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## Idiopathic myocardial fibrosis in captive chimpanzees (*Pan troglodytes*)

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Abstract:	Cardiovascular disorders and, predominantly idiopathic myocardial fibrosis, are frequently associated with mortality among zoo-housed chimpanzees ( <i>Pan troglodytes</i> ). Formalin-fixed whole hearts of deceased chimpanzees housed in zoos (n=33) or in an African sanctuary (n=2) underwent detailed macroscopic and histopathologic examination using a standardized protocol. Archived histological slides from the hearts of 23 additional African sanctuary-housed chimpanzees were also examined. Myocardial fibrosis (MF) was identified in 30/33 (91%) of the zoo-housed chimpanzees but none of the 25 sanctuary-housed chimpanzees MF was shown to be characterized by both interstitial and replacement fibrosis. Immunophenotyping demonstrated that these fibrotic lesions were accompanied by the increased presence of macrophages, alpha smooth muscle actin-positive myofibroblasts and a minimal to mild T-cell dominant infiltration. There was no convincing evidence of cardiotropic viral infection, or suggestion that diabetes mellitus or vitamin E or selenium deficiency are associated with the presence of the lesion. However, serum vitamin D concentrations among zoo-housed chimpanzees were found to be lower in seasons of low UV light levels.

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**Idiopathic myocardial fibrosis in captive chimpanzees (*Pan troglodytes*)**

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**ABSTRACT:**

Cardiovascular disorders and, predominantly idiopathic myocardial fibrosis, are frequently associated with mortality among zoo-housed chimpanzees (*Pan troglodytes*). Formalin-fixed whole hearts of deceased chimpanzees housed in zoos (n=33) or in an African sanctuary (n=2) underwent detailed macroscopic and histopathologic examination using a standardized protocol. Archived histological slides from the hearts of 23 additional African sanctuary-housed chimpanzees were also examined. Myocardial fibrosis (MF) was identified in 30/33 (91%) of the zoo-housed chimpanzees but none of the 25 sanctuary-housed chimpanzees MF was shown to be characterized by both interstitial and replacement fibrosis. Immunophenotyping demonstrated that these fibrotic lesions were accompanied by the increased presence of macrophages, alpha smooth muscle actin-positive myofibroblasts and a minimal to mild T-cell dominant infiltration. There was no convincing evidence of cardiotropic viral infection, or suggestion that diabetes mellitus or vitamin E or selenium deficiency are associated with the presence of the lesion. However, serum vitamin D concentrations among zoo-housed chimpanzees were found to be lower in seasons of low UV light levels.

**KEYWORDS:** Cardiac fibrosis, fibrosing cardiomyopathy, Chimpanzee, Great ape, heart, interstitial myocardial fibrosis, diabetes mellitus, vitamin D

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3 Cardiovascular disease has been cited as a significant cause of mortality among  
4 captive chimpanzees.<sup>23,32,37,46,48</sup> A chronic, degenerative heart disease characterized  
5 by fibrosis of the cardiac muscle is the predominant condition diagnosed on post-  
6 mortem examination, and is associated with sudden death presumably due to  
7 dysrhythmia.<sup>11,24,49</sup> As yet, however, the cause(s) of idiopathic myocardial fibrosis  
8 (IMF) in great apes (also referred to as interstitial myocardial fibrosis<sup>23</sup> and fibrosing  
9 cardiomyopathy<sup>43</sup>), remains unknown.

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11 Myocardial fibrosis (MF) is a non-specific response to cardiac insult or injury and one  
12 of the most common histologic features of the diseased heart. In humans, there are  
13 two histological subtypes of MF, the pathophysiology of which differ.<sup>28,39</sup>

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15 Replacement fibrosis (RF) is the irreversible response to cardiomyocyte damage and  
16 loss, which occurs for example following myocardial infarction or myocarditis.

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18 Necrotic cardiomyocytes are removed and replaced by scar tissue to preserve  
19 structural and functional integrity of the myocardium.<sup>15,52</sup> In contrast, interstitial  
20 fibrosis (IF) can occur even in the absence of cardiomyocyte damage or death and is  
21 an adaptive response which, at least initially, allows cardiac structure and function to  
22 be preserved. Its formation is typically reactive, occurring in response to underlying  
23 (sometimes remote) disease processes such as renal disease, hypertension,  
24 diabetes mellitus, primary cardiomyopathies, secondary pressure/volume  
25 overloading and inflammation.<sup>1,4,5,7,17,21,30,34,56</sup> IF is also a well-established feature of  
26 age-related remodeling of the human heart.<sup>18</sup> Irrespective of the cause, IF is  
27 characterized by excessive deposition of extracellular matrix (ECM) proteins by  
28 cardiac myofibroblasts, which expand the interstitial (and often, also, perivascular)  
29 space.<sup>52</sup> Importantly, IF is reversible and, given that its formation can precede that of

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3 the irreversible RF subtype, its extent and severity can also be used as a marker of  
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5 disease progression.<sup>29,31,39</sup>  
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8 Trauma and infectious disease are cited as the most frequent causes of death  
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10 among wild chimpanzees, with MF being identified in only two (out of 11)  
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12 chimpanzees from Gombe National Park, Tanzania between 2004-2010.<sup>51</sup> This  
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14 raises the question as to whether the high prevalence of cardiovascular disease and  
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16 MF seen in zoos and research laboratories is a feature and/or consequence of life in  
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18 captivity. There are differences in the life expectancy and the infectious, dietary and  
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20 environmental factors to which wild versus captive chimpanzee populations are  
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22 exposed, each of which require careful consideration and investigation. One such  
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24 difference is their exposure to sunlight and therefore, presumably, their serum  
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26 vitamin D levels.<sup>10,53</sup> An inverse relationship between serum vitamin D  
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28 concentrations and cardiovascular disease risk is recognized in people,<sup>42</sup> and  
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30 vitamin D has also been shown to have anti-fibrotic properties in the heart and other  
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32 organs.<sup>38</sup> Other diseases and deficiencies relating to metabolic status and  
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34 micronutrient levels in zoo-housed chimpanzees might also be significant. Type 2  
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36 diabetes has been diagnosed commonly in captive chimpanzee populations,<sup>36</sup> and in  
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38 humans is associated with a cardiomyopathy which is characterized by myocardial  
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40 fibrosis and dysfunctional remodeling.<sup>1,40</sup> Additionally, both vitamin E and selenium,  
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42 which possess anti-oxidant properties, are believed to have protective effects against  
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44 myocardial ischemic damage.<sup>6,13,44</sup> Deficiencies in both micronutrients have been  
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46 implicated in the development of myocardial necrosis in various animal species,<sup>14,22</sup>  
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48 and vitamin E deficiency specifically has been associated with cardiomyopathy in  
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50 gorillas<sup>35</sup> and other zoo-housed primates.<sup>26,27</sup> Captivity is also associated with  
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52 greater human-chimpanzee interaction than would occur in the wild. This, combined  
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3 with our genetic similarity, suggests that captive chimpanzees are at risk of  
4 becoming infected with human pathogens. Inflammation (albeit mild) has been  
5 described as a feature of MF in chimpanzees and gorillas,<sup>2,9,43,24</sup> and is recognized  
6 as being linked to cardiac fibrosis and cardiomyopathy in people.<sup>12,41,50</sup> The potential  
7 that IMF in chimpanzees might occur due to infectious myocarditis or viral  
8 persistence therefore also warrants exploration.  
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18 Until recently, much of the investigative work aimed at enhancing understanding  
19 about great ape cardiovascular disease has been carried out in North American zoos  
20 (driven by the Great Ape Heart Project, based at Zoo Atlanta) or US research  
21 facilities. In 2013, however, a European taskforce was formed, bringing together  
22 specialists from the fields of human and veterinary cardiovascular medicine and  
23 pathology. The Ape Heart Project is a collaborative initiative based at Twycross Zoo  
24 (United Kingdom), which aims to promote and drive the study of great ape  
25 cardiovascular disease across European zoological collections. Its work to date has  
26 resulted in mortality reviews for each of the great ape genera<sup>46,48</sup> a retrospective  
27 study of great ape cardiovascular disease and epidemiology and pathology<sup>49</sup>, and  
28 the generation of guidelines for the consistent examination, sampling and reporting  
29 of great ape cardiovascular lesions.<sup>47</sup>  
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46 This study, a part of the Ape Heart Project, describes the cardiac lesions affecting  
47 European zoo-housed chimpanzees, characterizes chimpanzee IMF, and  
48 investigates the possible associated dietary, infectious and environmental factors.  
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## **MATERIALS AND METHODS:**

