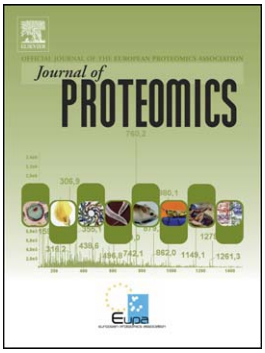

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Proteomic identification of putative biomarkers for early detection of sudden cardiac death in a family with a *LMNA* gene mutation causing dilated cardiomyopathy

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Abstract

Dilated cardiomyopathy (DCM) is a severe heart disease characterized by progressive ventricular dilation and impaired systolic function of the left ventricle. We recently identified a novel pathogenic mutation in the *LMNA* gene in a family affected by DCM showing sudden death background. We now aimed to identify potential biomarkers of disease status, as well as sudden death predictors, in members of this family. We analysed plasma samples from 14 family members carrying the mutation, four of which (with relevant clinical symptoms) were chosen for the proteomic analysis. Plasma samples from these four patients and from four sex- and age-matched healthy controls were processed for their enrichment in low- and medium-abundance proteins (ProteoMiner™) prior to proteomic analysis by 2D-DIGE and MS. 111 spots were found to be differentially regulated between mutation carriers and control groups, 83 of which were successfully identified by MS, corresponding to 41 different ORFs. Some proteins of interest were validated either by turbidimetry or western blot in family members and healthy controls. Actin, alpha-1-antytripsin, clusterin, vitamin-D binding protein and antithrombin-III showed increased levels in plasma from the diseased group. We suggest following these proteins as putative biomarkers for the evaluation of DCM status in *LMNA* mutation carriers.

Keywords: dilated cardiomyopathy, LMNA, sudden cardiac death, plasma biomarkers

Significance

We developed a proteomic analysis of plasma samples from a family showing history of dilated cardiomyopathy caused by a *LMNA* mutation, which may lead to premature death or cardiac transplant. We identified a number of proteins augmented in mutation carriers that could be followed as potential biomarkers for dilated cardiomyopathy on these patients.

1. Introduction

Dilated cardiomyopathy (DCM) is a severe disease of heart muscle characterized by progressive ventricular dilation and impaired systolic function of the left ventricle (LV) [1], although dilatation of both ventricles may also occur [2]. DCM is a major cause of heart failure and sudden cardiac death (SCD), characterized by genetic and clinical heterogeneity [3]. Familial DCM is now estimated to account for close to 50% of all cases of "idiopathic" DCM [4]. Advances in molecular genetics have allowed the identification of the gene defect for some forms of DCM. Genetic biomarkers provide insights into disease susceptibility, supplying no indication of whether or not clinical disease has been developed. Furthermore, imaging biomarkers detect the presence of subclinical cardiac disease but are of little value for characterizing earlier stages, when subclinical disease is not apparent yet. Since 2009, American Heart Failure guidelines include recommendations with regard to genetic counselling and genetic testing in patients and families with certain cardiomyopathies [5]. Circulating biomarkers may be informative either early or late in the disease process, with some of them reflecting activity in biological pathways that precede disease and others triggered by the presence of subclinical cardiovascular disease. In this regard, proteomics is a valuable tool for biomarker discovery. Indeed, in the field of SCD, a recent study – based on 2-DE and MS – identified potential plasma biomarkers in a family with Brugada Syndrome patients sharing the same mutation in the SCN5A gene [6].

In a recent work, we identified a novel pathogenic mutation in the *LMNA* gene in a family affected by DCM showing SCD familial background. This novel mutation induces that, at nucleotide 871 in exon 5 of the *LMNA* gene, the Glutamic acid (Glu-E-) changes to Lysine (Lys -K-) in the first position of the codon [7]. Mutations in the *LMNA* gene may be the most common genetic cause of familial DCM (together with mutations in the *TTN* gene [8]) and their pattern of heritance is primarily autosomal dominant with high penetrance, almost 100% at 60 years old [9].

The natural course of *LMNA*- cardiomyopathy can be aggressive, often leading to premature death or cardiac transplant [9-11]. The high incidence of conduction disturbances and arrhythmogenic events has made cardiologists conscious about this cardiomyopathy. As reported by Morales et al, this

DCM is preceded by an asymptomatic stage followed by a symptomatic one [12], with a progressive temporal concatenation.

LMNA gene encodes for the proteins Lamin A and C, that are intermediate filament proteins present in the nuclear membrane and the nucleoplasm. It has been postulated that stress signals generated in the cytoplasm can be transmitted to the nucleus affecting the chromatin organization and, subsequently, altering gene expression. Thus, it has been hypothesized that a disordered nuclear membrane caused by *LMNA* mutations provokes mechanical instability in the contracting muscle, leading to nuclear damage and eventual myocyte loss; alternatively, it has been also proposed that *LMNA* mutations impairs lamin modulation of gene expression, leading to complex, downstream organ dysfunction [13].

LMNA mutations cause a DCM that can or cannot be accompanied by different skeletal dysfunctions. In our population, we used all the clinical tools at our disposal, and five of our patients have been implanted an ICD (implantable cardiovascular defibrillator).

For all the above reasons, the identification of molecular biomarkers that allowed the follow-up of mutation carriers would be crucial for an early identification of individuals at risk of suffering *LMNA*-DCM and SCD**.**

Thus, based on our previous experience in cardiovascular proteomics [14], in the present study we focused on the above family to complement the genomic study with a proteomic analysis of plasma samples from *LMNA* mutation carriers affected by DCM in comparison to healthy donors. Our goal was to identify putative biomarkers for DCM and early detection of SCD in mutation carriers.

2. Materials and methods

2.1. Clinical presentation and diagnostic methods

The *LMNA* gene mutation was detected in our proband (III-33) and in other 19 members of the same family (**Figure 1).** This family has several members who suffered sudden cardiac death (SCD). We

established genotype-phenotype correlation based on the novel pathogenic mutation in the *LMNA* gene mentioned above [7]. Written informed consent was obtained from all family members and controls included in our study. The study was conducted according to the Declaration of Helsinki and was supported by the Ethics Committee of Puerta del Mar University Hospital, Cádiz, (Spain).

It is important to point out that we could obtain plasma samples from only 14 of the 21 members of the *LMNA* gene mutation family due to subjects' rejection to participate in the study. The other major limitation was the heterogeneity of the cohort of mutation carriers, which is an expected consequence of studying subjects from the same family. To manage this issue, we decided to perform the proteomic analysis in a small homogeneous cohort of 4 patients with established DCM in comparison to healthy controls in order to detect the most consistent differences and then validate them in all family members.

Detailed clinical information was obtained from each subject, including family history, age of presentation, initial symptoms of heart failure, physical examination, serum creatine kinase levels, electrocardiogram (ECG), transthoracic echocardiogram (ECC), Holter monitoring, and treadmill testing. Those patients who showed any impairment in the ECC underwent a cardiac-MRI. Routine 2D-ECC, Doppler and Tissue Doppler Imaging (TDI) was performed according to the standards of the American Society of Echocardiography [15,16].

