UNIVERSITA' DEGLI STUDI DI MILANO

PhD Course in Veterinary and Animal Science

Class XXXIV

MYXOMATOUS MITRAL VALVE DISEASE IN CAVALIER KING CHARLES SPANIEL: CLINICAL, GENETIC, AND CARDIAC BIOMARKERS STUDY

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List of abbreviations

A peak velocity (A-Vmax); Actual number of ancestors (f_a); Actual number of founders (f_e); Actual number of founding genomes (N_g); Actual size of the population (N_e); Allelic richness (A_r); American College of Veterinary Internal Medicine (ACVIM); Anatomic regurgitant orifice area (AROA); Angiotensin II (A-II); Angiotensin-converting enzyme (ACE); Angiotensin-converting enzyme inhibitors (ACEI); Angle between aortic annulus and anterior leaflet (AAo-AP); Annulus height (AnH); Annulus height to commissural width ratio (AHCWR); Anterior mitral valve area (AMVA); Anterior mitral valve length (AMVL); Anterior mitral valve width (AMVW); Area of regurgitant jet/left atrium area ratio (ARJ/LAA); Area under the curve (AUC); Argonaute (ago); Atrial natriuretic peptide (ANP); Average relatedness coefficient (AR); B-type natriuretic peptide (BNP); Black and tan (B&T); Blenheim (B); Body condition score (BCS); Body length (BL); Body mass index (BMI); Body surface area (BSA); Canine chromosomes (CFA); Cardiac troponin I (cTnI); Cardiorenal syndrome (CRS); Cavalier King Charles spaniels (CKCS); Circulating Exosomal miRNA (ex-miRNA); Chronic kidney disease (CKD); Coefficients of variation (CV %); Complete blood count (CBC); Congestive heart failure (CHF); Congestive heart failure due to MMVD (MMVD-CHF); Cross-population extended haplotype homozygosity (XP-EHH); Dogs born from the selection program (BP); Dogs not born from the selection program selection (not-BP); Dorso-ventral (DV); E peak velocity (E-Vmax); Effective regurgitant orifice area (EROA); Electrocardiography (ECG); End-diastolic volume index (EDVI); End-systolic volume index (ESVI); Ente Nazionale della Cinofilia Italiana (ENCI); Epidermal growth factor receptor (EGFR); European Society of Veterinary Cardiology (ESVC); E-Vmax to A-Vmax ratio (E/A); Expected heterozygous rate (H_e); Exportin-5 (EXP-5); Extracellular matrix (ECM); Fixation index (F_{IS}); Fractional shortening (FS%); Genome-wide association study (GWAS); Glomerular filtration rate (GFR); Head length (HL); Head-nose length (HNL); Head width

(HW); Head stop angle (HA); Height at withers (WH); Heterozygosity (H); Inbreeding coefficient (F); Inbreeding rate for generation (ΔF); International Renal Interest Society (IRIS); Inverse probability weighting analyses (IPW); Kinship coefficient (Φ); Left atrium (LA); Left atrium to aorta ratio (LA/Ao); Left lateral (LL); Left ventricle (LV); Left ventricular end diastolic volume (EDV); Left ventricular end systolic volume (ESV); Left ventricular internal diameter at end-diastole (LVIDD); Left ventricular internal diameter at end-systole (LVIDS); Left ventricular internal diameter in diastole normalized for body weight (LVIDdN); Left ventricular internal diameter in systole normalized for body weight (LVIDsN); Left ventricular normalized dimensions in diastole (LVIDad); Left ventricular normalized dimensions in systole (LVIDas); Livre des Origines Français (LOF); Long-axis left atrial dimension indexed to the long-axis aortic valve annulus diameter (LAD/AoD_Lx); Median survival time (MST); MicroRNAs (miRNAs); Minor allele frequency (MAF); Mitral valve (MV); Mitral regurgitation (MR); Mitral valve annulus in diastole (MVAd); Mitral valve annulus in systole (MVAs); Mitral valve prolapse (MVP); Myxomatous mitral valve disease (MMVD); Modified version of vertebral left atrial size (M-VLAS); N-terminal pro-B-type natriuretic peptide (NTproBNP); Natriuretic peptides (NPs); Next-generation sequencing (NGS); New York Heart Association (NYHA); Nonplanar angle (NPA); Normalized left ventricular internal diameter in systole (LVIDsN); Normalized tenting volume (nTnV); Nose length (NL); Number of founders (f); Observed heterozygous rate (H_o); Precursor miRNA (pre-miRNA); Primary miRNA (pri-miRNA); Pro-atrial natriuretic peptide (NT-proANP); Proximal isovelocity surface area (PISA); Pulsed-wave Doppler (PWD); Quantitative real-time PCR (RT-qPCR); Radiographic left atrial enlargement (RLAD); Receiver operating characteristic (ROC); Regurgitant jet area signal (ARJ); Renin angiotensin aldosterone system (RAAS); Right lateral (RL); RNA-induced silencing complex (RISC); ROH-based inbreeding coefficient (FROH); Ruby (R); Runs of homozygosity (ROH); Serum creatinine (sCr); Short-axis LA indexed to the short-axis aortic

root (LA/Ao_Sx); Single-nucleotide polymorphisms (SNPs); Single-stranded RNAs (ssRNAs); Symmetric dimethylarginine (SDMA); Sphericity index (SI); Stabilized inverse probability weights (SIPW); Tenting area (TnA); Tenting height (TnH); Thoracic anterior or axillary circumference (TC1); Thoracic depth – thoracic width ratio (TD/TW); Thoracic lower or basal circumference (TC3); Thoracic mean or papillary circumference (TC2); Thorax height (TH); Thorax length (TL); Thorax width (TW); Transforming growth factor β (TGF-β); Transthoracic echocardiographic (TTE); Tricolor (T); Two-dimensional echocardiography (2DE); Urinary aldosterone (UAldo); Urinary aldosterone-to-creatinine ratio (UAldo:C); Vertebral heart atrial size (VLAS); Vertebral heart score (VHS); Width at chest (CW); Wingless and Int-1 (Wnt); Wright's fixation index (FST); 3D trans thoracic echocardiography (RT–3DTTE);

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1. Introduction

The dog represents the pet par excellence, the domestication of this species going back to ancient times, almost 32,000 years ago [1]. Following initial domestication from wolves, semi-controlled breeding enabled humans to shape dog varieties each of which was mentally and physically suited for widely different tasks. More recently, during the past 200–300 years, guidelines with the purpose of reinforcing desirable traits have formalized this process by promoting strictly controlled breeding in closed populations. While this process has created an extraordinary diversity in dog morphology and behavior, bottlenecks and restricted gene flow associated with breed formation have also resulted in a loss of genetic variation and random development of individual disease mutations [2]. Consequently, it is now likely that relatively few common variants with serious effects are contributing to the high risk of certain diseases in particular breeds. Cardiovascular disorders are among these [1].

Unintentionally, sublethal/lethal genes are selected, such as genes that in nature would be eliminated by natural selection. Some of these genes can be phenotypically expressed as the presence of hereditary cardiovascular diseases and the severity, linked to the degree of the expression of the pathology, can be variable and can affect the quality and lifespan of the affected dog.

The considerable incidence of cardiovascular diseases in some breeds has prompted the scientific community to improve medical knowledge and to seek appropriate therapies for effective treatment [3,4]. Satisfactory therapeutic success is not always achieved, and this leads us to seek useful preventive methods that can limit the appearance and/or the development of these pathologies. Genetic selection and reproductive programs are part of these strategies [5]. Inevitably, the prevention involves both breeders and owners. They take on an essential role and can make the difference in the selection programs. Breeders and owners can transform the secondary prevention (e.g., screening) into primary prevention, by having an active role

together with veterinarians in the genetic selection. The indications of the screenings must be followed and those subjects suffering from the pathology and with certain genetic profiles must be excluded from reproduction.

To date, there have been few scientific studies of molecular genetics that provide us with indications regarding the transmission mode of hereditary heart diseases [1]. Since they are very expensive and complex to carry out, it is very difficult to rapidly obtain large-scale genetic tests. To provide specific guidance on genetic selection, the collection of data related to the incidence of heart disease in different breeds and affected bloodlines can help us to conduct retrospective studies on genealogies, which useful to identify the hereditary mechanisms. Myxomatous mitral valve disease (MMVD) predominantly affects Cavalier King Charles spaniels (CKCS) and is an acquired pathology with an hereditability base in this breed [6], which manifests itself at varying unpredictable degrees. This is essential to consider. While congenital diseases are present at birth, and a diagnosis can usually be done before breeding, the MMVD in CKCS might only be defined at one year of age or later in life, greatly complicating the breeding programs. For these reasons, this PhD project proposes a multimodal screening modality for MMVD in CKCS. Our goal is to identify subjects affected early on, without clinical signs, and therefore possible carriers of the worst degree and/or more rapid evolution of the disease. We have attempted to reach this goal through a genetic, echocardiographic, morphological and biomarker study of the subjects included in this project.

We would like to support breeders in their targeted selection programs, in order to obtain healthier subjects with a good life expectancy, thus ensuring the protection of the genetic pool of the breed, which represents an important national and international goal.

In these three years, since the project began, important data have been obtained. These are reported in the individual studies described below in this PhD thesis.

The clinical examination of the same subject over time enables identification of the age of onset of MMVD and consequently the precocity of its appearance. In view of this, we scheduled various controls over time, mainly annual or even six-monthly, so as to have more clinical and instrumental data to analyze, and better identify any changes in the mitral valve apparatus.

In this project breeders and owners played a fundamental role, such as the choice to screen their dogs, especially breeding animals, because this can make a long-term difference in reducing the incidence of MMVD. The follow-up of these subjects will be fundamental, and it will certainly take many years to obtain precise answers and to further improve the screening protocols.

2. Abstract

One hundred and sixty-five CKCS subjects, both healthy and affected by different stages of MMVD, were examined from November 2018 to June 2021 at the Veterinary Teaching Hospital in Lodi – University of Milan – Cardiology Section. Each dog was submitted to a clinical examination and echocardiographic, radiographic, and morphometric data were collected. Whole blood and plasma were collected for genetic analysis and biomarker (miRNA) evaluation.

The objectives of this study were: 1) To describe breed-specific reference values for vertebral heart score (VHS), vertebral heart atrial size (VLAS), M-VLAS, and radiographic left atrial enlargement (RLAD) in healthy adults CKCS; 2) To conduct a genomic study on a population of Italian CKCS; 3) To characterize echocardiographic features of the mitral valve in this breed, focusing on dogs classified as American College of Veterinary Internal Medicine (ACVIM) B1, without clinical signs and without left atrial and ventricular enlargement; 4) To analyze the relationships and the prognostic value of morphometric variables in CKCS affected by MMVD; 5) To analyze the expression of miRNAs described in the literature as being involved in the pathophysiology of MMVD, and identified in dogs' plasma.

Results: 1) Healthy CKCS had a median VHS of 10.08 ± 0.56v, a VLAS, M-VLAS and RLAD respectively of 1.79 ± 0.3v, 2.23 ± 0.44v and 1.2 ± 0.34v; 2). The top 1% single-nucleotide polymorphisms (SNPs) of both Wright's fixation index (FST) and cross-population extended haplotype homozygosity (XP-EHH) analyses localized 10 consensus genes on chromosomes 3-11-14-19; 3) Within class B1, older subjects showed significantly higher values of anterior mitral valve area (AMVA), width (AMVW), mitral valve annulus in diastole (MVAd) and systole (MVAs) and lower sphericity index (SI); 4) A more severe mitral regurgitant jet size and a thicker anterior mitral valve leaflet were observed in CKCS smaller than standard proposed by the Ente Nazionale della Cinofilia Italiana (ENCI) and with morphometric

characteristics tending to brachycephalism; 5) miR-30b-5 was significantly higher in ACVIM B1 compared to healthy subjects (ACVIM class A) and the AUC was 0.79. According to the age of dogs, the expression of miR-30b-5p remained significantly higher in group B1<3y (2.3 folds p=0.03), B1 3-7y (2.2 folds p=0.03), and B1>7y (2.7 folds p=0.02) than in stage A. The AUCs were fair in discriminating group B1<3y and A (AUC 0.78), and B1 3-7y and A (AUC 0.78), and good in discriminating group B1>7y and A (AUC 0.82).

Conclusions: 1) Findings supported previous studies recommending the use of breed-specific reference values for VHS, VLAS, M-VLAS and RLAD and provided background data for future radiographic evaluations of CKCS dogs with clinical signs of cardiac disease; 2) This genetic analysis expands the knowledge of the genetic basis of MMVD by identifying genes involved in the early onset of MMVD in CKCS; 3) This is the first study that describes measurements of the anterior mitral valve leaflet and the mitral valve annulus in the CKCS affected by MMVD at different stages. Differently aged B1 dogs have different clinical and echocardiographic patterns. Further investigations with a larger study population and an appropriate follow-up would highlight prognostic factors related to disease worsening within this heterogeneous ACVIM class; 4) The morphological study of CKCS showed that a more severe regurgitant jet size was observed in subjects with a shorter head and nose. Subjects with a smaller head stop angle had thicker anterior mitral valve leaflets. Dogs with cephalic morphology more similar to the King Charles spaniel breed, that is with a brachycephalic morphotype, showed a more severe regurgitant jet size and valvular characteristics related to worse forms of MMVD (thicker anterior mitral valve leaflet, greater mitral valve annulus and lower sphericity index); 5) miR-30b-5p increases in the plasma of asymptomatic CKCS and this can be considered a potentially promising biomarker even at an asymptomatic stage of disease, particularly at a young age.

3. Literature review

3.1. The Cavalier King Charles spaniel

3.1.1. History of the breed

The modern Cavalier King Charles spaniels have as their direct ancestors the small English Toy spaniels seen in many paintings and illustrations of the 16th, 17th, and 18th centuries. During the reigns of the Tudors, it was commonplace for court ladies to keep Toy spaniels as pets. However, in England it was the Stuart dynasty during which the dog obtained the Royal title of 'King Charles spaniel'. King Charles II was said to be rarely seen without two or three of his beloved pets following behind [7,8].

Over the years, particularly with the arrival of the Dutch Court of William III, Toy spaniels became no longer fashionable and were substituted in popularity by the Pug. Toy spaniels almost ceased to be household animals until the 18th and 19th centuries. This was the period when a special variety of red and white Toy spaniels were bred at Blenheim Palace by the Dukes of Marlborough. These dogs acquired a certain reputation for their sporting characteristics and reobtained their position as ladies' companions (Fig 1) [7,8].

Figure 1. Duke of Marlborough Family with their spaniels. John Singer Sargent, 1905 [7].

Until the 19th century there were no dog shows. Consequently, no recognized breed standards existed, resulting in a wide range of variability in terms of type and size of dog. Breeding was in no way carefully planned or organized. It was not until the Victorian period that dog breeders

started to hold shows and enthusiasts began to breed dogs to very precise phenotypes that corresponded to what people required. This brought about a new fashion for dogs with a shorter face, which eventually evolved into the typical flat face of the King Charles Spaniel of today. A number of several very proficient breeders were successful in breeding very high quality dogs, with a flat face, high forehead, and with very long ears set low. This type is still popular and a very attractive breed for the public [8].

In the 1920s, Mr Roswell Eldridge, an ardent enthusiast of Toy Spaniels, came from the USA to visit England and was struck by the absence of the small, long-nosed version of Toy spaniels. His solution was to offer prize money at Cruft's Dog Show in London, to those exhibiting King Charles Spaniels with long noses, just like those in King Charles II's time (Fig 2). The offer was for three years, later extended to five years. The prize money was £25 for the best male and female breeders. As reported by Cruft's catalogue, he attempted to obtain dogs "As shown in the pictures of King Charles II's time, long face, no stop; flat skull, not inclined to be domed and with the spot in the center of the skull" [8].

Figure 2. Sir Edwin Landseer, Dash, 1836. Oil on panel [8].

The King Charles breeders showed little enthusiasm for these recommendations. They had worked specifically for years to eliminate the long nose, so Eldridge's suggestion was highly unpopular. In the following years, only a few enthusiasts continued the breeding plans. Mrs Hewitt Pitt was the most important King Charles breeder among them. After five years little

had been achieved and the Kennel Club maintained that there was not a sufficient number of standardized to warrant a separate breed registration [8].

However, 1928 saw the foundation of a club, and the title "Cavalier King Charles spaniel" was chosen for the dogs produced. At the first meeting, held the second day of Cruft's Dog Show, in the same year of the foundation, the standard of the breed was established, and it was the same as it is today. The live pattern was Ann's Son, belonging to Miss Mostyn Walker (Fig 3). Club members came up with all the reproductions of illustrations and paintings of the 16th, 17th, and 18th centuries that they could collect together. It was agreed among members that the dog should in no way be considered a fashionable breed and there was to be no modification of its coat [7,8].

Figure 3. Ann's Son (property of Miss Mostyn Walker) [8]**.**

Little progress was made for several years, and Kennel Club breed recognition was still not forthcoming. In the absence of Challenge Certificates, few people were sufficiently interested in breeding a dog with no sales value. These King Charles 'pioneers' entered for Open classes at shows, obtaining classes for the spaniels at the shows where Show Secretaries were co-operative. Usually, there were no money prizes, but at least the public saw and they became increasingly popular. Gradually, the public became aware that the movement had come to stay [7,8].

In 1945, the Kennel Club classified the dogs as a unique breed, and the first set of Challenge Certificates were issued a year later. The first Cavalier champion was owned by Mrs Pitt's daughter Jane (Mrs Bowdler). He was Ch Daywell Roger and had been bred by Lt. Col. and

Mrs Brierly. In the middle of the century, Daywell Roger was very widely used as a stud, and was a major contribution to the development of the breed in the mid-1900s (Fig 4) [7,8].

Figure 4. Ch. Daywell Roger, 1946, first Cavalier Champion [8].

By 1960, annual Cavalier registration at the Kennel Club was over a thousand and more than sixty had received the major awards as champions. The breed's future was assured, and this was further highlighted in 1963 when Mrs Cryer's Blenheim Ch Amelia of Laguna won the Toy Group at Crufts. In 1965 the first Club yearbook was published, containing the activities of 1964. It was a tiny red volume with a single page dedicated to the prefixes and affixes of all Club members. With registrations increasing, the number of Challenge Certificates (C.C.) offered at Championship shows also rose, together with the size of classes. Exactly ten years after Amelia's winning performance, another Blenheim subject obtained the highest possible result and became Supreme Best in Show at Crufts. On winning this maximum award, Messrs. Hall & Evans' Alansmere Aquarius was a young dog, but soon became a Champion. Public attention focused still further on the breed both in UK and overseas after his success. There were already well-established Cavalier Clubs in the U.S.A., Australia, and New Zealand, and new clubs in Finland and Sweden [7,8].

By the end of the seventies, interest in shows was so high that Cavaliers were always led the Toy Group entries at championship events. In 1978, the club had its golden jubilee, celebrating with a social event at Royal Leamington Spa and a championship show, which attracted a large number of competitors at the village of Stoneleigh, in Warwickshire. The president was Amice Pitt. It was her last public appearance, and the many members attending were to see this

important figure shortly before she died in December 1978. Still today, each year the Amice Pitt Rally is held in by the various Cavalier clubs and is designed as a commemoration and to recognize the contribution she made to the history of the breed [7].

Early in the eighties, registrations reached 10,000. There was the need to have a separate judge for each sex at most of the championship shows. This was not seen as a positive development, was generally recognized as inevitable. That a Cavalier could win at the highest level was certain, and this was reaffirmed at Crufts in 1981 when Mr & Mrs Newton's Ch. Jia Laertes of Tonnew won the Toy group. Meanwhile, regional clubs and rescue groups increased in number, the latter assisting individual Cavaliers in difficulty. In 1988, on the occasion of the club's Diamond Jubilee, the championship show had 777 entries, and a total of 363 champions has been recorded[7].

In the 1990's, Cavaliers regularly won top awards in the Toy group entries at annual championship shows. Cavaliers continued to be successful at group level, and several went on to Reserve B.I.S. In 1993, Messrs. Hall & Evans' Ch. Spring Tide at Alansmere broke the breed C.C. record (set by Ch. Aloysius of Sunninghill in the 1960's) and finished the year on 23 C.C.s. The record was broken again by Rix & Berry, with their Ch. Lymrey Royal Reflection, and in the bitches category with Ch. Lymrey Top of the Pops [7].

3.1.2. Breed standard

FCI-Standard N° 136 (Fig 5) [9].

Figure 5. M. Davidson, illustrator. NKU Picture Library [9]**.**

Origin: Great Britain.

Date of publication of the official valid standard: 04.11.2008.

Utilization: Companion and Toy.

FCI-classification: Group 9 Companion and Toy Dogs. Section 7 English Toy spaniels. Without working trial.

GENERAL APPEARANCE: Active, graceful, and well balanced, with gentle expression.

BEHAVIOUR / TEMPERAMENT: Sporting, affectionate, absolutely fearless. Gay, friendly, non-aggressive; no tendency towards nervousness.

HEAD:

Cranial region:

Skull: Almost flat between ears.

Stop: Shallow.

Facial region:

Nose: Nostrils black and well developed without flesh marks.

Muzzle: Length from base of stop to tip of nose about 1 1/2 ins. (3.8 cm). Well tapered. Face well filled below eyes. Any tendency to snipiness undesirable.

Lips: Well developed and not pendulous.

Jaws/Teeth: Jaws strong, with a perfect, regular and complete scissor bite, i.e., the upper teeth closely overlapping the lower teeth and set square to the jaws.

Eyes: Large, dark, round but not prominent; spaced well apart.

Ears: Long, set high, with plenty of feather.

NECK: Moderate length, slightly arched.

BODY:

Back: Level.

Loin: Short-coupled.

Chest: Moderate; good spring of ribs.

TAIL: Length of tail in balance with body, well set on, carried happily but never much above the level of the back. Docking previously optional when no more than one-third was to be removed.

LIMBS:

Forequarters:

General appearance: Legs moderately boned, straight.

Shoulders: Well laid back.

Forefeet: Compact, cushioned and well feathered.

Hindquarters:

General appearance: Legs with moderate bone.

Stifle: Well turned.

Hocks: No tendency to cow- or sickle-hocks.

Hind feet: Compact, cushioned and well feathered.

GAIT / MOVEMENT: Free-moving and elegant in action, plenty of drive from behind. Fore- and hindlegs move parallel when viewed from in front and behind.

COAT:

Hair: Long, silky, free from curl. Slight wave permissible. Plenty of feathering. Totally free from trimming.

Color: Recognized colors are:

- Black and Tan: Raven black with tan markings above the eyes, on cheeks, inside ears, on chest and legs and underside of tail. Tan should be bright. White marks undesirable.
- Ruby: Whole colored rich red. White markings undesirable.
- Blenheim: Rich chestnut markings well broken up, on pearly white ground. Markings evenly divided on head, leaving room between ears for much valued lozenge mark or spot (a unique characteristic of the breed).
- Tricolor: Black and white well-spaced, broken up, with tan markings over eyes, cheeks, inside ears, inside legs, and on underside of tail.

Any other color or combination of colors highly undesirable.

WEIGHT: 5.4 - 8 kg (12 - 18 lbs.). A small, well-balanced dog well within these weight desirables.

FAULTS: Any departure from the foregoing points should be considered a fault and the seriousness with which the fault should be regarded should be in exact proportion to its degree and its effect upon the health and welfare of the dog.

DISQUALIFYING FAULTS:

- Aggressive or overly shy dogs.
- Any dog clearly showing physical of behavioral abnormalities.

N.B.:

• Male animals should have two apparently normal testicles fully descended into the scrotum.

• Only functionally and clinically healthy dogs, with breed typical conformation should be used for breeding [9].

3.1.3. Genetic diversity in Cavalier King Charles spaniels

The breeds are closed populations subjected to selection and therefore the individuals of the same breed are on average more closely related to each other than a couple randomly chosen in the entire population. The genetic diversity of individuals of the same breed can be evaluated using genealogical or genomic data.

3.1.3.1. Genealogical evaluation

The inbreeding coefficient (F) is one of the most frequently used genealogical parameters and is defined as the probability that both alleles inherited from an individual are copies of a single allele from an ancestor common to both parents. This value is always greater than 0 if the parents have common ancestors and grows if the latter increase in number in the most recent generations. Inbreeding rate for generation (ΔF) is directly related to rate of loss of genetic variability in the population, rate of loss of heterozygosity, impact of inbreeding depression, the rate at which unknown deleterious alleles can spread to the population, and the probability of loss of favorable mutations in the population. It is clear, therefore, the importance of avoiding that ΔF increases excessively because of genetic selection. Indeed, the appearance of negative effects due to inbreeding can be expected with values higher than 0.01 [10]. The inbreeding coefficient of the hypothetical offspring of two individuals is called kinship or kinship coefficient (Φ) [11].

Another important parameter is the actual size of the population (N_e), that is the number of individuals of an ideal population that would face a loss of genetic diversity from inbreeding equal to that of the population of interest. It is also influenced by the unequal use of males and

females in reproduction [11-13]. The loss of genetic diversity increases dramatically when N_e falls below the value of 100 and a population is considered at high risk of incurring the deleterious effects of inbreeding when N_e is less than 50 [14].

Number of founders (f) is the number of individuals in a population whose parents are not known. The actual number of founders (f_e), on the other hand, is calculated as the reciprocal probability that two randomly extracted genes from the population derive from the same founder. The values of f and f_e would be the same if all founders had contributed equally to the genetic pool. However, f_e is often smaller as they have been used differently in reproduction [13,15]. This parameter considers the selection rate, that is the probability of reproduction, and the variation in the size of the family, that is the production of progeny per individual, but not the possible gene loss in the following generations [16].

The actual number of ancestors (f_a), whereby ancestors are considered as individuals, founders or not, who are estimated to have made the greatest genetic contribution to the population, is calculated as the reciprocal probability that two genes randomly extracted from the population are derived from the same ancestor. Unlike f_e, this parameter considers the potential bottlenecks in pedigree, the main cause of genetic loss in some populations [16].

The actual number of founding genomes (N_g) is described as the probability that a given gene in the founders (founding gene) is still present in the studied population. This parameter considers all causes of genetic loss during segregation, so it will always be less than f_e and f_a [16].

In 2015 Lewis et al. analyzed the pedigrees of dogs belonging to all 215 breeds recognized by the United Kingdom (UK) Kennel Club, registered in the period from 1980 to 2014. Regarding the CKCS, it was seen that F, which was below 0.01 in 1980, increased progressively until 2002, when it reached values above 0.06. The subsequent descent allowed this coefficient to be reduced by one percentage point, but since 2009 it has risen again. On the official website of

the UK Kennel Club, it is indicated that the value of F calculated on CKCS breed dogs born in 2018 is 0.063 [17]. The trend of F is closely related to that of ΔF, which in this study was calculated on blocks of 5 years: in the first decade considered, it stood at values of 0.01, while since 2000 it has decreased to even negative values. This trend has been observed in almost all breeds considered. The estimated N_e based on ΔF for the entire period covered by the study is 111.2. Finally, the considerable use of *popular sires* was evident, that is a small number of very prolific breeding males; however, this practice seems to have subsided since 2000 [11,17].

Another study that covered a considerable amount of time, particularly from 1958 to 2009, focused on Australian dogs of 32 different breeds. Regarding the CKCS, pedigrees of 29855 subjects were analyzed, resulting in: F = 0.035, with values of less than 0.01 for 89% of the dogs; ΔF = 0.002, both considering the entire time interval and the period 2000-2009; N_e = 204; f_e = 258; f_a = 113; N_g = 55.9 [18]. The f_e/f ratio was equal to 0.31, rather low, as in many other breeds that counted a high number of subjects, for the unequal contribution provided by the founders. The value of 0.44 for the f_a/f_e ratio indicates that in this case the breed was subjected to a bottleneck [18].

In Belgium, in 2016, Wijnrocx conducted a study in 23 breeds, evaluating pedigrees recorded from 1965 to 2013. The 4783 belonging to CKCS produced a calculation of: F = 0.009; Φ = 0.5; N_e = 106,6; f_e = 294; f_a = 157; N_g = 0.41. The CKCS stood out because, among all breeds evaluated, it was characterized by the lowest value of Φ and the highest N_g. The f_e/f ratio was 0.41 and the f_a/fe ratio was 0.54 [19].

Finally, a study on French dogs of 61 breeds born between 2001 and 2005 was published by Leroy et al. in 2009. The pedigrees of the CKCS analyzed were 27392 and the parameters calculated: F = 0.003; Φ = 0.014; N_e = 150, while the average for all breeds was 226; f_e = 200; $_{fa}$ = 61. The 0.31 f_a/f_e ratio is the lowest of all the above studies (Table 1) [15].

Table 1. Summary of genealogical parameters expressing CKCS's genetic diversity in the literature.

	UK 1980-2014	Australia 1958-2009	Belgium 1965-2013	France 2001-2005
F (%)		3.5	0.9	3.3
Φ			0.5	1.4
f_e		258	294	200
f_a		113	157	61
N_e	111.2	204	106.6	150
N_g		55.9	0.41	
Authors	[11]	[18]	[19]	[15]

F = Inbreeding coefficient; Φ = Kinship coefficient; f_e = Actual number of founders; f_a = Actual number of ancestors; N_e = Actual size of the population; N_g = Actual number of founding genomes.

3.1.3.2. Genetic marker evaluation

The most recent genomic approach uses genetic markers to evaluate molecular genetic diversity, which is expressed as heterozygosity (H) and allelic richness (A_r). H represents the total frequency of heterozygosity for a given locus and thus estimates the probability that two randomly selected alleles in the population are different. The calculation can be performed on the population as observed heterozygous rate (H_o) or based on the Hardy-Weinberg equilibrium as expected heterozygous rate (H_e) [20,21]. A_r, on the other hand, corresponds to the number of alleles in the population and may indicate that the population has undergone a reduction in size or bottlenecks [20,21]. The fixation index (F_{IS}) is the correlation between two alleles taken from a population in relation to a subpopulation. When F_{IS} is positive, the heterozygosity of the population is significantly lower than the Hardy-Weinberg equilibrium, thus indicating the preferential coupling between related individuals [22,23].

Two of the studies mentioned above also carried out a genomic analysis of genetic diversity. In particular, the Wijnrocx et al. study in 2016 estimated 375 CKCS born between 2008 and 2013, thus obtaining the values of: H_e = 0.516; H_o = 0.505; A_r = 2.76; F_{IS} = 0.021, which was not significantly different from 0 [19].

Leroy et al., in 2009, had instead championship 30 CKCS and calculated: H_e = 0.47, while the average value for all dogs was 0.62; H_o = 0.45; A_r = 3.1, with an average for all breeds of 4.56; F_{IS} = 0.041 [15].

Slightly different, however, was the approach followed by Mellanby et al. (2013), who analyzed the DNA of dogs of 26 different breeds, sampled during clinical practice or, as for CKCS only, in 25 individuals, during a dog show. The results of the analysis on the CKCS were: H_e = 0.55, while the average value for all dogs was 0.62; H_o = 0.55; F_{IS} = 0.001. The authors concluded that there was no evidence that the breed had met a bottleneck (Table 2) [24].

Table 2. Summary of genomic parameters expressing CKCS genetic diversity in the literature.

	Belgium 2008-2013	France 2001-2005	UK 2013
Number of CKCS	375	30	25
H_e	0.52	0.47	0.55
H_o	0.51	0.45	0.55
A_r	2.76	3.1	
F_{IS} (%)	2.1	4.1	0.1
Authors	[19]	[15]	[24]

H_e = Expected heterozygous rate; H_o = observed heterozygous rate; A_r = Allelic richness; F_{IS} = Fixation index.

3.2. Epidemiology and factors affecting the onset of mitral valve disease

MMVD represents 70% of the dog's heart disease, with a prevalence, in small-sized older dogs, of close to 100% [16,25].

Whitney, in 1974, subdivided the necropsy findings of 200 dogs referable to this pathology into four degrees depending on severity: types 1 and 2, recognizable only at autopsy, were present in 35% of dogs under 5 years of age, but clinically significant in less than 3%; types 3 and 4, instead, were found in 97% of dogs over 9 years old and were clinically significant in 40% of animals (Fig 6) [26,27]. It is evident, therefore, that the pathology has a chronic and progressive course and that its prevalence, as well as its severity, increases considerably with the advancement of age [28].

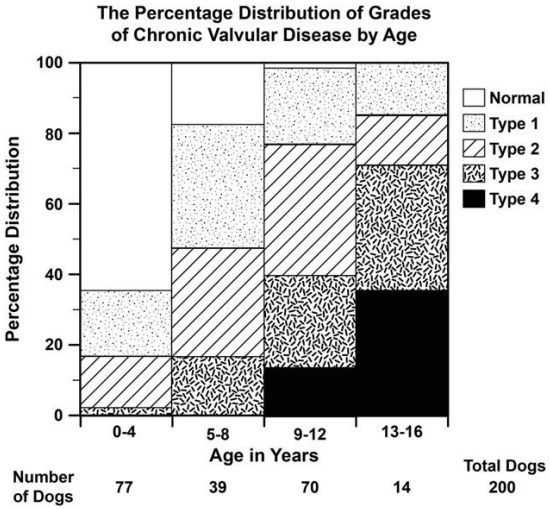

Figure 6. Bar chart showing the percentage distribution of the four types of MMVD by age in 200 dogs [27].

A study of 942 adult dogs, over 1 year old, belonging to 6 small breeds, showed a prevalence of 14.4 ± 2.2% [29], which, compared with that of the entire dog population, reported by previous authors, of 4.5 ± 0.5% and 3.5 ± 0.3% [30,31], confirms that these breeds present a 3-4 times greater risk in the development of MMVD. It has been established that among the most affected breeds there are CKCS, Dachshund, Poodle, Yorkshire terrier, Chihuahua, Miniature

and Standard schnauzers, Cocker spaniel, Pinscher, Pekingese, Shih-Tzu, Beagle and Whippet [32,33].

It has been estimated that about 50% of CKCS are affected by MMVD at the age of 6-7 years and almost 100% at the age of 10 years [34,35]. The presence of a left apical systolic murmur, a good indicator of the presence of this disease, is detectable in 29.4% of dogs, in 17.2% of those under 4 years of age, 48.3% above that age and 100% of dogs of 9-11 years of age [36]. A Swedish study revealed that, in more than 400,000 claims for compensation for dogs who died less than 10 years of age, the first ten breeds for heart-related mortality rates were all large breeds, excluding the CKCS, second only to the Irish Wolfhound. Of the 840 CKCS requests, only 32 (4%) were classified as "cardiomyopathy", while the others were referred to MMVD. The authors considered that mortality rates were much lower in other breeds susceptible to MMVD because the disease is characterized by a later onset, so rarely at ten years old does it cause symptoms such as to cause the death of the animal or make euthanasia necessary due to cardiac disorders [37]. It is curious to note that in Australia the prevalence in the breed is lower, or perhaps the pathology occurs later, affecting only 25% of dogs aged 4 or more. It is plausible that these differences are because, in different countries, breeders prefer different genetic lines [35]. A particularity of this breed, besides the high prevalence of the pathology, is therefore its early onset.

A study based on 3D echocardiography showed that the mitral valve morphology of healthy CKCS differs from that of other healthy dogs, as it is flatter and shorter, with a lower tenting of the flaps and a smaller posterior flap. These anatomical abnormalities, which are similar to those observed in dogs affected by the disease, could be the factor that predisposes the breed to an early onset of the disease, given that such abnormalities would lead to increased valvular stress [38-40].

At least in this breed, a genetic predisposition with a polygenic character exists. Puppies from parent dogs with more intense heart murmurs at a younger age are more likely to experience the same symptoms [41]. Furthermore, there is a high heritability of early mitral prolapse, this being estimated based on the presence of murmurs in 4-year-old dogs [42].

Considering these numbers, clinically healthy CKCS that do not present heart murmurs and that not present evidence of MMVD at the echocardiographic examination are included in ACVIM class A. These dogs are at high risk of disease development, and it would be appropriate to subject them regularly to cardiological examination [43].

As reported in several studies on CKCS and Dachshund, even within the same breed, body weight seems to have a negative correlation with the presence and severity of mitral valve prolapse (MVP) [28,44]. In the Dachshund, it was also observed that the circumference of the thorax correlates similarly (negatively) to the MVP [44].

Finally, at least as regards the small breeds, it has been observed that, if at a young age the sex seems irrelevant as regards the probability of developing a mitral prolapse, after 5 years the pathology is more frequent and more serious in the male dogs [41]. Similarly, the prevalence of a left apical systolic murmur is 1.9 times higher and increases faster in males than females [29].

3.2.1. Hereditability in Cavalier King Charles spaniels

Many authors have recorded a high prevalence of MMVD in CKCS and a much more rapid progression of the pathology in this breed than in others [34,36]. The exact etiology has not yet been established, but given the high prevalence of the disease in some breeds, it has been assumed to have a hereditary component of a polygenic type. This etiology implies that more genes are involved and that a certain threshold of expression must be reached before the disease manifests itself [45]. This would explain why the offspring of two CKCS suffering from a more serious disease are more frequently affected by heart murmurs. Heart murmurs are also more

intense than those manifested by the offspring of two subjects with a milder form of the disease [41].

Recent studies have yielded mixed results on the subject. A study carried out on CKCS in 2011 from five European countries was able to identify two loci significantly associated with the development of MMVD on canine chromosomes (CFA) 13 and 14. Specifically, on Cfa13q2.2.3 a region of 2 gene coding proteins was highlighted by analyzing the entire genotyped canine population, while this region rises to 20 genes (1.68 Mb) considering only dogs from Denmark and Sweden. On the CFA14q1.3, however, a region of 29 genes (1.58 Mb) was detected significantly associated with MMVD, a region which drops to 9 genes when considering only dogs from Sweden and Denmark. The allele present on CFA14 appears to have a pathogenic effect, while the allele present on CFA13 has a protective effect [46]. In contrast, a study of 36 CKCS in 2012 divided into cases and controls did not highlight a region significantly related to the development of MMVD in this breed, nor an intensity in heart murmur. The analysis carried out in the study therefore showed that the familiarity of the pathology in the CKCS is not because of a specific gene. For this reason, it is not possible to devise selection strategies to eliminate the disease based on currently available genomic information [47].

Although there is therefore no certainty of which are precisely the genes involved in the etiopathogenesis of MMVD, the genetic predisposition of CKCS was demonstrated by Swenson et al. in 1996. In support of the hypothesis, it is reported that about the 50% of the subjects of this breed have a heart murmur at 6-7 years of age, while the probability of finding a murmur at auscultation rises to 100% in subjects over 11 years old [35]. It is not yet certain whether males tend to develop the pathology at an earlier age than females and to exhibit higher intensity murmurs, since in some studies this is asserted [41,42], whereas in others it is confuted [35,42,48].

