Cardiovascular Renal Axis Disorder and Inflammatory Response in Feline Congestive Heart Failure Due to Primary Cardiomyopathy

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Declaration

I hereby declare that the work included in this thesis has been composed by myself, except where the acknowledgements have been made. This work has not been, and will not be submitted for any other degree or academic qualifications.

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List of Abbreviations

2D	two dimensional
ACE	angiotensin-converting enzyme
ACVIM	American College of Veterinary Internal Medicine
ADMA	asymmetric dimethylarginine
AGP	alpha-1-acid glycoprotein
AKI	acute kidney injury
ANOVA	one-way analysis of variance
APC	atrial premature complex
APP	acute phase protein
ARVC	arrhythmogenic right ventricular cardiomyopathy
ATE	arterial thromboembolism
AV	atrioventricular
BNP	brain natriuretic peptide
BP	blood pressure
bpm	beat per minute
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CHF	congestive heart failure
CI	confidence intervals
CKD	chronic kidney disease
CM	cardiomyopathy
Cr	creatinine
CRP	C-reactive protein
CvRD	cardiovascular-renal axis disorder
DCM	dilated cardiomyopathy
dl	decilitre
DMVD	degenerative mitral valve disease
DSH	domestic short hair
ECG	electrocardiography
ECM	extracellular matrix
ECVIM	European College of Veterinary Internal Medicine
FIP	feline infectious peritonitis

FS	left ventricle fraction shortening
GFR	glomerular filtration rate
h	hour
HCM	hypertrophic cardiomyopathy
HOCM	hypertrophic obstructive cardiomyopathy
Hp	haptoglobin
HRP	horseradish peroxidase
ICU	intensive care unit
ID	identification
IL-6	interleukin-6
IQR	interquartile range
IRIS	International Renal Interest Society
ISACHC	International Small Animal Cardiac Health Council
IVS	interventricular septum
IVSd	interventricular septum thickness end diastole
kg	kilogram
LA	left atrium/atrial
LA/Ao	left atrial to aortic root ratio
l	litre
LRG1	leucine-rich alpha-2-glycoprotein1
LV	left ventricle/ventricular
LVFWd	left ventricular free wall thickness end diastole
LVIDd	left ventricular internal diameter end diastole
LVIDs	left ventricular internal diameter end systole
LVOTO	left ventricular outflow tract obstruction
mg	microgram
min	minute
ml	millilitre
mm	millimetre
mmHg	millimetre of mercury
MST	median survival time
MYBPC	myosin binding protein C
NAG	N-acetyl-beta-glucosaminidase

ng	nanogram
NGAL	neutrophil gelatinase-associated lipocalin
NO	nitric oxide
NOS	nitric oxide synthase
NT-proBNP	N-terminal pro-brain natriuretic peptide
PCT	procalcitonin
PE	point estimate
pmol	picomole
proANP	prohormone atrial natriuretic peptide
RAAS	renin-angiotensin-aldosterone-system
RCM	restrictive cardiomyopathy
ROC	receiver-operating characteristics
rpm	revolutions per minute
SAA	serum amyloid A
SAM	systolic anterior motion
SDMA	symmetric dimethylarginine
SNS	sympathetic nervous system
SPARCL	spatial proximity analyte reagent capture luminescence
T4	thyroxine
TGF-β	transform growth factor-beta
TMT	transient myocardial thickening
TNF-α	tumour necrosis factor-alpha
UCM	unclassified cardiomyopathy
ug	microgram
ul	microlitre
umol	micromole
VPC	ventricular premature complex

Abstract

Congestive heart failure (CHF) is a common cardiac condition in cats, which usually results from primary cardiomyopathy (CM). It is a complex syndrome with many pathophysiological processes involved. The aim of this study was to investigate two aspects i.e. cardiovascular renal axis disorder (CvRD) and systemic inflammatory response in feline CHF due to primary CM.

The study population included 25 CHF cats, 12 asymptomatic CM cats and 20 healthy control cats. Two panels of biomarkers were tested as reflections of CvRD (cardiac biomarker NT-proBNP, renal biomarker SDMA and creatinine) and inflammatory response (7 acute phase proteins) respectively. The biomarker expressions in the three cat groups, correlations between the biomarkers and selected clinical variables, and risk factors in the CHF cats were analysed. Following the baseline studies, pilot longitudinal studies were carried out to investigate biomarker changes in 12 CHF cats over 12 months.

The results showed both CvRD and systemic inflammatory response occurred in feline CHF. Considering the high prevalence of CvRD, it is recommended that the novel renal biomarker SDMA should be used as a renal function monitor in CHF management. Moreover, similar to NT-proBNP, it potentially carries prognostic value for feline CHF. Three acute phase proteins (APPs) showed significant changes in CHF (i.e. LRG1, ceruloplasmin and SAA) and may help to better understand the pathogenesis of feline CHF as well as predict the disease outcome. Positive correlations between APPs and cardiac variables suggested their intrinsic involvement in cardiac disease. In addition, serum AGP appeared to carry independent prognostic value for feline CHF. In combination with other clinical information, the APPs may be promising novel clinical biomarkers for feline CHF. Additionally, some clinical parameters were found to have the ability to stage CM and provide prognostic information. Preliminary results from longitudinal measurements showed that NT-proBNP measurements persistently higher than 1500 pmol/l, or a single SDMA measurement higher than 20 ug/dl appeared to be associated with a poorer outcome. Most APP expressions appeared to decrease over time. Future work will be needed to consolidate these findings.

This study has identified novel CvRD and inflammatory biomarkers involved in a feline CHF study population. Current findings can be potentially implemented in future research and in clinical management of feline CHF and CM.

Highlights of the Study

- This is the first study reporting CvRD in cats with CHF due to primary CM and that SDMA and NT-proBNP both potentially have prognostic value in feline CHF.
- This is the first study demonstrating APPs involved in feline CHF; moreover, serum AGP appeared to be an independent predictor for poor prognosis in CHF.
- This is the first study reporting LRG1 expression in cats.

Chapter 1: Introduction

1.1 Feline Cardiomyopathies

Cardiomyopathies (CMs) are common cardiac diseases in cats; they are defined as myocardial diseases associated with cardiac dysfunction (Richardson et al., 1996). Traditionally, based on anatomical and functional abnormalities, primary CMs are categorized into hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular cardiomyopathy (ARVC) and unclassified cardiomyopathy (UCM) (Ferasin et al., 2003; Fox et al., 2000).

Initial identification of feline CM can be traced back to the 1970s (Liu, 1970). In the early 1980s feline CM was first proposed to be a comparable animal model for human HCM with preserved ejection fraction (Liu and Tilley, 1980). Subsequent studies on feline CM have been mainly focused on HCM, with other forms of CMs relatively under-investigated. Today, the majority of our understanding of feline CM is largely based on the HCM phenotype.

1.1.1 Aetiology and Pathogenesis

The aetiology and pathogenesis of feline CM, particularly idiopathic CM, remains unclear, however familial heritability has been observed in both feline and human CM patients. In human medicine, HCM is considered as a genetic heart disease caused by mutation of sarcomeric protein encoding genes. There are 1500 known mutations of 11 genes that have been identified to associate with human HCM (Maron and Fox, 2015). Similar to human, feline HCM may also have a genetic cause associated with heart muscle sarcomere mutations. Causative mutations of myosin binding protein C3 (MYBPC3) have been identified in the Maine Coon (Meurs et al., 2005; Fries et al., 2008) and Ragdoll cats (Meurs et al., 2007; Borgeat et al., 2014a). There are other cat breeds found to have familial HCM, including British Short Hair (Granstrom et al., 2011), Sphynx (Silverman et al., 2012) and Norwegian Forest cats (Marz et al., 2015). Familial inherited RCM has also been reported in humans, and accounted for one third of total RCM cases in one study (Kaski et al., 2008). In cats, so far there is no causative mutation identified for other CM phenotypes but familial inheritance or a genetic cause has been proposed in previous DCM and ARVC studies (Lawler et al., 1993; Fox et al., 2000). The significance of genetic aetiology in feline CM is yet to be fully understood, however it has been hypothesized that different clinical CM phenotypes may be caused by variations of the mutations within specific functional domains of the sarcomeric disease gene (Côté et al., 2011b).

Apart from genetic factors, other causative factors have been reported in feline CM such as taurine deficiency associated DCM (Lawler et al., 1993) and myocardial change secondary to systemic diseases such as hyperthyroidism and hypertension (Ware, 2011b). Recently, there was an interesting report of transient myocardial thickening (TMT) and CHF in cats; this has been described as a reversible condition, most likely due to myocardial acute inflammation or oedema in response to some antecedent events such as general anaesthesia or traumatic injuries, although no definitive evidence has been proven for this hypothesis (Novo Matos et al., 2018). Metabolic disorders such as obesity and diabetes mellitus have been found to cause CMs in human (Alpert, 2001; Boudina and Abel, 2010). As the two species share many similarities in metabolic disorders, the cat has been proposed to be a useful model for research into human obesity and diabetes (Hoenig, 2012). In cats, so far obesity and diabetes mellitus have not been proven as CM causes, but certain links were established in previous studies. For example, obesity has been found as a risk factor in inherited HCM in the Maine Coon breed; a theory of environmental factors such as nutritional modification of genes has been hypothesized to explain the diverse presentations of familiar inherited HCM in the Maine Coon (Freeman et al., 2013). A later randomised trial in HCM cats suggested diet may play a role in the disease (Freeman et al., 2014). Differential serum fatty acid profiles were observed in HCM cats compared with the normal controls, which suggested HCM cats may have altered fatty acid metabolism potentially linked with cardiovascular conditions (Hall et al., 2014a). Also in previous studies, body weight and body condition score have been found to be associated with the incidence and prognosis of HCM (Payne et al., 2015a; Finn et al., 2010). A few studies have investigated the links between diabetes mellitus and cardiac dysfunction in cats. A recent publication demonstrated reduced diastolic function occurred in diabetic cats, irrespective of diabetic control status; the diastolic dysfunction appeared to progress in those cats in a 6 month follow up (Pereira et al., 2017). Another study has shown heart failure was common in cats with diabetes mellitus and was responsible for approximately 40% of deaths in diabetic cats in their study population (Little et al., 2008). Another endocrinological condition, acromegaly (hypersomatotropism) is known to be associated with myocardial hypertrophy in cats and the echocardiographic changes were found to be reversible with effective treatment of the acromegaly (Borgeat et al., 2018).

1.1.2 Pathology and Histopathology

The macroscopic features of each CM phenotype are relatively distinguishable, however, despite different phenotypic diagnosis, the histopathological changes of feline CM are largely similar. In most of the feline CMs, myocardial and extracellular matrix (ECM) remodelling, coronary arteriosclerosis and inflammatory cell infiltration are commonly reported histopathological features (Fox, 2003; Fox, 2004; Kittleson et al., 1999; Aupperle et al., 2011; Khor et al., 2015).

The gross pathological findings in HCM are characterized by variable degrees of left ventricular (LV) hypertrophy, papillary muscle hypertrophy and reduced LV cavity diameter (Fox, 2003). A moderately to severely dilated left atrium (LA) is commonly seen (Fox, 2003). Histopathological hallmarks of HCM have been described as myofibre disarray, small intramural coronary arteriosclerosis, interstitial fibrosis and replacement fibrosis (Kittleson et al., 1999; Liu et al., 1993). In Liu's study in 1993, 30% of HCM cats had evidence of myofibre disarray and, 74% had abnormal intramural coronary arteries, while in Fox's later study, more than 60% of cats had the above two abnormalities and concurrent ECM remodelling (Fox et al., 1995). Myocardial collagen deposition and inflammatory cell infiltration has been found in pre-clinical HCM cats, which may suggest ECM remodelling and inflammation occur at an early stage of the disease (Khor et al., 2015). Male cats, which are reported to be more predisposed to HCM, have myocardial changes in a pro-inflammatory stage even when they are free from overt cardiac disease (Fonfara et al., 2015).

RCM shares many similarities with HCM (Spalla et al., 2016) and can be further classified in to a myocardial form (Fox et al., 2014) and an endocardial form (Fox, 2004). Macroscopically, bi-atrial dilation can be seen in both forms, and there is no apparent myocardial hypertrophy (Fox et al., 2014). Endocardial RCM is characterised by endocardial and myocardial interstitial fibrosis in the left ventricle, presence of inflammatory cells consistent with endomyocarditis, and occasional arteriosclerosis in the intramural coronary arterioles (Fox, 2004; Kimura et al., 2016). In the myocardial form of RCM, myofibre disarray and replacement fibrosis of the myocardium were reported features (Fox et al., 2014).

DCM is characterized typically by a dilated LA and ventricle and in the more advanced cases all four cardiac chambers are dilated (Côté et al., 2011c). Histopathological findings of DCM have been described as variable; myocytolysis, myofibre degeneration and fibrosis may be observed (Côté et al., 2011c).

ARVC is uncommon in cats. The most consistent post-mortem cardiac change in ARVC is right ventricular dilation (Harvey et al., 2005; Fox et al., 2000). Microscopically, it is characterized by cardiomyocyte degeneration, adipose and fibrous tissue replacement of the myocardium and inflammatory cell infiltration in the right ventricle. Myocarditis can be a common feature. Similar histopathological changes were also observed in the left ventricle in most ARVC cats in Fox and colleagues' study (Fox et al., 2000).

UCM has been defined as a type of CM that cannot readily fit any of the above classifications, and may possess a combination of non-specific changes (Ferasin, 2009). So far no specific histopathological changes are reported for UCM.

Phenotype transition of feline CM may occur; notably a DCM may be an end-stage consequence of other CM types such as a primary HCM (Côté et al., 2011b). This confounds accurate macroscopic classification and thus histopathological characterization for individual CM phenotypes. Considering this issue, possibly a broad term, such as feline CM, would be more appropriate to use when describing the disease.

1.1.3 Clinical Presentation, Diagnosis and Prognosis

The clinical presentation and progression of CM is variable among individual cats. While a large proportion of affected cats remain asymptomatic, other cats develop significant clinical consequences including CHF, arterial thromboembolism (ATE) and arrhythmia associated sudden death. The symptomatic patients generally have a much poorer prognosis compared with asymptomatic ones (Atkins et al., 1992). Common clinical findings include a heart murmur, audible gallop sounds (the 3rd and 4th heart sounds, which are not normally audible in small animals), arrhythmias, pulse deficit, tachypnoea, dyspnoea, tachycardia, syncope, collapse, hypothermia, anorexia, lethargy and signs of pain. Most of the above signs are non-specific, although certain parameters have been proven to associate with disease outcome (Côté et al., 2011b). Primarily focusing on the HCM phenotype, the disease epidemiology, diagnostic and prognostic features are discussed below:

HCM

HCM is the most common form of feline CM, which represented 57.5% of cases in one study of 106 CM cats (Ferasin et al., 2003). The prevalence of HCM in clinically healthy cats is reported to be approximately 15% (Payne et al., 2015b; Paige et al., 2009; Wagner et al., 2010).

The mean age at presentation is reported as around 5-6 years, with male cats over-represented (Ferasin et al., 2003; Payne et al., 2015b; Rush et al., 2002; Atkins et al., 1992). The Maine Coon and Ragdoll cats with homozygous MYBPC3 mutations have been shown to have earlier onset and a more severe form of HCM (Kittleson et al., 1999; Meurs et al., 2007). Common diagnostic criteria of HCM are increased LV wall thickness > 6 mm at the end of diastole and/or evidence of focal hypertrophy in the ventricles, and papillary muscle hypertrophy on 2 dimensional (2D) echocardiography (Ferasin, 2009; Payne et al., 2015b). LA enlargement is a common echo feature in HCM cats (48.8%), especially in the advanced stage of disease (Payne et al., 2010). The most common physical examination findings in asymptomatic HCM cats are a systolic heart murmur and/or gallop sounds (Côté et al., 2011b). When CHF develops, dyspnoea is usually the first obvious clinical sign (Côté, 2017). The most common electrocardiographic (ECG) finding with HCM is left anterior fascicular block and intermittent ventricular premature complexes (VPCs) (Ferasin et al., 2003).

Hypertrophic obstructive cardiomyopathy (HOCM) is a form of HCM when there is narrowing of the LV outflow tract and also frequently has systolic anterior motion (SAM) of the septal cusp of the mitral valve (Ferasin, 2009; Côté et al., 2011b). SAM is usually associated with LV outflow tract obstruction (LVOTO), which is possibly due to focal hypertrophy of the interventricular septum (IVS), abnormal anatomy of the mitral valve apparatus or physiological sympathetic activation (Abbott, 2010). It has been considered as a positive outcome indicator in feline HCM (Fox et al., 1995; Payne et al., 2010).

Apart from CHF (which will be discussed in Section 1.2), another common clinical outcome of HCM is ATE. Incidence of ATE in HCM cats has been reported as ranging from 12-17% (Rush et al., 2002; Atkins et al., 1992; Peterson et al., 1993). LA enlargement and dysfunction appear to be major risk factors for ATE in HCM cats (Laste and Harpster, 1995; Payne et al., 2015b). In fact, ATE is not unique to HCM; LA enlargement and evidence of increased thromboembolic risk (i.e. spontaneous echo contrast) are also seen in feline RCM, UCM and DCM (Peck et al., 2016). Acute lameness (especially hindlimbs) and pain are common clinical signs for ATE. Similar to CHF, the long term prognosis of ATE is generally poor (Côté et al., 2011d).

The current treatment principle for feline HCM is mainly targeting the symptomatic cases, i.e. CHF, ATE or arrhythmia. In asymptomatic HCM cats, therapeutic intervention is controversial (Ferasin, 2009). Diltiazem, atenolol and angiotensin converting enzyme (ACE) inhibitors have

been advocated for use in asymptomatic HCM cats, but there is lack of scientific evidence to prove their definite benefits (Ferasin, 2009). Anti-platelet medication clopidogrel may be more justified to use in asymptomatic CM cases to reduce the risk of future thrombotic events when there is evidence of LA enlargement, spontaneous echo contrast or an intra-cardiac thrombus (Hogan et al., 2015). Treatment of CHF will be discussed in Section 1.2.3.

The prognosis for HCM largely depends on the underlying cause and whether the cats are symptomatic (Spalla et al., 2016; Atkins et al., 1992; Rush et al., 2002). CM secondary to hyperthyroidism or systemic hypertension have better outcomes (Spalla et al., 2016). Reported median survival time (MST) in asymptomatic HCM ranges from 1129-3671 days. Cats with ATE had a shorter MST, ranging from 61-184 days (Atkin et al., 1992; Rush et al., 2002; Payne et al., 2010). Survival of HCM cats with CHF is discussed in Section 1.2.4.

Other CM Phenotypes

The other feline CM phenotypes include RCM, DCM, ARVC and UCM. The clinical signs and treatment strategy share a lot of common features with symptomatic HCMs. Regarding disease epidemiology, presentation and prognosis, each of these have some unique characteristics.

RCM is the second most common CM in cats, accounting for 15-20% of total CM according to previous studies (Ferasin et al., 2003; Locatelli et al., 2018). The echocardiographic diagnosis of RCM is characterized by severely dilated LA or bi-atrial dilation, usually with endocardial fibrotic lesions and impaired diastolic function (Ferasin, 2009). Most RCM cats at diagnosis were symptomatic with respiratory distress as the most common clinical presentation (Ferasin et al., 2003; Locatelli et al., 2018) and it appears to be a more severe form of CM compared with HCM (Côté et al., 2011b). Reported MST of feline RCM is between 132-466 days (Ferasin et al., 2003; Spalla et al., 2016; Locatelli et al., 2018) and once respiratory distress develops the prognosis is very poor (Locatelli et al., 2018).

DCM is reported to represent 4.4-10.4% of total CM in cats (Ferasin et al., 2003; Locatelli et al., 2018). The echocardiographic diagnostic criteria are severe LV dilation with reduced LV systolic function (Ferasin et al., 2003). Taurine deficiency plays a role in feline DCM (Pion et al., 1987), but it cannot fully explain the aetiology in all cases (Côté et al., 2011c). The incidence of CHF in DCM is high, and in one previous study cardiomegaly and CHF occurred in 100% of DCM cats (Ferasin et al., 2003); the prognosis for DCM is grave (Ferasin et al., 2003; Côté et al., 2011c). One study has shown treatment with pimobendan improved the MST

in DCM cats to 49 days; without pimobendan, the MST was only 12 days (Hambrook and Bennett, 2012).

ARVC is an uncommon phenotype in cats and one previously reported incidence was 0.5% of feline CM cases (Locatelli et al., 2018); male cats appear to be over-represented (Fox et al., 2000; Harvey et al., 2005). The cardinal echocardiographic feature of ARVC is right ventriclar dilation with thinning of the myocardium, right atrial dilation and an apical ventricular aneurism may also be present (Fox et al., 2000). ARVC is most commonly associated with right sided CHF, and syncope may be observed in affected cats. Arrhythmia is common in feline ARVC, and variable supraventricular and/or ventricular arrhythmias and 3rd degree atrioventricular (AV) block have been identified (Fox et al., 2000; Harvey et al., 2005). The reported prognosis for ARVC in cats is very poor (Fox et al., 2000).

UCM has been defined as those cardiomyopathies that cannot easily be recognized and categorized into the current known phenotypes based on echocardiographic criteria (Ferasin et al., 2003). The reported incidence is 2.9-10.4% of all feline CMs (Ferasin et al., 2003; Locatelli et al., 2018). It should be noted that this phenotype may be a transition phenotype to others (Côté et al., 2011b). The reported MST of UCM cats was 925 days (Ferasin et al., 2003).

1.2 Feline Congestive Heart Failure

The first documentation of heart failure in human history goes back to 2000 BC and cardiogenic congestion was recorded in both ancient Egypt and China (Ferrari et al., 2016). For quite a long period of time, heart failure was considered simply as a circulatory disorder, in which the heart could not pump out enough blood to satisfy the body's requirements. Nowadays, by definition, heart failure is considered to be a complex clinical syndrome in which the heart fails to produce enough cardiac output to meet the body's metabolic demands or is compromised to achieve so at the cost of increased filling pressure (Ware, 2011a).

1.2.1 Aetiology and Pathogenesis

Congestive heart failure (CHF) is characterized by venous congestion or fluid accumulation in body cavities due to high cardiac filling pressures (Côté et al., 2011a). Based on the nature of the primary cause, heart failure can be classified into systolic failure (also called reduced ejection fraction) or diastolic failure (also called preserved ejection fraction). In feline medicine, the most common cause of CHF is one of the primary CMs, and they are often associated with diastolic failure (Côté et al., 2011a).

