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Research Article

Novel Characterisation of Baseline Post-Mortem Myocardial Lymphocytic Infiltrate to Avoid Misdiagnosis of Lymphocytic Myocarditis

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Abstract

In current pathology literature there is no established baseline for lymphocytic infiltrate in myocardial tissue. In post-mortem cases, pathologists may misdiagnose viral or other lymphocytic myocarditis where lymphocytic infiltrate is identified. An established baseline for myocardial lymphocytic infiltrate would help pathologists avoid over- or misdiagnosis of lymphocytic myocarditis. To our knowledge this is the first study to undertake this investigation. Sections were from formalin-fixed paraffin-embedded post-mortem heart muscle (n = 100; 50 heart disease and 50 non-heart disease cases). Immunohistochemistry was applied to identify lymphocyte sub-types including T-cells, T-helper cells, Natural Killer cells, B-cells and degree of myocardial fibrosis (CD3, CD4, CD8, CD79a and CD34, respectively). No cases had clinical or laboratory evidence of viral myocarditis or other diseases that cause lymphocytic myocarditis. Cases were blindly reviewed by consultant histopathologist and senior medical scientist for number of lymphocytes/mm^2 away from areas of fibrosis. Fibrosis was graded from 1+ (mild) to 3+ (marked). Chi-Square and T-tests were performed. Results confirm a baseline range lymphocytic infiltrate in non-lymphocytic myocarditis cases. There was no statistically significant difference between heart disease and non-heart disease cases. There was a trend for more CD8-positive Natural Killer T-cells and CD34-positive interstitial fibrosis in heart disease cases. The range and average of CD3, CD4, and CD8-positive T lymphocytes are similar in heart disease and non-heart disease and establish an upper threshold of 21-40 T lymphocytes per mm^2 with an average of 12.5 T lymphocytes per mm^2. While further research should include a larger sample and ideally include cases of viral or other lymphocytic myocarditis for comparison, this study reports a baseline range of lymphocytes in myocardial tissue above which viral or other lymphocytic myocarditis can be increasingly considered in post-mortem cases.

Keywords: Lymphocytic Myocarditis; Lymphocytic Infiltrate; Immunohistochemistry; T Cell Subsets; Heart Disease; Post-Mortem Myocardium

Introduction

Lymphocytic myocarditis is a general term given to inflammation of the myocardium and can be clinically either acute or chronic. It is caused most commonly by viral infection including enterovirus, adenovirus, influenza, HHV-6, and coxsackievirus. Non-viral causes include bacterial and fungal infections, such as *Corynebac-*

terium diphtheria, *Neisseria meningococcus*, *Borrelia* and *Candida.* Other causes include status post viral and streptococcal infections, systemic lupus erythematosus (SLE), drug hypersensitivity, and cardiac transplant rejection. Chronic heart disease is often associated with chronic inflammatory myocardial infiltrate, including chronic myocardial ischaemia. It should be noted that lymphocytes may be seen in myocardium in lymphoma cases [1]. Lymphomas typically are comprised of B lymphocytes whereas lymphocytic infiltrates are typically T lymphocytes, for which immunohistochemistry is necessary for distinction.

Lymphocytic myocarditis is not a common diagnosis whereas heart disease is one of the leading causes of death in the world [2]. Both will cause extravasation of lymphocytes into myocardium. Lymphocytic myocarditis and sub-chronic and chronic ischaemic heart disease can be similar in histologic appearance and true cases of lymphocytic myocarditis can be over- or underestimated [3]. A standardised method needs to be followed to definitively diagnose lymphocytic myocarditis and this should include knowledge of baseline lymphocytes in myocardium in both heart disease and non-heart disease cases. In the current literature there is no established baseline for lymphocytic infiltrate in myocardial tissue, and pathologists may misdiagnose viral or other lymphocytic myocarditis in post-mortem cases where lymphocytic infiltrate is identified [4]. An established baseline for myocardial lymphocytic infiltrate in non-lymphocytic myocarditis cases would be extremely beneficial to pathologists when assessing post-mortem myocardium.

