Obesity-induced activation of JunD promotes myocardial lipid accumulation and metabolic cardiomyopathy

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Aims

Metabolic cardiomyopathy (MC)—characterized by intra-myocardial triglyceride (TG) accumulation and lipotoxic damage—is an emerging cause of heart failure in obese patients. Yet, its mechanisms remain poorly understood. The Activator Protein 1 (AP-1) member JunD was recently identified as a key modulator of hepatic lipid metabolism in obese mice. The present study investigates the role of JunD in obesity-induced MC.

Methods and results

JunD transcriptional activity was increased in hearts from diet-induced obese (DIO) mice and was associated with myocardial TG accumulation and left ventricular (LV) dysfunction. Obese mice lacking JunD were protected against MC. In DIO hearts, JunD directly binds PPARγ promoter thus enabling transcription of genes involved in TG synthesis, uptake, hydrolysis, and storage (i.e. Fas, Cd36, Lpl, Plin5). Cardiac-specific overexpression of JunD in lean mice led to PPARγ activation, cardiac steatosis, and dysfunction, thereby mimicking the MC phenotype. In DIO hearts as well as in neonatal rat ventricular myocytes exposed to palmitic acid, Ago2 immunoprecipitation, and luciferase assays revealed JunD as a direct target of miR-494-3p. Indeed, miR-494-3p was down-regulated in hearts from obese mice, while its overexpression prevented lipotoxic damage by suppressing JunD/PPARγ signalling. JunD and miR-494-3p were also dysregulated in myocardial specimens from obese patients as compared with non-obese controls, and correlated with myocardial TG content, expression of PPARγ-dependent genes, and echocardiographic indices of LV dysfunction.

Conclusion

miR-494-3p/JunD is a novel molecular axis involved in obesity-related MC. These results pave the way for approaches to prevent or treat LV dysfunction in obese patients.

Keywords

Obesity • Myocardial steatosis • Lipotoxic damage • Metabolic cardiomyopathy • MicroRNAs • Epigenetics

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Translational perspective

Prevalence of obesity is skyrocketing across the globe and continues to climb. Metabolic cardiomyopathy (MC) is an emerging cause of left ventricular dysfunction in obese patients. However, its mechanisms remain to be deciphered. In cultured cardiomyocytes, gain- and loss-of-function mutant mice and myocardial biopsies from obese and non-obese patients, we show that the AP-1 transcription factor JunD derails key metabolic pathways in the obese heart, thus fostering lipid accumulation, lipotoxic damage, and cardiac dysfunction. In hearts from obese mice and patients, miR-494-3p orchestrates JunD levels, and its overexpression blunts JunD-dependent lipid accumulation and lipotoxic damage. These translational findings improve our understanding of the pathophysiology of MC, and may set the stage for novel therapeutic strategies to prevent or treat lipotoxic heart failure in obese patients.

Introduction

The International Diabetes Federation has recently estimated that—by the year 2040—almost 500 million people will be overweight and insulin resistant while 642 million will be affected by Type 2 diabetes (T2D).1 Obesity and T2D have many deleterious effects on the heart, including changes in substrate utilization, tissue metabolism, oxidative stress, and inflammation that are believed to promote cardiac dysfunction, and eventually heart failure (HF).2–4 A growing body of evidence indicates that patients with glomerulosclerotic perturbations are at risk of developing a metabolic cardiomyopathy (MC) phenotype, which is unrelated to the presence of coronary artery disease, hypertension, and other comorbidities.5,6 In these patients, MC mainly results from intracellular accumulation of lipotoxic by-products leading to myocyte apoptosis and contractile dysfunction.7 Over 80% of patients affected by HF are either obese or diabetic.8,9 Notably, HF represents one of the leading cause of mortality in these patients.8,9 Although MC prevalence is expected to rise considerably over the next decades, the underlying mechanisms remain poorly understood, and breakthrough therapies have yet to be developed in this setting.5

