**Since 2010, I am developing in collaboration with several groups some programs in order to identify human susceptibility genes for chronic Chagas cardiomyopathy.**

# 1. OUR INTERNATIONAL CONSORTIUM

## 2. Research program

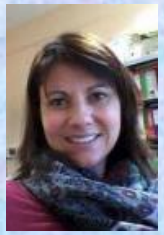
Introduction
Concept
Gene expression analysis
CardioChip microarrays/Agilent microarrays/RNA-sequencing
Common variant association studies.
Candidate gene case/control studies/
Genome-wide association studies (GWAS)/
Exome sequencing
Epigenetic
MiRNA, lncRNA, methylation

## 3. General Information

The International consortium
Chagas disease is a Neglected Tropical Disease
History
Leonel Messi is also fighting against this disease
Life cycle
Vectors and reservoirs
Transmission
Acute and chronic phases
Epidemiology
In Brazil / In Europe
Diagnosis during acute phase
Diagnosis during chronic phase
Treatment

# OUR INTERNATIONAL CONSORTIUM







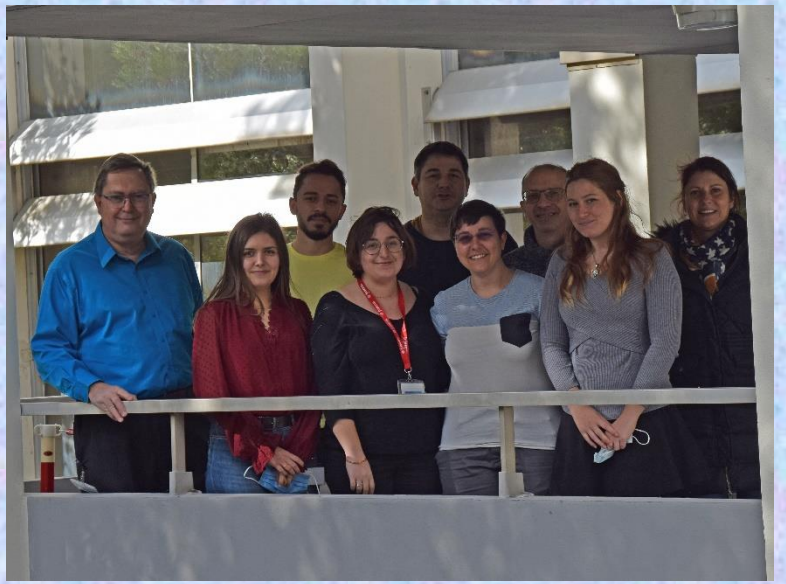


## IN FRANCE

**Inserm UMR U1090 (Marseille)** Christophe Chevillard, Lionel Spinelli, Sandrine Marquet, Magali Torres, Joao Nunes, Frédéric Gallardo, Pauline Andrieux, Pauline Brochet, Serena Renoliet, Ambre Borie.
Previous members: Amanda Farage Frade, Laurie Laugier, Sandrine Cabantous, Maryem Ourhache.

**Inserm UMR U1163 (Paris)** Aurélie Cobat, Laurent Abel.

**Inserm UMR U1083 (Angers)** Vincent Procaccio, David Goudenege.








# OUR INTERNATIONAL CONSORTIUM

## IN BRAZIL

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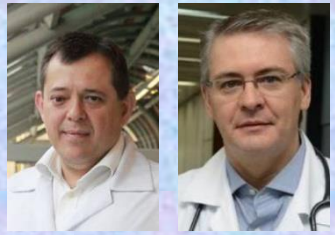

### Hospital das Clínicas (São Paulo)

**Barbara Maria Ianni,** Alexandre Pereira, Charles Mady, Paula Buck.

### Laboratório de Anatomia Patológica do InCor (São Paulo)

**Luiz Alberto Benvenuti**

### The Instituto de Cardiologia Dante Pazzanese (São Paulo)

**Mario Hiroyuki Hirata**, Abílio Fragata, Marcelo Sampaio, Bruno Saba, Hui Tzu Lin-Wang.

# OUR INTERNATIONAL CONSORTIUM

## IN BRAZIL

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Hospital das Clínicas and School of Medicine, Universidade Federal de Minas Gerais (Belo Horizonte)

Maria Do Carmo Nunes, Luiz Ribeiro Antonio.

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Ariela Mota Ferreira

Faculdade de Medicina (Ribeirão Preto)

**Edouardo Donadi**, André Schmidt, José Antonio Marin-Neto, Fabricio Dias.

# OUR INTERNATIONAL CONSORTIUM

## IN BRAZIL

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**Martino Martinelli**, Sergio Siqueira, Giselle de Lima Peixoto.

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**Joseli Lannes-Vieira**

Centro de Pesquisas Aggeu Magalhães - CPqAM-FIOCRUZ-PE Recife)

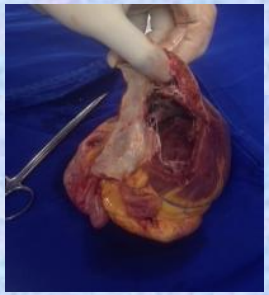
**Luydson Vasconcelos,** Virginia Maria Barros de Lorena, Clarice Neuenschwander, Lins de Morais Fonseca

# Cohort

Our project is involving multiple groups that have done several epidemiological, immunological and genetic analyses on parasitic diseases. Our project, which will benefit of the partners expertise has a high rate of success. Feasibility of this project is excellent and there is no major step that should raise problem.

The Laboratory of Immunology at Incor started a Chagas patient DNA bank in the mid 90s, in collaboration with the Cardiomyopathies clinical group of the Institute. Then a multicenter arrangement involving additional clinical partners in Brazil was established.

To our knowledge, we have the largest cohort of Chagas patients for genetics, immunological studies and so on.



**Fresh heart tissue: n=58**

- Chronic Chagas Cardiomyopathy (CCC) n=25
- Non inflammatory dilated cardiomyopathy (CDM) n=16
- Ischemic patients (CI) n=10
- Healthy control n=7

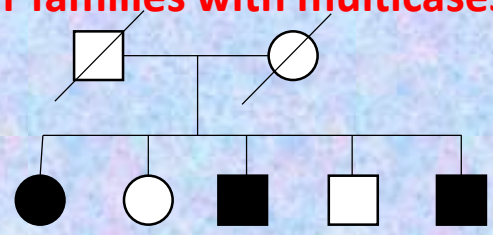
**DNA samples: n=3306**

- Asymptomatic subjects n=886
- Chronic Chagas Cardiomyopathy (CCC) n=1882
- EMF patients n=40
- Ischemic patients (CI) n=9
- LVNC patients n=50
- Chagas Mega Oesophagus patients n=15
- Idiopatic Mega Oesophagus patients n=9
- Digestive form patients n=9

**FFPE samples: n=228**

- Chronic Chagas Cardiomyopathy (CCC) n=168
- Non inflammatory dilated cardiomyopathy n=60

**Nuclear families with multicases: n=22**



**CCC patients with arrhythmia n= 362**

patient with pacemaker n=210

patient with defibrillator n=98

**CCC patients**

Severe CCC (EF≤0.4%) n=686

Moderate CCC (EF≥0.4%) n=974

# Animal models

## Mouse model

7 week old

100 trypomastigote infection

CTL

15 DPI

30 DPI

45 DPI

DPI: day post infection

MPI: month post infection

## Syrian hamster model

10-12 week old

3.5 × $10^4$ to $10^5$ trypomastigote infection

CTL

4 MPI

8 MPI

12 MPI

## Introduction

A variety of parasites cause chronic infections that last for long periods of time in their human host without much clinical symptoms; in some subjects, however, parasites cause severe disease. These pathological disorders may become apparent after 10 to 20 years of infection as in subjects infected by *Schistosoma mansoni* or by *Trypanosoma cruzi*, or within a few weeks of infection in patients affected by *Leishmania donovani* or by *Plasmodium falciparum*.

Various studies have attempted to identify the factors that cause disease to develop in only a fraction of the population exposed to parasites. Much attention has been given to the environment because parasite transmission depends markedly on environmental factors including vector density, vector distribution, and parasite virulence. However, host genetic polymorphisms are also factors that determine infection and disease phenotypes.

Dissecting the relative contribution of the environment, the parasite and the host in pathogenesis often requires large population studies that are costly and difficult to carry out. A major difficulty to be faced by the geneticists is the large genetic variability of certain parasite populations, which may complicate the detection of host genetic factors since parasite variability is not easy to evaluate. Despite these difficulties, major advances have been made in the field of genetics of some of the parasitic diseases, and provided important insights into the mechanisms of pathogenesis. So far, several susceptibility loci were mapped on the human genome and some susceptibility genes were identified. It is likely that these studies will yield extremely useful information for drug and vaccine development.

Chagas disease is a major public health problem in Latin America, resulting from lifelong infection with the protozoan parasite Trypanosoma cruzi.

Up to 30 years after acute infection, approximately 30% of the 6 million infected people eventually develop chronic Chagas cardiomyopathy (CCC), a life-threatening inflammatory dilated cardiomyopathy (*Bocchi EA et al. 2017; Chevillard C et al 2018*). Most other T. cruzi-infected patients will remain asymptomatic for life (60%) or develop digestive disease, which causes less deaths (approx. 10%) (*Bocchi EA et al. 2017*).

Chagas disease is the most common cause of non-ischemic cardiomyopathy in Latin America, causing approximately 10,000 deaths/year, mainly due to heart failure and severe arrhythmia/sudden death (*Bocchi EA et al. 2017*). Migration turned Chagas disease into a global health problem, with an estimated 400,000 infected persons living in non endemic countries, mainly the United States and Europe. Current anti–T. cruzi drugs have shown to be unable to block progression toward CCC (*Morillo CA et al. 2015*). After acute infection, parasitism is partially controlled by the immune response, and low-grade parasite persistence fuels the systemic production of inflammatory cytokines like IFN-γ and TNF-α, which is more intense in CCC than ASY patients (*Abel LC et al. 2001; Gomes JA et al. 2005; Sousa GR et al. 2014*).

**Bocchi EA**, Bestetti RB, Scanavacca MI, Cunha Neto E, Issa VS. Chronic Chagas Heart Disease Management: From Etiology to Cardiomyopathy Treatment. J Am Coll Cardiol. 2017 Sep 19;70(12):1510-1524.

**Chevillard C**, Nunes JPS, Frade AF, Almeida RR, Pandey RP, Nascimento MS, Kalil J, Cunha-Neto E. Disease Tolerance and Pathogen Resistance Genes May Underlie Trypanosoma cruzi Persistence and Differential Progression to Chagas Disease Cardiomyopathy. Front Immunol. 2018 Dec 3;9:2791.

**Morillo CA**, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A Jr, Rosas F, Villena E, Quiroz R, Bonilla R, Britto C, Guhl F, Velazquez E, Bonilla L, Meeks B, Rao-Melacini P, Pogue J, Mattos A, Lazdins J, Rassi A, Connolly SJ, Yusuf S; BENEFIT Investigators. Randomized Trial of Benznidazole for Chronic Chagas' Cardiomyopathy. N Engl J Med. 2015 Oct;373(14):1295-306.

**Abel LC,** Rizzo LV, Ianni B, Albuquerque F, Bacal F, Carrara D, Bocchi EA, Teixeira HC, Mady C, Kalil J, Cunha-Neto E. Chronic Chagas' disease cardiomyopathy patients display an increased IFN-gamma response to Trypanosoma cruzi infection. J Autoimmun. 2001 Aug;17(1):99-107.

**Gomes JA**, Bahia-Oliveira LM, Rocha MO, Busek SC, Teixeira MM, Silva JS, Correa-Oliveira R. Type 1 chemokine receptor expression in Chagas' disease correlates with morbidity in cardiac patients. Infect Immun. 2005 Dec;73(12):7960-6.

**Sousa GR**, Gomes JA, Fares RC, Damásio MP, Chaves AT, Ferreira KS, Nunes MC, Medeiros NI, Valente VA, Corrêa-Oliveira R, Rocha MO. Plasma cytokine expression is associated with cardiac morbidity in chagas disease. PLoS One. 2014 Mar 6;9(3):e87082.

CCC is characterized by a monocyte and T cell-rich myocarditis (*Fonseca SG et al. 2007; Higuchi Mde L et al. 1993*) with cardiomyocyte damage and hypertrophy, and prominent fibrosis; *T. cruzi* parasites are very scarce. IFN-γ producing Th1 cells accumulate in the myocardium of CCC patients (*Abel LC et al. 2001; Reis MM et al. 1997; Rocha Rodrigues DB et al. 2012)* in response to locally produced chemokine ligands CXCL9 and CCL5 (Nogueira LG et al. 2012). Accordingly, IFN-γ was found to be the most highly expressed cytokine mRNA in CCC myocardium using a 13-cytokine panel (*Nogueira LG et al. 2014*).

Both heart-crossreactive (*Cunha-Neto E et al. 1996*) and *T. cruzi*-specific T cells (*Fonseca SG et al. 2005*) have been found in CCC heart tissue, and both and may play a role in the myocarditis of CCC. Together, evidence suggests that myocarditis and IFNγ signaling plays a major pathogenic role in CCC development and severity, although downstream events leading to the heart disease phenotype are still obscure.


**Fonseca SG**, Reis MM, Coelho V, Nogueira LG, Monteiro SM, Mairena EC, Bacal F, Bocchi E, Guilherme L, Zheng XX, Liew FY, Higuchi ML, Kalil J, Cunha-Neto E. Locally produced survival cytokines IL-15 and IL-7 may be associated to the predominance of CD8+ T cells at heart lesions of human chronic Chagas disease cardiomyopathy. Scand J Immunol. 2007 Aug-Sep;66(2-3):362-71.

**Higuchi Mde L**, Gutierrez PS, Aiello VD, Palomino S, Bocchi E, Kalil J, Bellotti G, Pileggi F. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. Virchows Arch A Pathol Anat Histopathol. 1993;423(3):157-60.

**Reis MM**, Higuchi Mde L, Benvenuti LA, Aiello VD, Gutierrez PS, Bellotti G, Pileggi F. An in situ quantitative immunohistochemical study of cytokines and IL-2R+ in chronic human chagasic myocarditis: correlation with the presence of myocardial Trypanosoma cruzi antigens. Clin Immunol Immunopathol. 1997 May;83(2):165-72.

**Rocha Rodrigues DB**, dos Reis MA, Romano A, Pereira SA, Teixeira Vde P, Tostes S Jr, Rodrigues V Jr. In situ expression of regulatory cytokines by heart inflammatory cells in Chagas' disease patients with heart failure. Clin Dev Immunol. 2012;2012:361730.

**Nogueira LG**, Santos RH, Ianni BM, Fiorelli AI, Mairena EC, Benvenuti LA, Frade A, Donadi E, Dias F, Saba B, Wang HT, Fragata A, Sampaio M, Hirata MH, Buck P, Mady C, Bocchi EA, Stolf NA, Kalil J, Cunha-Neto E. Myocardial chemokine expression and intensity of myocarditis in Chagas cardiomyopathy are controlled by polymorphisms in CXCL9 and CXCL10. PLoS Negl Trop Dis. 2012;6(10):e1867.

**Nogueira LG**, Santos RH, Fiorelli AI, Mairena EC, Benvenuti LA, Bocchi EA, Stolf NA, Kalil J, Cunha-Neto E. Myocardial gene expression of T-bet, GATA-3, Ror-γt, FoxP3, and hallmark cytokines in chronic Chagas disease cardiomyopathy: an essentially unopposed TH1-type response. Mediators Inflamm. 2014;2014:914326.

**Cunha-Neto E**, Coelho V, Guilherme L, Fiorelli A, Stolf N, Kalil J. Autoimmunity in Chagas' disease. Identification of cardiac myosin-B13 Trypanosoma cruzi protein crossreactive T cell clones in heart lesions of a chronic Chagas' cardiomyopathy patient. J Clin Invest. 1996 Oct 15;98(8):1709-12.

**Fonseca SG**, Moins-Teisserenc H, Clave E, Ianni B, Nunes VL, Mady C, Iwai LK, Sette A, Sidney J, Marin ML, Goldberg AC, Guilherme L, Charron D, Toubert A, Kalil J, Cunha-Neto E. Identification of multiple HLA-A*0201-restricted cruzipain and FL-160 CD8+ epitopes recognized by T cells from chronically Trypanosoma cruzi-infected patients. Microbes Infect. 2005 Apr;7(4):688-97.

We hypothesize that specific gene/protein expression profiles and activation of specific disease pathways in affected myocardial tissue are fundamental factors for disease progression.

We hypothesize that expression of pathogenetically relevant genes and proteins in the myocardial tissue of CCC patients is controlled by genetic polymorphisms.

We are able to set up a systems biology approach, that couldn't be done anywhere else.

Systems biology is an approach that aims to model and discover interactions and emergent properties of complex biological systems, which is addressed using quantitative measures and by rigorous integration of "omics" data. It will lead to the identification of host genetic factors that predispose individuals to chronic disease. In addition, results of this analyses may provide novel therapeutic targets, as well as diagnosis and prognosis.

# Concept

Most **pathogen resistance genes** (**PRG**) inhibit infection by directly reducing pathogen burden, and are related to immune-driven mechanisms—which, when in excess, can lead to death. Disease tolerance is an alternative strategy to avoid death after infection, whereby the pathogen's damaging effect on the host is mitigated. Disease tolerance is defined as the situation where an organism can bear a pathogen load without tissue damage and in the absence of a disease state. **Disease tolerance genes** (**DTG**)—which do not limit infection, but reduce its fitness costs—operate to minimize tissue damaging effects of the pathogen, leading to stress and damage reduction responses; DTG can also operate by counteracting excessive, tissue-damaging PRG activity.

We here hypothesize that the absolute need for **DTG** to control potentially lethal **PRG** activity against *T. cruzi* leads to parasite persistence and establishment of chronic infection. Our second hypothesis is that **PRG** and **DTG** also determine the differential progression of chronic Chagas disease toward tissue damage (CCC). According to this hypothesis, ASY patients are disease tolerant, while tissue damage in CCC is a consequence of insufficient **DTG** and/or excessive **PRG** activity.


**Chevillard C**, **Nunes JPS**, Frade AF, Almeida RR, Pandey RP, Nascimento MS, Kalil J, Cunha-Neto E. Disease Tolerance and Pathogen Resistance Genes May Underlie Trypanosoma cruzi Persistence and Differential Progression to Chagas Disease Cardiomyopathy. Front Immunol. 2018 Dec 3;9:2791. doi:10.3389/fimmu.2018.02791. PMID: 30559742; PMCID: PMC6286977.

# Pathogen resistance genes PRG in *T. cruzi* infection

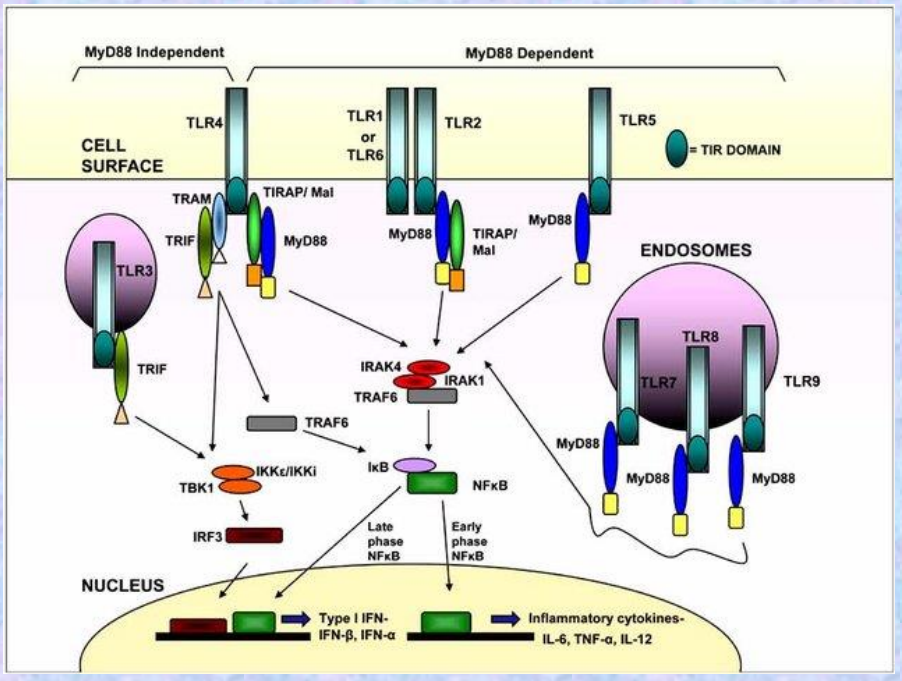
Most known pathogen resistance mechanisms against *T. cruzi* are immune-driven, directed at the intracellular forms of the parasite, and can be harmful if excessive.

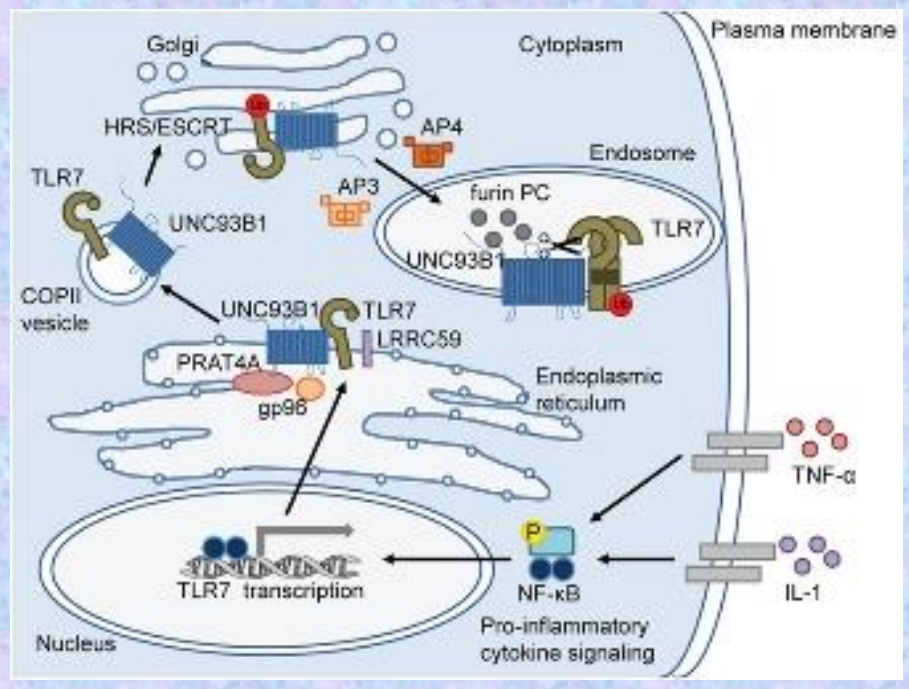
**PRG** were defined as genes essential for control of T. cruzi parasitism and needed for survival of infection; operationally, we identified as **PRG** those genes whose knockout led to increased pathogen load and mortality.

Most **PRG** belong to the TLR-MYD88-IL12-IFNG pathway, *IL17* pathway, cell migration, inflammasome and other pathways involved in restriction of intracellular pathogen growth.

Along with **PRG** genes, we can notice: TLR4, TLR7, TLR9, UNC93B1, MYD88, IL6, IL12, IL12A, IL12B, IL17A, IFNG, STAT1, STAT4, NOS1, NOS2, CASP1, NCF1, CCL2, ICAM1, CD28, IRGM1, PTAFR, LGALS1, PNPLA8, PI3KCG.

| Symbol | Name |
|---|---|
| **PATHOGEN RESISTANCE GENES** | |
| *tlr4* (8) | Toll-like receptor 4 |
| *tlr7* (9) | Toll-like receptor 7 |
| *tlr9* (9, 10) | Toll-like receptor 9 |
| *unc93b1* (9) | Unc-93 homolog B1, TLR signaling regulator |
| *myd88* (9, 11–13) | Myeloid differentiation primary response 88 |
| *il6* (14) | Interleukin 6 |
| *il12* (15) | Interleukin 12A |
| *il12a* (16) | Interleukin 12B |
| *il12b* (17) | Interleukin 17A |
| *il17a* (18, 19) | |
| *ifng* (13, 15, 20–23) | Interferon-γ |
| *stat1* (24) | Signal transducer and activator of transcription 1 |
| *stat4* (25) | Signal transducer and activator of transcription 4 |
| *tnfrsf1a* (26) | TNF receptor superfamily member 1A |
| *nos1* (27) | nitric oxide synthase 1 |
| *nos2* (15, 22) | nitric oxide synthase 2, inducible |
| *casp1* (28) | caspase 1 |
| *pycard* (28) | Asc/PYD and CARD domain containing |
| *ncf1* (29) | P47phox/neutrophil cytosolic factor 1 |
| *ccl2* (30) | C-C motif chemokine ligand 2 |
| *ccr5* (31, 32) | C-C motif chemokine receptor 5 |
| *icam1* (33) | intercellular adhesion molecule 1 |
| *cd28* (34) | CD28 antigen |
| *irgm1* (23) | immunity-related GTPase family M member 1 |
| *ptafr* (35) | platelet-activating factor receptor |
| *lgals1* (36) | Galectin-1/lectin, galactose binding, soluble 1 |
| *pnpla8* (37) | Phospholipase A2γ (iPLA2γ)/patatin-like phospholipase domain containing 8 |
| *pi3kcg* (38) | phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma |




## Mice genetically deficient on TLR4, TLR7, and TLR9, MYD88, and UNC93B1 display increased blood parasitism and mortality *(*Oliveira *et al. 2013;* Caetano *et al. 2013;* Bafica *et al. 2013*; Campos *et al. 2013;* Koga *et al. 2013;* Gonçalves *et al. 2013*).

**Oliveira AC**, de Alencar BC, Tzelepis F, Klezewsky W, da Silva RN, Neves FS, Cavalcanti GS, Boscardin S, Nunes MP, Santiago MF, Nóbrega A, Rodrigues MM, Bellio M. Impaired innate immunity in Tlr4(-/-) mice but preserved CD8+ T cell responses against Trypanosoma cruzi in Tlr4-, Tlr2-, Tlr9- or Myd88-deficient mice. PLoS Pathog. 2010 Apr 29;6(4):e1000870. doi: 10.1371/journal.ppat.1000870. PMID: 20442858; PMCID: PMC2861687.

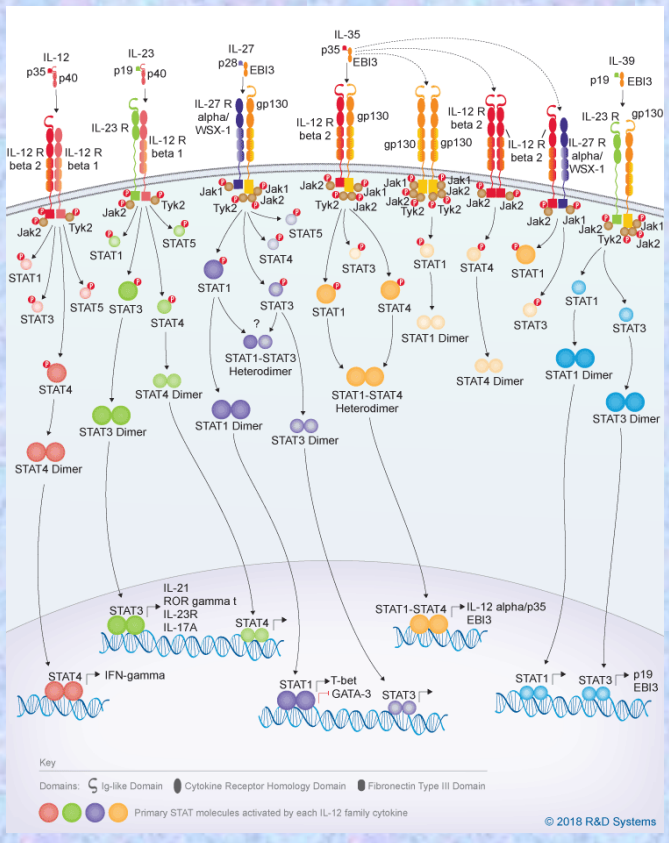
**Caetano BC**, Carmo BB, Melo MB, Cerny A, dos Santos SL, Bartholomeu DC, Golenbock DT, Gazzinelli RT. Requirement of UNC93B1 reveals a critical role for TLR7 in host resistance to primary infection with Trypanosoma cruzi. J Immunol. 2011 Aug 15;187(4):1903-11. doi: 10.4049/jimmunol.1003911. Epub 2011 Jul 13. PMID: 21753151; PMCID: PMC3150366.

**Bafica A**, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. J Immunol. 2006 Sep 15;177(6):3515-9. doi: 10.4049/jimmunol.177.6.3515. PMID: 16951309.

**Campos MA**, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, Ropert C, Gazzinelli RT. Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88. J Immunol. 2004 Feb 1;172(3):1711-8. doi: 10.4049/jimmunol.172.3.1711. PMID: 14734753.

**Koga R**, Hamano S, Kuwata H, Atarashi K, Ogawa M, Hisaeda H, Yamamoto M, Akira S, Himeno K, Matsumoto M, Takeda K. TLR-dependent induction of IFN-beta mediates host defense against Trypanosoma cruzi. J Immunol. 2006 Nov 15;177(10):7059-66. doi: 10.4049/jimmunol.177.10.7059. PMID: 17082622.

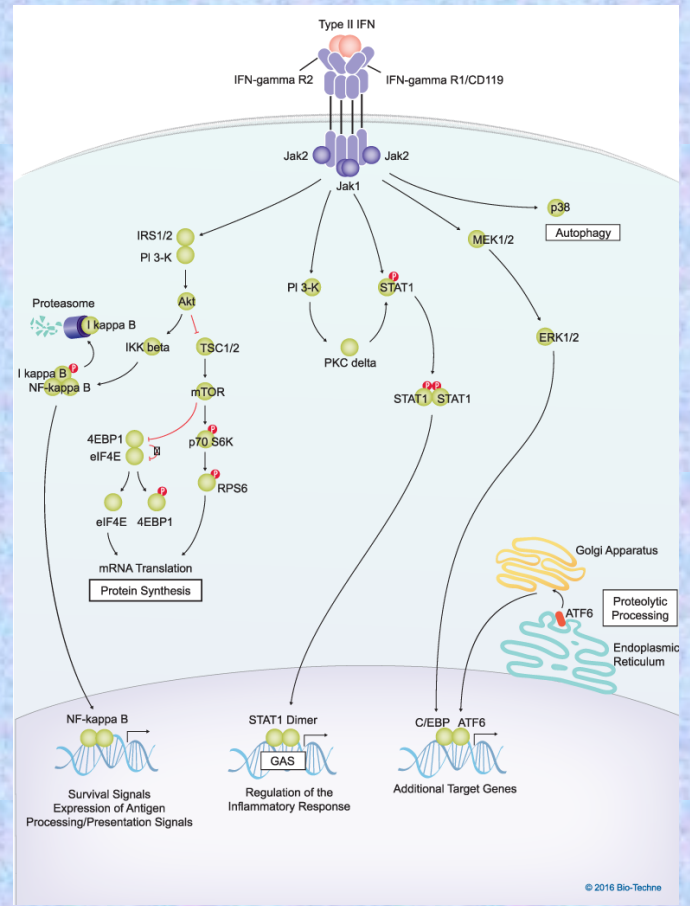
**Gonçalves VM**, Matteucci KC, Buzzo CL, Miollo BH, Ferrante D, Torrecilhas AC, Rodrigues MM, Alvarez JM, Bortoluci KR. NLRP3 controls Trypanosoma cruzi infection through a caspase-1-dependent IL-1R-independent NO production. PLoS Negl Trop Dis. 2013 Oct 3;7(10):e2469. doi: 10.1371/journal.pntd.0002469. PMID: 24098823; PMCID: PMC3789781.




Mice genetically deficient of IL12A, IL12B, and STAT4, essential for the differentiation of IFN-γ-producing Th1 cells, also display intense tissue and blood parasitism with increased mortality *(Michailowsky et al. 2001 and 2004)*.

**Michailowsky V**, Silva NM, Rocha CD, Vieira LQ, Lannes-Vieira J, Gazzinelli RT. Pivotal role of interleukin-12 and interferon-gamma axis in controlling tissue parasitism and inflammation in the heart and central nervous system during Trypanosoma cruzi infection. Am J Pathol. 2001 Nov;159(5):1723-33. doi: 10.1016/s0002-9440(10)63019-2. PMID: 11696433; PMCID: PMC3277321.

**Michailowsky V**, Celes MR, Marino AP, Silva AA, Vieira LQ, Rossi MA, Gazzinelli RT, Lannes-Vieira J, Silva JS. Intercellular adhesion molecule 1 deficiency leads to impaired recruitment of T lymphocytes and enhanced host susceptibility to infection with Trypanosoma cruzi. J Immunol. 2004 Jul 1;173(1):463-70. doi: 10.4049/jimmunol.173.1.463. PMID: 15210806.




IFNG is one of the main PRG involved in T. cruzi parasite control. Mice genetically deficient on IFNG or STAT1 display drastically augmented T. cruzi parasitism and 100% mortality 13 days after infection *(Martins et al. 1999; Cummings et al. 2004; Kulkarni et al. 2015)*.

**Martins** GA, Vieira LQ, Cunha FQ, Silva JS. Gamma interferon modulates CD95 (Fas) and CD95 ligand (Fas-L) expression and nitric oxide-induced apoptosis during the acute phase of Trypanosoma cruzi infection: a possible role in immune response control. Infect Immun. 1999 Aug;67(8):3864-71. doi: 10.1128/IAI.67.8.3864-3871.1999. PMID: 10417150; PMCID: PMC96666.

**Cummings** KL, Tarleton RL. Inducible nitric oxide synthase is not essential for control of Trypanosoma cruzi infection in mice. Infect Immun. 2004 Jul;72(7):4081-9. doi: 10.1128/IAI.72.7.4081-4089.2004. PMID: 15213153; PMCID: PMC427393.

**Kulkarni** MM, Varikuti S, Terrazas C, Kimble JL, Satoskar AR, McGwire BS. Signal transducer and activator of transcription 1 (STAT-1) plays a critical role in control of Trypanosoma cruzi infection. Immunology. 2015 Jun;145(2):225-31. doi: 10.1111/imm.12438. PMID: 25545325; PMCID: PMC4427387.

T. cruzi amastigotes themselves dephosphorylate STAT1 serine residues, inhibiting IFN-γ signaling; evasion of IFN-γ signaling is further proof of the importance of the IFN-γ in the control of intracellular parasitism *(*Stahl *et al. 2014*).

Moreover TNFA and NOS2, play a major role in resistance to T. cruzi *(*Silva *et al. 1995;* Vila-del Sol *et al. 2008*). TNFA-receptor 1 knockout mice (TNFRSFA1$^{-/-}$), which display an increased number of blood and tissue parasites and shortened survival time *(*Pérez *et al. 2007*). Platelet-activating factor (PAF) KO mice are more susceptible to T. cruzi infection than wildtype mice.

IFN-γ, and TNF-α synergistically induce NF-kB activation to control T. cruzi parasitism and mortality in mice, by upregulating the expression of the PRG inducible nitric oxide synthase (NOS2), leading to the production of large amounts of NO and microbicidal reactive nitrogen species *(*Silva *et al. 1995;* Vila-del Sol *et al. 2008*).

IFN-γ increases ROS generation through induction of NADP oxidases (NOX2) and mitochondrial ROS via NF-kB activation *(*Hölscher *et al. 1998;* Wu *et al. 2011*).

IFN-γ-induced ROS enhances peroxynitrite anion ($ONOO^-$) production, a strong oxidant arising from the reaction of NO with superoxide radical($O^-_2$) *(*Rakshit *et al. 2014*).


**Stahl P**, Ruppert V, Schwarz RT, Meyer T. Trypanosoma cruzi evades the protective role of interferon-gamma-signaling in parasite-infected cells. PLoS One. 2014 Oct 23;9(10):e110512. doi: 10.1371/journal.pone.0110512. PMID: 25340519; PMCID: PMC4207753.

**Silva JS**, Vespa GN, Cardoso MA, Aliberti JC, Cunha FQ. Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect Immun. 1995 Dec;63(12):4862-7. doi: 10.1128/IAI.63.12.4862-4867.1995. PMID: 7591147; PMCID: PMC173696.

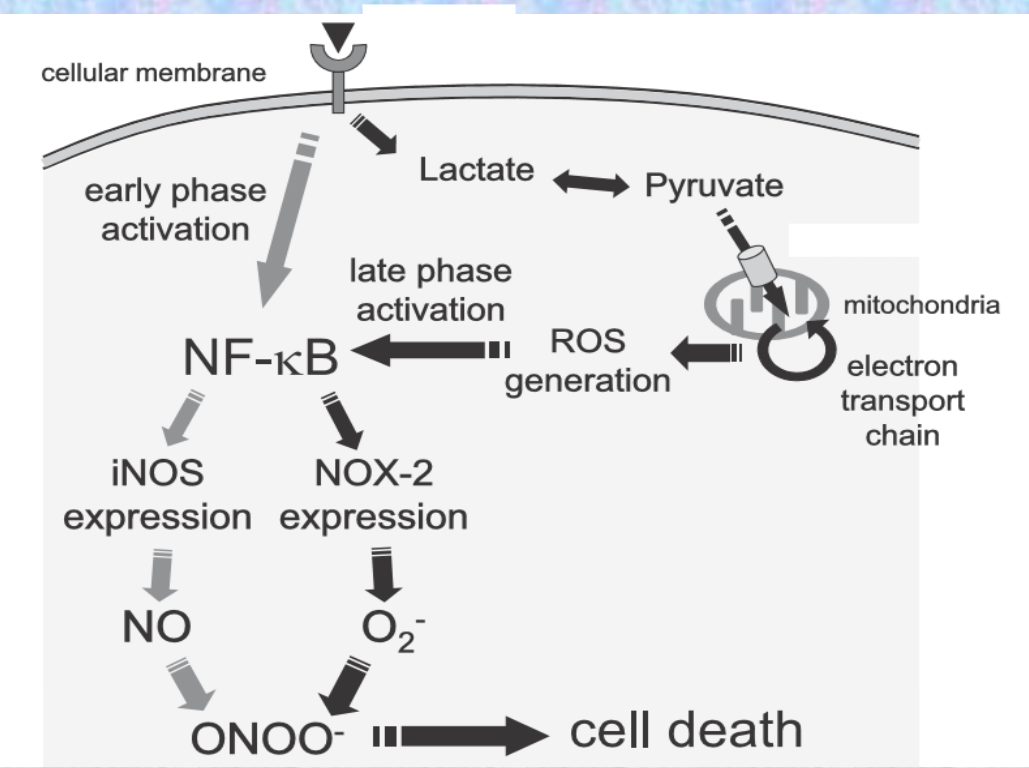
**Vila-del Sol V**, Punzón C, Fresno M. IFN-gamma-induced TNF-alpha expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages. J Immunol. 2008 Oct 1;181(7):4461-70. doi: 10.4049/jimmunol.181.7.4461. PMID: 18802049.

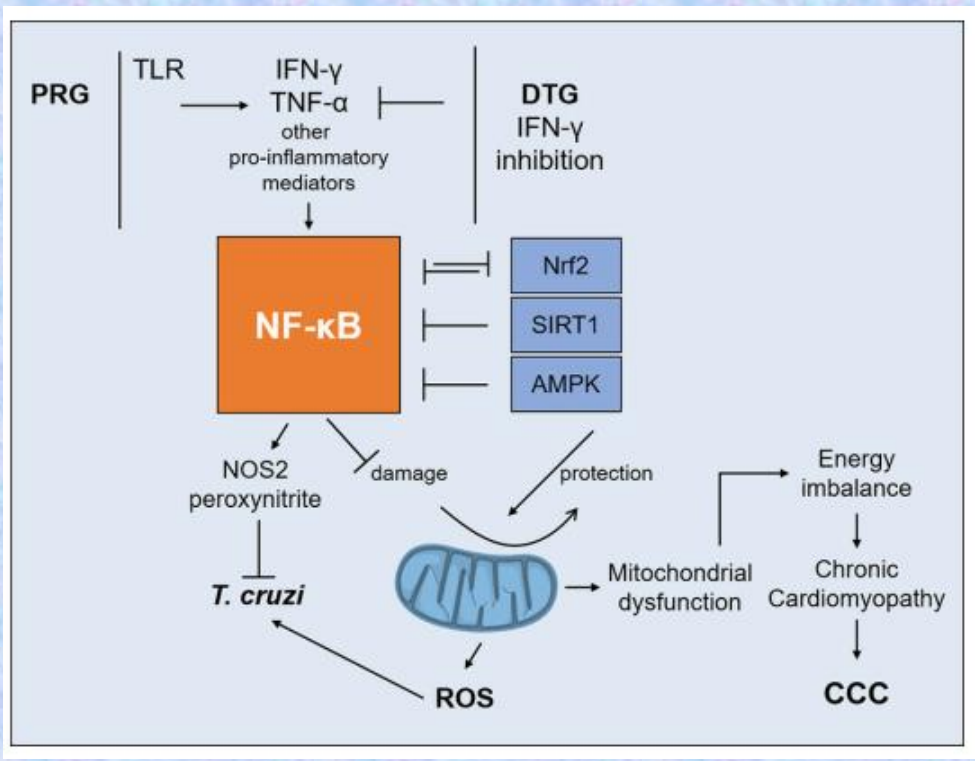
**Pérez AR**, Roggero E, Nicora A, Palazzi J, Besedovsky HO, Del Rey A, Bottasso OA. Thymus atrophy during Trypanosoma cruzi infection is caused by an immuno-endocrine imbalance. Brain Behav Immun. 2007 Oct;21(7):890-900. doi: 10.1016/j.bbi.2007.02.004. Epub 2007 Apr 6. PMID: 17412557.

**Hölscher C**, Köhler G, Müller U, Mossmann H, Schaub GA, Brombacher F. Defective nitric oxide effector functions lead to extreme susceptibility of Trypanosoma cruzi-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. Infect Immun. 1998 Mar;66(3):1208-15. doi: 10.1128/IAI.66.3.1208-1215.1998. PMID: 9488415; PMCID: PMC108035.

**Wu Y**, Antony S, Juhasz A, Lu J, Ge Y, Jiang G, Roy K, Doroshow JH. Up-regulation and sustained activation of Stat1 are essential for interferon-gamma (IFN-gamma)-induced dual oxidase 2 (Duox2) and dual oxidase A2 (DuoxA2) expression in human pancreatic cancer cell lines. J Biol Chem. 2011 Apr 8;286(14):12245-56. doi: 10.1074/jbc.M110.191031. Epub 2011 Feb 14. PMID: 21321110; PMCID: PMC3069428.

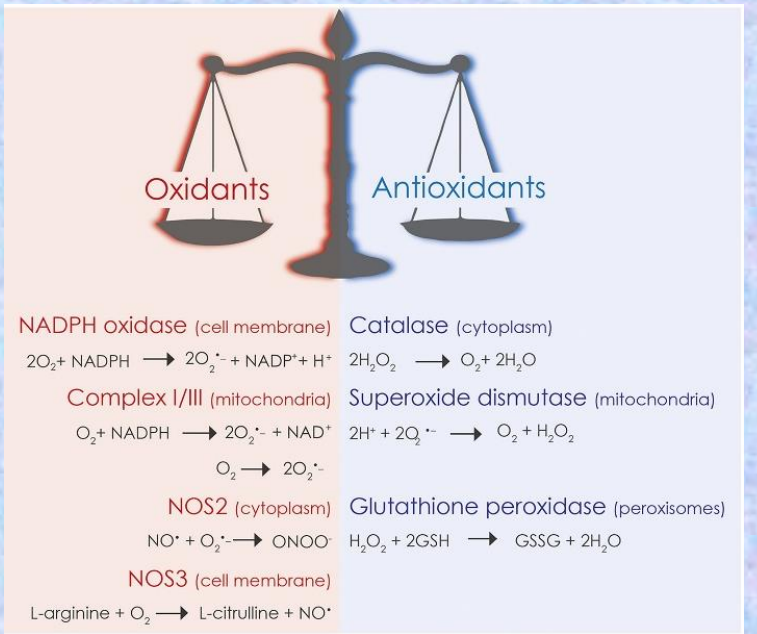
**Rakshit S**, Chandrasekar BS, Saha B, Victor ES, Majumdar S, Nandi D. Interferon-gamma induced cell death: Regulation and contributions of nitric oxide, cJun N-terminal kinase, reactive oxygen species and peroxynitrite. Biochim Biophys Acta. 2014 Nov;1843(11):2645-61. doi: 10.1016/j.bbamcr.2014.06.014. Epub 2014 Jun 28. PMID: 24983769.




ONOO⁻ induces damage to multiple molecules and is one of the ultimate effectors of parasite killing.

