The role of Caveolin-1 in canine Intervertebral disc degeneration

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ABSTRACT:

Intervertebral disc (IVD) degeneration is common in both Chondrodystrophic (CD) and Non-chondrodystrophic dogs (NCD) and can lead to pain and neurological deficits (IVD disease). Current treatment options do not stop the degeneration process, but (new) regenerative treatments could perhaps prevent this problem in the future. The peptide Caveolin-1 (Cav-1) could be an important regenerative treatment option. It is known to regulate the WNT/β-catenin signaling- pathway, which is suspected to play an important role in IVD degeneration.

In this study we tried to further elucidate the role of Cav-1 in canine IVD degeneration. Two immunohistochemical stainings (Cav-1 and Tunel) were performed on 37 cadaveric IVDs to assess the relation between Cav-1 expression and cell apoptosis during different stages of IVD degeneration (different Thompson-, Pfirrmann- and Modified Boos scores). Our hypothesis that Cav-1 expression decreases during early IVD degeneration and increases during late IVD degeneration, was confirmed during this study. Also, a positive correlation was found between Cav-1 expression and cell apoptosis, especially in CD dogs during the end stages of degeneration. The contradictory findings between cell apoptosis and Cav-1 expression in Notochordal- (NC) and Chondrocyt-like cells (CLC) in NCD dogs suggests a different role for Cav-1 in these two cell types.

INTRODUCTION:

Intervertebral disc (IVD) degeneration is a pathological condition which is commonly seen in dogs without any clinical symptoms.[1] However, when IVD disease (e.g. herniation) occurs, heavy pain and neurological symptoms – sometimes even paralysis – can develop.

Hansen et al. described two types of herniation.[2] Hansen type 1 herniation comprises sudden extrusion of the nucleus pulposus through the annulus fibrosus into the vertebral canal.[3] Chondrodystrophic (CD) dogs, which have disproportional short limbs because of their abnormal endochondral ossification, are predisposed to develop this type of herniation. In CD dogs, many IVDs show degenerative signs, but Hansen herniation type 1 mostly develops in the cervical and thoracolumbar regions of young dogs (> 2 years old.)[4]

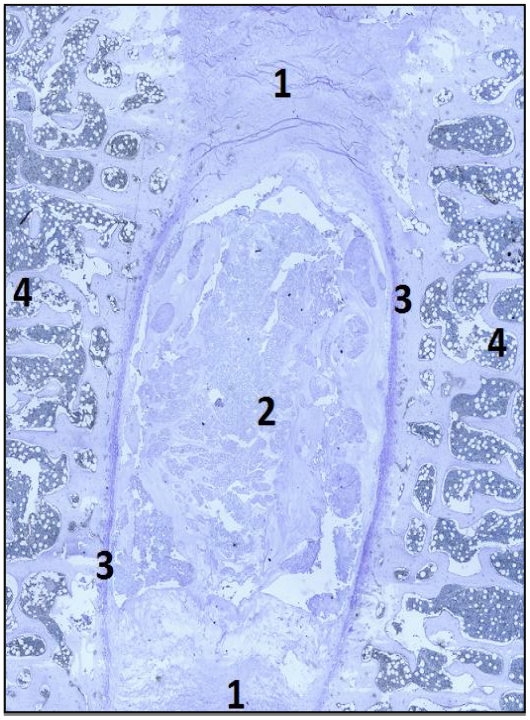


Figure 1. 1.Annulus fibrosus, 2.Nucleus pulposus, 3.Endplate, 4. Bone marrow

Non-chondrodystrophic (NCD) dogs, which have limbs of normal length, more commonly develop Hansen type 2 herniation. This type of herniation is known for its slower protrusion of both the AF and NP and is mostly seen in older aged dogs (2-6 years old) in the caudal cervical- and lumbosacral regions.[3] [4] Both types of herniation show comparable histological changes.[4] [5] [6]

Herniated IVDs can be treated in different ways. The most commonly performed chirurgic treatment is partial discectomy. Also spinal fusion of two vertebral bodies, (prophylactic) fenestration and the implantation of a new (synthetic) NP are chirurgic options, but appear to be less successful.[7] [8] [9-11] The chirurgical measures are exclusively based on the herniated IVD and the degeneration in other discs is not inhibited or prevented. Ideally, IVD degeneration is prevented before clinical symptoms develop. Therefore, the ultimate aim of regenerative treatments is to intervene before IVD degeneration starts and to prevent the development of IVD disease.

As mentioned previously, others have already shown that the same histological changes take place during IVD degeneration in both types of herniation.[4-6] The nucleus pulposus, normally stabilized by the protecting annulus fibrosus (fig 1), becomes less vicious and the notochordal cells (NCs) disappear.[1] Chondrocyt-like cell (CLC)-clusters appear in the nucleus pulposus and annulus fibrosus and the glycosaminoglycan (GAG) content decreases. These changes make it impossible for the IVD to optimally perform its normal function (stabilization and mobility of the vertebral column).[1]

Affected IVDs can be divided, based on their macroscopic or histological changes. Pfirrmann made a mapping based on the macroscopic changes that become visible with magnetic resonance imaging (MRI).[12] In human medicine, the Thompson- (macroscopic) and BOOS score (based on histological changes) are widely used.[13] [14]
Because of the known histological and morphological differences between human- and dog IVDs, Bergknut et al. developed a new canine IVD histological grading system: The Modified Boos score.[4, 5]

The cause of IVD degeneration is multi-factorial and genetic components are considered to play an important role. The biomolecular processes are not well defined yet. Smolders et al. found some changes in the canonical WNT/β-catenin signaling- pathway during IVD degeneration.[15, 16] This important pathway is regulated by the peptide Caveolin-1 (Cav-1). Cav-1 has an effect in many different tissues during regeneration and degeneration and is mainly localized in membranes of many different cells. Smolders et al. discovered that during the first stages of IVD degeneration (when the CLC's appear and the NCs disappear), a significant down-regulation of Cav-1 gene and protein expression occurs.[8] Heathfield et al. studied this phenomenon during the end stages of human IVD degeneration. [17] In these stages a positive correlation between the stage of degeneration and the level of Cav-1 was found. So, stage 5 scored discs showed significantly more Cav-1 expression than discs of stage 3 and 4. Also cellular senescence was positive correlated with the gene expression of Cav-1 during these end stages of IVD degeneration.[17]

AIM OF THIS STUDY:

We hypothesize that also in the canine species, the expression of Cav-1 decreases during the first stages of IVD degeneration and ultimately increases during the end stages. We furthermore hypothesize that during the IVD degeneration process, a positive correlation will be found between Cav-1 expression and apoptosis of the NP resident cells. Furthermore, we expect to find an reversed correlation between Cav-1 expression and cell proliferation during the IVD degeneration process.

MATERIALS & METHODS:

MATERIALS:

- **Study population**

The total study population exists of 37 cadaveric canine IVDs from different Thompson scores, originating from 16 dogs (table 1). The dogs were offered for pathological research or euthanized for other unrelated studies approved by the Ethics Committee on Animal Experimentation (DEC) of Utrecht University. On each IVD, the Pfirrmann- and Thompson gradation, and Modified Boos score was determined by three different specialists.
The dogs were of different genders and comprised both CD (5) and NCD (11) breeds (5 Beagles, 3 Foxhounds, 3 Kerry Beagles, 2 Mongrels, 1 Welsh Terrier, 1 Flatcoated Retriever and 1 Bouvier). Age ranches between 17 and 192 months (mean 75.6 months). Weight ranches between 8.5 and 44 kg (mean 23.8 kg).

Thompson score	1	2	3	4	5	**Total:**
Number of IVD's	8	7	8	7	7	37

Table 1

METHODS:

- **Immunohistochemical Cav-1 staining**

This cytoplasmatic/membrane staining was used to stain the Cav-1 quantity on each slide. First the slides were dewaxed in xylene (2x 5min) and rehydrated in graded alcohols (96%, 80%,70% and 60%). Then, after 3 minutes washing in PBS, the slides were incubated for 60 minutes in citrate in a water bath (37C° → 70C°). After 20 minutes of cooling and two washes in PBS 0.1%Tween (2x5min), the peroxidase activity was blocked (5 min, Ready-to-use reagent-DAKO). After another 5 minutes of washing in PBS 0.1%Tween, the slides were blocked for 30 minutes with NorGoatserum (10% + 1% BSA in 0.1%Tween in 1% PBS). Then the slides were incubated overnight with diluted mouse anti-caveolin (DB Bioscienses, 610406) on 4C°.

The next day, the slides were first washed another 3 times with PBS 0.1%Tween (3x5min) and after that the 2nd antibody (Dakocytomation envision anti-mouse, K4001) was added for 30 minutes at room temperature. After two washes with PBS (2x5min) the slides were incubated with DAB substrate for peroxidase (DAKO) at room temperature for 10 minutes. Then, after washing with PBS (2x5min) the slides were covered by hematoxylin drops for 5-10 seconds and flushed by tapwater for 10 minutes.
Afterwards, the slides were dehydrated in graded alcohols (60%, 70%, 80% and 96% (2x)) and waxed again (2x5min xylene).