The accumulation of triglycerides (TG) in the heart, caused by a mismatch between uptake and oxidation of fatty acids, is emerging as a powerful determinant of cardiac damage mainly via accumulation of free radicals and subsequent activation of apoptosis.7 Myocardial TG content is increased in T2D patients, and associated with impaired left ventricular (LV) diastolic function, regardless of age, body mass index (BMI), heart rate, visceral fat, and blood pressure.10,11 Of note, a consistent proportion of patients with non-ischaemic HF display intramyocardial TG overload and changes in gene expression which are associated with contractile dysfunction.12

The Activator Protein 1 (AP-1) complex has emerged as a key modulator of cellular growth and metabolism.13 The AP-1 dimer is composed by three different Jun proteins (c-Jun, JunB, and JunD) and four different Fos proteins (c-Fos, FosB, Fra-1, and Fra-2). These members assemble to form AP-1 transcription factors with activities that are strongly influenced by their specific components and their cellular environment.14 The AP-1 member JunD has shown to regulate insulin signalling and hepatic TG metabolism.15,16 Whether JunD participates in obesity-related MC is unknown. In neonatal rat ventricular myocytes, gain- and loss-of-function mutant mice and myocardial biopsies from obese and non-obese patients, we show that the AP-1 transcription factor JunD regulates PPARγ signalling, thus leading to myocardial TG accumulation, lipotoxic damage, and LV dysfunction. Our findings may set the basis for the design of new approaches to prevent MC in obesity.

Methods

A detailed description of JunD knockout mice (JunD−/−), cardiac-specific JunD overexpressing mice (α-MHC–JunD10), in vitro experiments in neonatal rat myocytes, experiments in human heart samples, transcriptomic and lipidomic analyses, and bioinformatics and statistical methods is reported in Supplementary material online. All primers used for real-time PCR are shown in Supplementary material online, Table S1. Experiments in JunD−/− and α-MHC–JunD10 mice were approved by the Kommission für Tierversuche des Kantons Zürich, Switzerland (licence number ZH156/2014). The clinical study was approved by Local Ethics Committee (Sant’ Andrea Hospital, University of Rome ‘Sapienza’, Rome, Italy, Prot. n. 191 SA_2016) and, in accordance with institutional guidelines, all participants were aware of the investigational nature of the study and gave written consent for their participation.

Statistical analysis

Comparisons of continuous variables were performed using unpaired two-sample t-test and Mann–Whitney U test, as appropriate. Categorical variables were compared using the χ2 test. Multiple comparisons were performed by one-way analysis of variance, followed by Bonferroni correction. Adjusted P values were calculated by multiplying raw P values by g, where g indicates the number of comparisons. Between variable correlations were assessed by Spearman’s test. A multiple t-test using the Benjamini–Hochberg false discovery rate procedure was employed for the analysis of gene expression data (real-time PCR array). Probability values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism Software (version 7.03).

Results

JunD is required for the development of obesity-induced cardiomyopathy

Diet-induced obesity was employed to investigate the role of the AP-1 transcription factor JunD in MC. In line with other reports,17 body weight almost doubled, and blood glucose levels increased by 1.5-fold after feeding 60 kcal% high-fat diet (HFD) for 20 weeks (Supplementary material online, Table S2). Cardiac JunD expression was increased in HFD-fed as compared with control diet (CD)-fed mice, as shown by real-time PCR, western blot, and
JunD in metabolic cardiomyopathy

immunohistochemistry (Figure 1A and B). Together with increased expression, JunD transcriptional activity was significantly enhanced in nuclear extracts from obese as compared with control hearts (Figure 1C). To understand the contribution of JunD in obesity-related MC, we studied CD-fed and HFD-fed mice, in the presence or in the absence of JunD genetic deletion (Supplementary material online, Figure S1). Control diet-fed WT and JunD−/− mice were similar for anthropometric parameters and lipid fractions, whereas HFD-fed JunD−/− showed decreased body weight and heart weight as compared with HFD-fed WT mice (Supplementary material online, Table S2). Transthoracic echocardiography was employed to assess cardiac function in the four experimental groups. When compared with CD-fed controls, HFD-fed mice showed similar diastolic and systolic LV diameters, whereas LV mass was increased in the latter group (Figure 1D–F). Ejection fraction (EF) and fractional shortening (FS)—both indices of systolic LV function—were impaired in obese as compared with lean animals (Figure 1G and H).

