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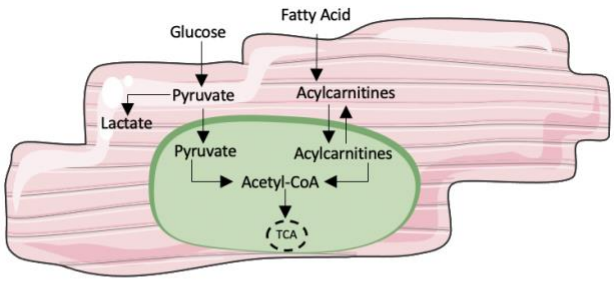

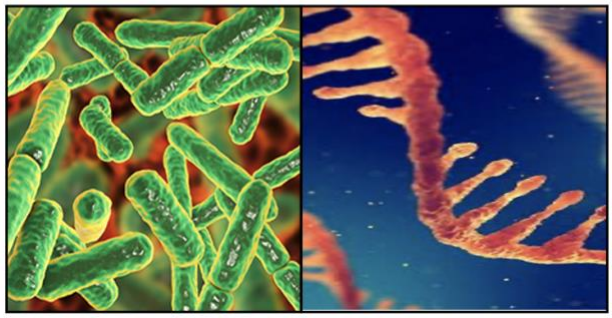

New translational strategies for heart failure treatment

PhD thesis

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New translational strategies for heart failure treatment

PhD THESIS

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Chapter 1

Introduction

a. General introduction

Despite important advances in diagnosis and treatment, heart failure (HF) remains a relentless syndrome with substantial morbidity and dismal prognosis. Although implementation and optimization of existing technologies and drugs may lead to better management of HF, new or alternative strategies are desirable. In this regard, basic science is expected to give fundamental inputsto accomplish these goals, by collecting evidence that may lay the foundations for or allow a better understanding of novel diagnostic, prognostic or therapeutic tools[1]. Here, we discuss our recent basicscience insights with potential clinical impact on HF. In the effort of providing a comprehensive overview, the main domains of translational research in HF are covered: cardiac metabolism, gut microbiota and systemic inflammation and therapeutic strategies (Figure 1)[2].

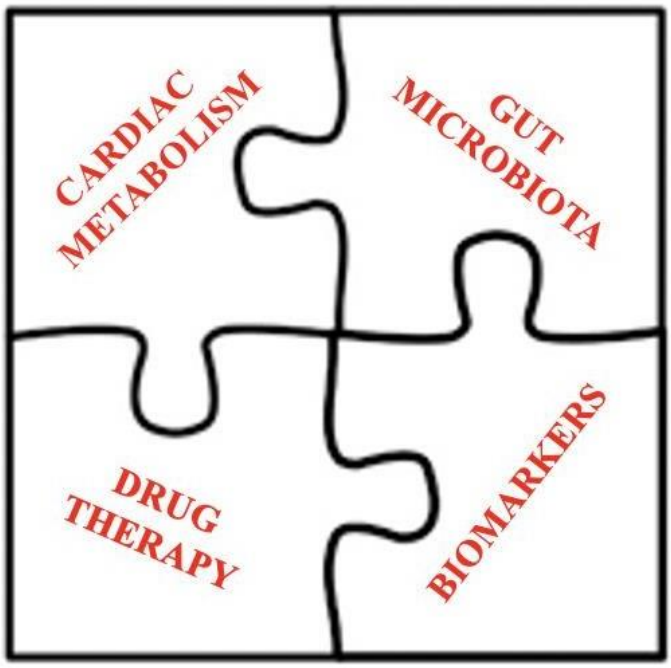


Figure 1. Topics of basic science research in heart failure covered by this thesis

HF is associated with profound metabolic changes that cause a progressive impairment of cardiac muscle function. As a consequence of impaired cardiac metabolism, other processes are activated in the failing heart that further exacerbate the progression of HF. Reduced production of high-energy phosphates might have important implications for both systolic and diastolic function in HF. The heart has a very high energy demand and must continuously produce large amounts of

ATP to sustain contractile function[3]. Thus, the continuous production of ATP must occur to maintain cardiac function. The heart achieves this by metabolizing a variety of fuels (including fatty acids, glucose, lactate, ketones, pyruvate, and amino acids), but primarily fatty acids by mitochondrial oxidative phosphorylation. This process requires large amounts of oxygen, resulting in the heart consuming more oxygen/unit weight than any other organ in the body. Any disruptions in the energy metabolic pathways that produce ATP, or in oxygen supply to the heart, can have catastrophic consequences on cardiac function. As a result, compromised energy production by the heart is an important contributor to most forms of heart disease. Impaired mitochondrial function in the failing heart can occur due to a variety of reasons, including (I) increased reactive oxygen species (ROS) production and dysregulation of mitochondrial Ca^{2+} homeostasis, (II) impairments in mitochondrial dynamics, sustained mitophagy, increased autophagic cell death of cardiomyocytes, and (III) alterations in transcriptional regulation of mitochondrial proteins and increases in post-translational protein modifications. The energy metabolic changes occurring in HF are generally accepted to include reductions in mitochondrial fatty acid oxidation. A compensatory response to reduced mitochondrial oxidative metabolism and ATP production in HF is an induction of glycolysis. However, this increase in glycolysis is insufficient to completely compensate for the energy deficit in HF or to restore cardiac function. Recently, it has become apparent that Ataxia-Telangiectasia Mutated (ATM) protein kinase is well known to play a significant role in oxidative stress and as DNA damage sensor, controlling and regulating cardiomyocyte metabolic homeostasis and protecting against cardiac dysfunction induced by stress. Due to its involvement in these processes, therapeutic activation of ATM could potentially be a novel approach for the prevention or treatment of cardiovascular diseases. Metabolic abnormalities promoting all the mechanisms leading to cardiac dysfunction include resistance to the metabolic actions of insulin in heart tissue (eg, insulin resistance), compensatory hyperinsulinemia, and progression of hyperglycemia. Reduced cardiac output and peripheral vasoconstriction in HF can induce intestinal hypoperfusion, disrupt intestinal barrier function, promote systemic inflammation and affect gut microbiota composition. In addition, gut microbiota-derived molecules, either structural components or bioactive products, can exert remote effects through the activation of different signalling pathways. Mammalian gut microbiota is composed by a diverse selection of microorganisms colonizing the gastrointestinal tract, generating a complex ecosystem with remarkable effects on nutrition, gut epithelial cell health, immunity, and inflammation. Different variables can affect microbiota composition, including genetics, age, diet, environmental factors and several human diseases. Interestingly, various experimental strategies have been described to modulate gut microbiota composition, including administration of antibiotics, probiotics, prebiotics, postbiotics, and faecal microbiota

transplantation, associated to temporary or prolonged restoration of gut function, microbiota composition and immune response. The latest scientific discoveries in the field have led to a significant incremental advance toward potential new cardiovascular therapies. Identifying novel and early-stage biomarkers of cardiovascular diseases is of paramount importance in predicting and preventing the major morbidity and mortality associated with these diseases. About 99% of the human genome do not encode proteins, but are transcriptionally active representing a broad spectrum of non-coding RNAs (ncRNAs) with important regulatory and structural functions. Non-coding RNAs have been identified as critical novel regulators of cell functions and are thus important candidates to improve diagnostics and prognosis assessment. The simultaneous integration of multi-omics approaches including but not limited to genomics, epigenomics, transcriptomics, proteomics, and metabolomics represents a powerful approach for understanding the mechanisms connecting identified genetic variations to cardiovascular diseases with gene causality, where many sources of variability are integrated into statistical models to identify key drivers and pathways that have the largest contribution to the disease[4].

b. Outline of the thesis

The thesis is address three major aspects in heart failure pathophysiology, prognosis and treatment:

I) Metabolic modulation of cardiac function in heart failure.

In the first part of the thesis, I report results in the field of cardiovascular diseases focusing on the role of Ataxia-Telangiectasia Mutated (ATM) in cardiac metabolism during heart failure and how metabolic abnormalities promoting cardiovascular disease as diabetic cardiomyopathy.

II) Role of gut microbiota in cardiovascular diseases.

In the second part of the thesis, I will report 2 research projects focusing on importance of gut function and microbiota composition in the pathophysiology of several cardiovascular diseases, including HF.

III) Novel diagnostic and therapeutic approaches in heart failure.

This section of the thesis is a collection of our research projects aimed at investigating novel diagnostic, prognostic or therapeutic targets for cardiovascular diseases.

Chapter 2

Metabolic modulation of cardiac function in heart failure

2.1 Role of Ataxia-Telangiectasia Mutated (ATM) protein in pressure overload-induced HF

a. Background and study hypothesis

Pressure overload-induced cardiac hypertrophy is associated with increased reactive oxygen species (ROS), inducing DNA damage and activating the protein kinase Ataxia-Telangiectasia Mutated (ATM) (Figure 2). Post-mitotic cells, neurons and cardiomyocytes cannot repair DNA lesions withDNA replication and rely for their survival on efficient sensors and effectors that sense and orchestrate the DNA damage response (DDR)[5,6]. Ataxia Telangiectasia Mutated (ATM) protein kinase is the major sensor of DNA damage and oxidative stress, variously implicated in metabolism[7,8]. Recently, with higher survival rates, metabolic and cardiovascular dysfunctions appear more frequently in *Atm*$^{-/-}$ patients[9]. This latency is evident also in *Atm*$^{-/-}$ mice, which presentat a later age ataxia and cardiovascular alterations[10]. Previous studies on ATM function(s) in heart have produced conflicting results. For example, cardiomyocyte-specific conditional *Atm* knockoutin mice or pharmacological inhibition prevented pressure-overload or cardiac hypertrophy[11]. Also, ATM was shown to protect the heart against diabetic cardiomyopathy[12]. We hypothesized that ATM, as exquisite oxidative stress and DNA damage sensor, controls and regulates cardiomyocytemetabolic homeostasis and protects against cardiac dysfunction induced by transverse aortic constriction (TAC).

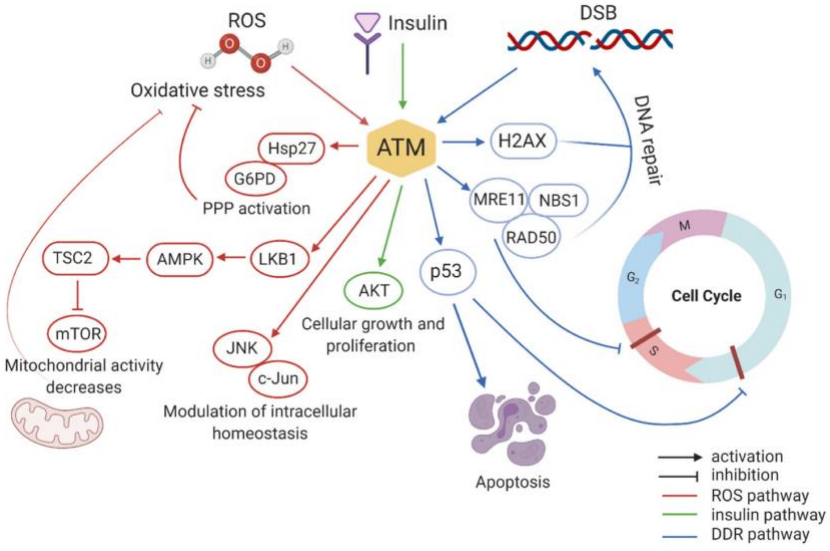


Figure 2. ATM signalling network.

A schematic representation of the different ataxia-telangiectasia mutated (ATM) signalling pathways.

b. ATM protects the heart against heart failure induced by mechanical stress.

To investigate the role of ATM in cardiac metabolism in response to mechanical stress, cardiac hypertrophy was induced by TAC in *Atm*$^{-/-}$ and *Atm*$^{+/+}$ mice. In sham conditions (same surgical procedure without aortic ligation) *Atm*$^{-/-}$ mice appeared healthy by morphometric and histological analysis, although the Left Ventricle Weight/Body Weight (LVW/BW) and cardiomyocyte cross sectional area were higher than the controls (Figure 3 A-B). Furthermore, LVs in *Atm*$^{-/-}$ hearts reprogrammed gene expression (Figure 3 C-E). After TAC, both *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mice displayed higher LVW and LVW/BW ratio (Figure 3 A), *Atm*$^{+/+}$ cardiomyocytes underwent hypertrophy andreprogrammed gene expression, while *Atm*$^{-/-}$ were not further modified by TAC (Figure 3 B-E).Furthermore, LV in *Atm*$^{-/-}$ hearts reprogrammed gene expression. The constitutive stressed phenotype of *Atm*$^{-/-}$ LV suggests that these hearts are more prone to HF after TAC. To this end wemeasured cardiac function by transthoracic echocardiography after TAC in all the groups. After TAC, *Atm*$^{-/-}$ mice displayed a significantly lower fractional shortening (% FS) (Figure 3F). After TAC, ATM inactivation induced a rapid deterioration of cardiac function and heart failure (HF) (Figure 3 F).

