TTN missense variants as a cause of familial DCM.

Short Title: Missense TTN variants can cause DCM

Fernando Domínguez, MD, PhD[1,2,3]; fdominguezrodriguez@gmail.com

Laura Lalaguna, MSc[2]; laura.lalaguna@cnic.es

Inés Martínez-Martín, MSc[2]; ines.martinez@cnic.es

Jesús Piqueras-Flores, MD, PhD[4,5]; jesus.piqueras.flores@gmail.com

Torsten Bloch Rasmussen MD, PhD[6]; torsten.bloch.rasmussen@gmail.com

Esther Zorio, MD, PhD[3,7]; zorio_est@gva.es

Giovanna Giovinazzo PhD[2]; ggiovinazzo@cnic.es

Belen Prados, PhD[2]; bprados@cnic.es

Juan Pablo Ochoa, MD, PhD[2]; jpo.cardio@gmail.com

Belen Bornstein, MD, PhD[1]; belen.bornstein@salud.madrid.org

Esther González-López, MD, PhD[1,3]; esthgonzalez@hotmail.com

Diana Velázquez-Carreras, MSc[2]; dianacarmen.velazquez@cnic.es

Maria Rosaria Pricolo, PhD[2]; mariarosaria.pricolo@cnic.es

Francisco Gutiérrez-Agüera, PhD[2]; fgutierrez@carrerasresearch.org

Juan Antonio Bernal, MD, PhD[2]; juanantonio.bernal@cnic.es

Elías Herrero-Galán, PhD[2]; elias.herrero@cnic.es

Jorge Alegre-Cebollada, PhD[2]; jalegre@cnic.es

Enrique Lara-Pezzi, PhD[2,3]; elara@cnic.es

Pablo García-Pavía, MD, PhD[1,2,3,8]; pablogpavia@yahoo.es

Affiliations:

[1]Heart Failure and Inherited Cardiac Diseases Unit, Department of Cardiology, Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain;

[2]Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain;

[3]CIBER Cardiovascular Diseases (CIBERCV), Madrid, Spain;

[4] Unidad de Cardiopatías Familiares. Servicio de Cardiología . Hospital Universitario de Ciudad Real, Ciudad Real, Spain.

[5] Facultad de Medicina de Ciudad Real. Universidad de Castilla La Mancha.

[6] Department of Cardiology, Aarhus University Hospital, Aarhus, Denmark

[7] Unidad de Cardiopatías Familiares, Muerte Súbita y Mecanismos de Enfermedad (CaFaMuSMe), Instituto de Investigación Sanitaria La Fe, Servicio de Cardiología, Hospital Universitario y Politécnico La Fe, Valencia, Spain.

[8] Universidad Francisco de Vitoria (UFV), Pozuelo de Alarcón, Spain;

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Address for correspondence

Fernando Dominguez MD, PhD and Pablo Gracia-Pavia, MD, PhD

Department of Cardiology,

Hospital Universitario Puerta de Hierro

Manuel de Falla, 2, Majadahonda, Madrid, 28222, Spain

Tel: +34-911917297, Fax: +34-911917718

Email: fdominguezrodriguez@gmail.com and pablogpavia@yahoo.es

Twitter Handle:

@fernidom

@drpavia

@cardiopdh

@CNIC_CARDIO

Non-standard Abbreviations and Acronyms

CD: Circular dichroism

DCM: Dilated cardiomyopathy

hiPSC: Human Induced Pluripotent Stem Cell

LVEDD: left ventricular end-diastolic diameter

LVEF: left ventricular ejection fraction

TTN: titin

High throughput sequencing technologies have revolutionized the identification of genetic variants responsible for genetic diseases such as dilated cardiomyopathy (DCM). However, a causal genetic variant is still not identified in approximately 60% of DCM[1]**.** Although truncating variants in *TTN* (TTNtv) are the main genetic cause of DCM, the role of rare missense variants in *TTN* as cause of DCM remains unknown. We describe two families with DCM in which clinical and *in vitro* investigations support that missense variants affecting a conserved cysteine of *TTN* are causative of DCM. The study was approved by the local Ethics Committee.

A Spanish family with 12 individuals with DCM was studied. Three phenotype-positive distant relatives underwent exome sequencing and shared a variant (Chr2(GRCh38):g.178741559A>T) in *TTN* predicted to cause the substitution of a cysteine by a serine: p.Cys3892Ser (NM_001267550.2:c.11674T>A). The affected amino acid is highly conserved (PhastCons100way score of 1.000, PhyloP100way score of 9.293). Several in silico predictors suggest that the change is deleterious, including the meta predictor (score: 0.708)[2]. Exome sequencing did not reveal additional shared rare variants in other cardiomyopathy-associated genes.

