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Heart diseases

# IMMUNOREGULATORY MOLECULES AND miRNAS AS TARGETS IN CORONARY HEART DISEASE AND ACUTE CORONARY SYNDROME

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## 1. Summary


### Background

Coronary artery disease (CAD), the most frequent cardiovascular disease (CVD), a chronic inflammatory disorder that may lead to acute coronary syndrome (ACS), is often caused by the rupture of an atherosclerotic plaque. Inflammation contributes to all stages of CAD, from the initial lesion to plaque rupture, and oxidized low-density lipoproteins (oxLDL) are the main trigger. There is evidence about the imbalance of Th17 lymphocytes and regulatory T cells in the immunopathogenesis of this disease as well as the importance of microRNAs (miRNAs) in this process. Results obtained by our group have shown that oxLDL bind the T lymphocyte immunoregulatory molecule CD69, thus inducing the expression of members of the NR4A family of nuclear receptors. The lymphocytic molecule CD69 is both a negative regulator of Th17 response and a promoter of Treg cells.

### Objectives

i) To assess CD69 as a novel lymphocyte receptor for oxLDL and its impact in the immunopathogenesis of CAD, and to elucidate the role NRA4 receptors in this process using in vitro and in vivo models**;** ii) to analyze circulating levels of Th17 and Treg cells in patients with CAD and the miRNA profiling of T lymphocytes in response to oxLDL and iii) to study the plasma miRNA profile in patients with CAD searching key miRNAs relevant for CAD and ACS.

### Methods

To accomplish these objectives, human T cells expressing or not CD69 (JKCD69 and Jurkat wt [JKwt] respectively) as well as CD69 deficient mice ($CD69^{-/-}$) were used. For the atherosclerotic model, these mice were crossed with LDL receptor-deficient mice and subjected to a high fat diet. To elucidate the CD69 regulatory pathways in ACS, we analyzed CD69-deficient and proficient mice after permanent occlusion of the left anterior descending coronary artery (LAD-ligation). Next generation sequencing was performed to determine the gene and miRNA expression profile induced in human T cells as a consequence of CD69/oxLDL interaction.

**Results**

After a high fat diet, mice lacking CD69 on lymphoid cells developed large atheroma plaques along with an increased Th17/Treg cell ratio in blood. OxLDL was shown to bind specifically and functionally to CD69 on human T lymphocytes, inhibiting the development of Th17 cells and inducing the expression of NR4A nuclear receptors. The binding of CD69 to oxLDL induced the expression of PD1 in human T cell lymphocytes, and miR-663a that regulates the expression the amino acid transporter SLC7A5. Patients with subclinical atherosclerosis displayed a significant CD69 and NR4A1 downregulation in peripheral blood leukocytes compared with healthy subjects. On the other hand, $CD69^{-/-}$ mice developed T cell gamma delta ($T\gamma\delta$) IL17+ responses early after cardiac ischemia inducing an increase in myocardial inflammation. The adoptive transfer of CD69+ Tregs to $CD69^{-/-}$ mice after LAD-ligation restored survival and recovery from heart inflammation and failure. Our data also showed an increase in CD69+ Treg cells and miR-155 in the plasma of mice after LAD-ligation. In humans, the lack of CD69+ Treg cells or miR-155 in plasma is an indicator of patients with bad prognosis after myocardial infarction.

**Conclusions**

This study identifies CD69 as an oxLDL receptor in T lymphocytes that contributes to the regulatory action of the adaptive immune system, preventing atherosclerosis development. Induction of PD1 through the binding of oxLDL to CD69 may be involved in the anti-inflammatory effects of CD69. CD69 expression is an independent marker of early subclinical disease in humans. Our data show that CD69 expression in Tregs is pivotal to maintaining immune homeostasis after myocardial infarction and the circulating levels of miR-155 might be a prognosis biomarker for ACS.

