concentrations of H1-488 for 24h, and one condition with both 10 µg/mL H1-488 treatment and blocking endocytosis by incubating cells on ice. After, the cells were harvested and the FITC mark within cells was analysed by flow cytometry. (B) HUVEC cells were incubated in the presence of 400 µg/mL of core histones for 4h at 37 °C in the case of control condition and on ice in the condition of blocking endocytosis. After, the cells were harvested and labelled using an Annexin V kit, and subsequently the survival rate was analysed by flow cytometry. Mean survival values are presented as bars, expressed as a percentage $\pm$ standard deviation ($n=3$), with statistically significant differences considered when $p<0.05$, calculated using (A) ANOVA one-way respect 0 or (B) t-test respect control, indicated by “n.s.” when is non-significant, * from $p<0.05$, and *** when $p<0.001$.

2.6 Effect of Modulation of Autophagy on Histone-Mediated Cytotoxicity and Colocalization with LC3B

To elucidate the role of autophagy in histone-mediated cytotoxicity and provide insights into the mechanisms underlying histone-induced cellular responses, specifically focusing on the interplay between histones and the LC3B autophagy marker and considering the link between the administration of extracellular histones and the induction of autophagy in endothelial cells, as suggested by previous results of our group (67), we continued to study the effect of the modulation of autophagy on cell survival and histone internalization in endothelial cells.

Firstly, we evaluated the incubation times with autophagy modulators. For autophagy inhibition, a mixture of 20 mM NH_4Cl and 100 nM leupeptin was used, which inhibits phagosome-lysosome fusion. For autophagy activation, Krebs-Henseleit salt medium, lacking nutrients for the cells, was employed. The cells were cultured and treated with the autophagy inhibitor mixtures for 1, 2, 3, and 5 hours; and with the autophagy activator for 5 and 24 hours. Subsequently, cells were collected, proteins were extracted, and the presence of LC3B and p62, a protein located in the

autophagosome, was assessed by Western blot, as described in sections 2.1 and 2.3 of the *Materials and Methods* section. The band intensities were quantified using densitometry. Results are shown in *Figure 27*. As depicted in the graphs (*Figure 27A*), LC3B II decreases when the activation treatment is extended to 24 hours, whereas it increases when autophagy is inhibited for 3 or 5 hours, resulting in decreased LC3B I levels. Furthermore, in *Figure 28B*, it can be observed that the p62 protein follows the same pattern as LC3B, decreasing when autophagy is activated for 24 hours and increasing when it is inhibited. This is due to the fact that when autophagy is inhibited, the compounds to be degraded and the components of the autophagy pathway accumulate in cells although autophagy never resolves. Based on these findings, the chosen autophagy modulation conditions for subsequent experiments were a 3-hour incubation with 20 mM NH₄Cl and 100 nM leupeptin for inhibition, and a 24-hour incubation with Krebs-Henseleit medium for autophagy activation.

Then, we wanted to determine the role of autophagy in histone-mediated cytotoxicity, so the impact of this process and its modulation on the survival capacity of endothelial cells was examined (*Figure 29*). In order to achieve this, cultured HUVEC cells were subjected to treatment for 3 hours with NH₄Cl (20 mM) and Leupeptin (100 nM). Alternatively, cells were cultured for 24 in the presence of Krebs-Henseleit medium. Subsequently, the cells were treated with 50 and 200 µg/ml of core histones for 4h (*Figure 29(A)*) or with histone H1-488 at a concentration of 50 µg/ml for 24 hours (*Figure 29(B)*), based on previous results. Cells were labelled using an Annexin V kit, and the cell survival was analysed by flow cytometry.

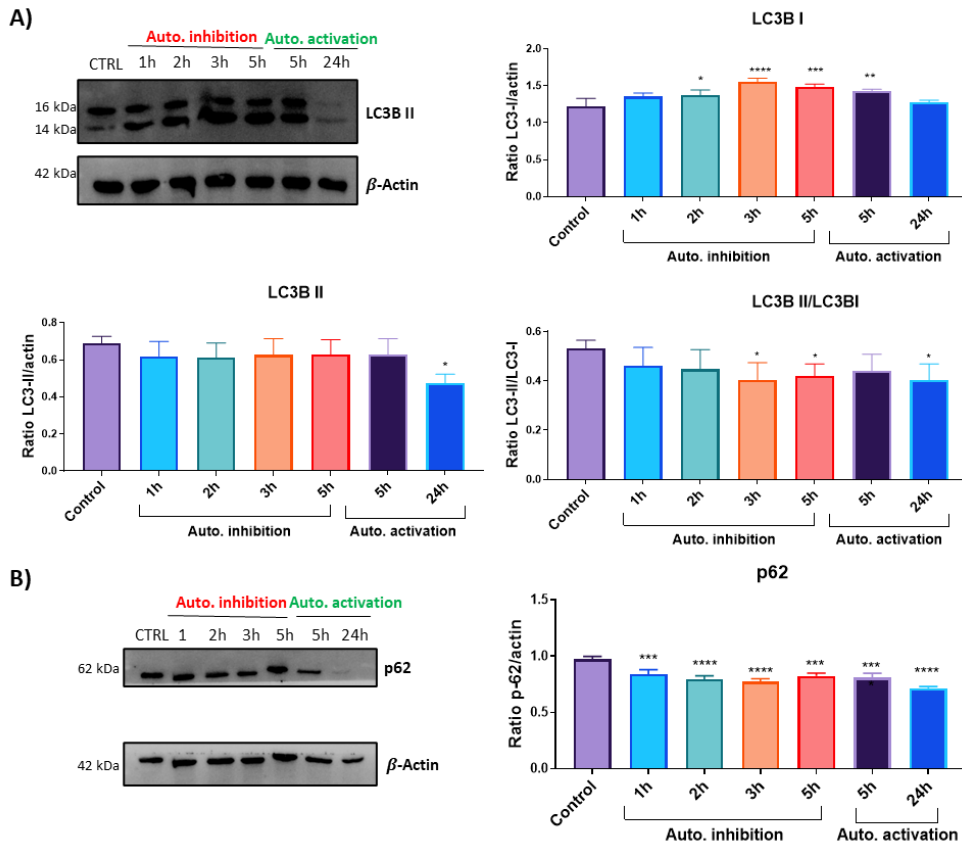


Figure 27. LC3B-II and p62 protein responses after autophagy modulation. HUVEC cells were treated with autophagy inhibitors (20 mM NH₄Cl and 100nM Leupeptin) and examined at 1, 2, 3 and 5 hours post-treatment; or autophagy upregulator (Krebs-Henseleit medium) and examined at 5 and 24 hours post-treatment. Western blot analysis is presented for (A) LC3B-I/II and (B) p62 levels, with a β -actin loading control. The histograms are the quantification of the bands shown analysed by densitometry in respect to actin. The mean values are shown as bars, expressed as a ratio $\pm$ the standard deviation ($n=3$) with statistically significant differences considered when $p<0.05$, calculated using ANOVA one-way respect control, indicated by * from $p<0.05$, *** when $p<0.001$ and **** when $p<0.0001$.

In the case of core histone treatment, as previously demonstrated, treatment with concentrations up to 200 μ g/mL does not decrease cell viability (Figure28A). Furthermore, we can observe that modulation of autophagy, whether increased or decreased, does not significantly affect cell survival. However, there is a trend indicating that at higher histone treatment doses, autophagy activation appears to be more beneficial for cell survival than inhibition.

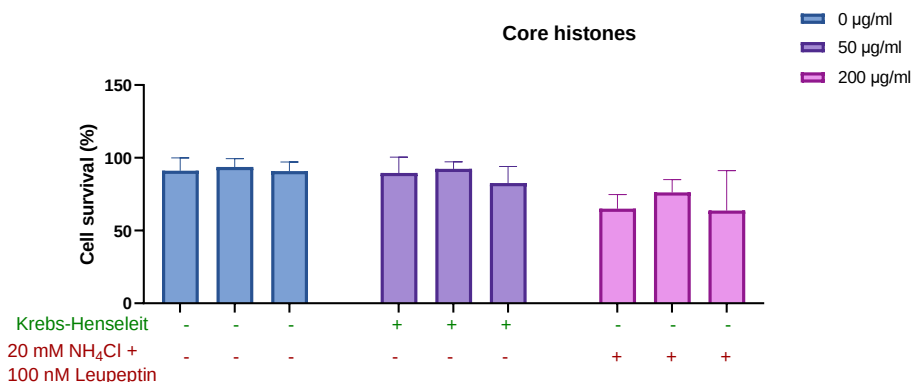
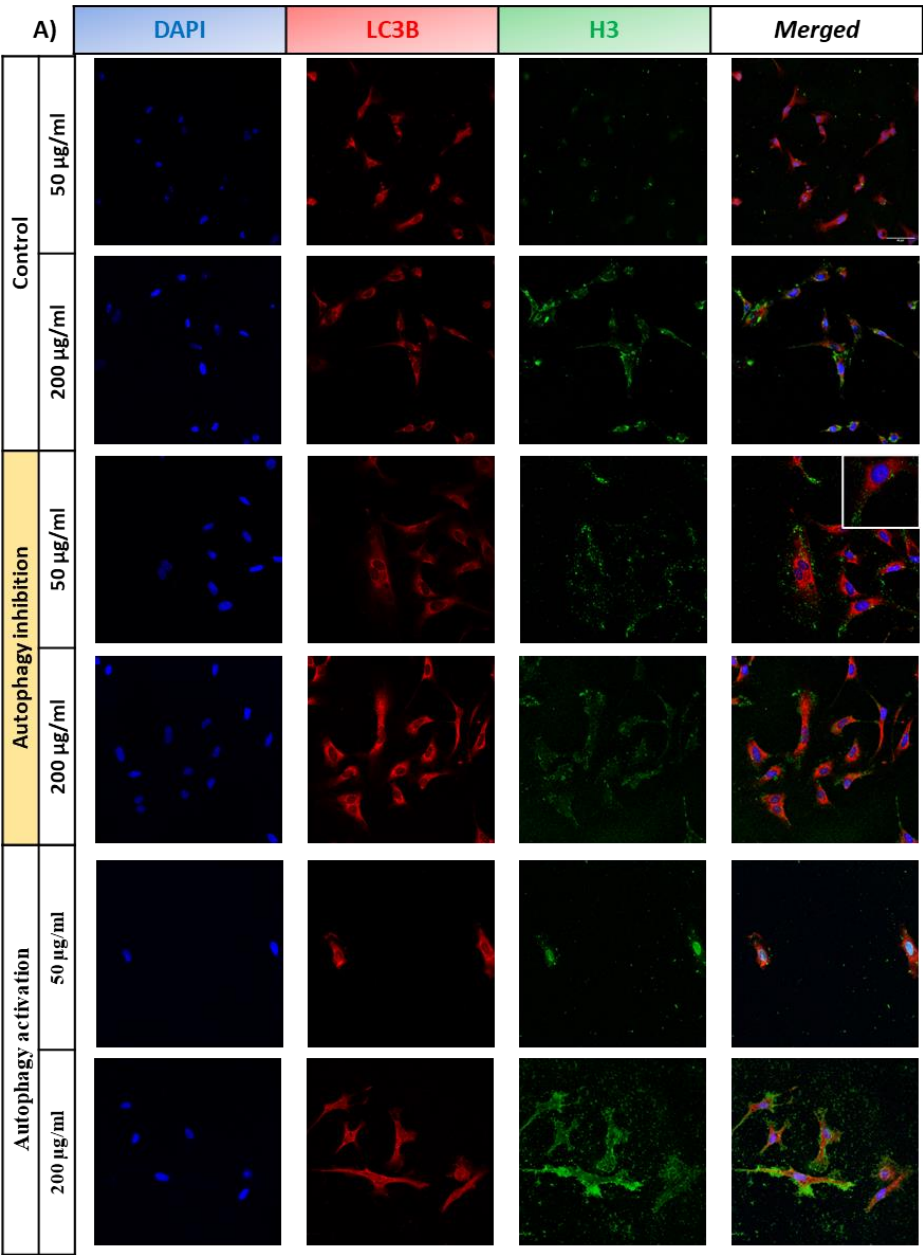


Figure 28. Effect of autophagy modulation on histone-mediated cytotoxicity. Cells cultured in the absence or presence of 50 or 200 µg/mL of core histones for 4h. In the case of autophagy inhibition cells were treated with 20mM NH₄Cl and 100nM Leupeptin during the last 3h of histone treatment, instead, for autophagy activation cells were incubated with Krebs-Henseleit medium for 24h and the histone treatment was added during the last 4h. The mean values are represented as bars, expressed as a percentage ± standard deviation (n=3).

Next, we aimed to analyse the impact of autophagy modulation on the colocalization between histones and LC3B protein using immunofluorescence and confocal microscopy (*Figures 29 and Figure 30*). Initially, HUVEC cells were cultured for 3 hours with NH₄Cl (20 mM) in combination with Leupeptin (100 nM). Alternatively, for autophagy induction, cells were incubated for 24 hours with Krebs-Henseleit medium. At the same time, core histones were administered at concentrations of 50 and 200 µg/ml for 4 hours (based on previous results) (*Figure 29*), or H1-488 histone was administered at a concentration of 50 µg/ml for 24 hours (*Figure 30*). In both cases, LC3B protein and histone were labelled using secondary antibodies conjugated to a fluorophore, and cell nuclei were stained with DAPI (see section 3.2 of *Materials and Methods* section). After obtaining and quantifying the images from the experiments with both core histone treatment and H1-488 histone treatment using the confocal microscopy software TCS SP8 X (Leica Microsystems; Germany), as shown in the confocal microscopy images (*Figure 29A* and *Figure 30A*), the inhibition of autophagy resulted in a significant decrease in the colocalization between histone H3 and LC3B protein. Conversely, increasing autophagy also led to a decrease in colocalization, although not as pronounced as in the case of inhibition (*Figure 29B*). Furthermore, in the case of H1-488 histone treatment, as observed in *Figure 30B*, the inhibition of autophagy only caused a significant reduction in colocalization when cells were also subjected to histone treatment. In contrast, when autophagy was increased, the

colocalization between H1-488 and LC3B protein decreased regardless of whether cells were treated with histone or not.



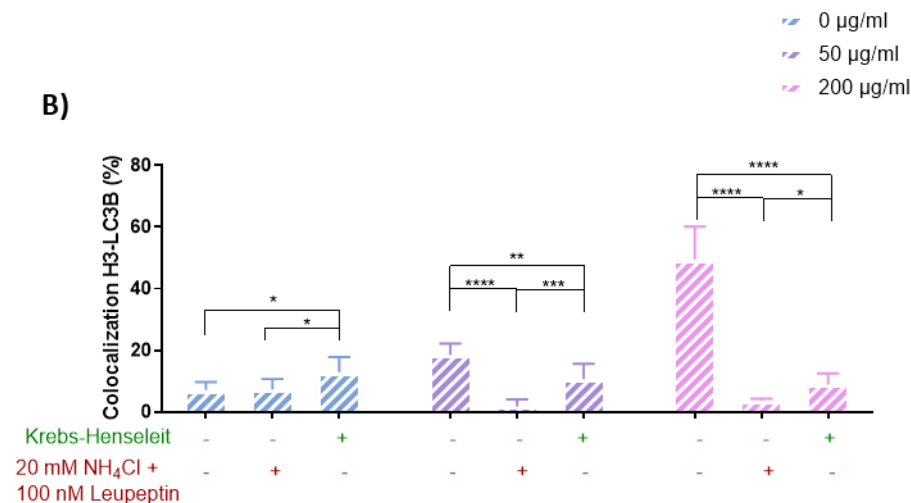
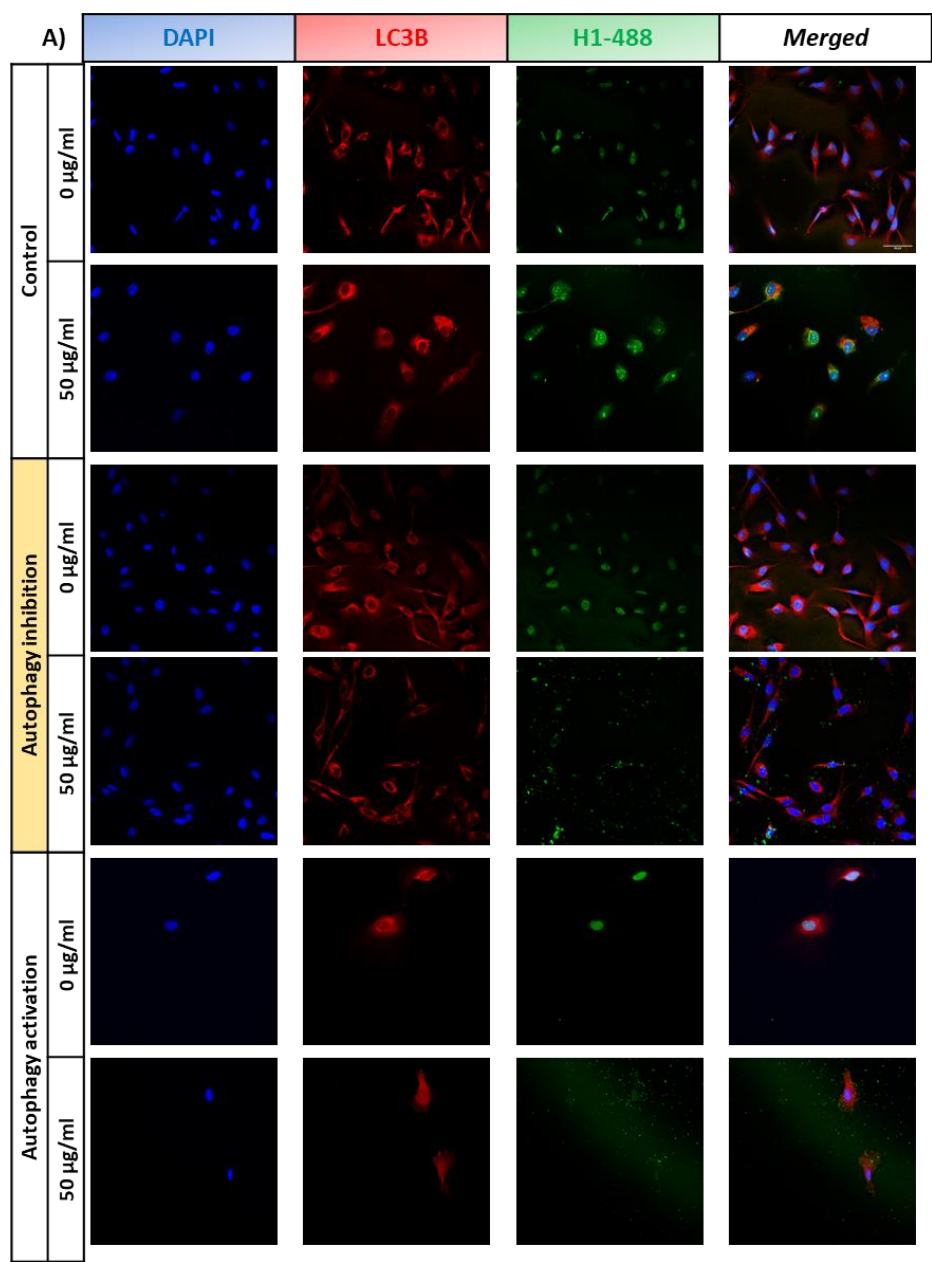


Figure 29. Effect of autophagy modulation on colocalization between histone H3 and LC3B. (A) Confocal microscopy images of HUVEC cells from selected analysed conditions. Shown are images corresponding to two of the control conditions used (50 and 200 µg/mL of core histones), cells treated with autophagy inhibitors NH₄Cl (20 mM) and Leupeptin (100 nM) for 3 hours and treated with 50 and 200 µg/mL of core histones for 4 hours, and cells treated with autophagy activator (Krebs-Henseleit medium) for 24 hours with the last 4 hours including treatment with 50 and 200 µg/mL of core histones. Each row represents images from the same field of view. Blue fluorescence corresponds to cell nuclei stained with DAPI, red fluorescence represents LC3B protein, and green fluorescence represents histone H3 labelled with secondary antibodies conjugated to Texas Red and Alexa488, respectively. Additionally, a fourth column shows the merged image of all three channels. Magnification, 40X. Scale bar 45 µm. The insert was obtained applying a 4x zoom of the indicated image. (B) Quantification of colocalization between histone H3 and LC3B in different conditions using the software of the TCS SP8 X confocal microscope (Leica Microsystems; Germany) (see section 3.5.2 Materials and Methods). The mean values are represented as bars, expressed as a percentage $\pm$ standard deviation (n=3), with statistically significant differences compared to the control (without autophagy modulation) indicated by " * " for p<0.05, " ** " for p<0.01, " *** " for p<0.001, and " **** " for p<0.0001, calculated using two-way ANOVA.



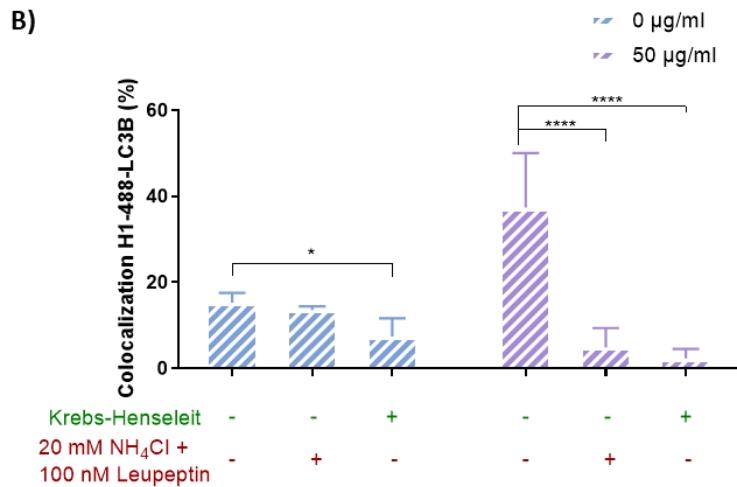


Figure 30 Effect of autophagy modulation on colocalization between histone H1-488 and LC3B. (A) Confocal microscopy images of HUVEC cells from selected analysed conditions. Shown are images corresponding to two of the control conditions used (50 µg/mL of histone H1-488 for 24h), cells treated with autophagy inhibitors NH₄Cl (20 mM) and Leupeptin (100 nM) for 3 hours, with 50 µg/mL histone H1-488 treatment for 24 hours, and cells treated with autophagy activator (Krebs-Henseleit medium) for 24 hours with treatment with 50 µg/mL of histone H1-488. Each row represents images from the same field of view. Blue fluorescence corresponds to cell nuclei stained with DAPI, red fluorescence represents LC3B protein labelled with secondary antibodies conjugated to Texas Red, and green fluorescence represents histone H1-488. Additionally, a fourth column shows the merged image of all three channels. In control condition (0 µg/mL of histone H1-488), endogenous histone was labelled in order to obtain colocalization data. Magnification, 40X. Scale bar 45 µm. (B) Quantification of colocalization between histone H3 and LC3B in different conditions using the software of the TCS SP8 X confocal microscope (Leica Microsystems; Germany) (see section 3.5.2 Materials and Methods). The mean values are represented as bars, expressed as a percentage $\pm$ standard deviation ($n=3$), with statistically significant differences compared to the control (without autophagy modulation) indicated by " * " for $p<0.05$ and " *** " for $p<0.0001$, calculated using two-way ANOVA.

Taken together, these results indicate a significant subcellular interaction between different extracellularly applied histones and the autophagy marker LC3B in HUVEC cell cultures, as well as the presence of these added histones in acidic lysosomal vesicles. This interaction is dramatically reduced under conditions of endocytosis inhibition and autophagy modulation, suggesting the existence of mechanisms for histone entry into endothelial cells and their processing through the autophagy pathway.

2.7 Changes in release of HMGB1 upon core histone treatment and autophagy modulation.

Lastly, we aimed to investigate the presence of the nuclear protein HMGB1 in the culture medium of HUVEC cells exposed to different histone treatments. It is known that HMGB1 is also associated with sepsis, functioning as a pro-inflammatory cytokine, and since HMGB1 can be actively secreted as part of the inflammatory response, it can be found in the plasma of septic patients as a late predictor of developing septic shock and mortality (80,82). To achieve this objective, HUVEC cells were treated with core histones for 4 hours or histone H1-488 for 24 hours. After completing the treatments, the medium of the culture plates was collected and analysed using an HMGB1 ELISA kit, as briefly described in Section 6 of the *Materials and Methods* section.

The results shown in *Figure 31* demonstrate that when HUVEC cells were treated with increasing concentrations of core histones (*Figure 31A*), the secretion of HMGB1 into the medium increases in a dose-dependent manner. In the same way, when HUVEC cells were exposed to histone H1-488 (*Figure 31B*), a dose-dependent increased in the concentration of the protein in the medium was observed which could point to that extracellular histones induce the active secretion of HMGB1. These results reinforce the possibility of using HMGB1 as a sepsis biomarker and provide insights into the interaction between extracellular histones and HUVEC cells.

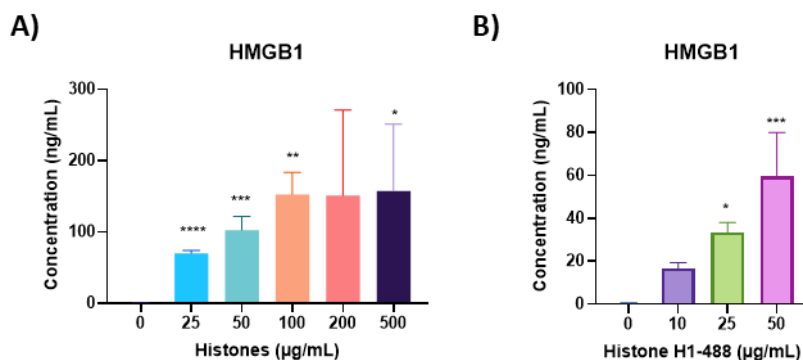


Figure 31. Quantification of HMGB1 on culture medium of HUVEC cells treated with different extracellular histones. HUVEC cells were treated with several concentrations of (A) core histones for 4h, or (B) histone H1-488 for 24h. After the treatments, the medium was collected and analyzed by ELISA. Bars represent mean values of concentration $\pm$ the standard deviation ($n=3$), considering significant differences with respect to the control (0 $\mu\text{g/mL}$) when $p<0.05$, calculated using ANOVA one-way indicated by * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$.

3. *Blood Vessel Organoid model*

3.1 Cytotoxic effects of extracellular histones in blood vessel organoids

Given the previous results obtained using different two-dimensional models of monolayer cell cultures, the relationship between cytotoxic potential of extracellular histones and the involvement of the autophagy pathway in these processes have been observed. Furthermore, it has been demonstrated that blocking endocytosis prevents histone entry into cells, leading to improvement in cell survival. However, the culture of HUVEC cells, despite being a more physiological model due to its endothelial origin, does not resemble the reality of an endothelium as it lacks three-dimensional structure. Therefore, to gain a more insightful perspective on the biological consequences of the interaction between circulating nucleoproteins and the endothelium, resembling the pathophysiological consequences observed in sepsis, and to validate the previously obtained results on cell culture models, we transitioned to a blood vessel organoid model.

The generation of vascular organoids is extensively described in section 1.2 of *Materials and Methods* section. Briefly, BVO cultures were generated from human iPSCs, which were differentiated into mesodermal lineage through the addition of differentiation factors, followed by vascular lineage differentiation. Once in this stage, the organoids were embedded in a Matrigel/collagen matrix, allowing vessel sprouting. Finally, mature organoids were individually cultured in 96-well plates. The process of organoid generation and maturation can be observed in the *Annex I* section, *Figure A1*.

After obtaining the vascular organoids, we proceeded to analyse the cytotoxicity induced by different extracellular histones in this more complex model (*Figure 32*). For this purpose, at least 16 organoids per condition were subjected to treatments with core histones for 4 hours (*Figure 32A*) or with histone H1-488 for 24 hours (*Figure 32B*). After the treatment, the organoids were collected and dissociated into individual cells, followed by staining with a viability dye. Cell survival was analyzed using flow cytometry, as described in section 4.1 of *Materials and Methods* section. The results indicate that core histones (*Figure 32A*) can cause a significant reduction in survival at high concentrations, starting from 500 µg/mL. However, histone H1-488 (*Figure 32B*) is unable to reduce viability under the analysed conditions. These results are consistent

with those previously obtained with the HUVEC cell model, with the organoids showing slightly more resistance to histone-mediated toxicity.

