White blood cell differentials in dogs with congestive heart failure (CHF) in comparison to those in dogs without cardiac disease.

Objectives: To determine if dogs with congestive heart failure have different white blood cell differentials in comparison to dogs without cardiac disease.

Methods: 72 canine CHF patients (mitral valve disease or dilated cardiomyopathy; cases) and 143 dogs without cardiac disease (controls) were retrospectively recruited. Signalment, white blood cell differentials and echocardiography data were retrieved. Basic statistical analysis was performed on white blood cell differentials. Principal components analysis was performed to compare white blood cell differentials in cases and controls with age, sex and case/control status as supplementary variables. Cases and controls were compared with binary logistic regression for the principal components identified and individual WBC differentials. Principal components analysis of cases alone was conducted with age, sex, weight, mitral E wave:isovolumic relaxation time as supplementary variables. Linear regression analysis was used to explore the association between mitral E wave:isovolumic relaxation time and the principal components, weight, sex, age and diagnosis (mitral valve disease or dilated cardiomyopathy).

Results: Amongst cases and controls, the largest variance in data (component 1) associated with neutrophils, band neutrophils, monocytes and case status ($p<0.01$). The odds of an individual being a case increased 2.5 (95% confidence interval: 1.4-4.4) fold for each unit increase in component 1. Amongst the cases, mitral E wave:isovolumic relaxation time associated with neutrophil count and diagnosis..

Conclusion: Dogs with CHF have statistically significant increases in neutrophils, band neutrophils and monocytes in comparison to those without cardiac disease however these changes remain within normal reference ranges.

Introduction

Congestive heart failure (CHF) is a common pathological syndrome in canine patients and a frequent consequence of advanced structural or functional cardiac disease, including myxomatous mitral valve disease (MVD) and dilated cardiomyopathy (DCM) (Bonnett et al. 2005). However, the underlying pathophysiology is incompletely understood. Cardiac remodelling leading to CHF is a complex process involving alterations in gene expression and molecular, cellular and interstitial changes influenced by haemodynamic loading, neurohormonal activation and activation of the immune system (Cohn at al. 2000).

In humans, it is well established that immune activation is an important component of CHF and inflammatory mediators including pro-inflammatory cytokines (e.g. interleukin 1ß, interleukin 6, C-reactive protein (CRP) and nitric oxide contribute to progression and prognosis in both acute and chronic disease (Deswal et al. 2001; Anker and Von Haehling 2004; Eisen et al. 2014; Li et al. 2014). More recently, activation of the immune system has been documented in canine CHF. Several studies have reported increased levels of acute phase proteins (e.g. CRP and haptoglobin), pro-inflammatory cytokines (e.g. pro-inflammatory interleukin-1β) and anti-fibrotic matrix metalloproteinase 1 in canine MVD patients with CHF (Rush et al.2006, Cunningham et al. 2012; Fonfara et. al 2012; Reimann et al. 2015; Polizopoulo et al. 2016) which is thought to support an inflammatory component to canine CHF.

In human CHF, leukocytosis is associated with CHF development (Pfister et al. 2012; Eisen et al. 2014) and mortality (Rudiger et al. 2005; Arruda-Olson et al. 2009; Riad et al. 2013) and is predictive of future hospitalisation (Engström et al. 2009). Monocytosis (Pfister et al. 2012; Shantsila et al. 2012), relative lymphopenia (Ommen et al. 1998; Acanfora et al. 2001; Rudiger et al. 2006) and increased neutrophil to lymphocyte ratio (Duffy et al. 2006; Uthamalingam et al. 2011; Zhang et al. 2014) are also related to disease severity and mortality. The association between leukocyte alterations and prognosis persists in human studies despite heterogeneous causes of CHF and study designs (Vaduganathan et al. 2012).

Alterations in white blood cell subpopulations have previously been described in canine CHF. Farabaugh and colleagues (2004) reported that significant leukocytosis with neutrophilia but not lymphopenia exists in canine CHF patients relative to controls, although differential leukocyte values remained in the reference ranges for most cases. In that study, however, the population size was small and further work was recommended to validate these findings. The objective of this study was

therefore to support previous evidence of alterations in WBC differential counts in canine CHF patients in comparison to controls with a larger population and, as secondary objectives, whether underlying disease type (MVD or DCM) or mitral E wave to isovolumic relaxation time correlate with WBC differential changes in CHF patients.

Materials and Methods

This retrospective study had ethical approval from the local Veterinary Research Ethics Committee (VREC84).

The medical records of all canine CHF patients diagnosed at a companion animal referral hospital from 2008 to 2015 were reviewed for suitability. Dogs which had undergone echocardiography and coded as CHF and either DCM or MVD in the Echopac (GE, Buckinghamshire, UK) archiving system were eligible for inclusion provided they had haematology performed within 24 hours of the echocardiogram. Echocardiography and analyses had been generated by cardiology diplomates or residents under direct supervision of a diplomate. The underlying diagnosis (MVD or DCM) was recorded. An echocardiographic diagnosis of MVD was based on the presence of mitral valve thickening, mitral valve regurgitation, left atrial and/or left ventricular dilation on 2-dimensional, M-mode and colour-flow Doppler echocardiography. An echocardiographic diagnosis of DCM was based on the presence of left atrial and left ventricular dilation with systolic dysfunction as per published guidelines (Dukes-McEwan et al. 2003) after active exclusion of other cardiac or systemic disease which may result in secondary myocardial failure. The echocardiographic measurement, mitral E wave velocity to isovolumic relaxation time ratio (E/IVRT) was documented for each case as an estimate of left sided filling pressures as it is considered the best echocardiographic indicator for predicting the presence of left sided congestive heart failure in dogs with MVD or DCM (Schober et al. 2010) however a definitive diagnosis of CHF was based on the presence of pulmonary oedema on thoracic radiography.

