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Cardiomyopathy and aging integrally contribute to the unfolded protein response collective pathways

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ABSTRACT

Proteins are essential elements controlling cellular processes. Their synthesis and assembly are vital to the cell as their defect would have deleterious consequences. A finely tuned conserved biological machinery known as the protein quality control (PQC) is in place to correct the defect. The PQC includes the unfolded protein response (UPR), devoted to recognizing, unfolding and refolding abnormally arranged proteins, and the clearance apparatuses composed of the Ubiquitin Proteasome System (UPS) and the Endoplasmic Reticulum Associated Protein Degradation (ERAD). Abnormally folded proteins accumulate in idiopathic dilated cardiomyopathy (iDCM). In this study we investigated the transcriptional and translational landscapes of the UPR and UPS systems using polymerase chain reaction (PCR) and western blotting (WB) of myocardial tissues from iDCM patients and age, ethnicity and biological sex matched control cases.

Our results show an increase of the three main UPR axis at the transcription and translational levels, suggesting an activation/inactivation of all axes, with altered PTM/cleavage/splicing eliciting abnormal downstream function. Notably, aging independently affects this machinery in diseased and control individuals. In addition, mutation in presenilin gene associated with Alzheimer's disease led to post-translational changes of the UPR components suggesting that genetic risk may exacerbate the natural age and disease-driven protein dyshomeostasis.

In conclusion, our findings highlight that abnormalities of UPR are a still largely unexplored feature in heart failure to be view in its entirely. The combined alteration of several target proteins of these pathways configures defective proteostasis as a condition of misfolded peptides accumulation ultimately exhausting the cell survival capabilities.

1. Introduction

Dilated cardiomyopathy (DCM) is regarded as a heterogenous group of heart diseases in light of the wide range of possible driving pathological mechanisms. These result in "structural and functional" abnormalities of the "heart muscle, in the absence of coronary artery disease

Abbreviations: AD, Alzheimer's Disease; CAD, Coronary Artery Disease; CHD, Congenital Heart Disease; iDCM, Idiopathic Dilated Cardiomyopathy; ERAD, Endoplasmic Reticulum Associated Protein Degradation; ER, Endoplasmic Reticulum; FDR, False Discovery Rate; iDCM, Idiopathic Dilated cardiomyopathy; KDEL, ER lumen protein retaining receptor-2; PAO, Pre-Amyloid Oligomers; PQC, Protein Quality Control; PTM, Post Translational Modifications; UPR, Unfolded Protein Response; UPS, Ubiquitin Proteasome System.

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(CAD), hypertension, valvular disease, and congenital heart disease (CHD) sufficient to cause the observed myocardial abnormality'" per the European Society of Cardiology guidelines 2023 [1]. Thus, in those cases, where no known etiology can be identified after performing clinical, laboratory and imaging assessments [2] patients are diagnosed with idiopathic DCM (iDCM) representing the second largest subset of cases.

At the subcellular level, various non-mutually exclusive pathways drive the molecular, cellular and tissue alterations in iDCM including cytoskeleton disarray, Ca^{2+} dishomeostasis, myocytes apoptosis and death [3]. Additionally, one of the most recent abnormalities described in iDCM is the underestimated finding of misfolded proteins aggregates in the myocardium forming small deposits of pre-amyloid oligomers (PAO), protofibrils and amyloid fibers [4].

Abnormal folding of proteins is a relatively common event in every cell. Folding of proteins is in fact a high error-prone process requiring prompt corrective actions in order to avoid the occurrence of aggregated proteins buildup and the resulting toxic damages broadly called "proteotoxicity" [5]. A finely tuned biological system - the protein quality control (PQC) machinery, which includes the unfolded protein response (UPR), is in place to maintain protein's health. It recognizes mal/misfolded proteins and refolds them in the cytosol or in the endoplasmic reticulum (ER), the sites of synthesis, post-translational modifications and folding of cytosolic, secreted or transmembrane proteins respectively.

While is still unknown if the accumulation of misfolded proteins is a primary or secondary pathogenic event, activation of individual components of the UPR have been described in a number of diverse cardiovascular conditions affecting the atria (atrial fibrillation) [6] or the ventricles (diabetic cardiomyopathy, ischemic heart disease and in murine models of aortic constriction, myocarditis or Alzheimer's Disease) [4,7–10] where it serves as a protective first defense against misfolded protein accumulation [11]. In selected cases of DCM - like the ones caused by mutations in the ER lumen protein retaining receptor-2 (KDEL receptor) - individual players of the UPR (rev in [12]) have been reported [13–16]. However, in iDCM, in light of the complex orchestrated interactions among different pathogenic mechanisms, the entire signaling has not been tested and we thought to survey the major known pathways to understand how UPR is altered in disease. Thus, in this study, we tested the expression and activation of all three major known UPR network pathways in human iDCM heart samples at transcription and translational levels.

Since aging per se decreases the competence of the PQC as age affects all proteins including the PQC ones, we addressed the aging- vs. disease-related changes of this system.

Our study also tested the effect of genetic risks on the UPR activation by comparing the changes in UPR in two iDCM cases with mutation in the presenilin-1 and two iDCM cases with mutation in the presenilin-2 gene (*PSEN1, PSEN2*), the first genes associated with Alzheimer's Disease (AD), one of the most commonly studied disease of protein folding. Notably, *PSEN1* is known to affect Ca^{2+} homeostasis [17–19] a known key defect in iDCM [20–22], indirectly hinting on the role of Ca^{2+} dishomeostasis in the response to proteostatic defects and linking two key defects of DCM.

Taken together, our study begins to shed light on the UPR landscape associated with iDCM, providing essential information for the diagnosis and potentially new therapeutic targets.

2. Methods

2.1. Sample collection

Failing discarded left ventricular tissue was collected at time of heart transplant. Non-failing hearts were obtained from the National Disease Research Interchange supported by the National Institute of Health. All donations were made with family consent. Donor hearts ruled out from transplantation were used if no macroscopic, laboratory or instrumental signs of cardiac diseases were present. Control hearts were found not suitable for transplantation due to lack of identification of a compatible recipient, blood transfusion while in the emergency department, age of donor or need for resuscitation. Failing and donor hearts were cardioprotected using Wisconsin cold oxygenated cardioplegic solution during cross clamp and transportation to the laboratory to preserve, at best, the tissue structural and molecular integrity. Samples were matched for age, sex and ethnicity when possible. The hearts were dissected in regions and the tissue flash frozen in liquid nitrogen and stored at −80 °C. For this study we used myocardial tissue collected from the anterior wall of the left ventricle of explanted iDCM failing hearts ($n = 13$) and from non-failing donor hearts ($n = 12$) (Table S1). Two sets of samples were run in separate gels. The study was approved by the Institutional Review Committee.

