

## Scientific letters

**Ageing-related ILK levels are associated with calcified aortic valve and circulating miR 199-3p levels*****La expresión ILK asociada a la edad se relaciona con la calcificación de la válvula aórtica y niveles plasmáticos del miR 199-3p*****To the Editor,**

Calcific aortic valve disease (CAVD) is a leading cardiovascular disease in the elderly, which results in the failure of valvular function.<sup>1</sup> Endothelial dysfunction, inflammation, and oxidative and mechanical stress lead to valve and myocardial remodeling, although mechanosensory pathways that promote calcific changes are yet to be identified. Integrin-linked kinase (ILK) is a key protein that regulates vascular tone and cardiac contractility, acting as a mechano-transducer of hemodynamic forces in the myocardium.<sup>2</sup>

We previously found that ILK expression in endothelial cells plays a pivotal role in regulating vasomotor tone by preventing uncoupling of endothelial nitric oxide synthase (eNOS). We described a disruption of this association with a clear correlation between ILK inhibition and atherosclerosis.<sup>3</sup> As part of the inflammatory response, cytotoxic nitric oxide (NO) levels from inducible nitric oxide synthase (iNOS) promote endocytosis of ILK followed by lysosomal degradation, leading to atherosclerotic progression.<sup>4</sup>

Endothelial dysfunction and atherosclerosis are 2 complications of aging in which ILK plays a significant role by still unexplored mechanisms. To test whether ILK may regulate CAVD, we used young and aged mice expressing ILK. Von Kossa staining of aortic valves from aged mice exhibited extensive calcium deposition compared with young animals, together with expression of osteogenic proteins osteopontin and Runx2 (figure 1A-B), with high levels of bone morphogenetic protein 2 (BMP2) (figure 1B). Aged hearts showed extensive fibrosis (figure 1C), but neither left ventricle diastolic nor systolic dysfunction, although end systolic interventricular septum thickness and left ventricular ejection fraction were reduced when compared with those in young mice (figure 1D-G).

Protein nitration, indicative of nitrate stress, as result of high levels of cytotoxic NO from increased iNOS expression and

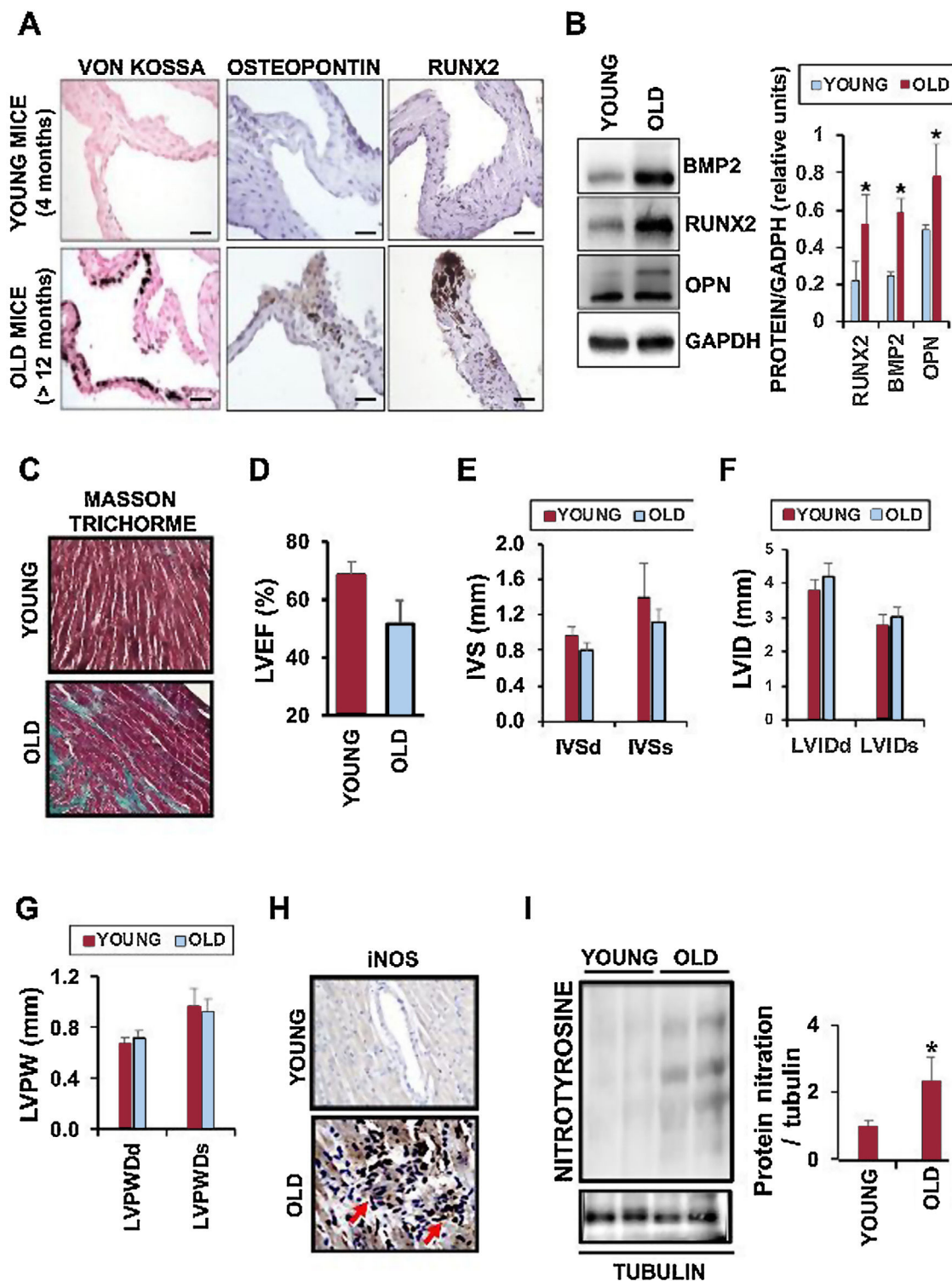
superoxide anion formation, were found in the hearts of aged mice (figure 1H-I), and endothelial ILK was significantly reduced in the coronary arteries compared with levels in young adults (figure 2A). Accordingly, acetylcholine stimulation of murine coronary arteries from aged mice lead to eNOS-dependent superoxide generation, instead of endothelial derived NO, as inhibition of eNOS with L-NG-nitroarginine-methyl ester (L-NAME) was sufficient to efficiently revert this effect (figure 2B).