### **Cardiac samples from African sanctuary chimpanzee**

Archived Hematoxylin Eosin (HE)-stained histologic sections of the heart of 23 African sanctuary chimpanzees (8 to 27y; median age 12y; 8 male, 15 female) were examined by a board certified veterinary pathologist (KB). These slides were produced from small, often single-site myocardial (unspecified location) samples taken at post-mortem examination at a single rescue and rehabilitation center in Sierra Leone, Africa. Two entire formalin fixed hearts from the same African sanctuary (cases C12 and C13; 11 and 13y; male) underwent detailed examination as described below. All animals from the sanctuary were housed in a large mixed indoor/outdoor forested enclosure and were fed a diet comprised of local mixed vegetables, fruits, cereals and nuts.

### **Cardiac samples from European zoo chimpanzees**

**Hearts** from European Association of Zoos and Aquaria (EAZA) member zoo-housed chimpanzees (n=33; 9 to 65y; median age 32y; 19 male, 14 female) chimpanzees (cases C1-C11, C14-C35) were collected post-mortem, submerged in 10% neutral buffered formalin and sent to the Ape Heart project for further investigation. Data relating to the age, sex, weight and circumstances of death for these animals are presented in Supplemental Table S1. All hearts were fully submerged in 10% neutral buffered formalin for at least 72 hours prior to examination and sampling.



### Detailed cardiac examination

Formalin fixed entire hearts underwent detailed macroscopic examination and sampling according to a protocol which is based on guidelines for the investigation of sudden cardiac death in humans<sup>3,45</sup> and has been published for use in great apes.<sup>47</sup> A minimum of twelve cardiac samples were taken from pre-determined and consistent locations including myocardium from the anterior, posterior and lateral left and right ventricular walls, anterior and posterior portions of the interventricular septal wall and right ventricular outflow tract. Samples of the aorta, sino-atrial nodal region and atrioventricular nodal region were also taken. Additional tissue samples of anomalies or lesions detected as part of the examination were taken as indicated. Histologic sections were prepared from formalin-fixed paraffin-embedded blocks and routinely stained with HE (n=35) and Masson's trichrome to highlight collagenous connective tissue fibers (n=13; C1, C3-4, C7, C10, C12-13, C15, C19-20, C23, C33-34). The histopathology slides were evaluated by a board-certified veterinary pathologist (KB) and a subset of cases additionally reviewed by an expert in medical (human) cardiovascular pathology (MS). Pathologic diagnoses were generated for each case and lesions graded according to severity. IF was used to describe the accumulation of excessive extracellular matrix (ECM) proteins expanding the interstitial space between cardiomyocytes; RF being used when the scar tissue replaced cardiomyocytes. Those cases which were affected by myocardial fibrosis (IF and/or RF) for which no cause could be identified, were given a diagnosis of idiopathic myocardial fibrosis (IMF).

## Immunophenotyping

Of those hearts that had undergone detailed cardiac examination, samples from eight zoo-housed chimpanzees (C1, C3, C4, C7, C10, C15, C19, C20) with variable degrees of IMF and two African sanctuary chimpanzees (C12, C13) without fibrosis, were also subjected to immunohistochemical examination. Tissue sections from the anterior left ventricular wall were immunolabelled for CD3, CD20, IBA-1 and  $\alpha$ -SMA (Alpha-Smooth Muscle Actin) (Supplemental Table S2) (Veterinary Laboratory Service, Liverpool, UK) using anti-human antibodies. Chimpanzee splenic tissue was used as a positive control (Supplemental Figures S1-S3). Within cardiac slides, vascular smooth muscle cells in arterial walls were used as an internal positive control for the  $\alpha$ -SMA antibody. The negative control consisted of substitution of the primary antibody with isotype matched murine immunoglobulin (Supplemental Figure S4).

## Detection of viruses

Fresh frozen heart tissue samples from six chimpanzees (C4, C8, C9, C11, C15, C20, Supplemental Table S3) taken at post mortem examination were tested for the presence of cardiotropic viruses at the Primate Biomedical Research Centre (Netherlands; <https://www.bprc.nl/en/our-services#diagnostics>; AAALAC accreditation). Nucleic acids were extracted and PCR and RT-PCR carried out to amplify the sequences of encephalomyocarditis virus, human adenovirus, parvovirus virus B19, human pan-enteroviruses, human herpesvirus 6 and pan-herpesviruses. Subsequent IHC for herpesvirus antigen (human herpes simplex viruses 1 and 2) and human cytomegalovirus was carried out at an accredited medical commercial

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3 diagnostic laboratory (University College London Hospitals, HSL Advanced  
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5 Diagnostics; <http://www.hsl-ad.com/>; UKAS to ISO 15189:2012 accreditation  
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7 standards) on those samples for which a positive PCR result was obtained.  
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### 10 11 12 13 14 **Vitamins D and E, selenium and glycosylated hemoglobin**

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17 Whole blood was collected via femoral venipuncture from 20 chimpanzees as part of  
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19 the routine preventative healthcare program at a UK zoological collection, under the  
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21 Veterinary Surgeons Act 1966. Of these 20 chimpanzees, 11 (cases AC1-11) were  
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23 still alive and 9 (cases C2-4, C8-9, C15, C20, C30, C35) subsequently died and were  
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25 examined as described above. Due the opportunistic nature of sample collection,  
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27 time between blood sampling and death for these nine chimpanzees varied from 0d  
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29 to 8.5y. Serum and EDTA-preserved whole blood samples were frozen and shipped,  
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31 on ice, to a United Kingdom Accreditation Service (UKAS)-accredited laboratory  
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33 (Laboratory Medicine-Central Manchester University Hospitals) where vitamins D, E  
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35 and selenium and glycosylated hemoglobin (HbA1c) were measured. In the absence  
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37 of established chimpanzee normal values, human reference intervals were used to  
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39 interpret results: vitamin E 11.6- 34.8  $\mu\text{mol/l}$ , selenium 0.8-1.5  $\mu\text{mol/l}$ , 25-OH-vitamin  
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41 D  $>50\text{nmol/l}$ , glycosylated hemoglobin (HbA1c)  $<48\text{mmol/mol}$ .  
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### 49 **Statistical analyses**

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52 Statistical analyses were carried out using SPSS Statistics (v.24). Data were  
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54 assessed for normality using a Shapiro-Wilk test. Age at death among European zoo  
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56 versus African sanctuary chimpanzees, and vitamin D levels during high (May to  
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58 October) versus low (November to April) ultraviolet (UV) light seasons were  
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3 compared using a Mann-Whitney U test. Confidence intervals were set at 95%,  
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5 p<0.05 were considered statistically significant.  
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**RESULTS:****Cardiac samples from African sanctuary chimpanzees**

Multiple acute myocardial infarcts, thromboemboli and sepsis were evident in the heart section of one adult female sanctuary-housed chimpanzee. No evidence of interstitial or replacement fibrosis was identified in any of the remaining 22 chimpanzees for which archived histological sections were examined.