The pathogenesis of heart failure is complex and involves a number of pathophysiological mechanisms such as inflammation, neurohormonal activation and myocardial remodelling (Dick and Epelman, 2016; Kemp and Conte, 2012). Neurohormonal activation is considered as one of the most important responses, particularly activation of the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone-system (RAAS) activation (Hartupee and Mann, 2017). SNS and RAAS activation is a compensatory response when cardiac function is impaired; however, in the long term they may lead to circulatory congestion and myocardial remodelling. The SNS responds earlier in cardiac decompensation and decreased cardiac output or pressure change sensed by arterial baroreceptors can activate the SNS to increase heart rate and contractility in order to improve cardiac output (Côté et al., 2011a). Activation of the RAAS pathway causes sodium and water retention to increase renal blood flow through a series of mechanisms. It can also induce myocardial inflammation and remodelling through cellular activation and cytokine release (Sciarretta et al., 2009). Neurohormonal responses also occur in the kidney and peripheral vasculature, which decrease renal sensitivity to natriuretic peptide, increase vasopressin and endothelin expression and impair nitric oxide (NO) release in the peripheral vasculature, resulting in vasoconstriction (Chinnaiyan et al., 2005). Inflammation has been considered to play a key role in heart failure and more details are discussed in Section 1.4. Overall, the neurohormonal and inflammatory responses are both

consequences of and causes for progression in heart failure, with anatomical cardiac remodelling occurring at the same time (Van Linthout and Tschope, 2017).

The myocardial remodelling in heart failure includes maladaptive cardiomyocyte hypertrophy and apoptosis, myocardial fibrosis, remodelling of the ECM and regional ischemia (Cohn et al., 2000; Fonfara et al., 2012). In a recent study of feline cardiac pathology, myofibre disarray, interstitial fibrosis, sub-endocardial fibrosis and occasional intramural arteriosclerosis were identified in CHF hearts (Wilkie et al., 2015).

1.2.2 Classification, Clinical Presentations and Diagnosis

Classification of CHF in small animals is mainly based on the clinical signs of the patient (Fox et al., 1999). The International Small Animal Cardiac Health Council (ISACHC) classification is one of the most common methods used in staging feline CHF (Finn et al., 2010). There are three major classes in this system, depending on whether the patient is symptomatic or not. CHF is classified as asymptomatic (Class I), mild to moderate CHF (Class II) or; severe CHF (Class III). Sub-classification is based on whether there is evidence of cardiac remodelling (class Ia vs. class Ib), or to what extent the clinical signs affect the patient's quality of life and whether home management is sufficient (class IIIa) or hospital stabilization is required (class IIIb) (Fox et al., 1999).

Antecedent events such as recent fluid therapy, general anaesthesia or recent corticosteroid administration may trigger CHF development in CM cats (Rush et al., 2002). The common clinical presentations of feline CHF include pulmonary oedema, pleural effusion, ascites, jugular distension and pericardial effusion (Ferasin and Defrancesco, 2015). Typically, feline CM induces left sided CHF, which most commonly presents as pulmonary oedema and/or pleural effusion (Côté et al., 2011a). Dyspnoea and tachypnoea are considered as cardinal physical examination findings of acute onset cardiac decompensation in cats (Ferasin and Defrancesco, 2015). Signs of dyspnoea, such as paradoxical breathing, are the most commonly observed abnormalities by the owners (Côté, 2017). Open-mouth breathing or labouring breathing are also common descriptions. The respiratory rate has been found to increase in CHF cats compared with cats with asymptomatic CM (Ljungvall et al., 2014). A resting or sleeping respiratory rate higher than 30 breaths per minute in a cat with cardiac disease warrants investigation for CHF (Ljungvall et al., 2014; Porciello et al., 2016). Heart rate changes in CHF cats can be highly variable according to previous publications; tachycardia should theoretically

be more common but this was an absent feature in many CHF cats (Dickson et al., 2018; Rush et al., 2002; Smith and Dukes-McEwan, 2012). Heart murmurs are often absent or inconsistent in CHF (Smith and Dukes-McEwan, 2012; Goutal et al., 2010) and do not appear to be a reliable indicator for significant cardiac disease in cats (Abbott, 2010). Gallop sounds, due to increased ventricular stiffness have been shown to have more pathognomonic significance in feline cardiac disease. Previous studies have suggested that 20%-50% of CHF cats have gallop sounds and their presence has been found to be a mortality risk factor in feline CHF (Payne et al., 2015b). Hypothermia is also reported in CHF cats, especially at initial presentation (Dickson et al., 2018; Goutal et al., 2010). Arrhythmias can be a concurrent problem with feline CHF, with ventricular premature complex, atrial fibrillation and ventricular tachycardia are common examples (Smith and Dukes-McEwan, 2012; Côté et al., 2004). Other signs may include pale mucous membrane, weak femoral pulses, abdominal distension, general weakness, anorexia and lethargy (Côté et al., 2011a).

Diagnosis of CHF mainly relies on imaging techniques, i.e. radiology and/or ultrasonography. Thoracic radiography is the gold standard for diagnosing pulmonary oedema (Ferasin and Defrancesco, 2015), however findings of pulmonary ultrasonography can also be suggestive (Boysen and Lisciandro, 2013). Pleural effusion, pericardial effusion and ascites may be appreciated on radiographs, however ultrasonography is a more sensitive and specific technique for the diagnosis of body cavity effusions.

Routine haematology and biochemistry may give non-specific changes; azotaemia and electrolyte imbalance, particularly hypokalaemia, are common findings in CHF cats (Goutal et al., 2010). Concentrations of both circulating cardiac biomarkers, N-terminal pro-brain natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI), have been reported to be increased in CHF cats compared with asymptomatic CM patients (Connolly et al., 2008; Fox et al., 2011; Herndon et al., 2002; Hori et al., 2008), although elevated cTnI is not a consistent finding (Connolly et al., 2003).

1.2.3 Therapeutic Management

Accurate recognition of CHF and the underlying cause is essential in CHF management (Côté, 2017). A CHF therapeutic plan is targeting at opposing the neurohormornal response, reducing preload and afterload, improving cardiac output and cardiac function and reducing maladaptive cardiac remodelling. In acute CHF, minimising stress, oxygen supplement, intravenous loop

diuretic administration and draining body cavity centesis are common procedures required for providing immediate relief; in chronic CHF, the underlying cause should be identified and addressed if possible, loop and other diuretics, pimobendan, ACE inhibitors and spironolactone are drugs commonly used and the treatment plan is tailored for the individual (Côté, 2017).

Furosemide is the most commonly used diuretic in treating both acute and chronic CHF. It is a loop diuretic and effective in reducing intravascular fluid accumulation (Ferasin and Defrancesco, 2015). The drawbacks of furosemide include a hypovolemic effect, impaired kidney function and a brake effect (diuretic tolerance) can develop during treatment (Côté, 2017). Nevertheless, it is still the first line choice in managing feline CHF. Torasemide is also a loop diuretic, which is ten times more potent than furosemide. It is a relatively new diuretic in small animal cardiology and has attracted great attention recently. It has been reported to have less brake effect than furosemide (Uechi et al., 2003; Hori et al., 2007), and has been suggested as a replacement for furosemide in managing feline CHF, especially when tolerance develops (Côté, 2017). Additionally, torasemide appears to have additional beneficial effects, such as aldosterone receptor blockage and an anti-myocardial fibrosis effect (Yamato et al., 2003; López et al., 2007). The thiazide diuretics, such as hydrochlorothiazide, and aldosterone blocker spironolactone may also be used as complementary diuretics to furosemide. Spironolactone is also thought to have anti-fibrotic effects in cardiac patients but this was not evident in one Maine Coon HCM study (MacDonald et al., 2008). The recent SEISICAT study suggest spironolactone is well tolerated and of a potential benefit for administration to cats in CHF (James et al., 2018).

ACE inhibitors block the production of vasoconstrictor angiotensin II and increase the vasodilator bradykinin, which act to oppose RAAS activation (Davies et al., 2000). They are commonly used in feline chronic CHF management, although so far there is still a lack of clear evidence for their benefit in feline CHF (Ferasin and Defrancesco, 2015).

Pimobendan is an inodilator that may improve vasodilation and cardiac contractility in CHF, but so far it is not licenced for use in cats. It has been reported that cats with CHF treated with pimobendan have increased survival compared with controls (Reina-Doreste et al., 2014).

As arrhythmias are not uncommon in CHF cats, anti-arrhythmics may be considered for use in certain cases. Digoxin is recommended when there is concurrent poor LV systolic function and a fast supraventricular tachycardia. Beta-blockers, such as atenolol, are contraindicated in acute CHF, and in patients treated with beta-blocker prior to CHF development, the dose may need

to be adjusted after CHF develops based on individual status (Côté, 2017). Antithrombotic prophylaxis, such as clopidogrel, should be used in CHF cats with LA enlargement and/or spontaneous echo contrast, as they are at high risk of developing thromboembolism (Ferasin and Defrancesco, 2015).

A well-known complication of CHF treatment, particularly with furosemide use, is renal dysfunction, with azotaemia and electrolyte disturbance commonly seen (Ferasin and Defrancesco, 2015). Routine monitoring of kidney function and electrolyte levels is recommended in treated CHF patients.

1.2.4 Survival and Prognosis

In general, CHF is considered to have a poor long-term prognosis. Cats with CHF secondary to DCM, RCM and ARVC are reported to have worse survival compared with HCM (Atkins et al., 1992; Fox et al., 2000; Rush et al., 2002; Ferasin et al., 2003; Payne et al., 2010; Locatelli et al., 2018). The MST in HCM cats with CHF has been reported to be from 92-536 days (Atkins et al., 1992; Rush et al., 2002; Payne et al., 2010). In CHF secondary to DCM, ARVC and RCM, the MST has been reported to be 11 days (Ferasin et al., 2003), 30 days (Fox et al., 2000) and 64-132 days (Ferasin et al., 2003; Locatelli et al., 2018). One exception is TMT induced CHF, which was shown to have a better prognosis than CHF due to HCM (Novo Matos et al., 2018).

Prognostic factors have been investigated extensively in feline CHF associated with primary CM. The presence of LA enlargement based on echocardiography is the most consistent negative prognostic factor (Fox et al., 1995; Rush et al., 2002; Payne et al., 2010; Payne et al., 2015a). Other reported risk factors in cats with CHF and CM include decreased LV fractional shortening (FS) (Petersen et al.,1993; Payne et al., 2015a), increased LV wall thickness (Fox et al., 1995), hypothermia at presentation (Goutal et al., 2010; Dickson et al., 2018), the presence of gallop sounds (Dickson et al., 2018), older age (Payne et al., 2015b; Rush et al., 2002), the presence of arrhythmias (Payne et al., 2015b), the presence of spontaneous echo contrast/intra-cardiac thrombus (Payne et al., 2015a; Peck et al., 2016), LA dysfunction (Payne et al., 2015a), the need for thoracocentesis (Rush et al., 2002), right ventricular enlargement (Rush et al., 2002) and abnormal body weight (Finn et al., 2010).

The CM cats (including those in CHF) with SAM and normal LA size are reported to have a better prognosis (Petersen et al., 1993; Fox et al., 1995; Rush et al., 2002; Payne et al., 2010;

Payne et al., 2015b). In Fox and colleagues' study HOCM was associated with better survival in CHF cats (Fox et al., 1995), whereas in Payne and others' study, SAM seemed only to be associated with asymptomatic status (Payne et al., 2010).

1.3 Cardiovascular-renal Axis Disorder

1.3.1 Definition of Cardiovascular-Renal Axis Disorder

Cardiovascular-renal Axis Disorder (CvRD), or cardio-renal syndrome, is defined as acute or chronic cardiovascular and renal disease, with the dysfunction of one organ system subsequently inducing impaired function or injury to the other organ system (Pouchelon et al., 2015). This disorder was first described in human medicine, with the term largely reserved for renal dysfunction in heart failure (Ronco et al., 2008). Since a CvRD consensus statement was released in 2015 (Pouchelon et al., 2015), it has gain more attention in veterinary medicine. CvRD in small animals has been divided into 3 classes; the $CvRD_H$ class is of particular interest in characterizing renal dysfunction caused by primary cardiovascular diseases. The potential underlying mechanisms of renal disorder in $CvRD_H$ include decreased cardiac output, reduced renal perfusion, neuroendocrine activation, reactive oxygen species production and passive renal congestion (Pouchelon et al., 2015). $CvRD_H$ can be further sub-classified into $CvRD_H$ (unstable disease), also called Acute Cardiorenal Syndrome, and $CvRD_H$ (stable disease), also called Chronic Cardiorenal Syndrome (Orvalho and Cowgill, 2017). Unstable $CvRD_H$ is associated with rapid cardiovascular decompensation such as acute heart failure, with acute renal injury occurring as a consequence of the event. Stable $CvRD_H$ results from chronic heart failure where the long term effect of inadequate cardiac output reduces renal perfusion and the activation of neurohormonal systems such as the SNS and RAAS eventually leads to progressive chronic renal disease (Ronco et al., 2008). Apart from the intrinsic interaction between the two organ systems, therapeutics agents used in CHF management, such as loop diuretics, also can exaggerate neurohormonal activation, reduce glomerular filtration rate (GFR) and worsen renal function (Francis et al., 1990; Lazzarini et al., 2012). Effective decongestion with diuretics in heart failure appears to be beneficial for patient survival, however, even at the cost of worsening renal function (Testani et al., 2010).

In the 2015 CvRD consensus statement, the importance of addressing the renal issue at the same time as CHF management achieved strong agreement between veterinary cardiologists and nephrologists (Pouchelon et al., 2015). The endeavor should be to focus on maximally preserving both cardiac and renal function, which will mutually benefit the two organ systems and eventually improve overall clinical outcomes.

1.3.2 Biomarkers for CvRD Detection

The detection of CvRD relies on cardiac and renal specific biomarkers in conjunction with diagnostic imaging tests such as ultrasonography (Orvalho and Cowgill, 2017). A cardiorenal biomarker panel for assessing cardiorenal syndrome has been proposed (Pouchelon et al., 2015; Orvalho and Cowgill, 2017). These biomarkers include renal function markers, novel kidney injury or stress markers and cardiac biomarkers (Orvalho and Cowgill, 2017). A combination of renal and cardiac biomarkers could be a substitute for current clinical diagnostics. Potentially they can provide more accurate information on both cardiac and renal function in a 'timely manner', serve as disease monitors and guide the clinician in making decisions on a therapeutic plan (Orvalho and Cowgill, 2017).

Currently in veterinary medicine the most well established renal functional marker is creatinine. It is an end-product of muscle metabolism, which has been found to be correlated with GFR and it is increased in renal disease when there is 75% kidney functional loss (Finco et al., 1995; Relford et al., 2016). Traditionally, the serum creatinine concentration was used for kidney disease staging, an increase in creatinine of more than 0.3mg/dl within 48 hours was considered to be indicative of acute kidney injury (AKI) (http://www.iris-kidney.com/pdf/4_ldc-revised-grading-of-acute-kidney-injury.pdf). According to the International Renal Interest Society (IRIS) guidelines, the absolute value of creatinine in non-azotaemic renal disease is defined as <140 μmol/l, and in mild AKI as 140-220 μmol/l. These values largely overlap with the normal reference range, which suggests that serum creatinine is not sensitive for detecting early AKI (Pouchelon et al., 2015). Moreover, the increase in creatinine concentration is largely not specific for renal disease; its expression is affected by lean muscle mass and potentially affected by a number of other factors including age, diet, body weight, medication usage and disease conditions associated with dehydration (Baxmann et al., 2008; Miyagawa et al., 2010; Watson et al., 1981; Yerramilli et al., 2016).

Considering that early recognition of AKI is essential for taking prompt action to protect the kidney, more sensitive renal biomarkers are needed for early diagnosis (Ronco et al., 2008). For this purpose, novel renal biomarkers have been identified recently in veterinary medicine, including symmetric dimethylarginine (SDMA), neutrophil gelatinase-associated lipocalin (NGAL), inosine, clusterin, cystatin B, kidney injury molecule-1, N-acetyl-beta-glucosaminidase (NAG) and retinol binding protein (Bland et al., 2014; Chacar et al., 2017; Lapointe et al., 2008; Orvalho and Cowgill, 2017; De Loor et al., 2013; Segev et al., 2013; Yerramilli et al., 2016).

SDMA has attracted attention as an alternative renal functional biomarker to creatinine in small animal medicine (Kielstein et al., 2006; Relford et al., 2016). It is a form of by-product of amino acid arginine methylation (Kakimoto and Akazawa, 1970), which is primarily eliminated by the kidney (McDermmott, 1976). In cats, it has been shown SDMA has a strong inverse correlation with GFR, therefore it can indirectly reflect renal function (Braff et al., 2014). Previous research in cats showed SDMA started to increase when GRF decreased by 25%, and its response to kidney disease was on average 17 months earlier than creatinine (Hall et al., 2014b). Unlike creatinine, SDMA is not affected by body lean mass, and thus may be more accurate as a renal marker particularly in cachexia or geriatric patients with muscle loss (Hall et al., 2014c). In the 2016 modified IRIS staging of chronic kidney disease (CKD) statement, SDMA was recommended to be used in assisting the previous creatinine based staging system. i.e. persistent increase in SDMA more than 14 ug/dl should be considered significant despite a creatinine value still in the normal range.

The value of using SDMA to detect early renal impairment in cats has been investigated in a few studies. A good example is a recent study investigating SDMA in feline hyperthyroidism before and after I131 therapy (Peterson et al., 2018). Initially, all cats prior to I131 treatment were non-azotaemic and after treatment azotaemia was revealed in 16% cats and a third of those cats had high SDMA prior to I131 treatment, which suggested SDMA has the ability to detect CKD masked by feline hyperthyroidism. They also assessed the diagnostic value of SDMA for masked CKD, showing that SDMA had a sensitivity of 33% and a specificity of 97.7%. Another study has suggested that SDMA is a superior marker for early renal disease detection compared with conventional creatinine and urea (Hall et al., 2016). They compared 6 monthly changes in serum SDMA and creatinine in a population of 80 geriatric cats fed with different types of diet; 28.8% IRIS stage I CKD was detected by elevated SDMA (>14 μg/dl) but not by creatinine, which confirms SDMA is a more sensitive marker for detecting early renal insufficiency.

In general, the specificity of SDMA as a renal marker is superior to creatinine, although its expression was found to be higher in older cats (Hall et al., 2014c). Apart from primary renal disease, it may also increase in other systemic conditions associated with reduced GFR and secondary renal dysfunction, such as feline hyperthyroidism, general anaesthesia and neoplasia (Peterson et al., 2018; Befu et al., 2018; Coyne et al., 2018).

In the past, SDMA was considered as a non-functional molecule, purely serving as a renal functional tracer. More recent human studies suggested SDMA may also act as a pro-inflammatory factor in chronic renal disease (Schepers et al., 2011). *In vitro* experiments showed it enhanced intracellular expression of inflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in monocytes. The same study also established an inflammatory analysis model in clinical CKD patients, in which SDMA was found closely correlated with conventional inflammatory markers (Schepers et al., 2011). The isomer of SDMA, asymmetric dimethylarginine (ADMA), has been recognized for decades as an inhibitor of nitric oxide synthase (NOS) and is involved in endothelial dysfunction (Vallance et al., 1992). Traditionally SDMA was not thought to be involved in the same pathological pathways, but newer evidence has shown it may be a mediator in endothelial dysfunction by competing with NOS and stimulating reactive oxygen species production when there is vascular injury (Closs et al., 1997; Kielstein et al., 2006; Schepers et al., 2011). Multiple studies have consistently demonstrated SDMA has an independent risk prediction value in cardiovascular disease (Schlesinger et al., 2016), which suggest an intrinsic link between SDMA and cardiovascular disorders.

The second essential part of a CvRD biomarker panel is cardiac biomarkers such as NT-proBNP and cTnI. The properties of the cardiac biomarkers will be discussed in detail in Section 1.5. A combination of renal and cardiac biomarkers would potentially allow sensitive detection and monitoring of CvRD and would be valuable for future clinical practice.

1.3.3 Role of CvRD in CHF

In humans, renal dysfunction is a common condition in heart failure and it has been found as an independent poor prognostic factor for heart failure survival (Hillege et al., 2000; Hillege et al., 2006; McClellan et al., 2002). In a veterinary 3-month survival analysis study, it was shown that dogs and cats that developed azotaemic AKI had at least a 3 times higher risk of mortality (Harison et al., 2012). Although CvRD is a relatively new concept in small animal medicine, the high prevalence of azotaemia in CHF has been well recognized (Gouni et al., 2008; Goutal et al., 2010; Nicolle et al., 2007; Yu et al., 2016; Martinelli et al., 2016). In dogs in CHF due to degenerative mitral valve disease (DMVD), the prevalence of azotaemia was reported as being 32%-71% in different studies, with a consistently higher incidence of azotaemia observed in more advanced heart failure in all of these studies (Nicolle et al., 2007; Yu et al., 2016;

Martinelli et al., 2016). A preliminary investigation of CvRD in DMVD dogs showed that chronic renal disease severity was positively correlated with the severity of CHF. The treated CHF dogs had a significantly higher incidence of CKD compared with untreated dogs. Moreover, the DMVD dogs that developed CvRD had a significantly shorter survival time compared with those without CvRD (Martinelli et al., 2016). In feline patients, to the author's knowledge, CvRD has not yet been reported. One retrospective study reported that azotaemia occurred in 58.8% of feline HCM cases; interestingly there was no significance difference between CHF cases and non-CHF cases in creatinine and urea levels (Gouni et al., 2008). Another study looked at azotaemic parameters in cats developing acute CHF during hospitalization and found that increased creatinine occurred in 53% of the CHF cats (Goutal et al., 2010). The high prevalence of azotaemia in heart failure is thought to be a reflection of CvRD, whether it is a result of intrinsic organ interactions or of iatrogenic origin (e.g. loop diuretics), it should not be neglected (Orvalho and Cowgill, 2017).

SDMA has been recently investigated as a CvRD marker in advanced stage CHF in dogs, finding that a progressively elevated SDMA level was correlated with increased heart failure severity (Choi et al., 2017). Renal injury marker Cystatin-C demonstrated similar changes to SDMA in the same study, and both markers were well correlated with the traditional renal parameters creatinine and urea.

Early detection and careful management of CvRD will influence the outcome of CHF. For example, worsening renal function and excessive use of diuretics in acute CHF management may lead to diuretic resistance, which is detrimental to both cardiac and renal function (Liang et al., 2008). Therefore, from a clinical point of view, CvRD should be detected and addressed as soon as possible in CHF patients.

