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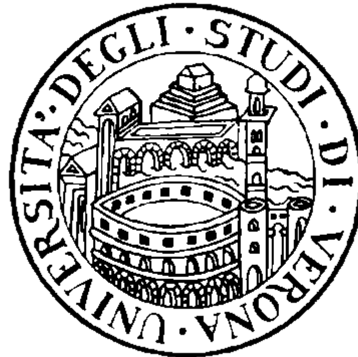

PhD School of Life and Health Sciences

PhD in Biomolecular Medicine

Curriculum in Clinical Genomics and Proteomics

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Understanding Sickle Cell Cardiomyopathy

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1. ABSTRACT

Sickle cell disease (SCD) is an inherited disorder of red blood cells caused by a point mutation in the beta-globin chain, leading to the formation of sickle-shaped erythrocytes. One of the leading causes of sudden death in young adult patients with SCD is cardiovascular disease, which pathogenesis is only partially elucidated.

Here, we study the effect of aging on sickle cell-related cardiomyopathy. In SCD mice, we observed age-dependent cardiomegaly, associated with myocardiocyte loss and degradation of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA2a), a key system in cardiomyocyte homeostasis. The increased activity of MMP9 is involved in the degradation of SERCA2a, suggesting a possible pro-inflammatory and pro-fibrotic environment characterizing the hearts from SCD mice. Indeed, we found increased collagen deposition associated with activation of TGF-β1 signaling pathways, with subsequent pathological myocardial remodeling cascade. This was associated with activation of NF-kB, an inflammatory and redox-related transcriptional factor, and up-regulation of markers of inflammatory vasculopathy. Along with these data, we found heart infiltration of the lymphocyte subpopulation of Th17, which is linked to both pro-inflammatory cytokines (CCL2, CCXL2, IL17) and activation of the TGF-b1 pathway, contributing to the progression of heart fibrosis.

Since the therapeutic options to treat SCD and sickle cell organ complications are still limited, we investigated the effects of colchicine (0.1 mg/Kg/day) an anti-inflammatory and immune-modulatory drug, recently tested in different models of heart diseases. Colchicine-treated SCD mice showed (i) lower heart NFkB activation, (ii) reduced cardiac Th17 infiltration, (iii) decrease pro-inflammatory and pro-fibrotic cytokines (iv) reduced activation of TGF-b1system and remodeling pathways.

In conclusion, our data contribute to going behind the state of the art and generating the rationale to consider transferring colchicine to clinical trials as a new therapeutic option to prevent/limit sickle cell-related cardiomyopathy.

2. ABBREVIATIONS

ANF - Atrial Natriuretic Factor

ARE - Antioxidant Response Element

BAFF/Blys - B-cell Activating Factor/B Lymphocyte Stimulator

BAFFR - B-cell Activating Factor

BNP - Brain Natriuretic Peptide

CCL-2 - C-C Motif Ligand 2

CCL19 - Chemokine (C-C motif) ligand 19

CCL21 - Chemokine (C-C motif) ligand 21

CXCL-1 - Chemokine (C-X-C motif) Ligand 1

CXCL-2 - Chemokine (C-X-C motif) Ligand 2

CXCL13 - Chemokine (C-X-C motif) ligand 13

ET-1 - Endothelin-1

FGF - Fibroblast Growth Factor

GM-CSF - Granulocyte Macrophage-Colony Stimulating Factor

HbS - Hemoglobin S

HIF - Hypoxia-Inducible Factor

HIF1a - Hypoxia-Inducible Factor 1, Alpha Subunit

HIF2a - Hypoxia-Inducible Factor 2, Alpha Subunit

HMOX1 - Heme Oxygenase 1

IL-1b - Interleukin 1 beta

IL17 - Interleukin 17

IL6 - Interleukin 6

IκB - Inhibitor Of Nuclear Factor Kappa B

LTβR - Lymphotoxin β receptor

LV - Left Ventricle

MCP1 - Monocyte Chemoattractant Protein 1

MI - Myocardial Infarction

MMP2 - Matrix Metalloproteinase-2

MMP8 - Matrix Metalloproteinase-8

Myo-Tg - Myotrophin-Overexpressed Transgenic Mouse

NFkB - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NLRP3 - NOD-Like Receptor Family, Pyrin Domain Containing 3

NO - Nitric Oxide

NQO1 - NAD(P)H Quinone Dehydrogenase 1

Nrf2 - Nuclear Factor (Erythroid Derived 2) Like 2

NT-pro-BNP - N-terminal Prohormone of Brain Natriuretic Peptide

PDGFB - Platelet Derived Growth Factor Subunit B

RANK - Receptor Activator of NFkB

RBC - Red Blood Cells

SCD - Sickle Cell Disease

SDNN - Standard Deviation of NN Intervals

SERCA2a - Sarco/Endoplasmic Reticulum Ca2+-ATPase

Smad2 - Mothers Against Decapentaplegic Homolog 2

TGF-b1 - Transforming Growth Factor Beta 1

TGF-β - Transforming Growth Factor Beta

Th17 - T Helper 17 Cells

TINN - Triangular Interpolation of NN Interval

TNF-α - Tumor Necrosis Factor Alpha

VCAM-1 - Vascular Cell Adhesion Protein 1

VEGF - Vascular Endothelial Growth Factor

VOC - Vaso-Occlusive Crisis

3. INTRODUCTION

3.1 Sickle cell disease

Sickle cell disease (SCD) is an autosomal recessive red cell disorder, caused by mutation in β-globin gene for hemoglobin (Hb) [1].

According to the World Health Organization (WHO), SCD is one of the most common worldwide monogenetic disorder, becoming a global problem of public health in the last decades [2] [3]. Epidemiologic studies have shown that SCD has spread all over the world from their common endemic areas, which include sub-Saharan Africa, Middle East, and tribal regions of India [4].

Sickle Hb (HbS) results from a point mutation, leading to a single nucleotide substitution (Val-> Glutamic acid) in the gene encoding the erythrocyte β-globin chain. HbS polymerizes when deoxygenated with the formation of sickled and dense red cells [5]. The two main clinical manifestations of SCD are: (i) chronic hemolysis and (ii) acute vaso-occlusive crisis (VOCs) [1].

In the last decade, growing evidence indicate that inflammatory vasculopathy is important in the pathogenesis of sickle cell related acute and chronic organ damages. Among them a crucial role has been recognized for (i) free heme and free Hb that binding to NO favors local reduced nitric oxide (NO) bioavailability [3] [6] [7] [8] [9]; (ii) amplified inflammatory response with the release of pro-inflammatory and vaso-active cytokines such as endothelin-1 (ET-1) or Il-1b [10] [11] [12]; (iii) increased expression of pro-adhesive molecules as vascular VCAM-1 or selectins [13] [14]; and (iv) impairment of pro-resolving events [15]. In addition, recent studies have highlighted a novel link between amplified inflammatory response and modulation of innate immunity in SCD patients during acute VOCs [16] [17]. Thus, the combination of dense, rigid red cells that are easily entrapped in peripheral microcirculation, and the entrapment of neutrophils through p-selectin mechanism, is responsible for the generation of hetero-thrombi, which sustain SCD microvascular occlusion [14] [18].

Organs such as kidney, liver, spleen, brain or heart are susceptible to ischemic/reperfusion damage [5] [19] [15] [20]. In addition, the recurrence of VOCs

in the same districts is responsible for chronic organ damage, ending in end-organ dysfunction that negatively impacts patient quality of life and survival [1] [5] [21] [22] [23] [24].

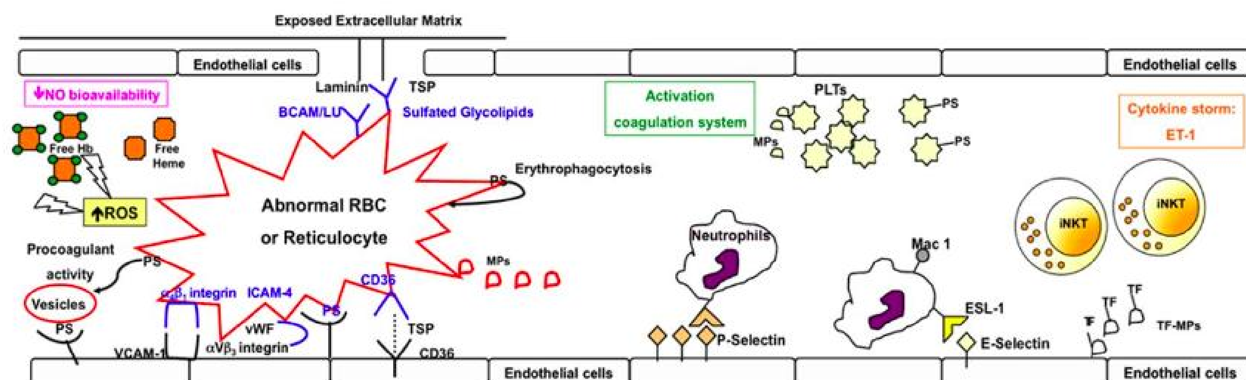

Figure 1. Schematic diagram of the mechanisms involved in the pathogenesis of acute sickle cell related vaso-occlusive events. These involve the adherence of sickle red blood cells (RBCs) or reticulocytes and neutrophils to the abnormally activated endothelial cells, with the participation of activated and phosphatidyl- Serine (PS)-rich platelets (PLTs), activation of the coagulation system, and activation of a cytokine storm. PS: Phosphatidyl-Serine; TSP: thrombospondine; vWF: von Willebrand factor; BCAM/LU: Lutheran blood group protein; ICAM-4: Landstein-Weiner (LW) blood group glycoprotein; MPs: microparticles; Mac1: β2 integrins (αMβ2 or CD11b/CD18); ESL-1: neutrophil E-selectin ligand-1; Hb: hemoglobin; ROS: reactive oxygen species; iNKT: invariant natural killer T cells; ET-1: endothelin-1; NO: nitric oxide (modified from Matte A, et al. Mediterr J Hematol Infect Dis 2019).