White blood cell differentials were generated by a haematology analyser and subsequently checked by manual count; only the manual counts were collated for statistical analysis. Exclusions were made for congenital heart disease (diagnosed by echocardiography) or concurrent systemic disease, based on physical examination and a routine minimum database of haematology and serum biochemistry testing. DCM patients with concurrent features of MVD (mitral valve thickening) were excluded from the study to allow comparison of these diseases in isolation. In total, seventy-two dogs were recruited. A population of dogs without cardiac disease (designated as the control population) was selected from the referred hospital population, in which haematology had been carried out as part

of health screening or for investigation of their presenting complaint. The population was not randomly selected however an effort was made to include patients without a strong suspicion for cardiac or primary inflammatory disease. Some patients were recruited from a previous cardiac screening study and were therefore known to have no cardiac, or other, disease whilst others presented for complaints which were not considered primarily inflammatory in nature. All control patients had no history or clinical evidence of underlying cardiac disease based on the absence of a heart murmur or reported clinical signs associated with congestive heart failure e.g. tachypnoea, dyspnoea, exercise intolerance or collapse. A subset of the control patients had undergone echocardiography as part of a previous study and were all found to be normal. Dogs were excluded from both groups if neoplasia was diagnosed or if there was confirmed or suspected primary inflammatory disease.

For the CHF and control groups, patient signalment, final diagnosis and haematology records were retrieved from the hospital computer system (Tristan Veterinary Software, Ltd, 2010). From the haematology reports, total white blood cell count and respective differential manual count absolute numbers with percentagesof neutrophils, lymphocytes , monocytes , eosinophils and basophils were retrieved.

Statistical Analysis

Case-Control Comparison

Data were initially entered into *Excel* spreadsheets (Microsoft Corporation, Redmond, WA) and statistical analyses were performed using *Sigma Plot 12* (SYSTAT Software, 2013). Shapiro-Wilk Normality Testing was used to establish the distribution of data sets to enable selection of the appropriate parametric or non-parametric methods for statistical comparisons. Basic descriptive statistics (mean and standard deviation or median with interquartile range; the latter if non-normal distribution) were applied to the haematological results. It was suspected that there could be an over-representation of certain breeds between the two groups, especially due to the presence of the Great Dane control group and so demographic comparisons were performed. To compare groups (e.g. haematological results, age, body-weight), Student's T-test or Mann-Whitney (if normal or non-normal data distribution, respectively) were used. To compare categorical data (e.g. sex, breeds) Chi-squared analysis was used. WBC differentials were compared between the control group subgroups using one-way ANOVA. P values of <0.05 were accepted as representing statistical significance.

As WBC differentials are likely to be correlated, to some extent, within individuals, principal components analysis (PCA) was used to investigate these possible associations as well as

investigating the effect of CHF as a variable of interest. PCA is a statistical procedure that transforms a set of (observed and possibly correlated) variables to a new set of uncorrelated variables called principal components (Husson et al. 2010). The transformation is conducted so that the first principal component (component 1) contains the largest variance (accounting for as much of the variability as possible), with each successive principal component accounting for less variation. In this way, it is often possible to explain much of the variation in the original data with a smaller set of uncorrelated variables. The PCA approach adopted in this study enabled the relationships between the multiple hematological variables to be explored and these patterns, in turn, to be compared with other explanatory variables of interest (such as mitral E/IVRT, age) and with the outcomes (case or control status) under study. By examining how each variable contributes to the principal components, the principal components can be interpreted in terms of variables of interest.

PCA was performed using the R language for statistical computing (R Core Team, 2016) using the *FactorMineR* package (Husson et al. 2010). Analysis was undertaken in two stages. Firstly, PCA was conducted using all data (cases combined with controls) regarding the WBC differentials, and with age, sex and case/control status as additional, supplementary variables. Supplementary variables are compared with but do not contribute to calculation of the principal components. Weight was not considered as a supplementary variable in the case-control group due to the risk of bias given the over-representation of Great Danes (known to be a heavy breed) in this group. Cases and controls were then compared with regards to the components identified using PCA and individual WBC differentials. These comparisons were made using binary logistic regression (R Core Team, 2016) with the dependent variable set as case or control and the independent variable as the principal component of WBC differential of interest as well as age.

Subsequently, analysis of the cases alone was conducted. PCA was used to explore the WBC differential data, with age, sex, weight and Mitral E/IVRT measurements included as supplementary variables. Linear regression analysis was used to explore the association between Mitral E/IVRT and the main principal components, weight and age. The association between Mitral E/IVRT and diagnosis (MVD or DCM) was also explored. Prior to analysis, Mitral E/IVRT was log10 transformed to improve normality of the data. In all cases, the relationship between each dependent and independent variable was examined using generalised additive models (conducted using the R Language for statistical computing with the 'mgcv' package).

Results

The CHF group (n=72) consisted of 44 MVD cases and 28 DCM cases. The control group (n= 143) included a group of healthy Great Danes (n=35) presented for cardiac screening, dogs presenting to the orthopaedic service for chronic lameness investigations (n=24), dogs with a diagnosis of idiopathic epilepsy (n=68), and a small number presenting for elective procedures e.g. castration (n=3). Further population breakdown is illustrated in Table 1. Comparison of signalment between the CHF and control groups (Table 1) found a non-normal distribution of data for weight in both groups. The controls were significantly younger ($p<0.001$) and heavier ($p=0.013$) than the CHF group. Great Danes were over-represented in the controls ($p<0.001$) and Cavalier King Charles Spaniels in the CHF group ($p<0.001$).

Basic statistical analysis of the WBC differentials (Table 2) revealed that the CHF group had a significantly higher median total WBC count (CHF group 11.96 $x10^9$/L, control group 9.0 $x10^9$/L, $p <0.001$), neutrophil count (CHF group 8.9 $x10^9$/L, control group 6.20 $x10^9$/L, $p <0.001$), monocyte count (CHF group 0.5 $x10^9$/L, control group 0.37 $x10^9$/L, $p=0.008$) and number of band neutrophils (CHF group 0.29 $x10^9$/L, control group 0.2 $x10^9$/L, $p=0.003$) than the control group. Basic statistical analysis and comparison of the WBC differentials in the control subgroups found that the Great Danes had a significantly lower lymphocyte count (median lymphocyte count 1.2$x10^9$/L; $p = 0.001$, idiopathic epilepsy 1.8, chronic lameness 1.9, elective procedures 1.3) than the other sub populations however no other significant differences were identified.

PCA *of cases and controls*

The main component of variability (component 1) was associated with neutrophils, band neutrophils and monocytes but not with lymphocytes, eosinophils or basophils. In contrast, component 2 was associated with lymphocytes, eosinophils and basophils with little contribution from neutrophils, bands or monocytes. Component 3 mainly associated with basophils whereas component 4 associated with eosinophils and, to some extent, monocytes.