## 2. Results

Transfected Jurkat T cells stably expressing CD69 (JKCD69) on their surface bound oxLDL much more efficiently than control untransfected JK (JKwt) cells and this binding was able to induce CD69 internalization in JKC69 cells. Functionally, this interaction inhibited the expression of IL-8 and IFN-gamma produced by activated JKCD69 cells, but not in JKwt cells, and diminished Th17/Th1 differentiation in primary cultures of human cells, but favored Treg cell polarization. We assessed whether oxLDL regulate

the expression of NR4A nuclear receptors (NR4A1 and NR4A3) in human CD4+ T cells. OxLDL enhanced NR4A1 and NR4A3 mRNA expression in human primary CD4+ T cells. An early induction of NR4A3 was also produced by oxLDL in JKCD69 cells, but not in JKwt cells which was blocked by anti-CD69 antibodies or by RNA interference, confirming the CD69-dependent effect of oxLDL. Finally, to evaluate the gene and miRNA expression profile induced in T cells by CD69/oxLDL interaction, JKCD69 and JKwt cells were incubated with native LDL and oxLDL. Next generation sequencing data showed an induction of PD1 that we confirmed by qPCR in human T cells, associated with an upregulation of PD1 protein levels. By luciferase assays, we confirmed that the oxLDL-CD69 binding activates the PD1 promoter. Moreover, we found that the oxLDL-CD69 interaction increased the mir-663a which regulates the expression of the amino acid transporter SLC7A5.

To analyze the role of CD69 in atherosclerosis in vivo, we generated mixed bone marrow chimeras proficient or deficient for CD69 either in the lymphoid or myeloid compartments in LDL receptor deficient mice ($LDLR^{-/-}$) that were subjected to a high fat diet (HFD) to induce atherosclerosis. A significant enhanced Th17 response in the peripheral blood and lower percentages of Foxp3 cells were observed in the group of $CD69^{-/-}$ mice specific for the lymphoid compartment. Moreover, atherosclerotic lesions were significantly more advanced with more extensive necrotic cores in these mice compared to the $CD69^{+/+}$ group (Figure 1). On the contrary, we did not detect any significant differences in Th17, Treg cells or atheroma plaque formation in myeloid compartment $CD69^{-/-}$ mice. Interestingly, NR4A1 and NR4A3 expression was decreased in $CD69^{-/-}$ mice after HFD. Moreover, expression of CD69 and NR4A1 transcripts gradually declined in peripheral blood leucocytes (PBLs) in $LDLR^{-/-}$ mice during HFD administration indicating that these receptors are dynamically regulated in PBLs during the development of atherosclerosis.

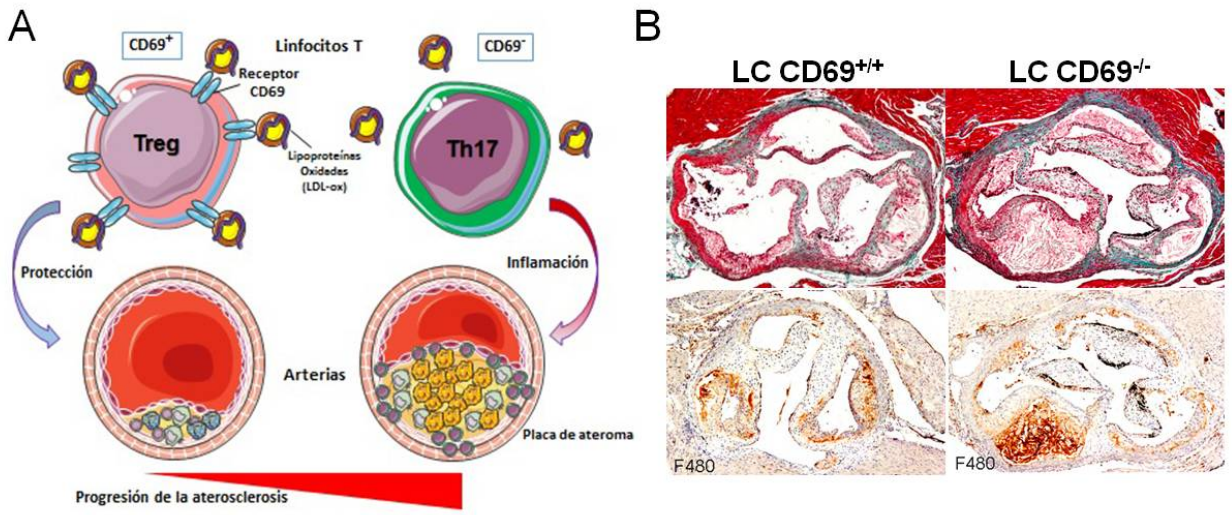


**Figure 1**. The deficiency of CD69 in lymphoid cells (LC CD69$^{-/-}$) induces a Th17 response favoring atherosclerosis development. **B**. Atherosclerotic plaque and necrotic core in aortic valves from mice lacking CD69 on lymphoid cells.