To establish a baseline lymphocytic infiltrate in myocardial tissue we evaluated sections from formalin-fixed paraffin-embedded post-mortem heart muscle in chronic ischaemic heart disease and non-heart disease related deaths (n = 100; 50 heart disease and 50 non-heart disease cases). No cases had clinical or laboratory evidence of viral or other infectious myocarditis, connective tissue disease, collagen vascular disease/vasculitic syndromes, or lymphoma. No cases had recent new medications prescribed. Immunohistochemistry was applied to myocardial tissue to identify lymphocyte sub-types including T-cells, T-helper cells, Natural Killer cells, B-cells and degree of myocardial fibrosis (CD3, CD4, CD8, CD79a and CD34, respectively). Cases were blindly reviewed by consultant histopathologist and senior medical scientist for number of lymphocytes/mm^2 away from areas of fibrosis. Fibrosis was graded from 1+ (mild) to 3+ (marked). Chi-Square and T-tests were performed. Results confirm a baseline lymphocytic infiltrate in non-lymphocytic myocarditis cases and establish an upper threshold of 40 lymphocytes per mm^2. While there was no statistically significant difference between heart disease and non-heart disease cases, there was a trend for more CD8-positive Natural Killer T-cells in heart disease cases. These findings will benefit pathologists and decrease the incidence of diagnostic error, specifically overdiagnosis of lymphocytic myocarditis as a cause of death.

Methods

A total of 100 postmortem cases were selected and grouped as heart disease related and non-heart disease related deaths. Heart disease cases showed evidence of chronic ischaemic heart disease including scarring fibrosis and chronic inflammatory infiltrate. Non-heart disease related deaths were suicides and road traffic accidents. Of these 97 (heart disease n = 54, non-heart disease n = 43) were suitable for study. Samples were chosen using a database search of the laboratory information system (LIS). Heart disease patients had a cause of death relating to chronic cardiovascular disease and non-heart disease patients had cause of death unrelated to cardiovascular disease. Sections were cut from blocks containing left ventricular wall myocardium at the level of anterior papillary muscles at 3-4μm and baked at 60°C and subsequently stained on the automated Ventana XT platform using routine antibodies. The antibody protocols have been optimised locally to ensure unmasking of antigen binding sites, masked by the covalent bonds formed during fixation in 10% formalin. CD3 (2GV6) Rabbit Monoclonal Primary antibody was used to identify T cells. CD4 (SP35) Rabbit Monoclonal Primary antibody was used to identify helper induced T-lymphocytes. CD8 (SP57) Rabbit Monoclonal Primary antibody was used to detect the glucoprotein CD8 on cytotoxic/suppressor T-lymphocytes. CD79a (SP18) Rabbit Monoclonal Primary antibody was used to identify proteins expressed on the surface of B lymphocytes. CD34 (QBEnd/10) Mouse Monoclonal antibody was used to qualitatively identify CD34 which highlights fibrous tissue. Post immunohistochemistry the slides were washed in detergent and dipped in 99% ethanol before being placed on the DRS® stainer for dehydrating and clearing post immunohistochemistry. Total quantity of lymphocytes was counted for CD3, CD4, CD8, CD79, using a magnification of X40 and examining four fields, (1mm^2). The CD34 results were graded 1+ (mild), 2+ (moderate), 3+ (severe) fibrosis. Data was anonymised and reviewed blindly. Statistical analysis was performed using R studio (version 1.3.1073) run-

ning R (version 4.0.2). Data manipulation was performed using the dplyr package (version 1.0.1) and graphing performed using ggplot2 (version 3.3.2).

