Aus dem Institut für Tierpathologie

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Evaluation and analysis of canine digital squamous cell carcinoma - Histological grading correlation to microscopic features of malignancy in the invasive front and copy number variation of the KIT ligand

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

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Argiñe Cerezo Echevarría

Tierärztin aus Madrid, Spanien

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For my family and loved ones

“Do not conform to the pattern of this world, but be transformed by the renewing of your mind”

Romans 12:2

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LIST OF ABBREVIATIONS

CDSCC	Canine digital squamous cell carcinoma
CN	Copy number
CNV	Copy number variation
ddPCR	Droplet digital polymerase chain reaction
DSCC	Digital squamous cell carcinoma
GS	Giant Schnauzer
HE	Hematoxylin & eosin
HPF	High-power field
IFGS	Invasive front grading system
KITLG	KIT ligand
OSCC	Oral squamous cell carcinoma
PV	Papilloma virus
SCC	Squamous cell carcinoma
SS	Standard Schnauzer
STF	Stem cell factor
TCBGS	Tumor cell budding grading system

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1. INTRODUCTION

Cutaneous squamous cell carcinoma (SCC) is a common, locally invasive neoplasm that arises from the epidermis with squamous differentiation. The development of this neoplasia is often associated with long-term UV-induced damage. As expected, sparse or lack of hair and/or light haircoat makes the area more susceptible to the development of cutaneous SCC. In dogs, this makes light-coated breeds more prone to suffer this neoplasia, with the exception of one location, the digit. Interestingly enough, it has been shown that, unlike in other locations, dark-coated breeds are predisposed to the development of squamous cell carcinoma arising from the digit or nailbed. Additionally, this different location seems to have implications concerning the neoplasm´s clinical behavior. For instance, digital squamous cell carcinoma (DSCC) is biologically more aggressive than its cutaneous counterpart, particularly noticeable in certain breeds such as the giant Schnauzer. This collection of particularities may suggest an underlying histological disparity which could partially explain its biological behavior.

Currently histological comparison of DSCC is challenging as there is no widely accepted grading system in dogs. Despite this, attempts have been made for grading this neoplasm in other locations (Nagamine et al., 2017), adapting the current human grading systems. This has been done by paying special attention to the neoplastic-stromal interphase, also known as “invasive front”. Human medicine has numerous, different grading schemes for SCC at different locations focusing on this feature, including the larynx and hypopharynx (Boxberg et al., 2019; H. Zhang et al., 2020), head and neck skin and mucosa (Caruntu et al., 2021), esophagus (Jesinghaus et al., 2019) or uterine cervix (Jesinghaus et al., 2018).

As shown in canines, there is a marked predisposing disparity based on haircoat and location of SCC. However, there are currently no extensive studies correlating the histological characteristics and the haircoat color to the development of this neoplasm at the digit. This would allow to potentially determine one of many potential underlying causes. With this pretense, recent studies have suggested a genetic abnormality predisposing to canine digital squamous cell carcinoma. This alteration corresponds with an increased number of copies of the KIT ligand gene (KITLG), correlating it with the development of digital squamous cell carcinoma in dogs. Given that KITLG is, among other things, associated with melanogenesis, this may also partially explain the predisposition of dark-haired breeds to this neoplasm.

The goals of this study are to: 1. investigate a correlation between the histological features of canine digital squamous cell carcinoma and the animal´s haircoat color and 2. correlate between the histological features associated with tumor malignancy of digital squamous cell carcinoma and a copy number variation of KIT ligand gene.

2. LITERATURE REVIEW

2.1. Squamous cell carcinoma; Comparative review between human and canine

2.1.1. Comparison of clinical and epidemiological differences between human and canine squamous cell carcinoma

Squamous cell carcinoma is a malignant neoplasia arising from the epidermal layer with varying degrees of keratinocyte differentiation. As expected, it can arise from any organ which is normally lined by squamous epithelium. This includes haired and glabrous skin, lips, oral cavity, esophagus, urinary tract (Martin et al., 2016), prostate (Hanna et al., 2020) and vagina (Yan et al., 2010).

In human medicine, despite the wide array of potential locations, the vast majority of SCC are included in four major sites; non-melanoma skin cancer, head and neck cancer, esophageal cancer and non-small cell lung cancer (Yan et al., 2010). The former, non-melanoma skin cancer, which includes both basal cell and squamous cell carcinoma is the most frequent neoplasia in humans (*Basal & Squamous Cell Skin Cancer Statistics*, 2023; Christenson et al., 2005). The second most common location is “head and neck” which comprises nasal and oral cavity, sinuses, nasopharynx, oropharynx, hypopharynx and larynx. As one can imagine, most of these locations are often intimately associated with the habit of smoking (Even et al., 2021). In addition, the incidence of SCC in humans is often driven by many other factors such as personal habits, environmental exposures, infections and/or ethnicity (Yan et al., 2010). However, interestingly enough, its exact incidence is unknown as is often not recorded in most cancer registries, unlike other neoplasias (Even et al., 2021; Siegel et al., 2023; Wong et al., 2003). Some factors for this lack of registry, include death outside the hospital (like dying at home), physician´s inexperience in death certification or lack of appropriate training (Even et al., 2021). However, other locations, such as nailbed SCC is considered a rare neoplasia in human medicine (Starace et al., 2018).

For all these locations, human pathology has tried to observe and create different histological grading systems to better characterize this neoplasia attending to its unique location. This is the case for SCC of the oral cavity (Bryne et al., 1989, 1992; Mohtasham et al., 2021), head and neck (Boxberg et al., 2019), uterine cervix (Jesinghaus et al., 2018) and skin (Schwab, 2011).

On the other side, squamous cell carcinoma in dogs represents roughly 5% of all epithelial tumors which, by itself, is the most common tumor type (38.45%), according to the Swiss canine registry (Grüntzig et al., 2015). Similar to humans, the most common reported site is the skin, followed by the oral cavity, which is occasionally compared with human´s SCC

of the head and neck (Liu et al., 2015). These different localizations are also related to a different biological behavior. For example, most often cutaneous SCC are less aggressive than their oral or digital counterpart (Nagamine et al., 2017). Different from humans, digital SCC is a common tumor seen in dogs, especially those predisposed (Grassinger et al., 2021; Wobeser et al., 2007). Unfortunately, unlike human pathology, there is no widely accepted grading system that is systematically used in canine SCC. Despite this, some attempts have been made, especially for canine oral SCC because of its more malignant behavior (Nagamine et al., 2017). Regrettably, up to date, there is no generalized consensus and acceptance.

