RESEARCH GROUP CTS-101: INTERCELLULAR COMMUNICATION

CENTRO DE INVESTIGACIÓN BIOMÉDICA

INSTITUTO DE BIOTECNOLOGÍA

DEPARTMENT OF PHYSIOLOGY

FACULTY OF MEDICINE

UNIVERSITY OF GRANADA

CHRONODISRUPTION: MELATONIN DEFICIENCY AND INNATE IMMUNITY. CONNECTION WITH MITOCHONDRIAL DYSFUNCTION DURING AGING

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DOCTORAL THESIS

PhD Program in Biomedicine

Granada, 2021

Editor: Universidad de Granada. Tesis Doctorales
Autor: Marisol Fernández Ortiz
ISBN: 978-84-1306-989-0
URI: http://hdl.handle.net/10481/70150

This doctoral Thesis has been carried out at the Research Group CTS-101, "Intercellular Communication", Instituto de Biotecnología, Centro de Investigación Biomédica (CIBM), University of Granada.

While performing her PhD work, Marisol Fernández Ortiz, graduate in Biochemistry, was supported by the following sources:

1. Formación de Profesorado Universitario (FPU) predoctoral fellowship from the Spanish Ministry of Science, Innovation and Universities, from November 2016 to March 2021.
2. A Short-Term Scientific Mission from the International Mobility Scholarship for PhD Students. European Cooperation in Science & Technology (COST). COST Action: Mitochondrial mapping: Evolution - Age - Gender - Lifestyle - Environment (MITOEAGLE). Place: Medical University of Innsbruck (Innsbruck, Austria). Duration: October 2017 - November 2017.
3. International Mobility Scholarship for PhD Students. Short foreing stays FPU. Place: University of Texas Health Science Center at San Antonio (San Antonio, Texas). Duration: April 2019 - July 2019.

Research grants that have supported the PhD work:

Project Title: Red temática de investigación cooperativa en envejecimiento y fragilidad (RETICEF)
Financial Entity: ISCIII
Participants Entities: University of Granada
Project Reference: RD12/0043/0005
Duration, From: 2013 **To:** 2016
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 4

Project Title: MicroRNAs, déficit de melatonina y activación del inflamasoma NLRP3 durante el envejecimiento
Financial Entity: ISCIII
Participants Entities: University of Granada
Project Reference: PI13-00981
Duration, From: 2014 **To:** 2016
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 6

Project Title: Looking for the connection between clock genes and mitochondrial impairment in aging and age-related loss of muscle fibers. Ciber de Fragilidad y Envejecimiento
Financial Entity: ISCIII
Participants Entities: University of Granada
Project Reference: CB/10/00238
Duration, From: 2016 **To:** 2020
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 4

Project Title: Nrf2 y déficit de melatonina, la conexión entre cronoinflamación y disfunción mitocondrial en el envejecimiento y pérdida muscular
Financial Entity: ISCIII
Participants Entities: University of Granada
Project Reference: PI16/00519
Duration, From: 2016 **To:** 2020
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 5

Project Title: The clock genes-melatonin-mitochondria connection in sarcopenia
Financial Entity: ISCIII
Participants Entities: University of Granada
Project Reference: PI2019-01372
Duration, From: 2020 **To:** 2022
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 6

Project Title: Análisis de la conexión entre genes reloj, melatonina y mitocondria en el modelo de parkinson en el pez cebra
Financial Entity: Junta de Andalucía
Participants Entities: University of Granada
Project Reference: P18-RT-698
Duration, From: 2020 **To:** 2022
Principal Investigator: Acuña-Castroviejo D
Number of Participating Researchers: 8

Some of the results presented in this Doctoral Thesis have been published in international journals or they are in preparation:

Fernández-Ortiz, M., Sayed, R.K.A., Fernández-Martínez, J., Cionfrini, A., Aranda-Martínez, P., Escames, G., de Haro, T., and Acuña-Castroviejo, D. (2020). Melatonin/Nrf2/NLRP3 Connection in Mouse Heart Mitochondria during Aging. Antioxidants (Basel); doi: 10.3390/antiox9121187.

Sayed, R.K.A., Fernández-Ortiz, M., Rahim, I., Fernández-Martínez, J., Aranda-Martínez, P., Rusanova, I., Martínez-Ruiz, L., Escames, G., and Acuña-Castroviejo, D. (2021). NLRP3 inflammasome deletion and / with melatonin supplementation mitigate age-dependent morphological and ultrastructural alterations in murine heart. J Gerontol A Biol Sci Med Sci 2021 (under review).

Fernández-Ortiz, M., Sayed, R.K.A., Román-Montoya, Y., Escames, G., and Acuña-Castroviejo, D. (2021). Aging and chronodisruption in mouse cardiac tissue. Effect of the NLRP3 inflammasome and melatonin therapy (in preparation).

Other publications related to this Doctoral Thesis:

Sayed, R.K., Fernández-Ortiz, M., Fernández-Martínez, J., Aranda Martínez, P., Guerra-Librero, A., Rodríguez-Santana, C., de Haro, T., Escames, G., Acuña-Castroviejo, D., and Rusanova, I. (2021). The Impact of Melatonin and NLRP3 Inflammasome on the Expression of microRNAs in Aged Muscle. Antioxidants (Basel)*;* doi: 10.3390/antiox10040524.

Rahim, I., Sayed, R.K., Fernández-Ortiz, M., Aranda-Martínez, P., Guerra-Librero, A., Fernández-Martínez, J., Rusanova, I., Escames, G., Djerdjouri, B., and Acuña-Castroviejo, D. (2021). Melatonin alleviates sepsis-induced heart injury through activating the Nrf2 pathway and inhibiting the NLRP3 inflammasome. Naunyn Schmiedebergs Arch Pharmacol; doi: 10.1007/s00210-020-01972-5.

Rusanova, I., Fernández-Martínez, J., Fernández-Ortiz, M., Aranda-Martínez, P., Escames, G., García-García, F.J., Mañas, L., and Acuña-Castroviejo, D. (2019). Involvement of plasma miRNAs, muscle miRNAs and mitochondrial miRNAs in the pathophysiology of frailty. Exp Gerontol; doi: 10.1016/j.exger.2019.110637.

Sayed, R.K.A., Fernández-Ortiz, M., Diaz-Casado, M.E., Aranda-Martínez, P., Fernández-Martínez, J., Guerra-Librero, A., Escames, G., López, L.C., Alsaadawy, R.M., and Acuña-Castroviejo, D. (2019). Lack of NLRP3 Inflammasome Activation Reduces Age-Dependent Sarcopenia and Mitochondrial Dysfunction, Favoring the Prophylactic Effect of Melatonin. J Gerontol A Biol Sci Med Sci; doi: 10.1093/gerona/glz079.

Rusanova, I., Diaz-Casado, M.E., Fernández-Ortiz, M., Aranda-Martínez, P., Guerra-Librero, A., García-García, F.J., Escames, G., Mañas, L., and Acuña-Castroviejo, D. (2018). Analysis of Plasma MicroRNAs as Predictors and Biomarkers of Aging and Frailty in Humans. Oxid Med Cell Longev; doi: 10.1155/2018/7671850.

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Chahbouni, M., López, M.D.S., Molina-Carballo, A., de Haro, T., Muñoz-Hoyos, A., Fernández-Ortiz, M., Guerra-Librero, A., and Acuña-Castroviejo, D. (2017). Melatonin Treatment Reduces Oxidative Damage and Normalizes Plasma Pro-Inflammatory Cytokines in Patients Suffering from Charcot-Marie-Tooth Neuropathy: A Pilot Study in Three Children. Molecules; doi: 10.3390/molecules22101728.

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Contributions to scientific meetings related to this doctoral thesis:

Oral communication

I Symposium de Medicina de precisión. De la Cronodisrupción a la sarcopenia. 2019. Granada (España). **Fernández-Ortiz, M.**, Sayed, R.K.A., Rahim, I., Fernandez-Martinez, J., Rusanova, I., Acuña-Castroviejo, D.

I Congreso de Investigadores del PTS. Inflammaging as a cause of sarcopenia: effects of melatonin treatment. 2019. Granada (España). **Fernández-Ortiz, M.,** Sayed, R.K.A., Acuña-Castroviejo, D.

17th International Congress of Anti-aging Medicine. Pineal versus extrapineal melatonin in aging. 2018. Granada (España). Acuña-Castroviejo, D., Venegas, C., **Fernández-Ortiz, M.,** Martinez, P.A., Fernandez-Martinez, J., Rusanova, I., Escames, G.

13th Conference on Mitochondrial Physiology: The role of mitochondria in health, disease and drug Discovery – COST MitoEAGLE perspectives and MitoEAGLE WG and MC Meeting. Mitochondria, melatonin, and neuroinflammation in Parkinson's disease. 2018. Jurmala (Latvia). Acuña-Castroviejo, D., Lopez, A., Diaz-Casado, M.E., **Fernández-Ortiz, M.**, Fernandez-Martinez, J., Escames, G.

13th Conference on Mitochondrial Physiology: The role of mitochondria in health, disease and drug Discovery – COST MitoEAGLE perspectives and MitoEAGLE WG and MC Meeting. Generating mitochondrial respirometry reference values from permeabilized mouse soleus muscle fibers. Working Group 2 report. 2018. Jurmala (Latvia). Garcia-Roves, P.M., Gama-Perez, P., Dahdah, N., Doerrier, C., Gnaiger, E., Lemieux, H., Holody, C.D., Carpenter, R.G., Tepp, K., Puurand, M., Kaambre, T., Dubouchaud, H., Chabi, B., Cortade, F., Ost, M., Pesta, D., Calabria, E., Casado, M., **Fernández-Ortiz, M.**, Acuña-Castroviejo, D., Villena, J., Grefte, S., Keijer, J., O'Brien, K., Sowton, A., Murray, A.J., Campbell, M.D., Marcinek, D.J.

XXXVIII Congreso de la Sociedad Española de Ciencias Fisiológicas (SECF). Aging and Sarcopenia. Roles of the innate immunity and melatonin therapy. 2016. Granada (España). **Fernández-Ortiz, M.,** Sayed, R.K., Pozo, J.A.R., Martinez, P.A., Acuña-Castroviejo, D.

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7ª Reunión nacional de la Sociedad Español de Medicina Geriátrica (SEMEG). 6ª Reunión de la Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF). Efecto del inflamasoma NLRP3 sobre la sarcopenia y terapia con melatonina. 2016. Granada (España). **Fernández-Ortiz, M.**, Sayed, R.K.A., Rahim, I., Escames, G., Acuña-Castroviejo, D.

ACKNOWLEDGMENTS / AGRADECIMIENTOS

The defense of this doctoral thesis closes what I consider to be one of the most exciting chapters of my life. Throughout these five years I have had the opportunity to develop myself on a scientific, academic and personal level. I would be lying if I said developing this Thesis was an uncomplicated and effortless process. As every researcher knows, patience is the mother of Science. However, what I can say is that I have always had people by my side who have supported me in those difficult times and with whom I have also shared in my achievements. This section is dedicated to them:

First of all, thank my supervisor and thesis tutor, Prof. Darío Acuña Castroviejo. Thank you for accepting me on your team, for trusting me, and for always being willing to share your knowledge and wisdom. To me, you have always been and will, a role model, as a teacher and as a researcher.

Thanks to Germaine, for always entering the laboratory with a smile, for helping me whenever I have needed it, and for infecting me with your passion for teaching and Science.

To Luis Carlos, always willing to listen and solve doubts related to some scientific protocol and the endless bureaucratic processes.

Thanks to Professor Yolanda Román, for her infinite patience, for her closeness and for her enormous help with the statistics of the clock genes.

Thanks to Prof Erich Gnaiger and Prof Yidong Bai, for accepting me as a part of their labs, giving me the opportunity to expand my knowledge and making my research stays one of the best experiences of my life.

To all my colleagues in the CTS-101 Group. So many hours in the lab have made me consider you part of my family. Each one of you has given me something unique and wherever I go I will always carry you with me in my memory and in my heart.

To my Salita 39's friends, for always brightening my day and infusing it with energy.

To my lifelong friends, for always having time to play board games, for your entertaining and endless talks, and for your field trips.

Last but not least, thank you to my family. Dad and Mom ... thank you for always being there, always supporting me, giving me strength, encouragement, and teaching me how important discipline and perseverance are. This would not have been possible without your help. To my grandmothers, for your love and for always giving me moments of peace and tranquility. To my grandparents, that wherever you are I know that you would be proud of me. Finally, thank you to my treasure, for just being you.

To all of you… THANK YOU.

Con la Defensa de esta Tesis doctoral se cierra lo que considero una de las etapas más emocionantes de mi vida. A lo largo de estos cinco años he tenido la oportunidad de desarrollarme a nivel científico, académico y personal. Mentiría si dijese que el desarrollo de la Tesis ha sido un proceso sencillo y fácil, pues todo investigador sabe que la paciencia es la madre de la Ciencia. Sin embargo, lo que sí puedo decir es que siempre he tenido a mi lado a personas que me han dado apoyo en esos momentos difíciles y con las que también he compartido mis logros. A ellas va dedicado este apartado:

En primer lugar, agradecer a mi director y tutor de tesis, el Prof. Darío Acuña Castroviejo. Gracias por aceptarme en tu equipo, por confiar en mí, y por estar siempre dispuesto a compartir tus conocimientos y sabiduría. Para mí siempre has sido y serás un modelo a seguir, como docente y como investigador.

Gracias a Germaine, por entrar al laboratorio siempre una sonrisa, por ayudarme siempre que lo he necesitado, y por contagiarme con tu pasión por la enseñanza y la Ciencia.

A Luis Carlos, siempre dispuesto a escuchar y resolverme dudas relacionadas con algún protocolo científico y con los interminables procesos burocráticos.

Gracias a la Profesora Yolanda Román, por su infinita paciencia, por su cercanía y por su enorme ayuda con la estadística de los genes reloj.

Gracias a los Profesores Erich Gnaiger y Yidong Bai, por aceptarme como un miembro más de sus laboratorios, por darme la oportunidad de expandir mis conocimientos y hacer de mis estancias de investigación una de las mejores experiencias de mi vida.

A todos mis compañeros del Grupo CTS-101. Tantas horas en el laboratorio han hecho que os considere parte de mi familia. Cada uno de vosotros me ha aportado algo único y allá donde vaya siempre os llevaré conmigo en la memoria y en el corazón.

A mis amigos de la Salita 39, por alegrarme siempre el día e inyectarlo de energía.

A mis amigos de toda la vida, por tener siempre tiempo para jugar a juegos de mesa, por vuestras charlas amenas e interminables, y por vuestras excursiones en el campo.

Por último, pero no por ello menos importante, gracias a mi familia. Papá y Mamá…gracias por haber estado siempre ahí, apoyándome en todo momento, dándome fuerzas, ánimos, y enseñándome lo importante que es la disciplina y perseverancia. Esto no habría sido posible sin vuestra ayuda. A mis abuelas, por vuestro cariño y por brindarme siempre momentos de paz y tranquilidad. A mis abuelos, que allá donde estéis sé que estaréis orgullosos de mí. Finalmente, gracias a mi tesoro, simplemente por ser tú.

A todos vosotros…GRACIAS.

ABBREVIATIONS

AANAT: Arialkylamine N-Acetyltransferase
ADP: adenosine diphosphate
AFMK: N1-acetyl-N2-formil-5-methoxykirunamine
AKT: protein kinase B
AMK: N1-acetyl-5-methoxykinuramine
AMP: adenosine monophosphate
AMPK: AMP-activated protein kinase
aMT: melatonin
AREs: Antioxidant Response Elements
ASC: Apoptosis-associated Speck-like protein containing a caspase recruitment domain
ASMT: N-acetylserotonin O-methyltransferase
ATP: adenosine triphosphate
Bax: BCL2-associated X protein
Bcl-2: B-cell lymphoma protein 2
Bmal1: brain and muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like 1
CAT: catalase
CCGs: clock-controlled genes
Chrono: ChIP-derived Repressor of Network Oscillator
Clock: circadian locomotor output cycles kaput
COX: cyclooxygenase
CREB: IP3R-cAMP response element binding protein
CRP: C-reactive protein
Cry: Chryptochrome
CSA: cross-sectional area
CVDs: cardiovascular diseases
DAMPs: Damage-Associated Molecular Patterns
DBD: DNA-binding domain
DR: death receptor
Drp1: dynamin-related protein 1
ECM: extracellular matrix
$FADH_2$: flavin adenine dinucleotide
G6PD: glucose-6-phosphate dehydrogenase
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GPx: glutathione peroxidase
GR: glutathion reductase
GSH: reduced glutathione
GSSG: oxidized glutathione
H_2O_2: hydrogen peroxide
Hmox1: heme oxygenase 1
IFN: interferon
IGF: insulin-like growth factor
IL: interleukin
IMF: intermyofibrillar
iNOS: inducible nitric oxide synthase
ipRGCs: intrinsically photosensitive retinal ganglion cells
IκB: inhibitor of kappa B
Keap1: kelch-like ECH-associated protein 1
L/D: light/dark
LBD: ligand-binding domain
LC3: microtubule-associated protein 1 light chain 3
LPS: lipopolysaccharide
LRR: leucine-rich-repeat
LV: left ventricle
MAO: monoamine oxidase
MAPK-ERK: Mitogen Activated Protein Kinase- Extracellular signal Regulated Kinase
Mfn2: mitofusin 2
miRNA: microRNA

MOMP: mitochondrial outer membrane permeabilization
MPTP: mitochondrial permeability transition pore
mtDNA: mitochondrial DNA
mTOR: mammalian target of rapamycin
NACHT: Nucleotide-binding oligomerization domain, leucine-rich-repeat family, Apoptosis inhibitory protein, Class II, major histocompatibility complex transactivator, Het-E incompatibility locus protein from Podospora anserina, Telomerase-associated protein 1
NAD: NACHT-associated domain
NADH: nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NAS: N-acetyl-serotonin
NF-κB: Nuclear Factor Kappa B
NLRP3: NLR family pyrin domain containing 3
NO: nitric oxide
Nqo1: NAD(P)H quinone dehydrogenase 1
Nrf2: nuclear factor, erythroid derived 2, like 2
$O_2^{\cdot-}$: superoxide anion
$OH^{\cdot}$: hydroxyl radical
$ONOO^-$: peroxynitrite
Opa1: optic atrophy 1
OXPHOS: oxidative phosphorylation
p53: transformation related protein 53
PAMPs: Pathogen-Associated Molecular Patterns
Per: Period
PGC-1α: PPARγ co-activator 1 alpha
P_i: inorganic phosphate
PI3K: phosphatidylinositol 3-kinase
pNrf2: phosphorylated Nrf2
PPARγ: peroxisome proliferator-activated receptor gamma
PRRs: Pattern Recognition Receptors
PVN: paraventricular nucleus
PYD: Pyrin Domain
Rev-erbα: reverse strand of protein ERB alpha
RISK: reperfusion injury salvage kinase
RNS: reactive nitrogen species
ROR/RZR: retinoid-related orphan receptor/retinoid Z receptor
ROREs: retinoic acid-related orphan receptor response elements
Rorα: Retinoic acid-related orphan receptor alpha
ROS: reactive oxygen species
SAMP: Senescence-Accelerated Mouse Prone
SASP: senescent-associated secretory phenotype
SIRT: sirtuin
SCN: suprachiasmatic nucleus
SOD: superoxide dismutase
TLRs: Toll-like receptors
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
TXNIP: thioredoxin-interacting protein
VDAC: voltage dependent anion channel
VSMCs: vascular smooth muscle cells
β-MHC: Myosin, heavy polypeptide 7, cardiac muscle, beta
γ-Gclc: gamma-glutamate-cysteine ligase, catalytic subunit

“The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge”

Stephen Hawking

“Education is the most powerful weapon which you can use to change the world”

Nelson Mandela

SUMMARY

Cardiovascular diseases are the leading cause of death in the world, with aging being the main risk factor associated with these pathologies (Global Health and Aging, 2019). The world's population is aging at an unprecedented rate, and study of the mechanisms underlying this process is vitally important from a health, economic and social point of view.

Aging is characterized by a deregulation of the immune system resulting in a subclinical, sterile, asymptomatic and chronic pro-inflammatory state known as inflammaging (Franceschi and Campisi, 2014). This inflammatory condition, coupled with oxidative stress, leads to mitochondrial dysfunction and subsequent apoptosis, facilitating the release of reactive species, ATP, and mtDNA. These hazard signals are recognized by the NLRP3 inflammasome, a multiprotein complex responsible for the maturation of pro-inflammatory cytokines dependent on NF-κB, including IL-1β. In this way, this process perpetuates a vicious cycle that results in systemic inflammation that is accompanied by symptoms of immunosenescence and activation of the innate immune pathway. Alterations in the regulation of mitochondrial homeostasis, including mechanisms of mitochondrial dynamics, autophagy, apoptosis, as well as decreased antioxidant defense that occurs with aging, such as the Nrf2-dependent pathway, may be necessary for activation of NLRP3 inflammasome. In addition, the effect this inflammasome may have on mitochondrial function or on the antioxidant pathway of Nrf2 during cardiac aging remains unknown.

Interestingly, numerous scientific studies relate inflammaging to the disruption of circadian rhythms, which allow the organism to adapt and anticipate environmental changes to ensure optimal physiological performance (Acuña-Castroviejo et al., 2017; Acuña-Fernández et al., 2020; Volt et al., 2016). In mammals, circadian rhythms are regulated by a central clock, located in the suprachiasmatic nucleus, and by peripheral clocks, located in virtually all tissues, including the heart. While there seems to be a connection between aging, clock genes and innate immune response, the molecular mechanisms that link these processes remain a mystery. To date, the influence of NF-κB on the disruption of circadian rhythms during aging has been demonstrated. However, little is known about NLRP3's involvement in aging-associated chronodisruption.

It should be noted that aging manifests the progressive loss of strength and muscle mass. This process is defined as sarcopenia and is considered one of the main causes of reduced physical performance and impaired cardiorespiratory function in patients with heart failure (Curcio et al., 2020). Numerous clinical and experimental studies have shown that aging is associated with histological, structural, and functional changes in cardiac tissue (Lakatta, 2002). Our group demonstrated that the absence of the NLRP3 inflammasome reduced sarcopenia in skeletal muscle

(Sayed et al., 2019). Given these results, we consider it of interest to analyze the involvement of NLRP3 inflammasome activation in structural alterations in the aging heart.

Melatonin is a hormone synthesized by the pineal gland, as well as by most organs and tissues, including the heart. Pineal melatonin has chronobiotic actions and its production decreases with age. This decline has been linked to changes in circadian rhythms, increased inflammation, and development of cardiac pathologies (Hardeland, 2012). Extrapineal melatonin has antioxidant and anti-inflammatory properties (Hawthorn et al., 2012). In experimental models that include chronic and acute inflammation as well as aging in the mouse heart, melatonin decreased innate immune response, counteracted oxidative stress, and improved the activity of cardiac mitochondria (García et al., 2015; Rodríguez et al., 2007). In addition, melatonin administration has been shown to improve muscle function and reduce inflammation in athletes (Leonardo-Mendonca et al., 2017).

Considering this theoretical framework, our work focused on the study of the causal relationship between chronodisruption, melatonin deficiency, and innate immunity, as well as the involvement of the NLRP3 inflammasome-mediated immune response during cardiac aging.

To accomplish this, the aims were to evaluate cardiac tissue of wild type C57/Bl6 mice and mice with a C57/Bl6 background knocked-out for NLRP3 inflammasome ($NLRP3^{-/-}$) of 3, 12 and 24 months of age in the following parameters:

1.- Mitochondrial pathway: mitochondrial dynamics, autophagy, apoptosis and mitochondria ultrastructure.

2.- Antioxidant pathway dependent on Nrf2.

3.- Biological clocking: expression of clock genes, rhythmicity, acrophase, amplitude and mesor.

4.- Histological study and MRI of heart muscle.

5.- Effects of melatonin treatment on the parameters mentioned above.

The results of this doctoral thesis show the deleterious effect NLRP3 inflammasome has on mitochondrial function during aging, as its absence prevented damage to mitochondrial dynamics and structure. Melatonin treatment also reestablished mitochondrial dynamics, had an anti-apoptotic action, restored the Nrf2 dependent antioxidant pathway, and preserved mitochondrial structure during aging.

With reference to the biological clock pathway, it could be found that aging, melatonin, and presence of the NLRP3 inflammasome had significant effects on expression observed in the clock

genes, except for the *Rev-erbα* gene, which was not affected by the mouse genotype. Small phase changes were observed in the *Clock* gene, loss of rhythmicity in *Per2 and Rorα* and a tendency for mesor to decrease with aging. The NLRP3 inflammasome influenced the acrophase of *Clock, Per2* and *Rorα,* suggesting some negative impact on the function of the myocardium. Melatonin restored rhythms and acrophases in cardiac tissue, highlighting its clinical potential in the prevention and treatment of chronodisruption. Besides these changes, the results indicate that the local chronobiotic system of the heart is highly protected against aging.

Finally, it was concluded that NLRP3 is involved in cardiac sarcopenia, as 24-month-old mutant mice had less thickening of the ventricular wall, less fibrosis, lower expression of inflammatory cytokines, and lower mitochondrial damage compared to wild type mice. Again, we observed a prophylactic effect of melatonin in preserving the structure and number of cardiomyocytes and reducing pro-inflammatory and hypertrophic markers as well as apoptosis. It is therefore inferred that suppression of the NLRP3 inflammasome and implementation of melatonin therapy may be beneficial therapeutic approaches to ameliorate cardiac aging and sarcopenia.

RESUMEN

Las enfermedades cardiovasculares constituyen la primera causa de muerte en el mundo, siendo el envejecimiento el principal factor de riesgo asociado a estas patologías (Global Health and Aging, 2019). En una población mundial que envejece a un ritmo sin precedentes, el estudio de los mecanismos que subyacen al proceso de envejecimiento resulta de vital importancia desde un punto de vista sanitario, económico y social.

El envejecimiento está caracterizado por una desregulación del sistema inmune que resulta en un estado pro-inflamatorio de carácter subclínico, estéril, asintomático y crónico conocido como inflammaging (Franceschi and Campisi, 2014). Esta condición inflamatoria, sumada al estrés oxidativo, desemboca en una disfunción mitocondrial y consecuente apoptosis, favoreciendo la liberación de especies reactivas, ATP y mtDNA. Estas señales de peligro son reconocidas por el inflamasoma NLRP3, un complejo multiproteico responsable de la maduración de las citoquinas pro-inflamatorias dependientes de NF-κB, entre ellas la IL-1β. De este modo, mediante este proceso se perpetúa un círculo vicioso que da lugar a una inflamación sistémica que se acompaña de síntomas de inmunosenescencia y activación de la vía inmune innata. Las alteraciones en los procesos que regulan la homeostasis mitocondrial, entre ellos los mecanismos de dinámica mitocondrial, autofagia, apoptosis, así como la disminución de la defensa antioxidante que ocurre con el envejecimiento, tales como la vía dependiente de Nrf2, podrían ser necesarias para la activación del inflamasoma NLRP3. Asimismo, se desconoce el papel que este inflamasoma puede ejercer sobre la función mitocondrial o sobre la vía antioxidante de Nrf2 durante el envejecimiento cardiaco.

Numerosos estudios científicos relacionan el inflammaging con la disrupción de los ritmos circadianos, los cuales permiten al organismo adaptarse y anticiparse a los cambios ambientales, asegurando así un rendimiento fisiológico óptimo (Acuña-Castroviejo et al., 2017; Acuña-Fernández et al., 2020; Volt et al., 2016). En los mamíferos, los ritmos circadianos están regulados por un reloj central, situado en el núcleo supraquiasmático, y por relojes periféricos, localizados prácticamente en la totalidad de los tejidos, entre ellos el corazón. Si bien parece existir una conexión entre el envejecimiento, los genes reloj y la respuesta inmune innata, los mecanismos moleculares que relacionan estos procesos continúan siendo un misterio. Hasta la fecha, se ha demostrado la influencia de NF-κB en la disrupción de los ritmos circadianos durante el envejecimiento. No obstante, poco se sabe acerca de la participación de NLRP3 en la cronodisrupción asociada al envejecimiento.

Cabe añadir que con el envejecimiento se pierde de manera progresiva la fuerza y la masa muscular. Este proceso se define como sarcopenia y está considerada una de las principales causas de reducción del rendimiento físico y función cardiorrespiratoria en pacientes con fallo cardiaco (Curcio et al., 2020). Numerosos estudios clínicos y experimentales han demostrado que el envejecimiento está

asociado a cambios histológicos, estructurales y funcionales del tejido cardiaco (Lakatta, 2002). Nuestro grupo demostró que la ausencia del inflamasoma NLRP3 redujo la sarcopenia en el músculo esquelético (Sayed et al., 2019). Teniendo en cuenta esos resultados, consideramos de interés analizar el papel del inflamasoma NLRP3 en los cambios estructurales relacionados con el envejecimiento en el corazón.

La melatonina es una hormona sintetizada por la glándula pineal, así como por la mayoría de los órganos y tejidos, incluido el corazón. La melatonina pineal tiene acciones cronobióticas y su producción disminuye con la edad. Este descenso se ha relacionado con la alteración de los ritmos circadianos, patologías inflamatorias y cardíacas (Hardeland, 2012). La melatonina extrapineal posee propiedades antioxidantes y antiinflamatorias (Espino et al., 2012). En modelos experimentales que incluyen inflamación crónica, aguda y envejecimiento en el corazón de ratón, la melatonina disminuyó la respuesta inmune innata, contrarrestó el estrés oxidativo y mejoró la actividad de las mitocondrias cardíacas (García et al., 2015; Rodríguez et al., 2007). Además, la administración de melatonina ha demostrado mejorar la función muscular y reducir la inflamación en atletas (Leonardo-Mendonça et al., 2017).

Teniendo en cuenta este marco teórico, nuestro trabajo se centró en el estudio de la relación causal entre la cronodisrupción, déficit de melatonina e inmunidad innata, así como de la respuesta inmune mediada por el inflamasoma NLRP3 durante el envejecimiento cardiaco.

Para ello, los objetivos fueron evaluar en el tejido cardiaco de ratones *wild type* de la cepa C57/Bl6 y los correspondientes deficientes en el inflamasoma NLRP3 ($NLRP3^{-/-}$) de 3, 12 y 24 meses de edad los siguientes parámetros:

1.- Vía mitocondrial: dinámica mitocondrial, autofagia, apoptosis y ultraestructura de la mitocondria.

2.- Vía antioxidante dependiente de Nrf2.

3.- Vía del reloj biológico: expresión de genes reloj, ritmicidad, acrofase, amplitud y mesor.

4.- Estudio histológico y resonancia magnética del músculo cardiaco.

5.- Efectos del tratamiento con melatonina en los parámetros mencionados anteriormente.

Los resultados de esta tesis doctoral muestran la influencia del inflamasoma NLRP3 en la función mitocondrial durante el envejecimiento, ya que su ausencia evitó daños en la dinámica y estructura mitocondrial. El tratamiento con melatonina también recuperó la dinámica mitocondrial, tuvo una acción antiapoptótica, restauró la vía antioxidante dependiente de Nrf2 y mejoró la estructura mitocondrial durante el envejecimiento.

En referencia a la vía del reloj biológico, se pudo comprobar que el envejecimiento, la melatonina y el inflamasoma NLRP3 tuvieron efectos significativos en los cambios de expresión observados en los genes reloj, a excepción del gen *Rev-erbα*, que no se vio afectado por el genotipo del ratón. Se observaron pequeños cambios de fase en el gen *Clock*, pérdida de ritmicidad en *Per2* y *Rorα* y una tendencia a la disminución del mesor con el envejecimiento. El inflamasoma NLRP3 influyó en la acrofase de *Clock*, *Per2* y *Rorα*, sugiriendo cierto impacto negativo en la función del miocardio. La melatonina restauró los ritmos y acrofases en el tejido cardiaco, poniendo de manifiesto su potencial clínico en la prevención y tratamiento de la cronodisrupción. En cualquier caso, los resultados indican que el sistema local cronobiótico del corazón está muy protegido frente al envejecimiento.

Por último, se concluyó que NLRP3 está implicado en la sarcopenia cardiaca, pues los ratones mutantes de 24 meses presentaron un menor engrosamiento de la pared ventricular, menor fibrosis, menor expresión de citoquinas inflamatorias y menos daño mitocondrial en comparación con los ratones *wild type*. Una vez más, observamos un efecto profiláctico de la melatonina al preservar la estructura y número de los cardiomiocitos, reducir marcadores proinflamatorios y de hipertrofia, así como la apoptosis. Se deduce por tanto que la supresión del inflamasoma NLRP3 y la terapia con melatonina pueden ser buenas aproximaciones terapéuticas para el envejecimiento cardiaco y la sarcopenia.

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INTRODUCTION

1. AGING

1.1. WHAT IS AGING?

In 1959, Bertrand Strehler defined the aging of metazoans as a "*process of gradual and irreversible disorganization that occurs over time, causes a progressive loss of bioenergetics capacity and functional performance and makes the survival of human and other species of metazoans impossible, even in an optimal habitat*" (Strehler, 1959). Thus, any change in the organism associated with age has to meet the following requirements (Strehler, 1977): 1) to be harmful, adversely affecting physiological functions; 2) to have a progressive nature and consequently build gradually; 3) to have intrinsic character, excluding those alterations derived from environmental factors; 4) to be universal, manifesting in all individuals of every species.

The world population is aging at an unprecedented rate, and this change is expected to accelerate in the coming decades (Global Health and Aging, 2019). In 2018 for the first time in recorded history, people aged 65 and over surpassed children under the age of 5 (Figure 1). Globally, the population over 65 is growing at a faster rate than the other population segments. The demographics of this group are expected to double between 2019 and 2050, while the number of inhabitants under 25 will peak then decrease slightly (Figure 2). Europe and North America had the oldest population in 2019, with 18% of people over 65 years of age. In these regions, the 65 year-old population will overcome those 25 years and younger by 2050.

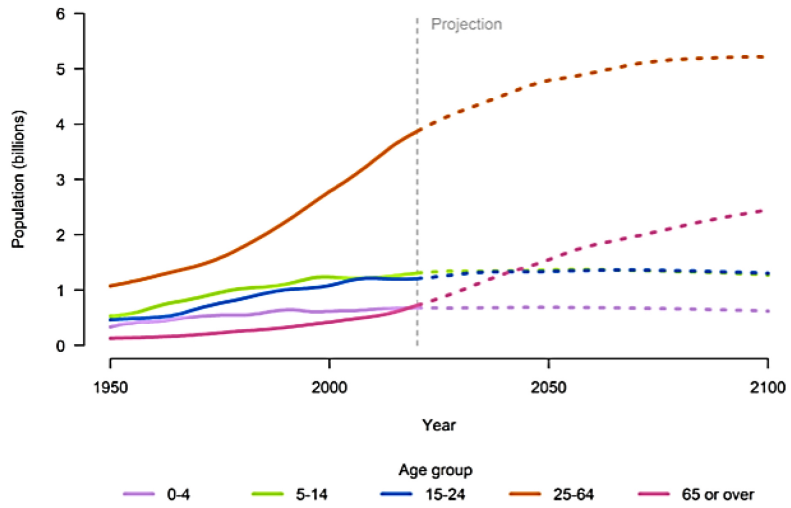


Figure 1. Estimated and projected global population by broad age group, 1950-2100. *People aged 65 years or over make up the fastest-growing age group. From Global Health and Aging, 2019.*

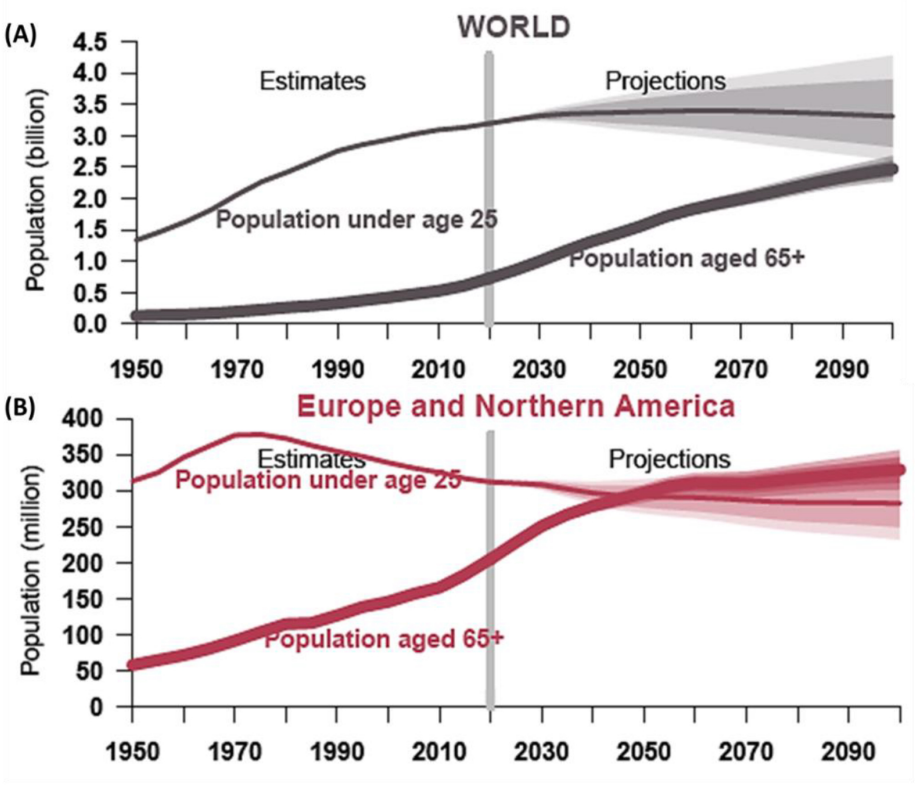


Figure 2. Estimated and projected population changes. *(A) Global population. (B) Europe and Northern America population. From Global Health and Aging, 2019.*

Given recent advances in medicine and technology, the resulting rise in life expectancy demands a focused on understanding the aging process. Unlike most diseases and disorders, age-related bodily decline is an inevitable consequence of living a long life. With the projected increase in the percentage of the global population over 65 years, research in this subject will remain an expanding frontier.

1.2. HALLMARKS OF AGING

López-Otín et al. classified and proposed the cellular and molecular hallmarks that contribute to the development and phenotype of aging (López-Otín et al., 2013). Each hallmark should satisfy the following prerequisites: 1) it should appear during aging; 2) its empirical exacerbation should advance aging and 3) its empirical amelioration should delay aging and extend healthy lifespan. Hallmarks of aging can be grouped into three main categories: primary, antagonistic and integrative (Figure 3).

Primary hallmarks damage cellular functions and could be the initiating factors whose harmful effects gradually accumulate with age. These hallmarks include genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. Antagonistic hallmarks, such as deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence, have consequences that depend on their intensity. Initially, at low levels, they are beneficial and become progressively deleterious as their severity increases. Finally, integrative hallmarks, which include stem cell exhaustion and altered

intercellular communication, manifest when mechanisms of tissue homeostasis are insufficient to counteract the detriments caused by primary and antagonist hallmarks.

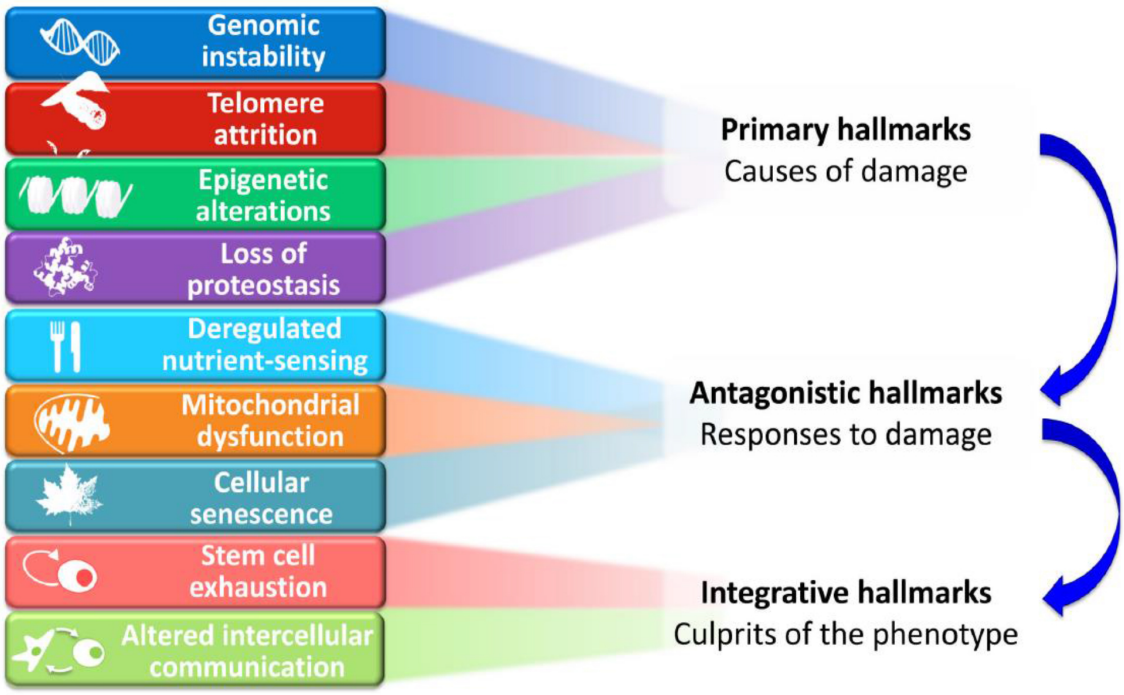


Figure 3. Hallmarks of aging and their functional interconnections. *Primary hallmarks are considered to be the leading causes of cellular damage. Antagonistic hallmarks are part of the compensatory responses to the damage. These responses initially diminish the damage, but eventually, if chronic or exacerbated, they become harmful themselves. The integrative hallmarks are the result of the two previous hallmarks and are responsible for the functional decline associated with aging. From López-Otín et al., 2013.*

1.2.1. Genomic instability

Integrity of genome can be affected by external physical, biological and chemical elements, as well as by internal elements that includes spontaneous hydrolytic reactions, DNA replication errors and reactive oxygen species (ROS) (Hoeijmakers, 2009).

Numerous investigations have unveiled an accumulation of somatic DNA mutations in cells from aged humans and models organism (Moskalev et al., 2013). It has been suggested that these DNA alterations may contribute to the functional impairment of B lymphocytes (Zhang et al., 2019a), satellite cells (Franco et al., 2018) and neurons (Lodato et al., 2018) in the elderly.

Mitochondrial DNA (mtDNA) is believed to be a major target for somatic mutations associated with aging due to the lack of histone protection, limited efficacy of mtDNA repair machinery compared to those of nuclear DNA, and unrepaired oxidative damage (Kauppila et al., 2017; Linnane et al., 1989). Mutations and deletions in mtDNA accumulate with aging and have been related to tissue dysfunction, severe decline of cellular energy, and age-associated diseases (Chinnery, 2015; Payne and Chinnery, 2015).

1.2.2. Telomere attrition

Telomeres are repetitive tandem sequences that protect chromosome ends from interchromosomal fusion and nucleolytic degradation. During each cell division, telomeres shorten due to the incapacity of DNA polymerases to fully replicate the end of the telomeric DNA (Allsopp et al., 1992). This telomere depletion leads to replicative senescence of some types of *in vitro* cultured cells (Greider, 1998). Telomeres have been considered a "molecular clock" and shortened telomeres have been linked to aging processes and risk of mortality (Vera et al., 2012). Telomerase can restore the lost telomeric DNA. However, most mammalian somatic cells do not express this enzyme, which only remains active in some tissues, such as activated lymphocytes, certain types of stem cell populations, and male germ cells (Wright et al., 1996). Telomerase deficiency in humans is involved with early development of some diseases, like aplastic anemia, pulmonary fibrosis and dyskeratosis congenita (Armanios and Blackburn, 2012). Genetic reactivation of telomerase reverted aging in aged telomerase-deficient mice (Jaskelioff et al., 2011). Additionally, aging was delayed in adult and old mice with pharmacological and systemic viral transduction of telomerase without increasing cancer incidence (Bernardes de Jesus et al., 2012).

1.2.3. Epigenetic alterations

Epigenetic alterations are heritable changes in gene expression that are achieved without affecting the nucleotide sequence. Epigenetic changes involve mainly DNA methylation, histone modification, and non-coding RNAs. Epigenetic modifications affect all cells and tissues during life (Talens et al., 2012). The *theory of developmental origins of health and disease* postulates that epigenetic alterations is a short-term adaptive mechanism that may become harmful in either short or long term, and therefore causing chronic disease and aging phenotype (Barker et al., 1989; Pembrey et al., 2014).

In mammals, DNA methylation happens predominantly at CpG islands, which are linked with about half of all human gene promoters. DNA methylation is emerging as one of the most robust predictor of human age (Horvath, 2013). Recently, it has been found a correlation between DNA methylation age and increased risk of death in elderly population. Interestingly, this association was independent of life-course predictor of aging and death, such as diabetes, smoking or hypertension (Marioni et al., 2015).

Histone modifications have been related to influencing longevity in several organisms through the regulation of DNA damage repair, transcriptional control and chromosome packaging (Wątroba et al., 2017). In this sense, sirtuins have been extensively studied as possible post-translational histone modulators during aging since its activity is age-dependent. The deficiency of mammalian nuclear

sirtuins (SIRT), such as SIRT1, SIRT6 and SIRT7, has been associated with accelerated aging, degenerative diseases and shortening of lifespan.

Non-coding RNAs have an important function in gene regulation and silencing. The most investigated are microRNAs (miRNA). These short-length sequences may regulate up to 80% of all genes expressed in humans (Kozomara and Griffiths-Jones, 2011). MiRNAs are involved in regulation of signaling pathways related to aging, among them the insulin/insulin-like growth factor (IGF) 1 and mammalian target of rapamycin (mTOR). Their role in the age-associated decline in the immune system is also accepted.

1.2.4. Loss of proteostasis

Proteostasis is a set of cellular mechanisms that maintain the homeostasis of the proteome, the synthesis and turnover of human proteins (Eisenstein, 2014). The accumulation of dysfunctional proteins and their consequent formation of insoluble aggregates is the principal cause of some age-related disorders like Alzheimer's and Parkinson's disease, and cataracts (Clark et al., 2012; Labbadia and Morimoto, 2015). Therefore, the loss of proteostasis is highly related with aging (Koga et al., 2011). The proteostasis system consists of chaperone-mediated folding, proteasomal degradation and autophagy.

Molecular chaperones are small proteins that support native polypeptide chains in the folding of functional protein conformations. The most relevant group of chaperones is the heat-shock factor (HSF) family proteins. Mutant mice with a deficiency of these chaperones exhibited accelerated aging phenotypes, while up-regulation showed an increase in longevity (Min et al., 2008; Swindell et al., 2009). In mammalian cells, the transcription factor HSF-1 is recognized as the master regulator of the heat-shock response, which consists in boosting the transcription of chaperones as an effect of a rise in unfolded proteins (Labbadia and Morimoto, 2015).

Proteasome activation is considered a conserved process of aging and longevity control, and its activity is known to decline with aging (Rubinsztein et al., 2011; Tomaru et al., 2012). Furthermore, it has been demonstrated that proteasomal action retards aging *in vivo* and *in vitro* models (Chondrogianni et al., 2014). People over the age of one hundred showed persistent proteasome functionality (Chondrogianni et al., 2000).

Autophagy is the intracellular digestion of alien cytosolic components and dysfunctional proteins and organelles, a process vital for homeostasis (Glick et al., 2010). As organisms age, their autophagic capacity decreases (Cuervo and Macian, 2014), which facilitates age-related diseases including senile osteoporosis, osteoarthritis and neurodegeneration (Caramés et al., 2015; Tan et al., 2014). Likewise, restoring or increasing autophagic capacity is sufficient to extent lifespan in nematodes, flies and mice model (Madeo et al., 2015).

1.2.5. Deregulated nutrient-sensing

Efficient detection and utilization of nutrients are of vital importance for cellular homeostasis, and overall development throughout the life of an organism. Insulin sensitivity is an evolutionarily preserved regulator of metabolism and age-related decline in its efficacy has been of great interest in scientific research. Insulin regulation is mediated by the actions of growth hormone (GH) and IGF-1, both of which decline with age (Schumacher et al., 2008). Intriguingly, this downregulation has also been linked to increased longevity (Fontana et al., 2010). This paradox has been theorized to be a defensive response to reduce cell growth and metabolism in the context of age-induced cellular damage (Garinis et al., 2008). Moreover, excessive reduction of these regulators becomes lethally deleterious, as demonstrated by mouse null mutations of the downstream factors PI3K (phosphatidylinositol 3-kinase) and AKT (protein kinase B) (Renner and Carnero, 2009).

Dietary or caloric restriction, which consists of low-caloric intake throughout life, has been linked with IGF-1 inhibition thereby decreasing mTOR activation by impairing the activity of downstream factors PI3K and AKT (Houtkooper et al., 2010). The mTOR kinase is implicated in virtually all facets of anabolic metabolism. Additionally, dietary restriction leads to adenosine monophosphate (AMP)-activated protein kinase (AMPK) and SIRT1 nutrient sensing upregulation, impairing mTOR action and activating peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1 alpha (PGC-1α), respectively (Alers et al., 2012). The resulting increase in autophagy and mitochondrial biogenesis are associated with the enhanced longevity following caloric-restriction in both simple and complex organisms (Anton and Leeuwenburgh, 2013).

1.2.6. Mitochondrial dysfunction

Mitochondria are subcellular, dynamic, self-autonomous, pleomorphic and double-membraned organelles found in nearly all eukaryotic cells (Kurland and Andersson, 2000). The main function of mitochondria is production of adenosine triphosphate (ATP) for cellular utilization by oxidative phosphorylation (OXPHOS) in cellular respiration. This system consists of four respiratory complexes (I- nicotinamide adenine dinucleotide (NADH) dehydrogenase, II- succinate dehydrogenase, III- cytochrome c reductase and IV- cytochrome c oxidase) located in the inner mitochondria membrane, two mobile carriers (coenzyme Q and cytochrome c) and ATP synthase.

On complexes I and II, the reduced coenzymes NADH and flavin adenine dinucleotide ($FADH_2$), which are generated by dehydrogenases in tricarboxylic acid, are oxidized. The electrons released by the oxidation of reduced coenzymes are transported through the respiratory chain to the molecular oxygen, supplying energy to pump protons across the inner mitochondria membrane. The proton-motive force creates an electrochemical potential that is used by ATP synthase to form ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Benard et al., 2006).

Along with energy production, mitochondria also generate ROS and play an essential role in regulation of apoptosis, lipid and amino acid metabolism, calcium homeostasis, thermogenesis and cell cycle regulation (Friedman and Nunnari, 2014).

Mitochondrial dysfunction has long been recognized as a main contributor to aging and age-related disorders (Acuña Castroviejo et al., 2011). As organisms age, OXPHOS capacity tends to decline, thus raising electron leakage and diminishing ATP production. Biogenesis and mitochondrial dynamics also decrease with aging, while mtDNA impairment, production of ROS, oxidation of mitochondrial proteins and triggering of apoptosis lead to a progressive cellular damage.

1.2.6.1. Mitochondria theory of aging

The *mitochondria theory of aging* has been a leading theory on aging. It was first proposed by Harman in 1956 and postulates that the production of intracellular ROS is the main driving force of the aging process (Harman, 1956). This theory was further refined and developed by Miquel et al. in 1980, who suggested that aging was associated with free radical reactions initiated in the mitochondria and changes in their redox status (Miquel et al., 1980).

Mitochondria are the primary intracellular site of oxygen consumption. Nearly 85% of the oxygen consumed by cells goes into the production of ATP through OXPHOS. Therefore, mitochondria are the major source of ROS, most of them derived from the mitochondrial respiratory chain (Grimm and Eckert, 2017) (Figure 4). As a natural by-product of respiration, 0.2-2% of molecular oxygen is one-electron reduced to form superoxide anion ($O_2^{\cdot-}$). Superoxide may be catalyzed by superoxide dismutase (SOD) resulting in the production of a longer-lived and membrane permeant hydrogen peroxide (H_2O_2). Although $O_2^{\cdot-}$ and H_2O_2 can also be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and monoamine oxidases (MAO) in mitochondria, these oxygen species are mainly generated by mitochondrial complexes I and III during electron transfer (Wong et al., 2019). Hydrogen peroxide may be fully reduced to water by the antioxidant defense systems glutathione peroxidase (GPx), glutathion reductase (GR) and catalase (CAT) (Murphy, 2009). Additionally, H_2O_2 may react with reactive iron (Fe^{2+}), from the degradation of iron-containing macromolecules, such as ferritin, myoglobin and cytochrome c, to produce hydroxyl radical (OH^-) via Fenton-type reaction (Terman and Kurz, 2013). Hydroxyl radicals are short-lived ubiquitin oxidants that are extremely harmful to biological material. Other potentially damaging molecules that are also produced by mitochondria are reactive nitrogen species (RNS), specifically nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$). Nitric oxide competitively inhibits mitochondria complex IV activity by binding its oxygen site (Brown and Borutaite, 2002). Peroxynitrite deteriorates protein functions by nitration (Goldstein and Merényi, 2008).