Results

Lymphocyte quantities were grouped as follows: 0, 1-20 and 21-40. They were quantified at a magnification of x40 examining four fields = 1mm^2. Fibrosis was graded 1+, 2+, and 3+ (Table 1). Although heart disease cases had a greater number of lymphocytes this was not statistically significant. CD8 showed a strong trend just under the level of significance. The CD79a and the CD34 positivity were not significant. In relation to counting cells, slightly greater CD3, CD4 and CD8 positivity was seen in heart disease versus non-heart disease cases. Increased fibrosis was more prominent in heart disease cases than in non-heart disease cases. There were marginally more cases lacking B cells (CD79a) in heart disease cases compared with non-heart disease. In non-heart disease cases lymphocytes per mm^2 were found to be CD3 m = 12, CD4 m = 7, CD8 m = 5, CD79a m = 1, CD34 grade m = 1+, and averages of CD3 m = 13, CD4 m = 7, CD8 m = 7, CD79a m = 2, CD34 grade m = 2+ in heart disease patients.

	CD3		CD4		CD8		CD79a		CD34	
Lymphocytes	**HD**	**NHD**	**HD**	**NHD**	**HD**	**NHD**	**HD**	**NHD**	**HD**	**NHD**
0	-	-	-	-	3	2	19	18		
1-20	44	40	53	44	48	41	24	26		
21-40	10	4	1	-	3	1	-	-		
Fibrosis 1+									19	38
Fibrosis 2+									26	6
Fibrosis 3+									9	-

Table 1: Lymphocytes quantified, heart disease (HD) and non-heart disease (NHD) cases.

In heart disease cases the number of CD3+ lymphocytes present (n) ranged from 1-39, with a mean of m = 13. There were 0 cases with no lymphocytes, 44 cases where n = 1-20 and 10 cases where n = 21-40. The number of CD4+ lymphocytes ranged from 1-27, m = 7. There were 0 cases with no lymphocytes, 53 cases where n = 1-20 and 1 case where n = 21-40. The number of CD8+ lymphocytes ranged from 0-35, m = 7. There were 3 cases with no lymphocytes, 48 cases where n = 1-20 and 3 cases where n = 21-40. CD79a positive cells were consistently low or absent, m = 2, 19 cases where n = 0, 24 cases where n = 1-20. CD34 showed evidence of fibrosis at 1+ in 19 heart disease cases, 2+ in 26 heart disease cases and 3+ in 9 heart disease cases, m = 2+ (Figures 1-3).

The number of CD3+ lymphocytes present (n) in non-heart disease cases ranged from 5-34, and m = 12. There were 0 cases with no lymphocytes, 40 cases where n = 1-20 and 4 cases where n = 21-40. The number of CD4+ lymphocytes ranged from 2-15, m = 7. There were 0 cases with no lymphocytes, 44 cases where n = 1-20.

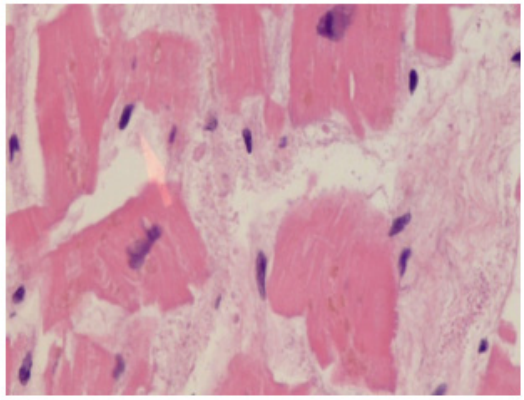

Figure 1: Lymphocytes in heart disease myocardium (H and E) (x 40).

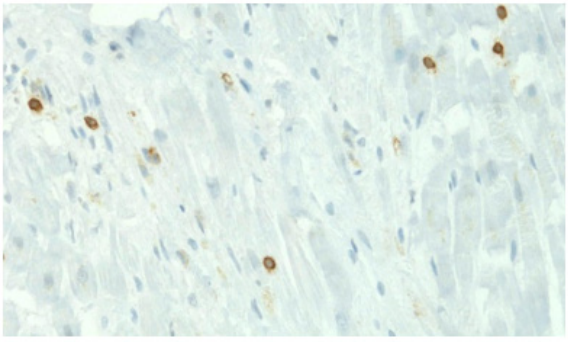

Figure 2: CD3 + Lymphocytes in heart disease myocardium (x 40).

