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ELABORATO DI LAUREA

**APREMILAST MIGLIORA LA COESIONE DEI CARDIOMIOCITI
E L'ARITMIA IN DIVERSI MODELLI DI CARDIOMIOPATIA
ARITMOGENA**

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ABSTRACT

La cardiomiopatia aritmogena (ACM) è una rara patologia cardiaca ereditaria caratterizzata da una ridotta coesione tra cardiomiociti, aritmie ventricolari e aumentato rischio di morte cardiaca improvvisa. Le attuali strategie terapeutiche sono principalmente sintomatiche, ma recentemente apremilast ha dimostrato la capacità di migliorare l'adesione cellulare nei cheratinociti affetti da pemfigo volgare.

Nello studio oggetto di questa tesi è stato valutato il potenziale terapeutico di apremilast nell'ACM mediante l'utilizzo di cardiomiociti derivati da cellule staminali pluripotenti indotte di una paziente affetta da ACM (ACM-hiPSC-CMs), portatrice di una mutazione eterozigote nel gene della desmoplachina (*DSP*), oltre che di modelli murini e linee cellulari cardiache.

I cardiomiociti ACM-hiPSC-CMs hanno mostrato un'accentuata riduzione della coesione cellulare, recuperata in seguito al trattamento farmacologico con apremilast. Nelle cellule HL-1, questo effetto è risultato associato alla fosforilazione della placoglobina (JUP) in corrispondenza della serina 665 e all'attivazione della via di segnalazione ERK1/2. Questi risultati sono stati confermati da ulteriori esperimenti condotti su sezioni cardiache di topi knockout per la placoglobina e di topi portatori della mutazione fosfodeficiente S665A.

Nel complesso, i risultati indicano che apremilast è in grado di rafforzare l'adesione tra i cardiomiociti e che potrebbe perciò rappresentare un'eventuale strategia terapeutica per il trattamento dell'ACM.

1. INTRODUZIONE

1.1 Cardiomiopatia aritmogena (ACM)

La cardiomiopatia aritmogena (*Arrhythmogenic Cardiomyopathy*, ACM) è una rara patologia cardiaca ereditaria a trasmissione autosomica dominante (Tadros et al., 2023). Di conseguenza, la malattia può colpire maschi e femmine con uguale probabilità ed è sufficiente la presenza di una sola copia mutata del gene per determinare la manifestazione clinica della patologia. Tuttavia, l'ACM è caratterizzata da penetranza incompleta, il che implica che non tutti gli individui portatori di una mutazione genetica responsabile della malattia sviluppano effettivamente la patologia (Van Der Voorn et al., 2020).

Inizialmente si riteneva che la malattia coinvolgesse prevalentemente il ventricolo destro (RV), giustificando così il nome di “cardiomiopatia aritmogena del ventricolo destro” (ARVC). Tuttavia, studi successivi hanno dimostrato che la patologia può interessare anche il ventricolo sinistro (LV) o presentarsi con un fenotipo biventricolare, portando così all'introduzione del termine più generale “cardiomiopatia aritmogena” (Van Der Voorn et al., 2020).

La prevalenza stimata dell'ACM varia da 1:1000 a 1:5000 individui, a seconda della popolazione. Tuttavia, questo dato è probabilmente sottostimato, poiché la morte cardiaca improvvisa rappresenta la prima manifestazione della patologia in circa il 5-10% dei casi, soprattutto in individui giovani e asintomatici, inclusi atleti.

L'ACM generalmente si manifesta in tre fasi cliniche principali: (I) la fase precoce nascosta, caratterizzata da sintomi minimi, ma associata a rischio di morte cardiaca improvvisa; (II) la fase elettrica, caratterizzata da aritmie ventricolari e anomalie strutturali; (III) la fase terminale, con gravi cambiamenti strutturali, dilatazione ventricolare e disfunzione che possono progredire verso l'insufficienza cardiaca (Kohela & van Rooij, 2022).

La principale caratteristica istopatologica della cardiomiopatia aritmogena è la progressiva perdita del miocardio sano, probabilmente a causa di atrofia e morte cellulare dei cardiomiociti, e la sua conseguente sostituzione con tessuto fibroadiposo. Tale sostituzione si estende generalmente dall'epicardio verso

l'endocardio (Fig. 1). Con il progredire della malattia, il tessuto fibroadiposo può contribuire ad una maggiore suscettibilità alle aritmie ventricolari e ad un maggiore rischio di morte cardiaca improvvisa.

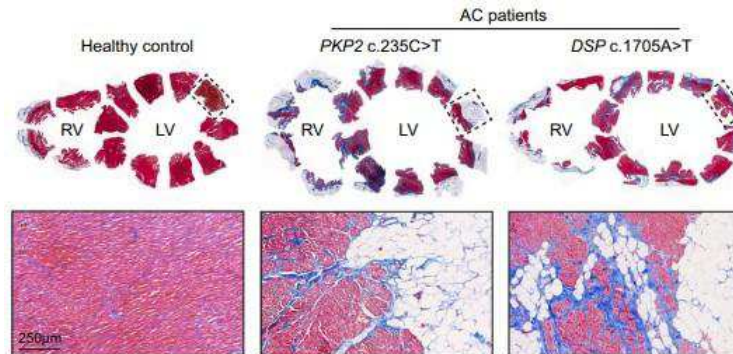


Figura 1. Rimodellamento fibroadiposo del tessuto cardiaco nei pazienti affetti da ACM (Kohela & van Rooij, 2022).

Le sezioni trasversali mostrano un cuore di controllo (a sinistra) e cuori di pazienti con mutazioni nei geni della plakofilina 2 (*PKP2*, al centro) e desmoplachina (*DSP*, a destra), causative della patologia. A differenza del cuore sano, nei campioni patologici è evidente una sostituzione del miocardio (in rosso) con tessuto fibroso (in blu) e con tessuto adiposo (in bianco).

Le immagini inferiori rappresentano ingrandimenti delle sezioni trasversali.

1.2 Dischi intercalari

Nel cuore, i cardiomiociti sono connessi tra loro da strutture altamente specializzate denominate dischi intercalari, costituiti da giunzioni meccaniche ed elettriche (Nielsen et al., 2023). I dischi intercalari svolgono tre funzioni principali: garantire l'ancoraggio meccanico tra le cellule, consentire la trasmissione della forza contrattile e permettere la propagazione del segnale elettrico.

Essi sono costituiti da tre principali tipologie di giunzioni: giunzioni aderenti, giunzioni gap e desmosomi (Fig. 2).

Le giunzioni aderenti sono complessi multiproteici che mantengono unite le cellule durante la contrazione cardiaca, collegando i filamenti di actina di cardiomiociti adiacenti. Questo consente la trasmissione della forza contrattile, permettendo una contrazione coordinata del cuore. Inoltre, le giunzioni aderenti contribuiscono al mantenimento dell'integrità strutturale delle cellule in condizioni di stress meccanico (Nielsen et al., 2023).

Le giunzioni gap rappresentano canali di comunicazione diretta tra cellule adiacenti, consentendo il passaggio di ioni e piccole molecole (fino a 1 kDa) tra i

citoplasmici di cardiomiociti vicini. In questo modo, permettono la propagazione del segnale elettrico e garantiscono una contrazione sincronizzata del cuore. Esse si formano dall'allineamento di connessioni appartenenti a cellule vicine; ciascuna connessione è costituita da sei subunità proteiche chiamate connesine (Nielsen et al., 2023).

I desmosomi sono strutture multiproteiche che assicurano l'ancoraggio meccanico tra cardiomiociti, collegando i filamenti intermedi di desmina (IF) di cellule adiacenti e contribuendo al mantenimento dell'integrità del tessuto durante la contrazione (Fig. 3). Nei desmosomi cardiaci, le cellule sono connesse tramite le caderine desmosomiali desmogleina 2 (DSG2) e desmocollina 2 (DSC2). Le loro porzioni intracellulari interagiscono con la placoglobina (JUP) e la plakofilina 2 (PKP2), che a loro volta si associano alla desmoplachina (DSP), collegando così le caderine desmosomiali alla desmina (Nielsen et al., 2023).

Nel cuore dei mammiferi sono inoltre presenti complessi ibridi, chiamati *areae compositae*, che integrano proteine dei desmosomi e delle giunzioni aderenti, contribuendo a garantire la resistenza meccanica del tessuto cardiaco (Nielsen et al., 2023).

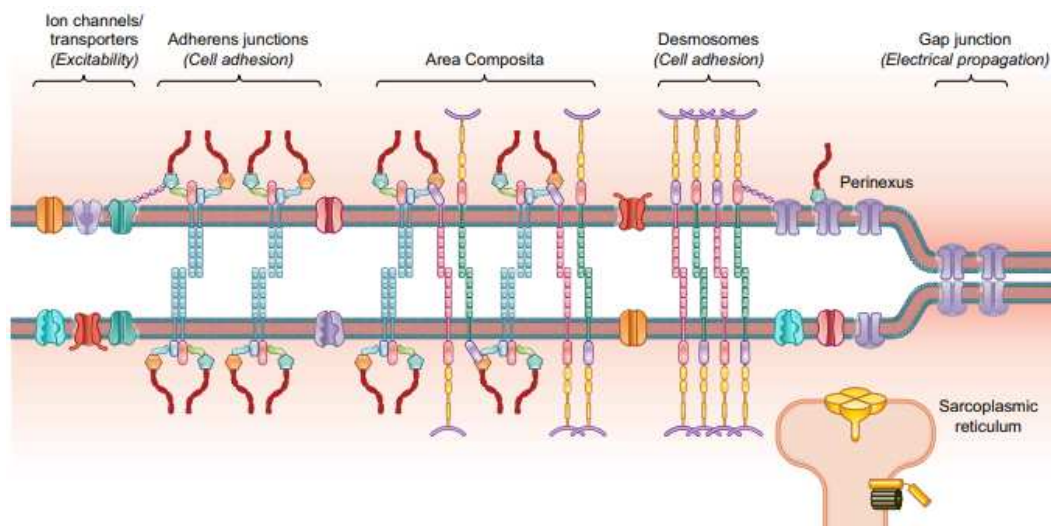


Figura 2. Rappresentazione di un disco intercalare dei cardiomiociti (Nielsen et al., 2023).

Da sinistra sono rappresentati: i canali ionici che consentono l'ingresso e l'uscita di ioni al fine di regolare l'eccitabilità elettrica delle cellule, le giunzioni aderenti, l'area composita, i desmosomi e le giunzioni gap.

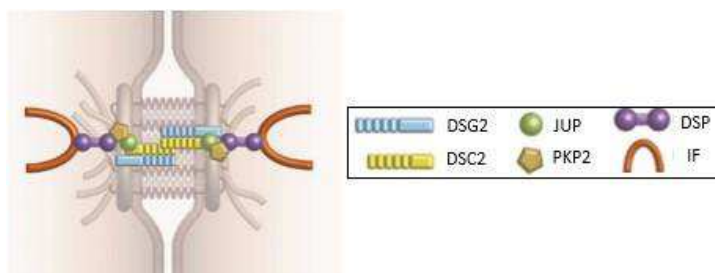


Figura 3. Struttura di un desmosoma (Delmar & McKenna, 2010).

Nello spazio extracellulare DSG2 e DSC2 si legano tra loro mentre nel lato citoplasmatico sono collegate ad una serie di proteine di ancoraggio (JUP, PKP2), le quali tramite DSP legano i IF.

1.3 Genetica della cardiomiopatia aritmogena

Circa il 60% dei pazienti affetti da cardiomiopatia aritmogena presenta una o più mutazioni in geni che codificano proteine desmosomiali. In particolare, sono state identificate varianti patogene in cinque geni che codificano i principali componenti dei desmosomi: plakofilina 2 (*PKP2*), desmoplachina (*DSP*), placoglobina (*JUP*), desmogleina 2 (*DSG2*) e desmocollina 2 (*DSC2*). Tali mutazioni compromettono le interazioni all'interno dell'area composita destabilizzando le connessioni intercellulari e alterando sia le funzioni di ancoraggio sia i meccanismi di segnalazione del disco intercalare, con conseguente riduzione della coesione tra cardiomiociti (Austin et al., 2019).

Anche mutazioni in geni non desmosomiali sono state associate all'ACM, sebbene le evidenze relative alla loro patogenicità siano più limitate (Gerull & Brodehl, 2021).

Nella maggior parte dei casi, l'ereditarietà è autosomica dominante con penetranza incompleta. Tuttavia, sono state identificate anche forme autosomiche recessive (Austin et al., 2019). Inoltre, i casi più gravi o a esordio precoce sono frequentemente associati a eterozigosi composta, in cui due alleli dello stesso gene presentano mutazioni diverse, o eterozigosi digenica, se sono presenti mutazioni in due geni distinti (Asimaki et al., 2015).

Queste evidenze sottolineano il ruolo centrale dell'integrità strutturale e funzionale dei desmosomi nella patogenesi della cardiomiopatia aritmogena.

1.4 Scopo della ricerca

La diagnosi precoce dell'ACM è cruciale per ridurre il rischio di morte cardiaca improvvisa, soprattutto nei pazienti giovani e negli atleti. Tuttavia, essa risulta spesso complessa a causa dell'ampia variabilità fenotipica della malattia (Corrado et al., 2017).

Inoltre, attualmente non esistono terapie in grado di contrastare le alterazioni miocardiche progressive tipiche dell'ACM; pertanto, le opzioni terapeutiche disponibili includono cambiamenti nello stile di vita (come l'astensione da attività fisica intensa o di resistenza), trattamento farmacologico con β -bloccanti o farmaci antiaritmici, ablazione mediante catetere, impianto di defibrillatori cardiaci e, nei casi più gravi, trapianto cardiaco.

Studi precedenti hanno dimostrato che l'aumento dei livelli intracellulari di cAMP, attraverso la fosforilazione della placoglobina (PG) mediata da PKA in serina 665, favorisce il rafforzamento della coesione tra cardiomiociti mediata da DSG2, fenomeno definito “adesiotropia positiva” (Shoykhet et al., 2020). Questi risultati suggeriscono che l'adesione cellulare a livello del disco intercalare cardiaco sia finemente regolata e possa essere potenziata tramite farmaci in grado di aumentare il cAMP intracellulare.

Recentemente è stato osservato che apremilast, un inibitore della fosfodiesterasi-4 (PDE-4) già impiegato nel trattamento della psoriasi e della malattia di Behçet, è in grado di contrastare la perdita di coesione cellulare indotta da autoanticorpi, promuovendo la stabilità delle strutture desmosomiali.

Alla luce di queste evidenze, questo studio (Stangner et al., 2025) si propone di valutare se l'apremilast sia in grado di potenziare la coesione desmosomiale dei cardiomiociti e di esplorarne il potenziale utilizzo terapeutico nell'ACM.

2. MATERIALI E METODI

2.1 Modelli sperimentali

Nello studio di Stangner et al. (2025) sono stati utilizzati diversi modelli biologici per analizzare gli effetti della cardiomiopatia aritmogena e del trattamento farmacologico con apremilast.

In particolare, sono stati utilizzati cardiomiociti derivati da cellule staminali pluripotenti indotte umane ottenute da una paziente affetta da ACM e da due controlli sani. Queste cellule rappresentano un modello in vitro della patologia e consentono di studiare gli effetti della mutazione in un background genetico umano.

Inoltre, sono state impiegate cellule cardiache murine HL-1 come modello cellulare cardiaco per l'analisi dei meccanismi molecolari.

Infine, sono state analizzate sezioni cardiache ex vivo di topi, che mantengono l'architettura tissutale, le connessioni cellulari e l'organizzazione tridimensionale del tessuto.

Questo approccio consente di confermare i risultati ottenuti nei modelli cellulari in un contesto fisiologico.

2.2 Campioni biologici per generare cellule staminali pluripotenti indotte umane

Sono stati utilizzati tre differenti tipi di tessuti umani per la generazione di cellule staminali pluripotenti indotte umane (hiPSCs): (I) fibroblasti della vena umana (hVFs) derivati dalla probanda affetta da cardiomiopatia aritmogena (NP0151-11 F), utilizzati come modello cellulare patologico; (II) fibroblasti dermici umani (hDFs) isolati da un campione di pelle di un soggetto maschile sano (NP0040-8), utilizzati come controllo sano non correlato; (III) cellule mononucleate del sangue periferico (PBMCs) della madre sana della probanda (NP0147-4), utilizzate come controllo genetico familiare.

La probanda è una ragazza di 14 anni affetta da ACM, deceduta per morte cardiaca improvvisa. Il sequenziamento del DNA della paziente ha rivelato una variante non

senso in eterozigosi nel gene *DSP* (c.2854G > T), che porta alla formazione di un codone di stop prematuro (p.Glu952*) (Fig. 4).

La madre della probanda, invece, non presenta mutazioni nel gene *DSP*.

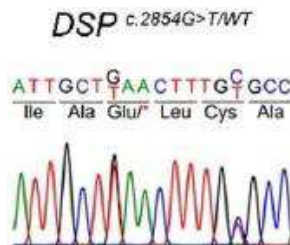


Figura 4. Sequenziamento del DNA delle hiPSCs della probanda (Stangner et al., 2025).

La paziente nel gene *DSP* presenta la mutazione c.2854G > T e una variante eterozigote silente c.2862C > T.

2.3 Coltura cellulare e crioconservazione

I tessuti ottenuti dalla probanda (hVFs) e dal donatore sano (hDFs) sono stati frammentati, posti in piastre di coltura e mantenuti in Dulbecco's Modified Eagle Medium (DMEM), contenente siero fetale bovino, glutammina, amminoacidi non essenziali e antibiotici.

Le cellule sono state coltivate fino al raggiungimento della confluenza e successivamente sono state raccolte tramite dissociazione ed espanse. Infine, i fibroblasti ottenuti sono stati crioconservati in azoto liquido per utilizzi futuri.

Analogamente, le cellule della madre sana (PBMCs) sono state isolate mediante centrifugazione dal sangue periferico e successivamente conservate in azoto liquido.

2.4 Generazione delle cellule staminali pluripotenti indotte umane (hiPSCs)

Le cellule conservate in azoto liquido sono state scongelate e riprogrammate in cellule staminali pluripotenti indotte umane (hiPSCs).

Le cellule hVFs e le PBMCs sono state trasdotte mediante vettori basati su virus Sendai, appartenenti alla famiglia dei *Paramyxoviridae*. Si tratta di virus a RNA a singolo filamento non integrativi, che entrano nel citoplasma delle cellule senza integrarsi nel genoma cellulare. Una volta all'interno, i vettori esprimono fattori di trascrizione esogeni che vengono tradotti dalla cellula ospite inducendo la pluripotenza e determinando la riprogrammazione dell'espressione genica. In

questo modo, hVFs e PBMCs vengono convertite in cellule staminali pluripotenti. Dopo la riprogrammazione, il materiale virale viene progressivamente diluito ed eliminato dalle cellule durante le successive divisioni cellulari.

