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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-κB-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 ± 8%). At 6-month follow-up ($n = 23$), PAI-1 levels decreased (36 ± 14 ng/mL, $P < 0.01$ vs. BL) and LVEF (49 ± 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-1β 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-κB 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-κB/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-κB-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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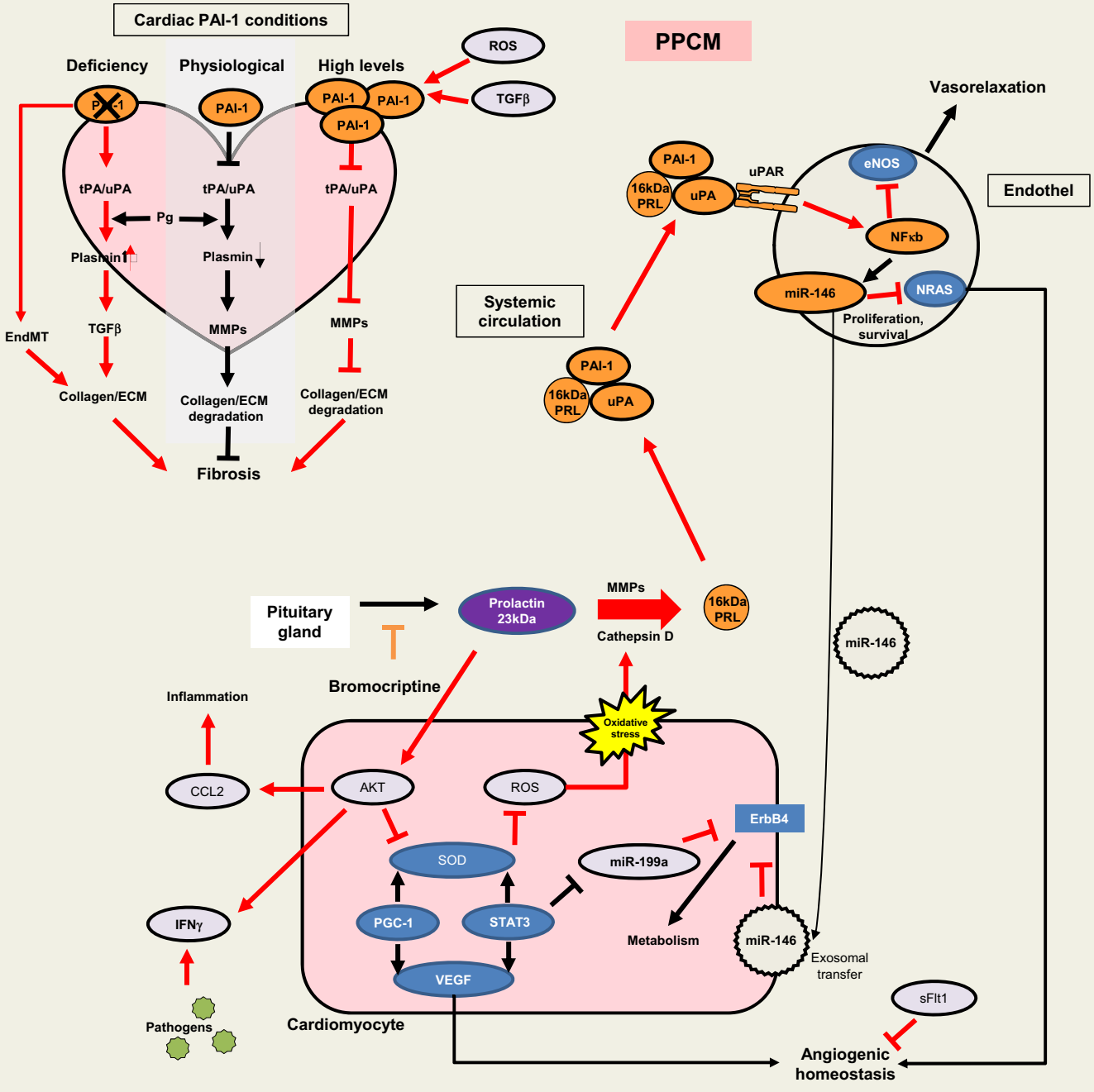
Graphical Abstract



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 pro-apoptotic 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-κB) 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-κB 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-κB 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-κB 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-κB 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-1β 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 *αMHC-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