1.4 Inflammatory Responses and Acute Phase Proteins

1.4.1 Overview of Inflammation and Heart failure

The involvement of inflammation in heart failure has been recognized, however the complex underlying mechanism is yet to be fully understood. Whether inflammation is a cause or consequence of heart failure has been extensively explored; the most prevailing understanding suggested that inflammation and heart failure reciprocally affect each other, namely forming a vicious cycle (Van Linthout and Tschope, 2017). Inflammation can trigger or exaggerate heart failure in a number of ways, through either the systemic circulation or local organ effects. Documented mechanisms include triggering cardiac cellular apoptosis and maladaptive hypertrophy, endothelial dysfunction, abnormal ECM remodelling and endothelial mesenchymal transition, eventually leading to macroscopic cardiac remodelling and dysfunction. In heart failure, induced local or systemic sterile inflammation can also occur as a response to cardiac cytokine release in the decompensated heart (Dick and Epelman, 2016; Van Linthout and Tschope, 2017). To investigate the role of inflammation in heart failure, a biomarker-based approach shows its merits; it potentially can help to understand the aetiology and pathogenesis of heart failure and may identify therapeutic targets and aid in disease surveillance and prognosis (Bozkurt et al., 2010). Currently the main inflammatory mediators investigated in heart failure include pro-inflammatory cytokines and acute phase proteins (Bozkurt et al., 2010; Braunwald, 2008; Mavropoulou et al., 2016).

1.4.2 Acute Phase Proteins

The acute phase response has been described as an innate immune response to stimuli occurring in trauma, infection, stress, neoplasia and inflammation processes (Cray et al., 2009). Acute phase proteins (APPs) are important biological reactants released during the acute phase response. So far more than 200 APPs have been reported, but their full functions are still under investigation (Cray et al., 2009). The APPs are predominantly produced by hepatocytes, although other organs such as adipose tissue have also been found to have the ability to produce APPs (Ahmed et al., 2012). Depending on the response to stimulation, the APPs are classified into negative and positive groups. The former decrease during the inflammatory response, and the latter (positive APPs) showing an increase in expression during inflammation (Ceron et al., 2005). Common negative APPs in humans and in dogs and cats include albumin and transferrin; positive APPs include C-reactive protein (CRP), serum amyloid A (SAA), alpha-1-acid

glycoprotein (AGP), haptoglobin (Hp), fibrinogen, ceruloplasmin and leucine-rich alpha-2-glycoprotein1 (LRG1) (Ceron et al., 2005; Shirai et al., 2009). Depending on their differential rate and pattern of response to stimuli, the positive APPs can be further classified into three groups: major, moderate and minor (Eckersall and Bell, 2010). Major APPs have the fastest response rate, usually increasing within the first 48 hours to reach a peak, then declining rapidly. The levels of increased expression can be 10-100 fold compared to normal. Moderate APPs have slower response rates and less marked rises (5-10 fold) compared with major APPs, usually peaking after 2-3 days before starting to decline. Minor APPs have the slowest response and degradation rates and also the lowest rise in concentration (Eckersall and Bell, 2010). There are variations between different species in APP expression. For example, in dogs the most important major APP is considered to be CRP, while in cats, CRP seems to be less useful (Kajikawa et al., 1999). The most well established major APPs in cats are SAA and AGP, and Hp is the most common minor feline APP (Ceron et al., 2005).

It should be noted that APPs are highly sensitive biomarkers and they lack specificity, as they can be involved in multiple disease processes. Therefore the interpretation of APPs should be done with caution, especially when trying to clarify their roles in specific diseases. An editorial review was published in 2008 in which guidelines for APPs interpretation were proposed in small animal medicine; this provides valuable guidance for clinical application as well as for APP related scientific study design (Ceron et al., 2008). According to the guidelines, species specific tests should be used whenever possible and an APP profile should include at least one major and one moderate APP. Although it is essential to obtain baseline data on APPs in associated disease processes, in order to investigate disease pathophysiology, investigations of APP changes in a temporal manner would provide information for disease monitoring and prognosis (Ceron et al., 2008; Eckersall and Schmidt, 2014). Additionally, it has been suggested that an increase in APPs in a clinical healthy animal may potentially indicate the presence of subclinical disease (Ceron et al., 2008).

Although the APPs were discovered a long time ago, they only become popular in veterinary medicine in last two decades. Most APP studies in dogs and cats focus mainly on infectious conditions, traumatic/inflammatory events and neoplasia (Kajikawa et al., 1999; Eckersall and Schmidt, 2014). In feline medicine, it is known that AGP has good diagnostic value in feline infectious peritonitis (FIP) (Paltrinieri et al., 2007; Hazuchova et al., 2017) and SAA can be a prognostic factor in sick or hospitalized cats with various disease conditions (Kajikawa et al., 1999; Kann et al., 2012; Tamamoto et al., 2013). The role of APPs in specific systemic

inflammatory diseases, such as pancreatitis and anaemia secondary to inflammation and chronic renal disease, has also been investigated (Tamamoto et al., 2009; Ottenjann et al., 2006; Javard et al., 2017). Currently, several APPs are available for assessing and monitoring infectious or inflammatory disease status. Common APP profiles in dogs are CRP/Hp and in cats are SAA/AGP/Hp (Ceron et al., 2008). Apart from these, a huge knowledge gap exists regarding other APPs in small animal patients.

1.4.3 APPs in Cardiovascular Diseases

APPs have been studied in human cardiovascular disease research. A good review article from 1999 discussed the role of CRP in cardiovascular disease; by then the circulating CRP level was already known as an independent risk factor for cardiovascular events (Lagrand et al., 1999). Over the past 20 years more APPs were identified as having connections with cardiovascular diseases, and their interactions with the circulatory system seem to go beyond straightforward inflammation (Ahmed et al., 2012; Kumagai et al., 2016; Watson et al., 2011). For example, LRG1 was found to protect against adverse myocardial and vascular remodelling in heart disease, potentially by interfering with the transforming growth factor-beta (TGF-β) pathway (Song and Wang, 2015; Kumagai et al., 2016). Ceruloplasmin is a metalloprotein that binds most of the copper in plasma and has documented functions including iron detoxification and an association with oxidative stress (Ceron et al., 2005; Cao and Hill, 2014). Both LRG1 and ceruloplasmin were found to be increased in heart failure in humans (Watson et al., 2011; Hammadah et al., 2014; Cabassi et al., 2014; Dadu et al., 2013). SAA, AGP, Hp and procalcitonin (PCT) are other examples of APPs that have been found to be associated with cardiovascular disease (Singh-Manoux et al., 2017; Johnson et al., 2004; Fischer et al., 2014; Holme et al., 2009; Suleiman et al., 2005; Canbay et al., 2015; Mockel et al., 2017) and to date, more investigations are still needed to characterize their full functions.

Regarding APPs in small animal cardiovascular diseases, published data were mainly related to dog DMVD. CRP is the most widely investigated APP in canine mitral valve disease, and several studies have reported CRP to be increased in canine CHF and that its elevation amplitude was correlated with CHF severity (Cunningham et al., 2012; Domanjko Petric et al., 2018; Polizopoulou et al., 2015; Reimann et al., 2016). Although CRP does not differentiate DMVD groups at different severities (Ljungvall et al., 2010; Rush et al., 2006), consistently serum CRP appears to positively correlate with cTnI in DMVD dogs (Polizopoulou et al., 2015;

Ljungvall et al., 2010). Moreover, in symptomatic DMVD dogs, CRP was also found to associate with echocardiographic changes such as increased left atrium to aortic root ratio (LA/Ao) and LV diameter during diastole, increased mitral valve inflow E wave velocity and decreased LV ejection fraction (Reimann et al., 2016; Domanjko Petric et al., 2018). Other APPs were investigated in canine DMVD, including Hp and ceruloplasmin, with Hp found to have significantly higher expression in the severe disease group compared with asymptomatic patients (Polizopoulou et al., 2015).

There is less information regarding the involvement of APPs in feline cardiovascular disease. A very recent scientific report showed SAA to be increased in asymptomatic HCM cats with global LV hypertrophy, which suggested an involvement of SAA in feline CM (Van Hoek et al., 2018a). In one FIP focused study, CHF cats that developed body cavity effusions were evaluated for SAA, Hp and AGP expression (Hazuchova et al., 2017). Compared with FIP, the CHF cats had significantly lower SAA, Hp and AGP levels, however no healthy control cats were used in the study for comparison.

1.5 Biomarkers in Cardiology

The original definition of a biomarker was established in 2001: 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Atkinson et al., 2001). It has been proposed that biomarkers could contribute to both research and clinical medicine. From upstream in vitro and in vivo studies to downstream clinical application, they may serve as valuable tools in understanding disease pathogenesis, aiding disease diagnosis and staging, indicating disease prognosis and monitoring patients' response to therapeutics (Atkinson et al., 2001).

Given the above merits, biomarkers have emerged in the past decade in cardiovascular medicine. Extensive research work has been carried out on developing novel biomarkers for cardiovascular diseases. A rationale for assessing novel clinical cardiovascular biomarkers was summarized in one review. In general, three criteria were proposed: (1) whether the biomarker is measurable; a biomarker test should be accurate, reproducible and readily accessible with reasonable cost; (2) whether the biomarker provides additional information supplementary to existing clinical tests; (3) whether the biomarker can help to make clinical decisions in patient management, such as guiding a therapeutic plan and risk assessment etc. (Morrow and De Lemos, 2007).

In humans, biomarkers in heart failure have been developed in relation to inflammation, oxidative stress, ECM remodelling, neurohormonal regulation, myocardial injury and fibrosis, myocardial stress, CvRD and other pathophysiological pathways (Braunwald, 2008; Senthong et al., 2017). Genomic, metabolomic and proteomic techniques were also implemented recently to explore novel biomarkers for heart failure.

In small animal cardiology, current clinical biomarkers are restricted to NT-proBNP and cTnI. Both biomarkers are reasonably well established in cats and they have been shown to have value in feline cardiac disease management (Borgeat et al., 2015a).

NT-proBNP is an inactive form of cleaved product from the prohormone brain natriuretic peptide (Harris et al., 2017b). Brain natriuretic peptide (BNP) is secreted by atrial and ventricular myocytes in response to myocardial stretch, its main function being to regulate plasma volume and promote natriuresis when there is volume or pressure overload in the ventricle, which can counteract RAAS activation (Volpe et al., 2016). NT-proBNP has a longer half-life than BNP, thus it is more stable for assessment as a biomarker of myocardial stress

(Braunwald, 2008). In previous feline CM studies, NT-proBNP has demonstrated reasonably good ability consistently in differentiating healthy, asymptomatic HCM from CHF cats (Connolly et al., 2008; Wess et al., 2009; Fox et al., 2011). With a cut off at 100 pmol/l, the reported sensitivity and specificity of detecting sub-clinical HCM was 94% and 81% respectively (Wess et al., 2009); the sensitivity and specificity of detecting asymptomatic feline cardiac disease (primary CM) was 84.6% and 100% respectively (Harris et al., 2017a). It is also a valuable tool in differentiating cardiogenic respiratory distress from primary respiratory disease in cats (Connolly et al., 2009; Fox et al., 2009; Hassdenteufel et al., 2013). One recent study investigated NT-proBNP in CHF cats with short term follow up, and it has shown that greater reduction of NT-proBNP in response to hospitalization may be a positive indicator for survival in cats (Pierce et al., 2017). The prognostic potential of NT-proBNP in feline heart disease has been noticed for a long time (Borgeat et al., 2014b), but so far there is no evidence supporting its use as an independent risk predictor. A partial reason for this may be the high biological variability of NT-proBNP in individual cats, which makes it challenging to interpret changes in a temporal manner (Harris et al., 2017b). Prohormone atrial natriuretic peptide (proANP) is another myocardial stress marker which is similar to NT-proBNP. Increased concentrations seem to correlate with severity of HCM especially when the disease is in a more advanced stage (Zimmering et al., 2009; Parzeniecka-Jaworska et al., 2016). In general, it has not shown superior characteristics to NT-proBNP in diagnosing and staging feline CM (Connolly et al., 2008).

cTnI and cardiac troponin T (cTnT) are biomarkers of myocardial injury, with cTnI currently more widely used in the feline field (Borgeat et al., 2015a). Similar to NT-proBNP, cTnI also has some value in detecting asymptomatic CM and differentiating cardiogenic respiratory signs, but it has been considered less useful than NT-proBNP in these two aspects (Borgeat et al., 2015a). In a previous study, an increased concentration of cTnI was found to be associated with a risk of death in feline HCM, but this did not stay in the final survival analysis model, while cTnT turned out to be a significant prognosticator (Langhorn et al., 2014).

Both NT-proBNP and cTnI are known to increase in other systemic disease conditions. For example, NT-proBNP and cTnI have been found to increase in feline hyperthyroidism (Sangster et al., 2014); an increased concentration of NT-proBNP has been associated with systemic hypertension in CKD cats (Lalor et al., 2009); increased concentrations of cTnI were reported in anaemia (Lalor et al., 2014). Additionally, since both NT-proBNP and cTnI are partially eliminated by the kidneys, it has been proposed that renal function should be taken

into account when interpreting these two biomarkers, especially if a patient has concurrent cardiac and renal disease (Orvalho and Cowgill, 2017).

Recently, other potential cardiac biomarkers have been investigated in small animal medicine, particularly in association with CM and CHF. These include inflammatory cytokines (Reimann et al., 2016; Cunningham et al., 2012; Fonfara et al., 2012), ECM remodelling markers (Borgeat et al., 2015b; Fonfara et al., 2013; Aupplerle et al., 2011), markers of CvRD (Choi et al., 2017; Orvalho and Cowgill, 2017), metabolic markers (Fonfara et al., 2017), haemostatic factors (Tablin et al., 2014; Tarnow et al., 2007) and more advanced global discovery approaches including genomics (Lu et al., 2015; Oyama and Chittur, 2006) and proteomics (Locatelli et al., 2017).

There is a great need to develop novel biomarkers for disease diagnosis, management and prognosis in veterinary cardiology. A multiple biomarker-based scoring approach for risk stratification has been advocated in human medicine in the past 10 years, and the evaluation outcome looks very promising (Senthong et al., 2017; Braunwald, 2008). This could also be a future direction in veterinary medicine - a new era of multiple biomarker-based medicine.

1.6 Aims, Scope and Hypotheses

Aims

The aims were to evaluate whether CvRD and an inflammatory response occur in feline CHF due to primary CM, and to identify novel biomarkers for feline CHF and CM.

Scope

The study population contained CM cats diagnosed at the Cardiology Service of the University of Glasgow from 2014 to 2017, the majority of enrolment being done from 2016 to 2017. Serum samples were collected from cats and stored for batch analysis, and clinical records of the CM cats were collated. Serum biomarker assays were performed by collaborating IDEXX reference laboratories and Life Diagnostics Inc., USA. A panel of markers for CvRD and a panel of APPs were assessed respectively in CM cats with CHF, CM cats without clinical signs and healthy controls.

A preliminary longitudinal study was carried out in CHF cats, when biomarker changes were tracked 12 months for each individual.

Hypotheses

1. CvRD occurs in feline CHF due to primary CM; NT-proBNP and renal function markers are associated with survival in CHF cats.
2. Inflammatory responses occur in feline CHF due to primary CM; selected APPs are associated with survival in CHF cats.
3. By measuring biomarkers at a series of planned time points, optimal time points for predicting disease prognosis may be determined in CHF cats.

Chapter 2: Materials and Methods

2.1 Summary of Study Design

The object of this study was to investigate whether CvRD and inflammatory responses occur in feline CHF secondary to CM. Two panels of serum protein markers were assessed, and the aim was also to identify whether these markers could be used as novel biomarkers for CHF management and prognosis.

The study populations included three cat groups: cats diagnosed with CHF due to primary CM (the CHF group), cats diagnosed with asymptomatic CM (the asymptomatic group) and healthy controls. Inclusion and exclusion criteria, definitions of terms and classifications used in the study were established during the study design.

There were two parts to this study (Figure 2.1): Part 1-baseline study. The intrinsic differences in the expression of the biomarkers were compared in the three cat groups. Additionally, clinical parameters, correlation of biomarkers and clinical variables were also assessed, and a survival analysis was performed in CHF cats. Part 2-longitudinal study. This was carried out in the CHF group. After initial sample collection, CHF cats which were enrolled during this time period had follow-up blood sampling at subsequent revisits. Serial biomarker levels were measured in follow-up samples and the trends of biomarker changes were assessed over time.

Clinical data and blood samples were collected from candidate CM cats in the Cardiology Service of the Small Animal Hospital, University of Glasgow. Serum samples were stored for batched biomarker tests. Healthy cat serum samples obtained from a commercial source were used as controls.

CvRD biomarker analysis was performed in IDEXX reference laboratories; the profile included NT-proBNP, SDMA and creatinine. Inflammatory response was assessed by analysing a panel of APPs including AGP, CRP, Hp, LRG1, SAA, PCT and ceruloplasmin. The APP work was performed in a collaborating laboratory in the USA.

After data collection, statistical analysis was performed. One-way analysis of variance (ANOVA) and Student's T test were used to compare quantitative data between groups. Chi-square and Fisher exact tests were used to compare categorical data between groups. Spearman's rho test was used to assess correlations among variables. Cox proportional hazards models were used for survival analysis in the CHF cats.

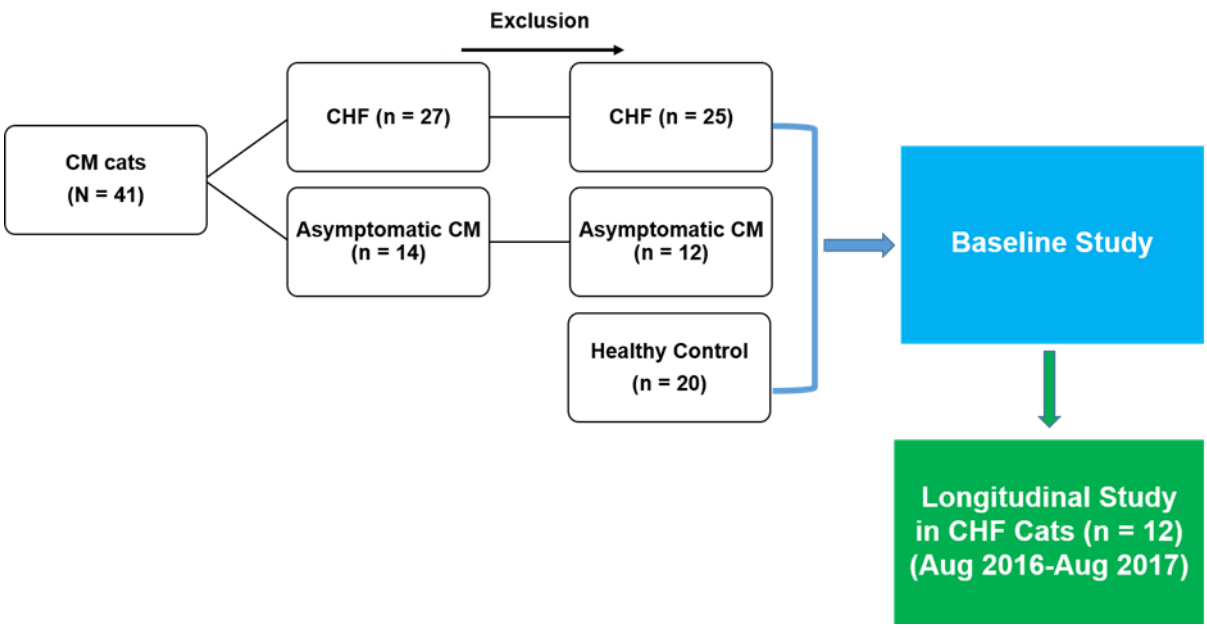

Figure 2.1. Illustration of cat enrolment for the biomarker baseline and longitudinal study. Originally 41 CM cats entered in the study; after initial exclusion, 25 CHF and 12 asymptomatic cats were enrolled and used for baseline characteristics analysis.

2.2 Study Population

2.2.1 CM Cats

From January 2014 to August 2017, 41 cats diagnosed with CMs were recruited to the study in the Cardiology Service of the Small Animal Hospital at the University of Glasgow with full owner consent. Among the candidate cats, 27 cats were in CHF and 14 cats had asymptomatic CM. The following inclusion and exclusion criteria were used for candidate selection:

Inclusion criteria

- Any type of primary CM diagnosed by echocardiography including HCM, HOCM, DCM, UCM, RCM and ARVC.

Exclusion criteria

- CM secondary to hyperthyroidism or hypertension. For hyperthyroidism, cats older than 6 years were screened based on clinical records. In cats older than 6 years old without thyroid function test results, serum thyroxine (T4) was tested retrospectively before a decision on final enrolment was made. Cats less than 6 years of age were exempted from thyroid function testing, as they were unlikely to be affected. For hypertension screening, a consistent reading of systolic blood pressure (BP) $\geqslant$ 180 mmHg or $\geqslant$ 160 mmHg with retinal changes were considered as systemic hypertension (Payne et al., 2013).
- Primary CKD history-IRIS stage $\geqslant$ II (http://www.iris-kidney.com/pdf/3_staging-of-ckd.pdf) (Table 2.1) at the time CM was first diagnosed.
- A diagnosis of other conditions that have been reported to trigger an acute phase reaction, including any active infectious diseases, recent inflammatory events (surgery or trauma) or certain systemic diseases (pancreatitis, neoplasia and anaemia secondary to chronic inflammatory disease).

Four cats were further excluded from the study for the following reasons: in the CHF group, one cat was later found to have hyperthyroidism and for another cat, it was not possible to collect sufficient blood to run the planned biomarker tests; in the asymptomatic CM group, one cat had echo data missing, and another cat died due to the sudden onset of ATE, but clinical CHF development could not be ruled out. Finally, 37 CM cats (25 CHF cats and 12 asymptomatic CM) were enrolled in the study.