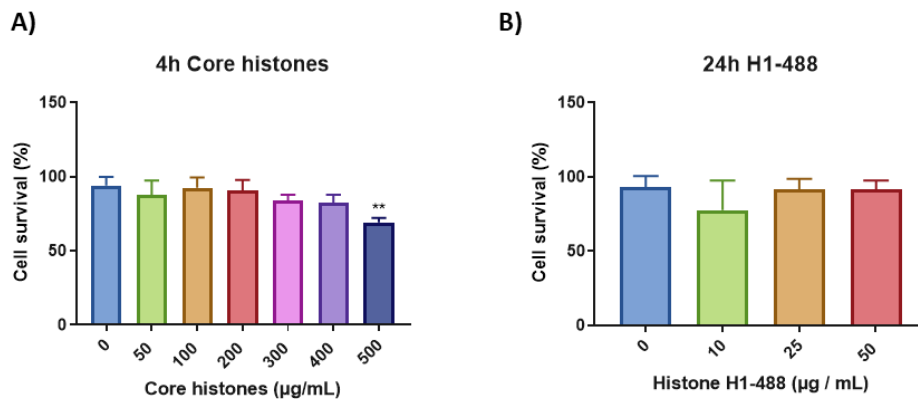


Figure 32. Analysis of histone-mediated cytotoxicity in blood vessel organoids (BVOs). BVOs were incubated in the absence or presence of several concentrations of (A) core histones for 4h or (B) histone H1-488 for 24h. After the indicated incubation times, the BVOs were harvested, disaggregated into single cells, and labelled using a viability dye, and subsequently analysed by flow cytometry. The mean values are shown as bars, expressed as a percentage $\pm$ the standard deviation ($n=3$). Statistical significance was considered at a p -value < 0.05 , calculated in all cases using a one-way ANOVA statistical test indicated by ** when $p < 0.01$.

3.2 Localization of extracellular histones and autophagy flux marker LC3B in BVOs

After observing that extracellular histones also exhibit cytotoxic potential in the vascular organoid model, we aimed to investigate the localization of histones within the organoid and their potential relationship with the autophagy pathway, as previously demonstrated in endothelial cells. To accomplish this, once the organoids were differentiated and matured, they were treated with core histones for 4 hours (Figure 33) or with histone H1-488 (Figure 34) for 24 hours, at different concentrations with a minimum of five organoids per condition. Subsequently, the organoids were collected and processed for immunofluorescence staining, as described in section 3.1.2 of *Materials and Methods* section, using LC3B, H3 and CD31 (an endothelial transmembrane protein used as a marker of endothelial cells since in the organoid several cell types are found) antibodies followed by a species-specific secondary antibody conjugated to a fluorescent tag, and in the case of H1, through its own autofluorescence. Confocal microscopy images of at least three organoids per condition

were captured. The average fluorescence intensity of the green signal corresponding to histone H3 (Figure 33B) or H1-488 (Figure 34C) and the red signal corresponding to the autophagic flux protein LC3B (Figure 33C and 33D) were quantified using ImageJ, by measuring the average fluorescence of each image and subtracting the background.

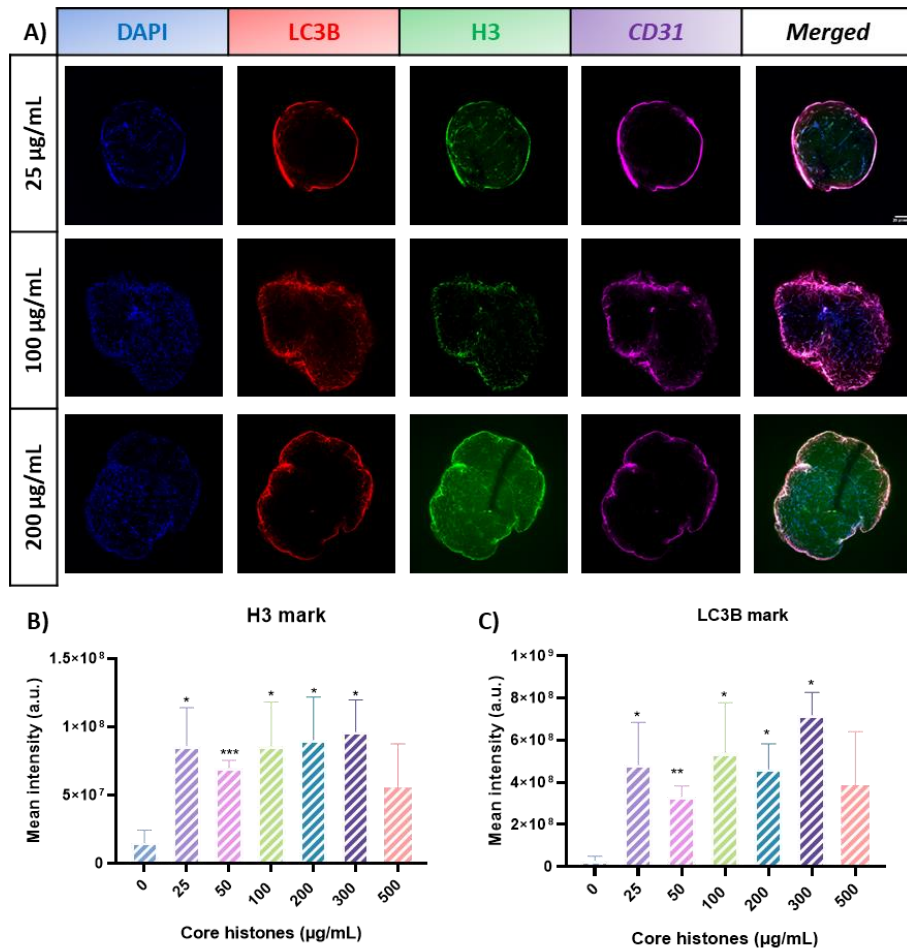


Figure 33. Analysis of histone H3 and LC3B on blood vessel organoids (BVOs) treated with core histones. (A) Confocal microscopy images of BVOs that were subjected to treatment with several concentrations of core histones for 4 hours. The blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one to histone H3 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the fourth channels used. Magnification, 10X. Scale bar 20 µm. (B) Quantification of the mean fluorescence intensity of green channel (H1-488) by Image J. (C) Quantification of the mean fluorescence intensity of red channel (LC3B) by ImageJ. The histogram's bars represent the mean values $\pm$ the standard deviation of the percentage of colocalization of each condition (n=3). Statistically significant differences were considered with respect to the control (0 µg/mL), and between the different conditions when $p < 0.05$, calculated using ANOVA one-way indicated by * when $p < 0.05$, ** from $p < 0.01$ and *** when $p < 0.001$.

In *Figure 33*, some of the tested conditions are shown (the rest can be observed in *Annex I* section *Figure A.7*) and it can be observed that even at low doses, the signal corresponding to histone H3 no longer coincides with the nuclear signal (which would correspond to endogenous histone H3), but instead shows a more homogeneous distribution throughout the organoid, particularly at the organoid's edge and centre. Furthermore, in the quantification of green fluorescence (*Figure 33B*) a significant increase in fluorescence intensity is observed starting from the lowest dose (25 µg/mL), which is maintained at all concentrations except for the highest dose (500 µg/mL), possibly due to the cytotoxic effects previously observed. Regarding the signal of the autophagic flux marker protein LC3B, it is also predominantly distributed at the organoid's edge (*Figure 33A*), and the fluorescence intensity follows the same pattern, increasing when exposed to core histone treatment, except at the highest dose (*Figure 33C*).

Then, the same experiment was performed but using histone H1-488 as treatment instead of core histones, to ensure that the green mark observed corresponded to the treatment with extracellular histones and not the endogenous ones. As observed in the images, even at low concentrations of histone H1-488, histone staining is evident in the organoid, predominantly localized at the outer edge and centre of the organoid (*Figure 34B*). In the quantification analysis (*Figure 34C*), a significant dose-dependent increase in the intensity of the histone H1-488 signal can be observed. Additionally, the red signal is also distributed along the organoid edge, colocalizing with the green signal. The same behaviour is observed with the red signal corresponding to LC3B, which increases with higher histone treatment concentrations (*Figure 34D*), suggesting that the treatment at not very high doses enhances autophagy while treatment with high doses (500 µg/mL) leads to cell death and not the activation of autophagy.

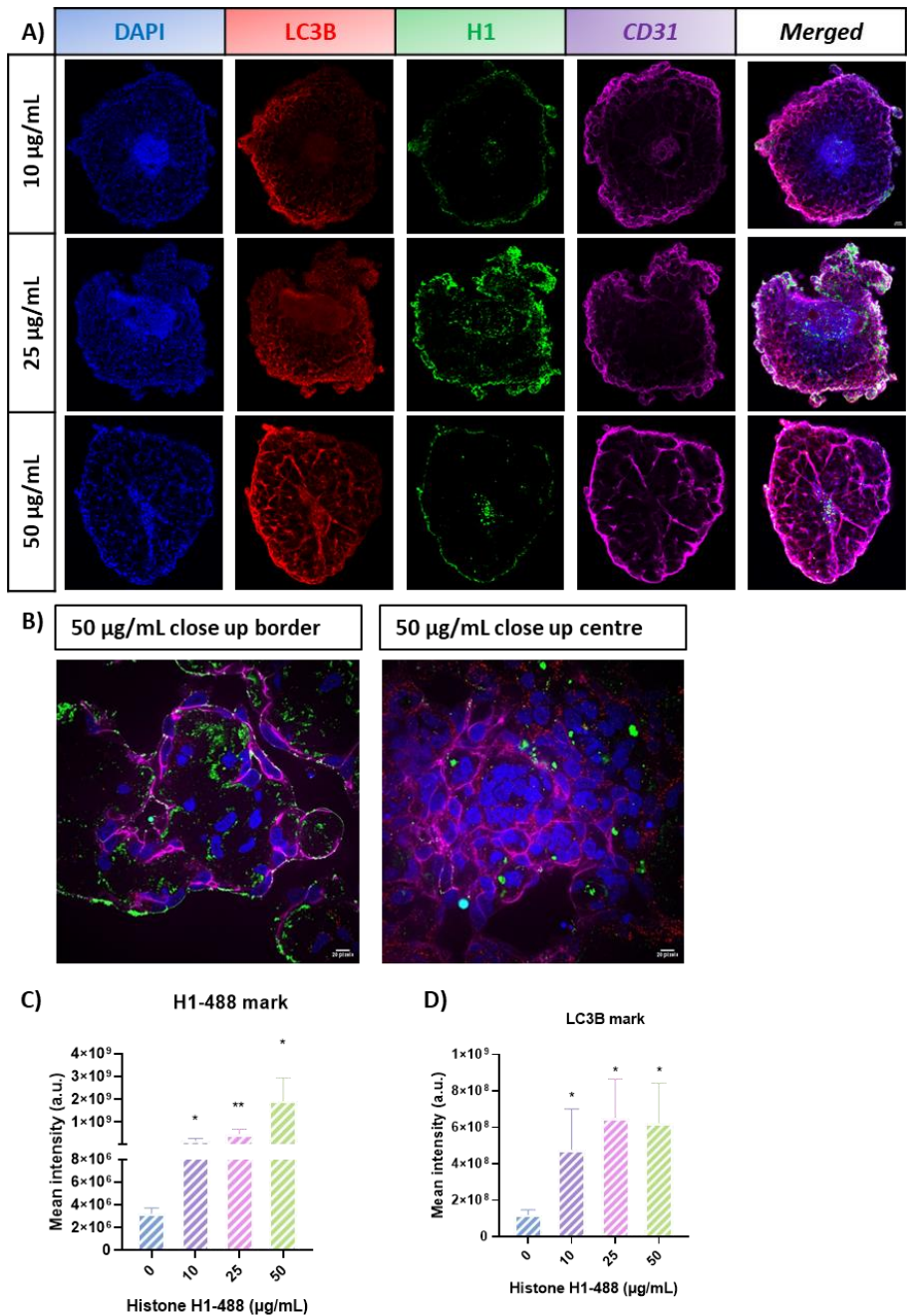


Figure 34 Analysis of histone H1-488 and LC3B on blood vessel organoids (BVOs) treated with histone H1-488. (A) Confocal microscopy images of BVOs that were subjected to treatment with several concentrations of histone H1-488 (10, 25 and 50 $\mu\text{g/mL}$) for 24 hours. The blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one with the autofluorescence of histone H1-488 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the fourth channels used. Magnification, 10X. Scale bar 60 μm (B) Images from the border and the center of the organoid treated with 50 $\mu\text{g/mL}$ of histone H1-488 showing a fusion of the fourth channels used with a

magnification of 40X. Scale bar 20 μm (C) Quantification of the mean fluorescence intensity of green channel (H1-488) by Image J. D) Quantification of the mean fluorescence intensity of red channel (LC3(B) by ImageJ. The histogram's bars represent the mean values $\pm$ the standard deviation of the percentage of colocalization of each condition ($n=3$). Statistically significant differences were considered with respect to the control (0 $\mu\text{g/mL}$), and between the different conditions when $p<0.05$, calculated using ANOVA one-way indicated by * when $p<0.05$ and ** from $p<0.01$.

3.3 Effect of Modulation of Autophagy on Histone-Mediated changes in BVOs.

As previously observed, the addition of sublethal concentrations of extracellular histones has been shown to increase the fluorescent signal of LC3B, thereby leading to the induction of the autophagy pathway due to the treatment. In order to provide insights into the mechanisms underlying histone-induced cellular responses, we sought to investigate the effect of autophagy modulation using a more physiological experimental model, such as BVOs.

Firstly, the impact of autophagy modulation on BVO survival was evaluated. To achieve this, differentiated and matured BVOs were exposed to a treatment with 10 $\mu\text{g/mL}$ of histone H1-488 for 24 hours. In some experimental conditions, BVOs were also treated with NH_4Cl (20 mM) and Leupeptin (100 nM) during the last 3 hours of histone treatment or cultured for 24 hours in the presence of Krebs-Henseleit medium. Subsequently, BVOs were collected and dissociated into individual cells, followed by staining with a viability dye. The survival of cells was then analysed using flow cytometry, as described in section 4.1 of *Materials and Methods* section. *Figure 35* illustrates the results, indicating that the modulation of autophagy had no significant impact on the survival of organoids.

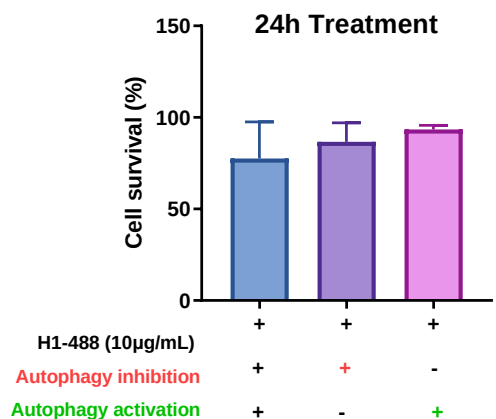


Figure 35. Effect of autophagy modulation on histone-mediated cytotoxicity. Blood vessel organoids (BVOs) were cultured in the presence of 10 µg/mL of histone H1-488 for 24h. In the case of autophagy inhibition BVOs were treated with 20mM NH₄Cl and 100nM Leupeptin during the last 3h of histone treatment, instead, for autophagy activation BVOs were incubated with Krebs-Henseleit medium for 24h. The mean values are represented as bars, expressed as a percentage ± standard deviation (n=3), with statistically significant differences compared to the control (without autophagy modulation).

Subsequently, we aimed to analyse the impact of autophagy modulation on the localization of histones and the protein LC3B, as well as their expression levels, utilizing immunofluorescence and confocal microscopy techniques (Figure 36 and Figure 37). Initially, BVOs were cultured for 3 hours in the presence of NH₄Cl (20 mM) combined with Leupeptin (100 nM). Alternatively, to induce autophagy, cells were incubated with Krebs-Henseleit medium for 24 hours. Concurrently, core histones were administered at concentrations of 200 and 500 µg/mL for 4 hours (Figure 36), while H1-488 histone was administered at a concentration of 10 µg/mL for 24 hours (Figure 37). After the treatments, organoids were collected and subjected to immunofluorescence staining, as outlined in section 3.1.2 of *Materials and Methods* section. The staining utilized antibodies specific to LC3B, H3, and CD31, followed by incubation with a species-specific secondary antibody conjugated to a fluorescent tag. In the case of H1, its location was assessed by its autofluorescence. Confocal microscopy was employed to capture images from at least three organoids per experimental condition. The average fluorescence intensity of the green signal corresponding to histone H3 (Figure 36B) or H1-488 (Figure 37B), as well as the red signal corresponding to the autophagic flux protein LC3B (Figure

36C and 36C), was quantified using *ImageJ*. This was accomplished by measuring the average fluorescence intensity of each image and subtracting the background signal.

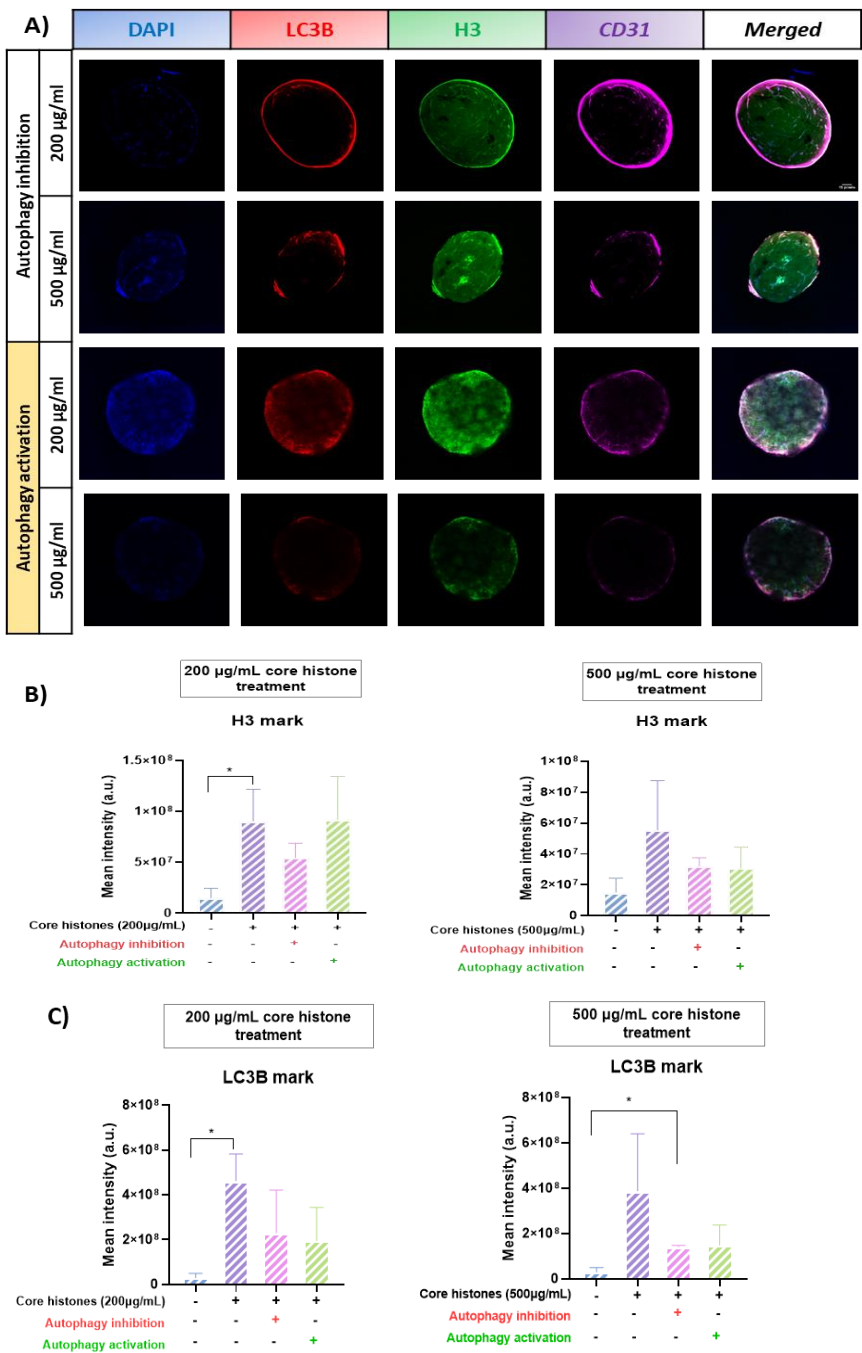


Figure 36 Effect of autophagy modulation on fluorescence of histone H3 and LC3B. (A) Confocal microscopy images of blood vessel organoids (BVOs) from selected analysed conditions. Images are shown BVOs treated

with autophagy inhibitors NH_4Cl (20 mM) and Leupeptin (100 nM) for 3 hours and treated with 50 and 200 $\mu\text{g/mL}$ of core histones for 4 hours, and cells treated with autophagy activator (Krebs-Henseleit medium) for 24 hours with the last 4 hours including treatment with 50 and 200 $\mu\text{g/mL}$ of core histones. The blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one to histone H3 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the fourth channels used. Magnification, 10X. Scale bar 15 μm . (B) Quantification of the mean fluorescence intensity of green channel (H1-488) by Image J. (C) Quantification of the mean fluorescence intensity of red channel (LC3(B) by ImageJ. The bars represent the mean values $\pm$ the standard deviation of the percentage of colocalization of each condition ($n=3$). Statistically significant differences were considered with respect to the control (0 $\mu\text{g/mL}$). and between the different conditions when $p<0.05$, calculated using ANOVA one-way indicated by * when $p<0.05$.

As depicted in *Figure 36*, the modulation of autophagy seems to be reduced the average fluorescence intensity of both LC3B and H3 when BVOs are treated with 200 or 500 $\mu\text{g/mL}$ of core histones compared to the control group treated with histones but without autophagy modulation, although not significantly. Furthermore, under the conditions of 500 $\mu\text{g/mL}$ treatment, it can be observed that even the fluorescence of the nuclei changes and decreases due to the toxicity induced by these high doses (shown in *Figure 32*), making it difficult to draw definitive conclusions. When focusing on the 200 $\mu\text{g/mL}$ dose, it can be observed that, although not statistically significant, the fluorescence intensity of H3 decreases when autophagy is inhibited and remains unchanged when activated. Regarding the fluorescence intensity of LC3B, it decreases whether autophagy is activated or inhibited, but the changes are not statistically significant. However, further assays should be needed in order to draw conclusions.

In *Figure 37*, the impact of autophagy modulation on organoids treated with H1-488 histone for 24 hours is demonstrated. A clear change in the localization of the H1-488 signal is observed, shifting from being present throughout the organoid when autophagy is inhibited to being predominantly localized at the borders when autophagy is enhanced. However, in terms of signal intensity, no significant differences are observed between the two groups or compared to the control group treated with histone without autophagy modulation (*Figure 37B*). On the other hand, the LC3B signal shows a reduction, albeit not statistically significant, when autophagy is modulated, with a more pronounced decrease when autophagy is activated.

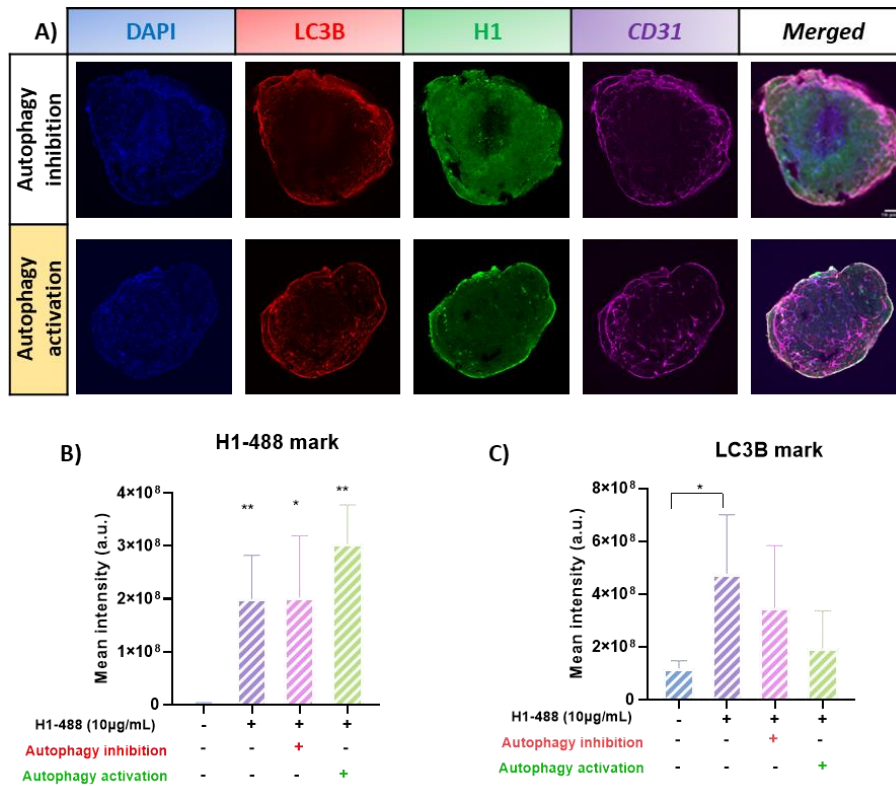


Figure 37. Effect of autophagy modulation on fluorescence of histone H1.488 and LC3B. (A) Confocal microscopy images of blood vessel organoids (BVOs) from selected analysed conditions. Images are shown BVOs treated with autophagy inhibitors NH_4Cl (20 mM) and Leupeptin (100 nM) for 3 hours and treated with 10 $\mu\text{g}/\text{mL}$ of histone H1-488 for 24 hours, and cells treated with autophagy activator (Krebs-Henseleit medium) for 24 hours with 10 $\mu\text{g}/\text{mL}$ of histone H1-488. The blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one to histone H1-488 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the fourth channels used. Magnification, 10X. Scale bar 20 μm (B) Quantification of the mean fluorescence intensity of green channel (H1-488) by Image J. (C) Quantification of the mean fluorescence intensity of red channel (LC3B) by ImageJ. The bars represent the mean values $\pm$ the standard deviation of the percentage of colocalization of each condition ($n=3$). Statistically significant differences were considered with respect to the control (0 $\mu\text{g}/\text{mL}$), and between the different conditions when $p < 0.05$, calculated using ANOVA one-way indicated by * when $p < 0.05$ and ** when $p < 0.01$.

In summary, the modulation of autophagy had a non-significant impact on the fluorescence intensity of LC3BII and H3 when BVOs were treated with core histones. Higher doses of histones induced toxicity, affecting nuclear fluorescence. Autophagy modulation altered the localization of H1-488 histone but did not significantly affect its fluorescence intensity. Overall, these findings provide preliminary insights into the relationship between autophagy modulation and histone-induced cellular responses, highlighting the need for further investigation.

3.4 Effect of endocytosis blockade on the histone mediated changes in BVOs

Based on previous evidence in the literature and our previous findings with HUVEC cells, we sought to investigate whether extracellular histones could penetrate the interior of organoids, potentially leading to reduced viability, by utilizing the endocytic pathway as previously postulated. Additionally, we aimed to determine whether blocking endocytosis would prevent histone entry and have beneficial effects on vascular organoid survival. To accomplish this, differentiated and matured BVOs were subjected to a 15-minute incubation on ice at 4°C, as described in studies by Wang et al. (189) and as previously shown in *section 5 of Chapter 2*, to block endocytosis. Subsequently, BVOs were treated with various concentrations of histone H1-488 (0, 10, 25, and 50 µg/mL) for 24 hours at 37°C under standard treatment conditions (*Figure 38A*). In another condition, BVOs were treated with 10 µg/mL of H1-488 and endocytosis was blocked or not as previously explained (*Figure 38B*). Furthermore, BVOs were treated with 500 µg/mL of core histones for 4 hours at both 37°C and 4°C to block endocytosis (*Figure 38C*). Subsequently, the cells were collected, and the FITC signal resulting from histone treatment was analysed using flow cytometry. Additionally, the cell survival rate was analysed using flow cytometry for the core histone treatment, as previously described. As observed in *Figure 38A*, even the lowest concentration of histone H1-488 significantly increased the FITC signal within the cells compared to the condition without histone (0 µg/mL) and this signal remained consistent at higher treatment doses when BVOs were cultured under standard conditions. These findings indicate that histone H1-488 is indeed present within the organoids. Conversely, in the condition treated with 10 µg/mL of histone H1-488 but with blocked endocytosis (*Figure 38B*), a minimal FITC signal was observed, with highly significant differences compared to BVOs treated with the same concentration of histone H1-488 but with normal endocytic function. In addition, *Figure 38C* shows that blocking endocytosis prevents histone mediated cytotoxicity, since when BVOs are treated with 500 µg/mL of core histones the survival is highly decreased but when the organoids were incubated at 4°C for blocking endocytosis, survival is recovered being not significant the difference between BVOs which were not exposed to any histone treatment. Taken together these results suggest that histones enter vascular organoids via the endocytic pathway.