3.2 Cardiomyopathy and sickle cell disease

Cardiovascular disease has been described to contribute to morbidity and mortality of young adults with SCD (e.g.: sudden deaths in up to 30% of patients with SCD) [25]. Although progress in the knowledge of mechanisms involved in both acute and chronic SCD complication has been made, much remains to be investigated in sickle cell related cardiomyopathy. Indeed, limited studies are available on patients with SCD and cardiomyopathy. Abnormally high cardiac troponin-I and galectin-3, respectively markers of myocardial ischemia and fibrosis, have been reported in patients with SCD during acute VOCs [26] [27]. This is complemented by the observation of increase plasma NT-pro-BNP in SCD patients [28] [29] [30] [31] [32]. Finally, Niss O et al. have recently reported a positive correlation between diastolic dysfunction and extracellular volume fraction, used to evaluate the degree of myocardial fibrosis in patients with SCD [29].

In clinical management of SCD patients, simple transfusion or erythrocytapheresis might be used, Bratis K et al, excluded chronic transfusion as risk factor for cardiomyopathy in patients with SCD. This study showed occurrence of myocardial ischemia, diffuse myocardial fibrosis in presence of non-pathologic iron heart content, while severe iron overload has been observed in liver of young adults with SCD [33].

Similar data in association with left ventricular dilation and diastolic dysfunction were also reported by Desai et al [34]. Collectively, these data suggest that recurrent vaso-occlusive crisis, impaired vasoreactivity, and reperfusion injury might play a key role in development of sickle cell cardiomyopathy rather than the iron overload.

Using a mouse model for SCD (Berkley SCD mice), fibrosis and loss of cardiomyocytes have been linked to inflammation, vascular dysfunction and pro-fibrotic micro-environment [32]. This results in a unique hyperdynamic restrictive cardiomyopathy, indicating myocardial fibrosis as crucial event in worsening sickle cell related cardiomyopathy. In SCD mice, cardiomyocyte loss and abnormal mitochondrial function might result from the combination of focal microvascular occlusion, oxidative stress and local pro-inflammatory environment [34] [35] [36] [33]. Noteworthy, Bakeer N et al have recently reported that up-regulation of genes involved in angiogenesis, extracellular matrix remodeling, lipid metabolism, and neurotransmission may also be involved in cardiomyocyte loss observed in SCD mice [37]. In humanized SCD mice (Townes' knocked-in transgenic SCD mice), a correlation between inflammation and cardiomyopathy has been proposed, linking free heme with upregulation of heart IL-6 mRNA expression. This is associated with the up-regulation of genes associated with cardiac hypertrophy, such as atrial natriuretic factor (Nppa) and β-Myosin heavy chain 7 (Myh7) [38]. In addition, up-regulation of pro-inflammatory and pro-fibrotic IL-18 was associated with activation of NF-kB and increased cardiac fibrosis [39].

3.3 Transcription factors involved in hypertrophic cardiomyopathy

Studies in different models of hypertrophic cardiomyopathy have highlighted the important role of inflammatory and redox related transcription factors such as Nrf2, NF-kB or HIF.

Nuclear factor erythroid-2–related factor 2 (Nrf2)

Nuclear factor erythroid-2–related factor 2 (Nrf2), a ubiquitously expressed basic leucine zipper (b-Zip) transcription factor, plays a crucial role in regulating the expression of antioxidant system and in protecting organisms against oxidation [40] [41]. Under steady state, Nrf2 resides in the cytoplasm, where it is ubiquitinated by the Keap1-Cul3 ubiquitin ligase complex. In response to oxidation, reactive sulfhydryls located on Keap1 can easily be oxidized, thereby releasing Nrf2 from the inhibitory complex. Thus, Nrf2 moves to the nucleus, where it activates target genes while binding to a common DNA sequence called antioxidant response element (ARE) [42] [43] [44].

Different studies have shown that enzymes regulated by Nrf2 are involved in the pathogenesis of cardiovascular diseases (Table 1) [45]. Li et al. reported the protective effect of Nrf2 activation in a mouse model of cardiac hypertrophy. In this study, Nrf2-knockout mice develop myocardial fibrosis and cell apoptosis in a model of cardiac stress induced by transverse aortic constriction [46]. Shanmugam G et al performed a study in Nrf2-/- mice exposed to repeated endurance exercise designed to induce cardiac stress, followed by cardiac adaptation. In this model, the down-regulation of ARE antioxidant gene expression was associated with up-regulation of hypertrophy genes [47].

In SCD, chronic hemolysis results in plasma free heme, inflammatory vasculopathy and severe oxidative stress. Thus, activation of Nrf2 has been reported in both vascular endothelial cells and in target organs for SCD such as kidney, lung or brain [48] [49] [50] [51].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is another transcription factor important in inflammatory and redox-related response [52] [53]

[54]. NF-kB is a family of dimeric transcription factors responsible for: (i) cytokine production and cell survival; (ii) innate and adaptive immunity; (iii) cell differentiation, proliferation and survival, which has been found in most multicellular organisms [55]. In steady-state, NF-kB is blocked by inhibitory proteins, such as IκB family and other related proteins containing ankyrin repeats [56]. NF-kB activation occurs throughout canonical or noncanonical (alternative) pathways. Canonical pathway. NF-kB activation is related to pro-inflammatory cytokines such as IL-1β and TNF-α, as well as various cellular stresses such as oxidative stress, shear stress,, DNA damage [57] [58]. Non-canonical pathway. NF-kB is activated by TNF receptor family ligands such as LTβR, BAFFR, CD40, and RANK [59] [60]. Translocation to the nucleus and binding to its specific regions of DNA promotes the expression of downstream proteins such as certain chemokines (CXCL13, CCL21, CCL19) and cytokines (BAFF/Blys) [58] [61] [62].

In animal models of cardiovascular disease, NF-κB activation has been linked to cardiac remodeling, hypertrophy, and heart failure (Table 1) [63]. In a gain-of-function transgenic mouse model with enhanced IKK/NF-κB activation, Maier H. J et al. showed that chronic activation of IKK/NF-κB promotes CD11b+ cell infiltration, fibrosis, and myocyte atrophy. This ultimately results in inflammatory dilated cardiomyopathy and heart failure [54]. In another transgenic mouse model overexpressing myotrophin (Myo-Tg – transgenic mice with overexpression of myotrophin in the heart under the regulation of α-MHC promoter), the activation of NF-kB results in myocardial hypertrophy with subsequent development of heart failure. The hallmarks of the model were left ventricular hypertrophy, multiple focal fibrosis, myocyte necrosis, and impaired cardiac function. As a proof of concept of the key role of NF-kB in the pathogenesis of hypertrophic cardiomyopathy NF-kB-p65 was silenced by RNA interference. This improves cardiac function and decrease heart mass, supporting the detrimental role of over and sustained activation of NF-kB in development of hypertrophic cardiomyopathy [64].

In SCD, activation of NF-kB has been shown in different sickle cell target organs as well as in vascular endothelial cells, resulting in up-regulation of pro-inflammatory cytokines and adhesion molecules such as IL-6, MCP-1 and VCAM-1 [65].

Hypoxia inducible factor HIF

Cardiac fibrosis has been also linked to the activation of HIF transcriptional factors (HIF1a, 2a) [66]. HIFs play a crucial role in processes that aim to minimizing hypoxic cellular damage [67] [68]. HIF-1 is involved in cellular adaptation to hypoxia [69] [70]. HIF-2 is involved in mechanisms that facilitate iron absorption in response to hypoxia [71].

Previous studies have shown that HIF1 activates the stress responsive trophic and mitotic factors, activating pro-angiogenic pathways (e.g.: VEGF) and pro-fibrotic signaling throughout the TGF-β receptor signaling pathways [72] [73]. This later largely contributes to cardiac fibrosis [74] [75].

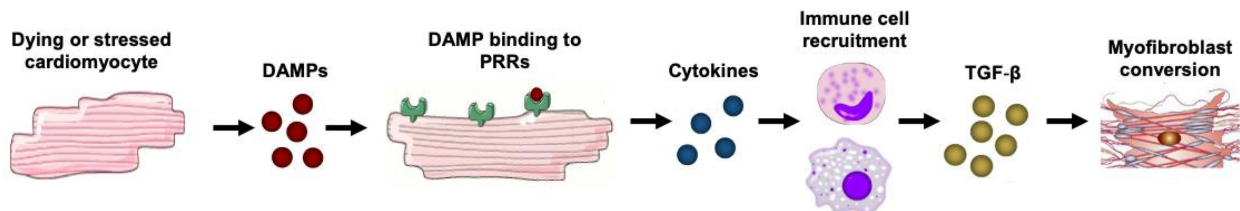

Figure 2. Schematic diagram of the mechanism involved in cardiac fibrosis through TGF-β. Cardiac injury events such as myocardial infarction trigger the cardiomyocytes to produce damage-associated molecular patterns (DAMPs), which in turn bind to pattern recognition receptors (PRRs) on neighboring cells such as cardiomyocytes, fibroblasts, resident immune cells, endothelial cells. In response, they releasee multiple cytokines that attract immune cells to the site of injury, first neutrophils and then macrophages. The latter, upon engulfment of apoptotic neutrophils, secrete TGF-β. TGF-β promotes fibrosis through the proliferation and migration of fibroblast which begin to differentiate into myofibroblast, the main source of the extracellular matrix components and which contributes greatly to the remodeling of cardiac tissue (modified from Thomas TP et al., Front Physiol. 2020).