As in the coronary arteries, endothelial ILK levels found in the aortic valves from aged mice were also lower than those in young adults (figure 2C). The causative role of ILK in valvular calcification was assayed in human valvular endothelial cells, in which ILK expression was decreased by transfecting with a specific small (si) interfering RNA (siILK). ILK silencing inhibited NO production by human valvular endothelial cells (hVECs) (figure 2D) and correlated with an increased expression of BMP2 (figure 2E), as in the aortic valves of aged mice (figure 1B).

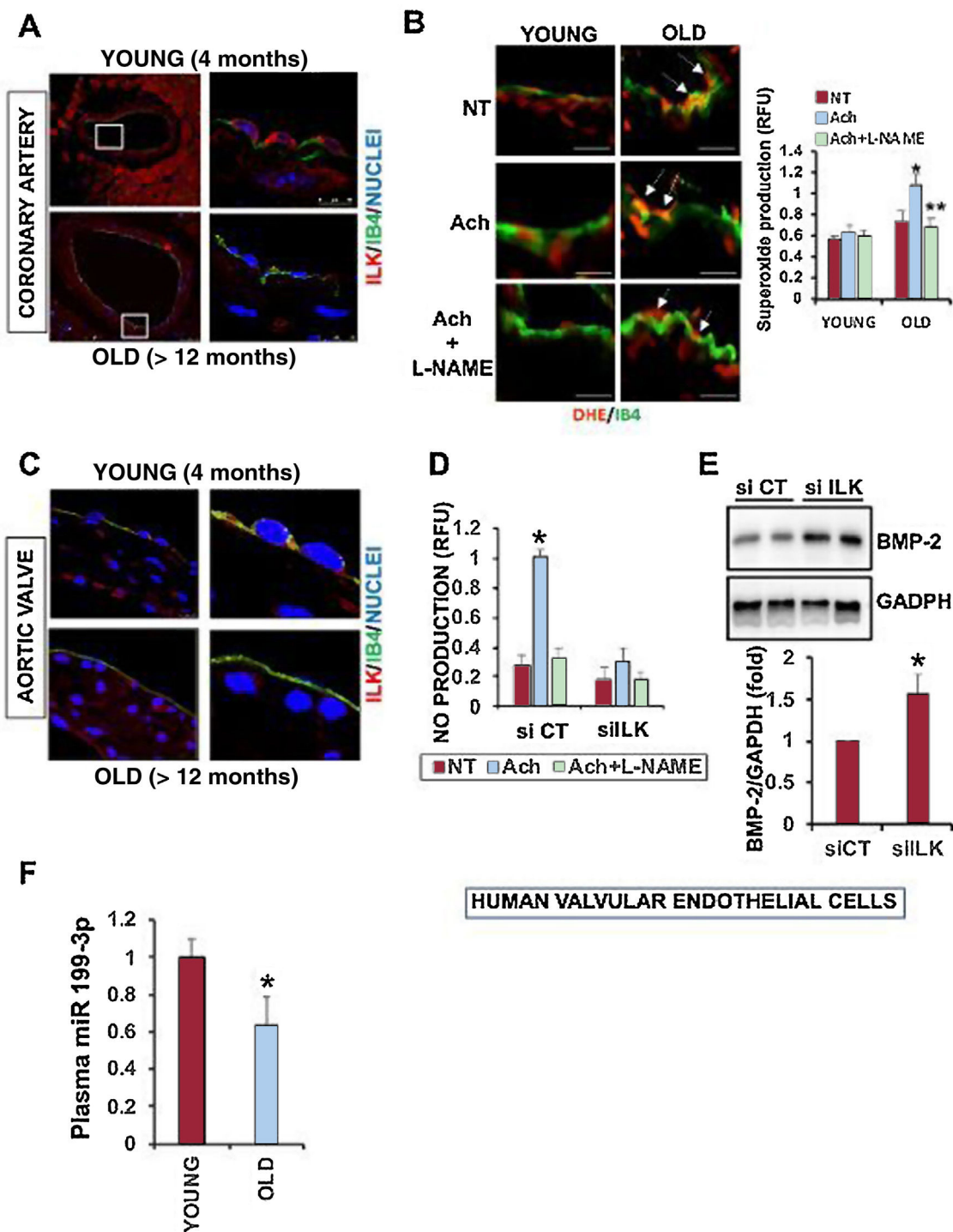
We analyzed the expression of several microRNAs (miRNAs) involved in CAVD.<sup>5</sup> Out of 11 miRNAs differentially expressed in young vs aged mice, miR 199-3p was related to the expression of Notch and eNOS, both genes directly related to CAVD (Notch) and vascular tone (eNOS). Silencing of Notch-1 increased the levels of Runx2, promoting valvular calcification. We unexpectedly found a correlation between downregulation of ILK with decreased levels of miR-199-3p in aged mice (figure 2F), suggesting a Notch-independent mechanism of aortic calcification by ILK, although Notch in CAVD is under debate, since it also promotes pro-osteogenic responses in human aortic valve interstitial cells.<sup>6</sup>

In conclusion, we found for the first time a significant association between decreased endothelial ILK, eNOS uncoupling and valvular calcification in aged mice, suggesting that ILK may prevent valvular calcification through miRNA199-3p. Further studies including the use of endothelial-specific conditional knockout mouse models will be crucial to validate the specific contribution of ILK and surrogated miRNAs as targets in CAVD.

All animal procedures were approved by the University of Alcalá Animal Care Committee and Autonomous Community of Madrid (experimental procedure 231.2/20) and conformed to the EU Directive on the protection of animals used for experimental and other scientific purposes (enacted under Spanish law 1201/2005).



**Figure 1.** Aging-related calcification of aortic valves. A: Von Kossa and immunohistochemical detection of osteopontin and Runx2 in aortic valves from young and old mice. B: immunoblot detection of BMP2, Runx2, and osteopontin in the aortic valves (n = 4/group; mean ± standard deviation; asterisk indicates  $P < .05$  young vs old). C: Masson-trichrome staining of heart sections from the same hearts; left ventricular ejection fraction (D), end diastolic and systolic interventricular septum thickness (E), end diastolic and systolic left ventricular diameter (F), end diastolic and systolic left ventricle posterior wall thickness (G), expression of iNOS (H), and detection of protein nitration with antinitrotyrosine antibody in the hearts (I); n = 4/group; mean ± SD; asterisk indicates  $P < .05$  young vs old.