Among the numerous small dog breeds that are affected by MMVD, the CKCS has a much higher and younger age prevalence of the disease [34,35]. It is reasonable to assume that this breed is subjected to two different forms of MMVD: a form with an early onset and a rapid progression and the typical form of MMVD which occurs in the adult-elderly age with a long evolution, which affects also the other breeds [42]. Therefore, taking into consideration subjects between 4 and 5 years of age, Lewis et al. in 2011 attempted to estimate the heritability of the presence and degree of heart murmur in affected CKCS regarding the early onset of MMVD. Of the 1,252 dogs subjected to auscultation, 108 were reported to have the typical systolic murmur on the mitral valve and the average degree of intensity of the murmur of these 108 patients was found to be 1.731. The estimated heritability values in the study were 0.33 ± 0.072 as regards the presence of the murmur and 0.67 ± 0.071 for the degree of intensity. The study also highlighted a significant presence of higher-grade murmurs in males than females [42].

Both inherited values obtained were very high and significant, therefore demonstrating that the presence and the intensity of a murmur are heritable factors. This indicates that the MMVD manifested by the CKCS at an early age (in this case 4-5 years) has a genetic origin [42].

Based on the results obtained in this study, it can therefore be assumed that the CKCS can be divided into 3 classes:

1. Dogs not genetically predisposed to the development of MMVD.
2. Dogs predisposed to the development of the degenerative form of MMVD, which is manifested in adult-old age.
3. Dogs presenting the genetic predisposition of CKCS to the development of an early onset form of MMVD [42].

The very high heritability results obtained from the study above suggest that, if performed correctly, genetic selection on subjects predisposed to early onset of the disease would lead to effective results [42]. One of the limitations of the study of Lewis et al. was the low specificity

of auscultation. In fact, the identification of an innocent murmur or a murmur caused by a different pathology could lead to an incorrect diagnosis of MMVD. However, it has been demonstrated that the identification of a mitral systolic murmur in the CKCS due to causes different from MMVD is extremely rare [48].

Auscultation is the clinical approach which is routinely applied during the physical examination of the patient. The identification of a murmur is common in subjects suffering from a heart disease and the murmur is staged by the clinician in a system that provides 6 different degrees of intensity [49].

In CKCS affected by MMVD, as in any other dog suffering from this disease, a correlation between the intensity of the murmur, the severity of mitral regurgitation and, therefore, the severity of the disease itself has been demonstrated [50,51]. Some authors have shown that the intensity of the murmur increases with the worsening of some echocardiographic parameters, such as the left atrium to aorta ratio (LA/Ao), the internal diameter of left ventricle in diastole, the thickness of the mitral flaps and the mitral regurgitant volume. It is not surprising that a correlation between the intensity of the murmur and the age of the subject, the MMVD being a progressive pathology, can be aggravated with the increase in age [48]. An increase in the intensity of the heart murmur therefore represents an important characteristic of the pathology that reflects a possible worsening of the disease and a clinical manifestation of left heart failure both in CKCS and in other affected breeds [45,50,52]. Over the years, different selection programs have been implemented for CKCS. The Swedish, English, and Danish programs are those with the most significant results. The Swedish and the English programs based the selection of the breeding dogs solely on the identification of a heart murmur. The Danish program, instead, associated the identification of a heart murmur to the echocardiographic examination. Of the three screening programs, only the English one was on a voluntary basis [53-55].

The Swedish breeding program started in 2001 with the aim of reducing the prevalence of mitral regurgitation (MR) caused by MMVD in CKCS. In this program, dogs were not allowed to breed until four years of age and needed a heart auscultation without murmurs within eight months before mating. However, dogs were allowed to breed at an age of 24 months if the dog and its parents were examined and no murmurs were detected. Male dogs that had a heart auscultation at seven years of age without murmurs were allowed to breed without further heart evaluation. Breeding animals whose parents had heart murmurs before four years of age were not allowed to breed [56]. The aim of Lundin's study was to analyze the prevalence of heart murmurs in six-year-old CKCS and to estimate if the prevalence had decreased since the introduction of the Swedish breeding program in 2001 [53]. Of the 132 CKCS born in 2001, only 56 subjects with an average age of 6.2 years met the inclusion criteria and were included. Similarly, of the 221 CKCS born in 2003 only75 subjects of average age of 5.9 years were included. The prevalence of the heart murmur was calculated from these 131 subjects. Of the 56 dogs born in 2001, 52% had a heart murmur. There were no differences between the prevalence and the intensity of the murmurs between males and females. Of the 75 subjects born in 2003, 55% had a heart murmur. There was no significant difference in the prevalence of the murmur between males and females, while the murmurs presented by males were significantly higher than those presented by females. Finally, subjects born in 2001 and 2003 showed no differences between them that were statistically significant either in the prevalence or in the intensity of the heart murmur.

This study showed that there was no improvement in the presence and intensity of heart murmurs among the dog population of CKCS born in 2001 and born in 2003 in Sweden, despite in those years the Swedish selection program being active [53]. In addition, the prevalence of 52% found in 2007 and that of 55% found in 2009 agreed with the results from other studies [35]. These results can be justified by the fact that the breeding program might have had a slow

effect on disease prevalence over a longer period, especially in dogs less than four years of age. The underlying causes for the findings of the study published by Lundin were unclear [53]. Possible explanations included several factors such as an excessively low age limit for breeding, importation of breeding dogs with an unknown background, a lower inheritance of MMVD than previously estimated, inadequate compliance to the breeding programs among breeders, and insensitive screening methods. Furthermore, the screening program only encouraged breeders to screen dogs up to a certain age and many dogs developed MMVD after that age. Therefore, continued screening of dogs used for breeding until they develop a heart murmur should be beneficial in order to obtain a complete view of the onset of heart murmurs within the breed and thereby facilitate breeding against MMVD.

The UK selection program, from 1991 to 2010, was entirely voluntary and based the choice of the breeders on the exclusive presence/absence of a heart murmur. The selection program suggested that any dog used for breeding should be at least 5 years old and free from an audible murmur consistent with MMVD. Dogs over 2.5 years could be used for breeding if their parents were over 5 years before they developed a murmur. The scheme was based on the presence or absence of a murmur suggestive of MMVD. The results of this testing were recorded on a database. A list of dogs that were over 5 years of age when they first developed a murmur was published by the UK Cavalier Club. This was refined in 2006 so that only dogs tested by a cardiologist could be included in the "over 5 list" and breeders were encouraged to have their dogs tested at least once in the first 5 years by a cardiologist [55]. It was hoped that by breeding from dogs who develop murmurs later in life, or whose parents do, the average age at which a CKCS develops MMVD would increase. The aim of the study published by Swift was to analyze the results of the UK CKCS database developed during breed screening to examine whether the screening was having an impact on the age at which murmurs were first detected [55]. The results suggested that the age incidence of murmurs associated with MMVD might

be increased by application of breeding guidelines based on auscultation alone. However, this benefit was only seen in a subgroup of subjects and compliance of breeders with this voluntary scheme was poor. Therefore, the results of the UK selection program must be considered as only partially successful.

At the same time as the breeding programs described above, from 2001 a breeding scheme has been ongoing in Denmark as a collaboration between the Danish Kennel Club Association, The Cavalier Club in Denmark, and the University of Copenhagen. The aim of the study published by Birkegard was to evaluate if a mandatory breeding scheme based on echocardiography and cardiac auscultation results in decreased MMVD severity in CKCS after an 8- to 10-year time period [54].

For each subject, reproduction was prohibited if the murmur was 3/6 and the mitral leaflets prolapse was considered severe. Subjects with a 2/6 murmur were excluded if the prolapse was moderate-severe. Reproduction was allowed in subjects with a 1/6 heart murmur associated with a moderate leaflet prolapse, or with a 2/6 murmur associated with a mild mitral valve prolapse [54]. Between 2002 and 2011, 997 dogs (689 females and 308 males) were evaluated for a total of 1380 cardiac evaluations (1.4 average evaluations per subject). Age was found to be positively related to the presence of a mitral murmur, but the risk of having a heart murmur in 2010-2011 compared to 2002-2003 had decreased by 73% for dogs born from the selection program (BP). For subjects not born from the selection program selection (not-BP), however, the risk of developing a heart murmur was no different between 2002- 2003 and 2010-2011. Between the two groups, BP subjects had a 69% decreased risk of developing a murmur compared to non-BP. As regards the degree of MVP, however, a correlation with both age and sex was observed. Again, BP subjects presented a 36% decreased risk of having MVP compared to non-BP. The risk of having MVP between 2010-2011 and 2002-2003, however, did not appear to be significantly reduced. The Danish program has shown that a breeding program that

combines auscultation with an echocardiographic exam and that establishes mandatory rules for breeding can successfully reduce the risk of having a mitral murmur caused by MMVD. It is important that the participation in the program is not on a voluntary base, because the number of owners willing to implement it is likely to be fewer and therefore affect the final outcome [54].

3.2.2. Association between canine morphometry and mitral valve prolapse

Several echocardiographic studies have documented that MVP (i.e., leaflet protrusion back into the left atrium during systole secondary to the myxomatous degeneration) is an important consequence of the development of MMVD in dogs [36,57-60]. The pathology of this canine disease is known to be very similar to that of primary MVP in humans [61,62]. In both humans and dogs, the disease is characterized by progressive myxomatous degeneration, which results in valvular deformation and regurgitant flow across the mitral valve, the MR [30]. Several small and medium-size breeds are predisposed to developing MR, and consequently heart murmur, due to MMVD [31]. Almost all CKCS older than 10 years will have developed murmurs due to MR [6,57,63,64], and although the prevalence is lower in Dachshunds, the Dachshund is still one of the breeds known to be predisposed to MR secondary to MMVD [31,34,57,60,63,65]. Focusing now mainly on the MVP, we can state that the prevalence of MVP among young CKCS and Dachshunds without murmurs is 87% and 47%, respectively [58,60,66]. Little is known about the epidemiology of MVP in dogs. In a study of mostly young CKCS, a positive correlation was found between age and MVP, but gender had no influence on prevalence and severity [36]. In humans, especially among young and middle-aged individuals, MVP has been found in some studies to be more prevalent among women than among men [67]. In other studies, however, gender had no influence on MVP prevalence [60]. Among older people, the prevalence of severe MR due to MVP is significantly higher in men than in women [68]. In humans, an asthenic habitus (i.e., low anteroposterior thorax diameter) is associated with a

markedly increased risk of MVP [69,70]. In dogs, to our knowledge, no studies have evaluated whether the prevalence and severity of MMVD is related to the shape or size of the thorax. Based on studies published in the veterinary literature, we can only declare that generally, small breeds are known to develop heart valve incompetence and MR more often than large breeds, suggesting a negative correlation with body weight [31]. Some other studies have shown that there is a negative correlation between body weight and MVP, even within the same breed [36]. In humans, low body weight is consistently found to be associated with MVP [71]. Results of echocardiographic studies indicate that MVP constitutes a major part of the genetic background of the disease, but the inheritance of MVP in dogs has not been investigated [36,44,58]. In humans, some researchers have suggested that MVP is an autosomal dominant trait with age and sex-dependent expression, but arguments have also been made in favor of a polygenic inheritance [71-73].

In 1999 Olsen et al. carried out a study on the Dachshund aimed at assessing the influence of age, sex, coat type, body weight, body condition score (BCS), heart rate and thoracic size on the severity of MMVD. At the precordial impulse, the circumference of the thorax was measured using a measuring tape. Also at the precordial impulse, the height of the thorax, and the width of the thorax at points 5 and 10 cm dorsal to the sternum were measured using a custom-made sliding gauge. The degree of obesity was subjectively estimated to the nearest 5%. To date, it is the only study in literature that has taken into consideration the hypothesis of a correlation between thoracic morphology and MMVD in dogs [74].

Of the 190 Dachshunds examined, the thoracic circumference was negatively related to the development of MVP. In addition, there was a negative correlation between the thoracic circumference and both the severity of MR and the heart murmur intensity. These results are in accordance with studies carried out in human medicine showing an association between MVP and patients with asthenic habitus [69,70].

A further negative correlation was described between the severity of MVP and body weight, demonstrating that smaller dogs have more severe MMVD and the BCS appears negatively related to the degree of thickening of the mitral leaflets.

Finally, the coat type influenced the presence and the gravity of the MVP in the long-haired Dachshunds. In fact, the prevalence of MVP was very high, while it was lower in the short-haired Dachshunds and even less in the hard-haired variant. Usually, reproduction takes place between subjects with the same coat type, which is why there could be justification for a different genetic predisposition to MVP [74].

Although the results of the previously described studies are very interesting and highlight the interdependence between canine morphology and the echocardiographic aspect of the mitral valve we must attempt to be critical. The first significant limit is that most of the studies assessing the presence of MVP and MR have used the long axis 4-chamber right parasternal view, which could lead to an incorrect prevalence due to the saddle shape morphology of the mitral valve [28]. Furthermore, jet size found by color flow mapping should only be regarded as a semiquantitative measure of the degree of MR [75]. Several factors such as the quality of the imaging window, the distance to the flow being imaged, gain settings, the immobility of the patient, and the experience of the operator have an influence on this measure [75]. For these reasons, the left apical 4-chamber view must be used because the degree of MR may be underestimated if color flow mapping is performed from the right side of the thorax [28].

Until now, no study has assessed the influence of morphometry on the echocardiographic aspect of the mitral valve in the CKCS.

3.3. Pathophysiology applied to the echocardiographic aspect of the mitral valve in Cavalier King Charles spaniels

The mitral valve (MV) annulus is a hyperbolic paraboloid (commonly described as "saddle-shaped") [39,76,77]. This geometry, consistent across several mammalian species [78], confers a mechanical advantage and is important for optimizing leaflet curvature, reducing leaflet stress, and maintaining valve competency [78-81]. Differences in MV apparatus morphology and presumably, the mechanical forces acting on the valve, have been suggested as a stimulus for signaling pathways that contribute to myxomatous degeneration and its progression [82-84]. In addition, abnormal in vitro stresses on the canine MV have been directly linked to an increased expression of myxomatous effector proteins [85]. Therefore, a breed-specific difference in MV apparatus morphology might alter mechanical stresses on valve leaflets that can activate signaling pathways that contribute to myxomatous degeneration and its progression. Data from the literature show that the analysis of the canine MV using real time 3D trans thoracic echocardiography (RT–3DTTE) is feasible and repeatable, and that healthy CKCS have an elliptical, saddle-shaped MV [39,77,86]. In contrast, dogs affected by MMVD have a more circular and flatter MV [40].

Based on these studies, Menciotti et al. in 2018 analyzed the MV morphology of healthy CKCS compared to the MV morphology of healthy dogs of other breeds [39]. They hypothesized and demonstrated that the MV of CKCS has morphologic differences compared to dogs of other breeds. Specifically, the MV of CKCS is flatter than in dogs of other breeds, with a reduced annulus height (AnH) and reduced leaflet tenting, as demonstrated by reduced tenting height (TnH), tenting area (TnA), and normalized tenting volume (nTnV). Furthermore, relative to dogs of other breeds, the angle between the aortic annulus and anterior leaflet (AAo-AP) of CKCS is greater.

The morphology of the MV influences the stress acting on the leaflets and on the tensioning apparatus [39,78,81], and therefore an abnormal valvular shape and annular configuration could alter the forces applied on the MV during each cardiac cycle. Although most experimental evidence suggests that annular flattening is the source of increased valvular stress [78,81], it is implied that this increase in stress is mediated through changes in leaflet geometry. Indeed, an increased valvular stress associated with annular flattening has been demonstrated to be related to the degree of flatness of the leaflets, not to flatness of the annulus [78,81]. Therefore, a primary alteration in the spatial configuration of the leaflets very probably results in abnormal stresses that are independent of changes in annular configuration. These abnormal stresses on the canine MV are postulated to play a role in the pathogenesis and progression of MMVD in dogs, because they have been directly linked in-vitro to an increased expression of myxomatous effector proteins (such as α-smooth muscle actin and matrix metalloproteinases), chondrogenic markers (such as bone morphogenic protein and collagen type 2), and endogenous serotonin synthesis (up-regulation of tryptophan hydroxylase 1) [39,85]. Moreover, Menciotti et al. reported that the differences between the MV morphology of CKCS and dogs of other breeds do not include 3D indexes of annular nonplanarity, such as annulus height to commissural width ratio (AHCWR) or nonplanar angle (NPA), which are associated with increased leaflet stress [78,80]. Furthermore, the spatial configuration of the leaflets is another fundamental characteristic that potentially minimizes valvular peak stress [78,87]. Interestingly, the four variables found to differ between healthy young adult CKCS and healthy dogs of other breeds (AnH, TnH, TnA, nTnV), are the same four MV morphologic variables that the group of Menciotti found to be altered in a mixed population of dogs affected by MMVD without cardiac remodeling (i.e., ACVIM Stage B1) [40]. This finding supported the hypothesis that morphologic alterations of the valve could represent factors that predispose to the development of the disease, rather than the changes resulting from a diseased status, as has been proposed in

humans [39,88]. The differences in age between CKCS and dogs of other breeds should not have had a significant impact on the results of Menciotti's study. In fact, simple linear regression analysis failed to identify a significant linear relationship between MV morphologic variables and the age of the dogs. Interestingly, this might also suggest that the morphologic variables measured by Menciotti et al. and by previous studies are intrinsic characteristics of the MV and of its apparatus that do not change throughout the life of the dogs, at least in dogs with ages comparable to the ones analyzed in this study [39,40,77].

3.3.1. Echocardiography and advanced diagnostic of the mitral valve in Cavalier King Charles spaniels

Standardized echocardiographic criteria distinguish between normal and abnormal mitral valve morphology in humans, but despite the high prevalence of MMVD in dogs, similar criteria for use in canine echocardiography have not been established, not even for the CKCS [89-91]. The lack of objective or quantitative echocardiographic criteria has hampered systematic investigation of the relationships between structural mitral valve abnormalities and clinical assessments of disease severity. Additionally, the prognostic relevance of abnormal mitral valve anatomy associated with MMVD in dogs has not been fully evaluated, particularly in CKCS. In humans, mitral valve leaflets measuring 5 mm or greater in width during diastole are known to be a predictor of complications associated with MMVD [92]. The prognostic relevance of systolic thickness of the anterior mitral valve leaflet has been investigated in Dachshunds, but this variable is not predictive of increases in the left atrial and left ventricular end diastolic diameters over a 3-year period [93]. Further study of prognostic indicators related to mitral valve anatomy in dogs, and in CKCS, is needed.

The image plane most utilized for evaluation of the mitral valve in people, especially regarding diagnosis of mitral valve prolapse, is the parasternal long axis view. This view corresponds most closely to the veterinary image plane known as the right parasternal long-axis left

ventricular outflow view, which enables visualization of the aorta in addition to the left atrium, left ventricle and mitral valve leaflets [94]. The right parasternal long-axis 4-chamber view, though not a standard image plane in human cardiology, is also considered a standard echocardiographic view for assessment of the canine mitral valve [94-96]. Both views provide visualization of the mitral valve apparatus; however, the optimal image plane for assessment of the mitral valve in dogs has not been critically assessed.

As reported by Wesselowski et al., measurements of the anterior mitral valve leaflet and the mitral valve annulus in the dog can be indexed to body weight based on the allometric relationship between mitral valve dimensions and body size. Reference intervals have been proposed over a range of body sizes. Relative to normal dogs, the diameter of the mitral valve annulus as well as the thickness, length and area of the anterior mitral valve leaflet are greater in patients with advanced MMVD [97]. These data are not available specifically for CKCS.

Current classification of the severity of MMVD in dogs is based on both clinical signs and identification of cardiac remodeling by radiographs and echocardiography [43]. In humans, classification of the severity of MMVD is mainly based on echocardiographic criteria with particular attention paid to quantification of MR [98-100]. In fact, in humans, the severity of MR is associated with prognosis in patients with MMVD [99,101]. It is unclear whether the severity of MR is an independent predictor of survival in dogs with MMVD, but several studies indirectly suggest that this is also true in this species [49,97,102]. One of the main factors affecting MR severity is the size of the orifice through which MR occurs [103,104]. Cardiac magnetic resonance imaging is considered the gold standard to assess the mitral regurgitant orifice in humans, but this technique has several limitations in veterinary medicine. These include a requirement for general anesthesia, time, and specific personnel and equipment, therefore resulting in elevated costs and poor applicability in clinical settings. Two-dimensional echocardiography (2DE), in combination with Doppler-based techniques is currently the

method of choice for non-invasive evaluation of MR in both humans and dogs [98,102,103,105]. However, obtaining information about the size of the orifice using 2DE and Doppler techniques can be time-consuming, and is demonstrated to suffer from limitations that could arise from misalignment of the scanning plane with the regurgitant jet, and shape and direction of the MR jet [103]. It is important to know that color flow Doppler echocardiography is a semiquantitative estimate influenced by factors such as the echocardiographic equipment and technical settings [106]. However, other methods of estimating MR size, such as calculation of the effective regurgitant orifice area (EROA) using the proximal isovelocity surface area (PISA) or vena contracta, are not always possible to achieve in mild cases of MR [105]. In humans, measuring the area of the regurgitant orifice during systole, i.e., planimetry of the anatomic regurgitant orifice area (AROA), using RT–3DTTE, has proved to be a feasible and accurate way to assess MR [104,107]. Moreover, analysis of the AROA has demonstrated several advantages compared with other conventional techniques as it is relatively rapid and non-invasive [98,104,105]. To the best of our knowledge, no attempt has been made in veterinary medicine to calculate the AROA using RT–3DTTE in dogs affected by MMVD, particularly in CKCS.

Furthermore, regarding CKCS, interestingly, the presence of echocardiographic intermittent MR is associated with increased cardiac mortality [106]. The nature of intermittent MR is generally poorly characterized, and the clinical importance of this phenomenon has not previously been studied in dogs and has not been described in human patients [108-109].

3.4. Early biomarkers of myxomatous mitral valve disease

3.4.1. miRNAs

In recent years there has been a significant development of new diagnostic methods dedicated to the diagnosis of animal diseases and to a better understanding of their underlying mechanisms. Genetics is a very important aspect of dog breeding, and another significant reason why we have observed a great interest in the development of new genetic and genomic markers of specific diseases. In veterinary cardiology, one group of the new markers could be miRNAs. MicroRNAs are defined as 21–25 nucleotide single-stranded RNAs (ssRNAs), which are produced from hairpin-shaped precursors [110]. miRNA transcripts are then processed after their synthesis. In recent years, there have been significant efforts to investigate the processing of miRNAs in animals. Genes for miRNAs are transcribed to a primary miRNA (pri-miRNA). The pri-miRNA is processed within the nucleus to a precursor miRNA (pre-miRNA) by Drosha, a class 2 RNase III enzyme. Next, the transport of pre-miRNAs to the cytoplasm is mediated by exportin-5 (EXP-5). In the cytoplasm, they are further processed to become mature miRNAs by Dicer, an RNase III type protein and loaded onto the Argonaute (ago) protein to produce the effector RNA-induced silencing complex (RISC).

miRNAs have key roles in the regulation of distinct processes in mammals. They provide a key and powerful tool in gene regulation and thus a potential novel class of therapeutic targets. They play an evolutionarily conserved developmental role and diverse physiological functions in animal and largely exhibit limited complementarity with their target mRNAs in animals, but this is still sufficient to regulate several physiological processes. It has been suggested that they repress the initiation step of the translation process, which may be followed by mRNA degradation [111].

Several studies have reported miRNAs in platelets, nucleated blood cells, erythrocytes and in plasma. Moreover, plasma miRNAs were found to be unexpectedly stable even under

conditions such as long-time storage at room temperature and low or high pH, whereas exogenously added synthetic miRNAs are quickly degraded because of RNAse activity in the plasma [112,113]. This means that endogenous miRNAs present in plasma must be shielded in some way from degradation. Recent studies have shown that miRNAs are packaged in lipid vesicles (exosomes, microvesicles, apoptotic bodies) or associated with RNA-binding proteins or lipoprotein complexes [114-118]. These properties make miRNAs ideal stable novel diagnostic biomarkers, which can be measured in easily accessible samples and can be used to diagnose different pathological conditions. Plasma and serum miRNAs are currently being intensively investigated and specific miRNA expression patterns are being reported for various pathological conditions in humans [119-122]. The results of recent studies show that circulating miRNAs are potential biomarkers for the detection of cancer and heart disease [119-125]. However, in veterinary medicine the situation is quite different. There are few reports about circulating miRNAs research in animal diseases and particularly in dogs with heart disease [126-129].

Hulanicka et al., in 2014 [126], analyzed the expression of 9 miRNAs described in the literature as being involved in cardiovascular pathology in the plasma of Dachshunds suffering from MMVD. Dogs included in the study were assigned to ACVIM B (n = 8), ACVIM C (n = 8) or ACVIM A–unaffected control group (n = 8) according to the ACVIM classification [126]. Expression analysis using the Real-time PCR method revealed that two out of nine miRNAs were significantly downregulated: the expression of miR-30b differed between ACVIM stage B and stage A (control) dogs; the expression of mi-133b distinguished between ACVIM stage C and stage A dogs. 5 miRNAs (miR-125, miR-126, miR-21, miR-29b and miR-30b) showed a trend of downregulation in the ACVIM C group, whereas the levels of miR-423 were the same in healthy and diseased dogs [126]. Expression of miR-208a and 208b was not detected. The Authors concluded that miR-30b could be a potential biomarker of ACVIM stage B heart

failure in Dachshunds with MMVD and miR-133b could be a potential biomarker of ACVIM stage C [126].

The study of Li et al., in 2015 [127], investigated the miRNA expression profile in dogs with MMVD. 277 miRNAs were quantified using RT-qPCR from six normal dogs (ACVIM A), six dogs with MMVD mild to moderate cardiac enlargement (ACVIM Stage B1/B2) and six dogs with MMVD and congestive heart failure (ACVIM Stage C/D) [127]. Eleven miRNAs were differentially expressed. Dogs in Stage B1/B2 or C/D had four upregulated miRNAs, including three cfa-let-7/cfa-miR-98 family members, while seven others were downregulated, compared to Stage A. Expression of six of the 11 miRNAs were also significantly different between dogs in Stage C/D and those in Stage B1/B2. The expression changes were greater as disease severity increased. The Authors concluded that these miRNAs may be candidates for novel biomarkers and may provide insights into genetic regulatory pathways in canine MMVD [127]. Dogs and humans share some similarities in MMVD, including degenerative valvular structure, expression patterns of extracellular matrix proteins, and some common signaling pathways [83,127]. Therefore, results from this study, including changes in the cfa-let-7/cfa-miR-98 family members, may be relevant to the study of human MMVD [127].

Yang et al., in 2017 [128], studied the association between circulating exosomal miRNA (ex-miRNA) content and MMVD, congestive heart failure due to MMVD (MMVD-CHF) and ageing, which is strongly associated with MMVD. Ex-miRNA was isolated from old normal/healthy dogs (n = 6), young normal dogs (n = 7), dogs with MMVD (n = 7) and dogs with MMVD-CHF (n = 7). Separately, total plasma miRNA was isolated from normal dogs (n = 8), dogs with MMVD (n = 8) and dogs with MMVD-CHF (n = 11) [128]. Using qRT-PCR, exosomal miR-181c ($p = 0.003$) and miR-495 ($p = 0.0001$) significantly increased in dogs with MMVD-CHF compared to the other three groups [128]. Exosomal miR-9 ($p = 0.002$) increased in dogs with MMVD, and MMVD-CHF compared to age-matched (old) normal dogs [128].

Exosomal miR-599 ($p = 0.002$) decreased in dogs with MMVD compared to old normal dogs. In total plasma, 58 miRNAs were deemed significantly different ($p < 0.04$) between normal dogs, dogs with MMVD and dogs with MMVD-CHF. However, in contrast to ex-miRNA, none of the miRNA in total plasma remained statistically significant if the false discovery rate was <15% [128]. Changes in ex-miRNAs were observed in dogs as they age (miR-9, miR-495 and miR-599), develop MMVD (miR-9 and miR-599) and progress from MMVD to CHF (miR-181c and miR-495). Ex-miRNA expression-level changes appear to be more specific to disease states than total plasma miRNAs [128]. The present observations suggest that dysregulation of miR-9 and miR-599 chronologically precedes CHF, suggesting that these miRNAs warrant further investigation as putative initiating factors for CHF, while others were found only at the time of CHF (miR-181c and miR-495), which denotes a role in initiation, progression, or consequence of CHF. The present data are inconclusive with respect to causality but permit novel hypotheses to be advanced concerning the epigenetic mechanisms of MMVD, left heart enlargement and progression to heart failure [128]. Jung et al., in 2017 [129], characterized the expression profiles of circulating miRNAs via genome-wide sequencing for dogs with CHF due to MMVD. The study involved 9 healthy client-owned dogs and 8 age-matched client-owned dogs with CHF secondary to MMVD. Blood samples were collected before administering cardiac medications for the management of CHF [129]. Isolated microRNAs from plasma were classified into microRNA libraries and subjected to next-generation sequencing (NGS) for genome-wide sequencing analysis and quantification of circulating microRNAs [129]. qRT-PCR assays were used to validate expression profiles of differentially expressed circulating microRNAs identified from NGS analysis of dogs with CHF [129]. 326 microRNAs were identified with NGS analysis. Hierarchical analysis revealed distinct expression patterns of circulating microRNAs between healthy dogs and dogs with CHF. Results of qRT-PCR assays confirmed up-regulation of 4 microRNAs (miR-133, miR-1, miR-let-7e, and miR-125) and

downregulation of 4 selected microRNAs (miR-30c, miR-128, miR-142, and miR-423) [129]. Results of qRT-PCR assays were highly correlated with NGS data and supported the specificity of circulating microRNA expression profiles in dogs with CHF secondary to MMVD. These results suggested that circulating microRNA expression patterns were unique and could serve as molecular biomarkers of CHF in dogs with MMVD. miRNAs also play an important role in mediating transcriptional changes observed in humans with CHF [129]. This raises the possibility that microRNAs may potentially serve as molecular biomarkers for CHF in dogs and that altered expression of microRNAs may characterize different stages of heart disease. A distinct and validated panel of circulating microRNAs may serve as a predictor for the risk of CHF in dogs. However, it is possible that the expression profile of microRNAs identified in the present study may have been specific for small-breed dogs and MMVD. Effects of various types and stages of heart disease, biological variability, medical treatment, or other confounding cardiopulmonary risk factors on miRNA expression profiles will need to be investigated to overcome diagnostic limitations on the use of circulating microRNA concentrations in dogs [129]. In the study reported here, microRNA expression patterns were distinct in dogs with CHF, compared with the expression patterns for healthy dogs. However, before wide clinical application, additional studies are needed to assess the predictive value with regard to identifying the canine patients that are likely to develop CHF by comparing microRNA profiles at different stages of heart disease. Quantification of circulating miRNAs can be challenging because of the lack of proper endogenous control miRNAs for normalization. Expression of several endogenous circulating miRNAs can change because of cardiovascular disease or other risk factors [123]. Preliminary experiments conducted by Jung's research group with SNORD95 and RNU6-2 as endogenous circulating miRNAs failed to reveal accuracy in quantitative profiles (unpublished data) [129]. Caenorhabditis elegans miR-39 lacks sequence homology with canine miRNAs. An alternative approach with C elegans miR-39 as a control

sample has successfully yielded microRNA expression profiles consistent with NGS data [124-125]. Absolute quantification via the NGS platform has yielded significant differential expression of 93 miRNAs, with custom-designed primers specific for canine miRNAs for qRT-PCR assays. Small sample size and biological variability in microRNA copy numbers for each dog could have affected significant differences in the study reported here. The use of NGS enabled comprehensive analysis of sequencing and quantification of miRNAs as an epigenetic regulator of heart disease. Data for the present study suggested that microRNA expression profiles were distinct for dogs with CHF, compared with the expression profiles for clinically normal dogs. Further validation of circulating microRNA expression profiles may lead to the discovery of novel diagnostic biomarkers for CHF in dogs. Circulating miRNAs may help guide treatment and lead to improved outcomes in dogs with heart disease. Based on what has been published in the literature about miRNAs and MMVD in dogs, the need arises to create studies with a greater number of subjects belonging to the same breed. We must also try to classify patients unambiguously and correctly, focusing mainly on those very inhomogeneous ACVIM classes, such as the B1 and B2 classes.

3.4.2. Natriuretic peptides

After discussing miRNAs, which, as we have seen, can be considered as new early biomarkers for MMVD in dogs, it is correct to point out that several studies have investigated concentrations of other biomarkers in dogs at different stages of MMVD, such as natriuretic peptides (NPs). NPs are used as indicators of cardiac health in humans and have been suggested to be of diagnostic and prognostic value in cardiac disease in dogs. B-type natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NTproBNP) tests are currently widely used as diagnostic and prognostic tools for CHF and left ventricular dysfunction in acute myocardial infarction in humans [130]. Both BNP and atrial natriuretic peptide (ANP) are released by myocardial tissue in response to volume or pressure overload and consecutive

increased ventricular and atrial wall stretch [131-132]. In circulation, BNP and ANP increase natriuresis and diuresis, and decrease systemic vascular resistance [133]. BNP is synthesized as proBNP and further cleaved into BNP and the biological inactive Nterminal end NT-proBNP [134]. ANP is encoded as a 126- amino acid precursor molecule, which is cleaved into its 98-amino acid fragment amino-terminal pro-atrial natriuretic peptide (NT-proANP) and ANP, similar to BNP. NT-proANP is further cleaved into the 3 molecules proANP 1-30, proANP 31-67, and proANP 68-98 [135], which all appear to have physiologic functions like those of ANP [136], while some authors do not describe any biological activity [137]. Importantly, the half-lives of NT-proBNP and NT-proANP fragments are significantly longer than those of BNP and ANP, which make them preferable as diagnostic markers [138,139]. Furthermore, studies in humans suggest that proANP is more sensitive compared to ANP in detecting mild increases in atrial filling pressures and that proANP is the most sensitive marker among ANP, BNP, C-type NP, and NTproBNP in discriminating New York Heart Association (NYHA) Class I individuals (patients with no limitations of physical activity) from healthy individuals [140,141]. Interestingly, NP concentrations in people seem to be dependent on body mass index (BMI), age, and androgen levels [142-144], but no consistent veterinary reports about age-, gender- and weight related variations in healthy dogs exist [145-148]. So far, there has been no consensus regarding the diagnostic power of NP concentrations to discriminate dogs without cardiac disease from dogs with subclinical stages of MMVD, and to differentiate between dogs in different asymptomatic stages [149-153]. Due to the low sensitivity and specificity of the calculated cut-offs, NP measurements are not useful to differentiate between healthy and asymptomatic patients with MMVD. NP testing could be useful to monitor disease progression, as NP concentrations increase with severity of disease. Serial testing of NPs might be useful for monitoring disease progress and identifying patients with clinically significant heart disease;, although the reported weekly or daily variability of NPs might be a limiting factor [154]. An

interesting and potentially clinically relevant result obtained by Wolf et al. in 2013 is the statistically significant differences in NT-proBNP and proANP concentrations between intact male and female dogs [155]. These results agree with several studies in humans, where BNP, ANP, and NT-proBNP concentrations were significantly higher in women than in men [156-158]. The reason for these results is not completely clear. One study suggests that androgens are mediating sex-related differences in BNP and NT-proBNP levels [159]. In Wolf's study no difference in NP concentrations between different body size and age groups are described, which is consistent with findings in other canine studies [145,146,154]. However, in one study including only healthy CKCS, NT-proANP was lower in larger breeds and higher in older dogs [147], and in another study, a population of overweight dogs had significantly lower NT-proANP concentrations than controls with normal BMI [160]. Also, a study in Doberman Pinschers showed significantly increased NT-proBNP concentration in dogs > 8 years of age [161]. These results are like those of human studies, where NP levels decrease with increasing BMI and rise in older populations [156,162]. These discrepancies might be explained by the fact that the dogs were classified according to their absolute weight and age. In summary, NT-proBNP and proANP could differentiate dogs with CHF from patients with MMVD not yet affected by CHF. However, due to low sensitivity and specificity, NT-proBNP and proANP testing is not useful to detect asymptomatic dogs with MMVD, and to differentiate ACVIM stages B1 and B2 in asymptomatic dogs. A strong association was found between plasma NT-proBNP concentration and clinical progression only in presumed stage B dogs [163]. Furthermore, significant differences between healthy intact male and female dogs suggest that sex-specific reference intervals should be determined to help interpret concentrations of NTproBNP and proANP.

3.4.3. Cardiac troponin I

Recent research in dogs with MMVD has investigated the clinical utility of the cardiac biomarker cardiac troponin I (cTnI) [164-167]. Cardiac troponin-I is an intracellular myocyte protein that has been established as a marker of myocyte injury [168], although it provides no information on the cause of injury or the mechanism of troponin release [169]. Conventionally, the cardiac troponins have been used for diagnosis of acute myocardial infarction in humans and have become the gold standard biomarkers for this indication. They have become increasingly recognized as an objective measure of cardiomyocyte status in both cardiac and noncardiac disease, supplying additional information to that provided by echocardiography and electrocardiography (ECG). Injury to cardiomyocytes can occur through a variety of mechanisms with subsequent release of troponins [169,170]. Irrespective of the underlying disease or the mechanism of troponin release, the presence of myocardial injury is associated with an increased risk of death. As increasingly sensitive assays are introduced, the frequent occurrence of myocardial injury is becoming apparent, and our understanding of its causes and importance is constantly evolving. Presently, troponins are valuable for detecting a subgroup of patients with higher risk of death. Future research is needed to clarify whether troponins can serve as monitoring tools guiding treatment, whether administering more aggressive treatment to patients with evidence of myocardial injury is beneficial, and whether normalizing of troponin concentrations in patients presenting with evidence of myocardial injury is associated with reduced risk of death. In veterinary medicine, the role of cTnI in the development and evolution of canine MMVD has not yet been clarified. One study reported an increased chance of developing advanced MMVD associated with elevated cTnI concentrations, suggesting a potential value of cTnI in longitudinal evaluation in dogs. Changes in cTnI have been studied in human patients with acquired cardiac disease [165,171,172], and biologic variability has been recognized as an important routine aspect when interpreting serial changes in biomarker

concentrations [173]. The accurate assessment of changes in cTnI concentrations in both healthy people and those with cardiac disease relies in part on knowledge of the biological variability, while an evaluation of cardiac biomarker biological variability is limited in veterinary medicine [154,167]. Inaccurate interpretation of longitudinal changes in cTnI values may occur if biological variability is not considered. Elevations of cTnI concentration of > 134% in healthy dogs and > 110% in dogs with MMVD may indicate the onset or progression of disease, warranting further clinical evaluation. However, more studies are needed, especially because human ischemic cardiomyopathy and canine MMVD have very different pathophysiological mechanisms. Furthermore, as reported by Borgarelli et al. for a population of asymptomatic dogs affected by MMVD, cTnI levels are not affected by treatment, confirming that there is no significant underlying myocardial damage during the preclinical phase of MMVD [164,174,175].