In humans, cutaneous SCC is one of the cancers with the highest mutation rates (Fania et al., 2021), often associated with UV-exposure and damage. The most common pathogenesis underlying UV-related SCC lies on chronic UV-exposure, leading to p53 gene (TP53) mutations, which alters its pathways. P53 is a transcription factor that regulates the cell cycle and maintains genomic stability by indirectly inducing cycle arrest when necessary, among other functions (Fania et al., 2021). This UV-induced mutation likely renders p53 functionally obsolete, thus causing the uncontrolled multiplication of cells with damaged DNA (Fania et al., 2021; Howell & Ramsey, 2022). However, this is not the only possible pathogenesis, as other complex mutations have been described in human literature, such as CDKN2A gene, hTERT promoter, NOTCH1, NOTCH2, TP63, RIPK4, among others (Fania et al., 2021). Presently, these mutations have not been widely researched in the canine population.

2.1.2. Comparison of proposed grading systems for squamous cell carcinoma in humans and canines

Attending to SCC histological appearance, there are published, yet to some degree outdated, gradings for dogs. Currently, the most widely accepted is Broder´s grading system (Schwab, 2011), based on human medicine. This grading system pays particular attention to the tumor´s overall morphological features to determine its level of differentiation. The result is the classification of the neoplasia as well-differentiated (grade 1), moderately-differentiated (grade 2-3) or poorly-differentiated (grade 4). However, this adapted classification system, although established, is not routinely used, given that the differentiation may be dependent on the area evaluated (Belluco et al., 2013), and unclear prognostic characterization. The end result is the pathologist´s somewhat skepticism on its true value during routine examination, thus not being frequently reported.

Presently, several other different adapted grading systems have been attempted, most of them based on human medicine. This has been performed in order to classify squamous cell carcinoma at different locations, such as oral (Almangush et al., 2020), larynx/hypopharynx (Boxberg et al., 2019) or uterine cervix (Jesinghaus et al., 2018). The location most studied, given its malignant biological behavior, is canine oral squamous cell carcinoma (Mestrinho, Pissarra, et al., 2017; Nagamine et al., 2017). Unfortunately, the grading system is currently not widely used in veterinary pathology.

More recently, a special interest has been placed in the so-called “invasive front” and “epithelial-mesenchymal transition”, as it may potentially correlate with SCC clinical behavior (Boxberg et al., 2019; Bryne et al., 1989, 1992; Jesinghaus et al., 2018, 2019; Mohtasham et al., 2021; Nemec et al., 2012; Shimizu et al., 2018; Yamakawa et al., 2019) better than the classic, more widely used, aforementioned Broder´s grading system (Anneroth & Hansen, 1984; Arthur & Farr, 1972; Eneroth et al., 1972). The invasive front (IF) is defined as the tumor-host interface, where neoplastic cells interact with the surrounding stroma, infiltrating and expanding. This unique feature reveals a certain pattern of invasion and, therefore, potential malignancy. Within this IF there is a histological parameter known as “tumor budding” widely used in many modern grading systems in several neoplasms (Hiratsuka et al., 2022; Kim et al., 2022; Yamaguchi et al., 2010), including squamous cell carcinoma (Acharya et al., 2020; Boxberg et al., 2019; Caruntu et al., 2021; Jesinghaus et al., 2018, 2019; Joshi et al., 2020; Mohtasham et al., 2021; Shimizu et al., 2018; Yamakawa et al., 2019; H. Zhang et al., 2020). These “buds” are defined as small aggregates (groups of 5 or fewer cells) that detach from the main neoplasm, infiltrating into the surrounding tissue (Boxberg et al., 2019; Jesinghaus et al., 2018; Nagamine et al., 2017).

An interesting and novel veterinary grading system was developed by Nagamine et al. for canine oral and cutaneous squamous cell carcinoma which includes the evaluation of the IF (Nagamine et al., 2017). This new system may render promising results and opens the door to potentially base newer histological gradings on this feature. As a downfall, this grading system currently does not have any supporting, strong, prognostic evidence. However, it does show that oral squamous cell carcinoma, often associated with a poorer prognosis, has generally a higher grade than its cutaneous, generally more benign, counterpart (Nagamine et al., 2017).

In another line of investigation in veterinary medicine, several immunohistochemical strategies, such as survivin (Bongiovanni et al., 2009), cyclo-oxygenase-2 (de Almeida et al., 2001), proliferating cell nuclear antigen (PCNA) (Mestrinho, Faísca, et al., 2017), p63 (Mestrinho et al., 2015), E-Cadherin (Mestrinho et al., 2015), periostin (Mineshige et al., 2018), pS6 and p-mTOR (Delgado et al., 2022) have been attempted to try and establish a correlation

between malignancy of canine SCC in various locations. These include mainly the skin (Bongiovanni et al., 2009; de Almeida et al., 2001; Mineshige et al., 2018) and oral cavity (Mestrinho et al., 2015; Mestrinho, Faísca, et al., 2017; Nagamine et al., 2017). Yet, several studies are still pending to ensure a widely accepted panel of true prognostic value.

As seen, even if human and canine SCC have some similarities, there are clear, major disparities like underlying inciting causes (e.g. smoking causing human head and neck SCC (Even et al., 2021), unlikely in dogs), associated researched mutations, histological characterization and grading. This, as expected, impacts greatly the potential therapy and prognosis.

2.2. Canine cutaneous squamous cell carcinoma

2.2.1. Etiology, breed predisposition, age, sex distribution and pathogenesis

Squamous cell carcinoma in dogs, as already explained, is most commonly seen in the skin (dermal), digit and oral cavity. Each location has shown to have a slightly different prognosis and biological behavior (Fulton et al., 2013; Marconato et al., 2021; Willcox et al., 2019) (Table 1). Squamous cell carcinoma reaches its peak of incidence in dogs between 6 and 13 years of age. (Goldschmidt & Goldschmidt, 2017) with no sex predisposition (Goldschmidt & Goldschmidt, 2017; Mauldin & Peters-Kennedy, 2016).