Figure 3: CD34 + Fibrosis in heart disease myocardium (x 40).

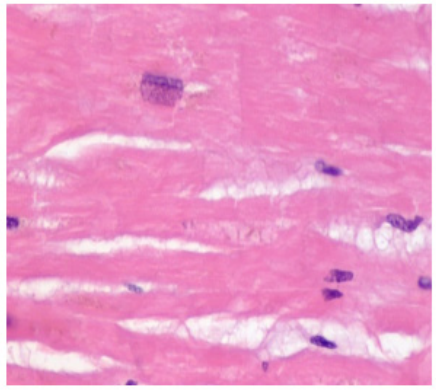

Figure 4: Lymphocytes in non-heart disease myocardium (H and E) (x 40).

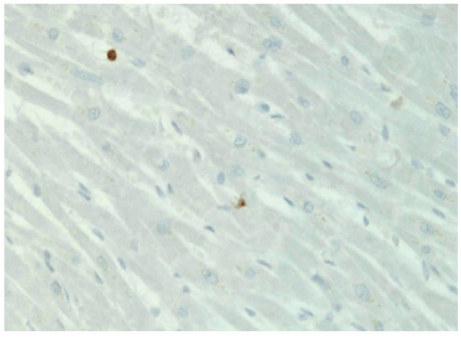

Figure 5: CD3 + Lymphocytes in non-heart disease myocardium (x 40).

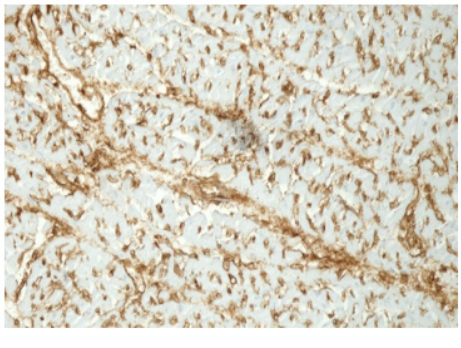

Figure 6: CD34 + Fibrosis in non-heart disease myocardium (x 40).

The number of CD8+ lymphocytes ranged from 0-22, m = 5. There were 2 cases with no lymphocytes, 41 cases where n = 1-20 and 1 case where n = 21-40. The CD79a positive cells showed 18 cases where n = 0, 26 cases where n = 1-20, m = 1. CD34 positivity is scored at an average of 1+ for non-heart disease cases, with 38 cases scoring 1+ and 6 cases scoring 2+, m = 1+ (Figures 4-6).

Patients were matched for age and gender (Table 2). From the above groups 24 matched pairs were found and these provide the basis for the statistical analysis. General descriptive statistics were calculated for each cell count (CD3, CD4, CD8 and CD79a number per category, mean and standard deviation). Reference ranges of expected values with a 95% confidence interval (CI) were constructed for each cell type (Table 3). Statistical analysis

showed very little difference between heart disease and non-heart disease groups. There is a trend to suggest that CD8 positive cells are higher in diseased states and CD4 positive cells are lower.

	Age group	HD/Sex (male, female)	NHD/Sex (male, female)
Group 1	20-29	0	6 (m = 6)
Group 2	30-39	0	9 (m = 6, f = 3)
Group 3	40-49	4 (m = 3, f = 1)	6 (m = 1, f = 5)
Group 4	50-59	7(m = 7)	7 (m = 7)
Group 5	60-69	16 (m = 12, f = 4)	7 (m = 7)
Group 6	70-79	18 (m = 11, f = 7)	7 (m = 3, f = 4)
Group 7	80-89	8 (m = 3, f = 5)	2 (m = 2)
Group 8	90-99	1 (f = 1)	0

Table 2: Heart Disease (HD) and non-heart disease (NHD) groups, age- and gender- matched.