2.2. Sample collection

We selected 14 patients carrying the *LMNA* mutation (see **Figure 1** and **Table 1**), including several children. Due to biological variability in terms of age and clinical symptoms, we selected a homogenous group of four family members presenting relevant clinical symptoms for the proteomic analysis. A healthy sex- and age-matched control group was chosen for the proteomic study.

Blood samples were collected under fasting conditions in k_2EDTA-containing tubes. Plasma was obtained by centrifugation at 800 g for 10 min. Samples were aliquot, snap frozen in liquid nitrogen and stored at -80 º C until further analysis.

2.3. ProteoMiner™ enrichment and protein quantification

Plasma samples to be analysed by proteomics (III-33, III-35, III-27, III-13 and four sex- and age-matched healthy controls) were processed for the enrichment in medium- and low-abundance proteins. One millilitre of each plasma sample was pre-fractioned using a bead-based library of combinatorial peptide ligands (ProteoMiner™ kit, Bio-Rad Laboratories) and eluted in 200µl of DIGE buffer (65 mM CHAPS, 5 M urea, 2 M thiourea, 0.15 M NDSB-256, 30 mM Tris, 1 mM sodium vanadate, 0.1 mM sodium fluoride, and 1 mM benzamidine).

Protein sample concentration was determined with the Coomassie plus reagent (Thermo Scientific, Asheville, NC) according to the Bradford method protocol. Equal amounts of protein were pooled for each condition (DCM patients and healthy donors).

2.4. 2D-DIGE

Five gels (technical replicates) were run with a total of 150 µg of mixed labelled protein per gel. These protein mixtures contained 50 µg of protein from each sample group (pool of 4 patients and pool of 4 healthy donors) randomly labelled with 400 pmol minimal CyDye DIGE fluors (Cy3 or Cy5) and 50 µg of the two pools mix (25 µg from patients´ pool and 25 µg from healthy donors´ pool) labelled with 400 pmol Cy2.

Labelling was performed for 30 min on ice in the dark. The reaction was stopped with 1 µl of 10 mM lysine acting for 10 min on ice in the dark. After labelling step, the three samples were pooled and an equal volume of 2X sample buffer was added (65 mM CHAPS, 2 M thiourea, 5 M urea, 0.15 M NDSB-256, 130 mM DTT, 4 mM tributylphosphine, 1 mM sodium vanadate, 0.1 mM sodium fluoride, and 1 mM benzamidine). After mixing, the tube was left for 15 min on ice in the dark. Before reswelling of immobilized pH gradient (IPG) strips, samples were diluted in 2D Sample buffer (5 M urea, 2 M thiourea, 2 mM tributylphosphine, 65 mM DTT, 65 mM CHAPS, 0.15 M NDSB-256, 1 mM sodium vanadate, 0.1 mM sodium fluoride, and 1 mM benzamidine) to a final volume of 500 µl, and ampholytes (Servalyt 4–7) were added to a final concentration of 1.6% (v/v).

IPG strips were rehydrated with the samples for 16h in the dark. Isoelectric focusing (IEF) was run on 24 cm, pH 4–7 IPG strips (GE Healthcare) powered by a Multiphor II (GE Healthcare) for 64.9 kVh at 17°C. Following focusing, IPG strips were immediately equilibrated for 15 min in reduction buffer (6 M urea, 50 mM tris pH 8.8, 30% glycerol, 2% w/v SDS, 65 mM DTT and traces of bromophenol blue) and then for 15 min in alkylation buffer (6 M urea, 50 mM tris pH 8.8, 30% glycerol, 2% w/v SDS, 135 mM iodoacetamide and traces of bromophenol blue) with gentle agitation; all steps in the dark. IPG strips were washed out with ultrapure water and placed on top of the second dimension gels, embedded with 0.5% melted agarose.

Second dimension protein separation was based on SDS-polyacrylamide gel electrophoresis (PAGE). Five 11% polyacrylamide gels were run at 10°C (20 mA per gel for 1 h, followed by 40 mA per gel for 4 h) by using an Ettan Dalt 6 system (GE Healthcare). Following electrophoresis gels were scanned directly in a Typhoon FLA 7000 (GE Healthcare).

2.5. Differential image analysis

Gels images were processed with Progenesis SameSpots (v 4.5) from Nonlinear Dynamics Ltd. (Newcastle, UK) in order to find real differences between both conditions of study. Both manual and automatic alignments were used to align the images. All gels were compared with each other and fold values as well as p-values of all spots were calculated by SamesSpots software using one way ANOVA analysis. Differential expression of a protein present in the gels was considered significant when the fold change was at least 2 and the p value was below 0.05.

2.6. MS analysis

Differentially regulated spots were excised manually from the gels and in-gel digested with trypsin following the protocol defined by Shevchenko et al [17]. Identifications were by MALDI-TOF/TOF, in a 4800 Plus MALDI-TOF/TOF analyser (Applied Biosystems), and by LC–MS/MS on an EASY-nLC (Proxeon, Bruker Daltonik) and a Bruker Amazon ETD ion trap. MALDI-MS and MS/MS data were combined through the GPS Explorer Software Version 3.6 to search a non-redundant protein database (Swissprot 2014_02) using the Mascot software version 2.1 (Matrix Science). For LC-

MS/MS, database search was performed with the Mascot v2.3 search tool (Matrix Science, London, UK) screening SwissProt (release version 2014_10). Further information on MS analysis can be found in Supplementary methods.

2.7. Interaction network analysis

STRING v10 software [18] was used to investigate possible interactions among all the identified proteins. Interactive pathways were generated to observe potential direct and indirect relations among the differentially regulated proteins.

2.8. Western blotting

1D-western blotting was performed to analyse individual samples of either ProteoMiner enriched-plasma (4 patients and 4 healthy controls) or crude plasma (14 mutation carriers and 7 healthy donors). 1D-western blotting on depleted plasma samples was carried out due to the potential interference of the high-abundant plasma IgGs in the weight range of the validated proteins. 1D-western blotting was used for validating actin in crude plasma from all mutation carriers, owing to the divergence between actin and IgGs' molecular weights.

For 1D-western blotting of ProteoMiner enriched- plasma samples, 10 µg of protein dissolved in Laemmli sample buffer (2% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol, 25mM Tris, pH 6.8) were loaded per lane in a 11% SDS-PAGE gel. For 1D-western blotting of crude plasma samples, 0.5µl of plasma dissolved in Laemmli sample buffer were loaded per lane on 10% SDS-PAGE gels (this volume corresponds approximately to 25 µg of protein).

Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare) according to the manufacturer protocol. For 1D-western blotting of crude plasma samples, the membranes were blocked in 5% skim milk powder in TBS-T (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20) for 1h at room temperature and incubated overnight at 4°C with mouse anti- actin antibody (MAB 1501, Merck Millipore Ltd. Tullagreen, Carrigtwohill Co. CORK IRL) dilution 1:1000. For 1D-western blotting of depleted- plasma samples, the membranes

were blocked in 5% BSA in TBS-T (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20) overnight at 4°C and incubated for 90 min at room temperature with the following Santa Cruz Biotechnology, Inc. (Delaware, CA, USA) antibodies: mouse anti- clusterin (sc-166907) dilution 1:1000 or mouse anti- vitamin D-binding protein (sc-365441) dilution 1:1000.

