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PhD thesis

Unveiling cardiac IL-33/ST2L pathway deregulation in animal model of obesity and cardiovascular disease

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Table of contents

Abstract

Obesity has become an increasing public health issue worldwide; it is estimated than over 65% of American adult population is overweight and 35% are actually obese. Criteria for obesity definition is traditionally linked to Body Mass Index (BMI). Although an important correlation exists between BMI and body fat deposit, the BMI criteria cannot actually predict body composition in any given individual due to variability determined by age, sex, race and ethnicity. Besides the weight of an individual, both fat localization and type represent more important estimators of cardiovascular disease. Importantly, the accumulation of visceral fat – more than subcutaneous fat – is linked to cardiovascular disease severity and mortality. Among ectopic fat depots, pericardial and epicardial fat accumulation are intriguingly important. Epicardial adipose tissue (EAT) represents a biological active tissue that secretes adipokines known to promote low-grade inflammation and diabetic vascular complications. The cause is due to the shared blood supply between EAT and myocardium, as indeed EAT provides the 50–70% of the energy used for contraction trough the release of free fatty acids (FFAs). It is intuitive that molecular/metabolic alteration of EAT will be reflected on its secretome and on cardiac metabolism. EAT thickness seems to be associated to ventricular myocardial mass and importantly to myocardial steatosis. A plethora of works highlighted the role of IL-33 and its receptor ST2 within cardiac dysfunction and inflammation associated with obesity. Once secreted in activated or damaged cells, IL-33 modulates the functions of various immune cells through ST2 binding, affecting the proliferation of T cells, macrophages, and innate lymphoid cells. Accordingly, IL-33/ST2 pathway is involved in lipid metabolic diseases and - following its role as immune sensor to infection and stress – it is linked to pro-fibrotic remodeling of myocardium and more in general in cardiovascular diseases. Our group demonstrated in coronary artery disease (CAD) a direct correlation between EAT thickness and IL-33/ST2 signalling imbalance further highlighting a role for EPAC protein involved in cAMP signal transduction

In this PhD Project we aimed to further investigated the IL-33/ST2 effects on cardiac remodeling in obesity, focusing on the molecular pathway that links adipose/cardiac-derived IL-33 to development of fibrosis or hypertrophy. We choose Zucker Fatty rats (ZF) and Zucker Diabetics Fatty rats (ZDF) to overcome the problems linked to tissue availability and to the homogeneity of human EAT samples that need to be collected during cardiac surgery. Since genetic animal models do not fully recapitulate human pathology, we developed *in*

vitro models to mimic adipose and myocardial relationship in vitro. Indeed, we evaluated the effects of visceral adipose tissue (VAT)-derived cellular medium in the modulation of adipose secretome and how they affect myocardial gene expression. Finally, we compared obtained results with those derived from Diet induced obesity (DIO) mice, a naturally occurring model of obesity that reliably resembles human disease. Following both molecular and proteomic analysis, we demonstrated a dysregulation of IL-33/ST2 signalling in both adipose and cardiac tissue, where they affected myocardial gene expression and determined a pro-fibrotic signature. In Zucker rats, pro-fibrotic effects were counteracted by ghrelin-induced IL-33 secretion, whose release influenced transcription factor expression (such as MEF2a), but also increased sST2 and not cardioprotective ST2L form. In this context we observed a reduction of EPAC signalling, that is promoted by VAT secretome and linked to ST2 isoforms balancing regulation. Similar results were obtained in ZDF rats, as both models did not develop evident alteration of cardiac architecture, although we described the increase of the pro-fibrotic signature. To avoid the genetic bias linked to leptin mutation and ghrelin up-regulation we repeated our analysis in DIO mice that fully recapitulates human obesity and presents gradual appearance of hyperglycaemia and progression of metabolic syndrome. Thanks to the analysis of cardiac proteome, we observed an enrichment in proteins and networks involved in extra-cellular matrix remodeling and ventricular function. Interestingly, IL-33 was considered among the possible up-stream regulators of this process. In conclusion, this PhD project demonstrated a dysregulation of IL-33/ST2 signalling in obesity, that directly correlate with Epac expression in ZF rats. The alteration of these pathways in adipose tissue could influence IL-33/ST2 expression and hypertoric/fibrotic response in cardiac tissue. Importantly the effect of IL-33 signalling could be modulated by hormones (such as Ghrelin) and other stimuli.

Importantly, the final effect of IL-33 signalling activation is dependent on several factor, as cell types' origin and balancing of ST2 isoforms. Noteworthy, extreme importance has to be ascribed to the animal models used in *in vivo* experiments, the chronic or acute condition as such as the time of IL-33 secretion. This way, it is reasonable that to define a unique protective role of IL-33 is over-simplistic and further studies are needed to confirm and unveil mechanisms of IL-33 as a gene expression regulator in cardiac obesity.

Disclosure for research integrity

This research was conducted following the European Code of Conduct for Research Integrity.

Abbreviations

American Heart Association (AHA)

AMI (acute myocardial infarction)

Body Mass Index (BMI

Cardiovascular disease (CVD)

Coronary artery disease (CAD),

Cyclic adenosine monophosphate (cAMP)

Diastolic Blood Pressure (DBP)

Diet induced obesity mice (DIO)

Epicardial adipose tissue (EAT)

Exchange proteins directly activated by cAMP-1 and 2 (EPAC1 and EPAC2)

Free fatty acids (FFAs).

Heart failure (HF)

High endothelial venule (NF-HEV)

IL-1 receptor-like 1 (IL1RL1)

Ingenuity pathway analysis (IPA)

Interferon-γ (INF-γ)

Interleukin-33 (IL-33)

Knock out (KO)

Matrix metalloproteinase (MMP)

Metabolically healthy overweight/obese (MHOO)

Myocyte enhancing factor 2 A (MEF2A)

Non-ST elevation myocardial infarction (NSTEMI)

Peroxisome proliferator-activated receptor-α,γ (PPARα and PPARγ)

Phosphoenolpyruvate carboxykinase (PCK-1)

Reactive oxygen species (ROS)

Receptor for advanced glycation end products (RAGE)

Single nucleotide polymorphisms (SNPs)

ST-elevation myocardial infarction (STEMI)

Subcutaneous adipose tissue (SAT)

Suppression of Tumorigenicity 2 protein (ST2)

Tumour necrosis factor α (TNF-α)

Type 2 Diabetes mellitus (T2DM)

Visceral adipose tissue (VAT)

Ying Yang 1 (YY1)

Zucker fatty diabetics rats (ZDF)

Zucker fatty rats (ZF)

Introduction

The IL-33/ST2 signalling pathway

IL-33 structure

IL-33 is a cytokine belonging to the IL-1 family discovered in 2003 by Baekkevold *et al*. Firstly named nuclear factor of high endothelial venule (NF-HEV) (1). The protein in humans encompasses 270 amino-acids and it is composed of two domains as represented in Fig.1: the C-terminal domain, responsible for the ST2-dependent signalling and the N-terminal domain - that contains a chromatin-binding motif and it is supposed to function as a transcriptional repressor (2). The two domains are separated by a “protease sensor domain” that contains multiple cleavage sites. C-terminal domain – also called IL-1 like cytokine domain – also possess an important cleavage site for caspase 3/7 (3).

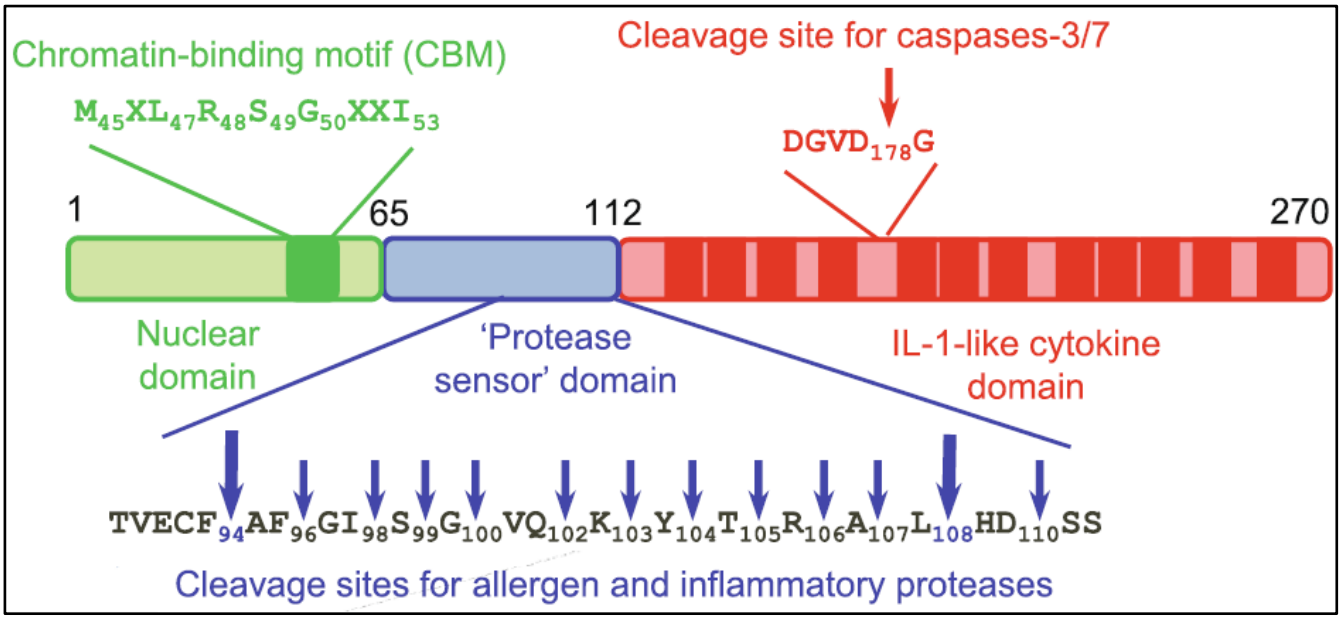

Fig 1 IL-33 primary structure and its functional domains (from (3))

The C- domain binds its receptor ST2, forming a ternary complex with the co-receptor IL-1 receptor accessory protein IL-1RAcP: this complex activates MyD88- dependent signalling pathways (4). The N-terminal domain contains the sequence for nuclear localization that is essential for its regulation. In fact, IL-33 is sequestered in the nucleus and the deletion of nuclear signals provokes the continuative release of cytokines in the extracellular space, leading to multi-organs inflammation and death (5). These lethal effects are mediated by

ST2 signalling as its genetic deletion preserved mice from death. Although a new role for IL-33 has been postulated as a regulator of transcription, the effect of intranuclear IL-33 is largely unknown and controversy, depending on cell types and stimulation (6, 7). Accordingly, Travers and colleagues reported that induced expression of nuclear IL-33 in esophageal epithelial cells lacking ST2 had no impact on gene expression (8).

IL-33 cell expression

In humans, the endothelium is the predominant source of IL-33: at the steady state the IL-33 is abundantly expressed in the nuclei of blood vessel in almost all human organs (9, 10) while differences exist in mice where constitutive expression is lower (11-13). Furthermore IL-33 is strongly expressed in epithelial cells of barrier tissues (bronchus, nasopharynx, skin, stomach, salivary gland, vagina, etc…) where they respond to stimuli from the environment. In human lungs the major sources of IL-33 are airway basal epithelial cells and endothelial cells. In this sense, damage to the endothelium and subsequent release of IL-33 is a rapid and important sensor and mediator of inflammation (10, 14).

IL-33 and disease

The epithelium-derived IL-33 is involved in several tissues and disease processes. Thanks to animal models suffering from conditional knock out (KO) of the IL-33 gene in endothelial cells, the effect of endothelium-derived IL-33 have been elucidated. In particular in a pressure overload model IL-33 induced systemic inflammation and similarly it contributed to kidney and liver ischemia–reperfusion injury (15). IL-33 has a role in allergy such as in mouse models of gastrointestinal allergy (16). Epithelial derived IL-33 in involved in asthma, since genome-wide association studies revealed that IL-33 represents a major susceptibility *locus* in this pathology (17). IL-33 is up-regulated in a multitude of disease and inflammatory process (autoimmune or allergic) and following infections (parasite, virus) and in sepsis. Often the activation of an autocrine/paracrine pathway is responsible for the up-regulation of IL-33, as its release stimulates its production in the IL-33 secreting cells (3). However, danger stimulation determines the over-expression of IL-33 in different cells (18, 19).

Bioactive forms of IL-33: full-length protein and mature forms

Firstly, it was though that IL-33 required cleavage for activation after extracellular release. On the contrary it was demonstrated by 11 that the full-length isoform, IL-33FL, (IL-33aa1-270) has biological activity and is able to activate ST2L signalling, whilst once cleaved by

caspase 1 or 3-7 it became inactivated (20, 21). This finding is in line with its biological role as an alarmin, that does not need to be activated in a condition of programmed cell death as apoptosis, in which the activation of inflammation and immune system is not required. On the other hands, cleavage from endogenous or infection-derived proteases – such as neutrophil elastase and cathepsin G, mast cell chymase and tryptase – produce a 18–21 kDa proteins considered as the mature forms. These mature forms contained the C-terminal IL-1-like cytokine domain and show a ~30- to 60-fold higher biological activity than that of IL-33 (17, 22, 23).

This rapid and powerful activation of IL-33 after allergen protease is responsible for the importance of IL-33 signalling in allergic disease (24). However, allergen and other proteases are also fundamental mechanisms of IL-33 regulation (17). Indeed, intensive proteases processing determines IL-33 inactivation. Another mechanism of protein degradation is mediated by oxidation that regulates the formation of two intra-molecular disulfide bonds that disrupt the ST2L binding site. Besides, during condition of increased reactive oxygen species (ROS) production, IL-33 degradation is increased (25). On the other hand, de-ubiquitination of IL-33 is involved in nuclear stability whereas ubiquitination in transcriptional activation (26, 27).