Table 2.1. Staging of feline CKD based on blood creatinine concentration

Stage	Blood Creatinine (µmol/l)	Comments
At risk	< 140	History suggests the animal is at risk of developing CKD in the future due to various factors.
1	< 140	Non-azotaemic. Some renal abnormality is present e.g. inadequate concentrating ability, abnormal renal palpation or imaging findings.
2	140-250	Mild azotaemia. Clinical signs usually mild or absent.
3	251-440	Moderate renal azotaemia. Many extra renal clinical signs may be present.
4	>440	Increased risk of systemic clinical signs and uremic crises

2.2.2 Healthy Control Cats

Commercial serum samples (Biobest, UK) from 20 clinically healthy cats were used as controls, with age and sex information available. Four cats were older than 6 years, and a retrospective T4 test ruled out hyperthyroidism in these cats. As echocardiography data was not available, all the healthy control cats were screened using NT-proBNP to eliminate occult heart disease. One cat had an NT-proBNP value of 29 pmol/l, but the NT-proBNP concentrations for the other 19 cats were below the lowest level of the reference range (<24 pmol/l). IDEXX reference laboratory's recommended cut off to differentiate healthy and asymptomatic cardiac diseased cats is 100 pmol/l (Cardiopet proBNP) (Wess et al., 2009; Harris et al., 2017a). Therefore, these cats were believed to be free from occult CM.

2.3 Diagnoses of CM and CHF

2.3.1 CM Diagnosis and Classification

CMs were diagnosed with echocardiography performed by a European College of Veterinary Internal Medicine (ECVIM) Board-certified Cardiologist, or ECVIM residents in Cardiology or a Cardiology Certificate trainee working under the supervision of an ECVIM Board-certified Cardiologist. For cats that had multiple echocardiographic examinations performed, the results obtained closest to the time of 1st blood sample collection were used for the CM diagnosis and echo variable analysis. Based on 2D, M-mode and Doppler echocardiography parameters, candidate cats were diagnosed with one of the following conditions at study entry: HCM (including HOCM), DCM, UCM, RCM or ARVC. Details of the echocardiography diagnostic criteria are listed in Table 2.2. Final echo measurements and the CM classification for each individual cat were approved by an ECVIM Board-certified cardiologist.

Table 2.2. Classification and diagnostic criteria for feline CM

CM Classification	Diagnostic Criteria Based on Echocardiography
HCM	Left ventricular hypertrophy was defined as an end-diastolic maximal thickness of the left ventricular free wall (LVFWd) or interventricular septum (IVSd) of ⩾ 6 mm (Fox et al., 1995); and/or evidence of focal hypertrophy in the left ventricle (Ferasin, 2009). HOCM was included in this category in the current study, which was characterized by presence of LVOTO.
RCM	Characterized by marked bi-atrial enlargement without significant myocardial hypertrophy, normal or mildly reduced myocardial systolic function, and diastolic dysfunction i.e. a restrictive left ventricular filling pattern confirmed with Doppler echocardiography (Fox et al., 2014).
DCM	Characterized by dilation of the left ventricular lumen and decreased myocardial function. In the current study the following cut-offs were used: left ventricular internal diameter end diastole (LVIDd) > 18 mm, left ventricular internal diameter end systole (LVIDs) > 12 mm, LV FS < 30%. Normal ranges vary considerably in the literature (Côté et al., 2011c; Pion et al., 1992; Ferasin, 2009).
ARVC	Marked right side cardiac chamber enlargement particularly right ventricular dilation with thinning of the myocardium, right atrial dilation and an apical ventricular aneurism may also be present. Mild tricuspid valve regurgitation may be seen (Côté et al., 2011e; Fox et al., 2000).
UCM	Myocardial abnormalities that do not readily fit into any of the classifications defined above (Ferasin et al., 2003).

2.3.2 CHF Diagnosis and Classification

CHF was diagnosed based on clinical history, presentation and evidence on thoracic radiographs and/or on ultrasonography. Presentations of CHF include pulmonary oedema, pleural effusion, pericardial effusion and ascites.

The cardiac decompensation status of CM cats was staged based on the ISACHC classifications (Table 2.3) (Fox et al., 1999). CHF group in the current study refers to 'clinical CHF', which contained cats belonging to ISACHC Class II, IIIa or Class IIIb; the asymptomatic group contained cats belong to ISACHC Class Ia or Ib.

Table 2.3. ISACHC classification

Class	Criteria
I asymptomatic patient	**Ia**-heart disease present, no clinical signs, no evidence of cardiac remodelling; **Ib**-heart disease present, no clinical signs, evidence of cardiac remodelling is present.
II mild to moderate heart failure	Clinical signs evident at rest or with mild exertion, adversely affected quality of life, home treatment indicated.
III advanced heart failure	**IIIa**-heart disease with clinical signs of advanced CHF at rest, home treatment indicated. **IIIb**-heart disease with clinical signs of advanced CHF at rest, hospitalization required for management.

2.4 Clinical Record Collection

Relevant clinical records of CM cats were documented during the study period (i.e. by November 2017). History records were checked through hospital archives or the electronic medical records system (Excelicare, AxSys Technology, UK) installed in the Small Animal Hospital. The following details were recorded for candidate cats at entry to the study: animal name, case number, date of birth, sex, breed, date of initial diagnosis (Table 2.4), age at initial diagnosis, CM diagnosis, CHF diagnosis, serum T4 level, systolic BP and comorbidities. Echo variables (Table 2.5) which have been reported to be associated with severity and prognosis of feline CM were recorded, including LA diameter (mm), LA/Ao ratio, LVFWd (mm), IVSd (mm), LV FS %, presence of spontaneous echo contrast ('smoke') or intra-cardiac thrombus (Payne et al., 2013; Payne et al., 2015a).

The following clinical information was recorded at the time of each blood sampling: sampling date, age at sampling, body weight, heart rhythm during auscultation, presence of gallop sounds, heart murmur, pulse deficit, heart rate, respiratory rate, ECG, CHF severity based on ISACHC classification (Table 2.4), cTnI, haematocrit and cardiac medication status.

Prior to final statistical analysis in November 2017, CM cat survival information was collected. The information was mainly collected through the Excelicare system, with the rest of the information clarified by contacting the relevant referring veterinary practice or client. The following information was recorded for survival analysis: survival status (alive or dead), date of death (if applicable), cause of mortality (if applicable) and date of last contact (if applicable) (Table 2.4).

Additionally, the following information was collected in the CHF cats: overall CHF stability during the study, times of thoracocentesis, total days in the intensive care unit (ICU) during the study, concurrent cardiac arrhythmia, concurrent thrombotic/pro-thrombotic events (i.e. spontaneous echo contrast, intra-cardiac thrombus observed on echocardiography and ATE) and cardiac medical treatments during the study.

Table 2.4. Definition of terms used in clinical record

Term	Explanation
Initial diagnosis date	For a CHF cat it was defined as the date on which both primary CM (diagnosed based on echocardiography) and CHF (based on radiography and/or ultrasonography) were definitely diagnosed; for an asymptomatic CM cat it was defined as the date when primary CM diagnosis was made based on echocardiography.
Survival days	Survival status of all CM cats was recorded during the study period. For non-survival cats, it was defined as date difference between initial diagnosis and date of death. For cats still alive by the end of the study or lost to follow up, date of last contact or last time the cat known to be alive was used and censored in statistical analysis.
Cause of mortality	(1) Cardiac cause: a cat which died naturally or euthanized due to refractory/uncontrolled CHF, or other cardiac disease related death such as arterial or pulmonary thromboembolism; (2) Non-cardiac cause: a cat which died naturally or euthanized due to unrelated reason (s) to cardiac disease.
Overall CHF status (stable/unstable):	'Stable' status was defined as during study period- (1) a ISACHC IIIa animal, alive, home management for CHF was sufficient (Porciello et al., 2016) and there was no known record of apparent CHF deterioration-i.e. progression to IIIb; OR (2) a ISACHC IIIb animal, alive, after initial hospital management for CHF, home management for CHF was sufficient and no re-hospitalization due to CHF; 'Unstable' status was defined as during the study period- (1) a CHF cat which died naturally or was euthanized due to cardiac reason; OR (2) there was apparent CHF deterioration (e.g. a cat with disease progressed from home management to hospital management) or hospital management always required at each visit.
CHF severity grade (1-4 scales)	CHF severity was graded based on ISACHC classification: 1 = no evidence of cardiac remodelling (ISACHC class IIa); 2 = evidence of cardiac remodelling (ISACHC class IIb); 3 = advanced CHF, home management (ISACHC class IIIa); 4 = advanced CHF, need hospitalization (ISACHC class IIIb)

Table 2.5. Echocardiography variables and evaluation method

Echo Variable	Evaluation method
LA diameter	2D mode. Right parasternal short axis view at the heart base. Measured at the end of diastole with all aortic valve leaflets closed, at the onset of the QRS complex.
LA/Ao ratio	2D mode. Right parasternal short axis view at the heart base. Measured at the end of diastole with all aortic valve leaflets closed, at the onset of the QRS complex.
LVFWd	2D or M-mode. Right parasternal views. Measured for maximal LV free wall thickness at the end of diastole, with leading edge to leading edge technique.
IVSd	2D or M-mode. Right parasternal views. Measured for maximal interventricular septal thickness at the end of diastole, with leading edge to leading edge technique.
LV FS%	M mode. Right parasternal short axis of the left ventricle at the level of the chordae tendineae. Measured with leading edge to leading edge technique.
Spontaneous echo-contrast/intra-cardiac thrombus	2D mode. Right parasternal views and left cranial views of the left auricular appendage. Looked for evidence of cloud-like swirling of intra-cardiac blood or a thrombus.

2.5 Blood Sample Collection, Processing and Storage.

CM cat blood samples were collected as soon as possible after the initial diagnosis was made. A volume of 0.3-1.5 ml of blood was sampled via venepuncture (in most case using jugular vein). Following collection, samples were allowed to clot and centrifuged at 9000 rpm for 3 minutes at room temperature; serum was separated and collected into a new collection tube and stored in a -20°C freezer temporarily then transferred to a -80°C freezer for long term storage before batch analysis. Commercial healthy control feline serum samples followed the same storage protocol.

2.6 Experimental Design

Due to serum sample volume limitations and different sample volume requirements in each test, a prioritization of the biomarker tests was arranged prior to laboratory testing. Based on research interest, sample volume availability and laboratory test feasibility, tests were prioritised in the following order (with priority in descending order): NT-proBNP, SDMA, creatinine and APPs (LRG1, Hp, AGP, SAA, CRP, PCT and ceruloplasmin).

2.6.1 Baseline Study in Three Cat Groups

Blood samples from 35 CM cats (23 CHF cats and 12 asymptomatic CM cats) and 20 healthy control cats were used for the baseline study. A detailed allocation plan is illustrated in Figure 2.2.

2.6.2 Longitudinal Study in CHF Cats

Sample collection for the longitudinal study was carried out in CHF cats between August 2016 and August 2017. After initial baseline sample collection, cats enrolled during this time period had blood samples taken at subsequent revisits. By August 2017, 12 CHF cats had follow-up blood samples after the initial baseline sample collection. Among them, 12 cats were tested for CvRD markers and 9 cats were tested for APPs in the serial study

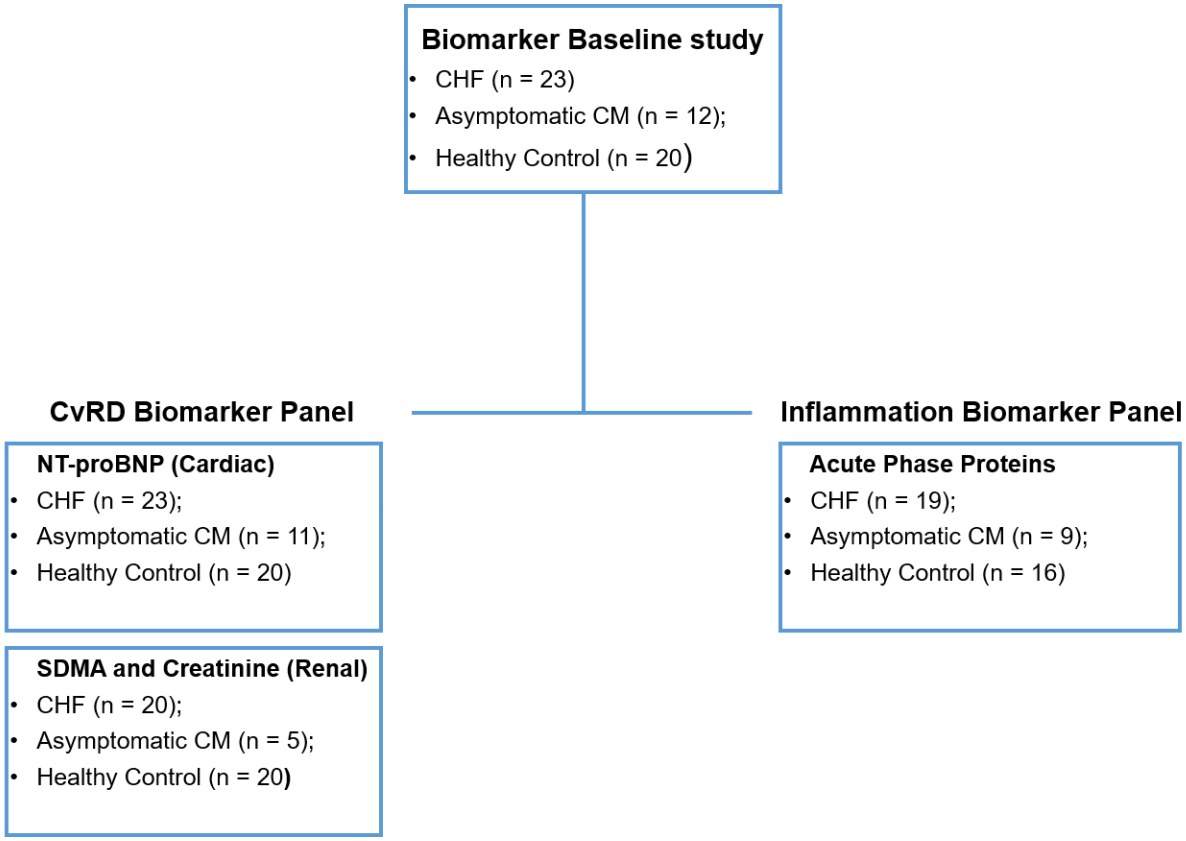

Figure 2.2. Study subject allocation plan for the biomarker baseline study. Two panels of biomarkers were evaluated i.e. CvRD and Inflammation. Note: different numbers of cats were used for different analyses; this was due to serum sample volume limitations to meet all test requirements.

2.7 Measurements of CvRD Biomarkers

For samples collected between 2016 and 2017, volumes of 200-330 ul serum were shipped to IDEXX Reference Laboratory (Germany) for biomarker analysis; NT-proBNP concentration was measured using the Cardiopet proBNP assay. SDMA was measured using the IDEXX SDMA assay and creatinine was measured using a kinetic colour test. Assay methods are listed in Table 2.6. Additionally, 9 samples (3 CHF and 6 asymptomatic CM) collected in 2014 had serum NT-proBNP values measured at time of collection using the same methodology at the IDEXX Reference Laboratory (UK). The NT-proBNP data from these cats was kindly shared by Iñigo Sanz González. These retrospective data were combined with the new NT-proBNP results in 2017 for statistical analysis.

Table 2.6. Detection methods for NT-proBNP, SDMA and creatinine

Biomarker	Detection Method	Detection Range
NT-proBNP	Cardiopet proBNP (Enzyme-linked immunosorbent assay)	24-1500 pmol/l
SDMA	Enzyme immunoassay	1-100 µg/dl
Creatinine	Kinetic colour test (compensated Jaffé reaction)	5-2200 µmol/l

2.8 Measurements of APPs

Pre-frozen serum samples collected between 2014 and 2017 were shipped on dry ice to Dr Christopher Chadwick at Life Diagnostic Inc., USA for APPs tests. Spatial Proximity Analyte Reagent Capture Luminescence (SPARCL^{TM}) assays were used for APP measurements (Table 2-7). The principle of a SPARCL™ assay is as follows: each assay contains two specific antibodies with one conjugated to horseradish peroxidase (HRP) and the other conjugated to chemiluminescent substrate -acridan. Both antibodies target the same biomarker. In a reaction system, when the two conjugated antibodies bind to their target biomarker, HRP and acridan react and cause chemiluminescence which is proportional to target biomarker concentration.

Prior to sample testing, APP standard solutions were prepared. The lyophilized APP stock was reconstituted with diluent (CSD50-1, Life Diagnostic Inc. USA) and mixed gently until dissolved. Eight standards were prepared for each APP. Depending on the specific APP, the following serial dilutions were used respectively: For AGP: 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 ng/ml; CRP: 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.195 and 0.098 ng/ml; Hp: 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 ng/ml; SAA: 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml; PCT: 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.20 ng/ml; LRG1: 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.813ng/ml; and ceruloplasmin: 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56 ng/ml.

Serum samples were diluted to different concentrations with the diluent provided (Table 2.7), depending on the nature of each assay. In the wells of a 96 well plate, 25 µl of mixed HRP and acridan conjugated antibodies were added, followed by 50 µl of standard solution or diluted sample. The plate was incubated for 30 min at 25°C and 150 rpm on a microplate incubator. After incubation, the plate was placed in a BMG LUMI star Omega luminometer (BMG LABTECH, USA). The luminometer was set up at a gain of 3600, primed with 1ml of trigger solution and the injection needle was positioned in the injection port in advance. Luminescence was immediately measured after injecting 37.5 µl trigger solution. A standard curve was calculated by plotting luminescence for the standards versus log 10 of each APP concentration using graphing software. Sample APP concentration was calculated from the standard curve by converting the luminescence data antilog.

Table 2.7. SPARCL™ assays used in the APP measurements

APP	Catalogue Number	Species	Serum Sample Dilution (fold)
AGP	AGP-SP-8	Feline	20,000
CRP	CRP-SP-8	Feline	125,000
Hp	Hapt-SP-8	Feline	50,000
SAA	SAA-SP-8	Feline	1000 or 50,000
PCT	PCT-SP-4	Canine	40
LRG1	LRG-SP-8	Feline	100
Ceruloplasmin	CER-SP-8	Feline	10,000

2.9 Data Analysis

All biomarker test results and clinical data were summarized and organized in one Microsoft Office Excel Datasheet and, basic descriptive data analysis was performed using Microsoft Office Excel. Statistical analysis was kindly performed and shared by Professor Geoffrey Fosgate in the Faculty of Veterinary Sciences, University of Pretoria, Onderstepoort, South Africa.

Prior to statistical analysis, natural logarithm, square root or rank transformations were employed to improve the data normality distribution. All quantitative data were presented descriptively as the median and interquartile range (IQR). For NT-proBNP data analysis, a result >1500 pmol/l was assigned the value 1501pmol/l; a result < 24 pmol/l was assigned the value 23pmol/l (Sangster et al., 2014). ANOVA and t tests were used to compare quantitative data between groups. Significant ANOVA results were followed by multiple pairwise t tests employing Bonferroni correction of post hoc P values. Categorical data were described using frequencies, proportions, and 95% mid-P exact confidence intervals (CI). Chi-square and Fisher exact tests were used to compare categorical data among groups. The correlation between variables was assessed using scatter plots and calculating Spearman's rho. Cox proportional hazards models were used to estimate the association between biomarkers and other cat-level variables (Appendix 1) on survival time in CHF cats. Univariate screening models were used and all variables significant ($P < 0.20$) were selected in a backwards stepwise process for evaluation in multivariable models. Spearman's rho was used to evaluate collinearity between selected variables and the variable with the weaker univariate association was excluded when $\rho < 0.70$. Biomarkers with $P < 0.20$ in the univariate screening models were analysed in multivariable models; receiver-operating characteristics (ROC) analysis was used to identify the most accurate cut-off for identifying non-surviving cats using the largest Youden index. The variables were removed one-by-one based on the largest Wald P value until all remaining variables were significant at $P < 0.05$. Interaction terms were not evaluated.

MINITAB Statistical Software Release 13.32 (Minitab Inc, State College, Pennsylvania, USA) was used to evaluate the normality assumption for quantitative data, plot histograms, calculate descriptive statistics and perform the Anderson-Darling normality test. Categorical data analysis was performed in available freeware Epi Info, version 6.04 (CDC, Atlanta, GA, USA) and all remaining analyses were performed using commercial software IBM SPSS Statistics Version 24 (International Business Machines Corp., Armonk, NY, USA). Statistical findings were interpreted at the 5% level of significance. Descriptive plots for the longitudinal study

were created using the ggplot2 package (Wickham, 2009) within R (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

Chapter 3: Results

3.1 Study Population

In total 37 CM cats were enrolled in the study, with 25 cats in CHF and 12 cats showing no clinical signs i.e. asymptomatic CM. Signalment and body weight are summarized in Table 3.1. Healthy control cats were significantly younger than the CM cats ($P < 0.01$). Domestic short hair was the predominant breed for both CHF and asymptomatic CM cats, accounting for 84% and 100% of the populations respectively. Breed information in the healthy control group was not available. For both CHF and asymptomatic cats, males were over-represented; overall the male proportion was 74%. There were no significant differences in sex, age and body weight between CHF and asymptomatic cats ($P > 0.05$). All the enrolled cats were free from hyperthyroidism and no evidence of systemic hypertension was documented in any of the enrolled cats; systolic BP records were available in 14 CHF cats and 9 asymptomatic CM cats, the values being 131 $\pm$ 26 (range 80-170) mmHg vs 133 $\pm$ 14 (range 110-160) mmHg respectively.

Recorded comorbidities in CHF cats were diabetes mellitus (n = 1), concurrent respiratory disease (n = 2), suspected disseminated carcinomatosis (n = 1), tricuspid dysplasia due to suspected storage disease (n = 1) and 3rd degree AV block with pacemaker implanted (n = 1). In the asymptomatic CM cats one had a chronic benign cervical mass, two had mild periodontal disease and one cat had eosinophilia of unknown aetiology.

Table 3.1. Signalments and body weight in the study populations

	CHF (n = 25)	**Asymptomatic (n = 12)**	**Healthy Control (n = 20)**
Age (years)	8.1 ± 4.7 (1.0-15.3)	6.6 ± 4.4 (0.7-14.3)	4.3 ± 3.0 (0.6-11.3)
Breed	DSH (n = 21); British blue (n = 1); Ragdoll (n = 1); Siamese (n = 1); Bengal (n = 1)	DSH (n = 12)	NA
Sex	Female (n = 8); Male (n = 17)	Female (n = 3); Male (n = 9)	Female (n = 10); Male (n = 10)
Weight (kg)	4.9 ± 1.7 (2.3-9.5)	5.7± 1.4 (3.7-8.0)	NA

Statistical analyses between CHF and asymptomatic CM cats were performed for age (P = 0.35), sex (P = 1.0) and weight (P = 0.177). Values of variables are presented as mean + standard deviation (range). DSH, domestic short hair; NA, not available.