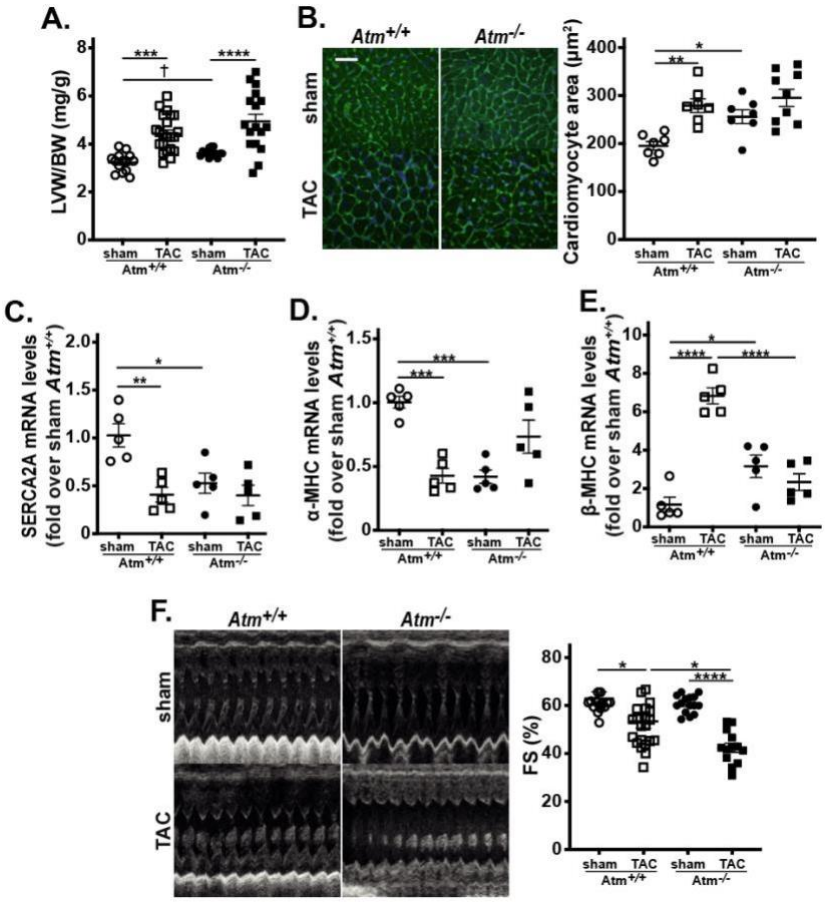


Figure 3. ATM protects against HF induced by mechanical stress.

(A) Left ventricular weight (LVW) to body weight (BW) ratio in $Atm^{+/+}$ and $Atm^{-/-}$ mice after 1w sham orTAC procedures, ($Atm^{+/+}$ sham n=15; $Atm^{-/-}$ sham n=13; $Atm^{+/+}$ TAC n=23; $Atm^{-/-}$ TAC n=17). (B) Left: Representative images of Wheat Germ Agglutinin (WGA) staining of cardiac transversal sections in sham or TAC $Atm^{+/+}$ or $Atm^{-/-}$ left ventricles (LVs). Scale bar: 20 μm. Right: quantitative analysis of WGA from sham or TAC $Atm^{+/+}$ or $Atm^{-/-}$ LVs ($Atm^{+/+}$ sham n=7; $Atm^{-/-}$ sham n=8; $Atm^{+/+}$ TAC n=7; $Atm^{-/-}$ TAC n=9). (C-E) Relative mRNA levels, expressed as fold change over $Atm^{+/+}$ *sham of Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA2) and isoforms of Myosin Heavy Chain (respectively, a-MHC and β-MHC) in sham or TAC* $Atm^{+/+}$ *and* $Atm^{-/-}$ *LVs (*$Atm^{+/+}$ *sham n=5;* $Atm^{-/-}$ *sham n=5;* $Atm^{+/+}$ *TAC n=5;* $Atm^{-/-}$ *TAC n=5). (F) Left: representative echocardiographic M-mode images of LVs from $Atm^{+/+}$ and $Atm^{-/-}$ mice after sham or TAC procedures. Right: % fractional shortening (FS%) in $Atm^{+/+}$ and $Atm^{-/-}$ mice after sham or TAC ($Atm^{+/+}$ sham n=15; $Atm^{-/-}$ sham n=13; $Atm^{+/+}$ TAC n=23; $Atm^{-/-}$ TAC n=17). Data are presented as mean ±SEM. The data were analyzed by Student unpaired t test for each pair of four groups (cross, †, p<0.05) and subsequent multiple comparison were made by two-way ANOVA with Tukey correction *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

c. ATM regulates glucose metabolism and pyruvate trafficking

Since ATM inactivation is broadly associated with altered glucose and lipid metabolism, we hypothesized that ATM modifies cell metabolism to prepare the cell for DNA repair. We analyzed mRNA and protein levels of key enzymes involved in glucose and lipid metabolism in LVs from all the groups. Glycolytic enzyme hexokinase-2 (HK2) was induced by TAC in $Atm^{+/+}$ LVs while in sham $Atm^{-/-}$ LVs, HK2, was constitutively higher than $Atm^{+/+}$ sham, (Figure 4 A). The higher basal levels of HK2 suggest a loss of regulation of this enzyme and an imbalance in glycolytic steps in $Atm^{-/-}$ LVs. To complement and validate this analysis, we performed a targeted metabolomic profiling of LVs from both genotypes during TAC. Pyruvate accumulated in sham or TAC $Atm^{-/-}$ LVs (Figure 4 B), while lactate levels were induced by TAC only in $Atm^{+/+}$ mice (Figure 4 C). High pyruvate levels in $Atm^{-/-}$ LVs suggest a block in pyruvate metabolism linked to mitochondrial dysfunction or import impairment. However, in $Atm^{-/-}$ LVs we found a significant reduction of mRNA levels of the mitochondrial pyruvate carriers 1 (MPC1) and 2 (MPC2), beforeand after TAC (Figure 4 D-E). TAC in $Atm^{+/+}$ hearts altered MPC1/2 ratio and reduced pyruvate entry in the mitochondria. Pyruvate did not accumulate in the cytosol because it was rapidly converted to lactate (Figure 4 B-C). However, notwithstanding reduced carrier levels, a fraction of pyruvate can enter the mitochondria and can be metabolized by pyruvate

dehydrogenase A1 (PDHA1) to acetyl-CoA. PDHA1 protein level was downregulated by TAC in *Atm*$^{+/+}$, not in *Atm*$^{-/-}$ LVs (Figure 4 F), suggesting that ATM during TAC reduces PDHA1 protein level. However, PDHA1 regulation by stress only in part seems dependent on ATM because the levels of the enzyme were constitutively low in *Atm*$^{-/-}$ LVs and were not further downregulated by TAC (Figure 4 F).

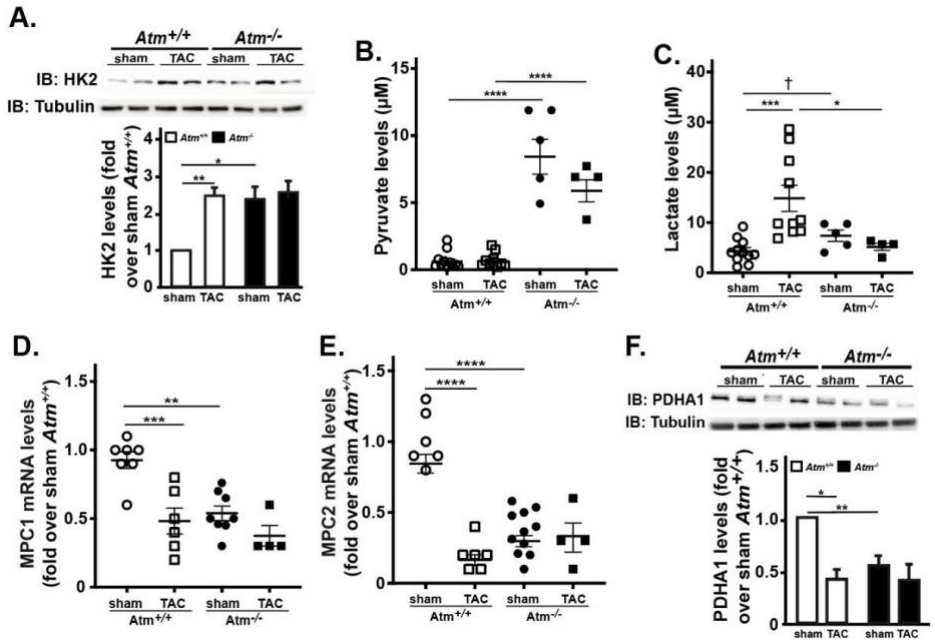


Figure 4. ATM regulates glucose metabolism and Pyruvate trafficking.

(A) Representative western blots and quantifications of Hexokinase-2 (HK2) in left ventricles (LVs) from sham or TAC Atm$^{+/+}$ *and Atm*$^{-/-}$ *mice, (Atm*$^{+/+}$ *sham n=6; Atm*$^{-/-}$ *sham n=6; Atm*$^{+/+}$ *TAC n=6; Atm*$^{-/-}$ *TAC n=6). (B-C) Pyruvate and Lactate (μM) levels in LV samples from Atm*$^{+/+}$ *and Atm*$^{-/-}$ *mice after sham or TAC 1w procedure, (Atm*$^{+/+}$ *sham n=11; Atm*$^{-/-}$ *sham n=5; Atm*$^{+/+}$ *TAC n=8; Atm*$^{-/-}$ *TAC n=4). (D-E) Relative mRNA levels of Mitochondrial Pyruvate Carrier 1 and 2 (MPC1 and MPC2) in left ventricles (LVs) from sham or TAC Atm*$^{+/+}$ *and Atm*$^{-/-}$ *mice (Atm*$^{+/+}$ *sham n=6-7; Atm*$^{-/-}$ *sham n=8-10; Atm*$^{+/+}$ *TAC n=6; Atm*$^{-/-}$ *TAC n=3-4). (F) Representative western blot and quantification PDHA1 protein levels in LVs from sham or TAC Atm+/+ and Atm-/- mice (Atm*$^{+/+}$ *sham n=4; Atm*$^{-/-}$ *sham n=4-5; Atm*$^{+/+}$ *TAC n=4-6; Atm*$^{-/-}$ *TAC n=4-6). Data arepresented as mean ±SEM. The data were analyzed by Student unpaired t test for each pair of fourgroups (cross, †, p<0.05) and subsequent multiple comparison were made by two-way ANOVA with Tukey correction *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.*

d. ATM affects mitochondrial metabolism of energy substrates

Pyruvate accumulation suggests that the glycolytic flux and the subsequent conversion of pyruvate to acetyl-CoA in the TCA cycle were altered in *Atm*$^{-/-}$ LVs. In sham conditions, notwithstanding the reduction of pyruvate mitochondrial transport and oxidative decarboxylation, specific metabolites of Krebs cycle such as succinate and malate accumulated in *Atm*$^{-/-}$ LV. (Figure 5 A-B). Since pyruvate is trapped in the cytosol and PDHA1 protein levels are reduced, in *Atm*$^{-/-}$ LVs, acetyl CoA entering Krebs cycle derives from fatty acids oxidation (FAO). In fact, *Atm*$^{-/-}$ sham LVs were characterized bya remarkable increase of free carnitine, acyl carnitines (AC) containing short, medium and long chainfatty acids (FA) (Figure 5 C-F). FA profiling revealed that ATM deletion increased sham and TAC levels of lauric (12C) and myristic (14C) (Figure 5 G-H). Also, aminoacids (AA), including non-polar, aromatic, basic and acid chain,the majority of which derive from pyruvate and TCA intermediates, accumulated in *Atm*$^{-/-}$ sham LVs(Figure 5 I-K). Serum AA and AC levels were comparable in all different groups of mice. These dataimply that mitochondria in *Atm*$^{-/-}$ LVs are functional, and that altered FAO is the consequence of pyruvate trapping in the cytosol. Downregulation of acetyl-CoA, derived from pyruvate, stimulates FAO, which becomes the main source of mitochondrial acetyl-CoA. Over time, further pyruvate depletion in mitochondria unbalances TCA metabolites and slows down TCA. FAO outpaces the TCA, resulting in accumulation and release of FAO intermediates, such as ACs (Figure 5) The accumulation of FAO intermediates is the ultimate cause of insulin resistance shown in Figure 6.