One of the TGF-b1 downstream proteins, Smad2, up-regulates the expression of MCP1, a key attractant for monocytes and macrophages. MCP1 promotes cardiac fibrosis by recruitment of myocardial fibroblasts [76] [77]. In mice at a young age, constitutive expression of cardiac HIF-1α has been shown to be protective against myocardial infarction [78]. M. Hölscher et al, showed that the constitutive expression of HIF-1α does not have a long-term beneficial effect on murine hearts, since it stimulates transverse aortic constriction, which in turns, induces cardiac stress. This

observation is similar to that observed in patients with dilated cardiomyopathy in ischemic cardiomyopathy, increased HIF-1α proteins in left ventricular [79].

Noteworthy, up-regulation of HIF1a has been demonstrated in SCD under hypoxic conditions, oxidative stress and chronic inflammatory state in lungs [80] [81], kidney [82], brain [83].

Table 1. Transcriptional factors and hypertrophic cardiomyopathy		
Transcriptional factor	**Key Findings**	**References**
Nrf2	In a mouse model of pressure overload-induced cardiac hypertrophy was observed: - Protective mechanisms of Nrf2 activation upon increased cardiac stress; - Nrf2-knockout mice show reduced cardiac efficiency, associated with severe myocardial fibrosis and cell apoptosis.	Li, J., et al. 2009 [46]
	Nrf2 knockout mice display: - downregulation of antioxidant gene expression; - upregulation of markers indicating cardiac hypertrophy such as ANF, BNP; - Aged Nrf2-/- mice display ventricular remodeling and diastolic dysfunction.	Shanmugam, G., et al. 2017 [47]
NF-kB	In a mouse model of cardiomyocyte-specific expression of constitutively active IKK2 was observed: - Chronic activation of IKK/NF-κB is associated with to dilated cardiomyopathy; - Infiltration of the heart with CD11b+ cells, fibrosis formation, and myocyte atrophy.	Maier, H.J. et al. 2012 [54]
	In a mouse model of myotrophin overexpression in the heart was observed: - Enhanced activation of the NF-kB pathway through myotrophin (Myo-Tg) overexpression; - Activated NF-kB led to left ventricular hypertrophy, multiple focal fibrosis, myocyte necrosis, and impaired cardiac function; - Silencing NF-kB showed an improvement of cardiac function and a decrease of heart mass.	Gupta, S., et al.. 2008 [64]
	HIF-1α transgenic mouse model display: - Short-term protective effect during myocardial infarction.	Kido, M., et al. 2005 [78]
	Transgenic HIF-1α mouse model showed that:	

HIF	- Constitutive expression of HIF-1α stimulates transverse aortic constriction, induce cardiac stress in mice; In agreement, was observed: - Elevated HIF-1α protein levels in patients with dilated cardiomyopathy and ischemic cardiomyopathy.	Hölscher, M., et al. 2012 [79]
Nrf2: Nuclear factor erythroid-2–related factor 2; NF-kB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; HIF: Hypoxia-Inducible Factor.		

Taken together, the revision of the literature highlights that inflammatory and redox related transcription factors play an important role in the pathogenesis of hypertrophic cardiomyopathy. Thus, the study of the correlation of these mechanisms in the context of sickle cell related cardiomyopathy is of great interest and cover an unmet need.

3.4 Role of calcium pump SERCA2a in hypertrophic cardiomyopathy

The sarcoplasmic reticulum is the main calcium storage in muscle cells and plays a crucial role in coordinating contraction-relaxation cycles in cardiomyocytes [84]. These cycles are tightly controlled by the activity of the cardiac sarco(endo)plasmic reticulum, calcium pump SERCA2a [85] [86]. Failure to remove Ca^{2+} from the cytosol prevents muscular relaxation, contributing to the development of diastolic dysfunction [87]. Several groups reported the involvement of different mechanisms affecting SERCA2a function. Among them, changes in the phosphorylation level of phospholamban, a key regulator of SERCA2a, reduces its activity [88] [89] [90]. Roczkowsky A et al demonstrated that damage of the sarcoplasmic reticulum related to ischemic reperfusion injury, results in the malfunctioning of SERCA2a. This is sustained by reduction of Serca2 activity, due to degradation of SERCA2a. This is caused by the matrix-metalloproteinases, MMP2. Upon the ischemia MMP2 activity is increased by released reactive oxygen species whereupon MMP2 cleaves Serca2 in multiple sites causing Serca2 degradation and dysfunction [91]. Noteworthy, SERCA2a expression is placed under control of Nrf2. Indeed, $Nrf2^{-/-}$ mice showed left ventricular diastolic dysfunction and cardiac hypertrophy, which correlated with significant downregulation of SERCA2a protein expression, compared to control animals. Despite this finding, pro-BNP levels and the levels of

inflammatory cytokine TNF-alpha, evaluated in the same study, were not elevated, suggesting a direct link between Nrf2 activation, calcium pump function and hypertrophic cardiomyopathy [92].

3.5 Cardiomyopathy and colchicine

Colchicine is a well-known anti-inflammatory agent. Colchicine is a tropolone alkaloid, which can be found in many plants, but is generally extracted from the *Colchicum autumnale* (autumn crocus) [93].

Studies have shown that colchicine's mechanisms of action is multimodal by (i) inhibition of microtubule assembly, impaired attachment of microtubule protofilaments, destabilization and depolymerization of microtubules; (ii) downregulation of NLRP3 inflammasomes and subsequent release of inflammatory cytokines (such as IL-1β and IL-18) from them; (iii) prevention of adhesion of neutrophils to the endothelium; (iv) decreasing activity of TNF-alpha [94] [95].

Approved by the U.S. Food and Drug Administration (FDA) in 2009 to treat gout and familial Mediterranean fever, nowadays is used extensively in the treatment of autoimmune and inflammatory disorders. In the late 20th century, colchicine's beneficial effects and ability to reduce inflammation gave impetus to its use in cardiovascular diseases.

Recent studies have shown that colchicine improves heart function, restores cardiomyocyte function and ameliorates the survival of patients with pericarditis, atherosclerosis, atrial fibrillation and heart failure [96].

Therefore, recent studies on the use of colchicine in the context of attenuation of the inflammatory response and restorative cardiac function has been considered (Table 2).

Table 2. Colchicine and Heart		
Model/Trial	**Key findings**	**Reference**
	- Oral administration of colchicine significantly improves survival of in acute phase of MI. - Colchicine favors heart remodeling at 4 weeks after MI;	

Mouse Model Myocardial ischemia/reperfusion injury	- B-type natriuretic peptide mRNA expression in the non-infarct and infarct areas was attenuated in colchicine treated mouse group 4 weeks after MI; - Colchicine inhibits the increase in LV scar size; - Colchicine inhibits the up-regulation of TNF-α, IL-1β, IL-18, MCP-1, and CXCL2 mRNA at the infarct site; - Colchicine attenuates the increase of NLRP3 inflammasome after MI; - Colchicine suppresses chemotaxis, adhesiveness, mobilization and degranulation of lysosomes of neutrophils, monocytes and macrophages.	Fujisue K et al., 2017
Mouse Model Myocardial ischemia/reperfusion injury	- A low dose of colchicine oral administration before reperfusion reduces myocardial damage and infarct size 24 hours after acute MI; - 24 hours after ischemia-reperfusion, colchicine reduces systemic pro-inflammatory cytokines. IL6, IL10, MCP-1, IL1β; - Colchicine improves the hemodynamic parameters of the heart in the long-term perspective with an amelioration in the cardiac output; - Markedly reduced area of myocardial fibrosis in the colchicine group.	Akodad M et al., 2017
Mouse Model Myocardial ischemia/reperfusion injury	- Colchicine injection before reperfusion improve the Heart rate variability parameters (SDNN, TINN) as well as HRV index; - Colchicine prevents sympathetic denervation after MI in distant areas of the heart. it has been shown that denervation in the area adjacent to the scar increases the risk of sudden death.	Huet F et al., 2020

Patients First large-scale study of the effects of colchicine in patients with myocardial infarction	- First large-scale study of colchicine's interest in secondary prevention after acute MI; - Low-dose colchicine daily resulted in a significant reduction in the risk of ischemic cardio-vascular events in patients with recent MI; - The risk of death from cardiovascular causes, resuscitation, cardiac arrest, MI, stroke is lower in patients receiving colchicine therapy;	Bouabdallaoui N et al., 2020

4. AIM OF THE STUDY

To understand the mechanisms involved in sickle cell related cardiomyopathy.

