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# Age- and gender-dependent myocardial transcription patterns of cytokines and extracellular matrix remodelling enzymes in cats with non-cardiac diseases

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ABSTRACT

*Background:* Age, gender and systemic diseases all influence cardiac function and remodelling. In cats, age and gender associated myocardial remodelling and the effect of systemic diseases on the myocardium have so far not been studied. The aim of the study was therefore to investigate whether relevant cytokines and extracellular matrix (ECM) remodelling enzymes are expressed in the myocardium of cats with non-cardiac diseases and whether transcription levels are influenced by age and gender.

*Methods:* The study was performed on myocardial samples from 26 cats aged between 2 and 19 years that had died with non-cardiac diseases. Seventeen cats were female (2 entire) and nine were male (1 entire). Of these, nine cats were diagnosed with diseases unlikely to affect the myocardium (control cats). The remaining 17 cats suffered from diseases with likely systemic effects. All hearts were assessed for any pathological changes, and the myocardium was analysed for interleukin (IL)-1, -2, -4, -6, -18, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)-2, -3, -13, tissue inhibitor of MMP (TIMP)-1, -2 and -3 transcriptions using quantitative RT-PCR assays.

*Results:* Despite the absence of any histological evidence of myocardial damage, inflammation and fibrosis, the myocardium of all the cats was found to constitutively transcribe cytokines and ECM remodelling enzymes, with generally higher mRNA concentrations in the atria than in the ventricles. The young and male cats exhibited higher transcription levels throughout the myocardium in comparison to the older and female cats. Furthermore, age-associated transcription pattern differed between male and female cats.

*Conclusion:* The constitutive transcription of ECM remodelling enzymes suggests continuous myocardial remodelling throughout the entire life of a cat. The myocardium of young and male cats appears to be in a pro-inflammatory state, whereas in older and female cats the myocardium exhibits a reduced inflammatory reaction to systemic disease. Age-associated cardiac remodelling seems to be influenced by non-hormonal factors in male and female cats.



## 1. Introduction

In the human heart, ageing is characterised by progressive ventricular fibrosis and cardiomyocyte density, resulting in increased ventricular stiffness and diastolic dysfunction (Kitzman and Edwards, 1990). In the ageing human population heart failure with preserved ejection fraction is a frequent presentation. Gender differences are well known, with female species exhibiting favourable cardiac pathology (Chua et al., 2011; Pavon et al., 2012). Furthermore, systemic diseases such as diabetes mellitus, renal diseases, hypertension, and obesity, are known comorbidities and contribute to impaired cardiac function (Ather et al., 2012). Similar presentations are seen in cats and dogs. Age associated increased ventricular stiffness is observed in pet animals (Santilli and Bussadori, 1998; Schober and Fuentes, 2001; Saunders, 2012), retrospective studies suggest a male predisposition for hypertrophic cardiomyopathy in cats (Atkins et al., 1992; Abbott, 2010; Payne et al., 2010) and older animals develop systemic diseases that are known heart failure comorbidities in human patients (Metzger and Rebar, 2012; Saunders, 2012).

Increased ventricular stiffness is caused by imbalanced extracellular matrix (ECM) remodelling and ventricular fibrosis (Spinale, 2007). The composition of the ECM is regulated by enzymes, the matrix metalloproteinases (MMP), which degrade the ECM components, and their inhibitors, tissue inhibitors of MMP (TIMP-1 to -4), which indirectly lead to ECM deposition (Vanhoutte and Heymans, 2010).

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Matrix metalloproteinase and TIMP imbalance and ECM degradation and deposition are associated with cardiac diseases and dysfunction (Spinale, 2007), but only little is known about their role in age-related cardiac changes. Mouse models of ageing exhibit varying MMP, TIMP and transforming growth factor (TGF)-β mRNA and protein production (Brooks and Conrad, 2000; Lindsey et al., 2005; Tian et al., 2007; Kandalam et al., 2010; Wang et al., 2010; Givvimani et al., 2013; Uchinaka et al., 2014).

Cytokines play an important role in the activation of MMPs and TIMPs. They contribute to the regulation of inflammatory responses and are elevated in cardiac and systemic diseases (Hedayat et al., 2010; Fonfara et al., 2012; Fonfara et al., 2013a). In particular interleukin (IL)-6 and tumour necrosis factor (TNF)-α are linked to ageing processes and were found to be elevated in patients with loss of muscle mass and cachexia (Morley and Baumgartner, 2004; Walston et al., 2009). However, their main role during ageing is not yet clear (Maggio et al., 2006).

No studies exist in investigating the influence of age or gender on the myocardium in cats. Gaining knowledge of the myocardial ageing and remodelling processes in cats is of particular interest, not only because the systemic diseases that often affect ageing cats are similar to known human comorbidities (i.e. diabetes mellitus, renal diseases, systemic hypertension), but also because cats are commonly neutered, which allows the investigation of non-hormonal gender associated cardiac remodelling.

The objective of the present study was to investigate the constitutive, i.e. non-cardiac disease associated transcription of a range of cytokines and ECM remodelling enzymes known to be relevant for cardiac remodelling in other species, in different cardiac regions and in cats of different genders and ages. A proportion of cats had systemic diseases that potentially affected cardiac function or resulted in a systemic inflammatory response. The transcription patterns of these cats were therefore compared to the baseline expression of cats with diseases unlikely to affect the heart.