3.2 Clinical Record Comparison in CM Cats

Overall CM phenotypes were significantly different between CHF and asymptomatic cats (Figure 3.1). Compared with the asymptomatic group (n = 12), the CHF group (n = 25) shows more diverse types of cardiomyopathies, including 44% HCM, 28% UCM, 24% RCM and 4% DCM; in the asymptomatic group 92% cats were diagnosed with HCM, except one UCM. Additionally, HOCM was present in 4 CHF cats, and 2 cats had SAM; in asymptomatic CM cats, 8 had HOCM and 3 had SAM.

Figure 3.1. Comparison of CM phenotypes in CHF and asymptomatic CM cats. The differences were statistically significant (P = 0.011).

Baseline cardiovascular parameters including physical examination findings, ECG and selected echocardiographic parameters were recorded in both the CHF and asymptomatic groups at each sample point (Table 3.2). Compared with the asymptomatic group, cats in CHF showed a significantly higher respiratory rate (P = 0.009), increased LA diameter (P <0.001), increased LA/Ao ratio (P <0.001) and decreased LV FS% (P = 0.001) (Table 3.2). No significant differences were found for the presence of an arrhythmia (either by auscultation or on ECG) or gallop sounds, heart murmur status, pulse deficit, heart rate, LVFWd or IVSd.

Table 3.2. Comparison of clinical parameters in CM cats at admission

	CHF (n = 25)		**Asymptomatic (n = 12)**		
Variable	**n/d**	**PE* (Interval†)**	**n/d**	**PE* (Interval†)**	**P value**
Irregular heart rhythm	10/18	0.56 (0.33, 0.77)	1/7	0.14 (0.01, 0.53)	0.090
Gallop sounds audible	4/24	0.17 (0.06, 0.35)	0/12	0.00 (0.00, 0.22)	0.278
Murmur present	12/19	0.63 (0.40, 0.82)	11/12	0.92 (0.65, 1.0)	0.108
Murmur grade ≥ 3	9/19	0.47 (0.26, 0.69)	6/12	0.50 (0.23, 0.77)	0.886
Pulse deficit	3/11	0.27 (0.07, 0.58)	1/8	0.13 (0.01, 0.48)	0.603
Abnormal ECG	13 /20	0.65 (0.43, 0.83)	3/9	0.33 (0.09, 0.67)	0.226
Heart rate (per min)	24/25	180 (165, 200)	12/12	169 (160, 195)	0.398
Respiratory rate (per min)	25/25	44 (36, 60)	12/12	30 (20, 55)	0.009
LA diameter (mm)	25/25	20.0 (16.5, 22.0)	12/12	13.0 (11.3, 15.0)	<0.001
LA/Ao ratio	25/25	2.34 (1.84, 2.56)	12/12	1.32 (1.26, 1.39)	<0.001
LVFWd (mm)	25/25	6.38 (5.21, 7.41)	12/12	6.15 (5.03, 7.67)	0.987
IVSd (mm)	25/25	6.03 (4.54, 7.53)	12/12	5.23 (4.67, 7.73)	0.463
LV FS (%)	25/25	37.0 (23.5, 47.0)	12/12	53.0 (44.5, 63.0)	0.001

n/d, numerator/denominator; *PE, point estimate, corresponding to the proportion for categorical variables and the median for quantitative data. †Interval-95% CI for categorical data and IQR for quantitative data.

3.3 Circulating Biomarker Expressions in CM and Health Cats

Ten biomarkers were examined in the current study, including three associated with CvRD (i.e. NT-proBNP, SDMA and creatinine) and seven APPs (i.e. AGP, CRP, Hp, LRG1, SAA, PCT and ceruloplasmin) (Table 3.3).

Serum NT-proBNP, SDMA and creatinine levels were increased in CHF cats. NT-proBNP clearly differentiated the three groups. Cats in CHF showed significantly higher NT-proBNP levels than asymptomatic CM and healthy cats ($P < 0.05$). In the asymptomatic CM population, three cats had an NT-proBNP concentration less than 100 pmol/l. In serum samples from healthy controls, NT-proBNP was generally undetectable using the current test i.e. <24 pmol/l, except one healthy cat had a value of 29 pmol/l. Renal markers SDMA and creatinine were also significantly increased in CHF cats compared with healthy controls ($P < 0.05$). SDMA was significantly higher in CHF cats compared with asymptomatic cats ($P < 0.05$) but was not different between asymptomatic and healthy groups ($P > 0.05$); creatinine neither differentiated CHF cats from asymptomatic cats, nor differentiated asymptomatic cats from healthy controls ($P > 0.05$). In the CHF group, 61% cats demonstrated abnormal SDMA levels according to the IDEXX laboratory reference (SDMA >14 ug/dl), while for creatinine, 44% had abnormal serum concentration (creatinine >168 µmol/l). One asymptomatic cat and two cats in the control group showed serum SDMA values higher than 14 ug/dl; another asymptomatic CM cat had a creatinine value above the reference range. The remaining cats in both asymptomatic and healthy groups had normal renal marker values. SDMA and creatinine were not measured in 2 CHF cats due to insufficient sample volume.

CHF cats had significantly different expressions of APPs LRG1, SAA and ceruloplasmin compared with other groups. LRG-1 was significantly higher in CHF cats compared with healthy controls ($P < 0.05$), although no significant differences were detected between the CHF and asymptomatic groups or the asymptomatic and healthy groups; SAA was significantly increased in CHF cats compared with both asymptomatic CM cats and healthy cats ($P < 0.05$); ceruloplasmin was significantly increased in CHF cats compared with asymptomatic and healthy counterparts ($P < 0.05$). Regardless of cardiac disease status, there were no significant differences in AGP, CRP, Hp and PCT between groups. PCT concentration was zero in most cats (24/37), for the rest it was above zero, of which six cats were in CHF, two were asymptomatic CM and five were healthy controls. Additionally, serum troponin I levels (measured as part of their clinical investigations) were higher in CHF cats ($n = 5$) than in asymptomatic CM cats ($n = 4$) ($P = 0.01$).

Table 3.3. Comparison of serum biomarker concentrations in CHF, asymptomatic CM and healthy control cats

	CHF		Asymptomatic		Healthy Control		
Biomarker	**n**	**Median (IQR)**	**n**	**Median (IQR)**	**n**	**Median (IQR)**	**P value***
CvRD Profile							
NT-ProBNP (pmol/l)	19	1293[a] (780, >1500)	11	282[b] (76, 532)	20	<24[c] (<24, <24)	<0.001
SDMA(ug/dl)	14	18.5[a] (12.0, 23.0)	5	11.0 [b] (8.5, 13.5)	19	9.0[b] (8.0, 12.0)	<0.001
Creatinine(µmol/l)	14	159[a] (117, 195)	5	127[a, b] (115, 181)	19	95[b] (80, 120)	<0.001
APPs							
AGP (µg/ml)	15	302 (240, 626)	9	187 (147, 325)	16	290 (187, 454)	0.327
CRP (µg/ml)	15	360 (226, 509)	9	290 (187, 454)	16	316 (218, 388)	0.356
Hp (µg/ml)	15	239 (32, 321)	9	141 (117, 217)	16	163 (57, 231)	0.887
LRG1 (µg/ml)	15	8.9[a] (6.4, 10.8)	9	5.3[a, b] (4.3, 7.0)	16	3.3[b] (2.7, 4.4)	0.037
SAA (µg/ml)	15	1.34[a] (1.00, 11.27)	9	0.79[b] (0.56, 1.00)	16	0.75[b] (0.64, 1.07)	0.009
PCT (ng/ml)	15	0 (0, 50.6)	9	0 (0, 5.7)	16	0 (0, 20.0)	0.644
Ceruloplasmin (µg/ml)	15	326[a] (272, 419)	9	227[b] (194, 268)	16	283[b] (241, 312)	0.001
Myocardial Injury							
cTnI (ng/ml)	5	1.11[a] (1.03, 1.69)	4	0.37[b] (0.12, 0.64)	0		0.010

* Medians without superscripts in common were significantly different (P <0.05) based on paired t test with Bonferroni correction of P value.

3.4 Correlations Between Biomarkers and Clinical Variables

Biomarker expression was compared between different diagnostic phenotypes in the 37 CM cats. No apparent differences in the expression of 10 serum biomarkers were observed between HCM, UCM and other CM phenotypes ($P \geq 0.05$) (Table 3.4).

Table 3.4. Comparison of serum biomarker concentrations among different CM phenotypes

	HCM (n = 22)		UCM (n = 8)		Other* (n = 7)		
Biomarker	**n**	**Median (IQR)**	**n**	**Median (IQR)**	**n**	**Median (IQR)**	**P value**
CvRD							
NT-ProBNP (pmol/l)	20	536 (214, 1426)	8	1397 (900, >1500)	6	1089 (721, >1500)	0.054
SDMA (ug/dl)	11	14.0 (11.0, 23.0)	7	20.0 (13.0, 21.0)	5	13.0 (11.0, 19.5)	0.599
Creatinine (µmol/l)	11	154 (104, 185)	7	194 (161, 201)	5	116 (98, 151)	0.050
APPs							
AGP (µg/ml)	18	252 (165, 629)	7	297 (239, 520)	3	407 (302, 416)	0.840
CRP (µg/ml)	18	325 (234, 402)	7	394 (360, 711)	3	476 (193, 490)	0.577
Hp (µg/ml)	18	187 (104, 332)	7	292 (32, 472)	3	286 (272, 492)	0.544
LRG1 (µg/ml)	18	7.0 (5.0, 10.3)	7	8.9 (6.4, 10.8)	3	8.4 (6.4, 10.8)	0.768
SAA (µg/ml)	18	1.0 (0.79, 3.21)	7	1.10 (0.73, 11.83)	3	1.48 (1.12, 1.70)	0.695
PCT (ng/ml)	18	0 (0, 11.6)	7	0 (0, 12.4)	3	0 (0, 96.0)	0.980
Ceruloplasmin (µg/ml)	18	272 (226, 391)	7	326 (263, 481)	3	468 (294, 652)	0.129

*Combined group of DCM (n = 1) and RCM (n = 6). ‡Two group comparison was performed between HCM and non-HCM diagnoses.

Markers of interest in the current study showed various correlations with each other and with clinical variables (Table 3.5). Major findings are summarized as following:

(1) All three CvRD markers were significantly correlated with each other, with moderate positive correlations (Rho = 0.401-0.615, P <0.01). Seven APPs showed variable correlations with each other, with most of them having significantly weak to moderate positive correlations (Rho = 0.288-0.610, P <0.05); significantly negative correlation was only observed between PCT and Hp.

(2) Significant positive correlations were found between biomarkers of myocardial stress, APPs and myocardial injury. NT-proBNP showed weak to moderate correlations with AGP, LRG1, SAA and ceruloplasmin; also showed a strong positive correlation with cTnI. AGP and Hp showed significantly strong positive correlations with cTnI level.

(3) Significant correlations were found between several biomarkers and clinical echo variables. In general, most of the biomarkers positively correlated with LA diameter and LA/Ao ratio and negatively correlated with LV FS. In particular, NT-proBNP showed moderate positive correlations with LA diameter, LA/Ao ratio and a negative correlation with LV FS; LRG1 demonstrated similar correlation patterns to NT-proBNP with the above echo variables, and also moderate positive correlations with LA diameter and with LA/Ao ratio, and a negative correlation with LV FS. Ceruloplasmin was positively correlated with LA diameter and LA/Ao ratio. Hp and SAA were positively correlated with LA diameter. Creatinine was negatively correlated with LVFWd and LV FS; PCT was negatively correlated with LVFWd.

(4) Significant positive correlations were found between the three biomarkers and disease severity: NT-proBNP had a moderate positive correlation with CHF grades (Rho = 0.489, P <0.001); SAA and ceruloplasmin was weakly to moderately correlated with CHF grades.

(5) In these cat populations, age appeared to have a moderate effect on creatinine (Rho = 0.505, P <0.001) and a weak effect on LRG1 (Rho = 0.296, P = 0.028) and SAA (Rho = 0.363, P = 0.006). No significant correlations were found between age and other biomarkers.

(6) Significant correlations were found between several APPs and two of the renal parameters. SDMA was found to have a moderate positive correlation with CRP and Hp, and a moderate negative correlation with PCT; creatinine was found to have a positive correlation with CRP, LRG1 and SAA.

Table 3.5. Spearman's rank correlation between measured biomarkers and clinical variables at each sampling period in all cats participated in the study

	NT-proBNP	SDMA	Creatinine	AGP	CRP	Hp	LRG1	SAA	PCT	Ceruloplasmin
Biomarker										
NT-proBNP	1	**0.615** (<0.001)	**0.454** (<0.001)	**0.269** (0.049)	0.240 (0.080)	0.242 (0.078)	**0.456** (0.001)	**0.317** (0.020)	-0.168 (0.224)	**0.439** (0.001)
SDMA	**0.615** (<0.001)	1	**0.401** (0.002)	0.277 (0.065)	**0.543** (<0.001)	**0.417** (0.004)	0.210 (0.167)	0.254 (0.092)	**-0.385** (0.009)	0.174 (0.254)
Creatinine	**0.454** (<0.001)	**0.401** (0.002)	1	0.116 (0.448)	**0.312** (0.037)	0.174 (0.253)	**0.470** (0.001)	**0.472** (0.001)	-0.043 (0.778)	0.232 (0.124)
AGP	**0.269** (0.049)	0.277 (0.065)	0.116 (0.448)	1	0.145 (0.291)	**0.421** (0.001)	**0.288** (0.033)	**0.474** (<0.001)	-0.053 (0.702)	**0.610** (<0.001)
CRP	0.240 (0.080)	**0.543** (<0.001)	**0.312** (0.037)	0.145 (0.291)	1	0.257 (0.058)	**0.394** (0.003)	**0.402** (0.002)	-0.134 (0.331)	**0.291** (0.031)
Hp	0.242 (0.078)	**0.417** (0.004)	0.174 (0.253)	**0.421** (0.001)	0.257 (0.058)	1	0.193 (0.158)	**0.314** (0.019)	**-0.325** (0.015)	**0.327** (0.015)
LRG1	**0.456** (0.001)	0.210 (0.167)	**0.470** (0.001)	**0.288** (0.033)	**0.394** (0.003)	0.193 (0.158)	1	**0.481** (<0.001)	**0.420** (0.001)	**0.342** (0.011)
SAA	**0.317** (0.020)	0.254 (0.092)	**0.472** (0.001)	**0.474** (<0.001)	**0.402** (0.002)	**0.314** (0.019)	**0.481** (<0.001)	1	-0.022 (0.872)	**0.527** (<0.001)
PCT	-0.168 (0.224)	**-0.385** (0.009)	-0.043 (0.778)	-0.053 (0.702)	-0.134 (0.331)	**-0.325** (0.015)	**0.420** (0.001)	-0.022 (0.872)	1	0.085 (0.538)
Ceruloplasmin	**0.439** (0.001)	0.174 (0.254)	0.232 (0.124)	**0.610** (<0.001)	**0.291** (0.031)	**0.327** (0.015)	**0.342** (0.011)	**0.527** (<0.001)	0.085 (0.538)	1
cTnI	**0.730** (0.017)	0.667 (0.219)	0.700 (0.188)	**0.786** (0.036)	-0.071 (0.879)	**0.893** (0.007)	0.750 (0.052)	0.559 (0.192)	0.236 (0.610)	0.643 (0.119)
Echo Variables										
LA diameter	**0.528** (0.001)	0.324 (0.132)	0.165 (0.453)	0.300 (0.121)	0.297 (0.125)	**0.422** (0.025)	**0.537** (0.003)	**0.420** (0.026)	0.053 (0.790)	**0.429** (0.023)
LA/Ao ratio	**0.547** (0.001)	0.404 (0.056)	0.110 (0.617)	0.150 (0.447)	0.313 (0.105)	0.309 (0.110)	**0.445** (0.018)	0.147 (0.456)	0.146 (0.460)	**0.376** (0.048)
LVFWd	0.133 (0.453)	0.030 (0.893)	**-0.453** (0.030)	0.174 (0.376)	0.099 (0.616)	0.208 (0.289)	-0.109 (0.581)	0.243 (0.213)	**-0.562** (0.002)	0.204 (0.299)
IVSd	0.084 (0.636)	0.072 (0.743)	-0.004 (0.986)	0.224 (0.251)	0.239 (0.221)	-0.007 (0.972)	-0.011 (0.956)	0.204 (0.297)	-0.216 (0.270)	0.193 (0.326)
LV FS	**-0.428** (0.012)	-0.362 (0.090)	**-0.468** (0.024)	-0.171 (0.384)	-0.333 (0.084)	-0.302 (0.118)	**-0.388** (0.042)	-0.229 (0.242)	0.003 (0.988)	-0.201 (0.306)
Others										
CHF grade	**0.489** (<0.001)	0.305 (0.052)	-0.035 (0.827)	0.309 (0.059)	0.044 (0.793)	0.221 (0.182)	0.213 (0.198)	**0.345** (0.034)	0.079 (0.636)	**0.405** (0.012)
Age (sampling)	0.183 (0.123)	0.178 (0.175)	**0.505** (<0.001)	0.059 (0.666)	0.175 (0.201)	0.162 (0.238)	**0.296** (0.028)	**0.363** (0.006)	0.136 (0.321)	0.247 (0.069)
Diagnosis days	0.058 (0.683)	-0.076 (0.635)	-0.055 (0.734)	-0.028 (0.863)	-0.073 (0.657)	0.023 (0.890)	-0.270 (0.096)	-0.214 (0.191)	-0.049 (0.767)	0.288 .075)

Data presented as spearman's rank correlation (P Value). Significant correlations are presented in bold font.

3.5 Survival Analysis in CHF Cats

Regarding presentation of CHF, 13 cats were diagnosed with pulmonary oedema and 17 cats with pleural effusion. Pericardial effusion and ascites were present in 6 and 4 cats respectively. Based on the ISACHC system, 64% cats were in ISACHC Class IIIa and 36% cats were in Class IIIb at admission. During the study period, 10 cats in CHF groups had thoracocentesis procedures (from 1-4 times). Seventeen CHF cats required stabilization in the ICU, with total stay time 4.3 $\pm$ 3.6 days (range from 0.5-15 days). Eleven cats were considered as overall stable without apparent CHF deterioration during the study, and the other 14 CHF cats were classified as unstable which either died or had CHF deterioration during the study time. Concurrent arrhythmias in CHF cats included VPCs, atrial fibrillation, ventricular tachycardia, atrial premature complexes (APCs) and 3rd degree AV block. In total 11 cats had a risk or episode of thrombotic event: 9 cats had spontaneous echo contrast and 1 cat had an intra-cardiac thrombus on echocardiography; ATE occurred in one cat. All CHF cats received various combinations of cardiac medications prior to 1st blood sampling, including furosemide (1.0-13.8 mg/kg/24h), torasemide (0.2-1.0 mg/kg/24h), benazepril (0.2-1.0 mg/kg/24h), spironolactone (1.3-2.6 mg/kg/24h), pimobendan (0.3-0.7 mg/kg/24h), clopidogrel (1.9-8.9 mg/kg/24h), aspirin (3.6-8.0 mg/kg/72h), diltiazem (5.2-7.7 mg/kg/24h), sotalol (4.3-4.8 mg/kg/24h) and potassium supplementation. By November 2017, 12 cats in CHF were alive and the other 13 had died. Causes of mortality include 8 cardiac deaths, 1 suspected death from neoplasia and concurrent CHF, and 2 cats died with reasons unknown.

Survival analysis was performed to identify potential prognostic factors for all causes of mortality in CHF cats. Initial analysis suggested five potential prognostic indicators for survival status in the CHF group (Table 3.6) i.e. LA diameter, LA/Ao ratio, LV FS, NT-proBNP and SDMA ($P < 0.05$). Significantly increased LA diameters ($P = 0.003$), increased LA/Ao ratio ($P < 0.001$) and decreased LV FS ($P = 0.036$) were found in cats that died compared with cats that survived. Of the biomarkers, NT-proBNP was significantly higher in non-survivors, with a median concentration of >1500 pmol/l (IQR 1308->1500) in non-survivors and 780 pmol/l (IQR 578-1477) in survivors. Similarly, significantly higher SDMA was found in the serum of non-surviving cats with median concentration at 21ug/dl (IQR 16.5-23.0), while in cats that survived the median SDMA level was 13.0 ug/dl (IQR 11.0-20.0) ($P = 0.007$). No significant differences ($P > 0.05$) were found between survivors and cats that died in creatinine and APP concentrations.

Table 3.6. Comparison of potential prognostic indicators determined at the time of admission for CHF cats.

	Survived (n = 12)		Died (n = 13)		
Variable	**Median (n)**	**IQR**	**Median (n)**	**IQR**	**P value***
Age at diagnosis (years)	9.6 (12)	4.9, 12.2	5.1 (13)	4.1, 13.1	0.906
Weight (kg)	4.9 (11)	4.3, 6.8	3.8 (13)	3.3, 5.4	0.050
Echo Variables					
LA diameter (mm)	18.0 (12)	16.3, 21.0	21.0 (13)	17.5, 22.5	0.003
LA/Ao ratio	2.11 (12)	1.72, 2.54	2.36 (13)	2.03, 2.86	<0.001
LVFWd (mm)	6.15 (12)	5.44, 7.37	6.56 (13)	4.60, 7.70	0.729
IVSd (mm)	6.37 (12)	4.37, 8.94	6.00 (13)	4.68, 7.21	0.892
LV FS (%)	37.5 (12)	26.5, 48.3	30.0 (13)	21.0, 46.5	0.036
CvRD Biomarkers					
NT-ProBNP (pmol/l)	780 (11)	578, 1477	>1500 (12)	1308, >1500	0.001
SDMA (ug/dl)	13.0 (9)	11.0, 20.0	21.0 (9)	16.5, 23.0	0.007
Creatinine (µmol/l)	157 (9)	114, 201	161 (9)	111, 191	0.922
APPs					
AGP (µg/ml)	302 (9)	234, 477	459 (10)	248, 770	0.090
CRP (µg/ml)	476 (9)	274, 544	368 (10)	267, 439	0.937
Hp (µg/ml)	272 (9)	145, 361	307 (10)	24, 641	0.526
LRG1 (µg/ml)	9.4 (9)	7.8, 11.9	8.6 (10)	5.5, 11.3	0.940
SAA (µg/ml)	1.70 (9)	1.23, 14.09	1.11 (10)	0.84, 3.45	0.844
PCT (ng/ml)	0 (9)	0, 54.0	0 (10)	0, 21.9	0.938
Ceruloplasmin (µg/ml)	391 (9)	283, 487	372 (10)	289, 496	0.932

Univariate Cox proportional hazard analysis was performed to evaluate the effect of potential prognostic factors on survival time in the 25 CHF cats considering all causes of mortality (Table 3.7). Significant univariate predictors included unstable CHF (P = 0.018), arrhythmia detected by auscultation (P = 0.015), audible gallop sounds (P = 0.029), LA/Ao ratio (P = 0.007) and serum AGP level (P = 0.007). Cats with unstable CHF had an increased hazard ratio of 12.0; presence of an arrhythmia and gallop sounds gave an increased hazard ratio of 14.1 and 5.1 respectively. Insignificant (P <0.20, but > 0.05) univariate predictors included LA diameter, heart rate, murmur grade ≥ 4, presence of pulse deficit, arrhythmia on ECG, NT-proBNP and SDMA level.