Diastolic function—assessed by both pulse wave Doppler and tissue Doppler imaging (TDI)—was also affected in obese mice, as shown by decreased E/A ratio and prolongation of isovolumic relaxation time (Figure 1I and J, Supplementary material online, Table S3). Along the same line, LV myocardial performance index—a well-established index of systolic-diastolic performance—was impaired in obese as compared with control mice (Figure 1K). Interestingly, HFD-fed JunD−/− mice displayed reduced LV mass and were protected against LV dysfunction, thus suggesting a protective role of JunD deletion in obesity-induced cardiomyopathy (Figure 1F–K, Supplementary material online, Table S3).

JunD regulates PPARγ signalling in the obese heart

Given the pivotal role of peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ) in the pathogenesis of MC, their expression was investigated in CD-fed and HFD-fed WT and JunD−/− mice. Myocardial gene and protein expression of both PPARα and PPARγ were increased in WT obese as compared with lean mice, while they were almost blunted in HFD-fed JunD−/− animals (Figure 2A–D). Next, we asked whether JunD directly regulates the transcription of PPARα and PPARγ. Chromatin immunoprecipitation (ChiP) assay showed that JunD binds PPARγ (region 100/-199) but not PPARα promoter in the heart of obese mice (Figure 2E). A custom real-time PCR array revealed a profound dysregulation of PPAR-dependent genes implicated in fatty acid synthesis (i.e. fatty acid synthase, Fas), uptake [i.e. Cd36, fatty acid binding protein 3 (Fabp3), and 4 (Fabp4)], hydrolysis (i.e. lipoprotein lipase, Lpl), and storage (i.e. perilipin 5, Plin5) in obese as compared to control hearts (Figure 2F–H). Notably, such maladaptive changes were not observed in the heart of HFD-fed JunD−/− mice. Figure 2G and H shows significantly down- and up-regulated PPAR-dependent genes across the four experimental groups, respectively. The full list of PPAR-dependent genes is provided in Supplementary material online, Table S4.

JunD−/− mice display reduced intra-myocardial triglyceride accumulation

Based on transcriptomics data, JunD likely modulates genes involved in myocardial TG metabolism. Hence, we investigated intra-myocardial TG content in CD-fed and HFD-fed WT and JunD−/− mice. Total TG levels were increased in heart specimens from obese as compared with control mice (Figure 3A). In contrast, myocardial TG content was not significantly affected in obese mice lacking JunD (Figure 3A). The latter finding was confirmed by mass spectrometry (MS)-based quantification of cardiac TG species across the different experimental groups (Figure 3B).

Intracellular lipid accumulation leads to oxidized fatty acids and de novo synthesis of ceramides, a class of sphingolipids involved in oxidative stress and apoptosis.

Hence, we performed MS-based ceramide profiling in heart samples from CD-fed to HFD-fed WT and JunD−/− mice. We found an increase of [Cer(d18:1/16:0)] and [Cer(d18:1/24:1)] in the heart of obese as compared with lean mice, whereas both ceramides were reduced in HFD-fed JunD−/− as compared with WT animals (Figure 3C). In line with these findings, 3-nitrotyrosine (3-NT) as well as DNA and lipid peroxidation were increased in obese WT but not in JunD−/− hearts (Figure 3D–F). Apoptosis, assessed by activation of Caspase-3, was also significantly reduced in hearts from obese JunD−/− mice (Figure 3G and H).