					Reference Range with 95% CI	
Antibody	Disease state	n	Mean	SD	Lower	Upper
CD3	NHD	24	11.46	5.35	9.32	13.60
	HD	24	12.96	8.34	9.62	16.30
CD4	NHD	24	6.42	2.48	5.43	7.41
	HD	24	5.83	3.32	4.50	7.16
CD8	NHD	24	4.08	3.41	2.72	5.44
	HD	24	6.50	6.27	3.99	9.01
CD79a	NHD	24	0.88	1.08	0.45	1.31
	HD	24	1.08	1.35	0.54	1.62

Table 3: Statistical analysis of 24 matched pairs and lymphocyte immunohistochemistry.

A series of paired *t*-tests were performed on the cell counts between the heart disease and non-heart disease groups. As there were less than 30 cases per group a Shapiro-Wilk normality test was performed on the differences between paired samples to ensure that these differences were normally distributed. In one comparison (CD3) normality was violated and the non-parametric equivalent of the *t*-test, the Wilcoxon signed-rank test was performed. In the other comparisons the results of their Shapiro-Wilk normality tests were not significant, so *t*-tests were performed as appropriate (Table 4). The non-heart disease cases contained many patients < 40 years of age while the heart disease group contained mostly patients > 40 years of age. It was possible to match 24 pairs. CD8 showed a strong trend associated with heart disease.

	p-value		
Antibody	Shapiro-Wilk	Paired *t*-test	Wilcoxon signed rank
CD3	0.03*		0.64
CD4	0.19	0.45	
CD8	0.09	0.06	
CD79	0.12	0.57	

Table 4: Statistical analysis of 24 matched pairs for Paired *t*-test. * p < 0.05 invalidating normality requiring the use of the non-parametric Wilcoxon signed-rank test.

Boxplots with overlying scatter plots were generated to visualise the distribution of cell counts for a given disease state (Figures 7-10). Values beyond the ends of the whiskers are considered outliers. In relation to CD34 the distribution of cell counts in diseased groups is not statistically significant but there is a trend to suggest that increased CD3 counts with increasing CD34 grade. There is an inverse relationship with CD8 and CD4 positive cells, with a wider distribution of CD8+positive cell counts with increasing CD34 grading and the opposite with CD4 (lower counts in higher CD34). There was no increase seen with CD79a positive cells and CD34 grade.

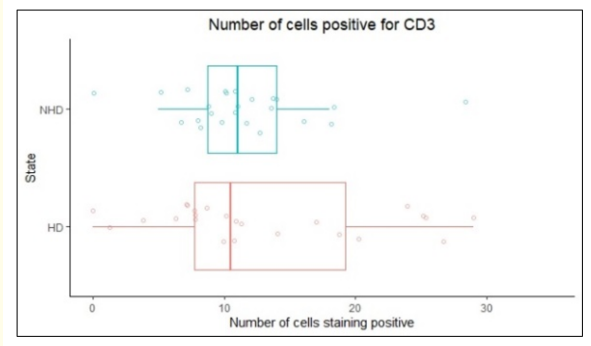

Figure 7: CD 3 positivity in heart disease (HD) and non-heart disease (NHD) myocardium.

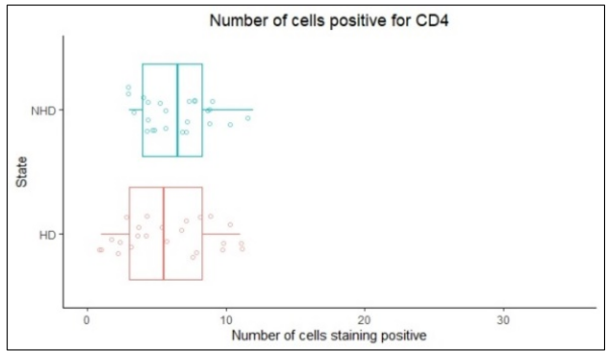

Figure 8: CD 4 positivity in heart disease (HD) and non-heart disease (NHD) myocardium.