The two African sanctuary chimpanzees (C12 and C13; 11y and 13y; male) which underwent a comprehensive cardiac examination, showed no gross heart lesions and no histologic evidence of IF or RF.

**Cardiac samples from European zoo chimpanzees**

Macroscopic examinations revealed a mild to moderate left ventricular hypertrophy (concentric thickening of the left ventricular free wall and septum) and atrial dilation in 6/33 (18%) adult zoo-housed chimpanzees (Supplemental Table S1). Histological examination revealed mild to moderate degrees of fiber and nuclear hypertrophy within the mid-myocardium of the left ventricular free wall and septum of these animals. All affected hearts also showed variable degrees of IF, perivascular fibrosis and RF affecting multiple or all examined myocardial sections.

One case (C33) was diagnosed with hypertrophic cardiomyopathy (HCM) based on marked, random myofiber disarray. One adult female chimpanzee (C29) had a mild dilation of the left ventricle and atrium and a mildly increased mitral valve circumference with an overall enlarged heart. Histologically in this case, moderate to marked interstitial fibrosis compressed mid-myocardial myofibers into thin and elongated, wavy fibers within the left ventricle and septum. Other adjacent areas

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3 nearby had fiber and nuclear hypertrophy. Multifocally, the subepicardial and  
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5 subendocardial spaces contained variably sized areas of RF. The findings were  
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7 interpreted as dilated cardiomyopathy (DCM). One elderly, female chimpanzee  
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9 (C27) showed a mild to moderate aortic valve stenosis with a secondary mild left and  
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11 right ventricular hypertrophy (likely due to chronic pressure overload and to  
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13 pulmonary hypertension, respectively).  
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17 Histopathological examination of consistent areas of each heart showed a diffuse,  
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19 mild variation in myocardial nuclear size in all investigated chimpanzees; interpreted  
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21 by the authors to be a normal finding in hominids. There were mild to severe fibrotic  
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23 changes affecting one to all four chambers in 30/33 (91%; age range: 9 to 65 years)  
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25 of the European zoo-housed chimpanzees investigated (Supplemental Table S1). IF  
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27 was the most consistent feature; it varied from minimal to marked (Figures 1, 2) and  
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29 was identified in all 30 of the chimpanzees with MF. Perivascular fibrosis (expansion  
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31 of the vascular adventitial matrix) was also identified in these 30 zoo-chimpanzees,  
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33 its severity closely mirroring that of the IF and therefore interpreted as being part of  
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35 the same change and/or process. IF appeared to begin in very mild cases within the  
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37 mid-myocardium of the left ventricular wall and interventricular septum and with  
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39 increasing severity involved the entire myocardium of all four chambers. Minimal to  
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41 severe, randomly distributed myocardial RF which occasionally extended into the  
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43 subepicardial space and involved the epicardium, was also identified in 29/30  
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45 animals with MF (Figures 3, 4). Masson's trichrome staining of a subset of cases  
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47 (n=13; C1, C3-4, C7, C10, C12-13, C15, C19-20, C23, C33-34) confirmed the  
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49 presence and extent of the IF, RF and perivascular fibrosis (Figures 1, 2).  
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57 A minimal to mild, occasionally moderate, mixed leukocytic infiltration was also  
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59 identified in the myocardium of chimpanzees with myocardial fibrosis. It was  
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3 consistently associated with areas of replacement fibrosis and more pronounced in  
4 granulation tissue. IHC, carried out on 10 chimpanzees, demonstrated the leukocyte  
5 population to be composed mainly of CD3-positive T lymphocytes and IBA-1-positive  
6 macrophages (Figure 5) with fewer neutrophils, eosinophils, and CD20-positive B  
7 lymphocytes (Supplemental Figures S5, S6 and S7). Hearts unaffected by MF  
8 showed no or low numbers of  $\alpha$ -SMA reactive myofibroblasts (Supplemental Figure  
9 S8). Greater numbers of scar-related  $\alpha$ -SMA reactive myofibroblasts, as well as  
10 IBA1-reactive macrophages and scar-related, T-cell dominant, minimal to mild  
11 leukocyte populations were present in all MF-affected hearts, with their numbers  
12 often seemingly mirroring the severity of myocardial changes (Figure 6). The extent  
13 of eosinophil and neutrophil infiltration was greater in cases of sudden death and in  
14 association with foci of acute myocytolysis (Figure 7). Additional changes in the  
15 myocardium of cases of acute cardiac failure included acute hemorrhages and  
16 variably-sized areas of contraction band necrosis (Figure 8). Areas of RF which  
17 appeared to be older due to a more organized collagen deposition, lack of  
18 vascularization and leukocytes, often showed increasing numbers of mature  
19 adipocytes within the center of the lesion (so-called 'fibrofatty core'; Figure 4). Other  
20 chronic changes identified in two chimpanzees were scattered fiber calcifications  
21 indicating dystrophic calcification of previous cardiomyofiber degeneration.  
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### 51 **Detection of viruses**

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53 Three chimpanzees (C9, C11, C15) of the six tested were positive for pan-  
54 herpesviral nucleic acid on PCR (Supplemental Table S3). Subsequent IHC testing  
55 of cardiac tissue from these animals was negative for herpes simplex virus 1 and 2  
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3 and cytomegalovirus. PCR testing was negative for encephalomyocarditis virus,  
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5 human adenovirus, parvovirus virus B19, human pan-enteroviruses and human  
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7 herpesvirus 6.  
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### 10 11 12 13 **Vitamins D and E, selenium and glycosylated hemoglobin** 14