Our negative control slide was treated with another first antibody (goat anti –mouse IgG, R&D, HAF007) and the same second antibody. Our positive control references were the endothelial cells in the same samples.

- **Immunohistochemical Ki67 staining**

With this protocol we tried to stain the core-peptides that are specific for proliferation. We modified our Cav-1 protocol but ultimately didn't obtain a good staining for canine IVDs (Supplementary A).

- **Immunohistochemical Tunel staining**

This core staining was used to determine which cells were in apoptotic stages. We stained the apoptotic DNA strand breaks by enzymatically labeling the free 3'-OH termini with modified nucleotides by using the ApopTag ® Plus Peroxidase *In Situ* Apoptosis Detection Kit (S701) from Millipore. The following protocol was used:
First we dewaxed the slides in xylene (2x 5min) and rehydrated the slides in graded alcohols. (96%, 80%,70% en 60%). After 5 minutes washing in PBS we covered each IVD for 9 minutes with proteinase K. Then, after 2 x 2minutes washing with MilliQ we blocked the peroxidase activity by adding Ready-to-use reagent (DAKO) for 10 minutes. Then, after 2x 5 minutes washing in PBS, we added 30 microliter of equilibration buffer for at least 1 minute. After 1 minute we took off as much buffer as we could, added the TDT enzyme and incubate on 37Cº for 60 minutes. Then we washed the slides another 4 times. First 10 minutes in stop-and-washbuffer, then 3x1 minute in PBS. After that, antidigoxignenin conjugaat was added for 30 minutes. Then we washed 4x2 minutes in PBS and added the peroxidase substrate (DAB) for 5 minutes. The last washes were then performed: first 3x1minute in MilliQ and then 1x5 minute in MilliQ. The slides were covered with diluted (1:2) hematoxilin for 30 seconds and flushed for 10 minutes by tap water. Afterwards, the slides were dehydrated in graded alcohols (60%, 70%, 80% and 96% (2x) and waxed again (2x5min xylene).

Our negative control was treated with another first antibody (goat anti –rabbit IgG, R&D HAF008) and the same second antibody. Mammae tissue of a rat was used for positive control. Also bone marrow in our own samples was used as positive control reference.

- **Digitalization of the immunohistochemical stainings**

We used the Olympus BX60 microscope and the computer program Leica Application Suite (LAS V4.2) to make photographs from our samples.
With microscope 4x objective we took several photographs of the nuclei pulposi from each Cav-1 positive stained sample. We stitched these photographs by using the same computer program to get a clear overview of the whole nucleus pulposus.

After that, we used Adobe photoshop CS6 to make semi-quantitative measurements of the total nucleus pulposus surface (cells and matrix) and the Cav-1 positive stained area. The stained area was determined with the integrated option "color range". The ratio between the stained area and the total nucleus pulposus surface (=Cav-1 ratio) was used for further statistical analyses.

We also took photographs with microscope 20x objective from all Tunel- and Cav-1 stained samples. On each sample, 4 specific areas of the nucleus pulposus were selected (fig 2). These areas were first photographed in the Cav-1 sample, thereafter we photographed exactly the same areas in the Tunel stained sample. Again, on each Cav-1 photograph, the ratio between nucleus pulposus (only cells) and Cav-1 positive area (=Cav-1 ratio) were measured with photoshop CS6 (fig 2).

The Tunel photographs were used to find out the ratio between the total number of nuclei and the positive nuclei. (=Tunel ratio) This was counted by hand by using the integrated option "count tool" from Photoshop CS6 (fig 2). All the data were used for further statistical analysis.

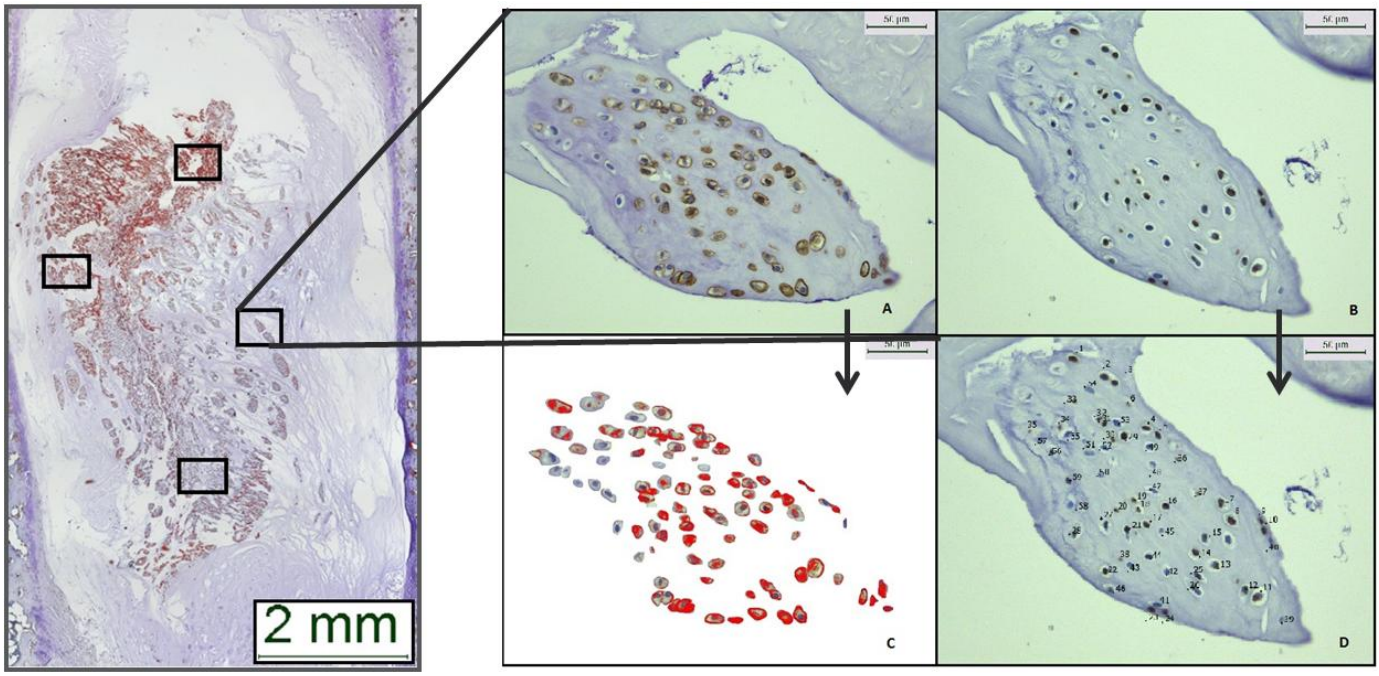


Figure 2

- **Statistical analyses**

Statistical analysis on the differences in average positive Cav-1/Tunel area between the different Thompson/Pfirrmann grades was performed using RStudio (version 0.96, http://www.rstudio.com) and R (version 2.15.2).
Before we determined whether the differences were statistically significant, the data were examined for normal distribution; the Cav-1 ratio data were not normally distributed, whereas the Tunel ratio data were. Therefore, we performed statistics with the log-value of the Cav-1 data. The mixed model was optimized per comparison. First, the random part of the model was determined (e.g. with random slopes and/or random intercepts). Thereafter, the fixed part of the model was optimized.

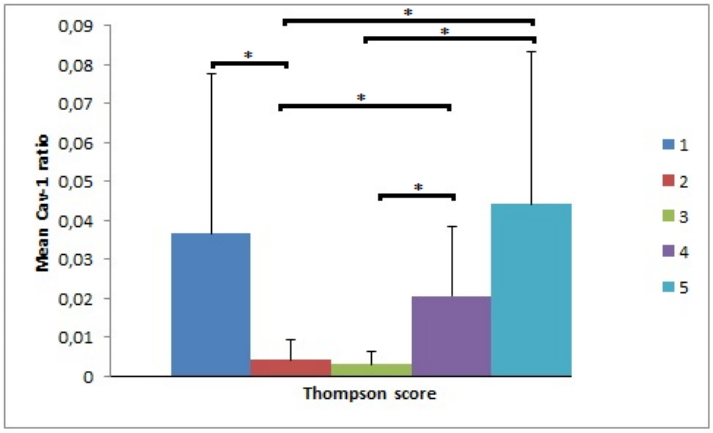
To find (significant) correlations between on the one hand the Cav-1 ratio or Tunel ratio and the Thompson/Pfirrmann/Modified Boos score, we used the IBM SPSS statistics 20 program. The Pearson correlation was used for measuring with the Tunel ratio because these results were distributed normally. Because Cav-1 ratio were not normally distributed, the Spearman correlation was used for measuring all data that consists Cav-1 ratio.

