**Aus dem Institut für Tierpathologie des Fachbereichs Veterinärmedizin**

**der Freien Universität Berlin**

# Age- and tumor- dependent senescence in canine testis and eye

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dogs, testes, eyes, neoplasm, biochemical markers, kinases, spermatogenesis, Leydig cells, fibroblasts, retina, eye lens

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## 2 Abbreviations

| | |
|---|---|
| Adenoma of PHG | Adenoma of the perianal hepatoid gland |
| ACA of PHG | Adenocarcinoma of the perianal hepatoid gland |
| BPH | benign prostatic hyperplasia |
| cat | cataract |
| CB | ciliary body |
| CBA | ciliary body adenoma |
| conj Mel | conjunctival melanoma |
| crypto | cryptorchid |
| d | diameter |
| DNA | deoxyribonucleic acid |
| f | female |
| FFPE | formalin-fixed and paraffin-embedded |
| Fig | Figure |
| GCL | ganglion cell layer |
| HE | haematoxylin and eosin |
| HPF | high power field |
| IHC | immunohistochemistry |
| INL | inner nuclear layer |
| IQR | interquartile range |
| ITT | intertubular triangle |
| KOH | Potassium hydroxid |
| LCT | Leydig cell tumor |
| m | male |
| M | median |
| ME | microenvironment |
| MV | mean value |
| na | not accessible |
| neut | neutered |

| | |
|---|---|
| nk | not known |
| No | number |
| ONL | outer nuclear layer |
| PC | prostatic cysts |
| pH | power of hydrogen or *potentia hydrogenii* |
| PH | perineal hernia |
| pigment | melanin pigmentation |
| rc | reproduction control |
| pRB | retinoblastoma protein |
| RPE | retinal pigment epithelium |
| SA- βGal | senescence-associated beta-galactosidase |
| SASP | senescence-associated secretory phenotype |
| SD | standard deviation |
| SEM | seminoma |
| SERT | Sertoli cell tumor |
| T | tumor |
| UV | ultraviolet |
| uv Mel | uveal melanocytoma |
| UM | uveal melanocytoma |
| w | with |
| w cat | with cataract |
| w/o | without |
| w/o cat | without cataract |

# 3 Introduction

## 3.1 Aging

Aging is the continuous loss of normal cell function with time that affects most living organisms.[1] There have been several attempts to explain aging. Antagonistic pleiotropy, also known as the pay later theory, states that some genes give you a benefit in young (re)productive years whilst having deleterious effects that manifest at an age beyond the reproductive phase.[2] It therefore contributes to aging whilst having no evolutionary consequences. An example is the testosterone production in male humans. Whilst increasing the fertility in young age it may increase the risk for prostate cancer with age whilst other studies even attribute testosterone a protective effect[3,4] Another example is the expression of p53, one of the best known tumor-suppressors. Whilst a high expression of p53 decreases the risk for tumor development in mice, it also significantly limits their life span in comparison to their conspecifics with a lower expression of p53.[5,6] Aging is thus the result of the declining force of natural selection with age.

Aging occurs at the molecular, cellular, tissue and organismal level and is dependent on a variety of factors including metabolic rate, sensitivity to DNA damage, efficiency of DNA repair, effectiveness of antioxidant systems or rate of prooxidant generation, immune function and cellular proliferation.[7]

The pace of aging is extremely variable between species, populations and individuals. Some organisms like the sea anemone are thought to not age at all.[8] In many animals, including the elephant and the mouse, a positive relationship between body size and longevity can be observed. This has always been explained with the higher metabolic rate of smaller animals and the correlating higher level of oxidative stress.[7] Dogs, however, have an inverse relationship between life expectancy and body weight as small pure breed dogs live longer than large pure breed dogs.[9] It is known that smaller dogs have a higher metabolic rate which challenges the hypothesis behind elephant and mouse. To date, it remains hypothetical which factors are responsible for the inverse relationship of body size and life span in dogs.

In 2013, Lopez et al. proposed nine hallmarks of aging as important contributors to aging and the aging phenotype: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion, altered intercellular communication and cellular senescence.[10]

## 3.2 Cellular senescence

Cellular senescence can be defined as irreversible cell cycle arrest coupled with an altered, enlarged morphology and the expression of a senescence-associated secretory phenotype

(SASP).[10] The term senescence was first introduced by Hayflick and Moorhead in 1965, when they discovered the replicative senescence.[11] They described the limited lifespan of serially passaged human fibroblasts in cell culture, termed the Hayflick limit. The effect they observed seems to be caused by telomere shortening. Today, we know that there are also telomere-independent stimuli that trigger senescence including radiation, oxidative stress and genotoxic drugs.[12]

The accumulation of senescent cells in aged tissues has been shown in mice, men and baboons with investigated tissues being skin, brain, liver, pancreas and hematopoetic stem cells.[13-18] If this accumulation is the result of an increased generation or a decreased clearance of senescent cells remains unknown. The relationship of senescent cells and age remains controversial. The effects of senescent cells can be diverse as senescent cells exhibit the SASP with numerous paracrine activities including the secretion of pro-inflammatory cytokines, growth factors and proteases.[1] They have been associated with both deleterious, e.g. atherosclerosis, as well as beneficial, e.g. restriction of fibrosis, effects.[1]

### 3.3 Senescence markers

Characterization of senescent cells in tissues and organs has proven to be rather challenging due to the absence of senescence specific markers.[19] This may be due to the multifunctional nature of senescent cells. There is more than one kind of senescent cell strongly dependent on the cell type and the senescence-inducing stressor. One must also consider that senescence may not be a static endpoint but rather a multi-step evolving process, starting with chromatin modifications that lead to cell cycle arrest and progression to specific but variable gene upregulations that result in the expression of the SASP.[20]

The gold standard is the senescence-associated beta-galactosidase (SA-βGal), a lysosomal enzyme that is overexpressed in correlation with the senescent phenotype at pH 6.[19] It has, however, several limitations including the fact that it can also be induced by stresses such as confluence in cell culture (confluence-associated senescence).[21] Also, it is only applicable in cell culture or on vitrificated tissues (liquid nitrogen).[22]

Senescence-associated growth arrest often begins with the DNA-damage response (e.g. induced by telomere shortening, UV-light) and follows the p53/p21 or p16INK4a/pRB (retinoblastoma protein) tumor suppressor pathways.[21] Markers based on the initial DNA-damage include the phosphorylated histone protein H2AX (termed γH2AX), one of the earliest responses to DNA-double strand breaks.[23] Markers based on the tumor suppressor pathways include cyclin-dependent kinase inhibitors p21, p16 and p53, all of which ultimately inhibit the phosphorylation of the retinoblastoma protein (pRB) thus inactivating the transcription factor E2F, which is required for cell cycle proliferation.[21]

## 3.4 Senescence and tumor development

Age is the primary risk factor for the development of many cancer types.[24] There is increasing evidence that senescent cells accumulate not only in aged tissues but also at sites of age-related degenerative and hyperplastic pathologies including premature aged skin, neurodegeneration, joint diseases, benign prostate hyperplasia and atherosclerotic lesions.[25-31] It is proposed that an increase of senescent cells with age leads to a change in tissue milieu that is supportive of tumor development and growth.[24] This change is supposedly brought about by the expression of SASP, which includes factors that disrupt tissue architecture and/or stimulates nearby cells to proliferate. This theory has been supported by co-culture as well as xenograft studies. Senescent cells stimulate the proliferation of pre-neoplastic and neoplastic but not normal epithelial cells in cell culture.[32] Likewise, the injection of senescent fibroblasts significantly promotes the growth of mouse and human epithelial tumor cells in immunocompromised mice.[32] Additionally, the co-cultivation of carcinoma-associated fibroblasts (i.e. from the microenvironment of the tumor) with canine mammary cancer cells displayed an up-regulation of 23 genes in the cancer cells associated with cell adhesion, angiogenesis and epithelial-to-mesenchymal transition.[33]

## 3.5 Tumors of the canine testis

The most common testicular tumors in dogs are Leydig cell tumors (interstitial cell tumors), seminomas and Sertoli cell tumors.[34] Canine testicular tumors are a common and often incidental finding in old dogs.[35] Cryptorchid dogs are predisposed to develop seminomas and Sertoli cell tumors.[36] Leydig cell tumors originate from the endocrine Leydig cells. They may be associated with tumors of the perianal glands, tail gland hyperplasia and benign prostatic hyperplasia which could be the result of androgen production by the tumor cells or the resident Leydig cells. Seminomas originate from spermatogenic cells possibly spermatogonia. They produce no hormones and thus cause no hormone-associated paraneoplastic effects. Sertoli cell tumors originate from the intratubular Sertoli cells and may produce hormones including estrogen and inhibin. This hormone production may lead to the feminization syndrome with bone marrow depletion, penis and testicular atrophy, gynecomastia, galactorrhea, bilateral alopecia and squamous metaplasia of the prostate. Most canine testicular tumors are benign. However, up to 15 % of the Sertoli cell tumors and seminomas are considered malignant and may metastasize.[37] Fertility of dogs with testicular neoplasm is strongly variable and dependent on the size, multiplicity and hormone production of the tumor.[35]

## 3.6 Tumors of the canine eyes

The most common intraocular tumors in dogs are uveal melanocytic tumors and ciliary body adenomas.[38] Uveal melanocytic tumors are mostly pigmented and located anterior rather than

choroidal and are mostly benign. Only 4-6 % of uveal melanocytic tumors are malignant with local invasion and metastasis.[39] Histological differentiation of uveal melanomas from uveal melanocytomas may be challenging as it has been shown that mitotic index and tumor extension are not always reliable predictors in biological behavior.[40] The most commonly used malignancy criteria remains the mitotic index which in malignant uveal melanomas is > 4 / 10 HPF (high power field) in addition to anaplastic cellular characteristics and tissue invasiveness.[38,39] Ciliary body adenomas are benign, typically unpigmented tumors that arise from the ciliary body epithelium.[38] Their malignant counterpart is rare and shows invasion of the sclera as its major criteria of malignancy. Other, even less common intraocular tumors include lymphoma, peripheral nerve sheath tumor and metastasis. Tumors of the optic nerve are rare and include astrocytomas and meningiomas. Although most intraocular tumors are considered benign, they increase the risk for glaucoma and enucleation is mostly inevitable.[38]

Most periocular tumors are benign and include Meibomian gland adenomas, papillomas and melanocytomas (eyelid or limbal). Conjunctival melanocytic neoplasms, however, are mostly malignant. They arise most commonly from the nictitans, followed by bulbar and palpebral conjunctiva. They are variably pigmented and have the potential for invasion and metastasis. Not all conjunctival melanomas show traditional cytological criteria of malignancy including anisokaryosis and a high mitotic index.[39]

### 3.7 Rationale and hypotheses

Dogs are one of our most favorite companion animals and we share more than just a roof. They are the shadow to our lifestyle, particularly concerning exercise, diet and environmental pollutants and other influences. The aging process is strongly influenced by our way of life as well as genetics. But concerning the latter we share at least one thing: diversity. Whereas our laboratory animals have been selected for similarity dogs have been bred for phenotypic diversity. An additional advantage to rodent models is that recent studies have shown that canine gene products concerning DNA damage response are more closely related to their human homologs than those of mice.[41,42] Research conducted on aging of dogs is scarce and there are huge knowledge gaps in need of filling. The presence of cellular senescence and its potential role in tumor development has not been confirmed in aged dogs.

Chosen were two organ systems that are known to show age-dependent pathologies: the canine testis and the canine eye. Canine testis are prone to developing tumors with age. Canine eyes show a variety of age-associated changes including corneal changes like calcareous degeneration and indolent ulcers, nuclear sclerosis and retinal degeneration.

Validated and established senescence markers p21 and γH2AX were tested for their applicability outside the classical culture plate and mouse model.

The following hypotheses were established:

Hypotheses concerning the canine testis:

1. The expression of senescence markers (p21, γH2AX) increases in aged canine testis as well as a sign of premature aging in cryptorchid testis and their expression can be correlated with morphological signs of aging in the canine testis.
2. The expression of senescence markers (p21, γH2AX) increases in the microenvironment of testicular tumors in comparison to controls.
3. The expression of p21 and γH2AX differs in tumor cells between testicular tumors.

Hypotheses concerning the canine eye:

1. The expression of senescence markers (p21, γH2AX) increases in any canine ocular structure with age.
2. The expression of senescence markers (p21, γH2AX) increases in the microenvironment of uveal melanocytomas and ciliary body adenomas in comparison to controls.
3. The expression of p21 and γH2AX differs in tumor cells of benign uveal melanocytomas versus malignant conjunctival melanomas.

To evaluate hypotheses regarding the testis, the expression of p21 and γH2AX was analyzed in young, old and cryptorchid canine testes. Subsequently, the number of positive cells was correlated with other age-dependent morphological changes. Furthermore, the expression of these markers in the microenvironment and tumor cells of testicular tumors was analyzed.

To evaluate the hypotheses regarding the eye, the same markers were tested in different structures of the canine eye of young and old dogs, as well as in the microenvironment and tumor cells of uveal melanocytomas and ciliary body adenomas. Finally, possible differences between the expression of the markers in benign versus malignant melanocytic ocular tumors were investigated and compared.

# 4 Materials and Methods

## 4.1 Sample selection

### 4.1.1 Defining old dogs

In dogs, a negative correlation can be observed between weight and life expectancy. The Goldston method (Table 1) was used to determine whether a dog could be considered old. Goldston correlated weight with the age at which dogs are considered geriatric.[35,43]

| Goldston Table | | | |
|---|---|---|---|
| **Dog size** | **Weight [kg]** | **Age [years]** | **SD** |
| small | 0 - 9 | 11.5 | ± 1.9 |
| medium | 10 - 23 | 10.9 | ± 1.6 |
| large | 24 – 40.5 | 8.9 | ± 1.4 |
| giant | > 40.5 | 7.5 | ± 1.9 |

**Table 1. Goldston Table**

Goldston table correlating weight with age at which dogs are considered geriatric.

Abbreviations: SD, standard deviation.

### 4.1.2 Testis samples

Clinically and pathologically healthy testes of 15 young dogs (age range: 9-18 months; mean: 12.1 months; 9 pure-breeds, 6 mixed-breeds) and of 15 old dogs (age range: 7-15 years; mean: 11.5 years; 12 pure-breeds, 3 mixed-breeds) were collected. Young dogs had been neutered to prevent uncontrolled reproduction or testes were collected during routine necropsy of animals with no testicular pathology. Old dogs had been therapeutically neutered due to perineal herniation, benign prostatic hyperplasia or testicular tumor or epididymitis of the contralateral testis. Only healthy testes were included in this study. Additionally, cryptorchid testes from 3 dogs (≤ 2 years of age; 2 pure-breeds, 1 mixed-breed) were included. Reasons for neutering were the cryptorchidism for two dogs and one cryptorchid testis was collected from a dog during routine necropsy.

Also, routine diagnostic tissue samples (whole testes) from 48 canine testicular tumors were included with 15 Leydig cell tumors (age range: 8-13 years; mean: 10.5 years; 10 pure-breeds, 5 mixed-breeds), 15 seminomas (age range: 7-15 years; mean: 10.1 years; 13 pure breeds, 2 mixed-breeds) and 15 Sertoli cell tumors in descended testes (age range: 6-16 years; mean: 10.3 years; 12 pure-breeds, 3 mixed-breeds), and 3 Sertoli cell tumors of cryptorchid testes (age range: 8-11 years; mean: 10 years; 1 pure-breed, 2 mixed-breeds).

Tissue samples were longitudinally cut, formalin-fixed and paraffin-embedded (FFPE) and 4 µm thick sections were stained with hematoxylin and eosin (HE) for independent blinded evaluation. Size and symmetry of testes were measured but data not included in this study due to breed-differences and effect of formalin fixation on testicular size. Consistency was not evaluated due to formalin fixation.