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16 Ante-mortem analysis of serum/blood vitamin D, vitamin E, selenium and  
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18 glycosylated hemoglobin (HbA1C) levels was carried out for 20 zoo-housed  
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20 chimpanzees, nine of which had also undergone detailed post-mortem cardiac  
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22 examination (Table 1). The glycosylated hemoglobin levels failed to show any  
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24 evidence of diabetes mellitus (Table 1). Two chimpanzees were shown to be vitamin  
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26 E deficient (AC2, AC7), whereas levels exceeded the upper limit of the (human)  
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28 reference interval in four animals. No deficiencies in selenium were identified and  
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30 levels exceeded the upper limit of the (human) reference interval in five animals.  
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32 Vitamin D levels were significantly higher in samples collected during the high (n=19)  
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34 versus low (n=12) UV-light season ( $105.0 \pm 33.92$  nmol/l [mean  $\pm$  SD] in May to  
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36 October versus vs  $54.56 \pm 22.47$  nmol/l in November to April;  $p < 0.0001$ ). Using  
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38 human reference ranges, vitamin D levels were inadequate (25-50nmol/l; n=4) or  
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40 deficient (<25nmol/l; n=2) in 6/12 (50%) low UV season samples, but inadequate in  
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42 only one of the 19 high UV season samples tested (Table 1). This animal was C4, a  
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44 male chimpanzee which died suddenly after the subsequent winter and was  
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46 diagnosed with severe idiopathic myocardial fibrosis on post-mortem examination  
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48 (Supplemental Table S1).  
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### Age at death

The age at death of the European zoo-housed chimpanzees (n=33; 32y [median], 28.02-37.98y [95% confidence interval]) was significantly higher ( $p<0.001$ ) than that of the African Sanctuary (n=25; 12y, 11.28-14.64y) housed chimpanzees

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## DISCUSSION:

Our findings demonstrate that European zoo-housed chimpanzees exhibit a similar spectrum of cardiovascular lesions as those described in humans and other species including primary cardiomyopathies such as HCM and secondary ventricular hypertrophy. Given that many, if not all, of these conditions are associated with pathological remodeling caused by the excessive deposition of extracellular matrix (ECM) proteins,<sup>52</sup> this suggests a great need for care when interpreting of the presence of MF in the chimpanzee. It also demonstrates the need for a comprehensive cardiac post-mortem examination and the histologic evaluation of consistent samples taken from multiple areas of the heart, to ensure adequate diagnoses can be made.

A considerable proportion (n=22/35; 63%) of chimpanzees in this study did, however, die due to or with cardiac fibrosis, which was not accompanied by macroscopic changes and for which the cause, despite extensive whole-heart examination, remains unclear. It has long been known that this pathological entity, referred to here as idiopathic myocardial fibrosis (IMF), is a frequent post-mortem finding among captive chimpanzees and other great apes.<sup>16,45,46</sup> Inconsistencies in post-mortem examination and sampling between zoological collections has, however, previously resulted in a lack of good quality comparable data available for research on this topic.<sup>16,45,46</sup>

This study therefore also aimed to expand upon current descriptions of chimpanzee idiopathic myocardial fibrosis and to investigate potential causes. It utilized a consistent, detailed and published methodology to examine and subsequently characterize the macroscopic, histopathologic and immunohistochemical features of

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3 chimpanzee IMF. The changes associated with IMF in chimpanzees closely  
4 resembled a condition of the same name which is associated with sudden cardiac  
5 death in humans.<sup>19</sup> In the chimpanzees examined as part of this study, however, the  
6 degree of inflammation was mildly more prominent than would typically be observed  
7 in humans with the same condition (M. Sheppard, personal communication). This led  
8 to speculation that chimpanzee IMF may in fact be the sequela of an acquired,  
9 inflammatory, and perhaps infectious condition.  
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20 This possibility was investigated by IHC and more detailed characterization of the  
21 inflammatory response. The findings, however, were instead suggestive that the  
22 observed scar-related, minimal-to-mild lymphocyte and macrophage population was  
23 in fact related to a mild, chronic reaction and did not appear to be the inciting cause  
24 of myocardial damage. This is further supported by our inability to demonstrate the  
25 involvement of viral infection in IMF in this study. Since chimpanzee blood samples  
26 are commonly pan-herpesvirus-positive on PCR, the three positive PCR results for  
27 pan-herpesvirus in cardiac tissue were interpreted as likely being the result of blood  
28 contamination. The possibility that other, less known viruses might be involved in the  
29 pathogenesis of IMF, however, cannot be ruled out and warrants further  
30 investigation.  
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46 Interestingly, granulocytes were often present within the lesions in those  
47 chimpanzees that suffered acute, sudden cardiac deaths. These lesions also often  
48 had acute cardiomyofiber lysis and/or contraction bands that were not seen in  
49 chimpanzees that had been euthanized. Given that such myofiber lysis can occur in  
50 the event of terminal dysrhythmic events, these findings therefore support the  
51 previously held notion that death in affected chimpanzees might be caused by the  
52 pro-arrhythmogenic effect of MF.<sup>11,24 20</sup>  
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3 Myocardial fibrosis (MF) is a non-specific response to cardiac insult or injury and one  
4 of the most common histologic features of the diseased heart. The fact that MF is a  
5 feature in primary and secondary cardiomyopathies, as well as many other cardiac  
6 and non-cardiac diseases, has previously made definitive interpretation of its  
7 presence in the chimpanzee heart difficult. Given that the pathophysiology of the two  
8 types of fibrosis (replacement and interstitial) differ,<sup>28,39</sup> determining which is  
9 predominant in chimpanzee IMF could provide powerful insight into its pathogenesis.

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20 This study demonstrated that both IF and RF are histopathological features in  
21 chimpanzee MF and may be part of the same disease process, with RF developing  
22 following chronic, progressive and/or severe IF. Given that IF can be halted or even  
23 reversed, this finding has implications for the potential treatment of affected  
24 individuals and therefore reduction of mortality.

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32 In humans, one of the most common causes of non-ischemic IF is systemic  
33 hypertension.<sup>18</sup> Left ventricular hypertrophy was indeed diagnosed in five adult  
34 chimpanzees as part of this study and can result from systemic hypertension.

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Review of the clinical history supplied for these animals, revealed that these animals  
had a history of chronic renal disease, suggesting that systemic hypertension might  
have been the cause of their left ventricular hypertrophy. To which degree these  
chronic secondary changes caused or contributed to the MF present in these  
animals is unclear. The chimpanzee diagnosed with DCM (C29) also had histologic  
lesions of chronic, marked renal disease. This animal's DCM phenotype was  
therefore interpreted as being the result of terminal cardiac decompensation  
following a pressure overload and prior undiagnosed left ventricular hypertrophy.

These findings therefore highlight the need for extensive post-mortem examination of  
not just the heart, but the whole body to be carried out, to rule out all possible