***Table 1**. Publications of larger collections of canine squamous cell carcinoma at non-digital locations*

Reference	Location	Total number dogs included with SCC	Dogs with follow-up	Median Survival time	Metastasis
(Strafuss et al., 1976)	Skin	112	NA	Not determined	7
(Fulton et al., 2013)	Mouth	31	31	54 days untreated; 365 days if underwent surgery	9
(Willcox et al., 2019)	Skin	193	148	1004 days	27/123
(Sharma et al., 2021)	Mouth	25	25	Not reached	0
(Treggiari et al., 2023)	Tonsil	102	102	126 days (including dogs and other carcinomas NOS*)	Not disclosed specific to SCC

* Not otherwise specified

In dogs, cutaneous squamous cell carcinoma represents one of the most common dermal neoplasias. This high neoplastic incidence is closely related to its main risk factor, skin UV-damage due to prolonged sunlight exposure (Hargis & Thomassen, 1979; Madewell et al., 1981; Morrison, 2012; Nikula et al., 1992). As sunlight is considered as probably the most

important carcinogenic stimulus (Mauldin & Peters-Kennedy, 2016), SCC in dogs is often associated with the precancerous actinic keratosis and solar dermatosis (Hargis & Thomassen, 1979; Nikula et al., 1992; Willcox et al., 2019). For this very reason, in dogs, environmental factors such as latitude or altitude may play a role for the development of SCC. As it can be deduced, this tumor is more prevalent in tropical countries (D. Alves et al., 2022; Mauldin & Peters-Kennedy, 2016).

As expected with UV-damage, less pigmented regions, sparse hair or denuded areas like the belly (Hargis et al., 1977; Nikula et al., 1992) are at greater risk of sun-induced damage and, therefore, SCC development. As anticipated, outdoor animals or those with a light haircoat are prone to this tumor. This predisposition includes Dalmatians, boxers, bull terriers, beagles, and pointers, among others (Er & Sutton, 1989; Strafuss et al., 1976; Villamil et al., 2011; Willcox et al., 2019). Additionally, some studies have potentially linked the presence of papillomavirus (PV) and the development of this neoplasia (Alves et al., 2020; Chang et al., 2020; Luff et al., 2016). Other, less common factors that have been suggested include previous history of trauma or burns (Gourley et al., 1982).

2.2.2. Clinical picture, histopathological features

Clinically, cutaneous SCC appears as single or occasionally multiple lesions often involving the head, abdomen, limbs and perineum (Goldschmidt & Goldschmidt, 2017) (digital squamous cell carcinoma will be discussed separately). Its appearance may be diverse, ranging from a raised plaque to a centrally ulcerated mass (Goldschmidt et al., 2018). It is locally invasive, commonly slowly progressive, and low (up to 5%) metastatic rate at later stages of the disease, according to some studies in beagles (Hargis et al., 1977). Its metastatic rate is often directly related to its location and the possibility of complete excision (Goldschmidt & Goldschmidt, 2017; Willcox et al., 2019). Other additional features such as the presence or absence of actinic change, may significantly change the prognosis and survival time of cutaneous SCC. Dogs with this change had a longer survival time than those that did not have it (Willcox et al., 2019).

Currently, histopathology is considered the gold standard for the diagnosis of this cutaneous squamous cell carcinoma, often subdividing it according to their histological morphology, invasiveness and differentiation (Goldschmidt et al., 2018). Within the least invasive type, there is squamous cell carcinoma *in-situ.* This subtype, as its name suggests, is confined to the basement membrane, without dermal infiltration (yet) (Mauldin & Peters-Kennedy, 2016). The other, more malignant type is invasive squamous cell carcinoma. Unlike

its *in-situ* counterpart, the neoplasia extends into the underlying dermis, thus surpassing the epidermal basement membrane and infiltrating the surrounding stroma. Also, invasive SCC is further subdivided into several subtypes as described: well-differentiated/ conventional, poorly differentiated, acantholytic, spindle cell and verrucous variants (Mauldin & Peters-Kennedy, 2016). Other sources additionally describe other, less common, papillary and clear cell subtypes (Goldschmidt et al., 2018). Typically, conventional acantholytic and spindle cell variants are considered to have a markedly infiltrative biological behavior, thus making the complete excision often challenging (Goldschmidt et al., 2018).

2.3. Canine digital cell carcinoma

2.3.1. Etiology, breed predisposition, age, sex distribution and pathogenesis

When talking about digital neoplasia, squamous cell carcinoma is the most commonly diagnosed tumor, accounting for 47.4% to 63.5% of all malignancies in the dog at this location (Grassinger et al., 2021; Wobeser et al., 2007). It is most often seen in animals between 6 and 13 years of age, varying between studies, with no sex predisposition. (Goldschmidt & Goldschmidt, 2017; Grassinger et al., 2021; Wobeser et al., 2007). As a peculiar feature, is occasionally seen multicentrically, affecting several digits within the same animal (Aupperle-Lellbach et al., 2023 (1); Belluco et al., 2013; Chiu et al., 2022; Frese et al., 1983; Paradis et al., 1989; Wobeser et al., 2007). Chiu and colleagues, presently the largest cohort of DSCC available, established a risk of developing additional DSCC in giant Schnauzers (GS) and standard Poodles (SP) up to 56% within two years after first diagnosis (Chiu et al., 2022). Later, similar conclusions of increased risk in GS and standard Schnauzer (SS) were drawn by Aupperle-Lellbach and colleagues when assessing the risk of DSCC development in Schnauzers according to their size (Aupperle-Lellbach et al., 2023 (1)). Additionally, unlike cutaneous SCC, digital squamous cell carcinoma is not associated with UV-damage. Also, almost counter-intuitively, it is most often seen in dark-haired breeds such as Rottweiler, giant Schnauzer, standard Poodle and Dachshund, among others (Aupperle-Lellbach et al., 2023 (1); Belluco et al., 2013; Chiu et al., 2022; Grassinger et al., 2021; O'Brien et al., 1992). However, in some larger studies, certain breeds such as Newfoundland dogs, Bernese Mountain dogs and German shepherd dogs seem not to be predisposed to this neoplasia (Chiu et al., 2022). Contrary to some canine cutaneous SCC (C. D. B. T. Alves et al., 2020; Chang et al., 2020; Luff et al., 2016) or some human DSCC (Alam et al., 2003; Gormley et al., 2011) papillomavirus DNA has not been definitely identified consistently in canine DSCC (Munday, 2013).