For background information on collected testes samples see Table 2 and 3.

| **Young canine testes** | | | | | | |
|---|---|---|---|---|---|---|
| **No** | **Breed** | **Age [months]** | **Indication** | **Contralateral testes** | **Body weight [kg]** | **Goldston category** |
| 1 | German Shepherd | 14 | rc | nl | 35.0 | large |
| 2 | Pomeranian | 12 | rc | nl | 4.2 | small |
| 3 | Jack Russell Mixed-breed | 12 | rc | nl | 6.9 | small |
| 4 | Terrier | 12 | rc | nl | 7.2 | small |
| 5 | Rottweiler | 12 | rc | nl | 45.0 | giant |
| 6 | Labrador-Dalmatian Mixed-breed | 12 | rc | nl | 23.4 | medium |
| 7 | Shepherd Mixed-breed | 12 | rc | nl | 26.0 | large |
| 8 | Miniature Poodle | 12 | rc | nl | 5.8 | small |
| 9 | Chinese Crested Dog | 12 | rc | nl | 11.0 | medium |
| 10 | Irish Wolfhound | 16 | rc | nl | 27.0 | large |
| 11 | Mixed-breed | 18 | rc | nl | 17.1 | medium |
| 12 | Labrador | 11 | rc | nl | 23.7 | large |
| 13 | Mixed-breed | 9 | rc | nl | 10.7 | medium |
| 14 | Bichon Frise | 9 | rc | nl | 5.3 | small |
| 15 | Miniature Pinscher | 9 | rc | nl | 2.7 | small |
| | **mean value** | **12.1** | | | **16.7** | |
| | **standard deviation** | **2.4** | | | **12.3** | |
| | **minimum** | **9** | | | **2.7** | |
| | **maximum** | **18** | | | **45** | |
| | | | | | | |
| **Old canine testes** | | | | | | |
| **No** | **Breed** | **Age [years]** | **Indication** | **Contralateral testes** | **Body weight [kg]** | **Goldston category** |
| 1 | Poodle | 13 | BPH | nl | 8.5 | small |
| 2 | Dachshound Mixed-breed | 11 | tumor | tumor | 7.0 | small |
| 3 | Griffon | 12 | epididymitis | epididymitis | 4.8 | small |
| 4 | Perro de Agua Espanol | 10 | BPH; PH | fibrosis | 22.2 | medium |
| 5 | Rhodesian Ridgeback | 7 | unknown | nl | 37.0 | large |
| 6 | Pekingese | 12 | BPH; PH | nl | 6.0 | small |
| 7 | Labrador Retriever | 10 | tumor | LCT | 41.0 | large |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | Terrier Mixed-breed | 11 | PH | nl | 10.3 | medium |
| 9 | Wire-haried dachshound | 11 | suspicion of tumor | SERT | 5.2 | small |
| 10 | Beagle | 13 | tumor | LCT | 18.5 | medium |
| 11 | Pinscher Mixed-breed | 14 | tumor | LCT | 7.5 | small |
| 12 | Wire-haried dachshound | 15 | tumor | SEM | 6.1 | small |
| 13 | Beagle | 12 | tumor | SERT | 14.0 | medium |
| 14 | Rhodesian Ridgeback | 10 | tumor | LCT | 46.8 | large |
| 15 | Hovawart | 11 | tumor | SERT | 37.0 | large |
| | **mean value** | **11.5** | | | **18.7** | |
| | **standard deviation** | **1.9** | | | **14.8** | |
| | **minimum** | **7.0** | | | **4.8** | |
| | **maximum** | **15.0** | | | **46.8** | |
| | | | | | | |
| **Cryptorchid canine testes** | | | | | | |
| **No** | **Breed** | **Age [years]** | **Indication** | **Contralateral testes** | **Body weight [kg]** | **Goldston category** |
| 1 | Rhodesian Ridgeback | 2 | routine necropsy | nl | 48.6 | giant |
| 2 | Rhodesian Ridgeback | 1 | crypto | na | 34.9 | giant |
| 3 | Mixed-breed | 2 | crypto | na | 24.5 | large |
| | **mean value** | **1.7** | | | **2.1** | |
| | **standard deviation** | **0.5** | | | **0.5** | |
| | **minimum** | **1** | | | **1.5** | |
| | **maximum** | **2** | | | **2.8** | |

**Table 2. Sample collection of young, old and cryptorchid canine testes**

Sample collection including background information of young, old and cryptorchid canine testes.

Abbreviations: BPH, benign prostatic hyperplasia; crypto, cryptorchidism; na, not accessible; nl, no lesion; No, number; LCT, Leydig cell tumor; PH, perineal hernia; SEM, seminoma; SERT, Sertoli cell tumor; rc, reproduction control.

| **Leydig cell tumor** | | | | | |
|---|---|---|---|---|---|
| **No** | **Contralateral testes** | **Breed** | **Age [years]** | **Associated lesions** | **Crypto** |
| 1 | LCT | Mixed-breed | 11 | no | no |
| 2 | nl | Labrador Mixed-breed | 12 | BPH | no |
| 3 | SEM | Canadian Shepherd | 9 | no | no |
| 4 | na | Golden Retriever | 8 | no | no |
| 5 | LCT | West Highland White Terrier | 11 | alopecia | no |
| 6 | LCT | Australian Kelpie | 12 | BPH; PC | no |
| 7 | LCT | Golden Retriever | 10 | no | no |
| 8 | LCT | Mixed-breed | 12 | PC | no |
| 9 | LCT | Jack Russel Terrier | 11 | PC | no |
| 10 | LCT | Appenzell Mountain Dog | 10 | no | no |
| 11 | na | Foxterrier | 8 | no | no |

| | | | | | |
|---|---|---|---|---|---|
| 12 | na | Mixed-breed | 13 | BPH | no |
| 13 | na | German Shepherd Mixed-breed | 12 | no | no |
| 14 | LCT | White Shepherd | 9 | no | no |
| 15 | LCT | Wire-haired dachshound | 10 | dermatitis of the croup | no |
| | | | | | |
| | | **mean value** | **10.5** | | |
| | | **standard deviation** | **1.5** | | |
| | | **minimum** | **8** | | |
| | | **maximum** | **13** | | |
| | | | | | |
| **Sertoli cell tumor** | | | | | |
| **No** | **Contralateral testes** | **Breed** | **Age [years]** | **Associated lesions** | **Crypto** |
| 1 | na | Labrador | 9 | no | no |
| 2 | na | Collie | 9 | no | no |
| 3 | nl | Yorkshire Terrier | 12 | no | no |
| 4 | na | Boxer | 9 | no | no |
| 5 | na | Mixed-breed | 11 | no | no |
| 6 | na | Boxer | 11 | no | no |
| 7 | nl | Border Terrier | 11 | no | no |
| 8 | nl | Beagle | 12 | no | no |
| 9 | na | Airedale Terrier | 10 | no | no |
| 10 | na | Mixed-breed | 9 | no | no |
| 11 | nl | Collie | 6 | BPH | no |
| 12 | SERT | Engl. Setter | 9 | no | no |
| 13 | crypto | Cocker Spaniel | 11 | no | no |
| 14 | nl | Mixed-breed | 16 | no | no |
| 15 | nl | German shepherd | 9 | no | no |
| | | | | | |
| | | **mean value** | **10.3** | | |
| | | **standard deviation** | **2.1** | | |
| | | **minimum** | **6** | | |
| | | **maximum** | **16** | | |
| | | | | | |
| 16 | na | Mixed-breed | 8 | no | yes |
| 17 | na | Mixed-breed | 11 | no | yes |
| 18 | na | Samoyed | 11 | hyperestrogenism | yes |
| | | | | | |
| | | **mean value** | **10** | | |
| | | **standard deviation** | **1.4** | | |
| | | **minimum** | **8** | | |
| | | **maximum** | **11** | | |
| | | | | | |
| **Seminoma** | | | | | |

| No | Contralateral testes | Breed | Age [years] | Other Lesions | Crypto |
|---|---|---|---|---|---|
| 1 | na | Mixed-breed | 10 | no | no |
| 2 | LCT | Canadian Shepherd | 9 | no | no |
| 3 | SEM | German Shepherd | 7 | no | no |
| 4 | nl | Eurasier | 9 | BPH | no |
| 5 | nl | Labrador Retriever | 9 | no | no |
| 6 | nl | Yorkshire Terrier | 8 | no | no |
| 7 | nl | Wire-haired dachshound | 15 | ACA of PHG | no |
| 8 | LCT | Golden Retriever | 8 | no | no |
| 9 | LCT | Golden Retriever | 14 | Adenoma of PHG | no |
| 10 | SEM | Rhodesian Rigdeback | 9 | no | no |
| 11 | nl | Standard Poodle | 11 | no | no |
| 12 | nl | Terrier Mixed-Breed | 11 | no | no |
| 13 | nl | Jack Russell Terrier | 12 | no | no |
| 14 | nl | German Wirehaired Pointer | 10 | BPH | no |
| 15 | nl | Small Münsterländer | 9 | no | no |
| | | | | | |
| | | **mean value** | **10.1** | | |
| | | **standard deviation** | **2.1** | | |
| | | **minimum** | **7** | | |
| | | **maximum** | **15** | | |

**Table 3. Sample collection of canine testicular tumors**

Sample collection including background information of dogs affected by Leydig cell tumor, Sertoli cell tumor or seminoma.

Abbreviations: Adenoma of PHG, Adenoma of perianal hepatoid glands; ACA of PHG, Adenocarcinoma of perianal hepatoid glands; BPH, benign prostatic hyperplasia; crypto, cryptorchidism; LCT, Leydig cell tumor; na, not accessible; nl, no lesion; No, number; PC, prostatic cysts; SEM, seminoma; SERT, Sertoli cell tumor.

### 4.1.3 Eye samples

Clinically and pathologically healthy eyes of 10 young dogs (age range: 9-24 months; 6 pure-breeds and 4 mixed-breeds) and of 9 older dogs (age range: 9.5-12.4 years; 5 pure-breeds and 4 mixed-breeds) were collected from routine diagnostics. Dogs had died for reasons independent of the eyes and were verified free of ocular lesions by three board certified pathologists (OK, RK, AB).

In addition, 13 uveal melanocytomas were included (age range: 5-15 years; 10 pure-breeds, 3 mixed-breeds). Five of these eyes showed signs of cataract including fibre liquefaction with formation of Morgagni globules, epithelial hyperplasia and capsular thickening. Two of the melanocytomas were partially amelanotic and one of these also had a mitotic index of 7 / 10 HPF which qualifies as a malignancy criteria. Three melanocytomas were ≤ 0.1 cm in diameter.

Additionally included from routine diagnostic were 9 ciliary body adenomas (age range: 4-11 years; 6 pure-breeds and 3 mixed-breeds) and 8 conjunctival melanomas (age range: 6-13 years; 5 pure-breeds and 3 mixed-breeds), of which one was macroscopically and microscopically free of melanin pigmentation. This amelanotic conjunctival melanoma was, however, immunohistochemically positive for S100. Tumor diagnoses were confirmed by two board certified pathologists (OK, RK) on the basis of the WHO nomenclature [44] and current literature [39].

Tissue samples were either formaldehyde-fixed (4 %) or fixed with Davidson solution (330 ml ethanol 96 %, 330 ml Aqua dest., 220 ml formaldehyde 37 %, 110 ml ethanoic acid and one drop eosin) and paraffin-embedded. 4 µm thick sections were bleached (3 % $H_20_2$ and 2.5 % KOH) and stained with haematoxylin and eosin (HE). Size of eye bulbs were not measured due to differences in formalin fixation (duration, amount) with influence of shrinkage (retrospective study).

For background information on collected eye samples see Table 4 and 5.

| **Canine eyes** | | | | | |
|---|---|---|---|---|---|
| **Young canine eyes** | | | | | |
| **No.** | **Breed** | **Age [months]** | **Sex** | **Body weight [kg]** | **Goldston category** |
| 1 | Labrador Mixed-breed | 21 | m | 22.7 | medium |
| 2 | Labrador Mixed-breed | 18 | f neut | 10.0 | medium |
| 3 | Irish Wolfhound | 15 | m | 27.0 | large |
| 4 | Mixed-breed | 18 | m | 17.1 | medium |
| 5 | Schnoodle | 24 | f | 4.3 | small |
| 6 | Dalmatian | 10 | m neut | 20.5 | medium |
| 7 | Border Collie | 9 | female | 8.8 | small |
| 8 | Mixed-breed | 24 | m | 30 | large |
| 9 | German Shepherd | 9 | m | 30 | large |
| 10 | Yorkshire Terrier | 24 | f | 4.9 | small |
| | **mean value** | **17.2** | | **17.5** | |
| | **standard deviation** | **5.9** | | **9.5** | |
| | **minimum** | **9** | | **4.3** | |
| | **maximum** | **24** | | **30** | |
| | | | | | |
| **Old canine eyes** | | | | | |
| **No.** | **Breed** | **Age (months)** | **Sex** | **Body weight (kg)** | **Goldston category** |
| 1 | Mixed-breed | 12 | m | 7.2 | small |
| 2 | Mixed-breed | 12 | f neut | 7.5 | small |
| 3 | Boston Terrier | 12 | f neut | 8.2 | small |
| 4 | German Shepherd | 12 | female | 35.0 | large |
| 5 | Mixe-breed | 11 | m | 40.0 | large |

| | | | | | |
|---|---|---|---|---|---|
| 6 | Wire-haired dachshound | 12 | f | 8.6 | small |
| 7 | Husky | 10 | m | 35.0 | large |
| 8 | Labrador Mixed-breed | 12 | m neut | 57.0 | giant |
| 9 | Dobermann | 9 | f neut | 30.0 | large |
| | **mean value** | **11.3** | | **25.4** | |
| | **standard deviation** | **1.1** | | **17.1** | |
| | **minimum** | **9** | | **7.2** | |
| | **maximum** | **12** | | **5** | |

**Table 4. Sample collection of young and old eyes**

Sample collection including background information of eyes of young and old dogs.

Abbreviations: f, female; m, male; neut, neutered; No, number.

| **Canine ocular tumors** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Uveal melanocytoma** | | | | | | | |
| **No** | **Breed** | **Age [years]** | **Body weight [kg]** | **Sex** | **Cataract** | **Pigment** | **Mitoses/10 HPF (400x)** |
| **1** | Weimeraner | 5 | nk | f neut | no | yes | 0 |
| **2** | Beagle | 12 | nk | m neut | no | sparce | 7 |
| **3** | Jack Russell Terrier | 11 | nk | f neut | no | yes | 0 |
| **4** | Labrador | 11 | nk | f neut | no | yes | 0 |
| **5** | Dachshound Mixed-breed | 10 | nk | m neut | no | variable | 1 |
| **6** | German Shepherd | 8 | nk | m | yes | yes | 0 |
| **7** | Mixed-breed | 18 | nk | m neut | yes | yes | 0 |
| **8** | Bearded Collie | 8 | nk | f | yes | yes | 0 |
| **9** | Poodle | 13 | nk | f neut | yes | yes | 0 |
| **10** | Mixed-breed | 14 | nk | f neut | yes | yes | 2 |
| **11** | German Hound | 13 | 39.0 | m | no | yes | 0 |
| **12** | Shih Tzu | 15 | 7.5 | m neut | no | yes | 0 |
| **13** | Rottweiler | 13 | 25.3 | f | no | yes | 0 |
| | **mean value** | **11.6** | | | | | |
| | **standard deviation** | **3.2** | | | | | |
| | **minimum** | **5** | | | | | |
| | **maximum** | **18** | | | | | |
| | | | | | | | |
| **Ciliary body adenoma** | | | | | | | |
| **No** | **Breed** | **Age [years]** | **Body weight [kg]** | **Sex** | **Cataract** | | |
| **1** | Dachshound | 9 | nk | f | no | | |
| **2** | Rottweiler | 8 | nk | m | no | | |
| **3** | Mixed-breed | 11 | nk | m | no | | |
| **4** | Great Dane | 5 | nk | m neut | no | | |
| **5** | Gordon Setter | 6 | nk | m | no | | |
| **6** | German Shepherd | 11 | nk | f neut | no | | |
| **7** | Irish Wolfhound | 4 | nk | m neut | no | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **8** | Flat Coated Mixed-breed | 10 | nk | m | no | | |
| **9** | Mixed-breed | 11 | nk | m neut | yes | | |
| | **mean value** | **8.3** | | | | | |
| | **standard deviation** | **2.6** | | | | | |
| | **minimum** | **4** | | | | | |
| | **maximum** | **11** | | | | | |
| | | | | | | | |
| **Conjunctival melanoma** | | | | | | | |
| **No** | **Breed** | **Age [years]** | **Body weight [kg]** | **Sex** | | **Pigment** | **IHC** |
| 1 | unknown | 13 | nk | f neut | | yes | |
| 2 | Cocker Spaniel | 11 | nk | m | | yes | |
| 3 | Small Münsterländer | 11 | nk | f | | no | S 100 positive |
| 4 | Rhodesian Ridgeback | 9 | nk | f neut | | sparce | |
| 5 | Mixed-breed | 10 | nk | m neut | | sparce | |
| 6 | Mixed-breed | 6 | nk | m neut | | sparce | |
| 7 | Bernese Mountain Dog | 9 | nk | m | | sparce | |
| 8 | Shepherd-Husky Mixed-breed | 12 | nk | f | | sparce | |
| | **mean value** | **10.1** | | | | | |
| | **standard deviation** | **2** | | | | | |
| | **minimum** | **6** | | | | | |
| | **maximum** | **13** | | | | | |

**Table 5. Sample collection of ocular tumors**

Sample collection including background information of canine eyes affected by uveal melanocytoma, ciliary body adenoma and conjunctival melanoma.