The PESA (Progression of Early Subclinical Atherosclerosis) study is a prospective study that uses advanced imaging techniques to assess the presence of atheroma plaques in the main arteries of healthy individuals. We compared CD69 and NR4A1 mRNA expression in PBLs from PESA participants with focal (n= 55) or generalized subclinical atherosclerosis (n= 128) to that of PESA participants without any evidence of subclinical atherosclerosis (n= 122). CD69 levels correlated significantly with NR4A1 levels, supporting the notion of a common regulation pathway. Multivariable logistic regression analysis determined that CD69 expression is an independent predictor of atherosclerosis at an early stage (OR=0.62, p=0.0056) (Figure 2).

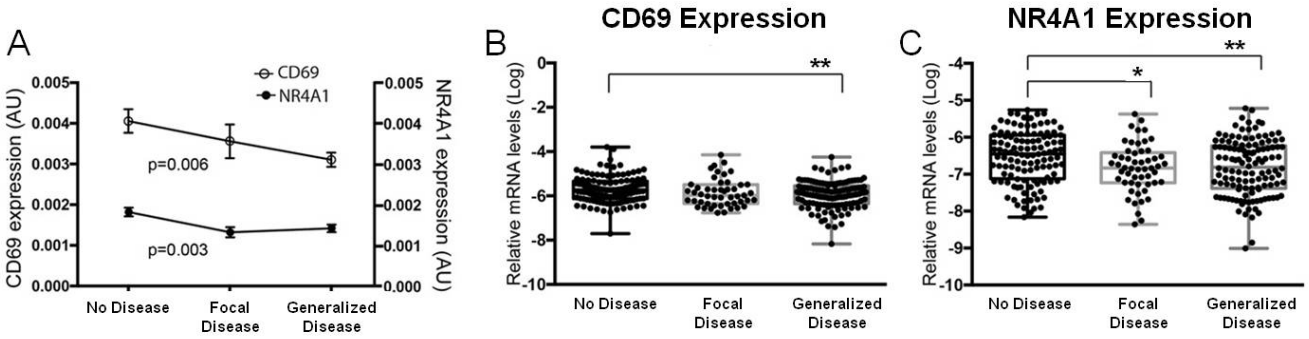


**Figure 2**. CD69 and NR4A1 expression in peripheral blood lymphocytes from subjects with subclinical atherosclerosis classified as focal or generalized disease and in those from healthy individuals. **A,**

Progressive decrease of CD69 and NR4A1 expression during atherosclerosis development in humans**. B-C,** Reduction of CD69 and NR4A1 expression in individuals with subclinical atherosclerosis compared with healthy subjects.

To assess the role of CD69 and microRNAs in the acute coronary syndrome (ACS), we analyzed the expression of CD69 by Tregs in 238 patients with ACS (both with ST-segment elevation myocardial infarction [STEMI] and with non ST-segment elevation myocardial infarction [NSTEMI]) after hemodynamic catheterization and 80 healthy volunteers. Regarding cardiovascular risk factors, in all cases the percentage of patients presenting at least one risk factor was always greater than 90%, the three most frequent being dyslipidemia, arterial hypertension, and former or current smoker. We defined two different populations based on CD69 expression, a group of patients with > 50% of cells CD69+ and a group of patients with < 50% of cells CD69+ in both STEMI and NSTEMI groups. CD4+ and Tregs expressing CD69 significantly correlate with the levels of troponin (TnT), creatine kinase (CK) and cardiac function abnormalities. Moreover, patients with high CD69 expression levels correlate with higher TnT indicating a correlation between CD69 expression and severity of the heart damage in patients with an acute myocardial infarction (AMI). Interestingly, we found that lack of CD69 expression in Treg cells in blood is an indicator of patients with bad prognosis after an AMI, suggesting that CD69 expression in circulating Treg cells detected early after the ischemic event could be a prognostic marker (Figure 3 A-B).