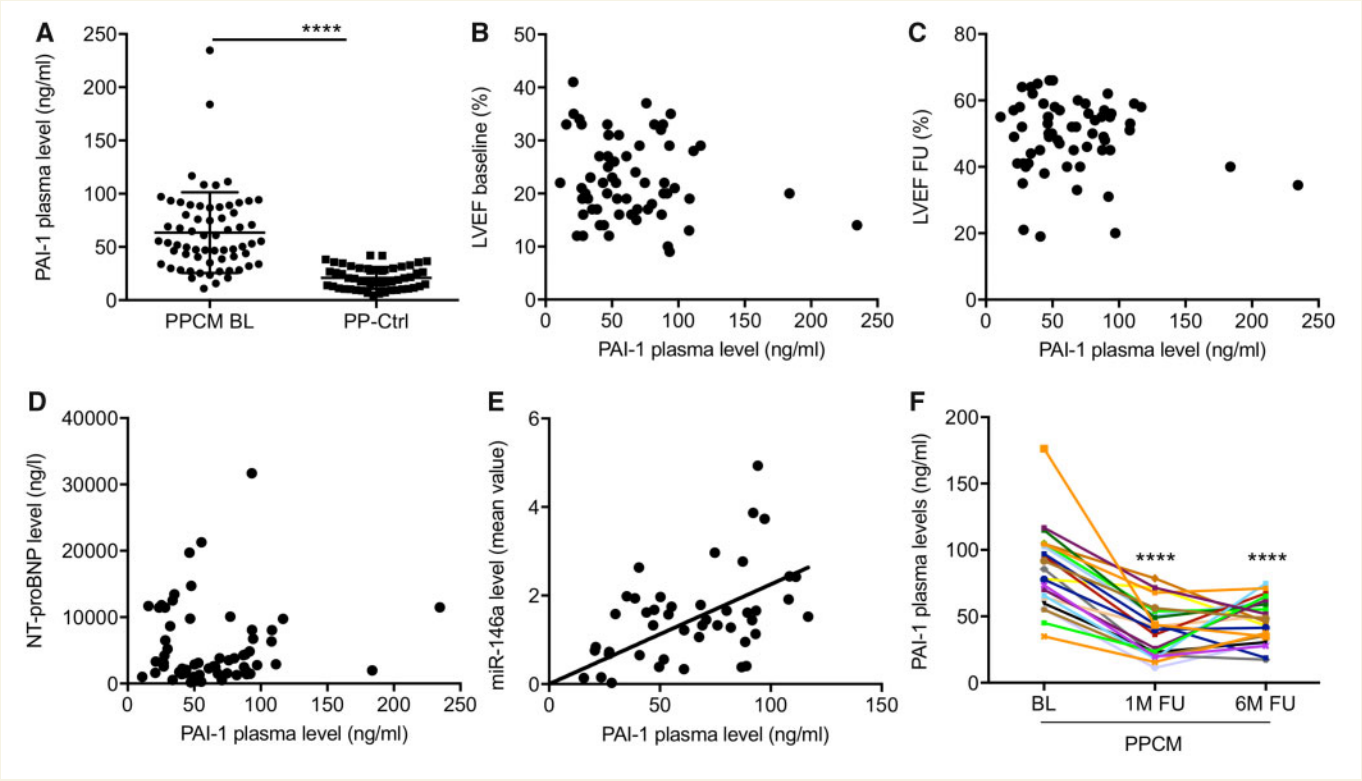


Figure 1 Plasma PAI-1 levels in healthy postpartum women and in patients with PPCM. (*A*) PAI-1 plasma levels (ng/mL) of PPCM patients at BL ($n = 64$) compared with PP-Ctrl ($n = 53$, $P < 0.0001$). Correlation analysis of PAI-1 levels from PPCM at BL with (*B*) LVEF at BL ($n = 64$; Pearson *r*: -0.1454, *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 ± 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 ± 8% and at 6-month follow-up: 49 ± 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-κB 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-κB 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-κB 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-κB 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-κB 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