Given the lack of histological UV-related damage in DSCC, the presence of occasional multiple digits recurrence, along with the black-haired and breed predilection (Belluco et al., 2013; Chiu et al., 2022; Karyadi et al., 2013; Paradis et al., 1989; Wobeser et al., 2007), an underlying genetic predisposition has been hypothesized (Belluco et al., 2013; Karyadi et al., 2013; Paradis et al., 1989). Karyadi and colleagues followed up on that hypothesis and have linked the presence of a copy number variant in the chromosome 15 in the region of KIT ligand (KITLG) locus with a higher risk of DSCC development (Karyadi et al., 2013). However, other genetic factors may play a role, suggesting possible multiple pathways that lead to final oncogenesis of DSCC (Karyadi et al., 2013).

2.3.2. Clinical picture, histopathological features and proposed gradings

Clinically, DSCC is seen as a mass or lesion arising from the distal portion of the distal phalange (P3), or the nailbed, with frequent loss of the associated claw and secondary infection (Goldschmidt & Goldschmidt, 2017). This may be grossly seen as digital swelling and lameness (Marconato et al., 2021), more commonly in the forelimbs (Aupperle-Lellbach et al., 2023 (2); Belluco et al., 2013; Chiu et al., 2022; Marconato et al., 2021). Given its location and proximity to the phalanges, it is common P3 invasion and osteolysis. Occasionally, it will further extend proximally, thus reaching the articular cartilage, joint, bursae, related tendons and more proximal phalanges (Goldschmidt et al., 2018), which in 80% of cases may show radiographical evidence of osteolysis (Marino et al., 1995). Of the affected animals, there is a recorded 5% to 25% metastatic prevalence to regional lymph nodes and distant organs (Table 2) (Aupperle-Lellbach et al., 2023 (2); Goldschmidt et al., 2018; Liu & Hohn, 1968; Marino et al., 1995; O'Brien et al., 1992; Wobeser et al., 2007). Therefore, DSCC may have a more aggressive, metastatic behavior than those developing in other regions (Wobeser et al., 2007). Additionally, local recurrence is more frequently seen in those animals with related P3 destruction or incomplete excision of the tumor (Goldschmidt et al., 2018). This is the reason that affected toe amputation is the most common treatment choice (O'Brien et al., 1992).

***Table 2**. Publications of larger collections of canine digital squamous cell carcinoma, adapted table (Chiu et al., 2022)*

Reference	Country	Total number dogs with DSCC	Dogs with follow-up	Postoperatively local recurrence	Initial or eventual involvement of additional digits number (%)	Metastasis (of animals with follow-up) Number (%)
(Aupperle-Lellbach, et al., 2023 (1))	Europe	478	55	NA	54 (11%)	10 (20%)
(Marconato et al., 2021)	Italy	79	79	3	18 (23%)	14 (18%)
(Belluco et al., 2013)	France	154	49	0	11 (22%)	4 (8%)
(Wobeser et al., 2007)	USA/Canada	109	42	1	7 (6%)	10 (24%)
(Henry et al., 2005)	USA	33	33	0	3 (9%)	0
(Marino et al., 1995)	USA	25	19	0	Unknown	1 (5%)
(O'Brien et al., 1992)	USA	29	21	0	0	0

NA; non-applicable

Currently, there is no widely accepted histological grading scheme used in veterinary medicine to characterize DSCC. The most popular is the already mentioned, somewhat outdated, Broder´s grading system (Schwab, 2011). However, correlation between this grading system and prognosis is not well established for DSCC.

2.4. Haircoat pigmentation and related genes

In order to understand the potential link between pigmentation and the development of DSCC, one must understand the mechanism behind haircoat pigmentation and distribution. The current canine population has great diversity, with different sizes, shapes, haircoat colors and coat texture. Surprisingly, the dog´s nuclear DNA coding sequence only differs 0.04% from its predecessor, the wolf (Saif et al., 2020). To achieve this diversity in color, the different types and degrees of pigmentation for skin, hair and/or other structures, are determined by the density/amount, type and distribution of melanin (Matamá et al., 2015; Saif et al., 2020). All different tones and colors in pigmented hair are determined by a combination of eumelanin and pheomelanin. The former, highly polymerized eumelanin, results in a black-to-brown pigmentation. On the other hand, pheomelanin is less polymerized, with a higher sulfur content, which results in a light yellow-to-reddish-brown (Matamá et al., 2015). This production of one or the other type of melanin, is known as melanogenesis. This process of follicular

melanogenesis, only happens in the hair bulb during the active growth phase (also known as anagen phase) (Matamá et al., 2015). Melanogenesis within the hair follicle´s melanocyte is achieved through an intercellular signaling pathway. This results in the production of both eumelanin and pheomelanin by the melanosome, a specialized organelle. These melanocytic pathways are additionally controlled by paracrine factors, which are secreted by neighboring keratinocytes (Saif et al., 2020).

Canine haircoat color is determined by pigment dilution genes (C (colored/chinchilla) Locus, B (brown) Locus, D (dilute) Locus, M (merle) Locus, I (intensity) Locus and G Locus)) and the Pigment Switching genes (A (agouti) Locus, E (extension) Locus and K (dominant black) Locus, in addition to T (ticking), S (spotting), P (pink-eye dilution) and R (roan) loci which often interact with each other for the resulting color. Some of the genes associated, such as for C locus and G locus are not completely elucidated yet (Brancalion et al., 2022; Saif et al., 2020). The different expressions of these genes result in the variable production of eumelanin and phaeomelanin. Similarly, the absence of pigment gives a white color to the coat (Bannasch et al., 2021; Saif et al., 2020; Weich et al., 2020).