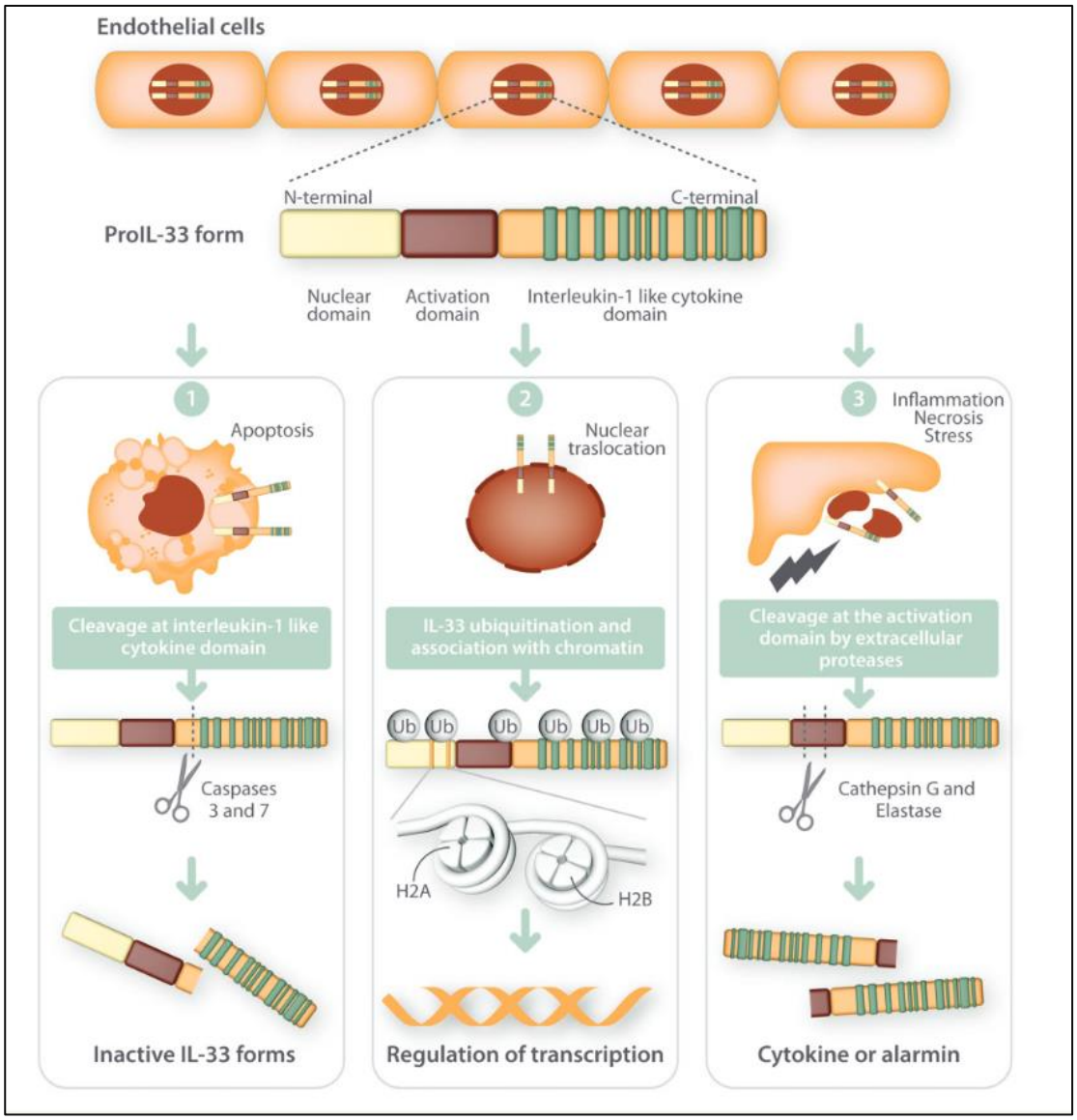

Figure 2: Pro-IL-33 processing and function (28)

Biology and structure of ST2

The ST2 gene was first described by Werenskiold and colleagues and named Suppression of Tumorigenicity 2 protein (ST2) due to its role in the induction of cell proliferation, a fundamental characteristic of cancer development (29). The ST2 gene, located on human chromosome 2q12, encodes a protein that is a member of the interleukin-1 receptor-like-1 (IL1RL1) family. Four protein isoforms of ST2 are produced as a result of alternative promoter splicing and 3′ post-transcription processing of ST2 mRNA (30, 31). The most important forms are the transmembrane one, ST2 ligand receptor (ST2L) with molecular weight of 67 kDa and three extracellular IgG domains, and a smaller secreted soluble form

(sST2) with molecular weight of 37 kDa, sharing similar extracellular structure but without the transmembrane and intracellular parts. The role of vST2, variant form, has still to be elucidated. The two isoforms are receptors that bind IL-33 and not IL-1α, IL-1β or IL-1R antagonists. The ST2L is expressed on membrane surface and activates MAP kinase cascade and NF-kB to modulate immune response. On the other hands, the soluble form of ST2 receptor lacks the transmembrane and intracellular domains and functions as decoy receptor, blocking IL-33 signalling (28).

ST2 is expressed by cells of different origin such as embryonic, hematopoietic, tumorigenic, and immune ones and its effects depend both on different cells' source and stimuli as well as on cell growth factors (platelet-derived, acidic fibroblast and primary fibroblast growth factor) (32). The first and most important role of ST2L is crucial for the development of T helper type 2 lymphocytes. In response to IL-33/ST2L signalling Th2 cells produced IL-4, IL-5 and IL-13 anti-inflammatory cytokines, promoting the switch of immune response from th1 towards th2. However, these cytokines are also involved in allergic reactions. On the contrary the over-expression of sST2 soluble form that blocks IL-33 signalling also blocks Th2 development, favouring the TH1 production of pro-inflammatory cytokines leading to Tumour necrosis factor α (TNF-α) and inflammation (33, 34). These process are sought to be involved in several inflammatory and autoimmune diseases (33-35) in different organs and biological systems such as in the heart (36-38).

Once released after injury in the extracellular space IL-33 reaches the surface of ST2 receptor and the complex is formed by means IL-1 receptor-like 1 (IL1RL1) and IL1RAcP (IL-1 receptor accessory protein), to initiate inflammatory pathways in immune cells or cell proliferation. This complex needs to recruit additional adaptors (including MyD88, IRAK1/4, TRAF6) and the cascade ends in the activation of different transcription factors such as ERK, NF-kB, p38, JNK. Importantly, as mentioned above, the effect changes in relation to cell type considered (immune cells, cancer cells) (39).

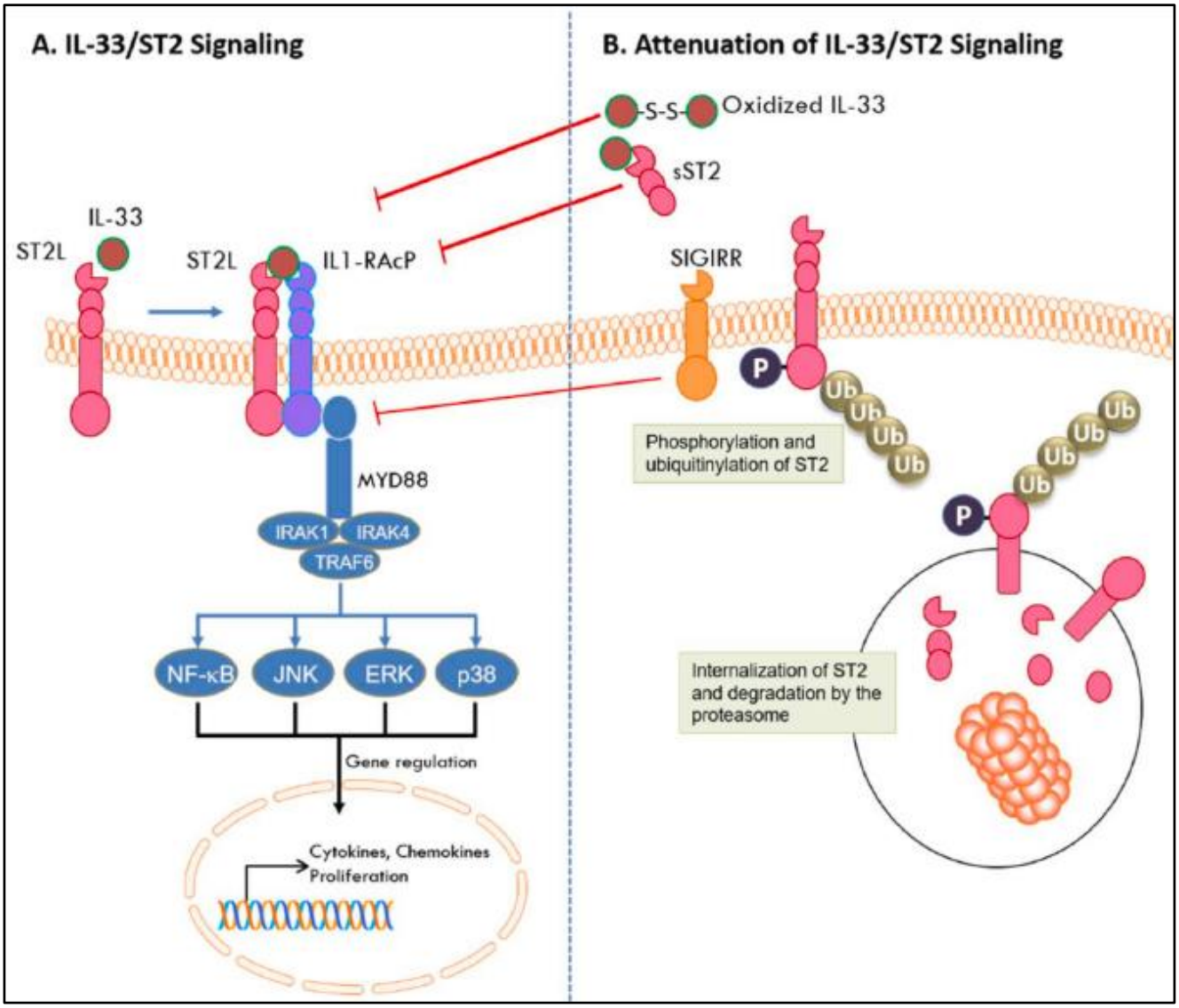

Figure 3: Activation and regulation of IL-33/ST2 signalling (64).

IL-33/ ST2 axis

The role of IL-33 is to alert the organism of a damage that is occurring through the activation of the immune system. Thus, it represents an alarm signal released from the nuclei of producing cells upon cell injury. Endothelial cell damage, mechanical injury or virus and parasite infection, allergic intranasal administration of ragweed pollen (40) all determine rapid release of endogenous IL-33 protein in the extracellular space in plasma, cerebrospinal fluids, and nasal lavage fluids. One-hour IL-33 rapid increase is transient, so that its level in the various fluids returned always to baseline within 24h (25).

Release of full-length IL-33 protein is evident following induction of cellular necrosis but its role during homeostasis needs to be clarified. It has been reported that IL-33 could be released from cells subjected to mechanical forces *in vivo* followed by cell recovery or death at later time points (24, 41). However chronic IL-33 stimulation such in asthma or autoimmune disease will probably follow different mechanism of release.

IL-33/ST2 and cardiovascular disease

A plethora of studies evidenced the importance of sST2/ST2L/IL-33 triad role in cardiovascular disease. As mentioned before, the effect is mostly beneficial and linked to ST2L activation, that favors cardiac repair reducing cardiovascular remodeling or atherosclerotic plaque progression. However, continuous and exaggerated IL-33 release will lead to excessive Th2 response, recruitment of mast cells or eosinophils and tissue damage. In this sense this view is probably over-simplistic as IL-33 administration is not always beneficial since both pro- and anti-inflammatory actions have been attributed to IL-33, neither a harmful role of sST2 is definitely established (28).

In the cardiovascular system IL-33 is constitutively expressed in the nuclei of cardiac fibroblasts, endothelial cells, cardiomyocytes, and coronary artery smooth muscle cells (42). It can be released both by living cells in response to mechanical stress during fiber stretching or following cell necrosis (43). Its expression in cardiac tissue is promoted by Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-c, and IL-1β. Conversely, ST2L and sST2 mRNA were reported to be expressed at low levels in cardiomyocytes in humans, but it can also be transiently induced in other cell types (44).
In the heart, the binding to ST2L receptor and its co-receptor IL-1RAcP recruits MYD88, IRAK1, IRAK4, and TRAF6, leading to activation of ERK1/2, JNK, p38 MAPK, and NF-kB: this condition determines the subsequent activation of multiple pathways that exert the beneficial effect of IL-33 regarding to cytokines' secretion, immunomodulation, cell proliferation, and cardiomyocytes' survival. In this sense, *in vitro* studies demonstrated that IL-33 favours the survival of neonatal rat cardiomyocytes from cell hypoxia and after anoxia/reoxygenation (45) by increasing the expression of anti-apoptotic proteins (XIAP, cIAP1, survivin, Bcl-xL, and Bcl-2) (46).
These findings were further confirmed *in vivo* as IL-33 protects the heart from fibrosis and reduced infarct size. However, IL-33 alone does not suppress collagen I/III or periostin production by adult rat cardiac fibroblast but - on the contrary - IL-33 stimulates expression of cytokines and chemokines (IL-6 and CCL-2) associated with cardiac inflammation and fibrosis. Accordingly, the beneficial effect of IL33 in the myocardium is probably indirect. Furthermore Abston *et al.* found a paradox effect of that IL-33 treatment in healthy mice, that promoted inflammation inducing eosinophilic pericarditis leading to impaired heart function. Indeed, the beneficial effect of Il-33 in infarct seems to be linked to reduced ROS-production and the cells involved are yet to be defined (47).

Interestingly a few reports indicated a role for sST2 itself beyond its blocking effect on IL-33 signalling. This effect seems to be related to direct anti-inflammatory actions on macrophages by downregulating Toll-like receptors.

Genetic variants in IL-33 and ST2 genes and relationship with CAD

Starting from the evidence that the binding ST2L/IL-33 determines a strong immune response leading to inflammatory conditions, Ho and colleagues investigated the variations of sST2 in plasma of 2,991 Framingham Offspring Cohort participants. Interestingly, they found that clinical and environmental features of patients – as age, cardiac evaluation, systolic blood pressure and other comorbidities – had a limited impact (15%) in the determination of sST2 concentrations (48). In opposite, more than 40% of sST2 variability was determined by genetic factors, as several single nucleotide polymorphisms (SNPs) or missense variants of *IL1RL1* gene caused dramatic alterations in IL-33/ST2-dependent pathways. Accordingly, it was demonstrated a pathological activation of different pro-inflammatory signalling pathways, as those dependent on IL-1β, NF-kB, MAP-kinases and mTOR (48). Similarly, other studies described the synergy between IL-1b and IL-33 (49, 50) and the association of IL1RL1 polymorphisms with enhanced CAD severity (51, 52). Interesting results were obtained through the study of IL-33 promoter region, since different SNPs were linked to IL-33 over-expression and elevated risk of CAD (52) or to down-regulation of IL-33 and lower probability to suffer from CAD and obesity (53).

IL-33 and sST2 as a clinical biomarker

A multitude of studies reported that serum levels of IL-33 decreased with increasing CVD severity. Its expression is opposite to those of IL-6 or the extracellular protease matrix metalloproteinase (MMP)-28 (54, 55). However, although CVD was present, serum IL-33 was undetectable in more than 50% of study participants. Often, sST2 is preferred to IL-33, due to its greater stability. Furthermore, ELISA assay for IL-33 lack sensitivity and specificity since Il-33 has a very short half-life and its level could be wrongly assessed due to interference of sST2 binding, protein degradation and oxidation. Oxidation could also modify the protein immunoreactivity thus conveying the antibody in the assay unable to recognize it. This finding has to be considered in condition of increased ROS production and oxidative stress such as obesity and cigarette smoke (56, 57).

On the opposite, all studies confirmed that circulating levels of sST2 increased with greater severity of CAD events so that it allows to discriminate patients among non-ST elevation myocardial infarction (NSTEMI) and ST elevation myocardial infarction (STEMI).

Importantly these studies evidenced the prognostic utility of sST2 that predicts worse outcome in acute myocardial infarction (MI) (58), systemic and pulmonary hypertension (59, 60), coronary artery disease (CAD) and heart failure (HF) (61), and type 2 diabetes mellitus (T2DM). (62). Furthermore - in the long-term - sST2 is an independent predictor of all-cause mortality. Comparing sST2 performance with well-known biomarkers as troponin or NT-proBNP levels revealed that sST2 positively correlated with infarct size This way, admittance levels were a significant predictor of cardiovascular death and predicts the risk of developing it. sST2 level could also predict left ventricular remodeling after infarction myocardial, being an important indicator of future cardiac function (63). Interestingly sST2 is less influenced by modifying factors as age, sex and by kidney failure and other comorbidities than classic used biomarkers (64). Thanks to all of these consideration sST2 is a promising new clinical biomarker for both acute and chronic heart failure.

