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Quantitative Expression of the Mutated *Lamin A/C* Gene in Patients With Cardiolaminopathy

Nupoor Narula, BS,* Valentina Favalli, BME,* Paolo Tarantino, BD,* Maurizia Grasso, PHD,* Andrea Pilotto, BS,* Riccardo Bellazzi, PHD,† Alessandra Serio, MD,* Fabiana I. Gambarin, MD,* Philippe Charron, MD,‡ Benjamin Meder, MD,§ Yigal Pinto, MD,‖ Perry M. Elliott, MD,¶ Jens Mogensen, MD,# Martino Bolognesi, PHD,** Michela Bollati, PHD,** Eloisa Arbustini, MD*

Pavia and Milan, Italy; Paris, France; Heidelberg, Germany; Amsterdam, the Netherlands; London, United Kingdom; and Aarhus, Denmark

Objectives The authors sought to investigate the gene and protein expression in *Lamin A/C* (*LMNA*)-mutated dilated cardiolaminopathy (DCM) patients ($DCM^{LMNAMut}$) versus *LMNA*-wild-type DCM (DCM^{LMNAWT}), and normal controls ($CTRL^{LMNAWT}$).

Background Dilated cardiolaminopathies are clinically characterized by high arrhythmogenic risk and caused by *LMNA* mutations. Little is known regarding quantitative gene expression (QGE) of the *LMNA* gene in blood and myocardium, as well as regarding myocardial expression of the lamin A/C protein.

Methods Using the comparative ΔΔCT method, we evaluated the QGE of *LMNA* (QGE^{LMNA}) in peripheral blood and myocardial RNA from carriers of *LMNA* mutations, versus blood and myocardial samples from DCM^{LMNAWT} patients and $CTRL^{LMNAWT}$ individuals. After generating reference values in normal controls, QGE^{LMNA} was performed in 311 consecutive patients and relatives, blind to genotype, to assess the predictive value of QGE^{LMNA} for the identification of mutation carriers. In parallel, *Lamin A/C* was investigated in myocardial samples from $DCM^{LMNAMut}$ versus DCM^{LMNAWT} versus normal hearts ($CTRL^{LMNAWT}$).

Results *LMNA* was significantly underexpressed in mRNA from peripheral blood and myocardium of $DCM^{LMNAMut}$ patients versus DCM^{LMNAWT} and $CTRL^{LMNAWT}$. In 311 individuals, blind to genotype, the QGE^{LMNA} showed 100% sensitivity and 87% specificity as a predictor of *LMNA* mutations. The receiver-operating characteristic curve analysis yielded an area under the curve of 0.957 ($p < 0.001$). Loss of protein in cardiomyocytes' nuclei was documented in $DCM^{LMNAMut}$ patients.

Conclusions The reduced expression of *LMNA* gene in blood is a novel potential predictive biomarker for dilated cardiolaminopathies with parallel loss of protein expression in cardiomyocyte nuclei. (J Am Coll Cardiol 2012;60:1916–20)

Dilated cardiomyopathy (DCM) is a primary myocardial disease with heterogeneous etiology, wherein genetic defects are found in up to 48% of DCM cases (1). *Lamin A/C* (*LMNA*) mutations occur in approximately 8% of familial cases (2); the most common cardiac phenotype associated with *LMNA* defects comprises DCM with conduction disease (80%), high arrhythmogenic risk, and progression to end-stage heart failure (3–5). Haploinsufficiency is a major mechanism of myocardial damage in $Lmna^{+/-}$ mouse models (6). In human laminopathy, the expression of the protein in the nuclear membrane of cardiac and skeletal myocytes may either be reduced or remain normal (4,7). Although quantitative gene expression (QGE) in peripheral blood has been suggested as a promising molecular signature of DCM (8), only scattered data exist of the mutated *LMNA* gene (QGE^{LMNA}) in patients with cardiolaminopathy (9).

