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Association between Aortoseptal Angle in Golden Retriever Puppies and Subaortic Stenosis in Adulthood

M.C. Belanger, E. Côté, and G. Beauchamp

Background: Predicting subaortic stenosis (SAS) in adult Golden Retriever dogs (GRs) by evaluating them as puppies is hampered by the progressive expression of the SAS phenotype in youth. In some children who develop SAS as adults, an abnormal aortoseptal angle (AoSA) precedes development of stenosis.

Objectives: To determine the normal AoSA in young adult GRs using echocardiography; to assess the value of AoSA in GR puppies for predicting development of the SAS phenotype.

Animals: Forty-eight 2- to 6-month-old GR puppies.

Methods: Prospective study. Puppies were recruited from clients and breeders. Puppies were evaluated with a physical examination and an echocardiogram, and this evaluation was repeated when they were 12–18-month-old adults. Puppies were classified as unaffected (WNL) or affected (SAS) retroactively, based on their results as adults.

Results: In WNL young adult GRs, mean ± SD AoSA was 152.3 ± 6.5°. Mean ± SD AoSA in SAS puppies (144.9 ± 8.6°) was significantly different from mean AoSA in WNL puppies (155.7 ± 8.8°, $P < .01$). No puppy with AoSA >160° had the SAS phenotype as a young adult; 93% (75.7–99.1%) of puppies with AoSA <145° had the SAS phenotype as young adults. Peak LVOT velocity increased significantly between evaluations ($P < .0001$) whereas AoSA did not ($P = .45$).

Conclusion and Clinical Significance: A steep AoSA in GR puppies is associated with the SAS phenotype in young adulthood. Some GR puppies have an abnormal AoSA that persists in young adulthood and is detectable before peak LVOT velocity reaches levels consistent with SAS.

Key words: Aortic; Cardiovascular; Dog; Echocardiography; Heart.

Subaortic stenosis (SAS) is the most common congenital heart defect of large-breed dogs and Golden Retrievers (GRs) are overrepresented.[1–4] Hereditary transmission through an autosomal recessive trait is suspected in this breed.[3] In GRs, a form of SAS has been described, where malalignment between the aortic root and the interventricular septum (IVS) is a striking feature of the morphologic abnormality.[5] However, the temporal evolution of this lesion is incompletely understood. In other breeds, the typical, discrete lesions of SAS are first noted in the left ventricular outflow tract (LVOT) of puppies at 4–8 weeks of age and the obstruction is progressive as the puppy grows.[6–11] Necropsy evaluation of affected dogs of other breeds indicates that the morphologic lesion of discrete SAS is more fully expressed in young adults[8] than it is in puppies, and current screening methods can produce ambiguous results that fail to discriminate between normal hearts and mild SAS in both puppies and adults.[2,6] Severe SAS can be fatal[9] and treatment is not curative.[12,13] Rather, removal of affected individuals from the breeding pool is an important part of strategies for reducing the prevalence of SAS.[14] For these reasons, efforts aimed at accurately identifying SAS in young GRs are justified.

Abbreviations:

2D	two-dimensional
AoSA	aortoseptal angle
GR	Golden Retriever
IVS	interventricular septum
LVOT	left ventricular outflow tract
SAS	subaortic stenosis
Vmax	peak velocity
WNL	within normal limits

Studies in humans and adult dogs have shown that SAS can be associated with a measurably abnormal aortoseptal angle (AoSA).[15–17] Such morphologic changes in the LVOT can generate shear stresses that induce proliferation of fibrous tissue, creating or exacerbating the discrete stenotic lesion.[18] Thus, aortoseptal malalignment can serve as the disease substrate, preceding the development of the fibrotic lesion in children with SAS.[18] If an abnormal AoSA is the primary lesion in SAS in some GRs, then it might be possible to identify it in such dogs when they are young, possibly before the emergence of Doppler echocardiographic abnormalities and other characteristics with which SAS is diagnosed at present.

The goals of this study were (1) to determine the normal value of AoSA in young adult GRs, and (2) to assess the diagnostic value of AoSA for predicting development of SAS in GRs by assessing GRs as puppies and again as adults.