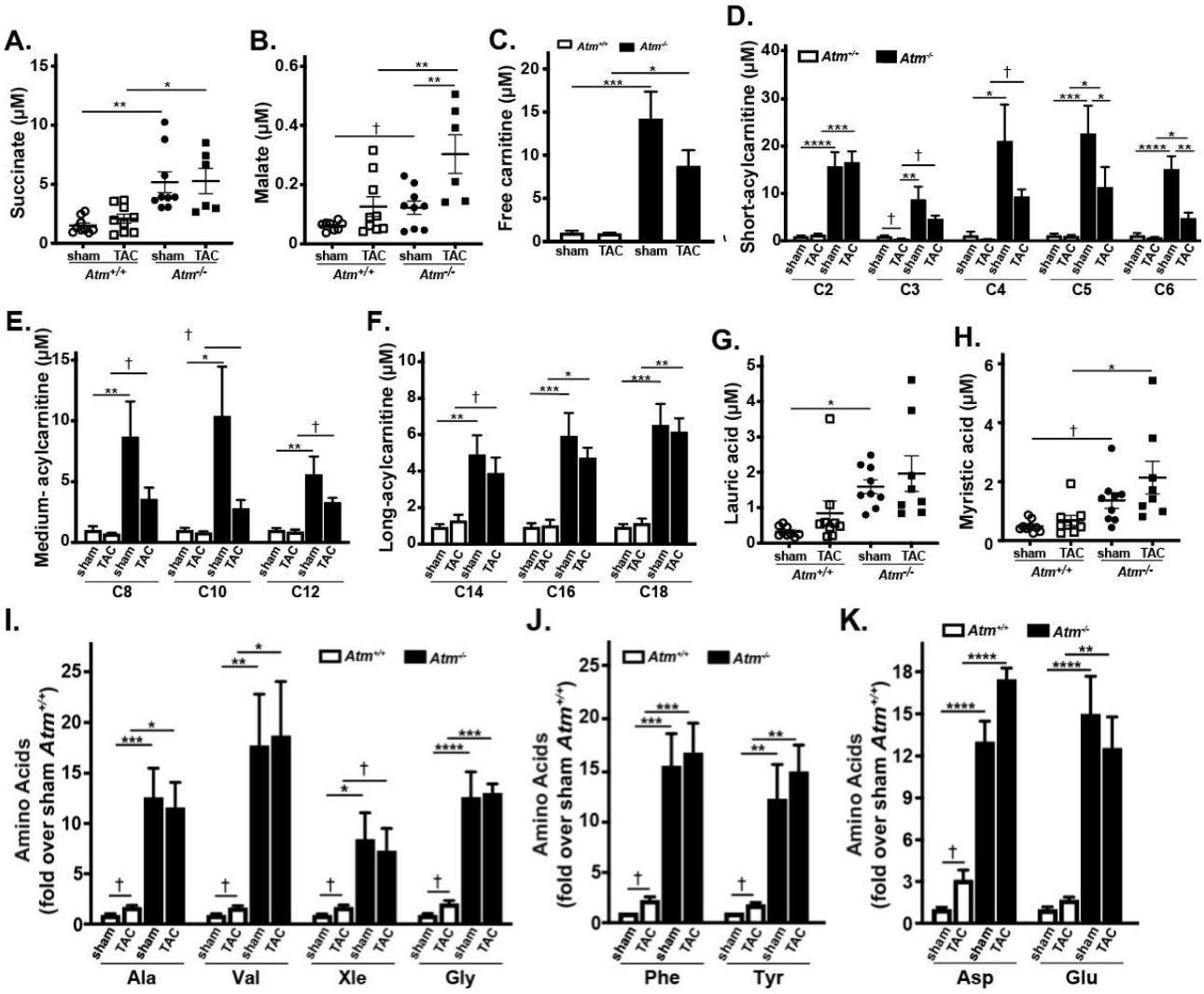


Figure 5. Mitochondrial metabolism of energy substrates

*Succinate (A), malate (B) levels in left ventricles (LVs) from sham or TAC $Atm^{+/+}$ and $Atm^{-/-}$ mice ($Atm^{+/+}$ sham n=9; $Atm^{-/-}$ sham n=9; $Atm^{+/+}$ TAC n=9; $Atm^{-/-}$ TAC n=6). Free carnitine (C), short (D), medium (E) and long (F) acylcarnitine (AC) levels in LVs from sham or TAC $Atm^{+/+}$ and $Atm^{-/-}$ mice ($Atm^{+/+}$ sham n=6; $Atm^{-/-}$ sham n=6; $Atm^{+/+}$ TAC n=6; $Atm^{-/-}$ TAC n=4). Lauric (G), myristic (H), levels in LVs from sham or TAC $Atm^{+/+}$ and$Atm^{-/-}$ mice ($Atm^{+/+}$ sham n=9; $Atm^{-/-}$ sham n=9; $Atm^{+/+}$ TAC n=8; $Atm^{-/-}$ TAC n=8). (I-K) Amino Acid (AA) levels in left ventricle tissue in $Atm^{+/+}$ and $Atm^{-/-}$ mice in sham andTAC procedure mice ($Atm^{+/+}$ sham n=6; $Atm^{-/-}$ sham n=6; $Atm^{+/+}$ TAC n=6; $Atm^{-/-}$ TAC n=4). Data are presented as mean ±SEM. The data were analyzed by Student unpaired t test for each pair of four groups (cross, $p<0.05$) and subsequent multiple comparison were made bytwo-way ANOVA with Tukey correction *$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$. Abbreviations: C0, carnitine; C2, Acetylcarnitine, C3 Propionylcarnitine, C3, Propionylcarnitine; C4, Butyrylcarnitine; C5, Isovalerylcarnitine; C6, Hexanoylcarnitine; C8, Octanoylcarnitine/Caprylylcarnitine; C10, Decanoylcarnitine/Caprylcarnitine; C12, Dodecanoylcarnitine/Laurylcarnitine; C14, Tetradecanoylcarnitine/ Myristylcarnitine; C16, Hexadecanoylcarnitine/Palmitoylcarnitine; C18, Octadecanoylcarnitine/Stearylcarnitine.*

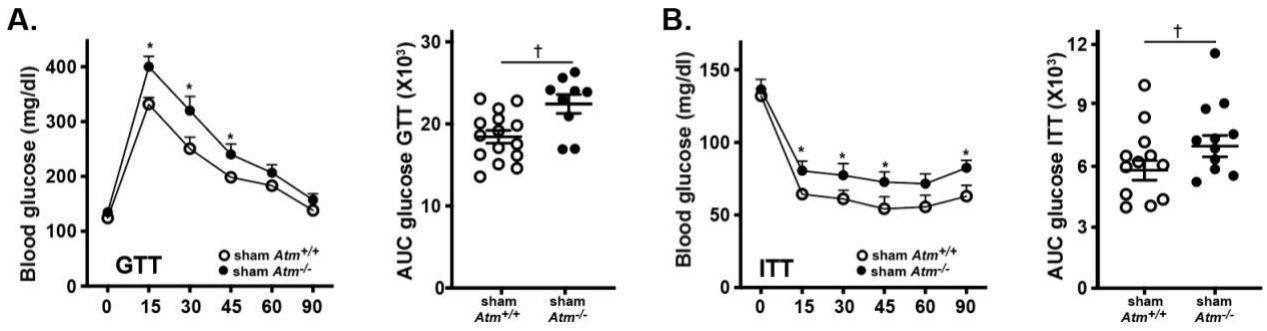


Figure 6. ATM deletion induces insulin resistance

*(A) Blood glucose concentrations and Area Under the Curve (AUC) during intraperitoneal glucose tolerance tests (GTT) in $Atm^{+/+}$ and $Atm^{-/-}$ mice after sham. (B) Blood glucose concentrations and Area Under the Curve (AUC) during intraperitoneal insulin tolerance tests (ITT) in $Atm^{+/+}$ and $Atm^{-/-}$ mice after sham mice ($Atm^{+/+}$ sham n=15; $Atm^{-/-}$ sham n=9). Data are presented as mean ± SEM. The data were analyzed by Student unpairedt test for each pair of four groups (cross, †, p<0.05) and subsequent multiple comparison were madeby two-way ANOVA with Tukey correction *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.*

e. Conclusions

The analysis of wild-type ($Atm^{+/+}$) or $Atm^{-/-}$ mice hearts after TAC shows that ATM protects against heart failure (HF) by rewiring glucose and mitochondrial metabolism of energy substrates, necessaryfor DNA repair. This study demonstrates, for the first time, that ATM regulates the levels of critical enzymes involved in pyruvate metabolism in the heart. ATM inactivation promotes a specific cardiac metabolic signature, with significant accumulation of pyruvate, branched amino-acids, short and medium fatty acids acyl-carnitines. The metabolic block due to ATM inactivation accelerates heart failure in stressed hearts. $Atm^{-/-}$ hearts are extremely vulnerable to HF due to mis-repaired DNA lesions and imbalanced glycolysis. These data explain the conflicting interpretations on ATM role inthe heart[13,14], and provide a novel framework linking DNA damage, metabolism, and HF. Due to its involvement in these processes, therapeutic activation of ATM could potentially be a novel approachfor the prevention or treatment of cardiovascular disease.

Chapter 3

Role of gut microbiota and gut-heart axis in cardiovascular diseases

Several cardiovascular diseases (CVD) have been commonly associated with remarkable changes in gut barrier function and microbiota composition through different, still largely undefined mechanisms, presumably acting at multiple levels[15,16]. A mutual gut-heart crosstalk has been recently proposed in heart failure (HF), even if messengers and underlying mechanisms are still not entirely defined[17]. Reduced cardiac output and peripheral vasoconstriction in HF can induce intestinal hypoperfusion, disrupt intestinal barrier function, promote systemic inflammation and affect gut microbiota composition[18]. The potential roles of specific bacteria in the pathogenesis of cardiometabolic disorders and their therapeutic implications are now starting to be elucidated[19,20]. In addition, gut microbiota- derived molecules, either structural components or bioactive products, can exert remote effects through the activation of different signalling pathways in CVD[21,22,23]. In this context, despite species- specific limitations, experimental systems including animal models are crucial to test for causal connections and provide novel insight into host–microbiota interactions modelling human health and diseases.

3.1 Transverse Aortic Constriction induces gut barrier alterations, microbiota remodelling and systemic inflammation

a. Background and study hypothesis

The murine model of transverse aortic constriction (TAC) is one of the most well-established and widely used preclinical models of pressure overload-induced cardiac hypertrophy and failure[14,16]. It has been recently demonstrated that choline diet and its gut microbe-derived metabolite Trimethylamine N-Oxide (TMAO) exacerbate TAC-induced HF, while nonlethal inhibition ofTMAO production improves cardiac function and remodelling after TAC, clearly indicating that gut microbiota can affect cardiac function and remodelling induced by pressure overload[17,18]. In the present study, we investigated the effects of TAC on intestinal barrier integrity, intestinal and serumcytokines, serum endotoxin levels and gut microbiota composition in C57BL/6 mice. Our results suggest that gut modifications might represent an important variable in the development andprogression of cardiac dysfunction in response to TAC, and support this murine model as a valuabletool to establish the role and mechanisms of gut-heart crosstalk in HF.

b. Intestinal permeability upon TAC leads to systemic LPS translocation and inflammation

TAC induced left ventricular hypertrophy and systolic dysfunction and immediately after TAC, abdominal aortic blood flow was significantly reduced in TAC mice compared to sham, resulting in intestinal hypoperfusion (Figure 7 A). Decreased intestinal perfusion in TAC 1w mice was associatedto a prompt and strong weakening of intestinal barrier, as shown by reduced mRNA expressionof *Ocln* and *Tjp1* and reduced immunostaining of zonula occludens-1 (zo-1) after 1w of pressure overload (Figure 7 B-C).

Colon expression levels of anti-inflammatory cytokine interleukin-10 (IL-10) were significantly reduced in TAC 1 w and TAC 4w colon samples compared to respective sham. Consistent with theseresults, serum levels of lipopolysaccharide (LPS) and proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), were rapidly and persistently enhanced after TAC surgery,while circulating levels of IL-10 were reduced in TAC mice (Figure 8 A-E).

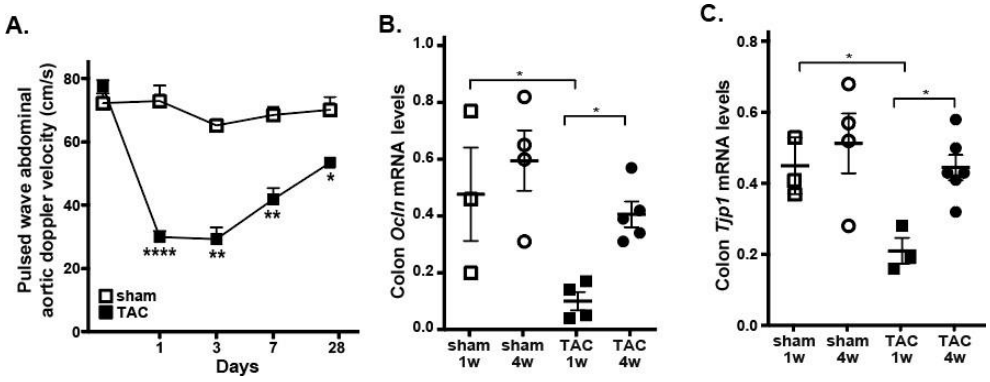


Figure 7. Effects of TAC or sham surgery on intestinal barrier integrity

(A) Abdominal aortic blood flow (cm/s) was evaluated at 1, 3, 7 and 28 days after surgical procedurein sham and TAC mice by Pulsed Wave Doppler (sham n= 6; TAC n = 10). (B-C) mRNA expression levels of occludin (Ocln) and tight junction protein ZO-1 (Tjp1) in colon samples from sham or TAC mice (sham 1w: n =3; sham 4w: n =4; TAC 1w: n= 3–4; TAC 4w: n= 5–6). Data are presentedas mean ±SEM. Statistical significances were assessed using one-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test (A) or Tukey's comparison test as appropriate (B-C).