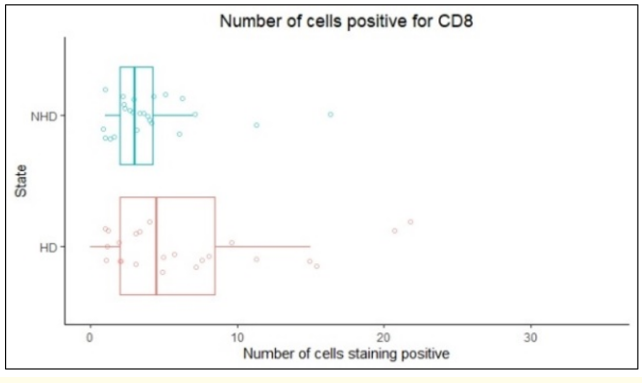

Figure 9: CD 8 positivity in heart disease (HD) and non-heart disease (NHD) myocardium.

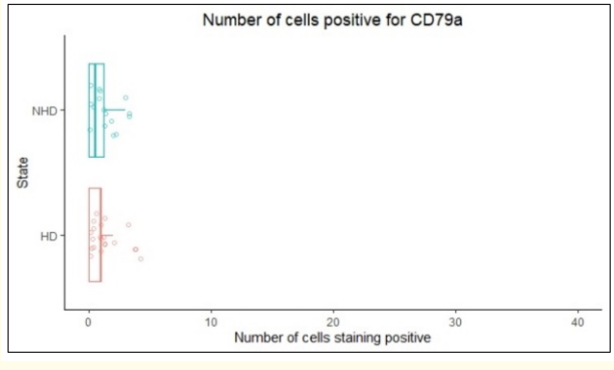

Figure 10: CD 79a positivity in heart disease (HD) and non-heart disease (NHD) myocardium.

Discussion

Lymphocytic myocarditis is diagnosed in part by assessing lymphocytic infiltrate in myocardial tissue. In difficult post-mortem cases where a more common or obvious cause of death is elusive, pathologists may be tempted to consider lymphocytic myocarditis sometimes based solely on the presence of lymphocytic infiltrate. This study has established a baseline range lymphocytic infiltrate in myocardial tissue, both heart disease and non-heart disease, such that misinterpretation of lymphocytic infiltrate in non- lymphocytic myocarditis cases can be avoided (Figure 11). This is the first study to our knowledge in the medical literature.

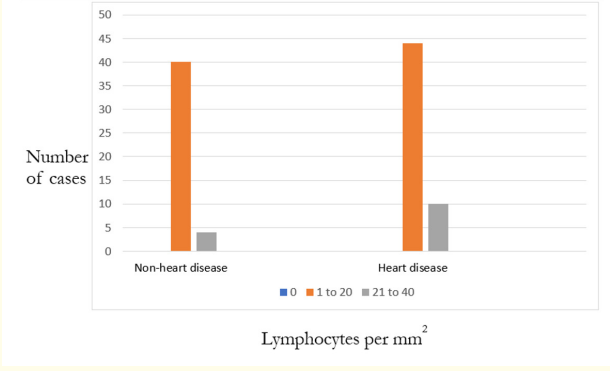

Figure 11: Range of myocardial lymphocytes in non-lymphocytic myocarditis.

The Dallas criteria, histological examination of endomyocardial biopsy, with or without associated myocyte necrosis [5], remains the gold standard in diagnosis of lymphocytic myocarditis, though it is not without criticism [6]. In 2012, Sagar., *et al.* [7] suggested that endomyocardial biopsy, histological evaluation and immunohistochemistry for T cells (CD3) and macrophages (CD68) at 14 or more per high power field would aid definitive diagnosis of lymphocytic myocarditis. The presence of lymphocytic infiltrate within the myocardium associated with myocyte necrosis of non-ischaemic origin with 14 leucocytes/mm^2 including up to 4 monocytes/mm^2 with the presence of CD3 positive T-lymphocytes > 7 cells/mm was found to be indicative of lymphocytic myocarditis [8]. Bonsignore., *et al*. 2018 [4] carried out a study on 48 cases which had a previous diagnosis of lymphocytic myocarditis and found that once these cases underwent immunohistochemistry staining, 21 of the cases should not have been considered as lymphocytic myocarditis. The number of lymphocytes seen in true lymphocytic myocarditis may overlap with cases of heart disease