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Materials and Methods

Dogs

Purebred, 2- to 6-month-old GR puppies were recruited from breeders and clients of 2 veterinary teaching hospitals between January 2009 and July 2012 to participate in this longitudinal, observational study. Owners provided informed consent in writing. The study was approved by both institutions' Animal Care Committees. All GRs were identified by tattoo or microchip readings at all visits. Each GR was evaluated with a physical examination, Doppler-derived measurement of arterial blood pressure,[a] and echocardiography, all on 2 occasions: at age 2–6 months ("first evaluation") and again at age 12–18 months ("second evaluation"). Heart murmurs, when noted, were graded conventionally.[19] Dogs were eliminated from the study if, at either evaluation, they demonstrated additional congenital cardiac malformations; systemic hypertension (defined as average systolic arterial blood pressure >160 mmHg); overt signs of illness; lack of identifiable tattoo or microchip, or inconsistency in identification from the first to the second evaluations; noncooperation during evaluation; failure to return for the second evaluation; or any combination of these factors.

Echocardiography

Echocardiographic examinations (2D, M-mode, and Doppler) were performed as previously described, with right-sided long-axis LVOT and subcostal views used for calculation of AoSA[15,20] and LVOT velocity, respectively.[21–23] Echocardiograms were performed with dogs in lateral recumbency using ultrasound units equipped with either 1.5–4.0 MHz or 3.5–8.0 MHz phased-array transducers[b,c] and simultaneous electrocardiographic display. No animal was sedated; to encourage cooperation through postprandial relaxation, owners were asked to feed each puppy in the 15 minutes that preceded the echocardiogram.

Measurement of AoSA on all echocardiograms was performed posthoc by a single evaluator (EC). Before blinding, the evaluator identified a brief (4- to 20 heartbeats) echocardiographic cine loop for each animal in each age group (puppy or young adult) that accurately demonstrated the LVOT and IVS, as previously described.[21] Then, the primary investigator (MCB) removed all clinical information (ie, name, date, and all other identifying marks) and assigned a number to each echocardiogram without the evaluator's knowledge. Finally, the evaluator determined the AoSA from the numbered echocardiograms.

The evaluator assessed AoSA both quantitatively and qualitatively. For quantitative evaluation, the previous definition of AoSA was retained: the angle formed by the long axis of the ascending aorta and the plane of the ventricular septum at end-diastole, measured from the right parasternal long-axis LVOT view.[15,20] Using a computer's[d] standard screenshot function, the evaluator captured 3 consecutive or nonconsecutive end-diastolic still frames from each cine loop. These digital images were imported into image processing software,[e] where the evaluator applied marks to define the interventricular septal axis and the axis of the aortic root as previously described (Fig 1).[15] Briefly, 2 lines spanned the aortic annulus and sinotubular junction, the joined midpoints of which created a line that served to bisect the aortic root and therefore identify the aortic axis. Similarly, 2 lines spanned the IVS approximately at the level of the maximal diastolic excursion of the septal leaflet of the mitral valve and a point 2 cm apical to it, the joined midpoints of which created a line that bisected the IVS and served to identify its long axis. The AoSA was then measured as the angle of intersection of these 2 lines, using open-source software.[f] Each dog's AoSA was determined twice (first and second evaluations), and the reported result was the average of the 3 measurements from each evaluation.

For qualitative evaluation, the evaluator reviewed the cine loops rather than the still frames, whereas remaining blinded to all information other than evaluation group (first or second evaluation). To reduce bias in preparation for qualitative assessment, each of 288 cine loops (3 cine loops for each dog × 2 evaluations for each dog × 48 dogs) was assigned a randomly generated number between 1 and 100,000.[g] Then, the evaluator performed the qualitative analysis by viewing the cine loops in numerical (ie, randomized) order. Qualitative assessment consisted of visual examination of each echo cine loop and a binary result: normal or abnormal.