Components 1 and 2 accounted for 49.22% of the variation in the data (28.98% and 20.24% respectively). Components 3 and 4 contributed 17.21% and 14.55% of the data respectively (Table 3). This was also illustrated as a factor map (Figure 1) which demonstrated the correlations between the white cell counts and also the supplementary variable, age. Two variables were significantly associated with component 1; case status (correlation 0.42, $p<0.010$) and age (correlation 0.17, $p=0.010$, see Figure 1). Hence, case animals were more likely to score highly on component 1 (which was characterized by relative neutrophilia, increased band neutrophils and relative monocytosis- Table 1, also illustrated in Figure 1). Component 2 (lymphocyte, eosinophils and, to a lesser extent,

basophils) was significantly negatively associated with case status (correlation -0.15 p = 0.02) and age (correlation -0.21, p<0.01). Sex was associated only with component 2 (correlation with males -0.13, p = 0.049) but not with component 1.

The relationship between component 1 and 2 is shown in Figure 2. Although there was considerable overlap between cases and controls with regards to the components, there was evidence that case animals are more likely to score highly on component 1 (neutrophils, band neutrophils and monocytes).

Generalised additive models indicated that the form of the relationship between the outcome (case status) and each dependent variable (principal components 1, 2 and age) was approximately linear, indicating that this assumption of generalised linear regression analysis was met. The association between case-control status and component 1 was further explored using univariable logistic regression (Table 4). Component 1 was significantly associated with case-control status (p=0.002) with the odds of an individual being a case increasing 2.5 (95% confidence interval: 1.4-4.4) fold for each single unit increase in component 1. This increase is a composite of the three WBC types incorporated in the principal component (neutrophils, band neutrophils and monocytes) and so it cannot be differentiated which cell line predominantly contributes to this. However, univariable logistic regression using the WBC differentials as explanatory variables again highlighted the increased odds of being a case associated with increases in neutrophils, band neutrophils and monocytes (Table 4). The association between case status and lymphocyte, and eosinophil count was not significant. Due to the correlation between some differentials, and because the co-relationship between the cell types and case status was explored through PCA, as shown above; multivariable analysis using all cell types was not performed. The association with component 1 remained significant (p=0.007) after controlling for age (odds ratio 2.4, 95% confidence interval: 1.4-4.9). Component 2 was not associated with case status, even after controlling for age.

Case only analysis

Among the cases only, similar to the PCA that combined case and control data, component 1 was characterised by neutrophils, band neutrophils and monocytes whereas component 2 was characterised by lymphocytes and eosinophils (Table 5, Figure 3). Additionally, Mitral E/IVRT most strongly correlated with component 1. Component 3 mainly correlated with basophils whereas component 4 was weakly correlated with eosinophils and monocytes. Among the cases only, PCA identified that components 1 and 2 accounted for 54.7% of the variation in the data (34.39% and 20.31% respectively). Components 3 and 4 explained an additional 16.92% and 12.63% respectively.

Linear regression identified no significant associations between mitral E/IVRT and component 1 or 2 or with individual WBC differentials, except for neutrophils (p=0.040; Table 6). Diagnosis (MVD or DCM) was significantly associated with E/IVRT (p=0.040; Table 6), with MVD having a higher E/IVRT than DCM. Controlling for age, sex and weight did not meaningfully affect these associations. Diagnosis was also incorporated as a variable into the case-only analysis however no significant difference was found between any of the first 4 components (the only components examined).

There were no significant differences in individual WBC differentials between DCM and MVD (data not shown).

Discussion

This study identified statistically significant differences between the differential WBC counts of canine CHF patients and those of controls. This finding is consistent with previous evidence (Farabaugh et al. 2004), however the present study had a larger study population and examined both underlying disease process and the relationship to the echocardiographic measurement, mitral E/IVRT (a marked of increased left ventricular filling pressures) as secondary objectives.

In humans, WBC count (specifically granulocyte count) is independently associated with increasing CRP levels and risk of heart failure (Pfister et al. 2012). In one study, leukocyte concentrations were elevated years before patients were hospitalised due to CHF, suggesting a predictive value (Engström et al. 2009). WBC count at hospital admission predicts mortality in human DCM patients with severe left ventricular dilation and is therefore an independent predictor of mortality in these patients (Riad et al. 2013). Whilst leukocytosis is associated with physiological stress in humans (Benschop et al. 1996, Chatterjee et al. 2005), the presence of a relative leukocytosis in human CHF is generally inferred to be part of a wider inflammatory response (Arruda-Olson et al. 2009, Engström et al. 2009, Riad et. al. 2013). It is postulated that leukocytes release pro-inflammatory cytokines e.g. tumour necrosis factor α, interleukin-6 and CRP which then have deleterious effects on the myocardium resulting in reduced left ventricular function (Uthamalingam et al. 2011; Shantsila et al. 2012). Activated neutrophils may also release proteolytic enzymes which facilitate tissue destruction; increased levels of myeloperoxidase have been shown in patients with CHF suggesting inflammatory activation (Vasilyev et al. 2005).

In our study, whilst most CHF patients' neutrophil, band neutrophil and monocyte counts were within accepted reference ranges, a relative neutrophilia, increased band neutrophil count and monocytosis (as captured by the first principal component) were associated with canine CHF patients in comparison to the control group. The clinical significance of these findings is unclear but

it is possible that the presence of a relative neutrophilia could be due to physiological stress in CHF patients. There is evidence of physiological stress in canine cardiac patients; Tidholm et al. (2005) reported no difference in plasma cortisol between controls and dogs with preclinical and clinical DCM, but the urine cortisol-creatinine and urine catecholamine-creatinine ratios were higher in DCM patients. We did not distinguish the underlying cause for the relative neutrophilia observed in the CHF cases and as other markers of systemic inflammation e.g. CRP were not assayed, we cannot conclude that our results are consistent with a systemic inflammatory response as is suggested in the human literature.

