Scientific Reports

https://doi.org/10.1038/s41598-026-38660-y

Article in Press

Identification of altered salivary microRNAs in Cavalier King Charles Spaniels affected by mitral valve disease at different ACVIM stages

Received: 16 September 2025

Accepted: 30 January 2026

Published online: 20 February 2026

Cite this article as: Ghilardi S., Salvi G., Bagardi M. *et al.* Identification of altered salivary microRNAs in Cavalier King Charles Spaniels affected by mitral valve disease at different ACVIM stages. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-38660-y

Sara Ghilardi, Gaspare Salvi, Mara Bagardi, Ombretta Balbi, Asia Balgera, Yasmine Dadi, Giovanni Romito, Michele Polli, Maddalena Arigoni, Raffaele Calogero, Paola G. Brambilla & Cristina Lecchi

Identification of altered salivary microRNAs in Cavalier King Charles Spaniels affected by mitral valve disease at different ACVIM stages

Sara Ghilardi[1], Gaspare Salvi[1], Mara Bagardi[1], Ombretta Balbi[1,4], Asia Balgera[1], Yasmine Dadi[1], Giovanni Romito[2], Michele Polli[1], Maddalena Arigoni[3], Raffaele Calogero[3], Paola G. Brambilla[1], Cristina Lecchi[1,4*]

[1]Department of Veterinary Medicine and Animal Science, University of Milan, Lodi, Italy

[2]Department of Veterinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Bologna, Italy

[3]Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy

[4]MOOVET DXC S.r.l., Precision Diagnostics LAB for Animal Health – Spin-off of the University of Milan, hosted at the Department of Veterinary Medicine and Animal Sciences, Lodi, Italy

[*]Corresponding author

E-mail: cristina.lecchi@unimi.it

Abstract

Myxomatous mitral valve disease (MMVD) in Cavalier King Charles Spaniels (CKCSs) can be diagnosed at a young age. Early and sensitive biomarkers related to the disease are currently lacking. MicroRNAs (miRNAs) are involved in the onset and progression of canine MMVD. This study aimed to profile the salivary miRNAs associated with MMVD severity in CKCSs and to identify their target genes. This was a pilot prospective, cross-sectional study. Saliva was collected from twenty-five CKCSs belonging to the American College of Veterinary Internal Medicine (ACVIM) stages A (n. 6), B1 (n. 13), and B2 (n. 6), and salivary miRNAs' expression was profiled by Next Generation Sequencing. miRNAs' target genes were identified using bioinformatic tools. Results showed that 25 miRNAs were differentially expressed (DE) between ACVIM A and B1 dogs; 35 DE-miRNAs were modulated in ACVIM B2 compared to B1 CKCSs; 4 miRNAs were up-regulated in ACVIM stage B1 patients compared to both ACVIM stages A and B2. These findings confirm that salivary miRNAs can be successfully quantified in CKCSs and that their expression differs across MMVD severity groups. The DE-miRNAs were associated with signalling pathways related to cellular metabolism, survival, and early inflammatory remodeling. Given the exploratory and cross-sectional design, these miRNAs should be considered candidate, non-invasive indicators associated with disease severity rather than established biomarkers. Further longitudinal and validation studies are needed to assess their diagnostic potential.

Keywords

Myxomatous mitral valve disease, microRNA, saliva, biomarkers, dog, Cavalier King Charles Spaniels.

Introduction

Myxomatous mitral valve disease (MMVD) is the most common acquired canine heart disease and the most frequent cause of congestive heart failure (CHF) in dogs[1]. The incidence and progression of the disease are strongly associated with age and breed[1]. The disease is hereditary in some breeds, like the Cavalier King Charles Spaniel (CKCS)[2], for which a polygenically inherited component has been hypothesized; indeed, the disease can be commonly diagnosed before 6 years of age in these dogs[2,3]. Transthoracic echocardiography is the gold standard for the ante-mortem, non-invasive diagnosis of MMVD in dogs[4]; however, its accuracy can be limited in the early stages when cardiac murmurs are soft and valvular changes are subtle[1,5]. As a result, reliable biomarkers could support earlier and more objective detection of the disease.

Although the pathogenesis of MMVD is not fully understood, accumulating evidence indicates that molecular and biochemical alterations accompany disease development and progression[6,7]. Among these, dysregulation of microRNAs (miRNAs) has been documented in the cardiac tissue, plasma, and serum of dogs affected by MMVD[5,7-13]. miRNAs play crucial roles in regulating cellular homeostasis, and changes in their expression have already been linked to various canine cardiovascular processes[8]. Several studies reported that miRNAs isolated from cardiac tissue, plasma, and serum are modulated in dogs affected by MMVD[8]. Moreover, some of these miRNAs are reported to be differentially expressed among the various American College of Veterinary Internal Medicine (ACVIM) stages of the disease[8-12]. However, to date, most studies on miRNAs in dogs have only evaluated one or a few miRNAs associated with MMVD[14], and only a few studies focused on specific breeds[5,10,13]. Recently,

a relationship between MMVD and circulating miR-30b-5p in CKCSs has been established, reporting that miR-30b-5p can discriminate among ACVIM A and B1 subjects, even at a young age[5], and that higher plasma miR-30-5p expressions are associated with forms of MMVD that are more stable over time[13].

At present, the search for circulating biomarkers still depends on blood sampling, which can pose challenges in certain clinical situations (i.e., anxious, young, or difficult-to-handle animals); therefore, the need for minimally invasive and repeatable diagnostic tools has stimulated interest in alternative matrices such as saliva[15,16]. In fact, although blood sampling is routinely feasible in dogs, saliva offers a minimally invasive and repeatable alternative that may facilitate serial monitoring or sampling. Salivary biomarkers have gained increasing relevance in human cardiology, and miRNAs are particularly promising due to their stability and abundance in this matrix[17,18]. Despite this potential, salivary miRNAs have not yet been investigated in veterinary cardiology, and no data are available regarding their expression in dogs with MMVD. For this reason, the absence of minimally invasive biomarkers for the early stages of MMVD highlights a significant clinical need, particularly in high-risk breeds such as CKCSs.

The present study aimed at assessing miRNAs' expression profiles in the saliva of CKCSs affected by MMVD at different ACVIM stages (A, B1, B2). We explored whether stage-related differences in salivary miRNA profiles could be identified and whether bioinformatic analyses of associated targets and pathways might provide preliminary insights into biological processes potentially involved in early MMVD progression, thereby supporting the identification of candidate, minimally invasive molecular patterns associated with the disease.

Materials and Methods

Subjects' enrollment and saliva collection

This was a pilot exploratory prospective, cross-sectional study. The study included owned CKCS (i.e., > 1 year) that were visited at the Cardiology Unit of the Department of Veterinary Medicine and Animal Science, Veterinary Teaching Hospital of the University of Milan, between May 2022 and January 2024. Sample collection procedures and patient management were conducted in accordance with the ARRIVE guidelines to ensure appropriate reporting and high standards of animal welfare. Informed consent was obtained from the owners, as per the University of Milan's ethical committee statement, number 2/2016, and a high standard of care was maintained throughout each examination.

For each CKCS, clinical history was registered, and clinical evaluation (complete with auscultation and non-invasive blood pressure measurement), complete blood exams (haematology and serum biochemistry), echocardiography, radiographic evaluation of the thorax, and electrocardiography were performed as part of a yearly breed screening program. A standard transthoracic echocardiography was performed by three experienced operators (a third-year PhD student in small animal cardiology, an ECVIM-CA cardiology resident-in-training, and a professor of veterinary cardiology with more than 20 years of experience) using a MyLab™FOX ultrasound machine (Esaote, Genova, Italy) equipped with multifrequency phased-array probes (2-9 MHz, 1-5 MHz). Probes were chosen according to the dog's size. Echocardiography was performed with continuous electrocardiographic monitoring, and video clips including at least three

cardiac cycles were obtained to make offline measurements. Animals were gently restrained in the right and left lateral recumbency, and no sedation was needed for the procedure. Echocardiographic measurements were obtained by the same operator who performed the transthoracic echocardiography. Parameters from 2D images were measured using the inner edge-to-inner edge method, including the left atrium-to-aortic root ratio (LA/Ao), which was obtained from a right parasternal short-axis view of the cardiac base as previously reported[19]. Measurements from M-mode images included left ventricular internal diameters in end-diastole and end-systole normalized for body weight (LVIDdN and LVIDsN, respectively) and were obtained from a 2D-directed image of the right short-axis view at the level of the papillary muscles using the leading edge-to-leading edge method[20]. Calculated measurements from M-mode parameters were subsequently obtained, specifically ejection fraction (EF%) and fractional shortening (FS%), by applying formulas previously reported[21]. Color flow Doppler was used to identify and describe the severity of mitral regurgitation (MR) using a right parasternal long-axis four-chamber view and a left apical view. MR severity was assessed by calculating the maximal ratio of the regurgitant jet area signal to LA area (ARJ/LAA ratio), and it was considered mild when ARJ/LAA ratio was < 20-30%, moderate when it was ≥ 20-30% and ≤ 70%, and severe when it was > 70%[21]. Finally, the diastolic flow profile of the left ventricle was obtained through pulsed-wave Doppler by placing the sample volume at the mitral leaflets' tips[21]; for the study, E wave peak velocity and E/A ratio were registered.

While inter- and intra-operator variability were not formally evaluated in the current study, the echocardiographic protocol and measurement procedures were consistent with those used in a recent study of our group involving healthy

French Bulldogs[22]. In that study, the intra-operator coefficient of variation (CV) ranged from 0.52% to 10.11%, and the inter-operator CV ranged from 1.28% to 14.19% for key M-mode, 2D, and Doppler parameters[22]. This prior validation supports the reliability of the echocardiographic measurements utilized in the present study. Based on the echocardiographic findings, dogs were assigned to an MMVD stage following the current guidelines[4], as follows: a) ACVIM stage A: absence of a left apical systolic heart murmur on cardiac auscultation, as well as echocardiographic lack of mitral valve thickening and/or prolapse and mitral regurgitation; b) ACVIM stage B: presence of a left apical systolic heart murmur on cardiac auscultation associated with echocardiographic findings of mitral valve thickening and/or prolapse and mitral regurgitation identified on color flow Doppler interrogation, in the absence of clinical signs related to left-sided CHF[4]. Additionally, ACVIM stage B CKCSs were divided into ACVIM stage B1 dogs (i.e., echocardiographic LA/Ao < 1.6[19], LVIDdN < 1.7[20], and breed-adjusted radiographic vertebral heart score (VHS) ≤ 10.5[4]), and ACVIM stage B2 dogs (i.e., echocardiographic LA/Ao ≥ 1.6[19], LVIDdN ≥ 1.7[20], and breed-adjusted radiographic VHS >10.5[4]). Since pharmacologic treatments may influence circulating or salivary miRNA profiles, all dogs receiving cardiac medications as suggested by the current ACVIM guidelines[4] were registered (i.e., pimobendan in stage B2 dogs). This variable was recorded. Treatment was not included as a covariate in the statistical model but was considered during interpretation of the results.