Abbreviations: f, female; HPF, high power field; IHC, immunohistochemistry; m, male; neut, neutered; nk, not known; No, number; pigment, melanin pigmentation.

## 4.2 Morphological quantification of the canine testes

Parameters that had been reported to show species- and study-dependent age-associated changes were identified.[35,45] These included the interstitial collagen content, tubular diameters, epithelial areas and number of Leydig cells per intertubular triangle (ITT) (Fig 1). An intertubular triangle is the area within a triangle formed by three touching tubuli. These four parameters were evaluated in all healthy testes of young and old dogs as well as in the 3 cryptorchid testes. In addition, the spermatogenetic activity of 10 young and 10 old testes as well as the 3 young cryptorchid testes was evaluated using the modified Johnsen score.[35,46]

For assessment of the interstitial collagen content slides were stained with picrosirius red (Morphisto staining kit according manufacturer's instructions), scanned (200 x magnification

with ScanScope CS2, Leica) and analyzed using image analysis software (eSlide Manager / Genie, Leica Biosystems Imaging Inc, DB Maarn, The Netherlands). Using this software a customized algorithm was created using stained sections and annotation of positive and negative areas in the whole slide image. The percentage of collagen tissue (stained red) was measured in relation to the total area including tubular lumina as well as in relation to the tissue area excluding tubular lumina. All artefacts were excluded prior to evaluation.

Tubular diameters as well as epithelial areas of 100 round tubular cross-sections of each testis were examined at 100 x total magnification with an Olympus BX41 microscope equipped with a Color View II Camera (Olympus Soft Imaging Solutions GmbH). Total areas of entire tubuli were determined and put in percentual relation to areas of tubular lumina (epithelial area / tubular area) and tubular diameters were measured using the CellSens software (Olympus Soft Imaging Solutions GmbH). 10 round cross-sectioned tubuli were evaluated in 10 randomly chosen representative fields.

Leydig cells were assessed in each testis in 10 random intertubular triangels (ITT). An ITT is the area within a triangle formed by three touching tubuli (Fig 1).


**Figure 1. Leydig cells per intertubular triangle (ITT)**

Leydig cells per intertubular triangle (ITT) in the canine testis. An ITT is the area within a triangle formed by three touching tubuli. Left picture: Young dog (1 year) with few Leydig cells / ITT. Middle picture: Old dog (11 years) with increased number of Leydig cells / ITT. Right picture: Young, cryptorchid dog (2 years) with high number of Leydig cells / ITT. HE stain.

Spermatogenetic activity was examined using the modified Johnsen score.[35] Johnsen (1970) scored each of overall 100 tubular sections from 1-10 (10 being the highest score) depending on the presence of the main cell types arranged in the order of maturity (Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa). The score was modified by Peters (2000) for transferability to canine testes (Fig 2). The score was applied on HE-stains of 10 young and 10 old canine testes as well as 3 young cryptorchid testes. For each testis 100 round tubular cross-sections were evaluated in 10 randomly chosen representative fields at 400 x total magnification.

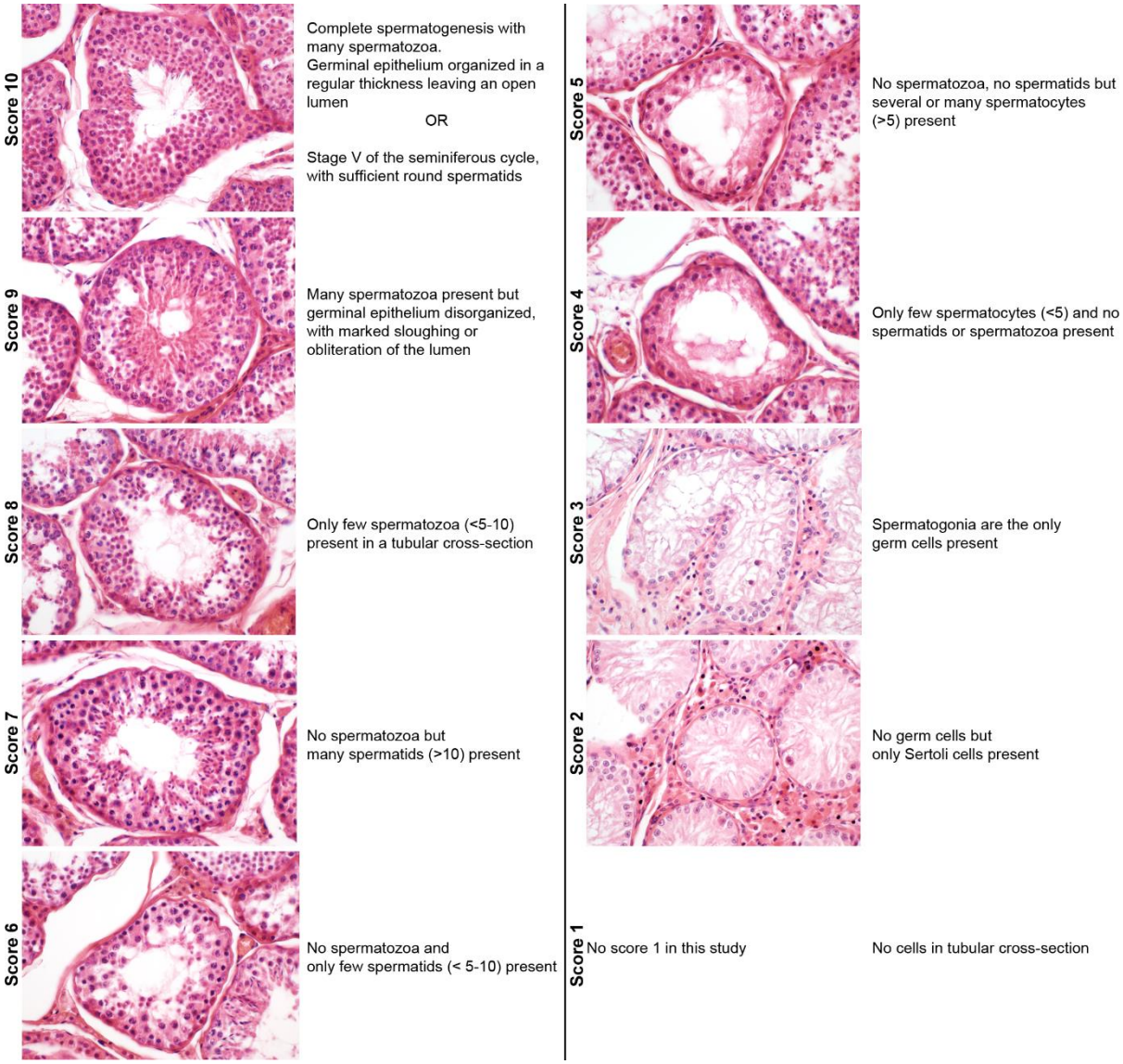


**Figure 2. Modified Johnsen scoring system for evaluation of spermatogenesis**

The modified Johnsen score is a scoring system ranging from 1 to 10 and is used for the evaluation of canine spermatogenesis. Presented are images of testicular tubular cross-sections and descriptions of each score except score 1 which was not detected in any testis of this study. The original Johnsen score (1970) was developed for human testis and gives each of overall 100 tubular sections a score from 1-10 depending on the presence of the main cell types arranged in the order of maturity (Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa). The Johnsen scoring system was modified by Peters (2000) for application to canine testes. HE stain.

## 4.3 Immunohistochemistry for testes and eyes

### 4.3.1 Protocol for immunohistochemistry

Paraffin-embedded tissues were cut at 4 µm thickness, mounted on adhesive glass slides, dewaxed in xylene, followed by rehydration in descending graded alcohols. Endogenous peroxidase was blocked with 3 % $H_2O_2$ in PBS for 30 minutes at room temperature. Antigen heat retrieval was achieved in citrate buffer (pH 6) for 12 minutes (600 Watt) in a microwave oven followed by a cooling period of 15 minutes. For prevention of non-specific antibody binding slides were blocked with 8 % Roti-Immunoblock (Roth, Karlsruhe, Germany) and 20 % goat serum for 30 minutes at room temperature. Monoclonal mouse anti-human γH2AX (phospho S139) antibody (dilution 1:1000; Abcam, England), monoclonal mouse anti-human p21 (WAF1/Cip1; dilution 1:50; DAKO, Denmark) and monoclonal rat-anti-mouse ki67 (Purified (MIB-1) dilution 1:75; DAKO, Denmark) were incubated for 1 hour at room temperature. For slides stained for p21, cultured primary canine fibroblasts from passage 11 were used as positive controls. Slides were incubated with a secondary biotinylated goat anti-mouse antibody (dilution 1:200; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Color was developed by incubating the slides with freshly prepared avidin-biotin-peroxidase complex (ABC) solution (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA), followed by repeated washes and exposure to diaminobenzidine tetrachloride (Merck; Darmstadt, Germany). The sections were counterstained with hematoxylin, dehydrated in ascending graded ethanols, cleared in xylene, and coverslipped. All three antibodies showed a nuclear staining. Negative controls were included in each run with commercial mouse immunoglobulins (Bio-Genex, Fremont, California, USA) instead of primary antibodies. Germ cells during chromatin remodeling, associated with double-strand breaks, served as internal positive control for γH2AX.[47,48]

### 4.3.2 Quantification in healthy testes and eyes

In healthy canine testes, positive γH2AX or p21 fibroblasts and Leydig cells were counted in 200 cells of that particular cell type from random high power fields (HPF, 400x).

In healthy canine eyes, the following compartments were evaluated separately: epithelium and stroma of the lens and cornea, ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL) and retinal pigment epithelium (RPE) of the retina, as well as iris and ciliary body, and, if available the optic nerve (young: 8/10; old: 5/9). For each compartment, positive γH2AX or p21 cells were counted in 200 cells from random high power fields (HPF, 400x).

### 4.3.3 Quantification in the microenvironment of testicular and intraocular tumors

The tumor microenvironment consists of all non-cancerous cells present in and adjacent to the tumor including fibroblasts, which are said to possibly promote tumor growth.[49] Cell culture

studies have shown that a maximum range of approximately 400 µm is needed for effective cell-cell-interactions.[50]

In testicular tumors, intratumoral stroma was examined as microenvironment. If the tumor contained only scarce amounts of stroma, fibroblasts directly surrounding the tumor at a maximum distance of 200 µm were considered microenvironment. In this microenvironment, positive γH2AX or p21 fibroblasts were counted in 200 cells from random high power fields (HPF, 400x).

In intraocular tumors, all ocular structures in contact with or supported by the aqueous humor presumably influence intraocular tumors and vice versa and can therefore be considered microenvironment. The aqueous humor is in direct contact with many ocular structures and even provides nutrition to avascular structures like the lens and cornea.[51] It allows inflammatory cells and mediators to circulate the eye as well as drug distribution, including to and through the retina.[52] That intraocular tumors and ocular structures communicate via the aqueous humor is supported by studies in humans and dogs which have shown that ocular tumors modify the cytokine expression including growth factors in the aqueous humor.[53-56]

The following compartments were assessed as microenvironment in intraocular tumors (uveal melanocytoma, ciliary body adenoma): epithelium and stroma of the lens and cornea, ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL) and retinal pigment epithelium (RPE) of the retina, as well as iris and ciliary body. The ciliary body was excluded in eyes with ciliary body adenomas as it was always affected by infiltration of tumors cells. In 5/13 eyes with uveal melanocytoma the iris was excluded for the same reason. For each compartment (here: microenvironment), positive γH2AX or p21 cells were counted in 200 cells (lens: 100 cells) from random high power fields (HPF, 400x). In some eyes not all compartments could be evaluated due to technical reasons. The RPE and the cornea were especially susceptible to technical destruction, which after multiple repeats was accepted as unavoidable for certain samples of eyes with ocular tumors.

#### 4.3.4 Quantification in tumors cells of testicular and ocular tumors

For testicular tumors and ocular melanocytic tumors (uveal melanocytoma, conjunctival melanoma), positive γH2AX or p21 positive tumor cells were counted in 200 cells from random high power fields (HPF, 400x).

A ki67 index for estimation of cell proliferation activity was determined additionally for testicular tumors. For the ki67 index, fields with highest positivity were selected and 5 random HPF analyzed per tissue. In each HPF, 200 tumor cells were examined and the percentage of positive cells was calculated. In accordance with current literature, the following scoring system

was applied: score 0 (0-10 % positive cells); score 1 (11-30 % positive cells); score 2 (31-50 % positive cells); score 3 (> 50 % positive cells).[57]

## 4.4 Statistical Analysis

Statistical significance analyses and graphical illustrations were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, USA, www.graphpad.com. Data were tested for Gaussian distribution using the Shapiro-Wilk test. Gaussian distributed data (ki67-index and morphological parameters of the testes except Johnsen score) were tested with unpaired t-test with Welch's correction controlling for unequal variances. Non-parametric data (Johnsen score, immunohistochemical data except ki67, all data of eyes) were tested using the Mann-Whitney U Test. Gaussian distributed data are expressed as mean ± standard deviation (SD) and non-parametric data are expressed as median with interquartile range (IQR; 75 % quartile – 25 % quartile). Due to low case numbers (n=3) data from cryptorchid testes were analyzed only descriptively.

The minimum amount of cells to examine for γH2AX or p21 positivity as well as the number of intertubular triangles and tubuli was calculated based on preliminary evaluations of selected samples.

# 5 Results

## 5.1 Testis

### 5.1.1 Morphological parameters in young, old and cryptorchid canine testis

The mean number of Leydig cells / ITT showed a significant, age-dependent increase from 4.2 ± 0.5 cells in young to 12.4 ± 1.8 cells ($p \leq 0.0001$) (Fig 3, Table 6). The median modified Johnsen score for spermatogenesis was significantly decreased with age from a median score of 9.76 to a median score of 9.17 ($p \leq 0.0001$, Fig 3, Table 6). Additionally, tubular irregularities (scores ≤ 8) were more commonly observed in old testes (Fig 4). Testis from young dogs had an average of 6 / 100 tubuli and testis from old dogs had an average of 16 / 100 tubuli with a score below 9. However, every accessible epididymis (young: 7/10; old: 8/10) contained spermatozoa. Mean tubular diameters showed a high variability (range 170.7 – 277.2 µm) between individuals. However, no significant age-dependent differences (young: mean of 228.7 ± 25.0 µm; old: 240.1 ± 20.1 µm) could be noted. No correlation could be identified between body weight and tubular diameter (Table 7). The epithelial area / tubular area showed no significant age-dependent changes (young: 89.9 ± 4.0 % of the tubular area; old: mean: 88.7 ± 4.3 % of the tubular area, Fig 3, Table 6). The mean interstitial collagen contents also showed no significant age-dependent changes in relation to the total area including tubular lumina (young: 20.1 ± 5.6 %; old: 22.9 ± 4.7 %) as well as in relation to epithelial tissue excluding tubular lumina (mean young: 27.5 ± 7.3 %; old: 28.9 ± 6.1 %, Fig 3, Table 6).

Young cryptorchid testis were compared to young healthy testis (Fig 3, Table 6). The mean number of Leydig cells / ITT was significantly increased (young: 4.2 ± 0.5 Leydig cells / ITT; cryptorchid: 26.7 ± 0.8 Leydig cells / ITT). The mean interstitial collagen content was increased (related to total area: young: 20.1 ± 5.6 %; cryptorchid: 29.1 ± 10.7 %; related to epithelial tissue: young: 28.9 ± 6.1 %; cryptorchid: 33.3 ± 8.1 %). The mean tubular diameter was decreased (mean young: 228.7 ± 25.0 µm; cryptorchid: 123.6 ± 25.0 µm). The mean epithelial area / tubular area was also decreased (mean young: 89.9 ± 4.0 %; cryptorchid: 43.0 ± 4.1 %; Fig 4). The low median modified Johnsen score resembled spermatogenetic inactive testis (median young: 9.76; cryptorchid: 2; Fig 4). None of the three cryptorchid testis contained spermatozoa in the epididymis.