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3 primary and secondary causes of MF before denoting it as idiopathic. It also  
4  
5 suggests a need for further research into the potential overlap between renal disease  
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7 and the condition reported as IMF, and (as has recently been described by Chilton  
8  
9 and colleagues)<sup>9</sup> cardiorenal syndrome in chimpanzees.  
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13 Dysfunctional myocardial remodeling, interstitial fibrosis and perivascular fibrosis are  
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15 also features of diabetic cardiomyopathy.<sup>1,40</sup> Whilst our study failed to demonstrate  
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17 any evidence of inadequate glycemic control in the animals that were tested, it is  
18  
19 notable that metabolic disease, diabetes mellitus, and its major risk factor obesity are  
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21 common in captive chimpanzees.<sup>36,54</sup> Indeed, one IMF-affected chimpanzee (C17)  
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23 which underwent post-mortem examination only, was declared by the submitting zoo  
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25 to have a clinical history of diabetes mellitus. Screening for diabetes (resting blood  
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27 glucose, fructosamine, glycosylated hemoglobin) should still therefore form part of  
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29 routine health assessment, especially for geriatric animals.  
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34 Despite previous reports that cardiovascular disease is more common among male  
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36 than female chimpanzees,<sup>24,49</sup> our paper showed cardiac disease and specifically  
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38 IMF to affect males and females with similar frequency (n=13/21 [62%] males versus  
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40 9/14 [64%] females). However, moderate-marked IMF was present in 7/13 (54%)  
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42 males but only 2/9 (22%) females and the mean ages differed between the sexes  
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44 (male: 27y vs female 39y). Although the number of animals limits this comparison,  
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46 the results suggest that males might be affected more severely and/or earlier in life.  
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51 The progressive accumulation of collagen in the heart is a recognized feature of  
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53 cardiac aging in humans, even in the absence of other risk factors such as  
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55 hypertension, smoking and high cholesterol.<sup>8,33</sup> The age difference between the  
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57 European zoo (median age 32y) versus African sanctuary (median age 12y)  
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3 chimpanzees in this study might therefore explain the observed difference in IMF  
4 frequency between them. However, IMF was observed in European zoo-housed  
5 chimpanzees as young as 10 years of age (C1) and in two sub-adults (C18, C32;  
6 19y), suggesting that age alone does not explain the apparent discrepancies in  
7 disease prevalence between the two study populations.  
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15 Another key difference between them was the samples examined. All European  
16 samples were subject to the comprehensive macroscopic and histopathologic  
17 examination protocol whereas only small, single-site cardiac sections were available  
18 for histopathologic evaluation for all but two of the African chimpanzees and so early  
19 degenerative changes might have been missed.  
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29 If the observed difference in the incidence of IMF between the sanctuary- versus  
30 zoo-housed chimpanzees is valid, then differences in their living conditions and  
31 management could be considered as possible cardiac disease risk factors. The two  
32 populations differ, in their geographical location, housing type (forest vs indoor  
33 enclosures), climatic conditions (including UV light exposure), diet, rearing history,  
34 and genetic lineage. Amongst other things, these factors are likely to influence  
35 serum concentrations of vitamin D.  
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45 Whilst there are no published papers documenting serum vitamin D concentrations  
46 for chimpanzees living in their natural environment, it has been shown that captive  
47 chimpanzees without regular access to unfiltered sunlight do indeed experience  
48 clinically significant deficiencies.<sup>10,53</sup> This finding was also substantiated by the  
49 results of our study, with clear seasonal differences and low levels of serum vitamin  
50 D during the low-UV season of November to April. Clinical investigations in humans  
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3 suggest an association between vitamin D deficiency and cardiovascular disease  
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5 risk.<sup>55</sup>  
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8 Prior ante-mortem vitamin D data was available for 9/35 (26%) chimpanzees  
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10 undergoing post-mortem cardiac examination, and of these, three cases appeared to  
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12 suggest a possible link between vitamin D deficiency and death due to IMF: Case C4  
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14 had inadequate vitamin D levels in the winter two years before death as well as  
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16 during his last summer; while case C3 had inadequate levels at the time of death in  
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18 winter but sufficient levels the previous summer. Case C20 was one of the only adult  
19  
20 zoo chimpanzees examined that was not affected by cardiac fibrosis and also had  
21  
22 the highest summer levels of vitamin D (182.9 nmol/L) of all chimpanzees examined.  
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24 No vitamin D levels were available for this animal during the low UV season, so it is  
25  
26 not possible to conclude whether a seasonal deficiency existed. Whilst there are a  
27  
28 number of possible explanations for this animal's comparatively higher vitamin D  
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30 levels, it is interesting that this chimpanzee was also affected with complete alopecia  
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32 and paler skin pigmentation, and it could be hypothesized, therefore, that this may  
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34 have facilitated better cutaneous vitamin D synthesis.<sup>25</sup>  
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41 The limitations in this preliminary dataset, not least the variation in time between  
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43 collection and death, means that these observations are only circumstantial. The  
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45 findings do, however, highlight this potential association as an important area for  
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47 further investigation.  
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51 This study showed, for the first time, an association between the presence of  
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53 myofibroblasts and the presence and severity of chimpanzee MF. Upon injury,  
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55 TGF $\beta$ 1 stimulates the transformation of human cardiac fibroblasts into pro-fibrogenic  
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57 myofibroblasts, a process which, in vitro, is inhibited by vitamin D.<sup>37</sup> The increase in  
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3 myofibroblast numbers producing excessive collagen seen in diseased versus  
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5 healthy myocardial tissues as demonstrated by IHC in this study, suggests that a  
6  
7 similar process might take place in the chimpanzee heart. This in turn suggests that  
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9 the low serum vitamin D concentrations reported above might promote the  
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11 progressive accumulation of collagen which is seen in chimpanzee IMF and  
12  
13 therefore be associated with an increased risk of disease or accelerated ageing in  
14  
15 these animals. Additional work would be needed to confirm this hypothesis but, if  
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17 confirmed, might suggest that correcting vitamin D deficiency could reduce disease  
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19 prevalence and progression. Given these findings and the fact that vitamin D is  
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21 known to play an immunoregulatory role in various diseases, its potential role in  
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23 cardiac and overall health of captive chimpanzees warrants further investigation.  
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### 29 **Conclusion:**

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32 In conclusion, our study shows that, as in laboratory and American zoo-housed  
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34 populations, a significant proportion of European zoo-housed chimpanzees also  
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36 exhibit myocardial fibrosis. In most instances, this fibrosis is not accompanied by any  
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38 macroscopic lesions. Despite extensive post-mortem examination, no obvious cause  
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40 could be identified and the pathogenesis is not yet understood. The results of this  
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42 study identify some important avenues for further exploration of the  
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44 aetiopathogenesis of chimpanzee myocardial fibrosis.  
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**Figure legends:**

**Figures 1-6** Idiopathic myocardial fibrosis, heart, chimpanzee. Figure 1: Mild interstitial fibrosis. Masson's trichrome. Figure 2: Marked interstitial fibrosis. Masson's trichrome. Figure 3: Mild replacement fibrosis (arrow). Hematoxylin Eosin (HE). Figure 4: Marked replacement fibrosis with a so-called 'fibrofatty' core, a presumed long-standing scar. HE. Figure 5: Resident macrophages throughout the myocardium are more numerous in areas of replacement fibrosis. Immunohistochemistry (IHC) for IBA-1. Figure 6: Affected myocardium with alpha smooth muscle actin ( $\alpha$ -SMA) positive myofibroblasts (arrows) within areas of replacement fibrosis. The arterial tunica media is also positive (asterisk). IHC for  $\alpha$ -SMA.

**Figures 7, 8:** Idiopathic myocardial fibrosis (IMF), heart, chimpanzee. Acute myocardial changes associated with sudden death in subjects with IMF. Figure 7: Acute myocytolysis. Hematoxylin Eosin (HE). Figure 8: Acute contraction band necrosis: Densely eosinophilic wavy contraction bands (arrow) extend across the fibers. Affected myofibers are fragmented, have loss of cross striations, and nuclei are not clearly visible or pyknotic. HE.

Table 1: Severity of myocardial fibrosis and concentration of serum and blood analytes for 20 chimpanzees screened for metabolic disturbances. Cases C2-C35 subsequently died and received a full postmortem examination, and cases AC1-AC11 remained alive. High UV and low UV samples were collected in May-October and November-April respectively.

