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Table of contents

Abbreviations

ADCC	antibody dependent cellular cytotoxicity
AF	atrial fibrillation
ACEI	angiotensin converting enzyme inhibitor
ARB	angiotensin II type 1 receptor blocker
ATM	adipose tissue macrophage
BA	bile acid
BAR	bile acid-activated receptor
BMI	body mass index
BSH	bile salt hydrolase
CA	cholic acid
CDCA	chenodeoxycholic acid
CVD	cardiovascular disease
DAMP	danger associated molecular pattern
DCA	deoxycholic acid
DHF	diastolic heart failure
ECM	extracellular matrix
EF	ejection fraction
GLP-1	glucagon-like peptide 1
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
IR	insulin resistance
LA	left atrium
LAVi	left atrial volume index
LCA	litocholic acid
LV	left ventricle
MetS	metabolic syndrome
MI	myocardial infarct
MMP	matrix metalloproteinase
NAFLD	non-alcoholic fatty liver disease
NK	natural killer

PAMP	pathogen associated molecular pattern
RAAS	renin-angiotensin-aldosterone system
SCFAs	short chain fatty acids
SHF	sistolic heart failure
T2D	type 2 diabetes
TCR	T-cell receptor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labelling

1. Introduction

1.1. Background of the study

1.1.1. Introduction to metabolic syndrome – definition and prevalence

According to the WHO report, cardiovascular diseases (CVDs) are number one cause of death globally, claiming 17,7 millions of deaths in 2015 which represented 31% of all global deaths (Roth et al., 2017). CVD comprises several disorders with atherosclerotic plaque formation underlying its pathology, most common of which are heart failure, myocardial infarct, stroke and arrhythmias.

Although it is agreed that abdominal obesity, abnormal cholesterol levels, hypertriglyceridemia, insulin resistance and hypertension all contribute to the development of diseases like CVD, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and obesity-related cancers (O'Neill and O'Driscoll, 2015), there is an urgent need to accurately identify those high risk individuals in order to introduce medical treatment in time and save lives.

Metabolic syndrome (MetS) can be diagnosed when at least three of the aforementioned risk factors are present. However, several definitions issued by different authorities with regards to the criteria and cut off points are available, as presented in Tab. 1. It is clear that the application of divergent criteria can lead to somewhat distinct prevalence estimates for the same sample group (Ford, 2005). What is remarkable, though, is that MetS prevalence varies with ethnicity (Balkau et al., 2003) while frequently showing little difference between male and female population (Beigh and Jain, 2012). Ford et al. demonstrate in their series of work an increase in Mets prevalence among US adults from period of 1988 to 2006 (Ford et al., 2002; Ford, 2005; Ford et al., 2010) and those findings were confirmed in a recent study (Moore et al., 2017). Although MetS prevalence clearly increases with age (Hildrum et al., 2007), there is an evidence that it reaches its peak in 60-69 age group in European men and women (Hu et al., 2004). Both of these aforementioned statements represent a major economic and public health concern, considering the fact that patients with MetS are at twice risk of developing CVD over the next 5 to 10 years compared to the individuals without the syndrome and 5 times more likely to suffer from type 2 diabetes (T2D) (Alberti et al., 2009). This calls for further investigation of its underlying pathophysiology as well as novel therapeutic approaches.

Table 1 MetS criteria and cut off points issued by different authorities.
IDF=International Diabetes Federation, NCEP-ATPIII=National Cholesterol Education Program-Adult Treatment Panel III, AHA/NHLBI=American Heart Association/National Heart, Lung and Blood Institute.

	Harmonized ('09.)	**IDF ('05.)**	**NCEP-ATPIII ('01.)**	**AHA/NHLBI ('05.)**
MetS Criteria	Any 3 or more of:	WC≥94 cm (M) WC≥80 cm (F)	Any 3 or more of:	Any 3 or more of:
WC	Ethnic specific cutpoints	And 2 or more of:	≥102 cm (M) ≥88 cm (F)	≥102 cm (M) ≥88 cm (F)
HDL	<1.03 mmol/L (M) <1.29 mmol/L (F) OR medication for reduced HDL	<1.03 mmol/L (M) <1.29 mmol/L (F) OR medication for reduced HDL	<1.03 mmol/L (M) <1.29 mmol/L (F) OR medication for reduced HDL	<1.03 mmol/L (M) <1.29 mmol/L (F) OR medication for reduced HDL
TG	≥1.7 mmol/L OR medication for elevated TG	≥1.7 mmol/L OR medication for elevated TG	≥1.7 mmol/L OR medication for elevated TG	≥1.7 mmol/L OR medication for elevated TG
BP	≥130/85 mm Hg OR BP-lowering medication	≥130/85 mm Hg OR BP-lowering medication	≥130/85 mm Hg OR BP-lowering medication	≥130/85 mm Hg OR BP-lowering medication
FBG	≥5.6 mmol/L OR antidiabetic medication	≥5.6 mmol/L OR antidiabetic medication	≥6.1 mmol/L OR antidiabetic medication	≥5.6 mmol/L OR antidiabetic medication

1.1.2. Heart failure and cardiac remodelling

As mentioned above, metabolic syndrome is a major risk factor for cardiovascular events, including heart failure, myocardial infarction (MI), atrial fibrillation (AF), arrhythmias, revascularization, stroke or CV death. Herein the focus goes to the aspects of heart failure.

Heart failure (HF) is a constellation of signs and symptoms caused by an inadequate performance of the heart, which can be caused by a variety of conditions damaging myocardium such as coronary artery disease, arterial hypertension, idiopathic cardiomyopathy and valvular heart disease, among others. In addition to differentiating between the right and left-side HF, reflecting the ability of the cardiac muscle to pump an adequate amount of oxygen rich blood to the lungs and rest of the body, respectively, HF can be designated as a systolic or diastolic one, reflecting dysfunctional contraction or relaxation rates, respectively (Federmann and Hess, 1994). Although not exclusively, systolic heart failure (SHF) is often found in post-MI stage

and is usually characterized by reduced ejection fraction (HFrEF) (approximately 40% or lower), whereas the diastolic heart failure (DHF) is associated but not restricted to hypertension, diabetes mellitus and obesity and is usually accompanied by a preserved (HFpEF) (Jessup and Brozena, 2003). A complex blend of structural, functional and biologic alternations are important in heart failure (Jessup and Brozena, 2003), one of which is remodelling. The distinctive features of remodelling as well as myocyte and matrix changes in DHF and SHF are presented by Chatterjee et al. (Chatterjee and Massie, 2007). In that work they state that the myocyte hypertrophy and apoptosis as well as myocardial fibrosis may be observed in both SHF and DHF, however, the differences may appear on matrix metalloproteinases (MMPs) levels as well as in the extent of collagen cross-links.

Cardiac remodelling is defined as a group of molecular and interstitial changes that manifest clinically as changes in size, mass (hypertrophy and atrophy), geometry (wall thickness and shape) and function of the heart after an injury (Azevedo et al., 2016). Whereas an insult produces the initial decline in LV pumping capacity in SHF, seems like a prolonged wall stress may account for the progressive ventricular dilation in DHF (Chatterjee and Massie, 2007). In both instances, pathological progression which ensues results in a mismatch between cardiac oxygen supply and demand, a condition which is at first balanced by several compensatory mechanisms such as sympathetic nervous system, the renin-angiotensin-aldosterone axis, vasopressin and the cytokine system (Karayannis et al., 2008). Although these mechanisms initially successfully restore cardiovascular homeostasis causing patients to remain asymptomatic for a period of time (Jessup and Brozena, 2003), the development of disease calls for further compensatory mechanisms which seem to begin with a series of adaptive LV anatomical and functional changes known as ‘’LV remodelling’’ (Jessup and Brozena, 2003). Studies have shown that LV dysfunction is necessary but may not be sufficient for the development of the symptomatic HF and that patients with signs and symptoms of HF may have a normal EF (Steendijk, 2004). Further, there are studies highlighting the importance of left atrial remodelling in contribution to the progression of asymptomatic LV dysfunction to chronic symptomatic HF (Karayannis et al., 2008). On the other hand, although left atrial volume index (LAVi) was associated with the severity of diastolic dysfunction, it seems to be a poor marker of mild and moderate diastolic dysfunction in the general population (Pritchett et al., 2005).

1.1.3. Microbiome, bile acids and cardiovascular disease link

Ever since Turnbaugh et al. have identified an *obesity-associated microbiome with increased capacity for energy harvest*, thus implicating that the gut microbiota may be the influencing factor that further promotes obesity and metabolic disorders in some obese subjects, the microbiome field has been receiving an increasing attention and its role has been implicated in various health- and disease-related conditions (Turnbaugh et al., 2006). Studies have confirmed that obese individuals have an altered gut microbiota compared to lean controls, and this is often characterized by reduced number of *Bacteroidetes* strains (Greiner and Backhed, 2011). Similarly, obesity-related changes in the composition of the gut microbiota were found in lean versus obese Göttingen and Ossabaw minipig breeds (Pedersen et al., 2013). In both pig models diet seemed to be the defining factor that shapes the gut microbiota as observed by the changes in different bacterial divisions between the lean and obese pigs. Another study assessed predominant bacterial divisions in the distal gut of Meishan and Landrace pig breeds and concluded that manipulating gut microbial communities could control fat storage in pigs, however, more research is needed in this area (Guo et al., 2008). Furthermore, Greiner and Bäckhed summarize in their report how gut microbiota may result in an increased *de novo* lipogenesis through the production of short chain fatty acids (SCFAs), lead to altered fatty acid oxidation, promote inflammation and related adiposity or disrupt gut permeability (Greiner and Backhed, 2011).

Moreover, a recent study highlighted the importance of gut microbiota in CVD and metabolic disorders (Li et al., 2017). It is acknowledged there that the gut microbiota dysbiosis contributes to the development of hypertension as hypertensive human subjects were characterized by dramatically decreased microbial richness and diversity and *Prevotella*-dominated enterotype. In addition, researchers discovered that faecal transplantation from hypertensive human donors to germ-free mice elevated blood pressure which suggests that hypertension may be transferable through microbiota (Li et al., 2017).

A recent extensive report review on gut microbiota as a potential target of metabolic syndrome presents probiotic bacterial strains that have been linked with either reduced visceral fat, increased production of glucagon-like peptide-1 (GLP-1), lean phenotype, decreased gluconeogenesis and glucose homeostasis or even attenuated diet-induced hyper-cholesterolemia (He and Shi, 2017). For example, it is mentioned that administration of synbiotic food containing *L. Acidophilus* ATCC 4962, fructooligosaccharide, inulin and mannitol in hypercholesterolaemic pigs for 8 weeks resulted in reductions in serum

triglycerides and total LDL-cholesterol as well as an increased HDL-cholesterol concentration (He and Shi, 2017).

Finally, human bile acid pool is a subject to bacterial modifications. And while humans synthesize cholic acid (CA) and chenodeoxycholic acid (CDCA), an additional removal of 7α-hydroxyl group by intestinal bacteria represents a predominant (although not the only) modification pattern thus generating deoxycholic acid (DCA) and lithocholic acid (LCA), respectively (Staels and Fonseca, 2009). Bile acid system and its components are discussed further in text (*1.5. A novel mechanism and involvement of bile acids in pathogenesis of heart failure*). However, while the extent and overall impact of secondary and tertiary bile acids remains to be elucidated, it is considered that only unconjugated BAs are subject to these modifications (Long et al., 2017), implicating that the presence of intestinal organisms possessing bile salt hydrolase (BSH), an enzyme for deconjugation, represents an important factor in this process. Therefore, it is reasonable to say that the gut microbiome dysbiosis holds a significant potential to disrupt normal bile acid pool. In recent years, the role of bile acids has expanded well beyond fat solubilisation and nutrient absorption. Thus they have been linked to lipid and glucose homeostasis through their interaction with bile acid-activated receptors (BARs) (Trauner et al., 2010). Moreover, their role is implicated in cardiovascular function showing the ability to decrease myocyte contraction rate and induce bradycardia, reduce the duration of action potential or induce myocyte apoptosis, to name few (Khurana et al., 2011). In addition, bile acids are also shown to induce inflammatory genes in hepatocytes (Allen et al., 2011). Of particular importance seems to be TGR5, a bile acid-activated receptor with the ability to improve glucose homeostasis (Katsuma et al., 2005), increase energy expenditure in brown adipose tissue (Watanabe et al., 2006), protect gut intestinal barrier (Cipriani et al., 2011) as well as reduce inflammation and exert anti-atherosclerotic properties (Pols, 2014). Recently, TGR5 has been identified as a novel therapy target for heart failure (Moreshwar S Desai, 2014). In this study, researchers demonstrated that cholic acid (CA) feeding functionally activates TGR5 in mouse heart and they also showed that CA attenuates contractile failure and pathologic hypertrophy in mouse model of HF (Moreshwar S Desai, 2014).

1.1.4. Porcine model of metabolic syndrome

As the human race rapidly expands and major technological, cultural and other advancements continue to affect its well-being, there is an emerging need for biomedical research to keep pace with ever-increasing rates of health issues as well as the diversity and

severity of contemporary ailments. In line with that, animal testing has not only improved our understanding of the pathophysiology of numerous diseases but it has also contributed to many life-saving cures and prevention strategies such as polio vaccine or development of pacemakers. (Aquilina, 2006; Scrase, 2015).

According to the latest available report issued by European commission, in 2011 in the EU, the total number of animals used for experimental and other scientific purposes was just under 11,5 million; 80% of them were rodents and rabbits, whereas the number of pigs used in biomedical research across the EU in 2011, together with horses, cattle, sheep, donkeys and other *Artiodactyla* and *Perissodactyla* species, has not been rising significantly since 1996 and fluctuates around 1% (ec.europa.eu/environment/chemicals/lab_animals/reports_en.htm). And while there is no doubt that rodent models of cardiovascular disease have produced important findings, such as adverse renal effects of ACE inhibitors in rats with myocardial infarction (Westendorp et al., 2009), there are concerns regarding the translatability of the rodent models which is further emphasized by well-known structural and functional differences compared to humans (Lal et al., 2016). Advantages and disadvantages of many small and large animal models in cardiac contraction research are well reviewed by Nejad and Janssen (Milani-Nejad and Janssen, 2014). In this extensive report, they acknowledge that large heart/body weight and similarities to the human cardiovascular system make pig a valuable pre-clinical animal model in CVD studies.

Further, both humans and pigs are prone to the development of obesity and related cardiovascular diseases, showing similar plaque histology and pathogenesis pattern (Reddick et al., 1990; Thim, 2010). Atherosclerosis in pigs develops both spontaneously and when induced by an experimental atherogenic high cholesterol diet in streptozocin-induced diabetic animals (Gerrity et al., 2001) but it does not develop in previously healthy pigs treated with 6%-cholesterol diet for 6 weeks (Mihaylov et al., 2000). In addition, numerous research groups have now succeeded in developing metabolic syndrome in different pig strains by high caloric diets, mostly consisting of 15-25% (by weight) fatty acids (mainly lard supplemented with hydrogenated soya bean and coconut oil), 1-2% cholesterol, 40% refined sugars (commonly 20% sucrose and 20% fructose), 17% protein and 15% other carbohydrates like starches and fibres (Zhang and Lerman, 2016). In general, *ad libitum* feeding of a high calorie, high sugar/fat diet induces obesity within a period of 3–6 months (Koopmans and Schuurman, 2015). These porcine models, like humans, usually exhibit three or more of the clinical signs of metabolic syndrome including insulin resistance, high amounts of visceral fat and high blood pressure

(Spurlock and Gabler, 2008). Furthermore, female pigs seem to develop metabolic syndrome more readily than their counterpart male pigs (Larsen et al., 2001) as they present larger abdominal circumference and higher concentrations of plasma insulin, triglycerides, total cholesterol and leptin (Christoffersen et al., 2007). Some of the breeds frequently used to stimulate metabolic syndrome in pigs include Yucatan, Göttingen, Ossabaw, Yorkshire, Sinclair as well as Landrace (Zhang and Lerman, 2016). Landrace is a British domestic breed of pig recently successfully used by Schwarzl et al. to develop a porcine model that mimics cardiac phenotype of heart failure with preserved ejection fraction (HFpEF) (Schwarzl et al., 2015) while its Yorkshire crossbreed has been used as a model of metabolic syndrome (te Pas et al., 2013).