A saliva sample was collected using a sterile dry swab (Copan Diagnostics Inc., USA) placed in the mouth of the dog for about fifteen seconds. Swabs were stored at -80°C for further analysis.

Patients affected by systemic diseases (e.g., metabolic diseases, oncologic diseases, infectious diseases), congenital or acquired cardiovascular diseases different from MMVD, MMVD at ACVIM stages different from the aforementioned ones (i.e., stages C and D), rhythm disturbances, systemic hypertension, or pre-capillary pulmonary hypertension, and dogs with any clinical signs of oral disorders were excluded from the study. Dogs in ACVIM stages C and D were excluded from the study, as the analysis specifically targeted the early, preclinical stages of MMVD (A, B1, and B2). The presence of overt CHF, along with the increased clinical and therapeutic variability typical of stages C and D, would have introduced additional inconsistencies that were not compatible with the objectives of this pilot discovery study.

Small RNA extraction and sequencing

Total RNA was extracted using the MicroRNA Concentrator kit (A&A Biotecnology, Cat. No 035-25C) following the manufacturer's instructions. The RNA quality and quantity were verified according to MIQE guidelines[23]. RNA concentration was quantified by Qubit® 2.0 Fluorometer with Qubit® microRNA Assay Kit (Invitrogen, Cat. No. Q32880). Small RNA transcripts were converted into barcoded cDNA libraries. Library preparation was performed as previously reported[24] using the NEBNext Multiplex small RNA Library Prep Set (Cat. No. NEB#E7560) for Illumina and run on the NextSeq2000 (Illumina Inc., USA). Each library underwent quality analysis using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip. Only libraries that met the expected profile criteria were considered for sequencing. The output of the NextSeq2000 Illumina sequencer was demultiplexed using bcl2fastq Illumina software embedded in the docker4seq package[24,25]. miRNA expression

quantification was performed using the previously described workflow[24] and the implementation as described[26]. Sequences were mapped using BWA[27] to *Canis familiaris* precursor miRNAs available in miRBase 22.1 (http://www.mirbase.org/). Count tables and counts per million reads (CPM) tables were used to perform differential expression (DE) analysis with a threshold-adjusted *P* value ≤ 0.1 and an absolute $\log_2$ Fold Change ($\log_2$FC) ≥ 1, as this is aligned with discovery-stage biomarker research and with considerations related to false discovery rate control.

As this is a pilot discovery study, the miRNAs identified should be regarded as exploratory biomarker candidates that will require independent validation before clinical application (i.e., bigger cohort, different breeds).

miRNA target prioritization

The target genes of DE-miRNAs were predicted using MiRWalk 3.0[28] by targeting the entire gene sequence (including 5' untranslated region (UTR), codon sequence (CDS), and 3'UTR) and filtering for the 3 miRNA-target prediction programs (miRDB[29], miRTarBase[30] and Targetscan[31]). The list of target genes predicted by the three tools was included in further analysis, and functional mRNA enrichment was performed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatic resource[32,33] and biological pathways in the KEGG (Kyoto Encyclopedia of Genes and Genomes)[34] were examined for enrichment. Genes associated with cancer and infectious diseases have been deliberately excluded from further analyses.

Computational and statistical analysis

Raw reads quality-check, adapter clipping, and mapping were performed as previously reported[25]. After reads' mapping, a matrix of integer values was

created. The value in the i-th row and the j-th column of the matrix report how many reads have been unambiguously assigned to mature miRNA i in sample j. The unwanted variation in the data was estimated using the functions implemented in the SVA package[35]. The differential expression analysis was performed using DESeq2[36], setting a threshold-adjusted *P*-value < 0.1 and -1 ≥ $|\log_2 FC| \geq 1$.

Statistical analysis was carried out using SPSS 28 (SPSS Inc., Chicago, IL, USA). The distribution of data was assessed using the Shapiro-Wilk test. Normally-distributed continuous variables were reported as mean ± standard deviation (SD), while non-normally-distributed variables were reported as median and interquartile range (IQR) from the 25th to the 75th percentile ($IQR_{25\text{-}75}$). Categorical variables were expressed as frequencies and percentages.

Differences between continuous variables were analyzed among the ACVIM stages using the Kruskal-Wallis non-parametric test. Statistical significance was accepted at a *P* value ≤ 0.05.

Results

Demographics, clinical, and echocardiographic characteristics of the study population

Twenty-five adult CKCSs were included in the study. Six dogs were classified as ACVIM stage A, 13 as ACVIM stage B1, and 6 as ACVIM stage B2. Patients' clinical and echocardiographic findings are summarized in Table 1. Age was significantly higher in the ACVIM stage B2 dogs compared to healthy dogs (ACVIM stage A) (*P* = .006). Furthermore, as expected, ACVIM stage B2 dogs had some significantly higher echocardiographic values when compared to both

healthy and ACVIM stage B1 dogs. Specifically, LA/Ao (P_{A-B2} = .003; P_{B1-B2} = .004), E wave peak velocity (P_{A-B2} = .001; P_{B1-B2} = .006), LVIDdN (P_{A-B2} = .000; P_{B1-B2} = .011), and LVIDsN (P_{A-B2} = .010; P_{B1-B2} = .024).

Among the ACVIM stage B2 dogs, 2 (33.3%) were on medical treatment at the time of inclusion. Specifically, both were given Pimobendan (0.28 and 0.32 mg/kg q12 h). The complete dataset is available as Supporting Information.

RNA extraction from canine saliva and characterization of miRNome profile

To characterize miRNA expression profiles, small RNA-seq was performed on RNA extracted from the saliva of dogs. After RNA extraction, small RNAs were selected according to their size on a gel (≈146 bp band) and sequenced on the NextSeq2000 sequencer (Illumina). More than 1 million reads for each sample were generated. The counts table was used to detect DE-miRNAs via DESeq2 analysis[36]. This analysis depicted the expression of 453 *Canis familiaris* (cfa) miRNAs in the saliva.

Modulated salivary miRNAs in healthy and MMVD-affected dogs

To identify the common MMVD signature between A, B1, and B2 ACVIM stages, the Venn analysis was performed. A difference in miRNA profile among the MMVD stages was observed, with 25 DE-miRNAs between ACVIM stages A and B1 dogs and 35 DE-miRNAs between ACVIM stages B2 and B1 dogs; among the DE-miRNAs, four (i.e., cfa-miR-8857, cfa-miR-9, cfa-miR-206, cfa-miR-433) were dysregulated in both comparison groups (up-regulated in B1 compared to both A and B2), as shown in Figure 1 and Supplementary Table 1. Specifically, all the 25 DE-miRNAs were up-regulated in ACVIM stage B1 dogs when compared

to stage A; among the 35 DE-miRNAs between stage B1 and stage B2 CKCSs, 30 were up-regulated, and 5 were down-regulated in stage B1 compared to stage B2 dogs. No altered miRNAs were detected in ACVIM stage A dogs compared to B2 dogs (Supplementary Table 1). Absence of detectable differential expression may reflect molecular stabilization, cohort size limitations, or convergence of chronic disease phenotypes rather than complete biological equivalence.

miRNA target prediction and pathway enrichment

To assist the reader in contextualizing the pathway analyses, the main findings are summarized below:

- 25 miRNAs were up-regulated in B1 compared to A dogs. These miRNAs were predominantly associated with neurotrophin, AMPK, FoxO, and MAPK signaling pathways.
- When comparing B1 and B2 dogs, 35 DE-miRNAs were detected. The 30 DE-miRNAs that were up-regulated in B1 dogs were enriched in thyroid hormone signaling, efferocytosis, and adherens junction pathways. Conversely, the 5 down-regulated miRNAs in B1 dogs were connected to Th17 cell differentiation, adherens junction, and cellular senescence pathways.
- The four miRNAs that were up-regulated in B1 when compared to both A and B2 CKCSs converged on the PI3K–Akt, longevity-regulating, neurotrophin, and AMPK pathways.

A compared to B1 dogs

The predicted mRNA targets of the 25 DE-miRNAs, which were all up-regulated in ACVIM B1 CKCSs, were 211 (135 at 3’UTR, 17 at 5’UTR, and 83 at CDS).

Effect sizes (log2FC values) for all DE-miRNAs are reported in Supplementary Table 1 to contextualize the magnitude of modulation. KEGG pathway analysis was performed on the enriched genes using DAVID. The top significantly enriched KEGG pathways are reported in Figure 2a. The DE-miRNAs were identified to be primarily involved in the neurotrophin, the AMPK (AMP-activated protein kinase), the FoxO (Forkhead box transcription factor class O), and the MAPK (mitogen-activated protein kinase) signaling pathways. Subsequently, gene ontology (GO) analysis was performed, including the following categories: biological process (BP), cellular component (CC), and molecular function (MF). Most of the enriched MF included genes involved in the regulation of transcription and transduction processes and the negative regulation of the apoptotic process; the enriched BP included genes involved in RNA, DNA, and protein binding, while enriched CC genes mainly converged on the nucleus and the cytosol (Figure 2b).

B2 compared to B1 dogs

Thirty-five DE-miRNAs were detected comparing ACVIM stage B2 and stage B1 CKCSs. In detail, 30 DE-miRNAs were up-regulated, and 5 were down-regulated in ACVIM stage B1 dogs. Effect sizes (log2FC values) for all DE-miRNAs are reported in Supplementary Table 1 to contextualize the magnitude of modulation.

The predicted mRNA targets of the 5 down-regulated miRNAs in ACVIM stage B1 dogs were 93 (58 at 3'UTR, 4 at 5'UTR, and 37 at CDS). KEGG pathway analysis was performed using DAVID, and the top significantly enriched KEGG pathways are reported in Figure 3a. The miRNAs down-regulated in ACVIM stage B1 CKCSs were predominantly involved in the following pathways: thyroid hormones (THs) signaling pathway, efferocytosis, and adherens

junction pathways. GO analysis revealed that down-regulated miRNAs enriched MF included genes mostly involved in the regulation of the transcription and transduction processes and the positive regulation of the apoptotic process; the enriched BP included genes involved in RNA, DNA, and protein binding, while enriched CC genes mainly converged on the nucleus and the cytosol (Figure 3b).