Le cellule hDFs, invece, sono state riprogrammate mediante vettori plasmidici episomali, ossia plasmidi a DNA circolare non integrativi. Tali vettori consentono l'espressione transiente di fattori di riprogrammazione necessari alla conversione delle cellule hDFs in hiPSCs. Anche in questo caso, dopo la riprogrammazione, i plasmidi episomali vengono progressivamente diluiti ed eliminati durante le divisioni cellulari.

2.5 Differenziamento delle hiPSCs in cardiomiociti

Le cellule staminali pluripotenti indotte umane così ottenute vengono successivamente differenziate in cardiomiociti mediante molecole capaci di modulare la via di segnalazione Wnt/ β -catenina, un pathway fondamentale nello sviluppo embrionale del mesoderma e nella successiva specificazione in cardiomiociti.

In particolare, le hiPSCs sono state inizialmente trattate per 24 ore con CHIR99021, un fattore che attiva la via Wnt/ β -catenina e che induce la transizione delle hiPSCs verso il mesoderma primitivo.

Successivamente, le cellule sono state sottoposte ad un trattamento di 48 ore con XAV939 e IWP2, molecole che inibiscono la via Wnt/ β -catenina e che inducono il differenziamento delle cellule mesodermiche in cardiomiociti.

Dopo circa 7 giorni, le cellule cardiache ottenute dalle hiPSCs (hiPSC-CMs) iniziano a contrarsi spontaneamente.

2.6 RT-PCR

L'avvenuta eliminazione del virus Sendai, utilizzato per la riprogrammazione delle cellule, e l'espressione dei marcatori di pluripotenza delle hiPSCs sono state valutate mediante RT-PCR semi-quantitativa.

Per verificare la clearance virale, l'RNA è stato estratto con TRIzol da tre condizioni sperimentali: (I) cellule non infettate, utilizzate come controllo negativo, in cui non ci si aspetta alcun segnale di PCR se non per eventuale contaminazione;

(II) cellule appena infettate, impiegate come controllo positivo per confermare la funzionalità dei primer e la rilevazione del virus; (III) linee hiPSCs stabilizzate, ossia cellule riprogrammate coltivate per diversi passaggi, necessarie per valutare l'effettiva eliminazione del virus non integrativo.

Questa analisi ha confermato la progressiva scomparsa del materiale virale durante le divisioni cellulari.

Per la valutazione dell'espressione genica, l'RNA estratto con TRIzol è stato retrotrascritto in cDNA mediante trascrittasi inversa SuperScript II e primer random esameri. Il cDNA ottenuto è stato poi amplificato mediante PCR.

I prodotti di entrambe le amplificazioni per PCR sono stati infine analizzati mediante elettroforesi su gel di agarosio e visualizzati con bromuro di etidio, permettendo di confermare la corretta riprogrammazione delle hiPSCs pluripotenti prive di vettore virale.

2.7 Saggi di dissociazione

Nei tre differenti modelli sperimentali (cellule hiPSCs, cellule HL-1 e sezioni cardiache), sono stati eseguiti saggi di dissociazione per valutare la coesione cellulare, la stabilità delle strutture giunzionali e gli effetti dei trattamenti farmacologici sulle giunzioni intercellulari.

Nel contesto dell'ACM, i diversi modelli biologici sono stati sottoposti a stress meccanico mediante agitazione controllata, al fine di generare forze di taglio. Il grado di frammentazione del monostrato cellulare è stato utilizzato come indice inversamente proporzionale all'adesione intercellulare.

2.8 Western blot

Dopo i rispettivi trattamenti farmacologici, le cellule HL-1, le sezioni cardiache e gli hiPSC-CMs sono stati analizzati mediante Western blot per valutare l'espressione e la quantificazione di proteine coinvolte nei desmosomi e nei segnali cellulari correlati all'aritmia e alla coesione cellulare. In particolare, sono state studiate le proteine delle giunzioni cellulari DSP, DSG2, PKP2, PG e PG-pS665, mentre tra le proteine di segnalazione cellulare sono state valutate ERK1/2 e pERK1/2.

Il Western blot è stato impiegato per confrontare cellule sane e cellule con mutazioni causative dell'ACM, cellule trattate con apremilast e cellule non trattate.

2.9 Multielectrode array (MEA)

La tecnologia *multielectrode array* (MEA) registra, amplifica e analizza i segnali provenienti da campioni biologici in vitro, e i dati vengono elaborati da un software di acquisizione dedicato.

Questo strumento può essere utilizzato per registrare segnali da sezioni di cuore o cervello, cellule staminali, colture neuronali o cardiache (*In Vitro MEA Systems* | [Www.Multichannelsystems.Com](http://www.Multichannelsystems.Com), s.d.).

I sistemi MEA presentano quattro componenti principali: (I) il *microelectrode array* (MEA) è il dispositivo in cui vengono posizionati i tessuti o coltivate le cellule e contenente la camera di microelettrodi; (II) il *headstage* che contiene l'amplificatore e il sistema di stimolazione; (III) la scheda di interfaccia (*interface board*) che collega l'hardware al computer e riceve i dati dall'*headstage* per trasmetterli al software; (IV) il computer esegue il software di acquisizione e analisi dei dati, consentendo di visualizzare in tempo reale i segnali registrati (Fig. 5).

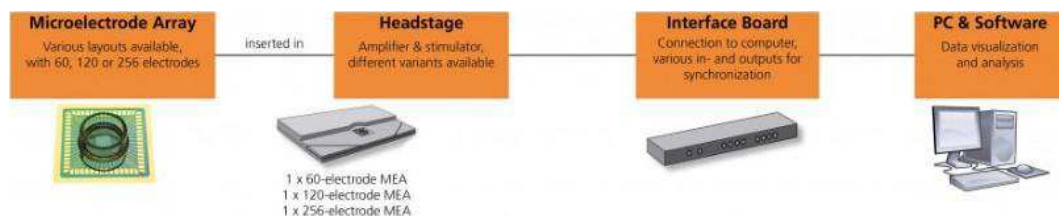


Figura 5. Schema semplificato dell'architettura di un sistema MEA (*In Vitro MEA Systems* | [Www.Multichannelsystems.Com](http://www.Multichannelsystems.Com), s.d.).

Da sinistra a destra sono rappresentati: il *microelectrode array* (MEA), il *headstage*, la scheda di interfaccia (*interface board*) e il PC con il software per l'acquisizione e l'analisi dei segnali.

Nello studio di Stangner et al. (2025), le sezioni cardiache ottenute da topi wild-type ($Jup^{+/+}$) e topi knockout per la placoglobina ($Jup^{-/-}$) sono state analizzate mediante il MEA2100-60-System per valutare la velocità di conduzione del segnale elettrico nel tessuto, prima e dopo il trattamento farmacologico con apremilast.

Per preservare l'architettura cardiaca e stabilizzare il tessuto, le sezioni sono state tagliate con uno spessore di 300 μm utilizzando un vibratomo e trasferite in medium Claycomb privo di norepinefrina e integrato con siero fetale bovino e

penicillina/streptomicina. Sono state quindi posizionate in camere MEA a 60 elettrodi e immerse nel medium per consentire la registrazione della propagazione del segnale elettrico attraverso il tessuto.

Dopo la registrazione iniziale, è stato aggiunto apremilast sia nelle sezioni $Jup^{+/+}$ sia nelle sezioni $Jup^{-/-}$, e le registrazioni sono state ripetute.

La variabilità del ritmo cardiaco è stata valutata mediante la deviazione standard degli intervalli battito-battito (SDNN), in cui valori elevati indicano un ritmo più variabile e valori ridotti un ritmo più regolare; tale parametro può essere alterato in presenza di aritmie.

La velocità di conduzione del segnale elettrico è stata calcolata come rapporto tra la distanza tra due elettrodi (200 μm) e il tempo medio di propagazione del segnale.

3. RISULTATI

3.1 Apremilast migliora la coesione dei cardiomiociti, la traslocazione di DSG2-DP, la fosforilazione di PG e l'attivazione di ERK1/2 nelle cellule HL-1

Inizialmente, le cellule HL-1 sono state trattate per 30 minuti a 37 °C con apremilast alle concentrazioni di 1 μ M e 10 μ M oppure con una combinazione di forskolina e rolipram (FR), utilizzata come controllo positivo. Lo scopo del trattamento era valutare la produzione intracellulare di cAMP. La forskolina stimola infatti la sintesi di cAMP mediante l'attivazione dell'adenilato ciclasi, mentre il rolipram e l'apremilast inibiscono la fosfodiesterasi-4 (PDE-4), impedendo la degradazione del secondo messaggero ciclico.

I risultati hanno mostrato che nelle cellule di controllo non trattate la concentrazione media di cAMP era pari a 14 pmol/ml, mentre aumentava a 334 pmol/ml dopo il trattamento con FR e a 25 pmol/ml e 27 pmol/ml dopo il trattamento con apremilast rispettivamente a 1 μ M e 10 μ M (Fig. 6a).

Questi dati indicano che l'apremilast determina un incremento della concentrazione intracellulare di cAMP rispetto al controllo non trattato, sebbene l'aumento risulti inferiore rispetto a quello osservato con il trattamento mediante FR.

Poiché le due concentrazioni di apremilast hanno determinato un incremento pressoché sovrapponibile della coesione cardiomiocitaria, gli esperimenti successivi sono stati condotti utilizzando la concentrazione di 1 μ M.

Successivamente, le cellule HL-1 (Fig. 6b) e le sezioni cardiache di topi wild-type (Fig. 6c) sono state trattate per 1 ora a 37 °C con FR o apremilast e poi sottoposte ad un saggio di dissociazione, con l'obiettivo di valutare la coesione tra i cardiomiociti dopo l'applicazione di uno stress meccanico.

Nei campioni di controllo non trattati è stato osservato il numero medio più elevato di frammenti del monostrato cellulare (nelle cellule HL-1) o di cardiomiociti isolati (nelle sezioni cardiache), a dimostrazione della minore adesione cellulare. Al contrario, il trattamento con FR o apremilast determina una significativa riduzione del numero di frammenti o di cellule isolate, evidenziando un aumento della coesione cellulare.

Inoltre, tramite colorazione immunohistochimica è stato dimostrato che, analogamente a FR, nelle cellule HL-1 l'apremilast favorisce la traslocazione delle proteine desmogleina 2 (DSG2) e desmoplachina (DP) verso le giunzioni cellulari, promuovendo la stabilizzazione dei desmosomi e aumentando l'adesione tra i cardiomiociti.

Infine, l'analisi mediante Western blot ha evidenziato che il trattamento con apremilast, analogamente a FR, nelle cellule cardiache murine favorisce la fosforilazione della placoglobina alla serina 665 (PG-pS665) e delle proteine ERK1/2 (pERK1/2) (Fig. 6d), dimostrando l'attivazione di vie di segnalazione coinvolte nel rafforzamento dei desmosomi.

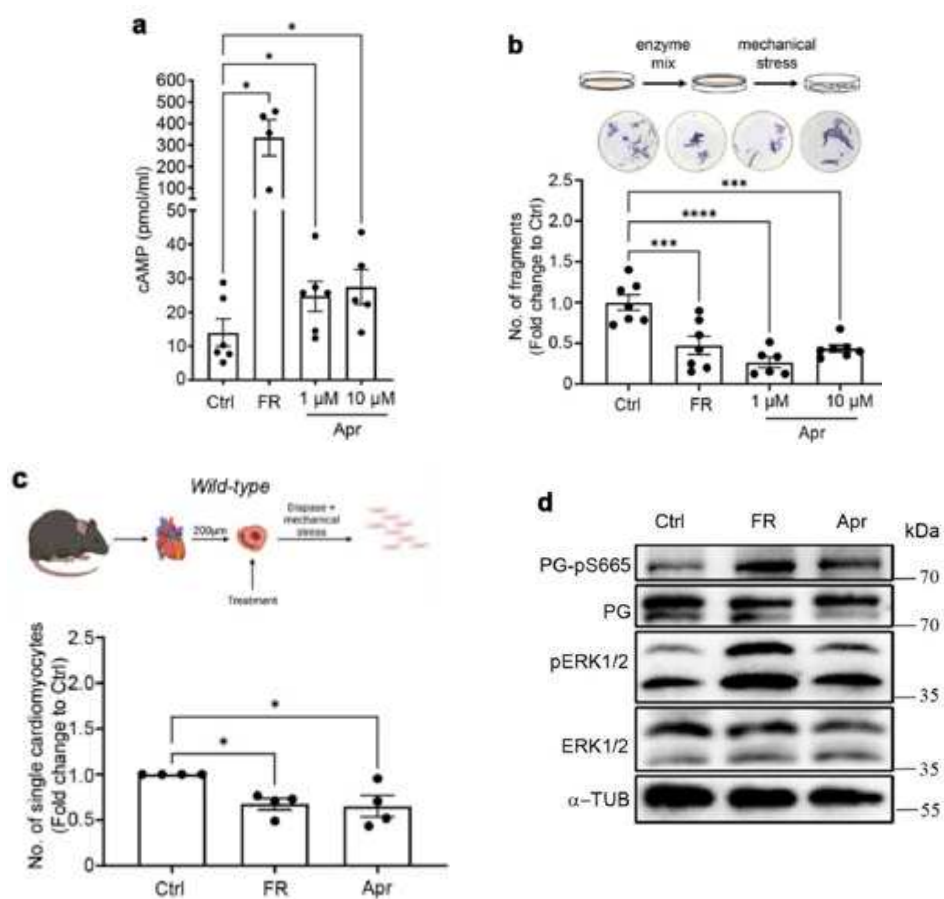


Figura 6. a. Apremilast (Apr) induce un aumento della produzione intracellulare di cAMP nelle cellule HL-1. b. Il saggio di dissociazione nelle cellule HL-1 evidenzia un aumento della coesione cellulare indotto da Apr. c. Il saggio di dissociazione nelle sezioni cardiache di topi wild-type evidenzia un aumento della coesione tra i cardiomiociti indotto da Apr. d. Analisi mediante Western blot dell'espressione di PG-pS665, PG totale, pERK1/2 ed ERK1/2 totale nelle cellule HL-1 dopo il trattamento con Apr.

3.2 La coesione cardiomiocitaria potenziata da apremilast richiede PG

Dopo aver dimostrato che l'apremilast aumenta i livelli intracellulari di cAMP, la fosforilazione della placoglobina in serina 665 e la coesione dei cardiomiociti, per valutare il ruolo della placoglobina nel meccanismo d'azione del farmaco sono state utilizzate sezioni cardiache provenienti da topi portatori della mutazione S665A della placoglobina (JUPS665A) e da topi knockout per la placoglobina (*Jup*^{-/-}).

Per valutare se la fosforilazione di PG fosse indispensabile per l'attività dell'apremilast, sono stati utilizzati i topi JUPS665A, il cui cuore presenta una morfologia e un'istologia normali, ma nei quali la placoglobina non può essere fosforilata a causa della sostituzione della serina 665 con un'alanina.

Nonostante questa mutazione, il saggio di dissociazione eseguito su sezioni cardiache provenienti da questi topi ha mostrato che l'apremilast migliora comunque la coesione dei cardiomiociti (Fig. 7a). Questo risultato dimostra che la fosforilazione della placoglobina in Ser665 non è strettamente indispensabile per l'effetto dell'apremilast, suggerendo che il farmaco possa agire anche attraverso meccanismi di segnalazione alternativi.

Per valutare se invece la presenza della placoglobina fosse necessaria per l'azione del farmaco, sono stati utilizzati topi knockout, nei quali il gene *Jup* è stato inattivato e, di conseguenza, la placoglobina non viene espressa.

Il saggio di dissociazione effettuato su sezioni cardiache provenienti da questi topi non ha evidenziato alcun miglioramento della coesione cardiomiocitaria in seguito al trattamento con apremilast. Questi risultati indicano che la presenza della placoglobina è indispensabile affinché l'apremilast possa esercitare il suo effetto (Fig. 7b).

Nel complesso, questi esperimenti dimostrano che la presenza della placoglobina è essenziale per l'aumento della coesione cardiomiocitaria indotto dall'apremilast, mentre la sua fosforilazione in Ser665 rappresenta un meccanismo di regolazione ma non è strettamente necessaria poiché il farmaco risulta efficace anche nei topi JUPS665A.

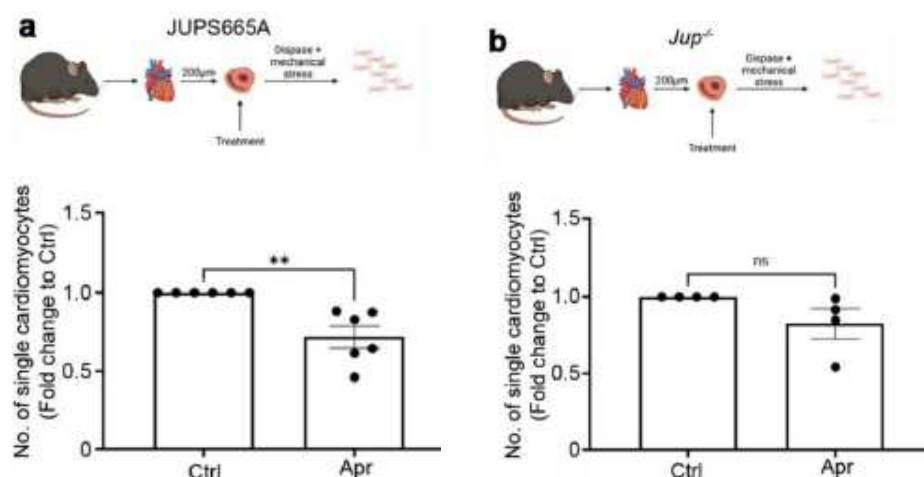


Figura 7. a. Il saggio di dissociazione nelle sezioni cardiache di topi JUPS665A evidenzia un aumento della coesione cellulare indotto da Apr. **b.** Il saggio di dissociazione nelle sezioni cardiache di topi *Jup*^{-/-} mostra l'assenza di un effetto significativo di Apr sulla coesione cardiomiocitaria.

3.3 L'attivazione di ERK1/2 è cruciale nella coesione dei cardiomiociti potenziata dall'apremilast

Successivamente, poiché l'apremilast aumenta la concentrazione intracellulare di cAMP e promuove l'attivazione della via di segnalazione di ERK1/2, ne è stato valutato il ruolo nella coesione cardiomiocitaria potenziata dal farmaco.

ERK1/2 (*Extracellular signal-Regulated Kinases 1 and 2*) sono due chinasi serina/treonina appartenenti alla famiglia delle MAP chinasi (*Mitogen-Activated Protein Kinases*). Questi enzimi trasducono segnali dall'esterno all'interno della cellula e regolano numerosi processi biologici, tra cui l'adesione cellulare, la progressione del ciclo cellulare, la migrazione, la sopravvivenza, il differenziamento, il metabolismo e la proliferazione cellulare (Roskoski, 2012).

Le cellule HL-1 sono state trattate per un'ora a 37 °C con 1 µM di apremilast oppure con una combinazione di apremilast e U0126. Quest'ultima molecola è un inibitore di MEK1/2, una chinasi situata a monte di ERK1/2 nella cascata di segnalazione delle MAP chinasi. L'inibizione di MEK1/2 impedisce la fosforilazione attivante di ERK1/2, determinando il blocco della via di segnalazione.