3.4.4. Urinary aldosterone to creatinine ratio

The renin angiotensin aldosterone system (RAAS) represents an important compensatory mechanism of heart failure. However, a chronic activation is maladaptive and contributes to the development of cardiovascular remodeling and congestive pattern because of the harmful cardiovascular and renal effects of angiotensin II (A-II) and aldosterone [176-184]. Indeed, higher aldosterone levels have been associated with cardiac remodeling and worse outcomes in humans with heart diseases [182,185-192], and urinary aldosterone (UAldo) concentration has appeared to be associated with greater ventricular remodeling and a worse prognosis in dogs with MMVD [193-194]. Moreover, the beneficial effects of angiotensin-converting enzyme inhibitors (ACEI) and spironolactone in patients with CHF have been shown in both species, indirectly proving the negative impact of chronic RAAS stimulation [177,195-198]. The trend of RAAS activity during MMVD is still uncertain. While it is fairly well established that RAAS is overstimulated after the onset of CHF secondary to various heart diseases [178,199-201],

there are conflicting data about the neurohormonal activation during the asymptomatic phase of MMVD [175,176,178,193,202-210]. Accordingly, whereas the administration of RAAS blockers (e.g., ACEI, spironolactone) is recommended in ACVIM stage C and D, their use in pre-clinical MMVD is still subject to debate [43,177]. Moreover, the aldosterone breakthrough (ABT) phenomenon suggests the possibility of RAAS overexpression even after the beginning of ACEI therapy [211-2012]. Therefore, the assessment of RAAS activity in course of MMVD could help optimize the follow-up and the therapeutic management of the patient. The urinary aldosterone-to-creatinine ratio (UAldo:C) seems to be a very useful parameter for the monitoring of RAAS activity in clinical practice. It has been proven to reflect RAAS activation and to be comparable to 24 h urinary aldosterone excretion, which, unlike serum/plasma aldosterone, is not affected by the pulsatile variations of aldosterone secretion. Secondly, it can be easily determined from a single "free-catch" urine sample, thus avoiding blood sampling and reducing the stress of in-hospital visits [212-213]. Moreover, compared to other RAAS components, aldosterone has the advantage of being the last effector of the cascade. Thus, its assessment also considers the alternative pathways of RAAS, such as the angiotensin-converting enzyme (ACE) or A-II independent ones [214].

It has been demonstrated that Ualdo:C is not significantly different among healthy, ACVIM stage B1, stage B2 and stage C dogs [176]. This parameter appeared to be influenced by individual factors, such as breed, sex and age, and therapy has probably added further variability [176]. This means that the use of median values of UAldo:C to interpret the RAAS activity of a single patient or of a specific MMVD stage might be misleading [176]. An individual monitoring of this parameter may be more appropriate and would help clarify its real diagnostic and prognostic value in dogs affected by MMVD [176].

Furthermore, UAldo:C is positively associated with LA/Ao, sustaining the mutual relationship between RAAS and cardiac remodeling and suggesting a possible role of UAldo:C as a marker of MMVD progression [176].

However, data about UAldo:C in healthy and MMVD populations are still not sufficiently consolidated and should be expanded before introducing it into the diagnostic routine.

3.4.5. Symmetric dimethylarginine

Symmetric dimethylarginine (SDMA) is a serum biomarker of renal damage in dogs. Moreover, SDMA concentration is an independent predictor of the development of severe heart failure in humans with cardiac disease. According to the ACVIM guidelines, the administration of certain cardiac drugs, such as pimobendan and spironolactone, has clinical usefulness for dogs in ACVIM stage B2, but the administration of different drugs, including diuretics, ACEI, pimobendan and spironolactone is strongly recommended for dogs in stage C and D [175,215-218]. Before starting and pursuing a long-term diuretic therapy, renal function should always be assessed by measuring renal parameters and electrolyte concentrations [220]. Indeed, renal dysfunction is common in older dogs such as those affected by MMVD [221]. In humans, the concomitant renal and cardiac dysfunction, whereby the dysfunction of one organ induces dysfunction of the other, is defined as cardiorenal syndrome (CRS) [222]. This condition has also gained interest in veterinary medicine and in 2015 a consensus group published the definition and classification of this condition in dogs and cats, that has been renamed as “cardiovascular-renal disorders” [223].

Chronic kidney disease (CKD) has a prevalence of about 60% in humans with CHF [224]. In dogs, the prevalence of CKD seems higher in those affected by MMVD compared to the aged-matched population [225], and the severity of renal impairment increases with the severity of heart disease [221]. Renal disorders are associated with a poor prognosis in their later stages, thus making their early diagnosis extremely important for treatment and to prevent progression

[226]. According to the International Renal Interest Society (IRIS), serum creatinine (sCr) and SDMA are the standard biomarkers used to diagnose CKD and grade its severity in dogs [227]. SDMA is a product of arginine methylation and is mainly excreted by the kidneys [228]. This parameter has been validated in dogs and cats and is now routinely available [229-230]. SDMA is inversely correlated with glomerular filtration rate (GFR) in both humans and dogs and is a valuable biomarker for early detection of kidney failure [231]. Moreover, its plasma concentration increases earlier than that of sCr and is less affected by extra-renal factors (e.g., age, body weight and muscular mass) [232-234].

In humans, SDMA gradually increases with the progression of heart disease and is an independent predictor of the development of severe HF; moreover, it is associated with a worse outcome and mortality [235]. In dogs with MMVD, two studies evaluated SDMA as a biomarker of heart disease severity using an “old” clinical classification or the less recent and stringent ACVIM guidelines [236,237].

The results of a study published by Valente et al. (2020) failed to demonstrate that renal function, evaluated by measuring serum SDMA concentration, is significantly impaired in dogs with MMVD [238]. Although some dogs in the ACVIM C+D group of MMVD had an increased concentration of the variables used to identify renal dysfunction, this was most likely due to pre-renal azotemia instead of representing a feature of the CRS described in humans. Furthermore, no correlation was found between SDMA concentration and radiographic and echocardiographic parameters associated with increased MMVD severity [238].

3.5. Prognosis in Cavalier King Charles spaniels

MMVD is a progressive disease with a slow onset of clinical signs, and many affected animals die of unrelated diseases. Many studies have reported survival time and prognostic indicators in dogs with MMVD. However, these studies were focused mainly on specific breeds and did not include large breed dogs [34,57,58,93,94,239,240] or focused on specific aspects of the disease, such as influence on survival after chordal rupture [241] or effect of therapy on survival time [196,242-244].

Borgarelli et al. in 2008, documented the long-term outcome and the influence of clinical and echocardiographic variables on survival in a large population of dogs of different breeds and weight affected by MMVD, demonstrating that about 70% of included dogs survived or died due to causes unrelated to MMVD during the observation period [244]. This finding indicates that MMVD is a comparably benign condition, as previously reported in both humans and dogs [242,245-250].

The literature reports a median survival time (MST) of between 5 and 14 months once CHF develops [25,243-245].

The knowledge of MST and prognostic factors could assist clinicians in communicating to the owners the prognosis of dogs with advanced heart failure secondary to MMVD [251]. It is important to understand the long-term outcome and the relations of some clinical, echocardiographic variables and therapeutic schemes on survival in a large series of dogs [251].

Previously, studies have proposed several clinical and echocardiographic prognostic indicators affecting survival in different breeds [108,252-254]. However, many of these have focused on severe classes of MMVD and it has been found that many of the echocardiographic variables do not change drastically until late stages of the disease [47].

In CKCS, as stated before, MMVD is associated with earlier onset and thus with potentially greater cardiac morbidity and mortality compared to other breeds [31,37,62,66]. The prevalence

and evolution of MMVD was studied in CKCS by Häggström et al. in 1992 [66]. Twenty eight percent of the included dogs which had previously had murmurs had been euthanized for signs of CHF, whereas none of the dogs which had been free of murmurs had died from CHF. Swedish animal insurance statistics from 1982 to 1990 (1983 excluded) for dogs less than 10 years old showed that claims for veterinary care or death or euthanasia were five times more common in the CKCS than in Dachshunds and eight times more common than the mean for all other insured breeds [66]. Nevertheless, the preclinical period often varies markedly between CKCS, making it challenging for clinicians to identify dogs that will eventually succumb because of the disease [113,244]. Identification of this subpopulation of dogs at high risk of developing CHF, preferably at an early age, would have several advantages such as more targeted monitoring, and owner education on recognition of clinical signs. As studies indicate an inherited component of the disease in at least some of the highly susceptible breeds, early predictors would also allow for improved breeding recommendations [41,42,44,51,54]. In addition, unnecessary owner concern regarding a short lifespan might be avoided for low-risk dogs [244]. Consequently, there is great interest in identifying the risk of factors and the prognostic indicators associated with onset, progression, and survival of CKCS affected by MMVD.

Very few studies have been conducted to investigate the prognostic factors at early stages of MMVD in CKCS. Reimann et al., in 2017, examined the predictive value of selected clinical and echocardiographic variables associated with MMVD in CKCS younger than 3 years and compared long-term cardiac mortality versus all-causes [106]. The results of this study show that the presence of more than mild MR, even if intermittent, on echocardiography in CKCS aged 1–3 years is associated with increased risk of CD. This is especially true for male dogs [106].

Furthermore, even though MMVD is generally considered a relatively benign condition, a relatively large proportion (more than one-fourth) of the CKCS deaths are due to cardiac causes [106]. More severe MR at echocardiographic examination at a young age is associated with increased cardiac mortality in CKCS [106]. This is not true if the MR diagnosis is based on murmur intensity on auscultation, underlining the importance of the echocardiography assessment of MR [29,106,254].

Intermittent, but not mild MR is associated with cardiac mortality, although the intermittent MR is mild in most heart beats. Reimann et al. hypothesized that the single heart beats with more severe MR mediate valvular shear stress, influencings disease progression, or that the atrium cannot handle the more severe MRs that suddenly occur [106]. Moreover, intermittent MR is associated with variations in R–R interval and cardiac mortality [109] and might represent moderate or severe MR masked by a variation in R–R interval or heart rate. These hypotheses would imply that intermittent MRs should be considered as serious as the maximal MR observed instead of the MR that is most often observed. Thus, intermittent MR or intermittent murmurs might be warning signs that warrant more close surveillance of a patient. It is unknown whether the size of the intermittent MR is important in relation to progression of MMVD, and whether intermittent MR is an early manifestation of progression to more advanced MR in this breed. Obviously, further studies are needed to clarify the importance of this phenomenon.

Mitral valve prolapse is also suggested to have prognostic significance [36,93,102] and should be used as an early risk factor for MMVD, predictive of cardiac death in CKCS, despite the fact that no association with survival has ever been discovered [106].

In addition, higher normalized left ventricular internal diameter in systole (LVIDsN) (and lower fractional shortening - FS%) is strongly associated with all-causes and cardiac mortality in CKCS [106].

Even though MMVD is considered to be a relatively benign condition, Reimann et al. referred a large proportion of deaths due to cardiac causes (26%) [106]. In this study, lifetime could not be estimated as dogs were included at 3 years old, meaning that the lifetime would be overestimated as dogs dying before 3 years of age were excluded. Moreover, cardiac mortality does not begin to increase before the age of 7 years and many dogs survive longer [106, 254-258] (Fig 7). In addition, Reimann stated that dogs with moderate to severe, even intermittent, MR had an increased risk of death and that this was especially true for male dogs [106]. This is in accordance with previous studies indicating that MMVD in males is more common and progresses more rapidly [41,44,259].

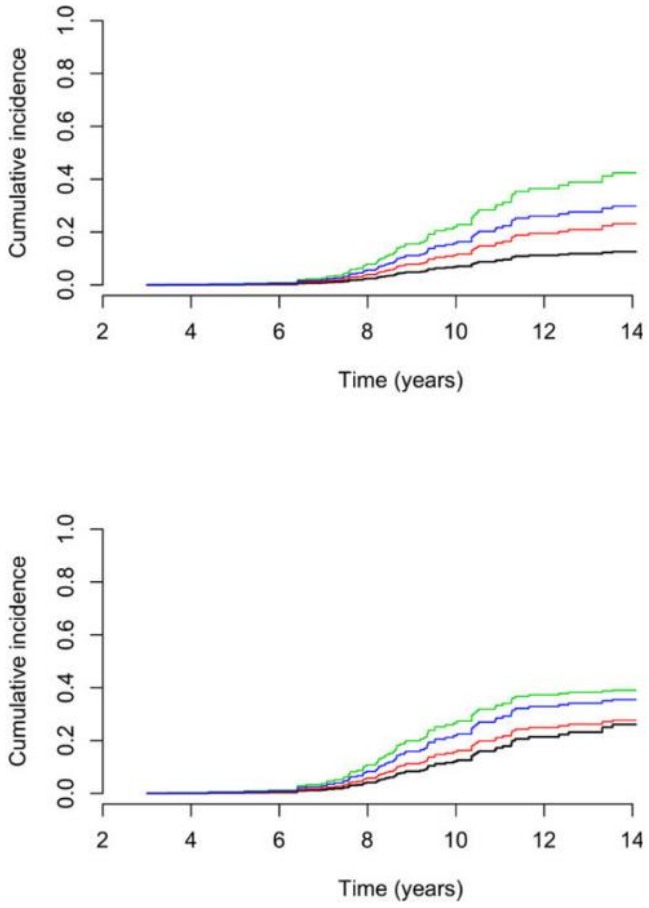

Figure 7. Cumulative incidence curves for cardiac death given for each combination of sex (top = females, bottom = males) and mitral regurgitation (MR) group where the black curve represents no MR = 0%, the red curve represents mild MR (50%) MR, and the blue curve intermittent MR (most often mild MR but some MR jets ≥ 20%). The remaining variables are set at their median values (LVIDsN = 0.93, LA/Ao = 1.2, and HR = 120) [106].

Furthermore, data suggested (Fig 8) that males have a trend toward higher all-cause mortality rate, with approximately 60% of male CKCS dying before the age of 10 years compared to only approximately 50% of the females [106].

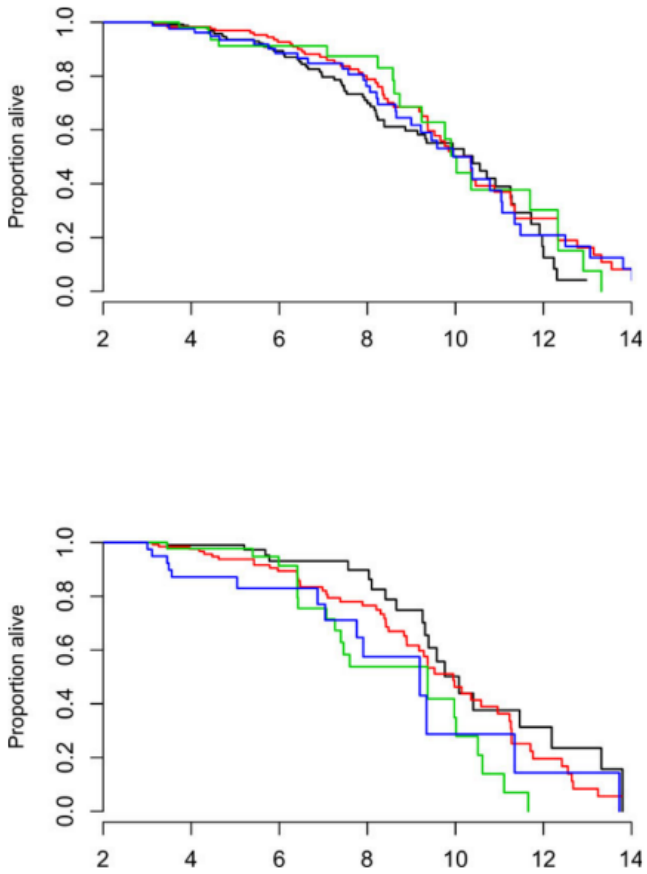

Figure 8. Kaplan-Meier curves for all-cause mortality given for each combination of sex (top = females, bottom = males) and mitral regurgitation (MR) group where the black curve represents no MR = 0%, the red curve represents mild MR (50%) MR, and the blue represents intermittent MR (most often mild MR but some MR jets ≥20%) [106].

Unquestionably, it is clear is that further prospective studies are needed to clarify the presence and significance of intermittent MR in CKCS, in order to relate them to the MST of the subjects. With this PhD thesis, through a prospective clinical study, based on what is already known about MMVD in the CKCS, we have attempted to lay the basis for this objective.

3.6. References of literature review

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4. Brief background

4.1. Radiographic study

In CKCS, thoracic radiographs are frequently used to screen for evidence of left-sided cardiomegaly secondary to MMVD. Only normal values of VHS in CKCS have been investigated and are reported to be higher than non-breed-specific reference ranges.

VLAS, M-VLAS, and RLAD have recently been proposed as new radiographic methods for quantifying left atrial size in dogs, but no published studies have evaluated these parameters in different breeds, and not even in the CKCS. The 2019 ACVIM MMVD consensus statement recommends, if available, the use of breed-specific radiographic normal values and this is the reason why in this PhD project this radiographic study has been included (Appendix 9.1. page 98).

4.2. Genetic study

In some breeds, such as CKCS, the polygenic hereditary component plays a predominant role in the pathogenesis of MMVD. Moreover, early-onset MMVD, typically found in CKCS, also appears to be highly heritable.

The peculiarities of the breed structure of dogs (e.g., a small breeding population and the use of popular sires) reduce the within-breed genetic variability, generally making even a small number of cases and controls useful to effectively detect genomic regions that are associated with a particular trait or disease. The literature reports several genomic studies on canine MMVD, also in CKCS.

At present, there is no genetic test for MMVD, and the breeding programs put in place in the past were not as effective as expected, especially if based only on auscultatory findings. Nevertheless, an accurate selection of animals for breeding is essential, since the high prevalence of the pathology in this breed makes the elimination of all the dogs diagnosed with MMVD from reproduction unfeasible. Discovering the genetic basis of MMVD may increase the effectiveness of breeding protocols, enabling an early identification of subjects predisposed to a severe form of this pathology. For this reason, during this PhD project a genomic study on an Italian population of CKCS was conducted. The selection of cases and controls is a crucial aspect of this study and has been based on a complete echocardiographic and genealogical examination, with the latter also being checked against the genomic data. The genomic analyses performed during this PhD project have never been used for the investigation of MMVD in CKCS. The examination of previously published data about the genes and pathway here identified enables a description of their possible role in the pathogenesis of the disease (Appendix 9.2. page 114).

4.3. Echocardiographic study

MMVD in CKCS involves almost all subjects over eleven years as well as many dogs under four years of age. To reduce the incidence of the disease in this breed, it would be advisable to distinguish CKCS in the three categories of non-genetically predisposed, predisposed to age-related MMVD (typical of many breeds), and with breed predisposition to its early onset. Up to now, there has been no genetic test able to discriminate among these three groups and for this reason, CKCS breeding plans are based only on the cardiac phenotype. However, the results of the screening programs are more promising if echocardiographic data are also considered.

Since the prevalence of MMVD is highly dependent on age, it is important to choose an age limit whereby dogs with early onset of MMVD can be excluded from reproduction. On the other hand, to place it at too advanced an age would result in the exclusion of an excessive number of breeders: it is not advisable to exclude more than 30% of dogs from the breeders for a screening program for a single disease. The identification, at a young age, of dogs at high risk of adverse outcomes in the future is desirable. Early predictors would also allow for improved breeding recommendations. For these reasons, the description of some echocardiographic valvular characteristics in CKCS could be useful in the identification of subjects predisposed to the development of the early and/or rapid form of the disease. Therefore, during this PhD program the echocardiographic features of MMVD-affected CKCS in ACVIM B1 divided according to their age at MMVD diagnosis were described with 2D echocardiography (Appendix 9.3. page 140).

4.4. Morphometric study

The same genes can affect both the dogs' body size and their heart development. Pedersen et al. (1999) showed that there is a negative correlation between body weight and MVP in CKCS. Furthermore, as stated before, MMVD is associated with earlier disease onset in this breed and with greater cardiac morbidity and mortality compared to other breeds. However, the preclinical period often varies markedly among subjects, making it challenging for clinicians to identify those that will eventually develop clinical signs. For these reasons, the early identification of a morphotype associated with a more severe MMVD can have several advantages. This could allow clinicians to monitor dogs in a very targeted way, as well as educating breeders regarding the selection of subjects without some phenotypical characteristics that are related to more severe MV degeneration and/or more rapid progression of the disease.

Only one study has evaluated the prevalence and severity of MVP in relation to the size of the thorax in dogs, particularly in Dachshunds, but no study has ever analyzed the association between MVP or MMVD severity and morphometric measurements in CKCS. For this reason, this PhD program includes a morphometric study aimed at investigating morphometric measurements in relation to the echocardiographic features of MMVD in ACVIM class B1 CKCS. This class includes most of the breeding population and is very heterogeneous from the clinical, morphological, and echocardiographic points of view and therefore this project has focused on this ACVIM class (Appendix 9.4. page 161).

4.5. miRNA study

Due to the lack of early signs, symptoms, and predictive biomarkers, an early diagnosis of MMVD is difficult. Identifying reliable specific biomarkers is desirable, especially for screening and breeding programs. Expression of miRNAs is associated with several human and veterinary disorders, including heart diseases. The dysregulation of circulating miRNAs has previously been investigated also in MMVD-affected dogs following different approaches, including quantitative real-time PCR (RT-qPCR), microarray, and next-generation sequencing (NGS). Most of the dogs enrolled in these studies were classified following ACVIM guidelines, as stage C and D, while only one study performed analysis also on dogs older than 8 years in ACVIM stage B1 and B2. For this reason, this PhD project carried out a study aimed at improving MMVD assessment in CKCS at the asymptomatic ACVIM stage B1. This was done by grouping the dogs according to their age at the time of diagnosis (younger than 3 years, between 3 and 7 years, and older than 7 years), by ascertaining whether three miRNAs, previously associated with MMVD, are modulated in the plasma of CKCS, and investigating their potential use as early biomarkers to identify asymptomatic dogs in ACVIM stage B1 (Appendix 9.5. page 188).

5. Aims

Due to the peculiarly early onset and rapid evolution of MMVD in CKCS, the identification of phenotypic and genotypic characteristics associated with more severe/rapid forms of MMVD could be useful for setting up breeding selection programs aimed at reducing the prevalence of the disease in this breed. The hypothesis of this PhD thesis is that CKCS with different genetic, echocardiographic, and morphometric patterns could have different prognostic profiles.

In particular, it would be very interesting to investigate if biomarkers, genetic pathways or morphometric characteristics in CKCS in ACVIM class B1 exist and if they can give an early prediction of the MMVD evolution in each subject, even before clinical and echocardiographic onset of the disease.

The specific aims of this PhD project are related to different aspects of MMVD in this breed and are here reported for each study:

1) Radiographic study:
    a. To describe breed-specific reference values for VHS, VLAS, M-VLAS and RLAD in a sample population of healthy adult CKCS and to compare those with the data already available from the literature.
    b. To investigate the effect of the aspects of recumbency, gender, body weight, and thoracic depth – thoracic width ratio (TD/TW) on the radiographic measurements.
2) Genetic study:
    a. To find genomic regions associated with early-onset MMVD predisposition in the CKCS.
3) Echocardiographic study:
    a. To analyze the echocardiographic features of MMVD-affected CKCS in ACVIM stage B1.

b. To provide a description of echocardiographic findings related to CKCS in different ACVIM classes.
c. To compare echocardiographic characteristics in ACVIM class B1 CKCS, allocated according to age at time of MMVD diagnosis, to highlight if different aged subjects had different echocardiographic patterns.

4) Morphometric study:
   a. To investigate morphometric measurements in relation to echocardiographic features of MMVD in ACVIM class B1 CKCS.
   b. To investigate whether the morphology of the subject can be related to the outcome and the echocardiographic appearance of the mitral valve apparatus and of the left ventricle morphology in this breed.

5) miRNA study:
   a. To identify specific miRNAs that enable us to obtain an earlier diagnosis and to predict the evolution of the disease in CKCS without clinical signs (subjects with MMVD in ACVIM B1 class allocated according to age).

6. Summary of the results

6.1. Radiographic study

The 30 CKCS included in this study (n. 22 females and n. 8 males; mean age: 2.66 ± 1.42 years; mean body weight: 7.92 ± 1.68 kg) have a lower VHS (10.08 ± 0.56) than the reference value of 10.6 ± 0.5 established by Lamb in 2001 for this breed (P = 0.002) and higher than the reference value of 9.7 ± 0.5 proposed by Buchanan and Bücheler in 1995 (P < 0.001). The VLAS, M-VLAS, and the RLAD of CKCS in our study are respectively 1.79 ± 0.3, 2.23 ± 0.44, and 1.2 ± 0.34. These are lower than the values previously reported by Malcolm (2.07± 0.25), Lamb (2.6 ± 0.3), and Salguero (1.97 ± 0.57) (P = 0.000). No differences in VHS were found between left lateral (LL) and right lateral (RL) recumbencies (P = 0.25), whereas VLAS, M-VLAS, and RLAD are significantly higher in the LL than the RL view (P < 0.001, P = 0.001, and P = 0.02, respectively). Both RL and LL VHS, VLAS, M-VLAS, and RLAD do not significantly differ between males and females (P > 0.05). All radiographic measurements do not significantly vary between different BCS groups (P > 0.05). Mean thoracic depth (TD) to thoracic width (TW) ratio is 0.91 ± 0.08. Fifteen dogs (50%) have a TD/TW ratio < 0.9 and n. 15 (50%) > 0.9. All CKCS in this study have an intermediate chest conformation (0.75 < TD/TW < 1.25). Thoracic depth to thoracic width ratio does not differ significantly between the sexes (P = 0.26). The VHS, VLAS, M-VLAS, and RLAD show no correlation with BCS (P > 0.05), and body weight (P > 0.05). Only LL VLAS show a moderately positive correlation with BCS (r = 0.38, P = 0.037). There is no significant correlation between the type of chest and VHS, VLAS, M-VLAS, and RLAD in all included dogs and no significant differences are observed for dogs with different TD/TW ratio (higher and lower than 0.9). Conversely, CKCS with TD/TW ratio lower than 0.9 have a greater LA/Ao_Sx ratio and lower sphericity index (both P = 0.001) (Appendix 9.1.2. page 114).

6.2. Genetic study

Following clinical and echocardiographic examinations, 33 not-directly-related CKCSs were selected and classified as cases (n = 16) if MMVD was present before 5 years of age or as controls (n = 17) if no or very mild MMVD was present after 5 years of age. Among the controls, two are ACVIM A and 15 ACVIM B1; fourteen cases belong to ACVIM class B1, one to class B2, and one to class D. This subset is composed of 22 females and 11 males. Mean age is 6.2 ± 2.6 years and is higher in controls (8.1 ± 0.5 years) than in cases (4.1 ± 0.5 years) ($r2 = 0.53$, $P < 0.0001$). It was possible to obtain the pedigree information of 20 out of 33 dogs (81% of cases and 41% of controls). The average relatedness coefficient (AR) and the inbreeding coefficient (F) coefficients are 0.06 and 0.01, respectively. Since pedigree information is not available for all the dogs included in the study, a genetic marker-based method for calculating inbreeding (FROH) was used. FROH is 0.24 ± 0.03 for the whole sample. There are no significant differences between FROH in cases (0.24 ± 0.04) and in controls (0.23 ± 0.02). These results demonstrate that the inbreeding of the two groups is similar and, therefore, could not influence the results of these analyses.

DNA was extracted from whole blood and genotyped with a Canine 230K SNP BeadChip instrument. Cases and controls were compared with three complementary genomic analyses (Wright's fixation index—FST, cross-population extended haplotype homozygosity—XP-EHH, and runs of homozygosity—ROH) to identify differences in terms of heterozygosity and regions of homozygosity. The top 1% single-nucleotide polymorphisms (SNPs) were selected and mapped, and the genes were thoroughly investigated.

There are ten consensus genes localized on chromosomes 3-11-14-19, partially confirming previous studies. The HEPACAM2, CDK6, and FAH genes, related to the transforming growth factor β (TGF-β) pathway and heart development, emerged from the ROH analysis (Appendix 9.2.2. page 130).

6.3. Echocardiographic study

Ninety CKCS were included: 60 females, and 30 males. Mean age is 5.67 ± 2.75 years and does not differ between males and females. Mean weight is 9.13 ± 1.94 kg and is slightly positively related to age (r2 = 0.18, P = 0.000). Males are heavier than females (P = 0.042).

Eighty-one (90%) dogs were affected by MMVD: nine (10%) in ACVIM class A, 64 (71%) in class B1, 11 (12%) in class B2, and six in class C/D (7%). In 59% of included dogs a heart murmur was detected at auscultation; heart murmur intensity is highly positively correlated with the ACVIM class (r2 = 0.75, P = 0.000) and moderately with age (r2 = 0.61, P = 0.000). The presence of a murmur does not discriminate between the A and B1 classes because in 44% of B1 subjects, a heart murmur is not audible. In 76% of dogs without a heart murmur, there is an echocardiographic diagnosis of MMVD.

Anterior mitral valve leaflet length (AMVL) is longer in class B2 (0.91, IQR_{25-75} 0.87-0.96) compared to A (0.75, IQR_{25-75} 0.66-0.79) and in classes B2 (0.91, IQR_{25-75} 0.87-0.96) and C/D (0.94, IQR_{25-75} 0.87-1) compared to class B1 (0.69, IQR_{25-75} 0.63-0.77) (P < 0.01). The same was observed for the anterior mitral width (AMVW) and area (AMVA): they are greater in classes B2 (0.19, IQR_{25-75} 0.18-0.21 and 0.13, IQR_{25-75} 0.11-0.15 respectively) and C/D (0.22, IQR_{25-75} 0.19-0.96 and 0.16, IQR_{25-75} 0.13-0.21 respectively) than in classes A (0.14, IQR_{25-75} 0.11-0.15; 0.09 and IQR_{25-75} 0.07-0.10 respectively) and B1 (0.15, IQR_{25-75} 0.12-0.17 and 0.09, IQR_{25-75} 0.07-0.11 respectively) (P < 0.01). Mitral valve annuli in diastole (MVAd) and systole (MVAs) are larger in class C/D (1.23, IQR_{25-75} 1.13-1.44 and 1.00, IQR_{25-75} 0.71-1.24 respectively) than in classes A (0.76, IQR_{25-75} 0.68-0.87 and 0.56, IQR_{25-75} 0.52-0.64 respectively) and B1 (0.85, IQR_{25-75} 0.75-0.93 and 0.62, IQR_{25-75} 0.55-0.73 respectively) (P < 0.01). There are no statistically significant differences in mitral valve measurements between the A and B1 classes and between the B2 and C/D classes (P > 0.05).

Subjects in class B1 were divided into age-related classes (age at time of MMVD diagnosis): up to 3 years (group 1), between 3 and 6 years (group 2) and over 6 years old (group 3). AMVW and AMVA are greater in group 3 (0.16 ± 0.03 and 0.10 ± 0.03 respectively) than group 1 (0.13 ± 0.02 and 0.08 ± 0.02 respectively) ($P < 0.01$). MVAd is greater in group 3 (0.90 ± 0.09) compared to group 1 (0.79 ± 0.12) and 2 (0.82 ± 0.11) ($P < 0.01$), whereas MVAs is greater in group 3 (0.69 ± 0.10) compared to group 1 only (0.62 ± 0.09) ($P < 0.01$). There are no significant differences in mitral valve measurements between different sexes in ACVIM class B1 ($P > 0.05$) (Appendix 9.3.2. page 155).

6.4. Morphometric study

The median age of the 52 dogs included is 4.16 years: 14 (27%) younger than 3 years, 25 (48%) between 3 and 6 years, and 13 (25%) older than 6 years. Eleven dogs (21.2%) are intact males, 2 (3.8%) neutered males, 34 (65.4%) intact females, and 5 (9.6%) neutered females. The median weight is 9.15 kg. Thirty-six subjects (69.2%) weigh more than the proposed breed standard (5–8 Kg). In 26 dogs (50%), no murmurs were found. There were soft murmurs (I–II/VI left apical systolic) in 20 dogs (38.5%), whereas in 6 dogs (11.5%) the murmurs were of moderate/loud intensity (III–IV/VI bilateral systolic). With reference to the 26 dogs with undetectable murmurs, 25 had mitral valve prolapse (MVP) (19 mild and 6 moderate) and 19 had mitral regurgitation (MR) (13 trivial, 3 trace, and 3 mild).

Forty-four (84.6%) dogs, 11 (21.1%) males and 33 (63.5%) females, have a height at the withers (29.20 cm, $IQR_{25\text{-}75}$ 27.78–31.58) lower than the breed standard (34–36 cm for males and 32–35 cm for females). In 6 (11.5%) subjects, the nose length is longer than the standard (3.8 cm), whereas in 45 (86.5%) it is shorter than 3.8 cm. In 36 CKCS (69.2%), the nose length is shorter than 3.5 cm and in 17 (32.7%) it is shorter than 3 cm.

The IPW analysis was performed including only 49 of the 52 subjects, due to the lack of some values for ordinal and continuous morphometric variables. The IPW analysis showed that body length ($P = 0.03$) and nose length ($P < 0.01$) have negative influences on heart murmur intensity (a shorter body length and shorter nose are associated with a higher murmur intensity). Furthermore, head length ($P < 0.001$) has a negative influence on jet size (shorter head is associated with larger jet size). However, morphometric measurements have no effects on MVP severity.

Head length has a positive influence on the anterior mitral valve length ($P < 0.001$) (a longer head is associated with a longer anterior mitral valve leaflet). The thorax width has a positive influence ($P = 0.01$) on the anterior mitral valve width, whereas thorax length has a negative

influence (P = 0.04): subjects with a larger or shorter thorax have a thicker anterior mitral valve leaflet. The variables of body length (P = 0.02), thorax width (P = 0.000), mean or papillary circumference (P < 0.001), head length (P = 0.000), and head stop angle (P = 0.000) have positive influences on mitral valve annulus in the diastole, whereas thorax height (P = 0.02), thoracic anterior or axillary circumference (P = 0.000), thoracic lower or basal circumference (P = 0.02), and nose length (P < 0.001) have a negative influence. Thorax width (P = 0.01) and head stop angle (P = 0.000) have positive influences on the mitral valve annulus in the systole, whereas thorax height (P = 0.002) have a negative influence. Anterior or axillary thoracic circumference (P = 0.01) and head length (P = 0.000) have a positive influence on the sphericity index (Appendix 9.4.2. page 180).

6.5. miRNA study

The mean age of the 44 included CKCS is 3.3 years, and the mean body weight is 8.1 Kg. Fourteen subjects (31.82%) are males, and 30 (68.18%) are females. Weight is lower in B1<3 years and A subjects compared with the B1>7 years group, whereas echocardiographic variables are not statistically different among age groups.

Small RNA was extracted from plasma, and the spike-in cel-miR-39 was quantified in all collected samples. Three miRNAs, namely miR-1-3p, miR-30b-5p, and miR-128-3p, were detected in all plasma samples. The comparative analysis shows that miR-30b-5p has a significant differential abundance in the plasma of MMVD-affected dogs compared to the healthy group (ACVIM A). If group B1 is further split according to the age of the dogs, the expression of miR-30b-5p remains significantly higher. Groups B1<3, B1 3-7, and B1>7 contain a higher level of miR-30b-5p than group A. There are no differences in the amount of miR-1-3p and miR-128-3p. The age is not correlated with the expression of analyzed miRNAs in the entire population and each age class.

The ability of miR-30b-5p to discriminate between groups A and B1 (AUC=0.793) is good. Dividing group B1 according to age, the ability to discriminate group A and group B1 <3 years (AUC=0.780) and group A and group B1 3-7 years (AUC=0.780) is good, while it is very good in discriminating group A and B1>7 years (AUC=0.822). Thus, miR-30b-5p can discriminate between healthy (stage A) and asymptomatic MMVD-affected dogs (stage B1) (Appendix 9.5.2. page 202).

7. General discussion and conclusion

7.1. Radiographic study

The radiographic study was carried out with the intent of describing the normal parameters of the atrial dimensions (VLAS, M-VLAS, and RLAD) in a population of healthy CKCS, which have never been described in the literature for this breed.

In this CKCS sample, the VHS was significantly higher than (10.08 ± 0.56) the not-breed specific reference values initially established by Buchanan and Bücheler in 1995 (9.7 ± 0.5) [6], but significantly lower than the breed standard proposed by Lamb et al. in 2001 (10.6 ± 0.5) [10]. In the study by Buchanan and Bücheler (1995) there were no significant differences between RL and LL recumbencies for VHS [6]. This is in accordance with our results. Lamb et al. in 2001 evaluated only RL view in their study with multiple breeds, but Greco et al. in 2008 reported a higher VHS value by 0.3 vertebra in RL recumbency compared to LL [10,35]. VLAS, M-VLAS and RLAD in our study were significantly higher in LL than RL view. This can be anatomically justified by a possible overlapping of the venous sinus of the cava veins, of the coronary venous sinus and of the caudal vena cava outflows.

Buchanan and Bücheler (1995) did not detect any differences in the VHS between males and females [6]. However, Lamb et al. in 2001 described lower VHS values in female dogs than in male dogs [10]. In the present study, VHS values of male and female dogs were not significantly different. A possible explanation could be that in our CKCS population there is no sexual dimorphism between male and females, as reported by breed standard (https://www.enci.it/media/2405/136.pdf) [38], whereas other studies report more variation in this breed [39].

The VLAS found in our study population is lower (1.79 ± 0.3) than the values proposed by Malcolm et al. (2.07± 0.25) and more similar to those reported by Puccinelli et al. (1.8 ± 0.2) [7,21]. The same difference from data reported by literature can be observed for M-VLAS and

RLAD (2.23 ± 0.44 versus 2.6 ± 0.3 and 1.2 ± 0.34 versus 1.97 ± 0.57 respectively) [8,9]. It is interesting to report that the control group (consisted of healthy subjects) from which normal values of VLAS and M-VLAS were derived, were composed of only 15 and 6 dogs respectively, with only one healthy CKCS included in Malcolm et al. study and no CKCS in Lam et al. study [7]. Furthermore, the control group in the study of Salguero et al. (RLAD) included only one healthy CKCS. Thus, the presence of other breeds could have raised the proposed VLAS and M-VLAS ranges and RLAD.

We must also emphasize that all the published data about VLAS, M-VLAS and RLAD reported a cut off able to discriminate among subject with or without left atrial enlargement and not the normal radiographic size of the left atrium. It is therefore likely that values obtained in our study are lower for this reason.

