BMC Veterinary Research

RESEARCH

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Inflammatory and immune variables as predictors of survival in dogs with myxomatous mitral valve disease

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Abstract

Background We aimed to investigate the association between selected inflammatory and immune variables and survival of dogs with myxomatous mitral valve disease (MMVD). We evaluated data of 62 client-owned dogs with MMVD, grouped into preclinical, stable congestive heart failure (CHF) and unstable CHF. Univariate Cox proportional hazards regression analysis was used to quantify the association of white blood cell count, concentrations and percentages of T lymphocytes and their subtypes (T helper lymphocytes, cytotoxic T lymphocytes, double positive T lymphocytes, double negative T lymphocytes) and B lymphocytes with survival. *P* values < 0.1 in individual groups and *P* values < 0.05 in the group of all patients were considered significant. Spearman correlation coefficients between significant covariates were calculated to assess the relationships among variables and with survival.

Results In the preclinical group, percentage of double positive T lymphocytes was negatively associated with survival (hazard ratio (HR) = 2.328; *P* = 0.051). In the unstable CHF, T lymphocyte (HR = 1.613; *P* = 0.085), cytotoxic T lymphocyte (HR = 1.562; *P* = 0.048), double positive (HR = 1.751; *P* = 0.042), and double negative T lymphocyte (HR = 1.613; *P* = 0.096) concentrations were negatively associated with survival, as well as cytotoxic T lymphocyte (HR = 1.502; *P* = 0.007) concentration in the group of all patients. The percentage of T helper lymphocytes was positively associated with survival in the unstable CHF (HR = 0.604; *P* = 0.053) and in the group of all patients (HR = 0.733; *P* = 0.044).

The concentration of cytotoxic T lymphocytes positively correlated with left atrial to aortic ratio (LA/Ao) (rho = 0.259, *P* = 0.037), and peak velocity of early diastolic mitral flow (rho = 0.259, *P* = 0.039), whereas the percentage of T helper lymphocytes negatively correlated with left atrial to aortic ratio (LA/Ao) (rho = -0.212, *P* = 0.090) and early to late mitral flow ratio (rho = -0.232, *P* = 0.072).

Conclusions Cytotoxic T lymphocytes, T helper lymphocytes, double positive and double negative T lymphocytes as well as biomarkers cardiac troponin I, N-terminal pro-B-type natriuretic peptide, C-reactive protein are implicated in the progression of MMVD.

Keywords Canine, CD4 T helper lymphocytes, CD8 cytotoxic T lymphocytes, Cox proportional hazards model, DPT double positive T lymphocytes, DNT double negative T lymphocytes, MMVD

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Background

Myxomatous mitral valve disease (MMVD) accounts for approximately 75% of acquired heart disease in dogs [1], with 30% of cases progressing to congestive heart failure (CHF) [2]. Several known pathophysiological mechanisms are involved in the development and progression of the disease, including the immune system [3, 4]. In dogs with MMVD, an increase in the total white blood cell count (WBC) was observed with an increase in neutrophil granulocyte and monocyte levels in the peripheral blood, especially in the advanced stage of the disease [4, 5]. In addition, a recent study found a higher proportion of cytotoxic T lymphocytes (CD3 + CD8 +) and a lower proportion of T helper lymphocytes (CD3 + CD4 +) in dogs with advanced stages of MMVD. The presence of double positive T lymphocytes (DPT) and double negative T lymphocytes (DNT) was reported for the first time in dogs with MMVD in the same study [6]. T lymphocytes react to myocardial antigens and lead to an inflammatory process through direct cytotoxicity and cytokine production, resulting in impaired cardiac function, damage to cardiac tissue, leading to cardiac fibrosis and ultimately heart failure [7]. In murine models, cytotoxic T lymphocytes have been found to recognize and kill normal cardiomyocytes by activating the Perforin/Granzyme pathway or activating death receptor molecules of the tumor necrosis factor receptor family, leading to a spread of damage and promoting further inflammation [8, 9]. In contrast, CD3 + CD4 + lymphocytes help to regulate the immune response and suppress excessive inflammation to prevent further damage to cardiac tissue [10]. The role of DNT and DPT lymphocytes in heart disease and heart failure is not well understood. Normally, they are known to play a role in autoimmune diseases and cancer [11, 12].

Inflammatory markers may be predictors of survival in heart failure, as higher levels of C-reactive protein (CRP) have been associated with worse outcome in people with heart failure [13, 14]. While some studies have reported the association of WBC and survival in dogs with heart failure [15–17], there are no published studies to date examining the association between lymphocytes of different subtypes (CD3 + CD4 +, CD3 + CD8 +, DPT, DNT) and survival in dogs with MMVD. In addition, there is currently no publication regarding the association of systolic function measured by global longitudinal strain (GLS) in dogs with MMVD with survival and the possible association of GLS with inflammatory and immune variables.

The aim of our study was to investigate the association of inflammatory and immune cells, in particular concentrations and percentages of T lymphocyte (CD3+) subtypes (CD3 + CD4 +, CD3 + CD8 +, DPT, DNT) with survival in dogs with MMVD. In addition, we investigated correlations between selected inflammatory and immune variables and echocardiographic parameters.

Results

In this retrospective study, we included 62 dogs with MMVD that were categorized into three groups according to the American College of Veterinary Internal Medicine (ACVIM) classification [1]: preclinical stage (ACVIM B2; 19), stable CHF (ACVIM C; 24), unstable CHF (ACVIM C and D; 19), comprising 24 females (38.7%) and 38 males (61.3%). At the time of the telephone interview, 12 patients (63.2%) in the ACVIM B2 group were still alive and 7 (36.8%) had died. Of these, 5 (71.4%) died or were euthanized due to heart failure. In the stable CHF group, 6 patients (25%) were still alive and 18 (75%) had died. Of these, 15 (83.3%) died or were euthanized due to heart failure. In the unstable CHF group, only 1 patient (5.3%) remained alive at the time of the telephone interview and 18 (94.7%) patients were dead, of those 17 (94.4%) died or were euthanized due to heart failure. Other causes of death or euthanasia were neoplastic disease (4), pyometra (1) and urethral obstruction (1). The mean age (± standard deviation) of dogs in different stages of MMVD was as follows: preclinical dogs – 10.0 ± 2.1 years (minimum (MIN)-maximum (MAX): 6.0 – 14.2 years), stable CHF – 11.0 ± 1.9 years (MIN–MAX: 7.6 – 14.3 years), unstable CHF – 10.7 ± 1.6 years (MIN–MAX: 7.4 – 13.5 years), and all patients together – 10.36 ± 1.92 years (MIN–MAX: 6.0 – 14.3 years). The median weight of dogs in the respective groups was as follows: preclinical dogs – 7.60 kg (MIN–MAX: 3.0 – 16.8 kg), stable CHF – 7.70 kg (MIN–MAX: 4.4 – 33.0 kg), unstable CHF – 7.60 kg (MIN–MAX: 2.4 – 44.7 kg), and all patients together – 7.60 kg (MIN–MAX: 2.4 – 44.7 kg). Dog breeds included in the study were: Cavalier King Charles Spaniel (13), mix breed dogs (12), Chihuahua (5), Pekingese (4), Maltese (3), Poodle (3), Tibetan Terrier (3), Yorkshire Terrier (3), English Cocker Spaniel (2), Shi Tzu (2), Whippet (2), American Pit Bull Terrier (1), Airedale Terrier (1), Chinese Crested Dog (1), Coton de Tulear (1), Dachshund (1), German Shepherd (1), Italian Greyhound (1), Japanese Chin (1), Pomeranian (1), Staffordshire Terrier (1). Univariate Cox proportional hazard regression models were constructed and tested for each covariate separately for each group (preclinical, stable CHF, unstable CHF) and for all patients. Covariates with either a P value of the Spearman-ρ statistic (with survival time) or the univariate Cox P value or the likelihood ratio P value < 0.1 were potentially linearly associated with log survival time. The results of the univariate Cox proportional hazards regression analysis are shown in Table 1 (preclinical group),

Table 1 Results of univariate Cox proportional hazards regression analysis, preclinical group