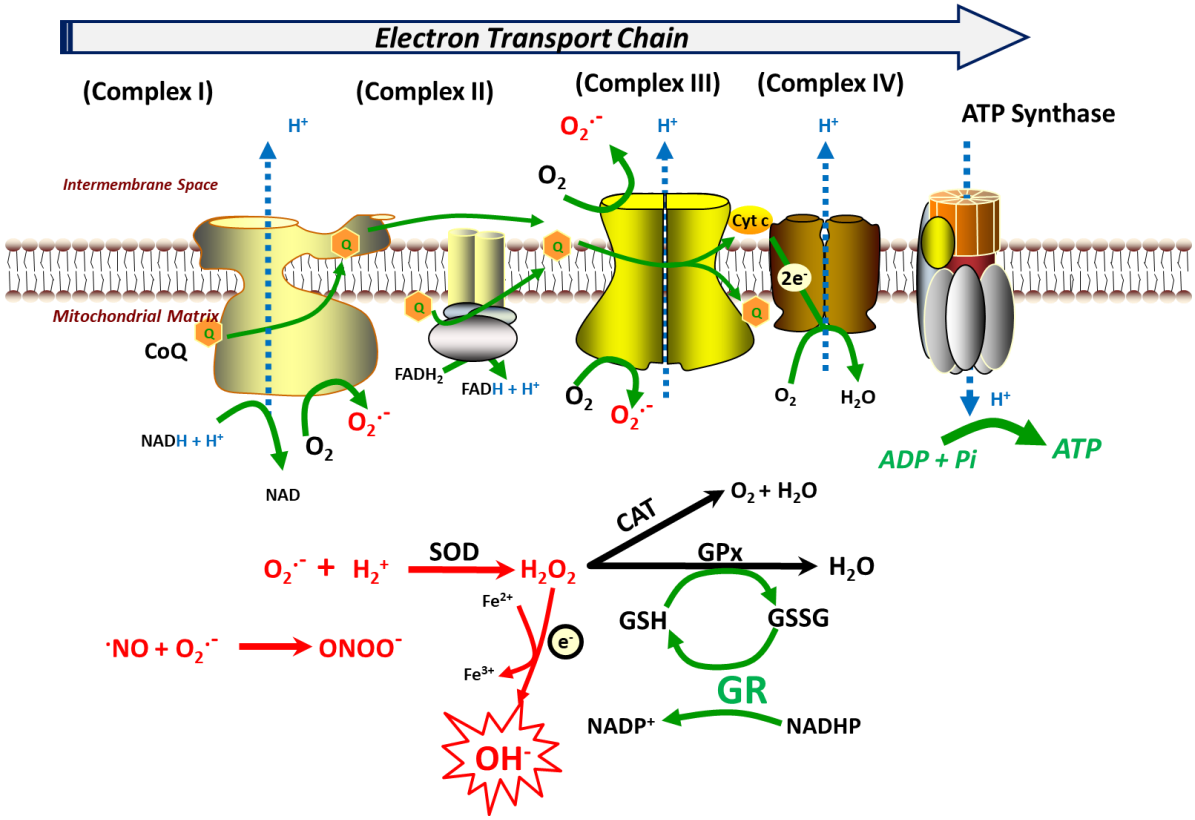


Figure 4. ROS production in mitochondria and endogenous systems of antioxidants enzymes. *Mitochondria are the major source of ROS production. Leakage of electrons from complexes I and III leads to the reduction of oxygen to form superoxide, which is the precursor of most ROS. Free radicals are maintained at physiological levels by endogenous systems of antioxidants enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. GSH/GSSG: reduced/oxidized glutathione.*

In physiological conditions, ROS production and antioxidant defense systems are in harmonious equilibrium (Grimm et al., 2016). It is worth mentioning that ROS are involved in physiological processes that include immune response, inflammation, synaptic plasticity, as well as signaling pathways involved in cell cycle, apoptosis, necrosis or senescence (Sena and Chandel, 2012). It has been proven that ROS activated the transcription factor hypoxia-induced factor 1 (HIF-1), which is linked to prolonged longevity (Bratic and Larsson, 2013). These findings suggest that ROS-induced stress response is vital for tissue homeostasis. However, there is an imbalance between ROS generation and antioxidant defenses during aging (Figure 5). This age-related chronic overproduction of ROS deteriorates mitochondria function by decreasing the activity of respiratory chain activity, membrane potential, and ATP production. The resulting oxidative damage leads to cell toxicity and apoptosis (Cedikova et al., 2016).

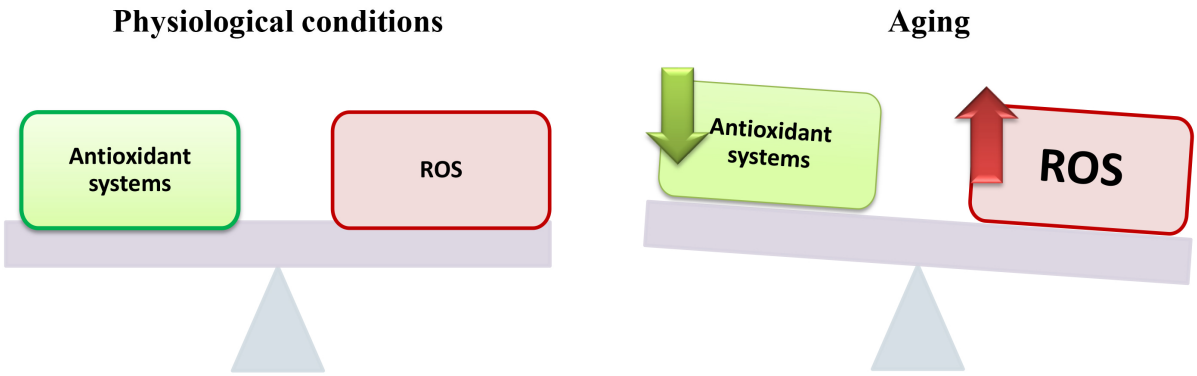


Figure 5. Equilibrium of antioxidant system and ROS at physiological and aged conditions. *In physiological conditions, ROS generation and antioxidant defense system are in balance. During aging, there is an increase in ROS production and a decline in the antioxidant systems, leading to an oxidative stress.*

1.2.6.2. Signaling pathways mediating mitochondrial effects of aging

Aging causes progressive increase in ROS that results in impairment and dysregulation of mitochondria-mediated signaling pathways. Paramount among them are mitochondria dynamics, autophagy (mitophagy), apoptosis, and antioxidant response.

1.2.6.2.1. Mitochondria dynamics

Central to metabolic homeostasis is the malleable nature of mitochondria dynamics that regulate the ever-changing bioenergetics of an organism (Liu et al., 2020). Regulation is maintained by a complex, context-dependent compensatory network of proteins responsible for mitochondria fusion, Mitofusin 2 (Mfn2) and Optic atrophy 1 (Opa1), and fission, Dynamin-related protein 1 (Drp1). Fusion facilitates the merging of healthy mitochondria with damaged adjacent mitochondria to share the energetic demands of a stressed cell. Mfn2 and Opa1 serve to increase the efficiency of bioenergetics by sustaining ATP production and maintain mitochondrial viability when resources are scarce. Fission proteins maintain homeostasis by separating irreparably damaged mitochondria from healthy ones for degradation, allowing for dispersal of mtDNA and the mitochondrial proteome during biogenesis. Drp1 decreases bioenergetics efficiency by increasing oxidative stress, membrane depolarization, and attenuating ATP production when utilization of excess resources is necessary for metabolic homeostasis. Mitochondrial dynamics has been closely linked to the regulation of autophagy (Villanueva Paz et al., 2016). The fission of mitochondria results in generation of healthy and damaged organelles. Mitochondrial depolarization prevents the fusion process, thus isolating damaged mitochondria and allowing their elimination by autophagy (mitophagy).

1.2.6.2.2. Autophagy (mitophagy)

Autophagy maintains the production of ATP generating metabolic intermediates and amino acids in the face of nutrient shortage. Furthermore, it is described as a cell survival mechanism through

which defective organelles are eliminated (Villanueva Paz et al., 2016). Thus, when damaged or non-functional mitochondria are selectively degraded by autophagy, which is called mitophagy (Lemasters, 2005). The exact mechanism that triggers mitophagy remains to be elucidated. It has been postulated that damaged mitochondria have a decreased mitochondria membrane potential (Twig and Shirihai, 2011). The uncoupled mitochondria are stabilized by the protein phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) at the surface of the mitochondrial outer membrane where it phosphorylates ubiquitin (Narendra et al., 2010). The ubiquitin phosphorylation recruits cytosolic E3 ubiquitin ligase Parkin, which polyubiquitinates mitochondrial proteins leading to the formation of autophagosome. Subsequently, LC3-mediated fusion with lysosomes proceeds to digest the damaged mitochondria (Narendra et al., 2008).

1.2.6.2.3. Apoptosis

Apoptosis, or programmed cell death, is generally characterized by caspase-mediated changes in cell structure, membrane, and DNA constitution (Kerr et al., 1972). These characteristics include cell shrinkage, budding of the membrane, chromatin condensation and DNA fragmentation. Apoptosis is not only critical during cell damage or stress events but is equally vital during embryonic and postembryonic development and morphogenesis, as well as in pathological and therapeutic settings (Nikoletopoulou et al., 2013). Moreover, disabled apoptosis is an event that participates in the genesis and progression of cancer (Kroemer et al., 2007). Finally, massive spikes in apoptotic events contribute to the pathophysiology of septic shock, intoxicants, infectious diseases and aging (Thompson, 1995).

There are two major signaling pathways that end in apoptotic cell death: the death receptor (DR) (extrinsic) and mitochondrial (intrinsic) pathways. The former, as the name implies, is initiated by external stimulus binding of ligands to DRs expressed on the cell surface, most commonly from a subset of the tumor necrosis factor (TNF) receptor (TNFR) family, such a Fas(CD95), TNFR1, and TNF-related apoptosis-inducing ligand (TRAIL) receptor (Green and Llambi, 2015). Cytotoxic lymphocytes, among other cell types, are known to kill transformed or infected cells by locally expressing DR ligands, like TNF-α, that leads to caspase-8 activation, making this pathway vital to immune system function and maintenance of homeostasis.

The mitochondrial pathway of apoptosis is an internal signaling cascade driven in a cell-autonomous manner that will manifest in conditions of cellular stress that exceed reparative capacity. These conditions may be caused by an increase in ROS, RNS, Ca^{2+}, mtDNA damage or UV radiation, genotoxic agents, and aggregation of unfolded proteins that result in DNA damage and endoplasmic reticulum stress (Green and Llambi, 2015). The intracellular event that will trigger the intrinsic pathway is mitochondrial outer membrane permeabilization (MOMP) that facilitates cytosolic translocation of pro-apoptotic factors, predominantly cytochrome c through the mitochondrial

permeability transition pore (MPTP). Cytochrome c release is regulated by expression of pro-apoptotic effector proteins BCL2-associated X protein (Bax) and BCL2 antagonist/killer 1 (Bak), anti-apoptotic Bcl-2 proteins, and BH3-only proteins such as BH3 interacting domain death agonist (Bid) that serve to activate the pro-apoptotic effector proteins and/or neutralize the anti-apoptotic B-cell lymphoma protein 2 (Bcl-2) and Bcl-X_L proteins (Llambi et al., 2011).

Regardless of the pathway initiated, either external DR binding or internal MPTP, the next step is facilitating caspase activation (Kroemer et al., 2007). Extrinsic DRs will recruit a caspase activating platform, known as the death-induced-signaling-complex (DISC), and intrinsic cytochrome c release will join with apoptotic peptidase activating factor 1 (Apaf-1) to induce assembly of a caspase activating complex, known as the apoptosome (Green and Llambi, 2015; Kroemer et al., 2007). The DR extrinsic pathway DISC will activate caspase 8 and the intrinsic pathway apoptosome will bind procaspase 9 and release active caspase 9, both caspases 8 & 9 will act on executioner caspases 3, 6 & 7 (Kroemer et al., 2007; Nikoletopoulou et al., 2013; Salvesen and Riedl, 2008). The intrinsic and extrinsic pathways of apoptosis were once thought to be exclusive from one another, though in recent years understanding how they converge on caspases 3, 6 & 7 activation is one of several connections elucidated. Bid is a link between the activation of both intrinsic and extrinsic pathways through its interaction with caspase 8. When cleaved by this caspase, Bid will translocate to the mitochondria and insert into the membrane where it activates BAX and BAK, initiating a signaling cascade leading to MOMP and thusly cytochrome c release (Haupt et al., 2003; Kroemer et al., 2007).

Regulation by transformation related protein 53 (p53) of genes that code for cell-surface DRs, including TNFR family proteins Fas and DR5, as well as genes that code for APAF-1 and Bcl-2 family proteins, Bax, Bak and Bid, that regulate the release of cytochrome c, shows the involvement of this transcription factor in both extrinsic and intrinsic apoptosis (Lohrum and Vousden, 1999). Regarding the intrinsic pathway, cytosolic p53 migrates to the mitochondria and promotes cytochrome c release by forming complexes with, and inhibiting, the action of anti-apoptotic Bcl-2 and Bcl-X_L (Vaseva and Moll, 2009). In the extrinsic pathway, p53 plays a key role by inducing expression of genes that code for members of the TNFR family, including Fas and DR5. Additionally, p53 not only induces expression of Fas, but will traffic Fas to the cellular membrane and facilitate rapid sensitization by increasing the amount of cell-surface DRs (Dickens et al., 2012; Green and Kroemer, 2009; Vaseva and Moll, 2009). Given these observations, p53 facilitates the convergence of DR-mediated and mitochondrial-mediated apoptosis.

1.2.6.2.4. Nrf2-antioxidant pathway

Oxidative stress is defined as a disruption of the balance between ROS production and the efficacy of endogenous antioxidant systems (Sohal and Orr, 2012). In this sense, the nuclear factor, erythroid derived 2, like 2 (Nrf2) signaling pathway is possibly the greatest cellular defense against

toxins and oxidizing agents, being referred as 'guardian of healthspan' (Lewis et al., 2010) and 'master regulator of aging' (Bruns et al., 2015). Nrf2 is a transcription factor that, under conditions of low cellular stress, is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1) binding, which constitutively targets Nrf2 for ubiquitin-mediated proteasomal degradation (Itoh et al., 1999). In the event of oxidative stress, Nrf2 is phosphorylated and released from Keap1, translocated to the nucleus where binds to the Antioxidant Response Elements (AREs) in promoter regions of genes involved in the maintenance of redox homeostasis (Hayes and Dinkova-Kostova, 2014). Among them are those that code for the heme oxygenase 1 (Hmox1), NAD(P)H quinone dehydrogenase 1 (Nqo1) and gamma-glutamate-cysteine ligase, catalytic subunit (γ-Gclc) proteins. Nrf2 influences mitochondria function by balancing reduction and oxidation processes and improving ATP production, membrane potential, fatty acid oxidation and structural integrity (Dinkova-Kostova and Abramov, 2015).

1.2.7. Cellular senescence

Cellular senescence is a stress response characterized by halted cell division and complex alterations in morphology, proteasome, secretome, and chromatin arrangement (Rodier and Campisi, 2011). Conditions that cause senescence involve genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, ROS, mitochondrial dysfunction, and other mechanisms yet to be elucidated (Childs et al., 2015, 2017). These conditions facilitate the activation of tumor suppressor genes $p16^{INK4a}$, p21, and p53 that will arrest cell cycle progression (Liu et al., 2009).

Historically, senescence was believed to be a protective mechanism against carcinogenesis by stopping proliferation of cells with damaged or unstable chromosomes (Dimri, 2005). Recently, it has been demonstrated that senescent cells can aggregate in tissues for several years by developing resistance to apoptosis, consequently interfering with tissue repair and regeneration if not cleared by the immune system (Kirkland and Tchkonia, 2017). Senescent cells manifest a senescent-associated secretory phenotype (SASP) that generate many compromising agents that include inflammatory cytokines and chemokines, matrix proteases, and growth factors (Andriani et al., 2016). Therefore, accumulation of the SASP and the resultant degeneration and dysfunction has been proposed as a primary driver of aging and age-related diseases, such as Alzheimer's disease, diabetes, pulmonary fibrosis, atherosclerosis, and osteoarthritis (Baker and Petersen, 2018; Palmer et al., 2015; Waters et al., 2018).

1.2.8. Stem cell exhaustion

Stem cells are characterized by their ability to self-renew. Stem cell exhaustion alludes to the progressive functional deterioration of tissue-specific adult stem cells that preserve homeostasis (de Haan and Lazare, 2018). The loss of the cellular regenerative potential leads to common features of

aging. The decline in hematopoietic stem cells results in immunosenescence, myeloid disorder, and anemia (Shaw et al., 2010). Mesenchymal stem cell dysfunction propagates osteoporosis and impairs fracture healing (Gruber et al., 2006). The decrease in muscle mass and strength, known as sarcopenia, is directly related to satellite cell deterioration with aging (Conboy and Rando, 2012). Intestinal epithelial stem cell depletion impairs intestinal function (López-Otín et al., 2013). Stem cell exhaustion is an integrative hallmark of aging that constitutes one of the primary malefactors of organismal aging via cell-intrinsic and –extrinsic mechanisms. There is much debate on which mechanism is most responsible. Recent reviews have postulated that there is a highly interdependent and interconnected relationship of extrinsic cellular microenvironment changes and intrinsic cellular compensatory processes (Kovtonyuk et al., 2016). Stem cell exhaustion is a complex multifaceted process whose age-related mechanisms are not fully understood. It is believed that this process encompasses several hallmarks of aging; among them are DNA damage, epigenetic changes, mitochondrial dysfunction, and senescence.

1.2.9. Altered intercellular communication

Aging implies changes in intercellular communication, including endocrine, neuroendocrine and neuronal pathways (Russell and Kahn, 2007). Renin-angiotensin system, adrenergic and insulin-IGF1 are examples of neurohormonal signals dysregulated with aging.

Aging-related immune system decline is another prominent factor that alters intercellular communications by promoting immunosenescence and inflammaging (López-Otín et al., 2013). Immunosenescence is the decline in immune system functionality as age increases. Aging occurs with an accumulation of senescent cells that continually generate pro-inflammatory cytokines, chemokines, and proteases that perpetuate a low-grade chronic inflammation, known as inflammaging, described in section 2.1 (Franceschi and Campisi, 2014). Additionally, senescent cells promote senescence in adjacent and local cells by processes that involve ROS and gap junctions-mediated cellular contacts (Nelson et al., 2012). Thus, the alterations in intercellular communication compound with aging as the derailment of mechanical and functional properties of all tissues are propagated by rampant inflammation and ineffectual immunosurveillance.

1.3. AGING AND HEART

Aging is the main risk factor for cardiovascular diseases (CVDs), including heart failure, stroke, peripheral vascular disease, and coronary heart disease (Obas and Vasan, 2018). CVDs are the leading cause of death world-wide (Global Health and Aging, 2019). The global population of individuals over the age of 65 is projected to nearly double in the next three decades, giving rise to the incidence of this disease. Though we know CVD is correlated with aging, the causation of this development remains to be understood.

1.3.1. Effect of aging on cardiac structure and function

As a heart ages, there is a steady decline in the number of cardiomyocytes and a compensatory increase in fibroblast proliferation, resulting in an accumulation of collagen that leads to fibrosis and hypertrophy of the left ventricle (LV) (Abdellatif et al., 2018). Left ventricular hypertrophy and fibrosis are hallmarks of age-induced cardiac structural remodeling by stiffening the myocardium. While this preserves ejection fraction at rest, the loss of LV contractility augments passive and active filling of the left atrium, which forces remodeling and atrial enlargement, producing another hallmark of cardiac aging: decline in diastolic function (Lakatta and Levy, 2003). Additionally, loss of sinoatrial node myocyte activity along with fibrosis-impaired electrical impulse conductivity leads to a decrease in maximal heart rate and ejection fraction, severely hampering cardiac output.

1.3.2. Molecular mechanisms of aging in heart

The heart is the first organ formed during embryonic development (Srivastava and Olson, 2000). At 22 days of development, primitive heart beats can be detected. From that moment, it performs the same essential function throughout life: transporting vital nutrients to and cellular waste from all parts of the organism. The ATP demand of the heart is the greatest in the body, consuming approximately 6 kg per day (North Brian J. and Sinclair David A., 2012). About 95% of this ATP is produced by mitochondrial OXPHOS; because of this, cardiomyocytes have higher mitochondria content than any other cell type, occupying around 40% of cytosolic volume (Schaper et al., 1985). As aging ensues mitochondria undergo structural changes such as swelling, matrix deformation, and loss of cristae; this not only produces more ROS but will consequently produce less ATP by ROS-mediated mtDNA damage to essential electron transport chain components. This constant high-energy demand, vast amounts of mitochondria, relatively low antioxidant defense, all perpetuating the ROS negative feedback loop makes the heart potently susceptible to oxidative stress as aging progresses (Woodall and Gustafsson, 2018). Particularly, an excess of ROS has shown to be involved in an impairment of intracellular contractility signaling and ventricular dysfunction, contributing to the pathogenesis of fibrosis, atherosclerosis, myocardial infarction and heart failure. This situation is aggravated by a loss of antioxidants enzymes of mitochondria cardiac tissue, among them SOD, CAT and GPx (Brown David I. and Griendling Kathy K., 2015). Oxidative stress generated in mitochondria is the main contributor to senescence in cardiomyocytes, resulting in high rates of apoptotic cell death that have been implied in the incidence of acute and chronic heart failure. The decline in cardiomyocytes reduces cardiac stem cells reserves, whose regenerative activity falls from 1 to 0.4% at the age of 20 and 75, respectively (Bergmann et al., 2009). Mitochondria function is severely hampered as a result of impaired signaling pathways that regulate mitochondria dynamics, autophagy and Nrf2. Altered mitochondria dynamics have been associated with cardiac hypertrophy and heart failure (Chen et al., 2011). The age-dependent deterioration in autophagy/mitophagy is implicated in cardiomyopathy

characterized by defective mitochondria, contractile dysfunction, higher fibrosis, cardiomyocyte apoptosis, hypertrophy and heart failure (Nakai et al., 2007). As the heart ages, Nrf2 antioxidant pathway is downregulated, correlating with age-related cardiomyopathy. Since Nrf2 has proved to confer cardioprotection in several disease models, this transcription factor is a promising target for therapeutic intervention in the anti-aging stratagem (Fernández-Ortiz et al., 2020; Rahim et al., 2021). As progressive loss of mitochondrial function occurs, ROS production increases with mutations in mtDNA (Acuña Castroviejo et al., 2011). The accumulation of mtDNA deletions and mutations in cardiac aging is depicted in the hearts of mice that have a deficient version of mtDNA polymerase γ proofreader. These mice exhibited early onset fibrosis, impaired diastolic and systolic function, and cardiac enlargement (Trifunovic et al., 2004). Moreover, mtDNA released from damaged mitochondria culminate in myocarditis and dilated cardiomyopathy by activating NLR family pyrin domain containing 3 (NLRP3) inflammasome and therefore propagating an age-related chronic low grade inflammatory environment, known as inflammaging (Fernández-Ortiz et al., 2020).

2. IMMUNE SYSTEM AND INFLAMMATORY RESPONSE DURING AGING

Taking into account the sensor and effector mechanisms used, as well as the speed and specificity of the triggered reaction, the immune response of our body has been classified as innate and adaptive or acquired (Medzhitov and Janeway, 1997). The innate immune response is the host's first line of defense against invasion. It is made up of physical, chemical and microbiological barriers, as well as elements of the immune system such as neutrophils, monocytes, macrophages, dendritic cells, eosinophils or cytokines. Recognition of the foreign particle is carried out by a limited number of germline encoded receptors that elicit an immediate, limited, and nonspecific response (Moreno and Sánchez-Ibarrola, 2003). The adaptive or acquired immune response is characterized by generating specific reactions against antigens in which a high number of receptors generated by gene rearrangement participate and are expressed on the surface of B and T lymphocytes. This type of immunity trades target specificity for a slower response time than that of innate immunity. Although, memory mechanisms make it possible to respond more quickly and powerfully when a foreign particle is reencountered (Flajnik and Kasahara, 2010).

At the beginning of the sixth decade of life, the human immune system begins an age-related functional decline that manifests a maladaptive state known as immunosenescence (Weyand and Goronzy, 2016). This dysregulation of the immune response translates into greater susceptibility to infectious processes, cancer, and a reduction in the response to vaccination in older populations.

Additionally, the inflammatory response mediated by the innate immune system gains intensity, contributing to a chronic inflammatory process known as inflammaging (Franceschi et al., 2000).

2.1. INFLAMMAGING

Inflammation is the local response of an organism's immune system to damage inflicted on vascularized cells and tissues by bacterial pathogens or any other aggressor of a physical, chemical, biological or mechanical nature (Nathan, 2002). The inflammatory cascade is initiated by innate immune cells that detect and signal the presence of damage to trigger classical signs of inflammation (swelling, redness, heat and pain). Subsequently, the adaptive system is activated to enhance these effects to neutralize the offending agent. In this way, inflammation is of enormous physiological value. So long as it is triggered in a rapid, coordinated and controlled way to mitigate damage while allowing for the elimination of the aggressor, resolution and repair of the damaged tissue, and recovery of organismal homeostasis. However, if the inflammatory response is not adequately controlled, not only does it fail in its purpose of eliminating the noxious stimulus, but it also persists in the body causing greater damage than the stimulus itself that originally initiated it (Drayton et al., 2006). An example of tissue damage associated with a perpetual inflammatory response is that which appears during aging (Figure 6).

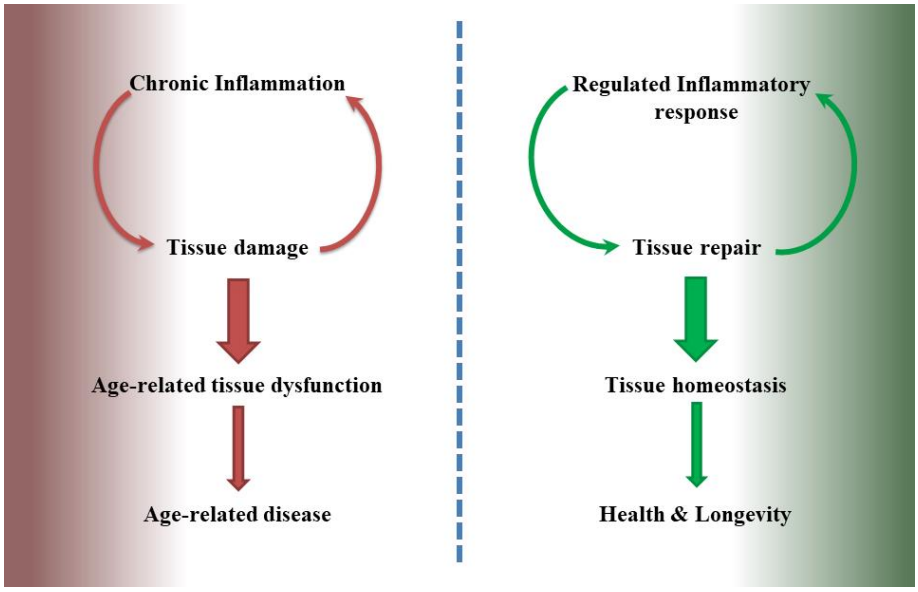


Figure 6. Inflammation response in young and old organisms. *In young healthy organisms (right, green) a regulated inflammatory response promotes tissue homeostasis and longevity. During aging (left, red), chronic inflammation and tissue damage drive the dysfunctional state that leads to age-related diseases.*

The aging process is accompanied by a state of subclinical, asymptomatic, chronic and systemic inflammation, a phenomenon known as 'inflammaging' (Franceschi et al., 2000). This chronic-low grade inflammation is characterized by a 2 to 4-fold increase in serum levels of pro-inflammatory mediators, including C-reactive protein (CRP), TNF-α or interleukin (IL)-6, in the older

population when compared to younger adults (Krabbe et al., 2004). These inflammatory mediators are strong predictors of all-cause mortality risk. Etiology of age-related inflammation remains to be elucidated and the mechanisms proposed so far are diverse and mutually non-exclusive. Among them, the accumulation of senescent cells and their acquisition of the SASP stand out (Franceschi and Campisi, 2014). Current studies point to changes in the composition of the gut microbiota with age as a source of inflammatory activation (Buford, 2017). Metabolic dysfunction as seen in obesity leads to inflammaging (Gregor and Hotamisligil, 2011). While adiponectin found in lean states favors the production of anti-inflammatory cytokines, increased leptin during obesity promotes the production of IL-6, IL-12 and TNF-α. Increased adipose tissue with age is known to be an underlying cause of higher levels of inflammatory cytokines in obesity. Recently, it has been suggested that innate immune cells have a memory driven by epigenetic changes that allow for enhanced responsiveness when pathogens are reencountered. This "trained immunity" has been put forward as a contributing factor to inflammaging (Franceschi et al., 2017). The sustained exposure to infectious agents throughout life, especially by virus such as cytomegalovirus (CMV), promotes a chronic stimulation of the immune system that induces a pro-inflammatory response. This situation is aggravated by the inability of the adaptive immune system to contain viral infections and eliminate exogenous antigens with aging, thereby extending the duration of the immune response and its adverse consequences (Deeks, 2011). Along with chronic antigenic overload, the presence of macromolecules and damaged organelles that accumulate in old organisms is a major stimulus activating inflammatory signaling during aging. The cell debris comes from dead and deteriorating cells and is produced at a higher rate in aged tissues, because the mechanisms responsible for its elimination, including autophagy and apoptosis, are altered with aging. The result of all these processes is induction of the innate immune response through Nuclear Factor Kappa B (NF-κB) pathway and NLRP3 activation (Youm et al., 2013). Thus, a better understanding of these pathways would help decipher the etiology of inflammaging and facilitate the identification of new therapeutic targets

2.2. INNATE IMMUNE RESPONSE: EFFECT OF AGING

The innate immune system recognizes highly conserved antigenic structures in various microorganisms that are called Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs are recognized by receptors of the innate immune system known as Pattern Recognition Receptors (PRRs). Structurally, all PRRs have a specific domain for the recognition of PAMPs and a protein-protein interaction region essential to initiate cell signaling processes (Medzhitov, 2007). PRRs can be classified according to their functional differences:

Secreted PRRs: They are present in body fluids and in the extracellular space. Given their location, these PRRs are in charge of binding to the cell surface of microorganisms, activating the complement system, initiating opsonization of pathogens, and participating in the transfer of PAMPs

to other receptors involved in the innate immune response (Lee and Kim, 2007). Examples of secreted PRRs are complement receptors, CRP, serum amyloid P component protein, and pentraxin-related protein PTX3.

Transmembrane PRRs: They are expressed on the cell membrane or on lysosomes / endosomes. They carry out immune recognition on uninfected cells to trigger an effector response (Iwasaki and Medzhitov, 2010). Toll-like receptors (TLRs) and C-type Lectin receptors stand out as transmembrane PRRs. TLRs have been shown to be involved in the inflammaging process, distinguishing in turn between a) membranous TLRs (TLR1, 2, 4, 5 and 6), responsible for the activation of the NF-κB pathway ; and b) Intracellular TLRs, responsible for detecting nucleic acids of viruses and bacteria that induce the activation of Interferon Regulatory Factor (IRF) (Uematsu and Akira, 2007).

Cytosolic PRRs: They act as intracellular sensors detecting the presence of viruses, microbial products, substances related to cellular stress or crystalline particles of non-infectious origin. Cytosolic PRRs mediate the immune response once the cell is infected and cooperate with transmembrane PRRs to give continuity to intracellular signaling associated with the presence of a foreign element (Pichlmair and Reis e Sousa, 2007). These PRRs include the retinoic acid-inducible gene I (RIG-I) - like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs). NLRs cooperate with transmembrane TLRs to activate the innate immune response and apoptosis involving the formation of signaling complexes, such as inflammasomes and Nucleotide-binding Oligomerization Domain (NOD) signalosomes (Martinon et al., 2002).

It should be noted that the immune response to a certain pathogen is not limited only to its recognition by a single PRR, but also involves complex cooperation between different receptors, immune cells and mediators. Furthermore, the same pathogen presents multiple PAMPs that will be recognized by different types of PRR. Therefore, the connection established between the different PRRs shows a quantitative effect (the synergistic responses between PRRs allow an efficient response to low concentrations of PAMP) and a qualitative effect (the activation of a PRR by its PAMP activates other related PRRs). In this way, the host increases its ability to detect any pathogen and respond efficiently (Ishii et al., 2008).

However, as already mentioned, a disproportionate inflammatory response can cause cell death and tissue damage in the host, releasing noxious cellular components into the extracellular environment. These components or danger signals are called Damage-Associated Molecular Patterns (DAMPs) and include cellular proteins like High mobility group protein B1 (HMGB1), IL-1 and Histone deacetylase dependent SIN3A (SAP130) and related proteins, as well as nucleic acids DNA, ATP or uric acid. These DAMPs are recognized by some PRRs mentioned above, which triggers a

greater inflammatory response to the foreign agent and a failure in the regulation of inflammatory pathways (Jounai et al., 2012).

The mechanisms underlying the basal inflammatory process associated with aging undergo quantitative and qualitative changes in innate immunity cells and alterations in the expression and signal cascades initiated by PRR activation. This ultimately leads to uncontrolled secretion of pro-inflammatory mediators. It has been observed that there is an age-related decrease in the expression of TLRs in innate immune cells (Shaw et al., 2013)). One of the first studies evaluating the function of TLRs in aged C57BL/6 mice showed a general decrease in gene expression of the receptors TLR1-TLR9, decreased protein content of TLR4, and an increase in production of TNF-α and IL-6 dependent on TLR in peritoneal and splenic macrophages (Renshaw et al., 2002). These data provide clear evidence of TLR functional deterioration and the consequent increase in inflammatory response brought about by increased production of inflammatory cytokines during aging.

On the other hand, very little is known about the effects that age has on the functioning of cytosolic PRR NLRs. It was not until 2013 that the involvement of the NLRP3 inflammasome in the systemic low-grade age-related "sterile" inflammation in adipose tissue and brain was demonstrated for the first time. The absence of this protein protected the mice from age-associated increases in innate immune activation, in addition to improving glucose tolerance, motor performance, cognitive function, as well as attenuate bone loss. While the connection between inflammaging and NLRP3 activation has been widely reported, some of the molecular mechanisms underlying this process remain unclear (Volt et al., 2016).

Together, all these findings relate the dysregulation of innate immunity activation and the perpetuation of pro-inflammatory processes that occurs with age (Figure 7).

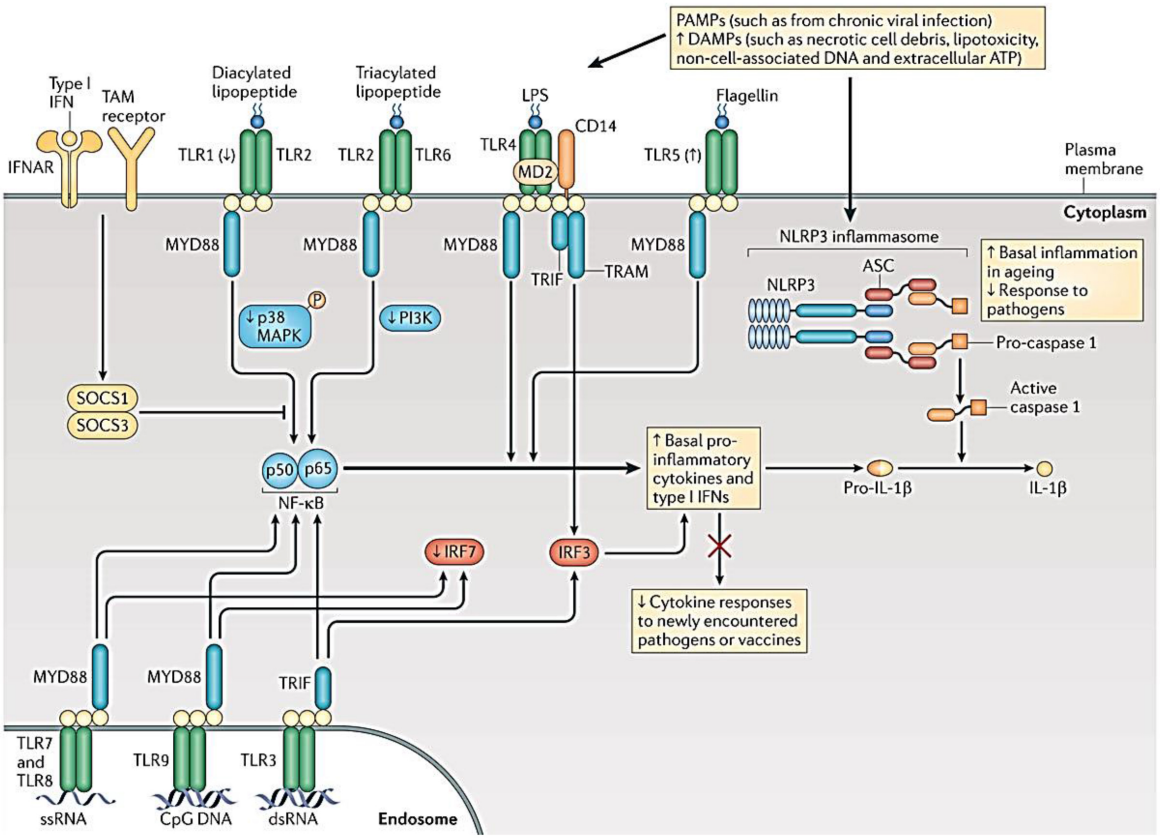


Figure 7. Effects of aging on PRR-dependent innate immunity signaling. *Representation of signaling pathways mediated by TLR and NLRP3 during aging, which occurs with high levels of PAMPs and DAMPs that contribute to an elevated pro-inflammatory state. dsRNA, double-stranded RNA; IFN, interferon; IFNAR, IFNα/β receptor; IL-1β, interleukin-1β; IRF, IFN-regulatory factor; LPS, lipopolysaccharide; MD2, myeloid differentiation factor 2; MYD88, myeloid differentiation primary-response protein 88; NF-κB, nuclear factor-κB; SOCS, suppressor of cytokine signalling; ssRNA, single-stranded RNA; TAM, TYRO3, AXL and MER; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing IFNβ. From Shaw et al., 2013.*

2.2.1. NF-κB as a regulator of immune and inflammatory response. Effect of NF-κB during aging

Despite the enormous variety of stimuli to which the immune system responds, the complicated task of communicating the presence of lesions or infections in the body is carried out by a relatively low number of signaling pathways, which ultimately depends on a common transcriptional activator known as NF-κB.

In mammals, the NF-κB family of transcription factors consists of five members identified as p65 (RelA), RelB, c-Rel, p50 (NF-κB 1 and its precursor p105) and p52 (NF-κB 2 and its precursor p100). These proteins possess a Rel-homology domain (RHD) that allows DNA binding, dimerization and nuclear translocation, whereas only p65, RelB and c-Rel have a transcriptional activation domain (TAD) for gene activation. NF-κB members can form any combination of homo- or heterodimers, each with different transcriptional ability. However, not every possible dimer combination could be demonstrated to occur *in vivo*. For example, p65/p50 heterodimers, which constitute the majority form,

strongly promote gene transcription, while p50/p50 homodimers repress gene transcription (Christian et al., 2016; Liu et al., 2017).

Members of the NF-κB family are activated by a wide variety of stimuli, including pro-inflammatory cytokines such as TNF-α or IL-1β, B- or T-cell mitogens, bacteria and bacterial products such as LPS, viruses, proteins, double-stranded RNA, or even physical or chemical stress situations (Karin and Ben-Neriah, 2000). The recognition of these ligands by their specific receptors leads to the activation of NF-κB through two possible pathways: the canonical pathway and the non-canonical pathway (Figure 8). In the canonical pathway, the nuclear translocation of the p50/p65 heterodimers occurs following phosphorylation mediated degradation of the inhibitor of kappaB (IκB). This pathway is mainly activated in response to bacterial products and pro-inflammatory signals, playing a key role in the pathogenesis of inflammatory diseases and exacerbation of the innate immune response. The non-canonical pathway is activated primarily by cytokines of the TNF-α family, although not by TNF-α itself. In it, the IKKα-dependent phosphorylation of p100:RelB leads to the processing of p100 and the consequent generation of the heterodimer p52:RelB that enters the nucleus and activates the transcription of its target genes (Oeckinghaus et al., 2011). The non-canonical pathway has been shown to play a major role in the adaptative immune responses, lymphoid organogenesis and B cell maturation (Sehnert et al., 2020).

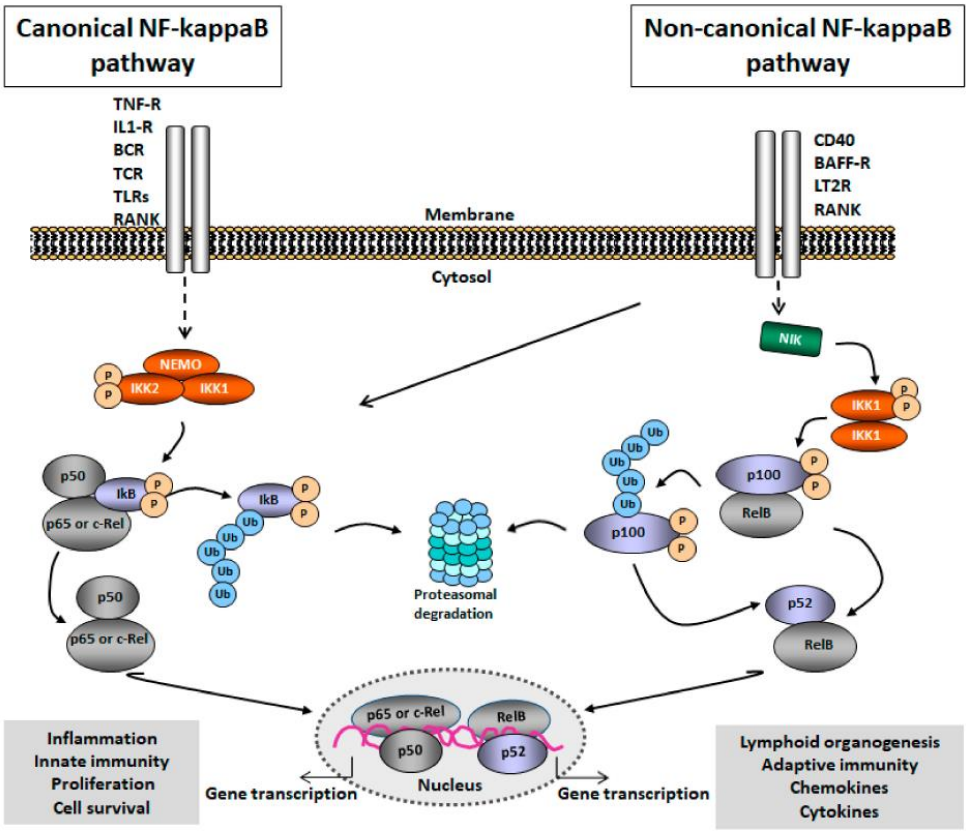


Figure 8. NF-κB signaling pathway. *The canonical pathway (left) is activated through different receptors including TNFR, IL-1 receptor (IL-1R), B-cell receptor (BCR), T-cell receptor (TCR), Toll-like receptor, RANK,*

and CD40. The non-canonical pathway (right) is activated through CD40, B-cell-activating factor receptor (BAFF-R), lymphotoxin beta receptor (LT2R) and RANK. From Sehnert et al., 2020.

An aberrant NF-κB expression contributes to the pathogenesis of chronic immune-mediated diseases including rheumatoid arthritis, multiple sclerosis, asthma and others (Gregersen et al., 2009; Makarov, 2001; Mishra et al., 2018). Numerous studies support a causal relationship between inflammaging and a deregulated NF-κB activation. The first of these took place more than two decades ago, when Helenius et al. evaluated the DNA-binding capacity of a series of transcription factors (NF-κB, AP-1 and Sp-1) in nuclear extracts from various tissues from young and old rodents. The results showed a significant increase in the DNA binding of the NF-κB complexes in all the tissues of the old animals, while the DNA-binding capacity of AP-1 and Sp-1 did not change with age. This finding demonstrated the increase in NF-κB activation with aging, unlike other transcription factors related to activation of the immune system. Recently, mouse model with gain of NF-κB function showed a shorter lifespan and signs of accelerated aging at middle age that included alopecia, osteoporosis, central nervous system alterations, enhanced cellular senescence and reduced regenerative capacity (Bernal et al., 2014). This study exhibits a causal relationship between inflammation linked to NF-κB overexpression and the deregulation of immune responses that leads to development of age-related phenotypes.

To date, no gold standard for the treatment of chronic inflammation during aging has been identified. In this sense, NF-κB is a very attractive therapeutic target due to its importance of pathogenic inflammatory pathways. However, NF-κB is known for its crucial role during immune responses, cell growth, development and survival (Hayden and Ghosh, 2008), suggesting that a systemic NF-κB inhibitor may cause serious adverse effects associated with severe immunosuppression and loss of homeostasis. Pioneering approaches must be developed to dampen NF-κB activity in a cell-type specific manner. Another very promising target for counteracting the effects of inflammaging and currently enjoying great scientific interest is the inhibition of another essential component of the innate immune system: the NLRP3 inflammasome.

2.2.2. NLRP3 as a regulator of immune and inflammatory response. Effect of NLRP3 during aging

Inflammasomes are defined as high molecular weight multiprotein complexes that regulate the activation of inflammatory caspases and, consequently, the maturation of their respective substrates (Martinon et al., 2002). These caspases are produced in the form of catalytically inactive proenzymes that, once the signal is detected, integrate and dimerize in the inflammasome, which triggers their activation mediated by proteolytic processing and subsequent maturation of the pro-inflammatory cytokines IL-1β, IL-18 and IL-33 (Martinon et al., 2009).

Among the different types of inflammasome, NLRP3 is the best characterized and studied one because of its implication in different autoinflammatory diseases, among them cold-induced familial autoinflammatory syndrome or Muckle-Wells syndrome. These pathologies are included within what are known as cryopyrin-associates periodic syndromes (CAPS) and are characterized by recurrent episodes of fever and systemic inflammation of the tissues. The cause of these diseases is due to mutations in the *Nlrp3* gene characterized by a disproportionate activation of the inflammasome, resulting in excessive release of pro-inflammatory cytokines, mainly IL-1β (Neven et al., 2008). In addition, it should be noted that, unlike other NLRs, the NLRP3 inflammasome can be activated in response to DAMPs, turning it into the most important sensor of endogenous stress signal (Feldman et al., 2015).

Structurally, the NLRP3 inflammasome is made up of the NLRP3 receptor, the Apoptosis-associated Speck-like protein containing a caspase recruitment domain (ASC) adapter protein and the cysteine protease caspase-1 (Agostini et al., 2004). The NLRP3 receptor consists of three domains (Figure 9): an N-terminal domain called Pyrin Domain (PYD), a NACHT-NAD (NACHT, Nucleotide-binding oligomerization domain, leucine-rich-repeat family, Apoptosis inhibitory protein, Class II, major histocompatibility complex transactivator, Het-E incompatibility locus protein from Podospora anserina, Telomerase-associated protein 1; NAD, NACHT-associated domain) core domain and a C-terminal leucine-rich-repeat (LRR) domain. In an inactive state, NLRP3 is constitutively expressed in the cytosol given the internal interaction that is established between the domains NACHT-NAD and the LRR of the receptor. In the presence of activating signals, the NLRP3 protein undergoes conformational modifications that expose the central domain NACHT-NAD, which allows the association of the adapter protein ASC through PYD-like interactions, and the interaction with pro-caspase-1 through interactions of type Caspase Activation and Recruitment Domain (CARD). As a consequence of these conformational modifications and interactions, the proteolytic activity of caspase-1 is induced and the subsequent processing of the pro-inflammatory cytokines pro-IL1β, pro-IL-18 and pro-IL-33 to their active forms IL- 1β, IL-18 and IL-33, respectively (Dunne, 2011).

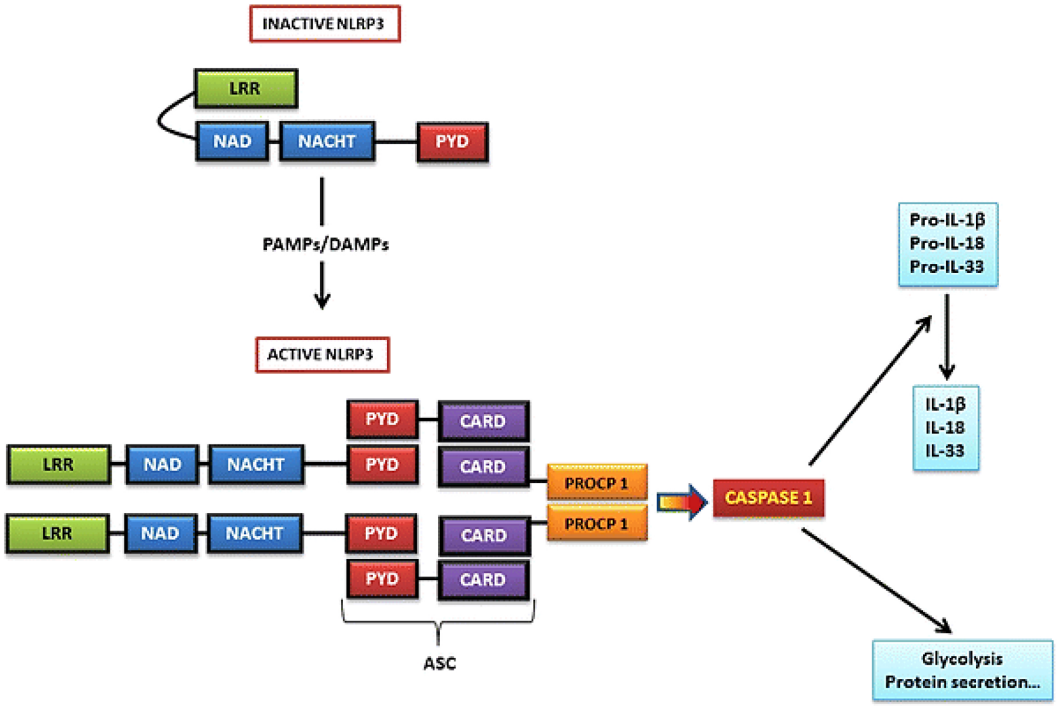


Figure 9. Schematic representation of the NLRP3 components and their assembly during the inflammasome activation. *In its inactive form, NLRP3 is constitutively expressed in the cytoplasm (upper). The recognition of a series of PAMPs/DAMPs induces conformational changes in NLRP3, recruiting ASC and pro-caspase-1 to the inflammasome (lower). Consequently, caspase-1 is activated facilitating the maturation of pro-inflammatory cytokines to their active forms. From Escames et al., 2012.*

NLRP3 inflammasome activation occurs in response to a wide variety of stimuli, including bacterial products (muramyl dipeptide, LPS, or bacterial RNA), bacterial toxins (nigericin, listeriolysin O, gramicidin), Gram-negative pathogenic bacteria, and Gram-positive, viruses and viral products, fungal pathogens or signs related to the existence of damage in the body (silica or cholesterol crystals, aluminum hydroxide, monosodium urate, β-amyloid plaque, extracellular ATP, cytoplasmic DNA or ROS) (Bauernfeind et al., 2011). Various mechanisms have been suggested that trigger the activation of the NLRP3 inflammasome, although none of them alone can explain the response to the enormous diversity of stimuli mentioned above (Figure 10).

