**Update on biomarkers and use in myxomatous mitral valve disease in dogs**

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Biomarkers are characteristics that are objectively measured as indicators of health, disease, or a response to an exposure or intervention, including therapeutic interventions (FDA definition). <https://www.fda.gov/science-research/focus-areas-regulatory-science-report/focus-area-biomarkers>

Cardiac biomarkers are blood-based substances which can be assayed to give an indication of cardiac or cardiovascular health or disease. Most commonly used in clinical veterinary and cardiology specialist practice are:

* N-terminal proBNP (brain natriuretic peptide) (NTproBNP); synthesised and released from ventricular cardiomyocytes under conditions of myocardial wall stress.
* Troponin I: marker of cardiomyocyte injury (leakage marker from cardiomyocytes).

Myxomatous mitral valve disease (MMVD) is a valvular disease associated with volume overload with left sided chamber dilatation in presence of significant mitral regurgitation and therefore increased myocardial wall stress; increasing NTproBNP reflects this. In contrast, during phases of compensatory remodelling, there is no or minimal cardiomyocyte injury, so Troponin I is less useful for diagnosis or screening for the condition (in contrast to cardiomyopathies).

**N-terminal pro-B-type natriuretic peptide**

**Role of NTproBNP in screening for MMVD**

In primary care practice, where there may be no easy access for echocardiography, the significance of an acquired heart murmur consistent with mitral regurgitation can be assessed with NTproBNP. It can also be used to differentiate between a cardiac versus noncardiac cause of clinical signs, such as coughing. If concentration is normal, watchful monitoring of the murmur grade and serial evaluation of NT-proBNP (e.g. 6 monthly) can be considered. If values are increased (e.g. >900 pmol/L) further assessment and staging the MMVD based on the ACVIM consensus statement (Keene et al., 2019) is indicated; echocardiography and / or thoracic radiographs should be carried out, via referral if necessary. With NTproBNP values of >1500 pmol/L, there is increased risk of developing CHF within 6 – 12 months (Chetboul et al 2009; Serres et al. 2009, Borgarelli et al 2021)

If reliable echocardiography is available, NTproBNP level correlates with echo variables including LA/Ao, regurgitant fraction and normalised diastolic LV diameter or volume (Chetboul et al 2009) and so the addition of NTproBNP may not provide additional information.

**Can NTproBNP be used to stage MMVD?**

The increase in NTproBNP concentration with worsening stage of MMVD has been long recognised (Häggström et al 2000). There are certainly statistically significant population differences for dogs with different stages of MMVD. However, there is considerable overlap between groups, especially between ACVIM Stage B1 and B2 (Wolf et al, 2013), which means in primary care practice, the NTproBNP result cannot be used to determine when to start pimobendan. It is useful at identifying congestive heart failure as the cause of respiratory distress (cut-off >2447 pmol/L) (Fox et al. 2015). Higher values in MMVD dogs in Stage B indicate dogs which are more likely to progress (Mattin et al 2019a).

N-terminal proBNP concentration was associated with Stage B2, but predictive value improved when used in in conjunction with other physical examination and /or biochemical variables in a predictive model using multivariable logistic regression (Wilshaw et al. 2021). These authors (HAMLET study) proposed a future app which would be useful in primary care practice.

**What limits the value of NTproBNP assay?**

There can be considerable day to day biological variability of NTproBNP concentration, but usually not sufficient to affect decision making (Winter et al. 2017a). There are also some breed influences (Misbach et al 2013; Sjöstrand et al 2014 Gomart et al, 2020). There is a positive association between NTproBNP and age, independent of disease severity (Mattin et al. 2019b). Renal function (Miyagawa et al., 2013, Pelander et al 2017), exercise (Wall et al. 2018) and systemic hypertension (Jang et al. 2023) may also influence results. In some cases, NTproBNP can be increased for no identifiable reason (Misbach et al. 2013).

**Which NTproBNP assay?**

N-terminal proBNP assays are species specific. There is sample degradation after taking the sample, and with freeze-thaw cycles etc. recognised in the past (Hezzell et al. 2015). IDEXX now have developed a 2nd generation assay (Cahill et al. 2015), considered more stable, but the recommendation is that it should be received by the lab within 24 – 48 hours (Cardiopet). EDTA plasma is preferred as most stable (Cahill et al. 2015), and the sample should be centrifuged and separated as soon as possible after the taking sample. In recognition of the issue in sample degradation for the 1st generation assay in the past, the lab provided special protease inhibitor tubes, to put the EDTA plasma sample into prior to shipping.

The NT-proBNP assay is offered by other commercial labs, but it should be noted that this is the first generation assay and prone to sample degradation; they do not provide protease inhibitor tubes.