Targeting JunD blunts PPARγ signalling in neonatal rat ventricular myocytes

To further prove the mechanistic link between JunD and PPARγ, we performed in vitro experiments in neonatal rat ventricular myocytes (NRVMs) exposed to high concentrations of palmitic acid (PA), a condition mimicking obesity and insulin resistance. Concentration-effects experiments showed that 48-h treatment with PA at the dose of 200 μM led to JunD up-regulation in NRVMs (Figure 4A, Supplementary material online, Figure S2). Increased JunD levels in PA-treated NRVMs were associated with PPARγ up-regulation (Figure 4B). Gene silencing of JunD by siRNA was sufficient to abolish PPARγ expression in NRVMs exposed to PA (Figure 4B). Furthermore, PPARγ-dependent genes involved in TG synthesis, uptake, hydrolysis, and storage, such as Fas, Cd36, Lpl, and Plin5, were blunted in JunD-depleted NRVMs (Figure 4C). Consistently, gene silencing of PPARγ-prevented lipid uptake and lipotoxic damage in PA-treated NRVM (Supplementary material online, Figure S3).

miR-494-3p regulates cardiac JunD expression in obesity

Non-coding RNAs have recently emerged as key regulators of gene expression in cardiometabolic states. Therefore, we investigated whether microRNAs regulate JunD expression in our setting. To identify possible JunD-targeting miRNAs, we analysed the 3’-UTR of JunD with two different miRNA target prediction tools (http://www.targetscan.org/; http://www.microrna.org). A pool of six miRNAs conserved among mammals or vertebrates were predicted by the two computational tools. Among conserved miRs, miR-375,
Figure 1 Genetic deletion of JunD prevents cardiomyopathy development in obese mice. (A) Real-time PCR and western blot showing gene and protein expression of JunD in heart specimens collected from control diet-fed and high-fat diet fed mice. (B) Immunohistochemistry on heart cross sections showing JunD up-regulation in obese as compared with control hearts, n = 5/group. (C) JunD transcriptional activity in nuclear extracts from control and obese mouse hearts. (D–F) Left ventricular end-diastolic diameters, volumes, and left ventricular mass of the four experimental groups. (G) Ejection fraction and fractional shortening in control and obese WT and JunD−/− mice. (H) Representative images of M-mode scans in short-axis view. (I–K) E/A ratio, isovolumic relaxation time, and myocardial performance index. Data are expressed as mean ± standard deviation. Adjusted P values (p*g) are shown for multiple comparisons. CD, control diet; HFD, high-fat diet; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LV mass, left ventricular mass; IVRT, isovolumic relaxation time; MPI, myocardial performance index.
miR-206, and miR-494-3p were selected based on (i) site-type, (ii) context score, (iii) context score percentile, (iv) weighted context score, (v) conserved branch, and (vi) the probability of conserved targeting (Figure 4D). The expression of JunD-targeting miRNAs was assessed in the heart of HFD-fed vs. CD-fed mice. We found that miR-494-3p was down-regulated in obese as compared to non-obese hearts, whereas no significant differences were observed for miR-206 and miR-375 (Figure 4E). RISC(ago2)-immunoprecipitation