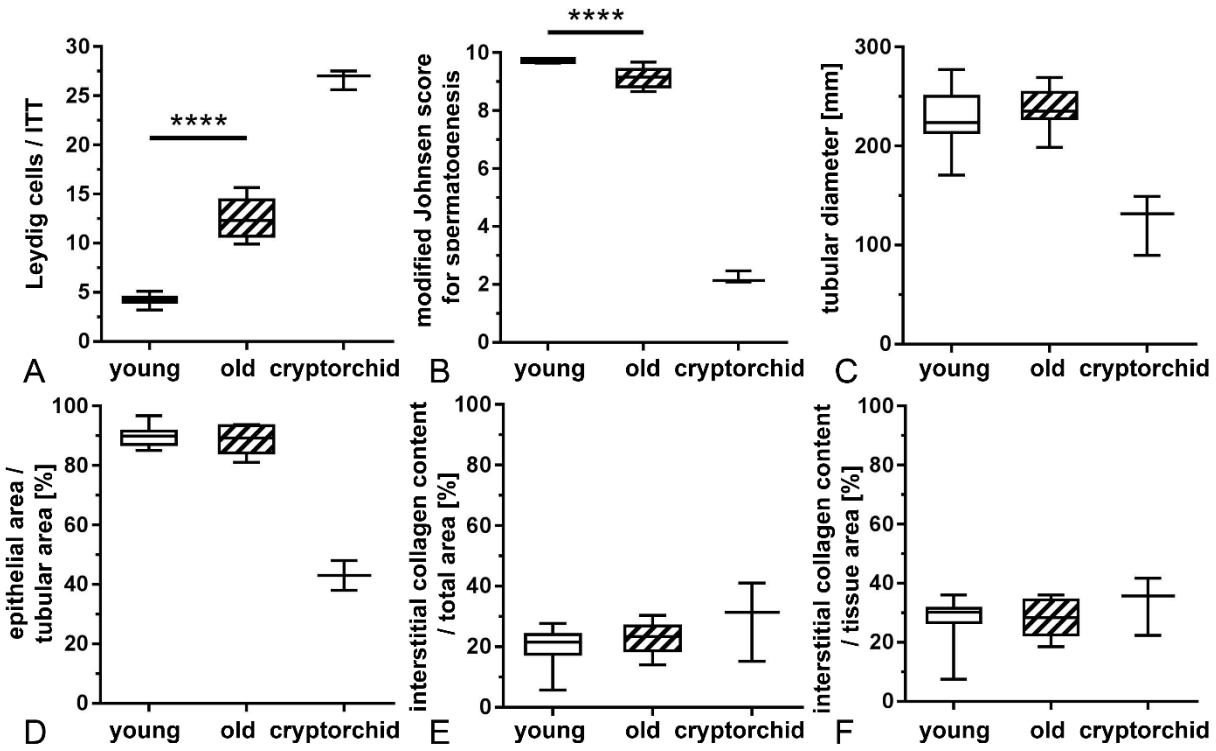


**Figure 3. Results of morphological parameters for young, old and cryptorchid canine testes.**

**A:** Leydig cells / intertubular triangle (ITT) increased with age. Cryptorchid testes showed even higher number of Leydig cells / intertubular triangle than old dogs. **B:** The modified Johnsen score (spermatogenesis score) decreased significantly with age. Cryptorchid testes showed very low modified Johnsen score in accordance with the known sterility for this condition. **C-F:** Tubular diameter, epithelial area / tubular area and interstitial collagen content showed no age-dependent significant changes. For cryptorchid testes, tubular diameters were smaller, epithelial area / tubular area lower and interstitial collagen content more in comparison to young and old healthy testes. Box and whisker plots, boxes are outlined by 25$^{th}$ to 75$^{th}$ percentiles, median as horizontal line, and whiskers draw minimum to maximum. Significance indicated by stars: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Abbreviations: ITT, intertubular triangle.

| | Young testes | | Old testes | | Cryptorchid testes | |
|---|---|---|---|---|---|---|
| | **MV** | **SD** | **MV** | **SD** | **MV** | **SD** |
| **Age [years]** | 1.0 | 0.2 | 11.5 | 1.9 | 2.1 | 0.6 |
| **Weight [kg]** | 16.7 | 12.3 | 18.7 | 14.8 | 36.0 | 9.9 |
| **Leydig cells / ITT** | 4.2 | 0.5 | 12.4[a] | 1.8 | 27.0 | 0.8 |
| **Johnsen score** | 9.8 (M) | 0.1 (IQR) | 9.2[a] (M) | 0.5 (IQR) | 2.1 (M) | 0.2 (IQR) |
| **Tubular diameter [µm]** | 228.7 | 25.0 | 240.1 | 20.1 | 123.6 | 25.0 |
| **Epithelial area / tubular area [%]** | 89.9 | 4.0 | 88.7 | 4.3 | 43.0 | 4.1 |
| **Interstitial collagen content / total area [%]** | 20.2 | 5.6 | 22.9 | 4.7 | 29.2 | 10.7 |
| **Interstitial collagen content / tissue area [%]** | 27.5 | 7.3 | 28.9 | 6.1 | 33.3 | 8.1 |

**Table 6. Results of morphological parameters in canine testes**

Overview of results of morphological parameters for young, old and cryptorchid canine testes.

Abbreviations: ITT, intertubular triangle; MV, mean value; SD, standard deviation; M, median; IQR, interquartile range.

[a] significantly different from young testes ($p \leq 0.0001$)

| Testes of young dogs | | | | |
|---|---|---|---|---|
| **No** | **Breed** | **Weight [kg]** | **Category** | **d Tubulus [µm]** |
| 1 | Miniature pinscher | 2.7 | small | 222 |
| 2 | Pomeranian | 4.2 | small | 224 |
| 3 | Bichon Frise | 5.3 | small | 278 |
| 4 | Miniature Poodle | 5.8 | small | 238 |
| 5 | Jack Russell Mixed-breed | 6.9 | small | 222 |
| 6 | Terrier | 7.2 | small | 252 |
| 7 | Mixed-breed | 10.7 | medium | 170 |
| 8 | Chinese Crested Dog | 11.0 | medium | 256 |
| 9 | Mixed-breed | 17.1 | medium | 218 |
| 10 | Labrador-Dalmatian Mixed-breed | 23.4 | medium | 214 |
| 11 | Labrador | 23.7 | large | 250 |
| 12 | Shepherd Mixed-breed | 26.0 | large | 230 |
| 13 | Irish Wolfhound | 27.0 | large | 212 |
| 14 | German Shepherd | 35.0 | large | 230 |
| 15 | Rottweiler | 45.0 | giant | 246 |
| | | | | |
| **Testes of old dogs** | | | | |
| **No** | **Breed** | **Weight [kg]** | **Category** | **d Tubulus [µm]** |
| 1 | Griffon | 4.8 | small | 209 |
| 2 | Wire-haried dachshound | 5.2 | small | 235 |
| 3 | Pekingese | 6.0 | small | 228 |
| 4 | Wire-haired dachshound | 6.1 | small | 233 |
| 5 | Dachshound Mixed-breed | 7.0 | small | 267 |
| 6 | Pinscher Mixed-breed | 7.5 | small | 269 |
| 7 | Poodle | 8.5 | small | 235 |
| 8 | Terrier Mixed-breed | 10.3 | medium | 268 |
| 9 | Beagle | 14.0 | medium | 254 |
| 10 | Beagle | 18.5 | medium | 246 |
| 11 | Perro de Agua Espanol | 22.2 | medium | 250 |
| 12 | Rhodesian Ridgeback | 37.0 | large | 199 |
| 13 | Hovawart | 37.0 | large | 224 |
| 14 | Labrador Retriever | 41.0 | large | 233 |
| 15 | Rhodesian Ridgeback | 46.8 | large | 252 |

**Table 7. Tubular diameters and body weights of young and old dogs**

Dogs sorted by body weight in ascending order. Tubular diameters were not correlated with body weight of dogs in this study. For definition of categories see Goldston Table in Figure 1.

Abbreviations: d, diameter.

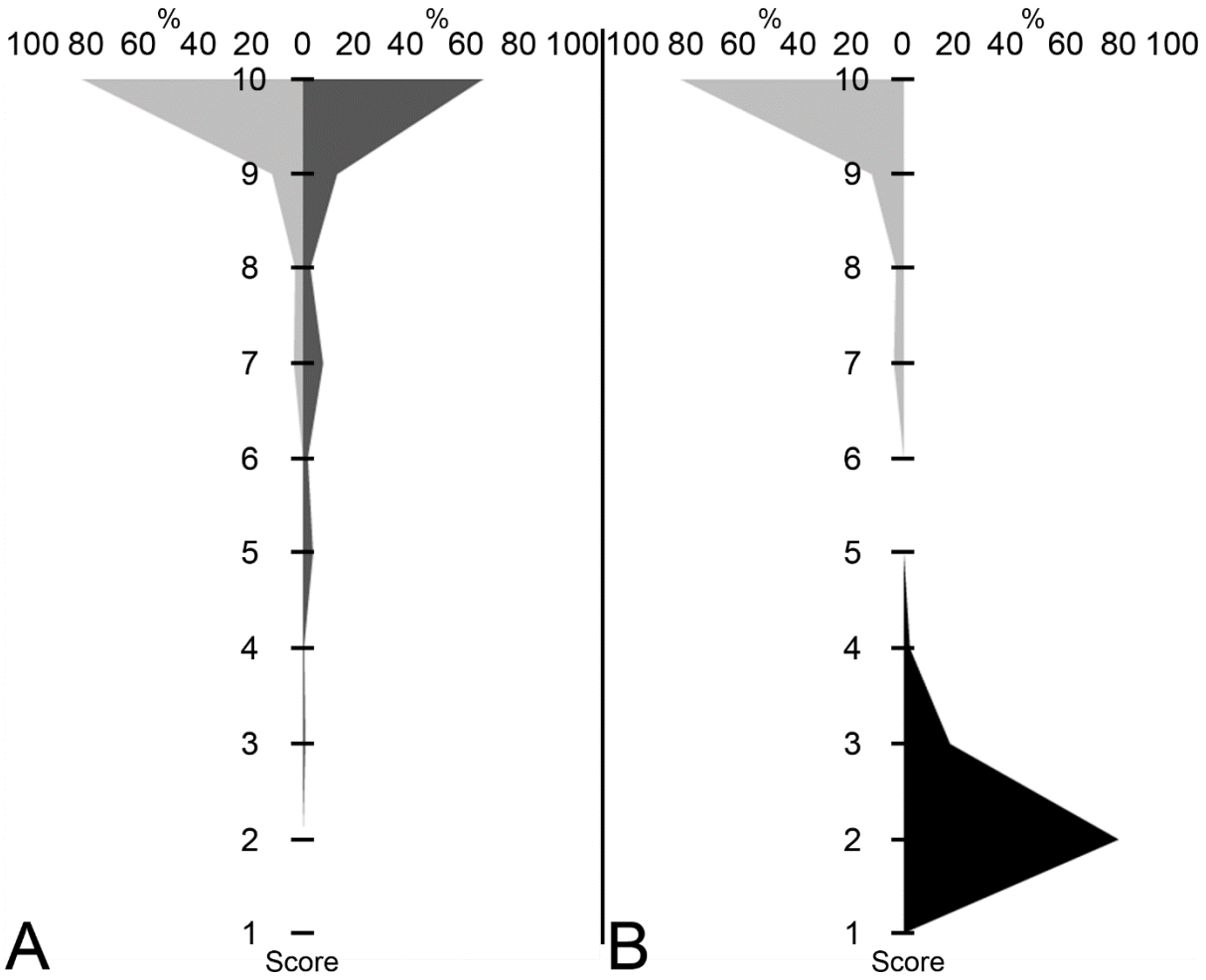


**Figure 4. Comparison of Modified Johnsen score in young, old and cryptorchid canine testes**

Comparison of mean percentages for each score (Modified Johnsen score for evaluation of canine spermatogenesis) for young (light grey), old (dark grey) and cryptorchid (black) testes. **A**: Comparison of young (light grey) versus old (dark grey) testes with a few more irregularities (scores ≤ 8) in old testes. **B**: Comparison of young (light grey) versus cryptorchid (black) testes with inverse low scores for cryptorchid testes.

### 5.1.2 Senescence marker expression in healthy young, old and cryptorchid canine testis

Both markers (γH2AX, p21) showed a nuclear signal (Fig 5). The expression of p21 showed an age-dependent increase in fibroblasts (median: young: 0.5 %; old: 2.0 % ($p \leq 0.0001$) and Leydig cells (median: young: 0.5 %; old: 4.0 % ($p \leq 0.001$) (Fig 6). Nonetheless, p21 expression was a rare event with only few positive cells. The expression of γH2AX was less than 1 % in fibroblasts and Leydig cells and no age-dependent changes could be noted (Fig 6). No expression of γH2AX and p21 could be detected in fibroblasts and Leydig cells of cryptorchid testes (Fig 6).

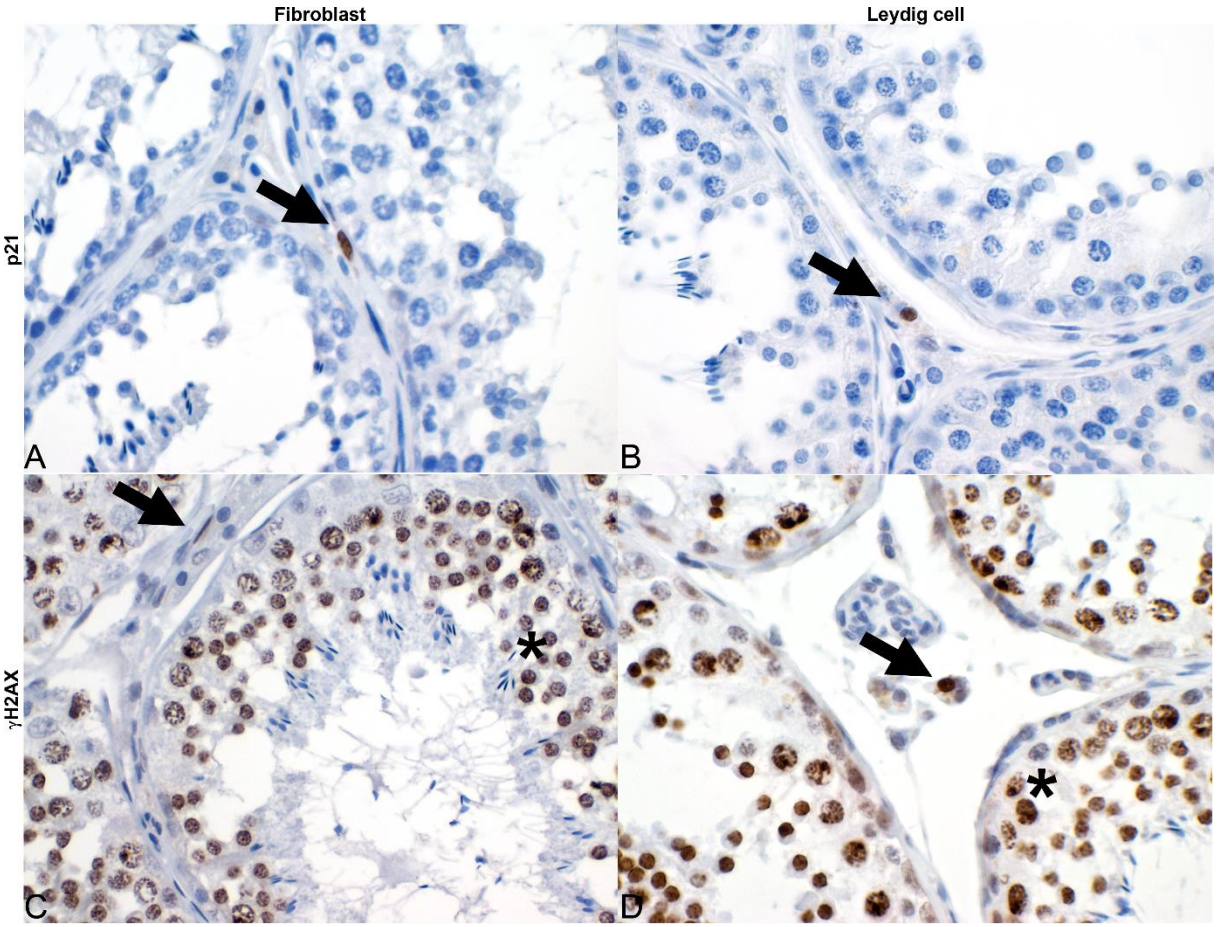


**Figure 5. Immunohistochemistry of p21 and γH2AX in canine testes**

Fibroblasts and Leydig cells positive for p21 (**A-B**) and γH2AX (**C-D**) (marked by arrows) in canine testicular interstitium. Germ cells (asterisk) functioned as an internal control for γH2AX as chromatin remodeling is associated with double strand breaks. Immunohistochemistry, ABC-method, hematoxylin counterstain.