To investigate the expression of lamin A/C protein and QGE^{LMNA} as well as its potential diagnostic contribution to

From the *Centre for Inherited Cardiovascular Diseases, Foundation IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy; †Dipartimento di Ingegneria Industriale e dell'Informazione, University of Pavia, Pavia, Italy; ‡UPMC University Paris 6, AP-HP, Centre de Référence des Maladies Cardiaques Héréditaires, Hôpital Pitié-Salpêtrière, Paris, France; §Department of Medicine III, University of Heidelberg, Heidelberg, Germany; ‖Heart Failure Research Center Academic Medical Center, Amsterdam, the Netherlands; ¶Inherited Cardiac Diseases Unit, The Heart Hospital, University College London, London, United Kingdom; #Department of Cardiology, Skejby University Hospital Aarhus, Aarhus, Denmark; and the **Department of Biomolecular Sciences and Biotechnology, University of Milan, Milan, Italy. Funded by EU INHERITANCE [Grant Number EU291924]. The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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the clinical work-up of cardiomyopathy, we determined immunohistochemical expression of the lamin A/C protein in the myocardium and QGE^{LMNA} in RNA from myocardial tissue and peripheral blood samples of DCM patients with *LMNA* mutations.

Methods

The study was designed to measure *LMNA* gene expression in DCM patients with known *LMNA* mutations (DCM$^{LMNA\text{Mut}}$) in comparison with both DCM patients with wild-type *LMNA* (DCM$^{LMNA\text{WT}}$) and healthy control subjects (CTRL$^{LMNA\text{WT}}$) (Online Fig. 1). Methods used for genetic testing of *LMNA* and for QGE^{LMNA} at both the myocardial and the peripheral blood levels are detailed in the Online Methods.

In the first part of the study, we established the normal QGE^{LMNA} values in 115 CTRL$^{LMNA\text{WT}}$ subjects for the evaluation of 96 DCM$^{LMNA\text{WT}}$ and 67 DCM$^{LMNA\text{MUT}}$ patients. In the second part, we aimed to assess the value of the QGE^{LMNA} assay for predicting *LMNA* mutations in 311 individuals, both DCM probands and relatives, blinded to genotype. Online Table 1 summarizes the mutations, the number of patients that were included in the first study, and the number of mutated patients and healthy carriers that were included in the second study. In the third part, directed at myocardial tissue, we also performed QGE^{LMNA} in endomyocardial biopsy (EMB) samples obtained from 25 DCM$^{LMNA\text{MUT}}$ prospective heart transplant recipients and compared them with 20 CTRL$^{LMNA\text{WT}}$ endomyocardial biopsies obtained from donor hearts before transplantation. In addition to QGE^{LMNA}, using anti-lamin

Abbreviations and Acronyms

DCM = dilated cardiomyopathy
EMB = endomyocardial biopsy
NLS = nuclear localizing sequence
PTC = premature termination codon
QGE = quantitative gene expression
ROC = receiver-operating characteristic