In canine haircoat color, the modulation of the relative synthesis of eumelanin or pheomelanin through different pathways are controlled by three major genes: melanocortin receptor 1 (MC1R), agouti signalizing pathway (ASIP) and canine beta-defensin 103 (CBD103). These three genes cross-interact, causing different haircoat color, known as pigment-type switching (Brancalion et al., 2022).

We here focus on the Extension (E) locus which is controlled by the MC1R gene. The MC1R is a G-coupled receptor in melanocytes, which along with melanocyte stimulating hormone, regulates eumelanin production (Brancalion et al., 2022). In this orchestra of genes, KITLG is related to the E locus pigment intensity gene for pigment saturation (Brancalion et al., 2022). Its tyrosinase receptor c-kit has a critical role in the migration, proliferation and differentiation of melanoblasts during embryogenesis (Botchkareva et al., 2001).

2.4.1. KIT ligand (KITLG/SCF) and KITLG gene

KIT ligand (KITLG) gene, located in chromosome 15 in dogs (Schmutz et al., 2003), is a proto-oncogene that encodes KITLG (also known as stem cell factor (SCF)). This is a resulting protein, found in both membrane bound and soluble forms, which binds to the c-Kit receptor (*KITLG Protein Expression Summary - The Human Protein Atlas*, n.d.; *Reactome | Signaling by SCF-KIT*, n.d.). This KITLG protein is a 274 amino acid cytokine (*KITLG - Kit Ligand - Canis Lupus Familiaris (Dog) | UniProtKB | UniProt*, n.d.), involved in cell-to-cell

communication through small protein signaling. KITLG is expressed by various cells within the body, making it a low specific ligand. Some examples of cells expressing KITLG include stromal cells, such as those in the bone marrow, regulating hematopoiesis (Ostronoff et al., 2008; Shull et al., 1992; Young et al., 2021), endothelial cells during repair and regeneration (Chen et al., 2017), interstitial cells of Cajal (Hulzinga et al., 1995; Rich et al., 2003), mast cells (Amagai et al., 2015; Costa Casagrande et al., 2015), sertoli cells (Rothschild et al., 2003), and melanocytes (Picardo & Cardinali, 2011). This last group of cells express both KITLG and c-KIT receptor interacting for the regulation in melanogenesis and differentiation. KITLG is involved in several functions such as regulation, cell survival and proliferation, hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, as well as melanogenesis.

On the other hand, c-Kit (also known as KIT or CD117) is a tyrosinase-kinase receptor (RTK), encoded by KIT gene. Tyrosinase-kinase receptors (RTKs), account for more than 60 molecules classified into 20 families depending on their structure and ligand. Of this, c-KIT belongs to the type III receptor tyrosine kinase family (Liang et al., 2013). They are transmembrane, cell-surface proteins which function as signal transduction of a wide array of metabolic processes such as proliferation, apoptosis and cell differentiation. They allow communication through the phosphorylation of tyrosine residue of certain intracellular substrate proteins within the cytosolic side of the cell (Esteban-Villarrubia et al., 2020). Unlike KITLG, which is only able to bind specifically to its receptor c-KIT, c-KIT receptor can additionally bind to other ligands to initiate other signaling pathways.

The binding of SCF to transmembrane c-KIT causes de dimerization of the receptor and subsequent activation of its intrinsic tyrosinase activity (Rönnstrand, 2004). Specifically in the case of melanogenesis, the SCF-KIT receptor tyrosine kinase pathway is accomplished through MAPK-ERK signaling and the activation of the MITF transcription factor (D'Mello et al., 2016).

As already mentioned, KITLG is physiologically involved in postnatal cutaneous melanogenesis and follicular epithelial melanocyte terminal differentiation, making it significant in canine haircoat. KIT ligand (KITLG) (also known as SCF) has a direct influence on melanocyte proliferation and distribution, as well as melanin synthesis in mammals, with variable expression across species. It is proposed to influence pigment saturation by different distribution along the hair shaft (Brancalion et al., 2022; Weich et al., 2020). Although it is not the only E locus modifier, it does influence it. To illustrate this, in dogs pigment intensity is associated with a copy number variant upstream of KITLG (Bannasch et al., 2021). The copy number of the KITLG is significantly associated with eumelanin intensity (phenotypically seen as darker or more intense color) in the Poodle and across breeds, less so in pheomelanin

(Bannasch et al., 2021). Similarly, a higher KITLG expression has been seen in goats (S. Wu et al., 2021) and mink (Song et al., 2017) with a darker, more intense haircoat color. And as expected, a reduced expression is associated with lighter hair color in Europeans (Guenther et al., 2014; Miller et al., 2007) and pigmentation in stickleback fish (Miller et al., 2007), as well as deafness and hypopigmentation in Bama miniature pigs (Xu et al., 2020).

Related to pigmentary alterations, in human medicine, this KITLG and KITLG/c-Kit pathway has been associated with familial pigmentary disorders. This is either by an aberrant expression or dysfunction, causing progressive hyper- or hypopigmentation (Table 3) (Amyere et al., 2011; Gorenjak et al., 2021; Picardo & Cardinali, 2011). Additionally, given the wide distribution of KITLG, in human medicine there are a number of mutations of KITLG associated with a number of cancers, like testicular germ cell cancer (Kanetsky et al., 2009), thymoma (Yang et al., 2020) or colorectal cancer (Yang et al., 2014), among others.