The current study utilizes Landrace pig organism and it models experimental procedures used by Schwarzl et al. Specifically, the experimental group (n=5) was fed for 12 weeks western chow diet supplemented with high quantities of salt (4%), cholesterol (4%), crude fat (25%), cholate (0.5%) and sugar (26%) to induce metabolic syndrome. The other 5 age and sex matched control animals were served a regular diet. All animals had free access to water. Morphological and systemic measurements performed previously in this laboratory confirmed the presence of signs and symptoms of metabolic syndrome, as well as characteristics of poor cardiac function in experimental animals, suggesting the presence of HFpEF (data not published).

Despite few differences (Crick et al., 1998; Milani-Nejad and Janssen, 2014) and practical requirements regarding the cost, pigs seem to be an appropriate biomedical model for the study of CVD and atherosclerosis as the morphology and physical function of cardiovascular system in swine are similar to humans (Swindle et al., 2012). In addition, comparable protein and lipid metabolism and similar postprandial responses (Nielsen et al., 2014) have made this animal a valuable model in nutritional and pharmacological studies. Other general advantages of using the pig in biomedical research are that it is possible to use standard medical technologies to image internal organs and vessels, repeatedly collect blood samples and obtain an adequate quantity of tissue sample at slaughter.

Moreover, in relation to the current study, the porcine immune system more closely resembles humans for >80% of analysed parameters, whereas mice were more similar to humans in <10% (Meurens et al., 2012). In addition, immune cells identified in humans are also present in pigs and their markers closely resemble those in humans. Guzylack and Salmon provide a comprehensive review on those markers in their work *Membrane markers of immune*

cells in swine: an update (Piriou-Guzylack and Salmon, 2008). Although chronic inflammation has been implicated in obesity and metabolic syndrome, its role in cardiac dysfunction has not yet been elucidated. Either way, comprehensive and integrated analyses of porcine genome sequence have proved this animal as a relevant model in immunological studies in relation to human physiology (Dawson et al., 2013; Mair et al., 2014; Dawson et al., 2017). Further, common mammalian genetics and physiology result in common cell cycle, which also includes events like apoptosis or normal cell death. Indeed, apoptosis has been identified in both human and porcine models of heart disease (Narula et al., 1996; Olivetti et al., 1996; van Empel et al., 2005; Li et al., 2012; Elmadhun et al., 2014). Moreover, two recent studies demonstrate that the porcine heart, like human, may as well go through remodelling. In the first one, Gyöngyösi et al. produce a porcine model of progressive cardiac hypertrophy and fibrosis (Gyongyosi et al., 2017), whereas in the second one Barallobre-Barreiro et al. reveal an early- and late-stage cardiac extracellular matrix remodelling in a porcine model of ischemia-reperfusion injury (Barallobre-Barreiro et al., 2012). In addition, a study on cardiac tissue in Ossabaw pig model of metabolic syndrome reveals a significant increase in fibrosis-related staining (Sirius red and Trichrome) compared to both lean and obese groups (Li et al., 2012). Finally, porcine bile acid pool composition, chemistry and transhepatic bile acid kinetics are comparable to human ones, showing similar postprandial responses and hepatic BA clearance (Kobayashi et al., 1998; Tsai et al., 2011; Eggink et al., 2017). For example, CDCA and CA are primary bile acids in many species, including humans and pigs and glycine conjugates are predominant forms in both (Burrin et al., 2013). However, hyodeoxycholic acid (HDCA) is a porcine BA not found in humans, and pigs do not have DCA, in contrast to humans (Eggink et al., 2017). This may be significant in relation to TGR5 since DCA shows high potency to activate this receptor. Further, a recent study showed that total BA concentrations in the portal vein were at least 6 times higher than in the other blood vessels (including LCA, the secondary BA with highest affinity for TGR5) and that peripheral exposure to the secondary BAs with the highest TGR5 affinity is low (Eggink et al., 2017). However, considering the before-mentioned link between bile acids and microbiota this may be altered in certain disease- and obesity-related states.

These observed similarities in human and pig physiology are important to note when considering pig as a model for human CVD and investigating potential treatment options. It is also important to remember here that established molecular and cellular mechanisms of cardiac dysfunction include, but are probably not limited to, cell's death (apoptosis, necrosis, autophagy), changes in energy utilization (shift from free fatty acid oxidation to glucose

oxidation), oxidative stress, inflammation, collagen accumulation, fibroblast proliferation, changes in composition and activity of contractile proteins (α- and β-myosin, troponin) resulting in decreased contractility and, finally, alternations in calcium transport (Azevedo et al., 2016). However, when it comes to metabolic syndrome and its association with progressive nature of HF, the extent and clinical significance of these and many other pathophysiological alternations, as well as their differential occurrence in the four chambers in heart, remain to be elucidated.

In that light, the first part of the current study focuses on three different mechanisms and their possible involvement in HF:

1. Inflammation
2. Apoptosis
3. Fibrosis

The second part of this study aims to assess the expression of cardiac TGR5, a bile acid-activated receptor, in order to investigate its involvement in the pathogenesis of HF.

1.2. Inflammation

1.2.1. Introduction to immune system and inflammation

Inflammation is a major defence reaction initiated by infection or tissue injury and stress. Initial step of the inflammation process represents immune cell's encounter with so called pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) as these are the structures endowed to trigger the activation of the innate immune system (Newton and Dixit, 2012). Recognition of these entities is achieved by an array of specialized receptors displayed on both membrane surfaces and intracellular spaces of immune cells (Takeuchi and Akira, 2010). In addition, immune response is always to be proportional to the level of threat, thus there is a need to neatly balance its protective and destructive features and this is being accomplished by immune cells communicating with each other and with non-immune cells within the body by means of cytokines and chemokines (Graves and Jiang, 1995; Geginat et al., 2001; Cameron MJ, 2003).

Immune system cells can roughly be divided into those of myeloid and lymphoid lineage with macrophages, monocytes, mast cells, dendritic cells, neutrophils, basophils and

eosinophils representing the former and T lymphocytes, B lymphocytes as well as natural killer (NK) cells representing the latter (Janeway CA Jr, 2001). Tissue-resident macrophages are frequently the first ones to sense the threat (Tay et al., 2014). These cells seem to be especially important due to their phagocytic ability and plasticity when it comes to receptors and subsequent signalling molecules they produce (Stout and Suttles, 2004). Furthermore, macrophages make an interesting cell type to study owing to their ability to exhibit both M1 (pro-inflammatory, critical in response to infection) and M2 (anti-inflammatory, facilitating tissue repair) phenotype (Mills, 2012) since M1 or M2 – dominant responses are observed in disease (Mills et al., 2015). And while M1 macrophages are irreplaceable in states of infection due to their antimicrobial pro-inflammatory properties (Wang et al., 2014), a subtype of M2-macrophages (M2c) favour more anti-inflammatory, wound-healing response through production of IL-10, TGFβ, IL1Ra. IL-4 (Gordon, 2003; Mantovani et al., 2004) and they contribute to the production and deposition of extracellular matrix components within the wound (Minutti et al., 2017). Although recognizing only two subsets of this diverse and extraordinary type of cells undoubtedly implies oversimplification, identifying one of those subsets in various conditions may help us to understand the nature of the ongoing local and systemic processes and to further establish possible therapeutic targets for manipulation in human disease. In addition, macrophages can also be derived from monocyte precursors that circulate in the blood stream for a number of hours before they undergo differentiation into macrophages at the inflammation site (Taylor and Gordon, 2003). The cytokines secreted by tissue-macrophages, especially TNFα and IL-1β have a particular role to increase the adhesiveness of the endothelial cells lining the blood capillaries closest to the site of inflammation through triggering the exposure of P and E selectins on these cells which then in turn bind carbohydrate ligands present on monocytes, with MCP-1 serving as major chemotactic factor (Deshmane et al., 2009). It is important to remind one that macrophages are professional phagocytes and are highly specialized in removal of dying cells and cellular debris thus they can ingest a pathogen invading our body, or neutrophil, which came of age after exerting its function in early stages of chronic inflammation, or oxidized cholesterol by binding it to their specific receptor (CD36) thus becoming an apoptotic foam cells contributing to the atheromatous plaque of atherosclerosis (Savill et al., 1989; Aderem and Underhill, 1999; Han et al., 1999). However, the incentive for a macrophage to phagocyte the target cell seems to be the (ab)normal molecular signal that the target cell displays on its surface (Callahan et al., 2000). Commonly, if the target cell is deemed to represent a threat (expressing an abnormal

molecular pattern/signal), upon phagocytosis, macrophage will process it and present the triggering molecular signal on its surface. It will subsequently wait for a T-cell to recognize it and to further trigger the adaptive immune response by activating the appropriate B-cell in the lymph node which will in turn start proliferating and producing the antibodies (immunoglobulins) that are specifically tailored to target other cells which express the initial molecular signal that triggered this response (Harvey et al., 2007). T and B lymphocytes are central players in the adaptive immune system with highly specific surface receptors responding to complementary molecular structures which represent the potential threat to the host and, once these cells are triggered, they release powerful cytokines aimed to drive host immune responses in a particular direction (Janeway CA Jr, 2001). Fig. 1 shows major T cell subsets, their triggering molecules, cytokines they produce and currently known roles in immune defence. Natural killer cells, seem to be important in both innate and adaptive immune responses as they inspect host cells for the expression of normal cell-surface molecules (MHC1) or sense if cells have been opsonized by (immunoglobulins) IgG and subsequently trigger the death-machinery resulting in killing the target cell (Mandal and Viswanathan, 2015) known as antibody-dependent cellular cytotoxicity (ADCC).

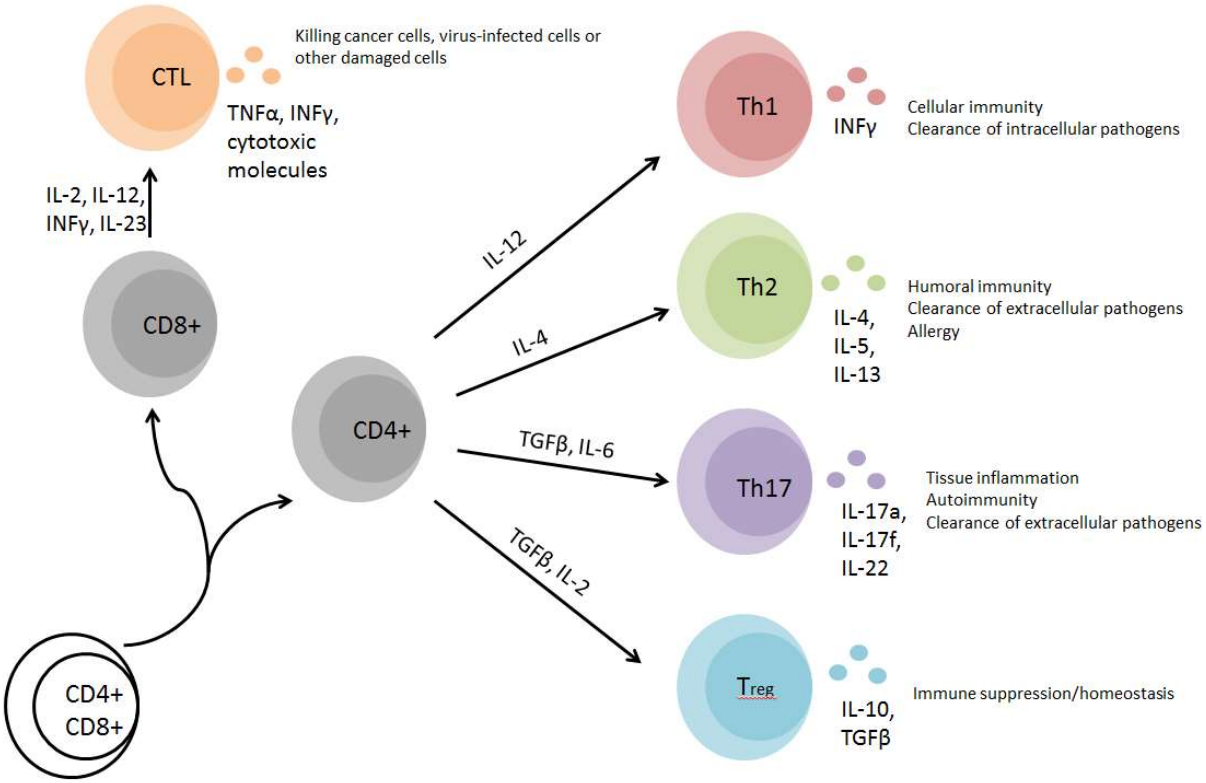


Figure 1. Major T-cell subsets. The figure shows major T-cell subsets, their triggering molecules, cytokines they produce and general roles in immune system defence. CTL=Cytotoxic T-cell, Th= T-helper cell, Treg= Regulatory T-cell.

1.2.2. Inflammation in metabolic syndrome and heart disease

Patients with MetS frequently exhibit pro-inflammatory and pro-thrombotic profiles (Odrowaz-Sypniewska, 2007; Kalupahana et al., 2012). Moreover, obesity and insulin resistance, two of its important hallmarks, have strongly been associated with chronic low-grade inflammation. For example, it was proven that pro-inflammatory cytokines such as TNFα and IL-6 can impair insulin signalling in insulin-sensitive tissues and can further give rise to local and systemic insulin resistance (IR) (Cai et al., 2005), as confirmed additionally by an established IR in human skeletal muscle after an acute TNFα infusion (Plomgaard et al., 2005). Over the recent years, evidence has emerged highlighting the link between low-grade inflammation in adipose tissue in obese individuals and IR (Xu et al., 2003; Wellen and Hotamisligil, 2005). Obesity has further been associated with macrophage accumulation in adipose tissue (Weisberg et al., 2003). It is believed that the obesity skews adipose tissue macrophages (ATMs) toward pro-inflammatory M1 phenotype thus triggering low-grade inflammation (Boutens et al., 2018). In addition, adipose tissue from obese animals expresses high levels of MCP-1, resulting in subsequent monocyte infiltration and differentiation into macrophages (Xu et al., 2003; Kanda et al., 2006). On the side of the adaptive immune responses, Nishimura et al. showed that the adipose tissue in high-fat fed mice is characterized by infiltration of cytotoxic CD8(+) T-cells, accompanied by reduction in CD4(+) helper and regulatory (Treg) T-cells (Nishimura et al., 2009) which also confirms the polarization paradigm with respect to T-cells and their activation pathways (Figure 1). Interestingly, this polarization toward CD8(+) subset occurs prior to adipose tissue infiltration with macrophages suggesting that the cytotoxic activity and CD8(+)-derived cytokines may be responsible for the macrophage infiltration and subsequent shift toward M1 phenotype. Adipose tissue remodelling via degradation of ECM has also been associated with obesity (Chavey et al., 2003).

On the other hand, immune system's is involvement in cardiac (patho)physiology is still not completely understood and is being rapidly explored over the last few years, thus the latest studies suggesting that cardiac macrophages may have a surprising role in heart electrical conduction (Fernandez-Ruiz, 2017), cardiac regeneration (Godwin et al., 2017) or heart attack (Nahrendorf and Swirski, 2016; King et al., 2017). A comprehensive review on protective and pathogenic functions of macrophage subsets, including conditions such as atherosclerosis and fibrosis is presented by Murray and Wynn (Murray and Wynn, 2011). In this report, they

acknowledge that, in atherosclerosis, it is thought that macrophages lodge in the intima and subintima of arteries, eventually leading to the formation of obstructive atherosclerotic plaques that are prone to rupture, leading to thrombosis, myocardial infarction or stroke. A more recent study assessed monocyte and macrophage contributions to cardiac remodelling and, among other noteworthy things, highlights that macrophages are an intrinsic part of the heart under physiological conditions, that cardiac macrophages expand in response to stress and that macrophage expansion through monocyte recruitment associates with cardiac remodelling (Hulsmans et al., 2016). They also state that this expansion occurs likely through both local macrophage proliferation and monocyte recruitment and that the transition of macrophage phenotypes from inflammatory to reparative could be a potential mechanism of cardioprotection after MI, but prolonged activation of reparative macrophages may eventually contribute to extensive cardiac fibrosis, increased stiffness and diastolic dysfunction. Finally, they acknowledge that, in the future, mammalian systems other than mouse may be needed to model complex neural, immune, endocrine and metabolic interactions during hypertension, obesity and dyslipidaemia, all contributing to human heart failure (Hulsmans et al., 2016). Another valuable review was given by Shen and Young, in which they discuss the role of immune cells in heart failure and hypertension with specific focus on mineralocortocoid receptors. They note that some experimental disease models suggest that M1 is subsequently replaced by profibrotic (M2) macrophage phenotype likely in response to tissue signals or T-cell cytokines and that M2-macrophages release profibrotic factors involved in cardiac remodelling such as TGFβ which promotes fibroblast activation and collagen deposition (Shen and Young, 2012).