Also in humans, a relative lymphopenia is independently associated with survival time in advanced heart failure (Ommen et al. 1998) and considered a prognostic indicator in elderly CHF patients (Acanfora et al. 2001). The relative lymphopenia documented in human CHF patients is thought to reflect neurohormonal activation (Thomson et al. 1980) and acts as an indirect marker of activation of the hypothalamic-pituitary-adrenal axis (Ommen et al. 1998). In contrast to the human literature, neither our study nor a smaller previous study (Farabaugh et al. 2004) identified significant lymphopenia (which is also an indicator of physiological stress in animals (Stockman et al. 2003)), in canine CHF patients in comparison to controls. However our control population did contain an over-representation of Great Danes with a significantly lower lymphocyte count in comparison to other controls which may have confounded our ability to identify significant differences in the lymphocyte counts between the CHF group and controls. Additionally, Farabaugh and colleagues (2004) did show significant differences between the lymphocyte sub-populations in dogs with CHF compared with controls, which was not investigated in our study

In this study, CHF patients had increased number of band neutrophils in comparison to control patients. The presence of band neutrophils is not typically associated with the classical stress leukogram and, rather, is considered an indicator of an inflammatory leukogram (Barger 2003, Stockham et al. 2003). This result may therefore support the hypothesis of an inflammatory process in canine CHF, as described in the veterinary literature (Cunningham et al. 2012; Fonfara et al. 2012; Reimann et al. 2015). To the authors' knowledge, the presence of immature (band) neutrophils has not been reported in human CHF.

Monocytosis in human CHF patients is associated with angiogenesis secondary to myocardial damage and elevated left-sided filling pressures (Apostolakis et al. 2010). Increased numbers of monocyte-derived endothelial progenitor cells have been demonstrated in heart failure, further supporting this (Shantsila et al. 2012). The results of the present study could be further explored in veterinary patients to establish if associations exist between monocyte count and cardiac

remodeling in canine heart disease. Relative monocytosis in canine CHF has not previously been reported in the veterinary literature.

In this study, WBC differentials were investigated in relation to mitral E/IVRT which is a marker of increased left ventricular filling pressures and therefore supportive of the presence of left-sided CHF (Schober et al. 2010), justifying further investigations e.g. thoracic radiography. There was a weak positive association between neutrophil count independent of other WBC differentials, and mitral E/IVRT. Mitral E/IVRT also correlated most strongly with component 1 (neutrophils, band neutrophils and monocytes), which is logical given that case status was associated with similar WBC differential changes. Despite these findings, analysis of the relationship between principal components 1 and 2 in all patients showed considerable overlap between our case and control results (Figure 2) and so measurement of neutrophil count could not be considered sufficiently discriminatory to justify further investigations in patients with suspected left sided congestive heart failure. The weak nature of this association may reflect pre-treatment with cardiac medications including diuretics, which may have affected results. It would, however be interesting to repeat this study with incorporation of other markers of disease severity e.g. left atrial size, left ventricular dimension in diastole and /or systole (indexed to body weight (Cornell et al. 2004)), or in DCM cases, variables reflecting systolic function such as Simpson's derived end systolic volume index,ejection fraction and fractional shortening especially as there is some evidence of progressive immune activation with increasing severity of cardiac disease in veterinary patients (Cunningham et al. 2012, Ljungvall et al. 2010, Polizopoulou et al. 2015).

No differences were found in WBC differentials between different types of cardiac disease (MVD and DCM), although this may reflect reduced statistical power. In humans, significant alterations in WBC differentials have been documented in a variety of ischaemic and non-ischaemic cardiac diseases but no condition is associated with significantly heightened inflammatory biomarkers in comparison to others (Engströem et al. 2009, Pfister et al. 2012). Therefore, the leucogram changes associated with CHF may reflect the CHF syndrome itself, rather than the primary cardiac condition leading to CHF.

Limitations

The control population recruited in this study was not truly representative of the population from which the cases were drawn and were not randomly selected so the potential affect of selection bias on results should be taken into consideration. Controls were recruited from a referred population and so could not be considered strictly healthy although their presenting complaints were not considered to be primarily inflammatory in nature. Unfortunately, blood sampling from completely

healthy pets without clinical justification to do so would be considered unethical and so the inclusion of a strictly healthy control population was not achievable. The orthopaedic cases included in the controls selection presented for chronic lameness investigations which could arguably have an inflammatory component however the patients were clinically well with no clinical suspicion for polyarthritis. Idiopathic epilepsy especially is not considered an inflammatory disease based on serum or cerebrospinal fluid CRP levels (Narkamura et al. 2007, Bathen-Noethen et al. 2008). Additionally, there was no statistical evidence of a significant relative leukocytosis, neutrophilia or increased band neutrophil count (features associated with an inflammatory state) in any of the control subgroups in comparison to the others. The relative lymphopenia in the Great Dane group may reflect breed variation however breed differences in WBC differentials have not been reported in the veterinary literature, except in the case of eosinophils (Bourges-Abella et al 340 2011). Concurrent testing of acute phase protein levels e.g. CRP to definitively exclude significant concurrent inflammatory disease may have improved the validity of the control population.

The case and control patients were not matched for age, sex, breed or weight and were known to include different breed proportions; Great Danes contributed a larger proportion of the control population whilst Cavalier King Charles Spaniels were over-represented in the cases. The Great Danes were included in the controls as they were known to be clinically healthy with normal echocardiographic results at the time of inclusion, which improved the validity of the control population. As the CHF population tended to be lighter than the control population, some of our findings may be a consequence of differences in breed and weight.

As the CHF group was a referred population, most were not treatment-naive cases, and may have received a variety of cardiac medications prior to echocardiography. We cannot exclude the possibility that treatment influenced the results, and serial haematologies would be indicated to investigate the effect of treatment. Additionally, as the CHF group was a referred population and cases were restricted to MVD and DCM only (this decision was made as these are the most common structural cardiac diseases in dogs (Bonnett et al. 2005)), we cannot state that our findings are reflective of all CHF cases managed in primary practice or of dogs with other types of cardiac disease (although disease type did not appear to correlate with white blood cell differentials).

There was overlap between the leukogram results of the case and control groups so, based on our population, haematology results in isolation are not sufficiently discriminatory in determining the presence of CHF in individual veterinary cardiac patients. Neither can they be considered of prognostic value in an individual dog, as an inflammatory leucogram should prompt exclusion of other systemic conditions rather than presuming it reflects the concurrent CHF.

However, our results do indicate that the potential role of inflammation should be further investigated in the syndrome of canine CHF.

Conclusions

This study shows that canine CHF patients with MVD or DCM have a statistically significant relative neutrophilia and monocytosis and increased band neutrophils in comparison to dogs without CHF, independent of underlying cardiac disease. Whilst our results are not of diagnostic or prognostic value to the practitioner in the individual case, they may be of interest in elucidating some of the complex pathophysiological processes underlying the development of canine CHF.