5. MATERIALS AND METHODS

5.1 Drugs and chemicals

NaCl, Na_3VO_4, TRIS, Tween 20, EDTA, choline, $MgCl_2$, MOPS, Na_2HPO_4, KH_2PO_4, NaF, bicine, β-mercaptoethanol, glycine, glycerol, potassium cyanide, bromphenol blue, sodium dodecil sulphate (SDS), albumin from bovine serum (BSA), May-Grunwald-Giemsa's Azur-Eosin-Methylene Blue solution, dithiotreithol (DTT), Type A porcine gelatine, Immobilon Classico and Immobilon Forte Western Hrp solution were from Merck Group (Darmstadt, Germany); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland); Triton X-100 was purchased from GE Healthcare (Little Chalfont, UK); 40% Acrylamide/Bis Solution, 37.5:1 was from BIO-RAD (Hercules, CA, USA); Full Range Rainbow Molecular Weight Marker, Temed were purchased from GE Healthcare Life Biosciences (Little Chalfont,UK); (San Diego, USA);; Dulbecco's Phosphate Buffered Saline (DPBS) was from Lonza (Belgium).

5.2 Mouse strains and design of the study

Experiments were performed on 3/4 and 7/8-months old male and female mice. Humanized mouse model for human normal hemoglobin (HbA, AA mice, $Hba^{tm1(HBA)Tow}$ $Hbb^{tm3(HBG1,HBB)Tow}$) and human sickle hemoglobin (Townes model for sickle cell anemia, HbS, SS mice, $Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1,HBB*)Tow}$) were used. Whenever indicated sickle mice were treated with colchicine (0.1 mg/kg daily) or vehicle (water) for 4 months by gavage, starting at 3 months of age [97]. Hematological parameters were measured every month. Animals were anesthetized with 3-4% isoflurane under oxygen flow and blood was collected by retro-orbital venipuncture with heparinized capillaries. Hemoglobin and hematocrit were determined by Drabkin's and standard manual methods, respectively [98, 99]; red cell indices were analyzed using Sysmex XN-1000 (Sysmex Corporation, Hyogo, Japan). Mouse weight was collected every 2 weeks. The animal protocol was approved by Animal Care and Use Committee of the University of Verona (CIRSAL). At the end of the treatments, animals were euthanized, and organs were

immediately harvested and either froze in liquid nitrogen or fixed in 10% formalin and embedded in paraffin for histology. Before freezing, hearts were ex-vivo perfused with PBS to completely remove blood [100]. Frozen organs were store at -80°C until usage [101]. Plasma was prepared by centrifuging blood at 3500 for 15 min at 4°C and then stored at -80°C [102].

5.3 Zymogram analysis of murine hearts

Frozen hearts were homogenized with the Tissue Master 125 Watt Lab Homogenizer (Omni International, Bedford, USA) in tris lysis buffer (50mM tris-HCl pH 7.4 and 0,5% Triton, without protease inhibitors) for MMP zymogram analysis. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to manual procedures. Proteins from homogenized mouse hearts were solubilized with non-reducing sample buffer (SB 2X: 50mM Tris pH6.8, 2% v/v glycerol, bromophenol blue) [103] [104], and loaded onto a 7.5% T polyacrylamide gel with type A porcine gelatine (2 mg/ml) polymerized within the matrix to detect the activity of MMPs. SDS-PAGE analysis was performed at a constant voltage of 90V, gels were then washed 4 times with washing solution (50 mM Tris-HCl pH 7.4, 2.5% Triton X-100, mM CaCl2, 1 μM ZnCl2), in order to remove SDS that interferes with the activity of the MMPs, and incubated in the reacting solution (50 mM Tris-HCl pH 7.4, 1% Triton X-100, mM CaCl2, 1 μM ZnCl2) at 37 °C for 48 hrs. After the incubation, the gels were washed with water and stained with Colloidal Coomassie; the de-staining brought out the light bands on a dark background that indicate the activity of MMPs [105].

5.4 Immunoblot analysis

Frozen hearts from each studied group were homogenized with the Tissue Master 125 Watt Lab Homogenizer in iced cold bicine buffer (20 mM Bicine pH 7.4, 1.5% Triton X-100, Roche Complete inhibitor cocktail, 1 mM Na3VO4) and incubated for 1h at 4°C. After centrifuging at 13.000g for 30 min at 4°C, the supernatant, containing proteins, was collected and used for further analysis. Total

protein content was estimated by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions, proteins were solubilized in sample buffer (SB: Tris-HCl 50mM, pH 6.8; 100mM beta mercaptoethanol; 2 % v/v SDS; 10% v/v glycerol and a few bromophenol blue) and analyzed by SDS-PAGE followed by immunoblot analysis onto nitrocellulose membranes (Thermo Fisher Scientific, Waltham, MA, USA). The following specific antibodies were used: Anti-MMP2, Anti-MMP9, Anti-MCP1/CCL2, Anti-PDGF Receptor, Anti-FGF Receptor (phospho Tyr653/654), Anti-PDGF Receptor (phospho Tyr740) from GeneTex (Ca, USA); Anti-GAPDH, Anti-SERCA-2 (F-1), Anti-TGF-β Receptor, Anti-β-Actin (2A3), Anti-ET-1, Anti-HIF 1α (H-206), Anti-HIF 2α, Anti-IL-6 from Santa Cruz Biotechnology (CA, USA); Anti-Phospho (Ser536) NF-kB p65, Anti-NF-kB p65 (clone C22B4) from Cell Signaling Technology (Danvers, MA, USA); Anti-P-Selectin from Abcam (Cambridge, UK); Anti-FGF Receptor from Thermo Fisher Scientific (Waltham, MA, USA) ; anti α-Actin from Merck Group; anti-VCAM-1 from R and D Systems (Minneapolis, MN, USA); Anti-Thromboxane synthase TBXAS-1 was from Cayman Chemical Company (Michigan, USA). The following secondary antibodies were used: donkey anti-rabbit or donkey anti-mouse IgG HRP conjugated from GE Healthcare Life Sciences (Chicago, IL, USA), or donkey anti-goat IgG HRP conjugated from Santa Cruz Biotechnology (CA, USA). Blots were developed using the Immobilon Western Chemiluminescent HRP Substrate and images were acquired using the Alliance Q9 Advanced Chemiluminescence Imager (Uvitec, Cambridge, UK).

5.5 Immunoprecipitation assay

Immunoprecipitation (IP) assays were carried out as previously reported, using 1 mg of proteins from mouse hearts in 600 μl of bicine buffer added of a mix of specific anti-Phospho Tyrosine antibodies (clone 4G10 from Mercks Group and clone PY99 from Santa Cruz Biotechnology, Inc) [106].

5.6 Quantification of plasmatic cytokines

ELISA assay: soluble NT-pro-BNP levels in plasma of AA and SS mice were measured using the Mouse NT-pro-BNP ELISA Kit (NBP2-76775, Novus Biologicals, Colorado, USA) following the manufacturer instructions [107].

Ella Automated Next Gen Immunoassay: ELLA (Bio-Techne, Minnesota, USA) is an automated next gen cytokine immunoassay. It is based on the simultaneous run of different single plex microfluidics ELISA assays (Ella Automated Immunoassay System Datasheets, Bio-Techne, Minnesota, USA) [108, 109], allowing the quantification of 1 to 8 analytes. Each sample is analyzed in triplicate within patented cartridges. We used a cartridge combination of 32x4M and quantified CCL-2, CXCL-2, CXCL-1, GM-CSF in plasma from each studied group of mice. Results were analyzed and graphed using the GraphPad Prism Software.

5.7 Flow cytometric analysis of Th17 CD4+ T cells heart infiltrate

Th17 CD4+ cells in the heart from AA, SS and SS treated with Colchicine mice were determined by flow cytometric analysis using the Mouse Th1/Th2/Th17 Phenotyping Kit (BD Biosciences, New Jersey, USA), following the manufacturer instructions (Technical Data Sheet, Mouse Th1/Th2/Th17 Phenotyping Kit, BD Biosciences) [110]. Briefly freshly harvested hearts were ex vivo perfused with PBS (320 mOsm), cut into small pieces, and placed into PBS BSA 1% collagenase II (0.5 mg/ml, Merck Group, Darmstadt, Germany) for 1h at 37°C. After the complete mechanical dissociation, using a GentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), hearts were washed 3 times with PBS-BSA 1% to completely remove collagenase. Then the entire homogenate was collected and incubated in PBS, BSA 1% added of specific anti CD3 PE-Cy7, anti CD8 APC-Cy7, anti CD45-V450 (Thermo Fisher Scientific), for 30 min at RT. The samples were then washed with PBS-BSA 1% and fixed in 1 ml of cold fixation solution (BD Cytofix buffer) for 20 min. Fixed cells were washed in PBS-BSA 1%, and permeabilized using the 1x BD Perm/Wash buffer for 15 minutes. The samples were then stained in BD Perm/Wash buffer added of the antibody mixed cocktail containing CD4 PerCP-Cy5.5; IL-17A PE; IFN-GMA FITC; IL-4 APC (Mouse Th1/Th2/Th17 Phenotyping

Kit, BD) for 30 min to specifically distinguish between Th1, Th2 and Th17 CD4+ subgroups of cells. Samples were washed with1× BD Perm/Wash buffer and resuspended in 300 μl of PBS BSA1%. Before acquisition 5 μl of counting beads (CountBright[T] Absolute Counting Beads, ThermoFisher Scientific) were added to each sample. Samples were acquired using BD LSRFortessa (BD Biosciences) Analysis of acquired samples were performed using FlowJo Software (Tree Star, Ashland, OR, USA).