Signalment variables were forced into multivariable models to account for potential confounding. Multivariable survival analysis (Table 3.8) suggested the following three variables are independent poor prognostic factors in the CHF cats (n = 19): body weight < 4.5 kg, LA/Ao ratio ≥ 2 and serum AGP level ≥ 600 µg/ml were found to increase hazard ratios significantly (P = 0.023, P = 0.013 and P = 0.009 respectively).

Table 3.7. Univariate Cox proportional hazards analysis evaluating the effects of potential prognostic factors for CHF cats. Results are presented for signalment and only those other variables with P <0.20.

Variable/level	n	Parameter estimate ($\hat{\beta}$)	Hazards ratio (95% CI)	P value (Wald)
Signalment				
Age				0.501
<5 years	8	0.751	2.12 (0.51, 8.90)	0.305
5-10 years	7	0.825	2.28 (0.50, 10.4)	0.287
>10 years	10	Referent		
Sex				
Male	17	-0.138	0.87 (0.28, 2.76)	0.815
Female	8	Referent		
Weight				
<4.5 kg	11	0.431	1.54 (0.49, 4.86)	0.463
≥4.5 kg	13	Referent		
Cardiac Variables				
LA diameter (mm)*	25	0.140	1.15 (0.99, 1.33)	0.064
LA/Ao ratio*	25	1.131	3.10 (1.36, 7.05)	0.007
Heart rate (bpm)*	24	0.019	1.02 (0.99, 1.05)	0.170
Unstable CHF	25	2.486	12.0 (1.53, 95.5)	0.018
Arrhythmia by auscultation	18	2.644	14.1 (1.68, 118)	0.015
Gallop sounds	24	1.631	5.11 (1.18, 22.1)	0.029
Murmur grade ≥4	19	1.349	3.85 (0.74, 20.0)	0.108
Pulse deficit	11	1.434	4.20 (0.70, 25.3)	0.118
Arrhythmia on ECG	20	1.792	6.00 (0.75, 48.4)	0.092
Biomarkers				
NT-ProBNP*	20	0.002	1.002 (1.000, 1.005)	0.077
SDMA*	18	0.088	1.090 (0.960, 1.250)	0.190
AGP*	19	0.005	1.005 (1.001, 1.0029)	0.007

*Variable analyzed as a continuous predictor. Bpm, beats per minute;

Table 3.8. Multivariable Cox proportional hazards analysis evaluating the effects of potential prognostic factors for CHF cats.

Variable/level	n*	Parameter estimate ($\hat{\beta}$)	Hazards ratio (95% CI)	P value (Wald)
Signalment				
Age				
<7 years	10	1.482	4.40 (0.39, 49.5)	0.230
≥7 years	9	Referent		
Sex				
Male	13	-0.528	0.59 (0.07, 5.19)	0.634
Female	6	Referent		
Weight				
<4.5 kg	10	3.891	49.0 (1.71, 1402)	0.023
≥4.5 kg	9	Referent		
Cardiac Variables				
LA/Ao ratio†				
≥2	12	4.682	108 (2.66, 4395)	0.013
<2	7	Referent		
Biomarkers				
AGP†				
≥600 (µg/ml)	5	3.695	40.2 (2.53, 641)	0.009
<600 (µg/ml)	14	Referent		

*Six cats had missing data in one or more of the analysed variables and therefore could not be included. †Cut-off for evaluation determined using a ROC curve analysis

3.6 Longitudinal Biomarker Studies in CHF Cats

From August 2016 to August 2017, 12 CHF cats had serial samples taken for biomarker measurements, at the initial visit and then at subsequent revisits, with from 2-5 serial samples collected over a time interval from 7-321 days after the first sample collection date. Six of these cats had died by November 2017 (the data collection stop point), the other six remained alive.

Serial NT-proBNP Measurement in CHF Cats

Overall, the serial NT-proBNP measurements show inconsistent patterns among the 12 CHF cats (Figure 3.2), however cats that died appeared to have higher NT-proBNP values than survivors. Details of measurements and the trend of change in each cat are summarized in Table 3.9. Eight cats had a >1500 pmol/l NT-proBNP measurement at the initial visit, and all cats that died belonged to this sub-population. Knowing that NT-proBNP has high biological variability in cats, a more than 60% of change compared with the previous measurement was considered as a significant ‘increase’ or ‘decrease’ (Harris et al., 2017b). Despite varied revisit intervals and numbers of measurements, at subsequent revisits 67% of the cats that died had an NT-proBNP concentration persistently higher than 1500 pmol/l. In the survivor group, 80% of cats had stable or decreased NT-proBNP concentrations at the 1st follow up time point, with one cat as an exception with a high NT-proBNP value (Cat No.1).

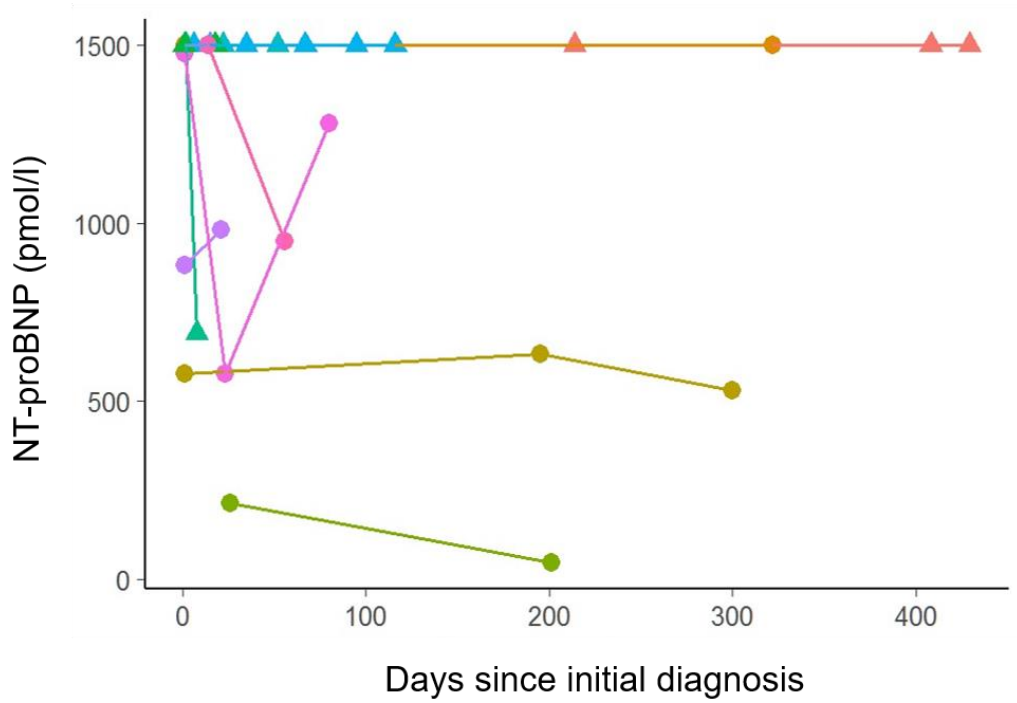

Figure 3.2. Serial NT-proBNP measurements in CHF cats. Serum biomarker changing trends are illustrated along the time axis post initial diagnosis. No consistent patterns were evident in NT-proBNP longitudinal changes between cats, except the majority of cats that died had NT-proBNP values persistently higher than 1500 pmol/l. Each colour represents a different cat; triangles represent cats that died and solid circles represent cats that survived.

Table 3.9. Longitudinal data summary in CHF Cats-NT-proBNP

CHF Survivor (n = 6)							
Cat ID	**Day 0**	**Day 1-14**	**Day 15-30**	**Day 31-90**	**Day 91-180**	**Day 181-365**	**Overall Trend**
No.1	>1500					>1500	Persistently high
No.2	578					633; 530	Stable
No.4	213				45		↓
No.7	882		982				Stable
No.10	1477	578		1282			↓↑
No.12	>1500			950			Stable or ↓
CHF Non-Survivor (n = 6)							
Cat ID	**Day 0**	**Day 1-14**	**Day 15-30**	**Day 31-90**	**Day 91-180**	**Day 181-365**	**Overall Trend**
No.3	>1500			950			Stable or ↓
No.5	>1500	692					Stable or ↓
No.6	>1500		>1500	>1500; >1500	>1500		Persistently high
No.8	>1500		>1500				Persistently high
No.9	>1500	>1500					Persistently high
No.11	>1500		>1500				Persistently high

Serum biomarker changing trends post initial diagnosis were illustrated. Day 0 was defined as the 1st day of blood sampling. For the overall trend summary, a NT-proBNP value >1500 pmol/l is described as ‘high’; a more than 60% positive or negative change from the previous measurement is described as ‘increased’ or ‘decreased’; less than 60% of concentration change from the previous measurement is described as ‘stable’; ↓, decreased; ↑, increased; ↓↑, firstly decreased then followed by an increase; ↑↓, firstly increased then followed by a decrease.

Serial SDMA and Creatinine Measurement in CHF Cats

Similar to NT-proBNP, 11 of the 12 CHF cats had serial measurements of SDMA and creatinine, with 1-4 follow up time points. In terms of trend of change in serial SDMA and creatinine measurement, there were no consistent patterns identified among the individual CHF cats for either of the markers (Figure 3.3).

More than 70% of cats had consistent overall trends of changes between SDMA and creatinine (Table 3.10). When looking at single data pairs, however, discrepancy between SDMA and creatinine interpretation was identified. According to IDEXX laboratory's SDMA and creatinine test reference ranges for detecting renal dysfunction (SDMA >14 ug/dl; creatinine value >165 umol/l), 9 pairs of conflicting data (* in Table 3.10) were identified between SDMA and creatinine. Among these 8 pairs showed abnormal SDMA but a normal creatinine value, the remaining one showed borderline normal SDMA but abnormal creatinine. Six cats were involved with those conflicting data pairs. Four cats were non-survivors and three of them showed high SDMA but normal creatinine at initial assessment.

Additionally, cats in the non-survivor group showed higher values of SDMA; cats with one SDMA measurement >20 ug/dl all died in the current study, regardless of the time point.

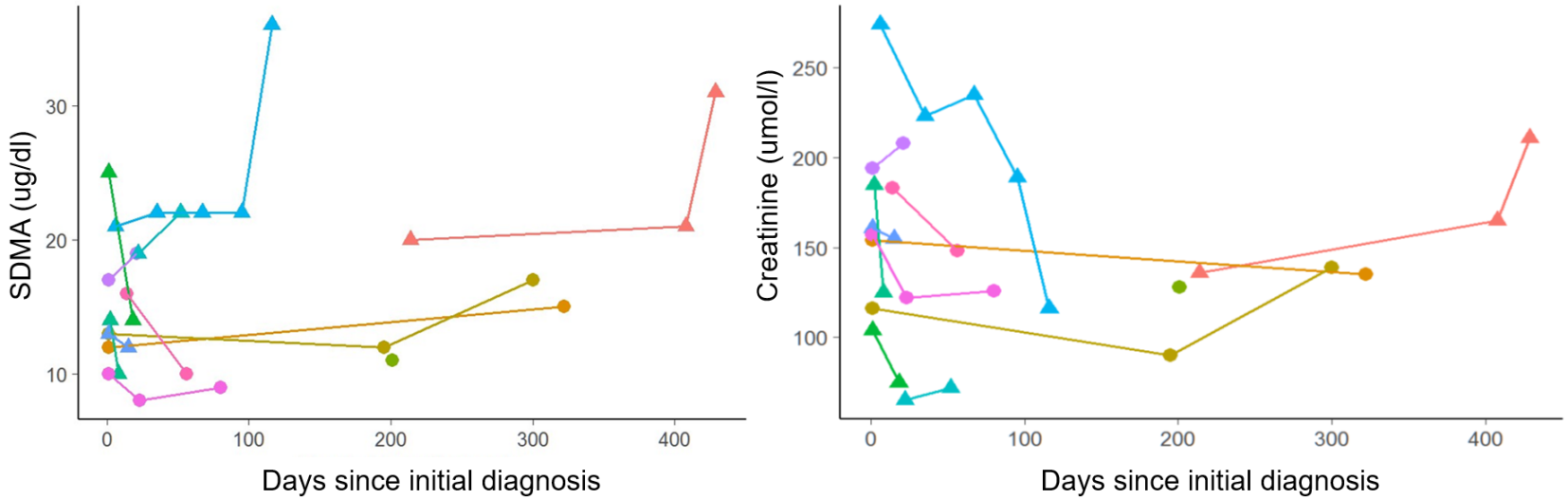

Figure 3.3. Serial SDMA and creatinine measurements in CHF cats. Serum biomarker changing trends are illustrated along the time axis post initial diagnosis. No consistent patterns were evident in either marker's longitudinal changes between cats. Each colour represents a different cat; triangles represent cats that died and solid circles represent cats that survived.

Table 3.10. Longitudinal data summary in CHF Cats-SDMA and creatinine

CHF Survivor (n = 6)															
Cat ID	**Day 0**		**Day 1-14**		**Day15-30**		**Day31-90**		**Day91-180**		**Day 181-365**		**Initial Assessment**		**Overall Trend**	
	SDMA	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**CR**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**
No.1	12	154									*15	*135	Normal	Normal	↑ to high	↓
No.2	13	116									12; *17	90; *139	Normal	Normal	↑ to high	↓ ↑
No.4	/	/							11	128			/	/	/	/
No.7	17	194			19	208							High	High	↑	↑
No.10	10	157	8	122			9	126					Normal	Normal	↓ ↑	↓ ↑
No.12	16	183					10	148					High	High	↓ to normal	↓ to normal
CHF Non-Survivor (n = 6)																
Cat ID	**Day 0**		**Day 1-14**		**Day15-30**		**Day31-90**		**Day91-180**		**Day 181-365**		**Initial Assessment**		**Overall Trend**	
	SDMA	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**	**SDMA**	**Cr**
No.3	*20	*136									*21; 31	*165; 211	High	Normal	↑	↑ to high
No.5	*14	*185	10	125									Normal	High	↓	↓ to normal
No.6	21	274			22	223	22; 22	235; 189	*36	*116			High	High	↑	↓ to normal
No.8	*19	*65			*22	*72							High	Normal	↑	↑
No.9	13	161	12	155									Normal	Normal	↓	↓
No.11	*25	*104			14	75							High	Normal	↓ to normal	↓

Unit: SDMA-ug/dl; creatinine-umol/l. * SDMA and creatinine conflicting data pairs. Cat No.4 Day 0 data missing was due to a technical reason. SDMA >14 ug/dl or creatinine >165 umol/l was defined as 'high'. ID, identification; ↓, decreased; ↑, increased; ↓ ↑, firstly decreased then followed by an increase; ↑ ↓, firstly increased then followed by a decrease.

Serial APPs Measurement in CHF cats

In total, 9 out of 12 cats had follow up samples measured for APP markers. Overall longitudinal changes of each APP were highly diverse among individual cats; no clearly consistent patterns were identified (Figure 3.4). Overall trends of change for each individual APP marker in each cat are summarized in Table 3.11. Each cat demonstrated a unique pattern of APP profile change. There was no apparent difference appreciated between survivors and non-survivors. For each APP change in the whole population: consistent with baseline measurements, PCT was largely undetectable in the longitudinal study with two cats as exceptions. For the rest of the biomarkers, Hp decreased or remain unchanged in the majority (7 out of 9) of cats especially in non-survivors; CRP, ceruloplasmin and LRG-1 decreased in 8 out of 9, 7 out of 9 and 6 out of 9 cats respectively at the 1st follow up time point. AGP and SAA showed diverse changes among individual cats.

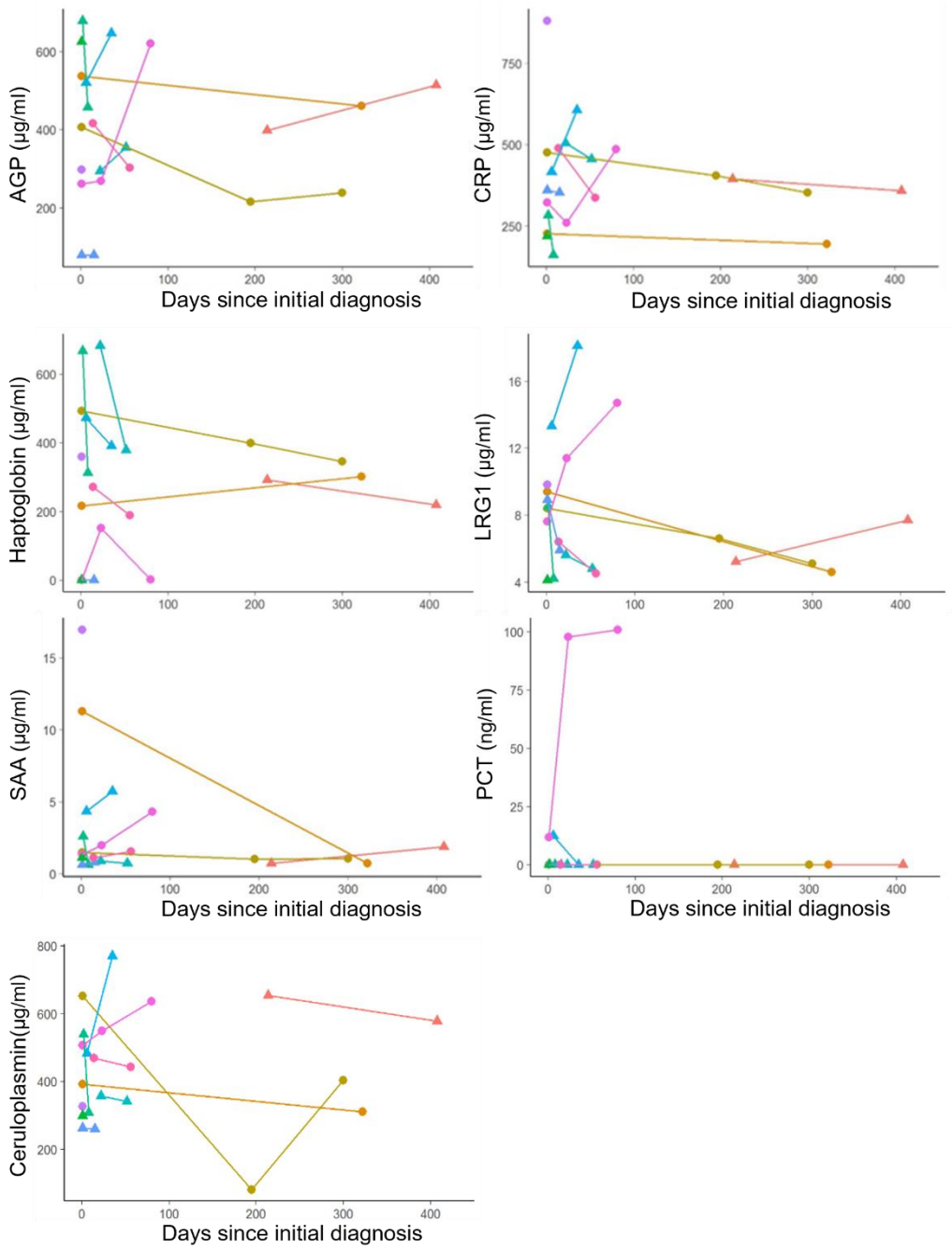

Figure 3.4. Serial APP measurements in CHF cats. Changing trends of seven APPs are illustrated along the time axis post initial diagnosis. In total, 9 out of 12 cats had follow up samples measured for APP markers. For the remaining three cats, two had one initial measurement, which was also included here. The changes of each APP were highly variable. Each colour represents a different cat; triangles represent cats that died and solid circles represent cats that survived.

Table 3.11. Longitudinal data summary in CHF Cats-APPs

CHF Survivor (n=6)							
Cat ID	**AGP**	**CRP**	**Hp**	**LRG1**	**SAA**	**PCT**	**Ceruloplasmin**
No.1	↓	↓	↑	↓	↓	_	↓
No.2	↓↑	↓	↓	↓	↓	_	↓↑
No.4	/	/	/	/	/	/	/
No.7	/	/	/	/	/	/	/
No.10	↑	↓↑	↑↓	↑	↑	↑	↑
No.12	↓	↓	↓	↓	↑	_	↓
CHF Non-Survivor (n=6)							
Cat ID	**AGP**	**CRP**	**Hp**	**LRG1**	**SAA**	**PCT**	**Ceruloplasmin**
No.3	↑	↓	↓	↑	↑	_	↓
No.5	↓	↓	↓	↓	↓	_	↓
No.6	↑	↑	↓	↑	↑	↓	↑
No.8	↑	↓	↓	↓	↓	_	↓
No.9	↓	↓	_	↓	↑	_	↓
No.11	/	/	/	/	/	/	/

Day 0 was defined as the 1st day of blood sampling. Trends of change are summarized for each APP in each individual cat. Note each cat demonstrated diverse changing patterns among APP expression; except in Cat No. 5, all the APPs decreased in subsequent measurements compared with the measurements at the initial time point. Cats No. 4, No. 7 and No. 11 could not have serial APPs measured due to serum sample volume limitations; cats No. 7 and No. 11 had the Day 0 concentration measured but not at subsequent time points. In the APPs longitudinal study, there were 1-2 follow up time points (not including Day 0). ↓, decreased; ↑, increased; ↓↑, firstly decreased then followed by an increase; ↑↓, firstly increased then followed by a decrease.

Chapter 4: Discussion

4.1 CvRD Occurs in Feline CHF

To the authors' knowledge, this is the first study demonstrating CvRD namely $CvRD_H$ to be present in CHF cats with primary CMs. The presence of this disorder is shown by significant over-expression of renal biomarkers SDMA and creatinine, and the cardiac biomarker NT-proBNP in the CHF cats compared with normal controls. Moreover, all three markers were significantly correlated suggesting a close relationship between the two organ systems.