1.2.3. Study aim 1: determining inflammation markers in cardiac tissue in a porcine model of metabolic syndrome

CDs (cluster of differentiation) are cell surface molecules expressed on leukocytes and other cells relevant for the immune system. CD molecules are commonly used as cell markers, allowing the identification and isolation of leukocyte populations, subsets, and differentiation stages. Indeed, specific identification of the various subpopulations of leukocytes enables improved investigations of the immune response, thus more recently these molecules have been recognized as invaluable tools for the treatment of several malignancies and autoimmune diseases (Engel et al., 2015).

a) CD163

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily and is exclusively expressed in monocytes and macrophages . It is involved in the clearance and endocytosis of haemoglobin/haptoglobin complexes by macrophages and may thereby protect tissues from free haemoglobin-mediated oxidative damage (Moestrup and Moller, 2004). A particularly high expression is seen in macrophages of the 'alternative activation' phenotype (M2) playing a major role in dampening the inflammatory response and in scavenging components of damaged cells (Moestrup and Moller, 2004). In their report on membrane markers of the immune cells in swine, Guzylack and Salmon confirm that this receptor is restricted to the cells of the porcine monocyte/macrophage lineage and that it shares a sequence similar to that of human (Piriou-Guzylack and Salmon, 2008). They also state that CD163+ monocytes produce more TNFα, express high levels of adhesion molecules and are better at presenting antigens to T-cells when compared to CD163- monocytes (Piriou-Guzylack and Salmon, 2008).

It has been reported that soluble CD163 is elevated and associated with subclinical atherosclerosis in men with and without HIV infection (Burdo et al., 2011; McKibben et al., 2015). Further, increased cardiac expression of CD163 has been found in a porcine model of metabolic syndrome developed by Li et al. (Li et al., 2012). Having in mind that CD163 is a marker mainly associated with the M2 subclass (Moestrup and Moller, 2004), one should be reminded of the roles linked to this subset, including the contribution in wound healing and tissue repair. M2-macrophages are critical players in tissue repair, and depletion of these cells results in impaired wound-healing (Leibovich and Ross, 1975; Mirza et al., 2009). However, these properties must be neatly balanced to avoid undesirable fibrotic changes in an injured tissue. Indeed, wound-healing macrophages may be detrimental to the host when their matrix-producing activity is enhanced, similarly to the deregulated activity of classically activated macrophages in autoimmunity (Mosser and Edwards, 2008). In addition to producing pro-fibrotic mediators, macrophages have also been shown to directly enhance the activation of myofibroblasts, the key ECM-producing effector cells (Wynn and Vannella, 2016).

b) CD16

CD16 or FcγRIII is a receptor for Fc (constant) region of immunoglobulin G (IgG) showing highest affinity for IgG3 subclass (Peter J. Delves and Roitt., 2017). This receptor comes in two isoforms; FcγRIIIa is highly expressed on macrophages, NK cells, γδ T-cells and some monocytes, whereas FcγRIIIb is mainly restricted to neutrophils and eosinophils (Peter J.

Delves and Roitt., 2017). CD16-expressing cells are able to lyse or phagocyte IgG-opsonized cells, promote antigen presentation and induce inflammation (Deo et al., 1997). Further, CD16 seems to be especially interesting since it is essential for antibody-dependent cellular cytotoxicity (ADCC) (Yeap et al., 2016). Although ADCC can be performed by all other above mentioned cells it is often associated with NK cells (Vivier et al., 2008). The process of ADCC requires IgG to be bound to a target cell (thus opsonising it) prior to CD16 binding to its Fc region and allowing NK cell to release cytotoxic factors that cause death of the target cell (Peter J. Delves and Roitt., 2017). Referring back to Guzylack-Salmon's report on swine membrane markers of the immune cells, it is stated that all blood monocytes and all NK cells bear CD16 but, unlike in humans, it is also found on immature and mature monocyte-derived dendritic cells (DCs) and blood DCs in pigs (Piriou-Guzylack and Salmon, 2008).

Increased levels of CD14++CD16+ blood monocytes have been reported in heart failure patients (Barisione et al., 2010) and aberrant expression of CD16 on human monocytes in patients with coronary heart disease was identified as a contributor to the development of atherosclerosis since enhancement of CD16 on monocytes was closely correlated to increased content of MMP-9 in aorta, which cleaves different bioactive molecules implicated in plaque destabilization (Huang et al., 2012). Prior to that finding, Huang et al. have reported that CD16 expression on human monocytes is increased in patients with coronary heart disease (Huang et al., 2012). Moreover, in addition to the fact that increased levels of CD16 were found on blood monocytes in HF patients in 3 studies mentioned, another study by Czepluch et al. examined CD16 expression in human myocardium after infarction (Czepluch et al., 2013). The study concluded that monocytes are preferentially present in the subacute phase after MI and contribute to cardiac repair process, in particular fibrosis (Czepluch et al., 2013).

c) CD3

The CD3 complex is an integral part of the T-cell receptor (TCR) as is therefore exclusively expressed on αβ- and γδ T-cells, therefore, neither NK cells nor B-cells express this molecule (Peter J. Delves and Roitt., 2017). Guzylack and Salmon confirm this expression pattern in swine (Piriou-Guzylack and Salmon, 2008). Moreover, CD3+ lymphoid cells are already detected at around day 30 of gestation in pigs (Rothkotter et al., 2002). When TCR is activated, signals are propagated via the CD3 co-receptor complex, which is made up of CD3 γ, ε, δ, and ζ chains (Birnbaum et al., 2014).

A study from 1997 found that CD3+ lymphocytes were more abundantly present in tissue obtained from patients with end stage heart failure (prior to transplantation) (Devaux et al., 1997). In this study Devaux et al. observed CD3+ cells in variable numbers in extracellular space of tissues obtained from patients who suffered myocarditis, ischemia and dilated cardiomyopathy (Devaux et al., 1997). This valuable study, which examined cells' adhesion molecules and the presence of low grade inflammation in human chronic heart failure, also confirms increased macrophage count in examined tissues. Consequently, authors conclude that the low-grade inflammation is present in the failing human myocardium and this may significantly contribute to the structural deterioration that is the basis of reduced cardiac function in congestive heart failure (Devaux et al., 1997). Further, activation of peripheral blood CD3+ T-lymphocytes was observed in patients with atrial fibrillation (Liu et al., 2012). In addition, Steffens et al. have found that the short-term treatment with anti-CD3 antibody (Ab) induces a regulatory T-cell phenotype and restores self-tolerance in a mouse model of atherosclerosis (Steffens et al., 2006). This was confirmed in a recent study which found that CD3-Ab treatment induced rapid regression of established atherosclerosis via reducing CD4+ T-cells and increasing proportion of regulatory T-cells (Kita et al., 2014). Authors in this article conclude that therapeutic intervention for T-cell mediated immune responses may represent a novel strategy to induce atherosclerosis regression in combination with lipid-lowering therapy (Kita et al., 2014).

It is for the above-mentioned reasons that the current study focuses on the assessment of CD163, CD16 and CD3 in cardiac tissue in a porcine model of metabolic syndrome, with the aim to investigate the inflammation pattern in the pathogenesis of HF and to, in future studies, target specific components of immune system in order to modulate the outcome of the disease.

1.3. Apoptosis

1.3.1. Introduction to apoptosis

Apoptosis is a genetically regulated form of death which describes the orchestrated collapse of a cell characterised by membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA followed by a rapid engulfment of the corpse by neighbouring cells (Renehan et al., 2001). Further, it is described as an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation, unlike necrosis

which implies uncontrolled release of inflammatory cellular contents (Fink and Cookson, 2005).

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Oberhaus, 2003). The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template (Kyrylkova et al., 2012). The current study utilizes this method to assess apoptotic rates in cardiac tissue.

1.3.2. Apoptosis in heart disease and cardiac remodelling

In their recent review, *Morphological aspects of apoptosis in heart diseases*, Teringova and Tousek have concluded that the apoptotic cell death represents a significant contributor to the myocardial damage in patients with acute myocardial infarction. They also acknowledge that the apoptosis accounts for the subsequent LV remodelling and the development of heart failure thus finding a sensitive marker of apoptosis would help to predict the prognosis of such patients (Teringova and Tousek, 2017).

As nicely described by Takemura and Fujiwara, when subjected to a chronic load, the heart maintains an appropriate functional level through cardiomyocyte hyperfunctionality and hypertrophy, however, if the load is excessive or long lasting, cardiac hypertrophy decompensates into cardiac dilation and failure and it is likely that these changes involve progressive cardiomyocyte degeneration and death (Takemura and Fujiwara, 2006).

1.3.3. Study aim 2: determining apoptosis rates in cardiac tissue in a porcine model of metabolic syndrome

Increased apoptosis rates have been consistently reported over the years in cases of chronic heart failure (CHF) and dilated cardiomyopathy (DCM), however, the rates may range depending on the primary disease, stage or even gender, as summarized by van Empel et al. (van Empel et al., 2005). In this report, it was also noted that cardiomyocyte apoptosis has been documented as a pivotal form of cell death in ischemia and reperfusion damage, with several other reports documenting apoptotic rates of 2-12% in the border zone of human myocardial infarcts (van Empel et al., 2005). However, when it comes to HF, it is also notable to say that not all models of HF have been associated with apoptosis (Kang and Izumo, 2000).

Further, Sharov et al. have identified cardiomyocyte apoptosis in left ventricular (LV) tissue in dogs with heart failure using TUNEL assay while there was no evidence of apoptotic cardiomyocytes in normal dogs (Sharov et al., 1996). Narula et al. identified cardiomyocyte nuclear DNA fragmentation by TUNEL technique on a small number of explanted failed human hearts (Narula et al., 1996). This study was followed another one by Olivetti et al. who examined 15 failed hearts after acute myocardial infarction and found that TUNEL positive cardiomyocyte nuclei ranged from 673 to 6549 nuclei/million (Olivetti et al., 1996). Pathophysiological triggers of apoptosis in heart failure are reviewed by Sabbah and these may include hypoxia, calcium overload, excess levels of angiotensin-II, excess levels of norepinephrine, free radicals or increased levels of specific cytokines such as TNFα (Sabbah, 2000). In addition, study by Li et al. revealed increased rates of apoptosis in cardiac tissue of MetS animals, however, this increase did not reach statistical significance (Li et al., 2012). Another study utilizing Ossabaw pig model of metabolic syndrome found that cell death was increased in MetS group compared to the control, as assessed by TUNEL staining (Elmadhun et al., 2014).

1.4. Fibrosis

1.4.1. Introduction to fibrosis

Fibrosis is a symptom that is present in multiple diseases. It is the common scarring endpoint that is associated with the excessive generation and accumulation of collagen and other matrix proteins and this increase in matrix causes an increase in tissue stiffness, which impacts normal tissue function (Murray, 2015). Cardiac fibroblasts are the main cells which produce structural proteins of extracellular matrix (ECM) and are important for normal cardiac function, however, their hyperactivity, *i.e*. transition to myofibroblasts, may result in excess deposition of ECM proteins in the myocardium (Fan et al., 2012). In addition to being the primary source of ECM proteins, fibroblasts produce a number of cytokines, peptides, and enzymes among which matrix metalloproteinases (MMPs) and their inhibitors (Fan et al., 2012).

As said, under certain conditions, fibroblasts are activated and undergo phenotypic transition into “myofibroblasts”. Although in healing wounds myofibroblasts are required for tissue repair, in pathologic conditions activated myofibroblasts become the cellular effectors of the fibrotic process (Biernacka and Frangogiannis, 2011). Like fibroblasts, myofibroblasts are

non-excitable cells and are likely to produce barriers to conduction as they intercalate themselves between myocytes (Baum and Duffy, 2011). Enhanced contractility of these cells is believed to be important in allowing them to contract while bound to matrix collagens and other proteins, thereby allowing for physical remodelling of the matrix itself (Arora and McCulloch, 1994).

1.4.2. Fibrosis in heart disease and cardiac remodelling

The extracellular matrix (ECM) maintains the structural integrity of the myocardium and allows the transmission of electrical and mechanical forces between the myocytes for systole and diastole (Segura et al., 2014). ECM molecules found in the heart include hyaluronan, fibronectin, fibrillin, proteoglycans, and collagens (Lockhart et al., 2011) and these are produced by cardiac fibroblasts, which comprise the largest cell population in the myocardium (Fan et al., 2012). During the ventricular remodelling, collagen increases and occupies the areas between the myocytes and the vessels (Segura et al., 2014). The resultant fibrosis is initially a compensatory mechanism occurring at sites of previous cardiomyocyte necrosis to preserve the structural integrity of the myocardium but may progress adversely (Segura et al., 2014) thus imposing a viscoelastic burden that compromises all of the diastole, including rate of relaxation, diastolic suction and passive stiffness (Burlew and Weber, 2002). Moreover, the effect of connective tissue on cardiac electrophysiology has been attributed to its non-excitability resulting in areas of conduction block (Rog-Zielinska et al., 2016).

Within the heart, this fibrosis is thought to be partially driven by transforming growth factor-β1 (TGF-β1), a potent stimulator of collagen-producing cardiac fibroblasts (Khan and Sheppard, 2006). However, a recent review suggests that reduced collagen degradation by matrix metalloproteinases (MMPs) may be more important than increased *de novo* synthesis in the pathogenesis of aging-associated fibrosis (Biernacka and Frangogiannis, 2011). Clearly, the extent of fibrosis is determined by the balance between ECM-degrading and ECM-producing mechanisms. Moreover, activation of renin-angiotensin II-aldosterone system (RAAS) has also been associated with the development of fibrosis in hypertensive heart disease and chronic heart failure (Brilla, 2000). *In vitro* experiments using adult rat cardiac fibroblasts showed that both angiotensin II (AngII) and aldosterone stimulate collagen synthesis in a dose-dependent manner while AngII additionally suppressed the activity of MMP-1 (Brilla et al., 1994). Thus, it is proven that the administration of an angiotensin II type 1 receptor blocker (ARB) or an angiotensin-converting enzyme inhibitor (ACEI) inhibits ventricular fibrosis to the same degree

(Yamamoto et al., 2005) whereas their combined effect decreased myocardial fibrosis and LV stiffness even more effectively compared to ARB or ACEI alone (Funabiki et al., 2004). However, due to the increased adverse effects risks, the combination of the two is not recommended.

1.4.3. Study aim 3: determining the extent of fibrosis in cardiac tissue in a porcine model of metabolic syndrome

Although to date, fibroblasts have been difficult to positively identify, they may be identified based on their spindle shape combined with positive staining for the mesenchymal marker vimentin (Goodpaster et al., 2008). Vimentin is a protein present in the intermediate filaments of fibroblasts and has been the most widely used marker for these cells, although it is also expressed in other cell types such as endothelial and myoepithelial cells (Fan et al., 2012), as well as in human monocytes and activated macrophages (Mor-Vaknin et al., 2003). Impaired mechanical stability, migration and contractile capacity are observed in vimentin-deficient fibroblasts (Eckes et al., 1998). The crosstalk between cardiac fibroblasts and myocytes seems to be important during cardiac development and remodelling in response to injury (Ottaviano and Yee, 2011). Further, vimentin remains expressed on activated form of fibroblasts, known as myofibroblasts, which are thought to be the key effector cells driving the fibrosis in tissue (Chaurasia et al., 2009). Therefore, semi-quantifying vimentin expression may represent one of the initial screening tests for fibrosis, although additional markers are necessary to confirm this further.

A recent study examined patients with congestive heart failure that suffer from diastolic dysfunction with persistent left ventricular function (HFpEF) and found significantly elevated interstitial fibrosis with significant increase of vimentin positive cardiac fibroblasts (M. Willmer, 2013).

Yet, a study assessing myofibroblast activation and connective tissue formation in a porcine model of atrial fibrillation (AF) and reduced ventricular function reported that the levels of vimentin-expressing cells were similar in experimental animals compared to the control group in sinus rhythm. However, they confirm increased atrial fibrosis through enhanced expression of collagens I and V in right atrial tissue after 14 days follow-up. In addition, they confirm that a fraction of α-smooth muscle actin, a key marker for myofibroblasts, was elevated in AF animals (Lugenbiel et al., 2017).