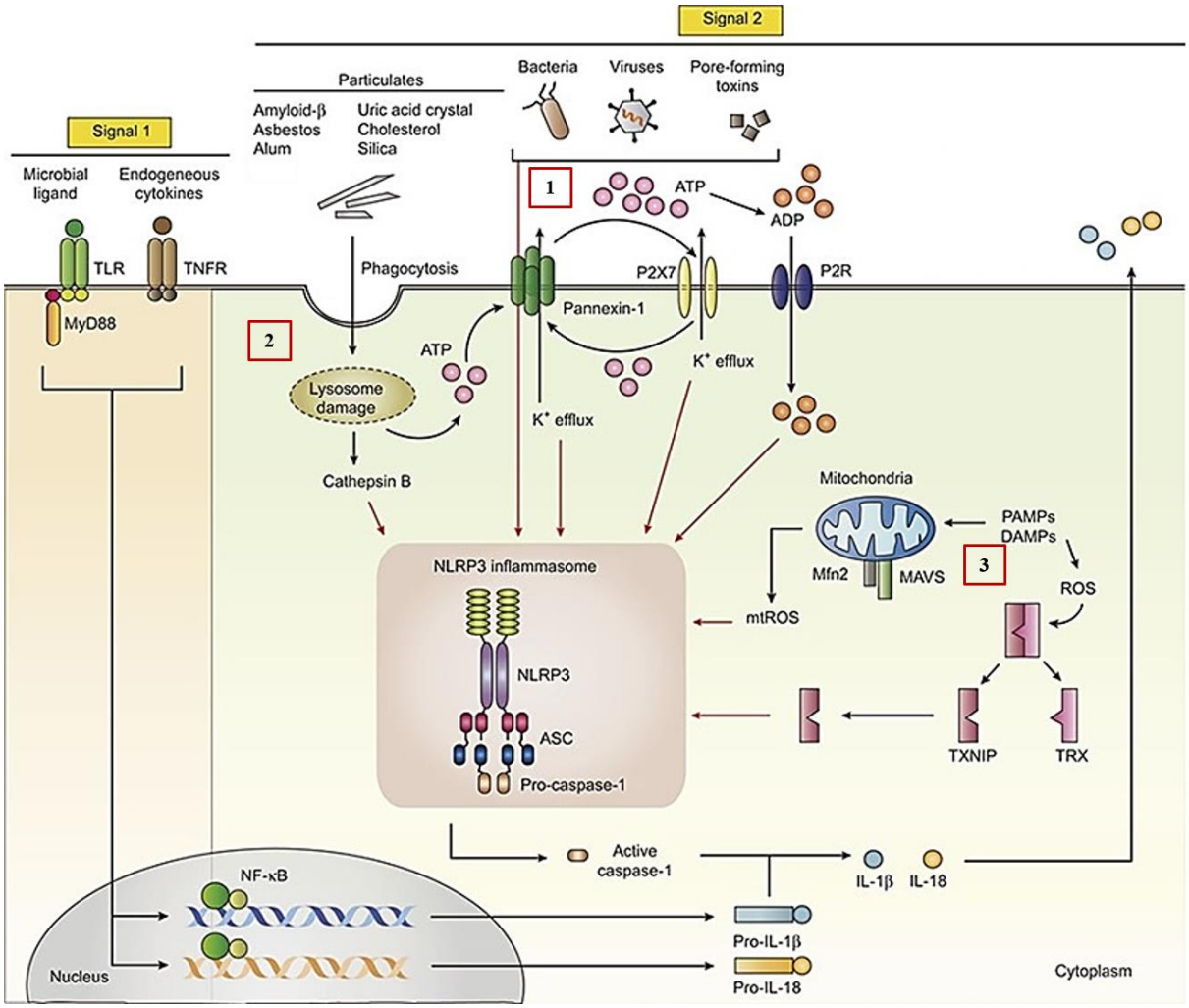


Figure 10. Models involved in the activation of the NLRP3 inflammasome. *Both signal 1 and 2 are required for NLRP3 inflammasome activation. Signal 1 activates the NF-κB pathway and increases NLRP3 levels. Signal 2 promotes triggering of the NLRP3 inflammasome complex mediated by potassium output (1), lysosomal content release (2) and ROS generation (3). Adapted from Jo et al., 2016.*

The first signal in inflammasome activation involves the priming signal, which is mediated by ligands recognized by TLRs and induces the NF-κB pathway to upregulate the expression of pro-IL-1β and NLRP3. The second signal is mediated by numerous PAMPs and DAMPs, named above, and promotes the assembly of ASC and pro-caspase 1. Several molecular pathways have been proposed for NLRP3 activation to induce caspase-1-activation and IL-1β maturation:

- One model suggests that the activation of the inflammasome occurs in response to the rapid output of potassium caused by each and every one of the above ligands (Lamkanfi et al., 2009). This model is supported not only by the fact that sub-physiological amounts of potassium induce spontaneous activation of the inflammasome, which suggests that it can detect small decreases in intracellular potassium levels, but also due to the profound inhibition of the NLRP3 inflammasome that is observed in response to the increase in potassium concentrations at the extracellular level, preventing potassium efflux form the cell (Pétrilli et

al., 2007). Potassium efflux occurs through pores formed by the action of certain bacterial toxins that alter cell integrity, or through P2X7 purinergic receptors in response to extracellular ATP (Mariathasan et al., 2006). The presence of ATP in this location is associated with cell damage, necrosis, as well as mechanical stimuli on certain cell types, mainly endothelial and epithelial cells (Martinon et al., 2009). Once out of the cell, ATP induces activation of P2X7 receptors, an ion channel regulated by ATP that acts in conjunction with the hemichannels of panexin-1, which can act as channels specific for ATP release. Consequently, not only is a pore produced in the cell membrane that facilitates the cytoplasmic entry of NLRP3, but the release of ATP mediated by panexin-1 amplifies the activation of the P2X7 receptor-dependent inflammasome.

- Another proposed mechanism for the activation of the NLRP3 inflammasome is based on the destabilization of the lysosome after phagocytosis of crystalline particles or structures, including particles of silica, cholesterol or β-amyloid. As a consequence of this lysosomal disintegration, its content is released into the cytoplasm, an event that is perceived as a sign of endogenous damage by the immune system (Dostert et al., 2008). In this model, the release to the cytosol of cathepsin B, a proteolytic enzyme responsible for catalyzing the hydrolysis of proteins to polypeptides, will play a key role in the production of IL-1β in response to a variety of mediators dependent on processes of phagocytosis. This fact is evidenced by the suppression exerted by the inhibitor of the biological activity of cathepsin B, Ca-074-me, on the activation of the inflammasome (Hornung et al., 2008).

- Recently, it has been observed that all known NLRP3 inflammasome activators induce ROS production, suggesting the use of antioxidants could effectively inhibit inflammasome activation (García et al., 2015). In this activation model, the thioredoxin-interacting protein (TXNIP) has an essential role in associating ROS production with inflammasome activation (Zhou et al., 2011). Under basal conditions, TXNIP is associated with thioredoxin oxidoreductase (TRX), a complex that dissociates when intracellular ROS levels increase. This dissociation is double-edged: while free thioredoxin can play its role as a ROS scavenger, TXNIP associates with NLRP3 in a ROS-dependent manner, ultimately inducing its activation. Although this causative effect of ROS has been known for a long time, it was not until relatively recently that the importance of mitochondria in modulating innate immunity through direct activation of the NLRP3 inflammasome was brought to light. In this sense, Zhou et al. observed that ROS production associated with mitochondrial dysfunction directly activates the NLRP3 inflammasome while Nakahira et al. demonstrated that mitophagy constitutes an important inhibitory mechanism of NLRP3 inflammasome (Nakahira et al., 2011). Likewise, the association between NLRP3 and mitochondria is sustained by:

- Subcellular location of the mitochondria during inflammatory processes. While the inactive NLRP3 and ASC proteins associate with the endoplasmic reticulum, the activation of the inflammasome induces its perinuclear distribution in conjunction with the mitochondria (Zhou et al., 2011).
- Mitochondria ROS production facilitates opening of the MPTP, consequently releasing mtDNA which is detected as a signal of damage and index the activation of the NLRP3 inflammasome and the secretion of IL-1β and IL-18 (Nakahira et al., 2011; Zhou et al., 2011). Interestingly, the magnitude of caspase-1 activation is directly related to the amount of mtDNA in the cytosol, suggesting its role as a caspase-1 coactivator.

Exposure throughout our lives to different antigens and stressful stimuli causes a state of chronic oxidative stress in our body (Baylis et al., 2013). This situation allows for mitochondrial ROS release, causing greater oxidative damage in the biomolecules of senescent cells, and strongly contributes to age-related cellular deterioration. The oxidative process that accompanies aging leads to the release and accumulation of DAMPs that initiate an inflammatory process through PRRs, such as TLRs and NLRs, triggering the activation of the NLRP3 inflammasome, an increase in IL-1β levels and a perpetual inflammatory response (Feldman et al., 2015). The aberrant activity of the NLRP3 inflammasome has been implied in a multitude of age-related pathologies, such as neurodegenerative diseases, obesity, diabetes, osteoarthritis and CVDs (Sebastian-Valverde and Pasinetti, 2020). All these data suggest that targeting NLRP3inflammasome may be potentially beneficial to reestablishing of immune competence and homeostasis state in the elderly.

2.3. INFLAMMAGING AS A CAUSE OF CARDIOVASCULAR DISEASES

Epidemiological studies reveal that CVDs emerge ensuing a chronic inflammatory state (Welsh et al., 2017). The increase in inflammatory markers with aging is considered CV risk factor (Scuteri et al., 2011; Ungvari et al., 2004). Thus, the increase in the pro-inflammatory cytokine IL-6 has been implicated in age-associated vascular disease. CRP was linked to increased arterial stiffness in middle and old-age subjects. Likewise, TNF-α has been found to be upregulated in coronary arteries, leading to endothelial dysfunction and proatherogenic inflammatory mediators. Ischemic cardiac injury is often caused by obstruction of arterial blood flow through the coronary artery by atherosclerotic lesions.

Atherosclerosis is defined as a chronic inflammatory disorder of the medium and large-size arteries distinguished by an endothelial accumulation of immune cells, extracellular matrix (ECM), cholesterol and lipids (Miteva et al., 2018). This cardiovascular event constitutes the main cause of CVD and NLRP3 inflammasome plays an important role in its etiology (Figure 11).

The high levels of ROS observed in aged individuals that come from the oxidative stress and mitochondrial dysfunction induce the oxidation of LDL. Oxidized LDL and cholesterol crystal act as DAMPs that initiate NLRP3 activation, which leads to the maturation of IL-1β. At the same time, IL-1β enhances the inflammatory reaction by increasing the expression of IL-6, IL-8, TNF-α, vascular cell adhesion molecule (VCAM)-1, and monocyte chemo-attractant protein-1 (MCP-1). These events facilitate the infiltration of neutrophils and monocytes. In addition, IL-1β promotes proliferation and migration of vascular smooth muscle cells (VSMCs), enables the development of foam cells, and boosts the metalloproteinase (MPP) expression and successive collagen degradation that promotes plaque instability. This situation is exacerbated by the release of more pro-inflammatory cytokines and more ROS, which further provoke LDL oxidation and aggravate atherosclerosis.

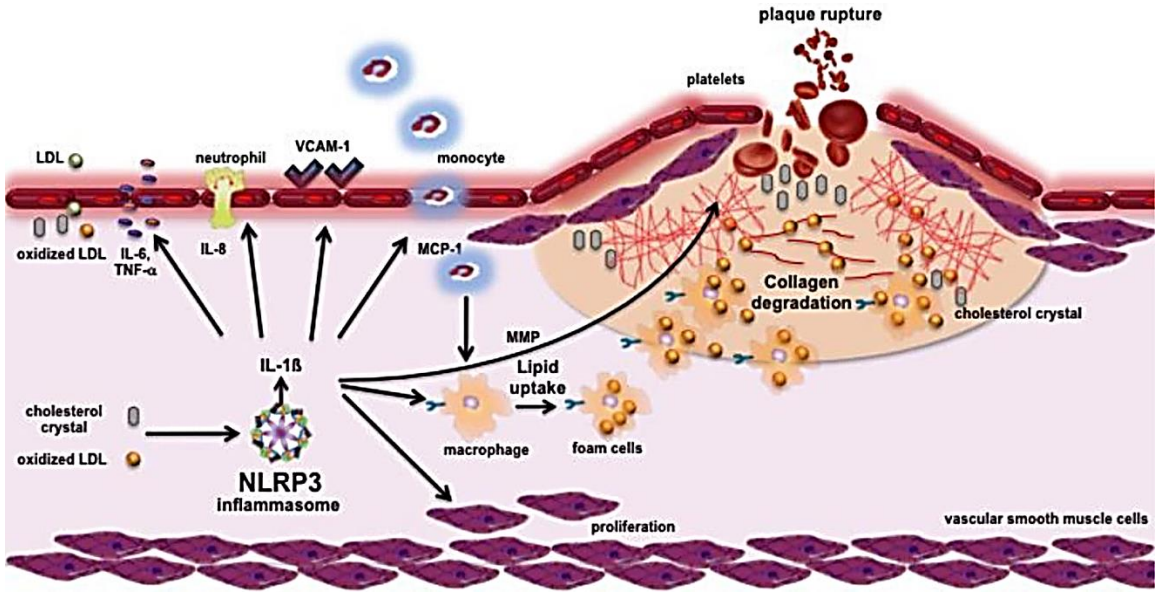


Figure 11. Role of the NLRP3 inflammasome in atherosclerosis. *Aging contributes to the development of atherosclerosis, a chronic inflammatory arterial disease characterized by the deposition of lipids and cholesterol crystals that act as DAMPs by triggering NLRP3 inflammatory response. From Miteva et al., 2018.*

Considering what is mentioned in this section, we can conclude that the innate immune system and especially NLRP3 inflammasome has an essential role in the maintenance of an age-associated chronic inflammatory state, because of its capability to sense many of the aging-related danger signals, thus orchestrating an immune response that promotes inflammation. Consequently, the NLRP3 inflammasome appears to be a tempting therapeutic target for the treatment of not only the cardiovascular events described here but also for the aging process.

3. CYRCADIAN RHYTHMS AND CLOCK GENES

The concept of circadian rhythms was defined by Halberg et al. in 1959 to detail the association between the Earth's daily rotation and the endogenous oscillation of many physiological factors (Halberg, 1959). In this way, circadian rhythms are regular cycles in a host of biochemical, functional and behavioral parameters that display periods of near 24 h (Dibner et al., 2010). Biologically, circadian rhythms allow organism to adapt and anticipate environmental changes and ensure optimal physiological performance. Sleep-wake cycles, food intake, body temperature, secretion of enzymes and hormones, neurological activity and glucose homeostasis are some of the myriads of biological processes that follow circadian rhythms. In mammals, circadian rhythms are regulated by one central or master clock and peripheral clocks.

The master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus and it consists of around 20.000 neurons in rodents and 50.000 in humans (Hastings et al., 2018). Photoperiod is the main *Zeitgeber* (from the German *time giver*) for the central clock, which is synchronized by the environmental lighting conditions to ensure that the organism is in phase with the environment. In vertebrates, circadian photoreception occurs via intrinsically photosensitive retinal ganglion cells (ipRGCs) due to the presence of melanopsin pigment, that is directly activated by blue light with a wavelength of 440 to 480 nm. These cells also integrate light signals from visual photoreceptors (rods and cones) to adjust the SCN to the external day-night cycle. The SCN then projects to a wide range of brain areas involved in the regulation of metabolic pathways; among them are the paraventricular nucleus (PVN) of the hypothalamus, sub-paraventricular zone (sPVZ), dorsomedial hypothalamus (DMH) and arcuate nucleus (ARC) (Brown et al., 2010). Through this retino-hypothalamic pathway, SCN conveys phase information to peripheral pacemakers, helping preserve homeostasis of the organism by maintaining internal synchrony. These peripheral clocks are found in almost all mammalian tissues including the retina, heart, kidneys, liver, lungs, ovaries, testis, gut, spleen, muscle, and immune cells, among others (Acuña-Castroviejo et al., 2017). Feeding time, physical exercise and availability of metabolites are the *Zeitgeber* for the peripheral clocks that synchronize their circadian rhythms (Schibler et al., 2015). Other than orchestrating metabolic pathways, the SNC regulates synthesis and release of pineal melatonin depending of the level of photoreception. Specifically, during night, PVN neuronal activity promotes melatonin secretion, that peaks between 2 am and 4 am, and is rapidly released into the blood and cerebrospinal fluid reaching all cells of the organism (Reiter, 1993). Light increases SCN electrical activity via ipRGCs photo-stimulation, which inhibits PVN neurons and, thus, melatonin secretion during the day. These qualities of melatonin characterize it as an endogenous synchronizer of circadian rhythms because this pineal hormone transmits the timekeeping signals from SCN and coordinates each cell of the body with a period of 24 hours (Pfeffer et al., 2018) (Figure 12).

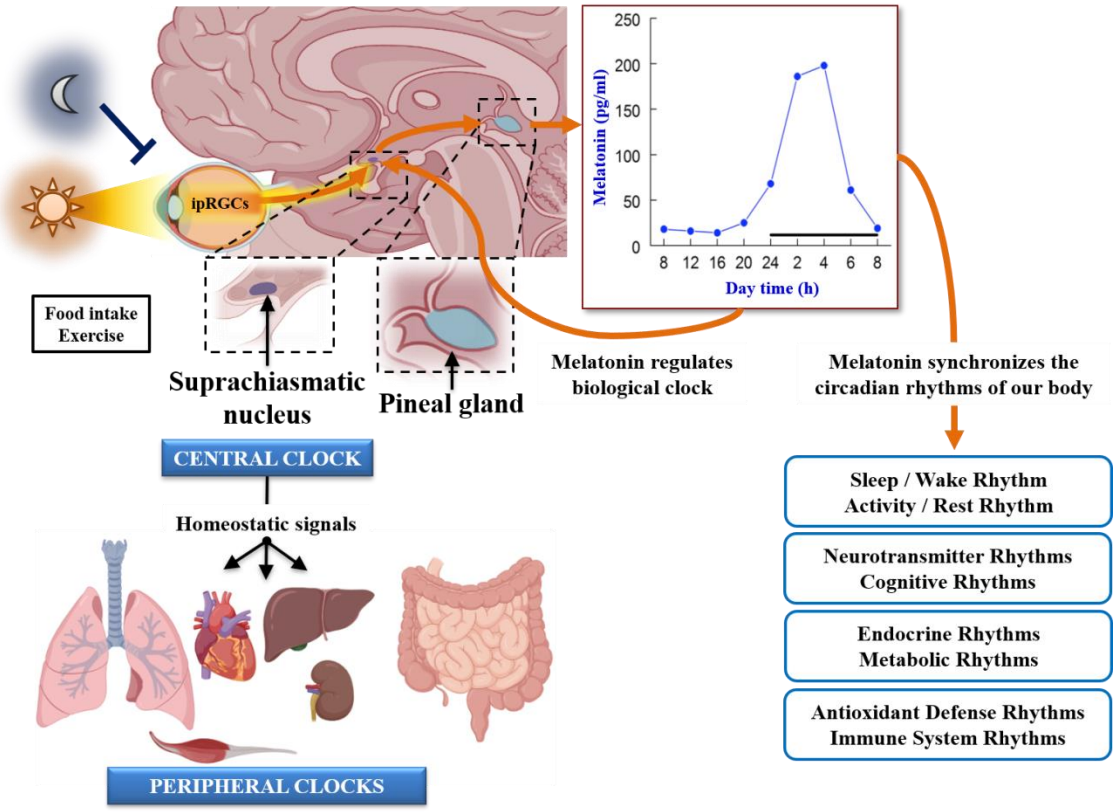


Figure 12. Mammalian circadian rhythms. *In mammals, circadian rhythms are regulated by circadian clocks. These clocks include a central clock and peripheral clocks. The central clock is located in the SCN of the hypothalamus and is synchronized mainly by photoperiod. The central clock regulates basic functions of the organism by two processes: 1) synchronization of peripheral clocks via humoral signals to maintain homeostasis; 2) regulating pineal production of melatonin. This hormone is the endogenous synchronizer of circadian rhythms.*

Central and peripheral clocks share the same molecular mechanism (Mohawk et al., 2012). It is based on positive and negative transcription-translation feedback loops (TTFL) (Figure 13). The transcription factors CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1) form a heterodimer that binds to Enhancer (E)-box sites located in promoter regions and induces the expression of the clock genes *Per* and *Cry* (Period and Chryptochrome, respectively), and also *Ror* (Retinoic acid-related orphan receptor)-α, *Rev-erb* (reverse strand of protein ERB)-α and other clock controlled genes (CCGs, which represent approximately 10-20% of the complete genome in mammals. Once translated the protein PER and CRY heterodimerize in cytosol and translocate to the nucleus, where they repress CLOCK:BMAIL1 heterodimer action. A new cycle starts when the PER:CRY complex decreases, owing to the low levels of CLOCK:BMAL. A second feedback loop consists of RORα and REV-ERBα, which compete to bind retinoic acid-related orphan receptor response elements (ROREs) in the promoter region of *Bmal1*. RORα activates the transcription of *Bmal1* while REV-ERBα represses it.

Recently, a new protein has been discovered, CHRONO (ChIP-derived Repressor of Network Oscillator), that functions as a core component of the mammalian circadian clock. Its overexpression leads to suppression of CLOCK:BMAL1 activity in a histone deacetylase-dependent manner (Goriki et al., 2014).

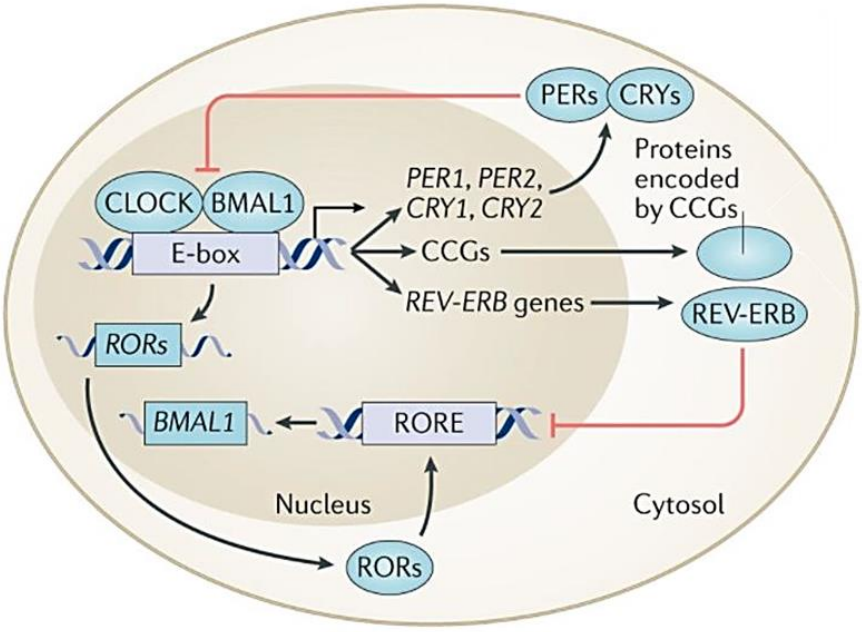


Figure 13. Mammalian clock system. *The molecular clock is comprised of interconnected transcription feedback loops: CLOCK and BMAL1 heterodimer on the activation loop, which promotes Per, Cry, Rorα, Rev-Erbα and CCGs transcription. PER:CRY heterodimer on the inhibition loop, that inhibits CLOCK:BMAL1. RORα and REV-ERBα activates and represses Bmal1 transcription, respectively. Adapted from Firsov and Bonny, 2018.*

3.1. CIRCADIAN RHYTHMS DURING AGING: CHRONODISRUPTION PROCESS

The biologist Pittendrigh, during the influential Cold Spring Harbor Symposium on Quantitative Biology in 1960 (XXV: Biological Clocks), postulated that "*circadian rhythms are inherent in and pervade the living system to an extent that they are fundamental features of its organization; and to an extent that if deranged they impair it*" (Pittendrigh, 1960). Consequently, chronodisruption was defined as a misalignment of internal clock with *zeitgebers*. This syndrome is frequent in people who work nightshifts, experience jet-lag, or are otherwise subjected to artificial lighting during the night (Rajaratnam and Arendt, 2001). Perturbation of circadian synchronicity predisposes individuals to several pathologies; among them are sleep disorders, cognitive impairments, cancer, metabolic syndrome and CVDs (Pauley, 2004). An inescapable source of chronodisruption is aging. The major circadian changes observed in the elderly include phase advancing of the daily rhythms, attenuation of the amplitude and fragmentation of the rhythms (Hood and Amir, 2017). Also described during aging are alteration in the phase relationship with environmental photoperiod cycle, a reduced response to resetting signals and a desynchrony of rhythms within an organism.

Aging affects light reception by impairing blue light transmission, the most crucial wavelength to prompt continuation of the circadian entrainment (Turner and Mainster, 2008). This fact is due to the gradual loss of ipRGCs. A 40-year-old adult has already lost 35% of these cells, and at 55 the individual will perceive less than half the circadian photoperiod a 25-year-old adult would. This circumstance progressively weakens light reception of the circadian system. Similarly, a reported impairment of master clock performance is the reduction of number and functionality of neurons and synaptic terminals (Tsukahara et al., 2005). In addition, the secretion pattern of the main output, melatonin, decreases and experiences phase advancement with aging (Srinivasan et al., 2005). On average, elderly individuals show a 50% decline in nocturnal melatonin levels (Touitou, 2001). The rhythmicity of this pineal hormone is dampened by pinealocyte secretion deficiency, pineal size reduction and calcification (Kunz et al., 1999). Moreover, SNC expression of MT1 melatonin receptor is diminished in aged humans. These findings indicate that circadian systems are not only less able to adjust to environmental time cues with age, but also that melatoninergic feedback to the SCN may be deteriorated in aging (Arellanes-Licea et al., 2014).

Melatonin release modulates the activity of the ipRGCs time-keeping signals to the SNC, regulates core body temperature, stimulates sleep onset and controls the circadian oscillator system (Arendt, 2006). Melatonin also contributes to the rhythmic control of the immune system, influencing the diurnal oscillations of leukocyte proliferation, cytokine production, and Natural Killer (NK) cell activity. Indeed, several studies have shown a connection between a decline in melatonin secretion and age-associated pathologies that occur with alterations in immune system and inflammation, such as Parkinson's or Alzheimer's disease (Cardinali et al., 2008).

Immune system activity is connected with the circadian clock, as it is shown by the daily variations of monocytes, macrophages, T and B lymphocytes in blood (Cermakian et al., 2014). These data suggest that innate immunity is in part controlled by the clock proteins (Figure 14). It has been reported that CLOCK phosphorylates and acetylates p65, increasing the transcriptional activity of NF-κB and consequently the inflammatory response (Spengler et al., 2012). Instead, BMAL1 recruits CLOCK, preventing the activation of innate immunity and acting as an anti-inflammatory agent. Furthermore, BMAL1 increases the expression of RORα, which can further suppress NF-κB through the increase of its inhibitor IκB and the deacetylation of p65 mediated by the binding of melatonin to ROR (Nguyen et al., 2013). REV-ERBα induces an inflammatory response by inhibiting BMAL1. PER and CRY also modulate inflammation. Among the three known PER proteins, PER2 has been proposed as the most important in the control of innate immunity, since it seems to act by stimulating it through BMAL1 repression. On the other hand, the absence of CRY causes an increase in IL-6, TNF-α and inducible nitric oxide synthase (iNOS), giving rise to a pro-inflammatory state dependent on NF-κB action (Narasimamurthy et al., 2012).

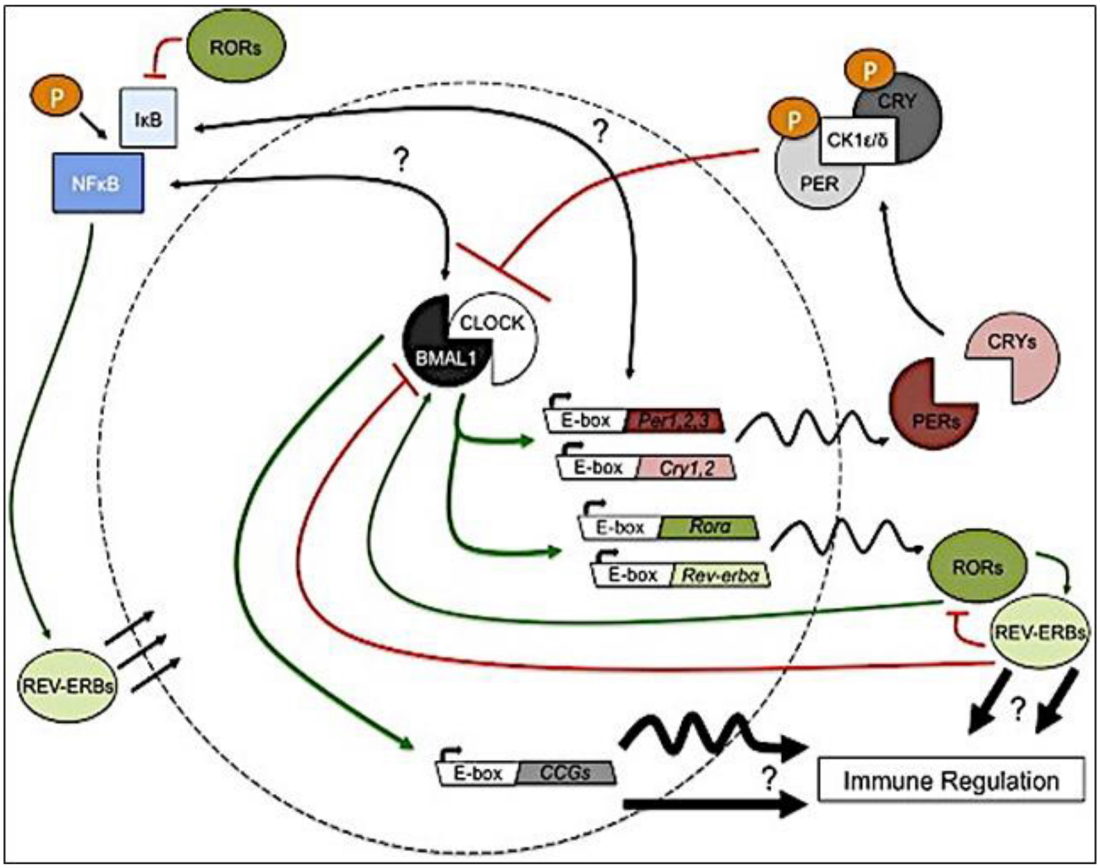


Figure 14. Interaction between clock genes and NF-κB to modulate innate immune activity.

Interestingly, inflammation also disrupts the molecular clock. In this sense, chronodisruption with aging promotes an increase in pro-inflammatory factors towards anti-inflammatory cytokines, causing a further alteration of the clock by exacerbating innate immune response (Volt et al., 2016). The underlying mechanisms involved in the alterations of clock genes by inflammaging are probably related to the inhibitory roles of TNF-α, IL-1β and NF-κB on the transcriptional activity of CLOCK:BMAL1 complex (Cavadini et al., 2007).

These studies suggest a relationship between clock genes, aging and innate immune response (Acuña-Castroviejo et al., 2017; Acuña-Fernández et al., 2020; Volt et al., 2016). However, this connection is not fully clarified since most research to date is focused on NF-κB, leaving the role of NLRP3 unknown. Understanding the involvement of this inflammasome during chronoinflammaging may reveal valuable information to mitigate this process.

Regarding the effect of aging on peripheral clocks, the consensus in the field is still unclear with many controversial results. Some tissues seem unaffected by phase shift while other tissues manifest phase discordance with light cycle or the lack of rhythms (Yamazaki et al., 2002). It is known that organs age at different rates and have tissue-specific impaired pathways in both mammals and *Drosophila* (Girardot et al., 2006). Some tissues may have functional roles in the entire circadian system, adding more complexity to the understating of aging influence on peripheral clocks. More

studies are required in this research area to elucidate the intricate mechanism of circadian rhythm regulation with aging.

3.2. CIRCADIAN RHYTHMS IN HEART. EFFECT OF AGE-RELATED CHRONODISRUPTION

Circadian rhythms are essential for a healthy cardiovascular system. The rhythm of heartbeat shows a diurnal oscillation with higher frequencies and cardiac output during the active phase and lowered activity during the sleep phase (Durgan David J. and Young Martin E., 2010). As a result, a concomitant rhythm appears in blood pressure and, thus, oxygen and nutrient supplementation. Additionally, the risk of serious cardiovascular events, such as stroke, acute myocardial infarction, and sudden death, exhibits circadian patterns characterized by marked peaks in the morning hours at the transition from sleep to wake phase (Willich et al., 1992). Recent investigations have demonstrated that disturbances of circadian rhythms are associated with risk of cardiovascular events (Martino and Sole, 2009).

Genetic mouse models of altered circadian clock function show a ranging degree of abnormalities in heart rate and other cardiac circadian rhythms depending on which distinct circadian clock component is modified (Bray et al., 2008) (Figure 15).

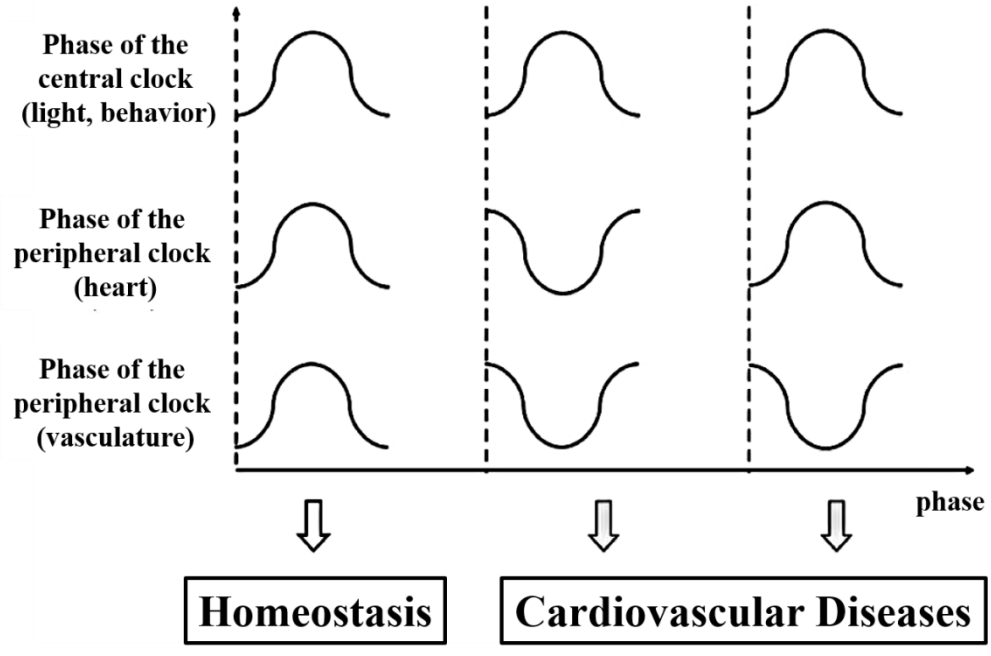


Figure 15. Phases of central and cardiac peripheral clocks. *Synchronization between central and cardiac peripheral clock is essential in keeping physiological function and organism homeostasis. Disruption between central and peripheral clock, or desynchrony among the peripheral clocks leads to cardiac dysfunction. Adapted from Takeda and Maemura, 2015.*

One study reported that a change in the light/dark (L/D) cycle length from 24 h (12/12 h L/D) to 20 h (10/10 h) increased disease severity of mice that had been subjected to the pressure-overload cardiac hypertrophy (transverse aortic constriction, TAC) model (Penev et al., 1998). A repeated phase shift of L/D cycles in hamster with cardiomyopathy considerably compromised their survival (Hurd and Ralph, 1998). These results conclude that disruption of external rhythmicity promotes cardiovascular pathology and affects longevity.

Loss of synchronization between the central and peripheral clocks also underlies cardiac dysfunction. Hamsters with a mutation in casein kinase-1ε are named *tau* mutants, and instead of having a period of 24 h, as seen in wild type hamsters, *+/tau* heterozygotes have shorter cycles with a duration of 22 h. Mutant animals showed cardiomyopathy, fibrosis, impaired systolic function and died at younger age. Interestingly, cardiac impairment disappeared in heterozygotes hamsters when they were kept under a 22 h period that fits their intrinsic rhythm (Martino et al., 2008).

The role of the internal clock in cardiovascular disorders has been studied in genetically modified mice. *Bmal1*-deficient mice displayed age-associated cardiomyopathy, with a thin myocardial wall, decreased cardiac function, and alterations in sarcomere structure in histology (Lefta et al., 2012). Intrinsically, circadian peripheral clocks in heart are present in cardiomyocytes, VSMCs and endothelial cells. Cardiomyocyte-specific *Clock* mutant mice exhibited increased bi-ventricular weight, cardiomyocyte size, and hypertrophy (Durgan et al., 2011). Similarly, cardiomyocyte-specific *Bmal1*-deficient mice had a decreased ejection fraction and developed dilated cardiomyopathy that ended in heart failure, severely reducing their life span (Young et al., 2014). Regarding vascular physiology, mice with *Per2* knocked out showed aortic rings with decreased endothelium-dependent relaxation activity and impaired vasodilation (Viswambharan et al., 2007).

The mechanism underlying aging regulation of circadian clock genes in the heart is unknown. However, in recent research, Gao et al. performed RNA sequencing in young (2 months) and old (18 months) mouse aorta to clarify age-associated changes in the transcriptome. Authors found that circadian rhythms genes were differentially expressed in young and old aorta. In the old aorta, *Clock* and *Bmal1* exhibited a 30% and 50% decrease, respectively, concurrent with an upregulation of *Per2*. In addition, the analysis showed most upregulated pathways in aged aortae were related to immune response, including inflammation (Gao et al., 2020). ECM organization, protein folding control and stress response were the top downregulated pathways. Altogether, the results support the concept that chronodisruption and inflammaging are major contributors in vascular cell senescence and age-related cardiovascular dysfunction (Volt et al., 2016) (Figure 16).

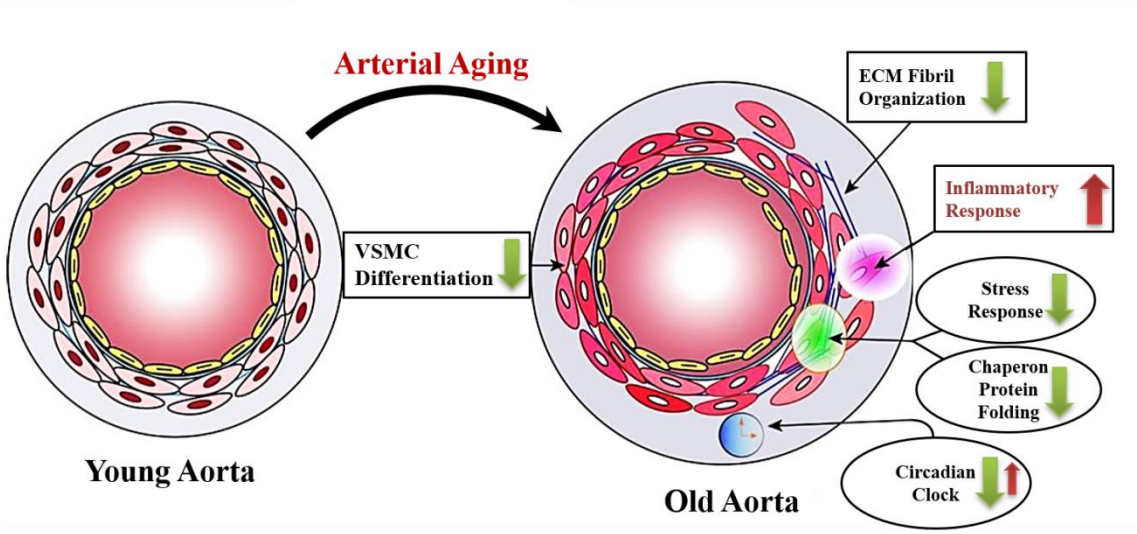


Figure 16. Proposed model of transcriptome regulated by arterial aging. *Arterial aging triggers inflammatory response, leads to circadian clock impairment, and reduction in extracellular matrix organization, chaperone-mediated protein folding control and stress response. Adapted from Gao et al., 2020.*

Pharmacological targeting of circadian rhythms regulators may provide a new opportunity for treating heart disease. The therapeutic implications could be particularly important for individuals who are subjected to chronodisruption, such as nightshift workers and individuals with sleep disorders in the aging population. In this sense, melatonin, as an endogenous synchronizer of central and peripheral circadian rhythms, may be an excellent candidate for that purpose.

4. MELATONIN

Melatonin, or N-acetyl-5-methoxytryptamine (from Greek *melas* = black, dark + *tonin*, from serotonin; melatonin is a hormone that can lighten skin color in some animals, and is derived from serotonin) (aMT), is a highly preserved indoleamine throughout evolution, being present from primitive organisms such as cyanobacteria, parasites such as *Tripanosoma cruzi* and single-celled algae to current organisms (Hardeland, 2008; Hardeland and Poeggeler, 2003; Macías et al., 1999). It was initially described by McCord and Allen in 1917 (McCord and Allen, 1917) and was first isolated from cow pineal gland extracts by Lerner in 1958 (Lerner et al., 1958). A year later, the same author identified the chemical structure of this molecule, giving it the name melatonin (Lerner et al., 1959) (Figure 17). From a chemical point of view, it is an organic crystal, white, with a molecular weight of 232.38g/mol, a melting point between 116-118˚C, not very soluble in water but very soluble in ethanol.

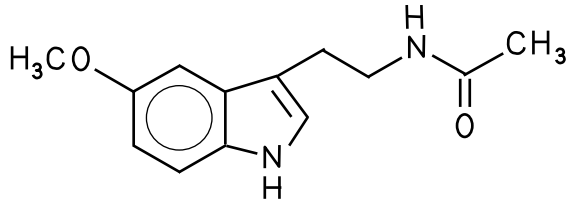


Figure 17. Chemical structure of melatonin.

Melatonin is ubiquitous, distributed throughout the body thanks to its amphipathic nature, which allows it to cross all cellular barriers. Evolutionarily, it is believed that its appearance is due to an adaptation of organisms to use oxygen, since melatonin can neutralize the free radicals produced by this gas' effect on different cellular structures.

Initially, melatonin was characterized as a regulatory element in circadian and reproductive physiology. However, later studies discovered an extra pineal melatonin with antioxidant and anti-inflammatory actions, as well as play a key role in maintaining mitochondrial homeostasis (Acuña Castroviejo et al., 2011; Acuña-Castroviejo et al., 2003; Escames et al., 2003; López et al., 2009; Martín et al., 2000a, 2000b).

4.1. MELATONIN SYNTHESIS

In mammals, the first place where melatonin synthesis was discovered was in the pineal gland which, connected to SCN, possesses the enzymes necessary for its synthesis from serotonin, and whose activity was apparently conditioned by light-dark cycles (Arendt, 2006). In this way, the synthesis of melatonin in the pineal gland acts as a coded message indicating the duration of darkness, reaching pico and nanomolar plasma concentrations (Reiter, 1991).

Melatonin is synthesized from the amino acid tryptophan, which is captured from the circulatory stream by the pinealocyte through an active transport mechanism (Zhao et al., 2019a). Tryptophan is first transformed into 5-hydroxytryptamine, or serotonin, following a process of hydroxylation and subsequent decarboxylation mediated by L-Tryptophan hydroxylase (TPH) and 5-hydroxytryptophan decarboxylase, respectively. Serotonin may suffer a deamination by action of MAO, producing N-acetyl-5-hydroxytryptamine, or acetylation by Arialkylamine N-Acetyltransferase (AANAT), in which case N-acetyl-serotonin (NAS) would be produced. Finally, NAS is methylated by N-acetylserotonin O-methyltransferase (ASMT), producing melatonin (Figure 18). Initially, AANAT was identified as the limiting enzyme in this process, as it has the same circadian rhythm as melatonin and its action is inhibited by light. However, recent studies have shown that melatonin synthesis is unaffected by a decrease or increase in AANAT activity, which points to ASTM as the possible limiting enzyme in the synthesis of this indolamine (Liu and Borjigin, 2005).

In addition to pineal synthesis, it has been shown that this synthesis of melatonin extends to most, if not all, organs and tissues of the body, in addition to non-endocrine cells such as eosinophils, platelets, endothelial cells and NK cells (Acuña-Castroviejo et al., 2014). Similarly, melatonin levels are known to vary within cell organelles, with the nuclear and mitochondrial concentrations of this molecule greater than plasma levels (Venegas et al., 2012). Unlike pineal melatonin, which is rapidly released into the vascular system to access fluids, cell compartments and tissues, extrapineal melatonin that occurs in tissues remains inside the cell and does not enter circulation. This fact suggests that its biological action is different from melatonin of pineal origin (Acuña-Castroviejo et al., 2014).

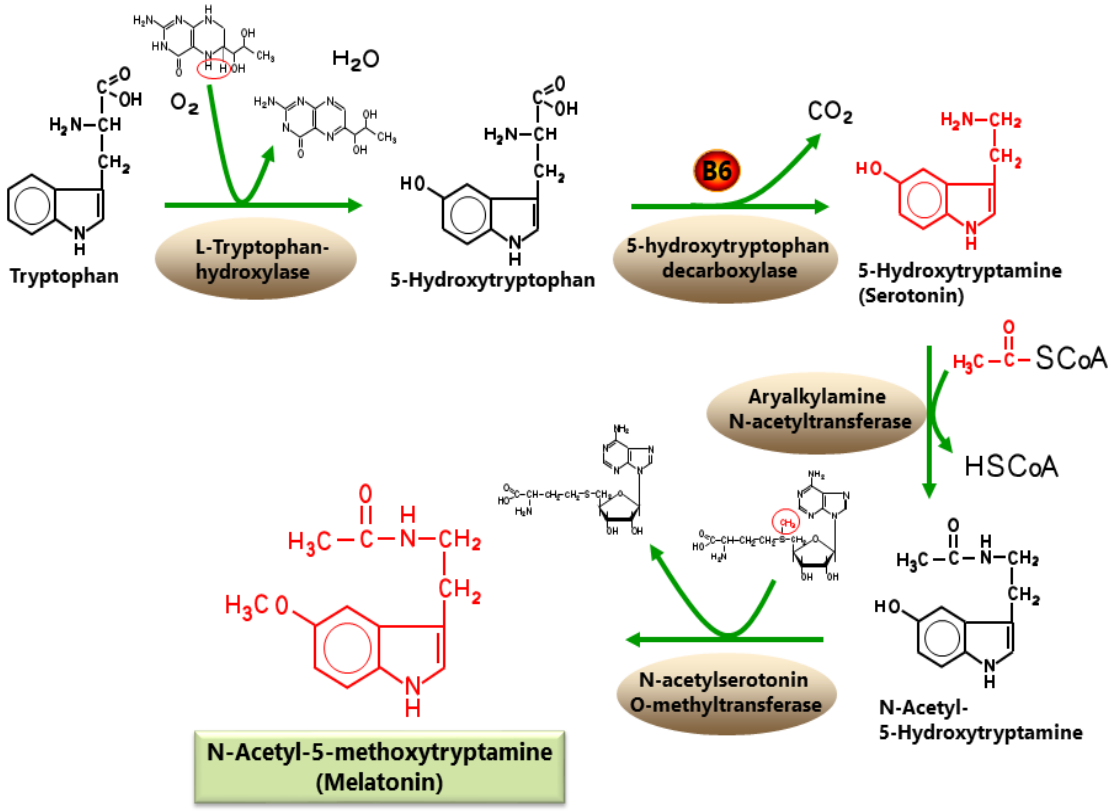


Figure 18. Synthesis of melatonin.

4.2. MECHANISMS OF ACTION

Given the lipophilic nature of melatonin, it was initially considered that this molecule could pass through biological membranes with ease. However, today it is known that plasma, nuclear and mitochondrial membranes have regulatory mechanisms for the entry of melatonin, avoiding an excess concentration of it inside the cell (Venegas et al., 2012). This regulatory mechanism is essential for the maintenance of organismal homeostasis. In view of the different cellular targets, the following mechanisms of action are attributed to melatonin:

- Binding to membrane receptors.
- Binding to nuclear receptors.
- Interaction with cytosolic proteins.
- Antioxidant with direct and indirect action.
- Anti-inflammatory.
- Maintenance of mitochondrial homeostasis.

4.2.1. Binding to membrane receptors

As membrane receptors, two receptors coupled to G proteins, called MT1 (Mel 1a) and MT2 (Mel 1b), have been identified. Despite sharing a homology of 60% in their amino acid sequence, there are differences between the two receptors (Reppert et al., 1995):

1. The MT2 receptor has a lower affinity (Kd = 160 pmol/L) for the radio ligand ^{125}I-melatonin compared to that showed by MT1 (Kd = 20-40 pmol/L). In any case, both receptors have a high affinity for melatonin (Jockers et al., 2016).

2. They are expressed in various tissues of the body, although the location of MT2 appears to be more limited to the brain. It should be noted that the expression of these receptors depends on various factors, such as species, tissue, endocrine status, the state of development in which the organism is located and environmental light (Vanecek, 1998).

3. Melatonin's interaction with each of these receptors causes different effects: MT1 is associated with G proteins that inactivate adenylate cyclase and activate phospholipase Cβ. MT2 also inhibits the pathway of soluble cyclase adenylate (von Gall et al., 2002).

Subsequently, this family of membrane receptors has been expanded with the discovery of the MT3 receptor, characterized as a quinone reductase 2. This receptor has been identified in hamster but not in humans (Nosjean et al., 2000).

Admittedly the mechanisms that regulate the expression of membrane receptors are very complex, so the responses mediated by melatonin depend on various factors such as the circadian

rhythm phase, duration of exposure, greater or lesser presence of endogenous melatonin and sensitivity of the receptor to melatonin.

4.2.2. Binding to nuclear receptors

The first indication of a possible interaction of melatonin with nuclear material was obtained when a high concentration of this hormone was found to be associated with chromatin (Withyachumnarnkul et al., 1986). Subsequently, the presence of melatonin in the nucleus was demonstrated by Acuña-Castroviejo et al. They tested their specific binding to the protein fraction of the rat liver nucleus using ^{125}I-iodomelatonine, suggesting the existence of nuclear receptors for melatonin (Acuña-Castroviejo et al., 1994).

In 1994, Becker-Andre et al. (Becker-André et al., 1994) demonstrated a genomic action of melatonin through RORα nuclear receptors, a subfamily of nuclear receptors or ligand-dependent transcription factors that provide organisms with the ability to control gene expression in response to physiological, developmental and environmental factors. However, there is currently some controversy as to whether ROR transcription factors are true nuclear melatonin receptors (Lardone et al., 2011), despite recent publications that support its existence (García et al., 2015).

The ROR/RZR family of receptors (retinoid-related orphan receptor/retinoid Z receptor) is divided into three subtypes: RORα (Becker-André et al., 1994), RORβ (Carlberg et al., 1994) y RORγ (Hirose et al., 1994). Due to variable splicing, each gene has several isoforms, which differ only at its terminal amino end. In humans, 4 RORα isoforms (RORα1-4) have been found, while only isoforms 1 and 4 have been described in mice. Of the two isoforms generated by RORβ, in humans only isoform 1 is expressed, and the two isoforms generated by RORγ are present in both species. Each of these isoforms show a specific expression of tissue and are involved in the control of different biological processes (Smirnov, 2001). RORα is expressed in virtually all peripheral tissues, RORβ in brain and retina, and RORγ in adipose tissue, skeletal muscle, liver, kidney and immune system.

From a structural point of view, the members of this subfamily have a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Jetten et al., 2001). DBD consists of two zinc fingers that allow the specific union of ROR monomers to DNA, recognition that takes place because of the existence of a RORE, consisting of a consensus sequence (AGGTCA) preceded by a sequence of 6 base pairs rich in AT. LBD is located at the C-terminal end and presents an activation function sequence (AF-2) responsible for the transcriptional activity of the nuclear receptor once it has joined its ligand.

Once attached to DNA through its response element, ROR mediates the regulation of numerous biological processes, including circadian rhythms, where the feedback loop involving ROR and REV-ERBα competitively bind to RORE, as detailed in the previous section. In addition, ROR

also controls embryonic development, cell differentiation and proliferation processes, metabolism and immune system response, and testicular development (Sayed et al., 2019a). In relation to the latter point, transcriptional activity of RORα has been shown to be related to inhibition of the enzyme 5-lipooxygenase, which is involved in the biosynthesis of pro-inflammatory leukotriene in human B lymphocytes (Steinhilber et al., 1995). RORα1 inhibits the expression of pro-inflammatory mediators IL-6, IL-18 and cyclooxygenase (COX)-2 due to the presence of response elements to RORα in the promoter of the Iκbα gen, acting as a negative regulator in the signaling pathways dependent on NF-κB (Delerive et al., 2001). These properties were subsequently confirmed by seeing that mutant mice for RORα had a greater LPS-induced inflammatory reaction compared to wild-type, although this fact was not related to a repression or lack of induction of Iκbα expression in mutant mice (Stapleton et al., 2005).

4.2.3. Interaction with cytosolic proteins

Melatonin is mainly bound to two cytosolic proteins: calmodulin and calreticulin (León et al., 2000). By interacting with calmodulin, melatonin is involved in processes such as modulation of cytoskeleton structure, or the activity of enzymes such as neuronal nitric oxide synthase (nNOS) and phosphodiesterase (León et al., 2006). Through its binding to calreticulin, melatonin regulates calcium metabolism (Macías et al., 2003).

4.2.4. Antioxidant with direct and indirect action

Melatonin, as well as some of its metabolites such as N1-acetyl-5-methoxykinuramine (AMK) and N1-acetyl-N2-formil-5-methoxykirunamine (AFMK), can directly neutralize ROS and RNS (Reiter et al., 2003a). Being an electron-rich molecule, this indoleamine can successively transfer them to OH^-, forming an indole radical; this, in turn, can neutralize another OH^- to generate cyclic 3-hydroxymelatonin. On the other hand, melatonin can also scavenge $O_2^{\cdot -}$ radical, resulting in the active metabolite AFMK. Both cyclic 3-hydroxymelatonin and the metabolite AFMK possess a high antioxidant capacity, increasing the action of melatonin as a powerful free radical scavenger (Reiter et al., 2003b). Melatonin is also able to react with RNS, such as $ONNO^-$ or peroxide radicals ($LOO\cdot$) (Zhang et al., 1999), preventing the spread of lipid peroxidation.

In addition to this intrinsic ability to purify free radicals, melatonin can stimulate the activity and expression of other antioxidant systems, indirectly reducing oxidative stress. Firstly, melatonin stimulates the glutathione cycle, thus regulating the balance GSSG/GSH. To do this, melatonin increases the activity of GPx, GRd, γ-glutamylcysteine synthase and glucose-6-phosphate dehydrogenase (G6PD) (Martín et al., 2000a; Urata et al., 1999). The enzime γ-glutamylcysteine is the limiting step in glutathione biosynthesis and the enzyme G6PD generates NADPH necessary for GRd.

The action of melatonin has also been described in other antioxidant enzymes, such as CAT and SOD (Acuna-Castroviejo et al., 2007).

Whether through direct or indirect action, melatonin is a powerful antioxidant and plays an important role in maintaining the balance of cellular redox state, especially at the mitochondrial level, as this organelle is the main source of ROS and RNS. In this way, melatonin protects macromolecules such as DNA, proteins and lipids from oxidative damage. In addition, thanks to the characteristics of its N-acetyl group, melatonin increases the effectiveness of other natural antioxidants, acting synergistically with vitamins C and E (Reiter et al., 2003b).

4.2.5. Anti-inflammatory

Inflammation and free radical production are reciprocally connected process. Therefore, the property of melatonin to activate endogenous antioxidant defense and its efficiency as ROS scavenger are crucial to its anti-inflammatory activity (Mauriz et al., 2013).

Melatonin and its metabolites AMK and AFMK also act directly as anti-inflammatory agents inhibiting the synthesis of prostaglandins and adhesion molecules. In addition, melatonin decreases the expression of COX-2 in macrophages, as well as adhesion and leukocyte migration on activated endothelial cells, thus avoiding the recruitment of polymorphonuclear cells and reducing inflammation (Cuzzocrea and Reiter, 2002).

Various investigations show that melatonin decreases pro-inflammatory cytokines and increases anti-inflammatory cytokines. In animals exposed to heat stress, stimulated with LPS or during aging, melatonin minimized the pro-inflammatory cytokines TNF-α, IL-12 and IFN-γ and increased the inflammatory cytokine IL-10. In senescent animals, melatonin diminished expressions of pro-inflammatory cytokines IL-16, IL-1o and TNF-α (Forman et al., 2011; Reiter et al., 2000).