In all above mentioned tests, a P-value <0.05 was considered significant.

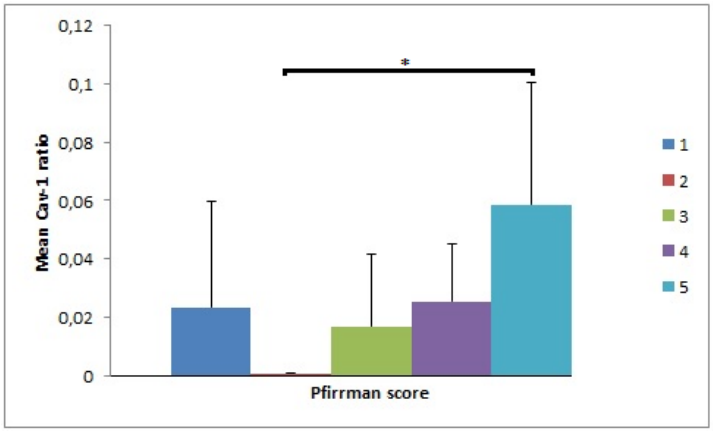
RESULTS:

- **Mixed model results**

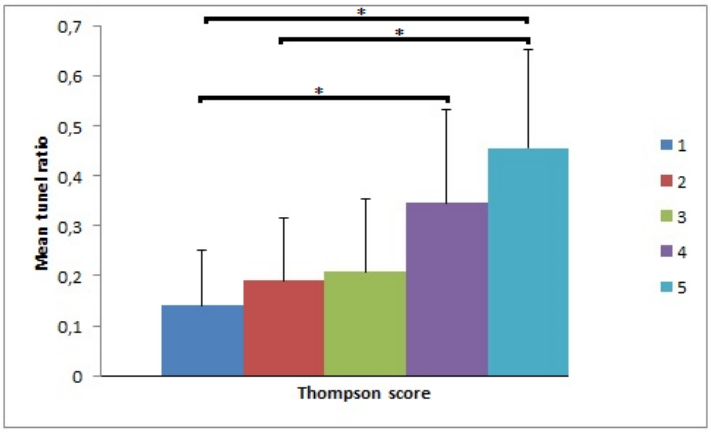
The differences in average positive Cav-1 and Tunel ratio per Thompson- and Pfirrmann score are shown in graph 1 till 4. Significant differences are indicated with an asterisk (*, p<0.05). The average expression of Cav-1 in Thompson score 1 is significantly higher than in Thompson score 2. The Cav-1 ratio in Thompson score 4 and 5 is also significantly higher than in Thompson score 2 and 3. Graph 2 and 4 appear to show the same trend, although not all differences in average are significant.
Graph 3 shows an undivided positive linear correlation between mean Tunel ratio and Thompson score.



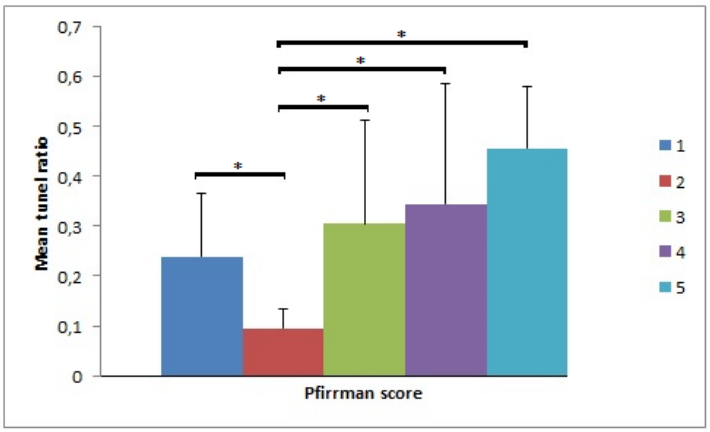
Graph 1



Graph 2



Graph 3



Graph 4

- **Immunohistochemical Cav-1 staining**

37 IVD samples were stained using the above mentioned protocol. One sample was lost during the washing steps. Analysis of the immunohistochemical results using the microscope 4x objective demonstrated cytoplasmic/membrane Cav-1 staining in 13 samples, whereas 35 positively Cav-1 stained samples were identified with the use of the 20x objective. NCs of Thompson score 1 appear to express most Cav-1 protein, but also CLCs (especially in CD dogs) show much Cav-1 protein expression in the Thompson- and Pfirrmann scores 4 and 5 (Fig 3 and 4).

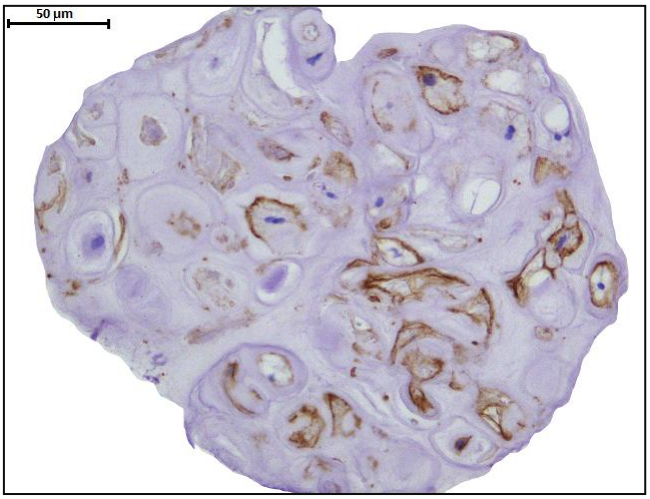


Figure 3

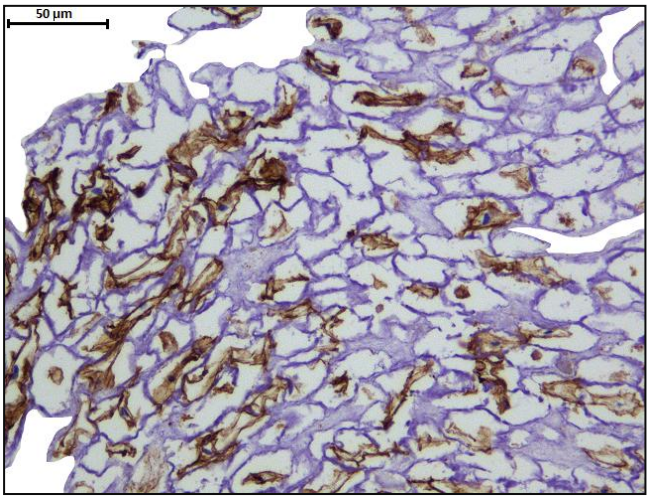
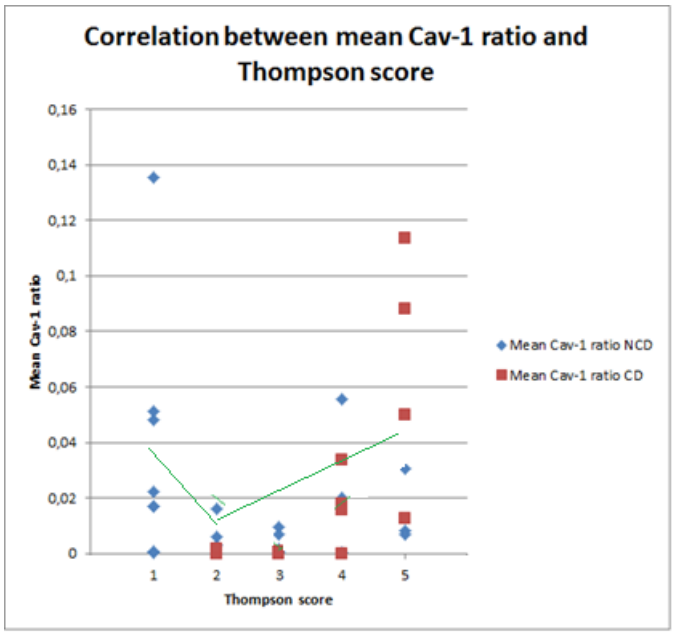
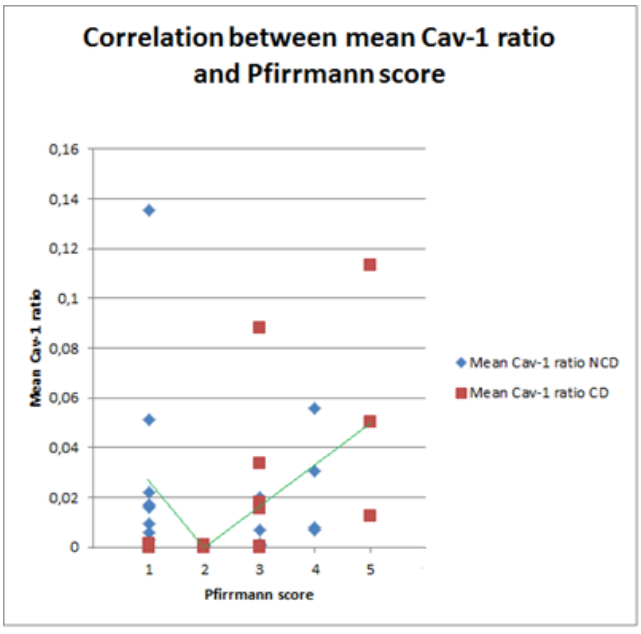


Figure 4

By analyzing the mean Cav-1 ratio (objective 20x) per Thompson- and Pfirrmann score, a divided correlation was found (Graph 5 and 6). A negative correlation between stage 1 and 2, and a positive correlation between stage 2,3,4 and 5. These two correlations were both significant ($p < 0.01$, table 2).