Parameter	N	Spearman's ρ statistic	Spearman's ρ statistic *P* value	PH-assumption *P* value	Likelihood-ratio *P* value	Cox HR exp(coef)	Cox HR 95% CI	Cox *P* value
Age (months)*	19	-0.469	0.043	0.61	0.002	3.849	1.539, 9.627	0.004
BCS	18	-0.019	0.941	0.53	0.9	0.973	0.492, 1.923	0.936
Heart rate	19	0.064	0.796	0.25	0.5	0.789	0.398, 1.564	0.497
Murmur	19	-0.193	0.430	0.07	0.4	1.396	0.646, 3.015	0.396
Sex	19	0.165	0.499	0.12	0.6	1.212	0.599, 2.453	0.593
Therapy	19	0.231	0.342	0.23	0.6	0.821	0.382, 1.764	0.612
Weight (kg)	19	0.206	0.397	0.59	0.8	0.898	0.426, 1.893	0.777
LA/Ao	19	-0.013	0.957	0.46	0.6	0.834	0.413, 1.684	0.613
LVIDd	19	-0.068	0.783	0.51	0.9	0.952	0.426, 2.129	0.905
LVIDdN	19	-0.165	0.498	0.56	0.8	0.925	0.428, 1.998	0.843
A (m/s)	19	-0.002	0.994	0.87	0.6	0.829	0.382, 1.799	0.636
E (m/s)+	19	-0.396	0.093	0.76	0.3	1.486	0.709, 3.114	0.295
E/A+	19	-0.442	0.058	0.25	0.1	1.713	0.878, 3.342	0.114
TR PG (mmHg)	14	-0.359	0.208	0.99	0.3	1.457	0.731, 2.905	0.285
CRP (mg/L)	19	0.026	0.920	0.07	0.8	1.087	0.481, 2.454	0.841
cTnI (ug/L)	19	-0.011	0.966	0.59	0.2	1.500	0.789, 2.851	0.216
NT-proBNP (pmol/L)	19	-0.302	0.209	0.88	0.2	1.665	0.886, 3.129	0.113
WBC	19	0.314	0.190	0.14	0.2	0.549	0.231, 1.305	0.174
Neutrophil (%)	19	0.357	0.134	0.92	0.6	0.840	0.434, 1.629	0.606
Neutrophil ($\times 10^9$/L)+	19	0.402	0.089	0.17	0.1	0.496	0.180, 1.362	0.173
Monocyte (%)	19	-0.101	0.681	0.23	0.8	1.099	0.543, 2.224	0.793
Monocyte ($\times 10^9$/L)	19	0.149	0.541	0.10	0.7	0.836	0.365, 1.915	0.671
Total lymphoycte (%)	19	-0.349	0.143	0.51	0.2	1.591	0.740, 3.42	0.234
Total lymphocyte ($\times 10^9$/L)	19	-0.086	0.726	0.73	1	0.999	0.473, 2.111	0.998
NLR	19	0.351	0.141	0.78	0.2	0.561	0.230, 1.368	0.204
CD3 + (%)	19	0.161	0.511	0.75	0.7	1.148	0.520, 2.534	0.733
CD3 + ($\times 10^9$/L)	19	0.014	0.957	0.63	1	1.004	0.493, 2.045	0.992
CD3 + CD4 + (%)	19	-0.207	0.394	0.08	0.7	1.172	0.471, 2.917	0.734
CD3 + CD4 + ($\times 10^9$/L)	19	-0.105	0.668	0.27	0.7	1.148	0.564, 2.338	0.703
CD3 + CD8 + (%)	19	0.175	0.471	0.05	0.9	1.065	0.474, 2.39	0.879
CD3 + CD8 + ($\times 10^9$/L)	19	0.174	0.475	0.35	0.8	0.918	0.436, 1.931	0.822
CD4/CD8	19	-0.188	0.440	0.06	0.2	1.922	0.774, 4.774	0.159
DPT(%)*	19	-0.457	0.049	0.85	0.04	2.328	0.996, 5.444	0.051
DPT ($\times 10^9$/L)	19	-0.361	0.129	0.59	0.4	1.339	0.715, 2.507	0.362
DNT(%)	19	0.276	0.252	0.50	0.2	0.523	0.193, 1.416	0.202
DNT ($\times 10^9$/L)	19	0.142	0.560	0.45	0.5	0.766	0.324, 1.813	0.545
CD45 + CD21 + (%)+	19	-0.404	0.086	0.84	0.4	1.352	0.749, 2.439	0.317
CD45 + CD21 + ($\times 10^9$/L)	19	-0.275	0.253	0.95	0.3	1.445	0.739, 2.826	0.282
GLS aplax (%)*	19	0.414	0.078	0.06	0.03	0.444	0.213, 0.928	0.031
GLS 4-ch (%)*	19	0.737	0.000	0.55	0.004	0.367	0.184, 0.734	0.005
GLS 2-ch (%)	19	0.219	0.369	0.11	0.4	0.734	0.369, 1.462	0.379
GLS Avg (%)*	19	0.553	0.016	0.82	0.004	0.216	0.061, 0.768	0.018

* Covariate with Cox *P* value < 0.1 and PH-assumption *P* value > 0.05, which are considered significant in the univariate Cox analysis

+ Covariates with Spearman's *ρ* statistic *P* value < 0.1, which may also be significantly monotonically associated with the length of survival

Legend: *A* Peak velocity of late diastolic mitral flow, *BCS* Body condition score, *CD3* + Total T lymphocytes, *CD3* + *CD4* + T helper lymphocytes, *CD3* + *CD8* + Cytotoxic T lymphocytes, *CD4/CD8* T helper lymphocytes to cytotoxic T lymphocytes ratio, *CD45* + *CD21* + B lymphocytes, *cTnI* Cardiac troponin I, *CRP* C-reactive protein, *E* Peak velocity of early diastolic mitral flow, *E/A* Early to late mitral flow ratio, *DPT* Double negative T lymphocytes, *DNT* Double negative T lymphocytes, *GLS Aplax* Global longitudinal strain in aplax view, *GLS Avg* Average global longitudinal strain, *GLS 4-ch* Global longitudinal strain in four chamber view, *GLS 2-ch* Global longitudinal strain in two chamber view, *HR* Hazard ratio, *LA/Ao* Left atrial to aortic ratio, *LVIDd* Left ventricular end-diastolic diameter, *LVIDdN* Indexed left ventricular internal end-diastolic diameter, *N* Number of dogs, *NLR* Neutrophil to lymphocyte ratio, *NT-proBNP* N-terminal pro-B-type natriuretic peptide, *PH* Proportional hazard, *TR PG* Tricuspid regurgitation derived pressure gradient, *WBC* White blood cell count

Table 2 Results of univariate Cox proportional hazards regression analysis, stable CHF group