The predicted mRNA targets of the 30 up-regulated miRNAs in ACVIM stage B1 dogs were 94 (58 at 3'UTR, 6 at 5'UTR, and 35 at CDS). DAVID was used to perform KEGG pathway analysis, and the top significantly enriched KEGG pathways are reported in Figure 4a. The up-regulated miRNAs in ACVIM stage B1 CKCSs were involved in the T helper 17 (Th17) cell differentiation, adherens junction, and cellular senescence pathways. GO analysis revealed that most of the up-regulated miRNAs enriched MF included genes involved in the regulation of the transcription and transduction processes and the positive regulation of the apoptotic process; the enriched BP included genes involved in RNA, DNA, and protein binding, while enriched CC genes mainly converged on the nucleus and the cytosol (Figure 4b).

DE-miRNAs **modulated in all the considered ACVIM stages (up-regulated in B1 compared to both A and B2 dogs)**

Target enrichment analyses were also performed on the four dysregulated miRNAs, namely cfa-miR-8857, cfa-miR-9, cfa-miR-206, and cfa-miR-433, that were up-regulated in B1 compared to both A and B2 dogs. The predicted mRNA targets of these 4 DE-miRNAs were 43 (25 at 3'UTR, 4 at 5'UTR, and 16 at CDS). KEGG pathway analysis was performed through DAVID, and the top significantly enriched KEGG pathways are reported in Figure 5a. The DE-miRNAs were identified to be primarily involved in the PI3K-Akt signaling

pathway, the longevity regulating pathway, and the neurotrophin and AMPK signaling pathways. Gene ontology analysis revealed that most of the enriched MF included genes involved in regulating transcription and apoptotic processes; the enriched BP included genes involved in RNA, DNA, and protein binding, while enriched CC genes mainly converged on the nucleus and the cytosol (Figure 5b).

Discussion

This pilot study provides the first description of the salivary miRNome in CKCSs across different MMVD severity groups. Results demonstrated that (I) the level of salivary miRNAs is modulated by MMVD in CKCSs; (II) ACVIM stage B1was associated with an enrichment of pathways commonly linked to compensatory and protective biological processes, whereas ACVIM stage B2 was associated with an enrichment of pathways related to inflammatory remodeling and cellular stress responses; (III) four DE-miRNAs could potentially represent the common signature of MMVD in CKCSs.

Changes in circulating miRNAs have been previously reported in dogs affected by MMVD using a targeted approach. Specifically, Li and coworkers (2015)[11] profiled the blood miRNA of dogs at ACVIM stages B1/B2, C/D, and A using a qPCR array approach, pointing out that 11 miRNAs out of 277 were differentially expressed. A different panel of 11 miRNAs was investigated in dogs with various heart diseases, including MMVD, by real-time-qPCR, demonstrating that cfa-miR-130b was up-regulated in MMVD-affected dogs at ACVIM stage B (comprising both B1 and B2), although no differences were observed in stage C and stage D compared to the healthy group[12]. miRNA delivered by small extracellular vesicles and isolated from whole plasma of dogs

with asymptomatic MMVD (ACVIM stages B1 and B2) and dogs with severe MMVD (ACVIM stages C and D) was profiled using a PCR Array, showing the differential expression of some miRNA also related to dogs' age; moreover, the study showed that small extracellular delivered miRNAs were more specific than miRNA isolated from whole plasma[9]. Our group investigated the expression of circulating miRNA in the plasma of CKCSs with early asymptomatic MMVD using a targeted strategy, highlighting that miR-30b-5p is up-regulated in MMVD-affected dogs at ACVIM stage B1 compared to stage A, potentially providing a candidate biomarker for the diagnosis of MMVD in this breed[5]. In contrast, findings from the present study did not indicate dysregulation of this miRNA in saliva, suggesting that miRNA signatures may vary substantially across biological matrices, even within the same pathological condition. These differences are consistent with the known matrix-specificity of miRNA profiles and the physiological barriers between blood and saliva[37].

The present study applied for the first time an untargeted approach to identify dysregulated miRNAs in association with MMVD in dogs' saliva, a non-invasive, cost-effective, and patient-friendly matrix for biomarker detection that can be collected over time. However, its clinical adoption in both human and veterinary medicine remains limited due to biological variability and the lack of standardized protocols for sample collection, processing, and analysis[38,39]. To enhance diagnostic accuracy and inform treatment decisions in veterinary cardiology, efforts should be made to harmonize and validate procedures across multiple centers. Identifying a panel composed of several miRNAs may improve diagnostic accuracy and guide treatment in veterinary cardiology, representing a potentially suitable tool for identifying asymptomatic dogs at ACVIM stage B1

and, ideally, for supporting future screening programs for the presence of MMVD[4].

Results from the present study showed that: (I) ACVIM stage B1 is characterized by an enrichment of pathways that are broadly associated with compensatory and protective signaling, such as AMPK, PI3K–Akt, and pathways related to neurotrophins and thyroid hormones; (II) ACVIM stage B2 exhibits an enrichment of pathways associated with inflammatory remodeling and cellular stress responses, including Th17 polarization and senescence-associated signaling; and (III) shared miRNAs converge on PI3K–Akt, AMPK, neurotrophin, and longevity-regulating pathways. This suggests a fundamental molecular background for early myxomatous mitral valve disease (MMVD) biology in this breed.

To examine the results in more depth, the 25 up-regulated miRNAs in ACVIM stage B1 dogs when compared to ACVIM stage A dogs were associated with neurotrophin, AMPK, FoxO, and MAPK signaling pathways. Neurotrophins are a family of proteins modulating nervous system activities and regulating the survival of endothelial cells, vascular smooth muscle cells, and cardiomyocytes, angiogenesis, and vasculogenesis[40]. Neurotrophins can bind to the tropomyosin kinase (Trk) receptors on endothelial cells, vascular smooth muscle cells, and cardiomyocytes, and to the p75 receptor, a neurotrophic factor receptor that can be found on endothelial cells, cardiomyocytes, and cardiac fibroblasts[40]. Trk receptors activate MAPK and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways, which lead to inflammation and ventricular remodeling[41,42]. The PI3K/Akt signaling pathway is critical in cardiovascular diseases, exerting an antiapoptotic effect and enhancing cardiomyocyte survival, angiogenesis, and repair processes[43]. On the contrary, the activation

of the p75 receptor mainly promotes pro-apoptotic processes[44], and the AMPK signaling pathway regulates energy metabolism, enhancing glucose uptake, glycolysis, and fatty acid oxidation, protecting cardiomyocytes under ischemic conditions in humans[45]. Taken together, the pathways associated with miRNAs up-regulated in B1 compared to A dogs are consistent with early compensatory and pro-survival responses in preclinical MMVD. The down-regulated miRNAs in ACVIM stage B1 compared to B2 CKCSs were predominantly involved in the THs and efferocytosis signaling pathways. THs can increase systolic and diastolic cardiac functions and reduce systemic vascular resistance[46,47]. In cardiomyocytes, THs upregulate genes encoding for sodium/potassium ATPase, α-myosin heavy chain, and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) and down-regulate β-myosin heavy chain and phospholamban (PLN) genes[48]. Furthermore, triiodothyronine (T3) can directly influence cardiomyocytes by upregulating β1-adrenergic receptors, with a positive inotropic effect[49]. Moreover, THs regulate key signaling pathways, such as PI3K/Akt, exerting an antiapoptotic effect[49]. Association of down-regulated miRNAs in B1 with TH-related pathways may therefore reflect mechanisms supporting myocardial performance during earlier stages of disease**.** The up-regulated miRNAs in ACVIM stage B1 compared to stage B2 CKCSs are involved in the Th17 cell differentiation and cellular senescence pathways. Th17 cells, a subset of CD4+ T lymphocytes, secrete IL-17 and IL-22 and repair-associated components, including matrix metalloproteinases (MMPs) and proteoglycans, enhancing inflammation during cardiac tissue remodeling[50]. A recent study in dogs reported that the release of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, increases with the progression

of mitral valve degeneration, and is associated with progressive volumetric overload caused by MMVD[51].

Cellular senescence, whose pathway is inhibited in ACVIM B1 CKCSs and promoted in ACVIM B2 dogs, refers to a state of irreversible growth arrest triggered by factors like telomere shortening, DNA damage, and oxidative stress. In humans, old cardiomyocytes adopt a senescent-like phenotype, and the myocardium acquires structural changes, including progressive cellular hypertrophy, interstitial fibrosis, and inflammation; the ultimate consequence is the impairment of both diastolic and systolic function[52]. The enrichment of Th17 and senescence-related pathways in B2 compared to B1 suggests a transition towards inflammatory remodeling and cellular stress in more advanced preclinical MMVD. The miRNAs identified comparing B1 and B2 dogs are linked to pathways that indicate a shift from compensatory signaling to stress- and inflammation-related signaling. No DE-miRNAs were detected in A compared to B2 dogs. Chronic MMVD in dogs is a disease characterized by both aging and stress. We hypothesize that at disease onset, tissues and immune cells activate compensatory and stress-response pathways leading to a strong and transient miRNA modulation, while, as the disease progresses, the compensatory pathways normalize even though structural damage continues. As reported in a different model[53], several miRNAs are dysregulated during myocarditis, exhibiting phase-dependent changes that correlate with viral infection, immune status, and fibrosis. Moreover, the progression of MMVD includes valve remodeling and a shift in the relative abundance of cell types; thus, the overall tissue or circulating miRNA signature could shift, dilute, or appear normalized[54].

Four DE-miRNAs, namely cfa-miR-8857, cfa-miR-9, cfa-miR-206, and cfa-miR-433, were up-regulated in B1 dogs when compared to both A and B2 dogs, potentially representing the common signature of MMVD in CKCSs. Yang and coworkers (2017)[9] associated the expression of cfa-miR-9 with the development of MMVD without any relationship with the progression of the disease toward CHF[9]. Cfa-miR-206 is modulated during canine atrial fibrillation[55]. In a recent study on MMVD, its expression level seemed to decrease in MMVD-affected dogs compared to healthy dogs, but the reduction was not significant[56].