Graph 5



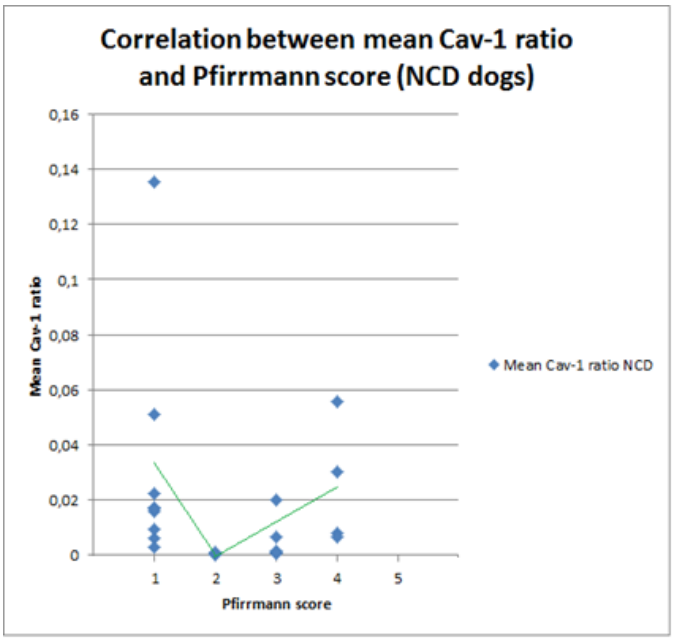
Graph 6

Cav-1 ratios per Thompson- and Pfirrmann score gathered with the 4x objective show the same pattern as with the 20x objective (Graph A and B in supplementary B). Due to the few data, however, other r-values were found and the divided correlations were not all significant (Table 2).

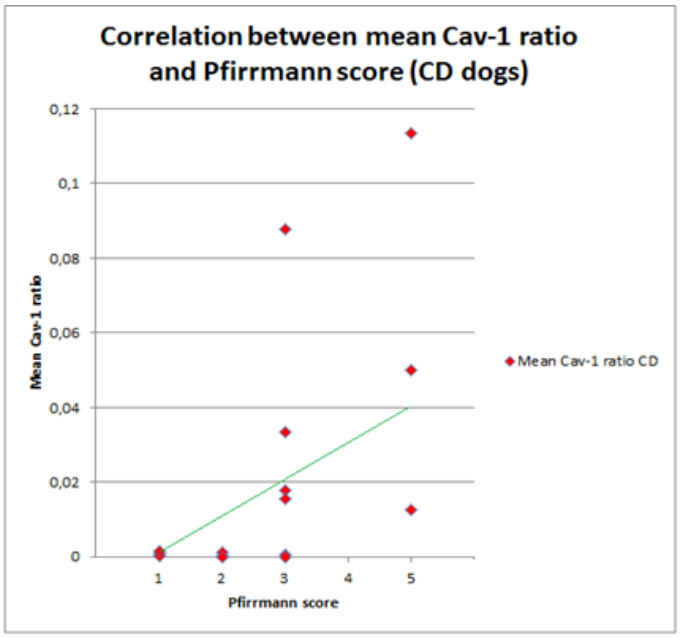
		Cav-1 ratio CD+NCD (obj.4)	Cav-1 ratio CD+NCD (obj.20)	Cav-1 ratio CD (obj.20)	Cav-1 ratio NCD (obj.20)	Mean Cav-1 ratio CD+NCD (obj.20)	Mean Cav-1 ratio CD (obj.20)	Mean Cav-1 ratio NCD (obj.20)	Tunel ratio CD+NCD (obj. 20)	Tunel ratio CD (obj. 20)	Tunel ratio NCD (obj. 20)	Mean Tunel ratio CD+NCD (obj. 20)	Mean Tunel ratio CD (obj. 20)	Mean Tunel ratio NCD (obj. 20)
Thompson	**(r)**	-.569	.124	.600	-.108	.174	.719	-.129	.439	.515	.392	.564	.682	.485
p-value		.043	.143	.000	.321	.311	.004	.567	.000	.000	.000	.000	.005	.030
Thomspon1+2	**(r)**	-.756	-.519	xxx	-.355	-.557	xxx	-.387	.144	xxx	.249	.205	xxx	.342
p-value		.030	.000	xxx	.018	.031	xxx	.239	.273	xxx	.103	.464	xxx	.304
Thompson2t/m5	**(r)**	-.132	.507	.600	.367	.593	.719	.349	.392	.515	.251	.525	.682	.343
p-value		.777	.000	.000	.006	.001	.004	.222	.000	.000	.092	.005	.005	.276
Pfirrmann	**(r)**	-.453	.143	.450	-.084	.190	.606	-.141	.290	.393	.178	.367	.521	.203
p-value		.139	.093	.000	.453	.273	.022	.542	.001	.002	.128	.033	.046	.404
Pfirrmann1+2	**(r)**	xxx	-.430	-.434	-.436	-.632	xxx	-.753	-.377	-.416	-.356	-.531	-.678	-.490
p-value		xxx	.000	0.056	.002	0.006	xxx	.005	.001	.043	.013	.023	.139	.106
Pfirrmann 2t/m5	**(r)**	.289	.484	.500	.452	.659	.558	.808	.420	.442	.389	.532	.572	.484
p-value		.638	.000	.001	.002	.001	.075	.001	.000	.002	.016	.011	.052	.156
Modified boos	**(r)**	-.461	.125	.538	-.084	.152	.685	-.142	.323	.437	.220	.400	.578	.233
p-value		.132	.151	.000	.464	.392	.007	.550	.000	.000	.068	.021	.024	.352
Modified boos<13	**(r)**	-.418	-.431	.565	-.255	-.454	.580	-.212	-.017	.136	-.025	-.025	.221	-.035
p-value		.350	.001	.009	.112	.089	.306	.556	.898	.569	.879	.930	.721	.923
Modified boos>13	**(r)**	.564	.247	.356	.129	.296	.416	.074	.126	.348	-.140	.168	.473	-.202
p-value		.322	.034	.033	.440	.219	.266	.839	.297	.028	.460	.506	.167	.631

Table 2. r- and p values of different correlations. In red are significant p-values ($p<0.05$), in blue are negative correlations.

The divided correlation seen in Cav-1 ratio per Pfirrmann score was not detected when we only look at the Cav-1 ratios from the CD dogs (Graph 7). However, the Cav-1 ratios per Pfirrmann score of NCD dogs per Pfirrmann score did show more or less the same divided correlation (Graph 8). This difference between CD- and NCD dogs was also seen in comparisons between Cav-1 and Thompson score (Graph C and D in supplementary B).



Graph 7



Graph 8

Although there is more dispersion when Cav-1 is compared with the Modified Boos score, it seemed like the same trends are found as seen per Thomspon- and Pfirrmann score (Graph E, F and G in supplementary B). However, the divided correlation in CD dogs appears not to be significant in this case (Table 2).

- **Immunohistochemical Tunel staining**

37 IVD samples were stained using the above mentioned protocol. One sample was lost during the washing steps. All samples showed more or less apoptotic nuclei (using the 20x objective) (Fig 5 and 6).