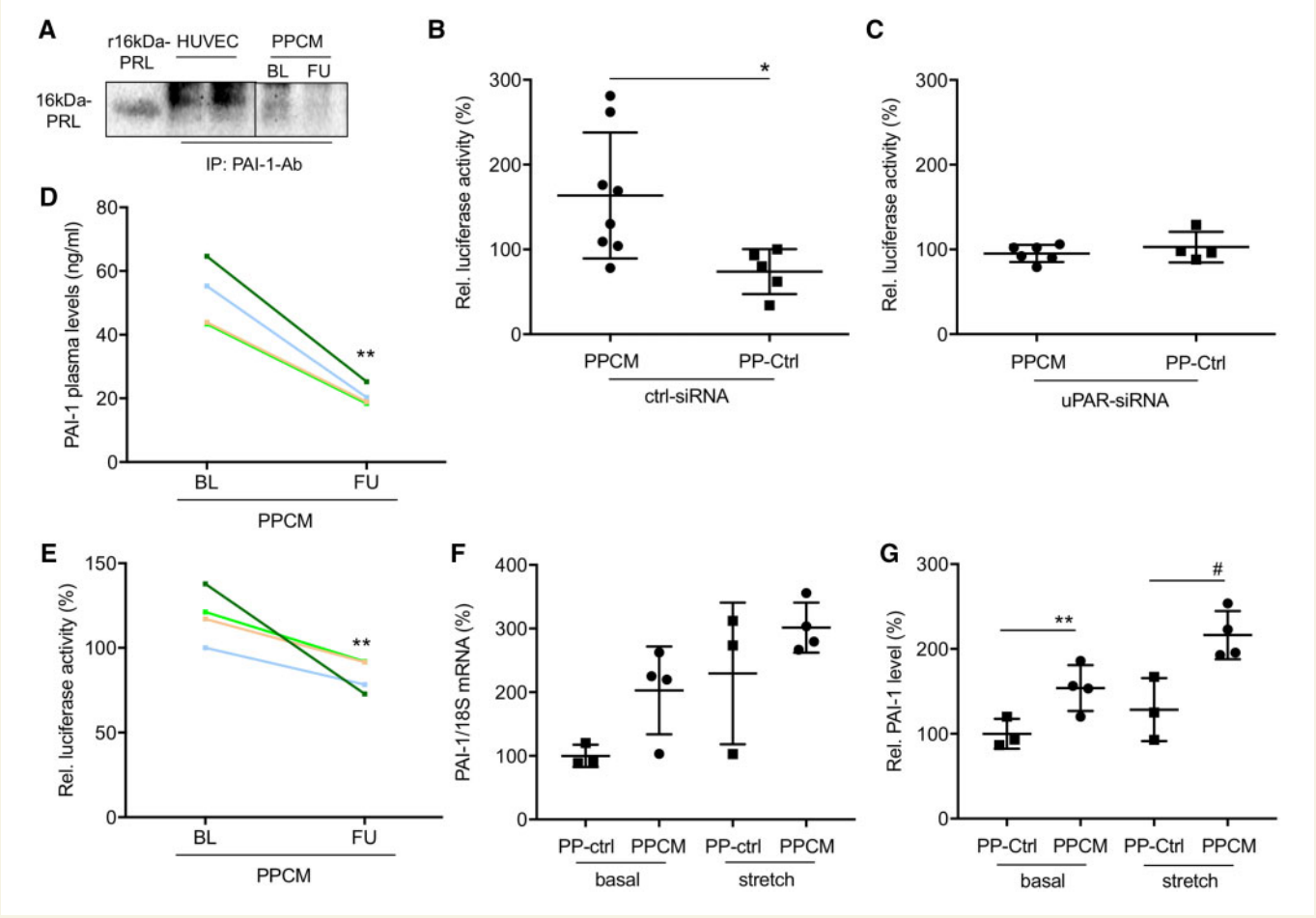


Figure 2 Formation of 16 kDa-PRL/PAI-1 and uPAR-mediated NF-κB activation induced by serum from PPCM and healthy postpartum women, and PAI-1 expression in dermal fibroblasts from PPCM patients and healthy postpartum relatives. (*A*) Representative western blot of 16 kDa-PRL protein levels after immunoprecipitation (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-κB 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 ± SD; unpaired two-tailed *t*-test. (*D*) PAI-1 plasma levels (ng/mL) at BL and 6-month follow-up and (*E*) NF-κB 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 ± 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-κB 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-κB 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 ± 512% vs. NF, $n = 11$: 193 ± 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-

