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14. ABSTRACT

Peripartum cardiomyopathy (PPCM) is marked by loss of cardiac contractile function in women late in pregnancy or soon after delivery. PPCM affects approximately 1:1000 births worldwide, and frequently leads to chronic heart failure, cardiac transplantation, or death. Our proposal was focused on understanding how mutations in TTN predispose to PPCM.

Aim 1: Human genetics: we collated a cohort of >600 clinically annotated women with PPCM, and performed deep sequencing of the TTN gene, followed by detailed phenotype/genotype correlations, including evaluation of correlation between TTN mutations and severity of disease on presentation, and recovery from PPCM. This work was published in Circulation in May of 2021. We also analyzed the UPenn and Geisinger Biobank data sets of >70,000 exomes for TTN truncating variants, and concluded that surprisingly large number of people carry these variants without having PPCM or other dilated cardiomyopathy. This work was published in Circulation in April of 2019.

Aim 2: Mouse genetics: we generated mice genetically modified to encode mutations in *ttn* that precisely match those found in three women with PPCM. Baseline phenotyping revealed little baseline dysfunction. Data with cardiotoxic stressors such as chemotoxic agents, transverse aortic banding, and adrenergic infusions, has revealed surprisingly little effect, compared to control animals. On the other hand, data indicated that repeated pregnancies (n=five) do worsen function in these animals, compared to controls. Works to test the impact of various pregnancy hormones on cardiac function in these *ttn* knock-in mice has not identified causal hormones.

Aim 3: Cellular studies: we generated iPSCs bearing the same TTN mutations as in mice above. We have also harvested adult human cardiomyocytes from post-transplant hearts of patients bearing TTN truncating variants (n=4 so far) and compared their biomechanical phenotype compared to patients with cardiomyopathy without TTN variants, and to nonfailing donor subjects. We have found surprisingly that TTNtv-bearing cells contract nearly as well as cells from nonfailing controls, and clearly better than cells from other failing hearts. We have also evaluated in detail tissue from these human hearts with TTN truncating variants (n=22), and have demonstrated conclusively that mutant protein can be found in these hearts, which has long been a controversial issue. This work was published in Science Translational Medicine in 2021. We have also demonstrated in explanted crushed cardiac tissue, using novel antibodies specific to the TTN truncated protein, that the truncated protein incorporates into the sarcomere in adult human hearts. This work is being prepared for submission.

15. SUBJECT TERMS

Peripartum Cardiomyopathy, Human genetics, Women's cardiovascular disease

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INTRODUCTION

Peripartum Cardiomyopathy (PPCM) is a disease characterized by systolic heart failure presenting in the last month of pregnancy or the 1st five months postpartum. It affects anywhere from 1:300 to 1:3000 births, with certain geographic “hot spots” like Haiti. In about half of the cases, heart failure persists, and often leads to cardiac transplantation or death. PPCM accounts for >50% of cases of maternal cardiogenic shock in the US, and it has become the leading cause of maternal peripartum death. The disease affects all socioeconomic and racial sectors, but is more common and bears significantly worse prognosis in women of African descent, a group that is also more highly represented in the US Armed Forces. Mortality rates are as high as 25% at 5 years in African American women in the US.⁴ The consequences of PPCM are thus often devastating for otherwise healthy young women and their newborn infants. The precise cause of PPCM remains elusive. There is limited ongoing research into the mechanisms underlying PPCM, and almost none in the US. Advocacy for PPCM has been poor, limiting public awareness and funding support. Recent work from our lab and others in Germany, however, has uncovered the novel notion that PPCM is a vascular disease. And most recently, we have made the additional breakthrough observation that many women with PPCM bear mutations in the large sarcomeric protein titin (the gene *TTN*). These observations have raised considerable excitement about potential novel ways to diagnose, prognose, and treat PPCM. The paradigm-shifting finding that women with PPCM frequently bear mutations in *TTN* has led to key unanswered questions:

Clinically: Does the presence of *TTN* mutations in women with PPCM predict severity of disease and/or clinical outcome? Based on *post-hoc* analyses of our initial study, we **hypothesize that the presence of *TTN* mutations correlates with diminished cardiac recovery and higher risk of recurrence with subsequent pregnancy.**

Mechanistically: How do mutations in *TTN* affect cardiomyocyte function and interact with pregnancy to cause PPCM? **We hypothesize that *TTN* mutations reduce the metabolic efficiency of contraction/relaxation, leading to contractile defects that become pathogenic in the metabolically vulnerable context of late pregnancy.**

KEYWORDS

Peripartum cardiomyopathy, heart failure, titin, TTN, pregnancy, women, vascular

ACCOMPLISHMENTS

What were the major goals of the project?

Guided by the hypotheses above, we proposed to take a comprehensive functional genomics approach to investigate in depth the contribution of *TTN* mutations to PPCM, including clinical, pre-clinical, and cellular studies:

Aim 1: Test in humans if the presence of *TTN* mutations predicts clinical outcomes.

Aim 2: Test in mouse models if and how *TTN* truncating variants predispose to PPCM.

Aim 3: Test in cell culture if and how *TTN* variants cause contractile and metabolic insufficiency.

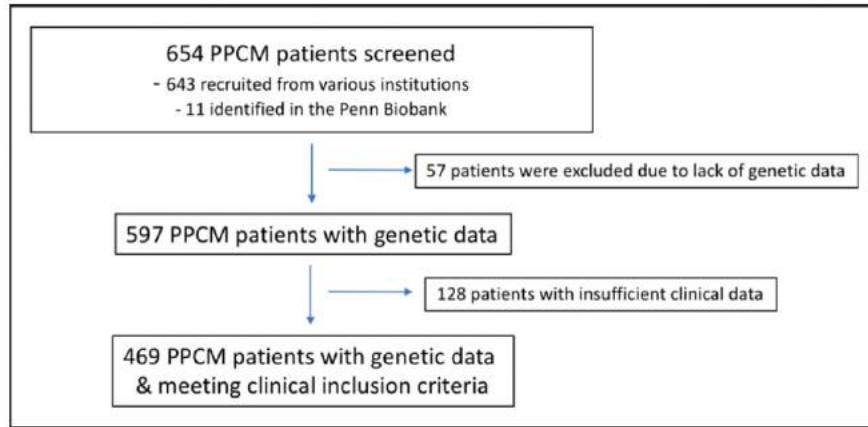
What was accomplished under these goals?

The following is organized following the outline of the original SOW:

Aim 1: Test in humans if the presence of *TTN* mutations predicts clinical outcomes.

Major Task 1 : Acquisition of PPCM DNA Samples

We began by expanding our multi-center PPCM Collaborative Network, and have assembled now more than 600 cases of PPCM, including blood samples and comprehensive clinical annotations. Women with PPCM were retrospectively identified from several US and international academic centers (**Subtask 1**), and clinical information and DNA samples were acquired (**Subtask 2**). Clinical information was collated in a RedCAP database and linked to subsequent sequencing data (**Subtask 3**). 654 women with PPCM were identified. Of these, 185 were excluded for further analyses due to insufficient clinical data or genetic data (figure 1).



The demographic and clinical data of the final 469 cases is shown in table 1:

Characteristics	University of Pennsylvania	Japan	IPAC	IMAC-2	Germany	Chicago	Pitt
No. of patients	106	8	82	31	108	4	25
Age, y	30.49±6.7	30.63±3.5	29.89±6.3	31.29±6.8	33.85±5.2	23.50±8.6	29.67±5.6
European descent, n (%)	48 (46.1)	0	52 (63.4)	20 (63.5)	47 (90.4)	0	16 (64)
African descent, n (%)	53 (51.0)	0	26 (31.7)	11 (35.5)	3 (5.8)	3 (75)	9 (36)
Other race, n (%)	3 (2.9)	8	4 (4.9)	0	2 (3.8)	1 (25)	0
Left ventricular ejection fraction	27.2±12.4	29.1±10.1	29.5±9.2	27.2±7.4	26.7±9.0	32.9±6.0	27.8±10.2
Gestational hypertension or pre-eclampsia, n (%)	42/104 (40.4)†	6/8 (75)	32/82 (39)	NA	24/64 (37.5)†	1/3 (33.3)	8/25 (32)

Hopkins	Washington University	Yale	Israel	Denmark	Total	USA	Non-USA	P value*
19	19	4	41	22	469	290	179	
32.47±4.8	27.67±6.9	27.75±5.9	32.15±6.3	30.59±6.7	30.94±6.4	30.13	32.64	1.3×10 ⁻⁵
12 (63.2)	10 (55.6)	1 (25)	35 (87.5)	22 (100)	263 (64.1)	159 (55.21)	62 (82.67)	2.62×10 ⁻⁵
5 (26.3)	8 (44.4)	2 (50)	5 (12.5)	0	126 (30.7)	118 (40.97)	5 (6.67)	1.87×10 ⁻¹⁹
2 (10.5)	0	1 (25)	0	0	21 (5.1)	11 (3.82)	8 (10.67)	
27.8±10.6	31.3±8.3	27.0±12.4	35±8.1	25.9±9.5	28.5	28.43	28.6	0.85
3/18 (16.7)†	9/19 (47.4)	1/3 (33.3)	6/41 (14.6)	10/22 (45.5)	142/389 (36.5)	96/254 (37.8)	46/135 (34.1)	0.51

Table 1: Demographic and clinical characteristics in each cohort of the current study. *some patients missing data on hypertensive disorder. ** P-value US vs non-US by Student's t-test for continuous variables and by Chi-squared test for discrete variables.

Major Task 2: DNA preparation, sequencing, analysis

Next-generation sequencing was performed on 67 genes, including *TTN*, and evaluated for burden of truncating and missense variants (**Subtask 1**). Where indicated, the variants were confirmed by Sanger sequencing (**Subtask 2**). As noted above, 469 women met inclusion criteria. 10.4% of women with PPCM bore *TTN*ts (Odds ratio [OR]=9.4 compared with 1.2% in

reference population; Bonferroni-corrected P [P^*] = 1.2×10^{-46}). We additionally identified overrepresentation of truncating variants in *FLNC* (OR=24.8, $P^*=7.0 \times 10^{-8}$), *DSP* (OR=14.9, $P^*=1.0 \times 10^{-8}$), and *BAG3* (OR=53.1, $P^*=0.02$), genes not previously associated with PPCM. The identified variants are listed in table 2:

Genes	Peripartum cardiomyopathy		Controls		Variant excess, %	Odds ratio (95% CI)	Nominal P value	Corrected P value*
	Cohort variants	Cohort frequency, %	Genome Aggregation Database variants	Genome Aggregation Database frequency, %				
<i>BAG3</i>	1	0.21	5	0.004	0.209	53.05 (6.19–454.96)	2.92×10^{-4}	1.96×10^{-2}
<i>DES</i>	1	0.21	28	0.025	0.189	8.72 (1.18–64.2)	3.36×10^{-2}	1
<i>DSP</i>	6	1.28	108	0.087	1.192	14.9 (6.51–34.06)	1.55×10^{-10}	1.04×10^{-8}
<i>FKTN</i>	1	0.21	57	0.048	0.165	4.46 (0.62–32.28)	0.14	1
<i>FLNC</i>	4	0.85	41	0.035	0.818	24.75 (8.83–69.38)	1.05×10^{-9}	7.02×10^{-8}
<i>ILK</i>	1	0.21	59	0.048	0.166	4.48 (0.62–32.4)	0.14	1
<i>MYH6</i>	2	0.43	153	0.123	0.303	3.47 (0.86–14.05)	0.08	1
<i>MYH7</i>	2	0.43	94	0.075	0.351	5.7 (1.4–23.21)	1.50×10^{-2}	1
<i>PLEC</i>	1	0.21	169	0.157	0.056	1.36 (0.19–9.73)	0.76	1
<i>TMPO</i>	1	0.21	87	0.069	0.144	3.08 (0.43–22.13)	0.26	1
<i>VCL</i>	1	0.21	59	0.047	0.166	4.54 (0.63–32.84)	0.13	1
<i>TTN</i>	49	10.45	1453	1.224	9.224	9.42 (6.97–12.71)	1.81×10^{-48}	1.21×10^{-46}
Summary	70	14.93	5103	3.619	11.306	2.25 (1.74–2.9)	5.18×10^{-10}	3.47×10^{-8}

Table 2: Prevalence of rare protein truncating variants in peripartum cardiomyopathy (PPCM). The prevalence of truncating variants in patients with PPCM is compared with controls from the Genome Aggregation Database (gnomAD). Nominal P values are from Odds Ratios, and Corrected P^* values are adjusted for testing 67 genes with the Bonferroni method.

This profile of genetic variants is highly similar to that found in non-ischemic dilated cardiomyopathy (DCM). The work reveals a degree of genetic similarity between PPCM and DCM, suggesting that gene-specific therapeutic approaches being developed for DCM may also apply to PPCM, and that approaches to genetic testing in PPCM should mirror those taken in DCM. The high similarity to DCM is shown in figure 2:

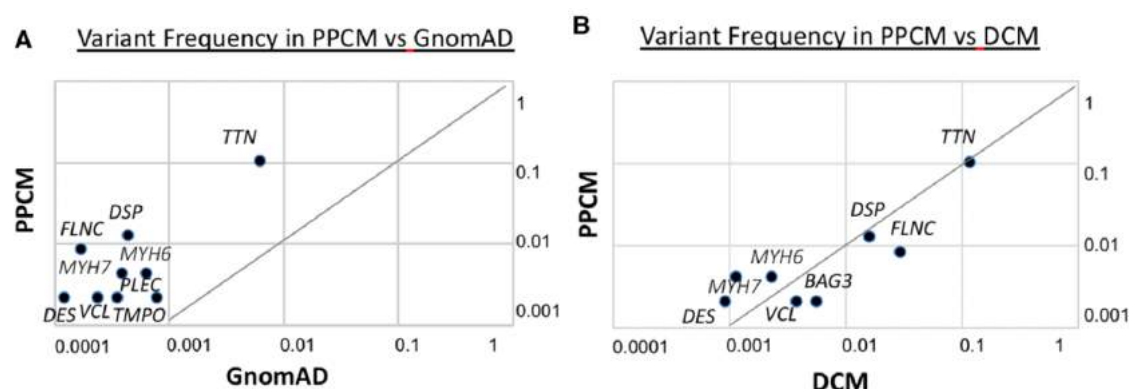


Figure 2: Comparison of the prevalence of rare truncating variants between peripartum cardiomyopathy (PPCM) and the Genome Aggregation Database (gnomAD, a), and between PPCM and non-ischemic dilated cardiomyopathy (DCM, b). Variant frequencies for DCM are from Mazarrotto et al. 2019, except for *FLNC*, which is from Ader et al. 2019. Diagonal line represents the equivalence line.

Next, the impact of TTNtvs on severity of clinical presentation, and on clinical outcomes, was evaluated (**Subtask 3**). Women with TTNtvs had lower left ventricular ejection fraction (LVEF) on presentation than did women without TTNtvs (23.5% vs 29%, $P=2.5 \times 10^{-4}$), but did not differ significantly in timing of presentation after delivery, in prevalence of preeclampsia, or in rates of clinical recovery, as outlined in table 3:

Characteristic	TTN truncating variant present (n=45)	TTN truncating variant absent (n=424)	P value
Age at diagnosis, y	30.40±7.04	31.03±6.28	0.41
Ejection fraction, %	23.51±8.99	29.08±10.02	2.52×10^{-4}
Race/ethnicity, % (No./total No.)			0.53
Black	24.32 (9/37)	31.38 (116/372)	
White	67.57 (25/37)	63.98 (238/372)	
Other	8.11 (3/37)	4.84 (18/372)	
Gestational hypertension,* % (No./total No.)	25 (9/36)	37.5 (132/352)	0.14
Left ventricular end-diastolic diameter,† cm	5.71±1.33	5.68±0.77	0.93
Left ventricular end-systolic diameter,† cm	4.97±1.34	4.70±0.90	0.34
Recovery, % (No./total No.)	67.5 (27/40)	59.6 (211/354)	0.33
Negative outcome,‡ % (No./total No.)	21.74 (5/23)	17.42 (27/155)	0.59

Table 3: Comparison of clinical characteristics between peripartum cardiomyopathy (PPCM) patients with high-percentage spliced-in (hiPSI) TTN truncating variants (TTNtvs), and those without. **Includes patients with gestational hypertension, mild and severe pre-eclampsia, and superimposed pre-eclampsia. *** Measured on TTE from initial presentation. # Negative outcome counted as EF≤35 at 12 months follow up, LVAD, heart transplantation, or death. P values are from Student's t-test for continuous variables and by Chi-squared test for discrete variables.

Overall, this study provides the first extensive genetic and phenotypic landscape of PPCM, and demonstrates that predisposition to heart failure is an important risk factor for PPCM. The clarification of genotype/phenotype associations has important implications for genetic counseling. The work is now published (**Subtask 4**) in *Circulation* (Goli et al. 2021), and the genetic implications further discussed also in *Circulation* (Arany, 2022).

In parallel work, we continued to leverage our large cohort of women with PPCM at the University of Pennsylvania to better understand the strikingly higher incidence of PPCM and worse outcomes in Black women (a group that is more highly represented in the US Armed Forces). We showed in 2017 that Black women with PPCM have persistent dysfunction at twice the rate of White women, and that even when recovery does occur, it takes twice as long. We then used geocoding and street-level US Census data to demonstrate that these racial inequities are in fact largely explained by socioeconomic inequities, most specifically education status. These observations are actionable and have high implications for clinical care, as they show that racial inequities in PPCM are not inherently genetic and thus can be addressed. This work was published in the *American Heart Journal* in 2021 (see Appendix).

Aim 2 (Major Task 3): Test in mouse models if and how *TTN* truncating variants predispose to PPCM.

We have used CRISPR/Cas9 to generate 3 knock-in mice bearing *TTN* mutations identical to those identified in women with PPCM (**Subtask 1**). Baseline phenotyping has revealed little baseline dysfunction, as measured by echocardiography and classic morphometric measurements. We proceeded to challenge the mice (**Subtask 2**) with various stressors, including chemotoxic agents, because we had found, in work with Drs. Jon and Cricket Seidman, that women with breast cancer and with *TTN* truncating variants were also predisposed to develop chemotherapy-induced cardiomyopathy. Interestingly, the knock-in mice bearing *TTN* mutations were overly sensitive to treatment with doxorubicin, as assessed by echocardiography (**Subtask 3**), demonstrating synergy between these genetic and environmental factors in vivo, but the mice were not sensitized to bortezomib, a proteasome inhibitor often used in the treatment of breast cancer. Part of this work was published in *Circulation* in July 2019 (see Appendix) and is shown in figure 3:

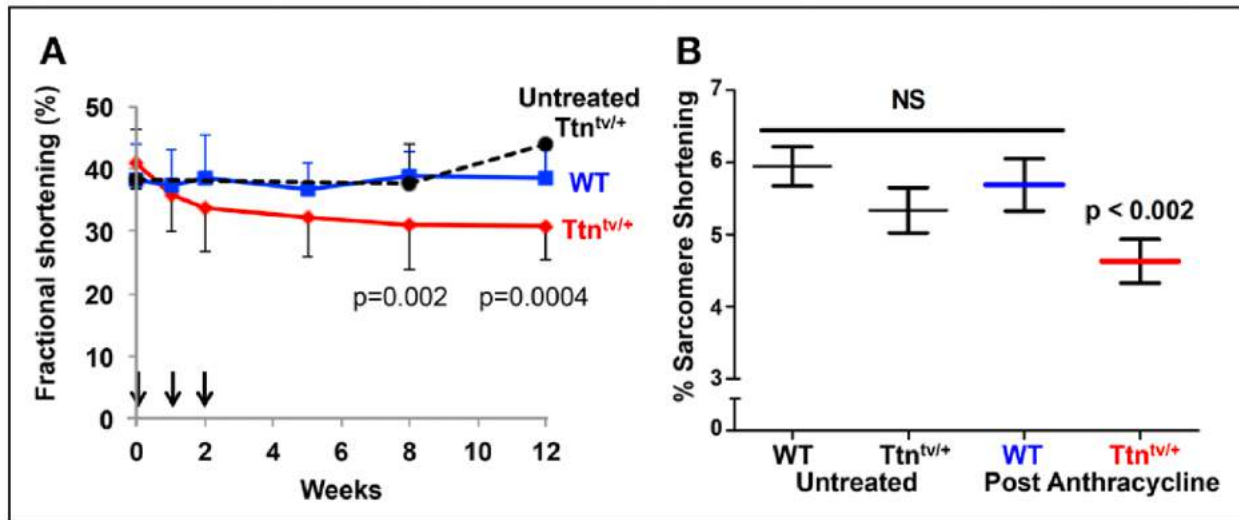


Figure 3: Persistent cardiac dysfunction in Tntn^{tv/+} mice after anthracycline treatment.

A, Untreated Tntn^{tv/+} mice have left ventricular function comparable with wild-type (WT) mice.^{25,26} Intraperitoneal doxorubicin (5mg/kg) was administered (arrows) to WT and Tntn^{tv/+} mice (n=15 per genotype) in 3 successive weekly doses (cumulative dose=45 mg/m²). Serial echocardiograms showed persistent significantly depressed systolic function (mean fractional shortening $\pm$ SD) in Tntn^{tv/+} in comparison with WT mice (P=0.0004). B, Isolated cardiomyocytes (n $\geq$ 52 per group) were studied 12 weeks after initial doxorubicin injection. Cardiomyocytes from doxorubicin-treated Tntn^{tv/+} mice had significantly depressed contractility (P<0.002) in comparison with cardiomyocytes from doxorubicin-treated WT mice or untreated mice. NS indicates not significant.

In addition, we find that repeated pregnancies (five) cause more cardiac dysfunction in mice bearing TTN truncating variants than in control mice, consistent with a model of PPCM. These data suggested that the repeated exposure to pregnancy hormones can trigger PPCM. We therefore generated multiple reagents to over-express pregnancy hormones in mice, via viral delivery to liver. However, none of these exacerbated cardiac function in our TTN knock-in mice. Treating the mice with transverse aortic banding surgery, or implantable continuous adrenergic delivery, also had no obvious negative impact on cardiac function. To try to understand why mice repeatedly displayed minimal phenotypes despite multiple insults, we tested if the truncated titin protein was expressed in TTN knock-in mice, knowing that the protein is expressed in human DCM hearts (see below). To do this, we developed proteomic techniques to detect the presence of allele-specific titin protein – i.e. determine which of the two TTN alleles is encoding for detected protein. This is a critical point, because it allows us to determine if peptides derived from the TTN^{tv}-encoding allele are detectable, even if the predicted sized protein is not apparent on gel electrophoresis. The concept for this approach is simple: we identify missense-encoding SNPs that differ between the two alleles, and then use targeted proteomic detection of predicted peptides encoding these SNPs to determine from which allele

these titin-derived peptides originate (see Fig4). In the case of detection in mice, we maximize the presence of discriminating SNPs by using F1 hybrids from different pure-background parents, one of whom bears the TTNtv. We have backcrossed our TTNtv mice into pure C57Bl/6N (importantly, not J), and we have determined that the maximum number of discordant missense variants in the TTN gene occurs when these mice are crossed to CAST/EiJ strains (93 amino acid differences). Proteomics on hearts from F1 from a cross between WT CAST/EiJ and TTNtv C57Bl/6N yield *no detectable peptides derived from the C57Bl/6N allele*, demonstrating absence of peptides from the truncated titin protein (Fig4), in sharp contrast to human TTNtv-bearing samples (see aim 3):

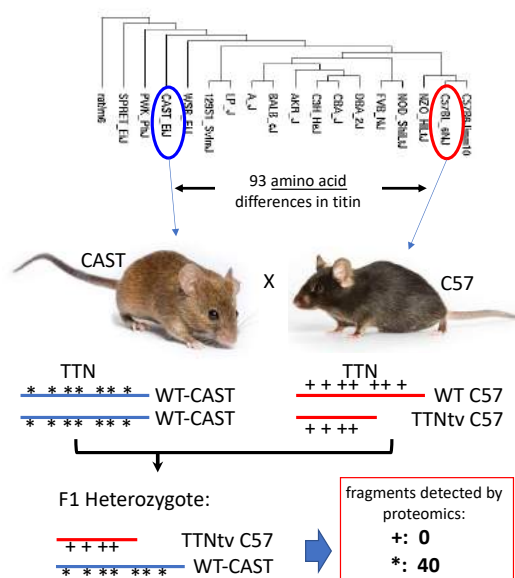


Figure 4: Proteomic detection of TTN truncated protein

We conclude that mice may not be a good model for TTNtv-bearing disease. Ongoing efforts are aimed at inhibiting proteostasis in order to test if such an approach can elicit the stability of the truncated protein and consequent phenotype.

Aim 3: Test in cell culture if and how *TTN* variants cause contractile and metabolic insufficiency.

Major Task 4: iPSCs

We have generated iPSCs bearing the same TTN mutations as in mice above (**Subtask 1**), differentiated them to cardiomyocytes (**Subtask 2**).

CHR POS	REF	VAR	TYPE	RACE	PE	EF BASE	EF 12M
179406990	C	G	Splice Site Donor	AA	no	14	21
179435628	A	T	Stop Gained	C	no	20	38
179436455	TA	T	Frame Shift	C	no	15	NA

Table 4: Sites of mutations used to generate knock-in mice and iPSCs. CHR POS: human chromosomal position. REFerent and VARiant base. AA: African American. C: Caucasian. PE: preeclampsia. EF: ejection fraction. 12M: 12 months. NA: not available, patient deceased.

Major Task 5: Measuring cardiomyocyte function

Because we found that iPSCs did not behave consistently, and that the differentiation of the cells remained quite immature (ie early fetal-stage), we decided instead to turn to adult human cardiomyocytes to measure cardiomyocyte function. We worked with Drs. Ken Margulies and Ben Prosser to harvest adult human cardiomyocytes from freshly harvested post-transplant hearts of patients bearing TTN truncating variants (n=4), and we compared their biomechanical phenotype compared to patients with cardiomyopathy without TTN variants, and to nonfailing donor subjects. This exciting work yielded the surprising observation that cells bearing TTN truncating variants derived from failing hearts performed in isolated contractile assays nearly as well as cells from nonfailing hearts, and significantly better than cells from failing hearts that did not bear TTN variants (Figure 5). This surprising observation suggests both a fundamental underlying difference between TTN variant positive and negative failing hearts, and that in hearts with TTN variants non-cell autonomous factors (e.g. preload or afterload, or extracellular milieu) play a critical role in determining heart failure.

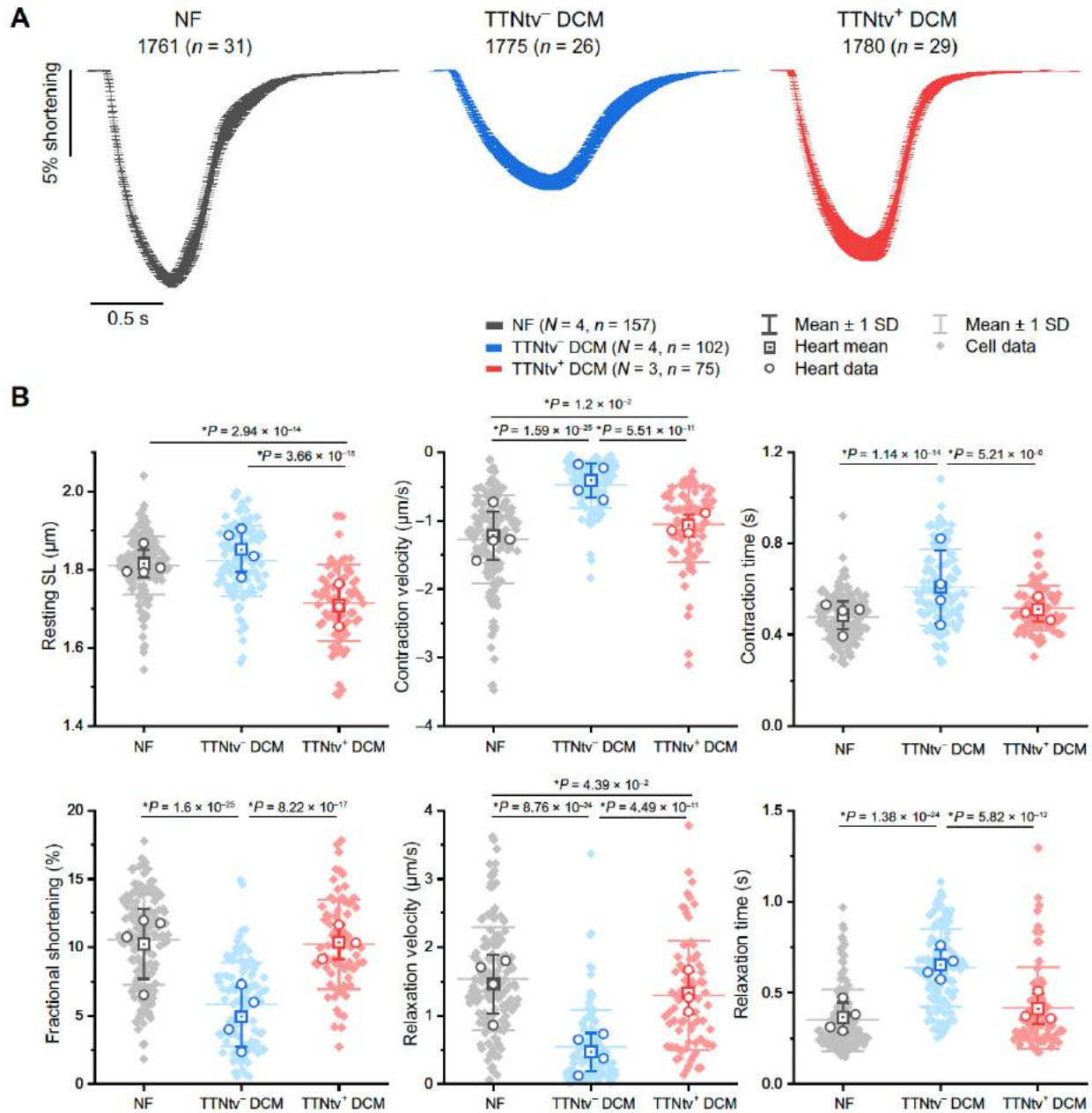


Figure 5. Contractility of intact adult cardiomyocytes isolated from TTNtv⁺, TTNtv⁻, and non-failing (NF) human hearts. ACM unloaded shortening was assessed from 7 NF donor hearts, 4 hiPSI TTNtv⁺ post-transplant hearts, and 6 TTNtv⁻ post-transplant hearts. Top, representative average ACM fractional shortening upon 0.5 Hz electrical stimulation from a NF, TTNtv⁻, and TTNtv⁺ heart. n = number of myocytes assessed per heart, data presented as mean shortening $\pm$ SE. Bottom, quantification of the indicated contractile parameters of pooled data from all assessed hearts. Statistical significance was determined by One-Way ANOVA with post-hoc Bonferroni comparisons between myocytes (closed diamonds). Between heart comparisons (open circles) were also performed, yielding similar results, and are reported in S. Data File S2, along with full contractility parameters for each individual heart.

In parallel, we further leveraged our collaborator's large collection of post-transplant human hearts to identify 19 hearts with TTNts, and we developed mass spectrometry and electrophoretic methodologies to detect truncated TTN proteins (biochemical work with TTN protein is very challenging, because the protein is >4megadaltons) to demonstrate consistent presence of truncated TTN proteins in these hearts (figures 6 and 7). This observation is of great importance, because truncated TTN protein has never been successfully detected before, and it was assumed not to exist. The work thus changes pathophysiological paradigms for both PPCM and DCM; explains why the mouse models insufficiently mirror the human disease; and expose new hypotheses about how TTNts cause cardiomyopathy (which we are addressing). This work was published in *Science Translational Medicine* in 2021 and was accompanied by highly similar work from the Linke lab in Germany, thus strongly reinforcing the conclusions.

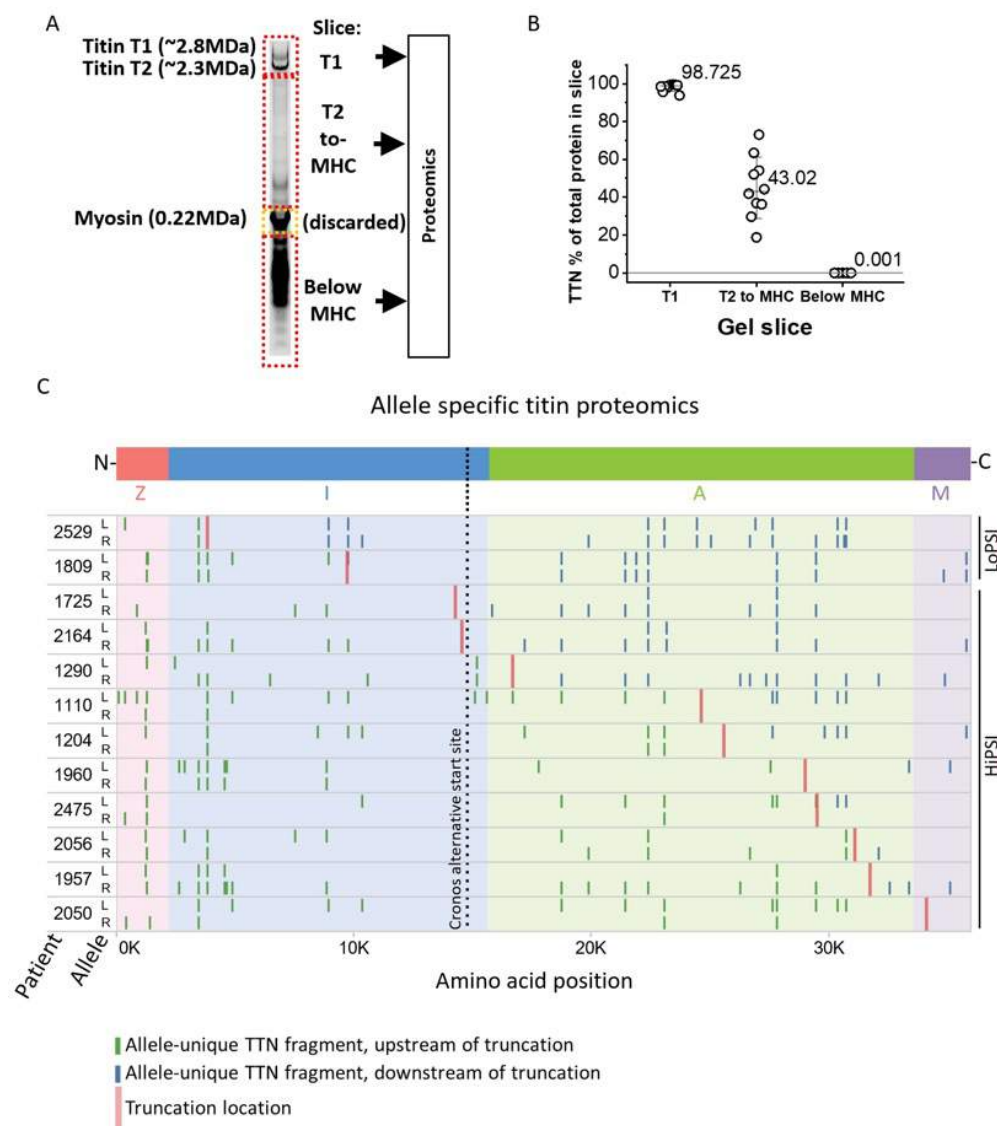


Figure 6: Proteomic detection of truncated titin proteins encoded by the allele bearing TTNtv in TTNtv heretozygous DCM hearts. (A): experimental scheme. Modified electrophoresis was used to excise three slices (labeled T1, T2-to-MHC, and below MHC), discarding the abundant myosin heavy chain (MHC). Slices were then subjected to proteomic evaluation. (B): relative abundance of peptides mapped to the TTN inferred complete isoform in each slice. (C): Mapping of identified TTN peptides to the titin protein predicted to be encoded by either allele (arbitrarily labeled L and R), using haplotype phasing and captured genomics sequences (one allele bears the TTNtv, but cannot be ascribed to L or R based on phasing). Patients 2529 and 1809 bear loPSI TTNtvs; the remainder are hiPSI TTNtvs. The Cronos alternative transcriptional start site is indicated.

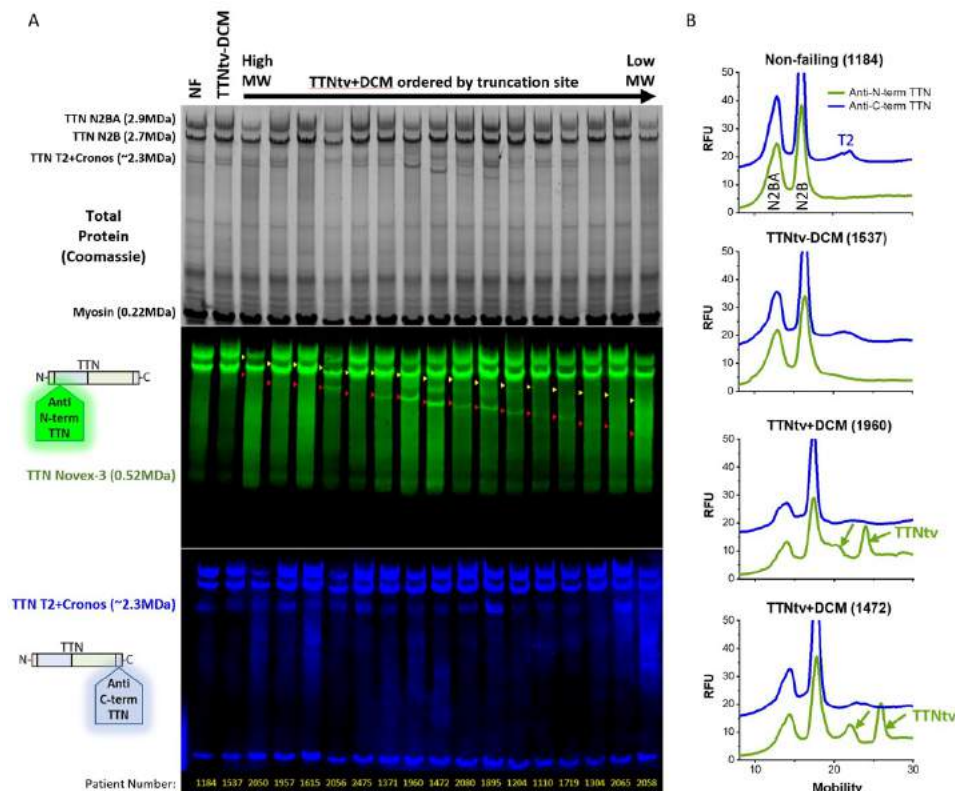


Figure 7: Detection of truncated titin proteins in TTNtv+ DCM hearts by gel electrophoresis and simultaneous two-color Western blotting. (A): Total protein Coomassie staining (top), and Western blotting with antibodies directed against the N-terminus (middle, green) and C-terminus (bottom, blue) of titin protein, of 16 TTNtv+ DCM hearts, ordered by size of predicted truncated titin protein. A nonfailing (NF) and a TTNtv- DCM heart are shown for comparison. TTN Novex: protein encoded by an alternatively spliced isoform that does not contain the epitope recognized by the C-terminus antibody. TTN Cronos: a TTN isoform encoded by the transcript originating at the alternative start site Cronos, and which does not contain the epitope recognized by the N-terminus antibody. Threshold and gamma are adjusted per channel for clarity of display. (B): Traces of Western blots with N- (green) and C- (blue) terminus antibodies with control NF and TTNtv- DCM hearts (top two) and two sample TTNtv+ DCM hearts (bottom two).

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The work we have reported has had major impact:

1. We have conclusively demonstrated and characterized the role of genetics in causing PPCM. Genetic testing of women with PPCM is, as a result, now increasingly routinely performed
2. We have conclusively demonstrated that truncated TTN proteins are present in hearts of patients with TTNtv-caused cardiomyopathy. This has fundamentally altered mechanistic understanding of the disease.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS.

Nothing to Report.

PRODUCTS

The following were funded in total or in part by this award and are appended:

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Genetic Variants Associated With Cancer Therapy–Induced Cardiomyopathy

BACKGROUND: Cancer therapy–induced cardiomyopathy (CCM) is associated with cumulative drug exposures and preexisting cardiovascular disorders. These parameters incompletely account for substantial interindividual susceptibility to CCM. We hypothesized that rare variants in cardiomyopathy genes contribute to CCM.

METHODS: We studied 213 patients with CCM from 3 cohorts: retrospectively recruited adults with diverse cancers ($n=99$), prospectively phenotyped adults with breast cancer ($n=73$), and prospectively phenotyped children with acute myeloid leukemia ($n=41$). Cardiomyopathy genes, including 9 prespecified genes, were sequenced. The prevalence of rare variants was compared between CCM cohorts and The Cancer Genome Atlas participants ($n=2053$), healthy volunteers ($n=445$), and an ancestry-matched reference population. Clinical characteristics and outcomes were assessed and stratified by genotypes. A prevalent CCM genotype was modeled in anthracycline-treated mice.

RESULTS: CCM was diagnosed 0.4 to 9 years after chemotherapy; 90% of these patients received anthracyclines. Adult patients with CCM had cardiovascular risk factors similar to the US population. Among 9 prioritized genes, patients with CCM had more rare protein-altering variants than comparative cohorts ($P \leq 1.98 \times 10^{-4}$). Titin-truncating variants (TTNtrvs) predominated, occurring in 7.5% of patients with CCM versus 1.1% of The Cancer Genome Atlas participants ($P=7.36 \times 10^{-8}$), 0.7% of healthy volunteers ($P=3.42 \times 10^{-6}$), and 0.6% of the reference population ($P=5.87 \times 10^{-14}$). Adult patients who had CCM with TTNtrvs experienced more heart failure and atrial fibrillation ($P=0.003$) and impaired myocardial recovery ($P=0.03$) than those without. Consistent with human data, anthracycline-treated TTNtrv mice and isolated TTNtrv cardiomyocytes showed sustained contractile dysfunction unlike wild-type ($P=0.0004$ and $P<0.002$, respectively).

CONCLUSIONS: Unrecognized rare variants in cardiomyopathy-associated genes, particularly TTNtrvs, increased the risk for CCM in children and adults, and adverse cardiac events in adults. Genotype, along with cumulative chemotherapy dosage and traditional cardiovascular risk factors, improves the identification of patients who have cancer at highest risk for CCM.

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Clinical Perspective

What Is New?

- This is the first study to consider the association between rare genetic variants in a large set of cardiomyopathy genes and the occurrence of cancer therapy–induced cardiomyopathy (CCM).
- We demonstrated an increased prevalence of rare variants in cardiomyopathy genes, in particular, truncating variants in the *TTN* gene, in adult and pediatric patients who have cancer with CCM.
- We confirmed human genetic data with experimental analyses, showing that anthracyclines induced protracted left ventricular dysfunction in mice with titin-truncating variants, but not in wild-type mice.

What Are the Clinical Implications?

- Our findings show that variants in cardiomyopathy genes contribute to CCM susceptibility among adult and pediatric patients with cancer.
- The identification of genetic risk factors opens new opportunities to define patients at high risk for CCM and associated adverse outcomes.
- Future investigations to define patients who have cancer with high risk for CCM through genetic testing can assess the efficacy of prophylactic cardioprotective drugs and treatment regimens to reduce CCM while providing effective cancer therapy.

Considerable advances in cancer therapies have led to major improvements in long-term survival for many malignancies, but also to unintended side effects, including cardiotoxicity.^{1,2} Cancer therapy–induced cardiomyopathy (CCM), identified as reduced left ventricular ejection fraction (LVEF) with or without signs and symptoms of overt heart failure,³ can occur during, shortly after, or many years beyond cancer treatments and affects the long-term prognosis of patients.^{1,4,5}

Anthracyclines, which are commonly used to treat both solid tumors and hematologic malignancies in children and adults,² cause cardiotoxicity in up to 10% of patients with cumulative dosages of 250 mg/m² but in 65% of patients receiving cumulative dosages >550 mg/m².⁶ Combining anthracyclines with other therapies, such as trastuzumab (an antibody targeting HER-2), can provoke greater cardiotoxicity with depressed LVEF occurring in ≈18% to 34% of treated individuals, and severe, symptomatic heart failure in 2% to 4%.^{3,7} Additional clinical parameters are recognized to contribute to CCM, including female sex, extremes of age, and preexisting cardiac risk factors.² Even when accounting for these factors, predicting individual susceptibility to CCM remains challenging.

Several candidate gene and genome-wide association studies have identified common genetic variants that are associated with CCM through candidate gene analyses and genome-wide association studies.^{2,8–12} Although a recent systemic literature review concluded that the overall evidence supporting variant associations with CCM was limited, genetic data were robust for one intergenic variant (rs28714259) and variants in proximity to 4 other genes.¹³ Rare variants in genes that cause familial cardiomyopathies¹⁴ have also been identified in several small case series and isolated patients with CCM.^{13,15–17}

To better understand the clinical and genetic determinants in CCM, we studied 3 CCM cohorts comprising adult and pediatric patients with diverse malignancies, of whom 90% received anthracyclines. We then corroborated our human findings through cardiac phenotyping of anthracycline-treated mice. From these analyses, we demonstrate the direct and prevalent involvement of variants in genes associated with dilated cardiomyopathy and, in particular, titin-truncating variants (*TTN*ts) in CCM.

METHODS

The data that support the findings of this study are available within the article, the online supplementary files, and publicly available databases. Additional requests, from qualified researchers trained in human subject confidentiality protocols, for anonymized data may be sent to the corresponding authors.

CCM Cohorts, Healthy Volunteer, and Population Controls

Research protocols were reviewed and approved by the institutional ethics board at each participating site. Adult patients with CCM (cohorts A and B), parents of minor patients with CCM (cohort C), and healthy volunteers provided written informed consent. Cohort A includes non-Finnish European patients with CCM retrospectively collected from 6 European heart failure or cardiac transplantation clinics in Spain and the United Kingdom. Cohort B includes prospectively enrolled patients with breast cancer, participating in cardiotoxicity studies of cancer treatments (clinicaltrials.gov NCT01173341). Cohort C includes pediatric patients with newly diagnosed acute myeloid leukemia, enrolled in a clinical therapy trial therapy (AAML1031; clinicaltrials.gov NCT01371981). Cohorts B and C are US patients with non-Finnish European, African, or Asian ancestry, who had prespecified clinical assessments with cardiac imaging (echocardiograms or multigated acquisition scans) before, during, and after chemotherapy. Table 1 provides additional demographic profiles on these cohorts.

CCM was diagnosed irrespective of symptoms based on LVEF to <50 (cohort B) or <53% (cohorts A and C)^{3,18,19} and ≥10% reduction from baseline by echocardiography or <50% and ≥10% reduction from baseline by radionuclide ventriculography, in the absence of established coronary artery disease, cardiomyopathy, primary valvular disease, or uncontrolled hypertension.

Table 1. Clinical Characteristics at Baseline and Follow-Up in Patients With CCM in Study Cohorts

Characteristics	Cohort A (n=99)	Cohort B (n=73)	Cohort C (n=41)
Baseline			
Age at chemotherapy onset, y	48.7±17.1	49.6±10.8	10.8±5.6
Male sex, n (%)	33 (33)§	0 (0)§	17 (41)§
Family history of cardiomyopathy, n (%)	3 (3)	0 (0)	NA
Ethnicity, n (%)			
White	99 (100)§	46 (63)§	25 (61)§
Black	0 (0)§	23 (32)§	9 (22)§
Asian	0 (0)§	4 (5)§	3 (7)§
Others	0 (0)§	0 (0)§	4 (10)§
Comorbidities, n (%)			
Current or past smokers	33 (33)	25 (34)	0 (0)
Hypertension	29 (29)	29 (39)	0 (0)
Hypercholesterolemia	27 (27)	17 (23)	0 (0)
Diabetes mellitus	20 (20)	7 (10)	0 (0)
Oncological disease, n (%)			
Breast cancer	51 (52)§	73 (100)§	0 (0)§
Hematological malignancy	38 (38)§	0 (0)§	41 (100)§
Other solid tumor	10 (10)§	0 (0)§	0 (0)§
Exposure to anthracycline, n (%)	91 (91)	58 (79)	41 (100)
Cumulative anthracycline dose, * mg/m ²	300.7±153.2	240±0	488.3±23.4
Exposure to anti-HER2, n (%)	20 (20)§	36 (49)§	0 (0)§
Radiation therapy to chest, n (%)	32 (32)§	47 (64)§	0 (0)§
LVEF before chemotherapy,† %	62.1±5.4§	55.0±4.7§	64.5±5.8§
At CCM diagnosis			
Time from chemotherapy to diagnosis, y	3 (1–9)§	0.7 (0.4–1.2)§	0.3 (0.2–0.4)§
LVEF at CCM diagnosis, %	33.0±10.0§	41.5±5.5§	44.8±6.7§
NYHA functional class III–IV, n (%)	44 (44)§	1 (1.3)§	NA§
Follow-up			
Duration of follow-up, y	4 (1–7)§	1.7 (0.7–3.3)§	2.3 (1.1–3.2)§
Final LVEF, %	47.3±13.2	49.3±8.2	54.8±13.0
LVEF recovery,‡ n (%)	50 (50)	33 (45)	28 (68)
Cardiac transplantation, n (%)	9 (9)	0 (0)	0 (0)
Cardiac death, n (%)	3 (3)	0 (0)	2 (5)

Data are expressed as numbers (%) or mean±SD, except for time from chemotherapy to cardiotoxicity diagnosis and duration of follow-up, which are expressed as median (Q1–Q3). Comorbidities are those present at diagnosis of CCM. Patient characteristics which are significantly different (adjusted *P*-value <0.05 using Fisher exact test for categorical variables and Welch *t* test for continuous variables) between adult cohorts A and B are shown in bold. CCM indicates cancer therapy-induced cardiomyopathy; HER-2, human epidermal growth factor receptor-2; LVEF, left ventricular ejection fraction; and NYHA, New York Heart Association.

*Cumulative anthracycline dose for 7 patients in cohort A is unknown; these patients were excluded from calculation. Fifty-eight patients in cohort B who underwent anthracycline treatment received 240 mg/m². Planned cumulative anthracycline dose for cohort C (492 mg/m² for 40 patients and 342 mg/m² for 1 patient) is noted in the table, but actual dose may have varied, because 17 patients underwent dose modification of unknown quantity during their treatment period.

†LVEF before chemotherapy was available to review in 31, 73, and 41 subjects in cohorts A, B, and C, respectively.

‡LVEF recovery was defined as an absolute increase in LVEF ≥5% with final LVEF ≥50% in the adult cohorts and ≥53% in the pediatric cohort.

§Significant values.

Additional clinical information including follow-up duration and adverse outcomes was obtained from medical and clinical trial records and patient reports. Where prechemotherapy cardiac imaging was absent, patients were included when LVEF was ≤45% and no alternative cause for cardiac dysfunction other

than chemotherapy was identified. LVEF recovery was defined by a final LVEF ≥50% with ≥5% LVEF increase or restoration of LVEF to the baseline value.¹⁸

Healthy volunteers of European ancestry (n=445) were prospectively recruited participants into the U.K. Digital Heart Project

(<https://digital-heart.org/>)²⁰ with no cardiovascular disease or risk factors by self-report and normal cardiac magnetic resonance imaging.

Next-Generation Sequencing and Variant Analysis

Genomic DNA extracted from peripheral blood samples was used to produce DNA-sequencing libraries that were captured and sequenced on Illumina TruSight Cardio Sequencing kit and a custom Agilent array (DCMv5) as described.^{20,21} Variants were identified using the Genome Analysis Tool Kit (GATK) HaplotypeCaller tool following GATK Best Practices.²² Rare variants (minor allele frequency $<1.0 \times 10^{-4}$, assessed in ancestry-matched subjects in the Genome Aggregation Database [gnomAD]²³) were annotated by SnpEFF and GRCh37.68 (also see the [online-only Data Supplement](#)). The cumulative burden of rare variants in cardiomyopathy genes was compared in CCM cohorts with all patients who had breast ($n=1042$) and lung ($n=1011$) cancer participating in The Cancer Genome Project (TCGA),²⁴ healthy volunteers, and gnomAD²³ subjects with non-Finnish European, African, and Asian ancestries (combined and in ancestry-specific analyses).

Anthracycline Treatment of Mice

Protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Harvard Medical School (Boston, MA). Wild-type and heterozygous C57BL/6N mice with a titin A-band truncation ($Ttn^{tv/+}$)^{25,26} received 3 doses of intraperitoneal doxorubicin (5 mg/kg) at weekly intervals (≈ 45 mg/m²). Cardiac function was assessed in vivo at baseline (age=10–14 weeks) and weekly using a digital ultrasound system (Vevo 2100 Imaging System and M5550D transducer; FujiFilm VisualSonics) by an experienced observer blinded to mouse genotype and treatment. Cardiomyocytes from treated and untreated wild-type and $Ttn^{tv/+}$ mice were isolated and sarcomere contractility was measured (see the [online-only Data Supplement Methods](#)).

Statistical Analyses

Cohort and subgroup analyses, and comparisons with TCGA genomic data,²⁴ healthy volunteers, and reference populations²³ were performed by Fisher exact test (2-tailed), binomial test, or Pearson χ^2 test of association for categorical values. Welch t test and Kruskal-Wallis rank sum tests were used to assess numerical data. Analyses were conducted using either the Stata SE package (version 14, StataCorp) or the R statistical package (version 3.4.0; <http://www.R-project.org/>).

Additional method details are provided in the [online-only Data Supplement](#).

RESULTS

Patients With CCM

We studied 3 CCM cohorts (Table 1). Cohort A includes 99 patients of European ancestry with hematologic, breast, or other solid-tumor cancer (mean age at treatment=48.7 $\pm$ 17.1 years), recruited from heart failure and

cardiac transplant clinics. Two US cohorts were identified through prospective longitudinal cardiac evaluations obtained throughout cancer therapy: Cohort B comprised 73 patients (mean age at treatment=49.6 $\pm$ 10.8 years) with European, African, or Asian ancestry, enrolled from breast cancer clinics as part of a prospective study of who developed CCM during treatment; Cohort C comprised 41 pediatric patients with newly diagnosed acute myelogenous leukemia (mean age at treatment=10.8 $\pm$ 5.6 years) of diverse ancestries. Although individual treatments varied, 90% of all patients with CCM received anthracycline and 33% of adults received trastuzumab. After normalizing anthracycline doses²⁷ the cumulative equivalent dose was <400 mg/m² in 93.9% of patients in cohort A, 100% of patients in cohort B, and 2.3% of patients in cohort C.

We assessed clinical risk factors for CCM in these cohorts. Seventy-six percent of all patients were CCM were females, predominantly treated for breast cancer. In cohorts A and B the prevalence of cigarette smoking, hypertension, and diabetes mellitus was comparable (P =not significant) to that of the general US population,²⁸ but hypercholesterolemia in patients with cancer was less common ($P=3.0 \times 10^{-9}$). Three patients in cohort A, without prechemotherapy imaging studies, had family histories of cardiomyopathy of unknown cause. Patients in cohort C were considerably younger (mean age=10.8 $\pm$ 5.6 years), without cardiovascular risk factors, and all had normal LVEF at study entry.

The median time after the initiation of cancer treatment to CCM diagnosis in cohort A was 3.0 (range=1–9) years, but 0.3 to 0.7 years for cohorts B and C because of cardiac surveillance during treatment in these 2 cohorts. At CCM diagnosis, the mean LVEF decrease was 23.4 $\pm$ 9.2% in cohort A, 13.5 $\pm$ 3.3% in cohort B, and 19.7 $\pm$ 6.0% in cohort C. Across all cohorts, treatment with high cumulative anthracycline dose (>400 mg/m²) was not associated with poorer left ventricular (LV) dysfunction at CCM diagnosis (mean LVEF=42.0 $\pm$ 9.6%). Patients (cohorts A and B) who received trastuzumab without anthracycline had similar cardiovascular risk factors and no significant differences in either baseline or postchemotherapeutic LVEF (mean LVEF decrease=13.9 $\pm$ 3.6%) in comparison with patients receiving anthracyclines with or without other agents (mean LVEF decrease=16.7 $\pm$ 7.5%). Cardiac recovery occurred in approximately half of patients with CCM from each cohort, but 9% of patients in cohort A underwent cardiac transplantation. Cardiac deaths occurred in 3% of patients in cohort A and in 5% of patients in cohort C.

Gene Variants in Patients With CCM

We previously identified 9 genes with an excess of rare missense and in-frame insertions/deletion or truncating variants among patients with cardiomyopathy.²⁹ Within

Table 2. Burden Analysis of 9 DCM Genes in CCM Cohorts

Gene	Cohort A (n=99)	Cohort B (n=73)	Cohort C (n=41)	All CCM (n=213)	TCGA* Breast/ Lung (n=2053)	HVOL† (n=445)	P Values Comparisons of			
							All CCM Versus			NFE‡ CCM (n=170) Versus NFE gnomAD
							TCGA	HVOL	gnomAD‡	
BAG3	1 (1.0%)	2 (2.7%)	0 (0.0%)	3 (1.4%)	18 (0.9%)	4 (0.9%)	0.44 (1)	0.69 (1)	0.44 (1)	0.37 (1)
DSP	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (0.2 %)	0 (0.0%)	1 (1)	1 (1)	1 (1)	1 (1)
LMNA	0 (0.0%)	1 (1.4%)	0 (0.0%)	1 (0.5%)	16 (0.8%)	2 (0.4%)	1 (1)	1 (1)	1 (1)	1 (1)
MYH7	3 (3.0%)	0 (0.0%)	0 (0.0%)	3 (1.4%)	35 (1.7%)	5 (1.1%)	1 (1)	0.72 (1)	1 (1)	0.75 (1)
SCN5A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (0.2%)	1 (0.2%)	1 (1)	1 (1)	1 (1)	1 (1)
TCAP	1 (1.0%)	0 (0.0%)	1 (2.4%)	2 (0.9%)	2 (0.1%)	0 (0.0%)	0.05 (0.45)	0.10 (0.90)	0.07 (0.62)	1 (1)
TNNC1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.1%)	0 (0.0%)	1 (1)	1 (1)	1 (1)	1 (1)
TNNT2	0 (0.0%)	1 (1.4%)	0 (0.0%)	1 (0.5%)	7 (0.3%)	0 (0.0%)	0.55 (1)	0.32 (1)	0.50 (1)	0.34 (1)
TTN	10 (10.0%)	4 (5.5%)	2 (4.9%)	16 (7.5%)	22 (1.1%)	3 (0.7%)	7.36e–08 (6.62e–07)	3.42e–06 (3.08e–05)	5.87e–14 (5.28e–13)	2.03e–10 (1.82e–09)
8 genes (no TTN)	5 (5.1%)	4 (5.5%)	1 (2.4%)	10 (4.7%)	86 (4.2%)	12 (2.7%)	0.72	0.25	0.01	0.21
9 genes	15 (15.1%)	8 (11%)	3 (7.3%)	26 (12.2%)	108 (5.3%)	15 (3.4%)	1.98e–04	3.90e–05	1.78e–06	6.98e–06

The number of subjects with variants in each gene is noted in the table, and percentages are noted in parentheses (%). Types of variants analyzed for each gene: all protein-altering variants (BAG3, LMNA, TCAP, TNNC1, TNNT2), missense variants and in-frame deletion or insertion only (MYH7), frameshift variant, stop-gained, splice-donor, and splice-acceptor variants only (DSP, SCN5A, TTN). P values were calculated via Fisher exact test for all CCM vs breast and lung cancer TCGA participants (TCGA) and healthy volunteers (HVOL) and binomial test for all CCM vs gnomAD.²³ Bonferroni corrections are provided in parentheses. Significant values are shown in bold. CCM indicates cancer therapy-induced cardiomyopathy; and DCM, dilated cardiomyopathy.

*TCGA denotes all breast and lung cancer participants (n=2053) in The Cancer Genome Atlas.²⁴
†HVOL denotes 445 healthy volunteers without cardiovascular disease based on detailed evaluations.
‡gnomAD denotes reference population with African American, non-Finnish European, and East Asian ancestries.
§NFE denotes non-Finnish Europeans ancestries. The subanalyses of NFE patients with CCM are compared with NFE gnomAD reference population n≥113482.
||Significant values.
See [Table II in the online-only Data Supplement](#) for subanalyses of patients with CCM and reference populations stratified by ancestry.

these prespecified genes, we examined rare variants (defined as minor allele frequency <1.0e–4) among ancestry-matched reference populations,²³ CCM cohorts, healthy volunteers, and all breast and lung cancer participants in TCGA²⁴ (Table 2). Because anthracyclines are highly effective and widely used to treat these malignancies,⁶ we expect that most TCGA participants received this chemotherapeutic agent. The prevalence of rare protein-altering variants across all 9 genes was significantly higher in a combined CCM cohort than in unselected lung and breast cancer TCGA participants ($P=1.98\text{e}–04$), healthy volunteers ($P=3.90\text{e}–05$), and reference populations ($P=1.78\text{e}–06$). Although patients with CCM had rare variants in several established dilated cardiomyopathy (DCM) genes (*BAG3*, *LMNA*, *MYH7*, *TCAP*, *TNNT2*, and *TTN*), only variants in *TTN*, which encodes titin, were significantly increased. TTNts were highly enriched in all patients with CCM (n=16; 7.5%) in comparison with unselected breast or lung TCGA participants (combined, $P=7.36\text{e}–08$ and [Table I in the online-only Data Supplement](#)), healthy volunteers ($P=3.42\text{e}–06$), and the reference population ($P=5.87\text{e}–14$). Subanalyses of patients with CCM and the reference population stratified by ancestry ([Table II in the online-only Data Supplement](#)), although limited by small numbers, confirmed the observed enrichment of TTNts in all patients with CCM. TTNts that are significantly

increased in patients with DCM^{20,30} reside in exons that are highly expressed in LV tissues, especially those that encode the A-band and distal I-band.³¹ TTNts identified in patients with CCM shared these characteristics (Table 3). We extended these analyses to include 40 other genes that have been implicated in cardiomyopathies.²⁹ Variants in these genes account for a very small fraction of unselected patients with cardiomyopathy. There was no significant difference in the prevalence of all rare protein-altering variants (minor allele frequency <1.0e–4; [Tables III and IV in the online-only Data Supplement](#)) or variants predicted as damaging ([Tables V and VI in the online-only Data Supplement](#)) in patients with CCM in each cohort or the combined CCM cohort, in comparison with healthy volunteers or in the reference population. For individual genes, the prevalence of rare variants was nominally increased only in *FKRP* (encoding fukutin-related protein); recessive *FKRP* mutations cause several forms of muscular dystrophies with cardiac involvement.³²

Clinical Outcomes in Adult Patients Who Have CCM With or Without TTNts

Patients with CCM in cohorts A and B were predominantly women (81%), with breast cancer (73%), with traditional cardiovascular risk factors, who received

Table 3. Summary of TTNtv Identified From All 3 Cohorts With CCM

Variant	Impact	TTN Band	Affected Exon	PSI of Affected Exon	Cancer Type	Chemotherapy	LVEF at CCM (Change)	Follow-Up, y	Adverse Outcome
chr2:179399704 c.101638G>T	Nonsense (p.Glu33880X)	M	359	1	Breast	Epirubicin	30% (NA)	14	HF
chr2:179400742 c.100731dupA	Frameshift (p.Ser33578IlefsTer15)	A	358	0.99	Breast	Doxorubicin + Trastuzumab	37% (24%)	4	VT, HF
chr2:179410112 c.95722+2delT	Splicing	A	345	0.99	Endometrial	Doxorubicin	20% (NA)	5	HF
chr2:179414849 c.91715dupA	Frameshift (p.Asn30572fs)	A	338	0.98	Breast	Epirubicin	42% (NA)	5	HF
chr2:179422284 c.60512-2A>C	Splicing	A	330	0.96	Breast	Doxorubicin + Trastuzumab	43% (17%)	2.2	None
chr2:179425091 c.85768C>T	Nonsense (p.Arg28590X)	A	327	0.95	Breast	Doxorubicin	35% (NA)	2	HF, VT, transplantation
chr2:179428124 c.82734dupA	Frameshift (p.Val27579SerfsTer15)	A	327	0.95	Non- Hodgkin lymphoma	Doxorubicin	29% (NA)	2	None
chr2:179432234 c.78625G>T	Nonsense (p.Glu26209X)	A	327	0.95	Non- Hodgkin lymphoma	Doxorubicin	34% (NA)	3	HF
chr2:179435679 c.75179delA	Frameshift (p.Asn25060fs)	A	327	0.95	Breast	Doxorubicin + Trastuzumab	44% (12%)	3.4	None
chr2:179441250 c.69715+6T>C	Splicing	A	326	0.95	Breast	Trastuzumab	38% (12%)	0.4	None
chr2:179446855 c.66240delA	Frameshift (p.Asp22081MetfsTer31)	A	316	0.86	Bone sarcoma	Doxorubicin	25% (NA)	6	HF
chr2:179453355 c.63096delT	Frameshift (p.His21032fs)	A	305	0.97	AML	Daunorubicin+ Mitoxantrone	36% (22%)	2.8	None
chr2:179478777 c.49345+2T>C	Splicing	A	263	0.89	AML	Daunorubicin+ Mitoxantrone	50% (18%)	0.8	None
chr2:179571683 c.29042-2A>C	Splicing	I	102	0.88	Breast	Doxorubicin	35% (15%)	1.6	None
chr2:179604819 c.13141G>T	Nonsense (p.Glu4381X)	I	49	0.95	Breast	Doxorubicin	31% (30%)	6	HF
chr2:179631116 c.9693_9694delCT	Frameshift (p.Tyr3232CysfsTer5)	I	41	1	Breast	Epirubicin	45% (NA)	6	HF

Variants are defined based on the meta-transcript (LRG_391_t1 / ENST00000589042) that incorporates all exons in described TTN isoforms (including fetal and noncardiac isoforms) with the exception of exons that are unique to the novex transcripts.

AML indicates acute myeloid leukemia; CCM, cancer therapy–induced cardiomyopathy; HF, heart failure hospitalization; LVEF, left ventricular ejection fraction; NA, not available; PSI, proportions spliced in; TTNtv, titin-truncating variant; and VT, ventricular tachycardia.

anthracyclines (86.6%) or trastuzumab (33%), and with follow-up between 8.4 months and 18 years (Table 1). We defined the clinical courses among patients who have CCM with TTNtv and compared risk factors for CCM and outcomes among patients with and without TTNtv (Table 4 and [Tables VII through IX in the online-only Data Supplement](#)). At diagnosis of CCM, the mean LVEF of patients with (34.9±7.4) and without TTNtv (36.8±9.5; *P*=not significant) were comparable; however, patients with TTNtv had more heart failure hospitalizations and atrial fibrillation (*P*=0.003 for each) than those without TTNtv. Recovery occurred in both groups, although the final mean LVEF was more depressed in patients with TTNtv (39.6±14.2 versus 48.9±10.8; *P*=0.03).

Modeling CCM in TTNtv Mice

Given the multiple variables that can influence cardiotoxicity in human patients, we assessed whether TTNtv increased susceptibility to anthracycline-induced cardiomyopathy in an experimental model. Doxorubicin was administered (3 doses of 5 mg/kg at weekly intervals; cumulative=45 mg/m²) to genetically identical mice, with the exception of the absence (wild-type) or presence (Ttn^{tw/+}) of a heterozygous A-band titin truncation in one gene copy.^{25,26} Untreated Ttn^{tw/+} mice have normal LV function (not significantly different from wild-type mice) and anthracycline administration comparably depressed LV function in both genotypes at week 4 after treatments (Figure). However, at week 8, LV function recovered to baseline in wild-type mice but remained

Table 4. Comparisons of Risk Factors and Outcomes in Adult Patient Who Has CCM With and Without TTNtv

Clinical Characteristics	TTNtv (n=14)	Non-TTNtv (n=158)	P Value*
Baseline			
Male sex, n (%)	2 (14.3)	31 (19.6)	1
Comorbidities, n (%)			
Current or past smokers	3 (21.4)	55 (34.8)	0.39
Hypertension	4 (28.6)	54 (34.2)	0.78
Hypercholesterolemia	6 (42.9)	38 (24.1)	0.20
Diabetes mellitus	2 (14.3)	25 (15.8)	1
Oncological treatments, n (%)			
Doxorubicin ± other	13 (92.9)	136 (86.1)	0.70
At CCM diagnosis			
LVEF at CCM diagnosis, %	34.9±7.4	36.8±9.5	0.38
NYHA functional class III–IV, n (%)	6 (42.9)	39 (24.7)	0.2
Follow-up			
Last LVEF, %	39.6±14.2	48.9±10.8	0.03‡
LVEF recovery, n (%)	6 (42.9)	77 (48.7)	0.78
On neurohormonal blockers,† n (%)	10 (71.4)	121 (76.6)	0.74
Atrial fibrillation, n (%)	5 (35.7)	10 (6.3)	0.003‡
Heart failure–related hospitalization, n (%)	9 (64.3)	39 (24.7)	0.003‡
Cardiac transplantation, n (%)	2 (14.3)	7 (4.4)	0.16
Aborted sudden cardiac death, n (%)	1 (7.1)	6 (3.8)	0.45
Cardiac death, n (%)	0 (0.0)	3 (1.9)	1
Death from all cause, n (%)	0 (0.0)	5 (3.2)	1

CCM indicates cancer therapy–induced cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; and TTNtv, titin-truncating variant.

*P values were calculated using the Fisher exact test for categorical variables and the Welch t test for continuous variables.

†Number of patients who were taking at least one of β -blockers, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, or mineralocorticoid receptor antagonists. No data were available to review for 1 of the patients with TTNtv.

‡Significant values.

depressed through week 12 in $Ttn^{tv/+}$ mice ($P=0.0004$ versus wild-type). Functional analyses in isolated cardiomyocytes confirmed that LV dysfunction reflected cell autonomous effects of anthracyclines (Figure B). Histological analysis of cardiac tissues from anthracycline-treated wild-type or $Ttn^{tv/+}$ mice were comparable and showed no significant increase in fibrosis or apoptosis in comparison with untreated mice.

DISCUSSION

We demonstrate an increased prevalence of DCM-associated gene variants, predominantly TTNtv, in adult patients who have cancer and pediatric patients who have acute myelogenous leukemia with CCM relative to

controls. Although the majority of the patients with CCM have European ancestry, the frequency of cardiomyopathy variants in other patients with CCM who have other ancestries was not significantly different (Table II in the online-only Data Supplement). TTNtv were identified in 16 of 213 CCM cases (7.5%), a considerably higher prevalence than in unselected breast and lung cancer TCGA participants (1.1%, $P=7.36e-08$) or healthy volunteers (0.7%, $P=3.42e-06$) and enriched in comparison with ancestry-matched reference populations ($P=5.87e-14$). Because cardiac status is not recorded for TCGA participants, these data provide conservative estimates of the burden of TTNtv in CCM. Further support that TTNtv contribute to CCM is derived from a mouse model of CCM: anthracycline-treated $Ttn^{tv/+}$ mice and isolated cardiomyocytes had protracted LV and cellular dysfunction in comparison with wild-type.

Clinical outcomes among patients with CCM showed considerable variability, but cardiac function improved in 45% to 68% of adult and pediatric patients. Recovery occurred in 83 adults and 28 children, and was not significantly correlated ($P\geq 0.5$) with preexisting cardiovascular risk factors in adults, TTNtv, high (>400 mg/m²) anthracycline dose, or trastuzumab therapy. However, adult patients who have CCM with TTNtv had more heart failure hospitalizations and atrial fibrillation, as occurs in patients with DCM caused by TTNtv,^{33,34} and cardiac function was worse in patients with than in patients without these variants.

In addition to TTNtv, our analyses identified rare protein-altering variants in 5 genes previously studied in patients with DCM.²⁹ Mutations in *BAG3*, *LMNA*, *MYH7*, and *TNNT2* are established autosomal dominant causes of DCM.^{14,35} *TCAP* mutations are occasionally identified in patients with DCM,³⁶ but more commonly cause a recessive form of limb-girdle muscular dystrophy.³⁷ Despite the low prevalence of variants in these genes across all CCM cohorts (4.7%), their critical roles in myocyte biology imply that variants identified here may contribute to an individual's risk for CCM.

The increased burden of rare variants, including TTNtv, indicate that genetics is an important component in CCM susceptibility and adverse outcomes. We demonstrate that genetics is associated with CCM susceptibility across different cancer types and treatment regimens, in particular, those including anthracycline and trastuzumab (Table 1). Genetic variants in previously identified cardiomyopathy genes were increased among adult cancer survivors with overt CCM and severe clinical courses, and among prospectively studied adult and pediatric patients with mild CCM identified during ongoing cancer treatment. It is notable that heart failure, cardiac transplantation, aborted sudden death, and cardiac death occurred years after completion of chemotherapy regimens in some patients with CCM (Table 4), an observation that underscores the

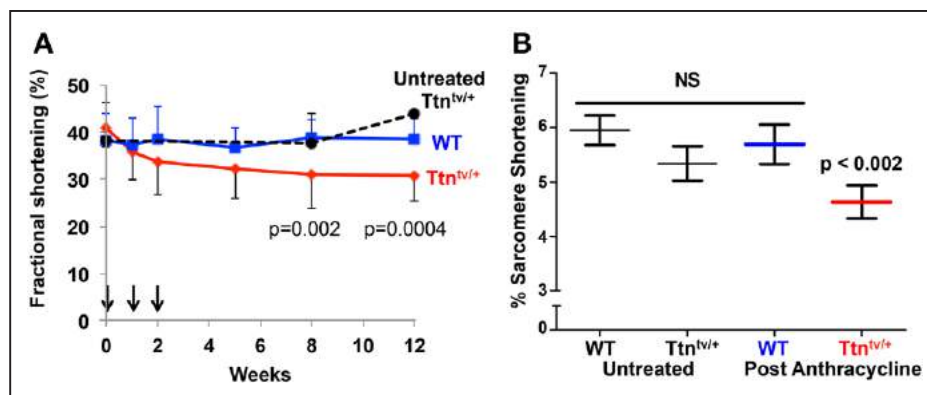


Figure. Persistent cardiac dysfunction in Ttn^{lv/+} mice after anthracycline treatment.

A, Untreated Ttn^{lv/+} mice have left ventricular function comparable with wild-type (WT) mice.^{25,26} Intraperitoneal doxorubicin (5mg/kg) was administered (arrows) to WT and Ttn^{lv/+} mice (n=15 per genotype) in 3 successive weekly doses (cumulative dose=45 mg/m²). Serial echocardiograms showed persistent significantly depressed systolic function (mean fractional shortening $\pm$ SD) in Ttn^{lv/+} in comparison with WT mice ($P=0.0004$). **B**, Isolated cardiomyocytes (n $\geq$ 52 per group) were studied 12 weeks after initial doxorubicin injection. Cardiomyocytes from doxorubicin-treated Ttn^{lv/+} mice had significantly depressed contractility ($P<0.002$) in comparison with cardiomyocytes from doxorubicin-treated WT mice or untreated mice. NS indicates not significant.

need for continued cardiac surveillance in patients with CCM.

These data establish a genetic relationship between DCM and CCM. Cardiomyopathy variants were found in 12.2% of patients with CCM (Table 2), whereas these occur in $\approx$ 40% of patients with familial and sporadic DCM.^{31,38–40} Whether broader genomic analyses may uncover additional genetic contributors to CCM is worthy of study. TTNts are significantly prominent in DCM, occurring in 15% of ambulatory and 25% of end-stage patients,^{30,31,34,38} but are rarely identified in childhood-onset DCM,⁴¹ whereas here we identified TTNts in 8.1% of adults and 5.0% of children with CCM. TTNts found in patients with CCM, like those in patients with DCM, disrupted exons that are constitutively expressed in the heart and are overrepresented in the A-band (Table 3). TTNts also occur in $\approx$ 15% of patients with peripartum cardiomyopathy²¹ and in $\approx$ 10% of individuals with alcoholic cardiomyopathy,⁴² findings that imply additional cardiovascular stress can unmask the deleterious cardiac effects of TTNts. Consistent with this supposition, in vitro analyses of human isogenic cardiomyocytes (derived from induced pluripotent stems cells) demonstrate that titin provides an essential mechanical connection that propagates diastolic traction stresses from β -cardiac myosin during sarcomere formation. Cardiomyocytes with TTNts have diminished reassembly of sarcomeres after stress in comparison with cells without TTNts.⁴³ We suggest that chemotherapy, like pregnancy and excessive alcohol, is an important provocation that is poorly tolerated by TTNts, a conclusion that is supported both by these human data and by analyses of anthracycline-treated TTNt mice.

We recognize several limitations in this study. Given the demographic profiles of the cohorts studied here, further analyses of patients with diverse ancestries are needed. Cohort A was retrospectively recruited

after diagnosis of CCM, and these patients had more severe phenotypes and longer durations of follow-up than the prospectively identified patients in cohorts B and C. Because breast cancer was the most common diagnosis in adult patients with CCM and all pediatric cases had acute myelogenous leukemia, these findings may not be relevant to other cancers and other treatment regimens. All patients had individual chemotherapy dosages and additional treatments based on clinical practice and treatment protocols. These and other variables may influence susceptibility to CCM. This study compared the frequency of TTNts among patients with CCM to the frequency of TTNts in a large cohort of patients with cancer participating in TCGA, some fraction of whom likely developed CCM. A limitation of our study is that we do not know which TCGA subjects developed CCM, potentially affecting the accuracy of the TTNt frequency estimate in the CCM-free cancer cohort. A more ideal comparison group would have been patients treated with chemotherapy who did not develop cardiomyopathy. While recognizing these issues, we suggest that enrichment of protein-altering variants and TTNts across all cohorts strongly supports the conclusion that genetics, like high-dose anthracycline and combination therapy, and cardiovascular risk factors contribute to CCM.

Current strategies to diagnose CCM focus on imaging and circulating biomarkers^{1,2,18,44–46} and treatment guidelines are limited, often recommending interruption or discontinuation of chemotherapy that can negatively impact the survival of patients with cancer. The identification of genetic risk factors opens new opportunities to identify patients with cancer at high risk for CCM and to assess the efficacy of prophylactic cardioprotective drugs and treatment regimens.^{47–49} Future investigations will determine if early recognition of patients who have cancer with high CCM risk through genetic testing

can optimize cancer and cardiovascular treatments to reduce CCM while providing effective cancer therapy.

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Drs C. E. and J. G. Seidman are founders and own shares in Myokardia Inc, a startup company that is developing therapeutics that target the sarcomere. James S. Ware receives grant support and honoraria from Myokardia. Myokardia had no involvement in this study. The other authors report no conflicts.

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ORIGINAL RESEARCH ARTICLE

Genomics-First Evaluation of Heart Disease Associated With Titin-Truncating Variants

BACKGROUND: Truncating variants in the Titin gene (TTNtvs) are common in individuals with idiopathic dilated cardiomyopathy (DCM). However, a comprehensive genomics-first evaluation of the impact of TTNtvs in different clinical contexts, and the evaluation of modifiers such as genetic ancestry, has not been performed.

METHODS: We reviewed whole exome sequence data for >71 000 individuals (61 040 from the Geisinger MyCode Community Health Initiative (2007 to present) and 10 273 from the PennMedicine BioBank (2013 to present) to identify anyone with TTNtvs. We further selected individuals with TTNtvs in exons highly expressed in the heart (proportion spliced in [PSI] >0.9). Using linked electronic health records, we evaluated associations of TTNtvs with diagnoses and quantitative echocardiographic measures, including subanalyses for individuals with and without DCM diagnoses. We also reviewed data from the Jackson Heart Study to validate specific analyses for individuals of African ancestry.

RESULTS: Identified with a TTNtv in a highly expressed exon (hiPSI) were 1.2% individuals in PennMedicine BioBank and 0.6% at Geisinger. The presence of a hiPSI TTNtv was associated with increased odds of DCM in individuals of European ancestry (odds ratio [95% CI]: 18.7 [9.1–39.4] {PennMedicine BioBank} and 10.8 [7.0–16.0] {Geisinger}). hiPSI TTNtvs were not associated with DCM in individuals of African ancestry, despite a high DCM prevalence (odds ratio, 1.8 [0.2–13.7]; $P=0.57$). Among 244 individuals of European ancestry with DCM in PennMedicine BioBank, hiPSI TTNtv carriers had lower left ventricular ejection fraction ($\beta=-12\%$, $P=3\times 10^{-7}$), and increased left ventricular diameter ($\beta=0.65$ cm, $P=9\times 10^{-3}$). In the Geisinger cohort, hiPSI TTNtv carriers without a cardiomyopathy diagnosis had more atrial fibrillation (odds ratio, 2.4 [1.6–3.6]) and heart failure (odds ratio, 3.8 [2.4–6.0]), and lower left ventricular ejection fraction ($\beta=-3.4\%$, $P=1\times 10^{-7}$).

CONCLUSIONS: Individuals of European ancestry with hiPSI TTNtv have an abnormal cardiac phenotype characterized by lower left ventricular ejection fraction, irrespective of the clinical manifestation of cardiomyopathy. Associations with arrhythmias, including atrial fibrillation, were observed even when controlling for cardiomyopathy diagnosis. In contrast, no association between hiPSI TTNtvs and DCM was discerned among individuals of African ancestry. Given these findings, clinical identification of hiPSI TTNtv carriers may alter clinical management strategies.

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Clinical Perspective

What Is New?

- Genetic variants causing premature truncation of the sarcomere protein, Titin, are highly prevalent in numerous forms of nonischemic cardiomyopathy, but little is known about the consequences of these Titin variants ascertained independently of cardiomyopathy (ie, genomics-first).
- We report that individuals of European ancestry identified through a genomics-first approach have much greater odds of developing dilated cardiomyopathy, and have lower left ventricular function than their peers whether a clinical diagnosis of dilated cardiomyopathy is present or not.
- We also find that the association of Titin variants and dilated cardiomyopathy is much weaker in individuals of African ancestry.

What Are the Clinical Implications?

- Truncating genetic variants in Titin have a measurable effect in large clinical populations, with respect to both strong associations with cardiomyopathy and arrhythmia and quantitative differences in cardiac structure and function, although these associations appear strongest in individuals of European ancestry.
- Identification of such variants, either through targeted genetic testing in the setting of cardiomyopathy or screening for secondary findings, may provide clinical value for individualized risk stratification and preventive cardiology.

Titin is a large sarcomeric protein that is vital for cardiac contraction and relaxation.¹ Genetic sequencing of large cohorts of patients ascertained based on the diagnosis of familial or idiopathic dilated cardiomyopathy (DCM) has identified heterozygous variants in *TTN* that are predicted to lead to protein truncation (*TTN*-truncating variants, or *TTN*ts) in ~15% to 20% of these cases.^{2–7} Subsequent work has demonstrated that this association is driven by *TTN*ts in exons that are constitutively expressed (proportion spliced in [PSI]>0.9, or hiPSI) in the primary cardiac isoforms of *TTN* (N2BA and N2B).³ However, the prognostic significance of identifying a *TTN*ts, in particular, as an incidental finding, is uncertain because of substantial challenges in variant interpretation and the high frequency of *TTN*ts in the general population.^{2,8}

A critical step toward enhancing the clinical utility of identifying a *TTN*ts is to define the phenotypic impact of *TTN*ts independent of DCM-based ascertainment.⁹ This is important because DCM may represent an extreme on a spectrum of *TTN*ts-related disease that includes milder, but still clinically relevant forms.¹⁰ For example, a recent study found that a small group (n=15)

of healthy volunteers incidentally identified with *TTN*ts (*PSI*>0.15) had mild increases in left ventricular (LV) volumes by cardiac MRI, suggesting possible deleterious effects of *TTN*ts in the absence of clinically recognized DCM.¹¹ In addition, little is known about the associations of *TTN*ts with disease in non-European populations, despite some suggestion for differential effects in individuals of African ancestry.³

Linking large-scale genomic sequence data to electronic health records (EHRs) provides a powerful opportunity to leverage a genomics-first approach to answer such questions, including the possible identification of novel genotype-phenotype associations, and deep characterization of heterogeneous disease based on individual genotype. Therefore, we comprehensively evaluated phenotypes associated with *TTN*ts in 2 large biobanks with whole exome sequence data linked to EHRs. These 2 biobanks represent informative extremes of clinical contexts: the Geisinger MyCode project¹² represents a general clinical population with high engagement and continuity; in contrast, the PennMedicine Biobank (PMBB) represents a tertiary care center with a high referral rate, and hence a higher disease prevalence. We hypothesized that individuals with *TTN*ts would demonstrate a distinct clinical phenotype characterized by associations of clinical diagnoses and differences in ventricular size and function in comparison with individuals without *TTN*ts.

