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http://dx.doi.org/10.1016/j.jacc.2014.12.049

Please note: This study was supported by a research grant from St. Jude Medical. Dr. Wong is the recipient of the Keith Goldsbury Postgraduate Research Scholarship Award (ID: PC11M 6218) from the National Heart Foundation of Australia. Dr. Kalman has received research support from St. Jude Medical, Medtronic Inc., Biosense Webster, and Boston Scientific. Dr. Pedagogos has received honoraria and research grants from Shire and Amgen. Dr. Sanders has served on the advisory boards of Biosense Webster, Medtronic, St. Jude Medical, Sanofi, and Merck, Sharpe and Dohme; has received lecture fees from Biosense Webster, Medtronic, St. Jude Medical, Boston Scientific, Biotronik, Sanofi, and Merck, Sharpe and Dohme; has received research funding from Medtronic, St. Jude Medical, Boston Scientific, Biotronik, and Sorin; is supported by a Practitioner Fellowship from the National Health and Medical Research Council of Australia; and is supported by the National Heart Foundation of Australia. Dr. Morton has received research support from St. Jude Medical. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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RNA Sequencing Analysis Identifies New Human Collagen Genes Involved in Cardiac Remodeling

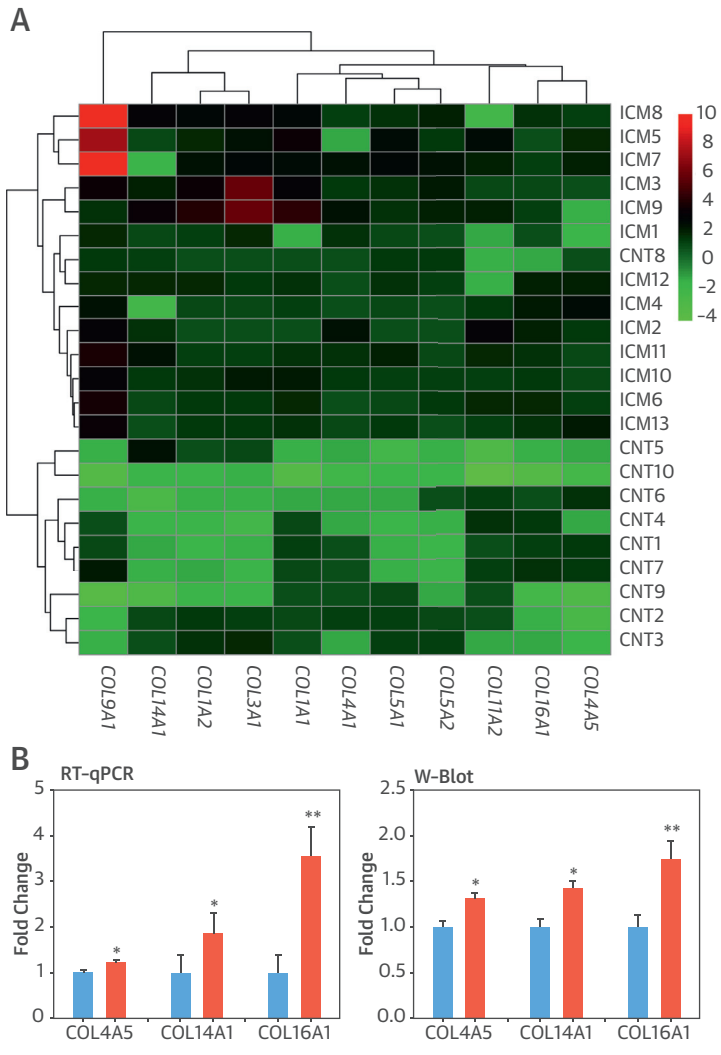
Ventricular remodeling, a process involving morphological changes in cardiomyocytes and the extracellular matrix (ECM), has been linked to worsening of clinical status and heart failure (HF) development. In HF, remodeling initially occurs as a compensatory response to preserve the structural integrity of the myocardium, but progressive collagen deposition can lead to cardiac fibrosis and impairment of diastolic and systolic function (1). Thus, we analyzed changes in the expression profile of collagen-related genes in patients with ischemic cardiomyopathy (ICM) and examined the relationships with left ventricular (LV) dysfunction.