Parameter	N	Spearman's ρ statistic	Spearman's ρ statistic *P* value	PH-assumption *P* value	Likelihood-ratio *P* value	Cox HR exp(coef)	Cox HR 95% CI	Cox *P* value
Age (months)[+]	24	-0.440	0.032	0.04	0.2	1.438	0.847, 2.440	0.178
BCS[*]	20	0.346	0.135	0.83	0.07	0.588	0.327, 1.057	0.076
Heart rate[*]	24	-0.369	0.076	0.50	0.05	1.705	1.003, 2.897	0.049
Murmur	23	0.071	0.749	0.30	0.5	0.825	0.501, 1.359	0.450
Sex[+]	24	-0.536	0.007	0.04	0.02	1.796	1.121, 2.878	0.015
Therapy	23	-0.207	0.344	0.63	0.1	1.559	0.843, 2.882	0.157
Weight (kg)	24	0.030	0.889	0.67	0.8	1.063	0.667, 1.694	0.798
LA/Ao	23	-0.270	0.212	0.85	0.2	1.478	0.777, 2.811	0.234
LVIDd	23	0.021	0.925	0.26	0.8	1.066	0.592, 1.921	0.832
LVIDdN	23	-0.077	0.726	0.11	0.6	1.172	0.595, 2.309	0.646
A (m/s)	22	0.214	0.338	0.04	0.7	0.892	0.534, 1.490	0.663
E (m/s)	23	0.242	0.267	0.13	0.3	0.756	0.440, 1.311	0.323
E/A	22	-0.022	0.924	0.34	0.5	0.786	0.385, 1.606	0.510
TR PG (mmHg)	21	-0.063	0.786	0.47	0.7	1.120	0.626, 2.003	0.704
CRP (mg/L)	24	-0.220	0.300	0.45	0.4	0.832	0.530, 1.306	0.424
cTnI (ug/L)[*]	24	-0.278	0.187	0.95	0.1	1.592	0.979, 2.588	0.061
NT-proBNP (pmol/L)[*]	24	-0.253	0.232	0.28	0.04	1.887	1.067, 3.337	0.029
WBC	24	0.296	0.168	0.30	0.4	0.816	0.531, 1.253	0.352
Neutrophil (%)	24	0.094	0.661	0.58	0.4	0.828	0.524, 1.308	0.418
Neutrophil ($\times 10^9$/L)	24	0.270	0.201	0.46	0.3	0.793	0.516, 1.219	0.291
Monocyte (%)	24	-0.146	0.495	0.64	0.7	1.097	0.700, 1.719	0.687
Monocyte ($\times 10^9$/L)	24	0.070	0.746	0.86	0.7	0.909	0.564, 1.466	0.696
Total lymphocyte (%)	24	-0.140	0.513	0.64	0.3	1.306	0.799, 2.134	0.287
Total lymphocyte ($\times 10^9$/L)	24	-0.112	0.601	0.54	0.8	1.069	0.712, 1.605	0.748
NLR	24	0.126	0.556	0.73	0.4	0.807	0.496, 1.313	0.387
CD3 + (%)	24	-0.164	0.441	0.99	0.4	1.228	0.780, 1.933	0.376
CD3 + ($\times 10^9$/L)	24	-0.309	0.142	0.68	0.2	1.459	0.871, 2.444	0.151
CD3 + CD4 + (%)	24	0.117	0.586	0.89	0.5	0.845	0.520, 1.372	0.495
CD3 + CD4 + ($\times 10^9$/L)	24	-0.188	0.378	0.96	0.4	1.280	0.762, 2.147	0.351
CD3 + CD8 + (%)	24	-0.110	0.609	0.65	0.7	1.108	0.712, 1.725	0.650
CD3 + CD8 + ($\times 10^9$/L)	24	-0.145	0.497	0.32	0.3	1.289	0.812, 2.048	0.282
CD4/CD8	24	0.103	0.632	0.52	0.9	0.949	0.523, 1.720	0.863
DPT (%)	24	0.011	0.961	0.43	1.0	1.001	0.589, 1.702	0.996
DPT ($\times 10^9$/L)	24	-0.189	0.376	0.44	0.2	1.481	0.826, 2.654	0.188
DNT (%)	24	-0.130	0.546	0.72	0.9	1.039	0.679, 1.588	0.861
DNT ($\times 10^9$/L)[+]	24	-0.375	0.072	0.84	0.3	1.257	0.829, 1.906	0.281
CD45 + CD21 + (%)	24	-0.146	0.496	0.08	0.7	1.112	0.684, 1.806	0.669
CD45 + CD21 + ($\times 10^9$/L)	24	-0.106	0.621	0.94	0.9	1.014	0.685, 1.500	0.946
GLS aplax (%)[*]	15	0.307	0.265	0.71	0.06	0.457	0.195, 1.069	0.071
GLS 4-ch (%)	16	0.229	0.391	0.86	0.5	0.756	0.363, 1.575	0.455
GLS 2-ch (%)[*]	15	0.511	0.054	0.93	0.05	0.480	0.221, 1.041	0.063
GLS Avg (%)[*]	16	0.341	0.196	0.60	0.04	0.391	0.151, 1.013	0.053

[*] Covariate with Cox *P* value < 0.1 and PH-assumption *P* value > 0.05, which are considered significant in the univariate Cox analysis

[+] Covariates with Spearman's *ρ* statistic *P* value < 0.1, which may also be significantly monotonically associated with the length of survival

Legend: *A* Peak velocity of late diastolic mitral flow, *BCS* Body condition score, *CD3* + Total T lymphocytes, *CD3* + *CD4* + T helper lymphocytes, *CD3* + *CD8* + Cytotoxic T lymphocytes, *CD4/CD8* T helper lymphocytes to cytotoxic T lymphocytes ratio, *CD45* + *CD21* + B lymphocytes, *cTnI* Cardiac troponin I, *CRP* C-reactive protein, *E* Peak velocity of early diastolic mitral flow, *E/A* Early to late mitral flow ratio, *DPT* Double negative T lymphocytes, *DNT* Double negative T lymphocytes, *GLS aplax* Global longitudinal strain in aplax view, *GLS Avg* Average global longitudinal strain, *GLS 4-ch* Global longitudinal strain in four chamber view, *GLS 2-ch* Global longitudinal strain in two chamber view, *HR* Hazard ratio, *LA/Ao* Left atrial to aortic ratio, *LVIDd* Left ventricular end-diastolic diameter, *LVIDdN* Indexed left ventricular internal end-diastolic diameter, N number of dogs, *NLR* Neutrophil to lymphocyte ratio, *NT-proBNP* N-terminal pro-B-type natriuretic peptide, *PH* Proportional hazard, *TR PG* Tricuspid regurgitation derived pressure gradient, *WBC* White blood cell count

Table 2 (stable CHF group), Table 3 (unstable CHF group), and Table 4 (all patients).

The hazard ratio may be zero despite the statistical significance (small *P* value) of the corresponding covariate. This can arise from large differences in the units of the (non-normalized) covariates and survival time, so our interpretation was primarily based on the P values. This is explained in more detail in the description of the statistical analyses.

The Kaplan–Meier survival curves for the three groups of patients (preclinical, stable CHF, and unstable CHF) are presented in Fig. 1. Every event of interest (death) during the study period is represented by a vertical curve drop. The plotted survival curves, along with the calculated log-rank test *P* value, which is less than 0.0001, clearly confirm the expected outcome, namely that the three groups of patients differ significantly in the probability of death at any given time point. For instance, the Kaplan–Meier curves in Fig. 1 indicate that the probability of surviving 750 days, which is about 25 months, is 89.5% for the preclinical group (17 out of 19 patients), only 50.0% for the stable CHF group (12 out of 24 patients), and merely 15.8% for the unstable CHF group (3 out of 19 patients).

Age was negatively associated with survival in the preclinical group and when all patients were considered as a group.

Total white blood cell count, as well as relative and absolute counts of neutrophils, monocytes, and total lymphocytes, showed no significant association with survival in any group. Similarly, B lymphocytes (CD45 + CD21 +) were not associated with survival in any group.

The concentration of CD3 + lymphocytes was significantly negatively associated with survival in the unstable CHF group (Table 3), whereas no significant association was found in the other groups. The concentration of CD3 + CD8 + lymphocytes was significantly negatively associated with survival, with a higher concentration of CD3 + CD8 + lymphocytes increasing the risk of death by 56.2% (HR = 1.562; 95% CI = 1.003, 2.433; $P = 0.048$) in dogs with unstable CHF and by 50.2% (HR = 1.502; 95% CI = 1.116, 2.022; $P = 0.007$) in the group of all patients (Table 4). In contrast, CD3 + CD4 + lymphocyte percentage was significantly positively associated with survival in both the unstable CHF group and in the group of all patients. In the unstable CHF group, a higher percentage of CD3 + CD4 + lymphocytes reduced the risk of death by 39.6% (HR = 0.604; 95% CI = 0.363, 1.006; $P = 0.053$). In the group of all patients, a higher percentage of CD3 + CD4 + reduced the risk of death by 26.7% (HR = 0.733; 95% CI = 0.542, 0.991; $P = 0.044$).

The percentage of DPT was significantly negatively associated with survival in the preclinical group (Table 1), with higher proportion of DPT lymphocytes increasing the risk of death by 132.8% (HR = 2.328; 95% CI = 0.996, 5.444; $P = 0.051$). In addition, both DPT and DNT lymphocyte concentrations were significantly negatively associated with survival in the unstable CHF group (Table 3), with a higher DPT concentration increasing the risk of death by 75.1% (HR = 1.751; 95% CI = 1.021, 3.003; $P = 0.042$) and a higher DNT concentration increasing the risk of death by 61.3% (HR = 1.613; 95% CI = 0.918, 2.834; $P = 0.096$).

A significant association between cardiac troponin I (cTnI) concentration and increased risk of death was observed in patients with MMVD, with higher cTnI concentration significantly increasing the risk of death in the stable CHF group (Table 2), unstable CHF group (Table 3), as well as in the group of all patients (Table 4). N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration showed a significant negative association with survival only in the stable CHF group. C-reactive protein concentration also had a significant negative association with survival in the all patients group.

Echocardiographic parameters such as left atrial to aortic ratio (LA/Ao), left ventricular end-diastolic diameter (LVIDd), indexed left ventricular internal diameter at end-diastole (LVIDdN), peak velocity of early diastolic mitral flow (E), and early to late mitral flow ratio (E/A) were all significantly negatively associated with survival in the group of all patients (Table 4), whereas E/A and LVIDd were also significantly negatively associated with survival in the unstable CHF group (Table 3).

Global longitudinal strain in aplax view (GLS aplax) and GLS in four chamber view (GLS 4-ch), as well as average GLS (GLS Avg), were significantly positively associated with survival in the preclinical group (Table 1). Similarly, in the group of stable CHF patients (Table 2), GLS aplax, GLS in two chamber view (GLS 2-ch), and GLS Avg were significantly positively associated with survival. Furthermore, in the group of all patients (Table 4), GLS aplax, GLS 4-ch, GLS-2-ch, and GLS Avg were also significantly positively associated with survival. Conversely, GLS 2-ch and GLS Avg were significantly negatively associated with survival in patients with unstable CHF (Table 3). Descriptive statistics of measured parameters are presented in supplementary Table 1.