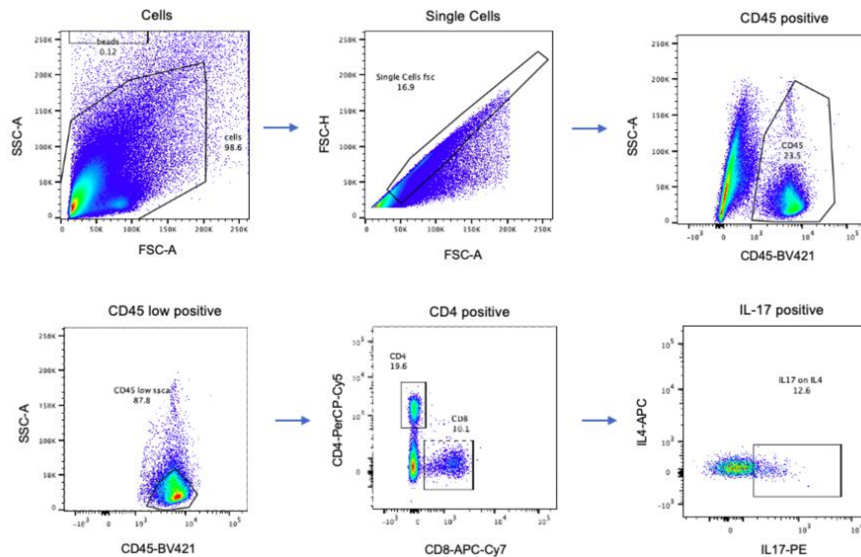

Figure 3. Representative scatter plots of the flow cytometric gating strategy to analyze IL-17 secreting T-lymphocytes in the heart of healthy controls and SCD mice.

5.8 Histopathology of mouse hearts

Formalin fixed and paraffin embedded (FFPE) mouse hearts were cut in multiple (at least three) 3-micron whole mount sections for histological analysis as previously described [111]. Briefly heart slices were dewaxed, rehydrated, and subjected to haematoxylin-eosin staining to assess the area of cardiomyocytes[15]. PicroSirius Red staining to analyze interstitial fibrosis.[112]. Two pathologists independently and blindly evaluated the tissue architecture and changes using a 63x equipped microscope.

5.9 Statistical Analysis

Data were analyzed using either *t*-test or one-way ANOVA for repeated measures. A difference with a $p < 0.05$ was considered significant.

6. RESULTS AND CRITICAL DISCUSSION

6.1 Humanized sickle cell mice show an age dependent hypertrophic cardiomyopathy and degradation of SERCA2a

In the present study, we used humanized mouse model for SCD to understand the mechanism(s) involved in the pathogenesis of SCD related cardiomyopathy. To address the question, we first evaluated the impact of aging on heart function of SCD mice. As shown in Figure 4A, SCD mice displayed cardiomegaly already at 3 months of age when compared to healthy (AA) animals. This was associated with age dependent increased cardiomyocyte areas, as attempt to compensate for loss of cardiomyocyte function/damage in aged SCD mice (Figure 4B). This agrees with the up-regulation of plasma pro-BNP, used as marker of ventricular dysfunction, when compared to either AA mice or 3 months-old SCD animals (Figure 4C).

Taking together these data suggest an age dependent development of cardiomyopathy in SCD mice. This is also supported by the age dependent increased expression of β-actin in SCD mice when compared to healthy animals (Figure 4D), which is a cytoskeletal protein expressed in all four chambers of the heart and is reported to be overexpressed in the setting of hypertrophic cardiomyopathy as compensatory mechanisms before the development into dilated cardiomyopathy [113] [114].

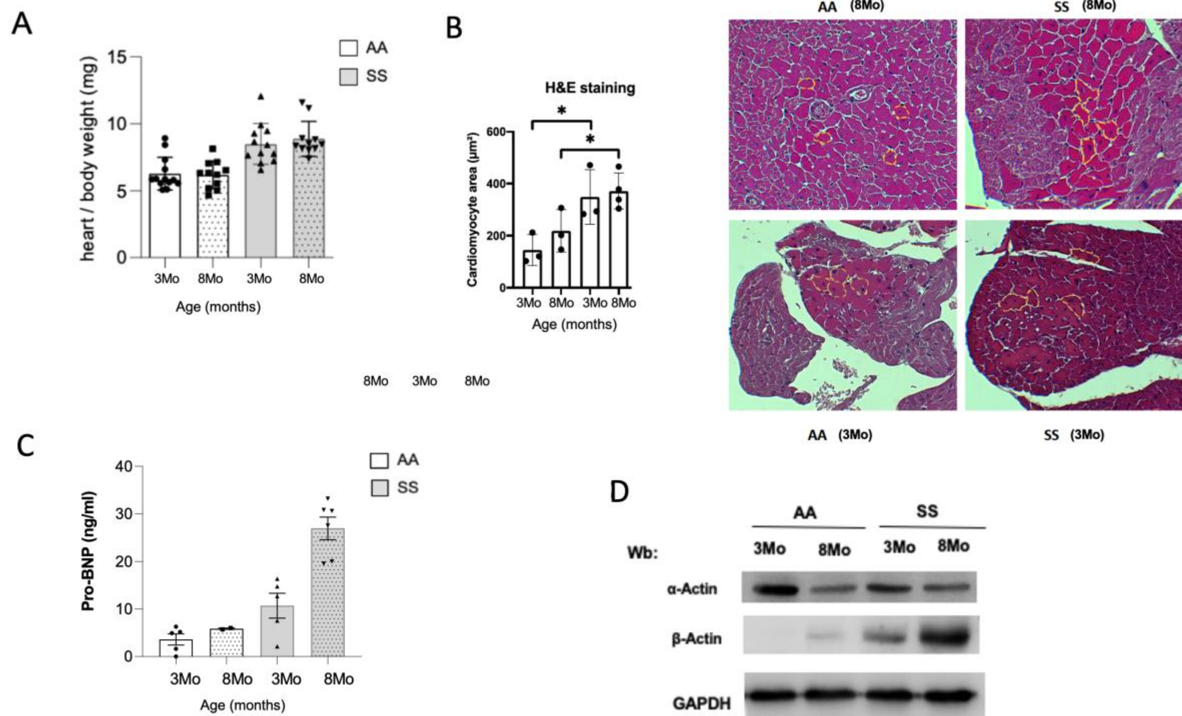

Figure 4. The Townes humanized mouse model of sickle cell disease develop an age-dependent hyperthrophic cardiomyopathy. Upper panel: (A) Heart weight and body weight ratio of control (AA) and sickle (SS) mice at 3 and 8 months of age; bar graphs show means ± SD (n=10); * P<0.05 vs AA. **(B)** H&E staining of heart sections from control (AA) and sickle (SS) mice at 3 and 8 months of age; bar graphs show means ± SD (n=10); * P<0.05 vs AA. Original magnification, ×100. One representative image from 3-4 with similar results. **Lower panel**: **(C)** Soluble NT-pro-BNP plasma levels in control (AA) and sickle (SS) mice at 3 and 8 months of age; Data are presented as means ± SEM (*n*=5). *p< 0.05 compared to AA mice by two-way ANOVA for multiple comparisons. **(D)** Immunoblot analysis with specific antibodies against α-Actin and β-Actin in control (AA) and sickle (SS) mice at 3 and 8 months of age. GAPDH was used as loading control. One representative of other three with similar results.

Recent studies in both animal models and patients with different cardiovascular diseases have highlighted the importance of sarcoplasmic reticulum calcium ATPase cardiac isoform 2 (SERCA2a), a calcium transport system important for myocardial performance [115]. We then decided to evaluate the SERCA2a expression in our model [116] [117]. As shown in Figure 5A, we found protein degradation SERCA2a (SERCA2a proteolytic residues between 60-75 kDa), in hearts from SS mice when compared to healthy controls [91]. This agrees with previous reports, showing an association between a reduction in SERCA2a activity and adverse cardiac remodeling [118, 119].

SERCA2a is degraded by metalloproteinases (MMPs) that participate to myocardial remodeling as collagenases [91]. As shown in Figure 5B, we found an age dependent increased expression of MMP9 compared to healthy mice. While MMP2 expression was significantly higher only in 8 months-old SS mice than in either younger SS or heathy animals (Figure 5B). To link SERCA2a degradation with MMP9, we carried out MMPs zymograms and we observed increased MMP9 activity in 8 months-old SS mice (Figure 5C).

Taken together these data suggest that degradation of SERCA2a contributes to reduce cardiomyocyte performance, ending in hypertrophic cardiomyopathy observed in SCD mice.