Another advantage of the 2nd generation assay is that the upper limit of quantification is ≥10,000 pmol/L (1st generation ≥3,000) so there is broader dynamic range. Comparison of the first and second assays showed similar results, so similar cut-offs from original publications can still be used (Cahill et al. 2015).

NTproBNP would be much more useful if available as a patient side point-of care test, especially in an animal with clinical signs. So far, there is no SNAP test similar to the feline assay for use by veterinarians. However, there is a recent point of care test (V-check) with good correlation with the IDEXX assay, although the lower limit of quantification is 650 pmol/L (Harr et al. 2022)

**Can we use NT-proBNP to guide Treatment?**

In human cardiology, UK NICE guidelines recommend that general practitioners use NTproBNP to assess every patient with suspected heart failure, with guidance when and how urgently to refer to a cardiologist based on the results (2023 update):

 <https://www.nice.org.uk/guidance/qs9/chapter/Quality-statement-1-N-terminal-pro-B-type-natriuretic-peptide-measurement#rationale> However, current NICE guidelines about monitoring treatment for all type of heart failure on recommend “NT-proBNP as part of a treatment optimisation protocol only in a specialist care setting for people aged under 75 who have heart failure with reduced ejection fraction and an eGFR above 60 ml/min/1.73 m2” (2018) <https://www.nice.org.uk/guidance/ng106/chapter/Recommendations#monitoring-treatment-for-all-types-of-heart-failure> .

A Cochrane review compared the approach of NTproBNP (or BNP) guided therapy to conventional management and monitoring of congestive heart failure, based on symptoms. It suggested that there was a reduced relative risk (RR) for heart failure mortality and heart failure admissions in patients with NP guided therapy (RR 0.84 and 0.70 respectively), although this was less clear for all-cause mortality (RR 0.87) or all-cause admissions (RR 0.93) (McLellan et al. 2016). The benefit of NTproBNP guided therapy in reducing hospitalisations is only significant in patients less than 75 years old (Davarzani et al 2017). Continued high NT-pro-BNP levels predicts poor quality of life in human patients (Zelenak et al. 2019).

Veterinary cardiology has not so far embraced serial monitoring of canine NTproBNP, possibly because of the cost. However, decrease in NTproBNP concentration (to <965 pmol/L) in response to treatment is a good prognostic indicator (Wolf et al. 2012). Rate of increase of NTproBNP is associated with death (Hezzell et al. 2012). Serial monitoring to guide treatment was proposed to be potentially useful a decade ago (Oyama et al. 2013). Hezzell and colleagues (2018) reported on intensification of treatment in dogs with recently decompensated CHF based on NTproBNP concentration (>1500 pmol/L), showing NTproBNP could be reduced. This study was just over 21 days, so longer-term follow up and direct comparison between dogs monitored and treated based on clinical signs such as sleeping respiratory rate (SRR) as well as those based on NTproBNP concentration is indicated. Increase in SRR is an early indicator of further decompensation, but intervening based on serial NTproBNP concentration may actually prevent this decompensation.

**Troponin I**

Troponin I, a marker of heart muscle cell injury, is usually not increased in early (pre-symptomatic) stages of MMVD. However, Troponin I increases in more advanced disease, and it correlates with clinical status (Polizopoulou et al 2014; Chan et al 2019). Persistently high or increasing values indicate poor prognosis (Hezzell et al. 2012). However, treatment may result in decrease of Troponin I concentration (Polizopoulou et al 2014; Chan et al. 2019). Surprisingly, in the study by Chan and colleagues (2019), greater decrease in Troponin I over time was associated with worse prognosis.

The reason for increase in Troponin I in a non-myocardial disease may reflect the remodelling changes associated with volume overload. This may result in cardiomyocyte death and replacement fibrosis. Furthermore, arteriosclerosis has been reported in MMVD, resulting in ischaemic injury and fibrosis. In a longitudinal study of dogs with MMVD and subsequent post-mortem examination, the last measured Troponin I concentration was found to correlate with the severity of myocardial fibrosis on histopathology (Falk et al. 2013). Troponin I concentration also correlates with the acute phase protein, CRP, in dogs with MMVD (Ljungvall et al 2010), with CRP associated with severity of disease; further evidence of cardiac disease and congestive heart failure being associated with inflammation (Polizopoulou et al. 2015).

Troponin I may increase with systemic disease affecting the myocardium, so increased concentration is not specific for cardiac disease. Some studies also show an association with ageing (Ljungvall et al 2010). Troponin I therefore predicts all mortality rather than just specifically cardiac mortality (Hezzell et al 2012). There is also considerable biological variability in Troponin I in both healthy dogs and those with MMVD. It has been recommended that only changes in concentration of >110% in dogs with MMVD are likely to reflect changes in status during serial monitoring; smaller changes may merely reflect this biological variation (Winter et al. 2017b).