Spearman's rank correlation coefficients (rho) between significant covariates were calculated to assess the relationships among variables in all groups. However, due to the sample size, we only report the Spearman's rank correlation coefficients in the group of all patients, as shown in Fig. 2. The analysis showed that age weakly positively correlated with cTnI (rho = 0.307, $P = 0.012$) and CD3 + CD8 + lymphocyte concentrations (rho = 0.254,

Table 3 Results of univariate Cox proportional hazards regression analysis, unstable CHF group

Parameter	N	Spearman's ρ statistic	Spearman's ρ statistic *P* value	PH-assumption *P* value	Likelihood-ratio *P* value	Cox HR exp(coef)	Cox HR 95% CI	Cox *P* value
Age (months)$^{+}$	19	-0.429	0.067	0.016	0.2	1.488	0.807, 2.743	0.203
BCS^{+}	16	0.539	0.031	0.047	0.1	0.627	0.355, 1.106	0.107
Heart rate	19	-0.210	0.388	0.37	0.5	1.215	0.724, 2.038	0.462
Murmur	19	-0.326	0.173	0.71	0.1	1.572	0.863, 2.862	0.139
Sex	19	0.000	1.000	0.74	0.8	0.955	0.593, 1.537	0.850
Therapy^{+}	19	-0.407	0.084	0.18	0.2	1.303	0.829, 2.047	0.251
Weight (kg)*	19	0.131	0.594	0.29	0.1	1.758	0.938, 3.294	0.079
LA/Ao	19	-0.345	0.148	0.77	0.1	1.461	0.927, 2.302	0.103
LVIDd*	18	-0.072	0.775	0.10	0.05	1.939	0.972, 3.867	0.060
LVIDdN	18	-0.240	0.338	0.35	0.1	1.651	0.885, 3.082	0.115
A (m/s)	16	0.354	0.179	0.15	0.1	0.542	0.259, 1.133	0.103
E (m/s)	18	0.214	0.393	0.01	0.7	1.090	0.662, 1.796	0.734
E/A*	16	-0.288	0.278	0.86	0.02	2.593	1.143, 5.881	0.023
TR PG (mmHg)	17	0.218	0.400	0.20	0.5	0.834	0.500, 1.391	0.487
CRP (mg/L)$^{+}$	19	-0.335	0.160	0.04	0.06	1.775	1.045, 3.013	0.034
cTnI (ug/L)*	19	-0.533	0.019	0.14	0.01	2.108	1.227, 3.620	0.007
NT-proBNP (pmol/L)	19	-0.236	0.331	0.10	0.1	1.465	0.895, 2.398	0.129
WBC	19	-0.010	0.969	0.52	0.8	1.071	0.602, 1.905	0.816
Neutrophil (%)	19	0.148	0.545	0.76	0.5	0.848	0.500, 1.437	0.540
Neutrophil ($\times 10^9$/L)	19	0.036	0.884	0.44	0.9	1.043	0.574, 1.896	0.891
Monocyte (%)	19	-0.253	0.297	0.01	0.8	1.050	0.662, 1.666	0.835
Monocyte ($\times 10^9$/L)	19	-0.198	0.416	0.05	0.7	1.105	0.634, 1.927	0.725
Total lymphocyte (%)	19	-0.157	0.521	0.69	0.5	1.230	0.717, 2.108	0.453
Total lymphoycte ($\times 10^9$/L)	19	-0.102	0.678	0.40	0.5	1.175	0.730, 1.891	0.508
NLR	19	0.136	0.579	0.38	1.0	0.987	0.528, 1.845	0.968
CD3 + (%)$^{+}$	19	-0.641	0.003	0.004	0.1	1.688	0.865, 3.294	0.125
CD3 + ($\times 10^9$/L)*	19	-0.480	0.038	0.41	0.1	1.613	0.936, 2.779	0.085
CD3 + CD4 + (%)*	19	0.448	0.054	0.70	0.05	0.604	0.363, 1.006	0.053
CD3 + CD4 + ($\times 10^9$/L)	19	-0.264	0.275	0.46	0.8	0.939	0.601, 1.469	0.784
CD3 + CD8 + (%)	19	-0.365	0.124	0.79	0.2	1.435	0.875, 2.353	0.152
CD3 + CD8 + ($\times 10^9$/L)*	19	-0.509	0.026	0.76	0.1	1.562	1.003, 2.433	0.048
CD4/CD8 $^{+}$	19	0.413	0.079	0.68	0.1	0.638	0.362, 1.126	0.121
DPT (%)	19	0.017	0.944	0.32	0.7	1.080	0.699, 1.670	0.729
DPT ($\times 10^9$/L)*	19	-0.351	0.141	0.94	0.05	1.751	1.021, 3.003	0.042
DNT (%)	19	-0.068	0.783	0.77	0.6	1.223	0.630, 2.371	0.552
DNT ($\times 10^9$/L)*	19	-0.389	0.100	0.98	0.1	1.613	0.918, 2.834	0.096
CD45 + CD21 + (%)$^{+}$	19	0.447	0.055	0.01	0.3	0.738	0.436, 1.249	0.257
CD45 + CD21 + ($\times 10^9$/L)	19	0.384	0.105	0.003	0.6	0.874	0.503, 1.520	0.634
GS aplax (%)	14	0.218	0.454	0.19	0.7	0.886	0.486, 1.617	0.694
GS 4-ch (%)	14	-0.301	0.295	0.15	0.2	1.588	0.841, 3.001	0.154
GS 2-ch (%)*	14	-0.560	0.040	0.70	0.03	2.465	1.000, 6.076	0.050
GS Avg (%)*	14	-0.402	0.155	0.13	0.06	2.237	0.861, 5.815	0.098

* Covariate with Cox *P* value < 0.1 and PH-assumption *P* value > 0.05, which are considered significant in the univariate Cox analysis

$^{+}$ Covariates with Spearman's *ρ* statistic *P* value < 0.1, which may also be significantly monotonically associated with the length of survival

Legend: *A* Peak velocity of late diastolic mitral flow, *BCS* Body condition score, *CD3* + Total T lymphocytes, *CD3* + *CD4* + T helper lymphocytes, *CD3* + *CD8* + Cytotoxic T lymphocytes, *CD4/CD8* T helper lymphocytes to cytotoxic T lymphocytes ratio, *CD45* + *CD21* + B lymphocytes, *cTnI* Cardiac troponin I, *CRP* C-reactive protein, *E* Peak velocity of early diastolic mitral flow, *E/A* Early to late mitral flow ratio, *DPT* Double negative T lymphocytes, *DNT* Double negative T lymphocytes, *GLS aplax* Global longitudinal strain in aplax view, *GLS Avg* Average global longitudinal strain, *GLS 4-ch* Global longitudinal strain in four chamber view, *GLS 2-ch* Global longitudinal strain in two chamber view, *HR* Hazard ratio, *LA/Ao* Left atrial to aortic ratio, *LVIDd* Left ventricular end-diastolic diameter, *LVIDdN* Indexed left ventricular internal end-diastolic diameter, *N* Number of dogs, *NLR* Neutrophil to lymphocyte ratio, *NT-proBNP* N-terminal pro-B-type natriuretic peptide, *PH* Proportional hazard, *TR PG* Tricuspid regurgitation derived pressure gradient, *WBC* White blood cell count

$P=0.040$) and weakly negatively with CD3+CD4+lymphocyte percentage (rho=-0.293, $P=0.017$). The left atrium to aorta ratio (rho=-0.212, $P=0.090$), E/A (rho=-0.232, $P=0.072$) and cTnI (rho=-0.288, $P=0.019$) weakly negatively correlated with CD3+CD4+lymphocyte percentage, whereas LA/Ao (rho=0.259, $P=0.037$), LVIDd (rho=0.231, $P=0.067$), E (rho=0.259, $P=0.039$) and cTnI (rho=0.293, $P=0.017$) weakly positively correlated with CD3+CD8+lymphocyte concentration. Furthermore, cTnI concentration moderately positively correlated with CRP (rho=0.673, $P<0.001$). The left atrium to aorta ratio also moderately positively correlated with LVIDdN (rho=0.402, $P=0.001$), E (rho=0.474, $P<0.001$), and E/A (rho=0.469, $P<0.001$).

Discussion

In this study, we investigated the prognostic significance of inflammatory and immune cells and their association with selected cardiac biomarkers and echocardiographic variables. Main findings of our study are a significant negative association of the CD3+CD8+lymphocyte concentration with survival, a significant positive association of CD3+CD4+lymphocyte percentage with survival in both unstable CHF group and in all patient group. In addition, we found a significant negative association with survival for DPT lymphocyte percentage in the preclinical group, as well as DPT and DNT lymphocyte concentration in the unstable CHF group. All these immune cells may be of prognostic importance in dogs with MMVD, particularly in the advanced stage of heart failure.