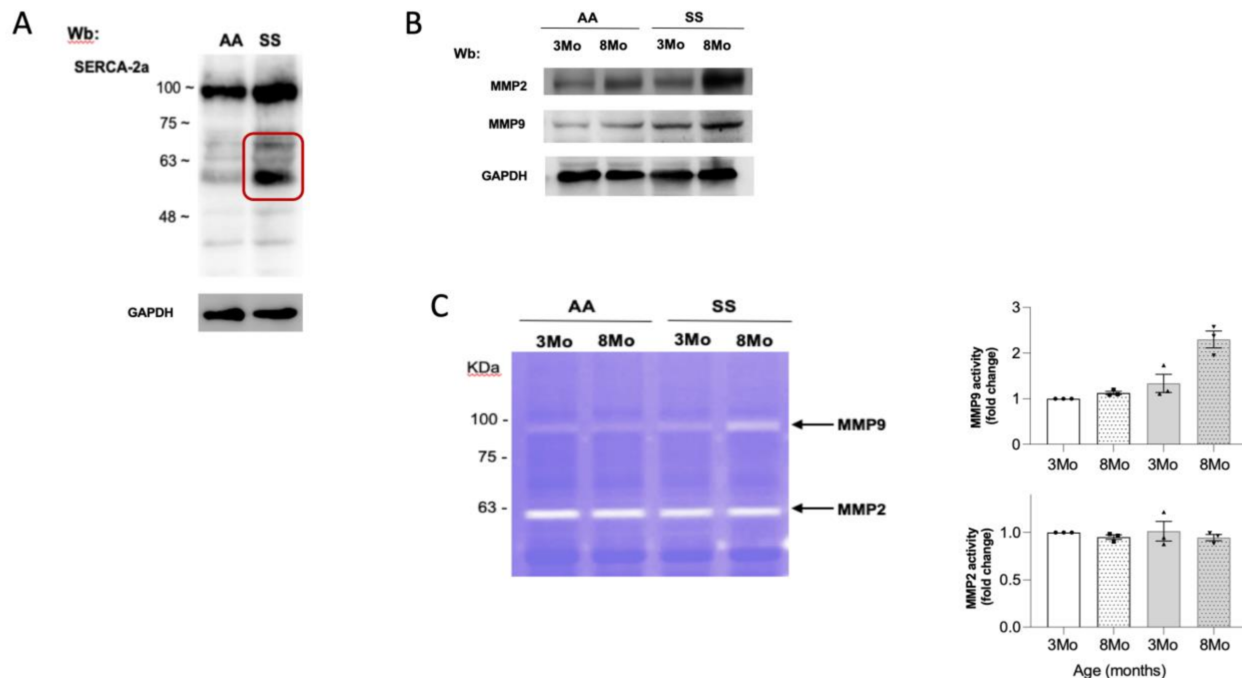

Figure 5. SERCA2a degradation by metalloproteases contributes to SCD cardiomyopathy. (A) Immunoblot analysis of hearts from healthy (AA) and sickle cell disease (SS) mice at 8 months of age with specific antibody against SERCA2a. Red box: SERCA2a degradation products at 70, 65, and 50 kDa. One representative image of other three with similar results. **(B)** Immunoblot analysis of hearts from control (AA) and sickle (SS) mice at 3 and 8 months of age with specific antibodies against MMP2 and MMP9. GAPDH was used as loading control. One representative image of other three with similar results. **(C)** Gel-zymogram with representation of MMP2 and MMP9 proteolytic activity in the hearts of control (AA) and sickle (SS) mice at 3 and 8 months of age. **Right panel.** Densitometric analysis of MMP2 and MMP9 bands. Bar graphs show means ± SD (n=3); * P<0.05 vs AA.

Collectively our data indicate that SS mice develop an age-dependent hypertrophic cardiomyopathy, associated with SERCA2a degradation and increased MMP9 activity that contributes to myocardial remodeling.

6.2 Cardiac inflammation promotes inflammatory vasculopathy and cardiac remodeling

Since vascular vulnerability and chronic inflammation characterized SCD, we evaluated the activation of the transcription factor NF-kB p65 in heart from AA and SS mice. Indeed, previous studies have shown that NF-kB plays a crucial role in acute phase response to oxidation and to ischemic/reoxygenation stress [120]. In SCD mice, we observed age induced activation of NF-kB p65 in the heart from SS mice compared to healthy controls (Figure 6A). This was associated with increased expression of P-selectin and ET-1 (Figure 6B), both participating to inflammatory vasculopathy and vascular dysfunction [10, 121]. Collectively our data suggest that local sustained inflammatory response with vascular dysfunction contributes to the pathogenesis of sickle cell related cardiomyopathy.

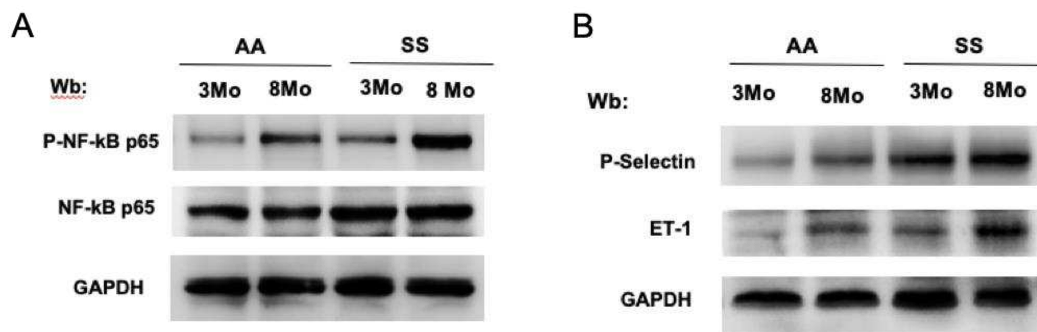

Figure 6. Activation of NFkB and increased expression of the adhesion molecules ET-1 and P-Selectin sustain inflammatory vasculopathy in the SCD hearts (A) Immunoblot analysis of hearts from control (AA) and sickle (SS) mice at 3 and 8 months of age with specific antibodies against phospho-(Ser536) NF-kB, NF-kB p65. GAPDH was used as loading control. One representative image of other three with similar results. **(B)** Immunoblot analysis of hearts from healthy (AA) and sickle cell (SS) mice at 3 and 8 months of age with specific antibodies against P-selectin, Endothelin-1 (ET-1). GAPDH was used as loading control. One representative image of other three with similar results.

Previous studies have shown that TGF-β1 plays a crucial role in promoting tissue repair, myocardial remodeling and cardiac fibrosis [122]. Here, we evaluated collagen deposition and TGF-β1 pathway in hearts from SS mice. As shown in Figure 6A, Picrosirius Red staining of mouse hearts show the presence of cardiac fibrosis in SS old mice compared to AA animals (Figure 7A). This was associated with increased activation of TGF-β1 receptor, evaluated as amount of Tyr—phospho TGF-β1 receptor in heart from older SS mice when compared to younger SS animals or healthy controls (Figure 7B). The pro-fibrotic signature of sickle cell related cardiomyopathy, was also supported by increased expression of PDFG-B and activation of the related receptor, PDGF-B Rec. This agrees with higher amount of active FGF receptors, both involved in extracellular matrix remodeling and cardiac fibrosis (Figure 7C) [123] [124] [125]. Taken together these data indicate that chronic inflammation combined with endothelial dysfunction promotes cardiomyocyte loss, sustains pro-fibrotic environment resulting in over-activation of TGF-β1 signaling pathway.

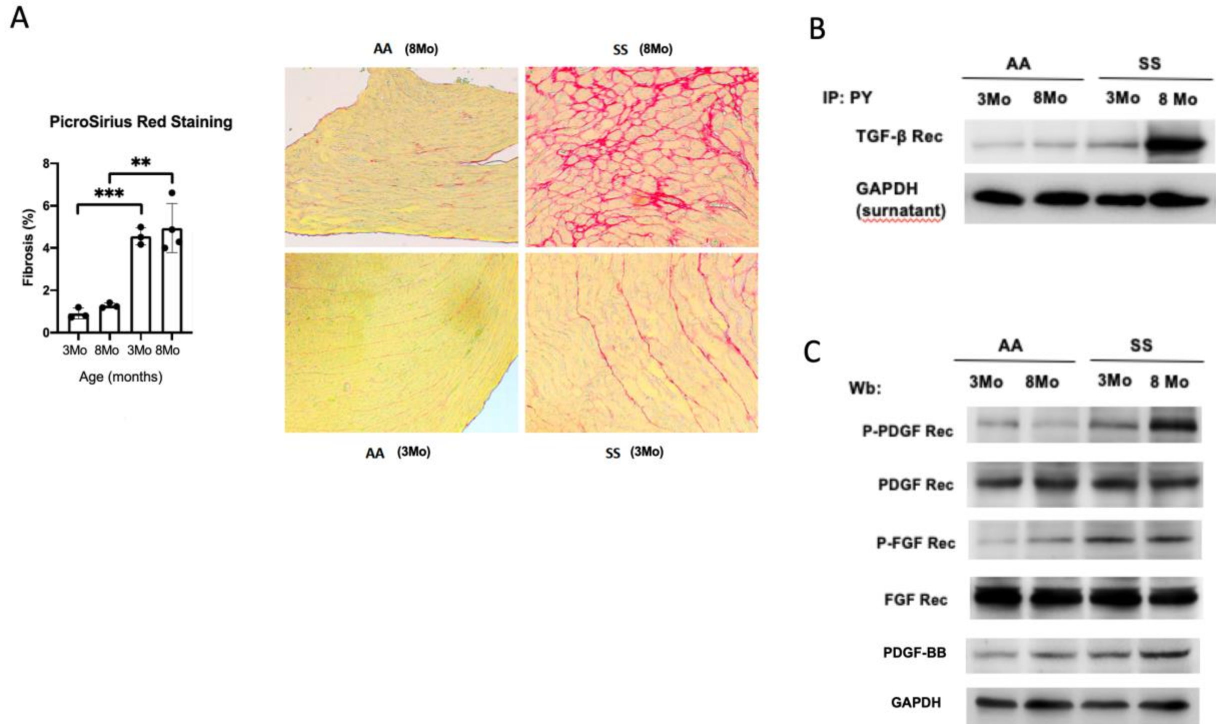

Figure 7. Humanized sickle mice show adverse cardiac remodeling and excessive collagen deposition with age. (A) Micropicture of PicroSirius Red staining heart sections from AA and SS mice at 3 and 8 months of age. Original magnification, ×100. One representative image from 4 with similar results; **(B)** Immunoprecipitation assay with anti Phospho Tyrosine (PY) and immunoblot with specific antibodies for TGF-β receptor from the hearts of control (AA) and sickle (SS) mice at 3 and 8 months of age; GAPDH on the corresponding supernatant was used as loading control; **(C)** Immunoblot analysis with specific antibodies against PDGF-BB, PDGF receptor and FGF receptor and the total forms PDGF receptor, FGF receptor control (AA) and sickle mice (SS) at 3 and 8 months of age. GAPDH was used as loading control.