The first report demonstrating the anti-inflammatory properties of melatonin was that of Crespo et al. (Crespo et al., 1999), showing the efficacy of the indoleamine to prevent endotoxemia in rats. Multiple studies of the same group further reported the potent anti-inflammatory efficacy of melatonin against systemic innate immune activation. This indolamine suppressed NF-κB activity in Senescence-Accelerated Mouse Prone (SAMP)8 mice and other conditions such as ischemia/reperfusion injury, Alzheimer's disease, pulmonary inflammation, diabetes, cancer and exercise stress. It was recently proved that melatonin dampened the NF-κB/NLRP3 connection and activation during cardiac sepsis and aging (García et al., 2015; Rahim et al., 2017; Volt et al., 2016). This research provides the basis for further research on the value of melatonin in protecting against myocardial damage associated to inflammation.

4.2.6. Maintenance of mitochondrial homeostasis

Melatonin contributes to the maintenance of mitochondrial homeostasis thanks to its antioxidant effect, discussed above, and to the direct action it exerts on this organelle. In particular, melatonin interacts with the electron transport chain, increasing the activity of respiratory complexes in healthy mitochondria and, above all, in damaged mitochondria. Given the high redox potential of melatonin (0.94 V), it is thought that this indoleamine not only stimulates the activity of the complexes, but could also donate electrons by itself by increasing the electron flow (Tan et al., 2000).

Martin et al. was the first reporting the outstanding role of melatonin to maintain mitochondrial homeostasis (Martín et al., 2000a). The same group reportedly showed the interaction between melatonin and mitochondria, the specificity of melatonin to act for maintaining mitochondrial function, demonstrating that mitochondria is the main target for melatonin in the cell (Doerrier et al., 2015; Escames et al., 2012; García et al., 2015; Ortiz et al., 2014). Melatonin also has other effects at the mitochondrial level, including increased mitochondrial biogenesis following chronic administration, internal mitochondrial membrane stabilization, increased ATP production and prevention of apoptosis by modulating mitochondrial membrane potential, regulating calcium homeostasis and inhibiting MPTP (López et al., 2006). All these properties make melatonin a possible therapeutic agent in pathologies where mitochondrial function is aggravated and threatens cell survival, including CVDs.

4.3. MELATONIN MECHANISMS OF ACTION IN CARDIAC MUSCLE

Melatonin protects cardiac muscle through interconnected receptor and non-receptor pathways (Fu et al., 2020) (Figure 19):

The related pathways by which melatonin shelters myocardium via **membrane receptors** are: 1) reperfusion injury salvage kinase (RISK) pathway; 2) SAFE pathway, and 3) Notch pathway.

1. The **RISK pathway** has an intracellular biological role through MT1 and MT2 melatonin receptors. In turn, this via is constituted by three downstream signal pathways, namely: a) Mitogen Activated Protein Kinase- Extracellular signal Regulated Kinase (MAPK-ERK) signal pathway; b) AMPK pathway, and c) PI3K-Akt signaling pathway.

The MAPK-ERK signaling pathway upregulates the antioxidant factor Nrf2, which couples with DNA AREs to upregulate the expressions of Hmox, Nqo1, and glutathione s-transferase 1 (GST1), and reduce the expression of apoptotic proteins p38 and p21. ERK inhibits activity of the voltage dependent anion channel (VDAC) and the transcription factor of IP3R-cAMP response element binding protein (CREB). An extreme activation of VDAC and CREB would cause intracellular calcium overload, which would lead to mitochondria dysfunction and

cardiomyocyte necrosis (Zhou et al., 2018). Downstream effects of this pathway also involve the activation of endothelial nitric oxide synthase (eNOS) and downregulation of apoptotic factors such as Bax, Bad, and phosphorylation of caspases that avoid the opening of MPTP (Acuña Castroviejo et al., 2011).

AMPK pathway is interrelated with MAPK-ERK through Nrf2 and has a synergistic action in antioxidant stress and anti-apoptotic processes (Yu et al., 2016). AMPK promotes mitochondria fission by activation of Drp1, thereby opening the MPTP. SIRT3 is a downstream target of PGC-1α, which is stimulated by AMPK. This pathway enhances the activity of GPx and SOD, decreases the transfer of Bax to mitochondria and boots the biosynthesis of this organelle (Lochner et al., 2018).

The primary effect of PI3K-Akt pathway is to reduce the cellular oxidative stress. Akt is activated by Nrf2. It is known that melatonin modulates the activity of ERK throught Akt. Signaling molecules that participates in this pathway are Zrt/Irt-like protein 1 (Zip1), brain-derived neurotrophic factor (BDNF) and PPARγ (Zhang et al., 2019b). 2. In the SAFE pathway, melatonin phosphorylates JAK2-STAT3 via TNF cell membrane receptor. Downstream molecular effects include the expression of TNF-α, Bcl-2, antioxidant genes, mcl1 and FAS, and the inhibition of Bax, caspase-3, cytochrome c, cyclin-dependent kinase (cyclin D1) and MPTP opening. The phosphorylation of STAT3 activates ERK and Akt pathways (Yang et al., 2013).

2. In the **SAFE pathway**, melatonin phosphorylates JAK2-STAT3 via TNF cell membrane receptor. Downstream molecular effects include the expression of TNF-α, Bcl-2, antioxidant genes, mcl1 and FAS, and the inhibition of Bax, caspase-3, cytochrome c, cyclin-dependent kinase (cyclin D1) and MPTP opening. The phosphorylation of STAT3 activates ERK and Akt pathways (Yang et al., 2013).

3. Regarding the **Notch pathway**, melatonin activates the expression of Hairy and enhancer of split 1 (Hes-1) via Notch 1-Notch Intracellular area (NICD). Hes-1 inhibits the negative regulatory effect of chromosome 10 (PTEN) on PI3K. Furthermore, Notch pathway decreases the effects of cardiomyocyte apoptosis by modulating mitophagy with the mitochondrial fusion protein Mfn2 (Pei et al., 2016).

In the **nuclear receptor** signaling pathway, melatonin interacts with RORα to regulate autophagy, cytochrome c release, and to promote the expression of genes related to calcium processing that will contribute to reduce the stress injury to and apoptosis of the cardiomyocytes. Some of these

genes are myocardial sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2α, sodium-calcium exchange 1 (NCX1), Ryanodine receptor 2 (RyR2) and Ca^{2+}-calmodulin-dependent kinase II (CAMKII) (Yeung et al., 2008).

Finally, melatonin also acts directly inside the cells and has biological effects through **non-receptor mediated pathways**. In cytosol, this indolamine promotes the release of NO, stimulates the activity of iNOS and the expression of SIRT3 via PKB-Akt. Another sirtuin activated by melatonin is SIRT1, which regulates oxidative stress, mitophagy and apoptosis by enhancing the expression of Bcl2 and weakening Bax and caspase 3. The ultimate effects of these pathways are to reduce oxidative stress, inflammatory responses, and to protect mitochondrial function of cardiomyocyte (Mauriz et al., 2013).

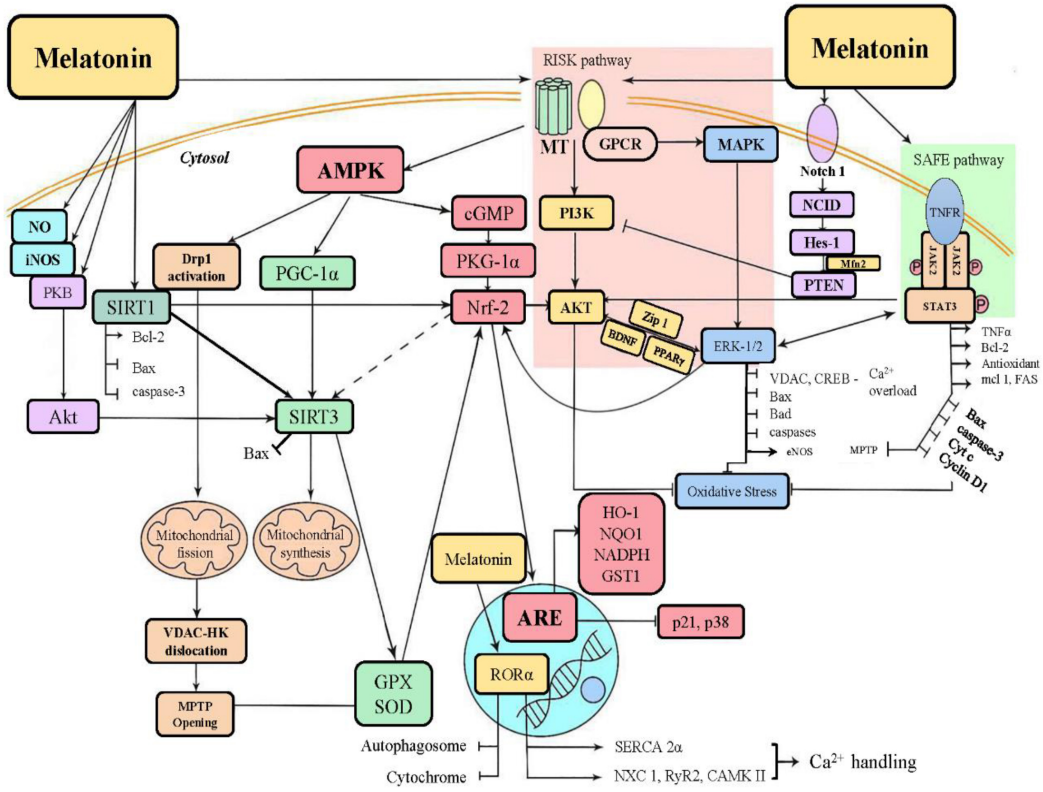


Figure 19. Melatonin mechanism of action mediated by receptor and non-receptor pathways in cardiac muscle. *Melatonin protects cardiac muscle mainly by being bound to membrane and nuclear receptors. Membrane receptors pathways include 1) RISK pathway, mediated by MT1 and MT2 receptors; 2) SAFE pathway, mediated by TNFR; and 3) Notch pathway. There are three downstream signal pathways belonging to RISK: a) MAPK-ERK; 2) AMPK; and 3) PI3K-Akt. Melatonin also acts through the nuclear receptor RORα. In addition, melatonin can enter into cells, where it has direct biological effects. Adapted from Fu et al., 2020.*

HYPOTHESIS AND AIMS

Aging is the main risk factor for CVDs, which are the leading cause of death world-wide. Previous studies have proved that aging is associated with a low-grade pro-inflammatory state characterized by a subclinical, sterile, asymptomatic and chronic inflammation, called inflammaging. This phenomenon produces free radicals that cause oxidative damage and amplify the release of pro-inflammatory cytokines, propagating a vicious cycle that ends in systemic inflammation, immunosenescence and innate immune activation, whose main components are NF-κB and NLRP3 inflammasome. Atherosclerotic lesions are considered an underlying consequence of inflammaging and are the major cause of serious cardiovascular events such as ischemic cardiac injury, heart attack or stroke. Recent works have pointed to NLRP3 as a key player in the etiology of these diseases.

It is established that the main driving force of the aging process is oxidative stress caused by an imbalance between ROS generation and antioxidant defenses. The age-related increase in free radicals results in impairment and dysregulation of mitochondria, which are the primary source of ROS, most of them derived from the mitochondrial respiratory chain. In addition, ROS production induces deleterious mitochondria changes that directly activate the NLRP3 inflammasome and may also activate the Nrf2 pathway, establishing a connection between inflammaging, mitochondrial dysfunction and endogenous protective response by Nrf2-dependent antioxidant response. The fact that the heart has a high-energy demand, vast amounts of mitochondria and relatively low antioxidant defense, makes this organ particularly susceptible to oxidative stress as aging progresses.

Increasing evidence supports a connection between aging and alterations in the biological clock that control the circadian rhythms. This chronodisruption process has been linked to a decline in melatonin synthesis. This indolamine is the endogenous synchronizer of central and peripheral circadian rhythms. Circadian rhythm disruption and the decrease in melatonin levels have both been connected with the risk of cardiovascular events. Furthermore, inflammation has been found to disrupt the molecular clock. Therefore, chronodisruption with aging may increase pro-inflammatory factors towards anti-inflammatory cytokines, causing a further alteration of the clock by exacerbating innate immune response.

In addition to its chronobiotic effects, melatonin has anti-oxidative and anti-inflammatory properties that depend on high levels of extrapineal melatonin. Its main target is mitochondria, boosting their bioenergetic properties, enhancing ATP levels and mitigating the formation of free radicals. In numerous experimental conditions including acute and chronic inflammation, and aging in mouse heart, melatonin significantly prevented oxidative stress, reduced activation of innate immunity, and improved cardiac mitochondria function.

As a result, our hypothesis is:

1. NLRP3 inflammasome plays an essential role in cardiac aging, promoting mitochondria dysfunction and impairing antioxidant defense. Given the antioxidant and anti-inflammatory properties of melatonin, this indolamine is able to counteract aging and NLRP3 effects.
2. Nrf2 response may be insufficient to counteract the damage induced by NLRP3 response; the effects of melatonin to activate this pathway may be of utility in aged heart.
3. NLRP3 inflammasome leads to chronodisruption during cardiac aging. Since chronodisruption is caused by a decrease in melatonin, the endogenous synchronizer of the biological clock, we believe that by restoring the circadian melatonin pattern in older animals we can resynchronize the broken biological clock to, partially, or fully, offset deficits associated with age-related amelioration of melatonin synthesis.
4. Aging and NLRP3 inflammasome promote morphometrical and ultrastructural alterations in heart muscle. We placed confidence in melatonin to prevent age-related cardiac sarcopenia.

To test the hypothesis, we will address the following aims:

General aim:

To study the causal relationship between chronodisruption, melatonin deficiency, and innate immune response mediated by NLRP3 during aging in heart tissue.

Specific aims:

To evaluate in cardiac muscle of 3, 12 and 24 month old C57/Bl6 and NLRP3 deficient mice:

- Mitochondria pathway: fission, fusion, mito/autophagy, apoptosis and mitochondria ultrastructure.
- Nrf2-dependent antioxidant pathway.
- Biological clock pathway: clock gene expression, rhythmicity, acrophase, amplitude and mesor.
- Histology, ultrastructure and magnetic resonance imaging of cardiac muscle.
- The effects of melatonin treatment on the aforementioned parameters.

METHODS

1. ANIMALS AND TREATMENT

Wild-type C57BL/6J and NLRP3-knockout mice NLRP3$^{-/-}$ (B6.129S6-NLRP3^{tm1Bhk}/J) on a wild-type C57BL/6J background (>10 backcrosses) aged 3 weeks were purchased from Charles River (Barcelona, Spain) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. Mice were housed in the animal facility of the University of Granada under a specific pathogen-free barrier and were kept under controlled temperature (22 ºC ± 1 ºC). Room illumination was on automated 12 h L/D cycle (lights on at 08:00 h). Animals had *ad libitum* access to tap water and pelleted rodent chow.

This study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Sciences, Bethesda, MD, USA), the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS #123), and the Spanish law for animal experimentation (R.D. 53/2013). The protocol was approved by the Andalusian Ethical Committee (05/07/2016/130).

Wild-type (WT) and NLRP3$^{-/-}$ mice were divided into five experimental groups (Figure 20): (I) young (Y, 3-months old), (II) early-aged (EA, 12-months old), (III) early-aged plus melatonin (EA + aMT), (IV) old-aged (OA, 24-months old), and (V) old-aged plus melatonin (OA + aMT) mice. Melatonin was orally administered at 10 mg/kg/day in the chow during the last two months before early and old-aged treated mice were sacrificed (EA + aMT at the age of 10 months and OA + aMT at the age of 22 months). The other groups of animals (Y, EA and OA) were fed with normal chow without melatonin. The melatonin pelleted chow was prepared by the Diet Production Unit facility of the University of Granada.

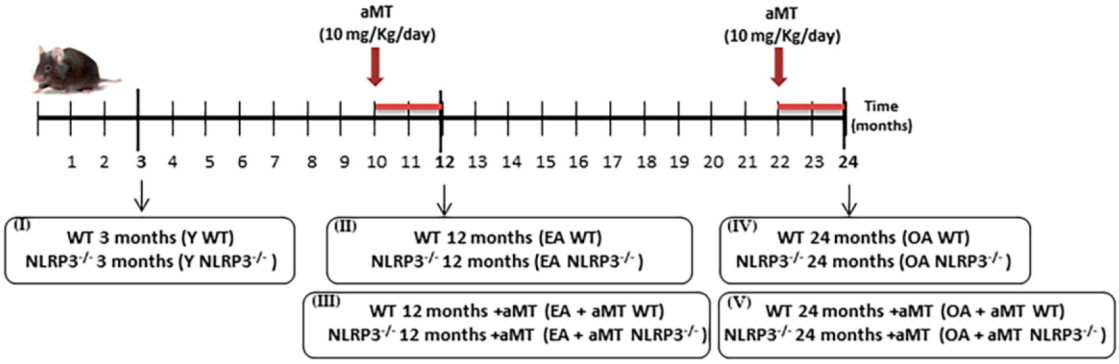


Figure 20. Study design summary: experimental groups and melatonin treatment.

Animals were sacrificed by cervical dislocation after equithesin administered via intraperitoneal injection (1 mL/kg), and hearts were collected. The LV was dissected and divided into two parts. One part was washed in saline, and rapidly fixed in 2.5% glutaraldehyde for transmission electron microscopy analysis, while the other part was stored at -80 ˚C for further

western blot analysis. To assess the cardiac muscle fibers architecture, animals were transcardially perfused with trump´s fixative and hearts were processed for light microscopic examination. For these experiments, animals were always sacrificed at the same time of day to avoid circadian variations in the immune response (Curtis et al., 2014).

To study the circadian rhythm, mice were sacrificed by cervical dislocation after equithensin anesthesia via intraperitoneal injection (1 mL/kg), at 24:00, 06:00, 12:00 and 18:00 h under a 12 h light/dark cycle. At night, the sacrifice of animals was performed under a dim red light which does not influence endogenous melatonin production. Hearts were collected, washed in cold saline and freshly store at -80 ºC for PCR analysis.

2. ISOLATION OF CYTOSOL FRACTION

Pure cytosolic subcellular fraction was isolated from heart tissue according to Dimauro et al. (Dimauro et al., 2012) with some adjustments described in Rahim et al. (Rahim et al., 2017). Heart tissue was homogenized on ice at 800 rpm in 500 µL of STM buffer containing 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 5% phosphatase inhibitor buffer (125 mM NaF, 250 mM β-glycerophosphate, 250 mM p-nitrophenyl phosphate, and 25 mM $NaVO_3$), and a protease inhibitor cocktail (Cat. 78429, Thermo Fisher Scientific, Waltham, MA, USA) with a Teflon pestle (Stuart Scientific, mod. SS2). The homogenate was maintained on ice for 30 min, and then centrifuged at 800 g for 15 min at 4 ºC. The supernatant was labeled as S0 and used for subsequent isolation of cytosolic fractions. S0 was centrifuged at 800× g for 10 min at 4 ºC and the supernatant S1 was centrifuged at 11.000× g for 10 min. The resulting supernatant S2, containing cytosol and microsomal fraction, was precipitated in cold 100% acetone at -20 ºC for 1 h followed by centrifugation at 12.000× g for 5 min. The pellet was resuspended in 300 µL STM buffer and labeled as cytosolic fraction, which was aliquoted and frozen at -80 ºC.

3. SAMPLE PREPARATION AND ANALYSIS OF PROTEIN CONTENT BY WESTERN BLOT

Western blot analysis was performed on cytosolic fractions of mice hearts. Samples were prepared for western blot analysis with a denaturation process that followed the following steps:

The cytosolic fraction was thawed and, after measuring its protein content, it was treated with a buffer composed of 2.5% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol and 0.01% bromophenol blue. The mixture was heated at 99 ºC for 5 minutes, and subsequently incubated for

another 5 minutes at 4 ºC. Sample treatment was completed with a short centrifugation at maximum speed.

Denatured protein samples (40 µg/fraction) were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 12% or 15% acrylamide/bis-acrylamide gels. Electrophoretic separation was performed at 100 V, 400 mA for 2 h at room temperature (RT). Proteins were then wet transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Life Science S.L.U., Madrid, Spain) at 100V during 90 min at 4 ºC. The membrane was blocked in 5% bovine serum albumin (BSA) in PBS-T (phosphate-buffered saline, composed by 0.01 M K_2HPO_4, 0.15 M NaCl, pH 7.4, with 0.01% Tween-20) for 1.5 h at RT, then incubated overnight at 4 ºC with the primary antibodies diluted in blocking buffer as per manufacturer's specification. The primary antibodies used are shown in Table 1:

Antibody	Reference	Company
Anti-Bax	sc-7480	Santa Cruz Biotechnology (Heidelberg, Germany)
Anti-Bcl2	sc-7382	
Anti-Casp9	sc-56076	
Anti-p53	sc-126	
Anti-GAPDH	sc-166574	
Anti-Nrf2	sc-722	
Anti-Nqo1	sc-32793	
Anti-γ-Gclc	sc-390811	
Anti-Keap1	10503-2-AP	Proteintech (Manchester, United Kingdom)
Anti-Hmox1	70081s	Cell Signaling Technology (Leiden, The Netherlands)
Anti-pNrf2 (Ser40)	bs-2013R	Bioss Antibodies (Woburn, MA, USA)
Anti-LC3	NB100-2220	Novus Biologicals (Centennial, CO, USA)
Anti-Opa1	CPA3687	Quimigen (Madrid, Spain)
Anti-Drp1	PA5-43802	Fisher Scientific (Madrid, Spain)
Anti-Mfn2	TA344104	OriGene Technologies (Rockville, MD, USA)

Table 1. List of primary antibodies used in western blot analysis. *Bax: BCL2-associated X protein; Bcl2: B-cell lymphoma protein 2; Casp9: caspase 9; p53: transformation related protein 53; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Nrf2: nuclear factor, erythroid derived 2, like 2; Nqo1: NAD(P)H quinone dehydrogenase 1; γ-Gclc: gamma-glutamate-cysteine ligase, catalytic subunit; Keap1: kelch-like ECH-*

associated protein 1; Hmox1: heme oxygenase 1; pNrf2: phosphorylated Nrf2 (Ser40); LC3: microtubule-associated protein 1 light chain 3; Opa1: optic atrophy 1; Drp1: dynamin-related protein 1; Mfn2: mitofusin 2.

Membranes were washed with PBS-T 3 × 10 min and incubated for 1 h at room temperature with anti-mouse (BD Biosciences Pharmigen, San Jose, CA, USA) or anti-rabbit (Thermo Scientific, Madrid, Spain) IgG-horseradish peroxidase conjugated secondary antibodies diluted according to the manufacturer's instructions. After washing with PBS-T, immunoreaction was detected using ClarityTM Western ECL Substrate (Bio-Rad, Madrid, Spain) and revealed in Kodak Image Station 4000MM PRO (Carestream Health, Rochester, NY, USA). Bands were analyzed and quantified using Kodak Molecular Imaging Software v. 4.5.1 (Carestream Health, Rochester, NY, USA).

The percentage of acrylamide / bis-acrylamide gel used in electrophoresis, as well as the dilutions of primary and secondary antibodies used for each protein are shown in Table 2**.**

	Mfn2	**Opa1**	**Drp1**	**LC3I**	**LC3II**	**Nrf2**	**pNrf2**	**Keap1**
% GEL	12%	12%	12%	15%	15%	12%	12%	12%
1º Ab	1:1000	1:500	1:1000	1:500	1:500	1:500	1:1000	1:1000
2º Ab	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)	Anti-rabbit (1:5000)

	Hmox1	**Nqo1**	**γGclc**	**p53**	**Casp9**	**Bax**	**Bcl2**	**GAPDH**
% GEL	15%	15%	12%	15%	12%	15%	15%	12/15 %
1º Ab	1:1000	1:200	1:100	1:1000	1:1000	1:1000	1:500	1:200
2º Ab	Anti-rabbit (1:5000)	Anti-mouse (1:5000)	Anti-mouse (1:5000)	Anti-mouse (1:1000)	Anti-mouse (1:1000)	Anti-mouse (1:1000)	Anti-mouse (1:1000)	Anti-mouse (1:5000)

Table 2. Western blot conditions for the analysis of the content of our target proteins.

4. GENE EXPRESSION ANALYSIS

4.1. RNA extraction

RNA was isolated from frozen mouse hearts using the NZY Total RNA Isolation kit (Nzytech gene & expression, Lisbon, Portugal), following the protocol provided by the company. An initial proteinase K digestion step was performed to improve the yield of RNA (20 mg/mL proteinase K, 600 mAU/mL) (Qiagen, Hilden, Germany). RNA was quantified in a Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its integrity was confirmed by 2% agarose gel electrophoresis. RNA was aliquoted and stored at -80 ºC for its later reverse transcription into cDNA.

4.2. Reverse transcription reaction

RNA was reverse transcribed to cDNA with qScript™ cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, MD, USA). The samples were loaded into a Techne Thermal Cycler / PCR model FTGene2D (Techne, Cambridge, UK). The reverse transcription reaction conditions are specified in Table 3. After finishing the program, the cDNA obtained was aliquoted and stored at -20 ºC until later use.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (ºC)	22	42	85	4
Time (min)	5	30	5	∞

Table 3. Conditions programmed into the thermal cycler for reverse transcription.

4.3. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Amplification was performed by quantitative real-time polymerase chain reaction (RT-PCR) in a Stratagene Mx3005P QPCR System (Agilent Technologies, Madrid, Spain) with SYBR® Premix Ex Taq™ (Takara Bio Europe, Saint-Germain-en-Laye, France). The PCR mix contained 80 ng of cDNA, 10 µM of each primer and 10 µL of SYBR Green of the kit in a final volume of 20 µL. Primer sequences are showed in the Table 4 and they were designed using the Beacon Designer software 4.0 (Premier Biosoft Inc., Palo Alto, CA, USA). Thermal profile of RT-PCR is indicated in Table 5. Output data were analyzed according to the standard curves generated from increasing amounts of cDNA (0.05, 0.5, 5, 50, and 500 ng). Beta-actin housekeeping gene was used as an endogenous reference gene. Template-free (water) sample was used as a negative control and 3 months-old wild type mice were used as a calibrator sample.

Gene Symbol	Gene Description	Forward primer	Reverse primer
Clock	Circadian Locomotor Output Cycles Kaput	GGTGGTGACTGCCTATCCTAC	CTGCTGTTGTTGTTGCTGTTG
Bmal1	Brain and muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like 1	GAAGACAATGAGCCAGACAAC	CCATAGATTTCACCCGTATTTCC
Per2	Period circadian clock 2	ATCTATCTGTGCTGCTGGTC	ACTGGTGATGTCTCGTTCC
Chrono	Circadian associated repressor of transcription	GCATTGGTGTCATCCTTGTC	TTAGTCATCTCTCTGTCTGTGG
Rev-erbα	Reverse strand of protein ERB alpha	ACACACTCTCTGCTCTTC	GACCTTGACACAAACTGG
Rorα	Retinoic acid-related orphan receptor alpha	AGGTGGTGTTTATTAGGATGTG	TCTTCTCGGTGGTTCTTCT
β-MHC	Myosin, heavy polypeptide 7, cardiac muscle, beta	CAAGCGGAAGCTGGAGGGA	CCTCGATGCGTGCCTGAAG
IL-1α	Interleukin 1 alpha	AGCCCGTGTTGCTGAAGGAGT	CCGACTTTGTTCTTTGGTGGCA
IL-6	Interleukin 6	AAAGCCAGAGTCCTTCAGAGAGA	GGAGAGCATTGGAAATTGGGGTA
TNF-α	Tumor necrosis factor alpha	AGCCCACGTCGTAGCAAACC	GGTGAGGAGCACGTAGTCGG
β-actin	Beta-actin	GCTGTCCCTGTATGCCTCTG	CGCTCGTTGCCAATAGTGATG

Table 4. List of primers used in RT-PCR assay.

Number of cycles	Step	Duration	Temperature
1	Denaturalization	10 min	96 ºC
40	DNA Replication	15 s	95 ºC
		1 min	55 ºC

Table 5. RT-PCR cycle program

5. MAGNETIC RESONANCE IMAGING

The magnetic resonance experiments were carried out on a small-animal horizontal 7 Tesla USR Bruker BioSpec TM 70/20 USR magnet (Ettlingen, Germany). Before imaging, mice were anesthetized with isoflurane (1.5% in air), and the breathing rate was monitored using an air balloon placed on top of the lungs (SA Instruments, Inc., New York, NY). The respiration rates between animals were similar for every experiment. For heart imaging, the animals were placed in prone and supine positions, with placement of non-magnetic metallic or carbon-fiber Electrocardiogram (ECG) electrodes on the front paw and limbs. Coronal CINE was acquired using a CINE sequence with the following parameters: Time to Echo (TE) = 1.6 ms, Time to Repetition (TR) = 8 ms, number of averages = 1, flip angle = 15.0, slice thickness = 0.8 mm, image size = 192x192, field-of-view = 25x25 mm^2. Analysis of heart length, left ventricular lumen length and left ventricular wall thickness were applied on the acquired images.

6. TISSUE PREPARATION FOR LIGHT MICROSCOPY

For histological analysis, animals were weighed and anaesthetized by intraperitoneal injection of equithesin (1 mL/kg). After confirming complete anaesthetization through loss of all reflexes, animals were transcardially perfused with warm saline followed by trump's fixative (3.7% formaldehyde plus 1% glutaraldehyde in saline buffer). The heart was carefully dissected, weighted after removal of excessive connective tissues, and was fixed in the trump's fixative. Part of the LV was immersed in bouin's solution for further histological analysis, while other part was fixed for further transmission electron microscopy (TEM) analysis.

After proper fixation, samples of the LV were washed in ethanol 70% (3 times x 24h each time), dehydrated in ascending graded concentrations of ethanol, cleared in xylene, and then embedded in the paraffin wax. Sections 4μm-thick were cut with a SLEE Mainz Cut 5062 microtome, dewaxed in xylene (2 x 30 minutes), rehydrated in descending concentrations of ethanol (100%, 95%, 80% and 70%), washed with distilled water, and were stained with Hematoxylin and Eosin (H&E) stain for general histological analysis and Van Gieson stain for differentiation of connective tissue and cardiac

muscle fibers. Sections were dehydrated in an ascending series of ethanol (70%, 95%, and 100%), cleared in xylene (2 x 10 minutes), and mounted with Dibutyl Phthalate Xylene (DPX). The sections were examined by a Carl Zeiss Primo Star Optic microscope, and digital images were acquired using a Magnifier AxioCamICc3 digital camera (BioSciences, Jena, Germany).

7. TRANSMISSION ELECTRON MICROSCOPY

Small pieces from the LV of experimental groups were fixed in a 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and post fixed in 0.1 M cacodylate buffer-containing 1% osmium tetraoxide with 1% potassium ferrocyanide for 1 hour. The samples were then immersed on 0.15% tannic acid for 50 seconds, incubated in 1% uranyl acetate for 1.5 hour with shaking, dehydrated in ethanol, and embedded in resin. Ultrathin sections of 65 nm thickness were cut using a Reichert-Jung Ultracut E ultramicrotome, doubled stained with uranyl acetate and lead citrate, and finally examined on a Carl Zeiss Leo 906E electron microscope.

8. FLUORESCENT DETECTION OF APOPTOTIC NUCLEI

For analysis of nuclear apoptosis in the heart, paraffin sections of 4μm-thickness were immersed in xylene, rehydrated in a descending series of ethanol, washed with distilled water, air-dried, rinsed in PBS 1X (2 x 5 minutes), and finally stained with 33258 Hoechst dye (H6024, Sigma-Aldrich, Madrid, Spain). After staining, sections were washed in PBS (5 x 5 minutes), air-dried, mounted, and examined with LEICA DM5500B fluorescent microscope. Acquired images were used for detection of the percentage of apoptotic nuclei. Hoechst dye is a fluorescent dye that penetrates the cellular nucleus and binds to DNA, enabling detection of apoptotic nuclei. Under a 350-nm wavelength light, this dye emits blue fluorescence that allows visibility of the nuclear DNA and observation of nuclear fragmentation or chromatin condensation.

9. MORPHOMETRICAL ANALYSES

Morphometrical analysis of cardiomyocytes number and cross-sectional area (CSA), as well as the percentage of the fibrotic area were performed using images of Van Gieson-stained paraffin cross sections (10 images, 40x objective, per animal). Moreover, intermyofibrillar (IMF) mitochondrial number, CSA, and Feret's diameter, as well as the percentage of the mitochondrial damage (as number of damaged mitochondria/total mitochondrial number·100) were analyzed on electron micrographs (5 images, 10000x, per animal). All these morphometrical analyses were analyzed by two double-blinded

investigators using Image J processing software and were represented as a percentage compared with the young group.

10. PROTEIN QUANTIFICATION

Determination of protein quantity was accomplished by utilizing the Bradford method (Bradford, 1976). This technique is based on the binding of a hydrophobic dye, Coomassie Blue G-250, to proteins. In acidic solution, the dye exists in two forms: orange, whose maximum absorption is at a wavelength between 465 and 470 nm, and blue, whose maximum absorption corresponds to a wavelength of 595 nm. Proteins bind to the blue form to establish a protein-dye complex, with an extinction coefficient greater than the free dye and which is easily detectable by spectrophotometric analysis. The absorbance variation of Coomassie Blue-G is proportional to the amount of protein bound dye, and consequently proportional to the protein concentration in a solution.

To determine the concentration of total protein present in the sample, a standard curve was made using BSA dissolved in 20 mM Tris at concentrations between 0.05 and 0.6 mg / mL. 10 µL of buffer (blank), 10 µL of each concentration of the standard curve, and 10 µL of sample are deposited in the respective wells of a microplate. A final addition of 200 µL of Bradford reagent (1/5 dilution) is added to each well and the plate is shaken for 15 min at RT. Lastly, the absorbance is measured at 595 nm in a plate spectrophotometer (Power-Wavex Microplate Scanning Spectrophotometer, BioTek Winooski, USA), expressing the results in mg of protein / mL.

11. STATISTIC ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). One-way ANOVA with a Tukey's post hoc test was used to compare the differences between experimental groups. The values were found to be significantly different when $p < 0.05$.

For circadian rhythm studies, Cosinor analysis (Nelson et al., 1979) was performed with the Time Series Analysis-Seriel Cosinor 6.3 Lab View software (TSASC 6.3; Expert Soft Technologies Inc, BioMedical Computing and Applied Statistics Laboratory, Esvres, France). Rhythm characterization includes the average level of three parameters, calculated with 95% confidence limits: the mesor (acronym for midline estimating statistic of rhythm, it is the mean of the oscillation), the amplitude (half the difference between the minimum and maximum of the fitted cosine function) and the acrophase (the timing of the cosine maximum). This procedure allows testing the null hypothesis that the amplitude of the cosine function is equal to zero. Rhythm detection was considered

statistically significant at $p < 0.05$. In addition, to evaluate the effect of the time point of mice sacrifice, age, melatonin treatment, and genotype in the expression of clock genes transcripts, multifactorial ANOVA analysis was performed with R software 2020 (RStudio, Inc., Boston, MA). Different post hoc test were performed according to the normality of the data.

RESULTS

CHAPTER 1: MELATONIN/NRF2/NLRP3 CONNECTION IN MOUSE HEART MITOCHONDRIA DURING AGING

1. NLRP3 deficiency prevents, and melatonin treatment restores cardiac muscle mitochondrial dynamics altered by aging

Anomalies in mitochondrial dynamics (fusion/fission) are typical of aged cardiac muscle (Wu et al., 2019). Here, we showed that aging induced a decrease in the levels of proteins involved in mitochondrial dynamics, including Mfn2, Opa1, and Drp1, in WT mice, an effect absent in NLRP3$^{-/-}$ mice (Figure 21 A-C). Melatonin supplementation counteracted the decline of Mfn2, Opa1, and Drp1 caused by aging in WT mice. Interestingly, no significant effect of melatonin was observed in fusion proteins Mfn2 and Opa1 in NLRP3$^{-/-}$ mice at the age of 12 and 24 months (Figure 21 A, B). A slight, but not significant enhancement in fission protein Drp1 was noted in EA and OA NLRP3$^{-/-}$ mice with melatonin supplementation (Figure 21 C).

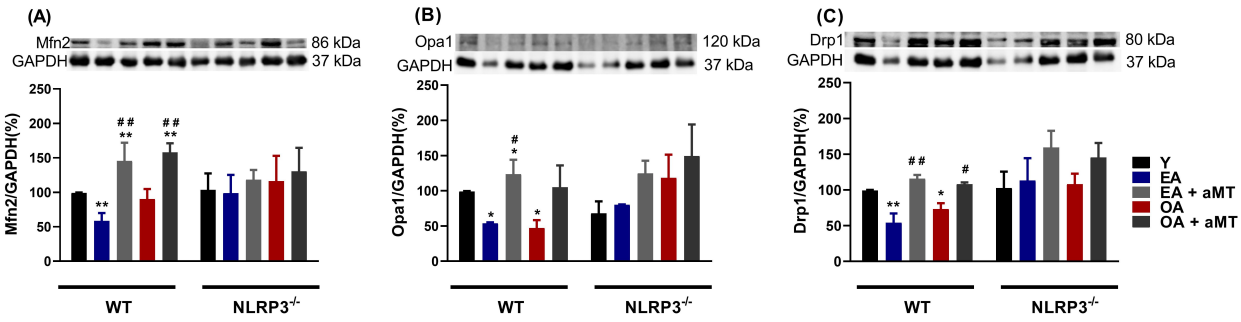


Figure 21. Changes in mitochondrial dynamics (fusion/fission) in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. ***(A)*** *Protein levels of Mfn2.* ***(B)*** *Protein levels of Opa1.* ***(C)*** *Protein levels of Drp1. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01 vs. Y; #p<0.05, ##p<0.01 vs. group without melatonin treatment.*

2. NLRP3 deficiency and melatonin therapy had minimal effects in autophagy in cardiac muscle during aging

A drop in the autophagic capacity observed in cardiac aging is associated with the accumulation of dysfunctional mitochondria, exaggerated ROS production, and mtDNA release (Wu et al., 2019; Zhou et al., 2011). Unsurprisingly, the conversion of LC3I to LC3II, a hallmark of autophagy (Lee et al., 2011), was significantly reduced in WT mice during aging, as reflected in the decrease in the LC3II/LC3I ratio in WT EA and OA mice (Figure 22). LC3II/LC3I ratio trends to increase in NLRP3$^{-/-}$ EA and OA mice, which may explain the attempt to restore autophagy events. Melatonin administration had minimal effects on the LC3II/LC3I ratio in all cases.

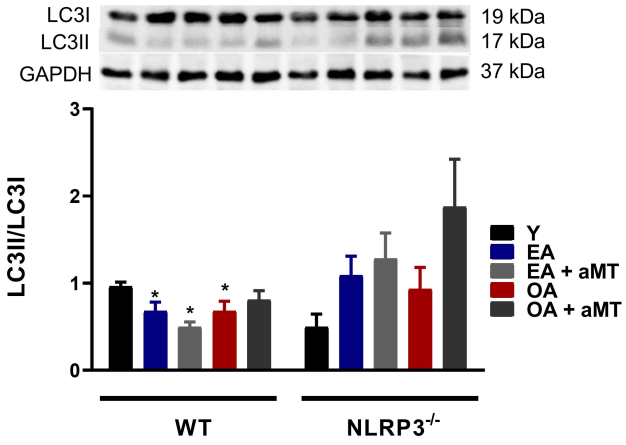


Figure 22. Changes in autophagy in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. *LC3II/LC3I ratio. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05 vs. Y.*

3. Melatonin treatment and, to a lesser extent NLRP3 deficiency, reduced apoptosis in cardiac muscle during aging

Despite being intensively studied over the past three decades, many of the mechanisms of apoptotic cell death remain unknown. Although the relationship between aging and apoptosis have been a subject of controversy in scientific community, there seems to be consensus that apoptosis plays a significant role in cardiac aging (Quarles et al., 2015). Here, we showed that aging induced a rise in the levels of some proteins involved in apoptotic processes, including p53 and caspase 9 in both WT and NLRP3$^{-/-}$ mice. Melatonin treatment significantly diminished the levels of p53 and caspase 9 in EA WT mice and in EA and OA mutant mice (Figure 23 A, B). The pro-apoptotic protein Bax and the anti-apoptotic Bcl2 were significantly enhanced by aging in WT mice. Mutant mice only showed Bcl2 increased in OA animal's group (Figure 23 C, D). We observed a slight rise in Bax/Bcl2 ratio in EA and a significant increase in WT OA mice (Figure 23 E). The absence of NLRP3, however, prevented the apoptotic process associated with aging since Bax/Bcl2 ratio remained at similar levels as that of Y mutant mice. Melatonin supplementation significantly decreased the Bax/Bcl2 ratio in EA and OA WT mice, but had no effect in NLRP3$^{-/-}$ mice.

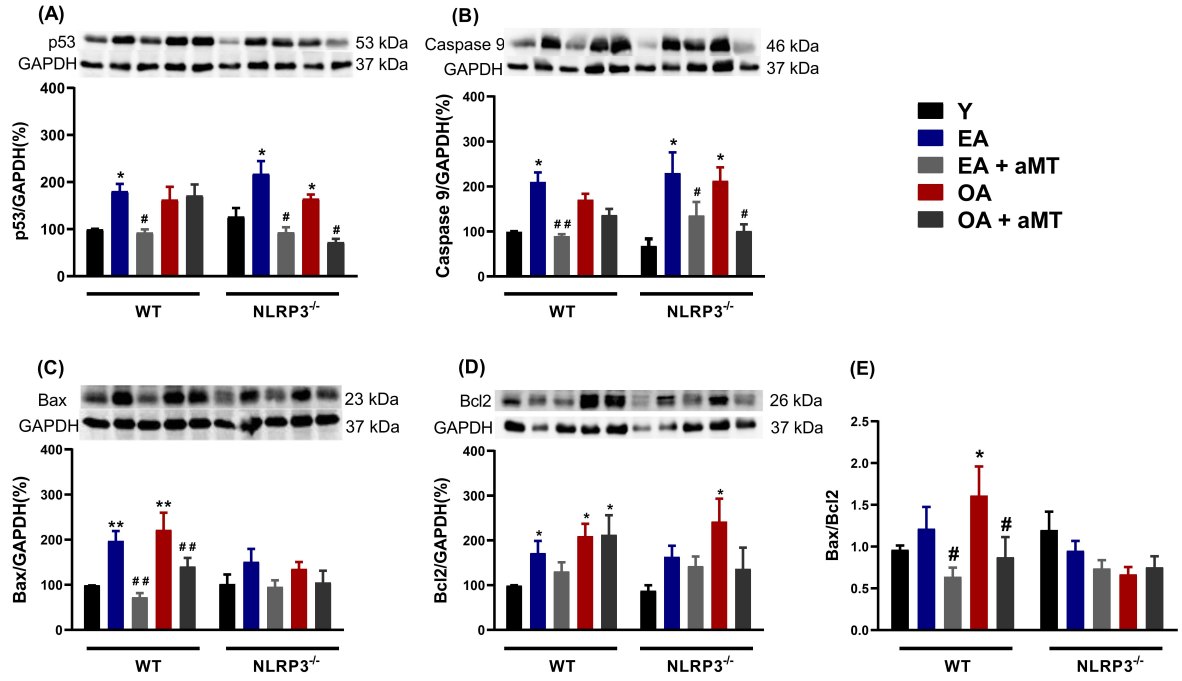


Figure 23. Changes in apoptosis in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. ***(A)*** *Protein levels of p53.* ***(B)*** *Protein levels of caspase 9.* ***(C)*** *Protein levels of Bax.* ***(D)*** *Protein levels of Bcl2.* ***(E)*** *Bax/Bcl2 ratio. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01 vs. Y; #p<0.05, ##p<0.01 vs. group without melatonin treatment.*

4. Melatonin treatment, but not NLRP3 deficiency, recovered the Nrf2-dependent antioxidant capacity in cardiac muscle declined by aging

In recent years, emerging evidence has indicated that aging leads to a gradual reduction of the Nrf2-dependent antioxidant response, which in turn contributes to the accumulation of oxidative stress (Schmidlin et al., 2019; Zhang et al., 2015). Our results showed a significant decrease in the protein levels of Nrf2 and its active form phosphorylated Nrf2 (pNrf2) (Ser40) in WT and NLRP3$^{-/-}$ mice with age, suggesting that NLRP3 deficiency was unable to ameliorate the age-related decline of Nrf2 and pNrf2 (Ser40) in these animals (Figure 24 A, B). Melatonin supplementation markedly recovered the levels of Nrf2 and pNrf2 (Ser40) in both WT and mutant EA and OA mice. Aging and melatonin therapy did not significantly modify the levels of the Nrf2 inhibitor, Keap1, in either mouse strain (Figure 24 C). Hmox1, Nqo1, and γGclc, three cytoprotective enzymes transcriptionally regulated by Nrf2, also remarkably decreased in WT OA mice (Figure 24 D-F). The levels of Hmox1 and γGclc significantly dropped in NLRP3$^{-/-}$ EA and OA mice (Figure 24 D, F). Protein content of Nqo1 enzyme was not modified by aging in mutant animals (Figure 24 E). Again, melatonin treatment greatly enhanced the levels of Hmox1, Nqo1, and γGclc in WT and NLRP3$^{-/-}$ mice.

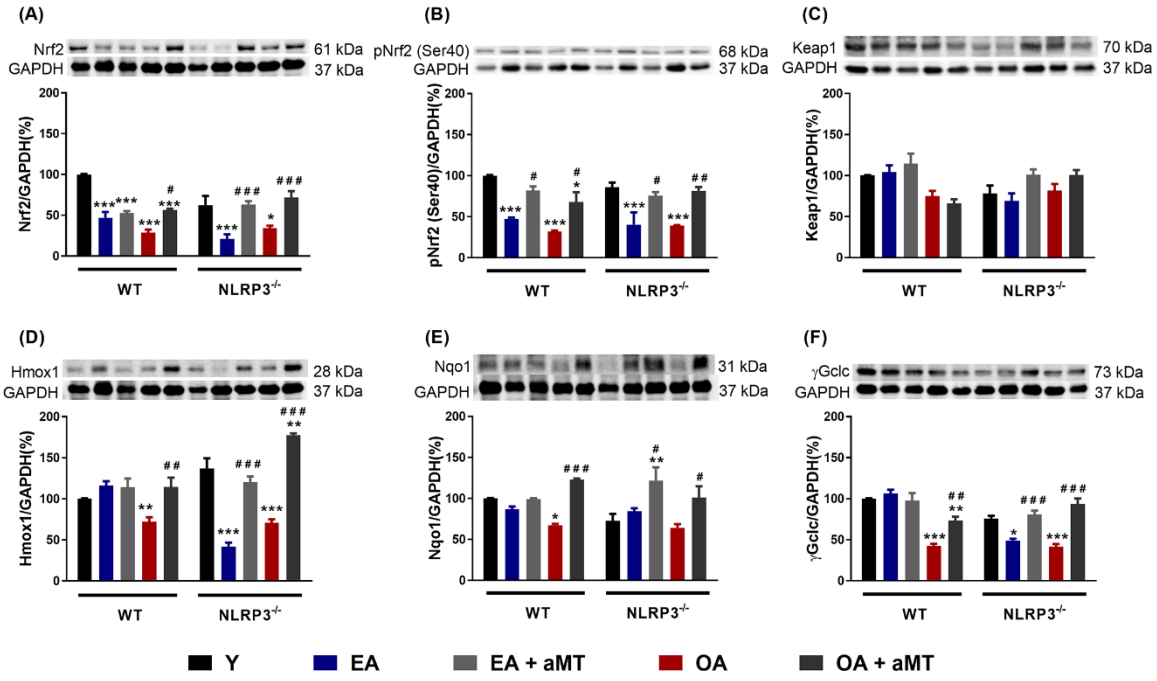


Figure 24. Changes in the Nrf2-dependent antioxidant pathway in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. ***(A)*** *Protein levels of Nrf2.* ***(B)*** *Protein levels of pNrf2 (Ser40).* ***(C)*** *Protein levels of Keap1.* ***(D)*** *Protein levels of Hmox1.* ***(E)*** *Protein levels of Nqo1.* ***(F)*** *Protein levels of γGclc. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01, ***p<0.001 vs. Y; #p<0.05, ##p<0.01, ###p<0.001 vs. group without melatonin treatment.*

5. NLRP3 deficiency and melatonin therapy improved mitochondria ultrastructure altered by age in cardiac muscle

Transmission electron microscopy of the cardiac muscles of Y WT mice revealed presence of normally intact and compacted mitochondria with clearly organized cristae distributed in the intermyofibrillar spaces (Figure 25 A, B). At the age of 12 months (EA), most of these mitochondria were found to be normal; however, a few showed cristae damage (Figure 25 C, D). These changes were exacerbated, and numerous mitochondria were severely damaged, hypertrophied, and vacuolated with completely destroyed cristae in WT OA mice (Figure 25 G, H). Melatonin supplementation, however, preserved the normal ultrastructure of the cardiac mitochondria in EA (Figure 25 E, F) and OA WT mice (Figure 25 I, J) maintaining their healthy and compact appearance.

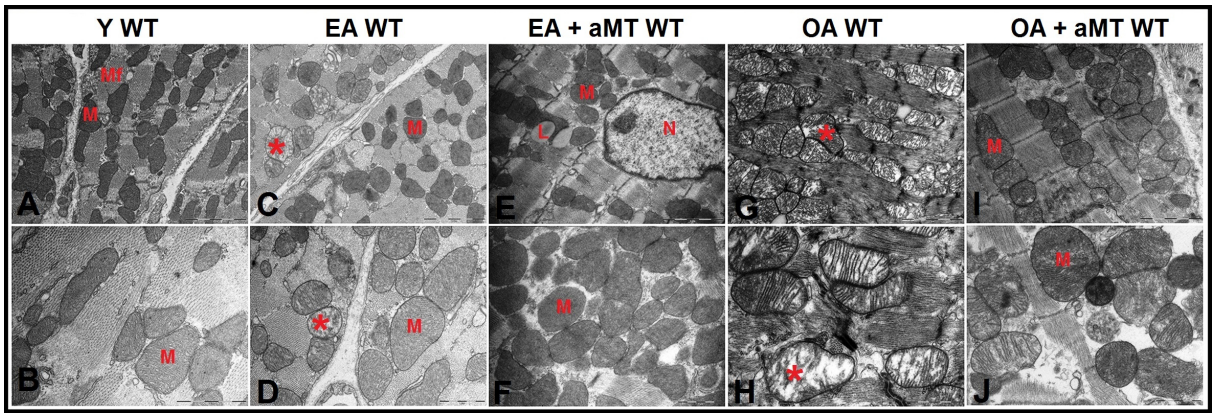


Figure 25. Age-associated ultrastructural changes of mitochondria in cardiac muscle fibers of WT mice and melatonin treatment. ***(A, B)*** *Electron micrographs of cardiac muscle fibers of Y WT mice revealing presence of normally intact and compacted mitochondria (M) distributed among myofibrils (Mf).* ***(C, D)*** *Electron micrographs of cardiac muscle fibers of EA WT mice demonstrating presence of normal mitochondria (M) with few demonstrating cristae damage (asterisk).* ***(E, F)*** *Electron micrographs of cardiac muscle fibers of EA + aMT WT mice showing the protective effect of melatonin supplementation in preserving normal mitochondrial structure (M) with presence of lipid droplets (L), N; nucleus.* ***(G, H)*** *Electron micrographs of cardiac muscle fibers of OA WT mice clarifying the presence of numerous severely damaged hypertrophied vacuolated mitochondria with completely destructed cristae (asterisk).* ***(I, J)*** *Electron micrographs of cardiac muscle fibers of OA + aMT WT mice exhibiting the beneficial effect of melatonin supplementation in keeping normal mitochondrial architecture (M). (A, C, E, G, I): bar = 2 µm and (B, D, F, H, J): bar = 1 µm.*

Cardiac muscle fibers of NLRP3$^{-/-}$ Y mice presented normal highly compacted mitochondria with densely packed cristae (Figure 26 A, B). Mitochondrial structure did not change in EA mice, except one that showed damage in peripheral cristae (Figure 26 C, D). The mitochondrial damage was less prevalent at 24 months in comparison with WT OA mice. Mitochondria were characterized by their widely separated and organized cristae, with presence of small-sized membranous vacuoles of possibly autophagic nature (Figure 26 G, H). Melatonin treatment exhibited an obvious protective effect at the age of 12 (Figure 26 E, F) and 24 months (Figure 26 I, J), where it kept normal mitochondrial architecture with aging, in addition to formation of multivesicular bodies, which reflect the induction of the autophagic processes.