Il saggio di dissociazione ha evidenziato che il trattamento combinato con apremilast e U0126 non ha determinato il potenziamento della coesione cardiomiocitaria osservato nelle cellule HL-1 trattate esclusivamente con

apremilast. Questo risultato indica che l'attivazione di ERK1/2 è necessaria per l'effetto dell'apremilast sulla coesione cellulare (Fig. 8a).

Inoltre, mediante un'analisi Western blot, è stata valutata la fosforilazione della placoglobina in corrispondenza della serina 665 (PG-pS665). L'apremilast aumenta la fosforilazione della placoglobina, mentre in presenza di U0126, che blocca l'attivazione di ERK1/2, tale modificazione non viene più rilevata. Questi risultati indicano che il potenziamento della coesione cellulare indotto dall'apremilast dipende dall'attivazione della via ERK1/2 e dalla conseguente fosforilazione della placoglobina (Fig. 8b).

Infine, mediante immunocolorazione, è stato osservato che la traslocazione di DSG2 e DP verso i bordi cellulari mediata da apremilast viene annullata dall'inibizione di ERK1/2 indotta da U0126. Questi risultati dimostrano che ERK1/2 svolge un ruolo fondamentale nella corretta organizzazione e stabilizzazione dei desmosomi.

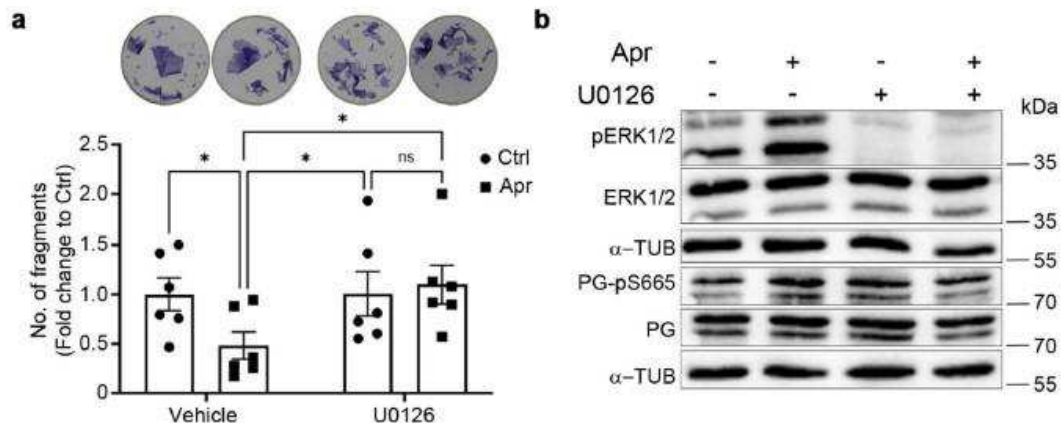


Figura 8. a. Saggio di dissociazione nelle cellule HL-1 trattate con Apr in presenza o assenza di U0126: il trattamento con l'inibitore di MEK1/2 abolisce il potenziamento della coesione cardiomiocitaria indotto da Apr. **b.** Analisi mediante Western blot dell'espressione di pERK1/2, ERK1/2 totale, PG-pS665 e PG totale in cellule HL-1 di controllo e dopo il trattamento con Apr, U0126 o la combinazione di Apr e U0126.

3.4 Isolamento e caratterizzazione di hiPSCs da una paziente con ACM (ACM-hiPSCs)

Dopo aver dimostrato che l'apremilast potenzia la coesione dei cardiomiociti nelle cellule cardiache murine e nelle sezioni cardiache murine, è stato valutato se l'effetto del farmaco potesse essere riprodotto anche in un modello umano di ACM.

A tale scopo, sono state generate cellule staminali pluripotenti indotte umane (hiPSCs) a partire da cellule di una paziente affetta dalla patologia (NP0151-11 F) e dalla madre sana (NP0147-4).

L'analisi istologica del tessuto cardiaco post-mortem della probanda, deceduta per morte cardiaca improvvisa, ha evidenziato perdita di cardiomiociti e presenza di adiposi e fibrosi, alterazioni tipiche nei cuori di soggetti affetti da ACM (Fig. 9a).

La pluripotenza della linea NP0151-11 F è stata confermata mediante l'analisi della morfologia cellulare, dell'espressione di marcatori specifici della pluripotenza e della capacità di differenziarsi nei derivati di tutti e tre i foglietti embrionali.

Le hiPSCs presentano la morfologia tipica delle colonie pluripotenti, caratterizzata da cellule piccole, strettamente adese tra loro, caratterizzate da nuclei di grandi dimensioni, nucleoli ben evidenti e ridotti spazi intercellulari (Kato et al., 2016) (Fig. 9b).

Mediante immunofluorescenza sono stati analizzati i marcatori della pluripotenza OCT4, NANOG e SSEA4. La loro espressione conferma che la linea NP0151-11 F mantiene le caratteristiche tipiche delle cellule staminali pluripotenti (Fig. 9c).

Infine, la pluripotenza è stata ulteriormente confermata dalla capacità delle hiPSCs di differenziarsi nei tre foglietti embrionali: mesoderma, endoderma ed ectoderma, come dimostrato dall'espressione dei rispettivi marcatori TBXT, SOX17 e OTX2 (Fig. 9d).

Le hiPSCs sono state differenziate in cardiomiociti (hiPSC-CMs), i quali presentano le tipiche striature trasversali delle cellule muscolari cardiache ed esprimono le proteine desmosomiali DSG2, PKP2 e PG, la proteina delle giunzioni aderenti N-CAD, la proteina delle giunzioni gap Cx43 e il marcatore sarcomerico cardiaco F-actina. L'analisi mediante immunostaining degli hiPSC-CMs derivati dalla paziente con ACM (ACM-hiPSC-CMs) ha evidenziato una colorazione della DP molto fiavole, discontinua e poco distribuita lungo le giunzioni cellulari, indicando un'alterata localizzazione della proteina desmosomiale conseguente alla mutazione c.2854G >T nel gene *DSP* (Fig. 9e). Al contrario, la distribuzione delle altre proteine giunzionali analizzate è risultata conservata.

Nel complesso, queste analisi confermano che gli ACM-hiPSC-CMs della linea NP0151-11 F riproducono le principali caratteristiche molecolari della cardiomiopatia aritmogena e costituiscono un modello sperimentale idoneo per valutare gli effetti dell'apremilast.

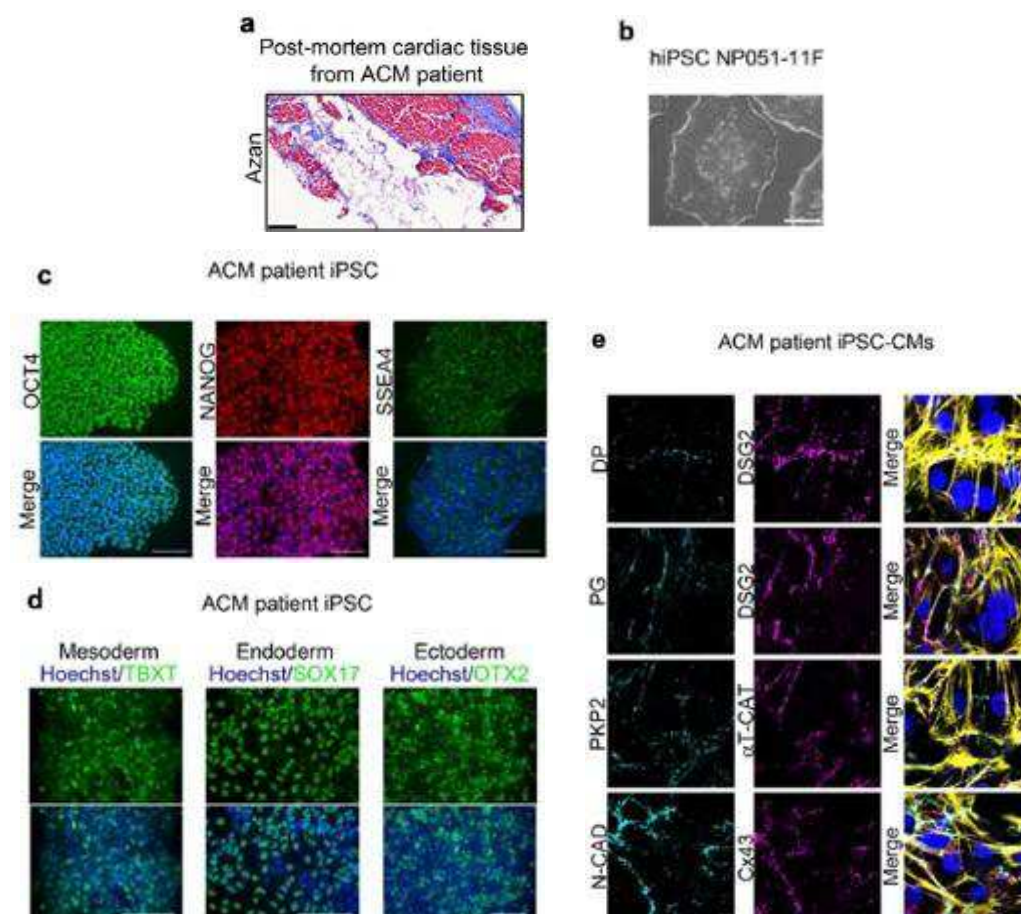


Figura 9. **a.** Analisi istologica post-mortem del ventricolo sinistro della probanda affetta da ACM. La colorazione Azan evidenzia la sostituzione fibroadiposa del miocardio: i cardiomiociti sono visualizzati in rosso, il tessuto fibrotico in blu e il tessuto adiposo in bianco. **b.** Morfologia delle colonie della linea di hiPSC NP0151-11 F derivata dalla paziente con ACM. **c.** Analisi immunocitochimica dei marcatori di pluripotenza nella linea ACM-hiPSC NP0151-11 F. **d.** Analisi immunocitochimica dei marcatori specifici dei tre foglietti embrionali nella linea ACM-hiPSC NP0151-11 F. **e.** Analisi immunostaining delle proteine delle giunzioni intercellulari e dei desmosomi nei cardiomiociti derivati dalla linea ACM-hiPSC NP0151-11 F: α -T-catenina, DP, DSG2, PG, PKP2, N-CAD e Cx43.

3.5 Apremilast attenua la perdita di coesione negli ACM-hiPSC-CMs

Successivamente è stato valutato se la coesione fosse alterata in cardiomiociti derivati da cellule staminali pluripotenti indotte umane ottenute dalla paziente affetta da ACM (ACM-hiPSC-CMs).

Mediante saggi di dissociazione sono stati confrontati gli hiPSC-CMs della madre sana della paziente indice e di un controllo sano non imparentato. Da queste analisi è emerso che, dopo agitazione meccanica, gli ACM-hiPSC-CMs hanno prodotto un numero significativamente maggiore di frammenti cellulari, causato da una ridotta coesione tra i cardiomiociti, rispetto ai cardiomiociti derivati dalla madre sana e dal controllo non imparentato (Fig. 10a).

In seguito, allo scopo di stabilire se l'aumento intracellulare di cAMP potesse stabilizzare la coesione dei cardiomiociti come già osservato nelle cellule HL-1 e nelle sezioni cardiache murine, gli ACM-hiPSC-CMs sono stati trattati per un'ora e 24 ore a 37 °C con FR e apremilast. Dopo il trattamento, i saggi di dissociazione hanno evidenziato una maggior coesione cardiomiocitaria rispetto al controllo non trattato (Fig. 10b).

Mediante analisi di Western blot, dopo il trattamento con FR e con apremilast, negli ACM-hiPSC-CMs è stato osservato un aumento della fosforilazione della placoglobina in Ser665, mentre i livelli delle altre proteine analizzate (DP, DSG2, PKP2 e N-CAD) e la fosforilazione di ERK1/2 sono rimasti invariati. La quantificazione del Western blot ha confermato un aumento significativo di PG-pS665 in seguito al trattamento con apremilast, senza evidenziare variazioni significative dei livelli di pERK1/2 (Fig. 10c). Questi risultati suggeriscono che, negli ACM-hiPSC-CMs, l'effetto del farmaco non è associato a un aumento dell'attivazione della via ERK1/2, ma all'incremento della fosforilazione di PG in Ser665 conseguente all'aumento intracellulare di cAMP determinato dall'inibizione della PDE-4.

Infine, l'immunostaining ha evidenziato che, nelle cellule di controllo, il segnale di PG-pS665 è debole, mentre aumenta dopo il trattamento con FR o apremilast. Inoltre, PG-pS665 e DSG2 risultano distribuite in modo continuo lungo le giunzioni

intercellulari dei cardiomiociti, suggerendo una migliore organizzazione e stabilizzazione dei desmosomi.

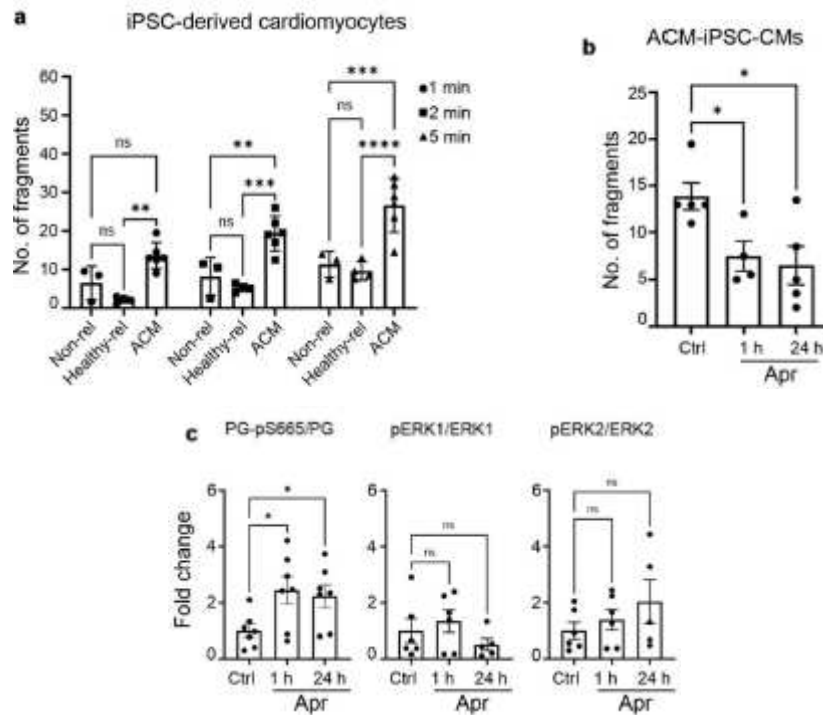


Figura 10. a. Saggio di dissociazione eseguito su cardiomiociti derivati da hiPSCs di un controllo sano non imparentato (*Non-rel*), della madre sana della paziente (*Healthy-rel*) e della paziente affetta da ACM (*ACM*). **b.** Saggio di dissociazione eseguito sugli ACM-hiPSC-CMs dopo trattamento con Apr. **c.** Quantificazione del Western blot relativa al trattamento con apremilast: la fosforilazione della placoglobina in Ser665 aumenta significativamente, mentre non si osservano variazioni significative della fosforilazione di ERK1/2.

3.6 Apremilast riduce le aritmie nel modello murino ex vivo di ACM

Infine è stato indagato se il miglioramento della coesione cardiomiocitaria si traducesse anche in una riduzione delle aritmie, una delle principali manifestazioni cliniche dell'ACM. A tale scopo è stato utilizzato il modello murino.

In particolare, sono stati utilizzati topi wild-type (*Jup^{+/+}*), in cui la placoglobina è normalmente presente, e topi knockout in omozigosi per la placoglobina (*Jup^{-/-}*), che sviluppano un fenotipo simile a quello dell'ACM, rappresentando quindi un modello murino della malattia.

Utilizzando sezioni cardiache ex vivo ottenute da questi due modelli murini, mediante analisi con MEA, è stata registrata l'attività elettrica del tessuto cardiaco

calcolando la SDNN (*Standard Deviation of Normal-to-Normal intervals*), ossia la deviazione standard del tempo tra due battiti consecutivi.

Per i topi $Jup^{+/+}$ la SDNN è risultata molto bassa sia prima che dopo il trattamento con l'apremilast, a indicare che il cuore batte regolarmente.

Per i topi $Jup^{-/-}$, invece, la SDNN è risultata molto elevata prima del trattamento, indice di una maggiore variabilità del ritmo cardiaco e del maggior rischio di aritmie. Al contrario, dopo il trattamento con l'apremilast, la SDNN si riduce (Fig. 11a).

Successivamente sono stati eseguiti esperimenti di perfusione Langendorff. In questo modello sperimentale il cuore viene espantato dal topo e mantenuto vitale mediante perfusione retrograda attraverso l'aorta con una soluzione ricca di ossigeno e nutrienti. L'analisi ha evidenziato che la frequenza cardiaca rimane invariata prima e dopo il trattamento con apremilast sia nei topi $Jup^{+/+}$ che $Jup^{-/-}$ (Fig. 11b). Questi risultati indicano che l'apremilast non modifica la frequenza cardiaca, ma riduce la variabilità del ritmo, esercitando quindi un effetto antiaritmico senza alterare la velocità del battito.

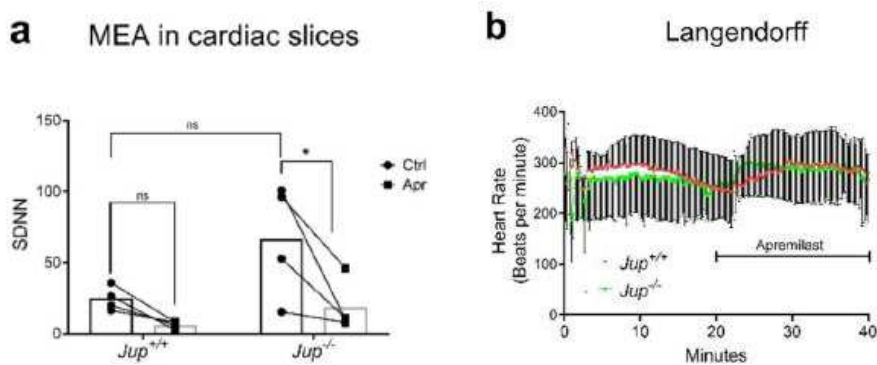


Figura 11. a. Analisi mediante MEA eseguita su sezioni cardiache ventricolari di topi $Jup^{+/+}$ e $Jup^{-/-}$ prima e dopo il trattamento con Apr. **b.** Esperimento di perfusione cardiaca secondo Langendorff eseguito su cuori isolati di topi $Jup^{+/+}$ e $Jup^{-/-}$, prima e dopo il trattamento con Apr.