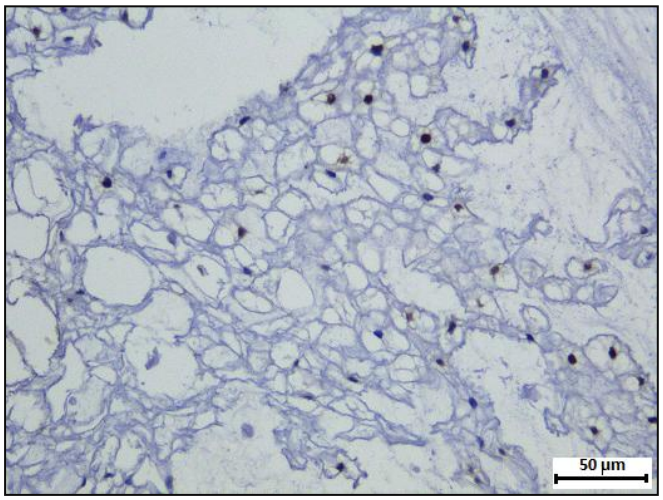


Figure 5

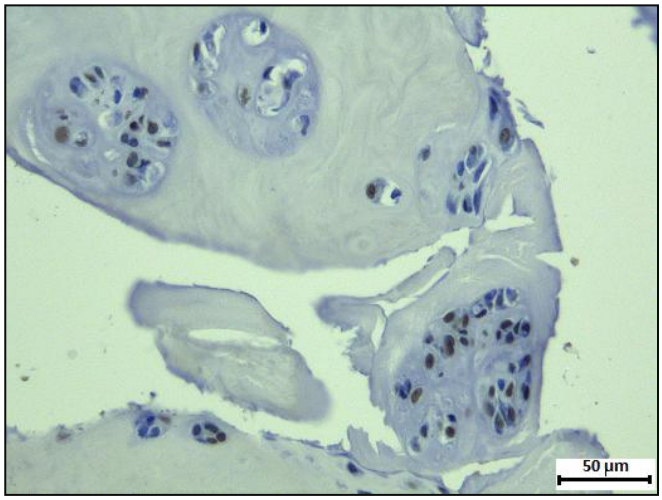
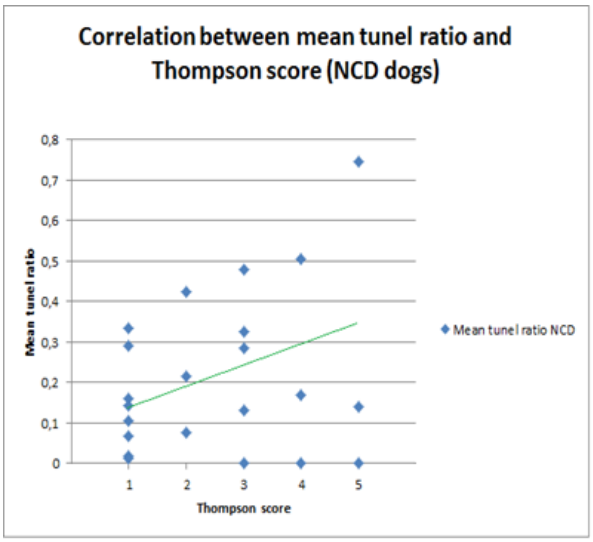
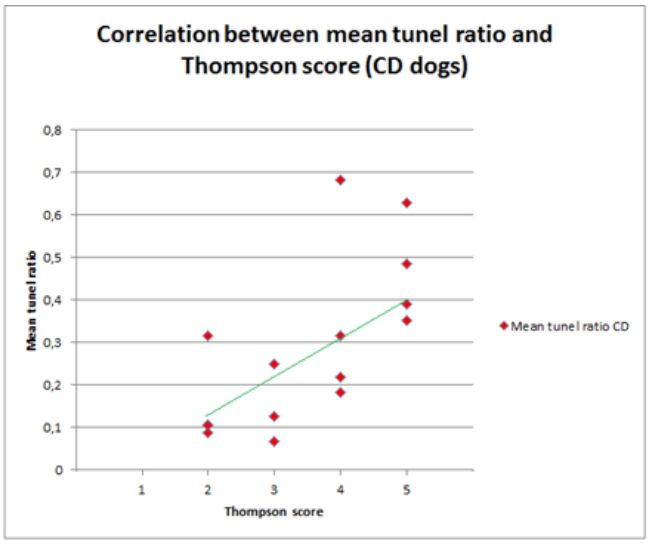


Figure 6

Mean Tunel-ratios per Thompson score in NCD dogs seem to give a different correlation than what was seen in Cav-1 ratio per Thompson score (Graph 7.) A significant (undivided) positive correlation was found in this case ($P<0.01$). The Tunel ratio per Thompson score in CD dogs showed the same trend as seen in Cav-1 ratios (Graph 8).



Graph 7

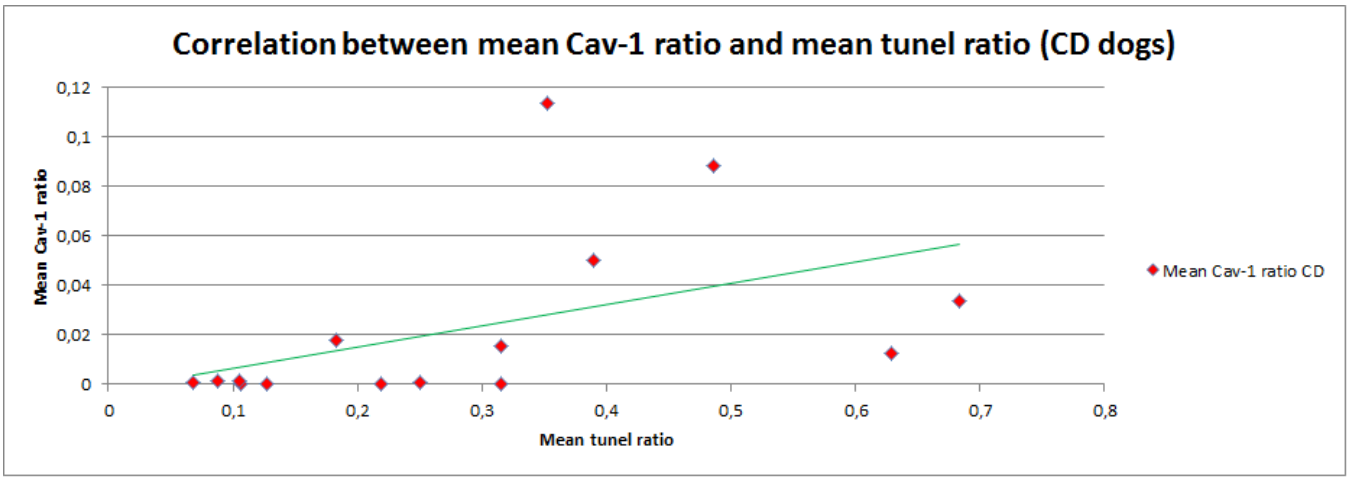


Graph 8

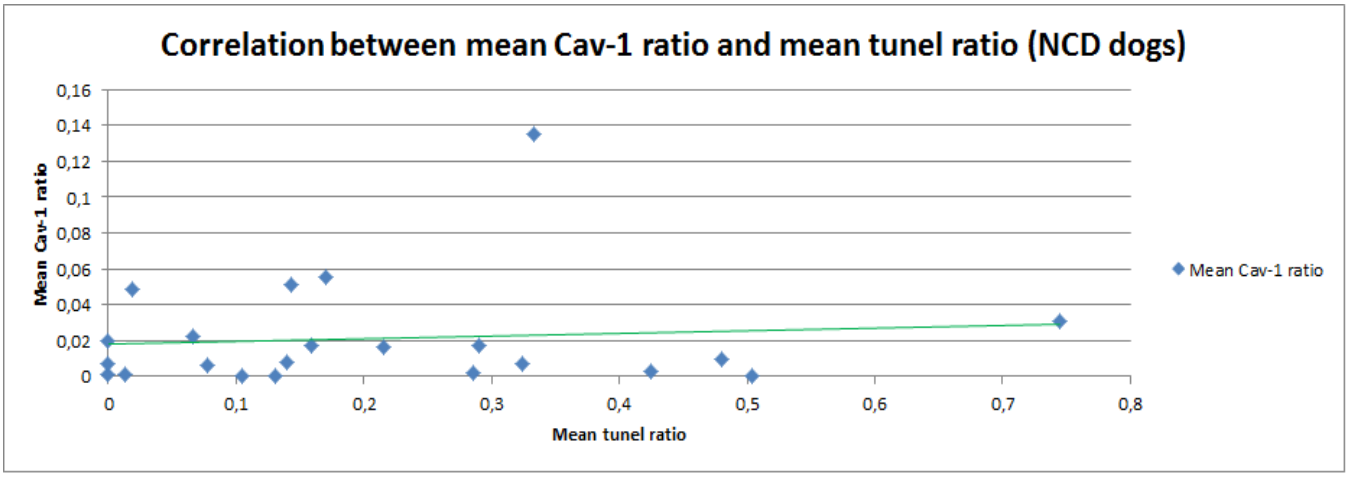
When the Tunel ratio per Pfirrmann- and Modified Boos score was compared with Cav-1 ratios per Pfirrmann and Modified Boos score the same trends were seen (Graph H and I in supplementary B).

- **Correlation between Cav-1 ratio and Tunel ratio**

To determine if there was a (significant) correlation between the Cav-1 ratio and the Tunel ratio we also compared these data for both CD- and NCD dogs (Graph 9 and 10).