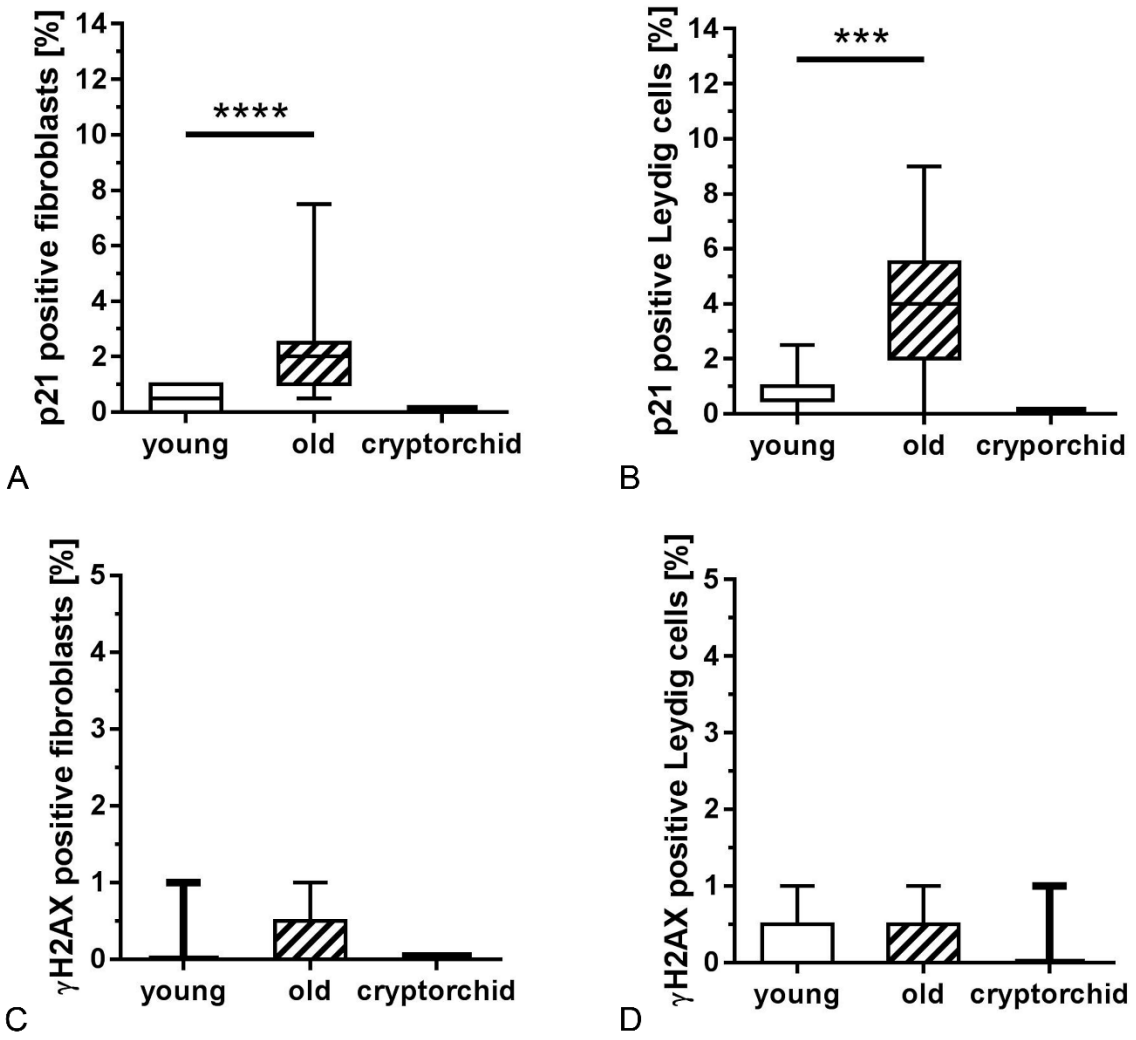


**Figure 6. Age-dependent expression of p21 and γH2AX in canine testes**

Age-dependent expression of p21 or γH2AX positive fibroblasts and Leydig cells, as well as in cryptorchid testes. **A, B:** P21 positive fibroblasts and Leydig cells showed an age-dependent increase. Cryptorchid testes showed no p21 positive fibroblasts or Leydig cells. **C, D:** γH2AX positive fibroblasts and Leydig cells showed no significant age-dependent increase. Cryptorchid testes showed no γH2AX positive fibroblasts and Leydig cells.

Box and whisker plots, boxes are outlined by 25$^{th}$ to 75$^{th}$ percentiles, median as horizontal line, and whiskers draw minimum to maximum. Significance indicated by stars: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

### 5.1.3 Senescence marker expression in the microenvironment of testicular tumors

In the microenvironments of all examined testicular tumors (Sertoli cell tumor, seminoma, Leydig cell tumor) expression of γH2AX and p21 in fibroblasts was ≤ 1 % (Fig 7). P21 expression in the microenvironments was never increased in comparison to fibroblasts from young or old canine testis. Even contrary, there was a significantly less expression of p21 in

the microenvironment of Leydig cell tumors and seminomas in comparison to fibroblasts from old dogs (old: median of 2.0 %; microenvironment: median of 0.5 %; p ≤ 0.05, Fig 7).

The only increases of γH2AX expression in microenvironments were seen for Sertoli cell tumors and seminomas (Fig 7). γH2AX expression of fibroblasts in microenvironment of Sertoli cell tumors was significantly increased in comparison to fibroblasts from young dogs (young: median: 0 %; microenvironment: median: 0.5 %, p ≤ 0.0001). This was even seen in comparison to fibroblasts from old testes (p ≤ 0.05). γH2AX expression of fibroblasts in microenvironment of seminomas was significantly increased in comparison to fibroblasts from young dogs (young: median: 0 %; microenvironment: median: 0.5 %, p ≤ 0.01). The microenvironments of cryptorchid Sertoli cell tumors showed a similarly low marker expression as the microenvironments of non-cryptorchid Sertoli cell tumors.

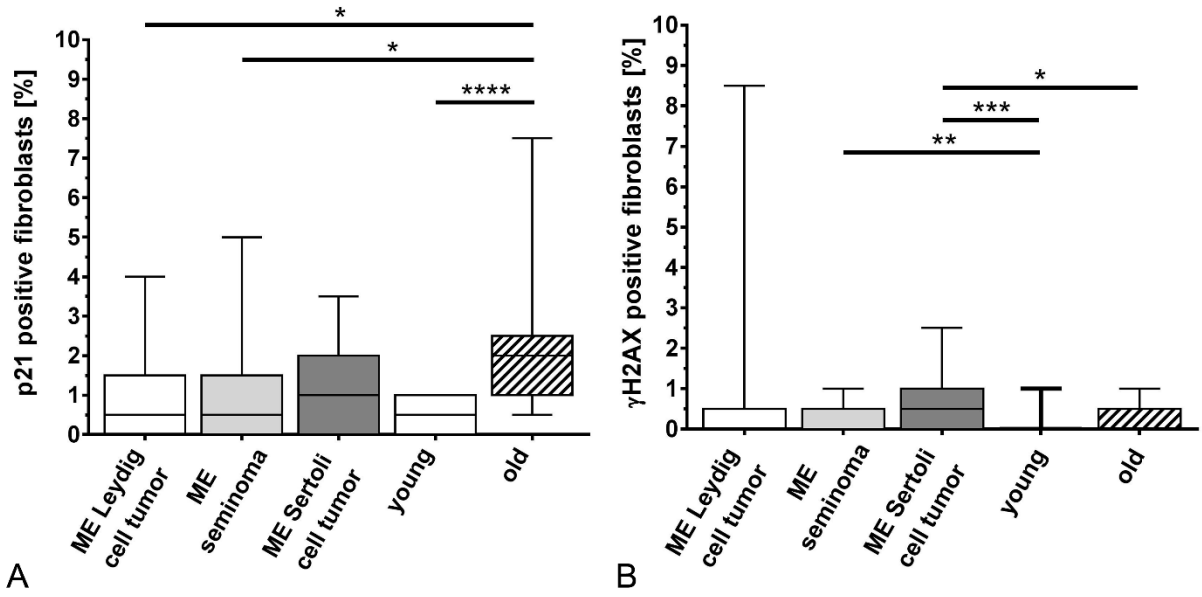


**Figure 7. Expression of p21 and γH2AX in microenvironment of canine testicular tumors**

Percentages of p21 and γH2AX positive fibroblasts in the microenvironment of canine testicular tumors in comparison to fibroblasts from testes of young and old dogs. **A:** A small yet significant increase of 21 positive fibroblasts could be noted with age. However, no increase could be noted in the microenvironment of testicular tumors. In contrast, p21 positive fibroblasts are even significantly lower in relation to fibroblasts from old testes for Leydig cell tumors (p ≤ 0.05) and seminomas (p ≤ 0.05). **B:** No increase of γH2AX positive fibroblasts could be noted with age. However, γH2AX positive fibroblasts increased in the microenvironment of seminomas (p ≤ 0.01) and Sertoli cell tumors (p ≤ 0.001) in relation to fibroblasts from young testes. Box and whisker plots, boxes are outlined by 25$^{th}$ to 75$^{th}$ percentiles, median as horizontal line, and whiskers draw minimum to maximum. Significance indicated by stars: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001.

Abbreviations: ME, microenvironment.

### 5.1.4 Marker expression in tumor cells of canine testicular tumors

P21 expression in tumor cells was low in all examined testicular tumors (Table 8, Fig 8). Leydig cell tumors showed the highest expression of p21 with a median of 1.5 %, followed by seminomas median of 0.5 % and Sertoli cell tumors with a median of 0.5 %.

γH2AX expression in tumor cells was inverse to p21 expression in tumor cells (Table 8, Fig 8). Leydig cell tumors showed the lowest expression of γH2AX with a median of 0.5 %, followed by seminomas with a median of 5.5 % and Sertoli cell tumors with a median of 30.0 %.

Intertumor variance of marker expression was low with two exceptions (Fig 8, Fig 9). Sertoli cell tumors showed two expression patterns of γH2AX. 10 Sertoli cell tumors showed a high expression of γH2AX positive tumor cells with a median of 32.5 % whereas five Sertoli cell tumors showed a low expression of γH2AX with a median of 1 %. Leydig cell tumors showed two expression patterns for p21. 12 Leydig cell tumors showed a low expression with a median of 1.5 % p21 positive tumor cells. Three Leydig cell tumors contained foci of p21 positive tumor cells with a median of 47.5 % positive tumor cells. These p21 rich foci had showed no γH2AX or ki67 staining.

None of the above-mentioned staining patterns could be correlated with body weight, breed, age of dog. In addition all Leydig cell tumors showed a solid growth pattern, all Sertoli cell tumors showed a intratubular growth pattern and none showed metastasis or invasive growth.

Ki-67 indices showed a great intertumor variability (Table 8, Fig 8) Leydig cell tumors showed the lowest staining with a mean of 5.3 ± 3.7 % (score 0), followed by Sertoli cell tumor with a mean of 15.1 ± 7.8 % (score 1) and seminomas with a mean of 49.7 ± 5.0 %.

Cryptorchid Sertoli cell tumors showed similar marker expression to non-cryptorchid Sertoli cell tumors (Table 8, Fig 8).

| | **p21** | | **γH2AX** | | **ki67** | |
|---|---|---|---|---|---|---|
| | **median [%]** | **IQR [%]** | **median [%]** | **IQR [%]** | **median [%]** | **IQR [%]** |
| **Leydig cell tumor** | 1.5 | 17.8 | 0.5 | 0.3 | 5.3 | 3.7 |
| **Seminoma** | 0.5 | 1.5 | 5.5 | 1.5 | 49.7 | 5.0 |
| **Sertoli cell tumor** | 0.5 | 0.8 | 30.0 | 32.0 | 15.1 | 7.8 |
| **Sertoli cell tumor[c]** | 0.8 | 0.3 | 35.5 | 5.3 | 19.7 | 4.8 |

**Table 8. Overview of marker positive canine testicular tumor cells**

p21, γH2AX with median and interquartile range (IQR) in %; ki67 with mean percentage (MV) of positive cells ± standard deviation (SD) in %.

[c] cryptorchid testes

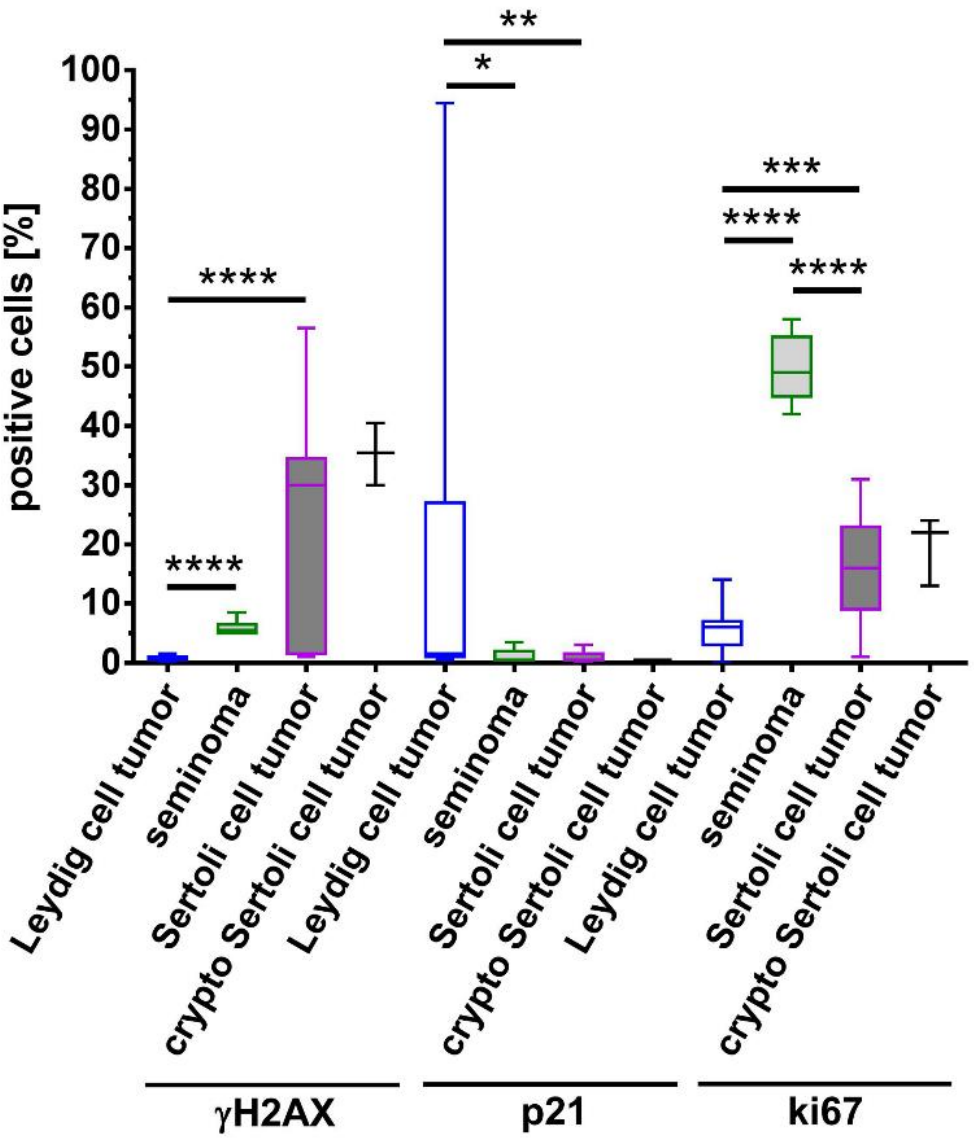


**Figure 8. γH2AX, p21 and ki67 positive tumor cells in canine testes**

Leydig cell tumors had significantly less γH2AX positive cells than seminomas ($p \leq 0.0001$) and Sertoli cell tumors ($p \leq 0.0001$). Leydig cell tumors had significantly more p21 positive cells than seminomas ($p \leq 0.05$) and Sertoli cell tumors ($p \leq 0.01$). Leydig cell tumors had significantly less ki67 positive cells than seminomas ($p \leq 0.0001$) and Sertoli cell tumors ($p \leq 0.001$). Seminomas had significantly more ki67 positive cells than Sertoli cell tumors ($p \leq 0.0001$). Box and whisker plots, boxes are outlined by $25^{th}$ to $75^{th}$ percentiles, median as horizontal line, and whiskers draw minimum to maximum. Significance indicated by stars: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Abbreviation: crypto, cryptorchid.