2.2. RT-qPCR

Total RNA from flash frozen samples was extracted and purified as described in the RNeasy Mini Kit product datasheet (Qiagen, #74004) and processed with the RNase-Free DNase Set to remove possible DNA contamination (Qiagen, #79254). The first strand cDNA was synthetized using the RT2 First Strand Kit (Qiagen, #330404). The material was analyzed with the RT^2 Profiler™ PCR Array Human Unfolded Protein Response (Qiagen, # PAHS-089ZA-24) and by qPCR using RT^2 SYBR Green ROX Master mix (Qiagen, #330523).

2.3. Immunoblotting

Human cardiac tissue was lysate with buffer containing 20 mM TrisHCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA 1% Triton, 2,5 mM with protease and phosphatase inhibitors (Thermo Fisher, # 78442) added. The protein concentration of tissue lysate preparations was measured using the Bradford method [23] or Micro BCA™ Protein Assay Kit (Thermo Fisher #23235). All immunoblots were performed under reducing conditions on gradient gels, using standard techniques. All primary antibodies used were incubated in 5% milk or 5% bovine serum albumin (for phosphoproteins) for 1 h at RT or overnight at 4 °C. Primary and secondary antibodies used are listed in Table S2. The blots were then incubated in a solution containing HRP conjugated IgG (Goat-anti Rabbit IgG HRP B-1215, Rabbit anti-mouse HRP P-0161, Rabbit Anti-goat HRP P0449). The signal was detected by enhanced chemiluminescent substrate ECL (full blots are shown in Fig. S1). Target protein normalization was performed dividing the target's signal by protein load signal (using BioRad stain-free gels). Data obtained from the target protein/protein load ratio have been plotted based on age. Each data point represents the abovementioned ratio in respect to the reported protein target for all samples, colour-coded for group and trends over time. Correlation coefficient and significance are reported. The same myocardial samples used for the transcriptional profiling were employed for translational analysis of the UPR pathway by SDS page in a first set of samples (Table S1 Set 1). A replication cohort of samples with representative samples overlapping for rigor was run on a separate gel under the same conditions to increase the overall number of samples (Table S1 Set 2).

2.4. Statistical analysis

Data and statistical analysis are presented in Tables S3 and S4 Data were analyzed using an unpaired, 2-tailed Student's *t*-test. Statistical significance was defined as a P value of less than 0.05. Single datapoints are reported in within the graphs. Bar charts and error bars represent means and standard deviation respectively.

Associations between each RNA with the different categorical characteristics were evaluated using a series of two sample *t*-tests. Statistical assumptions were checked graphically, and transformations were

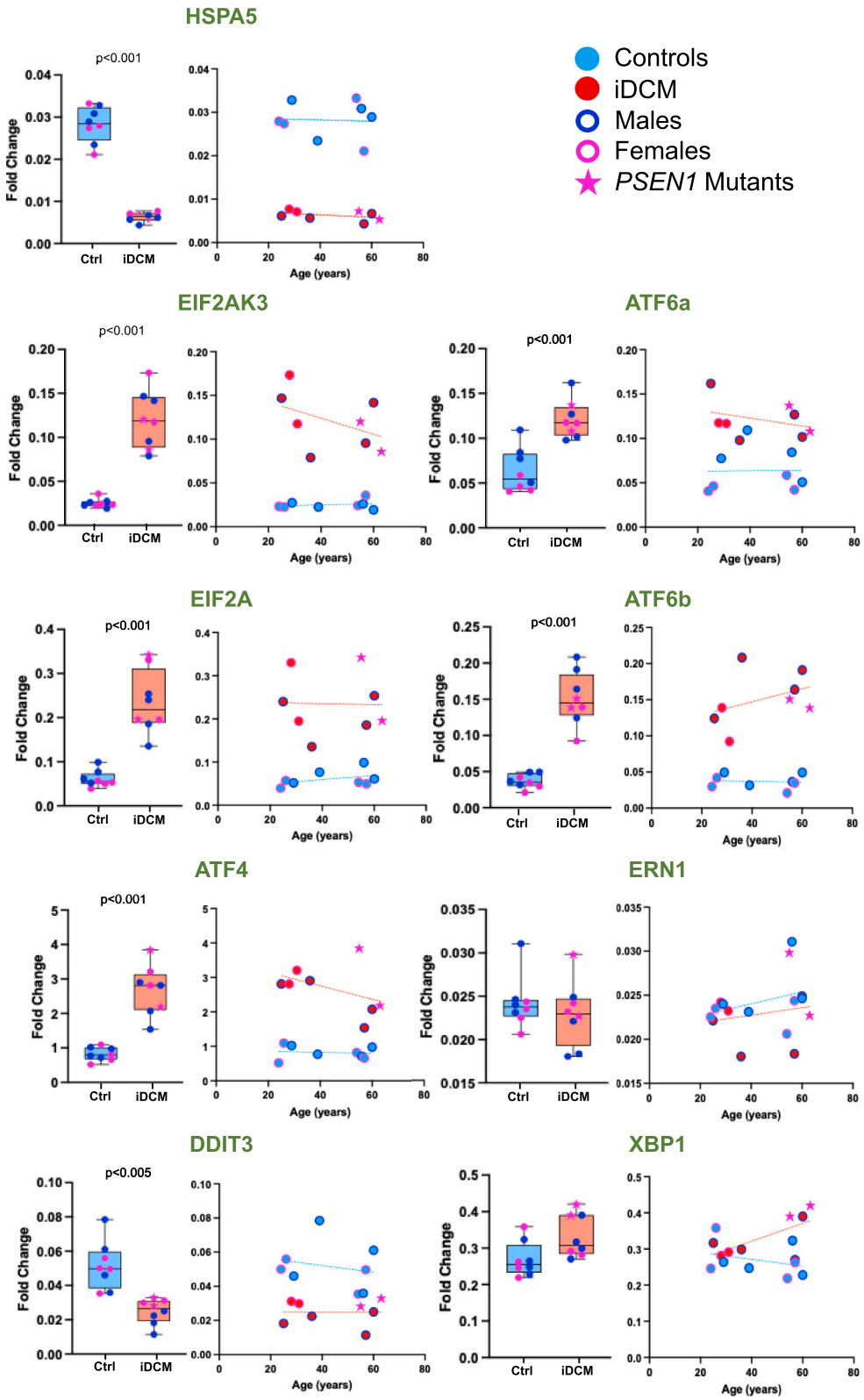

Fig. 1. Gene expression of the UPR signaling: Box plot comparison of gene expression for all samples (left panels). Right panels represent the scatterplot charts showing the individual datapoints for target expression distributed by subjects' age (blue dots = control; red dots = iDCM). Samples from male and female are shown by blue/pink individual datapoint in the box plots and blue pink circles around the data point in the scatterplots. All analyses are expressed as fold change representing relative expression normalized to housekeeping genes. Boxplot data are expressed as Means ± Standard Deviation. With the exception of the HSPA5 (encoding for the chaperone GRP78) and DDIT3 (encoding for CHOP) genes the box plot data show an overall increase gene expression. Age tends to decrease gene expression in iDCM while the ERN-XBP1 gene expression shows a stronger response in older iDCM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