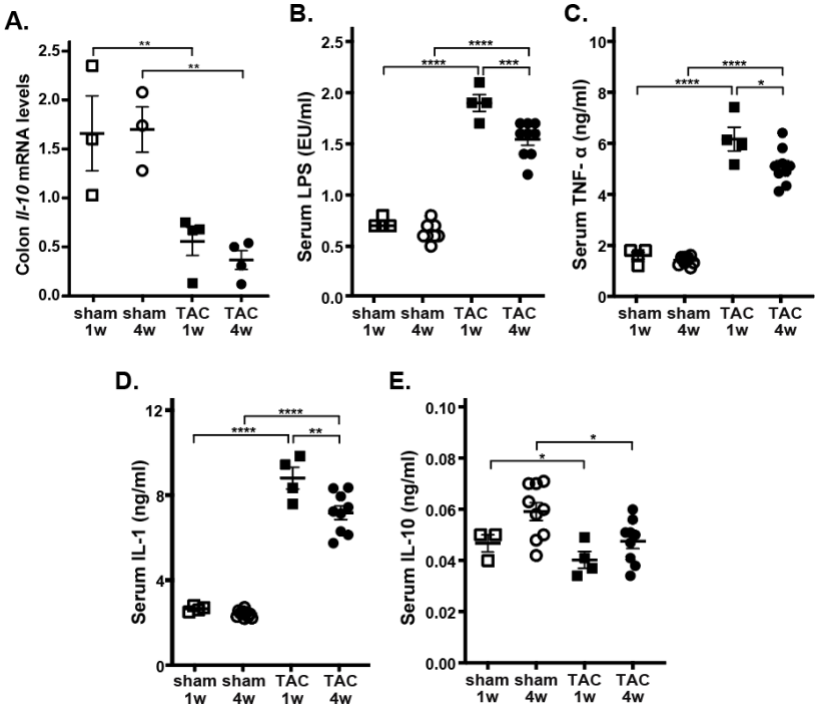


Figure 8. Effects of TAC or sham surgery on inflammation

(A) mRNA expression levels of Interleukin-10 (Il10) in colon samples. Serum levels of (B) lipopolysaccharide (LPS), (C) tumor necrosis factor-α (TNF-α), (D) interleukin-1 (IL-1) and (E) IL-10 in all experimental groups (sham 1w: n =3; sham 4w: n =3-9; TAC 1w: n=4; TAC 4w: n= 4–9). Results are presented as mean ± SEM. Statistical significances were assessed using one-way ANOVA followed by Tukey's comparison test as appropriate.

c. TAC impacts on faecal microbiota composition

Gut barrier integrity is closely linked to gut microbiota composition, and they can be reciprocally affected, especially in response to external pathological causes. Comparison of faecal gut microbiota communities among groups revealed significant changes of bacterial genera inside Actinobacteria, Firmicutes, Proteobacteria and TM7 phyla. Specifically, intergroup differences at genus and specieslevels were analyzed by the linear discriminant analysis (LDA) effect size (LEfSe), identifying the genera *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, unclassified genus (u.g.) of RF32 and u.g. of F16 as characteristic of TAC mice, whereas the genus *Oscillospira* was significantly abundant in TAC mice compared to sham at specific time windows (Figure 9).

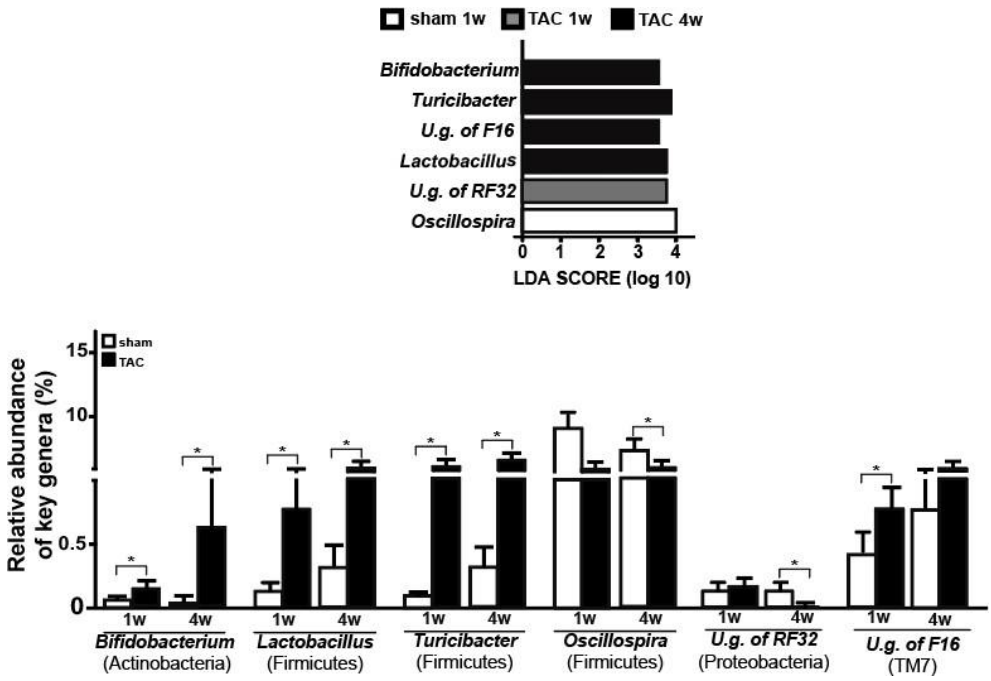


Figure 9. Gut microbiota composition after sham or TAC surgery in mice

*LDA scores (top) and relative abundance (bottom) of key phylotypes discriminating sham and TAC bacterial communities are reported (sham: n = 8; TAC: n = 9). Statistical significances were assessed using LEfSe analysis withalpha values of 0.05 for both Kruskal-Wallis and pairwise Wilcoxon tests and a cutoff value of LDAscore (log10) above 2.0 (*p<0.05 and **p<0.01 vs. correspondent sham).*

d. Conclusions

Our results suggest that gut modifications might represent an important variable in the development and progression of cardiac dysfunction in response to TAC, and support this murine model as a valuable tool to establish the role and mechanisms of gut-heart crosstalk in HF. Collectively, these data indicate that a significant remodelling of specific bacterial species abundance within identified key genera occurs soon after TAC, identifying a clear effect of the surgery on microbiota profiles and, possibly, on microbiota functionality. Alterations of gut structure/function and dysbiosis may represent important elements to be considered in the development or progression of cardiac dysfunction in response to pressure overload induced by TAC. Whether restoration of gut function and microbiota composition might exert a beneficial effect on cardiac remodelling and dysfunction is still unknown and will require further investigations. However, our findings clearly support the murine model of TAC as a valuable tool to establish the importance of gut barrier function and microbiota composition in HF, suggesting novel important avenues of research in this field, including administration of single or defined cocktails of bacterial species to counteract alterations in gut barrier integrity during HF.

3.2 Partial loss of *Akap1* promotes cardiac dysfunction, gut barrier dysfunction and alterations of gut microbiota composition during aging

a. Background and study hypothesis

Mitochondrial A-kinase anchoring proteins (mitoAKAP) encoded by the *Akap1* gene promote Protein Kinase A mitochondrial targeting, regulating mitochondrial structure and function, reactive oxygen species production and cardiomyocyte survival. Whether mitoAKAP levels play a role in cardiac aging, gut barrier integrity and gut microbiota composition is currently unknown. Exist a connectionbetween mitochondria and the gut microbiome provided by reactive oxygen species (ROS). Maintaining a diverse gut microbiome is generally associated with organismal fitness, intestinal health and resistance to environmental stress. In contrast, gut microbiome imbalance, termed dysbiosis, is linked to a reduction in organismal well-being. ROS are essential signalling molecules but can be damaging when present in excess. Increasing ROS levels have been shown to influence human health, homeostasis of gut cells, and the gastrointestinal microbial community's biodiversity. Reciprocally, gut microbes can affect ROS levels, mitochondrial homeostasis, and host health (Figure 10). The aim of this study was to highlight the complex interplay between cardiac dysfunction, gut barrier integrity, gut microbiota composition and aging in young (6-month-old, 6m) and old (24- month-old, 24m) *wild type* (*Akap1*$^{+/+}$) and *Akap1* heterozygous mice (*Akap1*$^{+/-}$).

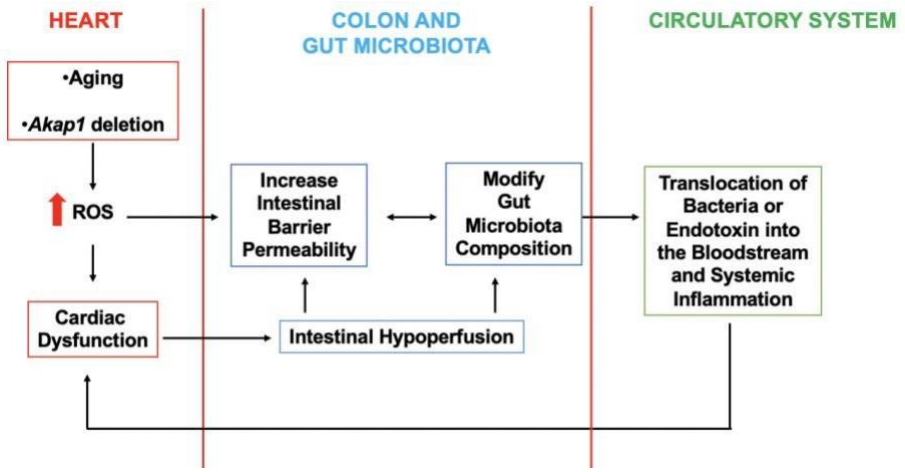


Figure 10. Role of age and Akap1 deletion in heart-gut axis.

b. Partial deletion of *Akap1* gene accelerates cardiac dysfunction and induce colon permeability and systemic inflammation in agedmice.

Cardiac function was noninvasively analyzed by echocardiography in 6m and 24m *Akap1*$^{+/+}$ and *Akap1*$^{+/-}$ mice. Partial loss of *Akap1* accelerated the progression of cardiac dysfunction in 24m mice,as demonstrated by a significantly lower % fractional shortening (%FS) compared to 24m *Akap1*$^{+/+}$ mice (Figure 11).

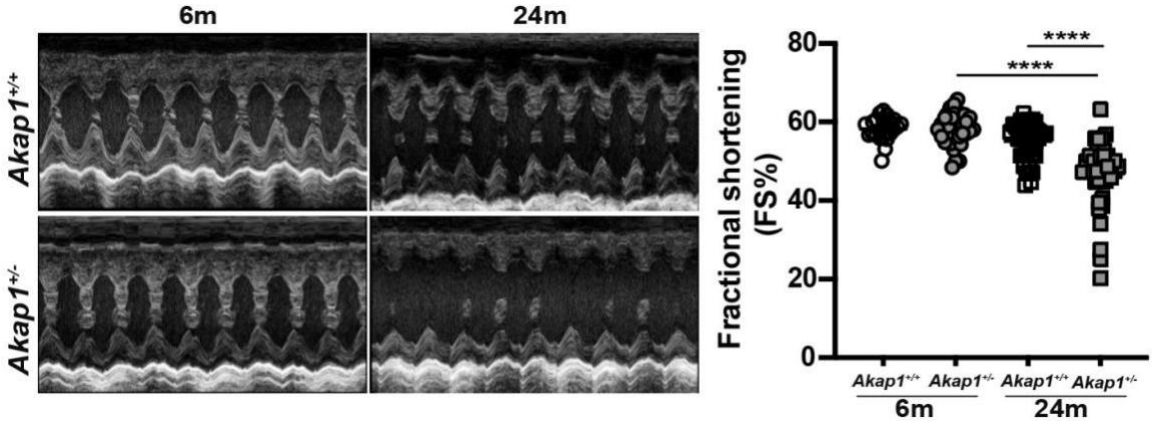


Figure 11. Cardiac function in aged Akap1$^{+/+}$ and Akap1$^{+/-}$ mice.

*Representative images echocardiography (left) and FS% (right) of LV from 6m and 24m Akap1$^{+/+}$ and Akap1$^{+/-}$ mice (6m Akap1$^{+/+}$n =20; 24m Akap1$^{+/+}$ n =20; 6m Akap1$^{+/-}$ n =20; 24m Akap1$^{+/-}$ n =20). Data are presented as mean ±SEM. The data were analyzed by two-way ANOVA with Tukey correction *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.*

Next, the effects of age on intestinal barrier integrity and systemic inflammation were evaluated in *Akap1*$^{+/-}$ mice by Tight junction protein ZO-1 (Tjp1) and Occludin (Ocln) mRNA expression analysisand by inflammatory interleukins analysis, in colon and serum samples. In 24m *Akap1*$^{+/-}$ mice, agingwas associated to enhanced colon permeability, as shown by reduced mRNA expression of *Ocln* and *Tjp1* (Figure 12 A-B). Compared to 24m *Akap1*$^{+/+}$ mice, 24m *Akap1*$^{+/-}$ mice showed long-lasting decrease of colon anti-inflammatory cytokine levels and significant increases of serum levels of bacterial lipopolysaccharide and proinflammatory cytokines (Figure 12 C-I)