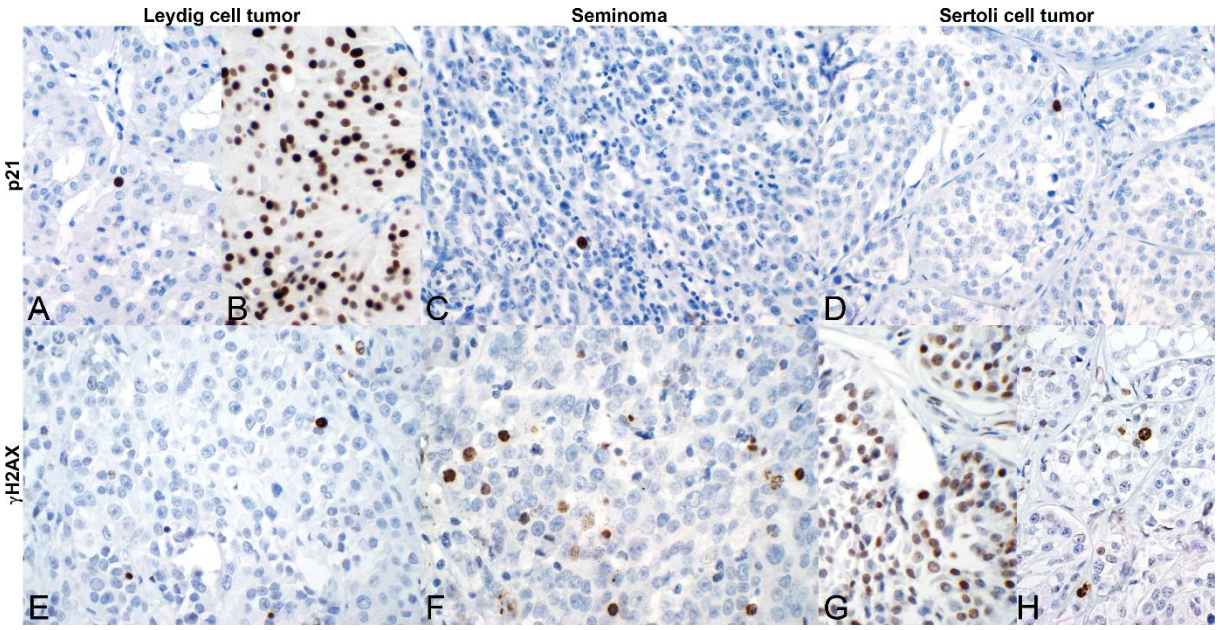


**Figure 9. Immunohistochemistry of p21 and γH2AX in tumor cells of canine testis**

**A**: Most Leydig cell tumors (12/15) with only few p21 positive tumor cells. **B**: Some Leydig cell tumors (3/15) contained p21 rich nests. **C**: Seminomas with only few p21 positive tumor cells. **D:** Sertoli cell tumors with only few p21 positive tumor cells**. E:** Leydig cell tumors with only few γH2AX positive tumor cells. **F:** Seminomas with few to moderate amounts of γH2AX positive tumor cells. **G:** Most Sertoli cell tumors (10/15) with many γH2AX positive tumor cells. **H**: Some Sertoli cell tumors (5/15) with only few γH2AX positive tumor cells. Immunohistochemistry, ABC-method, hematoxylin counterstain.

## 5.2 Eye

### 5.2.1 Senescence marker expression in healthy young and old eyes

Both markers (γH2AX, p21) showed a nuclear signal. Positive cells posed a rare event and no age-dependent changes could be noted for both markers in any of the 11 examined compartments (epithelium and stroma of the lens and cornea, GCL, INL, ONL, RPE, iris, ciliary body, optic nerve) of the canine eye (Fig 10). For both markers the median was 0 in all compartments with the only exception being γH2AX in the lens stroma (median: young: 1.5 %; old: 1.0 %) and GCL of retina (median: young: 0.3 %; old: 1.0 %).

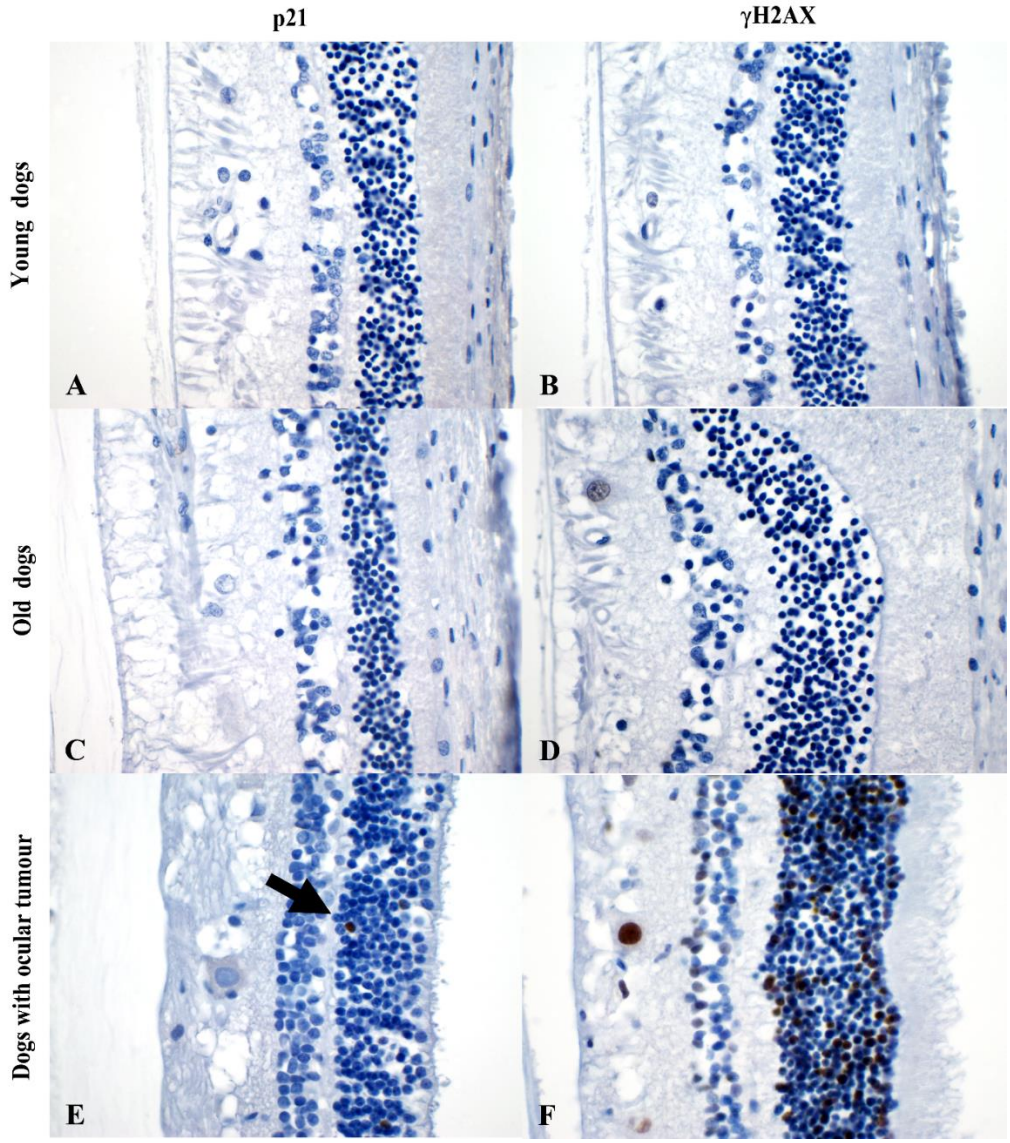


**Figure 10. Immunohistochemistry of p21 and γH2AX in canine retina**

P21 and γH2AX expression in retinas of young and old dogs (**A-D**) and retinas of dogs with ocular tumor (uveal melanocytoma, **E-F**), immunohistochemistry for p21 and γH2AX. **A-D:** Positive cells show diffuse brown, nuclear signal. No positive cells detectable in young and old eyes. **E, F**: In eyes with uveal

melanocytoma, few positive cells for p21 (arrow, **E**) and many positive cells for γH2AX in the retina (especially GCL and ONL, **E**) can be seen.

### 5.2.2 Senescence marker expression in the microenvironment of ocular tumors

Marker (γH2AX, p21) expression was compared in all compartments of eyes with ocular tumors (i.e. microenvironment, see definition in materials and methods) with equivalent compartments of young as well as old healthy eyes (Table 9).

| | | γH2AX | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | UM all | | UM > 0.1 cm | | UM ≤ 0.1 cm | | UM w cat | | UM wo cat | | CBA | | Young | | Old | |
| | | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR |
| Lens | Epithelium | 0 | 2.0 | 0* | 1.8 | 1.0 | 1.5 | 0 | 1.0 | 0 | 2.0 | 2.0 | 15.0 | 0 | 2.0 | 0 | 1.0 |
| | Stroma | 5.0 | 3.0 | 5.0 | 2.8 | 3.0 | 5.5 | 6.0 | 1.0 | 4.0 | 2.0 | 0 | 2.0 | 1.5 | 3.5 | 1.0 | 1.0 |
| Retina | GCL | 21.5*** | 23.3 | 26.8*** | 8.9 | 3.5 | 4.0 | 26.8 | 7.3 | 25.5 | 14.3 | 33.0* | 24.5 | 0.3 | 1.9 | 1.0 | 1.0 |
| | INL | 0.5 | 1.0 | 0.3 | 0.9 | 0.5 | 4.5 | 0.5 | 1.0 | 0 | 0.5 | 0.5 | 1.5 | 0 | 0.4 | 0 | 0 |
| | ONL | 31.0*** | 17.0 | 33.5**** | 8.4 | 0 | 0 | 36.0 | 2.5 | 31.0 | 4.0 | 34.5*** | 19.5 | 0 | 0 | 0 | 0 |
| | RPE | 0 | 0.8 | 0 | 0.8 | 0 | 0.5 | 0.8 | 9.9 | 0 | 0 | 0.3 | 1.0 | 0 | 0 | 0 | 0 |
| Uvea | Iris | 1.0* | 1.5 | 0.5* | 1.4 | 1.3* | 0.3 | 1.0 | 0.75 | 0 | 3.3 | 1.3*** | 1.3 | 0 | 0 | 0 | 0.5 |
| | CB | 0.5* | 1.0 | 0.3* | 1.0 | 0.5* | 0.8 | 0 | 1.0 | 0.5 | 1.0 | T | T | 0 | 0 | 0 | 0.5 |
| Cornea | Epithelium | 1.0 | 12.5 | 6.8* | 48.3 | 0 | 2.8 | n < 3 | 7.3 | 0.5 | 3.9 | 35.0** | 18.0 | 0 | 0 | 0 | 0 |
| | Stroma | 0 | 0.5 | 0 | 1.9 | 0 | 0.3 | 0 | 0 | 1.25 | 2.5 | 0 | 0.5 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | | | |
| | | p21 | | | | | | | | | | | | | | | |
| | | UM all | | UM > 0.1 cm | | UM ≤ 0.1 cm | | UM w cat | | UM wo cat | | CBA | | Young | | Old | |
| | | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR | M | IQR |
| Lens | Epithelium | 0* | 1.0 | 0.5* | 1.0 | 0 | 0 | 1.0 | 1.0 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Stroma | 0 | 2.0 | 0 | 2.0 | 0 | 0 | 2.0 | 2.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Retina | GCL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.3 | 0.5* | 1.5 | 0 | 0 | 0 | 0 |
| | INL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5* | 2.5 | 0 | 0 | 0 | 0 |
| | ONL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | RPE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0.1 | 0 | 0 | 0 | 0 |
| Uvea | Iris | 0.3* | 0.8 | 0.5* | 1.1 | 0 | 0 | 0.5 | 0.5 | 0 | 1.8 | 0.5* | 1.0 | 0 | 0 | 0 | 0 |
| | CB | 2.0*** | 3.0 | 2.0* | 2.1 | 0.5* | 2.5 | 2.0 | 1.0 | 2.0 | 2.5 | T | T | 0 | 0 | 0 | 0.5 |
| Cornea | Epithelium | 0 | 0.5 | 0 | 0.5 | 0 | 0.3 | n < 3 | 0 | 0 | 0.1 | 0 | 0.5 | 0 | 0 | 0 | 0 |
| | Stroma | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 9. Results of expression of γH2AX and p21 in the microenvironments of ocular tumors**

Overview of the results of marker expression γH2AX and p21 in the microenvironment (i.e. compartments of the eye, see above) of uveal melanocytomas (all, n = 13; > 0.1 cm, n= 10; ≤ 0.1 cm, n = 3) and ciliary body adenomas compared to eyes of young and old dogs. Percentages in median with interquartile range (IQR). Significance in comparison to young eyes indicated by stars: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001. Uveal melanocytomas with and without cataract (eyes > 0.1 cm) were compared internally and not to young and old eyes.

Abbreviation: cat, cataract; CB, ciliary body; CBA, ciliary body adenoma; UM, uveal melanocytoma; T, tumor; w, with; wo, without.

#### 5.2.2.1 Uveal melanocytomas

The amount of γH2AX positive cells increased in eyes with uveal melanocytomas in the retina, specifically the GCL (*vs* young and old: both $p \leq 0.001$, Fig 10) and the ONL (*vs* young and old: both $p \leq 0.001$, Fig 10), the iris (*vs* young: $p \leq 0.05$), the ciliary body (*vs* young: $p \leq 0.05$), the lens stroma (*vs* old: $p \leq 0.001$) and the corneal epithelium (*vs* old: $p \leq 0.05$). Including only uveal melanocytomas > 0.1 cm in diameter (n=10) an additional increase was noted in the corneal epithelium (*vs* young and old: both $p \leq 0.05$). Including only uveal melanocytomas ≤ 0.1 cm, there were less significant increases. These were only detected in the iris ($p \leq 0.05$) and the ciliary body ($p \leq 0.05$).

The amount of p21 positive cells increased in eyes with uveal melanocytomas also in the iris (*vs* young: $p \leq 0.05$) and the ciliary body (*vs* young: $p \leq 0.001$; *vs* old: $p \leq 0.05$). In addition, p21 positive cell increases could be detected in the lens epithelium (*vs* young: $p \leq 0.05$). Including only uveal melanocytomas > 0.1 cm in diameter (n=10) an additional increase was noted in the lens epithelium (*vs* old: $p \leq 0.05$) and the iris (*vs* old: $p \leq 0.05$). Including only uveal melanocytomas ≤ 0.1 cm the only increase was noted in the ciliary body (*vs* young: $p \leq 0.05$). Single cell positivity was observed in other compartments of the eye (e.g. ONL, Fig 10).

Expression of markers in the microenvironment were not significantly different in melanocytomas with cataract (n = 5) versus those without cataract (n = 5).

The RPE of three samples and the cornea of four samples were lost during processing.

#### 5.2.2.2 Ciliary body adenomas

The amount of γH2AX positive cells increased in eyes with ciliary body adenomas in the retina specifically the GCL (*vs* young and old: both $p \leq 0.05$) and the ONL (*vs* young: $p \leq 0.001$; *vs* old: $p \leq 0.01$), the iris (*vs* young: $p \leq 0.001$; *vs* old: $p \leq 0.05$) and the epithelium of the cornea (*vs* young and old: both $p \leq 0.01$).

The amount of p21 positive cells increased in eyes with ciliary body adenomas also in the GCL (*vs* young and old: both $p \leq 0.05$) and the iris (*vs* young: $p \leq 0.05$). In addition, p21 positive cell increases could be detected in the retinal INL (*vs* young: $p \leq 0.05$; *vs* old: $p \leq 0.01$).