For 2D western blotting, 25µg of each depleted- plasma pool (patients and healthy donors) were loaded on 7cm, pH 4-7 IPG strips and proteins were separated first by IEF and secondly by SDS-PAGE in a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). The membranes were blocked in 5% BSA in TBS-T (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20) overnight at 4°C and incubated for 90 min at room temperature with the following Santa Cruz Biotechnology, Inc. (Delaware, CA, USA) antibodies: mouse anti- α1 antitrypsin (sc-59438) dilution 1:1000; mouse anti-clusterin (sc-166907) dilution 1:1000 and mouse anti- vitamin D-binding protein (sc-365441) dilution 1:1000.

Following washes in TBS-T, membranes were exposed to horseradish peroxidase labelled goat anti- mouse antibody 31430 (Pierce, Rockford, IL) dilution 1:5000. Membranes were washed again and processed using an enhanced chemiluminiscence system (ECL, Pierce, Rockford, USA).

For 1D-western blotting, protein bands intensity was quantified by densitometry using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.47. Statistical analysis was performed with GraphPad Software, Inc. (La Jolla, CA, USA) comparing the data by the Mann-Whitney test. Associations between actin levels and clinical parameters were evaluated using univariate linear regression. A post hoc power analysis was carried out at an alpha level of 0.05, two tailed.

2.9. Turbidimetry

Antithrombin-III and Alpha-1-antitrypsin were validated by a turbidimetric assay using a BS200E analyser (Mindray Bio-Medical Electronics Co., Ltd.).

Previously to sample processing, calibration and quality control of the equipment was performed with Prot Cal and Prot Control reactives (Spinreact, Girona, Spain). Crude plasma samples from 14 mutation carriers and 6 healthy donors were analysed with the quantitative turbidimetric tests ATROM-III and α1-ATRYP (Spinreact, Girona, Spain) according to the manufacturer protocol. Results data are expressed as the median and the standard deviation, and were compared by Mann-Whitney test using GraphPad Software, Inc. (La Jolla, CA, USA). Associations between protein levels and clinical parameters were evaluated using univariate linear regression. A post hoc power analysis was carried out at an alpha level of 0.05, two tailed.

3. Results

3.1. Clinical characteristics of patients

Out of the 14 family members carrying the *LMNA* mutation, we selected the four most symptomatic with DCM (III-13, III-27, III-33 and III-35) for proteomic analysis **(Figure 1, Table 1**). Plasma samples of four sex- and age- matched healthy controls were also collected.

It has been established that DCM is characterized by depressed ejection faction (EF), considered lower than 50%, and lightly dilated and rounded left ventricle (LV). The alteration of diastolic pattern precedes the left ventricle dysfunction in these patients. As *LMNA*-DCM is very arrhythmogenic and most of SCD are due to malignant arrhythmias, the presence of ICD means the progression of the disease. Symptomatic patients have been treated with angiotensin-converting-enzyme inhibitors and beta-blockers, as Clinical Guidelines suggest.

The first patient included in the proteomic study (III-33) is a 34-year-old man, clinically symptomatic. He showed a depressed left ventricular ejection fraction (LVEF). The LV was rounded-shape and slightly dilated, as shown by ECC. He was implanted a defibrillator (ICD). One of his sisters (III-27) is a 43 years-old lady, who presented at the time several episodes of both syncope and palpitations. She was implanted an ICD because of some syncope episodes. Her LV size was slightly

increased and systolic function was lightly decreased according to transthoracic ECC, tissue Doppler and cardiac–MR. Coronary artery disease was ruled out as the cause of the arrhythmia. III-35, their 35 years-old sister, suffered from both palpitations and occasionally shortness of breath. She showed a depressed systolic function by ECC and diastolic pattern type I. This information was confirmed by cardiac-MR. A patients´ cousin, III-13, is a 43 years old woman, who suffered an episode of syncope. The ECC showed a rounded-shape and slightly dilated LV confirmed by cardiac-MR. She also showed a lightly depressed ejection fraction (48%). Nowadays, the patient III-35 and III-13 have been implanted an ICD. All patients´ and mutation carriers' data are shown in **Table 1.**

3.2. Differential proteomic analysis

Plasma samples from patients with DCM- and *LMNA* mutation- and healthy controls, with no mutation, were compared (patient and control groups). Firstly, we checked the enrichment efficiency of the ProteoMiner^{TM} approach by running 10 µg of protein sample before and after ProteoMiner processing, as well as column flow-through, in a 10% SDS-PAGE gel. After staining the gel with Sypro Ruby, we could demonstrate the method was efficient to achieve enrichment on proteins of low and medium abundance (**Supplementary Figure 1**).

All samples were processed and grouped in the DCM patients or healthy control group. Following the differential 2D-DIGE-based proteomic analysis, 111 protein spots were identified as differentially regulated between patients and control groups (fold change cut-off ≥ 2; $p < 0.01$) (**Figure 2, Supplementary data**). From those, 71 were up-regulated in patients whereas 40 were down-regulated. A principal components analysis (PCA) showed a very good separation between groups based on the proteome profile (not shown). We could successfully identify 83 protein features by MS which corresponded to 41 different ORFs, 33 of which were up-regulated in patients´ samples (**Table 2; Supplementary Table 1)**. Fifty-six per cent of the proteins identified are known to be secreted by a classical pathway (i.e. have a signal peptide), whereas 32% are known to be secreted by non-classical pathways, such as microvesicles or exosomes (data obtained from SecretomeP 2.0 [19] and UniProt [20] servers).

Regarding protein function, 14 of the identified proteins play a central role in the activation of the complement system and are involved in inflammatory response (e.g. C4b-binding protein alpha chain and complement factor H, Alpha-1-antitrypsin, fetuin A). Other important group represented includes proteins essential for the maintenance of haemostasis and platelet activation, blood coagulation, and thrombus formation (e.g. antithrombin-III, fibrinogen, prothrombin). Besides the above, another classical pathway secreted protein identified is clusterin, an ubiquitous anti-apoptotic protein involved in oxidative-stress protection. Regarding proteins secreted by a non-classical pathway, the main group corresponds to essential components of the cytoskeleton and cell signalling proteins (e.g. actin isoforms or vitamin-D binding protein).

We chose for validation studies five proteins related to cardiovascular events that were up-regulated in the DCM group: actin, vitamin-D binding protein (VTDB), alpha-1-antytripsin (A1AT), clusterin (CLUS), and antithrombin-III (ANT3).

3.3. Actin, VTDB, A1AT, CLUS and ANT3 are augmented in plasma from *LMNA* mutation carriers.

Some of the identified proteins were validated due to its potential as cardiovascular disease overcome predictors. We chose actin (the most important cytoskeleton component), A1AT (as an inflammation marker), CLUS (a protective protein), VTDB (involved in haemostasis and inflammation) and ANT3 (essential for haemostasis maintenance). Regarding the validation strategy, we combined western blotting and turbidimetry. The latter was done in individual crude plasma whereas for western blotting crude plasma was only used for validations in those cases where there was no interference of high abundant proteins; otherwise, depleted samples were used.