The KEGG analysis identified the PI3K-Akt, longevity, neurotrophin, and AMPK signaling pathways as potentially modulated by these 4 DE-miRNAs. The neurotrophin, the PI3K-Akt, and the AMPK signaling pathways have already been discussed. The longevity-regulating pathway promotes cellular longevity by encompassing genes that regulate autophagy, mitochondrial function, stress defense mechanisms, and survival pathways while attenuating pro-inflammatory mediators[57]. These pathways regulate cardiac cell survival by promoting energy metabolism and antiapoptotic processes, possibly counteracting the negative consequences of MMVD progression, such as myocardial modification, cell death, and fibrotic replacement. Thus, the shared miRNAs may capture a core molecular signature of early MMVD in CKCSs, involving pathways related to survival, metabolism, and long-term tissue adaptation.

From a translational perspective, these findings indicate that salivary miRNA panels could potentially (I) aid in the early identification of at-risk CKCSs before overt clinical and echocardiographic changes are detected, (II) contribute to molecular monitoring of progression from stage B1 to stage B2, and (III) provide a minimally invasive and repeatable sampling method in clinical

situations where blood draws are challenging or frequent follow-ups are necessary.

This study has some limitations: first, the cohort consisted of a relatively small number of dogs from a single breed (CKCS) enrolled at a single centre, which is appropriate for an initial discovery effort but limits the generalizability of the findings to other breeds and populations. Moreover, aside from the limited sample size, it should be considered that working with saliva implies that miRNAs related to the oral cavity are included in the analysis. However, this matrix is widely accepted in human medicine since studies consistently show that salivary biomarkers can reflect general health[58]. We tried to minimize this bias by selecting a homogenous group of MMVD-affected subjects belonging to the same breed without any clinical signs of oral disorders and excluding genes associated with cancer and infectious diseases in the GO analysis, although some degree of oral mucosal and salivary biological variability cannot be completely ruled out and may contribute to signal heterogeneity, and some bias may have been introduced by restricting the GO analysis to non–cancer- and non–infection-related genes.

In addition, the present work is based on a cross-sectional design, which provides a snapshot of disease-associated molecular signatures but does not allow inference of temporal progression or causality. Longitudinal studies will be required to clarify how salivary miRNA profiles evolve as dogs transition between ACVIM stages.

Not all stages of the disease were represented in our sample; dogs belonging to the ACVIM stages C and D were excluded from this pilot study, as it specifically focuses on characterizing molecular alterations occurring during the early, asymptomatic phases of MMVD. In fact, once dogs reach these

clinical stages of the disease, the diagnosis is generally straightforward due to the presence of evident clinical signs of CHF. However, the exclusion of stages C and D limits inferences regarding late-stage disease, and future work including dogs in stages C and D will be necessary to define later-stage molecular trajectories.

The potential modulatory effects of pimobendan on salivary miRNA expression are currently unknown, as there are no available studies that specifically investigate the effect of pimobendan on miRNAs in the context of MMVD in dogs. Consequently, the inclusion of two treated B2 dogs in our study population may represent a confounding factor for the results. The inclusion of treated dogs was maintained to prevent a further reduction in the statistical power of this small pilot study. Additionally, biomarker-based tests would likely be used in clinical practice for dogs already receiving cardiac therapy; therefore, keeping these two dogs in the study provides a more accurate representation of the target population.

Age is known to influence miRNA expression in dogs affected by MMVD[9], and this study did not take into account its potential confounding effect. However, it is important to recognize that MMVD is a progressive, age-associated disease. In clinical practice, dogs in more advanced ACVIM stages are typically older than those classified at stages A or B1. Consequently, age and disease severity are often intertwined in naturally occurring MMVD. From a translational standpoint, an ideal biomarker should remain informative across the full spectrum of age and disease stages encountered in clinical settings, rather than relying on age-adjusted models that may not accurately reflect clinical realities. Furthermore, due to the exploratory design of the study and its modest sample size, age adjustment in a multivariable model was not feasible; however, this

point is acknowledged as a potential confounder and will be addressed in future validation cohorts.

Finally, as this is an exploratory sequencing-based discovery study, the identified salivary miRNAs have not yet been validated using an independent orthogonal technique (e.g., qPCR) in a separate cohort. Independent technical and biological validation will be an essential next step toward clinical translation and toward assessing the robustness and reproducibility of the proposed biomarker panel across larger and more diverse canine populations.

In this pilot cross-sectional study, we identified a set of salivary miRNAs that differ across MMVD severity groups in CKCSs and are associated with target genes involved in cell survival and functions, repair processes, and inflammation. These findings support the feasibility of salivary miRNA profiling in dogs and suggest that saliva may provide a complementary matrix for exploring molecular changes associated with MMVD. However, given the exploratory nature of this work, the identified miRNAs should be regarded as candidate markers rather than established biomarkers, and no diagnostic performance can be inferred from the present data. Future studies, including technical and biological validation, longitudinal monitoring, and evaluation in larger and more heterogeneous canine populations, will be essential to determine whether salivary miRNAs could eventually assist clinical decision-making, for example, by supporting early screening or facilitating repeated, non-invasive molecular assessments alongside standard clinical and echocardiographic evaluations.

Data Availability Statement

The datasets generated during the current study are available from the corresponding author upon reasonable request.

The datasets generated and analysed during the current study are available in the GEO repository, Series record GSE307507.

Funding Declaration

The study was partially funded by the Seed4Innovation Scouting Program 2023 of the Università degli Studi di Milano – project title: ‘MOOVET – Molecular Diagnostic Tools in Veterinary Medicine’, and partially by the ‘Piano di Sostegno alla Ricerca (PSR 2023) Linea 2 - Dotazione annuale per attività istituzionali’.

Acknowledgements

The content is the object of Italian Patent Application n. 102025000001866 filed on 03/02/2025.

References

1. Borgarelli, M. & Buchanan, J. W. Historical review, epidemiology and natural history of degenerative mitral valve disease. *J. Vet. Cardiol.* **14**, 93–101 (2012).
2. Pedersen, H. D., Lorentzen, K. A. & Kristensen, B. Echocardiographic mitral valve prolapse in cavalier King Charles spaniels: epidemiology and prognostic significance for regurgitation. *Vet. Rec.* **144**, 315–320 (1999).
3. Lewis, T., Swift, S., Woolliams, J. A. & Blott, S. Heritability of premature mitral valve disease in Cavalier King Charles spaniels. *Vet. J.* **188**, 73–76 (2011).
4. Keene, B. W. et al. ACVIM consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs. *J. Vet. Intern. Med.* **33**, 1127–1140 (2019).
5. Bagardi, M. et al. Circulating MiR-30b-5p is up-regulated in Cavalier King Charles Spaniels affected by early myxomatous mitral valve disease. *PLoS One.* **17**, e0266208 (2022).
6. Aupperle, H. & Disatian, S. Pathology, protein expression and signaling in myxomatous mitral valve degeneration: comparison of dogs and humans. *J. Vet. Cardiol.* **14**, 59–71 (2012).
7. Lu, C. C. et al. Gene network and canonical pathway analysis in canine myxomatous mitral valve disease: a microarray study. *Vet. J.* **204**, 23–31 (2015).
8. Reis-Ferreira, A., Neto-Mendes, J., Brás-Silva, C., Lobo, L. & Fontes-Sousa, A. P. Emerging Roles of Micrornas in Veterinary Cardiology. *Vet. Sci.* **9**, 533 (2022).

9. Yang, V. K. et al. Circulating exosome microRNA associated with heart failure secondary to myxomatous mitral valve disease in a naturally occurring canine model. *J. Extracell. Vesicles* **6**, 1350088 (2017).
10. Hulanicka, M., Garncarz, M., Parzeniecka-Jaworska, M. & Jank, M. Plasma miRNAs as potential biomarkers of chronic degenerative valvular disease in Dachshunds. *BMC Vet. Res.* **10**, 205 (2014).
11. Li, Q., Freeman, L., Rush, J. & Laflamme, D. Expression Profiling of Circulating MicroRNAs in Canine Myxomatous Mitral Valve Disease. *Int. J. Mol. Sci.* **16**, 14098–14108 (2015).
12. Ro, W.-B. et al. Identification and Characterization of Circulating MicroRNAs as Novel Biomarkers in Dogs With Heart Diseases. *Front. Vet. Sci.* **8**, 729929 (2021).
13. Ghilardi, S. et al. Prospective pilot study on the predictive significance of plasma miR-30b-5p through the study of echocardiographic modifications in Cavalier King Charles Spaniels affected by different stages of myxomatous mitral valve disease: The PRIME study. *PLoS One* **17**, e0274724 (2022).
14. Varvil, M. S. & dos Santos, A. P. A review on microRNA detection and expression studies in dogs. *Front. Vet. Sci.* **10**, 1261085 (2023).
15. Park, N. J. et al. Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection. *Clin. Cancer. Res.* **15**, 5473–5477 (2009).
16. Setti, G. et al. Salivary MicroRNA for Diagnosis of Cancer and Systemic Diseases: A Systematic Review. *Int. J. Mol. Sci.* **21**, 907 (2020).

17. Floriano, P. N. et al. Use of Saliva-Based Nano-Biochip Tests for Acute Myocardial Infarction at the Point of Care: A Feasibility Study. *Clin. Chem.* **55**, 1530–1538 (2009).
18. Michael, A. et al. Exosomes from human saliva as a source of microRNA biomarkers. *Oral. Dis.* **16**, 34–38 (2010).
19. Hansson, K., Häggström, J., Kvart, C. & Lord, P. Left atrial to aortic root indices using two-dimensional and M-mode echocardiography in cavalier King Charles spaniels with and without left atrial enlargement. *Vet. Radiol. Ultrasound* **43**, 568–575 (2002).
20. Cornell, C. C. et al. Allometric Scaling of M-Mode Cardiac Measurements in Normal Adult Dogs. *J. Vet. Intern. Med.* **18**, 311–321 (2004).
21. Chetboul, V. & Tissier, R. Echocardiographic assessment of canine degenerative mitral valve disease. *J. Vet. Cardiol.* **14**, 127–148 (2012).
22. Bagardi, M., Ghilardi, S., Locatelli, C., Romussi, S. & Brambilla, P. G. Effects of animal position on M-mode, two-dimensional, and Doppler echocardiographic measurements in heatlhy French bulldogs. *Vet. Radiol. Ultrasound.* **64**, 758-767 (2023).
23. Bustin, S. A. et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **55**, 611–622 (2009).
24. Pardini, B. et al. microRNA profiles in urine by next-generation sequencing can stratify bladder cancer subtypes. *Oncotarget* **9**, 20658–20669 (2018).