We obtained 23 human LV tissue samples from patients with ICM (13 men; mean age, 54 ± 8 years) with end-stage HF undergoing heart transplantation and control donors (CNT) (8 men, 2 women; mean age, 47 ± 16 years). All control donors had normal LV function (ejection fraction >50%) and no history of cardiac disease. As an established condition for inclusion in the study, all selected samples displayed a 260/280 nm absorbance ratio >2.0 and RNA integrity number ≥9. Transcriptome-level differences between ICM and CNT samples were investigated by means of large-scale screening of 23 heart samples with the use of RNA-sequencing technology and further validated by means of real-time (RT-PCR) and Western blot analysis (**Figure 1**). These data have been deposited in the NCBI Gene Expression Omnibus (GEO) (retrieved by use of the GEO Series accession No. GSE55296). We performed quantitative RT-PCR assays in duplicate with the use of TaqMan technology in the ViiA7 The Fast Real-Time RT-PCR System (Applied Biosystems, Foster City, California); collagen IV (COL4A5, Hs00166712_m1), collagen XIV (COL14A1, Hs00964045_m1), and collagen XVI (COL16A1, Hs00156876_m1) was obtained from TaqMan. Housekeeping genes *GAPDH* (Hs99999905_m1), *PGK1* (Hs99999906_m1), and *TFRC* (Hs00951083_m1) were used as reference. Regarding the Western blot analysis, tissue samples were transferred into Lysing Matrix D tubes designed for use with the FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, California). Protein samples were separated by tris-acetate electrophoresis on 3% to 8% polyacrylamide gels under no-reducing conditions and transferred to a PVDF membrane with the use of the iBlot Gel Transfer Device (Life Technologies, Carlsbad, California) for Western blot analyses. The primary detection antibodies used were anti-COL4A5 rabbit polyclonal (sc-11360) obtained from Santa Cruz Biotechnology, anti-COL14A1 rabbit polyclonal (ab-101464) obtained from Abcam, and anti-COL16A1 rabbit polyclonal (A-96190) obtained from Sigma; monoclonal anti-GAPDH antibody (ab-9484) from Abcam was used as a loading control. We must note that patients with end-stage HF are under heavy medical treatment, and some therapies might influence mRNA levels.

We compared ICM and CNT samples, and we found significantly increased mRNA levels of 11 collagen genes, such as *COL9A1*, *COL11A2*, *COL14A1,* and *COL16A1* ($p < 0.05$ for all), not previously described in the cardiac remodeling process. A heat map and

JACC VOL. 65, NO. 12, 2015
MARCH 31, 2015:1263–71

FIGURE 1 Effects of Heart Failure on Collagen Gene Expression Levels



(A) Hierarchical clustering shows the separation of both ischemic cardiomyopathy (ICM) and control donor (CNT) groups. **(B)** The differential expression of *COL4A5*, *COL14A1*, and *COL16A1* was validated by means of quantitative RT-PCR and Western (W) blot, respectively, in ischemic heart **(salmon bars)** versus control (fold change). Results were considered statistically significant at *$p < 0.05$, **$p < 0.01$, versus CNT. **Bars** represent mean ± SEM values.

hierarchical clustering analysis identified 2 divergent gene expression profiles, showing a clear separation of the ICM and CNT groups (Figure 1). We also found significant relationships between LV dysfunction and the gene expression levels of *COL4A5* (fractional shortening, $r = -0.694$, $p < 0.05$) and *COL16A1* (LV end-systolic diameter and LV end-diastolic diameter, $r = 0.678$, $p < 0.05$, and $r = 0.687$, $p < 0.05$, respectively).

For validation of the novel regulated transcripts, we performed quantitative RT-PCR and Western blot analysis, focusing on those related significantly with the LV function: *COL16A1* and *COL4A5*, and *COL14A1*, newly described. These results (Figure 1) confirm that patients with ICM showed direction changes in expression identical to what we found in the RNA-Seq analysis.

Collagens types IX, XIV, and XVI belong to the fibril-associated collagens with interrupted triple helices class, highly expressed in tissues that have high mechanical stress, such as heart tissue. Collagen IX is thought to be required for several processes, including eye and heart development, and its overexpression has been observed in pathologies such as pectus excavatum (2). In light of these studies, our results suggest that overexpression of *COL9A1* could lead to increased cardiac tissue stiffness and may be potentially involved in human heart development and remodeling.

Previous studies suggest that collagen XIV is required to establish and maintain an organized ECM environment in the developing myocardium and plays an important role in regulating cardiomyocyte growth and cardiac fibroblast survival (3). During replacement fibrosis, cardiomyocytes undergo hypertrophic adaptive changes, whereas myofibroblasts remain at the site of injury; this results in collagen deposition and scar formation. We propose that collagen XIV expression could promote this process.