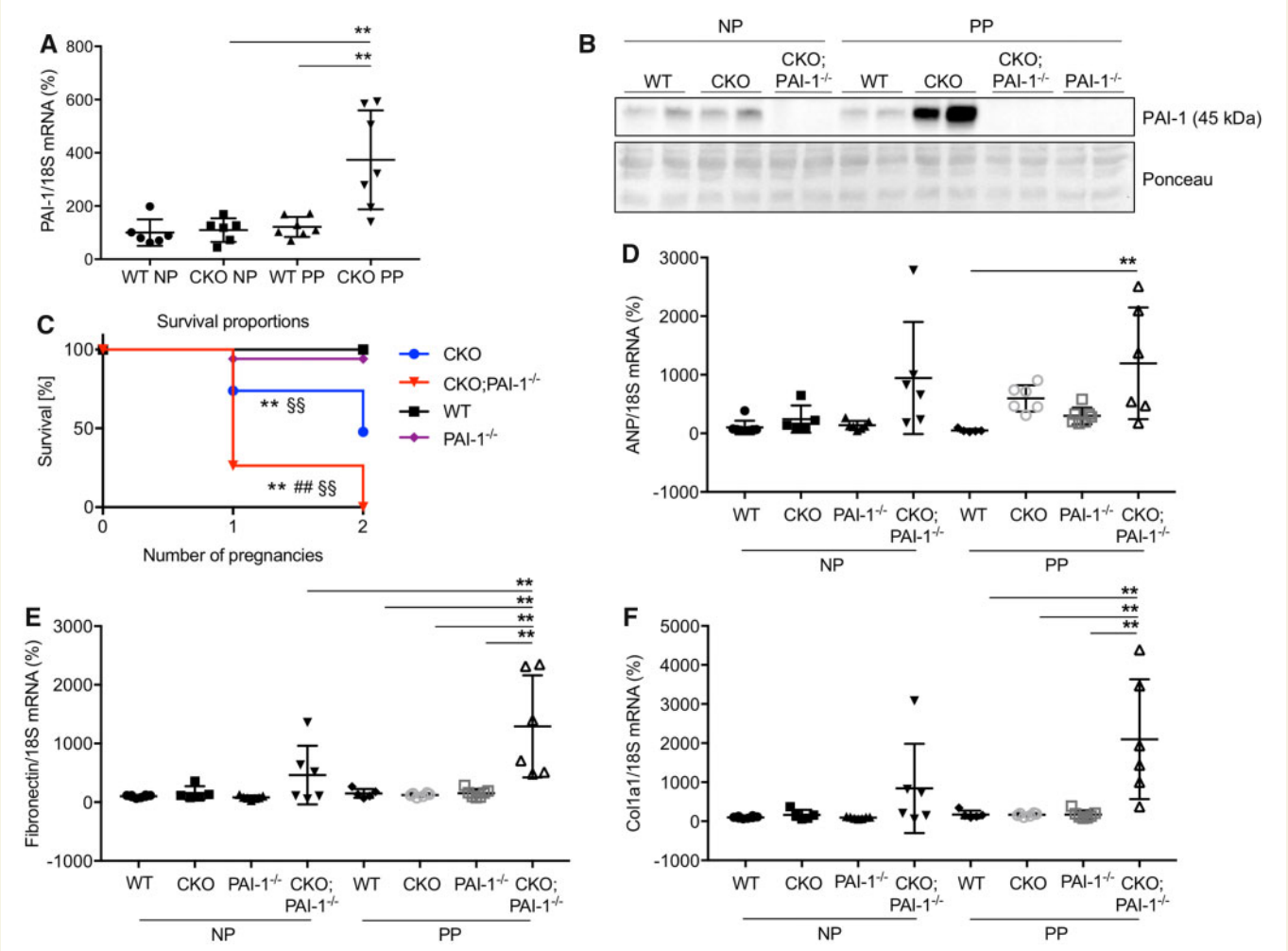


Figure 3 Effect of pregnancy and nursing in WT, CKO, and CKO; PAI-1$^{-/-}$ mice. (*A*) PAI-1 mRNA by qRT-PCR in cardiac tissue from WT and CKO mice, nulli-pari (NP, WT $n = 6$; CKO $n = 6$) and after two pregnancies and nursing periods (PP, WT $n = 7$; CKO $n = 7$), $^{**}P < 0.01$ vs. CKO PP. (*B*) 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, $^{\S\S}P < 0.01$ PAI-1$^{-/-}$. (*D*) ANP, (*E*) fibronectin, and (*F*) Col1α1 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 $n = 8$	CKO NP $n = 6$	PAI-1$^{-/-}$ NP $n = 8$	CKO; PAI-1$^{-/-}$ NP $n = 6$	WT PP $n = 6$	CKO PP $n = 7$	PAI-1$^{-/-}$ PP $n = 6$	CKO; PAI-1$^{-/-}$ PP $n = 5$
%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.