4. DISCUSSIONE

Ad oggi, le opzioni terapeutiche per i pazienti affetti da cardiomiopatia aritmogena sono prevalentemente sintomatiche, ossia finalizzate ad alleviare i sintomi, quali aritmie e insufficienza cardiaca, e a prevenire la morte cardiaca improvvisa.

Nella maggior parte dei casi vengono prescritti β -bloccanti per il controllo delle aritmie, in particolare delle tachicardie ventricolari. Tuttavia, questi farmaci possono risultare inefficaci o mal tollerati a causa dei loro effetti collaterali. Di conseguenza, risulta necessario individuare nuove strategie terapeutiche per il trattamento dell'ACM.

Studi precedenti hanno dimostrato che gli inibitori delle fosfodiesterasi possono essere efficaci nel trattamento dell'insufficienza cardiaca e dei processi infiammatori.

In questo contesto, l'apremilast, un inibitore della fosfodiesterasi-4 (PDE-4), si è dimostrato efficace nel trattamento del pemfigo volgare, una malattia autoimmune desmosomiale della cute caratterizzata dalla perdita di adesione tra i cheratinociti. Inoltre, ulteriori studi hanno evidenziato che la stabilizzazione dell'adesione cellulare può rappresentare una strategia terapeutica efficace nelle malattie desmosomiali.

Alla luce di queste evidenze, Stangner et al. (2025) hanno valutato se l'apremilast fosse in grado di migliorare la stabilità dei desmosomi a livello delle giunzioni intercellulari, aumentare la coesione dei cardiomiociti e ridurre le aritmie in un modello murino di ACM.

Grazie a modelli sperimentali, in particolare la linea cellulare murina HL-1 e sezioni di tessuto cardiaco murino, è stato osservato un aumento della coesione dei cardiomiociti associato all'incremento dei livelli intracellulari di cAMP.

Come modello umano in vitro della patologia sono stati utilizzati cardiomiociti derivati da cellule staminali pluripotenti indotte (hiPSC-CMs). Mediante un saggio di dissociazione è emerso che gli hiPSC-CMs derivati da una paziente affetta da ACM (ACM-hiPSC-CMs) presentano una coesione cellulare compromessa rispetto agli hiPSC-CMs ottenuti dalla madre sana e da un individuo sano non imparentato.

Questi risultati supportano l'ipotesi che la perdita di adesione cellulare rappresenti uno degli eventi chiave nella patogenesi dell'ACM e suggeriscono che il ripristino della coesione dei cardiomiociti possa costituire una potenziale strategia terapeutica.

A tal proposito, Stangner et al. (2025) hanno dimostrato che, negli ACM-hiPSC-CMs, l'apremilast aumenta i livelli intracellulari di cAMP, promuove la fosforilazione di PG-pS665 e la traslocazione di DSG2 a livello delle giunzioni intercellulari, determinando un aumento della coesione dei cardiomiociti.

Tali risultati sono in accordo con quanto osservato sia nelle cellule HL-1 e nelle sezioni cardiache murine sia negli studi precedenti che hanno impiegato forskolina e rolipram (FR).

Tuttavia, l'apremilast induce un incremento dei livelli intracellulari di cAMP circa dieci volte inferiore rispetto a FR, pur producendo un effetto comparabile sulla coesione cellulare. Questo dato suggerisce che il rafforzamento dell'adesione tra cardiomiociti possa essere ottenuto anche senza raggiungere elevate concentrazioni intracellulari di cAMP. In questo modo si ha la possibilità di ottenere un beneficio terapeutico mantenendo bassa l'attivazione della via di segnalazione, con una potenziale riduzione degli effetti collaterali.

Nel loro studio, Stangner et al. (2025) hanno inoltre osservato che, nei topi JUPS665A, l'apremilast aumenta la coesione dei cardiomiociti anche in assenza della fosforilazione di PG. Tuttavia, il farmaco promuove comunque la traslocazione di DSG2 e PG a livello delle giunzioni intercellulari, indicando che l'effetto dell'apremilast è mantenuto anche in assenza della fosforilazione di PG.

Al contrario, nei topi *Jup*^{-/-}, privi di PG, l'apremilast non è in grado di esercitare il proprio effetto, suggerendo che la presenza di PG sia essenziale per l'azione del farmaco.

Una differenza significativa tra le cellule HL-1 e gli ACM-hiPSC-CMs riguarda la via di segnalazione ERK1/2. Mentre nelle cellule HL-1 l'attivazione di ERK1/2 si è dimostrata essenziale per gli effetti dell'apremilast, negli ACM-hiPSC-CMs il farmaco non attiva questa via, suggerendo l'esistenza di meccanismi differenti, nei cardiomiociti murini e umani, alla base dell'incremento della coesione cellulare.

Poiché la maggior parte dei pazienti affetti da ACM soffre di aritmie, che in alcuni casi possono portare a morte cardiaca improvvisa, Stangner et al. (2025) hanno indagato se l'apremilast potesse stabilizzare la ritmicità del battito cardiaco mediante esperimenti MEA e Langendorff. Da questi esperimenti è emersa una riduzione delle aritmie nei topi *Jup^{-/-}*, nei quali tuttavia l'apremilast non aumenta la coesione dei cardiomiociti. Questo dato suggerisce che il miglioramento della ritmicità cardiaca possa dipendere da effetti dell'inibizione della PDE-4 sulla contrattilità o sul potenziale d'azione, indipendenti dalla presenza di PG.

In sintesi, lo studio di Stangner et al. (2025) ha il merito di aver dimostrato per la prima volta che l'adesione tra i cardiomiociti derivati da una paziente affetta da ACM è compromessa e che l'apremilast è in grado di ripristinare efficacemente la coesione cellulare mediata dai desmosomi, oltre a ridurre le aritmie. Questi risultati suggeriscono che l'apremilast possa rappresentare una promettente strategia farmacologica per il trattamento della cardiomiopatia aritmogena. Tuttavia, sono necessari ulteriori studi preclinici e clinici per confermarne l'efficacia e la sicurezza nell'uomo.

Nonostante i risultati incoraggianti, lo studio presenta alcuni limiti. Gli esperimenti sono stati condotti prevalentemente su modelli cellulari in vitro e su sezioni di tessuto cardiaco murino che, sebbene rappresentino strumenti fondamentali per lo studio dei meccanismi molecolari della patologia, non riproducono la complessità del microambiente cardiaco. In particolare, i cardiomiociti derivati da hiPSCs sono cellule immature rispetto ai cardiomiociti adulti; ne deriva che tali modelli non consentono di valutare le interazioni tra diversi tipi cellulari presenti nel cuore, quali fibroblasti, cellule endoteliali, cellule immunitarie e progenitori cardiaci (Risato et al., 2024).

Un ulteriore limite è rappresentato dal ridotto numero di linee cellulari umane analizzate: i risultati ottenuti derivano da hiPSCs generate da una sola paziente affetta da ACM, pertanto andrebbero confermati mediante esperimenti condotti su un numero maggiore di pazienti portatori di differenti varianti patogene.

Per superare tali limitazioni, negli ultimi anni la ricerca si è orientata sempre più verso l'impiego di modelli animali in vivo, tra cui il topo e lo zebrafish.

I modelli murini presentano un'elevata somiglianza anatomica e fisiologica con il cuore umano, consentendo di studiare il ruolo dei geni coinvolti nella patogenesi dell'ACM, gli effetti di specifiche varianti genetiche, la progressione della malattia e le interazioni tra le diverse vie di segnalazione (Risato et al., 2024).

Lo zebrafish, invece, rappresenta un modello sperimentale più economico e facilmente manipolabile; inoltre, la sua struttura cardiaca e l'organizzazione delle proteine desmosomiali consentono di riprodurre efficacemente il fenotipo dell'ACM (Risato et al., 2024).

In conclusione, sebbene nessun modello sperimentale sia in grado di riprodurre completamente la complessità della malattia umana, i modelli animali rappresentano strumenti indispensabili per approfondire i meccanismi molecolari alla base della cardiomiopatia aritmogena e per valutare l'efficacia e la sicurezza di nuove strategie terapeutiche. Pertanto, futuri studi condotti in modelli animali di ACM e successivi trial clinici saranno indispensabili per confermare il potenziale terapeutico dell'apremilast e valutarne l'effettiva applicabilità.

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RESEARCH

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Apremilast improves cardiomyocyte cohesion and arrhythmia in different models for arrhythmogenic cardiomyopathy

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Abstract

Background Arrhythmogenic cardiomyopathy (ACM) is a genetically inherited desmosome heart disease leading to life-threatening arrhythmias and sudden cardiac death. Currently, ACM treatment paradigms are merely symptom targeting. Recently, apremilast was shown to stabilize keratinocyte adhesion in the desmosomal disease pemphigus vulgaris. Therefore, this study investigated whether apremilast can be a therapeutic option for ACM.

Methods Human induced pluripotent stem cells from a healthy control (hiPSC) and an ACM index patient (ACM-hiPSC) carrying a heterozygous desmoplakin (*DSP*) gene mutation (c.2854G > T, p.Glu952Ter), confirmed by whole exome sequencing (WES), were established. Cyclic-AMP ELISA, dissociation assay, immunostaining, and Western blotting analyses were performed in human iPSC-derived cardiomyocytes (hiPSC-CMs), murine HL-1 cardiomyocytes, and cardiac slices derived from wild-type (WT) mice, plakoglobin (PG, *Jup*) knockout (*Jup*^{-/-}) (murine ACM model) or PG Serine 665 phosphodeficient (JUP-S665A) mice. Microelectrode array (MEA) analyses in ventricular cardiac slices and Langendorff heart perfusion were performed to analyze heart rate variability and arrhythmia.

Results ACM-hiPSC derived cardiomyocytes (ACM-hiPSC-CMs) revealed a significant loss of cohesion, which was rescued by apremilast. Further, treatment with apremilast strengthened basal cardiomyocyte cohesion in HL-1 cells and WT murine cardiac slices, paralleled by phosphorylation of PG at Serine 665 in human and murine models. In HL-1 cells, apremilast in addition activated ERK1/2, inhibition of which abolished apremilast-enhanced cardiomyocyte cohesion. Further, dissociation assays in slice cultures from JUP-S665A and *Jup*^{-/-} mice revealed that PG is crucial

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for apremilast-enhanced cardiomyocyte cohesion. In parallel to enhanced cell adhesion, MEA and Langendorff measurements from WT and *Jup*^{-/-} mice demonstrated decreased heart rate variability and arrhythmia after apremilast treatment.

Conclusions Apremilast improves loss of cardiomyocyte cohesion, enhances localization of DSG2, and reduces arrhythmia in human and/or murine models of ACM ex vivo and in vitro, providing a novel treatment strategy for ACM by preserving desmosome function.

Keywords Apremilast, Arrhythmogenic cardiomyopathy, Arrhythmia, Desmoplakin and desmoglein 2

Translational perspective

The current therapeutic options for patients with arrhythmogenic cardiomyopathy (ACM) include lifestyle changes, treatment with anti-arrhythmic drugs, catheter ablation, implantable cardiac defibrillators, and, ultimately, heart transplantation for patients who are having therapy-refractory arrhythmia or have developed heart failure. However, lifestyle changes, such as refraining from physical endurance activities and β -blocker therapy, are most commonly used in patients carrying genetic variants coding for proteins of the desmosomal complex. Recent advancements hint that strategies to enhance intracellular cAMP could be beneficial in treating desmosomal diseases and can be effective therapeutics, which would be highly relevant for ACM patients.

In this study, we show that apremilast improves loss of cardiomyocyte cohesion, enhances localization of desmosomal proteins, and reduces arrhythmia in human and murine models of ACM ex vivo and in vitro, providing a novel treatment strategy for ACM by preserving desmosome function.

Introduction

Arrhythmogenic cardiomyopathy (ACM) is an uncommon hereditary heart condition that can lead to sudden cardiac death (SCD), particularly among young athletes (Corrado, Basso et al. [9], Corrado, Link et al. [10]). The estimated prevalence of this condition ranges from 1 in 1000 to 1 in 5000 individuals (Peters, Trummel et al. [37], Groeneweg, Bhonsale et al. [4]). Notably, SCD can even occur in the initial concealed phase without observable structural changes in the ventricles, and it may be the first manifestation of the disease in approximately 5–10% of cases (Peters, Trummel et al. [37], Bhonsale, Groeneweg et al. [4], Groeneweg, Bhonsale et al. [4]). Approximately 60% of ACM patients carry pathogenic variants in genes encoding desmosomal proteins encompassing both plaque proteins like plakophilin 2 (PKP2), desmoplakin (DSP), and plakoglobin (JUP), as well as cadherin-type adhesion molecules such as desmoglein 2 (DSG2) and desmocollin 2 (DSC2) [14], Marcus, McKenna et al. [30], Vimalanathan, Ehler et al. [55], Costa, Cerrone et al. [12], Gasperetti, Carrick et al. [19]. Hence, ACM is

recognized primarily as a hereditary condition affecting the desmosome.

Pathogenic variants in desmosome genes such as *DSG2*, *DSP*, and *PKP2* associated with ACM often disrupt intercellular desmosomal connections, thereby reducing cellular cohesion [61]. Various pathways regulating cardiomyocyte cohesion have been identified Schinner, Vielmuth et al. [43], Shoykhet, Trenz et al. [49], Yeruva, Kempf et al. [58], Yeruva, Korber et al. [59, 61]. Additionally, mounting evidence suggests that desmosomes play a role in cellular cohesion and can serve as signaling centers and maintain tissue integrity (Spindler, Eming et al. [52], Yeruva and Waschke [61], Spindler, Gerull et al. [53], Vielmuth, Radeva et al. [54], Perl, Pokorny et al. [35]). Thus, cardiac desmosomes are integral components of an intricate signaling network that can both influence and be influenced by cellular signaling processes.

The current therapeutic options for patients with ACM include lifestyle changes, treatment with anti-arrhythmic drugs, catheter ablation, implantable cardiac defibrillators, and ultimately, heart transplantation for patients who are having therapy refractory arrhythmia or developed heart failure (Corrado, Basso et al. [9], Gasperetti, Peretto et al. [20]). However, in patients carrying genetic variant coding for proteins of the desmosomal complex, lifestyle changes, such as restraining from physical endurance activities and β -blocker therapy, are most commonly used (Corrado, Basso et al. [9], Corrado and Zorzi [11], Grasso, Bondavalli et al. [22]).

In our previous work, we discovered increased cAMP levels via PKA-mediated PG phosphorylation at serine 665 (PG-pS665) as a driving force for DSG2-mediated enhanced cardiomyocyte cohesion, which is referred to as positive adhesiotropy (Schinner, Vielmuth et al. [43], Shoykhet, Trenz et al. [49], Yeruva and Waschke [61]). This indicated that cardiomyocyte adhesion at the intercalated disc (ICD) is precisely regulated and can be enhanced via cAMP-increasing drugs. We have recently demonstrated that apremilast, a PDE-4 inhibitor, clinically used in treating psoriasis and Behçet's disease, abrogated pemphigus autoantibody-induced loss of keratinocyte cohesion via PG-pS665, inhibition of autoantibody-mediated keratin retraction and DP assembly in the desmosomal plaques (Sigmund, Winkler et al. [50]).

Further, two recent case report studies showed that apremilast treatment in a pemphigus patient led to a decrease in DSG-specific autoantibody levels and the healing of lesions (Meier, Holstein et al. [32], Delvaux, Bohelay et al. [15]).

These recent advancements hint that strategies enhancing intracellular cAMP could be beneficial in treating desmosomal diseases and can be effective therapeutics, which would be highly relevant for ACM patients. Therefore, this study investigated whether apremilast can enhance desmosomal-mediated cardiomyocyte cohesion and be used as an ACM therapeutic agent.

Materials and methods

Reagents

The supplementary file details all the important reagents, mediators, primers, and antibodies used in this study and the missing methods in the main manuscript.

HL-1 cell culture

As described in our previous studies, the murine atrial cardiac myocyte cell line HL-1 was maintained in Claycomb medium (Sigma-Aldrich, #51800 C) containing 100 μ mol/ml norepinephrine (NE, Sigma-Aldrich, #0937) (Shoykhet, Trenz et al. [49], Yeruva, Korber et al. [59], Yeruva, Stangner et al. [60]). Cells seeded for experiments were incubated in Claycomb medium without norepinephrine to avoid basal adrenergic stimulation.

Dissociation assays in HL-1 cells

For dissociation assays, experiments were performed as explained previously (Shoykhet, Trenz et al. [49], Yeruva, Korber et al. [59], Yeruva, Stangner et al. [60] and in supplementary methods.

Murine models

All mice lines used in this study were generated and bred as described previously for *Jup*^{S665A} (Sigmund, Winkler et al. [50], *Jup*^{-/-} (Schinner, Vielmuth et al. [43], Schinner, Erber et al. [40], Schinner, Olivares-Florez et al. [42], Shoykhet, Trenz et al. [49], Yeruva, Korber et al. [59], Shoykhet, Dervishi et al. [48] and *Pkp2*^{-/-} (Shoykhet, Dervishi et al. [48]). For experiments age- and sex-matched littermates, 8-14-week-old mice, were used. Euthanasia was performed using isofluran (Iso-Vet, 1000 mg/g, #469860) in an E-Z anesthesia system (#EZ-SA800-OS, World precision instruments, Germany). Mice were briefly anesthetized with 5% isoflurane until the mouse became immobile, mostly within 1 to 2 min. A toe pinch was applied to ensure that the animal was anesthetized, then cervical dislocation was performed, and hearts were excised for further analysis. All the animal experiments confined to the principles outlined in the Declaration of Helsinki. Ethics statement was provided in the section

'Study approval'. All the experiments performed with mice have been reported in line with the ARRIVE guidelines 2.0.

Sex as a biological variable

Both male and female mice were included, and similar findings were found in both sexes.

Murine ventricular cardiac slice culture and dissociation assay from mice

Murine ventricular cardiac slice cultures, dissociation assays, and lysates for Western blots were obtained exactly as published previously (Shoykhet, Dervishi et al. [48]). Details of this can be found in the supplementary methods.

Human tissue collection for hiPSC generation

Human vein fibroblasts (hVFs) were isolated at the Institute of Human Genetics of the LMU Munich from the vein of the index patient (ID-number NP0151; named as NP151-11 F in the manuscript) collected after the autopsy at the Pathology Department of the Munich Clinic Schwabing. The human dermal fibroblasts (hDFs) used to generate the NP0040-8 hiPSC line were isolated from a full-thickness skin sample obtained aseptically from a healthy male subject by punch biopsy. The tissue specimens were placed into cell culture dishes, cut into small pieces, and cultured in the Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, #61965-026) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA, # F9665), 2% Glutamax (Thermo Fisher Scientific, #35050-061), 1% non-essential amino acids (NEAA, Thermo Fisher Scientific, #11140-035), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 100 μ M β -mercaptoethanol (β -ME, Thermo Fisher Scientific, #31350-010) until confluent outgrowing fibroblasts appeared. The hVFs or hDFs were then collected by dissociation with TrypLE Express (Thermo Fisher Scientific, #12604021), expanded for an additional 2–3 passages and cryopreserved in aliquots for future use.