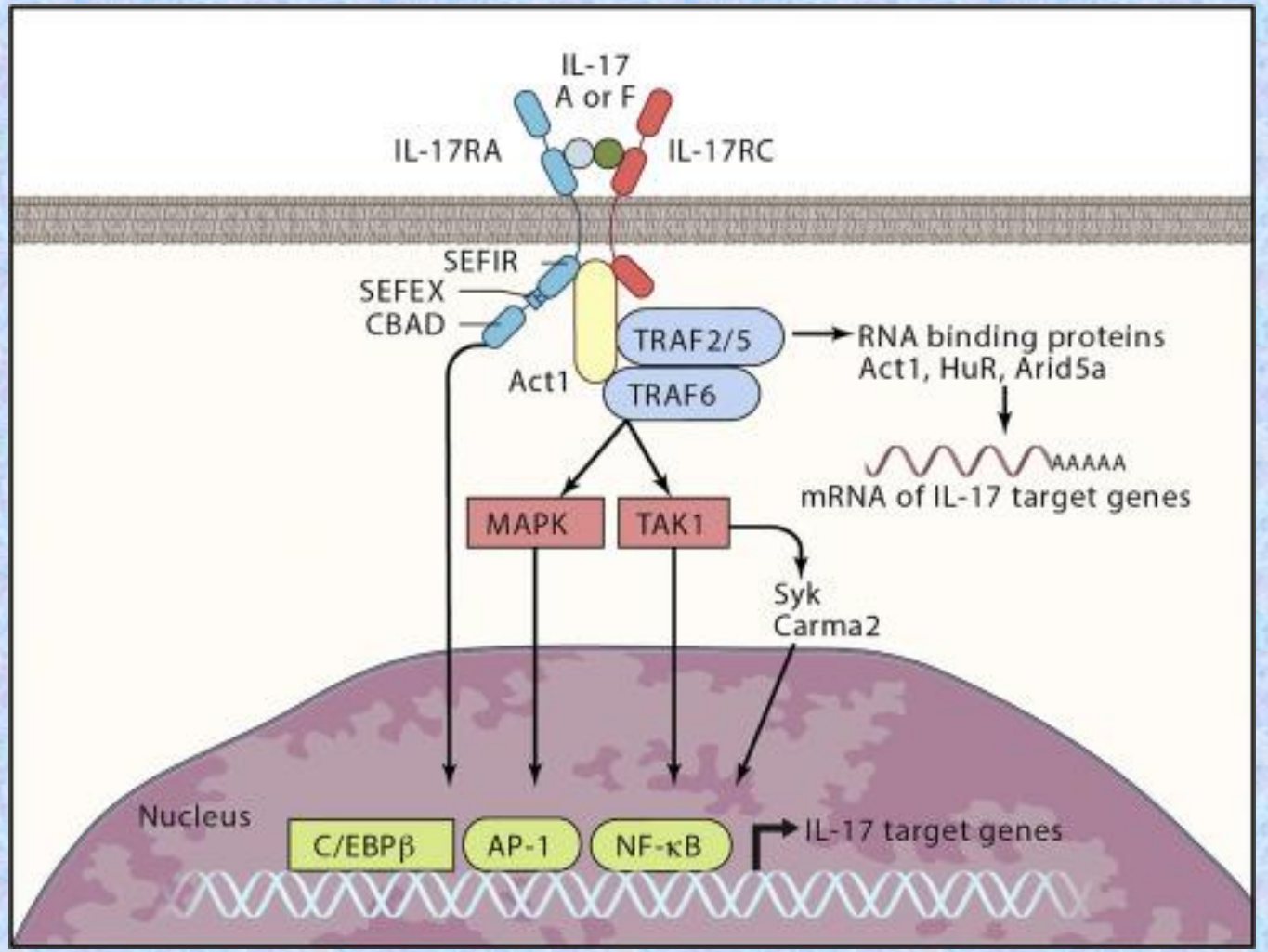
Peroxynitrite promotes morphological disruption of internalized parasites, and induces severe alterations of energy metabolism, calcium homeostasis, and trypanothione depletion, severely impairing parasite redox homeostasis *(Alvarez et al. 2011;* Koo *et al. 2016*).




**Alvarez MN**, Peluffo G, Piacenza L, Radi R. Intraphagosomal peroxynitrite as a macrophage-derived cytotoxin against internalized Trypanosoma cruzi: consequences for oxidative killing and role of microbial peroxiredoxins in infectivity. J Biol Chem. 2011 Feb 25;286(8):6627-40. doi: 10.1074/jbc.M110.167247. Epub 2010 Nov 23. PMID: 21098483; PMCID: PMC3057850.

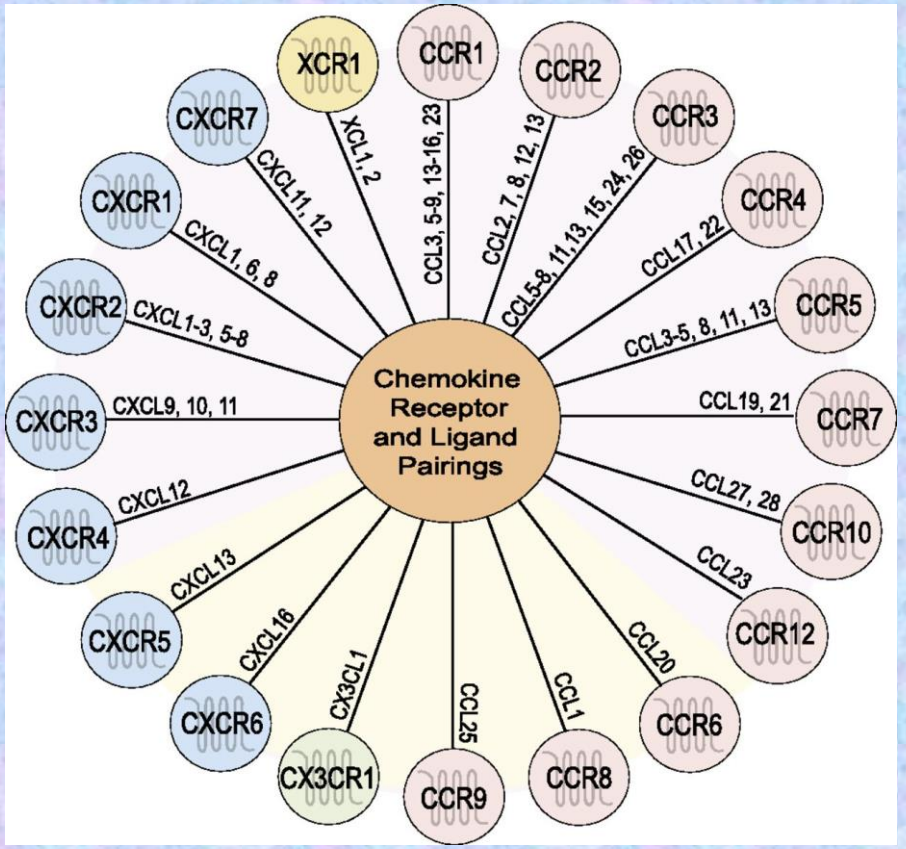
**Koo SJ**, Chowdhury IH, Szczesny B, Wan X, Garg NJ. Macrophages Promote Oxidative Metabolism To Drive Nitric Oxide Generation in Response to Trypanosoma cruzi. Infect Immun. 2016 Nov 18;84(12):3527-3541. doi: 10.1128/IAI.00809-16. PMID: 27698021; PMCID: PMC5116729.

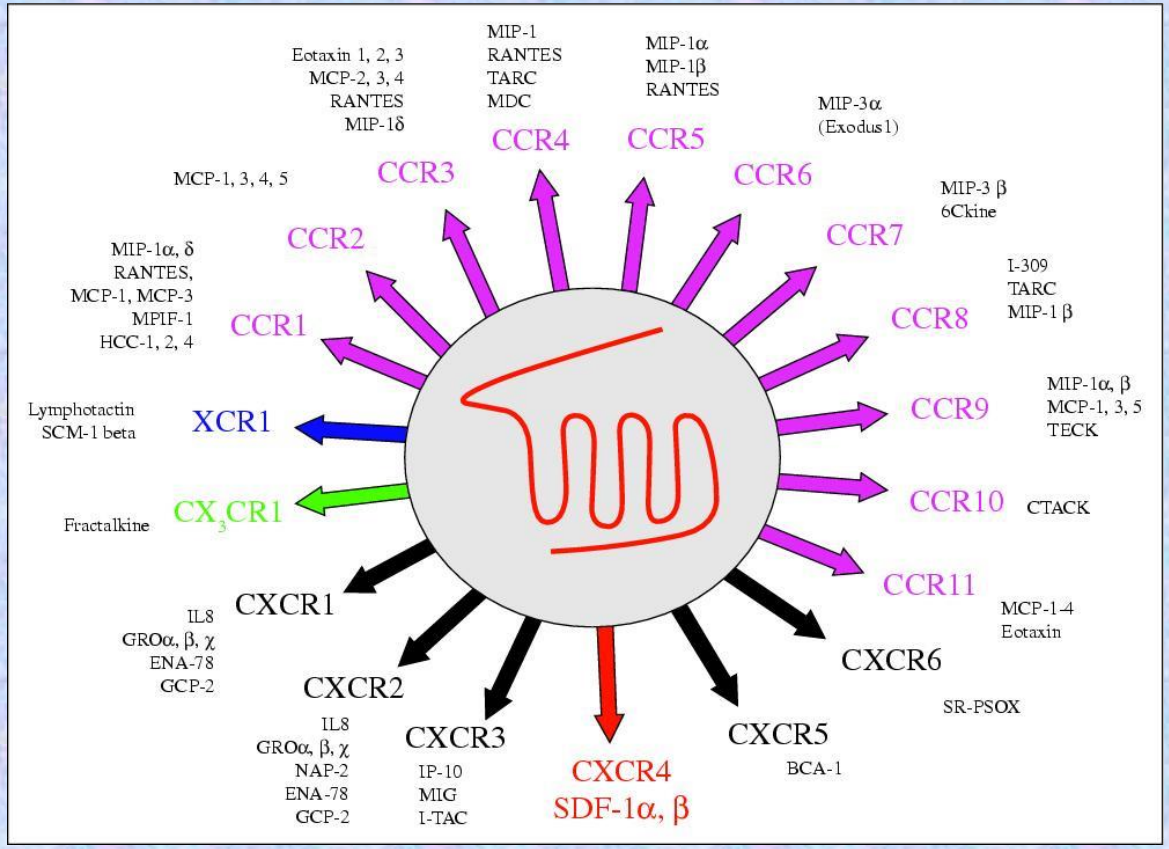
IL17A is also a key **PRG**. IL-17A signaling is mainly dependent on TNF-α receptor associated factor 6 (TRAF6), but it can strongly promote TNF-α-induced NF-kB signaling by stabilizing proinflammatory mRNAs *(Gu et al. 2013)*.
IL-17A also increases the persistence time of T. cruzi in the parasitophorous vacuole, enhancing exposure time of T. cruzi to the antimicrobial environment of endolysosomes, which can be further enhanced by IFN-γ-induced mechanisms *(Erdmann et al. 2013)*.




**Gu C**, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. Cytokine. 2013 Nov;64(2):477-85. doi: 10.1016/j.cyto.2013.07.022. Epub 2013 Sep 3. PMID: 24011563; PMCID: PMC3867811.
**Erdmann H**, Roßnagel C, Böhme J, Iwakura Y, Jacobs T, Schaible UE, Hölscher C. IL-17A promotes macrophage effector mechanisms against Trypanosoma cruzi by trapping parasites in the endolysosomal compartment. Immunobiology. 2013 Jun;218(6):910-23. doi: 10.1016/j.imbio.2012.10.005. Epub 2012 Oct 26. PMID: 23182712.

Mice knockout for genes involved in migration pathways, like chemokines/receptors CCL2, CCR5, and adhesion molecule ICAM1 also develop increased parasitism and decreased survival, in line with the impaired recruitment of leukocytes to sites of parasite replication *(*Paiva *et al. 2009;* Machado *et al. 2005;* Hardison *et al. 2006 ;* Michailowsky *et al. 2004*).




**Paiva CN**, Figueiredo RT, Kroll-Palhares K, Silva AA, Silvério JC, Gibaldi D, Pyrrho Ados S, Benjamim CF, Lannes-Vieira J, Bozza MT. CCL2/MCP-1 controls parasite burden, cell infiltration, and mononuclear activation during acute Trypanosoma cruzi infection. J Leukoc Biol. 2009 Nov;86(5):1239-46. doi: 10.1189/jlb.0309187. Epub 2009 Jul 29. PMID: 19641038.

**Machado FS**, Koyama NS, Carregaro V, Ferreira BR, Milanezi CM, Teixeira MM, Rossi MA, Silva JS. CCR5 plays a critical role in the development of myocarditis and host protection in mice infected with Trypanosoma cruzi. J Infect Dis. 2005 Feb 15;191(4):627-36. doi: 10.1086/427515. Epub 2005 Jan 13. PMID: 15655788; PMCID: PMC7109658.

**Hardison JL**, Wrightsman RA, Carpenter PM, Kuziel WA, Lane TE, Manning JE. The CC chemokine receptor 5 is important in control of parasite replication and acute cardiac inflammation following infection with Trypanosoma cruzi. Infect Immun. 2006 Jan;74(1):135-43. doi: 10.1128/IAI.74.1.135-143.2006. PMID: 16368966; PMCID: PMC1346647.

**Michailowsky V**, Celes MR, Marino AP, Silva AA, Vieira LQ, Rossi MA, Gazzinelli RT, Lannes-Vieira J, Silva JS. Intercellular adhesion molecule 1 deficiency leads to impaired recruitment of T lymphocytes and enhanced host susceptibility to infection with Trypanosoma cruzi. J Immunol. 2004 Jul 1;173(1):463-70. doi: 10.4049/jimmunol.173.1.463. PMID: 15210806.

# Disease tolerance genes DTG in *T. cruzi* infection

Remarkably, all T. cruzi **DTG** (IL10, Ebi-IL27p28, IL17RA, IL23, IL6) shared as a common feature the ability to reduce IFN-γ production or Th1 differentiation.

| Symbol | Name |
|---|---|
| **DISEASE TOLERANCE GENES** | |
| *il6* (39) | Interleukin 6 |
| *il10* (40, 41) | Interleukin 10 |
| *il23* (39) | Interleukin 23 |
| *il17ra* (39, 42) | Interleukin 17 receptor A |
| *ebi3* (39) | Epstein-Barr virus induced gene 3/ Interleukin-27p28 |

Infection of IL-10 deficient mice is accompanied by increased release of IFN-γ, TNF-α, IL-12, and RNS *(*Hölscher *et al. 2013*). Mechanistically, IL-10 is a potent inhibitor of monocyte-macrophage activation and NK cell activity and can inhibit the synthesis of TNF-α and IL-12 and IFN-γ *(*Abrahamsohn *et al. 2013;* Couper *et al. 2013*).

Mice genetically deficient of IL-17RA or IL-23 showed increased mortality due to a shift to a Th1 profile after infection and augmented IFN-γ and TNF-α levels in the heart *(*Medina *et al. 2013;* Tosello Boari *et al. 2013 ;* da Matta Guedes *et al. 2013*).


**Hölscher C**, Mohrs M, Dai WJ, Köhler G, Ryffel B, Schaub GA, Mossmann H, Brombacher F. Tumor necrosis factor alpha-mediated toxic shock in Trypanosoma cruzi-infected interleukin 10-deficient mice. Infect Immun. 2000 Jul;68(7):4075-83. doi: 10.1128/iai.68.7.4075-4083.2000. PMID: 10858224; PMCID: PMC101698.

**Abrahamsohn IA**, Coffman RL. Trypanosoma cruzi: IL-10, TNF, IFN-gamma, and IL-12 regulate innate and acquired immunity to infection. Exp Parasitol. 1996 Nov;84(2):231-44. doi: 10.1006/expr.1996.0109. PMID: 8932773.

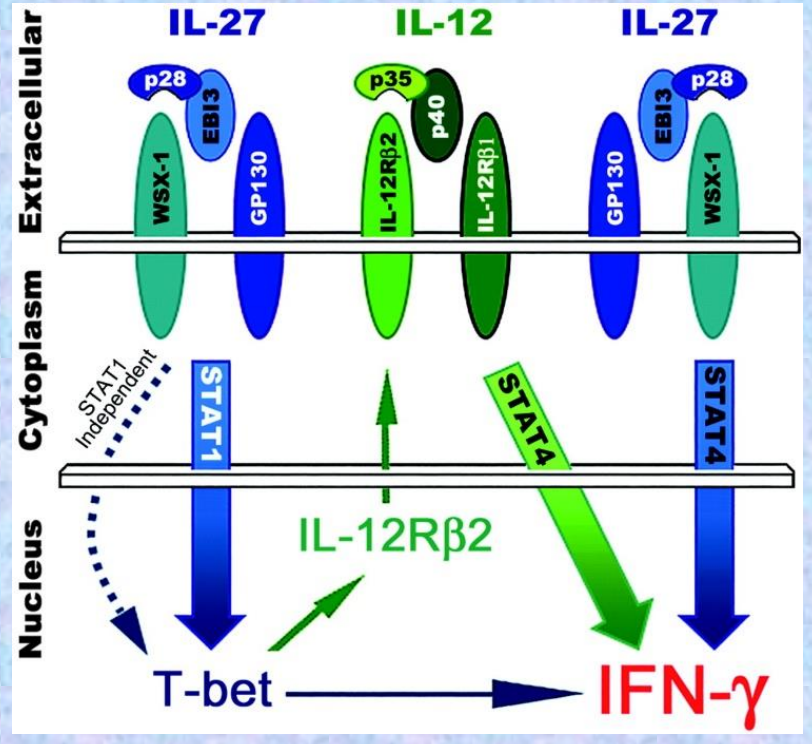
**Couper KN**, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol. 2008 May 1;180(9):5771-7. doi: 10.4049/jimmunol.180.9.5771. PMID: 18424693.

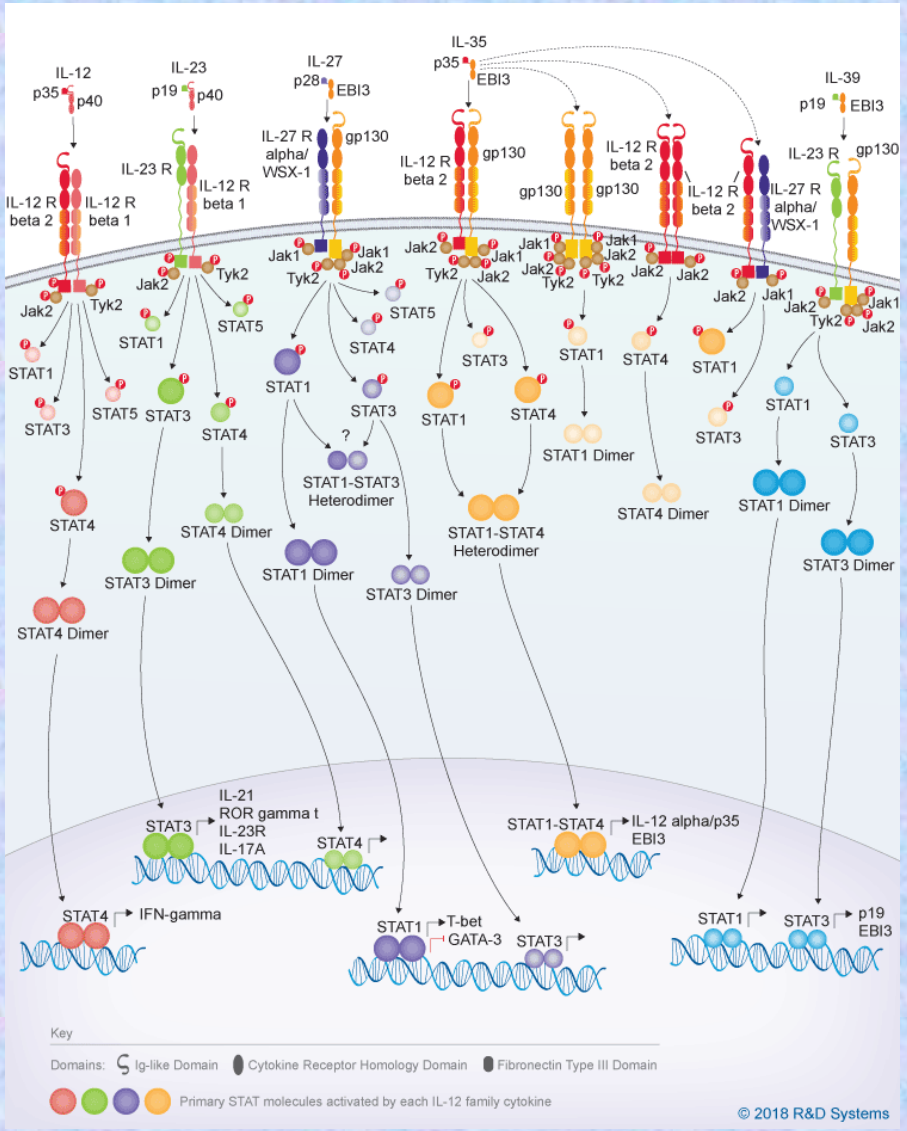
**Medina TS**, Oliveira GG, Silva MC, David BA, Silva GK, Fonseca DM, Sesti-Costa R, Frade AF, Baron MA, Ianni B, Pereira AC, Chevillard C, Cunha-Neto E, Marin-Neto JA, Silva JS. Ebi3 Prevents Trypanosoma cruzi-Induced Myocarditis by Dampening IFN-γ-Driven Inflammation. Front Immunol. 2017 Sep 26;8:1213. doi: 10.3389/fimmu.2017.01213. PMID: 29033934; PMCID: PMC5626942.

**Tosello Boari** J, Amezcua Vesely MC, Bermejo DA, Ramello MC, Montes CL, Cejas H, Gruppi A, Acosta Rodríguez EV. IL-17RA signaling reduces inflammation and mortality during Trypanosoma cruzi infection by recruiting suppressive IL-10-producing neutrophils. PLoS Pathog. 2012;8(4):e1002658. doi: 10.1371/journal.ppat.1002658. Epub 2012 Apr 26. PMID: 22577359; PMCID: PMC3343119.

**da Matta Guedes** PM, Gutierrez FR, Maia FL, Milanezi CM, Silva GK, Pavanelli WR, Silva JS. IL-17 produced during Trypanosoma cruzi infection plays a central role in regulating parasite-induced myocarditis. PLoS Negl Trop Dis. 2010 Feb 16;4(2):e604. doi: 10.1371/journal.pntd.0000604. PMID: 20169058; PMCID: PMC2821906.

IL-23 is a key stimulatory cytokine for Th17 and innate “type 17” cells that can respond immediately to pathogenic insults; IL-23 may thus also suppress IFN-γ by promoting IL17RA signaling *(Gaffen et al. 2013)*. Infection of Ebi3/IL-27p28 deficient mice is accompanied by increased IFN-γ production, with augmented Th1 immune response *(Böhme et al. 2013)*. Mechanistically, Ebi3 signaling modulates overproduction of IFN-γ, by inducing a population of IL-10 producing Tr1 T cells *(Medina et al. 2013)*.




**Gaffen SL**, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol. 2014 Sep;14(9):585-600. doi: 10.1038/nri3707. PMID: 25145755; PMCID: PMC4281037.

**Böhme J**, Roßnagel C, Jacobs T, Behrends J, Hölscher C, Erdmann H. Epstein-Barr virus-induced gene 3 suppresses T helper type 1, type 17 and type 2 immune responses after Trypanosoma cruzi infection and inhibits parasite replication by interfering with alternative macrophage activation. Immunology. 2016 Mar;147(3):338-48. doi: 10.1111/imm.12565. PMID: 26694585; PMCID: PMC4754611.

**Medina TS**, Oliveira GG, Silva MC, David BA, Silva GK, Fonseca DM, Sesti-Costa R, Frade AF, Baron MA, Ianni B, Pereira AC, Chevillard C, Cunha-Neto E, Marin-Neto JA, Silva JS. Ebi3 Prevents Trypanosoma cruzi-Induced Myocarditis by Dampening IFN-γ-Driven Inflammation. Front Immunol. 2017 Sep 26;8:1213. doi: 10.3389/fimmu.2017.01213. PMID: 29033934; PMCID: PMC5626942.

## Immune dynamics during infection

T. cruzi subverts a highly conserved cellular pathway for the repair of plasma membrane lesions and explores endogenous cellular machinery for invasion, escape from the parasitophorous vacuole, which allows intracytoplasmic survival, and replication.

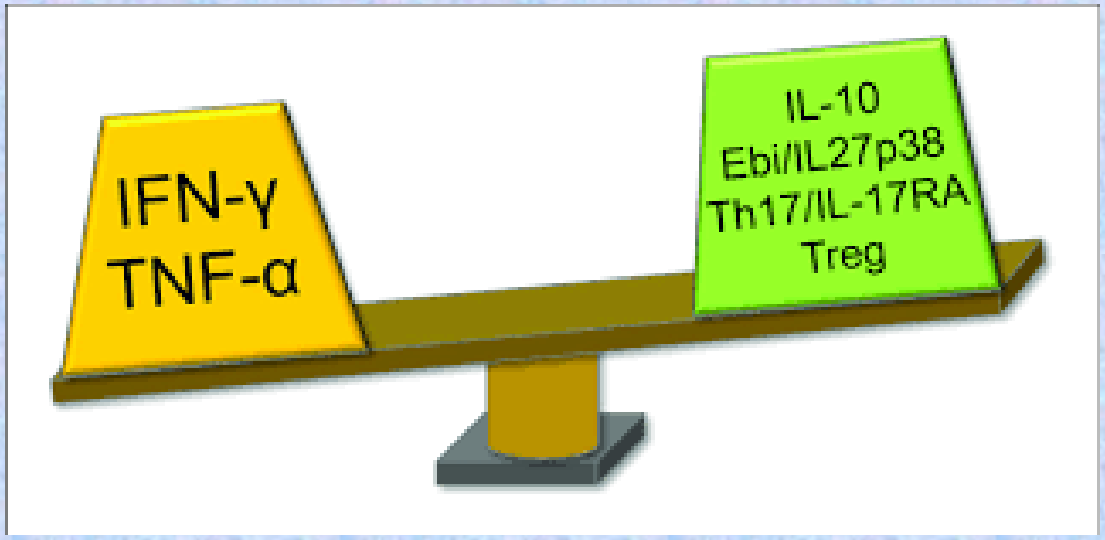
Trypanosoma cruzi is internalized by several different mechanisms, but end up in the phagolysosomal compartment, where T. cruzi DNA and RNA engage TLR7 and TLR9. TLR engagement promotes the Myd88-mediated activation of NF-kB. Lysosome acidification promotes escape of T. cruzi to the cytoplasm, where it differentiates into the replicative amastigote forms.

Replication in the cytoplasm leads to activation of inflammasomes that can induce inflammatory cytokines and NF-kB activation. This induces pro-inflammatory cytokines including IL-12, a PRG which elicits differentiation of IFN-γ-producing Th1 cells soon after infection, promoting Th1 cell differentiation. IFN-γ induces expression of multiple other PRGs, such as TNFA and NOS2.

The immune response that occurs during acute infection leads to partial parasite control. T. cruzi evades complete eradication, leading to the establishment of a chronic persistent infection with low parasitism. T. cruzi-infected individuals maintain increased production of inflammatory/Th1 cytokines like IFN-γ and TNF-α as compared to healthy individuals, as a result of persistent stimulus of innate and specific immunity.

CCC patients show an increased number of IFN-γ- producing Th1 T cells and plasma TNF-α levels as compared with ASY. Conversely, numbers of IL-10-producing $CD4^+$ $CD25^+$ regulatory T cells (Tregs) $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs and Th17 cells, as well as Ebi3/IL-27p28 levels are lower as compared with ASY *(Cai et al. 2016; Sousa et al. 2017)*.

The exacerbated Th1 response observed in the peripheral blood of CCC patients is reflected on the Th1-rich inflammatory infiltrate predominantly secreting IFN-γ and TNF-α. A lower, but significant, production of IL-4, IL-6, IL-7, IL-15, IL-18 was found in their heart tissue *(Cunha-neto et al. 2005; Higuchi Mde et al. 1993; Reis et al. 2013; Reis et al. 2013)*.




**Cai CW**, Blase JR, Zhang X, Eickhoff CS, Hoft DF. Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. PLoS Pathog. 2016 Oct 3;12(10):e1005902. doi: 10.1371/journal.ppat.1005902. PMID: 27695083; PMCID: PMC5047564.

**Sousa GR**, Gomes JA, Damasio MP, Nunes MC, Costa HS, Medeiros NI, Fares RC, Chaves AT, Corrêa-Oliveira R, Rocha MO. The role of interleukin 17-mediated immune response in Chagas disease: High level is correlated with better left ventricular function. PLoS One. 2017 Mar 9;12(3):e0172833. doi: 10.1371/journal.pone.0172833. PMID: 28278264; PMCID: PMC5344340.

**Cunha-Neto E**, Dzau VJ, Allen PD, Stamatiou D, Benvenutti L, Higuchi ML, Koyama NS, Silva JS, Kalil J, Liew CC. Cardiac gene expression profiling provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. Am J Pathol. 2005 Aug;167(2):305-13. doi:10.1016/S0002-9440(10)62976-8. PMID: 16049318; PMCID: PMC1603558.

**Higuchi Mde L**, Gutierrez PS, Aiello VD, Palomino S, Bocchi E, Kalil J, Bellotti G, Pileggi F. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. Virchows Arch A Pathol Anat Histopathol. 1993;423(3):157-60. doi: 10.1007/BF01614765. PMID: 7901937.

**Reis DD**, Jones EM, Tostes S Jr, Lopes ER, Gazzinelli G, Colley DG, McCurley TL. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A+, CD8+ lymphocytes. Am J Trop Med Hyg. 1993 May;48(5):637-44. doi: 10.4269/ajtmh.1993.48.637. PMID: 8517482.

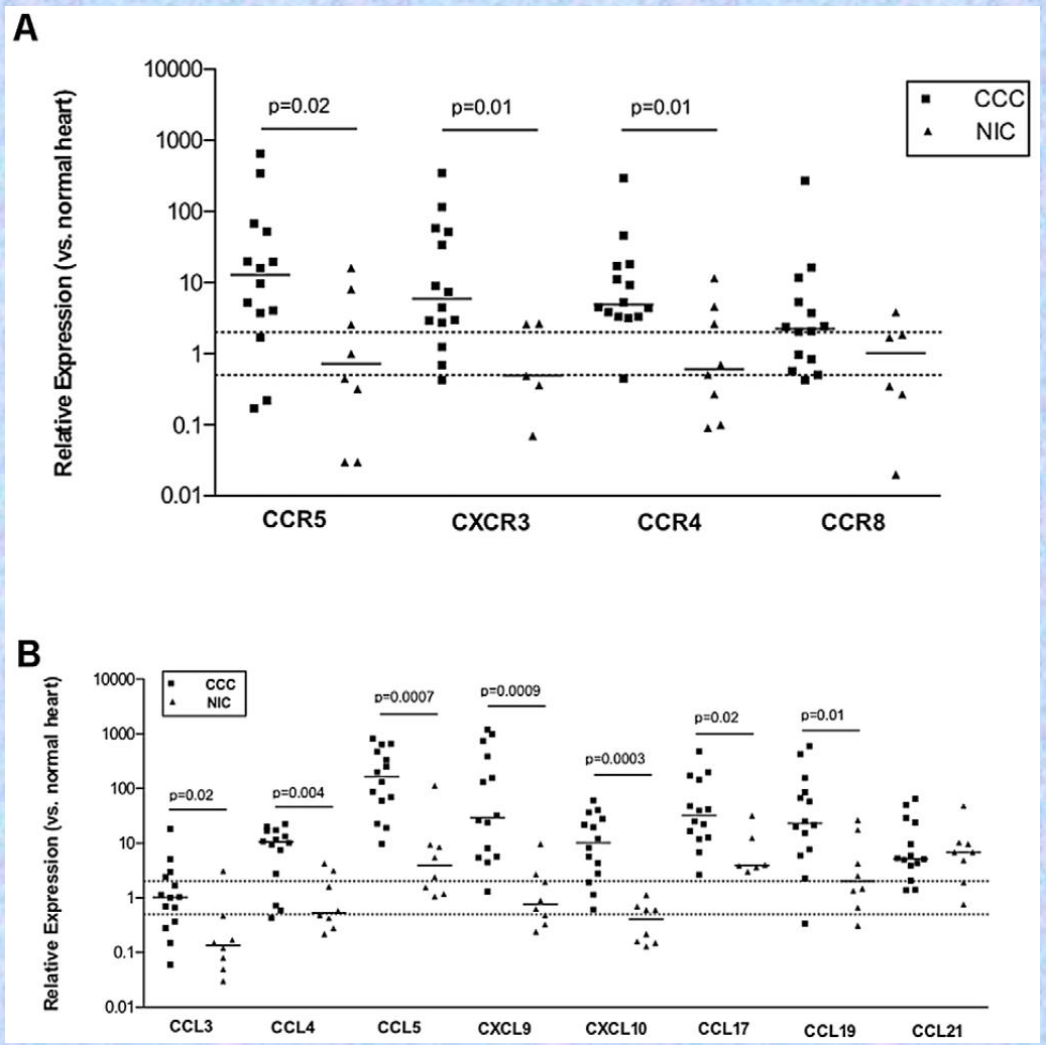
**Reis MM**, Higuchi Mde L, Benvenuti LA, Aiello VD, Gutierrez PS, Bellotti G, Pileggi F. An in situ quantitative immunohistochemical study of cytokines and IL-2R+ in chronic human chagasic myocarditis: correlation with the presence of myocardial Trypanosoma cruzi antigens. Clin Immunol Immunopathol. 1997 May;83(2):165-72. doi: 10.1006/clin.1997.4335. PMID: 9143377.

A positive correlation between Tbet expression and left ventricular dilation, corroborating the pathogenic role of Tbet positive/IFN-γ producing T cells toward CCC.

IFN-γ-producing CCR5+CXCR3+ Th1 T cells are more abundant in CCC than ASY, and the same cells were identified in CCC heart tissue, along with their chemokine ligands (CCL3, CCL4, CCL5, CXCL9, and CXCL10, respectively). CCL5 and CXCL9 were the most highly expressed chemokine mRNAs, and the intensity of the myocardial inflammation was positively correlated with CXCL9 mRNA expression *(*Nogueira *et al. 2017*).

The lack of regulation could explain the destructiveness of the inflammatory infiltrate, most likely due to excessive collateral damage by IFN-γ-producing T cells.

The immunomodulatory profile of ASY patients, with increased levels of DTG and lower levels of the PRG IFN-γ, indicates that ASY patients are in a state of disease tolerance. It is interesting to notice that the majority of chronic Chagas disease patients −60% are disease-tolerant ASY patients.



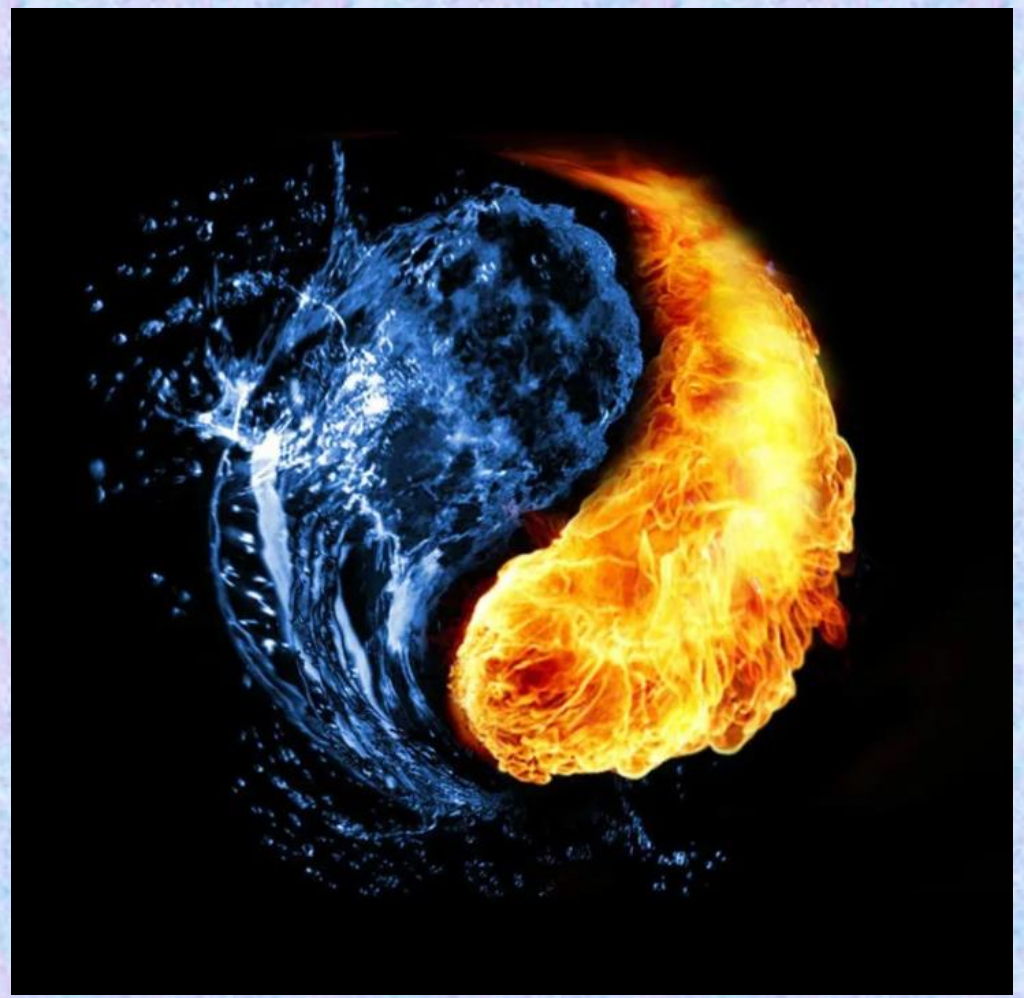

**Nogueira LG**, Santos RH, Ianni BM, Fiorelli AI, Mairena EC, Benvenuti LA, Frade A, Donadi E, Dias F, Saba B, Wang HT, Fragata A, Sampaio M, Hirata MH, Buck P, Mady C, Bocchi EA, Stolf NA, Kalil J, Cunha-Neto E. Myocardial chemokine expression and intensity of myocarditis in Chagas cardiomyopathy are controlled by polymorphisms in CXCL9 and CXCL10. PLoS Negl Trop Dis. 2012;6(10):e1867. doi: 10.1371/journal.pntd.0001867. Epub 2012 Oct 25. PMID: 23150742; PMCID: PMC3493616.

## IFN has a Yin-Yang effect

While IFN-γ can control parasites, excessive levels can cause tissue damage and death in the acute and chronic phases.

It regulates more than 1,000 genes through activation of Janus tyrosine kinase (JAK) and phosphorylation of transducer and activator of transcription 1 (STAT-1) pathway.

Among key IFN-γ-inducible inflammatory genes are TNF-α, and several other inflammatory cytokines and chemokines, interferon-inducible factor 1 (IRF1) and other PRG, including inducible nitric oxide synthase (NOS2)

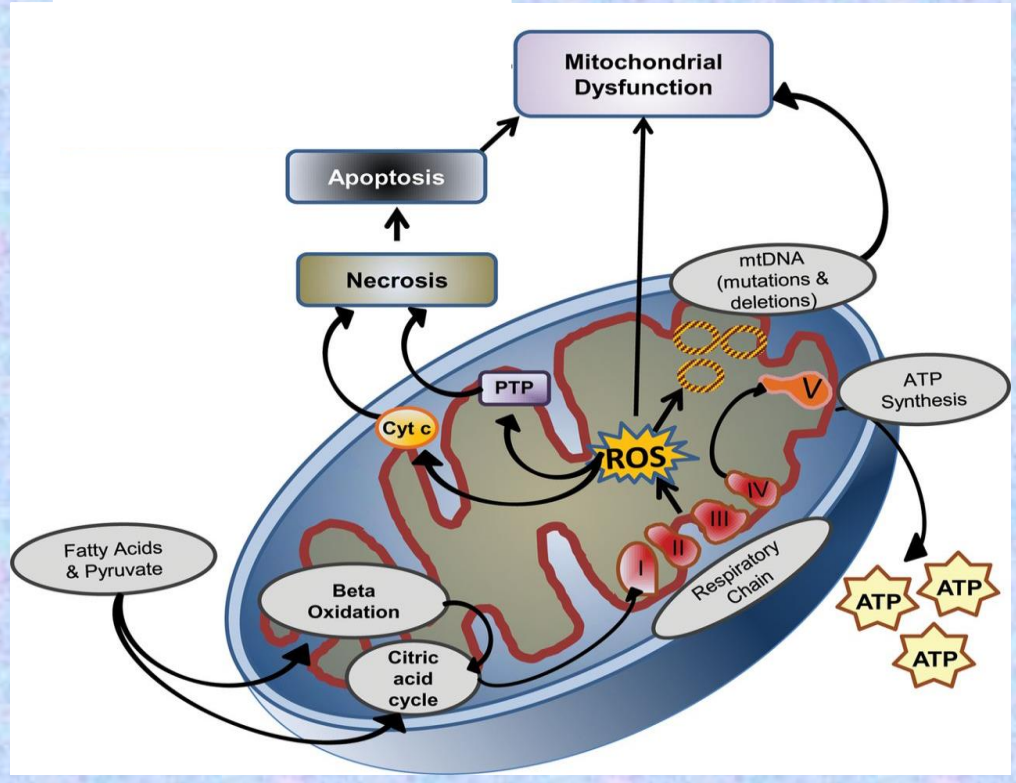


Systemic T. cruzi persistence drives continued production of IFN-γ by T cells, important for parasite control through NOS production, as well as activating ROS through induction of NADP oxidases and mitochondrial ROS through NF-kB *(Hölscher et al. 1998)*. However, T. cruzi is highly resistant to ROS; IFN-γ and the accompanying NOS and ROS can also induce severe disturbances of heart function.

**Hölscher C**, Köhler G, Müller U, Mossmann H, Schaub GA, Brombacher F. Defective nitric oxide effector functions lead to extreme susceptibility of Trypanosoma cruzi-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. Infect Immun. 1998 Mar;66(3):1208-15. doi: 10.1128/IAI.66.3.1208-1215.1998. PMID: 9488415; PMCID: PMC108035.

In cardiomyocytes, IFN-γ treatment reduced contractility, induced NO/peroxynitrite-dependent cardiomyocyte apoptosis, reduced cardiomyocyte area, and also induced atrial natriuretic factor and production of chemokines CCL3, CCL5, and CXCL1. IFN-γ regulates cardiac fibrosis by increasing fibroblast proliferation, production of hyaluronan and metalloproteinases 2 and 9.

Evidence of mitochondrial dysfunction has been found in hearts of animal models of Chagas disease, as well as the myocardium of CCC patients. This is especially relevant for CCC pathogenesis, since mitochondrial dysfunction is a paramount feature of heart failure of diverse etiologies *(*Brown *et al. 2013;* Wan *et al. 2013*).



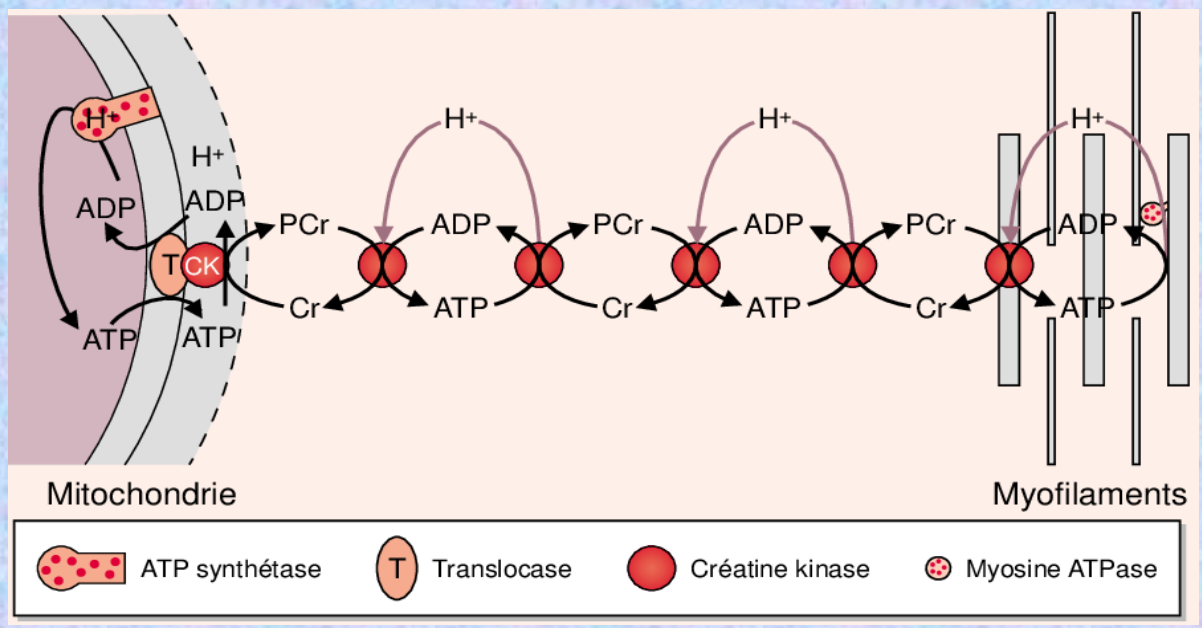

**Brown DA**, Perry JB, Allen ME, Sabbah HN, Stauffer BL, Shaikh SR, Cleland JG, Colucci WS, Butler J, Voors AA, Anker SD, Pitt B, Pieske B, Filippatos G, Greene SJ, Gheorghiade M. Expert consensus document: Mitochondrial function as a therapeutic target in heart failure. Nat Rev Cardiol. 2017 Apr;14(4):238-250. doi: 10.1038/nrcardio.2016.203. Epub 2016 Dec 22. PMID: 28004807; PMCID: PMC5350035.

**Wan X**, Wen JJ, Koo SJ, Liang LY, Garg NJ. SIRT1-PGC1α-NFκB Pathway of Oxidative and Inflammatory Stress during Trypanosoma cruzi Infection: Benefits of SIRT1-Targeted Therapy in Improving Heart Function in Chagas Disease. PLoS Pathog. 2016 Oct 20;12(10):e1005954. doi: 10.1371/journal.ppat.1005954. PMID: 27764247; PMCID: PMC5072651.

## Chronic phase is associated to mitochondrial dysfunction in hearts

- Cunha-Neto *et al.* described altered expression of mitochondrial genes and 16S mitochondrial rRNA in CCC heart lesions (Cunha-neto *et al. 2005*).

- A selective reduction of protein expression of ATP synthase and creatine kinase activity—key mitochondrial energy metabolism enzymes-in CCC heart lesions (Teixeira *et al. 2005*).




- mitochondrial DNA content was found to be reduced in CCC heart tissue, further indicating that mitochondrial function is compromised in CCC.

**Cunha-Neto E**, Dzau VJ, Allen PD, Stamatiou D, Benvenutti L, Higuchi ML, Koyama NS, Silva JS, Kalil J, Liew CC. Cardiac gene expression profiling provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. Am J Pathol. 2005 Aug;167(2):305-13. doi:10.1016/S0002-9440(10)62976-8. PMID: 16049318; PMCID: PMC1603558.

**Teixeira PC**, Santos RH, Fiorelli AI, Bilate AM, Benvenuti LA, Stolf NA, Kalil J, Cunha-Neto E. Selective decrease of components of the creatine kinase system and ATP synthase complex in chronic Chagas disease cardiomyopathy. PLoS Negl Trop Dis. 2011 Jun;5(6):e1205. doi: 10.1371/journal.pntd.0001205. Epub 2011 Jun 28. PMID: 21738806; PMCID: PMC3125151.

**Wan** X, Gupta S, Zago MP, Davidson MM, Dousset P, Amoroso A, Garg NJ. Defects of mtDNA replication impaired mitochondrial biogenesis during Trypanosoma cruzi infection in human cardiomyocytes and chagasic patients: the role of Nrf1/2 and antioxidant response. J Am Heart Assoc. 2012 Dec;1(6):e003855. doi: 10.1161/JAHA.112.003855. Epub 2012 Dec 19. PMID: 23316324; PMCID: PMC3540675.