Findings from this study can be used as a background for future thoracic radiographic assessments in CKCS. The purpose of this study was to determine breed specific reference values for VHS, VLAS, M-VLAS and RLAD in healthy adult CKCS [6-9]. Based on literature review, this is the first study proposing the reference intervals in this breed for VLAS, M-VLAS and RLAD (Appendix 9.1.3. page 116, 9.1.5. page 120).

7.2. Genetic study

The purpose of the genetic study was to identify selection signatures that can distinguish between subjects with a diagnosis of MMVD at a very young age (before 5 years) and subjects in which this pathology may appear at an older age (after 5 years) or otherwise persist at a milder stage for a long time (after 8 years). Our results were superimposable to those found in other studies on various canine breeds [44–46]. The identification of consensus genes was reached using two independent methods that analyse different genomic characteristics: FST highlights genomic differences between groups in terms of expected heterozygosity, whereas XP-EHH is based on the comparison of regions of homozygosity that differentiate the groups. Moreover, ROH analysis on the regions surrounding these genes showed a different presence of long homozygous portions of DNA between the cases and controls. From this analysis, we observed that ROH containing the HEPACAM2 and CDK6 genes were present in 50% of cases and only 5% of controls. It could be hypothesized that these genes may be involved in predisposition to rapidly progressing MMVD rather than its early onset. A follow-up of the dogs included in the case group may clarify if the progression of the disease in subjects in which ROH were found around the genes is quicker than in subjects in which they were not present. Another possible reason is that the cases in which ROH were absent are heterozygous carriers of the allele that contributes to MMVD predisposition. KIAA1024 and FAH genes were also relevant, because ROH flanking them were found in the genome of 80% of cases but only in approximately 40% of controls. A possible explanation of the presence of controls showing a ROH around these genes is that they might have been misclassified due to the mitral valve pathophysiology, which makes it difficult to detect a real control. To better describe their possible role in MMVD onset and progression, an accurate investigation about consensus genes was performed. Some of them were shown to be directly or indirectly related to mechanisms already supposed to be involved in the disease's pathogenesis, such as the TGF-β signaling

pathway, or to processes related to heart development or functionality. The most relevant consensus genes are described below. HEPACAM2 interacts with FGFR1 (fibroblast growth factor receptor 1), which is associated with abnormal heart development. Moreover, during adult life, valves maintain a pool of mesenchymal cells responsive to FGF and producing proteoglycans, which are also increased during MMVD [47]. It should be noted that HEPACAM2 may localize on the region (CFA 14q1.3) that Madsen et al. (2011) found to be associated to MMVD [3]. FAH interacts with ADAMTSL4 (ADAMTS like 4), which is supposed to facilitate FBN1 (fibrillin 1) microfibril biogenesis [48]. FBN1 is one of candidate genes for MMVD predisposition, because it regulates TGF-β signaling and is associated with Marfan syndrome, which represents one of the syndromic forms of human mitral valve prolapse [49]. CDK6 prevents cell proliferation and negatively regulates cell differentiation but is required for the proliferation of specific cell types. Moreover, it interacts with CDKN2B (cyclin-dependent kinase inhibitor 2B), whose expression was found to be induced by TGF-β and is associated with coronary heart disease [50]. EPB41L4B promotes cellular adhesion, migration, and motility in vitro and may play a role in wound healing [51]. This gene interacts with the CASQ2 (calsequestrin 2) gene, which encodes a protein localized in cardiac muscle cells that stores calcium for muscle function [52]. Mutations in this gene cause catecholaminergic polymorphic ventricular tachycardia [53]. It is interesting to note that some genes were associated with height (CDK6 and ZRANB3) or body mass (FRRS1L, EPB41L4B, and ZRANB3) in humans. It has been well established that small dog breeds are predisposed to MMVD [54,55]. Moreover, people affected by mitral valve prolapse tend to have a low body mass index and be leaner and shorter than other individuals [56,57]. A morphometric evaluation of CKCS could allow identification if the selection for specific physical body features is related to the predisposition to the disease. In fact, the circumference of the thorax has already been negatively correlated with mitral valve prolapse in Dachshunds [8]. For the other genes able to

significantly distinguish between cases and controls, no evident correlation with MMVD was found. The main pathways associated with the genes identified by this study appear to be involved in processes related to heart development and homeostasis, as reported by several studies on humans, mice, and dogs.

The genetic study carried out in this PhD project is the first that through the careful selection of cases and controls made it possible to identify genes and pathways potentially involved in the pathogenesis of early-onset MMVD in CKCS (Appendix 9.2.3. page 137, 9.2.5. page 142).

7.3. Echocardiographic study

In the echocardiographic study, 80% of included CKCS were referred for echocardiographic screening and this may explain the high number of B1 subjects presented at visit by breeders. All symptomatic subjects were at least 7.5 years old. Despite the small number of subjects in ACVIM class B2 and above, as expected, presence and severity (ACVIM class) of the disease were positively related to the age [7,14]. Patients aging over 7 years were all affected, but the disease was also diagnosed in very young dogs (50% of those under 2 years of age, all classified as ACVIM B1). These results are consistent with those reported by other authors, which indicated valvular alterations in 67% of dogs aged from 6 months to 3 years and 95% of older dogs [7]. The weight was positively related to ACVIM class ($P = 0.013$). This result contrasts with two different studies, which observed a negative correlation [15]. However, it should be considered that in these studies the significance of the correlation was mild and in our case the weight explained only 9% of the variability of the ACVIM classification. This association was probably affected by the positive correlation between weight and age of the subjects ($r2 = 0.42$, $P = 0.000$). It is very interesting to note that 64% of the included dogs weighted over 8 kg, upper limit accepted by the ENCI standard for the CKCS [35]; however, it was not possible to distinguish overweight dogs due to a lack of evaluation of subjects' body condition score (BCS). The observation of a weight greater than the ENCI standard may be due to the recruitment also of companion subjects and not only of those bred for reproduction and exposition; however, it would be advisable to investigate this issue by increasing the sample size, to determine whether the values obtained are associated with an overweight problem or the morphology of many CKCS differs from the breed standard.

In this study, contrarily to those reported in literature [1], the disease had not a higher prevalence in males and affected males were not significantly younger than affected females. This could be justified by the higher number of females presented for breeder screening. Nevertheless,

despite the small number of subjects in ACVIM classes B2 and C/D, the risk of remodelling in affected males was higher than in females: there were more males than females in ACVIM class B2 or above and affected males showed higher values of LA/Ao and LVIDdN. As reported by Misbach et al. (2014) [31], there were no significant differences between sexes in healthy dogs. These data are also to be considered only as descriptive and not as statistically significant, given the scarcity of healthy subjects included in this study.

In 59% of the dogs, it was possible to hear a heart murmur during auscultation. Despite the association between murmur intensity and ACVIM class, the auscultation was not an eligible method for discriminating between A and B1 subjects. These data underline the importance of performing echocardiographic screening, especially in this breed. In support of this, it should be noted that the selection protocols put in place in several countries proved to be ineffective when based only on auscultatory findings, while when consider the echocardiographic data the results were more promising [11–13].

The AMVL and AMVW obtained in this study were greater than those reported by Wesselowski et al. (2015) for each ACVIM class [19]: this is in accordance with the valvular characteristics peculiar to CKCS [16]. This finding is even more relevant considering that annular measurements were consistent with Wesselowski's, underlining a mitral valve dimension proportionally greater than heart one. Furthermore, the results of this study underline how the valvular measurements varied considerably within the same class (ACVIM B1), which showed clinical and echocardiographic characteristics of extreme heterogeneity. Firstly, heart murmur severity was associated with the age of the subjects and with the severity of mitral valve morphology alterations. Secondly, annular dilation was not expected in ACVIM B1 subjects, as this group was defined by the absence of chamber enlargement, but a modification of the annulus in older dogs was observed although remaining within the standard limits. Further investigations are needed to understand if this would predict a worst evolution of the

disease. Similarly, in older B1 CKCS, a significant reduction of the SI was observed (more spherical left ventricle), although it was not associated with a significant increase in the left atrial and ventricular dimensions. This, as described by Sargent et al. in 2015 for dogs affected by MMVD at different ACVIM stages, would be predictive of cardiac mortality.

In this echocardiographic study, the description of the mitral valve anterior leaflet in each ACVIM class, and particularly in the B1 class, delineated the quantitative echocardiographic characteristics of an Italian population of CKCS affected by this pathology. This is the first study that describes measurements of the anterior mitral valve leaflet and the mitral valve annulus in the CKCS affected by MMVD at different stages. It must be highlighted that, due to the small sample size of ACVIM class A, B2 and C/D, only data regarding B1 subjects can be considered as statistically significant. Thus, data concerning A, B2 and C/D classes have to be considered as purely descriptive of the Italian population examined. Regarding the results obtained for the ACVIM class B1, the diameter of the mitral valve annulus in systole and diastole, as well as the thickness and area of the anterior mitral valve leaflet and the sphericity of left ventricle, are greater in patients with MMVD diagnosed at an advanced age, although they are not associated with a significant increase in the left atrial and ventricular dimensions. Furthermore, the results of this study underline how the valvular measurements vary considerably within the same class (ACVIM B1), which shows clinical and echocardiographic characteristics of extreme heterogeneity. Further investigations with a larger study population could help to clarify whether additional echocardiographic valvular changes can be identified in CKCS in ACVIM class B1 and an appropriate follow-up would allow us to highlight prognostic factors related to disease worsening within this ACVIM class (Appendix 9.3.3. page 162, 9.3.5. page 167).

7.4. Morphometric study

The idea of the morphometric study originated because till now very little has been discovered regarding the relationship between echocardiographic indicators of the severity of MMVD (i.e., MVP severity, jet size and indexed echocardiographic measurements) and the morphometric measures, in all breeds To the best of the authors' knowledge, this study is the first ever on the relationship between morphometric data and echocardiographic and color Doppler measures in CKCS to have been carried out.

The highlighting of any association between morphometric data, the severity of echocardiographic lesions, clinical symptoms, and evolution time of the disease will be defined by a prospective longitudinal study, and these results could be the basis.

In the present study, is clear that subjects with a larger thorax width or a shorter thorax length (more barrel-shaped) and a shorter head had thicker anterior mitral valve leaflets. Mitral valve annulus in the diastole has been observed to be larger in subjects with a smaller thorax height (reduced dorso-ventral thoracic dimension), larger thorax width, and greater mean or papillary thoracic circumference (TC2). The same was observed in subjects with shorter noses.

Regarding mitral valve annulus in systole, the results are superimposable to the diastole: the annulus has been observed to be greater in subjects with a smaller thorax height and larger thorax width. It is also interesting to underline the positive influence of anterior or ancillary thoracic circumference (TC1) and head length on the sphericity index: the ventricular shape was more spherical in subjects with a smaller TC1 and shorter head. These findings are similar to those observed for human medicine, in which the MVP is associated with an asthenic habitus, corresponding to a reduced antero-posterior thorax diameter [46,47]. Furthermore, in the study carried out by Olsen in Dachshunds [3], the thoracic circumference was found to be negatively correlated with the severity of MVP.

Obviously, from the results obtained, the authors can only speculate about the influence of some morphometric measures on the heart murmur intensity and jet size, but not the influence on the MVP. However, as observed by Olsen [3], the authors may suppose that the results obtained may indicate an echocardiographic phenotype that is more easily associated with mitral valve disease (shorter or thicker anterior mitral valve leaflets, greater mitral valve annulus in the systole and diastole, and lower sphericity index).

Only one study in CKCS had shown a negative correlation between the severity of MVP and body weight, demonstrating that smaller dogs have more severe forms of MMVD [9]. With these results, as stated before, the authors are not able to demonstrate the same; on the other hand, the association between the cranial morphology of the subjects, the severity of the heart murmur, the jet size dimension, and other valvular characteristics is still relevant. The authors observed that subjects with a shorter head are more likely to have a higher jet size. Furthermore, subjects with a shorter body and nose length have a higher heart murmur intensity. Thus, given all the results discussed, CKCS with a shorter nose and head and a more barrel-shaped thorax are likely to have worse valvular characteristics than subjects with longer and narrower skulls and bodies. This means that a brachycephalic morphotype, much more similar to the King Charles spaniel breed in cephalic morphology, is related to more severe jet size and to worse valvular characteristics. Therefore, the observations on morphotype can be useful prospectively to conduct future breeding programs (Appendix 9.4.3. page 188, 9.4.5. page 193).

7.5. miRNAs study

Although miRNAs are currently intensively investigated in human medicine because of their diagnostic potential in many different conditions, there are only few reports related to circulating miRNAs studies in dogs affected by MMVD and no study about the early diagnosis of this disease in a predisposed breed such as CKCS.

Yang and colleagues (2017) investigated the cargo of exosomes purified from the plasma of MMVD-affected dogs using an array-based approach, demonstrating that miR-9 and miR-599 were dysregulated, while no differences were detectable analyzing whole plasma [25]. The array-based approach used by the authors may have caused a high False Discovery Rate (FDR), set at 20%, thus limiting the power of detection [25]. Another study, including old dogs (range, 8.2 to 13.8 years) with congestive heart failure (CHF) secondary to MMVD (ACVIM stage C), reported that 326 miRNAs were modulated comparing healthy (ACVIM stage A) and CHF (ACVIM stage C) affected dogs; the validation step, performed by RT-qPCR, demonstrated the overexpression of miR-133, miR-1, let-7e, and miR-125, and the down expression of miR-30c, miR-128, miR-142, and miR-423 [28]. Although they focused on a group of animals affected by a severe disease with clinically detectable signs, the results appeared worthy to be further studied even in younger patients, prompting to include miR-1 and miR-128 to be included in our investigation. Based on results reported by Hulanicka and co-workers (2014) [39], who however included in the study old dogs (range, 10.17 ± 3.36 years), we identified miR-30b as a potential marker to be further investigated in a younger cohort of MMVD ACVIM stage B1 affected CKCS.

Since the molecular background of MMVD is not yet fully elucidated, the identification of any specific markers (prognostic and/or therapeutic) would be of great value and importance for identifying asymptomatic patients, especially at a young age.

This study carried out on the evaluation of plasmatic miRNA levels also demonstrated the relationship between high levels of circulating miR-30b-5p and the presence of MMVD, even in young CKCS. The concentration of miR-30b-5p is significantly upregulated in asymptomatic MMVD-affected (ACVIM stage B1) subjects compared to healthy (ACVIM stage A) dogs and its dysregulation is detectable also in young dogs (age < 3y, ranging from 6 months to 2.4 years), even in subjects without audible heart murmurs.

The results of this study confirm that even in dogs, particularly in CKCS, as already demonstrated in humans, there is a differential expression of miRNAs and suggests that their expression profiles are distinct for dogs with MMVD, compared to expression profiles for healthy dogs.

This study identified a biomarker that may have an impact in both implementing preventing programs through genetic selection and in clinical practice, confirming that in CKCS as well, as already demonstrated in humans, there is a differential expression of miRNAs, suggesting that their expression profiles are distinct for dogs with MMVD compared to expression profiles of healthy dogs.

For these reasons, miRNAs may be considered new biomarkers and may provide the basis for further investigations to assess the follow-up of this cohort of subjects, aimed at the characterization of the evolution of the disease in the CKCS. Considering the results obtained, this study lays the basis to set up more focused breeding programs and a targeted selection, to obtain healthier subjects with a good life expectancy, and at the same time ensuring the protection of the genetic pool of the breed, which represents an important national and international goal (Appendix 9.5.3. page 206, 9.5.5. page 211).

The CKCS is a fashionable breed that has a range of qualities suited for living in an apartment, together with children and elderly people, and which is likely to remain one of the most popular dog breeds for many years. MMVD is unfortunately a widespread disease in this breed and often causes premature death. These were the main reasons why this PhD research project was focused on CKCS, and why so many diagnostic techniques have approached the issue of MMVD in this particular breed.

The general organization of this PhD thesis might be seen as a study pathway regarding the MMVD in the CKCS. This project is aimed at the identification of any possible clues useful to predict the beginning and the evolution of the MMVD in this breed, starting from subjects belonging to ACVIM class B1. Therefore, the thesis has been organized in chapters, and each one is a small piece of a puzzle that hopefully might be expanded in the future. This puzzle includes the knowledge of genetics, echocardiography, morphology, and a hint of radiology. The final goal is to distinguish dogs not genetically predisposed to the development of MMVD from dogs predisposed to the development of the degenerative form of MMVD and from dogs presenting the genetic predisposition to the early onset form. Obviously, further investigations with a larger study population would help to clarify whether additional genetic, echocardiographic, and morphometric characteristics can be identified particularly in CKCS in ACVIM class B1 and an appropriate follow-up would enable us to highlight prognostic factors related to the worsening of the disease within this ACVIM class.

I believe that the results of this PhD thesis can be useful to clinicians and breeders as a support to echocardiographic screening, which is the gold standard for the diagnosis, so as to reduce the incidence of more serious and more rapid forms of MMVD in this breed.

During this PhD project the breeders and the owners played a fundamental role, because their willingness to screen their dogs, especially breeding animals, and can make a long-term difference in reducing the incidence of MMVD. Prevention based on different approaches is

essential to reduce the incidence of hereditary heart disease, and this is what we have attempted to achieve with this project. To study the follow-up of our population it is fundamental to identify dogs that will develop more serious or rapid forms of the disease. Many years will be needed to obtain precise answers, although improving a breed is undoubtedly achievable.

The magnitude of the heritability suggests that selection against early onset of MMVD would be successful and, to that end, the production of early biomarkers for premature MMVD would be a useful addition. However, care must be taken to ensure that the selection from early and severe MMVD does not result in a concomitant increase in other notable diseases in the CKCS, such as syringomyelia, or that too high a selection intensity leads to a drastic loss of the genetic pool variability, thereby reducing the general robustness of the breed and potentially leading to another genetic disease in the future.

8. Limitations

This PhD thesis is a clinical study based on the voluntary membership and the availability of CKCS' breeders and owners during these three years and this work is certainly not without limitations.

The main limit was the uneven numbers of the ACVIM classes, particularly in the echocardiographic and morphometric studies. There was, in fact, a considerable disproportion between the number of subjects in ACVIM class B1 (the one most subjected to screening) and all the other classes. Increasing the number of subjects in the different classes, especially in more advanced stages, could have enabled a more appropriate delineation of the clinical and echocardiographic profile of each class. Secondly, there was a disproportion in the number of females compared to males. This was probably due to the voluntary adherence to the screening program and to the greater participation of breeders compared to private owners.

Furthermore, the qualitative nature of some analyzed echocardiographic parameters should be interpreted with caution. Jet size detected by color flow mapping in the echocardiographic study should only be regarded as a semiquantitative measure of the degree of MR. Several factors, such as the quality of the echocardiographic machine, the quality of the imaging window, the distance to the flow being imaged, gain settings, pulse repetition frequency setting for the color Doppler, the immobility of the patient, and the experience of the operator have an influence on this measure. The left apical 4-chamber view was used because the degree of MR may be underestimated if color flow mapping is performed from the right side of the thorax. Furthermore, it has long been known for humans that, given the 3D morphology of the mitral valve, long axis images that do not include the left ventricular outflow tract (LVOT) greatly overestimate the presence of MVP. Considering recent publications on canine 3D mitral valve morphology, both in multi-breed populations and specifically in CKCS, this cannot be ignored any longer in future studies. Another possible limit of the echocardiographic and morphometric

studies was the failure to use the left-parasternal long axis two chamber view for the visualization of all the mitral scallops and the exact prolapsing portion.

Regarding the biomarker study, furthermore, we must declare that the clinical application of miRNAs as biomarkers is still limited. One of the most significant obstacles is the difficulty concerning the normalization of circulating miRNAs. Spiked in synthetic miRNAs are widely used to normalize serum and plasma miRNA expression, but this approach does not include effects of pre-analytic variables on circulating miRNA measurement. It must also be pointed out that the sole presence of specific miRNAs is a biomarker of disease, infection, or inflammation. There are no measuring units, there are no possibilities of disease grading based on miRNAs concentration and there are no reference levels of specific miRNAs in specific tissues, neither healthy nor diseased. For these reasons we must consider miRNAs as research biomarkers.

Finally, in general, for all the studies completed in these three years, the impossibility of having a long-term follow-up was a considerable problem because it did not allow us to correlate the genetic data, echocardiographic and morphometric measurements, and miRNA expression profiles with the prognosis of each subject.

9. Appendix - Description of the studies

9.1. Breed-specific vertebral heart score, vertebral left atrial size, and radiographic left atrial dimension in Cavalier King Charles spaniels: reference interval study

Bagardi, M.; Locatelli, C.; Manfredi, M.; Bassi, J.; Spediacci, C.; Ghilardi, S.; Zani, D.D.; Brambilla, P.G.
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Myxomatous mitral valve disease (MMVD) is a cardiovascular disease affecting dogs, progressing to mitral regurgitation and eventually heart failure [1]. The incidence is age-related and is particularly high in some breeds such as the Cavalier King Charles spaniels (CKCS) [1,2]. Fifty percent of CKCS are affected by the age of 6-7 years, and almost 100% are affected by the age of 11 [1,2]. In this breed, radiographs are frequently used to screen for evidence of left-sided cardiomegaly secondary to MMVD [3,4]. Thoracic radiography has an important role in the evaluation of the cardiovascular system and is often utilized as a screening tool to objectively assess heart size in dogs through VHS, VLAS, M-VLAS, and RLAD [3,5-9]. The VHS is influenced by different morphotypes, and several studies have described breed-specific reference range [10-20]. Only normal values of VHS in CKCS have been investigated in this study and are reported to be higher than non-breed-specific reference ranges (respectively 10.6 ± 0.5 vs 9.7 ± 0.5) [3,10]. VLAS, M-VLAS and RLAD have recently been proposed as new radiographic methods for the evaluation of left atrial size in dogs [7-9]. However, no studies have evaluated VLAS, M-VLAS and RLAD in different breeds, except for a recent study on VLAS in Chihuahuas [21]. Breed-specific VLAS, M-VLAS and RLAD reference values have not been reported for CKCS, although the 2019 ACVIM MMVD consensus statement recommends, if available, the use of breed-specific radiographic normal values [22]. The first aim of this study was to describe breed-specific reference values for VHS, VLAS, M-VLAS and RLAD in a sample population of healthy adult CKCS and to compare those with the data

already available from the literature. The second aim was to investigate the effect of the aspects of recumbency, gender, body weight, and thoracic depth – thoracic width ratio (TD/TW) on the VHS, VLAS, M-VLAS and RLAD measurements. We hypothesized that radiographic measures in CKCS would differ from previously published reference values and that they would not be significantly affected by sex, body condition score (BCS), or chest conformation, but influenced by the recumbency, particularly the atrial measurements. The results of this study could be useful for clinicians, especially when echocardiography is not available, in order to monitor the cardiac size during the progression of heart enlargement in this widespread breed.

9.1.1. Materials and Methods

Case selection

All included CKCS underwent a complete physical examination, thoracic radiographs, and echocardiography. Dogs were considered healthy based on the absence of prior clinical conditions/abnormalities documented by the owners and unremarkable physical examination, cardiovascular assessment, and transthoracic echocardiogram [21]. Dogs with cardio-structural heart diseases and cardiac chamber enlargement identified on echocardiogram were excluded from the study, as well as dogs aged <12 months because of the possible influence of young age and skeletal immaturity on the radiographic vertebral-based measurements. Thoracic radiographs that revealed an overt malposition of the patient (as the considerable rotation, the movement due to excessive breathing for tachypnea or the brachial muscles superimposition on the cranial aspect of the thorax) or the presence of thoracic vertebral abnormalities (e.g., hemivertebrae) were not included in the study [23,24]. All decisions for dog inclusion or exclusion were made by two veterinarians with more than fifteen years of clinical experience in radiology and cardiology respectively, based on a consensus opinion.

Echocardiographic examination

All echocardiographic examinations were performed in not sedated dogs, using an ultrasonographic unit [MyLab50 Gold cardiovascular ultrasound machine (Esaote, Genova, Italy)] equipped with multi-frequency phased array probes (3.5–5 and 7.5–10 MHz), chosen according to the weight of the subject. Each dog underwent a complete echocardiographic examination, which included transthoracic two-dimensional, M-mode, and Doppler imaging [25]. An average of 3 cardiac cycles was used for each measurement. Left ventricular internal diameter at end-diastole (LVIDD) and left ventricular internal diameter at end-systole (LVIDS) were measured on 2-dimensional-guided M-mode right parasternal short axis images. End-diastole of the left ventricle (LV) chamber was defined as the internal dimension at the onset of the QRS complex on the echo-timing ECG. The end-systolic LV chamber internal dimension was defined as the minimum chamber dimension. The measurements were made from inner edge (blood-tissue interface) to inner edge. LVIDD was normalized to body size (LVIDDN) as previously described, and left ventricular diastolic dimension was considered normal with a LVIDDN <1.7 [22,26]. Echocardiographic left atrial (LA) size was determined using two different body size-indexed linear measurements: (a) short-axis LA indexed to the short-axis aortic root (LA/Ao_Sx) and (b) long-axis left atrial dimension indexed to the long-axis aortic valve annulus diameter (LAD/AoD_Lx). LA/Ao_Sx was measured by the 2-dimensional right parasternal short axis view as previously described [27]. LA/Ao_Sx was calculated from left atrial and aortic root diameters. Left atrial and aortic root dimensions were measured from inner edge to inner edge, timed after the end of the T wave, in the earliest frame in which the aortic valve cusps were closed. For the evaluation of LAD/AoD_Lx, maximum long-axis left atrial dimension was determined from a right parasternal long-axis four chamber view where a line is drawn from the mid atrial septum, that is, region of the fossa ovale, to the internal reflection of the bright pericardium in the far field. This bisected the long axis LA area and is approximately parallel to the mitral annulus [28,29]. Left atrial size was considered normal

when the LA/Ao_Sx was < 1.6 and the LAD/AoD_Lx was < 2.4 [22,29]. The operator performing the echocardiographic measurements was blinded to the VHS, VLAS, M-VLAS and RLAD measurement. Only CKCS belonging to ACVIM class A were included in the study [30,31].

Radiographic examination

Radiographic examinations and measures by radiologist that were blinded to echocardiographic findings [23]. The radiographic examination included right lateral (RL), left lateral (LL), and dorso-ventral (DV) views. The dogs were conscious and carefully contained to prevent an abnormally positioned thoracic vertebral column and trachea. All radiograms were taken at the time of full inspiration. The thoracic radiographic studies were obtained with a digital system (RX D-VET G35i, FUJIFILM Italia S.P.A., Milano, Italia) and radiographic exposure factors for each dog were based on patient body size.

A radiological reading workstation (iMac Retina 5K, 27-inch, 2014 with OsiriX© MD v. 8.0.2, Pixmeo SARL, Switzerland) was used for all radiographic measurements, performed with digital caliper. The radiographic evaluation of VHS, VLAS, M-VLAS and RLAD were performed in both RL and LL radiographic views [6-9,32]. Each length was expressed in vertebral body units (v) to the nearest 0.1 vertebra for all the described radiographic measurements.

Specifically for VHS, the long-axis dimension (L) was measured from the ventral border of the largest of the main stem bronchi seen in cross section to the most ventral point of the cardiac apex. The short-axis dimension (S) was drawn perpendicular to the long axis dimension from the caudal border of the cardiac silhouette at the dorsal aspect of the caudal vena cava to the cranial border of the cardiac silhouette. The two lengths (L and S) were then repositioned over the thoracic vertebrae, parallel to the vertebral canal, beginning to the fourth thoracic vertebrae (T4). The VHS was the sum of the two lines in vertebral body units (Fig 1A).

For VLAS, the length between the center of the most ventral aspect of the carina to the caudal aspect of the left atrium at point of intersection with the dorsal border of the caudal vena cava was measured. A line equal in length to this measurement was drawn from the cranial border of the T4 and extended caudally parallel to the vertebral canal. The reported VLAS was the length of this line in vertebral body units (Fig 1B). Modified VLAS (M-VLAS) was calculated starting from VLAS, as originally described [7], and a second dimensional measurement was made by placement of the digital caliper at the most distal LA border excluding the pulmonary vein orifice and extended to perpendicularly intersect with the first line. As before, the line was transposed on the vertebral column from the cranial edge of the T4 body and the M-VLAS was defined as length of this measurement in vertebral body units [8] (Fig 1C). The computer software was used to ensure a line bisecting the 90° angle formed by the intersection of the VHS L and S axes connecting this point with the radiographic projection of the dorsal edge of the LA, both for RL and LL projection. This length was then drawn starting from the cranial edge of T4 and used as RLAD (Fig 1D). In cases where it was difficult to differentiate the dorsal anatomical boundaries of the LA and the neighboring pulmonary veins, the most dorsal aspect of the soft tissue opacity seen at this level was routinely used for all measurements [9]. Thoracic conformation was determined from the TD/TW ratio, as described by Buchanan and Bücheler [6]. The depth of thorax was measured in the RL radiographic view from the cranial edge of xiphoid process to the ventral border of vertebral column along a line perpendicular to vertebral column (Fig 2A). The width of the thorax was measured on a DV radiograph as the distance between medial borders of eighth ribs at their most lateral curvatures [17] (Fig 2B).

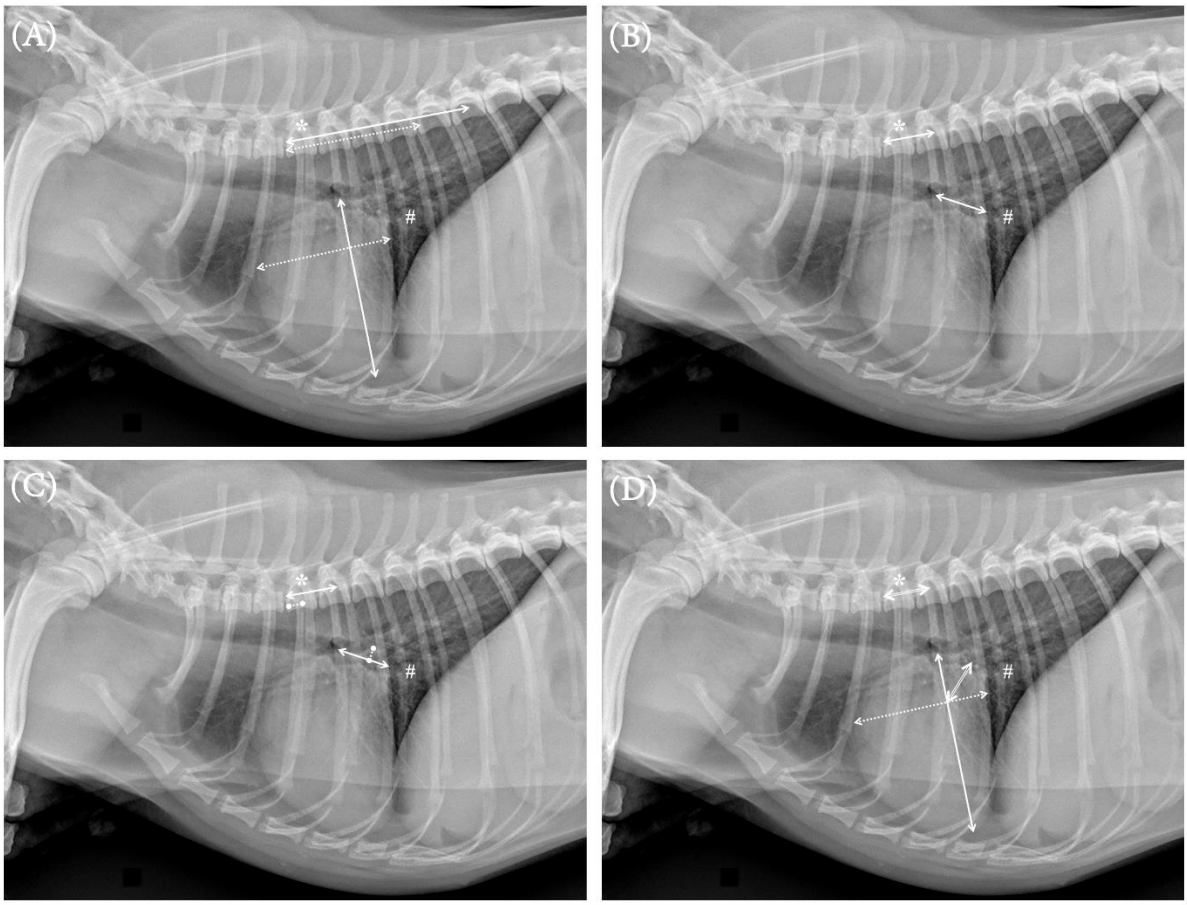

Figure 1. Right lateral thoracic radiographic image (kVp 75, mAs 2.5) of a CKCS demonstrating the radiographic measurements of vertebral heart size (VHS), vertebral left atrial size (VLAS), it's modified version (M-VLAS), and radiographic left atrial dimension (RLAD) performed in this study.

A, For VHS, a line is drawn from the central and ventral border of the carina to the most distant point of the cardiac apex (solid line). The short-axis (dotted line) line was measured at the widest part of the cardiac silhouette within the central one-third region, typically near the ventral border of the caudal vena cava (#), and perpendicular to the long-axis. The measurements of the two axes were then indexed to the thoracic vertebral bodies starting at the cranial edge of T4 (*) and summed (9.6 vertebrae in this example). B, For VLAS, a line was drawn from the central and ventral border of the carina to the caudal most border of the left atrium, where it intersected with the dorsal border of the caudal vena cava (#). This line was indexed to the thoracic vertebral bodies starting at the cranial edge of T4 (*) and summed (1.7 vertebrae in this example). C, For MVLAS — an initial line (solid line)— was drawn from the center of the most ventral aspect of the carina to the intersection between the most caudal aspect of the left atrium and the dorsal border of the caudal vena cava (#). A second additional line (dotted line) was then drawn from the most distal border of the left atrium towards the first line, intersecting it perpendicularly. Two separate straight lines corresponding to the lengths of the first 2 lines were then drawn from the cranial edge of the T4 (*) and summed (2 vertebrae in this example). D, For RLAD the computer software was used to ensure a line (double line) bisecting the 90° angle formed by the intersection of the VHS long (solid line) and short (dotted line) axes connecting this point with the radiographic projection of the dorsal edge of the LA, both for RL and LL projection. This length was then drawn starting from the cranial edge of T4 (*), summed, and used as RLAD (1.4 vertebrae in this example).

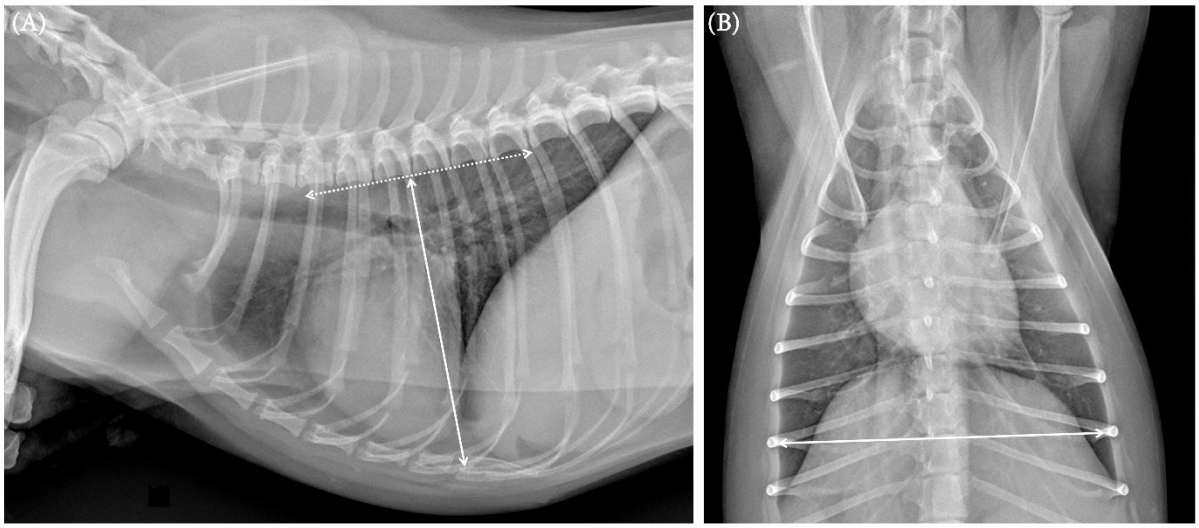

Figure 2. A, Thoracic depth (solid line) measured from xiphoid process to the perpendicular of vertebral column in right lateral recumbency (kVp 75, mAs 2.5). B, Thoracic width measured as the distance between medial borders of eighth rib at their most lateral curvatures in dorso-ventral recumbency (kVp 75, mAs 2.5).

Statistical analysis

Statistical analyses were performed using SPSS^{TM} 27.0 (IBM, SPSS, USA). Descriptive statistics were generated. The distribution of data for continuous variables was assessed for normality by means of the Kolmogorov-Smirnov test. Variables were normally distributed, and results were reported as mean ± standard deviation (SD) unless otherwise specified. A Paired Student's t-test was used to compare the VHS, VLAS, M-VLAS and RLAD between right and left lateral recumbency. An unpaired Student's t-test was used to compare the VHS, VLAS, M-VLAS and RLAD between males and females, between dogs with different TD/TW ratio, particularly TD/TW higher or lower than 0.9, and between dogs with different BCS and body weight. The TD/TW 0.9 cut off was arbitrary chosen due to the intermediate CKCS' chest conformation. A Pearson correlation coefficient (r) was applied to study the correlation between all radiographic measurements, clinical (age, weight, BCS) and echocardiographic data (LVIDDN, LA/Ao_Sx, LAD/AoD_Lx, sphericity index). The correlation was considered weak, moderate, strong, or perfect respectively when the value of the correlation coefficient was 0.1-0.3, 0.4-0.6, 0.7-0.9 or 1 [33]. A correlation analysis between VHS, VLAS, M-VLAS, RLAD and TD to TW ratio was performed to determine whether chest conformation was responsible

for variation in these radiographic parameters. To compare obtained VHS, VLAS, M-VLAS and RLAD measurements, the values from Malcolm's study expressed as median and interquartile range were converted to mean and standard deviation (2.07 ± 0.25 vertebrae) [7]; the same was for M-VLAS (2.6 ± 0.3 vertebrae) and RLAD (1.97 ± 0.57 vertebrae) [8,9,34]. A one sample t-test was then used to test whether the VHS, VLAS, M-VLAS and RLAD in our population of CKCS differed from the mean reference values proposed by literature [6-9,32]. A P value < 0.05 was considered significant for all analyses.