The mechanism of CvRD has been proposed previously in a veterinary consensus statement (Pouchelon et al., 2015). In cardiac decompensation, reduced cardiac output leads to inadequate renal perfusion, activation of neurohormonal responses, endothelial dysfunction and passive venous congestion and these are suggested as potential factors contributing to renal dysfunction (Pouchelon et al., 2015). Renal insufficiency has been reported to be a common comorbidity in human heart failure (McClellan et al., 2002, Akhter et al., 2004). CvRD has been reported in dog mitral valve disease, particularly associated with CHF. The severity of heart disease was found to be significantly correlated with the severity of chronic renal disease, moreover the presence of CvRD was associated with poorer survival (Martinelli et al., 2016). Although CvRD has not been reported in feline CHF, renal azotaemia is known as a common feature in feline heart disease. One previous study showed that in a HCM cat population, the prevalence of azotaemia was around 60% (Gouni et al., 2008). Apart from the cardio-renal interplay, therapeutic agents for CHF, notably loop diuretics and ACE inhibitors are also thought to induce renal dysfunction through their pharmacological effect on intra-renal haemodynamics (Orvalho and Cowgill, 2017).

Considering the high prevalence and significance of CvRD in CHF, monitoring renal function is essential especially for medically treated patients. Ideally renal function should be evaluated by measuring GFR, however the technique is not very practical in small animal medicine. Traditionally creatinine is used for renal functional staging. One major drawback of creatinine is that it only shows a significant increase in primary renal disease when there is 75% nephron functional loss (Relford et al., 2016). Moreover, it may be affected by a number of biological variables such as age and lean body mass (Miyagawa et al., 2010, Hall et al., 2014b). Novel renal markers with better sensitivity and specificity need to be developed in small animal medicine.

SDMA is a metabolite of amino acid arginine methylation (Kakimoto and Akazawa, 1970) and, like creatinine, it is associated with GFR in cats (Hall et al., 2014b) but is more sensitive in detecting early renal changes. In the current study the median SDMA level was found to be increased in CHF cats but the median creatinine level remained in the reference range; 61% CHF cats showed evidence of reduced GFR by increased SDMA while only 41% of the same population had increased creatinine. These findings were consisted with Hall and others' study in 2014, in which SDMA was shown to increase when there was 25% loss of GFR and its detection of renal dysfunction was 17 months earlier than creatinine (Hall et al., 2014a). In a recent study on feline hyperthyroidism evaluating the ability of SDMA to detect 'masked' early renal disease without azotaemia, the sensitivity and specificity of SDMA was reported to be 33% and 97% respectively (Peterson et al., 2018). In the most recent IRIS CKD guidelines, SDMA was included as a complementary test to creatinine for CKD staging (http://www.iris-kidney.com/pdf/3_staging-of-ckd.pdf). In Hall and others' study in 2014 (Hall et al., 2014b), SDMA appeared to increase in older cats with low GFR; in this study however, there was no age correlation with SDMA. It should also be noted that SDMA can increase in pre-renal azotaemia associated GRF reduction (such as dehydration and general anaesthesia), therefore when using SDMA as a renal functional marker, other clinical information should be considered for SDMA interpretation.

Renal dysfunction is an independent prognostic factor in human heart failure (Hillege et al., 2000; Hillege et al., 2006). Early recognition of kidney injury is essential for preserving kidney function. Therefore, SDMA should be used in feline CHF management, where early detection of renal impairment allows the clinician to adjust the treatment protocol.

NT-proBNP is a useful marker in feline cardiology; the main function of NT-proBNP, which is released with myocardial stretch, is to protect against RAAS activation (Volpe et al., 2016). Previous studies suggested it can distinguish cardiogenic respiratory stress from primary respiratory disease and also differentiate different stages of cardiomyopathies (Borgeat et al., 2015a). Consistent with findings in the literature (Connolly et al., 2008; Wess et al., 2009; Fox et al., 2011), in the current study NT-proBNP clearly differentiated CHF cats, occult CM cats and healthy controls. NT-proBNP was positively correlated with left atrial size; this has been reported by Connolly and others (Connolly et al., 2008) and can be explained by increased myocardial stretch in the left atrium during the disease process. More interestingly, NT-proBNP was also found to be negatively correlated with left ventricular systolic function; it has been shown that low left ventricular systolic function is associated with decreased survival time

in feline HCM (Payne et al., 2013). Together with positive correlations observed between NT-proBNP and CHF severity, these indicate that high NT-proBNP might be associated with poorer outcome in feline CM. Indeed, in this study both NT-proBNP and SDMA were significantly higher in CHF cats that died compared with survivors, which suggested both markers potentially could predict survival, although neither remained significant in univariate or multivariate survival analyses models. The prognostic value of NT-proBNP is less investigated in cats compared with humans. A previous study had similar findings to ours in that NT-proBNP seemed to be a promising prognostic marker but did not show statistical significance in the multivariate survival analysis (Borgeat et al., 2014b). Investigation of the prognostic value of SDMA in small animal heart disease is very limited. One previous cat study compared SDMA expression in feline HCM, CKD and diabetes mellitus. SDMA seemed to be irrelevant in feline HCM, although the cardiac cat population used in that study was largely asymptomatic (Langhorn et al., 2017). In the current study, there were no significant SDMA changes between the asymptomatic CM cats and healthy controls. In a recent dog study, it was found to be increased in advanced stages of CHF in DMVD dogs (Choi et al., 2017). It should be noted that apart from reflecting GFR as a renal function marker, SDMA may play a role as a pro-inflammatory factor associated with endothelial dysfunction in feline CHF, based on previous human evidence (Schepers et al., 2011). The role and significance of SDMA in feline CM and CHF requires further investigation. Interestingly NT-proBNP is mainly eliminated by the kidney, thus the increased NT-proBNP may be a consequence of renal dysfunction rather than reflecting heart disease severity (Lalor et al., 2009), which further complicates the story. Future studies are necessary to clarify the prognostic characteristics of NT-proBNP and SDMA in feline CHF. It should be noted that, as all the CHF cats had loop diuretic treatment at entry to the study, this would have an effect on biomarker expression particularly on the renal markers; this should be considered when interpret the results. However, regardless of whether treated for heart failure or not, elevated SDMA and creatinine should not be ignored in CHF cats.

In summary, increased SDMA and creatinine supported CvRD being present in feline CHF. The results suggested SDMA should be added to kidney function monitoring protocols in CHF management. NT-proBNP and SDMA may have prognostic potential in feline CHF.

4.2 Inflammatory Biomarker APPs Increased in Feline CHF

In this study seven positive APPs were screened as biomarkers of systemic inflammation. Four of them were found to be linked with feline CHF. LRG1 and SAA were significantly increased in CHF cats compared with control cats; ceruloplasmin was significantly higher in CHF cats compared with asymptomatic CM cats; serum AGP level was found to be associated with CHF cat survival. Moreover, those APPs showed significant positive correlations with cardiac biomarkers, echocardiographic variables such as LA size and CHF severity based on the ISACHC system. All these findings suggest systemic inflammation plays a role in feline CHF.

Although inflammation has been recognized in heart failure patients for a long time, the mechanism was not well defined (Dick and Epelman, 2016). A recent review suggested that a reciprocal relationship might exist between heart failure and inflammation (Van Linthout and Tschope, 2017). Circulating inflammatory mediators such as APPs have been found to be predictive of adverse outcome in a number of human heart failure studies (Dick and Epelman, 2016). APPs by definition are a group of by-products released during acute phase reactions in response to pathological insults, helping to restore physiological haemostasis (Ceron et al., 2005). They are highly sensitive in response to tissue injury but lack specificity, therefore a multiple APP profile based approach is considered more accurate than single marker based investigation (Ceron et al., 2008). This pilot study is the first report using a multiple APP screening approach to demonstrate their involvement in feline CHF and our findings potentially indicate directions for future research, especially in improving understanding of the pathogenesis of this complex syndrome.

There were discrepancies in the APP expression patterns in the CHF group. For example, LRG-1 and ceruloplasmin were significantly higher in CHF cats, whereas CRP and Hp were not significantly different in CHF cats. Apart from knowing that there are species differences in APPs (Ceron et al., 2005), the differential expression of the APPs may also suggest that potential pathophysiological pathways differ in feline CHF. In particular, the correlations of LRG1, ceruloplasmin, SAA and AGP with cardiac biomarkers and echo variables such as left atrial size indicate their specific cardiac involvement, rather than purely serving as systemic inflammatory markers.

LRG1 is a relatively novel APP in cardiovascular research. The detailed biological function of LRG1 is not very clear. It has been found to be associated with inflammation, neutrophil differentiation and may potentially play a role in angiogenesis and myocardial fibrosis by

interacting with TGF-β (Song and Wang, 2015; Kumagai et al., 2016). It was found to be increased in human diastolic heart failure, independently detected heart failure and was found to be superior to BNP (Watson et al., 2011). In an experimental induced myocardial infarct study in mice, LRG1 was shown to have a protective function against adverse cardiac remodelling (Kumagai et al., 2016). In the current study, the positive correlations between LRG1, left atrial size and NT-proBNP suggested an intrinsic cardiac involvement of LRG in feline CHF and CM. The specific role of this marker in the disease requires further investigation.

Ceruloplasmin is a copper transporter involved in iron detoxification. It also has multiple links with oxidative stress (Ceron et al., 2005; Cabassi et al., 2014). In humans, this APP was found to predict adverse cardiovascular events (Ahmed et al., 2012) and it has been shown to associate with heart failure development and can independently predict all causes of mortality in heart failure patients (Kaya et al., 2013; Hammadah et al., 2014). In the current study ceruloplasmin was significantly higher in CHF cats compared with asymptomatic CM and healthy controls and it was positively correlated with CHF severity. Similar to LRG-1, veterinary data on ceruloplasmin are very limited. One interesting feline heart worm study found ceruloplasmin was significantly increased in symptomatic cats that were seropositive for *Dirofilaria immitis* (Silvestre-Ferreira et al., 2017).

SAA is a small serum hydrophobic apolipoprotein, traditionally considered to be secreted in the liver, but more recent research suggests it can also be produced by adipose tissue and it may play a role in adipose inflammation (Yang et al., 2006). It has been considered as an inflammatory marker in cats for more than a decade and it is the most rapidly responsive major APP in cats (Sasaki et al., 2003). It has been found to increase in sick cats, hospitalized cats and cats with various disease conditions (Kajikawa et al., 1999; Kann et al., 2012; Tamamoto et al., 2013). One study suggested SAA has independent prognostic value in diseased cats with any diagnosis (Tamamoto et al., 2013). In the current study, SAA differentiated the CHF cats from asymptomatic CM cats and healthy control cats but failed to differentiate asymptomatic CM from healthy cats. This suggests SAA up-regulation particularly associated with feline CHF rather than compensated CM. Considering all the CHF cats were in advanced stages of heart failure i.e. ISACHC III and more than a third of the CHF cats required hospitalization at the time of blood sampling, it is not surprising SAA increased in this population. However, the specific connection of SAA with feline heart disease remains unclear. A recent research abstract reported SAA was significantly increased in asymptomatic feline HCM, specifically in 11 cats with generalized left ventricular hypertrophy, which indicated an involvement of

SAA in the early stages of feline CM (Van Hoek et al., 2018a). In humans, SAA has been associated with atherosclerosis and in a selected study population with coronary artery disease, it turned out to be an independent predictor for adverse cardiovascular events (Johnson et al., 2004). In Kann et al, 2012's study, age has been pointed out as a factor that can contribute to SAA increase (Kann et al., 2012), in the current study, a weak correlation was found between age and SAA level. Our healthy controls were significantly younger than the CHF and asymptomatic CM cats, but there was no significant difference in SAA expression between healthy control and asymptomatic CM, thus age is unlikely to have a dominant effect. Similarly, a gender influence was pointed out in the same study, but it was not evaluated in this study.

AGP is one the three best studied APPs in cats and it is well known as being useful for discriminating FIP (Paltrinieri et al., 2007; Hazuchova et al., 2017). In this study, serum AGP was not found to be significantly elevated in the CHF cats compared with controls, however a strong correlation with cTnI and a weak correlation with NT-proBNP was also identified for serum AGP, which suggested a potential cardiac connection. A more exciting finding was in hazard risk analyses, where it seemed to be an independent risk predictor for all causes of mortality in the CHF cat population. In humans, this APP has been shown as an independent prognosticator for cardiovascular mortality as well as all causes of mortality (Fischer et al., 2014), although in a later study it was suggested that AGP was not superior to CRP in predicting all causes of mortality (Singh-Manoux et al., 2017). A recent cat study investigated the value of AGP in distinguishing pleural effusions caused by feline FIP, cardiac disease or neoplasia. Lower AGP expression, together with lower Hp and SAA was found in cardiogenic effusion cases compared with infectious disease and neoplasia. A limitation of that study was that no healthy controls were included (Hazuchova et al., 2017).

CRP is the most commonly studied major APP in humans and in the dog, however its value seems limited in feline patients (Ceron et al., 2005). In human medicine, it is well accepted as a prognostic indicator for predicting mortality risk in heart failure patients (Singh-Manoux et al., 2017). Previous dog studies also showed CRP to be increased in CHF and its elevation was associated with severity (Cunningham et al., 2012; Domanjko Petric et al., 2018). In the current study it did not detect any link between CRP and feline CHF.

Hp is considered to be a moderate APP in cats (Ceron et al., 2005). Biologically it is involved in detoxification of haemoglobin as well as being an inflammatory reactant (Wassell, 2000). A specific Hp genotype in humans has been found to be associated with cardiovascular risk in

diabetes mellitus (Suleiman et al., 2005). In a large scale prospective human study, Hp was found to be a risk factor in heart failure (Holme et al., 2009). Its significance in veterinary cardiovascular diseases is not well established. In one canine DMVD study, Hp elevation was found in dogs that developed CHF (Polizopoulou et al., 2015).

PCT is an acute phase reactant most commonly associated with sepsis (Giunti et al., 2006), but there is no available cat data for this APP. In this study, the PCT assay was developed for canine use, the species differences in this marker between dogs and cats are unknown. The fact that PCT was largely undetectable in the current study population could therefore be either a genuine biological feature or due to a technical limitation. PCT has been recently linked with heart failure in humans (Canbay et al., 2015; Mockel et al., 2017). In a small case controlled retrospective study, it showed reasonable sensitivity and specificity for CHF diagnosis (Canbay et al., 2015).

The APPs provided valuable information for feline CHF, however the findings should be interpreted with caution for the following reasons: Firstly, APPs are well known as sensitive biomarkers but very non-specific. In this study, relatively strict exclusion criteria were used, however as most CHF cats were relatively old and commonly had multiple comorbidities, the possibility of occult comorbidity cannot be eliminated, which therefore might affect the current results. Secondly, biological variables may affect the APP expression; for example, both LRG and SAA showed a weak positive correlation with age in the current study. Given the fact cats in the CHF group were significantly older than healthy controls, age effects might have contributed to the differential expression between CHF cats and healthy controls for these two markers. More studies are necessary to clarify the effect of biological variations in APP expression. An age matched control population would be ideal to use for consolidating current findings on these two APPs. Lastly, most feline APP tests are not currently standardised, which results in inconsistent cut-off values between different techniques (Kann et al., 2012). In the current study the hazard analysis model predicted AGP >600 µg/ml had significantly higher risk than cats with lower AGP values. Based on the results, AGP is a promising risk predictor for feline CHF, however it is not recommended using this marker as a sole test of feline CHF prognosis at this stage, particularly in using the 600 µg/ml cut-off for clinical prognostic assessment when a different test method is used. APP assays used in this study were validated in cats. Test standardization is essential to establish in the future for using a specific APP for disease diagnosis and prognosis.

In summary, an inflammatory response appears to play a role in feline CHF and LRG1, SAA, ceruloplasmin and AGP may be used as novel biomarkers for feline CHF. The detailed role of these APPs in feline CHF requires further investigation, but they might help to understand the disease pathogenesis and be potentially useful for disease prognosis in combination with other clinical assessments.

4.3 The Relationships Between CvRD biomarkers and APPs

In this study, it has shown that there were correlations between the CvRD biomarkers and APPs, which suggested CvRD and the inflammatory process may potentially interact with each other in feline CHF. The significant correlations between the two panels of markers included: NT-proBNP positively correlated with AGP, LRG, SAA and ceruloplasmin; cTnI had strong positive correlations with AGP and Hp; SDMA had moderate positive correlations with CRP and Hp and creatinine positively correlated with CRP, LRG1 and SAA.

The complex relationship between CvRD and inflammation is yet to be fully understood. Possible mechanisms include RAAS response and activation of cellular immunity (Schefold et al. 2016). A recent study showed systemic inflammation plays a role in feline chronic renal disease (Javard et al., 2017).

It should be noted that some biomarkers may have additional functions besides serving as a marker of CvRD or inflammation. For example, apart from reflecting renal function, SDMA is also considered to play a part in the NO pathway and endothelial dysfunction (Dimitrow et al., 2007; Cunningham et al., 2012; Mommersteeg et al., 2016) and serve as a pro-inflammatory factor in kidney disease (Schepers et al., 2011). Further investigations on specific biomarker function would be necessary to define their precise roles in heart disease.

4.4 Feline CM Phenotype and Biomarker Expression

One interesting finding in this study was that feline CM phenotype did not affect the expression of any investigated biomarker. This information is valuable for future feline CM studies, as these biomarkers can potentially serve as universal tools in studying different types of cardiomyopathy.

It is well accepted that the current feline CM phenotypic classification system is equivocal. As the classification system is based on anatomical change and the disease course is dynamic, sometimes it is difficult to reach a definitive diagnosis of CM subtype based on echocardiography (van Hoek et al., 2018b). This is particularly a challenge in CHF patients. In this study, heterogeneity was observed in CM diagnosis, with more diverse phenotypes in the CHF than in the asymptomatic group. Once a cat develops CHF, regardless of the primary CM phenotype, the treatment strategy is similar. In terms of disease prognosis however certain CM types are reported to have a worse prognosis than others (Pion et al., 1992; Ferasin et al., 2003). The equivocal classification system may hinder accuracy of disease prognosis. Most previous studies have focused on HCM which is the most common and readily recognized phenotype, while limited work has been done on RCM, UCM or ARVC. Findings in HCM studies may not be always translatable to other types of CM.

Using serum biomarkers can potentially overcome the above limitations related to CM diagnosis and research focus, as they have less bias and thus may give a more accurate reflection of disease status and progression.

4.5 Clinical Examination Parameters Can Be Helpful Tools for Monitoring Feline CHF

Apart from serum biomarkers, some relevant clinical parameters were also evaluated in the diseased population. Some of the cardiovascular physical examination parameters can be useful for disease monitoring and prognosis.

Respiratory rate during physical examination was found significantly higher in CHF cats than in asymptomatic CM cats in the current study. Considering the 'white coat' effect during hospital visits (Quimby et al., 2011), respiratory rate is a more valuable monitoring tool in cats for CHF surveillance at home. Previous reports suggested a sleeping or resting respiratory rate persistently higher than 30 breaths per minute at home should be investigated for potential progression from sub-clinical heart disease to decompensated heart failure (Ljungvall et al., 2014). The same cut-off also can be used to assess whether CHF is well controlled in cats (Porciello et al. 2016). In a recent study investigating feline RCM, respiratory distress was identified as an independent poor prognosticator in RCM which emphasizes the importance of this physical variable in cardiac disease clinical management (Locatelli et al., 2018). Arrhythmia and gallop sounds detected by cardiac auscultation showed potentially prognostic value for feline CHF in this study. These findings were consistent with previous studies (Smith and Dukes-McEwan, 2012; Payne et al., 2015a). Audible gallop sounds have been considered a relatively specific indicator for feline cardiac disease; in CHF cats, the prevalence was reported to be approximately 20-50% (Goutal et al., 2010; Smith and Dukes-McEwan, 2012; Dickson et al., 2018). Other physical examination parameters such as heart rate and heart murmur were not significant prognostic factors in feline CHF in the current study.

Another significant physical examination parameter which potentially has prognostic value is body weight. Body weight was an independent risk predictor in CHF cats in the final multivariate analysis. CHF cats with a body weight less than 4.5 kg had at least 1.7 times higher risk of death than those heavier than 4.5 kg. In humans, loss of weight and body lean mass are significantly associated with poorer outcome in chronic heart failure (Pocock et al., 2008). In Finn and others' 2010 study, body weight was a significant predictor of survival time in CHF cats (Finn et al., 2010). Weight loss associated with cardiac cachexia is considered to be a logical explanation for poorer survival, however it was suggested in the same study that both highest and lowest body weight may be associated with poorer clinical outcome in CHF cats (Finn et al., 2010). Balanced nutrition management and having a weight target maintained in

the normal physiological range is probably the most sensible strategy for feline chronic disease patients (Freeman et al., 2016).

The value of echocardiographic parameters in feline CM prognosis has been well recognized in previous studies (Peterson et al., 1993; Fox et al., 1995; Payne et al., 2010; Payne et al., 2013). Consistent with most of the previous reports, CHF cats in this study had more severe LA enlargement and poorer LV systolic functions than asymptomatic CM cats; moreover, these parameters were significantly higher in CHF cats that died, suggesting the two parameters correlated with disease severity. LA/Ao ratio particularly remained significant in both univariate and multivariate survival analyses. LA/Ao ratio higher than 2:1 was an independent risk factor in CHF cats. This finding fits the current clinical impression that LA remodelling has generally been considered as a very important marker for disease severity and progression in feline CM (Payne et al., 2013).

In summary, several clinical variables showed prognostic value for feline CHF. With more advanced diagnostic techniques becoming available in small animal cardiology, the power of the basic cardiovascular examination should not be neglected (Dickson et al., 2018). A combination of objective clinical parameters and biomarkers could be very helpful for disease risk assessment.

4.6 Longitudinal Biomarker Studies in CHF Cats- What Did We Learn?

In the second part of this study, longitudinal investigations were carried out in 12 CHF cats to assess the biomarker changes. Preliminary data were obtained, and no definitive conclusion can be drawn at this stage, but findings suggest some directions for future work such as that temporal monitoring of some biomarkers can potentially predict survival in CHF cats.