References

Acanfora, D., Gheorghiade, M., Trojano, *et al*. (2001) Relative lymphocyte count: A prognostic indicator of mortality in elderly patients with congestive heart failure. *American Heart Journal* 142, 167-173

Anker, S. D., & Von Haehling, S. (2004) Inflammatory mediators in chronic heart disease: an overview. *Heart* 90, 464–470.

Apostolakis, S., Lip, G.Y., Shantsila, E. (2010) Monocytes in heart failure: relationship to a deteriorating immune overreaction or a desperate attempt for tissue repair? *Cardiovascular Research* 85 (4) 649-660

Arruda-Olson, A. M., Reeder, G. S., Bell, M. R., *et al.* (2009) Neutrophilia predicts death and heart failure after myocardial infarction: A community-based study. *Circulation: Cardiovascular Quality and Outcomes* 2, 656-662

Barger A.M. (2003) The complete blood cell count: a powerful diagnostic tool. *Veterinary Clinics of North America: Small Animal Practice* 33, 1207-1222

Bathen-Noethen, A., Carlson, R., Menzel, D. *et al.* (2008) Concentrations of acute-phase proteins in dogs with steroid responsive meningitis-arteritis. *Journal of Veterinary Internal Medicine* 22, 1149-1156

Benschop R.J., Rodriguez-Feuerhahn M., Schedlowski M. (1996) Catecholamine-induced leukocytosis: early observations, current research and future directions. *Brain, Behaviour and Immunity* 10, 77-91

Bonnett, B. N., Egenvall, A., Hedhammar, *et al.*(2005) Mortality in over 350,000 insured Swedish dogs from 1995-2000: breed-, gender-, age-and cause-specific rates. *Acta Veterinaria Scandinavica* 46, 105–120.

Bourges-Abella, N., Geffre, A., Concordet, D. *et al.* (2011) Canine reference intervals for the Sysmex XT-2000iV haematology analyzer. *Veterinary Clinical Pathology* 403, 303-315

Chatterjee, K. (2005) Neurohormonal activation in congestive heart failure and the role of vasopressin. *American Journal of Cardiology* 95, 8-13

Cohn, J. N., Ferrari, R., Sharpe, N. (2000) Cardiac remodeling-concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. *Journal of the American*

College of Cardiology. 35, 569-82

Cornell, C. C., Kittleson, M. D., Della Torre, P., *et al.* (2004) Allometric scaling of M-Mode cardiac measurements in normal adult dogs. *Journal of Veterinary Internal Medicine* 18, 311–321

Cunningham, S. M., Rush, J. E., Freeman, L. M. (2012) Systemic inflammation and endothelial dysfunction in dogs with congestive heart failure. *Journal of Veterinary Internal Medicine* 26, 547-557

Deswal, A., Petersen, N. J., Feldman, A. M., *et al.* (2001) Cytokines and cytokine receptors in advanced heart failure an analysis of the cytokine database from the Vesnarinone Trial (VEST). *Circulation* 103, 2055-2059

Duffy, B. K., Gurm, H. S., Rajagopal, V., *et al.* (2006) Usefulness of an elevated neutrophil to lymphocyte ratio in predicting long-term mortality after percutaneous coronary intervention. *The American Journal of Cardiology* 97, 993-996

Dukes-McEwan, J., Borgarelli, M., Tidholm, A., *et al.* (2003) Proposed guidelines for the diagnosis of canine idiopathic dilated cardiomyopathy. *Journal of Veterinary Cardiology* 5 (2), 7-19

Eisen, A., Benderly, M., Behar, S., *et al.* (2014) Inflammation and future risk of symptomatic heart failure in patients with stable coronary artery disease. *American Heart Journal* 167, 707-714

Engström, G., Melander, O., Hedblad, B. (2009) Leukocyte count and incidence of hospitalizations due to heart failure. *Circulation: Heart Failure* 2, 217-222

Farabaugh, A. E., Freeman, L. M., Rush, J. E., *et al*. (2004) Lymphocyte subpopulations and hematologic variables in dogs with congestive heart failure. *Journal of Veterinary Internal Medicine* 18, 505–509

Fonfara, S., Tew, S. R., Cripps, P., *et al.* (2012) Increased blood mRNA expression of inflammatory and anti-fibrotic markers in dogs with congestive heart failure. *Research in Veterinary Science* 93, 879-885

Freeman, L. M., Rush, J. E., Kehayias, J. J., *et al.* (1998) Nutritional alterations and the effect of fish oil supplementation in dogs with heart failure. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine* 12, 440-448

Husson, F., Le, S., Pages, J. (2010) Exploratory multivariate analysis by example using R 1st edition, Boca Raton FL, CBC Press

Li, X., Chen, C., Gan, F., *et al.* (2014) Plasma NT pro-BNP, hs-CRP and big-ET levels at admission as prognostic markers of survival in hospitalized patients with dilated cardiomyopathy: a single-center cohort study. *BMC Cardiovascular Disorders* 14, 67

Ljungvall, I., Höglund, K., Tidholm, A., *et al.* (2010) Cardiac troponin I is associated with severity of myxomatous mitral valve disease, age, and C-reactive protein in dogs. *Journal of Veterinary Internal Medicine* 24, 153-159

Narkamura, M., Takahashi, M., Ohno, K., *et al.* (2007) C-reactive protein concentration in dogs with various diseases. *Journal of Veterinary Medical Science* 70, 127-131

Ommen, S. R., Hodge, D. O., Rodeheffer, R. J., *et al.* (1998) Predictive power of the relative lymphocyte concentration in patients with advanced heart failure. *Circulation* 97, 19-22

Polizopoulou Z.S.; Koutinas C.K.; Ceron J.J., *et al.* (2015) Correlation of serum cardiac troponin I and acute phase protein concentrations with clinical staging in dogs with degenerative mitral valve disease. *Veterinary Clinical Pathology* 44, 397-404

Pfister, R., Sharp, S. J., Luben, R., *et al.* (2012) Differential white blood cell count and incident heart failure in men and women in the EPIC-Norfolk study. *European Heart Journal* 457, 523-530