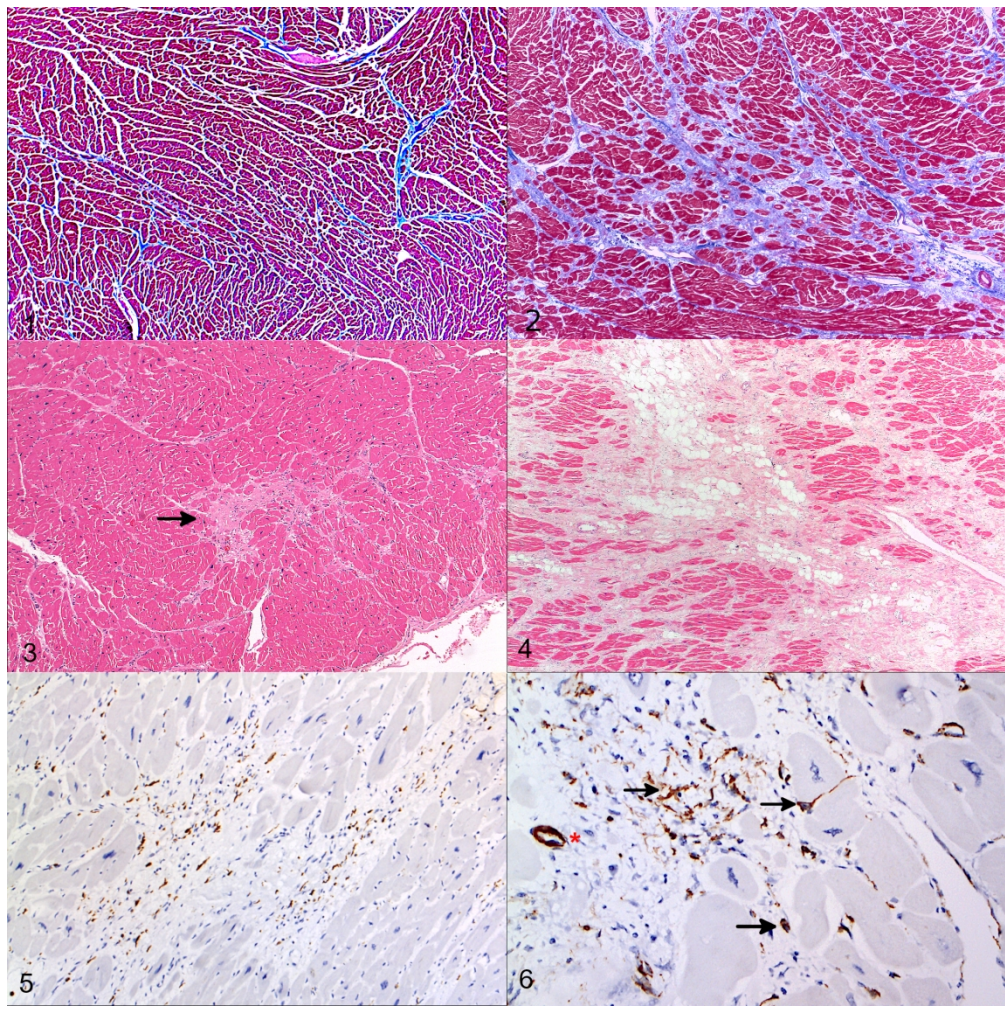
Study ID	Fibrosis severity	Total 25-OH Vitamin D		Vitamin E	Selenium	HbA1c
		<i>nmol/l</i>		<i>umol/l</i>	<i>umol/l</i>	<i>mmol/mol</i>
		<i>High UV</i>	<i>Low UV</i>			
<b>C2</b>	Mod	104.7	-	-	-	-
<b>C3</b>	Mod to marked	66.7	43.7*	18.2	0.93	-
<b>C4</b>	Marked	45.4*	46.7*	20.4	1.5	-
<b>C8</b>	Mod	93.1 – 121	84.2	-	-	-
<b>C9</b>	Mild	66.7	-	-	-	-
<b>C15</b>	Mod to marked	110	59	12.3	1.78**	-
<b>C20</b>	None	182.9	-	19.4	1.73**	22
<b>C30</b>	Mild to mod	89.2	-	37.2**	1.45	22
<b>C35</b>	Mild to mod	109	-	16.7	1.69	24

<b>AC1</b>	-	99.5	-	18.3	1.88	23
<b>AC2</b>	-	131	-	10.9*	1.73	16
<b>AC3</b>	-	154	20.1* - 72	18.9	1.43	19
<b>AC4</b>	-	156	43*	24.8	1.11	-
<b>AC5</b>	-	-	67.2	40.1**	1.69**	-
<b>AC6</b>	-	72	15.1*	33.7	0.86	-
<b>AC7</b>	-	-	32.9*	4.5*	1.26	-
<b>AC8</b>	-	120	74.1	30.2	1.26	-
<b>AC9</b>	-	89.2	-	37.2**	1.45	22
<b>AC10</b>	-	94.3	-	25.3	1.39	25
<b>AC11</b>	-	-	72.7	38.9**	1.78**	24

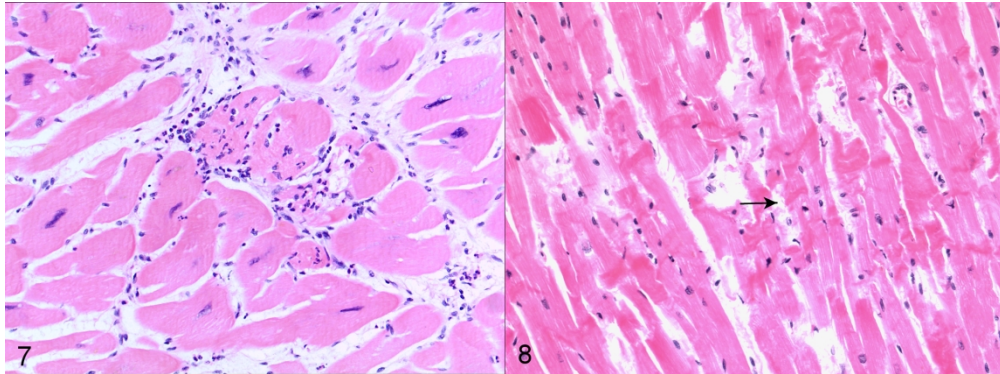
For animals where samples collected on more than one occasion were tested, values are provided as a range.

\*: deficient, result beneath lower limit of (human) reference interval; \*\*: result above upper limit of (human) reference interval. Mod.: moderate.