25. Cordero, F., Beccuti, M., Arigoni, M., Donatelli, S. & Calogero, R. A. Optimizing a Massive Parallel Sequencing Workflow for Quantitative miRNA Expression Analysis. *PLoS One* **7**, e31630 (2012).
26. Beccuti, M. et al. SeqBox: RNAseq/ChIPseq reproducible analysis on a consumer game computer. *Bioinformatics* **34**, 871–872 (2018).
27. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
28. Sticht, C., De La Torre, C., Parveen, A. & Gretz, N. miRWalk: An online resource for prediction of microRNA binding sites. *PLoS One* **13**, e0206239 (2018).
29. Wong, N. & Wang, X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res.* **43**, D146–D152 (2015).
30. Hsu, S.-D. et al. miRTarBase: a database curates experimentally validated microRNA–target interactions. *Nucleic Acids Res.* **39**, D163–D169 (2011).
31. Agarwal, V., Bell, G. W., Nam, J.-W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**, e05005 (2015).
32. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).
33. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).

34. Kanehisa, M., Goto, S., Sato, Y., Furumichi, M. & Tanabe, M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* **40**, D109–D114 (2012).
35. Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E. & Storey, J. D. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* **28**, 882–883 (2012).
36. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
37. El-Mogy, M. et al. Diversity and signature of small RNA in different bodily fluids using next generation sequencing. *BMC Genomics* **19**, 408 (2018).
38. Li, Y., Ou, Y., Fan, K. & Liu, G. Salivary diagnostics: opportunities and challenges. *Theranostics* **14**, 6969-6990 (2024).
39. Nunes, L. A., Mussavira, S. & Bindhu, O. S. Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. *Biochem. Med. (Zagreb)* **25**, 177-192 (2015).
40. Caporali, A. & Emanueli, C. Cardiovascular Actions of Neurotrophins. *Physiol. Rev.* **89**, 279–308 (2009).
41. Huang, E. J. & Reichardt, L. F. Trk Receptors: Roles in Neuronal Signal Transduction. *Annu. Rev. Biochem.* **72**, 609–642 (2003).
42. Zhang, W., Elimban, V., Nijjar, M. S., Gupta, S. K. & Dhalla, N. S. Role of mitogen-activated protein kinase in cardiac hypertrophy and heart failure. *Exp. Clin. Cardiol.* **8**, 173–183 (2003).
43. Caporali, A. et al. Identification of the prosurvival activity of nerve growth factor on cardiac myocytes. *Cell. Death. Differ.* **15**, 299–311 (2008).

44. Elshaer, S. L. & El-Remessy, A. B. Implication of the neurotrophin receptor p75 ^{NTR} in vascular diseases: beyond the eye. *Expert Rev. Ophthalmol.* **12**, 149–158 (2017).
45. Dyck, J. R. B. & Lopaschuk, G. D. AMPK alterations in cardiac physiology and pathology: enemy or ally? *J. Physiol.* **574**, 95–112 (2006).
46. Klein, I. & Ojamaa, K. Thyroid Hormone and the Cardiovascular System. *N. Engl. J. Med.* **344**, 501–509 (2001).
47. Kranias, E. G. & Hajjar, R. J. Modulation of Cardiac Contractility by the Phopholamban/SERCA2a Regulatome. *Circ. Res.* **110**, 1646–1660 (2012).
48. Holt, E., Sjaastad, I., Lunde, P. K., Christensen, G. & Sejersted, O. M. Thyroid Hormone Control of Contraction and the Ca2+-ATPase/phospholamban Complex in Adult Rat Ventricular Myocytes. *J. Mol. Cell. Cardiol.* **31**, 645–656 (1999).
49. Yamakawa, H. et al. Thyroid Hormone Plays an Important Role in Cardiac Function: From Bench to Bedside. *Front. Physiol.* **12**, 606931 (2021).
50. Yang, K. et al. Th17 Cells in Cardiovascular Disease. *Cell. Biochem. Funct.* **43**, e70069 (2025).
51. Piantedosi, D. et al. Pro-Inflammatory and Immunological Profile of Dogs with Myxomatous Mitral Valve Disease. *Vet. Sci.* **9**, 326 (2022).
52. Luan, Y. et al. Cardiac cell senescence: molecular mechanisms, key proteins and therapeutic targets. *Cell. Death. Discov.* **10**, 78 (2024).
53. Wang, J. & Han B. Dysregulated CD4+ T Cells and microRNAs in Myocarditis. *Front. Immunol.* **11**, 539 (2020).
54. Dai, B. et al. The Cell Type-Specific Functions of miR-21 in Cardiovascular Diseases. *Front. Genet.* **11**, 563166 (2020).

55. Zhang, Y. et al. MicroRNA Profiling of Atrial Fibrillation in Canines: MiR-206 Modulates Intrinsic Cardiac Autonomic Nerve Remodeling by Regulating SOD1. *PLoS One* **10**, e0122674 (2015).
56. Palarea-Albaladejo, J. et al. Assessing the use of blood microRNA expression patterns for predictive diagnosis of myxomatous mitral valve disease in dogs. *Front. Vet. Sci.* **11**, 1443847 (2024).
57. Salas-Pérez, F. et al. DNA methylation in genes of longevity-regulating pathways: association with obesity and metabolic complications. *Aging* **11**, 1874–1899 (2019).
58. Sullivan, R. et al. Confounding Factors Impacting microRNA Expression in Human Saliva: Methodological and Biological Considerations. *Genes (Basel)* **13**, 1874 (2022).

Authors contribution

Conception: Sara Ghilardi, Mara Bagardi, Giovanni Romito, Michele Polli, Paola G. Brambilla, Cristina Lecchi

Methodology: Sara Ghilardi, Gaspare Salvi, Mara Bagardi, Ombretta Balbi, Asia Balgera, Yasmine Dadi, Maddalena Arigoni, Raffaele Calogero, Paola G. Brambilla, Cristina Lecchi

Data acquisition: Sara Ghilardi, Mara Bagardi, Paola G. Brambilla

Data analysis: Sara Ghilardi, Gaspare Salvi, Mara Bagardi, Ombretta Balbi, Asia Balgera, Yasmine Dadi, Maddalena Arigoni, Raffaele Calogero

Interpretation of data: Sara Ghilardi, Mara Bagardi, Giovanni Romito, Michele Polli, Maddalena Arigoni, Raffaele Calogero, Cristina Lecchi

Writing—original draft preparation: Sara Ghilardi, Cristina Lecchi

All authors reviewed and revised the manuscript.

Additional information

The authors declare no competing interests.

Table 1. Clinical and echocardiographic data of the 25 CKCSs included in the study, grouped by their ACVIM stage

ACVIM stage	Gender (M/F)	Age (years)	Heart murmur intensity	Body weight (kg)	LA/Ao	E wave peak velocity (m/s)	E/A ratio	Ejection fraction (%)	Fractional shortening (%)	LVIDdN (cm/kg)	LVIDsN (cm/kg)	MR severity
A (n. 6)	3/3	2.61 ± 1.87	Absent (n. 6)	8.85 ± 2.13	1.15 ± .12	.70 (.59-.71)	1.33 ± .33	66.67 ± 9.59	35.50 ± 7.18	1.33 ± .09	.82 ± .10	0 (n. 6)
B1 (n. 13)	9/4	5.34 ± 3.67	1-2/6 (n. 11) 3-4/6 (n. 2)	10.08 ± 1.99	1.22 ± .17	.73 ± .09	1.29 ± .28	67.23 ± 8.81	37.31 ± 5.81	1.46 ± .12	.88 ± .13	1 (n. 9) 2 (n. 4)
B2 (n. 6)	1/5	9.11 (8.79-9.67)*	3-4/6 (n.5) 5-6/6 (n. 1)	9.25 ± .96	1.71 (1.69-1.73)*†	1.06 ± .13*†	1.25 (1.20-1.30)	74.33 ± 4.13	42.67 ± 3.72	2.00 (1.96-2.05)*†	1.05 (1.05-1.19)*†	2 (n. 4) 3 (n.2)

Continuous variables are reported as mean ± standard deviation or median and IQR_{25-75} according to their distribution (normal or non-normal, respectively). Differences among ACVIM stages are indicated where present.
ACVIM: American College of Veterinary Internal Medicine; F: females; LA/Ao: left atrium-to-aortic root ratio; LVIDdN: left ventricular internal diameter in diastole normalized for body weight; LVIDsN: left ventricular internal diameter in systole normalized for body weight; M: males; MR: mitral regurgitation. For MR severity, the legend is as follows: 0 = absent; 1 = regurgitant jet area signal to left atrium area (ARJ/LAA) ratio < 20-30%; 2 = ARJ/LAA ratio ≥ 20-30% and ≤ 70%; 3 = ARJ/LAA ratio > 70%.
*Different from ACVIM stage A ($P \leq 0.05$)
†Different from ACVIM stage B1 ($P \leq 0.05$)

Figure 1. Venn diagram showing the overlap of DE-miRNAs among the ACVIM stages. 25 miRNAs were altered when comparing A with B1 CKCSs, while 35 miRNAs were altered when comparing B2 with B1 CKCSs. The graph shows the overlapping of four DE-miRNAs (i.e., cfa-miR-8857, cfa-miR-9, cfa-miR-206, cfa-miR-433) that were shared between both comparison groups and may therefore represent the MMVD signature in this population of CKCSs. The direction of miRNA regulation (up- or down-) is reported in the text but not shown in the figure.

Figure 2. Target prediction, gene ontology, and pathway enrichment analysis of the 25 up-regulated miRNAs in ACVIM stage B1 compared to stage A CKCSs. Target genes of the differentially expressed miRNAs were predicted using miRWalk 3.0 and retained only when supported by all three databases (miRDB, miRTarBase and TargetScan). Functional enrichment of the resulting gene list was performed using DAVID. The top significantly enriched items are shown. a) KEGG pathway enrichment analysis showing the top significantly enriched pathways (P values expressed as −log10). b) Gene Ontology (GO) enrichment analysis at the level of biological process, cellular component, and molecular function. These findings highlight the main molecular pathways potentially associated with the miRNAs up-regulated in B1 compared to A dogs.