Once all measurements for the first and second evaluations were completed, unblinding occurred and dogs were classified as unaffected (Group WNL) or affected (Group SAS) based on the absence or presence, respectively, of the SAS phenotype on the second evaluation only. Therefore, a puppy's group assignment was made retroactively according to whether or not it satisfied

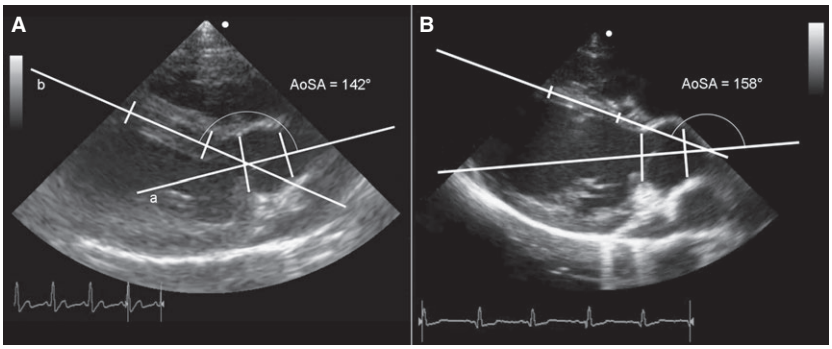

Fig 1. **(A)** Aortoseptal angle measurement in a Golden Retriever puppy that developed SAS as a young adult. The angle is formed by the long axis of the ascending aorta and the plane of the IVS. The midline axis of the aortic root is constructed by bisecting the aortic root at the level of the annulus and above the sinotubular junction (line a). The midline axis of the IVS is constructed by bisecting the septum at the level of the mitral leaflet tips and 2 cm apically from that point (line b). AoSA = 142°. Note the rightward deviation of the aortic axis (right side = top of image) causing this lower/steep AoSA. This 2-month-old puppy had an LVOT Vmax of 1.5 m/s on this echocardiogram, but 2.8 m/s on the echocardiogram performed at age 14 months. **(B)** Aortoseptal angle measurement in a normal Golden Retriever puppy. AoSA = 158°. AoSA, aortoseptal angle; IVS, interventricular septum; LVOT Vmax, peak left ventricular outflow tract velocity; SAS, subaortic stenosis.

the auscultatory and Doppler echocardiographic criteria for SAS later, as a young adult. The SAS phenotype was defined as the presence of a basilar systolic ejection murmur and a Doppler echocardiographic subcostal LVOT peak velocity (LVOT Vmax) ≥2.3 m/s on the second evaluation.[24] This diagnosis was not based on any other imaging criteria.

To attempt to separate SAS GRs from WNL GRs with greater accuracy, a secondary analysis was planned a priori. In this additional analysis, an equivocal group was empirically defined as GRs with Vmax = 2.0–2.5 m/s at the second evaluation.[3,17] For this secondary analysis, GRs in the equivocal group were excluded; GRs with an LVOT Vmax <2.0 m/s were categorized as free of the SAS phenotype (WNL-UNEQ), and GRs with a murmur as described above and LVOT Vmax >2.5 m/s were categorized as having the SAS phenotype (SAS-UNEQ).

Statistical Analyses

Target sample size was calculated using Lehr's formula (sample size = 16/[standard difference/standard deviation]^2), where the standard difference is the smallest difference of interest, and the probabilities of type I and II errors are 0.05 and 0.2, respectively.[25] The standard deviation (SD) was extrapolated from similar work on Boxers dogs (SD = 4.17°).[15] Using a smallest difference of interest of 2° in AoSA, a sample size of 16/(2/4.17)^2 = 69 individuals was projected to generate results that met these statistical criteria.

The reliability of AoSA measurements was assessed by determining an intraclass correlation coefficient obtained from a linear mixed model of the triplicate measurements for adults and puppies. A 2-way repeated measures ANOVA with group as between-subject factor and age as within-subject factor was used for determining the effect of group and age and their interaction on angle and LVOT Vmax values. The model for LVOT Vmax also took into account the unequal variances between the 2 groups. Pearson's correlation coefficient was used for examining the relationship between the angle and LVOT Vmax values for each group separately. Receiver-operator characteristic (ROC) curve analysis was used for evaluating sensitivity and specificity with respect to disease status at different thresholds of AoSA or LVOT Vmax values for puppies and adults separately. Ninety-five percent confidence intervals (95% CI) were calculated for the various estimates of sensitivity and specificity. A P value <.05 was considered statistically significant.