9.1.2. Results

The sample population consisted of 30 adult healthy CKCS. The sample size was based on convenience sampling. There were n. 22 (73.3%) females (n. 3 neutered) and n. 8 (26.7%) males (n. 1 neutered); with a mean age of 2.66 ± 1.42 years (range: 1-6 years) and a mean body weight of 7.92 ± 1.68 kg (range: 5.1-12 kg). Twelve subjects (40%) weighted more than the proposed breed standard (5-8 Kg). The mean BCS was 5/9 ± 1/9 (range: 4/9-7/9). Nineteen dogs (63.33%) had Blenheim coat color type, n.1 (3.33%) ruby, n. 3 (10%) black and tan and n. 7 (23.34%) tricolor. The small number of subjects with coats other than Blenheim did not allow to perform an adequate statistical analysis against this physical parameter. All the clinical and the echocardiographic data are reported in Table 1.

Table 1. Clinical and echocardiographic data of all included healthy CKCS.

	Overall population	Females	Males
N. of dogs	30	22	8
Sex	22F (3NF) 8 M	22 F 3NF	8 M
Age y	2.66 ± 1.42	2.79 ± 1.42	2.32 ± 1.47
Weight kg	7.92 ± 1.68	7.74 ± 1.67	8.42 ± 1.72
BCS	5/9 ± 1/9	5/9 ± 1/9	5/9 ± 1/9
Coat color type	19 Blenheim 1 Ruby 3 Black and tan 7 Tricolor	14 Blenheim 1 Ruby 2 Black and tan 4 Tricolor	5 Blenheim 1 Black and tan 3 Tricolor
LA/Ao_Sx	1.20 ± 0.17 1.22	1.22 ± 0.16*	1.07 ± 0.11
LAD/AoD_Lx	2.01 ± 0.19	2.08 ± 0.13*	1.81 ± 0.19
LVIDDN	1.30 ± 0.16	1.28 ± 0.16	1.33 ± 0.18
SI	1.57 ± 0.2	1.55 ± 0.21	1.65 ± 0.17

F: females; NF: neutered females; M: Males; BCS: Body condition score; LA/Ao_Sx: Short-axis left atrium indexed to the short-axis aortic root; LAD/AoD_Lx: Long-axis left atrial dimension indexed to the long-axis aortic valve annulus diameter; LVIDDN: Normalized left ventricular internal diameter in diastole; SI: sphericity index. *Parameters significantly higher in females compared to males ($P < 0.05$).

In our study, the CKCS had a significantly lower VHS (10.08 ± 0.56; 95% range 9.87-10.29) than the reference value of 10.6 ± 0.5 established by Lamb et al. in 2001 for this breed ($P = 0.002$) and higher than the reference value of 9.7 ± 0.5 proposed by Buchanan and Bücheler in 1995 ($P < 0.001$) [6,10]. The VLAS, M-VLAS and the RLAD of CKCS in our study were respectively 1.79 ± 0.3 (95% range, 1.68-1.9), 2.23 ± 0.44 (95% range, 2.06-2.39) and 1.2 ± 0.34 (95% range, 1.07-1.33). These were lower than the values previously reported by Malcolm et al. (2.07± 0.25; $P = 0.000$), Lam et al. (2.6 ± 0.3; $P = 0.000$) and Salguero et al. (1.97 ± 0.57; $P = 0.000$).

Table 2 reports the radiographic values of the VHS, VLAS, M-VLAS and RLAD in RL and LL view from our study. No significant differences in VHS were found between LL and RL recumbencies ($P = 0.25$), whereas VLAS, M-VLAS and RLAD were significantly higher in LL than RL view ($P < 0.001$, $P = 0.001$ and $P = 0.02$ respectively).

Table 2. Vertebral heart score, vertebral left atrial size, modified vertebral left atrial size and radiographic left atrial dimension in 30 healthy Cavalier King Charles spaniels.

	Recumbency	n	Mean ± SD	95% range	P
VHS	RL	30	10.08 ± 0.56	9.87-10.29	0.25
	LL	30	10.00 ± 0.41	9.85-10.17	
VLAS	RL	30	1.79 ± 0.3	1.68-1.90	0.000
	LL	30	1.99 ± 0.25	1.90-2.09	
M-VLAS	RL	30	2.23 ± 0.44	2.06-2.39	0.001
	LL	30	2.48 ± 0.28	2.38-2.59	
RLAD	RL	30	1.20 ± 0.34	1.07-1.33	0.02
	LL	30	1.37 ± 0.20	1.29-1.44	

Both RL and LL VHS, VLAS, M-VLAS and RLAD did not significantly differ between males and females ($P > 0.05$). All radiographic measurements did not significantly differ between different BCS groups ($P > 0.05$). Mean TD to TW ratio was 0.91 ± 0.08 (95% range 0.88-0.94). Fifteen dogs (50%) had a TD/TW ratio < 0.9 and n. 15 (50%) > 0.9. All CKCS of this study had an intermediate chest conformation ($0.75 < TD/TW < 1.25$) [6]. TD to TW ratio did not differ significantly between the sexes ($P = 0.26$).

The VHS, VLAS, M-VLAS and RLAD showed no correlation with BCS ($P > 0.05$), and body weight ($P > 0.05$). Only LL VLAS showed a moderate positive correlation with BCS ($r = 0.38$, $P = 0.037$).

There was no significant correlation between the type of chest and VHS, VLAS, M-VLAS and RLAD in all included dogs and no significant differences were observed for dogs with different TD/TW ratio (higher and lower than 0.9). Conversely, CKCS with TD/TW ratio lower than 0.9 had greater LA/Ao_Sx ratio and lower sphericity index (both $P = 0.001$).

9.1.3. Discussion

The purpose of this study was to determine breed specific reference values for VHS, VLAS, M-VLAS and RLAD in healthy adult CKCS [6-9]. Based on literature review, this is the first study proposing the reference intervals in this breed for VLAS, M-VLAS and RLAD.

In this CKCS sample, the VHS was significantly higher than (10.08 ± 0.56) the not-breed specific reference values initially established by Buchanan and Bücheler in 1995 (9.7 ± 0.5) [6], but significantly lower than the breed standard proposed by Lamb et al. in 2001 (10.6 ± 0.5) [10]. In the study by Buchanan and Bücheler (1995) there were no significant differences between RL and LL recumbencies for VHS [6]. This is in accordance with our results. Lamb et al. in 2001 evaluated only RL view in their study with multiple breeds, but Greco et al. in 2008 reported a higher VHS value by 0.3 vertebra in RL recumbency compared to LL [10,35]. Similarly, other studies found a higher VHS in RL recumbency than in LL recumbency [12,15,17,18]. Disagreement between studies may be explained first by differences in thoracic morphotypes among breeds. It was also hypothesized that the larger VHS in RL recumbency may be due to the divergent X-ray beam and the larger distance of the heart from the cassette in RL recumbency [35]. In addition, possible variations in radiographic cardiac size during the cardiac cycle (diastolic vs systolic dimensions) need to be considered. In fact, while respiratory cycle can be controlled when radiographs are taken, cardiac cycle cannot [36,37]. The previously reported mean VHS ± SD ranges from 9.9 ± 0.8 to 10.4 ± 0.8 vertebrae between end-diastolic and end-systolic measurements with fluoroscopy at peak inspiration for dogs positioned in right lateral recumbency [37]. On average, mean VHS ± SD is 0.3 ± 0.3 vertebrae greater in diastole than in systole at peak inspiration, with VHS varying up to 0.97 vertebral units over the cardiac cycle in some individuals [36]. Similar influence of cardiac cycle is observed on VLAS but however the same has never been described for RLAD [37]. Furthermore, without further studies, we can only deduce that M-VLAS, being a derivative of VLAS, might also show similar influence by cardiac cycle. VLAS, M-VLAS and RLAD in our study were significantly higher in LL than RL view. This can be anatomically justified by a possible overlapping of the venous sinus of the cava veins, of the coronary venous sinus and of the caudal vena cava outflows.

Buchanan and Bücheler (1995) did not detect any differences in the VHS between males and females [6]. However, Lamb et al. in 2001 described lower VHS values in female dogs than in male dogs [10]. It should be noted, however, that the difference between males and females in Lamb et al. study has been observed in the general population and not for each breed [10]. In the present study, VHS values of male and female dogs were not significantly different. A possible explanation could be that in our CKCS population there is no sexual dimorphism between male and females, as reported by breed standard (https://www.enci.it/media/2405/136.pdf) [38], whereas other studies report more variation in this breed [39]. Finally, no correlation was found between the VHS, BCS, and body weight. This result is in accordance with previous studies in Spitzs, mixed breeds, and Labrador retrievers [17], but not with the results found in Lhasa Apsos and Norwich Terriers [16,20]. Disagreement between studies may be explained by possible variations in the amount of pericardial fat in different breeds [18].

The VLAS found in our study population is lower (1.79 ± 0.3) than the values proposed by Malcolm et al. (2.07± 0.25) and more similar to those reported by Puccinelli et al. (1.8 ± 0.2) [7,21]. The same difference from data reported by literature can be observed for M-VLAS and RLAD (2.23 ± 0.44 versus 2.6 ± 0.3 and 1.2 ± 0.34 versus 1.97 ± 0.57 respectively) [8,9]. It is interesting to report that the control group (consisted of healthy subjects) from which normal values of VLAS and M-VLAS were derived, were composed of only 15 and 6 dogs respectively, with only one healthy CKCS included in Malcolm et al. study and no CKCS in Lam et al. study [7]. Furthermore, the control group in the study of Salguero et al. (RLAD) included only one healthy CKCS. Thus, the presence of other breeds could have raised the proposed VLAS and M-VLAS ranges and RLAD.

We must also emphasize that all the published data about VLAS, M-VLAS and RLAD reported a cut off able to discriminate among subject with or without left atrial enlargement and not the

normal radiographic size of the left atrium. It is therefore likely that values obtained in our study are lower for this reason.

Further studies including a larger population of healthy and affected by MMVD at different stages CKCS are needed to better clarify the normal reference interval of the VLAS, M-VLAS and RLAD in this breed and a cut-off value useful for discriminate left atrial enlargement. In addition, breed-specific differences in the VLAS, M-VLAS and RLAD, like the VHS, should be considered.

The present study was not without limitations. The main limit was the sample size. A larger population could have led to possible differences in the proposed reference range. In fact, the CKCS breed is very inhomogeneous in terms of size and morphotype. Body weight in CKCS may vary with different genetic lineages; however, our range of body weight was wide (5-12 kg), and no correlation was found between the VHS and body weight. Thus, it is reasonable to believe that even increasing the sample population, similar results would be found. In addition, most studies reporting breed-specific reference values of the VHS included a maximum of 30 cases per breed, as the present study [10,11,15-17,19].

Finally, like previous studies on radiographic vertebral-based measurement [7,16,41,42], our sample population did not include dogs younger than 12 months.

9.1.4. Conclusion

The results of this study support previous research indicating that breed-specific reference values for the VHS are needed. Furthermore, as underlined by 2019 ACVIM MMVD guidelines, VHS, VLAS, M-VLAS and RLAD breed-specific reference values should be introduced in the evaluation of thoracic radiograms, also in healthy subjects. In CKCS, the VLAS, M-VLAS and RLAD values found in this study can be used as references to avoid misinterpretation of cardiomegaly in this breed. Further studies evaluating the VLAS, M-VLAS and RLAD in different canine breeds are warranted.

9.1.5. References

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9.2. A genomic study of myxomatous mitral valve disease in Cavalier King Charles spaniels

Bionda; A.; Cortellari, M.; Bagardi, M.; Frattini, S.; Negro, A.; Locatelli, C.; Brambilla, P.G.; Crepaldi, P. Animals (Basel). 2020 Oct 16;10(10):1895. doi: 10.3390/ani10101895.

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease in dogs, accounting for approximately 75% of all dogs with heart disease [1], particularly in older dogs and smaller dog breeds [2]. Cavalier King Charles spaniels (CKCS) show the earliest onset and the highest incidence of MMVD when compared with other breeds [1,3–5]. The primary clinical finding in dogs affected by MMVD is a systolic heart murmur, but no murmur might occur in mild cases [6]; thus, an echocardiography is considered as the gold standard for the confirmation and staging of this disease [7]. There is evidence from the literature that, at least in some breeds, such as CKCS and Dachshunds, the hereditary component plays a predominant role in the pathogenesis of MMVD [8–10]. This disease has been suggested to be inherited as a polygenic trait. In fact, the proportion of offspring with heart murmurs and the intensity of these murmurs are both significantly greater with increased parental severity [8,10]. Moreover, early-onset MMVD, typically found in CKCS, also appears to be highly heritable. In particular, the heritability is 0.67 ± 0.07 for the degree of the heart murmur and 0.33 ± 0.07 for the presence or absence of the murmur, considering dogs exclusively aged between 4 and 5 years [9]. Previous studies have demonstrated that the linkage disequilibrium in dogs is 10 to 100 times more extensive than in the human genome; therefore, the number of single-nucleotide polymorphism (SNP) markers required for genomic association studies in dogs is considerably lower than in humans [11–14]. Moreover, the peculiarities of breed structure of dogs (e.g., a small breeding population and the use of popular sires) reduce the within-breed genetic variability, generally making even a small number of cases and controls (from 20 to 100, depending on the type of trait [15]) useful to effectively detect genomic regions that are

associated with a particular trait or disease when genotyping just 15000 SNPs [11,14], which is 10 times less than those contained in the SNP chip used in the present study. In the literature, it is possible to find several genomic studies on canine MMVD, as summarized in Table 1. Madsen et al. (2011) identified two loci on canine chromosomes (CFA) 13 and 14 that are weakly associated with the development of MMVD via a genome-wide association study (GWAS) of CKCS [3]. French at al. (2012), in contrast, did not find any evidence for loci associated with a mitral valve murmur with a GWAS in this breed, nor regions of highly discrepant homo/heterozygosity. The authors concluded that the familial occurrence of mitral valve murmurs in the CKCS breed is not due to a single major gene effect [16]. Meurs et al. (2017) performed a whole-genome sequencing of 10 CKCS and 10 Dachshunds. They filtered the variants of canine gene orthologs of the human genes known to be associated with MMVD against a database of variants derived from whole-genome sequencing of 98 medium to large dog breeds. The latter were chosen, assuming that the prevalence of MMVD in medium to large dogs is very low, but their phenotypes were not assessed. No variant was found in any of the genes evaluated that were present in at least eight of 10 affected samples, but a single coding variant, predicted to be benign, was found in the COL5A1 gene in nine of the 10 affected CKCS and in, at most, 5% of the other breeds examined [17].

Table 1. Summary of previous genomic studies about myxomatous mitral valve degeneration (MMVD).

Authors	Results	Dog breed	Sample size	Criteria for the inclusion		Diagnostic techniques	Genomic analysis
				Age	Diagnosis		
Madsen et al., 2011	CFA 13q2.2.3 CFA 14q1.3	CKCS	139 cases 102 controls	< 4,5 years < 8 years > 8 years	Murmur ≥ 1/6 and ARJ/LAA ≥ 20% Heart failure symptoms Murmur ≤ 2/6 and ARJ/LAA ≤ 50%	Auscultation Echocardiography	GWAS
French et al., 2012	No mutations at a single genetic locus were found	CKCS	3 cases 18 early onset 18 late onset	No restrictions < 5 years > 7 years	Detectable murmur Detectable murmur Detectable murmur	Auscultation	Homozygosity mapping GWAS
Stern et al., 2015	FSTL5, EEF1a1a, NAF1, NPY1R, NPY5R, TMA16, March1 ARHGAP26	Whippet	138 dogs	5 years cut-off	Scoring based on age, presence and degree of mitral valve prolapse, regurgitation and left heart enlargement	Auscultation Echocardiography Cumulative echocardiographic score system	GWAS
Torres-García et al., 2016	Allele T of the rs22372411 variant of COL1A2	Poodle	50 cases 80 controls	No restrictions > 8 years	Diagnosis of MMVD MMVD absent or mild	Auscultation Echocardiography	Candidate gene polymorphisms
Meurs et al., 2018	A missense mutation of COL5A1, predicted to be benign, was present in CKCS	CKCS and Dachshunds	10 CKCS e 10 Dachshunds as cases 98 medium-large breed dogs as controls	No restrictions No restrictions	Diagnosis di MMVD Phenotype not evaluated; low prevalence of MMVD	Auscultation Echocardiography	Candidate gene approach, whole genome sequencing
Lee et al., 2018	SERT (SLC6A4): c.1193delT (p.Val397Gly)	Maltese	20 cases 10 controls	No restrictions No restrictions	Diagnosis of MMVD Echocardiographically healthy	Echocardiography	Candidate gene polymorphisms
Lee et al., 2019	PDZD2, CTNNA3, LDLRAD4, ARVCF	Maltese	32 cases 16 controls	No restrictions > 10 years	Diagnosis of MMVD Echocardiographically healthy	Echocardiography Radiography	GWAS

CFA: canine chromosome; CKCS: Cavalier King Charles spaniel; ARJ/LAA: area of regurgitant jet/left atrium area ratio; GWAS: genome-wide association study.

The Maltese breed was the object of two genomic studies. Lee et al. (2018) applied a candidate gene polymorphism approach and identified six polymorphisms of the SERT gene in samples with MMVD [18]. The GWAS performed by Lee et al. (2019), instead, revealed significant SNPs in several genes associated with cardiac function, including PDZD2, ARVCF, CTNNA3, and LDLRAD4 [19]. Torres-García et al. (2016) studied the polymorphisms of the COL1A2 gene, which interestingly localizes on the region of CFA 14 (identified by Madsen et al.) in poodles and found an association between the rs22372411 variant and susceptibility to MMVD [20]. The problem of all GWAS case-control studies concerning MMVD is the late onset of the disease, making the identification of a real control which will not develop the disease at a later age more difficult. In 2015, Stern et al. tried to solve this problem by associating their results with the whippet dog breed with a continuous variable that included the age of onset of the MMVD (considered early if under 5 years of age) and a score of severity. In this study, a genome-wide significant association was identified on the region of CFA 15, containing FSTL5, and of CFA 2, containing ARHGAP26 [21]. At present, there is no genetic test for MMVD, and the breeding plans for CKCS put in place so far have not been as effective as expected, especially if based only on auscultatory findings [22–24]. Nevertheless, an accurate selection of animals for breeding is essential, since the high prevalence of this pathology in this breed makes the elimination of all the dogs diagnosed with MMVD from reproduction unfeasible. Bagardi et al. [4] clinically and echocardiographically evaluated a representative sample of the Italian population of CKCS, identifying signs of myxomatous degeneration in 90% of them, also widely present in young dogs. Discovering the genetic basis of MMVD may increase the effectiveness of breeding protocols, allowing an early identification of subjects predisposed to a severe form of this pathology. For this reason, here, we conduct a genomic study on the Italian population analysed by Bagardi et al. [4]. The selection of cases and controls was a crucial aspect of our study and was based on a complete echocardiographic and

genealogic examination, with the latter also being checked against the genomic data. The genomic analyses we performed here have never been used for the investigation of this pathology, and the examination of previously published data about the genes and pathway we identified here also allowed us to describe their possible role in the pathogenesis of the disease. Therefore, the aim of this study was to find genomic regions associated with early-onset MMVD predisposition in the CKCS dog breed.

9.2.1. Materials and Methods

This study was carried out with 33 privately owned CKCS. The main selection criteria were the ACVIM classification and age. Our samples were grouped as follows:

• Cases (n = 16): Dogs with MMVD (class B1 or more severe) diagnosed before the age of 5 or with severe disease (class C or D) before the age of 8;

• Controls (n = 17): Dogs without MMVD (class A) or with extremely mild signs of MMVD (class B1 with a trivial mitral regurgitation characterized by a maximal ratio of the regurgitant jet area signal to left atrium area ≤ 20%) [25] over 5 years of age or those suffering from a mild form of disease (class B1) over 8 years of age.

For example, subjects in ACVIM class B2 that were older than 5 years or in more severe classes that were older than 8 years could not be considered as either cases or controls; thus, they were excluded from this study. Moreover, to limit the rate of consanguinity among the dogs as far as possible, in cases of close kinship, the sample with more "extreme" characteristics (the youngest affected or the oldest healthy dog) was chosen. These dogs were selected among a larger group of 90 CKCS, examined at the Cardiology Unit of the Department of Veterinary Medicine of University of Milan between December 2018 and September 2019.

Information regarding birthdates was verified by checking each animal's microchip number in the regional registry, while the genealogical study was derived from the consultation of the online genealogy book of the Italian Kennel Club (ENCI) (http://www.enci.it/libro-

genealogico/libro-genealogico-online#) or the Centrale Canine site (https://www.centra-canine.fr/lofselect). The cardiovascular system was evaluated by checking the presence/absence of a murmur and, if present, its intensity (grade I–VI/VI) and point of maximum intensity. Since auscultation in dogs with echocardiographic evidence of this disease has often proven to be normal, in addition to clinical data, all subjects underwent a complete echocardiographic examination that was performed by three well-trained investigators using a MyLab50 Gold cardiovascular ultrasound machine (Esaote, Florence, Italy). The exams were carried out according to a standard procedure with concurrent continuous electrocardiographic monitoring [26]. Dogs were staged according to the ACVIM guidelines [7]. Peripheral venous blood sampling was performed at the end of the examination. Blood was collected from the jugular vein into 2.5 mL EDTA tubes after a 12-h fasting period.

Statistical analysis

The statistical analysis was performed using JMP® 15.0.0 (SAS Institute Inc., Cary, NC, USA, 1989–2019). Appropriate descriptive analyses were applied. Variables were reported as the mean ± standard deviation if they were normally distributed after an Anderson–Darling test; otherwise, they were reported as median and interquartile range values. Individual and clinical data of the subjects included in different groups were compared with linear regression and χ 2 tests. The statistical methods applied to the genealogical and genomic analyses are reported below.

Genealogic analysis

Pedigree information was analysed using Optisel [27]. The inbreeding coefficient (F) and average relatedness coefficient (AR) were both calculated. F is the probability of an individual receiving, at one locus, two identical-by-descent alleles that are copies of a single allele carried by a common ancestor of the parents. AR is the probability that an allele randomly chosen from the whole population in the pedigree belongs to a given animal.

DNA extraction and genomic analysis

The whole-blood samples, collected in EDTA tubes, were stored at −20 ◦C. DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions. The concentration and quality of the DNA of each sample were both assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific®, Waltham, MA, USA). The quality and quantity of the DNA extracted were suitable for the downstream analyses. All 33 samples were genotyped via outsourcing by Agrotis S.r.l., Laboratory of Genetics and Services, using Canine 230K SNP BeadChips (containing over 230,000 SNPs) on an iScan System (Illumina®, San Diego, CA, USA). Raw genotype data (in the .ped and .map formats) were processed for quality control using the PLINK 1.9 software package [28] as follows: SNPs were excluded if they had a call rate of FST is the proportion of genetic diversity due to allele frequency differences between cases and controls groups. Thus, loci showing unusually large amounts of differentiation (high FST values) may identify regions of the genome that have been subject to diversifying selection in the two groups, whereas loci showing unusually small amounts of differentiation (low FST values) may identify regions that have been subject to stabilizing selection [30,31]. FST was calculated using PLINK 1.9. All markers ranking in the top 1% of the empirical distribution of FST values were considered as relevant. This threshold, which corresponds to an usual statistical threshold, was used in other similar studies [32,33] to retain only the highest signals. XP-EHH is a linkage disequilibrium-based method that compares the lengths of haplotypes (consistent with the allele under selection and the neighbor variants in linkage disequilibrium) at each marker between two different populations, allowing the detection of strong, directional selection of one allele in one of the two populations while remaining polymorphic in the other [34,35]. XP-EHH was calculated using the SELSCAN 1.1.0 software package [36]. Similarly to the FST analysis, all markers within the top 1% of the empirical distribution of normalized XP-EHH values [37–39] were

considered as relevant. The top 1% SNPs of both the analyses were mapped to the reference genome assembly CanFam3.1. Since combining multiple independent tests increases power and resolution [35,40], we then compared the results of the aforementioned analyses in order to identify the SNPs found by both of them, namely, the ones that most significantly differentiated between the cases and controls. The genes containing the relevant SNPs were examined for pathways using Enrichr [41], which looks for associations in various libraries, including KEGG Human 2019, WikiPathways Human 2019 and BioPlanet 2019. Information about the identified genes was obtained via the GeneCards database (https://www.genecards.org/) [42]. Genomic regions subjected to selection may show a reduced nucleotide diversity and increased homozygosity around the selected locus if compared to the rest of genome [43]. Therefore, on a subset of genes consisting of consensus genes and genes involved in the most relevant pathways, runs of homozygosity (ROH) were investigated using a sliding window approach in PLINK 1.9. The sliding window was 50 SNPs long, and a maximum of five missing genotypes and no heterozygous SNPs were tolerated. An ROH was called if the following criteria were fulfilled: (1) 50 or more consecutive homozygous SNPs; (2) a minimum length of 1 Mb; (3) a minimum density of one SNP per 50 kb; (4) a maximum gap between two consecutive SNPs of 100 kb. For each group, the proportion of dogs showing a ROH was calculated. The ROH-based inbreeding coefficient (FROH) was calculated for each animal, dividing the total length of all ROH in its genome by the length of the autosomal genome covered by SNPs on the chip.

9.2.2. Results

Our sample consisted of 33 subjects chosen among 90 CKCS previously investigated by Bagardi et al. [4]. Thirty-one out of 33 (94%) dogs had signs of mitral valve degeneration. Among the controls, two were classified as A and 15 as B1; fourteen cases were included in ACVIM class B1, one in class B2, and one in class D. This subset was composed of 22 females and 11 males. Age ranged from 0.8 to 11.4 years (6.2 ± 2.6 years). Distributions in the ACVIM

classes, age, and gender were not significantly different to the whole sample of 90 dogs. According to the selection criteria, age was significantly higher in controls (8.1 ± 0.5 years) than in cases (4.1 ± 0.5 years) ($r2 = 0.53$, $P < 0.0001$). It was possible to get the pedigree information of 20 out of 33 (61%) dogs (81% of cases and 41% of controls). The average relatedness (AR) and average inbreeding (F) coefficients calculated for the dogs with a pedigree were 0.06 and 0.01, respectively. Since pedigree information was not available for all the dogs included in our study, we also used a genetic marker-based method for calculating inbreeding (FROH). F and FROH are not directly comparable, because the pedigree-based coefficient measures the mean expected autozygosity of an individual (identity by descent), whereas the latter measures the realized autozygosity (identity by state). FROH was found to be 0.24 ± 0.03 for the whole sample. No significant differences were found between FROH in cases (0.24 ± 0.04, ranging from 0.18 to 0.28) and in controls (0.23 ± 0.02, ranging from 0.19 to 0.28). These results demonstrate that the inbreeding of the two groups was similar and, therefore, could not influence the results of our genetic analyses. Moreover, the discrepancy between F and FROH was consistent with that observed in other studies on dogs [44–46].

Genomic analysis

After the quality control, which excluded the SNPs with a low call rate and MAF and those localized on the sex chromosomes, 103,606 SNPs remained for downstream analyses. None of the 33 animals were excluded. Comparing the case and control groups, there were 291 SNPs characterized by the top 1% values of FST (0.19–0.43), mapping regions containing 157 different genes (Fig 1). There were 152 SNPs characterized by the top 1% values of XP-EHH (2.73–3.82), mapping regions containing 45 different genes (Fig 2). The SNPs shared between both the analyses were mapped to genomic regions containing 10 genes (hereafter called "consensus genes" for conciseness), as reported in Table 2.

Table 2. Consensus genes common to the FST and XP-EHH analyses: names and chromosomal coordinates.

Gene name	CFA	Start	End	Complete name
KIAA1024	3	57739740	57748234	KIAA1024
TBC1D14	3	59003766	59097331	TBC1 domain family member 14
FAH	3	57300583	57326453	Fumarylacetoacetate hydrolase
FRRS1L	11	64281895	64447650	Ferric chelate reductase 1 like
EPB41L4B	11	64312862	64447436	Erythrocyte membrane protein band 4.1 like 4B
CDK6	14	18188429	18420100	Cyclin dependent kinase 6
HEPACAM2	14	18695744	18735539	HEPACAM family member 2
RAB3GAP1	19	37861985	37957007	RAB3 GTPase activating protein catalytic subunit 1
ZRANB3	19	38002194	38302593	Zinc finger RANBP2-type containing 3
UBXN4	19	38519927	38568223	UBX domain protein 4

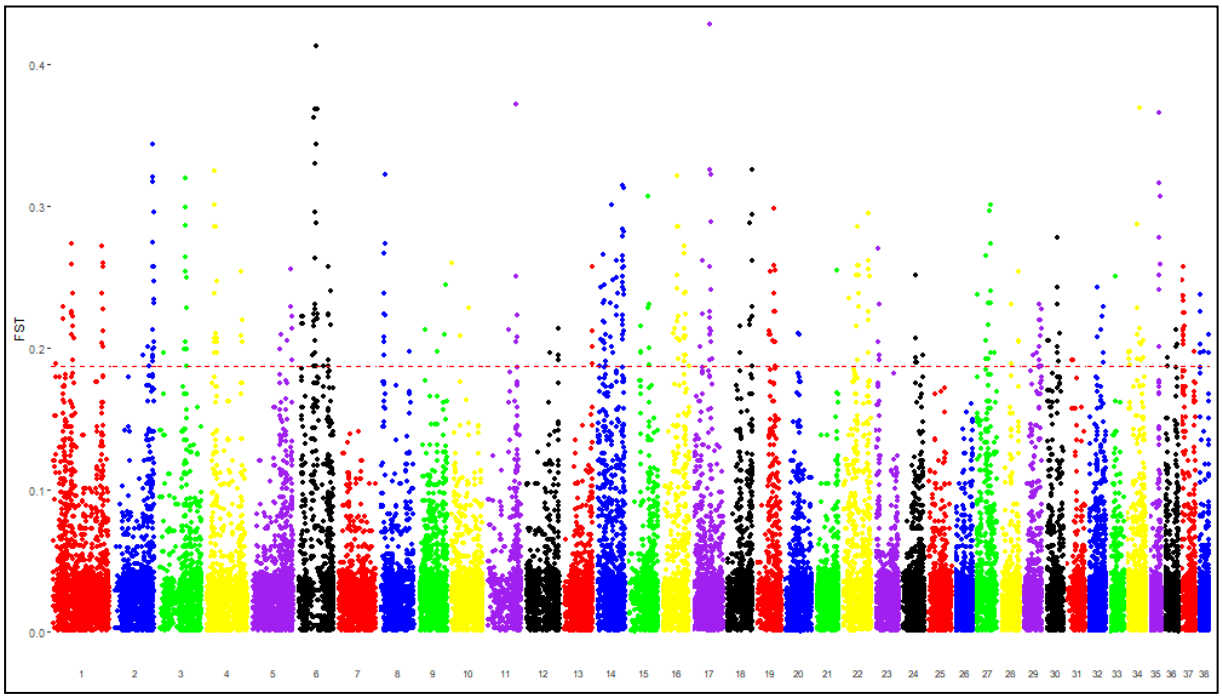

Figure 1. Manhattan plots of the Wright's fixation index (FST) analysis comparing cases vs. controls. Each single-nucleotide polymorphism (SNP) is represented by a dot. Each chromosome is represented by a different color. The dotted red line represents the cut-off value of the top 1%, equal to 0.19. The genomic regions in which SNPs were mapped over the dotted red line were considered as significantly different between the two compared groups.

ROH analysis revealed that at least 50% of cases and no more than 9% of controls showed homozygous regions around the STEAP2, HEPACAM2, and CDK6 genes. The ROH data, including ARNT2, KIAA1024, and FAH were also interesting, because they were present in the genome of almost 80% of cases but only in approximately 40% of controls. No ROH was found in any group for the genes ADCY9, AXIN1, CACNA1H, CREBBP, PDPK1, SLC8A2, and TRAP1. All details about the proportion of cases and controls in which ROH were found are shown in the Table 3. Fig 3 represents the most relevant genes identified by our analysis.

Table 3. Proportion of cases and controls showing a run of homozygosity in regions around selected genes.

Gene	Cases (n. 16)	Controls (n. 17)	Difference between cases and controls
STEAP2	0.55	0.09	0.46
HEPACAM2	0.50	0.05	0.45
CDK6	0.50	0.06	0.44
ARNT2	0.80	0.42	0.38
KIAA1024	0.79	0.41	0.38
FAH	0.80	0.42	0.38
BCAR1	0.44	0.10	0.34
RAB10	0.38	0.12	0.26
PPP2R2C	0.65	0.40	0.25
LATS1	0.57	0.34	0.23
PDE1A	0.62	0.43	0.19
NRG1	0.50	0.32	0.18
TBC1D14	0.59	0.44	0.15
RAB3GAP1	0.08	0.05	0.03
UBXN4	0.04	0.01	0.02
ZRANB3	0.04	0.02	0.01
PLCB2	0.19	0.17	0.01
PDE3A	0.32	0.32	0.00
ADCY9	0.00	0.00	0.00

AXIN1	0.00	0.00	0.00
CACNA1H	0.00	0.00	0.00
CREBBP	0.00	0.00	0.00
PDPK1	0.00	0.00	0.00
SLC8A2	0.00	0.00	0.00
TRAP1	0.00	0.00	0.00
TCF7L1	0.26	0.27	-0.01
SMAD3	0.20	0.24	-0.04
TLN2	0.30	0.35	-0.05
WNT2	0.08	0.18	-0.10
CTNNAL1	0.18	0.27	-0.10
ADCYAP1R1	0.06	0.18	-0.11
FRRS1L	0.17	0.28	-0.12
EPB41L4B	0.17	0.29	-0.12
LPAR1	0.17	0.29	-0.12
CTNNA3	0.06	0.20	-0.14
PRKD1	0.22	0.38	-0.17
ADCY2	0.25	0.43	-0.18
ITPR2	0.35	0.57	-0.22

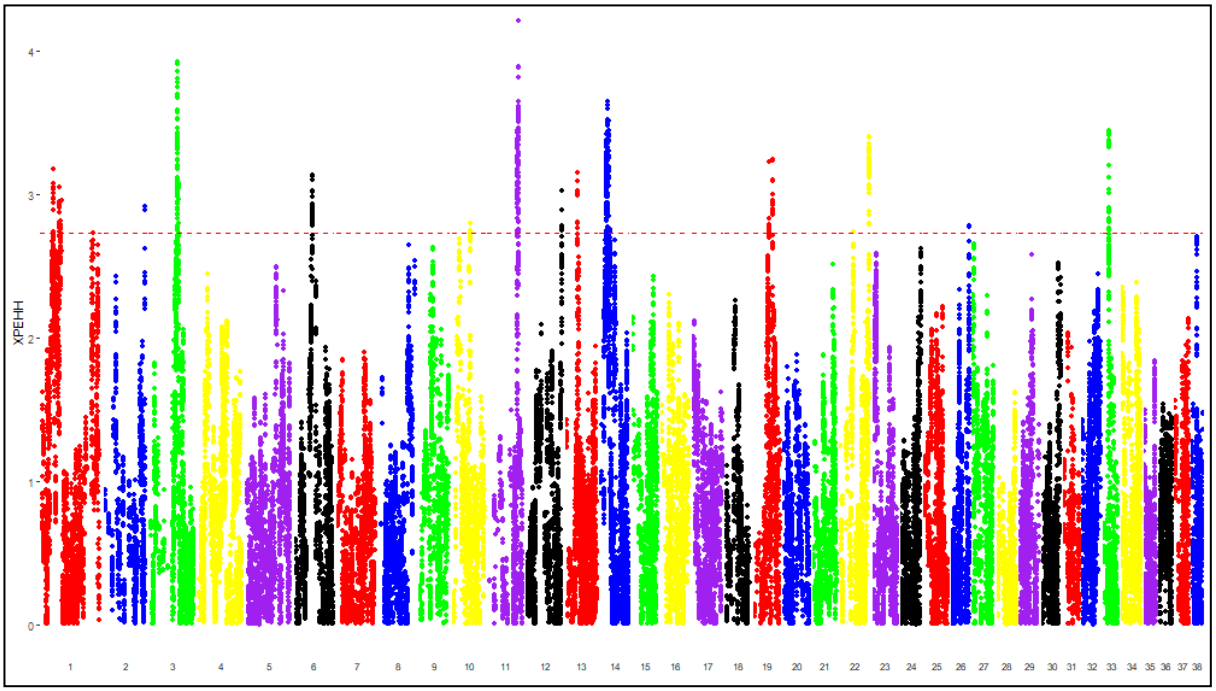

Figure 2. Manhattan plots of the cross-population extended haplotype homozygosity (XP-EHH) analysis comparing cases vs. controls. Manhattan plot of log10 XP-EHH values. Each SNP is represented by a dot. Each chromosome is represented by a different color. The dotted red line represents the cut-off value of the top 1%, equal to 2.73. The genomic regions in which SNPs were mapped over the dotted red line were considered as significantly different between the two compared groups.

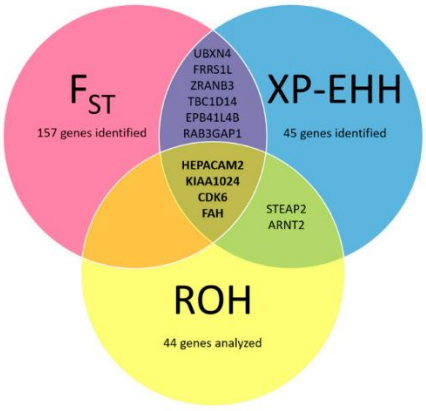

Figure 3. Venn diagram representing the genes identified by the FST, XP-EHH, and runs of homozygosity (ROH) analyses. The SNPs with the top 1% values of FST and XP-EHH were mapped on genomic regions containing 157 and 45 genes, respectively. The intersection between the two circles represents the consensus genes. For a subset of 44 genes, highlighted by the aforementioned analyses (see Supplementary Materials, Table S1), ROH were investigated: ROH including the STEAP2, HEPACAM2, and CDK6 genes were found in at least 50% of cases and no more than 9% of controls; ROH including the ARNT2, KIAA1024, and FAH were present in almost 80% of cases but only in approximately 40% of controls.

Pathway analysis

To better understand the functions performed by each identified gene, single genes were evaluated in relation to the pathways they are involved in. This allowed classification of 34 of the aforementioned genes in the following most relevant pathways: the Wnt signaling pathway, apelin pathway, hippo signaling pathway, ErbB and epidermal growth factor receptor (EGFR), transforming growth factor β (TGF-β) signaling pathway, endothelins, aldosterone, renin, and body mass index. All details are reported in Table 4.

Table 4. Pathways and heart diseases associated by Enrichr to the genes identified by our genomic analyses.