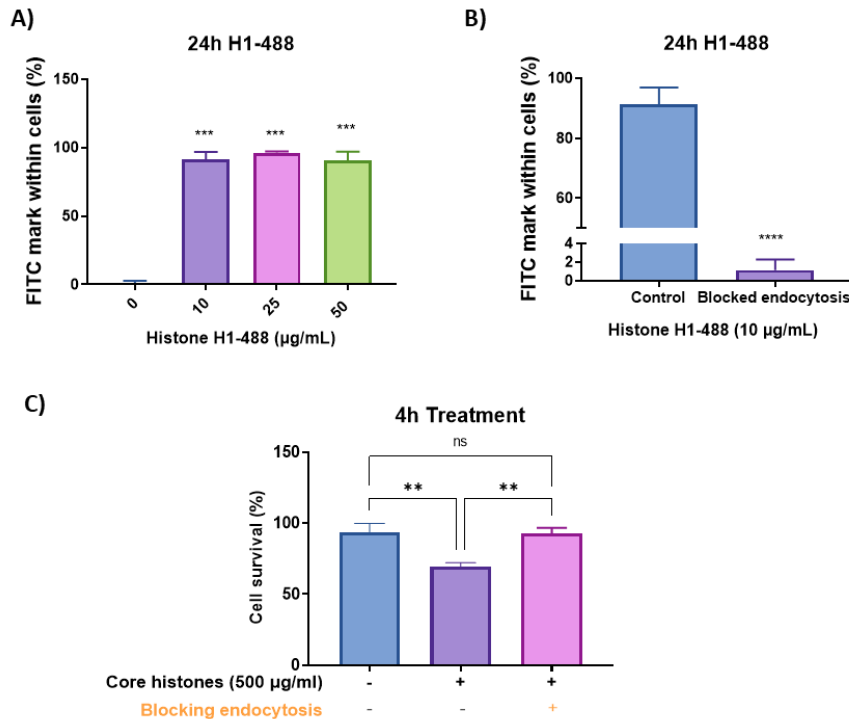


Figure 38. Blocking endocytosis prevents histone entry to blood vessel organoids (BVOs) and reduces histone-induced cytotoxicity. (A) BVOs were incubated in the absence or presence of several concentrations of H1-488 for 24h and then they were harvested and the FITC mark within cells was analyzed by flow cytometry. (B) BVOs were in both conditions treated with 10 µg/mL of histone H1-488 and in the case of control they were incubated at standard conditions (37°C) for 24h and the condition of blocking endocytosis BVOs were incubated on ice. After, the BVOs were harvested and the FITC mark within cells was analyzed by flow cytometry. (C) BVOs were incubated or not in the presence of 500 µg/mL of core histones for 4h at 37 °C in the case of control condition and on ice in the condition of blocking endocytosis. After, the BVOs were harvested and labeled using a viability dye, and subsequently the survival rate was analyzed by flow cytometry. Mean survival values are presented as bars, expressed as a percentage $\pm$ standard deviation ($n=3$), with statistically significant differences considered when $p<0.05$, calculated using ANOVA one-way (A) and (B) or t-test (C) indicated by ** from $p<0.01$, *** when $p<0.001$ and **** when $p<0.0001$.

To further investigate the effect of endocytosis blockade on histone internalization and its downstream actions, we aimed to determine the localization of histone treatment in organoids when the endocytic pathway was blocked, as well as its potential impact on the autophagy pathway. Differentiated organoids were incubated on ice for fifteen minutes, followed by treatment with 10 µg/mL of histone H1-488 for 24 hours at 4°C, as previously described. Organoids treated with 10 µg/mL of histone H1-488 for 24 hours at 37°C were used as controls. Subsequently, the organoids were collected and processed for immunofluorescence staining using LC3B and CD31 antibodies, followed by a species-specific secondary antibody conjugated to a fluorescent tag. H1-488 histone was detected through its own autofluorescence.

Confocal microscopy images of at least three organoids per condition were captured. Figure 39A illustrates examples of fluorescence microscopy images for the analysed conditions.

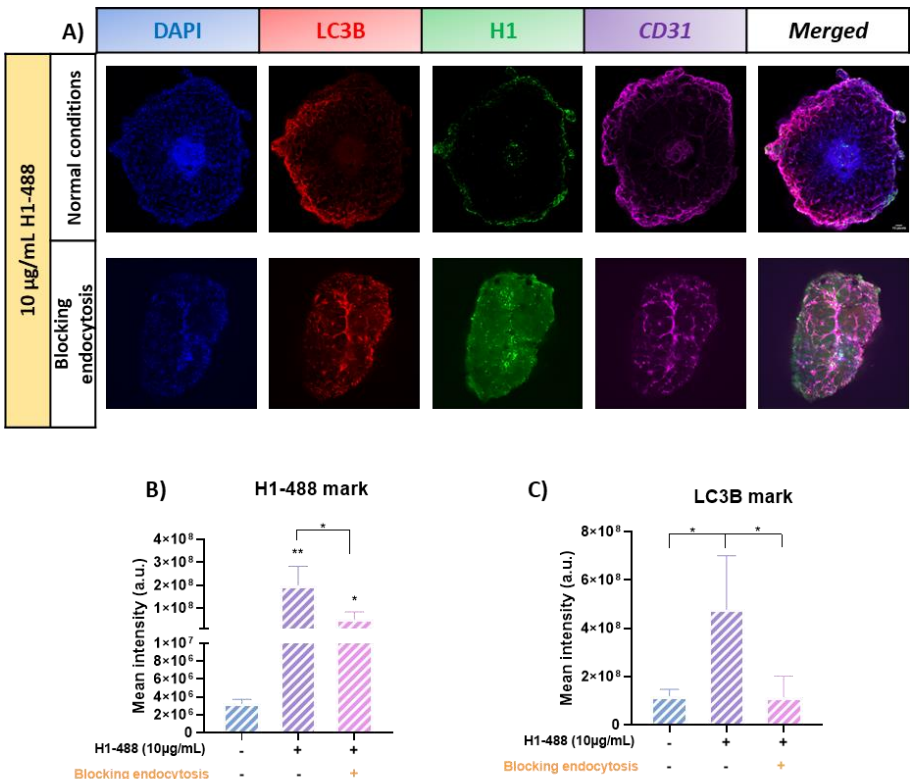


Figure 39 Effect of blocking endocytosis on localization and expression of histone H1-488 and LC3B. (A) Confocal microscopy images of blood vessel organoids (BVOs) treated with histone H1-488 at 10 µg/ml and subjected or not to endocytosis blocking conditions. Each row shows images from the same field of view, with the blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one to histone H1-488 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the fourth channels used. Magnification, 10X. Scale bar 10 µm. (B) Quantification of the mean fluorescence intensity of green channel (H1-488) by ImageJ. (C) Quantification of the mean fluorescence intensity of red channel (LC3B) by ImageJ. The bars represent the mean values ± the standard deviation of the mean intensity value of fluorescence of each condition (n=3). Statistically significant differences were considered with respect to the control (0 µg/mL). and between the different conditions when $p < 0.05$, calculated using ANOVA one-way indicated by * when $p < 0.05$ and ** when $p < 0.01$.

The average fluorescence intensity of the green signal corresponding to H1-488 (Figure 39B) and the red signal corresponding to the autophagic flux protein LC3B (Figure 39C) were quantified using ImageJ, measuring the average fluorescence of each image, and subtracting the background. As shown in Figure 39A, when endocytosis was blocked, the H1-488 histone marker was observed on the surface of the organoid but not inside the cells. The green dots were clustered around the organoid surface,

indicating that when endocytosis is blocked, histone H1-488 remains stacked at the organoid membrane and does not enter the cells. This explains why the fluorescence intensity of the histone marker (*Figure 39B*) is higher in the endocytosis-blocked condition compared to the non-histone treatment condition, but significantly lower than in the condition where histone treatment occurs under normal conditions, allowing for internalization of the histones. Regarding the LC3B marker (*Figure 39C*), when endocytosis was blocked, no increase in LC3B fluorescence intensity was observed, as seen when treatment was carried out under normal conditions. Instead, the values were like those of the non-histone-treated organoids, significantly lower than when organoids were treated with histones under normal conditions.

Based on these findings, we can propose that, similar to HUVEC cell cultures, histones are internalized via the endocytic pathway in BVO, and that this internalization is necessary for histones to exert their cytotoxic effects on the organoids.

3.5 Changes in gene expression and miRNA upon chromatin-derived molecules treatments.

Given that, as shown above, extracellular histones produce cytotoxic effects on vascular organoids and induce an increase in the autophagy pathway, we sought to investigate the potential changes associated with their treatment in terms of the expression of various proinflammatory cytokines secreted in the context of sepsis (25), as well as certain vascular parameters that have also been reported to be affected by extracellular histones (74). To achieve this objective, differentiated vascular organoids were obtained and treated with various concentrations of core histones (0, 50, 100, 200, and 500 $\mu\text{g/mL}$) for 4 hours. Subsequently, at least 12 organoids were collected for each condition, and RNA was extracted using affinity columns. Finally, the gene expression of eNOS, iNOS, IL-1 α , IL-6, IL-1 β , HMGB1, VCAM-1, ICAM-1, and E-SELECTIN were analyzed by RT-qPCR using specific primers indicated in *Table 4*, as described in section 5 of *Materials and Methods* section. Results presented in *Figure 40*. indicate that IL-6, VCAM-1, HMGB1, E-selectin, and ICAM-1 show a dose-dependent increasing trend in response to histone treatment. However, due to data dispersion, no significant differences are observed except for the 100 $\mu\text{g/mL}$ concentration of IL-6 (*Figure 40D*) and the 500 $\mu\text{g/mL}$

concentration of VCAM-1 (Figure 40G). On the other hand, there are also dose-dependent decreasing trends in the genes IL-1 α , IL-1 β , and eNOS, with a significant decrease observed in the 100 and 500 $\mu\text{g}/\text{mL}$ concentrations of eNOS (Figure 40A). The expression levels of the genes iNOS and ICAM-1 remain stable regardless of histone treatment. In conclusion, extracellular histone treatment in vascular organoids showed dose-dependent effects on certain proinflammatory cytokine expression and vascular parameters. Significant changes were observed for IL-6 and VCAM-1, while IL-1 α , IL-1 β , and eNOS exhibited dose-dependent decreases. These findings contribute to our understanding of histone-mediated effects in sepsis and its associated vascular dysfunction.

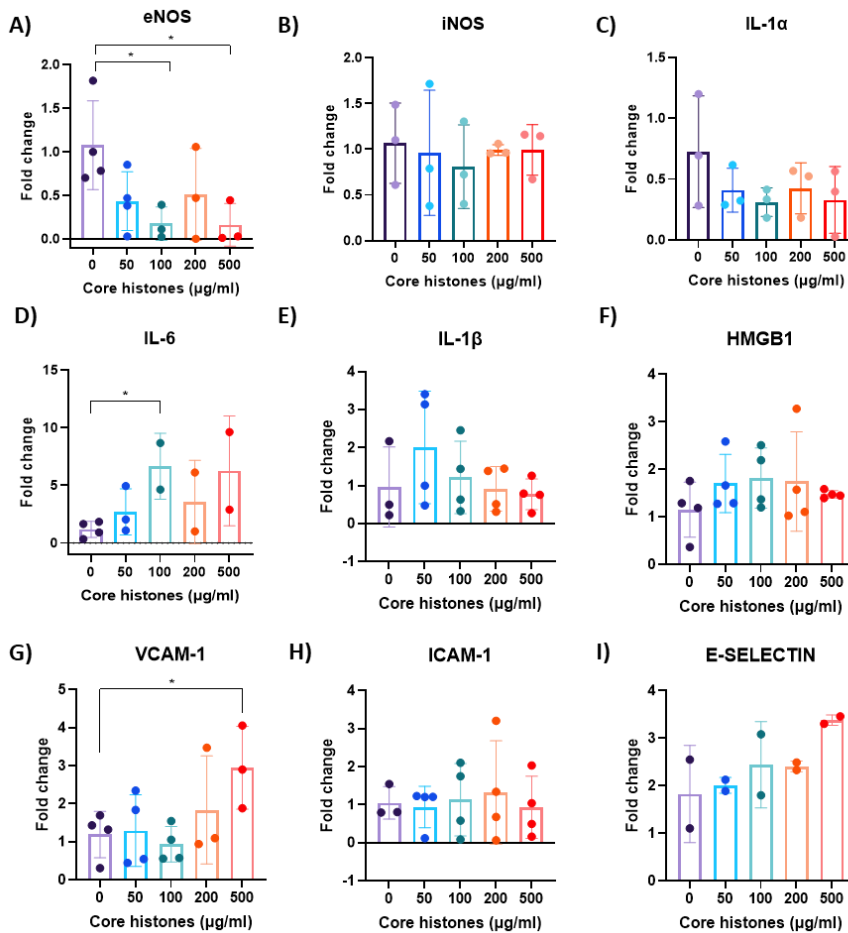


Figure 40. Histone induced gene expression alterations on proinflammatory and vascular genes in blood vessel organoids (BVOs). Blood vessel organoids were treated with several concentrations of core histones (0, 50, 100, 200 and 500 $\mu\text{g}/\text{mL}$), the RNA was extracted using affinity columns and then the gene expression of

(A) eNOS (B) iNOS (C) IL-1 α (D) IL-6 (E) IL-1 β , (F) HMGB1 (G) VCAM-1 (H) ICAM-1 (I) E-SELECTIN was analyzed by RT-qPCR using specific primers shown in Table 4. The bars represent the mean values $\pm$ the standard deviation of fold change expression respect to housekeeping gene *EEF1A* of each condition ($n=3$). Statistically significant differences were considered with respect to the control (0 $\mu\text{g/mL}$), and between the different conditions when $p<0.05$, calculated using ANOVA one-way indicated by * when $p<0.05$.

On the other hand, an attempt was made to determine the miRNA values in the organoid model when subjected to the effects of histones, similar to what was done in other cellular models, in order to obtain a miRNA signature that could be potentially used as a biomarker in the future. To achieve this, vascular organoids (BVOs) were treated with core histones at concentrations of 50, 100, 200, and 500 $\mu\text{g/mL}$, following established protocols. Subsequently, the BVOs were collected, and RNA extraction was performed using affinity columns. The expression levels of the specific miRNAs of interest were analysed using individual reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays for each miRNA, as described in Section 5 of the *Materials and Methods* section.

In the case of miR-155 (Figure 41A), it can be observed that its expression tends to be upregulated when vascular organoids are exposed to core histones, with a significant increase at 50 $\mu\text{g/mL}$, which then switches to a decreasing trend. Regarding miR-146a (Figure 41B), BVOs do not exhibit any significant alterations in expression in response to core histone treatment. The expression levels tend to be downregulated at lower concentrations and remain at similar levels to the control condition without histone treatment at higher concentrations.

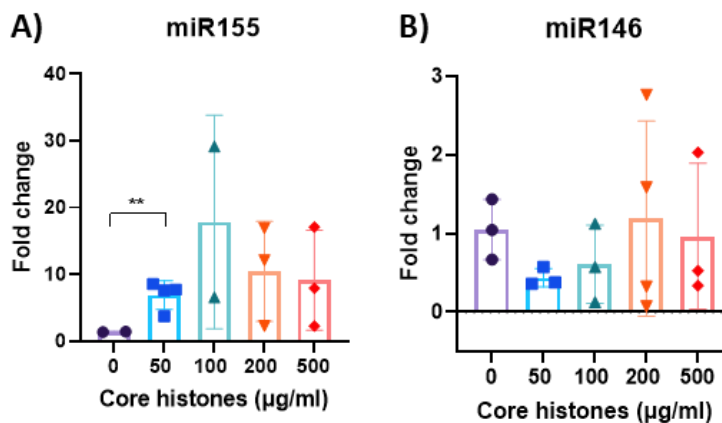


Figure 41. Effect of core histone treatment on miRNA expression in blood vessel organoids (BVOs). The bars represent the ratio of expression of (A) miR55 and (B) miR-146 miRNA as relative expression measured by RT-qPCR referenced to the expression of U6 as housekeeping gene. BVOs were treated with several core histones (0,50,100,200 and 500 $\mu\text{g/mL}$), the RNA was extracted using affinity columns and then specific miRNA RT-

qPCR. Bars represent mean values as a percentage $\pm$ the standard deviation ($n=3$), considering significant differences with respect to the control (0 $\mu\text{g/mL}$) when $p<0.05$, calculated using ANOVA one-way indicated by ** for $p<0.01$.

In summary, core histone treatment could modulate miR-155 expression in vascular organoids, showing an upregulation at 50 $\mu\text{g/mL}$; however, miR-146a expression remains unchanged. These findings contribute to our understanding of miRNA regulation in vascular organoids and their potential as biomarkers, warranting further investigation.

One of the objectives of our project was to investigate the role of proinflammatory nuclear protein HMGB1 in the context of the cytotoxicity mediated by nuclear macromolecules; with this aim, we focused on the expression of certain genes previously analysed in relation to sepsis. To accomplish this, differentiated vascular organoids were obtained and subjected to various concentrations of HMGB1 (0, 50, 100, and 200 ng/mL) for a duration of 24 hours. Subsequently, at least 12 organoids were collected for each condition, and RNA was extracted using affinity columns. Finally, the gene expression levels of eNOS, iNOS, IL-6, HMGB1, VCAM-1, ICAM-1, and E-SELECTIN were analysed via RT-qPCR, utilizing specific primers as outlined in Table 4, as described in section 5 of the *Materials and Methods* section. The results are depicted in Figure 42.

Results appear to exhibit a similar trend to those obtained with core histone treatment. We observed a clear dose-dependent downregulation of eNOS, and although iNOS did not significantly increase, there was a substantial elevation at the highest concentration (*Figure 41A and B*). However, we did not observe any changes in the expression of the proinflammatory cytokines (IL-6 and HMGB1 (*Figure 42C and D*)). Regarding the vascular markers (*Figure 42 E, F and G*) only E-selectin exhibited decreased expression, in a dose-dependent manner, when organoids were treated with HMGB1. However, VCAM-1 tended towards overexpression, and ICAM-1 displayed downregulation.

In conclusion, the treatment with the proinflammatory protein HMGB1 in BVOs showed dose-dependent effects on the expression of several genes in differentiated vascular organoids. Surprisingly, there were no significant changes observed in the expression of proinflammatory cytokines IL-6 and HMGB1. These findings suggest that

HMGB1 may play a role in modulating vascular gene expression in sepsis, warranting further investigation into its mechanism of action.

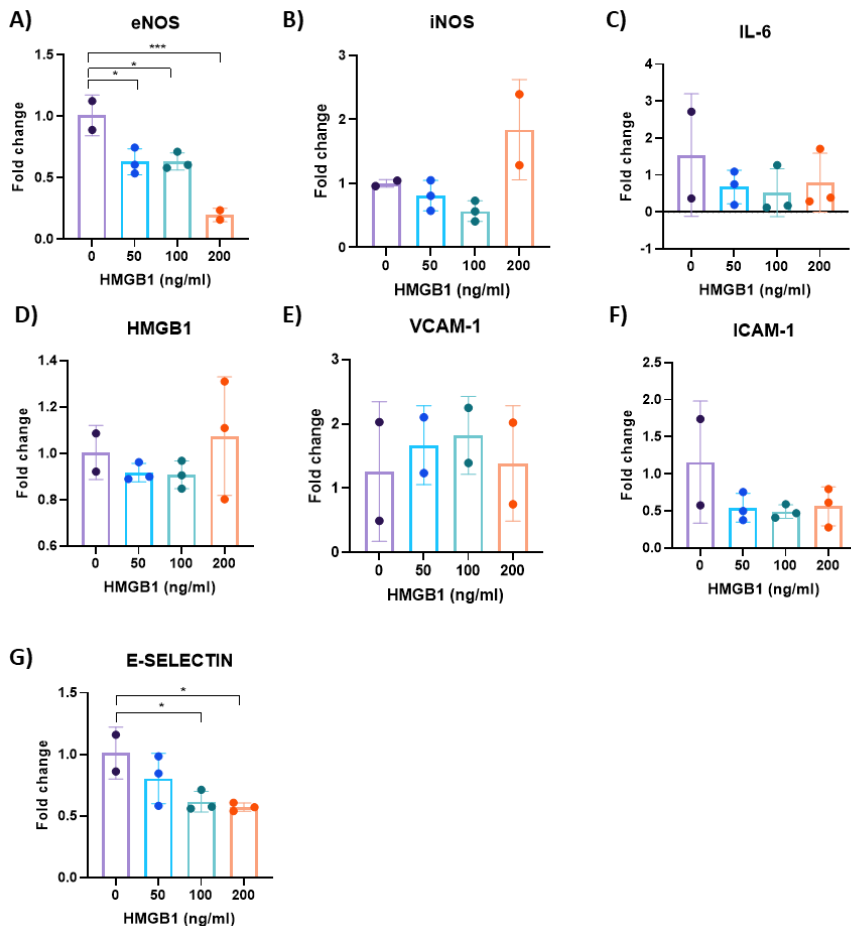


Figure 42 HMGB1 induced gene expression alterations on proinflammatory and vascular genes in blood vessel organoids (BVOs). Blood vessel organoids were treated with several concentrations of HMGB1 (0, 50, 100 and 200 ng/ml), the RNA was extracted using affinity columns and then the gene expression of A) eNOS B) iNOS C) IL-6 D) HMGB1 E) VCAM-1 F) ICAM-1 G) E-SELECTIN was analysed by RT-qPCR using specific primers shown in Table 4. The bars represent the mean values ± the standard deviation of fold change expression respect to housekeeping gene *EEF1A* of each condition (n=3). Statistically significant differences were considered with respect to the control (0 µg/mL). and between the different conditions when $p < 0.05$, calculated using ANOVA one-way indicated by * when $p < 0.05$.

3.6 Changes in release of HMGB1 upon core histone treatment and autophagy modulation.

Lastly, we aimed to investigate the presence of the nuclear protein HMGB1 in the supernatants of organoids exposed to different histone treatments and autophagy

modulation as it was done with HUVEC cells since there was a dose-dependent relationship between the secretion of HMGB1 and the exposure to extracellular histones. To achieve this objective, differentiated vascular organoids were obtained and treated with various concentrations of core histones for 4 hours or histone H1-488 for 24 hours. Additionally, these organoids were also treated with NH_4Cl (20 mM) and Leupeptin (100 nM) during the last 3 hours of histone treatment or cultured for 24 hours in the presence of Krebs-Henseleit medium. After completing the treatments, the supernatant from at least 10 organoids per condition was collected and analysed using an HMGB1 ELISA kit, following the manufacturer's instructions, as briefly described in Section 6 of the *Materials and Methods* section. The results shown in *Figure 43* demonstrate that when organoids are treated with increasing concentrations of core histones (*Figure 43A*), the secretion of HMGB1 into the supernatant increases in a dose-dependent manner, with a significant increase observed from the lowest tested dose (25 $\mu\text{g/mL}$).

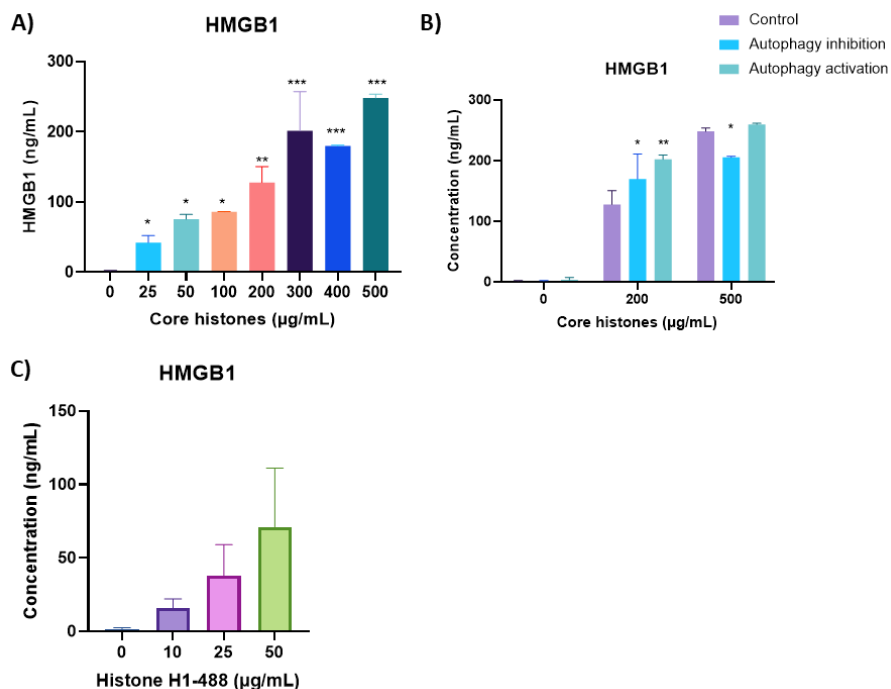


Figure 43. Quantification of HMGB1 on supernatant of blood vessel organoids (BVOs) treated with extracellular histones and autophagy modulators. BVOs were treated with several concentrations of (A) core histones, or (B) autophagy was modulated using autophagy inhibitors NH_4Cl (20 mM) and Leupeptin (100 nM) for 3 hours and with autophagy activator (Krebs-Henseleit medium) for 24h and (C) treated with histone H1-488. After the treatments, supernatant was collected and analyzed by ELISA. Bars represent mean values of concentration $\pm$ the standard deviation ($n=3$), considering significant differences with respect to the control (0

*μg/mL for A and C and control for (B) when $p < 0.05$, calculated using ANOVA one-way for (A) and (C) and two-way ANOVA for (B) indicated by * for $p < 0.05$, " ** " for $p < 0.01$ and *** for $p < 0.001$.*

Furthermore, when autophagy is modulated concurrently with intermedium doses of histone treatment (200 $\mu\text{g/mL}$; *Figure 43B*) the secretion of HMGB1 is further enhanced. However, when organoids are treated with cytotoxic doses such as 500 $\mu\text{g/mL}$, inhibition of autophagy leads to a decrease in HMGB1 secretion compared to the control condition, while activation of autophagy does not produce changes compared to the control. In addition, the secretion of HMGB1 into the medium was also analysed when BVOs were treated with histone H1-488. The results indicate a similar dose-dependent increasing trend in HMGB1 secretion as observed with core histones, although the changes were not significant.

In summary, our findings demonstrate that extracellular histones induce the secretion of the nuclear protein HMGB1 in vascular organoids. Modulating autophagy enhances HMGB1 secretion, particularly at intermediate histone treatment doses. These results contribute to our understanding of the interplay between histones, autophagy, and HMGB1 in sepsis-related processes and confirm the usefulness of BVOs as a biological model for the study of the molecular pathophysiology of sepsis.

4. *Potential of HMGB1
as sepsis biomarker
in human plasma*

4.1 Evaluation of HMGB1 as a biomarker for sepsis and septic shock

As stated above and described in the literature, HMGB1 is present in the plasma of patients with sepsis and septic shock (190). However, given the symptomatic heterogeneity in these pathologies and the difficulties in reproducibility of this type of analysis, it was decided to first verify the levels of HMGB1 in plasma samples previously analysed in our laboratory to detect citrullinated histone H3 and circulating nucleosomes (82), and if these parameters showed any type of correlation. The analysis was performed using a commercial ELISA kit for HMGB1 determination, as explained in the *Materials and Methods* section. After reading the absorbance of all samples, the protein concentration in plasma was quantified using the standard curve. The levels of HMGB1 in both sepsis and septic shock patients were significantly higher than in the control group, which included patients from the intensive care unit (ICU) with non-infectious conditions (*Figure 44*). It is worth noting that although the differences between the controls and patients (both sepsis and septic shock) are substantial, they did not reach statistical significance between the patient groups (sepsis and septic shock). However, it can be observed that HMGB1 values are higher in patients with sepsis than in those with septic shock (*Figure 44B*).