Table 3. *Publications in human medicine associated to a KITLG mutation with a melanin-related disease*

Publication	Alteration on KITLG gene or protein expression	Disease/abnormality
(Amyere et al., 2011) (Wang et al., 2021) (Zhang et al., 2006)	Gene Mutation- substitution/missense	Familial progressive hyper- and hypopigmentation
(Ogawa et al., 2017)	Gene Mutation	Waardenburg syndrome type 2
(Vona et al., 2022)	Gene Mutation- substitution	Hypomelanosis and sensorineural hearing loss
(Richards et al., 2001) (Hamadah et al., 2019)	Gene Mutation	Piebaldism
(Hachiya et al., 2009)	Increased transcription and Protein overexpression in keratinocytes	UV-B melanosis
(Kang et al., 2006)	Protein overexpression in keratinocytes	Melasma
(Shishido et al., 2001)	Protein Overexpression in fibroblastic tumor cells	Epidermal hyperpigmentation in dermatofibroma

Currently, the molecular expression of c-KIT in veterinary medicine is often confined to canine mast cell tumors (Bowlt Blacklock et al., 2018; Costa Casagrande et al., 2015; Patruno et al., 2014; Preziosi et al., 2004; Webster et al., 2006), gastrointestinal tumors (Gregory-Bryson et al., 2010; Morini et al., 2022), and less frequently with melanoma (Chu et al., 2013; Newman et al., 2012; Tani et al., 2021). Similarly, KITLG is rarely investigated in veterinary medicine, with few studies in canine melanoma (Conrad et al., 2022) and digital squamous cell carcinomas (Karyadi et al., 2013). The latter remains particularly promising, given that DSCC

is known to develop most often in dark breeds (Karyadi et al., 2013) which, in turn, have the potential of a higher KITLG copy number (Bannasch et al., 2021).

3. AIMS OF STUDY

Currently, there is mounting literature describing the unique clinical and predisposing features of canine DSCC, its potential correlation of gene KITLG, to a darker haircoat and the presence of the tumor. This hypothesis made it imperative to explore its possible relationship.

Therefore, the overall aim of the studies carried out was to correlate the presence and histological features of DSCC based on a modified grading system. Later on, this was contrasted with the animals' haircoat color and KITLG gene copy number. This was to further explore a correlation between the genetic anomaly and tumor development.

In study 1, the following questions were addressed based on 94 canine DSCC:

- Can two preexisting grading schemes for squamous cell carcinoma be adapted for a digital location in dogs? Can these two grading schemes be challenged on a set-number of DSCC yielding comparable results in their final grade?

- Is the histological grade of DSCC associated with the dog´s phenotypical haircoat color? This is under the premise that animals with a dark haircoat are predisposed to the development of this neoplasia and that certain breeds, such as Schnauzers, have a more malignant behavior.

Study 2. Paired EDTA blood along with histological samples of DSCC from 70 dogs were evaluated to answer the following question:

- Is there a potential correlation between the histological grade (carried out on study 1) and copy number values in KITLG gene?

4. SUBSUMING THE PUBLISHED WORK AND CONTRIBUTION DESCRIPTION

In order to conduct the study, the material was initially selected from Laboklin GmbH & Co. KG histopathological archive within the Pathology department, as well pairing blood samples from the Clinical Laboratory Diagnostics department. The blood samples were then taken to the Genetics department (Labogen), where further genetic testing was performed,

My contribution to both studies carried out include the selection of cases, blinded histological evaluation and grading of those accepted. Additionally, I was responsible for the investigation, analysis and data curation of the final results. Afterwards, under the supervision of Mrs. PD Dr. Aupperle-Lellbach, I was the primary writer of the manuscript, with its subsequent review and editing.

Prof. Dr. Robert Klopfleisch from the Institute for Animal Pathology at Freie Universität Berlin was the director and expert advisor of both studies conducted. He was involved in the conceptualization of the study, as well as supervision of the final review prior to publication.

Mrs. PD Dr. Heike Aupperle-Lellbach from Laboklin GmbH & Co. KG was the primary responsible in the planning of the studies and closely supervised them both. She was in charge of the studies´ conceptualization, validation, supervision and project administration. Additionally, she was responsible for the funding acquired for the development of this project.

Alexandra Kehl from Laboklin GmbH & Co. KG was responsible for carrying out and validate the genetic analysis included in the study. Both her and Dr. Cristoph Beitzinger from Laboklin GmbH & Co. KG were involved in the publication editing and expert advice concerning to the genetic analysis.

Julia M. Grassinger from Laboklin GmbH & Co. KG and Dr. Tobias Müller Institut für Bioinformatik, Universität Würzburg aided for the statistical analysis of study 1 and 2, respectively.

4.1. Study 1: Evaluating the Histologic Grade of Digital Squamous Cell Carcinomas in Dogs with Dark and Light Haircoat — A Comparative Study of the Invasive Front and Tumor Cell Budding Systems

CEREZO-ECHEVARRIA A, GRASSINGER J M, BEITZINGER C, KLOPFLEISCH R, AUPPERLE-LELLBACH H (2020). Evaluating the Histologic Grade of Digital Squamous Cell Carcinomas in Dogs with Dark and Light Haircoat—A Comparative Study of the Invasive Front and Tumor Cell Budding Systems. *Veterinary Sciences*, *8*(1), 3.

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Evaluating the Histologic Grade of Digital Squamous Cell Carcinomas in Dogs with Dark and Light Haircoat—A Comparative Study of the Invasive Front and Tumor Cell Budding Systems

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Simple Summary: This study compares two different adapted grading systems for Canine digital squamous cells carcinomas, taking into account the animals' haircoat color and focusing on the tumor's invasive front. In general, dark-haired breeds develop more poorly differentiated DSCC than their light-haired counterparts. Additionally, both grading systems challenged are in agreement when grading well differentiated CDSCC in both populations but are discordant when assessing tumors with poorly differentiated features. To our knowledge, this is the first study comparing CDSCC in dogs by two histological grading systems, taking into account their phenotypical and presumed genotypical haircoat color and demonstrating that digital squamous carcinomas are not only more common in dark-haired dogs, but potentially more aggressive.

Abstract: Canine digital squamous cell carcinomas (CDSCC) are particularly aggressive when compared to their occurrence in other locations. Although these neoplasms are more frequently seen in dark-haired dogs, such as Giant Schnauzers, there are no data checking whether these tumors are histologically different between breeds. We histologically evaluated DSCC from 94 dogs. These were divided into two groups, namely, (1) dark-haired (N = 76) and (2) light-haired breeds (N = 18), further subdividing Group 1 into three subgroups, (1a) black breeds (n = 11), (1b) Schnauzers (n = 34) and (1c) black & tan breeds (n = 31). Adaptations from two different squamous cell carcinomas grading schemes from human and veterinary literature were used. Both systems showed significant differences when compared to Groups 1 and 2 in terms of final grade, invasive front keratinization, degree of invasion, nuclear pleomorphism, tumor cell budding, smallest tumor nest size and amount of tumor stroma. Group 2 was consistently better differentiated CDSCC than Group 1. However, there were no significant differences among the dark-haired breeds in any of the features evaluated. This study represents the first attempt to grade CDSCC while taking into account both phenotypical and presumptive genotypical haircoat color. In conclusion, CDSCC are not only more common in dark-haired dogs, they are also histologically more aggressive.