Evidence indicates that many damaging effects of IFN-γ are secondary to promoting peroxynitrite-dependent and independent mitochondrial dysfunction and oxidative stress.

IFN-γ effects on mitochondria include

inhibition of the oxidative metabolism (Luss *et al. 1995*).
an increased rate of ATP depletion (Wang *et al. 1996*).
an inhibition of creatine kinase expression (Kalovidouris *et al. 1993*).

NF-KB activation is one of the main mechanisms of mitochondrial damage induced by IFN-γ.

IFN-γ/TNF-α-driven NF-kB activation is known to cause dissipation of the proton gradient and impairment of the mitochondrial membrane potential (MMP) and ATP synthesis, leading to apoptosis (Lee *et al. 2007*; Zorava *et al. 2017*).

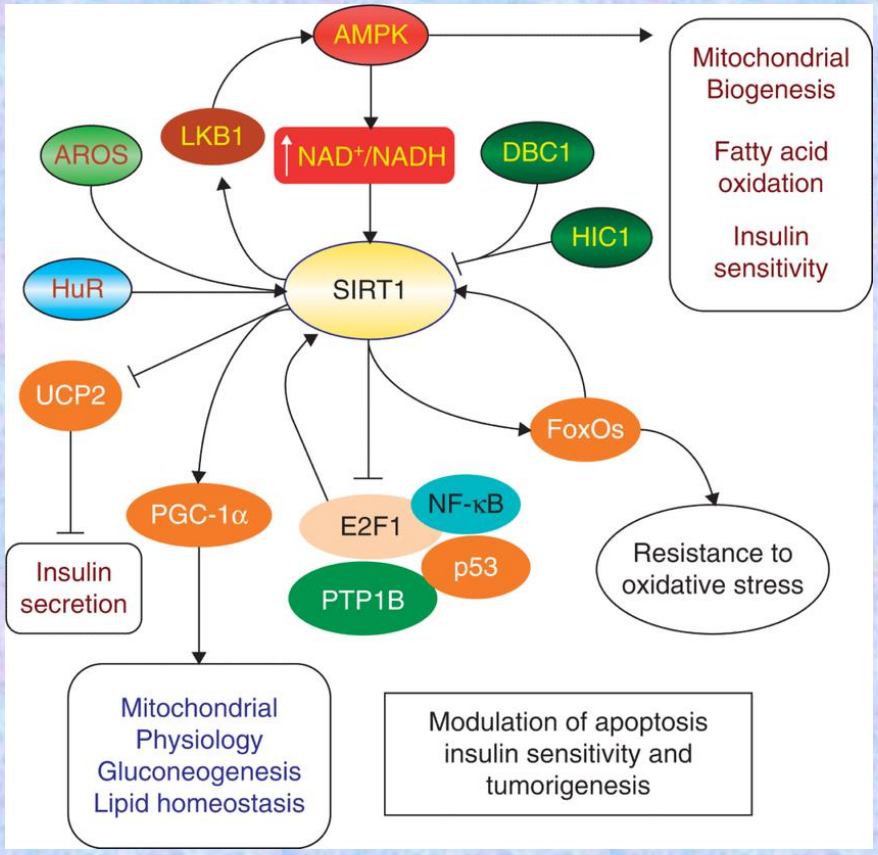
inhibition of NF-kB has been shown to improve MMP with substantial decrease of NOS2/NO induction and ROS release. Excessive mitochondrial ROS production by cardiomyocytes is considered as a central cause of heart failure

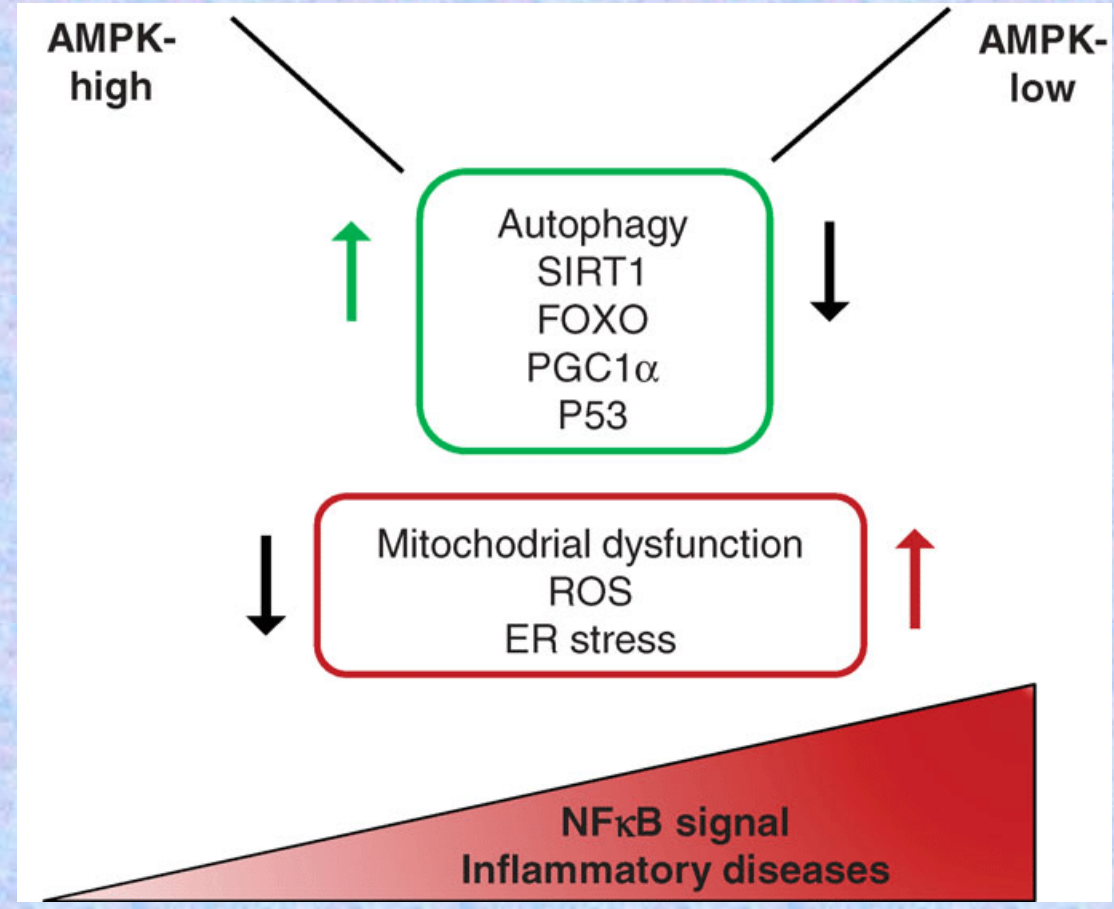
**Luss H**, Watkins SC, Freeswick PD, Imro AK, Nussler AK, Billiar TR, Simmons RL, del Nido PJ, McGowan FX Jr. Characterization of inducible nitric oxide synthase expression in endotoxemic rat cardiac myocytes in vivo and following cytokine exposure in vitro. J Mol Cell Cardiol. 1995 Sep;27(9):2015-29. doi: 10.1016/0022-2828(95)90023-3. PMID: 8523461.
**Wang D**, McMillin JB, Bick R, Buja LM. Response of the neonatal rat cardiomyocyte in culture to energy depletion: effects of cytokines, nitric oxide, and heat shock proteins. Lab Invest. 1996 Dec;75(6):809-18. PMID: 8973476.
**Kalovidouris AE**, Plotkin Z, Graesser D. Interferon-gamma inhibits proliferation, differentiation, and creatine kinase activity of cultured human muscle cells. II. A possible role in myositis. J Rheumatol. 1993 Oct;20(10):1718-23. PMID: 8295184.
**Lee HJ**, Oh YK, Rhee M, Lim JY, Hwang JY, Park YS, Kwon Y, Choi KH, Jo I, Park SI, Gao B, Kim WH. The role of STAT1/IRF-1 on synergistic ROS production and loss of mitochondrial transmembrane potential during hepatic cell death induced by LPS/d-GalN. J Mol Biol. 2007 Jun 15;369(4):967-84. doi: 10.1016/j.jmb.2007.03.072. Epub 2007 Apr 1. PMID: 17475277.
**Zorova LD**, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, Babenko VA, Zorov SD, Balakireva AV, Juhaszova M, Sollott SJ, Zorov DB. Mitochondrial membrane potential. Anal Biochem. 2018 Jul 1;552:50-59. doi: 10.1016/j.ab.2017.07.009. Epub 2017 Jul 12. PMID: 28711444; PMCID: PMC5792320.

A significant crosstalk occurs between NF-kB and mitochondrion-protecting proteins. NF-kB signaling down-regulates sirtuin-1 (SIRT1) activity through the expression of IFN-γ, ROS, and NO (Kauppinen *et al. 1995*).

SIRT1, an antioxidant and anti-inflammatory protein, regulates the oxidative respiration and cellular survival and is highly expressed in the heart, acting as an inhibitor of NF-kB inflammatory signals (Caruso *et al. 1995*).

SIRT1 enhances mitochondrial oxidative metabolism through 5'AMP-activated protein kinase (AMPK) resulting in the resolution of inflammation (Kauppinen *et al. 1995*).



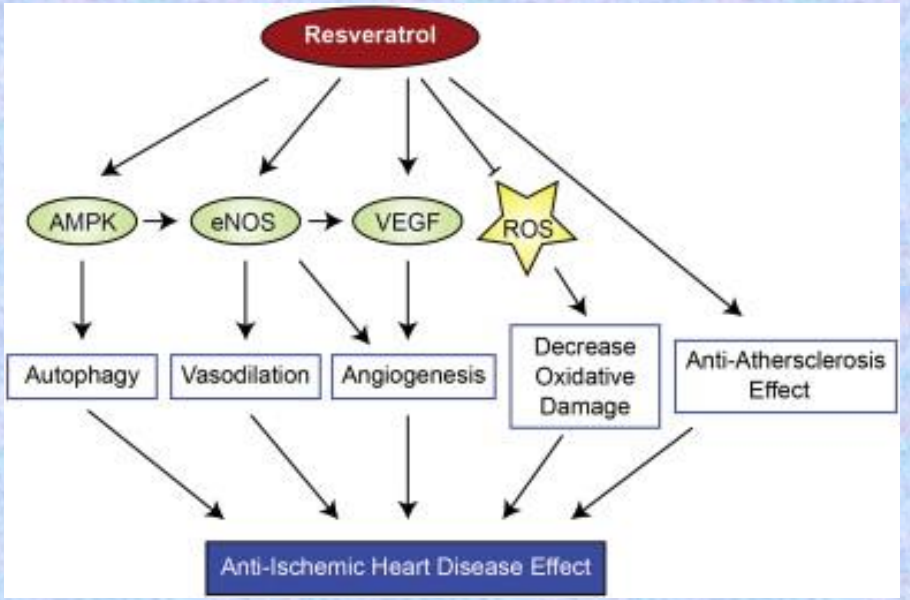



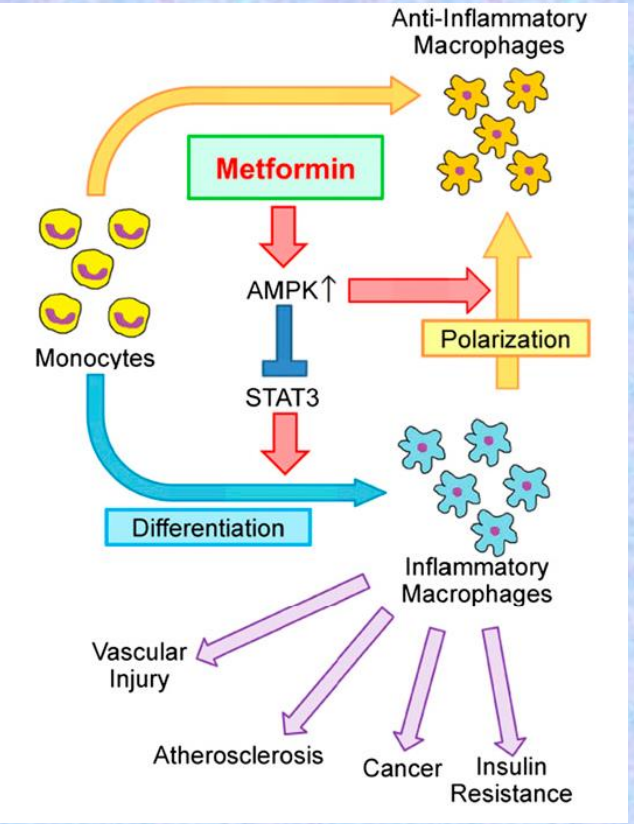

**Kauppinen A**, Suuronen T, Ojala J, Kaarniranta K, Salminen A. Antagonistic crosstalk between NF-κB and SIRT1 in the regulation of inflammation and metabolic disorders. Cell Signal. 2013 Oct;25(10):1939-48. doi: 10.1016/j.cellsig.2013.06.007. Epub 2013 Jun 11. PMID: 23770291.

**Caruso R**, Marafini I, Franzè E, Stolfi C, Zorzi F, Monteleone I, Caprioli F, Colantoni A, Sarra M, Sedda S, Biancone L, Sileri P, Sica GS, MacDonald TT, Pallone F, Monteleone G. Defective expression of SIRT1 contributes to sustain inflammatory pathways in the gut. Mucosal Immunol. 2014 Nov;7(6):1467-79. doi: 10.1038/mi.2014.35. Epub 2014 May 21. PMID: 24850427.

Treatment of T. cruzi-infected mice with SIRT1 and/or AMPK agonists SRT1720, resveratrol and metformin reduced myocardial NF-kB transcriptional activity, inflammation and oxidative stress, resulting in beneficial results for restoration of cardiac function (Wan *et al. 2016*; Vilar-Pereira *et al. 2015*).

Preserving Nrf2 activity was shown to arrest the mitochondrial and cardiac oxidative stress, cardiac fibrosis, and heart failure in murine T. cruzi infection (Wen *et al. 1995*). Nrf2 is the master regulator of the antioxidant response, a transcription factor controlling expression of hundreds of genes (Kovac *et al. 2015*), and promotes mitochondrial biogenesis (Dinkova-Kostova *et al. 2015*).




**Wan X**, Wen JJ, Koo SJ, Liang LY, Garg NJ. SIRT1-PGC1α-NFκB Pathway of Oxidative and Inflammatory Stress during Trypanosoma cruzi Infection: Benefits of SIRT1-Targeted Therapy in Improving Heart Function in Chagas Disease. PLoS Pathog. 2016 Oct 20;12(10):e1005954. doi: 10.1371/journal.ppat.1005954. PMID: 27764247; PMCID: PMC5072651.

**Vilar-Pereira G**, Carneiro VC, Mata-Santos H, Vicentino AR, Ramos IP, Giarola NL, Feijó DF, Meyer-Fernandes JR, Paula-Neto HA, Medei E, Bozza MT, Lannes- Vieira J, Paiva CN. Resveratrol Reverses Functional Chagas Heart Disease in Mice. PLoS Pathog. 2016 Oct 27;12(10):e1005947. doi: 10.1371/journal.ppat.1005947. PMID: 27788262; PMCID: PMC5082855.

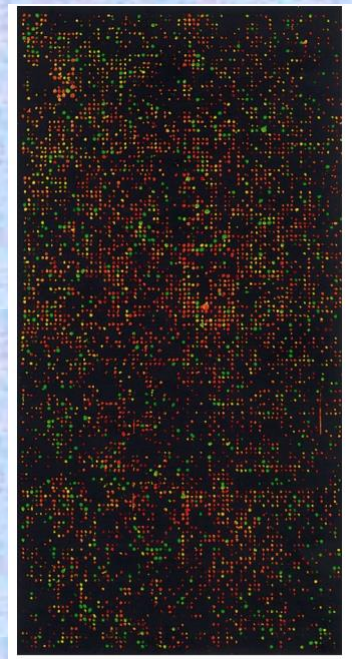
**Wen JJ**, Porter C, Garg NJ. Inhibition of NFE2L2-Antioxidant Response Element Pathway by Mitochondrial Reactive Oxygen Species Contributes to Development of Cardiomyopathy and Left Ventricular Dysfunction in Chagas Disease. Antioxid Redox Signal. 2017 Sep 20;27(9):550-566. doi: 10.1089/ars.2016.6831. Epub 2017 Jul 13. PMID: 28132522; PMCID: PMC5567598.

**Kovac S**, Angelova PR, Holmström KM, Zhang Y, Dinkova-Kostova AT, Abramov AY. Nrf2 regulates ROS production by mitochondria and NADPH oxidase. Biochim Biophys Acta. 2015 Apr;1850(4):794-801. doi: 10.1016/j.bbagen.2014.11.021. Epub 2014 Dec 5. PMID: 25484314; PMCID: PMC4471129.

**Dinkova-Kostova AT**, Abramov AY. The emerging role of Nrf2 in mitochondrial function. Free Radic Biol Med. 2015 Nov;88(Pt B):179-188. doi: 10.1016/j.freeradbiomed.2015.04.036. Epub 2015 May 11. PMID: 25975984; PMCID: PMC4726722.

# Our results

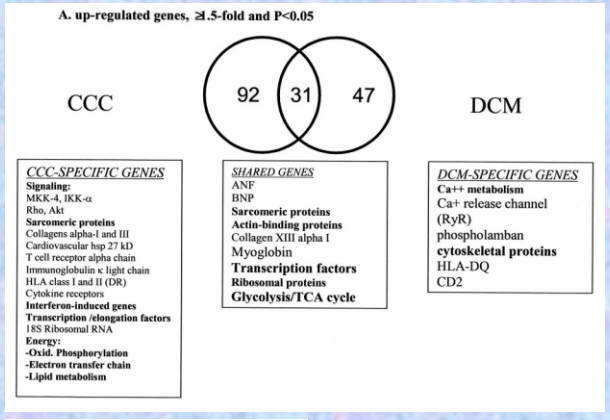
# Gene expression analysis

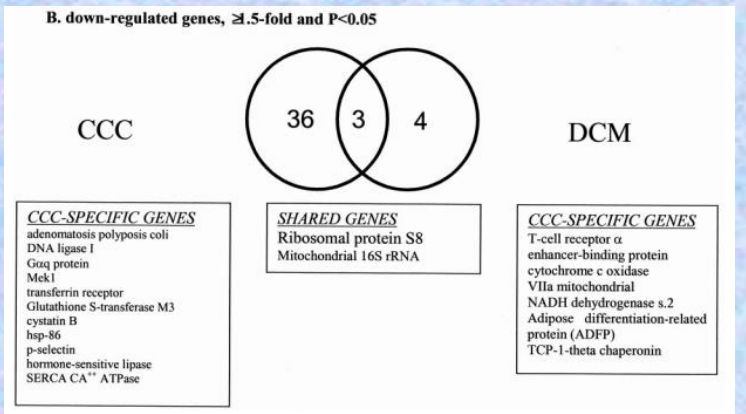


## CardioChip microarrays

To obtain **the first human genomic portrait** of heart failure derived from end-stage chagas patients, Cunha-Neto E. et al. explored expression analysis using the CardioChip microarray constructed in-house *(Cunha-Neto et al. 2005)*. Compared the gene expression fingerprint of CCC (n=7) with that of DCM (n=9), using non failing adult hearts as expression controls (n=4), and found that gene expression patterns are markedly different in CCC and DCM, with significant activity of IFN-inducible genes in CCC patients.

A hierarchical cluster analysis of all of the CCC, and most of the DCM and normal heart samples clustered together with other members of their groups. 582 and 364 genes were significantly up-regulated in CCC and DCM, respectively, including 126 genes that were up-regulated in both conditions. Similarly, 465 genes were significantly down-regulated in CCC and 32 in DCM.




Among genes selectively up-regulated in CCC, they observed that the participation of cell defense and metabolism categories was substantially higher than the frequency of normal heart samples. Cell defense genes up-regulated specifically in CCC are immune response genes.

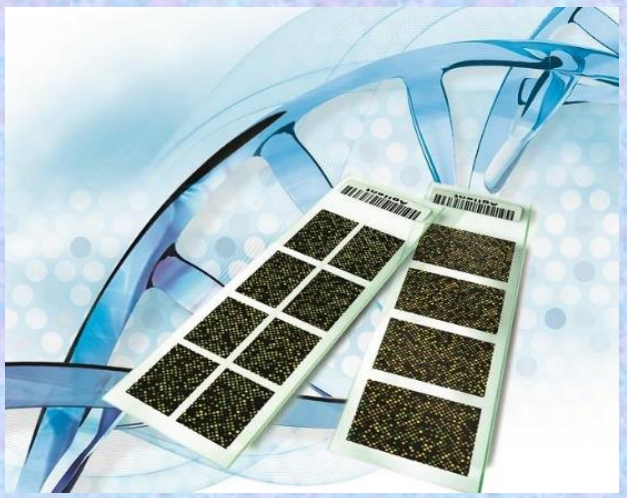

**Cunha-Neto E**, Dzau VJ, Allen PD, Stamatiou D, Benvenutti L, Higuchi ML, Koyama NS, Silva JS, Kalil J, Liew CC. Cardiac gene expression profiling provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. Am J Pathol. 2005 Aug;167(2):305-13. doi:10.1016/S0002-9440(10)62976-8. PMID: 16049318; PMCID: PMC1603558.

- Among cell defense genes, they found the MHC class I/class II molecules, IFN-γ receptor, IL-7 receptor, T-cell antigen receptor, immunoglobulin chains, cytotoxic granule nucleolysin, and IFN-inducible genes.
- Among metabolism genes, up regulated genes were related to energy metabolism, including seven proteins of the oxidative phosphorylation and proton transfer chains, and eight enzymes involved in lipid metabolism.
- Up-regulated structural genes comprise sarcomere-building contractile and cytoskeletal proteins, and collagens I and III.
- For signaling genes already associated to heart failure, they found angiotensin II receptor 2, G protein β polypeptide 2, several low-molecular weight GTPases such as rhoB, RAB1, RalB, along with IKK-α and mitogen-activated kinase MKK4, protein kinase H11, and its induced anti-apoptotic gene Akt-1.

Given the prominent local production of IFN-γ in CCC heart **fifteen percent of the 78 IFN-induced genes** present in the Cardiochip cDNA microarray, were up-regulated in CCC. Significantly, along with inflammatory response genes likely to be expressed by infiltrating inflammatory cells (eg, immunoglobulin, T-cell receptor genes, cytokine receptors), they observed several genes not known to be expressed by inflammatory cells (cardiovascular 27-kd hsp, angiotensin II receptor 2, fatty acid-binding protein 5). Of interest, the SERCA Ca++-ATPase, involved in cardiac calcium metabolism and down-modulated in CCC hearts, is also repressible by IFN-γ.

These data suggests, for the first time, that locally produced T-cell-dependent IFN-γ, MCP-1, and possibly other inflammatory cytokines may directly up-regulate the myocardial expression of ANF, a hypertrophy-related gene, in addition to inflammatory effector-mediated myocardial cell death. Authors postulate that this cytokine-induced gene modulation of myocardial gene expression may lead to the increased ventricular remodeling, morbidity, and mortality of CCC patients as compared to other cardiomyopathies. It is likely that the unique properties of the myocardium from CCC patients altered energy metabolism.

Transcriptome analysis was also performed on myocardial samples using Agilent SurePrint G3 Human GeneExpression v1 8x60K arrays. We found 1535 genes to be differentially expressed (DEG) between CCC and control myocardium, of which 1105 (72%) are upregulated in CCC. IPA canonical pathways analysis showed that the most enriched pathways are mainly immune-related, such as Th1 and Th2 T cells, dendritic cells/antigen presentation, leukocyte extravasation, NK and B cells; this is consistent with the high number of upregulated genes from the incoming inflammatory cells present in CCC but not in control heart tissue.



We also classified genes in additional relevant pathobiological processes and pathways such as inflammation, IFNγ-modulated genes/Th1 response, extracellular matrix, fibrosis, hypertrophy, contractility of heart, hypertrophy, arrhythmia, oxidative stress/antioxidant response, mitochondria, and mitochondria-related genes using IPA Knowledge Base (IKB) gene lists, which were in some cases merged with other published gene lists. The IFNγ-dependent/Th1 response gene list was merged with published IFNγ-induced/repressed gene lists, and the oxidative stress gene list was merged with Nrf2-modulated genes. The NF-kB-modulated gene list was obtained from Yang et al. *(Yang et al. 2016)*. The mitochondrial gene list was a combination of all genes contained in the Mitochondrion Gene Ontology term and Mitocarta 2.0 *(Calvo et al. 2015)*.

As expected, inflammation and IFNγ-dependent/Th1 response processes show the highest number of DEGs (361 and 148, respectively), followed by fibrosis (82) and hypertrophy (53). Of interest, we found a significant number of DEGs belonging to mitochondria and oxidative stress functions/processes (42 and 35, respectively). Some DEGs are shared by several biological functions/processes.

**Yang Y**, Jian Wu, Jinke Wang: A database and functional annotation of NF-κB target genes. Int J Clin Exp Med 2016;9(5):7986-7995.
**Calvo SE**, Clauser KR, Mootha VK. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res. 2016 Jan 4;44(D1):D1251-7. doi: 10.1093/nar/gkv1003. Epub 2015 Oct 7. PMID: 26450961; PMCID: PMC4702768.

**Among these processes inflammation may be specific to CCC as shown in previous gene expression studies** *(Nogueira et al. 2012, Nogueira et al. 2014)***.**

For the other processes, they have been also described in dilated cardiomyopathies of other etiologies. Upstream regulator analysis performed by IPA examines how many targets of each given transcriptional regulator are present in the DEGs - as well as the direction of change – based on the literature and IPA knowledge base; putative regulators are ranked according to overlap with expected targets and directionality (z-score). It indicated that IFNγ is the top upstream regulator, followed by other cytokines like TNFα, IL-18 and EBI3/IL27Rβ chain, the chemokines CCL5 and CXCL10, the transcription factors NF-kB and Ap1, and the PI3K enzyme.

27 cytokines and chemokines were upregulated in CCC heart tissue. Significantly, the 7 most upregulated among them were chemokines, including chemokine ligands of CCR5 (CCL5, CCL4) and CXCR3 CXCL9 and CXCL10). Multiple cytokines and chemokines that were top upstream regulators like IFNγ, CCL5, CXCL10, IL-18, IL-7, EBI3/IL-27b and IL-4 were found to be upregulated to different degrees in CCC myocardium. **This indicates that these cell types infiltrate the myocardium of CCC patients.**

**Nogueira LG**, Santos RH, Fiorelli AI, Mairena EC, Benvenuti LA, Bocchi EA, Stolf NA, Kalil J, Cunha-Neto E. Myocardial gene expression of T-bet, GATA-3, Ror-γt, FoxP3, and hallmark cytokines in chronic Chagas disease cardiomyopathy: an essentially unopposed TH1-type response. Mediators Inflamm. 2014;2014:914326. doi: 10.1155/2014/914326. Epub 2014 Jul 24. PMID: 25152568; PMCID: PMC4134835.

**Nogueira LG**, Santos RH, Ianni BM, Fiorelli AI, Mairena EC, Benvenuti LA, Frade A, Donadi E, Dias F, Saba B, Wang HT, Fragata A, Sampaio M, Hirata MH, Buck P, Mady C, Bocchi EA, Stolf NA, Kalil J, Cunha-Neto E. Myocardial chemokine expression and intensity of myocarditis in Chagas cardiomyopathy are controlled by polymorphisms in CXCL9 and CXCL10. PLoS Negl Trop Dis. 2012;6(10):e1867. doi: 10.1371/journal.pntd.0001867. Epub 2012 Oct 25. PMID: 23150742; PMCID: PMC4134835.


**Laugier L**, Ferreira LRP, Ferreira FM, Cabantous S, Frade AF, Nunes JP, Ribeiro RA, Brochet P, Teixeira PC, Santos RHB, Bocchi EA, Bacal F, Cândido DDS, Maso VE, Nakaya HI, Kalil J, Cunha-Neto E, Chevillard C. miRNAs may play a major role in the control of gene expression in key pathobiological processes in Chagas disease cardiomyopathy. PLoS Negl Trop Dis. 2020 Dec 22;14(12):e0008889. doi: 10.1371/journal.pntd.0008889. PMID: 33351798; PMCID: PMC7787679.

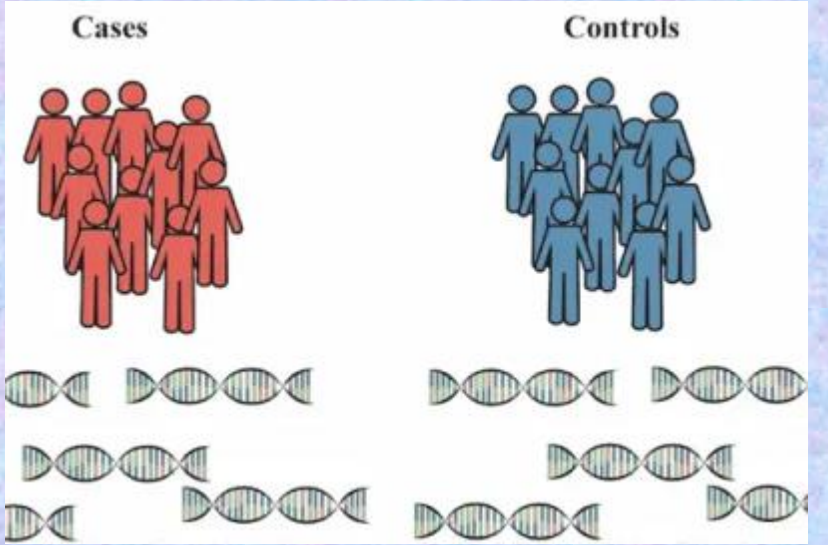
Conversely, genes down-regulated in CCC myocardium when compared to controls were enriched with signatures of cardiac muscle cells. **This result is most likely a consequence of reduced representation of cardiac mRNAs in CCC myocardium that was replaced by inflammatory cells.**

Our study pointed out IFN-γ is the top gene expression regulator with ca. 10% of DEGs being modulatable by it, in all pathobiological processes. Indeed, several studies have shown a negative impact of IFN-γ on the myocardium, leading to reduced contractility, release of chemokines and increased production of atrial natriuretic factor. IFN-γ-induced cardiac fibrosis with increased fibroblast proliferation, production of hyaluronan and metalloproteinases 2 and 9 has also been demonstrated. The role of IFN-γ, TNF-α and NF-kB as top upregulators are also in line with data in genetically modified murine models. Mice transgenic to IFN-γ developed a TNF-α-dependent inflammatory dilated cardiomyopathy with fibrosis and heart failure, and a very similar phenotype was developed by mice constitutively expressing active IKK2.

Mechanistically, IFN-γ induces TNF-α and potentiates TNF-α-mediated NF-kB signaling and upregulation of NOS2, leading to cardiomyocyte contractile dysfunction and apoptosis. This is mediated at least in part by NADPH-and NOS2-dependent production of reactive oxygen and nitrogen species (ROS and RNS, respectively), with oxidative and nitrosative stress. IFN-γ -induced RNS leads to inhibition of mitochondrial oxidative metabolism and ATP depletion in cardiomyocytes with ensuing mitochondrial dysfunction. Of interest, 169 DEGs, or ca 10% of DEGS are potentially modulated by NF-kB in CCC myocardium. Our data point towards IFN-γ and NF-kB-mediated signaling as a major player in Chagas cardiomyopathy; we believe they may have a central role in orchestrating the molecular processes that contribute to heart failure**.**


**Laugier L**, Ferreira LRP, Ferreira FM, Cabantous S, Frade AF, Nunes JP, Ribeiro RA, Brochet P, Teixeira PC, Santos RHB, Bocchi EA, Bacal F, Cândido DDS, Maso VE, Nakaya HI, Kalil J, Cunha-Neto E, Chevillard C. miRNAs may play a major role in the control of gene expression in key pathobiological processes in Chagas disease cardiomyopathy. PLoS Negl Trop Dis. 2020 Dec 22;14(12):e0008889. doi: 10.1371/journal.pntd.0008889. PMID: 33351798; PMCID: PMC7787679.

# Common variant association studies




## Candidate gene case/control studies

Chagas disease has a multifactorial etiology that involves complex host-parasite interactions governed by parasite and host genetics. Immune system mediators have been described to participate in driving heart and/or gut tissues inflammation, either through response to the parasite presence and, to a certain level, by autoimmune reactions.

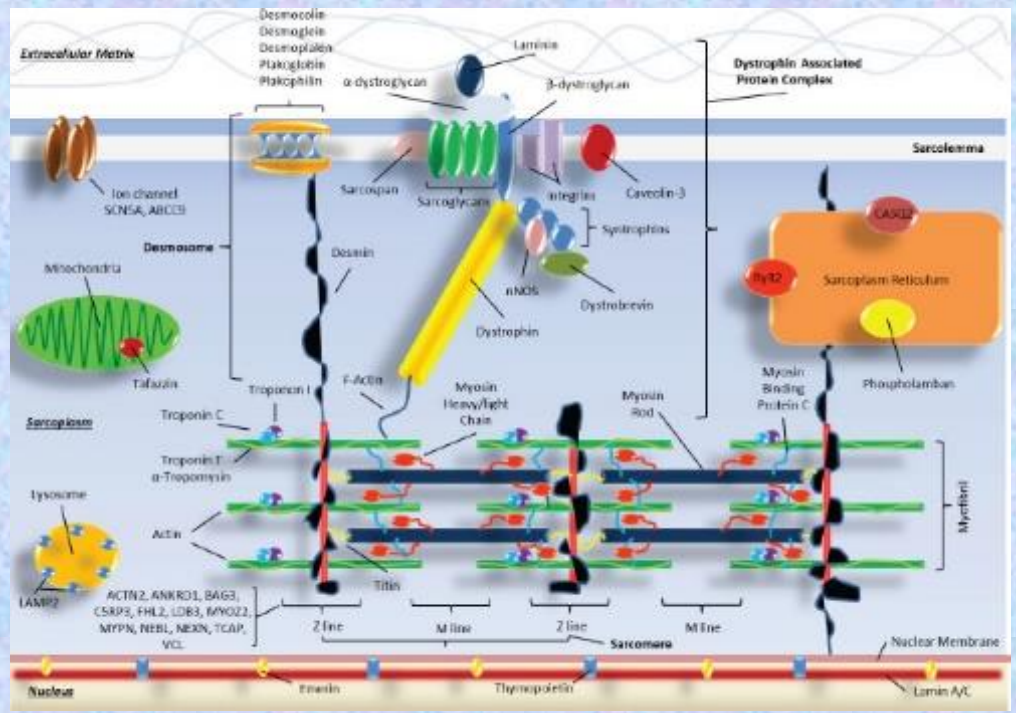
Most of the genetic studies performed so far have searched for sequence variations that could be associated to chronic Chagas cardiomyopathy susceptibility in immune system related genes. These searches followed a hypothesis-driven approach to find single nucleotide polymorphisms in genes known or suspected to play a role in those inflammatory phenomena. Amongst the genes studied there are: human leukocyte antigen (HLA) class I and class II alleles, cytokines (e.g.: IL-1β, IL-10, TNF-α, IL-17, IL-18) and chemokines and their receptors (MCP1/CCL2, CCR5, MIG/CXCL9,IP10/CXCL10) as well as inflammasome genes.

Some of these studies led to inconclusive results that may be explained in different ways: a) the use of seronegative subjects as controls which are inadequate controls, since it is unknown whether they were exposed to the pathogen; b) the relatively small size of the study groups which affected the power (the probability) to detect an association; c) the number of tested SNPs; d) the highly heterogeneous genetic background of the study population due to admixture; e) the sex ratio known to exist has not been taken in consideration.

**Last review on case control studies:**

**Acosta-Herrera M**, Strauss M, Casares-Marfil D, **Martín J**; Chagas Genetics CYTED Network. Genomic medicine in Chagas disease. Acta Trop. 2019 Sep;197:105062. doi: 10.1016/j.actatropica.2019.105062. Epub 2019 Jun 12. PMID: 31201776.

We showed that a single nucleotide polymorphism in the promoter region of the **alpha-cardiac actin gene (*ACTC1*)** associated with CCC influences transcription factor binding, implying that the polymorphism may influence myocardial transcriptional levels of the highly relevant ACTC1 gene. These results were obtained on a Brazilian population including 315 CCC patients and 118 asymptomatic individuals, and the same trend of association was found on a second independent cohort including 102 CCC patients and 36 asymptomatic individuals.





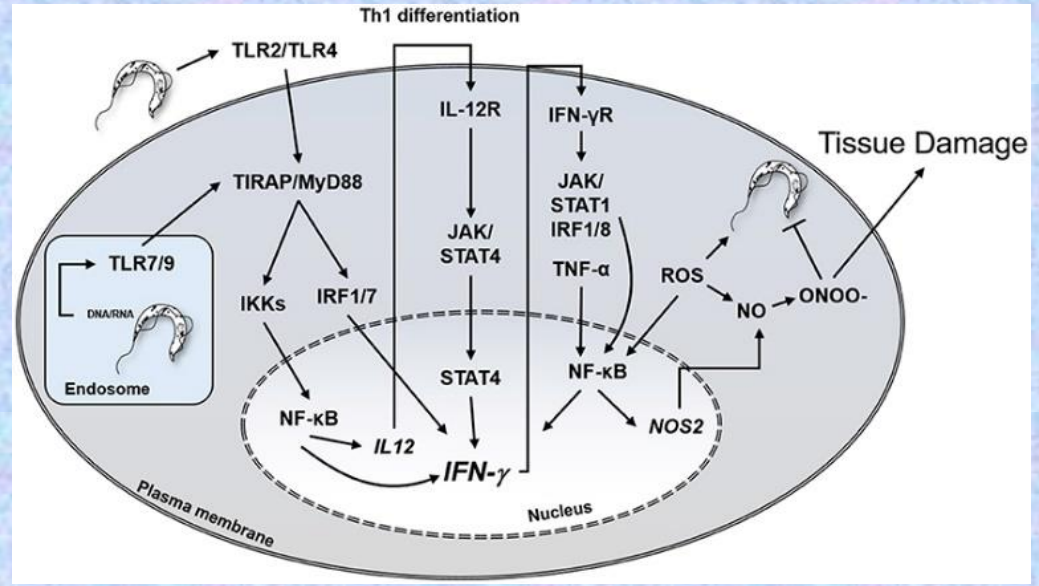
**Frade AF,** Teixeira PC, Ianni BM, Pissetti CW, Saba B, Wang LH, Kuramoto A, Nogueira LG, Buck P, Dias F, Giniaux H, Llored A, Alves S, Schmidt A, Donadi E, Marin-Neto JA, Hirata M, Sampaio M, Fragata A, Bocchi EA, Stolf AN, Fiorelli AI, Santos RH, Rodrigues V, Pereira AC, Kalil J, Cunha-Neto E, Chevillard C. Polymorphism in the alpha cardiac muscle actin 1 gene is associated to susceptibility to chronic inflammatory cardiomyopathy. PLoS One. 2013 Dec 19;8(12):e83446. doi: 10.1371/journal.pone.0083446. PMID: 24367596; PMCID: PMC3868584.

During acute *T. cruzi* infection, *T. cruzi* pathogen-associated molecular patterns (PAMPs) trigger innate immunity in multiple cell types, which release proinflammatory cytokines and chemokines, such as IL-1, IL-6, IL-12, IL-18, TNF-α, CCL2, CCL5, and CXCL9 activating and mobilizing migration of cascades of inflammatory cells. Antigen-presenting cells subsequently elicit a strong T cell and antibody response against *T. cruzi,* where IL-12 and IL-18 drive the differentiation of IFN-γ-producing *T. cruzi*–specific Th1 T cells which migrate to sites of *T. cruzi*-induced inflammation, including the myocardium, in response to locally produced chemokines. Th1 T cell and antibody responses lead to control but not complete elimination of tissue and blood parasitism, establishing a low-grade chronic persistent infection by *T. cruzi*.

As a result of persistent infection, both CCC and ASY chronic Chagas disease patients show a skewed Th1-type immune response, but those who develop Chagas cardiomyopathy display a particularly strong Th1-type immune response with increased numbers of IFN-γ-producing T cells in peripheral blood mononuclear cells (PBMC) as well as plasma TNF-α in comparison with uninfected or ASY patients.

We conducted a study focusing on **TIRAP, CCL2 and CCL5.** Thorough genetic analysis, testing multiple tag SNPs per gene and thus detecting any possible relevant genetic variants in a large Brazilian population and ASY subjects as controls we could have a sensitive assessment of the contribution of genetic variants in prognosis to CCC either confirming or finding additional associated SNPs in the mentioned genes. The CCL2rs2530797A/A and TIRAPrs8177376A/A were associated to an increase susceptibility whereas the CCR5rs3176763C/C genotype is associated to protection to CCC.

These associations were confirmed when we restricted the analysis to severe CCC, characterized by a left ventricular ejection fraction under 40%. Our data show beyond reasonable doubt that polymorphisms affecting key molecules involved in several immune parameters (innate immunity signal transduction and T cell/monocyte migration to inflammatory regions) play a role in genetic susceptibility to CCC development. However, the functional impact of these markers remains unknown. This also points out to the multigenic character of CCC, each polymorphism imparting a small contribution.





**Frade AF,** Pissetti CW, Ianni BM, Saba B, Lin-Wang HT, Nogueira LG, de Melo Borges A, Buck P, Dias F, Baron M, Ferreira LR, Schmidt A, Marin-Neto JA, Hirata M, Sampaio M, Fragata A, Pereira AC, Donadi E, Kalil J, Rodrigues V, Cunha-Neto E, Chevillard C. Genetic susceptibility to Chagas disease cardiomyopathy: involvement of several genes of the innate immunity and chemokine-dependent migration pathways. BMC Infect Dis. 2013 Dec 12;13:587. doi: 10.1186/1471-2334-13-587. PMID: 24330528; PMCID: PMC3866603.

The control of IFN-γ production by Th1-type T cells may be a key event for progression towards CCC. A genetic component to disease progression was suggested by the familial aggregation of cases and the association of gene polymorphisms with CCC development. We here investigate the role of gene polymorphisms (SNPs) in several genes involved in the control of IFN-γ production and Th1 T cell differentiation in CCC development.

We found 2 IL12 SNPs (rs2546893, rs919766) and a trend of association for a IL10 SNP (rs3024496) to be significantly associated with the ASY group. these associations were confirmed by multivariate analysis and allele tests. The rs919766C, 12rs2546893G and rs3024496C alleles were associated to an increase risk to CCC development. Our data show that novel polymorphisms affecting IL12B and IL10, but not IFNG or IL4 genes play a role in genetic susceptibility to CCC development. This might indicate that the increased Th1 differentiation and IFN-γ production associated with CCC is genetically controlled.


**Frade-Barros AF,** Ianni BM, Cabantous S, Pissetti CW, Saba B, Lin-Wang HT, Buck P, Marin-Neto JA, Schmidt A, Dias F, Hirata MH, Sampaio M, Fragata A, Pereira AC, Donadi E, Rodrigues V, Kalil J, Chevillard C, Cunha-Neto E. Polymorphisms in Genes Affecting Interferon-γ Production and Th1 T Cell Differentiation Are Associated With Progression to Chagas Disease Cardiomyopathy. Front Immunol. 2020 Jul 7;11:1386. doi: 10.3389/fimmu.2020.01386. PMID: 32733459; PMCID: PMC7358543.

## Genome-wide association studies (GWAS)

The first genome-wide association study (GWAS) on Chagas disease was published in 2013. This analysis included 600 Brazilian *T. cruzi* seropositive blood donors of different clinical forms and 488 Brazilian seronegative donors. Several phenotypes were analyzed, in addition to cardiomyopathy considered as the main trait. Authors also evaluated a limited number of specific parameters, including ejection fraction, PR interval, QRS duration (QRS), corrected QT interval (QTc), EIA signal/cutoff levels, and *T. cruzi* PCR status. Of the 600 *T. cruzi* seropositive donors cases, 221 were classified as having CCC, 311 had no cardiomyopathy, and 68 were inconclusive. The first genome-wide association study (GWAS) on Chagas disease was published in 2013 *(Deng et al. 2013).*

For cardiomyopathy, two trends of association (after multiple comparison corrections) were detected for markers located around SLCO1B1 gene *(Deng et al. 2013).* SLCO1B1 is a membrane transporter that belongs to a solute carrier family and plays a role in drug metabolism. It is expressed in the liver, brain, heart, and kidney and transports organic anions, such as digoxin, bilirubin, methotrexate, and statins. In addition, loss-of-function mutations may be associated with impaired drug action in target tissues *(Ishikawa T 2012)*. Moreover, a cluster of 12 SNPs within introns of COL14A1 was associated with PCR positivity. COL14A1 is a fibril-associated collagen which interacts with the fibril surface and regulates fibrillogenesis *(Ansorge HL et al. 2009; Birk DE et al. 1990)*. Probably all these markers at this locus are in linkage disequilibrium. Furthermore, HSPB8 is a small heat shock protein whose heart specific overexpression induces myocardial hypertrophy *(Depre C et al. 2002)*. HSPB8-transgenic mice bearing the K141N mutation expressed myocardial hypertrophy, ventricular dysfunction, and apical fibrosis—the latter being a hallmark of heart involvement in CCC *(Sanbe A et al. 2013)*. Significantly, expression of HSPB8 is selectively increased in myocardial tissue from CCC patients, rather than in idiopathic dilated cardiomyopathy patients *(Cunha-Neto E et al. 2005).* However, these indications remain suggestive due to the limited size of the studied cohort for a GWAS study. Surprisingly, no polymorphism in immune-related genes was found associated.


**Deng X**, Sabino EC, Cunha-Neto E, Ribeiro AL, Ianni B, Mady C, Busch MP, Seielstad M; REDSII Chagas Study Group from the NHLBI Retrovirus Epidemiology Donor Study-II Component International. Genome wide association study (GWAS) of Chagas cardiomyopathy in Trypanosoma cruzi seropositive subjects. PLoS One. 2013 Nov 20;8(11):e79629. doi: 10.1371/journal.pone.0079629. PMID: 24324551; PMCID: PMC3854669.

**Ishikawa T**. Genetic variants in the human SLCO1B1 gene and individualvariations in methotrexate clearance. Pharmacogenomics. 2012 Jul;13(9):993-4.

**Ansorge HL**, Meng X, Zhang G, Veit G, Sun M, Klement JF, Beason DP, Soslowsky LJ, Koch M, Birk DE. Type XIV Collagen Regulates Fibrillogenesis: premature collagen fibril growth and tissue dysfunction in null mice. J Biol Chem. 2009 Mar 27;284(13):8427-38.