CLUS and VTDB were validated by 1D-Western Blotting on depleted- plasma samples from the 4 patients and 4 healthy controls included in the proteomic study, confirming an increased presence of these proteins in patients' samples (**Figure 3A and 3B)**. 1D-SDS-PAGE western blotting on crude plasma samples was discarded due to IgG chains interference on the proteins weight range. Additionally, a quadruplicate of depleted plasma pools of the two conditions was run, showing

augmented levels of CLUS, VTDB and A1AT in patients´ samples in line with the proteomics data (**Figure 3C)**.

Antithrombin-III (ANT3) and Alpha-1-antitrypsin (A1AT) were validated by turbidimetry in crude plasma samples from the 14 *LMNA*- mutation carriers and 6 healthy controls. This method is widely used in clinics but a limited number of commercial kits are available. ATROM-III test showed a significant elevation of 21.2 % in ANT3 levels in mutation carriers when compared to control samples (**Figure 4A**) and α1-ATRYP test showed an elevation tendency of A1AT in patients´ samples although no statistical significance was found (**Figure 4B**).

Actin validation by 1D-Western Blotting on individual crude plasma samples showed a significant elevation of this protein in samples from mutation carriers when compared to healthy controls, with high degree of variability among the former (**Figure 5**).

From all the clinical parameters available, we selected the most relevant for determining the disease status and examined its possible correlation with plasma levels of the putative identified biomarkers. Interestingly, a linear regression analysis of each clinical parameter performed on all mutation carriers showed a positive relation between highest plasma levels of actin and pathological values of LVEF, ($F(1,9) = 9.57$, p value = 0.019). No further associations were seen between actin levels and the other clinical parameters analysed, and no associations were found between clinical parameters and the other validated proteins either. Moreover, the statistical analysis showed no association between the pharmacological treatment of the patients and plasma levels of the validated proteins.

3.4. Interaction network analysis

String software was used to investigate possible interactions between all the proteins identified in order to highlight predominant networks, pathways, and connections to pathophysiological processes. Lamin A was included in the protein list in order to find potential connections between the differential

regulated proteins and the primary mutation. Interestingly, 21 proteins are connected in a network related to response to stress, 12 are involved in a haemostasis network and 7 in a negative regulation of programmed cell death network (**Figure 6**).

4. Discussion

We have performed an extensive proteomic analysis of plasma samples from members of a family carrying a novel mutation in the *LMNA* gene we recently characterized [7]. This study led to the identification of relevant proteins with potential interest for being followed in these family members affected by the mutation with the aim of evaluating the risk of suffering DCM and SCD.

The total number of mutation carriers included in the study is low for biomarker discovery studies but is restricted by the number of family members. Regarding the proteomic analysis, a homogenous group of 4 symptomatic mutation carriers with DCM were selected in order to increase the chances of identifying early predictors of disease progression. Interestingly, several recent cardiopathies' studies, dealing with no so prevalent pathologies, also used small cohorts of patients for the proteomic analysis [6,21].

The 20 most abundant proteins in plasma represent about 98% of the total protein content, indicating the broad concentration range of proteins in plasma, calculated in approximately 11 orders of magnitude. The challenge of covering this order of magnitude in protein detection highlights the need of depletioning high-abundance proteins to reduce complexity and dynamic range of proteins before further analysis of samples. ProteoMiner™ was used to enrich medium- and low-abundance proteome getting thus rid of the noise-generating highly present proteins (e.g. albumin, transferrin). ProteoMiner™ technology uses a combinatorial library of hexapeptides bound to a chromatographic support that act as unique binders to proteins, effectively concentrating the low-abundance proteins.

Several proteomic analyses of plasma, applied to biomarker discovery in cardiovascular disease, made use of this technology for sample preparation [22].

After the proteomic analysis, a total of 83 differentially regulated protein spots were successfully identified but they came from only 41 ORFs, highlighting the relevance of post-translational modifications for many of these proteins. Most proteins identified are related to inflammatory response and haemostasis maintenance. Further, many of them could be located in networks related to response to stress and negative regulation of programmed cell death, as shown in **Figure 6**. The majority of identified proteins have a signal peptide, as expected; however, an important proportion of proteins secreted by non-classical pathways were also identified.

Besides Western Blot, turbidimetry was used as a valuable technique for results validation. Immunoturbidimetric analyses have become clinical routine tests for determining protein levels in plasma throughout the last decades. Moreover, this technique has been also successfully applied for validating the results of plasma proteomic studies recently [23].

In general, there was a good correlation between proteomic and validation data for those proteins selected, independently of the method used and if the plasma was crude or depleted. All validated proteins (actin, VTDB, A1AT, CLUS, and ANT3) are related to cardiovascular events and were found to be altered in different pathological conditions, as indicated below.

Cardiac hypertrophy leads to augmented protein synthesis in the myocardium; this partly occurs as a consequence of fetal transcription control reactivation [24,25]. Interestingly, genetic expression of α- skeletal actin (an actin isoform highly present in cardiac tissue) was found elevated in cardiac hypertrophy [26,27]. This protein, as well as other cytoskeleton components, is known to cooperate in early stress generation and in intracellular hypertrophic signal transduction [28]. Despite the low power of the biostatistical analysis, owing to the limited number of patients available for the study, we found an association between actin highest levels on plasma and pathological values of FEVI, indicating the potential usefulness of actin in the follow-up of these patients. Further analyses with larger number of patients, from different families, would be needed to develop regression analyses with more statistical power. Actin presence in extracellular space can be a consequence of

stress-induced microparticles secretion by the cardiomyocytes [29] or a consequence of liberation of intracellular components after cell death.

Vitamin-D binding protein (VTDB) is a plasmatic protein related to coagulation and thrombus formation that plays a role in the inflammatory response [30,31]. VTDB also acts as an extracellular actin scavenger system [32]. On the other hand, the serine protease inhibitor alpha-1-antitrypsin (A1AT) is an acute phase response (APR) protein that has been found to exhibit anti-inflammatory effects against TNF-α [33]. Interestingly, increased plasma levels of VTDB and A1AT have been associated to cardiovascular death [34].

Secreted clusterin (sCLU) acts as a chaperone for misfolded proteins and its expression is increased in the myocardium of the infarcted heart [35,36]. Reactive oxygen species (ROS) generation plays a central role in cardiac apoptotic cell death due to ischemia and reperfusion [37] and clusterin exerts cardioprotection against this oxidative stress. The activation of Akt/GSK-3β signalling pathway plays an important role in mediating this anti-apoptotic effect [38].

Finally, Antithrombin-III (ANT3) acts as a physiological thrombin inhibitor. Elevated levels of thrombin-antithrombin III complex were found in patients showing hypertrophic cardiomyopathy and dilated cardiomyopathy in comparison to healthy individuals [39,40]. Thrombin-antithrombin (TAT) complex is also thought to be a marker of thrombin generation [41] and it has been reported that high levels of thrombin generation are associated with unfavourable outcome in cardiovascular disease [42].