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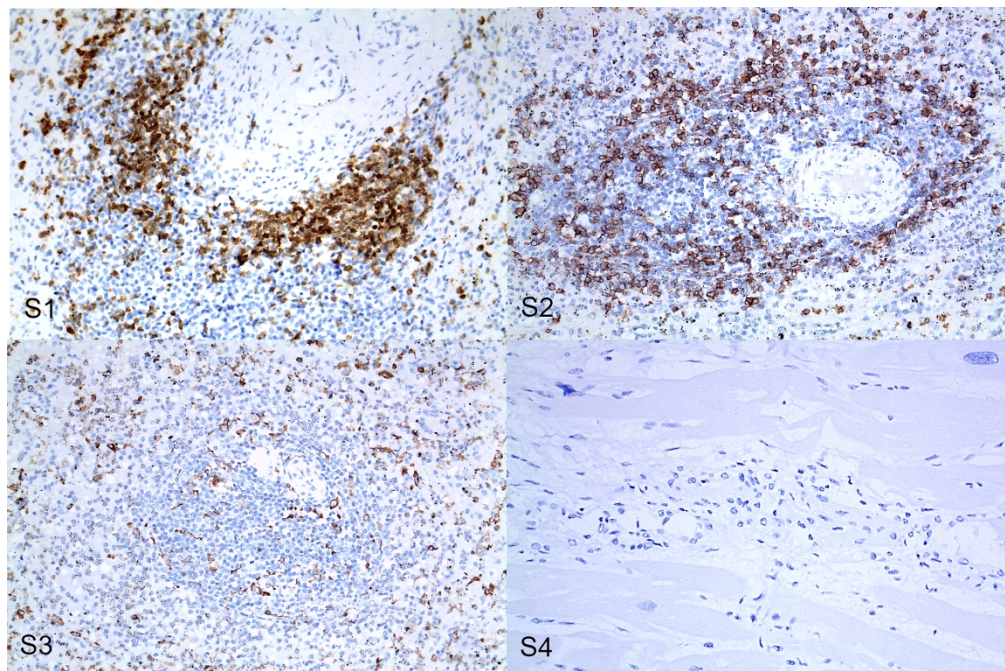
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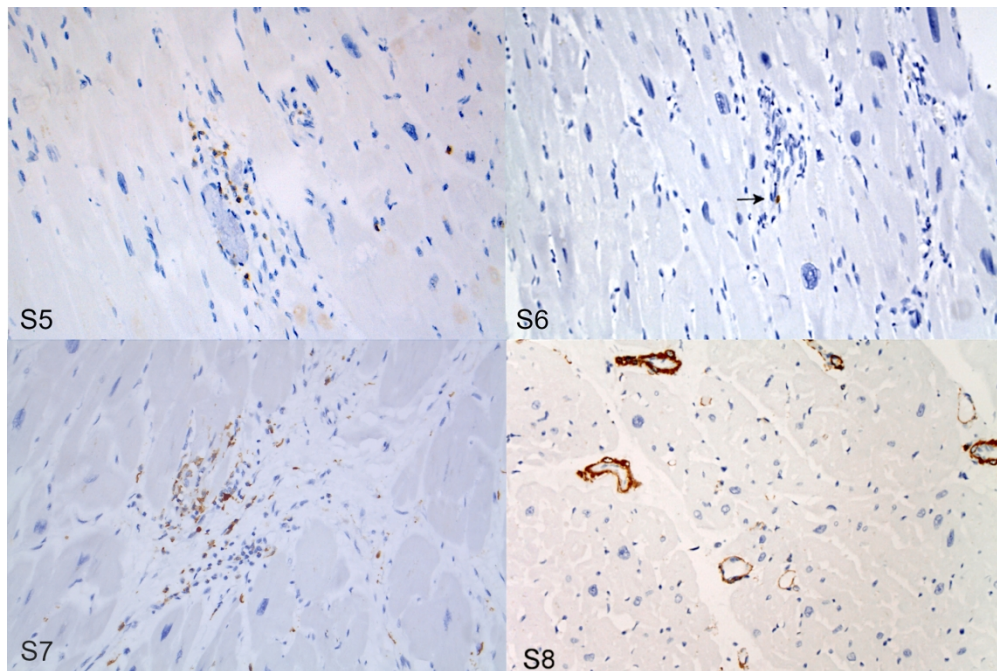


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3 **Supplemental Figure S1:** Spleen, chimpanzee, case C25, positive control.

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5 Immunohistochemistry (IHC) for CD3.

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7 **Supplemental Figure S2:** Spleen, chimpanzee, case C25, positive control. IHC for

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9 CD20.

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11 **Supplemental Figure S3:** Spleen, chimpanzee, case C25, positive control. IHC for

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13 Iba1.

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15 **Supplemental Figure S4:** Myocardium, chimpanzee, case C4, negative control. IHC

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17 for CD3.

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26 **Supplemental Figure S5:** Idiopathic Myocardial Fibrosis (IMF), heart, chimpanzee,  
27 case C10. There is a mild leukocytic perivascular infiltrate and multifocal  
28 immunolabeling. Immunohistochemistry (IHC) for CD3.

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32 **Supplemental Figure S6:** IMF, heart, chimpanzee, case C10. There is a mild  
33 leukocytic perivascular infiltrate and scattered immunolabeling (arrow). IHC for CD20.

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37 **Supplemental Figure S7:** IMF, heart, chimpanzee, case C10. There is a mild  
38 leukocytic perivascular infiltrate and prominent immunolabeling. IHC for IBA 1.

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42 **Supplemental Figure S8:** ~~Heart, chimpanzee, case C20. Normal tissue. Internal~~  
43 ~~positive control of immunolabeled IHC for  $\alpha$ -SMA (with internal positive control of~~  
44 ~~immunolabeled smooth muscle cells of the vascular tunica media and capillary~~  
45 ~~pericytes. IHC for  $\alpha$ -SMA.~~ Heart, chimpanzee C20. Normal tissue. Internal positive  
46 control of immunolabeled smooth muscle cells of the vascular tunica media and  
47 capillary pericytes. IHC for  $\alpha$ -SMA.  
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Supplement 1 (S1): Demographic data, circumstances and cause of death (if identified), key histopathological features (+ for mild, ++ for moderate and +++ for marked changes) and final cardiovascular diagnoses for 35 (33 European-zoo and 2 African sanctuary) chimpanzees which underwent macroscopic and histopathologic examination according to the comprehensive protocol.

Study ID	BW (kg)	Sex	Age (yrs)	Circumstances +/- cause of death	Histopathological features				Final cardiovascular diagnoses
					Chronic		Acute		
					RF	IF	AM	CBN	
<b>C1</b>	58	M	10	Accidental death	+ - ++	+	+	+	Mild IMF Acute, multifocal myocardial necrosis
<b>C2</b>	72	M	37	Perianesthetic death	+++	+ - ++	++	++	Mod LVH Marked myocardial fibrosis Acute, multifocal myocardial necrosis
<b>C3</b>	59	M	33	Perianesthetic death	++ - +++	++	+	+	Moderate to marked IMF Acute, multifocal myocardial necrosis
<b>C4</b>	61	M	33	Sudden cardiac death	+++	+++	+	+++	Marked IMF Acute, multifocal myocardial necrosis
<b>C5</b>	69	M	28	Sudden death history of diabetes	+++	++ - +++	+	+	Marked IMF Acute multifocal myocardial necrosis
<b>C6</b>	45	M	31	Perianesthetic death , bronchopneumonia	+	+	-	-	Mild IMF
<b>C7</b>	67	M	22	Sudden cardiac death	++ - +++	++	+	+++	Moderate to marked IMF Acute, multifocal myocardial necrosis
<b>C8</b>	69	M	39	Accidental death	++	+	++	+++	Moderate IMF Acute, multifocal myocardial necrosis
<b>C9</b>	48	F	32	Accidental death	+	+	++	+++	Mild IMF Acute, multifocal myocardial necrosis
<b>C10</b>	40	F	25	Perianesthetic death, pleuritis, pneumonia	++	+++	++	-	Mod LVH Marked myocardial fibrosis,