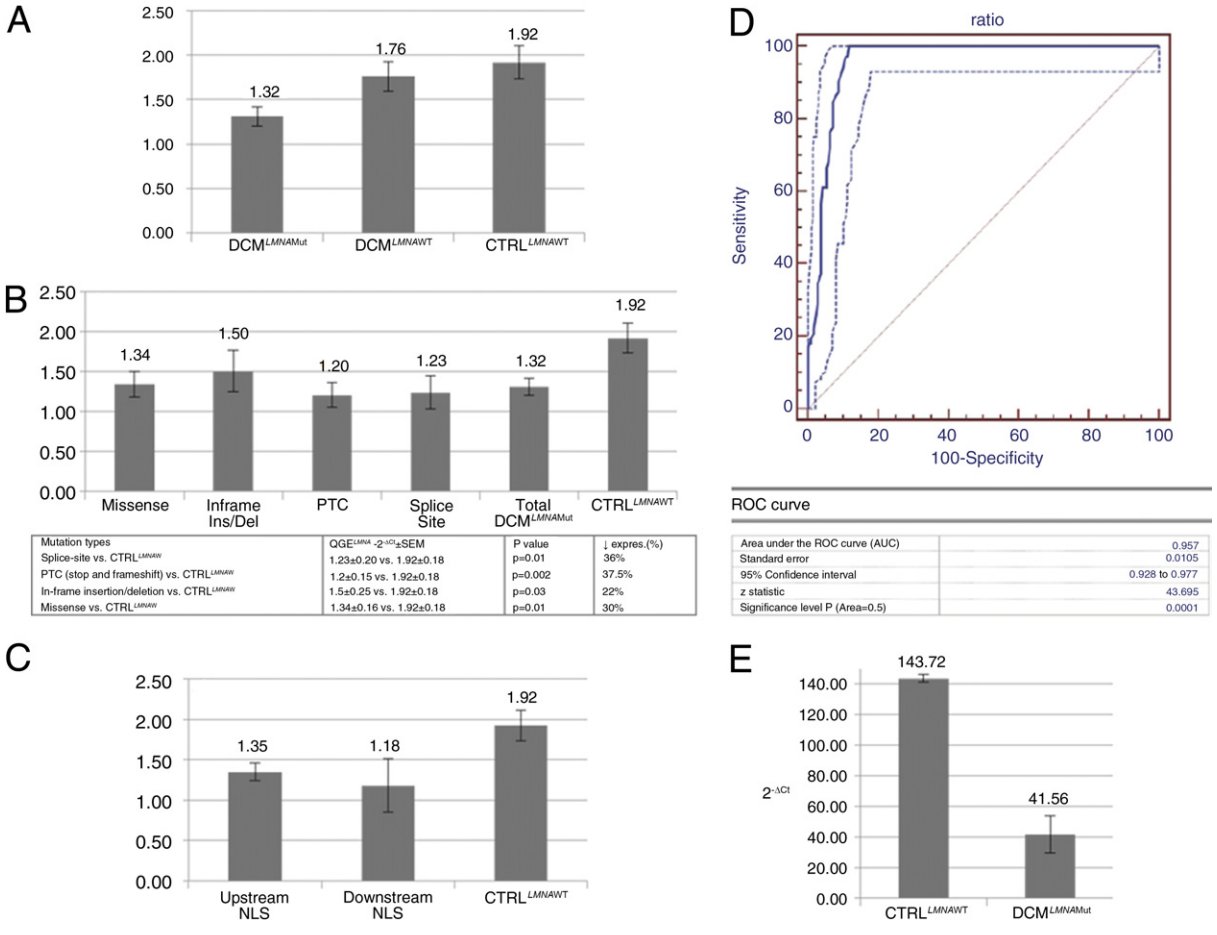

Figure 1 QGE^{LMNA} Study

(A to C) First part of the study: QGE^{LMNA} ($2^{-\Delta Ct}$ ± SEM) in total RNA from peripheral blood of 67 DCM$^{LMNA\text{Mut}}$, 96 DCM$^{LMNA\text{WT}}$, and 115 CTRL$^{LMNA\text{WT}}$. **(B)** QGE^{LMNA} levels did not vary in patients with different types of mutations, and **(C)** with mutations localized upstream or downstream of the nuclear localizing sequence (NLS). **(D)** Second part of the study: receiver-operating characteristic (ROC) curve analysis for the QGE^{LMNA} in the peripheral blood RNA from the 311 consecutive cases. At the threshold value with the highest sensitivity, the area under the curve (AUC) was 0.957 ($p < 0.001$). **(E)** Third part of the study: myocardial samples of DCM$^{LMNA\text{Mut}}$ versus CTRL$^{LMNA\text{WT}}$. CTRL = normal control; DCM = dilated cardiolaminopathy; PTC = premature termination codon; QGE = quantitative gene expression.

A/C antibodies (4), the protein expression was determined in 25 myocardial samples of patients with $DCM^{LMNAMut}$, 20 DCM^{LMNAWT}, and 20 $CTRL^{LMNAWT}$. Western blot analysis was undertaken in 12 $DCM^{LMNAMut}$ and 9 control heart samples with wild-type *LMNA* (Online Methods). We managed to collect appropriate samples for RNA in 22 patients with known laminopathy on 2 different occasions to assess whether the QGE^{LMNA} would change during follow-up. The study complies with the Declaration of Helsinki and was approved by the local ethics committee. All study participants provided written informed consent for genetic testing and anonymous data publication for scientific purposes.

Statistical analysis. For QGE^{LMNA}, relative quantification of the *LMNA* RNA at each time point was determined using the comparative ΔΔCT method (10) (Online Materials). The Kruskal-Wallis nonparametric test was used for multiple comparisons, using Mann-Whitney rank sum test with post hoc Bonferroni correction. The Mann-Whitney rank sum test was used for nonmultiple comparisons. A p value <0.05 was considered significant. Stata 10.1 (StataCorp, College Station, Texas) was used for computation. The receiver-operating characteristic (ROC) curve analysis (11) was performed in the 311 cases blind to mutations.