The proband was a male who underwent cardiac transplantation aged 57. Among 36 family members, 14 were carriers and 12 had DCM (86%) (Figure 1A). Median age at DCM diagnosis was 33 years (IQR:18-45). Mean left ventricular ejection fraction (LVEF) was 43±6% and mean left ventricular end-diastolic diameter (LVEDD) was 57±4 mm. The two carriers without DCM exhibited LVEF in the lower limit of normal range. 22 relatives were non-carriers and showed normal phenotype (median age 51 years, IQR:24-60, LVEF: 62±5%, LVEDD 46±2 mm). Family specific two-point logarithm of the odds (LOD) score was 3.96 (dominant model, 80% penetrance).

A Danish family with the same mutated residue but with Cys replaced by Arg (NM_001267550.2:c.11674T>C) was identified. In silico evaluation also predicted the variant to be pathogenic, with a REVEL score of 0.688. The proband was a female (LVEF of 20%, LVEDD 75 mm) who underwent heart transplantation aged 17. Her father (obligate carrier) was diagnosed with DCM at 54 and died aged 73. A 52-year-old half-sister exhibited the variant without DCM (Figure 1B).

To further characterize the p.Cys3892Ser variant, iPSC lines were generated using CRISPR/Cas9 to introduce a point mutation T>A c:11,674 in the TTN allele of a wild-type hiPSC line (HDF-iPS-SV10 -Spanish National Stem Cell Bank), wild type hiPSC, hetero and homozygous hiPSC-TTN^{Cys3892Ser} lines were differentiated to cardiac lineage. Video for single cell contraction amplitude showed deficient contraction in homozygous p.Cys3892Ser cardiomyocytes (Figure 1C). We did not detect decreased contraction in heterozygous p.Cys3892Ser cells, consistent with other DCM iPSC lines[3].

Circular dichroism (CD) spectroscopy was used to study thermal denaturation of recombinant purified TTN I21 domains with and without the p.Cys3892Ser variant. CD signal at 215nm was monitored as temperature increased from 25 to 85 ºC at a rate of 30ºC/h. The recombinant TTN I21 domain containing p.Cys3892Ser preserved the global fold of the wild-type protein (Figure 1D), but was not stable at physiological temperatures (Figure 1E). We also attempted to study variant p.Cys3892Arg by CD. However, this was not possible due to the insolubility of the mutant domain, which is probably caused by the higher destabilizing nature of the Cys to Arg substitution (data not shown).

We provide strong evidence supporting that *TTN* missense variants involving the conserved cysteine p.Cys3892 can cause DCM. Cysteine is one of the least abundant and most conserved amino acids. Cys3892 corresponds to a conserved cysteine of the cardiac

specific I21 domain, present in 27 species ranging from zebrafish to humans. None of the two variants have been described in the literature or in ClinVar, and they are not reported in gnomAD. Regarding molecular mechanisms of pathogenicity, it is tempting to speculate that titin domain destabilization could lead to reduced titin levels (haploinsufficiency) and/or saturation of protein quality control systems, both of which have been linked to pathogenicity in DCM *TTN* truncating variants. The Spanish family exhibited a LOD score of 3.96. LOD scores of 3 support odds 20:1 in favor of linkage, and a variant strongly related to the phenotype.

Our study is the first to unequivocally confirm that *TTN* missense variants can cause DCM. Gerull et al. reported that a missense *TTN* mutation could cause DCM[5] in a moderately large family with a LOD score of 2.73, which is below the value of 3 usually considered to prove association.

A recent study showing that in 530 DCM, almost 7% had rare TTN missense variants predicted to be deleterious by bioinformatics filtering. However, they were not enriched in DCM compared with ExAC and authors concluded that TTN missense variants should be classified as likely benign[4]. Our results confront with this conclusion, as we proved that missense variants involving Cys3892 are causative of DCM supporting the pathogenic role of certain missense TTN variants.