1.5. A novel involvement of bile acids in the pathogenesis of heart failure

1.5.1. Introduction to bile acids

Bile acids (BAs) are steroid molecules (Hofmann and Hagey, 2014) with the ability to facilitate nutrient absorption and biliary secretion of lipids, toxic metabolites and xenobiotics (Chiang, 2013). In addition, it is now well appreciated that BAs are also singling molecules with pleiotropic effects owing to their interaction with one of the eight currently known bile acid activated receptors (BARs), as shown in Tab. 2.

BAs are synthesized from cholesterol in a multistep process *via* one of the two major pathways (Javitt, 1994). Classical pathway of BA synthesis accounts for more than 90% of BA production in humans (Schwarz et al., 1996) and takes place in hepatocytes with 7α-hydroxylation representing the initial and rate limiting step (Li and Chiang, 2014). Alternative pathway, on the other hand, is initiated by CYP27A1 adding a hydroxyl group in α position on C27 and can occur in liver, steroidogenic tissues, macrophages and brain (Duane and Javitt, 1999; Chiang, 2017). Following the synthesis in hepatocytes, BAs are conjugated with either glycine or taurine (ratio 3:1 in humans) by a single enzyme, Bile acid-CoA:amino acid N-acyltransferase (BAT) (Falany et al., 1994); a process which increases their solubility at physiological pH, prevents Ca^{2+}precipitation, minimizes passive absorption and protects BAs from cleavage by pancreatic carboxypeptidases (Russell, 2003; Chiang, 2013). Although the exact reason for the preference toward glycine conjugation in primates is not clear (Vessey, 1978) and seems to represent a novelty from evolutionary standpoint (Hofmann et al., 2010), dietary intake of taurine, primarily in meat, seems to account for the size of taurine-conjugated BA pool in humans (Hardison, 1978) as hepatocytes do not produce this amino acid. These, now called, bile salts are then transported to the gallbladder where they remain stored until cholecystokinin, an enterohormone released upon digestion, stimulates gallbladder contraction and subsequent secretion of the bile into the duodenum (Chandra and Liddle, 2007). Once these amphipathic molecules have solubilized dietary fat and allowed for its absorption through the brush border membrane of cells lining the intestine, 95% of BAs are efficiently reabsorbed in the apical membrane of the terminal ileum, transported across the enterocyte to the basolateral membrane and secreted into the portal blood circulation to be taken up the liver, a process designated as enterohepatic circulation (Chiang, 2013). The remaining amount of BAs, about 200-600 mg daily, is secreted *via* faeces and replenished by cholesterol *de novo* synthesis to maintain a constant (3 g) bile acid pool (Chiang, 2013).

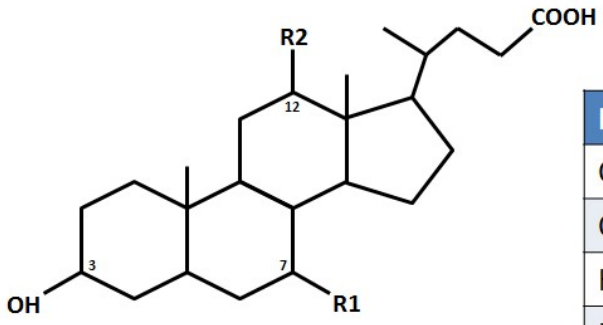
Hepatic CYP7A1 and BA biosynthesis are under negative feedback control by bile acids in a process controlled by hepatic farnesoid X receptor (FXR) (Chiang, 2015). Human intestinal FGF19 represents another important avenue to maintain BA homeostasis. FGF19 is released by enterocytes into the blood circulation in response to postprandial efflux of BAs and activates hepatic FGF receptor 4 (FGFR4) signalling to inhibit CYP7A1 mRNA expression and thereby acts to inhibit BA synthesis in the liver (Wu et al., 2011).

The principal human bile acids are CA, CDCA and DCA in ratios 2:2:1 whereas LCA is usually found in trace amounts. As shown in Fig.2 cholic acid, due to three hydroxyl groups in its molecular structure, is highly hydrophilic BA. LCA is, on the other hand, highly hydrophobic owing to only one OH-group in its structure. While their overall similar molecular structure holds the affinity to BARs, different electron distribution across the molecule accounts for the different conformational changes of the receptor upon ligand binding and subsequent triggering or halting intracellular signalling within the cell expressing that BAR (known as agonism and antagonism).

As mentioned above, there are several currently known bile acid responsive receptors as presented in Tab. 2. Thus, bile acids are ligands for at least five nuclear receptors: FXR, CAR, PXR, LXRα and VDR. In addition, secondary bile acids show high affinity to membrane bound G-protein-coupled receptors, including TGR5, S1P receptor and muscarinic receptors. The current study focuses on TGR5 and explores its possible involvement in the pathogenesis of HF.

Table 2. List of currently known bile acid-activated receptors (BARs) and their triggering molecules in the order of potency. CDCA=Chenodeoxycholic acid, LCA=Litocholic acid, DCA=Deoxycholic acid, CA=Cholic acid, HCA=Hyocholic acid, HDCA=Hyodeoxycholic acid, T=taurine conjugate.

BAR	Natural ligands
FXR (Farnesoid X receptor)	CDCA>LCA=DCA>CA
PXR (Pregnane X receptor)	LCA>DCA>CA
VDR (Vitamin D receptor)	LCA>DCA>CA
LXR (Liver X receptor)	HCA and HDCA
TGR5 (Takeda G-protein receptor)	DCA>LCA>CDCA>CA
Muscarinic receptor 2, 3	TLCA>TDCA>TCA
S1P2 Sphingosine-1-phosphate receptor)	Taurine or glycine conjugates



R1	R2	Bile acid
OH	OH	Cholic acid (CA)
OH	H	Chenodeoxycholic acid (CDCA)
H	OH	Deoxydcholic acid (DCA)
H	H	Litocholic acid

Figure 2. Molecular structure of human bile acids.

1.5.2. Bile acids in heart disease and cardiac remodelling

Discovery of bile acid-activated receptors has further resulted in efforts to link these receptors and physiological roles of BAs with conditions such as diabetes, NAFLD, dyslipidaemia or cardiovascular dysfunction (Prawitt et al., 2011; Porez et al., 2012; Li et al., 2013).

Indeed, FXR expression was detected in cardiovascular organs such as coronary arteries, aorta, heart and atherosclerotic arteries (Bishop-Bailey et al., 2004; Wang et al., 2008), whereas VDR has been identified in endothelial and vascular smooth muscle cells (Mathew et al., 2008). PXR expression has been discovered in mesenteric arteries and subsequently linked to vascular adaptation during pregnancy (Hagedorn et al., 2007). Muscarinic receptor stimulation by acetylcholine plays an important role in mediating parasympathetic control of cardiac functions such because heart rate, conduction, and contractility (Wang et al., 2001) thus studies indicate that CT (taurocholate) might regulate cardiac function due to its interaction with M2 muscarinic receptors (Sheikh Abdul Kadir et al., 2010). TGR5 has also been identified in hepatic sinusoidal endothelial cells and cardiomyocytes (Keitel et al., 2007; Khurana et al., 2011). In addition to receptors, BAs have also been shown to interact with Ca^{2+}-activated K^{+} (BKCa) channels in vascular smooth muscle tissue which then leads to vessel relaxation and preservation of tissue perfusion (Dopico et al., 2002). Further, what is interesting is that BA transporters such as BSEP, MDR3 and OATP are found to be expressed in a human foetal cardiomyocyte culture (Gorelik et al., 2006), however, their role in cardiac function is also not clear.

A comprehensive review on the effects on bile acids and their conjugated forms on cardiovascular tissue has been presented by Khurana et al in their article *Bile Acids Regulate Cardiovascular Function* (Khurana et al., 2011). To mention few, BAs and their synthetic forms

may decrease myocyte contraction rate and induce bradycardia (DCT, CA), reduce the duration of action potential (CT), induce myocyte apoptosis (UDCA), reduce allograft rejection (UDCA), cause arterial vasodilation (DCT, CDCT) or NOS up-regulation in endothelial cells (CDCA), reduce inflammation (6EDCA, GW4064), and even induce (CDCA) or inhibit (UDCA) angiogenesis in different tissue models.

Although the information on the possible involvement of BAs and their receptors in cardiac remodelling and heart failure is still lacking, it would be interesting to explore this avenue and assess the extent to which BAs may play a role in pathogenesis of this disease, especially considering microbiome changes seen in these states and their impact on BA pool composition.

1.5.3. Study aim 4: determining TGR5 expression in cardiac tissue in a porcine model of metabolic syndrome

Since its identification in 2002 (Maruyama et al., 2002), TGR5 (GP-BAR1, M-BAR, Takeda-G-protein-receptor 5) is found to be prominently expressed in gallbladder and enteric nervous system (Keitel et al., 2009; Poole et al., 2010) as well as in in several other tissues including spleen, lymph nodes, liver, adipocytes, kidney, skeletal muscle, pancreas and macrophages (Duboc et al., 2014). TGR5 is not expressed in hepatocytes but has been detected in hepatic resident macrophages (Kupffer cells) (Keitel et al., 2008). Although it is possible that physiological concentrations of circulating bile acids outside of the enterohepatic system do not trigger TGR5 activation (Li and Chiang, 2012), it has been demonstrated that secondary bile acids, DCA and LCA exhibit superior affinity for TGR5 compared to CDCA and CA regardless of the state of conjugation (Maruyama et al., 2002; Sato et al., 2008). This may imply that increased secondary to primary bile acids ratio due to variations in microbiome composition during MetS could be sufficient to activate this receptor in certain tissues.

TGR5 activation in enteroendocrine cell line shows high potency to stimulate glucagone-like-peptide 1 (GLP-1) production, which is known to promote insulin secretion thus regulating glucose homeostasis (Katsuma et al., 2005). In addition to antidiabetic effects due to GLP-1 effects, TGR5 has also sparked researchers' interest due to its ability to induce type 2 deiodinase in brown adipose tissue. This enzyme works by causing the conversion of thyroxin (T3) to active triiodothyronine (T4), which then stimulates mitochondrial oxidative phosphorylation and energy expenditure (Watanabe et al., 2006). This may be significant considering that a recent study found that the amount of brown adipose tissue is inversely correlated with body mass index (BMI), especially in older people (Cypess et al., 2009).

Further, TGR5 also seems to protect the integrity of intestinal barrier function (Cipriani et al., 2011).

Finally, an accumulating body of evidence now suggests that TGR5 also has a role in processes linked to inflammation (Pols, 2014), which may be relevant since low-grade inflammation is believed to be a major contributor to the development of metabolic syndrome. A high expression of TGR5 is found on monocytes and macrophages (Duboc et al., 2014) and may therefore be identified in human spleen (Kawamata et al., 2003), Kupffer cells (Keitel et al., 2008), alveolar macrophages (Kawamata et al., 2003), as well as human CD14+ monocytes (Ichikawa et al., 2012). Stimulation of isolated rat Kupffer cells with taurolithocholic acid (TLC), powerful TGR5 agonist, resulted in reduced expression of IL-1α, IL1-β, IL-6 and TNFα after LPS treatment (Keitel et al., 2008). This data suggests anti-inflammatory role of TGR5. Indeed, in one study TGR5 has been demonstrated to exert anti-inflammatory and anti-atherosclerotic actions by reducing macrophage inflammation and lipid loading (Pols et al., 2011). Another study also reports that the activation of TGR5 could inhibit inflammation in liver and stomach (Guo et al., 2015). In liver, TGR5 inhibits the expression of inflammatory mediators by interfering with NF-κB pathway in wild type (WT), but not in TGR5-deficient mice (Wang et al., 2011; Guo et al., 2015). Another study suggests that TGR5 attenuates liver ischemia/reperfusion injury *via* the inhibition of toll-like receptor 4 signalling in mice (Yang et al., 2017).

When it comes to the cardiovascular disease, it seems that the majority of studies have been focusing on the anti-inflammatory function of TGR5 and its ability to decrease production of pro-inflammatory cytokines and to therefore, attenuate atherosclerotic plaque formation. However, its expression and role in cardiac tissue remain vastly unexplored. Recently TGR5 has been identified as a novel therapy for heart failure (Moreshwar S Desai, 2014). In this study, researchers demonstrate that cholic acid (CA) feeding functionally activates TGR5 in mouse heart. They also show that CA attenuates contractile failure and pathologic hypertrophy in mouse model of HF and it also leads to enhanced glucose oxidation, a crucial step in cardiac adaptation to stress (Moreshwar S Desai, 2014).

2. Study outline

CVDs are the leading cause of death in the modern world, accounting for over one third of global deaths, according to the data from 2015. Patients who are diagnosed with metabolic syndrome, as evident by abdominal obesity, abnormal cholesterol levels, hypertriglyceridemia, insulin resistance and/or hypertension, are at twice risk of developing CVD over the next 5 to 10 years. Heart failure, often encountered within this group of patients, is a syndrome caused by an inadequate performance of the heart which, over the course of time, leads to series of adaptive molecular and interstitial changes known as cardiac remodelling, which poses a further threat to patient's well-being and results in symptomatic heart failure. Inflammation, apoptosis and/or fibrosis have been established in multiple conditions linked with poor cardiac function. In addition, bile acids are increasingly being recognised as signalling molecules involved in glucose and lipid metabolism. Moreover, their role has also been implicated in cardiovascular function and inflammation.

To investigate the extent and contribution of inflammation, apoptosis, fibrosis and bile acids to progressive decrease in cardiac function observed in MetS patients, the current study examined left atrial (LA) and left ventricular (LV) tissue of pigs with established metabolic syndrome (MetS), as well as of control animals (n=5 by group). M2-macrophage (CD163) and T-lymphocyte (CD3) markers, as well as CD16, a monocyte/NK cell receptor whose increased expression was linked with CVD, were used to assess inflammation. TUNEL staining was used to detect apoptotic cells, whereas vimentin was used as a marker of (myo)fibroblasts which might provide an initial screening for cardiac fibrosis. Finally, the expression of bile acid-activated receptor, TGR5, was assessed to determine the contribution of bile acid pool to the pathogenesis of heart failure (HF).

2.1.1. Working hypothesis

1. LA and LV tissue in MetS animals is characterized by increased amount of inflammation markers.
2. LA and LV tissue in MetS animals is characterized by higher apoptosis rates.
3. LA and LV tissue in MetS animals is characterized by higher fibrosis rates and myofibroblast expansion.
4. TGR5 might be involved in the pathogenesis of heart failure.

2.1.2. Objective

Identifying the ongoing processes in the cardiac muscle during the heart failure and modifying some of them might hold a potential to prevent the progressive loss of cardiac function seen in HF. The objective is to identify the expression of the above-mentioned markers in LA and LV tissue of MetS compared to the control animals, as these chambers seem to be the most relevant in transition to symptomatic HF. This information would reveal molecular targets for novel therapeutics to treat CVD.

3. Methods

3.1. Immunofluorescence staining for inflammation, apoptosis and fibrosis markers and TGR5 expression in cardiac tissue in MetS animals compared to the control group

3.1.1. OCT embedding and cryostatting

Upon collection, left atrial (LA) and left ventricular (LV) tissue was immediately embedded in OCT, put on a dry ice for a temporary temperature maintenance and transportation and stored in a freezer at -80 °C. Using Leica cryostat, blocks were sectioned as 5 µm thick slides and attached to coated Menzel Superfrost slides.

3.1.2. Staining

Slides were thawed at room temperature for 10-20 minutes and allowed to completely dry. Tissue was then fixed for 10 minutes in chilled methanol (CD163, CD16, CD3) or for 20 minutes 4% paraformaldehyde at room temperature (TGR5, TUNEL, vimentin). Slides were washed in PBS for 5 min 3 times in a row. The excess liquid was then aspirated and the blocking buffer (10% goat serum in PBS-Tween20) was applied for 1 hour at room temperature. Blocking buffer was aspirated and primary antibody was applied in an appropriate dilution in the incubation buffer (CD163 1:1000, CD16 1:750, CD3 1:200, TGR5 1:1000, vimentin 1:500; Tab. 3) and incubated overnight at 4 °C in a humid slide box. The next day, primary antibody was aspirated, and slides were washed in PBS for 5 min (x3) and incubated with an appropriate secondary antibody diluted in incubation buffer (goat-anti mouse IgG1 1:1000 or goat-anti rabbit IgG1 1:1000) for 1 hour at room temperature. Slides were then washed in PBS for 5 min (x3), DAPI (1 µg/ml solution in PBS) was applied for 10 minutes at room temperature. After the final washing with PBS, slides were covered with anti-fade mounting media and coverslipped. 1.5% Sudan Black solution was used to decrease autofluorescence in TGR5-stained tissues prior to the DAPI treatment. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) for assessing apoptosis in tissue was permeabilised with 0.1% Triton in water for 4 minutes and further staining was performed following the kit instructions and using provided (enzyme) solutions.