**Which Troponin I assay?**

Troponin I is not species specific, so there are a range on assays available. In healthy dogs, the standard Immulite assay is often below the limit of detection. Therefore, use of the standard assays is not recommended, but they can be useful (and have a wider range) when levels are high. There are a range of high- or ultra- sensitivity assays now commercially available and they are more likely to be useful for serial monitoring, as long as the biological variability is noted (Winter et al. 2017b). They will have different reference ranges, lower and upper limits of detection, and range of values.

**Use of both cardiac biomarkers together?**

Hezzell and colleagues (2012) showed that, when excluding echocardiographic variables, high sensitivity Troponin I and NTprobBNP (as well as heart rate and age) were all independently associated with survival. If both NTproBNP and high sensivity Troponin I were above cut-off values (>0.025 ng/mL and >524 pmol/L respectively), there was reduced survival compared to just one being increased and better survival when both biomarkers were below these cut-offs. This study showed that Troponin I was independently associated with survival when echocardiography variables were included, whereas NTproBNP was associated with the echo variables.

It makes sense that use of both cardiac biomarkers provides additional information, as they reflect different things.

**MicroRNAs**

MicroRNAs (or miRNAs) are small, non-coding RNAs (about 22 nucleotides long). They are transcribed in the same way as mRNA, but when processed (by endonucleolytic cleavages), the silence or down-regulate the expression of their gene targets. They are therefore important in numerous biological processes, and may be altered in various pathophysiological processes. MicroRNAs can be found in tissue (e.g. heart valves; Yang et al 2018) and can also be circulating. They can be cell-free in the plasma or within exosomes (microvesicles) (Yang et al 2017). If free in the plasma, they are protein bound with argonaute-2 or high density lipoproteins. They are very stable, so could be an ideal biomarker if specific for a certain disease or stage of a disease such as MMVD. An excellent review article about the role of miRNAs in veterinary cardiovascular disease has recently been published (Reis-Ferreira et al 2022). Nomenclature is not intuitive. Most are preceded by “miR”, with the capitalisation indicating the mature form of the miRNA, whereas “mir” is indicative of a precursor or primary miRNA (pre-miRNA and pri-miRNA). This is a newer nomenclature system, and many miRNAs have been renamed to adhere to this system, something which must be considered with older literature. However, some older sequences, preceded by “let” or “lin”, have been left unaltered for historical reasons. There then follows a number sequential to the order of discovery. A final suffix may be included, which may represent slight differences in sequence, with the number may be followed by a letter (a, b etc) or hyphenated number (-1, -2, etc), or may indicate sequence direction (5p or 3p). There may be a species specific prefix (e.g. cfa for *Canis familiaris* if canine), however miRNAs conserved between species will all carry the same main sequential numerical identifier. Due to the rapidly evolving nature of the field and constant identification of new miRNAs, future revisions and further standardisation of the current nomenclature is likely.

Plasma or serum miRNAs associated with MMVD include:

1. In Dachshunds with various stages of MMVD: miR-30b differed in Dachshunds with MMVD; down-regulated in Stage B compared with Stage A dogs (marker of preclinical MMVD). miR-133b was down-regulated in Stage C compared with Stage A dogs (so possible marker of CHF) (Hulanicka et al 2014)
2. In a study looking at differential expression of miRNAs between dogs of various breeds with MMVD and controls (Li et al. 2015), 7/11 miRNAs showed decreased expression in MMVD (Stage B or C/D compared with controls: cfa-miR-302d, cfa-miR-380, cfa-miR-874, cfa-miR-582, cfa-miR-490, cfa-miR-329b, and cfa-miR-487b. 4/11 showed increased expression in dogs with B or C compared with controls: cfa-miR-103, cfa-miR-98, cfa-let-7b, and cfa-let-7c). There were six which showed different expression between Stages B1/B2 and C/D: cfa-miR-582, cfa-miR-487b, cfa-miR-103, cfa-miR-98, cfa-let-7b.
3. When 9 healthy dogs were compared with 8 dogs with MMVD and CHF, of 326 miRNAs identified, principle component analysis (PCA) identified 47% of differential expression for component 1 and only an additional 9% for component 2. Of the 326 miRNAs, 5 canine specific miRNAs were significantly upregulated in CHF dogs; miR-133, miR-1, miR-139, cfa-let7e, miR-125a. There were 88 miRNAs down regulated in CHF (Jung & Bohan 2018)
4. In a study with 72 dogs with various heart diseases (35 with MMVD), and 10 healthy dogs, cfa-miR-130b was up-regulated in Stage B MMVD, but this is down-regulated again in Stages C/D, without any significant difference from control dogs. cfa-miR-130b was claimed to be more accurate (based on ROC AUC) than NT-proBNP for discriminating dogs with heart diseases from healthy dogs (but only Stage B2 MMVD) (Ro et al 2021).
5. CKCS specifically with Stage B1 MMVD, in different age categories, were addressed (Bagardi et al. 2022). MiR-30b-5p was significantly higher in CKCS with Stage B1 compared with stage A; with a trend to increasing up-regulation in Stage B1 if dogs were older (Bagardi et al 2022). Why this is different from the finding in Dachshunds (see no. 1 above) is not certain. However, miR-30b-5p could identify Stage B1 confirmed on echocardiography, but without any audible murmur.
6. Ghilardi et al (2022) further studied 35 CKCS with MMVD in different stages and the predictive value of miR-30b-5p (PRIME study). This showed that miR-30b-5p levels declined with advancing disease, evidenced by LV diameters or volumes, both in diastole and systole, LA/Ao, mitral regurgitation severity, MINE score, heart murmur grade. Higher miR-30b-5p therefore indicated more stable MMVD and less severe progression. However, this decline does show that miR-30b-5p is a marker of early MMVD, not advanced disease.