6.3 Heart Th17 inflammatory cell infiltrates and increased IL17 characterizes sickle cell related cardiomyopathy

We then explore pro-inflammatory and pro-fibrotic chemokines in aging SS mice compared to heathy controls. As shown in Figure 8A-B, we found higher heart expression of MCP1 (CCL-2), a monocyte chemoattractant protein, in SS mice than in AA animals. This was associated with increased soluble pro-inflammatory chemokines CXCL1 and CXCL2, involved in leukocytes (neutrophils, lymphocytes, and monocytes) recruitment as well as in heart remodeling (Figure

8B) [126]. Noteworthy, we found significant increase of IL17, which promotes CXCL1 expression and is a key downstream mediator of TGF-β1 pathway [127]. This is associated with increased IL-6 levels, supporting the pro-inflammatory and pro-fibrotic environment as characterizing profile of SS mice (Figure 8C).

Previous studies have shown that IL-17 contributes to migration of leukocytes and of lymphocytes Th17 as well as activation of endothelial cells and proliferation of fibroblasts [128] [129]. Here, we looked for possible Th17 cell infiltration in hearts from SS mice compared to AA animals. Indeed, we observed increased Th17 cell heart infiltration in SS mice compared to healthy animals (Figure 8D). This suggests participation of IL-17 and lymphocyte Th17 to heart fibrosis, characterizing SS mice [130] [131].

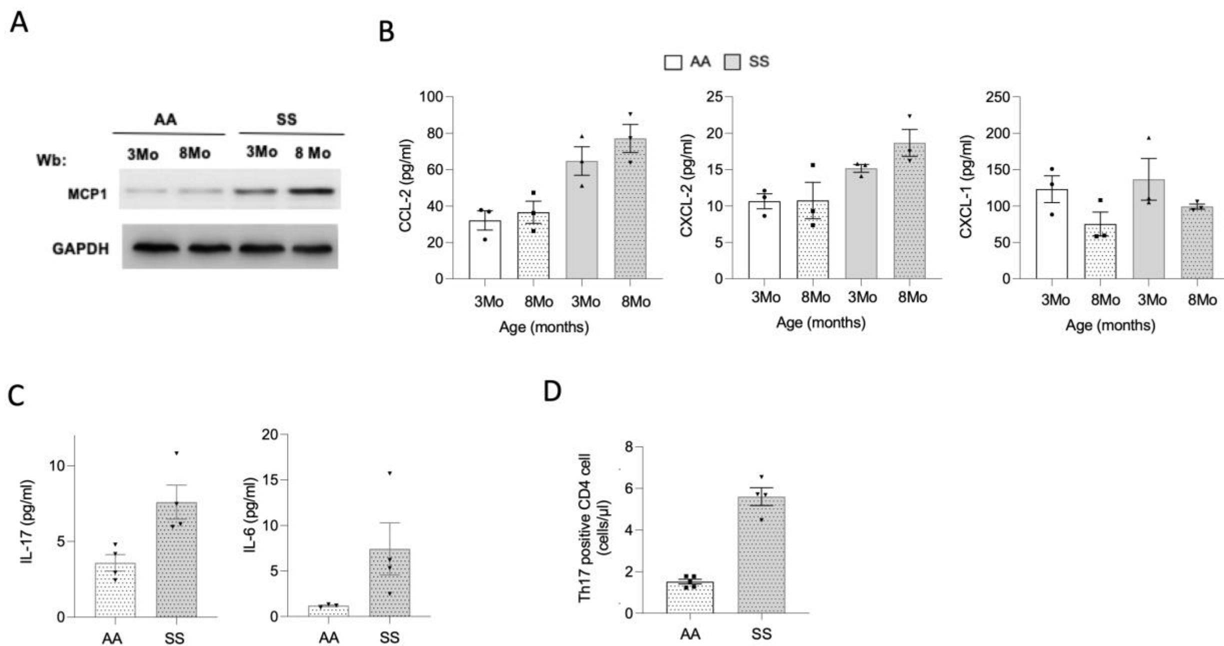

Figure 8. Th17 inflammatory infiltrates contribute to cardiac remodeling and fibrosis in sickle mice. Upper panel: (A) Immunoblot analysis of hearts from control mice (AA) and humanized mice with SCD (SS) at 3 and 8 months of age with specific antibodies against MCP1. GAPDH was used as loading control; **(B)** CCL-2, CXCL-2 and CXCL-1 plasma levels in control (AA) and sickle mice (SS) at 3 and 8 months of age; Data are presented as means ± SD (*n*=3). *p< 0.05 compared to AA mice by two-way ANOVA for multiple comparisons and T-Test. **Lower panel**: **(C)** IL-17 and IL-6 plasma levels in control and SCD mice at 8 months of age. Data are presented as means ± SD (*n*=4). *p< 0.05 compared to AA mice by two-way ANOVA for multiple comparisons and T-Test. **(D)** Levels of positive Th17 cell subpopulation of CD4 positive cells in heart homogenate from control (AA) and SCD (SS) mice at 8 months of age. Data are presented as means ± SD and each replicate (*n*=5).

All together our results indicate a key role of pro-inflammatory and pro- fibrotic cytokines associated with novel evidence of Th17 cell infiltration in the pathogenesis of sickle cell related cardiomyopathy.

6.4 Colchicine reduces cardiac inflammation and vascular dysfunction

Growing evidence highlights the crucial role of inflammation in different cardiomyopathies such as acute myocardial infarction, heart failure or atrial fibrosis associated with atrial fibrillation [132] [133]. Colchicine is an old drug with general anti-inflammatory effects, modulating local immune response (see introduction section). Noteworthy, colchicine has been reported to interfere with collagen deposition and heart fibrosis by interfering with TGF-b1 and IL-17 signaling pathways [133] [134] [132]. Thus, we chose it as an ideal candidate to study its effect on inflammation and the development of cardiomyopathy in the setting of sickle cell disease.

Colchicine was administrated at the dosage of 0.1 mg/Kg/d by gavage based on revision of the literature. In SCD mice, colchicine prevented the sickle cell related activation of NF-kB p65 compared to vehicle treated SS animals (Figure 9A). This was associated with down-regulation of markers of endothelial inflammatory activation such VCAM-1, P-selectin or ET-1 or thromboxane synthase-1 as well as pro-inflammatory cytokines such as IL-6 when compared to vehicle treated animals (Figure 9B). Noteworthy, we observed a significant reduction in soluble CCL-2 and CXCL-2 compared to vehicle treated SS mice (Figure 9C). Our results indicate that colchicine reduces heart inflammatory response and inflammatory vasculopathy in SS mice.

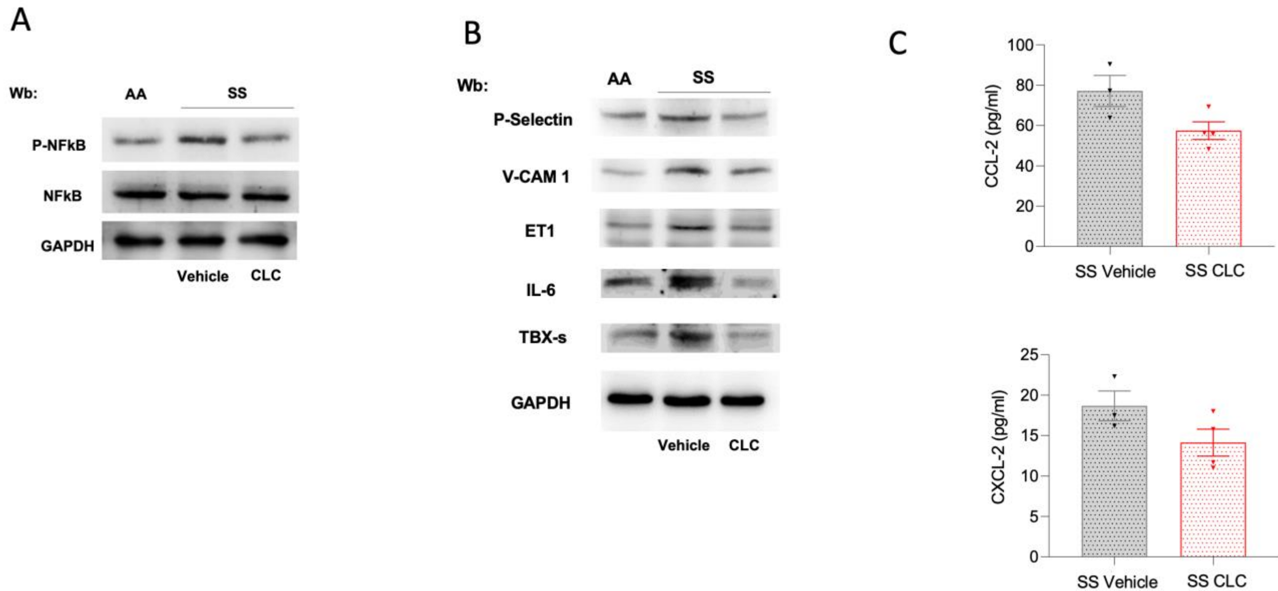

Figure 9. Colchicine reduces inflammatory vasculopathy in a SCD mouse model. (A) Immunoblot analysis of hearts from control (AA) and SCD (SS) mice treated with either vehicle or colchicine at the dosage of 0.1 mg/Kg/d with specific antibodies against phospho (Ser536) NF-kB, NF-kB p65. GAPDH was used as loading control; **(B)** Immunoblot analysis with specific antibodies against P-selectin, V-CAM1, ET-1, IL-6, TBX-s hearts from control (AA) and SCD (SS) mice treated with either vehicle or colchicine. GAPDH was used as loading control; **(C)** CCL-2 and CXCL-2 plasma levels in control (AA) and SCD (SS) mice treated with either vehicle or colchicine. Data are presented as means ± SD and each replicate (*n*=5). *p< 0.05 compared to SS control mice by two-way ANOVA for multiple comparisons and T-Test.