IL33/ST2 and obesity

Several studies investigated the relationship between IL33 and obesity with sometimes contradictory results. IL-33 is expressed in the endothelium of adipose tissue, and it can be upregulated. For example, the expression of IL-33 mRNA was found to be increased in adipose tissue of obese Wistar rats (65) or it was up-regulated following TNF-α stimulation

(66). On opposite, IL-33 seems to be upregulated to counteract inflammation and metabolic disorders caused by obesity (67). In fact, IL-33 could be related to low-grade inflammation of adipocytes that is characteristic feature of obesity (65). Conversely in obese Zucker rats Ragusa et al. showed that the mRNA level of IL-33 in adipose tissue was significantly reduced (68). Similar to animal models, in humans Circulating levels of Il-33 and sST2 in obesity are controversy. Lower levels of serum IL-33 were observed in non-lean individuals in comparison to those who are lean. IL-33 level was also negatively correlated to BMI in lean subject but this was not confirmed in obese subjects (69). Other studies observed increased serum levels of IL-33 in metabolically unhealthy overweight/obese subjects compared to healthy subject while no difference could be detected in healthy subject versus metabolically healthy overweight/obese (MHOO) in chinese population. In addiction the increase of serum IL-33 was positively associated to several metabolic syndrome risk factor such as diastolic blood pressure (DBP) and alanine transferase levels (70). On the contrary, Zeyda showed no significant alteration in the IL-33 level in serum of severely obese people versus non-overweight subjects (71).

The distribution and the consistency between adipose and serum levels of IL-33 is still a matter of debate and several explanations exists for conflicting results. IL-33 levels could depend on the different murine models employed, condition used for developing obesity (as the type of diet) and also on IL-33 assay used for measurement. In any case it is generally accepted that IL-33 has a beneficial role in obesity, by regulating homeostasis and promoting beige adipogenesis in white adipose tissue through activation of ILC2. In this context, IL-33 acts to modulate inflammation associated to obesity and to limit adiposity by increasing caloric expenditure. IL-33 is produced and released by adipocytes and endothelial cells in adipose tissue and its production is increased by the sympathetic nervous system.

All these effects are mainly mediated by the modulation of immune response (72). Kai et al treated intravenously DIO mice with recombinant IL-33. They showed that IL-33 did not alter the weight of the animals, however the weight of SAT and epididimal (VAT) tissue was reduced. Furthermore, adipose tissue accumulation and ballooning of liver were reduced in treated mice. IL-33 treatment restored elevated level of PPARγ and acetyl-CoA synthetase 1 (AceCS1) seen in HFD mice. Difference arose in SAT and VAT regarding IL-33 expression that was increased in SAT and reduced in VAT treated with High Fat diet and restored by exogenous IL33 treatment. These findings lead to the possibilities that resident cell could

play different roles in SAT and VAT (67). Similarly, Miller et al showed both *in vitro* and *in vivo* that IL-33 treatment promotes adipose tissue release of cytokines (IL-5, IL-13, IL-10) and enhances adipose macrophages' differentiation towards Th2 phenotype. Furthermore, IL-33 treatment of Ob/Ob mice influenced both mRNA expression of gene associated to lipid metabolism such as *C/EBPα, SREBP-1c, PPARγ* and circulating level of cytokines (with marked over-expression of IL-5, IL-6, IL-10 and IL-13). On the other hand, the treatment did not influence liver expression of genes involved in glucose and lipid metabolism - phosphoenolpyruvate carboxykinase (PCK-1), aminotransferase enzymes, SREBP-1c, FAS fatty acid synthase (FAS), LXRα, LXRβ, and PPARγ coactivator-1α (PGC-1α) - but it caused the polarization of liver macrophages/Kupffer cells toward an M2 phenotype (73). Other demonstration of the anti-inflammatory properties of IL-33 treatment in obesity came from Han et al., as they demonstrated that High fat diet induced $ST2^+$ Tregs reduction in VAT of DIO mice. IL-33 completely restored the proportion of Tregs and reduced TNF-α and IL-6 expression in VAT tissue but not in the spleen, confirming a local anti-inflammatory effect (74).

The beneficial effects of IL-33 treatment seemed to be linked to ST2L signalling as those are abolished in model lacking ST2L. Interestingly $ST2^{-/-}$ mice are more prone to develop obesity and increased their body weight in response to a high fat diet; in addition, they were not insulin resistant but showed altered pancreatic insulin secretion in response to glucose overload (73). Recently sST2 was identified as a potent regulator of adipose T_{reg} and ILC2 homeostasis as AAV-mediated elevation of sST2 is sufficient to determine diminished adipose tissue T_{regs}, increased adipose tissue inflammation and insulin resistance. In line with these evidence, plasma sST2 level was also associated with metabolic characteristics of human diabetes (75). Finally, TNFα was identified as a potent inducer of sST2 gene expression in adipocytes further linking inflammation and adipose tissue dysregulation (76).

Cardiac steatosis: a bridge between cardiovascular disease and obesity

Obesity has been traditionally linked to cardiovascular disease development (77). However body mass index and weight alone cannot actually predict the risk for having a cardiovascular event due to individual differences (race, sex age) (78). Furthermore, visceral or subcutaneous fat depots are not strictly biologically identical in term of fat storage and release, metabolism and secretome. Thus, research is increasing focusing on more reliable markers than BMI and waist circumference. Increasing interest has been developed on

ectopic fat accumulation such as pericardial and epicardial fat accumulation – called cardiac steatosis. As it was well-known that visceral adipose tissue has role in cardiovascular disease (CVD) pathogenesis by provoking insulin resistance and through the release of active adipokines in the circulation (79), the role of Epicardial tissue in cardiac remodeling was investigated. Eat has different physiological roles further than be a fat storage, it regulates free fatty acid release preventing cardiac lipotoxicity (80) and it protects the heart and coronary arteries from mechanical stress and hypothermia (81); and it contains the ganglia innervating myocardium (82). EAT and myocardium share common blood supply thus it can influence myocardium metabolism trough both a paracrine and vasocrine mechanisms. Physiologically, EAT secretes adipokines that exert protective effects on the myocardium as adiponectin (83) and orosomucoid (84) that mediates protection from apoptosis and antioxidant effects. For example, adiponectin improves nitric oxide bioavailability and endothelial function in the coronary vasculature (79). In pathological conditions it contributes to cardiac remodeling through secretion of adipokines, that enhance low-grade inflammation and diabetic vascular complications. EAT was shown to directly favoring atherogenesis by activating monocytes (85) and through the deposit and the oxidation of LDL (86). In this sense, EAT thickness is associated to ventricular myocardial mass and steatosis. And several studies have considered EAT as a quantifiable CVD risk marker (87, 88) through imaging. Although with still unknown mechanism, cardiac adiposity appears to promote alteration of cardiovascular profile increasing the risk of cardiovascular disease (89). The cardiomyocytes damage could be mediated by the production of lipotoxic intermediates (90) such as retinol binding protein-4 or activin A that negatively affect cardiac metabolism (91). In CAD, macrophages and T-cells proliferation determine a switch of EAT towards an inflammatory phenotype. Similarly in diabetes EAT is increasingly infiltrated by CD14+ monocytes (92). Elegant work from Venteclef *et al*. showed that EAT secretome in enriched in pro-fibrotic adipokines. In particular compared to other fat depots EAT secretes higher level of thrombospondin 2, vascular endothelial growth factor, activin A, TGF-*β*1 and matrix metalloproteinase isoforms, and induce extensive fibrosis of rat atria in organo-culture models (93). Intriguingly, recent findings suggested a bidirectional cross-talk between myocardium and EAT with dysfunctional myocardium that can provoke an inflammatory switch in gene expression in EAT tissue (94). There are few works regarding EAT-mediated expression of IL-33/ST2 pathway. Gruzdeva *et al.* investigated IL-33 system in relation to EAT thickness and visceral obesity in a cohort of post-AMI patients. They showed that EAT thickness increased with visceral obesity and correlates with fibrosis and maladaptive

remodeling one-year post-AMI. The degree of cardiac fibrosis was negatively related to the IL-33 levels and positively related to the ST2 levels (95).

Previous study of our group analyzed 55 patients presenting cardiovascular disease including CAD and non-CAD patients demonstrating a disbalance in IL-33/ST2 expression characterized by increased expression of ST2 in dysfunctional EAT tissue that correlates with ventricular remodeling and Left ventricle dysfunction (96). The pathological EAT locally produced exchange proteins directly activated by cAMP-1 and 2 (EPAC1 and EPAC2) proteins, and the latter positively correlated with the expression of ST2 whereas it does not correlate with IL-33 alarmin expression (96).

Aim of the thesis

In this project we aimed to investigate cardiac remodelling in obesity, both *in vivo* and *in vitro*, and - in particular - the pathways that link ectopic fat accumulation and detrimental fibrotic remodelling of the heart. A reliable approach to uncover the effectors of these pathways is to identify the molecules that are dysregulated in these tissues in conditions of cardiovascular risk such as type 2 diabetes, CAD and obesity. EAT trascriptome in CAD patients revealed an up-regulation of inflammatory pathways consisting in cytokine production (adipokynes, TNFα, IL-1β) and leukocytes migration. Indeed, our group already demonstrated a direct correlation between EAT and fibrosis in cardiac tissue of CAD patients related to the imbalanced IL-33/ST2 molecular pathway. The ST2/IL-33 pathway exerts a protective role in cardiac tissue, by reducing fibrosis and infarct size while increasing cardiac survival. Interestingly, ST2/IL-33 signalling is also related to EAT thickness. In obese individuals, plasma levels of sST2 are increased, partially due to the endothelium release of leptin in adipose tissue. In this sense, the chronic increase of ectopic fat body determines alterations - such as low-grade inflammation and leptin increase - that affect the activities of distant organs through IL-33/ST2 signalling modulations. Here we planned to evaluate ST2/IL-33 expression in VAT and heart in an *in vivo* model of obesity to correlate ST2/IL-33 system expression with the most important genes involved in cardiac remodeling. We investigated fibro-cytokine ST2 and IL-33 alarmin protein expression in obese ZF, ZDF and in control rats and we evaluated their correlation with both fibrotic gene pathways and cardiac matrix remodeling. Secondly, we planned to investigate the molecular mechanisms by which IL-33/ST2 signalling affects myocardium homeostasis, focusing on important cellular second messenger as cAMP, its regulators and effectors. We also concentrated our efforts in resolving the effects of adipose tissue secretome on cardiomyocytes by modeling *in vitro* the relationship between adipose tissue and myocardium. Using VAT conditioned medium, we planned to evaluate the effect of VAT-released IL-33 on cardiomyocytes' metabolism. Finally, as this pathology is a multifactorial disease that could arise both genetically or as a consequence of wrong lifestyle, we characterized DIO mice, whose obesity depend on the maintenance of a high fat diet. By exploiting cardiac proteomic analysis in these mice, we devised to globally evaluate the influence of fat accumulation, to confirm the importance of IL-33/ST2 pathway and – more importantly – to unveil new molecular target or enriched networks.

Material and Methods

Animal Models included in this study

Obesity in Zucker rats arose from a spontaneous mutation (a missense A to C mutation) in the leptin receptor gene on chromosome 5 ($Lepr^{fa}$) that determines the production of a non-functional receptor. The mutation leads to a state of hyperphagia, due to impaired satiety reflex that leads to obesity (97). Zucker fatty rat, and Zucker Diabetic rat are among the most used animal models for T2DM research today (98). Although over 4000 publications are present on PubMed, these animals do not fully recapitulate human disease. Diet induced obesity models are more reliable However, naturally developing obesity models do not need time-consuming diet scheme. In humans T2DM is slow and progressive while in ZF obesity is early - starting from 3 weeks - and becoming severe by 5 weeks. Importantly Zucker fatty rats are not hyperglycaemic (99) and showed only moderate hypertension. Finally, this model does not develop premature atherosclerosis or important cardiac lesion nor dysfunction (100).

Further Crossbreeding of Zucker rats for hyperglycaemia eventually developed a less obese, but diabetic subtype, called the Zucker Diabetic Fatty (ZDF) rat. They harbour an autosomal recessive mutation in β-cell transcription (independent from the Lepr mutation) (101). However only Male ZDF rats are more prone to the development of T2DM. Similar to ZF, ZDF rats do not develop hypertension or severe cardiovascular diseases (102).

DIO animal models are considered a particularly good model to study obesity in humans. It closely recapitulates the mechanisms promoting obesity and metabolic syndrome in humans. It gradually develops hyperinsulinemia, hyperglycaemia, and hypertension as in humans (103) analogue to metabolic syndrome in humans and it is reversible . Many researchers demonstrated that in these mice fat accumulates preferentially in the mesentery, paralleling abdominal obesity and promoting diabetes (104). High fat diet induced gradual accumulation of fat with hyperglycaemia that develops within 1 month and by 16 weeks fat mass increased by 93% accompanied by hyperplasia and hypertrophy of adipocytes. These animals are usually used to study the role of diet, pathophysiology and aetiology of the disease, as well as pharmacological tests. They also developed atherosclerosis (105) and compromised immune function (106).

Procedures involving living animals

Procedures involving living animals were conformed to Italian law (D.L.vo 116/92 and to the European Union guidelines.. and approved by local ethics committees and the Italian Ministry of Health (Number 5AD83.N.G1Q) For DIO mice experiments: fourteen six-week-old male C57BL/6N mice (Charles River Laboratories, Calco, Italy) were divided into two groups and fed for 20 weeks as follows: (1) normal chow diet (10% fat, CTR) and (2) high-fat (HF) diet (60% fat) (Charles River Laboratories). The mice were housed at constant room temperature (22± 2°C) and humidity (60± 5%) with a light–dark cycle of 12 h each with water ad libitum. At the age of 26 weeks, the mice were sacrificed through exposure to atmosphere saturation of carbon dioxide for 15 min. Cardiac and adipose tissues were collected and immediately snap-frozen in liquid nitrogen, and stored at -80°C until analyses or fixed in 10% buffered formalin, routinely processed, and paraffin-embedded and collected. Serum was collected and stored at-80°C. For Zucker rats' experiments: the use of animals in this study was authorized by the National Ministry of Health (protocol number (N°325/2015PR of 2015/04/05). Ten obese non-diabetic male Zucker rats (OB) (fa/fa-) and 10 lean littermates (L) (Fa/+) were provided by Charles River. All animals were housed in a controlled ambient environment (12 h light/dark cycle) at a temperature between 21 and 23°C. Cage population was limited to a maximum of four animals each to ensure the health and welfare of animals. The rats had free access to clean water and fed with standard diet. At 25 weeks of age rats were deeply anesthetized with Zoletyl (20mg/kg), then sacrificed by cervical dislocation. One animal of Lean group died, thus the organs were not collected. Heart, adipose tissue and serum were collected. 5 hearts for each group were formalin fixed and paraffin-embedded, 5 heart and 5 adipose tissue biopsies for each group were frozen in Allprotect Tissue Reagent (QIAGEN at -20°C. Mutated Zucker rats were called Obese group, while wild-type healthy rat were called Lean. For Zucker Diabetics rats' experiments: the use of animals in this study was authorized by the National Ministry of Health (protocol number N°325/2015PR of 2015/04/05).

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Total RNA Extraction and Reverse Transcription

Disruption and homogenization of cardiac and VAT samples (*n* =5 for each experimental group) were performed with the TissueLyser II* equipment (QIAGEN, Milan, Italy) through high-speed shaking in plastic tubes with5 mm stainless steel beads. Then, total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN), according to the manufacturer's instructions. RNA concentration was quantified with NanoDrop (Thermo Fisher Scientific, Monza, Milan, Italy). RNA samples (1 µg) were first treated with a genomic DNA elimination step (5 min/42 °C and kept on ice at least 1 min) and then reversely transcribed using the RT^2 First Strand Kit (15 min/42 °C and 5 min/95 °C) (QIAGEN). Samples were stored at −20 °C until real-time polymerase chain reaction (qPCR) analysis. Coculture cells were harvested from 48wells centrifuged at and cells pellet were frozen at -80 for further use. RNA was extracted from harvest cells with RNeasy Microkit (Ref 74004 QIAGEN). cDNA was generated using the Reverse Transcriptase (Kit RT^2 First Strand Kit Cat. No. / ID: 330404) followed by the SYBR-Green reaction to quantify the expression of the genes in Table 1. (Qiagen RT2 Sybr green qPCR Mastermix fast) All the cDNA samples were tested in triplicated, and the threshold cycles (Ct) of target genes were normalized against a housekeeping gene, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative transcript levels were calculated from the Ct values as $X = 2^{-\Delta\Delta ct}$ where X is the fold difference in the amount of target gene versus GAPDH and $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{GAPDH}$. The efficiency of primers used was calculated between 95.2% and 98.3%.