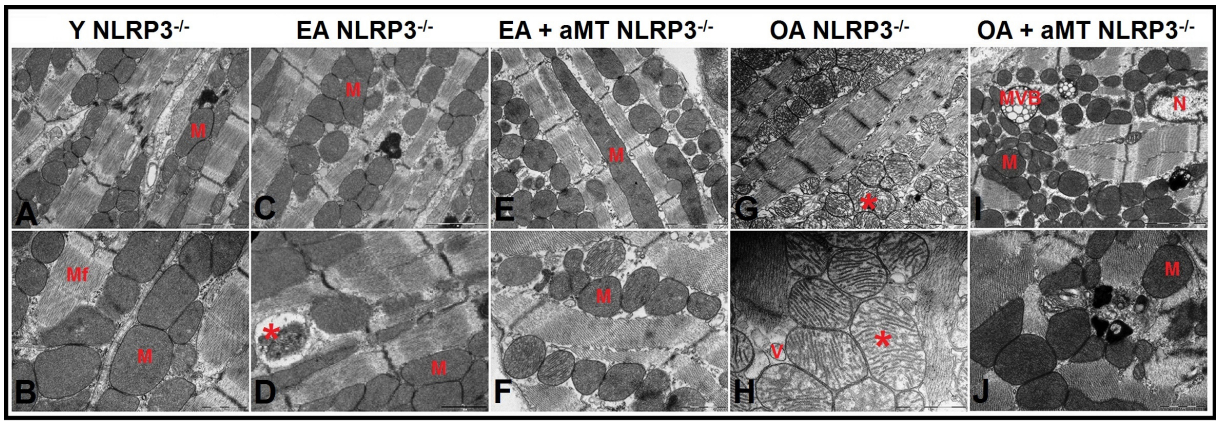


Figure 26. Age-related ultrastructural changes of mitochondria in cardiac muscle fibers of NLRP3$^{-/-}$ mice and melatonin treatment. ***(A, B)*** *Electron micrographs of cardiac muscle fibers of Y NLRP3$^{-/-}$ mice showing presence of normally highly compacted mitochondria with densely packed cristae (M) distributed among myofibrils (Mf).* ***(C, D)*** *Electron micrographs of cardiac muscle fibers of EA NLRP3$^{-/-}$ mice demonstrating intact mitochondria (M) with individual ones depicting damaged peripherally cristae (asterisk).* ***(E, F)*** *Electron micrographs of cardiac muscle fibers of EA + aMT NLRP3$^{-/-}$ mice revealing the clearly apparent prophylactic effect of melatonin supplementation in keeping normal mitochondrial architecture (M) with aging.* ***(G, H)*** *Electron micrographs of cardiac muscle fibers of OA NLRP3$^{-/-}$ mice indicating less detectable mitochondrial damage compared with WT mice, with presence of numerous mitochondria showing widely separated organized cristae (asterisk) and small-sized membranous vacuoles of possibly autophagic nature (V).* ***(I, J)*** *Electron micrographs of cardiac muscle fibers of OA + aMT NLRP3$^{-/-}$ mice showing the protective effect of melatonin supplementation in preserving normal mitochondrial structure (M), with formation of multivesicular bodies (MVB), which reflect the induction of the autophagic processes, N; nucleus. (A, C, E, G, I): bar = 2 μm and (B, D, F, H, J): bar = 1 μm.*

6. Lack of NLRP3 reduced mitochondria number loss and mitochondrial damage, an effect shared by melatonin

Morphometric analysis of cardiac mitochondria revealed that mitochondrial number exhibited initial non-significant decline in cardiac muscles of WT and NLRP3$^{-/-}$ EA mice. Nevertheless, mitochondrial number was significantly decreased in OA, being more pronounced in WT mice than NLRP3$^{-/-}$ one, an effect significantly counteracted after melatonin therapy (Figure 27 A). Furthermore, the percentage of the mitochondrial damage was significantly increased in aged mice, especially in WT animals, and it was counteracted by melatonin supplementation (Figure 27 B). Morphometrical analysis of the mitochondrial CSA illustrated a non-significant increase in cardiac muscle of WT and NLRP3$^{-/-}$ EA mice, whereas the former increased in aged animals (Figure 27 C). Mitochondrial diameter showed non-significant increase in WT EA mice, increasing in OA animals. NLRP3$^{-/-}$ mice revealed non-significant changes in mitochondrial diameter among all experimental groups (Figure 27 D).

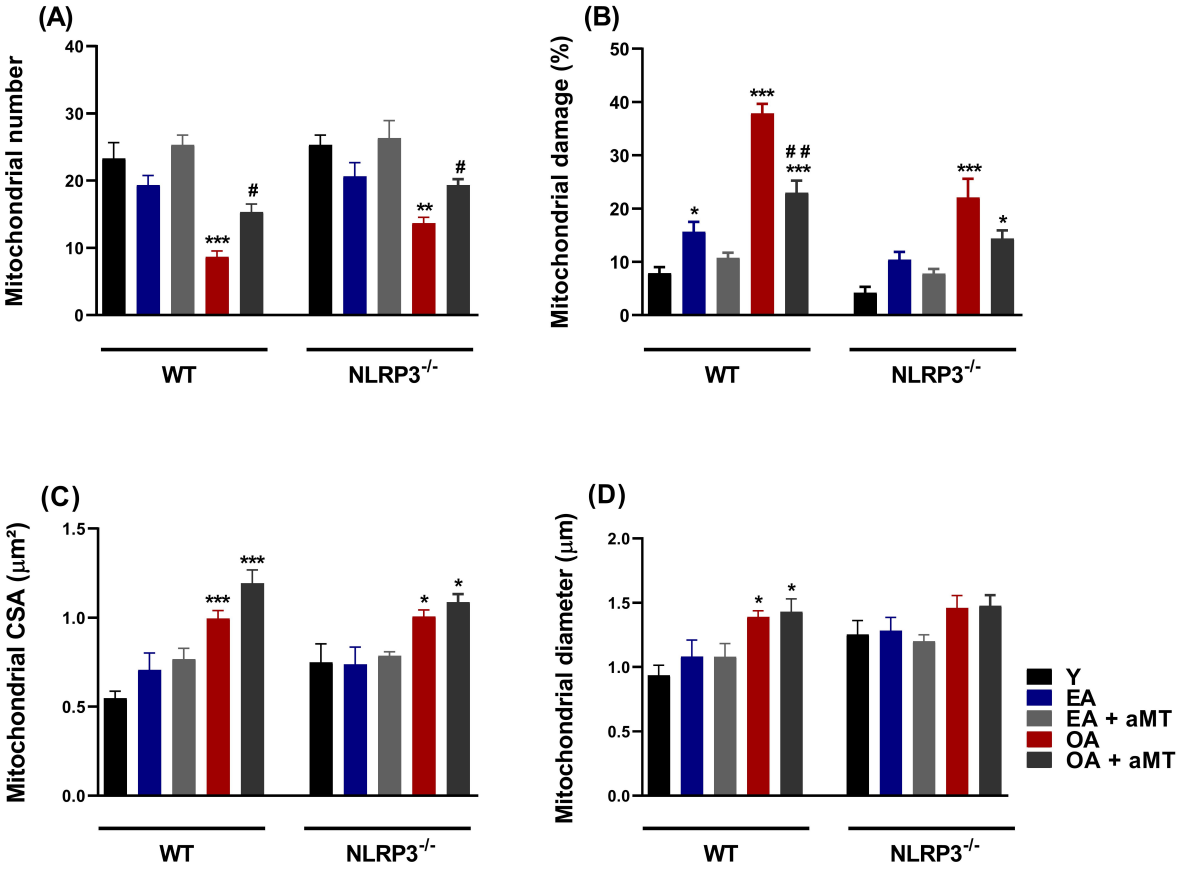
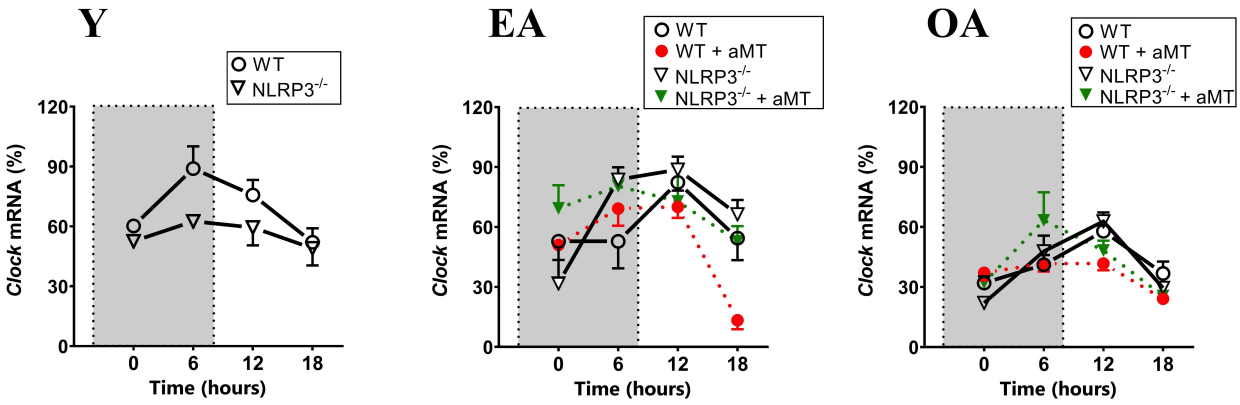


Figure 27. Age-associated morphometrical changes of intermyofibrillar mitochondria in cardiac muscle fibers of WT and NLRP3$^{-/-}$ mice and melatonin treatment. ***(A)*** *Analysis of mitochondrial number.* ***(B)*** *Analysis of mitochondrial damage percentage.* ***(C)*** *Analysis of cross section area (CSA, µm^2).* ***(D)*** *Analysis of mitochondrial Feret's diameter (µm). Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01, ***p<0.001 vs. Y; #p<0.05, ##p<0.01 vs. group without melatonin treatment.*

CHAPTER 2: AGING AND CHRONODISRUPTION IN MOUSE CARDIAC TISSUE. EFFECT OF THE NLRP3 INFLAMMASOME AND MELATONIN THERAPY

1. Time point, aging, melatonin treatment and, to a lesser extent NLRP3 inflammasome, had significant effects on the expression of clock genes in cardiac tissue

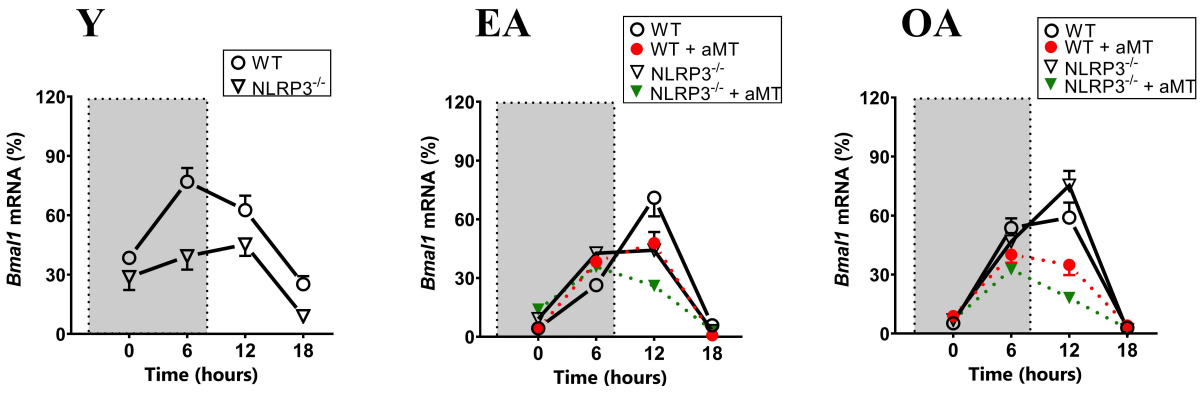
The Multifactorial-ANOVA evaluation of the expression of the gene *Clock* revealed a main effect of time point ($F_{(3,59)} = 81.80$, $p < 0.001$), age ($F_{(4,59)} = 47.60$, $p < 0.001$), genotype ($F_{(1,59)} = 9.87$, $p < 0.01$), and a significant time point x age x genotype interaction ($F_{(12,59)} = 6.33$, $p < 0.001$) in the heart (Figure 28). Data of significant differences in the relative expression of *Clock* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.



TRANSCRIPT: *Clock*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***		***Time point x Age x Genotype Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	$F_{(1,59)}$	P value	$F_{(12,59)}$	P value
81.80	<0.001	47.60	<0.001	9.87	<0.01	6.33	<0.001

Figure 28. Changes in the relative expression of the *Clock* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Clock transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.*

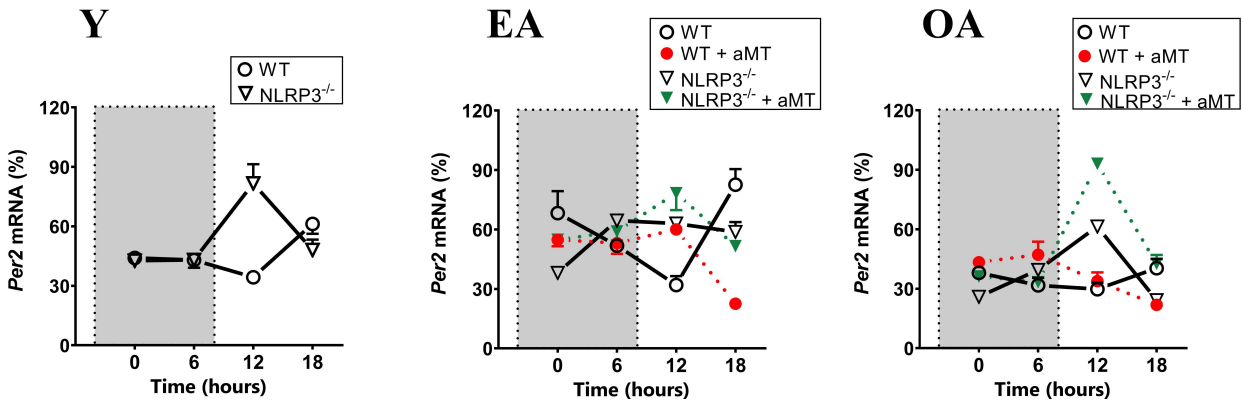
The Multifactorial-ANOVA analysis of *Bmal1* gene expression showed a main effect of time point ($F_{(3,59)} = 781.11$, $p < 0.001$), age ($F_{(4,59)} = 83.88$, $p < 0.001$), genotype ($F_{(1,59)} = 9.14$, $p < 0.05$), and a significant time point x age x genotype interaction ($F_{(12,59)} = 8.37$, $p < 0.001$) in cardiac muscle (Figure 29). Data of significant differences in the relative expression of *Bmal1* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Tukey multiple comparison of mean post-hoc test following ANOVA and are detailed in Annex 1.



TRANSCRIPT: *Bmal1*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***		***Time point x Age x Genotype Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	$F_{(1,59)}$	P value	$F_{(12,59)}$	P value
781.11	<0.001	83.88	<0.001	9.14	<0.05	8.37	<0.001

Figure 29. Changes in the relative expression of the *Bmal1* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Bmal1 transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Tukey multiple comparison of mean post-hoc test following ANOVA and are detailed in Annex 1.*

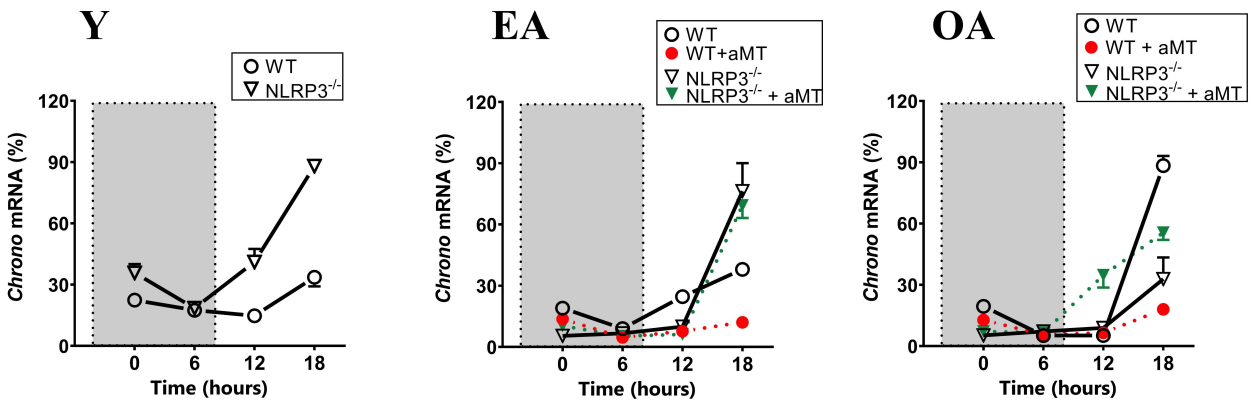
The Multifactorial-ANOVA calculation of *Per2* gene expression revealed a main effect of time point ($F_{(3,59)} = 23.31$, $p < 0.001$), age ($F_{(4,59)} = 38.11$, $p < 0.001$), genotype ($F_{(1,59)} = 42.17$, $p < 0.001$), and an important time point x age x genotype interaction ($F_{(12,59)} = 10.34$, $p < 0.001$) in the heart (Figure 30). Data of significant differences in the relative expression of *Per2* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Least Significant Difference post-hoc test and are detailed in Annex 1.



TRANSCRIPT: *Per2*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***		***Time point x Age x Genotype Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	$F_{(1,59)}$	P value	$F_{(12,59)}$	P value
23.31	<0.001	38.11	<0.001	42.17	<0.001	10.34	<0.001

Figure 30. Changes in the relative expression of the *Per2* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Per2 transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.*

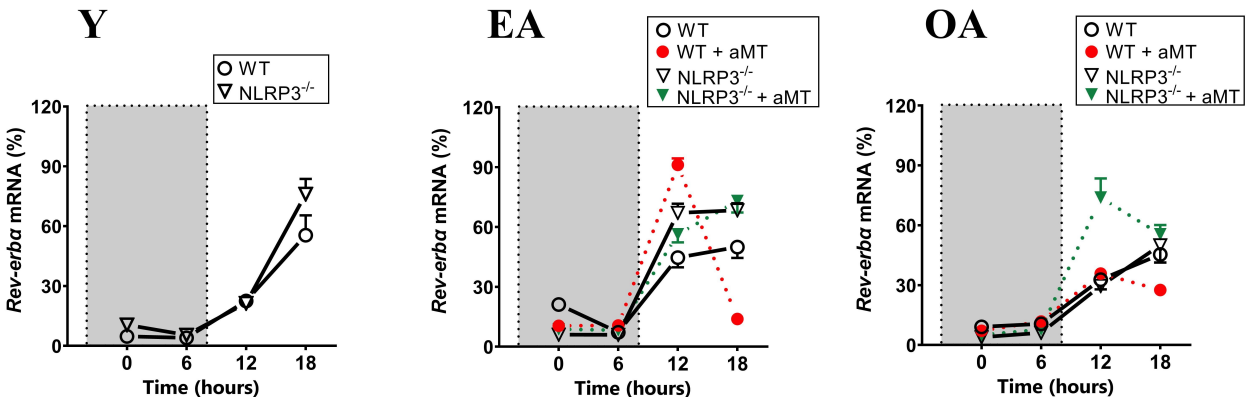
The Multifactorial-ANOVA analysis of *Chrono* gene expression indicated a main effect of time point ($F_{(3,59)} = 744.15$, $p < 0.001$), age ($F_{(4,59)} = 179.12$, $p < 0.001$), genotype ($F_{(1,59)} = 33.91$, $p < 0.001$), and a significant time point x age x genotype interaction ($F_{(12,59)} = 34.34$, $p < 0.001$) in cardiac muscle (Figure 31). Data of significant differences in the relative expression of *Chrono* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Least Significant Difference post-hoc test and are detailed in Annex 1.



TRANSCRIPT: *Chrono*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***		***Time point x Age x Genotype Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	$F_{(1,59)}$	P value	$F_{(12,59)}$	P value
744.15	<0.001	179.12	<0.001	33.91	<0.001	34.34	<0.001

Figure 31. Changes in the relative expression of the *Chrono* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Chrono transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.*

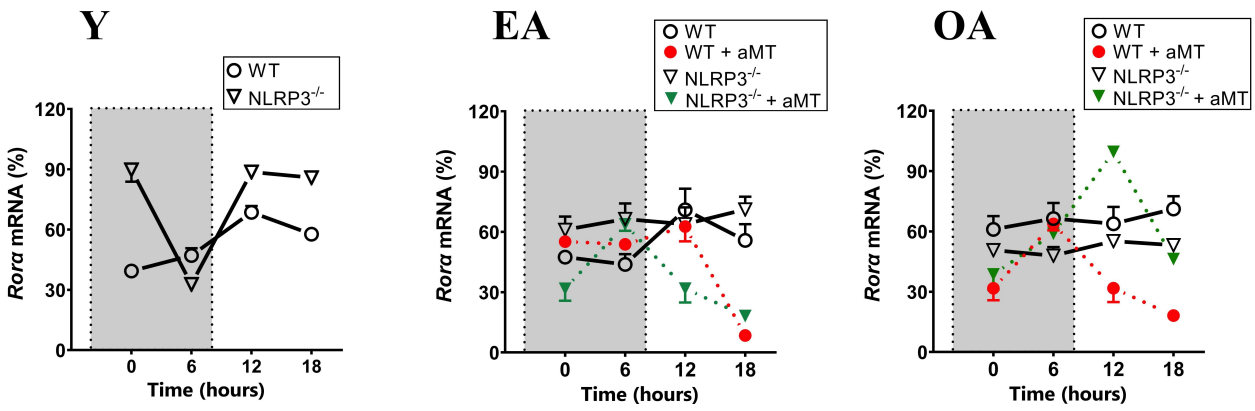
The Multifactorial-ANOVA evaluation of *Rev-erbα* gene expression revealed a main effect of time point ($F_{(3,59)} = 342.37$, $p < 0.001$), and age ($F_{(4,59)} = 8.38$, $p < 0.001$). Genotype had no impact in the changes of expression observed in *Rev-erbα* gene. Therefore, a significant time point x age interaction ($F_{(12,59)} = 8.02$, $p < 0.001$) was found in the myocardium (Figure 32). Data of significant differences in the relative expression of *Rev-erbα* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Least Significant Difference post-hoc test and are detailed in Annex 1.



TRANSCRIPT: *Rev-erbα*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***	***Time point x Age Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	No effect	$F_{(12,59)}$	P value
342.37	<0.001	8.38	<0.001		8.02	<0.001

Figure 32. Changes in the relative expression of the *Rev-erbα* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Rev-erbα transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.*

The Multifactorial-ANOVA analysis of the expression of *Rorα* gene expression showed a main effect of time point ($F_{(3,59)} = 9.51$, $p < 0.001$), age ($F_{(4,59)} = 16.34$, $p < 0.001$), genotype ($F_{(1,59)} = 19.92$, $p < 0.001$), and a significant time point x age x genotype interaction ($F_{(12,59)} = 14.02$, $p < 0.001$) in cardiac muscle (Figure 33). Data of significant differences in the relative expression of *Rorα* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment at every time point were evaluated by Least Significant Difference post-hoc test and are detailed in Annex 1.



TRANSCRIPT: *Rorα*. MULTIFACTORIAL ANOVA							
Main effect of Time point		***Main effect of Age***		***Main effect of Genotype***		***Time point x Age x Genotype Interaction***	
$F_{(3,59)}$	P value	$F_{(4,59)}$	P value	$F_{(1,59)}$	P value	$F_{(12,59)}$	P value
9.51	<0.001	16.34	<0.001	19.92	<0.001	14.02	<0.001

Figure 33. Changes in the relative expression of the *Rorα* transcript in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. Main effect of time point, age, genotype and their interaction. *Relative expression of the Rorα transcript in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice and their daily patterns at 4 time points (00:00, 06:00, 12:00 and 18:00 h) under a 12 h/12 h light/dark cycle. The shaded region on each graph represents constant darkness. Data are expressed as means ± SEM (n = 6 animals/group) (upper). The effect of time point, age, genotype and interactions were analyzed by Multifactorial-ANOVA (lower). Data of significant differences among the experimental groups were evaluated by Least Significant Difference post-hoc test following ANOVA and are detailed in Annex 1.*

2. Cosinor analysis revealed that aging and NLRP3 inflammasome did not have a great impact on the rhythm and changes in acrophase of clock genes in mice heart. Melatonin therapy restored the loss of rhythm and acrophase

The gene *Clock* presented an acrophase at 7.53 h in WT Y mice (Figure 34). This acrophase was significantly delayed until 12.2 h in WT EA mice and 11.4 h in WT OA. In both cases, melatonin was able to counteract this delay in acrophase, putting it back at 7 h. Rhythm was observed in all experimental groups, although it is remarkable that it decreased in EA and OA (Annex 2). Melatonin reestablished the rhythm in EA mice, to values like those of Y mice. A similar trend to that of WT mice was observed in NLRP3$^{-/-}$ mice. The acrophase of *Clock* in Y mice averaged at 7.80 h. A phase delay was also observed with EA and OA mice. This phase delay was not as pronounced as in WT mice, with an acrophase at 10.90 h in EA mice and at 10.40 h in OA mice. In both cases, melatonin corrected this phase, restoring it to around 7 h (Figure 34). Rhythm was appreciated in all experimental groups (Annex 2).

The acrophases of *Bmal1* showed some similarity with respect to the acrophases of the gene *Clock*. In WT Y mice, the acrophase of *Bmal*1 is located at 8 h, it is slightly delayed in EA mice until 11 h, and it is corrected again with melatonin, where it is located around 9 h. It was observed that both the acrophase and the rhythm of *Bmal1* barely change with aging or with melatonin treatment (Figure 34). The acrophase of *Bmal1* also appeared at 8 h in NLRP3$^{-/-}$ Y. Although some tendency to phase delay was observed in EA and OA mice, aging did not cause significant changes in the acrophase of *Bmal1* in the mutant mice (Figure 34). The treatment with melatonin advanced the acrophase in both cases, placing it closer to that of the Y mice. The existence of rhythm was observed in all the experimental groups, both in WT and in NLRP3$^{-/-}$ (Annex 2).

The *Per2* gene had an acrophase close to 20 h in WT Y mice, remaining constant with age in EA and OA. Interestingly, melatonin significantly advanced this acrophase at 12 and 24 months, with values close to 7 hours and 5 hours, respectively (Figure 34). It is also observed that *Per2* lost its rhythm in WT OA mice, which was restored with melatonin (Annex 2). In mice deficient in NLRP3, no prominent changes were observed with age or with melatonin therapy (Figure 34). It should be noted that the acrophase in this case was different with respect to the WT mice, occurring around 12 h. There was rhythm in all the experimental groups (Annex 2).

The acrophase of the gene *Chrono* was around 20 h in the WT Y mice. No notable changes were observed with aging or with melatonin treatment (Figure 34). The rhythm was maintained in all the experimental groups (Annex 2). In the mutant mice, the acrophases showed a trend like that observed in the WT mice. In this case, acrophase was found at around 18 h in Y mice, remaining constant throughout aging and with melatonin therapy (Figure 34). Mutant mice treated with

melatonin showed a phase advanced regarding WT. There was rhythm in all the experimental groups (Annex 2).

The gene *Rev-erbα* had an acrophase around 17 h in WT Y mice (Figure 34). This acrophase remained constant during aging. Interestingly, melatonin produced a phase advance in EA and OA. Rhythm was observed in all experimental groups (Annex 2). The acrophase values for *Rev-erbα* in the NLRP3$^{-/-}$ mice were quite like those in the WT mice (Figure 34). With aging, there appears to be a trend towards phase advancement. Melatonin had little effect on mutant mice. Again, there was rhythm in all experimental groups (Annex 2).

Rorα acrophase occurred at nearly 12 h in WT Y mice (Figure 34). Rhythm was lost in WT EA and WT OA mice. Melatonin recovered the rhythm and advanced the acrophase by 5-6 h in both experimental groups (Annex 2). In the case of NLRP3$^{-/-}$ mice, acrophase appeared at 18 h in Y mice (Figure 34). As in WT mice, the rhythm was lost at 12 and 24 months. Again, melatonin regained rhythm and produced a phase advance of an equivalent duration to that observed in WT mice (Annex 2).

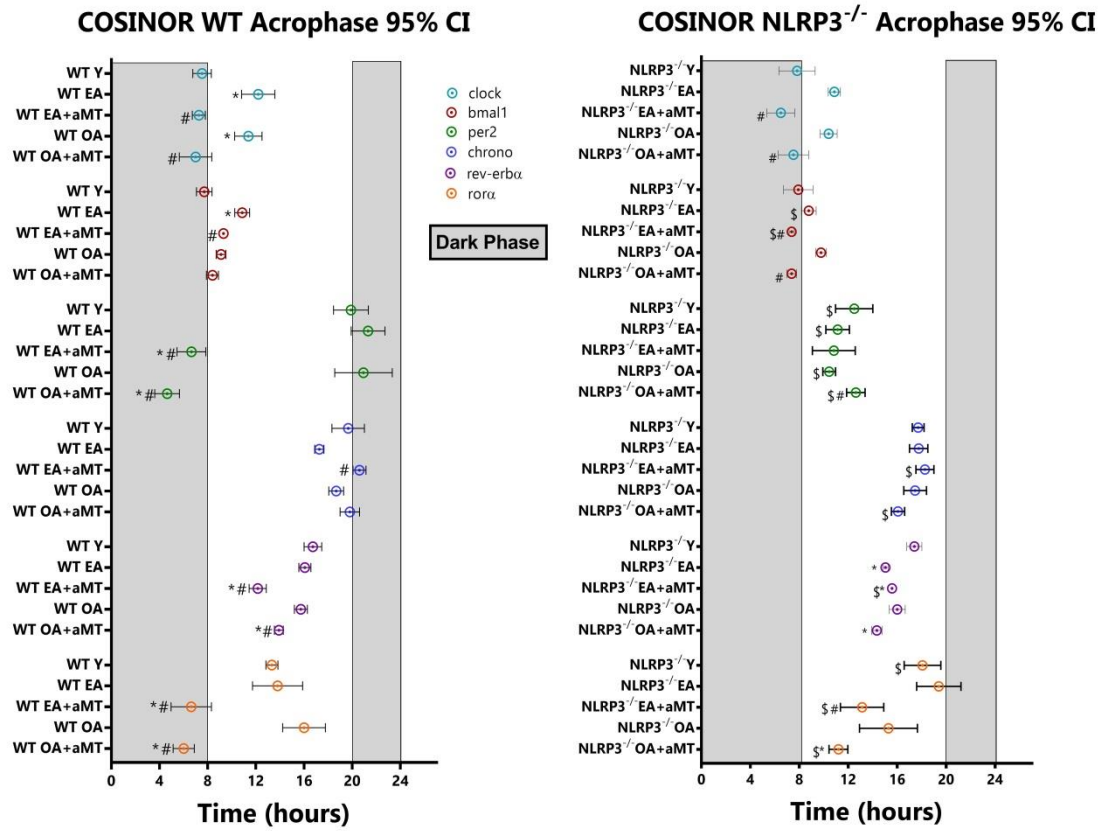


Figure 34. Acrophase charts showing peaks of fitted 24 h cosine for clock genes analyzed in heart of WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. *Acrophase of clock genes Clock (light blue plots), Bmal1 (red plots), Per2 (green plots), Chrono (dark blue plots), Rev-erbα (purple plots) and Rorα (orange plots) in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged*

*with melatonin (OA + aMT) wild type (left chart) and NLRP3$^{-/-}$ mice (right chart). The shaded region on each graph represents constant darkness. Data are expressed as means ± confidence interval (CI) of the Acrophase when p was ≤ 0.05 from the non-zero amplitude. Data of significant differences among the experimental groups were evaluated by overlapping of confidence interval and are detailed in Annex 2. *p<0.05 vs. Y; # p<0.05 vs. group without melatonin treatment; $ p<0.05 vs. WT mice.*

3. Cosinor analysis revealed that aging, the absence of NLRP3 inflammasome, and melatonin treatment had little impact on the amplitude of clock genes in heart muscle

The amplitude of the gene *Clock* remained constant with age and with melatonin treatment in WT mice (Figure 35). In mutant mice, the amplitude increased significantly in EA and in OA vs Y mice. Melatonin decreased this amplitude to values like Y group in NLRP3$^{-/-}$ EA mice (Figure 35).

The amplitude of *Bmal1* was invariable with aging in WT mice (Figure 35). It decreased significantly in WT OA + aMT vs WT OA mice. In NLRP3$^{-/-}$ mice, the amplitude increased at 24 months and melatonin decreased the amplitude to values like Y mice (Figure 35).

No changes were observed in the amplitude of the *Per2* gene in WT or NLRP3$^{-/-}$mice (Figure 35). This parameter remained constant with age, melatonin treatment, and the absence of the NLRP3 inflammasome.

The amplitude of *Chrono* increased in WT OA vs WT Y. Melatonin restored this amplitude (Figure 35). However, in the mutant mice, the amplitude decreased in NLRP3$^{-/-}$ OA vs NLRP3$^{-/-}$ Y. Again, melatonin reestablished this amplitude (Figure 35). It should be added that the amplitude of the mutant mice was higher than WT mice.

No noteworthy changes were observed with aging or melatonin treatment in the amplitude of the *Rev-erbα* gene in WT mice or NLRP3$^{-/-}$ mice (Figure 35).

The amplitude of *Rorα* gene did not undergo significant changes with age or with melatonin treatment in WT mice (Figure 35). A decrease in the amplitude was observed in NLRP3$^{-/-}$ OA vs NLRP3$^{-/-}$ Y mice, which was mended with melatonin (Figure 35).

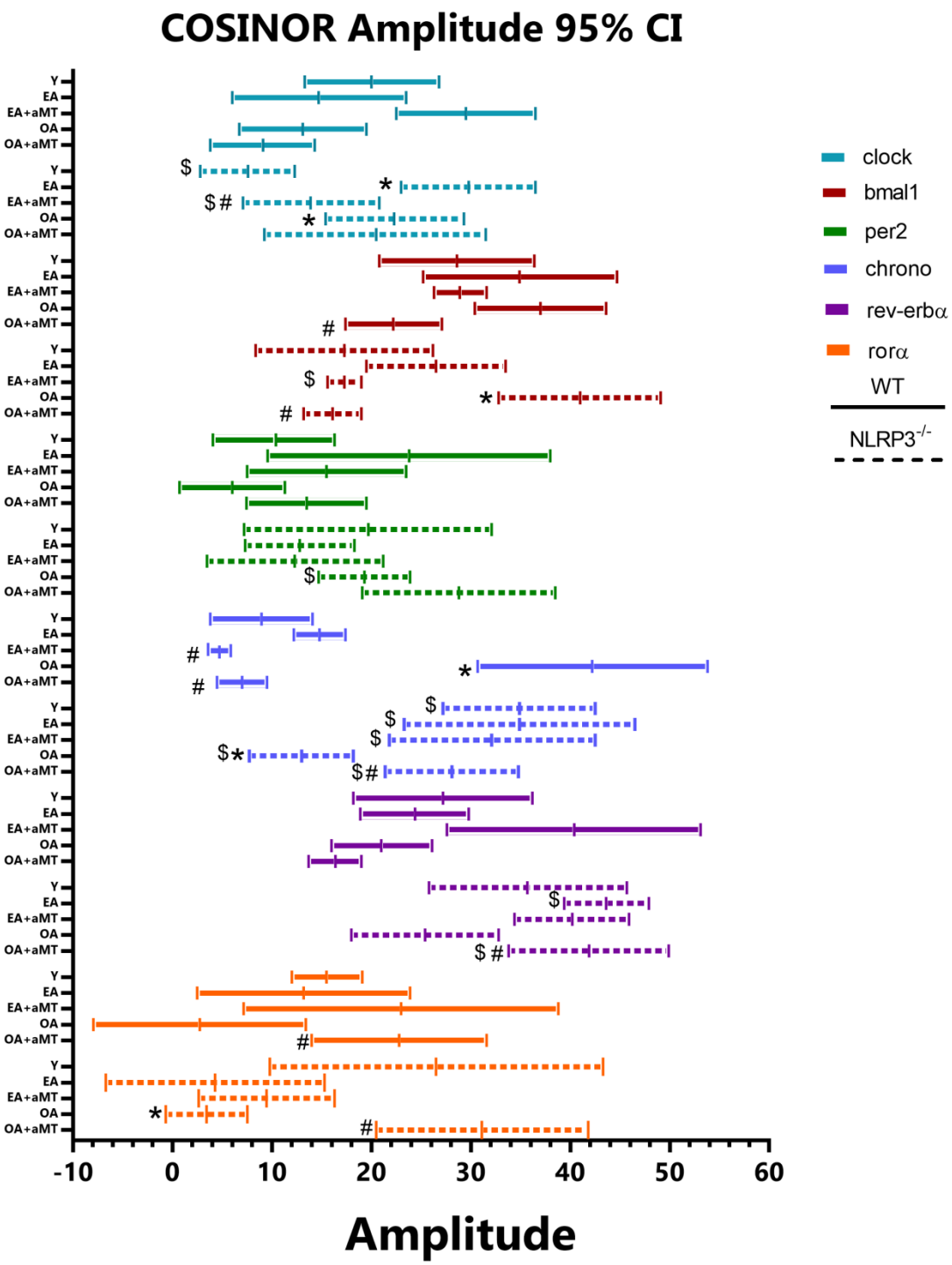


Figure 35. Amplitude chart showing peaks of fitted 24 h cosine for clock genes analyzed in heart of WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. *Amplitude of clock genes Clock (light blue lines), Bmal1 (red lines), Per2 (green lines), Chrono (dark blue lines), Rev-erbα (purple lines) and Rorα (orange lines) in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type (solid line) and NLRP3$^{-/-}$ mice (dashed line). The shaded region on each graph represents constant darkness. Data are expressed as means ± confidence interval (CI) of the Amplitud when p was ≤ 0.05 from the non-zero amplitude. Data of significant differences among the experimental groups were evaluated by overlapping of confidence interval and are detailed in Annex 2. *p<0.05 vs. Y; # p<0.05 vs. group without melatonin treatment; $ p<0.05 vs. WT mice.*

4. Cosinor analysis showed that aging caused significant changes in clock genes mesor, generally decreasing with age. Treatment with melatonin and the absence of NLRP3 had little effect on this parameter

The mesor of the gene *Clock* decreased with aging in WT mice (Figure 36). Melatonin had no effect. In NLRP3$^{-/-}$ mice, the mesor increased in group EA and decreased in OA (Figure 36). Melatonin therapy had no action.

In WT mice, the mesor of *Bmal1* declined with age. Melatonin did not counteract this outcome (Figure 36). The mesor remained constant with aging in mutant mice. Melatonin decreased mesor in NLRP3-deficient mice (Figure 36).

The mesor of *Per2* decreased with aging in WT OA vs WT Y mice. No effect of melatonin was observed (Figure 36). In the NLRP3$^{-/-}$ mice, a decrease in the mesor was again seen in the 24-month-old mice, although in this case melatonin restored the mesor to similar values to those of Y mice (Figure 36).

The mesor of the gene *Chrono* remained constant during aging in WT mice (Figure 36). Interestingly, melatonin significantly decreased this mesor. Mesor diminished with aging in NLRP3-deficient mice (Figure 36). Melatonin had no effect in mutant mice. It was further appreciated that, in general, the mesor had higher values in NLRP3$^{-/-}$ mice vs WT mice.

No changes were observed in the mesor of *Rev-erbα* gene derived from aging, treatment with melatonin or absence of the NLRP3 inflammasome (Figure 36).

The mesor of the *Rorα* increased in the WT OA vs WT Y mice (Figure 36). Melatonin decreased the mesor significantly in the OA group. In contrast, mesor was decreased in NLRP3$^{-/-}$ OA vs NLRP3$^{-/-}$ Y mice (Figure 36). Melatonin had no effect in mutant mice. Again, higher mesor values were seen in NLRP3 deficient mice compared to WT mice.

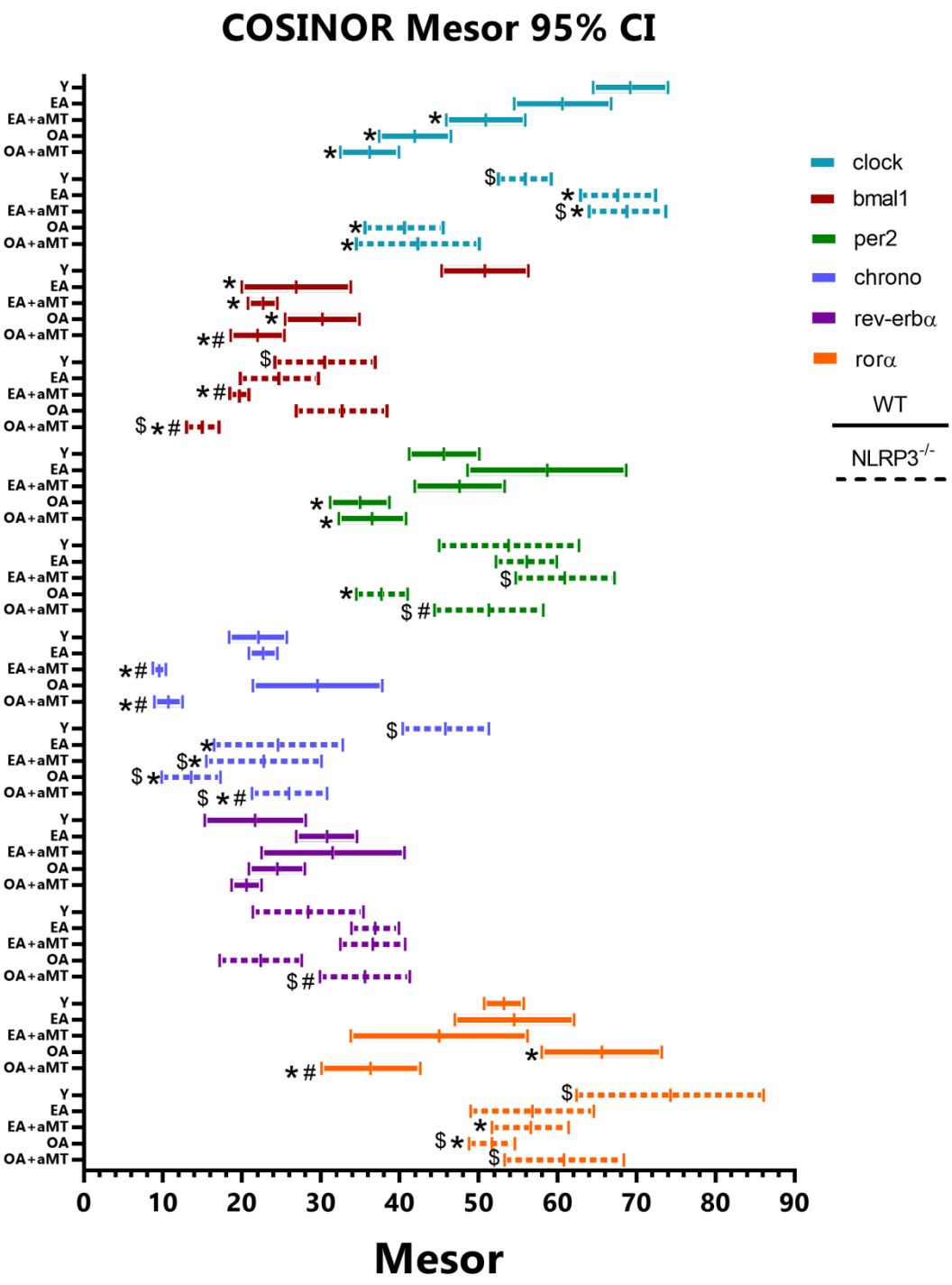


Figure 36. Mesor chart showing peaks of fitted 24 h cosine for clock genes analyzed in heart of WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. *Mesor of clock genes Clock (light blue lines), Bmal1 (red lines), Per2 (green lines), Chrono (dark blue lines), Rev-erbα (purple lines) and Rorα (orange lines) in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type (solid line) and NLRP3$^{-/-}$ mice (dashed line). The shaded region on each graph represents constant darkness. Data are expressed as means ± confidence interval (CI) of the Mesor when p was ≤ 0.05 from the non-zero amplitude. Data of significant differences among the experimental groups were evaluated by overlapping of confidence interval and are detailed in Annex 2. *p<0.05 vs. Y; # p<0.05 vs. group without melatonin treatment; $ p<0.05 vs. WT mice.*

CHAPTER 3: NLRP3 INFLAMMASOME DELETION AND / WITH MELATONIN SUPPLEMENTATION MITIGATE AGE-DEPENDENT MORPHOLOGICAL AND ULTRASTRUCTURAL ALTERATIONS IN MURINE HEART

1. NLRP3 absence and melatonin administration restored left ventricular lumen and inhibited thickening of its wall during aging

Magnetic resonance imaging of the heart of the Y, EA, and OA WT (Figure 37 A-E) and NLRP3$^{-/-}$ mice (Figure 37 F-J), as well as the beneficial effect of melatonin supplementation was illustrated in Figure 37. With aging, there were no changes in the length of the heart of the EA WT and NLRP3$^{-/-}$ mice, while the cardiac length of OA WT mice displayed a significant decline. However, melatonin therapy significantly increased the cardiac length in the EA NLRP3$^{-/-}$ animals, where its effect was more detectable than in WT mice (Figure 37 K). Moreover, aging induced a significant reduction in the length of the left ventricular lumen in WT and NLRP3$^{-/-}$ mice, and this decline was less detectable in the NLRP3$^{-/-}$ than in the WT mice. Melatonin administration recovered the luminal length of the LV in the EA and OA WT and NLRP3$^{-/-}$ mice (Figure 37 L). Aging was also accompanied with an increase in the thickness of the left ventricular wall in the WT and NLRP3$^{-/-}$ mice, and this increase was less remarkable in the NLRP3$^{-/-}$ than in the WT mice. Interestingly, this increase in the left ventricular wall thickness was countered in the OA animals by melatonin therapy, which revealed a more noticeable beneficial effect on NLRP3$^{-/-}$ mice (Figure 37 M).

2. NLRP3 deficiency and melatonin therapy enhanced cardiac anthropometric parameters during aging

Aging displayed a significant increase in the body weight and heart weight of both WT and NLRP3$^{-/-}$ mice, an effect enhanced by melatonin administration. While this rise in the body weight revealed no changes between WT and NLRP3$^{-/-}$ mice, the increase of heart weight was higher in the NLRP3$^{-/-}$ mice than WT ones with aging. Furthermore, melatonin therapy showed more considerable effects in the NLRP3$^{-/-}$ mice than in WT animals (Figure 37 N and O). The ratio of the heart weight to the body weight however, reported an age-mediated decline, which was higher in the OA WT mice than in the NLRP3$^{-/-}$ ones. This reduction was significantly countered by melatonin supplementation (Figure 37 P).

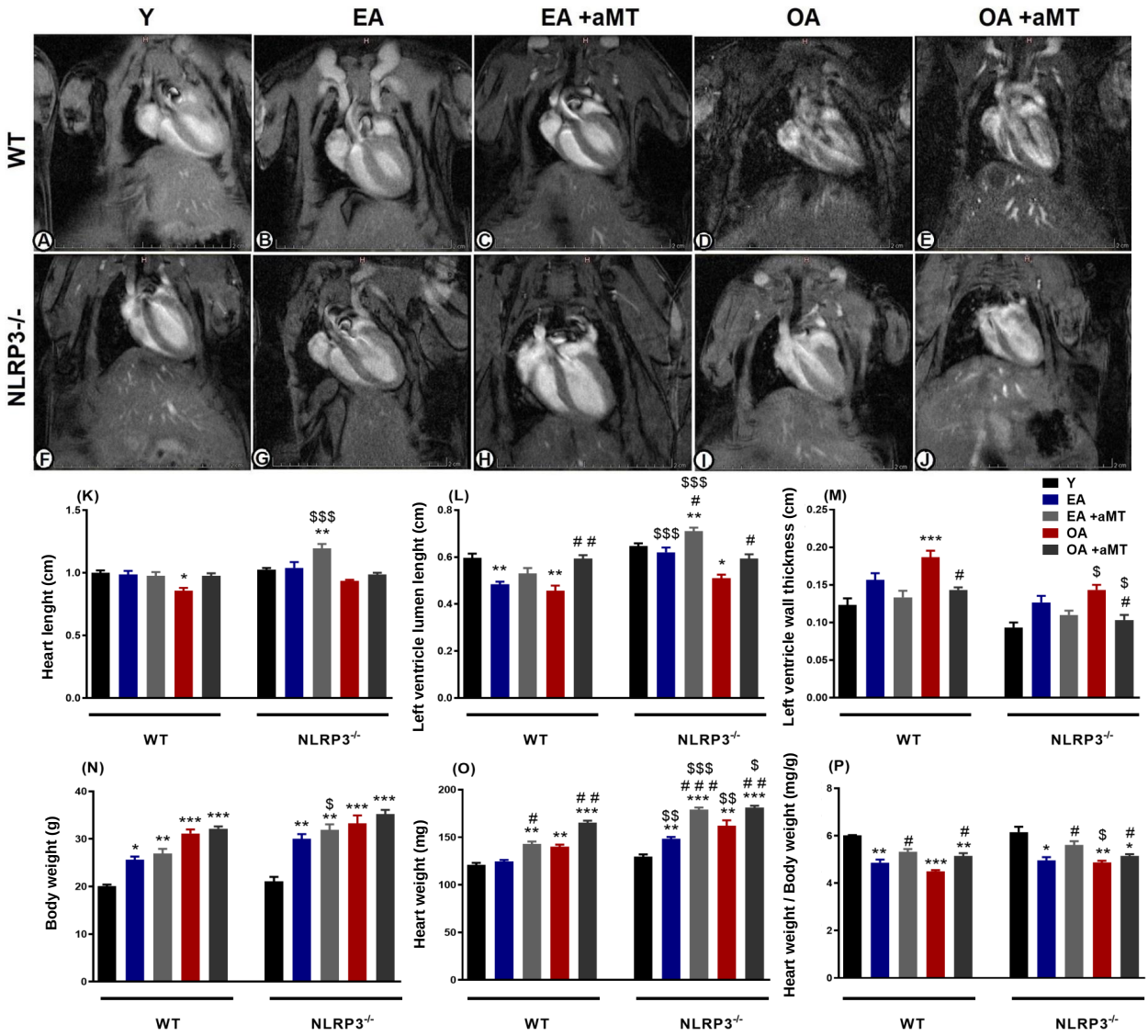


Figure 37. Impact of NLRP3 deficiency and melatonin therapy on cardiac magnetic resonance imaging and anthropometric parameters during aging. ***(A-E)*** *Magnetic resonance imaging of the heart of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) WT mice.* ***(F-J)*** *Magnetic resonance imaging of the heart of Y, EA, EA + aMT, OA, and OA + aMT NLRP3$^{-/-}$ mice.* ***(K)*** *Analysis of the heart length (cm).* ***(L)*** *Analysis of the luminal length of the left ventricle (cm).* ***(M)*** *Analysis of the thickness of the left ventricular wall (cm).* ***(N)*** *Analysis of the body weight (g).* ***(O)*** *Analysis of the heart weight (mg).* ***(P)*** *Analysis of the ratio of the heart weight to the body weight (mg/g). * p<0.05, ** p<0.01 and *** p<0.001 vs. Y; # p<0.05, ## p<0.01 and ### p<0.001 vs. group without melatonin treatment; $ p<0.05, $$ p<0.01 and $$$ p<0.001 vs. WT mice.*

3. NLRP3 deletion and melatonin supplementation reduced age-related histological and morphometrical alterations of cardiac myocytes, and minimized hypertrophy-associated genes as well as inflammatory cytokines genes

Histological analysis of the LV of the Y WT mice showed normal architecture of the cardiac muscles, which consisted of cardiomyocytes in different orientations; longitudinal, transverse and oblique. These cardiac fibers were separated from each other by narrow interstitial tissues, composed

of blood capillaries, less collagenous tissue, and fibroblasts. The nucleus was centrally located (Figure 38 A and B).

Cardiomyocytes of the EA WT mice revealed an initial degree of necrosis associated with lymphocytic infiltrates and widening of the interstitial spaces (Figure 38 C and D). These alterations were progressive in the heart of OA animals, where the myocardium demonstrated severe degrees of necrotic damage, associated with lymphocytic infiltrations and excessive collagen deposition, an indicator of fibrosis. Also, disorganization of the cardiac fibers and large interstitial spaces were illustrated (Figure 38 G, H). Melatonin administration, however, elucidated a protective effect on the cardiac muscle fibers of both EA (figure 38 E and F) and OA (Figure 38 I and J) WT mice. The fibers conserved their normal architecture with narrow interstitial spaces, less collagenous tissue infiltrations and absence of necrotic fibers.

Histological examination of the cardiac muscles of the Y NLRP3$^{-/-}$ mice (Figure 38 K and L) showed the normal organization of the cardiomyocytes, which demonstrated no changes in the EA animals, except for a widening of interstitial tissues (Figure 38 M and N). Less necrotic changes as well as reduced collagen deposition were observed in OA mutants (Figure 38 Q and R), compared with those of WT mice. Interestingly, melatonin therapy induced a more preservative effect on the cardiomyocytes of EA (Figure 38 O and P) and OA (Figure 38 S and T) NLRP3$^{-/-}$ mice than on WT mice, sustaining the normal architecture of muscle fibers with narrow interstitial spaces.

Aging also induced a significant loss of cardiac muscle fibers associated with hypertrophy of individual cardiomyocyte (increased CSA of individual cardiomyocyte). This decline in cardiac fiber number and increase in cardiomyocyte CSA were more remarkable in WT mice than NLRP3$^{-/-}$ ones. Melatonin therapy restored the number of cardiomyocytes and minimized muscle fiber hypertrophy in EA and OA WT and NLRP3$^{-/-}$ mice (Figure 38 U and V). Aging associated with increased β-MHC expression in WT and NLRP3$^{-/-}$ mice; however, the expression was more detectable in WT animals. Melatonin supplementation reduced β-MHC expression in both mice strain, with more considerable effect on NLRP3$^{-/-}$ mice (Figure 38 W).

Morphometrical analysis of the percentage fibrotic area revealed age-mediated induction of cardiac fibrosis (Figure 38 X), associated with increased expressions of IL-1α, IL-6 and TNF-α in the old-aged animals, while early-aged mice showed a non-significant increase (Figures 38 Y-ZZ). These incidence of fibrosis and expression of inflammatory cytokines were less remarkable in the NLRP3$^{-/-}$ mice. Melatonin supplementation however, reduced cardiac fibrosis and significantly decreased the expression of IL-1α and IL-6 in WT and NLRP3$^{-/-}$ mice, with non-significant decline of TNF-α.