**Birk DE**, Fitch JM, Babiarz JP, Doane KJ, Linsenmayer TF. Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci. 1990 Apr;95 (Pt 4):649-57.

**Depre C**, Hase M, Gaussin V, Zajac A, Wang L, Hittinger L, Ghaleh B, Yu X, Kudej RK, Wagner T, Sadoshima J, Vatner SF. H11 kinase is a novel mediator of myocardial hypertrophy in vivo. Circ Res. 2002 Nov 29;91(11):1007-14

**Sanbe A**, Marunouchi T, Abe T, Tezuka Y, Okada M, Aoki S, Tsumura H, Yamauchi J, Tanonaka K, Nishigori H, Tanoue A. Phenotype of cardiomyopathy in cardiac-specific heat shock protein B8 K141N transgenic mouse. J Biol Chem. 2013 Mar 29;288(13):8910-21.

**Cunha-Neto E**, Dzau VJ, Allen PD, Stamatiou D, Benvenutti L, Higuchi ML, Koyama NS, Silva JS, Kalil J, Liew CC. Cardiac gene expression profiling provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. Am J Pathol. 2005 Aug;167(2):305-13.

In order to better elucidate the genetic basis of Chagas disease and chronic Chagas cardiomyopathy, Marialbert Acosta-Herrera, Javier Martin et al. perform a large GWAS and a meta-analysis of Latin American populations that, in combination with complementary *in-silico* functional evidence, would provide further insights into the pathogenesis of this neglected disease.

Samples from three different Latin American countries: Colombia, Bolivia and Argentina were included in this study and meta-analyzed with data from the previous GWAS in a Brazilian population, comprising a total of 3,699 genomic DNA recruited samples. The strongest association (rs2458298; *p-value*=$3.27x10^{-08}$, OR=0.90, 95% CI 0.87-0.94), is located in chromosome 11 in an intronic region of the *NAALADL1* gene. This signal is followed by several proxy variants in high or moderate LD ($r^2$>0.4) located nearby *NAALADL1*, *SAC3D1* and *SNX15* genes. Regarding the rest of the suggestive signals, they are located in intergenic regions closed to *CDH8* and *KLF4* genes.

*SAC3D1*, also known as *SHD1*, has been identified as a transcriptional regulator of *STAT5 (Nakajima H et al. 2008)*, also associated with cardioprotection in humans *(Heusch G et al. 2011)*. Additionally, the STAT-5 signaling by IL-2, IL-7 and IL-15 receptors has been shown to be perturbed in peripheral and heart-infiltrating T cells in chronic Chagas cardiomyopathy *(Albareda MC et al. 2015)*.


**Casares-Marfil D**, Strauss M, Bosch-Nicolau P, Lo Presti MS, Molina I, Chevillard C, Cunha-Neto E, Sabino E, Ribeiro AL, González CI, Martín J, **Acosta-Herrera M**. A genome-wide association study identifies novel susceptibility loci in chronic Chagas cardiomyopathy. Clin Infect Dis. 2021 Feb 4:ciab090. doi: 10.1093/cid/ciab090. Epub ahead of print. PMID: 33539531.

**Nakajima H**, Tamura T, Ito M, Shibata F, Kuroda K, Fukuchi Y, Watanabe N, Kitamura T, Ikeda Y, Handa M. SHD1 is a novel cytokine-inducible, negative feedback regulator of STAT5-dependent transcription. Blood. 2009 Jan 29;113(5):1027-36. doi: 10.1182/blood-2008-01-133405. Epub 2008 Oct 6. PMID: 18838617.

**Heusch G**, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. Circ Res. 2012 Jan 6;110(1):111-5. doi: 10.1161/CIRCRESAHA.111.259556. Epub 2011 Nov 23. PMID: 22116817.

**Albareda MC**, Perez-Mazliah D, Natale MA, Castro-Eiro M, Alvarez MG, Viotti R, Bertocchi G, Lococo B, Tarleton RL, Laucella SA. Perturbed T cell IL-7 receptor signaling in chronic Chagas disease. J Immunol. 2015 Apr 15;194(8):3883-9. doi: 10.4049/jimmunol.1402202. Epub 2015 Mar 13. PMID: 25769928; PMCID: PMC4391971.

# Exome sequencing on Chagas families

High-impact rare gene variants altering protein structure and function underlie Mendelian disease and contribute to complex multifactorial disease. Approximately 10% of acute viral myocarditis (AVM) patients carried rare pathogenic homozygous variants in genes implicated in familial cardiomyopathy (Belkaya S et al. 2017) suggesting an overlap between genetic and acquired forms of myocarditis and cardiomyopathy.

We hypothesize here that rare genetic variants may lead to progression towards CCC by increasing cardiomyocyte susceptibility to inflammatory damage. Whole exome sequencing (WES) studies in families with multiple disease cases are an unbiased approach that has been used to identify rare pathogenic variants in Mendelian genetic disorders and complex multifactorial diseases. We used WES to search for rare, high impact gene variants linked to CCC in nuclear families containing multiple cases of Chagas disease and involved in pathobiological processes involved in inflammatory cardiomyopathy.

**Belkaya S**, Kontorovich AR, Byun M, Mulero-Navarro S, Bajolle F, Cobat A, Josowitz R, Itan Y, Quint R, Lorenzo L, Boucherit S, Stoven C, Di Filippo S, Abel L, Zhang SY, Bonnet D, Gelb BD, Casanova JL. Autosomal Recessive Cardiomyopathy Presenting as Acute Myocarditis. J Am Coll Cardiol. 2017 Apr 4;69(13):1653-1665. doi: 10.1016/j.jacc.2017.01.043. PMID: 28359509; PMCID: PMC5551973.

We performed whole exome sequencing and assessed rare pathogenic gene variants associated with CCC in six nuclear families containing multiple cases of Chagas disease families (25 patients) and in a group of unrelated ASY patients (n=14) who came from CD-endemic rural areas in Brazil.

Seropositive ASY patients

Seropositive CCC patients

**Family #1**

IC250E IC252E IC253E IC254E

**Family #2**

6156

6151 6154 6152 6157 6153 6155

**Family #3**

6087 6088 6086

**Unrelated ASY patients**

IC45EI 6097
IC49EI 6129
IC69EI 6143
IC95EI IC33EI
IC111EI IC92EI
6083 IC106EI
6091 6102

**Family #4**

6078 6075 6076

**Family #5**

6133 6136

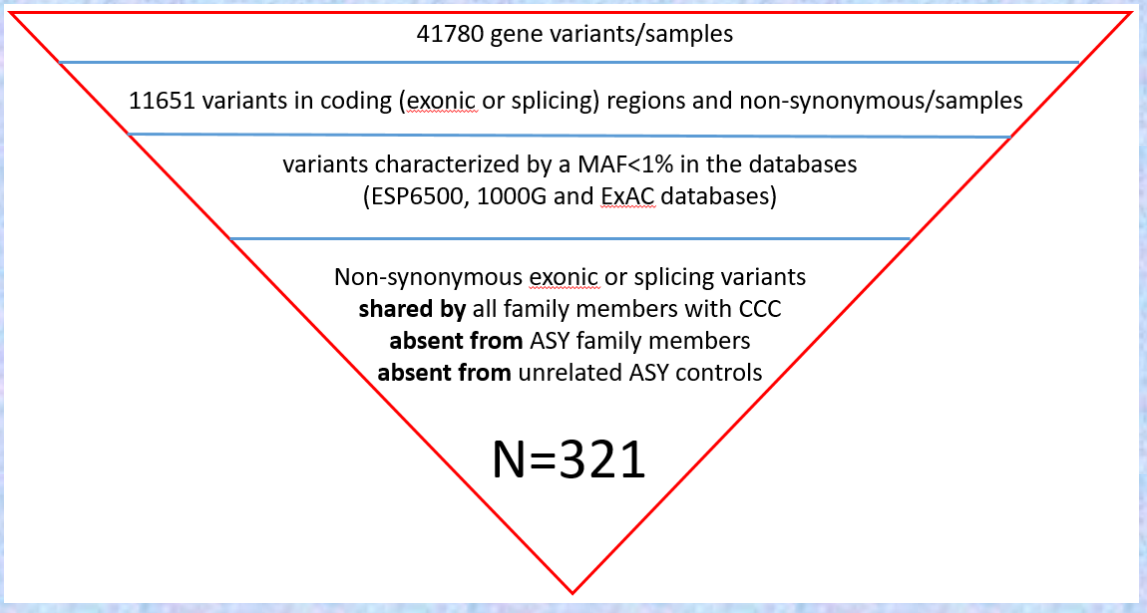
6131 6135 6134

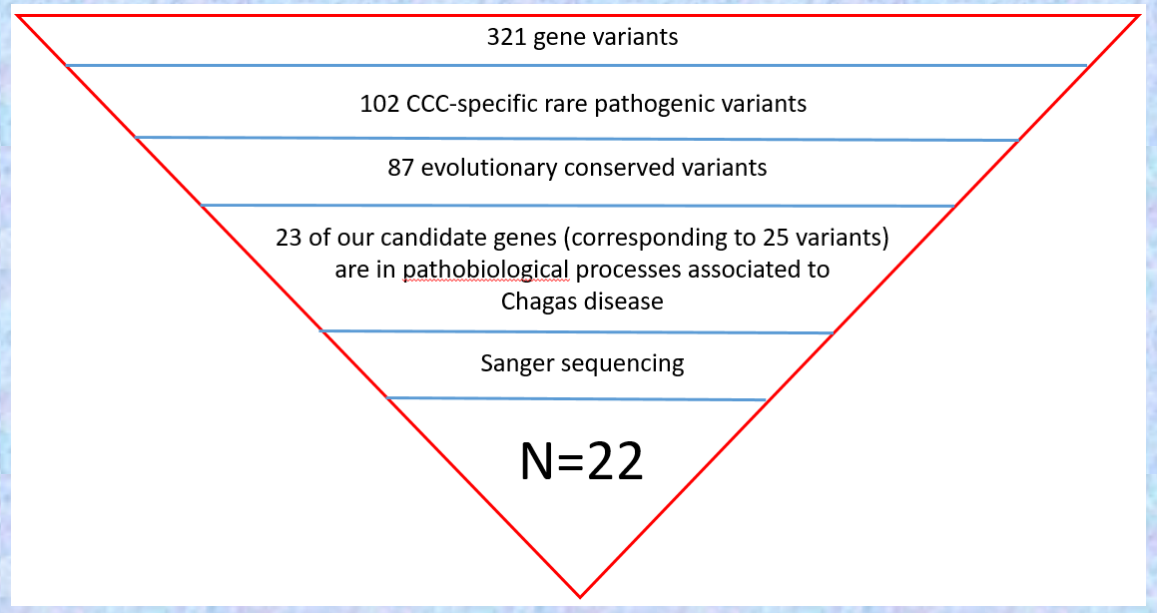
**Family #6**

IC191EC

IC174EC IC190EC

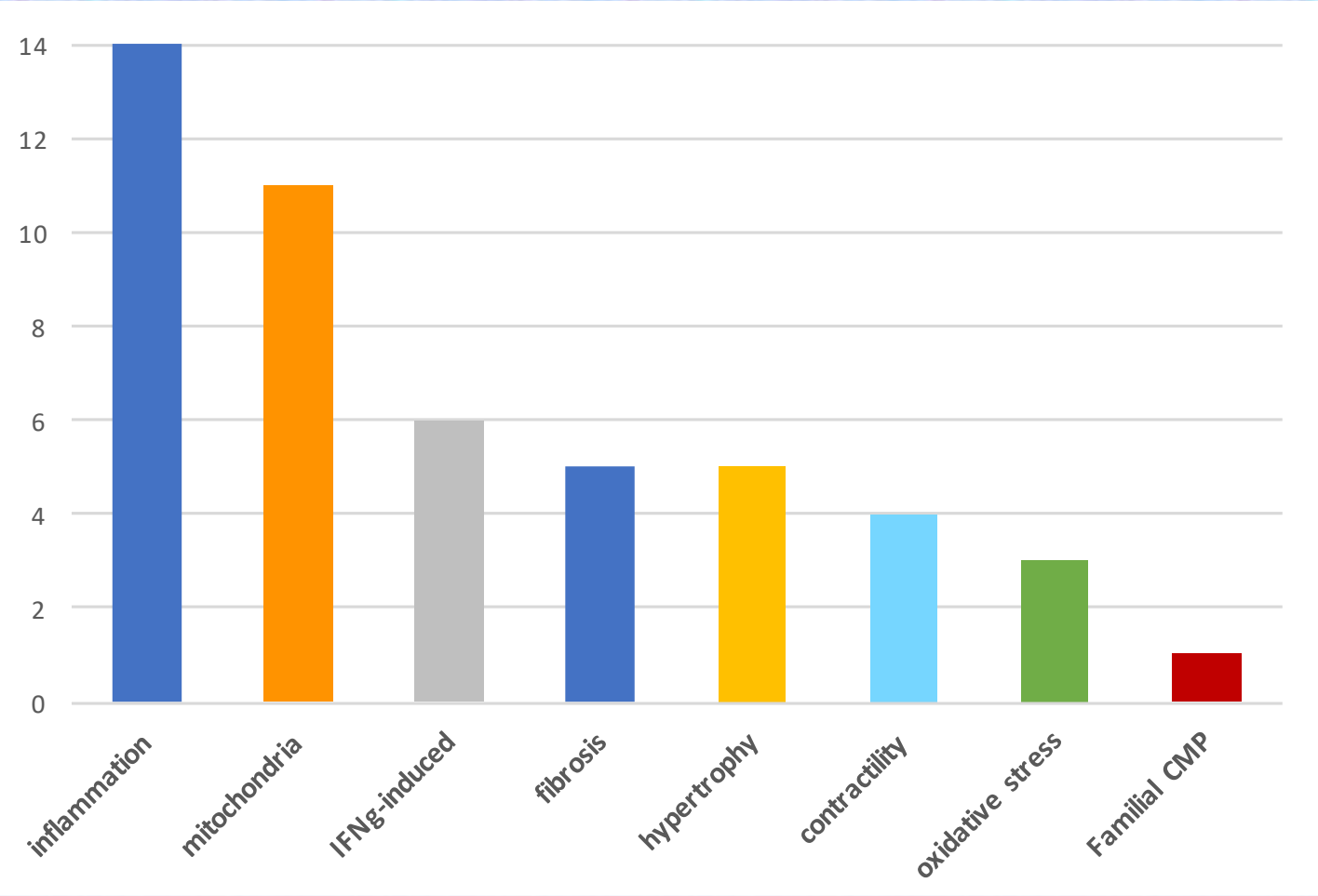
# Gene variant prioritization




Whole exome sequencing disclosed that on average, each patient sample contained 41780 gene variants. Among them, on average 11651 variants were located in coding (exonic or splicing) regions and non-synonymous. We focused on variants characterized by a minor allele frequency <1% in the databases (ESP6500, 1000G and ExAC). Under a hypothesis of complete penetrance, for each given family, we selected nonsynonymous exonic or splicing variants shared by all family members with CCC, but absent from ASY family members as well as by the unrelated ASY controls. A total of 321 CCC-specific nonsynonymous exonic rare variants were selected. After application of our pathogenicity filter with multiple algorithms, we found 102 CCC-specific rare pathogenic variants. After filtering for evolutionary conservation, we found 87 variants. At this point, we prioritized the 87 gene variants in 9 pathobiological processes associated to Chagas disease. It highlighted 23 of our candidate genes (corresponding to 25 variants). From these variants, 88% (22/25, contained in 20 genes) were confirmed by Sanger sequencing.

It shows the number of genes containing associated variants in each pathophysiological process. Some genes may be common to several pathways. We found a striking accumulation of CCC-associated variants in inflammation-related and mitochondrial genes (17 out of the 20 genes). All families carried at least one variant in mitochondrial or inflammation associated genes; five families carried variants in mitochondrial genes, and 5 in inflammation related genes.

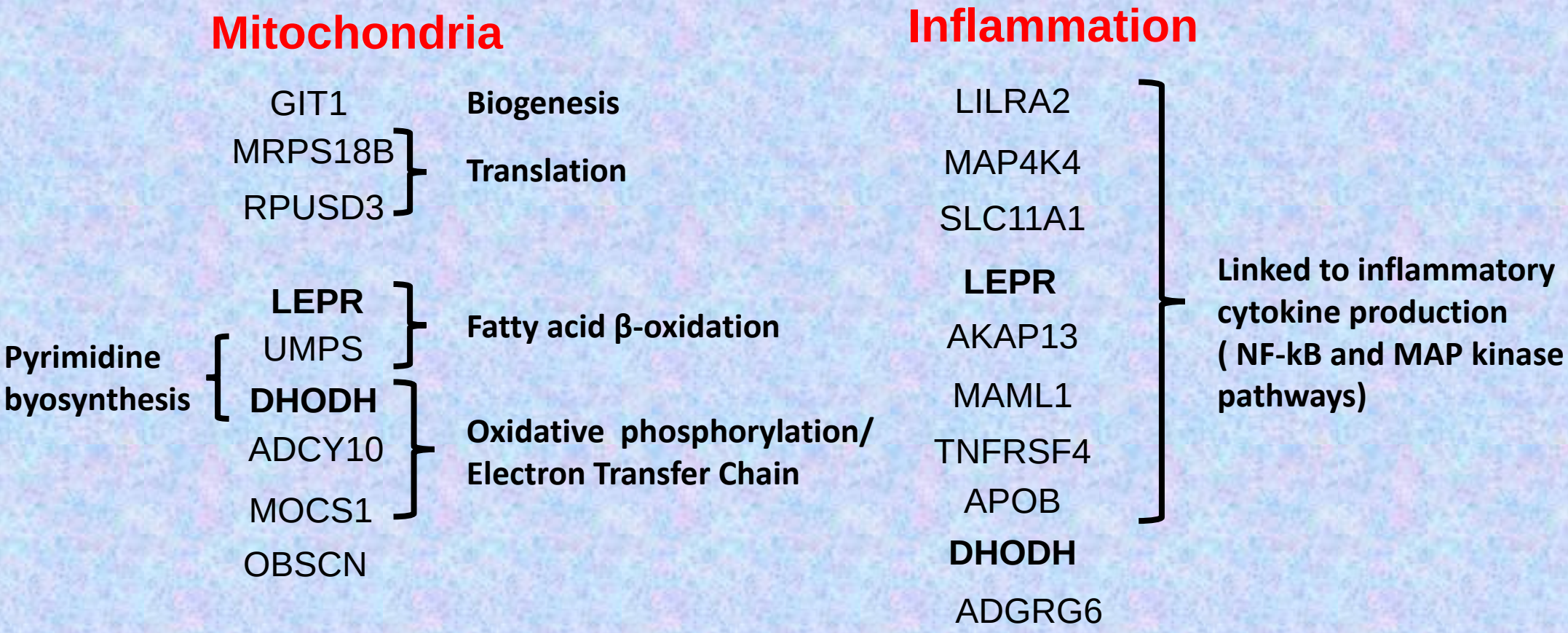



Each family displayed pathogenic variants in different genes

All families: mitochondrial or inflammation related genes

- 5/6 variants in inflammation genes
- 5/6 variants in mitochondrial genes
- 4/6 variants in inflammation and mitochondrial genes

Ten pathogenic variants were found in 9 mitochondrial genes (ADCY10, DHODH, GIT1, MRPS18B, RPUSD3, LEPR, UMPS, MOCS1 and OBSCN). Eleven pathogenic variants were located in 10 inflammation-associated genes (ADGRG6, AKAP13, LEPR, LILRA2, MAML1, MAP4K4, SLC11A1, TNFRSF4, APOB and DHODH).



In this study of whole exome sequencing of six nuclear families with multiple cases of CD, we found 22 CCC-associated rare heterozygous nonsynonymous high-impact pathogenic variants in 20 genes belonging to pathways relevant to inflammatory cardiomyopathy. Only individuals that were both seropositive and carriers of the heterozygous pathogenic variants developed CCC, but not seropositive patients carrying the wild-type sequences, nor seronegative siblings carrying the pathogenic variant.

Among the 9 mitochondrial genes showing CCC-specific pathogenic variants, 8 are involved in processes leading to mitochondrial ATP production (biogenesis, translation, fatty acid oxidation-FAOx and the electron transfer chain/oxidative phosphorylation (OXPHOS).

Interestingly, patients carrying mutations or animals genetically deficient in 6 genes (DHODH, UMPS, MRPS18B, GIT1, OBSCN, and LEPR) developed cardiac phenotypes.

up to 30% of mitochondriopathy patients develop cardiomyopathy, heart conduction defects, ventricular arrhythmia or sudden cardiac death, and autonomic nervous system imbalance, while up to 15% develop gastrointestinal motility disorders including achalasia/megaoesophagus and megacolon. The striking similarity between the clinical presentation and proportion of cardiac and digestive disorders in mitochondriopathies and the clinical spectrum of Chagas disease suggested the pathogenesis of CCC may be dependent on mitochondrial dysfunction.

Results indicate that the genetic contribution to CCC is polygenic and driven by several rare variants in genes that differ between families, but are related to mitochondria and inflammation. Results imply that mitochondrial dysfunction and inflammation, key processes in the pathophysiology of CCC, are at least in part genetically determined. To our knowledge, this is the first report that rare variants in mitochondrial and inflammation-related genes are linked to complex multifactorial cardiomyopathy.

Our results also support the notion of a two-hit mechanism where IFN-γ and proinflammatory cytokines induced by chronic infection trigger mitochondrial dysfunction and clinical disease in carriers of heterozygous mitochondrial gene variants. Indeed, modulation of mitochondrial damage induced by IFN-γ and other cytokines could perhaps be a suitable therapeutic target in CCC.

Treatment with mitochondria-protective agents such as antioxidants or agonists of Sirtuin-1 and AMP-activated protein kinase (AMPK) was found to attenuate or even reverse cardiac damage in mouse models of CCC, by reducing NF-kB activation and the intensity of chronic myocarditis. To conclude, it is possible that a similar two-hit mechanism, whereby genetic variants may increase mitochondrial susceptibility to inflammatory cytokine-induced dysfunction, may be relevant for the pathogenesis of other inflammatory cardiomyopathies and degenerative diseases associated with mitochondrial dysfunction.

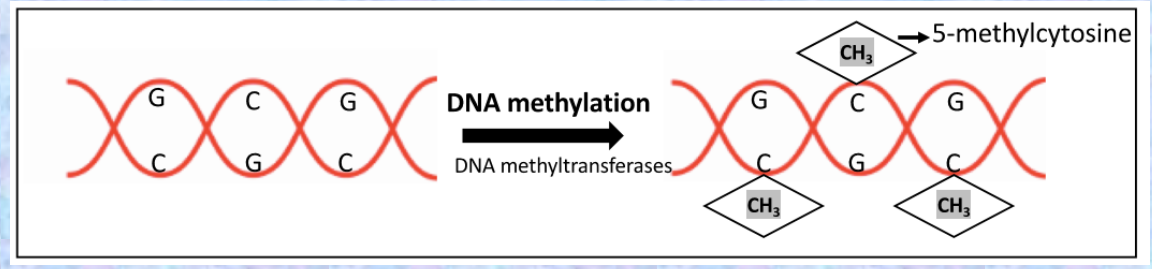

**Ouarhache M**, Marquet S, Frade AF, Ferreira AM, Ianni B, Almeida RR, Nunes JPS, Ferreira LRP, Rigaud VO, Cândido D, Mady C, Zaniratto RCF, Buck P, Torres M, Gallardo F, Andrieux P, Bydlowsky S, Levy D, Abel L, Cardoso CS, Santos- Junior OR, Oliveira LC, Oliveira CDL, Nunes MDC, Cobat A, Kalil J, Ribeiro AL, Sabino EC, Cunha-Neto E, Chevillard C. Rare Pathogenic Variants in Mitochondrial and Inflammation-Associated Genes May Lead to Inflammatory Cardiomyopathy in Chagas Disease. J Clin Immunol. 2021 Mar 3. doi: 10.1007/s10875-021-01000-y. Epub ahead of print. PMID: 33660144.

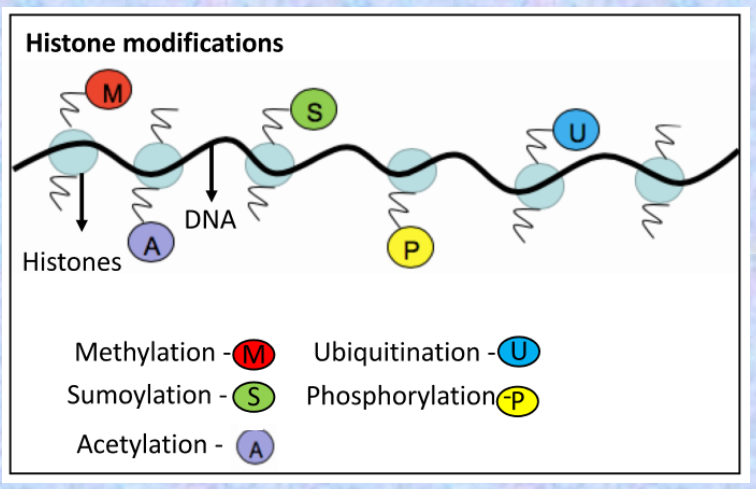
# Epigenetic in Chagas families

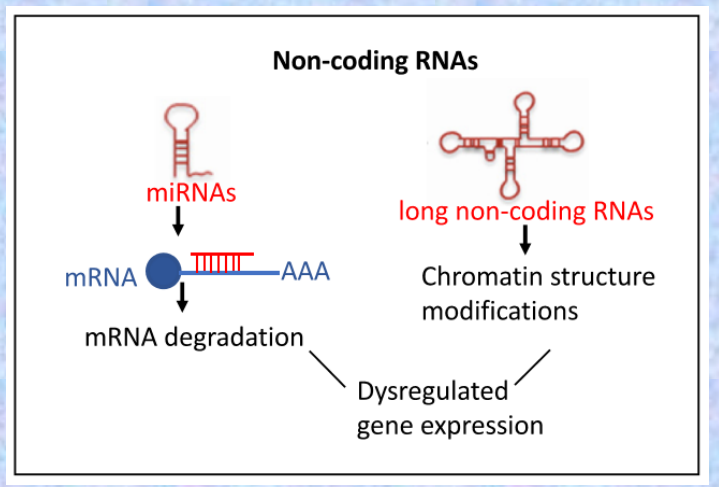
Epigenetics is currently one of the most rapidly developing fields of biological research. The belief that the genetic code is the only basis for biological inheritance has been challenged by the discovery of the epigenome. It is now known that DNA bases can be modified without altering the nucleotide sequence and that this “epigenome” can be modulated by a variety of environmental factors, including chemicals, nutrition, early environment, stress and ageing.

Known epigenetic processes include: (i) DNA methylation, (ii) histone modification (including diverse processes such as methylation, acetylation, phosphorylation, ubiquitination and SUMOylation) and (iii) RNA-based mechanisms including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs).

These processes modify gene expression and, given their chemical stability, influence the propagation of gene activity from one generation of cells to the next providing another mechanism of biological inheritance and variability.



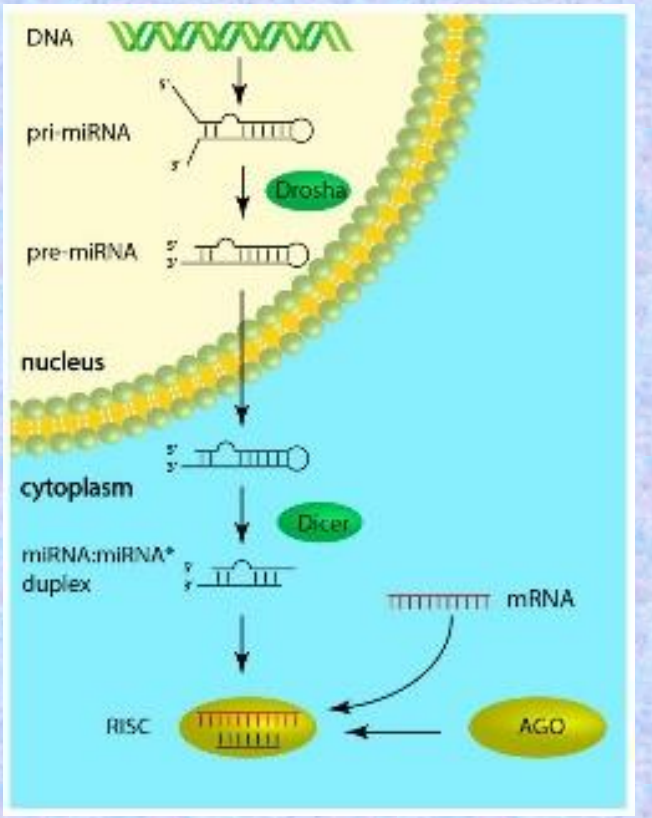



# MicroRNAs act as gene expression regulators

MicroRNAs (miRNAs) are involved in differential gene expression in cardiac diseases. MiRNAs are noncoding RNA molecules (~22 nucleotides long, single-stranded) that regulate gene expression through imperfect base-pairing with complementary sequences in their target mRNA leading to translational repression or transcript degradation.

Most miRNA genes are transcribed by RNA polymerase II from intergenic, intronic or polycistronic loci as a long primary miRNA transcript (pri-miRNA), which is then cleaved by the Drosha endoribonuclease to a 70-nt-long hairpin structure with 2-nt-3′ overhangs (pre-miRNA) (O'Brien J et al 2018). Pre-miRNA is subsequently exported to the cytoplasm and processed by a second endoribonuclease, Dicer, to form a 22-nucleotide-long miRNA:miRNA* duplex with imperfect complementarity. One strand of this duplex, the guide strand, then combines with the Argonaute (AGO) protein into the RNA-induced silencing complex (RISC), while the passenger strand gets degraded (Treiber T et al. 2018). The targeting of a mRNA occurs through imperfect base-pairing between the transcript and the so-called seed sequence in the miRNA, usually covering the nucleotides in positions 2–7 of the latter (Bartel DP 2009). As a consequence, a single miRNA can regulate multiple mRNA targets involved in diverse biological processes and, vice versa, a single mRNA can be regulated by several miRNAs.




**O'Brien J**, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol (Lausanne). 2018 Aug 3;9:402. doi: 10.3389/fendo.2018.00402. PMID: 30123182; PMCID: PMC6085463.

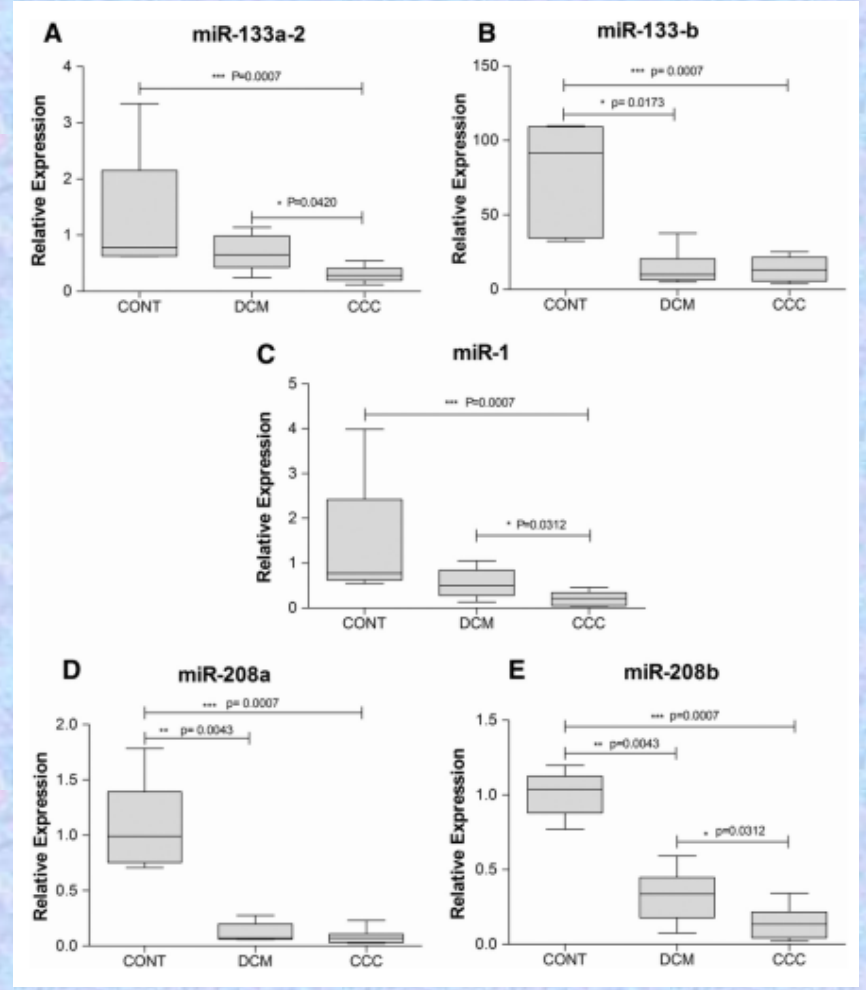
**Treiber T**, Treiber N, Meister G. Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. Nat Rev Mol Cell Biol. 2019 Jan;20(1):5-20. doi: 10.1038/s41580-018-0059-1. Erratum in: Nat Rev Mol Cell Biol. 2018 Dec;19(12):808. Erratum in: Nat Rev Mol Cell Biol. 2019 May;20(5):321. PMID: 30228348.

**Bartel DP**. MicroRNAs: target recognition and regulatory functions. Cell. 2009 Jan 23;136(2):215-33. doi: 10.1016/j.cell.2009.01.002. PMID: 19167326; PMCID:PMC3794896.

Over the past decade, the central role of miRNAs has been established in numerous biological and pathological processes, including cell differentiation, apoptosis and carcinogenesis across different species, from Drosophila to humans (Ambros V 20014; Carrington JC et al. 2003; Karp X et al. 2005).

We hypothesized thatmiRNAs could play a dominant role in regulating gene and protein expression in CCC myocardial tissue. We have chosen to analyze nine miRNAs: (miR-1, miR-133a-2, miR-133b, miR-208a, miR- 208b, miR-214-3p, miR-146a-5p, miR-155-5p and miR-150-5p), that had previously been described as playing important roles in cardiovascular disorders, to test our hypothesis that there is a dysregulated miRNA expression in the CCC myocardium.

From those, five miRNAs: miR-1, miR-133a-2, miR-133b, miR-208a, and 208b were significantly down-regulated in CCC samples as compared to controls (p = 0.0007). Three of them (miR-133b, miR-208a and miR-208b) were significantly reduced in dilated cardiomyopathy samples as compared to controls.





**Ambros V**. The functions of animal microRNAs. Nature. 2004 Sep 16;431(7006):350-5. doi: 10.1038/nature02871. PMID: 15372042

**Carrington JC**, Ambros V. Role of microRNAs in plant and animal development. Science. 2003 Jul 18;301(5631):336-8. doi: 10.1126/science.1085242. PMID: 12869753.

**Karp X**, Ambros V. Developmental biology. Encountering microRNAs in cell fate signaling. Science. 2005 Nov 25;310(5752):1288-9. doi: 10.1126/science.1121566. PMID: 16311325.



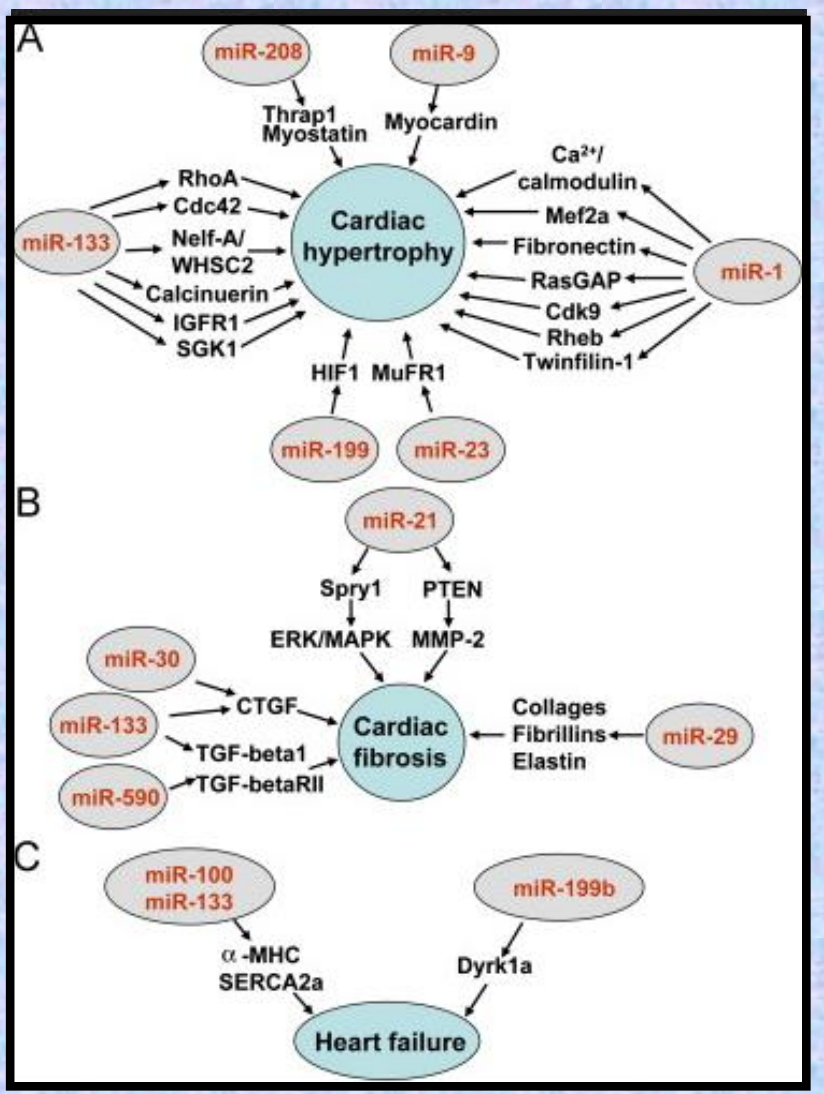

**Ferreira LR**, Frade AF, Santos RH, Teixeira PC, Baron MA, Navarro IC, Benvenuti LA, Fiorelli AI, Bocchi EA, Stolf NA, Chevillard C, Kalil J, Cunha- Neto E. MicroRNAs miR-1, miR-133a, miR-133b, miR-208a and miR-208b are dysregulated in Chronic Chagas disease Cardiomyopathy. Int J Cardiol. 2014 Aug 20;175(3):409-17. doi: 10.1016/j.ijcard.2014.05.019. Epub 2014 May 17. PMID: 24910366.


MiR-208 has 2 subfamilies,miR-208a andmiR-208b, which are encoded within an intron of a-cardiac muscle myosin heavy
chain gene (a-MHC, MYH6) and intron of b-cardiac myosin heavy chain gene (b-MHC, MYH7).

A recent study suggested that the expression levels of miR-208 showed its association with cardiac hypertrophy by negatively regulating SOX6 in cardiomyocytes (Huang X et al. 2015).

Another study proved that nemo-like kinase (NLK) is a direct target of miR-208 and this acts indirectly during Ginsenoside Rb1 protection of hypoxia/ischemia (Yan X et al. 2016).

Interestingly, a recent study revealed that the antagomir-208a could reduce the expression of endoglin and collagen I induced by mechanical stretch in H9C2 cells (Shyu KG et al. 2013).

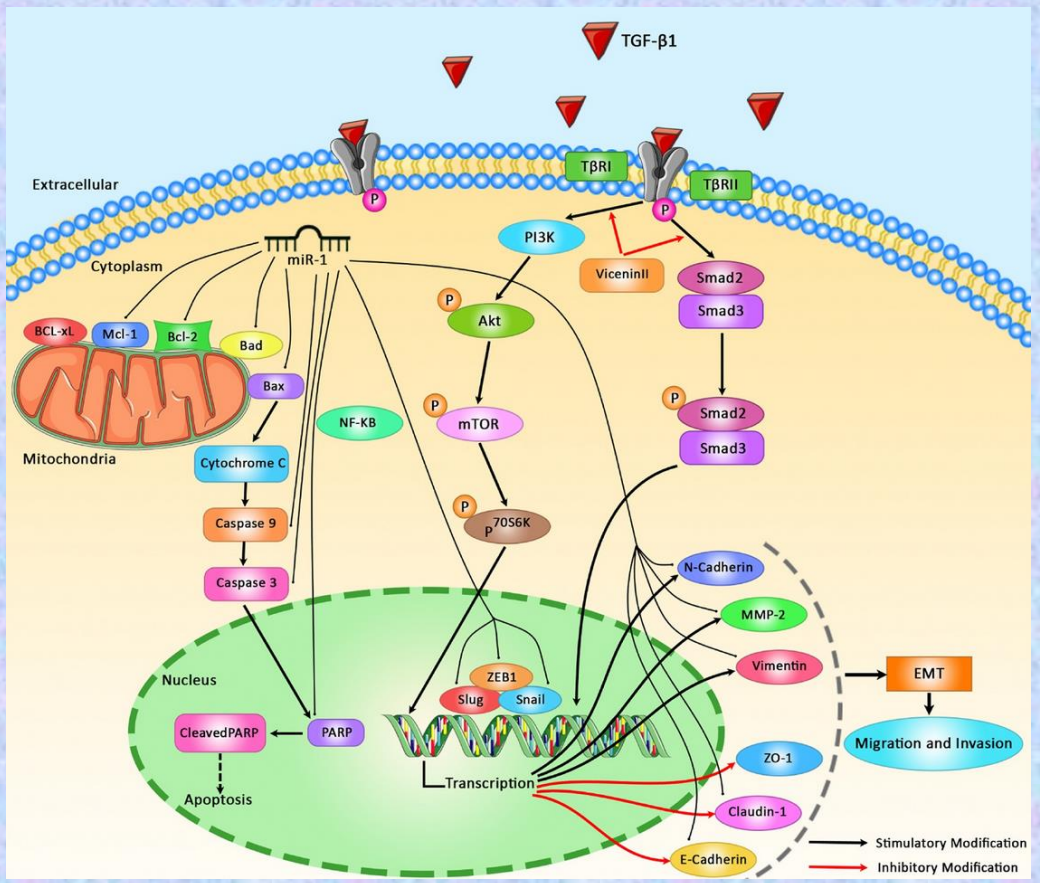



Huang X, Li Z, Bai B, Li X, Li Z. High expression of microRNA-208 is associated with cardiac hypertrophy via the negative regulation of the sex-determining region Y-box 6 protein. Exp Ther Med. 2015 Sep;10(3):921-926. doi: 10.3892/etm.2015.2645. Epub 2015 Jul 17. PMID: 26622415; PMCID: PMC4533156.

Yan X, Liu J, Wu H, Liu Y, Zheng S, Zhang C, Yang C. Impact of miR-208 and its Target Gene Nemo-Like Kinase on the Protective Effect of Ginsenoside Rb1 in Hypoxia/Ischemia Injuried Cardiomyocytes. Cell Physiol Biochem. 2016;39(3):1187-95. doi: 10.1159/000447825. Epub 2016 Sep 1. PMID: 27577116.

Shyu KG, Wang BW, Wu GJ, Lin CM, Chang H. Mechanical stretch via transforming growth factor-β1 activates microRNA208a to regulate endoglin expression in cultured rat cardiac myoblasts. Eur J Heart Fail. 2013 Jan;15(1):36-45. doi: 10.1093/eurjhf/hfs143. Epub 2012 Aug 31. PMID: 22941949.

miR-1 is a conserved miRNA with high expression in the muscle tissues particularly the heart muscle. It has a regulatory role on a number of genes such as heat shock protein 60 (HSP60) and Kruppel-like factor 4 (KLF4) at the post transcriptional level. miR-1 is associated to PI3K/AKT/mTOR/NFκB Pathway




Pinchi et al. have confirmed down-regulation of this miRNA in postmortem hearth samples of AMI patients compared with sudden cardiac death and controls (Safa A et al. 2020).

Wu et al. have judged the effects of Wenxin Granules on prevention of fatal arrhythmia by modulating gap junctions and miR-1 after MI in a rat model constructed by coronary artery ligation. Wenxin Granules preserved the configuration of the gap junctions and their fundamental Cx43 levels by modulating miR-1 (Wu A et al. 2017). miR-1 has a vital role in the maintenance of cardiac rhythms (Zhao Y et al. 2007) and its suppression may cause cardiac hypertrophy and arrhythmia.

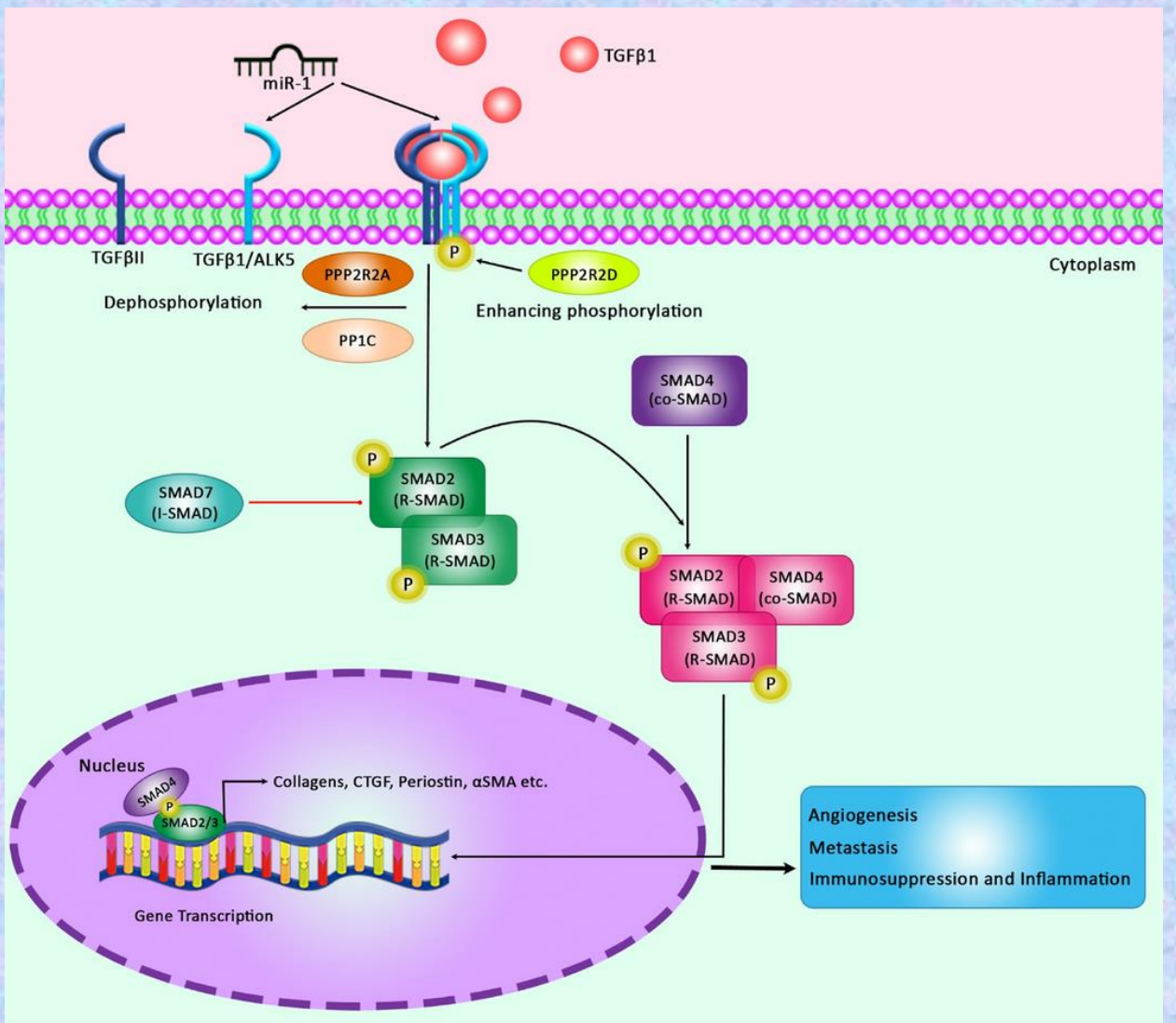
miR-1 has also been down-regulated in persons with symptomatic heart failure in association with the severity of this condition. Expression level of this miRNA has been suggested as a biomarker for predicting heart failure exacerbation (Sygitowicz G et al. 2015).