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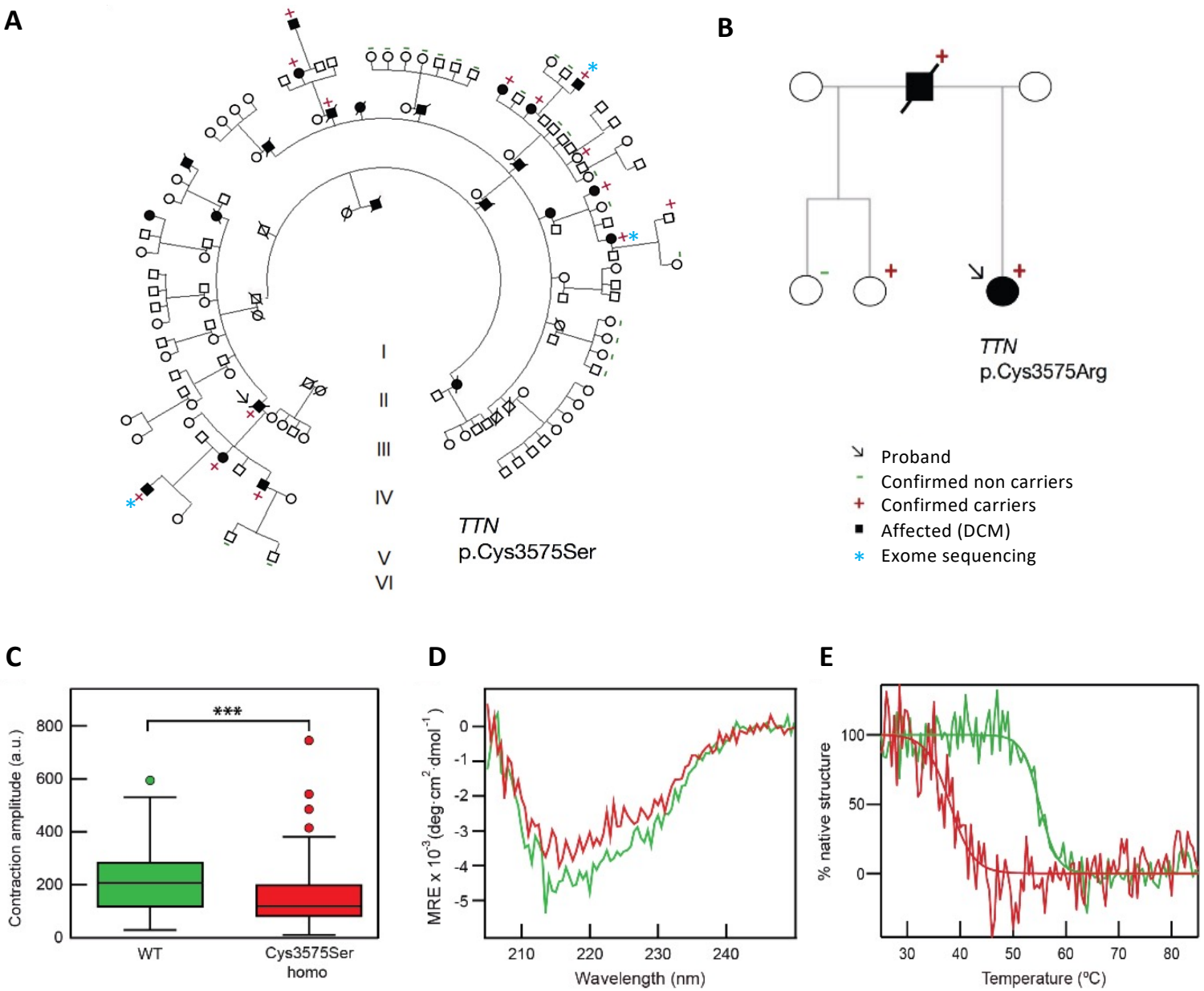
Figure 1. **A:** Pedigree of a large Spanish kindred with 14 TTN p.Cys3575Ser carriers (12 with DCM) and 22 non-carriers. Blue asterisks show individuals who underwent Exome-sequencing. **B:** Pedigree of the Danish family with the TTN p.Cys3575Arg variant, with 3 carriers (2 with DCM) and 1 non carrier. **C:** Amplitude of contraction of wild-type (green) and homozygous Cys3575Ser (red) iPSC-induced cardiomyocytes (*** p=0.0001, Mann-Whitney). **D:** Far-UV circular dichroism spectra at 25ºC of wild-type (green) and Cys3575Ser (red) recombinant I21 protein domains. **E:** Thermal unfolding curves of recombinant I21 WT (green) and Cys3575Ser (red) domains.


A
I
II
III
IV
V
VI
TTN
p.Cys3575Ser
B
TTN
p.Cys3575Arg
Proband
Confirmed non carriers
Confirmed carriers
Affected (DCM)
Exome sequencing
C
***
Contraction amplitude (a.u.)
800
600
400
200
0
WT
Cys3575Ser
homo
D
MRE x 10-3 (deg·cm2·dmol-1)
0
-1
-2
-3
-4
-5
210
220
230
240
Wavelength (nm)
E
% native structure
100
50
0
30
40
50
60
70
80
Temperature (ºC)