Persistently high NT-proBNP (>1500 pmol/l) appeared to be a risk factor in feline CHF. At the initial visit, eight out of 12 CHF cats had serum NT-proBNP >1500 pmol/l, six of these died within 321 days. Over 65% of the cats that died had persistently high NT-proBNP at subsequent visits. This suggested high NT-proBNP may serve as a disease severity alert in CHF. NT-proBNP has been used for heart disease prognosis in human medicine; as mentioned previously, its prognostic value in feline heart disease has not been well established. One recent longitudinal study in CHF cats showed a better outcome was linked with a greater reduction of NT-proBNP during hospitalization (Pierce et al., 2017). The challenge of NT-proBNP study design is the high biological variability in the cat. A 39.8% daily variability and 60.5% weekly variability has been reported to occur in adult healthy cats, for longitudinal assessment only a change above 60% should be considered as significant (Harris et al., 2017b). This should be considered in study design and data interpretation.

The longitudinal study of SDMA further confirmed the baseline study finding. It increased earlier than creatinine and high SDMA was associated with poor outcome. Although SDMA and creatinine values were compatible in most cases, nine pairs of conflicting data were spotted between SDMA and creatinine, among which eight pairs showed abnormal SDMA but normal creatinine. The longitudinal study showed SDMA detected renal injury earlier than creatinine in chronic CHF management. Cat No. 3 in the non-survivor CHF group is one classic example. At initial diagnosis the cat had a high SDMA value but normal creatinine value. After at least six months, creatinine started to increase. According to IDEXX recommendations, SDMA greater than 20 ug/dl requires immediate action (www.idexx.com/SDMAalgorithm). In the current SDMA temporal follow up, cats that had SDMA higher than 20 ug/dl all died regardless of measuring point. This further emphasized that renal protection should be carried out promptly when there is diagnostic evidence of kidney dysfunction. A previous study has shown SDMA started to increase when there was 25% glomerular damage. From the current results, it seems the renal injury at this stage is potentially reversible. Two cats (Cat No. 11 and Cat No. 12) demonstrated significant reduction of SDMA over time. In summary, preliminary data suggests longitudinal monitoring of SDMA is beneficial for heart failure management. It is a

valuable alternative kidney function marker to creatinine in CHF management, especially in cases where body lean mass is low.

The APP profile measurements were also tracked in a longitudinal manner. In general, the pilot data suggested most APPs seem to decrease over time, but APP changes in individual cats were very heterogeneous. Each cat also demonstrated a unique pattern of APP profile change. This reflected the sensitive and dynamic nature of serum APP expression. The longitudinal study of APPs was particularly challenging because: (1) they are known to be very sensitive biomarkers; (2) their full biological functions are not clear; (3) biological variability may affect interpretation; (4) they might be affected by occult/concurrent inflammatory conditions so their changes may be non-specific; (5) the timing of sample collection may be critical as major APPs peak and decline rapidly after insult so inconsistent long term time points will inevitably cause inconsistent results. There is a need for APP longitudinal studies (Eckersall and Schmidt, 2014); they could be used as valuable surveillance tools in the future and it would be worth looking at the correlations between clinical signs and patient's biomarker levels in a temporal manner (Tamamoto et al., 2009). For example, it would be interesting to assess if there is correlation between relapse of CHF and APP changes at subsequent re-visits compared with initial measurements. This future direction will potentially assist more advanced understanding of the role of APPs in feline CHF. A larger scale study, with fixed assessment time points associated with a particular CHF event would be helpful for assessing APP's role in surveillance and prognosis.

Apart from the small number of CHF cats in the study (n = 12), there were other limitations in the current longitudinal study design. The varied sampling points were not expected in the original study design. In the research proposal, fixed time points-Day 0, Day 14, Day 90 and Day 180 were planned for longitudinal data sampling, however, owners' compliance with scheduled revisit times was very poor. In a future study it may be worth thinking of ways to improve this. The discrepancy of initial diagnosis and initial sampling dates in several animals confused survival analyses; ideally the two dates should be identical. Additionally, short term changes may be worth evaluating prior to a long term dedicated study. For example, biomarker levels at admission compared with at discharge will give information on how biomarkers respond to changing disease status. More work with a larger cat population and more comprehensive time points are needed for a longitudinal study.

4.7 Limitations

There are several limitations in the current study; based on their associations, they can be divided into three aspects: study population, experimental techniques and study design.

In terms of study population, firstly it is a small-scale study with clinical cases collected in one cardiology referral service. The study population may not be fully representative, although the study population characteristics showed classic features of feline CM which were reported by previous relevant studies. A statistical power calculation was not performed in the current study, as it was unrealistic to achieve due to limited sample collection time and availability. Although the current sample number is justified for statistical comparisons, the small sample size could affect statistical power in particular in the survival analysis model. Power calculation would worth performing in the work arising from this pilot study. Due to the limitation of the current CM classification system and the dynamic nature of disease progression, accurate diagnosis of CM phenotype was difficult to achieve; both intra-observer and inter-observer agreement may be inconsistent. To best overcome this issue, the final phenotypic classification was ratified by one senior ECVIM boarded cardiologist. Despite the classification issue, in the current study there were no significant differences in targeted biomarkers among different CM diagnosis categories.

The exclusion criteria were mainly based on clinical records, which was not ideal particularly for ruling out renal disease and inflammatory comorbidities. Ideally complete urinalysis should be performed to look for inappropriate urine specific gravity, proteinuria or other evidence of kidney disease. Occult inflammatory conditions were challenging to detect unless all the candidate cats went through exhaustive clinical diagnostic testing, which was unrealistic to achieve in the current study. The known comorbidities of the study population might have effects on biomarker expression; for example two cats in the asymptomatic group had mild periodontal disease, which may potentially affect the inflammatory marker expression. Ideally these cats should be excluded. Another flaw of the current study was that the systolic BP record was missing in 11 CHF and three asymptomatic CM cats. Systemic hypertension was one of the exclusion criteria in this study; unfortunately not all the cats had a record available, which is not an uncommon scenario in the clinical setting. Therefore the exclusion for hypertension was performed as far as was practically possible. The reasons for missing BP values include cats being too stressed for BP measurements at presentation, but having no other clinical signs indicating systemic hypertension, or cats had a BP measured by the referring veterinary surgeon. Also most of these cats were younger than eight years, when they were considered

less likely to be hypertensive (Borgeat et al., 2014b). However, systemic hypertension cannot be completely ruled out in those cats.

Another limitation of the current study population is that the effect of medications on biomarker expression was not assessed. This was an issue particularly for the CvRD study, as it is known that loop diuretics can affect renal function, which can contribute to CvRD biomarker elevation. It should be noted that the effect of loop diuretics on renal function in CHF patients is like a "double edged sword": effective decongestion with diuretics would be beneficial for the kidney (Orvalho and Cowgill, 2017). Ideally an untreated CHF population should be used to assess CvRD; however, this is difficult to achieve in a cardiology referral clinic. Also, from a practical point of view, regardless of whether medical therapy was used or not, kidney function monitoring should be addressed in CHF cats.

Lastly, the serum samples from healthy control cats used in the current study were obtained from a commercial source; therefore, the full clinical history of these cats was not accessible. Echocardiography was not performed in this group, although the serum NT-proBNP test results suggested that occult heart disease was unlikely. The presence of other non-cardiac occult disease cannot be ruled out, however. In fact, the sporadic high values in SDMA and several APPs in some cats might indicate the presence of underlying conditions, but this pitfall is less likely to confound the current conclusions. Another issue with the healthy control population was that the cats were significantly younger than the CHF and asymptomatic groups. The age effect on biomarker expression cannot be eliminated in the current study.

One major limitation related to serum samples was retrospective acquisition in some animals. For the baseline APP study, nine serum samples were selected retrospectively from service archives. These samples were originally collected in 2012-2014 and long term storage effects on biomarker expression was not evaluated in the current study. Inadequate serum sample volume was another limitation; three candidate cats could not have biomarkers measured due to this issue. In future studies, fluid from a body cavity (e.g. pleural effusion) could be considered as an alternative for systemic biomarker measurement (Hazuchova et al., 2017), which may be easier to collect, especially in critical patients. For NT-proBNP tests, values were not completely accurate in the CHF group and in healthy controls due to the limitation of the assay detection range (24-1500 pmol/l), resulting in the median value in CHF cats being underestimated and in healthy controls possibly overestimated. Also partial NT-proBNP data were collected retrospectively (nine cats collected from 2012-2014). As exactly the same

techniques were used and both were performed in IDEXX Laboratories, the results should be compatible with the new test results from 2017. The APP tests used in the current study were newly developed commercial assays. So far, feline APP tests lack standardization; tests performed with different methodology in different laboratories may give different values (Sasaki et al., 2003). Therefore, the current APP cut-offs should be interpreted with caution for future studies if a different assay is used. Also, a dog PCT assay was used in the current study. Knowing that APP expression may be species specific, it is difficult to clarify whether the varied PCT expression in the current study was a genuine biological feature or due to the species difference. Additionally, the timing of measuring the APPs would in theory affect the absolute values of current measurements. This is particularly the case for major APPs such as SAA and AGP. The cut-off values may be potentially underestimated for those markers.

Limitations were also present in study design particularly for survival analysis. Some cats were euthanized rather than dying naturally, so survival days may not accurately reflect the disease severity, as multiple factors are involved in making a euthanasia decision, such as owner's choice. Also, it should be noted there was a discrepancy between initial diagnosis and initial blood sampling dates in some cats. Cats that have been controlled with long term medication prior to first biomarker measurement may benefit from medication and this might therefore mask the intrinsic disease status. Other limitations for the longitudinal study design are described in Section 4.6.

Overall, the current study did suggest SDMA, LRG-1, SAA, ceruloplasmin and AGP could be promising novel biomarkers for feline CHF and CM. Given the discussed limitations of this pilot study, future work needs to be carried out for further validation.

4.8 Final Discussion

Feline CMs have been recognized for more than 40 years and, the understanding of the disease has improved dramatically especially in the last two decades. Prevailing research has focused on understanding disease pathogenesis, developing and optimising methods for more cost-effective diagnosis, management and prognosis. A biomarker study can potentially meet all the above criteria and provides an opportunity to see the disease from a broader perspective.

This study focused on two mechanisms which were hypothesized to be associated with feline CMs particularly in the CHF stage: CvRD and systemic inflammation. By evaluating the two panels of serum protein biomarkers, it was aimed to answer the following questions: (1) Are these two pathophysiological processes involved in feline CM, particularly with CHF? (2) If yes, can any of these biomarkers be useful for disease diagnosis, management and prognosis?

The theory base of CvRD is that in heart disease renal perfusion is compromised secondary to low cardiac output and in the acute decompensation scenario, it can cause acute renal injury. The neurohormonal activation of the RAAS and SNS which temporarily compensate for reduced renal perfusion can in the long term cause adverse consequences (Ronco et al., 2008). Cytokines released during endothelial injury and passive renal congestion can also cause oxidative injury of the kidney (Pouchelon et al., 2015). Cardiac therapeutics such as loop diuretics also lead to GFR reduction and further impair kidney function (Francis et al., 1990; Lazzarini et al., 2012). This type of 'Cardiorenal Syndrome' has been firstly established in human medicine and more recently has drawn veterinary attention. The term cardiovascular–renal axis disorder i.e. CvRD was proposed in small animal medicine in 2015 by a group of veterinary cardiologists and nephrologists (Pouchelon et al., 2015). Studies in the small animal cardiology field since then include a few reports on chronic mitral valve disease (Martinelli et al., 2016; Choi et al., 2017). To the author's knowledge, this is the first reported study investigating CvRD in cat cardiac disease.

In the current study, renal functional marker SDMA and creatinine were significantly higher in CHF cats compared with controls. Cardiac biomarker NT-proBNP was found increased in CHF cats compared with CM cats without clinical signs and healthy cats in a reciprocal manner. High SDMA and NT-proBNP appeared to associate with poorer outcome in feline CHF. The significant correlations of renal biomarker with cardiac biomarker NT-proBNP supported the hypothesis that there was cardio-renal interaction in CHF.

The recognition of CvRD in feline CM with CHF is essential, as this would help cardiologists to make clinical decisions on whether and when action on protecting renal function should be carried out. Renal dysfunction is a poor prognostic indicator in human heart failure (Hillege et al., 2000; Hillege et al., 2006), earlier recognition of renal impairment in heart failure patient is therefore important, especially when the renal change is reversible. Knowing CvRD has developed in certain cardiac patients, clinicians can tailor those patients' medical treatment protocol and add extra consideration for kidney protection. In other words, it allows more elaborate control in balancing the two organ systems, which potentially can improve the long-term outcomes (Orvalho and Cowgill 2017). Traditionally, serum creatinine level was used for kidney disease grading, for both AKI and CKD. However, as a renal biomarker, neither sensitivity nor specificity of creatinine is ideal (Yerramilli et al., 2016). SDMA is a novel renal biomarker and became popular in veterinary medicine recently (Relford et al., 2016). In this study, SDMA appeared to be a more sensitive renal biomarker than creatinine, therefore it should be added to a renal function monitoring panel in feline CHF management. It should be noted that both SDMA and creatinine are considered as renal functional markers. To detect early renal injury, more sensitive renal biomarkers are needed. Currently, investigated renal injury markers in veterinary medicine include NGAL, inosine, clusterin, cystatin B, kidney injury molecule-1, NAG and retinol binding protein (Lapointe et al., 2008; De Loor et al., 2013; Bland et al., 2014; Chacar et al., 2017; Orvalho and Cowgill, 2017). These could be useful to detect earlier stages of CvRD in the future, and address the kidney issue when it is in an earlier reversible stage.

One limitation in the current study was it did not have a CHF population without medical treatment. Analysis of CvRD markers in such a population would be ideal for evaluating intrinsic CvRD in CHF in the absence of diuretic interference. However, in a referral practice, this is difficult to achieve as the majority of CHF cats have had some medical stabilization before they are referred for further management.

Inflammatory infiltration of the myocardium has been found in feline CM previously (Fox, 2003; Fox, 2004; Aupperle et al., 2011; Khor et al., 2015), both in asymptomic CM and in CHF states. The association between inflammation, the immune system and heart failure has been noticed for a long time and, their interaction and mutual cause-consequence relationships are very complex (Celis et al., 2008; Dick and Epelman, 2016; Van Linthout and Tschope, 2017). Circulating inflammatory mediators carry great value as inflammatory biomarkers in heart failure studies, apart from assisting identifying aetiology and pathogenesis, they have potential

as disease monitors, prognosticators and therapeutic targets (Bozkurt et al., 2010). APPs are good examples; they have been studied extensively in human cardiovascular diseases and some of them have showed promising prognostic value in human heart failure (Ahmed et al.,2012). In the current study, positive APPs SAA, LRG1 and ceruloplasmin were found increased in CHF cats compared with controls. Furthermore, these over-expressed APPs were significantly correlated with the cardiac biomarker NT-proBNP, LA size and CHF severity. Interestingly, serum AGP stood out in both univariate and multivariate analyses as an independent prognostic factor in feline CHF. All these findings supported the fact that APPs play a role in feline CHF. It should be noted that apart from serving as inflammatory biomarkers, APPs also have other biological functions. For example, LRG1 was found to have a protective effect against adverse cardiac remodelling (Kumagai et al., 2016) and ceruloplasmin has been found to associate with oxidative stress (Cao and Hill, 2014; Hammadah et al., 2014). These would be potentially useful in identifying causative pathological pathways in CHF and to develop therapeutic targets in the future studies.

It should be noted that the APP results should be interpreted cautiously. Because these biomarkers are highly sensitive and non-specific, unknown occult inflammatory comorbidities may contribute to their elevations (Ceron et al., 2005; Ceron et al., 2008). Also, so far most feline APP assays have not been not standardised, which may result in discrepancy in cut-offs between different studies (Kann et al., 2012). When used with other clinical information, they can be promising biomarkers for feline CHF.

There are three criteria for assessing usefulness of a biomarker: (1) whether it can be measured accurately and repeatable with short turnaround times and reasonable cost; (2) whether it can provide additional information to other established clinical assessments; (3) whether its quantification can assist in making clinical decisions (Morrow and de Lemos, 2007; Gandhi and Pinney, 2014). Serum SDMA fits these criteria as being reasonably accurate and repeatable with reasonable cost, it recently became available as an in-house test; it can provide information on kidney status that is superior to creatinine. The feline APPs assays currently still need more standardization. Serum AGP, SAA and Hp are relatively available. The APPs are useful on indicating if there is ongoing systemic inflammation; also they can potentially provide information for disease pathogenesis and prognosis. High AGP is a potential novel biomarker in predicting survival in CHF cats, although it cannot be used as a sole marker for prognosis; test standardization will help to set up accurate cut-offs. In summary, SDMA, SAA, LRG1, ceruloplasmin and AGP carry promise as novel biomarkers for feline CHF.

One exciting secondary finding was all the examined biomarkers were independent from CM sub-phenotypes. This is very useful information which means the biomarkers are free from phenotypic dependent bias, which can be a problem considering the current equivocal classification system. Several clinical parameters showed their power in differentiating feline CM stages and helping prognosis. A multiple assessment approach incorporating clinical diagnostic parameters and biomarkers would be interesting to develop in the future. Current well accepted heart failure classification systems in small animal medicine include American College of Veterinary Internal Medicine (ACVIM) classification and ISACHC classification system, however both classifications are not ideal (Pouchelon et al., 2015)-they fail to reveal underlying disease aetiology and cannot accurately and sensitively reflect disease severity and progression. A combination of clinical parameters, together with biomarkers possibly can give a more accurate picture of the disease, for example a scoring system containing clinical parameters known to be associated with prognosis, may be more beneficial to get objective views of individual animal's disease status. In human medicine, there are several scoring systems for risk assessment in heart failure patients (Ross, 2012; Pocock et al., 2013). A clinical scoring system has been developed to assess canine mitral valve disease severity and it managed to differentiate the patient groups with different clinical outcomes (López -Alvarez et al., 2015). In the future, a clinical scoring system could be developed for risk stratification in feline CHF. It would be more objective and helpful to reveal a global picture of the disease and would be a more accurate approach for giving prognosis compared with single factor based assessment.

Lastly, it should be clarified that this study primarily focused on CHF rather than CMs. The pathogenesis of feline primary CM remains unclear. When the disease is in an advanced CHF stage, it further complicates the story. Current understanding of CHF in veterinary field is a consequence rather than a cause for CM. In humans, 'maladaptive hypertrophy' also called cardiomyopathy of overload has been considered caused by heart failure (Katz, 2002). In a failing heart, neurohormonal systems are activated in response to decreased cardiac output and increased intra-cardiac pressure. The cardiomyocyte becomes hypertrophied as a response to pressure overload and to neurohormonal activation. In the end, evidence of myocardial remodelling is seen in any CHF patient, regardless of the primary cause either if it is a primary CM or mitral valve dysplasia. Knowing that humans and cats share similarities in many cardiovascular conditions, it would not be surprising if similar maladaptive mechanisms can occur in the feline patient. This provides extra challenges to solve the puzzle of feline primary

CM pathogenesis especially in the CHF case. For feline CM pathology and pathogenesis study, pre-clinical stage patients would be interesting to investigate, in terms of getting a clearer idea of what is going on in the heart before it gets decompensated.

4.9 Future Work

Apart from potential future work aspects mentioned previously, there are other further research directions extended from this project which can be summarized as following:

(1) Further characterize the prognostic values of SDMA and NT-proBNP in CHF cats. Longitudinal monitoring of SDMA and NT-proBNP values in larger number of CHF cats could yield more information. A combination of cardio-renal marker profile will also be interesting to assess, which may improve the power of disease prognosis.

(2) Investigate other cardiac and renal biomarkers for CvRD. Cardiac marker cTnI previously was found to have prognostic value for feline HCM (Borgeat, et al.,2014b), preliminary work in the current study also showed the cTnI level was significantly higher in CHF cats compared with compensated CM. The cardiac biomarkers cTnI/NT-proBNP combined with acute renal injury markers would be an interesting direction for future CvRD investigation, especially for monitoring acute CHF episodes.

(3) The APPs may be potentially used for disease monitoring and therapeutic response assessment. In future longitudinal studies, it would be interesting to see if the up-regulated APPs LRG1, SAA and ceruloplasmin have differential expressions in different CHF stages; if their changes are associated with clinical symptoms (e.g. recurrent CHF); if they are responsive to therapeutics. Additionally, pro-inflammatory or anti-inflammatory cytokines such as TNF-α, IL-6 might be worth evaluating to assess the extent of a systemic inflammatory response in feline CHF (Bozkurt et al., 2010).

(4) Knowing that apart from serving as inflammatory mediators, the APPs also have other biological functions, certain detailed pathophysiological pathways would be worthy to investigate for understanding the disease pathogenesis. For example, LRG1 is known to play a role in preventing adverse cardiac remodelling via TGF-β pathway in a mice model (Song and Wang, 2015; Kumagai et al., 2016), it would be interesting to examine the same pathway in feline CHF and develop potential therapeutic targets based on the pathway findings.

(5) Besides the biomarkers investigated in the current study, there is a bigger biomarker galaxy out there for further exploration. Advanced technology such as proteomic techniques will be useful for global discovery of the biomarkers in feline CHF and CM. In fact, the logical approach of clinical biomarker development is global biomarker discovery then followed by verification and validation (Frantzi et al., 2014). A proteomic study is therefore a promising future direction for systemic development of clinical biomarkers in feline CM.

4.10 Conclusion

In summary, the current findings support the presence of CvRD and systemic inflammation in cats with CHF caused by primary CM. SDMA is a promising biomarker in feline CHF and should be considered for use in practice for renal function monitoring in CHF management. The APPs LRG1, SAA and ceruloplasmin are believed to be helpful in understanding feline CHF pathogenesis and AGP can potentially be used as a complementary test for prognosis. A combination of clinical tests and biomarkers appears to be a promising future direction for risk stratification in feline CHF.

This study provides new insights into feline cardiac biomarker research and the findings will potentially benefit clinical management and prognosis, as well as fundamental research into the disease.

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Appendix 1: Parameters Considered in Univariate Analysis

Animal Information
Sex
Age at initial diagnosis
Body weight
Echo parameters
LA diameter
LA/Ao ratio
LVFWd
IVSd
LV FS%
Spontaneous Echo Contrast (present or absent)
Intracardiac thrombus (present or absent)
Clinical information
Previous ATE history (Yes or No)
Overall CHF status during study (stable or unstable)
Heart Rate
Arrhythmia by auscultation (present or absent)
Gallop sounds (present or absent)
Heart murmur grades
Pulse deficit (present or absent)
Arrhythmia on ECG (present or absent)
Respiratory rate
Biomarker Level
NT-ProBNP
SDMA
Creatinine
AGP
CRP
Hp
LRG1
SAA
PCT
Troponin