Figure 2 JunD regulates PPARγ signalling in the obese heart. (A) Real-time PCR and western blot show PPARγ expression in heart specimens from control and obese mice, in the presence or in the absence of JunD deletion. (B) Immunohistochemistry showing PPARγ expression in paraffin-embedded heart cross sections from the four experimental groups, n = 5/group. (C) Cardiac gene and protein expression of PPARγ in control and obese WT and JunD−/− mice. (D) PPARγ immunohistochemistry in the different groups, n = 5/group. (E) Chromatin immunoprecipitation assay showing the interaction of JunD with PPARγ (region 1001−1999) but not PPARγ promoter in heart specimens from obese as compared with control mice. Data are expressed as mean ± standard deviation. Adjusted P values (p*g) are shown for multiple comparisons. (F) Scatter plot from real-time PCR array showing fold changes (log2 values) for PPAR-dependent genes in obese vs. control hearts. Significantly down- and up-regulated genes are represented by green and red dots, respectively. A two-fold change was considered to indicate significant variations in gene expression. (G, H) Bars show significantly down- and up-regulated PPAR-dependent genes among the four experimental groups, n = 5/group. *P < 0.05 vs. WT CD; **P < 0.05 vs. WT HFD. CD, control diet; HFD, high-fat diet.
was employed to demonstrate whether miR-494-3p affected the interaction of JunD mRNA with Argonaute 2 (Ago2), a major component of the RISC complex. The association of JunD mRNA with Ago2 was assayed by real-time PCR of JunD mRNA in Ago2 immunoprecipitates (IP). We found that JunD mRNA in Ago2 IP samples was enriched by miR-494-3p, thus validating that miR-494-3p facilitates endogenous JunD mRNA association with RISC (Figure 4F). To confirm that miR-494-3p directly represses JunD expression, a luciferase gene reporter assay was performed in NRVMs. To assess the effects of miR-494-3p on JunD 3'-UTR, luciferase reporter plasmids containing full length JunD 3'-UTR (WT) or 3'-UTRs with a mutation in miR-494-3p ‘seed’-binding site (JunD 3'-UTRmut) were constructed. In the luciferase reporter assay, co-transfection of JunD 3'-UTR with miR-494-3p caused a significant reduction of 3'-UTR JunD luciferase activity as compared to allStar negative control. In contrast, cells transfected with JunD 3'-UTRmut construct were not sensitive to miR-494-3p treatment (Figure 4G).

We then overexpressed miR-494-3p in PA-treated NRVMs to investigate its putative role in preventing lipotoxic damage through modulation of JunD levels. Consistent with our observations in the mouse, PA treatment significantly reduced miR-494-3p expression in cultured NRVMs (Supplementary material online, Figure S4).

Figure 3 JunD /mice are protected against obesity-induced cardiac steatosis and lipotoxic damage. (A) Triglyceride content in heart homogenates from control and obese WT and JunD /mice. (B) Lipidomic-based quantification of triglyceride species among the different experimental groups (n = 5/group). (C) Ceramide profiling shows altered levels of Cer[(18:1/16:0)] and Cer[(d18:1/24:1)] in WT obese but not in JunD /mice. (D–F) Oxidative stress marker 3-nitrotyrosine, 8-hydroxy-2-deoxyguanosine levels, and lipid peroxidation in heart specimens from the different experimental groups. (G, H) Cardiac activity and expression of apoptotic protein Caspase-3, as assessed by ELISA and immunohistochemistry, respectively. n = 5/group. Data are expressed as mean ± standard deviation. Adjusted P values (p*g) are shown for multiple comparisons.
Figure 4 miR-494-3p orchestrates cardiac JunD expression in obesity. (A) JunD gene and protein expression in neonatal rat ventricular myocytes exposed to vehicle or palmitic acid (200 μM) for 48 h. (B) Real-time PCR and western blot showing PPARγ expression in palmitic acid-treated neonatal rat ventricular myocytes, in the presence or in the absence of JunD knockdown by small interfering RNA. A scrambled siRNA was used as a negative control. (C) Expression of PPAR-dependent genes involved in triglyceride metabolism among the different experimental groups. \( n = 5-6 / \) group. (D) Diagram showing conserved JunD-targeting microRNAs (miR-494-3p, miR-206, and miR-375) based on in silico prediction analysis. (E) Effects of miR-494-3p overexpression on JunD levels in palmitic acid-treated neonatal rat ventricular myocytes. NC-mimic was used as a negative control for miR-494-3p mimic. (F) Luciferase activity assessed in neonatal rat ventricular myocytes transfected with (i) 3’-UTR JunD + NC-mimic, (ii) 3’-UTR JunD + miR-494-3p, and (iii) 3’-UTR JunDmut + miR-494-3p. (H) Effects of miR-494-3p overexpression on JunD levels in palmitic acid-treated neonatal rat ventricular myocytes. Fas, fatty acid synthase; Lpl, lipoprotein lipase; Plin5, perilipin 5.
Conversely, miR-494-3p overexpression attenuated PA-induced JunD up-regulation (Figure 4H). Overexpression of miR-494-3p also reduced myocardial TG uptake, oxidative stress and apoptosis, thus confirming its role in the regulation of JunD/PPARγ axis (Figure 4I–K).