Table 3. List of antibodies used in the current study.

Primary Antibody	**Dilution**
CD163 – Mouse anti pig CD163 (Biorad)	1:1000
CD16 – Mouse anti pig CD16 (Biorad)	1:750
CD3 – Anti-CD3 antibody [SP7] (abcam)	1:200
Vimentin – Anti-vimentin antibody [V9] – Cytoskeleton marker (abcam)	1:500
TGR5 – Anti-GPCR TGR5 antibody (abcam)	1:1000
TUNEL – In situ cell death detection fluorescein (Roche)	NA
Secondary Antibody	
Goat Anti-rabbit IgG H&L (Alexa Fluor 488) (Thermo Fisscher Scientific)	1:1000
Goat Anti-Mouse IgG H&L (Alexa Fluor 488) (Thermo Fisscher Scientific)	1:1000

3.1.3. Imaging and data analysis

60x pictures were taken with the confocal microscope (Nikon D-Eclipse). Pictures were analysed using NIS-Elements BR 3.0 software and statistical analysis was performed using GraphPad prism 5.0. Differences between groups were analysed with student test followed by Fisher's test with significance set at $P<0.05$.

4. Results

4.1. MetS animals show an increase in CD163+ macrophages in LA and LV tissue

To test whether the events in the failing heart were aimed toward tissue repair and functional recovery through increased activity of M2-macrophages (anti-inflammatory), anti-CD163 antibody was used. CD163 is exclusively expressed in monocytes and macrophages, preferentially on M2 subgroup. Spleen was used as a positive control (Fig. 3A).

An increase in CD163+ macrophages was observed in both left atrial (LA) and left ventricular (LV) tissue in MetS animals compared to the control group (respectively, p=3.5E-19, p=1E-23, Fig. 3B, C).

The observed increase of CD163+ cells in MetS animals was approximately 2-fold in the percentage of CD163+ macrophages in both examined tissues: LA (9.4 ± 0.5 vs. 18.1 ± 0.7) and LV (10.1 ± 0.3 vs. 19.3 ± 0.6).

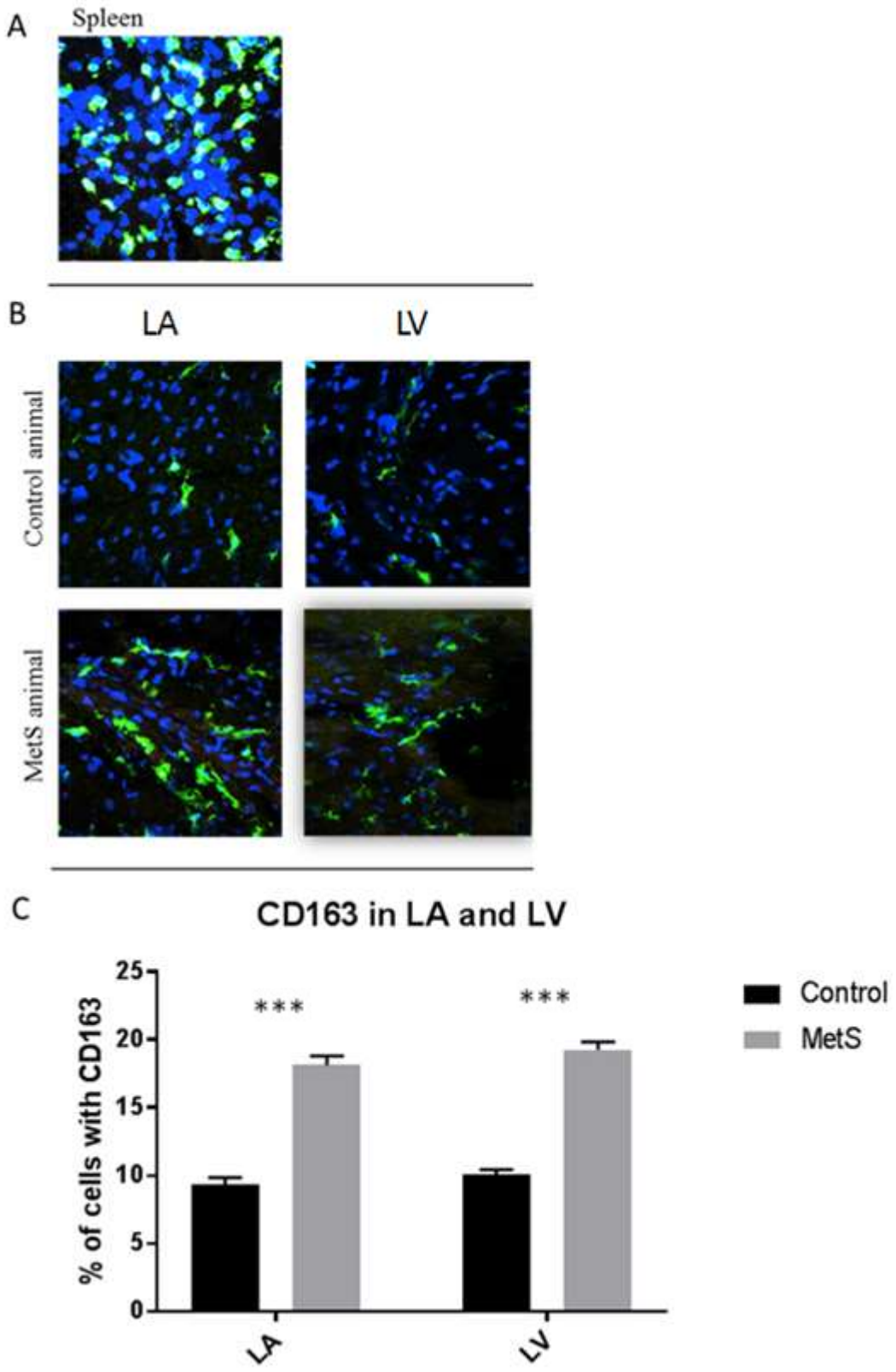


Figure 3 CD163 expression in LA and LV (CD163=green, DAPI=blue). A) Positive control tissue. C. B) representative 60x pictures of an OCT embedded heart tissue sections showing CD163 expression in LA and LV tissue (CD163=green, DAPI=blue). C) Semi-quantitative analysis of OCT heart tissue sections showing CD163 expression in LA and LV chambers. Number of CD163+ cells is expressed as a percentage over the percentage of total nuclei per picture. Quantification shows a significant increase of CD163+ cells in LA and LV tissue in MetS animals compared to the control group.

4.2. MetS animals show an increase in CD16+ cells in LA and LV tissue

To test whether the antibody dependent cellular cytotoxicity (ADCC) may play a role in heart failure, CD16-antibody was used. CD16, (FcγRIIIA) is an immunoglobulin receptor essential for ADCC by natural killer (NK) cells and is also expressed by a subset of human blood monocytes and neutrophils. Spleen was used as a positive control (Fig. 4A).

An increase in total number of CD16+ cells was observed in both left atrial (LA) and left ventricular (LV) in MetS animals compared to the control group (respectively, p=5E-19, p=4.6E-06, Fig. 4B, C).

The observed increase in MetS animals in the percentage of CD16+ cells was 8.7 ± 0.3 vs. 15.6 ± 0.5 in LA, and 6.3 ± 0.5 vs. 10.8 ± 0.7 in LV, compared to the control animals (Fig. 4B, C).

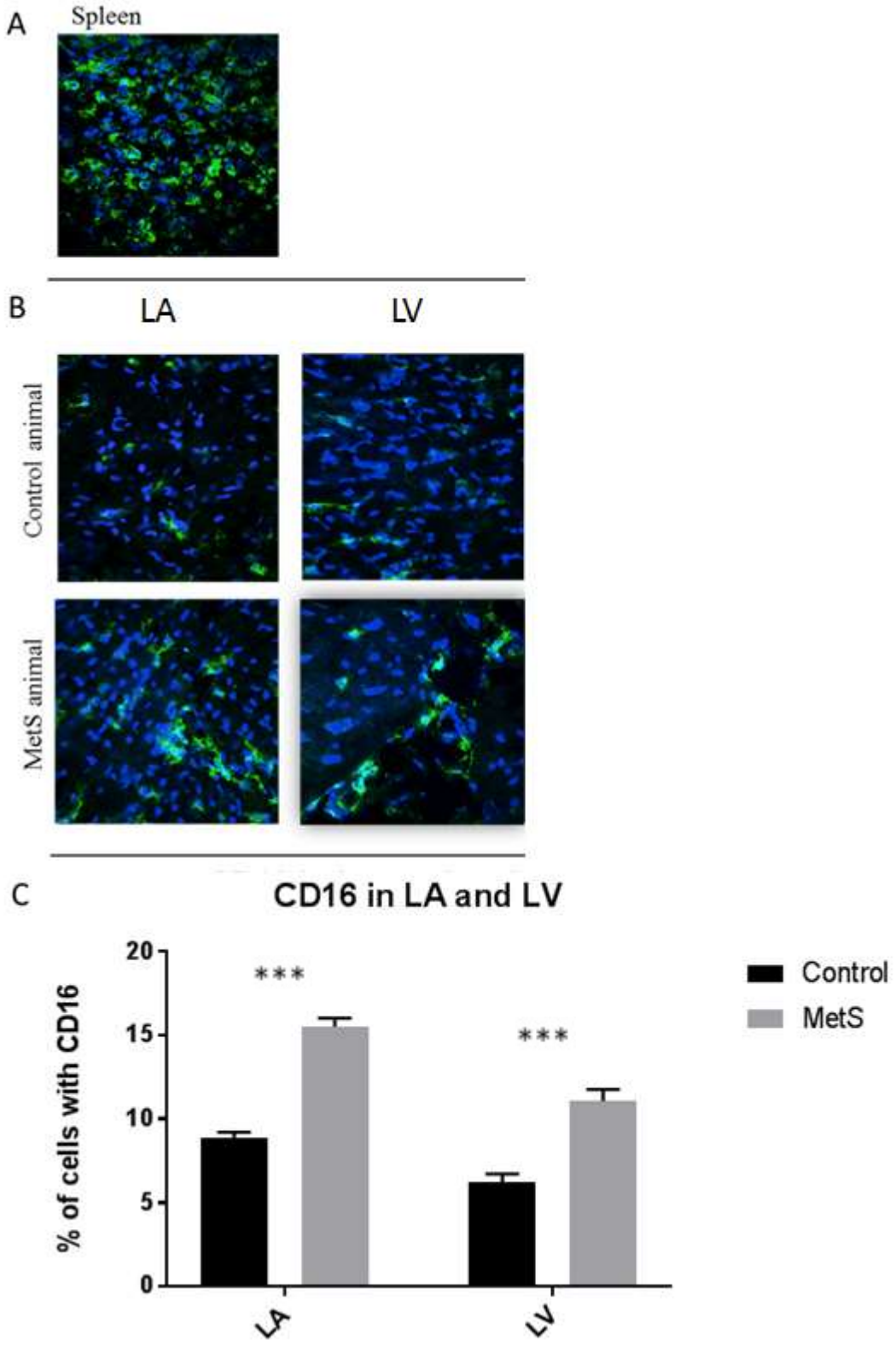


Figure 4 CD16 expression in LA and LV tissue (CD16=green, DAPI=blue). A) Positive control tissue (spleen). C, B) representative 60x pictures of an OCT embedded heart tissue sections showing CD16 expression in LA and LV tissue. C) Semi-quantitative analysis of OCT heart tissue sections showing CD16 expression in LA and LV tissue. Number of CD16+ cells is expressed as a percentage over the percentage of total nuclei per picture. Quantification shows significant increase of CD16+ cells in LA and LV tissue in MetS animals compared to the control group.

4.3. MetS animals do not show significant increase in CD3+ lymphocytes in LA and LV tissue

To assess whether the adaptive immune responses in terms of increased number of lymphocytes may be responsible for deleterious events in the failing heart, we used CD3-antibody. CD3 is a specific marker of T lymphocytes. Spleen was used as a positive control (Fig. 5A).

An increase in CD3+ lymphocytes was observed in left atrial (LA) and left ventricular (LV) tissue in MetS animals compared to the control group (respectively p=0.0002, p=0.045, Fig. 5B, C).

Statistical analysis showed an increase in CD3+ lymphocytes in LA (0.6 ± 0.1 in the control vs. 1.2 ± 0.1 in MetS animals) and LV (1.1 ± 0.1 in control *vs*. 1.4 ± 0.1 in MetS animals). However, due to the scarce number of stained cells included in analysis and small difference between means in LA (approximately 0.5), the result in this tissue was not regarded as a biologically significant increase in the number of T-lymphocytes in MetS animals, compared to the control group. The increase observed in LV tissue did not reach statistical significance.

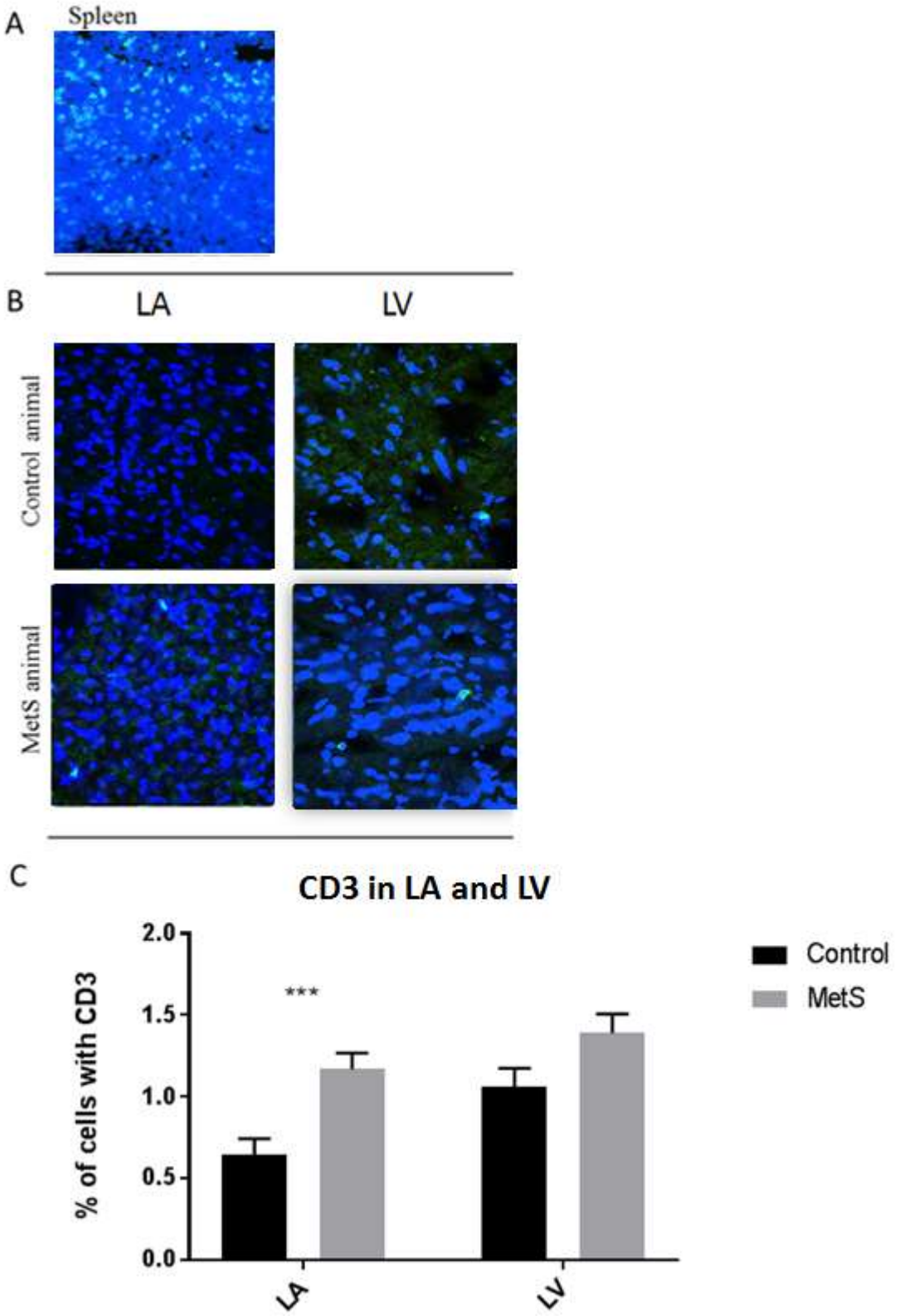


Figure 5 CD3 expression in LA and LV tissue (CD3=green, DAPI=blue). A) Positive control tissue (spleen). B) Representative 60x pictures of an OCT embedded heart tissue sections showing CD3 expression in LA and LV tissue. C) Semi-quantitative analysis of OCT heart tissue sections showing CD3 expression in LA and LV tissue. Number of CD3+ cells is expressed as a percentage over the percentage of total nuclei per picture. Quantification shows mild increase of CD3+ cells in LA and LV tissue in MetS animals compared to the control group.