The RPE of five samples and the cornea of four samples were lost during processing.

### 5.2.3 Marker expression in tumor cells of uveal melanocytomas and conjunctival melanomas

The expression of p21 was significantly different in uveal melanocytomas and conjunctival melanomas. P21 expression was significantly higher in conjunctival melanomas with a median

of 36.3 % than in uveal melanocytomas with a median of 1.5 % ($p \leq 0.001$) (Fig 11). This significance was independent of tumor size.

The expression of γH2AX was greatly variable for both tumors (Fig 11). The only significantly difference could be noted when comparing conjunctival melanomas with a median of 20.8 % with small uveal melanocytomas ≤ 0.1 cm with a median of 0.5 % ($p \leq 0.05$).

Uveal melanocytomas > 0.1 cm had significantly more γH2AX tumor cells with a median of 65.0 % than uveal melanocytomas ≤ 0.1 cm with a median of 0.5 % ($p \leq 0.05$). This could also be noted for p21 expression ($p \leq 0.01$).

See Figure 12 for marker expression differences in tumor cells of these two ocular melanocytic tumors.

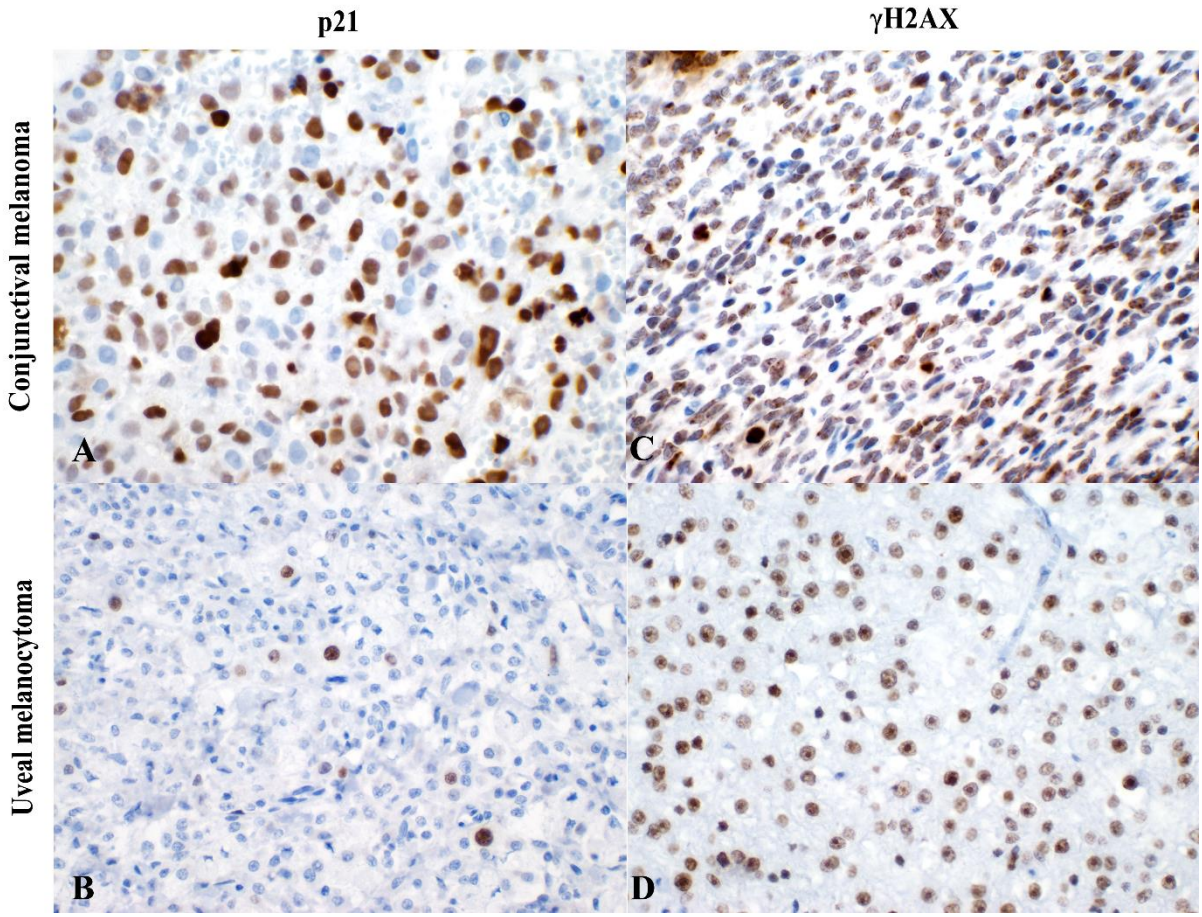


**Figure 11. Immunohistochemistry of p21 and γH2AX in tumor cells of conjunctival melanomas and uveal melanocytomas**

Positive tumor cells showed dotted (γH2AX in **C**) to diffuse brown nuclear signal. Malignant conjunctival melanomas showed high expression of p21 (**A**) in contrast to low expression of p21 in benign uveal melanocytomas (**B**). **C, D**: Both tumors showed highly variable but by trend high expression of γH2AX.

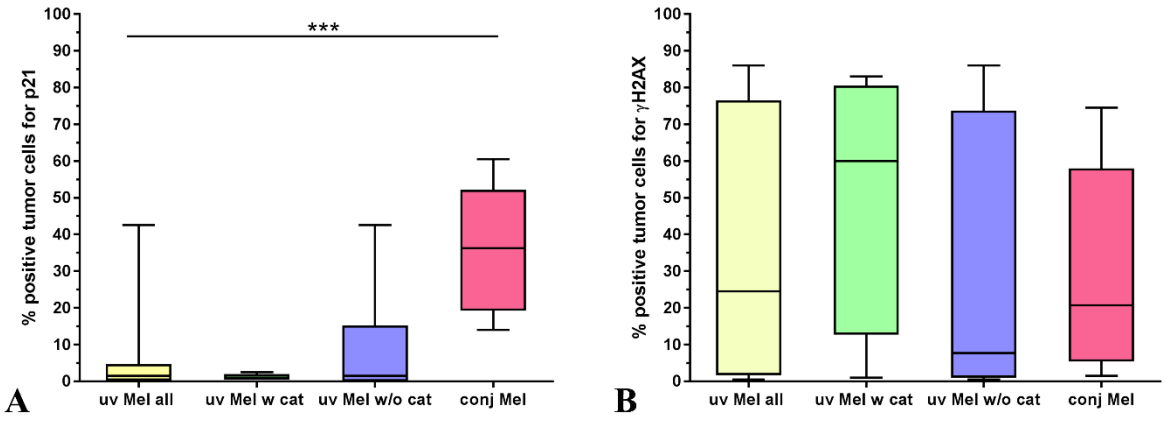


**Figure 12. γH2AX and p21 positive tumor cells in uveal melanocytomas and conjunctival melanomas**

**A:** Malignant conjunctival melanomas showed significantly more positive tumor cells than benign uveal melanocytomas. **B:** Tumor cells were highly variable in the expression of γH2AX. **A, B:** No significant differences could be observed between uveal melanocytomas with and without cataract for both markers. Box and whisker plots, boxes are outlined by 25$^{th}$ to 75$^{th}$ percentiles, median as horizontal line, and whiskers draw minimum to maximum. Significance indicated by stars: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Abbreviations: conj Mel, conjunctival melanoma; uv Mel, uveal melanocytoma, w cat, with cataract; w/o cat, without cataract.

# 6 Discussion

The aim of this study was to evaluate whether senescence markers γH2AX and p21 increase in old canine testes and eyes. In addition, the expression of these markers was correlated with age-associated morphological changes in the canine testes.

Additionally, the expression of senescence markers γH2AX and p21 was evaluated in the microenvironment of testicular (seminoma, Sertoli cell tumor, Leydig cell tumor) and ocular tumors (uveal melanocytoma, ciliary body adenoma).

Finally the expression of γH2AX and p21 in tumor cells was compared between benign uveal melanocytomas and malignant conjunctival melanomas, as well as between testicular tumors.

## 6.1 Senescence marker expression in aged canine testis and eyes

In the canine testis, an increased number of p21 positive fibroblasts ($p \leq 0.0001$) and Leydig cells ($p \leq 0.001$) was detected. However, it must be clarified that even though statistically significant these findings still pose rare events. The amount of p21 positive fibroblasts increased from median 0.5 % in young to median 2.0 % in old dogs. The amount of p21 positive Leydig cells increased from median 0.5 % in young to median 4.0 % in old dogs. When studying published data it seems that increasing of alleged senescent cells with age mostly poses rare events. In accordance with our data Wang et al. identified a significant yet minor increase in γH2AX positive cells in the dermis of mice from 1.1 ± 0.2 % in young animals to 4.8 ± 2.5 % in old animals.[58] Similarly, for human skin Ressler et al. detected an increase in p16INK4a positive cells in the dermis from 0.6 cells per mm (millimeter of basement membrane) in young donors, 2.9 cells per mm for middle aged donors and 6.3 cells per mm for old donors.[18] Supporting the assumption of only minimal increase of detectable senescent cells with age are images of alleged senescent cells in previous reports that only show single cells.[14,18,59,60] The biological significance of these single cells stays unclear. It remains speculative if these cells possess the secretory growth-stimulating secretory phenotype associated with senescent cells in vitro. Also, the amount of senescent cells needed to achieve certain effects remains unknown.[61]

However, γH2AX could not verify this finding as an increase in γH2AX positive fibroblasts or Leydig cells in the aged canine testis was not identified. There are possible explanations for an age-dependent p21 but not γH2AX increase. γH2AX is a sensor of DNA damage and whereas many, including p21, senescence pathways are DNA-damage induced, some are independent of DNA-double-strand breaks. Other triggers of senescence include oncogenic signaling, reactive oxygen species (ROS) or cell fusion.[62] These DNA-damage independent pathways may however still activate p21.[21]

Cryptorchid testes showed neither significant p21 nor γH2AX expressionIt was hypothesized that premature aging possibly due to intraabdominal higher temperature could be considered a cause for predisposition of tumor development. This could neither be verified nor ruled out considering that p21 and γH2AX showed no convincing increase in aged testis.

In the canine eye, neither p21 nor γH2AX showed an age-dependent increase in any of the examined ocular compartments. Our findings support Wang et al. who detected no age-dependent increase of γH2AX at least in murine lens epithelial cells.[58] However, our results are in contrast to studies that found an accumulation of senescent cells in the RPE of monkey (*in vivo)* and humans (*in vitro).*[63] There could be several reasons for our finding. First, the extent and progress of aging possesses a certain tissue and cell-type variability and some tissues age at a faster pace than others.[58] In primates, for example, an increase of senescence marker expression was found in the dermis but not in the skeletal muscle.[64] Second, it is unclear how long senescent cells persist before they are disposed of and this might also limit possibilities to seize them.[32] And third, considering that most studies on the cellular senescence have been conducted in cell culture the *in vitro* to canine *in vivo* transferability may be problematic.[12,65]

In addition to the above mentioned, it must be repeated that to date no marker of cellular senescence is completely specific. Thus, any lack of marker expression may be underestimating senescence. However, considering recent data, to our knowledge, two of the most promising markers of the field were used. The DNA-damage induced senescence pathway which includes γH2AX and p21 is one of the most important senescence pathway associated with aging. Additionally, the p21 pathway is linked and activated by other pathways, including p16, via other stimuli than damage response. Thus, γH2AX and p21 possibly encompass a broad spectrum of cellular senescence.

## 6.2 Morphological changes in the aged canine testis

Only few histomorphological changes were identified in aged canine testes. Adding to this the meager to non-existing senescence marker expression raises the question whether the canine testis ages at all. No significant decrease in epithelial area and tubular diameter and no significant increase in interstitial collagen content was detected. These parameters have been discussed controversially for the canine testis. Some studies show the same results[35,66]. However, some textbooks (i.e., JUBB/Kennedy[67]) still state the opposite, likely based on presumptions as in humans epithelial area and tubular diameter are known to decrease with age.[68-73] The only significant finding in the aged canine testis was an increase in mean number of Leydig cells / ITT ($p \leq 0.0001$). Leydig cell hyperplasia is common in aged dogs.[45] In man and rat, however, the number of Leydig cells decrease with age.[73-75] Additionally, a slight yet

significant decrease in spermatogenesis was detected in old testes ($p \leq 0.0001$) and a minor increase in tubular irregularities (scores ≤ 8). This challenges Peters' findings that spermatogenesis does not decrease in old dogs.[35] However, aged canine testes still possessed a mean score above 9 and ample morphologically normal spermatozoa. Needless to say no conclusions can be made on the functional quality (vitality, motility) of the spermatozoa produced. In humans, for example, it was shown that advanced paternal age has a negative effect on sperm quality, fertility and reproductive outcomes.[76] Also, numerous studies have shown that old canine testes are disposed to develop age-associated tumors with a prevalence of up to 65 % depending on the study quoted.[77] In our present study, the average age was 10.2 years for dogs with testicular tumors, 10.5 years for dogs with Leydig cell tumors, 10.1 years for seminomas and 10.2 years for Sertoli cell tumors. Given these facts it is rather unlikely that the canine testis does not age at all on any level. However, the mechanisms might vary between tissues or aging may strike at ages beyond the groups studied here.

## 6.3 Senescence marker expression in the microenvironment

### 6.3.1 Canine testicular tumors: Seminomas, Sertoli cell tumors, Leydig cell tumors

No significant increase of p21 positive fibroblasts could be noted in any of the testicular tumors in comparison to fibroblasts from young dogs. In comparison to fibroblasts from old dogs, a significant decrease of p21 positive fibroblasts could even be noted for Leydig cell tumors and seminomas. A possible explanation are simply variances in p21 expressing cells amongst individuals.

A minor yet significant increase of γH2AX positive cells was found in the microenvironment of seminomas and Sertoli cell tumors in relation to young fibroblasts. However, it must be considered that positive fibroblasts were indeed still a rare event and data could not be verified by p21 labelling. This renders a senescence-associated γH2AX expression less likely. An alternative explanation for γH2AX expression in the microenvironment of testicular tumors could possibly be tumor-induced secretion of growth factors. These would stimulate proliferation of cells in the microenvironment, resulting in faster turnover with increased probability of double-strand-breaks.

Taken together with the minor (p21) or lacking (γH2AX) increase of expression with age, our finding make a connection between an accumulation of p21 and γH2AX positive fibroblasts and tumor development in the canine testis less likely. However, hypothetically, an increase in senescent cells could not be seen in all aged individuals and some could be more prone to accumulation of senescent cells than others, including the individuals studied here. Also, those with accumulations of senescent cells could have pathologies that were not studied here. And

supposedly senescent cells are removed by effects of the tumor once tumor development has been triggered.

### 6.3.2 Canine ocular tumors: Uveal melanocytomas and ciliary body adenomas

The microenvironment of uveal melanocytomas and ciliary body adenomas showed significantly increased senescence marker expression. γH2AX expression was most prominent in the retina and the uvea. The examined tumors commonly develop adjacent to the iridocorneal angle and thus predispose for obstruction of humor drainage. Drainage obstruction ultimately raises the intraocular pressure with the possibility of resultant glaucoma. This subsequently damages the intrabulbar cells, which may lead to DNA damage with expression of γH2AX, primarily a marker of double-strand-breaks.[23] In this case, a damage-induced increased expression can be hypothesized.

An increased expression of p21 is also noted in the uvea and the retina in both tumors as well as in the lens in eyes with uveal melanocytomas. Co-culture studies have shown that senescent fibroblasts adopt a secretory phenotype that stimulates melanoma cells to proliferate.[78] Substantial data in vivo or on canine tissue is however lacking. The actual number of p21 positive fibroblasts found in our study was relatively small. It is not known how many senescent cells are needed to create an impact on the environment in vivo and how many of these alleged senescent cells even express the necessary secretory phenotype. Therefore, considering the small number of potentially senescent cells detected, any interpretation has to be cautious.

It must also be considered possible, that the preneoplastic phase i.e. potentially higher in senescence marker expression phase of the microenvironment was not seized with our case selection. However, three uveal melanocytomas of very small size (≤ 0.1 cm) were included and these did not show a high expression of senescence markers in their microenvironment. Contrary, they showed even less senescence marker expression in comparison to their larger counterparts. This further underlines the significance of tumor size in association with increased ocular pressure as a major contributing factor.