Results

Peripheral blood study: *LMNA* mRNA levels in known laminopathy and controls. EXPRESSION OF *LMNA* mRNA LEVELS IN PATIENTS WITH KNOWN $DCM^{LMNAMUT}$. The QGE^{LMNA} referral value in $CTRL^{LMNAWT}$ individuals was 1.92. *LMNA* was underexpressed by 30% in $DCM^{LMNAMut}$ patients as compared with $CTRL^{LMNAWT}$ individuals ($2^{-\Delta Ct} \pm SEM = 1.32 \pm 0.10$ vs. 1.92 ± 0.18, respectively; $p < 0.007$) (Fig. 1A), and by 26% ($2^{-\Delta Ct} \pm SEM = 1.32 \pm 0.10$ vs. 1.76 ± 0.16; $p = 0.02$) as compared with DCM^{LMNAWT} patients. Values observed in DCM^{LMNAWT} versus $CTRL^{LMNAWT}$ were not significantly different ($p = 0.71$).

LMNA mRNA LEVELS AND MUTATION TYPE. Comparative evaluation of *LMNA* mRNA levels among groups of patients with different types of *LMNA* mutations (missense, n = 37; in-frame insertion/deletion, n = 7; nonsense mutations and frameshift insertions/deletions, predicting a premature termination codon [PTC] and truncated lamin A/C protein [n = 12]; and splice site [n = 11]) did not demonstrate significant differences ($p = 0.93$) (Fig. 1B).

LMNA mRNA LEVELS AND MUTATION POSITION. QGE^{LMNA} did not differ in carriers of mutations up- and downstream of the nuclear localizing sequence (NLS) ($2^{-\Delta Ct} \pm SEM = 1.35 \pm 0.10$ and 1.18 ± 0.33; $p = 0.75$), all showing significantly lower levels when compared with controls (upstream NLS $2^{-\Delta Ct} \pm SEM = 1.35 \pm 0.10$ vs. $CTRL^{LMNAWT} = 1.92 \pm 0.18$; $p = 0.01$) and 39% (downstream NLS $2^{-\Delta Ct} \pm SEM = 1.18 \pm 0.33$ vs. $CTRL^{LMNAWT} = 1.92 \pm 0.18$; $p = 0.007$), respectively (Fig. 1C).

Table 1 Sensitivity and Specificity of QGE^{LMNA} in 311 Consecutive Individuals Blinded to Genotype

Threshold	%	Mutation +	Mutation −	Total
Threshold value 0.75				
≤0.75		72	30	102
>0.75		0	209	209
Total for threshold 0.75		72	239	311
Sensitivity	100			
Specificity	87			
Threshold value 0.7				
≤0.70		68	24	92
>0.70		4	215	219
Total for threshold 0.7		72	239	311
Sensitivity	94			
Specificity	90			
Threshold value 0.6				
≤0.60		56	17	73
>0.60		16	222	238
Total for threshold 0.6		72	239	311
Sensitivity	78			
Specificity	93			

Peripheral blood study: *LMNA* mRNA levels in 311 consecutive patients and relatives, blinded to genotype. The ROC analysis (Fig. 1D) yielded an area under the curve of 0.957 (95% confidence interval: 0.928 to 0.977) with significance level $p < 0.001$, confirming the QGE^{LMNA} value as a robust possible predictor of mutation presence. On the basis of the sensitivity and specificity of the assay, we tested 3 possible ratios as threshold values: 0.60, 0.70, and 0.75 (Table 1). Since our major aim was to predict LMNA mutations, we selected the threshold value of 0.75 that showed the highest sensitivity (100%) but the lowest specificity (87%). Using this threshold, the QGE^{LMNA} assay predicted not only all mutation carriers, but also 30 patients/individuals with the wild-type *LMNA* gene. (Online Table 2 lists QGE^{LMNA} data in the 311 samples, blind to genotype before QGE^{LMNA}.)

In the 22 LMNA mutation carriers with at least 2 samples obtained at different intervals (2.18 ± 1.43 years), *LMNA* gene expression showed a trend to decrease with time (Online Table 3). A possible future relevance of comparative evaluation over time is related to the potential use of QGE^{LMNA} for monitoring the evolution of the disease or correlation with functional data or medical treatment.