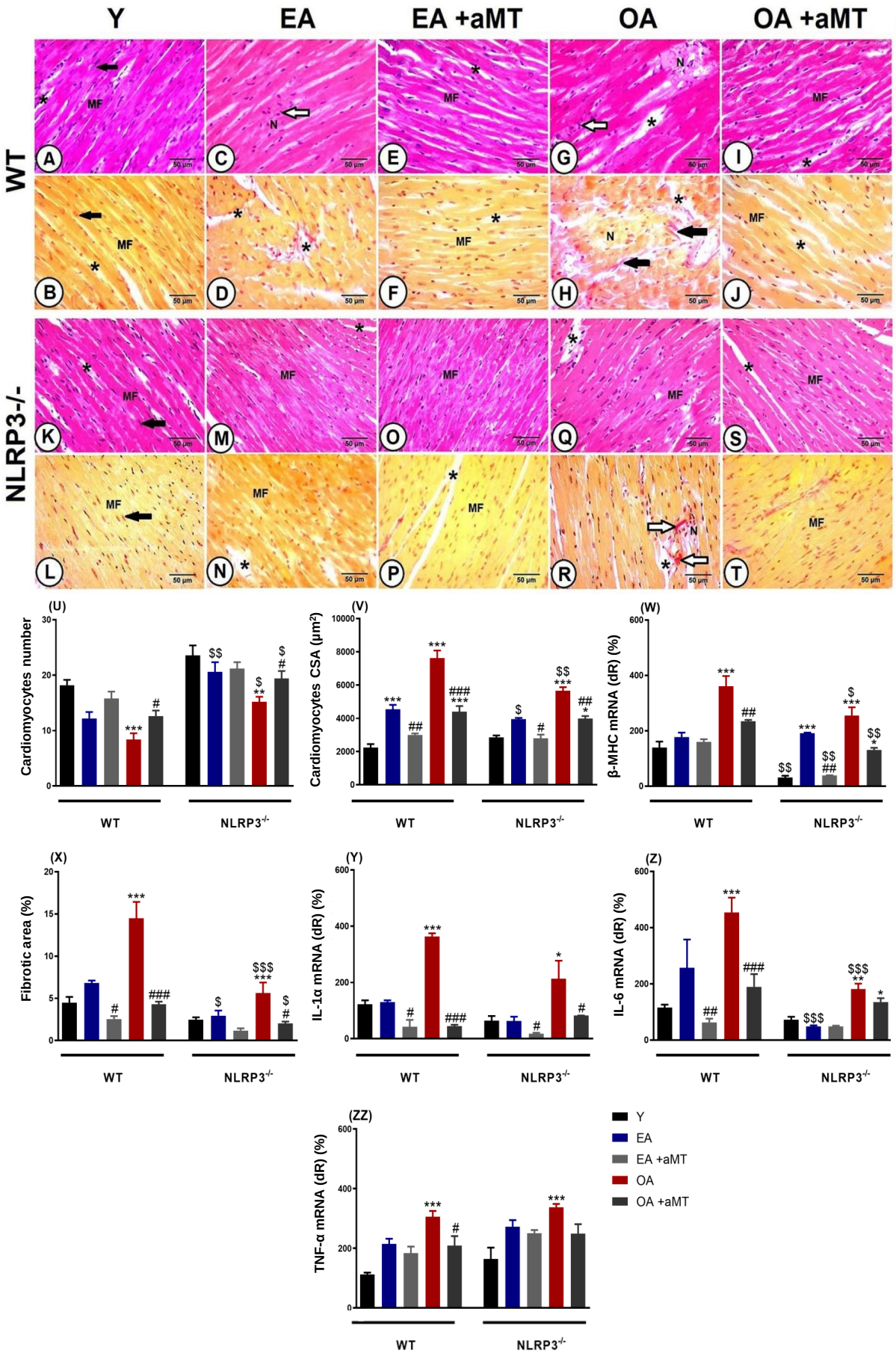

Y
EA
EA +aMT
OA
OA +aMT
WT
NLRP3-/-
MF
N
50 μm
(U)
Cardiomyocytes number
(V)
Cardiomyocytes CSA (μm²)
(W)
β-MHC mRNA (dR) (%)
(X)
Fibrotic area (%)
(Y)
IL-1α mRNA (dR) (%)
(Z)
IL-6 mRNA (dR) (%)
(ZZ)
TNF-α mRNA (dR) (%)
WT
NLRP3-/-
Y
EA
EA +aMT
OA
OA +aMT

Figure 38. Effect of NLRP3 deletion and melatonin supplementation on age-associated histological and morphometrical changes of cardiac muscle fibers. ***(A** and **B)** Left ventricle (LV) of the Y WT mice showing the normal cardiac muscle fibers (MF) architecture, with central nucleus (black arrows), and narrow interstitial spaces (black asterisks).* ***(C** and **D)** LV of the EA WT mice revealing an initial necrotic degree (N), with lymphocytic infilterates (white arrow) and wide interstitial spaces (black asterisks).* ***(E** and **F)** The conservative effect of melatonin on maintaining normal muscle fibers (MF) of EA mice, with narrow interstitial spaces (black asterisks).* ***(G** and **H)** LV of the OA WT mice demonstrating severe necrotic changes (N), with lymphocytic infiltrations (white arrow), excessive collagen deposition (black arrows), and wide interstitial spaces (black asterisks).* ***(I** and **J)** The protective effect of melatonin on improving cardiac muscle fibers (MF) and minimizing interstitial tissues (black asterisks) in OA animals.* ***(K** and **L)** LV of the Y NLRP3$^{-/-}$ mice showing the normal cardiac muscle fibers organization (MF), with centrally located muscle (black arrows) and interstitial spaces (black asterisks).* ***(M** and **N)** LV of the EA NLRP3$^{-/-}$ mice revealing wide interstitial spaces (black asterisks) without necrosis.* ***(O** and **P)** The preservative effect of melatonin on keeping normal structure of cardiac myocytes (MF) in EA mice.* ***(Q** and **R)** LV of the OA NLRP3$^{-/-}$ mice demonstrating less necrotic changes (N), interstitial spaces (black asterisks), and collagen deposition (white arrows).* ***(S** and **T)** The beneficial effect of melatonin on improving cardiac architecture, with narrow interstitial spaces (black asterisk). Bar=50 μm.* ***A, C, E, G, I, K, M, O, Q** and **S** stained with H&E stain, while **B, D, F, H, J, L, N, P, R** and **T** stained with Van Gieson stain.* ***(U and V)** Age-associated morphometrical changes in cardiac muscle fibers number (per 100 μm^2) and cross-sectional area (CSA).* ***(W)** mRNA expression level of β-MHC.* ***(X)** Morphometrical analysis of cardiac fibrosis during aging.* ***(Y)** mRNA expression level of IL-1α.* ***(Z)** mRNA expression level of IL-6.* ***(ZZ)** mRNA expression level of TNF-α. * p<0.05, ** p<0.01 and *** p<0.001 vs. Y; # p<0.05, ## p<0.01 and ### p<0.001 vs. group without melatonin treatment; $ p<0.05, $$ p<0.01 and $$$ p<0.001 vs. WT mice.*

4. NLRP3 ablation and melatonin administration conserved cardiac muscle ultrastructure during aging

Electron microscopy analysis of the LV of the Y WT mice clarified the normal ultrastructure of the cardiac muscle fibers, which are composed of centrally located nuclei, and are formed as well-organized longitudinally arranged myofibrils that illustrated cross and longitudinal striations with sarcoplasmic reticulum in between. Each myofibril consists of thread-like myofilaments, actin and myosin. The sarcomeres are well-aligned between each two successive Z-lines. Cardiomyocytes branched repeatedly and attached strongly at the intercalated disc (Figure 39 A). The mitochondria were intact and compacted with clearly organized cristae and were gathered in different orientations; clusters in between cardiac myofibrils as intermyofibrillar, around the nucleus, and beneath the sarcolemma as subsarcolemmal (Figure 39 B). Cardiac fibers had a better structure in Y NLRP3$^{-/-}$ mice than in WT. The myofibrils depicted an organized sarcomere, and highly compacted mitochondria with densely packed well-arranged cristae, and narrow interstitial spaces with a normally oriented sarcoplasmic reticulum (Figure 39 C and D).

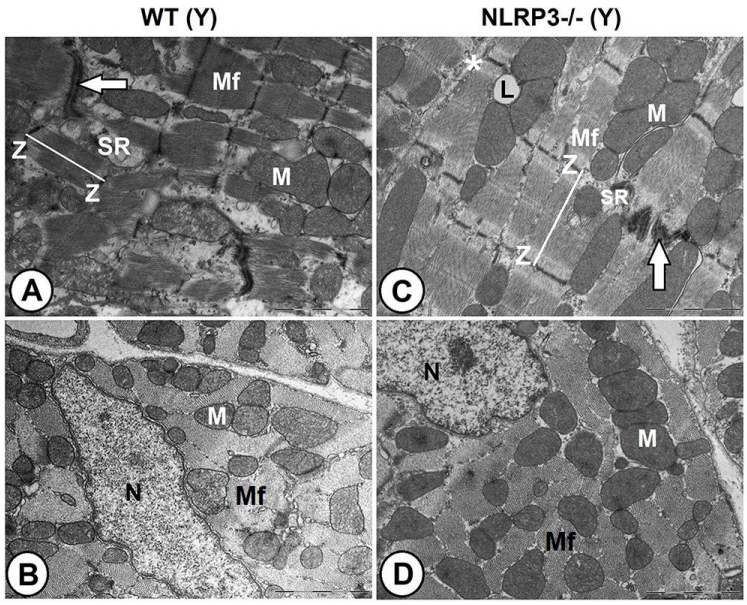


Figure 39. Ultrastructural architecture of cardiac muscle fibers in young WT and NLRP3$^{-/-}$ mice. ***(A** and **B)** Transmission electron micrographs of the LV of the Y WT mice clarifying well-organized longitudinally arranged myofibrils (Mf), with cross and longitudinal striations, centrally located nucleus (N), and intermyofibrillar sarcoplasmic reticulum (SR). Sarcomeres (line) are well-alignment between each two successive Z-lines (Z), and cardiomyocytes branched repeatly and attached strongly at the intercalated disc (arrow). The mitochondria were intact and compacted with clearly organized cristae, (M).* ***(C** and **D)** Transmission electron micrographs of the LV of the Y NLRP$^{-/-}$ mice showing cardiac myofibrils (Mf) of better structure with organized sarcomere between Z-lines (Z), highly compacted mitochondria (M), narrow interstitial spaces (asterisk), intermyofibrillar distribution of sarcoplasmic reticulum (SR), and lipid droplets (L). Nucleus (N) was centrally located. Bar=2 μm.*

At the early stage of aging, cardiac muscle fibers of WT mice showed disorganized myofibrils and sarcoplasmic reticulum. Some mitochondria displayed vacuolation and cristae damage. Appearance of small-sized multivesicular bodies, termed autophagosomes, were also detected (Figure 40 A and B). However, melatonin supplementation preserved the cardiomyocytes, maintained the normal orientation of the myofibrils and the intact contents of mitochondria, and reduced the residual bodies (Figure 40 C and D). In contrast, cardiac muscle fibers of the EA NLRP3$^{-/-}$ did not show age-related alterations in the myofibril architecture and mitochondrial composition, except presence of individual disorganized myofibrils with indistinct striations (Figure 40 E and F), which was improved with melatonin therapy that revealed a better preservative effect in EA NLRP3$^{-/-}$ mice than in WT (Figure 40 G and H).

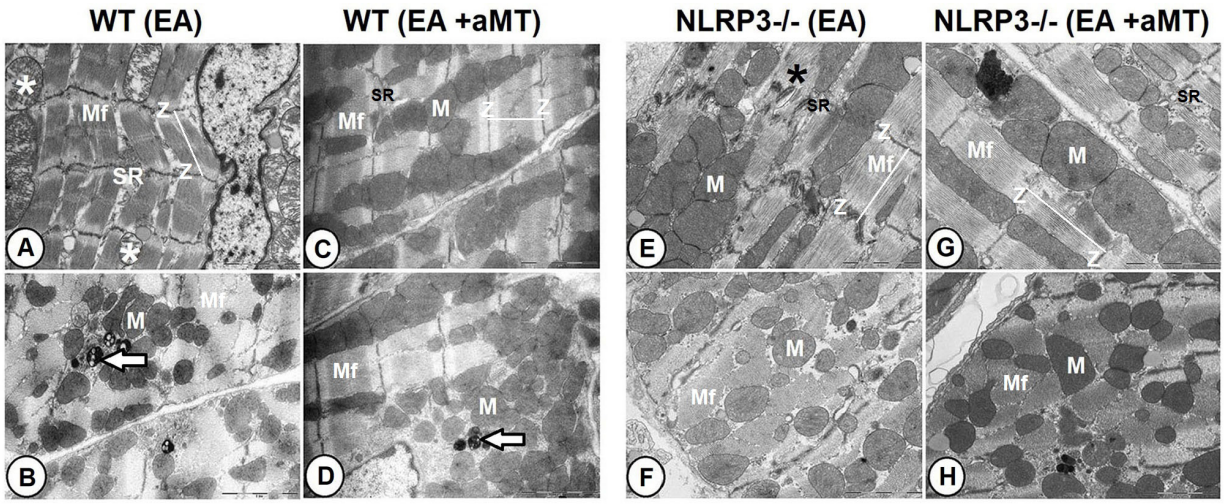


Figure 40. Impact of NLRP3 abscence and melatonin treatment on the ultrastructure of early-aged cardiac myocytes. ***(A** and **B)** Transmission electron micrographs of the LV of the EA WT mice showing disorganized myofibrils (Mf) and sarcoplasmic reticulum (SR). Some mitochondria displayed vacuolation and cristae damage (white asterisk), with appearance of small-sized multivesicular bodies (arrow).* ***(C** and **D)** Transmission electron micrographs of the LV of the EA WT mice after melatonin supplementation. The cardiac myofibrils (Mf) maintained the normal orientation, with intact mitochondrial contents (M), organized sarcoplasmic reticulum (SR), and reduced residual bodies (arrow).* ***(E** and **F)** Transmission electron micrographs of the LV of the EA NLRP3$^{-/-}$ mice revealing absence of age-related alterations in the myofibrils architecture (Mf), sarcoplasmic reticulum (SR), and mitochondrial composition (M), except presence of individual disorganized myofibrils with indistinct striations (black asterisk).* ***(G** and **H)** Transmission electron micrographs of the LV of the EA NLRP3$^{-/-}$ mice with melatonin therapy demonstrating improvement of myofibrils (Mf) and their sarcoplasmic reticulum (SR) and mitochondrial contents (M). Note sarcomeres arrangement between each two successive Z-lines (Z). Bar=2 μm.*

The cardiac muscle fibers of the OA WT mice demonstrated severe myofibrillar damage, with widening of interstitial spaces and disruption of the sarcoplasmic reticulum. Some mitochondria revealed a normal structure, while others were hypertrophied and demonstrated different stages of cristae damage and presence of inclusion bodies on their matrix. Splitting of the nucleus into two or three parts was mostly detected, with formation of autophagosomes (Figure 41 A-C). Meanwhile, melatonin supplementation conserved the cardiac muscle constitutions and protected nuclear and mitochondrial contents, excepting individual mitochondria that displayed structural damage. Small-sized residual bodies were also found (Figure 41 D-F).

The severe age-associated changes detected in cardiomyocytes were less evident in OA NLRP3$^{-/-}$ mice than WT ones. The cardiac muscles of the OA NLRP3$^{-/-}$ had less prevalent muscular damage with lipid droplets. Some mitochondria were normal and intact, while others were characterized by widely separated and disorganized cristae (Figure 41 G and H). Melatonin administration induced a beneficial effect, where it maintained normal muscular structure and mitochondrial architecture during aging. Most of the myofibrils and mitochondria were intact, while individual fibers revealed residual bodies and lipid infiltrations (Figure 41 I and J).

Aging induced an early non-significant decline of IMF mitochondrial number in cardiac muscle fibers of EA WT and NLRP3$^{-/-}$ mice. This decline was followed by a significant reduction in the OA animals of both groups; however, the decline in mitochondrial number was less prevalent in OA NLRP3$^{-/-}$ mice than WT mice. Melatonin administration maintained the integrity of the IMF mitochondria number with aging (Figure 41 K). Aged cardiomyocytes were also associated with non-significant increase in the IMF mitochondria CSA of EA WT and NLRP3$^{-/-}$ mice, while this induction of mitochondrial CSA was more considerable in OA animals (Figure 41 L).

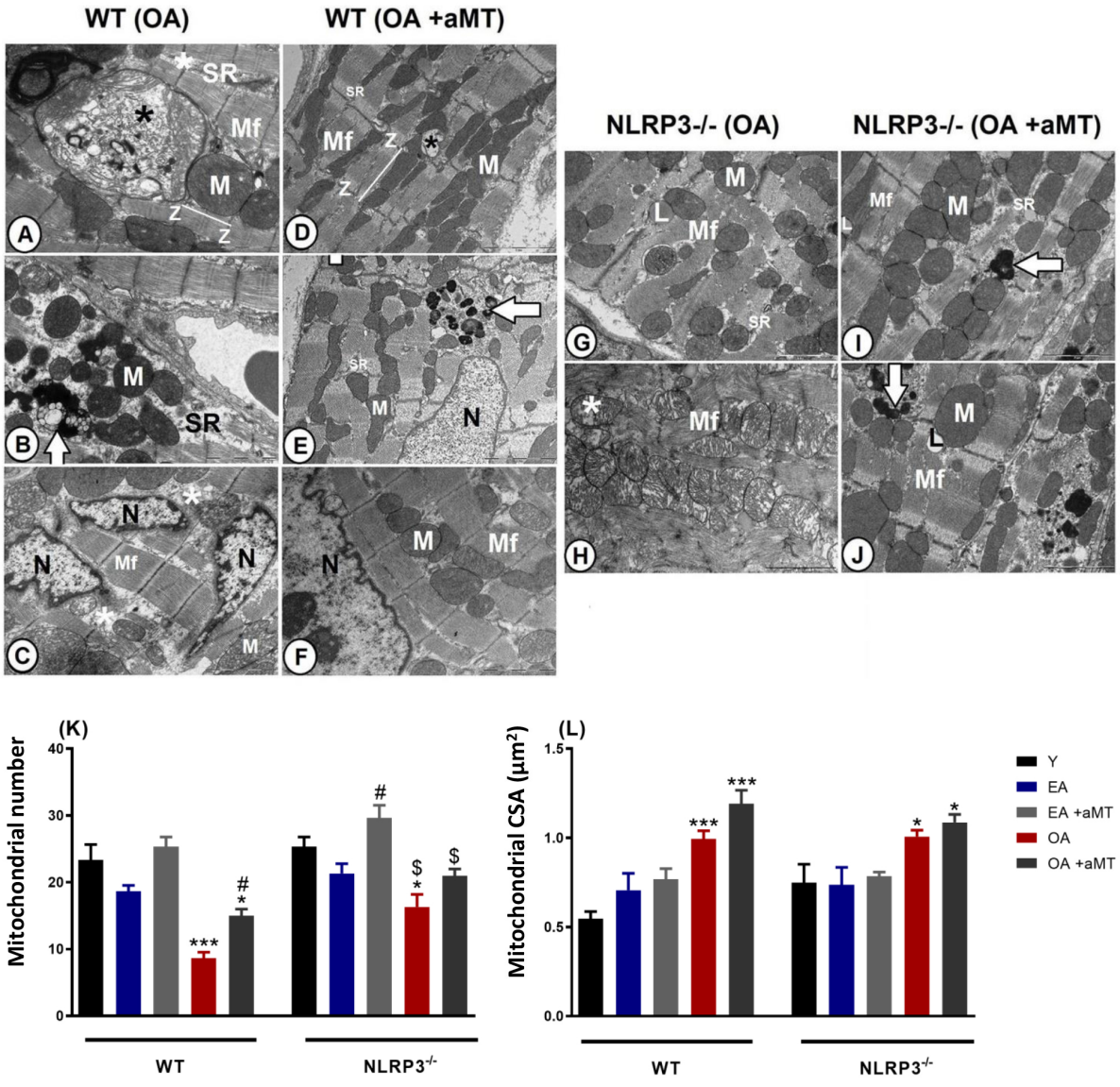


Figure 41. Effect of NLRP3 ablation and melatonin administration on age-associated ultrastructural and morphometrical alterations of cardiac muscle fibers and cardiac mitochondria. ***(A-C)*** *Transmission electron micrographs of the LV of the OA WT mice illustrating widening of interstitial spaces (white asterisk) and disruption of the sarcoplasmic reticulum (SR) between myofibrils (Mf). Some mitochondria revealed a normal structure (M), while others were hypertrophied and demonstrated different stages of cristae damage with presence of inclusions bodies on their matrix (black asterisks). Splitting of the nucleus (N) was mostly clarified, with formation of autophagosomes (arrow).* ***(D-F)*** *Transmission electron micrographs of the LV of the OA WT mice after melatonin supplementation showing conservation of cardiac myofibrils (Mf), nuclear structure (N),*

sarcoplasmic reticulum (SR), and mitochondrial contents (M), except of individual mitochondria displayed destructed damage (black asterisk), in addition to the presence of small-sized residual bodies (arrow). ***(G** and **H)** Transmission electron micrographs of the LV of the OA NLRP3$^{-/-}$ mice illustrating less prevalent damage of cardiac myofibrils (Mf), with well-organized sarcoplasmic reticulum (SR), and lipid droplet (L) infiltrations. Some mitochondria were normal, and intact, while others were characterized by their widely-separated and disorganized cristae (asterisk).* ***(I** and **J)** Transmission electron micrographs of the LV of the OA NLRP3$^{-/-}$ mice after treatment with melatonin depicting the maintenance of normal myofibrils (Mf) structure, sarcoplasmic reticulum (SR) organization, and mitochondrial architecture (M) during aging. Individual fibers containing residual bodies (arrows) and lipid infiltrations (L) were observed. Note organization of sarcomeres between each two successive Z-lines (Z). Bar=2 μm.* ***(K)** Age-mediated morphometrical changes of cardiac intermyofibrillar mitochondrial number (per 5 μm^2).* ***(L)** Age-associated morphometrical changes of mitochondrial cross-sectional area (CSA). Aging induced reduction of mitochondrial number, which was more detectable in WT than NLRP3$^{-/-}$ mice, and also was recovered by melatonin administration. Aging was also associated with increased CSA. * p<0.05 and *** p<0.001 vs. Y; # p<0.05 vs. group without melatonin treatment; $ p<0.05 vs. WT mice.*

5. NLRP3 absence and melatonin treatment diminished cardiac apoptosis during aging

Hoechst fluorescent analysis of the cardiac muscle fibers nuclei showed the normal nuclear appearance in Y WT and NLRP3$^{-/-}$ mice (Figure 42 A and B). With aging, cardiac muscle fibers of EA WT and NLRP3$^{-/-}$ animals illustrated signs of apoptosis, where apoptotic cells illustrated cell shrinkage, chromatin condensation, and nuclear fragmentation (Figure 42 C and D). These age-related apoptotic changes were more pronounced in the OA groups (Figure 42 G and H) and were more considerable in cardiac myocytes of WT mice than NLRP3$^{-/-}$ one (Figure 42 K). Interestingly, this age-mediated induction of nuclear apoptosis was countered by melatonin treatment in EA (Figure 42 E and F) and OA (Figure 42 I and J) WT and NLRP3$^{-/-}$ mice respectively (Figure 42 K).

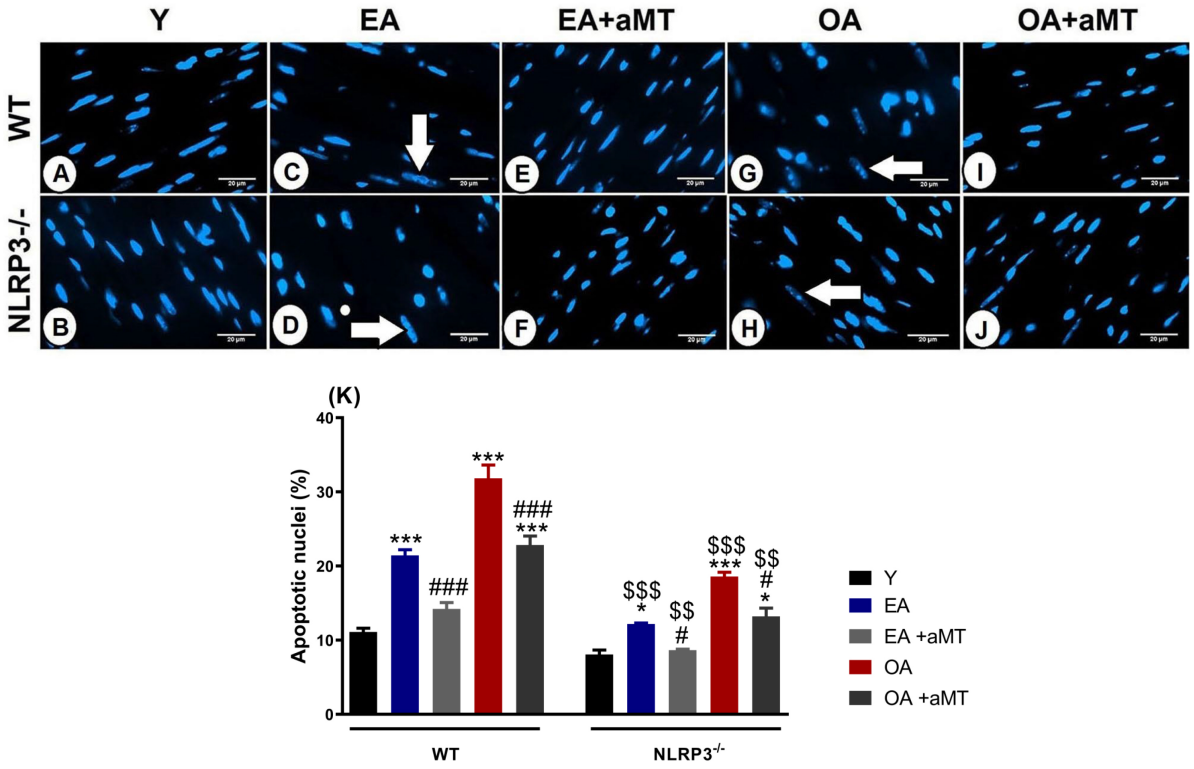


Figure 42. Impact of NLRP3 deletion and melatonin therapy on age-dependent cardiac apoptosis. ***(A** and **B)** Hoechst fluorescent analysis of the nuclear apoptosis in the LV of the Y WT and NLRP3$^{-/-}$ mice, respectively showing normal nuclear appearance. **(C** and **D)** Analysis of the nuclear apoptosis in the LV of the EA WT and NLRP3$^{-/-}$ mice, respectively revealing that aging induced nuclear apoptosis and fragmentation (arrows). **(E** and **F)** Analysis of the nuclear apoptosis in the LV of the EA WT and NLRP3$^{-/-}$ mice, respectively after melatonin therapy clarifying the protective effect of melatonin on reducing cardiac apoptosis. **(G** and **H)** Analysis of the nuclear apoptosis in the LV of the OA WT and NLRP3$^{-/-}$ mice, respectively exhibiting more detectable nuclear apoptosis. **(I** and **J)** Analysis of the nuclear apoptosis in the LV of the OA WT and NLRP3$^{-/-}$ mice, respectively after melatonin treatment confirming the beneficial effect of melatonin against age-related cardiac apoptosis. **(K)** Morphometrical analysis of apoptotic nuclei of cardiomyocytes during aging revealing that cardiac apoptosis was more considerable in cardiac myocytes of WT mice than in those of NLRP3$^{-/-}$ one. Bar=20 μm. * $p<0.05$ and *** $p<0.001$ vs. Y; # $p<0.05$ and ### $p<0.001$ vs. group without melatonin treatment; $$ $p<0.01$ and $$$ $p<0.001$ vs. WT mice.*

DISCUSSION

CHAPTER 1: MELATONIN/NRF2/NLRP3 CONNECTION IN MOUSE HEART MITOCHONDRIA DURING AGING

Immunosenescence and inflammaging are caused by persistent activation of NF-κB/NLRP3 inflammasome pathways generates chronic low-grade inflammation which leads to, among other detriments, accumulation of cardiac mitochondrial dysfunction, characterized by dysregulation of mitochondrial dynamics, autophagy, apoptosis, Nrf2 antioxidant pathway, and maintenance of ultrastructure of mitochondria (Franceschi et al., 2000). Another hallmark of aging is a decline in melatonin levels and its protective roles (Reiter et al., 1998). This brings about increased oxidative damage, chronodisruption, upregulation of pro-inflammatory cytokines, and down regulation of anti-oxidant/-inflammatory processes that contribute to inflammaging by facilitating mitochondrial disruption (Hardeland, 2019). The role of the NLRP3 inflammasome and melatonin levels in regulation of mitochondrial dysfunction, associated with cardiac aging, is not fully understood. Our results suggest direct involvement of this inflammasome by marked amelioration of some mitochondrial dysfunctions with NLRP3 ablation both involved with, and independent of, melatonin supplementation in EA and OA mice (Figures 43, 44).

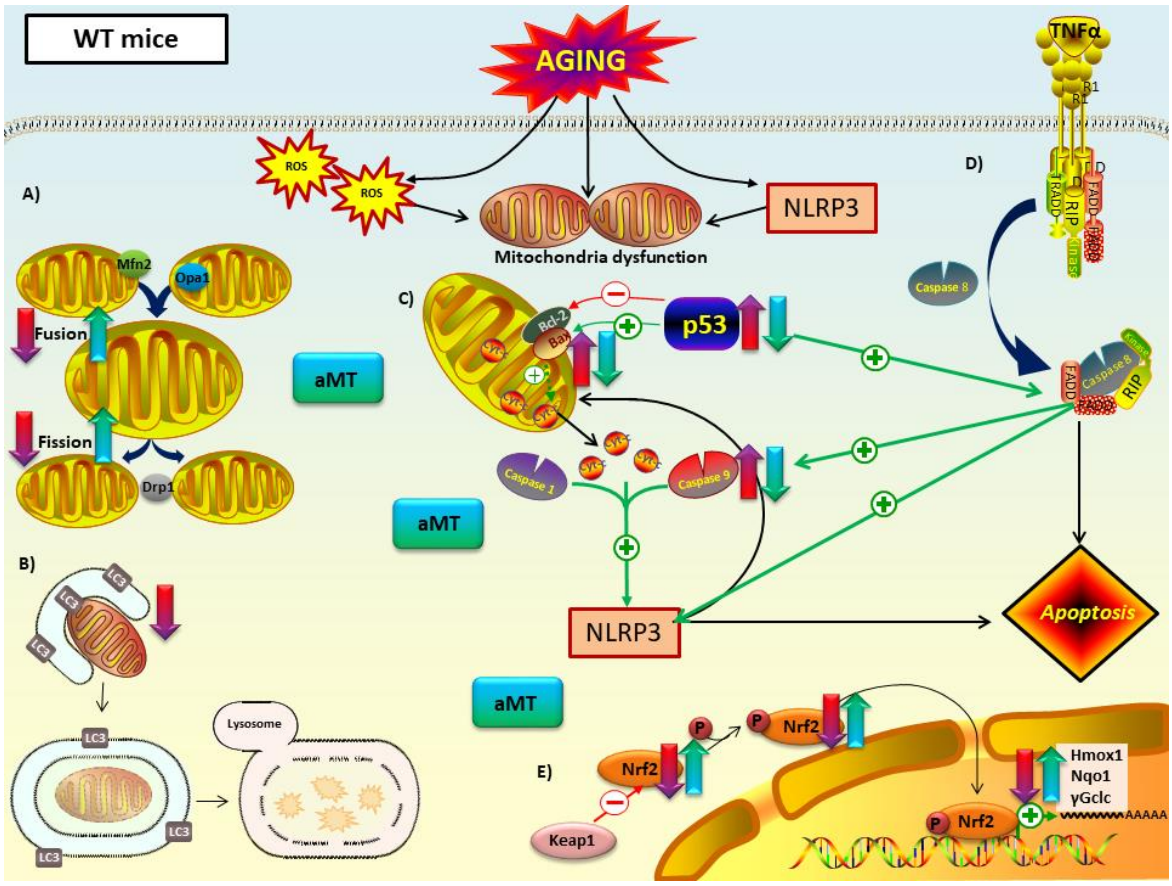


Figure 43. Proposed mechanism of melatonin in mitochondria of WT mice during cardiac aging. ***(A)*** *Mitochondrial dynamics: aging led to a decline in fusion (Mfn2 and Opa1) and fission proteins (Opa1). Melatonin treatment counteracted this decrease.* ***(B)*** *Autophagy (mitophagy): autophagic capacity dropped in aged myocardium. Melatonin therapy had minimal impact on this pathway.* ***(C)*** *Intrinsic and* ***(D)*** *extrinsic*

apoptosis: WT mice have intrinsic and extrinsic pathways mediated by p53 and caspase 9. Those apoptotic markers, as well as Bax/Bcl2 ratio, increased with aging and are related with NLRP3 activation. This inflammasome seemed to have a regulatory effect on the intrinsic apoptotic pathway, which depends on mitochondria cytochrome c release. Melatonin supplementation had an anti-apoptotic effect in both intrinsic and extrinsic apoptosis. ***(E)*** *Nrf2-dependent antioxidant response: Nrf2 and pNrf2 (Ser40) were reduced with aging. This loss was linked to the decrease of the cytoprotective enzyme transcriptionally regulate by Nrf2: Hmox1, Nqo1 and γGclc. Melatonin recovered this antioxidant pathway. No changes in Keap1 were reported. Red-purple arrow: impact of aging. Green-blue arrow: effect of melatonin treatment.*

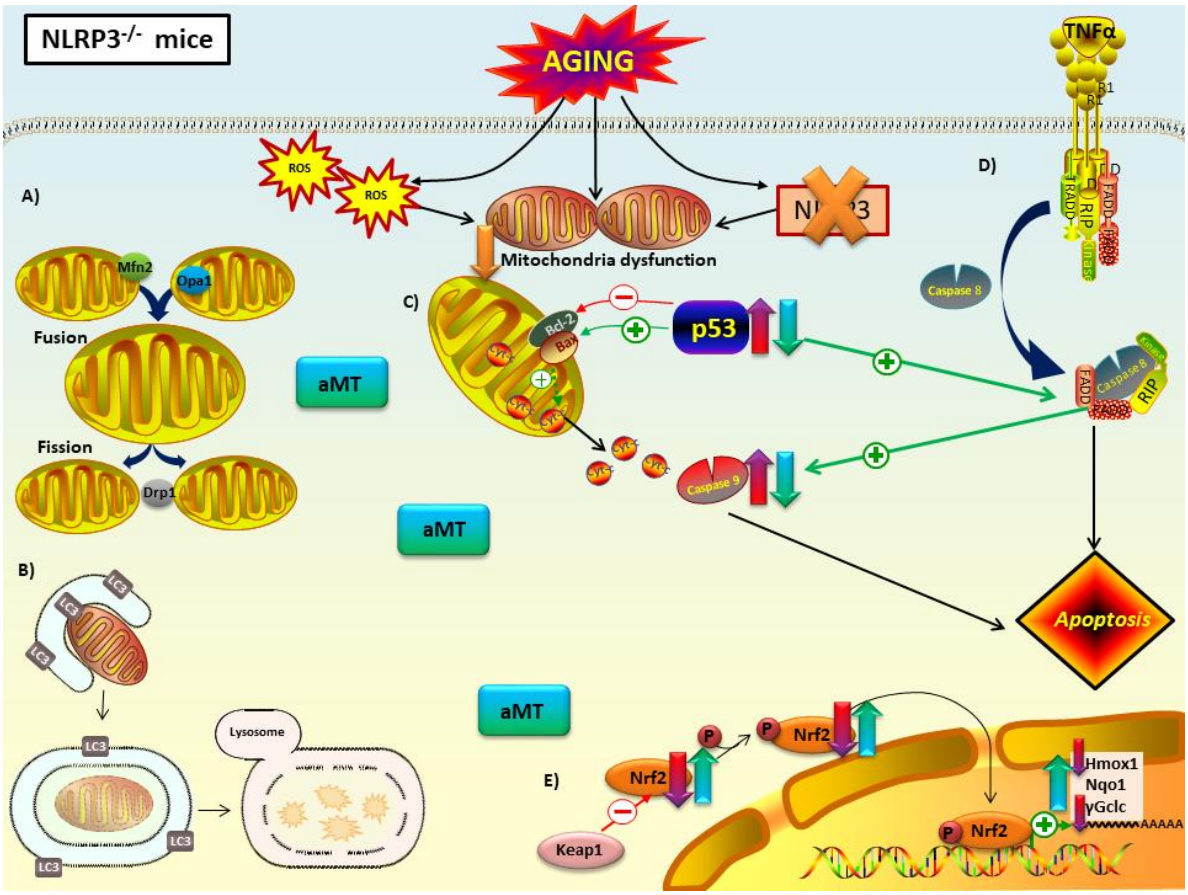


Figure 44. Proposed mechanism of melatonin in mitochondria of NLRP3$^{-/-}$ mice during cardiac aging. *Lack of NLRP3 inflammasome reduced mitochondria dysfunction.* ***(A)*** *Mitochondrial dynamics: the absence of NLRP3 prevented the decline in fusion (Mfn2 and Opa1) and fission proteins (Opa1) with aging. Melatonin treatment had no effect on these mice.* ***(B)*** *Autophagy (mitophagy): autophagic capacity was restored by NLRP3 deficiency. Melatonin therapy had minimal impact on autophagic capacity.* ***(C)*** *Intrinsic and* ***(D)*** *extrinsic apoptosis: loss of NLRP3 had an anti-apoptotic effect in Bax/Blc2 ratio, but not in p53 or caspase 9. The ablation of this inflammasome could trigger extrinsic apoptosis mediated by TNF-α binding to death receptor. Melatonin supplementation had an anti-apoptotic effect in p53 and caspase 9.* ***(E)*** *Nrf2-dependent antioxidant response: lack of NLRP3 did not recover the decrease of this antioxidant pathway with aging. Only Nqo1 were not diminished in mutant mice. Melatonin improved this antioxidant pathway. No changes in Keap1 were reported. Red-purple arrow: impact of aging. Green-blue arrow: effect of melatonin treatment.*

Mitochondria fusion (Mfn2 and Opa1) and fission (Drp1) proteins decrease naturally with aging, as seen in WT mice (Figure 43 A). Findings in the literature link declines in regulatory proteins of mitochondrial dynamics and age-related development of CVD (Ahuja et al., 2013; Rosca and

Hoppel, 2010; Stotland and Gottlieb, 2016; Zhao et al., 2014). Cardiomyocytes of Mfn2-deficient mice showed cardiac hypertrophy (Papanicolaou et al., 2011). Low levels of Opa1 have been reported in failing human heart (Chen et al., 2009). Loss of Drp1 in adult mice results in lethal dilated cardiomyopathy (Song et al., 2015). Our study also concluded that the absence of NLRP3 prevented the decrease in fusion and fission processes associated with aging that were observed in WT mice (Figure 44 A). This cardioprotective effect observed in NLRP3$^{-/-}$ mice supports the existence of a close relationship between mitochondrial dynamics and inflammaging. Our results are in line with scientific evidence that connects impaired mitochondria dynamics, stimulation of innate immune response and inflammasome activation (Horng, 2014; Ichinohe et al., 2013; Park et al., 2015; Szabadkai et al., 2004; Wang et al., 2014; Yasukawa et al., 2009). On the other hand, melatonin's mechanism of action in mitochondria dynamics and aging remains unclear. We indicate herein that melatonin promotes fusion by increasing the expression of the Mfn2 and Opa1 proteins in WT EA and OA mice (Figure 43 A). Most investigations support that this indolamine stimulates mitochondria fusion, contributing to the survival of cardiomyocytes and reducing mitochondria damage (Pei et al., 2016; Suwanjang et al., 2016; Zhang et al., 2019b). Moreover, numerous studies remark a melatonin-induced reduction of mitochondria fission with stressful stimuli (Chuang et al., 2016; Parameyong et al., 2013, 2015; Xu et al., 2016), showing a protective effect in cardiac function against ischemia/reperfusion injury and post-traumatic cardiac dysfunction *in vitro* and *in vivo* models, respectively (Ding et al., 2018a, 2018b; Zhou et al., 2017a). Conversely, we found that melatonin supplementation increased the levels of the Drp1 protein in EA and OA WT mice. Supporting our results, recent findings showed that increasement in Drp1 levels enhanced regulation of mitochondria homeostasis through mitophagy (Cho et al., 2019). Additionally, Drp1 overexpression in flies reversed age-related mitochondria dysfunction and age-onset pathologies (Rana et al., 2017). Taken together, our data suggests that melatonin enhances the response of mitochondria dynamics to maintain homeostasis during age-related metabolic stressors like inflammasome activation. It should be noted melatonin did not trigger significant changes in EA and OA NLRP3$^{-/-}$ mice either (Figure 44 A). This effect of melatonin has previously been related to its cytoprotective activity, since its effect will be greater the more cellular damage there is, while in situations of low damage or physiological conditions its response is minimal (Acuña Castroviejo et al., 2011).

The LC3II/LC3I ratio showed a significant decrease in autophagy in EA and OA WT mice compared to Y WT mice (Figure 43 B). Numerous findings indicate a loss of autophagy with aging in most organisms and tissues, including the heart (Cuervo, 2006; Rubinsztein et al., 2011; Shirakabe Akihiro et al., 2016; Zhou et al., 2017b). Changes in the expression of autophagic proteins such as Atg9, LAMP-1, and LC3II in aged mice and rats resulted in cardiac dysfunction (Hua et al., 2011; Taneike et al., 2010; Wohlgemuth et al., 2007). The consequent accumulation of altered organelles, mutated mtDNA, cristae disarray, and ROS, have been shown to propagate different age-related

cardiac pathologies (Boengler et al., 2009; Dai et al., 2014; Li et al., 2018) and produce risk-associated molecular pattern derived from mitochondria (DAMP) that activate NLRP3 inflammasome (Salminen et al., 2012a). Our results showed that the absence of NLRP3 prevented the drop in LC3II/LC3I ratio in mice during aging (Figure 44 B). Ablation of the NLRP3 inflammasome in old NLRP3$^{-/-}$ mice has been reported to improve the quality of autophagy by increasing the levels of ATG12, beclin 1 and LC3II and decreasing p62/SQSTM1 (Marín-Aguilar et al., 2020). Several studies have demonstrated the protective influence of melatonin by both increasing and decreasing autophagic capacity, in response to sterile and non-sterile inflammation (Kang et al., 2016; Lin et al., 2016; Ma et al., 2018; San-Miguel et al., 2015). Interestingly, in our results it is implied that melatonin had no effect on EA and OA WT mice compared to their corresponding controls (Figure 43 B). Similar results were obtained in the brain of SAMP8 mice, where melatonin did not cause changes in autophagy (Caballero et al., 2009). However, it is noteworthy that melatonin was able to increase autophagy of OA WT mice, thereby restoring levels like Y WT mice, but not in EA mice. This action suggests that melatonin and autophagy operate synergistically to increase cell survival, delay immunosenescence, and decrease oxidative stress. Thus, melatonin could act selectively, increasing autophagy only when antioxidant activity is severely impaired, or when sufficient loss of cellular homeostasis results in abnormal mitochondrial morphology and DR pathway activation (Houtkooper et al., 2013; Moore, 2008; Sebastián et al., 2016). Melatonin did not cause significant changes in the LC3II/LC3I ratio in NLRP3$^{-/-}$ mice (Figure 44 B), possibly due to the protective effect resulting from ablation of the inflammasome.

Apoptotic proteins p53 and caspase 9 were found to be increased in EA and OA vs Y mice in both WT and NLP3$^{-/-}$ mice (Figure 43 C, D, Figure 44 C, D). Oxidative stress that occurs during aging has been shown to induce apoptosis, mitochondria dysfunction in cardiomyocytes and ultimately heart failure (Aggarwal, 2000; D'Oria et al., 2020; Gustafsson and Gottlieb, 2003; Kannan and Jain, 2000). The Bax/Bcl2 ratio confirmed the increase in apoptosis with aging in WT mice. Interestingly, no changes were observed between ages in mutant mice. The ablation of NLRP3 had an anti-apoptotic protective effect during cardiac aging in Bax/Bcl2 ratio, but not in p53 or caspase 9. This finding suggests that NLRP3 is a direct regulator of the intrinsic apoptotic pathway in cardiac aging, which is dependent of the balance between Bax and Bcl2 and cytochrome c release (Figure 44 C). The absence of this inflammasome could trigger activation of extrinsic apoptosis with ligand-induced activation of several DRs since the participation of p53 and caspase 9 in this pathway has been reported in various tissues and cell models (D'Sa-Eipper et al., 2001; Haupt et al., 2003; McDonnell et al., 2003). In support of our hypothesis, recent studies revealed an increase in TNF-α in the serum of old NLRP3$^{-/-}$ mice compared to young mice (Cañadas-Lozano et al., 2020). This cytokine is linked to inflammaging (López-Otín et al., 2016) and induces extrinsic apoptotic pathway by binding to the cell DR TNFR1. On the other hand, findings have showed that caspase 8, which is key in extrinsic apoptosis, plays a

role in NLRP3 inflammasome priming and cytochrome c independent caspase 9 activation (Allam et al., 2014; McDonnell et al., 2008; Vince et al., 2018). Without NLRP3, cardiac aging-induced inflammation is favored and could start with extrinsic TNF-α apoptosis pathway preceding activation of caspase 8 which in turn activates caspase 9 (Figure 44 D). Further investigations centered of the impact of aging on the heart are required to elucidate the extent of the complex interactions between NLRP3 and apoptosis. In most cases, melatonin counteracted the high levels of p53 and caspase 9 associated with aging in WT and mutant mice and Bax/Bcl2 ratio in WT. This anti-apoptotic effect of melatonin during cardiac aging was evident in both extrinsic and intrinsic pathways (Figure 43 C, D, Figure 44 C, D) and can be explained due to its ability to restore the redox potential of the mitochondria membrane and reduce oxidative stress. These actions increase ATP production and decrease MOMP following release of cytochrome c (Acuna-Castroviejo et al., 2007).

Mitochondrial theory of aging (Harman, 1972; Miquel et al., 1980) postulates that an alteration in the redox state of the mitochondria, the main source of free radicals in the cell, causes oxidative damage that results in senescence, the primary driver of the aging process. In this sense, Nrf2 is defined as a 'guardian of health span' and a 'master regulator of aging' giving it enormous importance in the control of numerous antioxidant enzymes (Bruns et al., 2015; Lewis et al., 2010). It is well stablished that Nrf2 improves mitochondria function by balancing reduction and oxidation processes and influencing ATP production, membrane potential, fatty acid oxidation and structural integrity (Dinkova-Kostova and Abramov, 2015). However, changes in the levels of this protein during aging, as well as the antioxidant enzymes it regulates, have been the subject of debate in recent years. Controversial and even opposite results appear in many studies, which seem to depend on the species, strain, tissue, sex and experimental design. Our results in cardiac muscle indicate that cytosolic levels of Nrf2 and pNrf2 (Ser40) decrease with aging, both in WT and in $NLRP3^{-/-}$ mice at EA and OA (Figure 43 E, Figure 44 E). This may suggest translocation to the nucleus to activate transcription, to mediate age-related increases in ROS, decreasing cytosolic levels. The presence of pNrf2 in the cytosol could also be due to phosphorylation of Nrf2 by GSK-3β which translocates pNrf2 out of the nucleus (Tomobe et al., 2012). Our data agree with investigations showing that mice deficient in Nrf2 have a higher susceptibility to inflammation and oxidative stress (Ma et al., 2006). This alteration in the Nrf2 pathway is associated with CVDs (Howden, 2013; Reuland et al., 2013). $Nrf2^{-/-}$ mice were more prone to heart failure and their mortality increased ten days after suffering a myocardial infarction (Strom and Chen, 2017; Xu et al., 2014). Although most studies point to a decrease in Nrf2 in heart tissue with aging, the causes are unknown. Surprisingly, our results discarded Keap1 as the responsible of this declining since there were no changes in its levels between the different ages and experimental groups. In line with our findings, levels of Nrf2 and its mRNA were found to be reduced in the liver of 10-month-old SAMP8 mice compared to senescence-accelerated mouse resistant (SAMR)1 mice, while Keap1 mRNA and its protein levels remained unchanged with

age (Tomobe et al., 2012). The decrease in the antioxidant enzymes Hmox1, Nqo1 and γGclc during aging is possibly due to a less efficient Nrf2 signaling (Shih and Yen, 2007; Suh et al., 2004). Similar results using aortas of 24-month-old rats, whose Nrf2 levels were lower compared to 3-month-old young rats, resulted in a drop in the enzymes Hmox1, Nqo1 and γGclc (Ungvari et al., 2011a). However, the same group demonstrated that oxidative stress associated with aging did not induce significant changes in Nrf2 levels of carotid arteries in aged Rhesus macaques (20 years) compared to young individuals (10 years), and their respective antioxidant enzymes were not induced either (Ungvari et al., 2011b). Together, these data confirm that the expression of these antioxidant enzymes is linked to Nrf2. It also suggests the activation of this signaling pathway in the cardiovascular system during aging depends not only on the animal model but on the degree of oxidative stress as well. In this light, recent works described that there is a shift in Nrf2 target to Klf9 instead of Hmox1, Nqo1 and γGclc at excessive oxidative damage (Chhunchha et al., 2019; Zucker et al., 2014). This could explain the fact that Hmox1 and γGclc were decreased to a greater degree than WT by showing decline in EA while WT decreased only at OA. Interestingly, Nqo1 expression levels were not reduced in NLRP3$^{-/-}$ mice but were still upregulated by melatonin supplementation. Several studies show that Nqo1 is the prototype gene target for Nrf2 activation. In BV2 cells after cerebral ischemia reperfusion, Nrf2 ROS response was linked to Nqo1 expression (Xu et al., 2018). This could illuminate the limited decrease in cytosolic Nqo1 by being preferentially targeted by the ever-shrinking pool of Nrf2 and pNrf2 as aging ensues. This study also proved that scavenging of ROS by Nqo1 restrained NLRP3 inflammasome activation and IL-1β expression. Except for Keap1 expression levels, which remained unchanged during aging, treatment with melatonin counteracted the age-associated decline in expression of all the parameters of the Nrf2 signaling pathway, both in WT and NLRP3$^{-/-}$ mice (Figure 43 E, Figure 44 E). Melatonin has been shown to have a protective effect on the mitochondria by acting as a powerful antioxidant in a direct way, as a scavenger of free radicals, detoxifying ROS and RNS, and indirectly, increasing the rest of the Nrf2-dependent and independent antioxidant systems (Rahim et al., 2021; Reiter et al., 2001; Rodriguez et al., 2004; Tomás-Zapico and Coto-Montes, 2005).

Studies in animal models confirm that the ultrastructure of cardiac mitochondria changes with aging (Corsetti et al., 2008). Our study supported these results. A small number of isolated mitochondria had damaged cristae in EA mice, and severe mitochondrial damage, with destroyed, separated, vacuolated and hypertrophied cristae in OA mice. This mitochondrial impairment was more remarkable in WT mice than in mutants. These findings reveal age-induced cellular senescence and mitochondrial dysfunction (Shigenaga et al., 1994), as well as the cardioprotective effect linked to the ablation of the NLRP3 inflammasome (Wang et al., 2018b). Melatonin treatment maintained normal mitochondrial ultrastructure in all experimental groups. Multivesicular bodies increased in treated OA NLRP3$^{-/-}$ mice, which indicate autophagy induction. These results, once again, highlight the protective

role of melatonin against age-mediated mitochondria impairment and its ability to restore altered autophagic processes during cardiac aging (Rodríguez et al., 2008).

Various morphometric analyses show that the size and number of mitochondria per cell is impacted during cardiac aging (El'darov et al., 2015). Our results showed an increase in CSA and Feret's diameter in the mitochondria of OA WT mice, accompanied by a decrease in number of mitochondria. This mitochondrial hypertrophy has been related to a systemic demand from overload stress on the heart (Dobaczewski et al., 2011; Hefti et al., 1997), and our results suggest that it could also be an adaptive mechanism to compensate for the decrease in the amount of this organelle. The ablation of the NLRP3 inflammasome reduced cardiac hypertrophy, as there were no changes in Feret's diameter with age and less significant increase in CSA and decline in mitochondria number. To our knowledge, this is the first time that these morphometrical parameters are studied specifically in IMF during cardiac aging using a mice model. In line to our findings, CSA of cardiomyocytes from the LV of male Fischer 344 rats increased with aging, while the number of cardiomyocytes decreased (No et al., 2020). In Wistar rats, mitochondria volume fraction and mean size both in left and right ventricle were decreased in 2 years old vs 6 weeks old animals (Frenzel and Feimann, 1984). Our results showed that melatonin significantly increased the number of mitochondria in WT and $NLRP3^{-/-}$ mice, with no effect on CSA or Feret's diameter. It is possible that in this case two-months treatment is not enough to counter the age-related changes in CSA and Feret's diameter in the heart, one of the most energy-demanding organs of our body (Neubauer, 2007). This 'cardiac sarcopenia' has hardly been investigated since most studies focus on skeletal muscle. Indeed, our group previously performed the same analyses in gastrocnemius and morphometric alterations were observed earlier, in EA mice and protected in NLRP3 deficient mice (Sayed et al., 2019b). Our findings suggest that cardiac muscle and its mitochondria are physiologically more protected from age-related sarcopenia than skeletal muscle. Its ability to make a metabolic switch in favor of glycolysis instead of fatty acid oxidation during aging (Hyyti et al., 2010; Kates et al., 2003), being one of the organs where the NLRP3 inflammasome is expressed less (Huang et al., 2014; Ye et al., 2015), or the presence of resident macrophages with tissue protective function (Pinto et al., 2012) are some of many possible adaptations of the heart that could explain its greater resistance to sarcopenia.