Pathway or disease	P value	Adjusted P value	Associated genes	Library	Associated genes
Wnt signalling pathway	0.002	**0.040**	Top 1% F_{ST}	KEGG 2019 Human	CREBBP, TCFL1, SMAD3, AXIN1, WNT2, PLCB2
	0.0003	**0.031**	Top 1% F_{ST} + XP-EHH	Wikipathways 2019 mouse	CREBBP, TCFL1, PPP2R2C, AXIN1, PRKD1, WNT2
Hippo signalling pathway	0.002	**0.040**	Top 1% F_{ST}	KEGG 2019 Human	LATS1, TCF7L1, SMAD3, AXIN1, CTNNA3, WNT2
	0.001	**0.028**	Top 1% F_{ST} + XP-EHH	KEGG 2019 Human	LATS1, TCFL1, SMAD3, PPP2R2C, AXIN1, CTNNA3, WNNT2
Apelin	0.001	**0.026**	Top 1% F_{ST}	KEGG 2019 Human	ADCY9, SMAD3, ITPR2, ADCY2, PLCB2, SLC8A2
	0.002	**0.050**	Top 1% F_{ST} + XP-EHH	KEGG 2019 Human	ADCY9, SMAD3, ITPR2, ADCY2, PLCB2, SLC8A2
ErbB and EGFR	0.0002	**0.047**	Top 1% F_{ST}	BioPlanet 2019	ADCY9, PDPK1, PDE1A, ITPR2, NRG1, ADCY2
	0.0002	**0.047**	Top 1% F_{ST}	BioPlanet 2019	
TGF-β	0.009	1.000	Top 1% XP-EHH	BioPlanet 2019	ARNT2, **CDK6**, LPAR1, CTNNAL1, STEAP2
Body Mass Index	0.0002	0.313	Consensus	GWAS catalog 2019	**ZRANB3**, **EPB41L4B**, **FRRS1L**, **UBXN4**, **RAB3GAP1**
Endothelins	0.003	0.158	Top 1% F_{ST} + XP-EHH	BioPlanet 2019	ADCY9, ADCY2, PLCB2, BCAR1
Aldosterone	0.0001	**0.008**	Top 1% F_{ST}	KEGG 2019 Human	ADCY9, ITPR2, ADCY2, PRKD1, PLCB2, CACNA1H
Renin	0.0002	**0.008**	Top 1% F_{ST}	KEGG 2019 Human	ADCYAP1R1, PDE1A, PDE3A, ITPR2, PLCB2

Platelet activation	0.003	**0.046**	Top 1% F_{ST}	KEGG 2019 Human	ADCY9, ITPR2, ADCY2, TLN2, PLCB2

Significant adjusted p-values and consensus genes are indicated in bold. EGFR: epidermal growth factor receptor; TGF-β: transforming growth factor β. Adjusted p-values were automatically calculated by Enrichr using the Benjamini–Hochberg method for correction for multiple hypotheses testing.

9.2.3. Discussion

Among all dog breeds, CKCS stand out for being the breed with the highest prevalence and the earliest onset of MMVD [1,3]. Therefore, the purpose of this study was to identify selection signatures that can distinguish between subjects with a diagnosis of MMVD at a very young age (before 5 years) and subjects in which this pathology may appear at an older age (after 5 years) or otherwise persist at a milder stage for a long time (after 8 years). Following the calculation of FROH in cases and controls, we verified that the degree of inbreeding of the two groups was similar. Moreover, our results were superimposable to those found in other studies on various canine breeds [44–46]. The identification of consensus genes was reached using two independent methods that analyse different genomic characteristics: FST highlights genomic differences between groups in terms of expected heterozygosity, whereas XP-EHH is based on the comparison of regions of homozygosity that differentiate the groups. Moreover, ROH analysis on the regions surrounding these genes showed a different presence of long homozygous portions of DNA between the cases and controls. From this analysis, we observed that ROH containing the HEPACAM2and CDK6 genes were present in 50% of cases and only 5% of controls. It could be hypothesized that these genes may be involved in predisposition to rapidly progressing MMVD rather than its early onset. A follow-up of the dogs included in the case group may clarify if the progression of the disease in subjects in which ROH were found around the aforementioned genes is quicker than in subjects in which they were not present. Another possible reason is that the cases in which ROH were absent are heterozygous carriers of the allele that contributes to MMVD predisposition. KIAA1024 and FAH genes were also relevant, because ROH flanking them were found in the genome of 80% of cases but only in

approximately 40% of controls. A possible explanation of the presence of controls showing a ROH around these genes is that they might have been misclassified due to the mitral valve pathophysiology, which makes it difficult to detect a real control. To better describe their possible role in MMVD onset and progression, an accurate investigation about consensus genes was performed. Some of them were shown to be directly or indirectly related to mechanisms already supposed to be involved in the disease's pathogenesis, such as the TGF-β signaling pathway, or to processes related to heart development or functionality. The most relevant consensus genes are described below. HEPACAM2 interacts with FGFR1 (fibroblast growth factor receptor 1), which is associated with abnormal heart development. Moreover, during adult life, valves maintain a pool of mesenchymal cells responsive to FGF and producing proteoglycans, which are also increased during MMVD [47]. It should be noted that HEPACAM2 may localize on the region (CFA 14q1.3) that Madsen et al. (2011) found to be associated to MMVD [3]. FAH interacts with ADAMTSL4 (ADAMTS like 4), which is supposed to facilitate FBN1 (fibrillin 1) microfibril biogenesis [48]. FBN1 is one of candidate genes for MMVD predisposition, because it regulates TGF-β signaling and is associated with Marfan syndrome, which represents one of the syndromic forms of human mitral valve prolapse [49]. CDK6 prevents cell proliferation and negatively regulates cell differentiation but is required for the proliferation of specific cell types. Moreover, it interacts with CDKN2B (cyclin-dependent kinase inhibitor 2B), whose expression was found to be induced by TGF-β and is associated with coronary heart disease [50]. EPB41L4B promotes cellular adhesion, migration, and motility in vitro and may play a role in wound healing [51]. This gene interacts with the CASQ2 (calsequestrin 2) gene, which encodes a protein localized in cardiac muscle cells that stores calcium for muscle function [52]. Mutations in this gene cause catecholaminergic polymorphic ventricular tachycardia [53]. It is interesting to note that some genes were associated with height (CDK6 and ZRANB3) or body mass (FRRS1L, EPB41L4B,

and ZRANB3) in humans. It has been well established that small dog breeds are predisposed to MMVD [54,55]. Moreover, people affected by mitral valve prolapse tend to have a low body mass index and be leaner and shorter than other individuals [56,57]. A morphometric evaluation of CKCS could allow identification if the selection for specific physical body features is related to the predisposition to the disease. In fact, the circumference of the thorax has already been negatively correlated with mitral valve prolapse in Dachshunds [8]. For the other genes able to significantly distinguish between cases and controls, reported in Table 2, no evident correlation with MMVD was found. The main pathways associated with the genes identified by this study appear to be involved in processes related to heart development and homeostasis, as reported by several studies on humans, mice, and dogs. For example, during valvulogenesis, TGF-β is fundamental for the formation of endocardial cushions and epithelial-to-mesenchymal transition [58,59]. Cell migration and proliferation in these cushions requires the ErbB and Wnt canonical pathway, the latter being also important for maintaining the pool of undifferentiated mesenchymal cells responsive to FGF, even in adult life [47,60–62]. Valvular interstitial cell (VIC) activation to myofibroblasts, one the most accredited pathogenetic mechanisms of MMVD, seems to be stimulated by TGF-β and inhibited by apelin [63,64]. Canine VIC exposition to TGF-β3, in fact, has been shown to be able to regulate myofibroblast activation and proteoglycan synthesis in an in vitro system [65]. Moreover, the knockdown of ErbB or Axin2 (which represents one of the principal Wnt canonical pathway inhibitors) determined the development of hyperplastic and myxomatous valves in mice in some studies [66,67]. The TGF-β pathway is involved in many human cardiovascular diseases, such as the Marfan, Ehlers–Danlos, Loeys–Dietz, and aneurysms–osteoarthritis syndromes, whose phenotypes often feature mitral valve prolapse [68–70]. It is worth mentioning that a study demonstrated that the expression of TGF-β 1 and 3 in cells and the extracellular matrix (ECM) was increased in MMVD-affected canine mitral valves [71]. It has been speculated that the endothelium can

be involved in MMVD pathogenesis as well. Its damage upregulates endothelin and nitric oxide, which are involved in the production and alteration of ECMs [72,73]. Moreover, an increase in endothelin receptors has been proven to be associated with MMVD in canine mitral valves [74,75], and with age and mechanical stress in porcine ones [74,75]. Regarding renin and aldosterone, it is well documented that, when the stroke volume decreases due to MMVD progression, several neuro-endocrine compensatory mechanisms are activated, including the renin–angiotensin–aldosterone system (RAAS) [76]. Although this system contributes to the maintenance of blood pressure in heart failure, it is one of the main targets of MMVD therapy due to its role in the development of congestive heart failure itself [77,78]. Finally, the role of platelet activation has not yet been clarified. Human patients with a mitral valve prolapse seem to be more at risk of thromboembolism [79–81] and present increased platelet activation [82,83]. In dogs, instead, no significant association has been found between MMVD and thrombus formation [84], and, in most of the studies, platelet functionality has been decreased in canines [85–87].

9.2.4. Conclusion

Several mechanisms have been thought to contribute to the development of MMVD, such as the TGF-β signaling pathway [65,71], increases in serotonin receptors [88–91], alterations of ECM organization [65,71,89,92], endothelial damage [72,84,89,93–95], and oxidative stress [89]. The present genomic study involves a relatively small number of cases and controls. The high prevalence of MMVD in CKCS, even at a young age, as well as the variability in its progress, makes it difficult to recruit a great number of subjects that can be clearly categorizable as either cases or controls. However, the careful selection of cases and controls among a larger population of dogs made possible to identify genes and pathways potentially involved in the pathogenesis of early-onset MMVD in the CKCS dog breed. Particularly, our findings are consistent with the hypothesis that the role of TGF-β, ECM disruption, and/or endothelin may

be relevant and deserve further investigation. Genomic studies are not only important to identify genes associated with MMVD predisposition, but also to predict the loss of genetic diversity of the breed following the exclusion of reproduction of dogs with specific phenotypical features. Indeed, a breeding program should not exclude more than 50% of the dog population [96] and no more than 30% when screening for a single disease [97]. For example, since the prevalence of MMVD is highly dependent on age, it would be important to choose an age limit whereby dogs with early onset of MMVD can be excluded from reproduction. On the other hand, to place the limit at an advanced age would result in the exclusion of an excessive number of animals for breeding. The investigation of the genetic basis of canine MMVD would surely benefit from an increase in the number of samples, which would also permit the analysis of sexual chromosomes (a X-linked form of mitral valve prolapse has, in fact, been found in human [98]). Moreover, a follow-up study conducted on ACVIM B1 patients could allow us to evaluate how MMVD progresses in these dogs. This will be helpful to identify genomic haplotypes associated with early-onset and rapidly progressing MMVD predispositions that, together with morphometric, clinical, and echocardiographic characterizations, could be used as part of a screening program for CKCS, defining early selection criteria for the exclusion of subjects from breeding. In this respect, it would be important to include collaboration with the veterinary medical community, which may inform us about dogs that should particularly be included in further genomic studies as either a case or control sample.

9.2.5. References

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9.3. Echocardiographic evaluation of the mitral valve in Cavalier King Charles spaniels

Bagardi, M.; Bionda, A.; Locatelli, C.; Cortellari, M.; Frattini, S.; Negro, A.; Crepaldi, P.; Brambilla, P.G. Animals (Basel). 2020 Aug 19;10(9):1454. doi: 10.3390/ani10091454.

Cavalier King Charles spaniels (CKCS) show the earliest onset and the highest incidence of myxomatous mitral valve disease (MMVD), compared with other breeds [1–5]: it involves almost all subjects over eleven years [6] as well as many dogs under four years of age [7]. At present, there is no treatment for the disease itself that can be applied on a large population of dogs (i.e., valve replacement and repair surgery), so prevention is the only strategy to reduce its incidence.

The etiology of the MMVD has not been fully clarified yet, but in CKCS the hereditary component plays a predominant role. Other factors, such as exercise level, degree of obesity and diet could contribute to the development of the disease, although with a minor influence [8]. Parental MMVD status is an important factor influencing the probability of occurrence of heart murmurs and their intensity in offspring in this breed [9]. Myxomatous mitral valve disease development is a polygenic threshold trait, and the sex of the offspring influences threshold levels: more males than females develop murmurs [9]. This would explain why, in the same family, males generally present the disease at a younger age. Moreover, early MMVD, typically found in CKCS, also appears to be highly heritable [10]. In order to reduce the incidence of the disease, it would be advisable to distinguish CKCS in: non-genetically predisposed, predisposed to age-related MMVD (typical of many breeds), and with breed predisposition to its early onset [10]. Up to now, there is no genetic test able to discriminate among these three groups. For this reason, CKCS breeding plans are based only on the cardiac phenotype of the breeders. However, the selection protocols put in place so far in several

countries proved to be ineffective when based only on auscultatory findings, while the results were more promising when echocardiographic data were considered as well [11–13].

Since the prevalence of MMVD is highly dependent on age, it would be important to choose an age limit whereby dogs with early onset of MMVD can be excluded from reproduction. In fact, to place it at too advanced age would result in the exclusion of an excessive number of breeders, and it is not advisable to exclude more than 30% of the dogs from the breeding for a screening program for a single disease [14]. Furthermore, an estimation of the genetic value would allow to exclude predisposed dogs before their reproduction and to get a predictive evaluation on animals whose health status has not been recorded yet [15].

As reported by Menciotti et al. (2018) [16], a 3D transthoracic echocardiography of the mitral valve of healthy young adult CKCS showed that leaflet tenting and the posterior leaflet were reduced in comparison with dogs of other breeds. These morphologic differences could predispose this breed to the early onset of MMVD [16]. Identification at a young age of dogs at high risk of adverse outcome in the future is desirable [17]. Hence, this breed, in particular subjects in ACVIM class B1 which represent the majority of CKCS undergoing our voluntary recruitment screening program, should be the object of a dedicated study aimed at associating valve morphology with genomics, to determine the peculiarities of CKCS who will develop the MMVD earlier and will present an adverse event related to heart disease. Early predictors would also allow for improved breeding recommendations. Previously published data on the echocardiographic anatomy of the mitral valve in dogs are limited to normal valves in seven healthy Norfolk terriers [18] and in sixty MMDV-affected dogs of different breeds [19].

The main objective of this study was to analyze the echocardiographic features of MMVD affected CKCS in ACVIM B1. We also provided a description of echocardiographic findings related to dogs in different ACVIM classes. Two-dimensional (2-D) echocardiography was chosen for this study, due to its widespread diffusion and easy use by most echocardiographers.

Particularly, the mitral valve morphology and the degree of leaflets prolapse were described to characterize the echocardiographic anatomy of the CKCS mitral valve apparatus. Secondarily we compared echocardiographic data in ACVIM class B1 dogs divided according to age at time of MMVD diagnosis, to understand if different aged subjects had different echocardiographic patterns [20,21]. Our goal is to assess whether there were different echocardiographic patterns within the ACVIM class B1. This could lead us to classify valvular lesions and cardiac morphologies typical of the young CKCS predisposed to the early onset of MMVD compared with the older subjects.

9.3.1. Materials and Methods

This prospective cross-sectional study was carried out including 90 privately owned CKCS visited at the Cardiology Unit of the Veterinary Hospital - Department of Veterinary Medicine, University of Milan, between December 2018 and September 2019. The informed consent was signed by the owners, according to the ethical committee statement of the University of Milan number 2/2016. Each dog underwent a clinical and cardiological examination (including a complete echocardiographic exam). Dates of birth were verified by checking each animal's microchip number in the regional registry. Auscultatory findings were evaluated by three well-trained operators: the presence/absence, timing, intensity (0 = absent; 1 = I-II left systolic; 2 = III-IV bilateral systolic; 3 = V-VI bilateral systolic), and the point of maximum intensity of the murmur were recorded. Blood pressure was indirectly measured with a Doppler method according to the ACVIM consensus statement [22].

Echocardiographic examination (2-D, M-mode, spectral, and color-flow Doppler) was performed using MyLab50 Gold cardiovascular ultrasound machine equipped with multi-frequency phased array probes (3.5-5 and 7.5-10 MHz), chosen according to the weight of the subject. The exam was performed conforming to a standard procedure [23]. Video clips optimized for the visualization of mitral valve apparatus were acquired and stored using the

echo machine software for off-line measurements. All measurements of interest were repeated on three consecutive cardiac cycles, and the mean value was used in the statistical analysis [24]. Measurements of the same stored images were repeated in a randomly selected subset of six CKCS by MB one week after the initial measurements. These data were used to assess intra- and inter-observer repeatability. Diagnosis of MMVD was based on 2-D and color Doppler echocardiographic findings: typical lesions of the mitral valve apparatus and a demonstrated mitral regurgitation (MR) on the color Doppler echocardiogram were considered as the definitive diagnostic criteria [24]. The right parasternal four-chamber long axis view is considered the standard view for the assessment of the canine mitral valve [19,25–27] and of the mitral annulus [19].

The following measures were obtained from this view: the sphericity index (SI), the length (AMVL), width (AMVW) and area (AMVA) of the anterior mitral valve leaflet, and diameters of the mitral valve annulus in diastole (MVAd) and systole (MVAs). Moreover, the degree of MV prolapse and regurgitation were studied. The SI was calculated as the ratio of the left ventricle (LV) long-axis diameter to short-axis diameter in end-diastole [28] and used as indicator of LV remodelling. The AMVL, AMVW and AMVA were measured (in centimeters) during diastole, when the leaflet was fully extended, whereas the MVAd and MVAs in the first frame respectively after closing (end-diastole) and before opening (end-systole) of the leaflets [19]. Particularly, MVAd and MVAs were obtained measuring the distance from mitral valve hinge point to hinge point, with frame-by-frame advancement utilized to accurately identify their location [19]. All these measurements (AMVL, AMVW, AMVA, MVAd and MVAs) were indexed to body weight using the scaling exponents calculated by Wesselowski for each specific valve measure [19]. They were respectively: 0.37, 0.41, 0.78, 0.37 and 0.40.

Mitral valve prolapse was considered mild if the leaflets were prolapsing but did not cross the line joining their pivotal points (line P), moderate if protruded between the P line and the line

joining half of the echoic areas located in the lower part of the atrial septum at the level of atrioventricular junction (T line), severe if the leaflets exceeded the T line [29] (Fig 1). Lastly, MR was assessed by color Doppler, calculating the maximal ratio of the regurgitant jet area signal (ARJ) to left atrium area (LAA) (ARJ/LAA ratio) in left parasternal long axis view [24].

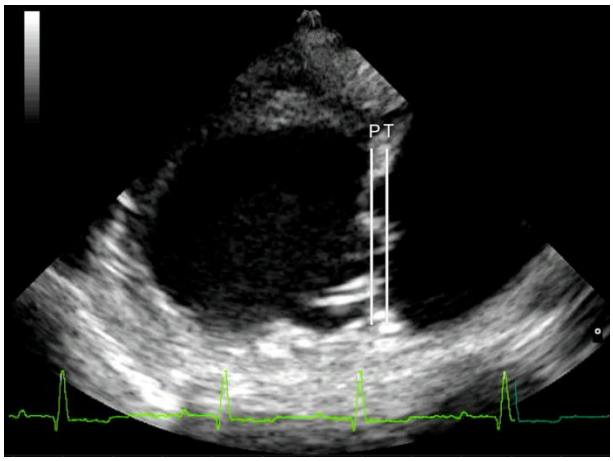

Figure 1. Right parasternal long axis four chamber view of a CKCS affected by MMVD in ACVIM class D; severe mitral valve prolapse was assessed by the protrusion of one or both leaflets over line T. The arrows outline the severity of mitral valve prolapse and the affected leaflet. Line P is drawn from the hinge point of the anterior leaflet to the hinge point of the posterior leaflet. Line T is drawn from the middle of the elliptical echogenic area at the lower part of the atrial septum to the atrioventricular junction, i.e. the junction between the left ventricular wall/annulus fibrosis and the left atrial wall.

Regurgitation was considered mild if it occupied less than one third of the atrium, moderate if between one and two thirds, severe if more than two thirds [24]. The following measurements were taken from the right parasternal short-axis view: the left atrial to aortic root ratio (LA/Ao) was obtained by 2-D technique, whereas the left ventricular diameters were measured in M-mode according to the leading-edge-to-leading-edge method [30,31].

Left ventricular end diastolic (EDV) and end systolic volumes (ESV) were calculated by the Teichholtz method and values were successively indexed for body surface area (BSA) to obtain the end-diastolic (EDVI) and the end-systolic (ESVI) volume indexes [32]. The left ventricular internal diameters in diastole and systole normalized for body weight (LVIDdN and LVIDsN respectively) were calculated using the allometric equation, as previously described [33]. Left ventricular fractional shortening (%FS) was calculated using the formula [(LVIDd-

LVIDs)/LVIDd] x 100. Trans-mitral flow [E peak velocity (E-Vmax), A peak velocity (A-Vmax), E-Vmax to A-Vmax ratio (E/A)] was measured using pulsed-wave Doppler (PWD) from the left four-chamber apical view.

Dogs were staged and stratified according to ACVIM guidelines [34]. Subjects in class B1 were also allocated in age-related groups, according to the age at time of MMVD diagnosis: up to three years (group 1), between three and six years (group 2) and over six years old (group 3).

Analysis of genealogical data

The pedigrees were obtained consulting the on-line genealogy book of the Ente Nazionale della Cinofilia Italiana (ENCI) (http://ww.enci.it/libro-genealogico/libro-genealogico-on-line#) or the Livre des Origines Français (LOF) select of the Centrale-canine site (https://www.centra-canine.fr/lofselect). The genealogical data were reported on an Excel worksheet and then analyzed with OPTISEL®, a library of free downloadable R® program (https://CRAN.R-project.org/package=optiSel). The following parameters were analyzed: inbreeding coefficient (F) (probability that both alleles inherited from an individual are copies of a single allele from an ancestor common to both parents), average relatedness coefficient (AR) (probability that a randomly chosen allele in the whole pedigree population belongs to a given animal), pedigree Completeness (proportion of known ancestors in each generation) and depth of the pedigree defined by the number of fully traced generations (in which all the ancestors are known) and the number of maximum generations traced (in which at least one ancestor is known).

Statistical analysis

Statistical analyses were performed using SPSSTM 26.0 (IBM, SPSS, USA). Descriptive statistics was generated. The distribution of data for continuous variables was assessed for normality by means of the Kolmogorov-Smirnov test. The variables were reported as mean ± standard deviation in case of normal distribution, otherwise as the median and interquartile range (IQR - 25th to 75th percentile). The correlation was considered weak, moderate, or strong

respectively when the value of the correlation coefficient was less than 0.3, between 0.3 and 0.7, or more than 0.7. The associations between continuous and categorical variables were investigated with $\chi 2$ test and their strength was evaluated with the coefficient of determination (r2). The comparison between continuous and categorical variables was assessed with Kruskal-Wallis test and the significance values have been adjusted according to the Bonferroni correction. Intra-observer and inter-observer repeatability of all variables obtained from the 4-chamber right parasternal long-axis image plane (AMVL, AMVW, AMVA, MVAd and MVAs) were further characterized through calculation of coefficients of variation (CV %).

9.3.2. Results

Clinical and genealogical results

Ninety CKCS were included; 28 dogs belonged to private owners and 62 to eight different breeders. The population consisted of 60 females (26 neutered), and 30 males (2 neutered). Age ranged from 0.5 to 11.7 years (5.67±2.75 y). Males' and females' mean ages were not significantly different. The weight ranged from 5.1 to 12.8 kg (9.13±1.94). Weight was mild positively related to age (r2 = 0.18, P = 0.000). Mean weight in males (9.72±0.35 kg) was significantly higher (P = 0.042) than in females (8.84±0.25 kg). Among the 90 dogs included in the study, 62 (68.9%) were registered into ENCI website or Centrale-canine database. For 28 subjects (31.1%) it was not possible to find any information about their genealogy. The pedigree analysis of the 62 pedigrees identified a total of 1207 animals, consisted of the dogs in our sample and all their known ancestors. The number of fully traced generations of the 62 pedigrees of our study's dogs was on average 3.77, ranging from 2 to 5 and the number of maximum generations traced was on average 10.61, ranging from 6 to 14. Average relatedness coefficient mean values of all 1207 subjects and of the 62 dogs included in this study were respectively 0.006 and 0.055. The mean F value for all 1207 subjects was 0.005, ranging from

0 and 0.25, whereas for the 62 dogs included in this study the mean F was 0.018. There was not a significant association between F and all analyzed variables.

Eighty-one (90%) dogs were affected by MMVD. The subjects were classified as follows: 9 (10%) in ACVIM class A, 64 (71%) in class B1, 11 (12%) in class B2, 6 in class C/D (7%). In 59% of included dogs a heart murmur was detected at auscultation; heart murmur intensity was highly positively correlated with ACVIM class ($r2 = 0.75$, $P = 0.000$) and moderately with the age ($r2 = 0.61$, $P = 0.000$). There were no discrepancies in the three operators' assessment of presence/absence and intensity of heart murmurs. The presence of the murmur did not discriminate between A and B1 classes ($P = 0.128$): in 44% of B1 subjects and heart murmur was not audible. In 76% of dogs without heart murmur there was an echocardiographic diagnosis of MMVD. Age was moderately positively related to ACVIM class ($r2 = 0.52$, $P = 0.000$). Although sex was not related to the presence of MMVD ($P = 0.456$) and ACVIM class ($P = 0.083$), heart chambers enlargement (class B2 and C/D) was statistically more frequent in males ($r2 = 0.93$, $P = 0.02$). The severity of mitral regurgitation was highly significantly related to the ACVIM class ($r2 = 0.78$, $P = 0.000$).

Echocardiographic data

Regarding the echocardiographic measurements, the intra-observer and the inter-observer (MB vs CL) coefficients of variation (CV range in %) were <10% and <20% respectively for each tested variable. Table 1 shows LA/Ao, E/A, EDVI, ESVI, FS%, LVIDdN and LVIDsN of all included subjects expressed in relation to ACVIM class. In affected dogs LA/Ao and LVIDdN were significantly greater (respectively $P = 0.011$ and $P = 0.017$) in males than in female; in healthy dogs no differences were observed between sexes.

Table 1. Clinical and echocardiographic parameters of all included subjects related to ACVIM classes.

Clinical and echocardiographic parameters of all included subjects													
		F	**M**	**Age (y)**	**Weight (kg)**	**HM**	**LA/Ao**	**E/A**	**ESVI (ml/m^2)**	**EDVI (ml/m^2)**	**%FS**	**LVIDdN (cm/kg)**	**LVIDsN (cm/kg)**
A (n. 9)	n.	7	2	9	9	0	9	9	9	9	9	9	9
	Mean			2.86	7.41						35.11		
	SD			1.89	1.13						9.23		
	Median						1.19	1.24	14.28	41.22		1.22	0.76
	IQR						1.11-1.33	1.09-1.35	9.95-18.2	37.88-53		1.17-1.33	0.67-0.84
B1 (n. 64)	n.	46	18	64	64	36	64	62	64	64	64	64	64
	Mean			5.36	9.23						34.42		
	SD			2.56	1.77						6.15		
	Median						1.23	1.30	20.99	55.28		1.36	0.89
	IQR						1.11-1.31	1.16-1.42	16.1-25.93	48.2-69.77		1.29-1.49	0.8-0.95
B2 (n. 11)	n.	4	7	11	11	11	11	11	11	11	11	12	12
	Mean			8.31	9.51						42.82		
	SD			1.61	2.29						6.78		
	Median						1.70	1.44	27.88	118.46		1.85	0.98
	IQR						1.59-1.65	1.31-1.46	22.72-34.93	104.65-113.64		1.82-1.94	0.90-1.07
C/D (n. 6)	n.	3	3	6	6	6	6	6	6	6	6	6	6
	Mean			8.49	10.02						46.16		
	SD			0.78	2.82						4.62		
	Median						1.96	1.59	39.28	182.74		2.21	1.13
	IQR						1.81-2.28	1.62-2.18	31.72-49.20	149.01-241.52		2.04-2.50	1.03-1.25

F = females; M = males; HM = n. of subject with heart murmur in each ACVIM class; LA/Ao = left atrium to aorta ratio; E/A = E and A waves ratio; ESVI = end-systolic volume index; EDVI = end-diastolic volume index; %FS = shortening fraction; LVIDdN = left ventricular internal diameter in diastole normalized for body weight; LVIDsN = left ventricular internal diameter in systole normalized for body weight. The variables are reported as mean and standard deviation (SD) in case of normal distribution, otherwise as the median and interquartile ranges (IQR - 25th to 75th).

In the whole population, 83 (92.2%) had a MV prolapse: 42 dogs showed a mild, 35 a moderate and 6 a severe prolapse. The anterior leaflet was always involved; in 77.1% of cases the prolapse also affected the posterior one. No significant correlations were found between prolapse severity and ACVIM class (P = 0.210) or MR severity (P = 0.310).

Table 2 shows all indexed mitral valve measurements and their significant differences in relation to each ACVIM class. Anterior mitral valve leaflet length (AMVL) was longer in class B2 compared to A and in classes B2 and C/D compared to class B1. The same was observed for AMVW and AMVA: they were greater in classes B2 and C/D than in classes A and B1. Mitral valve annulus in diastole (MVAd) and systole (MVAs) were larger in class C/D than in classes A and B1. No statistically significant differences were found for mitral valve measurements between A and B1 classes and between B2 and C/D classes. Moreover, MVAd (P = 0.004) and MVAs (P = 0.003) were larger in males and MVAd was significantly larger in neutered females than in entire females (P = 0.007).

Table 2. Indexed mitral valve measurements of all analyzed subjects related to ACVIM classes.

Indexed mitral valve measurements for each ACVIM class								
			AMVL (cm/kg)	**AMVW (cm/kg)**	**AMVA (cm/kg)**	**MVAd (cm/kg)**	**MVAs (cm/kg)**	**SI**
STAGE	**A**	n.	8	8	8	8	8	8
		Median	0.75†	0.14	0.09	0.76	0.56	1.38
		IQR	0.66-0.79	0.11-0.15	0.07-0.10	0.68-0.87	0.52-0.64	1.31-1.50
	B1	n.	62	61	61	64	64	64
		Median	0.69	0.15	0.09	0.85	0.62	1.38
		IQR	0.63-0.77	0.12-0.17	0.07-0.11	0.75-0.93	0.55-0.73	1.24-1.48
	B2	n.	11	11	11	11	11	11
		Median	0.91*	0.19*†	0.13*†	0.94	0.71	1.27
		IQR	0.87-0.96	0.18-0.21	0.11-0.15	0.85-1.03	0.64-0.79	1.17-1.36
	C/D	n.	6	6	6	6	6	6
		Median	0.94*	0.22*†	0.16*†	1.23*†	1.00*†	1.15*†
		IQR	0.87-1.00	0.19-0.31	0.13-0.21	1.13-1.44	0.71-1.24	1.03-1.25

All measurements were indexed to body weight using the scaling exponents calculated by Wesselowski, one for each specific valve measure (Wesselowski et al. 2015). They were respectively: 0.37, 0.41, 0.78, 0.37 and 0.40 for anterior mitral valve length (AMVL), width (AMVW), area (AMVA), mitral valve annulus in diastole (MVAd) and systole (MVAs). SI = sphericity index. The variables were not normally distributed and are reported as median and interquartile ranges (IQR - 25th to 75th). *Within a column, value differs significantly ($P < 0.01$) from ACVIM class B1. †Within a column, value differs significantly ($P < 0.01$) from ACVIM class A.

Clinical and echocardiographic parameters of subjects in ACVIM class B1 divided according to age-related classes are reported in Table 3. The results of the statistical analysis of these variables are reported in Table 4.

Table 3. Statistically significant differences between clinical findings, indexed echocardiographic measurements and SI of subjects in ACVIM class B1 categorized considering the age at time of MMVD diagnosis.

Clinical and echocardiographic parameters of subjects in ACVIM class B1 divided according to age-related classes																	
Sex	**Age (y)**	**Heart murmur severity**	**Weight (kg)**	**ESVI (ml/m^2)**	**EDVI (ml/m^2)**	**LA/Ao**	**E/A**	**FS%**	**LVIDdN**	**LVIDsN**	**MR**	**MVP**	**SI**	**AMVL**	**AMVW**	**AMVA**	**MVAd**	**MVAs**
Group 1																		
4eM, 6eF	1.90 ± 0.77	0 (n.9) 1 (n.1)	7.85 ± 1.25	20.73 ± 5.97	56.30 ± 18.40	1.13 ± 0.13	1.44 ± 0.24	32.20 ± 5.01	1.36 ± 0.17	0.88 ± 0.09	0 (n.3) 1 (n.7)	0 (n.1) 1 (n.7) 2 (n.2)	1.36 ± 0.27	0.70 ± 0.07	0.13 ± 0.02	0.08 ± 0.02	0.79 ± 0.12	0.62 ± 0.09
Group 2																		
15eF, 7nF, 7eM, 1nM	4.45 ± 1.03	0 (n.13) 1 (n.16) 2 (n.1)	9.36 ± 1.55*	20.32 ± 7.78	57.77 ± 16.24	1.21 ± 0.13	1.33 ± 0.24	34.23 ± 6.76	1.37 ± 0.16	0.86 ± 0.13	0 (n.4) 1 (n.25) 2 (n.1)	1 (n.22) 2 (n.7) 3 (n.1)	1.40 ± 0.21	0.71 ± 0.13	0.15 ± 0.04	0.09 ± 0.03	0.82 ± 0.11	0.60 ± 0.11
Group 3																		
6eF, 12nF, 5eM, 1nM	8.07 ± 1.63	0 (n.6) 1 (n.11) 2 (n.7)	9.97 ± 2.18*	22.80 ± 8.25	64.90 ± 19.96	1.26 ± 0.13	1.21 ± 0.21	35.58 ± 5.70	1.43 ± 0.17	0.90 ± 0.13	1 (n.17) 2 (n.6) 3 (n.1)	0 (n.1) 1 (n.9) 2 (n.14)	1.22 ± 0.3†	0.72 ± 0.11	0.16 ± 0.03*	0.10 ± 0.03*	0.90 ± 0.09*†	0.69 ± 0.10†

Age groups: Group 1) under 3 years (n.10 – 15.6%); Group 2) between 3 and 6 years (n. 30 – 46.9%); Group 3) over 6 years old (n.24 – 37.5%). The significance values have been adjusted according to the Bonferroni correction for more tests. Sex: eM = entire male, nM = neutered male; eF=entire female; nF = neutered female. Heart murmur severity: 0 = absent; 1 = I-II left systolic; 2 = III-IV bilateral systolic; 3 = V-VI bilateral systolic; LA/Ao = left atrium to aorta ratio; E/A = E and A waves ratio; ESVI = end-systolic volume index; EDVI = end-diastolic volume index; %FS = shortening fraction; LVIDdN = left ventricular internal diameter in diastole normalized for body weight; LVIDsN = left ventricular internal diameter in systole normalized for body weight; AMVL = anterior mitral valve length; AMVW = anterior mitral valve width; AMVA = anterior mitral valve area; MVAd = mitral valve annulus in diastole; MVAs = mitral valve annulus in systole; SI = sphericity index; MR = mitral regurgitation severity (0=absent; 1=regurgitant jet area (ARJ)/left atrial area (LAA)<1/3; 2 = 1/3<ARJ/LAA<2/3; 3 = ARJ/LAA>2/3); MVP = mitral valve prolapse severity (0 = absent, 1 = under P line, 2 = between P and T lines, 3 = over T line). The variables reported as mean and standard deviation (SD), and as number of subjects for each grade of severity for heart murmur, MR and MVP. *Within a column, value differs significantly ($P < 0.01$) from Group 1. †Within a column, value differs significantly ($P < 0.01$) from Group 2.

Table 4. Statistically significant differences between clinical findings, indexed echocardiographic measurements and SI of subjects in ACVIM class B1 categorized considering mitral regurgitation, mitral valve prolapse and heart murmur severity.

Pairwise comparison mitral regurgitation severity in ACVIM class B1			
	Severity class	P value	Adapted P value
Age (y)	0-2	0.001	0.008
Pairwise comparison mitral valve prolapse severity in ACVIM class B1			
	Severity class	P value	Adapted P value
Age (y)	1-2	0.002	0.009
Pairwise comparison heart murmur severity in ACVIM class B1			
	Severity class	P value	Adapted P value
Age (y)	0-1	0.005	0.016
	0-2	0.000	0.001
E/A	0-1	0.004	0.012
LVIDdN (cm/kg)	0-2	0.000	0.001
AMVW (cm/kg)	0-2	0.005	0.014
	1-2	0.007	0.021
AMVA (cm/kg)	0-2	0.001	0.003
	1-2	0.001	0.004
MVAd (cm/kg)	0-2	0.010	0.030
MVAs (cm/kg)	0-1	0.008	0.024
	0-2	0.002	0.006
SI	2-0	0.011	0.032

Mitral regurgitation severity: 0 = absent; 1 = regurgitant jet area (ARJ)/left atrial area (LAA)<1/3; 2 = 1/3<ARJ/LAA<2/3; 3 = ARJ/LAA>2/3. Mitral valve prolapse severity: 0 = absent; 1 = under P line; 2 = between P and T lines; 3 = over T line. Heart murmur severity: 0 = absent; 1 = I-II left systolic; 2 = III-IV bilateral systolic; 3 = V-VI bilateral systolic. The significance values have been adjusted according to the Bonferroni correction for more tests. LVIDdN = left ventricular internal diameter in diastole normalized for body weight; AMVW = anterior mitral valve width; AMVA = anterior mitral valve area; MVAd = mitral valve annulus in diastole; MVAs = mitral valve annulus in systole; SI = sphericity index.

Subjects in class B1 were divided in age-related classes (age at time of MMVD diagnosis): up to 3 years (group 1), between 3 and 6 years (group 2) and over 6 years old (group 3). Dogs resulted allocated 10 (15.6%) in group 1, 30 (46.9%) in group 2 and 24 (37.5%) in group 3. Anterior mitral valve width (AMVW) and area (AMVA) were greater in group 3 than group 1. Mitral valve annulus in diastole (MVAd) was greater in group 3 compared to group 1 and 2, whereas MVAs was greater in group 3 compared to group 1 only. No significant differences in mitral valve measurements were found between different sexes in ACVIM class B1.