Myocardial tissue studies. PATHOLOGICAL FEATURES. EMB of $DCM^{LMNAMut}$ patients revealed prominent nuclear changes, including irregularities of the morphological profile of myocyte nuclei and increased chromatin density, multifocal myofibrillar loss, and fibrosis of variable extent and severity. Ultrastructural study demonstrated severe changes in the nuclear membranes with blebs, pore clustering, nuclear microtubular structures, and pseudoinclusions (Fig. 2G).

LAMIN A/C IMMUNOSTAIN AND WESTERN BLOT. The EMBs of $CTRL^{LMNAWT}$ and of DCM^{LMNAWT} patients demonstrated homogeneous protein localization throughout the

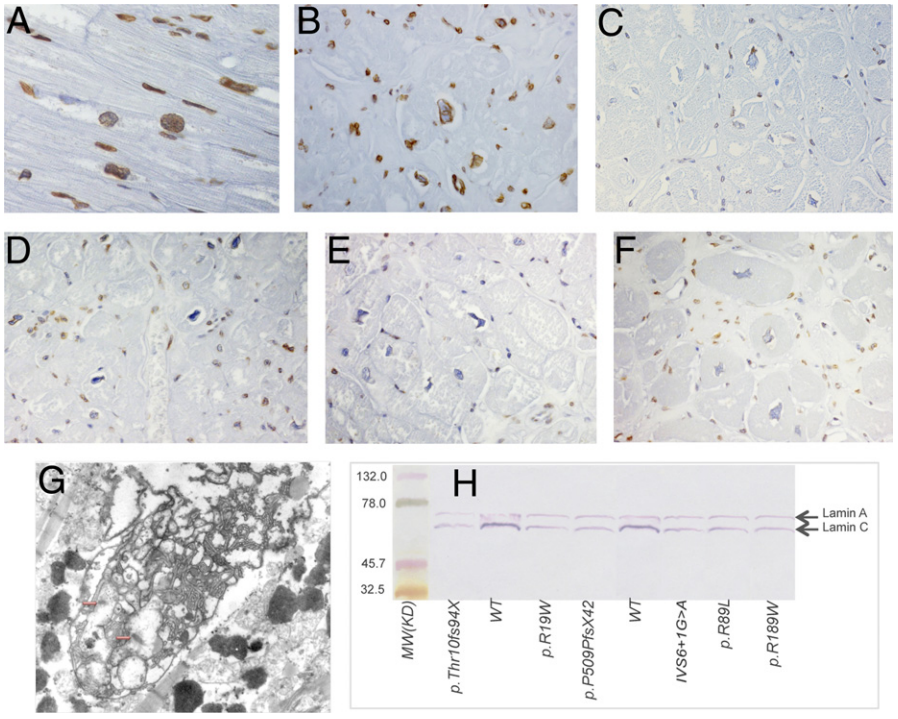

Figure 2 ***Lamin A/C*** **Expression Studies**

(A to F) Immunostain with anti-*Lamin A/C* antibodies: **(A)** normal nuclear expression of *Lamin A/C* in CTRL^{LMNAWT}; **(B)** DCM^{LMNAWT} showing abnormal morphology of the myocyte nuclei but preserved nuclear immunostain. **(C to F)** Loss of immunolabeling in nuclei of myocytes from DCM^{LMNAMut} patients harboring the following mutations: **(C)** IVS2-1G>A; **(D)** p.(Glu111X); **(E)** p.(Leu526_Arg527 insProAlafsX24); and **(F)** p.(Arg190Trp). **(G)** Electron micrograph of a cardiomyocyte in a patient harboring the *LMNA* p.(Arg225X) mutation, showing the prominent structural changes in myocytes' nuclei with pseudoinclusions **(arrows)**. **(H)** Western blot: myocardial samples obtained from hearts excised at transplantation. The decreased expression of the protein shows little variations in carriers of different *LMNA* mutations. MW(KD) = molecular weight (kilodaltons); WT = wild type; other abbreviations as in Figure 1.

nuclear membrane of the nuclei of myocytes (Figs. 2A and 2B). Myocytes of DCM^{LMNAMut} patients exhibited variable loss of lamin A/C expression (Figs. 2C to 2F), whereas interstitial and endothelial cells showed normal expression of the protein. Decreased protein expression was confirmed by Western blot analysis (Fig. 2H).