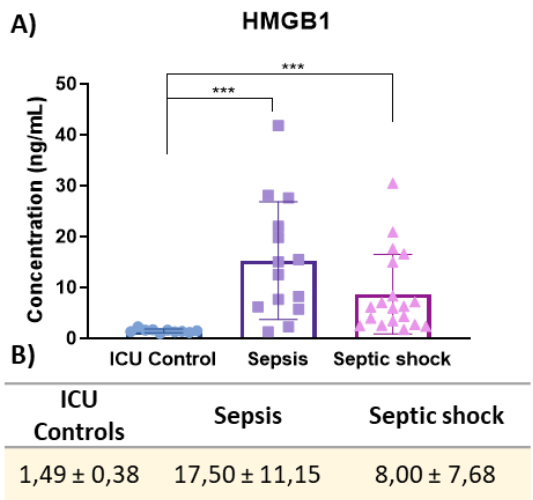
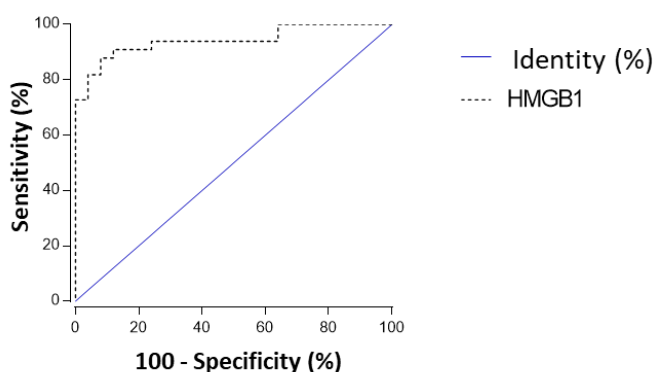


Figure 44. Quantification of HMGB1 using an ELISA kit in plasma samples from patients with sepsis and septic shock. (A) Plasma samples from patients with cerebral infarction (ICU control), patients with sepsis and septic shock were analyzed using an ELISA kit for HMGB1 determination. Bars represent mean values of concentration ± the standard deviation, considering significant differences with respect to the ICU control

when $p < 0.05$, calculated using ANOVA one-way indicated by *** for $p < 0.001$. (B) The mean values and standard deviation (SD) for each of the analyzed groups are presented in the table below.

Subsequently, a receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic power of HMGB1 levels as a biomarker to distinguish patients with septic shock or sepsis from severely ill patients with possible release of nuclear proteins due to traumatic or necrotic processes (cerebral ischemia) but unrelated to infectious processes (*Figure 45*). The area under the ROC curve (AUC), standard error, confidence interval (CI), optimal concentration cutoff value, and sensitivity and specificity percentages were calculated for differentiating between ICU control and cases (sepsis and septic shock patients) (*Figure 44B*). The results obtained demonstrate that our analysis exhibits high sensitivity (90.9%) as well as high specificity (88%) indicating that this HMGB1 ELISA kit can achieve a high diagnostic power to distinguish cases (sepsis and septic shock) from controls.

A)



B)

AUC	Standard error	IC 95%	p value	Cut off value	Sensitivity (%)	Specificity (%)
0,942	0,03	0,885-1	<0,0001	2,27	90,91	88,00

Figure 45 Diagnostic capacity of HMGB1 ELISA to differentiate cases from controls and septic patients. ROC curve for HMGB1 measured with HMGB1 ELISA as a biomarker for the diagnosis of septic processes. ROC curve analyses were performed by calculating the area under the ROC curve (AUC), standard error, confidence interval (CI), optimal concentration cutoff value, and sensitivity and specificity percentages for diagnostic and stratification purposes. Values of $p < 0.05$ were considered statistically significant.

4.2 Correlation studies between HMGB1 and sepsis clinical parameters

In line of the aforementioned results, we wanted to expand the study of the potential of HMGB1 as a biomarker for sepsis development. To do this, we performed a

comprehensive study by establishing correlation coefficients between HMGB1 and other biomarkers, as well as clinically significant variables registered in the analyzed cohorts. A greater number of correlations were found in the group of sepsis patients, according to the correlation analysis shown in *Annex I* section *Table A.1* and the most significant are shown in *Figure 46A*.

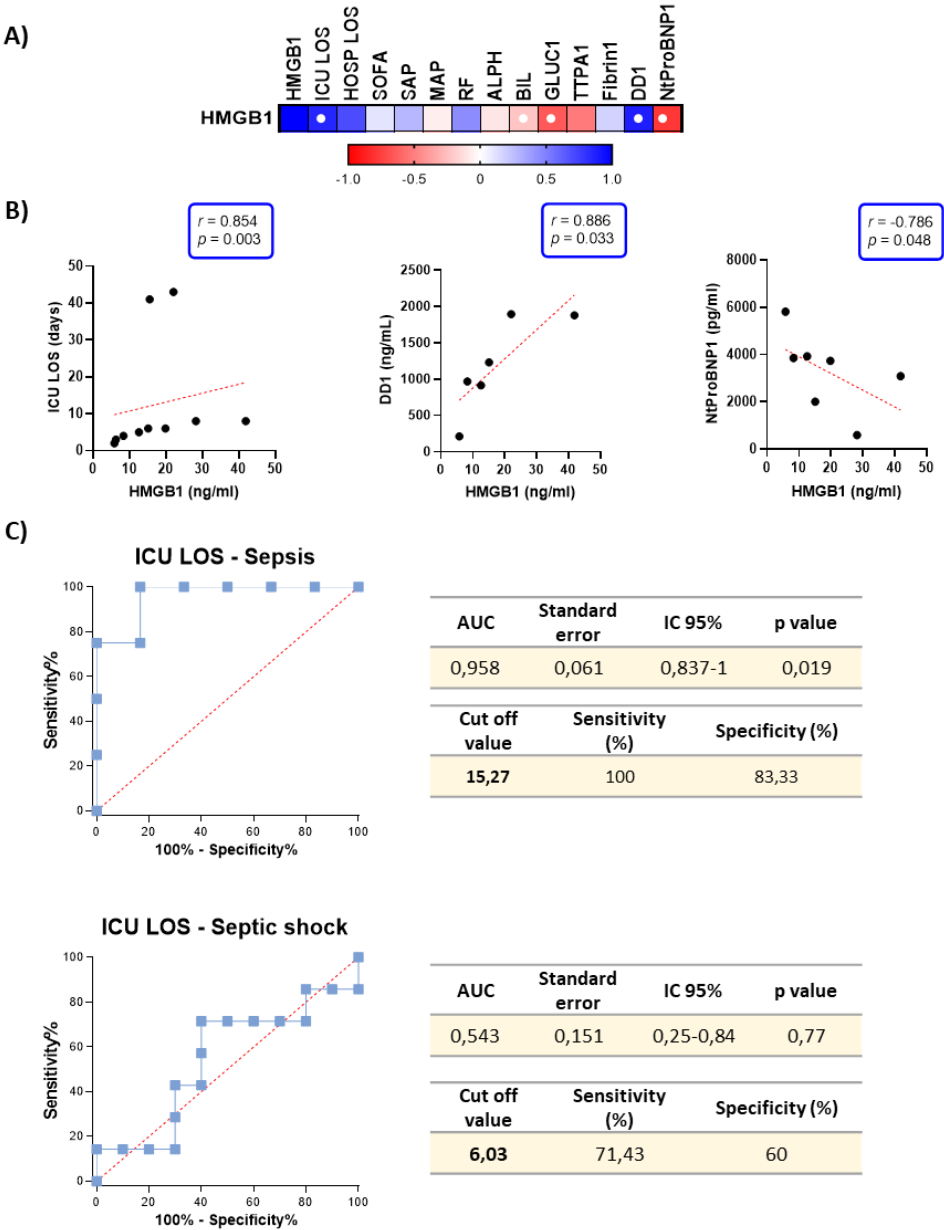


Figure 46. Correlation between biomarker levels, clinical parameters, and ROC curves for HMGB1 in sepsis and septic shock patients. (A) The correlation between clinical variables and biomarker levels as

determined by several ELISA kits. To analyze the associations between the variables, nonparametric Spearman correlation coefficients were generated. Referring to the legend, positive correlations are shown by blue cells, while negative correlations are shown by red cells. White dots represent significant relationships with a r value over 0.6 and a p 0.05 minimum level of significance. The thorough study emphasizes the relationships between CitH3 and HMGB1 that are most notable. ICU length of stay (ICU LOS), HOSP LOS (Hospitalization length of stay), respiratory frequency (RF), total SOFA score (SOF(A), total bilirubin (BIL), systolic arterial pressure (SAP), thromboplastin time (aPTT), mean arterial pressure (MAP), alkaline phosphatase (AP), D-dimer1 (DD1), glucose (GLU(C), activated partial fibrinogen (Fibrin), and amino-terminal fragment of the pro-natriuretic peptide type B1 (NtProBNP1) are some examples of clinical parameters. The values for each clinical parameter match those from day 1. For complete r and p values for each correlation, see Table A.1. (B) Graphs indicating high correlation between HMGB1 and ICU LOS (left), DD1 (middle) and NtProBNP1 (right) (C) Receiver Operating Characteristic (ROC) curves showing how HMGB1 levels can be used to forecast patients with sepsis or septic shock will need to stay in the ICU for more than five days.

Notably, HMGB1 demonstrated significant positive relationships with crucial clinical indicators such D-dimer1 (DD1) and ICU length of stay (LOS). The amino-terminal portion of the pro-natriuretic peptide type B1 (NtProBNP1), on the other hand, showed a substantial negative connection with HMGB1 (*Figure 46B*). These findings highlight the remarkable informative value of HMGB1 levels in defining the stage of sepsis and pertinent clinical indicators for sepsis care, along with our earlier analysis of diagnostic capacity and mean values derived from plasma samples of different patient groups. HMGB1 has in fact become a very promising predictive biomarker.

We created receiver operating characteristic (ROC) curves to further assess the HMGB1 levels' capacity to predict ICU stays longer than five days. An area under the curve (AUC) score of 0.9583 indicated that the analysis had a high degree of predictive power. Additionally, HMGB1 levels over 15.27 ng/ in sepsis showed 100% sensitivity and 83.33% specificity in predicting prolonged stays in the ICU. It is crucial to remember that in the patient group with septic shock, we were unable to find a significant cut-off value for accurate prediction (*Figure 46C*).

In conclusion, our study highlights the significant correlations between HMGB1 and important clinical indicators in sepsis. HMGB1 shows positive relationships with D-dimer1 and ICU length of stay, while exhibiting a negative connection with NtProBNP1. These findings emphasize the informative value of HMGB1 as a predictive biomarker for sepsis. Additionally, our analysis demonstrates that HMGB1 levels have a high degree of accuracy in predicting prolonged ICU stays.

DISCUSSION

1. Extracellular histones are cytotoxic for different experimental models

Sepsis is a frequently observed clinical condition characterized by an imbalanced immune response to infection, consisting of an initial inflammatory phase followed by an opposing anti-inflammatory phase. Simultaneous occurrence of these phases can result in persistent immune suppression. Histones found in the bloodstream during sepsis are recognized as one of the damage-associated molecular patterns (DAMPs) that can induce cellular damage through both receptor-dependent and receptor-independent mechanisms (42,48). These histones, released into the extracellular environment, contribute to the network of proinflammatory molecules that exhibit elevated levels during the early stage of sepsis (191).

Previous reports from our group have demonstrated that extracellular histones can activate various mechanisms depending on their concentration. These mechanisms include dose-dependent induction of cell death, specifically apoptosis (172). Additionally, histones can also modulate cellular processes such as autophagy (67), thereby influencing the equilibrium and stability of endothelial cells. Further studies conducted by our group have revealed that histone levels in the plasma of sepsis and septic shock patients reach levels capable of triggering apoptosis in HUVEC (Human Umbilical Vein Endothelial Cells) (64). Consequently, histones may exacerbate the symptoms during the early phase of sepsis by initiating endothelial apoptosis, given their procoagulant and proinflammatory properties. However, the molecular mechanisms underlying this potential cytotoxicity and its relationship with the autophagy pathway remain unknown. Therefore, the present study aims to characterize the cytotoxic potential of extracellular histones in various experimental models to elucidate how they interact with cells and induce apoptosis signals and activation of the autophagy pathway.

In recent years, the potential of extracellular histones as inducers of cell death has been demonstrated (50), both in endothelial cells (67) and in other models, such as various organs including the liver (53), heart (192), kidney (163), among others.

Our study revealed important insights into the cytotoxic potential of extracellular histones, specifically the core histones composed of H2A, H2B, H3, and H4, in different cell culture models as well as a fluorescent construct of histone H1-488 to perform direct colocalization studies. Initially, the HeLa and Hep3B cell lines were utilized to evaluate the effects of core histones on cell survival. The findings demonstrated that Hep3B cells exhibited greater sensitivity to extracellular histones compared to HeLa cells, both in terms of incubation time and concentration (*Figure 11*).

To further validate the observed cytotoxicity and its relationship with autophagy, the study model transitioned to the use of HUVEC cell culture, which is more relevant to the pathology under investigation. As well as immortalized cell cultures, HUVEC cells also showed histone induced cytotoxicity (*Figure 17*). Interestingly, the minimum dose required to induce cytotoxic effects in HUVEC cells is higher compared to immortalized cells, contrary to what would be expected since immortalized cells are typically more resistant. However, we believe this discrepancy is not due to higher tolerance of HUVEC cells to histone treatments but rather due to the variability in commercial histone batches. On the other hand, the results confirmed dose- and time-dependent cytotoxicity, with longer incubation times requiring lower concentrations to decrease cell survival. These findings reinforce the harmful impact of extracellular histones on endothelial cells, particularly in the context of sepsis.

Nevertheless, it is important to note that monolayer cell cultures, whether immortalized or not, do not adequately reflect the landscape of complex diseases such as sepsis. Therefore, to mimic human blood vessels, various models have been developed using stem cells or adult tissues. These models include two-dimensional co-culture systems of differentiated endothelial cells and pericytes, as well as three-dimensional cultures of early vascular progenitor cells (176–178). Additionally, self-organizing three-dimensional human blood vessel organoids derived from pluripotent stem cells accurately replicate the characteristics of human microvasculature, including endothelium, basement membrane, and mural cells. Blood vessel organoids (BVOs) offer a promising platform for studying vascular biology and disease modelling in a controlled environment (179,180). Therefore, to address this limitation and gain a more comprehensive understanding of the biological consequences of the interaction

between extracellular histones and the endothelium, a blood vessel organoid model was employed. We sought to investigate, for the first time, whether the addition of extracellular histones could model sepsis in the vascular organoid model. We found that indeed high doses of extracellular core histones caused cytotoxicity in this model (*Figure 32*), at a similar but slightly higher dose compared to the HUVEC endothelial cell model. This could be attributed to the vascular organoid model being more complex, with a 3D organization and diverse cell types that may better tolerate external aggressions. However, in none of the models tested, did the concentrations of histone H1-488 induce cell death, likely due to the failure to reach a sufficiently high dose of treatment. Additionally, it can be inferred that the combination of multiple histones, such as in the case of the histone core, act synergistically to increase toxicity.

In conclusion, the comprehensive analysis of extracellular histones in different cell culture models, including two-dimensional cell cultures and blood vessel organoids, provides valuable insights into their cytotoxic potential. These findings contribute to our understanding of the detrimental effects of extracellular histones, especially in the context of sepsis, and highlight the relevance of employing physiologically relevant models to study complex diseases.

2. Extracellular histones are internalized by endocytosis mechanisms and related to autophagy pathway

Previous studies have investigated the ability of histones to penetrate cells (193). However, few have elucidated the underlying mechanisms. In early studies, it was observed that when histones extracted from chicken were conjugated with pH-sensitive fluorophores and administered to Chinese hamster ovary cell monolayers, the fluorescence acquired by the cells increased upon neutralizing the lysosomal pH with chloroquine. This suggested that the labelled histones were located within the lysosomes (194). Additionally, in the same study, the kinetics of fluorescent histone labelling entering cells were examined, revealing two trends: a portion of the fluorescent label entered rapidly and localized to acidic organelles (likely lysosomes)

before rapidly diminishing, while another portion entered the cytoplasm at a much slower rate, without localizing to low-pH organelles, and took longer to diminish. Thus, a model was proposed to explain these observations, suggesting that histones initially entered the cell interior via saturable endocytic mechanisms that led them to lysosomes, while simultaneously diffusing more slowly directly through the plasma membrane into the cytoplasm (194). Contrarily, in studies conducted by Hariton et al. using HeLa cells treated with endocytosis inhibitors and subsequently with fluorescently labelled recombinant human histones, the kinetics of fluorescent labelling accumulation in the cytoplasm of the cells were found to be independent of endocytosis inhibition. This suggested that histones penetrated the cytoplasm exclusively through diffusion across the plasma membrane, rendering the role of endocytosis negligible (195). In this latter study, no data were collected regarding whether histones localized within or outside of lysosomes, thus the extent to which their results align with ours and those of Kaouass et al. (193) remains unknown. Considering all this evidence, the lack of knowledge surrounding the mechanisms by which histones enter cells becomes apparent, to the extent that the existence of mechanisms beyond simple diffusion across the membrane is questioned. In the present study, a methodology for immunofluorescent staining of the proteins of interest involved in this process was developed to generate an image bank that would allow the development and validation of an image analysis tool created for the HeLa and Hep3B models (*Figure 9*). This combined methodology has enabled the automated quantification, using fluorescence microscope images, of the level of overlap between histones labelled with fluorescent antibodies and a fluorophore marker with affinity for lysosomes (*Figure 10*). Furthermore, this work has revealed some effects of extracellular histones on immortalized human cells. Firstly, the results obtained suggest that free histones in the extracellular environment penetrate the interior of cells and are dose-dependently internalized into lysosomes (*Figure 13*). However, additional validations are required to confirm these findings with certainty, as two-dimensional colocalization studies do not provide conclusive evidence that histones are present inside lysosomes. To corroborate or refute this hypothesis, it would be necessary to repeat the study using confocal microscopy images, which can estimate all dimensions of cellular space and volume. In

fact, a complementary staining with plasma membrane markers would allow for a more precise measurement of the relative localization of histones.

In order to further investigate the potential entry routes and subsequent subcellular localization of extracellular histones and confirm the hypothesis proposed in the HeLa and Hep3B models, colocalization studies were conducted in HUVEC cells between extracellular histones and the LC3B protein (a well-known marker of the autophagic pathway, present on autophagosomal membranes) using different treatments with increasing concentrations of core histones (*Figure 18*), or with an extract mainly composed of H3-GFP histones (obtained from HeLa cells transfected with the pIRES-Neo-H3.3-GFP plasmid) (*Figure 19*). The results showed an increasing trend in colocalization between histone H3 and the LC3B protein as the concentration of histone treatment increased up to 200 µg/ml. However, higher concentrations of extracellular histones resulted in cell death (*Figure 18*), leading to a decrease in the observed colocalization. Furthermore, the increased colocalization with the GFP-tagged H3 histones from the extract obtained in the laboratory provides greater confidence that the previously obtained results are due to extracellular histones and not to those histones already present in the cellular nucleus. In other words, the dose-dependent overlap observed between the red fluorescence from the LC3B protein and the green fluorescence corresponding to extracellular histones suggests that, after penetrating the cells, histones are located inside autophagosomes. Similarly, to confirm these results, proximity ligation assays (PLA) were performed to analyze the interactions between LC3B protein and histone H3 (*Figure 20*), as well as with GFP-tagged histone H3 (*Figure 21*) obtained through transfection. In this case, there was not a clear dose-dependent increase in interactions as in the previous experiments, despite the observed upward trend. These results may be attributed to the sensitivity of the technique itself, as increasing the concentration of extracellular histone treatment progressively and thus increasing the number of interactions may surpass the system's sensitivity, reaching saturation. Although the previous results are more indirect due to the use of antibodies for visualizing the treatment with extracellular histones, an increase in colocalization was observed over time when treating cells with histone H1-488 (which emit their own fluorescence due to labelling with Alexa488) and quantifying their

colocalization with an acidic organelle marker, confirming the previous findings (*Figure 23*). These results are consistent with the work of Murphy et al.(194) and with the previously obtained results using HeLa and Hep3B cells, with the advantage that these images have been obtained through confocal microscopy, confirming that the corresponding histone and autophagic marker labels are indeed in the same subcellular location.

Regarding the relationship between vascular organoids (BVOs) and histone treatments in conjunction with autophagic markers, an interesting observation was made regarding the presence of histone labelling within the organoids when they were exposed to extracellular histone treatments (*Figure 33* and *Figure 34*). The mean intensity of fluorescence of the histone labelling exhibited a dose-dependent relationship, both for the core histone treatments (*Figure 33*) and the H1-488 histone treatment (*Figure 34*). This finding suggests that the organoids have the capacity to internalize and accumulate histones in a manner that is influenced by the administered dosage. However, it is important to acknowledge a limitation of the study, namely the inability to quantitatively assess the colocalization between the histone label and the LC3B marker. Despite this limitation, visual examination of the images indicates a partial colocalization of these two fluorescent marks, implying a potential association between histone presence and the activation of the autophagy pathway within the organoids. To gain further insight into the potential influence of histones on the autophagy pathway in the organoids, an alternative approach was employed. Specifically, the intensity of the LC3B label was quantified as a surrogate measure of autophagic activity. Interestingly, the results revealed that histone treatments induced the activation of the autophagy pathway within the organoids (*Figure 32C* and *Figure 34D*). This observation suggests that histones may play a role in modulating autophagic processes in the context of vascular organoids.

In summary, this study contributes to our understanding of the cellular uptake and subcellular localization of extracellular histones, as well as their potential influence on the autophagy pathway in both cell culture and vascular organoids. The results suggest that extracellular histones can penetrate cells, accumulate in lysosomes, and activate the autophagy pathway. These findings provide a foundation for further investigations

into the molecular mechanisms underlying these processes and their implications for cellular homeostasis, development, immune response and sepsis pathophysiology.

However, as mentioned earlier, there is discrepancy in the literature regarding whether histones enter cells through endocytosis or membrane diffusion. These discrepancies highlight the gaps in our understanding of the mechanisms involved in histone internalization and their cytotoxic effects. Recent studies by Wang et al. (189) have provided insights into the internalization of fluorescently labelled nucleosomes in HeLa cells. They demonstrated that nucleosomes initially interact with the cell membrane through nonspecific non-electrostatic interactions with the positively charged histone tails, and subsequently enter the cells through clathrin- or caveolin-dependent endocytosis (189). Another study showed that small polyanions (SPAs) can engage electrostatically with histones to counteract their harmful effects. It was discovered through in vitro research that SPAs prevented histones from having cytotoxic, platelet-activating, and erythrocyte-damaging actions. Mechanistic investigations showed that tiny polyanions prevent histones from rupturing lipid bilayers, demonstrating their capacity to counteract the negative effects of extracellular histones (196). Furthermore, research by Kleine and colleagues suggested that histone molecules immediately interact with membrane phospholipids to form channels, and both the protein's positive charge and tertiary structure are important in this process. This research shed light on the role of protein and membrane structure in the histone-induced damage to mammalian epithelium (197). Given the importance of the positive charges of histones for their potential internalization into cells, it was hypothesized in this study that histones could mediate their cytotoxic effects by binding to the plasma membrane through interaction with charges, followed by endocytic internalization. Our studies with HUVEC cells revealed the absence of increased colocalization between the LC3B protein and extracellular histones when endocytosis was blocked incubating cells at low temperature (4 °C) as previously confirmed by Wang et al. (189), supporting the idea that endocytosis is a possible route of histone entry into cells (*Figure 25*). To further confirm the endocytosis as the way of histone internalization, it was proved that in the absence of endocytosis blockage the mark corresponding to histone H1-488 treatment was internalized by HUVEC cells, but this effect was not observed when endocytosis was

blocked. In addition, it was demonstrated that the entry of histones by endocytosis mechanisms was necessary to induce the acute cytotoxic effects of extracellular histones (*Figure 26*).

Taken together, these observations could be explained based on the concept of "*non-canonical autophagy*, the recently described LC3B-mediated type of endocytosis" (119). During this process, components of the autophagic machinery are utilized to conjugate LC3 protein with membranes of clathrin-positive endosomes. Currently, this type of endocytosis has been associated with the recycling of receptors such as TREM-1 or TLR-4 in microglia (119), highlighting that, in the context of sepsis, these pattern recognition receptors are involved in the development of the disease's pathogenesis. However, to ensure the blockade of endocytosis, a specific inhibitor of these pathways should be tested.

Lastly, our studies with the more complex and physiologically relevant BVO model confirmed our previous findings in endothelial cells. In this model, it was observed that under normal culture conditions, the fluorescence signal corresponding to histone H1-488 was internalized within the organoids. However, when endocytosis was blocked, no detectable levels of internalized histones were found. Furthermore, the cytotoxic effects of core histones could be reversed when endocytosis was blocked (*Figure 38*). Additionally, it was observed that the localization of histone H1-488 changed after blocking endocytosis, with the histones remaining attached to the surface and cavities of the organoid without penetrating the interior. Finally, it was demonstrated that blocking endocytosis during histone treatments resulted in the absence of autophagy activation, and the levels of LC3B marker decreased to baseline levels (*Figure 39*). These results suggest that blocking endocytosis not only prevents the internalization of histones but also inhibits the activation of the autophagy pathway.

These results suggest that endocytosis plays a crucial role in the internalization of histones and the induction of their cytotoxic effects. Moreover, the possibility of non-canonical autophagy-mediated delivery of extracellular histones to autophagosomes in endothelial cells and complex models such as blood vessel organoids holds promising implications for understanding the inflammatory signalling cascade associated with

sepsis. Further experiments are warranted to validate and explore this intriguing research avenue.

Therefore, it could be stated a mechanism of action in which cells or organoids exposed to sublethal doses of extracellular histones internalize them and directs the extracellular histones to the autophagy pathway to be eliminated by the mechanism of non-canonical autophagy. Instead, if the concentration of extracellular histones gets over the cytotoxic limit, the autophagy pathway no longer can exert its function and cells or organoids are guided towards apoptosis.

3. Modulation of autophagy influences the alterations driven by extracellular histones

According to previous studies from our group about the relationship between extracellular histone-induced cytotoxicity and autophagy, the exposure to extracellular histones in HUVEC cells leads to the activation of cellular signalling pathways that ultimately result in increased levels of autophagic components. However, higher concentrations of histones lead to apoptosis activation (67). Collectively, these findings suggest that autophagy plays a protective role in the context of sepsis, as demonstrated by other studies linking hepatic autophagy activation to increased survival in septic mice (198). These results align with the well-known ability of autophagy to act as an intracellular defence mechanism that helps to maintain homeostasis in the face of various types of stress (92). However, contrasting evidence has emerged from a series of studies, associating autophagy with organ dysfunction in sepsis (199). Additionally, a dual role of autophagy in alveolar and endothelial lung epithelial cells following acute lung injury caused by sepsis has been proposed. Depending on the pathogen and the load, moderate and protective autophagy induction in response to oxidative stress has been observed (200,201), while excessive autophagy induction has been linked to increased programmed cell death (202). These findings highlight the controversy surrounding the role played by autophagy, particularly macroautophagy. Our results obtained using Hela and Hep3B cell models after subjecting the cells to modulators of autophagy showed that this modulation in cells treated with histones appears to indicate no changes in the localization of histones or the autophagic marker (*Figure A2*

in Annex I section). On the other hand, no changes in cytotoxicity mediated by these proteins were observed (*Figure 14*). However, these latter findings require additional validations to be confidently asserted, as there are certain limitations in the methodology employed as that fluorescent microscope has the disadvantage of only show images in two dimensions, making a stack of the z plane.

Regarding the experiments on autophagy modulation, it would be necessary to expand the range of concentrations and time points, as well as the variety of drugs used. Additionally, an analysis of protein expression would be required to assess the degree of actual activation or inhibition of cells under these conditions, both in the presence and absence of histones. Conversely, the results obtained in this study regarding histone-mediated cytotoxicity in HeLa and Hep3B cells treated with the autophagy modulators metformin and NH_4Cl do not seem to support the expected protective role of autophagy (*Figure 14*). It should be noted that the relationship between autophagy and cell death is not antagonistic in all cases, as autophagy plays a key role as a stress-sensing mechanism and can lead cells towards either survival or death (91). Thus, it would be more appropriate to state that autophagy is a protective mechanism against various types of stress that can tip the balance between cell death and survival in either direction depending on multiple conditions affecting the cells.