RT2 Profiler PCR Arrays

RT2 Profiler PCR Arrays allowed the detection of 84 key gene transcripts related to rat fibrosis (PARN-120Z, from QIAGEN) using qPCR. Each cDNA sample was diluted with nuclease-free

water and mixed with the RT2 SYBR green Mastermix (QIAGEN). Twenty-five µL of the same experimental mixture were automatically added to each well of the array (one array for each cDNA) using the QIAgility® equipment (Qiagen). qPCR was performed by the RotorGene-Q (Qiagen) and consisted of an initial activation of the Hot-start DNA Taq polymerase at 95 °C/10 min, which was followed by 40 cycles of 95 °C/15 s and 60 °C/30 s. Then, dissociation curves were performed to verify the specificity of the amplicons using the default melting curve program of the instrument. Data were analyzed using the RT2 Profiler PCR Array Data Analysis Web Portal (QIAGEN). A list of all the transcripts included in the fatty acid metabolism-array and in the fibrosis-array is included:

Pro-Fibrotic:

- ✓ Acta2, Agt, Ccl11 (Eotaxin), Ccl12, Ccl3 (Mip-1a), Ccn2, Grem1, Il13, Il13ra2, Il4, Il5, Snai1 (Snail, Sna).

Anti-Fibrotic:

- ✓ Bmp7, Hgf, Ifng, Il10, Il13ra2.

Extracellular Matrix (ECM) & Cell Adhesion Molecules

- **ECM Structural Constituents:** Col1a2, Col3a1.
- **ECM Remodeling Enzymes:** Lox, Mmp1, Mmp13, Mmp14, Mmp2, Mmp3, Mmp8, Mmp9, Plat (tPA), Plau (UPAM), Plg, Serpina1, Serpine1 (Pai-1), Serpinh1 (Hsp47), Timp1, Timp2, Timp3, Timp4.

Cell Adhesion Molecules:

- ✓ Itga1, Itga2, Itga3, Itgav, Itgb1, Itgb3, Itgb5, Itgb6, Itgb8.

Inflammatory Cytokines & Chemokines:

- ✓ Ccl11 (Eotaxin), Ccl12, Ccl3 (Mip-1a), Ccr2, Cxcr4, Ifng, Il10, Il13, Il13ra2, Il1a, Il1b, Il4, Il5, Ilk, Tnf.

Growth Factors:

- ✓ Agt, Ccn2, Edn1, Egf, Hgf, Pdgfa, Pdgfb, Vegfa.

Signal Transduction

TGFβ Superfamily Members:

- ✓ Bmp7, Cav1, Dcn, Eng (Evi-1), Grem1, Inhbe, Ltbp1, Smad2, Smad3, Smad4, Smad6, Smad7, Tgfb1, Tgfb2, Tgfb3, Tgfbr1 (Alk5), Tgfbr2, Tgif1, Thbs1 (TSP-1, Tsp1), Thbs2.

Transcription Factors:

- ✓ Cebpb, Jun, Myc, Nfkb1, Sp1, Stat1, Stat6.

Epithelial-to-Mesenchymal Transition (EMT):

- ✓ Akt1, Bmp7, Col1a2, Col3a1, Ilk, Itgav, Itgb1, Mmp2, Mmp3, Mmp9, Serpine1 (Pai-1), Smad2, Snai1 (Snail, Sna), Tgfb1, Tgfb2, Tgfb3, Timp1.

Other Genes related to Fibrosis:

- ✓ Bcl2, Faslg.

Western Blot Analysis

Total proteins from cardiac biopsies isolated from lean and obese rats were extracted in RIPA (Cell Signalling) lysis buffer 1x + 1% protease/phosphatase inhibitor (Cell Signalling) + dH_20, then disrupted and homogenized with TissueLyser II* (QIAGEN). Samples disruption were carried out in high-speed (20–30 Hz) shaking steps for 2 minutes for 2 consecutive cycles (in 1.2 ml micro tubes containing 5 mm stainless steel beads), then put on ice for 30' and centrifuged (at 4°C -13200rpm for 15') to collect the supernatant as described in QIAGEN Tissue Lyser Handbook. Cells were incubated with 250μL of Trypsin/EDTA for 5 minutes at 37°C. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. Trypsin was inactivated and lysis was stopped treating the cells with 250μL of cell culture medium. Total volume was transferred to a microfuge tube and the lysate was clarified by spinning for 8

minutes at 1200 rpm at room temperature. The cell medium was discarded and cellular pellet was immediately frozen at -80°C. Samples were resolved on polyacrylamide gels (BIO-RAD Mini- Protean TGX Stain –Free Gels ranging from 4% to 20%) and transferred (transfer system BIO-RAD Trans-Blot Turbo) to nitrocellulose membranes (BIO-RAD Trans-Blot Turbo 0,2µm nitrocellulose). Membranes were incubated with primary antibodies ON at 4°C, followed by washing, detection with horseradish peroxidase (HRP)-conjugated secondary antibodies (DakoCytomation, United States), and developed by enhanced chemiluminescence (ECL BIO-RAD Clarity™ Western substrate). Filters were incubated overnight. A list of all tested antibodies is included below. Bands were visualized using BIO-RAD Chemidoc™ Touch Image System. Densitometric analysis was performed using ImageJ software.

Cell culture and transfection

The rat H9C2 cell line was utilized in this study. All the cells were obtained from H9C2 Merck Cell Line from Rat BDIX heart myoblast-88092904. The cells were grown in DMEM high glucose (Euroclone) medium supplemented by 10% fetal bovine serum (FBS) and incubated at 37°C, with 5% CO_2 atmosphere. For transwell assay, cells were plated in a 24-well plate (30000cells/wells) with transwells insert (Costar code 5026649) in 0,5 mL of medium for each experimental time-point. 30 mg of fresh adipose tissue was plated on the inner chamber, on the top of the filter membrane while cells were plated on the bottom of the lower chamber in a 24-well plate (30000cells/wells). The peculiar pore size of the transwell membrane employed in our experiment did not allow direct cell-cell interaction between adipose tissue and H9C2, neither the passage of adipose cells. Cells and medium were collected after 3h, 6h 24, and 48h and RNA extraction was performed on lysed cells through Qiagen kit. H9C2 cells were also seeded for Ghrelin treatment (Ghrelin rat lyophilized powder G8902 Sigma-Aldrich) in 24well-plate (FALCON 353504) seeding density 30.000 cells in 0,5 ml of medium/well, treated at T0 and T48h and harvested every 24h till 72h. Following harvesting, cells were immediately lysed in Buffer RLT to prevent unwanted changes in the gene expression profile as suggested by Qiagen RNeasy Micro Kit protocol.

Shotgun Mass Spectrometry Analysis for Label-Free Proteomics

Heart tissues of 8 CTR mice and 8 mice fed with HF diet were analysed by a shotgun label-free proteomic approach for the identification and quantification of expressed proteins. The tissues were homogenized in 100 µL of extraction buffer (8M urea, 20mM Hepes pH 8.0, with Protease and Phosphatase Inhibitors cocktail). Prior to proteolysis, proteins were reduced with 13mM dithioerythriol (30 min at 55 °C) and alkylated with 26mM iodoacetamide (30 min at room temperature). Protein digestion was performed using sequence-grade trypsin (Promega) for 16 h/37 °C using a protein/enzyme ratio of 20:1 The collected peptides were desalted using Zip-Tip C18 before Mass Spectrometric (MS) analysis. NanoHPLC coupled to MS/MS analysis was performed on a Dionex UltiMate 3000 directly connected to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) by a nanoelectrospray ion source. Peptide mixtures were enriched on 75 µm ID X 150 mm EASY-Spray PepMap RSLC C18 column (Thermo Fisher Scientific) and separated using the LC gradient: 1% ACN in 0.1% formic acid for 10 min, 1–4% CAN in 0.1% formic acid for 6 min, 4–30% ACN in 0.1% formic acid for 147 min, and 30–50% ACN in 0.1% formic for 3 min at a flow rate of 0.3 µL/min. Orbitrap-MS spectra of eluting peptides were collected over an m/z range of 375–1500 at resolution of 120,000, operating in a data-dependent mode with a cycle time of 3 s between master scans. HCD MS/MS spectra were acquired in Orbitrap at resolution of 15,000 using a normalized collision energy of 35% and an isolation window of 1.6 m/z. Dynamic exclusion was set to 60 s. Rejection of +1 and unassigned charge states were enabled. A database search was conducted against the Mus Musculus Uniprot sequence database (https://www.uniprot.org/proteomes, accessed on 4 March 2019) with MaxQuant (version 1.6.0.1) software. The initial maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. Enzyme specificity was set to trypsin, defined as C-terminal to Arg and Lys excluding Pro, and a maximum of two missed cleavages were allowed. Carbamidomethylcysteine was set as a fixed modification, while N-terminal acetylation, Met oxidation, and Asn/Gln deamidation were set as variable modifications. Quantification in MaxQuant was performed using the built-in label-free quantification algorithms (LFQ) based on extracted ion intensity of precursor ions. False protein identifications (1%) were estimated by searching MS/MS spectra against the corresponding reversed-sequence (decoy) database. Statistical analysis was performed using the Perseus software (version 1.6.14.0). Only proteins that were present and quantified in at

least 75% of the repeats were positively identified in a sample and used for statistical analysis. Proteins were considered differentially expressed if they were present only in one condition or showed a significant t-test difference ($p \leq 0.05$). Bioinformatic analyses were carried out by Panther software (release 16.0) (107), CLUEGO software (Cytoskape release 3.8.2) (108), and Ingenuity Pathway Analysis (IPA 2021 release) to cluster enriched annotation groups of biological processes, pathways, and networks within the set of identified proteins (109). Functional grouping was based on Fischer's exact test p-value ≤ 0.05 and at least 3 counts. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (PXD031915). Thanks to kind collaboration with Prof. Gabriella Tedeschi.

ELISA assay

ELISA assays were performed to analyse protein expression in serum and VAT tissue of DIO mice ZF and ZDF rats. Rat IL-33 (Fine Test) for IL-33 analysis and Rat IL1RL1 (IL-1 Receptor Like1) (Fine Test) for ST2 were performed. Serum and VAT tissue homogenates were prepared following kit instruction. Serum was diluted 1:2 before assay testing.

Sircol Assay

Collagen amount was quantified in homogenates of cardiac biopsies by Sircol Collagen Assay (Sircol™, Soluble collagen assay Biocolor Ltd, UK). It measures mammalian collagen (I to V). the procedure was performed following kit instruction. 50µL of cardiac homogenates were analysed in this assay.

Histological analysis

Ematoxilin-Eosin staining and Trichrome Masson staining (Trichrome Stain Masson -SIGMA-HT15), were performed on serial section of cardiac biopsies from ZF and ZDF rats. 5 hearts for each group were formalin fixed and paraffin-embedded and cut on a microtome. The procedure and analysis were performed by Anatomic Pathology Service of IRCCS Policlinico San Donato Hospital, in collaboration with Prof. Clemente. Immunoistochemical Staining of Il-33 and ST2 were performed trough automated procedure Ventana Medical Systems (Roche). Antibodies

were used as follow: IL-33 Rabbit monoclonal 1:200 (Proteintech) and ST2 Rabbit (Enzo) Polyclonal antibody 1:500.

List of included antibodies

Collagen 3 alpha1 antibody, Cohesion biosciences, mouse monoclonal 1:1000 140KDa

Il-33 Enzo, rabbit polyclonal antibody, 1:1000 25kDa

Epac 1 (5D3), Cell signalling, Mouse mAb 1:1000 100 kDa

Epac 2 (5B1), Cell signalling Mouse, mAb 1:1000 115 kDa

FOXP3 (F-9) Santa Cruz Mouse monoclonal 1:200 48 kDa

ST2, Proteintech, polyclonal antibody Rabbit 63/37/30 kDa

ST2, Enzo, polyclonal antibody, 1:500

Tgf beta1, Cohesion biosciences, rabbit polyclonal, 1:500 43kDa

Vinculin, Cell Signalling, Rabbit mAb 1:1000 124kDa

Table 1: RT2qPCR Primer Assay QIAGEN

Prime Name	Primer Code	Amplicon length
Mouse-Rapgef4 (Epac2)	PPM32822A	115 bp
Mouse-Rapgef3 (Epac1)	PPM26532A	81 bp
Mouse-IL33	PPM32527A	134 bp
Mouse-IL1RL1	PPM03546A	132 bp
Mouse Gapdh	PPM02946E	140 bp
Rat Yy1	PPR53391A	99bp
Rat Socs3	PPR06602A	145bp
Rat Rapgef3 (Epac1)	PPR49530A	131bp
Rat Rapgef4 (Epac2)	PPR49522A	119bp
Rat Pparα	PPR44459A	110bp
Rat Mef2A	PPR62504B	100bp
Rat IL33	PPR564110A	97bp
Rar Hdac4	PPR47615A	89bp
Rat Ghsr	PPR51984A	121bp
Rat Ghrl	PPR49492A	107bp
Rat Gapdh	PPR06557B	200bp

Table 2: Custom LNA oligonucleotide (QUIAGEN):

Prime Name	Primer Code	Amplicon length
Forward Rat St2L	AGTTGTGCATTTACGGGAGAG	68bp
Reverse Rat St2L	GGATACTGCTTTCCACCACAG	
Forward Rat St2s	GGTGTGACCGACAAGGACT	119bp
Reverse Rat St2s	TTGTGAGAGACACTCCTTAC	

Availability of Data

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Statistics

Data were analyzed by GraphPad Prism™ and expressed as mean ± SD or mean ± SEM. To compare multiple groups means, one-way ANOVA followed by Tukey's multiple comparison test was used to determine significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ****$p < 0.0001$). To compare two groups, Student's t-test was applied assuming equal variances: difference was considered significant at $*p < 0.05$. To correlate protein's expression, linear regression and multivariate regression analyses were performed.

Results

Characterization of IL-33/ST2 signalling in Zucker rat

In this project we decided to investigate the IL-33/ST2 pathway expression in different models of obesity to highlight its effect in cardiac remodeling. Among them, we choose the Zucker Rat, that is a well-known model of obesity (97). This model has a mutation in leptin receptor gene, that determines the loss of satiety recognition causing a tendency to overeat. This model has been widely used to assess obesity effects.

Serum levels of IL-33 and sST2 in relation to obesity in ZR

At sacrifice (25 weeks of age), rats that harbour the homozygous mutation (Ob) developed obesity, and their weight was 3 times higher than those of controls (Lean 415.0± 11.67g; Obese 533.7± 17.08g with p<0.0001) as reported in literature (100) (Fig1a). To evaluate IL-33 and ST2 expression we measured IL-33 and the sST2 isoform levels in serum through ELISA and we observed a decrease of IL-33 levels (Lean 2.130±0.098 pg/ml; Obese 1.886±0.057 pg/ml with p=0.0627) and a statistically significative increase of sST2 (Lean:10370±0.1073; obese 1.632±0.05262 with p=0.0287) (respectively Fig1b and Fig2a). Intriguingly, we observed a positive correlation between weight and IL-33 serum level in Lean rats. However further increase of weight in Obese rats was associated to decreased level of serum IL-33 (p=0.024) (Fig2b).