Reimann, M. J., Ljungvall, I., Hillström, A., *et al.* (2015) Increased serum C-reactive protein concentrations in dogs with congestive heart failure due to myxomatous mitral valve disease. *The Veterinary Journal* 209, 113-118

Riad, A., Weitmann, K., Herda, L. R., *et al.* (2013) Initial white blood cell count is an independent risk factor for survival in patients with dilated cardiomyopathy. *International Journal of Cardiology* 168, 1207-1213

Rudiger, B., Burckhardt, O. A., Harpes, P. M., *et al.* (2006) The relative lymphocyte count on hospital admission is a risk factor for long-term mortality in patients with acute heart failure. *The American Journal of Emergency Medicine* 24, 451-454

Rush, J. E., Lee, N. D., Freeman, L. M., Brewer, B. (2006) C-reactive protein concentration in dogs with chronic valvular disease. *Journal of Veterinary Internal Medicine* 20, 635-639

Schober, K. E., Hart, T. M., Stern, J. A., *et al.* (2010) Detection of congestive heart failure in Dogs by Doppler echocardiography. *Journal of Veterinary Internal Medicine* 24, 1358-1368

Shantsila, E., Bialiuk, N., Navitski, D., *et al.* (2012) Blood leukocytes in heart failure with preserved

ejection fraction: impact on prognosis. *International Journal of Cardiology* 155, 337–338

Shantsila, E., Wrigley, B. J., Shantsila, A., *et al.* (2012) Monocyte-derived and CD34+/KDR+ endothelial progenitor cells in heart failure. *Journal of Thrombosis and Haemostasis* 10, 1252-1261

Stockham S.L.; Keeton K.S., Szladovits B. (2003) Clinical assessment of leukocytosis: distinguishing leukocytoses caused by inflammatory, glucocorticoid, physiologic and leukemic disorders or conditions. *Veterinary Clinics of North America: Small Animal Practice* 33, 1335-1357

Tidholm, A., Häggström, J., & Hansson, K. (2005) Vasopressin, cortisol, and catecholamine concentrations in dogs with dilated cardiomyopathy. *American Journal of Veterinary Research* 66, 1709-1717

Thomson, S.P, McMahon, L.J., Nugent, C.A. (1980) Endogenous cortisol: a regulator of the number of lymphocytes in peripheral blood. *Clinical Immunology and Immunopathology.* 17, 506–514

Uthamalingam, S., Patvardhan, E. A., Subramanian, S., *et al.* (2011) Utility of the neutrophil to lymphocyte ratio in predicting long-term outcomes in acute decompensated heart failure. *American Journal of Cardiology* 107, 433–438.

Vaduganathan, M, Ambrosy A.P., Greene, S.J., *et al.* (2012) Predictive value of low relative lymphocyte count in patients hospitalized for heart failure with reduced ejection fraction insights from the EVEREST Trial. *Circulation: Heart Failure* 5, 750–758

Vasilyev, N., Williams, T., Brennan, M. L., *et al.* (2005) Myeloperoxidase-generated oxidants modulate left ventricular remodeling but not infarct size after myocardial infarction. *Circulation* 112, 2812–2820.

Zhang, G. Y., Chen, M., Yu, Z. M., *et al.* (2014) Relation between neutrophil-to-lymphocyte ratio and severity of coronary artery stenosis. *Genetics and Molecular Research* 13, 9382-938

Table 1: Signalments of the CHF and Control groups

Population		CHF (n=72)	Control (n=143)
Breeds	Boxer	5	1
(p<0.001)	Cavalier	17	4
	Cocker Spaniel	6	2
	Crossbreed	4	13
	Doberman	8	0
	Great Dane	4	35
	Labrador	5	11
	Other Breeds	23	78
Age (Years)	0-4.5	4	67
(p<0.001)	4.6-8.5	25	49
	8.6-12.5	36	19
	12.6+	6	4
	Mean	8.89	5.44
	Standard Deviation	2.78	3.27
Sex	Female (Entire)	4	23
(p<0.001)	Female (Neutered)	22	29
	Male (Entire)	22	42
	Male (Neutered)	24	47
Weight (Kg)	0-9.9	21	5
(p = 0.013)	10-19.9	16	3
	20-29.9	10	12
	30-39.9	10	4
	40+	17	29
	Median	19.5	29.5
	25%	9.55	14.0
	75%	38.5	53.5

Abbreviations: CHF = Congestive Heart Failure, n = number, kg = kilograms

Table 2: White blood cell differentials in CHF and control populations

WBC Variable	CHF Dogs (n=72)			Control Dogs (n=143)			
	Median	25%	75%	Median	25%	75%	P Value
Total WBC (x 10^9/L)	11.96	9.68	15.58	9.00	7.41	10.55	<0.001
#N (x 10^9/L)	8.90	6.70	12.00	6.20	5.00	7.60	<0.001
#L (x 10^9/L)	1.50	0.96	2.21	1.60	1.10	2.16	0.700
#M (x 10^9/L)	0.50	0.26	0.82	0.37	0.21	0.61	0.008
#Eos (x 10^9/L)	0.24	0.01	0.48	0.32	0.17	0.49	0.040
#Bas (x 10^9/L)	0.01	0.01	0.01	0.01	0.01	0.01	0.008*
#bands	0.29	0.117	0.825	0.2	0.08	0.37	0.003
#Platelets(x 10^9/L)	287	208	379	277	230	329	0.432
%N	75.95	69.10	82.01	69.40	63.20	76.04	<0.001
%L	12.98	8.45	18.10	18.0	13.55	24.76	<0.001
%M	4.02	2.45	6.00	4.96	2.95	6.9	0.110
%Eos	1.97	0.16	3.97	3.10	1.65	5.60	0.002

Abbreviations: WBC = White Blood Cell, #N = neutrophil number, #L = lymphocyte number, #M = monocyte number, #Eos = eosinophil number, #Bas = basophil number, #platelets = platelet numbers, %N = percentage neutrophils, %L = percentage lymphocytes, %M = percentage monocytes, %Eos = percentage eosinophils

*medians and quartiles identical for #Bas, however the maximum value was 0.9 for controls and 0.1 for CHF

Table 3: Principal components analysis (PCA) showing WBC differential contributions to Components 1 & 2 (CHF cases and controls combined). Figures in brackets represent the correlation coefficient