METHODS

The data, analytic methods, and study materials from the University of Pennsylvania will be made available to other researchers for purposes of reproducing the results with approval of the University of Pennsylvania institutional review committee. The data and study materials from Geisinger will not be made available. The study was approved by the University of Pennsylvania institutional review committee and Geisinger institutional review committee, and all subjects gave informed consent.

Cohorts

The MyCode Community Health Initiative at Geisinger is a precision health project in which participants, with opt-in informed consent, provide biospecimens for broad research use, including permission to link associated data to EHRs.¹² Exome sequencing was performed through the DiscovEHR collaboration between Geisinger and the Regeneron Genetics Center.^{13–17} This study included the first 92 455 participants who underwent sequencing; this cohort is of predominantly European ancestry.

PMBB recruits patients from throughout the University of Pennsylvania Health System for genomic and precision medicine research. Participants actively consent to allow the linkage of biospecimens to their longitudinal EHR. Currently, >50 000 participants are enrolled in the PMBB; ~23% of these individuals self-identify as having African ancestry. This study included a subset of 10 289 individuals who have undergone

whole exome sequencing, performed through a collaboration with the Regeneron Genetics Center.

The Jackson Heart Study is a population-based observational study evaluating the etiology of cardiovascular, renal, and respiratory diseases among African Americans residing in the 3 counties (Hinds, Madison, and Rankin) that make up the Jackson, Mississippi metropolitan area. Data and biological materials have been collected from 5306 participants, aged 35 to 84 years. The study population is characterized by a high prevalence of diabetes mellitus, hypertension, obesity, and related disorders.

Genetic Sequencing, Variant Calling, and Genotype Assignment

Sample preparation and exome sequencing were completed per the standard Regeneron Genetics Center methodology, as described by Dewey et al¹⁴ and detailed in the [online-only Data Supplement Methods](#). Sequence quality was sufficient to provide >20 times read depth in >90% of samples for 96% of the bases in *TTN* for Geisinger and 82% in PMBB (83.6% in individuals of African ancestry and 82.1% in individuals of European ancestry).

After cohort sequencing, samples showing disagreement between genetically determined and reported sex, low-quality sequence data, genetically identified sample duplicates, and individuals with closer than third-degree relatedness were excluded. After exclusions, 61 040 samples from Geisinger and 10 289 samples from PMBB remained. Samples with quality scores at a given site, normalized by depth <3 (<5 for indels), site read depth <7 (<10 for indels), and alternate allele balance <25% (or <5 alternate reads) were removed. Sufficient positive predictive value for these thresholds was verified by Sanger sequence confirmation in selected samples.

Variant Annotation and Selection

We selected all *TTN* variants with minor allele frequency <0.001 and further selected all predicted loss of function, truncating variants (ie, stop gain, frameshift, splice site) using the Ensembl Variant Effect Predictor or ANNOVAR.^{18,19} Splice site variants included those affecting canonical donor and acceptor splice sites (ie, 2 bases flanking either side of each exon). PSL>0.9 (see below) was considered high.³

Phenotype Evaluation

For the Geisinger cohort, we collated available demographic (birthdate, age at last encounter, sex, race, vital status), diagnostic (*International Classification of Diseases* codes from clinical encounters), and quantitative data from the most recent echocardiogram with a physician-reported left ventricular ejection fraction (LVEF) from the Geisinger Phenomic-Initiative database as of April 19, 2018.

Within PMBB genetic ancestry was determined using principal components analysis based on HapMap3 reference populations.¹³ For individuals of European or African ancestry, quantitative ancestry estimates were performed using ADMIXTURE.²⁰ PMBB phenotype data were directly queried from Penn Data Store, which agglomerates EHR data from across the health system, as of January 18, 2018.

DCM was defined as ≥ 2 encounters with the I42.0 (Dilated cardiomyopathy) *International Classification of Diseases, 10th Revision* code; or ≥ 2 instances of nonspecific *International Classification of Diseases, Ninth Revision, Clinical Modification* or *International Classification of Diseases, 10th Revision* codes 425.4 (Other primary cardiomyopathies), 425.8 (Cardiomyopathy in other diseases classified elsewhere), 425.9 (Secondary cardiomyopathy, unspecified), I42.8 (Other cardiomyopathies), or I42.9 (Cardiomyopathy, unspecified) and mention of dilated or DCM in the underlying diagnosis code or on the left ventricular findings from echocardiography (Geisinger) or the phrases dilated cardiomyopathy or DCM in the free text encounter notes (PMBB). Individuals with only one occurrence of either specific or nonspecific codes, or free text, were excluded from analysis considering DCM as an outcome. Transthoracic echocardiographic parameters were extracted from structured reports (outpatient only for PMBB).

Because phenotype data on DCM were not directly collected in the Jackson Heart Study, DCM was determined based on cardiac morphology derived from echocardiography and cardiac MRI. Nonischemic DCM was defined based on age, sex, and body surface area standardized reductions in LVEF and increases in LV end-diastolic dimensions in individuals without a previous history of myocardial infarction or coronary revascularization.³

Statistics

Statistical analyses were performed using R (version 3.5).²¹ Individuals with hiPSI TTNtv were compared with the rest of their respective sequenced cohorts (excluding individuals with low PSI TTNtv) by using phenome-wide association (PheWAS), adjusted for age, sex, and principal components of ancestry (first 4 components for Geisinger, first 10 for PMBB).^{22,23} Logistic regression was used to specifically evaluate the association of TTNtv with DCM, adjusting for age, age,² sex, and principal components of ancestry. Because of the high percentage of individuals of African ancestry in PMBB, analyses were performed separately by genetic ancestry and combined with inverse variance weighted meta-analysis. Available echocardiography measurements were compared on a case/control basis, independently for the Geisinger, PMBB, and Jackson Heart Study cohorts, using the Welch *t* test or Wilcoxon test, as appropriate. Comparisons in patient subsets exclusively with (PMBB) and without (Geisinger) DCM were also made using robust linear regression²⁴, adjusting for age, sex, and ancestry. Bootstrapping with the adjusted percentile method (1000 resamples, with replacement) was used to calculate 95% CIs of the regression coefficients.²⁵ Controls were selected for echocardiography analyses in the Geisinger cohort from the sequenced population lacking a TTNtv and matched (5 controls per TTNtv individual) by sex and date of birth to the TTNtv cases.²⁶ In PMBB, the remaining sequenced population without a TTNtv was used for controls for all analyses.

RESULTS

Prevalence and Distribution of TTNtv

Four hundred ninety-two truncating variants were identified across *TTN* in the Geisinger cohort ([Table 1](#) in

the online-only Data Supplement). Of these, 208 were frameshifting insertions/deletions, 194 were stop gains, and 90 were essential splice site variants. One hundred eighty-nine TTNts were identified in PMBB, including 80 frameshifts, 66 stops, and 43 splice variants (Table II in the online-only Data Supplement). Figure 1 shows the distribution of variants within the *TTN* gene, and compares them with the Exome Aggregation Consortium (ExAC) reference population.²⁷ The variant distribution in the Geisinger cohort was similar to ExAC, whereas the distribution in PMBB strongly biased the A band and constitutively expressed exons (Figure 1, $P<0.005$). For Geisinger, 245 TTNts (49.8%) were located in high PSI (hiPSI) exons, whereas 126 variants in PMBB (75.1%) were hiPSI (Table III in the online-only Data Supplement).

Individuals and Demographic Details

A total of 501 individuals, representing 0.7% of the combined cohorts, were identified with a hiPSI TTNtv. Demographics are shown in Table 1 for the full cohorts, and shown by ancestry in Table IV in the online-only Data Supplement. The fraction of individuals of African ancestry was 2.8% and 21% in the Geisinger and PMBB cohorts, respectively. The prevalence of hiPSI TTNts in PMBB patients of European descent was 1.5%, significantly higher than in ExAC subjects of European descent (0.5%; $P<1\times10^{-6}$). In contrast, the prevalence of hiPSI TTNts in PMBB patients of African descent was

0.9%, not significantly different from that in ExAC subjects of African descent (0.7%; $P=0.14$).

Association With DCM

In the Geisinger cohort, 1.0% of all individuals carried a diagnosis of DCM, in contrast to 5.6% of PMBB, reflecting the large difference in disease prevalence between the cohorts. The distribution of TTNts associated with DCM diagnosis in both cohorts is indicated in red in Figure 1.

In the Geisinger cohort, 7.5% of individuals with a hiPSI TTNtv had a clinical diagnosis of DCM. The age- and sex-adjusted odds ratio for DCM in individuals with hiPSI TTNtv was 10.8 (95% CI, 7.0–16.0; $P=2\times10^{-29}$). Of all sequenced individuals with a DCM diagnosis, 4.2% had a hiPSI TTNtv. The age- and sex-adjusted odds ratio for DCM in individuals with hiPSI TTNtv was 10.8 (95% CI, 7.0–16.0; $P=2\times10^{-29}$).

In PMBB, 30% of individuals with a hiPSI TTNtv had DCM, representing 7% of individuals with DCM. Surprisingly, hiPSI TTNts were more prevalent in patients with DCM of European ancestry than in patients with DCM of African ancestry (40 [10%] of 397 patients versus 3 [1.4%] of 216 patients; $P=2\times10^{-5}$). The latter prevalence did not differ from the prevalence of hiPSI TTNtv in individuals of African ancestry in ExAC (0.5%, $P=0.15$). Furthermore, among individuals of European ancestry with TTNtv, the age- and sex-adjusted odds ratio for DCM was

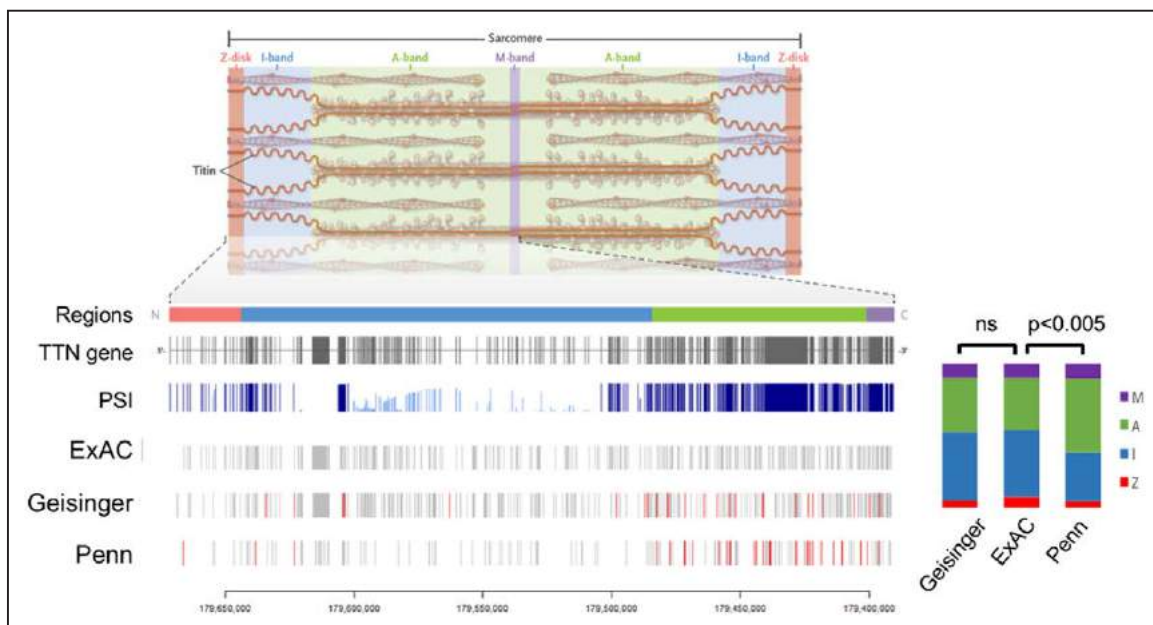


Figure 1. Location within the *TTN* gene of identified variants in the Geisinger and Penn cohorts.

Titin, a protein encoded by *TTN*, is an integral part of the cardiac sarcomere, spanning from Z-disk to M-line. Regions of the *TTN* gene and protein are designated according to their spatial domain in the sarcomere: Z-disk, I-band, A-band, M-band. The exons of the *TTN* gene and their associated proportion spliced in (PSI) are shown, and the distributions of truncating variants in those exons from the Exome Aggregation Consortium (ExAC) reference population and the Geisinger and Penn cohorts, as well. Variants found in patients with dilated cardiomyopathy are designated in red. The relative distribution of variants by *TTN* region is represented on the right stacked bar plots, showing that A-band variants are overrepresented in the Penn cohort in comparison with ExAC, whereas the Geisinger and ExAC distributions are comparable. ns indicates not significant.

Table 1. Cohort Demographics

Demographics	Geisinger			PMBB		
	Non-hiPSI Cohort	hiPSI	P Value	Non-hiPSI Cohort	hiPSI	P Value
Participants	60 681	359		10 147	142	
Male, n (%)	24 538 (40)	139 (39)	0.52	5997 (59)	95 (67)	0.07
EUR ancestry, n (%)	57 371 (95)	340 (95)	>0.99	8024 (79)	122 (86)	0.05
Age at last encounter	60 (45–71)	57 (43–70)	0.015	68 (59–77)	66 (57–74)	0.10
Diabetes mellitus, n (%)	11 528 (19)	55 (15)	0.08	3286 (32)	53 (37)	0.24
Hypertension, n (%)	32 214 (53)	172 (48)	0.06	7505 (74)	102 (72)	0.90
CAD, n (%)	11 005 (18)	93 (26)	3×10^{-4}	4449 (44)	55 (39)	0.79
Myocardial infarction, n (%)	924 (2)	10 (3)	0.08	2103 (21)	26 (18)	0.53
Heart failure, n (%)	5515 (9)	86 (24)	2×10^{-17}	3947 (39)	93 (66)	3×10^{-10}
Nonischemic DCM, n (%)	595 (1)	27 (8)	4×10^{-17}	570 (5.6)	43 (30)	2×10^{-21}
Heart transplant, n (%)	20 (<1)	1 (<1)	0.12	339 (3.3)	20 (14)	1×10^{-7}

Data are presented as number (%) or median (interquartile range). CAD indicates coronary artery disease; DCM, dilated cardiomyopathy; EUR, European; hiPSI, having variants within exons with high proportion spliced in to Titin transcripts; and PMBB, PennMedicine Biobank.

18.7 (95% CI, 9.1–39.4; $P=4 \times 10^{-15}$), whereas the odds ratio among individuals of African ancestry was only 1.8 (95% CI, 0.2–13.7; $P=0.57$) and was not statistically significant.

To further probe the interaction between ancestry and the association of TTNtv with DCM, we modeled the DCM outcome in the overall PMBB cohort while including both the principal components of genetic ancestry, and the percentage of African genetic ancestry from ADMIXTURE analysis, as well (Figure 1 in the online-only Data Supplement). With this approach, the odds ratio (OR) for the association of hiPSI TTNtv variants with DCM was 17 (95% CI, 9–31; $P=2.5 \times 10^{-21}$) in individuals with no African ancestry. Each 10% increase in percentage of African ancestry decreased the magnitude of this association by 22% (OR for interaction between hiPSI and 10% African ancestry on ADMIXTURE=0.78; 95% CI, 0.63–0.95; $P=0.02$; Figure 2 in the online-only Data Supplement). The median percentage of African ancestry in African Americans in the PMBB cohort is 85; based on this, we calculate the effect of carrying a hiPSI TTNtv on the risk of DCM in the PMBB African American individuals as only 2.2-fold increased. In contrast, the risk of DCM based on ancestry alone from our admixture models is higher, with African

Americans in PMBB being estimated to have a 3.4-fold increased risk of DCM than those without any African ancestry (OR, 1.13 per 10% African ancestry; 95% CI, 1.10–1.15; $P=0.02$).

To validate the observed lack of association between TTNtv and DCM in patients of African ancestry, we turned to the Jackson Heart Study, which conducted whole exome sequencing and extensive phenotypic evaluations of 2974 individuals, all of African descent. Among these individuals, we identified 29 (1%) with echocardiographic parameters specific for DCM (Table V in the online-only Data Supplement). Zero of these individuals were among the 18 identified with hiPSI TTNtv ($P=0.02$, assuming expected prevalence of 12%). There was no association between TTNtv carrier status and any echocardiographic or cardiac MRI parameter or incident heart failure in the participants in the Jackson Heart Study (Table VI in the online-only Data Supplement).

One possible explanation for a weaker/absent association of TTNtv with DCM in individuals of African ancestry may be an alternate exon-splicing pattern. In other words, the exons in *TTN* that are constitutively expressed (hiPSI) may vary by ancestry, such that the TTNtv in individuals of African ancestry in our analysis may be occurring in exons with low or intermediate PSI. To

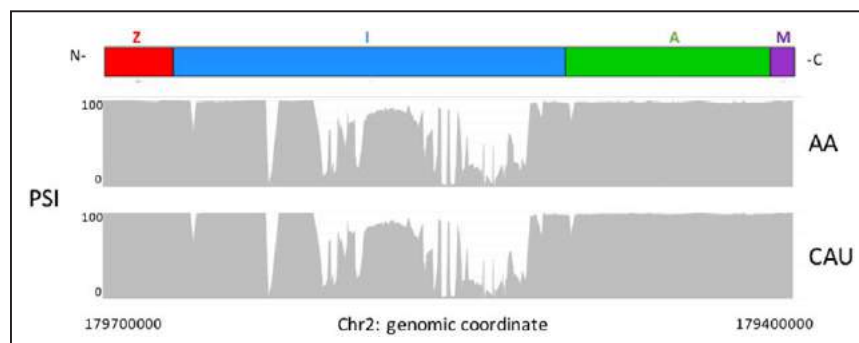


Figure 2. Proportion spliced in (PSI) exons in adult hearts from Caucasian (CAU) and African Americans (AA), explanted after cardiac transplantation for dilated cardiomyopathy.

No significant differences were noted between groups.

test this possibility, we performed RNA-sequencing of *TTN* mRNAs in 166 posttransplant hearts (89 of Caucasian descent, 77 of African descent), and 155 nonfailing discarded donor hearts (122 of Caucasian descent, 33 of African descent). As shown in Figure 2, PSI patterns were nearly identical in all 4 groups. Alternative splicing patterns thus do not differ between these races.

Disease Association

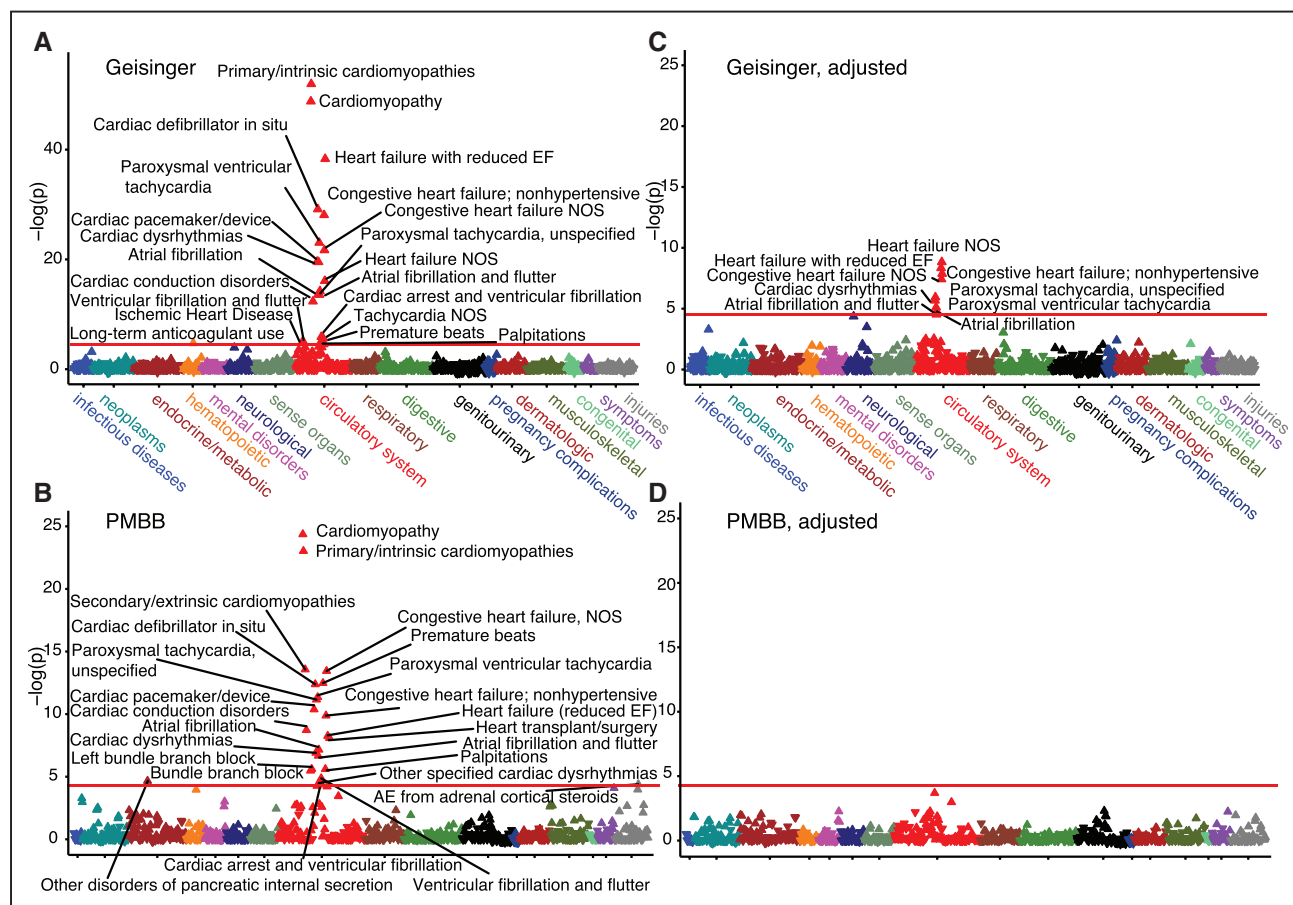
We performed a gene burden PheWAS in the Geisinger and PMBB cohorts to identify associations of broader disease diagnoses with hiPSI *TTN*tv (Figure 3A and 3B). In both cohorts, several diagnoses were significantly more common in the group with hiPSI *TTN*tv. These included cardiomyopathies (OR, 7–9), heart failure (OR, 4–8), arrhythmias (OR up to 13), and need for cardiac pacemaker or defibrillator (OR, 7–12; see Table VII in the online-only Data Supplement for OR and 95% CIs for individual phenotype codes by cohort). No associations with diagnoses were observed for low or intermediate PSI (<0.9) *TTN*tv (Figure III in the online-only Data Supplement), supporting the conclusion that tissue

expression level (PSI) is a critical factor in the pathogenesis of *TTN*tv-related disease.

Because many of these phenotypes are likely the downstream consequences of DCM, we repeated the PheWAS adjusting for the lead cardiomyopathy diagnoses. No associations were noted in the PMBB cohort after this adjustment. In the Geisinger cohort, however, several associations remained significant, including atrial fibrillation (OR, 2.4), other dysrhythmias (OR, 2–4), and heart failure (OR, 3–4; Figure 3C and 3D, Table VII in the online-only Data Supplement). Thus, in the Geisinger cohort, hiPSI *TTN*ts associate strongly with atrial fibrillation and arrhythmia even after adjustment for DCM, and the implications of hiPSI *TTN*tv (such as heart failure) exceed the extent of clinically recognized DCM.

Quantitative Phenotype

Measures of cardiac structure and function for individuals with a clinically obtained echocardiogram ($n=200$ [56%] for Geisinger, $n=67$ [40%] for PMBB) are provided in Table VIII in the online-only Data Supplement. Individuals with hiPSI *TTN*tv in both cohorts had lower



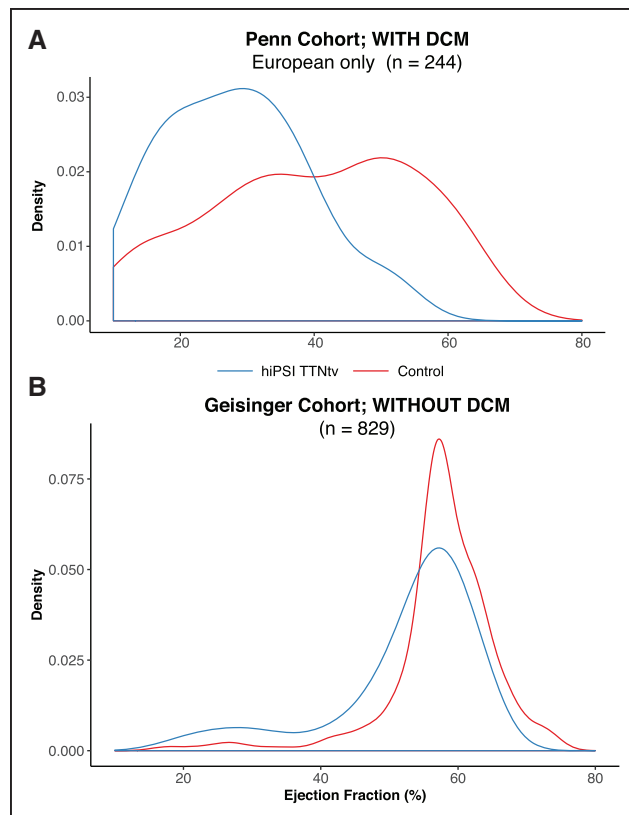


Figure 4. Density plots comparing left ventricular ejection fraction (LVEF) between hiPSI TTNtv (blue) and controls (red) among patients with (Penn, A) and without (Geisinger, B) dilated cardiomyopathy (DCM) diagnoses.

In both cases, LVEF was significantly lower in the group with TTNtv. hiPSI indicates having variants within exons with high proportion spliced in to Titin transcripts; Penn, PennMedicine BioBank; and TTNtv, Titin-truncating variants.

LVEF, decreased septal thickness and increased diastolic and systolic LV internal diameters (Table VIII in the online-only Data Supplement, Figure IV in the online-only Data Supplement). We also observed that LVEF was statistically different in individuals with hiPSI variants (>0.9) in comparison with individuals with intermediate (0.16 – 0.9) and low (0 – 0.15) PSI variants (Figure V in the online-only Data Supplement), further supporting the importance of PSI in determining disease association. Conversely, we did not observe variations in LVEF according to position (band) within the *TTN* gene (Figure VI in the online-only Data Supplement) for variants in hiPSI exons.

To examine the effect of hiPSI TTNtv within the subset of individuals with DCM, we focused on the PMBB cohort, where the number of individuals with DCM and hiPSI TTNtv was high ($n=43$). We restricted the analysis to patients who have DCM with available LVEF measurements, and of European ancestry, in light of the low number of hiPSI TTNtv-positive patients of African ancestry. Among individuals with DCM, we observed significantly lower LVEF and higher LV systolic diameter in patients with hiPSI TTNtv, in comparison with those without (Figure 4, Table 2). These differences persisted

after adjustments for age, sex, and ancestry (Table 2), and held whether evaluating median, minimum, or most recently measured LVEF (Figure VII in the online-only Data Supplement).

To examine the effect of hiPSI TTNtv among individuals without DCM, we focused on the Geisinger cohort, where the number of such individuals is high ($n=132$). For this analysis, DCM was defined broadly as any encounter coded with “dilated cardiomyopathy” (I42), “other (primary) cardiomyopathies” (425.4, I42.8), “cardiomyopathy in other diseases classified elsewhere” (425.8), “cardiomyopathy, unspecified” (I42.9), or “secondary cardiomyopathy, unspecified” (425.9). The lack of DCM was further confirmed through blinded physician chart review. After these exclusions, differences in LVEF, but not LV diastolic diameter, persisted adjusted for age, sex, and ancestry (Figure 4, Table 2). We further excluded any individuals with a heart failure diagnosis, and the association with lower LVEF persisted ($\beta=-2.3$ [95% CI, -3.3 to -1.2 ; $P=4\times 10^{-5}$).

DISCUSSION

In this genomics-first study, we combined *TTN* exome sequencing with extensive clinical phenotyping derived from EHRs to determine genotype-phenotype relationships in individuals bearing TTNtv. We leveraged 2 cohorts, a general clinical population (Geisinger) and patients from a tertiary referral center (PMBB). In both contexts, we found clear phenotypic differences in individuals with hiPSI TTNtv identified from a genomics-first approach. In a general clinical population (Geisinger), our findings demonstrate that: (1) hiPSI TTNtvs convey significantly increased odds for DCM and are also associated with other related cardiac phenotypes, such as systolic heart failure and arrhythmias, some of which may be independent of DCM; and (2) individuals with a hiPSI TTNtv and a clinically indicated echocardiogram have decreased LV function, even in the absence of a clinical diagnosis of nonischemic DCM. In a referral population (PMBB), our findings show that: (1) hiPSI TTNtvs are strongly associated with cardiac phenotypes, largely driven by DCM; (2) individuals with DCM and hiPSI TTNtv have markedly increased LV size and decreased LV function in comparison with patients with DCM who lack a TTNtv; and (3) the association between hiPSI TTNtvs and DCM in individuals of African ancestry is much weaker than in individuals of European ancestry.

Yield of DCM From Genomics-First TTNtv

Overall, 7.5% of Geisinger individuals and 30% of PMBB individuals with hiPSI TTNtv had DCM, corresponding with ORs of 10.8 and 18.7, respectively

Table 2. Echocardiogram Measures in hiPSI TTNtv+ Individuals and hiPSI TTNtv− Controls Without (Geisinger) or With (PMBB) DCM Diagnosis

Echocardiogram Measures	hiPSI TTNtv ⁺	hiPSI TTNtv [−]	Effect Estimate (95% CI)*	P Value
Geisinger (without DCM)				
LVEF, % (last)				
Median	57	57	−3.4 (−4.7 to −2.2)	1×10 ^{−7}
IQR	52–58	57–62		
n	123	706		
LVIDd, cm				
Median	4.6	4.6	0.04 (−0.09 to 0.16)	0.56
IQR	4.1–5.1	4.1–5.0		
n	107	604		
LVIDs, cm				
Median	3.2	3.0	0.24 (0.10 to 0.40)	1×10 ^{−3}
IQR	2.7–3.7	2.6–3.4		
n	95	501		
LVPWd, cm				
Median	1.0	1.0	−0.02 (−0.05 to 0.02)	0.44
IQR	0.9–1.2	0.9–1.2		
n	106	603		
IVSd, cm				
Median	1.1	1.1	−0.02 (−0.06 to 0.02)	0.33
IQR	0.9–1.2	0.9–1.2		
n	106	603		
MV E/A				
Median	1.0	1.0	−0.02 (−0.10 to 0.07)	0.70
IQR	0.8–1.2	0.8–1.2		
n	78	486		
LA dim†				
Median	3.7	3.7	0.05 (−0.08 to 0.19)	0.44
IQR	3.2–4.2	3.2–4.1		
n	90	547		
PMBB (with DCM; excluding transplant)				
LVEF, % (last)				
Median	29	40	−12.1 (−16.2 to −7.0)	3×10 ^{−07}
IQR	15	22.2		
n	26	218		
LVIDd, cm				
Median	5.67	5.42	0.37 (−0.01 to 0.77)	0.07
IQR	1.22	1.17		
n	25	193		
LVIDs, cm				
Median	4.82	4.12	0.65 (0.13 to 1.12)	0.009
IQR	1.25	1.32		
n	25	192		

(Continued)

Table 2. Continued

Echocardiogram Measures	hiPSI TTNtv ⁺	hiPSI TTNtv ⁻	Effect Estimate (95% CI)*	P Value
LVPWd, cm				
Median	0.92	1.02	−0.09 (−0.17 to 0.01)	0.07
IQR	0.22	0.24		
n	26	203		
IVSd, cm				
Median	0.92	1.04	−0.11 (−0.19 to −0.04)	0.004
IQR	0.21	0.28		
n	26	203		
MV E/A				
Median	91	0.92	0.05 (−0.18 to 0.30)	0.67
IQR	0.4	0.56		
n	16	165		
LA dim†				
Median	24.1	22.1	−0.09 (−3.75 to 4.72)	0.97
IQR	7.2	10.5		
n	21	162		

DCM indicates dilated cardiomyopathy; E/A, E-wave velocity/A-wave velocity; hiPSI, having variants within exons with high proportion spliced in to Titin transcripts; IQR, interquartile range; IVSd, interventricular septum thickness, diastole; LA, left atrium; LVEF, left ventricular ejection fraction; LVID (d/s), left ventricular internal diameter (diastole/systole); LVPWd, left ventricular posterior wall thickness, diastole; MV, mitral valve; PMBB, PennMedicine Biobank; and TTNtv, Titin truncating variants.

*Effect estimates computed from linear regression, adjusted for age, sex, and ancestry (principal components 1–4 [Geisinger] or 1–10 [PMBB]). 95% CIs defined with bootstrapping with 1000 samples.

†LA dimension (dim) represents parasternal diameter for Geisinger and 4-chamber area for PMBB.

(Europeans only for PMBB). The DCM/TTNtv association has been well documented through genetic sequencing of patients with DCM,^{2–4} but not previously documented through a genomics-first approach. The prevalence and OR differences likely reflect patient ascertainment into the respective biobanks. For Geisinger, participants are recruited across a large regional health system, comprising both primary care and specialty clinics, and are thus more representative of the population of central Pennsylvania. For PennMedicine, a tertiary care facility, a large percentage of participants have been referred for specialty care, consistent with the higher observed proportion of advanced disease. Hence, these cohorts represent opposite ends of the clinical spectrum for ascertaining patients with DCM, but our findings demonstrate that TTNtv have a meaningful and measurable impact in both settings.

Other Clinical Phenotype Associations

In addition to DCM, we found numerous other significant cardiac phenotype associations of TTNtv (in hiPSI exons) in individuals identified by using this genomics-first approach. These included diagnoses of cardiomyopathies; other common clinical manifestations associated with cardiomyopathies, such as heart failure, and ventricular and atrial dysrhythmias; and common interventions for cardiac arrhythmias and dysfunction, such

as defibrillator or pacemaker placement. Moreover, the fact that no phenotype is associated with low PSI TTNtv in our PheWAS reinforces the presumed benign nature of variants in low and intermediate PSI exons.

To evaluate whether TTNtv are associated with other diseases independent of DCM, we repeated the phenotype association while conditioning for cardiomyopathy diagnoses. Conditioning on cardiomyopathy in the PMBB cohort removed all other associations, suggesting they were all secondary to cardiomyopathy in this cohort with more advanced disease. However, several associations remained significant after adjustment in the Geisinger cohort, suggesting independence from DCM. These include atrial fibrillation, which is consistent with recent work showing increased early arrhythmic risk in patients who have DCM with TTNtv,²⁸ and genome-wide associations of loci in or near *TTN* and atrial fibrillation, as well.^{29–31} In addition, several systolic heart failure diagnoses remained significant. These findings are unlikely to be independent of DCM, but do suggest a clinical underrecognition of *TTN*-associated cardiomyopathy.

Quantitative Imaging Phenotype

Few data exist to address the question of whether an incidental finding of a hiPSI TTNtv is associated with structural cardiac dysfunction. The best evidence to date

comes from cardiac MRI data in 15 healthy individuals with intermediate or high PSI variants ($\text{PSI} > 0.15$) showing increased ventricular volumes.¹¹ Using our larger cohort of 132 individuals with a hiPSI TTNtv and a clinically indicated echocardiogram, but without a diagnosis of nonischemic DCM, we found that hiPSI TTNtv strongly correlated with lower LVEF, but no difference in diastolic diameter, suggesting a deficit in contractility. Although other heart conditions, such as ischemic disease, were present in our clinically based cohort, the extent to which they are related to or interacting with the TTNtv is unclear. Thus, clinical follow-up of incidental findings of hiPSI TTNtv, such as routine echocardiography, may be warranted to potentially identify underlying heart disease. Prospective studies will be needed to determine if subsequent early intervention before the development of overt DCM and heart failure, such as neurohormonal blockade, may prevent the development of symptoms in individuals with hiPSI TTNtv.

For patients with DCM, we find that hiPSI TTNtv were strongly associated with cardiac enlargement and as much as a 10% absolute reduction in LVEF, suggesting that hiPSI TTNtv DCM may represent a more severe form of disease. These findings contrast with recent findings in the Netherlands of a mild form of DCM associated with hiPSI TTNtv.³² In addition, a recent longitudinal study in the United Kingdom found that TTNtv were not associated with worse cardiac parameters in a DCM cohort.³³ This contrast between studies may reflect different patient populations, despite our adjustments for age, sex, and ancestry. For example, the mean age of patients with TTNtv in the Dutch cohort was 49 years, in comparison to 66 years in our cohort. The contrast between studies may also reflect patient ascertainment, acuity of presentation, or comorbidity profile. This heterogeneity between cohorts underscores the challenge of generalizing observations from one clinical population to another.

Ancestry-Stratified Differences of Prevalence of TTNtv in DCM

Among patients with DCM in the PMBB ($n=613$), the overall prevalence of hiPSI TTNtv (7%) was less than has been reported in ascertained DCM cohorts.^{2,33} Ancestry-specific carrier rates, however, likely explain this difference. Among patients with DCM of European ancestry the carrier rate of hiPSI TTNtv was 10%, which is akin to previously reported values.^{33,34} In striking contrast, the prevalence of TTNtv in individuals of African ancestry with DCM was 1.4%, which is not significantly different from that reported in a general population of African Americans (ExAC). In addition to this, hiPSI TTNtv were not associated with DCM in individuals of African ancestry in the PMBB cohort, nor with echocardiographic or cardiac MRI evidence of DCM in

subjects in the Jackson Heart Study. It is possible that we underestimated the true burden of DCM across Jackson Heart Study because some participants with echocardiographic or MRI findings close to the normal range might have been treated and had improvement in ejection fraction. However, based on the results in the PMBB cohort, we expect this be less likely to occur in patients with DCM carrying hiPSI TTNtv than those without variants. Unfortunately, previous DCM cohort studies have not reported on hiPSI TTNtv carrier status by genetic ancestry,^{2,5} although the presence of TTNtv in women with peripartum cardiomyopathy, a DCM-like disease, does seem to be equivalently high in women of European and African ancestry.⁷

A number of reasons may explain the lack of observed association between TTNtv and DCM in patients of African descent. It has been speculated that the development of DCM in the setting of hiPSI TTNtv requires a second hit (ie, extrinsic stress), such as volume overload or chemotherapy.^{7,11,35} Alternatively, environmental factors may be responsible for the lack of association, including access to health care and social determinants of health, both of which are worse in African American populations.^{36,37} In addition, patients of African descent have higher rates of DCM in general, such as perhaps higher rates of hypertension-associated DCM, and this high prevalence may dilute the measurable risk conferred by TTNtv. Also, it is possible that referral rates for advanced heart failure differ by race. Finally, it is possible that the genetic background in individuals of African ancestry limits the impact of TTNtv-induced risk of developing DCM. However, one possible such explanation, alternative splicing allowing more TTNtv to fall in low PSI exons, does not appear to be the case. It will be of future interest to evaluate the TTNtv/DCM relationship in cohorts of other genetic ancestries and with different approaches to ascertainment.

Limitations

The *TTN* gene is the largest and one of the most complex coding regions of the genome, and still presents technical challenges to next-generation sequencing. These technical limits may introduce confounding factors to our analysis, such as a higher than normal false discovery rate. We took steps to mitigate this potential, such as Sanger confirmation for a subset of samples to identify appropriate quality cutoffs, but these steps may not have removed all false-positive cases. However, because the likely effect of false-positive calls is to introduce noise in the signal, these potential errors are unlikely to explain our findings and may in fact lead to an underestimation of the effects in our observations.

Furthermore, the inability to detect an association between TTNtv and DCM in African American individu-

als may be the result of a relatively modest sample size. Although we cannot conclude that there is no association between TTNtv and DCM in African Americans, we can safely exclude an association on the order of magnitude we report in individuals of European ancestry. Based on our case and control numbers in African Americans, we have a >99% power to detect an OR of 18, that which we report individuals of European ancestry, and >90% power to detect an OR of at least 7, with a 2-sided α of 0.05.

In conclusion, our genomics-first evaluation of TTNtv in 2 disparate clinical populations demonstrates that hiPSI TTNtvs in individuals of European ancestry are associated with worse cardiac function in the presence of DCM and reduced (low-normal) cardiac function in the absence of a DCM diagnosis. Identification of TTNtv carriers of European ancestry may thus alter clinical management strategies, because individuals with hiPSI TTNtvs, but without clinical evidence of DCM, may harbor unrecognized cardiac dysfunction and dysrhythmia, whereas those patients with hiPSI TTNtv-mediated DCM may be at risk for more severe disease. Conversely, we did not detect any significant association between TTNtvs and DCM among individuals of African ancestry from 2 independent cohorts, suggesting the OR for DCM in those individuals is much less. Further ancestry-stratified analyses are therefore warranted to confirm this finding and evaluate these trends among other ethnic populations.

ARTICLE INFORMATION

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Importance of Early Diagnosis in Peripartum Cardiomyopathy

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See Editorial, pp 33–34

Abstract—Peripartum cardiomyopathy (PPCM) can lead to long-term systolic dysfunction, especially among black women. Hypertensive disorders of pregnancy (HDP) are the strongest risk factor for PPCM, but controversy remains on whether HDP predict a favorable outcome. Women with HDP are also often diagnosed with PPCM earlier than those without HDP. Our objective is to determine recovery of systolic function in patients with PPCM stratified by HDP, timing of diagnosis, and race. We conducted a retrospective cohort study of 220 patients (55% black) diagnosed with PPCM. Patients with PPCM and HDP were diagnosed earlier postpartum than patients without HDP ($P=0.013$), an effect that was most pronounced in nonblack patients. Rates of left ventricular ejection fraction (LVEF) recovery were similar among PPCM patients with and without HDP (68.4% versus 62.6%, $P=0.425$). In contrast, patients with PPCM diagnosed after 1-month postpartum had lower rates of LVEF recovery than patients diagnosed <1-month postpartum (53.7% versus 69.9%, $P=0.035$). LVEF at time of diagnosis is a strong predictor of LVEF recovery, and patients with PPCM diagnosed after 1-month postpartum had lower baseline LVEF compared to patients presenting earlier ($P=0.041$). The presence of HDP does not correlate with LVEF recovery in our racially diverse PPCM cohort. In contrast, early diagnosis portends a favorable outcome. Early diagnosis is associated with higher LVEF at presentation, likely explaining the improved outcomes in these women. These findings underscore the need for early monitoring and diagnosis, especially in at-risk and underserved populations. (*Hypertension*. 2020;75:91–97. DOI: 10.1161/HYPERTENSIONAHA.119.13291.) • [Online Data Supplement](#)

Key Words: cardiomyopathies ■ healthcare disparities ■ preeclampsia ■ pregnancy ■ women

Peripartum cardiomyopathy (PPCM) is an often severe form of cardiomyopathy diagnosed towards the end of pregnancy or in the months after delivery that affects at least 1 in 2000 deliveries in the United States.^{1,2} Recovery to normal systolic function occurs in 61% to 72% of patients in contemporary cohorts with ≈13% of patients having persistent severe dysfunction or major adverse cardiovascular events such as left ventricular (LV) assist device, heart transplant, or death.^{1,3,4}

Preeclampsia and gestational hypertension are hypertensive disorders of pregnancy (HDP) that are more common in black women and strongly predispose to the development of PPCM.^{1,5} At least 22% of patients with PPCM have preeclampsia and a third have any hypertension, including preeclampsia, gestational hypertension, or chronic hypertension.⁶ Preeclampsia and PPCM likely share pathophysiological mechanisms, including the upregulation of antiangiogenic and vasculotoxic hormones secreted by the placenta.^{1,7,8}

In patients in whom HDPs contribute to the development of PPCM, resolution of postpartum hypertension, and normalization of angiogenic imbalance may theoretically

accelerate resolution of PPCM. Consistent with this notion, PPCM cohorts in Germany and Japan have demonstrated a higher likelihood of LV recovery among patients with HDP compared to patients without HDP.^{9,10} In contrast, however, several studies in the United States have demonstrated no such relationship^{3,11} or conflicting outcomes.¹²

Timing of diagnosis may confound the relationship between HDP and PPCM since women with HDP are often treated with antihypertensives postpartum and require close follow-up in the days to weeks after delivery. Early postpartum follow-up may facilitate the early diagnosis and treatment of PPCM in women at risk. The prospective PPCM cohort in the Investigations of Pregnancy-Associated Cardiomyopathy registry suggested that earlier diagnosis is a predictor of recovery of systolic function, but interaction with HDP was not probed, and this relationship has not been explored in other cohorts.¹¹

Finally, we and others have shown that important racial disparities exist in PPCM outcomes in the United States and worldwide.^{4,11,13–16} In our cohort of 220 women with PPCM, black women were almost half as likely to recover and took

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twice as long to do so. Interestingly, we also noted that black women were diagnosed significantly later than nonblack women, typically a month or more after delivery.⁴ Together, these observations raise the question of how race, HDP, and timing of diagnosis interplay, if at all, to impact prognosis in PPCM. Our objective was, therefore, to understand how HDP and timing of postpartum diagnosis impact PPCM outcomes in one of the largest and racially diverse PPCM cohorts in the United States.

Methods

We conducted a retrospective cohort study of women diagnosed with PPCM between 1986 until 2016 treated within the University of Pennsylvania Health System (UPHS). This study was approved by the University of Pennsylvania Institutional Review Board. To minimize the possibility of unintentionally sharing information that can be used to reidentify patients, a subset of the data that support the findings of this study are available from the corresponding author on reasonable request.

Cohort

We queried the UPHS clinical data warehouse, Penn Data Store, to identify all patients with a potential diagnosis of PPCM, based on *International Classification of Diseases, Ninth Revision* diagnosis code of 674.5 or the presence of an echocardiogram within 6 months of delivery. We manually reviewed medical records, including echocardiography reports, consultation notes, and discharge summaries of all potentially eligible women. We included patients 18 years or older if they were diagnosed with PPCM or heart failure towards the end of pregnancy or in the several months following delivery and had no other identifiable cause for their heart failure.¹⁷ Inclusion criteria for PPCM diagnosis included (1) systolic dysfunction as determined by left ventricular ejection fraction (LVEF) <45% or fractional shortening <30% on echocardiogram at time of diagnosis; (2) documentation of systolic dysfunction in clinical notes at time of diagnosis; or (3) ongoing systolic dysfunction documented by echocardiogram or clinical notes among women transferred to UPHS after diagnosis. Exclusion criteria included a history of congenital heart disease, significant valvular disease predating PPCM diagnosis, and a history of radiation or cardiotoxic chemotherapy.

Covariates

We extracted relevant demographic and clinical information from Penn Data Store including diagnosis details, past obstetric and medical history, and clinical outcomes. Race was self-reported as documented in Penn Data Store. We dichotomized race as black versus nonblack as described previously⁴ and based upon reports that black women with PPCM have worse outcomes as described above. We included gestational hypertension, preeclampsia, and eclampsia as HDP as diagnosed on retrospective chart review. Timing of PPCM diagnosis was recorded as a categorical variable in the original cohort and was dichotomized into early (<1 month after delivery) versus late (≥1 month after delivery).

Outcomes

Our primary outcome was full recovery of LV function, defined as LVEF >50%, consistent with other contemporary cohorts.^{3,4,11} Secondary outcomes included time to recovery (days) for patients with documented dates of diagnosis and follow-up echocardiograms and adverse cardiovascular events, including LV assist device placement, heart transplantation, or death. Patients without follow-up echocardiogram data were censored. Patients were followed for the duration of time that they received care at UPHS, through 2016. As LVEF at time of diagnosis is one of the strongest predictors of subsequent LVEF recovery, we compared differences in baseline LVEF at time of diagnosis stratified by HDP and timing of PPCM diagnosis among the cohort of patients with available baseline echocardiographic reports.

Statistical Analysis

We presented continuous variables as mean with SD and median with interquartile range. We assessed continuous variables using the Student *t* test and Wilcoxon rank-sum tests as appropriate. Categorical variables are presented as counts and percentages and were compared utilizing χ^2 tests. We used Kaplan-Meier survival analysis for recovery time and group differences were compared using the Wilcoxon rank-sum test. We used logistic regression to analyze predictors of LVEF recovery. A *P*<0.05 was considered significant. We performed the analysis using SAS version 9.4 software (SAS Institute Inc, Cary, NC).

Results

We identified 220 PPCM patients (121 black patients, 55%) between 1986 and 2016. We were able to ascertain HDP status in 180 patients (81.8%) and timing of diagnosis in 193 patients (87.7%). The median duration of follow-up was 42.6 months (interquartile range 9.5–89.2 months).

HDP Baseline Characteristics

Baseline characteristics of patients with and without HDP stratified by race are presented in Table 1. Patients with HDP are younger, more likely to be nulliparous, and have twin gestation (known risk factors for HDP). These differences were observed in both black and nonblack patients, although statistical significance was not reached in the stratified analysis. Chronic hypertension was similar between patients with and without HDP (9.1% versus 8.2%, *P*=0.841) but was present almost exclusively in black patients. Both black and nonblack patients with HDP were more than twice as likely to be diagnosed in the first week postpartum compared to patients without HDP, as demonstrated in Figure. This effect was particularly notable for nonblack patients with HDP, among whom 72.7% presented in the first postpartum week. In comparison, only 27.9% of black patients with HDP were diagnosed in the first week after delivery (*P*=0.0001). There were no nonblack patients with HDP diagnosed during pregnancy.

In the 130 patients with complete echocardiographic data at baseline, LVEF at time of diagnosis was similar between patients with and without HDP in the entire cohort (median LVEF 28.8% versus 30%, *P*=0.636) with similar rates of EF<30% documented in the 2 groups (Table 1, Figure S1 in the [online-only Data Supplement](#)). Among black patients with HDP, 63.6% had a baseline LVEF<30% whereas only 33.3% of nonblack patients with HDP had an LVEF<30%.

Time to Diagnosis Baseline Characteristics

Patients diagnosed early (during pregnancy or within one month of delivery) trended towards being older and having higher rates of HDP, were more likely to have twin gestation and to undergo Cesarean delivery than women diagnosed later in the postpartum period (Table 2). In contrast to comparisons based on HDP above, patients with early postpartum diagnosis had a higher median LVEF compared to patients diagnosed 1 month or later postpartum (30% versus 22.5%, *P*=0.041) and more patients in the late diagnosis group had LVEF<30% (63.2% versus 44.8%, *P*=0.054), see Table 2 and Figure S1.

Table 1. Baseline Characteristics Stratified by HDP and Race

Characteristic	Entire Cohort (N=180)			Blacks (N=96)			Nonblacks (N=84)		
	No HDP	HDP	P Value	No HDP	HDP	P Value	No HDP	HDP	P Value
Mean age, y (SD)	30.4 (5.8)	28.0 (7.2)	0.012	29.2 (5.5)	26.0 (7.1)	0.016	31.7 (5.8)	30.6 (6.5)	0.442
Age >30 y, n (%) n=180	54 (54.0)	28 (35.0)	0.011	20 (40.0)	11 (23.9)	0.092	34 (68.0)	17 (50.0)	0.097
Timing of diagnosis, n (%) n=170									
During pregnancy	11 (11.7)	4 (5.3)	0.013	4 (8.7)	4 (9.3)	0.359	7 (14.6)	0 (0)	0.006
<1 wk PP	22 (23.4)	36 (47.4)		6 (13.0)	12 (27.9)		16 (33.3)	24 (72.7)	
≥1 wk and <1 mo PP	30 (31.9)	18 (23.7)		14 (30.4)	12 (27.9)		16 (33.3)	6 (18.2)	
1–5 mo PP	23 (24.5)	16 (21.1)		16 (34.8)	13 (30.2)		7 (14.6)	3 (9.1)	
>5 mo PP	8 (8.5)	2 (2.6)		6 (13.0)	2 (4.7)		2 (4.2)	0 (0)	
Early diagnosis PP, n (%) n=170	63 (67)	58 (76.3)	0.183	28 (65.1)	24 (52.2)	0.216	39 (81.3)	30 (90.9)	0.229
Chronic hypertension, n (%) n=172	9 (9.1)	6 (8.2)	0.841	8 (16.0)	6 (14.3)	0.820	1 (2.0)	0 (0)	0.424
Nulliparous, n (%) n=180	45 (45.0)	48 (60.0)	0.045	20 (40.0)	25 (54.4)	0.159	25 (50.0)	23 (67.7)	0.109
Twin pregnancy, n (%) n=180	8 (8.0)	14 (17.5)	0.053	2 (4.0)	4 (8.7)	0.342	6 (12.0)	10 (29.4)	0.046
Cesarean delivery, n (%) n=177	50 (51.2)	50 (63.3)	0.102	24 (49.0)	25 (55.6)	0.524	26 (53.1)	25 (73.5)	0.060
Median EF at diagnosis, % (IQR) n=130	30 (15–40)	28.8 (21.3–38.8)	0.636	30 (15–37.5)	25 (15–37.5)	0.867	32.5 (15–40)	32.5 (25–40)	0.429
EF at diagnosis <30%, n (%) n=130	34 (48.6)	30 (50.0)	0.871	16 (48.5)	21 (63.6)	0.215	18 (48.7)	9 (33.3)	0.221

EF indicates ejection fraction; HDP, hypertensive disorder of pregnancy; IQR, intraquartile range; and PP, postpartum.

PPCM Outcomes

Patients with HDP had similar rates of recovery of LV function compared with patients without HDP (68.4% versus 62.6%, $P=0.425$; Table 3). Black patients demonstrated similar rates of recovery despite HDP status, whereas non-black patients with HDP trended towards having higher rates of recovery (78.8% versus 66%, $P=0.209$). Among patients who recovered ($n=114$), 63.1% had documentation on time to recovery within 2 years of diagnosis. In this smaller subset, the variability of time to recovery was high and no significant difference existed between those with and without HDP. Rates of adverse outcomes (persistent dysfunction resulting in transplant, LV assist device, or death) were low and similar in patients with and without HDP (4% versus 7.1%, $P=0.378$).

Patients with early diagnosis (Table 3) had significantly higher rates of LV recovery (69.9% versus 53.7%, $P=0.035$). This trend was consistent in the analysis stratified by race. Patients with early diagnosis had a shorter time to recovery compared to patients with late diagnosis (4 versus 7 months, $P=0.047$). This result was driven by differences in time to recovery among black patients (4.3 versus 15.7 months, $P=0.013$). Rates of adverse outcomes were similar in patients diagnosed early and late (9.8% versus 14.8%, $P=0.323$). In univariate logistic regression analysis, nonblack race and early diagnosis were associated with a 2-fold increase in odds of LV recovery, whereas baseline LVEF $\geq 30\%$ was associated with an almost 4-fold increase in odds of recovery (Table 4).

We performed several sensitivity analyses to confirm the robustness of our findings. First, we repeat our analysis among women with documented preeclampsia and found similar trends. Second, given that our cohort spans 30 years,

during which time the standard of heart failure care has evolved, we repeated the analysis stratified by date of diagnosis (diagnosis date before 2006 or 2006 and later). Rates of recovery were similar in both cohorts when stratified by HDP and timing of diagnosis.

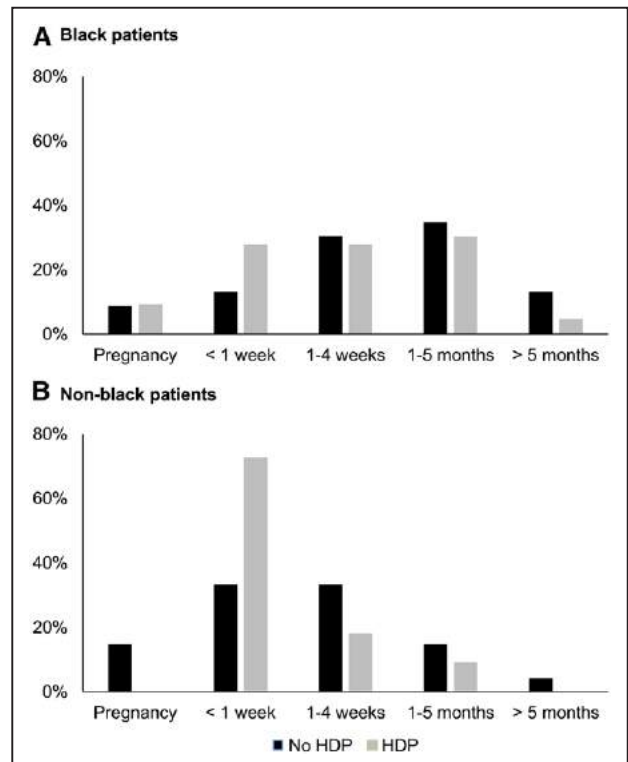


Figure. Timing of diagnosis by hypertensive disorder of pregnancy (HDP) and race. Timing refers to the postpartum period.

Table 2. Baseline Characteristics Stratified by Time to Diagnosis and Race

Characteristic	Entire Cohort (N=193)			Blacks (N=104)			Nonblacks (N=89)		
	Early	Late	P Value	Early	Late	P Value	Early	Late	P Value
Mean age, y (SD)	30.1 (6.8)	28.2 (5.8)	0.073	28.3 (6.8)	27.5 (6.3)	0.554	31.6 (6.5)	30.5 (3.0)	0.535
Age >30 y, n (%)	69 (50)	19 (34.6)	0.052	22 (35.5)	12 (28.6)	0.461	47 (61.8)	7 (53.8)	0.586
Timing of diagnosis, n (%)									
During pregnancy	17 (12.3)	0	<0.0001	10 (16.1)	0	<.0001	7 (9.2)	0	<0.0001
<1 wk PP	66 (47.8)	0		22 (35.5)	0		44 (57.9)	0	
≥1 wk and <1 mo PP	55 (39.9)	0		30 (48.4)	0		25 (32.9)	0	
1–5 mo PP	0	44 (80.0)		0	33 (78.6)		0	11 (84.6)	
>5 mo PP	0	11 (20.0)		0	9 (21.4)		0	2 (15.4)	
Hypertensive disorder of pregnancy n=170	58 (47.9)	18 (36.7)	0.183	28 (53.9)	15 (40.5)	0.216	30 (48.5)	3 (25.0)	0.229
Chronic hypertension, n (%) n=164	11 (9.4)	3 (6.4)	0.532	10 (19.6)	3 (8.6)	0.16	1 (1.5)	0	0.668
Nulliparous, n (%) n=193	72 (52.2)	25 (45.5)	0.399	28 (45.2)	17 (40.5)	0.636	44 (57.9)	8 (61.5)	0.805
Twin pregnancy, n (%) n=192	22 (15.9)	3 (5.6)	0.054	5 (8.1)	2 (4.9)	0.529	17 (22.4)	1 (7.7)	0.224
Cesarean delivery, n (%) n=188	82 (60.3)	21 (40.4)	0.014	30 (49.2)	16 (41.0)	0.425	52 (69.3)	5 (38.5)	0.032
Median EF at diagnosis, % (IQR) n=143	30 (20–40)	22.5 (12.5–37.5)	0.041	27.5 (18.8–38.8)	21.3 (12.5–32.5)	0.139	32.5 (20–40)	30 (15–41.3)	0.866
EF at diagnosis <30%, n (%) n=143	47 (44.8)	24 (63.2)	0.052	24 (54.6)	20 (66.7)	0.297	23 (37.7)	4 (50)	0.503

EF indicates ejection fraction; IQR, intraquartile range; and PP, postpartum.

Discussion

We have made several clinically important observations in our analysis. First, women with HDP are diagnosed with PPCM earlier in the postpartum period. This effect is largely driven by the very early presentation of nonblack patients. Second, early diagnosis (during or within 1 month of delivery) is strongly associated with recovery of LV function whereas HDP is not. Finally,

early postpartum diagnosis is independently associated with higher baseline LV function, which may represent the mechanism by which early diagnosis mediates improved recovery.

HDP and PPCM Outcomes

Prior studies exploring the prognostic impact of preeclampsia or any hypertensive disorder on PPCM outcomes have had

Table 3. Recovery Outcomes Stratified by Postpartum Time to Diagnosis, HDP, and Race

Outcome	HDP (n=175)			Early diagnosis (n=187)		
	Yes	No	P Value	Yes	No	P Value
Recovery of EF ≥50%, n (%)						
Entire cohort	52 (68.4)	62 (62.6)	0.425	93 (69.9)	29 (53.7)	0.035
Blacks	26 (60.5)	29 (59.2)	0.901	37 (63.8)	21 (51.2)	0.211
Nonblacks	26 (78.8)	33 (66.0)	0.209	56 (74.7)	8 (61.5)	0.327
Time to recovery, mo (IQR)						
Entire cohort (n=72/114)	3.5 (2.1–11.4)	4.5 (1.3–9.4)	0.918	4.0 (1.7–9.6)	7.0 (5.4–17.1)	0.047
Blacks (n=23/55)	2.9 (1.1–14.3)	7.3 (4.5–10.2)	0.379	4.3 (1.3–9.3)	15.7 (8.6–17.5)	0.013
Nonblacks (n=49/59)	3.8 (2.1–9.8)	4.0 (1.3–9.1)	0.599	4.0 (1.7–9.7)	5.4 (1.7–7.0)	0.886
Transplant, LVAD, or death, n (%)						
Entire cohort	3 (4.0)	7 (7.1)	0.378	13 (9.8)	8 (14.8)	0.323
Blacks	2 (4.7)	3 (6.1)	0.756	7 (12.1)	6 (14.6)	0.71
Nonblacks	1 (3.0)	4 (8.0)	0.352	6 (8.0)	2 (15.4)	0.393

HDP indicates hypertensive disorder of pregnancy; IQR, interquartile range; and LVAD, left ventricular assist device.

Table 4. Characteristics Associated With Recovery of LV Function

Characteristic	OR (95% CI)	P Value
Age >30 y	1.06 (0.60–1.85)	0.851
Nonblack race	1.90 (1.07–3.37)	0.029
Early diagnosis	2.0 (1.05–3.84)	0.036
Hypertensive disorder of pregnancy	1.29 (0.69–2.43)	0.426
Chronic hypertension	0.75 (0.25–2.22)	0.604
Nulliparous	1.76 (1.0–3.1)	0.05
Twin pregnancy	1.79 (0.72–4.46)	0.21
Cesarean delivery	1.01 (0.57–1.81)	0.965
Baseline EF $\geq$ 30%	3.91 (1.88–8.15)	0.0003

EF indicates ejection fraction; LV, left ventricle; and OR, odds ratio.

mixed results (Table S1). In a German cohort of 115 patients, HDP were highly prevalent and strongly associated with partial or full recovery of LV function compared to patients without HDP (49% versus 7%, respectively, $P=0.009$).⁹ Similarly in a Japanese cohort of 102 patients, HDP were associated with shorter hospitalization at time of diagnosis and improved LV recovery.¹⁰ Studies evaluating black patients in Soweto, South Africa have demonstrated improved PPCM outcomes with higher blood pressure at time of diagnosis but notably these studies excluded women with high blood pressure.^{14,15}

Race may mediate the relationship between preeclampsia and PPCM outcomes and explain why different outcomes are observed in cohorts based on geography and racial composition. In contrast to the German and Japanese cohorts, HDP have not been associated with favorable outcomes in the US-based cohorts (Table S1). The prospective Investigations of Pregnancy-Associated Cardiomyopathy cohort of 100 patients (30% black) demonstrated high rates of HDP but no association of HDP with recovery of LV function.¹¹ Goland et al³ reported no difference in LV recovery at up to 48 months of follow-up among 187 patients (24% black) with and without HDP. A small, predominately black cohort in St Louis (39 patients, 77% black) found that women with preeclampsia had lower 1-year rates of event-free survival, although among survivors with complete echocardiographic follow-up, women with preeclampsia had significantly higher rates of LV recovery (80% versus 25%).¹²

Our study here is one of the largest cohorts to date that addresses the question of how HDP impact outcomes and the first to evaluate the impact of race. We find that HDP are not associated with improved LV recovery. Moreover, this association was maintained in the analysis stratified by race. However, we observed a number of trends that should be further explored in future analyses with larger populations. Nonblack women with HDP were more likely to present in the first week after delivery compared to black women with HDP. Nonblack women with HDP also trended towards having higher baseline LV function, an important predictor of LV recovery, compared to women without HDP. Overall rates of LV recovery were similar between women with and without HDP, but recovery rates did trend towards being higher in those with HDP compared to those without among the 76 nonblack women (79% versus 66%, $P=0.285$), underscoring

the notion that different outcomes observed in international cohorts among PPCM women with HDP may be related to differences in racial composition of those cohorts. It is possible that a potentially protective effect of preeclampsia in nonblack populations is not present in black patients because of competing factors that adversely impact outcomes, perhaps related to socioeconomic or biological factors.

Time to Diagnosis and PPCM Outcomes

We have previously shown in this cohort that black women have lower rates of LV recovery and longer time to recovery compared to nonblack women. Here, we demonstrated that delayed time to diagnosis postpartum is associated with lower rates of LV recovery. Moreover, this observation was maintained in both black and nonblack women and was independent of HDP status. Thus, our study strongly suggests that timing of diagnosis is a key factor in overall outcome. Few studies have explored the relationship between timing of diagnosis and outcomes. In the Investigations of Pregnancy-Associated Cardiomyopathy cohort, black women were enrolled on average at 42 days postpartum, compared to 26 days for nonblack women. Longer time to postpartum diagnosis was significantly associated with worse outcomes in univariate analysis.¹¹ In the St Louis cohort, Lindley et al¹² demonstrated that none of the women diagnosed during pregnancy experienced LV recovery but did not evaluate timing of postpartum presentation.

Delayed diagnosis may explain part of the striking differences between outcomes in black versus nonblack women with PPCM. Socioeconomic factors can lead to the delayed recognition of heart failure symptoms by patients and physicians, leading to delays in the diagnosis and early treatment of PPCM. Adherence to evidence-based medications is lower in vulnerable patient populations and is associated with worse cardiovascular outcomes.¹⁸ The initiation of evidence-based therapies such as angiotensin-converting enzyme inhibitors or β -blockers in nonischemic cardiomyopathy is associated with LV reverse remodeling and even short-term changes in LVEF with medication are associated with prolonged survival.^{19,20} We previously demonstrated that prescription rates of angiotensin-converting enzymes inhibitors and β -blockers were similar between black and nonblack patients in our cohort at time of diagnosis.⁴ We would expect that women with a delayed diagnosis would also have delayed initiation of these evidence-based therapies, leading to worse LV function at baseline and, therefore, lower likelihood of recovery. None of the patients in our cohort were treated with bromocriptine.

Limitations

Our study also has limitations. Retrospective cohort data spanning 30 years may have incomplete data capture as patients were treated according to clinician discretion and not all patients had continuous follow-up within the UPHS system. Differences in follow-up frequency between patient groups could contribute to differences in time to recovery. Although our cohort is among the largest in the United States, our sample size limits our ability to interrogate subgroups for all clinically relevant variables that may impact

recovery. Referral bias may be present as sicker patients are more likely to be referred to a major academic medical center. Preeclampsia diagnoses may be underreported and misclassified; for this reason, we used HDP diagnoses more broadly with the assumption that some cases of gestational hypertension may represent preeclampsia. We included patients with a diagnosis of PPCM and LV systolic dysfunction based on echocardiogram reports and documentation in clinical records but had to exclude some patients due to inability to obtain full reports of all baseline and follow-up echocardiograms. Finally, although we have documentation of medication prescriptions at the time of diagnosis, we do not have more detailed data regarding the duration of treatment, dose titration, or adherence to medications, which may also impact recovery. Despite our study representing the largest clinical PPCM cohort reported in the United States, we are limited by sample size to generalize potentially important trends observed in the stratified analysis by race. Moving forward, efforts to combine clinical cohorts and create a prospective, longitudinal, and adjudicated PPCM registry should be prioritized to better understand how patient characteristics impact prognosis.

Conclusions

In a racially diverse cohort of patients with PPCM, early postpartum presentation is strongly associated with recovery of LV function whereas HDP are not. Later diagnosis is associated with lower EF at presentation, likely explaining the overall worse outcomes observed in black women. These findings underscore the need for early monitoring and diagnosis, especially in at-risk underserved populations.

Perspectives

The association between HDP and recovery of LV function in patients with PPCM is not clear. In this racially diverse cohort of women with PPCM, HDP were not associated with improved rates of LV recovery. Receiving a diagnosis of PPCM during pregnancy or within 1 month after delivery was associated with improved LV recovery compared to patients diagnosed later in the postpartum period. Early diagnosis was associated with higher LVEF, which likely mediates the relationship between late diagnosis and lower recovery rates. Black women are more likely to be diagnosed later in the postpartum period and have lower rates of recovery. Understanding and addressing factors that lead to later diagnosis in this population may lead to improved clinical outcomes.

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Disclosures

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Novelty and Significance

What Is New?

- In a racially diverse cohort of women with peripartum cardiomyopathy (PPCM) living in the United States, hypertensive disorders of pregnancy were not associated with improved recovery rates.
- Early diagnosis (within 1-month postpartum) was significantly associated with improved recovery compared to late diagnosis. This trend was consistent among black and nonblack patients.
- Patients with late diagnosis had a lower ejection fraction at time of presentation, which likely mediates the relationship between late diagnosis and lower recovery rates.

What Is Relevant?

- Earlier diagnosis of PPCM in black women may prevent the worse clinical outcomes currently observed in this population. Cardiac symptoms in women with PPCM risk factors should lead to prompt evaluation.

- Controversy exists in the literature about the association of HDP and PPCM recovery. Our study contributes to the growing literature in the United States demonstrating that women with PPCM do not experience improved recovery rates, differential effects based on race and geography may exist.

Summary

Recovery rates of PPCM in the United States are high but a significant proportion of women still develop severe cardiomyopathy or adverse outcomes. Black women are diagnosed significantly later than white women and have worse rates of recovery. Improving the early monitoring and diagnosis of PPCM in at-risk women may improve clinical outcomes in traditionally underserved populations.

THE PRESENT AND FUTURE

JACC STATE-OF-THE-ART REVIEW

Peripartum Cardiomyopathy

JACC State-of-the-Art Review



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ABSTRACT

Peripartum cardiomyopathy is a form of systolic heart failure affecting young women toward the end of pregnancy or in the months following delivery. Incidence is higher in African-American women and in women with older maternal age, hypertensive disorders of pregnancy, and multiple gestation pregnancies. Symptoms of heart failure mimic those of normal pregnancy, often resulting in a delay in diagnosis and preventable complications. Echocardiography showing decreased myocardial function is essential for the diagnosis. Medical management is similar to heart failure with reduced ejection fraction of other etiologies, but adjustments during pregnancy are necessary to ensure fetal safety. Variable outcomes include complete recovery, persistent heart failure, arrhythmias, thromboembolic events, and death. Subsequent pregnancy confers substantial risk of relapse and even death if there is incomplete myocardial recovery. Additional research about the etiology, optimal therapy including the use of bromocriptine, long-term outcomes, and duration of treatment after recovery are needed. (J Am Coll Cardiol 2020;75:207-21) © 2020 by the American College of Cardiology Foundation.

Peripartum cardiomyopathy (PPCM) is a form of systolic heart failure (HF) with reduced left ventricular ejection fraction (LVEF) affecting childbearing women during pregnancy or in the early postpartum period. Delays in diagnosis may occur because the symptoms and signs of PPCM can mimic the normal findings of late pregnancy and the peripartum period. Although some women have relatively mild disease and complete recovery, others experience significant morbidity and mortality. This clinical review will describe the definition, risk factors, etiology, pathophysiology, and prognostic factors and will provide recommendations for diagnosis, acute and chronic management, and important counseling considerations, including breastfeeding and subsequent pregnancies.

DEFINITION

PPCM is a diagnosis of exclusion in women presenting with HF due to left ventricular (LV) systolic dysfunction and should be considered when no other cause is evident. PPCM was previously defined as symptomatic HF presenting in the last month of pregnancy and up to 5 months postpartum (1,2); however, the definition has been broadened because women who presented prior to the last month of gestation were found to be clinically indistinguishable from patients with classically-defined PPCM (3). The 2010 Heart Failure Association of the European Society of Cardiology Working Group therefore revised the definition of PPCM to “an idiopathic cardiomyopathy presenting with HF secondary to LV systolic dysfunction towards the end of pregnancy or



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ABBREVIATIONS AND ACRONYMS

ACE = angiotensin-converting enzyme

BNP = brain natriuretic peptide

DCM = dilated cardiomyopathy

HF = heart failure

ICD = implantable cardioverter-defibrillator

LV = left ventricular

LVAD = left ventricular assist device

LVEF = left ventricular ejection fraction

PPCM = peripartum cardiomyopathy

in the months following delivery, where no other cause of heart failure is found” (4). The diagnostic criteria indicate that LVEF is <45% and there may or may not be ventricular dilatation (4,5). Outcomes are variable; women may have complete recovery, persistent myocardial dysfunction and HF, or rapid deterioration, leading to urgent need for temporary or durable mechanical circulatory support and cardiac transplantation (Central Illustration).

EPIDEMIOLOGY

Global estimates of the incidence of PPCM vary by regions, with reports as high as 1 in 100 deliveries in Nigeria (6) and 1 in 300 de-

liveries in Haiti (7), to as low as 1 in 20,000 deliveries in Japan (8). In the United States, reported incidence ranges from 1 in 1,000 to 1 in 4,000 (9–11) and may be increasing, due to the rise in maternal age, increased rates of multifetal pregnancies due to contemporary fertility techniques, and possibly to increased recognition of the disease. Many cases may moreover be unrecognized; thus, the true incidence is unknown.

RISK FACTORS

Several risk factors have been associated with PPCM. The incidence of PPCM is higher in women of African ancestry (10,12–15). In nationwide studies from the United States, >40% of the cases occurred in African-American women (9,10,16). Similarly, statewide studies report PPCM occurring 3 to 16 times as often in African-American women compared with white women (12–14). Pre-eclampsia and hypertension are strongly associated with PPCM (3,10,14,17–19). A meta-analysis of 22 studies including 979 cases of PPCM reported that pre-eclampsia was present in 22% of women with PPCM, compared with an average worldwide background rate of 5%, and other hypertensive disorders were present in 37% (18). Hypertension and pre-eclampsia can lead to HF and pulmonary edema due to predominant LV diastolic dysfunction. Although subclinical LV systolic dysfunction can be detected by speckle tracking strain imaging, LVEF is preserved (20–24). The majority of women with pre-eclampsia do not develop PPCM, which is only diagnosed when the systolic function is decreased. Multigestational pregnancies are reported in 7% to 14.5% of women with PPCM in the United States (17,18,25,26). A meta-analysis of 16 studies of PPCM showed an overall incidence of twin pregnancies in 9% of women, compared with the national average of 3% (18). Older maternal age also

HIGHLIGHTS

- Medications used to treat HF during pregnancy and lactation require special considerations.
- Severe HF may require advanced therapies and mechanical circulatory support.
- Subsequent pregnancies carry risk of relapse, and dedicated counseling and monitoring are essential.
- Future research about long-term outcomes, continued drug therapy, use of bromocriptine, device therapy, and genetics are needed.

appears to be associated with PPCM (3,12,14): one-half of cases of PPCM occur in women age >30 years, and 1 study reported that age >40 years had an odds ratio of 10 of developing the disease compared with women age <20 years (9). Although a minority of women with PPCM have a family history of cardiomyopathy (see the Genetics section), awareness of HF symptoms during and after pregnancy may improve detection of PPCM in these patients. Closer surveillance of women who are at highest risk during pregnancy could lead to earlier diagnosis and treatment.

PATHOPHYSIOLOGY

The etiology of PPCM is not fully understood and is likely multifactorial. Suggested but not proven mechanisms for the development of PPCM have included nutritional deficiencies (27,28), viral myocarditis (29,30), and autoimmune processes (31,32). Hemodynamic stress of pregnancy has been postulated as a potential etiology. However, the maximal cardiovascular changes occur in the second trimester (33), when most women with pre-existing cardiac disease develop symptomatic HF (34). In contrast, the majority of women with PPCM develop symptoms during late pregnancy or after the delivery (35).

The development of 2 vascular-hormonal animal models of pregnancy-associated cardiomyopathy suggested novel mechanisms for PPCM in humans. The first model was a STAT3 knockout mouse in which oxidative stress led to cleavage of the nursing hormone, prolactin. The 16-kDa prolactin fragment had vasculotoxic and pro-apoptotic properties and vascular and myocardial dysfunction (36). The mice treated with bromocriptine, a suppressor of prolactin secretion, had complete reversal of the

CENTRAL ILLUSTRATION Diagnosis, Management, and Outcomes for Peripartum Cardiomyopathy

Peripartum Cardiomyopathy (PPCM)

Definition:

- Non-ischemic cardiomyopathy with reduced LVEF (<45%)
- Commonly presents in the first months postpartum or towards the end of pregnancy

Risk Factors:

- African-American race, preeclampsia, hypertension, multigestational pregnancies, age >30 years

Symptoms:

- Heart failure symptoms can be confused with common symptoms of normal pregnancy

Management Options for PPCM



During Pregnancy:

- Beta-blockers, loop diuretics, hydralazine/isosorbide dinitrate, digoxin, low-molecular-weight heparin
- (No ACE/ARB/aldosterone receptor antagonists)
- MCS for severe heart failure/cardiogenic shock
- Consider early delivery if unstable



Delivery:

- Plan ahead with a Cardio-Obstetrics Team
- If unstable, consider hemodynamic monitoring and optimization
- Caution for fluid overload, especially after delivery



After Pregnancy:

- Heart failure management. Beta-blockers, enalapril, and spironolactone are compatible with breastfeeding.
- Anticoagulation for LV thrombus; consider if severe LV dysfunction (LVEF <35%)
- Consider a wearable cardioverter/defibrillator if severe LV dysfunction
- Discuss Contraception

Outcomes

Worse prognosis with lower LVEF, dilated LV, African-American race, and delayed diagnosis.

Long-term Outcomes

- After recovery, optimal duration of medication treatment is unknown
- In the case of stopping medications, wean gradually and observe closely
- Continue surveillance after recovery

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Diagnostic criteria, symptoms, and risk factors can aid in the diagnosis. Medications need to be tailored to pregnancy and breastfeeding status. Short- and long-term outcomes are variable and serial follow-up is important. ACE = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; LV = left ventricle; LVEF = left ventricular ejection fraction; MCS = mechanical circulatory support; PPCM = peripartum cardiomyopathy.

cardiomyopathy. Of note, human prolactin has been shown to be more resistant to cleavage than prolactin in rats (37), and extrapolations from rodent models may be limited. A second vascular-hormonal mouse model of PPCM was developed by cardiac-specific genetic deletion of proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), leading to vasculotoxicity by activation of the 16-kDa prolactin fragment and decreased expression of proangiogenic vascular endothelial growth factor (VEGF). In this model, the cardiomyopathy could be reversed only partly with bromocriptine and required the addition of VEGF for complete recovery (38). VEGF is also strongly antagonized in late gestation by the placental secretion of soluble Fms-like tyrosine kinase 1 (sFlt1), an antagonist of VEGF and placental growth factor. Exogenous sFlt1 was sufficient to cause profound systolic dysfunction in mice lacking cardiac PGC-1 α , even in the absence of pregnancy. Moreover, sFlt1 levels are markedly elevated in women with preeclampsia, likely in part explaining why preeclampsia is such a strong risk factor for PPCM. Elevated sFlt1 levels have also been found in a subset of women with PPCM and predict worse outcomes (39). In summary, 2 mouse models and epidemiological data support the notion that PPCM is in large part a vascular disease, triggered by the hormonal milieu of the peripartum (40,41).

GENETICS

Genetic differences may explain why only relatively few women develop HF in response to the vascular insults of late pregnancy. Observations of familial clustering of PPCM, as well as ~6% co-occurrence with idiopathic dilated cardiomyopathy (DCM), suggested early on the possibility of a genetic contribution to the disease (42-48). In a German registry, 15% of women with PPCM had a family history of cardiac disease in a first-degree relative (defined as PPCM, DCM, sudden death, or arrhythmias) (49). Genetic studies have subsequently supported the notion that genetics contribute to PPCM. A genome-wide association study of 79 patients with PPCM identified a single-nucleotide polymorphism near the *PTHLH* gene (50), and this gene may regulate vascular homeostasis (51). Evaluation of rare pedigrees that include both PPCM and DCM identified likely pathogenic variants in genes known to contribute to DCM such as *TTN* and *BAG3*. In a definitive genetic study of 172 patients with PPCM not pre-selected for family history, targeted sequencing of 43 genes known to associate with DCM revealed a 15% prevalence of truncating variants (i.e., nonsense, missense, or splicing variants) in patients

with PPCM (52). Two-thirds of the truncating variants were in the *TTN* gene, with the majority of these in the A band of *TTN*. The *TTN* gene encodes the largest human protein, titin, a critical structural component of sarcomeres in cardiac and skeletal muscle. The prevalence and location of these mutations in *TTN* overlap with other forms of DCM, again suggesting a genetic overlap between these 2 diseases. In post hoc analyses, *TTN* variants identified in the well-characterized subset of patients derived from the IPAC (Investigations of Pregnancy Associated Cardiomyopathy) cohort correlated with lower LVEF at 1 year, suggesting that the presence of *TTN* truncating variant may presage worse outcome (52). Overrepresentation of *TTN* truncating variants have likewise been identified in cohorts of cardiotoxicity associated with chemotherapy (53) and alcohol use (54), suggesting that pregnancy may similarly represent a “second hit” for patients predisposed by genetic variance. Importantly, however, >90% of individuals with *TTN* truncating variants do not develop DCM or PPCM (55), indicating that additional environmental, genetic, or epigenetic factors are at play. The fact that most women with PPCM do not have a family history of cardiomyopathy and that PPCM does not always recur with a subsequent pregnancy indicates that any genetic origin is incompletely penetrant and that additional factors contribute to disease incidence. Further research in diverse populations is needed to study the interaction of genetic variants with comorbidities as well as vascular, hormonal, and hemodynamic insults.

DIAGNOSIS

TIMING AND CLINICAL PRESENTATION. The majority of women with PPCM are diagnosed after delivery, typically in the first month postpartum (3). Frequent delays in diagnosis occur due to under-recognition of this disease and the overlap in signs and symptoms of normal pregnancy with those of HF. Unfortunately, delays in diagnosis are associated with increased incidence of preventable complications and worse outcomes (3,56,57). Most women present with signs and symptoms of HF including shortness of breath on exertion, fatigue, orthopnea, paroxysmal nocturnal dyspnea, edema, and chest tightness. Physical examination often reveals tachypnea, tachycardia, elevated jugular venous pressure, pulmonary rales, and peripheral edema. A minority of patients will present with cardiogenic shock, severe arrhythmias, and thromboembolic complications.