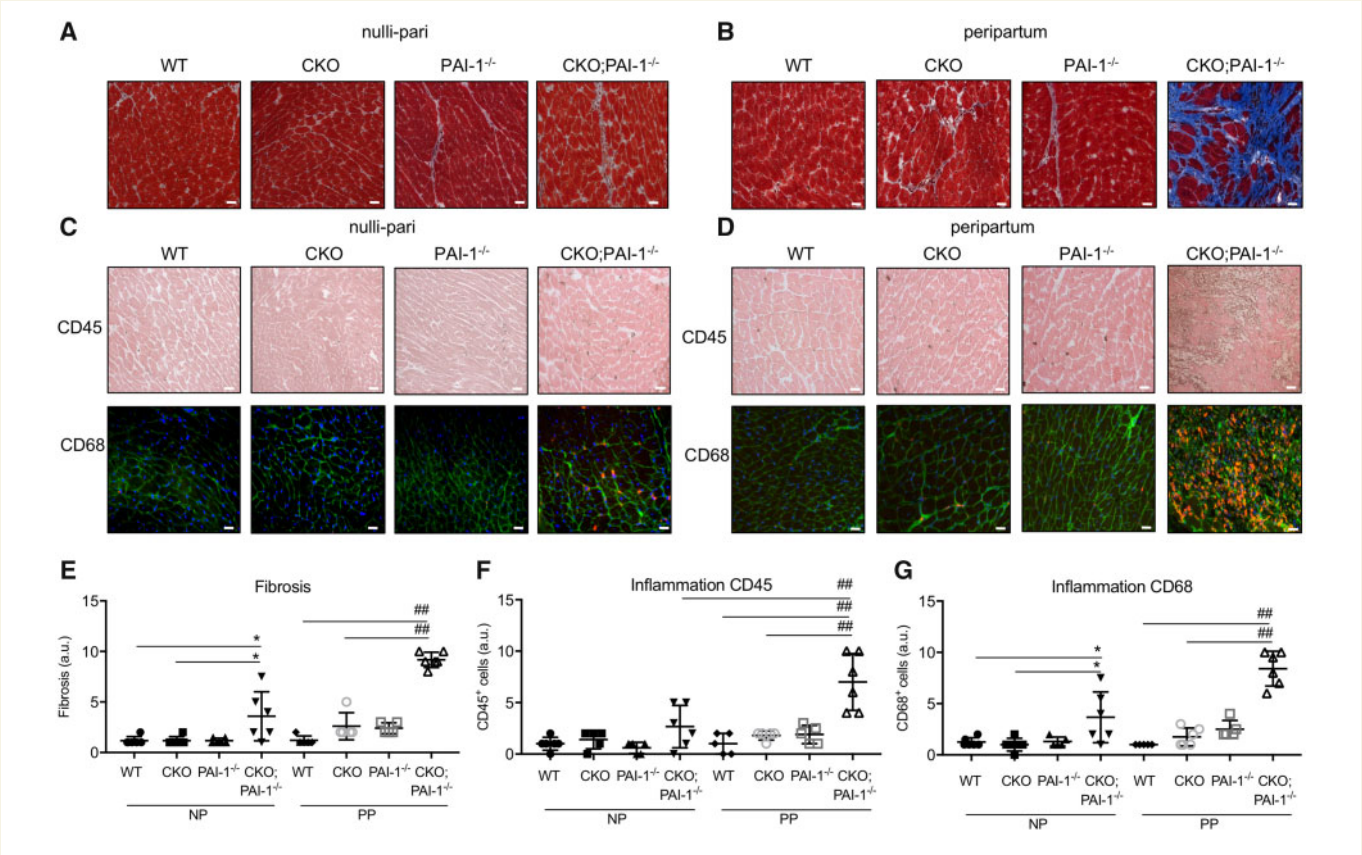


Figure 4 High degree of cardiac fibrosis and inflammation in CKO; PAI-1$^{-/-}$ mice in the peripartum phase of the first pregnancy. (*A* and *B*) Masson-trichrome staining visualizing fibrosis, (*C* and *D*) staining with the pan-inflammatory marker CD45 (brown, co-stained with haematoxylin, upper panels) 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 µm. 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 ± 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-κB activation. In contrast, PAI-1 does not correlate with NT-proBNP, the only clinical marker, which has so far found to been consistently up-regulated in PPCM, or with TNT which indicates myocardial damage. Neither CRP, IL-6 or IL-1β 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-κB 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-κB 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-κB activation further supporting the notion that a 16 kDa-PRL/PAI-1 interaction is responsible for non-canonical uPAR/NF-κB 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-κB/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-κB 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 levels[28] 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 Col1α1 and TGFβ2 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-κB 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-κB 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-κB 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.