6.5 In hearts from humanized SCD mice, Colchicine reduces heart Th17 inflammatory cell infiltrates and prevents TGF-b1 activation

Since we show that heart fibrosis and collagen deposition is part of sickle cell cardiomyopathy, we evaluated whether colchicine might interfere with TGFb1 pathway in heart of SS mice, modulating local inflammatory response and Th17 infiltrates. As shown in Figure 10A, colchicine significantly reduced plasma IL17 in SS mice compared to vehicle treated animals, without major change in IL6. This was associated with a significant reduction in Th17 lymphocytes heart infiltration compared to vehicle treated SS animals (Figure 10B). Being previously established a functional link between Th17, IL-17 and activation of TGF-b1 pathway towards fibrosis [135] [136], we evaluated activation of TGF-b1 receptor in hearts from SS treated with either vehicle or colchicine. As shown in Figure 10C, colchicine reduced

the activation of TGF-b-1 receptor when compared to vehicle treated SS animals. We then assessed the fibrosis marker PDGF-B and the activation of its receptor, a key component of extracellular matrix remodeling and fibrosis processes. Colchicine significantly reduced PDGF-B and the activation of PDGF-B receptor in colchicine treated SS animals when compared to vehicle treated mice (Figure 10C, lower panel).

Collectively, results demonstrate the beneficial effect of colchicine on progressive cardiomyopathy and fibrosis in humanized SS mice. The main events which lead to heart damage in terms of sickle cell disease are damage caused by reactive oxygen species (ROS) related to both hypoxia associated with vaso-occlusive crises (VOCs) and amplified inflammatory response mainly driven by pro-inflammatory and pro-fibrotic cytokines such as CCL2, CCLX2 and IL-17. These orchestrate local recruitment of lymphocytes Th17 subpopulations and activation of TGFb1 pathway. This is the most potent stimulation for collagen deposition and myocardial remodeling. Under healthy conditions, the Th17 subset has an essential role in assisting B cells, activating killing properties of macrophages, when it is in tightly regulated balance with Treg cells [137] [138]. And acquires pathogenic features and is involved in fibrotic processes in cardiomyopathies and act as an autoimmune agent, producing pro-inflammatory cytokines when this balance is disturbed [139]. At the same time, produced cytokines and TGF-B induces adverse cardiac remodeling and fibrosis, that also include the overexpression of PDGF B [140].

As additional new finding, we show that colchicine protects against sickle cell related cardiomyopathy by modulation of NF-kB activity resulting in downregulation of markers of inflammatory vasculopathy and extracellular matrix remodeling. Ultimately, this undermines T cell differentiation into a Th17 subset (Figure 10D). In conclusion, our study generates the rationale to consider colchicine for preventing/limiting sickle cell cardiomyopathy. The progress in the knowledge generated by our study will hope might be transferred to human subject with SCD in proof-of concept clinical trials.

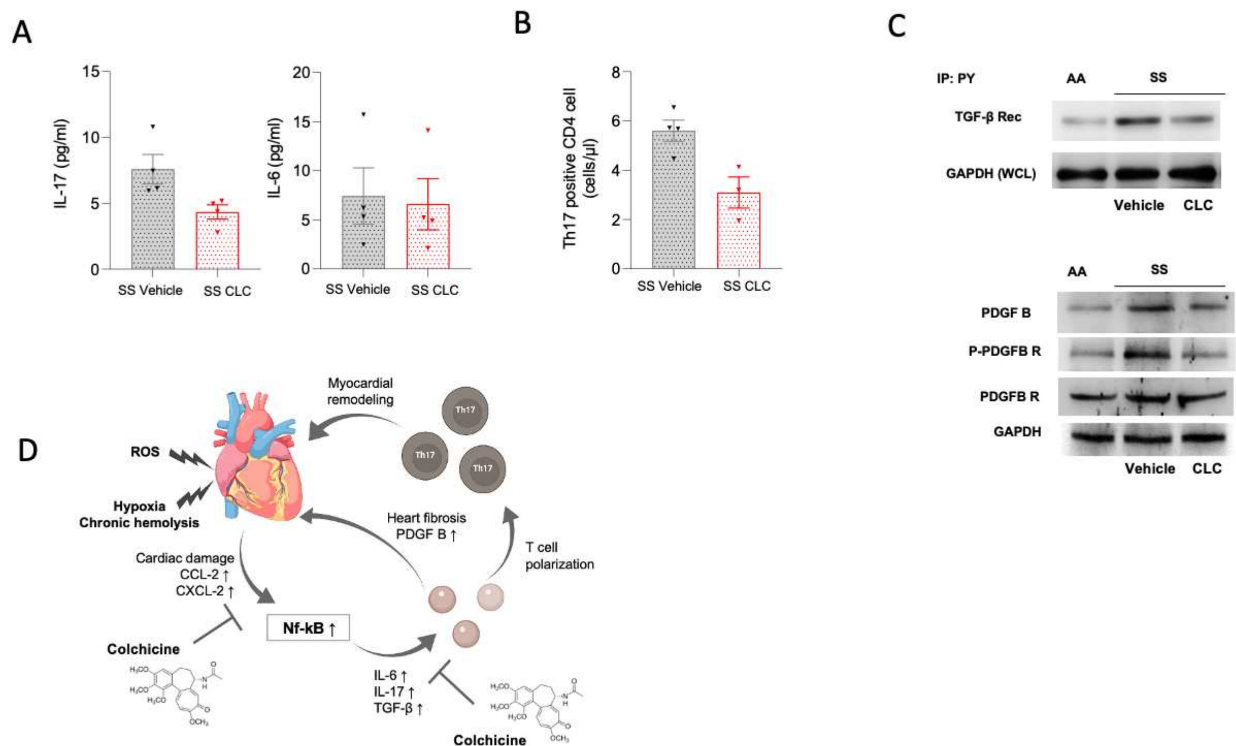

Figure 10. Colchicine treatment prevents inflammatory infiltration and adverse cardiac remodeling in sickle mice. (A) IL-17 and IL-6 plasma levels in hearts from SCD (SS) mice treated with either vehicle or colchicine at the dosage of 0.1 mg/Kg/d gavage. Data are presented as means ± SD and each replicate (*n*=4). *p< 0.05 compared to SS control mice by two-way ANOVA for multiple comparisons and T-Test. **(B)** Levels of positive Th17 cell subpopulation of CD4 cells in heart homogenate from control (AA) and SCD (SS) mice treated with either vehicle or colchicine. Data are presented as means ± SD and each replicate (*n*=4). **(C)** Immunoprecipitation assay with anti Phospho Tyrosine (PY) and immunoblot with specific antibodies for TGF-β receptor in the hearts of control (AA), sickle (SS) mice treated with either vehicle or colchicine; GAPDH on the corresponding supernatant was used as loading control; Immunoblot analysis with specific antibodies against PDGF-BB, PDGF receptor and the total forms PDGF receptor in control (AA), sickle mice (SS) and sickle mice treated with colchicine. GAPDH was used as loading control. **(D)** Schematic representation of molecular mechanisms involved in cardiac damage and remodeling in sickle cell disease related cardiomyopathy, that were studied in this work, and the effect of colchicine in this model

In the present study, we assessed in detail molecular mechanisms involved in sickle cell related cardiomyopathy and for the first time the treatment option with colchicine aimed to resolve the adverse events caused by a disease progression. Thereby, this work demonstrated a new understanding of the mechanisms leading

to sickle cell disease-associated cardiomyopathy and its management in terms of the beneficial effect of colchicine treatment.

6.6 Perspective and future steps

Based on our data, we plan to:

1. Further evaluate colchicine effects on SCD cardiomyopathy by:
   i) immunohistochemistry to assess heart lymphocyte Th17 infiltration;
   ii) echocardiography to assess diastolic function and cardiac hypertrophy;
   iii) single cell analysis of the cardiac inflammatory infiltrate to characterize leukocyte sub populations contributing to the progression of cardiomyopathy, notably Th17.
2. Moreover, we plan to combine colchicine treatment with other standard therapies for sickle cell disease such as hydroxyurea or innovative and promising approaches such the anti-P-Selectin antibody.

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