DIAGNOSTIC TESTING. Echocardiography should be performed in any suspected case of PPCM as the

TABLE 1 Differential Diagnosis for Heart Failure During Pregnancy

Differential Diagnosis	Considerations
Takotsubo cardiomyopathy	Echocardiogram may show classic apical ballooning
Familial cardiomyopathy	Family history, genetic testing
Pre-existing cardiomyopathy	History of HF prior to pregnancy; prior echo studies with low LVEF before pregnancy
Recurrent peripartum cardiomyopathy	Ask about symptoms of HF that occurred after a prior pregnancy
Pre-eclampsia	Preserved systolic function on echocardiogram
Hypertrophic cardiomyopathy	Left ventricular hypertrophy, LVOT obstruction, preserved systolic function, genetic testing
Myocarditis	Consider if viral prodrome, histological diagnosis, fulminant presentation
Arrhythmogenic right ventricular cardiomyopathy	Consider with family history, genetic testing, echocardiographic findings
Left ventricular noncompaction	Echocardiographic and CMR findings
Chemotherapy-related cardiomyopathy	History of chemotherapy, particularly doxorubicin
Valvular heart disease	Echocardiographic findings; congenital aortic stenosis; mitral stenosis from rheumatic heart disease in endemic country. Patients with PPCM may also have valve disease, i.e., mitral regurgitation
Congenital heart disease	May be diagnosed for the first time during pregnancy by echocardiography
Tachycardia-arrhythmia mediated cardiomyopathy	Consider if specific underlying rhythm abnormality. Note that sinus tachycardia may be secondary to heart failure during pregnancy
Hypertensive heart disease	Left ventricular hypertrophy; less common in young people unless very longstanding history of hypertension
Ischemic heart disease	Cardiovascular risk factors; angina; prior CAD; consider SCAD and MINOCA diagnoses
Cardiomyopathy related to other systemic medical diseases	Consider in the appropriate context, i.e., systemic lupus erythematosus, antiphospholipid syndrome, hemochromatosis
Cardiomyopathy related to other acute conditions	May consider if patient has other conditions such as sepsis, treatment in intensive care unit, post-respiratory arrest
Pulmonary embolism	Dyspnea, tachycardia with preserved LVEF

CAD = coronary artery disease; CMR = cardiac magnetic resonance imaging; HF = heart failure; LVEF = left ventricular ejection fraction; LVOT = left ventricular outflow tract; MINOCA = myocardial infarction in non-obstructive coronary arteries; PPCM = peripartum cardiomyopathy; SCAD = spontaneous coronary artery dissection.

LVEF is typically <45% (4). In addition to systolic dysfunction, the echocardiogram may demonstrate LV and right ventricular dilatation and/or dysfunction, functional mitral and/or tricuspid regurgitation, pulmonary hypertension, and left atrial or biatrial enlargement. Intracardiac thrombus may occur (17,58), and the LV apex should be clearly visualized particularly when the LVEF is severely reduced. Levels of brain natriuretic peptide (BNP) and N-terminal pro-BNP, which do not change significantly during normal pregnancy and may be mildly elevated in the setting of pre-eclampsia, are usually markedly elevated in PPCM (59–62). The electrocardiogram may show nonspecific abnormalities, but a normal electrocardiogram does not rule out PPCM (63). Chest x-rays show pulmonary venous congestion. Cardiac magnetic resonance imaging provides accurate ejection fraction and chamber measurements when the echocardiogram is inadequate, but gadolinium is avoided during pregnancy. Endomyocardial biopsy is only indicated if there is suspicion for an alternative diagnosis, such as giant cell myocarditis, that would necessitate a different management plan.

DIFFERENTIAL DIAGNOSIS. PPCM is a diagnosis of exclusion. To avoid overdiagnosis, careful attention

to possible pre-existing heart disease including cardiomyopathies and valvular disease is important. Severe pre-eclampsia can cause HF related to diastolic dysfunction, but PPCM is only diagnosed in the presence of systolic dysfunction. Potential causes of pregnancy-related HF are listed in [Table 1](#).

PROGNOSIS AND OUTCOMES

ADVERSE OUTCOMES. PPCM is associated with adverse outcomes, including brain injury, cardiopulmonary arrest, pulmonary edema, thromboembolic complications, mechanical circulatory support, cardiac transplantation, and death. In a study of 182 women with PPCM, the major adverse event preceded the diagnosis of PPCM in one-half of the patients, and cerebrovascular events and cardiopulmonary arrest were associated with residual brain damage (57). LV thrombus has been identified in as much as 10% to 17% of initial echocardiograms (17,64), and thromboembolic complications have been reported in 5% to 9% of women (65,66). The increased incidence of thromboembolic events in PPCM is likely related to the hypercoagulable state of pregnancy, cardiac dilatation and dysfunction, venous stasis, bed rest, and the post-operative status after cesarean section.

PROGNOSTIC FACTORS. Of various prognostic factors that have been studied, LVEF at the time of diagnosis is the most reliable predictor of adverse events or long-term recovery (3,25,57,67-69). In the IPAC cohort, LVEF <30% was associated with lower rates of recovery and increased risk of adverse events (70). Despite severe LV dysfunction at the time of diagnosis, some women will recover; thus, initial LVEF is not sufficient for determining an early and possible premature need for advanced therapies such as durable left ventricular assist device (LVAD), implantable cardioverter-defibrillator (ICD), cardiac resynchronization therapy, or transplant (25). Additional predictors of worse outcome include LV dilatation (17,25,67,70-72), LV thrombus (17), right ventricular systolic dysfunction (73,74), and obesity (75). African-American ethnicity is strongly associated with lower rates of recovery, longer recovery time, more adverse outcomes, and higher mortality (14,15,70,76). Concomitant pre-eclampsia has been associated with lower 1-year survival, but higher rates of LV recovery in survivors (77). Biomarkers associated with adverse outcomes include troponin (78), NT-proBNP (79), and sFlt1 (39). The presence of late gadolinium enhancement by cardiac magnetic resonance imaging may indicate fibrosis associated with less myocardial recovery; however, most patients with PPCM do not demonstrate late gadolinium enhancement (80,81).

MORTALITY. Mortality estimates differ significantly based on racial groups, geographical region, and duration of follow-up. At 1-year follow-up, mortality rates in the United States ranged from 4% in the IPAC study (70) to 11% in a population of 96% African-American women (82). Two-year mortality rates have been reported as 0% to 16% in different studies in the United States (3,64,68), 15% in Haiti (7), and 28% in Africa (83). Less data are available about long-term outcomes (>5 years), but U.S. mortality estimates have ranged from 7% to 20% (mean follow-up 6.3 to 8.6 years) (14,84-86).

RECOVERY. PPCM has been associated with a higher rate of recovery than other forms of HF with reduced LVEF (64), and recovery frequently occurs within the first 3 to 6 months (3,17,70). Delayed recovery can also occur, even up to 2 years following diagnosis (68,85-88). In the United States, recovery rates have varied depending on the patient population and the definition of recovery. Of the 100 prospectively enrolled women in the IPAC study, 72% recovered to LVEF >50% at 12 months (70). A lower recovery rate (45%) was reported in a study of 55 patients (51%

African-American) at follow-up time of 29 months (17), and among 40 indigent patients (87.5% African-American) where only 35% recovered with an average time of 54 months (68). A recent retrospective analysis of 59 women reported 43% recovered to LVEF of $\geq$ 50% by 12 months with a median time to recovery of 8 months (86).

MANAGEMENT

MEDICAL MANAGEMENT. Treatment of HF during pregnancy requires special modifications to ensure fetal safety. Following delivery, most HF medications are compatible with breastfeeding (Figure 1) (89-95).

Anticoagulation. Due to reports of increased incidence of LV thrombi and systemic thromboembolism in women with PPCM (9,40,57,96) and the hypercoagulable state of pregnancy and the early postpartum period (97), anticoagulation should be considered in the setting of severely decreased LVEF during late pregnancy and 6 to 8 weeks postpartum (40). Anticoagulation is suggested by the American Heart Association when the LVEF is <30% (98), whereas the European Society of Cardiology suggests using LVEF $\leq$ 35% as the threshold (99). No published data are available to guide the decision of therapeutic versus prophylactic anticoagulation. Warfarin crosses the placenta and is avoided during pregnancy for indications other than anticoagulation of mechanical heart valves. Low-molecular-weight heparin does not cross the placenta and can be used during pregnancy (92). Both warfarin and low-molecular-weight heparin are considered safe with lactation. The novel anticoagulants have not been studied during pregnancy or lactation and are generally avoided.

EXPERIMENTAL TREATMENT. Bromocriptine is a dopamine agonist and inhibits the release of prolactin. It was originally marketed for lactation suppression, but due to the association with myocardial infarction (100-103), stroke (104,105), and seizures (105), it is no longer approved for this indication. The concept that PPCM is driven by the antiangiogenic and proapoptotic 16-kDa form of prolactin led to experimentation using bromocriptine to inhibit prolactin secretion to prevent the development of PPCM in a mouse model (36). A pilot study compared the outcome of 10 South African patients with PPCM receiving bromocriptine in addition to standard HF therapy to that of 10 patients receiving standard therapy alone (106). Patients treated with bromocriptine (2.5 mg twice daily for 2 weeks, followed by 2.5 mg daily for 6 weeks) had greater improvement in LVEF at 6 months (27% to 58%; $p = 0.012$) than the

FIGURE 1 Heart Failure and Anticoagulant Medications: Indications and Safety in Pregnancy and During Lactation

MEDICATION	DURING PREGNANCY	POTENTIAL ADVERSE EFFECTS	INDICATIONS	DURING LACTATION
HEART FAILURE MEDICATIONS				
Loop diuretics	Yes	Caution for hypovolemia or hypotension that may lead to decreased placental perfusion	For signs and symptoms of congestion and fluid overload.	Yes, but over-diuresis can lead to decreased milk production.
Beta blockers (metoprolol tartrate used most commonly)	Yes	IUGR; fetal bradycardia and hypoglycemia	For standard treatment of HF; consider treatment of women with subsequent pregnancy.	Yes
Hydralazine/nitrates	Yes	Caution with hypotension	Use for afterload reduction during pregnancy (instead of ACE-I/ARB) when needed.	Yes, but ACE-I/ARB typically chosen post-partum
Digoxin	Yes	No associated congenital defects	Can be used with symptomatic heart failure and/or systolic dysfunction during pregnancy, or afterwards per guidelines.	Yes
ACE-I/ARB	No	Anuria, oligohydramnios, fetal limb contractures, craniofacial deformation, pulmonary atresia, fetal hypocalvaria, intra uterine growth restriction, prematurity, patent ductus arteriosus, stillbirth, neonatal hypotension and death	Cannot use during pregnancy. After delivery, should be used as part of guideline-directed medical therapy for afterload reduction and LV remodeling.	Enalapril and captopril can be used
Aldosterone receptor antagonists	No	Spironolactone has been associated with antiadrenergic activity, feminization of male rat fetuses and permanent changes in reproductive tract in both sexes	As per guideline-directed medical therapy for heart failure.	Spironolactone can be used
Sacubitril-valsartan	No	Same as ACE-I/ARB	As per guideline-directed medical therapy for heart failure.	No information in human, present in rat milk
Ivabradine	Scant data in humans; would avoid due to concerns in animal studies	Scant data in humans, animal data suggest risk	As per guideline-directed medical therapy for heart failure.	No information in human, present in rat milk
ANTICOAGULANTS				
Low molecular weight heparin	Yes	Caution at time of delivery and with neuraxial anesthesia; does not cross placenta; consider the need for monitoring anti-Xa levels	For prevention and treatment of thromboembolic complications during pregnancy and as bridge to warfarin postpartum.	Yes
Warfarin	Avoid	Warfarin embryopathy and fetopathy	For prevention and treatment of thromboembolic complications postpartum.	Yes

Legend:

Green box	Data or experience to support use
Red box	Caution with using this medication
Blue box	Data is limited or inconclusive

Safety of medications need to be considered during pregnancy and lactation. ACE-I = angiotensin-converting enzyme inhibitors; ARB = angiotensin receptor blocker; HF = heart failure; IUGR = intra-uterine growth restriction; LV = left ventricular; SSP = subsequent pregnancy.

control group (27% to 36%), and fewer experienced the composite endpoint ($n = 1$, 10%) (defined as death, New York Heart Association functional class III/IV, or LVEF $<35\%$ at 6 months) compared with the control group ($n = 8$, 80%; $p = 0.006$). A second African study compared the outcome of 48 patients with PPCM receiving HF therapy of furosemide and angiotensin-converting enzyme (ACE) inhibitors to an equal number of patients additionally receiving bromocriptine 2.5 mg twice daily for 4 weeks (107). LVEF was similar at entry but higher in the bromocriptine group at 2 weeks ($p = 0.01$) and at 3, 6, and 12 months ($p = 0.001$). Mortality at 6 months was high in both groups but lower in the bromocriptine-treated women (17% vs. 29%; $p = 0.0001$). The results of these 2 African studies and their general applicability were limited by the uncharacteristically high mortality rate in the control group compared with other studies (108). An observational registry of 115 German patients with PPCM reported that bromocriptine in addition to standard therapy was associated with a higher rate of improvement in LVEF, but there was no significant difference in overall rates of recovery (49). Recently, a randomized trial of 2 different regimens of bromocriptine (1 week in 27 patients vs. 8 weeks in 31 patients) in Germany found similar outcomes in both groups (109). Treatment was started on the average of 1.6 ± 1.6 months after the delivery, in addition to standard HF therapy including ACE inhibitors/angiotensin receptor blockers, beta-blockers, mineralocorticoid antagonists, and diuretic agents. The investigators postulated an association between bromocriptine and the favorable outcomes, but the absence of a control group not receiving bromocriptine makes the results inconclusive. No information is available for the use of this therapy in women with PPCM in the United States. Until more definitive information becomes available, bromocriptine should be considered experimental. The implications of not breastfeeding due to bromocriptine should be discussed with the patient. A randomized double-blind, placebo control study (REBIRTH [Randomized Evaluation of Bromocriptine In Myocardial Recovery Therapy]) to investigate the effect of bromocriptine on myocardial recovery and clinical outcome of 200 women with PPCM in the United States and Canada has been proposed by the IPAC group and is under evaluation. The 2018 European Society of Cardiology guidelines include a weak recommendation (Class IIb, Level of Evidence: B) for the use of bromocriptine (110). Due to the association with thrombotic complications, therapeutic anticoagulation is recommended in conjunction with bromocriptine.

TREATMENT OF SEVERE PPCM. Intravenous vasodilators, such as nitroglycerin, may be needed in the setting of acute decompensated HF during pregnancy. Nitroprusside is less desirable due to the theoretical risk of cyanide toxicity (92). Possible adverse effects of dobutamine were described in an observational study of 27 nonrandomized patients with PPCM and LVEF $\leq 25\%$ (111). The 7 women treated with dobutamine had worse outcomes, but there may have been selection bias in this small study. Levosimendan is an alternative inotropic medication (99), but was not shown to improve outcomes in a randomized study of 24 patients with PPCM (112), and is not available in the United States or Canada. A recent comparison of milrinone and levosimendan in 15 women with PPCM showed comparable hemodynamic improvement with both drugs (113).

Advanced therapies. A total of 60% of cases of cardiogenic shock during or shortly after pregnancy are caused by PPCM (114). Temporary mechanical circulatory support with intra-aortic balloon pump, percutaneous ventricular assist device therapy, and extracorporeal membrane oxygenation have been used successfully in PPCM and should be considered early in patients with hemodynamic instability despite inotropic support (115–119). Temporary or durable LVADs may also be needed. Of 99 women with PPCM who received LVAD, 6% recovered and 48% went on to cardiac transplantation (118). A study of 485 women with PPCM who received cardiac transplant between 1987 and 2010 reported higher rates of graft failure and lower age-adjusted survival, which may be explained by increased rejection, higher allo-sensitization, and higher pre-transplant acuity (120).

LABOR AND DELIVERY. Timing and mode of delivery in patients presenting with PPCM during pregnancy should be discussed with the patient and coordinated by a cardio-obstetrics team of experts from obstetrics, cardiology, maternal fetal medicine, anesthesiology, nursing, pharmacy, and social work (121). An attempt to stabilize the mother to avoid potential fetal complications of prematurity is reasonable. Hemodynamic instability despite medical therapy should prompt early delivery (or termination if prior to fetal viability). Stable patients are delivered vaginally unless there are obstetric reasons for cesarean section. Cesarean delivery is associated with a higher incidence of hemorrhage, infection, and thromboembolic complications (122). Detailed pre-delivery plans should involve the patient and an experienced multidisciplinary team. Unstable patients may benefit from invasive hemodynamic optimization prior to

FIGURE 2 Counseling and Management of Subsequent Pregnancies in PPCM

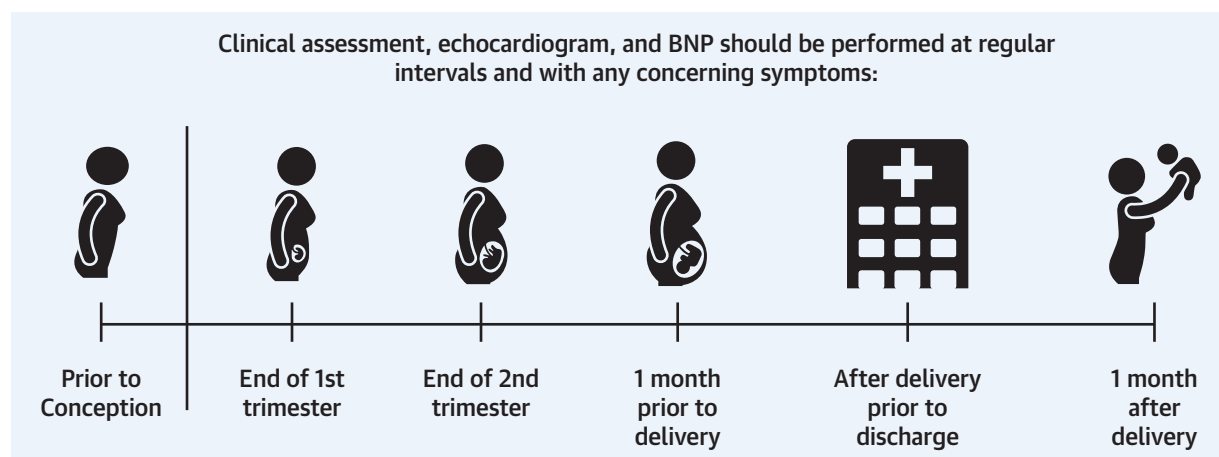
 Subsequent Pregnancy	Recovered (LVEF $\geq$ 50%)	Nonrecovered (LVEF <50%)
Preconception or First Visit	Preconception risk counseling and follow-up planning. Clinical and LVEF reassessment off renin-angiotensin blocking agents for 3 months. Baseline echocardiogram and BNP/NT-proBNP level.	Preconception risk counseling including discussion of alternative ways to build a family. If pregnant and not considering termination: Close follow-up planning, stop renin-angiotensin blocking agents and switch to hydralazine/isosorbide dinitrate. Baseline echocardiogram and BNP/NT-proBNP level.
Maternal Risks	~20% have a relapse Severe deterioration is rare Mortality unlikely Rate of subsequent recovery is high	Higher risk of relapse ~50% show further deterioration in LV dysfunction Increased morbidity and mortality Premature delivery and abortion more common
Medications	Continue beta blocker therapy (metoprolol tartrate preferred). Yield of starting prophylactic beta blocker therapy unclear. Diuretics and hydralazine/isosorbide dinitrate in case of clinical or LV functional deterioration.	Continue beta blocker therapy (metoprolol tartrate preferred). Hydralazine/Isosorbide dinitrate for hemodynamic and symptomatic improvement. Consider digoxin. Consider anticoagulation if severe LV dysfunction (LVEF <35%).
Follow-up	Close monitoring of symptoms during pregnancy and the postpartum period with repeat echocardiographic assessment of LV function and BNP/NT-proBNP level at the end of the 1st and 2nd trimesters, 1 month prior to delivery, after delivery prior to hospital discharge, 1 month postpartum, and at any time if symptoms develop.	
Labor and Delivery	Multidisciplinary team for planning; patient involved. Spontaneous vaginal delivery preferred unless fetal or maternal instability. Monitor for volume overload in the first 48 hours after delivery in cases of recurrent LV dysfunction.	Multidisciplinary team for planning; patient involved. Spontaneous vaginal delivery preferred unless fetal or maternal instability. Early delivery if further decrease in LV function and hemodynamic deterioration. Consider hemodynamic monitoring for optimization prior to delivery and monitoring during and after delivery. Monitor for volume overload in the first 48 hours after delivery.

Risks of a subsequent pregnancy differ based on the pre-conception recovery status. There is higher risk with nonrecovered myocardial function and pregnancy should be discouraged. Peripartum management options depend on the clinical status and myocardial function. ACE = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; LV = left ventricular; LVEF = left ventricular ejection fraction; PPCM = peripartum cardiomyopathy.

delivery and monitoring during delivery and the early postpartum period. Following delivery, removal of caval compression by the fetus, autotransfusion due to uterine contractions, and fluid mobilization and resorption contribute to an increase in venous return. The post-partum risk of fluid overload and pulmonary edema must be anticipated.

PRIOR TO HOSPITAL DISCHARGE

LACTATION. Breastfeeding confers multiple benefits for infants and mothers (123,124) and is recommended by the World Health Organization and the American Academy of Pediatrics (125). In developing countries where formula may be expensive and clean

FIGURE 3 Serial Monitoring During a Subsequent Pregnancy

Proposed outline for serial testing during a subsequent pregnancy. BNP = brain natriuretic peptide.

water is not readily available, not breastfeeding can pose a significant risk to the infant (126). Based on the prolactin hypothesis, the 2010 European statement on PPCM advised against breastfeeding (4). However, a small study of patients enrolled online in the United States indicated that women who breastfed actually had higher rates of recovery (26). Recent data from IPAC demonstrated that breastfeeding was not associated with adverse outcomes, inflammatory markers, or persistent myocardial dysfunction (127). The observation that breastfeeding seems to be safe in PPCM suggests that continued stimulation of prolactin secretion may not be harmful (128). Most HF medications can be given safely with breastfeeding (Figure 1) and should not be a reason to advise women against lactation.

SUDDEN DEATH PREVENTION. Only limited published data is available to guide the timing of ICD placement in women with PPCM. Premature placement should be avoided because a large proportion of women will recover to LVEF >35% within the first 6 months postpartum and will not meet criteria for ICD placement (129). Unfortunately, women with PPCM may experience cardiac arrest in the early months following diagnosis (130) and need to be protected. A recent analysis of administrative data of 9,841 hospitalizations with a primary diagnosis of PPCM revealed that 18.7% had an arrhythmia; of these, 4.2% had ventricular tachycardia, 1% had ventricular fibrillation, and 2.2% had cardiac arrest (131). In a small prospective study of women with

newly diagnosed PPCM, 3 of 7 patients with severely reduced LVEF who were compliant with a wearable cardioverter/defibrillator were found to have ventricular fibrillation that was appropriately shocked (130). A subsequent retrospective multicenter study by the same group reported that of 49 women with newly diagnosed PPCM and LVEF <35%, 12% (n = 6) had ventricular tachyarrhythmias (5 episodes of ventricular fibrillation, 2 with sustained ventricular tachycardia, and 1 with nonsustained ventricular tachycardia), and there were no inappropriate shocks (132). In contrast, a retrospective analysis of wearable cardioverter/defibrillators in 107 patients with PPCM found no shocks were delivered (either appropriate or inappropriate) during an average of 4 months of follow-up (133). Thus, despite conflicting data in small studies, and until more information becomes available, it may be reasonable to consider wearable cardioverter/defibrillators for women with new onset PPCM and severe LV dysfunction as a bridge to recovery or until an implantable ICD is indicated. Certain patients may benefit from cardiac resynchronization therapy. An anecdotal report demonstrated positive remodeling and improvement in LVEF in 2 women with PPCM treated with CRT after failing to improve on medical therapy (134).

CONTRACEPTION. Contraception should be discussed at the time of diagnosis or prior to hospital discharge. In the early postpartum setting with severe LV dysfunction, the increased risk of thromboembolism should dissuade the use of estrogen-containing

contraceptives. Progesterone-releasing subcutaneous implants or the Mirena intrauterine device are safe and effective choices. Injectable depot medroxyprogesterone acetate is less effective and is considered a second-line option. Nonhormonal barrier methods are less effective. Tubal ligation and vasectomy are other options. In a woman with persistent LV dysfunction, the risk of a subsequent pregnancy likely outweighs any risk associated with contraception. Therefore, women should be encouraged to select the method they will use most consistently. The importance of contraception should be emphasized by the cardiologist, as well as the obstetrician/gynecologist (135).

DURATION OF TREATMENT. In the presence of persistent cardiac dysfunction, cardiac medications should be continued indefinitely. After LV recovery, optimal duration of treatment is unknown. A rationale for continuation of medical therapy is supported by evidence of subclinical LV systolic dysfunction and anecdotal reports of late deterioration of LV function. Impaired LV global longitudinal and apical circumferential 2-dimensional strain were reported in 29 women with LV recovery (defined as LVEF $\geq 50\%$) at least 12 months after acute PPCM (136), supporting prior reports of decreased contractile reserve on dobutamine stress echocardiogram in women with PPCM and recovered LVEF (137). In addition, anecdotal reports have described deterioration of LV function after partial or complete recovery, even in the absence of a subsequent pregnancy (57,86,138). A recent study of patients with recovered DCM (LVEF $>50\%$) not related to pregnancy reported that 44% needed to restart cardiac medications within 6 months after discontinuation (139). It is not clear whether these findings can be extrapolated to patients with PPCM. If the patient is free from congestive symptoms, diuretic medications can be stopped. Additional HF medications, if stopped, should be weaned in a stepwise fashion with frequent clinical assessment and echocardiographic monitoring of LVEF (i.e., every 3 to 6 months). Reassessment of LV function is advised after drug discontinuation followed by annual clinical and echocardiographic assessment.

SUBSEQUENT PREGNANCY

The safety of a subsequent pregnancy is a frequent concern for patients and their families. Appropriate and accurate counseling is essential. The risks associated with a subsequent pregnancy depend primarily upon whether the myocardial function has fully recovered, and the pre-pregnancy LVEF is the

strongest predictor of outcomes (Figure 2). Detection of subclinical LV dysfunction by stress testing and strain imaging has been proposed (136,140), but further research about the predictive utility of these tests is needed.

RESIDUAL MYOCARDIAL DYSFUNCTION. If there is evidence of persistent myocardial dysfunction (i.e., LVEF $<50\%$), women should be advised on the reported high risk of recurrent HF, durable deterioration of cardiac function, and mortality. In a review of 93 women with persistent LV dysfunction (published between 2001 and 2010), nearly one-half had deterioration of LV function, which persisted in 39%, and 16% died (141). A more recent study of 16 European and African women with LV dysfunction prior to a subsequent pregnancy reported death in 25%, and only 31% recovered LV function $>50\%$ (142). Additionally, fetal outcomes tend to be worse among women with persistent LV dysfunction, with higher rates of stillbirth, abortion, and pre-term delivery (141). Based on these data, the 2018 European Society of Cardiology guidelines for the management of cardiovascular diseases during pregnancy discourage subsequent pregnancy if the LVEF is not $>50\%$ to 55% (110). After counseling, some women choose to proceed and should be followed closely during pregnancy.

RECOVERED MYOCARDIAL FUNCTION. Women who recover LVEF $>50\%$ have lower risk of complications during a subsequent pregnancy, but there is still increased risk of recurrent HF (141). An early study of 23 subsequent pregnancies in women with recovered LVEF reported no deaths, but 21% had a substantial ($>20\%$) decrease in LVEF and symptoms of HF, with 14% having persistent LV dysfunction (143). A prospective study of 18 women with subsequent pregnancies in Europe and Africa reported no mortality but an average of 15% reduction in LVEF after the delivery, which was persistent in almost one-half of them (142). More recently, a single-center study in the United States of 39 pregnancies in 24 women with LVEF of $\geq 50\%$ prior to subsequent pregnancy reported no maternal deaths and a relapse rate of 21% (LVEF nadir range of 35% to 45%) (144). All patients were reported to recover, although 3 women required admission to the intensive care unit. None of these patients were treated with bromocriptine, 79% breastfed their infants, and 44% were treated with beta-blockers (144). Pre-conception counseling should include discussion of the potential risk of recurrent myocardial dysfunction, which may persist after the pregnancy.

MONITORING AND TREATMENT DURING SUBSEQUENT PREGNANCY.

Our recommended protocol for monitoring women with a history of PPCM during a subsequent pregnancy is outlined in [Figures 2 and 3](#). In women with recovered LV function who are taking HF medications, ACE inhibitors/angiotensin receptor blockers, and aldosterone receptor antagonists should be discontinued prior to conception, and it may be prudent to ensure stability of LV function after at least 3 months off of these medications prior to considering the LV recovered. The prophylactic use of beta-blockers during subsequent pregnancies in women with recovered LVEF may be considered but there is a paucity of data to support its role. In the study by Codsì et al. (144), 19 of 43 women with subsequent pregnancies were treated with beta-blockers, 6 had a decrease in LVEF, and there were no instances of intrauterine growth restriction. In a recent series of patients with subsequent pregnancies, it was postulated that addition of bromocriptine to standard therapy led to higher rates of recovery and no deaths (142); however, this was a small, non-randomized study, and there was a significant difference in the baseline LVEF in the 2 arms of the study.

CONCLUSIONS

The diagnosis of PPCM should be considered in any pregnant or postpartum woman with symptoms concerning for HF. An elevated BNP level should always be followed by an echocardiogram to assess for systolic dysfunction. Prompt treatment with medications tailored for pregnancy and lactation may

prevent adverse outcomes. Limited studies suggest breastfeeding is safe. Acutely ill women should be managed by specialized multidisciplinary teams, and may require advanced HF therapies. Women considering a subsequent pregnancy should be counseled and monitored by physicians familiar with PPCM. Long-term follow-up is important, but the optimal duration of medications following recovery is unknown.

Despite many advances in our understanding of PPCM, questions remain about the pathogenesis and complex interaction of genetics with the vascular and hormonal milieu of late pregnancy. The role of bromocriptine remains unclear, and randomized clinical trials are needed for determining the risks and potential benefits. Important gaps in knowledge remain, such as the optimal anticoagulation strategy, timing of ICD implantation, risk prediction and management during a subsequent pregnancy, and the long-term duration of medications after myocardial recovery. Given the overall rarity of PPCM, collaboration among multiple centers will be needed to answer these questions.

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Neighborhood education status drives racial disparities in clinical outcomes in PPCM

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Background Peripartum cardiomyopathy (PPCM) disproportionately affects women of African ancestry. Additionally, clinical outcomes are worse in this subpopulation compared to White women with PPCM. The extent to which socioeconomic parameters contribute to these racial disparities is not known.

Methods We aimed to quantify the association between area-based proxies of socioeconomic status (SES) and clinical outcomes in PPCM, and to determine the potential contribution of these factors to racial disparities in outcomes. A retrospective cohort study was performed at the University of Pennsylvania Health System, a tertiary referral center serving a population with a high proportion of Black individuals. The cohort included 220 women with PPCM, 55% of whom were Black or African American. Available data included clinical and demographic characteristics as well as residential address georeferenced to US Census-derived block group measures of SES. Rates of sustained cardiac dysfunction (defined as persistent LVEF <50%, LVAD placement, transplant, or death) were compared by race and block group-level measures of SES, and a composite neighborhood concentrated disadvantage index (NDI). The contributions of area-based socioeconomic parameters to the association between race and sustained cardiac dysfunction were quantified.

Results Black race and higher NDI were both independently associated with sustained cardiac dysfunction (relative risk [RR] 1.63, confidence interval [CI] 1.13-2.36; and RR 1.29, CI 1.08-1.53, respectively). Following multivariable adjustment, effect size for NDI remained statistically significant, but effect size for Black race did not. The impact of low neighborhood education on racial disparities in outcomes was stronger than that of low neighborhood income (explaining 45% and 0% of the association with black race, respectively). After multivariate adjustment, only low area-based education persisted as significantly correlating with sustained cardiac dysfunction (RR 1.49; CI 1.02-2.17).

Conclusions Both Black race and NDI independently associate with adverse outcomes in women with PPCM in a single center study. Of the specific components of NDI, neighborhood low education was most strongly associated with clinical outcome and partially explained differences in race. These results suggest interventions targeting social determinants of health in disadvantaged communities may help to mitigate outcome disparities. (Am Heart J 2021;238:27–32.)

Peripartum cardiomyopathy (PPCM) is a sometimes severe form of cardiomyopathy occurring toward the end of pregnancy or months postpartum in women with previously normal hearts.^{1,2} The estimated incidence of PPCM is 1 in 2,000 pregnant women world-

wide, with hotspots such as Haiti and Nigeria.^{1,3} In the U.S., the prevalence of PPCM is higher in Black women than White women.^{4,5} Clinical outcomes in PPCM vary widely, with most women ultimately recovering cardiac function, but a significant minority of women developing worsening function, need for LVAD or cardiac transplant, or death.^{1,3} Again, Black women fare worse than White women: they are generally diagnosed later in the postpartum period and, when diagnosed, are less likely to have recovery of left ventricular (LV) function and more likely to experience adverse events compared to White women.^{6,7} We previously described the outcomes of 220 women with PPCM diagnosed and treated within the University of Pennsylvania Health System (Penn) and demonstrated that Black women were half as likely to recover, and took twice as long to do so when they did recover.⁶

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The reasons for differences in clinical outcomes between Black and White women with PPCM remain unclear, and may be related to underlying biology, genetics, or a complex interplay of individual and social factors. Social determinants of health impact the development of cardiovascular (CV) risk factors and disease, health behaviors, treatment patterns, and clinical outcomes in complex ways. Neighborhood disadvantage, for example, is associated with incident heart failure and risk of readmission, in addition to individual race/ethnicity and socioeconomic status.⁸⁻¹⁰ Neighborhood characteristics and associations with outcomes in PPCM have not previously been examined.

We therefore sought to determine: (1) if neighborhood level social determinants impact clinical outcomes in PPCM; and (2) to what extent the poor outcomes observed in Black women with PPCM may be attributed to neighborhood disadvantage. To do so, we leveraged our large, racially diverse cohort of PPCM patients, the majority of whom live in Philadelphia, a large city with noted differences in access to care and clinical outcomes based on race.¹¹ We further integrated our cohort with a spatially enabled data set to understand how neighborhood disadvantage impacts clinical outcomes in PPCM, and interacts with racial background.

Methods

The University of Pennsylvania Institutional Review Board approved the study and determined that informed consent was not required. We assembled a retrospective cohort of patients at Penn with a diagnosis of PPCM from January 1, 1986, through December 31, 2016 based on diagnostic codes (International Classification of Diseases, Ninth Revision codes 674.50-54) or echocardiographic evaluations obtained within 6 months of delivery.⁶ Patients with a history of congenital heart disease, valvular disease predating their PPCM diagnosis, history of radiation or cardiotoxic chemotherapy, or another explanation for their heart failure were excluded.

We abstracted demographic details, residential addresses, diagnosis, and clinical outcome information from the patient electronic medical records. We used ArcGIS Pro (Esri, Redlands, CA) to geocode addresses and identify corresponding Census block groups. A Census block group is the smallest geographic unit (average 1,500 people) in which sample data from decennial census and the American Community Survey are available. Using block groups as a proxy for neighborhoods, we spatiotemporally integrated a select set of variables from the 1990, 2000, and 2010 Census as well as the 2009 through the 2016 American Community Surveys.¹² A composite neighborhood concentrated disadvantage index (NDI) was computed for each patient as the sum of the proportions of unemployment, households receiving public assistance, persons below the federal poverty line,

adults without a high school diploma, female-headed households, renter-occupied residences, and residential tenure shorter than 1 year.¹³ Each of the components of the NDI were dichotomized at the threshold of their highest quartile for statistical analyses. Sustained cardiac dysfunction was defined as persistent LVEF <50%, cardiac transplant, LVAD placement, or death. Myocardial recovery was defined as at least one occasion of LVEF >50% on echocardiography.

Distributions of patient characteristics were compared by quartiles of block group level factors using χ^2 or Fisher exact tests. We examined the potential for a non-linear association between NDI and persistent cardiomyopathy with restricted cubic splines.¹⁴ We examined the cardiac outcomes (sustained cardiac dysfunction, and myocardial recovery) relative to NDI, and then separately to each component informing the NDI. Crude and multivariate log binomial regression models were used to estimate risk ratios (RR) and corresponding 95% confidence intervals (95% CI). Multivariable models were adjusted first for patient characteristics that were associated with the area-based exposures, or race. The proportional contribution of each area-based socioeconomic parameters to the effect of race on cardiac outcomes was quantified.¹⁵

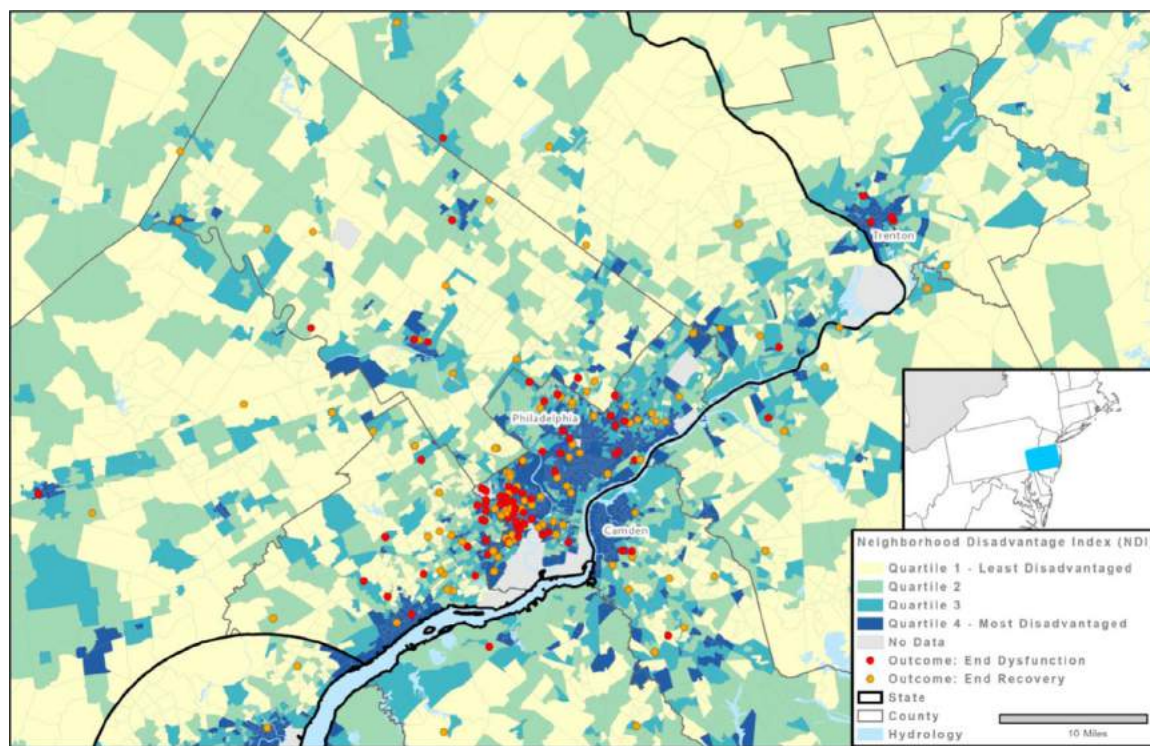
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Results

The cohort of 220 women diagnosed with PPCM consisted of 121 Black women and 99 non-Black women. Demographic and clinical characteristics at presentation and clinical outcomes of this cohort have been described previously.⁶ Figures 1 and S1 present the relational geographic distribution of the patients to the greater Philadelphia Metropolitan Area. Neighborhoods with greater disadvantage are concentrated in Philadelphia, Camden, and Trenton.

Table S1 provides the demographics and clinical characteristics for the study population overall, and subdivided by quartiles of NDI, median household income, and proportion of residents with less than high school education, according to block group. Patients in neighborhoods with greater disadvantage were younger (28 years in quartile 4 (Q4) vs. 32 years in quartile 1 (Q1), $P = .002$), and more likely to be Black (89% in Q4 vs 16% in Q1, $P < .001$). Patients from more disadvantaged neighborhoods tended to have a lower nadir LVEF compared to those from less disadvantaged neighborhoods.

Figure 1



Relational geographic distribution of PPCM patients to the greater Philadelphia Metropolitan Area, distinguishing between patients who recovered (in yellow) versus those who did not (in red), and stratified by neighborhood disadvantage index (NDI). To protect the privacy of the patients, their locations were spatially anonymized using areal filters and random perturbation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

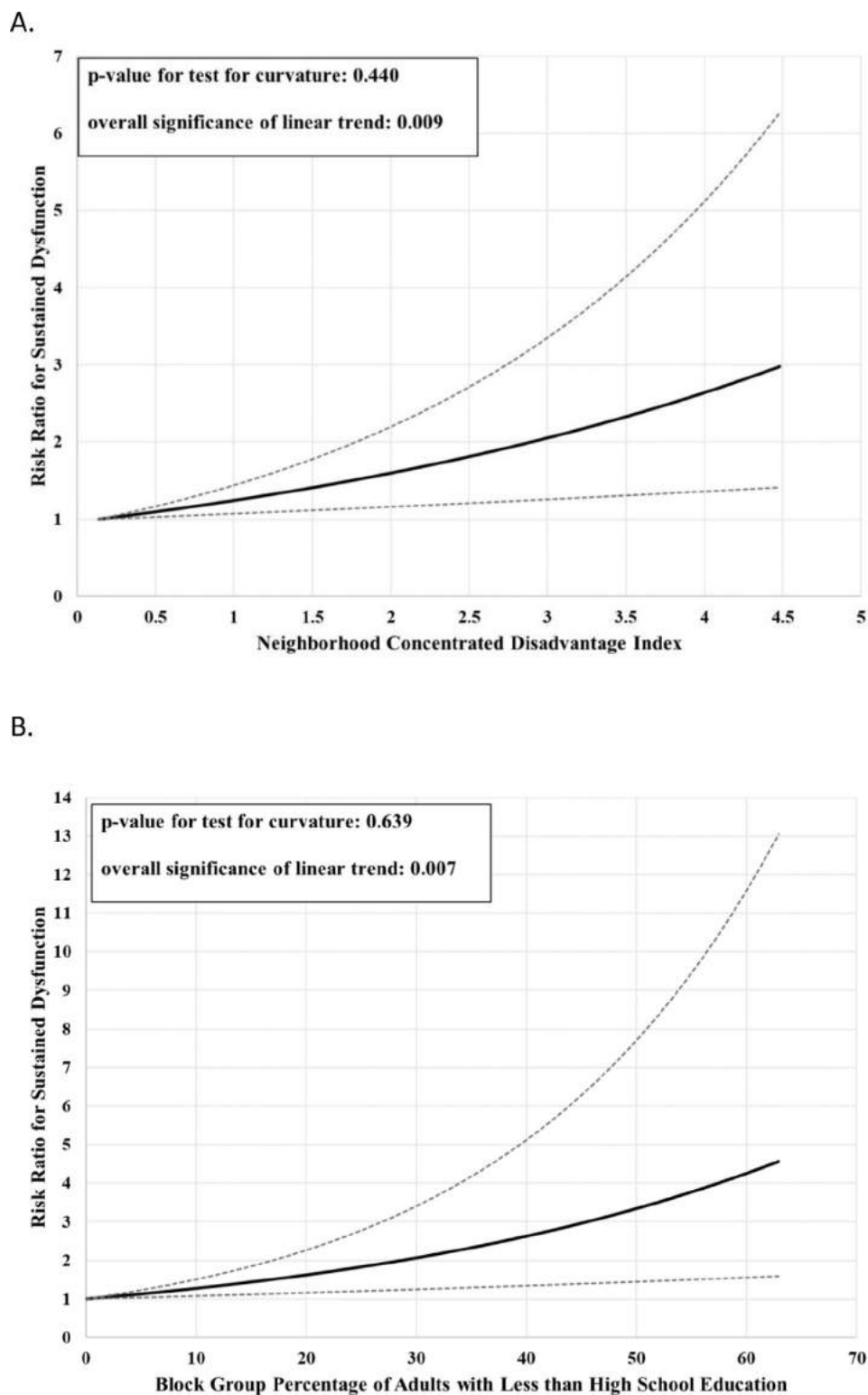
Overall, 36.3% ($n = 81$) of patients experienced sustained dysfunction. Both Black race (44% vs 28%; RR 1.63, 95% CI 1.13-2.36) and higher NDI (RR 1.29, 95% CI 1.08-2.53) were independently associated with sustained cardiac dysfunction (Table I). Restricted cubic spline regression revealed that the association between NDI and sustained dysfunction was linear (P value for test for curvature: 0.440; overall significance of linear trend 0.004; Figure 2). Every unit increase in NDI was associated with a 29% higher likelihood of sustained cardiac dysfunction. Effect estimates for both exposures were attenuated following adjustment for age, twin pregnancy, number of children, and timing of diagnosis, but only NDI remained statistically significant (Table I, M1).

Of the individual components of NDI, only area-based low education and high rental occupied housing were significantly associated with sustained cardiac dysfunction (Table I, first column), which persisted after adjusting for race (Table I, M2). Other components of NDI had adjusted risk ratios near null (Table I, M2). The effect estimate for race on sustained dysfunction was largely attenuated upon control for area-based low education: in fact,

low education explained approximately 45% of the effect of race on sustained dysfunction (Table I, M3 vs M2). In contrast, control for area-based low income had no impact on the effect estimate for race on sustained dysfunction (Table I, M5 vs M1). Overall comparable results were observed for myocardial recovery (Supplemental Table S2).

Discussion

We find here, in a large racially diverse cohort of women with PPCM, that the markedly worse average clinical outcome of Black women may be explained in large part by greater neighborhood disadvantage, specifically lower educational status at the block group level. The findings underscore the importance of social determinants of health as potential drivers of racial disparities in PPCM. Specifically, lower education status appears to impact PPCM outcomes independent of race, and may do so by creating barriers to timely access to high-quality medical care, communication with medical providers, understanding of symptoms, medication adherence, and

Figure 2

Linear relationships between (A) neighborhood-concentrated disadvantage index and occurrence of sustained cardiac dysfunction, and (B) proportion of adults in block group with less than a high school education and occurrence of sustained cardiac dysfunction.

Table I.

	Crude RR (95% CI)	p-value	Adjusted RR (M1)	p-value	Adjusted RR (M2)	p-value	Adjusted RR (M3)	p-value	Adjusted RR (M4)	p-value	Adjusted RR (M5)	p-value
Black race	1.63 (1.13, 2.36)	0.009	1.34 (0.88, 2.03)	0.171	1.19 (0.74, 1.92)	0.474	1.17 (0.76, 1.81)	0.419	1.18 (0.74, 1.78)	0.527	1.34 (0.87, 2.06)	0.185
Neighborhood concentrated disadvantage index	1.29 (1.08, 1.53)	0.004	1.23 (1.00, 1.51)	0.021	1.17 (0.91, 1.49)	0.217						
Assessments for the Individual Components of the Neighborhood Concentrated Disadvantage Index ^a												
>22.3% adults with less than HS education	1.73 (1.25, 2.39)	<0.001	1.57 (1.10, 2.24)	0.014	1.49 (1.02, 2.17)	0.040						
>31.8% Renter Occupied Housing	1.70 (1.20, 2.42)	0.003	1.57 (1.07, 2.30)	0.020	1.50 (1.00, 2.25)	0.049						
>28.5% HH w. annual income below poverty line	1.23 (0.86, 1.77)	0.265	1.09 (0.75, 1.60)	0.647	1.00 (0.68, 1.46)	0.999						
>31.5% Female Headed Households	1.23 (0.86, 1.77)	0.264	1.00 (0.68, 1.47)	0.991	0.95 (0.65, 1.39)	0.946						
>16.3% adults unemployed	1.14 (0.78, 1.67)	0.482	0.98 (0.66, 1.45)	0.915	0.90 (0.60, 1.33)	0.585						
>10.2% adults on public assistance	1.10 (0.75, 1.62)	0.621	0.96 (0.65, 1.42)	0.827	0.90 (0.60, 1.34)	0.900						
>28.4% with residential tenure less than 1 year	1.11 (0.76, 1.63)	0.576	1.08 (0.74, 1.59)	0.682	1.10 (0.74, 1.64)	0.616						

^a Each of the block group-level components of the neighborhood disadvantage index were dichotomized at highest quartile for statistical analyses (i.e. Q4 vs Q1-Q3). The numeric thresholds presented for each of the NDI components represent the cutoff value defining the highest quartile.

M1 = adjusted for age, twin pregnancy, number of children, and timing of diagnosis

M2 = race model adjusted for age, twin pregnancy, number of children, timing of diagnosis, and neighborhood concentrated disadvantage index (NDI); Models for individual NDI component area-based exposures adjusted for age, twin pregnancy, number of children, timing of diagnosis, and race

M3 = race model adjusted for age, twin pregnancy, number of children, timing of diagnosis, and neighborhood-based low education

M4 = race model adjusted for age, twin pregnancy, number of children, timing of diagnosis, and neighborhood-based high renter occupied housing

M5 = race model adjusted for age, twin pregnancy, number of children, timing of diagnosis, and neighborhood-based high poverty

self-advocacy, especially in the context of structural racism. Race and socioeconomic class are highly correlated in the U.S. Racial disparities in maternal health have been documented independent of education of the individual.¹⁶ The experiences of racism impact the quality of care delivered, patient care engagement, and communication between health care providers and patients. Women from lower SES groups report higher rates of perceived discrimination based on race and insurance status, which may further adversely impact maternal health outcomes.^{17, 18}

Despite being the largest mixed-race PPCM cohort reported to date, limitations of our study include being single-center with variable duration of follow-up, which reduces generalizability. Due to relatively small numbers, the analyses do not include Latino or Asian women. Additionally, data were assessed only at the block group level, rather than individual level. Although area-based measures of socioeconomic status may serve as crude proxies to individual SES, they may also have distinct independent and synergistic impacts on PPCM outcomes. Nevertheless, the NDI and component area-based exposures examined do not account for psychological distress, food insecurity, or delays in medical care, all of which may directly impact outcomes for women with PPCM. Data on the use of in vitro fertilization, or on medical treatments for PPCM including type, intensity, or duration were not captured in the manual chart abstractions completed for the cohort and thus are not accounted for in the presented analyses.

In summary, our finding that low area-based education was associated with worse outcomes in women with PPCM suggests a potential role for strategies aimed at improving access to care, patient-provider communication, and in particular health education in mitigating such disparities.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL RESEARCH ARTICLE

Genetic and Phenotypic Landscape of Peripartum Cardiomyopathy

BACKGROUND: Peripartum cardiomyopathy (PPCM) occurs in $\approx 1:2000$ deliveries in the United States and worldwide. The genetic underpinnings of PPCM remain poorly defined. Approximately 10% of women with PPCM harbor truncating variants in *TTN* (TTNtvs). Whether mutations in other genes can predispose to PPCM is not known. It is also not known if the presence of TTNtvs predicts clinical presentation or outcomes. Nor is it known if the prevalence of TTNtvs differs in women with PPCM and preeclampsia, the strongest risk factor for PPCM.

METHODS: Women with PPCM were retrospectively identified from several US and international academic centers, and clinical information and DNA samples were acquired. Next-generation sequencing was performed on 67 genes, including *TTN*, and evaluated for burden of truncating and missense variants. The impact of TTNtvs on the severity of clinical presentation, and on clinical outcomes, was evaluated.

RESULTS: Four hundred sixty-nine women met inclusion criteria. Of the women with PPCM, 10.4% bore TTNtvs (odds ratio=9.4 compared with 1.2% in the reference population; Bonferroni-corrected P [P^*]= 1.2×10^{-46}). We additionally identified overrepresentation of truncating variants in *FLNC* (odds ratio=24.8, $P^*=7.0 \times 10^{-8}$), *DSP* (odds ratio=14.9, $P^*=1.0 \times 10^{-8}$), and *BAG3* (odds ratio=53.1, $P^*=0.02$), genes not previously associated with PPCM. This profile is highly similar to that found in nonischemic dilated cardiomyopathy. Women with TTNtvs had lower left ventricular ejection fraction on presentation than did women without TTNtvs (23.5% versus 29%, $P=2.5 \times 10^{-4}$), but did not differ significantly in timing of presentation after delivery, in prevalence of preeclampsia, or in rates of clinical recovery.

CONCLUSIONS: This study provides the first extensive genetic and phenotypic landscape of PPCM and demonstrates that predisposition to heart failure is an important risk factor for PPCM. The work reveals a degree of genetic similarity between PPCM and dilated cardiomyopathy, suggesting that gene-specific therapeutic approaches being developed for dilated cardiomyopathy may also apply to PPCM, and that approaches to genetic testing in PPCM should mirror those taken in dilated cardiomyopathy. Last, the clarification of genotype/phenotype associations has important implications for genetic counseling.

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Clinical Perspective

What Is New?

- Women with peripartum cardiomyopathy bear a significantly high burden of loss-of-function variants in several genes, including *TTN*, *FLNC*, *DSP*, and *BAG3*.
- The identity and relative abundance of these variants is remarkably similar to that seen in idiopathic dilated cardiomyopathy, indicating that the genetic predisposition to peripartum cardiomyopathy and dilated cardiomyopathy may be the same.
- Although patients who have peripartum cardiomyopathy with *TTN* truncating variants present with a lower ejection fraction, no significant differences in rates of recovery were seen.

What Are the Clinical Implications?

- Genetic counseling and testing should be considered for women with peripartum cardiomyopathy, following guidelines developed for dilated cardiomyopathy.
- Gene-specific therapies established for dilated cardiomyopathy, such as low threshold for defibrillator devices in patients with *DSP* or *FLNC* variants, should be considered.

Peripartum cardiomyopathy (PPCM) is a rare disease manifesting as heart failure with reduced left ventricular ejection fraction (LVEF) that develops during the late peripartum or postpartum period.^{1,2} Women exhibit a range of presentations, including cardiogenic shock, and a range of outcomes, including the need for mechanical circulatory support or heart transplantation. The pathobiology of PPCM remains unclear, although there is a strong epidemiological relationship with preeclampsia,³ and evidence in rodents and clinical studies has implicated vasculohormonal pathways of late gestation, including prolactin, vascular endothelial growth factor, and soluble Fms-like tyrosine kinase 1, in the pathogenesis of PPCM.^{4,5} Genetic variation has been linked to the development of PPCM. PPCM incidence is higher in women of African American descent, and strikingly higher in populations from Haiti and Nigeria, suggesting a possible genetic contribution.^{2,6} A number of studies have reported familial clustering of PPCM, and co-occurrence, as well, with nonischemic dilated cardiomyopathy (DCM), a phenotypically similar disease, and which itself is linked to genetic variation.² Last, we showed previously that, among 172 women with PPCM, 15% bore truncating loss-of-function (LOF) variants (ie, frameshift, nonsense, and splice-altering) in the gene *TTN*, a prevalence significantly higher than in a reference population ($P=1.3\times 10^{-7}$), and similar to

a cohort of patients with DCM.⁷ At least a subset of PPCM thus has a genetic pathogenesis.

At least 2 critical questions were highlighted by previous studies. First, do LOF variants in genes other than *TTN* also predispose to PPCM? To date, PPCM cohorts have not been of sufficient size to allow assessment of the contribution of other DCM genes. And second, are there genotype/phenotype correlations between women with and without *TTN* truncating variants (TTNtvs)? To date, the largest PPCM group with both genotype and phenotype information was our post hoc analysis of 85 women from the IPAC cohort (Investigations in Pregnancy Associated Cardiomyopathy).⁷ In this small group, cardiac function at presentation was similar between women with and without TTNtvs, but at a 1-year follow-up, women carrying TTNtvs had worse cardiac function. By contrast, however, in the IMAC-2 group (Intervention in Myocarditis and Acute Cardiomyopathy 2), no differences were observed in ejection fraction at enrollment or follow-up ejection fraction.⁷ Thus, it remains unclear if TTNtvs in women with PPCM are associated with any aspects of clinical presentation or predict any clinical outcomes.

We therefore enrolled and examined a large, international cohort of 654 women with PPCM, who provided clinical information and DNA. Of these, 469 satisfied clinical and sequencing data quality metrics. We report here the identification of novel genetic determinants of PPCM and genotypic/phenotypic correlations.

METHODS

The authors declare that all supporting data are available within this article and its [Data Supplement](#).

Patient Recruitment and Exclusion

Eligible women with a diagnosis of peripartum cardiomyopathy were retrospectively recruited from international institutions, using the internationally accepted definition of PPCM, most recently updated by the Heart Failure Association of the European Society of Cardiology Working Group, as the development of heart failure and LVEF $\leq$ 45% toward the end of pregnancy or in the months after delivery, where no other cause is identified.⁸ In the United States, patients carrying an *International Classification of Diseases, 9th Revision* code of 674.54 or *International Classification of Diseases, 10th Revision* code of O90.3 were recruited from the identified academic centers. Patients were excluded for lack of baseline (ie, at time of diagnosis) LVEF data or a baseline LVEF $>$ 45%, and those with previous evidence of congenital heart disease or preexisting valvular heart disease were excluded, as well. Clinical and genetic data from these recruits were combined with data from the previously described cohort of 172 patients with PPCM.⁷ Chart reviews were performed for pertinent medical history including race and ethnicity, age at diagnosis, delivery date, mode of delivery, time from delivery to PPCM diagnosis, and history of gestational hypertension/preeclampsia/superimposed preeclampsia. Ancestry was

Table 1. Demographic and Clinical Characteristics, and Burden of Rare Protein-Truncating Variants, in Each Cohort of the Current Study

Characteristics	University of Pennsylvania	Japan	IPAC	IMAC-2	Germany	Chicago	Pitt
No. of patients	106	8	82	31	108	4	25
Age, y	30.49±6.7	30.63±3.5	29.89±6.3	31.29±6.8	33.85±5.2	23.50±8.6	29.67±5.6
European descent, n (%)	48 (46.1)	0	52 (63.4)	20 (63.5)	47 (90.4)	0	16 (64)
African descent, n (%)	53 (51.0)	0	26 (31.7)	11 (35.5)	3 (5.8)	3 (75)	9 (36)
Other race, n (%)	3 (2.9)	8	4 (4.9)	0	2 (3.8)	1 (25)	0
Left ventricular ejection fraction	27.2±12.4	29.1±10.1	29.5±9.2	27.2±7.4	26.7±9.0	32.9±6.0	27.8±10.2
Gestational hypertension or pre-eclampsia, n (%)	42/104 (40.4)†	6/8 (75)	32/82 (39)	NA	24/64 (37.5)†	1/3 (33.3)	8/25 (32)
All truncating variants	15	1	12	4	18	0	3
TTN truncating variants	9	1	10	4	13	0	0

(Continued)

established by patient self-report or by chart review. Cardiac data were reviewed for mode of PPCM diagnosis, ejection fraction, left ventricle end-systolic dimension, and left ventricle end-diastolic dimension at the time of diagnosis, presence of arrhythmias, use of cardiac medications, follow-up cardiac testing, and end point of PPCM diagnosis including but not limited to recovery, persistent dysfunction, left ventricular assist device, transplant, and death. The study was approved by the institutional review board at each study center. All patients provided written informed consent.

DNA Capture, Sequencing, and Analysis

DNA was extracted using Qiagen's Gentra Puregene kit; 1 µg of DNA was sheared with a Diagenode BioRuptor for 30 minutes set on high with 30 seconds on/off. Sheared DNA underwent library preparation using the Agilent SureSelect XT kit. Barcoded libraries were enriched for DCM-associated genes by using a custom Agilent SureSelect bait panel (Seidman DCMv5),⁷ which includes all 364 exons of *TTN*. Libraries were run on an Illumina NextSeq and variants were called using the Agilent SureCall.

Reference Population and Rare Variant Definition

The MyCode Community Health Initiative at Geisinger in Pennsylvania and New Jersey is a precision medicine initiative

that enrolls patient participants in a systemwide biobank designed to store blood and other samples for research use. We previously described TTN genotyping on the first 92 455 participants who underwent exomic sequencing,⁹ and use this population here as a control group of patients with TTNts but without a documented diagnosis of cardiomyopathy. The Genome Aggregation Database, GnomAD v2.1.1, was used as a reference population for pooled genetic analysis of the non-TTN genes. The data set spans 125 748 exome sequences and 15 708 whole-genome sequences from unrelated individuals. For all comparative analyses, we compared our sequencing data against the GnomAD v2 exomes for homogeneity of read depth and coverage, and used a minor allele frequency cutoff at $<1 \times 10^{-4}$ for rare variants, as used in previous studies in DCM and hypertrophic cardiomyopathy.¹⁰

Variant Analysis and Burden Testing

Protein-altering variants were defined as truncating (nonsense, frameshift, and splice site altering) or nontruncating (missense). We only included variants that mapped to regions in the GnomAD data frame with adequate coverage (defined as read depth ≥ 10 in at least 90% of individuals sequenced). All identified variants were passed through a filter for minor allele frequency $<1 \times 10^{-4}$ within the GnomAD database, and GnomAD flags including multinucleotide variant, low-complexity region, low-confidence predicted loss of

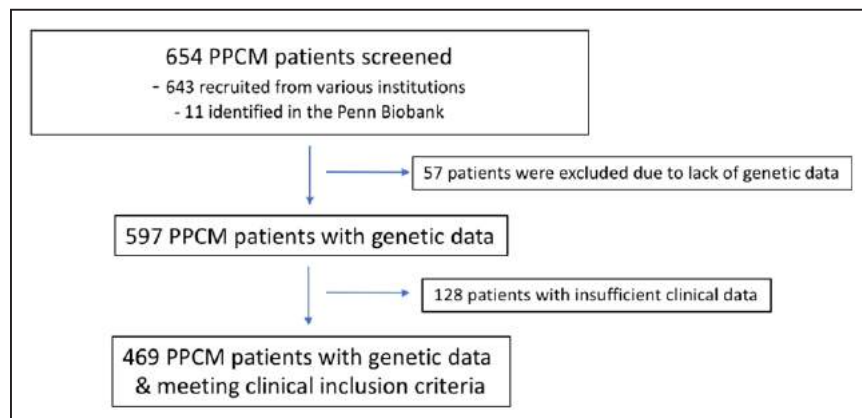


Figure 1. Inclusion criteria.
PPCM indicates peripartum cardiomyopathy.

Table 1. Continued

Hopkins	Washington University	Yale	Israel	Denmark	Total	USA	Non-USA	P value*
19	19	4	41	22	469	290	179	
32.47±4.8	27.67±6.9	27.75±5.9	32.15±6.3	30.59±6.7	30.94±6.4	30.13	32.64	1.3×10 ⁻⁵
12 (63.2)	10 (55.6)	1 (25)	35 (87.5)	22 (100)	263 (64.1)	159 (55.21)	62 (82.67)	2.62×10 ⁻⁵
5 (26.3)	8 (44.4)	2 (50)	5 (12.5)	0	126 (30.7)	118 (40.97)	5 (6.67)	1.87×10 ⁻¹⁹
2 (10.5)	0	1 (25)	0	0	21 (5.1)	11 (3.82)	8 (10.67)	
27.8±10.6	31.3±8.3	27.0±12.4	35±8.1	25.9±9.5	28.5	28.43	28.6	0.85
3/18 (16.7)+	9/19 (47.4)	1/3 (33.3)	6/41 (14.6)	10/22 (45.5)	142/389 (36.5)	96/254 (37.8)	46/135 (34.1)	0.51
4	2	0	7	4	70	40 (13.8)	30 (17.1)	0.46
2	0	0	6	4	49	25 (8.6)	24 (13.4)	0.14

Other race indicates non-White and non-Black.

IMAC-2 indicates Myocarditis and Acute Cardiomyopathy 2; and IPAC, Investigations in Pregnancy Associated Cardiomyopathy.

*P value United States vs non-United States by Student *t* test for continuous variables and by χ^2 test for discrete variables.

+Some patients missing data on hypertensive disorder.

function, and LOFTEE warning, as well. The National Center for Biotechnology Information ClinVar database was referenced for known pathogenic, unknown, or benign associations with cardiomyopathy listed in [Tables II and III in the Data Supplement](#).

Statistical Analysis

Comparisons of clinical and genetic characteristics were performed by using χ^2 and 2-sided Student *t* tests. Ejection fraction compared against time to diagnosis was evaluated using ANOVA and the Tukey method for multiple comparisons. Variant burden testing was performed for each gene, and case excess was defined by the difference in frequency between the PPCM cohort and GnomAD database. An odds ratio (OR) with 95% confidence interval was calculated using a presumed case and control ratio inferred by variant frequency of each gene in GnomAD. *P* values were adjusted by Bonferroni correction. All analyses were performed using Microsoft excel and R studio software.

RESULTS

Study Patients and DNA Sequencing

Six hundred fifty-four women with PPCM were identified and recruited from several US and international academic centers (Figure 1). Next-generation sequencing was performed on 67 genes, including *TTN* ([Table I in the Data Supplement](#)). Fifty-seven patients were excluded because of insufficient genetic data. Of the remaining 597 women, complete baseline cardiac function information was unavailable in 128, leaving 469 women with adequate genetic and clinical data. Demographic data ([Table 1](#)) indicated that 41% of US subjects were of African descent (compared with 13.4% of the US population,¹¹ OR, 4.43 [3.51–5.60], $P \leq 0.00001$), consistent with previous reports of elevated prevalence of PPCM in subjects of African descent. Of the total cases, 36.5%

were accompanied by gestational hypertension or pre-eclampsia (compared with estimated 5%–8% worldwide prevalence,¹² OR, 6.6–10.9 [5.4–13.4], $P \leq 0.00001$), again consistent with previous reports.³ No significant differences were noted in LVEF at presentation or presence of hypertensive disorder between US and non-US centers ([Table 1](#)). There was a higher prevalence of patients of African descent in US centers.

Genetic Variant Burden in PPCM

Variants were identified by next-generation sequencing of 67 genes previously associated with DCM using target capture methodology (see Methods). These sequence variants were filtered to include those with high sequence quality (read depth of ≥ 10 in 90% of sequenced individuals) and were rare in the reference GnomAD data set (mean allele frequency $< 1 \times 10^{-4}$). We focused on truncating variants, including nonsense, frameshift, and canonical splicing variants, because these variants are predicted to have a strong impact on protein function. Among the cohort of 469 women with PPCM, we identified 69 women who bore 70 different rare heterozygous truncating variants in 12 different genes ([Table 2](#) and [Table II in the Data Supplement](#)). There were no women bearing homozygous or compound heterozygous truncating variants. One woman bore a truncating variant in both MYH7 and FLCN ($P=0.81$ versus GnomAD, assuming a random distribution of truncating variants). The prevalence of 70 truncating variants in 469 women with PPCM (14.9%) demonstrates a 4.1-fold increased risk of disease compared with the GnomAD reference population (3.6%, Bonferroni-corrected $P [P^*]=3.5 \times 10^{-8}$).

Of the 70 truncating variants, 49 (70%) were in *TTN*, and are discussed further in the next section. Six truncating variants were in the desmoplakin (*DSP*) gene (OR, 14.9, compared with GnomAD; $P^*=1.0 \times 10^{-8}$), 4 were in

filamin C (*FLNC*, OR, 24.8; $P^*=7.0\times10^{-8}$), 1 was in *BAG3* (OR, 53.1; $P^*=0.02$), 2 were in each Myosin Heavy Chain 6 and 7 (*MYH6* and *MYH7*, $P^*>0.05$), and 1 was in *VCL* ($P^*>0.05$). Fifty-three of these variants were absent from the >125 000 exomes in GnomAD v2.1.1. This profile of genes with significant rare truncating variant enrichment is highly similar to that reported in a large cohort of patients with DCM,¹⁰ which identified significant variant enrichment in *BAG3*, *DSP*, *LMNA*, *TTN*, and *VCL* (Figure 2). Excess variant burden in *FLNC* was not reported in DCM, because *FLNC* is not included in most panels, including in that report. Of the 70 truncating variants identified in women with PPCM, 17 were identical to variants annotated as pathogenic or likely pathogenic for DCM in the ClinVar database (www.ncbi.nlm.nih.gov/clinvar).

The prevalence of missense variants in each of the 67 genes among the 469 women with PPCM was not significantly different from the burden found in GnomAD, after Bonferroni correction (Tables III and IV in the Data Supplement). *ANKRD1*, *DSP*, *LAMA2*, and *MYH7* reached nominal statistical significance. Excess burden of missense variants in *MYH7* has been reported in DCM.¹⁰ Two of the identified missense variants in *MYH7* were associated with dilated and hypertrophic cardiomyopathy in the ClinVar database.

There have been recent improvements in the prediction of noncanonical splicing variants that cause cryptic splicing, including the machine learning algorithm SpliceAI¹³ with a positive predictive value of $\approx 85\%$.¹⁴ Application of SpliceAI to our cohort revealed no splicing acceptor loss or donor loss variants in *TTN*, and 1 donor loss variant in *FLNC* ($P=0.022$ compared with prevalence in GnomAD). SpliceAI also identified 2 variants, 1 acceptor loss and 1 donor loss, in *DMD* ($P=0.008$ compared with GnomAD), in which we previously also noted a truncating variant in a woman with PPCM.⁷

Increased Risk of PPCM in Subjects With TTNtv

Forty-nine truncating variants were identified in *TTN* (10.45% of the cohort), OR=9.4 compared with 1.2% in GnomAD ($P^*=1.2\times10^{-46}$), and OR=14.5 compared with 0.8% in a collection of >60 000 exomes from volunteer participants in an outpatient clinical setting (Geisinger MyCode Community Health, ref 9, $P^*=1.1\times10^{-42}$). Some 19, 21, and 9 truncating variants were frameshift, nonsense, and canonical splice site variants, respectively, a distribution similar to that found in GnomAD, the Geisinger cohort, and various DCM cohorts (Table V in the Data Supplement and refs 9,10,15). Eighteen variants have previously been described, whereas 31 (63%) are new variants. Fifteen of the variants are identical to variants annotated as pathogenic or likely pathogenic for DCM in the ClinVar database.

TTN encodes 364 exons, only a subset of which are constitutively expressed in adult hearts.^{9,16} Exons expressed in a high percentage (>90%) of *TTN* transcripts are termed high Percent Spliced In (hiPSI); mutations in these hiPSI exons are likely to cause DCM.¹⁶ Of the 49 TTNtvs identified in women with PPCM, 46 (94%) were in hiPSI exons, a fraction similar to that found in the University of Pennsylvania Medical Biobank, representing a tertiary care center referral population enriched for disease prevalence (85%), but strongly enriched compared with the outpatient community Geisinger cohort⁹ and GnomAD reference cohorts (58%, $P=5.8\times10^{-6}$, and 51.2%, $P=5.1\times10^{-8}$, respectively). The burden of hiPSI TTNtvs was similar between geographic regions (Table 1), and between racial backgrounds (Table 3).

The location along the *TTN* gene of the 49 TTNtvs identified in women with PPCM is shown in Figure 3, and compared with the locations of TTNtvs identified in a primary outpatient cohort of 1040 patients with DCM from London and Singapore,¹⁰ and 2 reference cohorts: the University of Pennsylvania Medical Biobank, and the Geisinger cohort (the London/Singapore group only reported hiPSI TTNtvs). The majority of TTNtvs in women with PPCM mapped to the A band region of *TTN*, a distribution akin to that observed in the London/Singapore DCM cohort, and among patients diagnosed with DCM within the disease-enriched University of Pennsylvania Medical Biobank cohort, as well (dark red bars).

Clinical Characteristics of Patients Who Have PPCM With TTNtv

Patients who have PPCM with hiPSI (ie, likely pathogenic) TTNtvs presented at a similar age as did patients who have PPCM without TTNtvs (Table 3). The prevalence of women of African descent also did not differ between the 2 groups (24% and 31%, $P=0.5$), nor did the prevalence of TTNtvs in patients of African descent versus other descent (8.8% and 11%; $P=0.58$). In the reference GnomAD population, TTNtvs were found in 2.1% of persons of African descent ($P=1.3\times10^{-3}$) and in 1.1% of those of European descent ($P=5.4\times10^{-16}$). Patients with PPCM with hiPSI TTNtv, however, did have significantly lower LVEF at presentation than patients with PPCM without TTNtv (23.5% versus 29%, $P=2.5\times10^{-4}$; Table 3 and Figure 4A). We reported previously that LVEF tends to be lower with increasing time between delivery and diagnosis,¹⁷ and observed the same trend among the current cohort (Figure 4B; correlation coefficient -0.21 , $R^2=0.04$, $P=4.1\times10^{-5}$). The difference in presenting LVEF between patients who have PPCM with and without likely pathogenic TTNtvs was most pronounced among patients presenting in the first week after delivery (20.9% versus 31.3%, $P=2.3\times10^{-5}$; Figure 4B), compared with the subsequent 3 weeks (20.2% versus 31.3%; $P=0.035$), or thereafter (23.8% versus 25.2%;

Table 2. Prevalence of Rare Protein Truncating Variants in Peripartum Cardiomyopathy

Genes	Peripartum cardiomyopathy		Controls		Variant excess, %	Odds ratio (95% CI)	Nominal <i>P</i> value	Corrected <i>P</i> value*
	Cohort variants	Cohort frequency, %	Genome Aggregation Database variants	Genome Aggregation Database frequency, %				
<i>BAG3</i>	1	0.21	5	0.004	0.209	53.05 (6.19–454.96)	2.92×10 ⁻⁴	1.96×10 ⁻²
<i>DES</i>	1	0.21	28	0.025	0.189	8.72 (1.18–64.2)	3.36×10 ⁻²	1
<i>DSP</i>	6	1.28	108	0.087	1.192	14.9 (6.51–34.06)	1.55×10 ⁻¹⁰	1.04×10 ⁻⁸
<i>FKTN</i>	1	0.21	57	0.048	0.165	4.46 (0.62–32.28)	0.14	1
<i>FLNC</i>	4	0.85	41	0.035	0.818	24.75 (8.83–69.38)	1.05×10 ⁻⁹	7.02×10 ⁻⁸
<i>ILK</i>	1	0.21	59	0.048	0.166	4.48 (0.62–32.4)	0.14	1
<i>MYH6</i>	2	0.43	153	0.123	0.303	3.47 (0.86–14.05)	0.08	1
<i>MYH7</i>	2	0.43	94	0.075	0.351	5.7 (1.4–23.21)	1.50×10 ⁻²	1
<i>PLEC</i>	1	0.21	169	0.157	0.056	1.36 (0.19–9.73)	0.76	1
<i>TMPO</i>	1	0.21	87	0.069	0.144	3.08 (0.43–22.13)	0.26	1
<i>VCL</i>	1	0.21	59	0.047	0.166	4.54 (0.63–32.84)	0.13	1
<i>TTN</i>	49	10.45	1453	1.224	9.224	9.42 (6.97–12.71)	1.81×10 ⁻⁴⁸	1.21×10 ⁻⁴⁶
Summary	70	14.93	5103	3.619	11.306	2.25 (1.74–2.9)	5.18×10 ⁻¹⁰	3.47×10 ⁻⁸

The prevalence of truncating variants in patients with peripartum cardiomyopathy is compared with controls from the Genome Aggregation Database. Nominal *P* values are from odds ratios, and *corrected *P* values are adjusted for testing 67 genes with the Bonferroni method.

P=0.58). The prevalence of *TTN*ts did not correlate with time to diagnosis, despite the lower average LVEF in women presenting late after pregnancy.

We previously reported preliminary findings, in a limited post hoc analysis of a subset of 85 women with PPCM, that the presence of hiPSI, likely pathogenic *TTN*ts was associated with a lower prevalence of gestational hypertension and preeclampsia, and with worse clinical outcomes.⁷ In the current much larger cohort, these associations were not observed. Gestational hypertension and preeclampsia showed only a mild trend for lower prevalence among women with and without *TTN*tv (27% versus 38%; *P*=0.21). Recovery of LVEF (defined as >50%) was achieved in 68% and 60% of women with and without *TTN*tv, respectively (*P*=0.28), and adverse outcomes (death, advanced therapies including left ventricular assist device placement or heart transplantation, or persistent dysfunction with an LVEF≤35) occurred in 21% and 17%, respectively (*P*=0.68). Recovery of LVEF was overall less frequent in women of African versus European descent (53% versus 64%, *P*=0.041), but in neither group did *TTN*tv status affect rates of recovery (*P*=0.73 and 0.17, respectively). There was no significant difference in prevalence of hiPSI *TTN*tv between patients with follow-up and those lost to follow-up (Table VI in the Data Supplement).

DISCUSSION

We present here genotypic and clinical features in 469 patients with PPCM, the largest such cohort, and use this information to extend our understanding of

genotype-phenotype relationships in this disease. We confirm our previous observation that ≈10% of patients with PPCM carry a *TTN*tv and further identify a statistically significant excess burden of LOF variants in 3 genes previously associated with DCM, but not PPCM: *DSP*, *FLNC*, and *BAG3*.

The proteins encoded by these 4 genes have significantly different role in the cardiomyocyte. *TTN* is a major component of the sarcomere. *DSP* encodes desmoplakin, the primary force transducer between cardiac desmosomes and intermediate filaments. Truncating variants in *DSP* are associated with arrhythmogenic left ventricular cardiomyopathy.¹⁸ *FLNC* encodes for filamin C, a cytoskeletal protein expressed in the intercalated discs of cardiac cells. Truncating variants in *FLNC* have only recently been reported in DCM,^{19–22} and thus *FLNC* is still absent from most clinical genetic test panels. *BAG3* encodes a chaperone protein critical for chaperone-assisted selective autophagy.²³ Despite these differences, the profile of identified LOF variants in PPCM is remarkably similar to that seen in cohorts with DCM. The recent extensive analysis of a primary outpatient cohort of 1040 patients with DCM from London and Singapore revealed significant LOF burdens in *DSP*, *BAG3*, *VCL*, *LMNA*, and *MYH7*, in addition to the well-established prevalence of *TTN*ts.¹⁰ Excess LOF burdens in *VCL* and *MYH7* were also seen in our PPCM cohort but did not reach statistical significance after correction for multiple hypothesis testing, which may simply reflect insufficient statistical power. Moreover, the frequencies of LOF variants in each of these genes range widely (0.2%–10%) and

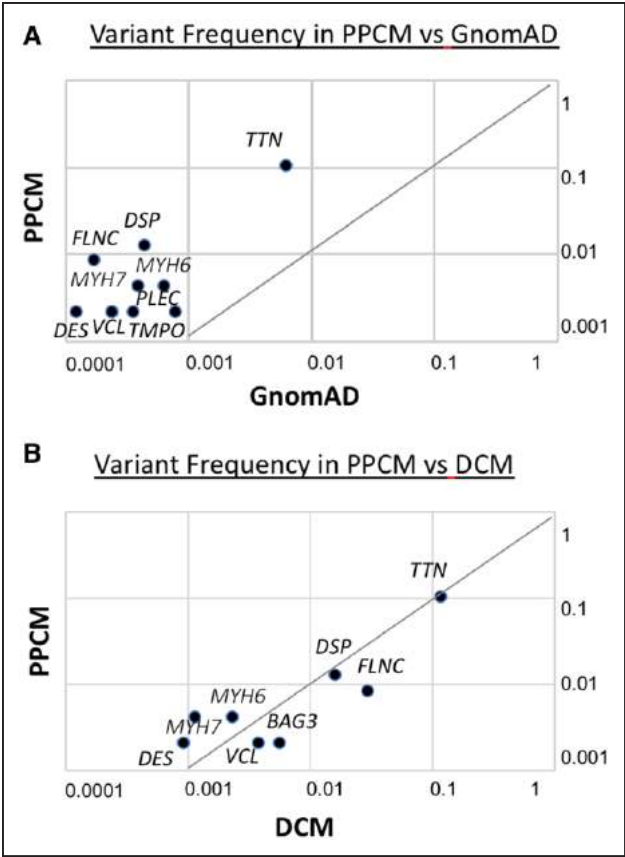


Figure 2. Comparison of the prevalence of rare truncating variants between peripartum cardiomyopathy (PPCM) and the Genome Aggregation Database (GnomAD, A), and between PPCM and nonischemic dilated cardiomyopathy (DCM, B). Variant frequencies for DCM are from Mazzarotto et al¹⁰ except for *FLNC*, which is from Ader et al.²² Diagonal line represents the equivalence line.

are remarkably similar between PPCM and DCM (Figure 2). There is thus a high degree of genetic similarity between PPCM and DCM, both in which genes bear LOFs and in the frequency with which LOFs are found in each gene.

A simple model to explain these observations invokes a 2-hit mechanism, whereby a genetic background, for example, a TTNtv, predisposes to DCM in response to an additional cardiac stressor, for example, pregnancy in the case of PPCM. In keeping with this notion, a high burden of TTNtvs has also been noted in cardiomyopathy induced by alcohol and cancer therapy, 2 other prominent cardiac stressors.^{24,25} Also in keeping with this notion, the penetrance of most LOF variants is low, suggesting that in the absence of additional cardiac stressors, cardiac function is preserved even in the presence of a genetic predisposition. For example, 95% of subjects in the outpatient Geisinger cohort who were identified to carry hiPSI TTNtvs did not have DCM, or significant cardiac dysfunction on echocardiography.⁹ In the context of PPCM, pregnancy is clearly the predominant stressor, often accentuated by preeclampsia, which is associated with 30% to 50%

Table 3. Comparison of Clinical Characteristics Between Patients With Peripartum Cardiomyopathy With High-Percentage Spliced-in TTN Truncating Variants (TTNtvs) and Those Without

Characteristic	TTN truncating variant present (n=45)	TTN truncating variant absent (n=424)	P value
Age at diagnosis, y	30.40±7.04	31.03±6.28	0.41
Ejection fraction, %	23.51±8.99	29.08±10.02	2.52×10 ⁻⁴
Race/ethnicity, % (No./total No.)			0.53
Black	24.32 (9/37)	31.38 (116/372)	
White	67.57 (25/37)	63.98 (238/372)	
Other	8.11 (3/37)	4.84 (18/372)	
Gestational hypertension,* % (No./total No.)	25 (9/36)	37.5 (132/352)	0.14
Left ventricular end-diastolic diameter,† cm	5.71±1.33	5.68±0.77	0.93
Left ventricular end-systolic diameter,† cm	4.97±1.34	4.70±0.90	0.34
Recovery, % (No./total No.)	67.5 (27/40)	59.6 (211/354)	0.33
Negative outcome,‡ % (No./total No.)	21.74 (5/23)	17.42 (27/155)	0.59

Other indicates non-White and non-Black.
*Includes patients with gestational hypertension, mild and severe preeclampsia, and superimposed preeclampsia.
†Measured on transthoracic echocardiography from initial presentation.
‡Negative outcome counted as ejection fractions≤35 at 12-month follow-up, left ventricular assist device, heart transplantation, or death. P values are by the Student t test for continuous variables and by the χ^2 test for discrete variables.

of cases of PPCM. We have shown previously that pregnancy triggers a vascular stress on the heart, which is accentuated in preeclampsia, and likely contributes to the pathogenesis of PPCM.^{5,26}

The genetic similarities between PPCM and DCM have implications for the care of women with PPCM. There are currently no recommendations for genetic testing in women with PPCM, and such testing is not reimbursed. In contrast, genetic testing for DCM is widely performed and usually reimbursed. Current recommendations for genetic testing in DCM vary, but, in general, it is recommended that probands and affected family members undergo genetic counseling and genetic testing, especially if there is evidence of conduction disease or family history of sudden death.^{27–29} The presence of truncating variants in *FLNC*, for example, has been associated with malignant arrhythmias, and may warrant more aggressive antiarrhythmic management (eg, automatic implantable cardioverter defibrillator).^{19,30} The American College of Medical Genetics also recommends cascade testing of at-risk family members for pathogenic and likely pathogenic variants.²⁸ Our study, which indicates that PPCM and DCM are often manifestations of similar underlying genetic predispositions, therefore suggests that approaches taken to genetic testing in PPCM should mirror those taken in DCM.