Keywords: digital squamous cell carcinoma; canine; cancer; tumor budding; digital; toe; squamous cell carcinoma; grading; haircoat color; genotype

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1. Introduction

Squamous cell carcinoma (SCC) is a fairly common, locally invasive and destructive neoplasm arising from the epithelium with keratinocyte differentiation. This neoplasia is known to metastasize in late stages of the disease, being variably prone to it depending

on its anatomical location [1,2]. Some of them, especially those arising from the nail bed or digits in dogs, are known to be particularly aggressive [1]. A number of factors are associated with the development of these tumors in veterinary species, including papilloma-induced neoplasms and chronic ultraviolet (UV)-damage, especially in poorly haired, light skin [1]. In proposed nondigital, UV-induced SCC, precancerous, actinic changes are often reported within the neighboring tissue [1,3]. This type of SCC is associated with slower progression and overall longer survival [3].

Curiously enough, canine subungual/digital squamous cell carcinomas (CDSCC) are most commonly seen in dark, large breeds such as Giant Schnauzers, black Labrador Retrievers and standard Poodles [1,4]. Papilloma virus was thought to be associated with CDSCC, but was not demonstrated by PCR positivity in affected digits [5].

Squamous cell carcinoma gradings are more widely explored in human medicine, conducting different grading schemes based on their location, such as the esophagus [6], uterine cervix [7], lung [8], larynx and hypopharynx [9], among others [10]. In veterinary medicine, a standardized grading system is not well characterized given its unclear prognostic value to date [11]. This is mainly because, in the toe, complete digit amputation is considered the only treatment option and, often curative [12]. Currently, the most used grading system is Broder's system [13] in which canine SCC is characterized as "well differentiated/I", "moderately differentiated/II and III" and "poorly differentiated/IV". The grading is based on its general morphologic features and its resemblance to normal squamous epithelium [14]. This, however, often ends up being the pathologist's subjective assessment (especially for tumors of grade II and III) and, with no proven prognostic correlation, discourages SCC subtyping in a diagnostic setting. Also, newer canine SCC gradings often focus on the oral cavity due to its more malignant behavior [15].

Recently, more research was conducted on the tumor invasive front and epithelial-mesenchymal transition, both in human [7,9,15,16] and veterinary medicine [2]. These features are associated with the pattern of invasion and, therefore, malignancy. The invasive front, as its name infers, is the tumor–host interface, in which neoplastic cells invade the surrounding stroma, spreading and infiltrating. The reason for studying invasive fronts in SCC is that within the same tumor, different grades of differentiation can be found but the invasive front consistently has more malignant features [2,11]. This suggests that it may be imperative for neoplastic infiltration and expansion. Features that are associated with more malignant behavior include tumor budding, which are small aggregates (less than five cells) or single tumor cells that detach from the primary tumor and invade into the surrounding stroma [2,7,9,10,14–16].

While CDSCC is the most common neoplasia in the canine digit (up to 47.4% of all malignant digital tumors) [17], there is not much literature available [11]. Canine squamous cell carcinoma, particularly that developing in dark-haired breeds, garnered special interest, suggesting an underlying genetic predisposition [11]. Different canine haircoat colors and distribution are due to eumelanin (dark) and pheomelanin (light) pigments, which are also responsible for the claw coloration. Dark-haired animals, such as Giant Schnauzers, black Labrador Retrievers and Poodles, normally have concurrent dark claws. However, light-haired dogs with recessive genotype e/e on the E-locus do not incorporate eumelanine into their hair or claws, hence the light appearance [18]. This presumably important gene locus is homozygous recessive (e/e) for some breeds (e.g., Golden Retriever), while completely absent and therefore homozygous wildtype (E/E) in others (e.g., black Russian Terrier). Interestingly enough, Poodles, Labrador Retrievers and some Schnauzer variants have individuals with either homozygous states (E/E or e/e) in their breed.

The KIT ligand (KITLG) locus, associated with postnatal cutaneous melanogenesis and follicular epithelial melanocyte terminal differentiation (among other functions), was shown to play a significant role in canine haircoat pigmentation [19]. One study identified a copy number variant at the KITLG locus in animals with CDSCC, which may predispose them to develop this neoplasia [20].

The objective of this study was (1) to compare two adapted grading schemes from both human and veterinary medicine for canine digital squamous cell carcinoma, and (2) to evaluate if there are significant characteristic disparities between light and dark coated dogs, based on the grading schemes discussed and taking into account their phenotypical haircoat color.

2. Materials and Methods

2.1. Samples

Out of the 2983 toes submitted between 2014 and 2019 for routine diagnostics to the pathology department of LABOKLIN GmbH and Co. KG, 53% (n = 1576) of them contained a tumor, of which 49% (n = 771) were CDSCC. Out of these, 39% were found in Schnauzer breeds and only 2.5% in Golden Retriever (unpublished data). Given that these samples were from dogs subjected to regular routine diagnostics and not sampled for pure research purposes, an Ethical Committee approval was not necessary before undertaking the research.

Histological samples of CDSCC from 94 dogs with a clear neoplastic invasive front, available breed and haircoat color were included in this study. All CDSCC from animals of unclear haircoat color, unknown breed or only including neoplastic fragments with no clear invasive front were excluded.

Dog grouping for this study followed the main color of their fur and claws (light/dark), as well as the presumed underlying genetics for the color loci A, K and E, which are most important in the distribution of light and dark pigment in hairs and claws.