CHAPTER 2: AGING AND CHRONODISRUPTION IN MOUSE CARDIAC TISSUE. EFFECT OF THE NLRP3 INFLAMMASOME AND MELATONIN THERAPY

The aging process leads to a chronic low-grade inflammatory environment that perpetuates innate immune response and is known as inflammaging (Ferrucci and Fabbri, 2018; Franceschi et al., 2000). The aggregation of pro-inflammatory signals impairs clock genes expression in both central and peripheral tissues (Cavadini et al., 2007; Gast et al., 2012). One of the key components of circadian rhythm regulation is the pineal melatonin. It is known that both melatonin synthesis and its receptor's expression decline with age (Waldhauser et al., 1998). Recent studies have shown the importance of clock gene regulation in maintaining cardiomyocyte function, growth, and renewal pathways as organisms age (Alibhai et al., 2017; Hergenhan et al., 2020). Our results clearly showed that not only does aging impact circadian gene expression in cardiac tissue, but melatonin treatment was also influential. Additionally, we found that, to a lesser extent, ablation of the NLRP3 inflammasome altered daily expression patterns of all clock genes investigated, excepting *Rev-erbα* where no effect was observed with genotype.

Interestingly, the role of *Rev-erbα* in the innate immune and, specifically, in the inflammatory response, has been a controversial topic in scientific research. On one hand, REV-ERBα exerts a pro-inflammatory action by binding competitively with RORα to the same RORE in the promoter of *Bmal1* (Acuña-Castroviejo et al., 2017). Among other processes, BMAL1 regulates *Nampt* expression, whose protein synthesizes NAD^+, the cofactor of SIRT1. This deacetylase inactivates NF-κB, therefore controlling the inflammatory response. On the other hand, the anti-inflammatory effect of REV-ERBα has been well-stablished through the downregulation of the expression of NF-κB signaling and related genes, such as IL-6, IL-1β, IL-18, Tnf-α, Ccl2 and Nlrp3 (Griffin et al., 2019; Guo et al., 2019; Wang et al., 2018a; Zhao et al., 2019b). It has been proposed that the inflammatory action of REV-ERBα and the mechanisms of repressing transcription may be tissue-specific (Griffin et al., 2019; Wang et al., 2020). Most studies reveal a protective role of REV-ERBα in cardiac tissue by inhibiting the expression of factors determining atherosclerosis risk, including: apoCIII protein (Raspé et al., 2001), PPAR nuclear receptors (Fontaine and Staels, 2007), and plasminogen activator inhibitor (PAI-1) (Vaughan, 2005). REV-ERBα activation, as well as selective agonists for REV-ERBα, have been shown to ameliorate heart failure and myocardial infarction in mice by down-regulation of NLRP3 inflammasome activity (Reitz et al., 2019; Stujanna et al., 2017; Zhang et al., 2017). The molecular mechanism of this anti-inflammatory effect remains poorly understood in heart. Using heart tissue chromatin immunoprecipitation sequencing (ChIP-Seq), a recent study proposed that *Rev-erbα* can colocalize with others transcription factors and coordinate the repression at thousands of loci in the genome mediated by multiple transcription factors, preventing a pathogenic switch of gene program (Zhang et al., 2017). The mentioned investigations highlight the anti-inflammatory effect of

Rev-erbα mediated inflammasome suppression in moderating vascular inflammation. The age-related increase of NLRP3 inflammasome activation is well established, and recent studies show increased *Rev-erbα* in murine cardiac tissue with age (Volt et al., 2016). Given our results illustrate that loss of *Nlrp3* did not alter the mRNA expression of *Rev-erbα*, this implies that while *Rev-erbα* can act directly on the inflammasome to suppress it, the presence of *Nlrp3* does not appear to influence *Rev-erbα* in murine cardiac tissue.

The core components of circadian mechanism *Clock* and *Bmal1* are vital determinant for cardiac physiology. Premature aging phenotype and age-associated cardiomyopathy is developed in global and cardiomyocyte-specific mutant mice for *Clock* and also for *Bmal1* gene (Alibhai et al., 2017; Bray et al., 2008; Ingle et al., 2015; Lefta et al., 2012; Young et al., 2014). Cosinor data reflects that rhythm of *Clock* and *Bmal1* is not affected by aging. Some debate has been found regarding the rhythmicity of the gene *Clock*. Some authors exposed no rhythm of gene *Clock* in heart of young Balb/c mice (Bonaconsa et al., 2014). However, our findings are in line with those who showed *Clock* rhythmicity and same acrophase that *Bmal1* in young age rodents (Herichová et al., 2007; Young et al., 2001). It should be noted that although the rhythm of gene *Clock* in heart was unaffected, the phase of the rhythms was delayed over 4 hours in WT EA and WT OA vs WT Y mice. Therefore, while peripheral oscillators may continue to function with rhythmic expression of core clock genes in heart, aging induced phase shifts and reorganization of rhythms in clock genes, as also observed in other peripheral tissues as kidney (Sellix et al., 2012; Yamazaki et al., 2002). Rhythms and acrophases of *Bmal1* were very preserved at all ages. Similar results were found in human skin fibroblasts and cortical area, as well as in mice brain and liver (Lim et al., 2013; Oishi et al., 2011; Pagani et al., 2011). This fact is extremely important for the maintenance of circadian rhythm in heart since *Bmal1* is the only obligate mammalian clock gene for rhythmicity (Bunger et al., 2000). Absence of *Nlrp3* preserved the acrophase in *Clock* and *Bmal1* genes in almost all ages, indicating the influence of this inflammasome on the age-related shifts in the acrophases of these genes. Melatonin treatment corrected the alterations in the acrophases of the gene *Clock*, probably by counteracting the disruption of CLOCK/BMAL1/NF-κB/SIRT1 in aged mice and reducing the activation of NLRP3 inflammasome (Volt et al., 2016).

Our results showed that *Bmal1* is expressed in antiphase with *Per2* gene in WT Y and EA, coinciding with prior studies in heart and other peripheral tissues like stomach and colon (Bonaconsa et al., 2014; Hoogerwerf et al., 2007). Acrophase time is maintained at the beginning of the dark phase (20-21 h), but rhythm is lost with aging in WT OA animals. Some findings reported no apparent changes in *Per2* resulting from the course of aging (Oishi et al., 2011; Tahara et al., 2017). However, our data are in agreement with others that revealed consistent effects in the *Per2* circadian pattern of aged animals (Chen et al., 2016; Driver, 2000; Kunieda et al., 2006; Zhao et al., 2018). Recent studies examining the role of *Per2* in cardiac function are conflicting. Some authors found *Per2*-mutant mice

had less severe injury in ischemia/reperfusion and non-reperfused myocardial infarction than control mice (Virag and Murry, 2003; Virag et al., 2010). Conversely, it has been widely established the cardioprotective effect of *Per2* as mediator of endothelial function, vascular senescence and angiogenesis (Eckle et al., 2012; Viswambharan et al., 2007; Wang et al., 2008). Melatonin restored the rhythm in WT OA mice and significantly advanced the acrophase in both WT EA and WT OA mice. The phase advance of *Per2* in control and hypertensive rat hearts was previously described after melatonin administration in drinking water during the dark phase for 6 weeks (Zeman et al., 2009). Melatonin may have a protective effect in cardiac tissue by extending the period, whose length decreases as a result of aging (McAuley et al., 2002; Pittendrigh and Daan, 1974; Weitzman et al., 1982; Witting et al., 1994). The shortening of circadian period has been linked to cardiomyopathies, fibrosis and decrease lifespan *in vitro* and *in vivo* experimental models (Klarsfeld and Rouyer, 1998; Krishnan et al., 2009, 2012; Martino et al., 2008; Pagani et al., 2011). Melatonin has been proposed to be a proteasome inhibitor in central and peripheral tissues, limiting the destruction of PER2 protein and therefore, increasing period length by advancing the phase (Vriend and Reiter, 2015). The contribution of this gene in immunity seems to be complex. Absence of *Per2* in mice reduced IL-1β and INFγ in sepsis, but it is also able to promote inflammation by reducing the activity of BMAL1 and REV-ERBα (Liu et al., 2006; Preitner et al., 2002). Inflammation, and especially IL-1β, disrupts the circadian rhythm of *Per2* in peripheral tissues (Yuan et al., 2019). Absence of NLRP3 maintained the rhythm in OA mice, possibly because this cytokine remains inactive without the action of this inflammasome. Unexpectedly, the acrophase of mutant mice remained constant with age and melatonin treatment. Interestingly, this acrophase was notably different from WT. The aged-associated increase of NLRP3 may cause this loss of rhythm in WT mice. The fact that acrophase was modified in the complete absence of this inflammasome suggests that NLRP3 may influence Per2 acrophase. Therefore, basal levels of NLRP3 could be necessary for maintaining Per2 rhythm. Similar findings were described regarding NF-κB (Hong et al., 2018).

As *Per2* gene, acrophase of *Chrono* occurred at 20 h in WT Y mice, being both genes repressor of the positive loop *Clock*/*Bmal1* and in antiphase with *Bmal1*, as previously described in SCN and other peripheral tissues (Goriki et al., 2014; Hatanaka et al., 2010). However, unlike *Per2*, the acrophase and rhythmicity of *Chrono* remained constant with aging and with melatonin therapy in WT mice. These data suggest that *Chrono* is an evolutionarily highly preserved gene. As a matter of fact, *Chrono* is known to be the gene that is rhythmically expressed in the largest number of tissues in diurnal primates (Mure et al., 2018). Lack of NLRP3 had no impact on rhythm and acrophase of *Chrono*, which were similar to WT observations. This data implies that, contrary to *Per2*, *Nlrp3* expression does not influence *Chrono* circadian activity. Overall, mechanisms of action and regulation of *Per2* and *Chrono* seem to be different. This result is in line with recent discoveries that found *Per2*

and *Chrono* to bind *Bmal1* N and C-terminus, respectively, and function distinctly as repressors in the mammalian circadian clock (Langmesser et al., 2008; Yang et al., 2020).

Rhythm and acrophase of *Rev-erbα* placed over 17 h persisted in the aged heart of WT mice, as formerly described in cardiac tissue of young and old mice (Bonaconsa et al., 2014), and young Winstar rats (Herichová et al., 2014; Szántóová et al., 2011), others peripheral tissues like mice small intestine and colon (Duez and Staels, 2008; Paulose et al., 2019), as well as experiments performed *in vitro* with young rat cardiomyocytes (Peliciari-Garcia et al., 2011). Melatonin treatment induced a phase advance of *Rev-erbα* in both WT EA and OA mice. The same phenomenon was observed in rat SCN (Masson-Pévet, 2007) but not in rat cardiomyocytes (Peliciari-Garcia et al., 2011). Although there is variation in the dosages and administration of melatonin, and experimental model across studies (Baburski et al., 2015), our results propose that the chronobiotic effect of melatonin may rely on *Rev-erbα* as an initial molecular target (Fontaine and Staels, 2007). There were no significant differences between NLRP3 deficient and WT mice regarding acrophase of *Rev-erbα*, which agrees with our previous comments regarding lack of effect of genotype in mRNA *Rev-erbα* expression. Aging seemed to cause phase advance in mutant mice like what is observed in WT melatonin treated mice. Additionally, melatonin had little impact on acrophase of $NLRP3^{-/-}$ mice. Based on these findings it appears that the therapeutic effect of melatonin on *Rev-erbα* acrophase is mimicked in the NLRP3 knock-out mice, corroborating that some protective effects of melatonin in WT cardiac tissue are dependent on suppression of NLRP3 inflammasome activation (Volt et al., 2016).

Rorα rhythm disappeared in heart of WT EA and WT OA mice. Conversely, neither rhythm nor systematic changes were found in *Rorα* expression of gastrointestinal tissues with aging (Paulose et al., 2019). Studies related to the age-associated changes in *Rorα* are very limited, but these discoveries imply there is a tissue-specific function, being this gene a molecular link between circadian rhythm and cardiac homeostasis (He et al., 2016a). Mice with a loss-of-function mutation in RORα ($Rora^{sg/sg}$) have impairments in the circadian oscillator and develop severe cardiomyopathies. Pharmacological activation of RORα ameliorated the deleterious cardiac changes and strengthened circadian oscillations (He et al., 2016b; Sato et al., 2004). In this sense, melatonin recovered the rhythm in WT EA and OA animals and, interestingly, advanced the phase of *Rorα* to be an hour or two before *Bmal1* acrophase, possibly enhancing its anti-inflammatory action during aging, as also observed in cardiac sepsis mice model (Volt et al., 2016). RORα is a known activator of the BMAL1/NAD+/SIRT1 anti-inflammatory pathway. *Bmal1* transcription increased the expression of *Nampt* gene and consequently NAD^+ levels, which is the substrate for SIRT1 deacetylase activity that inhibits p65 subunit of NF-κB (Donmez and Guarente, 2010; Yeung et al., 2004). Melatonin seems to modulate age-related inflammatory response through RORα. Although it remains a matter of debate whether RORα is a melatonin receptor, an increasing body of evidence suggests that RORα is essential as a mediator of some of the biological effect of melatonin, including the inhibition of the innate

immune response during chronic inflammation (Acuña-Castroviejo et al., 2017; Ding et al., 2019; García et al., 2015). RORα is known to induce the transcription of inhibitor of NF-κB (IκB) and inhibits the nuclear translocation of NF-κB. Lack of NLRP3 shifted the phase at 18 h in Y mice with less rhythmicity compared to WT Y animals. The chronodisruption has been previously observed in mutant mice (Acuña-Castroviejo et al., 2017), indicating that NLRP3 may have a role in maintaining circadian rhythm in heart. As in WT, rhythm was lost in NLRP3$^{-/-}$ EA and OA animals, and restored by melatonin, placing the acrophase respectively at 13 h and 11h, approaching WT Y mice phase. Melatonin was able to reestablish *Rorα* rhythm and advance acrophase to a similar degree in both strains of mice independent of NLRP3 inflammasome expression. These data could be due to the fact that there are no changes in *Nampt* and *Sirt1* expression in mutant mice (Rahim et al., 2017), limiting the activation of *Rorα* in Y mice. Taken together, these findings suggest that *Rorα* rhythm and acrophase are influenced by melatonin and NLRP3 inflammasome.

Lower amplitude has been linked with an augmented risk of CVD, and diminished mesor has been associated with a higher risk of coronary heart disease (Paudel et al., 2011). Circadian changes related to aging include the reduction of the amplitude in both central and peripheral tissues of mammals and *Drosophila melanogaster* (Chen et al., 2016; Nakamura et al., 2015; Rakshit et al., 2012; Roenneberg et al., 2007). However, a significant number of other studies showed no apparent effect of aging regarding these circadian parameters (Oishi et al., 2011; Pagani et al., 2011; Solanas et al., 2017; Yamaguchi et al., 2018). Our results suggest that aging, lack of NLRP3 inflammasome and melatonin treatment had low influence in the amplitude of clock genes in heart. Instead, mesor tended to decline with aging, and NLRP3 absence and melatonin did not restore this dampening. Contrary to our findings, Bonaconsa et al. found a tendency towards amplitude decrease and preservation of mesor in aged heart (Bonaconsa et al., 2014). Controversial results regarding amplitude have been also observed in rodent SCN and also in human leukocytes, mucosa and heart, cardiac tissue being the one with the widest range of amplitudes (Banks et al., 2016; Leibetseder et al., 2009). The intense metabolism and low rate of differentiation of myocardial cells were proposed by authors as a possible explanation of these results. Still, the exact reasons for these discrepancies in clock-gene amplitudes and mesor remain to be elucidated (Okamura, 2004; Tsinkalovsky et al., 2007).

CHAPTER 3: NLRP3 INFLAMMASOME DELETION AND / WITH MELATONIN SUPPLEMENTATION MITIGATE AGE-DEPENDENT MORPHOLOGICAL AND ULTRASTRUCTURAL ALTERATIONS IN MURINE HEART

This study describes for the first time the contribution of NLRP3 inflammasome to heart deterioration during aging. The NLRP3 inflammasome plays an essential role in the pathogenesis of various CVDs such as hypertension, atherosclerosis and myocardial infarction (Liu et al., 2018; Toldo and Abbate, 2018). To better understand the role of NLRP3 in cardiac aging and age-related cardiac sarcopenia, we examined the LV of differentially aged WT and NLRP3$^{-/-}$ mice. As observed here, the deletion of the NLRP3 inflammasome resulted in a better cardiac architecture with no age-associated changes in the EA mice, and with less necrotic and fibrotic changes in the OA animals when compared to WT mice. A recent study performed on NLRP3-knockout mice revealed a significant increase of heart weight / body weight ratio in the old WT mice, while non-significant increase were detected in old NLRP3$^{-/-}$ mice (Marín-Aguilar et al., 2020); however, the ratio of heart weight to body weight observed here reported an age-mediated reduction, confirmed previous findings reported elsewhere (Boyle et al., 2011), and this decline was higher in the OA WT mice than in OA NLRP3$^{-/-}$.

The reduction of heart weight to body weight ratio during aging was associated with a decline in cardiac fiber numbers, increased left ventricular wall thickness and an enhanced compensated cardiomyocyte hypertrophy of the remaining fibers with increased β-MHC mRNA expression. These alterations were less prominent in NLRP3$^{-/-}$ mice than in WT. Increased cardiomyocyte transverse CSA was reported in aged WT mice unlike NLRP3$^{-/-}$ (Marín-Aguilar et al., 2020). Our previous study in gastrocnemius muscle confirmed these results, where lack of NLRP3 inflammasome showed lower muscular decline and reduced collagen fibers in aged NLRP3$^{-/-}$ mice when compared with aged WT mice (Sayed et al., 2019b). The increased expression level of β-MHC during cardiac aging has been reported (Carnes et al., 2004). Other study suggested that β-MHC expression during aging is a marker of fibrosis rather than of cellular hypertrophy (Pandya et al., 2006).

Recently, a study revealed increased mass and collagen level of the LV, as well as the thickness of the septal wall in aged mice, associated with increased expressions of IL-1α, IL-6 and TNF-α, suggesting that cardiac structural and functional changes with age are closely graded with frailty and inflammation markers (Kane et al., 2021). NLRP3 inflammasome-related inflammaging has been reported to be activated during the aging phenomenon in many tissues and organs including heart (Liu et al., 2018; McBride et al., 2017). Pro-inflammatory cytokines induced through age-dependent inflammasome activation promote muscle tissue wasting and atrophy, whilst lack of NLRP3 protects against these inflammatory proceedings (Huang et al., 2017). The induction of fibrosis and IL-1α and IL-6 inflammatory cytokine genes observed here were less remarkable in the NLRP3$^{-/-}$ mice. Lately,

interstitial and perivascular cardiac fibrosis was described in aged WT mice, with non-significant changes in aged NLRP3$^{-/-}$ mice, where increased IL-6 serum and protein levels in the cardiac tissues of old WT and NLRP3$^{-/-}$ mice were observed (Marín-Aguilar et al., 2020). Therefore, the reduction of collagenous tissue infiltrations in the aged myocardium of NLRP3$^{-/-}$ mice assured the attenuation of fibrosis upon NLRP3 depletion (Kane et al., 2021).

Mitochondria play critical roles in cellular life and death, as it is important for maintaining cellular homeostasis, thus mitochondrial dysfunction with aging has been implicated in the deterioration in structure and function of skeletal and cardiac muscles (Hepple, 2016). The correlation between mitochondrial function and cardiovascular health was recently investigated, where lower mitochondrial oxidative capacity in aged individuals was associated with a positive previous history of CVD (Zampino et al., 2021). Furthermore, poorer mitochondrial function was recently proposed as a potential contributor of increased perceived fatigability (Liu et al., 2021). The current study demonstrated by electron microscopy that cardiac muscle aging is associated with an assortment of ultrastructural alterations, including mitochondrial swelling, cristae destruction and matrix vacuolization, increased lipid accumulation, and a decline in mitochondrial number. These observations of the mitochondrial ultrastructural alterations have been proposed as an indicator of cellular senescence and age-dependent loss of mitochondrial functions (Leonardo-Mendonça et al., 2017; Zhang et al., 2018), and were less considerable in aged cardiomyocytes of NLRP3$^{-/-}$ mice than WT (Marín-Aguilar et al., 2020). Our data confirm our previous findings in the skeletal muscle, where the lack of NLRP3 inflammasome reduced mitochondrial impairment during aging (Sayed et al., 2019b), and supporting the role of the NLRP3 inflammasome inhibition in prevention of cardiac aging (Marín-Aguilar et al., 2020).

Aging of cardiomyocytes was associated with formation of autophagosomes, which were more pronounced in aged cardiomyocytes of WT mice than NLRP3$^{-/-}$. Many studies have suggested the involvement of autophagy in the regulation of lifespan and aging (Madeo et al., 2015). It plays an essential role in mitigation of age-associated cardiac changes (Shirakabe Akihiro et al., 2016). The age-dependent decline of autophagy in the heart (Taneike et al., 2010) enhances impairments in cellular housekeeping functions that induce NF-κB signaling which, either directly or through inflammasomes, stimulates age-related pro-inflammatory events (Salminen et al., 2012b). Moreover, aging diminishes the autophagic/mitophagic capacity and leads to an accumulation of ROS, triggering activation of the NLRP3 inflammasome and induces inflammation in various tissues (Kane et al., 2021; Salminen et al., 2012b), while absence of NLRP3 improves mitochondrial dysfunction. Therefore, our results revealed reduction of autophagosome number and size in aged cardiac muscles of NLRP3$^{-/-}$ mice, confirming the beneficial effect of NLRP3 inhibition in improvement of autophagy quality during cardiac aging (Marín-Aguilar et al., 2020). Our previous study revealed that loss of NLRP3 had few impacts on age-dependent cardiac autophagic changes (Fernández-Ortiz et al., 2020).

A recent study confirmed the beneficial effect of NLRP3 inhibition via enhancing autophagy in aged mice. Moreover, this latter study proposed the impact of NLRP3 suppression on improving human health and age-dependent metabolic syndrome (Marín-Aguilar et al., 2020).

Aging of cardiac muscle fibers was associated with increased nuclear apoptosis, being more pronounced in cardiac myocytes of WT mice than NLRP3$^{-/-}$ ones. Apoptosis displays a key role in the muscular loss, where it has been defined in muscular denervation (Borisov and Carlson, 2000), as well as in chronic heart failure (Adams et al., 1999). It is an active process causing designed cellular death, and is associated with removal of apoptotic bodies without inflammation through phagocytosis of these bodies by surrounding cells or macrophages (Pollack and Leeuwenburgh, 2001). An increase in apoptosis and necrosis was previously observed in the myocardium of old animals (Kajstura et al., 1996). Our recent study detected less cardiac apoptosis during aging in the absence of NLRP3 (Fernández-Ortiz et al., 2020).

Besides its chronobiotic properties due to the low-level circadian release of melatonin by the pineal gland, melatonin is also produced in most organs and tissues of the body (Venegas et al., 2012). The so-called extrapineal melatonin exerts profound antioxidant and anti-inflammatory actions due to high levels that reach the cells. Recently, it has been showed that mouse hearts produce high amounts of melatonin that contributes to cardiac protection (Acuña-Castroviejo et al., 2018; García et al., 2015). Cardiac melatonin, however, decreases with age (Sanchez-Hidalgo et al., 2009), thus reducing this potential cardioprotective capacity. Here, melatonin supplementation to mice prevented the progress of age-related cardiac sarcopenia, where melatonin conserved the normal architecture of cardiomyocytes with narrow interstitium, less fibrosis and absence of necrotic fibers. Moreover, melatonin treatment recovered the thickness of the ventricular wall, improved the heart weight / body weight ratio through restoration of cardiomyocyte number and minimizing muscle fiber hypertrophy (decreased β-MHC expression) and apoptosis in EA and OA animals. Furthermore, melatonin supplementation significantly decreased the expression of IL-1α and IL-6 in WT and NLRP3$^{-/-}$ mice, confirming the anti-oxidative, anti-apoptotic, and anti-inflammatory effects of melatonin (Acuña-Castroviejo et al., 2014; Molpeceres et al., 2007). Melatonin administration was reported to inhibit cardiomyocyte apoptosis and improve organization of actin filaments, as well as maintain calcium homeostasis as protective mechanisms against myocardial reperfusion injury (Hu et al., 2018). The beneficial effects of melatonin treatment against age-related cardiac apoptosis and autophagic changes was more profound in NLRP3$^{-/-}$ mice than WT (Fernández-Ortiz et al., 2020).

Mitochondria are the key intracellular target of melatonin, which reduces free radical formation, and boosts the ATP production in both normal and pathological conditions (Acuña Castroviejo et al., 2011; Escames et al., 2003; López et al., 2009; Martín et al., 2000a). Melatonin therapy protected the cardiac muscle fibers against the age-dependent mitochondrial damage. It

maintained the normal ultrastructure of cardiac myocytes, preserved the mitochondrial contents, reduced residual and multivesicular bodies, and also kept the integrity of the IMF mitochondrial number with aging. These preservative effects of melatonin were more detectable in NLRP3$^{-/-}$ mice than in WT (Fernández-Ortiz et al., 2020), confirming our previous findings where melatonin protected muscles from age-related sarcopenia-dependent mitochondrial damages. Furthermore, NLRP3 inflammasome deletion reduced these alterations and induced the protective effects of melatonin (Sayed et al., 2018, 2019b). All of these findings support the essential impact of melatonin on preventing mitochondrial dysfunction, reducing oxidative stress, and minimizing sarcopenic alterations in patients (Coto-Montes et al., 2016).

CONCLUSIONS

1. Our results clarify the impact of NLRP3 inflammasome in the mitochondria during aging because the lack of NLRP3 prevents mitochondrial dynamics and ultrastructural mitochondrial impairments.

2. Similarly, melatonin treatment boosts the Nrf2-dependent antioxidant capacity of aged heart, making it able to improve mitochondrial structure.

3. Aging affects clock genes expression in mouse heart. Our results support that age caused small phase changes in *Clock*, loss of rhythmicity in *Per2* and *Rorα*, and a tendency towards dampening mesor.

4. NLRP3 inflammasome impacts clock gene expression in cardiac tissue, except for *Rev-erbα*, which was not affected by mice genotype. NLRP3 inflammasome activity, which increases with age, influenced the acrophase of *Clock*, *Per2* and *Rorα*, suggesting a negative impact in myocardial function.

5. Melatonin therapy restored acrophases and rhythm in cardiac tissue, giving it clinical potential in preventing and treating chronopathologies including those dependending on myocardial dysfunction.

6. Nevertheless, the changes in clock genes expression here reported support that the local chronobiotic system of the heart is highly protected from aging.

7. NLRP3 is involved in age-dependent sarcopenia in cardiac muscle, since NLRP3$^{-/-}$ mice show less thickening of the ventricular wall, less fibrosis in aged myocardium, lower expression of inflammatory cytokines and less mitochondrial damage compared to wild type mice.

8. Melatonin therapy prevents cardiac aging in a similar manner that the absence of NLRP3 inflammasome, suggesting that the former counteracts the inflammatory effects of this inflammasome, a property further demonstrated in our previous studies.

9. Overall, melatonin becomes an excellent cardioprotective agent against the deleterious effects of aging on mouse myocardial function.

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ANNEX

Annex 1. Aging, genotype, melatonin effect on clock gene expression in WT and NLRP3$^{-/-}$ mice. Sign post-hoc test after Multifactorial-ANOVA.

TRANSCRIPT: *Clock*. Post-hoc test: Least Significant Difference								
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***		
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value
0	Y vs OA	<0.05	0	NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ EA	<0.05	0	Y vs OA + aMT	<0.05
	EA vs OA	<0.05		NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ OA	<0.05	6	Y vs OA + aMT	<0.05
6	Y vs EA	<0.05		NLRP3$^{-/-}$ EA vs NLRP3$^{-/-}$ OA	<0.05		EA vs EA + aMT	<0.05
	Y vs OA	<0.05		WT EA vs NLRP3$^{-/-}$EA	<0.05	12	Y vs OA + aMT	<0.05
	EA vs OA	<0.05		WT OA vs NLRP3$^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05
12	EA vs OA	<0.05	6	NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ OA	<0.05	18	Y vs EA + aMT	<0.05
18	Y vs OA	<0.05		NLRP3$^{-/-}$ EA vs NLRP3$^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05
	EA vs OA	<0.05		WT EA vs NLRP3$^{-/-}$EA	<0.05		EA vs EA + aMT	<0.05
			12	NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ EA	<0.05		OA vs OA + aMT	<0.05
				NLRP3$^{-/-}$ EA vs NLRP3$^{-/-}$ OA	<0.05			
			18	NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ EA	<0.05			
				NLRP3$^{-/-}$ Y vs NLRP3$^{-/-}$ OA	<0.05			
				NLRP3$^{-/-}$ EA vs NLRP3$^{-/-}$ OA	<0.05			

TRANSCRIPT: *Bmal1*. Post-hoc test:Tukey multiple comparison of mean									
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***			
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	H
0	Y vs EA	<0.001	0	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.001	0	Y vs EA + aMT	<0.001	
	Y vs OA	<0.001		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.001		Y vs OA + aMT	<0.001	
6	Y vs EA	<0.001	6	WT Y vs $NLRP3^{-/-}$ Y	<0.01	6	Y vs EA + aMT	<0.05	
	EA vs OA	<0.05	12	$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	
18	Y vs EA	<0.001	18	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.001	18	Y vs EA + aMT	<0.001	
	Y vs OA	<0.001		$NLRP3^{-/-}$Y vs $NLRP3^{-/-}$OA	<0.001		Y vs OA + aMT	<0.001	
				WT Y vs $NLRP3^{-/-}$ Y	<0.001		EA vs EA+aMT	<0.001	
							EA + aMT vs OA	<0.001	
							EA + aMT vs OA + aMT	<0.001	

TRANSCRIPT: *Per2*. Post-hoc test: Least Significant Difference									
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***			
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	Hour
0	Y vs EA	<0.05	0	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05	6	Y vs EA + aMT	<0.05	0
	EA vs OA	<0.05		$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05	
6	Y vs OA	<0.05		WT EA vs $NLRP3^{-/-}$EA	<0.05	12	Y vs EA + aMT	<0.05	
	EA vs OA	<0.05		WT OA vs $NLRP3^{-/-}$ OA	<0.05		EA vs EA + aMT	<0.05	6
18	Y vs EA	<0.05	6	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	18	Y vs EA + aMT	<0.05	
	Y vs OA	<0.05		$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	12
	EA vs OA	<0.05		WT EA vs $NLRP3^{-/-}$EA	<0.05		EA vs EA + aMT	<0.05	
			12	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05		OA vs OA + aMT	<0.05	
				$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05				
			18	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05				
				$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05				
				WT Y vs $NLRP3^{-/-}$ Y	<0.05				
				WT EA vs $NLRP3^{-/-}$EA	<0.05				18
				WT OA vs $NLRP3^{-/-}$ OA	<0.05				

TRANSCRIPT: *Chrono*. Post-hoc test: Least Significant Difference									
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***			
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	Hour
6	Y vs EA	<0.05	0	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	0	Y vs EA + aMT	<0.05	0
	Y vs OA	<0.05		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	
	EA vs OA	<0.05		WT Y vs $NLRP3^{-/-}$ Y	<0.05		EA vs EA + aMT	<0.05	
12	Y vs EA	<0.05		WT EA vs $NLRP3^{-/-}$ EA	<0.05		OA vs OA + aMT	<0.05	
	Y vs OA	<0.05		WT OA vs $NLRP3^{-/-}$ OA	<0.05	6	Y vs EA + aMT	<0.05	
	EA vs OA	<0.05	6	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05		Y vs OA + aMT	<0.05	
18	Y vs OA	<0.05		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		EA vs EA + aMT	<0.05	6
	EA vs OA	<0.05		WT EA vs $NLRP3^{-/-}$ EA	<0.05	12	Y vs EA + aMT	<0.05	
				WT OA vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	
			12	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05		EA vs EA + aMT	<0.05	12
				$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05	
				WT Y vs $NLRP3^{-/-}$ Y	<0.05	18	Y vs EA + aMT	<0.05	
				WT EA vs $NLRP3^{-/-}$ EA	<0.05		Y vs OA + aMT	<0.05	
				WT OA vs $NLRP3^{-/-}$ OA	<0.05		EA vs EA + aMT	<0.05	

TRANSCRIPT: *Chrono*. Post-hoc test: Least Significant Difference (continued)									
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***			
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	Hour
			18	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05	18	OA vs OA + aMT	<0.05	18
				$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05				
				WT Y vs $NLRP3^{-/-}$ Y	<0.05				
				WT EA vs $NLRP3^{-/-}$ EA	<0.05				
				WT OA vs $NLRP3^{-/-}$ OA	<0.05				

TRANSCRIPT: *Rev-erbα*. Post-hoc test: Least Significant Difference									
Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***			
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	Hour
0	Y vs EA	<0.05	0	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	6	Y vs EA + aMT	<0.05	6
	EA vs OA	<0.05		$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	
6	Y vs EA	<0.05	6	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	12	Y vs EA + aMT	<0.05	12
	Y vs OA	<0.05		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		Y vs OA + aMT	<0.05	
12	Y vs EA	<0.05	12	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05		EA vs EA + aMT	<0.05	
	Y vs OA	<0.05		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05	
	EA vs OA	<0.05		$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05	18	Y vs EA + aMT	<0.05	18
							Y vs OA + aMT	<0.05	
							EA vs EA + aMT	<0.05	

TRANSCRIPT: *Rorα*. Post-hoc test: Least Significant Difference

Aging effect on WT			***Genotype effect***			***Melatonin effect on WT***				
Hour	Comparison	P value	Hour	Comparison	P value	Hour	Comparison	P value	Hour	
0	Y vs OA	<0.05	0	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	0	Y vs EA + aMT	<0.05	0	NL
6	Y vs OA	<0.05		$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05		NL
	EA vs OA	<0.05		WT Y vs $NLRP3^{-/-}$ Y	<0.05	6	Y vs OA + aMT	<0.05		NL
18	EA vs OA	<0.05	6	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05	12	Y vs OA + aMT	<0.05	6	NL
				$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05		N
				$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05	18	Y vs EA + aMT	<0.05	12	N
				WT Y vs $NLRP3^{-/-}$ Y	<0.05		Y vs OA + aMT	<0.05		N
				WT EA vs $NLRP3^{-/-}$ EA	<0.05		EA vs EA + aMT	<0.05		W
				WT OA vs $NLRP3^{-/-}$ OA	<0.05		OA vs OA + aMT	<0.05	18	NL
			12	$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ EA	<0.05					NL
				$NLRP3^{-/-}$ Y vs $NLRP3^{-/-}$ OA	<0.05					N
				$NLRP3^{-/-}$ EA vs $NLRP3^{-/-}$ OA	<0.05					W
				WT EA vs $NLRP3^{-/-}$ EA	<0.05					W
			18	$NLRP3^{-/-}$Y vs $NLRP3^{-/-}$OA	<0.05					
				$NLRP3^{-/-}$EA vs $NLRP3^{-/-}$OA	<0.05					
				WT Y vs $NLRP3^{-/-}$ Y	<0.05					
				WT EA vs $NLRP3^{-/-}$ EA	<0.05					

Annex 2. Cosinor analysis of clock gene expression in heart of WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment.

Group	Gene	P-value	PR	Acrophase (95% CI)	Amplitude (95% CI)	Mesor (95% CI)
WT Y	***Clock***	< 0.001	83,31	7.53 (6.21 – 8.87)	20 (13.30 - 26.80)	69.2 (64.50 - 74)
	Bmal1	< 0.001	73,48	7.67 (6.62 – 8.87)	28.60 (20.80 – 36.40)	50.80 (45.30 – 56.30)
	Per2	< 0.01	35,83	19.87 (17.40 – 22.40)	10.40 (4.07 – 16.30)	45.60 (41.20 – 50.10)
	Chrono	< 0.05	63,17	19.67 (17.33 - 22)	8.96 (3.80 – 14.10)	22.10 (18.40 – 25.70)
	Rev-erbα	< 0.001	65,24	16.73 (15.47 - 18)	27.20 (18.20 – 36.20)	21.70 (15.30 – 28.10)
	Rorα	< 0.001	83,25	13.33 (12.47 -14.20)	15.50 (12 – 19.10)	53.20 (50.70 – 55.70)
NLRP3$^{-/-}$ Y	***Clock***	< 0.05	48,22	7.80 (5.26 – 10.40)	7.60 (2.80 – 12.30)	55.90 (52.50 – 59.20)
	Bmal1	< 0.01	43,63	7.93 (5.84 - 10)	17.30 (8.37 – 26.20)	30.50 (24.20 – 36.90)
	Per2	< 0.05	58,63	12.47 (9.87 – 15.13)	19.70 (7.20 – 32.10)	53.80 (45 – 62.70)
	Chrono	< 0.001	81,06	17.73 (16.87 – 18.53)	34.90 (27.20 – 42.50)	45.80 (40.40 – 51.30)
	Rev-erbα	< 0.001	72,8	17.40 (16.33 – 18.47)	35.70 (25.80 – 45.70)	28.40 (21.40 – 35.40)
	Rorα	< 0.05	58,83	18.01 (15.47 – 20.67)	26.50 (9.79 – 43.30)	74.30 (62.40 – 86.10)
WT EA	***Clock***	< 0.01	37,08	12.20 (9.80 – 14.60)	14.70 (6.03 – 23.50)	60.60 (54.50 – 66.80)
	Bmal1	< 0.001	72,58	10.87 (9.80 - 11.93)	34.90 (25.20 – 44.70)	26.90 (20 – 33.80)
	Per2	< 0.05	61,46	21.33 (18.87 – 23.73)	23.80 (9.58 - 38)	58.70 (48.60 – 68.70)

	Chrono	< 0.001	86,97	17.27 (16.60 - 17.93)	14.80 (12.20 – 17.40)	22.70 (20.90 – 24.50)
	Rev-erbα	< 0.001	80,37	16.07 (15.20 - 16.93)	24.40 (18.90 – 29.80)	30.80 (26.90 – 34.60)
	Rorα	ns	23,86	13.80 (10.20 – 17.40)	13.20 (2.49 – 23.90)	54.50 (47 – 62.10)
NLRP3$^{-/-}$ EA	***Clock***	< 0.001	80,06	10.87 (10 - 11.73)	29.80 (23 – 36.50)	67.60 (62.90 – 72.40)
	Bmal1	< 0.001	74,63	8.80 (7.73 – 9.80)	26.50 (19.50 – 33.50)	24.70 (19.80 – 29.70)
	Per2	< 0.001	52,92	11.13 (9.47 – 12.80)	12.80 (7.32 – 18.30)	56.10 (52.20 – 59.90)
	Chrono	< 0.001	65,24	17.73 (16.47 – 19.07)	34.90 (23.30 – 46.50)	24.60 (16.50 – 32.80)
	Rev-erbα	< 0.001	95,62	15.07 (14.67 – 15.40)	43.60 (39.40 – 47.90)	36.90 (33.90 – 39.90)
	Rorα	ns	3,03	19.40 (16.27 – 22.53)	4.28 (-6.71 – 15.30)	56.80 (49 – 64.60)
WT EA + aMT	***Clock***	< 0.001	78,5	7.27 (6.34 – 8.20)	29.50 (22.50 – 36.50)	50.90 (45.90 – 55.90)
	Bmal1	< 0.001	96,16	9.33 (8.93 – 9.67)	28.90 (26.30 -31.60)	22.70 (20.80 – 24.50)
	Per2	< 0.01	43,64	6.65 (4.58 – 8.73)	15.50 (7.50 – 23.50)	47.60 (41.90 – 53.30)
	Chrono	< 0.001	77,88	20.60 (19.67 – 21.53)	4.73 (3.59 – 5.87)	9.55 (8.74 – 10.40)
	Rev-erbα	< 0.001	67,33	12.13 (10.93 – 13.40)	40.40 (27.60 – 53.10)	31.50 (22.50 – 40.60)
	Rorα	< 0.05	54,57	6.63 (3.73 – 9.53)	23 (7.17 – 38.80)	45 (33.80 – 56.20)
NLRP3$^{-/-}$ EA + aMT	***Clock***	< 0.01	46,18	6.48 (4.53 – 8.47)	13.90 (7.10 – 20.80)	68.80 (64 – 73.70)
	Bmal1	< 0.001	95,65	7.40 (7 - 7.73)	17.30 (15.60 - 19)	19.70 (18.50 – 20.90)
	Per2	< 0.05	41,03	10.80 (7.80 – 13.87)	12.30 (3.47 – 21.20)	60.90 (54.70 – 67.20)

	Chrono	< 0.001	66,5	18.27 (17 – 19.53)	32.10 (21.80 – 42.50)	22.80 (15.50 – 30.10)
	Rev-erbα	< 0.001	90,93	15.60 (15 – 16.13)	40.20 (34.40 – 45.90)	36.60 (32.50 – 40.70)
	Rorα	< 0.05	40,9	13.13 (10.07 – 16.20)	9.46 (2.65 – 16.30)	56.60 (51.70 – 61.40)
WT OA	***Clock***	< 0.01	46,41	11.40 (9.40 – 13.33)	13.10 (6.73 – 19.50)	41.90 (37.40 – 46.50)
	Bmal1	< 0.001	86,62	9.13 (8.40 - 9.80)	37 (30.40 – 43.60)	30.20 (25.50 – 34.90)
	Per2	ns	20,98	20.93 (16.80 – 25.07)	6.01 (0.72 – 11.30)	35 (31.20 – 38.70)
	Chrono	< 0.001	73,24	18.67 (17.60 – 19.73)	42.20 (30.70 – 53.80)	29.60 (21.40 – 37.80)
	Rev-erbα	< 0.001	77,94	15.73 (14.80 – 16.67)	21 (16 – 26.10)	24.50 (20.90 - 28)
	Rorα	ns	1,35	16 (12.93 – 19.07)	2.76 (-7.94 – 13.40)	65.60 (58 – 73.20)
NLRP3$^{-/-}$ OA	***Clock***	< 0.001	67,88	10.40 (9.20 – 11.60)	22.30 (15.40 – 29.30)	40.60 (35.60 – 45.50)
	Bmal1	< 0.001	83,88	9.80 (9 – 10.53)	41 (32.80 – 49.10)	32.70 (26.90 – 38.40)
	Per2	< 0.001	78,38	10.47 (9.53 – 11.33)	19.30 (14.70 – 23.90)	37.70 (34.50 - 41)
	Chrono	< 0.001	55,85	17.47 (15.87 – 19.07)	13 (7.74 – 18.20)	13.60 (9.86 – 17.30)
	Rev-erbα	< 0.001	70,96	16 (14.87 – 17.13)	25.40 (18 – 32.80)	22.40 (17.20 – 27.60)
	Rorα	ns	28,59	15.27 (11.20 – 19.40)	3.43 (-0.66 – 7.53)	51.70 (48.80 – 54.60)
WT OA + aMT	***Clock***	< 0.01	38,43	7 (4.66 – 9.33)	9.11 (3.80 – 14.30)	36.20 (32.50 – 39.90)
	Bmal1	< 0.001	81,19	8.40 (7.53 – 9.27)	22.20 (17.40 – 27.10)	22 (18.60 – 25.40)
	Per2	< 0.001	50,67	4.63 (2.86 – 6.41)	13.50 (7.45 – 19.50)	36.50 (32.30 – 40.80)

	Chrono	< 0.001	66,99	19.80 (18.40 – 21.20)	7 (4.48 – 9.51)	10.70 (8.92 – 12.50)
	Rev-erbα	< 0.001	93,08	13.93 (13.27 - 14.53)	16.40 (13.70 - 19)	20.60 (18.70 – 22.50)
	Rorα	< 0.001	79,2	6.01 (4.49 – 7.53)	22.80 (14 – 31.60)	36.30 (30.10 – 42.60)
NLRP3$^{-/-}$ OA + aMT	***Clock***	< 0.01	41,73	7.53 (5.37 – 9.67)	20.50 (9.25 – 31.50)	42.30 (34.50 – 50.10)
	Bmal1	< 0.001	86,25	7.40 (6.67 – 8.07)	16.10 (13.20 - 19)	15 (13 – 17.10)
	Per2	< 0.001	64,39	12.60 (11.33 – 13.93)	28.80 (19.10 – 38.50)	51.30 (44.40 – 58.20)
	Chrono	< 0.001	86,27	16.01 (15.13 - 17)	28.10 (21.40 – 34.80)	26 (21.30 – 30.80)
	Rev-erbα	< 0.001	84,81	14.33 (13.60 – 15.07)	41.90 (33.80 – 49.90)	35.60 (29.90 – 41.30)
	Rorα	< 0.001	82,95	11.20 (9.87 - 12.53)	31.10 (20.50 – 41.80)	60.80 (53.30 – 68.40)

Annex 3. Melatonin/Nrf2/NLRP3 connection in mouse heart mitochondria during aging.

Article
Antioxidants (Basel). 2020 Nov 27;9(12):1187. DOI:10.3390/antiox9121187.

Melatonin/Nrf2/NLRP3 connection in mouse heart mitochondria during aging

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Received: date; Accepted: date; Published: date

Abstract: Aging is a major risk for cardiovascular diseases (CVD). Age-related disorders include oxidative stress, mitochondria dysfunction and exacerbation of the NF-κB/NLRP3 innate immune response pathways. Some of the molecular mechanisms underlying these processes, however, remain unclear. This study tested the hypothesis that NLRP3 inflammasome plays a role in cardiac aging and melatonin is able to counteract its effects. With the aim of investigating the impact of NLRP3 inflammasome and the actions and target of melatonin in aged myocardium, we analyzed the expression of proteins implied in mitochondria dynamics, autophagy, apoptosis, Nrf2-dependent antioxidant response and mitochondria ultrastructure in heart of wild-type and NLRP3-knockout mice of 3, 12 and 24 moths-old, with and without melatonin treatment. Our results showed that the absence of NLRP3 prevented age-related mitochondrial dynamic alterations in cardiac muscle with minimal effects in cardiac autophagy during aging. The deficiency of the inflammasome affected Bax/Bcl2 ratio, but not p53 or caspase 9. The Nrf2-antioxidant pathway was also unaffected by the absence of NLRP3. Furthermore, NLRP3-deficiency prevented the drop in autophagy and mice showed less mitochondrial damage than wild-type animals. Interestingly, melatonin treatment recovered mitochondrial dynamics altered by aging and had few effects on cardiac autophagy. Melatonin supplementation also had an anti-apoptotic action in addition to restore Nrf2-antioxidant capacity and improve mitochondria ultrastructure altered by aging.

Keywords: melatonin; mitochondria; NLRP3 inflammasome; Nrf2; heart ultrastructure; apoptosis; mitochondrial dynamics

1. Introduction

Cardiovascular diseases (CVD) constitute the leading cause of death in the world, especially in industrialized countries [1]. Genetics, hypertension, diabetes, obesity, smoking, and physical inactivity have been identified as risk factors for these diseases [2]. However, aging is by far the major risk factor for cardiac dysfunction, since its prevalence increases dramatically in aged people. The connection between aging and these cardiac pathologies have been widely reported [3–5]. Cardiac aging correlates with hemodynamic and metabolic alterations together, with changes in the structure

and function of cardiovascular tissues. Furthermore, the increase in reactive oxygen species (ROS) and the activation of inflammation-related pathways have also been documented [6–8]. Aging is characterized by an increase in oxidative damage and persistent activation of innate immunity resulting in immunosenescence. This immune dysregulation results in a state of age-associated chronic inflammation termed 'inflammaging', which plays an important role in the onset and progression of cardiovascular diseases, in addition to other age-related disorders [9–12].

The main components of the innate immunity include NF-kB and NLRP3 inflammasome. Focusing on the NLRP3 inflammasome, it consists of the scaffold protein NLRP3, the adaptor protein ASC and caspase-1, forming a multiprotein complex [13]. The NLRP3 inflammasome is induced upon different signs of cellular 'danger' and is responsible for the maturation of the NF-kB-dependent pro-inflammatory cytokines including interleukin-1β (IL-1β), potentiating the inflammatory response [14]. Some of these danger signals, such as ROS and mitochondrial DNA (mtDNA), come from impaired mitochondria during inflammation [15]. Additionally, age-related alterations in processes that maintain mitochondrial homeostasis, including fusion, fission, autophagy (mitophagy), and mitochondrial biogenesis, have been described. The resulting accumulation of dysfunctional mitochondria enhances ROS production and mtDNA release [16,17]. Another fact that could contribute to NLRP3 inflammasome activation is the reduced endogenous antioxidant defense capacity which occurs during aging, in particular, the decline of transcription factor Nrf2 [18,19]. Thus, there seems to be a close relationship between aging, NF-kB/NLRP3 inflammasome response, cardiac and mitochondrial dysfunction, ROS formation, and decrease in Nrf2.

Melatonin (N-acetyl-5-methoxytryptamine, aMT) is an ubiquitous molecule that, aside from the pineal gland [20], is synthesized by most body organs and tissues, including the heart [21,22]. In addition to its chronobiotic effects, this indoleamine presents important anti-oxidative and anti-inflammatory properties that depend on the high levels of extrapineal melatonin [23–25]. Within the cell, melatonin acts on its main target, the mitochondria, boosting their bioenergetic properties, enhancing the ATP levels and reducing the formation of free radicals [26–30]. In multiple experimental conditions including acute and chronic inflammation, and aging in mouse heart, melatonin consistently prevented oxidative stress, reduced the innate immunity activation, and boosted cardiac mitochondria function [12,23,25,31].

The mechanisms by which NLRP3 contributes to cardiovascular disorders are still unclear [32]. We hypothesized that NLPR3 inflammasome has a role in aged cardiac muscle and we considered worthwhile to evaluate its association with molecular mechanisms underlying the development of cardiovascular diseases with age. Moreover, we also hypothesized that melatonin is able to counteract the age-related changes in the myocardium and we investigated where it exerts its action. For this purpose, we assessed age-associated disturbances regarding mitochondrial dynamics (fusion/fission), autophagy (mitophagy), apoptosis, Nrf2-dependent antioxidant response, and mitochondrial ultrastructure in the heart of the wild-type and NLRP3-knockout mice at 3, 12, and 24 months of age, with and without melatonin treatment.

2. Materials and Methods

2.1. Animals and Treatment

Wild-type C57BL/6J and NLRP3-knockout mice NLRP3$^{-/-}$ (B6.129S6-NLRP3tm1Bhk/J) on the wild-type C57BL/6J background (>10 backcrosses) aged 3 weeks, were purchased from Charles River (Barcelona, Spain) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. Mice were housed in the animal facility of the University of Granada under a specific pathogen-free barrier and were kept under controlled temperature (22°C ± 1°C). Room illumination was on automated 12h light/dark cycle (lights on at 08:00 h). Animals had ad libitum access to tap water and pelleted rodent chow.

This study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Sciences,

Bethesda, MD, USA), the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123), and the Spanish law for animal experimentation (R.D. 53/2013). The protocol was approved by the Andalusian's Ethical Committee (05/07/2016/130).

Wild-type (WT) and NLRP3$^{-/-}$ mice were divided into five experimental groups (n = 7 animals per group) (Figure 1): (I) young (Y, 3-months old), (II) early-aged (EA, 12-months old), (III) early-aged plus melatonin (EA + aMT), (IV) old-aged (OA, 24-months old), and (V) old-aged plus melatonin (OA + aMT) mice. Melatonin (aMT) was orally administered at 10 mg/kg/day in the chow during the last two months before early and old-aged treated mice were sacrificed (EA + aMT at the age of 10 months and OA + aMT at the age of 22 months). The other groups of animals (Y, EA and OA) were fed with normal chow without melatonin. The melatonin pelleted chow was prepared by the Diet Production Unit facility of the University of Granada. The amount of melatonin in the pellets was calculated according to the average daily food intake, number, weight and age of mice [33]. The use of 10 mg/kg/day was selected on the basis of previous studies that demonstrated the effectiveness of this dose on the aging process [11,34] and mitochondrial function [35,36]. C57/BL6A was reported to be a strain of mice that responds well to melatonin therapy [37,38]. Therefore, we deemed them suitable for the purpose of this study.

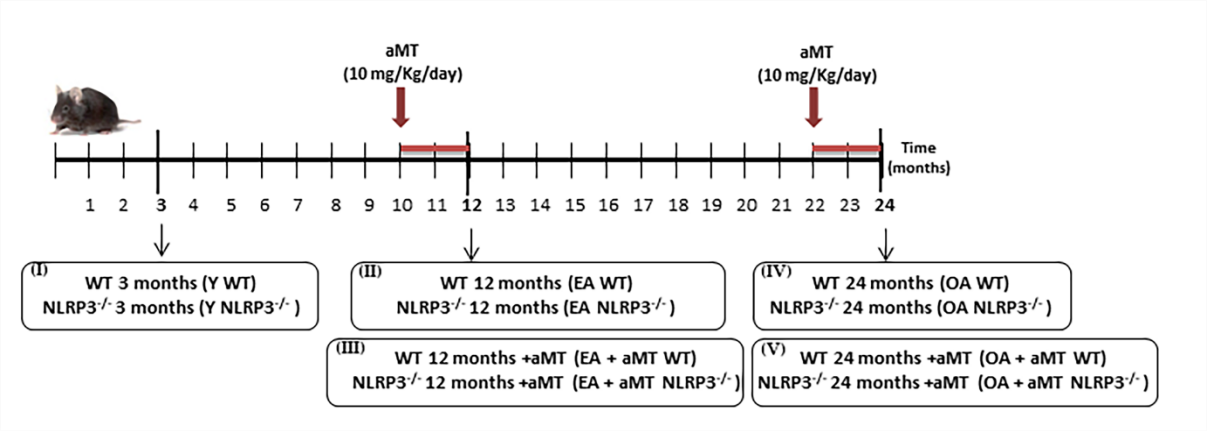

Figure 1. Study design summary: experimental groups and melatonin treatment.