Taken together, considering that both markers are not increased with age in any ocular compartment, and the small (p21) and possible pressure-damage-induced (γH2AX) expression, a tumor-induced triggering of increased marker expression is most likely.

## 6.4 Marker expression in tumor cells of testicular and ocular tumors

### 6.4.1 Testicular tumors

In humans, p21 staining is of prognostic relevance for the germ cell tumor, the most common testicular tumor in man. Invasive germ cell tumors are associated with loss of nuclear staining

and cytoplasmic p21 staining is associated with chemotherapeutic cisplatin resistance.[79-81] The cell cycle inhibitor p21 is normally active in the nucleus but can be phosphorylated and translocated to the cytoplasm where it inhibits apoptosis. There are no comparable studies for testicular tumors in dogs. P21 staining in our study was solely nuclear, making a true cell cycle arrest likely. Leydig cell tumors contained the most p21 positive cells followed by seminomas and Sertoli cell tumors. Most canine testicular tumors can be considered benign. Only few seminomas and Sertoli cell tumors metastasize. Leydig cell tumors which contained the most p21 positive cells, are not known to metastasize. None of the examined tumors showed signs of vascular invasion or other malignancy criteria.

Studies on γH2AX in testicular tumors are scarce in humans as well as in animals.[82,83] Leydig cell tumors showed the least expression of γH2AX, followed by seminomas and Sertoli cell tumors. One possible hypothesis could be that a high expression of γH2AX is associated with a fast turnover with insufficient cell division and repair. The ki67-indices of these tumors would support this theory. Seminomas and Sertoli cell tumors have high ki67-indices whereas Leydig cell tumors have low ki67-indices. Literature agrees on seminomas having the highest ki67 expression. However, literature disagrees whether Sertoli cell tumors or Leydig cell tumors have the higher ki67-index.[57,84,85]

### 6.4.2 Benign uveal melanocytoma versus malignant conjunctival melanoma

In humans and canines, a high p21 expression in melanocytic tumors is associated with malignancy in the majority of studies whilst others have conflicting results.[86-90] The exact mechanism is yet unknown. It seems that p21 has become dysfunctional and the cell cycle arrest overcome as metastasis is a common finding in melanocytic tumors overexpressing p21.[91] My findings support this data as benign uveal melanocytomas showed a low expression of p21 and malignant conjunctival melanomas displayed a high expression of p21. The use of p21 as a prognostic marker in canine melanocytic tumors should be considered.

γH2AX presented with extremely variable expression in both tumors. In addition to lacking literature data any interpretation would be far-fetched.

## 6.5 Limitations of this study

All retrospective studies have certain methodological limitation. Handling of samples and fixation may have varied slightly between the samples. However, both the testis and the eye are very sensitive indicators of even minor signs of decay, poor handling or fixation. This can be seen for example in epithelial detachment and poor immunoreactivity. In this study only samples with excellent morphological quality and no frozen tissues were included. Therefore, I presume the above-mentioned theoretical limitation to be of negligible influence.

Unfortunately, not all canine breeds could be included in this study and the results may thus not be fully representative for the entire dog population.

Samples of eyes had been processed with two different fixatives: formalin and Davidson solution. It is known that depending on the antibody the fixative can have an effect on the immunohistochemical staining quality.[92-95] Therefore I compared the two fixatives and their staining quality using the same sample (eye, one half formalin, one half Davidson solution) as well as a testis containing a Leydig cell tumor. No staining quality difference could be noted for both markers (p21, γH2AX) used in this study.

As with all retrospective studies certain limitation and uncertainties are inevitable especially concerning the anamnesis. I consulted the veterinarians of all animals included in this study for detailed information (see background information) as well as information of endocrinopathies and hormone therapies. Two animals of the old healthy group had received hormones for reproduction control. Dog 1 (poodle) had received Tardastrex on the 24$^{th}$ of January 2014 and was castrated on 04$^{th}$ of February. He was additionally diagnosed and treated for hypothyroidism (since 2012). Dog 6 (Pekinese) had received Ypozane in 2016 and was castrated shortly thereafter. Dog 11 (Pinscher Mixed-breed) had been diagnosed with Cushing and treated with Vetoryl. Although I cannot exclude a possible influence I saw no differences in staining quality and morphology compared to untreated animals. Also, not all canine breeds could be included in this study and the results may thus not be fully representative for the entire dog population.

# 7 Conclusions

The expression of alleged senescence markers γH2AX and p21 does not show a convincing age-dependent increase in the canine testis and eye. It is rather unlikely that canine testis and eye do not age as both show age-associated pathologies including morphological changes in the testes, nuclear sclerosis, corneal and retinal degeneration in the retina and tumor growth in both. It is possible, however, that dogs or these particular organ systems simply do not show an accumulation of senescent cells with age. Although rather unlikely, it could possibly be that an accumulation of senescent cells is not seen in these healthy organs and that any senescence marker increase would lead to pathologies which were excluded as only healthy controls were included. It is also conceivable that a different yet to be found biomarker is needed for canine tissue.

Morphological changes of the canine testes were fewer than expected. In contrast to humans, old dogs showed a significant increase of Leydig cells / intertubular triangle. Also a slight decrease of spermatogenesis (modified Johnsen score) could be detected. No decrease of tubular diameter or epithelial area / tubular area and no increase of interstitial collagen content could be noted.

Alleged senescence markers γH2AX and p21 were not highly expressed in the microenvironment of testicular tumors. This finding could mean that senescent cells are not involved in tumor development of the testis. It is also possible that the pre-neoplastic phase with accumulation of senescent cells is very short and was not included in our sample collection.

In contrast, alleged senescence γH2AX and p21 were increased in the microenvironment of ocular tumors in comparison to young control eyes. However a tumor-induced pressure increase with subsequent cell damage is likely. This is supported by the fact that age-associated increase could be noted in healthy eyes and small tumors without secondary glaucoma showed significantly less expression of markers.

Finally, tumors cells of malignant melanomas showed a significantly higher expression of p21 than benign uveal melanocytomas. This indicated that malignant melanocytic tumors can overcome senescence associated cell cycle arrest. P21 as tumor marker for malignancy in melanocytic tumors may be considered.

# 8 Summary

Cellular senescence was discovered in cell culture by Hayflick and Moorhead in 1965 in human fibroblasts. It is primarily characterized by irreversible cell cycle arrest and an altered, enlarged morphology. Senescent cells are thought to accumulate with age in a variety of species including mice, monkey and man. Also, they are thought to drive tumor growth and progression via their senescence-associated secretory phenotype.

No known biomarker for cellular senescence is completely specific. Senescence is often triggered by DNA damage, following classical tumor suppressor pathways and activation of cyclin-dependent kinase inhibitors. Looking towards current literature, two potential biomarkers for cellular senescence, DNA-damage marker γH2AX, and cyclin-dependent kinase inhibitor p21, were selected to be tested for transferability on formalin-fixed and paraffin-embedded canine tissue. Most studies on cellular senescence have been conducted in cell culture and to our knowledge none on canine tissues. Human gene products of the DNA damage response are more closely related to dog than to rodent which might make them a superior model.

The aim of this study was to test the hypotheses that senescence markers γH2AX and p21 increase with age and in the microenvironment of tumors. Two organ systems that are known to show age-dependent pathologies, testis and eye, were studies for age-dependent increase of senescent cells. For the testis, senescence markers expression was also correlated with age-dependent morphological changes (Leydig cells / intertubular triangle, modified Johnsen score for evaluation of spermatogenesis, interstitial collagen content, epithelial area / tubular area, tubular diameter). Additionally, the microenvironment of testicular (Leydig cell tumor, seminoma, Sertoli cell tumor) and ocular (uveal melanocytoma, ciliary body adenoma) tumors were examined for possible increase of senescent cells. As both used markers are also expressed in tumors, their role as tumor markers in ocular melanocytic tumors (benign uveal melanocytoma, malignant conjunctival melanoma) and testicular tumors was also studied.

In the canine testis an age-dependent significant increase could only be noted for p21 in fibroblasts and Leydig cells. However, the expression remained a rare event with only small amounts of cells. Morphological age-dependent changes were only seen in a significant increase of Leydig cells / intertubular triangle and a decrease in spermatogenesis (modified Johnsen score). However, contrary to my expectations, tubular diameters and epithelial areas / tubular areas did not decrease and interstitial collagen content did not increase with age.

In the microenvironment of testicular tumors, p21 and γH2AX expression was rare.

In testicular tumor cells, γH2AX expression was most prominent in Sertoli cell tumors, followed by seminomas and Leydig cell tumors, whereas p21 expression was most prominent in Leydig

cell tumors and low in Sertoli cell tumors and seminomas. These findings correlated with their ki67 indices, where Sertoli cell tumors and seminomas were mitotically more active than Leydig cell tumors.

In the canine eye, no age-dependent increase of senescence marker expression could be noted in any examined ocular compartment. In eyes with ocular tumors significantly more p21 and γH2AX expression could be noted in comparison to young eyes particularly in the retina, uvea and lens. However, a tumor-induced pressure increase is likely to have resulted in the higher expression due to cell damage.

Tumor cells of benign uveal melanocytomas contained more p21 positive cells than malignant conjunctival melanomas.

γH2AX and p21 could not show a convincing age-dependent increase in the canine testis and eye. Morphological changes, the tendency of tumor development and increase in age-related pathologies make it unlikely that the canine testis and eye do not age. Either these tissues do not show an accumulation of senescent cells or a different yet to be found biomarker is needed. A possible bidirectional cross-talk of tumor microenvironment and tumor cells could only be identified for ocular tumors, but this was most likely tumor-induced and not primary age-dependent senescence. P21 as tumor marker for malignancy in melanocytic tumors may be considered.

# 9 Zusammenfassung


**Alters- und tumorabhängige Seneszenz in kaninen Hoden und Augen**

Zelluläre Seneszenz wurde erstmals 1965 von Hayflick und Moorhead in humanen Fibroblasten in Zellkultur beschrieben. Zelluläre Seneszenz ist primär charakterisiert durch einen irreversiblen Zellzyklusarrest und eine veränderte, vergrößerte Morphologie. Seneszente Zellen akkumulieren im Alter in einer Vielzahl von Spezies unter anderem Maus, Affe und Mensch. Durch die Ausprägung eines sogenannten Seneszenz-assoziierten sekretorischen Phänotyps werden sie zudem mit der Förderung von Tumorwachstum und -progression in Verbindung gebracht.

Bis heute gilt kein bekannter Seneszenzmarker als spezifisch. Seneszenz wird meist über DNA-Schäden ausgelöst mit nachfolgender Aktivierung der Tumor-Suppressor Signalwege und deren Inhibitoren cyclin-abhängiger Kinasen (CDK). Mit Blick auf aktuelle Literatur wurden γH2AX, Marker für DNA-Schäden, und p21, CDK (cyclin-dependent kinase)-Inhibitor 1 als potentielle Biomarker der Seneszenz ausgewählt, um eine Übertragbarkeit von Zellkulturversuchen auf Formalin-fixiertes Paraffin-eingebettetes kanines Gewebe zu testen. Die meisten Studien zur Seneszenz wurden bislang in Zellkultur betrieben und soweit wir wissen keine an kaninem Gewebe. Humane Genprodukte der DNA-Schäden-Reaktion sind näher verwandt mit dem Hund als mit dem Nager, weshalb der Hund möglicherweise ein besseres Model darstellt.

Zweck dieser Studie war es die Hypothese zu testen, dass Seneszenzmarker γH2AX und p21 im Alter und in der Umgebung von Tumoren zunehmen. Hierfür wurden zwei Organsysteme, Hoden und Augen, ausgewählt, bei denen bekannt ist, dass sie altersabhängige Pathologien zeigen. Beim Hoden wurde die Expression der Seneszenzmarker auch mit altersabhängigen morphologischen Veränderungen (Leydig Zellen / intertubulärem Dreieck, modifizierter Johnsen Score zur Evaluierung der Spermatogenese, interstitieller Kollagengehalt, Epithelfläche / Tubulusfläche, tubulärer Diameter) korreliert. Zusätzlich wurde eine Zunahme seneszenter Zellen in der Tumorumgebung von testikulären (Leydigzelltumor, Seminom, Sertolizelltumor) und okularen Tumoren (uveale Melanozytome, Ziliarkörperadenome) überprüft. Da beide Marker auch in Tumorzellen exprimiert werden, wurde auch ihre Rolle als Tumormarker in okularen Tumoren (benigne uveale Melanozytome, maligne konjunktivale Melanome) und in testikulären Tumoren untersucht.

Im kaninen Hoden zeigte sich eine signifikante altersabhängige Zunahme p21 positiver Fibroblasten und Leydigzellen. Allerdings handelte es sich hierbei um wenige Einzelzellen. Die

einzigen signifikanten altersabhängigen morphologischen Unterschiede zeigten sich in einer Zunahme von Leydigzellen / intertubulärem Dreieck und einer Abnahme der Spermatogenese (modifizierter Johnsen Score). Entgegen der Erwartungen nahmen der tubuläre Diameter und die Epithelfläche / Tubulusfläche nicht ab und der interstitielle Kollagengehalt nicht zu.

In der Umgebung von testikulären Tumoren zeigte sich nur eine sehr geringe Expression von γH2AX und p21.

In testikulären Tumorzellen wiesen Sertolizelltumoren die stärkste Expression von γH2AX in auf, gefolgt von Seminomen und Leydigzelltumoren. Hingegen zeigte sich die stärkste Expression von p21 in Leydigzelltumoren und war sehr niedrig in Sertolizelltumoren und Seminomen. Diese Ergebnisse korrelierten mit denen der ki67-Indexe, bei denen sich Sertolizelltumoren und Seminome als mitotisch deutlich aktiver zeigten als Leydigzelltumoren.

In kaninen Augen zeigte sich in keinem der untersuchten Kompartimente des Auges eine altersabhängige Zunahme der Seneszenzmarker. In Augen mit okularen Tumoren zeigte sich im Vergleich zu jungen Augen eine signifikante Zunahme der p21 und γH2AX Expression besonders in der Retina, der Uvea und der Linse. Allerdings ist eine durch den Tumor ausgelöste Druckerhöhung im Auge wahrscheinlich verantwortlich für eine erhöhte Expression der Marker durch vermehrte Zellschäden.

Tumorzellen benigner uvealer Melanozytome zeigten eine deutlich höhere Expression p21 positiver Zellen als maligne konjunktivale Melanome.

Eine überzeugende altersabhängige Zunahme von γH2AX und p21 konnte in kaninen Hoden und Augen nicht nachgewiesen werden. Morphologische Veränderung, die Neigung zur Tumorentstehung und eine Zunahme altersabhängiger Pathologien machen es unwahrscheinlich, dass kanine Hoden und Augen nicht altern. Entweder zeigen diese Gewebe keine altersabhängige Akkumulation seneszenter Zellen oder ein anderer noch zu findender Biomarker ist hierfür nötig. Eine mögliche bidirektionale Beeinflussung von Tumorumgebung und Tumorzellen konnte nur in okularen Tumoren gesehen werden, jedoch ist hier eine durch den Tumor ausgelöste Druckerhöhung mit folgendem Zellschaden wahrscheinlicher als eine primär altersabhänge Seneszenz. P21 könnte als möglicher Tumormarker für Malignität in melanozytären Tumoren in Betracht gezogen werden.

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# 12 Publications

Posters:

S.E. Merz, A. Breithaupt, R. Klopfleisch, A.D. Gruber (2017)

**Senescence Markers in Canine Testes: P21$^{WAF1/Cip1}$ but not γH2AX is Increased with Age**

3rd Joint European Congress of ESVP, ECVp and ESTP, Lyon, Frankreich – 30.08.-02.09.2017

Abstract in: Journal of Comparative Pathology

S.E. Merz, A. Breithaupt, R. Klopfleisch, A.D. Gruber (2018)

**Alterung im Hundehoden: Signifikante Zunahme des Seneszenzmarkers p21**

61. Tagung der Fachgruppe Pathologie der DVG Fulda, Deutschland – 02.03.-04.03.2018

Abstract in: Tierärztliche Praxis

Journals:

Parts of this doctoral thesis will be published in the journal *Veterinary Pathology* (Summer 2019).

Parts of this doctoral thesis are currently under review in the *Journal of Comparative Pathology*.

# 13 Declaration of Originality

I hereby declare that the present doctoral thesis is my own work. I assure that I exclusively used the mentioned sources.

Berlin, 21.03.2019 Sophie Merz