**Safa A**, Bahroudi Z, Shoorei H, Majidpoor J, Abak A, Taheri M, Ghafouri-Fard S. miR-1: A comprehensive review of its role in normal development and diverse disorders. Biomed Pharmacother. 2020 Dec;132:110903. doi: 10.1016/j.biopha.2020.110903. Epub 2020 Oct 20. PMID: 33096351.

**Wu A**, Zhao M, Lou L, Zhai J, Zhang D, Zhu H, Gao Y, Shang H, Chai L. Effect of Wenxin Granules on Gap Junction and MiR-1 in Rats with Myocardial Infarction. Biomed Res Int. 2017;2017:3495021. doi: 10.1155/2017/3495021. Epub 2017 Sep 28. PMID: 29094045; PMCID: PMC5637836.

**Zhao Y**, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell. 2007 Apr 20;129(2):303-17. doi: 10.1016/j.cell.2007.03.030. Epub 2007 Mar 29. PMID: 17397913.

**Sygitowicz G**, Tomaniak M, Błaszczyk O, Kołtowski Ł, Filipiak KJ, Sitkiewicz D. Circulating microribonucleic acids miR-1, miR-21 and miR-208a in patients with symptomatic heart failure: Preliminary results. Arch Cardiovasc Dis. 2015 Dec;108(12):634-42. doi: 10.1016/j.acvd.2015.07.003. Epub 2015 Oct 21. PMID: 26498537.




**See this excellent review:**

Safa A, Bahroudi Z, Shoorei H, Majidpoor J, Abak A, Taheri M, Ghafouri-Fard S. miR-1: A comprehensive review of its role in normal development and diverse disorders. Biomed Pharmacother. 2020 Dec;132:110903. doi: 10.1016/j.biopha.2020.110903. Epub 2020 Oct 20. PMID: 33096351.

miR-1 can be regarded as a target for manipulation of inflammatory processes. This miRNA has a prominent role in inhibition of KLF4 and induction of NF-κB (Jiang F et al. 2019). NF-κB has been shown to activate expression of several pro-inflammatory proteins such as cytokines and chemokines, thus contributing in the control of inflammasome. Moreover, this nuclear factor regulates survival, function and differentiation of several immune cells. Therefore, NF-κB is involved in the pathogenesis of numerous inflammatory conditions (Liu T et al. 2017).

Modulation of expression of this miRNA might be regarded as a therapeutic option in some disorders.

Metformin has been shown to amend cardiac conduction defect through modulation of expression of miR-1 (Lv L et al. 2020).

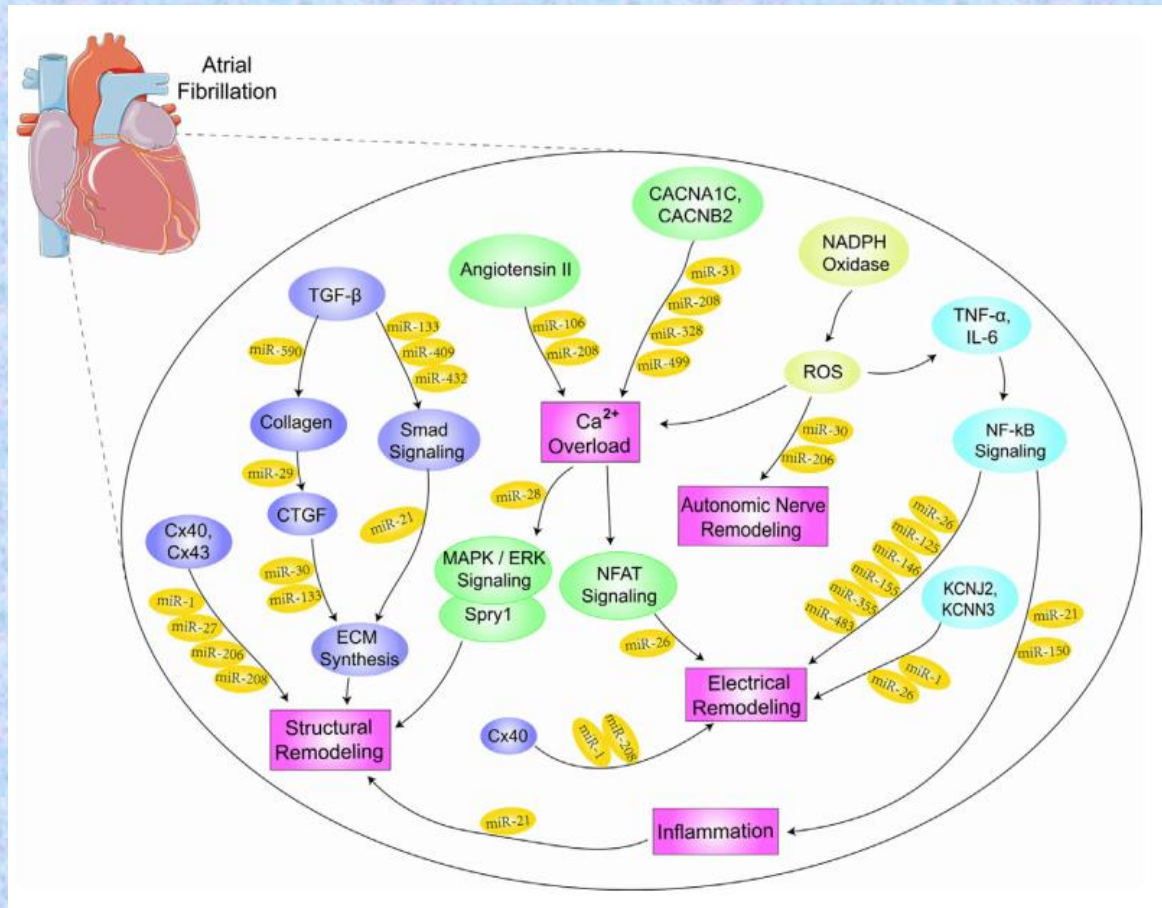
**Jiang F**, Chen Q, Wang W, Ling Y, Yan Y, Xia P. Hepatocyte-derived extracellular vesicles promote endothelial inflammation and atherogenesis via microRNA-1. J Hepatol. 2020 Jan;72(1):156-166. doi: 10.1016/j.jhep.2019.09.014. Epub 2019 Sep 27. PMID: 31568800.

**Liu T,** Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:17023–. doi: 10.1038/sigtrans.2017.23. Epub 2017 Jul 14. PMID: 29158945; PMCID: PMC5661633.

**Lv L**, Zheng N, Zhang L, Li R, Li Y, Yang R, Li C, Fang R, Shabanova A, Li X, Liu Y, Liang H, Zhou Y, Shan H. Metformin ameliorates cardiac conduction delay by regulating microRNA-1 in mice. Eur J Pharmacol. 2020 Aug 15;881:173131. doi: 10.1016/j.ejphar.2020.173131. Epub 2020 May 22. PMID: 32450177.

MiR-133 is expressed in the skeletal and cardiac muscles of mammals, birds and zebrafish. miR-133 has a profound influence on cardiac disorders. The aberrant expression of miR-133 was usually accompanied with cardiac hypertrophy, arrhythmogenesis and heart failure.

Several studies have demonstrated that miR-133 plays an important role in controlling structural changes in chronic atrial fibrillation (Li H et al. 2012). In accordance with the study that miR-133 was downregulated in atrial fibrillation group (Li H et al. 2012), Xu et al. also reported that miR-133 displayed an obvious downregulation tendency with aging in atrial fibrillation (Xu GL et al. 2013), suggesting the aberrantly expressed miR-133 may be responsible for the transition from adaptation to pathological atrial remodeling.



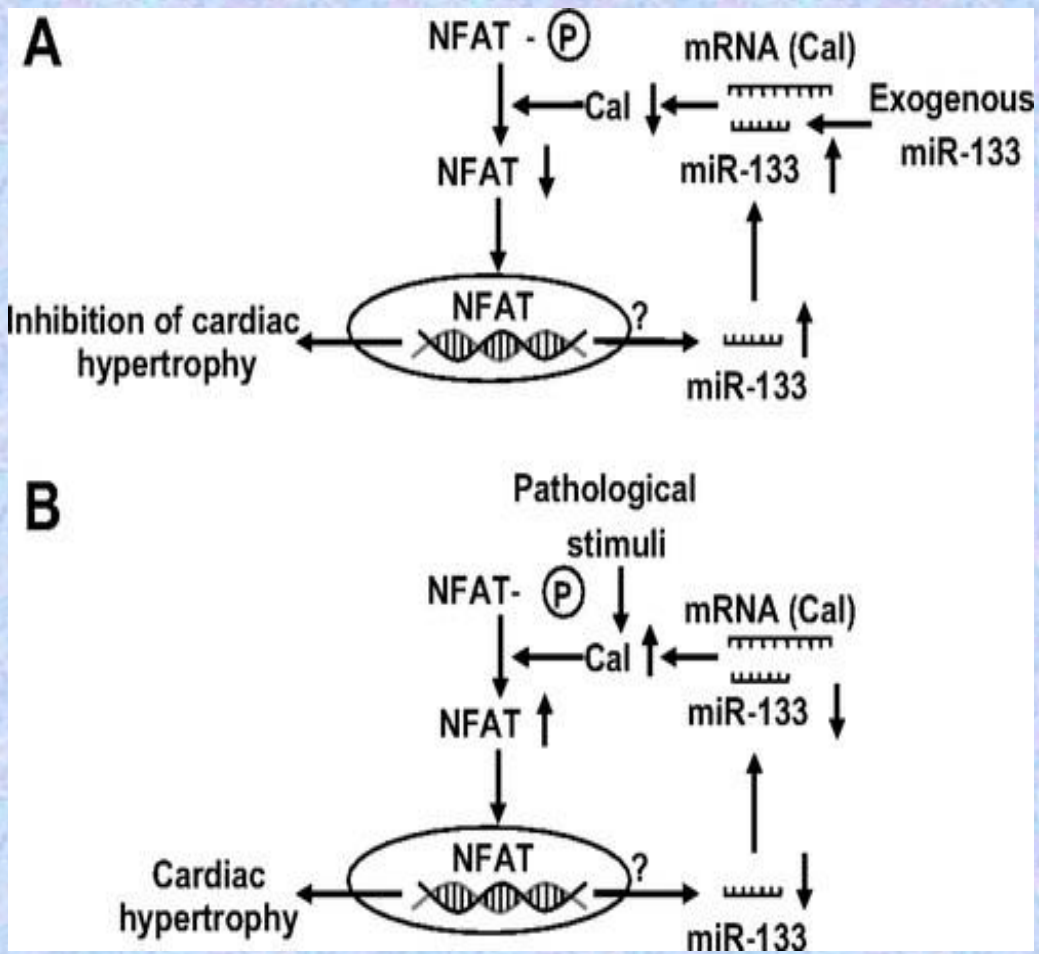

Nicotine promoted the protein levels of TGF-β1 and TGF-βRII and suppressed the expression of miR-133 and miR-590 in the detrimental atrial structural remodeling leading to atrial fibrillation (Shan H et al. 202009).


**Li H**, Li S, Yu B, Liu S. Expression of miR-133 and miR-30 in chronic atrial fibrillation in canines. Mol Med Rep. 2012 Jun;5(6):1457-60. doi: 10.3892/mmr.2012.831. Epub 2012 Mar 8. PMID: 22407060.

**Xu GJ**, Gan TY, Tang BP, Chen ZH, Ailiman M, Zhou XH, Jiang T, Song JG, Guo X, Li YD, Miao HJ, Zhang Y, Li JX. Changes in microRNAs expression are involved in age-related atrial structural remodeling and atrial fibrillation. Chin Med J (Engl). 2013;126(8):1458-63. PMID: 23595377.

**Shan H**, Zhang Y, Lu Y, Zhang Y, Pan Z, Cai B, Wang N, Li X, Feng T, Hong Y, Yang B. Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. Cardiovasc Res. 2009 Aug 1;83(3):465-72. doi: 10.1093/cvr/cvp130. Epub 2009 Apr 27. PMID: 19398468.

MiR-133 expression was inversely related to cardiac hypertrophy in murine models. Overexpression of miR-133 inhibited cardiac hypertrophy in vitro, while suppression of miR-133 induced the marked and sustained cardiac hypertrophy in vivo (Carè A et al. 2007).




Calcineurin is a calcium/calmodulin-activated serine-threonine phosphatase that dephosphorylates the transcriptional factor, nuclear factor of activated T cells (NFAT), which is translocated into the nucleus to bind to DNA and activate hypertrophic response genes (Molkentin JD et al. 1998; Dong D et al. 2003).

MiR-133 and calcineurin mutually regulated their expression via a positive feedback, and the reciprocal repression between miR-133 and calcineurin was observed in cardiac hypertrophy. Once the calcineurin/NFAT signaling was activated, miR-133 expression could be decreased with a loss of repression on calcineurin, and thereby the heart would be progressively hypertrophic (Dong D et al. 2010).

**Carè A**, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Høydal M, Autore C, Russo MA, Dorn GW 2nd, Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. MicroRNA-133 controls cardiac hypertrophy. Nat Med. 2007 May;13(5):613-8. doi: 10.1038/nm1582. Epub 2007 Apr 29. PMID: 17468766.

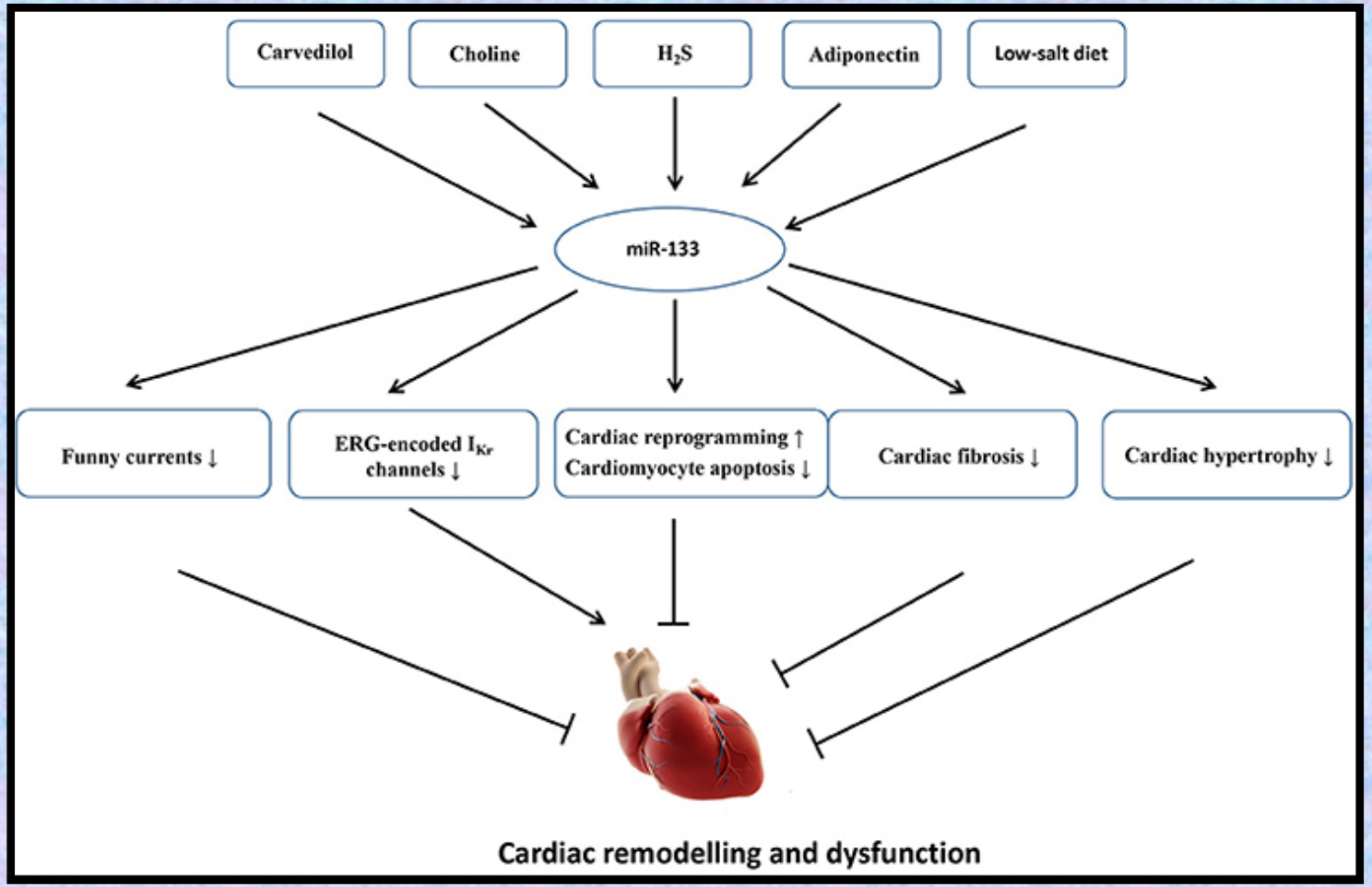
**Molkentin JD**, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998 Apr 17;93(2):215-28. doi: 10.1016/s0092-8674(00)81573-1. PMID: 9568714; PMCID: PMC4459646.

**Dong D**, Duan Y, Guo J, Roach DE, Swirp SL, Wang L, Lees-Miller JP, Sheldon RS, Molkentin JD, Duff HJ. Overexpression of calcineurin in mouse causes sudden cardiac death associated with decreased density of K+ channels. Cardiovasc Res. 2003 Feb;57(2):320-32. doi: 10.1016/s0008-6363(02)00661-2. PMID: 12566105.

**Dong DL**, Chen C, Huo R, Wang N, Li Z, Tu YJ, Hu JT, Chu X, Huang W, Yang BF. Reciprocal repression between microRNA-133 and calcineurin regulates cardiac hypertrophy: a novel mechanism for progressive cardiac hypertrophy. Hypertension. 2010 Apr;55(4):946-52. doi: 10.1161/HYPERTENSIONAHA.109.139519. Epub 2010 Feb 22. PMID: 20177001.

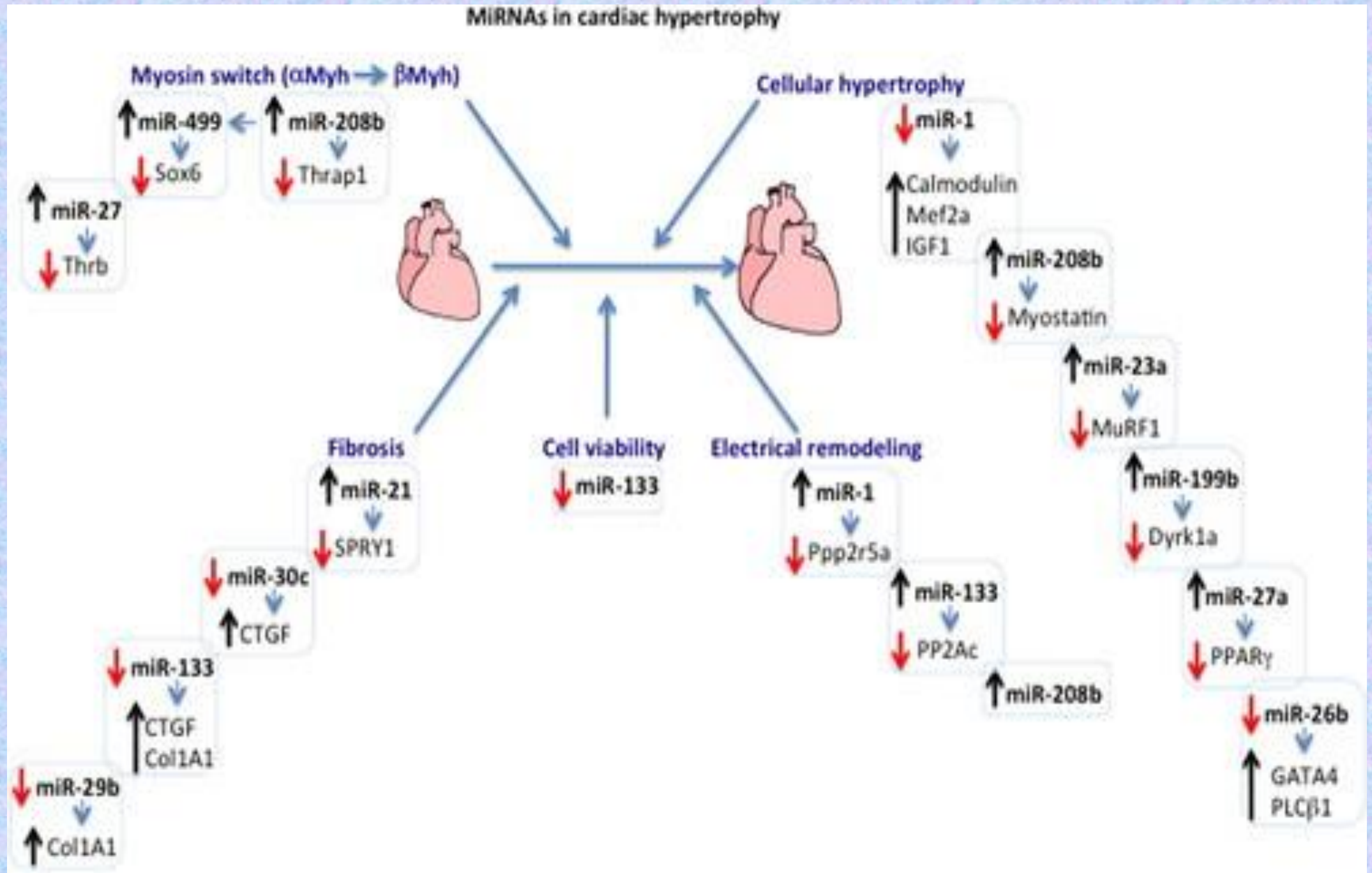
Acute myocardial infarction (AMI) indicates irreversible myocardial injury, leading to high mortality. A host of evidences revealed that muscle- and/or cardiac-specific miRNAs such as miR-1, miR-133a, miRNA-133b and miR-208 were involved in heart development and some cardiovascular diseases, such as myocardial infarction (Bostjancic E, et al. 2010).

Dysregulated miR-133 was found in the infarcted tissue in myocardial infarction patients. It might be a potential regulator of cardiac sarco/endoplasmic reticulum calcium ATPase-2 (SERCA2) which plays a central role in myocardial contractility (Bostjancic E, et al. 2012).




**Bostjancic E**, Zidar N, Stajer D, Glavac D. MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. Cardiology. 2010;115(3):163-9. doi: 10.1159/000268088. Epub 2009 Dec 21. PMID: 20029200.

**Boštjančič E**, Zidar N, Glavač D. MicroRNAs and cardiac sarcoplasmic reticulum calcium ATPase-2 in human myocardial infarction: expression and bioinformatic analysis. BMC Genomics. 2012 Oct 15;13:552. doi: 10.1186/1471-2164-13-552. PMID: 23066896; PMCID: PMC3532181.

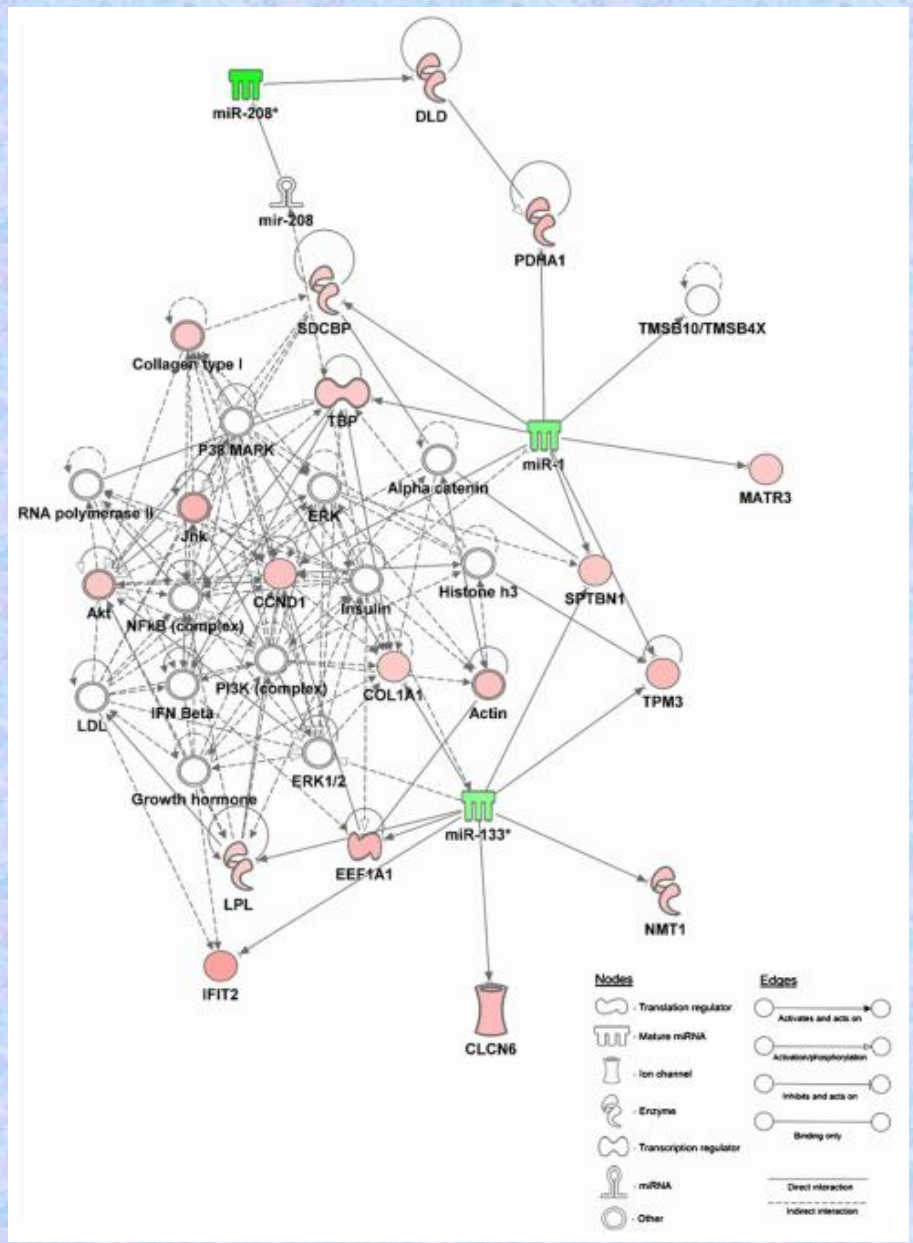

MiRNAs in cardiac hypertrophy
Myosin switch (αMyh → βMyh)
miR-499
Sox6
miR-208b
Thrap1
miR-27
Thrb
Cellular hypertrophy
miR-1
Calmodulin
Mef2a
IGF1
miR-208b
Myostatin
miR-23a
MuRF1
miR-199b
Dyrk1a
miR-27a
PPARγ
miR-26b
GATA4
PLCβ1
Fibrosis
miR-21
SPRY1
miR-30c
CTGF
miR-133
CTGF
Col1A1
miR-29b
Col1A1
Cell viability
miR-133
Electrical remodeling
miR-1
Ppp2r5a
miR-133
PP2Ac
miR-208b

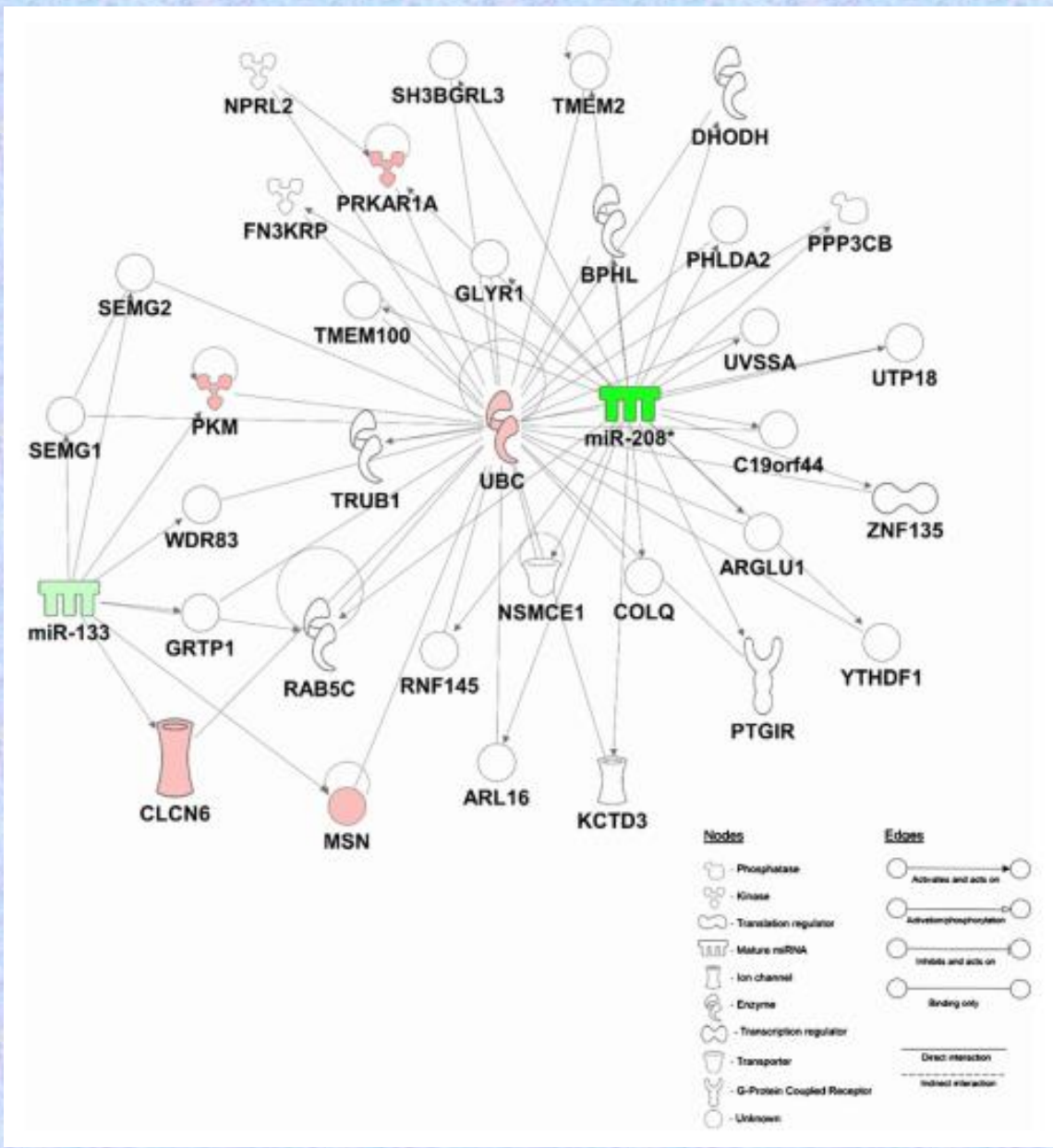
We used Ingenuity Pathway Analysis application for miRNA target prediction. Considering that miRNAs with the same seed sequence usually target the same RNAs, the IPA software clusters together the mature. The software identified a total of 2227 putative target transcripts of the five differentially expressed miRNAs in CCC: miR-1, miR-133a-2, miR-133b, miR-208a and miR-208b, where 1665 are high predicted and/or had already been experimentally validated as targets.

The next step was identifying upregulated target genes of the downregulated miRNAs (expression pairing) within the list of differentially expressed genes in CCC and DCM myocardium as assessed with the 10,386 element microarray Cardiochip

| Targets upregulated in CCC | | | | | | |
|---|---|---|---|---|---|---|
| Symbol | Fold change | Confidence | Gene ID | Symbol | Fold change | Entrez gene name |
| miR-1 | −6.39 | High (predicted) | **BC000076.2** | CCND1 | 1.8 | Cyclin D1 |
| miR-1 | −6.39 | High (predicted) | **M63485** | MATR3 | 1.5 | Matrin 3 |
| miR-1 | −6.39 | Moderate (predicted) | **M27166** | PDHA1 | 2.06 | Pyruvate dehydrogenase (lipoamide) alpha 1 |
| miR-1 | −6.39 | High (predicted) | **AF006636** | SDCBP | 1.52 | Syndecan binding protein (syntenin) |
| miR-1/miR-133a-3p | 6.39/-5.638 | High (predicted) | **L02897** | SPTBN1 | 1.6 | Spectrin, beta, non-erythrocytic 1 |
| miR-1 | −6.39 | High (predicted) | **6908** | TBP | 1.5 | TATA box binding protein |
| miR-1 | −6.39 | Experimentally Observed | **M17733** | TMSB10/TMSB4X | 1.5 | Thymosin beta 10 |
| miR-1/miR-133a-3p | 6.39/-5.638 | Experimentally Observed | **X04588** | TPM3 | 2 | Tropomyosin 3 |
| miR-133a-3p | −5.638 | High (predicted) | **D28475** | CLCN6 | 1.99 | Chloride channel, voltage-sensitive 6 |
| miR-133a-3p | −5.638 | High (predicted) | **Z74615** | COL1A1 | 1.5 | Collagen, type I, alpha 1 |
| miR-133a-3p | −5.638 | High (predicted) | **J04617** | EEF1A1 | 2.1 | Eukaryotic translation elongation factor 1 alpha 1 |
| miR-133a-3p | −5.638 | High (predicted) | **M14660** | IFIT2 | 2.7 | Interferon-induced protein with tetratricopeptide repeats 2 |
| miR-133a-3p | −5.638 | Moderate (predicted) | **M15856** | LPL | 1.5 | Lipoprotein lipase |
| miR-133a-3p | −5.638 | High (predicted) | **AF043324** | NMT1 | 1.7 | N-myristoyltransferase 1 |
| miR-133a-3p | −11.6 | High (predicted) | **J03620** | DLD | 1.8 | Dihydrolipoamide dehydrogenase |
| **Targets upregulated in DCM** | | | | | | |
| miR-133a-3p | −1.9 | High (predicted) | **D28475** | CLCN6 | 1.54 | Chloride channel, voltage-sensitive 6 |
| miR-133a-3p | −1.9 | High (predicted) | **M69066** | MSN | 1.6 | Moesin |
| miR-133a-3p | −1.9 | Experimentally Observed | **M26252** | PKM | 1.8 | Pyruvate kinase, muscle |
| miR-208a-3p | −7.8 | High (predicted) | **S54705** | PRKAR1A | 1.9 | Protein kinase, cAMP-dependent, regulatory, type I, alpha |
| **Targets upregulated in both CCC and DCM** | | | | | | |
| miR-133a-3p | −1.9 | High (predicted) | **D28475** | CLCN6 | 1.54 | Chloride channel, voltage-sensitive 6 |
| miR-1 | −6.39 | High (predicted) | **NM_053056.2** | CCND1 | 1.8 | Cyclin D1 |

IPA was used to identify molecular networks among the lists of differentially expressed genes in CCC and DCM that are also targets of the altered miRNAs.

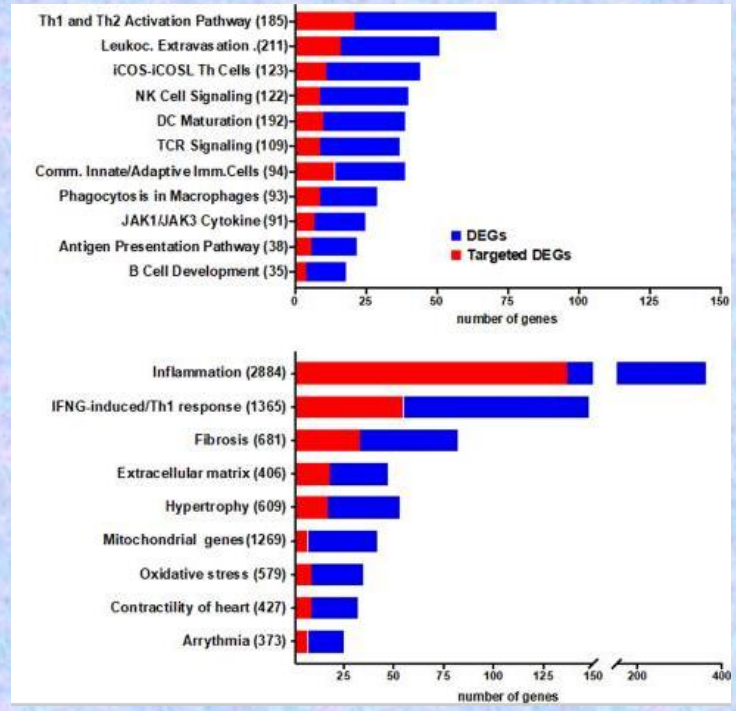


**We raised the hypothesis that mRNA expression and pathways/processes may to be heavily influenced by miRNA expression. To comprehensively address this issue, we performed an integrative genome-wide analysis of the role of miRNA in global gene expression in CCC.**

Whole genome expression analysis was done on SurePrint G3 Human GeneExpression v1 8x60K arrays (Agilent Technologies, Les Ulis, France) following the manufacturer's protocol. MiRNA profiling experiments were done for 754 miRNAs using preprinted TLDA microfluidic cards (Human MicroRNA Card Set v3.0), according to the manufacturer's protocols.

We found 1535 genes to be differentially expressed (DEG) between CCC and control myocardium, of which 1105 (72%) are upregulated, while 430 (28%) genes are downregulated in CCC. IPA canonical pathways analysis showed that the most enriched pathways are mainly immune-related, such as Th1 and Th2 T cells, dendritic cells/antigen presentation, leukocyte extravasation, NK and B cells; this is consistent with the high number of upregulated genes from the incoming inflammatory cells present in CCC but not in control heart tissue. Here is the number of genes in each pathobiological process relevant for the disease such as inflammation, IFNγ-modulated genes/Th1 response, extracellular matrix, fibrosis, contractility of heart, hypertrophy, arrhythmia, oxidative stress/antioxidant response, and mitochondria-related genes.

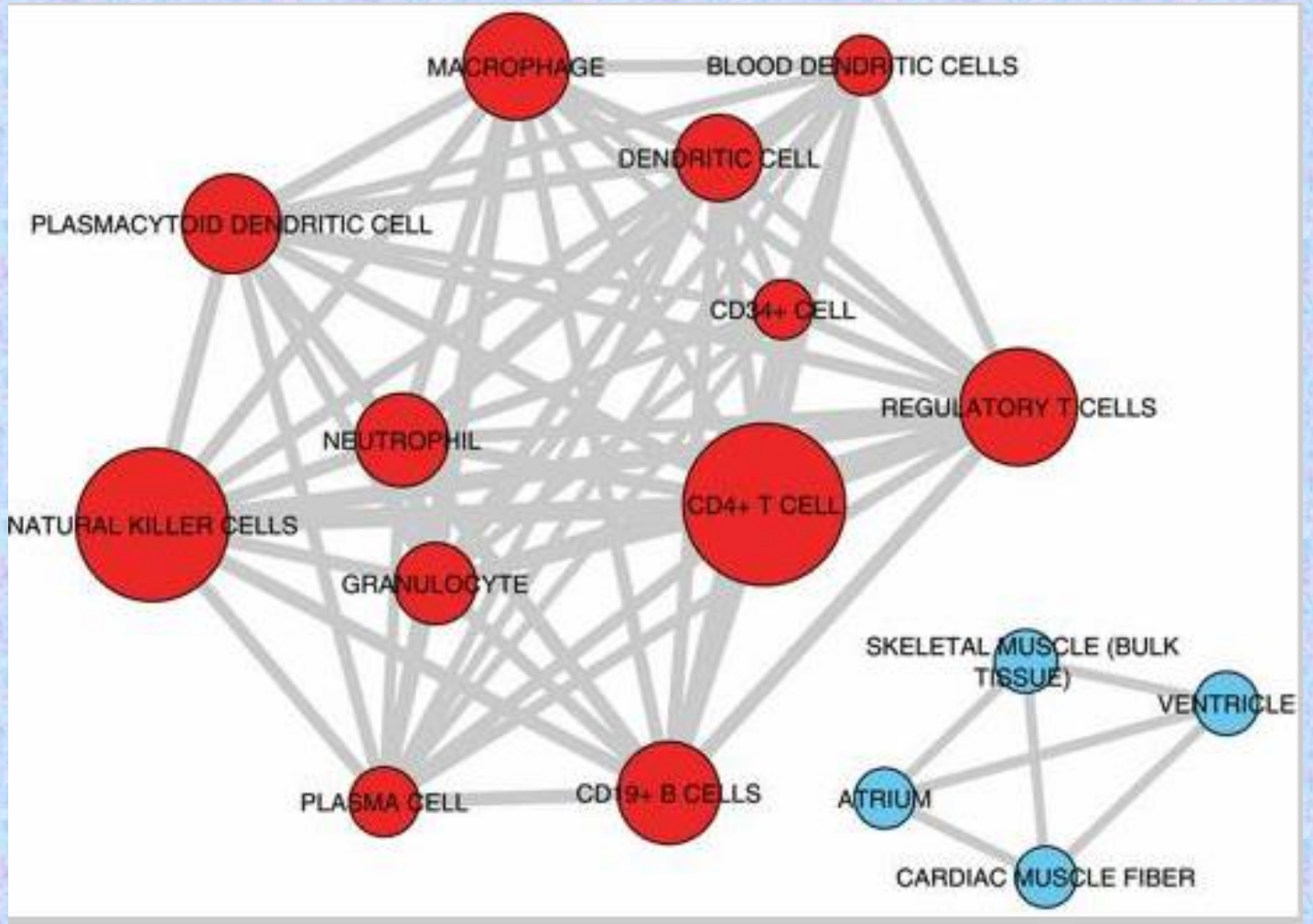




**Laugier L**, Ferreira LRP, Ferreira FM, Cabantous S, Frade AF, Nunes JP, Ribeiro RA, Brochet P, Teixeira PC, Santos RHB, Bocchi EA, Bacal F, Cândido DDS, Maso VE, Nakaya HI, Kalil J, Cunha-Neto E, Chevillard C. miRNAs may play a major role in the control of gene expression in key pathobiological processes in Chagas disease cardiomyopathy. PLoS Negl Trop Dis. 2020 Dec 22;14(12):e0008889. doi: 10.1371/journal.pntd.0008889. PMID: 33351798; PMCID: PMC7787679.

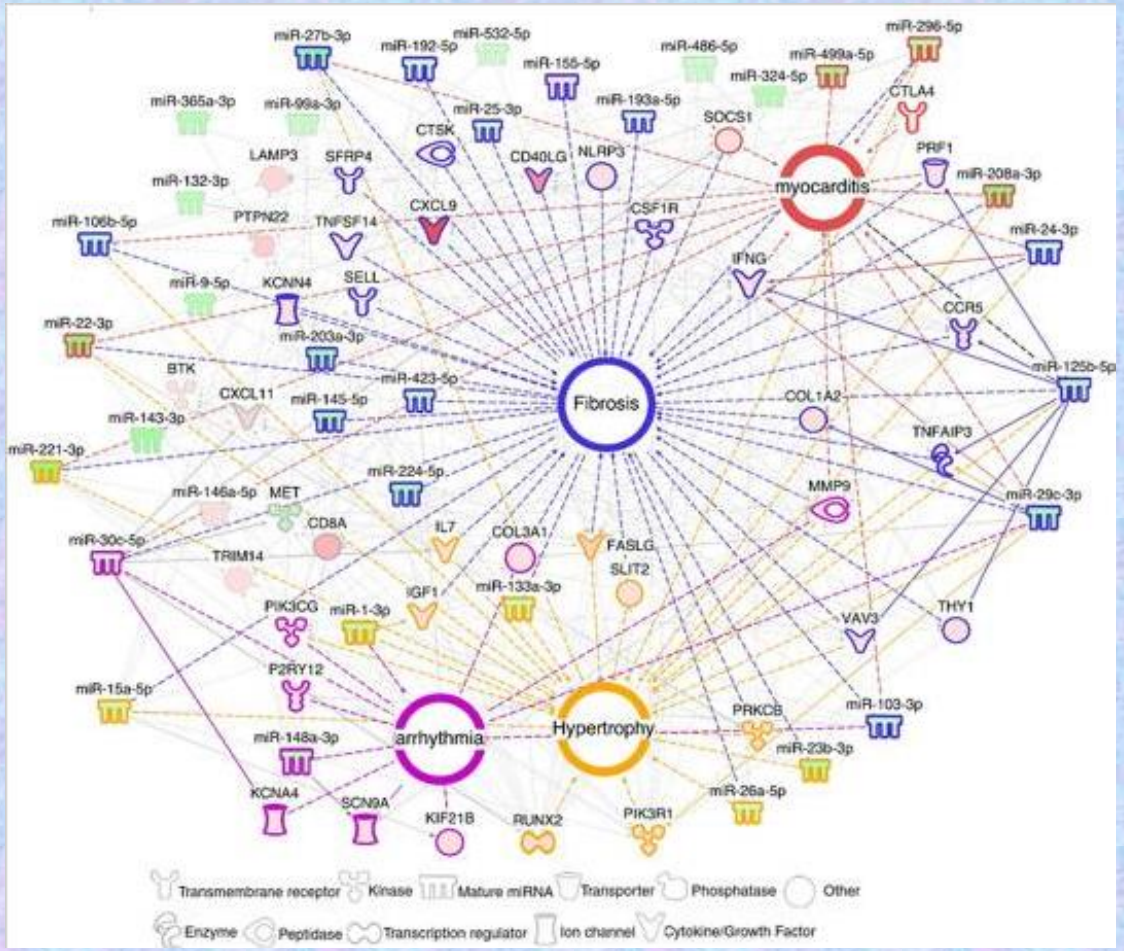
Upstream regulator analysis performed by IPA examines how many targets of each given transcriptional regulator are present in the DEGs—as well as the direction of change–based on the literature and IPA knowledge base; putative regulators are ranked according to overlap with expected targets and directionality (z-score). It indicated that IFNγ is the top upstream regulator, followed by other cytokines like TNFα, IL-18 and EBI3/IL27Rβ chain, the chemokines CCL5 and CXCL10, the transcription factors NF-kB and Ap1, and the PI3K enzyme

Deconvolution of immune cell type profiles in CCC myocardium revealed an enrichment of gene expression signatures of CD4+ T cells, NK cells, B cells/plasma cells, dendritic cells, plasmacytoid dendritic cells, regulatory T cells and granulocytes (red). This indicates that these cell types infiltrate the myocardium of CCC patients. Conversely, genes down-regulated in CCC myocardium when compared to controls were enriched with signatures of cardiac muscle cells (blue). This result is most likely a consequence of reduced representation of cardiac mRNAs in CCC myocardium that was replaced by inflammatory cells.