In our study, age was significantly negatively associated with survival in the preclinical group and the group of all patients. This is consistent with the previous study that has confirmed an association between older age and worse survival in dogs with MMVD [15]. When considering all patients together, we also found that age significantly positively correlated with cTnI and CD3+CD8+lymphocyte concentrations, while it significantly negatively correlated with CD3+CD4+lymphocyte percentage. It has been reported that cTnI concentration increases with myocardial damage, and was associated with age and disease progression [18]. A previous study confirmed that the proportion of CD3+CD8+lymphocytes increase in dogs with MMVD compared to healthy dogs and with the severity of the disease, while CD3+CD4+lymphocyte percentages decrease significantly with both age and the disease severity [6].

No significant association was found between WBC and relative or absolute numbers of neutrophils, monocytes, and total lymphocytes with survival in our study. Increased WBC, neutrophils [14] and decreased total lymphocytes [14, 19, 20] have been reported to be negatively associated with survival in humans with heart failure. In dogs with heart failure, one study confirmed a negative association of total WBC and survival [15] but this and another study found no significant association of neutrophils, monocytes, or total lymphocytes with survival [15, 21]. The lack of significant findings regarding WBC, neutrophils, monocytes and total lymphocytes in our study might be due to the small individual group sizes and/or greater variability in cell counts between individual dogs. High concentrations of inflammatory cells might be observed only in the acute decompensated phase of heart failure [4, 15], however even at this stage some dogs did not show extreme elevation of inflammatory cells, which we suspect is due to individual immune response and ability to react to unstable disease as well as the status of progression in each individual dog. In our study, we found no correlations between WBC and markers of MMVD progression. There is a single report that showed a positive correlation between the ratio of neutrophils to lymphocytes and monocytes to lymphocytes to LA/Ao and the vertebral heart score [17].

Cytotoxic T lymphocyte concentration was significantly negatively associated with survival in dogs with unstable CHF and in the group of all patients, whereas no significant associations of CD3+CD8+lymphocytes were observed in the preclinical and the stable CHF groups. This suggests that these cells may play an important role in disease progression, especially in the decompensated stage. Cytotoxic T lymphocytes play a critical role in the defense against viruses and neoplastic cells. Recently, their role in sterile, low-grade inflammatory diseases such as ischemic heart injury or aortic occlusion has sparked interest in murine models [8]. Experimental studies in mice have shown that CD3+CD8+lymphocytes infiltrate the myocardium as early as two weeks after injury [9], resulting in cardiomyocyte death by CD3+CD8+lymphocyte activation of the Perforin/Granzyme pathway or activation of death receptors molecules of the tumor necrosis factor receptor family [8]. Cardiotoxic T lymphocytes can recognize and kill normal cardiomyocytes, like the autoimmune response observed in viral myocarditis, leading to the spread of damage and promoting further inflammation [9]. Mice lacking functional CD3+CD8+lymphocytes had better cardiac function and overall survival [22].

In our study, a significant positive correlation was found between CD3+CD8+lymphocyte concentration and markers of MMVD progression, including cTnI, LA/Ao, LVIDd, and E, respectively, in the group of all patients. This suggests that CD3+CD8+lymphocytes might be associated with myocardial damage and contribute to cardiac remodeling. The correlation between

Table 4 Results of univariate Cox proportional hazards regression analysis, all patients

Parameter	N	Spearman's ρ statistic	Spearman's ρ statistic *P* value	PH-assumption *P* value	Likelihood-ratio *P* value	Cox HR exp(coef)	Cox HR 95% CI	Cox *P* value
Age (months)*	62	-0.385	0.002	0.63	0.002	1.667	1.200, 2.316	0.002
BCS	54	0.180	0.194	0.12	0.20	0.797	0.576, 1.104	0.172
Heart rate+	62	-0.469	0.000	0.02	0.0006	1.776	1.273, 2.478	0.001
Murmur+	61	-0.424	0.001	0.03	0.004	1.663	1.174, 2.356	0.004
Therapy+	61	-0.315	0.013	0.04	0.02	1.465	1.047, 2.050	0.026
Sex	62	-0.220	0.086	0.18	0.08	1.304	0.971, 1.753	0.078
Weight (kg)	62	0.092	0.479	0.80	0.40	1.161	0.814, 1.655	0.410
LA/Ao*	61	-0.522	0.000	0.39	0.00008	1.998	1.449, 2.754	0.000
LVIDd*	60	-0.257	0.048	0.95	0.01	1.625	1.137, 2.323	0.008
LVIDdN*	60	-0.425	0.001	0.63	0.0007	1.898	1.296, 2.779	0.001
A (m/s)	57	0.054	0.689	0.05	0.6	0.918	0.662, 1.272	0.606
E (m/s)*	60	-0.346	0.007	0.26	0.006	1.553	1.137, 2.121	0.006
E/A*	57	-0.374	0.004	0.24	0.0006	2.023	1.387, 2.951	0.000
TR PG (mmHg)	52	-0.246	0.079	0.74	0.1	1.282	0.951, 1.729	0.103
CRP (mg/L)*	62	-0.327	0.010	0.92	0.006	1.688	1.248, 2.284	0.001
cTnI (ug/L)*	62	-0.526	0.000	0.30	0.0001	1.846	1.432, 2.380	0.000
NT-proBNP(pmol/L)+	62	-0.675	0.000	0.03	0.000001	2.219	1.667, 2.953	0.000
WBC	62	-0.088	0.496	0.15	0.3	1.186	0.839, 1.677	0.335
Neutrophil (%)	62	0.101	0.437	0.56	0.4	0.895	0.674, 1.189	0.445
Neutrophil ($\times 10^9$/L)	62	-0.041	0.754	0.13	0.5	1.134	0.797, 1.614	0.486
Monocyte (%)	62	-0.231	0.071	0.08	0.3	1.171	0.887, 1.546	0.265
Monocyte ($\times 10^9$/L)	62	-0.179	0.164	0.02	0.2	1.273	0.923, 1.754	0.141
Total lymphocyte (%)	62	-0.147	0.255	0.32	0.2	1.235	0.905, 1.685	0.184
Total lymphocyte ($\times 10^9$/L)	62	-0.231	0.071	0.96	0.1	1.235	0.941, 1.621	0.128
NLR	62	0.132	0.307	0.23	0.5	0.882	0.618, 1.258	0.487
CD3 + (%)	62	-0.063	0.625	0.13	0.8	1.051	0.760, 1.454	0.762
CD3 + ($\times 10^9$/L)+	62	-0.251	0.049	0.14	0.1	1.297	0.955, 1.762	0.096
CD3 + CD4 + (%)*	62	0.270	0.034	0.52	0.05	0.733	0.542, 0.991	0.044
CD3 + CD4 + ($\times 10^9$/L)	62	-0.071	0.582	0.58	1	1.008	0.747, 1.361	0.956
CD3 + CD8 + (%)	62	-0.195	0.130	0.86	0.1	1.290	0.962, 1.731	0.089
CD3 + CD8 + ($\times 10^9$/L)*	62	-0.224	0.081	0.67	0.01	1.502	1.116, 2.022	0.007
CD4/CD8	62	0.229	0.074	0.93	0.3	0.832	0.574, 1.207	0.332
DPT (%)	62	0.014	0.914	0.63	0.8	1.047	0.763, 1.436	0.777
DPT ($\times 10^9$/L)	62	-0.173	0.179	0.99	0.3	1.205	0.884, 1.641	0.238
DNT (%)	62	-0.050	0.702	0.33	1	0.992	0.705, 1.397	0.964
DNT ($\times 10^9$/L)	62	-0.209	0.103	0.13	0.4	1.158	0.845, 1.586	0.362
CD45 + CD21 + (%)	62	-0.063	0.628	0.37	0.5	1.122	0.831, 1.515	0.453
CD45 + CD21 + ($\times 10^9$/L)	62	-0.129	0.318	0.21	0.3	1.151	0.892, 1.485	0.279
GS aplax (%)*	48	0.325	0.024	0.31	0.006	0.594	0.412, 0.857	0.005
GS 4-ch (%)*	49	0.259	0.073	0.05	0.05	0.709	0.506, 0.994	0.046
GS 2-ch (%)	48	0.216	0.140	0.80	0.08	0.735	0.524, 1.029	0.073
GS Avg (%)*	49	0.247	0.087	0.21	0.007	0.578	0.383, 0.872	0.009

* Covariate with Cox *P* value < 0.05 and PH-assumption *P* value > 0.05, which are considered significant in the univariate Cox analysis