We performed an exhaustive statistical analysis of all the validated proteins with the aim of detecting augmented levels of these proteins in the group of symptomatic individuals in comparison to asymptomatic mutation carriers, trying to associate these levels with clinical parameters. However, this association was only found in the case of actin, whose highest levels show association with pathological FEVI values. We aim to perform further studies in the future, including a bigger cohort of symptomatic and asymptomatic mutation carriers (from the same or different families presenting the

LMNA mutation), in order to confirm our results and with the purpose of finding an association between the identified putative biomarkers and the clinical status of the patients.

The *LMNA* mutation triggers an abnormal chromatin organization in the nucleus. The mechanism by which this leads to this type of cardiomyopathy is not known yet. In this study our aim was to identify potential plasma biomarkers which may help to identify symptomatic individuals at risk of suffering DCM or sudden cardiac death. In plasma we are able to detect secreted proteins, which are not necessarily directly connected with the nucleus of the cell. The association of these proteins to cardiovascular events illustrates their potential as predictors of possible negative cardiovascular outcomes. For determining the relation between this putative biomarkers and the *LMNA* mutation a comprehensive molecular study should be performed in the future.

In conclusion, we have developed a complex proteomic analysis of plasma samples from a family diagnosed with DCM caused by a *LMNA* mutation, in comparison with plasma from healthy controls with no mutation. We identified a number of proteins augmented in the diseased group that could be followed as putative biomarkers for the evaluation of *LMNA*-DCM status. However, a follow-up study of the still asymptomatic mutation carriers of the studied family, and further analysis in other families diagnosed with *LMNA*-DCM should be performed in order to prove the validity of such biomarker candidates. We believe the present study opens a new line of investigation for this relevant cardiovascular disease.

Study limitations

The present study has some limitations that should be considered. Firstly, it was a challenge to access all members of the family for sample collection; besides, the amount of blood donated was limited so we could not combine several proteomic approaches and validation methods. The number of family members was low for a typical biomarker discovery study so results should be taken cautiously and validated in the future in larger cohorts of patients when other families with the same or similar mutations are described and accessible. Besides the latter, in the future we also plan to extend our

validation and follow-up studies by collecting further samples from the same family members. In line with the above, shot gun approaches and other depleted methods would have probably provided complementary data and this is something to consider for the future.

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The authors declare no conflict of interest.

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Figure Legends

Figure 1. Pedigree of the studied family. Generations are indicated in the left column and all individuals are identified with a pedigree number. The proband is indicated by an arrow (III-33). *LMNA* mutation carriers are showed in black/grey round/square, and relatives without mutation are indicated with white round/square. The crossed individuals are deceased patients. Grey round/square marked individuals are mutation-carriers included in our study. +: clinically symptomatic patients. Males are marked with a square and female with a circle. (Modified from Pérez-Serra et al. 2015 [7]).

Figure 2. High-resolution 2D-DIGE-based differential proteomic analysis of depleted plasma from DCM patients carrying the *LMNA* mutation versus healthy donors. (**A**) Representative image of the 2D-DIGE analysis where the fluorescence emission from Cy3 and Cy5 dyes is superimposed. Red-orange colour spots are augmented in the patients group whereas green colour spots are augmented in healthy donor samples. (**B**) Representative image of the analysis in a grey scale. Differentially regulated spots are indicated. Further information about protein identifications can be found in Table 2 and Suppl. Table 1.

Figure 3. **CLUS, VTDB and A1AT levels are augmented in plasma from DCM patients carrying the *LMNA* mutation**. 1D-Western Blot images and densitometry graph representing the mean values

± SD of (**A**) CLUS and (**B**) VTDB in depleted- plasma samples from DCM patients and healthy matched- controls (*p<0.05). (**C**) Representative 2D-Western Blot images of the quadruplicate run for determining A1AT, VTDB and CLUS levels on pools of depleted plasma samples from DCM patients and healthy matched- controls. DCM: patients presenting dilated cardiomyopathy; C: healthy matched- controls; IB: Inmunoblot.

Figure 4. ANT3 and A1AT levels are increased in plasma samples from *LMNA* mutation carriers. (**A**) ANT3 and (**B**) A1AT concentration measurement by ATROM-III and α1-ATRYP turbidimetry quantitative tests respectively, where ‘’MC’’ means mutation carriers and ‘’C’’ healthy non-mutation carrier controls (N_{MC} =14 and N_C =6). Samples were analysed in duplicate. Results are presented as mean ± SD. **p<0.01.

Figure 5. **Actin is up-regulated in plasma from *LMNA* mutation carriers**. (**A**) Representative 1D-western blot images. (**B**) Densitometry graph representing the mean values ± SD of actin band intensities for all samples analysed (N_{MC}=14; N_C=7). MC, mutation carriers; C, healthy non-mutation carriers controls; all samples were analysed in duplicate. *p<0.05. IB: Inmunoblot

Figure 6. **STRING Interaction network analysis**. In grey, general interaction network of all proteins identified; in blue, protein network related to response to stress; in orange, protein network related to haemostasis and, in green, protein network related to negative regulation of programmed cell death.

TABLES

Table 1. Most relevant clinical outcomes of the family members included in the study.

Generation	Age/ Gender	LVEF<50%	Diastolic function impairment	ICD	ACEI	BB
II-7	60/♂	-	+	-	+	+
III-11	45/♀	-	-	-	-	-
III-13	43/♀	+	+	+	+	+
III-27	43/♀	+	+	+	+	+
III-33	34/♂	+	+	+	+	+
III-35	35/♀	+	+	+	+	+
III-39	25/♀	+	+	+	+	-
III-45	24/♀	-	-	-	-	-
IV-50	24/♀	-	-	-	-	-
IV-53	9/♀	-	-	-	-	-
IV-54	5/♂	-	-	-	-	-
IV-70	7/♂	-	-	-	-	-
IV-72	3/♀	-	-	-	-	-
V-77	1/♀	-	-	-	-	-

Table key: LVEF (%) left ventricle fraction ejection; ICD: implantable cardiovascular defibrillator; ACEI: Angiotensin-converting-enzyme inhibitor; BB: Beta blockers

"+" sign means the presence of the characteristic and "-" sign means its absence.

Table 2. 2D-DIGE analysis: List of proteins identified in spots that vary when comparing depleted plasma samples from DCM patients carrying the *LMNA* mutation and healthy donors. All variant protein spots have a fold change cut-off ≥2 and $p<0.05$. Red font numbers correspond to spots with increased intensity in patients´ depleted plasma whereas blue font numbers correspond to spots increased in the healthy controls group. Further information can be found in Supplementary Table 1.