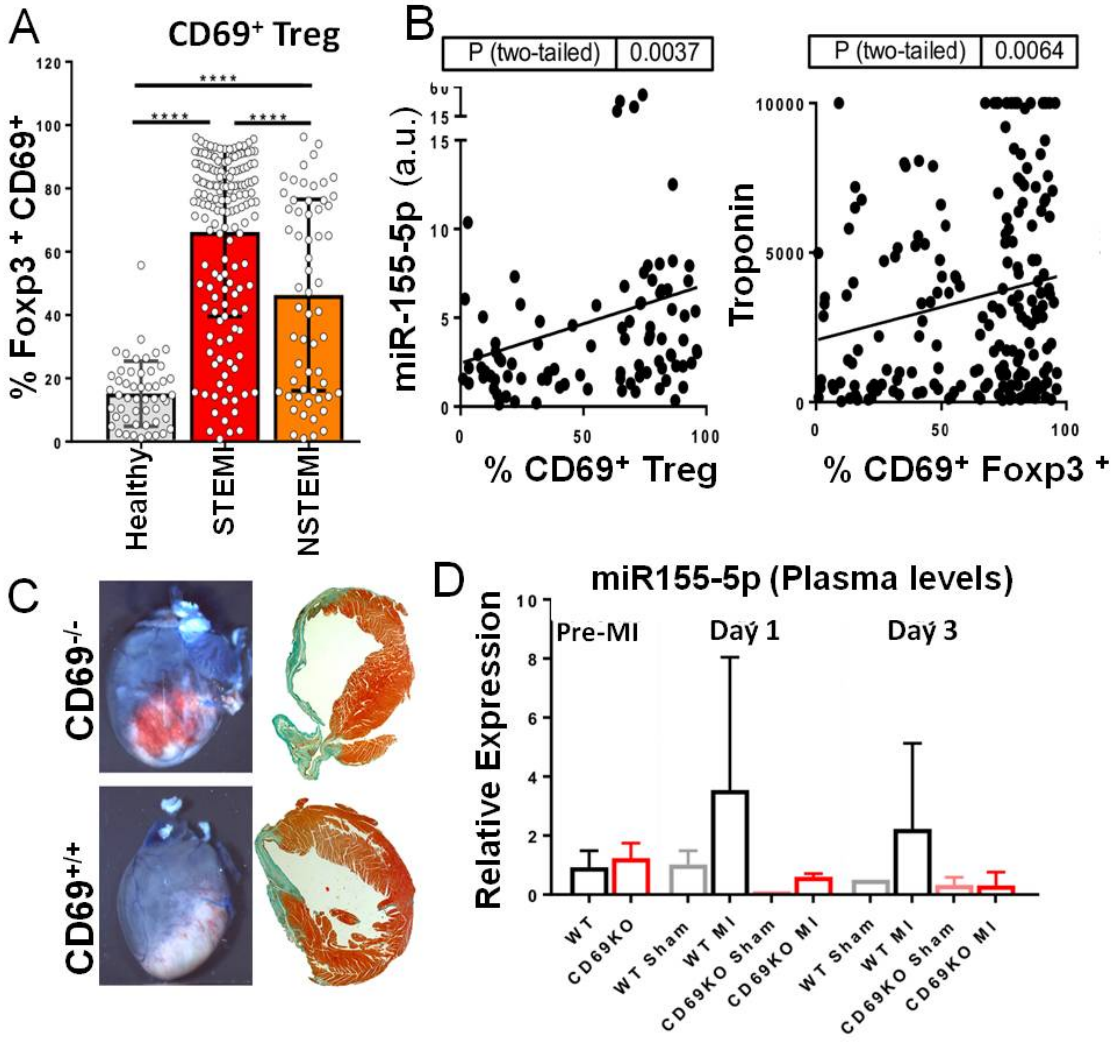


**Figure 3**. **A**, Circulating levels of CD69+ cells increase after an AMI, in particular in STEMI patients. **B**, A correlation between the levels of CD69+ cells and those of circulating miR-155 with troponin T was observed. **C**-**D**, In mice in which myocardial infarction was induced by LAD ligation, the infarct area was higher than that found in $CD69^{-/-}$ mice (**C**). miR-155 expression depends on CD69, and after infarction its levels increase in WT animals ($CD69^{+/+}$) but not in CD69 KO.