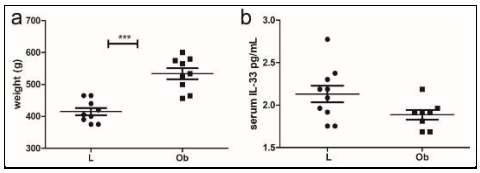

Figure 1: Characterization of IL-33 signalling in ZF rats: weight and serum IL-33 levels. (a) Graphs showing weight of rats at sacrifice (p=0.0001) and (b) ELISA assay of serum IL-33.

Figure 2: Characterization of IL-33 signalling in ZF rats: serum ST2 levels. (a) ELISA assay of ST2 serum levels (p=0.0287) and (b) graph showing opposite correlation between IL-33 and weight in Lean (p=0.0024) versus Obese (p=0.0373) rats

IL-33/ST2 pathway expression in visceral adipose tissue.

To investigate the source of serum IL-33/sST2 we analysed adipose tissue levels of IL-33 and ST2 by ELISA to verify whether their secretion is dependent on this tissue. Both IL-33 and total ST2 were increased in obese rats (mean IL-33 lean: 174.4±49.40pg/ml; obese: 346.2±38.10pg/ml with p=0.0141; ST2 lean 328.9±2898pg/ml; obese: 12.77±259.4pg/ml with p=0.0162). (Fig.3a,b respectively). However, ELISA analysis did not allow to discriminate between ligand or soluble ST2 isoform.

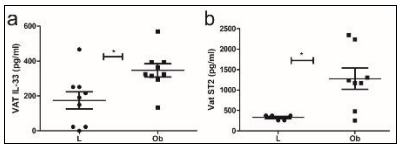

Figure 3: IL-33/ST2 expression in VAT tissue of ZF rats. Graphs showed up-regulation of IL-33 (p=0.0141) (a) and (b) ST2 (p=0.0162) expression in visceral adipose tissue (VAT) following qPCR experiments

IL-33/ST2 pathway expression in cardiac biopsies

In cardiac biopsies we observed a slight increase of IL-33 mRNA (Fig.4a), that became significant at protein level by WB analysis (Mean IL-33 lean: 0.5002±0.1503(AU); obese 0.9328±0.1237(AU) with p=0.0430) as shown in Fig4b. However, we did not detect significative differences in ST2L or sST2 isoforms as assessed through qPCR (Fig.4a). Western blot did not highlight any difference between ST2 and soluble isoforms probably due to high variability in ST2 expression (Fig4b). In fact, high variability of ST2 isoform expression could depend on cell localization (as in atria or in ventricula) and on cell types.

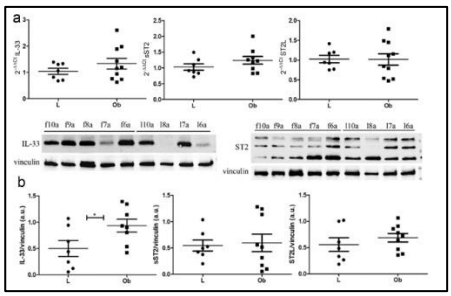

Figure 4. IL-33/ST2 pathway expression in cardiac biopsies of ZF rats. (a) qPCR graphs showed *IL-33* and *sST2* and *ST2L* expression. (b) Western blot showed increased IL-33 protein (p=0.0430) and no difference in ST2 isoforms levels.

Fibrosis characterization in cardiac tissue

We investigated whether IL-33/ST2 deregulation determined fibrotic remodeling in obese heart. To do this we analysed the expression of a panel of of 84 genes involved in the regulation of extracellular matrix (ECM) and cell adhesion molecules and in the fibrotic processes, which include both pro- and anti-fibrotic regulators, growth factors, inflammation-related molecules, signal transduction molecules, and regulators of epithelial-to-mesenchymal cell transition. We observed the activation of pro-fibrotic signalling, as indicated by the overexpression of Serpin Family E Member 1 (Serpin1) and Metallopeptidase 8 and 9 (MMP8 and MMP9). In particular all these genes codify for proteins involved in tissue remodeling, as Serpin1 is an inhibitor of tissue plasminogen activator and reduces the activity of matrix metalloproteinases while MMP8 and MMP9 are peptidases involved in the degradation of matrix. (Fig5a).

These data were further confirmed by WB analysis, assessing significant increased level of TGFβ - mean lean 1.206±0.03499; obese 2.347±0.04653 (AU) with $p<0.0001$ - and collagen 3. Sircol analysis demonstrated increased collagen fibers deposition in cardiac biopsies from obese rats compared to those of lean controls (Mean Lean 1076±26.21μg/ml; Obese: 2087±73.97μg/ml, with $p=0.0002$).

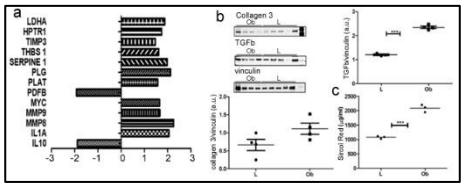

Figure 5: Fibrotic cardiac remodeling evaluation in ZF rat. (a) Graph showed fibrosis-related transcriptome in ZF rats. Genes that showed a fold change >2 or <−2 in the Ob vs. L group are represented and grouped according to their biological function ($p<0.01$). (b) Western blot showed upregulation of collagen 3 and TGFβ ($p=0.0001$) in Obese rats. (c) Graph showing Sircol red quantification demonstrated significant over-expression in Obese rats related to control ones ($p=0.0002$)

Investigating putative mediator of cardiac remodeling: Epac1

As exchange protein activated by cAMP (Epac1) is an important mediator of cardiac remodeling, we evaluated its expression in cardiac biopsies. Epac1 in heart tissue could act both as a pro-fibrotic and an anti-fibrotic agent depending on cell type. In fact, Epac1 KO mice appear to be protected from cardiomyocytes hypertrophy, fibrosis, and cardiac dysfunction induced by pressure overload and ischemia reperfusion (110) while in fibroblasts Epac1 seems to inhibit TGFβ-signalling (111). We observed a decrease of Epac1 in obese animals both at mRNA (Mean lean: 1.048±0.123 $2^{-\Delta\Delta CT}$; Obese 0.7496 ±0.05870 $2^{-\Delta\Delta CT}$, with p=0.0383) (Fig6a) and protein (Mean Lean 1.063 ±0.067 AU; Obese 0.8715±0.09691 AU, p=0.0695) levels in Ob rats (Fig6b). Interestingly, the amount of Epac1 was negatively correlated to IL-33 expression (Fig6c, p=0.0099).

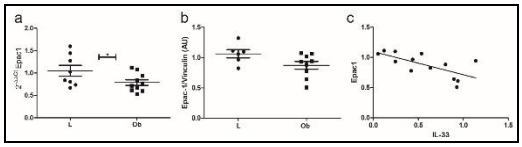

Figure 6: Epac1 expression in cardiac biopsies of ZF rats and its correlation with IL-33/ST2 signalling. Graphs showed *Epac-1* over-expression in (a) qPCR (p=0.0383) and (b) western blot. (c) Correlation between Epac1 and IL-33 expression (p=0.0099).

Characterization of obesity-induced-remodeling in obese rats

We performed morphologic analysis of rat heart on formalin-fixed section. Haematoxylin and Eosin and Masson's trichrome staining was performed to evaluate cardiomyocytes size and collagen deposition. Although some area of collagen deposition and fat infiltrates were detected in obese animals, we did not demonstrate a significant remodeling of cardiac architecture. (Fig.7)

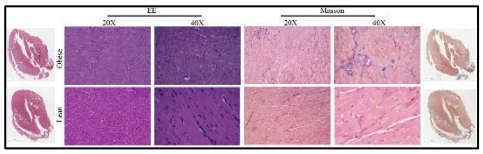

Figure 7: Pictures of Haematoxylin and Eosin and Masson's staining of cardiac biopsies of ZF rats.

Since histologic analysis did not evidence altered cardiac architecture, to go beyond, we performed immunohistochemical analysis of IL-33/ST2 expression in cardiac biopsies. According to literature (43), we observed endothelial expression of IL-33/ST2 – rarely in myofibers – however we did not assess significative differences between lean or obese rats in protein localization or in the amount of protein expression.(Fig8)

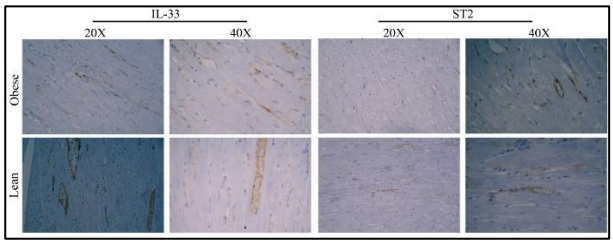

Figure 8: Pictures of IHC analysis of IL-33 and ST2 expression in heart biopsies of ZF rats.

FoxP3 evaluation in cardiac biopsies of ZR

We further decided to evaluate whether altered IL-33 expression could determine an accumulation of T-reg cells in cardiac tissue. In this sense IL-33 signalling could modulate Treg-FOXP3+ cells activity and phenotype on mucosal surface (112) but also in tissues, like in the lungs (113) and in different conditions such in tumor microenvironment (114). Foxp3 Treg cells are important in tissue repair and to avoid autoimmunity. Exogenous IL-33 administration given to normal or cardiac-transplanted mice, by shifting TH response towards TH2 also expands suppressive $CD4^+$ $Foxp3^+$ T regulatory cells (Treg). After MI, IL-33 could promote proliferation and expansion of Treg (115). Although the increase of IL-33

expression in cardiac biopsies of ZF Obese rats, we did not observe a significative increase of FOXP3 protein expression by means WB analysis. (Mean FoxP3/vinculin: Lean: 1.158A U and obese:1.036, p=0.1703) (fig.9).

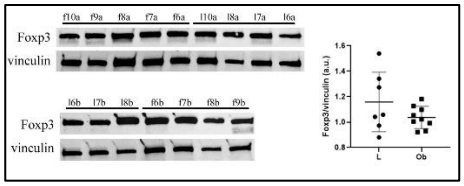

Figure 9: **Evaluation of FoxP3 expression in cardiac biopsies of ZF rats.** WB bands and histogram quantification of FoxP3 and vinculin expression.

Characterisation of Il-33/ST2 on cardiomyocites *in vitro*

Transwell coculture of adipose tissue and H9C2

To better elucidate the interplay among adipose tissue-derived signals and IL-33/ST2 expression in cardiac cells, we performed transwell experiments by plating H9C2 cells with VAT tissue derived from lean or obese animals for 48h**.** On lysed cells we analysed the expression of IL-33/ST2 pathway by qPCR. Adipose tissue stimulation determined the increase of IL-33 expression in H9C2 cells compared to control cells. More interestingly, we observed significant increase of IL-33 expression in cells cultured with adipose tissue from Ob rats, that was higher than those cultured with adipose tissue from Lean rats (Mean IL-33 Lean: 1.101± 0.1020 $2^{-\Delta\Delta CT}$, obese 1.489± 0.1383 $2^{-\Delta\Delta CT}$, with p=0.0468) (Fig.10a) Conversely, stimulation of adipose tissue with obese-derived VAT determined a slight reduction in the expression of ST2L while soluble sST2 did not vary, suggesting a dysregulation in IL-33/ST2L pathway due to obesity (Fig.10b,c). Finally, Epac1 expression was reduced in cells cultured with obese-derived VAT, accordingly to data obtained in cardiac biopsies and negatively correlated to ST2L expression (mean lean 0.8292±0.55560 $2^{-\Delta\Delta CT}$; obese 0.5567±0.06238 $2^{-\Delta\Delta CT}$, with p=0.0115) (Fig.10d). In last analysis, we observed a negative correlation of ST2L and Epac1 (p=0.0172) (Fig.10e).

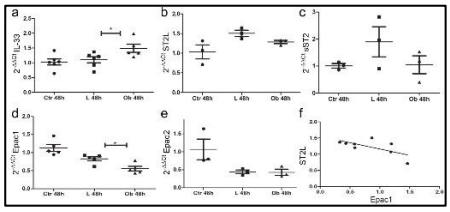

Figure 10: Conditioned medium with VAT tissue isolated by Lean/Obese ZF rats promotes cardiomyocytes gene expression alteration. qPCR experiments showed that fat stimulation from Obese rats promoted (a) *IL-33* expression (p=0.0468) while it reduced (b) *ST2L*. (c) *sST2* was not modified. Fat stimulation reduced (d) *Epac1* expression (p=0.0115), while (e) *Epac2* was unchanged. (f) Graph showed negative correlation between ST2L and Epac1 expression.

Pro-fibrotic and hypertrophic signalling in rat cardiomyocytes after adipose tissue stimulation

We further analysed whether fat stimulation could promote fibrotic or hypertrophic signalling in H9C2 cells: by analysing Yin Yang 1(YY1) and Myocyte-specific enhancer factor 2A (MEF2A). YY1 is a ubiquitous transcription factor, it belongs to the polycomb group protein family. It is a multifunctional zinc-finger transcription factor which can both activate or inhibit transcription, by acting as a transcriptional repressor, activator or initiator element binding protein in a context-dependent manner (116). A lot of of potential YY1 target genes have been identified which involve cell proliferation and differentiation including cancer development. In obesity it was showed that YY1 was up-regulated in hepatic tissue of both obese animals and NAFLD patients (117). Its up-regulation promotes triglyceride accumulation in adipocytes through repression of and Chop 10 16transcription and it increases serum triglyceride and free fatty acid levels. Interestingly, YY1 is increasingly reported to be relevant in pathologic conditions. Indeed it was found to be upregulated by kuster *et al* in the remodeled LV of swine post-MI (118) and in human heart failure, where Sucharov *et al* showed that YY1 acts as a negative regulator of αMyHC gene expression, leading to cardiac hypertrophy (119). Finally, the dual promoter region of ST2 allows the differential expression of the isoforms ST2L and sST2 as a result of a specific modulation due to cell-type or stress condition. The transcription is modulated by the binding of multiple transcription factors to as many consensus sites, including YY1 (120). When linked to the histone deacetylases HDAC4 they act as a co-repressor repressing the transcription of a given gene (121). Indeed, Ascension-Lopez *et al* demonstrated that YY1 and sST2 expression were correlated and up-regulated following myocardial infarct, and they are involved in pathological cardiac remodeling.

We observed a reduction in YY1 (Fig11a) due to VAT stimulation independently from the provenance of VAT tissue from lean (YY1 Mean CTR:1.060 ± 0.1479 $2^{-\Delta\Delta CT}$; mean Lean: 0.6352 ± 0.06292 $2^{-\Delta\Delta CT}$, p=0,0246). or from Obese animals (YY1 Mean ctr: 1.060 ± 0.14792$^{-\Delta\Delta CT}$ versus mean Ob: 0.6066 ± 0.06778 $2^{-\Delta\Delta CT}$, p= 0,0192) and similarly a reduction of MEF2A (Fig11b) expression transcription factor linked to cell differentiation and hypertrophic response (mean ctr: 0.9577 ± 0.1016 $2^{-\Delta\Delta CT}$ versus Lean 0.5371 ± 0.06221 $2^{-\Delta\Delta CT}$, p= 0,0055) and mean ctr 0.9577 ± 0.1016 $2^{-\Delta\Delta CT}$ versus Ob 0.4861 ± 0.05685 $2^{-\Delta\Delta CT}$, p=0.0023) while

no differences arose between cells treated with VAT from Lean or Obese rats in YY1or MEF2A expression.