It is widely described that the activation of Toll-like receptors (TLRs) in HUVEC cells leads to increased apoptosis through the induction of the $\text{NF}\kappa\text{B}$ /AP-1 signaling pathway (203), which raises doubts about the existence of survival-promoting pathways originating from TLRs beyond the autophagic pathway. Therefore, the most plausible explanation for the obtained results is that the levels of autophagy in the HeLa and Hep3B cells were not affected by NH_4Cl and metformin treatments, which would explain why no changes in histone-mediated cytotoxicity are observed among the different groups (*Figure 14*). There is also the possibility that the relationship between autophagy and histone-mediated cytotoxicity in HUVEC cells may not be the same as in HeLa and Hep3B cells, highlighting the need to repeat these experiments directly on HUVEC cells. This further reinforces the idea that studying the specific effect of extracellular histones on different experimental models can be of great interest.

For this reason, it was decided to switch to an experimental model using monolayer HUVEC cell cultures. For these experiments, Western blot analysis was initially performed to confirm that the chosen treatment times and conditions effectively inhibited or activated autophagy (*Figure27*). Histone-mediated cytotoxicity following the administration of autophagy inhibitors NH_4Cl and leupeptin was nearly absent, accompanied by a significant decrease in colocalization between LC3B protein and extracellular histones (*Figure28*). On the other hand, increasing autophagy also led to a decrease in colocalization, although not as pronounced as in the case of inhibition, and it was not dependent on histone treatment (*Figure29*). In these experiments we used sublethal doses of histones, as the objective was to assess the process of how histones exert its cytotoxic role and the importance of autophagy pathway in this process, because if the doses of histone treatment were too high the system would tend towards apoptosis and any of these changes could be observed.

Taken together, these results seem to support the idea that LC3-mediated endocytosis is responsible for the internalization of histones and, therefore, inhibition of autophagy prevents histones from exerting their cytotoxic effects on cells, resulting in improved cell survival. The possible mechanism of histone internalization and delivery to the non-canonical autophagy pathway to be degraded is described in *Figure 47*.

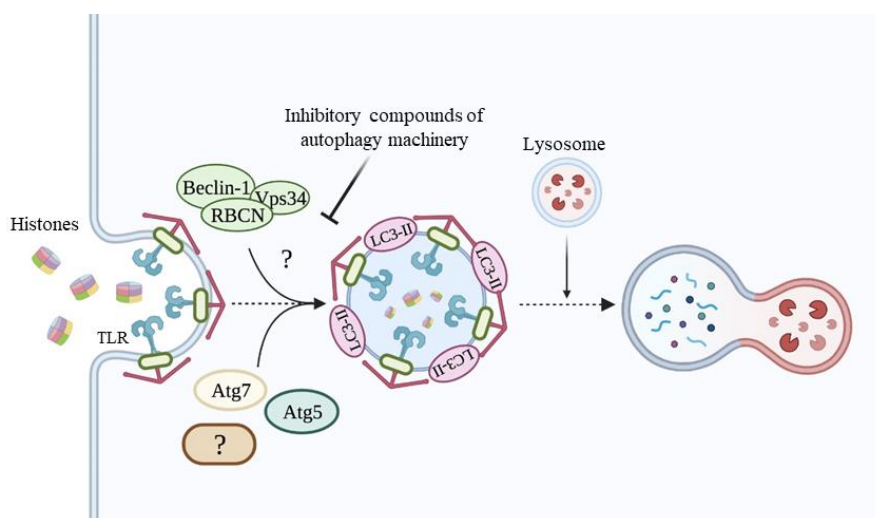


Figure 47. Possible entry mechanism of histones to cells through non-canonical autophagy. Histones interact with the plasma membrane of cells possibly, through the toll-like receptors and then the membrane is invaginated and the histone together with the receptor is internalized through a process called LC3 mediated endocytosis, a type of non-canonical autophagy. Finally, the endocytosed histones are directed to LC3 positive vesicles which will fuse to lysosomes to degrade the cargo, in this case, histones. LC3-II: Light chain 3B protein.

Atg7/5: Autophagy related protein 7/5- Vps34: class III phosphatidylinositol 3-kinase. RBCN: Rubicon Autophagy Regulator. TLR: Toll-like receptors Own elaboration figure using BioRender.com based on (119).

To further investigate the relationship between autophagy and extracellular histones, we proceeded to study this relationship in the more complex model of blood vessel organoids to corroborate previous results. In this case, we observed that modulation of autophagy does not cause a change in the survival of the organoids, which does not allow us to indicate a protective role of autophagy as described in some literature works. The reason of not observing a decreased on the survival rate could be because BVOs need very high doses of core histones to induce cell death and the doses used in these experiments even being sublethal were too much away from the cytotoxic ones. Also, the structure and complexity of the organoid make the entry of histones difficult which could also need more time to be effective. However, a limitation of this study is that the evaluation of survival was only performed under treatments with histone H1-488, so in the future, the effect of autophagy modulation should be tested in conjunction with core histone treatment to see if we observe the same effect of autophagy inhibition as observed in the HUVEC cell model.

Regarding the localization of histones when autophagy is modulated, in the case of treatments with core histones, the inhibition of autophagy tends to decrease the fluorescence intensity of histone H3, both at high and low concentrations, whereas this does not occur when treated with histone H1-488, probably because when H3 is assessed BVOs were treated with the mixture of core histones and not only with one histone as in the case of H1-488, being the possible reason of not showing the same effects. However, the modulation of autophagy had an insignificant impact on the fluorescence intensity of LC3BII in blood vessel organoids treated with core histones. When exposed to higher concentrations of histones, there was a toxic effect, leading to changes in nuclear fluorescence. Although autophagy modulation did alter the localization of H1-488 histone, it did not have a significant effect on its fluorescence intensity.

All these findings highlight the important role played by histones in the development of the complex pathogenesis of sepsis. These findings provide preliminary insights into the relationship between autophagy modulation and histone-induced

cellular responses. Therefore, it is vital to continue studying the pathways affected by extracellular histones, in order to elucidate the underlying mechanisms of histone internalization and cytotoxicity, and to develop therapies based on new therapeutic targets that address the diverse physiological processes affected during sepsis.

4. miRNA signature is altered by extracellular histones and autophagy modulation

Non-coding RNA molecules called miRNAs (miRNAs) range in size from 19 to 24 nucleotides (204). In response to stressors, cells release them actively by - cellular secretions like exosomes, ribonucleoprotein complexes, high density lipoproteins, and microvesicles - or passively upon cell death (through apoptotic bodies) (11,205). miRNAs have special qualities like specificity, selectivity, and high stability, that make them good biomarkers in a variety of disciplines (11).

Although a sizable portion of miRNAs are found intracellularly, circulating miRNAs provide evidence of their presence outside the cell. Biochemical fluids such blood, urine or saliva have been found to contain extracellular miRNAs (11,206). Cardiovascular disease, various cancers, pathophysiological dysfunction, substance poisoning, disorders of the central nervous system, metabolic disorders, immunological disorders, infections, and posttraumatic disorders are just a few of the conditions and diseases that have been linked to a variety of miRNA types (11,205,207). Due to their association with inflammation (186,187), miRNAs have been recognized as possible biomarkers in the setting of sepsis. Recent research has shown that sepsis causes expression patterns of a significant number of miRNAs to change. These results imply that miRNAs have potential as useful markers for sepsis diagnosis and surveillance (11). Therefore, we aimed to investigate the expression of several miRNAs described in the literature and previously analysed by our group in the context of sepsis (208–210), including miR-155, miR-146a, miR-223, miR-15, and miR122, in our various experimental models for studying the molecular mechanisms of sepsis, namely HeLa, Hep3B and BVOs.

miR-155 is a very flexible miRNA which targets a wide range of genes, including FOS, MAP3K14, NFAT5, IFNAR1, SoS1, and SMAD2 (211). Furthermore, recent studies by our team have shown changes in miR-155 and miR-146 in the context of neuroinflammation, highlighting their potential as biomarkers for disorders such as Lafora disease, which is characterized, among others, by neuroinflammation (186). Sepsis-related organ failure assessment score (SOFA) and sepsis severity in adult patients have been reported to be positively correlate with raised levels of miR-155 in the context of sepsis. This shows that in septic individuals, miR-155 may be a marker of a more serious disease and a worse prognosis (207). Various experimental models, including HeLa cells, Hep3B cells and vascular organoids, were used to evaluate the expression of miR-155 in this work. When exposed to core histones in HeLa cells under control circumstances, miR-155 expression was found to be downregulated. In Hep3B cells, a comparable trend was seen, however it was not statistically significant. Surprisingly, HeLa cells subjected to modulated autophagy lowered the expression of miR-155 in all examined settings, but Hep3B cells showed the reverse trend (*Figure 16A*). These results are inconsistent with prior literature, which describes an increase in the levels of this miRNA in septic patients. This discrepancy may be attributed to the involvement of numerous other factors in the context, apart from extracellular histones. Consequently, the observed picture may not capture the complete scenario. However, when vascular organoids are exposed to core histones, miR-155 expression tended to be elevated, with a significant increase seen at 50 µg/ml (*Figure 41A*). These results align with the findings described in the literature, indicating that this vascular organoid model is more complex and therefore more closely resembles the reality of patients. These results imply that core histone treatment affects miR-155 expression, especially when autophagy is active. These findings call for additional research since they shed information on how miR-155 is controlled in various cellular models and emphasize its potential as a biomarker.

miRNA 146a exhibits distinct expression patterns in different patient groups. Specifically, it is found to be upregulated in healthy individuals, while its expression is downregulated in patients with sepsis (11,212). miR-146a showed decreased expression levels in the sepsis group, according to research by Wang et al. on miRNA expression in critical septic patients (213). These findings demonstrate the potential of miR-146a as a

biomarker for identifying sepsis patients from healthy persons (11). Thus, the expression of miR-146a was also investigated in various experiments. In HeLa cells, under control conditions, the expression of miR-146a was clearly downregulated when treated with core histones. A similar downregulation trend was observed in Hep3B cells. Interestingly, when autophagy was modulated in HeLa cells, the dose-dependent downregulation of miR-146a was altered, and no differences were observed among the different core histone treatments. In contrast, in Hep3B cells, the downregulation of miR-146a in response to core histones remained unaffected by autophagy modulation (*Figure 17B*). These results are in line with the literature showing a downregulation of miR-146a when HeLa and Hep3B cells are exposed to core histone treatment and autophagy modulation. Similarly, in BVOs, there were no significant changes in miR-146a expression in response to core histone treatment (*Figure 40B*). The expression levels tended to be downregulated at lower concentrations but remained similar to the control condition without histone treatment at higher concentrations. Overall, these findings indicate the differential regulation of miR-146a in the different experimental models, suggesting complex mechanisms at play.

According to miRNA-122, research shows statistically significant alterations in sepsis patients compared to healthy people (214). Additionally, according to a work by García-Giménez et al., it has been shown to be increased in adolescent idiopathic scoliosis (215). However, we were unable to find amplification in the RT-qPCR for this miRNA in any of our experimental models. These findings imply that miR-122 is not expressed in vascular organoids under the investigated conditions.

Another miRNA object of study was miR-223 since it has been found to be downregulated in sepsis patients (213). Some studies, however, contend that miR-223 could not be a trustworthy biomarker for sepsis (11). Similar research by Benz et al. revealed no appreciable differences in the levels of miR-223 between sepsis patients and healthy people (216). The expression of miR-223 was shown to be lower in the sepsis group, according to Wang et al.'s investigation on miRNA expression in critically sick septic patients (213). These disparate findings point to the difficulty of employing miR-223 as a biomarker for sepsis and the potential involvement of a few variables that may affect its expression (11); in our results we reach the same conclusions, having no

significant changes were obtained in any of the models in which miR-223 was analysed (*Figure 16C*).

Finally, miR-15a was also analysed in our experimental models to provide insight into the relationship between extracellular histones and miRNAs. miR-15a is a possible biomarker for the early detection of neonatal sepsis (207). Additionally, Wang et al (217) hypothesized that the levels of miR-15a in adult patients can reliably separate sepsis from systemic inflammatory response syndrome (SIRS) and separate both illnesses from healthy people. These results shed light on the multiple functions of miR-15a in cellular processes and emphasize its potential as a diagnostic biomarker in various clinical settings (207). However, our findings revealed no discernible changes in the expression levels of miR-15a (*Figure 16D*) across all tested conditions in both HeLa and Hep3B cell models. This observation could potentially be attributed to the fact that these cell models may not closely resemble the sepsis environment. Furthermore, it is worth noting that the overexpression of this miRNA appears to be more prominent in the context of neonatal sepsis (207).

Finally, our study revealed that in response to core histone treatment and autophagy regulation, differential expression patterns for miR-155, miR-146a, and miR-223 were noticed in various cell cultures. These results imply that these miRNAs' expression might be controlled by intricate regulatory mechanisms. But miR122 did not exhibit any amplification in our experimental models, indicating that it was not present under the conditions tested. Additionally, HeLa and Hep3B cell models for miR-15 showed no discernible changes in expression levels, suggesting that these models might not effectively reflect the sepsis environment. Further research is warranted to elucidate the precise role and potential of these miRNAs as biomarkers in sepsis.

5. HMGB1 exhibits potential as a biomarker for sepsis progression and severity

HMGB1 has been characterized as a protein that performs various roles associated with inflammation and the transmission of cellular stress. This occurs when it translocates from the nucleus to the cytoplasm or is released into the extracellular space and subsequently enters the bloodstream. In extranuclear locations, HMGB1 functions as a damage-associated molecular pattern (DAMP) molecule and leads to inflammation. It has also been linked to the development of diverse disorders like trauma, autoimmune diseases, and sepsis (63).

HMGB1 has been proposed as a sepsis biomarker as it could be found in the plasma of patients with sepsis and septic shock (190). Then, to test if we could find HMGB1 in our cellular and organoid models, we exposed them to extracellular histones to model the pathology of sepsis and, indeed, we found that this stimulus was sufficient to induce the secretion of HMGB1 to the supernatant of the cultures in both HUVEC cells (*Figure 31*) and in BVOs (*Figure 43*). This would mean that extracellular histones are capable of inducing the translocation of HMGB1 from the nucleus to extracellular space as it happens in a context of inflammation such as sepsis, opening the possibility of using this protein as sepsis biomarker.

Therefore, we wanted to translate this knowledge to a more clinical point of view and analysing the levels of HMGB1 in sepsis and septic shock patients. However, given the symptomatic heterogeneity in these pathologies and the difficulties in reproducibility of this type of analysis, we first decided to verify the levels of HMGB1 in plasma samples. In our results (*Figure 44*), we observe that HMGB1 levels are significantly higher in patients with sepsis and septic shock compared to control individuals who stayed at ICU from a spontaneous intracerebral haemorrhage rather than being afflicted by an infection, autoimmune condition, or polytrauma injury. Interestingly, the highest values of HMGB1 were found in patients with sepsis rather than septic shock. This contrasts with the findings obtained for citrullinated histone H3 and circulating nucleoproteins (82), where the highest values of either biomarker always correspond to patients with septic shock. This fact could be relevant when considering

the combined use of HMGB1 and citrullinated H3 as biomarkers for sepsis and septic shock, as it would indicate an inverse correlation between them and facilitate the distinction between these two patient groups. However, when statistically analysing the correlation between both proteins, it was found that their inverse correlation was not robust enough to be significant (82). Taking all together, the analysis of HMGB1 using an ELISA kit can effectively differentiate patients from controls with high sensitivity and specificity. However, the differences between sepsis and septic shock patients are not sufficiently pronounced to be significant. The trend towards higher levels in sepsis compared to septic shock samples suggests that HMGB1 may be an earlier marker than other nucleoprotein-based markers. However, further analysis with a larger number of samples is required to corroborate this hypothesis. Nonetheless, the concurrent measurement of HMGB1 levels alongside other markers such as histone H3 using other methodology instead of ELISA - as for example the quantification of this nuclear proteins by more accurate techniques such as mass spectrometry - will enhance diagnostic capacity and assist in disease progression monitoring, allowing for the distinction between cases of sepsis that are close to septic shock and further confirming the relevance of HMGB1 as a nuclear protein present in the plasma of sepsis and septic shock patients. In fact, our group has developed a mass spectrometry method for measuring the histone levels in plasma of septic and septic shock patients (64) improving the results obtained with the ELISA kits for histones, HMGB1 and nucleosomes (82). Currently, we are working on the development of the mass spectrometry method for measuring also HMGB1 protein in the plasma of these patients which would confirm the importance of the protein in the context of sepsis.

If we focus on the clinical aspects of the patients and its relationship with our tested biomarker HMGB1, the results indicated that the groups of ICU control patients did not show significant variations in lactate or procalcitonin (PCT), two conventional sepsis indicators, and that the *exitus* rates were also similar. The diagnostic and prognostic usefulness of a biomarker significantly depends on their association with numerous clinical characteristics that make it possible to track the development and severity of diseases. Our tested biomarker HMGB1 showed a significant correlation between several clinical parameters, among other highlighting ICU length of stay, NtProBNP1

(amino-terminal fragment of the pro-natriuretic peptide type B1) and DD1 (D-dimer1). It has been previously proved the potential of HMGB1 on the worsening of sepsis, as it induces vascular permeability (75) and detrimental effects on the endothelium (78), possibly related to its different oxidative stress (69). Furthermore, high HMGB1 plasma levels have been reported in mice sepsis models (79). In sepsis patients HMGB1 exhibited prolonged elevation and reached its peak at a later stage following the onset of sepsis, setting it apart from other cytokines (80–82). This is in contrast to our results which point out the capacity of HMGB1 to predict, with very high specificity and sensitivity, ICU stays longer than five days for septic patients but not for septic shock ones (*Figure 46C*). One limitation of our analysis is that the samples from sepsis and septic shock patients were obtained six hours after admission to the ICU. Due to the characteristics of the pathology, not all patients would be at the same stage of infection development, which may have occurred not only hours but even days prior to admission. This could explain the large dispersion observed in the data (*Figure 44*), which hinders the establishment of robust correlations between protein levels and disease progression. Therefore, it would be advisable to repeat the HMGB1 analysis in these same patients at longer intervals after admission to observe its evolution and determine how HMGB1 levels are distributed over time. Nevertheless, HMGB1 does emerge as a promising biomarker for sepsis and septic shock due to its high specificity and sensitivity (*Figure 45*). Therefore, our study found that HMGB1 levels are significantly higher in patients with sepsis and septic shock compared to a control group. HMGB1 shows promise as a diagnostic tool, with high sensitivity and specificity, in distinguishing sepsis and septic shock cases from controls. We also observed correlations between HMGB1 and important clinical indicators, such as DD1 and ICU length of stay, highlighting its potential in defining the stage of sepsis. Furthermore, HMGB1 levels can predict prolonged ICU stays, exhibiting strong predictive power. However, it should be noted that a precise cut-off value for accurate prediction could not be determined for septic shock patients. Overall, our study supports the growing evidence of HMGB1 as a promising predictive biomarker for sepsis development. Further research and validation are warranted to fully understand its clinical implications and to enhance patient care in sepsis management.

6. Relevance and limitations of the present work

The thorough research conducted for this thesis clarifies the complex interactions between extracellular histones, cellular responses, and potential clinical consequences. Diverse cellular models using extracellular histones show dose-dependent cytotoxicity, with unique thresholds seen in diverse cell types and organoids. This study's innovative image analysis tool makes it possible to precisely identify and measure extracellular histones and lysosomal colocalization at the single-cell level. Notably, this technology disproved the idea of random cytoplasmic distribution by demonstrating a high concentration of the fluorescent histone marker on lysosomes in HeLa and Hep3B cells. The study also shows that extracellular histone exposure is associated with an increase in the colocalization of histones and LC3B in HUVEC cells, an effect that is diminished by blocking endocytosis. However, this association highlights a divergent trend in blood vessel organoids, emphasizing how complex autophagic interactions are. Histones' effect on miRNA expression in many cell types is further indication of their complicated regulatory role. A consequence that is replicated in blood vessel organoids with upregulated miR-155 is that core histone treatment differentially influences the expression of miR-155 and miR-146. Further highlighting the possible importance of these findings in the setting of clinical diseases is the connection between histone therapy and the secretion of HMGB1 in blood vessel organoids. The fact that HMGB1 levels in the plasma of septic patients are significantly higher and that these levels are also predictive of ICU outcomes underscores the clinical significance of this study's findings. The amino-terminal segment of the pro-natriuretic peptide type B1 and HMGB1 have been correlated with D-dimer, ICU stay duration, and HMGB1 to give useful markers for disease prognosis.

Our research has provided valuable insights, but there are several inherent limitations that must be considered when interpreting our findings. The variability in core histone batches used in our experiments is a significant concern, potentially influencing the unexpected resistance of HUVEC cells to histone-induced cytotoxicity compared to immortalized cells. This variability is likely due to inconsistencies in commercial histone batches. Monolayer cell cultures, whether immortalized or not, may not accurately represent the complex interactions between different cell types seen in

diseases like sepsis. Alternative models using stem cells or adult tissues better mimic human blood vessels' responses to histones, cautioning against direct extrapolation of our findings to in vivo settings. Furthermore, none of our tested models exhibited cell death in response to certain histone concentrations, suggesting that the doses used might not have been sufficiently high to induce significant cytotoxicity. The potential synergistic toxicity of multiple histones, particularly in the histone core, requires further investigation. Although our colocalization studies in Hela and Hep3B cells were informative, additional evidence is needed to confirm the presence of histones inside lysosomes. Two-dimensional colocalization studies have limitations in providing conclusive evidence, necessitating future research using confocal microscopy. Our investigations into autophagy modulation also have limitations, calling for expanded dose ranges, time points, and drug types, alongside protein expression assessments, to comprehensively understand the underlying mechanisms. In our study of organoids (BVOs), the absence of a decline in survival rate may be attributed to the need for extremely high histone doses or longer incubation time with histones and the structural complexity of the organoids hindering histone entry. Future research should explore the interaction between core histone therapy and autophagy regulation in this context. Lastly, our analysis of patient samples from sepsis and septic shock patients is constrained by the timing of sample collection, taken six hours after ICU admission when patients could be at various infection development stages. This temporal variability may have contributed to data dispersion and challenges in establishing robust associations between protein levels and disease progression. Future research should consider longer sample collection intervals and utilize more precise protein quantification methods like mass spectrometry to enhance analytical accuracy.

Overall, the results of this thesis open up exciting directions for further study and clinical applications. Further research into the underlying molecular processes driving histone-induced cytotoxicity and autophagic interactions is warranted in light of the complex interplay between extracellular histones and cellular responses that has been revealed here. Investigating the precise signalling mechanisms that mediate the diverse outcomes seen in different cell types and organoids may reveal new treatment targets for diseases impacted by histone release. The strong associations between histone-

related markers and clinical outcomes, from a translational perspective, lead to the potential use of these markers in disease prognosis and patient treatment. The prognostic value of these markers in larger patient cohorts could be further explored in other clinical research, as well as their potential as early markers of disease severity.

Finally, this thesis paves the way for additional research into the complex connections between histones and cells, as well as their molecular underpinnings and potential clinical applications. The complexity of extracellular histones' impact on numerous cellular functions provides a rich environment for more study and has the potential to aid in the creation of novel treatment approaches and diagnostic instruments.

CONCLUSIONS

CONCLUSIONS

1. Administration of core histones in all studied models induces cytotoxicity at high doses, starting from 200 $\mu\text{g/mL}$ in HeLa and Hep3B cells, 400 $\mu\text{g/mL}$ in HUVEC cells, and 500 $\mu\text{g/mL}$ in blood vessel organoids.
2. The developed image analysis tool allows recognition of extracellular histones and fluorescently labelled lysosomes, quantifying their overlap at the single-cell level using fluorescence microscopy images. In HeLa and Hep3B cells, the fluorescent histone marker is predominantly located on lysosomes rather than randomly distributed in the cytoplasm.
3. Treatment with extracellular histones (core histones and H1-488) leads to a dose and time-dependent increase in colocalization between extracellular histones and the autophagy-associated protein LC3B in HUVEC cells, as well as an increase in both histone and LC3B fluorescent markers in blood vessel organoids.
4. Under conditions of endocytosis blockade, entry of extracellular histones is prevented, and it is accompanied by a reduction in cytotoxicity caused by extracellular histones in HUVEC and blood vessel organoids.
5. Blocking endocytosis results in a decreased of colocalization between extracellular histones and the protein LC3B in HUVEC cells and in the case of blood vessel organoids, extracellular histones and LC3B fluorescent marks decrease with extracellular histone treatments.
6. Addition of autophagy inhibitors NH_4Cl and leupeptin, as well as Krebs-Henseleit medium as an autophagy activator, decreases colocalization between extracellular histones and the autophagic marker LC3B in HUVEC cells.
7. Core histone treatment induces downregulation of miR-155 and miR-146 in HeLa and Hep3B cells but in blood vessel organoids (BVOs) the expression of miR-155 is upregulated.
8. The expression of eNOs is downregulated in BVOs when they are exposed to extracellular histones or HMGB1, whereas the expression of VCAM-1 is

upregulated only in the presence of extracellular histones and the expression of E-selectin is decreased with HMGB1 treatment.

9. Treatment with core histones induces dose-dependent secretion of HMGB1 in blood vessel organoids and HUVEC cells.
10. HMGB1 levels are significantly higher in the plasma of patients with sepsis and septic shock compared to an ICU control group, demonstrating high sensitivity and specificity with positive correlations with D-dimer¹ and ICU length of stay, while exhibiting a negative association with amino-terminal fragment of the pro-natriuretic peptide type B1.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016 Feb 23;315(8):801.
2. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. *The Lancet*. 2020 Jan;395(10219):200–11.
3. Gauer RL. Early Recognition and Management of Sepsis in Adults: The First Six Hours [Internet]. Vol. 88, American Family Physician www.aafp.org/afp. 2013. Available from: www.aafp.org/afp.
4. Gauer R, Forbes D, Boyer N. Sepsis: Diagnosis and Management. *Am Fam Physician*. 2020 Apr 1;101(7):409–18.
5. Dolin HH, Papadimos TJ, Stepkowski S, Chen X, Pan ZK. A Novel Combination of Biomarkers to Herald the Onset of Sepsis Prior to the Manifestation of Symptoms. *Shock*. 2018 Apr;49(4):364–70.
6. Sadaka F, EthmaneAbouElMaali C, Cytron MA, Fowler K, Javaux VM, O'Brien J. Predicting Mortality of Patients With Sepsis: A Comparison of APACHE II and APACHE III Scoring Systems. *J Clin Med Res*. 2017;9(11):907–10.
7. Chakraborty RK, Burns B. Systemic Inflammatory Response Syndrome. 2022.
8. Fernando SM, Tran A, Taljaard M, Cheng W, Rochweg B, Seely AJE, et al. Prognostic Accuracy of the Quick Sequential Organ Failure Assessment for Mortality in Patients With Suspected Infection. *Ann Intern Med*. 2018 Feb 20;168(4):266.
9. Tian Y, Yao Y, Zhou J, Diao X, Chen H, Cai K, et al. Dynamic APACHE II Score to Predict the Outcome of Intensive Care Unit Patients. *Front Med (Lausanne)*. 2022 Jan 26;8.
10. Vieira Corrêa PCM, Carneiro DM, da Silva Valente L do S, Diogo FM, Lamarão LM, da Silva Maués JH, et al. Detection of Sepsis in Platelets Using MicroRNAs and Membrane Antigens. *Genes (Basel)*. 2021 Nov 25;12(12):1877.
11. Dumache R, Rogobete AF, Bedreag OH, Sarandan M, Cradigati AC, Papurica M, et al. Use of miRNAs as Biomarkers in Sepsis. *Analytical Cellular Pathology*. 2015;2015:1–9.
12. Benz F, Roy S, Trautwein C, Roderburg C, Luedde T. Circulating MicroRNAs as Biomarkers for Sepsis. *Int J Mol Sci*. 2016 Jan 9;17(1):78.
13. Tribolet L, Kerr E, Cowled C, Bean AGD, Stewart CR, Dearnley M, et al. MicroRNA Biomarkers for Infectious Diseases: From Basic Research to Biosensing. *Front Microbiol*. 2020 Jun 3;11.
14. Pierrakos C, Vincent JL. Sepsis biomarkers: a review. *Crit Care*. 2010;14(1):R15.
15. Gyawali B, Ramakrishna K, Dhamoon AS. Sepsis: The evolution in definition, pathophysiology, and management. *SAGE Open Med*. 2019 Jan 21;7:205031211983504.
16. Shoemaker WC, Appel PL, Kram HB, Waxman K, Lee TS. Prospective Trial of Supranormal Values of Survivors as Therapeutic Goals in High-Risk Surgical Patients. *Chest*. 1988 Dec;94(6):1176–86.

17. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock*. *Crit Care Med.* 2006 Jun;34(6):1589–96.
18. Rochwerf B, Alhazzani W, Sindi A, Heels-Ansdell D, Thabane L, Fox-Robichaud A, et al. Fluid Resuscitation in Sepsis. *Ann Intern Med.* 2014 Sep 2;161(5):347.
19. Bourgoin A, Leone M, Delmas A, Garnier F, Albanèse J, Martin C. Increasing mean arterial pressure in patients with septic shock: Effects on oxygen variables and renal function*. *Crit Care Med.* 2005 Apr;33(4):780–6.
20. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign. *Crit Care Med.* 2013 Feb;41(2):580–637.
21. Webster NR, Galley HF. Immunomodulation in the critically ill. *Br J Anaesth.* 2009 Jul;103(1):70–81.
22. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol.* 2008 Oct;8(10):776–87.
23. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent JL. Sepsis and septic shock. *Nat Rev Dis Primers.* 2016 Jun 30;2(1):16045.
24. Huang M, Cai S, Su J. The Pathogenesis of Sepsis and Potential Therapeutic Targets. *Int J Mol Sci.* 2019 Oct 29;20(21):5376.
25. Gotts JE, Matthay MA. Sepsis: pathophysiology and clinical management. *BMJ.* 2016 May 23;i1585.
26. Denning NL, Aziz M, Gurien SD, Wang P. DAMPs and NETs in Sepsis. *Front Immunol.* 2019 Oct 30;10.
27. Kumar S, Payal N, Srivastava VK, Kaushik S, Saxena J, Jyoti A. Neutrophil extracellular traps and organ dysfunction in sepsis. *Clinica Chimica Acta.* 2021 Dec;523:152–62.
28. Nedeva C, Menassa J, Puthalakath H. Sepsis: Inflammation Is a Necessary Evil. *Front Cell Dev Biol.* 2019 Jun 20;7.
29. Danielski LG, Giustina A Della, Bonfante S, Barichello T, Petronilho F. The NLRP3 Inflammasome and Its Role in Sepsis Development. *Inflammation.* 2020 Feb 18;43(1):24–31.
30. Biasizzo M, Kopitar-Jerala N. Interplay Between NLRP3 Inflammasome and Autophagy. *Front Immunol.* 2020 Oct 9;11.
31. Zheng X, Chen W, Gong F, Chen Y, Chen E. The Role and Mechanism of Pyroptosis and Potential Therapeutic Targets in Sepsis: A Review. *Front Immunol.* 2021 Jul 7;12.
32. Yipp BG, Kubes P. NETosis: how vital is it? *Blood.* 2013 Oct 17;122(16):2784–94.
33. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil Extracellular Traps Kill Bacteria. *Science (1979).* 2004 Mar 5;303(5663):1532–5.
34. Ait-Oufella H, Maury E, Lehoux S, Guidet B, Offenstadt G. The endothelium: physiological functions and role in microcirculatory failure during severe sepsis. *Intensive Care Med.* 2010 Aug 5;36(8):1286–98.

35. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol.* 2010 Dec 7;11(12):1136–42.
36. Lupu F, Keshari RS, Lambris JD, Mark Coggeshall K. Crosstalk between the coagulation and complement systems in sepsis. *Thromb Res.* 2014 May;133:S28–31.
37. Zetoune FS, Ward PA. Role of Complement and Histones in Sepsis. *Front Med (Lausanne).* 2020 Dec 23;7.
38. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol.* 2013 Dec 15;13(12):862–74.
39. Torres LK, Pickkers P, van der Poll T. Sepsis-Induced Immunosuppression. *Annu Rev Physiol.* 2022 Feb 10;84(1):157–81.
40. Delano MJ, Ward PA. The immune system's role in sepsis progression, resolution, and long-term outcome. *Immunol Rev.* 2016 Nov;274(1):330–53.
41. Campos EI, Reinberg D. Histones: Annotating Chromatin. *Annu Rev Genet.* 2009 Dec 1;43(1):559–99.
42. Allam R, Kumar SVR, Darisipudi MN, Anders HJ. Extracellular histones in tissue injury and inflammation. *J Mol Med.* 2014 May 6;92(5):465–72.
43. García-Giménez JL, Olaso G, Hake SB, Bönisch C, Wiedemann SM, Markovic J, et al. Histone H3 Glutathionylation in Proliferating Mammalian Cells Destabilizes Nucleosomal Structure. *Antioxid Redox Signal.* 2013 Oct 20;19(12):1305–20.
44. Talbert PB, Henikoff S. Histone variants — ancient wrap artists of the epigenome. *Nat Rev Mol Cell Biol.* 2010 Apr 3;11(4):264–75.
45. Zlatanova J, Bishop TC, Victor JM, Jackson V, van Holde K. The Nucleosome Family: Dynamic and Growing. *Structure.* 2009 Feb;17(2):160–71.
46. García-Giménez JL, Garcés C, Romá-Mateo C, Pallardó F V. Oxidative stress-mediated alterations in histone post-translational modifications. *Free Radic Biol Med.* 2021 Jul;170:6–18.
47. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. *Cell Death Dis.* 2014 Aug 14;5(8):e1370–e1370.
48. Silk E, Zhao H, Weng H, Ma D. The role of extracellular histone in organ injury. *Cell Death Dis.* 2017 May 25;8(5):e2812–e2812.
49. Cheng Z, Abrams ST, Alhamdi Y, Toh J, Yu W, Wang G, et al. Circulating Histones Are Major Mediators of Multiple Organ Dysfunction Syndrome in Acute Critical Illnesses. *Crit Care Med.* 2019 Aug;47(8):e677–84.
50. Xu J, Zhang X, Pelayo R, Monestier M, Ammolto CT, Semeraro F, et al. Extracellular histones are major mediators of death in sepsis. *Nat Med.* 2009 Nov 25;15(11):1318–21.
51. Moiana M, Aranda F, de Larrañaga G. A focus on the roles of histones in health and diseases. *Clin Biochem.* 2021 Aug;94:12–9.
52. Hirsch JG. BACTERICIDAL ACTION OF HISTONE. *Journal of Experimental Medicine.* 1958 Dec 1;108(6):925–44.

53. Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT. Extracellular Histones Are Mediators of Death through TLR2 and TLR4 in Mouse Fatal Liver Injury. *The Journal of Immunology*. 2011 Sep 1;187(5):2626–31.
54. Zhang X, Li X. The Role of Histones and Heparin in Sepsis: A Review. *J Intensive Care Med*. 2022 Mar 25;37(3):319–26.
55. PEREIRA LF, MARCO FM, BOIMORTO R, CATURLA A, BUSTOS A, CONCHA EGDELA, et al. Histones interact with anionic phospholipids with high avidity; its relevance for the binding of histone–antihistone immune complexes. *Clin Exp Immunol*. 2008 Jun 28;97(2):175–80.
56. Osca-Verdegel R, Beltrán-García J, Paes AB, Nacher-Sendra E, Novella S, Hermenegildo C, et al. Histone Citrullination Mediates a Protective Role in Endothelium and Modulates Inflammation. *Cells*. 2022 Dec 15;11(24):4070.
57. Gilthorpe JD, Oozeer F, Nash J, Calvo M, Bennett DL, Lumsden A, et al. Extracellular histone H1 is neurotoxic and drives a pro-inflammatory response in microglia. *F1000Res*. 2013 Jul 8;2:148.
58. Szatmary P, Huang W, Criddle D, Tepikin A, Sutton R. Biology, role and therapeutic potential of circulating histones in acute inflammatory disorders. *J Cell Mol Med*. 2018 Oct 7;22(10):4617–29.
59. Yokoyama Y, Ito T, Yasuda T, Furubeppu H, Kamikokuryo C, Yamada S, et al. Circulating histone H3 levels in septic patients are associated with coagulopathy, multiple organ failure, and death: a single-center observational study. *Thromb J*. 2019 Dec 14;17(1):1.
60. Ekaney ML, Otto GP, Sossdorf M, Sponholz C, Boehringer M, Loesche W, et al. Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. *Crit Care*. 2014 Oct 24;18(5):543.
61. Jackson Chornenki NL, Coke R, Kwong AC, Dwivedi DJ, Xu MK, McDonald E, et al. Comparison of the source and prognostic utility of cfDNA in trauma and sepsis. *Intensive Care Med Exp*. 2019 Dec 22;7(1):29.
62. Li Y, Wan D, Luo X, Song T, Wang Y, Yu Q, et al. Circulating Histones in Sepsis: Potential Outcome Predictors and Therapeutic Targets. *Front Immunol*. 2021 Mar 24;12.
63. Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. *J Immunol Methods*. 2009 Aug;347(1–2):3–11.
64. García-Giménez JL, Romá-Mateo C, Carbonell N, Palacios L, Peiró-Chova L, García-López E, et al. A new mass spectrometry-based method for the quantification of histones in plasma from septic shock patients. *Sci Rep*. 2017 Sep 6;7(1):10643.
65. García-Giménez JL, García-López E, Mena-Mollá S, Beltrán-García J, Osca-Verdegel R, Nacher-Sendra E, et al. Validation of circulating histone detection by mass spectrometry for early diagnosis, prognosis, and management of critically ill septic patients. *J Transl Med*. 2023 May 23;21(1):344.
66. Xu Z, Huang Y, Mao P, Zhang J, Li Y. Sepsis and ARDS: The Dark Side of Histones. *Mediators Inflamm*. 2015;2015:1–9.
67. Ibañez-Cabellos JS, Aguado C, Pérez-Cremades D, García-Giménez JL, Bueno-Betí C, García-López EM, et al. Extracellular histones activate autophagy and apoptosis via mTOR

- signaling in human endothelial cells. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 2018 Oct;1864(10):3234–46.
68. Javaherian K, Liu LF, Wang JC. Nonhistone Proteins HMG1 and HMG2 change the DNA Helical Structure. *Science* (1979). 1978 Mar 24;199(4335):1345–6.
 69. Deng M, Scott MJ, Fan J, Billiar TR. Location is the key to function: HMGB1 in sepsis and trauma-induced inflammation. *J Leukoc Biol*. 2019 Jun 27;106(1):161–9.
 70. Ugrinova I, Pasheva E. HMGB1 Protein: A Therapeutic Target Inside and Outside the Cell. In 2017. p. 37–76.
 71. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005 Apr;5(4):331–42.
 72. Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, et al. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *Journal of Experimental Medicine*. 2012 Aug 27;209(9):1519–28.
 73. Mullins GE, Sunden-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, et al. Activation of Human Umbilical Vein Endothelial Cells Leads to Relocation and Release of High-Mobility Group Box Chromosomal Protein 1. *Scand J Immunol*. 2004 Dec;60(6):566–73.
 74. Nawaz MI, Mohammad G. Role of high-mobility group box-1 protein in disruption of vascular barriers and regulation of leukocyte–endothelial interactions. *Journal of Receptors and Signal Transduction*. 2015 Jul 4;35(4):340–5.
 75. ZHENG YJ, XU WP, DING G, GAO YH, WANG HR, PAN SM. Expression of HMGB1 in septic serum induces vascular endothelial hyperpermeability. *Mol Med Rep*. 2016 Jan;13(1):513–21.
 76. Huang W, Liu Y, Li L, Zhang R, Liu W, Wu J, et al. HMGB1 Increases Permeability of the Endothelial Cell Monolayer via RAGE and Src Family Tyrosine Kinase Pathways. *Inflammation*. 2012 Feb 15;35(1):350–62.
 77. SHAO M, TANG ST, LIU B, ZHU HQ. Rac1 mediates HMGB1-induced hyperpermeability in pulmonary microvascular endothelial cells via MAPK signal transduction. *Mol Med Rep*. 2016 Jan;13(1):529–35.
 78. Ito T, Kawahara K ichi, Okamoto K, Yamada S, Yasuda M, Imaizumi H, et al. Proteolytic Cleavage of High Mobility Group Box 1 Protein by Thrombin-Thrombomodulin Complexes. *Arterioscler Thromb Vasc Biol*. 2008 Oct;28(10):1825–30.
 79. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a Late Mediator of Endotoxin Lethality in Mice. *Science* (1979). 1999 Jul 9;285(5425):248–51.
 80. Chavan SS, Huerta PT, Robbiati S, Valdes-Ferrer SI, Ochani M, Dancho M, et al. HMGB1 Mediates Cognitive Impairment in Sepsis Survivors. *Molecular Medicine*. 2012 Jun 16;18(6):930–7.
 81. Sundén-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, et al. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock*. *Crit Care Med*. 2005 Mar;33(3):564–73.

82. Beltrán-García J, Manclús JJ, García-López EM, Carbonell N, Ferreres J, Rodríguez-Gimillo M, et al. Comparative Analysis of Chromatin-Delivered Biomarkers in the Monitoring of Sepsis and Septic Shock: A Pilot Study. *Int J Mol Sci*. 2021 Sep 14;22(18):9935.
83. Huang W, Liu Y, Li L, Zhang R, Liu W, Wu J, et al. HMGB1 Increases Permeability of the Endothelial Cell Monolayer via RAGE and Src Family Tyrosine Kinase Pathways. *Inflammation*. 2012 Feb 15;35(1):350–62.
84. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proceedings of the National Academy of Sciences*. 2004 Jan 6;101(1):296–301.
85. Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H, et al. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum*. 2003 Jul;48(7):2052–8.
86. Stevens NE, Chapman MJ, Fraser CK, Kuchel TR, Hayball JD, Diener KR. Therapeutic targeting of HMGB1 during experimental sepsis modulates the inflammatory cytokine profile to one associated with improved clinical outcomes. *Sci Rep*. 2017 Jul 19;7(1):5850.
87. Wang H, Ward MF, Sama AE. Targeting HMGB1 in the treatment of sepsis. *Expert Opin Ther Targets*. 2014 Mar 6;18(3):257–68.
88. Lee W, Ku SK, Bae JS. Zingerone reduces HMGB1-mediated septic responses and improves survival in septic mice. *Toxicol Appl Pharmacol*. 2017 Aug;329:202–11.
89. Musumeci D, Roviello GN, Montesarchio D. An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. *Pharmacol Ther*. 2014 Mar;141(3):347–57.
90. Zheng S, Weng Q, Wu W, Ding G. Blood purification treatment initiated at the time of sepsis diagnosis effectively attenuates serum HMGB1 upregulation and improves patient prognosis. *Exp Ther Med*. 2017 Oct;14(4):3029–35.
91. Ravanani P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. *Life Sci*. 2017 Nov;188:53–67.
92. Qiu P, Liu Y, Zhang J. Review: the Role and Mechanisms of Macrophage Autophagy in Sepsis. *Inflammation*. 2019 Feb 7;42(1):6–19.
93. Deretic V, Kimura T, Timmins G, Moseley P, Chauhan S, Mandell M. Immunologic manifestations of autophagy. *Journal of Clinical Investigation*. 2015 Jan 2;125(1):75–84.
94. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nat Rev Immunol*. 2013 Oct 25;13(10):722–37.
95. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011 Jan 20;469(7330):323–35.
96. Kumar V. Targeting macrophage immunometabolism: Dawn in the darkness of sepsis. *Int Immunopharmacol*. 2018 May;58:173–85.
97. Gomes LR, Menck CFM, Cuervo AM. Chaperone-mediated autophagy prevents cellular transformation by regulating MYC proteasomal degradation. *Autophagy*. 2017 May 4;13(5):928–40.

98. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res.* 2014 Jan 24;24(1):24–41.
99. Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, et al. Inhibition of Macroautophagy Triggers Apoptosis. *Mol Cell Biol.* 2005 Feb;25(3):1025–40.
100. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol.* 2010 May;221(1):3–12.
101. Kirkin V, Lamark T, Sou YS, Bjørkøy G, Nunn JL, Bruun JA, et al. A Role for NBR1 in Autophagosomal Degradation of Ubiquitinated Substrates. *Mol Cell.* 2009 Feb;33(4):505–16.
102. Abounit K, Scarabelli TM, McCauley RB. Autophagy in mammalian cells. *World J Biol Chem.* 2012;3(1):1.
103. Dossou AS, Basu A. The Emerging Roles of mTORC1 in Macromanaging Autophagy. *Cancers (Basel).* 2019 Sep 24;11(10):1422.
104. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res.* 2014 Jan 24;24(1):24–41.
105. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Mol Cell.* 2008 Apr;30(2):214–26.
106. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett.* 2010 Apr 2;584(7):1287–95.
107. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria Supply Membranes for Autophagosome Biogenesis during Starvation. *Cell.* 2010 May;141(4):656–67.
108. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. *Autophagy.* 2009 Nov 16;5(8):1180–5.
109. Orsi A, Razi M, Dooley HC, Robinson D, Weston AE, Collinson LM, et al. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. *Mol Biol Cell.* 2012 May;23(10):1860–73.
110. Ge L, Melville D, Zhang M, Schekman R. The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife.* 2013 Aug 6;2:e00947.
111. Puri C, Renna M, Bento CF, Moreau K, Rubinsztein DC. Diverse Autophagosome Membrane Sources Coalesce in Recycling Endosomes. *Cell.* 2013 Sep;154(6):1285–99.
112. Ganley IG. Autophagosome maturation and lysosomal fusion. *Essays Biochem.* 2013 Sep 27;55:65–78.
113. Song C, Mitter SK, Qi X, Beli E, Rao H V., Ding J, et al. Oxidative stress-mediated NFκB phosphorylation upregulates p62/SQSTM1 and promotes retinal pigmented epithelial cell survival through increased autophagy. *PLoS One.* 2017 Feb 21;12(2):e0171940.

114. Djavaheri-Mergny M, Amelotti M, Mathieu J, Besançon F, Bauvy C, Souquère S, et al. NF- κ B Activation Represses Tumor Necrosis Factor- α -induced Autophagy. *Journal of Biological Chemistry*. 2006 Oct;281(41):30373–82.
115. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, et al. The TSC-mTOR Signaling Pathway Regulates the Innate Inflammatory Response. *Immunity*. 2008 Oct;29(4):565–77.
116. Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. *Am J Cancer Res*. 2012;2(2):214–21.
117. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol*. 2012 Feb 18;13(2):89–102.
118. Li Y, Guo Y, Tang J, Jiang J, Chen Z. New insights into the roles of CHOP-induced apoptosis in ER stress. *Acta Biochim Biophys Sin (Shanghai)*. 2014 Aug 1;46(8):629–40.
119. Jülg J, Strohm L, Behrends C. Canonical and Noncanonical Autophagy Pathways in Microglia. *Mol Cell Biol*. 2021 Feb 23;41(3).
120. Dupont N, Codogno P. Non-canonical Autophagy: Facts and Prospects. *Curr Pathobiol Rep*. 2013 Dec 8;1(4):263–71.
121. Codogno P, Mehrpour M, Proikas-Cezanne T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nat Rev Mol Cell Biol*. 2012 Jan 14;13(1):7–12.
122. Dupont N, Roccio F, Codogno P. Overview of noncanonical autophagy. In: *Non-Canonical Autophagy*. Elsevier; 2021. p. 41–67.
123. Codogno P, Mehrpour M, Proikas-Cezanne T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nat Rev Mol Cell Biol*. 2012 Jan 14;13(1):7–12.
124. Berryman S, Brooks E, Burman A, Hawes P, Roberts R, Netherton C, et al. Foot-and-Mouth Disease Virus Induces Autophagosomes during Cell Entry via a Class III Phosphatidylinositol 3-Kinase-Independent Pathway. *J Virol*. 2012 Dec;86(23):12940–53.
125. Arsov I, Adebayo A, Kucerova-Levisohn M, Haye J, MacNeil M, Papavasiliou FN, et al. A Role for Autophagic Protein Beclin 1 Early in Lymphocyte Development. *The Journal of Immunology*. 2011 Feb 15;186(4):2201–9.
126. Mestre MB, Fader CM, Sola C, Colombo MI. α -hemolysin is required for the activation of the autophagic pathway in *Staphylococcus aureus* infected cells. *Autophagy*. 2010 Jan 27;6(1):110–25.
127. Mauthe M, Jacob A, Freiburger S, Hentschel K, Stierhof YD, Codogno P, et al. Resveratrol-mediated autophagy requires WIPI-1-regulated LC3 lipidation in the absence of induced phagophore formation. *Autophagy*. 2011 Dec 28;7(12):1448–61.
128. Grottemeier A, Alers S, Pfisterer SG, Paasch F, Daubrawa M, Dieterle A, et al. AMPK-independent induction of autophagy by cytosolic Ca^{2+} increase. *Cell Signal*. 2010 Jun;22(6):914–25.
129. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. *Journal of Cell Biology*. 2005 Sep 26;170(7):1101–11.

130. Gui X, Yang H, Li T, Tan X, Shi P, Li M, et al. Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. *Nature*. 2019 Mar 6;567(7747):262–6.
131. Ra EA, Lee TA, Won Kim S, Park A, Choi H jin, Jang I, et al. TRIM31 promotes Atg5/Atg7-independent autophagy in intestinal cells. *Nat Commun*. 2016 May 24;7(1):11726.
132. Sharma V, Verma S, Seranova E, Sarkar S, Kumar D. Selective Autophagy and Xenophagy in Infection and Disease. *Front Cell Dev Biol*. 2018 Nov 13;6.
133. Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol*. 2007 Oct;7(10):767–77.
134. Sanjuan MA, Dillon CP, Tait SWG, Moshiah S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature*. 2007 Dec;450(7173):1253–7.
135. Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat Cell Biol*. 2011 Nov 16;13(11):1335–43.
136. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proceedings of the National Academy of Sciences*. 2011 Oct 18;108(42):17396–401.
137. Guo R, Wang H, Cui N. Autophagy Regulation on Pyroptosis: Mechanism and Medical Implication in Sepsis. *Mediators Inflamm*. 2021 Jun 24;2021:1–11.
138. Wang H, Bai G, Cui N, Han W, Long Y. T-cell-specific mTOR deletion in mice ameliorated CD4⁺ T-cell survival in lethal sepsis induced by severe invasive candidiasis. *Virulence*. 2019 Jan 1;10(1):892–901.
139. Kim JY, Paton JC, Briles DE, Rhee DK, Pyo S. *Streptococcus pneumoniae* induces pyroptosis through the regulation of autophagy in murine microglia. *Oncotarget*. 2015 Dec 29;6(42):44161–78.
140. Wang Z, Li Y, Yang X, Zhang L, Shen H, Xu W, et al. Protective effects of rapamycin induced autophagy on CLP septic mice. *Comp Immunol Microbiol Infect Dis*. 2019 Jun;64:47–52.
141. Ge Y, Xu X, Liang Q, Xu Y, Huang M. α -Mangostin suppresses NLRP3 inflammasome activation via promoting autophagy in LPS-stimulated murine macrophages and protects against CLP-induced sepsis in mice. *Inflammation Research*. 2019 Jun 29;68(6):471–9.
142. Liang H, Ding X, Li L, Wang T, Kan Q, Wang L, et al. Association of preadmission metformin use and mortality in patients with sepsis and diabetes mellitus: a systematic review and meta-analysis of cohort studies. *Crit Care*. 2019 Dec 18;23(1):50.
143. Saber S, El-Kader EMA. Novel complementary coloprotective effects of metformin and MCC950 by modulating HSP90/NLRP3 interaction and inducing autophagy in rats. *Inflammopharmacology*. 2021 Feb 27;29(1):237–51.
144. Pu Q, Gan C, Li R, Li Y, Tan S, Li X, et al. Atg7 Deficiency Intensifies Inflammasome Activation and Pyroptosis in *Pseudomonas* Sepsis. *The Journal of Immunology*. 2017 Apr 15;198(8):3205–13.

145. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature*. 2008 Nov 5;456(7219):264–8.
146. Kang R, Livesey KM, Zeh IHJ, Loze MT, Tang D. HMGB1: A novel Beclin 1-binding protein active in autophagy. *Autophagy*. 2010 Nov 16;6(8):1209–11.
147. Itakura A, McCarty OJT. Pivotal role for the mTOR pathway in the formation of neutrophil extracellular traps via regulation of autophagy. *American Journal of Physiology-Cell Physiology*. 2013 Aug 1;305(3):C348–54.
148. Boone BA, Orlichenko L, Schapiro NE, Loughran P, Gianfrate GC, Ellis JT, et al. The receptor for advanced glycation end products (RAGE) enhances autophagy and neutrophil extracellular traps in pancreatic cancer. *Cancer Gene Ther*. 2015 Jun;22(6):326–34.
149. Zhao H, Chen H, Xiaoyin M, Yang G, Hu Y, Xie K, et al. Autophagy Activation Improves Lung Injury and Inflammation in Sepsis. *Inflammation*. 2019 Apr 15;42(2):426–39.
150. Pérez-Cremades D, Bueno-Betí C, García-Giménez JL, Ibañez-Cabellos JS, Hermenegildo C, Pallardó F V., et al. Extracellular histones disarrange vasoactive mediators release through a COX-NOS interaction in human endothelial cells. *J Cell Mol Med*. 2017 Aug;21(8):1584–92.
151. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Crit Care Med*. 2001 Jul;29(7):1303–10.
152. Poli-de-Figueiredo LF, Garrido AG, Nakagawa N, Sannomiya P. EXPERIMENTAL MODELS OF SEPSIS AND THEIR CLINICAL RELEVANCE. *Shock*. 2008 Oct;30(7):53–9.
153. Dickson K, Lehmann C. Inflammatory Response to Different Toxins in Experimental Sepsis Models. *Int J Mol Sci*. 2019 Sep 5;20(18).
154. Fink MP, Heard SO. Laboratory models of sepsis and septic shock. *Journal of Surgical Research*. 1990 Aug;49(2):186–96.
155. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends Microbiol*. 2011 Apr;19(4):198–208.
156. Cherkasova MN. Experimental Modeling of Sepsis. *Biology Bulletin Reviews*. 2021 Dec 17;11(S1):65–77.
157. Page MJ, Kell DB, Pretorius E. The Role of Lipopolysaccharide-Induced Cell Signalling in Chronic Inflammation. *Chronic Stress*. 2022 Jan 8;6:247054702210763.
158. Leung CM, de Haan P, Ronaldson-Bouchard K, Kim GA, Ko J, Rho HS, et al. A guide to the organ-on-a-chip. *Nature Reviews Methods Primers*. 2022 May 12;2(1):33.
159. Caraballo C, Jaimes F. Organ Dysfunction in Sepsis: An Ominous Trajectory From Infection To Death. *Yale J Biol Med*. 2019 Dec;92(4):629–40.
160. Peerapornratana S, Manrique-Caballero CL, Gómez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. *Kidney Int*. 2019 Nov;96(5):1083–99.

161. Markewitz BA, Michael JR, Kohan DE. Cytokine-induced expression of a nitric oxide synthase in rat renal tubule cells. *Journal of Clinical Investigation*. 1993 May 1;91(5):2138–43.
162. Du C, Guan Q, Diao H, Yin Z, Jevnikar AM. Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. *American Journal of Physiology-Renal Physiology*. 2006 May;290(5):F1044–54.
163. Gómez H, Kellum JA. Sepsis-induced acute kidney injury. *Curr Opin Crit Care*. 2016 Dec;22(6):546–53.
164. Ye Z, Zhang L, Li R, Dong W, Liu S, Li Z, et al. Caspase-11 Mediates Pyroptosis of Tubular Epithelial Cells and Septic Acute Kidney Injury. *Kidney Blood Press Res*. 2019;44(4):465–78.
165. Lautz AJ, Zingarelli B. Age-Dependent Myocardial Dysfunction in Critically Ill Patients: Role of Mitochondrial Dysfunction. *Int J Mol Sci*. 2019 Jul 18;20(14):3523.
166. Chen D, Wang H, Cai X. Curcumin interferes with sepsis-induced cardiomyocyte apoptosis via TLR1 inhibition. *Revista Portuguesa de Cardiologia*. 2023 Mar;42(3):209–21.
167. Zhang G, Dong D, Wan X, Zhang Y. Cardiomyocyte death in sepsis: Mechanisms and regulation (Review). *Mol Med Rep*. 2022 Jun 15;26(2):257.
168. Zhang K, Wang Y, Chen S, Mao J, Jin Y, Ye H, et al. TREM2hi resident macrophages protect the septic heart by maintaining cardiomyocyte homeostasis. *Nat Metab*. 2023 Jan 12;5(1):129–46.
169. Lim GB. Cardiac-resident macrophages protect against sepsis-induced cardiomyopathy. *Nat Rev Cardiol*. 2023 Mar 23;20(3):141–141.
170. Dolmatova E V, Wang K, Mandavilli R, Griendling KK. The effects of sepsis on endothelium and clinical implications. *Cardiovasc Res*. 2021 Jan 1;117(1):60–73.
171. Ince C, Mayeux PR, Nguyen T, Gomez H, Kellum JA, Ospina-Tascón GA, et al. The Endothelium in Sepsis. *Shock*. 2016 Mar;45(3):259–70.
172. Pérez-Cremades D, Bueno-Betí C, García-Giménez JL, Ibañez-Cabellos JS, Hermenegildo C, Pallardó F V., et al. Extracellular histones disarrange vasoactive mediators release through a COX-NOS interaction in human endothelial cells. *J Cell Mol Med*. 2017 Aug;21(8):1584–92.
173. Pérez-Cremades D, Bueno-Betí C, García-Giménez JL, Ibañez-Cabellos JS, Pallardó F V., Hermenegildo C, et al. Extracellular histones trigger oxidative stress-dependent induction of the NF-κB/CAM pathway via TLR4 in endothelial cells. *J Physiol Biochem*. 2022 Dec 5;
174. Beltrán-García J, Osca-Verdegal R, Pérez-Cremades D, Novella S, Hermenegildo C, Pallardó F V, et al. Extracellular Histones Activate Endothelial NLRP3 Inflammasome and are Associated with a Severe Sepsis Phenotype. *J Inflamm Res*. 2022 Jul;Volume 15:4217–38.
175. Werschler N, Penninger J. Generation of Human Blood Vessel Organoids from Pluripotent Stem Cells. *Journal of Visualized Experiments*. 2023 Jan 20;(191).
176. Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerech S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. *Proceedings of the National Academy of Sciences*. 2013 Jul 30;110(31):12601–6.