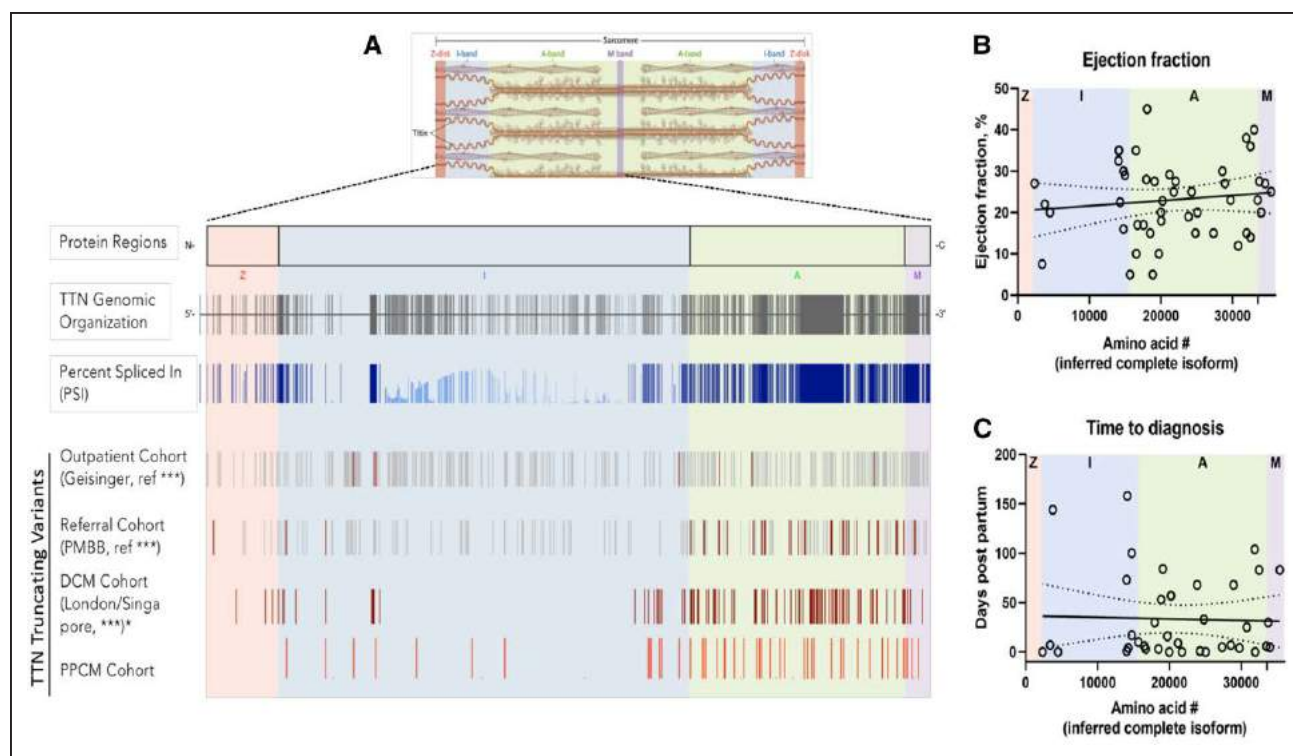


Figure 3. Genomic characteristics of truncating variants in *TTN*, and associated clinical phenotypes, in patients with peripartum cardiomyopathy.

A, Top. Titin is an integral part of the cardiac sarcomere, spanning from Z-disk to M-line. **Bottom.** Regions of the *TTN* gene and protein are designated according to their spatial distribution in the sarcomere: Z-disk, I-band, A-band, and M-band. The 364 exons of the *TTN* gene and their associated proportion spliced in (PSI) are shown, followed by the distribution of truncating variants in those exons identified in the Geisinger cohort (a general outpatient population), in the Penn Medicine Biobank (PMBB) cohort (a tertiary care referral population), the London/Singapore cohort of patients with idiopathic dilated cardiomyopathy (DCM; *only hiPSI TTNts are reported in this cohort), and the current peripartum cardiomyopathy (PPCM) cohort. Variants found in patients with DCM in the reference cohorts are designated in dark red. **B and C.** Lack of association between location of TTNtv and either left ventricle ejection fraction (**B**) or left time to diagnosis (**C**). Dark and dotted lines indicate linear regression and 95% confidence intervals, respectively. $P=0.41$ and 0.84 for difference from slope of zero in **A** and **B**, respectively. From the *New England Journal of Medicine*, Ware JS, Li J, Mazaika E, et al. "Shared genetic predisposition in peripartum and dilated cardiomyopathies," Volume 374. Copyright © 2016 Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society.

Because TTNts were found with high frequency in PPCM subjects ($\approx 10\%$), we were able to assess if clinical features of PPCM differ between patients with and without TTNts. LVEF at presentation was significantly lower in patients with TTNts, suggesting that TTNts may confer an initially more aggressive disease than other causes of PPCM. This observation is similar to that seen in DCM. For example, among patients with DCM in the University of Pennsylvania Biobank, we found that LVEF was significantly lower in patients with TTNts than in those without TTNts.⁹ LVEF is the best known predictor of clinical outcomes in PPCM,² but, surprisingly, despite the lower ventricular function on presentation, the presence of TTNts in women with PPCM did not correlate with worse rates of recovery, with lower ejection fraction at 1 year, or with adverse outcomes. The apparent equivalent final recovery rates in the context of lower LVEF at presentation could reflect a higher rate of recovery in TTNtv carriers, as has been suggested in DCM.^{31–33} These observations differ from those we made, post hoc, in the much smaller group of 85 women from the IPAC study alone,⁷ underscoring the importance

of recruiting large cohorts such as the current one to avoid spurious conclusions.

The prevalence of PPCM is at least 4-fold higher in women of African descent than European descent.^{2,34,35} However, the prevalence of TTNts among PPCM subjects was similar in these 2 groups, and the prevalence of TTNts in the GnomAD reference population is also similar in these 2 groups, indicating that TTNts cannot explain the higher predisposition to PPCM in women of African descent. This difference could reflect biological differences inherent to pregnancy as a cardiac stressor, or environmental differences, including access to health care and social determinants of health during pregnancy.

Preeclampsia and hypertension strongly predispose to PPCM,³ but the prevalence of TTNts also was equivalent in PPCM cases with or without a hypertensive disorder. In the context of the 2-hit hypothesis described above, these observations suggest that pregnancy as a cardiac stressor affects TTNtv carriers and noncarriers equivalently, regardless of race or the presence of hypertension. In addition, the findings indicate that the mechanisms by

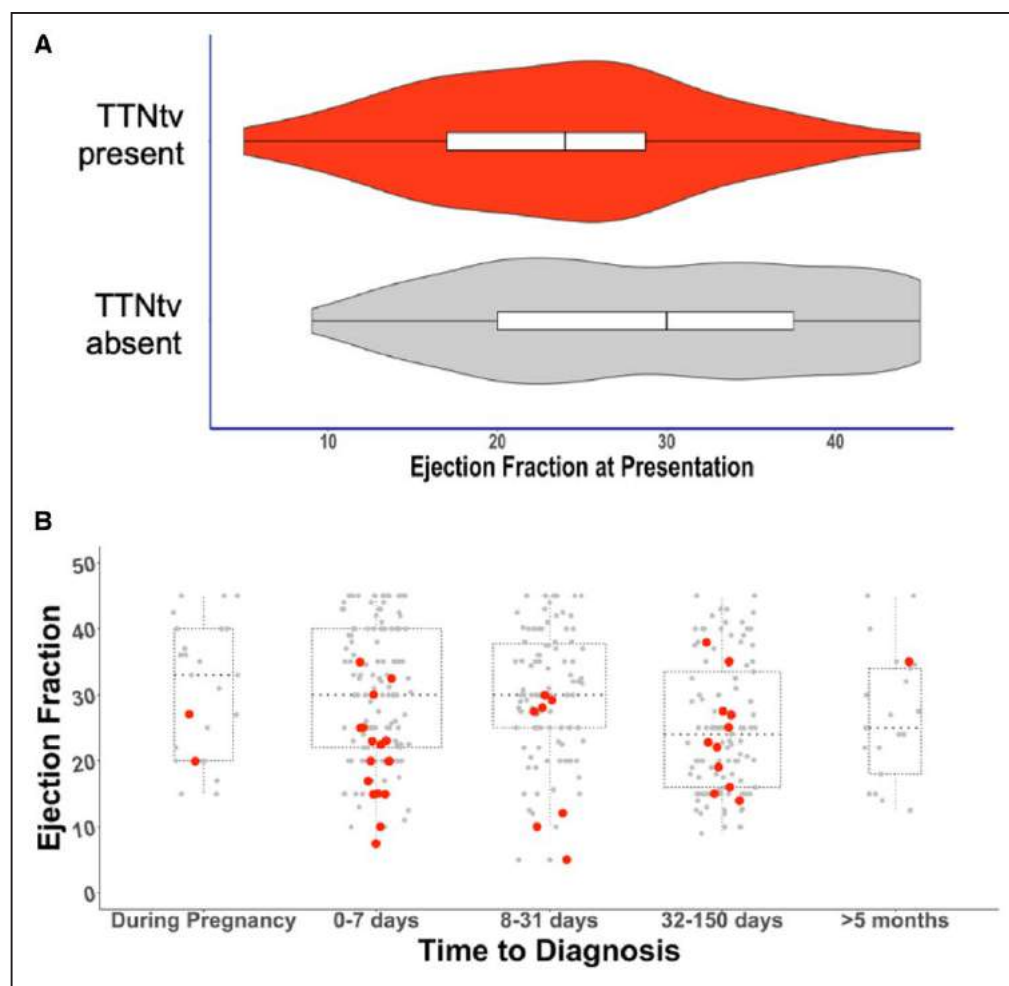


Figure 4. Impact of TTN truncating variants (TTNtv) on left ventricle ejection fraction at presentation, and time to diagnosis after delivery, in patients with peripartum cardiomyopathy (PPCM).

A, Comparison of left ventricular ejection fraction at the time of presentation between TTNtv-positive and -negative patients. $P=2.5 \times 10^{-4}$ by Student *t* test. **B**, Comparison of left ventricular ejection fraction with time to diagnosis after delivery. TTNtv-positive cases are indicated in red.

which gene mutations and hypertensive disorders stress the heart are distinct.

The mechanism by which TTNtv confer risk for PPCM or DCM remains unclear. We find that the location of truncation within the gene, and thus the predicted protein size, does not correlate with either LVEF at presentation, or time from delivery to diagnosis, indicating that the location of the TTNtv does not influence severity of disease. This observation suggests a pathogenic mechanism that is independent of truncated titin protein expression (eg, haploinsufficiency)³⁶ or, if integration of the truncated protein into the sarcomere contributes to disease, that integration of the N terminus is important, and that the loss of structures associated with the M-line is sufficient to cause disease.

Our study does have limitations. Because PPCM is a rare disease, our cohort size is necessarily limited, and lacks power to detect more subtle genetic predispositions. For example, we detected no significant increase in burden of missense variants in any gene tested, in contrast to much larger studies in DCM.¹⁰ Increasing

the number of PPCM cases studies will require a large international consortium. Our analyses are also limited to the panel of genes in our DNA capture platform. Whole exome sequencing or whole genome sequencing may elicit further genetic predispositions to PPCM, but such analyses will invariably suffer even further from power limitations. In addition, it is important to note that our conclusion of genetic similarity between PPCM and DCM is necessarily based on this limited panel of genes, previously associated with DCM, and thus genetic differences between DCM and PPCM may be uncovered with more comprehensive whole exome sequencing or whole genome sequencing. Our study is retrospective, and sampling bias may have occurred, although, considering the absence of dramatic phenotypic differences between TTNtv carriers and noncarriers, for example, it is unlikely that referral would have led to a bias with respect to genetic profile. Last, it is also worth noting that PPCM is a heterogeneous disease, and some women may have had some level of cardiac dysfunction before pregnancy.

Conclusions

In summary, we provide the first extensive genetic and phenotypic landscape of PPCM. We identify new truncating variants in *FLNC*, *DSP*, and *BAG3* with robust evidence of disease association. We report detailed associations between the highly prevalent truncating variants in *TTN* and clinical presentation and outcomes of PPCM. And we show that PPCM and nonischemic DCM closely share profiles of genetic predispositions, suggesting that approaches to genetic testing in PPCM should mirror those taken with DCM, and that pharmacological agents that are being developed for the treatment of DCM associated with mutations in these genes will also protect women with genetic PPCM.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

Expanded Methods

Data Supplement Tables I–VI

Appendices I and II

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CARDIOMYOPATHY

Truncated titin proteins in dilated cardiomyopathy

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Truncating variants in *TTN* (TTNtvs) are the most common known cause of nonischemic dilated cardiomyopathy (DCM), but how TTNtvs cause disease has remained controversial. Efforts to detect truncated titin proteins in affected human DCM hearts have failed, suggesting that disease is caused by haploinsufficiency, but reduced amounts of titin protein have not yet been demonstrated. Here, we leveraged a collection of 184 explanted post-transplant DCM hearts to show, using specialized electrophoretic gels, Western blotting, allelic phasing, and unbiased proteomics, that truncated titin proteins can quantitatively be detected in human DCM hearts. The sizes of truncated proteins corresponded to that predicted by their respective TTNtvs; the truncated proteins were encoded by the TTNtv-bearing allele; and no degradation fragments from protein encoded by either allele were detectable. In parallel, full-length titin was less abundant in TTNtv⁺ than in TTNtv⁻ DCM hearts. Disease severity or need for transplantation did not correlate with TTNtv location. Transcriptomic profiling revealed few differences in splicing or allelic imbalance of the *TTN* transcript between TTNtv⁺ and TTNtv⁻ DCM hearts. Studies with isolated human adult cardiomyocytes revealed no defects in contractility in cells from TTNtv⁺ compared to TTNtv⁻ DCM hearts. Together, these data demonstrate the presence of truncated titin protein in human TTNtv⁺ DCM, show reduced amounts of full-length titin protein in TTNtv⁺ DCM hearts, and support combined dominant-negative and haploinsufficiency contributions to disease.

INTRODUCTION

Titin, encoded by the gene *TTN*, is a large protein that spans the entire sarcomere, from the Z-disk to the M-band, and that is essential for sarcomere integrity and function (1). Heterozygous variants in *TTN* that are predicted to lead to truncated titin proteins (TTN-truncating variants or TTNtvs) can be found in 10 to 20% of patients with idiopathic or familial dilated cardiomyopathy (DCM), compared to a population prevalence of ~1% (2–6), making TTNtvs the most common known cause of DCM. More recent studies have also shown a high prevalence of TTNtvs in cardiomyopathy induced by alcohol (7), pregnancy (8), and cancer therapies (9), suggesting that TTNtv-bearing individuals are susceptible to cardiomyopathy in response to numerous insults.

How TTNtvs mechanistically give rise to disease has remained controversial. Various hypotheses have been proposed, ranging from haploinsufficiency, whereby insufficient titin protein is generated from the intact allele to compensate for the TTNtv allele (a process that might be aggravated by cardiac insults requiring accelerated titin production, for example, alcohol toxicity, cardiotoxic drugs, or pregnancy) to “poison peptide,” whereby TTNtv protein, the protein encoded by the TTNtv-bearing allele, incorporates into the sarcomere, causing dysfunction (2, 10). Determining whether TTNtv proteins are present in adult human DCM hearts is paramount to distinguishing between these mechanisms and to understanding how TTNtvs cause heart disease. TTNtv proteins can be detected in immature induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) (11), as well as in skeletal muscle of some patients bearing TTNtvs (12), and work with polysomes has demonstrated that mRNA

originating from the TTNtv allele can be found associated with ribosomes, suggesting that the allele can be translated (13). However, to date, repeated efforts to detect TTNtv proteins in affected human DCM hearts have failed (2, 4, 14). Moreover, proteomic studies in a rat model bearing an A-band TTNtv demonstrated complete absence of protein encoded by the TTNtv-bearing allele (13). The current leading theory is thus that haploinsufficiency of titin, rather than dominant-negative actions of the truncated peptide, leads to heart disease (2). However, reduced amounts of titin mRNA or protein expression in TTNtv human hearts have not been observed to date (2, 4, 14), challenging this theory.

In addition, the functional impact of TTNtvs on cardiomyocyte function has also remained undefined. Work with iPSC-CMs has demonstrated that mutant titin protein results in sarcomere insufficiency and impaired responses to stress (11), yet the extent to which these findings in immature iPSC cells reflects the impact of TTNtvs on adult cardiomyocytes remains unclear. To better understand the impact of TTNtvs on human hearts, we leveraged access to a large collection, accumulated over the past 20 years, of hearts collected after transplantation, as well as an ongoing procurement of such hearts, to evaluate the impact of TTNtvs on cardiac TTN mRNA and protein and on intact adult cardiomyocyte contractility.

RESULTS

Identification and characterization of TTNtvs in posttransplant DCM hearts

To evaluate the impact of TTNtvs on human hearts, we analyzed 184 hearts explanted from patients with nonischemic DCM and 14 nonfailing (NF) discarded donor hearts. Capture-based exome sequencing of *TTN* revealed 25 truncating variants in the DCM hearts (11 nonsense, 9 frameshift, and 5 splice site; table S1). Previous work demonstrated that, of the 364 exons encoded by *TTN*, only TTNtvs located in exons predicted to be spliced into the adult *TTN* transcripts

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[percentage spliced-in (PSI) >0.9, or hiPSI] are likely pathogenic (4, 5). Of the 25 TTNvs identified above, 22 were located in hiPSI exons (12% prevalence, $P < 2 \times 10^{-23}$ compared with 0.5% prevalence of hiPSI TTNvs in the reference gnomAD population). This prevalence is comparable to that reported in other DCM cohorts not requiring cardiac transplantation (4, 6). Seven of the TTNvs (all hiPSI) were identical to variants annotated as pathogenic, or probably pathogenic, for DCM in the ClinVar database (www.ncbi.nlm.nih.gov/clinvar). DNA from the 14 NF hearts encoded no TTNvs. No demographic differences or differences in cardiac morphology or hemodynamics were apparent between TTNtv⁻ and TTNtv⁺ patients (Table 1). All 22 hiPSI TTNvs were located in the C-terminal region of the I-band, A-band, or M-band region (Fig. 1A), akin to the distribution seen in DCM not requiring cardiac transplantation (Fig. 1A) (5, 6). Moreover, neither the age of the patient at time of transplant (Fig. 1B) nor pretransplant left ventricular ejection fraction (LVEF; Fig. 1C) correlated with the location of the TTNtv in the protein, suggesting that the location of the TTNtv does not influence severity of disease.

Impact of TTNvs on TTN mRNA allelic balance and splicing, and genomic mRNA expression

To investigate the impact of TTNvs on mRNA expression, we examined genome-wide RNA sequencing of myocardium from 138 of the 184 DCM hearts, of which 19 had hiPSI TTNvs and 10 were NF hearts. As noted above, the 364 exons of *TTN* are variably spliced, and alterations in *TTN* splicing caused by the presence of TTNvs could, in principle, cause disease. However, we identified no differences in *TTN* exon usage between TTNtv⁺ and TTNtv⁻ DCM hearts (Fig. 2A), suggesting that *TTN* alternative splicing is not an important driver of TTNtv-induced pathology. We next tested whether the presence of a TTNtv affected allelic balance, for example, by leading to nonsense-mediated decay of the truncated transcript. To do this,

we reconstructed each of the two individual alleles for each patient on the basis of the results of exome sequencing and by phasing known haplotypes (see Materials and Methods section). We detected no evidence of allelic imbalance in DCM, compared with NF hearts, and only marginal allelic imbalance in TTNtv⁺ DCM hearts compared with TTNtv⁻ DCM hearts (Fig. 2, B and C). The little allelic imbalance in TTNtv⁺ DCM hearts did not correlate with age at transplant, ejection fraction, or location of truncation in *TTN* (fig. S1A). Thus, the presence of TTNvs appeared to have little impact on the expression or splicing of *TTN*.

We next investigated the impact of TTNvs on global mRNA expression. Principal components analysis of the RNA-sequencing (RNA-seq) datasets differentiated both TTNtv⁺ and TTNtv⁻ DCM hearts from NF hearts but did not differentiate between the two forms of DCM (Fig. 2D). After Bonferroni correction, only one gene (*TPM3*; tropomyosin α -3 chain) (fig. S1B) was significantly altered in TTNtv⁺ compared to TTNtv⁻ DCM hearts. Of the top 20 most highly expressed genes, which mainly encode sarcomeric proteins, only *TTN* expression was nominally significantly reduced in TTNtv⁺ compared to TTNtv⁻ DCM hearts (fig. S1C). This mild reduction in *TTN* transcript may reflect the impact of mild allelic imbalance. Thus, no gross transcriptional differences were apparent between TTNtv⁺ and TTNtv⁻ DCM hearts.

Impact of TTNvs on human adult cardiomyocyte contractile function

Experimental data with iPSCs have indicated that TTNvs cause impairment of sarcomerogenesis (11), leading to the hypothesis that TTNvs may impair the ability to maintain or restore sarcomeres damaged by stresses such as pregnancy, alcohol, or chemotherapy (2, 8, 9). To identify putative defects of sarcomere function in adult DCM hearts with TTNvs, we isolated adult left ventricular

Table 1. Clinical and echocardiographic characteristics of 22 hiPSI TTNtv ⁺ versus 158 TTNtv ⁻ patients with DCM. For echocardiographic data, <i>n</i> represents the number of patients with complete echocardiography records. BMI, body mass index; VT/VF, ventricular tachycardia/ventricular fibrillation; LVEF, left ventricle ejection fraction; LVEDD, left ventricle end-diastolic dimension; LVESD, left ventricle end-systolic dimension.			
Patient statistics	TTNtv ⁻ DCM (<i>n</i> = 158)	TTNtv ⁺ DCM (<i>n</i> = 22)	<i>P</i>
Age (years)	51.77 ± 11.06	54.59 ± 6.62	0.25
Male (%)	93 (59)	16 (73)	0.21
African American (%)	71 (45)	6 (27)	0.12
Weight (kg)	78.52 ± 17.28	84.56 ± 20.43	0.14
Height (cm)	171.46 ± 10.53	174.43 ± 10.62	0.22
BMI (kg/m ²)	26.56 ± 4.67	27.67 ± 5.38	0.31
Heart weight (g)	504.56 ± 148.24	475.73 ± 96.00	0.38
History of comorbidity			
Atrial fibrillation (%)	69 (44)	13 (59)	0.18
VT/VF (%)	77 (49)	10 (45)	0.75
Diabetes mellitus (%)	41 (26)	5 (23)	0.77
Hypertension (%)	89 (56)	10 (45)	0.34
Echocardiography			
	(<i>n</i> = 108)	(<i>n</i> = 18)	
LVEF (%)	16.26 ± 7.40	15.38 ± 6.25	0.60
LVEDD (cm)	6.68 ± 1.35	6.77 ± 1.06	0.80
LVESD (cm)	6.10 ± 1.22	5.99 ± 1.03	0.73

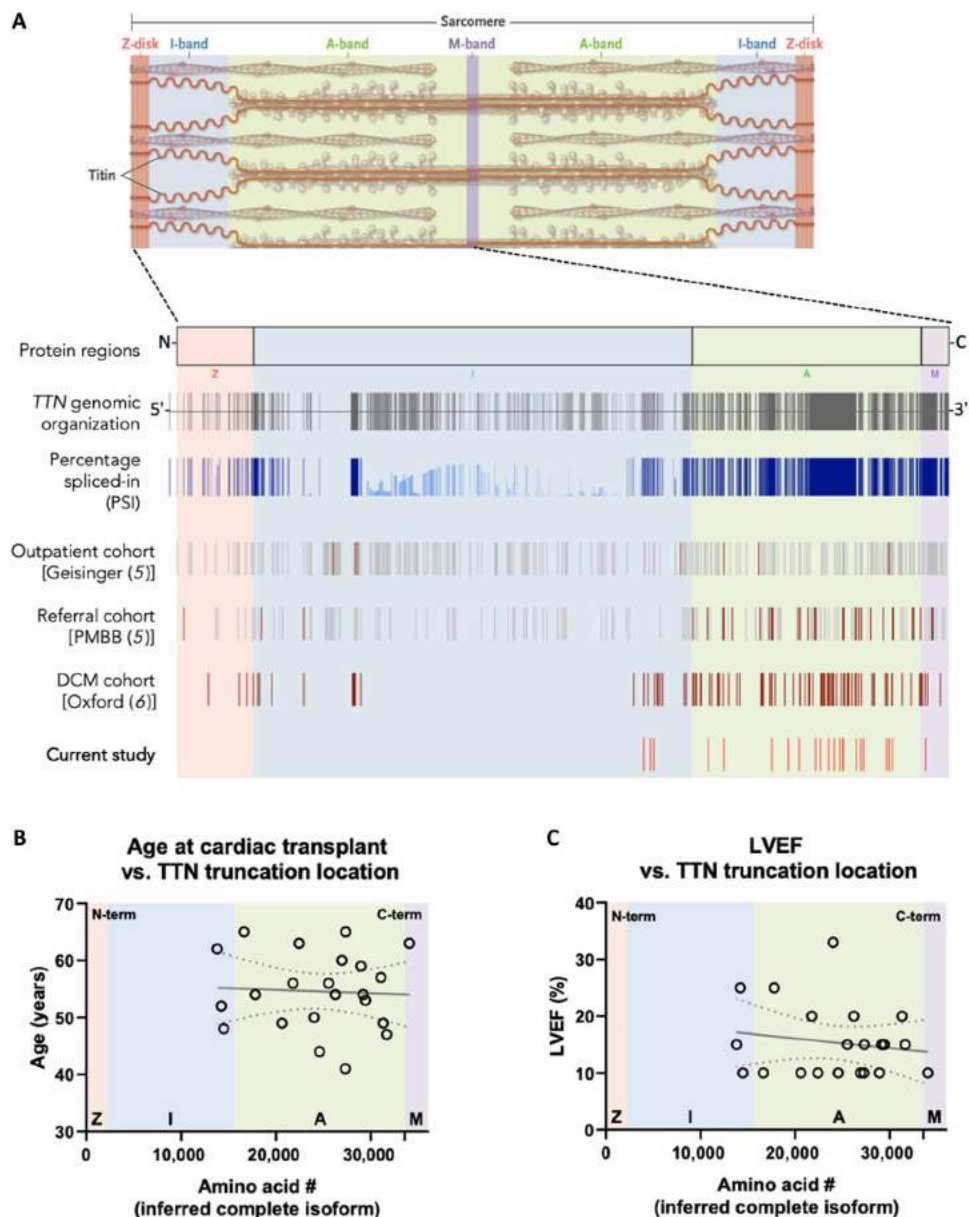


Fig. 1. Genomic characteristics of 22 hiPSI TTNts, and associated clinical phenotypes, in patients with DCM who underwent cardiac transplantation. (A): Top: Titin is an integral part of the cardiac sarcomere, spanning from Z-disk to M-line. Bottom: Regions of the *TTN* gene and protein are designated according to their spatial distribution in the sarcomere: Z-disk, I-band, A-band, and M-band. The 364 exons of the *TTN* gene and their associated percentage spliced-in (PSI) are shown, followed by the distribution of truncating variants in those exons identified in the Geisinger cohort (a general outpatient population) (5), in the Penn Medicine Biobank (PMBB) cohort (a tertiary care referral population) (5), a cohort of 1040 patients with idiopathic dilated cardiomyopathy (DCM) from London and Singapore (6), and the variants identified in the current study. For the latter two, only hiPSI variants are noted. Variants found in patients with DCM are designated in red. (B and C) Lack of association between location of TTNtv and either age (B) or left ventricular ejection fraction (LVEF; C) at the time of cardiac transplantation. Dotted lines indicate a 95% confidence interval about a linear regression, indicated by a solid line.

cardiomyocytes (ACMs) from three patients with DCM with hiPSI TTNts via collagenase perfusion at the time of transplant, as described previously (15), and compared to a group of similarly treated TTNtv⁻ DCM and NF controls (Fig. 3). Consistent with previous results (15), TTNtv⁻ DCM ACMs showed impaired contractile function upon electrical stimulation when compared to NF controls.

Unexpectedly, ACMs from TTNtv⁺ DCM hearts had at least equal or better fractional shortening, contraction and relaxation velocities, and contraction and relaxation times compared with TTNtv⁻ DCM hearts, more closely approximating the values of NF ACMs (Fig. 3). TTNtv⁺ ACMs had shorter resting sarcomere lengths than either TTNtv⁻ or NF ACMs. These data thus suggest that TTNts do not impair sarcomerogenesis in adult human hearts, in contrast to the case in iPSCs (11).

In light of this absence of overt worsening of contractility in TTNtv⁺ ACMs, we also sought to investigate whether other pathways were differentially affected between TTNtv⁺ and TTNtv⁻ DCM hearts. Alterations in autophagy have been suggested to contribute to disease progression in TTNtv⁺ DCM, including the observations of increased lipidated microtubule-associated protein 1 light chain 3B (LC3B-II) and p62 proteins, markers of activated autophagy, in rat models of TTNtv⁺ DCM (16, 17). However, Western blotting studies of proteins and posttranslational modifications involved in autophagy demonstrated no convincing differences in LC3, p62, or other proteins in TTNtv⁺ DCM compared to TTNtv⁻ DCM hearts (fig. S2). Thus, alterations in autophagy that occur in DCM appear not to be specific to TTNtv⁺ DCM.

Detection of abundant peptides encoded by the TTNtv-bearing allele in TTNtv hearts

The observed alterations in resting sarcomere length in TTNtv⁺ versus TTNtv⁻ hearts suggested that truncated titin proteins may be present and affecting cardiomyocyte function. To date, however, efforts at detecting truncated titin protein in human TTNtv⁺ DCM hearts have failed (4, 5, 14). Thus, existing evidence has been taken to support the paradigm that DCM induced by TTNts is caused by haploinsufficiency and not the dominant-negative actions of a truncated peptide (2). To probe more deeply into this question, we used proteomics to detect titin protein in TTNtv⁺ human DCM hearts.

Proteins were prepared from 10 hiPSI and 2 loPSI TTNtv⁺ DCM hearts, separated by SDS-agarose gel electrophoresis, extracted from the gel [excluding the highly abundant myosin heavy chain (MHC) band], subjected to trypsin or chymotrypsin fragmentation, and analyzed by unbiased proteomics (Fig. 4A).

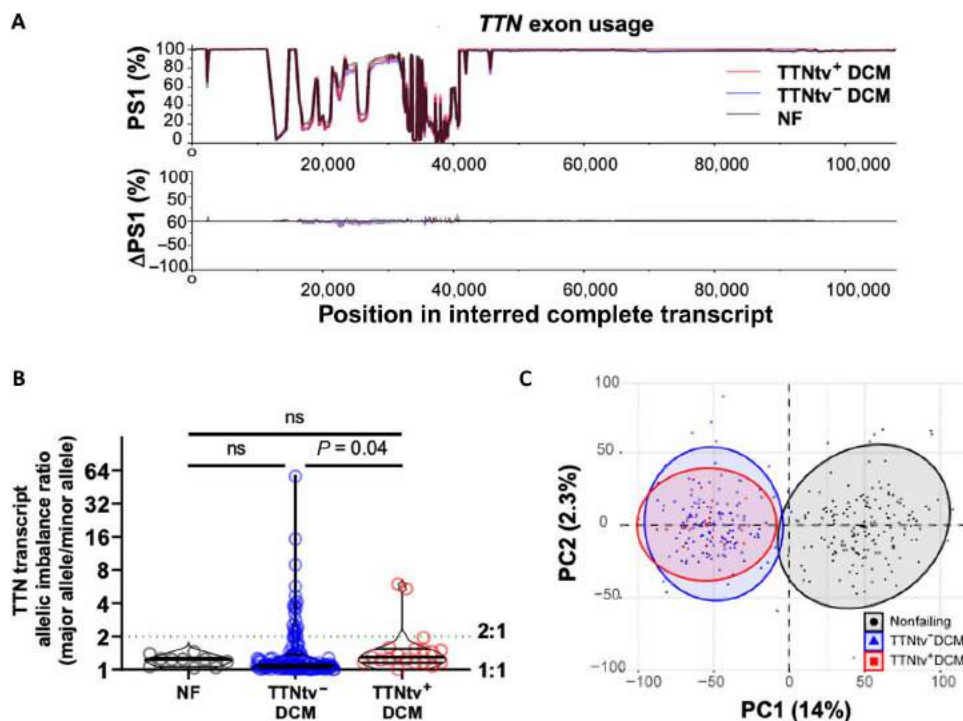


Fig. 2. Transcriptomic evaluation of hiPSI TTNtv⁺ versus TTNtv⁻ posttransplant DCM hearts. (A): Top: Minimal changes in *TTN* percentage spliced-in (PSI) exons between TTNtv⁺ DCM ($n = 19$), TTNtv⁻ DCM ($n = 119$), and nonfailing (NF) hearts ($n = 10$). Bottom: Change in PSI (Δ PSI) compared between types. Red shading: TTNtv⁺ DCM minus NF. Blue shading: TTNtv⁻ DCM minus NF. Gray trace: TTNtv⁺ DCM minus TTNtv⁻ DCM. Of note, splicing patterns of *TTN* do differ in Black versus White populations (5). (B) Mildly increased allelic imbalance in TTNtv⁺ versus TTNtv⁻ DCM heart ($P = 0.04$, $n = 10$, 129, and 19 in NF, TTNtv⁻ DCM, and TTNtv⁺ DCM, respectively), shown as allelic imbalance expressed as the ratio of the more common allele over the less common allele. P value determined by Kruskal-Wallis ANOVA. Error bars report median and interquartile ranges: NF, 1.24 (1.06 to 1.29), TTNtv⁻ DCM (1.03 to 1.37), TTNtv⁺ DCM (1.15 to 1.54). (C) Global transcriptomic profiling PCA separated NF ($n = 166$) from DCM hearts, but not TTNtv⁺ ($n = 19$) from TTNtv⁻ DCM ($n = 143$) hearts. ns, not significant.

We first tested the hypothesis that truncated titin may be actively cleaved or degraded into fragments that cause cellular proteotoxicity. However, we detected no titin peptides among proteins smaller than 200 kDa (about the size of myosin) in six of the samples and less than 1 in 100,000 peptides mapped to titin in the remaining samples (Fig. 4B). No difference in abundance of such peptides was seen in pathogenic hiPSI versus nonpathogenic loPSI TTNtv⁺ DCM hearts.

We next tested whether truncated titin could be detected among the larger proteins. The presence of heterozygous missense single nucleotide variants (SNVs) allowed assignment of peptide fragments to one allele or the other of each patient (arbitrarily labeled L and R alleles; Fig. 4C). Of note, there was a nonsignificant trend for higher total number of *TTN* missense variants per individual, and number of rare (<1%) missense variants, in TTNtv versus non-TTNtv individuals (13.5 versus 11.4, $P = 0.07$; and 5.6 versus 4.4, $P = 0.07$, respectively). Here, we used the missense variants as markers to identify the allelic source of detected peptides. Reconstruction of each complete *TTN* allele in each patient was achieved by phasing of the missense SNVs (see Materials and Methods). We identified 10 hiPSI TTNtv samples with sufficient number of missense variants to allow separate detection of proteins encoded by the two parental alleles. We also included two loPSI TTNtv samples to test the widely accepted notion that the reason loPSI variants are not pathogenic is because full-length protein is readily made by skipping the affected

exon. In all 12 hearts, including all 10 samples with hiPSI TTNtv, peptide fragments could be assigned to both alleles, demonstrating that the TTNtv-bearing alleles are translated into protein (Fig. 4C). In 7 of the 10 hiPSI hearts, fragments downstream of the truncations could only be assigned to one allele, consistent with translation proceeding up to and stopping at the truncation site. The frequency of detection of peptides assigned to the allele bearing the TTNtv in these seven hearts was roughly half that assigned to the non-TTNtv allele upstream of the truncating site (4.1 versus 9.4 per sample, $P = 0.007$), indicating that the truncated protein was less abundant than full-length titin (note that the TTNtv could not be mapped to the L or R allele and that assignment of the allele that bears the TTNtv is thus inferred). In 1 of the 10 hiPSI hearts (#2050), with the most C-terminal TTNtv, no peptides were detected downstream of the truncation in either allele, although in this case as well, one allele mapped 42% as many peptides upstream of the truncation site as the other allele, again suggesting that the truncated protein is less abundant than full-length titin. In the remaining 2 of the 10 hiPSI cases (#1725 and #2164), peptides could be assigned to both alleles even downstream of the predicted truncation, and in both these cases, the TTNtv occurred 5' to Cronos, a known alternative transcriptional start site for *TTN* (Fig. 4C), suggesting that these peptide fragments may be generated from the Cronos transcript. Last, in the two cases with a loPSI TTNtv (#2529 and #1809), fragments were detected from both alleles, both upstream and downstream of the truncation site, and at equivalent frequency, consistent with exclusion of the truncation-encoding exon. Together, these proteomic data indicate that fragments of truncated titin, encoded from the allele bearing a TTNtv, are present and abundant in TTNtv⁺ DCM hearts.

Detection of intact TTNtv protein in TTNtv⁺ hearts

The proteomic data above demonstrated the presence of titin at a molecular weight >200kD (that is, in the gel slices containing proteins larger than myosin) but could not be used to infer the presence of intact titin. Therefore, to determine whether intact TTNtv protein was present in TTNtv⁺ DCM hearts, as well as to confirm the presence of TTNtv protein by methods orthogonal to proteomics, we size separated protein from TTNtv⁺ and TTNtv⁻ DCM hearts, using modified SDS-agarose gel electrophoresis, followed by protein detection using either Coomassie blue staining or two-color fluorescence immunoblotting. Cardiac titin is typically detected in Coomassie stains as three clear bands: N2BA (2.9 MDa), N2B (2.7 MDa), and a Cronos-derived protein (2.3 MDa) (18). In most samples from TTNtv⁺ hearts, additional bands were clearly visible

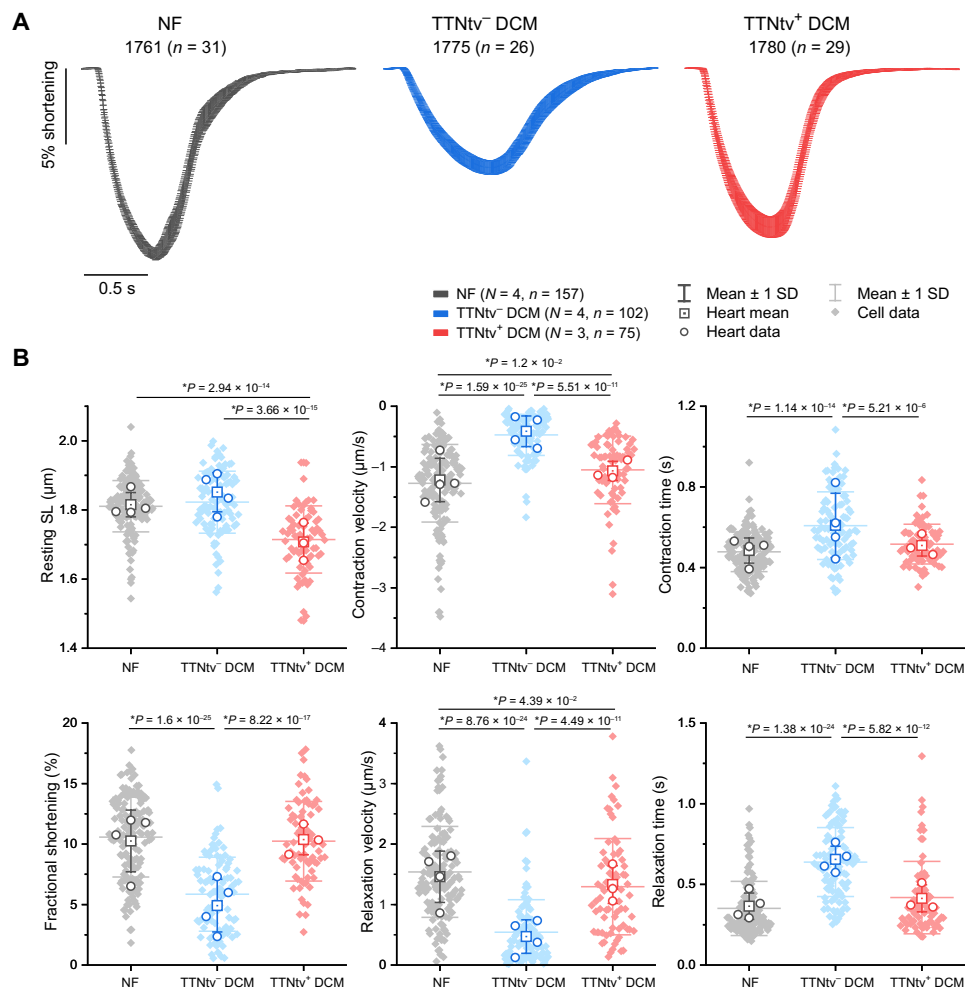


Fig. 3. Contractility of intact adult cardiomyocytes isolated from TTNtv⁺, TTNtv⁻, and NF human hearts. ACM unloaded shortening was assessed from four NF donor hearts, four TTNtv⁻ posttransplant hearts, and three hiPSI TTNtv⁺ posttransplant hearts. (A) Representative average ACM fractional shortening upon 0.5 Hz electrical stimulation from an NF, a TTNtv⁻, and a TTNtv⁺ heart. Patient ID is followed (in parenthesis) by n = number of myocytes assessed per heart, and data presented as mean shortening $\pm$ SD. (B) Quantification of the indicated contractile parameters of pooled data from all assessed hearts. N = number of hearts per group, n = number of cells evaluated per group. Statistical significance was determined by one-way ANOVA with post hoc Bonferroni comparisons between myocytes (closed diamonds). Between-heart comparisons (open circles) were also performed, yielding similar results, and are reported in data file S2, along with full contractility parameters for each individual heart. SL, sarcomere length.

(Fig. 5A, top). Immunoblotting with antibodies targeting the N- and C-terminal regions of titin demonstrated that all of these shorter titin proteins contained the N terminus (Fig. 5A, middle), whereas none of them contained the C terminus (Fig. 5A, bottom). Moreover, the observed bands migrated with a predicted size closely corresponding to the predicted molecular weight of the N2B isoform bearing the respective TTNtv (Fig. 5A, middle, red arrowheads). The more slowly migrating bands did not as reliably correspond to the predicted N2BA molecular weights, suggesting different processing of these isoforms. No distinct N terminus-containing proteins were detected in any sample below the predicted N2B TTNtv molecular weight, with the exception of Novex-3, a known 0.6-MDa cardiac titin isoform present in all samples. The intensity of the readily quantifiable lower N2B-derived TTNtv bands was considerably lower than that of full-length TTN proteins, ranging from 35% of

the sum of N2B + N2BA to nearly undetectable (average 4%), indicating the abundance of TTNtv proteins is less than that of intact titin (fig. S3). Upper N2BA-derived TTNtv bands, if present, were not quantified, due to overlap with full-length isoforms, and therefore, some TTNtv band intensity was unaccounted for in this analysis. These data demonstrate the presence of TTNtv proteins, at the predicted size, in TTNtv⁺ DCM hearts.

TTNtv proteins loosely associate with sarcomere-containing cellular fractions

To begin to address where in the cell TTNtv proteins can be found, we devised a method (fig. S4A) whereby cryopreserved cardiac tissue was first physically crushed on liquid nitrogen into a grossly cell-scale (~20 to 100 μm) powder (fig. S4B). The frozen powder was then added to relaxing buffer containing 0.5% Triton X-100, thus skinning cardiomyocytes and liberating soluble and membrane-bound proteins, but leaving large sarcomeric complexes intact and sedimentable by gentle centrifugation. Nonsarcomeric proteins, such as glyceraldehyde phosphate dehydrogenase (GAPDH), subjected to this protocol were found in the supernatant, whereas sarcomeric or sarcomere-associated proteins, such as MHC, desmin, and full-length titin, as well as Cronos titin, were found in the pellet (fig. S4C). A total of 99.7% of full-length titin partitioned to the sarcomere-associated fraction. In three evaluated TTNtv⁺ DCM hearts, TTNtv protein was largely (~80%) found in the pellet (fig. S4, C and D), suggesting active incorporation of truncated titin into the sarcomere, although it could not be ruled out that truncated titin is instead in non-sarcomeric aggregates that are also found

in the pellet. Despite >80% of truncated titin protein being retained in the sarcomere-containing fraction, TTNtv proteins were 49-fold more likely than full-length titin to dissociate from the sarcomere-containing fraction and partition to the soluble fraction. Thus, if truncated titin proteins do incorporate into the sarcomere, they are more loosely bound there than either full-length titin or Cronos titin. These data also demonstrated the consistent presence of appreciable quantities of Cronos titin protein in adult myocardium and that Cronos titin is tightly associated with sarcomere-containing fractions, consistent with its incorporation into the sarcomere.

Evidence of reduced full-length TTN protein expression in TTNtv⁺ hearts

Last, we sought to address the question of whether TTNtv⁺ patients demonstrated evidence of reduced total TTN protein expression. In

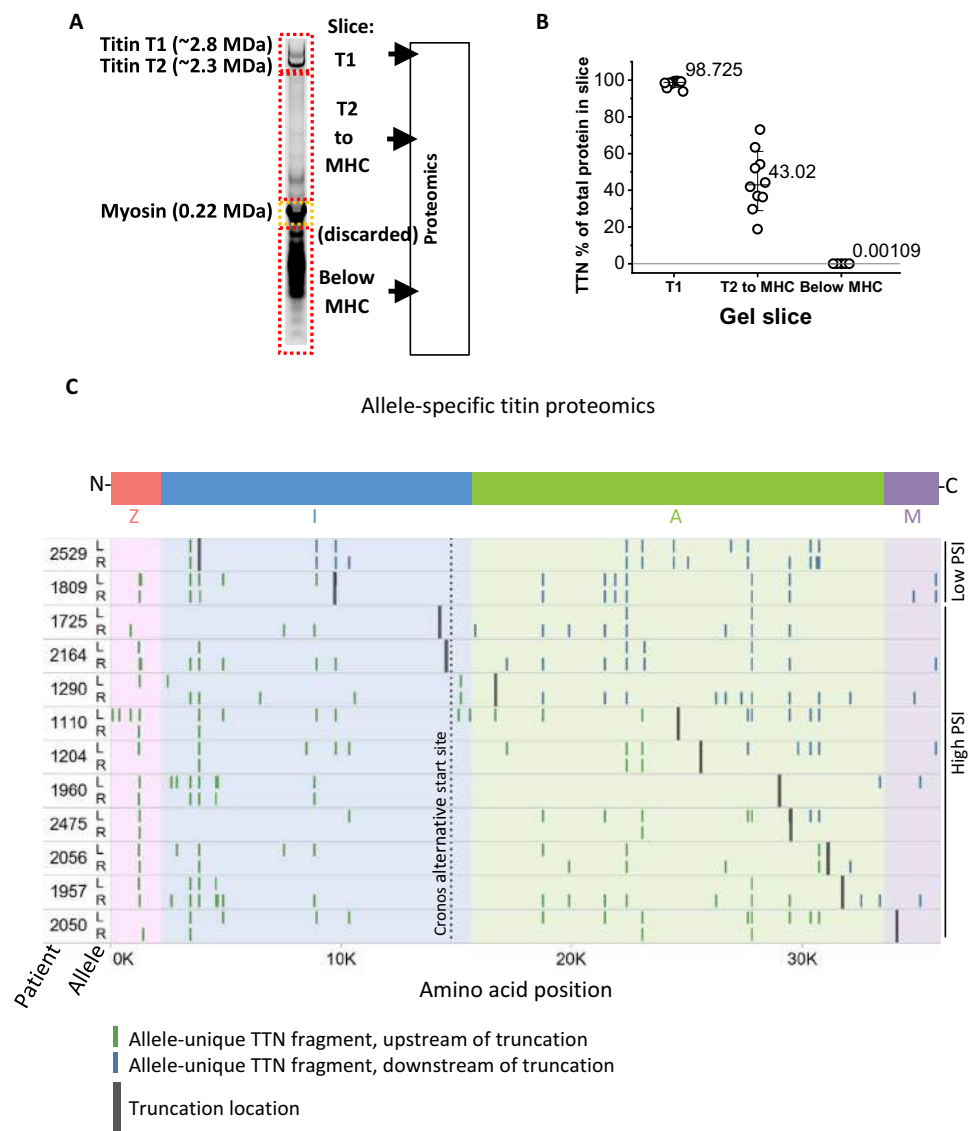


Fig. 4. Proteomic detection of truncated titin proteins encoded by the allele bearing *TTN*tv in *TTN*tv heretozygous DCM hearts. (A) Experimental scheme. Modified electrophoresis was used to excise three slices (labeled T1, T2-to-MHC, and below MHC), discarding the abundant myosin heavy chain (MHC) protein. Slices were then subjected to proteomic evaluation. (B) Relative abundance of peptides mapped to the *TTN* inferred complete isoform in each slice. (C) Mapping of identified *TTN* peptides to the titin protein predicted to be encoded by either allele (arbitrarily labeled L and R), using haplotype phasing and captured genomics sequences (one allele bears the *TTN*tv but cannot be ascribed to L or R based on phasing). Patients 2529 and 1809 bear nonpathogenic loPSI *TTN*tv; the remainder are hiPSI *TTN*tv. The Cronos alternative transcriptional start site is indicated.

other words, to what extent does full-length titin, encoded by the nontruncating allele, compensate quantitatively for the absence of full-length titin translated from the *TTN*tv allele, despite observed equivalent transcription of both alleles and active translation from the transcript that encodes the *TTN*tv? We therefore quantified full-length titin proteins in *TTN*tv⁺ hearts, measured from Coomassie-stained gels or Western blots (Fig. 6 and fig. S5). The sum of full-length N2A and N2B isoforms was about 30% lower in *TTN*tv⁺ hearts, compared to *TTN*tv⁻ hearts (Fig. 6 and fig. S5). This reduction was apparent whether detected *TTN* protein amounts were normalized to total tissue protein or to individual intracellular structural

proteins (MHC, dystrophin, or desmin). Thus, full-length titin protein, expressed from the non-*TTN*tv-bearing allele, does not fully compensate for truncated titin in *TTN*tv⁺ DCM hearts.

DISCUSSION

We show here, using independent orthogonal methods including unbiased proteomics, modified gel electrophoresis, and immunological detection, that truncated titin proteins, encoded by the allele containing a *TTN*tv, are present and relatively abundant in hearts from *TTN*tv⁺ DCM patients. The presence of truncated titin protein might suggest that a dominant negative or poison peptide mechanism contributes to DCM in these patients. We also provide quantitative evidence of reduced amounts of titin protein in *TTN*tv⁺ hearts. Work performed in parallel by Linke and co-workers (19) revealed similar evidence of both the presence of truncated titin protein and of reduced amounts of total titin protein in *TTN*tv⁺ hearts. We thus propose that disease initiation may be multifactorial, involving both haplo-insufficiency, as a consequence of reduced amounts of titin protein, and poison peptide mechanisms.

At least two possible poison peptide mechanisms can be envisioned: First, incorporation of the truncated proteins into the sarcomere, as suggested by our fractionation studies (fig. S4), may directly lead to sarcomeric dysfunction. We showed here that the size of the truncated protein does not appear to impact disease severity, as reflected in the need for transplantation (typically reflecting more severe disease), or LVEF or age at time of transplantation (Fig. 1). Thus, if integration of the truncated protein into the sarcomere contributes to disease, it can be inferred that integration of the N terminus is important and that the loss of structures associated with the M-line is sufficient to cause disease. The C terminus of titin encodes a putative kinase domain, mutations which are thought to cause skeletal muscle disease (12) and the absence of which in *TTN*tv protein could also be contributing to disease (2). On the other hand, integration of *TTN*tv protein into the sarcomere may not occur or may not be required for disease as suggested by the intact contractility of *TTN*tv⁺ ACMs (Fig. 3). For example, the *TTN*tv protein may be actively targeted for cleavage by the many proteases known to target titin, leading to disrupted protein homeostasis. Arguing against this model, however, is the absence of detectable small titin fragments in *TTN*tv⁺ hearts

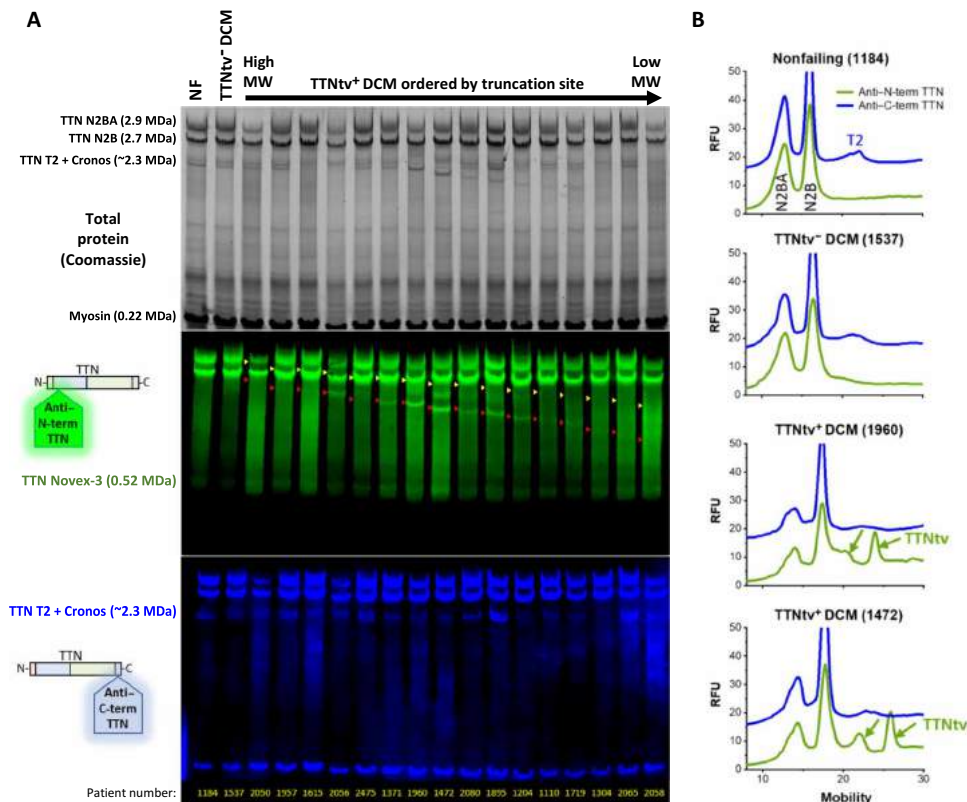


Fig. 5. Detection of truncated titin proteins at predicted locations in TTNtv⁺ DCM hearts by gel electrophoresis and simultaneous two-color Western blotting. (A) Total protein Coomassie staining (top), and Western blotting with antibodies directed against the N terminus (middle, green) and C terminus (bottom, blue) of titin protein, of 16 TTNtv⁺ DCM hearts, ordered by genetically predicted molecular weight (MW) of truncated titin protein. A nonfailing (NF) and a TTNtv⁻ DCM heart are shown for comparison. TTN Novex: Protein encoded by an alternatively spliced isoform that does not contain the epitope recognized by the C-terminal antibody. TTN Cronos: A TTN isoform encoded by the transcript originating at the alternative start site Cronos, which does not contain the epitope recognized by the N-terminal antibody. Threshold and gamma are adjusted per channel for clarity of display. Yellow and red arrowheads represent the predicted mobilities for truncated N2BA and N2B, respectively, on the basis of the truncation site location within the genome. The lowermost band in the blue TTN C-terminal channel is nonspecific binding (NSB) to MHC. (B) Traces of Western blot lane fluorescence intensities with N-terminal (green) and C-terminal (blue) antibodies with control NF and TTNtv⁻ DCM hearts (top two) and two sample TTNtv⁺ DCM hearts (bottom two). RFU, relative fluorescent units.

(Fig. 4A) or of substantially altered signatures of protein clearance such as macroautophagy in TTNtv⁺ hearts compared with TTNtv⁻ hearts (fig. S2). The disease penetrance of TTNtvs is low, suggesting that pathogenicity requires additional “hits” in addition to a TTNtv. We have previously shown, in a large outpatient population, that 95% of subjects with TTNtvs, including some annotated as pathogenic in ClinVar, showed no signs of cardiomyopathy (5). This concept is consistent with observations by us and others that TTNtvs are also prominently found in cohorts of pregnancy-induced, alcohol-induced, and oncoterapy-induced cardiomyopathies (7–9), all representing potential “second hits.” How these various cardiac stressors mechanistically interact with TTNtvs to cause disease remains unclear, but it is possible that they may synergize with a poison peptide mechanism, for example, by altering proteostasis.

We also showed here that the presence of TTNtvs appears to have little to no effect on mRNA processing. We used allelic phasing to show that there is little to no *TTN* allelic imbalance in excess to that seen in TTNtv⁻ hearts or any evidence for substantial alternative splicing of *TTN* in TTNtv⁺ hearts. In light of the lower detection of

TTNtv protein compared to full-length titin, these observations imply that either mRNA transcripts that contain TTNtvs are translated less efficiently, or there is increased degradation of TTNtv protein. Ribosome profiling studies have suggested that translation of the TTNtv-bearing mRNA is intact (13), supporting the notion that TTNtv proteins are actively degraded.

TTNtv⁺ ACMs appeared to more closely preserve an NF contractility profile when compared to TTNtv⁻ ACMs. These data differ from data acquired in TTNtv⁺ iPSC-derived CMs (11), in which contractility was affected, and more closely approximate data seen with isolated myofilaments from human TTNtv⁺ hearts (14), in which myofilament kinetics were more similar to NF than TTNtv⁻ samples. We did note a uniquely decreased resting sarcomere length in TTNtv⁺ ACMs. This could be due to structural changes to the sarcomere, increased diastolic calcium concentrations, or elevated myofilament calcium sensitivity, the latter of which would be consistent with the increased fractional shortening observed in these cells relative to TTNtv⁻ ACMs. Loss of ejection fraction in the context of seemingly preserved contractility could reflect other contextual factors, such as afterload, preload, and extracellular milieu. We also note that contractility was assessed in unloaded myocytes and thus may be insensitive to potential effects of TTNtvs on load-dependent regulation of myofilament function. The data also underscore the imperative to study adult cardiomyocytes.

Our data also support the conclusion that the presence of TTNtvs does not increase the likelihood of more severe disease, compared to patients with TTNtv⁻ DCM. No differences in demographic or echocardiographic characteristics were apparent in TTNtv⁺ versus TTNtv⁻ patients undergoing cardiac transplant. The 12% prevalence of hiPSI TTNtvs in our transplant cohort was no higher than that seen in cohorts of predominantly nontransplanted patients [for example, 11% in both a primary outpatient clinic DCM cohort and a secondary diagnostic referral DCM cohort (6)], indicating that TTNtvs do not increase the likelihood of undergoing transplantation, an indicator of disease severity. Last, global transcriptomic profiling revealed almost no substantial differences between TTNtv⁺ and TTNtv⁻ DCM hearts. Together, these data demonstrate that TTNtvs contribute to disease progression but suggest that a common pathogenic pathway is shared between most forms of DCM, regardless of their TTNtv genetic status.

Limitations of our study include the limited number of hearts available. For example, we did not have tissue from hearts bearing TTNtvs in the N-terminal hiPSI exons, which are also likely

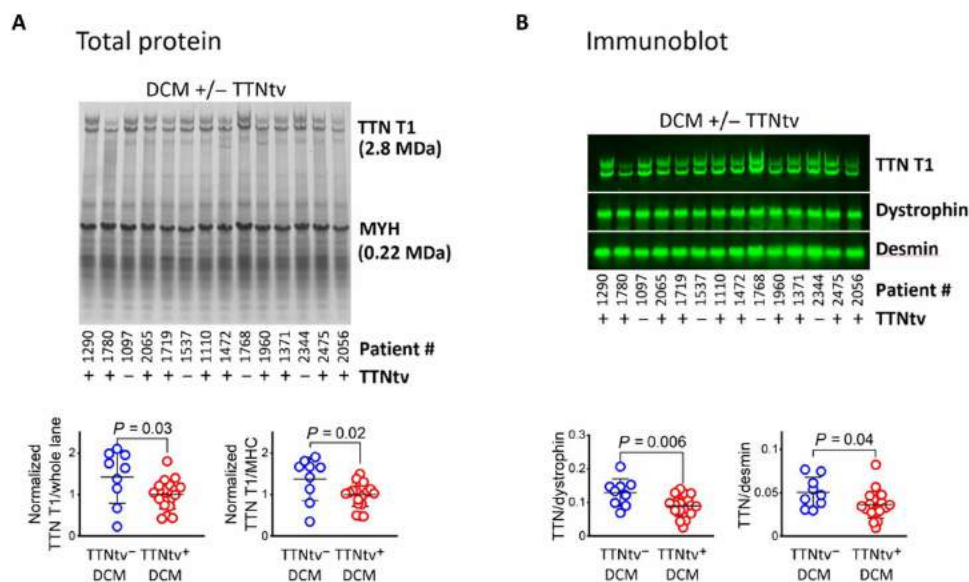


Fig. 6. Evidence of reduced titin protein in TTNtv⁺ DCM myocardium. Sample SDS–agarose gel and immunoblot of LV myocardium total protein samples from DCM hearts with and without TTNtv. **(A)** SDS–agarose gel stained with Coomassie blue, with total protein fluorescence detected at 700 nm. TTN relative abundance is expressed as normalized TTN total protein (T1, N2BA + N2B) band intensity divided by either whole lane or MHC (MYH) band intensity. **(B)** Immunoblot of replica gel of samples presented in (A), probed for titin, dystrophin, and desmin. TTN relative abundance is expressed as TTN total protein (T1, N2BA + N2B) band intensity divided by dystrophin or desmin band intensity. Quantifications of (A) and (B) are from gels displayed in this figure and additional gels present in fig. S5. For both gels and blots, there are a total of 8 TTNtv⁻ and 20 TTNtv⁺ hearts. Image gamma was adjusted for clarity of display. Statistical significance was determined using a one-way ANOVA, followed by a post hoc Tukey correction.

pathogenic. The high variability, for example, in the observed amount of truncated titin protein, also renders some analyses underpowered. In addition, as noted above, the fact that all hearts are in end-stage heart failure precludes conclusions about the early stages of pathogenesis and may introduce ascertainment bias. Another limitation of these studies is that mRNAs were acquired from terminally failing hearts, affected by a range of medications and devices, which may lead to the null effects by introducing large variability. Two important limitations to our ACM studies are the possibility of cell ascertainment bias, if, for example, the presence of TTNtvs accelerated the demise of poorly functioning cells during the cellular isolation procedure and the fact that we did not perform cellular challenges, for example, with imposed mechanical load or adrenergic stimulation. Further studies in loaded, intact myocardial tissue preparations from TTNtv⁺ hearts will complement these single-cell and myofilament examinations. Last, it is also important to note that all of these cells and mRNAs were acquired from terminally failing hearts, and the pathophysiology of advanced heart failure may have masked earlier differences in the pathogenicity of TTNtv⁻ versus TTNtv⁺ cardiomyopathy.

DCM is a multifactorial disease, for which to date no disease-specific therapies exist. Neurohormonal blockade and volume control remain the mainstays of therapy, but the prognosis of patients with DCM remains poor, with 5-year mortality approaching 50%. Our data, demonstrating the presence of truncated titin proteins in TTNtv⁺ DCM hearts, suggest that, in the subset of DCM patients with TTNtvs, targeting the truncated titin protein may provide a therapeutic opportunity.

MATERIALS AND METHODS

Study design

The overall goal of the study was to evaluate transcriptomic and proteomic differences between human failing hearts bearing truncating variants in the gene *TTN* and those not bearing truncating titin variants. Failing human hearts were acquired after orthotopic transplantation, and all available hearts underwent genomic sequencing to identify hearts bearing truncating variants in *TTN*. No hearts were excluded from the analysis. Sample size was determined by number of available cases of hearts with TTNtvs. All subsequent studies were performed blind to genotype. No randomization was performed. For the RNA studies, previously published RNA-seq data from a large subset of this collection of hearts were analyzed, with no exclusions. For the adult cardiomyocyte functional studies, we retrospectively analyzed data that had been acquired from fresh cell isolations, blind to genotype. For the protein studies, protein was prepared from hearts identified as bearing TTNtvs and evaluated by proteomics, gel electrophoresis, and Western blotting.

Procurement and characterization of human hearts

Procurement of human myocardial tissue was performed under protocols and ethical regulations approved by Institutional Review Boards at the University of Pennsylvania and the Gift-of-Life Donor Program (PA, USA) and as previously described (15). Briefly, failing human hearts of nonischemic origin were procured at the time of orthotopic heart transplantation at the Hospital of the University of Pennsylvania after informed consent from all participants. Only hearts from patients with nonischemic DCM were included; exclusion criteria included coronary artery disease, valvular disease, toxic agents, and anatomical abnormalities. NF hearts were obtained at the time of organ donation from cadaveric donors. In all cases, hearts were arrested in situ using ice-cold cardioplegia solution and transported on wet ice. TTNtv hearts were identified by genomic sequencing, and DCMs were classified by clinical diagnosis. The values reported for LVEF for patients with DCM in Table 1 are the last recorded LVEF measurement before heart transplant. These are derived via echocardiography. The most frequently used measurement approach is the modified Simpson's method (biplane method of disks).

Human left ventricular myocyte isolation and contractility measurements

Human left ventricular myocytes were isolated as described previously (15). For this study, myocytes were isolated from 11 hearts (4 NF, 4 TTNtv⁻ DCM, and 3 hiPSI TTNtv⁺: patients 1725, 1780, and 1719) for functional studies. Isolated myocytes were also used as part of separate, unrelated experiments that required short-term

(48 hours) culture and viral transduction with a control or experimental adenovirus; in this study, only cells transduced with control virus were used for analysis. Culture medium consisted of F-10 (1×) Nutrient Mixture (Ham) [+] L-glutamine (Life Technologies, 11550-043) supplemented with insulin-transferrin selenium-X (Gibco, 51500-056), 20 mM HEPES, Primocin (1 µg/µl; InvivoGen, ant-pm-1), 0.4 mM extra CaCl₂, 5% fetal bovine serum, and 25 µM cytochalasin D (Cayman, 11330). Viable myocytes were concentrated by gravity, and medium was added in culture so that neighboring cells were not in direct contact. Control viral constructs were permitted to express for 48 hours with a multiplicity of infection = 100 to 200.

Assessment of contractility by unloaded shortening was performed as in (15). Briefly, before contractility measurement, cultured human myocytes were transferred to fresh warm medium without cytochalasin D. Contractility was measured in a custom-fabricated cell chamber (IonOptix) mounted on an LSM Zeiss 880 inverted confocal microscope using either a 40× or 63× oil 1.4 numerical aperture objective and transmitted light camera (IonOptix MyoCam-S). Experiments were conducted at room temperature, and field stimulation was provided at 0.5 Hz with a cell stimulator (MyoPacer, IonOptix). After 10 to 30 s of pacing to achieve steady state, five traces were recorded and analyzed. Sarcomere length was measured optically by Fourier transform analysis (IonWizard, IonOptix). All experiments were performed and analyzed blind to genotype.

Tissue DNA and RNA-seq

DNA was extracted using Qiagen's Gentra Puregene kit. DNA (1 µg) was sheared with a Diagenode BioRuptor for 30 min set on high with 30 s on/off. Sheared DNA underwent library prep using Agilent's SureSelect XT2 kit. Barcoded exomes were enriched for DCM-associated genes using the Seidman DCM version 5.2 bait panel, which includes all 364 exons of *TTN*. Libraries were run on an Illumina NextSeq, and variants were called using Agilent's SureCall. Total RNA was extracted using Qiagen MiRNeasy kit with DNase on-column treatment; quality of RNA was verified by Agilent Bioanalyzer. RNA (1 µg) underwent library prep using Illumina's TruSeq Stranded mRNA kit. RNA-seq libraries were run on an Illumina HiSeq2000. Reads were aligned using STAR and normalized with surrogate variable analysis. Variants were identified with the use of the Genome Analysis Tool Kit (GATK) HaplotypeCaller tool on the basis of GATK Best Practices. All but one identified hiPSI truncating variant, a 5-base pair (bp) deletion in patient 2065, were confirmed by means of Sanger sequencing. A close review of NextGen sequencing results of patient 2065 showed extensive coverage (>100) at that site, with >40% prevalence of the 5-bp deletion, suggesting that the failure to confirm by Sanger sequencing resulted from amplification artifact. This case was therefore included in subsequent analyses.

DNA exome sequencing phasing and *TTN* allelic imbalance analysis

To discriminate *TTN* alleles on maternal and paternal chromosomes, we phased the genomes of 210 subjects using EAGLE2 (version 2.4.1) provided by the Sanger Institute (<https://sanger.ac.uk/science/tools/sanger-imputation-service>) (20). Because of the lack of intron information, we used a reference-based phasing method using the haplotype reference consortium, which consists of 64,976 haplotypes at 39,235,157 single-nucleotide polymorphisms (SNPs) (<http://haplotype-reference-consortium.org/>). Only phased heterozygous SNPs were used for the subsequent analyses. In the allelic counting step,

RNA-seq reads were aligned to the human reference genome hg19, and Samtools (version 1.10) mpileup (<http://htslib.org/doc/samtools-mpileup.html>) was used to generate a "pileup" of RNA-seq bases (or indels) aligned on each given heterozygous SNP on *TTN*. A customized script was then used to parse the heterozygous variations and compute how many reads were aligned to each haplotype (allele_0 and allele_1) on *TTN*. For each heterozygous SNP, the allelic imbalance was computed as $\text{number_of_bases_matched_on_allele0}/(\text{number_of_bases_matched_on_allele0} + \text{number_of_bases_matched_on_allele1})$. An allelic imbalance equal to 0.5 indicates exact 1:1 allelic ratio.

Myocardial protein isolation and gel electrophoresis

Previously frozen tissue samples stored at −80°C were handled on dry ice or liquid nitrogen. Roughly crushed fragments of myocardium free of epicardial fat of about 10 to 15 mg in mass were selected and further crushed on liquid nitrogen into a fine powder using a stainless steel rod as a pestle and a 2-ml stainless-steel microfuge tube as a mortar. Cryo-powdered heart tissue was resuspended in 40 volumes of titin homogenization buffer prepared according to (21, 22) and containing 8 M urea, 2 M thiourea, 3% SDS, 50 mM tris (pH 6.8), a small amount of phenol red, and 75 mM freshly added dithiothreitol. The tissue was then homogenized with a Potter-Elvehjem homogenizer until no fragments were visible, diluted 1:1 with 50:50 glycerol with 2× Roche cOmplete Mini protease inhibitor tablets, and briefly homogenized again. Samples were incubated for 30 min at 37°C and then spun at ~20,000g for 5 min at 10°C, and the pellet was discarded. Supernatants were aliquoted and stored at −80°C. For use, samples were thawed, briefly vortexed, and electrophoresed.

For SDS-agarose electrophoresis, total myocardial protein prepared as above was separated according to (22) with modifications according to a protocol provided by the Granzier laboratory. The running buffer was 50 mM tris, 0.384 M glycine (pH 8.3). Gels were cast from running buffer containing 1% Sea-Kem Gold agarose (Lonza) plus 30% glycerol. The gel was retained within the plates by a folded segment of nylon mesh placed between the plates. Fresh 2-mercaptoethanol was added to the upper buffer chamber at a dilution of 1:1000 just before sample loading. The gel apparatus was nearly completely filled with prechilled running buffer and placed in a slurry of ice during the run, which was conducted at a constant current of ~16 mA per gel. For total protein staining of SDS-agarose electrophoresis gels, gels were stained with GelCode blue (Thermo Fisher Scientific) according to the manufacturer's instructions and imaged on an Odyssey CLX (LiCor) infrared imager at 700 nm.

For immunoblotting of titin from SDS-agarose gels, protein was transferred to a nitrocellulose membrane using a Bio-Rad semidry transfer apparatus with a discontinuous buffer system according to a protocol provided by the Granzier laboratory. The anode I solution contained 20 mM tris, 0.05% SDS, and 10% methanol (pH 10.4). Anode II solution contained 25 mM tris, 0.05% SDS, 10% methanol (pH 10.4), and the cathode buffer solution contained 25 mM tris, 0.05% SDS, 10% methanol, 40 mM CAPS (pH 10.4), each with 10 mM 2-mercaptoethanol added just before assembly. The gel was soaked for 20 min before transferring in cathode buffer plus 10 mM 2-mercaptoethanol. Proteins were transferred at 1.3 mA per square centimeter of gel for 1 hour. Gels were stained after transfer with GelCode blue to verify absence of detectable untransferred protein, and membranes were stained with Ponceau S to verify even transfer. Membranes for fluorescence imaging were blocked and probed using Intercept (Li-Cor) blocking buffer and standard blotting methods.

Fluorescent blots were imaged using a Li-Cor Odyssey FC infrared imager. Fluorescent gels and blots were quantified using ImageStudio (Li-Cor).

For routine SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blotting of nontitin proteins, proteins prepared as for titin gels were run on standard 4 to 20% SDS-PAGE gels, transferred to a nitrocellulose membrane, stained for total protein using Ponceau S for transfer verification, probed according to standard immunoblotting methods, treated with chemiluminescent substrate, and exposed to film. Band quantifications were made using Image Studio or Empiria (Li-Cor) and normalized to indicated internal controls.

Myofibrillar physical isolation and immunohistochemistry

Cryo-crushed human myocardial tissue powder produced as described above was weighed in frozen form and then directly combined with 20 volumes (w/v) of modified skinning/relaxing buffer (23) composed of 20 mM imidazole (pH 7.0), 7.8 mM Na adenosine 5'-triphosphate, 12 mM magnesium propionate, 97.6 mM potassium propionate, and 0.5% Triton X-100, as well as Roche cOmplete Mini EDTA-free protease inhibitor tablets used at twofold indicated concentration. After brief agitation, the mixture was incubated for about 15 min on ice and then spun at 5000g for 5 min to partition soluble components to the supernatant and insoluble myofibrillar and other components to the pellet. A ~300- μ m pore size nylon mesh was used to exclude oversize fragments. The pellet was washed in the same buffer, and both supernatant and pellet were solubilized in titin-solubilizing buffer, bringing the final volume to 80-fold the original tissue input mass in titin solubilization buffer and 50:50 (v/v) glycerol, as described above. Complete partitioning of soluble (GAPDH) and insoluble markers (TTN T1, desmin, and myosin) were used to evaluate separation of soluble from insoluble components.

For cryo-crush immunofluorescence, cryo-crushed myocardium was added directly to neat methanol at -20°C and incubated for 8 min, quickly spun to pellet myocardial fragments, and resuspended in Seablock (Thermo Fisher Scientific) for continued processing for immunofluorescence staining, mounting, and imaging using standard methods.

Protein sequence analysis by liquid chromatography–tandem mass spectrometry

Hearts included in proteomic analysis were chosen by sample availability and preference for the largest number missense mutations to facilitate sensitive detection by proteomics and separation of proteins encoded by the two parental alleles. Excised gel bands were cut into about 1 mm³ pieces. Gel pieces were then subjected to a modified in-gel trypsin and chymotrypsin digestion procedure (24). Gel pieces were washed and dehydrated with acetonitrile for 10 min, followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces was with 50 mM ammonium bicarbonate solution containing modified sequencing-grade trypsin (12.5 ng/ μ l; Promega) at 4°C . After 45 min, the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. Samples were then placed in a 37°C room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 hour). The samples were then stored at 4°C until analysis.

Proteomics was performed by the Taplin Biological Mass Spectrometry Facility. On the day of analysis, the samples were reconstituted in 5 to 10 μ l of high-performance liquid chromatography (HPLC) solvent A (2.5% acetonitrile and 0.1% formic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 2.6- μ m C18 spherical silica beads into a fused silica capillary (100- μ m inner diameter by ~30-cm length) with a flame-drawn tip (25). After equilibrating the column, each sample was loaded via a Famos autosampler (LC Packings) onto the column. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile and 0.1% formic acid).

As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program Sequest (Thermo Fisher Scientific) (26). All databases include a reversed version of all the sequences, and the data were filtered to between a 1 and 2% peptide false discovery rate. Files containing raw proteomic have been uploaded to the PRIDE Database.

Statistical analyses

Statistical analyses were performed using OriginPro, GraphPad Prism, or Excel, except for bioinformatics, as noted. Multiple comparisons were made using a one-way analysis of variance (ANOVA), followed by a Tukey or Bonferroni multiple comparisons correction. In Fig. 2, multiple comparisons were made using a Kruskal-Wallis test followed by a Dunn's test.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abb7287

Fig. S1 to S5

Table S1

Data files S1 and S2

[View/request a protocol for this paper from Bio-protocol.](#)

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Abstract

One-sentence summary: Truncating mutations in *TTN* result in both truncated titin proteins and reduced full-length titin in patient hearts.

Editor's Summary:

Tracking titin in dilated cardiomyopathy

Truncating variants in *TTN*, the gene encoding the titin protein, underlie 15 to 25% of cases of nonischemic dilated cardiomyopathy (DCM), but whether the disease is caused by haploinsufficiency or the presence of truncated titin proteins is not yet clear. Here, using tissues from the hearts of patients with DCM and *TTN*-truncating variants as well as human induced pluripotent stem cells differentiated into cardiomyocytes, Fomin *et al.* and McAfee *et al.* identified both the presence of truncated titin proteins and less-abundant full-length titin protein. These findings suggest that the presence of truncated titin proteins as “poison peptides” and titin haploinsufficiency both contribute to the pathogenesis of disease and should support the investigation of targeted therapies to treat DCM caused by *TTN*-truncating variants.

Women with peripartum cardiomyopathy have normal ejection fraction, but abnormal systolic strain, during pregnancy

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Abstract

We report a case series of six women with peripartum cardiomyopathy (PPCM) who incidentally underwent echocardiography prior to the clinical presentation of PPCM. For comparison, we identified controls, matched 2:1 on age, race, body mass index, gestational age, and hypertensive disorder. Among the six cases, all were diagnosed with PPCM during the post-partum period. Pre-PPCM echocardiograms were performed between 17.7 weeks of gestation and 13 days post-partum. Baseline left ventricular ejection fraction and size were normal and similar to the 12 matched controls ($60\% \pm 6.6\%$ vs. $61.4\% \pm 6.3\%$, $P = 0.63$) or left ventricular end-diastolic dimension ($4.6 \text{ cm} \pm 0.2 \text{ cm}$ vs. $4.5 \text{ cm} \pm 0.4 \text{ cm}$, $P = 0.689$). There was a trend towards a less negative (more abnormal) mean global longitudinal strain in cases compared with controls ($-14\% \pm 4\%$ vs. $-18.3\% \pm 4.5\%$, $P = 0.0658$). Mean global circumferential strain was significantly less negative (more abnormal) in cases compared with controls ($-21.5\% \pm 5\%$ vs. $-29.3\% \pm 7.6\%$, $P = 0.0329$). We conclude that women who develop PPCM have normal left ventricular ejection fraction during gestation preceding PPCM, indicating that the disease develops acutely in the peripartum period. Abnormal strain can be detected, however, suggesting that strain imaging could represent a screening method in populations at high risk for PPCM if confirmed in future studies.

Keywords Peripartum cardiomyopathy; Strain echocardiography; Heart failure; Maternal morbidity

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Introduction

Peripartum cardiomyopathy (PPCM) is a form of systolic heart failure diagnosed towards the end of pregnancy or in the months following delivery in women without pre-existing cardiac disease.¹ Contemporary cohorts in the USA demonstrate that 65% to 72% have recovery of left ventricular (LV) function; however, recovery rates vary by population, and many women experience adverse outcomes, including persistent heart failure, arrhythmias, thromboembolic complications, or death.^{1–5} Whether women have normal LV ejection fraction (LVEF) and LV size during pregnancy and prior to development of PPCM has been disputed and has been difficult to study given the low incidence of PPCM and the infrequent use of echocardiograms during pregnancy. In addition, LV

strain has emerged as a powerful tool to detect subclinical LV dysfunction in the setting of cardiotoxic chemotherapy, prior to a subsequent decrease in LVEF.⁶ Whether changes in LV strain indices precede the drop in LVEF in PPCM has not been studied and may hold predictive ability to identify women at risk. Our aims are to describe baseline LV function, including LVEF and strain indices, of peripartum women prior to their development of PPCM and to compare these measures to peripartum women without PPCM.

Case report

We identified six cases of pregnant women with echocardiograms performed during pregnancy or post-partum period

and prior to the development of PPCM. We identified cases from a previously established and updated retrospective cohort of women with PPCM treated within the University of Pennsylvania Health System, as previously described.³ We included women who had also had an echocardiogram performed during pregnancy/post-partum period prior to the diagnosis of PPCM (pre-PPCM echocardiogram). We also compared baseline echocardiographic parameters of peripartum women who subsequently developed PPCM (cases) to peripartum women without PPCM (controls). Controls without known cardiac disease were matched 2:1 on maternal age (± 5 years), race, body mass index, gestational age (± 4 weeks), and hypertensive disorder. Control echocardiograms were selected from women who had enrolled in prior clinical research studies at our health system evaluating echocardiographic differences between women with hypertensive disorders of pregnancy and normotensive pregnancies. The primary outcomes were LVEF, LV end-diastolic dimension, global longitudinal strain (GLS), and global circumferential strain (GCS). LVEF was measured using modified Simpson bi-plane method. Strain was measured using speckle tracking (TomTec Imaging Systems, Unterschleissheim, Germany), as described previously.^{7,8} All echocardiograms were analysed by study staff (R.T. and Y.K.). Continuous variables were presented as means and standard deviations and assessed using the Student's *t*-test given normality of the data. Categorical variables were assessed using Fisher's exact test. We used univariate exact conditional regression to analyse outcomes to account for matched data.

The timing of pre-PPCM echocardiograms ranged from 17.7 weeks of gestation to 13 days post-partum. Indications for the pre-PPCM echocardiograms are presented in *Table 1*. For Cases 1, 2, and 3, indications for echocardiogram were first degree atrioventricular block, palpitations, and chronic hypertension, respectively. Case 4 had a known small, restrictive ventricular septal defect (VSD), and an echocardiogram was performed during pregnancy for routine screening. Of note, at the time of her subsequent PPCM diagnosis, it was thought that the drop in LVEF was not related to her known prior VSD. Case 5 had an echocardiogram performed as part of a workup for hypoxia and tachycardia, and she was ultimately diagnosed with a pulmonary embolism. Case 6 had an echocardiogram performed due to chest pain, and she was ultimately diagnosed with and treated for pericarditis.

Peripartum cardiomyopathy was diagnosed post-partum in all cases, ranging from 6 to 100 days after delivery. The timing between the pre-PPCM echocardiograms and PPCM diagnosis echocardiograms ranged from 19 to 222 days. At the time of PPCM diagnosis, LVEF ranged from 24% to 45%. The majority of the cases (5/6) ultimately had LVEF recovery (LVEF $\geq 50\%$). Genetic results were available for 4/6 cases, and none had a titin-truncating mutation.