+ Covariates with Spearman's ρ statistic *P* value < 0.05, which may also be significantly monotonically associated with the length of survival

Legend: A Peak velocity of late diastolic mitral flow, *BCS* Body condition score, *CD3* + Total T lymphocytes, *CD3* + *CD4* + T helper lymphocytes, *CD3* + *CD8* + Cytotoxic T lymphocytes, *CD4/CD8* T helper lymphocytes to cytotoxic T lymphocytes ratio, *CD45* + *CD21* + B lymphocytes, *cTnI* Cardiac troponin I, *CRP* C-reactive protein, *E* Peak velocity of early diastolic mitral flow, *E/A* Early to late mitral flow ratio, *DPT* Double negative T lymphocytes, *DNT* Double negative T lymphocytes, *GLS aplax* Global longitudinal strain in aplax view, *GLS Avg* Average global longitudinal strain, *GLS 4-ch* Global longitudinal strain in four chamber view, *GLS 2-ch* Global longitudinal strain in two chamber view, *HR* Hazard ratio, *LA/Ao* Left atrial to aortic ratio, *LVIDd* Left ventricular end-diastolic diameter, *LVIDdN* Indexed left ventricular internal end-diastolic diameter, *N* Number of dogs, *NLR* Neutrophil to lymphocyte ratio, *NT-proBNP* N-terminal pro-B-type natriuretic peptide, *PH* Proportional hazard, *TR PG* Tricuspid regurgitation derived pressure gradient, *WBC* White blood cell count

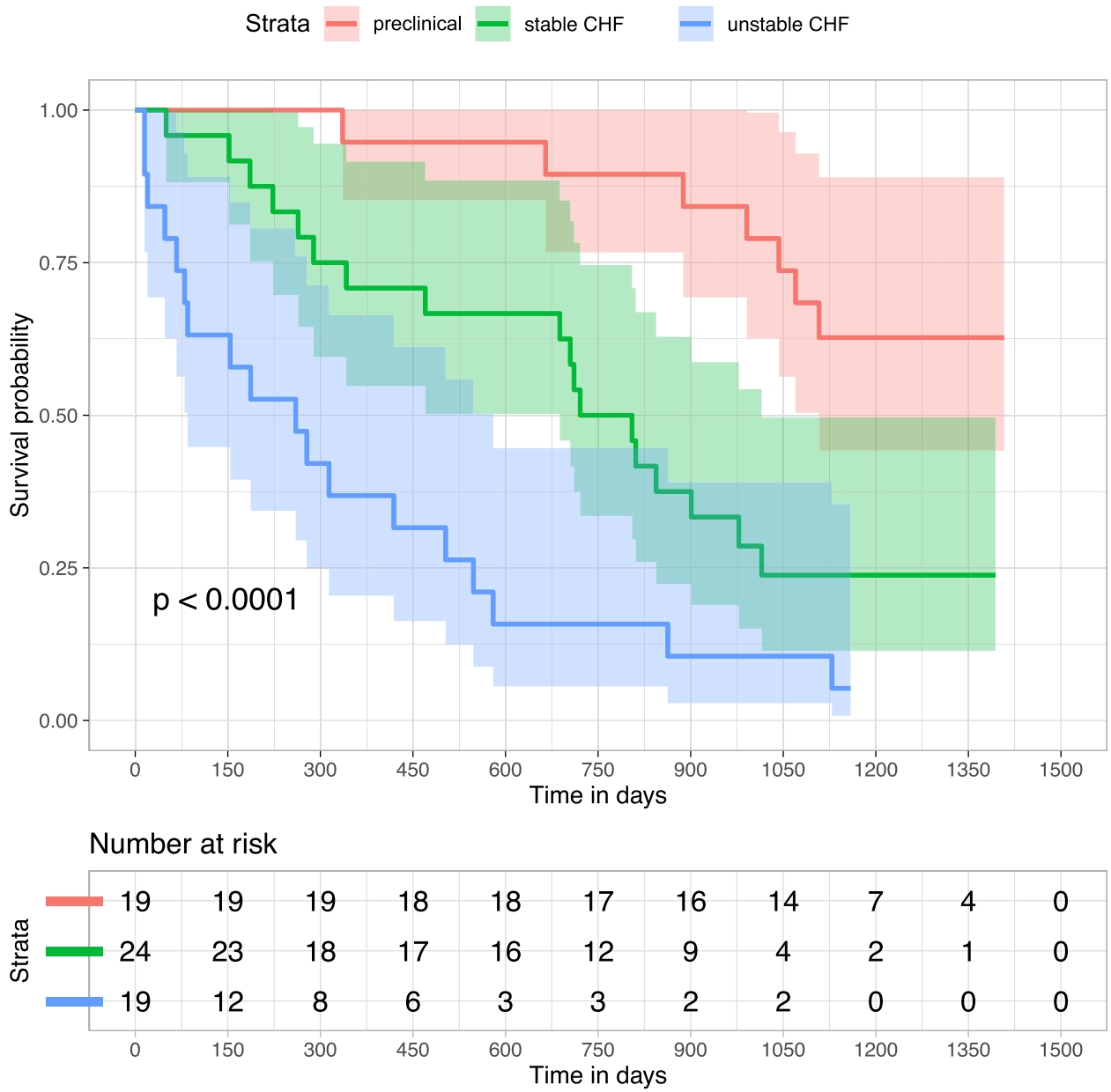

Fig. 1 Kaplan–Meier survival curves for three groups of dogs (with log-rank test P value and risk table)

cTnI and CD3+CD8+lymphocytes has previously been described in human medicine in acute myocardial infarction [23]. Cardiac troponin I is a specific marker of myocardial damage, and a higher cTnI concentration correlates with the severity of myocardial remodeling observed during the progression of heart disease [24]; however, cardiac therapy may affect cTnI levels [25].

On the opposite, CD3+CD4+lymphocytes, especially T regulatory lymphocytes help regulate immune response and suppress excessive inflammation to prevent further damage to the cardiac tissue. In chronic heart failure T regulatory cells can become less effective in controlling inflammation and can contribute to adverse remodeling and fibrosis [10].

In our study, we found a significant positive association between CD3+CD4+lymphocyte percentage and survival in dogs with MMVD in the group of unstable CHF, as well as in the group of all patients. T helper lymphocytes infiltrate the myocardium alongside CD3+CD8+lymphocytes in ischemic and non-ischemic cardiac pathologies and can contribute to fibrosis, hypertrophy, and remodeling of the left ventricle [26, 27]. In