	Component 1 (%)	Component 2 (%)	Component 3 (%)	Component 4 (%)
Neutrophils	36.98 (0.8)	9.60 (-0.34)	0.17 (-0.04)	6.13 (0.23)
Band Neutrophils	33.18 (0.76)	2.63 (0.18)	1.49 (-0.12)	9.80 (0.98)
Monocytes	22.43 (0.62)	1.74 (0.15)	15.46 (0.4)	4.22 (-0.19)
Lymphocytes	4.09 (0.27)	44.71 (0.74)	0.39 (-0.06)	32.76 (-0.53)
Eosinophils	1.49 (-0.16)	36.76 (0.67)	9.37 (-0.31)	36.47 (0.56)
Basophils	1.82 (-0.18)	4.54 (0.23)	73.12 (0.87)	10.62 (0.30)
Total variance explained	28.98	20.24	17.21	14.55
Cumulative Total	28.98	49.22	66.43	80.98

Table 4: Univariable binary logistic regression investigating the relationships between case/control status and the first two principal components and white blood cell counts.

	Coefficient	SE	OR	95% CI	P value
Component 1	0.905	0.297	2.5	1.4-4.4	0.002
Component 2	0.263	0.163	1.3	0.9-1.8	0.100
Neutrophils	0.373	0.067	1.5	1.3-1.8	<0.001
Band Neutrophils	1.533	0.428	4.6	2.1-11.2	<0.001
Lymphocytes	-0.051	0.164	1.0	0.7-1.3	0.800
Monocytes	0.773	0.338	2.2	1.1-4.3	0.020
Eosinophils	-0.389	0.420	0.7	0.3-1.5	0.400

Abbreviations: SE; standard error, OR; Odds Ratio, CI; Confidence Interval

Footnote: Basophils not included due to negligible numbers

Table 5: Principal components analysis (PCA) showing WBC differential contributions (correlations) to Component 1, 2, 3 and 4 (CHF cases only). Figures in brackets represent the correlation coefficient

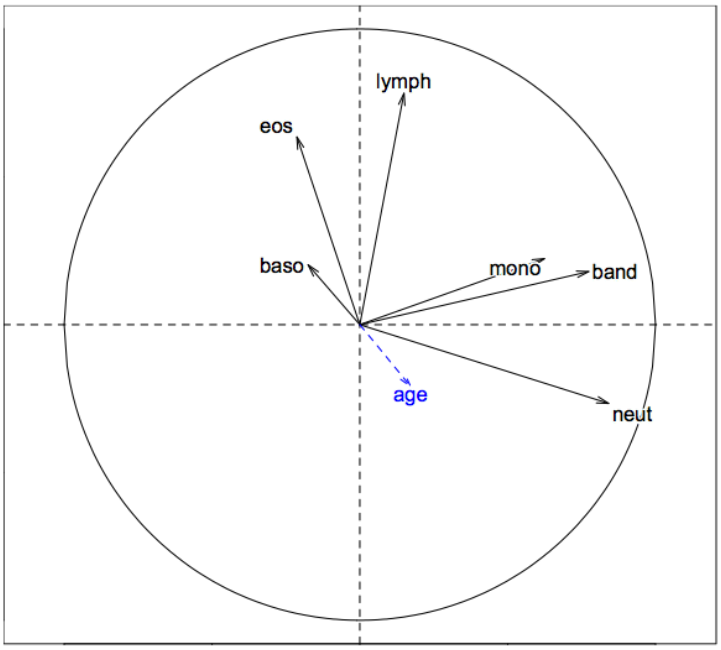
	Component 1 (%)	Component 2 (%)	Component 3 (%)	Component 4 (%)
Neutrophils	36.54 (0.87)	3.03 (0.19)	0.79 (-0.09)	2.17 (0.13)
Band Neutrophils	23.98 (0.7)	7.25 (0.30)	17.98 (-0.43)	16.41 (0.35)
Monocytes	15.91 (0.57)	10.68 (0.36)	3.19 (0.18)	62.44 (-0.69)
Lymphocytes	8.82 (-0.43)	41.72 (0.71)	0.00 (0.01)	0.38 (-0.05)
Eosinophils	11.32 (-0.48)	35.51 (0.66)	0.72 (-0.09)	3.46 (0.16)
Basophils	3.44 (0.27)	1.81 (0.15)	77.32 (0.89)	15.14 (0.34)
Total variance explained	34.39	20.31	16.92	12.63
Cumulative total variance explained	34.39	54.71	71.63	84.25

Table 6: Univariable linear regression results investigating, among the CHF cases, the association between E/IVRT (log_{10}) and principal components 1 and 2, white blood cell counts and diagnosis.

	Coefficient	SE	P value
Component 1	0.022	0.017	0.200
Component 2	0.022	0.020	0.300
Neutrophils	0.020	0.010	0.040
Band Neutrophils	0.122	0.088	0.200
Lymphocytes	-0.046	0.065	0.500
Monocytes	0.033	0.123	0.800
Eosinophils	0.086	0.146	0.600
Basophils	-1.939	3.708	0.060
Diagnosis **DCM** **MVD**	 Reference 0.224	 0.108	 0.040

Abbreviations: SE standard error , CI Confidence Interval, DCM Dilated Cardiomyopathy, MVD Mitral Valve Disease

Figure 1: Variable factor map demonstrating alignment of each WBC Differential (in black) in relation to Components 1 (x-axis) & 2 (y-axis) (from PCA using case and control data). The association with each component with the supplementary continuous variable age is shown in blue.