754 human miRNAs were screened on the heart samples and among them, 210 miRNAs were detected in every sample; these were quantified in each tissue sample. We have found that 80 out of 210 miRNAs were differentially expressed (DEMs) (absolute FC ≥1.5, p<0.05 without correction).

However, the list of the 80 miRNAs obtained without correction for multiple testing seems to be relevant as it contains miR-1 (p = 5,0E-03), miR-133a (p = 1,4E-02) and miR-133b (p = 1.3E-2) that we previously observed as under-expressed in CCC samples as compared to controls. MiR-208a, which was also previously described to be under expressed in CCC samples in the same study, is borderline in the present study (p = 5,5E-02).




In order to identify putative miRNA-target gene interactions among DEMs and DEGs, we performed inverse expression pairing of DEMs (80) and DEGs (1535). A total of 571 miRNA-mRNA interactions involving 67 DEMs and 396 DEGs were found by IPA**.**

We found that 5 miRNAs (hsa-miR-125b-5p, hsa-miR-15a-5p, hsa-miR-296-5p, hsa-miR-29c-3p and hsa-miR-103a-3p) each regulate more than twenty DEGs; moreover, each of them affects at least 6 of the 9 biological functions and processes analyzed. Moreover, several of these “master” miRNAs targeted multiple genes belonging to a given process at the same time, suggesting a synergistic action. A network built with DEM-DEG targets around the important pathobiological processes, myocarditis, fibrosis, hypertrophy and arrhythmia disclosed a strong focus on fibrosis, and several miRNAs and targets participated in various processes.

Our study was performed in whole heart tissue, containing several cell types, including cardiomyocytes, fibroblasts, endothelial and infiltrating inflammatory cells. We must thus keep in mind that results reflect the composite of mRNA and microRNA content of each cell type with its respective contribution.

Most of the RNA will come from cardiomyocytes, but inflammatory cell RNA will readily show up, since control tissue is free from inflammatory infiltrates, showing at most passenger leukocytes that are much less numerous. At any event, our results suggest that, by targeting multiple genes in relevant pathogenic disease pathways and processes, miRNAs can exert a combined regulatory effect that may be stronger than the effect of a single DEM-DEG interaction. In addition, we found a small number of key "high-ranking" differentially expressed miRNAs—those with the highest number of targets, overlapping with those with multiple targets involved in several pathological processes.

Our data identified specific molecular features in key pathogenic processes. Further investigation and validation of the more important miRNA-mRNA interactions involved in fibrosis, oxidative stress, and mitochondrial processes may reveal important insights into the pathogenesis of CCC and may translate in the identification of novel therapeutic targets.

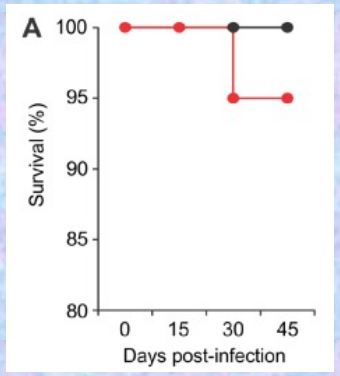
Our findings may have a bearing on myocarditis and inflammatory cardiomyopathy of distinct etiologies as well as to IFN-γ mediated age-related myocardial inflammation and functional decline as recently described.

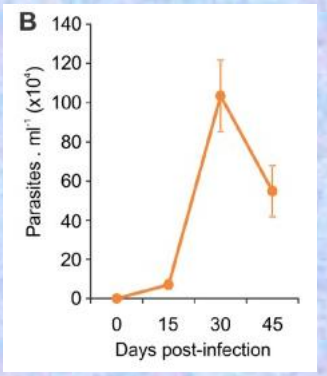

**Laugier L**, Ferreira LRP, Ferreira FM, Cabantous S, Frade AF, Nunes JP, Ribeiro RA, Brochet P, Teixeira PC, Santos RHB, Bocchi EA, Bacal F, Cândido DDS, Maso VE, Nakaya HI, Kalil J, Cunha-Neto E, Chevillard C. miRNAs may play a major role in the control of gene expression in key pathobiological processes in Chagas disease cardiomyopathy. PLoS Negl Trop Dis. 2020 Dec 22;14(12):e0008889. doi: 10.1371/journal.pntd.0008889. PMID: 33351798; PMCID: PMC7787679.

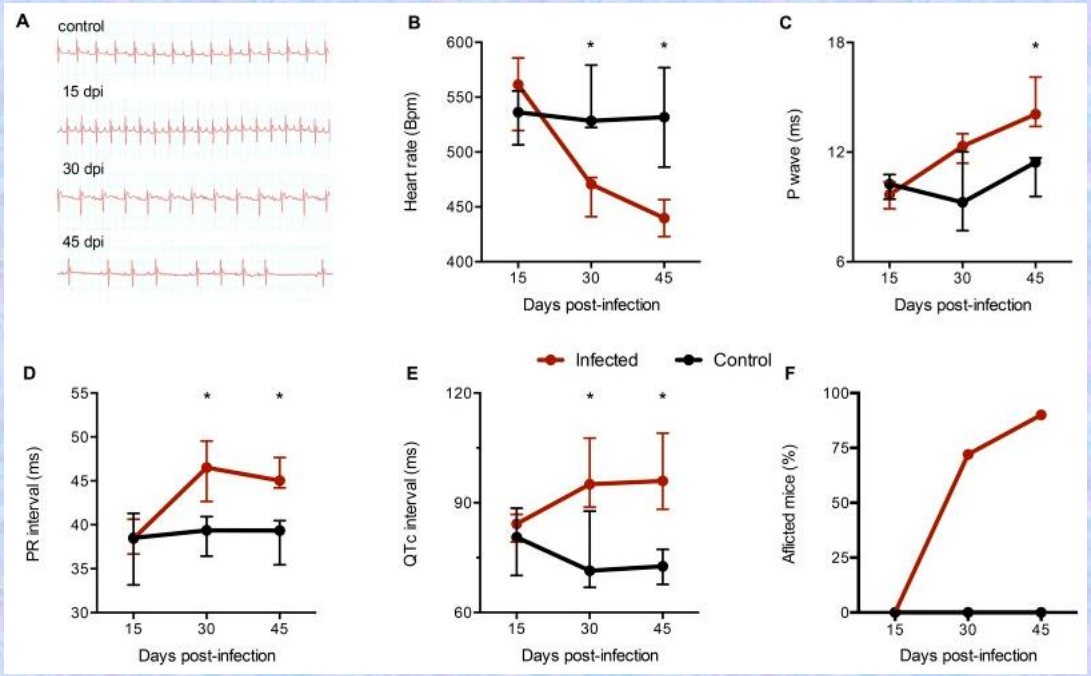
No study has approached the expression of miRNAs during the acute phase of Chagas disease. To investigate the consequences of acute *T. cruzi* infection in host miRNA expression, we used TaqMan Low Density Arrays (TLDA) to screen 641 miRNAs in mouse heart samples at 15, 30 and 45 days post *T. cruzi* infection (dpi).

C57BL/6 mice were intraperitoneally infected with 100 blood trypomastigotes of Colombian *T. cruzi* strain. This parasite strain was previously demonstrated to have a tissue tropism to skeletal muscle and myocardium and high pathogenicity. the ECG profiles of the uninfected controls and all three time points post infection showing the second-degree atrioventricular block and arrhythmia at 30 dpi and 45 dpi.

Infection with *T. cruzi* induced the first ECG alterations at 30 dpi with significant alterations in heart rate, prolongation of P wave and PR interval. QTc interval prolongation starts at 30 dpi. And finally, 75% and 90% of the infected mice present ECG alterations, at 30 and 45 dpi, respectively.

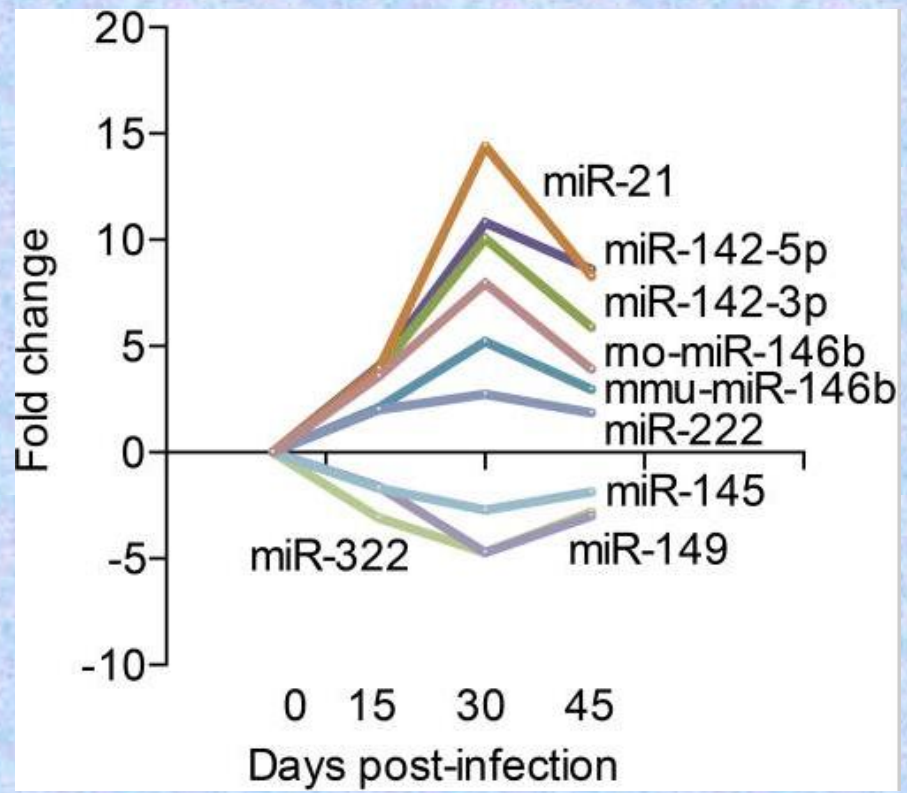


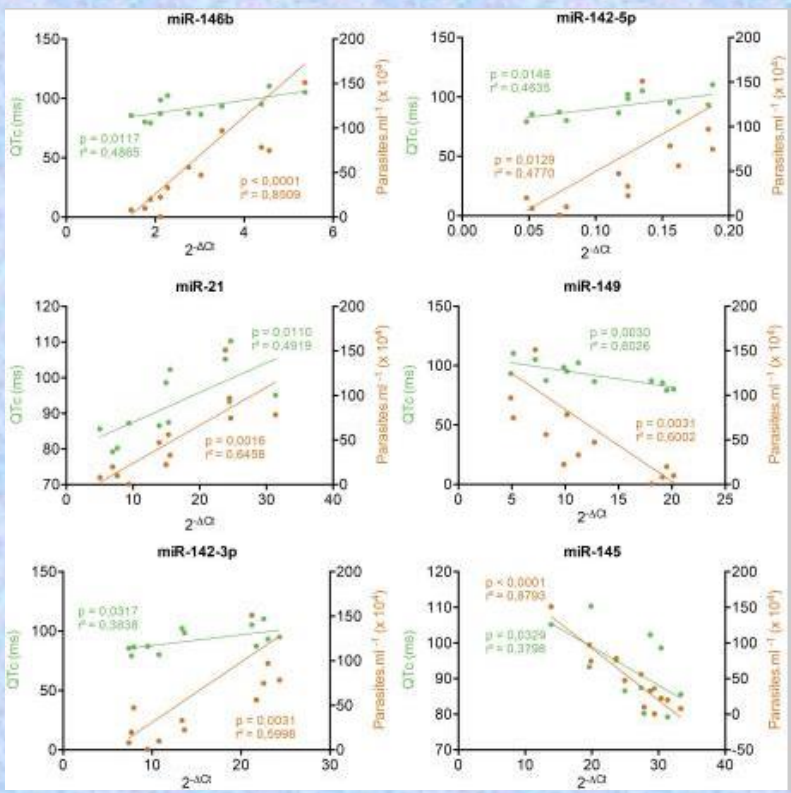



TLDA was used to screen 641 rodent miRNAs in heart samples from acutely *T. cruzi* infected mice at 15, 30 and 45 dpi (four mice per group). We have found 113 out of 641 miRNAs with significantly altered expression upon infection in at least one time point.

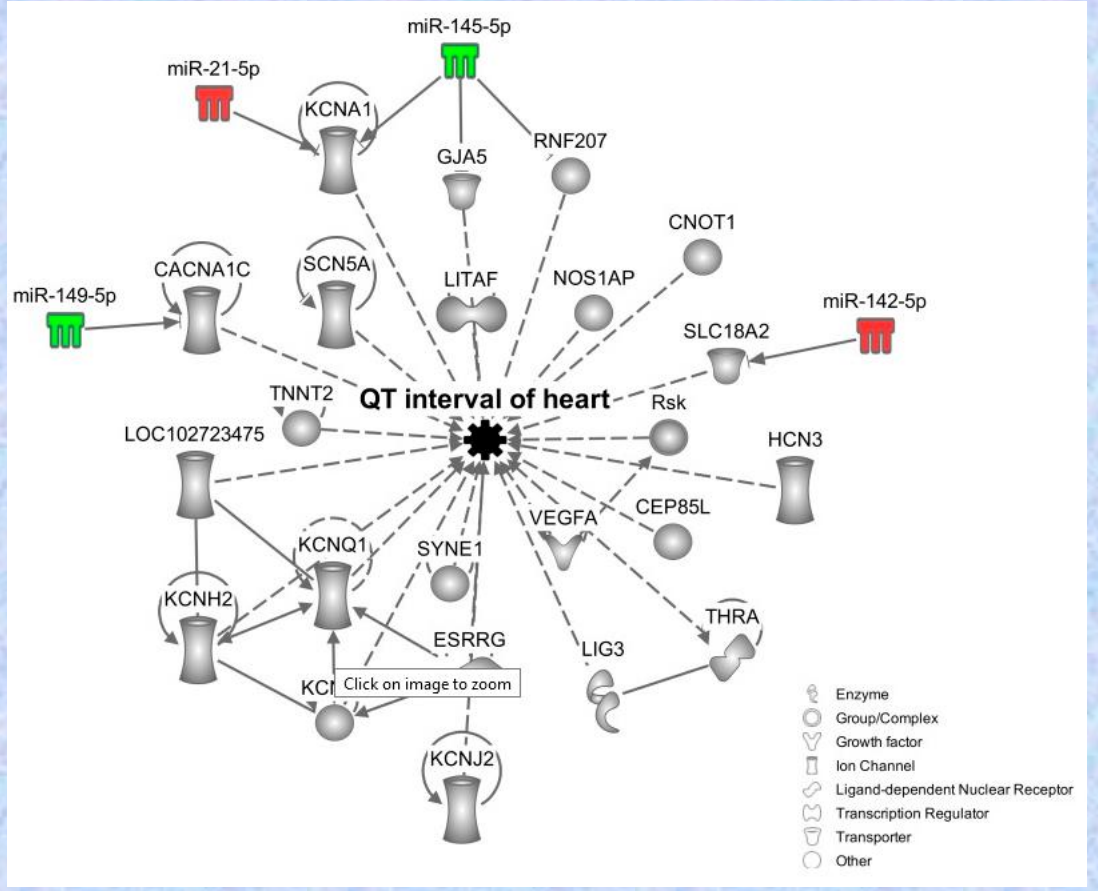
Seventeen miRNAs were significantly deregulated in all three time points post infection. The expression kinetics of nine miRNAs out of those 17 expressed in all three time points follow a similar profile to that of the parasitemia with a peak at 30 dpi. These findings suggest that this cluster of nine microRNAs might be, in some way, associated to the magnitude of the infection and indirectly to cardiac alterations.




In addition, six (out of nine) microRNAs were significantly correlated with changes in both parasitemia and QTc interval: miR-146b, miR-21, miR-142-3p miR-142-5p (positive correlation) and miR-145-5p and miR-149-5p (negative correlation)

Ingenuity Pathway Analysis (IPA) software was used to identify molecular networks and targets of the miRNAs miR-146b, miR-21, miR-142-3p miR-142-5p, miR-145 and miR-149, which were differentially expressed in all three time points post infection and were significantly correlated both with changes in parasitemia and QTc interval. A biological network was built in order to investigate the connection between those six miRNAs and the QTc interval.





**Navarro IC**, Ferreira FM, Nakaya HI, Baron MA, Vilar-Pereira G, Pereira IR, Silva AM, Real JM, De Brito T, Chevillard C, Lannes-Vieira J, Kalil J, Cunha-Neto E, Ferreira LR. MicroRNA Transcriptome Profiling in Heart of Trypanosoma cruzi-Infected Mice: Parasitological and Cardiological Outcomes. PLoS Negl Trop Dis. 2015 Jun 18;9(6):e0003828. doi: 10.1371/journal.pntd.0003828. PMID: 26086673; PMCID: PMC4473529.

Taken together, our results show that acute infection of mice with *T. cruzi* was able to modify the microRNA expression profile of the heart. The study of the acute phase of human Chagas disease is limited by the scarcity of samples, since most of the acute cases are not reported, because of the mild or absent symptomatology.

Correlation analysis identified potential miRNAs related to the clinical parameters. In addition, the pathway analysis revealed putative relationship between miRNAs and their targets, and how they could influence the ECG parameters.

CACNA1C is a calcium channel responsible for the L type current. This molecule is expressed in all excitable heart cells. A previous study showed this current is highly altered during experimental Chagas disease. This gene was highly predicted as a miR-149-5p target.

KCNA1 is a potassium channel massively expressed in neurons and its expression deregulation may facilitate the occurrence of atrial fibrillation. This gene was also highly predicted as target of both miRNAs miR-21-5p and miR-145-5p.

GJA5 codes for the gap junction connexin-40 (Cx40), molecule responsible for electrical impulse conduction in the heart and is associated with atrial fibrillation.

RNF207 is one of the main potassium channels related to repolarization of the cardiac action potential and it was recently associated with the regulation of humans QTc interval.

SLC18A2 codes for vesicular monoamine type 2 transporter, a molecule necessary for the vesicular release of the neurotransmitters. According to our network analysis, it was predicted as a miR-142-5p target.

**The alterations occurring in the host microRNA profile observed here reflect the role of these molecules in the acute phase of the infection and may highlight important aspects of the pathogenesis, opening a broad range of possibilities in the study of Chagas disease**

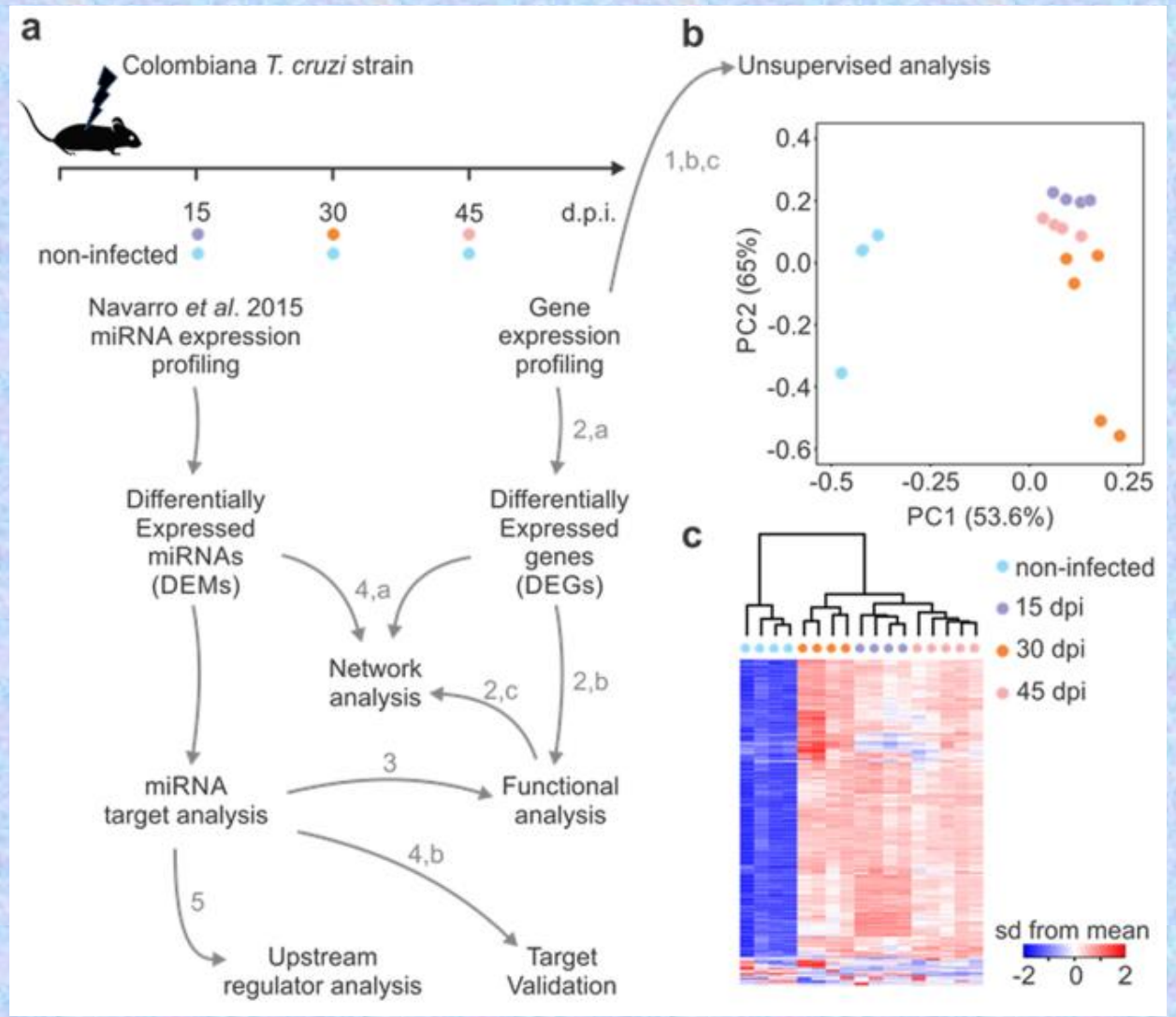

**Navarro IC**, Ferreira FM, Nakaya HI, Baron MA, Vilar-Pereira G, Pereira IR, Silva AM, Real JM, De Brito T, Chevillard C, Lannes-Vieira J, Kalil J, Cunha-Neto E, Ferreira LR. MicroRNA Transcriptome Profiling in Heart of Trypanosoma cruzi-Infected Mice: Parasitological and Cardiological Outcomes. PLoS Negl Trop Dis. 2015 Jun 18;9(6):e0003828. doi: 10.1371/journal.pntd.0003828. PMID: 26086673; PMCID: PMC4473529.

Our group has observed dysregulated miRNA expression in heart samples from CCC patients and acute *T. cruzi* infection in mice. In the mouse study, we found 113 differentially expressed miRs (DEMs) at 15, 30 and/or 45 days post infection. Several of the DEMs were significantly correlated with the clinically relevant parameters parasitemia and electrocardiography changes (QTc interval).

Although those results were suggestive of a role for miRNA in gene regulation and disease parameters, the relevance of miRNA control of the overall gene expression in heart of infected mice was still unknown.

In order to assess the role of miRNAs in the regulation of the transcriptional changes that occur during acute *T. cruzi* infection, we have performed an integrated genome-wide analysis of genes and miRNA expression changes in the hearts of acutely infected mice.

To this end, we performed mRNA expression analysis and used sequence-based miRNA target prediction and negative correlation of differentially expressed miRNA and mRNA in *T. cruzi*-infected heart tissue to identify regulated pathways.

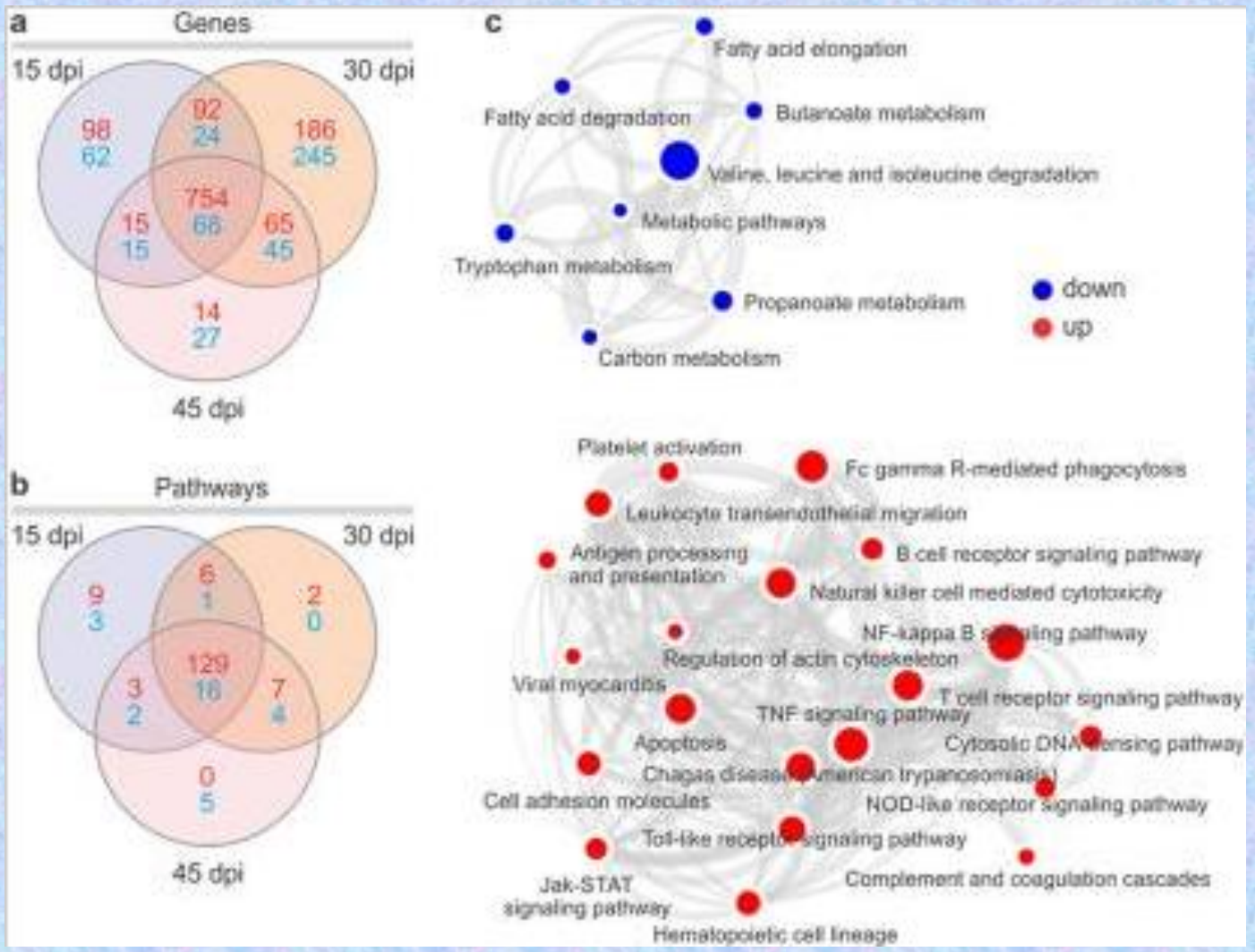


The total number of DEGs was 1685. The number of up- or downregulated DEGs at each time point, as well as those shared between them are depicted.

Pathways analysis of the DEGs in all time points was performed with gene set enrichment analysis (GSEA). Metabolic pathways such as fatty acid and amino acid degradation are predicted to be downregulated (blue) and pathways/biological functions related to innate and acquired immune response are predicted to be upregulated (red).

Since IFNγ is the most upregulated cytokine in all time points (30-50-fold versus uninfected heart).

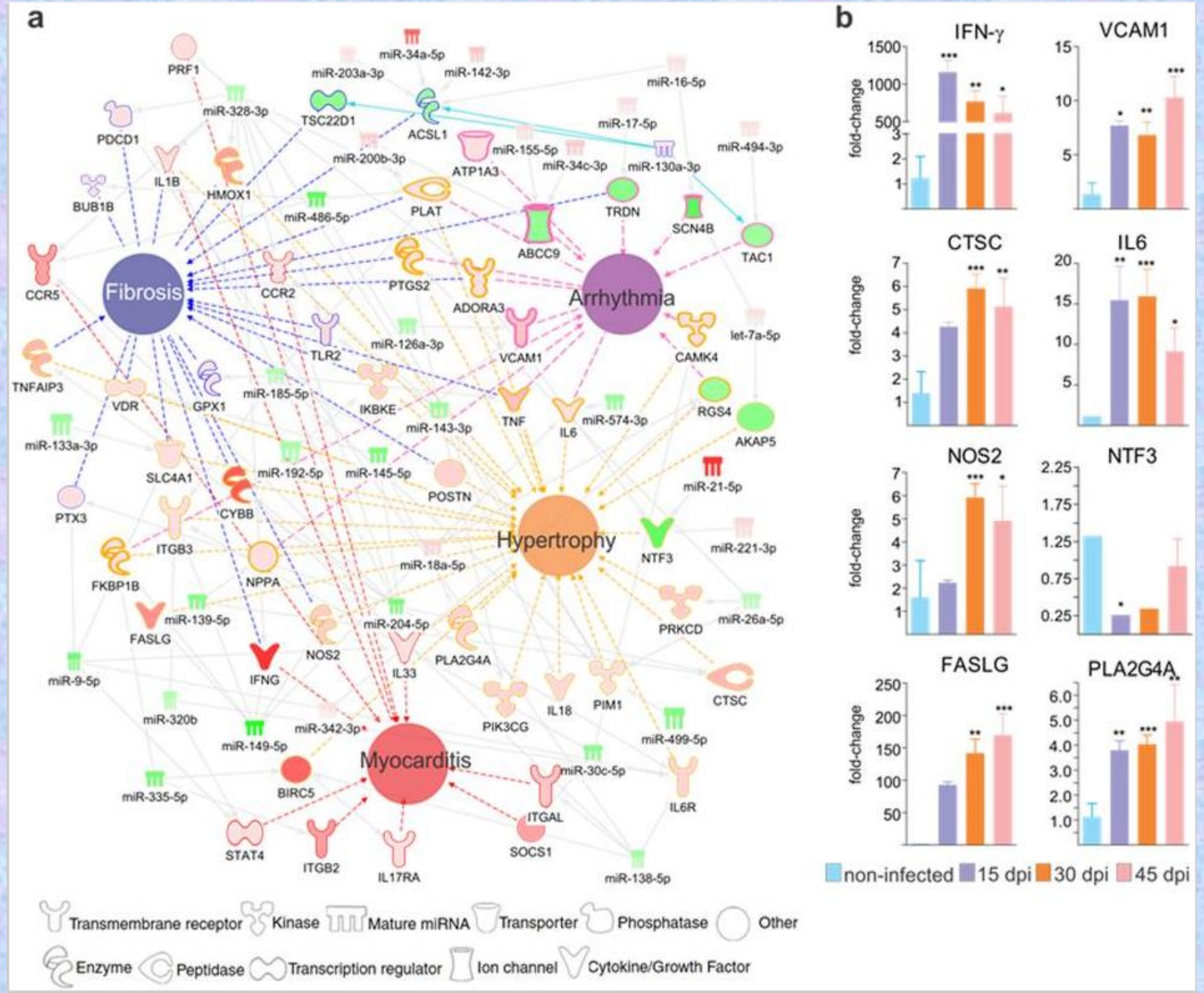
We found that 219/1067 IFNγ target genes are differentially expressed in all time points - approximately 20% of the IFNγ signature.



Our previous results have shown 113 DEMs upon *T. cruzi* infection at 15, 30 and 45 days post infection. The analysis of putative targets of DEMs within the DEGs list at each time point identified a total of 848 putative inversely paired targets of the 113 DEMs, using miRNA-target relationships predicted as high or experimentally validated.

In order to investigate the possible role of miRNAs in regulating the key pathophysiological processes in Chagas heart disease, we built networks with DEGs and DEMs in the 3 time points after *T. cruzi* infection, around each disease process (Arrhythmia, Fibrosis, Myocarditis and Hypertrophy of Heart).

qPCR validation of microarray expression results analysis on 8 miRNA targets present in the different networks and time points confirmed differential expression.



Our study was performed in whole heart tissue, containing several cell types, including cardiomyocytes, fibroblasts, endothelial and infiltrating inflammatory cells. This means we cannot be certain that all these miRNA and mRNA changes occurred in the same cell type. For instance, it is likely that many of the immune response-associated DEGs were expressed in the infiltrating inflammatory cells, although infection and inflammation can trigger expression of inflammatory genes in heart parenchymal cells as well.

This correlation approach has been used to provide an overview of miRNA-mRNA networks that involve a high number of differentially expressed genes. Indeed, a broad systematic analysis is limited due to the fact that correlation is not proof of causality. Our results suggest that, by potentially targeting multiple genes in each of several disease pathways and pathobiologic processes, microRNAs may exert a combined regulatory effect that may be stronger than the effect of a microRNA targeting a single mRNA in a pathway.

In addition, we found a small number of key “high-ranking” differentially expressed miRNAs - those with a high number of targets involved in several pathological processes. Our data identified specific molecular features of acute *T. cruzi* infection that may translate in the identification of novel therapeutic targets.

*In vitro* and *in vivo* testing of targeting of key miRNA to induce the amplification of anti-*T. cruzi* and tissue protection mechanisms, like the Nrf2 pathway or HMOX1; or reduction of maladaptive responses, like mitochondrial dysfunction, may establish the functional or therapeutic relevance of miRNA regulation in the context of *T. cruzi* infection.


**Ferreira LRP**, Ferreira FM, Laugier L, Cabantous S, Navarro IC, da Silva Cândido D, Rigaud VC, Real JM, Pereira GV, Pereira IR, Ruivo L, Pandey RP, Savoia M, Kalil J, Lannes-Vieira J, Nakaya H, Chevillard C, Cunha-Neto E. Integration of miRNA and gene expression profiles suggest a role for miRNAs in the pathobiological processes of acute Trypanosoma cruzi infection. Sci Rep. 2017 Dec 21;7(1):17990. doi: 10.1038/s41598-017-18080-9. PMID: 29269773; PMCID: PMC5740174.

# The Long Noncoding RNA (MIAT), Is Overexpressed During Dilated Cardiomyopathy Due to Chronic Chagas Disease.

A transcriptome analysis was performed on heart tissue biopsy specimens from 10 patients with CCC, 14 subjects with DCM, and 7 controls. Levels of 9 of 14 MIAT-specific probes were significantly different between patients with CCC and controls and between patients with CCC and patients with DCM. When we compared MIAT levels in patients with CCC were 3–49-fold greater than those in control subjects. MIATs were also upregulated in patients with CCC by 2–20-fold, relative to levels in subjects with DCM. To verify the robustness of our microarray results, MIAT was selected for validation by RT-PCR.

The second expression analysis was done on heart left ventricular tissue biopsy specimens from 18 patients with CCC, 17 subjects with DCM, and 7 controls. qRT-PCR confirmed the observed microarray expression changes in the gene.

The third analysis involved FFPE heart tissue samples from 111 patients. MIAT was detected in 78 samples (70%). The average fold change between patients with CCC and subjects with DCM was 4.8. To calculate the positive predictive value of elevated MIAT expression for FFPE samples from patients with CCC, we established a cutoff of $3 \times 10^{-7}$ in the level of MIAT expression. Levels of MIAT expression in 3 of 55 samples from subjects with DCM were above the cutoff, and 26 of 56 samples from patients with CCC had levels of MIAT expression above the cutoff. Therefore, elevated levels of MIAT expression in FFPE samples from patients with CCC had a positive predictive value of 89.6%.

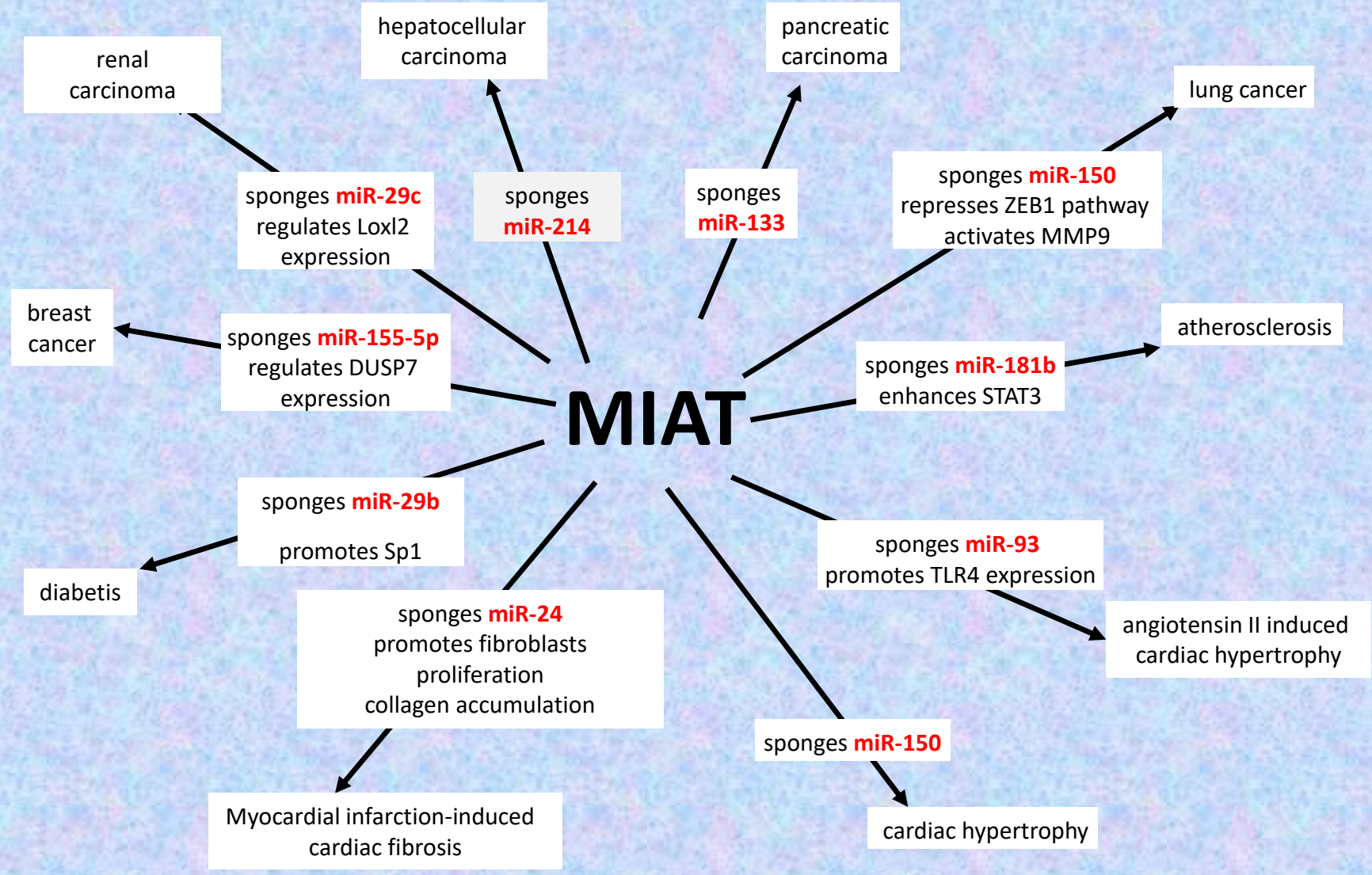
In the fourth analysis was done C57BL6 female infected mice 15, 30, and 45 days post infection. Significant differences were detected between groups. Linear regression with QTc intervals was detected but not with parasitemia levels.

The present study revealed that overexpression of MIAT was found specifically in myocardial samples from patients with CCC but not in those from subjects with DCM or control tissue, according to whole-transcriptome analysis of heart tissue samples. This overexpression was confirmed by qPCR analysis of a larger set of freshly frozen and FFPE heart tissue samples. Similar data were obtained in myocardial samples of *T. cruzi*–infected mice, where a correlation was found between the level of MIAT expression and QTc values. To our knowledge, this is the first study to provide evidence that lncRNAs are associated with chronic dilated cardiomyopathy.

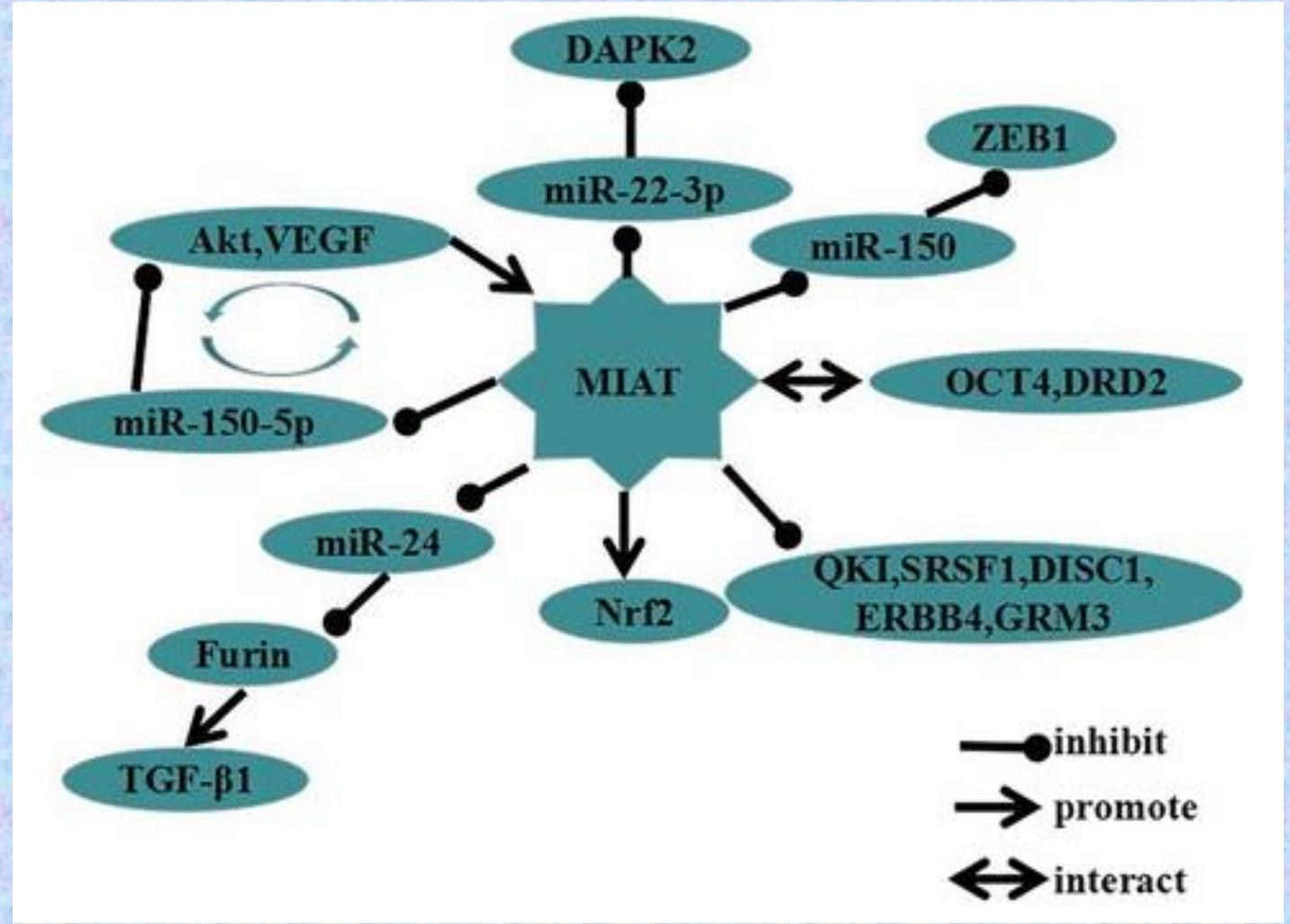
Ishii et al identified an MIAT haplotype associated with an increased risk of myocardial infraction in a Japanese population (Ishii N et al. 2006). Functional analyses revealed that the minor variant of 1 single-nucleotide polymorphism in exon 5 increased transcriptional levels of this lncRNA. Moreover, nuclear proteins bound more intensely to the allele associated with an increased risk than to the allele that did not increase the risk. The frequencies of the associated polymorphisms were too low in the Brazilian population to be able to detect significant association.

**Ishii N**, Ozaki K, Sato H, Mizuno H, Susumu Saito, Takahashi A, Miyamoto Y, Ikegawa S, Kamatani N, Hori M, Satoshi Saito, Nakamura Y, Tanaka T. Identification of a novel non-coding RNA, MIAT, that confers risk of myocardial infarction. J Hum Genet. 2006;51(12):1087-1099. doi: 10.1007/s10038-006-0070-9. Epub 2006 Oct 26. PMID: 17066261.