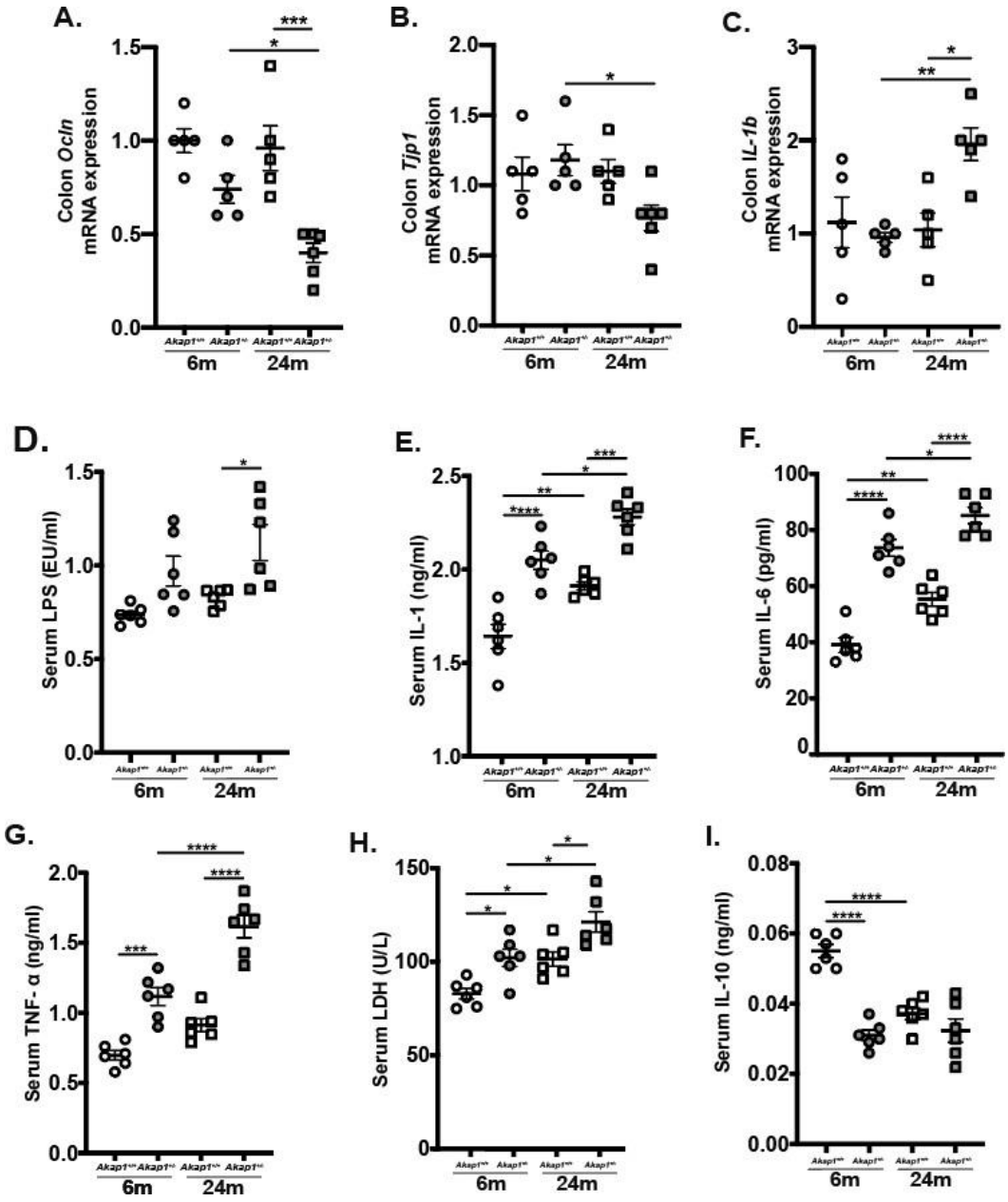
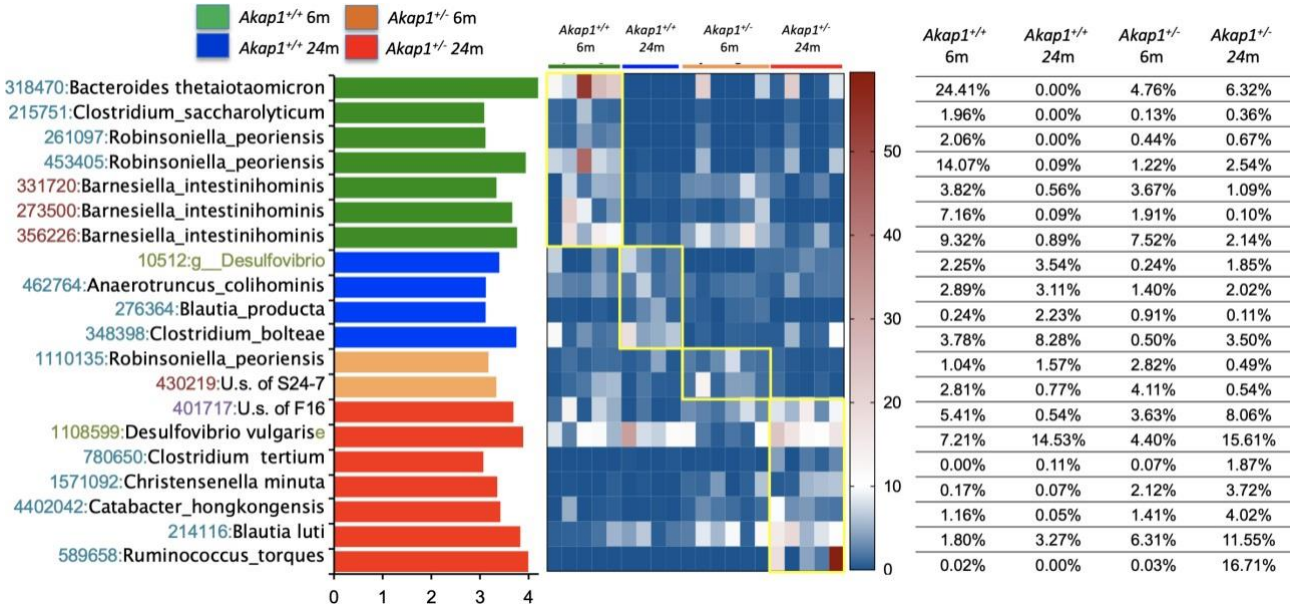


Figure 12. Colon permeability and inflammation in aged Akap1$^{+/+}$ and Akap1$^{+/-}$ mice. *(A, B) mRNA expression levels of occludin (Ocln) and tight junction protein ZO-1 (Tjp1) in colonsamples from sham or TAC mice. (C) mRNA expression levels of Interleukin-10 (Il10) in colonsamples. Serum levels of (D) lipopolysaccharide (LPS), (E) interleukin-1 (IL-1), (F) interleukin-6 (IL-6), (G) tumor necrosis factor-α (TNF-α), (H) lactate dehydrogenase (LDH) and (I) interleukin-10(IL-10) (6m Akap1$^{+/+}$n =5-6; 24m Akap1$^{+/+}$ n =5-6; 6m Akap1$^{+/-}$ n =5-6; 24m Akap1$^{+/-}$ n =6). Results are presented as mean ± SEM. Statistical significances were assessed using one-wayANOVA followed by Tukey's comparison test as appropriate.*

c. Partial deletion of *Akap1* gene impacts on faecal microbiota composition in aged mice.

A principal coordinate analysis of faecal samples suggesting that *Akap1*$^{+/-}$ 24m mice exhibit a different assortment of microbial communities (Figure 13) in all experimental groups. We identified high levels of *Desulfovibrio vulgaris, Clostridium tertium, Christensella minuta, Catabacter hongkongensis, Blautia luti and Ruminococcus torques* in 24m *Akap1*$^{+/-}$ compared to 24m *Akap1*$^{+/+}$.



Akap1+/+ 6m	Akap1+/+ 24m	Akap1+/- 6m	Akap1+/- 24m
24.41%	0.00%	4.76%	6.32%
1.96%	0.00%	0.13%	0.36%
2.06%	0.00%	0.44%	0.67%
14.07%	0.09%	1.22%	2.54%
3.82%	0.56%	3.67%	1.09%
7.16%	0.09%	1.91%	0.10%
9.32%	0.89%	7.52%	2.14%
2.25%	3.54%	0.24%	1.85%
2.89%	3.11%	1.40%	2.02%
0.24%	2.23%	0.91%	0.11%
3.78%	8.28%	0.50%	3.50%
1.04%	1.57%	2.82%	0.49%
2.81%	0.77%	4.11%	0.54%
5.41%	0.54%	3.63%	8.06%
7.21%	14.53%	4.40%	15.61%
0.00%	0.11%	0.07%	1.87%
0.17%	0.07%	2.12%	3.72%
1.16%	0.05%	1.41%	4.02%
1.80%	3.27%	6.31%	11.55%
0.02%	0.00%	0.03%	16.71%

Figure 13. Microbial communities in all experimental groups

Fecal microbiota composition in (green bar) Akap1$^{+/+}$ *6m, (blue bar) Akap1*$^{+/+}$ *24m, (orange bar) Akap1*$^{+/-}$ *6m and (red bar) Akap1*$^{+/-}$ *24m (6m Akap1*$^{+/+}$*n =5; 24m Akap1*$^{+/+}$ *n =4; 6m Akap1*$^{+/-}$ *n =6; 24m Akap1*$^{+/-}$ *n =5).*

d. Effects of faecal microbiota transplantation (FMT) on cardiac function and systemic inflammation in *Akap1*$^{+/+}$ mice.

Considering the microbiota alteration and potential contribution in cardiac function and systemic inflammation observed in in previous experiments, to further define this link, we analyzed whether exogenous bacteria could reshape the gut microbial composition. In the first experiment (Experiment 1) fecal microbiota from *Akap1*$^{+/-}$ 24m mice was transplanted in recipient *Akap1*$^{+/+}$ 6m and *Akap1*$^{+/+}$ 24m mice (Figure 14)

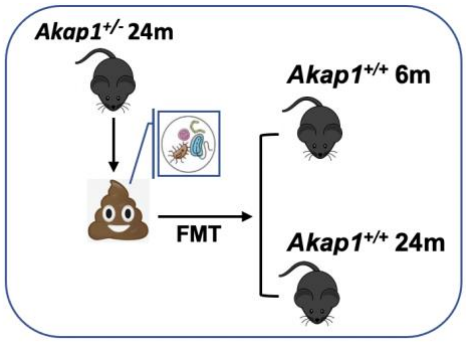


Figure 14. Schematic representation of FMT in experiment 1.

Antibiotics treatment was conducted for 7 days, and the transplant procedure was performed starting on day 8 (1 day after the end of antibiotics treatment) three times per week for 5 consecutive weeks. Fecal samples, for bacterial counts and 16S rDNA sequencing, were collected from all recipient and control mice at each time point that is, prior to antibiotic administration, immediately after the full antibiotic regimen and before the beginning of FMT, and at 5 weeks post-FMT. Transthoracic echocardiography was performed at different times, before the antibiotic treatment, after the end of antibiotic treatment and every week during the fecal transplantation period. Antibiotic solution (0.2 g/L ampicillin, neomycin, and metronidazole, and 0.1 g/L vancomycin) was freshly prepared every day in order to avoid degradation. Fecal samples were freshly prepared on the day of transplantantion administered within 3 h. Fecal supernatant used for FMT was given via single oral gavage (0.1 mL/mice). Mice treated with antibiotic, which did not receive FMT, received oral gavages of vehicle (phosphate-buffered saline).

After 5 weeks, cardiac function was evaluated in all the groups of mice by transthoracic echocardiography. Our results showed that FMT treatment had a substantial impact on cardiac function. Remarkably, Akap1$^{+/+}$ 6m and Akap1$^{+/+}$ 24m mice, that receiving feces from Akap1$^{+/-}$ 24m mice donors, had a significant reduction of fractional shortening (FS%) after 5w, respectively blue and light blue lines, (Figure 15 A) compared to respective vehicle treatment, respectively red and yellow lines. Moreover, FMT in *Akap1*$^{+/+}$ 6m and *Akap1*$^{+/+}$ 24m, from

$^{Akap1+/-}$ 24m donor mice, induced a marked alteration in systemic inflammation as showed by high levels of TNF-A, IL-1, IL- 6, IL-10 and LPS in *Akap1*$^{+/+}$ 6m and *Akap1*$^{+/+}$ 24m compared to respective vehicle treatment (Figure 15 B-F).

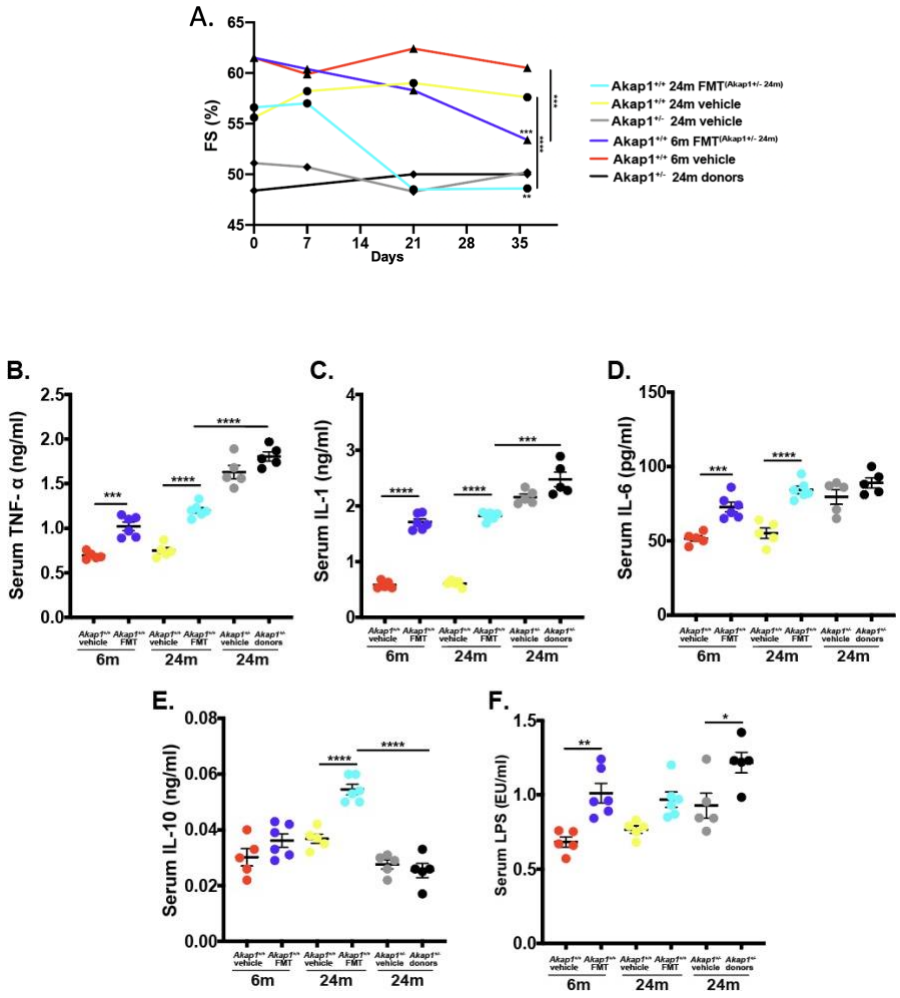


Figure 15. FMT modulates cardiac function and systemic inflammation in Akap1$^{+/+}$ 6m and Akap1$^{+/+}$ 24m

*(A) FS% of LV from FMT experimental mice groups. (B-F) Serum levels of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10) and lipopolysaccharide (LPS)(Akap1$^{+/+}$ 6m vehicle n=5; Akap1$^{+/+}$ 6m FMT n=6; Akap1$^{+/+}$ 24m vehicle n=5; Akap1$^{+/+}$ 24m FMT n=6; Akap1$^{+/-}$ vehicle n=5 and Akap1$^{+/-}$ donors n=5). Results are presented as mean ± SEM. Statistical significances were assessed using one-way ANOVA followed by Tukey's comparison test as appropriate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Furthermore, in the second experiment (Experiment 2) fecal microbiota from *Akap1*$^{+/+}$ 6m mice was transplanted in recipient *Akap1*$^{+/-}$ 24m mice as showed in the Figure 16.