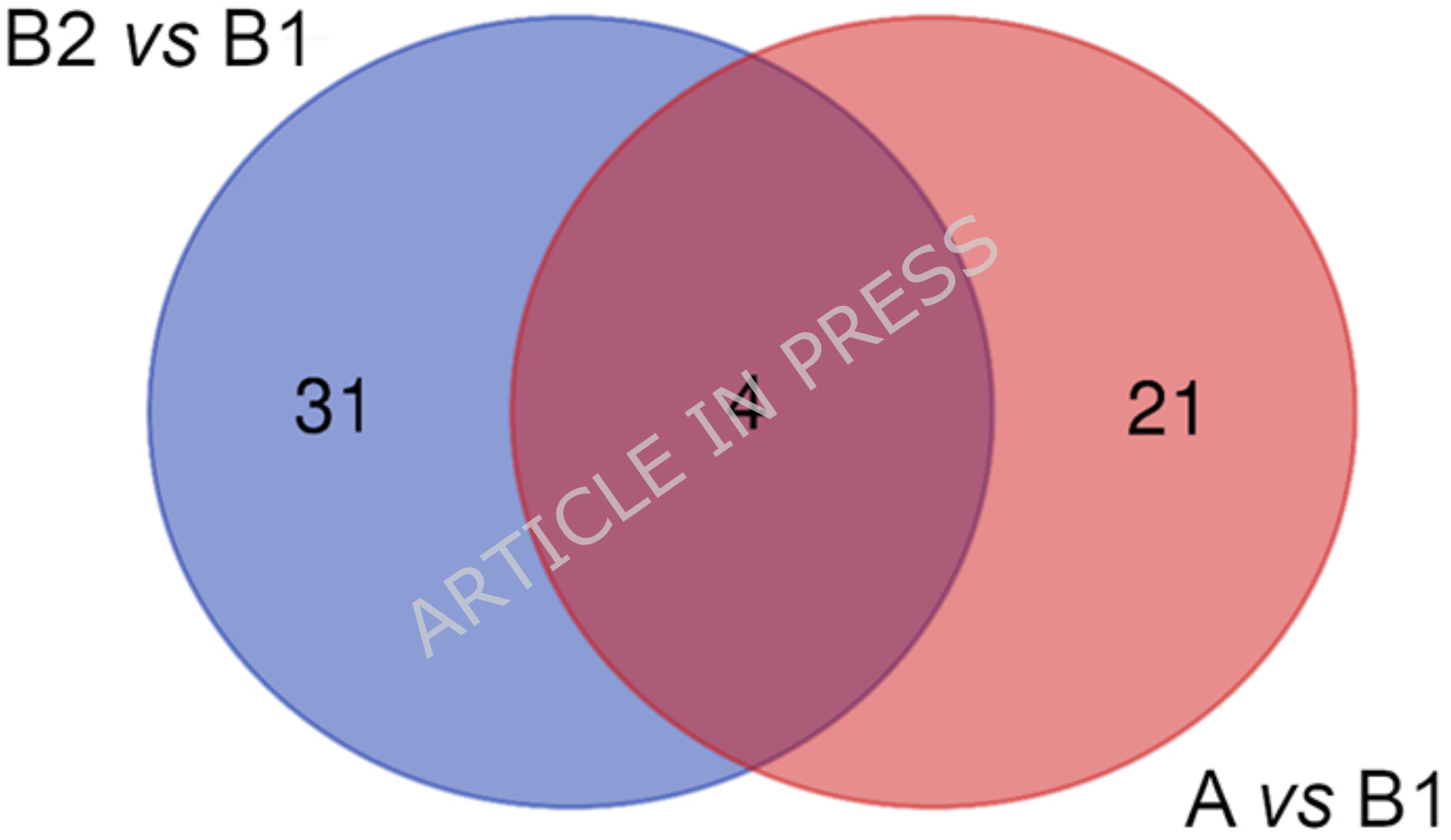
Figure 3. Target prediction, gene ontology and pathway enrichment analysis of the 5 down-regulated miRNAs in ACVIM stage B1 compared to stage B2 CKCSs. Target genes of the differentially expressed miRNAs were

predicted using miRWalk 3.0 and retained only when supported by all three databases (miRDB, miRTarBase and TargetScan). Functional enrichment of the resulting gene list was performed using DAVID. The top significantly enriched items are shown. a) KEGG pathway enrichment analysis showing the top significantly enriched pathways (P values expressed as −log10). b) Gene Ontology (GO) enrichment analysis at the level of biological process, cellular component, and molecular function. These findings highlight the main molecular pathways potentially associated with the miRNAs down-regulated in B1 compared to B2 dogs.

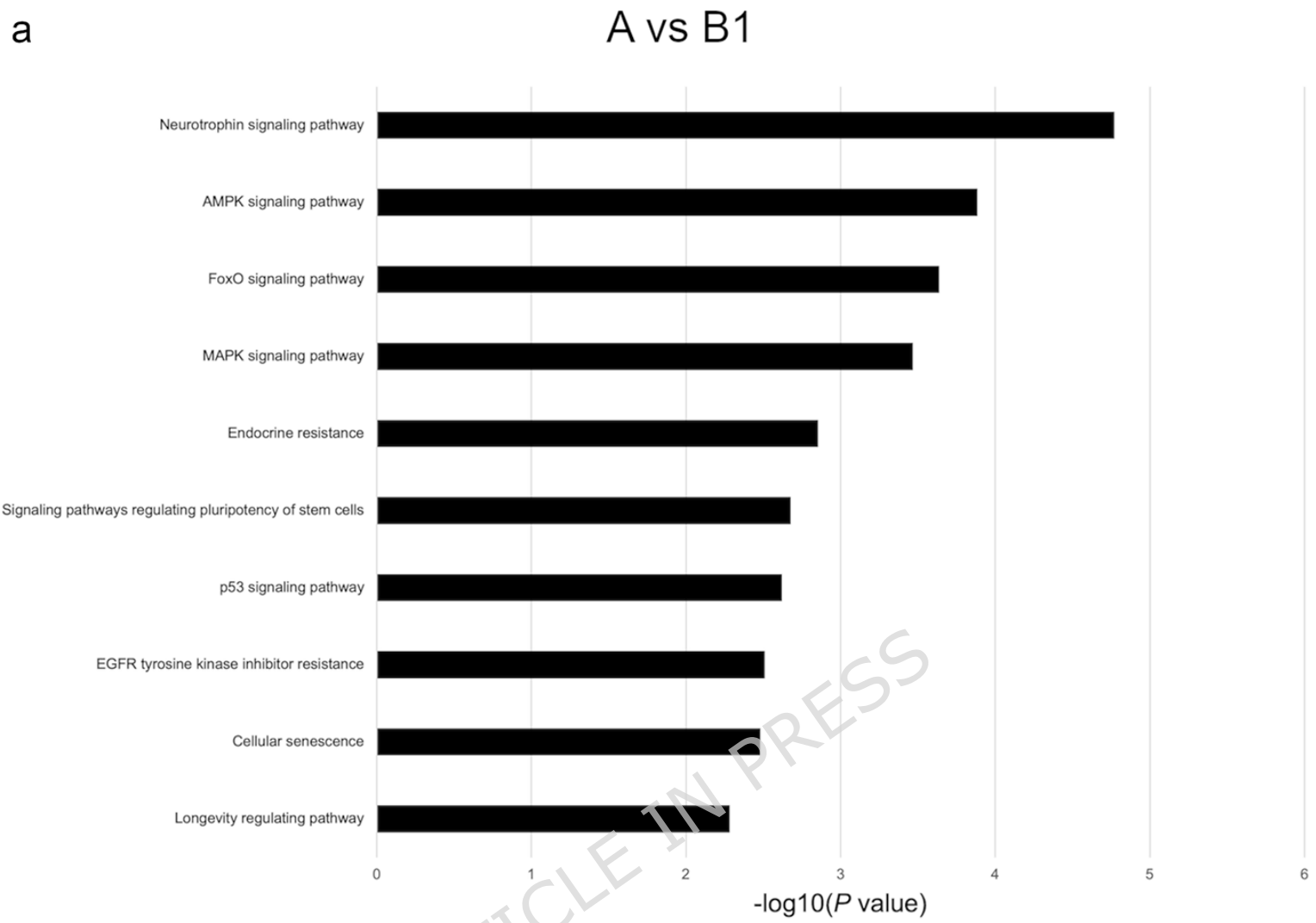
Figure 4. Target prediction, gene ontology, and pathway enrichment analysis of the 30 up-regulated miRNAs in ACVIM stage B1 compared to stage B2 CKCSs. Target genes of the differentially expressed miRNAs were predicted using miRWalk 3.0 and retained only when supported by all three databases (miRDB, miRTarBase, and TargetScan). Functional enrichment of the resulting gene list was performed using DAVID. The top significantly enriched items are shown. a) KEGG pathway enrichment analysis showing the top significantly enriched pathways (P values expressed as −log10). b) Gene Ontology (GO) enrichment analysis at the level of biological process, cellular component, and molecular function. These findings highlight the main molecular pathways potentially associated with the miRNAs up-regulated in B1 compared to B2 dogs.

Figure 5. Target prediction, gene ontology, and pathway enrichment analysis of the 4 up-regulated miRNAs in ACVIM stage B1 compared to both A and B2 CKCSs. Target genes of the differentially expressed miRNAs were predicted using miRWalk 3.0 and retained only when supported by all