Numerous microRNAs such as miR-214, miR-22-3p, miR-520d-3p, miR-203a, miR-29a-3p, miR-141, miR-150, miR-302, miR-29, and miR-155-5p have functional interactions with this lncRNA. Moreover, dysregulation of MIAT has been associated with abnormal activity of numerous cancer-related signaling cascades such as Hippo, PI3K/Akt/c-Met and Wnt/β-catenin.

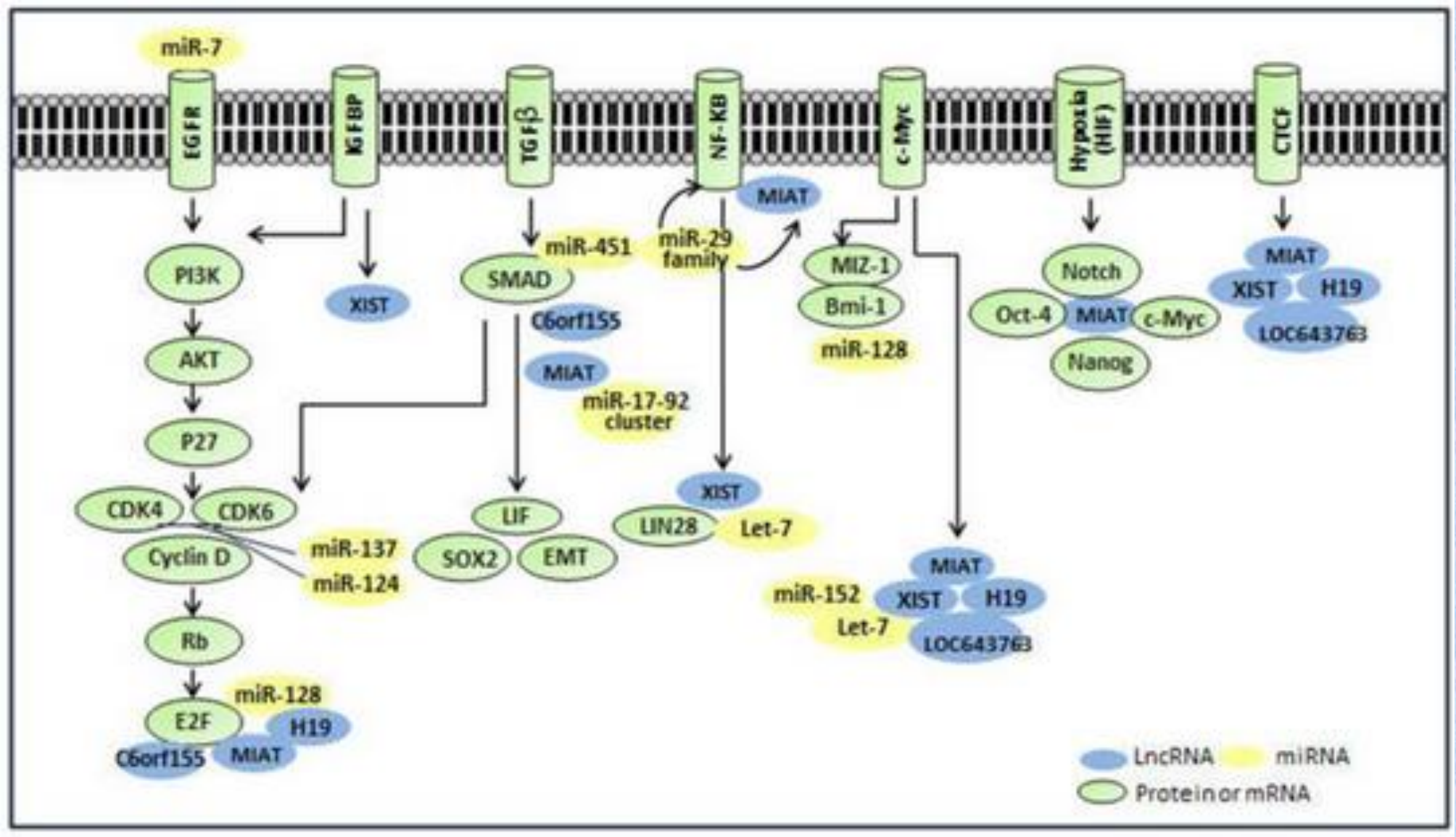


MIAT, a well-characterized disease-related lncRNA, can impact cellular functions such as proliferation, apoptosis, and invasion in various human diseases. The regulatory mechanisms of MIAT are extraordinarily complicated and involve multiple steps (Sun C et al. 2018). The aberrant expression of MIAT exhibits a critical role in disease development, and may serve as a potential biomarker for diagnosis and prognosis. Due to its strong disease specificity and reduced systemic toxicity, MIAT, which acts as a viable therapeutic target, is extremely promising. Taken together, the research that focuses on MIAT is still in the early stage, and some pivotal matters for its clinical application still need to be resolved. The detailed regulatory mechanisms upstream and downstream of MIAT should be paid attention to exploring, and consolidate the underlying mechanisms from the former. Undoubtedly, efforts to clarify the underlying mechanisms promise that MIAT will ultimately reach the clinic.




**Sun C**, Huang L, Li Z, Leng K, Xu Y, Jiang X, Cui Y. Long non-coding RNA MIATin development and disease: a new player in an old game. J Biomed Sci. 2018 Mar 13;25(1):23. doi: 10.1186/s12929-018-0427-3. PMID: 29534728; PMCID: PMC5851271.

# MIAT in various pathways




**Zhang X**, Kiang KM, Zhang GP, Leung GK. Long Non-Coding RNAs Dysregulation and Function in Glioblastoma Stem Cells. Noncoding RNA. 2015 Jun 3;1(1):69-86. doi: 10.3390/ncrna1010069. PMID: 29861416; PMCID: PMC5932540.

# DNA methylation and cardiomyopathy

## Evidences

Methylation of cytosine in CG dinucleotides within regulatory elements is believed to silence gene expression. These dinucleotides occur in certain important regulatory elements in the promoter region of the human beta-myosin heavy chain (beta-MHC) gene (Clifford CP et al. 1998). Clifford CP et al. found a reciprocal relationship between the level of beta-MHC mRNA expression in leucocytes and atrial myocardium and the degree of methylation of CG dinucleotides in the 5' regulatory elements of the gene.

Movassagh M et al. studied the pattern of DNA methylation, they undertook profiling with ischaemic and idiopathic end-stage cardiomyopathic left ventricular (LV) explants from patients who had undergone cardiac transplantation compared to normal control (Movassagh M et al. 2010). They identified 3 angiogenesis-related genetic loci that were differentially methylated. Moreover, for each individual LV tissue, differential methylation showed a predicted correlation to differential expression of the corresponding gene.

Meurs et al. evaluate the CpG methylation level of the exonic regions of the cardiac myosin binding protein C gene (MYBPC3), a common causal gene for hypertrophic cardiomyopathy. They also evaluated the methylation of the CpGs within the exonic regions of the skeletal muscle isoform of the myosin binding protein C gene (MYBPC2). The mean methylation level of CpGs was significantly higher in MYBPC3 than MYBPC2 (Meurs KM et al. 2011).

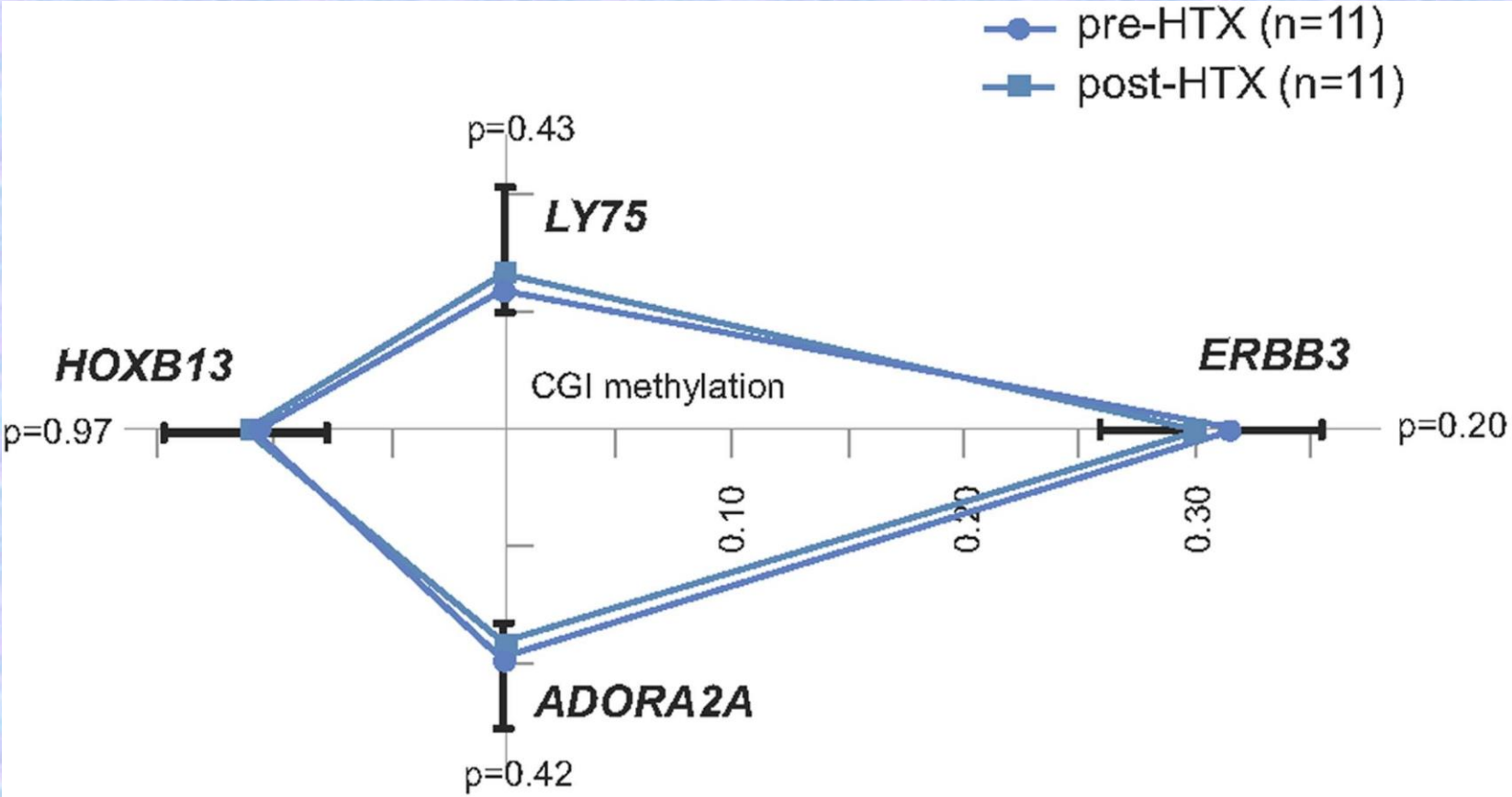
**Clifford CP**, Nunez DJ. Human beta-myosin heavy chain mRNA prevalence is inversely related to the degree of methylation of regulatory elements. Cardiovasc Res. 1998 Jun;38(3):736-43. doi: 10.1016/s0008 6363(98)00058-3. PMID: 9747442.

**Movassagh M**, Choy MK, Goddard M, Bennett MR, Down TA, Foo RS. Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure. PLoS One. 2010 Jan 13;5(1):e8564. doi: 10.1371/journal.pone.0008564. PMID: 20084101; PMCID: PMC2797324.

**Meurs KM**, Kuan M. Differential methylation of CpG sites in two isoforms of myosin binding protein C, an important hypertrophic cardiomyopathy gene. Environ Mol Mutagen. 2011 Mar;52(2):161-4. doi: 10.1002/em.20596. Epub 2010 Aug 25. PMID: 20740642.

The first exhaustive analysis was conductaed by Haas et al. (Haas et al. 2013). They studied genome-wide cardiac DNA methylation in DCM patients and controls to detect a possible epigenetic contribution to DCM. They detected distinct DNA methylation patterns in left ventricular heart tissue of DCM patients for several genes with previously unknown function in DCM, namely Lymphocyte antigen 75 (LY75), Tyrosine kinase-type cell surface receptor HER3 (ERBB3), Homeobox B13 (HOXB13) and Adenosine receptor A2A (ADORA2A).

They were able to show the functional relevance for the contribution of the identified genes in the pathogenesis of heart failure by using zebrafish as an *in vivo* model.

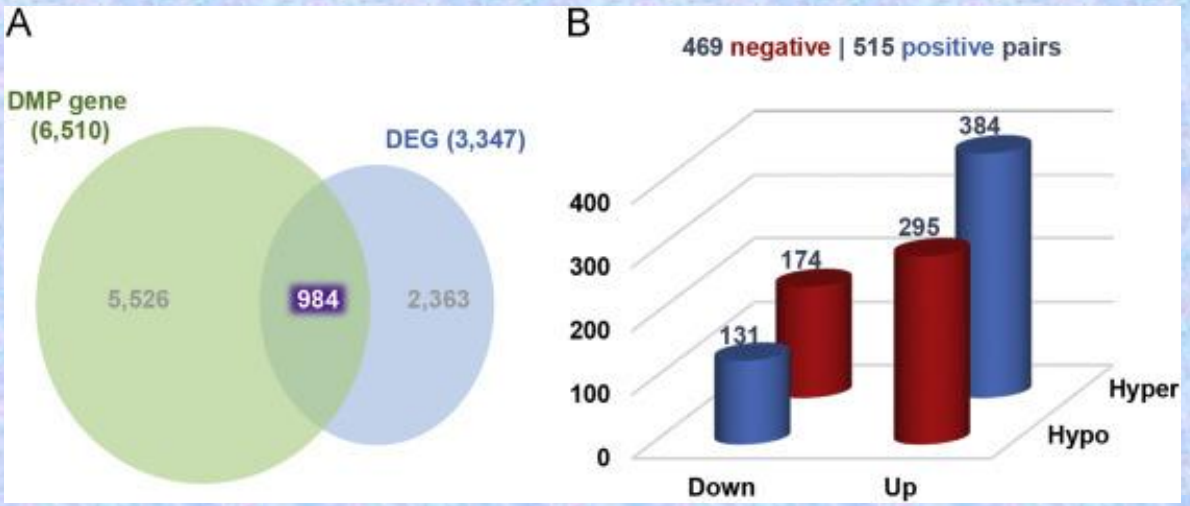



**Haas J**, Frese KS, Park YJ, Keller A, Vogel B, Lindroth AM, Weichenhan D, Franke J, Fischer S, Bauer A, Marquart S, Sedaghat-Hamedani F, Kayvanpour E, Köhler D, Wolf NM, Hassel S, Nietsch R, Wieland T, Ehlermann P, Schultz JH, Dösch A, Mereles D, Hardt S, Backs J, Hoheisel JD, Plass C, Katus HA, Meder B. Alterations in cardiac DNA methylation in human dilated cardiomyopathy. EMBO Mol Med. 2013 Mar;5(3):413-29. doi: 10.1002/emmm.201201553. Epub 2013 Jan 22. PMID: 23341106; PMCID: PMC3598081.

Jo BS et al. performed DNA methylation profiling using the Infinium 450K HumanMethylation BeadChip and mRNA expression profiling using the Human HT-12 v4 Expression BeadChip to identify the molecular alterations underlying the pathogenesis of DCM. They identified not only several genes already known to be associated with DCM but also novel genes whose DNA methylation and gene expression patterns were altered (Jo BS et al. 2016).

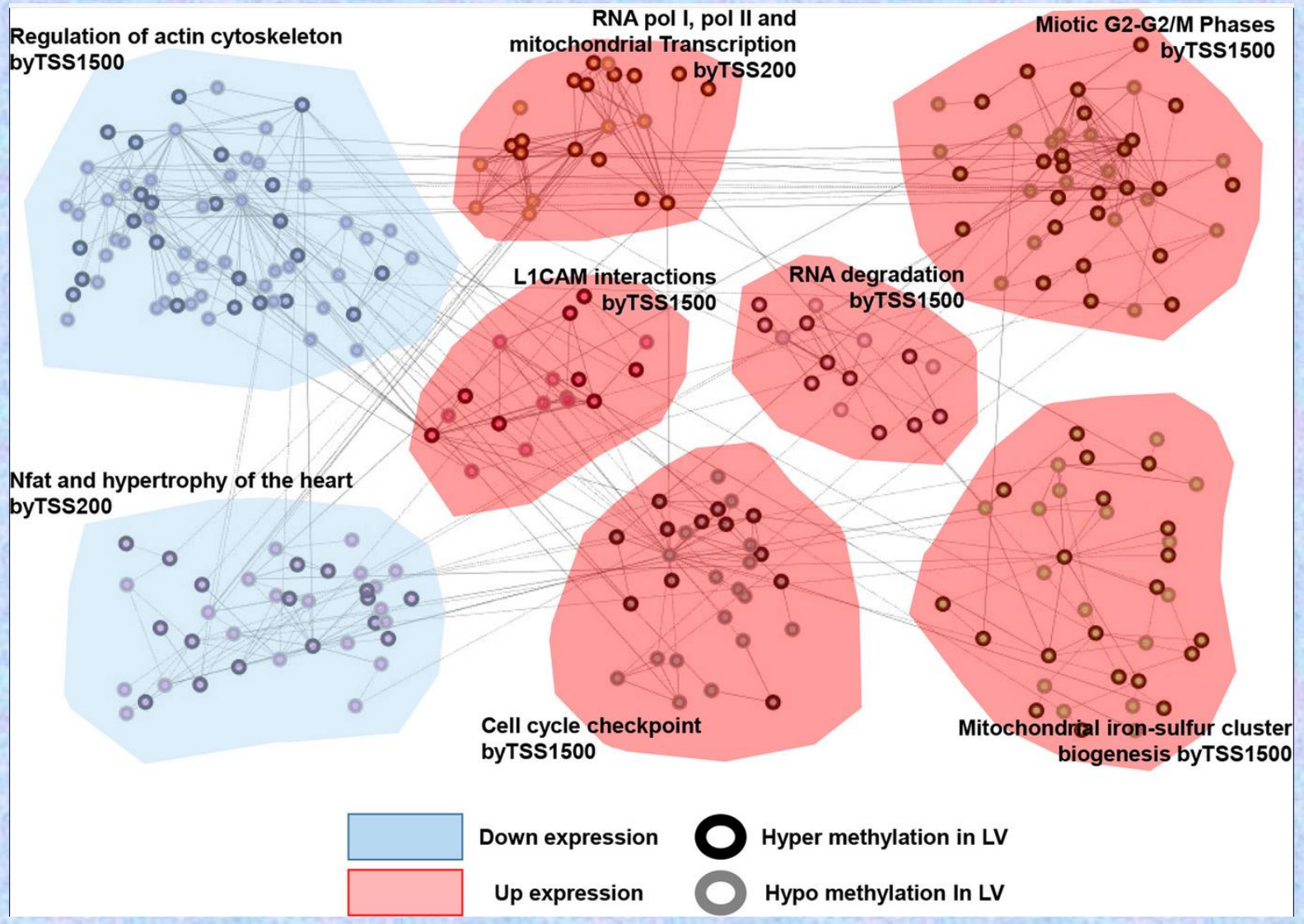
They found that the methylation alterations that occurred in promoter-upstream regions tended to be negatively linked to the gene expression changes as expected by conventional wisdom; the densities of 'Hypo-Up' and 'Hyper-Down' pairs were higher than those of 'Hypo-down' and 'Hyper-Up' pairs, respectively, in the upstream regions of genes such as TSS1500, TSS200, and 5'UTR.

The same analysis was also applied to the CpG sites located in the DHSs and enhancer regions. in the DHSs corresponded to the 'negative-' and 'positive-relation' group, respectively. Overall, the densities of 'Hypo-Up' and 'Hyper-Down' pairs were higher than those of the 'Hypo-Down' and 'Hyper-Up' pairs (i.e., the 'positive-relation' group) in the DHSs and enhancer regions.




**Jo BS**, Koh IU, Bae JB, Yu HY, Jeon ES, Lee HY, Kim JJ, Choi M, Choi SS. Methylome analysis reveals alterations in DNA methylation in the regulatory regions of left ventricle development genes in human dilated cardiomyopathy. Genomics. 2016 Aug;108(2):84-92. doi: 10.1016/j.ygeno.2016.07.001. Epub 2016 Jul 12. PMID: 27417303.

They established a functional interaction network using the 984 DMP-DEG pairs using the Reactome. The GREAT analysis also revealed that the significant categorical terms of the 45 genes included “cardiac left ventricle morphogenesis”.




**Jo BS**, Koh IU, Bae JB, Yu HY, Jeon ES, Lee HY, Kim JJ, Choi M, Choi SS. Methylome analysis reveals alterations in DNA methylation in the regulatory regions of left ventricle development genes in human dilated cardiomyopathy. Genomics. 2016 Aug;108(2):84-92. doi: 10.1016/j.ygeno.2016.07.001. Epub 2016 Jul 12. PMID: 27417303.

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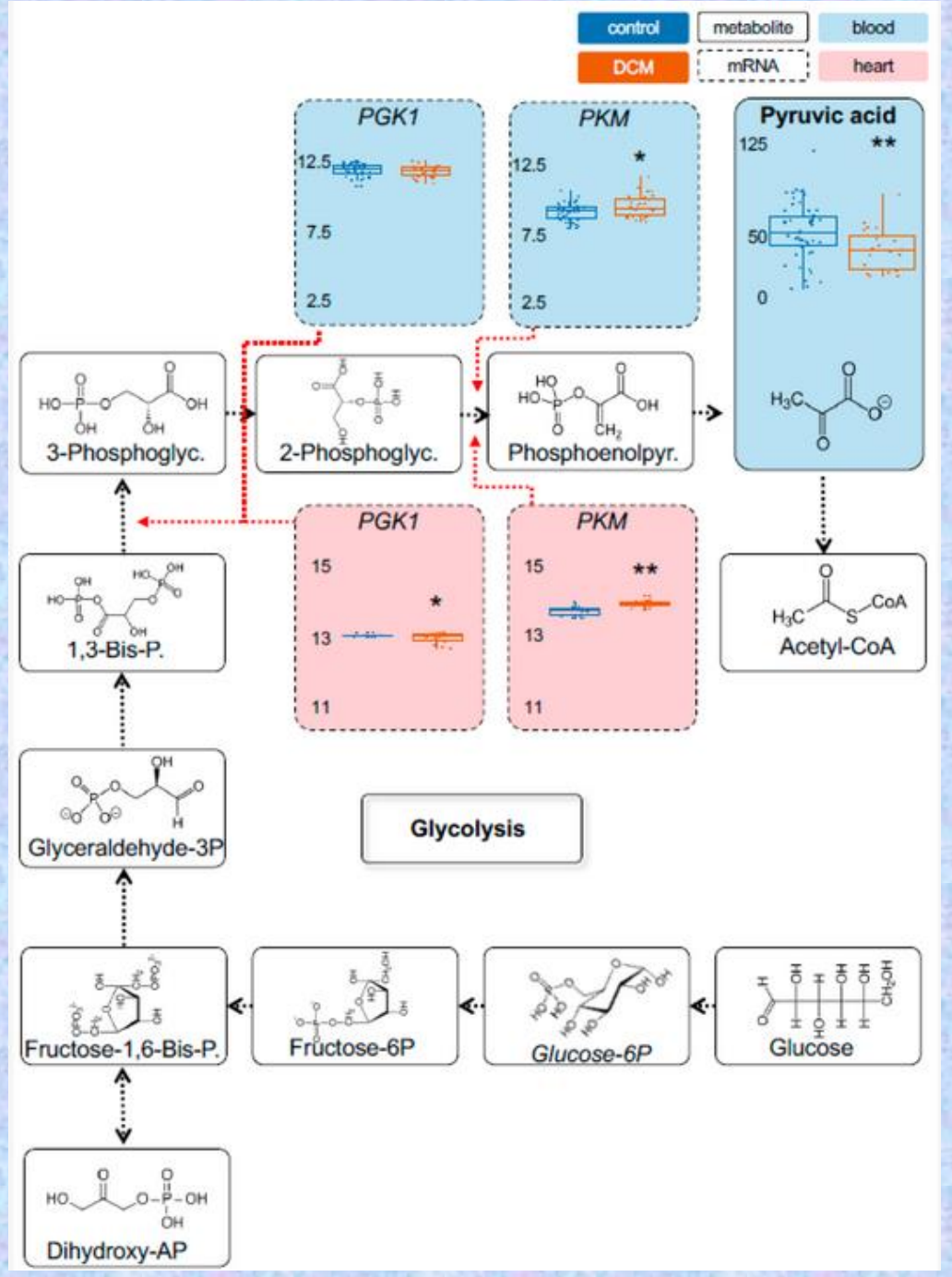
| Gene Symbol | Official Full Name |
|---|---|
| ALX4 | ALX homeobox 4 |
| ARHGEF10 | Rho guanine nucleotide exchange factor (GEF) 10 |
| ATP2A1 | ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 |
| BBC3 | BCL2 binding component 3 |
| BCL2 | B-cell CLL/lymphoma 2 |
| BRSK2 | BR serine/threonine kinase 2 |
| DNAJC10 | DnaJ (Hsp40) homolog, subfamily C, member 10 |
| EN1 | Engrailed homeobox 1 |
| FGF10 | Fibroblast growth factor 10 |
| FGF8 | Fibroblast growth factor 8 (androgen-induced) |
| FOXC1 | Forkhead box C1 |
| FOXC2 | Forkhead box C2 (MFH-1, mesenchyme forkhead 1) |
| FOXE3 | Forkhead box E3 |
| FOXF1 | Forkhead box F1 |
| GNB2L1 | Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 |
| HAND1 | Heart and neural crest derivatives expressed 1 |
| HOXA3 | Homeobox A3 |
| HOXA5 | Homeobox A5 |
| HOXD11 | Homeobox D11 |
| ISL1 | ISL LIM homeobox 1 |
| ITPR1 | Inositol 1,4,5-trisphosphate receptor, type 1 |
| MECOM | MDS1 and EVI1 complex locus |
| MSX2 | msh homeobox 2 |
| MYBPC3 | Myosin binding protein C, cardiac |
| MYL2 | Myosin, light chain 2, regulatory, cardiac, slow |
| NKX2-5 | NK2 homeobox 5 |
| NOTCH1 | Notch 1 |
| OSR2 | Odd-skipped related transcription factor 2 |
| PPP1R13L | Protein phosphatase 1, regulatory subunit 13 like |
| PTCD2 | Pentatricopeptide repeat domain 2 |
| RAPGEF3 | Rap guanine nucleotide exchange factor (GEF) 3 |
| RBPJ | Recombination signal binding protein for immunoglobulin kappa J region |
| RDH10 | Retinol dehydrogenase 10 (all-trans) |
| RYR2 | Ryanodine receptor 2 (cardiac) |
| SIX1 | SIX homeobox 1 |
| SMAD3 | SMAD family member 3 |
| TBX5 | T-box 5 |
| TFAP2A | Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) |
| TGFBR3 | Transforming growth factor, beta receptor III |
| THRA | Thyroid hormone receptor, alpha |
| TMBIM6 | Transmembrane BAX inhibitor motif containing 6 |
| TNFRSF10B | Tumor necrosis factor receptor superfamily, member 10b |
| TNNC1 | Troponin C type 1 (slow) |
| TWIST1 | Twist family bHLH transcription factor 1 |
| WNT7A | Wingless-type MMTV integration site family, member 7A |

Taken altogether, DNA methylation patterns are significantly altered between the LV and RV, and the alterations in turn affect mRNA expression patterns between two opposing statuses. Certainly, the study of global DNA methylation changes that occur under different physiological conditions can reveal the epigenomic dynamics that control gene expression during disease pathogenesis.

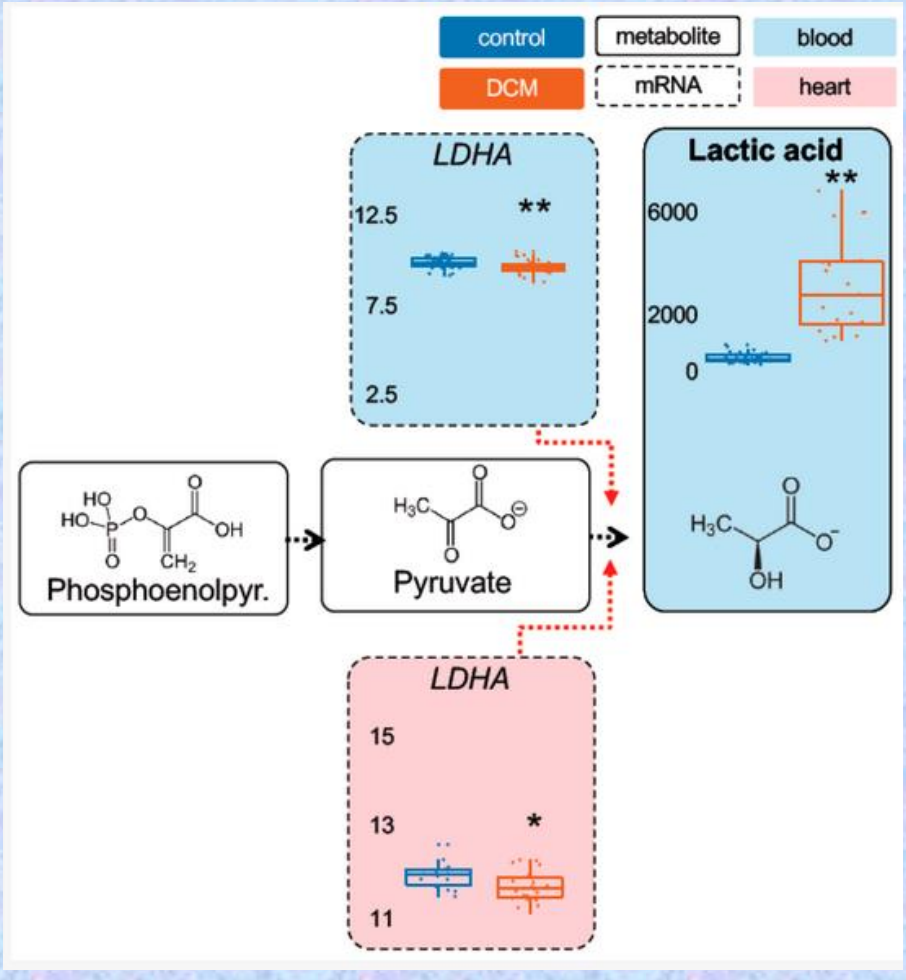
This year, Haas et al. Continue to increase knowledge on heart failure (Haas J et al. 2021). As energy production pathways are known to play a pivotal role in heart failure, we sought here to identify key metabolic changes in ischemic- and non-ischemic heart failure by using a multi-OMICS approach. Serum metabolites and mRNAseq and epigenetic DNA methylation profiles were analyzed from blood and left ventricular heart biopsy specimens of the same individuals.

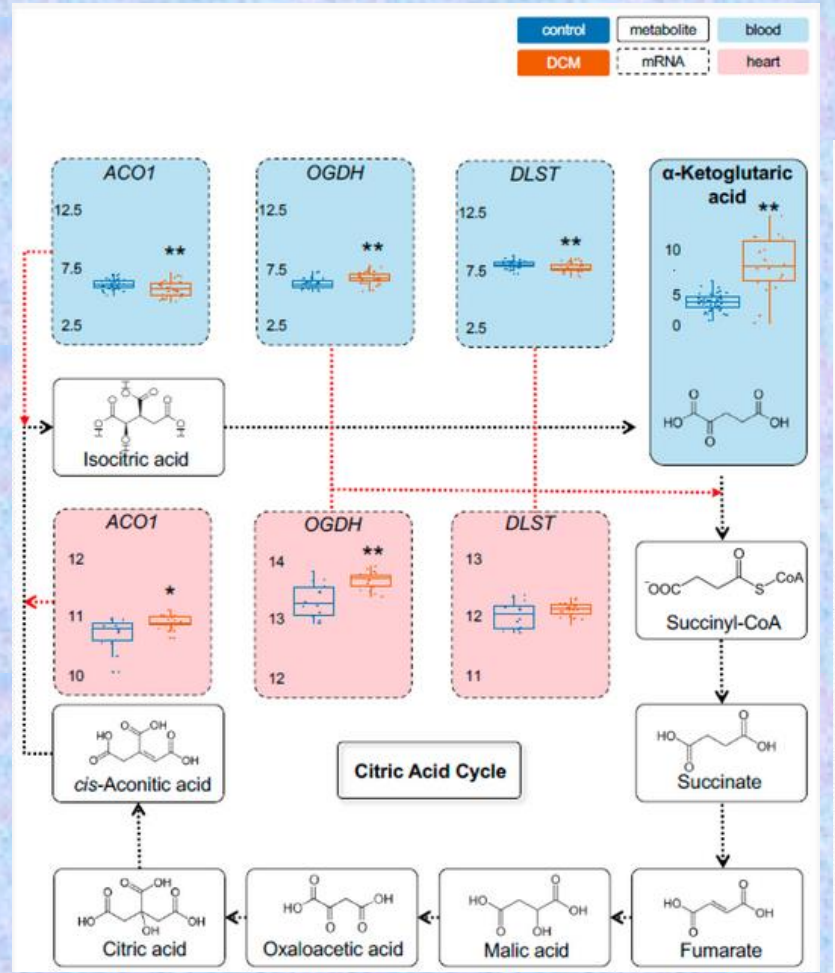
Metabolomic measurements were performed using Biocrates Metabolite assays for most important energy metabolites. While some metabolites showed unaltered levels in DCM compared to controls, such as pyruvic acid (+oxaloacetic acid), a range of other energy metabolites were significantly changed in their serum levels.

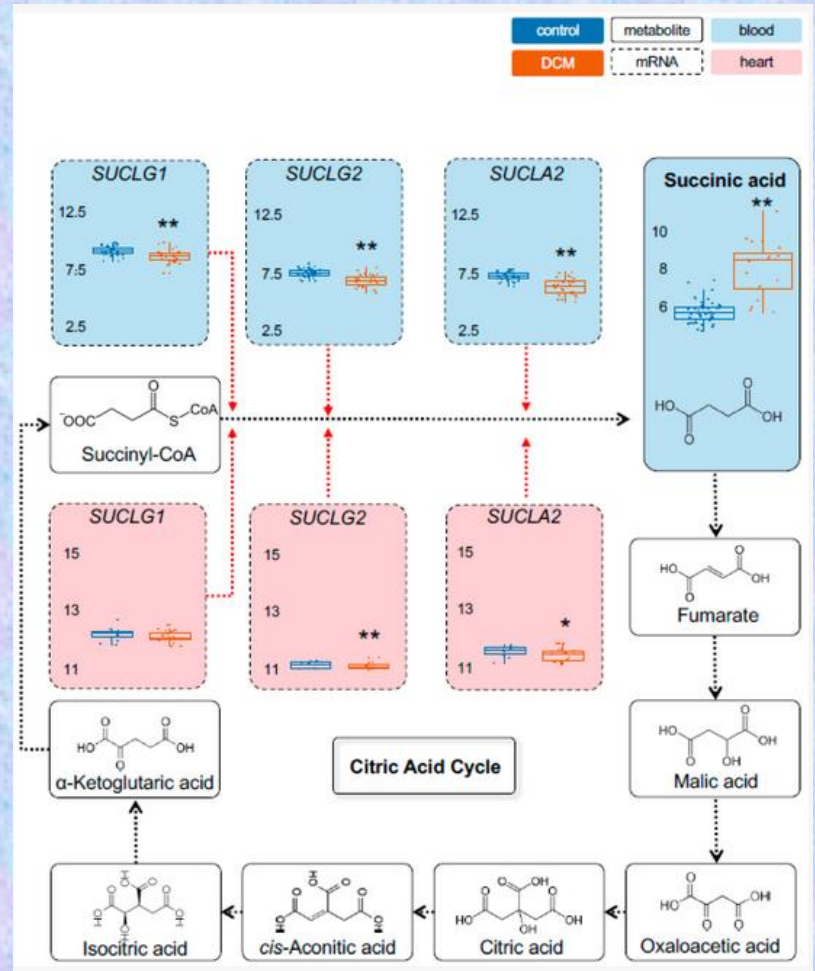
**Haas J**, Frese KS, Sedaghat-Hamedani F, Kayvanpour E, Tappu R, Nietsch R, Tugrul OF, Wisdom M, Dietrich C, Amr A, Weis T, Niederdränk T, Murphy MP, Krieg T, Dörr M, Völker U, Fielitz J, Frey N, Felix SB, Keller A, Katus HA, Meder B. Energy Metabolites as Biomarkers in Ischemic and Dilated Cardiomyopathy. Int J Mol Sci. 2021 Feb 18;22(4):1999. doi: 10.3390/ijms22041999. PMID: 33670449.



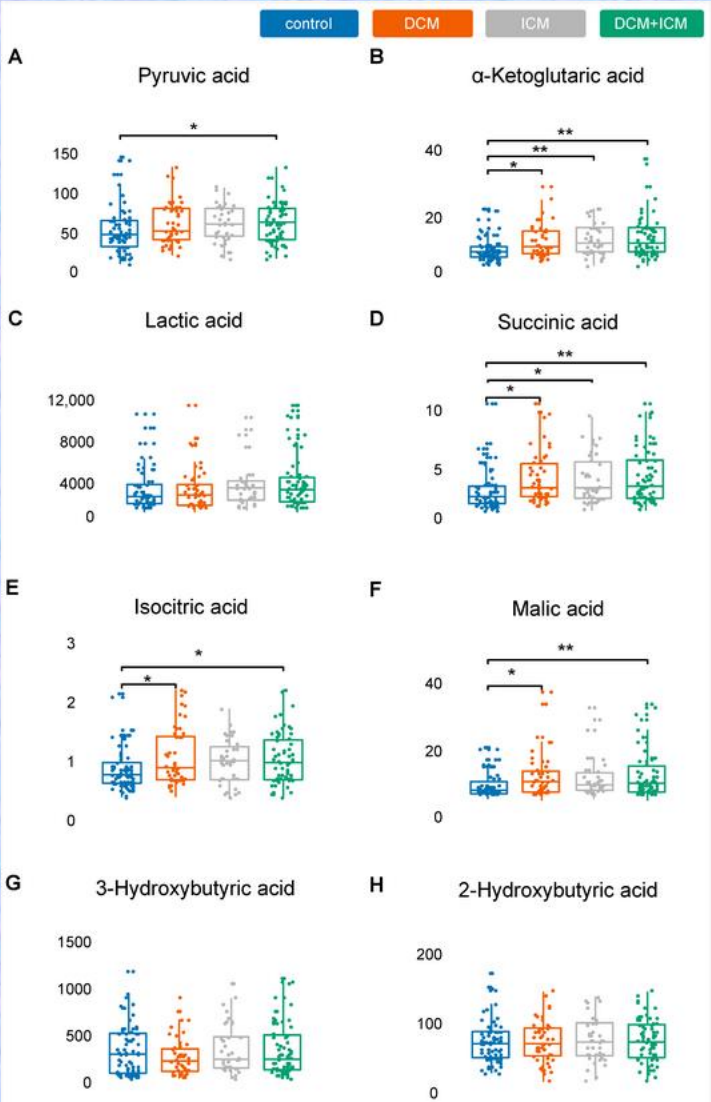
In case of lactate, DCM patients showed as much as a 5.7-fold higher level compared to controls ($p = 1.7 \times 10^{-6}$) similar results were obtained for alpha-ketoglutaric acid and succinic acid.



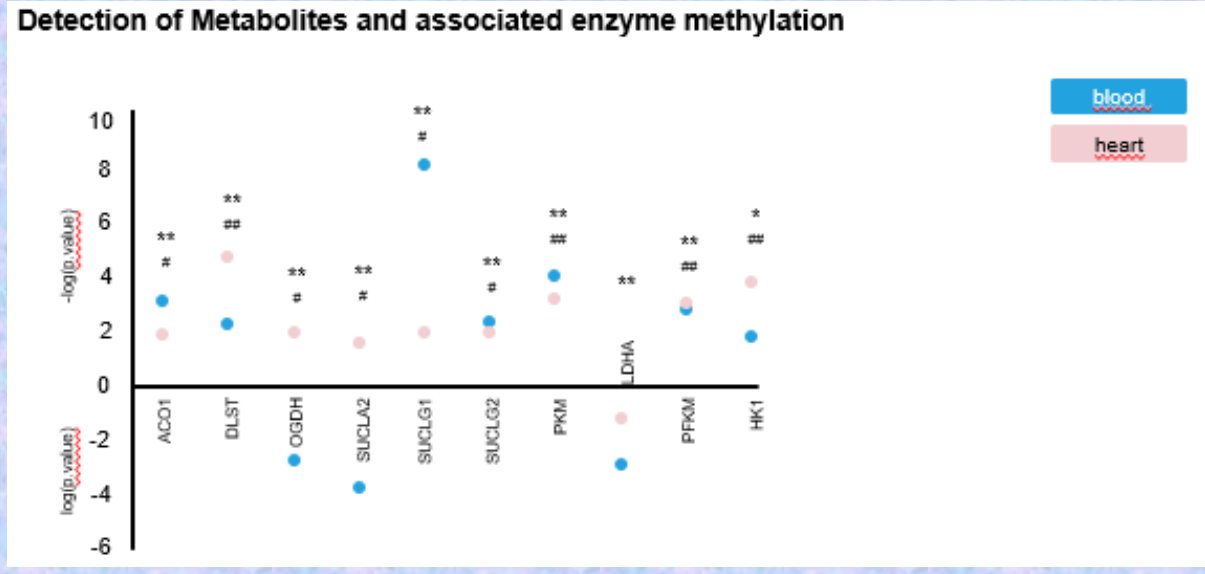



To explore whether the metabolic state-change is due to expression changes in key enzymes of the detected metabolites, we compared the metabolite data to whole-transcriptome RNA-sequencing data from blood and heart muscle tissue from the same cohort of DCM patients and compared them to controls.




In summary, we have detected significant dysregulation related to energy production pathways in HF at different OMICs-levels, showing its importance in disease onset and progression and suggest a possible use of distinct molecules like succinic acid as an (early) biomarker and interventional target in heart failure.




P-values were calculated for individual CpG sites between DCM and controls from the screening cohort. P-values have been aggregated per gene using Simes-method. For genes were mean methylation was higher in DCM compared to controls, negative logarithmic p-values for blood (blue) and heart tissue (pale red), were plotted. For genes were mean methylation levels were reduced in DCM compared to controls, logarithmic p-values were plotted. *: p<0.05 (blood); **: p<0.01 (blood). #: p<0.05 (heart); ##: p<0.01 (heart).

Alpha-ketoglutaric acid and succinic acid were significantly increased in DCM and ICM.

# and in chagas…..

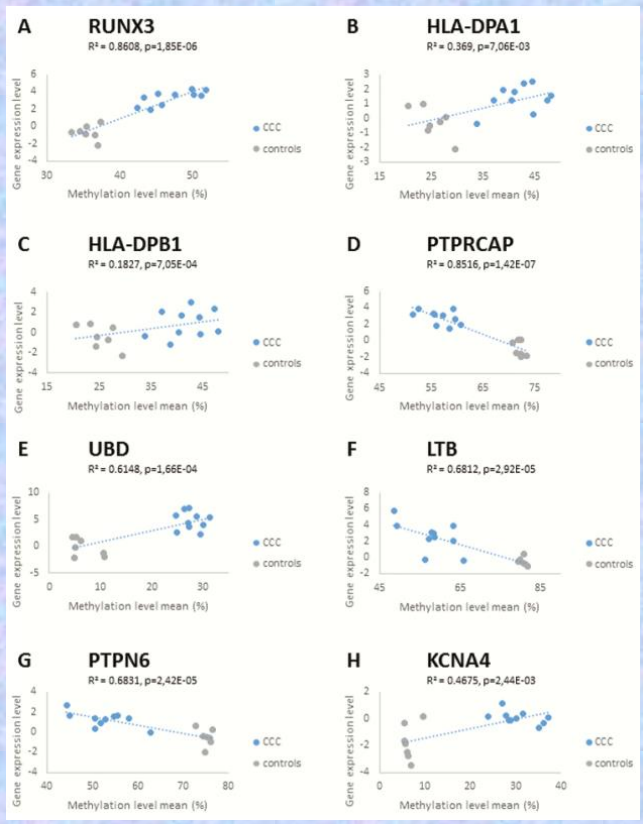
We investigated the impact of genome-wide cardiac DNA methylation on global gene expression in myocardial samples from end-stage CCC patients, compared to control samples from organ donors.

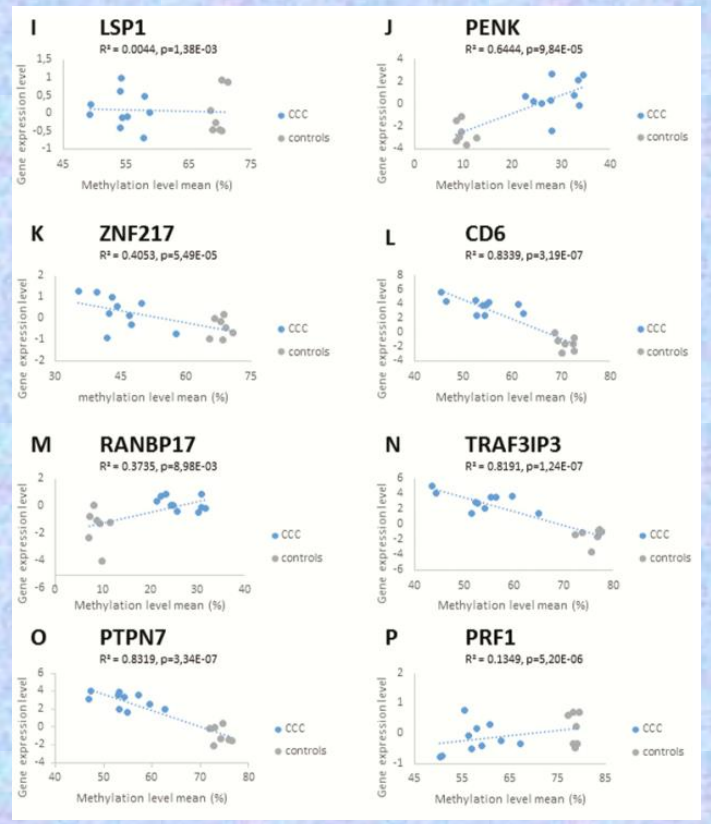
We identified 7595 differentially methylated sites between CCC and controls, 2649 (35%) were undermethylated and 4946 (65%) were overmethylated in CCC myocardium. Hierarchical clustering analysis based on the most significant CpG probes showed clear discrimination between the groups.