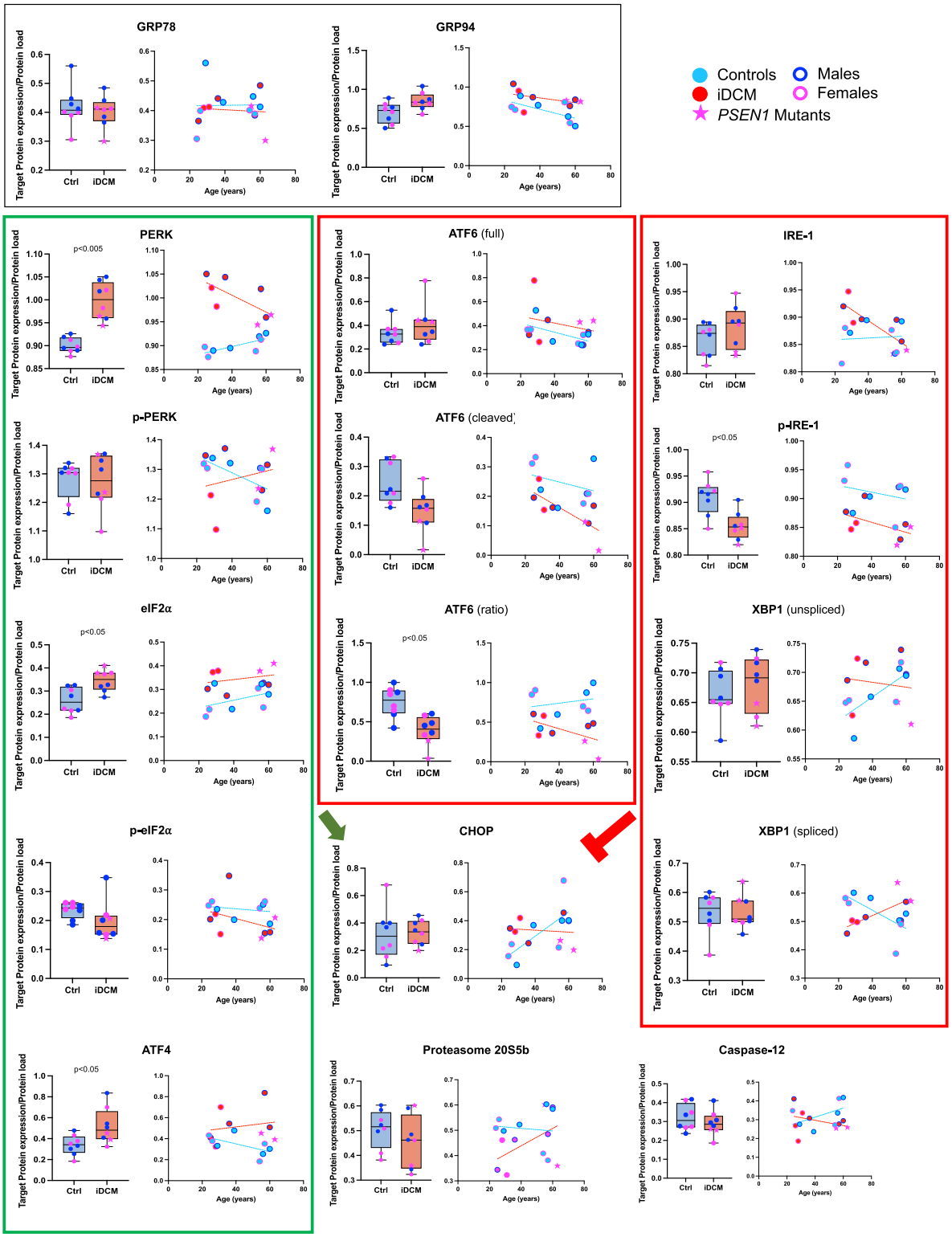

Fig. 2. Protein expression and age distribution of the UPR signaling: Box plot comparison of total protein expression for all samples (left panels). Right panels represent the scatterplot charts showing single datapoints for target protein expression distributed by subjects' age (blue dots = control; red dots = iDCM human cardiac tissue). Samples from male and female are shown by blue/ pink individual datapoint in the box plots and blue/pink circles around the data point in the scatterplots. Panel 2 A portraits the results of the first dataset; Panel 2B portraits the results of the second dataset. Data are expressed as Means ± Standard Deviation. The box plot data show an overall increase in the expression of the upstream effectors of the three main axis on the UPR while the downstream proteins and PTM appear overall downregulated with the exception of ATF4. Age affected protein expression with an overall decrease of PERK, ATF6 and IRE1 axes which tend promote cell death response to stress. However mutations modified the age trends as shown by the comparison of the set of samples with 2 (panel A) vs 4 mutants (panel B). Thus, it is important to consider age, genetic risk and disease duration in the evaluation of the UPR capacity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