Graph 9

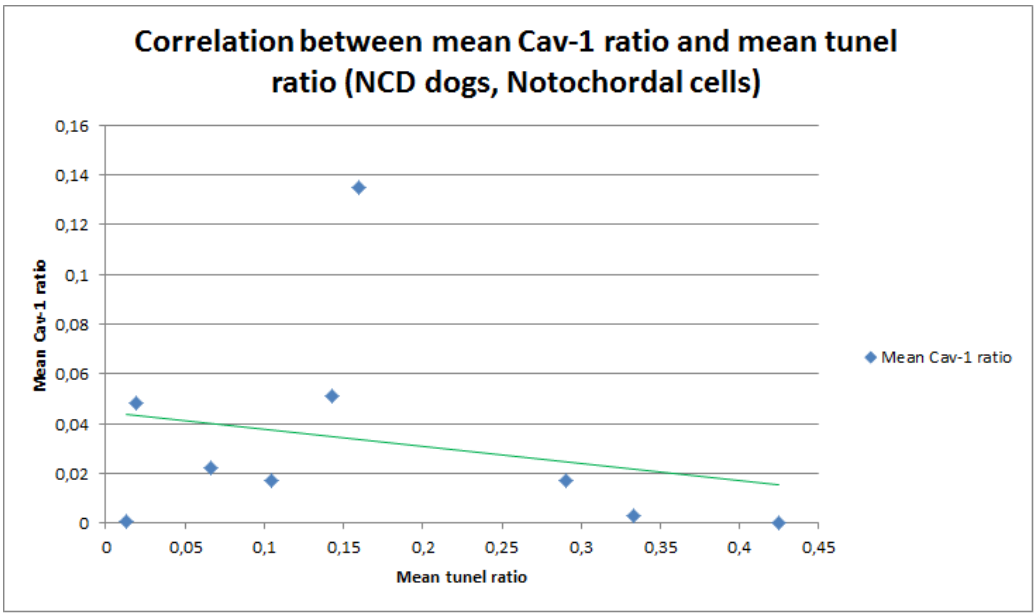


Graph 10

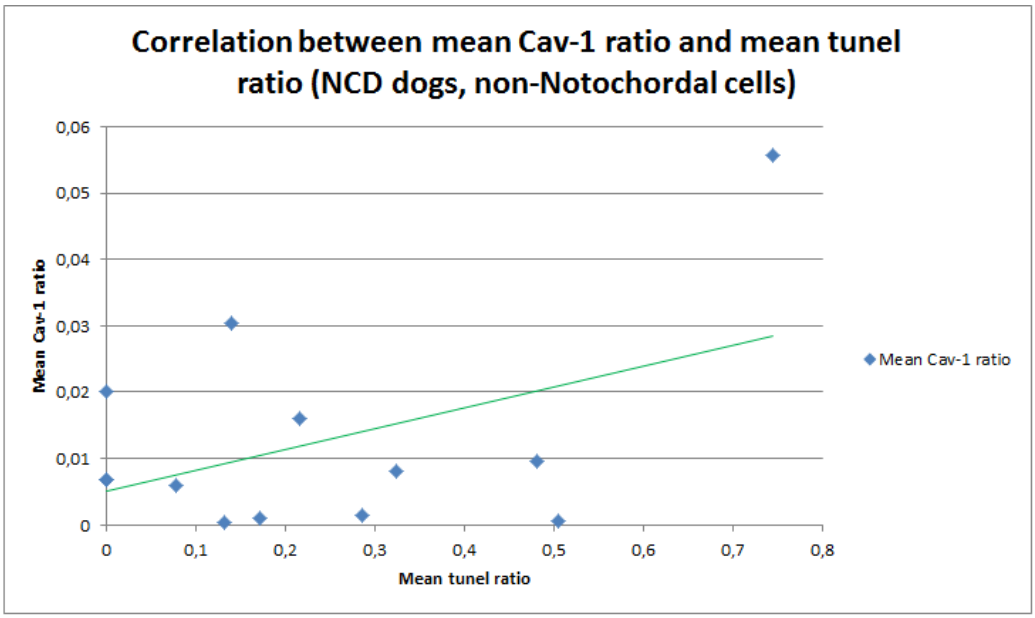
In CD dogs a significant correlation between Cav-1 ratio and Tunel ratio was found (P<0.05, Table 3), whereas NCD dogs didn't show a significant correlation. After we filtered out the data that contained NCs, NCD dogs also show more or less the same trend as seen between Cav-1 ratios and Tunel-ratios in CD dogs (Graph 11). Samples that contain NCs did not seem to show this trend (Graph 12).

		Tunel ratio CD+NCD (obj. 20)	Tunel ratio CD (obj. 20)	Tunel ratio NCD (obj. 20)	Mean Tunel ratio CD+NCD (obj. 20)	Mean Tunel ratio CD (obj. 20)	Mean Tunel ratio NCD (obj. 20)	Mean Tunel ratio NCD NC-cel coupes	Mean Tunel ratio NCD nonNC-cel coupes
Cav-1 ratio CD+NCD (obj.20)	**(r)**	.287							
p-value		.001							
Cav-1 ratio CD (obj.20)	**(r)**		.550						
p-value			.000						
Cav-1 ratio NCD (obj.20)	**(r)**			.109					
p-value				.342					
Mean Cav-1 ratio CD+NCD (obj.20)	**(r)**				.334				
p-value					.053				
Mean Cav-1 ratio CD (obj.20)	**(r)**					.565			
p-value						.035			
Mean Cav-1 ratio NCD (obj.20)	**(r)**						.185		
p-value							.435		
Mean Cav-1 ratio NCD NC-cel coupes	**(r)**							.167	
p-value								.668	
Tunel ratio NCD nonNC-cel coupes	**(r)**								.318
p-value									.340

Table 3. r- and p values of different correlations. In red are significant p-values (p<0.05).



Graph 11



Graph 12

DISCUSSION:

Samples of NCD- and CD dogs that were classified as Thompson or Pfirrmann stage 1 (and 2) showed huge differences in their Cav-1 ratio; NCD dog samples showed high Cav-1 ratios, while CD dog samples showed almost no Cav-1 expression. Most probably, this is because these few CD dog samples did not contain NCs. From earlier work, we know that gene expression of Cav-1 is significantly down regulated in the CLC-rich IVD compared with the NC-rich IVD in NCD dogs in the early IVD degeneration stages.[8, 16] This could probably be an explanation for the low levels of Cav-1 that we found in these samples.

Cav-1 and Tunel ratios per Modified Boos score gave more dispersion in the results than the Cav-1 and Tunel ratios per Thompson and Pfirrmann score did (Graph E,F and G in supplementary B). This most probably is because of the bigger range of scoring (1-29) in the Modified Boos score.[5] Bergknut et al. found a high correlation between Modified Boos- and Thompson score and also the Thompson and Pfirrman score appear to be highly correlated. [4, 18, 19] Bergknut et al. also found that the border area between Thompson score 2 and 3 matches with Modified Boos score 10 till 15.[5] Our finding that the correlation cutoff point is somewhere near Modified Boos score 13 confirms his finding.

Apoptosis is considered to play an important role in the process of IVD degeneration and has been thought to contribute to the degradation of extracellular matrix.[20] In this study the Tunel ratio is considered as the percentage of apoptosis.
Generally, the Tunel ratios per Thompson-, Pfirrmann, and Modified Boos score for CD- and NCD dogs showed comparable trends as the Cav-1 ratio per Thompson-, Pfirrmann, and Modified Boos score. However, the Tunel-ratio per Pfirrmann score for NCD dogs showed another pattern; here, a positive linear correlation was found instead of a divided correlation (seen in the Cav-1 ratio per Thompson score). However, a closer look to the samples that were scored 1 and 2 for the Thompson grade, reveals that all samples which contain NCs are scored as Thompson stage 1, except for 1 sample.
This sample is scored as Thompson stage 2 and contains most apoptosis of all Thompson stage 2 scored samples. Also, the Modified Boos- (8) and Pfirrmann score (1) showed that it was a sample that was actually in between stage 1 and 2. This could be the reason why we found a linear correlation instead of a divided correlation for Tunel ratio per Pfirrmann score in NCD dogs. When this specific sample was scored as Thompson stage 1, the same divided correlation was found as in all other comparisons.

The trend that we found in Cav-1 expression confirmed our hypothesis and the results that were found in earlier performed studies. Smolders et al. showed that WNT/β-catenin signaling as well as Cav-1 gene expression was down regulated in early (subclinical) degeneration in both CD- and NCD dogs.[16] He also showed increased Cav-1 gene expression in NCs compared with early degenerated CLCs.[16] Moreover, Heathfield et al. showed increased Cav-1 gene expression levels during the late human IVD degeneration stages, and found a positive correlation with genes that are linked with cell senescence ($p16^{INK4a}$).[17]

It could be that the decreased Cav-1 expression leads to degeneration of NCs. The subsequent increase in Cav-1 expression during the later degeneration stages could be an ultimate attempt to save the healthy NP phenotype. This is however doomed to fail, because the degenerative process is already in progress. Smolders et al. also suggests that Cav-1 can exert different actions depending on the cellular context and the stage of degeneration.[8, 21] The different r-values that we found between Cav-1 ratio and Tunel ratio for NCs and CLCs could confirm that assumption (Graph 11 and 12). Baker et al. also demonstrated that the role of Cav-1 in tissue repair may be negative or positive, depending on the tissue type and the nature of the repair process.[22] She also stated that a decreased Cav-1 expression could be required for activation of cell differentiation and/or proliferation.[22] Taking this into account, it could be that Cav-1 performs different functions in NCs and CLCs.