4.4. MetS animals have higher apoptosis rates in LA and LV tissue

To test whether the apoptosis was responsible for progressive decrease in cardiac function in MetS animals, a TUNEL apoptosis assay was used. For the TUNEL assay tissue was treated following the kit instructions and using heart tissue harvested in less than 12 hours period from a pig that suffered myocardial infarct (MI) as a positive control (Fig. 7A).

Statistically significant increase in apoptotic events was observed in both LA and LV tissue of MetS animals compared to the control group (respectively, $p=0.0061$, $p=0.0032$, Fig. 7B, C).

Total percentage of apoptotic cells in LA was 0.3 ± 0.20 for the control group and 6.7 ± 1.5 for MetS animals. Further, total percentage of apoptotic cells in LV was 0.2 ± 0.1 for the control group and 2.5 ± 0.2 for MetS animals.

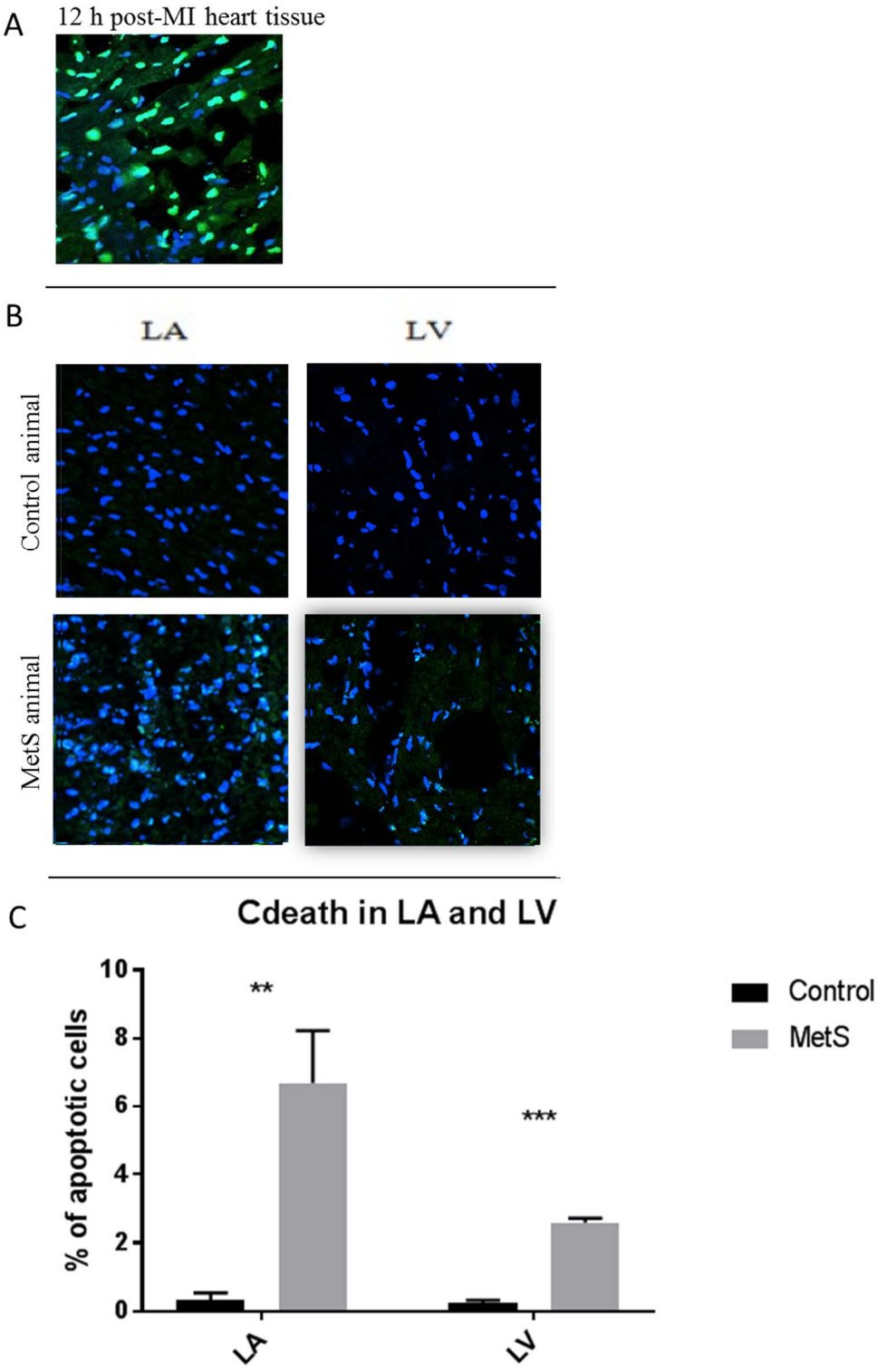


Figure 6. TUNEL apoptosis assay of LA and LV frozen tissue sections in MetS and control animals (TUNEL staining=green, DAPI= blue). A) Positive control tissue (12 h post-MI pig heart tissue). B, C) TUNEL stained LA and LV tissue in control and MetS animals. Number of apoptotic cells is expressed as percentage of TUNEL stained cells over total nuclei number. Quantification shows significant increase in the percentage of apoptotic cells in LA and LV tissue in MetS animals compared to the control group.

4.5. MetS animals show increase in vimentin expression in LV tissue but not in LA

To assess the signs of myocardial fibrosis in heart, anti-vimentin antibody was used. Vimentin is a protein expressed in the intermediate filaments of fibroblasts (FB) and has been most widely used marker for these cells, although it is also found on other cells types such as endothelial and myoepithelial cells. Kidney was used as a positive control (Fig. 8A).

No significant difference was observed in LA tissue between MetS and control animals, whereas the difference in LV was significant (respectively, p=0.2096, p=0.0001, Fig. 8B, C).

Further, percentage of vimentin on total percentage of cells in LA tissue was similar in both group of animals (38.5 ± 1.3 vs. 36.2 ± 1.2), whereas in LV tissue total percentage of vimentin+ cells increased from 21.5 ± 0.8 in the control group to 26.3 ± 0.9 in MetS group.

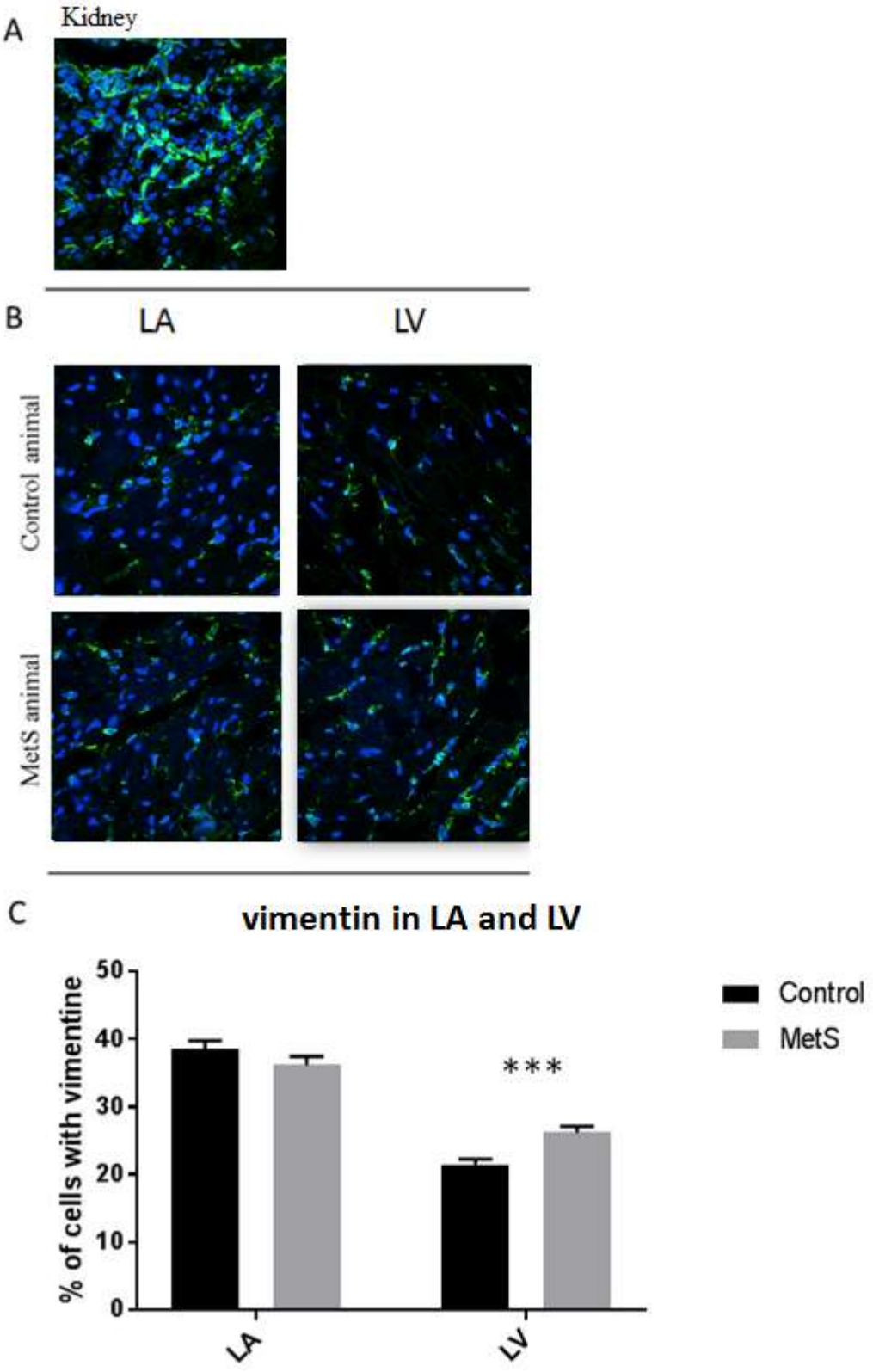


Figure 7. Vimentin expression in LA and LV tissue (vimentin=green, DAPI=blue). A) Positive control tissue (kidney). B) Representative 60x pictures of an OCT embedded heart tissue sections showing vimentin expression in LA and LV tissue. C) Semi-quantitative analysis of OCT heart tissue sections showing vimentin expression in LA and LV tissue. Number of vimentin+ cells is expressed as a percentage over the percentage of total nuclei per picture. Quantification shows no significant increase of vimentin+ cells in LA between the two groups, but significant increase in LV tissue in MetS animals compared to the control group.

4.6. MetS animals show higher expression of TGR5 in LA and LV tissue

To test whether an increase in serum bile acids observed previously in metabolic syndrome (data not showed) may be sufficient to activate TGR5 receptor expressed in the heart and whether that way bile acids may contribute to the heart failure and signs and symptoms of metabolic syndrome, we used anti-TGR5 antibody. Spleen was used as a positive control (Fig. 6A).

An increase in TGR5 expression was observed in MetS animals in LA and LV tissue compared to the control group (respectively, p=1.5E-14, p=1.0E-15, Fig. 6B, C).

Total percentage of TGR5-expressing cells in LA was 4.9 ± 0.3 for the control group and 9.3 ± 0.5 for MetS animals, whereas in LV that percentage was 5.5 ± 0.5 for the control group and 10.03 ± 0.3 for MetS animals.

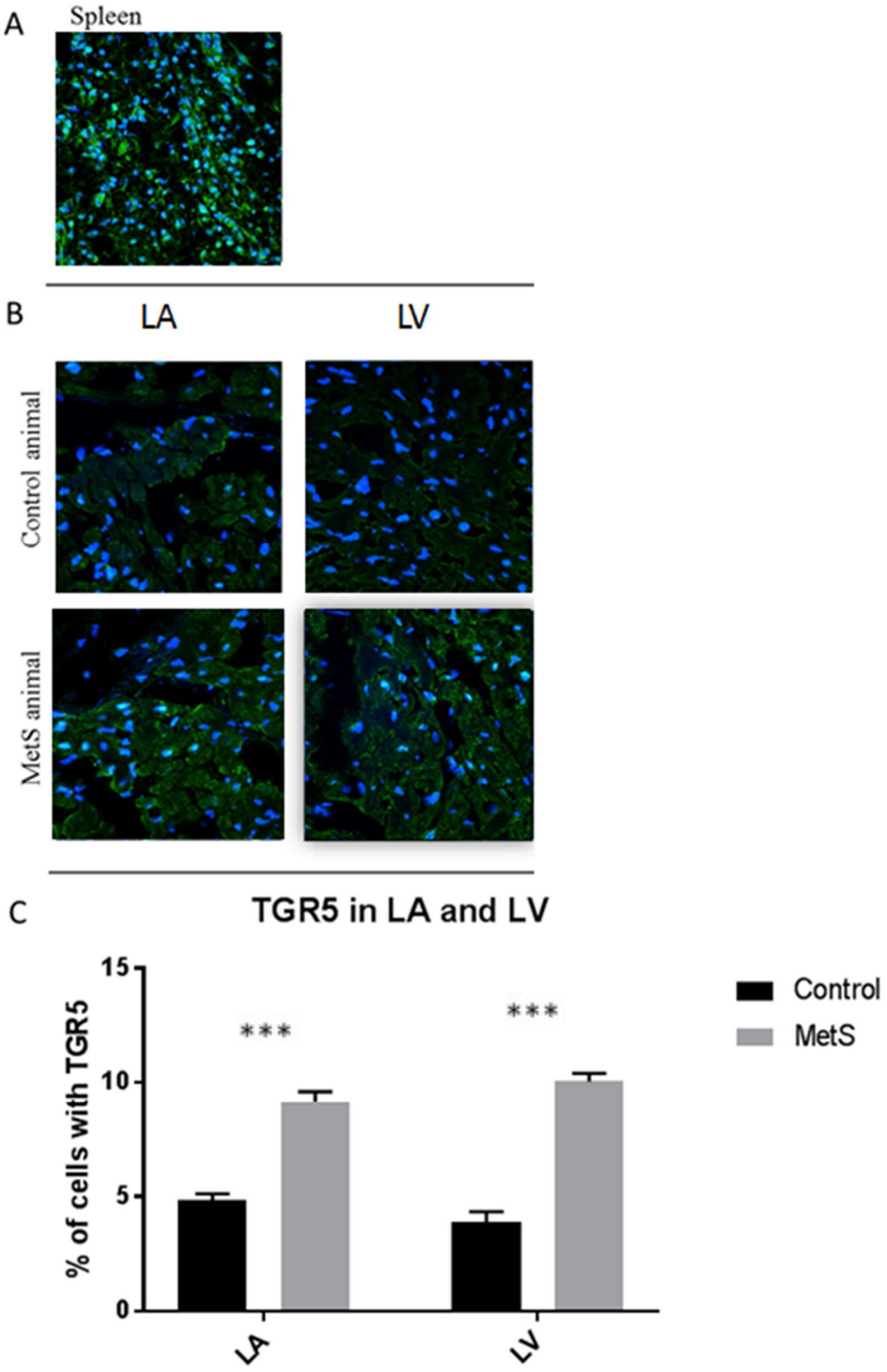


Figure 8. TGR5 expression in LA and LV tissue (TGR5=green, DAPI=blue). A) Positive control tissue (spleen). B) Representative 60x pictures of an OCT embedded heart tissue sections showing TGR5 expression in LA and LV tissue. C) Semi-quantitative analysis of OCT heart tissue sections showing TGR5 expression in LA and LV tissue. Number of TGR5+ cells is expressed as a percentage over the percentage of total nuclei per picture. Quantification shows significant increase of TGR5+ cells in LA and LV tissue in MetS animals compared to the control group.

5. Discussion

5.1. Inflammation in HF

5.1.1. Increase in CD163-positive cells

The data of the current study revealed a significant increase in CD163-expressing macrophages in cardiac tissue in LA and LV chambers.