The NP0147-4 hiPSC line was generated from peripheral blood mononuclear cells (PBMCs) that were obtained from the index patient's healthy mother. Whole blood (~30 ml) was collected by venepuncture into BD Vacutainer CPT vials (BD Biosciences, Heidelberg, Germany, #362753) and PBMCs were immediately isolated by centrifugation at RT following the manufacturer's protocol. PBMC aliquots were frozen in 10% dimethyl sulfoxide (DMSO, Applichem, Darmstadt, Germany, #A3672,0100) and stored in liquid nitrogen for future use.

Generation of hiPSCs

To generate insertion-free hiPSCs, cryopreserved hVFs from donor NP0151 were thawed and passaged in the human fibroblast (hF) medium (see above) for approximately two passages before reprogramming was initiated. PBMCs from donor NP0147 were thawed (day -4) and 5×10^5 cells were cultured in StemPro-34 SFM Medium (Thermo Fisher Scientific, #10639-011) freshly supplemented with the human recombinant IL-3 (ISOKine, ORF Genetics, #01-AA090), IL-6 (PreproTech, #AF-200-06), stem cell factor (SCF, ISOKine, ORF Genetics, #01-A0850) and FLT3-ligand (ISOKine, ORF Genetics, #01-A0120) in order to expand the erythroid progenitor cells. On day 0, the expanded erythroblasts (3×10^5 cells) and hVFs were transduced with Sendai vectors supplied in the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, # A16517) at the recommended multiplicities of infection (MOI) for each vector. The transduced hVFs were cultured in hF medium without antibiotics at 37 °C at 5% CO₂ under normoxic conditions for one day, and the medium was replaced with fresh hF medium. The cells were then cultured in hF medium until day 7, changing the medium every other day. On day 7, cells were dissociated with 0.05% trypsin/EDTA (Thermo Fisher Scientific, #25300054), plated at densities of 2, 5 and 10×10^4 cells per well of a 6-well plate coated with 0.5 µg/cm² vitronectin (VTN-N, Thermo Fisher Scientific, # A14700) and cultured in hF medium for a further 2 days. On day 8, the hF medium or the StemPro-34 SFM medium were replaced with the Essential 8 (E8) medium (Thermo Fisher Scientific, #A1517001) containing 100 ng/ml basic fibroblast growth factor (bFGF, Peprotech, # 100-18B), and cells were cultured with a medium change every two days until pluripotent stem cell-like colonies appeared, usually within 15–28 days after reprogramming. Initial hiPSC colonies were manually dissected and small cell clusters were transferred to fresh vitronectin-coated 6- or 12-well plates. Each harvested colony was cultured separately in complete E8 medium and after several passages, those clones that showed typical hiPSC morphology with no or low level of spontaneous differentiation were selected for further expansion, characterization and cryopreservation.

The hiPSC line NP0040-8 was generated from hDFs using the episomal plasmid vectors pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL that were developed by Shinya Yamanaka laboratory (Okita, Matsumura et al. [33]) and obtained from Addgene (plasmid IDs: 27077, 27078, and 27080, respectively). One million of hDFs were transfected with the 2.5 µg of each vector using the Neon Transfection System Kit 100 µl (Thermo Fisher Scientific, #MPK10096) in R Buffer (pulse voltage: 1700 V, pulse width: 10 ms, pulse number: 3).

After transfection, the cells were cultured in the hF medium until day 7, when the medium was changed to E8 medium. Cells were maintained in E8 with regular medium changes until hiPSC-like colonies appeared. Selected colonies were picked under a microscope using P10 pipette tips and several colony fragments of each clone were transferred to a new well of a 12-well plate coated with vitronectin. Cells were cultured in E8 medium until each clone was sufficiently expanded for characterization and cryopreservation.

Cultivation and cryopreservation of hiPSCs

hiPSCs were regularly passaged by Versene (Thermo Fisher Scientific, #15040066) upon reaching about 70–80% confluency at the split ratio of 1:4 to 1:6 and grown in complete E8 medium supplemented with 10 µM ROCK inhibitor Y27632 (Selleck Chemicals, Cologne, Germany) for the first 24 h. All hiPSC cultures were regularly checked for mycoplasma contamination using the MycoAlert® Mycoplasma Detection Kit (Lonza, Cologne, Germany). Master banks were prepared after hiPSC lines were confirmed to be SeV vector-free. Passage numbers at vector clearance varied between individual clones and usually occurred between passage numbers 7 and 19. Cells were banked as small cell clusters after dissociation with Versene in E8 medium supplemented with 10% DMSO and stored in liquid nitrogen until further use.

Maintenance of hiPSC lines

hiPSCs were maintained in Essential 8™ Medium (Gibco™, #A1517001) on Vitronectin (Gibco™, #A14700)-coated 6-well plates (Greiner, #657160) for NP0040-8 control hiPSC line or on Matrigel™ (Corning, #734–1440)-coated 6-well plates for NP0147-4 and NP0151-11 F hiPSC lines in a humidified incubator at 37 °C with 5% CO₂. Cells were passaged when 70–80% confluent (once every 3–5 days) by washing twice with DPBS without Mg²⁺ and Ca²⁺ (Gibco™, #14190250) and incubating with Versene (Gibco™, #15040066) for 3 to 5 min at RT. After removing Versene cell clusters were gently washed from the well with Essential 8™ Medium [for NP151-11 F and NP147-4 hiPSCs with 2 µM ROCK inhibitor (Y27632, Adooq, # A11001-5)] and transferred to a newly coated 6-well plate at split ratios between 1:6 to 1:20.

Differentiation of hiPSC lines

hiPSCs were differentiated into cardiomyocytes by using small molecules to modulate Wnt signaling pathways. When maintained cultures reached 80% confluency, hiPSCs were seeded as single cells by washing twice with DPBS without Mg²⁺ and Ca²⁺ (Fisher Scientific, #14040174) and incubating with TrypLE™ Express (Gibco™, #12604021) with 10 µl DNase I (2500 U/ml, Thermo Scientific™, #90083) per 1 ml TrypLE™ (Fisher

Scientific, #12604021) for 5 min at 37 °C. The reaction was stopped with Essential 8™ Medium supplemented with 10 µM ROCK inhibitor (Y27632, Adooq, #A11001-5) and cells were filtered through a 40 µm cell strainer (Corning®, #431750). After centrifugation and resuspension into fresh Essential 8™ Medium supplemented with 10 µM ROCK inhibitor, 0.6 to 0.8×10^6 single cells were seeded into each well of a 6-well plate. Cells were maintained in Essential 8™ Medium and differentiated when cells reached 90% confluency. First, cells were treated for exactly 24 h with 8 µM CHIR99021 (Sigma-Aldrich, #SML1046) in RPMI GlutaMAX™ (Gibco™, #61870010) with 50 µg/ml ascorbic-acid (WAKO Chemicals Europe, #013-12061), B27 supplement without insulin (Thermo Scientific™, #A1895601) and penicillin/streptomycin (Gibco, #15140-122). Cells were recovered in RPMI-B27minus insulin for 48 h before treated with 5 µM XAV939 (Sigma Aldrich, #X3004-5MG) and 5 µM IWP2 (Tocris, #3533/10) at day 3 for 48 h. At day 5 medium was changed to RPMI-B27minus insulin and from day 7, when beating monolayers started forming, medium was changed to fresh RPMI-B27plus insulin (Thermo Scientific™, #17504044) and refreshed every 2 days. Cells were replated for experiments between day 8 and 12.

RT-PCR

The clearance of the reprogramming SeV vectors in established hiPSC lines and expression of the pluripotency-associated markers OCT3/4, NANOG, SOX2, FOXD3 and DNMT3b at the transcript level was determined using semi-quantitative RT-PCR. Primers used for these analyses are listed in the Supplementary Table 1. For monitoring the vector clearance, total RNA was isolated using TRIzol (Thermo Fisher Scientific, #15596026) from uninfected cells (negative control), from cells freshly transduced with SeV vectors (positive control), and from established hiPSC clones at different stages of expansion. To determine the expression of the pluripotency-associated markers, RNA samples were isolated using the same method from an aliquot of banked cells. RNA was reverse transcribed into cDNA using random hexamers for priming and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, #18064071) following the manufacturer's recommendations. Negative control in RT reactions included all reaction components except RTase. Target sequences were amplified by PCR using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, #K1081) using the following cycling program: initial denaturation for 90 s at 95 °C followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing as specified in the Supplementary Table 1, 30 s extension at 72 °C, and 5 min final extension at 72 °C. GAPDH PCR-product was used as a house-keeping gene reference. PCR products were analyzed by

agarose gel electrophoresis and visualized using ethidium bromide.

Mutation analysis

To confirm the presence of the disease-causing pathogenic variant c.2854G>T in *DSP*, the genomic DNA was isolated from hiPSC lines using the DNeasy Blood & Tissue Kit (Qiagen, #69504) according to the manufacturer's recommendations. The gene segment harbouring the mutation was amplified by PCR using the primers listed in Supplementary Table 1, the PCR product was cleaned up using the QIAquick PCR purification Kit (Qiagen, #28104), and the eluted DNA samples were sent to Eurofins Genomics (Ebersberg, Germany) for sequencing using the forward and the reverse PCR primer.

Exome analysis

Sample preparation and bioinformatic analysis of the index patient NP0151-11 F and her mother [healthy-relative (NP147-4)] were performed at the Institute of Human Genetics at the Technical University of Munich. DNA was isolated from fibroblasts (patient) or blood lymphocytes (mother), respectively. Exome sequencing was performed using the Twist Human Exome 2.0 Plus Comprehensive Exome Spike-in and Mitochondrial Panel. Sequencing was performed on the NovaSeq 6000 as a 100 bp paired-end run (Illumina, San Diego, California).

Primary and secondary bioinformatic analysis were performed using the variant analysis pipeline EVAdb (<https://github.com/mri-ihg/EVAdb>). In particular, the Burrows-Wheeler Aligner (BWA) was used to map the generated data to the human genome sequence GRCh37. Single nucleotide variants (SNVs) and insertions/deletions (indels) were determined using SAMtools, PINDEL and the Genome Analysis Toolkit (GATK) package. Copy number variations (CNVs) were identified using ExomeDepth.

Exome-derived variant calls were annotated based on genomic coordinates, effect on gene product, and minor allele frequency within population control resources (gnomAD and in-house control exome dataset). Variants were further annotated for deleteriousness by calculating predictions from CADD, SIFT, and PolyPhen. To establish genetic causality and prioritize candidate genes, we considered various online repositories and in silico metrics such as ClinVar, HGMD, PubMed, OMIM, Uniprot Genotype-Tissue GTEx, Human Protein Atlas and gnomAD probability of being loss-of-function intolerant (pLI) and missense z-scores. After annotation, the variants were filtered using lists of sequencing artifacts, and calls with suboptimal quality parameters were eliminated. We applied a recessive filter for homozygous and compound heterozygous variants with a minor allele

frequency (MAF) < 1%. CNVs and mtDNA variants with a MAF filter < 0.01% or < 1% were evaluated. We applied a filter for variants that were listed as “pathogenic” or “likely pathogenic” in the ClinVar database without an additional MAF filter. Additionally, we explicitly filtered for variants in the *CDH2* gene without any MAF filter since our group previously showed that the autoantibodies in ACM patients cleaved and reduced N-CAD binding [60]. The Integrative Genomics Viewer (IGV) was used to inspect the filtered SNVs, indels, and CNVs visually.

Dissociation assays with hiPSC-CMs

Dissociation assay experiments were performed similarly to that of HL-1 cells with some modifications. hiPSC-CMs were seeded between days 8 and 12 of differentiation and plated with 2×10^5 cells per cm^2 density on Matrigel™-coated plates. hiPSC-CMs were grown until day 20 while changing medium every 2 days. hiPSC-CMs were treated with respective mediators for 1–24 h. Subsequently, the cells were washed with HBSS, treated with Liberase-DH (0.065 U/ml, Sigma-Aldrich, #5401054001) and Dispase II (2.5 U/ml, Sigma-Aldrich, #D4693-1G), and incubated at 37 °C until the cell monolayer detached from the wells (usually within 10–15 min). Then, the enzyme mix was carefully removed and replaced by HBSS. Mechanical stress was applied by horizontal rotation at 1250 rpm for 1 to 10 min. The number of fragments was determined utilizing a binocular stereomicroscope (Leica Microsystems, Wetzlar, Germany) and ImageJ analysis software.

Western blotting

After respective treatments, HL-1 cells, hiPSC-CMs or cardiac slices were lysed in SDS lysis buffer supplemented with protease and phosphatase inhibitors (cOmplete Protease Inhibitor Cocktail, Roche; #CO-RO and Phos-Stop, Roche, #PHOSS-RO). SDS-polyacrylamide gel electrophoresis was performed using 10–15 µg of protein for HL-1 cells or hiPSC-CMs and 40 µg for cardiac slices. Further details are provided in Supplementary materials. The membranes were incubated with antibodies of interest in 5% BSA/TBST or Roti-Block overnight at 4 °C (BSA: VWR; #422361V). Further details of primary antibody and secondary antibody concentrations are provided in Supplementary Table 2. Membranes were washed 3 times in TBST for 5 min and incubated in respective goat anti-mouse/rabbit-HRP conjugated secondary antibody at 1:20000 dilution in TBST for 1 h at room temperature. After incubation, the membranes were washed 3 x for 10 min in TBST and immunoreactive bands were detected with ECL solution Super Signal West (Thermo Scientific; #34577) after exposure in a FluorChemE system (ProteinSimple California, USA).

Immunostaining of HL-1 cells, hiPSC-CMs and murine cardiac slices

Immunostaining in HL-1 cardiomyocytes and murine cardiac slices was performed as described previously (Shoykhet, Trenz et al. [49], Shoykhet, Dervishi et al. [48] and explained further in supplementary file. Detailed information on immunostainings in hiPSC-CMs are provided in the supplementary file.

Multielectrode array (MEA) analysis in murine cardiac slices

MEA analysis in murine cardiac slices was performed as explained before (Schinner, Erber et al. [41], Schinner, Erber et al. [40] with some modifications. MEA2100-60-System equipped with 60MEA200/30iR-Ti-gr electrode chambers (both Multichannel Systems) was used for this analysis. 300-µm-thick cardiac slices were cut using Vibratome as indicated in the supplementary methods and were washed with HBSS buffer and transferred to Claycomb medium without norepinephrine, supplemented with 10% fetal bovine serum, and 10 µg/ml penicillin/streptomycin and incubated at 37 °C, 5% CO₂, for 15 min. Then cardiac slices were placed on MEA electrodes containing 500 µl of cardiac slice medium, and measurements were performed with MC_Rack software 4.6.2 (Multichannel Systems) at a sampling rate of 10 kHz with a gain of 1200. Ventricular Slices from either *Jup*^{+/+} or *Jup*^{-/-} hearts were analyzed by MEA for 5 min (Control) before the addition of 1 µM apremilast. Slices were then incubated at 37 °C and 5% CO₂ for 30 min and further measured for 5 min. SDNN was measured as the standard deviation of the beat-to-beat intervals for the last 60 s of the recordings. A minimum of 5 electrodes were analyzed and averaged for each measurement. Conduction of electrical signals (conduction velocity) between the cells was evaluated by dividing the distance between 2 electrodes (200 µm) by the mean time for propagation. Analysis was performed using heatmaps and tracings.

Langendorff heart perfusion

Adult murine hearts (from 12 to 14 weeks old mice) were used for Langendorff heart perfusion experiments using a commercially available apparatus (AD Instruments), and the results were analyzed using LabChart8 software as described previously (Schinner, Vielmuth et al. [43], Schinner, Olivares-Florez et al. [42]. Mice were sacrificed by cervical dislocation under isoflurane anesthesia and underwent subsequent preparation of the hearts in less than 5 min. Hearts were perfused in the retrograde Langendorff mode with heparin-free carbogen-gassed Krebs-Henseleit buffer supplemented with 18.8 nM norepinephrine at 37 °C with 60 mm H₂O constant pressure. ECG was recorded via needle electrodes. After recording the baseline for 20 min, 1 µM apremilast was added to

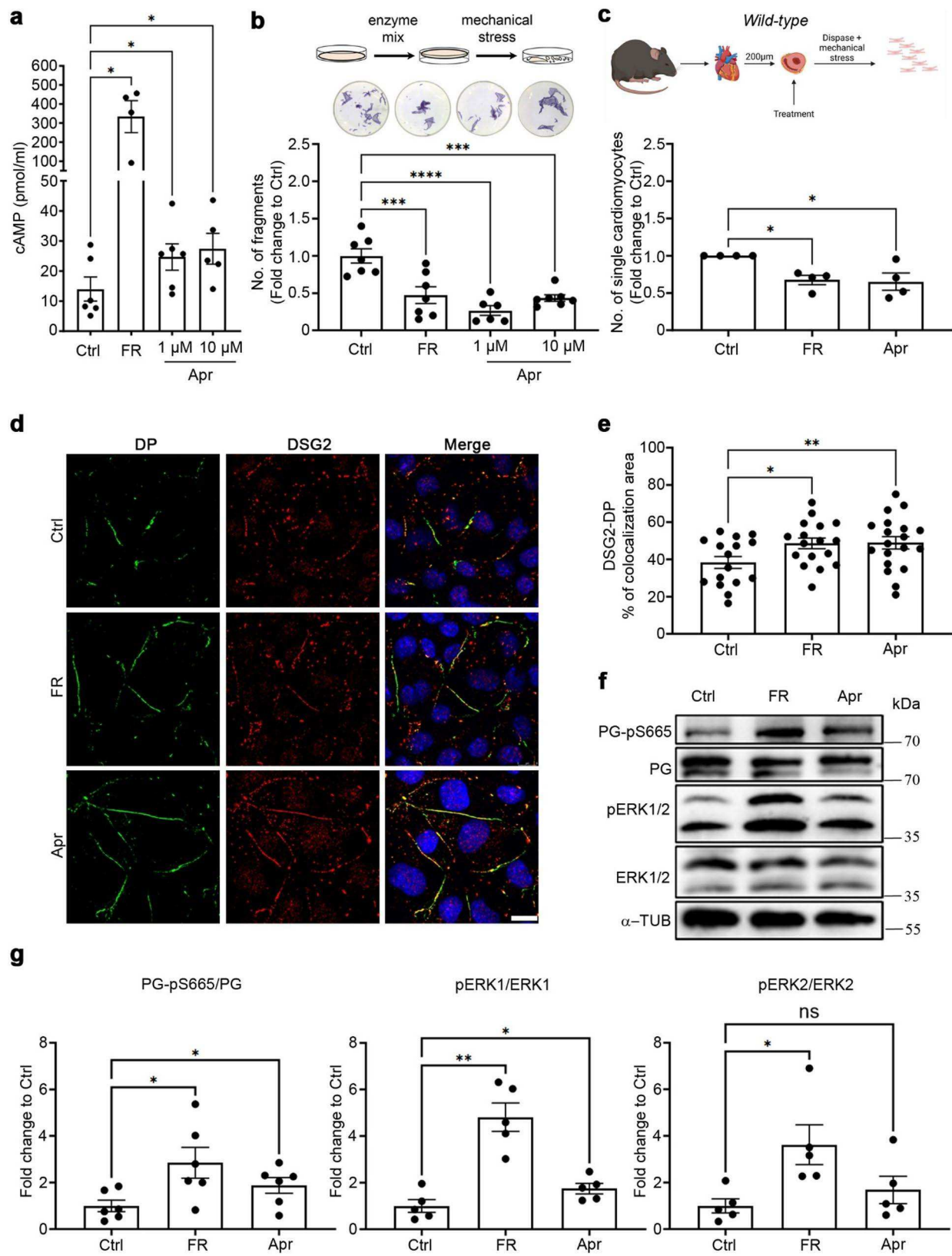


Fig. 1 (See legend on next page.)