Compared to most biomarkers, there are no units of measurement and miRNAs are simply given as severity of down- or up-regulation (expression profiling) compared with control animals or an internal control such as an endogenous or added miRNA.

The plethora of different miRNAs presented in the available literature likely indicates that no single one can be a gold-standard cardiac biomarker, but a more integrated approach is required for diagnosis and stage of the disease, such as principle component analysis.

This expression profiling approach of multiple miRNA markers is currently in development by a UK-based veterinary diagnostics company (MI:RNA Ltd, Edinburgh, www. mirna-diagnostics.com). In summary, a multiplexing detection assay is used to describe the unique profile or ‘fingerprint’ based on up to 30 miRNA markers for a particular patient’s sample, for which disease status can then be classified with bioinformatic modelling analysis based on a well described reference population. The modelling analysis has a continually improving supervised AI component, ensuring the accuracy of classification improves as the reference population increases, harnessing the power of so called ‘big data’. Results from a MMVD focused study are under submission, and the company has already applied the technology to infectious livestock disease, Johne’s disease (<https://doi.org/10.1101/2023.07.07.548088>).

**Other cardiac biomarkers**

**Copeptin**

Copeptin in the C-terminal fragment of pre-pro-vasopressin, which is much more stable than assaying vasopressin. It was found to be the best predictor of mortality, superior to NTproBNP (Düngen et al. 2018). Other studies have confirmed this, especially as an indicator of outcome in heart failure patients, recently reviewed in a metanalysis (Zimodro et al. 2022). Although salivary copetin has been investigated in dogs with separation anxiety, the author is unaware of any copeptin publications about heart failure in dogs.

**Galectin-3**

This is over-expressed in heart failure. It is produced by macrophages involved in tissue and fibrous remodelling. High levels predict morbidity in humans (need for hospitalisation) and mortality. It is inferior to NTproBNP in humans, but can be useful in risk stratification (Castiglione et al 2022). It has been investigated in dogs and is increased in dogs with MMVD, reflecting myocardial fibrosis (Sakarin et al. 2016; Lee et al. 2021). However, another study did not identity changes in Galectin-3 in dogs (Klein et al. 2022).

**Others**

A number of other biomarkers have been used both in the diagnosis of heart failure and risk stratification in humans (Castiglione et al 2022). These include Adrenomedullin (or fragment of its precursor – MR-proADM) and others. **Soluble suppression of tumorigenesis-2 (SsST2** – a marker of inflammation in heart failure) (Myre et al. 2022) has been investigated in dogs (interleukin-1 receptor like 1 protein (ST2) -which has a transmembrane (ST2L as well as the soluble form (sST2) (Klein et al. 2022)), but without significant differences from controls (Klein et al. 2022). **Osteopontin** modulates myocardial remodelling (hypertrophy) as well as remodelling of the extracellular matrix and is proinflammatory (Mamarzhakyov et al 2022). **Cartilage intermediate layer protein-1** **(CILP1)** has recently been recognised as being important in human heart disease, and is of prognostic value in heart failure (Wang et al. 2022). A very recent study showed serum levels increased with stage of MMVD in dogs (Kim et al. 2023). It is an extracellular matrix protein and is involved in myocardial fibrosis.

A panel of biomarkers may be useful in the future in veterinary cardiology in risk stratification.

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