The reduction of YY1 was directly related to the reduction of Epac1 signalling (Fig11c) ($p<0.001$). These data suggest that adipose tissue promoted a reduction in hypertrophic /fibrotic signalling mediated by IL-33-mediated repression of Epac1 transcription. ST2L expression was also negatively related to YY1 expression suggesting that the reduction of ST2L is linked to a switch towards a fibrotic response. (Fig11d)

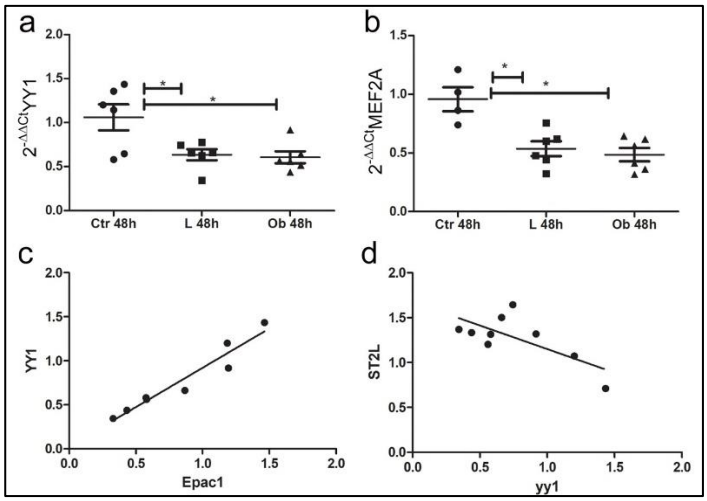

Figure 11: Conditioned medium from VAT tissue influenced hypertrophic/fibrotic signalling in cardiomyocytes. Graphs showed qPCR experiments with transwell between H9C2 cells and VAT tissue from Lean or Obese ZR. Fat stimulation reduced (a) *YY1* expression in both Lean ($p=0,0246$) and Ob ($p= 0,0192$) and (b) *MEF2A* expression in both Lean ($p=0,0055$) and (Ob $p=0.0023$) versus CTR. (c) Graph showed positive correlation between YY1 and Epac1 ($p<0.001$) while (d) negative correlation emerged between ST2L and YY1 ($p<0.00329$).

Modifiers of IL33/ST2 signalling:

In vitro Ghrelin stimulation of cardiomyocites

It is well accepted that Zucker rats present an increase in serum ghrelin (GHR) level (due to lacking of leptin signalling): GHR is principally secreted by gastric mucosa but also from cardiomyocytes, that normally present GHR receptor (122). This way, we decided to assess the effect of GHR stimulation on cardiomyocytes and whether GHR signalling could affect IL-33 release. We treated H9C2 cells GHR for 72h. We observed by means qPCR that - starting from 48h of treatment (Ghrelin group) when cells became confluent - GHR stimulated IL-33 expression in H9C2 cells (IL-33 Ctr: 1.033 ± 0.13012$^{-\Delta\Delta CT}$ Ghrelin 1.381 ± 0.07609 $2^{-\Delta\Delta CT}$ p=0,0396) (Fig11a) and up-regulated Epac2 inducible form (Epac2 Ctr: 1.088 ± 0.2116 $2^{-\Delta\Delta CT}$ Ghrelin 3.223 ± 0.1952 $2^{-\Delta\Delta CT}$ p<0.0001) – but not Epac1 (Fig12a,c,b respectively). We observed the up-regulation of MEF2A, a transcription factor linked to myogenesis and hypertrophy (MEF2A Ctr:0.9169 ± 0.07739 $2^{-\Delta\Delta CT}$ and Ghrelin: 1.298 ± 0.1275 $2^{-\Delta\Delta CT}$ p=0,0339) and of HDAC4 (HDAC4 Ctr:1.008 ± 0.05528 $2^{-\Delta\Delta CT}$ and Ghrelin:1.207±0.05009 $2^{-\Delta\Delta CT}$, with p=0.0279) that together with YY1 (a fibrotic transcription factor) can stimulate sST2 transcription. However, we did not assess any change in YY1 expression (Ctr 1.014 ± 0.07272 $2^{-\Delta\Delta CT}$ and Ghrelin:1.066 ± 0.06077 $2^{-\Delta\Delta CT}$, p= 0.5953). (Fig.12b)

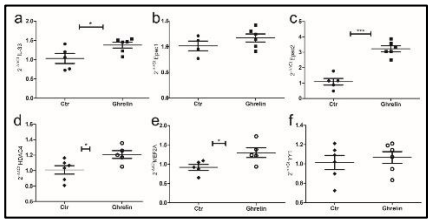

Figure 12: *in vitro* Ghrelin treatment of H9C2 cells modulated IL-33 signalling. Graphs showed qPCR of H9C2 cell treated with ghrelin or vector at 72h. Ghrelin treatment enhanced (a) *IL-33* (p=0.0396) and (c) *Epac2* expression (p<0.0001), while (b) *Epac1* was unchanged. (d) Ghrelin treatment also induced *HDAC4* (p=0.0279) and (e) *MEF2A* (p=0,0339) while it did not alter *YY1* expression.

Indeed, we observed increased expression of GHR receptor (GHSR Ctr:1.327 ± 0.4312 $2^{-\Delta\Delta CT}$ and Ghrelin: 3.494 ± 0.3827 $2^{-\Delta\Delta CT}$, with p=0.0044) and interestingly we showed an up-regulation of sST2 (sST2 Ctr:0.8200 ± 0.1071 $2^{-\Delta\Delta CT}$ and Ghrelin: 3.075 ± 0.2473 $2^{-\Delta\Delta CT}$, with p<0.0001) Conversely, we did not observe any difference in ST2 Ligand isoform (Ctr St2L: 1.077 ± 0.1904 and Ghrelin: 1.247 ± 0.05659 $2^{-\Delta\Delta CT}$ p=0,4176) (Fig13b.)

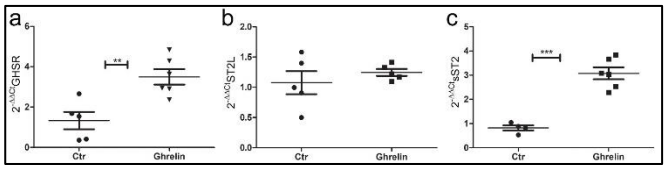

Figure 13: *in vitro* Ghrelin treatment of H9C2 cells modulated ST2 signalling. Graphs showed qPCR of H9C2 cell treated with ghrelin or vector at 72h. Ghrelin treatment enhanced (a) *GHSR* expression (p=0.0044), while (b) *ST2L* was unaffected. (c) The *sST2* mRNA was greatly increased by ghrelin treatment (p<0.0001).

Characterization of Ghrelin-IL-33/ST2 axis in Zucker rat cardiac tissue

As we observed a Ghrelin-dependent increase in IL-33 expression in cardiomyocytes in vitro, that was link to up-regulation of sST2 and of MEF2A, we decided to assess the mRNA levels of all these targets in cardiac biopsies of ZR to verify that this pathway in also active in vivo. Unexpectedly, we did not observe important difference in mRNA expression of YY1 or MEF2A in cardiac biopsies of Ob versus Lean rats (Fig.14a and b). However, the expression of these two factors is slightly positive correlated (p=0.0054). (Fig.14d). Finally we evaluated the expression of GhR receptor (GHSR) as it was demonstrated that GHSR is a marker of heart failure (123). In heart homogenates we observed an increase of GHSR expression, confirming the activation of ghrelin signalling in Ob rats (GHSR Lean: 0.8259 ± 0.1240 and Ob: 2.606±0.5754, with p= 0,0362) (Fig.14.c)

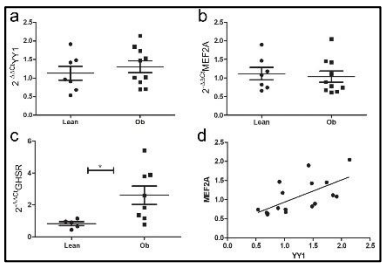

Figure 14: Ghrelin-induced pathway analysis in ZF cardiac biopsies. Graphs showed qPCR of (a) *YY1* and (b) *MEF2A* expression in cardiac biopsies of ZR. (c) *GHSR* mRNA was greatly increased *in vivo* in obese ZR (p=0,0362) while (d) a positive correlation was observed between MEF2A and YY1 in cardiac biopsies of ZR.

Characterization of IL-33/ST2 signalling in ZDF rats

Circulating level of Il33/St2 signalling

Further inbreeding of ZF rats resulted in new phenotypes as the Zucker Diabetic rats (ZDF) that spontaneously develop insulin resistance, T2DM, hyperlipidaemia and moderate hypertension. Although we assessed a slight increase of weight in diabetic rats, they did not develop obesity (Mean L: 394.0 ± 22.21g and Ob: 418.3 ± 10.27g) (Fig.15a).

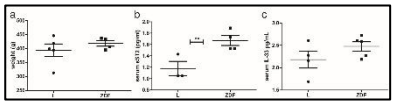

Figure 15: IL-33/ST2 pathway analysis in serum of ZDF rats. Graphs showed (a) weight of rats at sacrifice, and (b) ELISA assay of serum ST2 (p=0.0206) and (c) IL-33.

To evaluate IL-33 and ST2 expression, we measured these proteins in serum through ELISA. We observed a slight increase of serum IL-33 (Mean L: 2.181 ± 0.1902 pg/ml and Ob: 2.476 ± 0.1036pg/ml with p=0.1913 (Fig.15c) and a statistically significative increase of sST2 (Mean L: 1.177 ± 0.1267pg/ml and Ob: 1.670 ± 0.08718pg/ml, with p=0.0206) (Fig.15b). Finally, we did not observe any correlation between IL-33 circulating level and the weight of the rats.

Cardiac expression

In cardiac biopsies we observed a not significant decrease of IL-33 mRNA (mean ZDF: 0.8405 ± 0.08304 $2^{-\Delta\Delta CT}$ and mean lean: 1.025 ± 0.1145 $2^{-\Delta\Delta CT}$ p= 0,2543) (Fig.16a), as further confirmed in WB analysis (Mean Lean: 0.7460 ± 0.06043 and ZDF: 0.5549 ± 0.07911 with p=0,1033) (Fig.17). qPCR showed a not significant decrease of ST2, but, conversely we observed an increase of both Epac1 (Mean ZDF:1.279±0.06931, Lean: 0.9219±0.1057, with p=0.031) and Epac2 mRNA level (Mean ZDF:1.605±0.1115, Lean 1.042±0.1265, with p=0.0142) (Fig.16c,d).

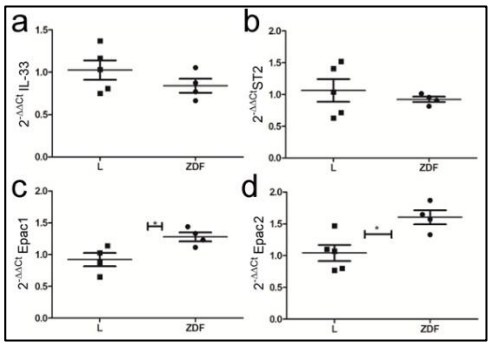

Figure 16: IL-33/ST2 pathway analysis in cardiac biopsies of ZDF rats. Graphs showed qPCR expression in cardiac biopsies of (a) *IL-33* and (b) *ST2*. Diabetics rats showed increased expression of both (c) *Epac1* (p=00301) and (d) *Epac2*.

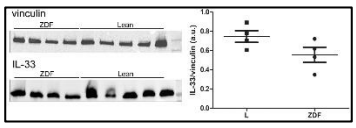

Figure 17: IL-33 protein expression in cardiac biopsies of ZDF rats. Graphs showed WB quantification of IL-33 and vinculin in cardiac biopsies of ZDF rats.

Interestingly, in WB analysis we observed a reduction of ST2L ligand form, while sST2 was not modified, suggesting a reduction of cardioprotection linked to diabetes (ST2L Mean L: 0.9145 ± 0.2521 AU and Mean ZDF: 0.2406 ± 0.09399 p= 0,0579) as shown in Fig.18.

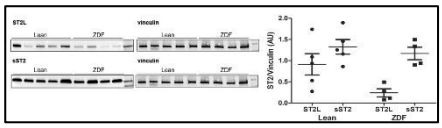

Figure 18: ST2 isoform expression in cardiac biopsies of ZDF rats. (a) Graphs showed WB quantification of ST2 isoforms and vinculin,in cardiac biopsies of ZDF rats.

Fibrotic remodeling of diabetic heart

We investigated whether the IL-33/ST2 deregulation determined fibrotic modulation in diabetic heart. We analysed a panel of gene involved in matrix remodeling and we observed the activation of pro-fibrotic genes (overexpression of MMP2 COL2A TGF etc.). However, neither histologic analysis or WB experiments confirmed these preliminary evidences. Both levels of collagen 3 and TGFβ were unchanged between Lean or diabetics cardiac biopsies. (Fig.19)

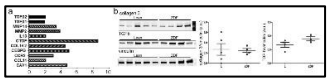

Figure 19: Analysis of fibrotic remodeling of cardiac biopsies of ZDF rats. Graphs showed (a) a panel of altered-profibrotic proteins and (b) WB quantification of TGFβ, collagen3 isoform and vinculin in cardiac biopsies of ZDF rats.

Similar to previous results obtained in ZF rats, haematoxylin and Eosin staining did not reveal important morphological alterations and trichrome Masson staining did not highlight any area of focused collagen deposition, fiber necrosis nor ventricular dilatation of diabetic heart as shown in Fig.20.

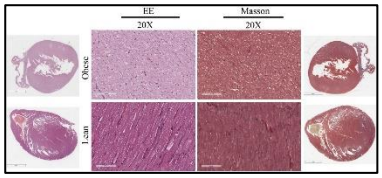

Figure 20: Pictures of Hematoxylin and Eosin and Masson's staining of cardiac biopsies of ZDF rats. Histological analysis did not reveal marked alteration of cardiac architecture in diabetics versus lean rats.

Characterisation of IL-33/ST2 pathway in Diet induced obesity (DIO) mice

To avoid the genetic bias due to leptin receptor mutation present in ZR, we decided to reproduce our experiments in a naturally occurring animal model of obesity. Thanks to these experiments we could better reproduce human disease, and – by comparing obtained results with previous ones – we could also highlight deeper mechanism linked to IL-33/ST2 pathway. Thus, we choose the DIO mice.

Circulating levels of IL-33/ST2 in DIO mice

Diet-induced obese (DIO) C57BL/6J mice are a model of pre-diabetic type 2 diabetes and obesity with mildly elevated blood glucose and impaired glucose tolerance. After 20 weeks of either normal chow diet (Lean group) or high fat diet (Obese group) we collected serum to analyze Il-33/ST2 signalling. In their serum we detected a decrease of sST2 and no difference in IL-33 circulating level. As expected, serum Leptin was increased in DIO mice (Mean L 40.22 ± 5.065pg/ml and mean Ob: and leptin 932.1 ± 71.0 pg/ml with p<0.0001), but no correlation existed with IL-33, while it negatively correlated to sST2 levels (Mean Lean 7.718 ± 0.06444 pg/ml; mean Ob: 6.635 ± 0.3007 pg/ml with p<0,0001); Spearman r= -0,8054 with p<0.0001) (Fig.21).

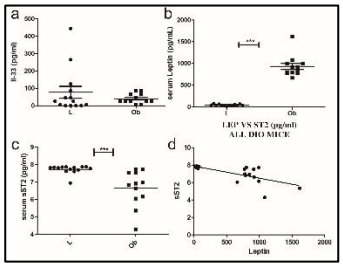

Figure 21: Circulating level of IL-33/ST2 and Leptin in DIO mice. Graphs (a) showed ELISA assay of serum IL-33 and (b) no significant correlation with leptin; (c) ELISA assay of ST2 (p=0.0206) and (d) negative correlation with leptin (p<0,0001).