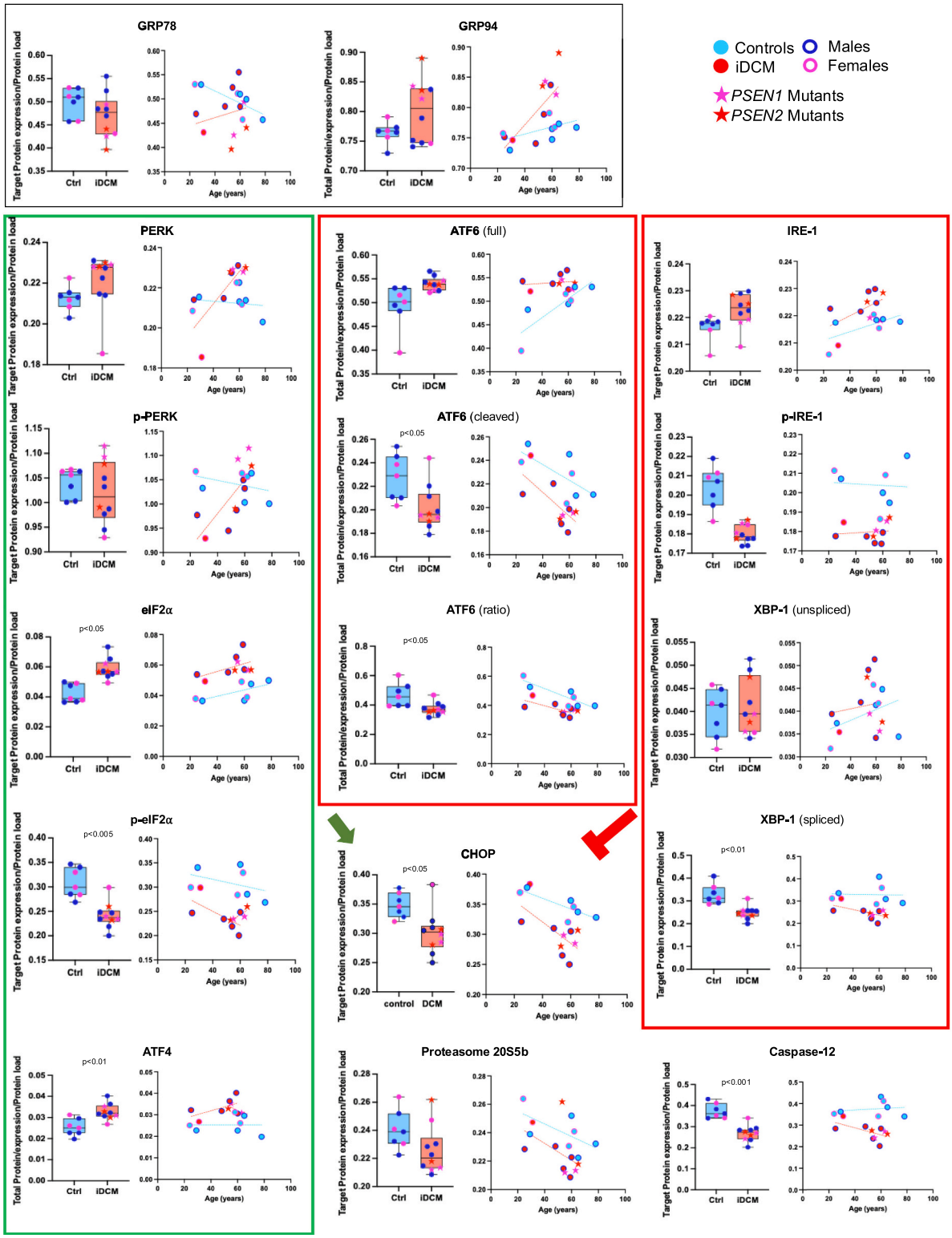

Fig. 2. (*continued*).

considered as needed. Statistical significance was reported both as unadjusted *P*-values and FDR corrected P-values to account for multiple testing. Mixed model analysis was used to analyze the two set of samples together. All analyses were run in R v 4.3.2.

Principal Component Analysis was performed using the FactoMineR (v2.8) package in R (v4.2.3). To quantify the biological and technical variation in both gene expression and protein experiments, we applied a linear mixed model by using the variancePartition (v1.28.9) package in R.

3. Results

3.1. Population demographics

Median ages and interquartile ranges of the samples from all control and iDCM subjects were 56.5 years (36.5–60.5) and 53.5.5 years (33.3–58.5) respectively. Male and female were equally represented in both groups (n = 6 M, 6F in the donor and n = 6F, 7 M in the iDCM group). The two cohorts were subsequently subdivided by age in two subgroups of lower and higher age. The four groups were matched for age and sex as possible. Samples from female and male are identified by colour of the individual data points in all boxplot graphs. In the age distribution graphs, sex was identified by colour of the symbol border while donor vs disease were identified by the colour of the dots. Mean age in years in the younger group was 29.5 for controls and 30 for iDCM; in the older group was 61.2 for controls and 57.1 for iDCM.

3.2. UPR transcriptional profiling

The overall transcriptional changes of the major effectors of the UPR pathway in the two matched groups is shown in Fig. S2 (panel A). Individual sample's gene expression values and means are shown in Table S3 (panel A) and statistical values are shown in Table S4 (panel A) that provides the *P*-values (unadjusted and FDR corrected) for all comparisons considered. There were no associations between RNA levels with patient's sex. By disease, in comparison to housekeeping genes (Fig. 1 box plot), most genes showed significant changes, mostly with increased values in the iDCM group except *HSPA5* and *DDIT3* ($P < 0.001$ for both before adjustment; $P < 0.001$ and $P < 0.005$ respectively after FDR adjustment) that were significantly decreased irrespective of age. By fold changes in comparison to controls, iDCM showed significant upregulation ($P < 0.001$) for ATF4, ATF6A, ATF6B, EIF2A and EIF2AK3). XBP1 changes were not significantly different after FDR correction ($P = 0.06$). *ERN1* was unchanged between the two cohorts.

3.3. UPR translational profiling

Protein expression and statistical values are shown in Table S3, S4. While the results of the two sets of samples could not be plotted together (Fig. S3), the quantification values of the separate gels with appropriate normalization (Fig. S3) could be analyzed together using a mixed model analysis (Table S4D).

Protein expression of the effectors of the UPR showed that the expression of the upstream regulators GRP78, ATF6 and IRE1 were not significantly different in iDCM vs controls in both sets separately (Fig. 2, S4B,C) and combined (Table S4D), while GRP94 ($P < 0.005$; $P < 0.01$ after FDR adjustment) and PERK ($P < 0.0001$ before and after FDR adjustment) were significantly increased in the combined sets (Table S4D).

3.4. Post translational modifications

Analysis of the post translational modifications (PTM) demonstrated that while the expression of the UPR effectors (significantly different for PERK, eIF2α,ATF4, and trending for ATF6 and IRE1) is increased to halt the unfolded protein load, the downstream PTM didn't increase indicating an important role of the PTM in the UPR in the failing heart (Fig. 2 box plot). Increased expression of PERK, eIF2α and IRE1 didn't match with an increase of their phosphorylation, but rather a decrease. Similarly, cleaved ATF6 ($P < 0.005$; before and after FDR adjustment) and its ratio to the full isoform ($P = 0.0005$ before and after FDR adjustment) were decreased in iDCM despite an unchanged full-length protein. Thus while the upstream signaling appears unaffected or upregulated, decreased activation was found for the downtream signaling.

Interestingly the overall UPR translational signaling indicates that the pathways leading to the activation of the programmed cell death (CHOP) showed a trend to activation in iDCM whereas the pathways leading to the inactivation of CHOP showed a trend to reduction in diseased samples with an overall effect of promoting cell death (Fig. 2 box plot).

3.5. Transcriptional and translational profiling of UPR

The comparison of the transcriptional vs. translational expression (Fig. S5) showed that protein expression paralleled the mRNA levels for most of the molecules except for GRP78, XBP1 and DDIT3/CHOP where age was the major contributor of the differences (Fig. S6).