Results

Dogs

Sixty-eight GRs underwent evaluation as puppies; of these, 48 (29 female; 19 male) were evaluated again as young adults (20 exclusions: lost to follow-up, 19; ventricular septal defect, 1) and thus constituted the study group. No dog had systemic hypertension at either evaluation. GRs were 3.33 months old (mean; range, 2–6 months) on first evaluation and 13.77 months old (mean; range, 12–18 months) on second evaluation. Based on results of the second evaluation, 27 dogs (56%; 18 female, 9 male) did not meet the criteria for the SAS phenotype and were included in Group WNL, and 21 dogs (44%; 11 female, 10 male) met the criteria for the SAS phenotype and were included in Group SAS. The 21 dogs in Group SAS were from several different litters and 8 separate breeders.

For the secondary analysis, 21 GRs had equivocal LVOT Vmax (2.0–2.5 m/s) and were excluded. Ten dogs had LVOT Vmax <2.0 m/s at young adulthood and were included in Group WNL-UNEQ; 17 dogs had both a heart murmur as described above and LVOT Vmax >2.5 m/s at young adulthood and were included in Group SAS-UNEQ.

Heart Murmurs

Twenty-three (48%) of the 48 GRs had a heart murmur on first evaluation as puppies compared to 33 as adults (69%) on second evaluation. In the SAS group, 14/21 puppies (67%) had a heart murmur on first evaluation (I/VI, 1; II/VI, 4; III/VI, 9), whereas the other 7 puppies had normal heart sounds on first evaluation and then had a grade I or II heart murmur on second evaluation. Nine puppies (33%) in the WNL group had a heart murmur (I/VI, 7; II/VI, 2) on first evaluation; normal heart sounds were evident in 3 of these on second evaluation at young adulthood, suggesting functional heart murmurs of juvenile patients in them, and functional heart murmurs of adult dogs in the other 6 in which the murmurs persisted. Of the 17 equivocal WNL young adults excluded from the second analysis, 6 (35%) had a heart murmur (I/VI, 6).

Echocardiographic Measurements

AoSA. In this sample, WNL young adult GRs had a mean (SD) AoSA of 152.3 ± 6.5°. Mean (SD) AoSA angle was significantly steeper, or lower, indicating aortoseptal malalignment, in the SAS group compared to the WNL group in both puppies (144.9 ± 8.6° versus 155.7 ± 8.8°, mean difference = −6.1, 95% CI: −10.9%, −1.4%, P = .01) and young adult GRs (146.2 ± 8.2° versus 152.3 ± 6.5°, P < .0001; mean difference = −10.8, 95% CI: −15.5%, −6.1%, P < .0001) (Fig 2). When analysis was confined to unequivocal dogs, the difference was greater: mean (SD) AoSA was 143.8 ± 8.1° in SAS-UNEQ puppies, whereas it was 158.0 ± 6.6° in WNL-UNEQ puppies (mean difference = −9.6, 95% CI: −15.4%, −3.7%, P = .002). AoSA values did not change significantly from the first to the second evaluation (P = .45) in either the WNL group or the SAS group (interaction between group and evaluation time, ie, first or second evaluation: P = .11). AoSA was inversely and significantly correlated with peak LVOT Vmax in young adults (r = −0.62, P < .0001).

An AoSA ≤145° in puppies was associated with a sensitivity of 57% (95% CI: 34.0–78.2%) and a specificity of 93% (95% CI: 75.7–99.1%) for the presence of the SAS phenotype in young adults. When only unequivocal dogs were included, specificity increased to 100% (95% CI: 75.3–100%). Conversely, ROC curve analysis indicated that maximal specificity for absence of SAS phenotype at young adulthood was observed at AoSA ≥155° (specificity 100% [95% CI 79–100%], sensitivity 54% [95% CI: 25–81%]) in unequivocal dogs.