LMNA **GENE EXPRESSION IN MYOCARDIAL TISSUE.** There was 71% underexpression of *LMNA* in the myocardial samples of DCM^{LMNAMut} patients ($2^{-\Delta Ct} \pm SEM = 41.56 \pm 12.06$) as compared with myocardium from CTRL^{LMNAWT} ($2^{-\Delta Ct} \pm SEM = 143.72 \pm 2.59$) with a ratio of mutated versus wild type of 29% ($p < 0.001$) (Fig. 1E).

Discussion

The present study documents that DCM^{LMNAMut} patients and healthy mutation carriers show decreased expression of the mutated gene in the mRNA in peripheral blood samples as well as the affected myocardium when compared with both normal controls and DCM patients with wild-type *LMNA*. In addition, the LMNA protein is underexpressed at the nuclear level in the cardiac myocytes of cardiolaminopathy patients.

The QGE^{LMNA} assay is a potential biomarker for cardiolaminopathies. *LMNA* gene expression decreases independently of mutation type in myocardial RNA. Although to a lower extent, a similar decrease is observed in the corresponding peripheral blood mRNA. Given the lower cost and the ease of performing the QGE compared with the genetic test, it could constitute a pre-genetic assay for guiding *LMNA* gene testing, which is the diagnostic gold standard. However, whereas the QGE^{LMNA} assay for the threshold ratio value of 0.75 recognized all mutated patients, it also showed a false-positive result in 30 patients with the wild-type *LMNA* gene.

The decreased gene and protein expression confirms haploinsufficiency as major disease mechanism in humans. The loss of lamin A/C in cardiac myocytes in vivo supports the haploinsufficiency mechanism previously shown in experimental models (6). Actively cycling cell types, including endothelial and interstitial cells, displayed normal *LMNA* immunostaining as compared with the gradual decrease in type-A lamins in the terminally differentiated cardiac myocytes. Low *LMNA* mRNA levels in the terminally differentiated, quiescent cells preclude the possibility of increased *LMNA* mRNA translation to stabilize the degradation rate,

whereas the increased mRNA levels in cycling cells moderately compensates for the progressive loss of protein (12).

In carriers of splice site and PTC mutations, the low levels of *LMNA* mRNA are likely explained by the rapid degradation of transcripts with PTC mutations by nonsense mediated decay as suggested in a family with dilated cardiolaminopathy harboring the p.Arg321X *LMNA* mutation (13). In carriers of missense *LMNA* mutations, the low levels of *LMNA* mRNA are likely due to modifications of the position and compaction of chromatin (14). The tethering of mammalian genes to the nuclear lamina leads to transcriptional repression possibly as a result of separation from the RNA Polymerase II machinery or formation of a repressive chromatin structure (9). The key role of lamins in the structure and function of nuclei suggests that gene-specific and genome-wide chromatin rearrangements may contribute to molecular mechanisms of pathology observed in patients with *LMNA* mutations (15).

Decreased expression of *Lamin A/C* in myocardial samples. Lamin A/C protein expression is decreased in myocyte nuclei of affected hearts, independently of mutation type. The underexpression of the protein in vivo has been previously shown in the myocardium of patients with dilated cardiolaminopathy (4), and is now confirmed, thus being a diagnostic contributor if EMB is performed. The decreased amount of lamin A/C is also confirmed by Western blot analysis of myocardial samples in which immunohistochemistry showed selective loss of protein in the nuclei of cardiac myocytes.

Study limitations. Since the inclusion criterion for the QGE^{LMNA} in the 311 cases blind to genotype was consecutive enrollment, the series included both patients and healthy relatives. This may be a limitation for the nonindependence of observations.

Conclusions

Both decreased expression of *LMNA* gene and variable loss of lamin A/C immunostain in EMB may contribute to the diagnostic algorithm in cardiolaminopathy. In particular, the reduced mRNA expression levels in blood offers to be a potential biomarker in patients for monitoring of the QGE^{LMNA} in *LMNA* mutation carriers.

Reprint requests and correspondence: Dr. Eloisa Arbustini, Centre for Inherited Cardiovascular Diseases, IRCCS Fondazione Policlinico San Matteo, Piazzale Golgi 19, 27100 Pavia, Italy. E-mail: e.arbustini@smatteo.pv.it.

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Key Words: biomarker ■ cardiolaminopathy ■ immunohistochemistry ■ lamin A/C ■ quantitative gene expressions.

APPENDIX

For an expanded methods and supplementary tables and figure, please see the online version of this paper.