and non-heart disease, and as Hazebroek., *et al*. 2014 [9] proposed, lymphocytic myocarditis should be suspected if the clinical presentation includes acute chest pain, ST/T wave changes, worsening, new onset or chronic heart failure in the absence of coronary artery disease, and other possible life-threatening conditions and if ECG or blood markers are suggestive of lymphocytic myocarditis. However, lymphocytic myocarditis should also be considered when the patient is asymptomatic [9] and PCR for viral genetic material would be useful in these cases.

Conclusion

This study confirms the presence of a baseline range lymphocytic infiltrate in non- lymphocytic myocarditis cases. There was no appreciable difference between heart disease and non-heart disease cases. The only noticeable albeit not statistically significant difference was the greater number of CD8-positive lymphocytes and greater CD34-positive fibrosis in heart disease cases. This would be expected as increasing fibrosis is consistent with increasing chronicity or greater chronic inflammation which sees an increasing CD8:CD4 ratio in general. No appreciable difference was seen with CD79a-positivity between heart disease and non-heart disease groups. B cells represent an early infiltrate and are subsequently replaced by T cells as injury persists. The range and average of CD3, CD4 and CD8-positve T lymphocytes are similar in heart disease and non-heart disease cases with the minority in both groups showing CD3-positive T lymphocytic infiltrate between 21 and 40 per mm^2 or greater and establishes a baseline range of 1-20 T lymphocytes with an average of approximately 12-13 T-cells per mm^2. Therefore, in cases where there is no clinical or microbiological correlate with lymphocytic myocarditis, it would be helpful to use the baseline range of ≥ 21-40 lymphocytes per mm^2 (m = 12.5) or greater as increasingly suggestive of true lymphocytic myocarditis.

Limitations of our study include only 24 data-matched pairs for statistical analysis. Also, matching data pairs on severity of heart disease and number of co-morbidities would perhaps help exclude other confounding variables influencing lymphocytic infiltrates in myocardium. While cases in this study were reviewed by a Consultant Pathologist and a senior medical scientist; it may be prudent to add a larger number of blind reviewers to lessen intra-observer bias. Sampling additional areas of myocardium such as right ventricular wall and interventricular septum with anterior, posterior, medial and lateral aspects would give a more holistic and accurate lymphocytic infiltrate assessment.

We present the first report of a baseline range lymphocytic infiltrate in post-mortem myocardial tissue from 54 heart disease and 43 non-heart disease cases with an upper threshold of ≥ 21-40 lymphocytes per mm^2 (m = 12.5). Heart disease cases showed greater CD8-positive Natural Killer T-lymphocytes and greater CD34-positive fibrosis compared with non-heart disease cases, as expected. Heart disease cases had myocardial lymphocytic infiltrate of > 20 T-lymphocytes per mm2 in 19% of cases (10/54) with none greater than 39/mm^2, and non-heart disease cases had myocardial lymphocytic infiltrate of > 20 T-lymphocytes per mm2 in only 9% of cases (4/44) with none greater than 34/mm^2. Since there is some overlap between cases of lymphocytic myocarditis and non-lymphocytic myocarditis myocardial lymphocytic infiltrate, lymphocytic myocarditis ideally should not be diagnosed in post-mortem tissue without additional supporting clinical and/or laboratory data. However, lymphocytic myocarditis can be increasingly considered as a benign lymphocytic myocardial infiltrate increases within the range of 21-40 lymphocytes per mm^2 (m = 12.5) or greater.

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