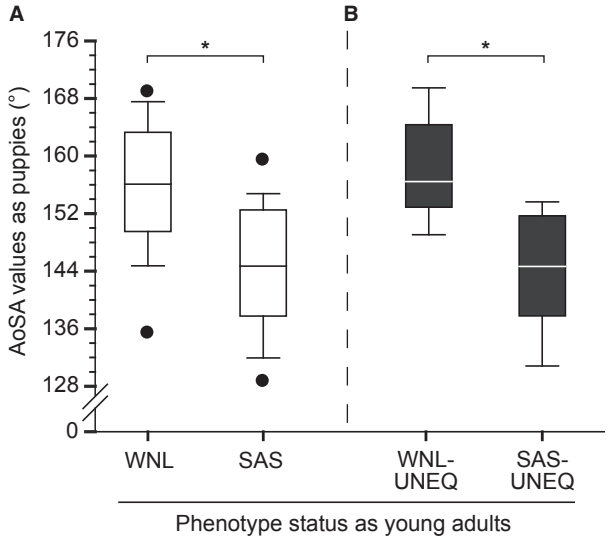

Fig 2. Box and whisker plots illustrating the AoSA values measured in **(A)** all GR puppies (n = 48) and **(B)** unequivocal GR puppies (n = 27), ie, with all puppies having an equivocal LVOT Vmax (2.0–2.5 m/s) at adulthood excluded from the analysis. The boxes represent the 25th and 75th percentiles and the central lines in the boxes represent the median values. The whiskers represent the 5th and the 95th percentiles. Values below and above the whiskers are drawn as dots. Group WNL: puppies that showed a normal phenotype at young adulthood (n = 27); Group SAS: puppies that developed the SAS phenotype at young adulthood (n = 21); Group WNL-UNEQ: puppies showing an unequivocally normal phenotype at young adulthood (n = 10); Group SAS-UNEQ: puppies showing an unequivocal SAS phenotype at young adulthood (n = 17). AoSA, aortoseptal angle; GR, Golden Retriever; Vmax, peak LVOT velocity (m/s); **P* value <.01 between groups.

The overall intraclass correlation coefficient for quantitative AoSA measurement was 62.5% (puppies, 77.6%; young adults, 41.7%). Qualitative AoSA assessment showed a sensitivity of 33.3% (95% CI: 14.6–57.0%) and a specificity of 96.3% (95% CI: 81.0–99.9%) to correctly classify GRs as normal or affected with the SAS phenotype based on subjective, blinded assessment of echocardiographic loops alone.

Vmax. The model revealed a significant increase in mean LVOT Vmax from the first to the second evaluation in both groups ($P < .001$): WNL GRs had mean (SD) LVOT Vmax = 1.66 ± 0.25 m/s as puppies and 1.99 ± 0.22 m/s as young adults (mean difference = 0.33, 95% CI: 0.19%, 0.45%, $P < .0001$), and SAS GRs had LVOT Vmax = 2.40 ± 0.91 m/s as puppies and 3.19 ± 0.87 m/s as young adults (mean difference = 0.79, 95% CI: 0.24%, 1.4%, $P = .006$). An LVOT Vmax >2.3 m/s on first evaluation was associated with a sensitivity of 52.4% (95% CI: 29.8–74.3%) and a specificity of 100% (95% CI: 87.2–100%) for identifying the SAS phenotype on second evaluation.

Combined Variables

The addition of AoSA to existing information (LVOT Vmax in this study) yielded the following results: GRs identified as having the SAS phenotype as young adults (Group SAS; n = 21) were identified correctly as having the SAS phenotype as puppies in 11/21 cases (52% [95% CI: 29.8–74.3%]) based on LVOT Vmax on first evaluation, but in 14/21 cases (67% [95% CI: 43.0–85.4%]) based on abnormal LVOT Vmax or abnormal AoSA. GRs identified as not having the SAS phenotype as young adults (Group WNL; n = 27) were identified correctly as such when they were puppies in 100% (95% CI: 87.2–100%) of cases based on LVOT Vmax and in 56% (95% CI: 35.3–74.5%) of cases based on AoSA.