To elucidate the CD69 regulatory pathways in ACS, we analyzed CD69-deficient and proficient mice after permanent occlusion of the left anterior descending coronary artery (LAD-ligation). $CD69s^{-/-}$ mice develop T cell gamma delta (Tγδ) IL17+ responses early after ischemia that induce an increase in myocardial inflammation and, as a consequence, a worsening of cardiac function. The adoptive transfer of CD69+ Tregs to $CD69^{-/-}$ mice after LAD-ligation restores survival and recovery from heart inflammation and failure, indicating that CD69+ Treg cells are pivotal for the maintenance of immune homeostasis after AMI. CD69+ Treg cells synthesize microRNA-155 (miR-155)

enhancing STAT-5 signaling and promoting Foxp3 and the regulatory phenotype of T cells. In accordance, we found an increase in CD69+ Treg cells and miR-155 in the plasma of mice after LAD-ligation (Figure 3 C-D). Further, CD69+ Treg cells and miR-155 circulating levels correlate with blood levels of TnT in patients who suffered an AMI, suggesting that CD69 expression is enhanced in parallel with heart damage to counteract the excess of inflammation (Figure 4). Interestingly, we found that lack of CD69+ Treg cells or miR-155 in plasma is an indicator of patients with bad prognosis after myocardial infarction.

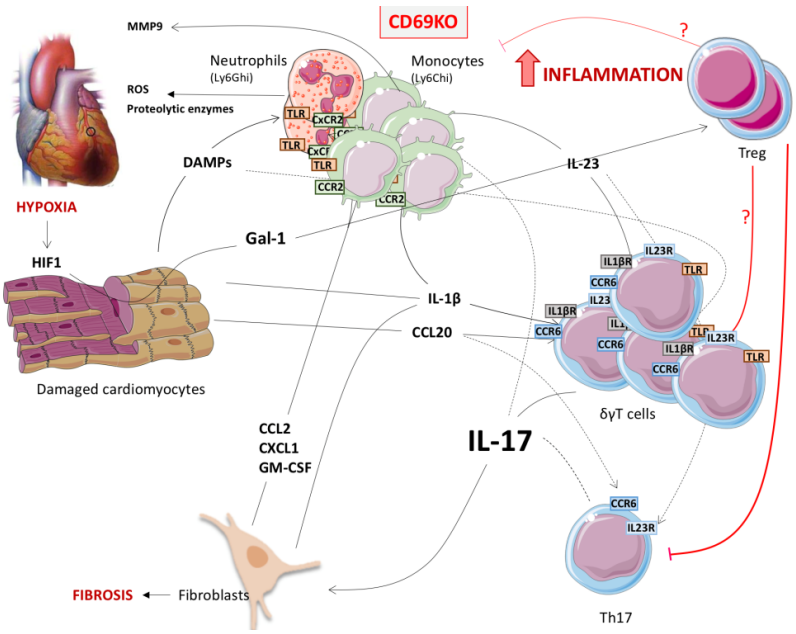


**Figure 4.** CD69 expression is fundamental for the maintenance of immune homeostasis after an acute myocardial infarction (AMI). The lack of CD69 in Treg cells induces an increased expression of IL-17 in gamma delta T cells aggravating inflammation and the adverse cardiac remodeling.

## 3. Relevance and future implications

The identification of molecules as biomarkers is key for early detection and diagnosis of patients with coronary artery disease. In this project, we have found that oxidized LDL binding to CD69 confers a regulatory phenotype to human and mouse T cells, dampening Th17 responses and ameliorating atherosclerosis. Expression of CD69 in circulating cells might serve as a new biomarker for the presence of subclinical

atherosclerosis. In addition, we have found that the expression of microRNA-155 and the levels of CD69+ Treg in blood are indicators of prognosis in patients with myocardial infarction.

## 4. Scientific bibliography generated


Tsilingiri K, de la Fuente H, Relaño M, Sánchez-Díaz R, Rodríguez C, Crespo J, Sánchez-Cabo F, Dopazo A, Alonso-Lebrero JL, Vara A, Vázquez J, Casasnovas JM, Alfonso F, Ibáñez B, Fuster V, Martínez-González J, Martín P, Sánchez-Madrid F. Oxidized low-density lipoprotein receptor in lymphocytes prevents atherosclerosis and predicts subclinical disease. *Circulation 2019;139:243-255.*

Sánchez-Díaz R, Blanco-Dominguez R, Lasarte S, Tsilingiri K, Martín-Gayo E, Linillos-Pradillo B, de la Fuente H, Sánchez-Madrid F, Nakagawa R, Toribio ML, Martín P. Thymus-Derived Regulatory T Cell Development Is Regulated by C-Type Lectin-Mediated BIC/MicroRNA 155 Expression. *Mol. Cell. Biol. 2017;37(9). pii: e00341-16*.