Animals were killed by cervical dislocation after ketamine plus xylazine anesthesia, and hearts were collected. The left ventricle was dissected and divided into two parts. One part waswashed in saline, and rapidly fixed in 2.5% glutaraldehyde for transmission electron microscopy analysis, while the other part was stored at -80ºC for further western blot analysis.

2.2. Western blot analysis

Pure cytosolic subcellular fraction was isolated from heart tissue according to Dimauro et al. [39] with some adjustments described in Rahim et al. [30]. Briefly, heart tissue was homogenized on ice at 800 rpm in 500 µL of STM buffer containing 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 0.5 mM DTT, 5% phosphatase inhibitor buffer (125 mM NaF, 250 mM β-glycerophosphate, 250 mM p-nitrophenyl phosphate, and 25 mM NaVO3), and a protease inhibitor cocktail (Cat. 78429, Thermo Fisher Scientific, Waltham, MA, USA) with a Teflon pestle. The homogenate was maintained on ice for 30 minutes, then centrifuged at 800 g for 15 minutes at 4ºC. The supernatant was labeled as S0 and used for subsequent isolation of cytosolic fractions. S0 was centrifuged at 800 g for 10 minutes at 4ºC and the supernatant S1 was centrifuged at 11000 g for 10 minutes. The resulting supernatant S2, containing cytosol and microsomal fraction, was precipitated in cold 100% acetone at −20°C for 1 hour followed by centrifugation at 12000 g for 5 minutes. The pellet was then resuspended in 300 µL STM buffer and labeled as cytosolic fraction.

Western blot analysis was performed on cytosolic fractions of mice hearts. Denatured protein samples (40 µg/fraction) were separated by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) using 12% or 15% acrylamide/bis-acrylamide gels. Proteins were then wet transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Life Science S.L.U., Madrid, Spain). The membrane was blocked in 5% bovine serum albumin (BSA) in PBST (PBS with 0.1% Tween-20) at room temperature and then incubated overnight at 4ºC with the primary antibodies (Table S1) diluted in blocking buffer per manufacturer´s specification. Membranes were washed with PBST 3 x 10 minutes and incubated for 1 hour at room temperature with anti-mouse (BD Biosciences Pharmigen, San Jose, CA, USA) or anti-rabbit (Thermo Scientific, Madrid, Spain) IgG-horseradish peroxidase conjugated secondary antibodies diluted according to manufacturer's instruction. After washing with PBST, immunoreaction was detected using ClarityTM Western ECL Substrate (Bio-Rad, Madrid, Spain) and revealed in Kodak Image Station 4000MM PRO (Carestream Health, Rochester, NY, USA). Bands were analyzed and quantified using Kodak Molecular Imaging Software v. 4.5.1 (Carestream Health, Rochester, NY, USA). GAPDH protein content was used to normalize the cytosolic subcellular fraction. Data obtained from early and old-aged mice were always compared to young mice of the same group. The value of WT Y mice group was defined as 100%.

2.3. Transmission electron microscopy (TEM)

Small pieces from the left ventricle of the heart were rapidly immersed in a 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for fixation, then post-fixed in 0.1M cacodylate buffer-with 1% osmium tetroxide and 1% potassium ferrocyanide for 1 hour. The specimens were then immersed in 0.15% tannic acid for 50 seconds, incubated in 1% uranyl acetate for 1.5 hour, dehydrated in ethanol, and embedded in resin. Ultrathin sections of 65 nm thickness were cut using a Reichert-Jung Ultracut E ultramicrotome. These sections were double stained with uranyl acetate and lead citrate [40], and examined by a Carl Zeiss Leo 906E electron microscope and digital electron micrographs were acquired.

2.4. Morphometric analyses

Using electron micrographs, mitochondrial number and percentage of the mitochondrial damage (as number of damaged mitochondria/ total mitochondrial number ·100) were analyzed in areas with a width and height of 5.24 μm and 3.99 μm, respectively. Moreover, some morphometric analyses, including cross sectional area (CSA) and Feret's diameter of the intermyofibrillar mitochondria, of cardiac muscle fibers were performed on images of electron microscopy using Image J processing software.

2.5. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) of n = 7 animals per group. All statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). One-way ANOVA with a Tukey's post hoc test was used to compare the differences between experimental groups. The values were found to be significantly different when $p < 0.05$.

3. Results

3.1. NLRP3 deficiency prevents, and melatonin treatment restores cardiac muscle mitochondrial dynamics altered by aging

Anomalies in mitochondrial dynamics (fusion/fission) are typical of aged cardiac muscle [16]. Here, we showed that aging induced a decrease in the levels of proteins involved in mitochondrial dynamics, including Mfn2, Opa1, and Drp1, in WT mice, an effect absent in NLRP3$^{-/-}$ mice (Figure 2A, B, C). Melatonin supplementation counteracted the decline of Mfn2, Opa1, and Drp1 caused by aging in WT mice. Interestingly, no significant effect of melatonin was observed in fusion proteins Mfn2 and Opa1 in NLRP3$^{-/-}$ mice at the age of 12 and 24 months (Figure 2A, B). A slight, but not significant

enhancement in fission protein Drp1 was noted in EA and OA NLRP3$^{-/-}$ mice with melatonin supplementation (Figure 2C).

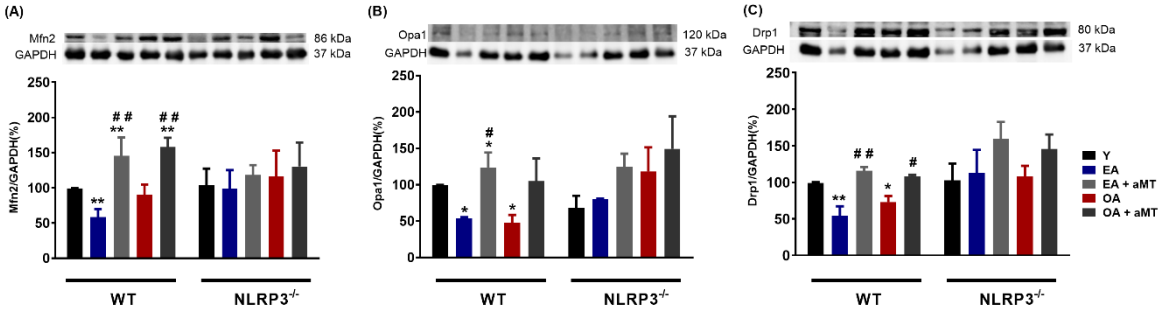

Figure 2. Changes in mitochondrial dynamics (fusion/fission) in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment**. (A)** Protein levels of Mfn2. **(B)** Protein levels of Opa1. **(C)** Protein levels of Drp1. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01 *vs.* Y; #p<0.05, ##p<0.01 *vs.* group without melatonin treatment.

3.2. NLRP3 deficiency and melatonin therapy had minimal effects in autophagy in cardiac muscle during aging

A drop in the autophagic capacity observed in cardiac aging, is associated with the accumulation of dysfunctional mitochondria, exaggerated ROS production, and mtDNA release [16,17]. Unsurprisingly, the conversion of LC3I to LC3II, a hallmark of autophagy [41], was significantly reduced in WT mice during aging, as reflected in the decrease in the LC3II/LC3I ratio in WT EA and OA mice (Figure 3). LC3II/LC3I ratio trends to increase in NLRP3$^{-/-}$ EA and OA mice, which may explain the attempt to restore autophagy events. Melatonin administration had minimal effects on the LC3II/LC3I ratio in all cases.

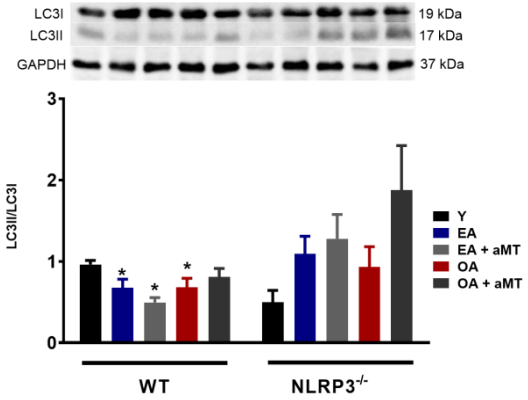

Figure 3. Changes in autophagy in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. LC3II/LC3I ratio. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01 *vs.* Y.

3.3. Melatonin treatment and, to a lesser extent NLRP3 deficiency, reduced apoptosis in cardiac muscle during aging

Despite being intensively studied over the past three decades, many of the mechanisms of apoptotic cell death remain unknown. Although the relationship between aging and apoptosis have been a subject of controversy in scientific community, there seems to be consensus that apoptosis

plays a significant role in cardiac aging [42]. Here, we showed that aging induced a rise in the levels of some proteins involved in apoptotic processes, including p53 and caspase 9 in both WT and NLRP3$^{-/-}$ mice. Melatonin treatment significantly diminished the levels of p53 and caspase 9 in EA WT mice and in EA and OA mutant mice (Figure 4A, B). The pro-apoptotic protein Bax and the anti-apoptotic Bcl2 were significantly enhanced by aging in WT mice. Mutant mice only showed Bcl2 increased in OA animal's group (Figure 4C, D). We observed a slight rise in Bax/Bcl2 ratio in EA and a significantly increase in WT OA mice (Figure 4E). The absence of NLRP3, however, prevented the apoptotic process associated with aging since Bax/Bcl2 ratio remained at similar levels that Y mutant mice. Melatonin supplementation significantly decreased the Bax/Bcl2 ratio in EA and OA WT mice, but had no effect in NLRP3$^{-/-}$ mice.

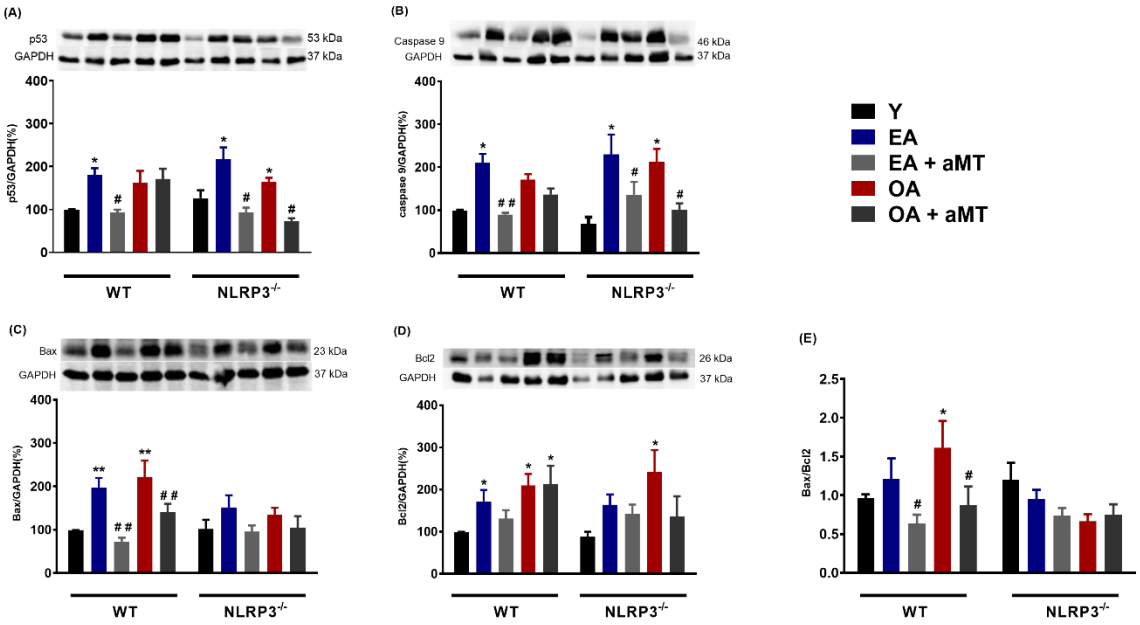

Figure 4. Changes in apoptosis in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. **(A)** Protein levels of p53. **(B)** Protein levels of caspase 9. **(C)** Protein levels of Bax. **(D)** Protein levels of Bcl2. **(E)** Bax/Bcl2 ratio. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01 *vs.* Y; #p<0.05, ##p<0.01 *vs.* group without melatonin treatment.

3.4. Melatonin treatment, but not NLRP3 deficiency, recovered the Nrf2-dependent antioxidant capacity in cardiac muscle declined by aging

In recent years, emerging evidence has indicated that aging leads to a gradual reduction of the Nrf2-dependent antioxidant response, which in turn contributes to the accumulation of oxidative stress [18,19]. Our results showed a significant decrease in the protein levels of Nrf2 and its active form pNrf2 (Ser40) in WT and NLRP3$^{-/-}$ mice with age, suggesting that NLRP3 deficiency was unable to ameliorate the age-related decline of Nrf2 and pNrf2 (Ser40) in these animals (Figure 5A, B). Melatonin supplementation markedly recovered the levels of Nrf2 and pNrf2 (Ser40) in both WT and mutant EA and OA mice. Aging and melatonin therapy did not significantly modify the levels of the Nrf2 inhibitor, Keap1, in either mouse strain (Figure 5C). Hmox1, Nqo1, and γGclc, three cytoprotective enzymes transcriptionally regulated by Nrf2, also remarkably decreased in WT OA mice (Figure 5D, E, F). The levels of Hmox1 and γGclc significantly dropped in NLRP3$^{-/-}$ EA and OA mice (Figure 5D, E). Protein content of Nqo1 enzyme was not modified by aging in mutant animals (Figure F). Again, melatonin treatment greatly enhanced the levels of Hmox1, Nqo1, and γGclc in WT and NLRP3$^{-/-}$ mice.

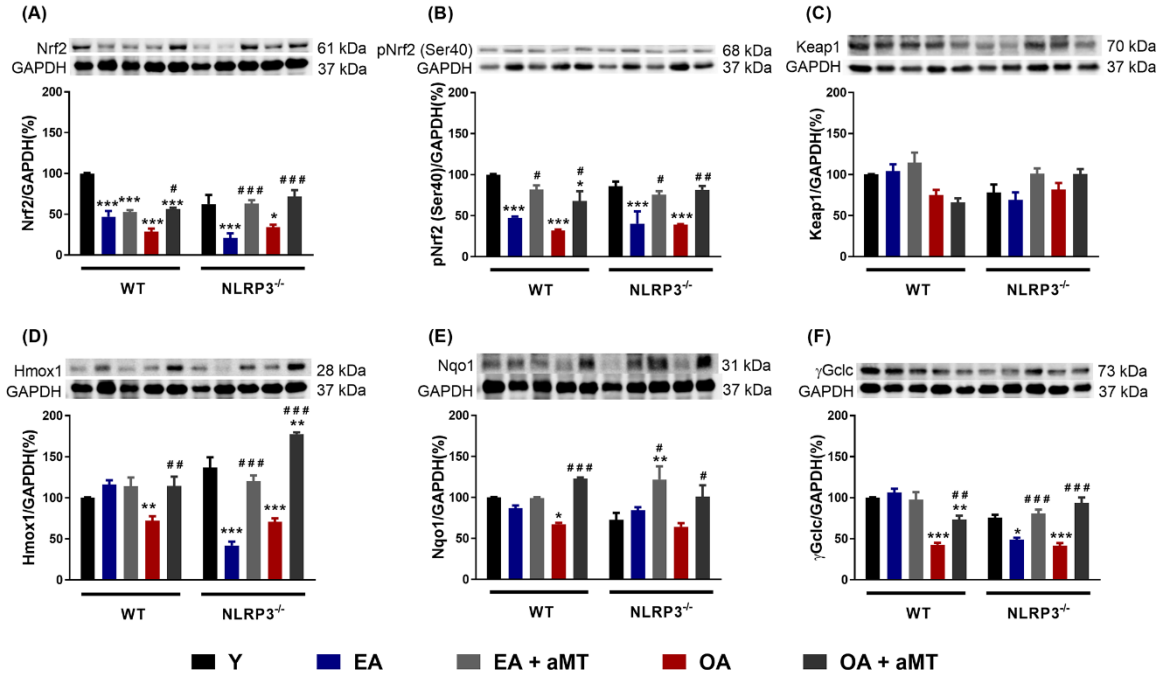

Figure 5. Changes in the Nrf2-dependent antioxidant pathway in WT and NLRP3$^{-/-}$ mice during aging and melatonin treatment. **(A)** Protein levels of Nrf2. **(B)** Protein levels of pNrf2 (Ser40). **(C)** Protein levels of Keap1. **(D)** Protein levels of Hmox1. **(E)** Protein levels of Nqo1. **(F)** Protein levels of γGclc. Experiments were performed in hearts of young (Y), early-aged (EA), early-aged with melatonin (EA + aMT), old-aged (OA), and old-aged with melatonin (OA + aMT) wild type and NLRP3$^{-/-}$ mice. Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01, ***p<0.001 *vs.* Y; #p<0.05, ##p<0.01, ###p<0.001 *vs.* group without melatonin treatment.

3.5. NLRP3 deficiency and melatonin therapy improved mitochondria ultrastructure altered by age in cardiac muscle

Transmission electron microscopy of the cardiac muscles of Y WT mice revealed presence of normally intact and compacted mitochondria with clearly organized cristae distributed in the intermyofibrillar spaces (Figure 6A, B). At the age of 12 months (EA), most of these mitochondria were found normally; however, a few showed cristae damage (Figure 6C, D). These changes were exacerbated and numerous mitochondria were severely damaged, hypertrophied, and vacuolated with completely destroyed cristae in WT OA mice (Figure 6G, H). Melatonin supplementation, however, preserved the normal ultrastructure of the cardiac mitochondria in EA (Figure 6E, F) and OA WT mice (Figure 6I, J) maintaining their healthy and compact appearance.

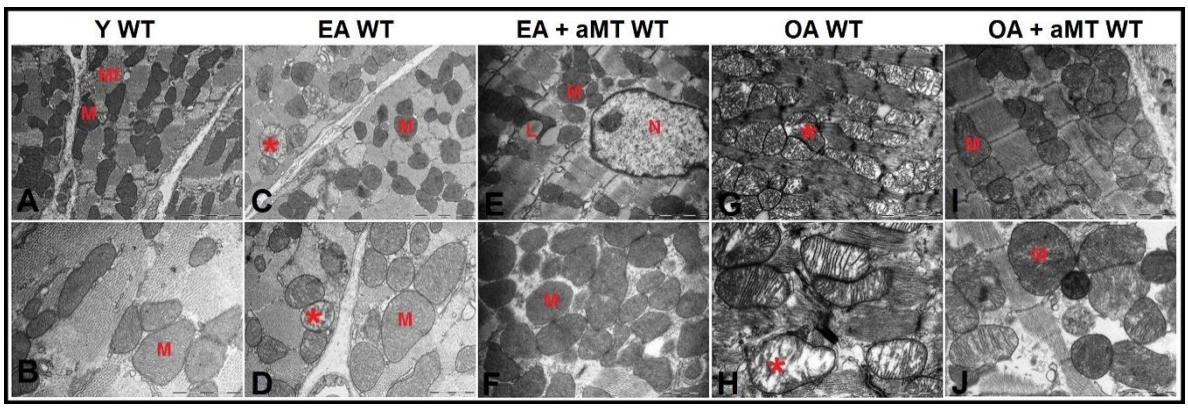

Figure 6. Age-associated ultrastructural changes of mitochondria in cardiac muscle fibers of WT mice and melatonin treatment. **(A, B)** Electron micrographs of cardiac muscle fibers of Y WT mice revealing

presence of normally intact and compacted mitochondria (M) distributed among myofibrils (Mf). **(C, D)** Electron micrographs of cardiac muscle fibers of EA WT mice demonstrating presence of normal mitochondria (M) with presence few ones demonstrated cristae damage (asterisk). **(E, F)** Electron micrographs of cardiac muscle fibers of EA + aMT WT mice showing the protective effect of melatonin supplementation in preserving normal mitochondrial structure (M) with presence of lipid droplets (L), N; nucleus. **(G, H)** Electron micrographs of cardiac muscle fibers of OA WT mice clarifying presence of numerous severely damaged hypertrophied vacuolated mitochondria with completely destructed cristae (asterisk). **(I, J)** Electron micrographs of cardiac muscle fibers of OA + aMT WT mice exhibiting the beneficial effect of melatonin supplementation in keeping normal mitochondrial architecture (M). A, C, E, G, I: bar = 2 μm and B, D, F, H, J: bar = 1 μm.

Cardiac muscle fibers of NLRP3$^{-/-}$ Y mice presented normal highly compacted mitochondria with densely packed cristae (Figure 7A, B). Mitochondrial structure did not change in EA mice, except one that showed damage in peripheral cristae (Figure 7C, D). The mitochondrial damage was less prevalent at 24 months in comparison with WT OA mice. Mitochondria were characterized by their widely-separated and organized cristae, with presence of small-sized membranous vacuoles of possibly autophagic nature (Figure 7G, H). Melatonin treatment exhibited an obvious protective effect at the age of 12 (Figure 7E, F) and 24 months (Figure 7I, J), where it kept normal mitochondrial architecture with aging, in addition to formation of multivesicular bodies, which reflect the induction of the autophagic processes.

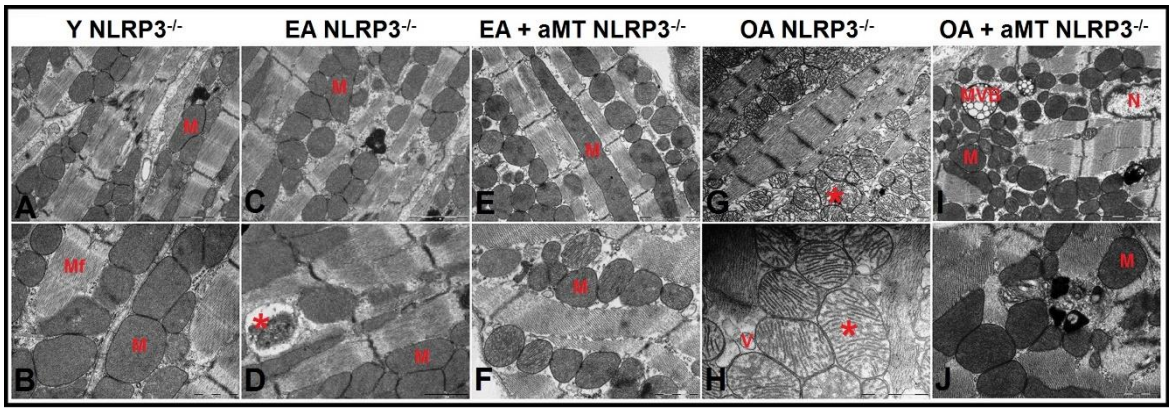

Figure 7. Age-related ultrastructural changes of mitochondria in cardiac muscle fibers of NLRP3$^{-/-}$ mice and melatonin treatment. **(A, B)** Electron micrographs of cardiac muscle fibers of Y NLRP3$^{-/-}$ mice showing presence of normally highly compacted mitochondria with densely packed cristae (M) distributed among myofibrils (Mf). (**C, D**) Electron micrographs of cardiac muscle fibers of EA NLRP3$^{-/-}$ mice demonstrating intact mitochondria (M) with individual ones depicting damaged peripherally cristae (asterisk). (**E, F)** Electron micrographs of cardiac muscle fibers of EA + aMT NLRP3$^{-/-}$ mice revealing the clearly apparent prophylactic effect of melatonin supplementation in keeping normal mitochondrial architecture (M) with aging. (**G, H)** Electron micrographs of cardiac muscle fibers of OA NLRP3$^{-/-}$ mice indicating less detectable mitochondrial damage compared with WT mice, with presence of numerous mitochondria showing widely-separated organized cristae (asterisk) and small-sized membranous vacuoles of possibly autophagic nature (V). (**I, J)** Electron micrographs of cardiac muscle fibers of OA + aMT NLRP3$^{-/-}$ mice showing the protective effect of melatonin supplementation in preserving normal mitochondrial structure (M), with formation of multivesicular bodies (MVB), which reflect the induction of the autophagic processes, N; nucleus. A, C, E, G, I: bar = 2 μm and B, D, F, H, J: bar = 1 μm.

3.6. Lack of NLRP3 reduced mitochondria number loss and mitochondrial damage, an effect shared by melatonin

Morphometric analysis of cardiac mitochondria revealed that mitochondrial number exhibited initial non-significant decline in cardiac muscles of WT and NLRP3$^{-/-}$ EA mice. Nevertheless,

mitochondrial number was significantly decreased in OA, being more pronounced in WT mice than NLRP3$^{-/-}$ one, an effect significantly counteracted after melatonin therapy (Figure 8A). Furthermore, the percentage of the mitochondrial damage was significantly increased in aged mice, especially in WT animals, and it was counteracted by melatonin supplementation (Figure 8B). Morphometrical analysis of the mitochondrial CSA illustrated a non-significant increase in cardiac muscle of WT and NLRP3$^{-/-}$ EA mice, whereas the former increased in aged animals (Figure 8C). Mitochondrial diameter showed non-significant increase in WT EA mice, increasing in OA animals. NLRP3$^{-/-}$ mice revealed non-significant changes in mitochondrial diameter among all experimental groups (Figure 8D).

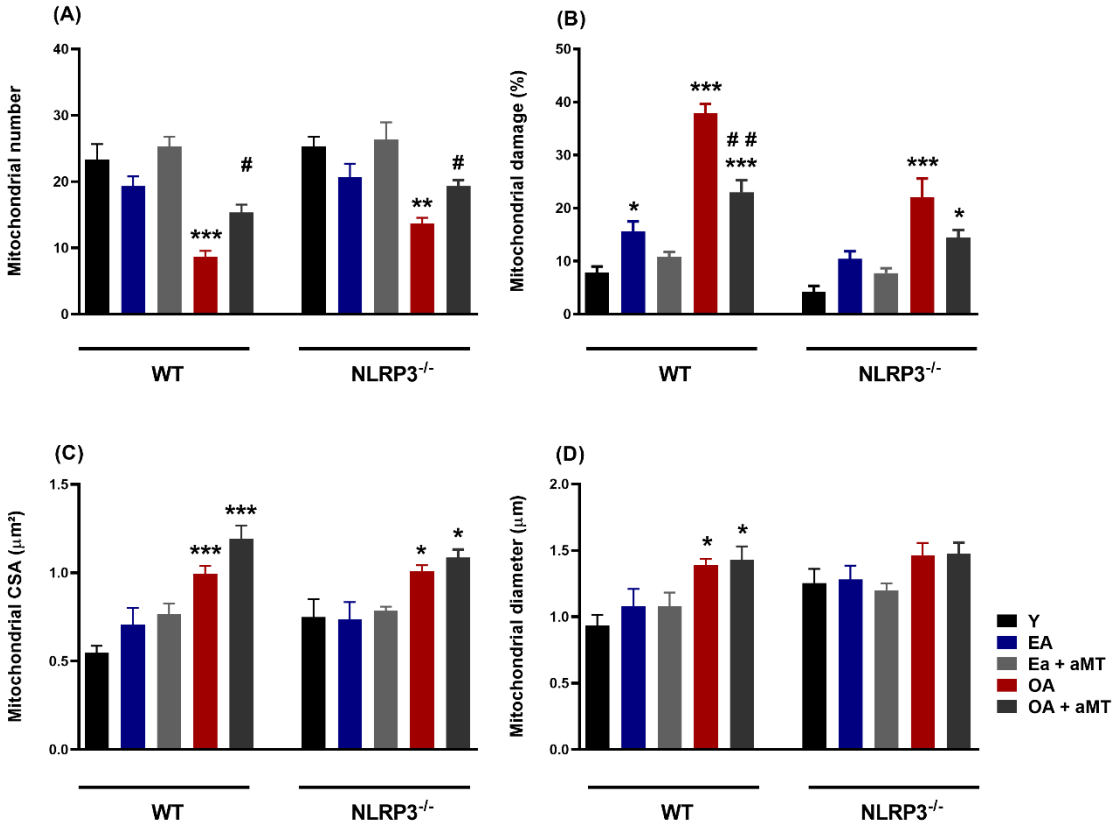

Figure 8. Age-associated morphometrical changes of intermyofibrillar mitochondria in cardiac muscle fibers of WT and NLRP3$^{-/-}$ mice and melatonin treatment. **(A)** Analysis of mitochondrial number. **(B)** Analysis of mitochondrial damage percentage. **(C)** Analysis of cross section area (CSA, μm²). **(D)** Analysis of mitochondrial Feret's diameter (μm). Data are expressed as means ± SEM (n = 7 animals/group). *p<0.05, **p<0.01, ***p<0.001 *vs.* Y; #p<0.05, ##p<0.01 *vs.* group without melatonin treatment.

4. Discussion

Immunosenescence and inflammaging are caused by persistent activation of NF-κB/NLRP3 inflammasome pathways generates chronic low-grade inflammation which leads to, among other detriments, accumulation of cardiac mitochondrial dysfunction, characterized by dysregulation of mitochondrial dynamics, autophagy, apoptosis, Nrf2 antioxidant pathway, and maintenance of ultrastructure of mitochondria [43]. Another hallmark of aging is a decline in melatonin levels and its protective roles [44]. This brings about increased oxidative damage, chronodisruption, upregulation of pro-inflammatory cytokines, and down regulation of anti-oxidant/-inflammatory processes that contribute to inflammaging by facilitating mitochondrial disruption [45]. The role of the NLRP3 inflammasome and melatonin levels in regulation of mitochondrial dysfunction, associated with cardiac aging, is not fully understood. Our results suggest direct involvement of this inflammasome

by marked amelioration of some mitochondrial dysfunctions with NLRP3 ablation both involved with, and independent of, melatonin supplementation in EA and OA mice (Figures 9, 10).

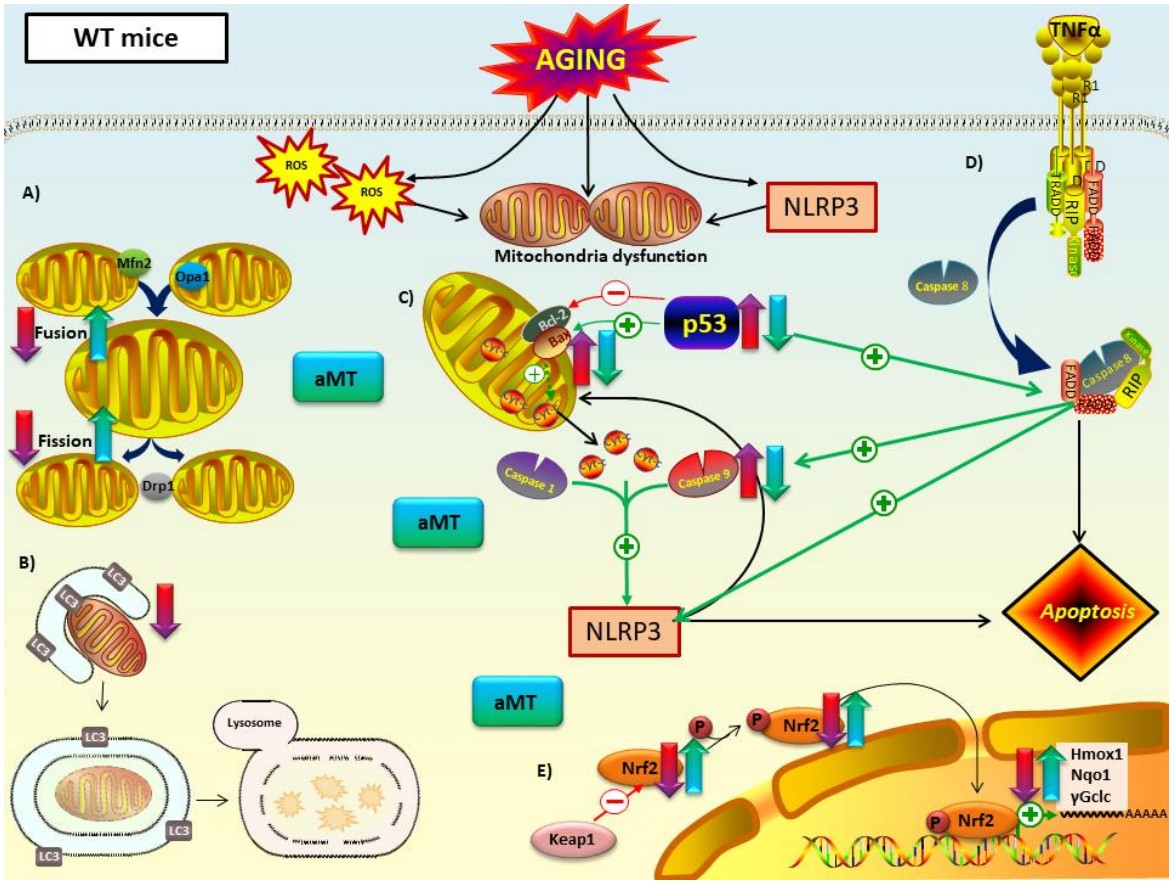

Figure 9. Proposed mechanism of melatonin in mitochondria of WT mice during cardiac aging. **(A)** Mitochondrial dynamics: aging led to a decline in fusion (Mfn2 and Opa1) and fission proteins (Opa1). Melatonin treatment counteracted this decrease. **(B)** Autophagy (mitophagy): autophagic capacity dropped in aged myocardium. Melatonin therapy had minimal impact on this pathway. **(C)** Intrinsic and **(D)** extrinsic apoptosis: WT mice have intrinsic and extrinsic pathways mediated by p53 and caspase 9. Those apoptotic markers, as well as Bax/Bcl2 ratio, increased with aging and are related with NLRP3 activation. This inflammasome seemed to have a regulatory effect on the intrinsic apoptotic pathway, which depends on mitochondria cytochrome c release. Melatonin supplementation had an anti-apoptotic effect in both intrinsic and extrinsic apoptosis. **(E)** Nrf2-dependent antioxidant response: Nrf2 and pNrf2 (Ser40) were reduced with aging. This loss was linked to the decrease of the cytoprotective enzyme transcriptionally regulate by Nrf2: Hmox1, Nqo1 and γGclc. Melatonin recovered this antioxidant pathway. No changes in Keap1 were reported. Red-purple arrow: impact of aging. Green-blue arrow: effect of melatonin treatment.

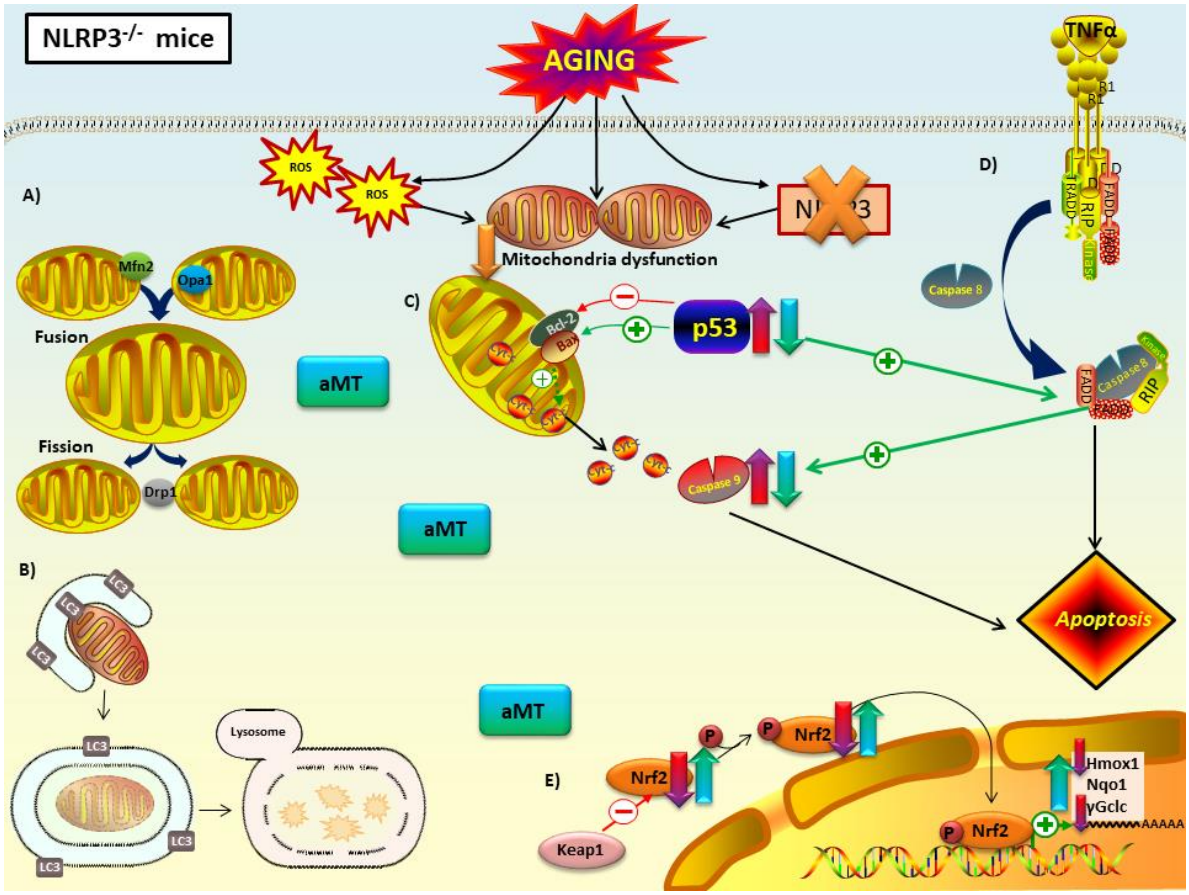

Figure 10. Proposed mechanism of melatonin in mitochondria of NLRP3$^{-/-}$ mice during cardiac aging. Lack of NLRP3 inflammasome reduced mitochondria dysfunction. **(A)** Mitochondrial dynamics: the absence of NLRP3 prevented the decline in fusion (Mfn2 and Opa1) and fission proteins (Opa1) with aging. Melatonin treatment had no effect on these mice. **(B)** Autophagy (mitophagy): autophagic capacity was restored by NLRP3 deficiency. Melatonin therapy had minimal impact on autophagic capacity. **(C)** Intrinsic and **(D)** extrinsic apoptosis: loss of NLRP3 had an anti-apoptotic effect in Bax/Blc2 ratio, but not in p53 or caspase 9. The ablation of this inflammasome could trigger extrinsic apoptosis mediated by TNFα binding to death receptor. Melatonin supplementation had an anti-apoptotic effect in p53 and caspase 9. **(E)** Nrf2-dependent antioxidant response: lack of NLRP3 did not recover the decrease of this antioxidant pathway with aging. Only Nqo1 were not diminished in mutant mice. Melatonin improved this antioxidant pathway. No changes in Keap1 were reported. Red-purple arrow: impact of aging. Green-blue arrow: effect of melatonin treatment.

Mitochondria fusion (Mfn2 and Opa1) and fission (Drp1) proteins decrease naturally with aging, as seen in WT mice (Figure 9A). Findings in the literature link declines in regulatory proteins of mitochondrial dynamics and age-related development of cardiovascular disease [46–49]. Cardiomyocytes of Mfn2-deficient mice showed cardiac hypertrophy [50]. Low levels of Opa1 have been reported in failing human heart [51]. Loss of Drp1 in adult mice results in lethal dilated cardiomyopathy [52]. Our study also concluded that the absence of NLRP3 prevented the decrease in fusion and fission processes associated with aging that were observed in WT mice (Figure 10A). This cardioprotective effect observed in NLRP3$^{-/-}$ mice supports the existence of a close relationship between mitochondrial dynamics and inflammaging. Our results are in line with scientific evidence that connects impaired mitochondria dynamics, stimulation of innate immune response and inflammasome activation [53–58]. On the other hand, melatonin's mechanism of action in mitochondria dynamics and aging remains unclear. We indicate herein that melatonin promotes fusion by increasing the expression of the Mfn2 and Opa1 proteins in WT EA and OA mice (Figure 9A). Most investigations support that this indolamine stimulates mitochondria fusion, contributing to

the survival of cardiomyocytes and reducing mitochondria damage [59–61]. Moreover, numerous studies remark a melatonin-induced reduction of mitochondria fission with stressful stimuli [62–65], showing a protective effect in cardiac function against ischemia/reperfusion injury and post-traumatic cardiac dysfunction in vitro and in vivo models, respectively [66–68]. Conversely, we found that melatonin supplementation increased the levels of the Drp1 protein in EA and OA WT mice. Supporting our results, recent findings showed that increasement in Drp1 levels enhanced regulation of mitochondria homeostasis through mitophagy [69]. Additionally, Drp1 overexpression in flies reversed age-related mitochondria dysfunction and age-onset pathologies [70]. Taken together, our data suggests that melatonin enhances the response of mitochondria dynamics to maintain homeostasis during age-related metabolic stressors like inflammasome activation. It should be noted melatonin did not trigger significant changes in EA and OA NLRP3$^{-/-}$ mice either (Figure 10A). This effect of melatonin has previously been related to its cytoprotective activity, since its effect will be greater the more cellular damage there is, while in situations of low damage or physiological conditions its response is minimal [71].

The LC3II/LC3I ratio showed a significant decrease in autophagy in EA and OA WT mice compared to Y WT mice (Figure 9B). Numerous findings indicate a loss of autophagy with aging in most organisms and tissues, including the heart [72–76]. Changes in the expression of autophagic proteins such as Atg9, LAMP-1, and LC3II in aged mice and rats resulted in cardiac dysfunction [77–79]. The consequent accumulation of altered organelles, mutated mtDNA, cristae disarray, and ROS, have been shown to propagate different age-related cardiac pathologies [74,75,80–82] and produce risk-associated molecular pattern derived from mitochondria (DAMP) that activate NLRP3 inflammasome [83]. Our results showed that the absence of NLRP3 prevented the drop in LC3II/LC3I ratio in mice during aging (Figure 10B). Ablation of the NLRP3 inflammasome in old NLRP3$^{-/-}$ mice has been reported to improve the quality of autophagy by increasing the levels of ATG12, beclin 1 and LC3II and decreasing p62/SQSTM1 [84]. Several studies have demonstrated the protective influence of melatonin by both increasing and decreasing autophagic capacity, in response to sterile and non-sterile inflammation [85–92]. Interestingly, in our results it is implied that melatonin had no effect on EA and OA WT mice compared to their corresponding controls (Figure 9B). Similar results were obtained in the brain of SAMP8 mice, where melatonin did not cause changes in autophagy [93]. However, it is noteworthy that melatonin was able to increase autophagy of OA WT mice, thereby restoring levels like Y WT mice, but not in EA mice. This action suggests that melatonin and autophagy operate synergistically to increase cell survival, delay immunosenescence, and decrease oxidative stress. Thus, melatonin could act selectively, increasing autophagy only when antioxidant activity is severely impaired, or when sufficient loss of cellular homeostasis results in abnormal mitochondrial morphology and death receptor pathway activation [94–98]. Melatonin did not cause significant changes in the LC3II/LC3I ratio in NLRP3$^{-/-}$ mice (Figure 10B), possibly due to the protective effect resulting from ablation of the inflammasome.

Apoptotic proteins p53 and caspase 9 were found to be increased in EA and OA vs Y mice in both WT and NLP3$^{-/-}$ mice (Figure 9C, D, Figure 10C, D). Oxidative stress that occurs during aging has been shown to induce apoptosis, mitochondria dysfunction in cardiomyocytes and ultimately heart failure [99–102]. The Bax/Bcl2 ratio confirmed the increase in apoptosis with aging in WT mice. Interestingly, no changes were observed between ages in mutant mice. The ablation of NLRP3 had an anti-apoptotic protective effect during cardiac aging in Bax/Bcl2 ratio, but not in p53 or caspase 9. This finding suggests that NLRP3 is a direct regulator of the intrinsic apoptotic pathway in cardiac aging, which is dependent of the balance between Bax and Bcl2 and cytochrome c release (Figure 10C). The absence of this inflammasome could trigger activation of extrinsic apoptosis with ligand-induced activation of several death receptors since the participation of p53 and caspase 9 in this pathway has been reported in various tissues and cell models [103–105]. In support of our hypothesis, recent studies revealed an increase in TNFα in the serum of old NLRP3$^{-/-}$ mice compared to young mice [106]. This cytokine is linked to inflammaging [107] and induces extrinsic apoptotic pathway by binding to the cell death receptor TNFR1. On the other hand, findings have showed that caspase 8,

which is key in extrinsic apoptosis, plays a role in NLRP3 inflammasome priming and cytochrome c independent caspase 9 activation [108–110]. Without NLRP3, cardiac aging-induced inflammation is favored and could start with extrinsic TNFα apoptosis pathway preceding activation of caspase 8 which in turn activates caspase 9 (Figure 10D). Further investigations centered of the impact of aging on the heart are required to elucidate the extent of the complex interactions between NLRP3 and apoptosis. In most cases, melatonin counteracted the high levels of p53 and caspase 9 associated with aging in WT and mutant mice and Bax/Bcl2 ratio in WT. This anti-apoptotic effect of melatonin during cardiac aging was evident in both extrinsic and intrinsic pathways (Figure 9C, D, Figure 10C, D) and can be explained due to its ability to restore the redox potential of the mitochondria membrane and reduce oxidative stress. These actions increase ATP production and decrease mitochondrial outer membrane permeabilization (MOMP) following release of cytochrome c [111].

Mitochondrial theory of aging [112,113] postulates that an alteration in the redox state of the mitochondria, the main source of free radicals in the cell, causes oxidative damage that results in senescence, the primary driver of the aging process. In this sense, Nrf2 is defined as a 'guardian of health span' and a 'master regulator of aging' giving it enormous importance in the control of numerous antioxidant enzymes [114,115]. It is well stablished that Nrf2 improves mitochondria function by balancing reduction and oxidation processes and influencing ATP production, membrane potential, fatty acid oxidation and structural integrity [116]. However, changes in the levels of this protein during aging, as well as the antioxidant enzymes it regulates, have been the subject of debate in recent years. Controversial and even opposite results appear in many studies, which seem to depend on the species, strain, tissue, sex and experimental design. Our results in cardiac muscle indicate that cytosolic levels of Nrf2 and pNrf2 (Ser40) decrease with aging, both in WT and in NLRP3$^{-/-}$ mice at EA and OA (Figure 9E, Figure 10E). This may suggest translocation to the nucleus to activate transcription, to mediate age-related increases in ROS, decreasing cytosolic levels. The presence of pNrf2 in the cytosol could also be due to phosphorylation of Nrf2 by GSK-3β which translocates pNrf2 out of the nucleus [117]. Our data agree with investigations showing that mice deficient in Nrf2 have a higher susceptibility to inflammation and oxidative stress [118]. This alteration in the Nrf2 pathway is associated with cardiovascular diseases [119,120]. Nrf2$^{-/-}$ mice were more prone to heart failure and their mortality increased ten days after suffering a myocardial infarction [121,122]. Although most studies point to a decrease in Nrf2 in heart tissue with aging, the causes are unknown. Surprisingly, our results discarded Keap1 as the responsible of this declining since there were no changes in its levels between the different ages and experimental groups. In line with our findings, levels of Nrf2 and its mRNA were found to be reduced in the liver of 10-month-old SAMP8 mice compared to SAMR1 mice, while Keap1 mRNA and its protein levels remained unchanged with age [117]. The decrease in the antioxidant enzymes Hmox1, Nqo1 and γGclc during aging is possibly due to a less efficient Nrf2 signaling [123,124]. Similar results using aortas of 24-month-old rats, whose Nrf2 levels were lower compared to 3-month-old young rats, resulted in a drop in the enzymes Hmox1, Nqo1 and γGclc [125]. However, the same group demonstrated that oxidative stress associated with aging did not induce significant changes in Nrf2 levels of carotid arteries in aged Rhesus macaques (20 years) compared to young individuals (10 years), and their respective antioxidant enzymes were not induced either [126]. Together, these data confirm that the expression of these antioxidant enzymes is linked to Nrf2. It also suggests the activation of this signaling pathway in the cardiovascular system during aging depends not only on the animal model but on the degree of oxidative stress as well. In this light, recent works described that there is a shift in Nrf2 target to Klf9 instead of Hmox1, Nqo1 and γGclc at excessive oxidative damage [127,128]. This could explain the fact that Hmox1 and γGclc were decreased to a greater degree than WT by showing decline in EA while WT decreased only at OA. Interestingly, Nqo1 expression levels were not reduced in NLRP3$^{-/-}$ mice but were still upregulated by melatonin supplementation. Several studies show that Nqo1 is the prototype gene target for Nrf2 activation. In BV2 cells after cerebral ischemia reperfusion, Nrf2 ROS response was linked to Nqo1 expression [129]. This could illuminate the limited decrease in cytosolic Nqo1 by being preferentially targeted by the ever-shrinking pool of

Nrf2 and pNrf2 as aging ensues. This study also proved that scavenging of ROS by Nqo1 restrained NLRP3 inflammasome activation and IL-1β expression. Except for Keap1 expression levels, which remained unchanged during aging, treatment with melatonin counteracted the age-associated decline in expression of all the parameters of the Nrf2 signaling pathway, both in WT and NLRP3$^{-/-}$ mice (Figure 9E, Figure 10E). Melatonin has been shown to have a protective effect on the mitochondria by acting as a powerful antioxidant in a direct way, as a scavenger of free radicals, detoxifying reactive oxygen and nitrogen species, and indirectly, increasing the rest of the Nrf2-dependent and independent antioxidant systems [130–133].

Studies in animal models confirm that the ultrastructure of cardiac mitochondria changes with aging [134]. Our study supported these results. A small number of isolated mitochondria had damaged cristae in EA mice, and severe mitochondrial damage, with destroyed, separated, vacuolated and hypertrophied cristae in OA mice. This mitochondrial impairment was more remarkable in WT mice than in mutants. These findings reveal age-induced cellular senescence and mitochondrial dysfunction [135], as well as the cardioprotective effect linked to the ablation of the NLRP3 inflammasome [136]. Melatonin treatment maintained normal mitochondrial ultrastructure in all experimental groups. Multivesicular bodies increased in treated OA NLRP3$^{-/-}$ mice, which indicate autophagy induction. These results, once again, highlight the protective role of melatonin against age-mediated mitochondria impairment and its ability to restore altered autophagic processes during cardiac aging [137].

Various morphometric analyses show that the size and number of mitochondria per cell is impacted during cardiac aging [138]. Our results showed an increase in CSA and Feret's diameter in the mitochondria of OA WT mice, accompanied by a decrease in number of mitochondria. This mitochondrial hypertrophy has been related to a systemic demand from overload stress on the heart [139,140], and our results suggest that it could also be an adaptive mechanism to compensate for the decrease in the amount of this organelle. The ablation of the NLRP3 inflammasome reduced cardiac hypertrophy, as there were no changes in Feret's diameter with age and less significant increase in CSA and decline in mitochondria number. To our knowledge, this is the first time that these morphometrical parameters are studied specifically in IMF during cardiac aging using a mice model. In line to our findings, CSA of cardiomyocytes from the left ventricle of male Fischer 344 rats increased with aging, while the number of cardiomyocytes decreased [141]. In Wistar rats, mitochondria volume fraction and mean size both in left and right ventricle were decreased in 2 years old vs 6 weeks old animals [142]. Our results showed that melatonin significantly increased the number of mitochondria in WT and NLRP3$^{-/-}$ mice, with no effect on CSA or Feret's diameter. It is possible that in this case two-months treatment is not enough to counter the age-related changes in CSA and Feret's diameter in the heart, one of the most energy-demanding organs of our body [143]. This 'cardiac sarcopenia' has hardly been investigated since most studies focus on skeletal muscle. Indeed, our group previously performed the same analyses in gastrocnemius and morphometric alterations were observed earlier, in EA mice and protected in NLRP3 deficient mice [144]. Our findings suggest that cardiac muscle and its mitochondria are physiologically more protected from age-related sarcopenia than skeletal muscle. Its ability to make a metabolic switch in favor of glycolysis instead of fatty acid oxidation during aging [145,146], being one of the organs where the NLRP3 inflammasome is expressed less [147,148], or the presence of resident macrophages with tissue protective function [149] are some of many possible adaptations of the heart that could explain its greater resistance to sarcopenia.

5. Conclusions

Results of this study clarify the impact of NLRP3 inflammasome and melatonin treatment in the mitochondria during cardiac aging. The main findings can be summarized as 1) NLRP3 knocking out and melatonin supplementation avoided mitochondrial dynamics changes of heart with aging; 2) loss of NLRP3 and melatonin treatment revealed few impact on cardiac autophagy during aging; 3) NLRP3 absence had less role on cardiac apoptosis during aging compared to melatonin therapy; 4)

melatonin restored aged-related Nrf2-dependent antioxidant capacity while NLRP3 inflammasome showed no effect on this pathway; 5) lack of NLRP3 as well as melatonin treatment enhanced mitochondria ultrastructure alterations in aged myocardium.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Table S1: List of primary antibodies used in western blot analysis; Fig.S1: Full western blot data.

Author Contributions: Conceptualization, D.A-C; methodology, M.F-O, R.K.A.S. and A.C.; formal analysis, J.F-M.; data curation, P.A-M.; writing—original draft preparation, M.F-O. and G.E.; writing—review and editing, D.A-C.; project administration, T.H.; funding acquisition, D.A-C. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partially supported by grants from the Instituto de Salud Carlos III (Ministerio de Economía y Competitividad, Spain), through the projects, PI16-00519, PI19-01372, and CB16-10-00238 (Co-funded by European Regional Development Fund/European Social Fund) "Investing in your future"), and from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (CTS-101), Spain. M F-O and J F-M are supported by a FPU fellowship from the Ministerio de Educación, Spain.

Acknowledgments: We are thankful to members of Electron Microscopy Unit (Assiut University, Egypt) for their technical support with transmission electron microscopy.

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

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