177. Orlova V V., Drabsch Y, Freund C, Petrus-Reurer S, van den Hil FE, Muenthaisong S, et al. Functionality of Endothelial Cells and Pericytes From Human Pluripotent Stem Cells Demonstrated in Cultured Vascular Plexus and Zebrafish Xenografts. *Arterioscler Thromb Vasc Biol.* 2014 Jan;34(1):177–86.
178. Orlova V V, van den Hil FE, Petrus-Reurer S, Drabsch Y, ten Dijke P, Mummery CL. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nat Protoc.* 2014 Jun 29;9(6):1514–31.
179. Wimmer RA, Leopoldi A, Aichinger M, Wick N, Hantusch B, Novatchkova M, et al. Human blood vessel organoids as a model of diabetic vasculopathy. *Nature.* 2019 Jan 16;565(7740):505–10.
180. Wimmer RA, Leopoldi A, Aichinger M, Kerjaschki D, Penninger JM. Generation of blood vessel organoids from human pluripotent stem cells. *Nat Protoc.* 2019 Nov 25;14(11):3082–100.
181. Salewskij K, Penninger JM. Blood Vessel Organoids for Development and Disease. *Circ Res.* 2023 Feb 17;132(4):498–510.
182. Wimmer RA, Leopoldi A, Aichinger M, Kerjaschki D, Penninger JM. Generation of blood vessel organoids from human pluripotent stem cells. *Nat Protoc.* 2019 Nov 25;14(11):3082–100.
183. Gustafsdottir SM, Schallmeiner E, Fredriksson S, Gullberg M, Söderberg O, Jarvius M, et al. Proximity ligation assays for sensitive and specific protein analyses. *Anal Biochem.* 2005 Oct;345(1):2–9.
184. Schmidt U, Weigert M, Broaddus C, Myers G. Cell Detection with Star-Convex Polygons. In 2018. p. 265–73.
185. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta C_T$ Method. *Methods.* 2001 Dec;25(4):402–8.
186. Romá-Mateo C, Lorente-Pozo S, Márquez-Thibaut L, Moreno-Estellés M, Garcés C, González D, et al. Age-Related microRNA Overexpression in Lafora Disease Male Mice Provides Links between Neuroinflammation and Oxidative Stress. *Int J Mol Sci.* 2023 Jan 6;24(2):1089.
187. Osca-Verdegel R, Beltrán-García J, Pallardó F V., García-Giménez JL. Role of microRNAs As Biomarkers in Sepsis-Associated Encephalopathy. *Mol Neurobiol.* 2021 Sep 23;58(9):4682–93.
188. Alam MS. Proximity Ligation Assay (PLA). *Curr Protoc Immunol.* 2018 Nov;123(1):e58.
189. Wang H, Shan X, Ren M, Shang M, Zhou C. Nucleosomes enter cells by clathrin- and caveolin-dependent endocytosis. *Nucleic Acids Res.* 2021 Dec 2;49(21):12306–19.
190. Sundén-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, et al. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock*. *Crit Care Med.* 2005 Mar;33(3):564–73.
191. Khakpour S, Wilhelmsen K, Hellman J. Vascular endothelial cell Toll-like receptor pathways in sepsis. *Innate Immun.* 2015 Nov 23;21(8):827–46.

192. Alhamdi Y, Abrams ST, Cheng Z, Jing S, Su D, Liu Z, et al. Circulating Histones Are Major Mediators of Cardiac Injury in Patients With Sepsis*. *Crit Care Med.* 2015 Oct;43(10):2094–103.
193. Kaouass M, Beaulieu R, Balicki D. Histonefection: Novel and potent non-viral gene delivery. *Journal of Controlled Release.* 2006 Jul;113(3):245–54.
194. Murphy RF, Jorgensen ED, Cantor CR. Kinetics of histone endocytosis in Chinese hamster ovary cells. A flow cytofluorometric analysis. *J Biol Chem.* 1982 Feb 25;257(4):1695–701.
195. Hariton-Gazal E, Rosenbluh J, Graessmann A, Gilon C, Loyter A. Direct translocation of histone molecules across cell membranes. *J Cell Sci.* 2003 Nov 15;116(22):4577–86.
196. Meara CHO, Coupland LA, Kordbacheh F, Quah BJC, Chang CW, Simon Davis DA, et al. Neutralizing the pathological effects of extracellular histones with small polyanions. *Nat Commun.* 2020 Dec 16;11(1):6408.
197. Kleine TJ, Lewis PN, Lewis SA. Histone-induced damage of a mammalian epithelium: the role of protein and membrane structure. *American Journal of Physiology-Cell Physiology.* 1997 Dec 1;273(6):C1925–36.
198. Carchman EH, Rao J, Loughran PA, Rosengart MR, Zuckerbraun BS. Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. *Hepatology.* 2011 Jun 2;53(6):2053–62.
199. Chen HR, Chuang YC, Chao CH, Yeh TM. Macrophage migration inhibitory factor induces vascular leakage via autophagy. *Biol Open.* 2015 Feb 15;4(2):244–52.
200. Lee SJ, Ryter SW, Xu JF, Nakahira K, Kim HP, Choi AMK, et al. Carbon Monoxide Activates Autophagy via Mitochondrial Reactive Oxygen Species Formation. *Am J Respir Cell Mol Biol.* 2011 Oct;45(4):867–73.
201. Tanaka A, Jin Y, Lee SJ, Zhang M, Kim HP, Stolz DB, et al. Hyperoxia-Induced LC3B Interacts with the Fas Apoptotic Pathway in Epithelial Cell Death. *Am J Respir Cell Mol Biol.* 2012 Apr;46(4):507–14.
202. Green DR, Levine B. To Be or Not to Be? How Selective Autophagy and Cell Death Govern Cell Fate. *Cell.* 2014 Mar;157(1):65–75.
203. Jiang F, Xu X, Li W, Xia K, Wang L, Yang X. Monotropein alleviates H2O2-induced inflammation, oxidative stress and apoptosis via NF- κ B/AP-1 signaling. *Mol Med Rep.* 2020 Sep 29;22(6):4828–36.
204. Abdelmohsen K, Srikantan S, Kang MJ, Gorospe M. Regulation of senescence by microRNA biogenesis factors. *Ageing Res Rev.* 2012 Sep;11(4):491–500.
205. Jones K, Nourse JP, Keane C, Bhatnagar A, Gandhi MK. Plasma MicroRNA Are Disease Response Biomarkers in Classical Hodgkin Lymphoma. *Clinical Cancer Research.* 2014 Jan 1;20(1):253–64.
206. Williams G, Uchimoto ML, Coult N, World D, Beasley E. Body fluid mixtures: Resolution using forensic microRNA analysis. *Forensic Sci Int Genet Suppl Ser.* 2013;4(1):e292–3.
207. Fatmi A, Chabni N, Cernada M, Vento M, González-López M, Aribi M, et al. Clinical and immunological aspects of microRNAs in neonatal sepsis. *Biomedicine & Pharmacotherapy.* 2022 Jan;145:112444.

208. Beltrán-García J, Osca-Verdegel R, Nácher-Sendra E, Cardona-Monzonís A, Sanchis-Gomar F, Carbonell N, et al. Role of non-coding RNAs as biomarkers of deleterious cardiovascular effects in sepsis. *Prog Cardiovasc Dis*. 2021 Sep;68:70–7.
209. Cánovas-Cervera I, Nacher-Sendra E, Osca-Verdegel R, Dolz-Andrés E, Beltrán-García J, Rodríguez-Gimillo M, et al. The Intricate Role of Non-Coding RNAs in Sepsis-Associated Disseminated Intravascular Coagulation. *Int J Mol Sci*. 2023 Jan 30;24(3):2582.
210. Beltrán-García J, Osca-Verdegel R, Romá-Mateo C, Carbonell N, Ferreres J, Rodríguez M, et al. Epigenetic biomarkers for human sepsis and septic shock: insights from immunosuppression. *Epigenomics*. 2020 Apr;12(7):617–46.
211. Dhas BB, Dirisala VR, Bhat BV. Expression Levels of Candidate Circulating microRNAs in Early-Onset Neonatal Sepsis Compared With Healthy Newborns. *Genomics Insights*. 2018 Jan 2;11:117863101879707.
212. WANG L, WANG HC, CHEN C, ZENG J, WANG Q, ZHENG L, et al. Differential expression of plasma miR-146a in sepsis patients compared with non-sepsis-SIRS patients. *Exp Ther Med*. 2013 Apr;5(4):1101–4.
213. Wang J feng, Yu M li, Yu G, Bian J jun, Deng X ming, Wan X jian, et al. Serum miR-146a and miR-223 as potential new biomarkers for sepsis. *Biochem Biophys Res Commun*. 2010 Mar;394(1):184–8.
214. Wang H, Yu B, Deng J, Jin Y, Xie L. Serum miR-122 correlates with short-term mortality in sepsis patients. *Crit Care*. 2014 Dec 12;18(6):704.
215. García-Giménez JL, Rubio-Belmar PA, Peiró-Chova L, Hervás D, González-Rodríguez D, Ibañez-Cabellos JS, et al. Circulating miRNAs as diagnostic biomarkers for adolescent idiopathic scoliosis. *Sci Rep*. 2018 Feb 8;8(1):2646.
216. Benz F, Tacke F, Luedde M, Trautwein C, Luedde T, Koch A, et al. Circulating MicroRNA-223 Serum Levels Do Not Predict Sepsis or Survival in Patients with Critical Illness. *Dis Markers*. 2015;2015:1–10.
217. Wang H, Zhang P, Chen W, Feng D, Jia Y, Xie L xin. Evidence for serum miR-15a and miR-16 levels as biomarkers that distinguish sepsis from systemic inflammatory response syndrome in human subjects. *Clin Chem Lab Med*. 2012 Jan 1;50(8).

ABBREVIATION LIST

2D – Two dimensions

AKI – Acute kidney injury

ALFY - Autophagy-linked FYVE protein

ANOVA - Analysis Of Variance

AP – Alkaline phosphatase

APACHE – Acute Physiology, Age and Chronic Health Evaluation

APC – Activated Protein C

APC – Allophycocyanin

aPTT - Thromboplastin time

ATCC – American Type Culture Collection

ATP – Adenosine Triphosphate

BCA – Bicinchoninic Acid

BIL – Total bilirubin

BMP-4 – Bone morphogenic protein 4

BSA – Bovine Serum Albumin

BVO – Blood Vessel Organoid

ChiR – 6-((2-((4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl) pyrimidin-2-yl) amino)ethyl)amino) nicotinonitrile

CI – Confidence interval

CO₂ – Carbon Dioxide

CT – cycle threshold

Cu⁺¹ – Copper +1

Cu⁺² – Copper +2

CXCR4 – Chemokine C-C-C type 4 receptor

DAMP – Damage-associated Molecular Pattern

DD1 – D-dimer1

DMEM – Dulbecco's Modified Eagle Medium

DNase – Deoxyribonuclease

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

EEF1A1 – Eukaryotic Translation Elongation Factor 1 Alpha 1

EGDT – Early Goal-Directed Therapy

EGM-2 – Endothelial Growth Medium2

ELISA – Enzyme-Linked Immunosorbent Assay

Fab 1 – Yeast orthologue of PIKfyve

FBS – Fetal Bovine Serum

FGF-2 – Fibroblast growth factor 2

Fibrin – Activated partial fibrinogen

FIO₂ – Fraction of Inspired Oxygen

FITC – Fluorescein isothiocyanate

FN-γ – Interferon-γ

FYVE – Fab1, YOTB, Yac1 and EEA1 domain

GA-1000 – Gentamicin sulphate/Amphotericin

GFP – Green Fluorescent Protein

GLUC – Glucose

GPX4 – Glutathione peroxidase 4

H1 – Histone 1

H1-488 – Histone 1 with a 488-fluorescent tag

H2A – Histone 2A

ABBREVIATIONS

H2B– Histone 2B	MgCl ₂ – Magnesium chloride
H3 – Histone 3	MIF – Macrophage migration Inhibitory Factor
H3-GFP – Histone 3 with GFP tag	MOF – Multiple Organ Failure
H4 – Histone 4	NaOH – Sodium hydroxide
HCl – Hydrochloric acid	NBR1 – Neighbour of BRCA1 gene 1
hEGF – Human Epidermal Growth Factor	NET – Neutrophil Extracellular Trap
HEPES – N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid	NH ₄ Cl – Ammonium Chloride
hFG-B – Human Basic Fibroblastic Growth Factor	NK – Natural Killer
HMGB1 – High Mobility Group Box 1	NLR – Nucleotide-binding and Oligomerization Domain-Like Receptor
HOSP LOS – Hospitalization length of stay	NLRLRP3 – Nucleotide-binding and Oligomerization Domain-Like Receptor-Pyrin domain-containing 3
hPSCs – Human pluripotent stem cells	NOD – Nucleotide-binding and Oligomerization Domain
HUVEC – Human Umbilical Vein Endothelial Cells	NtProBNP1 – Amino-terminal fragment of the pro-natriuretic peptide type B1
ICAM-1 – Intercellular adhesion molecule 1	P/S – Penicillin/Streptomycin antibiotic
ICU – Intensive Care Unit	p62/SQSTM1 - Sequestosome 1
ICU LOS – ICU length of stay	PAMP – Pathogen-associated Molecular Pattern
IF – Immunofluorescence	PaO ₂ – Partial Pressure of Oxygen
IL – Interleukin	PBS – Phosphate Buffer Saline
iPSC – Induced Pluripotent Stem Cells	PEI – Polyethyleneimine
JCR – Journal Citation Reports	PFA – Paraformaldehyde
KCl – Potassium chloride	PLA – Proximity Ligation Assay
LC3B – Light chain 3B protein	PRR – Pattern-Recognition Receptor
LPS – Lipopolysaccharide	PTM – Post-translational modifications
MAP – Mean Arterial Pressure	qSOFA – quick Sepsis-related Organ Failure Assessment
MAP – Mean arterial pressure	
MAPK – Mitogen-activated protein kinase	
MEM – Modified Eagle Medium	

R3-IGF-1 – Long Arg3 Insulin-Like Growth Factor- 1

RAGE - Receptor for Advanced Glycation End-products

RF – Respiratory frequency

RIG-I – Retinoic acid-Inducible Gene I

RLR – Retinoic acid-Inducible Gene I-like Receptor

RNase – Ribonuclease

ROC – receiver operating characteristic

ROC curve – Receiver operating characteristic curve

SAP – Systolic arterial pressure

SDS – Sodium Dodecyl Sulphate

SIRS – Systemic Inflammatory Response Syndrome

SOD2 – Superoxide dismutase 2

SOFA – Sepsis-related Organ Failure Assessment

SOFA – Total SOFA score

SPAs – Small polyanions

SS – Septic shock

TBS – Tris-Buffered Saline

TCA – Trichloroacetic Acid

TCR – T-cell receptor

Th2 – T-helper 2 cell

TLR – Toll-Like Receptor

TNF- α – Tumour Necrosis Factor- α

TREM-1 – Triggering Receptor Expressed on Myeloid cells 1

UK – United Kingdom

UPR – Unfolded Protein Response

USA – United States of America

Vac 1 – Vesicle transport protein

VCAM-1 – Vascular cell adhesion molecule 1

VEGF – Vascular Endothelial Growth Factor

WB – Western Blot

INDEX OF FIGURES

Figure 1. Summary of the pathogenic processes produced in the proinflammatory stage of sepsis.	6
Figure 2. Inflammatory positive feed-back loop in the pathogenesis of sepsis	9
Figure 3. Changes in immune cells during the immunosuppression phase of sepsis.	11
Figure 4. Pathogenic effects of extracellular histones	14
Figure 5. Schematic view of HMGB1 structure.	17
Figure 6. Releasing mechanism of HMGB1	19
Figure 7. Diagram of the different stages of autophagy.	22
Figure 8. Proximity Ligation Assay molecular basis scheme.	53
Figure 9. Image analysis tool workflow.	57
Figure 10. Schematic representation of the analysed images, as well as the quantified variables.	59
Figure 11. Analysis of histone-mediated cytotoxicity in HeLa and Hep3B cell models.	74
Figure 12. Fluorescence microscopy images of Hep3B and HeLa cells under core histone treatments.	76
Figure 13. Subcellular co-localization study of histones using the developed image analysis tool	78
Figure 14. Analysis of histone-mediated cytotoxicity following treatment of cells with autophagy modulators.	80
Figure 15. Analysis of HeLa cell cycle upon core histone treatment.	81
Figure 16. MiRNAs analysis of HeLa and Hep3B cells after modulation of autophagy and core histone treatments.	83
Figure 17. Analysis of histone-mediated cytotoxicity in HUVEC cells.	88
Figure 18. Analysis of colocalization between commercial H3 histone and LC3B.	89
Figure 19. Study of colocalization between histones H3-GFP and LC3B.	91
Figure 20. Analysis of interactions between commercial histones H3 and LC3B.	93
Figure 21. Analysis of interactions between H3-GFP histones and LC3B.	94
Figure 22. Cytotoxicity analysis of H1-488 histone.	96
Figure 23 Analysis of colocalization between histones H1-Alexa488 and LysoTracker Red DND-99.	97
Figure 24. Effect of blocking endocytosis on colocalization between histone H3 and LC3B.	99
Figure 25 Effect of blocking endocytosis on colocalization between histone H1-488 and LC3B.	100
Figure 26. Blocking endocytosis prevents histone entry to HUVEC cells and reduces histone-induced cytotoxicity.	102
Figure 27. LC3B-II and p62 protein responses after autophagy modulation.	104
Figure 28. Effect of autophagy modulation on histone-mediated cytotoxicity	105
Figure 29. Effect of autophagy modulation on colocalization between histone H3 and LC3B.	107
Figure 30 Effect of autophagy modulation on colocalization between histone H1-488 and LC3B.	109
Figure 31. Quantification of HMGB1 on supernatant of HUVEC treated with different extracellular histones.	110
Figure 32. Analysis of histone-mediated cytotoxicity in blood vessel organoids (BVOs).	114
Figure 33. Analysis of histone H3 and LC3B on blood vessel organoids (BVOs) treated with core histones.	115
Figure 34 Analysis of histone H1-488 and LC3B on blood vessel organoids (BVOs) treated with histone H1-488.	117
Figure 35. Effect of autophagy modulation on histone-mediated cytotoxicity	119
Figure 36 Effect of autophagy modulation on fluorescence of histone H3 and LC3B.	120
Figure 37.Effect of autophagy modulation on fluorescence of histone H1.488 and LC3B.	122
Figure 38. Blocking endocytosis prevents histone entry to blood vessel organoids (BVOs) and reduces histone-induced cytotoxicity.	124

<i>Figure 39 Effect of blocking endocytosis on localization and expression of histone H1-488 and LC3B..</i>	125
<i>Figure 40. Histone induced gene expression alterations on proinflammatory and vascular genes in blood vessel organoids (BVOs).</i>	127
<i>Figure 41. Effect of core histone treatment on miRNA expression in blood vessel organoids (BVOs).</i>	128
<i>Figure 42 HMGB1 induced gene expression alterations on proinflammatory and vascular genes in blood vessel organoids (BVOs).</i>	130
<i>Figure 43. Quantification of HMGB1 on supernatant of blood vessel organoids (BVOs) treated with extracellular histones and autophagy modulators.</i>	131
<i>Figure 44. Quantification of HMGB1 using an ELISA kit in plasma samples from patients with sepsis and septic shock.</i>	135
<i>Figure 45 Diagnostic capacity of HMGB1 ELISA to differentiate cases from controls and septic patients.</i>	136
<i>Figure 46. Correlation between biomarker levels, clinical parameters, and roc curves for hmgb1 in sepsis and septic shock patients.</i>	137
<i>Figure 47. Possible entry mechanism of histones to cells though non-canonical autophagy.</i>	151
<i>Figure A. 1. Timeline of the obtention of blood vessel organoids (BVOs).</i>	191
<i>Figure A. 2.. Fluorescence microscopy images of Hep3B and HeLa cells under autophagy modulation and core histone treatments.</i>	194
<i>Figure A. 3 Analysis of colocalization between commercial histones H3 and LC3B.</i>	195
<i>Figure A. 4 Analysis of interactions between commercial histones H3 and LC3B.</i>	196
<i>Figure A. 5 Evaluation of interactions between H3-GFP histones and LC3B.</i>	197
<i>Figure A. 6 Cytotoxicity induced by blocking endocytosis.</i>	198
<i>Figure A. 7 Blood vessel organoids (BVOs) treated with core histones.</i>	198

INDEX OF TABLES

<i>Table 1. SOFA Score parameters evaluation.</i>	2
<i>Table 2. Reagents used for cell culture treatments.</i>	45
<i>Table 3. Primary antibodies used.</i>	49
<i>Table 4. Primers used in gene expression analyses of human blood vessel organoids.</i>	63
<i>Table 5. Initial clinical characteristics of the patients with sepsis and the control group admitted to the intensive care unit (ICU).</i>	66
 <i>Table A. 1 Correlation values of levels of HMGB1 with clinical features of sepsis and septic patients..</i>	
	199

ANNEXES

ANNEX I: Supplementary figures and tables

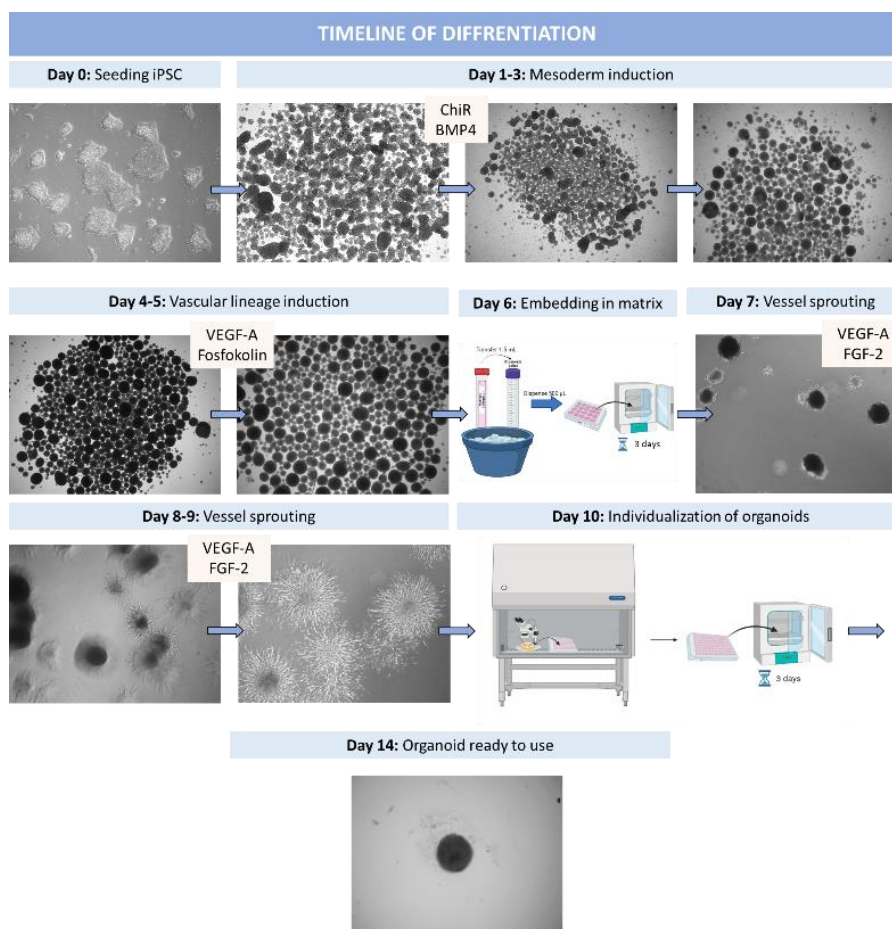
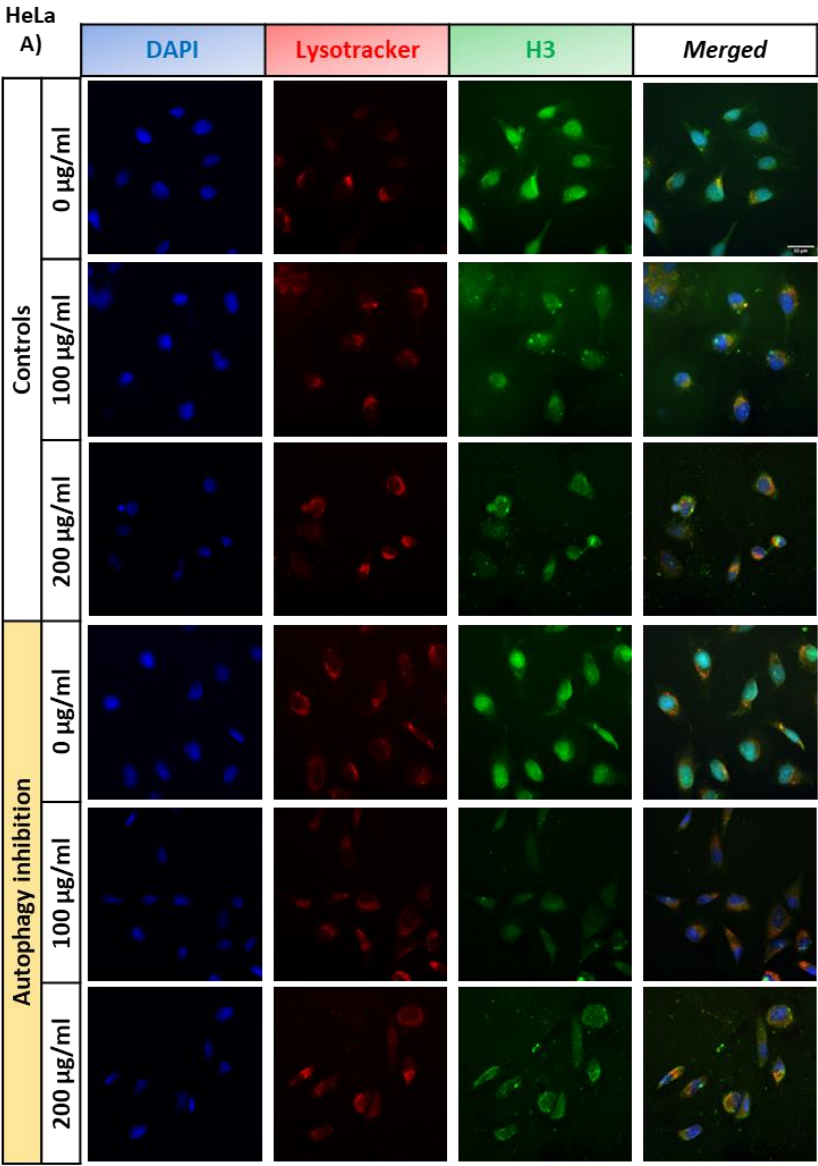
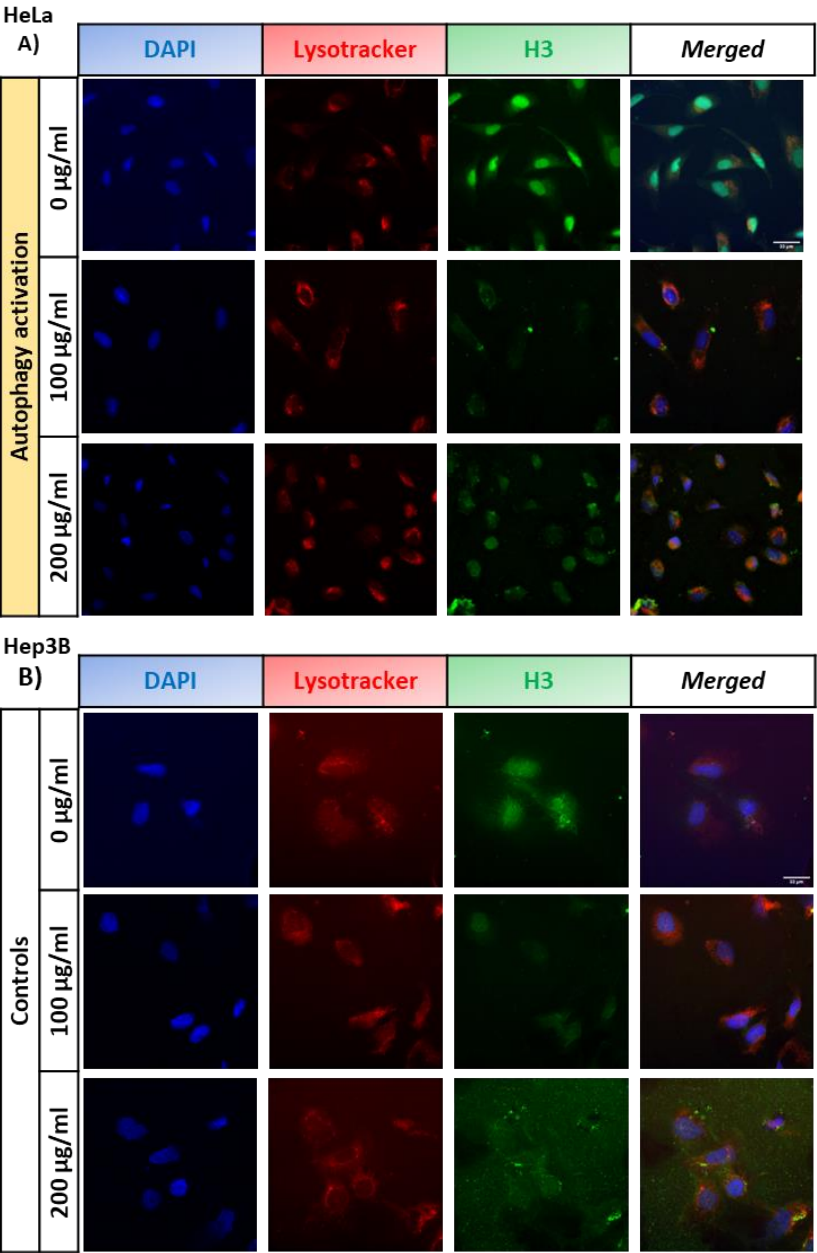


Figure A. 1. Timeline of the obtention of blood vessel organoids (BVOs). Human induced pluripotent stem cells (iPSC) are differentiated for fourteen days to obtain BVOs. The process of obtention is fully described in the section 1.2 of Materials and Methods. In the figure is shown the different factors added to the medium to differentiate iPSC to BVOs and pictures of the changes in morphology during the process.