The mean age was 32.8 years, and the majority (4/6) were black. There were no significant differences in baseline

Table 1 Cases of peripartum cardiomyopathy with echocardiograms performed prior to diagnosis

Case	Medical history	Pre-PPCM echocardiogram ^a			At time of PPCM diagnosis			Recovery	
		GA or days post-partum	LVEF	Clinical indication	Days post-partum	LVEF	Days between echocardiograms	Recovery to LVEF ≥ 50%	TTN mutation
1	T2 IDDM	17.7 weeks	52%	First degree AVB on ECG	73 days	33%	222	Yes	No
2	Crohn's disease	19.7 weeks	68%	Palpitations	9 days	37%	121	Yes	N/A
3	T1DM, chronic HTN	20.1 weeks	54%	Screening given chronic HTN	93 days	36%	153	No	No
4	Small restrictive VSD	32.9 weeks	67%	Screening given known VSD	6 days	45%	42	Yes	No
5	Asthma, rheumatoid arthritis	Post-partum 1 day	61%	Shortness of breath due to PE	20 days	29%	19	Yes	N/A
6	Asthma, hyperlipidaemia	Post-partum 13 days	58%	Chest pain, diagnosed with pericarditis	100 days	24%	87	Yes	No

AVB, atrioventricular block; ECG, electrocardiogram; GA, gestational age; HTN, hypertension; LVEF, left ventricular ejection fraction; N/A, not available; PE, pulmonary embolism; PPCM, peripartum cardiomyopathy; T1DM, type 1 diabetes mellitus; T2 IDDM, type 2 insulin-dependent diabetes mellitus; TTN, titin; VSD, ventricular septal defect.

characteristics between the matched cases and controls (Table 2). There was no significant difference in pre-PPCM LVEF ($60\% \pm 6.6\%$ vs. $61.4\% \pm 6.3\%$, $P = 0.63$) or LV end-diastolic dimension ($4.6 \text{ cm} \pm 0.2 \text{ cm}$ vs. $4.5 \text{ cm} \pm 0.4 \text{ cm}$, $P = 0.689$) between cases and controls. Strain analysis was technically able to be performed in 5/6 cases and all controls. There was a trend towards a less negative (more abnormal) mean GLS in cases compared with controls ($-14\% \pm 4\%$ vs. $-18.3\% \pm 4.5\%$, $P = 0.0658$). Mean GCS was significantly less negative (more abnormal) in cases compared with controls ($-21.5\% \pm 5\%$ vs. $-29.3\% \pm 7.6\%$, $P = 0.0329$) (Table 2).

Discussion

While it has been often assumed that women have normal LVEF and LV dimensions during pregnancy and prior to development of PPCM, this has not been previously confirmed with serial echocardiograms. Our findings support the conclusion that LVEF and LV dimensions are largely normal during pregnancy and early post-partum period in women who go on to develop PPCM and that changes in LVEF and LV dimensions occur acutely at the time of or shortly before the diagnosis of PPCM. Our study suggests that subclinical dysfunction, as measured by GLS and GCS, may be present prior to the development of symptomatic heart failure and LV dysfunction in women with PPCM. Therefore, subclinical strain abnormalities identified during pregnancy may help to identify women at increased risk of developing PPCM. Current thinking on the pathophysiology of PPCM suggests that hormones of late gestation, including those increased by pre-eclampsia, cause microvascular damage to the heart, leading to

PPCM.^{1,9} This abnormal hormonal milieu may manifest its cardiotoxic effect as subclinical dysfunction prior to causing an overt drop in LVEF, analogous to the cardiotoxic effect of chemotherapy.⁶ If our results are confirmed in larger cohorts, strain imaging could serve as a screening target in populations at increased risk for developing PPCM.

Cardiovascular disease, including PPCM, is a leading cause of maternal morbidity and mortality, disproportionately impacts women of African ancestry, and contributes to significant racial disparities in maternal outcomes observed in the USA.¹⁰ A limited echocardiogram during pregnancy to identify clinical or subclinical abnormalities in women with risk factors for PPCM, such as African ancestry, advanced maternal age, multi-gestational pregnancies, or hypertensive disorders, may identify women who need closer follow-up during pregnancy and the post-partum period to monitor for early signs or symptoms of heart disease. This is especially important for women who experience barriers to accessing care and may mistake cardiac symptoms for normal pregnancy symptoms.¹¹ Further research should examine the feasibility of obtaining limited maternal cardiac views during routine pregnancy care, such as the second trimester foetal ultrasound scan.

Our study has limitations to consider. First, normative values for strain during pregnancy have not been established, although some studies suggest that strain worsens during the third trimester.^{12,13} Average strain rates in our control cohort are similar to those reported in the literature. We aimed to account for pregnancy-related changes in strain by matching on gestational age and other clinical factors, although it was not possible to match on all relevant factors. Second, our sample size is small, and therefore, our results need to be confirmed in a larger

Table 2 Clinical and echocardiographic characteristics of peripartum women who subsequently develop peripartum cardiomyopathy and matched controls

	Cases (n = 6)	Controls (n = 12)	P-value
Baseline characteristics^a			
Age at delivery, mean (SD)	32.8 (8.3)	30.0 (8.3)	0.505
Race, n (%)			1.000
White	2 (33.3)	4 (33.3)	
Black	4 (66.7)	8 (66.7)	
BMI, mean (SD)	31.1 (4.9)	30.0 (4.7)	0.645
GA or days PP at time of echocardiogram	17.7 weeks to 13 days PP	19.1 weeks to 2 days PP	
Hypertensive disorder at time of echocardiogram, n (%)	2 (33.3)	3 (25.0)	1.000
GA at delivery, mean (SD)	35.5 (4.5)	36.6 (4.2)	0.619
Echocardiographic parameters, mean (SD)^b			
LV ejection fraction (%)	60.0 (6.6)	61.4 (6.3)	0.630
LV end-diastolic dimension (cm)	4.6 (0.2)	4.5 (0.4)	0.689
Global longitudinal strain (%)	-14.0 (4.0)	-18.3 (4.5)	0.066
Global circumferential strain (%)	-21.5 (5.0)	-29.3 (7.6)	0.033

BMI, body mass index; GA, gestational age; LV, left ventricular; PP, post-partum.

^aAnalysed using the Student's *t*-test for continuous variables and Fisher's exact test for binary variables.

^bAnalysed using univariate conditional exact regression.

study. Third, we do not have longitudinal strain data to determine whether these changes in strain persist or resolve over time in women with subsequent LV recovery. Finally, echocardiograms are not standard of care during pregnancy, and our cases and controls may represent selection bias. Pre-eclampsia and hypertension can impact strain values and are associated with PPCM, for which we aimed to control through matching. To our knowledge, the other indications for pre-PPCM echocardiograms (e.g. restrictive VSD and pulmonary embolism) do not significantly affect LV strain values.^{14–16}

Peripartum cardiomyopathy is a leading cause of maternal mortality in the post-partum period, especially among black women, and delayed diagnosis leads to worse clinical outcomes. Therefore, efforts focused on closer monitoring and earlier treatment of those at risk have the potential to improve maternal cardiovascular health and lessen racial disparities.

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Conflict of interest

None declared.

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ZFP36L2 suppresses mTORC1 through a P53-dependent pathway to prevent peripartum cardiomyopathy in mice

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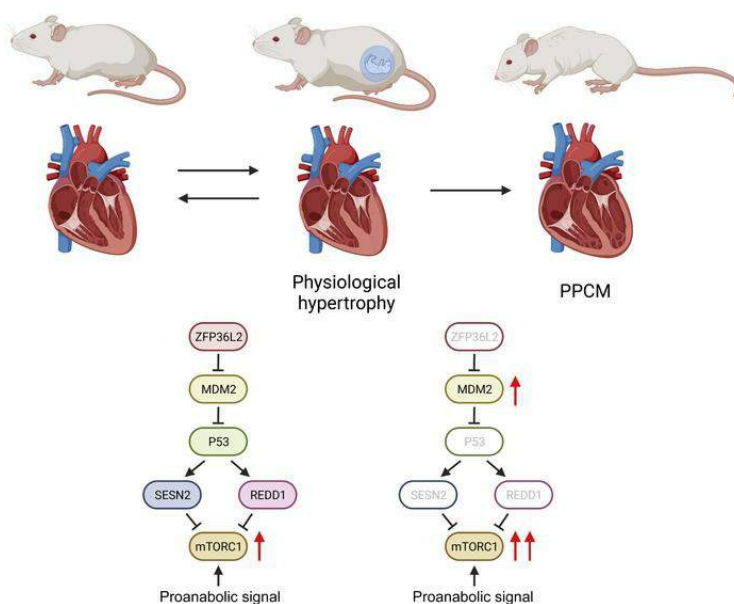
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ZFP36L2 suppresses mTORc1 through a P53-dependent pathway to prevent peripartum cardiomyopathy in mice

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Pregnancy is associated with substantial physiological changes of the heart, and disruptions in these processes can lead to peripartum cardiomyopathy (PPCM). The molecular processes that cause physiological and pathological changes in the heart during pregnancy are not well characterized. Here, we show that mTORc1 was activated in pregnancy to facilitate cardiac enlargement that was reversed after delivery in mice. mTORc1 activation in pregnancy was negatively regulated by the mRNA-destabilizing protein ZFP36L2 through its degradation of *Mdm2* mRNA and P53 stabilization, leading to increased SESN2 and REDD1 expression. This pathway impeded uncontrolled cardiomyocyte hypertrophy during pregnancy, and mice with cardiac-specific *Zfp36l2* deletion developed rapid cardiac dysfunction after delivery, while prenatal treatment of these mice with rapamycin improved postpartum cardiac function. Collectively, these data provide what we believe to be a novel pathway for the regulation of mTORc1 through mRNA stabilization of a P53 ubiquitin ligase. This pathway was critical for normal cardiac growth during pregnancy, and its reduction led to PPCM-like adverse remodeling in mice.

Introduction

Pregnancy is associated with a number of changes to cardiovascular function, including increases in cardiac output, arterial compliance, and extracellular fluid volume (1). In addition, left ventricular (LV) wall thickness and mass increase by 28% and 52%, respectively (2). These adjustments are made to meet the increased metabolic demands of both the mother and the fetus as well as to ensure adequate circulation for fetal growth and development (1). However, alterations to normal cardiovascular physiology in pregnancy can lead to pregnancy-associated heart disorders, including peripartum cardiomyopathy (PPCM). PPCM is a form of cardiomyopathy that occurs in the last trimester of pregnancy and up to 6 months postpartum; it is defined by a decrease of the LV ejection fraction (EF) and congestive heart failure (3). If untreated, PPCM can lead to death. The etiology of PPCM is unknown; however, several mechanisms, including autoimmunity, systemic viral illness, micronutrient deficiency, and genetics, have been proposed (4, 5). Recent studies have indicated that defects in cardiac angiogenic balance also contribute to PPCM (6, 7). However, a lack of robust rodent models has limited mechanistic insight into the etiology of this disease.

Zinc finger protein (ZFP) 36 like 2 (ZFP36L2, also known as TIS11D, ERF2, and BRF2) is a member of the ZFP family of mRNA

binding proteins, which also includes tristetraprolin (also known as ZFP36) and ZFP36L1. Members of this family bind to specific AU-rich elements (AREs) in the 3' untranslated region (UTR) of mRNA molecules, causing their degradation (8). Global deletion of *Zfp36l2* in mice does not lead to embryonic lethality (unlike deletion of *Zfp36l1*); however, newborn mice die within 2 weeks after birth and suffer from pancytopenia as a result of a deficiency in hematopoietic stem cells in the liver (9). Mutant mice expressing an N-terminal truncated form of ZFP36L2 exhibit early developmental defects and growth arrest at the 2-cell embryonic stage (10). Simultaneous deletion of *Zfp36l1* and *Zfp36l2* in thymus during early period of its development led to the development of Notch1-dependent T cell acute lymphoblastic leukemia (11). Furthermore, tissue-specific deletion of *Zfp36l1* and *Zfp36l2* in pro-B cells demonstrated that these two proteins are critical for quiescence after cellular expansion by precursor B cell receptor (12). Thus, ZFP36L2 appears to be critical in the development of certain cells in the body. However, its function in the heart and the mechanism by which it regulates cardiomyocyte physiology is not entirely understood.

The mechanistic target of rapamycin (mTOR) is a master regulator of protein synthesis, glucose metabolism, and autophagy (13). The role of mTOR in various murine models of heart disease has been studied extensively. A number of studies have looked at mTOR complex 1 (mTORc1) hyperactivation in the heart. Cardiac-specific deletion of *Tsc1* or *Tsc2* leads to death from heart failure at 6 months and 10 months of age, respectively (14, 15), while *Sesn1/2/3* triple-KO mice died during the neonatal period owing to failure to activate autophagy (16). These studies all indicate that mTORc1 hyperactivation is detrimental to normal embryonic development and cardiac function. For models of mTORc1

Authorship note: HK and YT contributed equally to this work.

Conflict of interest: HA serves as expert witness.

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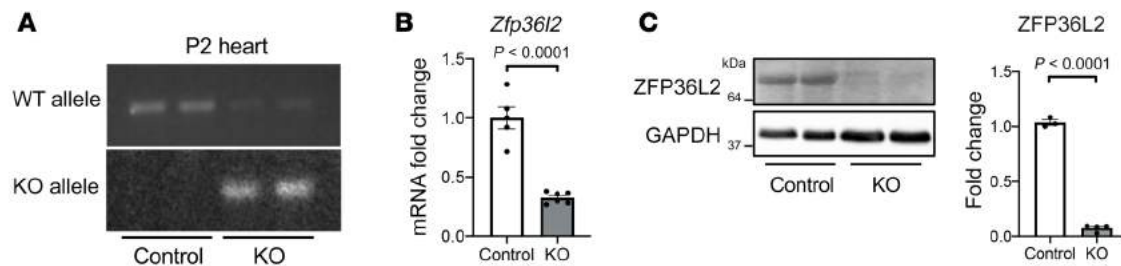


Figure 1. Successful cardiac *Zfp36l2* deletion in mice. (A) DNA ($n = 2$), (B) mRNA ($n = 5-6$), and (C) protein levels ($n = 3-4$) of *Zfp36l2* (A) at postnatal day 2 in the whole heart or (B and C) at 8 weeks old in isolated cardiomyocytes of cardiac-specific *Zfp36l2*-KO and control mice, confirming deletion of the gene in these animals. Data were analyzed by unpaired Student's *t* test.

suppression, both genetic and pharmacological approaches have been employed. Cardiac-specific deletion of *Mtor* causes lethality before the end of gestation (17) or within 3 weeks after birth (18), while deletion of the gene in the heart after birth causes lethal cardiomyopathy 5 weeks after induction (19). Treatment with rapamycin has been shown to extend life span in multiple organisms (20). Additionally, it regressed cardiac hypertrophy induced by trans-aortic constriction (TAC) and age-related cardiac hypertrophy (21, 22). Collectively, these studies demonstrate that the heart requires precise control of mTORc1 activity within a homeostatic range, and deletion of either the central mTORc1 components or its repressors leads to substantial mTORc1 dysregulation and severe damage to the heart.

In this paper, we studied the effects of cardiac-specific deletion of *Zfp36l2* on cardiac function. To our surprise, mice with deletion of this gene did not have a marked phenotype, except for in female mice after pregnancy, which displayed higher mortality and cardiac dysfunction. To define the mechanism for this phenotype, we undertook unbiased transcriptomic analyses, which showed alterations in the expression of key genes in the mTORc1 pathway with *Zfp36l2* deletion. Further studies narrowed this mechanism to a reduction in P53-mediated SESN2 and REDD1 expression, leading to chronic mTORc1 activation. Here, we showed that ZFP36L2 bound to and degraded the mRNA of the E3 protein-ubiquitin ligase MDM2; thus, *Zfp36l2* deletion leads to upregulation of MDM2 and P53 destabilization. We also showed that reversing mTORc1 hyperactivation in female mice with cardiac-specific *Zfp36l2* KO improved cardiac function. Overall, our results indicate that mRNA decay plays a major role in the regulation of the P53/mTORc1 pathway and that loss of ZFP36L2 in the heart leads to hyperactivation of mTORc1 and a PPCM-like phenotype in mice.

Results

Zfp36l2 deletion in the heart leads to a phenotype consistent with PPCM. Previous studies have demonstrated that 2 members of the ZFP36 family play a role in cardiac function; deletion of *Zfp36l1* and *Zfp36* (also known as *Ttp*) causes embryonic heart defects (23, 24) and cardiomyopathy in iron deficiency (25), respectively. While these proteins share structural homology (26), it is not clear whether deletion of *Zfp36l2* would have any effects on cardiac function and what major mRNA molecules ZFP36L2 targets. Therefore, we generated cardiac-specific deletion of *Zfp36l2* by crossing mice with LoxP sequences flanking exon 2 of *Zfp36l2* (27)

with α MHC-Cre mice. The control mice for our subsequent experiments were *Zfp36l2*^{fl/fl} Cre⁻. Cre expression resulted in deletion of *Zfp36l2* at birth, which was confirmed at the genomic, RNA, and protein level (Figure 1). As expected, hearts from *Zfp36l2*-KO mice did not display any change in the levels of ZFP36 or ZFP36L1 proteins (Supplemental Figure 1, A and B; supplemental material available online with this article; <https://doi.org/10.1172/JCI154491DS1>). Examination of the hearts showed no gross and histological abnormalities, and although the cross-sectional area (CSA) was slightly increased in the KO mice, the degree of fibrosis was similar between the groups (Supplemental Figure 1, C-E). Additionally, *Zfp36l2*-KO male and female mice did not display a reduction in EF at 10, 17, and 23 weeks of age (Supplemental Figure 2), supporting that the deletion of this gene does not alter cardiac function at baseline.

Pregnant female *Zfp36l2*-KO mice displayed a high mortality after delivery, and more than 90% of the mice died by 30 weeks of age (Figure 2A). The increased mortality in pregnant female *Zfp36l2*-KO mice was due to cardiac dysfunction, as the KO mice displayed markedly reduced cardiac function 7 days after delivery from first pregnancy (Supplemental Figure 3). Cardiac-specific deletion of *Zfp36l2* had no effect on the fertility of the mice, as both control and KO dams had a comparable number of deliveries and time to first delivery (Supplemental Figure 4, A and B). The KO mice also showed reduced EF and fractional shortening and increased LV diastolic and systolic diameters (LVDd and LVDs, respectively) 7 days after the first pregnancy (Figure 2B). Examination of the hearts after the first pregnancy revealed enlarged hearts (Figure 2C) and an increased heart weight/tibial length ratio (Figure 2D). Additionally, mRNA of markers of heart failure, *Anf* and *Bnp*, were increased in the hearts of *Zfp36l2*-KO mice after pregnancy (Figure 2E); however, there was no significant change in overall cardiac histologic structure or degree of fibrosis (Figure 2F). *Zfp36l2*-KO hearts also displayed higher CSA, consistent with possible cardiomyocyte hypertrophy (Figure 2G). The effects of second pregnancy were even more pronounced, as mice with *Zfp36l2* deletion displayed a more pronounced reduction in EF and fractional shortening and an increase in LVDd and LVDs (Supplemental Figure 5A), in addition to larger hearts (Supplemental Figure 5B). The rapid decline in cardiac function after pregnancy and the lack of tissue fibrosis indicate that deletion of *Zfp36l2* in mice leads to a phenotype consistent with PPCM, which is progressive and causes increased mortality. Although, all ZFP36

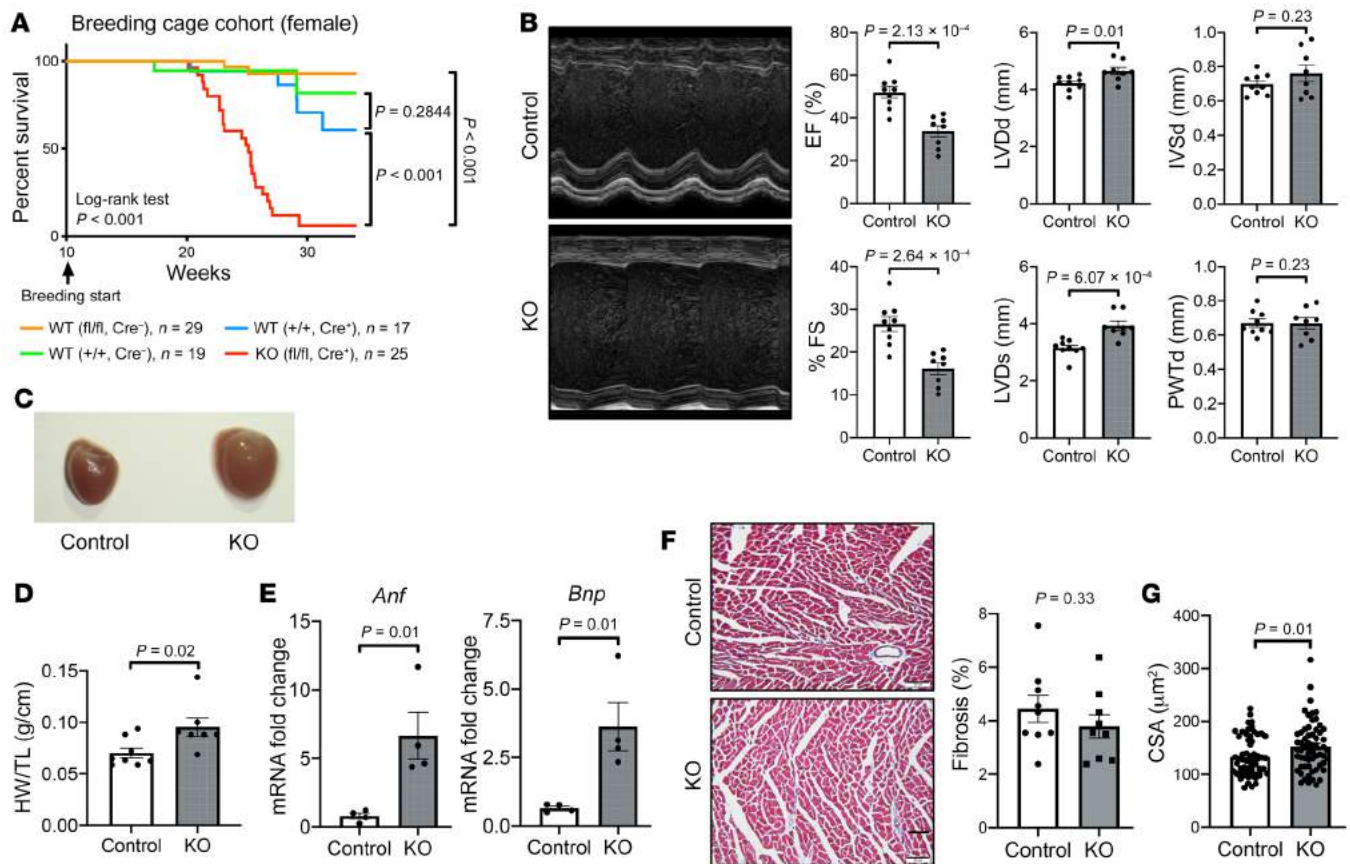


Figure 2. Cardiac *Zfp36l2* deletion induces heart failure. (A) Survival of *Zfp36l2*-KO mice and control mice after an average of 2 pregnancies and their deliveries. (B) Echo measurements in *Zfp36l2*-KO and control mice 7 days after first delivery ($n = 8-9$). EF, ejection fraction; FS, fractional shortening; LVDd, LV diastolic diameter; LVDs, LV systolic diameter. (C) Gross cardiac examination, (D) heart weight divided by tibial length (HW/TL) ($n = 7-8$), and (E) *Anf* and *Bnp* levels ($n = 4$) in *Zfp36l2*-KO and control mice 7 days after the first pregnancy. (F) Histology and assessment of the degree of fibrosis in *Zfp36l2*-KO and control littermates 7 days after first delivery ($n = 3$ mice and 3 sections per mouse). Scale bar: 50 μ m. (G) Cross-sectional area (CSA) in *Zfp36l2*-KO and control littermates 7 days after first delivery ($n = 3$ mice and 20 cardiomyocytes per mouse). Data were analyzed by log-rank test with Bonferroni's post hoc analyses for multiple group comparison (A) or unpaired Student's *t* test (B, D-F, and G).

family members recognize the same ARE sequences in the 3'-UTR of transcripts and can share targets, cardiac-specific deletion of *Zfp36* and *Zfp36l1* did not result in mortality after pregnancy, demonstrating that the PPCM phenotype is due to a specific function of ZFP36L2 (Supplemental Figure 6).

*Deletion of *Zfp36l2* in the heart leads to the activation of the mTORc1 pathway.* We next studied the mechanism by which *Zfp36l2* deletion causes pathological effects in the heart. We performed RNA-Seq in H9c2 cardiomyoblasts treated with control or *Zfp36l2* siRNA. The in vitro approach for this analysis was chosen over using intact hearts from the KO mice to avoid both the unwanted side effects of cardiomyopathy and contamination from noncardiac cells on gene expression. The efficiency of knockdown (KD) was confirmed at the mRNA and protein level (Supplemental Figure 7). It is predicted that ZFP36L2 binds to and causes degradation of a large number of mRNAs (27, 28); thus, we expected that the unbiased RNA-Seq analysis would yield changes in a large number of mRNAs. Indeed, RNA-Seq of the cells with *Zfp36l2* KD showed an increase in 9765 mRNAs and a decrease in 1435 mRNAs compared with control cells (Figure 3A and Supplemental Table 1). We then performed pathway analysis of the transcripts

that are significantly altered in the RNA-Seq data. This analysis revealed metabolic pathways, specifically genes involved in PI3K/mTORc1 signaling, as most significantly changed with *Zfp36l2* KD (Table 1 and Figure 3B). Additionally, analysis of the volcano plot revealed several positive regulators of the mTORc1 pathway to be upregulated, while a number of negative regulators to be downregulated (Figure 3). Thus, we further analyzed the RNA-Seq data for changes in mTORc1-related genes with *Zfp36l2* KD. Most of the genes demonstrated a significant increase in expression with downregulation of *Zfp36l2*, with the exception of 5 genes, including *Redd1* (also known as *Ddit4*), *Sesn2* and *Sesn3* (Figure 3B), which are negative regulators of mTORc1 and were decreased with *Zfp36l2* downregulation. Based on these results, we focused on the mTORc1 pathway for the mechanistic studies.

To validate that mTORc1 activity is altered upon *Zfp36l2* KO, we measured the phosphorylation of P70S6K. *Zfp36l2*-KO hearts at 8 weeks of age displayed significantly higher p-P70S6K^{T389} levels compared with control hearts (Figure 4, A and B), indicating higher mTORc1 activity. Consistent with hyperactivated mTORc1, *Zfp36l2* KD in H9c2 cells led to increased ³⁵S methionine incorporation into proteins (Supplemental Figure 8).

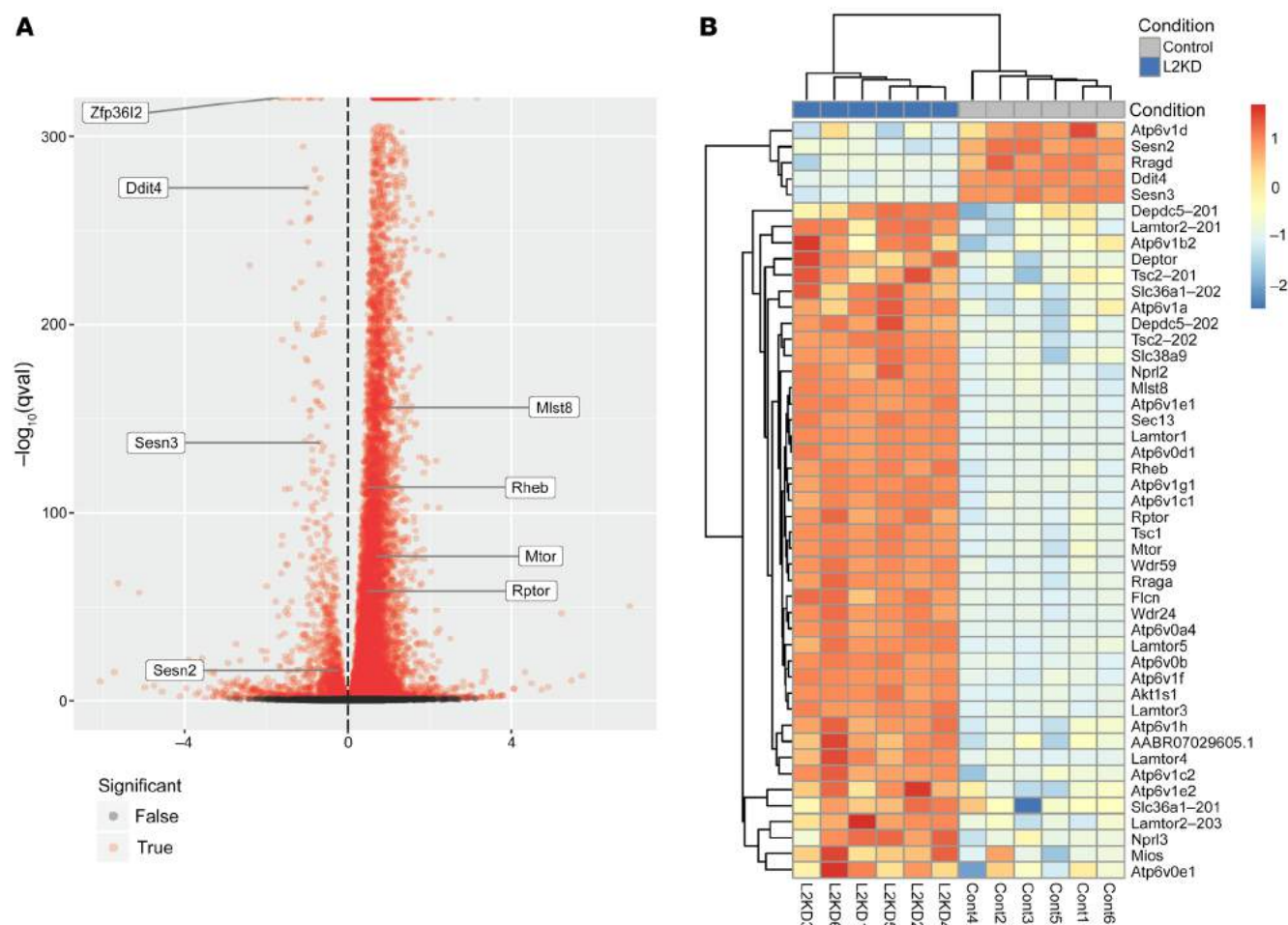


Figure 3. RNA-Seq of H9c2 cells treated with *Zfp36l2* or control siRNA. (A) Volcano plot of transcripts from the *Zfp36l2* KD versus control RNA-Seq experiment. Transcripts were normalized to *Actb* and *Hprt1*, and depicted are the $-\log_{10}$ transformation of the adjusted *P* values versus the "beta value" estimated by sleuth-ALR. Highlighted genes include *Zfp36l2* to confirm the KD, and other genes in the mTOR signaling pathway that had 1 major transcript significantly upregulated or downregulated. **(B)** Heatmap plot of differentially expressed transcripts from the mTOR signaling pathway estimated with *Zfp36l2* KD (*n* = 6).

Downregulation of *Zfp36l2* also resulted in reduced AKT^{S473} phosphorylation (Figure 4, A and C). AKT^{S473} is a direct target of mTORC2, which is subject to negative regulation by mTORC1. To determine if the decrease in p-AKT^{S473} was due to an independent decrease in mTORC2 activity or a result of feedback inhibition from hyperactivated mTORC1, we treated cells with rapamycin and measured AKT^{S473} phosphorylation in response to insulin. Rapamycin treatment rescued AKT^{S473} phosphorylation in *Zfp36l2*-KD cells (Figure 4, E and F), and it similarly suppressed P70S6K^{T389} phosphorylation (Figure 4, E and G), indicating that mTORC1 activity is regulated by ZFP36L2 and the decrease in p-AKT^{S473} is due to mTORC2 inhibition by mTORC1. These results collectively suggest that deletion of *Zfp36l2* increases mTORC1 activity.

mTORC1 regulation by ZFP36L2 is through TSC1/2 and amino acid sensing. The TSC1/2 complex is a major signaling hub for mTORC1 regulation by RHEB (29, 30). We first assessed whether the mechanism by which ZFP36L2 regulates mTORC1 activity requires a functional TSC complex by measuring P70S6K^{T389} phosphorylation in cells treated with *Tsc2* siRNA with and without concurrent *Zfp36l2* downregulation. *Tsc2* downregulation was suf-

ficient to diminish the effects of *Zfp36l2* KD on mTORC1 activation (Figure 4, H and I), indicating that the regulation of mTORC1 by ZFP36L2 is through TSC2. Because mRNA levels of *Redd1* (a positive regulator of TSC2) were reduced in the RNA-Seq of *Zfp36l2*-KD cells (Figure 3B), we then measured protein levels of REDD1 with *Zfp36l2* KD and showed that it was reduced (Figure 5, A and B). Additionally, TSC2 localization to lysosomes, a process required for mTORC1 inhibition, was significantly decreased in *Zfp36l2*-KD cells (Supplemental Figure 9). These findings collectively indicate that ZFP36L2 regulates mTORC1 through REDD1, a negative regulator of TSC complex.

To rule out influence of ZFP36L2 on other TSC-mediated upstream regulators of mTORC1 activity, we measured the activity of the AKT and AMPK pathways after downregulation of *Zfp36l2*. Our results indicated similar increases in p-P70S6K^{T389} and p-AKT^{S473} upon insulin stimulation in control and *Zfp36l2*-KD cells (Supplemental Figure 10, A–C), indicating that mTORC1 regulation by ZFP36L2 is independent of growth factor signaling. We also studied whether changes in cellular energetics, signaled through AMPK, mediate mTORC1 regulation by ZFP36L2.

Table 1. Pathway analysis with the top 10% of significantly altered transcripts with greatest absolute log₂ fold change

Biological process	FDR
Metabolic pathways	9.89×10^{-7}
Protein digestion and absorption	9.08×10^{-6}
Metabolism of xenobiotics by cytochrome P450	3.24×10^{-5}
Drug metabolism — other enzymes	8.78×10^{-5}
Steroid hormone biosynthesis	0.000114

p-AMPK^{T172} was not changed in the hearts of *Zfp36l2*-KO mice (Figure 4, A and D), suggesting that AMPK is also not required for the regulation of mTORC1.

mTORC1 activity is also modulated by the availability of amino acids (particularly leucine), which are sensed through the GATOR complexes and RAG GTPase (31, 32). Because regulation of mTORC1 by amino acids is independent of the TSC1/2 complex, we next determined whether ZFP36L2 also regulates mTORC1 through amino acid signaling. We first measured mTORC1 activity under total amino acid starvation and observed higher p-P70S6K^{T389} levels in *Zfp36l2*-KD cells (Figure 5, E and F). Additionally, leucine supplementation resulted in further hyperactivation of mTORC1 (Figure 5, E and F). Consistent with RNA-Seq data showing reduced *Sesn2* mRNA levels (Figure 3B), we observed reduced SESN2 protein levels in cells with ZFP36L2 downregulation (Figure 5, A and C). Additionally, ZFP36L2 is also regulated by amino acid starvation (Supplemental Figure 10D). Together, these findings indicate that loss of *Zfp36l2* regulates mTORC1 through amino acid sensing, in addition to the TSC1/2 pathway.

ZFP36L2 inhibits mTORC1 through a P53-mediated pathway. The results thus far indicate that ZFP36L2 inhibits mTORC1 independent of energy starvation (through AMPK) and growth factor signaling (through AKT) but involves the negative regulator of the TSC complex REDD1 and the leucine sensor SESN2. Because the levels of both REDD1 and SESN2 are reduced in cells with *Zfp36l2* KD, the regulation of these transcripts by ZFP36L2 is likely indirect. Previous studies implicated both SESN2 and REDD1 as downstream mediators of cellular stress response pathways (33, 34). To determine the protein(s) that mediate the ZFP36L2 signal to SESN2 and REDD1, we next studied the physiological consequences of this regulation and whether ZFP36L2 modulation alters cellular response to stress. Cells treated with *Zfp36l2* siRNA displayed increased cellular ROS and injury in response to H₂O₂ treatment (Supplemental Figure 11, A–C). Similar results were observed in cells treated with etoposide, which primarily induced DNA double-strand breaks (Supplemental Figure 11D). The increase in cell death with *Zfp36l2* KD was not due to higher levels of DNA damage, as the levels of phosphorylated histone H2AX, a marker of DNA strand breaks, were similar between control siRNA- and *Zfp36l2* siRNA-treated cells after doxorubicin (DOX) treatment (Supplemental Figure 12A).

The tumor suppressor protein P53, a major integrator of cellular stress signals, is induced following DNA damage as well as ROS insults, it is a key determinant of the balance between cellular repair versus cell death (35). Additionally, P53 is reported to

regulate both REDD1 and SESN2 expression (36–38). Thus, we next examined whether P53 plays a role in ZFP36L2 regulation of SESN2 and REDD1. In *Zfp36l2*-KD cells, we observed reduced P53 protein levels at baseline (Figure 5, A and D), which persisted after etoposide, H₂O₂, or DOX treatment (Supplemental Figure 12, B–D).

We next determined whether changes in P53 levels are responsible for altered SESN2 and REDD1 expression. We measured the mRNA levels of these 2 proteins after treating H9c2 cells with increasing concentrations of nutlin3 (a chemical that inhibits the interaction between MDM2 and P53, preventing degradation of P53). Nutlin3 increased *Redd1* and *Sesn2* mRNA levels in a dose-dependent manner (Figure 6A). Additionally, nutlin3 reversed the reduction in *Redd1* and *Sesn2* mRNA in response to *Zfp36l2* KD (Figure 6B). Nutlin3 also reversed the reduction in REDD1 and SESN2 protein levels and the increase in P70S6K phosphorylation in response to *Zfp36l2* KD (Figure 6, C and D), indicating that P53 acts upstream of REDD1, SESN2, and, eventually, mTORC1. Furthermore, P53-KO mouse embryonic fibroblasts (MEFs) displayed no change in the mRNA and protein levels of REDD1 and SESN2 following KD of *Zfp36l2*, and KD of *Zfp36l2* in P53 KO MEFs had no effect on mTORC1 activity (Supplemental Figure 13, A and B). Finally, we confirmed our findings by demonstrating that, in cardiomyocytes isolated from mice with cardiac deletion of *Zfp36l2*, protein levels of REDD1, SESN2, and P53 were decreased, while mTOR activity was increased (Figure 6, E and F), validating the RNA-Seq data in H9c2 cells (Figure 3). These results collectively indicate that P53 regulates ZFP36L2-mediated inhibition of mTORC1 through REDD1 and SESN2.

ZFP36L2 binds to and degrades the mRNA for Mdm2. Although P53 protein levels were reduced (Figure 5, A and D), P53 mRNA levels did not change in response to *Zfp36l2* downregulation (Figure 7A). These findings are consistent with ZFP36L2 regulating P53 at the posttranslational level. Therefore, we measured protein stability of P53 by treating cells with cycloheximide to halt new protein synthesis. P53 protein decayed more rapidly in *Zfp36l2*-KD cells compared with that in control cells (Figure 7, B and C), indicating that P53 protein stability is reduced with *Zfp36l2* KD. This mechanism is not unique to proliferating cells, as terminally differentiated neonatal rat cardiomyocytes (NRCMs) demonstrated similar destabilization of P53 with *Zfp36l2* downregulation (Figure 7B). MDM2 is an E3 protein-ubiquitin ligase that leads to P53 degradation (39). We found that *Mdm2* mRNA has a number of consensus ARE sequences in its 3'-UTR, suggesting that ZFP36L2 may bind to its mRNA (Figure 7D). Steady-state *Mdm2* mRNA levels and stability were increased with *Zfp36l2* KD (Figure 7, E and F), and its protein levels were also increased with *Zfp36l2* KD (Figure 7G). Additionally, *Mdm2* 3'-UTR coimmunoprecipitated with ZFP36L2 protein (Figure 7H), indicating that ZFP36L2 binds to and degrades *Mdm2* mRNA. Finally, P53 protein ubiquitination is increased with *Zfp36l2* KD (Figure 7I), consistent with increased MDM2 protein levels. Thus, ZFP36L2 controls P53 protein levels by regulating the mRNA stability of E3 protein-ubiquitin ligase MDM2.

mTORC1 is activated in murine pregnancy, and rapamycin treatment reverses the cardiomyopathy associated with Zfp36l2 deletion. Given that our data demonstrated a role of ZFP36L2 in pregnancy-mediated cardiac pathology and that mTORC1 is regulated in this pathway, we next conducted 2 sets of in vivo experiments to deter-

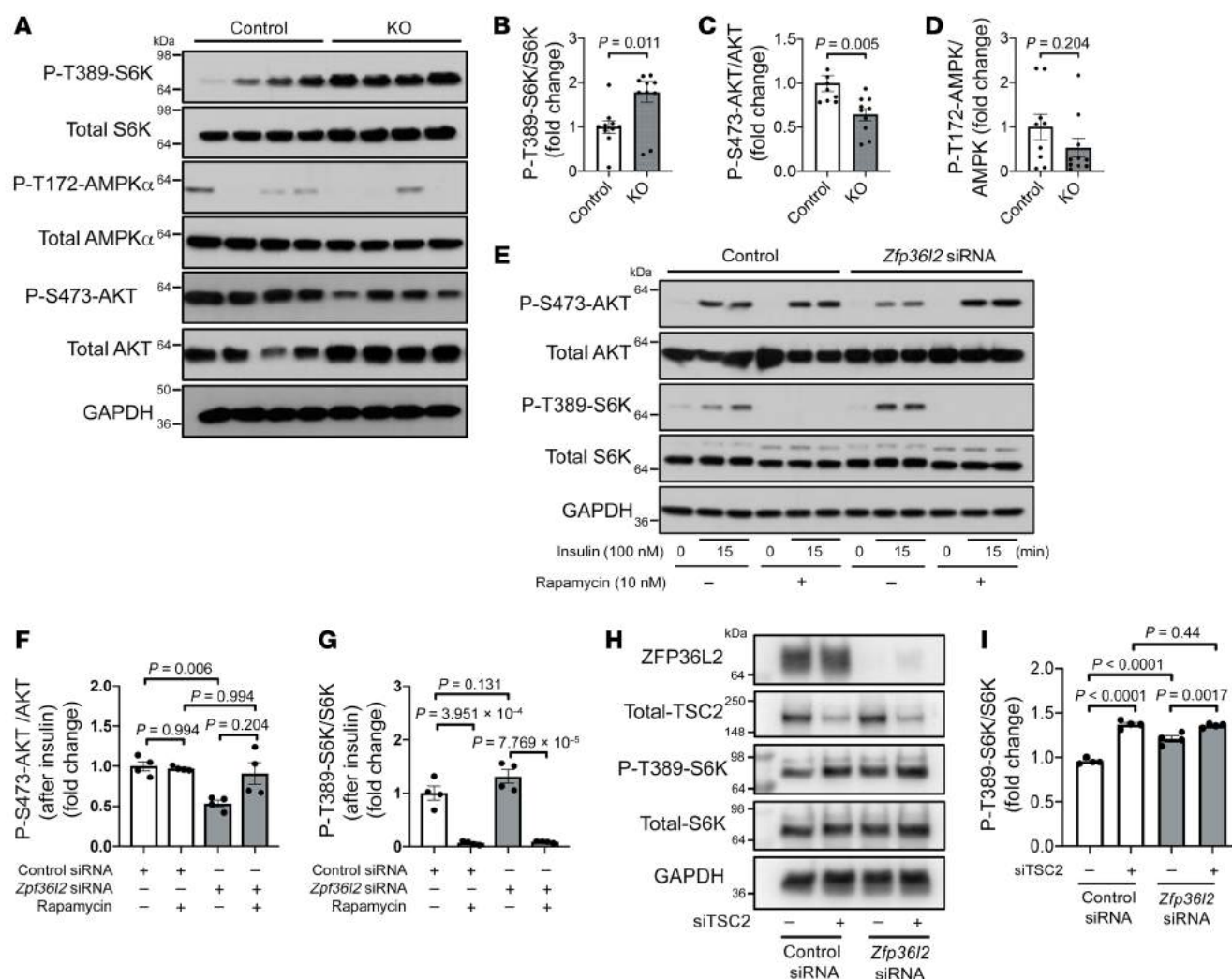


Figure 4. *Zfp36l2* deletion in the heart causes activation of the mTORC1 pathway. (A) Representative Western blot of heart extracts from control and *Zfp36l2*-KO mice, demonstrating increased mTOR activity with *Zfp36l2* deletion. (B–D) Summary of densitometry of Western blot analyses in (A), (B) demonstrating increased p-P70S6K^{T389} ($n = 10$ –11), (C) decreased p-AKT^{S473} ($n = 9$ –10), and (D) no change in p-AMPK^{T172} ($n = 9$ –10). (E) Representative Western blot of H9c2 cells treated with control or *Zfp36l2* siRNA in the presence and absence of insulin and 10 nM rapamycin. (F and G) Summary of densitometry of Western blot analysis in (E) ($n = 4$). (H) Representative Western blot of H9c2 cells treated with control or *Tsc2* siRNA with or without concurrent *Zfp36l2* KD. (I) Summary of densitometry of Western blot for p-P70S6K^{T389} in (H) ($n = 4$). Data were analyzed by unpaired Student's *t* test (B–D) or 2-way ANOVA with Tukey's test for multiple group comparison (F, G, and I).

mine (a) whether ZFP36L2 protein levels and mTORC1 activity are changed in pregnancy and (b) whether inhibition of mTOR can reverse the PPCM associated with *Zfp36l2* deletion. For the first set of experiments, we measured ZFP36L2 protein and mTOR activity in WT and *Zfp36l2*-KO mice in the third trimester of pregnancy and 1 and 4 weeks after pregnancy. Our results demonstrated that mTORC1 activity increased in the third trimester and continued to be elevated after 1 week of delivery, but it returned to normal 1 month after pregnancy (Figure 8, A and B). These results are consistent with those of previous reports that have shown mTOR activation during pregnancy (40). However, mTORC1 activity was substantially higher in *Zfp36l2*-KO mice during pregnancy and remained elevated after delivery (Figure 8, A and B). Cardiomyocytes from *Zfp36l2*-KO mice displayed larger CSA 1 week after pregnancy (Figure 2G). Additionally, ZFP36L2 levels

did not appear to change during pregnancy (Supplemental Figure 14). These results indicate that baseline expression of ZFP36L2 is necessary to keep the mTORC1 activation in check during pregnancy in mice.

ZFP36L2 binds to a number of mRNA molecules and thus alters the activity of a number of pathways. To determine whether the postpartum cardiomyopathy associated with *Zfp36l2* deletion is due to its specific effects on the mTORC1 pathway and its hyperactivation, we treated female pregnant mice with 1 mg/kg subcutaneous rapamycin every other day in the third trimester of the first pregnancy (Supplemental Figure 15A). We initiated the treatment at the third trimester (E12.5) because inhibition of mTORC1 prior to the third trimester detrimentally affects fetal development (41). As expected, rapamycin injection did not alter fetal development, as indicated by a similar gain in body weight in mice

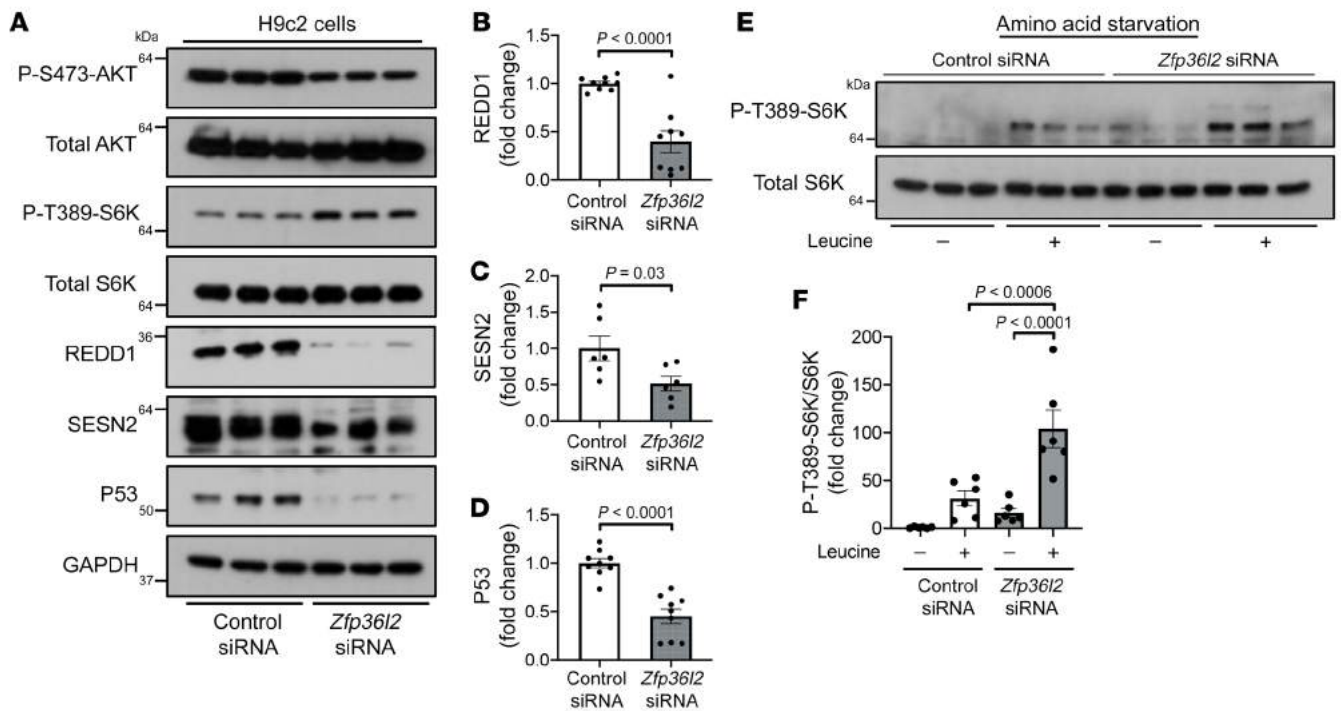


Figure 5. mTORc1 regulation by ZFP36L2 is through amino acid sensing and SESN2 and REDD1. (A) Representative Western blot of H9c2 cells treated with control or *Zfp36l2* siRNA and probed with p-AKT^{S473}, p-P70S6K^{T389}, SESN2, REDD1, and P53. (B–D) Summary of densitometry of Western blots in A, demonstrating decreased (B) REDD1 ($n = 9$), (C) SESN2 ($n = 9$), and (D) P53 ($n = 9$). (E) Representative Western blot and (F) summary of densitometry of p-P70S6K^{T389} ($n = 6$) in H9c2 cells with *Zfp36l2* KD under amino acid starvation and leucine supplementation. Data were analyzed by unpaired Student's *t* test (B–D) or 2-way ANOVA with Tukey's test for multiple group comparison (F).

receiving vehicle versus rapamycin (Supplemental Figure 15B). Hearts of pregnant female mice with *Zfp36l2* deletion sacrificed at E18–E19 of the first pregnancy displayed increased S6^{S240/244} phosphorylation (Figure 8C), while rapamycin treatment reduced mTORc1 activity (Figure 8D). Additionally, rapamycin significantly preserved LV systolic function in *Zfp36l2*-KO mice, as indicated by an increase in EF after the first pregnancy (Figure 8, E and F). The hearts of *Zfp36l2*-KO mice also displayed reduced LVDd and LVDs (Supplemental Figure 16, A–E). Finally, gross and histological examination of the hearts of pregnant mice with *Zfp36l2* deletion treated with rapamycin displayed reduced size, heart weight/tibial length, and markers of heart failure (*Anf* and *Bnp*) compared with mice given vehicle control (Figure 8, G–J). Rapamycin treatment also led to reduced cardiomyocyte CSA without altering cardiac fibrosis (Supplemental Figure 17). The effects of rapamycin were specific to pregnant *Zfp36l2*-KO mice, because nonpregnant *Zfp36l2*-KO mice and control pregnant mice did not show any change in their cardiac function after treatment with rapamycin (Supplemental Figure 18). These results indicate that increased mTORc1 activity can lead to the development of PPCM, and rapamycin treatment can be a potential therapy for this disease.

To determine whether changes in the components of the ZFP36L2/P53/REDD1/SESN2/mTOR pathway are specific to PPCM, we assessed the levels of these proteins in other forms of cardiomyopathy. Our results demonstrated that the components of this pathway were not altered in ischemic cardiomyopathy that occurred after myocardial infarction (Supplemental Figure 19) and pressure overload by TAC (Supplemental Figure 20). Final-

ly, we subjected *Zfp36l2*-KO mice and their littermate controls to TAC and assessed their cardiac function. There was no difference in cardiac function after TAC between control and *Zfp36l2*-KO mice (Supplemental Figure 21). Collectively, these results indicate that the effects of *Zfp36l2* deletion on cardiac function are specific to PPCM and not other forms of cardiomyopathy.

We next sequenced the coding region of the *ZFP36L2* gene in patients with PPCM to identify any potential mutations that might contribute to this disease in humans. It is important to note that this approach does not identify mutations within introns or the regulatory portion of the *ZFP36L2* gene. Although we identified some variants in *ZFP36L2* in several patients (Supplemental Table 2), these variations are not rare or predicted to be pathological, as determined on the gnomAD website (<https://gnomad.broadinstitute.org/>). Thus, we concluded that mutations within the coding region of the *ZFP36L2* gene are unlikely to cause PPCM.

We next obtained cardiac tissue samples from control participants and patients with PPCM to assess whether there was a change in the levels of ZFP36L2 protein in PPCM. Characteristics of patients from whom these samples were obtained are included in Supplemental Table 3. PPCM cardiac tissues displayed lower ZFP36L2 protein levels, and increased mTORc1 activity, as evidenced by increased p-P70S6K^{T389} (Figure 8K). We also measured the levels of ZFP36L2, P53, REDD1, and SESN2 proteins in hearts from patients with PPCM and other forms of cardiomyopathy and showed that the levels of these proteins were decreased only in PPCM samples (Figure 8K). These results suggest that alterations in the ZFP36L2/P53/REDD1/SESN2 pathway and

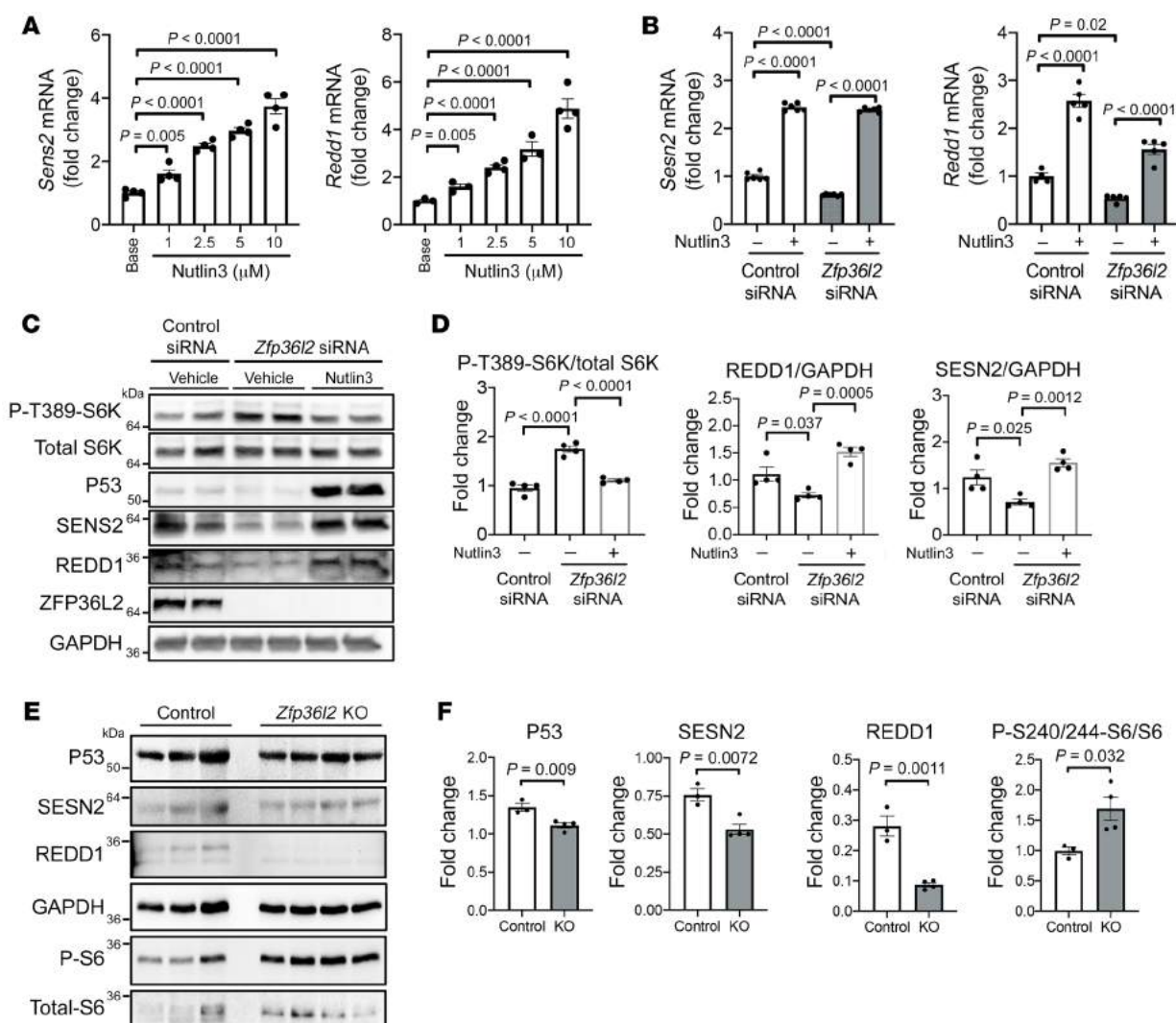


Figure 6. SESN2 and REDD1 regulation by ZFP36L2 is P53 dependent. (A) mRNA levels of *Sesn2* and *Redd1* after treating H9c2 cells with increasing concentrations of nutlin3, illustrating that nutlin3 increases both *Sesn2* and *Redd1* mRNA levels in a dose-dependent manner ($n = 3-4$). (B) *Sesn2* and *Redd1* mRNA levels in response to *Zfp36l2* KD and after treatment with nutlin3 ($n = 4-6$). (C) Representative Western blot of H9c2 cells treated with *Zfp36l2* siRNA in the presence and absence of nutlin3. (D) Summary of densitometry of Western blot analysis in C ($n = 4$), demonstrating that nutlin3 reverses the effects of *Zfp36l2* KD on the protein levels of P53, SESN2, and REDD1. (E) Western blot of P53, SESN2, REDD1, p-S6^{S240/244}, and total S6 in cardiomyocytes isolated from mice with cardiac deletion of *Zfp36l2* and (F) summary of densitometry ($n = 3-4$). Data were analyzed by unpaired Student's *t* test (A and F), 2-way ANOVA with Tukey's test for multiple group comparison (B), or 1-way ANOVA with Tukey's test for multiple group comparison (D).

mTORC1 activation are also potentially involved in the pathogenesis of PPCM in humans.

Discussion

mTORC1 is regulated through protein-protein interactions to exert rapid metabolic effects in response to various stimuli, including amino acid deprivation and growth factor signaling. Despite extensive work on the regulation of mTORC1, it has never been shown to our knowledge that the mTORC1 pathway can be regulated through mRNA stability by an mRNA binding protein. We discovered a potentially novel pathway that regulates mTORC1 activity through modulation of *Mdm2* mRNA stability by ZFP36L2, resulting in altered P53 levels and subsequent REDD1 and SESN2 expression. Our findings imply the existence of another layer of mTORC1 regulation through mRNA stability of its upstream reg-

ulators in addition to generally accepted protein level regulation. Additionally, we demonstrate that, in mice, moderate modulation of mTORC1 activity determines the switch between physiological and pathophysiological cardiac hypertrophy during pregnancy, a state where proanabolic signals converge on mTORC1. This potentially novel mTORC1 regulation pathway is likely enlisted in situations that require rheostat-like regulation of mTORC1 activity and halting of unchecked activation of mTORC1.

Pregnancy is associated with a number of hemodynamic changes to ensure adequate blood supply to the mother and to the fetus for proper fetal growth and development. One prominent change during pregnancy is the growth of the LV wall and mass, which return to prepregnancy levels after delivery. Although these changes have been demonstrated in mice and humans, the molecular basis of this phenomenon is not clear. Our data demon-

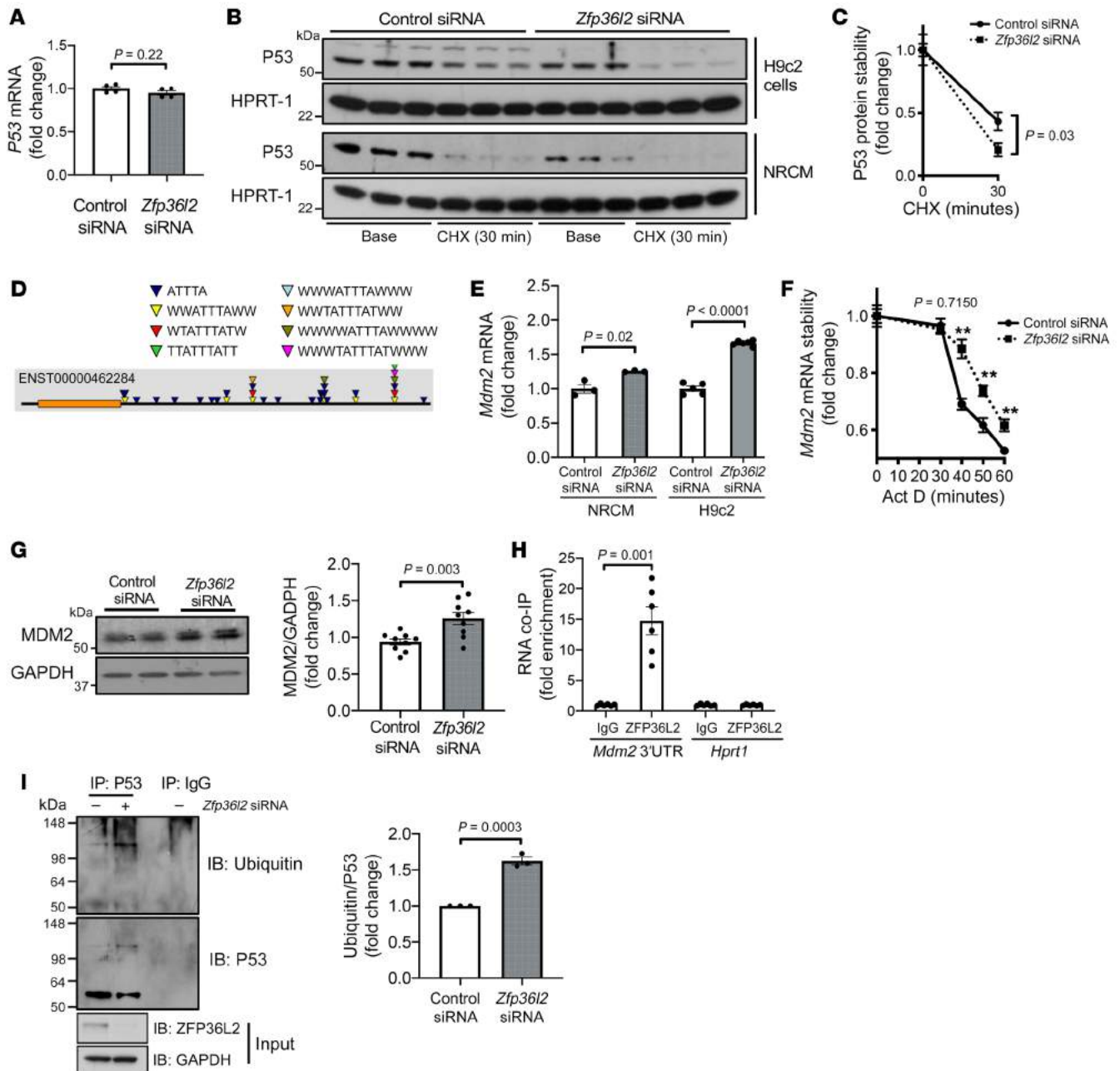


Figure 7. ZFP36L2 regulates P53 levels through its binding to and degradation of the *Mdm2* mRNA. (A) P53 mRNA levels in H9c2 cells treated with control or Zfp36l2 siRNA ($n = 4$). (B) Representative Western blot of P53 protein levels in H9c2 cells and neonatal rat cardiomyocytes (NRCMs) treated with control or Zfp36l2 siRNA 30 minutes after cycloheximide (CHX) treatment ($n = 3$). (C) Time course of P53 protein stability in H9c2 cells treated with control or Zfp36l2 siRNA after treatment with CHX ($n = 7$). (D) In silico analysis of the 3'-UTR of *Mdm2* mRNA demonstrated multiple consensus ARE sequences in its 3'-UTR. (E) Steady-state mRNA levels of *Mdm2* in H9c2 cells ($n = 5-6$) and NRCMs ($n = 3$) treated with control and Zfp36l2 siRNA. (F) Time course of *Mdm2* mRNA stability in H9c2 cells treated with control or Zfp36l2 siRNA and actinomycin D ($n = 8$). $^{**}P < 0.01$. (G) MDM2 protein levels in H9c2 cells treated with control and Zfp36l2 siRNA ($n = 9$). (H) RNA co-IP of *Mdm2* 3'-UTR with ZFP36L2 protein ($n = 6$). *Hprt1* was used as a negative control. (I) Assessment of P53 protein ubiquitination in H9c2 cells with control or Zfp36l2 siRNA ($n = 3$). Data were analyzed by unpaired Student's *t* test.

strate a central role for mTORc1 and P53 in this process and that the uncontrolled growth of the heart is halted by the regulation of mTORc1 activation by ZFP36L2. We also show that inadequate control of this pathway, as a result of lower expression of ZFP36L2 protein, can lead to PPCM in mice, which is partially rescued by rapamycin treatment. Because constitutive activation of mTORc1 leads to cardiomyopathy, it is not surprising that Zfp36l2 deletion

leads to a similar phenotype in pregnancy. However, this pathway plays a specific role in pregnancy because deletion of Zfp36l2 does not exacerbate heart failure induced by TAC in mice.

Prosurvival signaling pathways are also involved in the mechanism of cardiac hypertrophy during pregnancy. Chung et al. showed that AKT and ERK1/2 are activated in the hearts of pregnant mice, while p38 is decreased (40). Additionally, mice expressing a cardi-

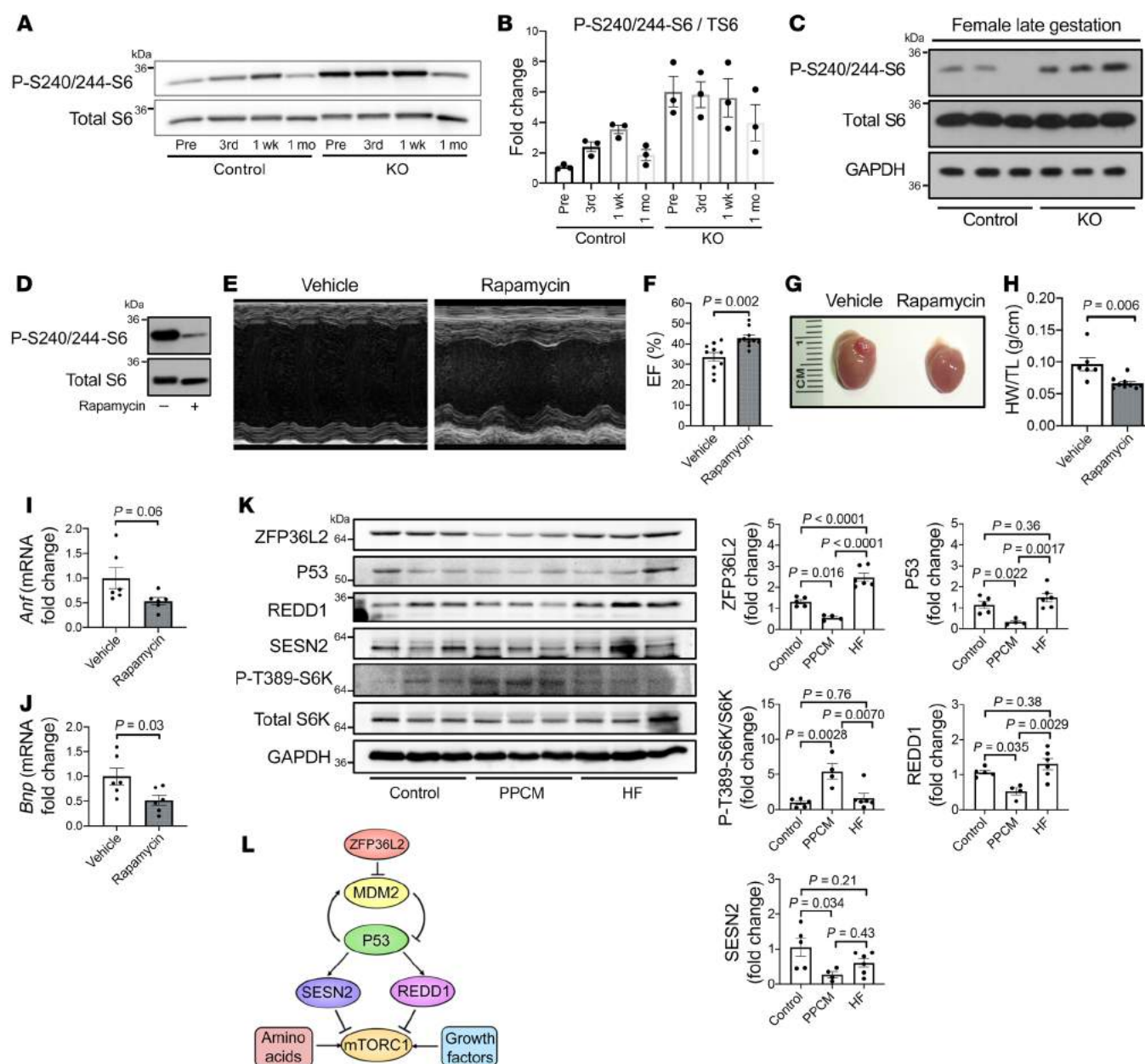


Figure 8. Rapamycin reverses the cardiac dysfunction that occurs in *Zfp3612*-KO mice. (A) Representative Western blot of p-S^{240/244} and total S6 in WT and *Zfp3612*-KO hearts in nonpregnant mice (Pre) as well as pregnant mice during the third trimester of the first pregnancy (3rd) and 1 week (1 wk) and 1 month (1 mo) after delivery. (B) Summary of densitometry of Western blot analysis in A ($n = 3$). (C) Western blot of p-S^{240/244} in the hearts of *Zfp3612*-KO and control mice in the late gestation phase, E18–E19 ($n = 3$). (D) Effective reduction of mTOR activity in the hearts of mice treated with rapamycin, as assessed by measurement of p-S^{240/244}. (E) 2D echo image and (F) summary bar graph of EF in *Zfp3612*-KO mice after treatment with rapamycin or vehicle according to the protocol in Supplemental Figure 15A ($n = 10$). (G) Gross examination of the hearts of *Zfp3612*-KO pregnant mice treated with a vehicle or rapamycin. (H) Heart weight to tibial length (HW/TL) ratio ($n = 6–9$) and (I and J) markers of heart failure (*Anf* and *BNP*) ($n = 6$) in *Zfp3612*-KO pregnant mice treated with a vehicle or rapamycin. (K) Protein levels of the components of the ZFP36L2/P53/REDD1/SESN2/mTOR pathway in patients with PPCM and other forms of heart failure (HF). (L) Graphic representation of the pathways through which ZFP36L2 regulates mTORC1 activity. Data were analyzed by unpaired Student's *t* test (F and H–J) or 1-way ANOVA with Tukey's test for multiple group comparison (K).

ac-specific myristoylated form of AKT (activated AKT) or constitutively active glycogen synthase kinase 3 β displayed an attenuated pregnancy-induced cardiac hypertrophy. They also demonstrated that progesterone increases cardiomyocyte cell size through ERK1/2 phosphorylation, and inhibition of this pathway blocked progesterone-induced hypertrophy. Because ERK1/2 and AKT can both function upstream of mTORC1, we propose that, during preg-

nancy, progesterone causes an increase in mTORC1 activation and cardiomyocyte hypertrophy. However, ZFP36L2 also works in parallel to keep this pathway under control and to impede unchecked activation of this process. Disruption of this regulation of mTORC1 contributes to uncontrolled cardiac growth during pregnancy and PPCM. In support of this model, we provide data showing that patients with PPCM have lower levels of ZFP36L2 protein.

A link between P53 and mTORC1 activity has been previously demonstrated. Three studies suggest that mTORC1 inhibition by genotoxic stress is dependent on P53 (36, 38, 42). A number of studies also implicated a role for P53 in mTORC1 inhibition by energy depletion (43–46); however, two other studies refuted a role for P53 in this process (47, 48). Two studies have implicated that P53 has protective effects under nonstress conditions (49, 50). Our results demonstrate that P53 plays an important role in regulating baseline mTORC1 activity through REDD1 and SESN2. Additionally, the role of P53 in cardiac physiology during pregnancy and the etiology of PPCM has not been described. Surprisingly, a significant association between PPCM and the risk of cancer was described in a cohort of 236 patients, and mutations in genes involved in DNA repair were identified in a subset of these patients (51). We show that P53 regulation of mTOR and cardiac growth during pregnancy has disease implications, as disruption of the pathway leads to mild mTORC1 hyperactivation and PPCM in mice.

We noted a reduction in ZFP36L2 protein levels and the corresponding increase in mTOR activity in the hearts of patients with PPCM (Figure 8K). Because the pathway involving ZFP36L2 regulation of mTOR activation is critical for normal cardiac growth during pregnancy and its disruption leads to PPCM in mice, alterations in ZFP36L2 levels and hyperactivation of mTORC1 potentially form part of the basis of PPCM in humans. We posit that these disruptions are triggered by the process of pregnancy. In our mouse model, the *Zfp36l2* gene is ablated from birth in mature cardiomyocytes, resulting in mTOR activation even in the absence of pregnancy, which is markedly exacerbated with the cardiac growth that occurs during pregnancy.

Our studies have not identified potential pathological mutations within the *ZFP36L2* gene in 37 patients with PPCM, suggesting that whole-body alterations of ZFP36L2 function may be pleiotropic in humans and thus evolutionarily disadvantageous. Complete deletion of the gene in mice leads to defective hematopoiesis and death within the first 2 weeks after birth (9). Two other murine models of PPCM, involving either STAT3 or PGC1 α deletion in the heart (7, 52), also lack human genetic correlates to date, likely for the same reasons.

The ZFP36L2/mTOR pathway is implicated as a critical process in the development of PPCM because we were able to reduce cardiac dysfunction with rapamycin in mice. We acknowledge that PPCM is a complex and likely multifactorial disease, involving multiple pathway and endocrine processes, and that our studies do not exclude other mechanisms, such as angiogenic imbalance, which have been proposed in other mouse models (6, 7). Ultimately, these pathways may synergize as part of a multifactorial etiology for PPCM. Thus, future work combining these models will test if there is a synergistic effect on cardiac function during pregnancy to establish a model that better recapitulates human PPCM. In addition, although the results of human samples suggest the involvement of the ZFP36L2/mTOR pathway in PPCM, further studies are needed to validate the extent to which this pathway is involved in the pathogenesis of PPCM in humans with larger cohorts.

In summary, our results demonstrate the existence of a pathway that regulates mTORC1 activity through an mRNA binding protein. This pathway alters both SESN2 and REDD1 through

P53 (Figure 8L). Additionally, disruption of this pathway leads to cardiac damage during the stress of pregnancy in mice, implying that inhibition of ZFP36L2-mediated regulation of mTORC1 causes context-specific effects on proanabolic processes. Our studies provide a model for understanding PPCM, and further studies are needed to validate our proposed mechanism in humans.

Methods

Cell culture and reagents. HEK293T cells, MEFs, and H9c2 cells were grown in Dulbecco's Modified Eagle's Medium (Corning) supplemented with 10% FBS (Atlanta Biologicals). HEK293T and H9c2 cells were purchased from ATCC, and P53 KO MEFs were a gift from Nissim Hay (University of Illinois, Chicago, Illinois, USA). NRCMs were isolated as described previously (53). Mouse adult cardiomyocytes were isolated as previously reported (54) with a slight modification: our collagenase buffer contained 1.5 mg/ml collagenase 2 (Worthington) and 40 μ M CaCl₂. All cells were maintained in a 37°C incubator with 5% CO₂ and were 70%–90% confluent when collected for analyses unless otherwise noted. To activate PI3K/Akt signaling, cells were first deprived of serum for 3 hours and then were treated with 100 nM insulin for 15 or 30 minutes. In another series of experiments, cells were pretreated with 10 nM rapamycin for 24 hours before serum deprivation. To evaluate the response of mTORC1 activity against the amino acid leucine, cells were first deprived of amino acid for 50 minutes in modified HBSS containing 25 mM glucose and 1.25 mM pyruvate and were then treated with 800 μ M L-leucine (MilliporeSigma) for 10 minutes. Nutlin3 (MilliporeSigma), an inhibitor of the interaction between P53 and MDM2, was used to activate P53 at a dose of 1–10 μ M for 2 to 6 hours depending on the experiment. To induce DNA damage, cells were treated with 200 μ M H₂O₂, 20 μ M etoposide, or 0.25 μ M DOX for indicated duration.

Mouse studies, survival studies after pregnancy, and rescue experiments with rapamycin. Mice were housed in the barrier facility at Northwestern University on a 12-hour-light/dark cycle. Generation of *Zfp36l2*-floxed (*Zfp36l2*^{fl/fl}) mice has been described previously (27). *Zfp36l2*^{fl/fl} mice were crossed with α MHC-Cre mice to generate *Zfp36l2*^{fl/ α} ; α MHC-Cre mice, which were then mated with *Zfp36l2*^{fl/fl} mice to generate cardiac-specific *Zfp36l2*-KO mice and floxed control littermates for experiments. α MHC-Cre mice and their nontransgenic littermates were also included as controls. Female mice were bred starting at 8 weeks of age. Pregnant KO mice were treated with rapamycin (1 mg/kg) or vehicle by subcutaneous injections every other day from day 12 of pregnancy until delivery. Nonpregnant mice were treated with the same amount of rapamycin or vehicle by subcutaneous injections every other day for 9 days starting from 9 weeks of age. Stock solution of rapamycin (20 mg/mL in ethanol) was suspended in 5% Tween 80, 5% PEG 400 dissolved in PBS for each injection.

Coronary ligation and TAC. The surgical protocol was performed as previously described (55). Briefly, mice were anesthetized with isoflurane, with induction at 3% and maintenance at 1.5%–2%. The animals were placed in a supine position, and ECG leads were attached. Body temperature was monitored using a rectal probe and was maintained at 37°C with heating pads throughout the experiment. A catheter was inserted into the trachea and was then attached to the mouse ventilator via a Y-shaped connector. The mice were ventilated at a tidal volume of 200 μ L and a rate of 105 breaths/min using a rodent ventilator. The chest was then opened by an incision in the left fourth inter-

costal space. The left anterior descending artery was occluded with an 8-0 silk suture. Ischemia was confirmed by pallor of the anterior wall of the left ventricle and by ST-segment elevation and QRS widening on the ECG. After confirmation of ligation, the chest was closed in layers. The mice were kept warm with heating pads and on 100% oxygen via nasal cannula. Animals were given buprenorphine for postoperative pain. Chronic pressure overload was induced by TAC in mice at 10–12 weeks as described previously (56).

In vitro [³⁵S]-methionine incorporation assay. Cells were first deprived of amino acid for 50 minutes in HBSS and then incubated with complete medium containing [³⁵S]-methionine (0.01 mCi/mL; Perkin-Elmer). After 1 hour, cells were lysed with RIPA buffer, and protein concentration was determined. Proteins were precipitated with trichloroacetic acid (10%) at 4°C overnight and washed with acetone, and radioactivity was determined by liquid scintillation counting. Radioactive levels were normalized to protein concentration.

Reverse transcription and quantitative real-time PCR. RNA was isolated from cells or heart samples using RNA-STAT60 (Tel-Test) and reverse transcribed with TaqMan Reverse Transcription Reagents (Invitrogen) according to the manufacturers' instructions. Quantification of relative gene expression was done using Fast SYBR Green Master Mix (Applied Biosystems) and run on 7500 Fast Real-Time PCR system (Applied Biosystems). The relative gene expression was determined using differences in Ct values between the gene of interest and housekeeping control genes, which were *Actb*, *Hprt1*, and/or *18S*, depending on the experiment.

mRNA stability assay and RNA co-IP. Cells were treated with 7.5 μM actinomycin D (MilliporeSigma) for indicated times, and RNA was harvested and processed as described above. RNA Co-IP experiments were conducted as described previously (57).

Transfection. siRNAs were purchased from Dharmacon. siRNAs were transfected using Dharmafect 1 Transfection Reagent (Dharmacon). Plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen), Lipofectamine 3000 (Invitrogen), or calcium phosphate transfection. Experiments were performed 24–48 hours after transfection. LAMP1-mGFP, which was used to express human LAMP1, was a gift from Esteban Dell'Angelica (Addgene plasmid 34831).

Western blotting. Proteins were resolved on 4%–12% Novex Bis-Tris poly-acrylamide gel (Invitrogen) and blotted onto nitrocellulose membrane (Invitrogen). Membranes were incubated with the following primary antibodies overnight, before the addition of HRP-conjugated secondary antibodies. P-P70S6K (T389), P70S6K, P-S6 (S240/244), S6, P-AKT (S473), AKT, P-AMPK (T172), AMPK, TSC2, P53 (1C12), Ubiquitin, P-H2AX, and GAPDH were from Cell Signaling Technology. HPRT-1, REDD1, SESN2, and MDM2 were from Proteintech. ZFP36L2 was obtained from Abcam, MilliporeSigma, and Santa Cruz Biotechnology. The presence of target protein was visualized using Super Surgical Western Pico ECL substrate (Pierce). Quantification of Western blotting images was done using ImageJ (NIH). Antibody suppliers and catalog numbers are shown in Supplemental Table 4.

P53 immunoprecipitation. After pretreatment with 20 μM MG132 for 5 hours, cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.5% Triton-X, and 10 mM N-ethylmaleimide. Precleared cell lysates (500 μg) were incubated with anti-P53 antibody or control IgG in lysis buffer at 4°C overnight with rotation. Antibody-protein complexes were collected with Protein G-Agarose beads (MilliporeSigma) and washed with ice-cold immunoprecipitation buffer according to the manufacturer's instructions. Immunoprecipitates were subjected to Western blotting to probe ubiquitination of P53.

Protein stability assay. Endogenous P53 protein degradation was analyzed by a protein stability assay. Briefly, H9c2 cells or NRCMs transfected with *Zfp36l2* siRNA for 48 hours were incubated with 50 μg/ml cycloheximide (MilliporeSigma) for 30 minutes. P53 protein levels were analyzed by Western blotting. The results from Western blot analysis were quantified by densitometry and normalized against the 0-minute time point.

Cell death analysis. Cells were stained with 5 μg/ml PI and in HBSS (Life Technologies) and Hoechst 33342 (Invitrogen) for nuclei counter stain. Images were acquired on a Zeiss AxioObserver.Z1 microscope, and cell death was defined as the number of PI-positive nuclei over total number of nuclei.

ROS measurements. Intracellular ROS levels were monitored by dichlorofluorescein (DCF) fluorescence (Invitrogen). After pretreatment with vehicle or 600 μM H₂O₂ for 90 minutes, cells were incubated in HBSS containing 1 μM DCF for 10 minutes according to the manufacturer's protocol. Cells were washed and stained with Hoechst 33342 for nuclei counter stain. DCF fluorescence was recorded by a fluorescent microscope.