Protein	UniProt Code	Spot No
Alpha-1-antitrypsin	A1AT_HUMAN	1129
Actin, aortic smooth muscle	ACTA_HUMAN	1343
Actin, cytoplasmic 1	ACTB_HUMAN	1347
Beta-actin-like protein 2	ACTBL_HUMAN	1347
Putative beta-actin-like protein 3	ACTBM_HUMAN	1347, 1348
Actin, alpha cardiac muscle 1	ACTC_HUMAN	1348
Actin, cytoplasmic 2	ACTG_HUMAN	1347, 1348
Actin, gamma-enteric smooth muscle	ACTH_HUMAN	1348
Alpha-actinin-1	ACTN1_HUMAN	705
Actin, alpha skeletal muscle	ACTS_HUMAN	1348
Serum albumin precursor	ALBU_HUMAN	1990
Antithrombin-III	ANT3_HUMAN	1067
Apolipoprotein A-I	APOA1_HUMAN	872, 873, 1894, 1900
Apolipoprotein A-IV	APOA4_HUMAN	1362
Apolipoprotein E	APOE_HUMAN	1567, 1612, 1649, 2025
Apolipoprotein L1	APOL1_HUMAN	1412
C4b-binding protein alpha chain	C4BPA_HUMAN	882, 883, 889, 891, 894
Complement factor H	CFAH_HUMAN	1367
Clusterin	CLUS_HUMAN	1507, 1512, 1539, 1541, 1542, 1550, 1561, 1981, 1983, 1989, 2019
Coagulation factor XIII B chain	F13B_HUMAN	761, 2034
Protein FAM176C	F176C_HUMAN	1550
EGF-containing fibulin-like extracellular matrix protein 1	FBLN3_HUMAN	1367
Ficolin-2	FCN2_HUMAN	1523, 1534, 1537, 1538
Alpha-2-HS-glycoprotein	FETUA_HUMAN	2032
Complement factor H-related protein 1	FHR1_HUMAN	1335, 1414
Complement factor H-related protein 5	FHR5_HUMAN	887
Fibrinogen alpha chain	FIBA_HUMAN	1990
Fibrinogen beta chain	FIBB_HUMAN	1119, 1122, 1123, 1144 1147, 1148, 1234, 1973 1976, 2031
Fibrinogen gamma chain	FIBG_HUMAN	670, 673, 676, 677, 679, 683, 684, 705, 1212, 1219, 1259, 1322, 1325, 1328
Histidine-rich glycoprotein	HRG_HUMAN	1498, 1499, 1508, 1514
Ig gamma-3 chain C region	IGHG3_HUMAN	1990
Ig kappa chain C region	IGKC_HUMAN	1786
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_HUMAN	1649
Ribosomal protein S6 kinase alpha-3	KS6A3_HUMAN	1134
Ig kappa chain V-II region Cum	KV201_HUMAN	1799
Ig kappa chain V-IV region Len	KV402_HUMAN	2011
Ig lambda-1 chain C regions	LAC1_HUMAN	1726
Ig lambda-7 chain C region	LAC7_HUMAN	1704
Protein midA homolog, mitochondrial	MIDA_HUMAN	1992, 1993
Prothrombin	THRB_HUMAN	1521
Vitamin D-binding protein	VTDB_HUMAN	1137, 1139, 1987

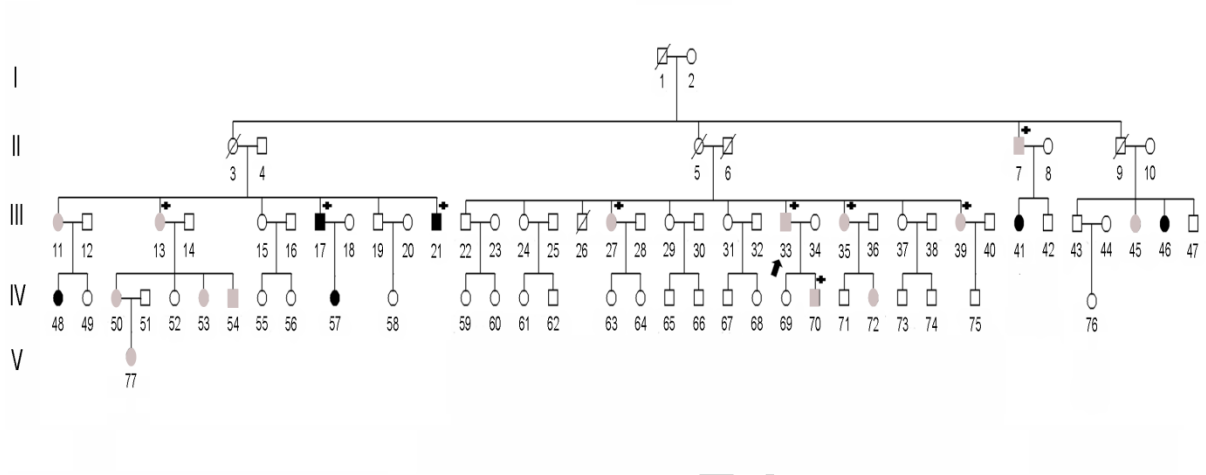

Figure 1

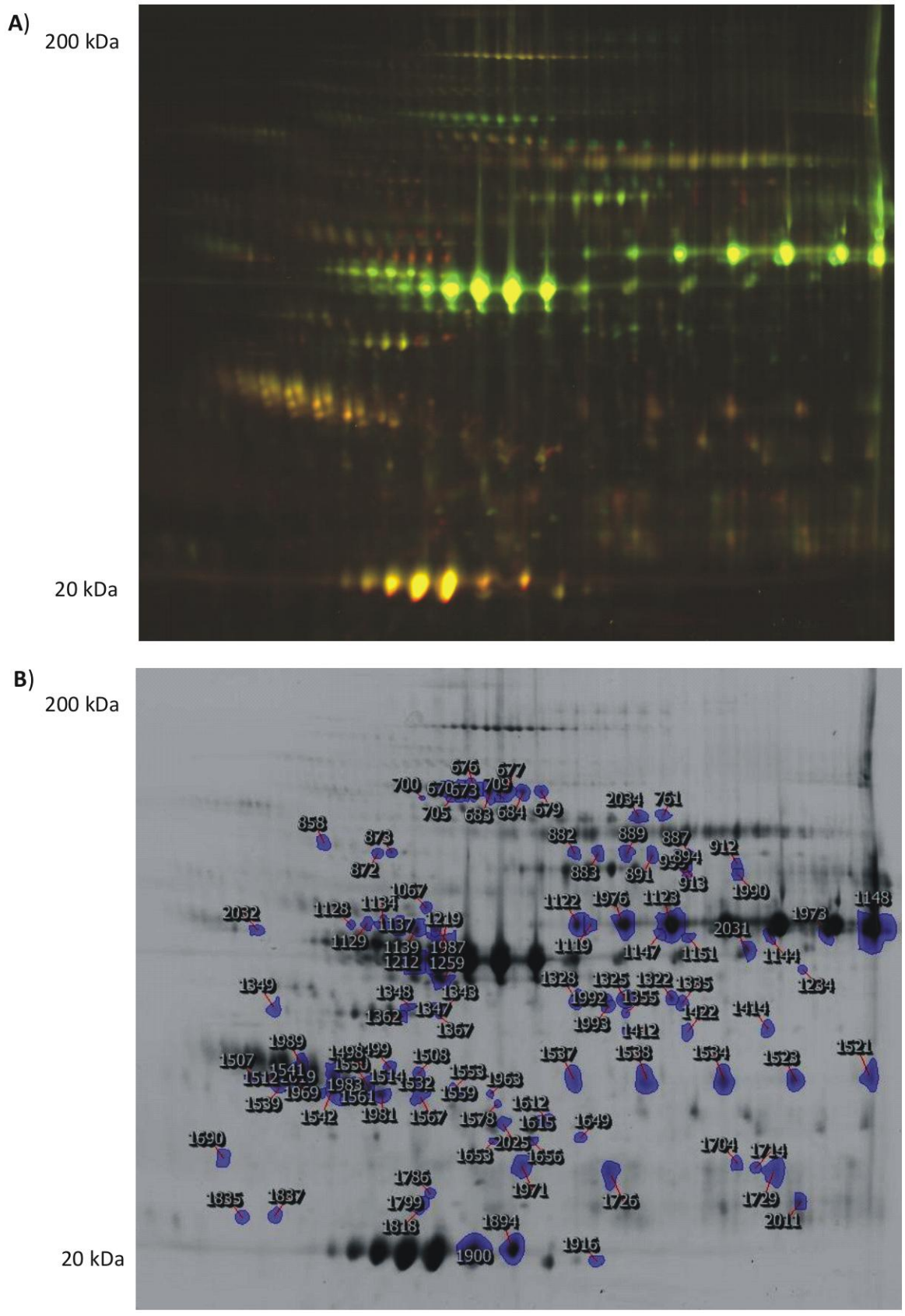
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Figure 2