3.6. Age-related scatterplots

While statistically there was no age effect both at the transcription and translational level (Table S4), age contributed to changes in individual UPR proteins affecting the overall changes when data from younger and older individuals were plotted together. The data obtained from the target mRNA and protein/protein load ratio plotted in correlation with subjects' ages (Figs. 1,2,S6) showed that at the upstream level, there was no difference in the expression of GRP78 for age both at mRNA and protein levels, while GRP94 showed a parallel trend increase in protein expression in iDCM and controls with age. However, at the protein level, iDCM from *PSEN2* mutant cases changed the trajectory as shown in the second cohort of samples (Fig. 2B, S7, S8). Age reduced the transcription of EIF2 AK3 in iDCM that equated the age related changes in protein expression (PERK) in the first set (Fig. S6). The full length ATF6 was overall higher in iDCM in both age subgroups while cleaved ATF6 decreased over time in controls, and even more in the iDCM group (Fig. 2A,B; S7). Corresponding in gene (ERN1) and protein expression, IRE1 had age discordant change with protein expression decresing with age in iDCM with a potential compensatory increase in mRNA levels (Fig. S6) in the first cohort.

For the downstream effectors, p-PERK showed an age dependent decrease in controls while it increased with age in iDCM samples (Fig. 2A,B, S7). The end effector of this axis, eIF2α showed a parallel small increase with age in controls and iDCM at the protein level with no age changes in the mRNA expression (EIF2A) (Fig. 1, S6). An age dependent decrease in p-eIF2α was present in particular for the iDCM samples (Fig. 2A,B,S7). There was an overall age dependent decrease in mRNA expression of ATF4 (Fig. 1,S6).

p-IRE1 showed a parallel overall decrease with age in iDCM and controls especially in the first cohort (Fig. 2A, S7, S8) possibly due to an effect of PSEN2 mutation affecting the trajectory in the second cohort being responsible for a similar discordance in the trajectory of XBP-1 unspliced and spliced between the two cohorts at the protein level (Fig. S7, S8A,B). Unspliced XBP1 showed a discordant increase mRNA and decrease protein expression with age in iDCM and the opposite effect in controls (Fig. S6).

Interestingly the lower expression of the proteasome, well described in the literature, was mostly present in the young group (Fig. 2A, S7) in the first cohort, with again a possible effect of *PSEN2* mutations worsening the expression in older iDCM in the second cohort (Fig. 2B, S7). Additionally, age had a discordant effect in the two cohorts with increased protein expression of CHOP in the control samples with no effect in diseases samples in the first set (Fig. 2A, S7) and a decreases protein expression in both controls and iDCM with a mutation effect in the protein expression in the second cohort (Fig. 2B,S7).

Overall although age didn't statistically significantly affect gene expression (Table S4A), it mitigated the differences with controls for EIF2AK3, ATF4 and CHOP (Fig. 1) while further increase the expression of ATF6B and XBP1 in iDCM vs controls. At the protein level it appears that there is an overall tendency for an age dependent decrease of the expression of all 3 axis (PERK, ATF6, IRE-1) in their distal signaling and caspases in iDCM with variability driven by the presence of *PSEN* mutations. Statistically, age significantly affected cleaved ATF6, eIF2α and

Caspase, altought the significance remained at $P < 0.1$ only for ATF6 after FRBackspaceDR adjustment (Table S4).

3.7. Loss of function mutation effect of UPR

Statistically, mutant status was associated with HSPA5, AFT6B ($P < 0.001$ before and $P < 0.01$ after FDR adjustment for HSPA5 before and $P < 0.05$ and $P < 0.1$ after FDR adjustment for ATF6B) at the gene expression level (Table S4A). Overall, mutant status was associated with GRP78 ($P < 0.05$ before and $P < 0.1$ after FDR adjustment); ATF6 cleaved and ratio ($P < 0.01$ before and $P < 0.1$ after FDR correction; $P < 0.005$ before and $P < 0.1$ after FDR correction respectively). eIF2α and p-eIF2α were significantly associated with the presence of mutation however, didn't remained significant after FDR correction (Table S4D).

3.8. Principal Component Analysis (PCA)

Through Principal Component Analysis we questioned the overall distribution of similarities/differences and the effect of variances within our set of patients. At a transcriptional level (Fig. 5A) iDCM patients were significantly distinct in comparison to their matched controls with iDCM probands showing a higher degree of heterogenicity. The divergences between the two groups were less profound at the translational level (Fig. 5B,C) an overlap that can be attributed to the influence of post translational modifications and aging. In fact, although the genotype played a significant role in protein expression, it was the major driver of the differences in RNA expression (Fig. 5D), whereas age also played a major role in protein expression possibly due to the effect on post-translational modifications (Fig. 5E-F).

4. Discussion

4.1. Proteotoxic molecular landscape of iDCM

Independent to the wide range of possible etiologies, DCM comprises a broad spectrum of flaws (with/without a known mono- or polygenic foundation) of molecular targets ranging from cytoskeletal architecture, sarcomeric array, nuclear envelope, ion channels, mitochondria, sarcoplasmic reticulum and desmosomes, among others [24]. Those changes would ultimately lead - alone or within the context of a multi-hit damage - to the exhaustion of the myocardial functional reserve. In addition to the forms with recognized origin, a group of cases where none of the known etiologies can be identified, populate a heterogeneous cluster of DCM labeled as “idiopathic” (iDCM).

Within this class, the discovery of the accumulation of misfolded proteins as plaque- and tangle-like structures in the myocardial tissue and cardiomyocytes, and of the mutations in the first gene associated with dementia of Alzheimer's (the presenilin gene - *PSEN*), led to the recognition of a group of sporadic and familial cardiomyopathies as part of the growing family of proteinopathies as an additional etiology for DCM [4,8–10]. These findings, together with mutations in desmin [25] and its chaperonin Aβ-crystallin genes [26,27] raise awareness on protein misfolding as an active driver of cardiac damage and an additional pathogenic mechanisms eliciting the cardiomyopathic morpho-functional phenotype.

In response to the accumulation of misfolded proteins, the cell activates the PQC, which, however, will eventually exhaust as a secondary adaptive response. While primary defects (mutations) of one or more of the UPR players have not yet been described as causative of cardiomyopathies (except for mutations of the KDEL gene), changes in individual proteins composing the UPR have been reported (rev in [12]). However, those may have broader consequences for the entire signaling cascade.

A main finding of this study is that, when viewed in its entirety, the changes in the three major arms of the UPR, at least at the end stage of disese, appear geared towards the activation of the apoptotic pathways with the partial PERK arm activating CHOP and inactivation of the ATF6, IRE1 arms inhibiting CHOP (Fig. 2A,B, S4, S7). However, the overall effect on the apoptotic pathway was statistically unchanged (Table S4), an effect potentially attributable to the differences in age progression between controls and disease.

4.2. Proteotoxic vulnerability of aging

Among the modifiers of the activation/alteration of the UPR, aging appered per se to be sufficient to change the unfolded protein response differently in health and disease. By plotting datapoints based on the age of subjects we were able to identify age dependent trends that could unravel possible targets associated with aging or disease alone, or changes present in disease that aging worsens (Figs. 1, 2, S6, S7). At the transcription level, the upstream effectors (EIF2αK3, ATF6a) - although overall remaining higher in diseased hearts - decline with age (Fig. 1, S6) while ERN1 differs (where the lack of decline could be driven by a mutant sample) (Fig. 1, S6).