Abbreviations: eos = eosinophils, baso = basophils, mono = monocytes, neut = neutrophils, lymph = lymphocytes

Figure 2: Individual factor map demonstrating individual response to components 1 (Dim 1) and 2 (Dim 2) (black dots = control patients, red dots = CHF patients)

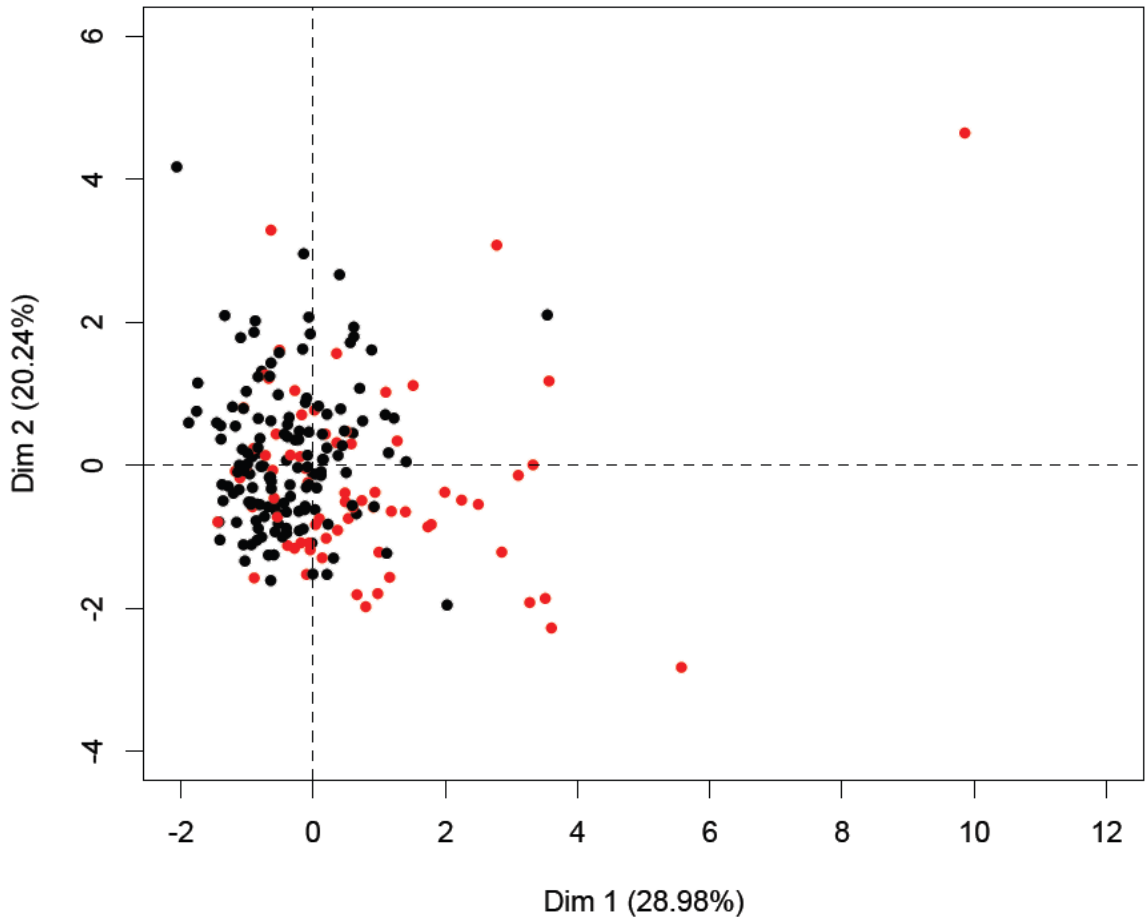
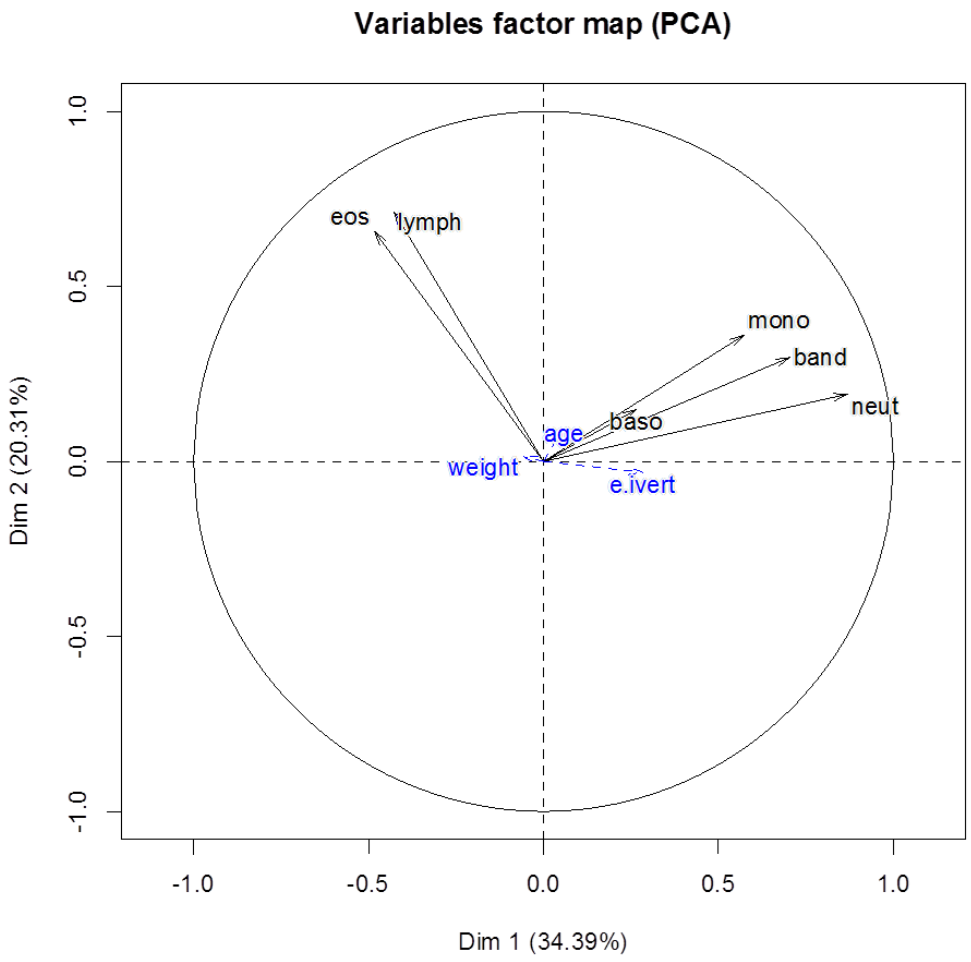

Dim 2 (20.24%)
6
4
2
0
-2
-4
-2
0
2
4
6
8
10
12
Dim 1 (28.98%)

Figure 3: Variable factor map demonstrating alignment of each white cell line in relation to Components 1 & 2 obtained from the PCA using CHF cases. The association with each component with the supplementary continuous variables age, weight and E/IVRT are shown in blue.



Abbreviations: eos = eosinophils, bas = basophils, mono = monocytes, neut = neutrophils, lymph = lymphocytes, E/IVRT = mitral E/IVRT