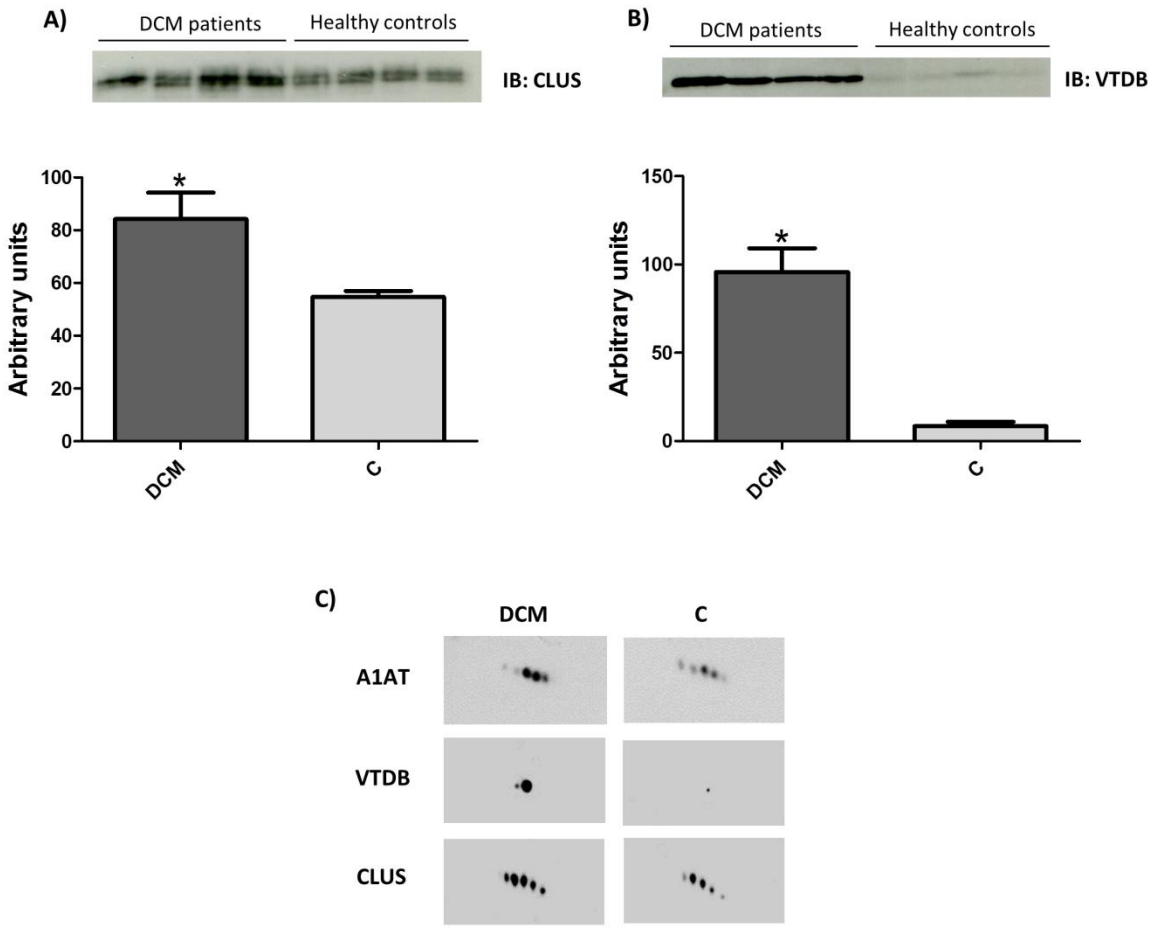


Figure 3

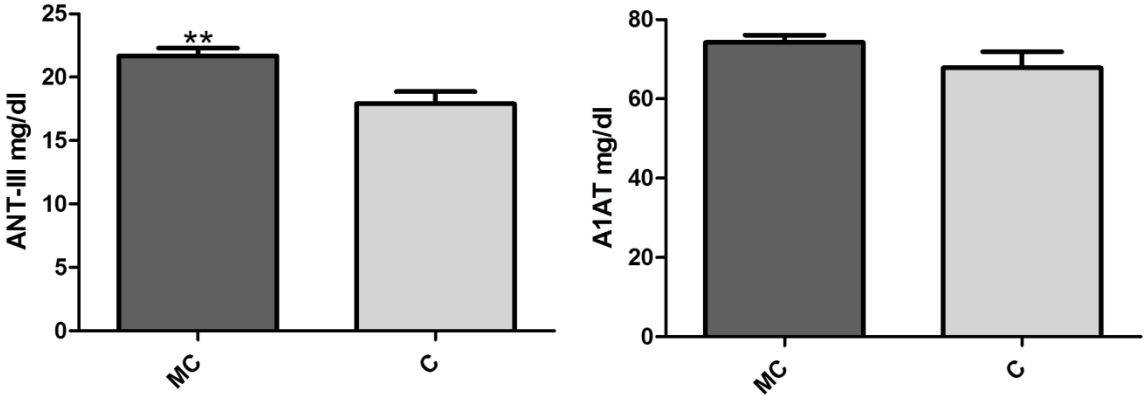


Figure 4

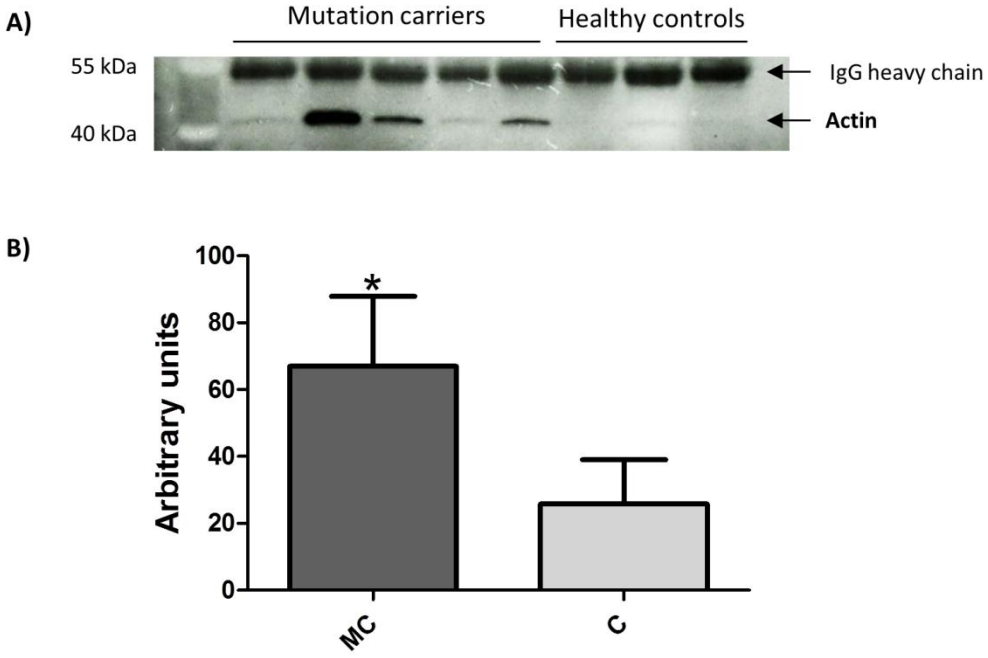


Figure 5

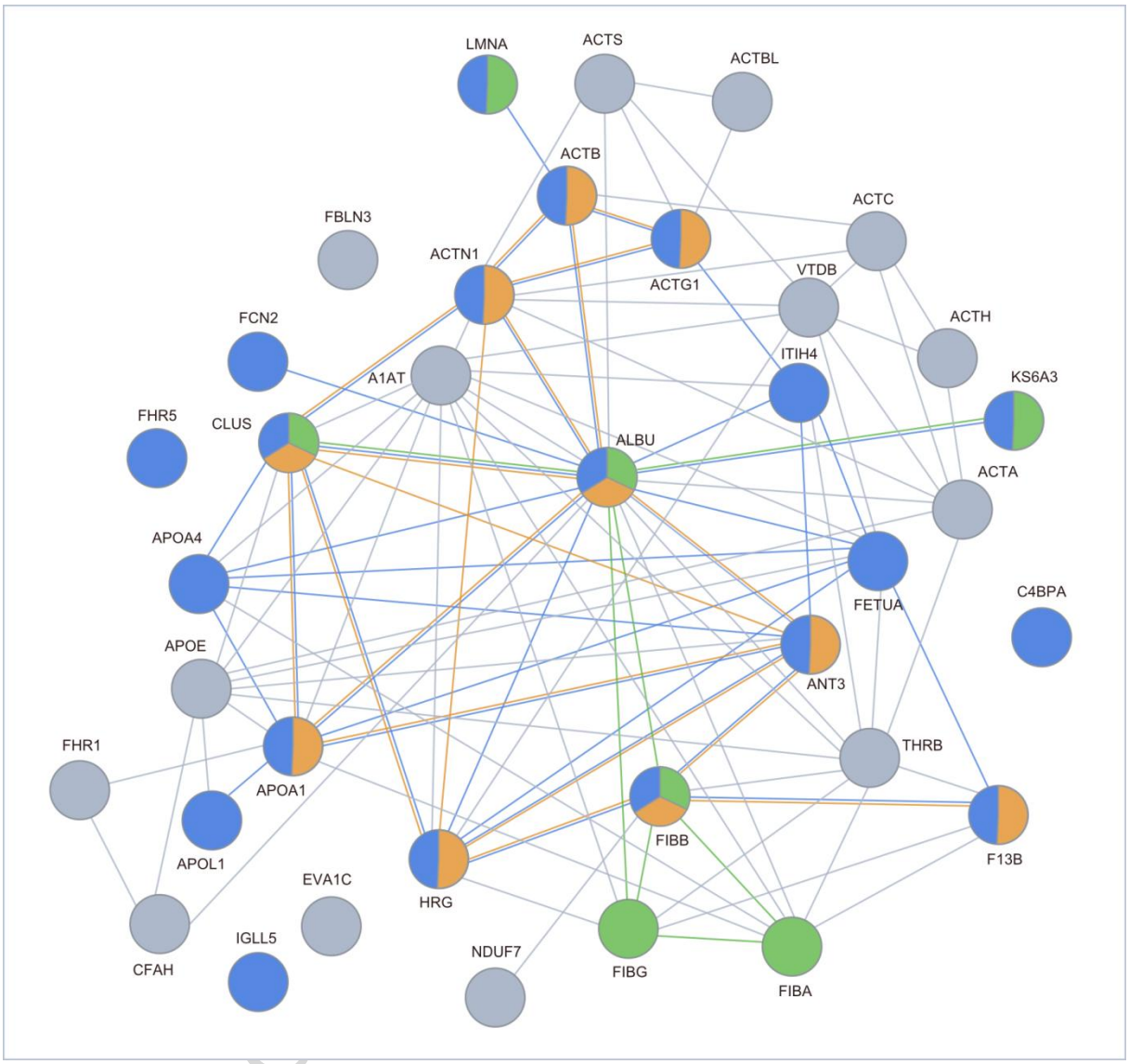
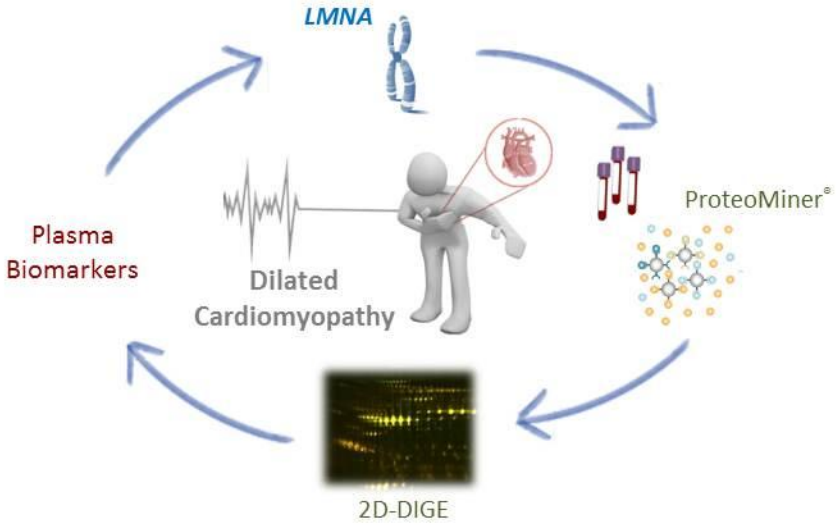


Figure 6

Graphical abstract



Highlights

- A proteomic analysis of plasma samples from members of a family with a recently described *LMNA* mutation, leading to dilated cardiomyopathy and sudden death, was performed.
- A number of cardiovascular-related proteins were found to be augmented in patients´ plasma.
- We propose these proteins as putative biomarkers of disease status in members of this family. Although the number of samples is limited, we believe the present study opens a new line of investigation for this relevant cardiovascular disease