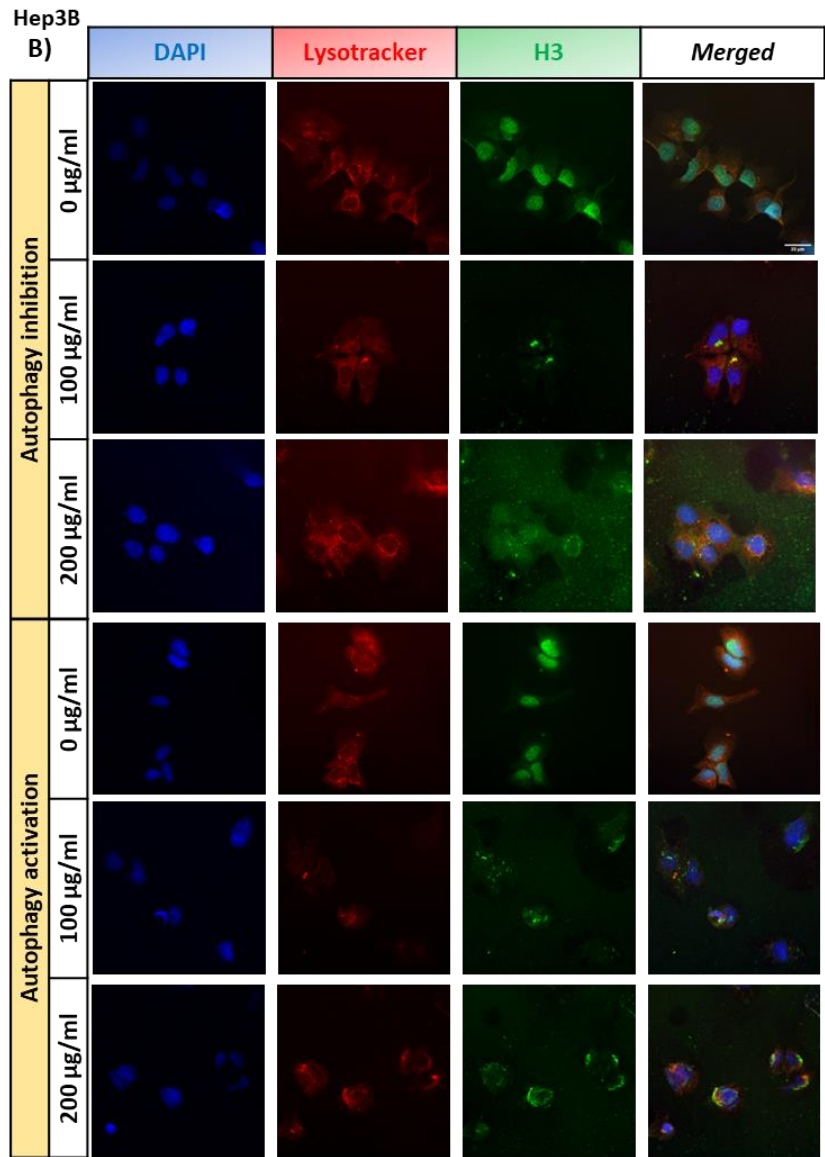


Figure A. 2.. Fluorescence microscopy images of Hep3B and HeLa cells under autophagy modulation and core histone treatments. Both HeLa (A) and Hep3B (B) were treated with 0, 100, and 200 µg/mL of core histones for 4 hours and within the last 30 minutes of incubation, 75nM of Lysotracker Red DND-99 was added to the cultures to mark lysosomes. Autophagy was increased using 2mM of metformin for 24h prior core histone treatment. Autophagy was inhibited using 20mM NH₄Cl and 100nM leupeptin 1.5h prior core histone treatment. After, an immunofluorescence was performed using a mouse anti-histone H3 primary antibody and a fluorescently labelled goat-anti-mouse secondary antibody with FITC were used to label histones, Lysotracker Red DND-99 was used to label lysosomes, and DAPI was used to label nuclei. The preparations were observed under a fluorescence microscope, specifically the DMI-8 model, and photographs were captured using a Leica-DFC 9000 GT camera attached to the microscope (see Materials and Methods). Each row corresponds to images from the same field of view, obtained by exciting the samples with lasers at specific wavelengths of 490 nm, 550 nm, or 365 nm to excite FITC, Lysotracker, or DAPI, respectively. The fourth column shows a fusion of the three corresponding images from a single field of view. Scale bar 33 µm

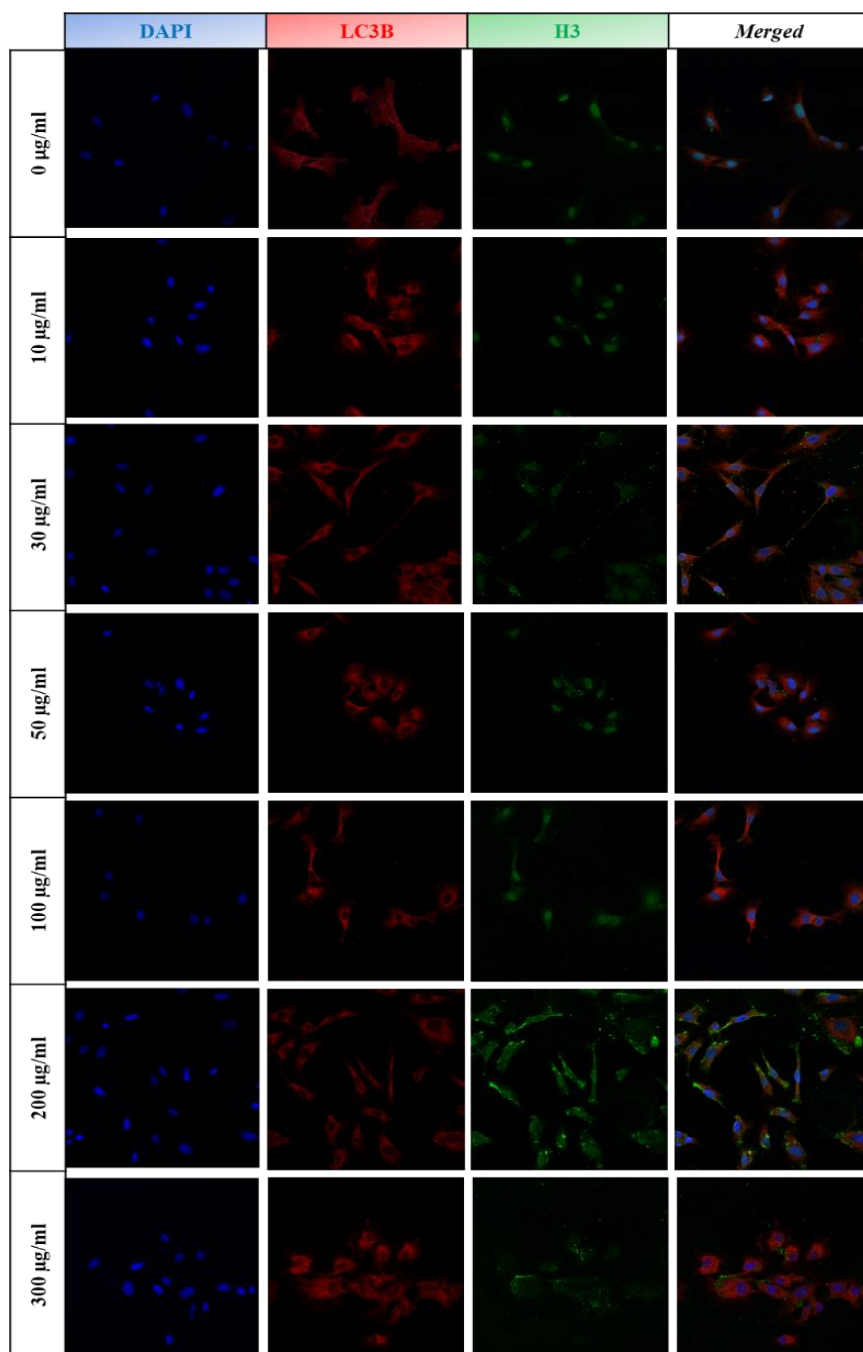


Figure A. 3 Analysis of colocalization between commercial histones H3 and LC3B. Confocal microscopy images of HUVEC cells subjected to treatment with commercial histones (a mixture of H2AB, H3, and H4 histone core) at concentrations ranging from 10 to 300 $\mu\text{g/ml}$. Each row represents images from the same visual field. Blue fluorescence corresponds to cellular nuclei stained with DAPI, red fluorescence represents LC3B protein through binding to a Texas Red-conjugated secondary antibody, and green fluorescence represents histone H3 through staining with an Alexa488-conjugated secondary antibody. Furthermore, a fourth column displays the merged image of the three channels used. Magnification, 40X.

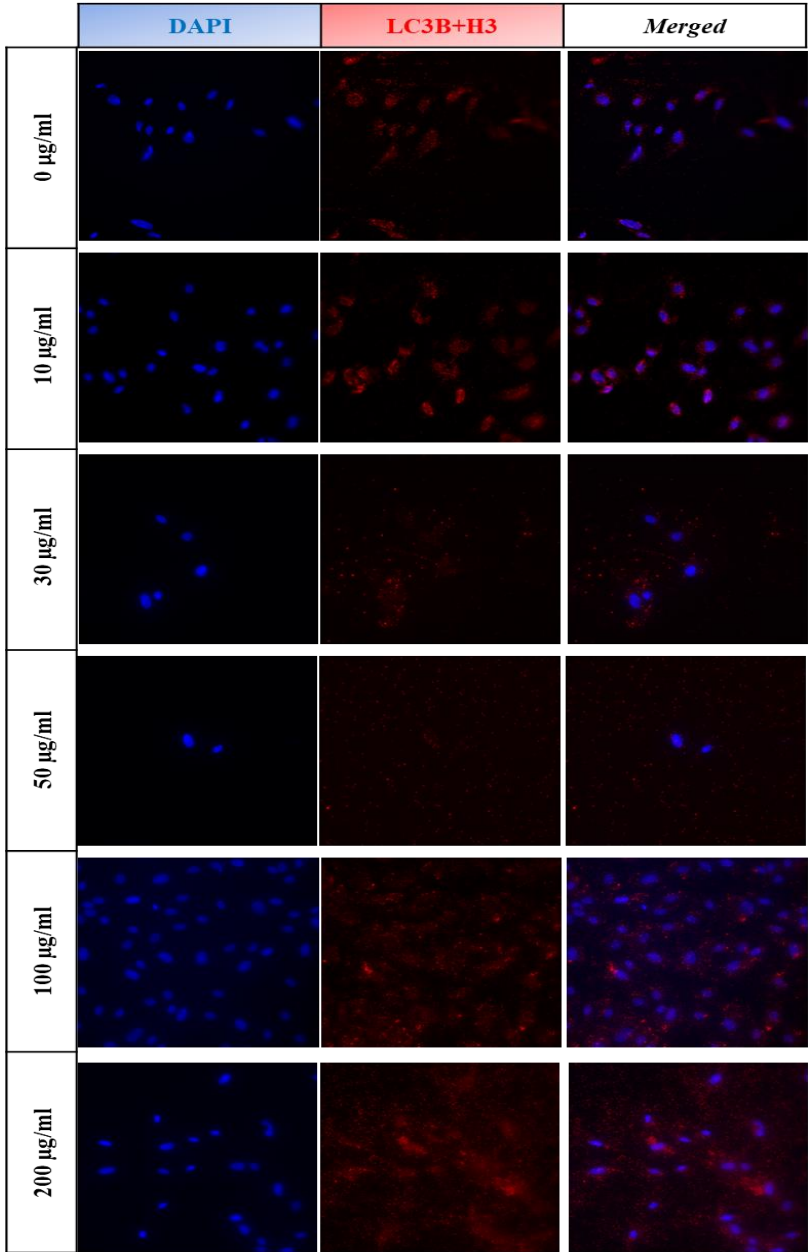


Figure A. 4 Analysis of interactions between commercial histones H3 and LC3B. Fluorescence microscopy images of HUVEC cells treated with increasing concentrations of core histones ranging from 10 to 200 $\mu\text{g/ml}$. Blue fluorescence corresponds to cellular nuclei stained with DAPI, while red fluorescence represents the interactions between histone H3 and LC3B protein (due to their physical proximity, enabling the binding of nucleotide tails attached to secondary antibodies by ligases and their amplification by polymerases with fluorescent nucleotides). The third column represents the merged image of the two channels used. Magnification 40X.

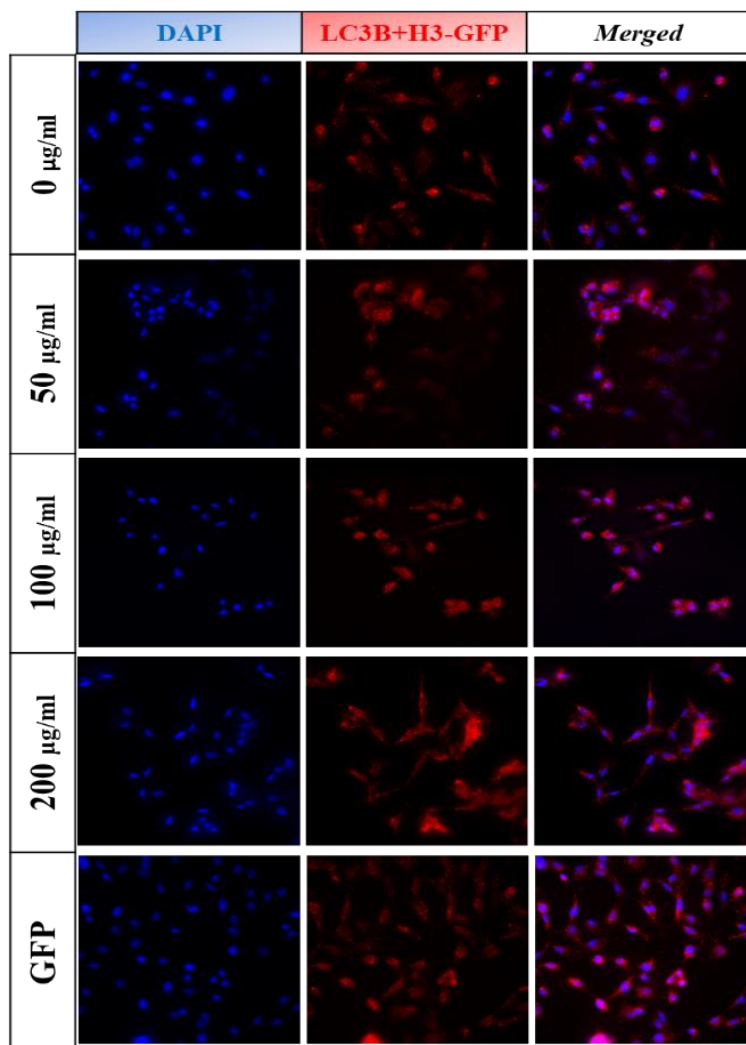


Figure A. 5 Evaluation of interactions between H3-GFP histones and LC3B. Fluorescence microscopy images of HUVEC cells treated with increasing concentrations of H3-GFP histone extract, along with the control using GFP vehicle. Blue fluorescence corresponds to cellular nuclei stained with DAPI, while red fluorescence represents the interactions between GFP protein attached to histone H3 and LC3B protein (due to their proximity within the intracellular space, leading to the binding of nucleotide tails conjugated to secondary antibodies by ligases and their amplification by polymerases with fluorescent nucleotides). The third column represents the merged image of the two channels used. Magnification 40X.

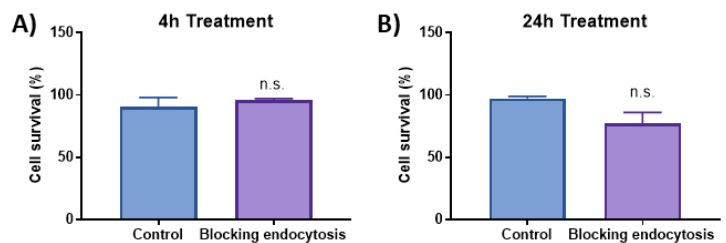


Figure A. 6 Cytotoxicity induced by blocking endocytosis. HUVEC cells were incubated for (A) 4h or (B) 24h at 4 °C in the condition of blocked endocytosis and at 37°C in the control condition. Then, cells were harvested and labeled using an Annexin V kit, and subsequently the survival rate was analyzed by flow cytometry. Mean survival values are presented as bars, expressed as a percentage $\pm$ standard deviation ($n=3$), with statistically significant differences considered when $p<0.05$, calculated using t-test indicated by n.s. as non-significant.

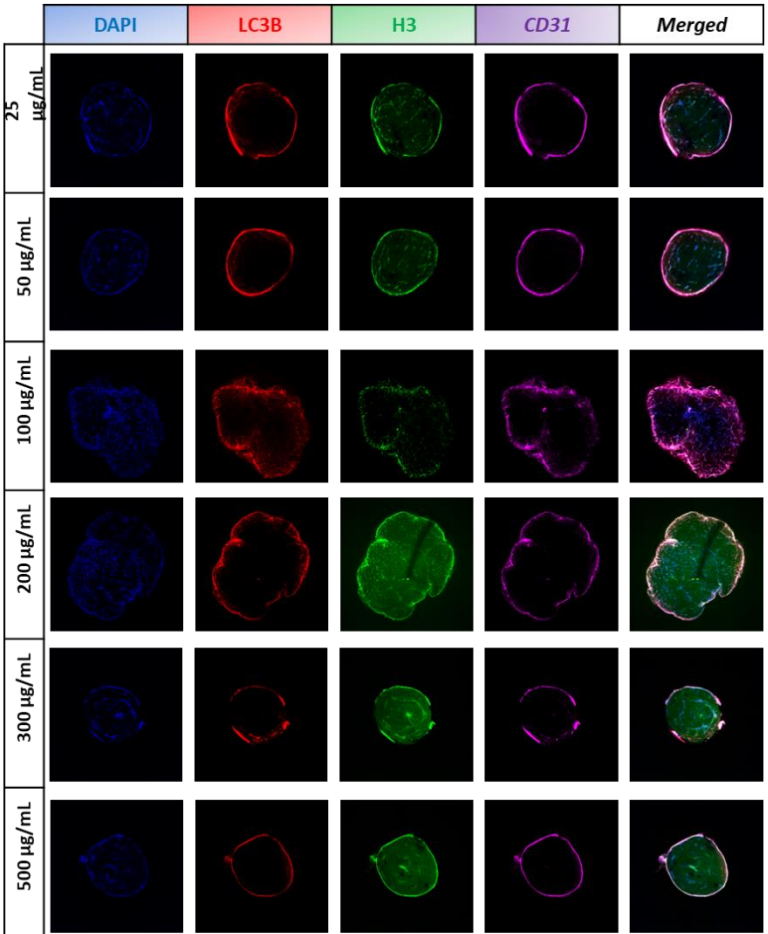


Figure A. 7 Blood vessel organoids (BVOs) treated with core histones. Confocal microscopy images of BVOs that were subjected to treatment with several concentrations of core histones for 4 hours. The blue fluorescence of each row shows cell nuclei by DAPI staining, red fluorescence corresponds to LC3B autophagy marker protein, the green one to histone H3 and purple one to CD31 endothelial cell marker, corresponding to the same visual field in the middle section of the organoid. In addition, a fifth column is shown with the fusion of the four channels used. Magnification, 10X.

Table A. 1 Correlation values of levels of HMGB1 with clinical features of sepsis and septic patients. (A) p-value correlation (B) Spearman r correlation.

(A)

Correlation P values	HMGB1	MBI	ICULOS	HOSPLOS	SOFA	SAP	MAP	RF	ALPH	BIL	GLUC1	TTPA1	Fibrin1	DD1	NtProBNP1
HMGB1		0,587	0,003	0,028	0,773	0,432	0,857	0,198	0,950	0,623	0,044	0,300	0,750	0,033	0,048
MBI	0,587		0,726	0,752	0,014	0,116	0,272	0,583	0,267	0,426	0,558	0,333	0,778	0,497	0,963
ICU LOS	0,003	0,726		2,690E-004	0,244	0,734	0,343	0,050	0,633	0,817	0,385	0,419	0,686	0,017	0,017
HOSP LOS	0,028	0,752	2,690E-004		0,239	0,415	0,352	0,043	0,950	0,813	0,915	0,342	0,956	0,033	0,016
SOFA	0,773	0,014	0,244	0,239		0,122	0,140	0,601	0,900	0,348	0,858	0,756	0,667	0,156	0,162
SAP	0,432	0,116	0,734	0,415	0,122		0,006	0,722	0,950	1,000	0,083	0,747	0,864	0,564	0,952
MAP	0,857	0,272	0,343	0,352	0,140	0,006		0,897	0,950	0,327	0,710	0,272	0,933	0,072	0,498
RF	0,198	0,583	0,050	0,043	0,601	0,722	0,897		0,567	0,408	0,815	0,150	0,150	0,250	0,152
ALPH	0,950	0,267	0,633	0,950	0,900	0,950	0,950	0,567		0,750	0,683	1,000	1,000	1,000	0,333
BIL	0,623	0,426	0,817	0,813	0,348	1,000	0,327	0,408	0,750		0,647	0,500	0,700	0,767	0,750
GLUC1	0,044	0,558	0,385	0,915	0,858	0,083	0,710	0,815	0,683	0,647		0,811	0,400	0,239	0,406
TTPA1	0,300	0,333	0,419	0,342	0,756	0,747	0,272	0,150	1,000	0,500	0,811		0,211	0,300	1,000
Fibrin1	0,750	0,778	0,686	0,956	0,667	0,864	0,933	0,150	1,000	0,700	0,400	0,211		0,433	0,378
DD1	0,033	0,497	0,017	0,033	0,156	0,564	0,072	0,250	1,000	0,767	0,239	0,300	0,433		0,083
NtProBNP1	0,048	0,963	0,017	0,016	0,162	0,952	0,498	0,152	0,333	0,750	0,406	1,000	0,378	0,083	

(B)

Correlation Spearman r	HMGB1	MBI	ICU LOS	HOSP LOS	SOFA	SAP	MAP	RF	ALPH	BIL	GLUC1	TTPA1	Fibrin1	DD1	NtProBNP1
HMGB1	1,000	0,195	0,854	0,701	0,105	0,280	-0,067	0,448	-0,100	-0,234	-0,657	-0,522	0,174	0,886	-0,786
MBI	0,195	1,000	-0,126	-0,113	-0,757	0,532	0,382	-0,198	0,667	-0,360	-0,208	-0,493	-0,145	-0,371	0,036
ICU LOS	0,854	-0,126	1,000	0,929	0,405	-0,122	-0,333	0,642	-0,359	-0,109	-0,306	-0,412	0,206	0,943	-0,873
HOSP LOS	0,701	-0,113	0,929	1,000	0,408	-0,287	-0,327	0,661	-0,100	0,109	-0,040	-0,471	-0,029	0,886	-0,873
SOFA	0,105	-0,757	0,405	0,408	1,000	-0,525	-0,503	0,188	-0,103	0,426	0,065	-0,194	-0,239	0,667	-0,599
SAP	0,280	0,532	-0,122	-0,287	-0,525	1,000	0,808	-0,129	0,100	0,000	-0,579	-0,176	-0,088	-0,314	0,036
MAP	-0,067	0,382	-0,333	-0,327	-0,503	0,808	1,000	0,049	0,100	0,432	-0,134	0,551	-0,058	-0,812	0,321
RF	0,448	-0,198	0,642	0,661	0,188	-0,129	0,049	1,000	-0,359	0,374	-0,086	0,708	0,708	0,577	-0,630
ALPH	-0,100	0,667	-0,359	-0,100	-0,103	0,100	0,100	-0,359	1,000	0,400	-0,300	-0,500	0,000	0,500	-0,800
BIL	-0,234	-0,360	-0,109	0,109	0,426	0,000	0,432	0,374	0,400	1,000	0,209	0,353	0,221	0,205	-0,174
GLUC1	-0,657	-0,208	-0,306	-0,040	0,065	-0,579	-0,134	-0,086	-0,300	0,209	1,000	0,132	-0,441	-0,580	0,378
TTPA1	-0,522	-0,493	-0,412	-0,471	-0,194	-0,176	0,551	0,708	-0,500	0,353	0,132	1,000	0,603	-0,616	0,000
Fibrin1	0,174	-0,145	0,206	-0,029	-0,239	-0,088	-0,058	0,708	0,000	0,221	-0,441	0,603	1,000	0,462	-0,435
DD1	0,886	-0,371	0,943	0,886	0,667	-0,314	-0,812	0,577	0,500	0,205	-0,580	-0,616	0,462	1,000	-0,900
NtProBNP1	-0,786	0,036	-0,873	-0,873	-0,599	0,036	0,321	-0,630	-0,800	-0,174	0,378	0,000	-0,435	-0,900	1,000