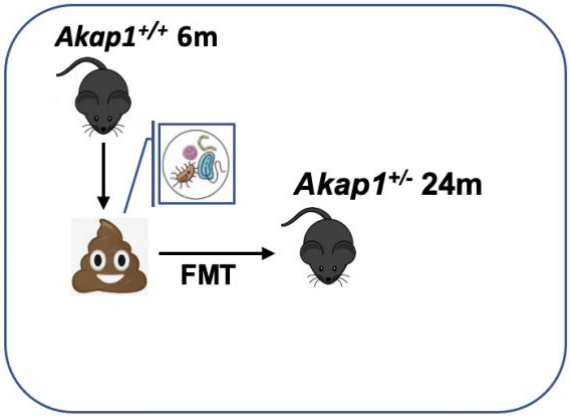


Figure 16. Schematic representation of FMT in experiment 2.

After 5 weeks, cardiac function was evaluated in all the groups of mice by transthoracic echocardiography. Our results showed that FMT treatment induced a significant increase of fractional shortening (FS%) in *Akap1*$^{+/-}$ 24m mice (red line) compare to vehicle *Akap1*$^{+/-}$ 24m mice (blue line) in the Figure 17.

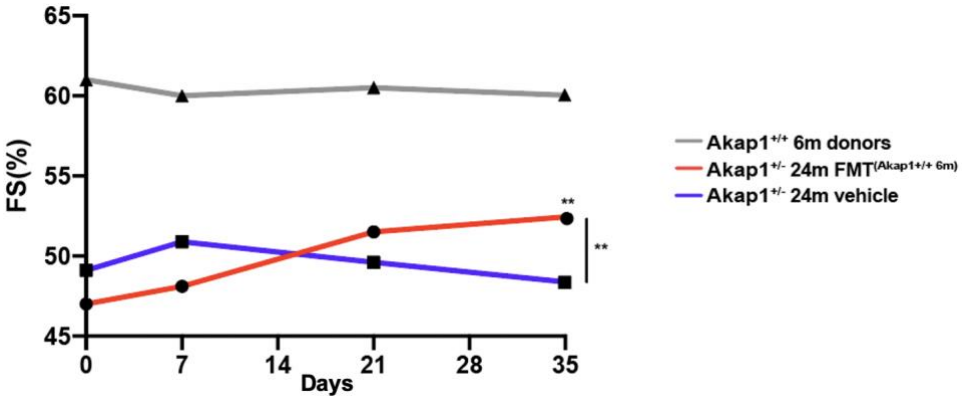


Figure 17. FMT modulates cardiac function in Akap1*$^{+/-}$ *24m mice.

FS% of LV from FMT experimental mice groups. (Akap1$^{+/-}$ *24m vehicle n=5; Akap1*$^{+/-}$ *24m FMT n=5 Akap1*$^{+/+}$ *6m donors n=5) Results are presented as mean ± SEM. Statistical significances were assessed using one-way ANOVA followed by Tukey's comparison test as appropriate and repeated measure ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

e. Conclusions

Partial *Akap1* deletion plays an important role in the progression toward HF and modulates colon permeability, systemic inflammation and gut microbiota composition during aging. Furthermore, weobserved that microbiota manipulation can modulate systemic inflammation and cardiac function. This work highlights the complex interplay between gut microbiota and development of cardiac dysfunction, and characterization of these processes might lead to the development of new diagnosticand therapeutic approaches for cardiac dysfunction.

Chapter 4

Novel diagnostic and therapeutic markers of heart failure

Biomarkers provide a low cost, low risk, and quick turnaround method to confirm or exclude a HF diagnosis, help to establish prognosis in the diagnosis, and more fundamentally, may provide substantial information on the complex pathophysiology that defines the syndrome of HF. Since then, hundreds of biomarkers have been described in HF; among these, the B-type natriuretic peptides (BNPs) remain the gold standard. The term biomarker refers to a broad subcategory of objective biological information, which can be measured accurately and reproducibly.

In order for a HF biomarker to be useful, it should possess certain qualities which we have previously published5 but briefly, the method by which a novel biomarker is judged should be thorough, assays used to measure the novel biomarker should be robust, the biomarker should reflect an important pathophysiologic pathway involved in the HF disease process, the biomarker should provide information other than what is already available by routine physical examination and laboratory evaluation, and finally, the biomarker should add to clinical judgment for understanding diagnosis, prognosis, or management of HF.

Several noncoding RNA molecules, that are closely involved in the transcriptional and post-transcriptional regulation of gene expression, have been demonstrated to be fairly accurate in the diagnosis of HF in small pilot studies. Al though less likely to emerge as a clinical tool, studies of microRNA have helped to identify biological pathways involved in remodelling and HF progression, which may in turn allow for development of novel treatment strategies.

HF is attributed mainly to four underlying conditions: hypertension, coronary artery disease, cardiomyopathy and valvular heart disease; however, genetic causes, particularly in dilated cardiomyopathy, also play an immense role. Still, HF could be partly prevented by improving lifestyle, while an improvement in lifestyle is also suggested when HF has already been diagnosed. Therapeutic lifestyle changes include adherence to a Mediterranean diet or exercise training which are able to reduce the risk of developing HF and improve endothelial function. In recent years, epidemiological studies and clinical trials have investigated the possibility that some dietary supplements and phytochemicals (overall referred to as natural products or nutraceuticals) can contribute to the improvement of HF-related symptoms.

4.1 Small Nucleolar RNA SNORD3A: A Potential New Biomarker and Molecular Player in Heart Failure.

a. Background and study hypothesis

Given the relative inaccessibility of myocardial human tissues, identification of circulating biomarkers mirroring myocardial pathological signalling pathways, especially in peripheral blood mononuclear cells (PBMC) is expected to be extremely relevant. Small Nucleolar RNAs (snoRNAs) have been shown to play important roles in various cellular physiological processes. However, the connection between snoRNAs and pathological dysfunction in the heart or PBMC is still poorly understood. The aim of this study was to identify novel circulating PBMC biomarkers linked to myocardial dysfunction and HF.

b. HF affects Snord3A expression levels.

Myocardial left ventricle (Lv) samples and PBMC were obtained from patients affected by heart failure (HF, n=13) undergoing heart transplantation and control donors (ctrl, n=7) and analyzed by RNA sequencing analysis (RNASeq). RnaSeq analysis identified a small set of genes differentially expressed in the Lv and PBMC from HF patients. To validate the RNAseq assays, expression levels, by real-time PCR analysis of different genes. Among these, SNORD3A was up-regulated in Lv and PBMC samples from HF patients compared to ctrl (Figure 18 A). Then, SNORD3A expression levels were tested in heart failure mice model and *in vitro* H9C2 cell line. Thus, HF was induced in 8-week-old wild type C57BL/6 mice by transverse aortic constriction (TAC, n=7) . Sham-operated mice (sham) were used as controls (n=7). After twelve-weeks (12w) TAC or sham operation, cardiac function was analyzed by echocardiography and Lv and PBMC samples were collected after sacrifice. In murine HF induced by 12w TAC, SNORD3A levels were increased both in the Lv and PBMC samples (Figure 18 B). On the other hand, in cell line, hypoxia was induced in cardiomyocyte cell line. Similarly, SNORD3A expression levels were also significantly increased in H9C2 cells exposed to in vitro hypoxia (Figure 18 C).

In order to test the role of SNORD3A in cardiomyocyte hypoxia, we next drew a sequence-specific antisense oligonucleotide (ASO) that block the function of SNORD3A. H9C2 cardiomyoblasts were transfected with SNORD3A-targeted antisense oligonucleotides (ASO) and cell survival was evaluated. Interestingly, H9C2 transfection with SNORD3A-specific ASO (100nM and 3h hypoxia) significantly reduced hypoxia-induced SNORD3A upregulation and increased hypoxia-induced cell death (Figure 18 D- E).

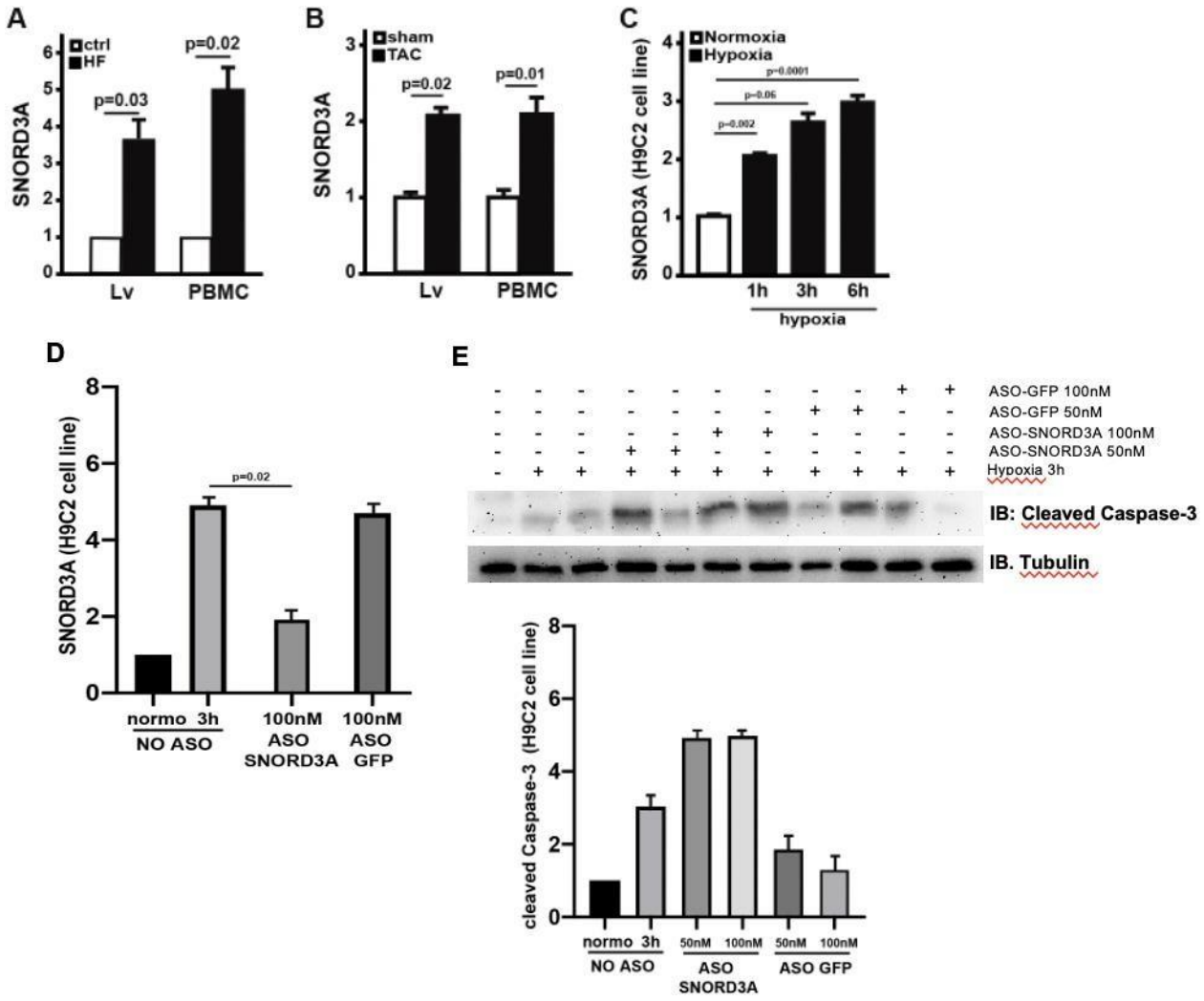


Figure 18. HF affects Snord3a levels.