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Fig. 1 Apremilast (Apr) enhances cardiomyocyte cohesion in HL-1 cells and wild-type murine cardiac slices. **a** HL-1 cells were treated with apremilast (Apr, 1 and 10 μ M) for 30 min at 37 °C, and cAMP was measured; FR (5 μ M/10 μ M; 1 h, 37 °C) was used as a positive control in all assays. Treatments were compared to non-treated Ctrl conditions. $N=4-6$. **b** Dissociation assays were performed in HL-1 cells treated with 1 or 10 μ M Apr for 1 h at 37 °C. $N=7$. The cartoon represents the dissociation assay method, and the panel below displays representative wells for each condition. MTT was used to show the viability of cells. **c** Dissociation assays were performed in wild-type murine cardiac slices treated with 1 μ M Apr for 1 h at 37 °C. $N=4$. The cartoon represents the dissociation assay method. **d** Immunostaining and confocal microscopy were performed for DP (green) and DSG2 (red) in HL-1 cells treated with FR (5 μ M/10 μ M) and 1 μ M Apr for 1 h at 37 °C. Scale bar: 10 μ m. **e** Quantification of DSG2-DP colocalization area. Each data point in the graph represents one confocal image performed across 3–4 experimental repeats. **f** Representative Western blots for PG-pS665, PG, pERK1/2, and ERK1/2 in HL-1 cells treated with FR (5 μ M/10 μ M) and 1 μ M Apr for 1 h at 37 °C. α -tubulin was used as a loading control. **g** Quantification of Western blots from **f**. $N=5-6$. All bar graphs in the figure represent mean $\pm$ SEM. One-way ANOVA with Holm-Šidák post-hoc analysis was performed to assess the statistical significance. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ and **** $p<0.00005$

the perfusion solution for the indicated time using a perfusion pump. Heartbeats and SDNN intervals were measured using LabChart8 software.

Statistics

Statistical analyses were performed using GraphPad Prism 8 or higher version. Two-tailed Student's t tests or 1- or 2-way ANOVA with Holm-Šidák post hoc tests were applied after outlier removal using the identify outliers (ROUT Method, with $Q=1\%$) from GraphPad Prism 10 and are explained in figure legends. Data are represented as mean $\pm$ SEM. Significance was assumed for $P<0.05$.

Results

Apremilast enhances cardiomyocyte cohesion, DSG2-DP translocation, PG phosphorylation and ERK1/2 activation in HL-1 cells

First, we measured cAMP production in HL-1 cells after treatment for 30 min at 37 °C with the combination of 5 μ M forskolin and 10 μ M rolipram (FR), as well as 1 μ M and 10 μ M apremilast. The treatment with FR served as a positive control. We observed an increase in cAMP from 14.02 ± 4.02 pmol/ml in control to 334.1 ± 83.72 , 24.68 ± 4.40 and 27.43 ± 5.11 pmol/ml after treatment with FR, 1 μ M apremilast, and 10 μ M apremilast, respectively, compared to control condition without any treatment (Fig. 1a). Further, we analyzed HL-1 cardiomyocyte cohesion utilizing dissociation assays. Treatment with FR and apremilast at both concentrations (1 μ M and 10 μ M) for 1 h at 37 °C led to a significant increase in basal cell cohesion as indicated by a lower number of monolayer fragments (Fig. 1b). Since 1 μ M apremilast corresponds to the plasma concentration of apremilast administered to humans (Sigmund, Winkler et al. [50]), we continued using 1 μ M apremilast as a final concentration in the medium across all the experiments. In addition to the HL-1 cells, we also performed dissociation assays in WT murine cardiac slice cultures and found that similar to HL-1 cells, apremilast enhanced cardiomyocyte cohesion in intact cardiac tissue (Fig. 1c).

As we have previously established that enhanced intracellular cAMP leads to enhanced DP and DSG2

translocation, PG-pS665 and ERK1/2 activation (Schinner, Vielmuth et al. [43], Shoykhet, Trenz et al. [49], Yeruva, Kempf et al. [58]), we investigated the same after treating HL-1 cells with apremilast and FR as a positive control. Immunostaining revealed that apremilast increased the translocation of DSG2 and DP to cell junctions (Fig. 1d and e). Western blot analysis showed that apremilast, similar to FR, enhanced phosphorylation of both PG at serine 665 and ERK1/2 (Fig. 1f and g).

Apremilast-enhanced cardiomyocyte cohesion requires PG

We recently established that apremilast rescues impaired keratinocyte adhesion by phosphorylating PG at serine 665 (Sigmund, Winkler et al. [50]). Therefore, we further investigated the role of PG serine 665 phosphorylation in cAMP-driven cardiomyocyte cohesion enhancement. For this, we utilized PG serine 665 phospho-deficient mice (JUPS665A)(Sigmund, Winkler et al. [50]). Hearts from these mice looked normal both morphologically and histologically (Suppl. Figure 1a). A dissociation assay performed in murine ventricular cardiac slices from JUPS665A mice revealed enhanced cardiomyocyte cohesion after apremilast (Fig. 2a), revealing that apremilast might work through different signaling mechanisms in the absence of PG S665. We further investigated whether apremilast can enhance cardiomyocyte cohesion in a murine ACM model for which we utilized cardiac-specific PG knockout ($Jup^{-/-}$) mice, which exhibited loss of cardiomyocytes leading to fibrosis (Suppl. Figure 1b) similar to that observed in ACM patients (Schinner, Vielmuth et al. [43], Yeruva, Kempf et al. [58]). Treatment of ventricular cardiac slices with 1 μ M apremilast did not enhance cardiomyocyte cohesion in $JUP^{-/-}$ mice (Fig. 2b). In contrast, in ventricular cardiac slices derived from a $Pkp2^{-/-}$ murine ACM model (Shoykhet, Dervishi et al. [48]), both apremilast (1 μ M, 1 h, 37 °C) and FR (5 μ M/10 μ M, 1 h, 37 °C) enhanced cardiomyocyte cohesion (Suppl. Figure 2), revealing that PG is necessary for apremilast mediated cardiomyocyte cohesion. We performed Western blot analysis to check for PG-pS665, which revealed that both apremilast and FR induced phosphorylation of PG at serine 665 in the wild-type JUPS665 mice. However, the PG-pS665 was absent in the

JUPS665A mice (Fig. 2c). We then asked whether apremilast would enhance DSG2 translocation to strengthen the ICD. To answer this, we performed immunostaining in ventricular cardiac slices from WT, JUPS665A and *Jup*^{-/-} mice and measured the thickness of staining for PG and DSG2 in the ICD, as described previously (Shoykhet, Dervishi et al. [48]). Apremilast increased the thickness of PG and DSG2 staining in both WT (Fig. 2d and e) and JUPS665A (Fig. 2f) mice cardiomyocyte ICDs, whereas we did not detect either PG or DSG2 in *Jup*^{-/-} mice cardiomyocyte ICDs, as observed in our previous studies (Schinner, Vielmuth et al. [43], Schinner, Olivares-Florez et al. [42], Shoykhet, Trenz et al. [49], Shoykhet, Dervishi et al. [48], and apremilast did not further enhance either PG or DSG2.

Activation of ERK1/2 is crucial in apremilast-enhanced cardiomyocyte cohesion

Furthermore, we investigated the role of ERK1/2, a serine/threonine kinase from the group of mitogen-activated protein kinases with important roles in signal transduction, in apremilast-enhanced cardiomyocyte cohesion, as we found earlier that the positive effects of cAMP to stabilize cardiomyocyte cohesion were dependent on ERK1/2 (Shoykhet, Trenz et al. [49]). Here, we utilized HL-1 cells and treated them with apremilast (1 μ M, 1 h, 37 °C) alone or in combination with U0126 (1 μ M, 30 min before apremilast treatment, 37 °C), a MEK inhibitor upstream of ERK1/2. Dissociation assays revealed that after inhibition of ERK1/2 activation by U0126, apremilast did not enhance cardiomyocyte cohesion (Fig. 3a), as observed with FR in our previous study (Shoykhet, Trenz et al. [49]). Under these conditions, PG-pS665 was not altered by apremilast (Fig. 3b and c). However, apremilast-mediated DSG2 and DP translocation to cell borders was abolished in the absence of ERK1/2 activation (Fig. 3d and e).

Isolation and characterization of hiPSCs from an ACM patient (ACM-hiPSCs)

So far, the results have established that apremilast enhanced cardiomyocyte cohesion in murine cardiomyocytes and cardiac slices. To further investigate whether apremilast will induce similar effects in human ACM patients, we established hiPSCs from a 14-year-old female ACM patient after she died of SCD (NP0151-11 F, index patient) and her mother (NP0147-4, healthy-relative). The index patient had a biventricular phenotype and confirmed ARVC in the family according to the Task Force Criteria of 2010 (Grasso, Bondavalli et al. [22]). The patient carried a heterozygous pathogenic variant in *DSP* c.2854G > T, leading to a truncation of desmoplakin protein at p.Glu952*, while the mother (NP147-4) has no mutation in *DSP* (Table 1). The premature termination

of the protein sequence at p.Glu952* affects DP interaction with desmin, whereas interaction domains for PG and PKP2 remained intact (Favre et al., 2018; Kowalczyk et al., 1997; Kowalczyk et al., 1999). Histological analysis of the post-mortem cardiac tissue of this ACM patient using H&E and Azan stainings revealed loss of cardiomyocytes, adipogenesis, and fibrosis of the cardiac tissue (Fig. 4a). Further, we performed immunostaining to analyze the proteins in the ICD. Analysis of desmosome proteins DP, DSG2, PG and the gap junction protein Cx43 in the post-mortem tissues revealed normal localization of the tested proteins at ICD except for DP, for which we could not detect any staining (Fig. 4b).

The pluripotency of this hiPSC lines was confirmed by demonstrating that these cells possess typical hiPSC colony morphology (Fig. 4c and Suppl. Figure 3a), express the selected hiPSC markers at the gene (Fig. 4d and Suppl. Figure 3b) and protein level (Fig. 4g and Suppl. Figure 3e), and have the ability to differentiate into derivatives of all three germ layers (Fig. 4h and Suppl. Figure 3f), including cardiomyocytes (Suppl. Video 1). The hiPSC-CMs exhibited cross-striations typical for cardiac muscle cells (Suppl. Figure 3g), and expressed all analyzed desmosomal proteins, adherens junction protein N-CAD, and the gap junction protein Cx43 (Fig. 4j). Using Sendai virus-specific PCR, we showed that the hiPSC lines NP0151-11 F and NP0147-4 are free of the Sendai virus reprogramming vectors (Fig. 4e and Suppl. Figure 3c), and the whole genome SNP profiling showed that these hiPSCs are genetically intact and retain the patient's original genetic background (Suppl. Figure 4). In addition, genomic DNA sequencing confirmed the presence of the pathogenic variant c.2854G > T in the *DSP* gene in this hiPSC line (Fig. 4f). Confirmation of the hiPSC line identity with the donor tissue was confirmed using Short Tandem Repeat (STR) genotyping using 14 markers, which revealed similar genetic relationship between NP0147-4 (mother) and NP0151-11 F (daughter) hiPSC-lines (Suppl. Figure 3h). In addition, DNA was isolated from fibroblasts of the ACM index-patient and from PBMCs of the healthy-relative (mother). Subsequently, the trio whole exome sequencing (WES) confirmed the pathogenic variant c.2854G > T in *DSP* (NM_001008844.3) in the index patient. A paternal inherited heterozygous variant of unknown significance was additionally identified in Laminin Subunit Alpha 4 (*LAMA4*). Moreover, two polymorphisms could be detected in *CDH2* and *CCR5* (Table 1). Western blotting revealed that ACM-hiPSC-CMs express lower levels of DP isoforms 1 and 2 than the healthy-relative-hiPSC-CMs. However, this difference did not reach statistical significance (Fig. 4i). Immunostaining in ACM-hiPSC-CMs appeared normal for desmosomal proteins DSG2, PKP2, PG, along with adherens junction protein N-CAD and gap junction protein Cx43.

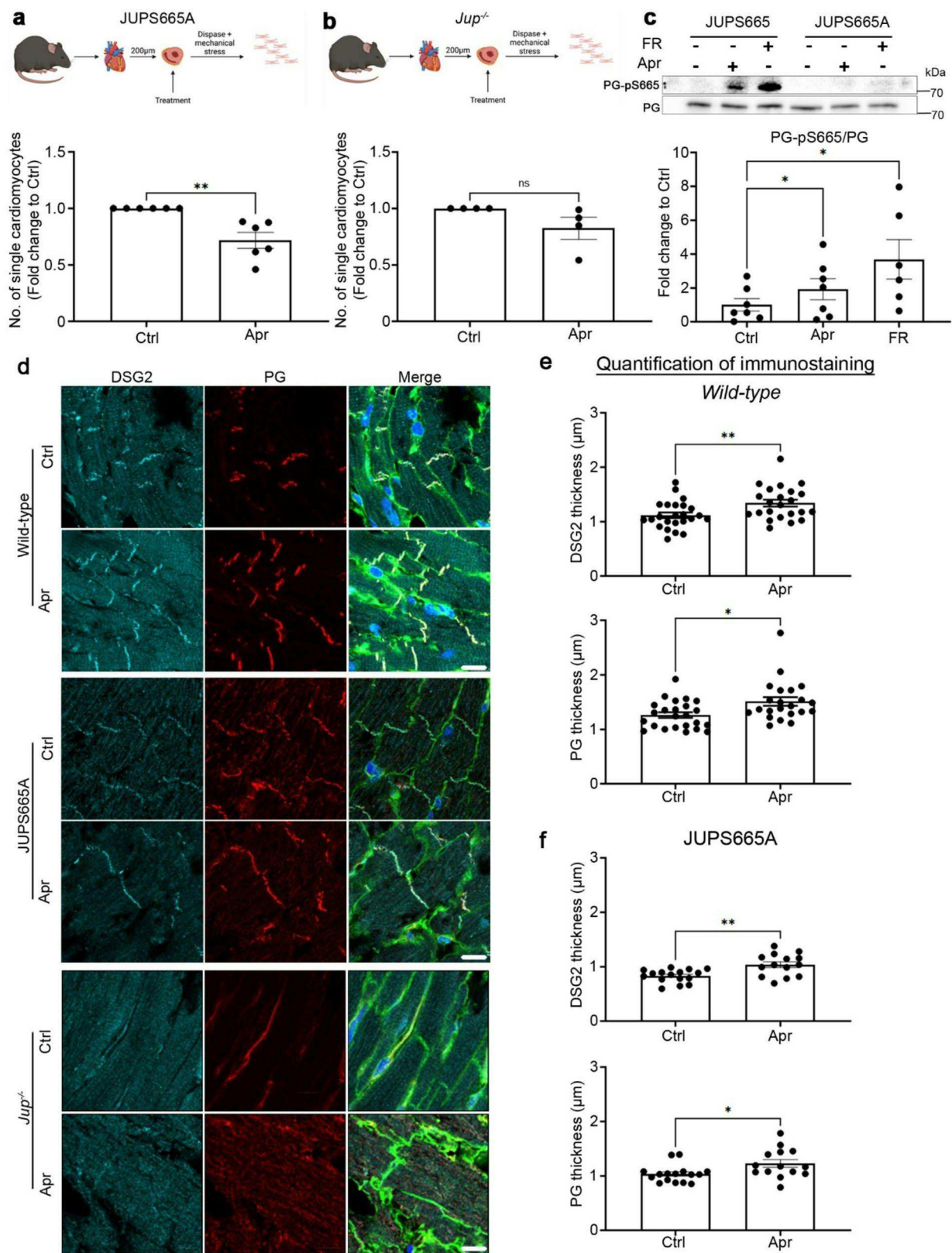


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Fig. 2 Apremilast requires plakoglobin to enhance cardiomyocyte cohesion. **a** Dissociation assays were performed with cardiac slices of PG-S665 phosphorylation deficient mice (JUPS665A), $N=5-6$, and **b** cardiomyocyte-specific deletion of PG ($Jup^{-/-}$) mice treated with apremilast (Apr, 1 μ M) for 1 h at 37 °C, $N=4$. The cartoons represent the dissociation assay method. **c** Representative Western blots for PG-pS665 and PG, in cardiac slices from JUPS665 (WT) and Jup S665A mice treated with Apr (1 μ M) and FR (5 μ M/10 μ M) for 1 h at 37 °C. The quantification of Western blots is displayed in the panel below. $N=6-7$. **d** Immunostaining and confocal microscopy were performed for DSG2 (Cyan), PG (red) and WGA (green, shown in the merge images) in cardiac slices from wild-type, JUPS665A and $Jup^{-/-}$ treated with Apr (1 μ M) for 1 h at 37 °C. Scale bar 10 μ m. **e** The thickness of DSG2 and PG staining in the ICD was measured and represented in μ m. Each data point in the graph represents the mean of multiple ICDs in one confocal image and measured across cardiac slices from 4–5 mice for each condition. Adjacent slices from a single mouse heart were used as a control for the respective treatments. All bar graphs in the figure represent mean $\pm$ SEM. Paired student t-test (**b**, **e**, and **f**) or One-way ANOVA with Holm-Sidak post-hoc analysis (**a** and **c**) was performed to determine the statistical significance. * $p<0.05$ and ** $p<0.005$

In contrast, DP staining was very faint and discontinuous along the ICD compared to DSG2 (Fig. 4j). In addition all the iPSC-CMs used in this study exhibited cardiac sarcomere marker F-actin (Suppl. Figure 5).

Apremilast alleviates loss of cardiomyocyte cohesion in ACM-hiPSC-CMs

As ACM is a disease of the desmosome and desmosome formation is essential for mechanically strengthening cardiac tissue by cardiomyocyte cohesion, we questioned whether cardiomyocyte cohesion is altered in ACM-hiPSC-CMs. We performed dispase-based dissociation assays to answer this question and compared the cardiomyocyte cohesion of healthy non-relative- and healthy relative-hiPSC-CMs with ACM-hiPSC-CMs. Upon applying similar mechanical forces, ACM-hiPSC-CMs showed an increased number of fragments compared to the healthy non-relative and healthy relative-derived cardiomyocytes. This loss of cohesion was enhanced by increasing the mechanical force applied for 5 min (Fig. 5a and Suppl. Figure 6a).