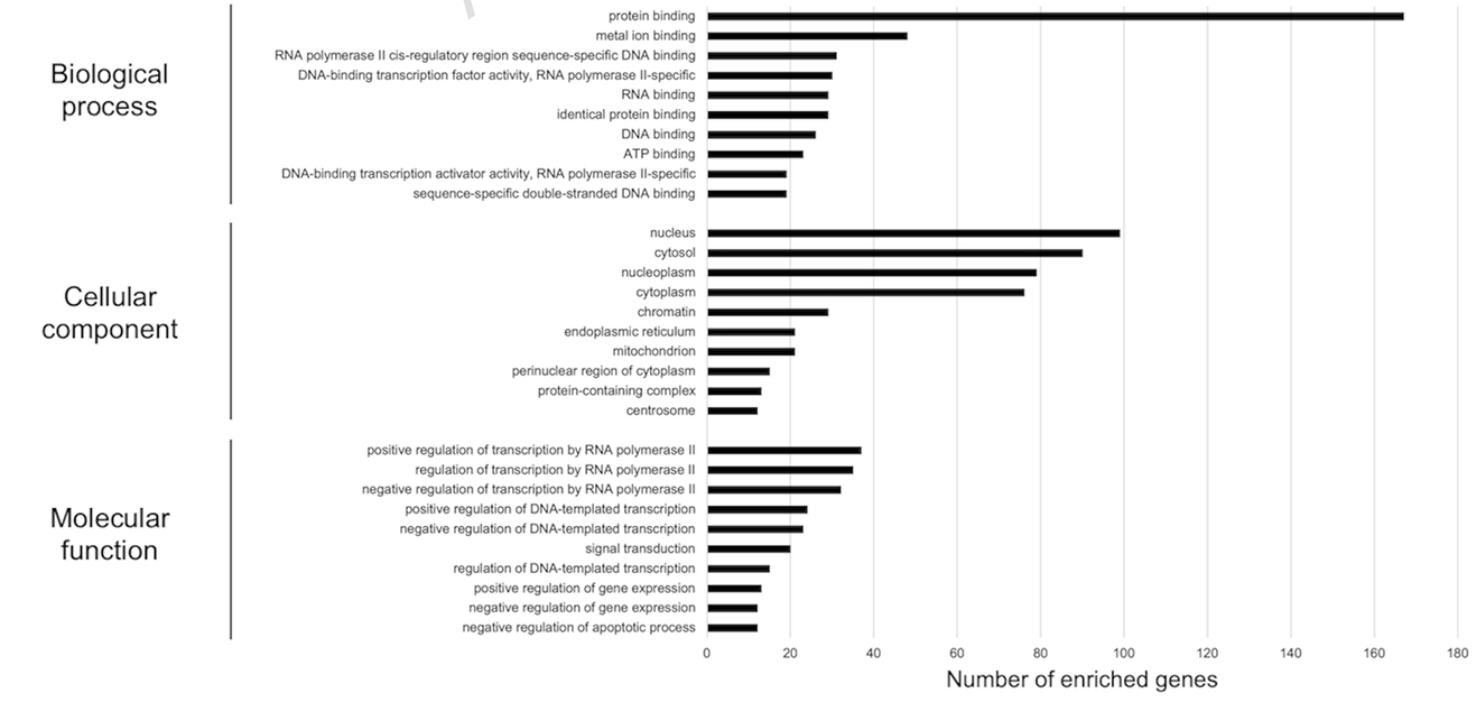
three databases (miRDB, miRTarBase and TargetScan). Functional enrichment of the resulting gene list was performed using DAVID. The top significantly enriched items are shown. a) KEGG pathway enrichment analysis showing the top significantly enriched pathways (P values expressed as −log10). b) Gene Ontology (GO) enrichment analysis at the level of biological process, cellular component, and molecular function. These findings highlight the main molecular pathways potentially associated with the miRNAs up-regulated in B1 compared to both A and B2 dogs.