Cardiac-specific JunD overexpression mimics obesity-induced metabolic cardiomyopathy

To determine whether JunD-dependent regulation of TG metabolism is cardiomyocyte specific, we used transgenic mice overexpressing JunD (JunDTg) via the cardiac α-myosin heavy chain (α-MHC) promoter.22 WT littermates were used as a control group. Western blot analysis and immunohistochemistry confirmed overexpression of JunD in the heart (Supplementary material online, Figure S5). Cardiac-specific overexpression of JunD was associated with an impairment of LV systolic function, as assessed by EF and FS (Figure 5A and B, Supplementary material online, Table S5). Indices of diastolic function and LV performance were also impaired in JunDTg as compared to WT littermates (Figure 5C–E). Of interest, PPARα and PPARγ expressions in the heart of WT and cardiac-specific JunDTg mice, as assessed by real-time PCR, western blot, and immunohistochemistry, confirmed JunD binding to PPARγ promoter (Figure 5J).

Chromatin immunoprecipitation assay showing JunD binding to PPARγ promoter. Cardiac expression of PPAR-dependent genes in WT and JunDTg mice, n = 5–6/group. (L–N) Intra-myocardial triglyceride content, oxidative stress, and Caspase-3 activity are enhanced in JunDTg as compared to WT littermates. Results are presented as mean ± standard deviation. IVRT, isovolumic relaxation time; MPI, myocardial performance index; Fas, fatty acid synthase; Plin5, perilipin 5; Lpl, lipoprotein lipase.

Figure 5
Cardiac-specific JunD overexpression mimics metabolic cardiomyopathy. (A) Left ventricular function in WT and cardiac-specific JunDTg mice, assessed by ejection fraction and fractional shortening. (B) Representative M-mode images of the left ventricle at the papillary muscle level in WT and JunDTg mice. (C–E) E/A ratio, isovolumic relaxation time, and myocardial performance index across the different groups. (F–I) PPARα and PPARγ expressions in the heart of WT and cardiac-specific JunDTg mice, as assessed by real-time PCR, western blot, and immunohistochemistry. (J) Chromatin immunoprecipitation assay showing JunD binding to PPARγ/PPARγ promoter. (K) Cardiac expression of PPAR-dependent genes in WT and JunDTg mice, n = 5–6/group. (L–N) Intra-myocardial triglyceride content, oxidative stress, and Caspase-3 activity are enhanced in JunDTg as compared to WT littermates. Results are presented as mean ± standard deviation. IVRT, isovolumic relaxation time; MPI, myocardial performance index; Fas, fatty acid synthase; Plin5, perilipin 5; Lpl, lipoprotein lipase.

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JunD/PPAR\(^c\) signalling in cardiac specimens from obese patients

In order to translate our experimental findings to human disease, JunD/PPAR\(^c\) signalling was investigated in right atrial myocardial specimens collected from obese patients and non-obese controls undergoing elective cardiac surgery. (Figure 6A) JunD gene and protein expression were higher in the myocardium of obese patients as compared to lean controls. (Figure 6B) Real-time PCR shows significant miR-494-3p down-regulation in the myocardium from obese patients as compared with non-obese controls. (Figure 6C) Expression of PPAR\(^c\), PPAR\(^a\), and lipid droplet-associated genes Cd36, Fas, Plin5, and Lpl in the two groups (\(n = 15\)–17/group). (Figure 6D) Myocardial triglyceride content is increased in obese as compared with non-obese patients. Results are presented as mean ± standard deviation. Fas, fatty acid synthase; Lpl, lipoprotein lipase; Plin5, perilipin 5.