Mostly paralleling the transcription changes, protein expression of upstream signals (PERK, ATF6, IRE1 (Fig. S7), declines with age in the first cohort, an effect not observed in the second cohort where the upstream signals increased with age in iDCM, an effect possibly driven by the mutations. The distal effectors (p-PERK, eIF2α, ATF4) showed age increase in iDCM while p-eIF2α, cleved ATF6 and spliced XBP-1 decline with age in disease. Age instead have minimal to no effect in controls with a trending decrease in p-PERK, p-eIF2α, p-IRE1 and spliced XBP-1 suggesting an exhaustion of these axes even in the absence of pathological morpho-functional alterations. This could be considered as a response to the chronic wear-and-tear process of proteins that would eventually lead to the accumulation of misfolded protein aggregates found in nearly 30% of myocardial samples at later age [4].

While the UPR overall decrease with age, the protein expression of the proteasome decreased in the young iDCM subjects as previously described [28–32], but increases with age although with high samples variability and with a possible effect of mutations especially in *PSEN2* (Fig. S7). Finally althought non significantly (Table S4) the expression of the cellular “stress sensor” CHOP decrease with age in iDCM suggesting a failure to regulate the proper response to ER stress in disease (Fig. S7).

4.3. iDCM heterogeneity

Whether aging per se hampers or stems from an altered UPR remains an unsolved modern scientific dilemma, yet it stands as a broadly accepted binomial. On the other hand, if pathological conditions, independently of age, impact on this molecular system is an intriguing topic of growing scientific interest, which will yield to a better understanding of diverse diseases – especially the chronic, age related ones - and are noteworthy for future investigations.

While the overall view of the intricacies of the UPR signaling appears complex, to simplify the interpretability of our dataset we applied the Principal Component Analysis (PCA) (Fig. 5) a dimensionality reduction method to capture patterns of similarity within complex cohorts. It allowed to identify a unique, yet expected, distribution of our probands which showed a predictable clustering of our cohorts of human samples, while appeared significantly divergent based on their group belonging, at a transcriptional level or at the translational level with a certain degree of overlap. Controls display a consistently homogenous expression of UPR machinery both at transcription and translation indicating a preserved and adequate control system which is either in function and not exhausted by internal and/or external perturbations, or it is simply unemployed beyond a physiological extent. In contrast, iDCM subjects appear to have a more heterogenous expression distribution suggesting the contribution of diverse factors either towards a protective or detrimental shift in terms of mechanisms, activated branches, effectiveness of molecular rescue and cellular fate. This distribution is in line with the discrepancy between transcription and translation products and perhaps one of the reasons limiting the full expression of proteins for salvage or

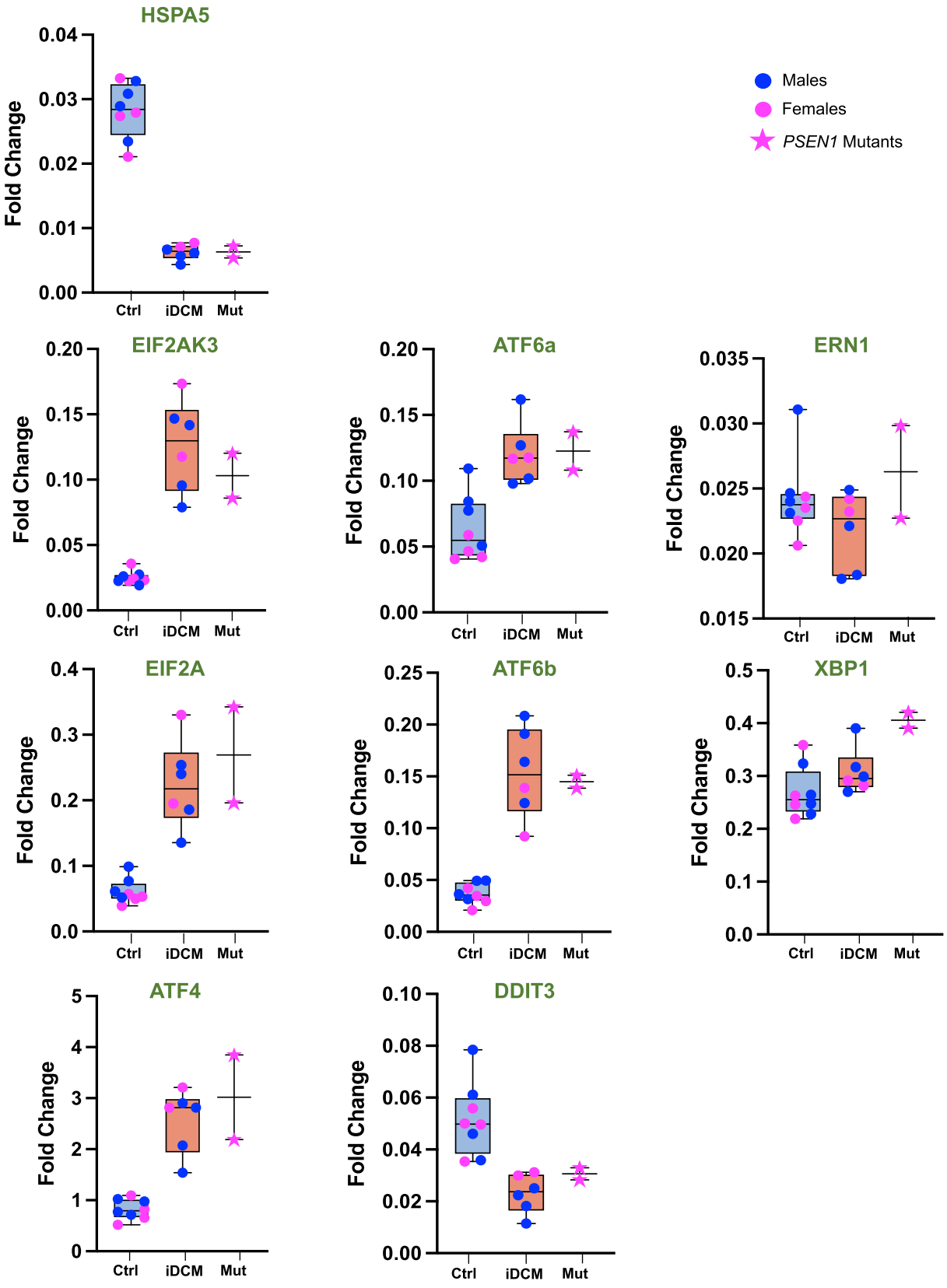

Fig. 3. Effect of *PSEN1* mutation on UPR transcriptional regulation: Box plot of the gene expression of the UPR in samples including the ones from patients with a mutation of *PSEN1* described in AD patients. Samples from male and female are shown by blue/pink individual datapoint in the box plots. All analyses are expressed as fold change representing relative expression normalized to housekeeping genes. The presence of mutations tend maintain or further parallel change the expression of UPR genes in iDCM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