**Figure 2.** Expression of ILK in the hearts of young and old mice. A: left, confocal microscopy detection of endothelial ILK (Alexa 488, red) in coronary arteries; IB4 (fluorescein isothiocyanate (FITC), green, as endothelial marker). Right, magnified sections (open boxes), n = 4/group. B: confocal microscopy detection of anion superoxide with dihydroethidium (DHE) fluorescence (red) in coronary arteries stimulated with 10  $\mu$ M acetylcholine (Ach), 30 minutes or in combination with 500  $\mu$ M of nitric oxide synthase (NOS) inhibitor L-NAME; n = 4/group. Mean  $\pm$  SD; asterisk indicates  $P < .05$  Ach young vs old; double asterisk indicates  $P < .001$  old Ach vs Ach plus L-NG-nitroarginine-methyl ester [L-NAME]). C: confocal microscopy detection of ILK (red) in the valves of the same mice; n = 4/group. D: NO production in human valvular endothelial cells stimulated as in B, in which the expression of ILK was reduced by RNA interference (siILK). A non ILK siRNA (siCT) was used as a negative control (n = 3; mean  $\pm$  SD; \* $P < .05$  Ach vs L-NAME). E: expression of BMP2 in the same cells; n = 3; mean  $\pm$  SD; asterisk indicates  $P < .05$  siCT vs siILK). F: plasma levels of miR199-3p analyzed by RT-qPCR; n = 4; mean  $\pm$  SD; asterisk indicates  $P < .05$  young vs old.

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## AUTHORS' CONTRIBUTIONS

S. Sánchez: experimentation, data acquisition. A. Cook: experimentation, data acquisition. P. Reventún: experimentation, data acquisition. C. Zaragoza: experimentation, review, editing. J. L. Zamorano: review, editing, funding acquisition. M. Saura: conceptualization, methodology, experimentation, original draft preparation, writing, review, editing, funding acquisition.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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## Virtual lipid clinic after acute coronary syndrome



### Consulta virtual de lípidos después de síndrome coronario agudo

#### To the Editor,

Treatments to reduce low-density lipoprotein cholesterol (LDL-C) are the best tool available for secondary prevention in patients who have had an acute coronary syndrome (ACS). Consequently, guidelines recommend increasingly stringent LDL-C levels, and the previous lipid target of LDL-C < 70 has been lowered to < 55 mg/dL.<sup>1</sup> Despite these recommendations, multicenter secondary prevention studies, such as EUROASPIRE<sup>2</sup> and DA VINCI,<sup>3</sup> show that these targets are reached in a low percentage of patients. Moreover, a recent study conducted in Spain reported that around 40% of postinfarction patients do not even meet the previous LDL-C target of < 70 mg/dL.<sup>4</sup> Failure to meet this goal is mainly due to underuse of the available treatments, whether the statin-ezetimibe combination or proprotein convertase subtilisin/kexin type 9 inhibitors (PCSK9i), indicated in fewer than 1% of patients.<sup>4–6</sup>

In an attempt to improve these results, a strategy was developed based on strict control and close follow-up for patients admitted to our department for ACS and designated the “post-ACS virtual lipid visit.” This study complied with the ethical principles established in the Declaration of Helsinki and was approved by the Research Ethics Committee under registration number 85.21. To summarize, the

following approach was used: post-ACS patients were discharged from the hospital with high-intensity statins (unless not tolerated) and lab work was ordered for 1 month after the infarction. One month later, the patient was contacted by phone and, following a telemedicine evaluation of the test results, the lipid-lowering treatment was titrated according to the therapeutic algorithm of the consensus document from the Spanish Society of Cardiology.<sup>5</sup> These visits were repeated each month until LDL-C was < 55 mg/dL. The time to achieve the lipid target was then computed, which was the primary endpoint of this study, and the patient was released from the virtual visit schedule to continue follow-up with conventional outpatient visits. In the virtual visits, a few patients had LDL-C levels > 55 mg/dL because their health condition made it inadvisable to pursue more aggressive strategies, such as the addition of PCSK9i to their therapy.

To provide some context, this “post-ACS virtual lipid visit” approach was first implemented in 2020 at the start of the COVID-19 pandemic, which halted the normal operation of cardiac rehabilitation programs, making group activities imprudent. This “post-ACS virtual lipid visit” retained the main tasks of these programs, such as health training and cardiovascular risk factor follow-up. Moreover, by focusing on risk factors and most particularly on LDL-C, stricter control and closer follow-up of these aspects became feasible.

A total of 388 patients were consecutively admitted to our Cardiology Department for ACS in 2020. The study excluded