9.3.3. Discussion

The prevalence of MMVD in our population was 90%. Despite this high prevalence, the subjects presenting cardiac remodeling and related symptoms were respectively 14% and 7%. Due to the small sample size dimension of subjects in ACVIM classes A, B2 and C/D, only the results obtained for ACVIM class B1 can be considered as statistically significant, whereas other data should be considered only descriptive for the screened population. Of included CKCS, 80% were referred for echocardiographic screening and this may explain the high number of B1 subjects presented at visit by breeders. All symptomatic subjects were at least 7.5 years old. Despite the small number of subjects in ACVIM class B2 and above, as expected, presence and severity (ACVIM class) of the disease were positively related to the age [7,14]. Patients aging over 7 years were all affected, but the disease was also diagnosed in very young dogs (50% of those under 2 years of age, all classified as ACVIM B1). These results are consistent with those reported by other authors, which indicated valvular alterations in 67% of dogs aged from 6 months to 3 years and 95% of older dogs [7]. The weight was positively related to ACVIM class ($P = 0.013$). This result contrasts with two different studies, which observed a negative correlation [15]. However, it should be considered that in these studies the significance of the correlation was mild and in our case the weight explained only 9% of the variability of the ACVIM classification. This association was probably affected by the positive correlation between weight and age of the subjects ($r2 = 0.42$, $P = 0.000$). It is very interesting to note that 64% of the included dogs weighted over 8 kg, upper limit accepted by the ENCI standard for the CKCS [35]; however, it was not possible to distinguish overweight dogs due to a lack of evaluation of subjects' body condition score (BCS). The observation of a weight greater than the ENCI standard may be due to the recruitment also of companion subjects and not only of those bred for reproduction and exposition; however, it would be advisable to investigate this issue by increasing the sample size, to determine whether the values obtained

are associated with an overweight problem or the morphology of many CKCS differs from the breed standard.

In this study, contrarily to those reported in literature [1], the disease had not a higher prevalence in males and affected males were not significantly younger than affected females. This could be justified by the higher number of females presented for breeder screening. Nevertheless, despite the small number of subjects in ACVIM classes B2 and C/D, the risk of remodelling in affected males was higher than in females: there were more males than females in ACVIM class B2 or above and affected males showed higher values of LA/Ao and LVIDdN. As reported by Misbach et al. (2014) [31], there were no significant differences between sexes in healthy dogs. These data are also to be considered only as descriptive and not as statistically significant, given the scarcity of healthy subjects included in this study.

Genealogy information could not be obtained for approximately 1/3 of the dogs included in the study. Although the number of unregistered subjects is not negligible, it should be noted that none of them was under five years of age: this could indicate that in Italy the purchase of dogs without pedigree has decreased in the last few years. The average inbreeding coefficient (F) of our sample population and their ancestors, equal to 0.5%, was lower than the one reported in the literature, which ranged from a minimum of 0.9% in Belgium to a maximum of 6.3% in CKCS born in 2018 in the UK [36]. The low relatedness among our subjects should ensure that our findings are not related to single families and popular sires' peculiarities.

In 59% of the dogs it was possible to hear a heart murmur during auscultation. Despite the association between murmur intensity and ACVIM class, the auscultation was not an eligible method for discriminating between A and B1 subjects. These data underline the importance of performing echocardiographic screening, especially in this breed. In support of this, it should be noted that the selection protocols put in place in several countries proved to be ineffective

when based only on auscultatory findings, while when taking into account the echocardiographic data the results were more promising [11–13].

The description of mitral valve anterior leaflet in each ACVIM class and particularly in B1 class allowed us to delineate quantitative echocardiographic characteristics of a population of CKCS affected by this pathology. Increases in thickness, length and area of the anterior mitral valve leaflet were evident in more severely affected patients (ACVIM classes B2 and C/D). These echocardiographic changes parallel the classic valvular remodeling reported on gross pathologic examination [37]. Additionally, there was an increase in the diameter of the MVAd in patients with more advanced MMVD, as reported by Wesselowski et al. (2015) [19]. All these findings are consistent with the expected changes associated with chronic mitral regurgitation, volume overload and enlargement of the left heart, despite the small size of B2 and C/D subjects [37]. The AMVL and AMVW obtained in this study were greater than those reported by Wesselowski et al. (2015) for each ACVIM class [19]: this is in accordance with the valvular characteristics peculiar to CKCS [16]. This finding is even more relevant considering that annular measurements were consistent with Wesselowski's, underlining a mitral valve dimension proportionally greater than heart one. Furthermore, the results of this study underline how the valvular measurements varied considerably within the same class (ACVIM B1), which showed clinical and echocardiographic characteristics of extreme heterogeneity. Firstly, heart murmur severity was associated with the age of the subjects and with the severity of mitral valve morphology alterations. Secondly, annular dilation was not expected in ACVIM B1 subjects, as this group was defined by the absence of chamber enlargement, but a modification of the annulus in older dogs was observed although remaining within the standard limits. Further investigations are needed to understand if this would predict a worst evolution of the disease. Similarly, in older B1 CKCS, a significant reduction of the SI was observed (more spherical left ventricle), although it was not associated with a significant

increase in the left atrial and ventricular dimensions. This, as described by Sargent et al. in 2015 for dogs affected by MMVD at different ACVIM stages, would be predictive of cardiac mortality. Further investigations with a larger study population could help to clarify whether additional echocardiographic valvular changes can be identified in CKCS in ACVIM class B1 and an appropriate follow-up would allow us to highlight prognostic factors related to disease worsening within this ACVIM class.

To sum up, in this study, 79% of the dogs were classified as B1 and this class included extremely different animals. In addition to the small heterogeneity in terms of MV morphology, ACVIM B1 subjects had significant variability in terms of age (from six months to over eleven years), presence and severity of mitral valve regurgitation and prolapse and presence and intensity of heart murmur. Therefore, in this breed the pathology can essentially follow two different paths. In the first case dogs are predisposed to age-related MMVD (typical of many breeds) and have a slow progression of the disease. In the second one, the disease onsets early and progresses rapidly, so that symptoms of heart failure could develop even before 8 years of age. It would therefore be useful to understand whether there are parameters that can early distinguish these two groups. In our opinion these parameters could be an association of morphological, echocardiographic, and genetic data. Therefore, the number of young symptomatic dogs and mildly affected old ones will be increased and the follow-up of ACVIM classes A and B1 will be performed in order to observe the pathology progression and to relate it also with a morphologic evaluation. The improvement of ACVIM class A subjects will be useful to identify reference intervals in healthy CKCS for the echocardiographic performed measures. Thanks to the characterization of mitral valve morphology and prolapse we were able to identify case and control subjects for a preliminary genetic analysis that will investigate MMVD related genes and pathways that are the base of the predisposition and early onset of

MMVD in this breed. In this way we will be able to address, based on clinical, echocardiographic, and genetic evidence, breeding programs that also include MMVD.

This study is not without limitations. The main limit was the uneven numerosity of the ACVIM classes. In this regard, increasing the number of subjects in the different classes would allow a more appropriate delineation of the clinical and echocardiographic profile of each of them. It must be highlighted that literature does not report reference intervals for healthy CKCS. Secondly, there was a disproportion in the number of females compared to males: this is due to the voluntary adherence to the screening program and to the greater participation of breeders compared to private owners. Furthermore, the lack of assessment of the subjects' BCS did not allow a proper evaluation of the weight and therefore a more precise indexing of the echocardiographic measurements, as well as the lack of morphometric data [15,38]. Lastly, the only use of right parasternal four chamber view for the identification of mitral regurgitation area in this study, is to be considered ad a limit.

9.3.4. Conclusion

In conclusion, this is the first study that describes measurements of the anterior mitral valve leaflet and the mitral valve annulus in the CKCS affected by MMVD at different stages. It must be highlighted that, due to the small sample size of ACVIM class A, B2 and C/D, only data regarding B1 subjects can be considered as statistically significant. Regarding ACVIM class B1 dogs, the diameter of the mitral valve annulus in systole and diastole, as well as the thickness and area of the anterior mitral valve leaflet and the sphericity of left ventricle, are greater in patients with MMVD diagnosed at advanced age. Essential will be the follow-up of these dogs to uniquely associate ventricular and valvular echocardiographic features with MMVD progression. A future aim will also be the evaluation of posterior mitral valve leaflet, both in healthy and affected CKCS.

9.3.5. References

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9.4. Echocardiographic mitral valve association with morphometric measurements in Cavalier King Charles spaniels via Inverse Probability Weighting analysis

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Myxomatous mitral valve disease (MMVD) is the most common acquired cardiac disease in canine patients [1]. Some studies indicate that there is a polygenically inherited component of the disease, and in at least some of the highly susceptible breeds, early predictors of MMVD development and progression, such as morphotype, could allow for improved breeding recommendations to be made [2-6]. As reported by Parker et al., the same genes can affect both dogs' body size and their heart development [7]. In fact, it is demonstrated that small breed dogs are more predisposed to MMVD development, especially CKCS [8]. Pedersen et al. (1999) showed that there is a negative correlation between body weight and mitral valve prolapse (MVP) in this breed [9]. Furthermore, in CKCS, MMVD is associated with earlier onset and thus with potentially greater cardiac morbidity and mortality compared to other breeds [8,10,11]. Yet, the preclinical period often varies markedly among subjects, making it challenging for clinicians to identify those that will eventually develop clinical signs [12,13]. For these reasons, the early identification of a morphotype associated with a more severe MMVD can have several advantages. This could allow clinicians to monitor dogs in a very targeted way and educate breeders regarding the selection of subjects without some phenotypical characteristics related to more severe MMVD and/or more rapid progression of the disease. This could be possible in the context of a breeding selection program, which should also consider all the heritable disorders of the CKCS.

To the best of authors' knowledge, only one study has evaluated the prevalence and severity of MVP in relation to the size of the thorax in dogs, in particular, in Dachshunds [3], but no study

has ever analyzed the association between MVP or MMVD severity and morphometric measurements in CKCS.

The aim of this study was to investigate morphometric measurements in relation to the echocardiographic features of MMVD in ACVIM class B1 CKCS [14]. This class includes the majority of the breeding population and is very heterogeneous from clinical, morphological, and echocardiographic points of view [15]. The identification of phenotypic characteristics associated with more severe forms of MMVD could be useful for setting breeding selection programs aimed at reducing the prevalence of the disease in this breed.

9.4.1. Material and Methods

Study population, research question and statistical framework

In this prospective clinical cross-sectional study, the authors carefully described the morphometry of a small Italian study population of CKCS and then evaluated the influence of body, thorax, and head dimension on different clinical features (i.e., heart murmur intensity) and echocardiographic measures/indexes of the severity of MMVD (MVP, semiquantitative evaluation of regurgitant jet size, and indexed mitral valve and annulus measurements). Furthermore, they investigated the severity of MMVD through a score assigned according to the degree of MVP, mitral regurgitation jet size, and age [8].

To investigate the association between morphometric measures and severity of MMVD, the authors used a method adopted from the causal inference framework [16]. The framework proposes methods to address causal questions accounting for confounding, which affects the association between exposure and the outcome of interest. In this study, they used inverse probability weighting (IPW) analyses that, via weighted regression modelling, adjust for confounders [17-18]. The confounders are used to estimate the probability of being exposed conditional on the values of the confounders; a function of this probability is used to construct weights. Weights are assigned to the subjects in the study population to balance them with

respect to the confounders used in the analysis. Balancing subjects in the study population allows to estimate an association that is unbiased from the confounders considered in the analysis while constructing the weights.

Inclusion Criteria and Clinical Examination

Fifty-two privately owned CKCS with asymptomatic MMVD and no cardiac enlargement (ACVIM stage B1) [14], belonging to different lineages and breeders, were recruited for enrollment in this prospective cross-sectional study. The dogs were examined during breed health screening at the Cardiology Unit of the Veterinary Teaching Hospital - Department of Veterinary Medicine - University of Milan.

All the dogs underwent physical examination, echocardiography, and morphometric evaluation. The data regarding the dates of birth and the genealogy were verified by checking each animal microchip number and family tree in the Italian regional registry and the ENCI (Ente Nazionale della Cinofilia Italiana) pedigree database. Cardiac auscultation was performed by two well-trained operators and the dogs were restrained in standing position in a quiet room by the owners. The detection of a left apical systolic murmur was not considered a mandatory inclusion criterion. The evaluated auscultatory findings were presence/absence, timing, and intensity (0=absent; 1=I-II/VI left apical systolic or soft murmur; 2=III-IV/VI bilateral systolic or moderate and loud murmur, respectively; 3=V-VI/VI bilateral systolic or palpable murmur) of murmur [19]. Unless otherwise stated, hereafter the term murmur refers to a left apical systolic murmur. The diagnosis of MMVD was based on the echocardio-graphic evidence of changes in the mitral valve leaflets (thickening and prolapse) and the presence of mitral regurgitation on color-flow Doppler [9]. To be included in the study, dogs must have no evidence of left atrial and left ventricle enlargement, defined as a left atrial-to-aortic root ratio (LA/Ao) $\geq$ 1.6 on a 2-dimensional echocardiography and as left ventricular normalized

dimensions in diastole (LVIDad) ≥ 1.7, respectively [14]. Blood pressure was indirectly measured using the Doppler method according to the ACVIM consensus statement [20,21].

Echocardiography and Assessment of Leaflet Measurements, MVP Severity and Jet Size

All echocardiograms were performed by the same operator using a MyLab50 Gold cardiovascular echocardiograph (Esaote, Genova, Italy) equipped with multi-frequency phased array probes (3.5-5 and 7.5-10 MHz), chosen according to the weight of the subject, with standardized settings. Video clips were acquired and stored using the echo machine software for off-line measurements. The exam was performed according to a standard procedure with concurrent continuous electrocardiographic monitoring [22].

The mitral valve was evaluated using both right and left parasternal long axis 4-chamber views [22,23]. Valve morphology and structures, including the presence/absence and grade of valvular prolapse, were defined. The right parasternal 4-chamber view was used for the morphological evaluation of the mitral anterior leaflet during its maximum distension in the diastole [23]. The anterior mitral valve length (AMVL), width (AMVW), and area (AMVA) were measured, as well as the mitral valve annulus in the diastole (MVAd) and systole (MVAs) in the first frame after the closing and before opening of the leaflets [23]. All the measurements were indexed according to the Wesselowski method [23]. Mitral valve prolapse was considered mild if the leaflets were prolapsing but did not cross the line joining their pivotal points (P line), moderate if they protruded between the P line and the line joining half of the echoic areas located in the lower part of the atrial septum at the level of the atrioventricular junction (T line), and severe if the leaflets exceeded the T line [24]. The sphericity index (SI) was calculated as the ratio of the LV long-axis diameter to short-axis diameter in end-diastole from the right parasternal 4-chamber long axis view; a value of SI <1.65 accounted for an increased sphericity and was considered abnormal according to the European Society of Veterinary Cardiology (ESVC) guidelines [25,26], despite the fact that the literature has not reported SI reference

values for small breed dogs, in particular for CKCS. Left ventricular end-diastolic (EDV) and end-systolic volumes (ESV) were calculated from the right parasternal long axis view using the Teichholtz method and the values were successively indexed for body surface area (BSA) in order to obtain the end-diastolic (EDVI) and end-systolic (ESVI) volume indexes [27]. The area length method was used for the calculation of 2-D-derived parameters: ejection fraction (EF%), indexed for BSA 2D-EDVI and 2D-ESVI for each patient [27]. Left ventricular normalized dimensions were calculated as described by Cornell et al. (2004) [28].

The following measurements were taken from the right parasternal short-axis view: LA/Ao was obtained in the two-dimensional view as described by Hansson et al. [29] and the left ventricular diameter was measured in M-mode with the leading edge to the inner edge method at the level of the papillary muscles. The color flow mapping of the mitral valve area was obtained from the left parasternal long axis 4-chamber view [30,31]. A pulse repetition frequency of 5 kHz was used, and the flow gain was adjusted to the maximal level without encountering background noise. The degree of MR (jet size) was assessed using color Doppler and by calculating the maximal ratio of the regurgitant jet area signal to the left atrium area (ARJ/LAA ratio) [32]. Regurgitant jet size was semi-quantitatively estimated as the percentage of the left atrial area (to the nearest 5%) that was occupied by the larger jet; it was considered to be trivial (<10%, not visible in all systolic events), trace (<10%, present in all systolic events), mild (between 10 and 30%), moderate (between 30 and 70%), or severe (>70%) [32,33]. Echocardiographic measurements were taken by one operator to reduce potential biases. All measurements of interest were repeated on 3 consecutive cardiac cycles and the mean value was used in the statistical analysis [32]. The within-day intra-observer variability in the studied variables was determined by reanalyzing the parameters measured by the same observer 3 times after the first measurement on a subset of 10 randomly selected blind exams from the database. The same

frames from the same videos were chosen for the evaluation of intra-observer variability. The intra-observer coefficients of variation (CV range in %) were <10% for each tested variable.

For descriptive purposes only, a severity score was assigned according to degree of MVP, mitral regurgitant jet size, and age of each subject, as re-ported by Stern et al. in 2015, according to the formula [(Mitral valve prolapse + Regurgitant jet size) x 5] / age [34]. Due to the age-related nature of disease severity, a continuous variable was constructed so that MMVD affecting younger dogs would be considered a more severe disease variant than the one affecting older dogs with the same level of degenerative change. The age of 5 years was chosen as a pivotal point in the breed, where dogs less than 5 years demonstrating clear evidence of disease were considered to be the most severely affected animals [34,35].

Morphometrics

Clinical and echocardiographic examinations were completed by a specific morphometric evaluation that included the assessment of the ENCI standard coat color type (Blenheim, ruby, tricolor, and black and tan) and the measurement of the body, thorax, and head of each dog. All the morphometric measurements taken, and their reference points are outlined in Table 1 [36-38]. Body size and cephalic, thoracic, and volume indexes were also calculated (Table 1) [36]. During the morphometric evaluation, the dogs were kept calm and in a standing position by their owners, with the four limbs perpendicular, and hand stacking as if they were in exposition. Morphometric evaluation was always performed by the same operator to reduce systematic errors, and on the left side of each dog to reduce potential biases. Intra-observer variability was < 10%. The circumference of the thorax was measured using a measuring tape. Body and thorax evaluations were performed using a custom-made sliding gauge (Fig 1). Figure 2 shows how morphometric measurements were performed and the reference points for each measurement.

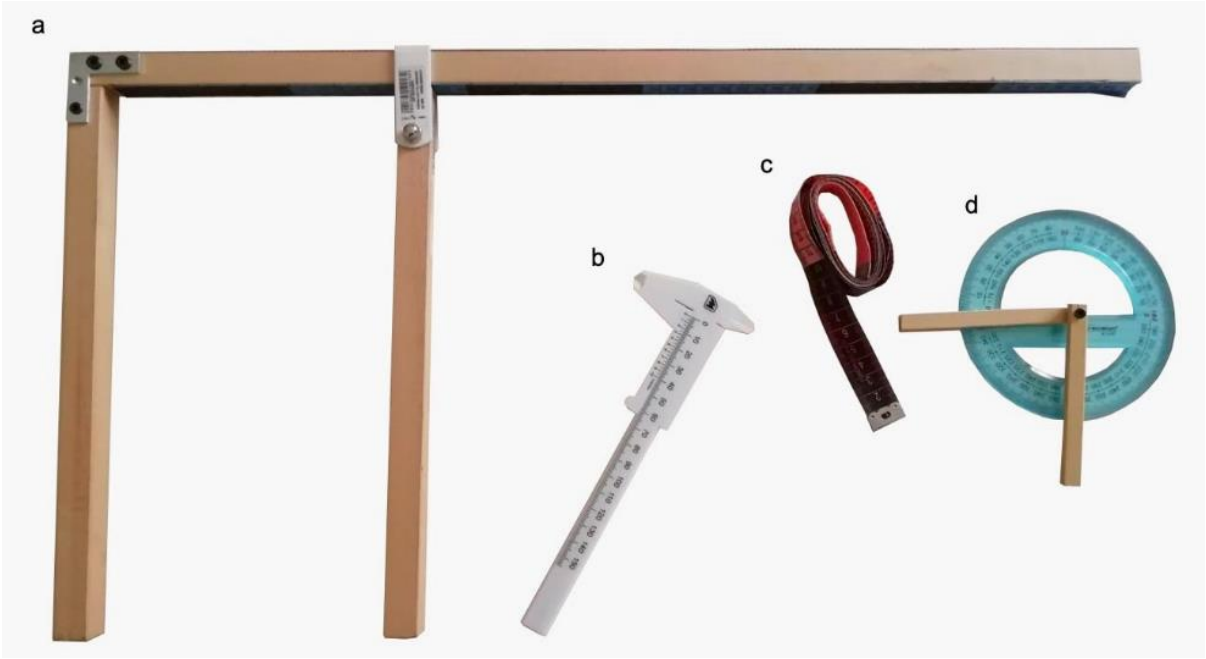

Figure 1. Measurements tools used to evaluate the thorax and the body dimensions.
a) The basal part of the custom-made sliding gauge used to measure the height at the withers and the width and height of the thorax and the mobile part that, together with the basal part, was used to measure the height at the withers and width and height of the thorax b) The gauge used to measure the head length, nose, and head width c) The measuring tape used to measure the three thorax circumferences d) The goniometer used to measure the head's stop angle.

Table 1. Morphometric measurements, body indexes and their reference points [35,37-39].

Body measurements	Thorax measurements	Head measurements
Height at the withers (WH): distance of the withers from the ground, measured at the top of the shoulder blades	Height (TH): distance between the back and the sternum, measured behind the shoulders	Head length (HL): measured from the top of the occipital ridge to the horizontal line joining the two inner corners of the eyelids
Body length (BL): distance between the tip of the shoulder and the tip of the buttock	Width (TW): measured just behind the shoulders	Nose length (NL): measured from the horizontal line joining the two inner corners of the eyelids to the cranial extremity of the truffle
Width at chest (CW): measured at the shoulder-humeral joints	Length (TL): distance between the shoulder tip and the midline of the last rib	Head + nose length (HNL): HL + NL
	Circumference (TC_1, TC_2, TC_3) - TC_1 (Anterior or axillary circumference): measured at the level of the anterior part of the axillary cable - TC_2 (Mean or papillary circumference): measured at the level of the first mammary nipples - TC_3 (Lower or basal circumference): measured at the level of the xiphoid process of the sternum, which corresponds above the spinous process of the seventh cervical vertebra	Head width (HW): measured at the zygomatic arches
		Head stop angle (HA): angle obtained, with the head seen in profile, by intersection of a line tangent to the frontal region (between the two orbits) and the line of the upper part of the nasal barrel
Body indexes		
Cephalic index: (HWx100)/HNL		
Craniofacial ratio (CFR): NL/HL		
Thoracic index: (TWx100)/TH; Height thorax index: (THx100)/WH		
Volume index: (Body weightx100)/WH		
Body size: (WHx100)/BL		

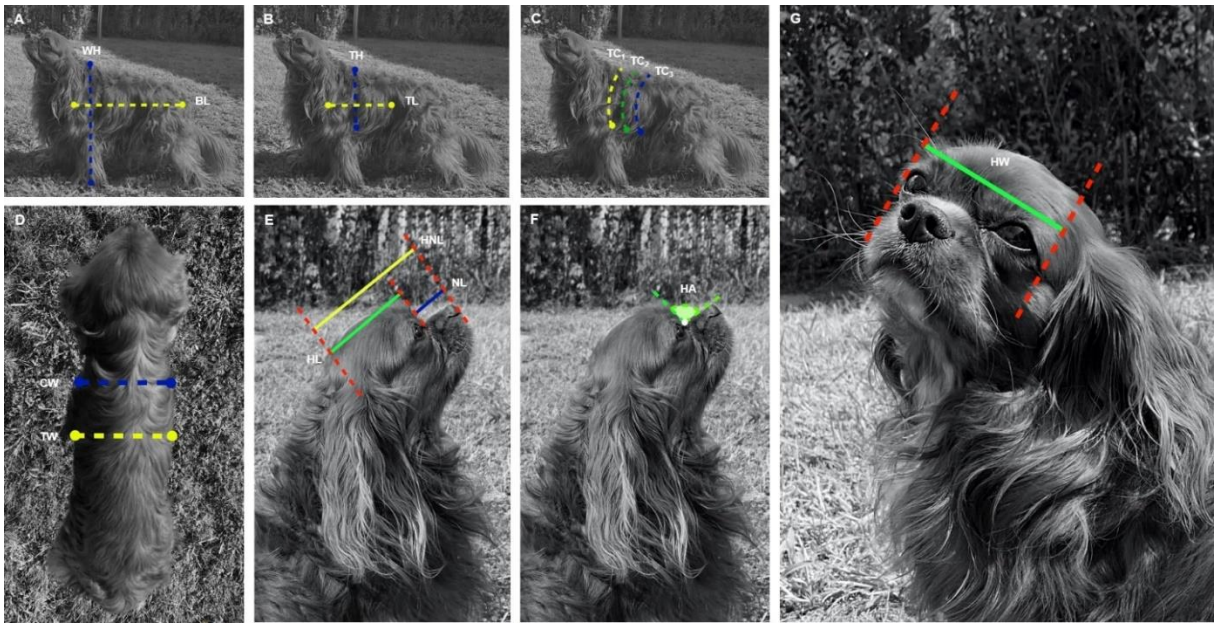

Figure 2. Demonstration of the instrumental and soft tape measurements.

Notes: Body and thorax dimensions were measured using a custom-made sliding gauge. Thoracic circumferences were measured on the dog standing and at rest with a firmly held soft tape measure. Head length, nose length, head and nose length, and head width were measured using a gauge. A goniometer was used to measure the head's stop angle. The detailed definitions of the measurements are shown in Table 1.33 The dogs here represented were included in the present study and photographed by the authors.

A) WH = height at the withers (cm) (blue line); BL = body length (cm) (yellow line).

B) TH = thorax height (cm) (blue line); TL = thorax length (cm) (yellow line).

C) TC1 = thoracic anterior or axillary circumference (cm) (yellow line); TC2 = thoracic mean or papillary circumference (cm) (green line); TC3 = thoracic lower or basal circumference (cm) (blue line).

D) CW = chest width (cm) (blue line); TW = thorax width (cm) (yellow line).

E) HL = head length (cm) (green line); NL = nose length (cm) (blue line); HNL = head-nose length (cm) (yellow line).

F) HA = head stop angle (°) (green).

G) HW = head width (cm) (red line).

The dogs were classified according to the standard values reported by ENCI (https://www.enci.it/media/2405/136.pdf). Standard nose length (NL) was "about 3.8 cm". It is important to note that the Italian breed guidelines do not give precise indications regarding the determination of the length of the nose (https://www.enci.it/media/2405/136.pdf). The body condition score (BCS) was recorded for each dog using a 1 to 9 score, and scores 4 and 5 were considered to be normal [39].

Exclusion Criteria

Healthy (n.16) and MMVD-affected CKCS at ACVIM stages B2 (n.15), C (n.15), and D (n.13) were not included, as well as those in stage B1 (n.5) with either left atrial or left ventricular enlargement but not both. Dogs with cardiac diseases other than MMVD, such as myocarditis

(n.1), congenital heart defects (n.2), cardiac tumors (n.1), and diagnosed arrhythmias (such as supraventricular/atrial premature contractions) (n.4), were not included in the study, as well as subjects with hypertension (n.3) or metabolic diseases (n.5) [21]. Dogs with facial, thoracic, and limb malformations (n.2) and dogs younger than one year of age (n.2) were also excluded.

Statistical Methods

The influence of morphometric measurements on MVP severity, jet size (ARJ/LAA ratio), murmur intensity, and echocardiographic indexed measurements (AMVL, AMVW, AMVA, MVAd, MVAs, SI) in the 52 CKCS was evaluated via IPW analyses. An IPW analysis requires the researcher to construct two regression models, one for the exposure and one for the outcome. The regression model used for the exposure is the propensity score (PS) model, which estimates, for all included subjects, the probabilities of being exposed to the risk factor of interest. For this study, the PS model included the 13 morphometric variables (exposures) as a multivariate response ("generalized multivariate propensity scores") [18], while age, sex, weight, and coat (covariates or confounders) were used as regressors. The probabilities associated with the predicted values that resulted from the estimated PS model were the propensity score estimates. Inverse probability weights were derived as "stabilized inverse probability weights" (SIPW)—i.e., the ratio between the multivariate marginal probability density of the exposures and the multivariate probability density of the exposures conditional to covariates [40].

The second regression model is the outcome model, the main analysis model, which aims to estimate the association of the exposures with the response variables. For each type of response variable (6 numerical and 3 ordinal outcomes), normal linear model or ordinal logistic regression models were estimated, with the morpho-metric variables as exposures, and the authors weighted the observations with the SIPW that had previously been estimated [41]. For the PS model, two alternative models were considered: an additive model (with no interaction between regressors) and an interaction model (with bivariate interactions between some of the

regressors). For the PS model and each of the responses, the bivariate interactions between regressors were explored using normal or ordinal regression models, each of them containing one regressor at the time and only one of the possible bivariate interactions between regressors. Finally, the interaction PS model was estimated by adding all bivariate interactions detected to be significant in the previous exploratory models. Interaction and additive models were then compared for the best performance. The best PS model out of the additive and the one including interactions was the model that provided a better balance of the values of the covariates across subjects and a better match for the positivity assumption [16]. The assumption of "positivity" implies that all participants have the potential to receive a particular level of exposure given any value of the confounders. This was checked by identifying the range of values of the exposures, where positivity is satisfied as the "multidimensional convex hull" (the smallest convex shape enclosing a given shape) calculated for the observed exposures values [18]. In the outcome model, robust standard errors were estimated to take the IPW into account [16].

The outcome models were checked for normality, linearity, variance homogeneity, and multicollinearity. Moreover, outcome ordinal models were also checked for the violation of the proportional odds assumption using surrogate residuals [42,43]. The effects of the morphometric indices that were not linear functions of some single morphometric measurements were derived from the parameters of the outcome models by means of the "delta method" [44]. All analyses were performed in an "R" environment [45].

9.4.2. Results

Clinical and Echocardiographic Results

The median age of the included dogs was 4.16 years (IQR25-75 2.91-6): 14 (27%) were younger than 3 years, 25 (48%) between 3 and 6 years, and 13 (25%) older than 6 years. Eleven dogs (21.2%) were intact males, 2 (3.8%) neutered males, 34 (65.4%) intact females, and 5 (9.6%) neutered females. Median weight was 9.15 Kg (IQR25-75 7.80-10.23). Thirty-six

subjects (69.2%) weighted more than the proposed breed standard (5-8 Kg), 8 (22.2%) of which were overweight (BCS > 5) and 4 (11.1%) were underweight (BCS < 4). No subjects weighted less than the standard (5 Kg). Neutered females showed higher body weight compared to intact females and intact males ($P < 0.05$).

In 26 dogs (50%), no murmurs were found. Soft murmurs (1) were present in 20 dogs (38.5%), whereas in 6 dogs (11.5%) murmurs were of moderate/loud intensity (2). With reference to the 26 dogs with undetectable murmurs, 25 had MVP (19 mild and 6 moderate) and 19 had MR (13 trivial, 3 trace, and 3 mild). Of these 26 subjects, 18 dogs presented MVP and MR, 1 MR only, and 7 MVP only. However, 51 (98.1%) of the 52 included subjects had MVP.

Sphericity index was lower than 1.65 in 48 (92.3%) subjects. Table 2a shows all clinical data (age, body weight and sex), indexed mitral valve measurements and MVP, jet size, murmur severity, and severity score of all included subjects. Moreover, MVAd was larger in subjects older than 6 years than in dogs younger than 3 years ($P = 0.03$), whereas MVAs was larger in subjects older than 6 years than in those between 3 and 6 years ($P < 0.001$). Sphericity index was lower in subjects older than 6 years compared to subjects with age between 3 and 6 years ($P = 0.01$).

Morphometric Measurements

Forty-four (84.6%) dogs, 11 (21.1%) males and 33 (63.5%) females, had a height at the withers lower than breed standard (34-36 cm for males and 32-35 cm for females). In 6 (11.5%) subjects the nose length was longer than the standard (3.8 cm), whereas in 45 (86.5%) was shorter than 3.8 cm. In 36 CKCS (69.2%) nose length was shorter than 3.5 cm and in 17 (32.7%) shorter than 3 cm. In only one subject the nose length was equal to the standard measure (3.8 cm). The morphometric measurements, coat color type, and BCS of overall included population are showed in Table 2b and 2c. The morphometric indexes are reported in Table 2d. Neutered females showed greater thorax height, thorax width, TC3, and volume index than intact females

and greater TC3 and volume index compared with intact males ($P < 0.05$). Furthermore, intact males showed greater height at withers, head length, and head-nose length compared with intact females ($P < 0.05$). No differences between nose length, head width, and head stop angle between sexes ($P > 0.05$) were found. Head stop angle was lower (i.e., closer to 90°) in subjects with a weight within standard (5-8 Kg) ($P = 0.04$). Subjects with tricolor coat type had larger head width than Blenheim ones ($P = 0.01$). Furthermore, younger subjects (<3 years) weighted less than older subjects ($P < 0.001$), showed a lower thorax length ($P < 0.05$) and volume index ($P < 0.01$), as well as higher severity score ($P < 0.05$).

Table 2. Clinical data, indexed mitral valve measurements, MVP, jet size, murmur severity, severity score and morphometric measurements, indexes, coat color type and BCS of all included subjects.

a. Clinical data, indexed mitral valve measurements and MVP, jet size, murmur severity, and score of severity

Age (y)	Body weight (Kg)	Sex	AMVL (cm)	AMVW (cm)	AMVA (cm)	MVAd (cm)	MVAs (cm)	SI	MVP	Jet size	Heart murmur severity	Score of severity
4.16 (2.91-6.00)	9.15*† (7.80-10.23)	F (n.35) NF (n.5) M (n.11) NM (n.2)	0.70 (0.63-0.79)	0.14 (0.12-0.16)	0.08 (0.06-0.11)	0.78 (0.74-0.88)	0.61 (0.55-0.66)	1.37 (1.24-1.50)	0 (n.1) 1 (n.31) 2 (n.19) 3 (n.1)	0 (n.6) 1 (n.18) 2 (n.7) 3 (n.14) 4 (n.7)	0 (n.26) 1 (n.20) 2 (n.6)	2.69 (2.01-3.45)

b. Body and thoracic morphometric measurements

Body morphometric measurements			Thoracic morphometric measurements					
WH	BL	CW	TH	TW	TL	TC_1	TC_2	TC_3
29.20 (27.78-31.58)	33.75 (29.70-35.85)	12.25 (11.45-13.53)	14.95 (13.58-16.13)	11.95 (11.28-13.00)	20.15 (18.68-22.10)	47.00 (45.00-49.63)	47.50 (46.00-50.63)	45.50 (43.58-49.50)

c. Head morphometric measurements and physical data

Head morphometric measurements					Physical data	
HL	NL	HNL	HW	HA	Coat color type	BCS
7.70 (7.20-8.13)	3.20 (2.80-3.50)	10.95 (10.20-11.85)	7.75 (7.50-8.10)	115.00 (110.00-120.00)	(n. 35) B (n. 4) B&T (n. 2) R (n. 11) T	(n. 6) 3 (n. 10) 4 (n. 26) 5 (n. 10) 6

d. Body indexes

Cephalic index	Craniofacial ratio (CFR)	Thoracic index	Height thorax index	Volume index	Body size
71.97 (68.08-75.50)	0.40 (0.36-0.44)	83.08 (75.54-86.83)	0.5 (0.49-0.53)	30.82 (25.97-34.31)	88.40 (83.54-93.09)

a. Note: The severity score formula was calculated as [(Mitral valve prolapse + Regurgitant jet size) x 5] / Age [35]. All echocardiographic measurements were indexed to body weight using the scaling exponents calculated by Wesselowski, one for each specific valve measure [24]. They were respectively: .37, .41, .78, .37, and .40 for AMVL, AMVW, AMVA, MVAd, and MVAs [21]. The variables are reported as median and interquartile ranges (IQR_{25-75}).
Abbreviations: F = intact females; NF = neutered females; M = intact males; NM = neutered males; AMVL = anterior mitral valve length; AMVW = anterior mitral valve width; AMVA = anterior mitral valve area; MVAd = mitral valve annulus in diastole; MVAs = mitral valve annulus in systole; SI = sphericity index; MVP = mitral valve prolapse severity (0 = absent, 1 = under P line, 2 = between P and T lines, 3 = over T line); Jet size = Mitral regurgitation severity (0=absent; 1=trivial, regurgitant jet area (ARJ)/left atrial area (LAA) <10% and not present in all cardiac cycles; 2=trace, ARJ/LAA <10% and present in all cardiac cycles; 3=mild, 10% < ARJ/LAA < 30%; 4=moderate, 30% < ARJ/LAA < 70%); Heart murmur severity: 0=absent; 1=I-II left systolic or soft murmur; 2=III-IV bilateral systolic or moderate and loud murmur respectively; 3=V-VI bilateral systolic or palpable murmur.
b. Note: The variables are reported as median and interquartile ranges (IQR_{25-75}). All morphometric measurements are reported in cm.
Abbreviations: WH = height at withers; BL = body length; CW = width at chest; TH = thorax height; TW = thorax width; TL = thorax length; TC1 = thoracic anterior or axillary circumference; TC2 = thoracic mean or papillary circumference; TC3 = thoracic lower or basal circumference.
c. Note: The variables are reported as median and interquartile ranges (IQR - 25th to 75th). All morphometric measurements are reported in cm, whereas HA is reported as °.
Abbreviations: HL = head length; NL = nose length; HNL = HL + NL; HW = head width; HA = head stop angle; B = Blenheim; B&T = Black and tan; R = Ruby; T = Tricolor; BCS = body condition score (scores 4 and 5 were considered as normal).
d. Note: The variables are reported as median and interquartile ranges (IQR_{25-75}).

Settings for IPW analysis

The IPW analysis was performed including only 49 of the 52 subjects due to the lack of some values for ordinal and continuous variables. Furthermore, the covariate “coat color type” was simplified in the categories “Blenheim” and “other colors” due to the small number of subjects with coats that were not Blenheim. For the same reason, the information "neutered/sterilized", which was originally incorporated in the covariate "sex", was not used.

The PS model with interactions between covariates/confounders provided a better balance in terms of the confounders observed between exposure groups (increasing comparability); accordingly, this model was used to build SIPW.

IPW analyses for ordinal variables

The results obtained were significant for two of the three considered ordinal variables, particularly for heart murmur intensity and jet size. The IPW analysis, in fact, showed that body length ($P = 0.03$) and nose length ($P < 0.01$) had negative influences on heart murmur intensity

(shorter body length and shorter nose were associated with a higher murmur intensity). Furthermore, head length ($P < 0.001$) had a negative influence on jet size (shorter head was associated to larger jet size). However, morphometric measurements had no effects on MVP severity. The results of the regression analysis for ordinal variables, applied to the included population, are summarized in Table 3a.

IPW analyses for continuous variables

Head length ($P < 0.001$) had positive influence on anterior mitral valve length (longer head was associated to longer anterior mitral valve leaflet).

Thorax width ($P = 0.01$) had positive influence on anterior mitral valve width, whereas thorax length ($P = 0.04$) had a negative one: subjects with larger or shorter thorax had thicker anterior mitral valve leaflet.