Cibrian D, Castillo-González R, Fernández-Gallego N, de la Fuente H, Jorge I, Saiz ML, Punzón C, Ramírez-Huesca M, Vicente-Manzanares M, Fresno M, Daudén E, Fraga-Fernandez J, Vazquez J, Aragonés J, Sánchez-Madrid F. *Targeting L-type amino acid transporter 1 in innate and adaptive T cells efficiently controls skin inflammation*. *J. Allergy Clin. Immunol.* 2020;145(1):199-214.e11.

Rafael Blanco-Domínguez, Raquel Sánchez-Díaz, Hortensia de la Fuente, …Francisco Sánchez-Madrid, and Pilar Martín. Circulating hsa-miR-Chr8:96, a new biomarker for acute myocarditis. Revised version currently in evaluation in *Circulation*.

Rafael Blanco-Domínguez, Raquel Sánchez-Días, Hortensia de la Fuente, Cristina Rodríguez …Fernando Alfonso, Albert Duran, Francisco Sánchez-Madrid, José Martinez-González and Pilar Martínez. *CD69 expression in CD4$^+$Foxp3$^+$ protects from immune-mediated damage after MI" Submitted.*

Clemente C, Rius C, Alonso-Herranz L, Martín-Alonso M, Pollán A, Camafeita E, Martínez F, Mota RA, Núñez V, Rodríguez C, Seiki M, Martínez-González J, Andrés V,

Ricote M, Arroyo AG. MT4-MMP deficiency increases patrolling monocyte recruitment to early lesions and accelerates atherosclerosis. *Nat. Commun*. 2018;9(1):910.

Alonso J, Cañes L, García-Redondo AB, García de Frutos P, Rodríguez C, Martínez-González J. The nuclear receptor NOR-1 modulates redox homeostasis in human vascular smooth muscle cells. *J. Mol. Cell. Cardiol*. 2018;122:23-33.

Martí-Pàmies I, Cañes L, Alonso J, Rodríguez C, Martínez-González J. The nuclear receptor NOR-1/NR4A3 regulates the multifunctional glycoprotein vitronectin in vascular smooth muscle cells.
*FASEB J*. 2017;31:4588-4599.

Cañes L, Martí-Pàmies I, Ballester-Servera1 C, Herraiz-Martínez A, Alonso J, Galán M, Nistal JF, Muniesa P, Osada J, Hove-Madsen L, Rodríguez C, Martínez-González J. Neuron-derived orphan receptor-1 modulates cardiac gene expression and exacerbates angiotensin II-induced cardiac hypertrophy. *Clin. Sci.* 2020;134:359-377

Rodríguez C, Martínez-González J. The role of lysyl oxidase enzymes in cardiac function and remodeling. *Cells.* 2019;8(12). pii: E1483.
Varona S, Orriols M, Galán M, Guadall A, Cañes L, Aguiló S, Sirvent M, Martínez-González J, Rodríguez C. Lysyl oxidase (LOX) limits VSMC proliferation and neointimal thickening through its extracellular enzymatic activity. *Sci. Rep*. 2018;8(1):13258.

Galán M, Varona S, Guadall A, Orriols M, Navas M, Aguiló S, de Diego A, Navarro MA, García-Dorado D, Rodríguez-Sinovas A, Martínez-González J, Rodriguez C. Lysyl oxidase over-expression accelerates cardiac remodelling and aggravates angiotensin II-induced hypertrophy. *FASEB J.* 2017;31:3787-3799.

Alonso J, Galán M, Martí-Pàmies I, Romero JM, Camacho M, Rodríguez C, Martínez-González J. NOR-1/NR4A3 regulates the cellular inhibitor of apoptosis 2 (cIAP2) in vascular cells: role in the survival response to hypoxic stress. *Sci. Rep*. 2016;6:34056.

Ferrán B, Martí-Pàmies I, Alonso J, Rodríguez-Calvo R, Aguiló S, Vidal F, Rodríguez C, Martínez-González J. The nuclear receptor NOR-1 regulates the small muscle protein, X-linked (SMPX) and myotube differentiation. *Sci. Rep*. 2016;6:25944.