Collagen XVI, a minor collagen component of connective tissues, is thought to act as a linker protein, helping to organize the large fibrillar networks that modulate ECM integrity and stability. A recent study proposed that in Crohn's disease, increased collagen XVI expression would promote formation and maturation of focal adhesion contacts on intestinal subepithelial myofibroblasts (4). This would retain the cells at the inflammation site, promoting fibrotic responses in the tissue and prolonging disturbances of cellular and ECM homeostasis. We suggest that dysregulated collagen XVI expression could play a similar role in cardiac tissue by keeping myofibroblasts at the inflammation site and promoting pathological remodeling.

Furthermore, collagen XI promotes the nucleation of type I and II fibrils and is required for myocardial morphogenesis (5). The overexpression of *COL11A2* may be related to the formation of heterotypic fibrils with collagen I, involved in cardiac remodeling.

Our findings propose that these collagen genes may have novel roles in the remodeling process, regulating the size increase of cardiomyocytes and the survival of myofibroblasts at the inflammation site and assisting the organization in fibers of other collagens, such as type I and III. All these processes jointly may facilitate the development of cardiac

fibrosis and, consequently, ventricular dysfunction. Inhibition of collagen remodeling can lead to improved cardiac function, demonstrating the relevance of new insights into the compensatory remodeling mechanism. Thus, our findings give new theoretical support for the treatment of patients with ischemic cardiomyopathy.

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http://dx.doi.org/10.1016/j.jacc.2015.01.029

Please note: This work was supported by grants from the National Institute of Health "Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III," Madrid, Spain [PI10/00275, PI13/00100]; European Regional Development Fund (ERDF); and RETICS, Madrid, Spain [RD 06/0003/1001, 12/0042/0003]. The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Omega-3 Highly Unsaturated Fatty Acids and Arrhythmia Risk

Influences of Load Conditions and a Differential Endogenous Metabolism

We read with great interest the recent report by Nigam et al. (1) about the AFFORD (Multi-Center Study to Evaluate the Effect of n-3 Fatty Acids [Omega-3] on Arrhythmia Recurrence in Atrial Fibrillation) trial. Supplementation of 4 capsules fish oil per day (containing a total of 1,600 mg eicosapentaenoic acid, 20:5n-3, and 800 mg docosahexaenoic acid [DHA], 22:6n-3) was shown not to reduce recurrence of atrial fibrillation. In parallel, markers of inflammation or oxidative stress were not affected. Up to now, numerous studies have provided a large variety of potential effects attributed to highly unsaturated fatty acid (HUFA) treatment ranging from marked prognostic improvements in heart failure and antiarrhythmogenic actions to no incremental effects. Due to the divergent results, the question arises whether mechanisms exist beyond an external HUFA intake.

In heart failure, an inverse shift of serum fatty acids occurs; particularly saturated and monounsaturated fatty acids were increased, whereas poly- and highly unsaturated omega-6 and omega-3 fatty acids were decreased (2,3). We have recently shown that increased ventricular wall stress is associated with reduced DHA levels (unpublished data, P. Alter, January, 2015). Similar, but less pronounced effects were found for eicosapentaenoic acid and arachidonic acid (20:4n-6). Because the liver is the major source of endogenous HUFA, pseudocholinesterase activity, a marker of hepatic metabolizing capacity, was examined and shown to be inversely correlated with increased end-diastolic and end-systolic ventricular wall stress, which emphasizes the hypothesis of a cardio-hepatic syndrome (e.g., influenced by congestion). In addition, local variances of the endogenous HUFA metabolism leading to inhomogeneities of HUFA concentrations and effects should be considered.

It was previously shown that systemic HUFA levels in red blood cells and plasma correlate with right atrial concentrations. Oral HUFA supplementation was incorporated into the atrial myocardium (4). However, little is known about cardiac influences on myocardial HUFA levels (5). We recently found significant differences of DHA concentrations among atrial and ventricular myocardium in experimental animals by using gas chromatography/mass spectrometry (atrium 4.69 ± 1.02% vs. ventricle 8.99 ± 2.05%; $p < 0.001$) (unpublished, P. Alter, January, 2015). It is suggested that different load conditions, in particular increased wall stress, are involved. Because DHA exhibits antiarrhythmogenic actions, the question arises whether reduced atrial DHA levels account for an increased risk of atrial fibrillation. Of note, intermediate DHA