Discussion

The present study demonstrates that the AP-1 transcription factor JunD participates to MC both in experimental and human obesity (Take home figure). Several lines of evidence support our conclusions: (i) JunD is up-regulated in the heart of obese mice, and contributes to transcriptional modulation of PPAR\(^c\) and PPAR\(^a\)-dependent genes implicated in TG uptake, storage, and hydrolysis; (ii) obese mice with genetic deletion of JunD do not show alterations of PPAR\(^c\) signalling and are protected against myocardial TG accumulation, lipotoxic damage, and LV systolic dysfunction; (iii) cardiac-specific overexpression of JunD in non-obese mice mimics molecular and functional features of MC; (iv) miR-494-3p directly targets JunD in the obese heart, and its overexpression prevents JunD/PPAR\(^c\) activation with the expression of PPAR\(^a\), PPAR\(^c\), and PPAR-dependent genes involved in myocardial TG accumulation (Supplementary material online, Figure S7). Negative correlations were found with indices of LV systolic function, such as EF and FS as well as with miR-494-3p (Figure 7E–G).
and lipotoxic damage in cultured NRVMs; and (v) miR494-3p/JunD/PPARγ pathway is dysregulated in the myocardium of obese patients as compared to non-obese controls, and correlates with cardiac TG content as well as with echocardiographic indices of cardiac function.

 Obesity currently affects 400 million people worldwide and it is emerging as a potent risk factor for the development of LV dysfunction, independently of hypertension, coronary artery disease, or valvular heart disease.23,24 Prevalence of obesity continues to escalate and will have an important impact on the development of HF in the decades to come.25 Of note, little diagnostic and therapeutic tools are available to fight obesity-related MC and its complications. Altogether these factors call for understanding the mechanistic basis underlying MC in obese patients. Here, we show that the AP-1 member JunD is an upstream regulator of PPARγ in the obese heart, leading to the transcription of PPAR-dependent genes (i.e. Cd36, Fabp3, Fabp4, Fas, Plin5, Lpl) involved in lipid uptake, hydrolysis, and storage. CD36 and FABP3/4 are pivotal mediators of myocardial lipid uptake, whereas LpL and Plin5 foster lipid droplet formation and storage.7 Along the same line, FAS amplifies lipid accumulation by promoting de novo synthesis of fatty acids in myocardial cells.26 We also observed that JunD deletion prevents obesity-induced accumulation of pro-apoptotic ceramides, namely Cer[(d18:1/16:0)] and Cer[(d18:1/24:1)]. The relevance of this finding is supported by the notion that both these ceramides predict HF and mortality in patients with cardiovascular disease.55 Although JunD activation was associated with the induction of both PPARα and PPARγ, ChIP assay showed that the former is not directly regulated by JunD. Increased PPARα expression in our setting may be interpreted as a compensatory but futile mechanism in response to increased TG content and up-regulation of PPARα-activating genes, namely FAS.7

The impact of JunD in the setting of obesity is supported by its ability to foster macrophage activity, cytokine secretion, and steatohepatitis.16,28 Key hallmarks of cardiometabolic disturbances. Consistent with our observations, JunD+/−// mice showed reduced hypertrophic response and decreased expression of brain natriuretic peptide after transverse aortic constriction (TAC), whereas cardiomyocyte-specific overexpression of JunD was associated with LV dilation and dysfunction.22 In contrast, other studies reported that JunD attenuates both phenylephrine and TAC-mediated cardiomyocyte hypertrophy,29,30 suggesting a dual role in hypertrophic response upon different stimuli and experimental conditions. Previous work showed that mice with JunD deletion have enhanced insulin signalling and reduced weight gain when fed a HFD.15,16 Similarly, in the present study we observed that obese JunD−/− mice had lower body weight as compared to obese WT animals. To rule out possible systemic effects of JunD deletion in obesity, we studied mice with cardiac-specific overexpression of JunD. The latter experimental model allowed us to conclude that cardiac JunD activation is sufficient to induce maladaptive metabolic remodelling and lipotoxic damage, thus phenocopying obesity-related MC.