Snord3A expression in human (A) and wild type C57BL/6 mice (B) in Lv and PBMC samples by rtPCR. (C) Snord3A expression in H9C2 cells. (D) SNORD3A-targeted antisense oligonucleotides (ASO) transfection (ASO-GFP as negative control). (E) Representative western blot and quantification of cleaved caspase-3 in H9C2 cell line after ASO transfection.

c. Conclusions

Our results have identified SNORD3A as a novel possible biomarker in human HF, similarly upregulated in the heart and PBMC, induced by hypoxia in vitro and putative protective role in heart failure.

4.2 Different age-independent effects of nutraceutical combinations on endothelium-mediated coronary flow reserve

a. Background and study hypothesis

Cardiac coronary system includes three different compartments, which are not well anatomically defined: a proximal compartment of epicardial coronary arteries, an intermediate compartment of pre- arterioles and a distal compartment of intramural arterioles, largely corresponding to coronary microcirculation. Dysfunction of one of these compartments can take place even in the absence of alterations of the other compartments. Coronary system function can be tested by transthoracic Doppler echocardiography through the non-invasive assessment of coronary flow reserve (CFR), which is the maximal increase in coronary flow above its resting value for a given perfusion pressure. It is well recognized that, in absence of significant stenosis of the epicardial coronary arteries, CFR represents an accurate expression of coronary microvascular function. Pharmacological agents used to induce maximal endothelium-independent hyperemia mainly include adenosine and dipyridamole. Hyperemia may even be provoked by a completely endothelium-dependent stimulus such as cold pressure test (CPT), which is performed by hand immersion in ice water for few minutes. Endothelium-mediated regulation of coronary vascular tone acts through the production and release of several vasoactive mediators such as nitric oxide (NO). CPT-derived CFR is largely influenced by traditional cardiovascular risk factors and predicts future coronary events.

Nutraceuticals (NUT) are diet supplements that deliver concentrated forms of bioactive agents, isolated or purified from food, that are used in dosages exerting healing properties and are well tolerated (hypoallergenic and digestible). NUT have shown clear beneficial effects on lipid profile. and can be used successfully either as alternatives or in addition to lipid-lowering drugs in patients with mild to moderate hypercholesterolemia. Some components of Nutraceuticals (NUT) such as red yeast rice and Morus alba have demonstrated positive effects on the endothelial function in hypercholesterolemic subjects[24,25]. Our aim was to compare the effects of two different NUT combinations on cold pressure test (CPT) derived coronary flow reserve (CFR) assessed by transthoracic echo-Doppler.

b. Effects of nutraceutical combinations on endothelium-mediated coronary flow reserve.

In a randomized, single-blind study, 28 consecutive patients with a variety of cardiovascular risk factors received NUT A (LopiGLIK®: berberine, red yeast rice powder, and leaf extract of Morus alba) or B (Armolipid Plus®: policosanol, red yeast rice, berberine, astaxantine, folic

acidandcoenzyme Q10). An echo-Doppler exam with evaluation of CFR was performed at baseline, 2 h (acute test) and 30 days after daily NUT assumption. Blood sampling for metabolic profile and platelet aggregometry was performed at baseline and after 30 days of daily NUT assumption. CFR was not significantly modified at the acute test. After 30 days, CFR improved with NUT A ($p < 0.0001$), because of the increase of hyperemic flow velocity ($p = 0.007$), but not with NUT B (Table 1).

Combination A	Time 0 ($n = 14$)	4 weeks CPT CFR ($n = 14$)	*p* value
Resting Systolic BP (mmHg)	124.6 ± 6.3	125.7 ± 8.9	0.865
Diastolic BP (mmHg)	79.3 ± 6.5	80.7 ± 8.0	0.391
Heart rate (bpm)	66.1 ± 13.1	71.2 ± 10.8	0.297
Coronary flow velocity (cm/s)	20.8 ± 4.0	20.4 ± 3.9	0.542
Post CPT Systolic BP (mmHg)	121.7 ± 15.8	125.7 ± 11.6	0.474
Diastolic BP (mmHg)	73.1 ± 9.1	75.5 ± 8.4	0.476
Heart rate (bpm)	69.5 ± 15.0	69.9 ± 7.7	0.521
Coronary flow velocity (cm/s)	28.9 ± 6.2	31.9 ± 6.2	**0.007**
CPT-derived CFR	1.39 ± 0.17	1.59 ± 0.14	**< 0.0001**
Combination B	Time 0 ($n = 13$)	4-weeks CPT CFR ($n = 13$)	*p* value
Resting Systolic BP (mmHg)	133.9 ± 7.8	131.2 ± 9.6	0.269
Diastolic BP (mmHg)	80.6 ± 9.5	79.4 ± 9.7	0.862
Heart rate (bpm)	69.9 ± 10.8	67.3 ± 6.1	0.351
Coronary flow velocity (cm/s)	19.3 ± 4.5	19.2 ± 3.7	0.820
Post CPT Systolic BP (mmHg)	120.8 ± 12.9	123.3 ± 14.9	0.600
Diastolic BP (mmHg)	75.7 ± 7.6	78.3 ± 10.9	0.417
Heart rate (bpm)	69.3 ± 10.1	70.3 ± 7.3	0.531
Coronary flow velocity (cm/s)	27.5 ± 5.2	28.3 ± 5.1	0.435
CPT-derived CFR	1.43 ± 0.12	1.48 ± 0.17	0.323

BP Blood pressure, *CFR* Coronary flow reserve, *CPT* Cold Pressure Test. Boldface= statistically significant *p* value

Table 1. CFR at baseline (Time 0) and after 4 weeks of daily NUT combination intake.

CFR was comparable between the two groups at baseline but became significantly higher after 30 days in NUT A ($p < 0.02$), with a higher CFR percent variation versus baseline ($p = 0.008$) (Figure 19). Total cholesterol and LDL-cholesterol were reduced with both NUT A ($p < 0.001$ and $p < 0.002$, respectively) and B (both $p < 0.02$), whereas platelet aggregation did not significantly change (Table 2).

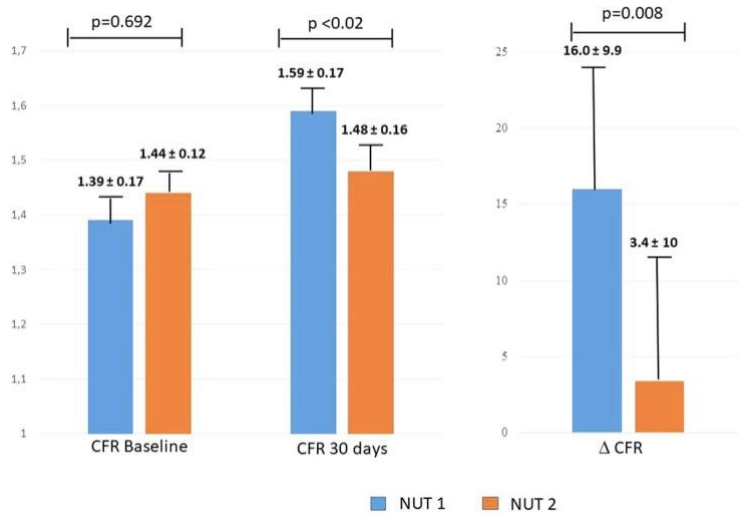


Figure 19. Comparison of baseline and 30 days CFR, and CFR percent changes (30 days versus baseline) in NUT A and NUT B patients.

Combination A	Time 0 ($n = 14$)	30 days CPT-CFR ($n = 14$)	p value
TC (mg/dL)	213.0 ± 39.4	197.1 ± 33.2	**< 0.001**
LDL-C(mg/dL)	138.2 ± 33.1	122.1 ± 25.9	**< 0.002**
Triglycerides (mg/dL)	130.1 ± 70.8	126.2 ± 52.7	0.828
Glycemia (mg/dL)	99.5 ± 24.3	98.2 ± 21.7	0.385
ADP(%)	66.1 ± 18.4	69.0 ± 20.1	0.700
Collagen(%)	60.1 ± 23.7	71.2 ± 25.9	0.124
ADP after insulin stimulation (%)	72.8 ± 12.1	74.5 ± 9.4	0.596
Collagen after insulin stimulation (%)	62.1 ± 26.2	67.7 ± 25.3	0.493
Combination B	Time 0 ($n = 13$)	30 days CPT-CFR ($n = 13$)	p value
TC (mg/dL)	209.6 ± 46.6	196.8 ± 35.6	**< 0.02**
LDL-C (mg/dL)	159.8 ± 42.9	148.5 ± 18.4	**< 0.02**
Triglycerides (mg/dL)	125.8 ± 17.7	103.6 ± 47.6	0.316
Glycemia (mg/dL)	91.6 ± 12.9	90.8 ± 7.4	0.861
ADP(%)	72.2 ± 25.4	77.3 ± 17.1	0.553
Collagen(%)	54.0 ± 39.4	69.8 ± 31.8	0.060
ADP after insulin stimulation (%)	71.5 ± 21.4	79.9 ± 11.0	0.167
Collagen after insulin stimulation (%)	55.4 ± 35.8	61.9 ± 38.6	0.156

ADP Adenosine diphosphate, *CPT* Cold Pressure Test, *LDL-C* Low density lipoprotein cholesterol, *TC* Total cholesterol. Boldface= statistically significant *p* value

Table 2 Blood assays at rest and after 4 weeks of daily NUT combination intake T-test for paired data)

c. Conclusions

The present study demonstrates a relevant effect of a novel NUT combination, LopiGLIK®, on CFR, in comparison with another combination which does not include Morus alba, an extract that has shown a recognized in vivo action on endothelial function[26]. The combination of NUT with dietary counseling has already shown the ability of improving lipid profile, glycemia, diastolic BP and risk scores, and of reducing the prevalence of metabolic syndrome in patients with moderate cardiovascular risk[27]. Our findings open additional new horizons on NUT therapy in blunting or even preventing the endothelial damage and thus the atherosclerotic progression in patients with a variable amount of cardiovascular risk factors, independently of TC levels and of the effect of aging. These results could have interesting implications on the prevention of age-related inflammatory diseases including coronary artery disease.

Chapter 5
Discussion and conclusions

In this long-lasting research journey, we investigated: a) the functional role of ATM protein in the regulation of critical enzymes involved in pyruvate metabolism in the heart and its inactivation accelerates heart failure in stressed hearts; b) the effects and role of gut microbiota composition, gut barrier disruption and systemic inflammation in murine models of cardiovascular disease; c) novel mechanisms in the regulation of cardiac function, novel therapeutic targets and biomarkers in HF. These studies significantly contributed to increasing the knowledge in their specific field and allowed the identification of novel potential targets or the proposal of novel treatments. Further studies will be needed to better explore these issues but these data have great translational potentiality with important clinical implications.

Several metabolic signatures reported in HF such as the switch from FA to glucose oxidation[28], the uncoupling of glycolysis from pyruvate oxidation[29] and PDH inhibition[30], are directly or indirectly dependent on ATM. In the heart, rewiring of glucose and FA metabolism protects cells from mechanical stress by stabilizing TCA, ROS, and by inducing glycolysis and gluconeogenesis. Conversely, Atm-/- LV cannot complete glycolysis, TCA and FAO, for the block of pyruvate metabolism, and they succumb to stress. Overall, DNA damage and stress drive cardiomyocyte hypertrophy by modifying the metabolism and reprogramming gene expression epitomized by switching on the expression of fetal genes. This is a general stress response characterized by metabolic switch from TCA oxidation (adult) to glycolysis (fetal), in which the heart uses lactate as main energy source. In HF, switching to glycolysis is an essential protective reaction of post-mitotic cells against HF[31,32]. Metabolic impairments play an important role in the development and progression of heart failure. The use of metabolic modulators, the number of which is steadily increasing, may be particularly effective in the treatment of heart failure. Recent evidence suggests that modulating cardiac energy metabolism by reducing fatty acid oxidation and/or increasing glucose oxidation represents a promising approach to the treatment of patients with heart failure. Increased understanding of the role of systemic and cardiac metabolism impairments in heart failure not only generates new pathophysiological concepts, but also stimulates the search for new therapies for patients with heart failure.

The combination of ATM and DNA-PKcs activation in cardiomyocytes, is indicative of DSBs. Collectively, there is a growing body of evidence suggesting that DDR is an important regulator of both physiological and pathological cardiomyocyte growth. In summary, we demonstrate that

pressure overload induces DNA DSBs in cardiomyocytes, resulting in activation of ATM. Importantly, our results suggest that disruption of DDR through pharmacological or genetic loss of ATM function can modulate pressure overload–induced cardiomyocyte hypertrophy.