This result is consistent with the recent report by Li et al. in which they report an increased CD163 expression in cardiac tissue in Ossabaw pigs with metabolic syndrome compared to the obese group of pigs (Li et al., 2012). In this study MetS-prone Ossabaw pigs were randomized to 10 weeks of standard chow or to 10 (obese) or 14 (MetS) weeks of atherogenic diet. Through the assessment of inflammation markers (CD163, CD8, TNFα, MCP-1, to name few), fibrosis and apoptosis (TUNEL) markers, this study concluded that the transition from obesity to MetS is associated with progressive changes of myocardial autophagy, apoptosis, inflammation and fibrosis (Li et al., 2012). Further, another study evaluated liver damage in children with non-alcoholic liver disease (NAFLD) by immunohistochemistry, and, among other findings, revealed that a significant increases in CD163+ and CD45+ cells, as well as a decrease in CD3+ cells, were correlated with the severity of liver damage in these patients as assessed by steatosis, ballooning and fibrosis (De Vito et al., 2012). Finally, a more recent study which utilized a rat model of atrial fibrosis and fibrillation revealed an enhanced expression of MCP-1 and ED-1 in left atrial tissue (Kume et al., 2017). MCP-1 serves as chemoattractant for circulating monocytes, which differentiate into macrophages upon infiltration in tissue, whereas ED-1 represents the rat homolog of human CD68, a marker of monocytes and macrophages. The study concluded that the left atrial (LA) inflammation and interstitial fibrosis contribute to the pathogenesis of atrial fibrillation (AF) (Kume et al., 2017).

The current results suggest that M2-macrophages play a role in HF, which is apparent through a significantly increased number of CD163-expressing macrophages in heart tissue in animals with poor cardiac function. The above-mentioned studies also suggest that the increased number of macrophages correlates with the severity of disease, regardless of the tissue or disease type. In addition, increased number of macrophages found in the current study confirms the ongoing inflammatory processes in the failing heart and suggests that the immune system plays a role in the pathogenesis of HF. However, it is still not clear whether the inflammatory tendencies in the failing heart are protective mechanisms or they are accelerating

the progression of disease, therefore, this question remains to be elucidated in future studies. Further, it is still not clear whether the increase in CD163+ macrophages in the failing myocardium is a consequence of gradual infiltration of these cells from bloodstream or it is a result of rapid proliferation of resident heart macrophages. However, considering that previously mentioned study of AF in rats found enhanced expression of MCP-1 in LA tissue, along with macrophage marker (CD68), it seems plausible that monocytes infiltrate cardiac tissue in HF. However, due to species and primary disease differences (AF *vs*. MetS), this premise needs to be explored and confirmed in a porcine or human model of HF.

To elucidate why M2-macrophages may increase in the failing tissue, their contribution to wound healing and tissue repair, as well as their phagocytic ability will be brought to attention in this discussion (see *Holistic approach to the results of the current study*).

5.1.2. Increase in CD16-positive cells

The current results indicate significantly increased activation of CD16 receptor in LA and LV tissue in MetS animals, compared to the control group.

Yin et al. have already reported that CD16 expression on human monocytes is increased in patients with coronary heart disease (CHD) (Huang et al., 2012) and that its aberrant expression on monocytes may contribute to the development of atherosclerosis (Huang et al., 2012). In the former study, levels of inflammatory cytokines, such as TNFα and IL-1, were elevated in sera in patients with CHD, along with increased expression of adhesion molecule ITAM-1 on CD14++CD16+ monocytes, whereas in the latter, the enhancement of CD16 on monocytes was closely correlated to the increased content of MMP-9 in aorta, which cleaves different bioactive molecules implicated in plaque destabilization. Moreover, increased levels of CD14++CD16+ blood monocytes have been reported in heart failure patients (Barisione et al., 2010). This study examined 30 male patients with congestive heart failure (CHF) and concluded not only that CD14++CD16+ populations were increased in CHF patients, but their levels also correlated positively with markers of severity of disease as well as CRP levels and neutrophil count. Moreover, in addition to the fact that the increased levels of CD16 were found on blood monocytes in HF patients in the three studies mentioned, another study by Czepluch et al. examined CD16 expression in human myocardium after infarction (Czepluch et al., 2013). The number of CD16+ cells was increased, although differences did not reach statistical significance. Although higher absolute numbers of CD16+ cells were found compared do CD14+ cells, the immunosignal was partially found to co-localise. Moreover, the number of

CD16+ and CD14+ cells correlated significantly with each other. The study concluded that monocytes are preferentially present in the sub-acute phase after MI and contribute to cardiac repair process, in particular fibrosis (Czepluch et al., 2013).

The repeatedly confirmed increase in CD16-expressing monocytes in blood stream in patients in HF and its correlation with the severity of disease makes it apparent that this receptor plays and important role in HF which is further confirmed by its correlation with atherosclerosis. On the other side, although an increase CD16 expression in study conducted by Czepluch et al. did not reach statistical significance, the sole nature of the condition studied (MI vs. MetS) in comparison to the current study may have contributed to this difference. Further, considering that CD16 is a crucial receptor for antibody-dependent cellular cytotoxicity mediated by natural killer (NK) cells or monocytes, this finding confirms ongoing inflammatory processes in cardiac tissue in MetS animals. ADCC is a mechanism activated to induce apoptosis in the target cell when it displays an appropriate signal allowing ADCC to happen. Although the identity of the target cell and the reason for displaying the 'death signal' are still not clear, this mechanism may represent a promising target pathway to modulate the outcome of the disease. Finally, one must also consider whether the increase in CD16-expressing cells in the failing myocardium observed in current study is causal or it represents a marker of HF progression caused by gradual infiltration of CD16+ monocytes or NK cells and whether this event is protective or harmful.

Therefore, if the premise that increased activation of CD16 receptor accounts for increased apoptosis rates, inhibition of CD16 or its signalling may represent a promising approach for the prevention and treatment of HF.

5.1.3. Elusive increase in CD3-positive cells

Statistical data showed small but significant increase in CD3+ lymphocytes in LA, but not in LV tissue in MetS animals, compared to the control group, however, this result became a subject of critical evaluation, thus it was concluded that the increase in LA does not seem biologically significant (less than 1 positively stained cell in MetS animals, n>144).

A recent study by Macri et al. evaluated several fibrosis and inflammation markers in the aging heart of Rhesus Macaque monkeys, among which were CD3 and CD8. Their results showed a significant increase in perivascular and interstitial fibrosis in the mid-myocardial region of each LV section between the young group of animals (0.33%) and both middle-aged

(1.7%) and the advanced age group. Further, they reported a significant increase in CD3-expressing cells in the middle-aged group compared to the young one, while there was no significant difference between the young and advanced age group. Surprisingly, they also reported that CD8 increased most significantly between the young group when compared to the advanced age group and that cells expressing CD8 significantly correlated with increasing age. Further, an older study found that CD3+ lymphocytes were more abundantly present in tissue obtained from patients with end stage HF (prior to transplantation), thus confirming the chronic low-grade inflammation (Devaux et al., 1997), however, the stage of development of disease may account for the differences observed in the current study. Finally, a recent study reported significant expansion of CD8+ and CD4+ T-cells in the failing myocardium in mice, with increased Th1, Th2, Th17 and Treg CD4+ subsets, marked reduction of Th1/Th2 ratio, augmentation of the Th17/Treg ratio and up regulation of Th2 cytokines. All of that sugessted that CD4+ lymphocytes are globally expanded and activated in chronic ischemic HF, with Th2 (versus Th1) and Th17 (versus Treg) predominance (Bansal et al., 2017). The study also reported that an adoptive transfer of splenic CD4+ T-cells (and, to a lesser extent cardiac CD3+ T-cells) from donor mice with HF induced long-term left-ventricular dysfunction, fibrosis and hypertrophy in naive recipient mice (Bansal et al., 2017). However, it is being more and more acknowledged that mice models of cardiovascular disease show poor translatability to human or large animal models, thus mice studies should be taken with extra caution. In contrast to that study, a review on post-MI myocardium T-cell subsets suggests that the conventional CD4+ T-cells in infarcted myocardium are mainly Th1 polarized, whereas Th2- and Th17-differentiated T-cells are barely detectable (Hofmann and Frantz, 2015). Finally, previously mentioned study on Ossabaw pigs with metabolic syndrome reported infiltration of CD8+ lymphocytes (those would also be stained as CD3+) in myocardial tissue of MetS animals compared to their both lean and obese counterparts (Li et al., 2012). Although this study suggests lymphocyte expansion in hearts of MetS pigs, unlike the current study, one must be reminded that different marker has been used, when compared to the current study. Indeed, since CD3 is a general T-lymphocyte marker reflecting the presence of both CD4+ (Th1, Th2 or Th17 helper cells) and CD8+ cells (cytotoxic T-cells), as well as γδ T-cells (these cells require no co-receptor in terms of CD4 or CD8 molecule), thus not differentiating between them, it seems possible that the polarization and expansion of one subclass of T-cells may have occurred simultaneously with the down regulation of the number of cells of another subclass, thus causing the global number of CD3+ lymphocytes to remain stable.

However, more studies are needed to elucidate whether the number of T-lymphocytes is altered in cardiac tissue in HF and which roles they may be having in pathogenesis and progression of disease.

5.1.4. Increase in apoptotic events

The current data revealed a 6% increase in apoptotic events in LA tissue of MetS animals, 2.3% increase in LV tissue.

This data is consistent with previous reports that metabolic syndrome promotes apoptosis in chronically ischemic myocardium (Li et al., 2012; Elmadhun et al., 2014). Specifically, the study on Ossabaw pigs comparing lean, obese and MetS animals revealed that apoptotic activity assessed by TUNEL and caspase-3 staining had not reached statistical significance in obese pigs, but was pronounced and significant in MetS animals (Li et al., 2012). In addition, cardiomyocyte apoptosis was also identified in LV tissue in dogs with heart failure by Sharov et al. (Sharov et al., 1996). Indeed, increased apoptosis rates have been consistently reported over the years in cases of patients with chronic heart failure (CHF) and dilated cardiomyopathy (DCM), as reviewed by van Empel et al. (van Empel et al., 2005). One of the studies mentioned there implies that the myocyte death in the failing human heart is gender dependent, occurring at lower rates in females (Guerra et al., 1999). Compared to the current results, Narula et al. reported similar apoptosis rates in end-stage heart failure patients and they further confirmed that the apoptosis was predominantly confined to the myocytes by performing double-labelling experiment with actin (Narula et al., 1996). However, it's notable to mention that not all models of HF are associated with apoptosis, as reviewed by Kang and Izumo (Kang and Izumo, 2000). In this article, they also highlighted important issues surrounding TUNEL method for assessing apoptosis, mainly because the apoptotic process is transient and so may be the window opportunity for detecting apoptotic cells. For example, in lymphocytes, the TUNEL-positive period is generally less than 12 hours and if the same holds true for cardiac myocytes, TUNEL staining may markedly underestimate the true prevalence of the apoptosis in HF which usually occurs over many months or years (Kang and Izumo, 2000).

Nevertheless, the current results and the afore-mentioned studies uniformly indicate that there is an increase in the number of dying cells in the failing myocardium. Therefore, it is essential to confirm the identity of dying cell and the reason they are going down the apoptosis

pathway in order to prevent this event from happening and possibly to halt the disease progression.

5.1.5. Increase in vimentin expression in LV tissue, but not in LA

Interestingly, the current study observed a significant increase in vimentin expression in LV tissue in MetS animals, compared to the control group, whereas this was not confirmed in LA tissue where there was no significant difference between the two groups.

To author's knowledge, this is the first report on differential LA/LV chamber-specific vimentin expression in HF. However, a study assessing molecular remodelling of left (LV) and right ventricular (RV) myocardium in chronic anthracycline cardiotoxicity found that vimentin expression on both mRNA and protein level was pronounced in LV tissue, whereas only a weaker changes were observed in RV (Lencova-Popelova et al., 2014). Further, they stated that the remodelling of ECM was almost exclusively found in the LV with particular induction of collagen 1 and 4 and they concluded that there is a marked asymmetry in molecular remodelling of myocytes, non-myocyte cells and ECM in response between LV and RV tissue subjected to chronic anthracycline treatment (Lencova-Popelova et al., 2014). Further, a study assessing myofibroblast activation and connective tissue formation in a porcine model of atrial fibrillation (AF) and reduced ventricular function reported that in the cardiac tissue obtained 14 days after the initiation of continuous atrial burst pacing, the levels of vimentin-expressing cells in right atrial (RA) tissue were similar in experimental animals compared to the control group in sinus rhythm (Lugenbiel et al., 2017). Further, Willmer et al. have observed a significant increase of vimentin positive cardiac fibroblasts in mice with diastolic HF, however, they do not specify the chamber (M. Willmer, 2013). In this study, they also confirm increased expression of collagen 1, 3, 4, 6 and 14, as well as an increase in several other proteins, which may be used to assess fibrosis. They conclude that angiotensin treatment induced cardiac fibrosis as apparent by an intensified fibrillogenesis (M. Willmer, 2013). Finally, a recent preliminary study found a higher amount of vimentin-positive cells in LA tissue of dogs with end-stage dilated cardiomyopathy (DCM) (Janus et al., 2016). However, considering that end-stage DCM animals were used in this study, which most certainly had advanced symptoms of disease, this study is not in contrast to the results of the current study. Indeed, it is generally accepted that fibrotic changes occur first in LV (Karayannis et al., 2008) and then in LA, and that the left atrial volume index (LAVi) is associated with the severity of diastolic dysfunction but seems to be a poor marker of mild and moderate diastolic dysfunction (Pritchett et al., 2005). This

may suggest that the experimental animals used in the current study were still in the asymptomatic stage of the development of disease thus showing fibrosis in LV which would with time affect LA as well.

Overall, considering that fibrosis is linked with poor organ function, it seems plausible that fibrotic changes might be happening in the hearts of MetS animals. However, a special caution needs to be taken when considering vimentin as a marker of fibrosis. Indeed, vimentin is a protein found on both fibroblasts and myofibroblasts. That said, while myofibroblasts are cells deemed to drive fibrotic changes through the enhanced production of collagen and cytokines, fibroblasts are normally found within healthy human and pig tissue. In addition, in order to completely identify the vimentin+ cells as fibroblasts or myofibroblasts, an exclusion of CD31 and CD45 antibody reactivity needs to be ensured (these are the markers of endothelial and myeloid cells, respectively). Therefore, no definite conclusion may be made on the presence of fibrosis and the identity of the vimentin+ cells observed in the current study, and further experiments are needed. However, it is tempting to speculate that these results do indeed suggest myofibroblast expansion in LV tissue. And although this might be an initial compensatory mechanism, prolonged stress leads to uncontrolled fibrotic changes. Further, considering that fibroblasts are inexcitable cells, their expansion in heart represents a serious issue. Therefore, preventing myofibroblast expansion and activation in cardiac tissue might represent a promising approach in halting the disease progression.

5.1.6. Increase in TGR5 expression

The current study observed an increased expression of TGR5 in LA and LV tissue in MetS animals, compared to the control group. In addition, it should also be noted that the current study confirmed the physiological expression of bile acid-activated receptor, TGR5, in cardiac muscle in control animals.

Although TGR5 is deemed an important metabolic regulator, with the ability to modulate energy expenditure, insulin sensitivity and particularly inflammation, the number of studies assessing its expression and role in HF are scarce. One previous study identified TGR5 as a novel therapy target for HF by observing the activation of TGR5 and its key RNA targets in mice fed cholic acid (CA) along with attenuated contractile failure and pathologic hypertrophy (Moreshwar S Desai, 2014). Interestingly, they also found that TGR5 deletion in heart accelerates transverse aortic constriction induced cardiomyopathy (Moreshwar S Desai, 2014). Although this result suggests that TGR5 activation in HF has a protective role, it should

be noted that the study was conducted on mice, which are imperfect model for human cardiovascular disease due to the known structural and physiological differences and that mice bile acid pool also differs from the human, which is further exaggerated by different dietary preferences. Further, in another study, researchers treated diabetic db/db mice with selective TGR5 agonist INT-777 and found decreased proteinuria, podocyte injury, mesangial expression, fibrosis, and CD68 macrophage infiltration in the kidney (Wang et al., 2016). They concluded that TGR5 activation induces mitochondrial biogenesis and prevents renal oxidative stress and lipid accumulation (Wang et al., 2016). This is, however, another TGR5 study conducted on mice and the results would need to be confirmed in human or large animal model studies. Interestingly, a recent study found that dendritic cells (DCs) derived from human peripheral blood monocytes cultured with specific TGR5 agonist produced less IL-12 and TNFα in response to commensal bacterial antigen (Ichikawa et al., 2012), whereas the same was not noted for stimulation through another BAR, nuclear receptor farnesoid X (FXR). They concluded that BAs induced differentiation of IL-12 hypo-producing DCs from monocytes via TGR5-cAMP pathway. Since IL-12 is a crucial factor for differentiation of Th1 lymphocytes, this might have further implications for the ongoing inflammation processes in heart since the current study found increased activation of TGR5 in hearts of MetS animals. However, it must be noted that the identity of cells bearing this activated TGR5 receptor is not yet elucidated. It would be interesting to perform a double staining with CD163 to elucidate whether TGR5 is expressed on activated cardiac macrophages or on cells of another type. It should also be noted that it was established that TGR5 activation protects against atherosclerosis by reducing macrophage infiltration and lipid loading in blood vessels (Pols et al., 2011).