RNA-Seq experiments. H9c2 cells were cultured as above and then transfected with either control or *Zfp36l2* siRNA (a mixture of 4 siRNAs for *Zfp36l2*, the target sequences of which are GCAAGUACG-GCGAGAAGUG, CAAAUCAACUCCACGCGCU, UGUCAGCU-UUCUACGAUUAU, and GCGCUGGUCAACAAGGAAA) using Dharmafect 1 Transfection Reagent (Dharmacon). The media were replaced after 24 hours, and then the cells were harvested 48 hours after transfection. Total RNA was extracted using NucleoSpin (Takara Bio) per the manufacturer's instructions. RNA quality was tested using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Six RNA samples each of control and *Zfp36l2* KD cells were submitted to the NU-Seq Core facility for library preparation and sequencing. Libraries were prepared using the TruSeq mRNA-Seq Library Prep Kit (Illumina) per the manufacturer's instructions, with each sample producing 50-bp single-end reads and given 1 index sequence. All samples were sequenced using 1 lane on a HiSeq 4000 (Illumina) and processed to FASTQ files using standard base-calling software and parameters.

RNA-Seq data analysis. The raw FASTQ files were used as input for kallisto (version 0.44.0) (58) to quantify transcript-level expression using the flags “-bias -b100.” This analysis used the annotations for the *Rattus norvegicus* transcriptome from Ensembl version 93 (July 2018). Next, the estimated expressions were used as input for *sleuth-CN* (<https://github.com/warrenmcm/sleuth-CN>, master branch, commit 461b95b), an extension of the *sleuth* package (59) that uses compositional data analysis and allows for the choice of “reference features” for normalization, similar to qPCR (60). When there is an expectation of a large number of features changing, such as in this study, this approach provides a more accurate interpretation of which features are changing and in what direction (60). The expression values were normalized to *Actb* and *Hprt1*, which have been used as reference genes or expression markers in previous human and rodent cardiomyocyte studies (61, 62). The transcripts per million were modeled for *sleuth*'s differential analysis using default settings, and the Wald statistic was used to test for differential expression at the transcript level, looking for differences between the *Zfp36l2*-KD and control groups. In this data set, absolute β values for the selected reference genes were

less than 0.1, and their variance (“sigma squared”) was less than 0.1, indicating that their normalized expression values were relatively consistent across samples and with each other.

Then, from the transcript-level differential analysis, we produced a gene-level differential analysis using the *P* value aggregation method developed by Yi et al. (63). This method does not attempt to estimate gene-level expression differences, but rather tests whether any isoform of a particular gene has changed, with a significant *P* value indicating that at least 1 isoform of that change has indeed changed. Genes were considered significantly regulated if the adjusted *P* value after aggregation was equal to or less than 0.05. From these results, we added 2 columns: “number of sig. transcripts,” which showed how many differentially expressed isoforms were present for that gene, as well as “direction of sig. transcripts,” which showed whether the DE isoforms had a concordant change (all “up” or “down”) or discordant (“mixed”) change.

To generate a heatmap, the sleuth-CN-normalized transcript expression values were first transformed to *z* scores (subtract from each expression value the mean expression value across samples and then divide by the standard deviation across samples). The transformed expression values are depicted on a scale from blue (most downregulated among samples) to red (most upregulated among samples), and the samples are annotated by condition (blue for KD samples; gray for control samples). Both the samples (columns) and transcripts (rows) were hierarchically clustered, with the clustering results depicted by the dendrograms. The samples cluster by condition, and the transcripts broadly cluster by upregulated transcripts and downregulated transcripts.

The volcano plots and heatmap plots were created using the standard plotting functions with sleuth; the ggrepel package (<https://slowkow.com/ggrepel>) was used for labeling the volcano plot. All of the sleuth-CN analysis and plotting was performed in R, version 3.5.1, and the pipeline for analyzing the data from start to finish was done using bioconda and snakemake. The full pipeline can be found on GitHub (https://github.com/warrenmccg/Zfp3612_paper_analysis, master branch, commit 4294996).

Pathway analysis. Pathway analysis was performed using the DAVID Bioinformatics Database (v6.8; <https://david.ncifcrf.gov>) with the top 10% of significantly altered transcripts with the greatest absolute log₂ fold change as input.

In silico analysis of AREs. AREs were identified by either of the following two criteria: (a) curated ARE entry in the AREsite Database (64) or (b) presence of AUUUA sequence in the 3'-UTR region of reference mRNA sequences by manual search.

Echocardiography, organ harvest, and histological analysis. Parasternal short- and long-axis views of the heart were obtained using a Vevo 770 high-resolution imaging system with a 30 MHz scan head. 2D and M-mode images were obtained and analyzed. Cardiac function was calculated as previously described (65). At the time of tissue harvest, mice were anesthetized with 250 mg/kg dose of freshly prepared Tribromoethanol (Avertin). Tissue was excised, rinsed in PBS to remove excess blood, and was then freshly frozen in liquid nitrogen and stored at -80°C. For histological analysis, immediately after making a small incision in the inferior vena cava, beating heart was perfused with relaxing buffer (100 mM KCl, 5 mM EGTA, and 5 mM Na pyrophosphate) until termination of beating, followed immediately with 30 ml of 4% paraformaldehyde using the peristaltic pump (7 mL/min) from the apex of left ventricle through a 22-gauge needle. The excised heart tissue was further fixed overnight in 4% paraformaldehyde

before graded dehydration in 70%, 80%, 90%, and 100% ethanol. The tissue sample was further dehydrated with xylene and embedded into paraffin. Sections were stained with hematoxylin and eosin for evaluation of general cardiac morphology and tissue organization. Masson's trichrome staining was used to visualize cardiac fibrosis.

Immunohistochemistry. Cells grown on a cover glass were fixed with 4% paraformaldehyde for 15 minutes. The cells were then washed with PBS, blocked with 5% goat serum and 0.1% Triton X-100 in PBS for 60 minutes, and incubated overnight with anti-phospho-Histone H2A.X antibody (Ser139; Cell Signaling Technology) or anti-TSC2 antibody (Cell Signaling Technology) in blocking buffer. The bound antibodies were labeled with an Alexa Fluor(R) 488 anti-rabbit secondary antibody or an Alexa Fluor(R) 594 anti-rabbit secondary antibody, and nuclei were stained with DAPI. The cover glass was mounted on a slide glass and was visualized using a fluorescent microscopy.

Human samples. All donors and transplant hearts received in situ cold cardioplegia to protect the myocardium. The control participants and patients with PPCM were age, sex, and race matched. Their medications are included in Supplemental Table 3. All donors had LV mass indexes of less than 95 g/m², consistent with MESA criteria (66). Heart weights were well below any pathologic level, as defined in the literature (67, 68). The control donors and donors with PPCM were not statistically different in age, height, weight, BMI, BSA, and the majority of echo parameters, except for LVEF.

Sequencing in the Zfp3612 gene in patients with PPCM. The extracted DNA samples from patients with PPCM were obtained in-house. We performed PCR with the DNA samples using primers flanking the Zfp3612 gene, followed by whole-exon sequencing of Zfp3612 with the Sanger sequencing technique. Variation in the sequence was determined by aligning the sequence of each sample with Zfp3612. Allele frequency in the variants was searched on the GnomAD website (<https://gnomad.broadinstitute.org/>).

Statistics. Data are presented as mean ± SEM. Single comparisons were assessed by an unpaired Student's 2-tailed *t* test. Statistical significance was assessed with ANOVA, with post hoc Tukey's test for multiple group comparison; a 1-way ANOVA was performed when the data set had 1 independent variable, while a 2-way ANOVA was done with 2 independent variables. Log-rank test was used for analysis of survival curves, with Bonferroni's post hoc analyses for multiple group comparison. A *P* value of less than 0.05 was considered statistically significant. The analysis was conducted using GraphPad Prism9 software.

Study approval. All animal studies were approved by the Institutional Animal Care and Use Committee at Northwestern University and were performed in accordance with guidelines from the NIH. Nonfailing and PPCM human heart samples were obtained from the University of Pennsylvania (IRB approval 848421). Failing human hearts (non-PPCM samples) were provided by the Clinical Trials Unit, Bluhm Cardiovascular Institute at Northwestern Memorial Hospital (Chicago, Illinois, USA) (IRB approval IRB STU00012288). Informed consent was obtained from all the transplant patients and from the families of the organ donors before tissue collection. Protocols for tissue procurement were approved by the University of Pennsylvania and Northwestern University, which is AAHRPP accredited.

Author contributions

HK designed, performed, and analyzed Western blotting and immunoprecipitation experiments, qPCR experiments, in vitro

radioactive amino acid incorporation assays, mRNA and protein stability assays, RNA co-IP experiments, cell death and ROS measurement assays, immunocytochemistry and mouse studies; performed cardiomyocyte isolation and RNA-Seq experiments; bred the mouse colonies; and drafted the manuscript. YT performed and analyzed Western blotting experiments, qPCR experiments, immunocytochemistry, and mouse studies. HCC, JSS, and WAM analyzed RNA-Seq experiments and performed pathway analyses. ADJ assisted with in silico analyses of AREs and organ harvest from mice. IBS provided critical expert guidance on the manuscript. CC managed the mouse colonies. ZA, JL, and PJB provided reagents. HK, YT, and HA wrote the manuscript, and HK, YT, JSS, and HA edited the final version. HA conceived and supervised the study and drafted the manuscript. All authors discussed the results and critically reviewed the manuscript.

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COMPENDIUM ON BASIC MODELS OF CARDIOVASCULAR DISEASE

Animal Models of Cardiovascular Complications of Pregnancy

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ABSTRACT: Cardiovascular complications of pregnancy have risen substantially over the past decades, and now account for the majority of pregnancy-induced maternal deaths, as well as having substantial long-term consequences on maternal cardiovascular health. The causes and pathophysiology of these complications remain poorly understood, and therapeutic options are limited. Preclinical models represent a crucial tool for understanding human disease. We review here advances made in preclinical models of cardiovascular complications of pregnancy, including preeclampsia and peripartum cardiomyopathy, with a focus on pathological mechanisms elicited by the models and on relevance to human disease.

Key Words: cardiovascular disease ■ hypertension ■ mortality ■ pregnancy ■ Women

Pregnancy and delivery are a high-risk period in a woman's life. As recently as 1920, 600 of every 100 000 women died in or around childbirth, usually from sepsis, hemorrhage, or hypertension.¹ The development of aseptic technique, antibiotics, and blood transfusions in the 20th century have dramatically decreased maternal mortality from these causes. Today, in the United States, the main causes of maternal mortality are cardiovascular in nature, including cardiomyopathy, preeclampsia, thromboembolic disease, strokes, and others.² Strikingly, maternal mortality in the United States has increased 3-fold in the last 40 years, likely a reflection of advancing maternal age, preexisting morbid conditions including obesity and diabetes, improved management of congenital heart disease allowing these patients to become pregnant, and environmental factors.² The United States is one of only 2 countries in which maternal mortality has increased since 2000. Equally strikingly, there are profound socioeconomic, geographic, and racial inequalities in outcomes. For example, Black women are 3× as likely to die during or after childbirth than White women.³ In addition, there is an emerging recognition that pregnancy complications presage substantial long-term risk of cardiovascular disease. For example, the life-long risk of cardiovascular disease (CVD) in women who have had preeclampsia is as much as tripled, on par with the

risk conferred by smoking or diabetes,⁴ and the American Heart Association now recommends that eliciting a history of preeclampsia be an integral component of routine clinic visits.⁵ Pregnancy-associated maternal disease also frequently affects the fetus or the newborn, both short and long-term. Therapeutic options for treating or preventing cardiovascular complications of pregnancy remain limited, with no existing disease-specific therapies for most of them. There is thus an urgent need to better understand the causes and pathophysiology of cardiovascular complications of pregnancy.

GENERAL CONSIDERATIONS

Before describing specific models of cardiovascular complications of pregnancy, it is useful to consider certain general concepts.

- Placental pregnancy is restricted to mammals (rudimentary placenta-like organs do exist in some fish).⁶ Models of cardiovascular complications of pregnancy must therefore be in mammals. To date, most models have used rodents, predominantly mice. Numerous parallels between rodent and human development and physiology, as well as the availability of genetic manipulations in mice, make rodents ideal model systems. Comparative

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Nonstandard Abbreviations and Acronyms

ANP	atrial natriuretic peptide
COMT	catechol-O-methyl transferase
CVD	cardiovascular disease
HELLP	hemolysis, elevated liver enzymes, low platelet
PGC-1α	PPAR γ -coactivator-1 α
PPCM	peripartum cardiomyopathy
RUPP	reduced uterine perfusion pressure model
sENG	soluble endoglin
sFLT1	soluble fms-like tyrosine kinase 1
STAT3	signal transducer of transcription 3
STOX1	storkhead box 1
VEGF	vascular endothelial growth factor

systems biology of human and mouse placental studies suggest that there is close to 90% concordance between proteins expressed in placental tissue from humans and mice.⁷ Most known hormonal changes of human pregnancy are recapitulated in the mouse and rat, though not all.⁸ Maternal changes in physiology, such as calorie retention via expansion of fat stores, and development of relative insulin resistance, also mirror those seen in human pregnancy.⁹ Many cardiovascular changes are also recapitulated, including decreased blood pressure throughout most of pregnancy, increase in plasma volume with consequent decrease in hematocrit, increase in cardiac output, and the development of cardiac hypertrophy.^{10,11} However, it is also important to recognize the many limitations of the rodent models. Unlike human pregnancies, rodent pregnancies are always multiparous, typically yielding 3 to 12 pups. Although primates and rodents share a hemochorial type of placentation, placentation in rodents is significantly different than in humans.⁸ For example, the invasion of the uterine wall by fetal trophoblasts is substantially shallower in mice, and trophoblast invasion in mice follows a perivascular route rather than an endovascular route noted in humans.¹² In addition, gestational length in rodents is significantly shorter (21 days) in contrast to $\approx$ 9 months in humans, and genome-wide expression profiling of mouse and human placentae revealed clusters of genes with distinct coexpression patterns across gestation, suggesting that the developmental timeline in mouse runs parallel to the first half of human placental development.¹³ Rodent hemodynamics are also significantly different from humans: the rodent heart, for example, beats 600 to 700 $\times$ per minute, an order of magnitude faster than the human heart.¹¹

- Hormones play a major role in disease. Pregnancy profoundly changes maternal homeostasis, and does so rapidly, creating numerous vulnerabilities. Parturition and the immediate postpartum period reverses many of these changes, and introduces new ones, at an even faster rate.¹⁴ These rapid changes are largely elicited and controlled by hormones such as progesterone emanating from the placenta. A simple way to conceptualize these profound effects is that the placenta, a tissue mostly of fetal origin, uses a panoply of hormones to developmentally reprogram maternal physiology to suit the needs of the fetus first, and then, anticipatorily at the end of gestation, of the newborn second. It is, therefore, not surprising that most uncovered mechanisms of cardiovascular complications of pregnancy involve aberrant hormonal effects, as discussed in detail below.
- Pregnancy and the postpartum period represent a unique relationship between 2 genetically distinct organisms. During pregnancy, this interaction largely occurs within the placenta. The placenta thus must be an immune-privileged setting, where neither maternal nor fetal immune system rejects the other.¹⁵ For the same reason, maternal immunity is generally suppressed during gestation, for example, studies have suggested that pregnancy is characterized by upregulation of T-regulatory cells that suppress immunity and maintain tolerance.¹⁶ This immune suppression, and the rebound immunologic activation postpartum, can also contribute to disease.

DISEASE MODELS OF CARDIOVASCULAR COMPLICATIONS OF PREGNANCY

Preeclampsia

Preeclampsia typically presents during the third trimester with hypertension and proteinuria and if left untreated can progress to end-organ damage in the kidney (acute kidney injury), liver (hemolysis, elevated liver enzymes, low platelet [HELLP] syndrome), and brain (eclampsia).¹⁷ Although delivery of placenta is the definitive treatment for this disease, preeclamptic women can have both short-term and long-term cardiovascular complications.¹⁸ In the short-term, severe hypertension in the postpartum period can occur and is often associated with repeat hospitalization and rarely seizures. Preeclampsia is also a significant risk factor for peripartum cardiomyopathy (see below) and for heart failure with preserved ejection fraction.¹⁹ In the long-term, preeclampsia is associated with 3-fold excess of CVD.¹⁸ Many pathways related to angiogenesis, inflammation, syncytiotrophoblast stress, and autoantibodies have been proposed to play a role in the pathogenesis of preeclampsia.¹⁷ Pathogenesis likely involves 2 distinct stages. The first, asymptomatic phase of the disease is associated with shallow invasion of the placenta into the decidua and myometrium and

decreased uteroplacental blood flow. The second stage occurs during the third trimester when women present with clinical features of hypertension, proteinuria, and microangiopathy in various organs such as kidney, liver, and brain. During this phase, there is syncytiotrophoblast stress that can be measured by high circulating levels of antiangiogenic factors, such as sFLT1 (soluble fms-like tyrosine kinase 1), sENG (soluble endoglin),²⁰ and increased urinary levels of prolactin and its antiangiogenic 16 kDa fragment.²¹ Animal models are critical to studying the cause/effect of the various pathways that have been proposed to play a role in preeclampsia. Tools to genetically modify animals, radiotelemetry to measure blood pressures noninvasively, and ultrasound/dopplers to evaluate uteroplacental blood flow, have enabled the continuous monitoring of healthy and preeclamptic pregnancies in rodents.²² Several animal models that recapitulate the first or second phase of the syndrome have been characterized. However, no single model fully recapitulates all the features of humans with preeclampsia, and therefore extrapolation of the experimental findings to the clinical situation should be interpreted with caution. Nevertheless, animal models have been invaluable in dissecting the pathogenesis of preeclampsia and validating potential targets for therapeutic intervention.²³ Here, we will summarize validated animal models currently used to study the pathophysiology of this disease (see Table 1).

Acute Placental Ischemia Models

Because the major defect in preeclampsia is placental ischemia related to poor placentation, researchers have modeled placental ischemia in rodents and primates using surgical approaches. The most common model is

induction of partial ischemia in pregnant rats using surgical clips of the uterine artery, referred to as reduced uterine perfusion pressure (RUPP) model.²⁴ Pregnant rats entering the RUPP procedure undergo clipping procedure at day 14 of gestation. Typically, a silver clip is placed around the aorta above the iliac bifurcation to reduce uterine perfusion pressure in the gravid rat by $\approx 40\%$. Because compensation of blood flow to the placenta occurs in pregnant rats through an adaptive increase in ovarian blood flow, additional clips on both right and left uterine arcades at the ovarian end right before the first segmental artery helps maintain the placental ischemia²⁵ (see Figure 1). RUPP rats develop reduced glomerular filtration rate, third-trimester hypertension, and variable degree of fetal growth restriction. Importantly, pathways implicated in humans with preeclampsia such as angiogenic imbalance, autoantibodies against angiotensin receptors, and proinflammatory factors have all been noted in this model.^{26–28,55} Many therapeutic compounds such as recombinant endothelial growth factors have been tested successfully in these models and provide valuable preclinical data needed before testing in pregnant humans.^{26,27} However, there are some caveats to this model. Preterm delivery has not been observed with this model, and therefore, it is difficult to assess prolongation of pregnancy with specific therapies. Also, RUPP is a model of nonsevere preeclampsia, as these animals have not been reported to develop HELLP syndrome or seizures to suggest eclampsia.

RUPP model has also been performed in mice by several groups, and preclinical studies to evaluate the safety and efficacy of nicotinamide and tadalafil were recently published.^{29,56} The advantage of using mice over rats is the

Table 1. Animal Models of Preeclampsia

Animal models	Species	Pathways	Limitations
Surgical ischemia of placenta ^{24–32}	Rats, mice, and baboons	Hypoxia, oxidative stress, inflammation, angiogenic imbalance	Lack of severe disease Cannot study trophoblast invasion
Angiotensinogen/renin transgenics ^{33–36}	Rats, mice	Oxidative stress, angiotensin receptor autoantibodies, Inflammation	Less relevant to humans as preeclampsia is characterized by suppressed renin and angiotensin II
sFLT1 overexpression ^{37–43}	Rats, mice	Angiogenic imbalance, Endothelial dysfunction	Short gestation and not optimal to study early placentation events
BPH/5 model ^{44,45}	Mice	Complement activation, Angiogenic imbalance	Lack of severe disease Short gestation
CBA/J $\times$ DBA/2	Mice	Complement activation, Angiogenic imbalance, immune dysfunction	Lack of severe disease Short gestation
COMT KO ⁴⁶	Mice	Hormonal and placental hypoxia	Lack of severe disease; no phenotype in rats; short gestation
STOX1 transgenic ⁴⁷	Mice	Inflammation, angiogenic imbalance	Relevance to humans is unclear as loss of function mutations variable depending on populations
RGS2 KO ⁴⁸	Mice	Histone deacetylation, cyclic AMP pathway	Relevance to humans is unclear as no evidence of placental ischemia
AVP infusion ^{49,50}	Mice, rats	Immune dysfunction, G protein signaling	Lack of severe disease; short gestation
L-NAME infusion ⁵¹	Mice, rats	Endothelial dysfunction	Partial phenotype
Dahl salt-sensitive rats ^{52–54}	Rats	Hypoxia, endothelial dysfunction	Lack of severe disease; not a model for de novo preeclampsia

AVP indicates arginine vasopressin; BPH/5, blood pressure high/5; COMT, catechol-O-methyltransferase; KO, knockout; L-NAME, L-NG-nitro arginine methyl ester; RGS2, regulator of G protein signaling; sFLT, soluble fms-like tyrosine kinase; and STOX1, storkhead box 1.

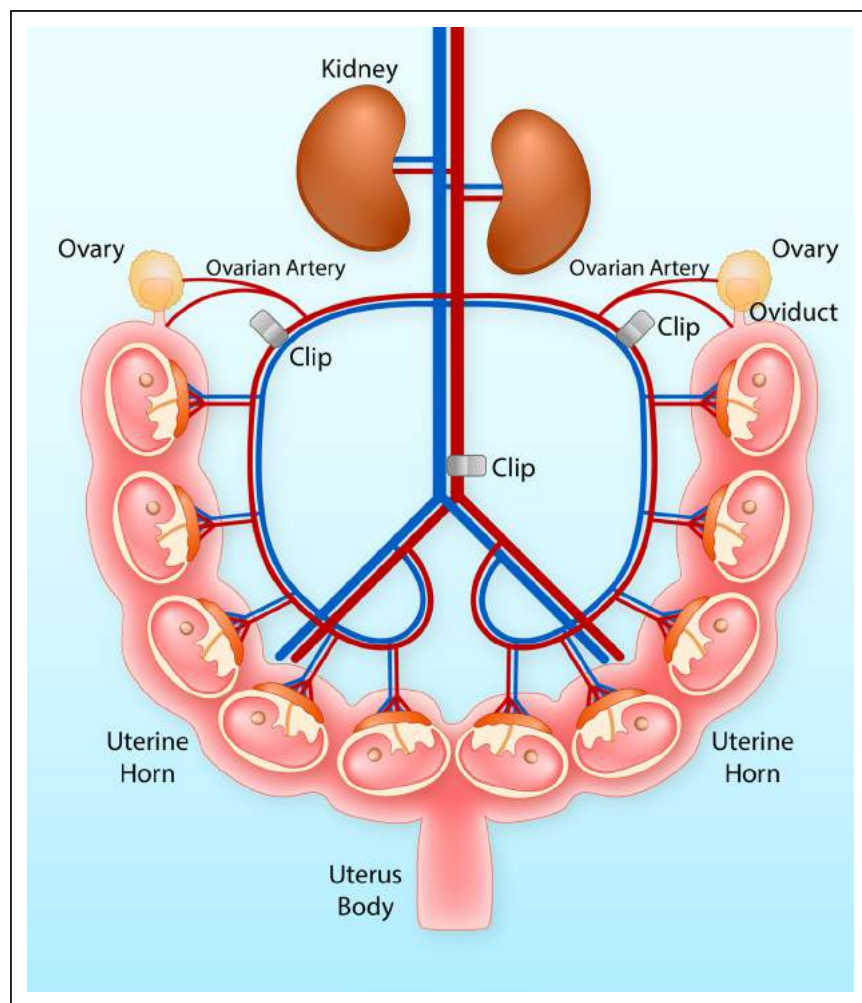


Figure 1. Schematic of the reduced uterine perfusion pressure (RUPP) model.

The RUPP model is made by clipping the ovarian arteries and abdominal aorta with silver clips during the third trimester in rats or in mice. Pregnant RUPP animals develop hypertension, decreased glomerular filtration rate, variable degrees of proteinuria, and fetal growth restriction. Illustration credit: Ben Smith.

availability of ready-to-use genetically modified mice from several repositories that can allow one to test the role of specific pathways in the pathogenesis of preeclampsia. To model ischemia, some groups have also exposed pregnant mice to relative hypoxia (9.5% O₂) from gestational day 7 to 17, inducing preeclampsia-like features including elevated preeclampsia biomarker levels.⁵⁷

Placental ischemia has also been modeled in nonhuman primates, typically with baboons.³⁰ Because pregnancy duration is significantly longer in baboons (180 days) than rodents, and because there is a single placenta and fetus, this surgical model closely approximates the clinical condition. Again, many therapies have been tested in this model that are now being pursued in clinical trials.^{31,58} Although there are several advantages to using nonhuman primates for preclinical proof-of-concept studies, a disadvantage is the inability to study early placentation events leading to preeclampsia because the induction of surgical ischemia may actually promote trophoblast invasion.⁵⁹

Renin-Angiotensin Models of Preeclampsia

Preeclampsia is characterized by enhanced angiotensin II sensitivity even before the onset of hypertension.⁶⁰ Models to study enhanced angiotensin II sensitivity and signaling

have largely used transgenic rodents in which the hypertension is induced by placental renin that acts on maternal angiotensinogen. When transgenic female mice expressing angiotensinogen are mated with transgenic males expressing renin, pregnant females display gestational hypertension during the third trimester due to secretion of placental renin into the maternal circulation.³³ Generalized seizures occur in 15% of these mice and the hypertension reverses after delivery of the placenta. Subsequently, other groups have reproduced this model in rats and reported that gestational hypertension was associated with the presence of agonistic antibodies against the angiotensin receptor^{34,61} (see Figure 2). Follow-up studies suggested that these agonistic autoantibodies not only induce angiotensin II sensitivity³⁵ but also may be sufficient to induce preeclampsia-like phenotypes when purified and injected into wild-type pregnant mice.^{32,62} Although several of the features of preeclampsia, such as gestational hypertension, albuminuria, and angiotensin II sensitivity are similar to humans with preeclampsia, this model has very high renin and angiotensin II which is in contrast to humans with preeclampsia, where renin and angiotensin II levels are suppressed. Nevertheless, the model has been particularly

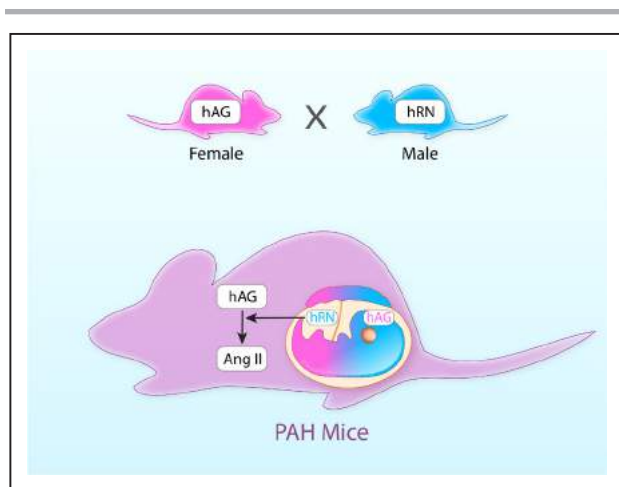


Figure 2. Ang II (angiotensin II) model of pregnancy-associated hypertension (PAH).

Female hAogen (human angiotensinogen) transgenic mice mated with male hRen (human renin) transgenic mice develop severe hypertension during pregnancy as human renin made from the fetus/placenta acts on maternal human angiotensinogen to induce excess Ang II that occurs only during pregnancy. Endogenous mouse renin will not cleave human angiotensinogen, making this a valuable model to study PAH. The opposite crosses (female hRen×male hAogen) can serve as controls as human angiotensinogen made in the fetus does not cross to the maternal circulation. Illustration credit: Ben Smith.

useful to dissect the early events of placentation. For example, studies suggest that enhanced angiotensin II signaling may block trophoblast invasion and that neutralization of the RAS may enhance placentation.^{36,63}

sFLT1 Overexpression Model

Clinical studies suggest that high levels of sFLT1 correlate with adverse maternal and fetal outcomes related to preeclampsia. Adenoviral-mediated expression of sFLT1 in pregnant mice and rats reproduces many of the features of preeclampsia including severe hypertension, proteinuria, glomerular endotheliosis, and fetal growth restriction^{22,37–40} (see Figure 3). In this model, sFLT1 is expressed ectopically from the liver and circulating concentrations achieved are higher than the concentrations noted in women with preeclampsia. To evaluate a more physiologically relevant model, transgenic overexpression of sFLT1 from the placenta was recently reported.⁴¹ In this model, the animals developed full-blown maternal disease as well as placental disease, including abnormal spiral artery remodeling and impaired blood flow to the fetoplacental unit.⁴² Other groups have also studied other synergistic antiangiogenic factors, for example, sENG. Transgenic expression of sENG during pregnancy leads to hypertension and fetal growth restriction.⁶⁴ Coexpression of sENG with sFLT1 either through adenoviral vector or as infusion of recombinant proteins has been used as a model of HELLP syndrome, a severe complication of preeclampsia.^{43,65} The sFLT1 overexpression model has been used to test several novel therapies, such as VEGF (vascular endothelial growth factor)-121

and statins.^{37,66} Kumasawa et al⁶⁷ showed that pravastatin upregulated PIGF (placental growth factor) and rescued preeclampsia-like features in a placental lentiviral sFLT1 overexpression model. Because clinical studies have reported that elevated circulating sFLT1 and low PIGF are present in most cases of preterm preeclampsia and that angiogenic imbalance correlates with adverse maternal and fetal outcomes related to preeclampsia,⁶⁸ there is strong interest in the sFLT1 model to test novel therapies, but also to understand downstream mechanisms by which sFLT1 induces vascular disease.

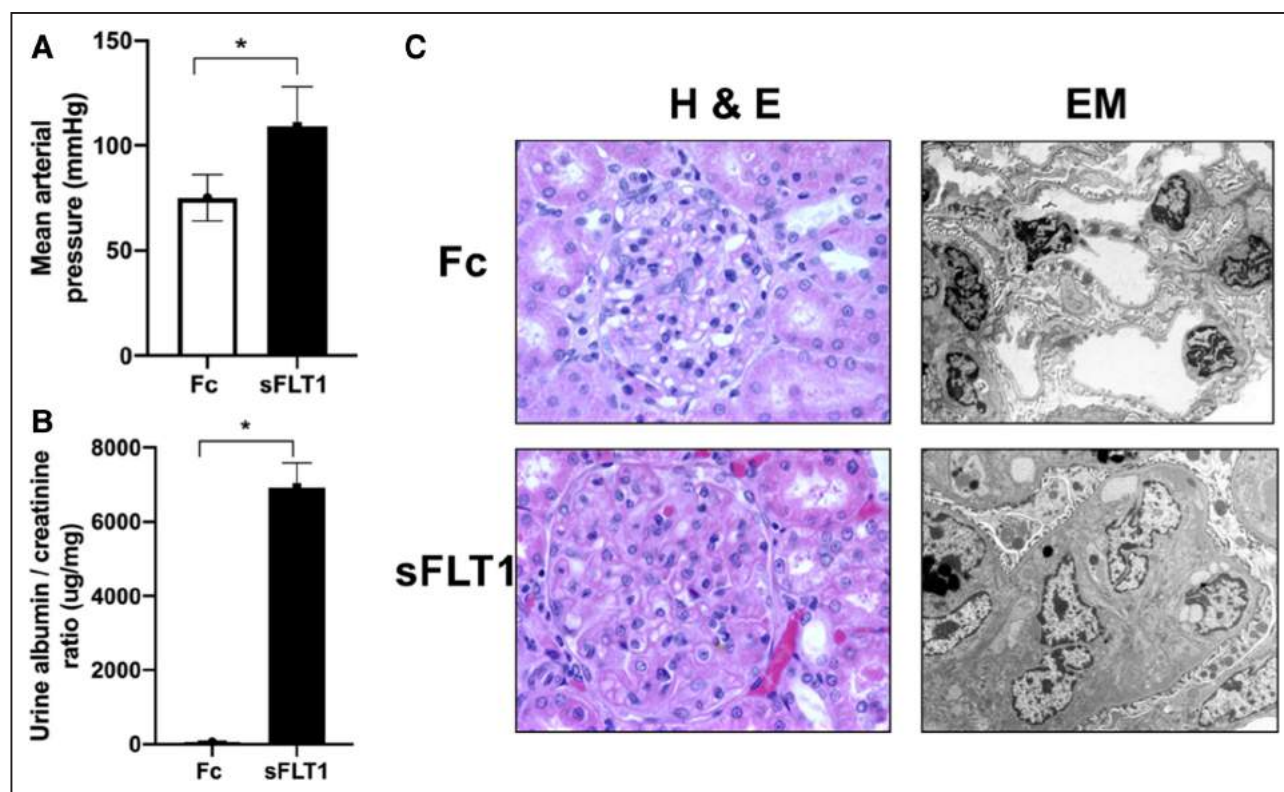
Genetic Models

BPH/5 Model

BPH/5 (blood pressure high/5) mice are an inbred subline derived from 20 generations of brother-sister matings of the inbred spontaneously hypertensive strain BPH/2.⁴⁴ During the nonpregnant states, BPH/5 has mildly elevated blood pressures when compared to the C57BL/6 strain but does not develop proteinuria. However, during pregnancy BPH/5 strain mice develop severe hypertension during third trimester that is accompanied by proteinuria and glomerular damage.⁴⁴ Interestingly, these maternal phenotypes are accompanied by low birth weight and smaller litter size in these mice. However, BPH/5 mice do not develop features of severe disease, such as HELLP syndrome.⁶⁹ Many groups have attempted to study the mechanisms of why this strain develops preeclampsia-like states. Sones et al⁷⁰ have suggested that antiangiogenic sFLT1 protein was upregulated in the BPH/5 placentas and that aberrant sFLT1 expression was responsible for the complement activation and poor placentation in this model. Interestingly, adenoviral delivery of VEGF121 in early pregnancy prevented the spontaneous development of preeclampsia in this model thus confirming that angiogenic imbalance was central to the pathogenesis of the maternal syndrome.⁴⁵

CBA/J×DBA/2 Model

Immunologic mechanisms have been hypothesized to play a central role in early placentation defects in preeclampsia. CBA/J×DBA/2 mating combinations have been extensively used to study miscarriages and preeclampsia.^{71,72} Initial studies reported embryos derived from mating CBA/J females with DBA/2 males showed an increased frequency of resorption and that surviving embryos showed consistent and significant intrauterine growth restriction. Administration of soluble CD200, an immune check point inhibitor has been shown to prevent abortions by augmenting T-regulatory cells.⁷³ Ahmed et al⁷⁴ characterized the maternal phenotypes of this mating combination and reported several features like albuminuria and glomerular fibrin deposits that are similar to humans with preeclampsia. Although these animals at baseline did not develop hypertension, they developed exaggerated sensitivity to angiotensin II. This animal model was explored to study several therapies such



as pravastatin and proangiogenic factor VEGF121 with beneficial effects and without inducing toxicity.

Catechol-O-Methyltransferase Knockout Mice

Kanasaki et al⁴⁶ first reported that mice lacking COMT (catechol-O-methyltransferase) in the placentas and in the mother developed third-trimester hypertension, proteinuria, and kidney damage. They further reported that the phenotype was mediated by a deficiency of 2-methoxyestradiol that is normally produced by COMT. Clinical studies suggest that genetic polymorphisms in the COMT pathway may be associated with the development of preeclampsia.⁷⁵ However, COMT knockout in rats was associated with no preeclampsia-like phenotype.⁷⁶ Whether this discrepancy is related to differences in placentation between mice and rats has not been resolved.

Ankyrin Repeat, SOCS Box-Containing 4 Knockout Mice

Rownley-Tilson et al⁷⁷ reported that mice genetically deficient in ubiquitin ligase ASB4 (Ankyrin repeat, SOCS box-containing 4) develop preeclampsia-like states including third-trimester hypertension and proteinuria. They further demonstrated that ASB4 promotes trophoblast differentiation through the degradation of ID2 (inhibitor of DNA binding). Aberrant expression of ID2 has been observed

in preeclamptic placentas, suggesting a role for this pathway in the human condition.⁷⁸ Li et al⁷⁹ reported that nicotinamide rescues the preeclampsia phenotypes and improves fetal survival in mice lacking ASB4.

Storkhead Box 1 Transgenic Mice

STOX1 (storkhead box 1) is a transcription factor belonging to the enlarged Forkhead Box gene family that has been associated with preeclampsia and is able to modulate trophoblast proliferation and migration. STOX1 is maternally expressed in a specific cell type of the placenta, the column extravillous trophoblasts. Overexpression of *STOX1* in human choriocarcinoma cells induces transcriptional alterations that mimic those of preeclamptic placentas.⁸⁰ Wild-type females crossed with males expressing STOX1 under a constitutive promoter were reported to develop severe gestational hypertension, proteinuria, and increased plasma level of soluble antiangiogenic factors, as well as kidney and placenta histological alterations.⁴⁷ Interestingly, aspirin use in this model was shown to reverse the hypertension phenotype, resembling the human situation.⁸¹ However, the role of STOX1 in human preeclampsia is still controversial. Although mutations in STOX1 gene were first reported in Dutch families,⁸² follow-up studies have not reported increased prevalence of STOX1 mutations in preeclampsia in other populations.^{83,84}

Corin Knockout Mice

Corin, also known as ANP (atrial natriuretic peptide)-converting enzyme, has been linked with trophoblast invasion and spiral artery remodeling.⁸⁵ Consistent with this hypothesis, pregnant Corin mice developed preeclampsia-like features including hypertension, proteinuria, and impaired spiral artery remodeling. However, mice lacking ANP, the Corin target, do not express any of the phenotypes of preeclampsia including hypertension or fetal growth restriction.⁸⁶ Furthermore, human studies have suggested that Corin levels may be elevated during preeclampsia,⁸⁷ suggesting that deficiency of Corin is unlikely to play significant role in preeclampsia.

RGS2 Knockout Mice

RGS (regulator of G protein signaling) proteins have been demonstrated to contribute to numerous cardiovascular diseases. Genetically modified mice with fetal and placental deficiency of RGS2 develop preeclampsia-like features, including hypertension and proteinuria during late gestation.⁴⁸ Interestingly, the RGS2 knockout model of preeclampsia does not have evidence of placental hypoxia or features of antiangiogenic factor upregulation. Reduced expression of RGS2 has been reported in some cases of human preeclampsia.⁴⁸ RGS2 deficient mice may thus be useful to study subclasses of preeclampsia that are not mediated by placental hypoxia or antiangiogenic factors.

Other Models of Preeclampsia

Pharmacological Compounds

Timed administration of compounds during pregnancy has been reported by some groups to model features of preeclampsia. Infusion of arginine vasopressin during pregnancy was reported to exhibit robust elevations in systolic blood pressure but without changes in diastolic blood pressure.⁴⁹ Changes in blood pressure were associated with increase in proteinuria and glomerular endotheliosis, both hallmarks of preeclampsia. Mechanistically, arginine vasopressin was shown to induce proinflammatory Th1 interferon-gamma and Th17 associated IL (interleukin) 17 in the placenta.⁵⁰ Human studies have suggested that copeptin, a surrogate biomarker of vasopressin, is elevated in humans with preeclampsia, but the differences in alterations were more apparent closer to the diagnosis of clinical preeclampsia.⁸⁸ Administration of L-NAME (L-NG-nitro arginine methyl ester), an inhibitor of eNOS (endothelial nitric oxide synthase), in pregnant rats also led to the development of hypertension, fetal growth restriction, and variable degrees of proteinuria.⁵¹

Dahl Salt-Sensitive Rats

Pregnant Dahl salt-sensitive rats have been used as a model of superimposed preeclampsia.⁵² Mean arterial blood pressures were elevated in the Dahl S rats before pregnancy.⁵³ However, during pregnancy these rats have elevated blood pressures that are not seen in other hypertensive rats (eg, spontaneously hypertensive rat [SHR]).

Dahl-S rats exhibited proteinuria, glomerulomegaly, and elevated sFLT1, all features of superimposed preeclampsia in humans. Sildenafil, a phosphodiesterase 5 inhibitor, ameliorated the maternal syndrome of preeclampsia and fetal growth restriction suggesting impaired nitric oxide signaling may be central to signs noted in this model.⁵² Consistent with this hypothesis, L-citrulline therapy in drinking water was also shown to reduce gestational hypertension and improved fetal growth in this model.⁵⁴ More work is needed to better characterize the early placental events in this model.

Preeclampsia Models to Study CVD

Because preeclampsia is associated with long-term hypertension and CVD, some investigators have used the preeclampsia animal models to study whether preeclampsia is directly involved in the CVD risk, in contrast to the alternative hypothesis that CVD and preeclampsia share underlying risk factors. Pruthi et al,⁸⁹ first reported that experimental preeclampsia using sFLT1 overexpression causes an enhanced vascular response to future vessel injury. Mice with prior exposure to preeclampsia or normal pregnancies were challenged with unilateral carotid injury. Preeclampsia-exposed mice had significantly enhanced vascular remodeling with increased vascular smooth muscle cell proliferation and vessel fibrosis compared with control pregnancy.⁸⁹ This new paradigm may contribute to the substantially increased risk of CVD in women exposed to preeclampsia. Bytautiene et al⁹⁰ reported that animals exposed to sFLT1 during pregnancy had no changes in blood pressure or vascular function six to eight months postpartum, but cardiovascular stressors such as angiotensin II or salt were not tested. In a follow-up study, the same group reported that sFLT1 exposure during pregnancy altered over 150 plasma proteins that could contribute to long-term cardiovascular disease.⁹¹ STOX1 transgenic mice develop, 8 months postpregnancy, cardiac fibrin deposits and mildly abnormal cardiac function in response to dobutamine stress.⁹² In summary, there is some evidence from mouse models that preeclampsia is directly involved in accentuating long-term CVD, but more work is needed to solidify this conclusion. Investigators have also used preeclampsia mouse models to study cardiovascular disease in offspring. For example, offspring of BPG/5 mice have white adipose tissue accumulation and evidence of hypertension and cardiomegaly.⁹³ Here too, more work is needed to better understand how in utero exposure to preeclampsia-like states may lead to CVD in the offspring.

Summary of Preeclampsia Models

In sum, although the use of animal models to study preeclampsia has increased dramatically in recent years, no one model faithfully recapitulates all the signs and symptoms of the human syndrome. Placental ischemia models in rodents have been most informative

as researchers have elucidated a pathogenic role for oxidative stress, antiangiogenic factors, and angiotensin autoantibodies in the pathogenesis of the maternal syndrome.⁹⁴ However, a significant limitation of ischemia models is that features of severe diseases, such as HELLP syndrome, eclampsia, and abruption are not noted. Transgenic or adenoviral overexpression of sFLT1 alone or in combination with sENG develops more robust preeclampsia phenotypes, including severe hypertension, nephrotic range proteinuria, cerebral edema, and HELLP like phenotypes.^{41–43,95} Taken together with the data that, in humans, high levels of sFLT1 and sENG correlate with adverse maternal and fetal outcomes,²⁰ there has been great interest in testing targeted therapies for preeclampsia in antiangiogenic overexpression model. A significant limitation of the rodent model, however, is lack of expression of all the isoforms of sFLT1 expressed in humans,⁹⁶ making it difficult to study molecular therapies such as antibodies that preferentially bind one isoform over others. It would, therefore, be critical to developing transgenic nonhuman primate models that express human sFLT1 isoforms and in whom the duration of pregnancy approximates the human situation. Furthermore, because there are no mechanical compromises to the uterine circulation in these transgenic models, the models can be used to evaluate whether therapies with maternal benefit would also benefit fetuses. Carter et al⁹⁷ have suggested that smaller primates such as common marmoset (a New World monkey) should be used to model preeclampsia; however, it is not known whether uterine invasion by trophoblasts occurs in marmosets to the same degree as in humans.

Peripartum Cardiomyopathy

Peripartum cardiomyopathy (PPCM) is characterized by systolic heart failure presenting in the last month of pregnancy or the first few months postpartum.^{98–103} It affects anywhere from 1:100 to 1:3000 births, with certain geographic hot spots like Haiti and Nigeria.^{98–103} PPCM accounts for >50% of cases of maternal cardiogenic shock in the United States¹⁰⁴ and is a leading cause of maternal peripartum death, with mortality rates in the United States as high as 25%.^{101,102,105–107} The disease affects all socioeconomic and racial sectors but is more common and bears significantly worse prognosis in women of African descent.^{108–110} The precise cause of PPCM remains elusive. However, work with preclinical models over the last 20 years, as outlined below (see also Table 2), has elucidated the current model that PPCM is in large part a vascular disease, triggered by aberrant hormonal changes of late pregnancy and early postpartum.^{111–115} In parallel, genetic studies in human cohorts have uncovered that rare genetic variants in various genes, including TTN, FLNC (filamin C), desmoplakin,

and others, can predispose to PPCM.^{137,138} The implications of these observations for building preclinical models are also discussed below.

Genetic Models of PPCM

Cardiac Expression of Gαq

The first mouse model of PPCM was described in 1998. Numerous hypertrophic signals in the heart, including angiotensin and adrenergic signals, are coupled to heterotrimeric guanine nucleotide-binding proteins of the Gq family. Transgenic models expressing various levels of Gαq in the heart were generated and shown to promote hypertrophy and subsequent systolic heart failure, a process that was markedly accelerated by repeated pregnancies.^{116,117} Subsequent work demonstrated that the systolic failure was caused by cardiomyocyte loss.^{118,119} This model had numerous limitations, however. First, the transgenic expression of Gαq was supraphysiologic, raising the possibility that the observed mechanism of cardiomyopathy is not relevant to human disease (transgenic lines with less induction of Gαq expression revealed no phenotype). Second, the transgenic mice revealed significant systolic dysfunction at baseline, even in the absence of pregnancy, whereas such dysfunction is not seen in women with PPCM,¹³⁹ and indeed the absence of such preexisting dysfunction is included in the definition of PPCM. This model was thus important as a first demonstration that pregnancy could accelerate cardiomyopathy in mice, but the model has not been used further. How much cardiomyocyte death contributes to PPCM in more faithful models and in the human disease remains uncertain.

Cardiac Deletion of Signal Transducer of Transcription 3

The most extensively studied model of PPCM is the *Myh6-Cre;Stat3^{lox/lox}* mouse, in which the transcription factor STAT3 (signal transducer of transcription 3) is selectively deleted in cardiomyocytes.¹¹² This model, although revealing no apparent dysfunction at baseline, develops systolic heart failure after 1 or 2 pregnancies. STAT3 is a ubiquitously expressed transcription factor that, in cardiomyocytes, regulates expression of antioxidant enzymes and proangiogenic factors. STAT3 is normally activated by phosphorylation in cardiomyocytes during pregnancy, and loss of cardiac STAT3 in the *Myh6-Cre;Stat3^{lox/lox}* model causes unrestrained production of reactive oxygen species and blunted angiogenesis.¹¹² The latter is in part caused by the reactive oxygen species–induced secretion of the peptidase cathepsin D, which cleaves the circulating nursing hormone prolactin to a 16 kDa fragment well-established to be vasculotoxic (often called vaso-inhibin; see Figure 4).¹¹² The 16 kDa prolactin fragment interacts with circulating PAI-1 (plasminogen activator inhibitor-1) to

Table 2. Mouse Models of Peripartum Cardiomyopathy

Model	Proposed mechanisms	Rescue experiments	Evidence for human relevance	Limitations
G _α Q transgenic ^{116–119}	Promotion of cardiomyocyte apoptosis			Supraphysiologic overexpression. Baseline dysfunction.
STAT3 cardiac KO ^{112,120–122}	Cardiac secretion of cathepsin D cleaves prolactin to vasculotoxic 16 KD fragment	Suppression of prolactin with bromocriptine	Some clinical support for treatment with bromocriptine. STAT3 is suppressed in cardiac PPCM tissue.	No genetic evidence for role of STAT3 in human PPCM
PGC-1α cardiac KO ^{113,123}	Blunted cardiac proangiogenic signals create vulnerability to vasculotoxic hormones, including sFLT1	VEGF injections, treatment with bromocriptine	sFLT1 elevations in PPCM. Epidemiological association with pre-eclampsia, and cardiac dysfunction.	No genetic evidence for role of PGC-1α in human PPCM
TTNtv knock-in ^{124–126}	Unknown		Incontrovertible evidence that variants in TTN contribute to human PPCM	Mild, if any, pregnancy-induced cardiac dysfunction
γ-secretase inhibition ¹²⁷	Suppression of Notch1 activation, causing blunted angiogenesis.			Unclear mechanism, pleiotropic effects of γ-secretase inhibition
Npr1 whole-body KO ¹²⁸	Indirect mechanism leads to lactation-dependent activation of aldosterone in CNS	CNS deletion of aldosterone receptor		Largely driven by elevated BP. Npr1 signaling is likely elevated in PPCM, rather than blunted. No genetic evidence in humans.
HSPB6/Hsp20 transgenic ^{129–131}	Cardiomyocyte apoptosis and suppressed autophagy			Unknown role of Hsp20 in human PPCM. High expression of mutant protein.
PERK transgenic ¹³²	Microvascular rarefaction			Unknown if PERK or the UPR is activated in PPCM hearts
EMCV infection ¹³³	Postpartum myocarditis			Unclear role of viral infection in PPCM
ErbB4 cardiac heterozygous KO ¹³⁴	ERBB4 protects maternal heart from peripartum stress		Multiple microRNAs that target <i>ErbB4</i> are elevated in serum of PPCM patients	Significant baseline cardiac dysfunction. Multiple microRNAs may have unpredictable targets. No genetic evidence in humans.
caAkt cardiac transgenic±STAT3 cardiac KO ¹³⁵	Enhanced oxidative stress and hypertrophy	Treatment with bromocriptine		Exuberant hypertrophy not reflective of normal pregnancy.
Zfp36l2 cardiac KO ¹³⁶	Disinhibition of mTORC1	Treatment with rapamycin	Some evidence of low Zfp36l2 in PPCM hearts	Baseline cardiac dysfunction. No genetic evidence in humans.

BP indicates blood pressure; caAkt, constitutively active Akt kinase; CNS, central nervous system; EMCV, encephalo-myocarditis virus; *ErbB4*, Erb-B2 receptor tyrosine kinase 4; HSPB6, heat shock protein family B (small) member 6; Hsp20, heat shock protein 20; KO, knockout; mTORC, mechanistic target of rapamycin kinase complex; npr1, natriuretic peptide receptor 1; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; PGC-1α, PPARγ-coactivator-1α; PPCM, peripartum cardiomyopathy; sFLT, soluble fms-like tyrosine kinase 1; STAT3, signal transducer of transcription 3; TTNtv, titin(TTN)-truncating variant; UPR, unfolded protein response; and Zfp36l2, zinc finger protein 36-like 2.

bind to the uPAR (urokinase-type plasminogen activator receptor) on endothelial cells,¹⁴⁰ triggering a cascade of cardiotoxic events, including endothelial apoptosis and vascular insufficiency, and endothelial secretion of exosomes containing microRNA-146a that, when taken up by adjacent cardiomyocytes, suppress prosurvival signaling by *ErbB4* (Figure 4). Thus, the *Myh6-Cre;Stat3^{lox/lox}* model was first to illustrate a vascular/hormonal paradigm to explain PPCM, whereby aberrant signaling by a pregnancy-induced hormone (prolactin) leads to vasculotoxicity and in turn cardiomyopathy. Most tellingly, suppression of prolactin secretion with the dopamine agonist bromocriptine prevented pregnancy-induced heart failure, demonstrating the critical role of prolactin in the pathology of the *Myh6-Cre;Stat3^{lox/lox}* model. The model has been used to study the role of the pregnancy hormone relaxin-2 on PPCM progression,¹²⁰ the effect of the metabolic modulator perhexiline,¹²¹ and the role of catecholamines, especially β1-adrenergic agonism.¹²²

In the latter case, β1-adrenergic stimulation substantially worsened cardiac function, mirroring observations in a small group of women with PPCM in whom treatment with dobutamine correlated with worse outcomes.¹²²

Numerous lines of evidence support the clinical relevance of the *Myh6-Cre;Stat3^{lox/lox}* model and the pathophysiological mechanism outlined above. Levels of STAT3 protein are decreased in left ventricular tissue from patients with PPCM, compared to nonfailing donors.¹¹² Circulating cathepsin D activity and levels of microRNA-146a are elevated in women with PPCM, compared to pregnancy-matched healthy women.^{112,141} And there is substantial, although still somewhat controversial, support for the use of bromocriptine in women with PPCM. Three randomized studies have suggested a benefit of bromocriptine use,^{142–144} but the studies had limitations including small size, patients lost to follow-up, and the lack of a placebo arm, respectively. There thus remains some equipoise on the use of bromocriptine for PPCM,

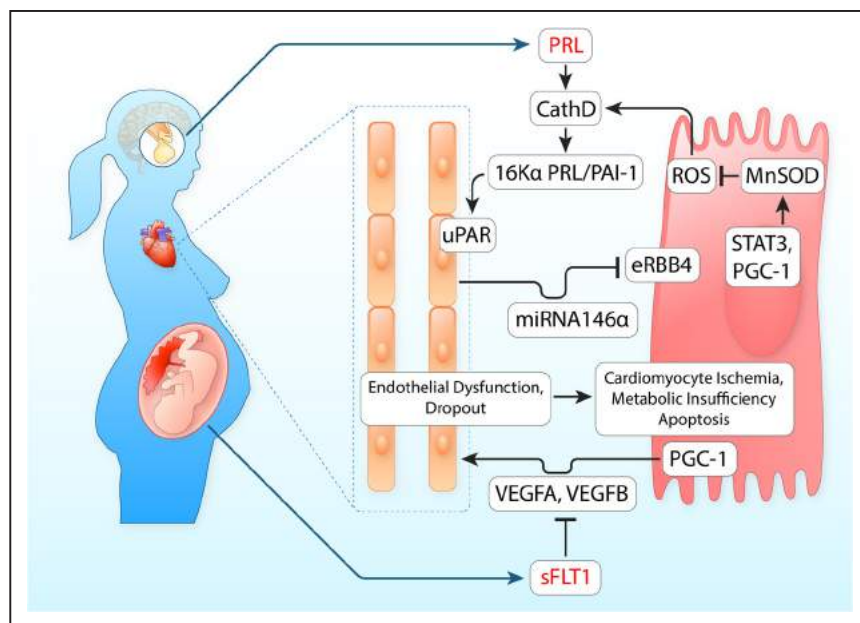


Figure 4. Vasculo-hormonal hypothesis of the pathophysiology of peripartum cardiomyopathy.

Pregnancy triggers the systemic secretion of hormones from the pituitary and placenta. Some of these, including PRL (prolactin) and sFLT1 (soluble FLT1), can be toxic to cardiac microvasculature, either via proteolytic conversion to vasculotoxic 16 Kd PRL or via inhibition of VEGF (vascular endothelial growth factor) signaling, respectively, ultimately leading to cardiac ischemia and apoptosis. See text for further details.

Illustration credit: Ben Smith.

and a large ($n=200$) randomized placebo-controlled study has now been initiated in the United States. In sum, there is substantial support for the human relevance of the *Myh6-Cre;Stat3^{lox/lox}* model of PPCM.

Cardiac Deletion of PGC-1 α

The next model of PPCM to garner attention was the *Myh6-Cre;Ppargc1a^{lox/lox}* mouse, in which the transcriptional coactivator PGC-1 α (PPAR γ -coactivator-1 α) is deleted selectively in cardiomyocytes.¹¹³ Like the STAT3 model, *Myh6-Cre;Ppargc1a^{lox/lox}* have minimal cardiac dysfunction at baseline but develop dilated cardiomyopathy after 1 or 2 pregnancies. PGC-1 α is a pleiotropic coactivator that coordinates multiple transcriptional programs, including the promotion of oxidative metabolism and angiogenesis, the latter in part via secretion of VEGF.^{145,146} Loss of cardiac PGC-1 α in the *Myh6-Cre;Ppargc1a^{lox/lox}* model leads to pregnancy-induced defects in cardiac microvasculature, cardiac ischemia, and cardiomyopathy.¹¹³ These effects were rescued by ectopic administration of VEGF and bromocriptine, but not either alone, supporting the likely role of prolactin in pregnancy-induced cardiomyopathy, but also indicating the presence of additional antiangiogenic factors. A search for the latter identified sFLT1, the placental-derived suppressor of VEGF activity described above (see preeclampsia section, and also Figure 4). sFLT1 was sufficient to trigger cardiomyopathy in *Myh6-Cre;Ppargc1a^{lox/lox}* mice in the absence of pregnancy.¹¹³ Activin-A, another placental-derived hormone, was also recently implicated in cardiomyopathy in this model: inhibition of Activin-A receptors partially reversed pregnancy-induced cardiomyopathy in *Myh6-Cre;Ppargc1a^{lox/lox}* mice.¹²³ Thus, as with the *Myh6-Cre;Stat3^{lox/lox}* model, the *Myh6-Cre;Ppargc1a^{lox/lox}* model illustrates the vascular/hormonal paradigm of PPCM, in

which pregnancy-induced hormones (sFLT1, activin-A) lead to vasculotoxicity and cardiomyopathy.

Numerous lines of evidence also support the clinical relevance of the *Myh6-Cre;Ppargc1a^{lox/lox}* model and the role of sFLT1, activin-A, and likely other placental hormones, in PPCM. PGC-1 α expression has been reported to be reduced in human cardiomyopathy, although not specifically in PPCM. Circulating levels of sFLT1 and activin-A are elevated in PPCM patients and correlate with heart failure severity and with adverse outcomes.^{113,123} Preeclampsia, in which sFLT1 and activin-A levels are markedly elevated (see above), is one of the strongest risk factors for PPCM, present in 25% to 50% of cases.^{101,102,147} In fact, even in the absence of PPCM, women with preeclampsia exhibit subclinical cardiac dysfunction that closely correlates with plasma sFLT1 levels.¹⁴⁸ It is unknown, however, if PGC-1 α is altered in cardiac tissue of human PPCM, and in fact has been reported as elevated in other forms of human cardiomyopathy.¹⁴⁹ Nevertheless, loss of cardiac PGC-1 α in mice is clearly a reliable model in which the murine heart is artificially made susceptible to pregnancy-induced vasculo-hormonal insults, allowing mechanistic studies of the latter.

TTN Knockin Mice

Recent work has demonstrated that 10% to 15% of women with PPCM harbor heterozygous truncating variants (ie, nonsense, frameshift, or splice site variants) in various genes known also to predispose to nonischemic dilated cardiomyopathy.^{137,138} Most prominent among these, present in $\approx 10\%$ of women with PPCM^{137,138} as well as patients with dilated cardiomyopathy,^{150,151} is *TTN*, encoding for the large sarcomeric protein titin. These observations have led to the generation of knockin mice, bearing heterozygous *Ttn* truncating variants analogous

to those seen in human patients. These models do not exhibit spontaneous cardiomyopathy, but cardiac stressors including minipump infusions with angiotensin II, treatment with doxorubicin, or acute volume overload, can elicit a mild decline in systolic function.^{124–126} The impact of pregnancy on these models has not been reported. Why these models are so mild, compared to the clinical disease, is unclear, but it should be noted that *TTN* truncating variants in humans are poorly penetrant for disease, with 95% of carriers exhibiting no cardiac dysfunction,¹⁵² strongly suggesting the requirement for second or third hits that may not be present in the mouse models. It is also worth noting that the truncated titin protein encoded by *TTN* variant-bearing allele is present in human hearts,^{153,154} but not in rat¹⁵⁵ or mouse (Z.A.) hearts, suggesting that protein quality control in the mouse may be better at clearing the mutated protein and thus averting disease. Thus, although mice bearing *Ttn* truncating variants reflect genetic underpinnings of the human disease more faithfully than do the *Myh6-Cre;Ppargc1a^{lox/lox}* and *Myh6-Cre;Stat3^{lox/lox}* models, the latter recapitulate better the disease phenotype. Mouse models with heterozygous deletions of other genes known to be mutated in cases of PPCM exist (*Bag3*,^{156,157} *Flnc*,¹⁵⁸ *Dsp*¹⁵⁹), but the impact of pregnancy on their cardiac function has not been reported.

Other Mouse Models of PPCM

Pharmacological Compounds

Several groups have recently reported other mouse models that develop cardiac failure during or after pregnancy. Treatment of pregnant wild-type mice during the last 3 days of gestation and first 3 weeks postpartum with an inhibitor of γ -secretase, which is required to activate Notch1 and normal angiogenesis, led to systolic cardiac failure, accompanied by blunted angiogenesis and lower circulating levels of cathepsin D and of sFlt1.¹²⁷ The study did not elucidate if γ -secretase inhibition elicits this cardiac phenotype primarily by acting on endothelial cells (where Notch1 plays a strong role) versus in cardiomyocytes (where cathepsin D is regulated) versus the placenta (the primary source of sFlt1 during pregnancy). Known effects of γ -secretase inhibition beyond suppression of Notch1 may also have contributed to the phenotype. Thus this model recapitulates some mechanistic aspects of the well-established *Myh6-Cre;Stat3^{lox/lox}* model, and benefits from not needing genetic modifications but the clinical relevance of Notch1 inhibition in human PPCM is not clear, and the pleiotropic effects of γ -secretase inhibition may render mechanistic investigations difficult.

Loss of Natriuretic Peptide Signaling

Another recent model of PPCM involved mice lacking *Npr1*, the receptor for the natriuretic peptides ANP and brain natriuretic peptide (BNP) that are secreted by the heart to modulate kidney function and blood pressure.¹²⁸ *Npr1^{-/-}* mice have marked elevations in blood pressure,

by ≈ 20 mmHg, accompanied by significant cardiac hypertrophy. After 2 pregnancies, the cardiac hypertrophy is accentuated, and the mice develop systolic heart failure. Mechanistic studies with various genetic crosses implicated lactation and a neuronal circuit involving signaling by aldosterone in the central nervous system.¹²⁸ The model appears to be driven largely by the elevated blood pressure and the ensuing cardiac hypertrophy, which is not typically observed in human PPCM. The model is also accompanied by activation of STAT3 phosphorylation in cardiac tissue, in contrast to loss of STAT3 in *Myh6-Cre;Stat3^{lox/lox}* mice or in human PPCM hearts.¹¹² Moreover, PPCM and other forms of heart failure are likely accompanied by elevated, rather than blunted, ANP/BNP signaling. Nevertheless, the study suggests that it may be worth testing aldosterone inhibition with readily available Food and Drug Administration–approved inhibitors in this and other models of PPCM, as well as in the human disease.

Protein Folding Defects

Two recent studies have suggested that aberrations in protein folding and the cellular response to misfolding may contribute to PPCM. HSPB6 (heat shock protein family B [small]member 6)/Hsp20 (heat shock protein 20) is a small heat-shock protein found primarily in muscle tissues, and protective in multiple models of heart failure. Female transgenic mice with cardiac-specific expression of a mutant form of HSPB6/Hsp20, identified in a small subset of patients with dilated cardiomyopathy, develop mild systolic failure after 2 to 4 pregnancies, accompanied by cardiomyocyte apoptosis and evidence of impaired autophagic flux.^{129,130} Male transgenic mice spontaneously develop similar dysfunction starting at 6 months of age, indicating that the developed heart failure is not specific to pregnancy.¹³¹ And *Myh6-Cre;Perk^{lox/lox}* mice, in which PERK (protein kinase R [PKR]-like endoplasmic reticulum kinase), one of the effectors of the unfolded protein response, is deleted selectively in cardiomyocytes, develop systolic heart failure after 3 to 4 pregnancies, accompanied by a marked decrease in VEGF expression, and microvascular rarefaction.¹³² Treatment with bromocriptine did not rescue pregnancy-induced systolic dysfunction in this model, but cotreatment with VEGF or other proangiogenic agents was not tested.¹³² Interestingly, treatment of hypoxic isolated cardiomyocytes with 23 kD prolactin promoted PERK activation, suggesting a role for PERK activation during lactation, but in vivo evidence for this conclusion remains lacking, nor is it known if PERK is activated in human PPCM hearts. Both of these studies thus suggest that aberrations in the unfolded protein response may contribute to PPCM, but further rescue studies will be needed to test this notion. The relevance of these models to the human disease is also difficult to gauge because there is little information available on the status of the unfolded protein response or of protein folding in human PPCM.

mTORC Hyperactivity

During mouse pregnancy, cardiac Akt and mTORC1 activity rise, concomitant with marked physiological hypertrophy and increasing cardiac mass, and this process reverses postpartum.^{10,160} Kouzu et al¹³⁶ recently demonstrated that preventing the postpartum suppression of cardiac mTORC1 activity may predispose to PPCM. *Myh6-Cre;Zfp36l2^{ox/ox}* mice lacking cardiac expression of Zfp36l2 (zinc finger protein 36-like 2), an mRNA-destabilizing protein that suppresses mTORC1 via an Mdm2 (mouse double minute 2)/p53/Redd1 (regulated in development and DNA damage response 1) pathway, develop profound mTORC1 hyperactivity and systolic dysfunction postpartum. The mice do spontaneously develop cardiac dysfunction in the absence of pregnancy, although pregnancy markedly accelerates the process. Importantly, systolic function is partially restored by treatment with rapamycin, a mTORC1 inhibitor. Interestingly, persistent mTORC1 activity can promote unfolded protein response,^{161,162} thus potentially linking to the mouse models described above. It will be of interest to evaluate mTORC1 signaling in other models of PPCM, as well as their response to rapamycin, to test the generalizability of this model. Vascularity or response to bromocriptine treatment was not tested in this model.

Summary of Models PPCM

Developing faithful models of PPCM is imperative to better study this still poorly understood disease. The variety of models associated with PPCM supports the notion that PPCM is a syndrome with multiple origins. Most clinical and preclinical evidence indicates that the disease is vascular and hormonal in nature, and 2 mouse models, the *Myh6-Cre;Stat3^{ox/ox}* and *Myh6-Cre;Ppargc1a^{ox/ox}* models, best capture these aspects of the disease, thereby also illustrating convergence of various pathologies on common pathways. However, both models are limited by the physiological differences between mouse and human pregnancies. Various other aspects of human PPCM are also poorly reflected in these models, including the observation that most women with PPCM recover cardiac function, whereas the mouse models do not. Better characterization of PPCM pheno- and genotypes in humans and the subsequent development of appropriate preclinical models of PPCM would significantly advance the field.

Models of Other Cardiovascular Complications of Pregnancy

Pregnancy-Associated Vascular Aneurysm/Dissection

Pregnancy is associated with increased risk of accelerated aneurysm progression and aortic dissection, as well as spontaneous coronary artery dissection. These catastrophic events often are seen late in pregnancy and in the immediate postpartum period. Habashi et al¹⁶³ recently reported

that mice with severe deficiency in fibrillin-1 expression, a model of Marfan syndrome, showed accelerated aortic growth during pregnancy, accompanied by high incidence of dissection and markedly decreased survival, which were blocked by oxytocin antagonists or by prevention of lactation. Similarly, pregnancy was noted to exacerbate aortic dissection in a mouse model of vascular Ehlers-Danlos syndrome that carried a dominant mutation in a major collagen (COL3A1; collagen type III alpha 1 chain) in blood vessels. Recently Zekavat et al¹⁶⁴ used whole-exome sequencing to identify rare variants in 10 fibrillar collagen genes that were predisposed to spontaneous coronary artery dissection and showed that mice with heterozygous deficiency in *Col5a1* or both *Col5a1* and *Col3a1* were prone to spontaneous aortic dissection, but they did not report if pregnancy accentuated the phenotype. Interestingly, similar to the Marfan mouse, oxytocin antagonists prevented pregnancy-associated aortic dissection in the COL3A1 mutant mice.¹⁶⁵ Since oxytocin is frequently used to support the delivery process, the present model may indicate more careful use of this hormonal treatment. Thus, as in preeclampsia and PPCM, pregnancy-associated hormones appear to play a dominant role in disease progression.

Pregnancy-Associated Thromboembolism

Pregnancy is characterized by a hyper-coagulable state and is associated with increased thrombosis and vascular complications such as recurrent miscarriages, venous thrombosis, and fetal growth restriction. Isermann et al¹⁶⁶ first reported that mice lacking thrombomodulin developed abortions due to activation of tissue-factor-initiated activation of a blood coagulation cascade at the maternal-fetal interface. Similarly, to model thrombosis related to aPL (antiphospholipid) antibody syndrome, several groups have performed passive transfer of the aPL antibodies purified from patients into pregnant mice. These aPL antibody-injected mice develop miscarriages or severe fetal growth restrictions due to placental thrombosis.¹⁶⁷ Other groups have suggested that mice immunized with β 2GPI (beta-2-glycoprotein I) also develop aPL-like syndrome.¹⁶⁸ β 2GPI is a phospholipid-binding protein, and it is thought that aPL antibodies are induced by binding of β 2GPI to endogenous phospholipids. Many groups have tested therapeutic compounds such as complement inhibitors and statins to ameliorate vascular thrombosis and improve adverse pregnancy outcomes in these aPL models.¹⁶⁹ Recently, aPL actions via apoE receptor 2 in placental trophoblasts were also shown to induce hypertension and fetal growth restriction in pregnant mice.¹⁷⁰ Interestingly, in this mouse model, protein phosphatase 2A inhibition was found to afford complete protection from aPL-associated pregnancy complications.

Pregnancy-Associated Cardiac Arrhythmias

Cardiac arrhythmias due to preexisting conditions may be exacerbated during pregnancy due to physiological tachycardia.¹⁷¹ El Khoury et al^{172,173} confirmed that pregnancy exacerbates supraventricular tachyarrhythmias using

programmed electrical stimulation protocols in anesthetized pregnant mice. Mechanistically, cell-intrinsic differences in sinoatrial node cell activity and increased L-type Ca^{2+} current and spontaneous Ca^{2+} release from sarcoplasmic reticulum could be demonstrated between nonpregnant and pregnant mice, and chronic administration of estrogen to nonpregnant mice was sufficient to increase heart rate, in a manner dependent on the estrogen receptor α .^{172,173} Thus the model suggests a hormonal, rather than hemodynamic, contribution to pregnancy-associated tachycardia.

CONCLUSIONS

Cardiovascular complications of pregnancy are frequent and long-lasting, but their pathophysiology remains poorly understood. Clinical studies are challenging, in part due to especially high regulatory hurdles in this patient population and to frequent skepticism and even disinformation. Preclinical studies in animal models are thus critical to advancing mechanistic insight. The placenta plays a dominant role in pathophysiology, and all animal models are thus in placental mammals. To date, most of these models are surgical, pharmacological, or genetic in nature. Few have incorporated environmental factors such as air pollution, or high-altitude modeling, which are also thought to contribute to cardiovascular complications of pregnancy. A potential role for infection, for example, myocarditis, has also been only minimally modeled.^{133,174} Nevertheless, animal models to date have provided substantial insight into disease pathogenesis, and have led to numerous potential treatments currently under evaluation, such as bromocriptine for PPCM (<https://www.clinicaltrials.gov>; Unique identifier: NCT05180773, NCT02590601, NCT00998556) or extracorporeal removal of sFlt1 for preeclampsia (<https://www.clinicaltrials.gov>; Unique identifier: NCT02923206, NCT03231657).

ARTICLE INFORMATION

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Disclosures

None.

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ON MY MIND

It Is Time to Offer Genetic Testing to Women With Peripartum Cardiomyopathy

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Peripartum cardiomyopathy (PPCM) is a form of systolic heart failure triggered by pregnancy that affects as many as 1 in 1000 women worldwide.¹ More than half of affected women recover cardiac function, but outcomes for the remainder can be poor. A substantial subset of women with PPCM die, require cardiac transplantation, or are afflicted with lifelong heart failure.¹ PPCM affects Black women at 4 times the rate it affects White women, and Black women recover less frequently and more slowly from the disease. The cause of PPCM remains poorly understood, but recent work has demonstrated a genetic contribution to PPCM in at least 15% of cases.² Genes affected include *BAG3*, *DSP*, *FLNC*, *VCL*, and most commonly *TTN*, the gene encoding for the large sarcomeric protein titin. Pathogenic or likely pathogenic variants are most commonly loss-of-function truncating mutations, including frameshift, nonsense, and splice-site variants. PPCM phenotypically resembles nonischemic dilated cardiomyopathy (DCM), which occurs with a prevalence similar to that of PPCM and for which a genetic contribution also can be demonstrated in at least 15% of cases. The relative prevalence of truncating variants in the genes noted here is nearly identical between PPCM and DCM, for example, 10% to 15% prevalence of variants in *TTN* and 2% to 3% prevalence of variants in *FLNC*.² Clustering of PPCM and DCM in some families has been reported.³ DCM and PPCM thus share numerous attributes and likely common pathophysiology, and PPCM may be part of a spectrum of DCM in general.

For patients with DCM and their families, genetic counseling and testing of a panel of genes linked to disease (ie, those genes most likely to yield clinically meaningful data) are recommended in some form by most major cardiology

associations, including the American Heart Association, the European Society of Cardiology, the American College of Medical Genetics, and the Heart Rhythm Society, and widely adopted.⁴ Should we not offer patients with PPCM the same as we offer patients with DCM? We should, for exactly the same reasons that genetic testing is recommended in cases of DCM (Figure):

1. Benefits for the patient: prognosis and risk stratification. A subset of variants identified in cases of DCM, including variants in *FLNC* and *DSP*, herald a high risk of arrhythmic complications, warranting close monitoring and possibly preventive measures such as implantation of an automatic cardioverter defibrillator.⁴ Although similar follow-up analyses are not available for PPCM, it is reasonable to think the same would be true for women with PPCM.
2. Benefits for family members: cascade testing when a pathogenic or likely pathogenic variant is identified in the proband (but not when the proband harbors no variant). Many women with PPCM have family members considering pregnancies. Identification of a pathogenic or likely pathogenic variant identical to that of the proband can inform their decision to undergo closer follow-up in pregnancy, to consider prenatal or preimplantation genetic testing, or in some cases, to move forward with pregnancy at all. In addition, current guidelines for DCM recommend yearly follow-up for variant-positive family members, with evidence that such follow-up can accelerate identification of affected relatives and improve survival. The same should be recommended for variant-positive family members of women with PPCM. Conversely, and perhaps most importantly,

Key Words: genetic testing ■ cardiomyopathies

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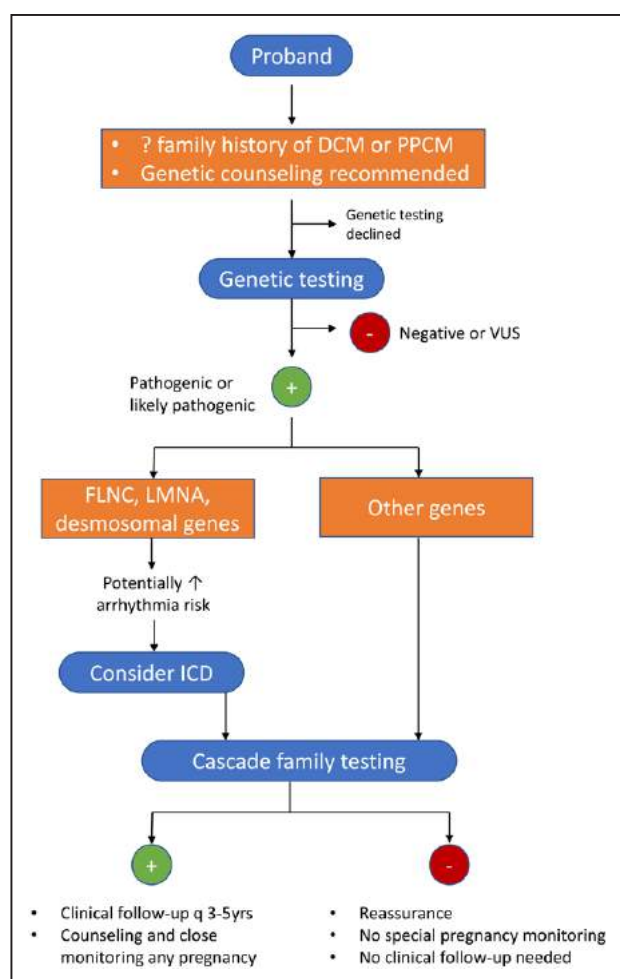


Figure. Proposed algorithm for managing genetic testing in a patient with PPCM.

See article for details.

a negative test when a pathogenic or likely pathogenic variant was identified in the proband can provide reassurance and is a cost-effective way to eliminate the need for such follow-up.

In addition to these indications, a codified protocol for genetic testing in PPCM would broaden our understanding of the phenotypic and prognostic similarities between DCM and PPCM. Standardizing protocols for genetic testing and counseling in PPCM also may help reduce the impact of bias on the care of underserved groups, in particular Black women, who are more likely both to develop PPCM and to have worse outcomes than non-Black women.¹ Numerous academic and commercial vendors provide testing of gene panels certified by the Clinical Laboratory Improvement Amendment. Although the panels differ, they all include the genes most commonly identified as mutated in both PPCM and DCM, and an argument has been made to achieve the highest predictive value by focusing on the dozen or so genes with the highest prevalence and certainty of causality, including *BAG3*, *DES*, *FLNC*, *LMNA*, *MYH7*, *PLN*, *RBM20*, *SCN5A*, *TNNC1*, *TNNT2*, *DSP*, and *TTN*.⁴

It is important to recognize risks and barriers for patients and family members. Access to genetic testing and out-of-pocket costs can be challenging for many patients. In addition, education of clinicians caring for these patients may be required. It is also imperative to precede and follow genetic testing with genetic counseling. Patient preferences are personal and best explored with a genetic counselor, as are the subtleties of identifying a pathogenic or likely pathogenic variant and its implications, including variable penetrance and expressivity, impact on insurability and lifestyle choices, identification of variants of unknown significance, and potential future changes in variant classifications. It is also important to recognize that, to date, it remains unproven that identifying genes associated with PPCM can translate into decreased morbidity or mortality. Last, although confirmatory genetic testing should be performed in cases of PPCM, at this point, predictive testing (ie, genetic screening) of all pregnant women would not be recommended. Many variants, especially truncating variants in *TTN*, are poorly penetrant,⁵ and identifying a variant in a subject not otherwise at risk does not offer sufficient positive predictive value for disease and would likely cause unnecessary follow-up studies and anxiety in patients and caretakers.

In short, from a genetics point of view, PPCM closely resembles DCM and may be part of a spectrum of disease. Recommendations for genetic testing and counseling for PPCM should thus mirror those for DCM.

ARTICLE INFORMATION

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Pathophysiology and risk factors of peripartum cardiomyopathy

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Abstract | Peripartum cardiomyopathy (PPCM) is a potentially fatal form of idiopathic heart failure with variable prevalence across different countries and ethnic groups. The cause of PPCM is unclear, but environmental and genetic factors and pregnancy-associated conditions such as pre-eclampsia can contribute to the development of PPCM. Furthermore, animal studies have shown that impaired vascular and metabolic function might be central to the development of PPCM. A better understanding of the pathogenic mechanisms involved in the development of PPCM is necessary to establish new therapies that can improve the outcomes of patients with PPCM. Pregnancy hormones tightly regulate a plethora of maternal adaptive responses, including haemodynamic, structural and metabolic changes in the cardiovascular system. In patients with PPCM, the peripartum period is associated with profound and rapid hormonal fluctuations that result in a brief period of disrupted cardiovascular (metabolic) homeostasis prone to secondary perturbations. In this Review, we discuss the latest studies on the potential pathophysiological mechanisms of and risk factors for PPCM, with a focus on maternal cardiovascular changes associated with pregnancy. We provide an updated framework to further our understanding of PPCM pathogenesis, which might lead to an improvement in disease definition.

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Peripartum cardiomyopathy (PPCM) is a form of heart failure that is associated with pregnancy^{1,2}. PPCM is defined as idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular (LV) systolic dysfunction in the peripartum phase (which encompasses the period leading up to the end of the pregnancy, the time of delivery and the months after delivery), in which no other cause of heart failure is found^{1,3–5}. Diagnosis generally follows the exclusion of other similar conditions; differential diagnoses include pre-existing dilated cardiomyopathy (DCM), familial cardiomyopathy, myocarditis, Takotsubo cardiomyopathy and valvular heart disease^{1,2,5,6}. PPCM outcomes are highly variable as evidenced by registry data from the ESC EURObservational Research Programme (EORP)⁴. Myocardial recovery (that is, LV ejection fraction (LVEF) >50%) was observed in 46% of patients 6 months after diagnosis of PPCM but persistent severe LV dysfunction or death occurred in 28%⁴. Patients with PPCM often have hypertension and heart palpitations and might have a long-term elevated risk of sudden death, arrhythmia and other cardiovascular complications⁷. A need for long-term drug therapy is common in this patient cohort, even among those with fully recovered LV function^{7,8}.

PPCM incidence seems to vary markedly depending on geographical region but inconsistent disease

definitions between different regions have precluded a direct comparison of studies. Countries with the lowest reported incidence of PPCM (per live birth) include Japan (1 in 16,667)^{9,10}, Denmark (1 in 10,000)¹¹ and Sweden (1 in 5,882)¹². Countries with the highest incidence of PPCM include Nigeria (1 in 100)¹³, Haiti (1 in 333)¹⁴, Pakistan (1 in 840)¹⁵ and South Africa (1 in 1,000)¹⁶. The estimated incidence of PPCM in Germany is 1 in 1,000–1,500 live births¹⁷, whereas the incidence of PPCM in the USA seems to have increased over the past 20 years from 1 in 1,200 live births to 1 in 910 live births¹⁸.

Although various pathophysiological mechanisms are proposed to contribute to the development of PPCM^{19–23}, their clinical relevance remains to be confirmed. PPCM is commonly thought to be a multifactorial syndrome, whereby several known and unknown factors related to pregnancy might result in PPCM; that is, a ‘multiple-hit model’. This hypothesis is supported by the observation that mice with a cardiac-specific knockout of either *Stat3* (encoding signal transducer and activator of transcription 3 (STAT3)) or *Pparg1a* (encoding peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α)) develop PPCM^{19,20}. These mice did not have any heart failure-related symptoms before pregnancy but developed severe heart failure postpartum^{19,20}.

Key points

- Physiological cardiovascular changes during pregnancy mediate the development of peripartum cardiomyopathy (PPCM) in women who are predisposed to the disease.
- Risk factors for PPCM include the presence of genetic variants that are common in other types of cardiomyopathy as well as low socioeconomic status, pre-eclampsia and a history of cancer.
- PPCM onset typically overlaps with the most extreme fluctuations in hormone levels during pregnancy that occur during the third trimester and at birth.
- Studies involving mouse models of PPCM demonstrate that the disease results from dysregulated metabolic pathways and angiogenic imbalance, possibly owing to aberrant hormonal signalling.
- Our understanding of the pathophysiology underlying PPCM is mainly derived from studies on animal models, which require confirmation in patient studies, but these preclinical data form the basis of current clinical guidelines and future experiments.

A 2020 study that examined human induced pluripotent stem cells (hiPSCs) derived from patients with PPCM highlighted a role for cardiomyocyte metabolism in the pathogenesis of PPCM²⁴. Several pregnancy-associated hormones, including fibroblast growth factor 21 (FGF21), progesterone, prolactin, oestrogen and soluble fms-like tyrosine kinase 1 (sFLT1), are involved in coordinating cardiac metabolic processes^{25–27}. Impaired metabolism in cardiomyocytes derived from patients with PPCM and the metabolic effects of pregnancy hormones together might indicate that the hearts of patients with PPCM cannot cope with the fluctuations in hormones and the downstream metabolic changes that occur during the peripartum period.