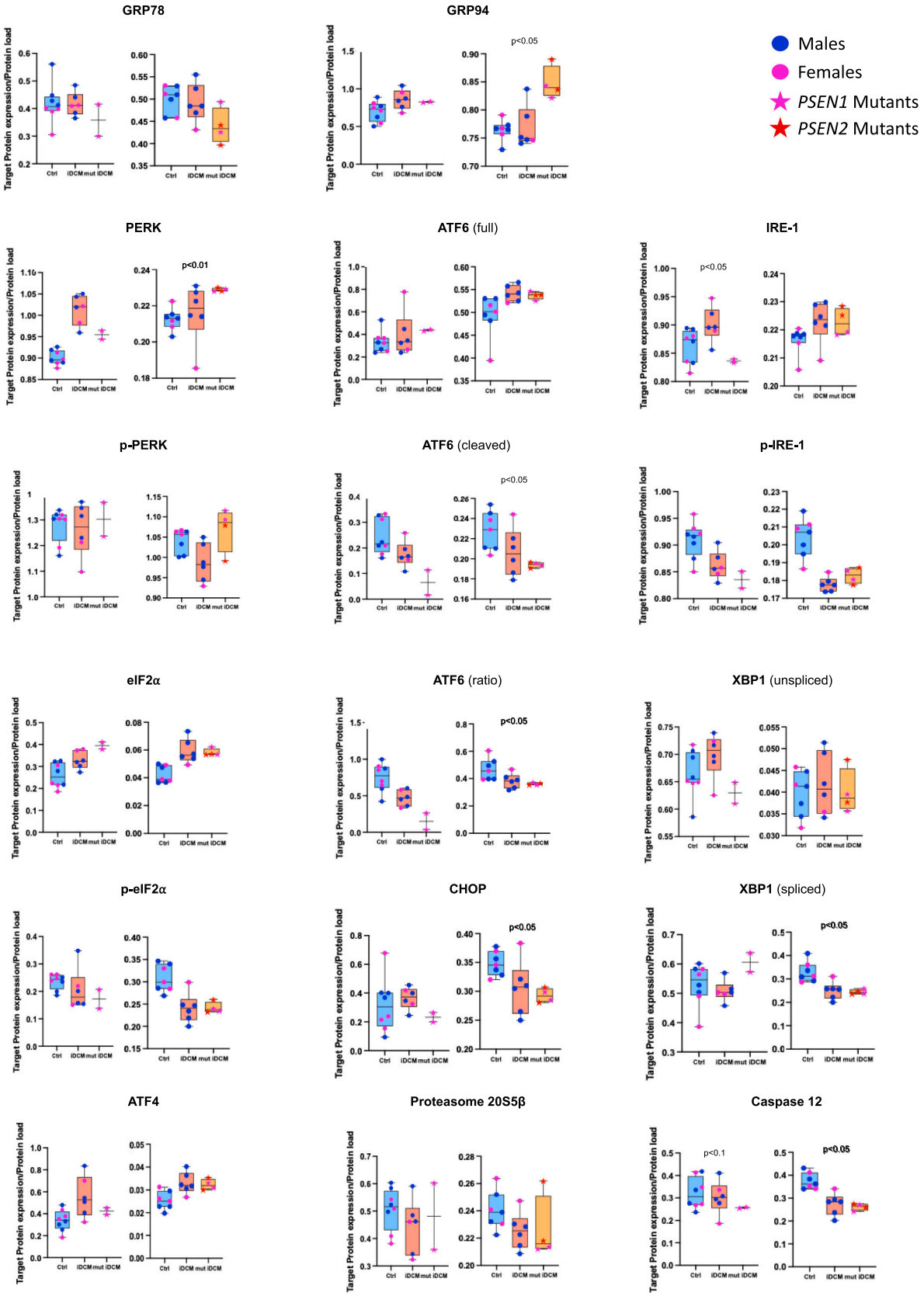

Fig. 4. Effect of *PSEN1/2* mutation on UPR translational regulation: Box plot expression of the UPR in samples including the ones from patient with mutation of *PSEN1* (first set) and/or *PSEN2* (second set) described in AD patients. Samples from male and female are shown by blue/pink individual datapoint in the box plots. *PSEN1* mutants are indicated by pink stars, *PSEN2* mutants are indicated by red stars. Mutations in *PSEN1/2* maintain or further modifies the UPR players in the same direction of non mutants in diseased patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