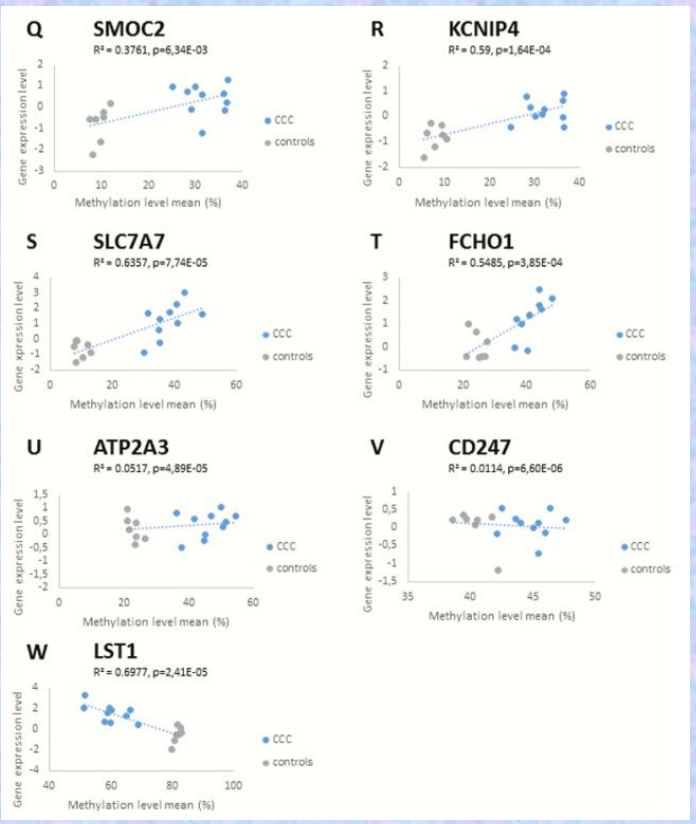
In total, 4720 genes were differentially methylated between CCC patients and controls, of which 399 were also differentially expressed. Several of them were related to heart function or to the immune response and had methylation sites in their promoter region. Reporter gene and in silico transcription factor binding analyses indicated promoter methylation modified expression of key genes. Among those, we found potassium channel genes *KCNA4* and *KCNIP4*, involved in electrical conduction and arrythmia, *SMOC2*, involved in matrix remodeling, as well as enkephalin and *RUNX3*, potentially involved in the increased T-helper 1 cytokine-mediated inflammatory damage in heart.

**Laugier L**, Frade AF, Ferreira FM, Baron MA, Teixeira PC, Cabantous S, Ferreira LRP, Louis L, Rigaud VOC, Gaiotto FA, Bacal F, Pomerantzeff P, Bocchi E, Kalil J, Santos RHB, Cunha-Neto E, Chevillard C. Whole-Genome Cardiac DNA Methylation Fingerprint and Gene Expression Analysis Provide New Insights in the Pathogenesis of Chronic Chagas Disease Cardiomyopathy. Clin Infect Dis. 2017 Oct 1;65(7):1103-1111. doi: 10.1093/cid/cix506. PMID: 28575239; PMCID: PMC5849099.

Methylation analysis was done 14 CCC patients and 7 controls whereas expression analysis was done on 10 CCC patients and 7 controls. We identified 399 genes that were simultaneously differentially expressed and differentially methylated between CCC and controls.

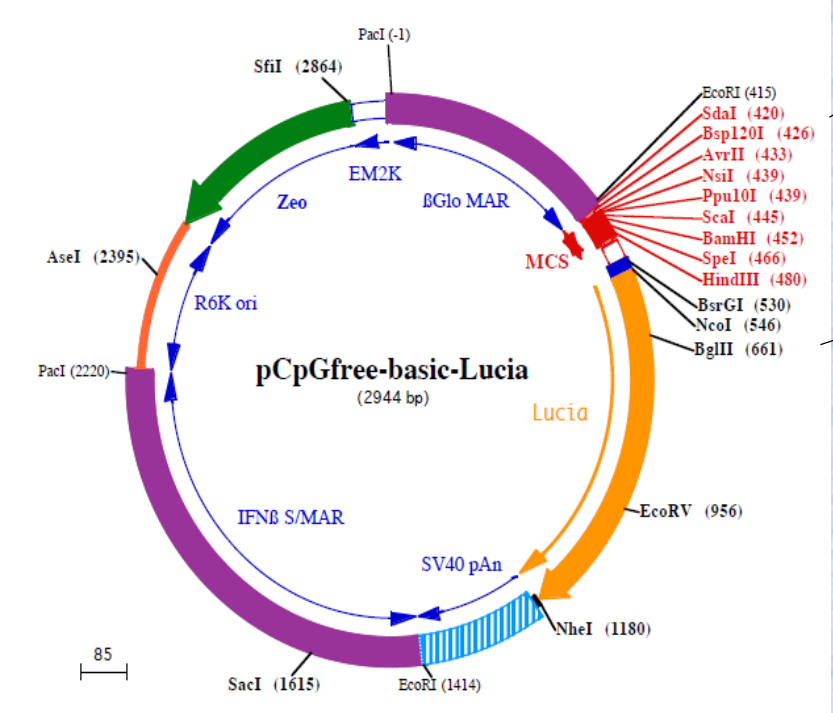



Most of these genes encode membrane components or receptors, and the main biological processes involved are linked to the immune response. Some of these differentially expressed genes were previously associated to Chagas disease (eg, *IL7*, *CCR7*, *CCL19*, *GATA4*, *HLA-DPB1*). Moreover, some contractile and metabolism genes present the same pattern in a previous study. We found that 34 genes are both differentially expressed and contain at least 5 differentially methylated sites, irrespective of their position; 23 of the 34 play a role in immune response or heart functions and were further investigated. Correlation between expression and methylation was found to be significant for all the genes.

Among the 23 immune- or heart-related genes, 12 possessed several differentially methylated CpG sites in their promoter region. We performed a luciferase promoter reporter assay of methylated or unmethylated promoter regions. The cloned promoter sequence of each gene (including differentially methylated CpG sites found in CCC myocardium) was inserted into a CpG free-basic plasmid; unmethylated plasmids were subsequently methylated, and unmethylated or methylated plasmids were transfected in HEK293 or AC16 cells.




CCTAGGAGCCCCTCCCCAGAGCAGGCGCTGCTCGCAGTGACAAGGGGT
CCTAGAGGCGCCCGCTGAGAGCCGCTGGCAACTCCCGGGGGGTGTCCTG
CCCAGCCCGAAGTCACCTCGGGTCTCTTTCGGGTCCATCTACTCTGTC
GCCAGAGGCACCGTGCCACCCGCGGCGCTTTCCTCTCCCCGCACCCCG
CCCCCCGCATCCCCAGTCCGGTGACTTGGCAGCAGGTGGCAGGGGCGG
CGCTCACGAGGGCGAGGGGCTGGGGTCCCCGCACTCGAGCCGCCCGTG
CAGCCCGCCCGCTTCCAGCCGCCGGCGCGAAGCTGCCGGCGCCTCGGC
CTCCCCCGCCACCCCGCGCGCCCCCGCCGCCAAGGGCCGCGCAGCGCC
CCCGGCGCCCCCCGCCGGACTAGT

**MssI methylase**
**+S-Adenosylmethionine**

CCTAGGAGCCCCTCCCCAGAGCAGGC$^{m}$GCTGCTC$^{m}$GCAGTGACAAGGG
GTCCTAGAGGC$^{m}$GCCC$^{m}$GCTGAGAGCC$^{m}$GCTGGCAACTCCC$^{m}$GGGGGT
GTCCTGCCCAGCCC$^{m}$GAAGTCACCTC$^{m}$GGGTCTCTTTC$^{m}$GGGTCCATC
TACTCTGTC$^{m}$GCCAGAGGCACC$^{m}$GTGCCACCC$^{m}$GC$^{m}$GGC$^{m}$GCTTTCCTC
TCCCC$^{m}$GCACCCC$^{m}$GCCCCCC$^{m}$GCATCCCCAGTCC$^{m}$GGTGACTTGGCA
GCAGGTGGCAGGGGC$^{m}$GGC$^{m}$GCTCAC$^{m}$GAGGGC$^{m}$GAGGGGCTGGGGTC
CCC$^{m}$GCACTC$^{m}$GAGCC$^{m}$GCCC$^{m}$GTGCAGCCC$^{m}$GCCC$^{m}$GCTTCCAGCC$^{m}$G
CC$^{m}$GGC$^{m}$GC$^{m}$GAAGCTGCC$^{m}$GGC$^{m}$GCCTC$^{m}$GGCCTCCCCC$^{m}$GCCACCCC$^{m}$
GC$^{m}$GC$^{m}$GCCCCC$^{m}$GCC$^{m}$GCCAAGGGCC$^{m}$GC$^{m}$GCAGC$^{m}$GCCCCC$^{m}$GGC$^{m}$GC
CCCCC$^{m}$GCC$^{m}$GGACTAGT

**Construct with the luciferase gene plus one gene promoter that is methylated**

In 11 of the 12 studied genes, methylation was inversely associated with gene expression, either in tissue or reporter gene analyses. Among those, we found potassium channel genes *KCNA4* and *KCNIP4*, involved in electrical conduction and arrythmia, *SMOC2*, involved in matrix remodeling, as well as enkephalin and *RUNX3*, potentially involved in the increased T-helper 1 cytokine-mediated inflammatory damage in heart.

## KCNA4

- encodes the potassium voltage-gated ion channel KV1.
- found to be overexpressed and overmethylated
- Promoter methylation increased transcription.
- It regulates of the fast repolarizing phase of action potentials in heart
- *Kcna4*-deficient mice display atrioventricular block and ventricular tachycardia, prominent life-threatening clinical features of CCC.
- The antiarrythmia drug amiodarone, commonly used in CCC treatment, increases *Kcna4* gene expression in mouse hearts.

## KCNIP4

- encodes a member of the family of voltage-gated potassium channel-interacting proteins (Kv4)
- found to be overexpressed and overmethylated.
- play important roles in regulating the excitability of myocytes and neurons.
- It modulates the shape of the action potential in heart.

## PENK

- encodes enkephalin, important in pain processing.
- is expressed predominantly in the brain and heart.
- Found overexpressed and overmethylated in CCC heart tissue.
- Encephalin is a ligand of δ opioid receptors.
- enkephalin signaling induces heart protection against ischemic.
- a novel immunoregulatory circuit for pathogenesis of CCC?

## RUNX3

- belongs to the Runt family of transcription factors, and can either activate or suppress transcription.
- found to be overexpressed and overmethylated.
- T-bet and Runx3 interact and functionally cooperate to induce maximal IFN-γ production in Th1 and Th17 T cells.
- T-bet and Runx3 have a major role in pathogenesis.
- RUNX3 methylation is associated with gastric and colorectal cancer.

Significantly, the target genes modulated by DNA methylation in our study of CCC myocardium were distinct from those identified in dilated cardiomyopathy in previous studies (Haas et al. 2013; Jo et al. 2016) suggesting that environmental/disease-specific factors regulating cardiac DNA methylation are different in the 2 cardiomyopathies, which may partially explain the worse prognosis observed in CCC. While our analysis started from differentially expressed genes that were also differentially methylated in the total gene body in CCC myocardial tissue as compared to controls, our reporter gene assays may have underestimated methylation effects in CpGs contained in other regions of the promoters, as well as methylation occurring in other parts of the gene body; on the other hand, methylase treatment of promoter sequences in plasmids also methylates CpG sites that were not found to be differentially methylated in vivo, which could also distinctly change expression patterns in a cell type-specific manner.

Our results suggest that DNA methylation modulates expression of pathogenetically relevant myocardium and immune system genes in CCC, especially those involving Th1 T cell activation, ion channels, myocardial remodeling, and protection. In-depth analysis of differentially methylated genes may help decipher the pathogenic mechanisms and provide therapeutic targets. Moreover, it will be essential to characterize the role of these epigenetic factors in CCC pathogenesis and progression to severe forms.

**Haas J**, Frese KS, Park YJ, Keller A, Vogel B, Lindroth AM, Weichenhan D, Franke J, Fischer S, Bauer A, Marquart S, Sedaghat-Hamedani F, Kayvanpour E, Köhler D, Wolf NM, Hassel S, Nietsch R, Wieland T, Ehlermann P, Schultz JH, Dösch A, Mereles D, Hardt S, Backs J, Hoheisel JD, Plass C, Katus HA, Meder B. Alterations in cardiac DNA methylation in human dilated cardiomyopathy. EMBO Mol Med. 2013 Mar;5(3):413-29. doi: 10.1002/emmm.201201553. Epub 2013 Jan 22. PMID: 23341106; PMCID: PMC3598081.

**Jo BS**, Koh IU, Bae JB, Yu HY, Jeon ES, Lee HY, Kim JJ, Choi M, Choi SS. Methylome analysis reveals alterations in DNA methylation in the regulatory regions of left ventricle development genes in human dilated cardiomyopathy. Genomics. 2016 Aug;108(2):84-92. doi: 10.1016/j.ygeno.2016.07.001. Epub 2016 Jul 12. PMID: 27417303.


**Laugier L**, Frade AF, Ferreira FM, Baron MA, Teixeira PC, Cabantous S, Ferreira LRP, Louis L, Rigaud VOC, Gaiotto FA, Bacal F, Pomerantzeff P, Bocchi E, Kalil J, Santos RHB, Cunha-Neto E, Chevillard C. Whole-Genome Cardiac DNA Methylation Fingerprint and Gene Expression Analysis Provide New Insights in the Pathogenesis of Chronic Chagas Disease Cardiomyopathy. Clin Infect Dis. 2017 Oct 1;65(7):1103-1111. doi: 10.1093/cid/cix506. PMID: 28575239; PMCID: PMC5849099.


## Nothing can be done without funders……

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The USP university

# Chagas disease is a Neglected Tropical Disease

**Neglected tropical diseases** (NTDs) a diverse group of communicable diseases that prevail in tropical and subtropical conditions in 149 countries – affect more than one billion people and cost developing economies billions of dollars every year. Populations living in poverty, without adequate sanitation and in close contact with infectious vectors and domestic animals and livestock are those worst affected.

**Neglected tropical diseases are:**

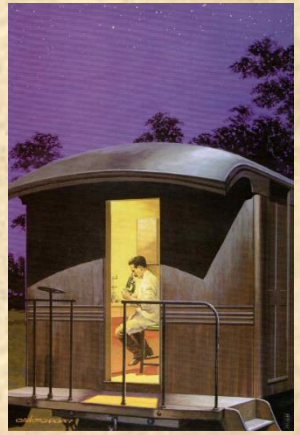
Buruli ulcer
Chagas disease
Dengue and Chikungunya
Dracunculiasis (guinea-worm disease)
Echinococcosis
Foodborne trematodiases
Human African trypanosomiasis (sleeping sickness)
Leishmaniasis
Leprosy (Hansen's disease)
Lymphatic filariasis
Mycetoma, chromoblastomycosis and other deep mycoses
Onchocerciasis (river blindness)
Rabies
Scabies and other ectoparasites
Schistosomiasis
Soil-transmitted helminthiases
Snakebite envenoming
Taeniasis/Cysticercosis
Trachoma
Yaws (Endemic treponematoses)

**One in 6 people on the planet... suffers from some kind of NTD**

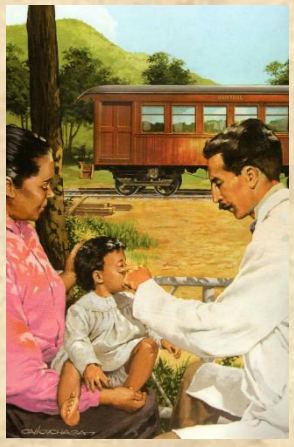
Joaquim Gascon, Director of the Neglected Tropical Diseases Initiatives, ISGlobal

# History

Carlos Chagas was born on 9 July 1878 in the farm “Bon Retiro” located close to the City of Oliveira in the interior of the State of Minas Gerais, Brazil. He started his medical studies in 1897 at the School of Medicine of Rio de Janeiro. In the late XIX century, the works by Louis Pasteur and Robert Koch induced a change in the medical paradigm with emphasis in experimental demonstrations of the causal link between microbes and disease. During the same years in Germany appeared the pathological concept of disease, linking organic lesions with symptoms. All these innovations were adopted by the reforms of the medical schools in Brazil and influenced the scientific formation of Chagas.



Chagas completed his medical studies between 1897 and 1903 and his examinations during these years were always ranked with high grades. Oswaldo Cruz accepted Chagas as a doctoral candidate and directed his thesis on “Hematological studies of Malaria” which was received with honors by the examiners. In 1903 the director appointed Chagas as research assistant at the Institute. In those years, the Institute of Manguinhos, under the direction of Oswaldo Cruz, initiated a process of institutional growth and gathered a distinguished group of Brazilian and foreign scientists. In 1907, he was requested to investigate and control a malaria outbreak in Lassance, Minas Gerais. In this moment Chagas could not have imagined that this field research was the beginning of one of the most notable medical discoveries. Chagas was, at the age of 28, a Research Assistant at the Institute of Manguinhos and was studying a new flagellate parasite isolated from triatomine insects captured in the State of Minas Gerais. Chagas made his discoveries in this order: first the causal agent, then the vector and finally the human cases. These notable discoveries were carried out by Chagas in twenty months. At the age of 33 Chagas had completed his discoveries and published the scientific articles that gave him world recognition and a deserved high place in medical history. After the publication of his classic article the world paid homage to Chagas who was elected member of the National Academy of Medicine of Brazil on 26 October 1910, and at the age of 31, of other National Academies of the continent. The Committee of Hygiene of the Society of Nations, precursor of the World Health Organization, was created in 1929. Chagas was elected member of this Committee from its inception until 1933.



***Moncayo A.*** Carlos Chagas: biographical sketch. Acta Trop. 2010 Jul-Aug;115(1-2):1-4.

# Once upon a time…..

A study of an Incan mummy from a German collection reveals the tragic story of a young woman suffering from a parasitic disease that ended with violent blows to the head.

**Panzer S**, Peschel O, Haas-Gebhard B, Bachmeier BE, Pusch CM, Nerlich AG. Reconstructing the life of an unknown (ca. 500 years-old South American Inca) mummy--multidisciplinary study of a Peruvian Inca mummy suggests severe Chagas disease and ritual homicide. PLoS One. 2014 Feb 26;9(2):e89528.

# Chagas disease

Chronic Chagas disease cardiomyopathy (CCC) is a chronic inflammatory cardiomyopathy occurring decades after infection with the protozoan *Trypanosoma cruzi*, endemic in Latin America (*Bocchi EA et al. 2017*). It is transmitted mainly by the reduviid insect vector, by blood transfusion, congenitally and by ingestion. Chagas disease is the most common cause of non-ischemic cardiomyopathy in Latin America, where 6 million people are infected, causing approximately 10,000 deaths/year, mainly due to cardiac compromise (*Bocchi EA et al. 2017 and Chevillard C et al. 2018*).

It has now become a worldwide health issue, with an estimated 400,000 infected persons living in nonendemic countries, mainly the United States and Europe (*Schmunis GA et al. 2010, Hotez PJ et al. 2013, Dias JCP et al. 2013, Coura JR et al. 2014 and Requena-Méndez A et al. 215*).


***Bocchi EA**, Bestetti RB, Scanavacca MI, Cunha Neto E, Issa VS. Chronic Chagas Heart Disease Management: From Etiology to Cardiomyopathy Treatment. J Am Coll Cardiol 2017;70:1510-1524.*

***Chevillard C**, Nunes JPS, Frade AF et al. Disease Tolerance and Pathoen Resistance Genes May Underlie Trypanosoma cruzi Persistence and Differential Progression to Chagas Disease Cardiomyopathy. Front Immunol 2018;9:2791.*

***Schmunis GA**, Yadon ZE. Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop. 2010 Jul-Aug;115(1-2):14-21.*

***Hotez PJ**, Dumonteil E, Betancourt Cravioto M, Bottazzi ME, Tapia-Conyer R, Meymandi S, et al. An unfolding tragedy of Chagas disease in North America. PLoS Negl Trop Dis. 2013 Oct;7(10):e2300.*

***Dias JCP**. Human chagas disease and migration in the context of globalization: some particular aspects. J Trop Med. 2013:789758.*

***Coura JR**, Viñas PA, Junqueira AC. Ecoepidemiology, short history and control of Chagas disease in the endemic countries and the new challenge for non-endemic countries. Mem Inst Oswaldo Cruz. 2014 Nov;109(7):856-62.*

***Requena-Méndez A**, Aldasoro E, Lazzari E, Sicuri E, Brown M, Moore DA, et al. Prevalence of Chagas disease in Latin-American migrants living in Europe: a systematic review and meta-analysis. PLoS Negl Trop Dis. 2015 Feb;9(2):e0003540.*

# Life cycle

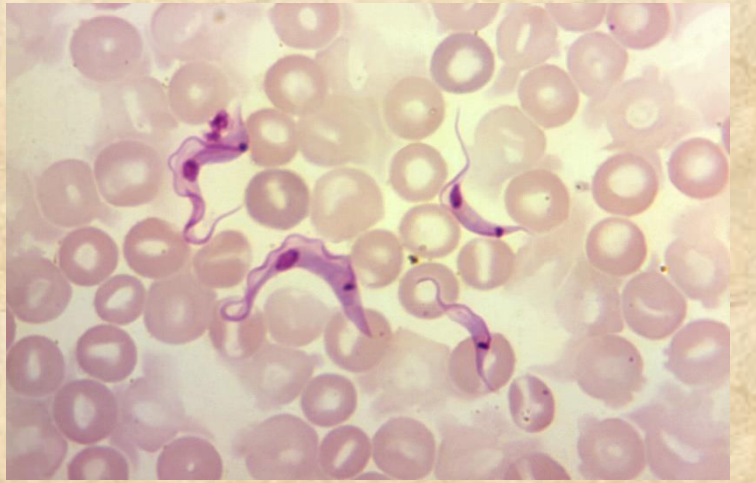
Chagas disease is caused by the parasite *Trypanosoma cruzi*. This protozoan can live in humans, mammals (>100 species), and the triatomine bug, which is the insect vector that spreads *T. cruzi* infection from one host to another. The triatomine bug lives in Latin America. Mammals infected with *T. cruzi* have parasites that circulate through the blood in the form of trypomastigotes (*Tyler KM et al. 2001*).

When the triatomine bug bites a mammal and feeds on its infected blood, it ingests parasites that subsequently mature in the intestine before being eliminated in faeces.

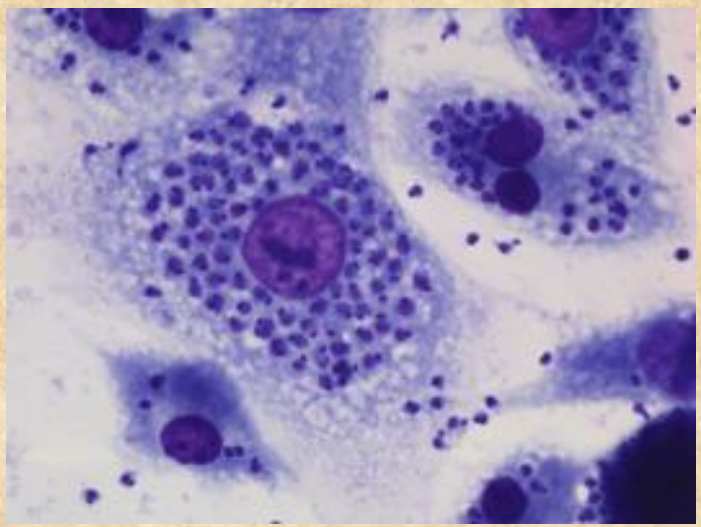
On biting the next mammal, the bug generally defecates near the site of the bite, allowing trypomastigotes in the faeces to enter the host through the wound, intact skin or mucous membranes, or the conjunctiva.

Once in the host, the parasite will invade the cells around the site of entry and develop into an intracellular form known as an amastigote, which can cause both direct and indirect tissue damage.

The amastigote then multiples, generating trypomastigotes, which are released into the blood, from where they infect other cells and tissues.



**trypomastigotes**



**amastigote**

***Tyler KM**, Engman DM. The life cycle of Trypanosoma cruzi revisited. Int J Parasitol 2001 31: 472–481.*

# Vectors and reservoirs

- *Paratriatoma hirsuta*
- *Triatoma gerstaeckeri*
- *Triatoma incrassata*
- *Triatoma indictiva*
- *Triatoma lecticularia*
- *Triatoma neotomae*
- *Triatoma protracta*
- *Triatoma recurva*
- *Triatoma rubida*
- *Triatoma rubrofasciata*
- *Triatoma sanguisuga*


# 5 ways of transmission

- Consumption of food contaminated with T. Cruzi through, for example, contact with infected triatomine bug faeces or urine.
- Blood transfusion from infected donors.
- Passage from an infected mother to her newborn during pregnancy or childbirth.
- Organ transplants using organs from infected donors.
- Laboratory accidents.

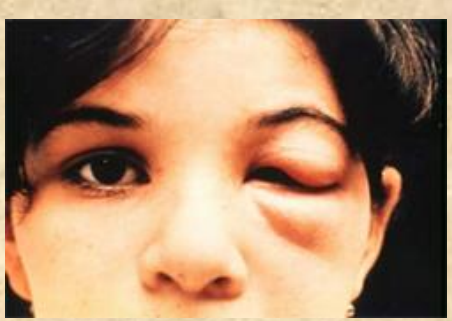



# Acute and chronic phases

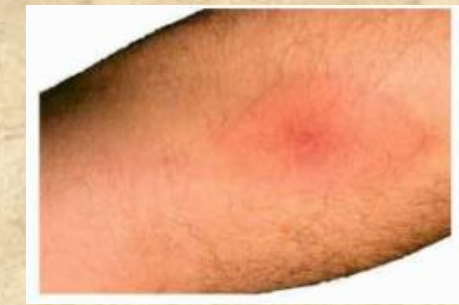
Chagas disease presents itself in 2 phases. The initial acute phase lasts for about 2 months after infection (Rassi A et al. 2007).

During the acute phase, a high number of parasites circulate in the blood but in most cases, symptoms are absent or mild and unspecific. In less than 50% of people bitten by a triatomine bug, characteristic first visible signs can be a skin lesion or a purplish swelling of the lids of one eye. Additionally, they can present fever, headache, enlarged lymph glands, pallor, muscle pain, difficulty in breathing, swelling, and abdominal or chest pain.

**Romana's sign**



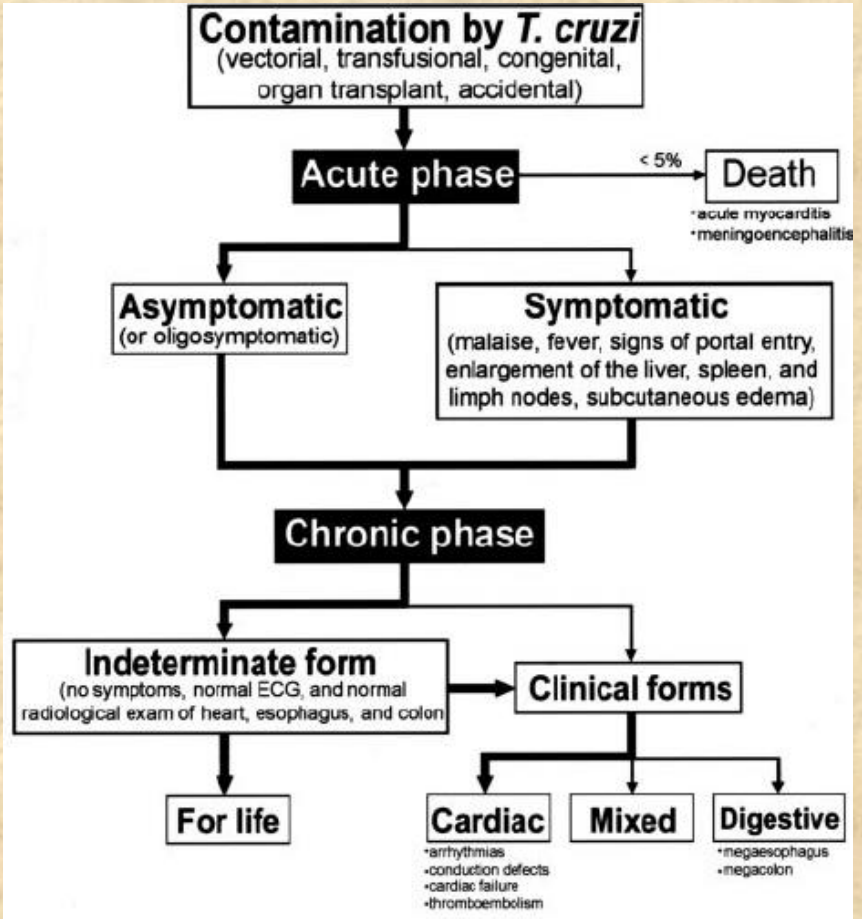
**Chagoma**



During the chronic phase, the parasites are hidden mainly in the heart and digestive muscles.

Up to 30% of patients suffer from cardiac disorders and up to 10% suffer from digestive (typically enlargement of the oesophagus or colon), neurological or mixed alterations.

In later years the infection can lead to sudden death due to cardiac arrhythmias or progressive heart failure caused by the destruction of the heart muscle and its nervous system.




***Rassi A Jr**, Rassi A, Rassi SG. Predictors of mortality in chronic Chagas disease: a systematic review of observational studies. Circulation. 2007 Mar 6;115(9):1101-8*

# Epidemiology

In the Americas, Chagas disease is responsible for ≈7.5 times as many disability-adjusted life-years lost as malaria (*Nunes MCP et al. 2018*).

Strategies targeting vector and transmission control have led to a substantial decline in global prevalence, now estimated at 6 million people compared with 8 million in 2005 and 18 million in 1990 (Rassi A et al.2010). Despite these improvements, in the 21 endemic countries, 13% of the population is thought to remain at risk.

The estimated national infection is highest in Bolivia (6.1%), followed by Argentina (3.6%) and Paraguay (2.1%), whereas the largest number of individuals living with Chagas disease, 42% of all cases, reside in Brazil (nearly 1.2 million people) and Argentina (1.5 million people).

Nearly 1.2 million people in these countries are thought to have Chagas cardiomyopathy (*Nunes MCP et al. 2018*).


# In Brazil

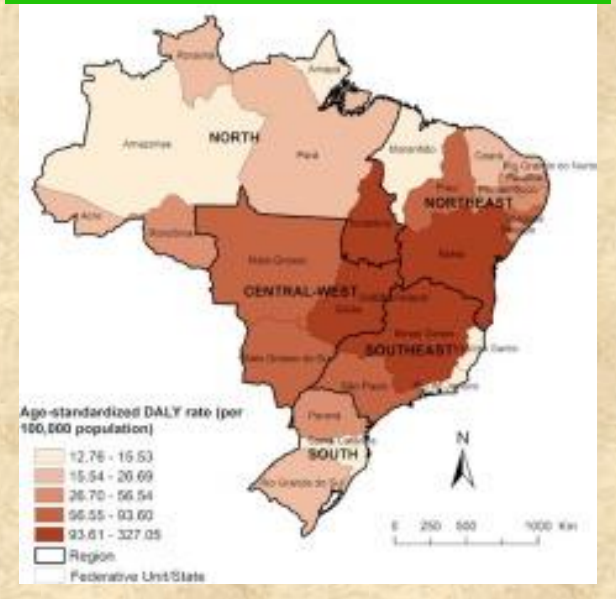
Only one triatoma species

**2 to 3 millions** of infected subjects

**1,1 millions** patients with chagas cardiomyopathy

**5 000** deaths per year

Annual cost: **130 dollars millions per year**





***Nunes MCP***, *Beaton A, Acquatella H, Bern C, Bolger AF, Echeverría LE, Dutra WO, Gascon J, Morillo CA, Oliveira-Filho J, Ribeiro ALP, Marin-Neto JA; American Heart Association Rheumatic Fever, Endocarditis and Kawasaki Disease Committee of the Council on Cardiovascular Disease in the Young; Council on Cardiovascular and Stroke Nursing; and Stroke Council. Chagas Cardiomyopathy: An Update of Current Clinical Knowledge and Management: A Scientific Statement From the American Heart Association. Circulation. 2018 Sep 18;138(12):e169-e209.*

***Rassi A***, *Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010; 375:1388–1402.*

***Martins-Melo FR***, *Carneiro M, Ribeiro ALP, Bezerra JMT, Werneck GL. Burden of Chagas disease in Brazil, 1990-2016: findings from the Global Burden of Disease Study 2016. Int J Parasitol. 2019 Mar;49(3-4):301-310.*

# In Europe *(Basile L et al. 2011)*

In 2008, more than 38 million migrants were living in Europe, of whom 11% came from Latin America *(Vasileva K et al. 2009)*. This figure did not include migrants without valid residency permit (irregular, undocumented migrants) *(European Commission 2009)*, people born outside Europe who have acquired citizenship of a European country, or children from foreign countries adopted by European families.

Currently, only a small number of persons infected with T. cruzi have been detected in Europe *(World Health Organization 2009)*. Several reasons account for this fact:

-Most European health professionals have little or no experience with the detection and management of Chagas disease *(Jackson J et al. 2009)*.

-Access to screening programmes for the communities at risk is very limited as only a few institutions offer screening, mostly in major urban areas.

-The delayed as most patients remain asymptomatic for many years *(Rassi et al. 2010)*.

The World Health Organization (WHO) set up in 2009 a working group of experts on Chagas disease from those European countries where T. cruzi-positive cases had been detected (Austria, Belgium, Croatia, Denmark, France, Germany, Italy, the Netherlands, Portugal, Romania, Spain, Sweden, Switzerland and United Kingdom).

**Distribution of the migrant population from countries endemic for Chagas disease resident in nine studied European countries, and estimated number of people infected in 2009.**

| Countries | Total (min-max) |
|---|---|
| Belgium | 683-921 |
| France | 2,148-2,823 |
| Germany | 1,123-1,481 |
| **Italy** | 6,464-12,036 |
| Netherlands | 967-1,773 |
| Portugal | 1,255 |
| **Spain** | 47,984-86,618 |
| Switzerland | 1,584-3,971 |
| **United Kingdom** | 6,111-12,201 |
| **total** | **68,318-123,078** |


***Basile L**, Jansa JM, Carlier Y, Salamanca DD, Angheben A, Bartoloni A, Seixas J, Van Gool T, Canavate C, Flores-Chavez M, Jackson Y, Chiodini PL, Albajar-Vinas P; Working Group on Chagas Disease. Chagas disease in European countries: the challenge of a surveillance system. Euro Surveill. 2011 Sep 15;16(37):19968.*

***World Health Organization** (WHO). Control and prevention of Chagas disease in Europe. Report of a WHO Informal Consultation (jointly organized by WHO headquarters and the WHO Regional Office for Europe) Geneva, Switzerland, 17-18 December 2009. Final report. Geneva: WHO; 2010. Report No.: WHO/HTM/NTD/IDM/2010.1. Available from: http://www. fac.org.ar/1/comites/chagas/Chagas_WHO_Technical%20 Report_16_06_10.pdf*

***Vasileva K.** Statistics in focus. Citizens of European countries account for the majority of the foreign population in EU-27 in 2008. Luxembourg: Eurostat; 2009. Report No.: 94/2009. Available from: http://epp.eurostat.ec.europa.eu/cache/ITY_ OFFPUB/KS-SF-09-094/EN/KS-SF-09-094-EN.PDF*

***European Commission** (EC). Size and development of irregular migration to the EU, Clandestino Research Project, Counting the Uncountable: Data and Trends across Europe. Bruxelles: EC; 2009 [Accessed: 27 Feb 2011]. Available from: http:// clandestino.eliamep.gr/wp-content/uploads/2009/12/ clandestino_policy_brief_comparative_size-of-irregular-migration.pdf*

***Jackson J**, Angheben A, Carrilero Fernández B, Jansa i Lopez del Vallado JM, Jannin JG, Albajar-Viñas P. Prise en charge de la maladie de Chagas en Europe. Expériences et défis en Espagne, Suisse et Italie. [Management of Chagas disease in* Europe. Experiences and challenges in Spain, Switzerland and Italy]. Bull Soc Pathol Exot. 2009;102(5):326-9.

**Rassi A Jr**, Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010;375(9723):1388-402.

# Diagnosis during acute phase

If you have the signs and symptoms of Chagas disease, blood tests can confirm the presence of the T. cruzi parasite or the proteins that your immune system creates (antibodies) to fight the parasite in your blood.

***Rassi A Jr**, Rassi A, Rassi SG. Predictors of mortality in chronic Chagas disease: a systematic review of observational studies. Circulation. 2007 Mar 6;115(9):1101-8*

# Diagnosis during chronic phase

If you're diagnosed with Chagas disease, you'll likely undergo additional tests to determine whether the disease has entered the chronic phase and caused heart or digestive complications. These tests may include:

**Electrocardiogram (ECG AND EKG),** a procedure that records the electrical activity of your heart

**Chest X-ray,** which lets your doctor see if your heart is enlarged

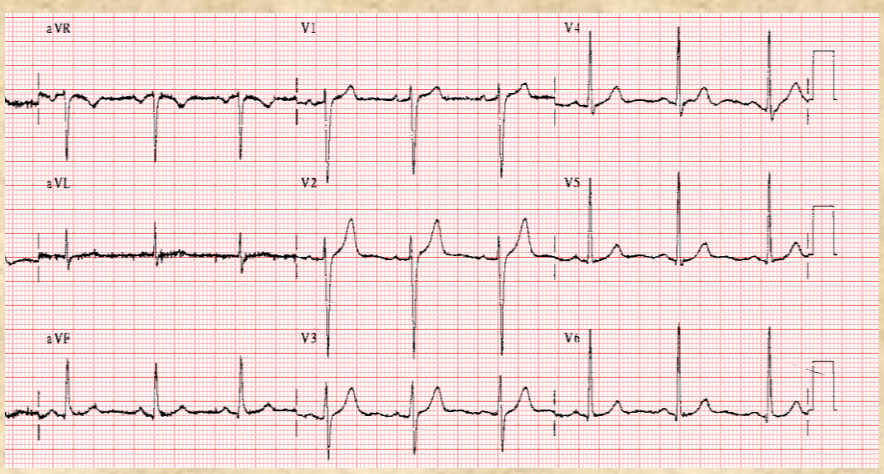
**Echocardiogram,** a test that uses sound waves to capture moving images of your heart, allowing your doctor to see any changes to the heart or its function

**Abdominal X-ray,** a procedure that uses radiation to capture images of your stomach, intestines and colon

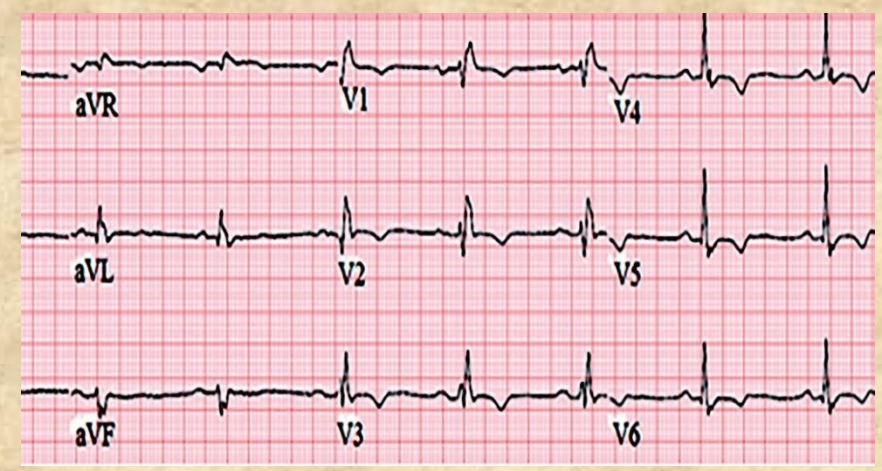
**Upper endoscopy,** a procedure in which you swallow a thin, lighted tube (endoscope) that transmits images of your esophagus onto a screen.

**Electrocardiogram ECG**

regular ECG




chronic chagas ECG

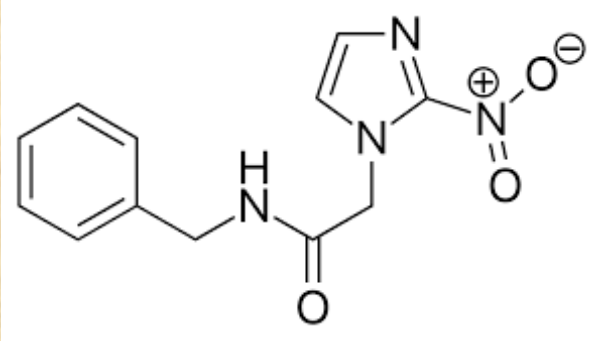


# Treatment

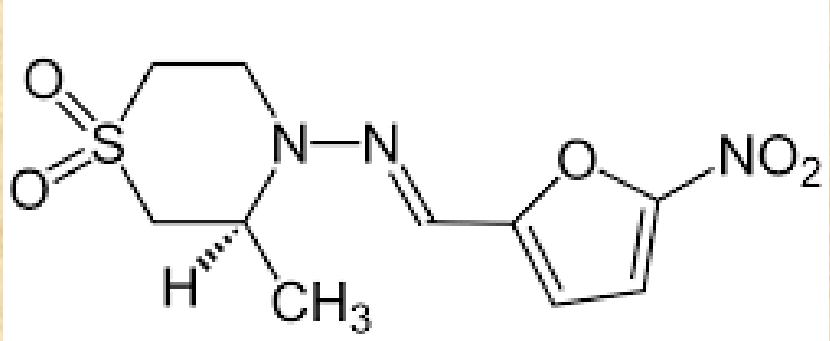
**All patients** with acute Chagas disease
with congenital infection
with chronic infections and immunosuppression
**should be treated with either benznidazole or nifurtimox**

If disease progresses to a **chronic phase**: it is no longer curable. The consensus among experts is that persons who have already developed cardiac or gastrointestinal symptoms should not be given anti-parasitic treatment.

All you can do for the patient is to provide symptom management and relief.

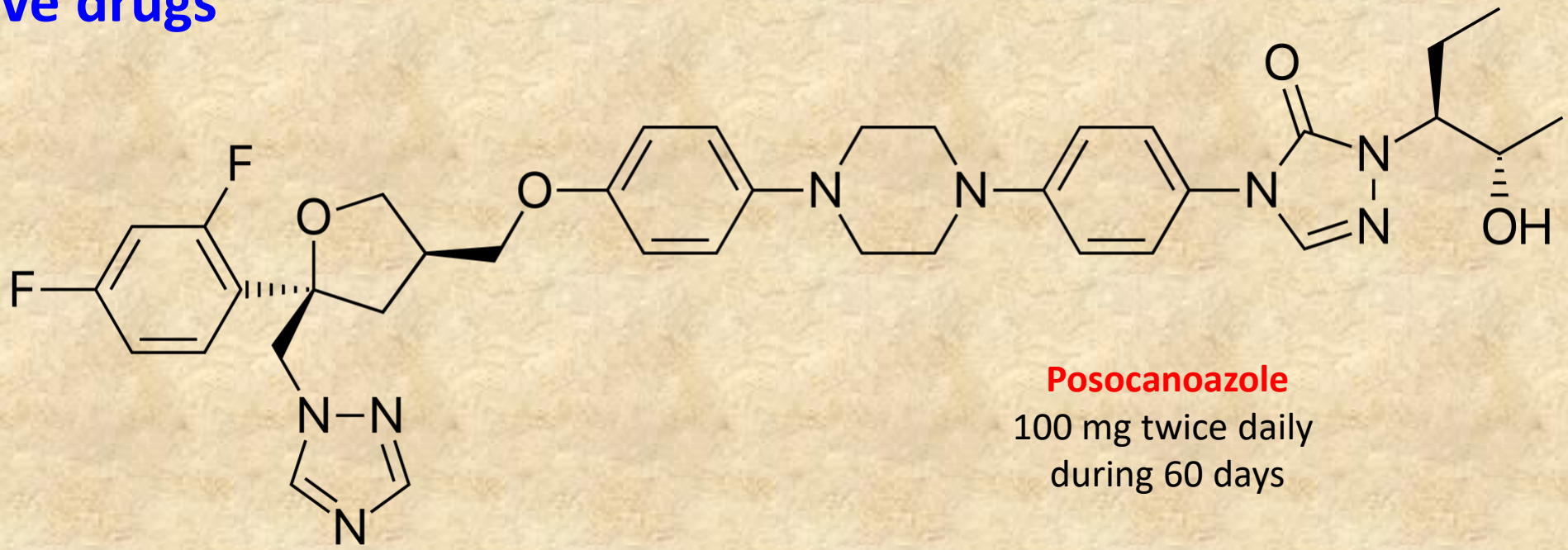



**Benznidazole**
5–10 mg/kg per day in two divided doses for 30–60 days

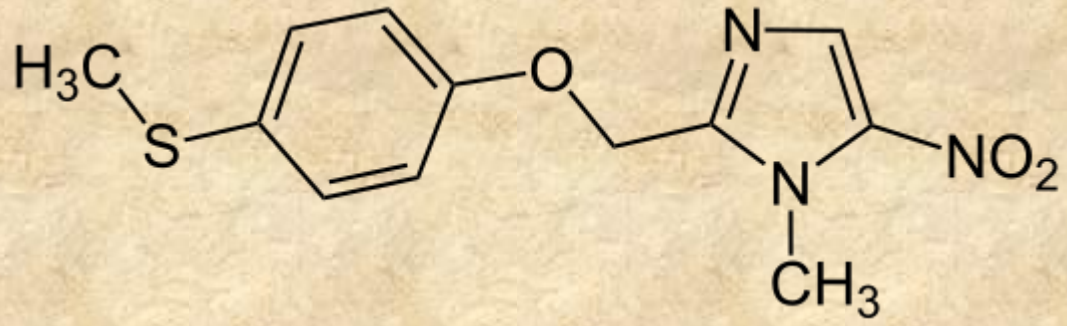



**Nifurtimox**
8–10 mg/kg in three divided doses after meals for 30–120 days

# Alternative drugs




**Posocanoazole**
100 mg twice daily
during 60 days




**Fexinidazole**
600 to 1200 mg
for 3, 7 or 10 days