In this Review, we discuss the latest research investigating the potential pathophysiological mechanisms and risk factors involved in PPCM, with a focus on the physiological changes that occur during pregnancy. We provide an updated framework to improve our understanding of PPCM pathogenesis, which might lead to better disease definition.

Cardiovascular adaptations in pregnancy

As described above, PPCM is hypothesized to result from a ‘multiple-hit model’, whereby external triggers and predispositions interact to cause disease. Although a putative predisposing risk factor remains elusive (but is most likely to be genetic), far more is known about the effects of pregnancy on the cardiovascular system that could trigger the development of PPCM. Hormones are the key regulatory elements that coordinate the physiological adaptations that occur during and after pregnancy. Cardiovascular adaptations to pregnancy include haemodynamic and structural changes, vascular remodelling and bioenergetic shifts. An impairment in these adaptive processes is thought to contribute to the pathogenesis of PPCM, but the exact mechanisms are still unclear, necessitating additional research^{5,20,28}. The following section provides a basis for such research by summarizing what is known about the physiological processes that occur during pregnancy from a cardiovascular perspective.

Haemodynamic changes

Pregnancy is associated with an increasing blood volume that leads to a chronically elevated cardiac volume load²⁹. As a result, cardiac output increases from the second

trimester to delivery, which corresponds with an increase in heart rate of approximately 20% and stroke volume of approximately 25%^{30–33}. Increases in stroke volume have been found to be higher in subsequent pregnancies than in the first pregnancy³⁰. Vascular resistance also decreases by approximately 30% in the first trimester and is restored to normal levels after delivery^{30–33}. In 2020, a multicentre, longitudinal study in 4,279 women demonstrated that median systolic and diastolic pressures briefly declined during early pregnancy but by late gestation rose by 7 mmHg and 9 mmHg, respectively, above nominal pressures³³. These changes seem to be small and could explain the inconsistency of the previous studies³³. Whether haemodynamic changes can lead to PPCM remains unknown but low systolic blood pressure and elevated heart rate have been associated with a worse outcome in patients with PPCM³³.

Structural changes

Alongside haemodynamic changes, the hearts of pregnant women undergo substantial structural remodelling. Both left and right ventricular end-diastolic diameters have been shown to increase by ~20% by late pregnancy, whereas left end-systolic diameter did not change between the third trimester and 3 months postpartum³⁴. A meta-analysis of 48 studies reported that LV mass was approximately 28% higher in the last trimester of normotensive pregnant women than in non-pregnant women³⁵. Together, these observations are indicative of gestational cardiac hypertrophy. Of note, cardiac dimensions and estimated weights during pregnancy were often compared at variable postpartum time points. Although these structural changes are known to be transient, whether heart dimensions can fully return to baseline (that is, pre-pregnancy levels), or the time required to do so, is unknown. Additionally, several histological studies in rodents have indicated that, unlike in pathological cardiac hypertrophy, the extensive cardiac remodelling that occurs during or after pregnancy does not involve fibrotic growth^{36–38}. However, similar histological studies have not been performed in healthy pregnant women at prepartum or postpartum given the risks associated with performing cardiac biopsies. Patients with PPCM can present with various cardiac phenotypes, including ventricular dilatation¹, borderline non-compaction cardiomyopathy^{39,40} and peripartum Takotsubo cardiomyopathy^{41,42}, whereas normal pregnancy is associated with reversible eccentric cardiac hypertrophy⁴³. This disparity might indicate that regulatory mechanisms involved in physiological cardiac remodelling during and after pregnancy in patients with PPCM could be impaired, leading to a decompensated phenotype. Genetic variants of structural genes have been linked with PPCM and are discussed in detail below.

Vascular remodelling

The balance between cardiac hypertrophy and vascular growth is crucial for the maintenance of adequate cardiac function during pregnancy. In concert with increased ventricular mass, the vasculature undergoes numerous adaptations during pregnancy. Of note, our understanding of vascular changes during pregnancy

is mostly derived from rodent studies. In mice, capillary density has been shown transiently to increase in late pregnancy^{19,36}. Pro-angiogenic genes, including *Angpt1*, *Fgf2*, *Ppargc1a* and *Vegfa*, are activated in early and mid-gestation but return to pre-pregnancy levels in late gestation⁴⁴. This finding is in line with the observed antiangiogenic environment associated with late gestation²⁰. These observations in rodents corresponded with the serum levels of placental growth factor (PlGF; a member of the vascular endothelial growth factor (VEGF) family that is predominantly expressed in the placenta) in humans, which reach a peak in the second trimester before returning to baseline levels in the last trimester⁴⁵. By contrast, circulating VEGF levels in humans seem to be stable in the first two trimesters before increasing near term⁴⁶. Like VEGF, serum levels of sFLT1 are also elevated in late pregnancy^{47–49}. Given that sFLT1 readily binds to circulating VEGF, whether the elevated level of VEGF is a reflection of levels of free VEGF or inactivated VEGF that is bound to sFLT1 is unknown. Therefore, this response might be a physiological adaptation to regulate and maintain angiogenic balance, both systemically and locally²⁰. Disruption of this delicate balance is a key factor in the development of pre-eclampsia⁵⁰ and is likely to be involved in the pathogenesis of PPCM. Vascular dysfunction and remodelling in the peripartum period are key aspects of PPCM pathophysiology and are discussed below.

Cardiac metabolism during pregnancy

The maternal heart undergoes unique bioenergetic changes during pregnancy, which are tightly regulated during each gestational phase. In a normal, non-pregnant, fasted state, the human heart utilizes free fatty acids (FFAs) as the primary source of energy^{51,52}. Other metabolic substrates include ketones, lactate and amino acids^{51,52}. Although glucose is one of the principal metabolic substrates for most human tissues, several studies have demonstrated that, in a healthy individual,

the heart consumes very little glucose, at least in the fasting state^{51,52}. As the pregnancy progresses, maternal metabolism shifts from a predominantly anabolic state with increased fat stores to a catabolic state with reduced fat mass and elevated levels of circulating FFAs to meet the energetic needs of the fetus⁵³. The shift from an anabolic state to a catabolic state is characterized by a profound increase in maternal basal metabolic rate by up to 60%⁵⁴. Insulin signalling has a pivotal role in coordinating this shift. Insulin resistance gradually develops with gestation and results in hyperglycaemia and hyperinsulinaemia by late pregnancy^{55,56}. Consequently, glucose uptake is limited in the mother and is shunted in the fetus. Very little is known about how cardiac metabolism changes during pregnancy in humans, but animal studies have provided insight into the associated molecular mechanisms. Early studies in rats showed a reduction in cardiac glucose oxidation by approximately 75% during pregnancy⁵⁷, and studies in dogs indicated a similar suppression of glucose utilization and a near-doubling of FFA oxidation during late pregnancy⁵⁸. Despite this metabolic shift towards FFA oxidation, and in contrast to insulin resistance in the liver and skeletal muscle, the hearts of mice in late pregnancy retain insulin sensitivity (defined as the capacity to activate the insulin signalling cascade)⁵⁹. The mechanisms underlying these metabolic changes are not well understood but are likely to include inhibition of glycolysis by high levels of FFA in late pregnancy according to the Randle cycle (a metabolic process involving the competition of glucose and FFA for substrates)⁶⁰ and specific cellular reprogramming through hormonal signalling such as progesterone induction via pyruvate dehydrogenase kinase 4, an endogenous inhibitor of pyruvate dehydrogenase and thus of carbohydrate use⁶¹ (FIG. 1). Determining how cardiac bioenergetics are regulated is crucial to understanding the underlying mechanisms of cardiovascular disease in general. Deletion of PGC1 α results in angiogenic imbalance²⁰ but, given that PGC1 α is also a key regulator of major metabolic pathways, especially those related to fatty acid oxidation, PGC1 α deficiency likely also results in metabolic imbalance⁶². Indeed, cardiomyocytes from patients with PPCM had reduced viability and metabolic flexibility upon inhibition of lipid metabolism²⁴. Therefore, impaired metabolic regulation might be a key component underlying the pathophysiology of PPCM.

Cardiovascular effects of hormones

Sex-related and pregnancy-related hormones are the key modulators of the various stages of pregnancy. Several hormones, including oestrogen and progesterone, are known to profoundly affect the cardiovascular system, but the specific molecular mechanisms underlying these effects in humans are largely unknown given that the majority of studies have been performed in animal models (FIG. 2).

Oestrogen. Oestrogens are a class of sex hormones that govern the development of the female reproductive system and have an important role during pregnancy. Oestrogen levels increase gradually as the pregnancy progresses and decrease immediately after delivery⁶³.

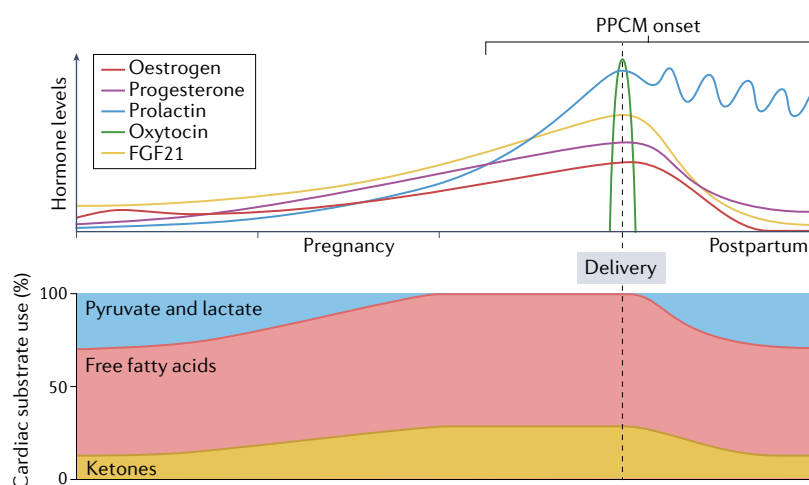


Fig. 1 | Pregnancy hormone levels correlate with cardiac bioenergetic profile. Fluctuating hormone levels correlate with changes in cardiac metabolic substrate utilization during pregnancy progression. Late gestation is associated with increased utilization of free fatty acids and ketones and reduced utilization of pyruvate and lactate. FGF21, fibroblast growth factor 21; PPCM, peripartum cardiomyopathy.

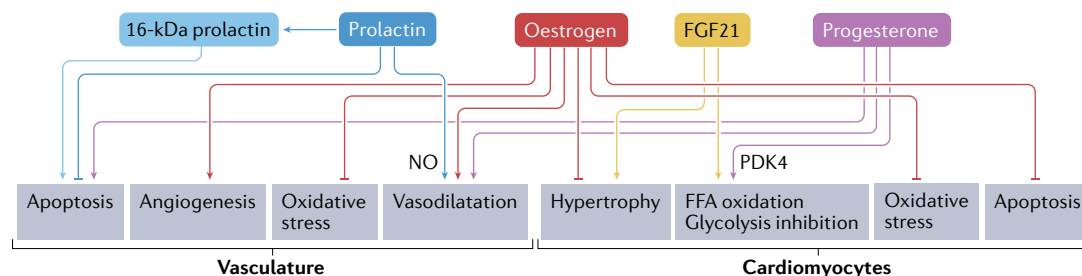


Fig. 2 | Pregnancy hormones elicit cardiovascular-specific effects. The pregnancy hormones prolactin (and its cleavage product 16-kDa prolactin), oestrogens, fibroblast growth factor 21 (FGF21) and progesterone have pleiotropic effects in the vasculature and in cardiomyocytes, including the promotion or inhibition of apoptosis, angiogenesis, metabolic signalling, vasodilatation, cardiac hypertrophy and oxidative stress. FFA, free fatty acid; NO, nitric oxide; PDK4, pyruvate dehydrogenase kinase 4.

Oestrogens exert mainly cardiovascular-protective effects⁶⁴ and induce angiogenesis and vasodilatation through increased nitric oxide (NO) synthesis^{65,66} as well as secretion of VEGF and PlGF^{67,68}. In addition, oestrogens can reduce inflammatory signalling and attenuate cardiac hypertrophy, and are protective against oxidative stress in endothelial cells and cardiomyocytes^{69–71}. Many of the protective effects of oestrogen result from its potent inhibition of apoptosis in cardiomyocytes and endothelial cells^{72,73}. A related class of receptors, the oestrogen-related receptors, have also been shown to widely regulate cardiac metabolism, contractility and conduction⁷⁴.

Progesterone. Like oestrogen, progesterone is primarily produced by the placenta during pregnancy, with increasing serum levels as the pregnancy progresses. Progesterone has been shown to protect against apoptosis by direct inhibition of the L-type voltage-dependent Ca^{2+} channel (in dogs) and via induction of *Bcl2l1* (in mice)^{75,76}. Furthermore, progesterone stimulation in the endothelium has been shown to trigger endothelial NO synthase-mediated synthesis of NO in both pregnant rats and pregnant women, which resulted in a marked reduction in vascular resistance^{77,78}. Numerous studies in animals have also demonstrated that progesterone can inhibit glycolysis via forkhead box protein O1-mediated pathways in tumours^{79,80}. In cardiomyocytes, progesterone can induce pyruvate dehydrogenase kinase 4 (PDK4) activity, which inhibits pyruvate dehydrogenase, an essential step in glycolysis⁵⁹.

Prolactin. The serum levels of prolactin peak at term and rapidly fall to pre-pregnancy levels after delivery if not repeatedly stimulated by breastfeeding⁸¹. The cardiovascular effects of prolactin in rats include a blunted response to angiotensin signalling⁸², protection of cardiomyocytes against intermittent hypoxia via activation of endothelial pro-survival signalling pathways⁸³ and regulation of endothelial vascular tone⁸⁴. However, these potential effects of prolactin are dose related, and the high dosages or concentrations often used in these rat studies preclude firm conclusions being drawn on the role of prolactin in humans. Prolactin signalling has been closely linked with PPCM pathogenesis and is discussed in detail below.

FGF21. A lesser-known pregnancy-related hormone is FGF21, which is mainly produced by the liver during pregnancy under the control of peroxisome proliferator-activated receptor- α ^{85–87}. Importantly, the heart is a target and a source of FGF21 (REF.⁸⁸). In mice, the downstream effects of FGF21 signalling in the heart were protective against pathological hypertrophy and prevented reactive oxygen species production^{88,89}. Furthermore, pregnant mice lacking FGF21 did not develop pregnancy-induced cardiac remodelling and had reduced levels of fatty acid oxidation, which might compromise cardiac function during pregnancy²⁶. These effects of FGF21 on the heart remain to be confirmed in humans, but FGF21 levels have been correlated with maternal BMI and adiposity⁹⁰. Moreover, fasting glucose levels were also inversely correlated with FGF21 levels, which might be an indication of maternal nutrient status in pregnancy⁹⁰. By extension, FGF21 has also been proposed as a biomarker for gestational diabetes mellitus and type 2 diabetes⁹¹.

Biomarkers and risk factors for PPCM

Although the exact cause of PPCM is currently unknown, several risk factors have been proposed, including heart failure-associated genetic defects⁹², ethnicity^{4,93}, hypertensive disorders⁹⁴, infections⁹⁵, twin and subsequent pregnancies⁹⁶ and history of cancer⁹⁷.

Biomarkers

A systematic review and meta-analysis of 31 case-control studies was conducted to compare the levels of 117 molecular biomarkers in patients with PPCM with those in healthy pregnant and postpartum women⁹⁸. Several biomarkers were identified as independent risk factors for PPCM (TABLE 1). Patients with PPCM had higher levels of natriuretic peptides, troponin, C-reactive protein and white blood cells but reduced levels of albumin and selenium compared with healthy pregnant controls⁹⁸. Of note, these biomarkers mainly reflect the presence of cardiomyopathy and are not sufficiently specific for the diagnosis of PPCM. However, other studies have also identified potential PPCM-specific biomarkers by comparing patients with PPCM with patients with other cardiovascular diseases. Serum levels of prolactin⁹⁹, microRNA-146a (miR-146a)^{21,100} and PlGF⁴⁹ were higher in patients with PPCM than in patients with heart failure who were not pregnant. Additionally, the ratio between

circulating sFLT1 and PlGF levels was proposed to have diagnostic value for PPCM⁴⁹. The identification of PPCM-specific biomarkers is a great unmet need and will substantially improve PPCM diagnosis and prognosis because targeted treatments can be started sooner.

Genetics

Given that PPCM and DCM share similar clinical characteristics, PPCM has been proposed to be part of the spectrum of DCM^{101–106}. Some patients diagnosed with PPCM might have had previously unrecognized DCM, although, in the few documented cases in which echocardiography was incidentally available before the clinical diagnosis of PPCM, LVEF was normal¹⁰². Genetic studies have indicated that variants of *BAG3* (encoding BAG family molecular chaperone regulator 3)^{92,106}, *DSP* (encoding desmoplakin)^{92,106}, *FLNC* (encoding filamin C)⁹², *LMNA* (encoding lamin A/C)^{92,106}, *MYH6* and *MYH7* (encoding myosin 6 and myosin 7, respectively)⁹², *TNNC1* (encoding cardiac troponin C)¹⁰¹, *TTN* (encoding titin)^{92,103–106} and *VCL* (encoding vinculin)^{92,106} have been identified in both patients with PPCM and patients with DCM. Specifically, truncating variants of *TTN* (the most common genetic cause of DCM) were found in approximately 10% of patients with PPCM¹⁰⁵. In addition, the frequencies with which variants in each of these genes are found in patients with PPCM closely mirror the same frequencies in patients with DCM, underscoring the similarity of genetic predispositions to both diseases^{105,107}. The association of variants in *BAG3* with PPCM has also prompted the hypothesis that various classes of molecular chaperones (such as heat shock proteins) could be involved in the pathogenesis of PPCM¹⁰⁸. A small genome-wide association study identified enrichment of a single nucleotide polymorphism near *PTHLH* (encoding parathyroid hormone-related protein) in 79 patients with PPCM, but this new locus requires further investigation before it can be linked to PPCM pathogenesis¹⁰⁹. Indeed, PPCM and DCM might be caused by similar gene variants, of which variants in *TTN* seem to be most prevalent. However, how these variants converge to cause PPCM remains to be investigated.

Geographical variation

Very little is known about the influence of geographical location on the incidence of PPCM despite the establishment of global initiatives such as the ESC EORP

PPCM registry. Of note, patients in this registry were not selected via consecutive screening but were chosen on the basis of PPCM diagnosis, and too few countries were included in this registry^{4,110}. An ideal registry should recruit patients via consecutive screening (that is, include all pregnant women and note the incidence of PPCM) and should involve as many countries as possible across all continents. However, such a registry is an ambitious endeavour. For now, conclusive data on the incidence of PPCM remain limited to regional studies. Consequently, the following section summarizes data from local studies (which employed non-consecutive screening of participants) and highlights the factors that should be considered in a global registry with consecutive patient recruitment.

A 2019 epidemiological report on the global incidence of PPCM indicated that the incidence was lowest in Japan and highest in Nigeria and Haiti¹⁰. The convergence of PPCM in these geographical hotspots supports the hypothesis that a specific genetic background predisposes individuals to PPCM^{92,108}, but environmental factors also have a role in increasing disease risk^{111,112}. Micronutrient deficiency and malnutrition are both examples of environmental factors that could predispose individuals to heart failure¹¹³ and PPCM. The 2020 PEACE registry, which consecutively recruited patients with PPCM from 22 hospitals in Nigeria, indicated that selenium deficiency and malnutrition were significantly associated with PPCM^{111,112} and that selenium supplementation could be beneficial for the treatment of PPCM¹¹⁴. Selenium deficiency is also common in other regions of sub-Saharan Africa (including Kenya, Mozambique and Zambia)¹¹⁵ and in the Keshan region in China¹¹⁶ but PPCM incidence in these regions is not known.

Race/ethnicity and socioeconomic status

Studies from the USA that include a diverse population cohort treated within the same health-care system corroborate that race is an important risk factor for PPCM. The patient cohort of numerous PPCM studies from the USA is comprised of >40% of African American individuals and 35% white individuals^{18,93,117,118}, despite African American individuals making up only 13.4% of the population¹¹⁹. Furthermore, compared with white patients with PPCM, African American patients with PPCM were younger, had a higher prevalence of gestational hypertension, a lower LVEF at diagnosis and slower functional recovery^{120,121}. However, given the well-established disparities in socioeconomic status between white and African American individuals in the USA, differences in the incidence and presentation of PPCM between the two populations might be attributable to environmental factors such as income or education level¹²². An interim report from the ESC EORP PPCM registry established that patients from countries with middle-to-high health expenditure were predominantly white (64.6% of the population versus 5.1% of Black individuals)¹²³. Conversely, patients from countries with low health expenditure were mostly Black or Asian (45.2% and 39.4%, respectively)¹²³. In this regard, low socioeconomic status (specifically lower education) has

Table 1 | Potential biomarkers of peripartum cardiomyopathy compared with normal pregnancy

Biomarker	Odds ratio	95% CI	Refs
Autoantibodies against β_1 adrenergic receptors and M_2 muscarinic receptors	18.8	1.9–183.3	¹⁶⁸
Anti-myocardial IgG	2.68	1.19–4.85	¹⁶⁹
N-terminal pro-B-type natriuretic peptide	1.92	1.12–4.15	¹⁷⁰
High-sensitivity C-reactive protein	1.86	1.08–4.02	^{169,170}
Uric acid	1.336	1.049–1.614	¹⁷¹
Presence of ACE polymorphism	0.253	0.114–0.558	¹⁷²

been associated with worse clinical outcomes in PPCM independent of race¹²².

Unsurprisingly, the incidence of PPCM is closely correlated with all-cause maternal mortality¹⁰. In general, maternal mortality is higher in low-income countries than in middle-to-high-income countries owing to reduced access to health care, suboptimal treatment regimens and low hospitalization rates¹⁰. In addition, birth rates are generally higher in low-income countries. Suboptimal health care (such as lack of genetic screening), a high birth rate and more subsequent pregnancies might indirectly contribute to the high incidence of PPCM in countries such as Nigeria^{124,125}.

Pre-eclampsia

Like PPCM, pregnancy-associated hypertension and pre-eclampsia can affect pregnant women during late gestation. Pre-eclampsia is defined as new-onset hypertension and proteinuria or new-onset hypertension with end-organ dysfunction with or without proteinuria after 20 weeks of gestation¹²⁶. The exact relationship between PPCM and pre-eclampsia is not fully understood but pre-eclampsia strongly predisposes patients to PPCM^{127,128}. Whether the increased cardiovascular risk of PPCM in patients with pre-eclampsia is attributable to direct consequences of the underlying cause of pre-eclampsia or to shared risk factors is currently unknown^{28,50,94}. A substudy of the ESC EORP PPCM registry described the differences in phenotypes and outcomes of patients with PPCM with or without hypertension and patients with PPCM plus pre-eclampsia⁹⁴. In comparison with patients with PPCM without hypertension, patients with PPCM plus pre-eclampsia presented with worse symptoms and more frequent signs of heart failure but had a greater likelihood of LV recovery (that is, LVEF $\geq 50\%$)⁹⁴. Patients with PPCM plus pre-eclampsia were also more likely to have peripheral oedema, pulmonary rales, short QRS duration and NYHA class IV heart failure symptoms than patients with PPCM without pre-eclampsia⁹⁴. A possible reason for improved outcomes in patients with both conditions might be that these patients are diagnosed and treated earlier than patients with PPCM alone¹²⁹.

Immune responses

PPCM is also associated with specific immune responses (possibly mediated by viral infections¹³⁰) that might increase susceptibility or result in worse outcomes. Patients with PPCM have higher serum levels of inflammatory biomarkers, such as C-reactive protein, tumour necrosis factor (TNF) and IL-6, than healthy controls¹³¹. A substudy of the IPAC trial demonstrated that levels of IL-22 and TNF were associated with adverse outcomes in patients with PPCM, whereas levels of IL-22 and IL-17 corresponded with disease severity, and levels of IL-2 and IL-4 correlated with recovered LVEF at 12 months postpartum¹³². Levels of circulating natural killer cells were also reduced in patients with PPCM during the early postpartum period (<6 weeks) compared with healthy pregnancy-matched controls, whereas levels of specific subsets of T cells were elevated¹³³. These immune cell levels normalized over time postpartum

but Black women had delayed recovery of natural killer cell levels compared with white women¹³³. Furthermore, autoantibodies against troponin I or cardiac sarcomeric myosin were also found in patients with PPCM, which correlated with lower baseline LVEF and a lower rate of full cardiac recovery at follow-up¹³⁴. Additional studies are required to determine the mechanistic pathways mediating these immune responses and their pathophysiological relevance for PPCM development and progression.

Subsequent pregnancies

Subsequent pregnancies pose an increased risk of worsening or recurring heart failure in patients with a history of PPCM^{135,136}. Unrecovered LV function at the time of a subsequent pregnancy has been associated with a higher risk of death regardless of age, blood pressure level, gravidity, parity and smoking status¹³⁶. Furthermore, subsequent pregnancies in patients with a history of PPCM have also been associated with significantly reduced LVEF, regardless of LVEF recovery after the index pregnancy^{135,136}. Mortality after the subsequent pregnancy was significantly higher in patients with persistently impaired LVEF (<50%) than in patients with recovered LVEF^{135,136}. Therefore, PPCM could result in persistent subclinical cardiac dysfunction, and subsequent pregnancies might exacerbate this dysfunction. Cardiac function is likely to decline further with each subsequent pregnancy^{137,138}.

Cancer

In a cohort of 236 patients with PPCM from Germany and Sweden, the prevalence of cancer was 16-fold higher than in age-matched women without PPCM⁹⁷. Of the 21 patients with cancer, 12 were diagnosed before PPCM presentation. Of note, 11 of these patients were treated with cardiotoxic cancer therapies, which are likely to have contributed to the deterioration of LV function when PPCM developed, resulting in delayed full cardiac recovery thereafter⁹⁷. Whole-exome sequencing revealed that 6 of 14 screened patients were carriers of potential pathogenic genetic variants associated with cardiomyopathy or cancer predisposition syndromes⁹⁷. However, in our study of South African patients with PPCM, we did not find any association between cancer and PPCM diagnosis (K.S., unpublished observations). Therefore, whether screening for genetic variants or cancer in patients with PPCM is warranted remains to be determined.

Pathophysiology of PPCM

Very little is known about the pathophysiology of PPCM in humans. The risk factors described above provide some insight into the condition of patients with PPCM at the time of diagnosis and at various follow-up periods but these data do not support inferences about the pathological mechanisms that eventually cause PPCM. To date, the majority of the mechanistic data available have been obtained from animal models that presented a disease phenotype that is similar to PPCM in humans. Although these animal models have helped to shape some of the clinical guidelines and hypotheses about

pathogenesis, most of the putative mechanisms remain to be confirmed in humans.

Mouse models of PPCM

The two main mouse models used to study PPCM are characterized by cardiomyocyte-specific deletion of *Stat3* or *Ppargc1a*^{19,20}. Mice with either genotype develop severe heart failure postpartum that closely resembles PPCM, with increasing severity in subsequent pregnancies^{19,20}. Abrogation of STAT3-mediated or PGC1 α -mediated signalling pathways in these mice resulted in reduced levels of the antioxidant manganese superoxide dismutase and impaired the physiological response to oxidative stress related to late pregnancy and early postpartum^{19,20,139}. The PI3K–AKT pathway is thought to be cardioprotective during pregnancy but transgenic overexpression of *Akt* in concert with *Stat3* knockout did not prevent the onset of PPCM in mice¹⁴⁰. Consequently, in mice with a *Stat3* knockout, stressed cardiomyocytes secrete the ubiquitous lysosomal protease cathepsin D in response to hypoxia, mechanical stretch and oxidative stress through regulated exocytosis^{19,141,142}. During the peripartum period, extracellular cathepsin D can cleave prolactin to form 16-kDa prolactin^{19,143,144}, which is classified as a vasoconstrictor and is part of a family of peptides that elicits antiangiogenic effects¹⁴⁵. The 16-kDa prolactin fragment can interact with the urokinase plasminogen activator surface receptor on the cell membrane of adjacent endothelial cells, and endocytosis can be induced by circulating plasminogen activator inhibitor 1 (REFS^{21,23,146}). This mechanism effectively inhibits migration and cell cycle progression and induces apoptosis in endothelial cells, subsequently impairing the cardiac microvasculature^{147–149}. Endothelial cells then secrete exosomes loaded with miR-146a, which are taken up by surrounding cardiomyocytes²¹. miR-146a has been shown to reduce protein expression

of receptor tyrosine-protein kinase erbB4 (ERBB4) and neuroblastoma RAS viral (V-ras) oncogene homologue (NRAS) in cardiomyocytes^{21,150}. NRAS expression is typically low in cardiomyocytes but, given that ERBB4 signalling is involved in cardiac development and metabolic processes, reduced expression of ERBB4 might have a role in the pathogenesis of PPCM^{151,152}. In addition, PGC1 α regulates the expression and production of VEGF and therefore facilitates angiogenesis. This pathway is likely to offset the antiangiogenic effects of elevated sFLT1 levels at term, and cardiac deletion of PGC1 α predisposes mice to cardiomyopathy even in the absence of pregnancy^{20,153}. Together, impaired STAT3 and PGC1 α signalling in mice have been shown to induce a phenotype similar to that of PPCM in humans by mediating angiogenic imbalance and abnormal metabolic regulation (FIG. 3).

Translating pathophysiology from mice to humans

The preclinical data described above provided a strong basis for the clinical assessment of prolactin inhibition in patients with PPCM^{154,155}. Bromocriptine is a dopamine agonist that suppresses prolactin release from the pituitary gland and was hypothesized to ameliorate the adverse effects of 16-kDa prolactin. However, translating the findings from mice to humans has proved difficult. For example, the mouse models of PPCM were characterized by cardiomyocyte-specific deletion of *Stat3* or *Ppargc1a*, and are not representative of patients with PPCM. Nevertheless, STAT3 is an important regulator of inflammation, and STAT3 activation and expression were greatly reduced in the hearts of patients with DCM or PPCM, which suggests that STAT3 is essential to mount an adequate response to cardiac stress^{19,156}. One study also showed that circulating prolactin levels during nursing were higher in patients with PPCM than in healthy pregnancy-matched controls⁹⁹ but these findings need further confirmation. A German cohort study demonstrated that serum levels of cathepsin D and miR-146a were also significantly elevated in patients with PPCM¹⁰⁰, whereas normal levels of miR-146a were observed in patients with PPCM who had received early bromocriptine treatment¹⁰⁰. In addition, increased circulating levels of 16-kDa prolactin have been shown in patients with PPCM¹⁹ but, given the lack of quantitative assays for vasoconstrictors, no reference ranges or serum levels for the disease have been established. The role of 16-kDa prolactin in the pathophysiology of PPCM remains a topic of debate because cathepsin D produces five distinct vasoconstrictors, four of which are potent antiangiogenic agents^{144,157}. Moreover, human prolactin is not readily cleaved by cathepsin D in most extracellular conditions, and enzymatic cleavage is dependent on pH levels^{158,159}.

Extrapolating the data on the role of prolactin in PPCM from animal models to patients remains difficult, but the notion of angiogenic imbalance in PPCM pathophysiology is promising¹⁶⁰. The systemic antiangiogenic state that is present during late pregnancy and early postpartum was negated by local VEGF production in the hearts of mice²⁰. Furthermore, excessive levels of sFLT1 were associated with vascular dysfunction and

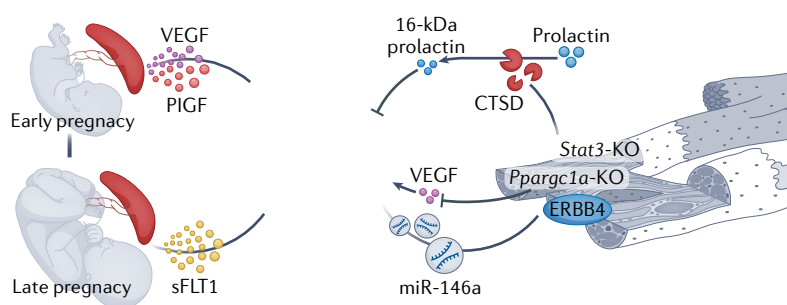


Fig. 3 | Proposed pathogenic pathways in peripartum cardiomyopathy. During early gestation, the placenta secretes high levels of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) into the maternal circulation, which stimulate vascularization and organ perfusion. As pregnancy progresses, circulating levels of VEGF and PIGF rise in concert with an increase in the secretion of the angiostatic soluble fms-like tyrosine kinase 1 (sFLT1). Alternatively, cardiomyocyte stress induces the exocytosis of the proteolytic enzyme cathepsin D (CTSD), which cleaves prolactin into a 16-kDa fragment that is cytotoxic to endothelial cells. Consequently, endothelial cells secrete exosomes loaded with microRNA-146a (miR-146a) that inhibit various cardiomyocyte processes, including receptor tyrosine-protein kinase ERBB4-mediated metabolism. Both mechanisms have a central role in the heart and might contribute to the development of peripartum cardiomyopathy. Of note, mice with a cardiac-specific knockout (KO) of either *Stat3* or *Ppargc1a* develop peripartum cardiomyopathy.

pre-eclampsia in humans¹⁶¹, which together support the hypothesis that PPCM might be a predominantly vascular disease of the heart. Therapies that promoted angiogenesis were effective in a rat model of pre-eclampsia and a mouse model of PPCM¹⁶². PPCM onset is generally later than pre-eclampsia onset in humans but angiogenic imbalance might be pivotal in both diseases. Serum levels of sFLT1 and PlGF and the ratio of sFLT1 to PlGF are used to diagnose pre-eclampsia and might also be useful for the diagnosis of PPCM^{48,49}. Therefore, from a vascular perspective, PPCM and pre-eclampsia might be part of a spectrum of cardiovascular diseases associated with vascular dysfunction.

Cardiac metabolism in PPCM

Pregnancy has evolved as a tightly regulated process; any disruptions to this process often result in major consequences for both the mother and child. Insights into specific aberrant metabolic pathways in patients with PPCM are scarce. Numerous genetic factors are hypothesized to cause cardiovascular distress that eventuates in PPCM. We have attempted to identify abnormal metabolic pathways present in PPCM by analysing cardiomyocytes obtained from hiPSCs (hiPSC-CMs) derived from patients with PPCM²⁴. To mimic pregnancy-related cardiac volume overload, cultured hiPSC-CMs were subjected to cyclic mechanical stretch. Although mechanical stretch resulted in differential expression of 2,647 genes, 1,248 of which were specific to the hiPSC-CMs, computational pathway analysis was ambiguous, suggesting that mechanical stretch did not elicit pathological effects in these cardiomyocytes²⁴. By contrast, 95 genes were differentially expressed in all stretched hiPSC-CMs and static hiPSC-CMs derived from patients with PPCM but not in static hiPSC-CMs derived from controls. The majority of enriched pathways were found to be related to lipid metabolism. Cardiac lipid metabolism is known to be abnormal during cardiac stress and disease^{51,52,163}. However, dysregulation of lipid metabolic pathways was also functionally confirmed in vitro in hiPSC-CMs from patients with PPCM and in isolated cardiomyocytes from mice with cardiac-specific *Stat3* knockout²⁴. The majority of differentially expressed genes were found to be controlled by several shared transcription factors, including nuclear transcription factor Y, transcription factor Sp1 and sterol regulatory element-binding protein 1. These results highlight a potential link between abnormal regulation of metabolism and endocrine-mediated regulation of cardiac metabolism during pregnancy. To date, no specific mutations have been found that directly link impaired regulation of metabolism to PPCM pathogenesis. However, increasing evidence on various mutations in cardiac-specific genes might support this hypothesis. Truncating variants in *TTN*, the gene encoding titin, are associated with abnormalities in both metabolic signalling¹⁰⁷ and PPCM^{92,104,105}, and have been shown to be pleiotropically detrimental to cardiac function¹⁶⁴. Truncating variants in *TTN* can lead to increased interstitial fibrosis and mitochondrial dysfunction in the heart¹⁰⁷. Additionally, mutations in the genes encoding the sarcomeric proteins troponin C and troponin T in mice have been shown

to modify calcium-binding affinity during contraction, which altered ATP consumption and increased energy demand^{165,166}. Whether these alterations in sarcomeric expression are also present in patients with PPCM is unknown but such changes might predispose patients to the disease.

Pregnancy gradually introduces various cardiovascular stressors. The steady increase in stress as the pregnancy progresses might be slow enough for the cardiovascular system to adapt and cope, but perhaps the reversal of most pregnancy-related changes immediately after delivery might overwhelm the heart. Furthermore, the conditions of late pregnancy might also present specific challenges for the cardiovascular system, which could explain the onset of PPCM during the last trimester. For example, high levels of progesterone and FGF21 might disrupt the metabolic balance in the heart and contribute to cardiac stress^{26,59}. Fluctuations in the levels of prolactin-derived vasoinhibins might also disrupt the delicate angiogenic balance, resulting in impaired vascular function^{20,21}. Given that vascular function is related to the supply of metabolic substrates and hypoxia, impaired vascular function could also induce cardiac metabolic stress¹⁶⁷.

Future directions

The majority of studies on PPCM have correlated the clinical characteristics of the disease with potential biomarkers but how these correlations relate to the underlying pathophysiology of PPCM is largely unknown. Importantly, representative animal models for a putative 'multiple-hit' disease like PPCM are difficult to create, especially given the numerous risk factors involved in PPCM development. The design of genetic studies to determine the pathological genetic factors involved in PPCM pathophysiology is also limited by inconsistent disease definitions between countries, which will dictate the nature of patients included in cohort studies. To standardize disease definition, the metabolic substrates utilized by the heart could be identified at the time of PPCM diagnosis^{51,52}. Furthermore, analyses could be expanded beyond metabolomics to also include (targeted) proteomics, which will establish the levels of hormones and other relevant endocrine factors. These analyses could be repeated during and after recovery from PPCM to elucidate molecular changes in the circulation. However, these studies would be mildly invasive and would be likely to be limited to patients with postpartum PPCM. Given that cardiac biopsy samples are often unobtainable in patients with PPCM, the molecular profile of other related tissues, such as skeletal muscle, might provide additional insight into disease pathophysiology. Further genetic screening could be performed to identify genetic variants in coding and non-coding genes but, at present, these studies are hampered by high costs, low sample availability, and technological and statistical limitations. Several genetic variants have been linked with PPCM⁹² but the differences in genetic variants between individuals and the number of identified genes, in addition to the scarcity of patients with PPCM, greatly limit the statistical power to improve genetic screening studies at present. When a potential

pathogenic factor for PPCM is identified, animal models could be developed. Of note, certain animal models are more suitable for epigenetic analyses, pregnancy studies or for determining analogous hormone regulation and function. Of note, most molecular aspects of pregnancy are conserved in placental mammals but duration and placentation differ greatly between species. Animals with a longer gestation period than that of mice and with organogenesis complete at birth, such as the guinea pig, might be preferable for use as a model for pregnancy research.

Conclusions

PPCM is a complex disease with many risk factors and proposed aetiologies. Given that the pathogenesis of PPCM in humans is poorly understood, diagnosis is difficult and early targeted screening is advised. Extensive cohort studies, such as the ESC EORP PPCM registry, are crucial for improving our understanding of the clinical presentation, risk factors and prognosis of PPCM⁴, but a global registry with consecutive patient selection would provide valuable incremental knowledge on the geographical incidence of PPCM. Moreover, insights into genetic factors that predispose women to PPCM from the past decade indicate that genetic evaluation might be advisable for patients with a family history

of cardiomyopathies. Importantly, the cardiovascular system undergoes fundamentally different changes in late pregnancy compared with early in the postpartum period, and the underlying cause for PPCM might be related to the changes that occur during these stages or by the transition caused by delivery. From a mechanistic perspective, PPCM seems to result from heart failure secondary to vascular dysfunction. Studies of two mouse models of PPCM have demonstrated that angiogenesis during the peripartum period is tightly regulated and sensitive to detrimental stimuli^{19,20}. Data from these animal models form the basis for several clinical studies and recommendations despite a poor understanding of how observations from these preclinical studies can be extrapolated to patients. Therefore, mechanistic studies in patients with PPCM are needed to gain further insight into the pathophysiology of PPCM. Our group has shown that hiPSC-CMs derived from patients with PPCM show metabolic impairment in vitro²⁴. Pregnancy hormones regulate maternal metabolism and angiogenesis during and after pregnancy. Taken together, pregnancy hormones might be the link between the delicately balanced angiogenic state and potentially unstable metabolic processes in the hearts of patients with PPCM.

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Research Letter

Clinical Predictors of Referral for and Yield of Genetic Testing in Peripartum Cardiomyopathy

The genetic architecture of peripartum cardiomyopathy (PPCM) and dilated cardiomyopathy (DCM) overlap, and 15% of patients with PPCM have a disease-causing variant in a DCM-associated gene.¹ This shared genetic pathobiology suggests that a subset of PPCM may be on the same spectrum as genetic DCM. Despite the impact of genetics on prognosis, risk stratification, and cascade screening, there are no consensus recommendations for genetic testing in PPCM.^{2,3}

We aimed to identify characteristics that affect referral of women with PPCM for clinical genetic testing and to describe factors associated with having a pathogenic variant in a gene causal for DCM. We conducted a retrospective study of 205 women with PPCM who received care within the University of Pennsylvania Health System (Philadelphia, Pennsylvania, USA) from 1986 to 2018 (ie, clinical cohort).⁴ We determined the frequency of referral for clinical genetic testing and examined differences in referral on the basis of age at PPCM diagnosis, race, pregnancy and comorbidity characteristics, family history, and referral to a heart failure specialist. We then examined these same characteristics among the subset of patients who underwent sequencing of 67 DCM genes as part of a separate research study¹ and stratified by the presence of a pathogenic variant (ie, research cohort).

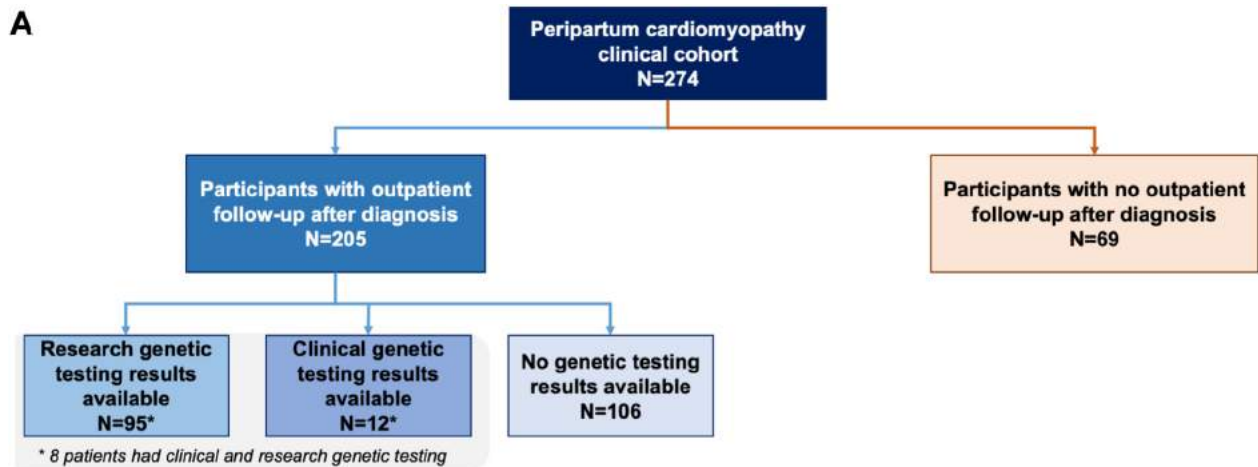
Descriptive statistics are reported as frequencies and percentages for categorical variables and median (IQR) for continuous variables with comparisons using Student's *t*-test and Pearson's chi-square test as appropriate. The α threshold was specified at 5% to determine statistical significance. All analyses were performed using SAS software version 9.4 (SAS Institute, Inc). The study was approved by the

University of Pennsylvania Institutional Review Board.

Of 205 patients with PPCM, 23 (11%) were referred for genetic testing and met with a genetic counselor (Figure 1). Individuals referred for genetic testing were more likely to have a family history taken at the first cardiology visit (78.3% vs 55.3%) and a first-degree relative with heart failure or cardiomyopathy (39.1% vs 12.6%; $P = 0.003$). Of the 23 patients referred for genetic evaluation, 12 (52%) completed testing. Two of these 12 (17%) patients had a pathogenic or likely pathogenic variant in *TTN* (titin) and were recommended for cascade screening.

In the cohort of 95 women with PPCM who underwent research testing, 13 (13.7%) had a pathogenic variant in a DCM-associated gene (10 in *TTN*, 1 in *DSP* [desmoplakin], 1 in *MYH7* [myosin heavy chain 7], 1 in *RBM20* [RNA binding motif protein 20]). Compared with those patients without a pathogenic variant, these women had a significantly lower left ventricular (LV) ejection fraction at diagnosis (15.0% vs 28.8%; $P = 0.008$). However, there were no differences between the 2 groups with regard to timing of PPCM diagnosis, frequency of pregnancy-related comorbidities, or presence of a family history of heart failure or cardiomyopathy.

Overall, we found that only about 50% of women with PPCM have any family history information documented in the medical record. A minority of patients were referred for clinical genetic counseling and testing, and the diagnostic yield of genetic testing in each of the clinical and research cohorts was approximately 15%, which is similar to the rate established in multiple previous reports.⁵ Referral for genetic evaluation was largely driven by recognition of a relevant family history; however, in unbiased genetic testing, there was no association between a positive family history and genotype-positive status. Thus, limiting genetic evaluation to women with a positive family history seems unfounded. We also found that severe LV systolic dysfunction at PPCM diagnosis was strongly associated with the presence of a pathogenic variant, thus indicating that patients with severely depressed LV function on PPCM presentation should be prioritized for genetic evaluation.

FIGURE 1 Study Inclusion Flowchart and Results

B

Characteristic	Clinical Cohort (N = 205)				Research Cohort (N = 95)			
	n	Genetics Referral (n = 23)	No Genetics Referral (n = 182)	P Value	n	Pathogenic Variant (n = 13)	No Pathogenic Variant (n = 82)	P Value
Median age at diagnosis, y	205	32 (26-24)	30 (26-34)	0.35	95	30 (20-34)	30 (26-33)	0.35
Race	205			0.76	95			0.77
Black		12 (52.2)	101 (55.5)			7 (53.8)	48 (58.5)	
Non-Black		11 (47.8)	81 (44.5)			6 (46.2)	34 (41.5)	
Timing of diagnosis	184			0.57	91			0.50
Pregnancy		3 (13.0)	13 (8.1)			1 (8.3)	10 (12.7)	
<1 mo postpartum		13 (56.5)	104 (64.0)			9 (75.0)	43 (54.4)	
≥1 mo postpartum		7 (30.4)	45 (28.0)			2 (16.7)	26 (32.9)	
Twin pregnancy	205	4 (17.4)	24 (13.2)	0.53	95	0 (0)	11 (13.4)	0.35
Pregnancy-related hypertension	188	14 (63.6)	74 (44.6)	0.11	92	8 (61.5)	35 (44.3)	0.37
Prepregnancy obesity	193	6 (26.1)	63 (37.1)	0.36	88	2 (15.4)	29 (38.7)	0.13
Chronic hypertension	194	4 (17.4)	26 (15.2)	0.76		0 (0)	10 (12.5)	0.35
Arrhythmia ^a	202	2 (8.7)	29 (16.2)	0.54	93	2 (15.4)	13 (16.5)	1.00
Median LVEF at diagnosis, %	176	22.5 (12.5-37.5)	27.5 (17.5-40.0)	0.18	81	15.0 (10.0-23.0)	28.8 (18.8-40.0)	0.008
LVEF <30% at diagnosis	176	15 (71.4)	78 (50.3)	0.10	81	10 (76.9)	34 (50.0)	0.13
No documented LVEF recovery	205	11 (47.8)	80 (44.0)	0.72	95	7 (53.8)	30 (36.6)	0.24
Median LVEF at recent cardiology visit, %	198	49.0 (45.0-56.0)	53.0 (40.0-58.5)	0.85	93	46.8 (40.0-52.5)	55 (42.5-57.5)	0.08
Referred to a heart failure specialist	205	14 (60.9)	109 (59.9)	1.00	95	10 (76.9)	49 (59.8)	0.46
Any family history information documented at first cardiology clinic visit	205	18 (78.3)	99 (55.3)	0.04	95	6 (46.2)	46 (56.1)	0.56
First-degree relative with HF or cardiomyopathy	205	9 (39.1)	23 (12.6)	0.003	95	1 (7.7)	10 (12.2)	1.00
First- or second-degree relative with HF or cardiomyopathy	205	10 (43.5)	32 (17.6)	0.01	95	2 (15.4)	13 (15.9)	1.00

(A) Patient flow diagram. Eight patients underwent clinical and research genetic testing. (B) Characteristics of patients with peripartum cardiomyopathy who underwent referral for clinical genetic testing and had pathogenic variants found on research genetic testing. Values are median (IQR) or n (%). ^aArrhythmia was defined as the presence of any of the following at first cardiology clinic visit: atrial fibrillation or flutter, supraventricular tachycardia, atrioventricular block, bundle branch block, premature ventricular contractions, and nonsustained or sustained ventricular tachycardia. HF = heart failure; LVEF = left ventricular ejection fraction.

Limitations of our study include its retrospective design, its small sample size, the potential lack of generalizability of single-center evaluation and testing practices, and low rates of documented family history. Recommendations for genetic testing in cardiomyopathy have changed over time,⁵ and this change may have affected genetic counseling

and testing referral rates for the clinical cohort. Involvement of a genetic counselor in the care of all patients with PPCM is not standard at our center, and this reflects contemporary clinical practice. The growing recognition of genetic causes of DCM coupled with decreased costs of clinical genetic testing has resulted in increased accessibility of

genetic evaluation; however, only about one-half of patients referred for genetic evaluation in our study completed clinical testing. Identifying barriers to completion of genetic counseling and testing is an important opportunity for future research.

We conclude that family history is a poor predictor of the presence of disease-causing variants, and additional patient-level characteristics, such as LV ejection fraction at PPCM diagnosis, may improve the identification of patients with pathogenic variants. In the absence of strong clinical predictors, genetic evaluation should be considered for all women with PPCM.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the [Author Center](#).

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Peripartum cardiomyopathy: from genetics to management

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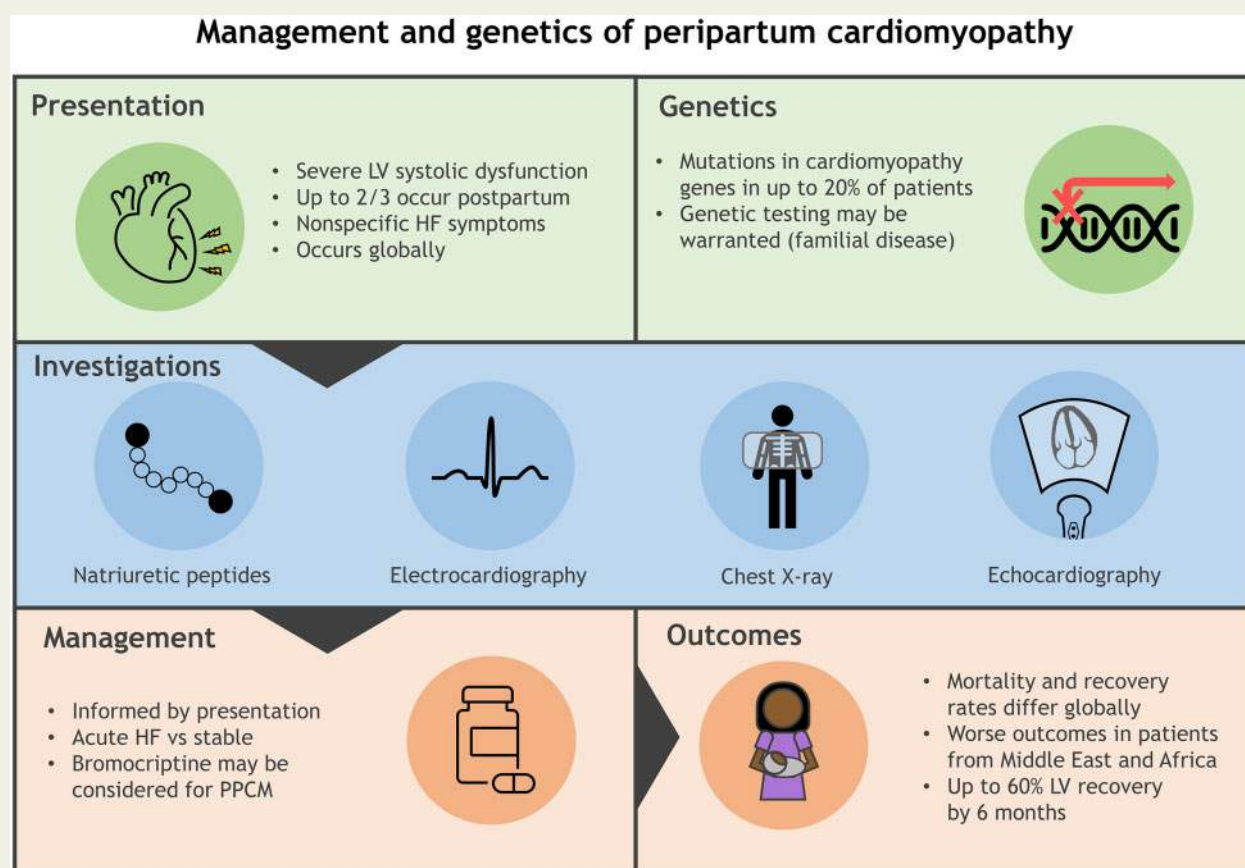
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Peripartum cardiomyopathy (PPCM) is a disease that occurs globally in all ethnic groups and should be suspected in any peripartum women presenting with symptoms and signs of heart failure, towards the end of pregnancy or in the months following delivery, with confirmed left ventricular dysfunction. After good history taking, all women should be thoroughly assessed, and alternative causes should be excluded. Urgent cardiac investigations with electrocardiogram and natriuretic peptide measurement (if available) should be performed. Echocardiography follows as the next step in investigation. Patients with abnormal cardiac investigations should be urgently referred to a cardiology team for expert management. Referral for genetic work-up should be considered if there is a family history of cardiomyopathy or sudden death. PPCM is a disease with substantial maternal and neonatal morbidity and mortality. Maternal mortality rates range widely, from 0% to 30%, depending on the ethnic background and geographic region. Just under half of women experience myocardial recovery. Remarkable advances in the comprehension of the pathogenesis and in patient management and therapy have been achieved, largely due to team efforts and close collaboration between basic scientists, cardiologists, intensive care specialists, and obstetricians. This review summarizes current knowledge of PPCM genetics, pathophysiology, diagnostic approach, management, and outcome.

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Graphical Abstract



Keywords

Peripartum cardiomyopathy • Heart failure • Pregnancy • Genetics

Introduction

Peripartum cardiomyopathy (PPCM) is increasingly recognized as an important medical condition that can complicate pregnancy. It is a life-threatening cardiomyopathy, characterized by acute or slowly progressing left ventricular (LV) dysfunction—late in pregnancy, during delivery, or in the first postpartum months—in women with no previously known cardiac disease.^{1,2} Over the past two decades a substantial amount of new knowledge on this condition has been published, including better understanding of the pathophysiology, the genetic predisposition for a portion of patients, on diagnostic tools, management and outcome. The Heart Failure Association of the European Society of Cardiology (ESC) Working Group on PPCM initiated the largest prospective global cohort study, under the umbrella of the EURObservational Research Programme (EORP), with >700 PPCM patients, providing novel data on presentation in various ethnic groups, as well as maternal and Foetal outcome.³

This review provides an update on new findings from genetics to management (*Graphical abstract*).

Presentation

Women often present with non-specific symptoms of heart failure late in pregnancy, during delivery or in the postpartum months, to medical professionals of various disciplines. The ESC EORP PPCM study has shown that the majority of women present postpartum in Africa (75%), Europe (69%), the Middle East (66%), and Asia-Pacific (57%) (*Figure 1*). At that stage, these women are usually no longer under obstetric care and present with their symptoms and signs to general practitioners or to the medical emergency services. Also, midwives may be the first to be addressed by postpartum women with symptoms and/or signs of heart failure. They should be aware of the potential presence of PPCM and trigger appropriate diagnostic clarification.

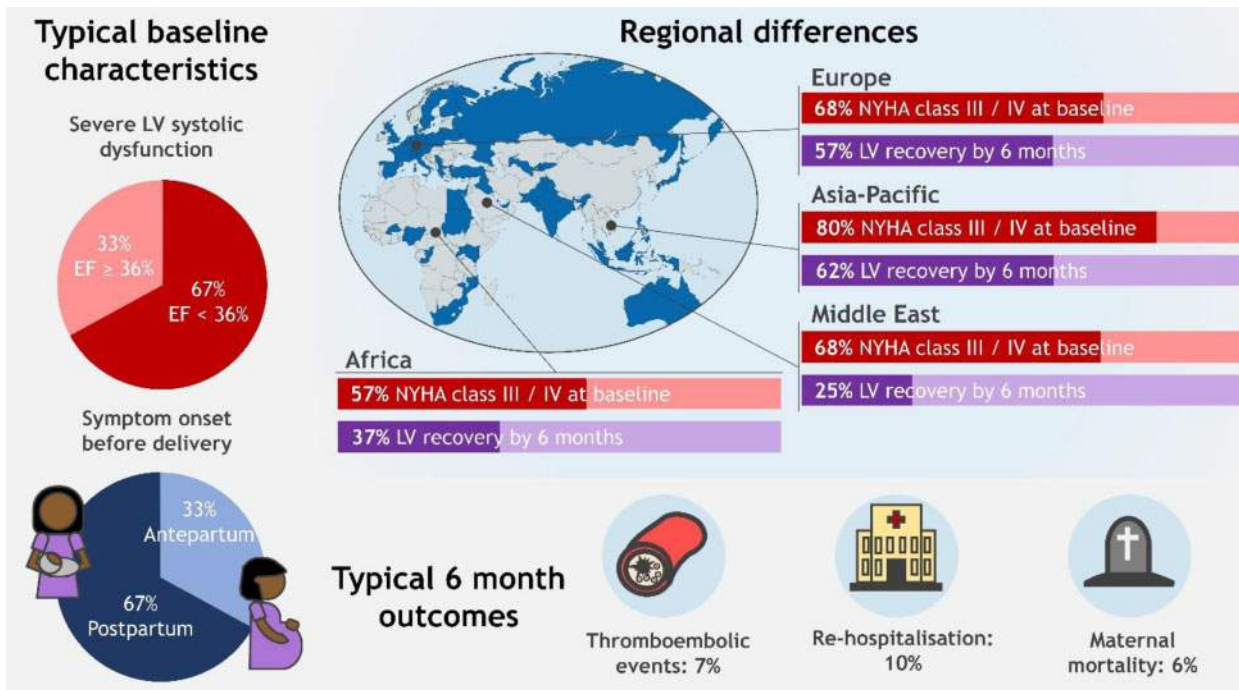


Figure 1 Baseline characteristics, 6-month outcomes, and regional differences observed in the peripartum cardiomyopathy (PPCM) EURObservational Research Programme (EORP). Analysis of 739 PPCM patients from 49 countries. In the map, countries in blue are those included in the EORP registry (countries not included in the illustration: Argentina, Canada, Honduras, Nicaragua, and the USA). EF, ejection fraction; LV, left ventricular; NYHA, New York Heart Association.

Distinguishing signs and symptoms of PPCM, from the spectrum of normal pregnancy or the common fatigue post-delivery, can be challenging. However, a low threshold of suspicion of heart failure is crucial in a woman with suggestive clinical features. A substantial proportion of women that present postpartum have few physical signs, despite substantial cardiac dysfunction (Figure 1). Shortness of breath, fatigue and mild ankle swelling are less specific symptoms of heart failure during pregnancy, whereas orthopnoea and paroxysmal nocturnal dyspnoea are more specific. The majority of women with PPCM present with severe symptoms (New York Heart Association class III or IV).⁴ PPCM is generally viewed as a diagnosis of exclusion. However, in a previously healthy woman presenting during the last month of pregnancy, or in the months after delivery, with signs and symptoms of heart failure, the diagnosis of PPCM is likely if an echocardiogram reveals significantly reduced LV ejection fraction (LVEF). A clearly detailed medical history is essential to exclude other conditions that may cause a dilated cardiomyopathy (DCM), including viruses, alcohol, LV non-compaction cardiomyopathy, and medications (particularly cardiotoxic chemotherapy). Hypertensive disorders complicate as many as 5–10% of pregnancies worldwide, with pre-eclampsia in ~3%.⁵ The prevalence of hypertensive disorders in women with PPCM is higher, with >20% having been reported to develop pre-eclampsia during the index pregnancy.⁶ The relationship between hypertensive disorders of pregnancy and PPCM is not well understood. Pathological pathways resulting in angiogenic imbalance and endothelial dysfunction have been identified in both conditions, suggesting that overlap may exist.⁷

Genetics

A role for genetics in the development of PPCM has been supported by multiple reports, with a genetic cause of disease identified in up to 20% of studied patients. The most notable example of this is mutations in the sarcomeric gene titin (*TTN*), a well-established disease gene for DCM. Several rare truncating (i.e. nonsense, frameshift, or splice site) mutations in *TTN* were reported in PPCM patients from European and American populations.^{8,9} These mutations comprised ~10–15% of a PPCM cohort, which was a similar *TTN* mutation yield to the 17% observed in a corresponding cohort of nonischaemic DCM patients.⁹

Combined with other discoveries of DCM gene mutations in PPCM patients,^{10–12} this suggests a substantial overlap in genetic susceptibility between PPCM and inherited forms of cardiomyopathy. Indeed, a number of PPCM patients have reported a positive first-degree family history for heart failure and cardiomyopathy, including those with cardiomyopathy-associated mutations.^{8,11,12} There have also been limited reports of PPCM occurring amongst carriers of X-linked forms of DCM, such as Duchenne muscular dystrophy and Danon disease.^{13–15}

A recent large study from the Arany group of 469 PPCM patients from around the world lent further weight to the notion that PPCM and DCM bear significant genetic overlap. The study confirmed a ~10% prevalence of pathogenic truncating variants in these women with PPCM, a ~20-fold higher prevalence than in control cohorts.¹⁶ In addition, statistically significantly enriched truncating variants were

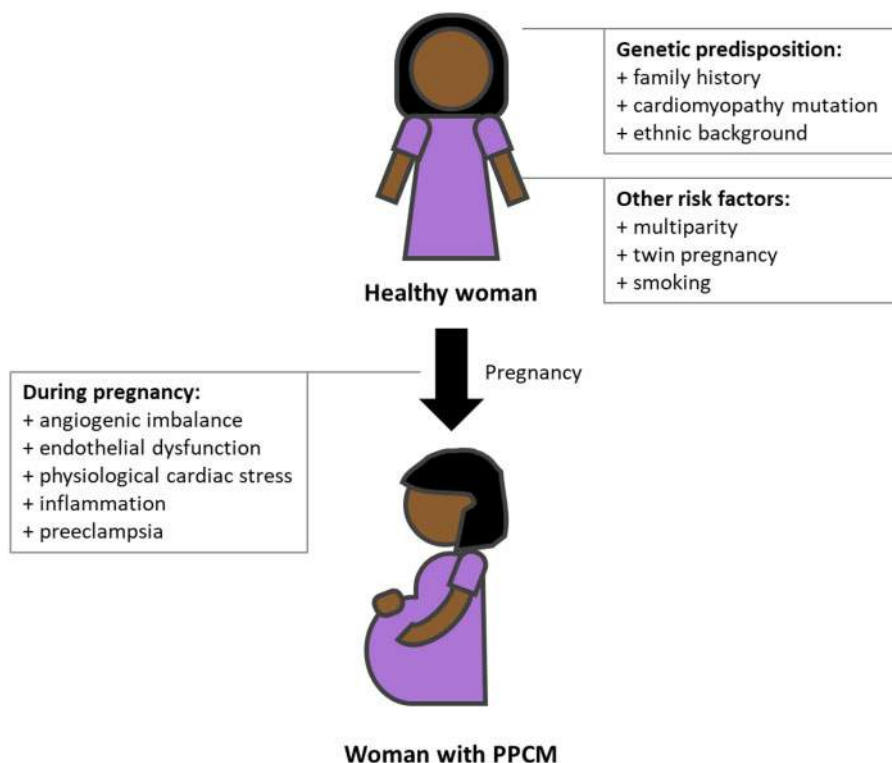


Figure 2 The 'multiple hit' model of peripartum cardiomyopathy (PPCM). Genetic predisposition, physiological changes during pregnancy, and other risk factors may act cumulatively to induce PPCM in otherwise healthy women. These include individuals with a family history of cardiovascular disease or carriers of cardiomyopathy-causing mutations, who do not manifest cardiomyopathy until late in pregnancy or shortly after delivery.

identified in the genes *DSP*, *FLNC*, and *BAG3*. The relative prevalence of truncating mutations in these four genes in women with PPCM (*TTN*: ~10%; *DSP* and *FLNC* ~1%; *BAG3*: ~0.2%) was nearly identical to the prevalence in cohorts with DCM, strongly supporting the idea that there is a high degree of genetic similarity between PPCM and DCM.

How these genetic mutations predispose to PPCM is unclear. The proteins encoded by these four genes have very different roles in the cardiomyocyte, including roles in the sarcomere (*TTN*), desmosome (*DSP*), intercalated discs (*FLNC*), and autophagy (*BAG3*). One possible explanation posits a 'multiple hit' model, in which accumulation of risk factors (genetic and environmental) increases the likelihood of disease (Figure 2). Consistent with this idea, a high prevalence of *TTN* mutations has also been seen in alcohol-induced and oncology-induced cardiomyopathies.^{17,18} Also, consistent with this idea of strong genetic/environment interaction, recent work has shown that 95% of people with truncating mutations in *TTN* show no evidence of cardiac disease, i.e. the penetrance of *TTN* truncations mutations is very low, indicating that cardiac function is preserved in the absence of additional cardiac stressors.¹⁹ The hemodynamic and vasculo-hormonal stressors of pregnancy or parturition likely represent such a stressor.¹

In addition to rare deleterious mutations, PPCM may be influenced by common variants, although fewer data are available to address this

possibility. A small study of 97 patients suggested that the C825T polymorphism in the gene *GNB3* may be associated with poorer outcomes, and may explain the increased prevalence of PPCM amongst individuals of African ancestry.²⁰ A similarly small genome-wide association study of 41 PPCM patients identified a single-nucleotide polymorphism in the parathyroid hormone-like hormone gene (*PTH1H*) that was significantly associated with risk of PPCM.²¹ Independent replication in larger studies will help elucidate the roles of these variants.

Genetic predispositions to PPCM may also be shared with other diseases. For example, the Hilfiker-Kleiner group suggested an enhanced risk for cancer in PPCM patients (16-fold), compared to age-matched women.²² The incidence of cancer occurred both prior to and after PPCM. Some of these patients carried known pathogenic or likely pathogenic variants, as classified according to the American College of Medical Genetics criteria, in genes associated with an increased cancer risk including *ATM*, *ERCC5*, *NBN*, *RECQL4*, and *SLX4*. Interestingly, all these genes are related to the DNA damage response (DDR) pathway,²² suggesting the possibility that mutations within the DDR pathway concomitantly increase the risks for cancer and for cardiotoxicity due to anticancer treatment and/or pregnancy. These findings will benefit from independent replication and may be limited to some demographic cohorts, as for example a review of the case records of a large South African cohort of >176 PPCM

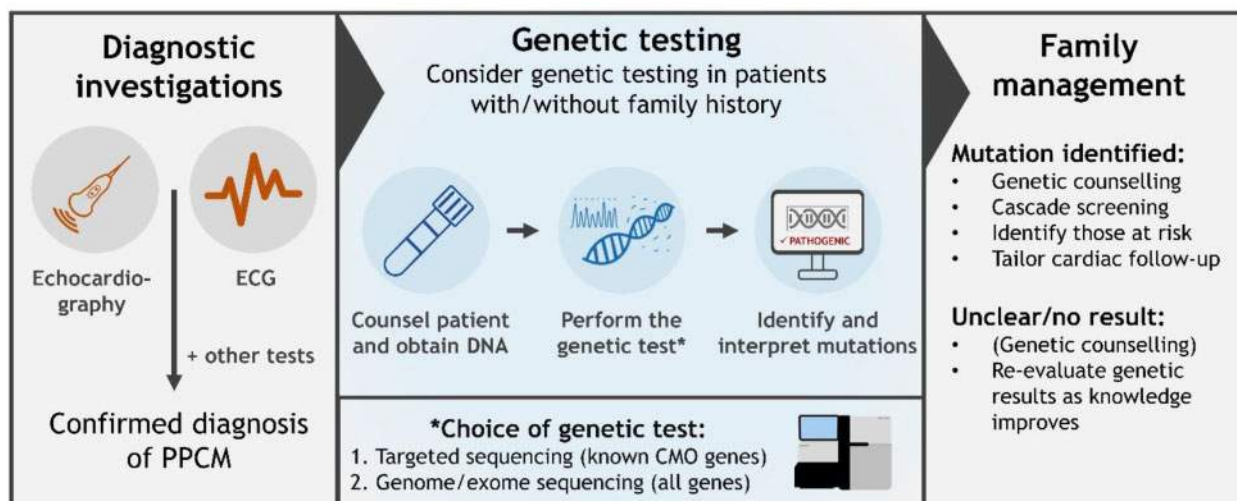


Figure 3 Overview of the genetic testing process. After a diagnosis of peripartum cardiomyopathy, genetic testing should be considered in health-care settings that offer it. Patients should be counselled before a DNA sample is obtained. Genetic tests include targeted sequencing of known cardiomyopathy genes and genome or exome sequencing. Once the results are interpreted, they should be reported to the patients and appropriate steps taken. In the event of a positive genetic result, cascade screening of her relatives may identify at-risk individuals who may require cardiac follow-up. CMO, cardiomyopathy; ECG, electrocardiography.

patients²³ did not detect any cases with cancer prior, or concomitant to, PPCM diagnosis (unpublished data).

The significant burden of identifiable genetic mutations in women with PPCM has implications for clinical care. Currently, there are no recommendations for genetic testing in women with PPCM. Initial observations suggest that mutations in *TTN* herald a worse prognosis,⁹ but more research is needed. In contrast, mutations in *FLNC* and *DSP* are associated with malignant arrhythmias in DCM and warrant aggressive anti-arrhythmic management. Moreover, in cases where a likely pathogenic variant is identified, testing of family members can provide reassurance or indicate closer monitoring. Genetic testing of patients with DCM, and of at-risk family members, is currently recommended.²⁴ In healthcare systems, which offer it, genetic testing may be considered in cases of PPCM, especially in cases of familial aggregation of cardiomyopathies, for prognosis and to facilitate family screening.

Inflammation, neurohormones, and cardiac signalling

PPCM is considered as a distinct entity of unknown aetiology,^{1,2} probably resulting from multiple pathomechanisms initiating and driving the disease. This also seems to be reflected by the variable cardiac presentation and risk factor profiles observed in PPCM patients and the different animal models that develop PPCM.

Amongst these are inflammation and autoimmune reactions with elevated levels of various circulating cytokines as potential factors inducing and driving PPCM.²⁵ Interleukin-6 (IL-6), tumour necrosis factor- α (TNF α), C-reactive protein (CRP), and interferon-gamma

(IFN γ) were found to be elevated and correlated with the severity of cardiac failure.^{26–28} Anti-inflammatory strategies, for example with pentoxifylline, a xanthine-derived agent known to reduce the release of TNF α and IL-6, improved the outcome of PPCM patients in an African cohort.²⁶ The origin of enhanced inflammation in PPCM patients is unclear and may be pathogen driven, or the result of autoimmune processes. In fact, several smaller studies reported elevated levels of autoantibodies (AABs), specifically against cardiac troponin I (TnI) and myosin heavy chain 7, which correlated with more severe LV dysfunction.²⁵ AABs against β 1-adrenergic receptors (β 1R) and the M2-muscarinic receptor (M2-R) were also described in PPCM patients.²⁹ These reports suggest that PPCM patients without AABs had a favourable outcome.³⁰ Respectively, plasmapheresis may improve cardiac function in PPCM patients.³¹ However, data are limited and it is unclear whether AABs are contributing to the pathogenesis of PPCM, or are a consequence of the disease.

An interesting aspect will also be the impact of the current COVID-19 pandemic on patients with PPCM, specifically because pregnancy suppresses the immune system, thus allowing viral infections to spread more efficiently. Moreover, after the delivery, the immune system is strongly activated, a feature that may, in view of the COVID-19 disease, be beneficial or may lead to an overactivation of the immune system with damaging effects.

However, there is accumulating evidence that several pathomechanisms in PPCM converge on a common pathway, which involves unbalanced oxidative stress and the generation of the anti-angiogenic 16-kDa prolactin.³² Along this line, experimental and clinical observation suggests central roles for protection of the maternal heart from oxidative stress by specific activation of major signalling pathways, including the signal transducer and activator of transcription 3 (STAT 3), the peroxisome proliferator-activated receptor γ coactivator 1 α

and phosphoinositid-3-kinase (PI3) and protein kinase B (AKT) as summarized by Hilfiker-Kleiner *et al.*³² and Ricke-Hoch *et al.*³³ Deregulation of any of these pathways is associated with increased oxidative stress, which promotes the cleavage of the nursing hormone prolactin, a hormone rising during pregnancy and, periodically, released from the pituitary gland during nursing, into shorter N-terminal 16-kDa prolactin fragment (16-kDa prolactin). The 16-kDa prolactin induces endothelial dysfunction and damage and subsequently leads to heart failure. Blocking prolactin with the dopamine D2 receptor agonist, bromocriptine, has emerged as a potential disease-specific therapy for PPCM, and it has been added to the ESC guidelines on cardiovascular diseases in pregnancy as a therapeutic option that should be considered.³⁴

More recently, impairment in neurohormonal signalling, specifically lower circulating levels of serotonin, has also been reported in PPCM patients.³⁵ Levels of the depression-associated microRNA-30e, a microRNA that impairs the function of the serotonin receptor 5-HT1AR, are elevated in PPCM patients.³⁵ These alterations were associated with an increased prevalence for major depressive disorders, post-traumatic-stress-disorder, and panic-disorder in PPCM patients, compared with postpartum women without a PPCM diagnosis.³⁵ However, it remains unknown if this observation is related to the severe stressors, these women undergo due to having severe heart failure linked to their pregnancy.

Diagnostic assessment

Urgent diagnostic assessment with prompt referral to a specialist is indicated in any peripartum woman with signs or symptoms of heart failure. Once a diagnosis of PPCM is reached, additional tests can be considered to allow more sophisticated phenotyping and prognostication.

The electrocardiogram (ECG) is a widely available and powerful diagnostic tool in any cardiac condition. It should form the basis of clinical work-up in all women with a potentially cardiac-related complaint and, particularly, in those with suspected PPCM. To a varying degree, at the time of diagnosis the ECG is abnormal in almost all women with PPCM. In a prospective study of women with PPCM in South Africa, >90% had at least one electrocardiographic abnormality and almost 50% had a significant electrocardiographic abnormality (e.g. Q-wave abnormality, ST-segment depression, T-wave inversion, bundle branch block, second- or third-degree atrioventricular block, frequent ectopy, or brady- or tachyarrhythmia).³⁶ The recently published paper on ECG abnormalities in >400 patients included in the EORP PPCM study confirmed this observation.³⁷

Chest X-rays can identify alternative causes for breathlessness or hypoxia, such as infection, effusion, or pneumothorax, but can be normal in PPCM, while commonly showing cardiomegaly and/or features of pulmonary congestion.

Echocardiography is the main diagnostic modality used to confirm the presence of cardiac dysfunction in PPCM and quantify severity. Furthermore, it excludes alternative causes of heart failure, such as congenital heart disease, primary valvular disease, and a number of inherited or acquired cardiomyopathies. LV end-diastolic and end-systolic volumes are commonly pathologically enlarged. The mean LVEF at the time of diagnosis in the global study on PPCM was around

30% in all ethnic groups.³ Commonly coexisting findings are functional mitral regurgitation and right ventricular dysfunction. Part of the routine echocardiogram must be a comprehensive assessment of the right heart, as a reduced baseline right ventricular function has been shown to be an independent predictor of a worse outcome.^{38,39}

Echocardiography is also an important tool in the identification of heart failure sequelae, such as intracardiac thrombi.

Access to cardiac magnetic resonance imaging (MRI) is often prohibited by cost and geographical location and, as such, it is not widely used as a standard investigation in the diagnosis of PPCM. Cardiac MRI can be useful to exclude other aetiologies, such as myocarditis, LV non-compaction cardiomyopathy, or infiltrative diseases, and to supplement information provided by echocardiography.

Routine haematological and biochemical testing can provide information on potentially reversible contributory factors, such as clinically significant anaemia, and help to establish the presence of end organ damage. Natriuretic peptide levels (where available) should be measured in women with suspected PPCM.

Although not currently routine medical practice, genetic testing may also be considered, especially in cases of familial aggregation of cardiomyopathies (Figure 3). Because PPCM frequently presents as acute heart failure (AHF), genetic testing may be secondary to more urgent clinical investigations that should take priority. However, the identification of disease-causing genetic mutations will be of clinical relevance to the patient and her family, including her offspring, who may be at the risk of genetic cardiomyopathy themselves. Patients would need to be counselled before performing genetic testing and, in the event that a positive result is obtained, on the methods of genetic testing and the potential implications of a positive result. Because genetic testing typically involves sequencing all well-established cardiomyopathy genes for mutations, identification of a pathogenic mutation in a PPCM patient should be followed by cascade screening of her relatives to determine other mutation carriers. Any mutation carriers may then require cardiological follow-up and routine screening for signs of deterioration in cardiac function.

Risk stratification

Risk stratification should be immediately performed to identify the appropriate level of care. The strongest parameters in identifying patients at risk of complications at the time of diagnosis are non-Caucasian race, ECG QT-interval prolongation, LVEF <30%, LV end-diastolic diameter of >60 mm, biventricular dysfunction, and delay of diagnosis. Other parameters including age >40 or <20 years, ante-partum diagnosis, haemodynamic parameters at presentation, and cardiac biomarkers might help refine the risk stratification.⁴⁰ Patients with a history of a previous diagnosis of PPCM have a particularly high risk of poor outcome and careful history taking must always be part of the first assessment.⁴¹ In all women with PPCM and a subsequent pregnancy, the key parameter is pre-pregnancy LVEF, since the risk of deterioration and even death is significant in cases of persistent LV dysfunction before subsequent pregnancies. However, even patients with recovered LV functions are still at risk for relapse.

PPCM is associated with substantial morbidity and mortality. Most of the life-threatening complications, including severe heart failure,

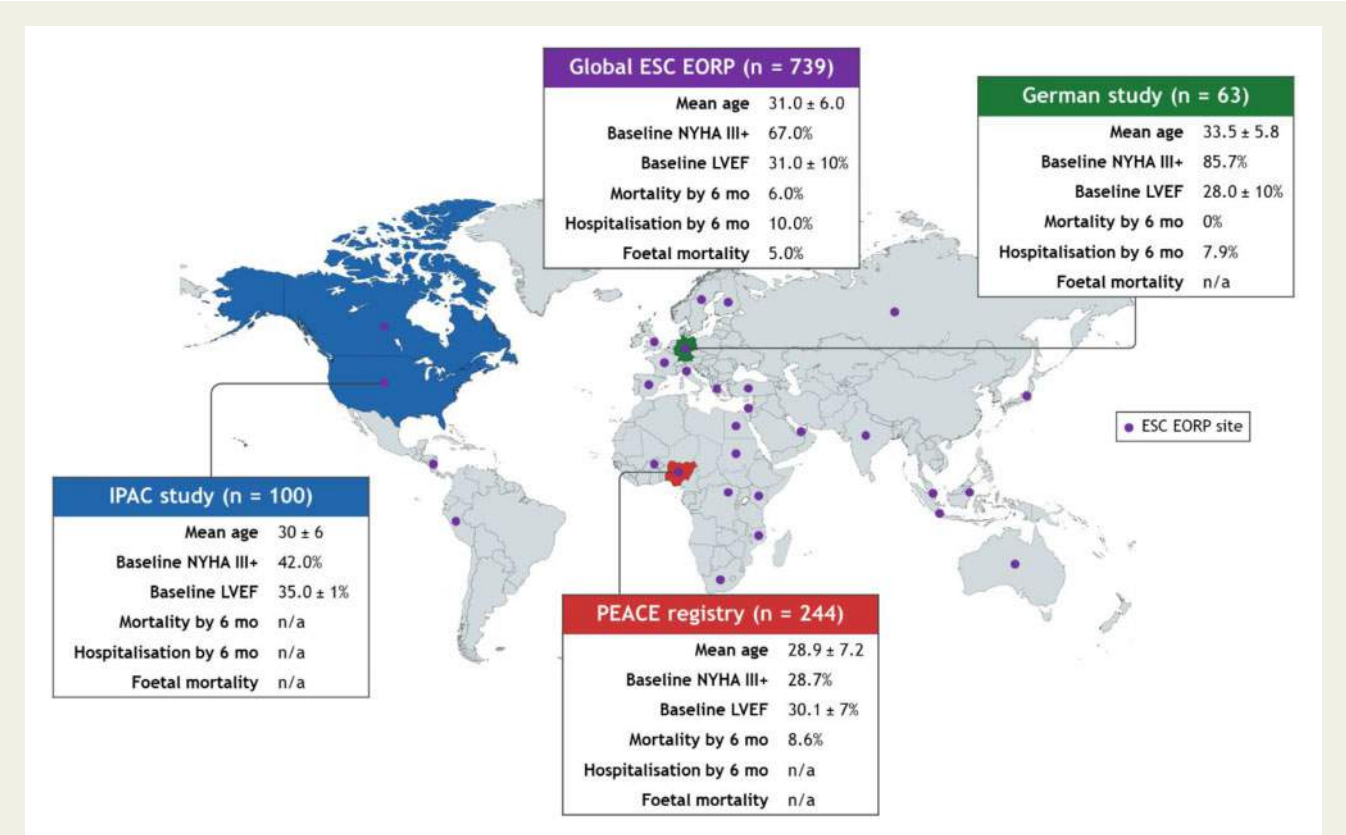


Figure 4 Comparison of clinical presentation and outcome of multi-centre peripartum cardiomyopathy studies. Data are taken from the North American Investigations of Pregnancy Associated Cardiomyopathy study,⁵³ PERipArtum Cardiomyopathy in NigEria registry,⁵⁴ a German multi-centre bromocriptine clinical trial,⁵¹ and the European Society of Cardiology EURObservational Research Programme global peripartum cardiomyopathy registry.³ Data that are not reported are marked n/a. LVEF, left ventricular ejection fraction; mo, month; n/a, not available; NYHA, New York Heart Association.

cardiogenic shock, arrhythmias, thromboembolic complications, and death, can be prevented by early referral to a centre with expertise in managing AHF and by appropriate use of medical and non-pharmacological therapy.²

Management

The management of PPCM differs according to the clinical scenario in which the patient presents; especially whether the patient is still pregnant or post-delivery, and whether the patient is stable or suffering from acute decompensated heart failure. A dedicated pregnancy heart team/task force for pregnant patients with AHF should be installed in all larger centres to ensure rapid decision-making processes regarding the best supportive care for mother and child, including (urgent) delivery, stopping lactation, and potential mechanical circulatory support (MCS) for patients in cardiogenic shock.^{1,34}

Mild heart failure may be treated with standard heart failure drugs on a normal ward, or even on an outpatient basis if regular follow-up visits can be ensured. Haemodynamically stable patients with respiratory insufficiency at rest will be treated with intravenous diuretics and oxygen supplementation on an intermediate care/heart failure unit, whereas women with severe forms of acute PPCM leading to

cardiopulmonary instability and cardiogenic shock are often in need of intubation and ventilation, as well as inotropic treatment and MCS implantation in an intensive care unit. Practical recommendations for treating AHF in the setting of PPCM are based on recent data and clinical experience.^{1,42-44}

To ensure optimal treatment according to current evidence and best experience, patients with severe distress should be transferred early to an experienced centre whenever possible. If hemodynamic instability persists despite medical treatment, implantation of temporary MCS should be considered early. As the probability of at least partial recovery of ventricular function is higher than in other cardiomyopathies, implantation of a durable ventricular assist device or cardiac transplantation is only rarely necessary.