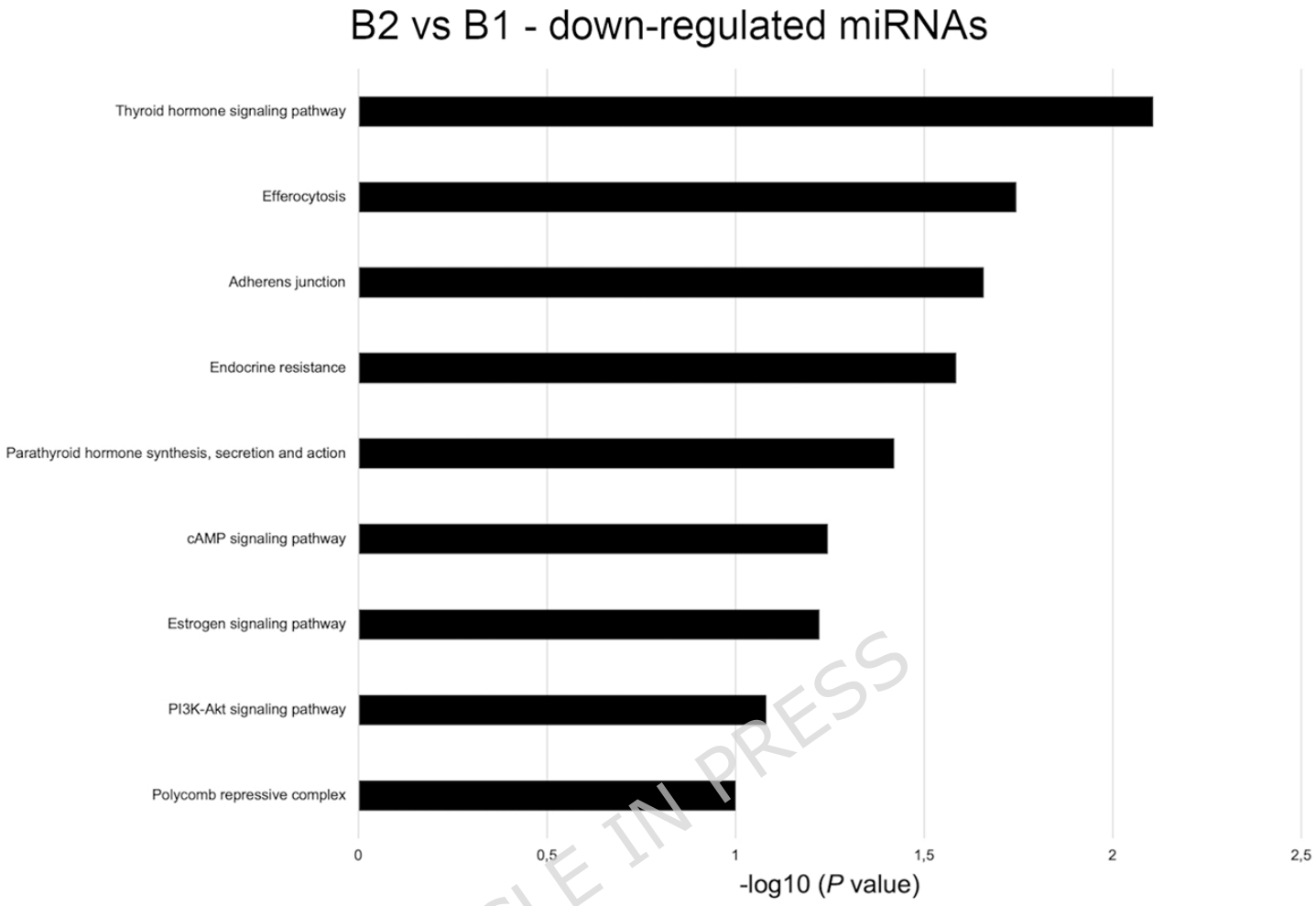
a



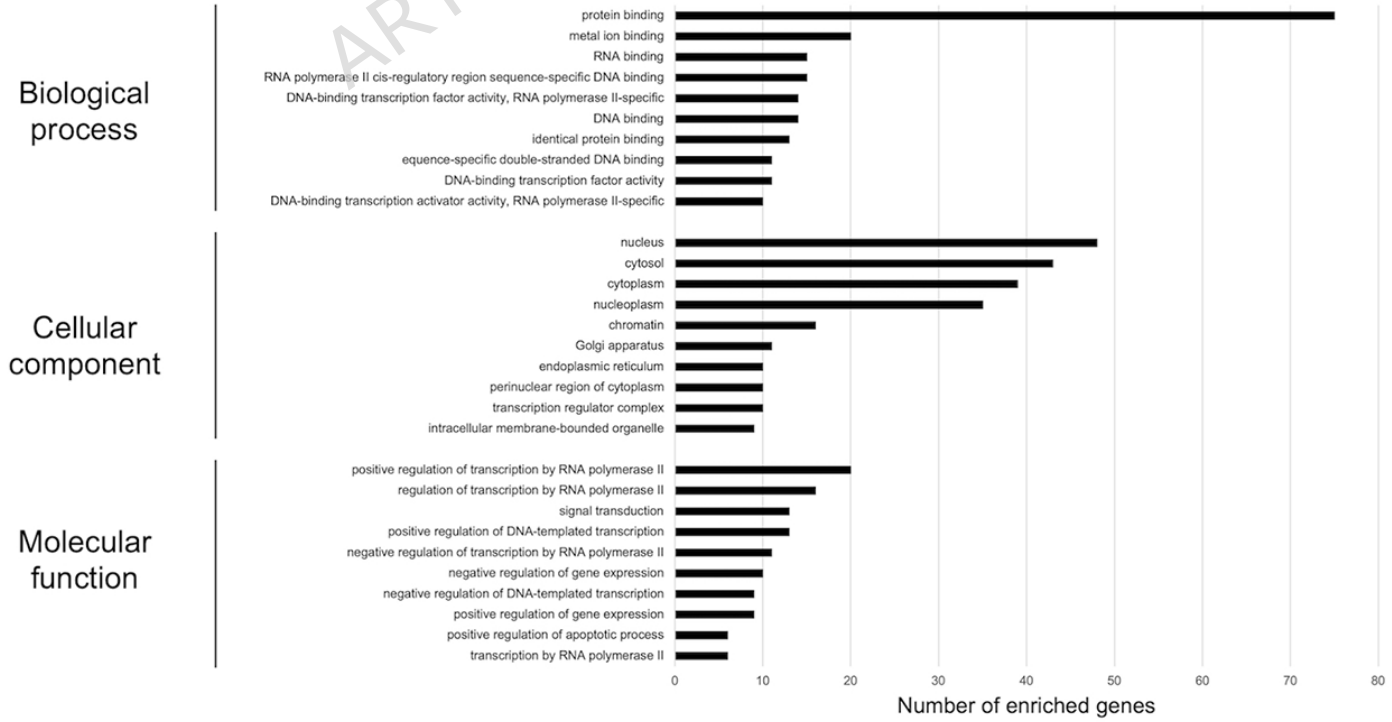
b



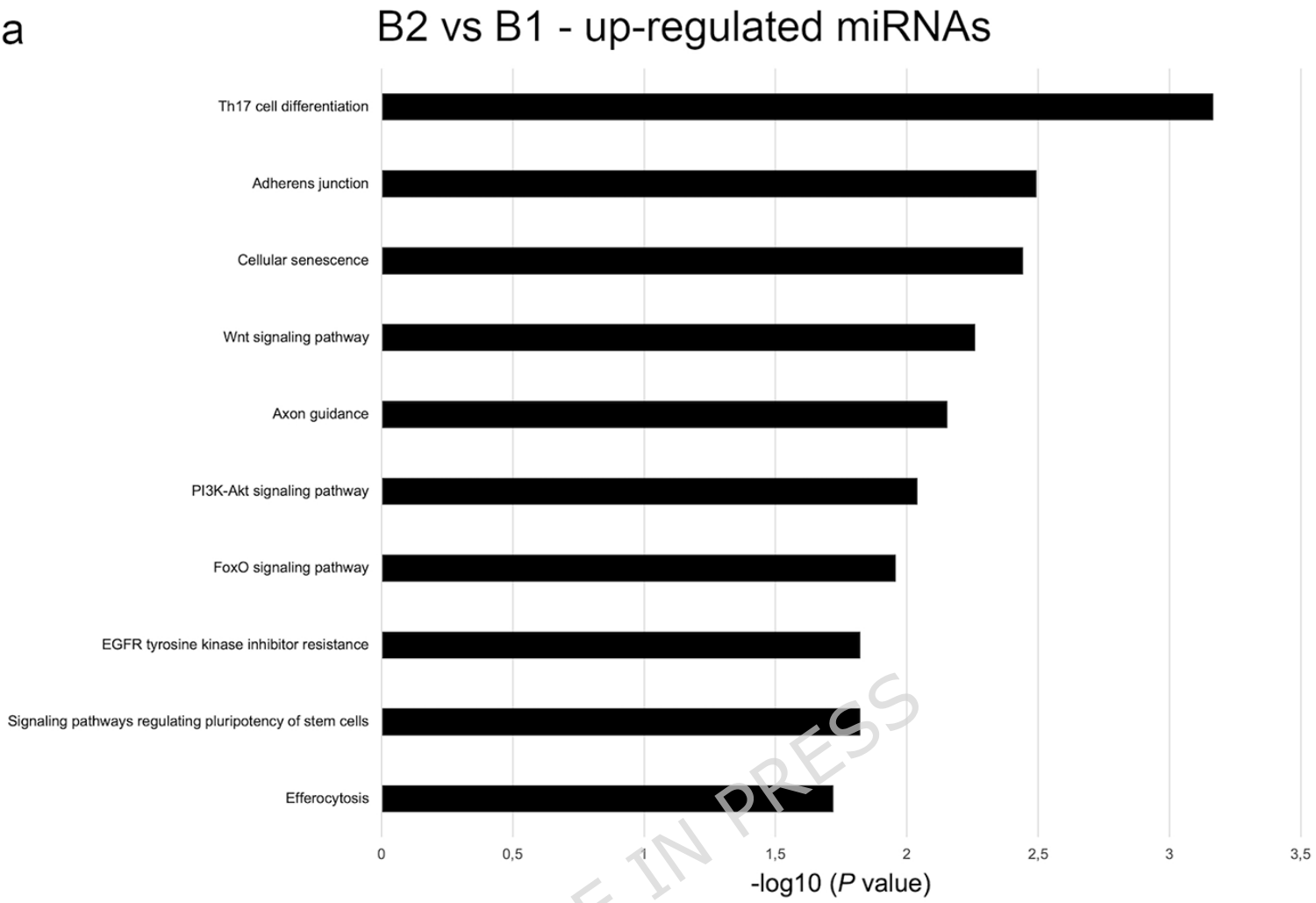
a



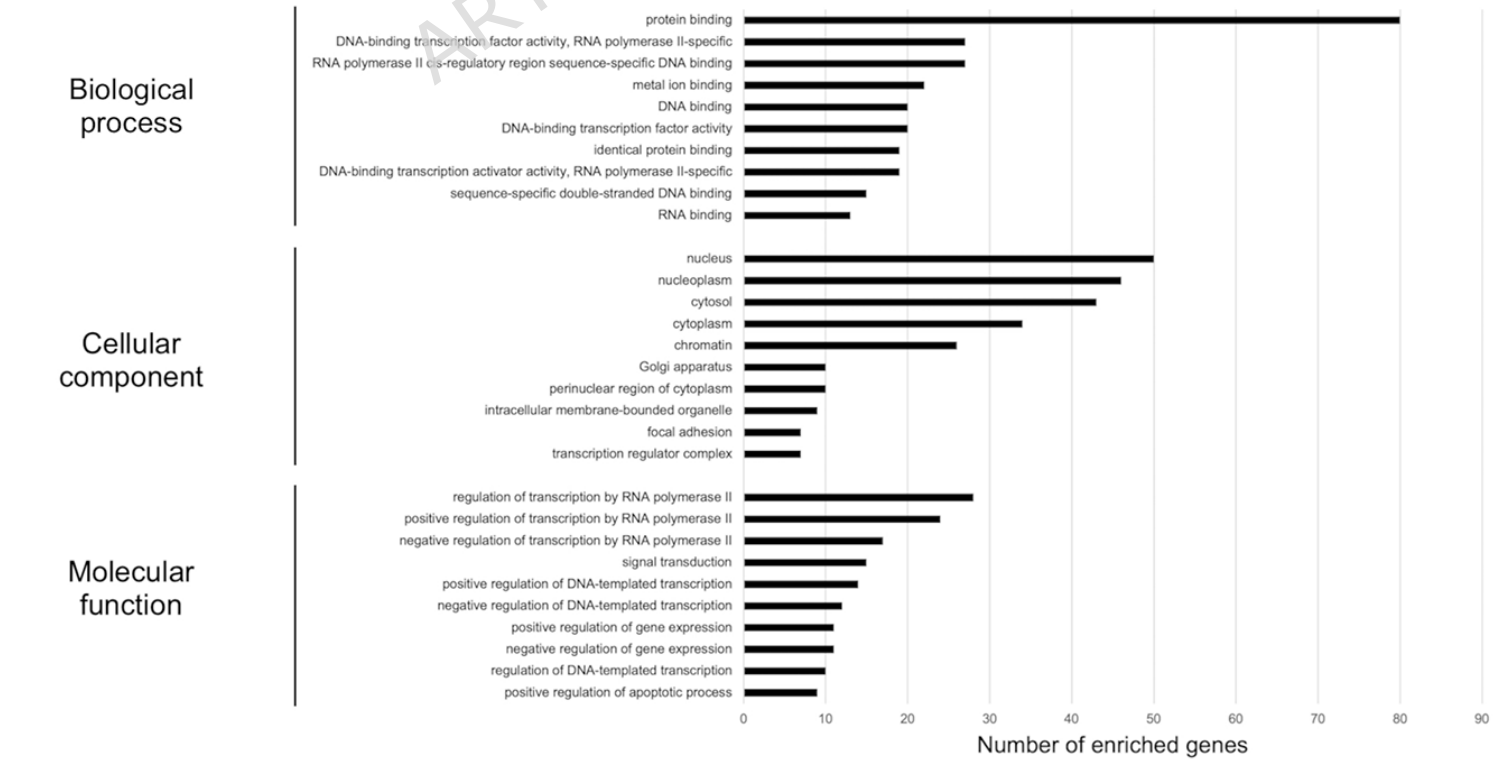
b



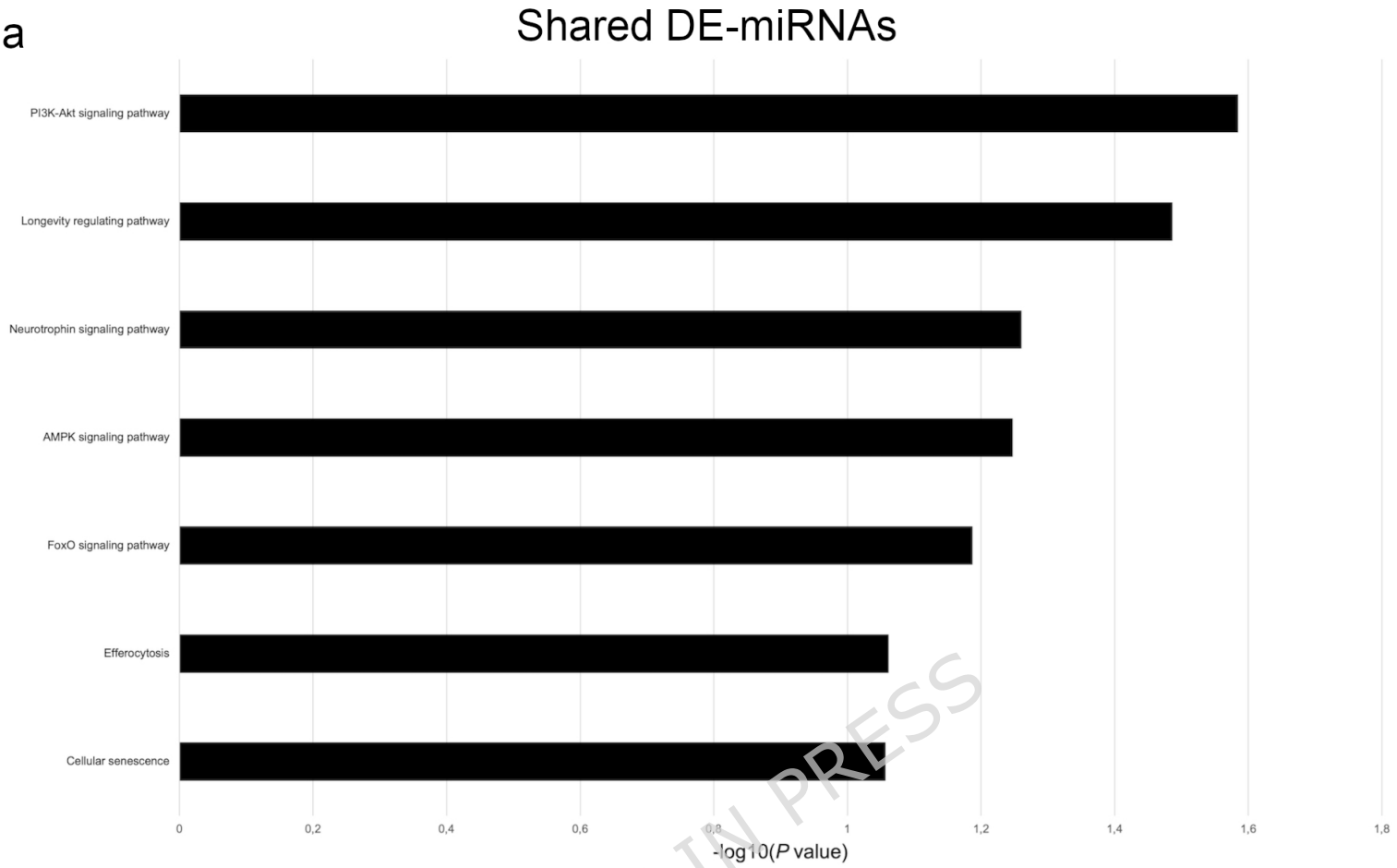
a



b



a



b