In the future, we need a more complete set of samples to get better results. We did not have any CD dog samples that contain NCs. That is probably why all graphs of these CD dogs showed a linear- instead of a divided correlation (found in NCD dogs). Young CD dogs (<1 year) do have IVDs which contain NCs and should ideally be included in future projects.[4]
Also samples of NCD dogs scored as Thompson stage 4 and 5 were underrepresented in this project. This made it more difficult to determine differences between NCD- and CD dogs in these stages.
Also the total number of samples need to be higher in a future study. This makes the coincidence factor smaller, gives better results when comparing with the bigger ranged Modified Boos score and would make the r-values more reliable.

CONCLUSION:

The present study investigates the role of Cav-1 during IVD degeneration in CD- and NCD dogs. We hypothesized that the expression of Cav-1 in canine IVDs decreases during the first stages of IVD degeneration and ultimately increases during the end stages. We furthermore hypothesize that during the IVD degeneration process, a positive correlation will be found between Cav-1 expression and apoptosis of the NP resident cells. Furthermore, we expect to find a reversed correlation between Cav-1 expression and cell proliferation during the IVD degeneration process.
Cav-1 ratios and Tunel ratios of 37 IVD samples were measured and correlations were made with Thompson, Pfirrmann and Modified Boos scores. Unfortunately, the Ki67 staining did not succeed during this study, so proliferation ratios could not be measured.
Our results confirmed our hypothesized pattern of Cav-1 during the degeneration process. We also found a positive correlation between Cav-1 expression and apoptosis, especially in CD dogs during the end stages of degeneration. However, NCs (of NCD dogs) did not show a correlation between Cav-1 expression and apoptosis. Perhaps, the Cav-1 peptide has different functions in different cell and/or tissue types.
The exact role of Cav-1 in IVDs should be further elucidated in order to confirm or reject if Cav-1 could be a good target for regenerative treatment in IVD degeneration.

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SUPPLEMENTARIES

A. Ki67 protocol:

With this protocol we tried to stain the core-peptides that are specific for proliferation. We used different methods for antigen retrieval, but in all cases, either the NP tissue was lost or the core-peptides were not stained appropriately. The problem is typed in **bold** in our protocol below.

First the slides were dewaxed in xylene (2x 5min) and rehydrated in graded alcohols. (96%, 80%,70% en 60%) Then, after 3 minutes washing in PBS, the slides were **incubated for 60 minutes in citrate in a water bath (37 Cº →98 Cº)**. After 20 minutes of cooling and two washes in PBS 0.1%Tween (2x5min) the peroxidase activity was blocked (5 min with Ready-to-use reagent-DAKO). After another 5 minutes of washing in PBS 0.1%Tween the slides were blocked for 30 minutes with NorGoatserum (10% + 1% BSA in 0.1%Tween in 1% PBS). Then the slides were incubated overnight with diluted anti-KI67 (thermo Scientific SP6 RM-9106-S) on 4Cº.
The next day the slides were first washed another 3 times with PBS 0.1%Tween (3x5min) and after that the 2nd antibody (Dakocytomation envision anti-Rabbit K4003) was added for 30 minutes at room temperature. After two washes with PBS (2x5min) the slides were incubated with DAB substrate for peroxidase (DAKO) at room temperature for 10 minutes. Then, after washing with PBS (2x5min) the slides were covered by hematoxylin drops for 5-10 seconds and flushed by tapwater for 10 minutes.
Afterwards, the slides were dehydrated in graded alcohols (60%, 70%, 80% and 96% (2x)) and waxed again (2x5min xylene).
Our negative control slide was treated with another first antibody (goat anti –mouse IGG, R&D HAF007) and the same second antibody.

We used canine duodenum as a positive control (this sample did not slip of the slides and showed massive proliferation using the above protocol). The nucleus pulposus (NP) cells didn't fix well enough to the slide and slipped off at 98Cº. That's why we tried to improve our protocol by changing incubation time and/or incubation temperature.

Modification on protocol	**Outcome**
30 min, 98 Cº	Duodenum +, NP slipped of
60 min, 80 Cº	NP slipped of
90 min, 80 Cº	Duodenum -, NP slipped of
120 min, 80 Cº	NP slipped of
Overnight, 80 Cº	Duodenum +, NP slipped of
90 min, 70 Cº	Duodenum -, NP -
120 min, 70 Cº	Duodenum -, NP -
180 min, 70 Cº	Duodenum -
240 min, 70 Cº	Duodenum -
Overnight, 70 Cº	Duodenum -

We also tried to modify the protocol using temperature-independent antigen retrieval methods (based on literature and in-house advice).

10 min Protein kinase (DAKO) (no watherbath)	Duodenum -
10 min Protein kinase (Quiagen) (no watherbath)	Duodenum -
90 min, 70 Cº + 10 min Protein kinase (DAKO)	Duodenum -
Incubation in 0,01 EDTA for 90 min, 70 Cº	Samples were totally gone

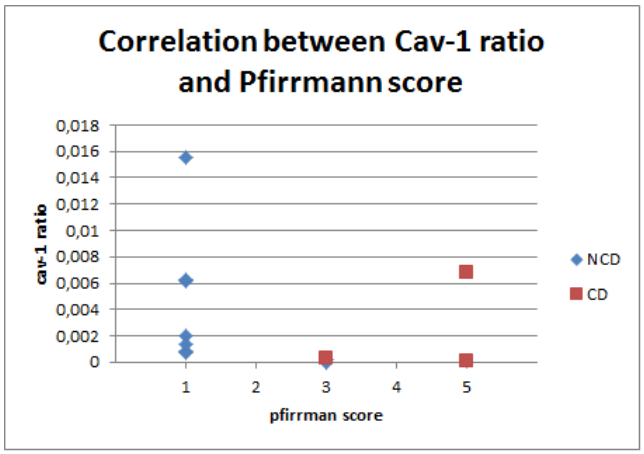
Ultimately, we tried to get better adhesion of the NP to the slide on different manners.

10 min, 98 Cº after 24 hrs in stove (56 Cº)	Duodenum -, NP slipped of
90 min, 80 Cº after 24 hrs in stove (56 Cº)	Duodenum -, NP slipped of
Overnight, 80 Cº after 24 hrs in stove (56 Cº)	Duodenum+, NP slipped of

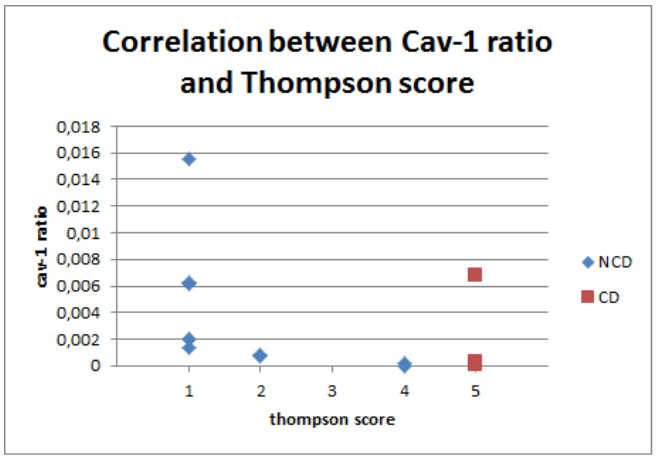
None of our modifications led to a good staining for canine IVDs. Perhaps other slides (e.g. coated slides) ensure better adhesion. However, due to time issues, we did not try this.