After establishing that ACM-hiPSC-CMs exhibited loss of cell cohesion, we investigated whether enhancing intracellular cAMP would stabilize cardiomyocyte cohesion, as previously established in murine cell lines and cardiac slices (Schinner, Vielmuth et al. [43], Shoykhet, Trenz et al. [49], Yeruva, Kempf et al. [58], Yeruva, Korber et al. [59]). Therefore, we treated ACM-hiPSC-CMs with FR (5 μ M/10 μ M) and apremilast (1 μ M) for 1 h and 24 h at 37 °C. Dissociation assays revealed that both FR (as a positive control) and apremilast increased cardiomyocyte cohesion compared to untreated control within 1 h to levels comparable to healthy hiPSC-CMs, which was stable even after 24 h (Fig. 5b compared to 5a, and Suppl. Figure 6b). In contrast, treatment of healthy relative-hiPSC-CMs (with enhanced cohesion compared to ACM-hiPSC-CMs) revealed that apremilast enhances cardiomyocyte cohesion only after 24 h (Suppl. Figure 6c and d). Western blot analysis of ACM-hiPSC-CMs lysates obtained after treatment with either FR (5 μ M/10 μ M, 37 °C, 1 h and 24 h) or apremilast (1 μ M, 37 °C, 1 h and 24 h) revealed increased PG-pS665 with varying intensities but no changes in ERK1/2 phosphorylation (Fig. 5c and d). FR stimulation led to a more robust PG phosphorylation

than apremilast stimulation. Desmosomal proteins DP, DSG2, PKP2 and the adherens junction protein N-CAD were unaltered (Fig. 5c and Suppl. Figure 7). Immunostaining revealed that upon apremilast and FR treatment for 1 h and 24 h both PG-pS665 and DSG2 were continuously localized at the cell junctions (Fig. 5e and f).

Apremilast reduces arrhythmia in ex vivo murine ACM model

ACM patients suffer from arrhythmias, which can cause SCD. Therefore, we further investigated whether apremilast, in addition to the rescue of cardiomyocyte cohesion, can reduce arrhythmias. We measured the standard deviation of R-R intervals (SDNN) for heart rate variability as a measure for arrhythmias. First, we performed MEA analysis using ex vivo murine cardiac slices from $Jup^{+/+}$ and $Jup^{-/-}$ mice. MEA analysis revealed a strong increase in SDNN of $Jup^{-/-}$ mice cardiac slices compared to $Jup^{+/+}$, which was not statistically significant due to the high variability of arrhythmia in $Jup^{-/-}$ tissue. Treatment with apremilast significantly decreased the SDNN in $Jup^{-/-}$ mice cardiac slices and increased conduction velocity (Fig. 6a and Suppl. Figure 8). To investigate the effects of apremilast in intact hearts, we performed Langendorff perfusion experiments from $Jup^{-/-}$ and $Jup^{+/+}$ mice. Apremilast treatment did not alter heart rate in both $Jup^{+/+}$ and $Jup^{-/-}$ mice (Fig. 6b and c), but ECG analysis revealed a high SDNN variability in $Jup^{-/-}$ hearts, which was reduced significantly after 10 min of apremilast treatment (Fig. 6d and e), which confirmed that apremilast effectively reduces arrhythmias.

Discussion

Treatment options for ACM patients exist mainly to relieve symptoms such as arrhythmia, heart failure and to prevent SCD. In most cases, β -blockers are used to control arrhythmia or ventricular tachycardia (Cappelletto, Gregorio et al. [6], Gasperetti, Targetti et al. [21]). However, in some patients, beta-blockers are either ineffective (Wichter, Borggreffe et al. [57], Marcus, Glidden et al. [31] or their use is limited due to side effects (Ermakov, Gerstenfeld et al. [17], Rolland, Badenco et al. [38]), which then requires invasive arrhythmic procedures. Therefore, the current treatment strategies urge

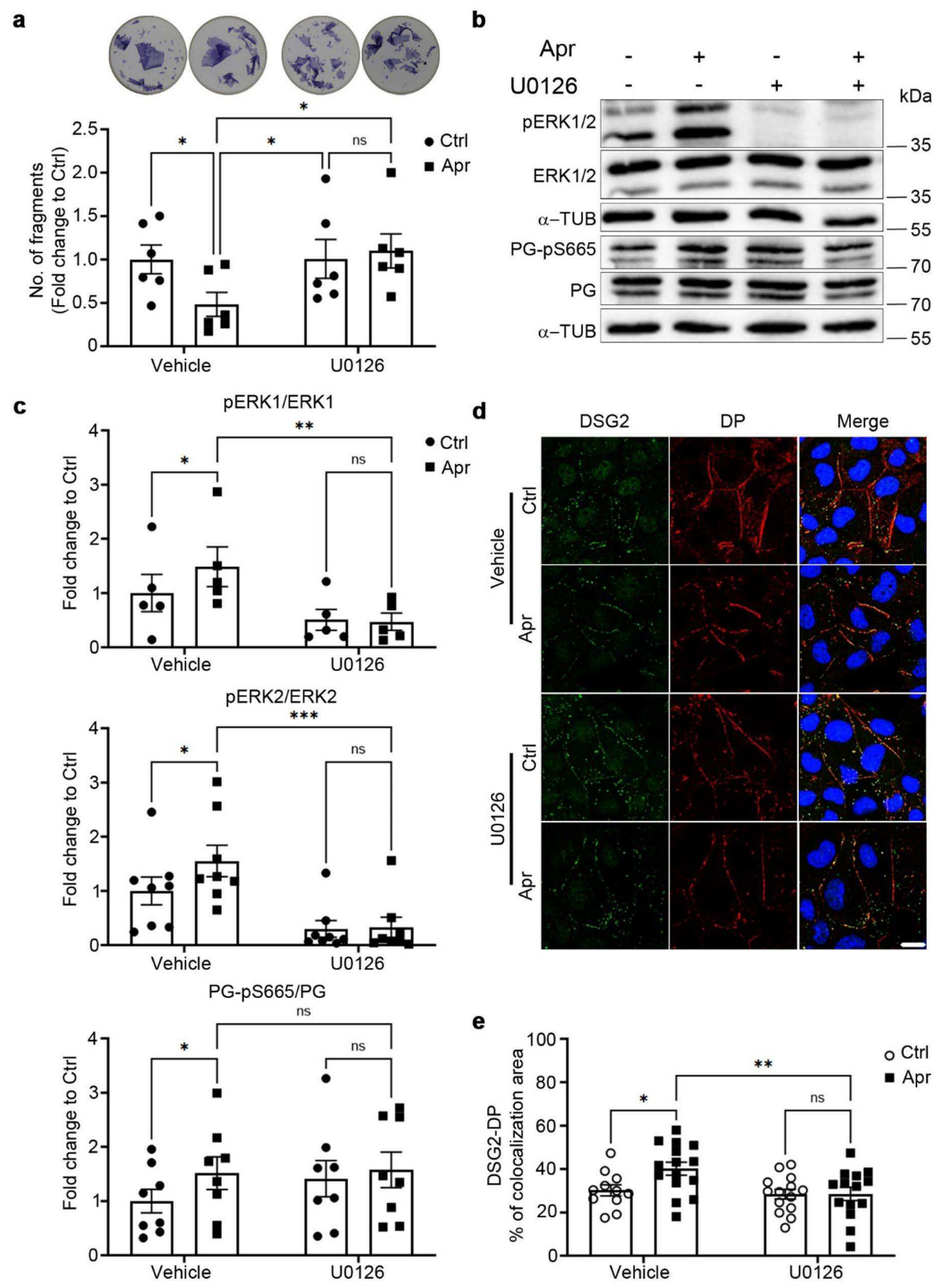


Fig. 3 (See legend on next page.)

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Fig. 3 Apremilast-enhanced cardiomyocyte cohesion depends on ERK1/2 phosphorylation. **a** Dissociation assays in HL-1 cells treated with Apr (1 μ M) with or without the MEK inhibitor U0126 (1 μ M) for 1 h at 37 °C. U0126 was added 30 min prior to Apr. $N=7$. The panel above displays representative wells for each condition **b** Representative Western blots for PG-pS665, PG, pERK1/2, and ERK1/2 in HL-1 cells treated with Apr (1 μ M) with or without U0126 (1 μ M) for 1 h at 37 °C. α -tubulin was used as a loading control. **c** Quantification of Western blots from **b**. $N=5-8$. **d** Immunostaining and confocal microscopy were performed for DP (green) and DSG2 (red) in HL-1 cells treated with Apr (1 μ M) with or without U0126 (1 μ M) for 1 h at 37 °C. Scale bar 10 μ m. **e** Quantification of DS2-DP colocalization area. Each data point in the graph represents one confocal image performed across 3–4 experimental repeats. α -tubulin (α -TUB) was used as a loading control. All bar graphs in the figure represent mean $\pm$ SEM. Two-way ANOVA with Holm-Šidák post-hoc analysis was performed to determine the statistical significance. * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$

new therapeutic options for ACM patients. In contrast to the β -blockers used in ACM patients, PDE inhibitors, which act by enhancing the ionotropic state of the heart, are very effective in treating heart failure [47], Amsalem, Kasparian et al. [1]). Moreover, PDE inhibitors were also shown to be effective in treating inflammation [24], Imam, Al-Harbi et al. [26]). In line with this, the PDE-4 inhibitor apremilast was applied effectively in patients suffering from the desmosomal autoimmune skin disease pemphigus vulgaris (Meier, Holstein et al. [32], Delvaux, Bohelay et al. [15]). Further, a recent study showed that apremilast mitigated the loss of keratinocyte cohesion induced by pemphigus autoantibodies (Sigmund, Winkler et al. [50]). In addition, recent evidence suggests that stabilization of cell adhesion could be beneficial in treating desmosome-related diseases (Schinner, Xu et al. [44], Waschke, Amagai et al. [56]). Based on the above observations, we hypothesized that apremilast can enhance the integrity and mechanical stability of desmosomes at cell junctions, enhance cardiomyocyte cohesion, and reduce arrhythmia in a murine ACM model.

We have previously reported that increasing intracellular cAMP levels and modulating other signaling mechanisms strengthen cardiomyocyte cohesion, primarily in murine models such as the HL-1 atrial cell line and murine cardiac slice cultures (Schinner, Vielmuth et al. [43], Schinner, Olivares-Florez et al. [42], Shoykhet, Trenz et al. [49], Yeruva, Kempf et al. [58], Yeruva, Korber et al. [59]. One significant challenge hindering progress in the study of ACM is the limited access to human tissues and suitable human cells for research. While animal models have provided valuable insights, an alternative approach involves hiPSCs, which can be differentiated into CMs, offering a robust in vitro model for studying ACM (Chelko, Asimaki et al. [8], Padron-Barthe, Villalba-Orero et al. [34], Kim, Trembley et al. [28], Maione, Meraviglia et al. [29], Seibert, Rubio et al. [45], Selgrade, Fullenkamp et al. [46]). After establishing ACM-hiPSCs and their differentiation into CMs, a dissociation assay was implemented to measure cardiomyocyte cohesion, comparing it to healthy non-relative and healthy mother hiPSC-CMs. For the first time, we demonstrate here that ACM-hiPSC-CMs exhibit impaired cohesion. These results were reinforced by another study using iPSC-CMs with a DSP truncated pathogenic variant, which also observed reduced cardiomyocyte cohesion under

contractile stress (Smith, Jin et al. [51]). The observations that cardiomyocyte cohesion is impaired in human iPSC-derived cardiomyocytes carrying a pathogenic DSP variant and that adhesion can be restored by pharmacological interventions similar to intact tissue cultures from a murine model of ACM with *Pkp2* deletion, indicates that loss of cell adhesion is a primary event in ACM pathogenesis similar to other desmosome diseases such as pemphigus [61], Spindler, Gerull et al. [53]) and that restoration of cell adhesion may be a primary goal of pharmacological therapy in ACM, as was recently observed in another study (Smith, Jin et al. [51]). Thus, increasing cAMP by apremilast or inhibiting EGFR by erlotinib might be promising new treatment options for ACM patients since they were proven to be effective in enhancing desmosome integrity and, thereby, cell adhesion in different experimental models of ACM and pemphigus, as shown in this study and previous reports (Shoykhet, Dervishi et al. [48], Sigmund, Winkler et al. [50], Egu, Schmitt et al. [16]).

Apremilast leads to PG-pS665, translocation of DSG2 and enhances cardiomyocyte cohesion in ACM-iPSC-CMs, mirroring observations in murine cell lines and cardiac slices and is in agreement with the effects of FR as reported before (Schinner, Vielmuth et al. [43], Shoykhet, Trenz et al. [49], Yeruva, Kempf et al. [58], Yeruva, Korber et al. [59, 61]. These data suggest that the mechanism of positive adhesiotropy is present in human cardiomyocytes, as well. However, since the increase of cAMP induced by apremilast was ten times less than that observed after FR treatment, whereas the effect on cell adhesion was comparable, it can be concluded that higher cAMP levels, which may cause side effects, are not required to strengthen cardiomyocyte adhesion. Therefore, we utilized 1 μ M of apremilast in all our experiments, as was done in our previous study as well (Sigmund, Winkler et al. [50]).

Interestingly, apremilast enhanced cardiomyocyte cohesion in the absence of PG serine phosphorylation in *Jup*S665A mice. Further, apremilast enhanced DSG2 and PG at the ICD, suggesting that apremilast can enhance cardiomyocyte cohesion by compensatory effects in a model where PG phosphorylation is genetically blunted. However, in the absence of PG in heart slice cultures from *Jup*^{-/-} mice, the effects of apremilast were abolished

Table 1 Clinical and genetic findings in the ACM patient

	155,469 (Index)	151,514 (Mother)
Clinical findings (TFC)	2 major criteria: pathogenic variant in <i>DSP</i> , fibrous replacement of the myocardium by autopsy (died from SCD at the age of 14 years)	Not affected
Main genetic finding		
Gene	<i>DSP</i>	n.a.
Reference sequence	NM_001008844.3	
Variant	c.2854G>T, p.(Glu952Ter)	
Status	heterozygous	
MAF	0	
Variant interpretation	pathogenic	
Possible genetic disease modifier		
Gene	<i>LAMA</i>	n.a.
Reference sequence	NM_001105206.3	
Variant	c.2917 C>G, p.(Leu973Val)	
Status	heterozygous	
MAF (gnomAD)	0.000003982	
Gene	<i>CCR5</i>	<i>CCR5</i>
Reference sequence	NM_000579.3	NM_000579.3
Variant	c.-301 + 246 A>G	c.-301 + 246 A>G
Status	homozygous	homozygous
MAF (gnomAD)	0.4800	0.4800
Gene	<i>CDH2</i>	n.a.
Reference sequence	NM_001308176.2	
Variant	c.62T>A, p.(Leu21Ter)	
Status	heterozygous	
MAF	0.2007	
Comment	The variant is not present in the MANE transcript	

MAF minor allele frequency, SCD sudden cardiac death, TFC task force criteria,

altogether, revealing that PG is strictly necessary for apremilast-mediated effects on cardiomyocyte cohesion.

Apremilast, similar to FR in our previous study (Shoykhet, Trenz et al. [49]), caused activation of ERK1 and required ERK1/2 for its positive adhesiotropic effect in HL-1 cells. In contrast, neither apremilast nor FR activated ERK1/2 in ACM-hiPSC-CMs, suggesting that the underlying mechanisms enhancing desmosomal adhesion may be at least in part different in human and murine cardiomyocytes. This should be considered when studying ACM pathogenesis in different experimental models, and it would suggest that human iPSC-CMs have advantages over murine models, in this context.

Most ACM patients suffer from arrhythmias, and in some, arrhythmias can lead to SCD [3], Bhonsale, Groeneweg et al. [4], Austin, Trembley et al. [2]). Therefore, we next investigated whether apremilast could stabilize the rhythmicity of spontaneous beating in different cardiac models. MEA and Langendorff experiments

revealed a significant reduction in arrhythmia of the PG-deficient ACM model (*Jup*^{-/-} mice) after apremilast treatment. Apremilast was effective in enhancing cardiomyocyte cohesion in all cell types, as well as in *Jup*^{+/-} mice, but not in *Jup*^{-/-} mice. Nevertheless, apremilast restored rhythmicity in *Jup*^{-/-} mice, which could be due to effects of PDE4 inhibition on other functional aspects of cardiomyocytes, such as action potential and contractility, which are not Pg-dependent. Indeed, previous studies have clearly established that PDE4 inhibitors have positive chronotropic and ionotropic effects [27], Huang, Xie et al. [25]). So far, there is no evidence for apremilast in the heart regarding its effect on the functional aspects of heart. Future studies focusing on these aspects are warranted for exploring apremilast potential in treating ACM.

In summary, we demonstrate here for the first time that cell adhesion was impaired in cardiomyocytes derived from an ACM patient and that apremilast effectively restored desmosome-mediated cardiomyocyte cohesion and reduced arrhythmia in ACM model. Encouragingly, apremilast was proven to inhibit doxorubicin-induced apoptosis and inflammation in the heart (Imam, Al-Harbi et al. [26]) and was also used as an anti-inflammatory drug in autoimmune and inflammation-related diseases such as psoriasis, arthritis and inflammatory bowel disease (Schett, Sloan et al. [39], Danese, Neurath et al. [13], Fan, Wang et al. [18]). Recently, autoimmunity and inflammation were observed as prominent features in ACM (Chatterjee, Fatah et al. [7], Chelko, Asimaki et al. [8], Caforio, Re et al. [5], Yeruva, Stangner et al. [60] and therapeutic modulation of inflammation in ACM hearts revealed an effective new mechanism-based therapy for ACM (Chelko, Asimaki et al. [8]). Further, clinical data from a large patient population revealed no adverse risks of major cardiac events in psoriatic arthritis patients (Persson, Hagberg et al. [36]). Overall, the studies mentioned above, combined with our data in the present study, support apremilast as a promising new therapeutic option for ACM.