In the present study we demonstrate that several murine models of cardiac disease induce prompt and strong weakening of intestinal barrier integrity, decrease of colon anti-inflammatory cytokine levels, increase of serum levels of LPS and proinflammatory cytokines, and significant differences in fecal bacterial genera inside Actinobacteria, Firmicutes, Proteobacteria and TM7 phyla. These dramatic alterations of gut barrier were associated with remarkable changes in gut microbiota composition. It is not surprising that significant interest is focused on the roles of the human gut microbiota, in cardiovascular disease and metabolic disorders, including heart failure. The gut microbiota plays a critical physiologic and metabolic role in the human body. The gut microbiota could be regarded as an endocrine organ because it not only releases its own products but also metabolizes host metabolites and external nutrients into hormone-like signals, which impact both normal physiologic processes and chronic diseases[33]. Our findings clearly support these murine models as valuable tools to establish the importance of gut barrier function and microbiota composition in HF, to address the mechanisms and mediators of gut-heart crosstalk, and to test novel potential therapeutic strategies. Currently, heart failure remains a major health burden. The rapid development of high-throughput sequencing technology enables us to uncover the previously unappreciated complexity of the gut microbiome. Since Wang and Tang et al's impressive research thoroughly revealed the interplay between gut microbes and atherosclerosis through the TMA/TMAO pathway, it has gradually become consensus that the gut microbiota contributes to cardiovascular pathophysiology via multiple metabolic and physiologic pathways. Through the identification of bacterial metabolites, it is possible for us to explore numerous microbial pathways that may be involved in the pathogenesis of cardiometabolic disorders and search for potential biomarkers for diagnosis and treatment. Except for the many clinical studies that have already demonstrated an association between TMA/TMAO and adverse outcomes of patients, a few studies based on 16S or metagenomics sequencing have discovered a reduction in SCFA-producing bacterial species in patients with heart failure, especially some butyrate-producing species such as *F. prausnitzii* and *E. rectale.* In addition to being major energy substrates of gut epithelial cells, SCFAs play essential roles in the maintenance of host glucose homeostasis and the immune system. A shift in the gut microbiota into a composition lacking in SCFA-producing bacteria might be a notable characteristic for patients with heart failure. Future studies are needed to explore the deep correlations between microbes, SCFAs and host cardiovascular health. By modulation of gut microbiota composition and function through diet,

pre/probiotics, FMT, and microbial enzyme inhibitors, it may become feasible for us to alter metabolic profiles in a preferred direction that is beneficial for host health in the long term. Transplantation of a defined group of bacteria or utilization of special microbial enzyme inhibitors, can probably adjust blood levels of biologically active microbial-derived metabolites by modulating gut microbial compositions or targeting specific microbial pathways, thus achieving a more personalized and accurate therapeutic intervention. However, neither approach has been studied to date in patients with heart failure. Future research is required to complement this gap. Finally, the use of pre/probiotics has shown great potential in treating heart failure. However, most studies have focused on a correlation between the oral uptake of probiotics and changes in heart failure phenotypes; only a few studies have explored the variations of gut microbial compositions and functions brought about by intervention with pre/probiotics, let alone the underlying metabolic and physiologic mechanisms. Pre/probiotics remain a cost-effective and practical option for intervention that is an area of active investigation, but mechanistic understanding is strongly needed. Although several studies revealed significant correlations between either the gut microbiota composition or their derived metabolites and the phenotypes of heart failure, the sample size of each study is not large enough. Heart failure is the end stage of cardiogenic diseases and is usually accompanied by multiple complications. Many factors including etiology, complications, drugs, host genetic heterogeneity, and lifestyles may contribute to confounding effects in the clinical study. Therefore, well-designed, prospective, and longitudinal clinical studies based on large cohorts are still needed to reveal the actual transformation of the gut microbiota composition and metabolic profile in heart failure. Cardiac disease has been a burden throughout history. With improved quality of life and prolonged life expectancy, the prevalence of cardiac diseases currently continues to escalate, which means that more people will enter the stage of heart failure. More innovative diagnostic and therapeutic approaches for heart failure are urgently in demand. As we gradually gain a deeper understanding of gut microbiota and heart failure interplay, the question of how to bring microbial information into clinical practice remains a major challenge. Of course, high-throughput technologies including 16S and metagenomics sequencing can provide profound information about a single patient's gut microbiota compositions, but such technologies are quite expensive and no evidence clearly clarified their utility in clinical practice as yet. Instead, studying metabolic profiles in blood and urine may be a practical way to guide personalized interventions. Undoubtedly, further investigations to explore the translational potential of mechanism research and the clinical application values of multiple therapeutic interventions are necessarily required.

HF still represent the number one cause of death worldwide. Cardiac-specific *in vivo* modulation of ncRNAs exhibit the potential to ameliorate cardiac dysfunction or diminish pathological

progression in the diseased heart, potentially making them new targets for the treatment of HF. NcRNAs may represent therapeutic targets, provided their expression can be modulated *in vivo*. The need for developing novel therapeutic strategies remains a major challenge in cardiovascular medicine. Here, we highlight the importance to identify novel circulating biomarkers linked to myocardial dysfunction and HF. In particular, the acquisition, integration and analysis of Big Data represents a remarkable scientific opportunity to improve the knowledge of biological phenomena and molecular networks activated in the heart by pathological overloads and to identify new molecular targets. Silencing of RNA molecules can be achieved by the use of sequence-specific antisense oligonucleotides (ASO) or RNA interference (RNAi) methods. Antisense drug therapies have already been applied in clinical trials targeting protein-coding mRNAs with one compound already on the market for the treatment of familial hypercholesterolemia (Mipomersen) and another approved by the Food and Drug Administration to treat Duchenne muscular dystrophy (Eteplirsen). In contrast to protein-coding mRNAs, ncRNAs may exert different functions with respect to their subcellular localization (nucleus or cytoplasm). This needs to be considered for the general targeting strategy. SiRNAs, for example, mainly function in the cytoplasm, therefore may be less effective against nuclear localized ncRNAs. In addition to subcellular localization, tissue and/or cell type-specific delivery of antisense therapeutics is crucial for targeted ncRNA modulation in different HF. In summary, a vast number of non-coding RNA are dynamically regulated upon initiation and progression of HF. Many have important biological functions and/or have the potential to serve as a novel class of circulating biomarkers. Several *in vivo* experiments have revealed that modulation of non-coding RNA offers a promising new therapeutic approach to treat cardiovascular diseases, albeit the silencing or overexpression approaches still require further refinements. Nevertheless, large screening approaches are often performed in animal models of HF and non-coding RNAs are not always conserved among species but this is a prerequisite for clinical translation. However, as the field of non-coding RNAs as potential therapeutic targets is still in its infancy it is not unlikely that in the near future non-coding RNAs will emerge as valuable new tools for the treatment of numerous diseases, including HF. Currently, GapmeRs are the most promising class of ASOs used for pharmacological silencing of ncRNAs in vivo, as they are able to enter the nucleus, thus, enable targeting of nuclear transcripts as well. GapmeRs consist of a DNA core flanked by two locked nucleic acids (LNA) sequences complementary to the target mRNA or ncRNA sequence. By chemically 'locking' the ribose backbone of the nucleotide structure, LNAs display a higher stability, target specificity and RNase H activation potential resulting in enhanced knockdown efficiency[34]. To date, no clinical trials targeting ncRNAs have been performed. This might be due to the relative novelty of ncRNAs been regarded as potential therapeutic targets compared

to proteins. However, therapeutic GapmeR injections have successfully been used to modulate ncRNAs in animal models of pressure overload[35] and MI[36]. In all of the mentioned studies the authors presented remarkably improved cardiac function upon therapeutic intervention, stressing the great potential of antisense drugs therapeutically targeting ncRNAs. Nevertheless, the use of LNA oligonucleotides may be associated with hepatotoxicity[37], highlighting the need for further chemical refinement of this novel class of drugs.

List of abbreviations

HF heart failure

ATM Ataxia-Telangiectasia Mutated

ROS reactive oxygen species

TAC transverse aortic constriction

SHAM sham operation

AGE glycated end-products

DM diabetes mellitus

HFpEF preserved ejection fraction

HFrEF reduced ejection fraction

CVD cardiovascular diseases

IL-10 interleukin-10

Tjp1 Tight junction protein ZO-1

Ocln Occludin

TNF-alpha Tumor Necrosis Factor-alpha

LPS Lipopolysaccharide

IL-1 Interleukin-1

LDA linear discriminant analysis

LEfSe effect size

mitoAKAP Mitochondrial A-kinase anchoring proteins

OTUs Operational Taxonomic Units

FMT fecal microbiota transplantation

NGS next-generation sequencing

ER Endoplasmic reticulum

PBMC peripheral blood mononuclear cells

snoRNAs

LV left ventricle

RNASeq RNA sequencing analysis

ASO antisense oligonucleotides

NUT Nutraceuticals

CPT cold pressure test

CFR coronary flow reserve

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PUBLICATIONS

Publications

-Boccella N, et al. *"Transverse Aortic Constriction Induces Gut Barrier Alterations, Microbiota Remodeling and Systemic Inflammation"*. Scientific reports 2021 Apr 1;11(1):7404.
-Ilardi F, et al. *"Impact of Chronic Kidney Disease on Platelet Aggregation in Patients with Acute Coronary Syndrome"* Journal of Cardiovascular Medicine 2020 Sep;21(9):660-666.
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-Paolillo R, et al. *"ATM Protects Heart from Stress-induced Failure by Rewiring Glucose and Lipid Metabolism"* . Nature metabolism (under reviewer)
-Piccolo R, et al. *"Platelet inhibition with Ticagrelor 60 mg versus 90 mg Twice Daily in Elderly Patients with Acute Coronary Syndrome: Rationale and Design of the PLINY THE ELDER trial"*. (under reviewer)
-Paolillo R, et al. *"Small Nucleolar RNA SNORD3A: A Potential New Biomarker and Molecular Player in Heart Failure"*. (in preparation)
-D'Apice S,et al. *"Partial loss of Akap1 promotes cardiac dysfunction, gut barrier dysfunction and alterations of gut microbiota composition during aging"* .(in preparation)

ORGANISATIONAL SKILLS

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Methods in cellular and molecular biology, biochemistry clinical, including Extraction of DNA / RNA, PCR, Electrophoresis, western blot, Rea-time PCR, immuno-preciptation.

Management of transgenic animal lines, job with animal model.

COMMUNICATION AND INTERPERSONAL SKILLS

Communication and interpersonal skills

Natural inclination and ability to work in a team, with people of different ethnicity, culture and language. Ability to speak in public, having exposed my university projects to an audience of students. Determination, autonomy, flexibility and a strong propensity to human relationship.

PRESENTATIONS

Presentations

- ESC Congress 2021:
Small Nucleolar RNA SNORD3A: A Potential New Biomarker and Molecular Player in Heart Failure. Paolillo R, D'Apice S, Schiattarella G.G, Holley C.L, Esposito G, Perrino C.
Partial loss of Akap1 promotes cardiac dysfunction, gut barrier dysfunction and alterations of gut microbiota composition during aging. D'Apice S, Paolillo R, Boccella N, Coretti L, Lama A, Avvedimento M, Esposito G, Lembo F, Perrino C.
-81° Congresso Nazionale della Società Italiana di Cardiologia
Ataxia-telangiectasia mutated protein protects cardiac cells from stress by rewiring glucose metabolism. Paolillo R, Caterino M, D'Apice S, Boccella N, Pezone A, Pirozzi M, Lombardi A, Gentile A, Esposito G, Ruoppolo M, Avvedimento VE, Perrino C.
Disfunzione Cardiaca associata all'invecchiamento e alla permeabilità intestinale in topi con delezione parziale del gene Akap1. D'Apice S, Paolillo R, Coretti L, Boccella N, Lama A, Avvedimento M, Mollica MP, Mattace Raso G, Esposito G, Lembo F, Perrino C.
- ESC Congress 2020:
Ataxia-Telangiectasia Mutated protein protects cardiac cells from stress by rewiring glucose metabolism. Paolillo R, Caterino M, D'Apice S, Boccella N, Pezone A, Pirozzi M, Lombardi A, Gentile A, Esposito G, Ruoppolo M, Avvedimento VE, Perrino C.
Transverse Aortic Constriction Impairs Intestinal Barrier Integrity, Promotes Inflammation and Alterations in Gut Microbiota Composition.
Boccella N, Paolillo R, Coretti L, D'Apice S, Lama A, Schiattarella GG, Cuomo M, Cavaliere G, Mollica MP, Mattace Raso G, Esposito G, Lembo F, Perrino C.
80° Congresso Nazionale della Società Italiana di Cardiologia
Ataxia Telangiectasia Mutated (ATM) ProteinKinase Regulates Cardiac Metabolism And Remodeling. Paolillo R, Caterino M, D'Apice S, Boccella N, Pezone A, Pirozzi M, Lombardi A, Gentile A, Esposito G, Ruoppolo M, Avvedimento VE, Perrino C.
Transverse Aortic Constriction Impairs Intestinal Barrier Integrity, Promotes Inflammation and Alterations in Gut Microbiota Composition.
Boccella N, Paolillo R, Coretti L, D'Apice S, Lama A, Schiattarella GG, Cuomo M, Cavaliere G, Mollica MP, Mattace Raso G, Esposito G, Lembo F, Perrino C.
-79° Congresso Nazionale della Società Italiana di Cardiologia
-78° Congresso Nazionale della Società Italiana di Cardiologia
-ESC Congress 2018

COURSES

Courses

-Prevention of risks in the laboratory
-Basic course on the use of statistics in Biomedical research

List of Publications

Partial loss of Akap1 promotes cardiac dysfunction, gut barrier dysfunction and alterations of gut microbiota composition during aging. D'Apice S, **Paolillo R**, Coretti L, Boccella N, Lama A, Avvedimento M, Mollica MP, Mattace Raso G, Esposito G, Lembo F, Perrino C. (in preparation)

"Small Nucleolar RNA SNORD3A: A Potential New Biomarker and Molecular Player in Heart Failure". **Paolillo R**, D'Apice S, Schiattarella GG, Holley CL, Esposito G, Perrino C. (in preparation)

"ATM Protects Heart from Stress-induced Failure by Rewiring Glucose and Lipid Metabolism". **Paolillo R**, Caterino M, D'Apice S, Boccella N, Pezone A, Pirozzi M, Lombardi A, Gentile A, Esposito G, Ruoppolo M, Avvedimento VE, Perrino C. Cell Metabolism (under reviewer)

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