Obesity alters inflammatory status in DIO mice

As it is known that obesity provokes a state of chronic low-grade inflammation, we analysed circulating level of both pro and anti-inflammatory cytokines. We did not observe significative differences in TNFα or IL-6 (data not shown); conversely, we observed an increase of FGF-21 (Mean L:184.0 ± 22.55pg/ml and Ob:11160 ± 1135pg/ml with p<0.0001) and a reduction

in IL-10 (Mean L:5.063 ± 0.6454pg/ml and Ob: 3.008 ± 0.6365 pg/ml with p=0.0326). On the contrary adiponectin and RAGE levels were unchanged. (Fig.22)

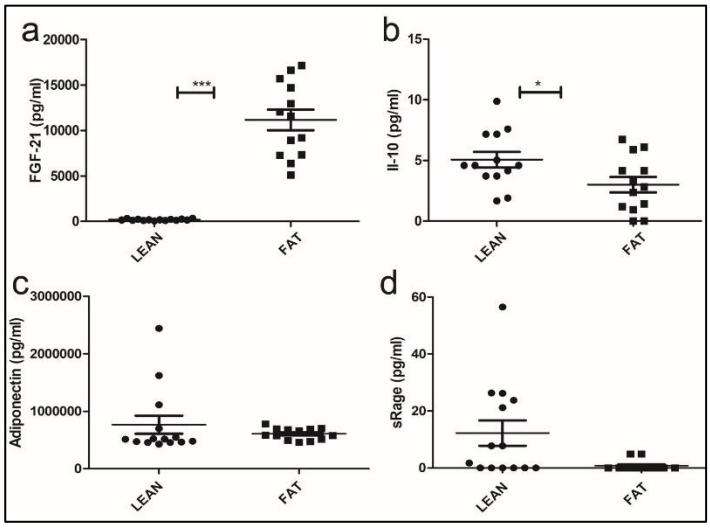

Figure 22: Circulating levels of pro- and anti-inflammatory cytokines in DIO mice. ELISA assay assessed up-regulation of serum (a) FGF-21 (p<0.0001) and (b) IL-10 (p=0.0326) in obese animals. (c) Adiponectin and (d) sRAGE were unchanged.

Adipose and cardiac expression of IL-33/ST2 pathway

HF diet altered the expression of IL-33/ ST2 pathway in visceral adipose tissue of obese mice. We observed a reduction of IL-33 expression (Mean L:0.3209 ± 0.03246 $2^{-\Delta\Delta CT}$; Ob:1.312 ± 0.4011 $2^{-\Delta\Delta CT}$, with p= 0,0140) and an increase in total ST2 (Mean L 2.696 ± 0.3184 $2^{-\Delta\Delta CT}$; Ob:1.094 ± 0.2204 $2^{-\Delta\Delta CT}$, with p=0,0023. Differently from cardiac tissue of obese ZR we observed an increase in Epac1 expression (Mean L 2.788 ± 0.5175 $2^{-\Delta\Delta CT}$; Ob:1.242 ± 0.3806 $2^{-\Delta\Delta CT}$, with p=0,0396). (Fig.23)

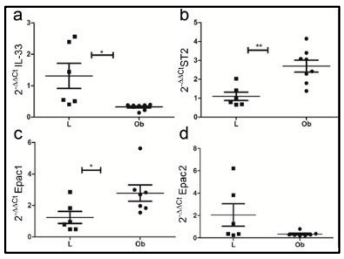

Figure 23: Analysis of visceral adipose expression of IL-33/ST2 pathway and Epac in DIO mice. qPCR graphs showed that fatty diet induced (a) reduction of *IL-33* expression (p=0,0140) while an increase of both (b) *ST2* (p=0,0023) and (c) *Epac1* (p=0,0396) in obese versus lean animals. (d) *Epac2* mRNA was unchanged.

These data were not confirmed in cardiac tissue of obese mice, further highlighting the important difference in IL-33/ST2 regulation depending on tissue and cell type considered. In cardiac tissue we observed an increase in Epac2 expression (Fig24) (Epac2 Mean L 2.466 ± 0.4844 $2^{-\Delta\Delta CT}$; Ob:6.398 ± 1.051 $2^{-\Delta\Delta CT}$, with p=0,0068)

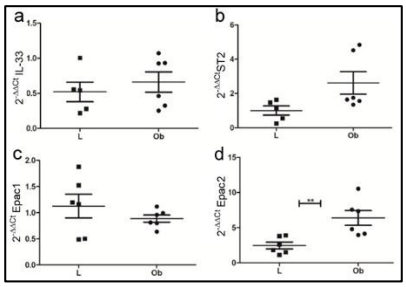

Figure 24: Cardiac expression of IL-33/ST2 pathway in DIO mice. Graph showed qPCR expression of (a) *IL-33*, (b) *ST2* and (c) *Epac1* that were not altered by fatty diet in DIO. (d) *Epac2* mRNA level was increased in Obese mice versus lean ones (p=0,0068).

Impact of fatty diet on cardiac proteome of DIO mice

To analyse the effect of fatty diet on cardiac metabolism, we performed proteomic analysis of whole cardiac tissue, in collaboration with the group of Prof. Tedeschi. Thanks to a quantitative shotgun label-free strategy we compared the entire proteome of the whole heart of lean and obese group. Among the proteins identified we observed some proteins that were differently expressed between the groups. Among the protein up-regulated in obese group we observed TGF, DCn, Collagen, ColGA3, MBL2, Col15A1 and Interleukin A2m. On the contrary among proteins down-regulated in the obese group we found Collagen sec61alpha-collagenIV, prostaglandin PTGR2 and interleukin TIMM50. Further analysis revealed proteins expressed only in the heart of obese mice although at low level: Adamts1, adamts16, adamts19 and adamts7. All of them are metalloproteinase involved in matrix remodeling (124). The bioinformatic analysis, carried out on these proteins by Cluego and Panther software, demonstrated a great impact of the fatty diet on cardiac proteome. (Fig.25)

Among the enriched networks identified by String software and Ingenuity Pathway Analysis (IPA) in HF hearts, we predict an impact on cardiac morphology as functional networks analysis shown an enrichment of pathways involved in fibrosis and left ventricular dilatation. The pathway involved are interconnected and regulates cardiovascular system development and disease. A further graphical representation of IPA analysis shows up-regulation of proteins involved in fatty acid metabolism and lipid transport, such as peroxisome proliferator-activated receptor-α,γ (PPARα and PPARγ), and their linkage to pathways involved in the organisation of cytoplasm and the formation of muscle cells underlying the impact of lipid excess on energy metabolism and cardiac cell homeostasis. The results of bioinformatics analysis were summarised in Figure 26.

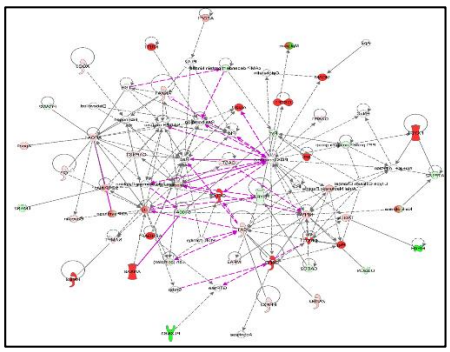

Figure 25: Cardiac proteome evaluation in DIO mice. Bioinformatic analysis by Ingenuity Pathway Analysis (IPA) of the proteins differentially expressed in the comparisons between the high-fat and normal chow group in mice. Image showed enriched networks in heart tissue of obese DIO mice.

IPA also identified the cascade of upstream transcriptional regulators that can explain the changes occurring in protein expression: interestingly, one of the upstream regulators was the IL-33. In our network IL-33 could be an inhibitor linked to ACAT1, ApoE, EPHX2, IGHM, ITIH1, MYLK, MARKS and UCP1 proteins, that are collectively involved in energy and cholesterol metabolism, inflammation and matrix remodeling. Interestingly IL33 was already known to regulate expression of genes involved in cholesterol metabolism and the same indirect interaction between ApoE and IL-33 was observed through IPA in serum from asthma patients (125), supporting our results.

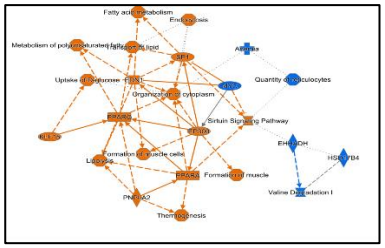

Figure 26: Graphical summary of the bioinformatic analysis of cardiac proteome in DIO mice. Increased proteins/processes are indicated in orange and decreased proteins/processes are indicated in blue

Discussion

Obesity is a multifactorial condition characterized by fat accumulation. Although there is a well-known association between obesity and cardiovascular pathology, the mechanism by which fat accumulation provokes heart remodeling is not definitely understood. In obesity there is an ectopic fat accumulation in the heart, called cardiac steatosis, that involves both the epicardium and the myofibers. Epicardial adipose tissue (EAT) shares blood supply with myofibers thus it represents a biologically active tissue that contributes to cardiac remodeling in pathological conditions, through secretion of adipokines. This fat storage physiologically protects myocardial homeostasis by the controlled release of FFA, adiponectin and orosomucoid (126) that exerts anti-oxidant effect. On the contrary, it enhances low-grade inflammation and diabetic vascular complications through the recruitment of monocytes and the release of retinol binding protein-4 or activin A (127). Recently, a few studies investigated the role of EAT in the regulation of myocardial metabolism. Firstly, Gruzdeva et al demonstrated a direct relation between EAT thickness and visceral obesity in a cohort of post-AMI patients. Indeed, they also determine a role for IL-33 system that correlates with fibrosis and maladaptive remodeling one year post IMA. Similarly, previous study from our group demonstrated a disbalance in IL33/ST2 expression in EAT tissue that correlates with ventricular remodeling and left ventricle dysfunction in CAD patients (96). In this sense, IL-33 signalling is known to exert a protective role in cardiovascular remodeling, in particular when released by necrotic cells after myocardial injury through the interaction with its receptor ST2L. On the contrary, the soluble form of ST2 receptor (sST2) binds IL-33, blocking its protective signalling. However, its role in obesity is still a matter of debate. Interestingly, IL33/ST2L signalling is involved in fat deposition: in obese human subjects, adipose tissue-resident endothelial cells increase expression of both IL-33 and ST2. However soluble ST2 serum levels are increased demonstrating a greater cardiovascular risk in human subject.

In this PhD project, we planned to evaluate the influence of adipose tissue on IL-33/ST2 pathway regulation and its role in cardiac steatosis and fibrotic remodeling in animal model of obesity. To deeply investigate the role of IL-33/ST2 signalling we decided to exploit different experimental models, both *in vitro* and *in vivo*. Indeed, we characterized different animal models such as Zucker rats and DIO mice. By comparing several results in animals that arose from

different type of obesity (naturally developed or genetically induced), we were allowed to discriminate between different mechanisms of gene expression and regulation. Firstly, we characterized IL-33/ST2 expression in serum, cardiac and adipose tissue of Zucker rats, which harbor a mutation in the leptin receptor gene that blocks leptin signalling. We showed that obesity determined a dysregulation of IL33/ST2 pathway characterized by a reduction of serum IL33 and an increase of serum ST2 in Obese animals compared to lean controls. An opposite relation emerged between weight and circulating IL-33 in Lean versus Obese animals. Indeed IL-33 seems to increase with weight till reaching certain level, but - with increased fat accumulation in obese rats - it tends to decrease. However, as introduced above, the measurement of IL-33 in serum suffers from some limitations. The first is due to its rapid short-half life and its rapid clearance from the circulation by proteases. IL-33 could also be oxidized by reactive species and it is of particular interest in disease state such in obesity or smokers (states of increased ROS production). The assay has technical limitation due to antibody used, as it varies in affinity and it is unable to bind modified or oxidized IL-33. Finally, IL-33 availability depends on its binding to sST2, whose concentration is increased in obese rats: it is possible that the true circulating IL-33 was actually different. On the other hand, the assay of sST2 is considered reliable and it is now included in clinic for cardiovascular risk assessment.

In Obese Zucker rats IL-33 protein increased in cardiac and visceral adipose tissue (VAT), while ST2 only in VAT. Unfortunately, we failed to demonstrate a significative difference in ST2 isoforms expression, possibly due to high variability observed among samples, as cardiac biopsies could have randomly ventricular or atrial origin and ST2 expression could depend on cell type. In this sense ST2L and sST2 mRNA are expressed at low levels in cardiomyocytes and cardiac fibroblast, but they are highly expressed by the endothelium and ST2L is expressed on most immune cells. As we observed a dysregulation in IL-33/ST2 signalling we evaluated whether this condition determined a cardiac fibrotic response. In cardiac biopsies, increased expression of pro-fibrotic genes such as MMP8, MMP9 and TIMP - that regulates matrix degradation - indicated fibrotic remodeling in obese rats (128). These findings were further confirmed by increased TGFβ and collagen deposition (measured by WB and Sircol red). However histological analysis (EE and Azan Mallory staining) showed comparably level of collagen deposition between lean ad obese rats and we could only observe some area of fat

infiltrates without significative modification of cardiac architecture. Similarly, we assessed the expression of IL-33/ST2 on cardiac tissue by immunohistochemical analysis, to evaluate difference in cell expression: we only confirmed the endothelial expression of IL-33 and ST2, while ST2 was also expressed on rare myofibers. Aberrant cAMP signalling contributes to pathological cardiac remodeling and heart failure by regulating intracellular calcium release (129). Besides the well-known PKA signalling, cAMP response could be mediated also by Epac protein (130). In the heart, Epac has been shown to play a role in Ca^{2+}-handling and excitation-contraction coupling via phospholipase C, CaMKII, and PKC dependent signalling. Epac proteins are guanine-nucleotide-exchange factors (GEFs) and two principal isoforms exist: Epac1 mRNA is nearly ubiquitous while Epac2 isoform is mainly expressed in the brain and endocrine tissues, such in adipose tissue where it controls adipogenesis (131).

In the heart, Epac1 could be both a pro-fibrotic and an anti-fibrotic agent depending on cell type. Epac1 KO mice appear to be protected from cardiomyocytes hypertrophy, fibrosis, and cardiac dysfunction induced by different stress conditions - pressure overload and ischemia reperfusion model - while in fibroblasts Epac1 seems to inhibit TGFβ-signalling. Interestingly they seem to have a role only in stress condition as Epac1- or Epac2-null mice did not show cardiac alterations (132, 133)

Several evidence indicated that CaMKII is a key effector of Epac1 hypertrophic signalling (134): following phosphorylation, it induces the nuclear export of HDAC4/5 thus relieving their inhibition on the pro-hypertrophic transcription factor MEF2A (135). Furthermore, in previous study from our group, we determined a positive correlation between Epac2 and IL-33 signalling in EAT tissue (96).