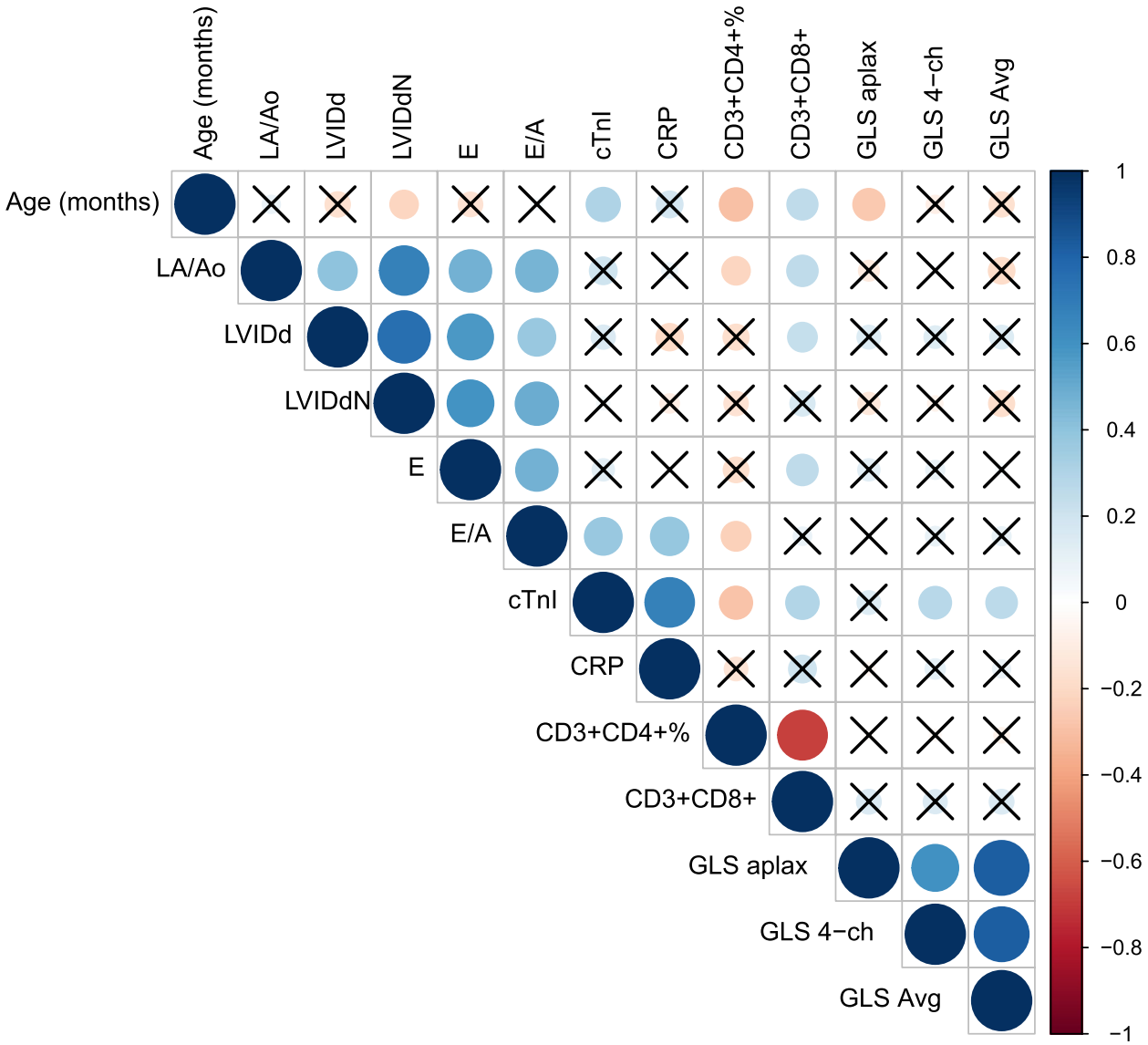

Fig. 2 Spearman's rank correlation coefficients for pairs of significant variables in the group of all patients. No significant correlation ($P > 0.1$) is indicated by a cross. Legend: CD3 + CD4 + %, percentage of T helper lymphocytes; CD3 + CD8 +, cytotoxic T lymphocyte concentration; CHF, congestive heart failure; cTnI, cardiac troponin I; CRP, C-reactive protein; E, peak velocity of early diastolic mitral flow; E/A, early to late mitral flow ratio; GLS aplax, global longitudinal strain in aplax view; GLS Avg, average global longitudinal strain; GLS 4-ch, global longitudinal strain in four-chamber view; LA/Ao, left atrial to aortic ratio; LVIDd, left ventricular end-diastolic diameter; LVIDdN, indexed left ventricular internal end-diastolic diameter

a mouse model of myocardial infarction, depletion of CD3 + CD4 + lymphocytes resulted in a higher total number of leukocytes with greater recruitment of pro-inflammatory monocytes and worse disease outcomes. Meanwhile, the recruitment of CD3 + CD4 + lymphocytes resulted in better wound healing and improved survival outcomes [28].

The CD3 + CD4 + percentage significantly negatively correlated with cTnI concentration, LA/Ao and E/A in the group of all patients. This results suggests downregulation or suppression of these cells with progression of MMVD.

There are no previous reports on the correlations between different lymphocyte subtypes and markers of MMVD progression in dogs. Our results suggest that CD3 + CD4 + and CD3 + CD8 + lymphocytes may play a role in cardiac remodeling and might be associated with the progression of heart disease and the development of heart failure.

Double negative T lymphocytes are a small population of CD3 + lymphocytes that are both CD4 and CD8 negative. While they constitute a small population of CD3 + lymphocytes, they play an important role in autoimmune diseases and cancer. Theoretically, DNT lymphocytes are early CD3 + lymphocytes that lack CD4 and CD8 receptors, and they escape the thymus before their final development. However, there is evidence that they can also develop from peripheral CD3 + CD8 + lymphocytes and produce proinflammatory cytokines such as interleukin (IL) 2, IL-4, IL-A17, and tumor necrosis factor α [12]. In contrast, there is a subset of DNT lymphocytes that exerts a similar regulatory function to CD3 + CD4 + lymphocytes and play a role in immune tolerance and immunomodulatory activity [29, 30].

On the other hand, DPT lymphocytes possess both CD8 and CD4 receptors, but their role is poorly defined. Studies in humans and mice have shown that their number is augmented in various diseases such as cancer, autoimmune diseases, rheumatoid arthritis, and chronic inflammatory diseases [11, 31, 32]. Their role is reportedly very similar to that of CD3 + CD4 + lymphocytes, but the population of DPT is very heterogeneous and can produce effector molecules, like tumor necrosis factor α and interferon γ, as well as some lytic enzymes such as perforin and granzyme B [32].

In our study, a significant negative association of DPT lymphocyte percentage with survival in the preclinical group was found. In addition, both DPT and DNT lymphocyte concentrations were significantly negatively associated with survival in the unstable CHF group. Apart from a previous study [6], which investigated peripheral lymphocyte subtypes, there are no other reports on the involvement of DNT and DPT lymphocytes in heart failure in dogs. This is the first report of their effects on survival in dogs with MMVD, and further studies are needed to better understand the onset of action of DPT and DNT lymphocytes in heart diseases.

In our study, the concentration of CRP was significantly negatively associated with survival in dogs with MMVD in the all patients group, which suggests that inflammation is a negative prognostic factor. No survival studies have been published on this topic in dogs, apart from some human studies that showed CRP concentration as a predictor of hospitalization and mortality in patients with heart failure [13, 14, 33]. In addition, CRP concentration significantly positively correlated with cTnI concentration in all patients group, which suggests that increased inflammation results in increased myocardial damage and disease progression. Our study found no significant correlation between CRP concentration and inflammatory cells in any group of dogs presumably due to variability of the results of the pertinent cells in individual groups as well as relatively small patients' groups. C-reactive protein is an acute-phase protein synthesized by the liver during an acute phase of the inflammatory process [34]. Studies in dogs with heart failure showed an increase in CRP concentration compared to healthy dogs [4, 35, 36].

The concentration of NT-proBNP was negatively associated with survival in the stable CHF group. Our results are consistent with previous studies showing a strong association between NT-proBNP concentration and survival in dogs with heart failure [3, 37]. However, we found no significant correlations between NT-proBNP and inflammatory and immune cells, which could be due to the large variation in NT-proBNP concentration between patients. Natriuretic neuropeptides are produced in the atria and ventricles of the heart and are stimulated and released in larger quantities at the time of cardiac wall stress [38]. The concentration of NT-proBNP can vary greatly between individual patients and has been shown that age, gender and renal function can influence the concentration of this blood cardiac marker [39, 40]. A certain intra-individual variability in NT-proBNP concentration has also been described, with the concentration in a single patient varying by up to 50% within a week of blood sampling [41].

In our study, we looked for a possible association between GLS and the survival of patients with different stages of MMVD. Our results showed that GLS measurements were significantly positively associated with survival in the preclinical, stable CHF group, and in the all patients group, whereas GLS becomes significantly negatively associated with survival with disease progression in the unstable CHF group where systolic function is worsening. No significant correlations between GLS and inflammatory or immunological variables were found in our study. Dogs in the moderate stage of MMVD exhibit increased left ventricular load dependent functional parameters such as shortening fraction, left ventricular ejection fraction, and GLS, whereas in severe stages of the disease, due to hemodynamic overload and an increase in left ventricular systolic diameter, these parameters show lower values, corresponding to a decrease in systolic function [42]. This was first described in a publication by Wess [43], indicating a dynamic change in left ventricular function at different stages of MMVD. In human patients with primary mitral regurgitation, preoperative GLS measurements have been shown to be predictors of long-term survival, with patients with reduced GLS having worse survival outcomes and worse ejection fraction after mitral valve surgery [44, 45]. In chronic ischemic cardiomyopathy in humans, reduced GLS was also found to be a predictor of worse survival outcome [46]. We have not found reports in the literature on the association of GLS and survival in dogs with MMVD.

Limitations

The small number of dogs in each group presents a limitation of this study. As the number of dogs in our study was rather small, the Cox analysis would not be performed reliably with too many variables/predictors at once.

Conclusion

In summary, our study has shown that T helper cells were positively associated with survival, whereas cytotoxic T cells, DPT, and DNT lymphocytes and markers of disease progression (NT-proBNP, cTnI) and marker of inflammation (CRP) were negatively associated with survival in dogs with MMVD.

We observed a significant positive correlation between cytotoxic T cells and echocardiographic variables of disease progression as well as cTnI, and a negative correlation between T helper cells and markers of disease progression and cTnI, further indicating the involvement of CD3+lymphocyte subtypes in the pathophysiology of MMVD.

This study suggests that the immune system may play an important role in canine MMVD and development of heart failure. Future studies could help to better understand the immune pathways involved in the progression of MMVD in dogs and potentially improve the prognosis and outcome of the disease.