5.1.7. Holistic approach to the results of the current study

a) CD163 and fibrosis

Along with the finding increased number of CD163+ macrophages, the current study speculates increased fibrosis rates as suggested by total vimentin expression in LV tissue, whereas there was no significant difference in vimentin expression in LA chamber. As previously mentioned, M2-macrophages are critical players in tissue repair, and depletion of these cells results in impaired wound healing (Leibovich and Ross, 1975; Mirza et al., 2009). A certain subclass of M2-macrophages expresses high levels of arginase-1 in response to IL-4, which allows these cells to generate precursors for collagen and fibroblast stimulating factor, thus supporting their role

in extracellular matrix deposition and wound closure (Ogle et al., 2016). However, again, undesirable fibrotic changes in an injured tissue may ensue if these functions are disbalanced, and this may be happening in the failing heart, which may be the reason why the current study observed an increased number of vimentin+ cells which are deemed to be fibroblasts. Further, in addition to producing pro-fibrotic mediators, macrophages have also been shown to directly enhance the activation of myofibroblasts, the key ECM-producing effector cells (Wynn and Vannella, 2016). Indeed, the previously mentioned studies also found an increased fibrosis was accompanied by an increased CD163-expression (De Vito et al., 2012; Li et al., 2012).

b) CD163 and apoptosis

Along with an increased number of CD163+ macrophages in LA and LV chamber in MetS animals, the current study found increased apoptosis rates in both mentioned chambers. If CD163+ macrophages are looked through their ability to phagocyte other cells, one may wonder which cells are their target. An appealing theory may be that an increased number of M2 macrophages in the failing myocardium may result from increased number of apoptotic cells and tissue repair tendencies. Indeed, in addition to observing an increase in CD163 expression, study by Li et al. of metabolic syndrome in Ossabaw pigs also found enhanced rates of apoptosis in cardiac tissue in MetS animals (Li et al., 2012), like the current study. Although CD163 has been correlated with apoptosis in different tissues, these studies did not provide an information on identity of the dying cells. It should, however, be acknowledged that the macrophages themselves may go through the apoptosis and that simply increased number of activated macrophages in the tissue accounts for the increased apoptosis rates observed. However, as mentioned in the introduction, cardiomyocyte apoptosis has been well established in multiple studies dealing with CVD. In one recent study, apoptosis of both myocytes and non-myocytes was clearly apparent in post-MI stage and this event was accompanied by significant decrease in CD80+ proinflammatory M1 macrophages and increase in CD163+ anti-inflammatory M2-macrophages in infarcted hearts (Rafatian et al., 2014). Although it is possible that multiple factors such as stress levels and hormones play a role in the longevity and resistance of the (cardiac) cells in our body, seems like a prolonged

wall stress and hypertension may account for the increased vulnerability of cardiomyocytes thus causing them to go down the apoptosis pathway. Considering that the current study also found increased apoptosis rates in LA and LV, macrophage infiltration seems plausible. However, more research is needed in this area to elucidate the link between cardiac macrophages and apoptosis in HF.

c) CD163 and TGR5

High TGR5 expression was observed in monocyte/macrophage lineage in one of the early studies which identified TGR5 and its tissue distribution (Kawamata et al., 2003). Specifically, in fractioned human leukocytes TGR5 mRNA was detected mainly in the resting CD14+ monocytes. This study also confirmed that TLCA and LCA, potent TGR5 agonists supressed phagocytic activity in activated macrophages and reduced the induction of pro-inflammatory cytokines upon LPS stimulation (Kawamata et al., 2003). An interesting study by Keitel et al. identified TGR5 and CD163 co-labelling in human term placentas, stating, thereby, that TGR5 was mainly localized in foetal macrophages and to a lower extent in trophoblasts (Keitel et al., 2013). They also report marked down-regulation of TGR5 mRNA expression in patients with intrahepatic obstructive cholestasis of pregnancy and unaffected cell-specific distribution of the TGR5 protein. Finally, CD163-TGR5 co-localization was also observed in Kupffer cell's (liver resident macrophages) (Keitel et al., 2008). These reports strongly indicate that CD163-expressing macrophages may express TGR5 and therefore be activated by bile acids. Considering that previous studies found that TGR5 stimulation inhibits pro-inflammatory cytokine production by macrophages, and the current study observed increased macrophage count in the failing heart, it would be interesting to closely determine the nature and significance of this mechanism in HF and to explore additional treatment options.

d) CD16 and apoptosis

Along with increased number of CD16-expressing cells in LA and LV tissue in MetS animals, the current study found an increased apoptosis rates in these chambers. Indeed, it seems plausible that increased activation of the receptor, which mediates antibody-dependent cellular citotoxicity (ADCC) may result in greater number of apoptotic cells, regardless of the cell type bearing this receptor (NK cells or

monocytes which infiltrated tissue). For that reason, elucidating the identity of the target cell and the reason it displays the *death signal*, whether in terms of being opsonized by immunoglobuline (IgG), not being able to express ubiquitously expressed MHC1 molecule or any other reason currently unknown, may represent an important clue in understanding the processes responsible for deteriorated cardiac function occurring in HF patients. Indeed, if the targeted cells are cardiomyocytes, then loss of these cells due to CD16 activation poses a serious threat and blocking this pathway may represent a promising therapeutic approach for patients with HF.

e) TGR5 and apoptosis

The current study observed increased apoptosis rates along with an increased TGR5 activation in LA and LV tissue. The previous reports on TGR5 contribution to apoptosis are not consistent (Yang et al., 2007; Reich et al., 2016). A recent study concluded that TGR5 activation by taurolithocholic acid or TGR5-selective agonist protects from death receptor-mediated apoptosis in cholangiocytes in mice. They suggested that this mechanism may protect cholangiocytes from BA toxicity under cholestatic conditions but may as well trigger proliferation and resistance to apoptosis in malignantly transformed cholangiocytes. (Reich et al., 2016). On contrary, a study performed on human hepatocytes found that TGR5 activation leads to c-Jun-N terminal kinsase (JNK) activation and reduced complex formation with caspase 8 thus facilitating caspase 8 recruitment to death-inducing signalling complex which promotes apoptosis (Yang et al., 2007). Thus, they suggest that TGR5 signalling blockage might have therapeutic application in cholestasis (Yang et al., 2007). The latter mechanism may give a link to increased apoptosis rates and TGR5 activation in cardiac tissue in MetS animals found in the current study.

6. Conclusion

The current study presents several important findings regarding the molecular and cellular changes happening in LA and LV tissue during HF in a porcine model of metabolic syndrome, which recapitulates human form of this disease. First, the heart tissue of MetS animals is characterized by a significant expansion and/or infiltration of M2-macrophage subset, as observed by an increased cellular expression of CD163. Secondly, cellular expression of CD16, monocyte and NK cells' receptor involved in ADCC, is significantly enhanced in the failing heart tissue of MetS animals. Further, no evidence of biologically significant expansion of T-lymphocytes was found in hearts of MetS animals, compared to the control group, as assessed by CD3 antibody reactivity. Moreover, an increase in apoptotic events is evident in MetS animals with poor cardiac function, whereas almost no cells' death could be observed in hearts of healthy animals. In addition, LV, but not LA tissue of MetS animals shows increase in vimentin+ cells. Finally, increased expression of TGR5, a bile acid-activated receptor, is evident in the failing heart tissue of MetS animals, compared to the control group.

These findings suggest that the cardiac remodelling in heart failure may be driven by immune cells such as M2-macrophages, monocytes and/or NK cells, and the accompanying (anti)inflammatory processes. Further, it was suggested that the increased apoptosis or cell death also contributed to the degenerative changes seen in HF. In addition, vimentin, as one of the markers of fibroblasts (although this identification is indefinite), might suggest that LV tissue is a subject to fibrotic changes to a greater extent and/or those changes occur prior to changes in LA. Finally, bile acids are introduced as a novel potential contributor to the pathogenesis of HF as the failing hearts showed an increased expression of TGR5, a bile acid-activated receptor.

In conclusion, the current study provides a comprehensive insight into the molecular and cellular changes in heart failure and offers an outline for the future studies exploring the pathways and mechanisms leading to cardiac dysfunction, which will then allow pharmaceutical companies to develop novel medicines targeting specific components of the pathways involved with aim to prevent the disease progression and decrease high mortality rates associated with HF.

7. Future perspectives

The current study represents an initial screening of the ongoing processes in heart tissue during HF with specific focus on inflammation, apoptosis and fibrosis, as well as bile acids.

An increase in M2-macrophages in the failing heart represents an event, which might contribute to the cardiac dysfunction. It would be interesting to confirm whether this increase is a result of monocyte infiltration, which might be revealed by an additional testing for CD11b, a marker for circulating monocytes, which differentiated into macrophages, as this might give a deeper insight into the processes by which immune cells mediate HF and how to modulate them. Further, additional monocyte/macrophage marker, such as CD14, would allow researchers to understand the nature (pro- or anti-inflammatory) of these cells. In addition, it is crucial to identify the apoptotic cells in the failing heart and the first step to this might be double labelling with α-sarcomeric actin (α-SA) to test whether those cells are cardiomyocytes. Preventing cardiomyocyte death might represent one of the key options to halt the disease progression. Further, it is necessary to identify the vimentin+ cells in order to make a definite conclusion on the extent of fibrosis and how to further modify it. Since there is still no ideal marker for either fibroblasts or myofibroblasts, a double labelling with an additional marker for each of these cell types might be an option. For that matter, double labelling of vimentin and fibroblast specific protein (FSP) would help to identify fibroblasts more closely, whereas a double labelling of vimentin and α-smooth muscle actin (αSMA) would more accurately identify myofibroblasts. Finally, it would be interesting to find out the identity of the cells expressing TGR5. For example, co-labelling with M2-macrophage marker could represent one of the initial screening tests, followed by co-labelling with vimentin, αSMA or αSA. In addition, quantifying and identifying heart tissue bile acids would represent an important insight into TGR5 activation processes. Last, but not least, if over the course of time bile acids show up as an important contributor to the pathogenesis of HF, more comprehensive approaches should be considered. For example, since bile acids are modified by the intestinal microbiota, which generates entities with different potential to trigger the activation of bile acid-activated receptors, one of the approaches to modify their action might be intervening with gut microbiome populations, through either probiotic supplementation or specific antibiotic and/or bacteriophage use.

8. List of figures

9. List of materials

Antibodies

Primary Antibody	Company
CD163 – Mouse anti pig CD163	Biorad
CD16 – Mouse anti pig CD16	Biorad
CD3 – Anti-CD3 antibody [SP7]	Abcam
Vimentin – Anti-vimentin antibody [V9] – Cytoskeleton marker	Abcam
TGR5 – Anti-GPCR TGR5 antibody	Abcam
TUNEL – In situ cell death detection fluorescein	Roche
Secondary Antibody	**Company**
Goat Anti-rabbit IgG H&L (Alexa Fluor 488)	Thermo Fischer Scientific
Goat Anti-Mouse IgG H&L (Alexa Fluor 488)	Thermo Fischer Scientific

Reagents

Reagent	Company
PBS	Sigma Aldrich
Tween20	Fisher Scientific
Triton	Sigma Aldrich
Goat serum	Sigma Aldrich
BSA	Sigma Aldrich
DAPI	Sigma Aldrich
Sudan Black	Sigma Aldrich
Methanol	Sigma Aldrich
Paraformaldehyde	Sigma Aldrich

Material

Material	Company
Superfrost Microscope Slides	Menzel
OCT	Thermo Scientific

Devices

Device	Company
Confocal microscope: Nikon D-Eclipse	Nikon
Cryostat	Leica

Software

Software	Company
NIS-Elements BR 3.0	Nikon

10. References

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Diplomski rad

MARKERI IMUNOLOŠKIH STANICA, APOPTOZE I FIBROZE U SVINJI KAO MODELU METABOLIČKOG SINDROMA I DOPRINOS RECEPTORA OSJETLJIVOG NA PRISUSTVO ŽUČNIH KISELINA PATOGENEZI SRČANOG ZATAJENJA

Lana Kitić

SAŽETAK

Srčano zatajenje (SZ) definirano je kao negativna promjena u funkciji srca kao crpke, a s vremenom vodi k adaptivnim molekularnim i intersticijalnim promjenama čiji je razmjer još slabo istražen.
Trenutna studija koristi tehniku imunofluorescencije kako bi ispitala tkivo lijevog atrija (LA) i lijevog ventrikula (LV) svinja koje su bile podređene hiperkaloričnoj i masnoj prehrani te razvile metabolički sindrom (MS) (n=5), kao i tkivo životinja kontrolne grupe (n=5). Cilj studije bio je korištenjem određenih staničnih markera utvrditi sudjelovanje upale (CD163, CD3, CD16), stanične smrti (TUNEL) i fibroze (vimentin) te receptora koji je osjetljiv na prisustvo žučnih kiselina (TGR5) u progresivnom smanjenju funkcije srčanog mišića.
Rezultati su pokazali značajno povećanje ekspresije markera CD163 i CD16, ali ne i CD3 u oba tkiva životinja s MS. Zabilježeno je i povećanje stope apoptoze u životinja s MS. Porast ekspresije vimentina zabilježen je u lijevom ventrikulu bolesnih životinja, ali ne i u lijevom atriju. Konačno, značajan porast ekspresije TGR5 receptora zabilježen je u oba analizirana tkiva životinja s MS .
Ova studija predstavlja važan test doprinosa žučnih kiselina i mehanizama kao što su upala, apoptoza i fibroza u patogenezi SZ koje prati MS. Dodatne studije potrebne su kako bi se točnije opisale promjene na staničnoj razini koje možebitno doprinose patogenezi SZ i time razvile nove terapijske opcije za ovu skupinu pacijenata.

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Rad sadrži: 75 stranica, 8 grafičkih prikaza, 3 tablice i 217 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: Srčano zatajenje, metabolički sindrom, upala, apoptoza, fibroza, žučne kiseline

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Diploma thesis

MARKERS OF IMMUNE CELLS, APOPTOSIS AND FIBROSIS MARKERS IN THE PORCINE MODEL OF METABOLIC SYNDROME AND A NOVEL INVOLVEMENT OF BILE ACID-ACTIVATED RECEPTOR

Lana Kitić

SUMMARY

Heart failure (HF) is defined as a syndrome caused by an inadequate performance of the heart as a pumping system. Over the course of time this leads to a series of adaptive molecular and interstitial changes, scope of which still remains to be elucidated.
The current study utilized immunofluorescence staining technique to examine left atrial (LA) and left ventricular (LV) tissue in high-fat fed pigs which developed metabolic syndrome (MetS) (n=5), as well as in their healthy counterparts (n=5). The goal was to assess the involvement of inflammation (CD163, CD3, CD16), apoptosis (TUNEL), fibrosis (vimentin) and a bile acid-activated receptor (TGR5) in a progressive decrease in cardiac function observed in MetS patients.
The results showed a significant increase in the expression of CD163 and CD16 but not CD3 in MetS animals. Further, there was an increase in apoptotic events in MetS animals compared to the control group. Vimentin expression was increased in LV tissue , but not in LA. Finally, a significant increase in TGR5 expression was evident in MetS animals compared to the control group.
This study provides an important test of the involvement of bile acids, inflammation, apoptosis and fibrosis in the pathogenesis of HF. Additional studies are needed to elucidate the exact cellular shifts which occur during the HF so new therapeutic options could be developed for this group of patients.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 75 pages, 8 figures, 3 tables and 217 references. Original is in English language.

Keywords: Heart failure, metabolic syndrome, inflammation, apoptosis, fibrosis, bile acids

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