B. Additional results

(r- and p values in table 2 and 3)



Graph A



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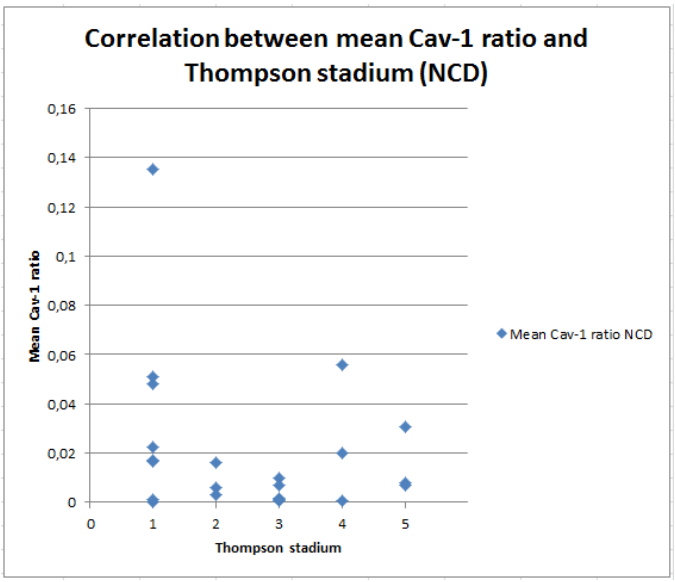

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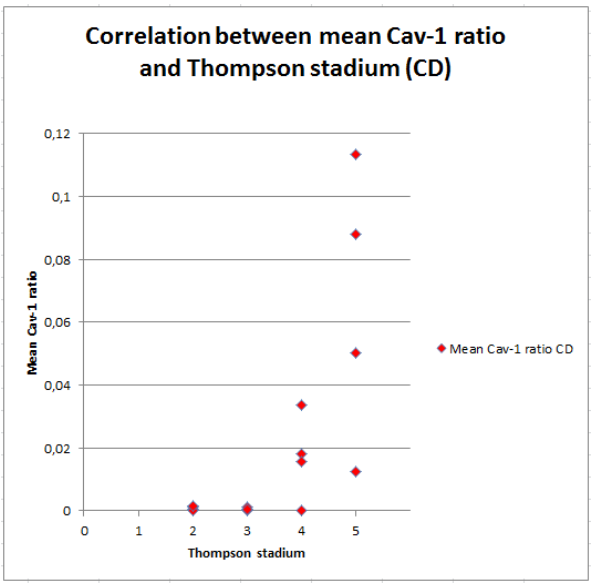

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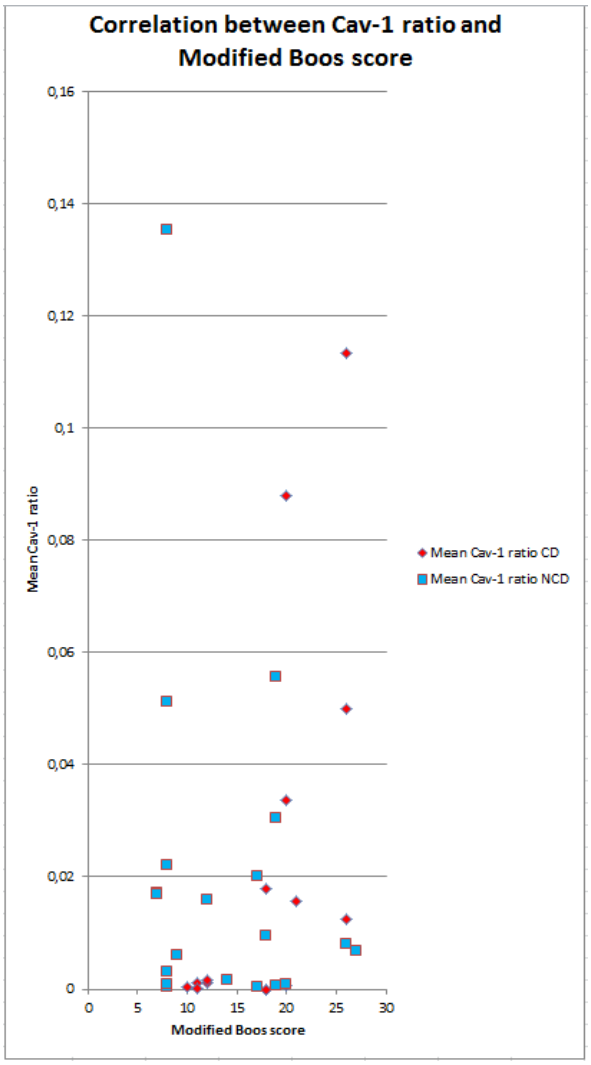

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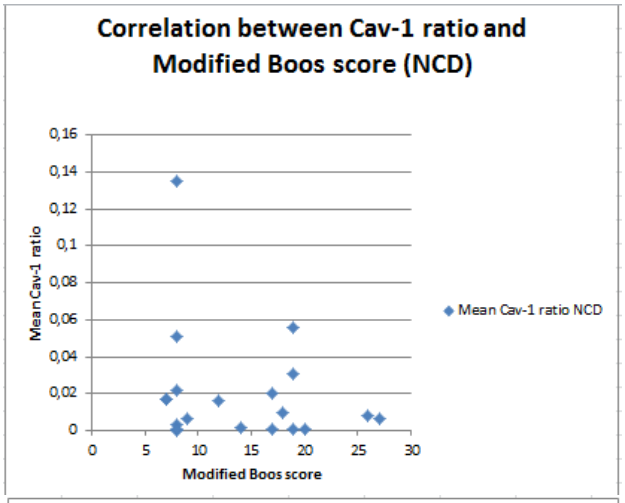
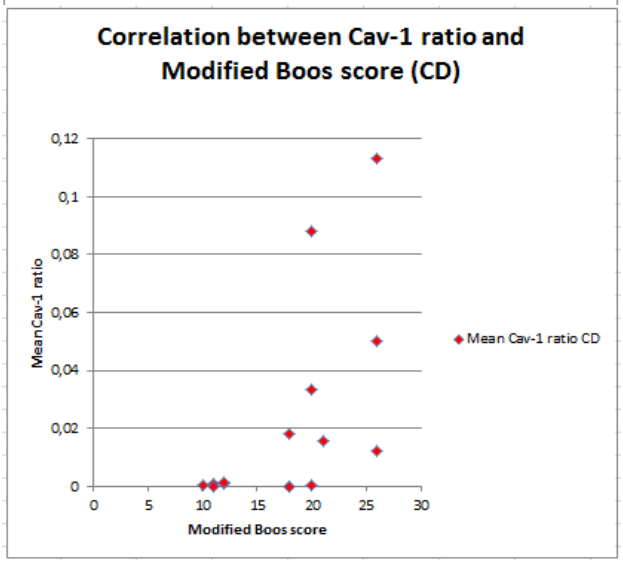
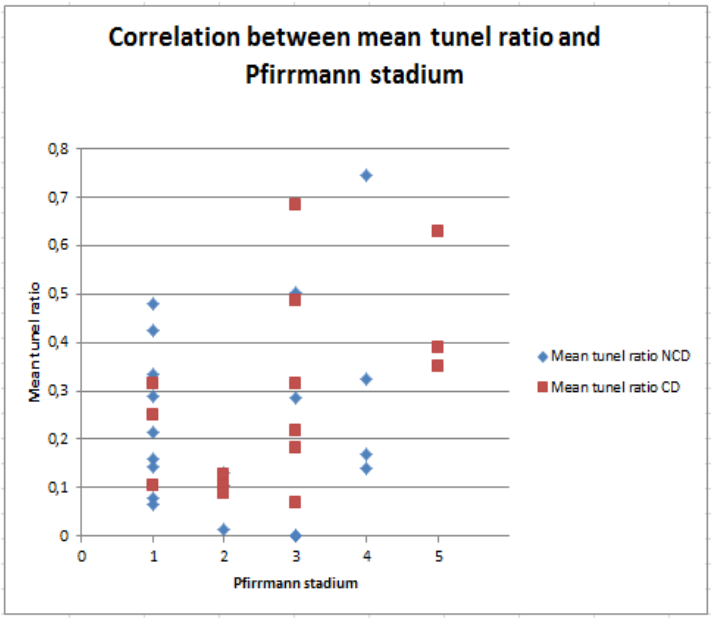
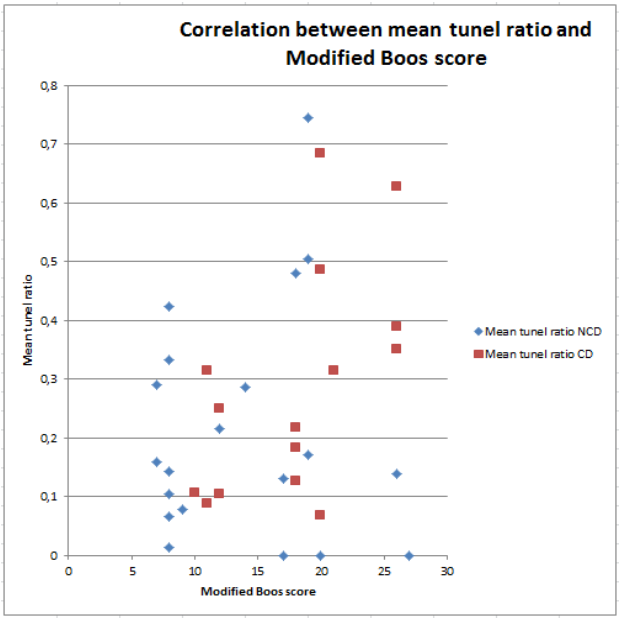




Graph F and G



Graph H



Graph I