In patients with stabilized heart failure not in need of, or weaned from, inotropics and vasopressors, standard drug treatment proven to ameliorate LV remodelling and clinical outcomes in heart failure with reduced ejection fraction should be installed and titrated to maximum guideline-based dosages.

Clearly, during pregnancy, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and angiotensin receptor-neprilysin inhibitors (ARNIs) are contraindicated because of foetal toxicity and hydralazine and nitrates may be used instead. Mineralocorticoid receptor antagonists (MRAs) should be avoided

during pregnancy and lactation but should be started postpartum in symptomatic women—especially in women with LVEF <35%.

Despite an increased risk of foetal growth restriction, beta-blockers are indicated in all stable patients with marked systolic dysfunction while pregnant, and in all patients postpartum.⁴³ Dosages of diuretics should be adapted to control the symptoms or signs of congestion but avoiding higher doses when possible.

Breastfeeding is controversial in peripartum women presenting with heart failure.⁴⁵ The 2018 ESC guidelines for the management of cardiovascular diseases during pregnancy suggests that preventing lactation may be considered in patients with severe heart failure to avoid the high metabolic demands of lactation and breastfeeding and to enable safe treatment with all established heart failure drugs.³⁴ There are, however, few published data that directly address the question of breastfeeding in PPCM. Only two small observational studies have directly addressed the question, and both have concluded that breastfeeding is not contraindicated in women with PPCM.^{46,47} The decision of whether to breastfeed with PPCM, in particular in women with moderate LV dysfunction, must also consider at least two other factors: (i) the benefit of breastfeeding to the infant, in particular in developing countries, where undernutrition accounts for most of childhood mortality,⁴⁸ and (ii) the safety of medications used to treat PPCM during lactation. A detailed description of medication safety during pregnancy and lactation is given in table 3 in Bauersachs *et al.*¹

Thromboembolic events

Around the time of PPCM diagnosis, the rate of peripheral arterial and venous thromboembolic events is rather high (7% in the first 30 days after delivery in the PPCM worldwide registry).^{3,4} Thus, the suspicion for thromboembolism must be high, and an appropriate diagnostic procedure performed. Initial anticoagulation in prophylactic dose should be considered in all patients with PPCM, whereas therapeutic anticoagulation is firmly recommended in patients with intracardiac thrombus, evidence of systemic thromboembolism, or paroxysmal or persistent atrial fibrillation.

Arrhythmias and ECG abnormalities

There is no specific ECG pattern for PPCM. However, the ECG is rarely normal in patients presenting with PPCM and sinus tachycardia, as well as repolarization abnormalities, are common.¹ In the EORP PPCM worldwide registry, ECG abnormalities have been analysed, including regional and ethnic differences and their correlation with echocardiographic features. Sinus tachycardia at baseline was associated with poor systolic function (LVEF <35%), whereas wide QRS, left bundle branch block and LV hypertrophy were associated with LV dilatation.³⁷

In a South African cohort, a poor outcome (defined as death, re-admission, NYHA class III/IV or LVEF ≤35% at 6- and 12-month follow-up) was independently predicted by a prolonged QTc interval or tachycardia at baseline.³⁶

PPCM patients are at risk for sudden cardiac death (SCD) in the months after diagnosis, especially when LVEF is <35%. As early implantation of an implantable cardioverter-defibrillator (ICD) is not necessary in most patients, given the high likelihood of ejection fraction improvement after several months of heart failure drug treatment, a wearable cardioverter-defibrillator may be useful to prevent

SCD due to ventricular fibrillation.^{1,49} Only in women with severe LV dysfunction >6 months after diagnosis despite optimal medical therapy, implantation of an ICD or cardiac resynchronization therapy should be considered.

Bromocriptine

Bromocriptine suppresses prolactin production and was used for many years to stop lactation postpartum. In a small proof-of-concept study on acute PPCM, the addition of bromocriptine to standard heart failure medical therapy was associated with improved LVEF and mortality.⁵⁰ A recent multi-centre randomized study comparing two different bromocriptine dosages (2.5 mg daily for one week vs. 5 mg daily for 2 weeks, followed by 2.5 mg daily for 6 weeks) in severe PPCM patients observed a high LV recovery rate at 6 months with no mortality, use of assist device or heart transplantation, indicating a beneficial association between the use of bromocriptine in acute PPCM and clinical outcome.⁵¹ The improvement in LVEF was similar in the short-term (one week) and long-term (eight weeks) bromocriptine groups, albeit a trend, not statistically significant, for more patients reaching full recovery at 6 months was found in the long-term group. Based on these data, the 2018 ESC Guidelines for the management of cardiovascular diseases during pregnancy states that bromocriptine may be considered in women with newly diagnosed PPCM.³⁴ Bromocriptine treatment should always be accompanied by anticoagulation at least at prophylactic dose, to reduce the thromboembolic risk.

To gain more evidence for bromocriptine treatment in PPCM, the North American Bromocriptine trial with funding from the NIH will randomize PPCM patients to 8 weeks of bromocriptine or placebo.

Long-term drug treatment/drug cessation

ARNI or ACE inhibitors, beta-blockers, and MRAs should probably be given in guideline-based dosages and not discontinued during the first year after complete recovery of LV function. Stepwise discontinuation of heart failure therapy might be considered if both complete recovery of ventricular function and normal exercise response are achieved. Since relapses have been observed after recovery in patients presenting with heart failure, tapering of the disease-modifying heart failure drugs should be performed under close observation of LV function.^{1,32} Diuretics should be tapered if patients no longer have symptoms or signs of congestion.

Outcome

Although the prognosis of PPCM patients is more favourable compared to other cardiomyopathies, outcomes (particularly recovery of LV function and mortality) significantly differ globally.^{2,41} Figure 4 summarizes the clinical presentation, 6-month maternal outcome including rehospitalization and death in four recently conducted PPCM cohort studies.^{3,51–53}

The highest maternal mortality was found in the 244 patients entered into the Nigerian PEACE study, with a median death of 18% within 17 months, and a 6-month mortality of 8%.

Mortality rates of PPCM patients range widely depending on geographical regions. The EORP global registry on PPCM, the largest

study to date, investigates clinical presentation, management and outcome of PPCM patients.⁵⁵ A total of 743 patients from > 40 countries in Europe, Africa, Asia-Pacific, and the Middle East were enrolled over a 6-year period. Initial results showed low mortality rates 1 month after the diagnosis (2.4% overall, 3.4% in ESC countries, and 1.4% in non-ESC countries).⁴ However, the 6-month mortality was 6%, with 42% due to heart failure and 30% due to sudden death.³

Mortality rates from other studies reported substantially higher mortality ranges such as 15–30% in Turkey⁵⁶ and 12–28% in South Africa.^{57,58}

Early mortality in the acute phase is often due to AHF, malignant arrhythmia, cardiogenic shock, and thromboembolic events.^{43,49} Later mortality is mainly due to deterioration of LV function, leading to heart failure.

Until recently it was thought that women with African ethnicity always experience worse outcome.^{59–61} However, data from the global ESC EORP demonstrated a worse outcome in women from Middle East compared to African patients.³ Currently, it is unknown if different genetic background plays a key role in defining the prognosis, or if the main determinants are also influenced by access to healthcare systems and heart failure therapy.

Most studies in patients with PPCM have only reported on the LV function at 6-month follow-up. Full recovery (mostly defined as LVEF >50–55%) ranges widely depending on racial background and geographic region. Data from China, Japan, Turkey, the USA, and Germany demonstrate recovery rates between 44% and 63% after 6 months.^{41,62–66} Women in Pakistan, the Philippines, Nigeria, and South Africa display a lower chance of full recovery rates ranging from 21% to 36%.^{23,27,41,67,68}

Prospective studies and registries that include patients with a follow-up ≥12 months demonstrate relatively high rates of LV recovery in PPCM patients. The multi-centre IPAC (Investigation of Pregnancy-Associated Cardiomyopathy) study analysed 100 American women with PPCM.⁵³ LV recovery was observed in 72% of women after 12 months. In a Turkish study, LV recovery was present in 47.6%.⁶² It is important to note that >50% of patients who recover did so beyond 12 months after initial diagnosis.

Only very few prospective data report on the long-term follow-up of PPCM patients beyond 5 years after initial diagnosis. A German study prospectively investigated outcome of 67 patients, with a mean follow-up of 63 ± 11 months.⁶⁹ The LV function improved from 26% at initial diagnosis to 50% after 12 months and 54% after 5 years. The number of patients experiencing full recovery was 48% after 6 months, further rising to 60% and 72% after 1 and 5 years, respectively.

Conclusion

PPCM is a global disease that is often associated with a delayed diagnosis, leading to significant morbidity and mortality. It is an important contributor to early (<42 days) and late (up to 1 year) postpartum maternal death. The reported 1-year mortality ranges from 5% to 25%.

In the last two decades, remarkable advances in the comprehension of the pathogenesis and the improvement in patient

management and therapy have been achieved, including a disease-specific therapeutic option, namely bromocriptine, which should be considered. This is largely due to team-effort and close collaboration between basic scientists, cardiologists, intensive care specialists and obstetricians. Recent data suggest that referral for genetic work-up should be considered for patients with a family history of a cardiomyopathy or sudden death.

With increasing awareness and better diagnostic tools the disease has moved from 'rare' to a 'relatively frequent' pregnancy complication, thereby raising research interest in this field.

Under the umbrella of the Heart Failure Association of the ESC, a PPCM Working Group was established in 2009, which led to several research projects advancing the knowledge about this condition.⁷⁰

Despite ongoing research, numerous uncertainties regarding the incidence, pathophysiology, treatment, and prognosis of PPCM patients persist, indicating the need for further investigation. The need is to create more awareness about this condition globally, but also to understand differences in mode and presentation.

The establishment of the international registry on PPCM, funded by the ESC under the umbrella of the EORP, which recruited >700 patients from >40 countries will conclude its follow-up in 2021. Many more lessons will be learned from this program but also from other studies, such as the multi-centre Nigerian PEACE PPCM registry^{54,71} and the large clinical service dedicated to women with PPCM in Iraq.⁷²

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In peripartum cardiomyopathy plasminogen activator inhibitor-1 is a potential new biomarker with controversial roles

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Aims

Peripartum cardiomyopathy (PPCM) is a life-threatening heart disease occurring in previously heart-healthy women. A common pathomechanism in PPCM involves the angiostatic 16 kDa-prolactin (16 kDa-PRL) fragment, which via NF- κ B-mediated up-regulation of microRNA-(miR)-146a induces vascular damage and heart failure. We analyse whether the plasminogen activator inhibitor-1 (PAI-1) is involved in the pathophysiology of PPCM.

Methods and results

In healthy age-matched postpartum women (PP-Ctrl, $n = 53$, left ventricular ejection fraction, LVEF $> 55\%$), PAI-1 plasma levels were within the normal range (21 ± 10 ng/mL), but significantly elevated (64 ± 38 ng/mL, $P < 0.01$) in postpartum PPCM patients at baseline (BL, $n = 64$, mean LVEF: $23 \pm 8\%$). At 6-month follow-up ($n = 23$), PAI-1 levels decreased (36 ± 14 ng/mL, $P < 0.01$ vs. BL) and LVEF ($49 \pm 11\%$) improved. Increased N-terminal pro-brain natriuretic peptide and Troponin T did not correlate with PAI-1. C-reactive protein, interleukin (IL)-6 and IL-1 β did not differ between PPCM patients and PP-Ctrl. MiR-146a was 3.6-fold ($P < 0.001$) higher in BL-PPCM plasma compared with PP-Ctrl and correlated positively with PAI-1. In BL-PPCM serum, 16 kDa-PRL coprecipitated with PAI-1, which was associated with higher ($P < 0.05$) uPAR-mediated NF- κ B activation in endothelial cells compared with PP-Ctrl serum. Cardiac biopsies and dermal fibroblasts from PPCM patients displayed higher PAI-1 mRNA levels ($P < 0.05$) than healthy controls. In PPCM mice (due to a cardiomyocyte-specific-knockout for STAT3, CKO), cardiac PAI-1 expression was higher than in postpartum wild-type controls, whereas a systemic PAI-1-knockout in CKO mice accelerated peripartum cardiac fibrosis, inflammation, heart failure, and mortality.

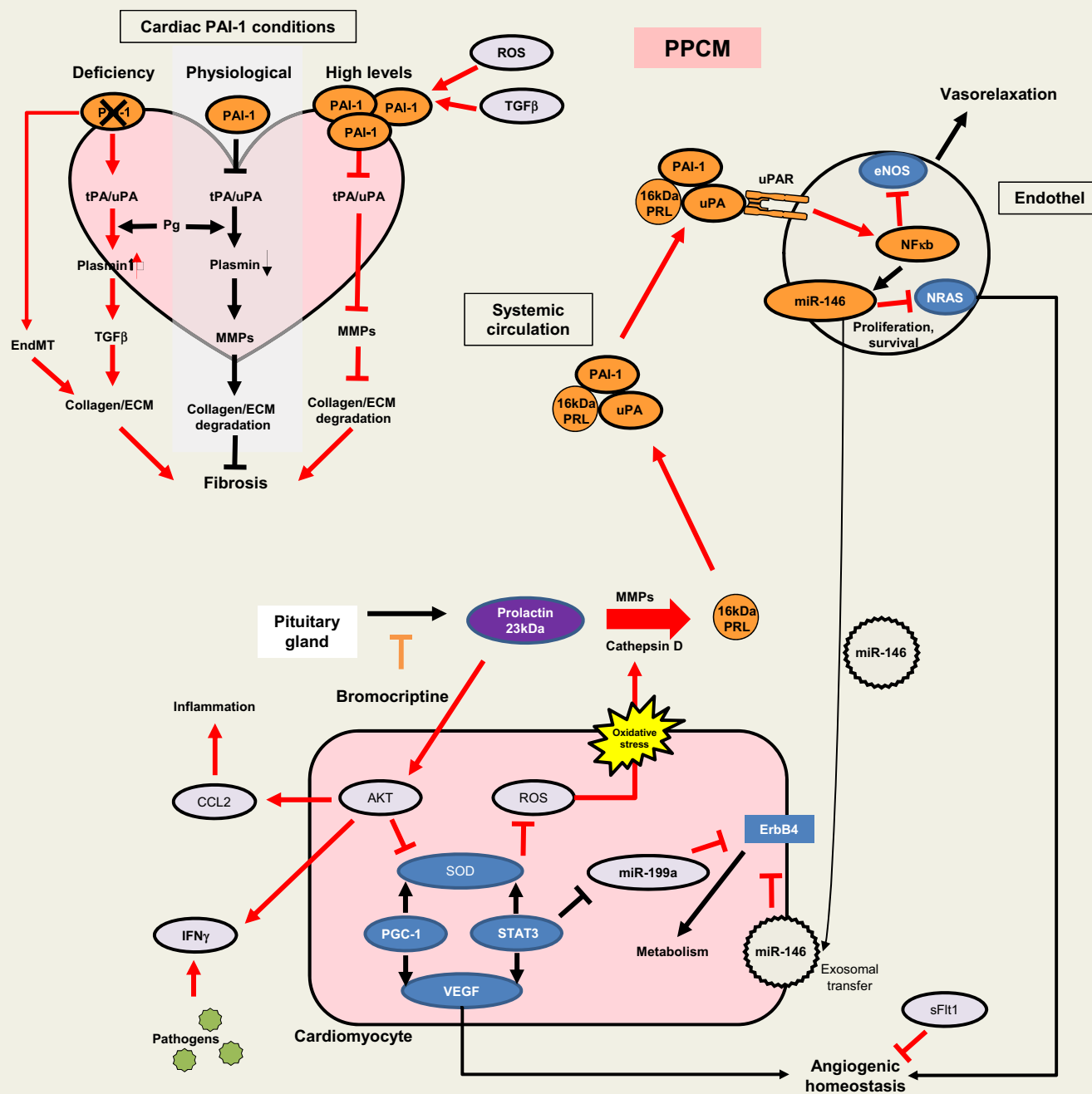
Conclusion

In PPCM patients, circulating and cardiac PAI-1 expression are up-regulated. While circulating PAI-1 may add 16 kDa-PRL to induce vascular impairment via the uPAR/NF- κ B/miR-146a pathway, experimental data suggest that cardiac PAI-1 expression seems to protect the PPCM heart from fibrosis. Thus, measuring circulating PAI-1 and miR-146a, together with an uPAR/NF- κ B-activity assay could be developed into a specific diagnostic marker assay for PPCM, but unrestricted reduction of PAI-1 for therapy may not be advised.

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Graphical Abstract



Keywords

PAI-1 • Heart failure • Peripartum cardiomyopathy • Biomarker • miR-146a

1. Introduction

Peripartum cardiomyopathy (PPCM) is defined as 'an idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular (LV) systolic dysfunction with an LV ejection fraction (LVEF) below 45% towards the end of pregnancy or in the months following delivery, where no cardiomyopathy or other cause of heart failure has been identified prior to the last month of pregnancy', and no other explanation for

peripartum heart failure has been found.^{1,2} Diagnosis of PPCM is often delayed because PPCM symptoms, i.e. dyspnoea, exercise intolerance, and oedema, are difficult to distinguish from normal peripartum-associated discomfort, and markers for cardiac injury, such as Troponin T (TNT) or C-reactive protein (CRP), are either frequently not elevated in PPCM, or are also elevated in healthy age-matched postpartum women.³ The only clinically confirmed biomarker for PPCM is N-terminal pro-brain natriuretic peptide (NT-proBNP), which, however, is not

specific for postpartum heart failure.^{1,4} Early diagnosis significantly improves the PPCM patient's chance of full cardiac recovery, whereas delays are associated with an increased rate of major adverse events and a greater likelihood for a transition to irreversible terminal heart failure.¹ Furthermore, undiagnosed and non-treated PPCM increases the risk for more severe heart failure in subsequent pregnancies.^{5,6}

The aetiology of PPCM is still unknown, but it has been proposed that oxidative stress and the production of the angiostatic and proapoptotic 16 kDa-prolactin (16 kDa-PRL) may represent a common pathomechanism in PPCM pathophysiology.^{7–11} We previously showed that 16 kDa-PRL mediates its adverse effects in PPCM by activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF- κ B) signalling in endothelial cells, which subsequently induces miR-146a. miR-146a mediates most of the adverse effects of 16 kDa-PRL in the vasculature.⁷ In addition, it is transferred to the cardiomyocytes via endothelial exosomes, inducing heart failure through alterations in cardiac erb-B2 Receptor tyrosine kinase 4 (ErbB4) signalling and metabolism.⁷ We previously showed that miR-146a is up-regulated in the plasma of PPCM patients.⁷ However, the precise signalling mechanisms upstream of the NF- κ B activation by 16 kDa-PRL in PPCM remain known. Research in tumour biology has shown that 16 kDa-PRL can integrate in a complex with plasminogen activator inhibitor-1 (PAI-1) (also called Serpin E1), urokinase (uPA), and the uPA receptor (uPAR) to induce non-canonical uPAR-induced NF- κ B activation and subsequent vascular damage.¹² In turn, PAI-1 is the principal inhibitor of the classical uPAR signalling through protein kinase B (AKT) and signal transducer and activator of transcription (STAT) and acts thereby as a pro-thrombotic factor that reduces fibrinolysis.¹³ Besides, PAI-1 has multiple additional biological roles including the regulation of tissue fibrosis. While in most tissues PAI-1 acts as a profibrotic factor, in the heart PAI-1 has opposing roles as it can promote or inhibit fibrosis.^{14–16} The present study investigated the expression of systemic and tissue PAI-1 and its potential role in PPCM. The study discovered that circulating PAI-1 is significantly higher (3.0-fold, $P < 0.01$) in PPCM patients compared with healthy postpartum controls (PP-Ctrl) and provides evidence that 16 kDa-PRL and PAI-1 physically interact to induce non-canonical uPAR-NF- κ B signalling in endothelial cells, thereby contributing to PPCM pathophysiology. PAI-1 expression is also higher in heart tissue and dermal fibroblasts from PPCM patients, as well as in cardiac tissue from mice with PPCM due to a cardiomyocyte-specific deletion of STAT3 (α -MHC-Cre^{tg}, STAT3^{flox/flox} mice; CKO).⁸ A systemic knockout of PAI-1 crossed into CKO mice, however, led to high peripartum mortality with accelerated cardiac fibrosis, inflammation and heart failure, indicating that cardiac PAI-1 expression is protective for the PPCM heart. Thus, PAI-1 is an independent marker for PPCM that, together with the uPAR-NF- κ B activity assay, could be developed into a disease-specific test system. However, due to its controversial role in PPCM, it is likely to be unsuitable as a therapeutic target.

2. Methods

Expanded methods are available in the [Supplementary material online](#).

2.1 Patients

The local ethics committee of Hannover Medical School approved this study. All patients provided written informed consent. The study conforms to the principles outlined in the declaration of Helsinki. All PPCM

patients ($n = 64$) enrolled in the study were diagnosed with PPCM according to the definition provided in position statement from the Heart Failure Association of the European Society of Cardiology Working Group on PPCM.^{1,2} The pregnancy-matched control collective consisted of healthy age- and postpartum-matched women (PP-Ctrl, $n = 53$) who displayed normal LVEF ($>55\%$). In addition, age-matched, non-pregnant healthy control women (either never pregnant or at least 24 months since their last delivery, $n = 8$) were used. LV tissue was obtained from three PPCM patients either at the time of assist device implantation or heart transplantation, and from donor hearts not suitable for transplantation ($n = 11$).

2.2 Patients and controls for dermal fibroblast isolation

Patients aged 18 years or above were eligible for the study if diagnosed with PPCM in line with the European Society of Cardiology guidelines 2012 as previously described.^{1,2} A total of four patients were included for dermal fibroblast isolation. The mother of one patient and the sister of another patient were included as healthy familial controls. Both control subjects were pregnant at least once without cardiac complications. All participants provided informed consent. This study was approved by the Medical Ethical Committee of the University Medical Center Groningen (application number: METc 2014.104). Further information on dermal fibroblast isolation is provided in the [Supplementary material online, Methods](#).

2.3 Blood tests

Blood samples were collected in S-Monovette[®] tubes containing ethylenediaminetetraacetic acid (EDTA, for plasma) or clot activator (for serum) at the time point of initial diagnosis (baseline, BL) and at the follow-up visits (1 month and 6 months after diagnosis) in PPCM patients. Blood samples were also obtained from non-pregnant and PP-Ctrl (PP-Ctrl: 1 day to 10.5 months postpartum) women. Plasma or serum was separated by centrifugation at 1500 rpm for 10 min and aliquots were stored at -80°C . Laboratory workup was performed as part of routine analysis by hospital laboratories for NT-proBNP, high sensitive CRP, TNT, and creatine kinase (CK). PAI-1 plasma levels were measured using the Quantikine ELISA for human total serpin E1/PAI-1 Immunoassay (R&D systems, DTSE100) according to the manufacturer's protocol. uPA, interleukin (IL)-6, and IL-1 β were measured in plasma using Bio-Plex Pro Assays (Human Cytokine Grp I Panel 27-Plex M50-0KCAT0Y, Human Cancer Biomarker Panel 2 18-plex 171AC600M, Bio-Rad, Hercules, USA) according to the manufacturer's instructions.

PAI-1 in cell culture supernatant from patients and healthy familial control dermal fibroblasts were analysed by multiplex analysis using the Bio-Plex Human cancer panel 2, 18-plex assays (171AC600M, Bio-Rad, Hercules, USA) as recommended by the manufacturer.

2.4 Animal experiments

The generation of mice with cardiomyocyte-restricted deletion of STAT3 α MHC-Cre^{tg/-}; STAT3^{fl/fl} mice (CKO) has been described previously.^{8,17} CKO mice were crossed to mice with a systemic PAI-1 knockout (PAI-1^{-/-}, Jackson laboratory Stock 002507 B6.129S2-Serpine1^{tm1Mlg/J}) to generate CKO; PAI-1^{-/-}, CKO; PAI-1^{+/+} or WT; PAI-1^{+/+} mice. Female mice were either subjected to pregnancy and analysed postpartum (PP) or were kept virgin and non-pregnant, referred as nulli-parous (NP). Echocardiography was performed in sedated mice and

Table 1 Summary of clinical data from PPCM patients at BL and postpartum controls

Parameters	PPCM patients (n = 64)	PP-Ctrl (n = 53)
Age (years), mean ± SD	34 ± 5	31 ± 5**
Parity, median (range)	2 (1–6)	1 (1–4)**
NYHA, median (range)	4 (1–4)	1 (1–1)****
LVEF (%), mean ± SD	23 ± 8	63 ± 5****
Heart rate (b.p.m.), mean ± SD	87 ± 15	
Systolic BP (mmHg), mean ± SD	109 ± 16	
Diastolic BP (mmHg), mean ± SD	70 ± 13	
Hypertensive complications	27% (16/59)	0
CRP (mg/L), median (range)	9 (0.5–180)	38 (10–164)**
hsTNT (ng/L), median (range)	19 (9–699)	2 (2–6)****
NT-proBNP (pmol/mL), median (range)	3087 (175–31 700)	59 (30–531)***
IL-1β (pg/mL)	n.d.	n.d.
IL-6 (pg/mL)	10.6 ± 6.8	7.1 ± 5.2
uPA (pg/mL)	307 ± 139	321 ± 151

New York Heart Association (NYHA), left ventricular ejection fraction (LVEF), blood pressure (BP), C-reactive protein (CRP), high sensitive troponin T (hsTNT), N-terminal pro-brain natriuretic peptide (NT-proBNP) were analysed in routine clinical lab tests, interleukin (IL)-1β (not detected, n.d.), IL-6 and urokinase type plasminogen activator (uPA) by multiplex analyses (BioRad). ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 PPCM patients at BL vs. healthy postpartum controls (PP-Ctrl). Comparison between the groups was performed using Student's *t*-test for Gaussian distributed data (presented as mean ± SD) and the Mann–Whitney *U* test where at least one column was not normally distributed (presented as median and range). Categorical variables are presented as frequencies (percentages) and were compared using Fisher's exact test.

determined in age-matched NP mice or in PP mice within the first 2 weeks after the first pregnancy (2% isoflurane inhalation, connected to a rodent ventilator) using a Vevo 770 (Visual Sonics) as described.^{8,17,18} All animal studies were undertaken in accordance with German Animal Welfare legislation and with the European Communities Council Directive 2010/63/EU for the protection of animals used for experimental purposes. All experiments were approved by the Local Institutional Animal Care and Research Advisory Committee and permitted by the relevant local authority for animal protection.

2.5 Statistical analyses

Statistical analysis was performed using GraphPad Prism version 5.0a or 7.0 for Mac OS X (GraphPad Software, San Diego, CA, USA). Normal distribution was tested using D'Agostino normality test. Continuous data were expressed as mean ± standard deviation or median and range, according to normality of distribution. Comparison between the groups was performed using Student's *t*-test for Gaussian distributed data and the Mann–Whitney *U* test where at least one column was not normally distributed. When comparing more than two groups, ANOVA and Bonferroni's *post hoc* test or Kruskal–Wallis and Dunn's *post hoc* test were used according to normality of distribution. Categorical variables are presented as frequencies (percentages) and compared using the Fisher's exact test. Survival data were analysed using the log-rank (Mantel–Cox) test. A two-tailed *P*-value of <0.05 was considered statistically significant. Correlation for NT-proBNP, CRP, TNT, CK, and miR-146a was analysed by ozone correlation analysis for Gaussian distributions by using Pearson correlation coefficients.

3. Results

3.1 Clinical characteristics

The clinical characteristics of all PPCM patients (*n* = 64) and the postpartum-matched healthy controls (PP-Ctrl, *n* = 53), including age, parity, and LVEF at BL and 6-month follow-up, are provided in Table 1. All PPCM patients included in the study were diagnosed postpartum.

3.2 Comparison of circulating levels of PAI-1, NT-proBNP, CRP, TNT, IL-6, and IL-1β in PPCM patients and healthy postpartum women

In line with previous reports,^{3,19} NT-proBNP plasma levels were significantly increased in PPCM BL, while TNT, a marker for cardiac injury, was only marginally elevated (Table 1). Both PPCM BL and PP-Ctrl showed moderate elevation in CRP, which in the present collective was in fact slightly higher in PP-Ctrl (Table 1). Other markers of inflammation and acute phase response, i.e. IL-6 and IL-1β were also determined, but no differences in IL-6 between PPCM at BL and PP-Ctrl were observed and IL-1β was beyond detection levels in either group (Table 1). In turn, circulating PAI-1 was significantly higher (3.0-fold, 64 ± 38 ng/mL, *P* < 0.01) in PPCM patients at BL compared with PP-Ctrl (Figure 1A). The mean of PAI-1 measured in PP-Ctrl (*n* = 53, 21 ± 10 ng/mL) was within the normal range for healthy women (published PAI-1 plasma levels: around 35 ng/mL,²⁰ PAI-1 levels measured in age-matched non-pregnant control women in the present study was 16 ± 10 ng/mL, *n* = 8). To analyse potential differences in PAI-1 levels in relation to the time after delivery, we compared PAI-1 levels in PP-Ctrl with blood sampling in the first 1–7 days after delivery (early PP-Ctrl) and with PP-Ctrl with blood sampling later than 1 week after delivery (blood sampling 1 week to 10.5 months postpartum, late PP-Ctrl). We did not observe any significant differences between these two groups [early PP-Ctrl, *n* = 40, PAI-1: 20.76 ± 10 ng/mL vs. late PP-Ctrl, *n* = 13: PAI-1: 21.35 ± 10 ng/mL, n.s.), indicating that PAI-1 plasma levels are not substantially affected by postpartum kinetics. In contrast to PAI-1, no differences in plasma levels of uPA were observed between PPCM at BL and PP-Ctrl (Table 1).

3.3 Baseline circulating PAI-1 levels positively correlated with circulating miR-146a plasma levels

PAI-1 levels in PPCM patients at BL did not correlate with BL LVEF, or with LVEF at 6-month follow-up (Figure 1B and C). No correlations were observed between PAI-1 levels and NT-proBNP (Figure 1D), TNT (data not shown, Pearson *r*: 0.02938; *P*-value: 0.8282) or CRP (data not shown, Pearson *r*: -0.02137; *P*-value: 0.8724). It has been shown that 16 kDa-PRL induces the expression of miR-146a in endothelial cells via NF-κB activation.⁷ At BL, the present PPCM collective displayed 3.6-fold (*P* < 0.001) higher miR-146a plasma levels compared with PP-Ctrl, confirming previous results of higher miR-146a levels in PPCM patients.⁷ Moreover, miR-146a plasma levels correlated positively with BL PAI-1 levels in the present PPCM collective (Figure 1E).

3.4 PAI-1 levels did not correlate with parity but were increased after delivery and prior to heart failure in PPCM patients with a subsequent pregnancy

High parity is a suspected risk factor for PPCM and parity was indeed significantly higher in PPCM patients compared with the healthy PP-Ctrl

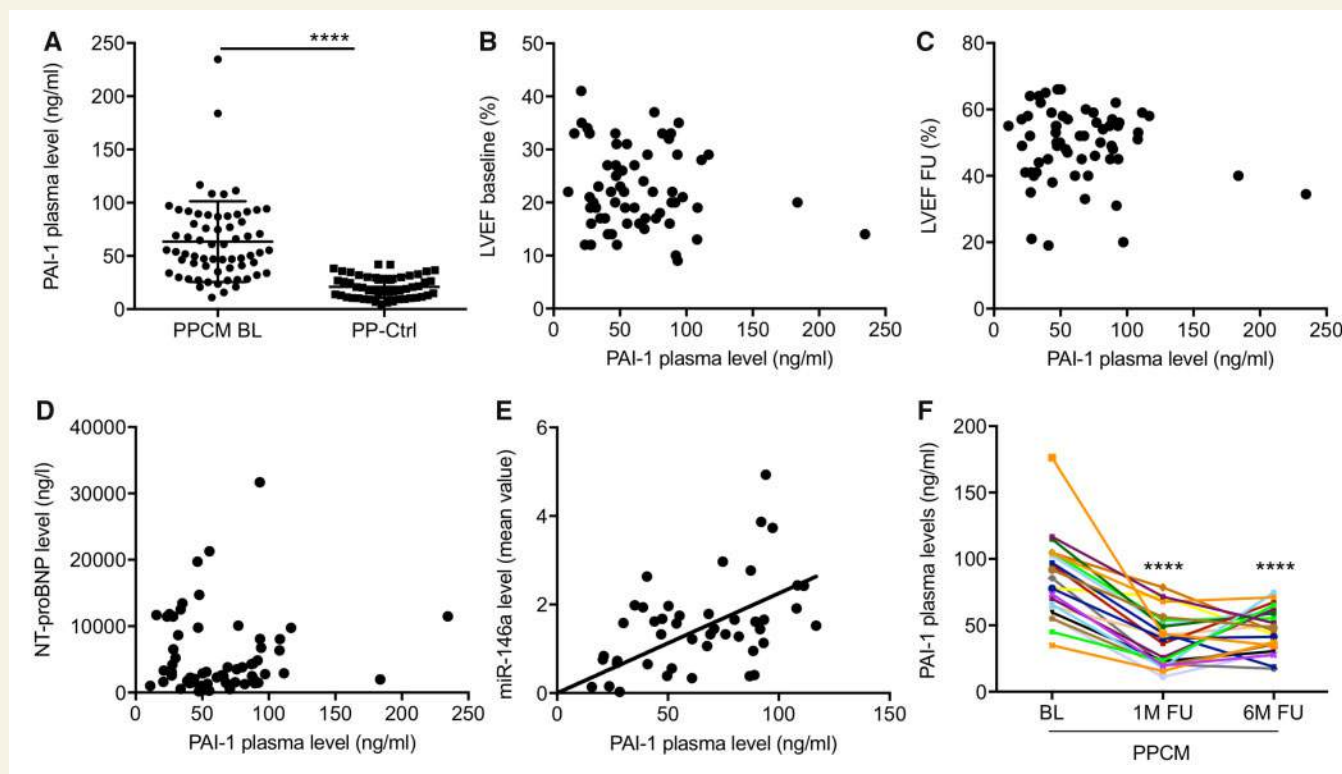


Figure 1 Plasma PAI-1 levels in healthy postpartum women and in patients with PPCM. (A) PAI-1 plasma levels (ng/mL) of PPCM patients at BL ($n = 64$) compared with PP-Ctrl ($n = 53$, $P < 0.0001$). Correlation analysis of PAI-1 levels from PPCM at BL with (B) LVEF at BL ($n = 64$; Pearson r : -0.1454 , P -value: 0.2517), (C) LVEF 6-month follow-up ($n = 61$, Pearson r : -0.1196 , P -value: 0.3586), and (D) NT-proBNP ($n = 60$, Pearson r : 0.03835 , P -value: 0.7711). (E) miR-146a levels positively correlated with PAI-1 plasma levels ($n = 45$; Pearson r : 0.4689 , P -value: 0.0012). (F) PAI-1 plasma levels (ng/mL) of PPCM patients at BL and at 1-month ($n = 23$; **** $P < 0.0001$ vs. BL) and 6-month follow-up ($n = 23$; *** $P < 0.001$ vs. BL); Data are presented as mean $\pm$ SD; unpaired two-tailed t -test (A), ozone correlation, Pearson correlation coefficients, two-tailed P -value (B–E). One-way ANOVA, Bonferroni's Multi Comparison Test (F).

(Table 1). However, no correlation between circulating BL PAI-1 levels and parity was observed in the PPCM patient cohort (Supplementary material online, Figure S1A). Interestingly, in three PPCM patients with subsequent pregnancies, PAI-1 levels measured after delivery of the subsequent pregnancy were all markedly above the mean of healthy postpartum controls prior to the worsening of cardiac function (Supplementary material online, Table S1).

3.5 Recovery in PPCM patients is associated with decreased circulating PAI-1 levels

All PPCM patients in the present study were treated according to the BOARD concept (Bromocriptine, Oral heart failure therapies, Anticoagulants, vasoRelaxing agents, and Diuretics^{19,21}) follow-up analyses were available for $n = 61$ patients (three PPCM patients were lost to follow-up). At 6 months after diagnosis (6 ± 0.5 months), a remarkable recovery in LV function was present in most PPCM patients with follow-up (mean LVEF BL: $23 \pm 8\%$ and at 6-month follow-up: $49 \pm 11\%$, $P < 0.001$ vs. PPCM BL). PAI-1 levels at 1-month and 6-month follow-up were available for $n = 23$ patients. In these patients, PAI-1 levels had already decreased significantly at 1-month follow-up and remained low at 6-month follow-up (Figure 1F).

3.6 In serum from PPCM patients, 16 kDa-PRL coprecipitates with PAI-1 and serum from PPCM patients induces higher uPAR-mediated NF- κ B activation in endothelial cells than serum from healthy postpartum women

It has been shown that PAI-1 in a complex with 16 kDa-PRL induces NF- κ B via the uPAR in tumour cells.¹² Here, we could demonstrate that 16 kDa-PRL could be coprecipitated with PAI-1 from human PPCM serum at BL but at 6-month follow-up 16 kDa-PRL protein levels were decreased and barely detectable (Figure 2A). To analyse whether the 16 kDa-PRL/PAI-1/uPAR complex activates NF- κ B in endothelial cells from PPCM patients, we used a rat heart endothelial cell line (RHE-A) in which human PAI-1 functionally interacts with the rat uPAR. These cells were stably transfected with an NF- κ B driven luciferase reporter plasmid and transduced either by lentiviral siRNA against uPAR or lentiviral control siRNA. Serum samples from PPCM patients taken at BL induced substantially more NF- κ B promoter activation compared with serum samples from PP-Ctrl in control siRNA transfected RHE-A cells, but no substantial differences were evident between PPCM and PP-Ctrl serum in siRNA-mediated uPAR knockdown RHE-A cells (Figure 2B and C). To investigate whether a reduction of PAI-1 in serum is associated with

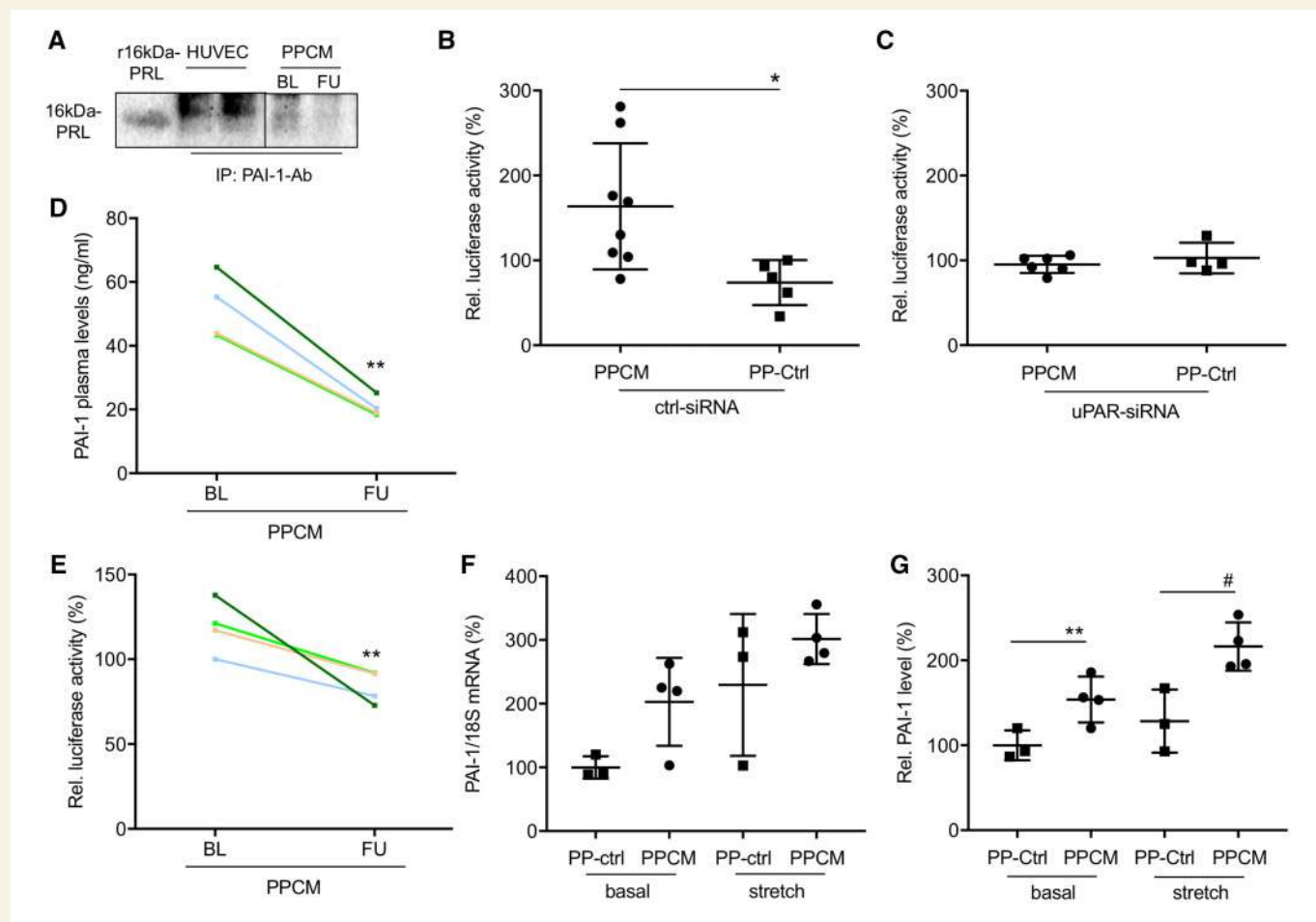


Figure 2 Formation of 16 kDa-PRL/PAI-1 and uPAR-mediated NF- κ B activation induced by serum from PPCM and healthy postpartum women, and PAI-1 expression in dermal fibroblasts from PPCM patients and healthy postpartum relatives. (A) Representative western blot of 16 kDa-PRL protein levels after immunoprecipitation (IP) with PAI-1 in conditioned HUVEC media with recombinant 16 kDa-PRL and human BL and 6-month follow-up PPCM serum. (B) NF- κ B activation measured by a relative luciferase reporter plasmid in RHE-A cells exposed to serum from PPCM women and healthy postpartum control women (PP-Ctrl) with control siRNA or (C) siRNA against the uPAR (serum from $n = 4$ to 8 individuals), assays performed in triplicates for each individual. $*P < 0.05$ vs. PPCM, all data are presented as mean $\pm$ SD; unpaired two-tailed t -test. (D) PAI-1 plasma levels (ng/mL) at BL and 6-month follow-up and (E) NF- κ B activation (luciferase reporter plasmid, in RHE-A cells) induced by BL and at 6-month follow-up serum (assays performed in triplicates for each individual. $**P < 0.01$ vs. PPCM BL) from the same PPCM patients ($n = 4$). PPCM BL was set at 100%, data are presented as mean $\pm$ SD; unpaired two-tailed t -test. (F) PAI-1 mRNA levels (qRT-PCR) under basal conditions or under 15% stretch (1 Hz) for 48 h in dermal fibroblasts from PPCM patients ($n = 4$) and healthy PP-Ctrl female relatives ($n = 2$ healthy familial controls, that were both pregnant at least once without cardiac complications. From one control patient, two independently generated cell lines were analysed). Basal controls were set to 100%, and (G) PAI-1 levels secreted into the cell culture supernatant under basal conditions or under 15% stretch (1 Hz) for 48 h in dermal fibroblasts from PPCM patients ($n = 4$) and healthy PP-Ctrl female relatives ($n = 2$ healthy familial controls, that were both pregnant at least once without cardiac complications. From one control patient, two independently generated cell lines were analysed. Basal controls were set to 100% (F and G), $**P < 0.01$ vs. ctrl basal; $\#P < 0.05$ vs. ctrl stretch, two-way ANOVA, Bonferroni's multiple comparison test.

reduced NF- κ B promoter activation in endothelial cells, we used serum from PPCM patients taken at BL and after 6-month follow-up when PAI-1 levels were significantly lower (Figure 2D). We observed that NF- κ B promoter activation induced by 6-month follow-up serum was significantly lower compared with BL serum (Figure 2E).

3.7 Expression of PAI-1 is elevated in cardiac tissue and in dermal fibroblasts from PPCM patients compared with respective healthy controls

Next, we measured PAI-1 expression in LV tissue from PPCM patients and from non-failing control hearts (NF). We observed that PAI-1

mRNA levels were significantly higher in LV tissue from PPCM patients compared with NF LV tissue from organ donors (PPCM, $n = 3$: $712 \pm 512\%$ vs. NF, $n = 11$: $193 \pm 35\%$, $P = 0.03$). To further analyse whether cells from PPCM patients produce more PAI-1 than healthy postpartum controls, dermal fibroblasts were isolated from PPCM patients ($n = 4$) and healthy female postpartum first-degree relatives (PP-Ctrl, $n = 2$, from two healthy familial controls, that were both pregnant at least once without cardiac complications. From one control patient, two different cell lines were generated and analysed separately). Dermal fibroblasts from PPCM patients displayed by trend higher PAI-1 mRNA expression and released significantly more PAI-1 under static and stretched conditions compared with PP-Ctrl fibroblasts (Figure 2F and G).

Table 2 PAI-1 plasma levels, LVEF at BL at follow-up in relation to the PAI-1 genotype

Genotype	4G/4G; A-844/A-844	4G/5G; A-844/-844G	4G/5G; A-844/A-844	5G/5G; -844G/-844G	5G/5G; A-844/-844G
n	9	8	2	3	1
LVEF BL (%)	24 ± 8	23 ± 8	17 ± 4	21 ± 2	35
LVEF follow-up (%)	47 ± 9	51 ± 14	38	49 ± 2	56
PAI-1 (ng/mL)	92 ± 43	66 ± 32	52 ± 12	57 ± 31	94

The 4G/5G polymorphism at position -675 and the base substitution of A to G at position -844 (A-844-G) were analysed either by GenoType PAI-1 assay (Hain Lifescience) and/or Sanger sequencing. PAI-1 plasma levels were analysed by ELISA, left ventricular ejection fraction (LVEF) at baseline (BL) and at 6-month follow-up. (One PPCM patient in the 4G/5G; A-844/A-844 was lost to follow-up).

3.8 Analyses of PAI-1 promoter polymorphisms in PPCM patients

It is known that polymorphism in the promoter of the PAI-1 gene, i.e. the 4G/5G polymorphism at position -675 and a base substitution of A to G at position -844 (A-844-G) (Supplementary material online, Figure S1B), influences the expression level of PAI-1 and thereby affects the risk for stroke, myocardial infarction, and miscarriages.^{13,22} We analysed the PAI-promoter region in *n*=23 PPCM patients of the German PPCM registry (Table 2) and observed that 39% (9/23) carried the high expression 4G/4G genotype, 48% (11/23) were heterozygous for 4G/5G and 17% (4/23) were of the lower expressing 5G/5G genotype. The reported incidence in healthy postpartum women for the high expression genotype is 24%, while 51% are heterozygous and 25% homozygous for the low expressing type.²³ For the high expressing A-844/A-844 genotype the prevalence in PPCM was 48% (11/23), while 39% (9/23) were heterozygous (A-844/844-G) and 13% (3/23) were homozygous for the low expressing 844-G/844-G polymorphism. The reported distribution of these variations in healthy postpartum women was 29% A-844/A-844, 51% A-844/844-G, and 20% 844-G/844-G.²³ LVEF at BL and at follow-up, as well as PAI-1 plasma levels for these *n*=23 PPCM patients in relation to the PAI-1 genotypes, are provided in (Table 2), showing that there were no substantial differences between the different PAI-1 genotypes in LVEF at BL and at 6-month follow-up. Patients who were homozygous for both high expressing gene variants (4G/4G; A-844/A-844) displayed the highest plasma PAI-1 levels (Table 2), however, the numbers were not high enough for a statistical evaluation of the different groups.

3.9 No difference in circulating PAI-1 levels in patients with confirmed genetic cardiomyopathy compared with PPCM patients not carrying such mutations

In the analysed collective, six patients carried cardiomyopathy-causing mutations (five in titin, one in Lamin A/C). PAI-1 levels in these patients (*n*=6, 49 ± 36 ng/mL) were not different (n.s) to those of PPCM patients not carrying such mutations (*n*=40, 68 ± 43 ng/mL).

3.10 PAI-1 expression is markedly increased in mice with PPCM due to a cardiomyocyte-specific deficiency of STAT3 (CKO)

We previously reported that STAT3 is reduced in the hearts of patients with PPCM and showed that female mice with a

cardiomyocyte-specific deficiency for STAT3 (CKO) develop PPCM.^{7,8} Here, we observed that PAI-1 mRNA and protein levels were significantly increased in cardiac tissue from CKO mice with PPCM compared with postpartum wild type (WT) mice, NP WT, and NP CKO mice (Figure 3A and B).

3.11 A homozygous systemic knockout of PAI-1 accelerates PPCM in CKO mice

To test whether targeting PAI-1 could be a therapeutic option in PPCM, we crossed a systemic deletion (PAI-1^{-/-}) into the CKO strain. We observed that female CKO mice homozygous for PAI-1 deletion (CKO; PAI-1^{-/-}) are born at the expected Mendelian ratio. At the age of 12 weeks, protein analyses reveal a complete absence of PAI-1 in the cardiac tissue of these mice (Figure 3B), which otherwise have normal cardiac function and dimensions (Table 3). When exposed to breeding, all CKO; PAI-1^{-/-} mice died at the end of the first pregnancy or immediately after the first delivery, while WT and PAI-1^{-/-} female mice survived two subsequent pregnancies and nursing periods well, and CKO mice displayed an increased mortality only after the second pregnancy and nursing period (Figure 3C). After the first pregnancy (PP), WT and PAI-1^{-/-} mice had normal cardiac function and dimensions, CKO mice showed slightly decreased cardiac function, while all CKO; PAI-1^{-/-} mice were in severe heart failure with substantial LV dilatation (Table 3). Heart failure in postpartum CKO; PAI-1^{-/-} was paralleled by an up-regulation of ANP, Fibronectin and Collagen alpha 1 (Col1α1) expression (Figure 3D–F). In age-matched nulli-pari WT, PAI-1^{-/-}, and CKO mice, normal cardiac morphology was observed, while a slight increase in fibrosis was visible in CKO; PAI-1^{-/-} mice (Figure 4A–G). After one pregnancy, no visible alterations in cardiac morphology, fibrosis, or inflammation were detectable in WT and PAI-1^{-/-} mice. CKO mice displayed moderately increased fibrosis and inflammation, while a high degree of fibrosis and inflammation was present in CKO; PAI-1^{-/-} mice (Figure 4A–G).

3.12 In PAI-1^{-/-} fibroblasts cell culture supernatant from STAT3 knockdown cardiomyocytes up-regulates the expression of Col1α1 and TGFβ2

In order to analyse why fibrosis was elevated in postpartum CKO; PAI-1^{-/-} but not in PAI-1^{-/-} mice and to a lesser extent in CKO mice after one pregnancy, cell culture supernatant from HL-1 cardiomyogenic cells with (STAT3-KD) and without (WT) shRNA-mediated knockdown of STAT3 was added to isolated fibroblasts from PAI-1^{-/-} mice. STAT3-KD supernatant induced a higher expression of Col1α1 and TGFβ2 in PAI-

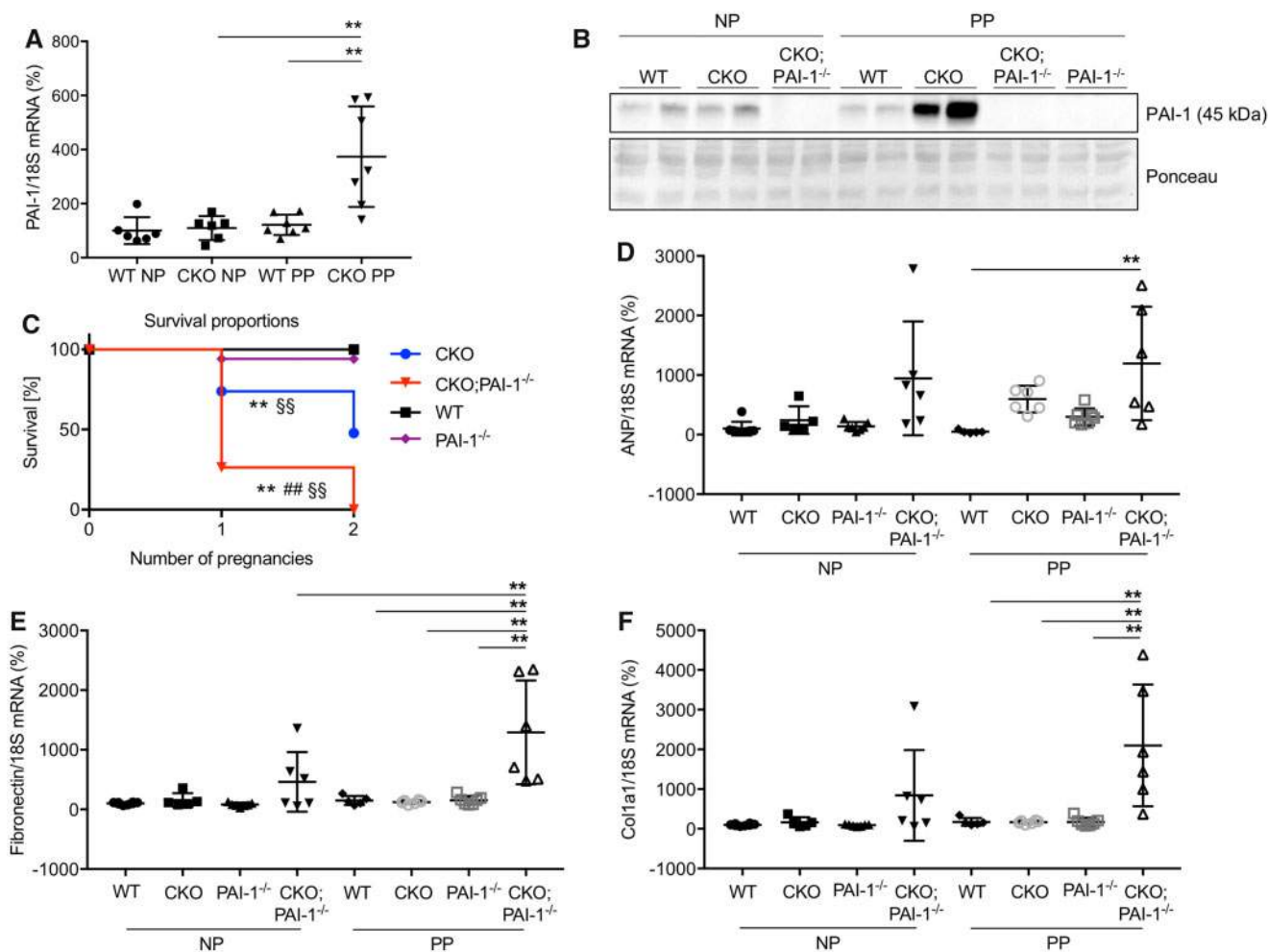


Figure 3 Effect of pregnancy and nursing in WT, CKO, and CKO; PAI-1^{-/-} mice. (A) PAI-1 mRNA by qRT-PCR in cardiac tissue from WT and CKO mice, nulli-pari (NP, WT *n* = 6; CKO *n* = 6) and after two pregnancies and nursing periods (PP, WT *n* = 7; CKO *n* = 7), ***P* < 0.01 vs. CKO PP. (B) Representative PAI-1 western blot in cardiac tissue from WT, CKO, and CKO; PAI-1^{-/-} mice, nulli-pari and after pregnancy. (C) Kaplan–Meier curve showing survival of WT (*n* = 31), CKO (*n* = 23), PAI-1^{-/-} (*n* = 17), and CKO; PAI-1^{-/-} (*n* = 19) mice exposed to pregnancy. Survival data were analysed using the log-rank (Mantel-Cox) test, ***P* < 0.01 vs. WT-PP, ##*P* < 0.01 vs. CKO-PP, §§*P* < 0.01 PAI-1^{-/-}. (D) ANP, (E) fibronectin, and (F) Col1a1 mRNA levels analysed by qRT-PCR in (D–F) WT (NP: *n* = 8; PP: *n* = 5), CKO (NP: *n* = 5; PP: *n* = 6), PAI-1^{-/-} (NP: *n* = 7; PP: *n* = 7), and CKO; PAI-1^{-/-} (NP: *n* = 6; PP: *n* = 6) mice nulli-pari and exposed to pregnancy. (D–F) ***P* < 0.01 vs. CKO; PAI-1^{-/-} PP, all data are presented as mean ± SD, two-way ANOVA, Bonferroni's multiple comparison test.

Table 3 Cardiac function and dimension in WT, CKO, PAI-1^{-/-}, and CKO; PAI-1^{-/-} mice nulli-parous and after the first pregnancy

	WT NP <i>n</i> = 8	CKO NP <i>n</i> = 6	PAI-1 ^{-/-} NP <i>n</i> = 8	CKO; PAI-1 ^{-/-} NP <i>n</i> = 6	WT PP <i>n</i> = 6	CKO PP <i>n</i> = 7	PAI-1 ^{-/-} PP <i>n</i> = 6	CKO; PAI-1 ^{-/-} PP <i>n</i> = 5
%FS	46 ± 8	36 ± 7	41 ± 3	46 ± 9	41 ± 6	28 ± 9*	37 ± 9	20 ± 5**.
LVEDD (mm)	3.51 ± 0.09	3.43 ± 0.15	3.59 ± 0.46	3.46 ± 0.21	4.17 ± 0.41	4.00 ± 0.48	3.79 ± 0.34	4.56 ± 0.9**.
LVESD (mm)	1.89 ± 0.29	2.18 ± 0.24	2.13 ± 0.29	1.85 ± 0.31	2.47 ± 0.26	2.90 ± 0.66	2.36 ± 0.30	3.67 ± 1.0*.
HR (b.p.m.)	548 ± 34	511 ± 50	537 ± 28	549 ± 27	487 ± 41	514 ± 41	523 ± 55	526 ± 20

Fractional shortening (FS), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), heart rate (beats per minute, b.p.m.), determined within the first 2 weeks after the first pregnancy. Data are presented as mean ± SD, **P* < 0.05, ***P* < 0.01 vs. NaCl WT-PP, ##*P* < 0.01 CKO; PAI-1^{-/-}-PP vs. CKO; PAI-1^{-/-} NP. Two-way ANOVA, Bonferroni's Multiple Comparison Test.

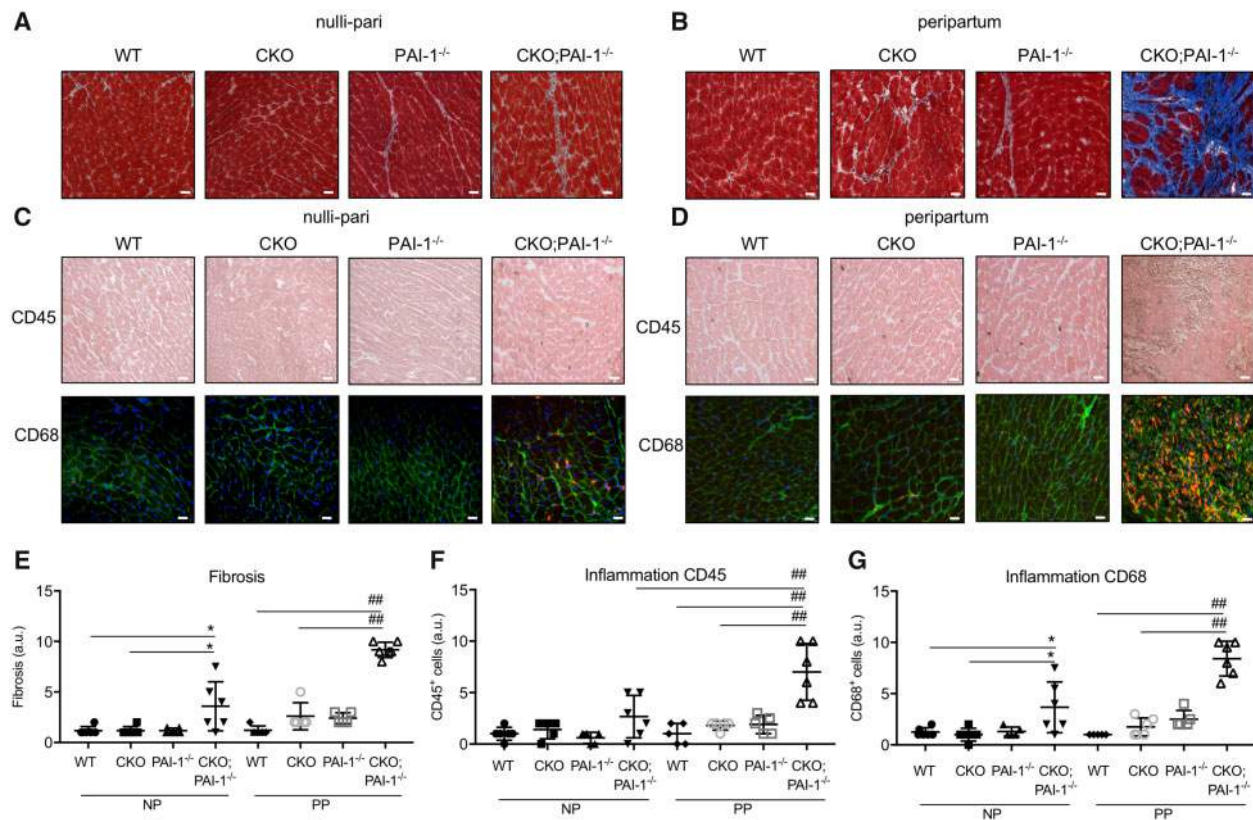


Figure 4 High degree of cardiac fibrosis and inflammation in CKO; PAI-1^{-/-} mice in the peripartum phase of the first pregnancy. (A and B) Masson-trichrome staining visualizing fibrosis, (C and D) staining with the pan-inflammatory marker CD45 (brown, co-stained with haematoxylin, upper panels) showing inflammation and the monocyte/macrophage marker CD68 (red, lower panels) co-stained with WGA (cell membranes, green) and DAPI (nuclei, blue) specifically depicting monocytes/macrophages in LV sections from WT, PAI-1^{-/-}, CKO, and CKO; PAI-1^{-/-} female mice nulli-pari (NP, A and C) and postpartum (PP, B and D), scale bars indicate 50 μ m. Dot blots summarizing (E) fibrosis in WT (NP: $n = 6$; PP: $n = 5$), CKO (NP: $n = 6$; PP: $n = 5$), PAI-1^{-/-} (NP: $n = 6$; PP: $n = 5$), and CKO; PAI-1^{-/-} (NP: $n = 6$; PP: $n = 6$) animals per group (F) global inflammation (CD45) in WT (NP: $n = 6$; PP: $n = 5$), CKO (NP: $n = 5$; PP: $n = 5$), PAI-1^{-/-} (NP: $n = 5$; PP: $n = 5$), and CKO; PAI-1^{-/-} (NP: $n = 6$; PP: $n = 6$) animals per group and (G) degree of monocytes/macrophage invasion (CD68) in WT (NP: $n = 6$; PP: $n = 5$), CKO (NP: $n = 6$; PP: $n = 6$), PAI-1^{-/-} (NP: $n = 5$; PP: $n = 5$), and CKO; PAI-1^{-/-} (NP: $n = 6$; PP: $n = 6$) calculated in arbitrary units (a.u.), * $P < 0.05$ vs. CKO; PAI-1^{-/-}-NP; ## $P < 0.01$ vs. CKO; PAI-1^{-/-}-PP. All data are presented as mean $\pm$ SD, two-way ANOVA, Bonferroni's multiple comparison test.

1^{-/-} fibroblasts compared with the WT supernatant (Supplementary material online, Figure S2AC).

4. Discussion

Here, we report that PPCM patients display markedly increased circulating levels of PAI-1 at the time of diagnosis compared with healthy postpartum controls. PAI-1 seems to be a novel marker for PPCM, which positively correlates with miR-146a levels, a marker for PPCM that is induced by 16 kDa-PRL-NF- κ B activation. In contrast, PAI-1 does not correlate with NT-proBNP, the only clinical marker, which has so far found to be consistently up-regulated in PPCM, or with TNT which indicates myocardial damage. Neither CRP, IL-6 or IL-1 β are specifically up-regulated in the present PPCM cohort. Circulating BL PAI-1 levels were not correlated with the severity of heart failure at diagnosis nor did they predict recovery at 6-month follow-up.

Although PAI-1 plasma levels seem suitable as a diagnostic biomarker for PPCM, relying solely on concentrations of PAI-1 without assessing its

function may be misleading, since this protein has different biological functions depending on its interaction partners.²⁴ Along this line, the canonical function of PAI-1 is to inhibit uPAR signalling.²⁴ Thereby, PAI-1 attenuates uPAR-mediated fibrinolysis and anti-thrombotic effects and thus acts as a prothrombotic factor.²⁴ More recently, it has been shown that PAI-1 binds to the 16 kDa-PRL to induces non-canonical activation of NF- κ B from the uPAR, thereby promoting the expression of miR-146a in endothelial cells, which is largely responsible for 16 kDa-PRL-induced impaired vascular function and heart failure in the pathophysiology of PPCM.^{7,12} Here, we showed that PAI-1 could be coprecipitate with 16 kDa-PRL from PPCM patients serum indicative for direct physical interaction of the two proteins. Additionally, we observed that BL serum from PPCM patients exhibited higher non-canonical uPAR-NF- κ B activation than serum from PP-Ctrl. In PPCM patients where PAI-1 plasma levels were reduced at 6-month follow-up, 6-month follow-up serum showed also reduced NF- κ B activation further supporting the notion that a 16 kDa-PRL/PAI-1 interaction is responsible for non-canonical uPAR/NF- κ B activation in PPCM patients. Moreover, we show that circulating levels of miR-146a are also higher in the present

PPCM cohort than in PP-Ctrl and that plasma PAI-1 levels correlated positively with plasma miR-146a. Based on these observations, we suggest that a 16 kDa-PRL/PAI-1/uPAR-NF- κ B/miR-146a signalling may contribute to the pathophysiology of PPCM. Therefore, we suggest developing a diagnostic combination test for PPCM, including measuring plasma PAI-1 levels, the activity of the uPAR-NF- κ B signalling module, and miR-146a in addition to NT-proBNP. Thereby, one could overcome the gap in diagnostic evidence for PPCM as the combination test identifies the presence of a PPCM-specific pathomechanism related to 16 kDa-PRL, thereby not only aiding diagnosis but also justifying the addition of the prolactin blocker bromocriptine to standard therapy for heart failure.^{19,21} Moreover, this test would also facilitate the diagnosis of PPCM arising from different aetiologies, since PAI-1 levels were also elevated in PPCM patients with confirmed cardiomyopathy-causing mutations. The test may also be suited to predict the risk for PPCM in patients with subsequent pregnancies as we found that plasma PAI-1 was also elevated in these patients after delivery of the subsequent pregnancy but prior to the onset of heart failure.

It has been shown that gene polymorphisms, i.e. the 4G/5G polymorphism at position -675 and the base substitution at position -844 (A-844-G), in the PAI-1 promoter affect PAI-1 expression and that the presence of high expression promoter variants are associated with a higher risk of thrombotic complications, miscarriages,^{13,22,25} and mild preeclampsia.²³ Hypertension and preeclampsia are risk factors for PPCM^{4,9} present in almost one quarter of the patients in the present PPCM cohort. In addition, PPCM patients have also a higher risk for thrombosis and frequently present with ventricular thrombi.²⁶ Both factors, high risk for pregnancy-associated hypertension and thrombosis, would be in line with the increased PAI-1 levels observed in PPCM patients. Moreover, a meta-analysis of several small studies has confirmed that elevated plasma PAI-1 levels are associated with major adverse cardiovascular events.²⁷ Here, we observed that the promoter combination 4G/4G; A-844/A-844, which is responsible for the highest PAI-1 expression, was indeed the most prevalent in PPCM patients and higher than in the normal population.²³ However, the number of sequenced patients in the present cohort is too small to be conclusive. In addition, different PAI-1 promoter polymorphism did not correlate with the severity of heart failure at BL, nor did they affect the chance for full recovery in the subgroup of genotyped PPCM patients. Follow-up analyses showed a rapid and persisting reduction in PAI-1 plasma levels at 1-month follow-up, when all PPCM patients of the present PPCM cohort received the BOARD treatment (Bromocriptine, Oral heart failure therapies, Anticoagulants, vasoRelaxing agents, and Diuretics), which also includes low-molecular weight heparin (LMWH) in addition to bromocriptine and standard therapy for heart failure. It has been shown that LMWH transiently reduces circulating PAI-1 levels²⁸ and there is evidence that 16 kDa-PRL, which is blocked by bromocriptine, induces PAI-1 expression in a positive feedback loop.²⁹ At 6-month follow-up, when all PPCM patients were only on standard heart failure therapy (no bromocriptine and no LMWH), PAI-1 levels had increased again in some patients. It is therefore possible that the two therapeutic components of the BOARD treatment concept, LMWH and bromocriptine may contribute to lower circulating PAI-1 levels in PPCM patients, a feature that has to be analysed in future studies, where also PPCM patients should be included who are not treated with the BOARD concept.

Besides higher circulating PAI-1 levels, we observed that PAI-1 expression was increased in cardiac tissue and in dermal fibroblasts from PPCM patients and also in LV tissue from CKO mice with PPCM, suggesting that PAI-1 may also have a direct impact in the PPCM heart.

Interestingly, it has been shown that PAI-1 can have opposing effects in the heart as both increased and reduced PAI-1 expression promotes cardiac fibrosis.¹⁴ To analyse the local effect of PAI-1 in hearts predisposed for PPCM, we introduced a systemic knockout of PAI-1 in our CKO PPCM mouse model. We observed that a lack of PAI-1 markedly aggravated PPCM in CKO mice, which was associated with extensive cardiac fibrosis and inflammation, heart failure, and high peripartum mortality. As neither CKO nor PAI-1^{-/-} mice showed a similarly severe PPCM phenotype, it seems that the combination of low STAT3 and lack of PAI-1 accelerates PPCM. The observation that supernatant from STAT3 knockdown cardiomyocyte cultures induce higher expression of Col1 α 1 and TGF β 2 in PAI-1^{-/-} knockout fibroblasts suggest that paracrine factors released from cardiomyocytes in CKO hearts are responsible for the strongly enhanced fibrosis observed in peripartum CKO; PAI-1^{-/-} mice. These data indicate that local up-regulation of PAI-1 in the heart of PPCM patients could have a protective effect as it prevents extensive fibrosis. Taken together with our previous findings that cardiac STAT3 expression is low in PPCM patients,^{8,30} the increased cardiac PAI-1 expression in human PPCM heart tissue may explain the overall low incidence of cardiac fibrosis in patients, and consequently the generally good prospects for recovery. Thus, these data reveal a paradox role of PAI-1 in the PPCM pathophysiology with a detrimental role in circulation but beneficial roles in the heart. Directly targeting PAI-1 in PPCM patients as a therapeutic avenue might therefore have unpredictable effects.

In conclusion, PPCM is a diagnosis of exclusion, where unexpected heart failure occurs in young women and where common blood tests for cardiac injury, i.e. TNT and acute phase response factors, are often negative or not specific. The present findings from this study suggest that, in addition to NT-proBNP, circulating PAI-1 levels in combination with an uPAR-NF- κ B activation assay may be a suitable biomarker combination to diagnose and predict PPCM in patients at risk. Furthermore, elevated circulating PAI-1 in PPCM patients may also go some way towards explaining the higher risk for thrombosis reported in these patients and support the use of at least prophylactic anticoagulation by LMWH, as recommended in the BOARD treatment regime. However, as outlined above, our data reveal that cardiac expression of PAI-1 may have also beneficial roles in PPCM by preventing cardiac fibrosis. Thus, PAI-1 seems to have multiple but, in part, opposing roles in PPCM, which include the described adverse effects in circulation and the local beneficial effects for the heart with the consequence that it may not be suited as a direct therapeutic target to treat PPCM.

4.1 Limitations of the study

As PPCM is a rare disease, limited numbers of patients are available, restricting group analyses and comparisons. For analyses in subsequent pregnancies, only three patients with blood samples taken within 24–48 h after delivery were available. Therefore, the data suggesting the potential use of PAI-1 plasma levels to predict risk for PPCM is preliminary and needs to be verified in higher numbers of patients. Similarly, the analyses of PAI-1 promoter polymorphism could only be performed in a low number of patients and need to be confirmed. With regard to the diagnostic specificity of PAI-1, in future studies comparison should also be performed with postpartum women with other cardiac conditions leading to cardiac dysfunction, e.g. women with congenital heart disease, myocarditis and/or dilated cardiomyopathy or pregnancy-associated complications such as preeclampsia or hypertensive disorders. In addition, since all patients with follow-up blood samples had been treated with the BOARD therapy concept after diagnosis, no follow-up data are present for patients obtaining other treatment concepts. Limitations in

the experimental model include that PPCM was induced by a cardiomyocyte-specific knockout of STAT3, which may not be representative for the condition in all PPCM patients.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

M.R.-H., M.F.H., and T.J.P. performed statistical analysis. D.H.-K. and P.v.d.M. handled funding and supervision. M.R.-H., M.F.H., T.J.P., S.S., J.N., S.H., N.B., M.S., B.S., E.S., Y.K., C.F., A.H., T.T., Z.A., and D.H.-K. acquired the data. M.R.-H., T.J.P., P.v.d.M., J.B., and D.H.-K. conceived and designed the research. M.R.-H., T.J.P., P.v.d.M., J.B., and D.H.-K. drafted the manuscript. M.R.-H., T.J.P., A.H., P.v.d.M., J.B., O.B., T.T., Z.A., and D.H.-K. undertook a critical revision of the manuscript for key content.

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Translational perspective

Our study suggests that PAI-1 adds 16 kDa-prolactin to induce the vascular damaging, non-canonical uPAR-NF- κ B signalling pathway as a central pathophysiology of PPCM. Circulating PAI-1 levels showed a positive correlation with miR-146a plasma levels. PAI-1, together with the uPAR-NF- κ B activity assay, emerged as a diagnostic marker for PPCM. The local cardiac up-regulation of PAI-1 in PPCM hearts seems to protect from cardiac fibrosis and inflammation, and may prevent permanent cardiac damage during acute PPCM. Due to its controversial role in PPCM, i.e. driving vascular damage but protecting the heart from fibrosis, PAI-1 seems unsuitable as a therapeutic target to treat PPCM.

EDITORIAL COMMENT

To Breastfeed or Not to Breastfeed With Peripartum Cardiomyopathy*



Zoltan Arany, MD, PhD,^a Arthur M. Feldman, MD, PhD^b

Peripartum cardiomyopathy (PPCM) is a disease of maternal cardiac systolic dysfunction, often accompanied by ventricular dilation, that afflicts ~1 in 2,000 births worldwide (1,2). PPCM accounts for most cases of cardiogenic shock in pregnancy (3) and is increasingly a leading cause of peripartum maternal death in the United States and abroad (4,5). Gestational hypertension, multiparous pregnancies, and African heritage are the strongest known risk factors for PPCM. Treatment mirrors that of dilated cardiomyopathy, focusing on supportive measures, neurohormonal blockade, and, when necessary, mechanical support or even cardiac transplantation. Prognosis is relatively favorable, with recovery of systolic function in the majority of women. However, a significant subset of women, who are typically otherwise young and healthy, and with a new infant to nurture, do not recover and are faced with prolonged cardiac insufficiency, need for cardiac transplantation, or premature mortality.

The cause of PPCM remains poorly understood. Approximately 10% of women with PPCM bear truncating mutations in the gene *TTN*, encoding for the sarcomeric protein titin, indicating a genetic cause to PPCM in at least a subset of cases (6). In addition, numerous studies in model organisms have advanced

the hypothesis that PPCM is caused by vasculotoxic hormones, released from the placenta and pituitary during late gestation and early postpartum periods (1,7-10). These toxic hormones damage the cardiac microvasculature, in turn leading to cardiomyocyte dysfunction and contractile failure. One of these potentially toxic hormones is prolactin (10). The maternal pituitary secretes prolactin late in gestation and continues to do so postpartum in response to breastfeeding. Prolactin acts directly on mammary glands to promote generation of milk. However, in some contexts, prolactin can be cleaved by extracellular proteases to yield a 16 kD peptide that is profoundly vasculotoxic. Studies in mice suggest that this action may occur in certain predisposed individuals, leading to loss of cardiac microvasculature, global ischemia, and cardiomyopathy.

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These studies have raised the possibility that suppressing prolactin production may benefit patients with PPCM by removing a key mechanistic driver of this disease. There are 2 readily available ways to suppress prolactin production: 1) treatment with dopamine agonists, such as bromocriptine, which act directly on the pituitary to suppress dopamine synthesis; or 2) cessation of breastfeeding. We refer the reader to recent sources for discussions on the use of dopamine agonists in PPCM (11-15); controversy remains on this topic.

What of breastfeeding in PPCM? On the basis of the preclinical experimental findings with prolactin, and of suggestive studies with bromocriptine, the 2010 European position statement on PPCM recommended cessation of breastfeeding in PPCM (16). A more recent European Society of Cardiology study group recommended that breastfeeding should be “encouraged in women with mild cardiac dysfunction” but is “not advisable in cases of severely

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impaired systolic function” (17). However, there are remarkably few published data that directly address the question of breastfeeding in PPCM, in part because most PPCM studies do not report on breastfeeding. A single, small ($N = 55$) retrospective Internet-recruited study in the United States that directly addressed the question suggested that breastfeeding was associated with better, rather than worse, maternal outcome (18); and a similar single-center retrospective study ($N = 27$) suggested no difference in outcome based on breastfeeding status (19). Both studies are retrospective and therefore may have been biased by ascertainment. Prospective studies were entirely lacking.

Enter the IPAC (Investigations of Pregnancy-Associated Cardiomyopathy) study. IPAC is a U.S.-based, multicenter prospective study that followed 100 women for 12 months immediately after the diagnosis of PPCM (20). A number of important studies have emanated from this cohort. In this issue of *JACC: Basic to Translational Research*, Koczo et al. (21) focus on the question of breastfeeding. Of 100 women, 15 were breastfeeding at entry, and 85 were not. This percentage is substantially below the U.S. national rate of breastfeeding at 6 months (57.6%; CDC report card [22]). Only 1 woman received a dopamine agonist. There were no obvious differences in demographic, hemodynamic, or obstetric parameters between women who breastfed and those who did not. The women who breastfed had a trend toward higher ejection fraction at presentation (breastfeeding 0.39 ± 0.06 vs. nonbreastfeeding 0.34 ± 0.10 ; $p = 0.06$). The key observation of the study, however, is that no difference was seen in mean change in left ventricular ejection fraction from entry to 6 months (breastfeeding 0.17 ± 0.09 vs. nonbreastfeeding 0.16 ± 0.11 ; $p = 0.46$) or 12 months (breastfeeding 0.18 ± 0.08 vs. nonbreastfeeding 0.17 ± 0.11 ; $p = 0.68$). In other words, breastfeeding seemed to have no impact whatsoever on recovery rates.

The strength of this study (21) lies in the fact that participants were followed up prospectively and all subjects underwent comprehensive phenotyping, and it thus provides important new data to instruct decision-making in a clinical setting that lacks clear guidelines. Nonetheless, the study has limitations. First, the small size of the trial provides limited power. It should be noted, however, that the lack of even a trend toward an adverse effect of breastfeeding makes it unlikely that a type II error biased the statistical outcome of the study. Second, the study is observational (i.e., it is not randomized). Is it possible, for example, that self-selection of a less ill cohort to breastfeeding could have biased the outcome to favor

breastfeeding? This possibility is suggested by the relatively low percentage of women breastfeeding, and the trend to higher ejection fraction at entry in this group. The only way to conclusively address this question is a prospective and randomized trial, an unlikely outcome. In sum, the study does not definitively report that breastfeeding is safe in women with PPCM, but it strongly suggests that it is so.

The decision of whether to breastfeed with PPCM must also consider the potential benefits of breastfeeding to both mother and infant. Critical nutrients and factors, both known and unknown, pass from mother to child via breast milk. In developing countries, where undernutrition and unsafe water supplies account for the majority of childhood mortality and where breast milk substitutes are expensive, discontinuation of breastfeeding can be catastrophic to the infant (23). Breastfeeding promotes bonding, immunoprotection, metabolic protection, an appropriate microbiome population, and profound protection against diarrheal and respiratory diseases and otitis media, while reducing risk of sudden infant death (24). Most of these findings are true in both high- and low-income countries. In addition, in high-income countries, breastfeeding is associated with protection from obesity and diabetes and with higher performance on intelligence tests. Strong evidence also implicates breastfeeding in maternal protection from breast and ovarian cancer. Prolactin itself has been implicated in many of these processes, in particular immunoprotection, and indeed Koczo et al. (21) show in their study that $CD8^+$ cytotoxic T cells were higher in breastfeeding women with PPCM (in contrast to $CD4^+$ helper cells, which tended to be lower). Both the World Health Organization and the American Academy of Pediatrics recommend exclusive breastfeeding for 6 months, and continued breastfeeding for at least 1 to 2 years. In short, discontinuation of breastfeeding should not be taken lightly.

Conversely, women with PPCM are a special case, because the majority are taking medicines for heart failure. Are these drugs transmitted to the fetus, and if so, are they safe? Levels of loop diuretics expressed in breast milk are likely too low to have an effect in the infant (25). Similarly, negligible amounts of bioactive derivatives of most angiotensin-converting enzyme inhibitors, mineralocorticoid receptor antagonists, beta-blockers, hydralazine, or nitrates are detected in milk, typically leading to $<1\%$ infant exposure on a weight-adjusted basis. With judicious choice of drugs, and appropriate monitoring of the infant, standard PPCM therapy thus seems safe for the infant and should not be a contraindication for breastfeeding.

Finally, returning to the context of the possible use of dopamine agonists such as bromocriptine, 2 points should be made about breastfeeding. First, once the decision has been made to use dopamine agonists, the question of whether to breastfeed is then obviously moot, as these drugs will suppress lactation. However, second, the observations that breastfeeding seems to be safe in PPCM suggests that continued stimulation of prolactin secretion into the maternal circulation is not harmful, somewhat calling into question the rationale for using dopamine agonists. Once again, only a placebo-controlled, randomized trial will adequately resolve this quandary. The lost benefits of breastfeeding should therefore be factored into the decision of using a dopamine agonist.

In conclusion, few direct clinical data exist to guide the decision of whether a woman with PPCM should

breastfeed. The study by Koczo et al. (21) provides substantially more data than existed in aggregate in the antecedent literature, but it still leaves us without certainty. To date, there is no direct evidence that breastfeeding in women with PPCM is harmful, and in fact increasing evidence that it is safe. Cessation of breastfeeding should thus be recommended only with caution.

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