Body length ($P = 0.02$), thorax width ($P = 0.000$), mean or papillary circumference (TC2) ($P < 0.001$), head length ($P = 0.000$), and head stop angle ($P = 0.000$) had positive influence on mitral valve annulus in diastole, whereas thorax height ($P = 0.02$), TC1 ($P = 0.000$), TC3 ($P = 0.02$), and nose length ($P < 0.001$) had a negative influence.

Thorax width ($P = 0.01$) and head stop angle ($P = 0.000$) had positive influence on mitral valve annulus in systole, whereas thorax height ($P = 0.002$) had a negative influence.

Anterior or axillary thoracic circumference ($P = 0.01$) and head length ($P = 0.000$) had a positive influence on sphericity index.

The derivation of the results of the IPW analysis with respect to the morphometric indexes showed that only thoracic index was negatively associated with mitral valve annulus in the systole and diastole. The results of the regression analysis for continuous variables, applied to the included population, are summarized in Table 3b.

The performance of the outcome models regarding normality, linearity, and variance homogeneity were acceptable according to the diagnostic plots, and there was no indication of

the violation of the proportional odds assumption for the ordinal models, whereas some multicollinearity problems were detected in the models, including interaction terms (data not shown).

Table 3. Results of the regression analysis applied to determine the influence of morphometric variables on clinical and echocardiographic parameters in all included subjects.

Regression analysis										
	BL	**TH**	**TW**	**TL**	**TC_1**	**TC_2**	**TC_3**	**HL**	**NL**	**HA**
a. Ordinal variables										
MVP										
Jet size								**n		
Heart murmur intensity	*n								**n	
b. Continuous variables										
AMVL								*p		
AMVW			*p	*n						
AMVA										
MVAd	*p	*n	***p		***n	***p	*n	***p	***n	***p
MVAs		**n	*p							***p
SI					**p			**p		

Note: All morphometric measurements are expressed in cm, whereas HA as °. Morphometric variables not influencing clinical and echocardiographic parameters are not reported in the table.
Level of statistical significance: *** = P < 0.001; ** = P < 0.01; * = P < 0.05.
p = positive association; n = negative association.
Abbreviations: MVP = mitral valve prolapse severity; Jet size = severity of mitral regurgitation; Anterior mitral valve: length (AMVL), width (AMVW), area (AMVA); MVAd = mitral valve annulus in diastole; MVAs = mitral valve annulus in systole; SI = sphericity index; BL = body length; TH = thorax height; TW = thorax width; TL = thorax length; TC1 = thoracic anterior or axillary circumference; TC2 = mean or papillary circumference; TC3 = thoracic lower or basal circumference; HL = head length; NL = nose length; HA = head stop angle.

9.4.3. Discussion

Very little is known regarding the relationship between echocardiographic indicators of the severity of MMVD (i.e., MVP severity, jet size, and indexed echocardio-graphic measurements) and the morphometric measures for all breeds. To the best of the authors' knowledge, this study is the first ever on the relationship between morphometric data and echocardiographic and color Doppler measures in CKCS to have been carried out. The highlight of any association between morphometric data, severity of echocardiographic lesions, clinical symptoms, and evolution time of the disease will be reached only through a long-term follow-up of the subjects and a longitudinal study. Through their results, the authors have tried to lay the basis for this.

In the present study, is clear that subjects with a larger thorax width or a shorter thorax length (more barrel-shaped) and a shorter head had thicker anterior mitral valve leaflets. Mitral valve annulus in the diastole has been observed to be larger in subjects with a smaller thorax height (reduced dorso-ventral thoracic dimension), larger thorax width, and greater mean or papillary thoracic circumference (TC2). The same was observed in subjects with shorter noses. Regarding mitral valve annulus in systole, the results are superimposable to the diastole: the annulus has been observed to be greater in subjects with a smaller thorax height and larger thorax width. It is also interesting to underline the positive influence of anterior or ancillary thoracic circumference (TC1) and head length on the sphericity index: the ventricular shape was more spherical in subjects with a smaller TC1 and shorter head. These findings are similar to those observed for human medicine, in which the MVP is associated with an asthenic habitus, corresponding to a reduced antero-posterior thorax diameter [46,47]. In fact, asthenic habitus in the dog could be considered as a reduction in the ventro-dorsal diameter, given the quadrupedal station. Furthermore, in the study carried out by Olsen in Dachshunds [3], the thoracic circumference was found to be negatively correlated with the severity of MVP.

Obviously, from the results obtained, the authors can only speculate about the influence of some morphometric measures on the heart murmur intensity and jet size, but not the influence on the MVP. However, as observed by Olsen [3], the authors may suppose that the results obtained may indicate an echocardiographic phenotype that is more easily associated with mitral valve disease (shorter or thicker anterior mitral valve leaflets, greater mitral valve annulus in the systole and diastole, and lower sphericity index).

Only one study in CKCS had shown a negative correlation between the severity of MVP and body weight, demonstrating that smaller dogs have more severe forms of MMVD [9]. With these results, as stated before, the authors are not able to demonstrate the same; on the other hand, the association between the cranial morphology of the subjects, the severity of the heart murmur, the jet size dimension, and other valvular characteristics is still relevant. The authors observed that subjects with a shorter head are more likely to have a higher jet size. Furthermore, subjects with a shorter body and nose length have a higher heart murmur intensity. Thus, given all the results discussed, CKCS with a shorter nose and head and a more barrel-shaped thorax are likely to have worse valvular characteristics than subjects with longer and narrower skulls and bodies. According to these results, the breeding of subjects with cranial morphology tending toward brachycephalism (wider and shorter head) may be counterproductive in view of the selected reproduction for MMVD, although additional studies are needed to confirm authors' findings.

In the literature, only one study has investigated the influence of the coat type (length) on MVP prevalence and severity, particularly in Dachshunds [3]. No association between MMVD and coat color has ever been described. In the present study, coat color, taken as a single factor, did not affect any of the MMVD indexes. Nevertheless, it should be considered that, in the sample included in this study, 68% of the subjects were Blenheim: in Italy, this is by far the most common color (65%), as reported by ENCI (https://www.enci.it/libro-

genealogico/razze/cavalier-king-charles-spaniel). The association between MMVD and coat color type should be evaluated in a larger population of dogs, including subjects in more advanced ACVIM classes.

Many of the dogs included had mild changes whose long-term significance is unknown due to the lack of data from a follow-up. In this study, as already stated, a high percentage of dogs without murmurs had mild to moderate MVP, and the authors report a high prevalence of echocardiographically detected MR in CKCS with no murmurs, reinforcing the notion that a purely clinical screening is unable to identify MMVD in this breed [48]. Similarly, subjects with slight mitral valve degenerations may have higher-intensity murmurs, indicating that there is no basis for proposing that murmur intensity is a valid indicator of disease severity. These findings highlight the need for additional research to follow up these subjects and understand which of them will develop disease with a faster course and which ones will remain stable with mild forms until advanced age. Furthermore, this will enable to understand if some physical characteristics can be related to the progression of MMVD and, given the results of this study, increase the focus on the evaluation of thorax and skull dimensions of the included subjects in following research. In the end, the morphometric data obtained, combined with the genetic analysis and echocardiographic evaluation of the subjects, could help to characterize some phenotypes related to more severe forms of MMVD.

Among the strengths of this study, the authors note that the accurate statistical approach used, based on IPW analyses, allowed them to account for the measured con-founding variables in the association between morphometric measures and MMVD.

However, this study also had some limitations. The qualitative nature of some echocardiographic parameters means that they should be interpreted with caution. Jet size detected by color flow mapping should only be regarded as a semiquantitative measure of the degree of MR, unlike methods using the proximal isovelocity surface area (PISA) and the vena

contracta [49]. Several factors, such as the quality of the imaging window, the distance to the flow being imaged, gain settings, pulse repetition frequency setting for the color Doppler, the immobility of the patient, and the experience of the operator, may influence this measure [50]. The left apical 4-chamber view was used because the degree of MR may be underestimated if color flow mapping is performed from the right side of the thorax [49]. Furthermore, it has long been known that, given the 3D morphology of the mitral valve, long-axis images that do not include the left ventricular outflow tract (LVOT) greatly overestimate the presence of MVP in humans. Considering the recent publications on canine 3D mitral valve morphology [51] in both multi-breed populations and CKCS, this cannot be ignored any longer. It must be highlighted that many of these dogs may not really have mitral valve prolapse. Therefore, specific studies assessing the association between quantitative echocardiographic severity measurements of MMVD, and the morphometry of subjects are necessary. To the best of authors' knowledge, SI cut-off reference values for small breed dogs have not been reported in the literature. Due to the limits of the cut-off value chosen, the obtained results suggest that CKCS have a ventricular morphology extremely different from all breeds described in the guidelines; thus, specific SI values are needed for CKCS [25,26].

Eventually, further investigations using a greater number of subjects with a non-Blenheim coat color type and in advanced ACVIM stages are needed, as well as with a higher number of males and subjects that are not overweight.

It is also necessary to point out that the subjects included were of different ages at the time of diagnosis. Via IPW analyses, this study attempted to find an association between morphometric measures and MMVD that was unbiased by the confounding factors of age, sex, weight, and coat type, but the presence of unmeasured relevant confounding is still possible.

9.4.4. Conclusion

In the CKCS included in the present study, MVP had an epidemiology resembling that known for MVP in humans and dachshunds [3,46,47]. In fact, MVP severity was significantly positively associated with measures of the degree of MMVD (e.g., jet size, leaflet length, and murmur intensity). In the present study, thorax height had a negative association with AMVL. Furthermore, thorax width and TC1 had positive associations with MVAd and SI, respectively. In particular, the most interesting result obtained is that subjects with a shorter head were associated with a higher jet size, while subjects with a shorter body and nose length had a higher heart murmur intensity. Regarding mitral valve and mitral annulus measurements, subjects with a more barrel-shaped thorax and a shorter nose had shorter and thicker anterior mitral valve leaflets and greater mitral valve annulus in the systole and diastole. This suggests that a brachycephalic morphotype, with dogs much more similar to the King Charles spaniel breed in cephalic morphology, is correlated with a more severe jet size and with valvular characteristics related to worse forms of MMVD; this may be counterproductive in view of the selected reproduction for MMVD. Studies focusing on the follow-up of B1 subjects, and the results of this study would allow to gain a better understanding of the morphological aspects that are often associated with the more severe and/or faster evolution of the disease in the CKCS. It could be useful to add information relating to more advanced ACVIM classes and older subjects. This, together with clinical and echocardiographic characterization, could be used as part of a screening program for CKCS defining early selection criteria for the exclusion of a subject from reproduction.

9.4.5. References

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9.5. Circulating miR-30b-5p is upregulated in Cavalier King Charles spaniels affected by early myxomatous mitral valve disease

Bagardi, M.; Zamarian, V.; Ceciliani, F.; Brambilla, P.G.; Lecchi, C. Submitted to Journal of Veterinary Cardiology

Myxomatous mitral valve disease (MMVD) is a cardiovascular disease affecting dogs, progressing to mitral regurgitation (MR) and eventually heart failure, giving rise to about 10% of all deaths in this species [1]. Although MMVD seems to be a genetic disorder, the mutation has not yet been identified [2]. The incidence is age-related and is particularly high in some breeds such as the CKCS, half of which are estimated to be affected by MMVD at the age of 6-7 years and almost all at 10 years [1,3-5]. Evidence from highly susceptible breeds such as CKCS and Dachshunds shows a strong inherited component to the disease and suggests a polygenic mode of inheritance [2,6,7]. Due to the lack of early signs, symptoms, and predictive biomarkers, the early diagnosis is difficult, and the identification of reliable specific biomarkers is desirable, especially for screening and breeding programs. MicroRNAs (miRNAs) are stable tissue-specific molecules with high sensitivity and specificity and are potentially suitable candidate markers of human cardiovascular diseases [8,9]. MiRNAs exert their function repressing target genes and regulating protein production through different mechanisms in several pathophysiological conditions, including myocardial infarction, hypertrophy, fibrosis, inflammation. MiRNAs can be secreted into extracellular fluids including plasma and serum, where they are relatively stable even under conditions such as long-time storage at room temperature, multiple freeze-thaw cycles, and low or high pH [10-14]. Aberrant expression of miRNAs is associated with several human and veterinary disorders including cancer and heart diseases [15-22]. The dysregulation of circulating miRNAs was previously investigated also in MMVD-affected dogs using different approaches, including quantitative real-time PCR (RT-qPCR), microarray, and next-generation sequencing (NGS) for genome-wide sequencing

analysis [23-28]. Most of the dogs enrolled in these studies were classified as American College of Veterinary Internal Medicine (ACVIM) stage C and D, while only one study performed analysis also on dogs older than 8 years in ACVIM stage B1 and B2 [26,29].

This study aimed at improving MMVD assessment in CKCS at the asymptomatic stage of the disease without cardiac remodelling (ACVIM stage B1) grouped according to the age at the time of diagnosis (younger than 3 years, between 3 and 7 years, and older than 7 years), by ascertaining whether three miRNAs, previously associated with MMVD, may be modulated in the plasma of CKCS, and investigating their potential use as biomarkers to identify as finely as possible asymptomatic dogs in ACVIM stage B1. The decision to focus the study on this ACVIM class was dictated by the fact that these dogs are those most subject to breed screening and that therefore will become selected breeders. It is therefore very interesting to identify with a biomarker, and not only with echocardiographic examination, the early presence of MMVD, especially in subjects that have no clinical signs and that do not present heart murmurs at the visit. Obviously, this cannot be separated from a follow-up of these dogs that will allow the evaluation of the more or less rapid evolution of the disease. There are currently no tests available to outline the type of evolution, but this work aims to lay the foundations.

9.5.1. Materials and methods

Clinical and echocardiographic examinations

The cardiological evaluation of the studied subjects was performed during a routine veterinary visit, in fasting dogs at least of 12 hours. The clinical data of the animals included: animal history, clinical and echocardiographic examinations. The cardiovascular system was evaluated by checking the presence/absence of murmur by two different well-trained operators. The evaluated auscultatory findings were presence/absence, timing, and intensity of murmur (0=absent; 1=I-II/VI left apical systolic or soft; 2=III-IV/VI bilateral systolic or moderate and loud respectively; 3=V-VI/VI bilateral systolic or palpable) [30]. Blood pressure was indirectly

measured with a Doppler method according to the ACVIM consensus statement [31,32]. Peripheral venous blood sampling was performed at the end of the examination. Blood was collected from the jugular or cephalic vein in two 2.5-mL EDTA tubes.

The echocardiographic exam was used to diagnose MMVD. A standard transthoracic echocardiographic (TTE) examination was performed with My Lab50 Gold Cardiovascular ultrasound machine (Esaote, Genova, Italy), equipped with multi-frequency phased array probes (3.5-5 and 7.5-10 MHz), chosen according to the weight of the subject. Video clips were acquired and stored using the echo machine software for off-line measurements. The exam was performed according to a standard procedure with concurrent continuous electrocardiographic monitoring [33]. All examinations were performed without pharmacological restraint. Dogs were classified according to the ACVIM classification scheme [29].

Inclusion criteria for dogs in the clinically normal group (ACVIM A) were: no echocardiographic evidence of heart disease, no clinical signs, no abnormalities on results of a complete blood count (CBC) and biochemical analyses, and no history of medical treatment within the previous 6 months. Inclusion criteria for dogs with MMVD at stage B1 were: echocardiographic evidence of a thickened or prolapsed mitral valve and mitral valve regurgitation, no evidence of left atrial dilatation, defined as a left atrial-to-aortic root ratio (LA/Ao) <1.6 on 2-dimensional echocardiography, and no left ventricle dilation, defined as left ventricular normalized dimensions in diastole (LVIDad) <1.7 [29]. The degree of MR (jet size) was assessed using color Doppler and calculating the maximal ratio of the regurgitant jet area signal to left atrium area (ARJ/LAA ratio) [34]. Regurgitant jet size was estimated, with the same echo-setting, as the percentage of the left atrial area (to the nearest 5%) that was occupied by the larger jet and it was considered as trivial or trace (<10%), mild (between 10 and 30%), moderate (between 30 and 70%) or severe (>70%) [34,35]. The trivial type was characterized by the absence of the regurgitant jet in all systolic events, whereas in the trace type the jet was

always present [35]. Totally, four groups of 11 client-owned dogs were included in the present study: group A or heathy control, group B1<3 with dogs younger than 3 years; group B1 3-7, with dogs older than 3 years and younger than 7 years, and B1>7 with dogs older than 7 years [36,37].

Dogs with asymptomatic MMVD and cardiac remodelling (ACVIM stages B2), dogs with symptomatic MMVD (ACVIM C and D) or with other systemic diseases such as systemic hypertension, uncontrolled hypothyroidism, hyperadrenocorticism, primary pulmonary hypertension, neoplasia, and other cardiac abnormalities such as dilated cardiomyopathy, congenital cardiac abnormalities, endocarditis, and severe arrhythmia were excluded from the study.

smallRNA isolation and RT-qPCR quantification

Blood samples for smallRNA isolation were collected in 2.5 ml EDTA-K3 tubes. Within 2 hours the samples were centrifuged at 800 g for 15 minutes. Plasma was stored at –80°C until RNA isolation.

SmallRNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, catalogue number 217184, Milan, Italy). An aliquot of 150 µL of plasma per sample was thawed on ice and centrifuged at 3000 × g for 5 min at 4 °C. RNA was extracted using miRNeasy Serum/Plasma Kits (Qiagen, catalogue number 217184, Milano, Italy) following the manufacturer's instructions. One milliliter of Qiazol (Qiagen) was added to an aliquot of 150 µl per sample. After incubation at room temperature for 5 min, 25 fmol of the exogenous synthetic spike-in control Caenorhabditis elegans miRNA cel-miR-39 (Qiagen, Cat. No. 219610) was spiked into samples at the beginning of the extraction procedure to check either the extraction of miRNAs and the efficiency of the cDNA synthesis. RNA extraction was then carried out according to the manufacturer's instructions. The RNA quality and quantity were verified according to MIQE guideline.38 To obtain cDNA, reverse transcription was performed using a TaqMan

Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007, Applied Biosystems) following the manufacturer's instructions.

Quantitative real-time PCR (RT-qPCR) was performed following the MIQE guidelines [38]. The small RNA TaqMan assays were performed according to the manufacturer's instructions using the selected primer/probe assays (ThermoFisher Scientific), including: cel-miR-39-3p (assay ID 478293_mir); miR-1-3p (assay ID 477820_mir);28 miR-30b-5p (assay ID 478007_mir);39 miR-128-3p (assay ID mmu480912_mir).28 The reference miRNA was miR-16-5p (assay ID rno481312_mir). Quantitation was performed in 15 µl in a CFX Connect Real-Time PCR Detection System (Bio-Rad) using 7.5 µl of 2X TaqMan Fast Advanced Master Mix (Cat. No. 4444557), 0.75 µl of miRNA-specific TaqMan Advanced assay reagent (20X), 1 µl of cDNA and water to make up the remaining volume. The thermal cycling profile was as follows: 50 °C for 2 min, 95 °C for 3 min and 40 cycles at 95 °C for 15 s and 60 °C for 40 s. No-RT controls and no-template controls were included. Data were normalized relative to the expression of miR-16. MicroRNA expression concentrations are presented in terms of fold change normalized to miR-16 expression using the formula $2^{-\Delta\Delta Cq}$ on Bio-Rad CFX Maestro Software.

Statistical analysis

Statistical analysis was performed using XLStat software for Windows (Addinsoft, New York, USA). Data were tested for normality using the Shapiro–Wilk test; as the data were not normally distributed, the nonparametric Kruskal-Wallis test was applied. Receiver operating characteristic (ROC) analysis was performed as previously reported to determine the diagnostic accuracy. The diagnostic value was calculated for miRNA that showed significant differential expression in the canine blood. Statistical significance was accepted at a P value of ≤ 0.05 and all the significance values were adjusted according to the Bonferroni post-hoc correction.

9.5.2. Results

Demographics and Characteristics of Study Subjects

The mean age of the 44 included CKCS was 3.3 years (IQR25-75 1.81-6.99), and the mean body weight 8.1 Kg (IQR25-75 7.48-9.68). Fourteen subjects (31.82%) were males and 30 (68.18%) were females. Study population characteristics (clinical and echocardiographic data), grouped according to the ACVIM classes and, for B1 class, to the age at the time of MMVD diagnosis are shown in Table 1. Weight was lower in B1<3 (P=0.04) and A (P=0.029) subjects compared with B1>7 group, whereas echocardiographic variables were not statistically different among age groups (P>0.05).

Table 1. Clinical and echocardiographic data of all included CKCS divided according to ACVIM classification and age at time of diagnosis of MMVD for subjects in ACVIM class B1. Note: All data are expressed as median and IQR_{25-75} ranges.

	Overall population	A	B1<3	B1 3-7	B1>7
N. of dogs	44	11	11	11	11
Sex	30F (8NF) 14M	8F (1NF) 3 M	6F 5M	6F (1NF) 5M	10F (6NF) 1M
Age y	3.3 (1.81-6.99)	1.96 (1.73-2.88)	1.52 (1.07-2.21)	3.88 (3.49-4.3)	8.14 (7.66-8.68)
Weight kg	8.10 (7.48-9.68)	7.75 (7.25-7.95)	7.8 (6.83-8)	9.4 (7.63-9.88)	10 (9.35-10.4)
SBP mmHg	135 (110-145)	125 (115-135)	130 (120-140)	130 (120-140)	140 (125-150)
Murmur	29 grade 0 10 grade 1 5 grade 2	11 grade 0	10 grade 0 1 grade 1	8 grade 0 3 grade 1	6 grade 1 5 grade 2
Regurgitant jet size	11 grade 0 5 grade 1 8 grade 2 15 grade 3 5 grade 4	11 grade 0	5 grade 1 6 grade 2	2 grade 2 9 grade 3	6 grade 3 5 grade 4
ESVI ml/m^2	17.95 (14.55-25.93)	16.60 (11.19-17.95)	16.69 (14.73-21.67)	19.48 (15.73-26.03)	22.15 (17.29-27.93)
EDVI ml/m^2	56.15 (48.28-71.91)	50.50 (45.54-61.87)	51.99 (41.97-62.64)	56.38 (52.42-85.29)	68.40 (58.98-76.59)
LA/Ao	1.15 (1.08-1.26)	1.21 (1.09-1.36)	1.08 (1.02-1.2)	1.17 (1.12-1.19)	1.15 (1.08-1.29)
E m/sec	0.73 (0.66-0.80)	0.76 (0.71-0.84)	0.68 (0.61-0.79)	0.7 (0.67-0.75)	0.68 (0.67-0.81)
E/A	1.3 (1.18-1.43)	1.29 (1.21-1.37)	1.47 (1.36-1.6)	1.21 (1.16-1.45)	1.22 (.99-1.34)
EF %	67 (58-73.25)	67 (62-77)	62 (59-70.50)	68 (57.5-74)	67 (57-71.5)
FS %	35 (29.75-40.25)	35 (32-43.5)	31 (30-38)	36 (29.5-41.5)	37 (28-39.5)
LVIDas	0.84 (0.77-0.95)	0.82 (0.7-0.84)	0.82 (0.78-0.90)	0.87 (0.79-0.96)	0.90 (0.81-0.98)
LVIDad	1.36 (1.29-1.51)	1.31 (1.26-1.42)	1.33 (1.22-1.43)	1.37 (1.33-1.62)	1.48 (1.40-1.55)

Abbreviations: Sex → F = female, NF = neutered female, M = male; SBP = systemic blood pressure; Murmur = left systolic heart murmur intensity → 0=absent, 1=I-II/VI left apical systolic or soft, 2=III-IV/VI bilateral systolic or moderate and loud; Regurgitant jet size → 0=absent, 1=trivial, 2=trace, 3=mild, 4=moderate; ESVI = end systolic volume index; EDVI = end diastolic volume index; LA/Ao = left atrium to aorta ratio; E = E wave velocity; E/A = E and A waves ratio; EF = ejection fraction; FS = shortening fraction; LVIDas = left ventricular normalized dimensions in systole; LVIDad = left ventricular normalized dimensions in diastole.

miR-30b-5p is dysregulated in MMVD affected dogs

Small RNA was extracted from and the spike in cel-miR-39 was quantified in all collected samples. Three miRNAs, namely miR-1-3p, miR-30b-5p, and

miR-128-3p, were detected in all canine plasma (Fig 1A-F). The comparative analysis demonstrated that one miRNA, namely miR-30b-5p, had a significant differential expression in the plasma of MMVD affected dogs compared to the healthy group. In detail, the abundance of miR-30b-5p increased 2.4 folds ($P = 0.0063$) in group B1 compared to A (Fig 1B). Splitting group B1 according to the age of dogs, the expression of miR-30b-5p remained significantly higher (Fig 1E). Group B1<3 (2.3 folds $P = 0.034$), B1 3-7 (2.2 folds $P = 0.028$), and B1>7 (2.7 folds $P = 0.018$) expressed a higher level of miR-30b-5p than group A. No differences were found in the amount of miR-1-3p (Fig 1A and D) and miR-128-3p (Fig 1C and F). The age proved not to be correlated with the expression of analyzed miRNAs in the entire population and in each age class ($P>0.05$).

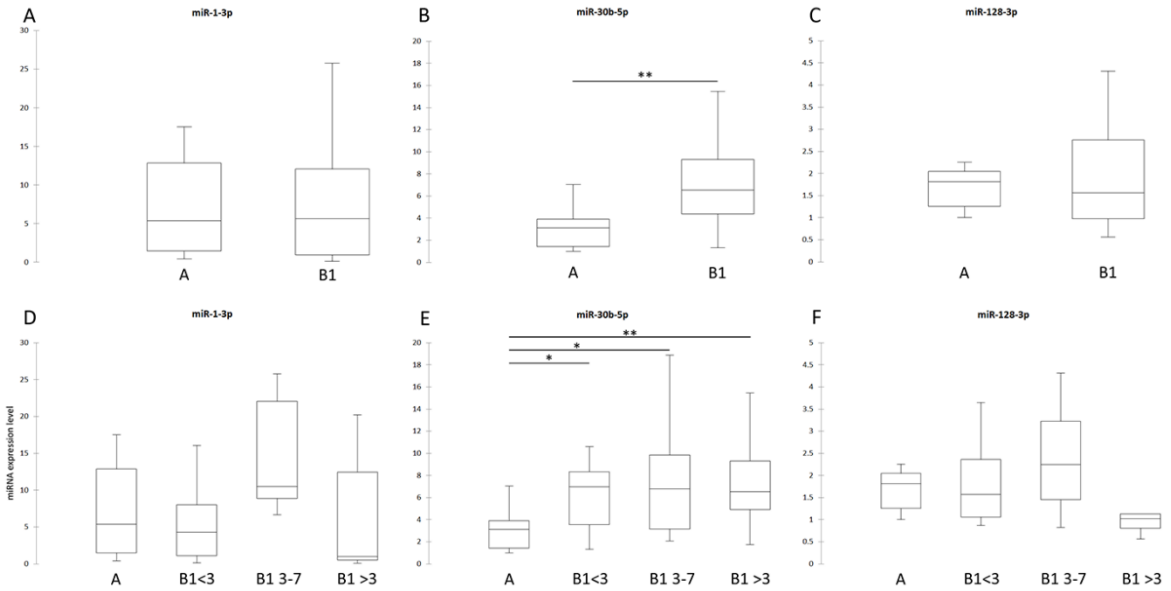

Figure 1. Expression level of miR-1-3p, miR-30b-5p, and miR-128-3p between group A and B1 (1A-C respectively) and between A and B1 divided according to age at MMVD diagnosis (1D-F respectively). miR-30b-5p increased 2.4 folds (P = 0.0063) in group B1 compared to A (1B). Splitting group B1 according to the age of dogs, the expression of miR-30b-5p remained significantly higher (1E). Group B1<3 (2.3 folds P = 0.034), B1 3-7 (2.2 folds P = 0.028), and B1>7 (2.7 folds P = 0.018) expressed a higher level of miR-30b-5p than group A. No differences were found in the amount of miR-1-3p (Fig 1A and D) and miR-128-3p (1C and 1F).

Diagnostic performance of miR-30b-5p discriminated between MMVD affected- and healthy dogs

To evaluate the diagnostic value of miR-30b-5p in plasma, ROC curve analysis was performed, and the associated AUC was used to confirm the diagnostic potency. Cut-off points were set to maximize the sum of sensitivity and specificity. The ability of miR-30b-5p to separate the tested samples into healthy (stage A) or MMVD affected (stage B1) is defined diagnostic accuracy and is measured by the area under the curve (AUC). The ability to discriminate group A and group B1 (AUC = 0.79; 95% CI 0.65-0.93) was good (Fig 2A). Dividing group B1 according to age, the ability to discriminate group A and group B1 <3 (AUC = 0.78; 95% CI 0.60-0.96) and group A and group B1 3-7 (AUC = 0.78; 95% CI 0.60-0.96) was good (Fig 2B and 2C respectively), while was very good in discriminating group A and B1>7 (AUC = 0.82; 95% CI

0.65-0.99) (Fig 2D) (Table 2). Thus, miR-30b-5p can discriminate between healthy (stage A) and asymptomatic MMVD-affected dogs (stage B1).

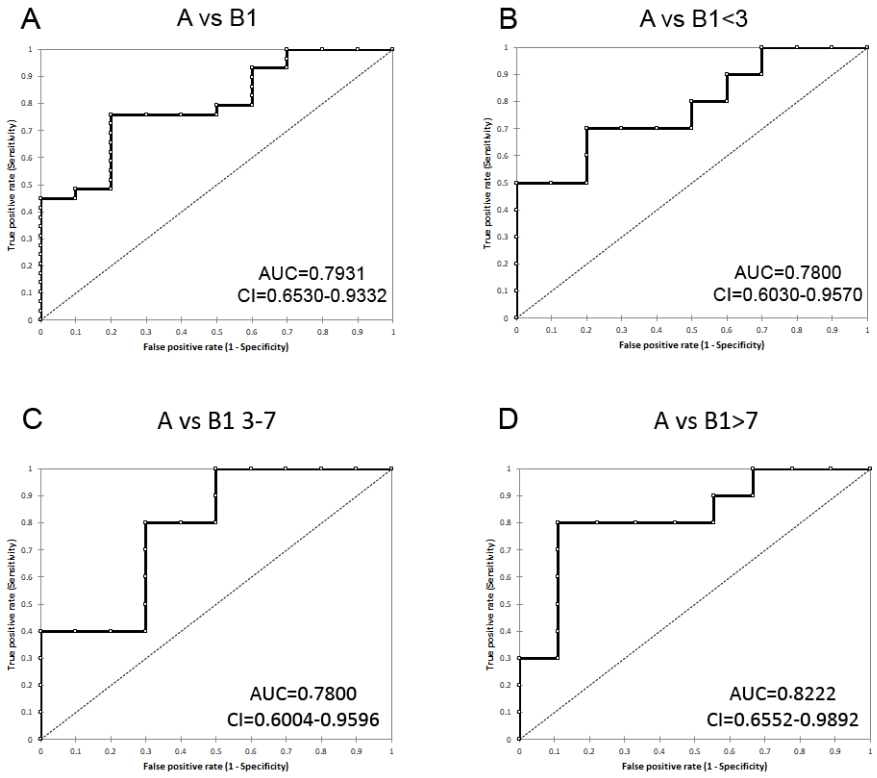

Figure 2. ROC curves for miR-30b-5p. Discrimination capacity between group A and group B1 (2A), group A and group B1 <3 (2B), group A and group B1 3-7 (2C), and group A and B1>7 (2D). miR-30b-5p can discriminate between healthy and asymptomatic MMVD-affected dogs.

Table 2: Area under the curve (95% confidence interval), cut off values and sensitivity and specificity of miR-30b-5p in CKCS's plasma.

	AUC	95% CI	P value	Cut off	Se-Sp
A vs B1	0.79	0.65 - 0.93	< 0.0001	3.98	0.76 - 0.80
A vs B1<3	0.78	0.60 - 0.96	0.0019	4.52	0.80 - 0.70
A vs B1 3-7	0.78	0.60 - 0.96	0.0023	4.37	0.80 - 0.70
A vs B1>7	0.82	0.66 - 0.99	0.0002	4.37	0.80 - 0.89

Abbreviations: AUC = area under the ROC curve; CI = confidence interval; Se = sensitivity; Sp = specificity.

9.5.3. Discussion

The present study reports the relationship between the abundance of circulating miR-30b-5p and the presence of MMVD, a pathology potentially leading to congestive heart failure and

associated clinical signs, even in young subjects, in CKCS breed. We found that the amount of miR-30b-5p is significantly upregulated in asymptomatic MMVD-affected (ACVIM stage B1) compared to healthy (ACVIM stage A) dogs and that its dysregulation is detectable also in young dogs (age <3, ranging from 6 months to 2.4 years), even in subjects without audible heart murmurs.

Yang and colleagues (2017) investigated the cargo of exosomes purified from the plasma of MMVD-affected dogs using an array-based approach, demonstrating that miR-9 and miR-599 were dysregulated, while no differences were detectable analyzing whole plasma [25]. The array-based approach used by the authors may have caused a high False Discovery Rate (FDR), set at 20%, thus limiting the power of detection [25]. Another study, including old dogs (range, 8.2 to 13.8 years) with congestive heart failure (CHF) secondary to MMVD (ACVIM stage C), reported that 326 miRNAs were modulated comparing healthy (ACVIM stage A) and CHF (ACVIM stage C) affected dogs; the validation step, performed by RT-qPCR, demonstrated the overexpression of miR-133, miR-1, let-7e, and miR-125, and the down expression of miR-30c, miR-128, miR-142, and miR-423 [28]. Although they focused on a group of animals affected by a severe disease with clinically detectable signs, the results appeared worthy to be further studied even in younger patients, prompting to include miR-1 and miR-128 to be included in our investigation. Based on results reported by Hulanicka and co-workers (2014) [39], who however included in the study old dogs (range, 10.17 ± 3.36 years), we identified miR-30b as a potential marker to be further investigated in a younger cohort of MMVD ACVIM stage B1 affected CKCS.

Since the molecular background of MMVD is not yet fully elucidated, the identification of any specific markers (prognostic and/or therapeutic) would be of great value and importance for identifying asymptomatic patients, especially at a young age.

The diagnosis of MMVD is based on the echocardiographic evaluation of mitral valve and leaflets' thickness that sometimes is hard to identify, since mildly affected valves work adequately, and the lesions apparently do not affect hemodynamic, given the absence of cardiac remodeling and clinical signs. Myxomatous mitral valve disease is age-related and the prevalence in old small breed dogs is up to 100%, in particular chondrodystrophic breeds including Cocker spaniels, Dachshunds, and Beagles. CKCS are more susceptible to develop congestive heart failure due to MMVD and at younger ages than other breed [40]. Thus, especially in highly susceptible breeds, such as CKCS, where MMVD starts at a very young age and progress over time, unfortunately, in different unpredictable grades. CKCS development is regarded as a hereditary character in this breed and has been associated with a multi-factorial polygenic transmission mode: therefore, several genes are involved, and a defined threshold of expression must be reached before the disease occurs [2,5-7].

Although miRNAs are currently intensively investigated in human medicine because of their diagnostic potential in many different conditions, there are only few reports related to circulating miRNAs studies in dogs affected by MMVD and no study about the early diagnosis of this disease in a predisposed breed such as CKCS.

This study identified a biomarker that may have an impact in both implementing preventing programs through genetic selection and in clinical practice, confirming that in CKCS as well, as already demonstrated in humans, there is a differential expression of miRNAs, suggesting that their expression profiles are distinct for dogs with MMVD compared to expression profiles of healthy dogs. We demonstrated that miR-30b-5p could discriminate CKCS at ACVIM stage A and young stage B1 (younger than 3 years), without heart murmurs, without clinical signs, but with an and echocardiographic diagnosis of MMVD. The identification of dogs with early asymptomatic MMVD with miR-30b-5p could help the clinicians and the breeders to better

focalized screening programs in this breed and to better choose the breeders, making use of a closer follow up in subjects with these characteristics.

For these reasons, miRNAs may be candidates for novel biomarkers and may provide the basis for further investigations to assess the follow-up and characterize the evolution of the disease in the CKCS.

This study presents some limitations. The utility of circulating miRNAs as biomarkers of many diseases has attracted considerable attention over recent years, however, it is also worthwhile to point out that the clinical application of miRNAs as biomarkers is still limited. One of the most significant obstacles is the difficulty concerning the normalization of circulating miRNAs. Spiked in synthetic miRNAs are widely used to normalize serum and plasma miRNAs expression, but this approach does not include effects of pre-analytic variables on circulating miRNAs measurement [41,42]. Since the difficulties associated with haemolysis and platelet contamination of plasma samples are also significant, strategies were proposed to minimize the degree of red blood cell derived miRNA contamination, as well as minimize platelet contamination of plasma samples [43]. It must also be pointed out that the sole presence of specific miRNAs is a biomarker of disease, infection, or inflammation. There are no measuring units, there are no possibilities of disease grading based on miRNAs concentration and there are no reference levels of specific miRNAs in specific tissues, both healthy and diseased. Despite all these deficiencies, miRNAs are still very promising tools in the process of disease diagnostics.

9.5.4. Conclusion

In conclusion, to the authors' knowledge, this work lays the basis for a breeding program that will help CKCS' breeders in their targeted selection, to obtain healthier subjects with a good life expectancy, thus ensuring the protection of the genetic pool of the breed, which represents an important national and international goal.

In this study the breeders and the owners played a fundamental role, as the choice to screen their dogs, especially breeding animals, can make a long-term difference on reducing the incidence of the MMVD. Screening is essential to reduce the incidence of hereditary heart disease, which brings enormous benefits, an objective that has been tried to achieve precisely with this project. The follow-up of these subjects will be fundamental to identify which subjects will develop more serious or rapid forms of the disease, and it will certainly take many years to get precise answers and to further improve the screening protocols.

The magnitude of the heritability estimates suggests that selection against premature MMVD would be successful and, to that end, the production of early biomarkers for premature MMVD would be a useful addition. However, care must be taken in any proposed breeding programme to ensure that breeding away from premature MMVD does not result in a concomitant increase in other notable diseases in the CKCS, such as syringomyelia, or that too high a selection intensity leads to a drastic loss of genetic diversity, thereby reducing the general robustness of the breed and potentially leading to another genetic disease in the future.

9.5.5. References

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10. Other publications

During my PhD studentship, I had the opportunity to work with several figures of our Veterinary Teaching Hospital staff (veterinary radiologists, pathologists, anesthesiologists, geneticists, surgeons), human cardiovascular surgeons and engineers. The other published studies in which I have been involved during my PhD studentship are reported below:

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11. Acknowledgements

"The highest reward for a person's toil is not what they get for it, but what they become by it"

John Ruskin

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