## 2. Material and methods

### 2.1. Animals and tissues

Twenty six cats were included in the study. Cats were patients that had been presented at the Universities of Helsinki and Bristol without clinical evidence of cardiac disease and had been euthanised upon the owner's request due to poor prognosis, impaired quality of life or financial constraints. Informed consent was obtained from owners prior to inclusion into the study and cats were assigned arbitrary numbers as identifiers. Institutional ethical approval was obtained.

From each cat, the signalement including breed, sex, age, weight and body condition score (BCS) was recorded. The heart was removed within 1 hour after death and grossly examined which confirmed the absence of any apparent cardiac disease. Myocardial samples from the interventricular septum, right atrium and ventricle, and left atrium and ventricle were collected for RNA extraction and stored in RNA stabilising solution (RNAlater; Ambion, Life Technologies, Paisley, UK) at −20 °C until analysed. Hearts were subsequently fixed in 10% formalin and samples from the same sites as those for RNA extraction were prepared and routinely paraffin wax embedded for histological examination. Sections (3–5 μm thick) were prepared and stained with haematoxylin–eosin (HE).

Twenty three cats subsequently underwent full necropsy to identify any relevant disease conditions. Samples were collected from all major organs as well as any gross abnormalities, fixed in 10% formalin and routinely paraffin wax embedded for histological examination of HE stained sections. For the remaining three cats (all from group "control cats") owners did not give consent for a full necropsy and only dissection and histological examination of the heart was permitted.

Based on the clinical and pathological findings, cats with diseases unlikely to have an effect on cardiac function and/or unlikely to have systemic effects, such as the systemic release of cytokines, that could have induced cytokine, MMP and/or TIMP production in the myocardium, were considered as control cats (group "control cats"); all remaining cats were allocated to group "systemic diseases", implying that they suffered from conditions with potential systemic effects, which might have influenced myocardial transcription processes (Fonfara et al., 2013a, 2013b).

### 2.2. RNA extraction

After removal from RNAlater, total RNA was extracted from the myocardial samples using a commercially available kit (Qiagen RNeasy Plus Universal mini Kit, Manchester, UK) according to the manufacturer's protocol. An initial step was added in which the tissue was placed in liquid nitrogen; the frozen tissue was then transferred into 900 μL of lysis reagent and ground thoroughly with a tissue pestle grinder. An on-column DNA digestion step was included. Final elution of the total RNA was performed using 30 μL of RNase-free water and repeated to maximise the amount of eluted RNA. The total RNA concentration of each sample was determined with a spectrophotometer.

### 2.3. Real time PCR

cDNA was synthesised from 300 ng total RNA using the I Skript cDNA Synthesis Kit (Bio-Rad, Hertfordshire, UK) according to the manufacturer's protocol and stored at −80 °C until use in the quantitative PCR. For the feline housekeeping gene GAPDH, as well as feline IL-1, -6, -18, TNF-α and TGF-β1, primers of previously published sequences were used (Kipar et al., 2001; Van Nguyen et al., 2006; Taglinger et al., 2008). Primers for feline IL-2, IL-4, IFN-γ, MMP-2, -3, 13, TIMP-1, -2 and -3 were designed using Primer Express software (Applied Biosystems, Life Technologies), and BLAST searches performed to confirm gene specificity. Primer sequences are shown in Table 1. Primers were synthesised by Eurogentec (Hampshire, UK). Primers were validated using a standard curve of eight serial dilutions and primer efficiencies were between 94% and 99%. PCR was performed according to standard protocols. Aliquots (1 μL) of cDNA were amplified in duplicates by PCR in 20 μL reaction volumes on a real time Stratagene 3500 (Applied Biosystems, Life Technologies) using Maxima SYBRGreen qPCR Master Mix (Fermentas, Cheshire, UK). Each assay well had a 20 μL reaction volume consisting of 12.5 μL SYBR Green PCR mastermix, each of 400 nM forward and reverse primers, and 1 μL of sample cDNA

**Table 1**
Primer sequences used for quantitative PCR.

| Primer | | Sequence (5′-3′) |
|---|---|---|
| IL-2 | Forward | CTGCTTCAAGCTCTACAAAGGAAAC |
| | Reverse | CTCCATTCAAAAGCAACCGTAAA |
| IL-4 | Forward | TCGTCCACGGCCAGAACT |
| | Reverse | TTTCTCGCTGTGAGGATGTTCA |
| IFN-γ | Forward | ATGATGACCAGCGCATTCAA |
| | Reverse | TTTACTGGAGCTGGTATTTAACAACTTATC |
| MMP-2 | Forward | TGGAGAGGCGGACATCATG |
| | Reverse | CCCGTCCTTGCCATCAAA |
| MMP-3 | Forward | GTTCTGGGCCATGAGAGGAA |
| | Reverse | GGAAAACCCAGGGTGTGGAT |
| MMP-13 | Forward | GACCCTCGACGCCATCAC |
| | Reverse | GCAGGCGCCAGAAGAATCT |
| TIMP-1 | Forward | GATACTTGCACAGGTCCCAGAAC |
| | Reverse | TCCGTCCCGCAGGTTTC |
| TIMP-2 | Forward | CTCCGGATGAATGTCTCTGGAT |
| | Reverse | GCAGAAGAACTTGGCCTGATG |
| TIMP-3 | Forward | CAACAAATACCAGTACCTGCTGACA |
| | Reverse | GGTCCCACCTCTCCACAAAGT |

IL: interleukin, IFN: interferon, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of matrix metalloproteinase.