Methods

Study population

We conducted a retrospective analysis of medical records from 62 client-owned dogs in various stages of MMVD that received treatment at the cardiology department of the University Small Animal Clinic between August 2018 and November 2019. These dogs had previously participated in a study investigating peripheral blood lymphocyte subtype concentrations and percentages. At the time of diagnosis, the dogs were divided into three groups: preclinical stage (ACVIM B2; 19), dogs with stable CHF (ACVIM C, 24), and dogs with unstable CHF (ACVIM C and ACVIM D, 19). All patients underwent a thorough clinical examination, echocardiography, electrocardiogram, chest radiographs (all dogs in CHF), and blood sampling at the time of enrollment in the study [6].

Dogs in stable CHF were clinically comfortable at rest and normal activity, had no presence of active pulmonary edema on radiographs at the inclusion in the study, but had a history of pulmonary edema. Dogs with unstable CHF had have active pulmonary edema on the radiographs and had showed clinical signs such as cough, dyspnea/tachypnea at the time of examination and were tachycardic. In the group of unstable CHF were also dogs with refractory MMVD, needing frequent adjustments of therapy due to high intracardiac pressure and persistence of pulmonary edema. Dogs that had received antibiotic treatment or other immunosuppressive medication in the month prior to blood sampling were excluded from participation in the study. In addition, dogs with other systemic diseases such as endocrine, metabolic or neoplastic diseases were not considered for participation in the study [6].

Echocardiography

Echocardiographic examinations were performed in all patients between August 2018 and September 2019 [VIVID E9, General Electric Healthcare]. All patients underwent 2D, M-mode, color Doppler, spectral and tissue Doppler analyses. Simultaneous ECG recordings were made for all cases, and measurements were performed offline [EchoPAC, General Electric Healthcare].

We additionally performed GLS measurements using archived echocardiographic images to assess systolic function of the left ventricle in three views: GLS aplax, GLS 4-ch, and GLS 2-ch. Endocardial margin was manually tracked for each of the three views, and dedicated software was used for calculations [AFI, GE Healthcare]. Global longitudinal strain average was calculated by the program using at least two of the three views if all three were not available in a patient. All strain analyses were performed by a single investigator (A.D.P.) to avoid inter-investigator variability.

Blood analyzes

Blood analyzes were performed at the time of enrollment in the previous study [6] and consisted of routine hematological and biochemical tests performed at the in-house laboratory using an automated laser-based hematology analyzer (ADVIA 120, Siemens, Munich, Germany) and an automated biochemistry analyzer (RX Daytona, Randox, Crumlin, United Kingdom).

N-terminal pro-B-type natriuretic peptide concentrations (pmol/L) were measured in IDEXX BioAnalytics Laboratory (IDEXX Laboratories, Ludwigsburg, Germany), cTnI concentrations (µg/L) with a high-sensitivity immunoassay (ADVIA Centaur TnI-Ultra; Siemens) and CRP concentrations (mg/L) with a canine-specific ELISA (Canine CRP ELISA; Alpco, Salem, New Hampshire).

Flow cytometry was performed on fresh whole blood samples withing 24 h of collection using a FACSCanto II flow cytometer (BD Biosciences, San Jose, California) with FACSDiva software, version 8.0.1 (BD Biosciences) to determine the percentages CD3+, CD3+CD4+, CD3+CD8+, DPT, DNT and CD45+CD21+lymphocytes. Monoclonal rat and mouse anti-canine antibodies against CD3 (clone CA17.2A12), CD4 (clone YKIX302.9), CD8 (clone YCATE55.9) in a mix (CD3:FITC/CD4:RPE/CD8:Alexa Fluor 647, ref: TC014), CD45 (clone

YKIX716.13; Alexa Fluor 488, ref: MCA1042A488), and CD21 (clone CA2.1D6; Alexa Fluor 647, ref: MCA1781A647) manufactured by Bio-Rad Laboratories Inc (Hercules, California), were used.

The blood sampling procedure and analyzes was performed at the time of inclusion in the previous study and are described in detail in the previous publication [6].

Patient status

Patient status (deceased/alive) and the cause of death were obtained from medical records at the small animal clinic or through a telephone interview with the owner. The telephone interviews with the owners took place from July 4, 2022 to July 19, 2022. Dogs that were alive at the time of our telephone conversation were censored.

Statistical analyzes

Survival analysis was performed in dogs with MMVD to examine survival from study entry to death. Dogs that were alive at the time of the telephone call were censored. Survival probabilities were estimated using the non-parametric Kaplan–Meier method, and survival functions/curves were compared using the non-parametric log-rank hypothesis test [47].

Univariate Cox proportional hazard regression analysis was performed in each of the three groups of patients with heart disease (preclinical, stable CHF, unstable CHF) and in the group of all patients [48]. A hazard ratio greater than 1 indicates a covariate that is positively associated with event probability (death) and thus negatively associated with survival. We subjected our Cox models to a likelihood ratio test, from which we concluded that a Cox model is statistically significant if the estimated *P*-value is below the chosen significance level. Since *P* values are larger when samples are small (as in our case), we chose to consider $P<0.1$ as significant within each group and $P<0.05$ in the group of all patients.

To ensure the validity of the Cox models, we checked (a) the linear relationship between log survival time and the covariates by estimating the *P* values of the Cox regression models and the Spearman correlation coefficients between survival time and each covariate, and (b) the fulfillment of the proportional hazards (PH) assumption of the covariates by testing the independence ($P>0.05$) between the corresponding set of scaled Schoenfeld residuals and time. It should also be noted that the covariates were normalized to z-scores (i.e., scaled by subtracting the mean and dividing by the standard deviation) before performing the Cox proportional hazard regression analyzes due to the very different means and spreads of the covariates and survival time. Z-score normalization rescales data to have a mean of 0 and a standard deviation of 1. Since normalization does not affect the significance (*P*-values) of the predictors—it only affects the point estimates (HR) of the regression coefficients and produces a smaller standard error of the estimates—our interpretation of the results of the Cox proportional hazard regression analyzes is mainly based on the *P*-values. When the HR values or the corresponding hazards of death are given as percentages, they always refer to the normalized values and therefore to the [-1,1] scale. Spearman correlation coefficients between significant covariates were calculated to assess the relationships among variables. The strength of the observed correlation rho was interpreted as written: 0.00–0.10: negligible; 0.10–0.39: weak; 0.40–0.69: moderate; 0.70–0.89: strong; and 0.90–1.00: very strong [49].

All the statistical analyzes were performed using the program R, version 4.3.2 [50].

Abbreviations

ACVIM	American College of Veterinary Internal Medicine
CD3 +	T lymphocytes
CD3 + CD4 +	T helper lymphocytes
CD3 + CD8 +	Cytotoxic T lymphocytes
CD45 + CD21 +	B lymphocytes
CHF	Congestive heart failure
CRP	C-reactive protein
cTnI	Cardiac troponin I
DNT	Double negative T lymphocytes
DPT	Double positive T lymphocytes
E	peak velocity of early diastolic mitral flow
E/A	Early to late mitral flow ratio
GLS	Global longitudinal strain
GLS aplax	Global longitudinal strain in aplax view
GLS Avg	Average global longitudinal strain
GLS 4-ch	Global longitudinal strain in four chamber view
GLS 2-ch	Global longitudinal strain in two chamber view
HR	Hazard ratio
IL	Interleukin
LA/Ao	Left atrial to aortic ratio
LVIDd	Left ventricular end-diastolic diameter
LVIDdN	Indexed left ventricular internal diameter at end-diastole
MMVD	Myxomatous mitral valve disease
NT-proBNP	N-terminal pro-B-type natriuretic peptide
WBC	Total white blood cell count

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04266-7.

Supplementary Table 1.

Acknowledgements

The authors would like to thank Mr. Phillip Nagel for language review of this manuscript.

Authors' contributions

M.C. organized the data, interpreted the results, wrote and revised the manuscript; N.D. interpreted the results, revised the manuscript; A.N.S. hypothesis generation and experimental design, interpreted the results, wrote and revised the manuscript; M.H. statistical analyses, interpreted the results, wrote and revised the manuscript; K.P. interpreted the results, revised the manuscript; A.I. revised the manuscript; A.D.P. hypothesis generation and experimental design, interpreted the results, wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials
Research data are available upon reasonable request.

Declarations

Ethics approval and consent to participate
All procedures complied with the applicable Slovenian governmental regulations (Animal Protection Act, The Official Gazette of the Republic of Slovenia, 43/2007). Authors declare human ethics approval was not needed for this study. All dog owners gave their informed consent to participate in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 7 June 2024 Accepted: 3 September 2024
Published online: 28 September 2024

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