										Acute, multifocal myocardial necrosis
<b>C11</b>	47	F	42	Euthanasia, infectious disease	+	-	-	-	-	Mild IMF
<b>C12</b>	45	M	11	Sudden death, unknown cause	-	-	-	-	-	No cardiac fibrosis
<b>C13</b>	50	M	13	Sudden death, systemic infection	-	-	+	-	-	No cardiac fibrosis Multifocal, acute myocardial necroses
<b>C14</b>	66	M	20	Perianesthetic death	-	+	+	-	-	Minimal to mild IMF Acute, multifocal myocardial necrosis
<b>C15</b>	44	F	46	Euthanasia, liver infection	++ - +++	++	++	+++	+++	Moderate to marked IMF Acute, multifocal myocardial necrosis
<b>C16</b>	50	M	52	Euthanasia, paresis	++	+ - ++	-	-	-	Moderate IMF
<b>C17</b>	58	M	46	Euthanasia, diabetes mellitus, heart failure	+	+ - ++	+	-	-	Mild to moderate IMF Focal dissecting coronary aneurysm
<b>C18</b>	61	M	19	Perianaesthetic death, air sacculitis	+++	++	+	-	-	Mod LVH Marked myocardial fibrosis Acute, multifocal myocardial necrosis
<b>C19</b>	51	F	25	Sudden death, end stage renal disease	++	++	-	-	-	Moderate IMF
<b>C20</b>	59	M	22	Perianaesthetic death, air sacculitis	-	-	-	-	-	No fibrosis
<b>C21</b>	47	F	59	Euthanasia , multiple age related disorders	+ - ++	+	-	++	++	Mild to moderate IMF
<b>C22</b>	50	M	22	Euthanasia, chronic colitis, debilitation	+ - ++	+	++	+	+	Mild IMF Acute, multifocal myocardial necrosis
<b>C23</b>	55	F	41	Sudden death	++ - +++	++ - +++	+	-	-	Mod LVH Mod to marked myocardial fibrosis
<b>C24</b>	38	F	47	Perianesthetic death	+ - ++	+ - ++	+	-	-	Mild to moderate IMF
<b>C25</b>	-	F	21	Euthanasia	-	-	-	-	-	No fibrosis
<b>C26</b>	75	M	28	Suspected tetanus; euthanasia	+ - ++	+ - ++	-	-	-	Mild LVH Mild to moderate myocardial fibrosis

<b>C27</b>	76	M	48	Syncopal episodes, sudden death	+ - ++	+	-	-	Mild to mod cardiomegaly Mild to moderate myocardial fibrosis Mod aortic stenosis
<b>C28</b>	12	M	3	Accidental death	-	-	-	-	No fibrosis
<b>C29</b>	50	F	44	Systemic infection, natural death	++ - +++	++ - +++	++	+++	DCM Mod to marked myocardial fibrosis, Acute, multifocal myocardial necrosis
<b>C30</b>	61	M	22	Perianesthetic death	+	++ - +++	+	++	Mild to moderate IMF Acute, multifocal myocardial necrosis
<b>C31</b>	63	F	65	Osteoarthritis, euthanasia	+	+ - ++	-	-	Mild IMF
<b>C32</b>	69	M	19	Perianesthetic death	+	+	-	-	Mild IMF
<b>C33</b>	44	F	47	Renal disease, euthanasia	+ - ++	+	-	-	HCM Mild to moderate myocardial fibrosis
<b>C34</b>	4	F	42	Thrombosis, euthanasia	+ - ++	+ - ++	-	-	Mild to moderate IMF
<b>C35</b>	82	F	40	Perianesthetic death	+ - ++	+	-	-	Mild to moderate IMF

Perianesthetic deaths occurred following anesthetic induction, intra-operatively or during anesthetic recovery, or when the animal did not regain full consciousness post anesthesia. Deaths were classified as sudden if they were non-violent and unexpected (i.e. did not follow a period of illness). Accidental deaths included for example, incidents in which animals died as the result of a fall or drowning.

Abbreviations: AM Acute myocytolysis; BW Body Weight; CBN Contraction Band Necrosis; DCM Dilated Cardiomyopathy; HCM Hypertrophic Cardiomyopathy; IMF Idiopathic Myocardial Fibrosis; IF Interstitial Fibrosis; LVH Left Ventricular Hypertrophy; RF Replacement Fibrosis

Supplemental Table 2, S2: Panel of human antibodies used for immunohistochemistry to characterize the scar-related minimal to mild leukocytic infiltrate and presence of myofibroblasts in idiopathic myocardial fibrosis in 10 (8 affected; 2 unaffected) chimpanzees.

Antigen	Antibody	Type	Dilution	Source
IBA-1	AIF1/IBA1	polyclonal goat	1:500	Lifespan Biosciences
$\alpha$ -SMA	Ab5695	polyclonal rabbit	1:500	Abcam
CD3	A0452	polyclonal rabbit	1:500	Dako
CD20	RB 9013-P	polyclonal rabbit	1:500	Thermo fisher

According to the commercial diagnostic lab, antigen retrieval was performed by calibrated water bath capable of maintaining the epitope retrieval solution in 10 mM sodium citrate buffer (pH 6.0) at 97°C for 30 min. Endogenous peroxidase was blocked using 100  $\mu$ L DakoREAL™ peroxidase blocking solution for 10 minutes (Dako, USA). Sections were then incubated with the primary antibody (Supplement 1) and evaluated by peroxidase conjugated polymer (EnVision plus Detection KIT; Dako) for 30 min and diaminobenzidine tetrahydrochloride was used as detection system (EnVision™ FLEX DAB+ Chromogen (DM827), Dako).

Supplemental table 3, S3: Overview of virological tests in cardiac tissues showing none to marked IMF with a variable, fibrosis related, minimal to mild, mixed leukocytic infiltrate.

		<b>C15</b>	<b>C20</b>	<b>C8</b>	<b>C9</b>	<b>C4</b>	<b>C11</b>
<b>Cardiac Pathology</b>	Histological diagnosis:	Moderate to marked IMF	None	Moderate IMF	Mild IMF	Marked IMF	Mild IMF
	RF associated leukocytic infiltrate:	Mild ly/mc/neu	-	Mild ly/mc/neu	Minimal ly/mc	Mild ly/mc/eos	-
<b>Viral PCR results</b>	Pan Herpes PCR	+	-	-	+	-	+
	EMCV RNA PCR	-	-	-	-	-	-
	Adenovirus DNA PCR	-	-	-	-	-	-
	Parvovirus (VP2) DNA PCR	-	-	-	-	-	-
	Enterovirus RNA PCR	-	-	-	-	-	-
	HHV6 DNA PCR	-	-	-	-	-	-
<b>IHC</b>	Human herpes simplex virus 1	-	n/d	n/d	-	n/d	-
	Human herpes simplex virus 2	-	n/d	n/d	-	n/d	-
	Cytomegalovirus	-	n/d	n/d	-	n/d	-

Abbreviations: IMF Idiopathic Myocardial Fibrosis; RF Replacement Fibrosis; ly lymphocytes; mc macrophages; neu neutrophils; eos eosinophils; PCR Polymerase chain reaction; IHC Immunohistochemistry; n/d no data.