Given the emerging link among environmental factors, obesity, and non-coding RNAs,31 we explored the role of miRNAs as modulators of JunD levels. Among JunD-targeting miRNAs, we found that miR-494-3p was down-regulated in the heart of obese mice as compared
to lean controls. Interestingly, overexpression of miR-494-3p blunted JunD levels and lipotoxic damage in NRVMs exposed to PA, a well-established model mimicking the effects of obesity. These findings are in line with previous work showing that cardiac-specific overexpression of miR-494-3p protects against ischaemia–reperfusion injury and myocardial apoptosis.

As a clinical translation, we also demonstrate that JunD signalling was profoundly altered in the myocardium of obese patients and was associated with up-regulation of lipid droplet-associated genes and TG accumulation. Interestingly, JunD gene expression correlated with echocardiographic indices of systo-diastolic function, such as EF, FS, and TDI-derived E/E' ratio, a sensitive indicator of diastolic dysfunction and cardiovascular events.

Our manuscript has some limitations. First, obesity-driven JunD up-regulation was not determined in an unbiased way. Our approach does not allow to appreciate the differential contribution of other transcription factors to the observed phenotype. However, transcriptomic analysis in T2D rat hearts has unveiled JunD as one of the top-ranking up-regulated genes, suggesting its pivotal role in comparison with other transcription factors. Second, a constitutive JunD

**Take home figure** Schematic showing JunD role in metabolic cardiomyopathy. Obesity-induced miR-494-3p down-regulation leads to increased JunD expression and binding to PPARγ promoter. JunD-dependent activation of PPARγ fosters transcriptional programmes enabling myocardial triglyceride accumulation, oxidative stress and apoptosis with subsequent development of metabolic cardiomyopathy. JunD deletion in obese mice prevents triglyceride accumulation and metabolic cardiomyopathy, whereas cardiac-specific overexpression (α-MHC-JunD^TG^) in lean mice mimics metabolic cardiomyopathy. Targeting miR-494-3p or JunD in neonatal rat ventricular myocytes blunts triglyceride accumulation and lipotoxic damage. NRVMs, neonatal rat ventricular myocytes.
knockout model was employed in our study, hence we cannot exclude the possibility that the absence of JunD in tissues other than the myocardium could have played a role in our setting. Nevertheless, experiments in mice with cardiac-specific overexpression of JunD and cultured myocytes suggest that cardiac JunD activation is sufficient to induce maladaptive metabolic remodelling and lipotoxic damage. Third, we did not investigate whether JunD impacts on myocardial insulin resistance, a key feature of obesity cardiomyopathy. Our findings are in line with previous work showing that JunD negatively regulates insulin signalling. Moreover, CD36 has been regarded as an insulin-resistance gene causing defective fatty acid and glucose metabolism. Here, we show that JunD leads to a PPARγ-dependent up-regulation of CD36, and this might contribute to dampen myocardial insulin signalling by shifting the metabolic substrate.

In conclusion, we have employed an advanced micro-ultrasound imaging system which allows to reduce speckle noise and artefacts while preserving and enhancing critical information. However, the echocardiographic assessment of cardiac function in small animals presents some limitations mainly due to elevated heart rate.

In conclusion, our translational study unveils a new molecular mechanism contributing to obesity-related MC. These findings improve our understanding of the pathophysiology of MC, and may set the stage for novel strategies to prevent or treat lipotoxic LV dysfunction in patients with obesity.

**Supplementary material**

Supplementary material is available at *European Heart Journal* online.

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