Thanks to these premises we decided to evaluate Epac1 expression in cardiac biopsies. In obese Zucker rats, we found reduced expression of Epac1 that was negatively related to IL-33 expression, suggesting a possible modulation of TGFβ/MEF2A regulation of transcription. To evaluate whether adipokines from adipose tissue could directly influence cardiomyocytes metabolism we performed transwell experiment by coculturing H9C2 cells (a line of fetal cardiomyoblasts) with VAT derived from Lean or Obese Zucker rats. Fat stimulation caused IL-33 up-regulation that was higher following stimulation with adipose tissue from Ob rats while

ST2L expression was greatly increased following stimulation from Lean animals. Finally, in line with results obtained in Zucker rats, Epac1 expression was reduced by fat stimulation, negatively related to ST2L expression. According to these results, we demonstrated that IL-33/ST2 signalling is deregulated in obesity and, importantly, incorrect ST2L/sST2 balancing drove the pro-fibrotic switch in gene expression. Furthermore, fat stimulation could influence the expression of YY1 and MEF2A, that are transcription factor involved in stress and drive hypertrophic or fibrotic response. The complex mechanism that regulates the interplay between YY1/MEF2A and IL-33/ST2 are not understood. However, a direct correlation was evidenced between YY1 and Epac1 while it was negatively related to ST2L. Indeed, in literature is reported that YY1 can increase the transcription of sST2 (thanks to the presence of a consensus sites in its dual promoter region) and that this is involved in fibrotic remodeling of perinfarctual zones (120). Importantly, YY1 has also been involved in obesity where it promotes triglyceride accumulation (117). However, the genetic background of Zucker rats could influence our results, as leptin signalling - that is lacking - determines central and peripheral effects. Indeed, the ZDF rat has high levels of plasma leptin. Leptin is a hormone that is mainly produced in white adipose tissue by the mature adipocytes but it can also be released by brown adipose tissue, skeletal muscle, placenta, ovaries, bone marrow, stomach and other tissues, although in a lesser extent. It has a central effect in the hypothalamus where it regulates in satiety and food intake. However, leptin signalling was also considered to be involved in a variety of physiological processes as the regulation of energy homeostasis, thermogenesis, skeletal growth (136). In this sense, altered leptin signalling could be responsible of a multitude of consequences depending by expression of short intracellular forms of Ob-R in various peripheral tissues, where it, activates JAK/STAT pathway after homodimerization.

To overcome this limitation, we performed *in vitro* experiment trying to partially reproduce ZR condition on cardiomyoblasts in culture. We knew from literature that Zucker rats also present an increase in serum ghrelin (GHR) level (due to lacking of leptin signalling): GHR is principally secreted by gastric mucosa but also from cardiomyocytes, that normally present GHR receptor. This way, we decided to assess the effect of GHR stimulation on cardiomyocytes and whether GHR signalling could affect IL-33 release. We observed that GHR stimulated IL-33 expression in H9C2 cells and upregulated Epac2 inducible form – but not Epac1. Indeed, we observed

increased expression of GHR receptor and the up-regulation of MEF2A, a transcription factor linked to myogenesis and hypertrophy whose regulation is Epac-dependent. Interestingly we showed an upregulation of sST2 and HDAC4, that together with YY1 (a fibrotic transcription factor) can stimulate sST2 transcription.

Thanks to these results we reasoned on the effect of the combination of fat stimulation and ghrelin signalling *in vivo* on cardiomyocytes. The up-regulation of GHSR in ZF obese rats demonstrated the activation of this signalling *in vivo*, but we did not observe any difference between MEF2A and YY1 expression. These findings are probably the result of a ghrelin compensative pathway that counteracts the dysregulation of IL-33/Epac signalling. Similar results were obtained in Zucker diabetic rats, that result from a further inbreeding of Zucker rats: they presented moderate hypertension and diabetes but developed a less obese phenotypes. In line with our data, we observed increased expression of cardiac IL-33 and of sST2 and conserved architecture of left or right ventricle. Similar to Zucker rats, histological analysis did not evidence altered cardiac architecture, although the presence of pro-fibrotic gene expression. Interestingly we observed an increase of both Epac1 and Epac2 mRNA levels. In this sense Epac2 is involved in cAMP regulation of insulin secretion. Epac2 seems to potentiate insulin secretion by pancreatic β-cells (137). Indeed, Epac2 agonists are now viewed as possible therapeutic targets for lowering hyperglycemia (138). Accordingly, increased Epac2 in ZDF rats than in ZF rats could reflect a compensative response to the development of hyperglycaemia.

To avoid the genetic bias due to leptin receptor mutation, we decided to reproduce our experiments in DIO mice. The obesity in these mice is the result of a high calories and fat diet, thus it basically reproduces human pathology. This way we eliminated genetic bias due to leptin mutation in Zucker rats. In obese DIO mice sera, we detected a decrease of sST2 and no difference in IL-33 circulating level. As expected in a naturally occurring model of obesity, serum Leptin was increased and it was negatively related to serum sST2. Fat accumulation was accompanied by an inflammatory status characterized by increased serum FGF-21 and reduced IL-10. In adipose tissue we observed a decrease in IL-33 and an increase in ST2 and Epac1 expression, but these findings were not confirmed in cardiac tissue where - on the opposite - we observed only an increase in Epac2 expression.

To further compare previous analysis in Zucker rats with DIO mice, we performed proteomic analysis of whole cardiac tissue. This analysis allowed us to analyze the effect of fatty diet not only IL-33/ST2 pathway but also on cardiac metabolism and functionality. Preliminary analysis through Cluego software revealed an up-regulation in HF mice of proteins involved in matrix remodeling (ADAMTS, MMP2). Among the enriched networks identified by String software and Ingenuity Pathway Analysis (IPA) in HF hearts, we detected cardiovascular system development and disease networks. In particular, functional networks analysis suggested the presence of fibrosis and left ventricular dilatation while up-regulated processes in metabolism (fatty acid metabolism and lipid transport, PPARα and PPARγ). IPA also identified the cascade of upstream transcriptional regulators that can explain the changes occurring in protein expression: interestingly, one of the upstream regulators was the IL-33. In our network IL-33 could be an inhibitor linked to ACAT1, ApoE, EPHX2, IGHM, ITIH1, MYLK, MARKS and UCP1 proteins, that are collectively involved in energy and cholesterol metabolism, inflammation and matrix remodeling. Interestingly IL-33 was already known to regulate expression of genes involved in cholesterol metabolism and the same indirect interaction between ApoE and IL-33 was observed through IPA in serum from asthma patients (125) supporting our results.

Conclusions

To investigate the IL-33/ST2 signalling dysregulation and its effect during cardiac adiposity accumulation, we took advantage of different models of obesity both *in vitro* and *in vivo*. Although IL-33 is traditionally considered to be cardioprotective through the activation of its membrane receptor ST2L, some controversies exist. Indeed IL-33 exerts a less known role of repressor of transcription, thanks to its chromatin-binding motif that is supposed to function as a transcriptional repressor (2). The role of intranuclear IL-33 signalling in cardiac disease and in obesity is still under investigation: in this sense it was observed that IL-33 administration in healthy mice determine fibrosis (28). Thus, both pro- and anti-inflammatory actions have been ascribed to nuclear or extracellular IL-33 and – moreover - in many cell types, the role of nuclear IL33 is still unknown. IL-33 overproduction could also be related to pro-inflammatory cytokine release and auto-immunity. Here we showed that obese adipose tissue promoted IL-33 production both in vivo and in vitro. In Zucker rats, this pathway led to a ST2L reduction, suggesting that cardioprotection is lost despite IL-33 increase. Further analysis demonstrate that the overproduction of IL-33 determined a reduction of Epac1. In our model, ghrelin up-regulation determined IL-33 over-expression: in this sense, IL-33 increase in cardiac tissue could represent a compensative pathway to counteract the excess of FFA or inflammation associated to increased adipocytes' size and mass. Although, chronic IL-33 overproduction will finally end in maladaptive remodeling, but in Zucker rats, ghrelin excess protects the heart by reducing apoptosis and promoting survival of cardiomyocytes. This pathway Is linked to the promotion of MEF2A. On the other hand, ghrelin stimulation enhanced sST2 production rather than ST2L, that will finally end in an increase of cardiovascular risk. Indeed, both ghrelin receptor and sST2 are involved in hypertrophic response related to stretch of cardiomyocytes in stress response. Furthermore, they are both considered as a marker of heart failure as they increased with severity of ventricular dysfunction in human disease (123). Obese Zucker rats presented a molecular signature of activated fibrotic response to fat accumulation but this is not translated in altered cardiac architecture due to the compensative response of IL-33 and ghrelin, although at the stage considered in these experiments. Thus, the effect of IL-33 signal in our model seems to be protective and increased following ghrelin stimulation, but its activation also promoted sST2 expression, counteracting the protective effect. In this sense it

was postulated that IL-33 is highly dependent on cellular and temporal expression, as an example in liver fibrosis, opposite effects of IL-33 were observed: tissue-protective mechanisms when acute and massive liver damage occurs and pro-fibrotic hepatic factor in cases of chronic injury (139).

On the other hands, obesity in DIO mice exerted different effects on IL-33/ST2 pathway. Indeed, we observed the IL-33 transcriptional role in promoting cardiac remodeling through the modulation of pathways linked to fatty acid oxidation, metabolism and matrix remodeling. In this sense several works reported that IL-33 influenced gene and protein expression, but it was recently demonstrated that it acts independently of its nuclear localization (3, 7, 140). However it was assessed that IL-33 could regulate interstitial cell extracellular matrix deposition in fibroblast injury (141) and that it is correlated to genes involved in DNA repair or mitochondrial function (142). Finally, as we mentioned above, IL-33 was linked to cholesterol regulation and ApoE in asthma patients.

Conflicting results in literature reported high or low level of IL-33 depending on animal model, tissue analyzed, acute or chronic condition considered. As an example expression of IL-33 mRNA was increased in adipose tissue of obese Wistar rats (65) while it was reduced in obese Zucker rats (68). Kai revealed opposite IL-33 mRNA expression in DIO mice between SAT and epididimal VAT, suggesting that tissue-resident IL-33 may play different roles. Exogenous administration of IL-33 for a limited time, seems to be protective in obesogenic condition in mice (67) where its effects are probably mediated by ST2L receptor and modulated by inflammation. However chronic uncontrolled IL-33 upregulation (endogenous or exogenous) could determine unexpected effects via nuclear signalling in different tissue such as the heart. In this sense, administration of IL-33 in healthy mice determines pericarditis (47). Interestingly IL-33 signalling could be referred to the “obesity paradox”, as being overweight or obese is associated with a favorable prognosis while having increased cardiovascular risk. Similarly, overweight condition will promote IL-33 signalling and ameliorate low-grade inflammation while increasing cardiovascular risk through increased sST2. Our data confirmed that therapeutic consideration cannot be postulated without considering the model used in the experiments and highlighted that the consideration of a protective role of IL-33 is probably over-simplistic.

In this project we further highlight a role for Epac protein in heart remodeling and their link to IL-33/ST2 pathway. However, in cardiac tissue the over-expression of IL-33 and the downregulation of Epac1 could affect not only muscular cells but also resident cells such as fibroblasts and endothelium. The role of Epac1 seems to depend on cell type, with opposite role in fibroblasts - where it has an anti-fibrotic role mediated by the inhibition of TGF-β - and in cardiomyocytes - where it activates MEF2A response: this way, further experiments are needed to evaluate their exact role in Obese Zucker rats cardiac remodeling or in other models of obesity, as proteomic analysis in DIO mice did not confirm their role.

Different limitations emerged during this PhD project. Firstly, this project arose from previous studies published in my lab about the deregulation of epicardial adipose tissue in obese human subjects. It was planned in the attempt of overcoming difficulties coming from the availability of human samples, in particular of healthy epicardial tissues. Accordingly, we evaluated this signalling pathway in animal models of obesity, the Zucker rat. Unfortunately, we realized soon during the Project that this model poorly recapitulates human pathology in terms of heart failure development. In fact, Zucker rats did not present an important expansion of epicardial adipose tissue nor significant dysfunctions of left ventricle. To overcame these issues, we performed in vitro study, coculturing adipose tissue and cardiomyoblasts to reproduce shared bloody supply between epicardial adipose tissue and myocardium in vivo. Finally, since Zucker rats presented a mutation in leptin receptor, we counteract this genetic bias switching to a different animal model: the Diet Induced Obesity mice. DIO mice avoid the background of altered leptin and GHR signalling - that could influence the IL-33/ST2 pathway thus representing the main goal of our study. A second point is the small sample size considered for Zucker and Zucker Diabetics rats, this fact was due to technical issues linked to Ministerial rules on ethical animal experimentation and to the need to perform multiple analysis (both histological and molecular analysis) that forced us to divided the animals in smaller groups. As this limitation was not surmountable during my PhD project we performed in vitro experiments, trying to reproduce our data in vitro.

Finally, this project is still ongoing to assess proteomic findings in DIO mice, in particular by confirming fibrotic and ventricular dilatation predicted by enriched network analysis and by deeply investigating the role of IL-33 as an up-stream regulator. In addition, to decipher the role

of Epac signalling in this context, we have planned to exploit pharmacological inhibitor of Epac1 in vitro.

In conclusion, with our approach, we had the opportunity to decipher the fundamental molecular pathways underlying IL33/ST2-obesity axis and provide the scientific community with valuable insights to counteract their pro-inflammatory effects.

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List of figures and tables

Dissemination of results

The results obtained in my research project have been shared with several colleagues according to the attendance in international conferences. In particular I presented our results with an oral presentation at SIPMeT Young Scientist Meeting (MOLECULAR PATHOLOGY: FROM BENCH TO BEDSIDE, 10-11 december 2021 in Perugia). This meeting was organised by SIPMET, the Italian society of Pathology and Translational Medicine, and included both academic and clinician members focused primarily on pathology but also on oncology and neuroscience. Thanks to this opportunity, we improved the dissemination of our work and – intriguingly – we created scientific collaborations. Furthermore, this year we presented our data through a poster at international SIPMeT Congress 2022, in Ancona, sharing our work to a greater platea. The results of this project will be included in a paper and submitted to an open access journal accordingly to the principles and actions of Open Science *(article in preparation)*. We are conscious that the organization of efficient and dynamic communication plan for project stakeholders is essential to gain visibility and to correctly inform all the scientific and non-scientific community concerning the milestones reached by the Project through mass communication devices (television, press, social media) and to allow interaction with other project-related topics. This way, the link to the publication will be included in the page of Phd program on principal social media (Facebook DMEM Page).

Lay summary

L'obesità è un problema di salute pubblica in continua crescita negli ultimi anni. Stili di vita sedentari e l'eccesso di grassi nella dieta sono i principali fattori che determinano obesità. L'obesità rappresenta un importante fattore di rischio di sviluppare diabete e malattie cardiovascolari, che rappresentano la prima causa di morte nei paesi occidentali. I meccanismi con cui l'accumulo di grasso determina un aumento del rischio cardiovascolare non sono del tutto chiari. Il nostro lavoro si propone di studiare in particolare come l'accumulo di grasso nel cuore e nella zona addominale alterino l'equilibrio metabolico del nostro organismo. Queste alterazioni nel lungo periodo si riflettono sul funzionamento cardiaco, modificandone la struttura

e alterandone le capacità di pompa. L'insufficienza cardiaca che ne risulta è l'incapacità del cuore di portare sangue e quindi ossigeno e nutrienti a tutte le cellule del nostro corpo. A questo scopo, utilizziamo diversi modelli di obesità come colture cellulari e modelli animali. Lo studio degli organi di questi animali ci permette di scoprire questi meccanismi ed in futuro di sviluppare nuovi farmaci e strategie per bloccare queste alterazioni prima che alterino la struttura cardiaca.

Obesity has emerged as an important risk factor for the onset of diabetes and cardiovascular diseases that constitute the main cause of mortality and morbidity in Western countries. The mechanisms by which fat accumulation causes an increase in cardiovascular risk are not really understood. The aim of this study is to investigate how adiposity accumulation in the heart and abdominal areas alters the metabolic balance of our body. These long-term changes affect the heart's function, altering its structure and pump competency. The resulting heart failure is the heart's inability to carry blood and therefore oxygen and nutrient to every cells of our organism. For this purpose, we use different models of obesity such as cell cultures and animal models. The study of the organs of these animals allows us to discover these mechanisms and in the future to develop new drugs and therapeutic strategies to block these alterations before they modify the cardiac structures.