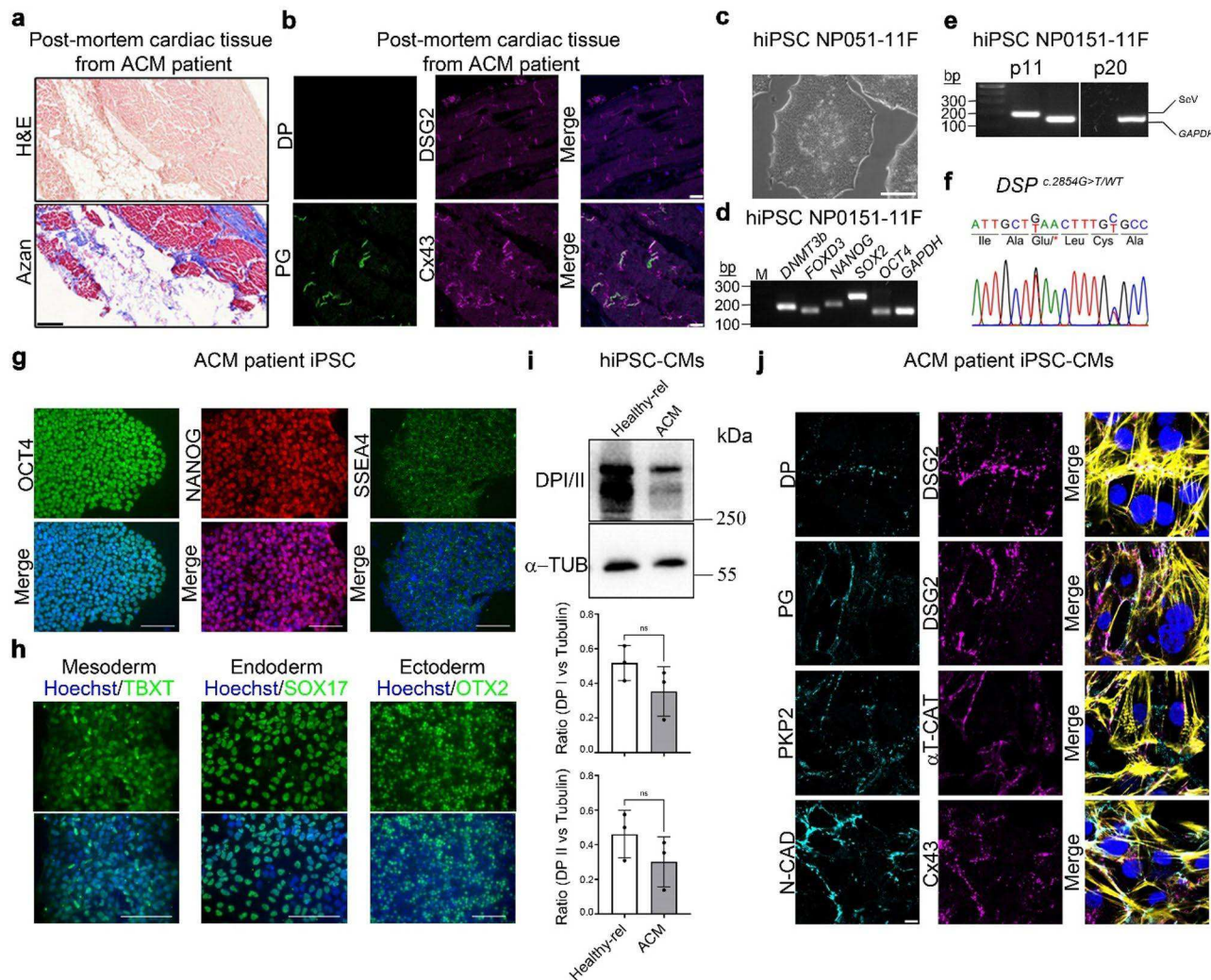


Fig. 4 Characterization of hiPSC line NP0151-11 F and hiPSC-CMs from ACM patient carrying the heterozygous mutation c.2854G>T in the *DSP* gene. **a** Post-mortem cardiac tissue (left ventricle) of ACM patient NP0151 showing large areas of cardiomyocyte loss, adipogenesis and fibrosis. Scale bar 1 mm. **b** Immunostaining for desmosomal proteins (DP, PG and DSG2) and gap junction protein Cx43 in the post-mortem cardiac tissue of ACM index patient revealed the absence of DSP at the ICD. Scale bar 10 μ m. **c** Colony morphology of NP0151-11 F ($DSP^{p.Glu952*/WT}$) hiPSC line. Scale bar 100 μ m. **d** Expression of indicated pluripotency-associated transcripts in ACM-hiPSC line NP0151-11 F as determined by semiquantitative RT-PCR. *GAPDH* was amplified as a positive control. **e** Confirmation of the absence of the reprogramming Sendai virus vector in NP0151-11 F hiPSC master banks (passage 20) using RT-PCR. Early passage NP0151-11 hiPSCs (passage 11), which still had the reprogramming vector, were used as a positive control for the SeV presence. **f** DNA sequencing revealed the presence of the *DSP* mutation c.2854G>T in a patient-specific hiPSC line NP0151-11 F. In addition, silent heterozygous variant c.2862 C>T in *DSP* was detected in NP0151-11 F hiPSCs. **g** Immunocytochemical detection of pluripotency markers OCT4, NANOG and SSEA4 in ACM-hiPSC line NP0151-11 F. Scale bars 100 μ m. **h** Tri-lineage differentiation potential of the ACM-hiPSC line NP0151-11 F as shown by immunocytochemical staining for TBXT (mesoderm), SOX17 (endoderm), and OTX2 (ectoderm). Scale bars 100 μ m. **i** Representative Western blots for DP I and II expression and its quantification normalized to α -tubulin in cardiomyocytes derived from healthy hiPSC line NP147-4 and ACM patient-specific hiPSC line NP0151-11 F. An unpaired student *t*-test was used to determine the statistical significance. **j** Immunostaining for α -T-catenin (α -T-cat), DP, DSG2, PG, PKP2, N-CAD and Cx43 in cardiomyocytes derived from the ACM-hiPSC-line NP0151-11 F. Actin was stained with Phalloidin Alexa Fluor 488 to show the sarcomere regions of cardiomyocytes and shown in yellow in the merged image. Scale bar 10 μ m. Nuclei in panels **g** and **h** were counterstained with Hoechst 33342 and in panel **b** and **j** with DAPI.

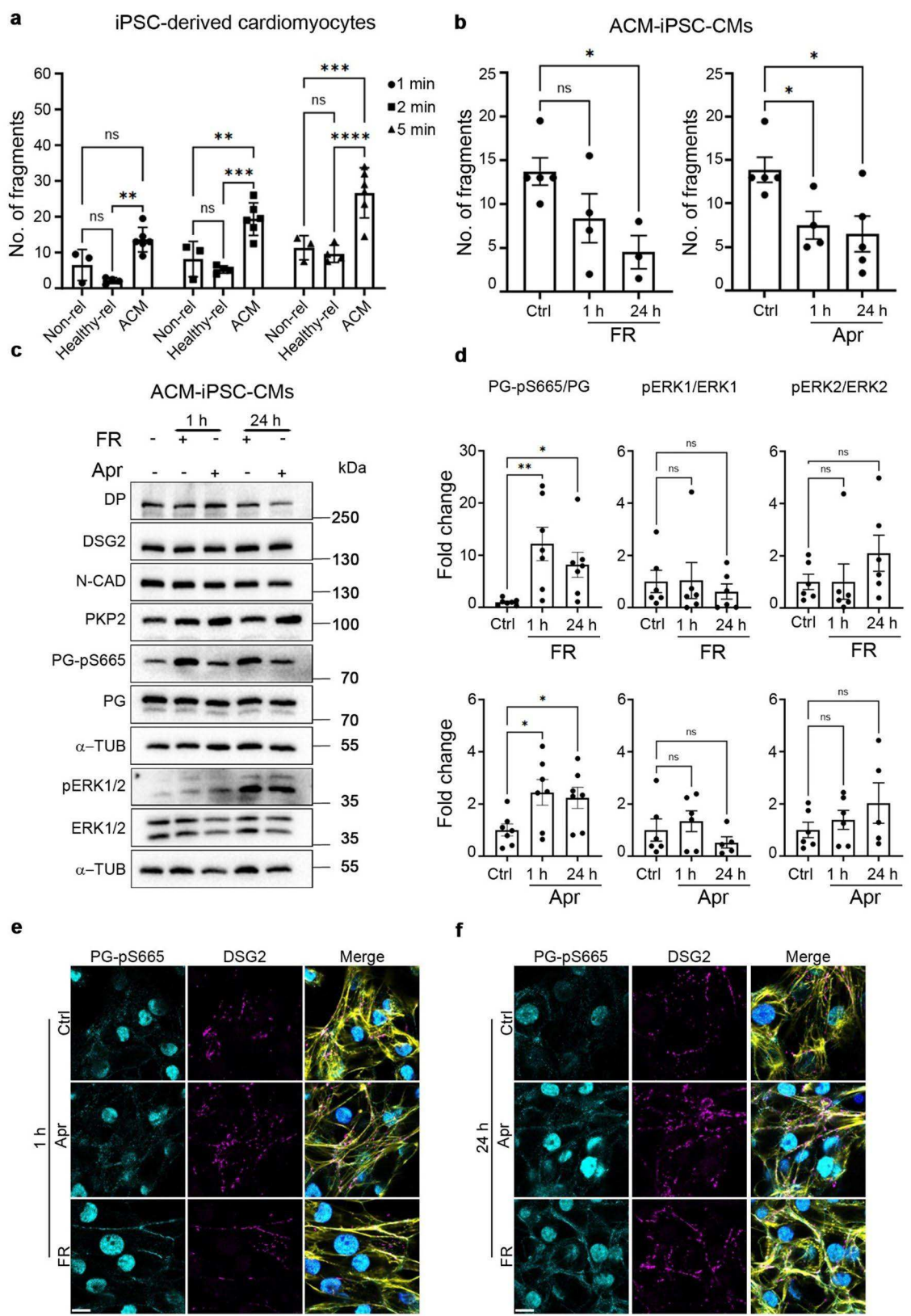


Fig. 5 (See legend on next page.)

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Fig. 5 Apremilast enhances cohesion of ACM-hiPSC-CMs. **a** Dissociation assays were performed in non-relative healthy (Non-rel), healthy mother (Healthy-rel) hiPSC- and ACM-hiPSC-derived cardiomyocytes for 1, 2, and 5 min to compare the cohesive strengths between healthy and ACM patient-derived cardiomyocytes. $N=3-6$. **b** Dissociation assays were performed in ACM-hiPSC-CMs treated with FR (5 μ M/10 μ M) and Apr (1 μ M) for 1 and 24 h at 37 °C. $N=4-5$. **c** Representative Western blots for desmosomal proteins DP, DSG2, PKP2, PG-pS665, PG, the adherens junction protein N-CAD, and the signaling molecule pERK1/2 and ERK1/2 in ACM-hiPSC-CMs treated with FR (5 μ M/10 μ M) and Apr (1 μ M) for 1 and 24 h at 37 °C. α -tubulin was used as a loading control. **d** Quantification of Western blots from **c**. $N=5-7$. **e** and **f** Immunostaining and representative confocal microscopy images of PG-pS665 (cyan) and DSG2 (magenta) in ACM-hiPSC-CMs treated with FR (5 μ M/10 μ M) and Apr (1 μ M) for 1 and 24 h at 37 °C. Merge images show actin staining using Phalloidin Alexa Fluor 488 (yellow) and nuclei staining using DAPI (blue). All bar graphs in the figure represent mean $\pm$ SEM. Scale bar 10 μ m. Two-way ANOVA (**a**) and one-way ANOVA (**b** and **d**) with Holm-Sidak post-hoc analysis was performed to calculate the statistical significance. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ and **** $p<0.00005$

Abbreviations

ACM	Arrhythmogenic cardiomyopathy
AJ	Adherence junctions
Apr	Apremilast
cAMP	Cyclic-adenosine monophosphate
Ctrl	Control
DES	Desmin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSC2	Desmocollin 2
DSG2	Desmoglein 2
DSP	Desmoplakin
ERK1/2	Extracellular-signal-regulated kinase 1/2
F	Forskolin
FBS	Fetal bovine serum

FLNC	Filamin-C
FR	Forskolin/rolipram
GJ	Gap junctions
hDF	Human dermal fibroblasts
hiPSC	Human induced pluripotent stem cells
hiPSC-CM	hiPSC-derived cardiomyocytes
HL-1	Murine atrial cardiac myocyte cell line
hVFs	Human vein fibroblasts
ICD	Intercalated disc
JUP	Junctional plakoglobin
MAF	Minor allele frequency
MEA	Multi-electrode/microelectrode array
Nav1.5	Sodium voltage-gated channel subunit 5 (SCN5A)
N-CAD	N-cadherin
NE	Norepinephrine

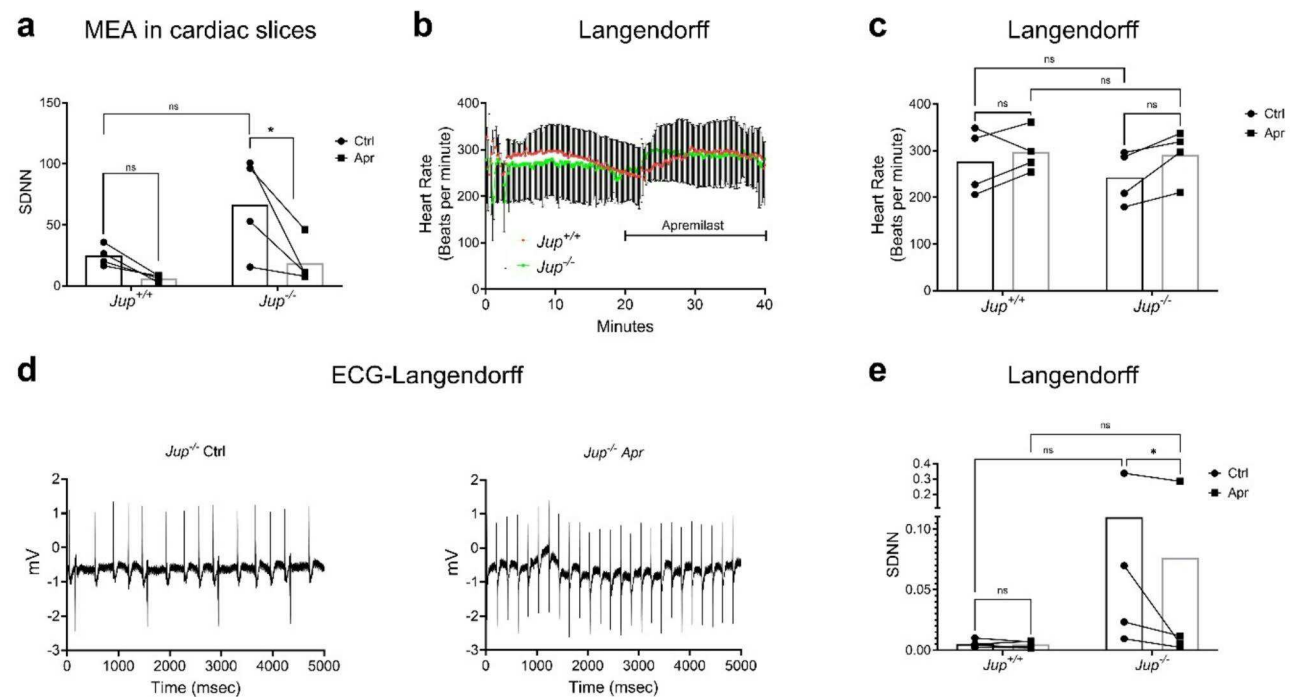


Fig. 6 Apremilast reduces arrhythmia in a murine ACM model. Heart rate variability was measured as the standard deviation of R-R intervals (SDNN), which was used to measure arrhythmia. For this purpose, **a** MEA was performed in the ventricular cardiac slices from $Jup^{+/+}$ and $Jup^{-/-}$ mice with or without Apr (1 μ M). Each data point represents one cardiac slice measured from a heart. Graph shows before-after effects of Apr, which is depicted with a connecting line between 2 data points measured on the same cardiac slice. $N=3$ mice per genotype. **b** Langendorff heart perfusion experiments were performed in isolated hearts from $Jup^{+/+}$ and $Jup^{-/-}$ mice. Hearts were perfused for 20 min and then treated with Apr (1 μ M) for 20 min. The graph shows the average heart rate measured across the whole experimental time with its corresponding SEM. $N=4$ mice per genotype. **c** The graph shows the average heart rate measured in **b** for 1 min before adding Apr (Ctrl) and after 10 min of Apr treatment. **d** Representative ECG curves (for 5 s) obtained from the Langendorff experiments in $Jup^{+/+}$ mice before (upper panel) and after 10 min of Apr (lower panel). $N=4$. **e** SDNN changes measured using the Langendorff perfusion experiments in the hearts of $Jup^{+/+}$ and $Jup^{-/-}$ mice. Bar graphs in **a**, **c**, and **e** represent before-after plots, and graphs in **b** represent mean $\pm$ SEM. Two-way ANOVA with Holm-Sidak post-hoc analysis was performed to find the statistical significance in **a**, **c**, **e** and **f**. * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$

NEAA	Non-essential amino acids
PBMCs	Peripheral blood mononuclear cells
PDE-4	Phosphodiesterase-4
PFA	Paraformaldehyde
PG	Plakoglobin
PKA	Protein kinase A
PKP2	Plakophilin 2
PLN	Phospholamban
R	Rolipram
SCD	Sudden cardiac death
SDNN	Standard deviation of R-R intervals
SNP	Single nucleotide polymorphism
TFC	Task force criteria
TMEM43	Transmembrane protein 43
WES	Whole exome sequencing
WT	Wild-type
α -TUB	α -Tubulin
β -ME	β -Mercaptoethanol

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Author contributions

KS, OD, JK, CW, SP, MS, SOF, SM, JO, NLCSW and SY acquired the data. KS, OD, TS and SY analyzed the data. EG handled the biosample, sequencing platform, and bioinformatics regarding the trio WES. DSW analyzed the trio WES data. RB provided the materials for hiPSCs and cardiac tissue. TW and BG provided hearts from *Pkp2*^{-/-} mice. hiPSCs were generated, and protocols to differentiate hiPSCs into cardiomyocytes were established in the lab of TS. SY drafted the manuscript. SY, TS and JW made critical revisions to the manuscript for important intellectual content and SY and JW designed the research. All authors proofread the manuscript.

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Data availability

The data corresponding to this manuscript can be obtained from the corresponding authors upon considerable request.

Declarations

Ethics approval and consent to participate

The Ethics Committee of the Medical Faculty of the University of Cologne (authorization number 14–306) approved patient tissue collection and use for generating hiPSCs. *Title of the project:* European Bank of pluripotent Stem Cells (EBISC). *Date of approval:* 28.10.2015. Further hiPSC study at the LMU was approved by the Ethics Committee approval at LMU (23–0203). *Title of the project:* Stabilization of cell adhesion in cardiomyocytes by adrenergic

stimulation. *Date of approval:* 13.03.2023. hiPSCs were isolated from individuals/relatives who gave written informed consent for participation in the study and/or the use of samples. Animal handling was done under the guidelines from Directive 2010/63/EU of the European Parliament and approved by the regional government of Upper Bavaria (Gz. ROB-55.2-2532. Vet_02-19-172, for *Jup* mice). *Title of the project:* Framework application for breeding transgenic/genetically modified mouse lines. The genetic modifications affect cell adhesion genes. *Date of approval:* 03.06.2020. The local ethics committee of the government of Lower Franconia (RUF-55.2.2-2532-2-663 for *Pkp2* mice). *Title of the project:* Role of a tandem peptide (TP) and the GSK-3 β inhibitor SB2 in arrhythmogenic cardiomyopathy (ACM) in a genetically modified mouse model. *Date of approval:* 05.04.2018.

Competing interests

The authors declare no competing interests.

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