Discussion

The results of the present study revealed that a propensity to develop the classically recognized echocardiographic phenotype of SAS can be suspected when a GR puppy has an abnormally steep AoSA. The results of the study also produced an expected range of AoSA values in healthy adult GRs, provided insights on the change in echocardiographic parameters in GRs as they grow, and revealed intraobserver variability in measuring AoSA and the value of assessing AoSA subjectively. The importance of these findings is multifaceted: this information supports the rheological concept of an SAS lesion, it mirrors findings in other breeds of dogs and in humans, and it could improve the sensitivity and specificity of the cardiac screening process in GR puppies.

The concept of SAS as a fixed obstruction to left ventricular outflow has existed for decades.[8,24,26,27] This notion was challenged when a new form of SAS was described in GR puppies.[5] The present study supports those findings and shows that, as suggested in the adult Boxer[15] and Dogue de Bordeaux,[17] GRs might suffer from SAS that is at least partially caused by an abnormal AoSA. The proposed mechanism implicates morphologic abnormalities of the LVOT, including a small aortic annulus, increased mitral-aortic valve separation and a steep AoSA that trigger nonlaminar flow in the LVOT. This turbulence causes shear stress in the LVOT and elicits a fibroblastic reaction that may form, or add to, the discrete lesion of SAS.[16,18,28] In humans, SAS does not appear during embryonic development of the heart and occurs very infrequently in the neonatal period; rather, it can be considered to be an acquired heart defect caused by altered blood flow patterns.[29,30] Shear stress in a genetically predisposed individual triggers stimulation of growth factors and cellular proliferation,[28] and in GRs, SAS has features to indicate that it is a heritable disorder.[3] One could hypothesize that a steep AoSA is genetically predetermined in some dogs and in such cases, is responsible for the flow disturbance that triggers or contributes to the development of the SAS phenotype.

Implicating an abnormal AoSA as the primary congenital malformation in dogs with SAS is supported by several observations: there is a direct relationship between AoSA and LVOT Vmax in adult dogs;[15,17]

the discrete lesion of SAS can appear, or grow, over time, and can recur after surgical excision[11,13]; and the point of maximal intensity of a murmur of SAS is sometimes not at the left base but at the right base, as might be expected with a steeper/lower AoSA that deviates the ascending aorta rightward (ie, toward the top of a 2D right-sided long-axis LVOT echocardiographic view). Further exploration of these notions in GRs is warranted, at least in part because a better understanding of the pathogenesis of the lesion could improve case selection and the accuracy of prognostication for GRs scheduled to undergo surgical or interventional treatment of SAS.[13]

Despite important earlier findings,[5,7,8,11] little is known about the morphologic evolution of the LVOT of retrievers as they grow to adulthood. Longitudinal evaluation of LVOT Vmax in this study population was consistent with previous reports in that all GRs with LVOT Vmax >2.3 m/s as puppies had the SAS phenotype as young adults. However, many puppies with LVOT Vmax <2.3 m/s had LVOT Vmax >2.3 m/s as young adults, which illustrates the variability in this measurement as dogs grow. Furthermore, adult dogs with LVOT Vmax >2.3 m/s can have such high velocities in the absence of detectable lesions of the LVOT, aortic valve, and ascending aorta, as demonstrated in other breeds.[17,26] Therefore, both the sensitivity and specificity observed in this study must be considered in light of the extensive limitation imposed by a diagnosis of SAS reached only through auscultation and Doppler echocardiography. Conversely, the findings of this study suggest that the static nature of AoSA during a dog's growth is different from the changes that occur with Doppler-assessed LVOT velocities: AoSA did not change significantly when puppies were re-examined as young adults, whereas LVOT Vmax was significantly higher in adult dogs compared to when the same dogs were puppies. This observation is consistent with the notion that LVOT Vmax is not a specific marker of SAS unless it is very high.[26,31] AoSA might therefore allow for an early, and more accurate, diagnosis of SAS in growing GRs.