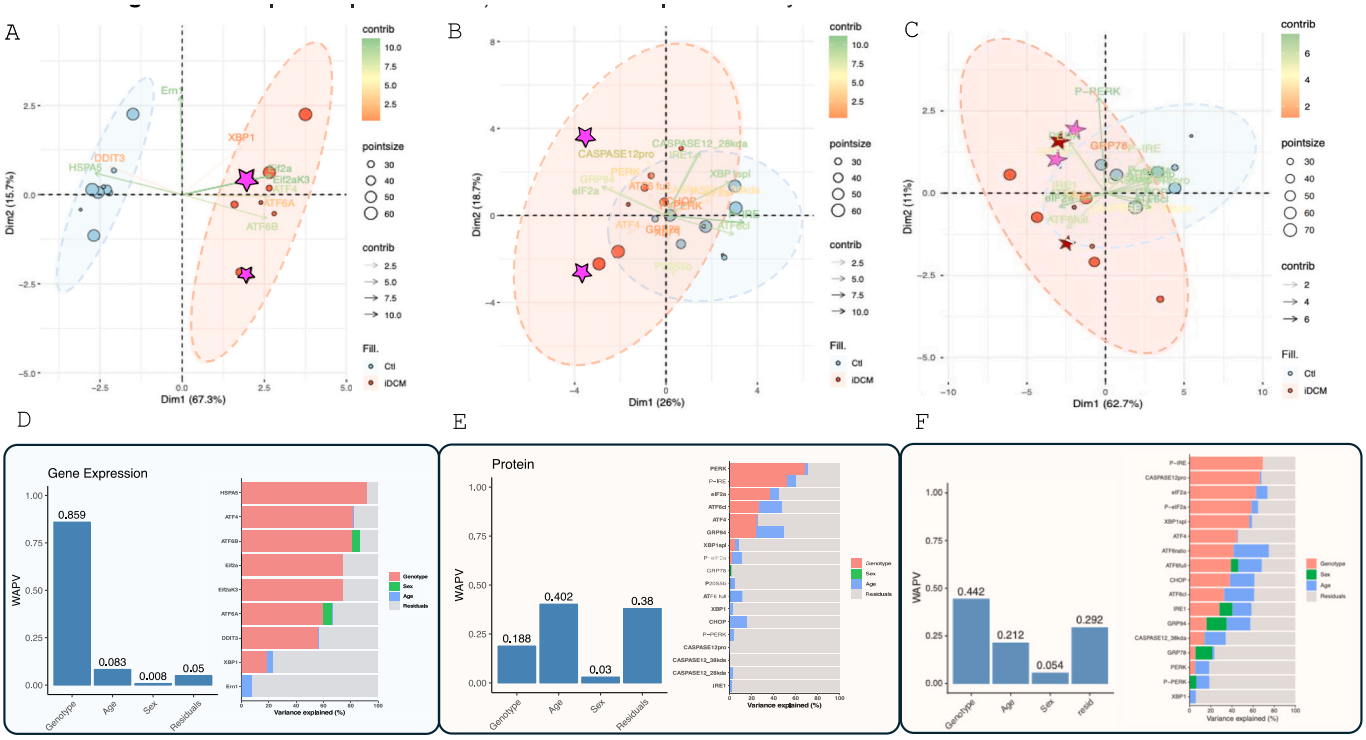

Fig. 5. Principal component (PCA) and variance explained analyses in iDCM and control human heart samples. A-C) Principal component analysis (PCA) plots for transcriptional and translational assessment of the first 16 (panels A, B) and translational of the second 16 (panel C) heart tissues were generated in R by using data obtained from RT-qPCR (a) and Western Blot (b). Each data point represents the samples included. iDCM samples are shown in red, control samples in blue. *PSEN1* mutants are indicated by a pink star, *PSEN2* mutants by a red star. Highlighted the major contributors of the variance explained by the two principal components. Gradient depicts the contribution level of each gene highlighted. D—F) Left side: Barplot depicting the variance explained by the covariates weighted across the first 5 principal components (WAPV = Weighted average proportion variance) for the RT-qPCR (D) and Western Blot data (E,F). Each bar corresponds to the % of variance explained by each variable (Genotype, Age, Sex). Right side: Stacked barplot showing the variance explained by each variable after correcting for all other variables using a linear mixed model. Genes are ranked by the variance explained by the Genotype (iDCM/CTL). Different colors correspond to the biological covariates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

simply indicating rescue system exhaustion. Notably, our results highlight the considerable role of post-translational modifications which can be missed by gene expression analysis only. Taken together, iDCM appears as a heterogenous group also at the level of the response to damage. This should be further characterized in a much larger cohort of patients with age/sex/ethnicity matched controls to identify adequate responders or subjects with reduced functional reserve, and possibly poorer prognosis underlying the need for personalized diagnostic and therapeutic approaches within the subgroup of proteotoxic iDCM beyond the subclassification as proteinopathy.

4.4. Proteotoxicity: a molecular switch towards further toxicity

Several ER protein folding helpers (such as the GRP chaperones) require Ca^{2+} for their activity making cardiomyocytes particularly susceptible to stress and accumulation of misfolded proteins. This is especially relevant in iDCM and heart failure where Ca^{2+} dishomeostasis, featuring sarcoplasmic reticulum (SR) Ca^{2+} depletion and cytosolic Ca^{2+} overload, is a well-known and an intensely investigated abnormality. Changes in SR [33] will have, simultaneously, the well known consequences for contractility, but also for the less investigated consequences on the proper folding of proteins and the correction of their defects. On the other hand, primary or secondary misfolded events or defects in the proteostatic machinery will, in turn, negatively impact ER/SR Ca^{2+} homeostasis reinforcing or initiating the overall defect.

4.5. Genetic risk for the response to stress

Within the group of cases tested here, four samples were obtained from patients carrying mutations in *PSEN1/2*. *PSEN1* and especially *PSEN2* mutations are known to affect Ca^{2+} homeostasis and to potentially alter the response of the Ca^{2+} dependent proteins such as the GRPs chaperones. In our dataset, *PSEN* mutations appear to affect the overall expression of the UPR molecules with the tendency to either maintain or further accentuate the direction of the overall expression changes found in the non-mutated iDCM cases (Figs. 3, 4, S8A,B). Albeit there were only four samples carrying *PSEN* mutation which were in the group of older cases, limiting the proper understanding of the role of such mutations for the disease per se, a counterintuitive finding was the effect on the translation of CHOP where there was less induction driven by the mutation (Fig. S8). Since *PSEN1* and even more *PSEN2* [19] loss of function mutation would lead to cytosolic accumulation of Ca^{2+}, a known activator of programmed cell death, further activation of the stress response was expected, with the effect of age related exhaustion as a possible explanation.

In conclusion our study provides, for the first time, an extended overview of the molecular landscape of the PQC in human myocardium in diseased and non diseased samples with additional consideration for the effect of aging and mutations. Our data broadly recognizes PTM as a critical step in the dynamic balance between protective and toxic drifts caused by protein misfolding [34]. As shown in our FDR analysis, aging itself plays a prominent role in worsening UPR response capability especially in the disease samples, with also a contribution of sex in particular for the chaperones and of the mutants although all mutant samples were from women patients. Similarly, mutations exacerbate the changes set by the disease. The results of the study provide key information for experimental and clinical studies evaluating the UPR as diverse, yet integrated responses to a variety of stressors.

5. Limitations

We acknowledge that the current dataset does not allow a mechanistic insight, requiring further investigations on a larger samples scale using omics (overcoming the technical limitation of the SDS page). This applies even more to the subset of *PSEN* mutants in light of their sample size, thus findings should be considered as preliminary. We acknolege that this initial study is not a longitudinal one, however the enrollment of subjects of younger and older ages had the intention of mimicking the course of aging in two different groups according to health status. Further studies are needed to understand the weight of these alterations and whether they could be considered as an age-related marker of cellular resistance. We acknowledge the partial availability of some of the clinical data especially for the donor controls. However the pathological status (iDCM or non cardiac disease) was determied by the advanced heart failure team and the donor organs obtained were deemed unusable for transplant for reasons not linked to cardiac disease. We also acknowledge the presence of one control sample from a patient whom received chemotherapy which however clustered by age and sex with the other donors (Fig. S9). Finally we acknowledge the presence of one donor with a reduced EF. It is possible that acute events or even protracted ICU-stay per se not reported in the available clinical data might have reduced the EF. All other available echo parameters are within the normal range and the values of the parameters we collected didn't show to be an outlyer within the control group.

Disclosures

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CRediT authorship contribution statement

Camilla Bacchin: Data curation, Formal analysis. **Marco Luciani:** Methodology, Investigation, Formal analysis, Data curation. **Luca Troncone:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Cristina Balla:** Investigation. **Stefano Berto:** Formal analysis. **Bethany Jacobs Wolf:** Formal analysis. **Federica del Monte:** Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in the development of this manuscript.

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Appendix A. Supplementary data

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