The study results also showed that LVOT Vmax could be within normal limits in some GR puppies and then be abnormally high in the same dogs as young adults, and that in such cases, an abnormal AoSA suggested the SAS phenotype when the GRs were still puppies (n = 3/10; 30%). Moreover, no dogs in Group SAS had a normal AoSA and an abnormal LVOT Vmax as puppies and then developed an abnormal AoSA (and retained an abnormal LVOT Vmax) as young adults. Specifically, in this study population, finding a normal AoSA (>155°) in a GR puppy strongly suggested that the animal would likely not develop the SAS phenotype as a young adult. Similarly, abnormal AoSA (<145°) had features that suggested it could serve as an early marker for SAS in some GRs, even when the traditional method of detection, LVOT Vmax, had not yet surpassed the threshold arbitrarily established for making the diagnosis of SAS. Such a conclusion is supported by similar AoSA values found in adult Boxer dogs with (AoSA = 142 ± 4.2°) and without (AoSA = 153 ± 4.8°) SAS.[15]

Intraclass correlation was lower (intraobserver variability was higher) than expected for AoSA measurement. Several reasons could explain this finding, including the unmasking of true variability when suggestion bias is removed (by the implementation of blinding), technical/operator limitations, and observer limitations. During an echocardiographic exam, the LVOT is dynamic, and the measurement of the AoSA from a still frame at 1 point in the cardiac cycle is an oversimplification of this complex morphology that could explain some variation. Interobserver variability for measurement of the AoSA is good in human patients,[16,32] but it was not assessed in the present study.

Subjective assessment of AoSA was a simple but potentially inconsistent approach to estimate AoSA in real time. However, this subjective assessment appeared clinically useful to recognize abnormal AoSA. The images in this study were processed using high-precision open-source software, but a clinical application could be facilitated by the existence of an angle-measuring feature in certain commonly used echocardiographs.[a]

The results of this study also fundamentally are limited by the absence of a true gold standard for the diagnosis of SAS in dogs. We chose to set the LVOT Vmax value at 2.3 m/s as proposed previously.[24] However, the suboptimal sensitivity and specificity of LVOT Vmax[26] pose a dilemma: the performance of echo-derived AoSA as a diagnostic criterion for SAS can be misrepresented if the point of reference is itself inexact, or if another disorder (eg, aortopathy) is present. We attempted to address this question by removing so-called equivocal results and then performing a secondary analysis. The greater separation of abnormal from normal individual results for AoSA with this approach suggested that the suboptimal sensitivity and specificity of LVOT Vmax were a limiting factor in the assessment of SAS, and that the diagnostic performance of AoSA for identifying SAS might be superior than can currently be identified.

In summary, a steep AoSA in this group of GR puppies was associated with the SAS phenotype in the same dogs when they were re-evaluated as young adults. Identification of an abnormal AoSA could contribute to making a conclusive and accurate diagnosis of SAS at an earlier stage of development of some GRs. However, AoSA measurement is unlikely to be a definitive single diagnostic marker for SAS; its value will likely be clearer when its performance is compared to that of other, more definitive tests, such as genetic analysis, necropsy studies, tridimensional imaging, or other reference points rather than LVOT Vmax alone.

Footnotes

[a] Model 811-B, Parks Medical Electronics Inc, Aloha, OR
[b] Vivid-7 ultrasound system, GE Medical, Wauwatosa, WI
[c] Logiq 7, GE Medical

[d] Apple MacBookPro: Apple Inc, Sunnyvale, CA
[e] Adobe Photoshop version 6.0: Adobe Systems, San Jose, CA
[f] National Institutes of Health: Image J. http://rsb.info.nih.gov/ij/ Accessed July 24, 2013
[g] Excel, Microsoft Office; Microsoft, Inc, Redmond, WA

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Conflicts of Interest: Authors disclose no conflict of interest.

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