In Group 1, the phenotypically "dark-haired breeds", composed of 76 dogs, was further divided into three subgroups: Group 1a (n = 11) was made by presumed genetically entirely black breeds (presumed KB/KB) including seven Russian Terriers, two black Briard, one black Giant Poodle, and one black Labrador Retriever. Group 1b (n = 34) (presumed KB/KB and KB/KY) were represented by 27 Giant Schnauzers and seven black standard Schnauzers. Group 1c (n = 31) consisted of genetically black & tan (presumed KY/KY + at/*) breeds, represented by 22 Rottweilers and 10 Gordon Setters. Group 2 (n = 18) were the light-colored breeds (presumed e/e) including 15 Golden Retrievers and three West Highland White Terriers. No genetic testing was performed to corroborate the presumed genotype in any of the groups.

The ages of the dogs ranged from 6 to 14 years, with a median of 10 years, as one animal's age was unknown. Sex was either unknown (n = 7), female intact (n = 18), female spayed (n = 18), male (n = 34) and male castrated (n = 18). Limb and toe affected, when available, was noted. Signalment is summarized in Table 1 and individual cases with more detailed information can be seen in Supplementary Table S1.

All digital samples were fixed in 10% phosphate-buffered formalin, routinely trimmed following laboratory standard procedures and decalcified on a mixture of ≥10–<20% hydrochloric acid (HCl) and formaldehyde (≥3%–<5%) (Osteomoll® rapid decalcifier solution for histology; catalogue no. 101736) over a period of 24–72 h, periodically assessing tissue until it was ready to be further processed. Afterward, longitudinal and sagittal sections were embedded in paraffin wax and cut at 4–5 µm thickness to then be stained with Hematoxylin–Eosin (HE). All slides were reviewed, selecting the most representative section. This was based on a good histological quality and clear invasive front with surrounding nonaffected stroma to evaluate the neoplastic–nonneoplastic transition. The most representative slide was scanned and analyzed through specialized image analysis software (NIS-elements software (Nikon, Tokyo, Japan); Aperio ImageScope (Leica, Wetzlar, Germany)).

Table 1. Dog signalment and affected region with digital squamous cell carcinoma (DSCC) in the present study.

Group Assignment	Phenotypic Haircoat Color	Breed	No. of Dogs	Mean Age (y.o.)	Sex					Number of Dogs with Affected Limb RF/RH/LF/LH/U
					M	MC	F	FS	U	
Group 1a (*n* = 11) No. 1–11	Black	Russian Terrier	7	9.5	2	0	1	2	2	1/0/3/0/3
		Briard	2		0	1	1	0	0	0/0/2/0/0
		Giant Poodle	1		0	1	0	0	0	0/0/1/0/0
		Labrador Retriever	1		0	1	0	0	0	0/0/1/0/0
Group 1b (*n* = 34) No. 12–45		Giant Schnauzer	27	8.5	10	8	4	5	0	9/1/10/2/5
		Standard Schnauzer	7		3	1	1	2	0	0/3/2/2/0
Group 1c (*n* = 31) No. 46–76	black & tan	Rottweiler	21	9.6	5	2	5	7	2	7/1/0/2/6
		Gordon Setter	10		6	1	2	1	0	3/2/0/0/5
Group 2 (*n* = 18) No. 77–94	Light	Golden Retriever	15	10.5	8	1	3	0	3	4/1/4/2/4
		WHWT	3		2	0	1	0	0	0/0/2/0/1

Abbreviations: y.o.: years old; M: male intact; MC: male castrated; F: female intact; FS: female spayed; U: unknown; RF: right forelimb; RH: right hindlimb; LF: left forelimb; LH: left hindlimb; U: unknown; WHWT: West Highland White Terrier.

2.2. Histological Grading

Two grading systems from human (Jesinghaus et al., (2018) [7] and Boxberg et al., (2019) [9]) and veterinary (Nagamine et al., (2017) [2]) medicine were adapted for the present study. The samples were assessed by a blinded diplomat of the American College of Veterinary Pathologists (ACVP) (AC), challenging the adapted systems.

2.2.1. Invasive front Grading System (IFGS)

An adaptation of Nagamine et al. (2017) grading system for canine oral SCC (OSCC) was used [2], following criteria of degree of keratinization, pattern of invasion, host response, nuclear pleomorphism and mitoses per high power field (HPF), as summarized in Table 2. All features were assessed, focusing exclusively on the invasive front.

Table 2. Invasive front grading system (IFGS) used in the present study of canine digital squamous cell carcinoma (CDSCC) (adapted from Nagamine et al., (2017) [2] for use in canine oral squamous cell carcinoma).

Morphological Feature	Score Value			
	1	2	3	4
Degree of keratinization	Highly keratinized (>50% cells)	Moderately keratinized (20–50% of cells)	Minimal keratinization (5–20% of cells)	No keratinization (0–5% of cells)
Pattern of invasion (bone or dermis)	Pushing, well-differentiated, infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups/cords of infiltrating cells ($n > 15$)	Widespread cellular dissociation in small groups and/or in single cells ($n < 15$)
Host response	Marked	Moderate	Slight	None
Nuclear pleomorphism	Mild (<25% anaplasia)	Moderate (25–50% anaplasia)	Marked (50–75% anaplasia)	Extreme (75–100% anaplasia)
Mitosis HPF (40×)	0–1	2–3	4–5	>5

Histological gradings are illustrated in Figures 1–5. Degree of keratinization (Figure 1a,b) was assessed from highly keratinized (>50% keratinization, 1 point) to no keratinization (0–5% keratinization, 4 points). Pattern of invasion (Figure 2a,b) ranged from well-differentiated, pushing and infiltrating borders (1 point) up to widespread dissociation into small groups, less than 15 cells (4 points). Host response (Figure 3a,b) was evaluated from a marked inflammatory reaction (1 point), to no inflammation (4 points). Nuclear pleomorphism (Figure 4a,b) was assessed, ranging from little pleomorphism with less than 25% anaplastic cells (1 point) up to extreme nuclear pleomorphism with poor differentiation (75–100% anaplastic cells) (4 points). Mitosis per high power field (HPF) (40×) (Figure 5a,b) ranged from minimal mitotic activity (0–1) (1 point) up to more than 5 mitoses (4 points). Mitosis per HPF was assessed in an overall area of 0.237 mm^2. Therefore, when assessing 10 HPF (400×), an overall area of 2.37 mm^2 was covered to guarantee standardization [21]. This procedure was decided for this study as challenged gradings often did not clarify the area covered.

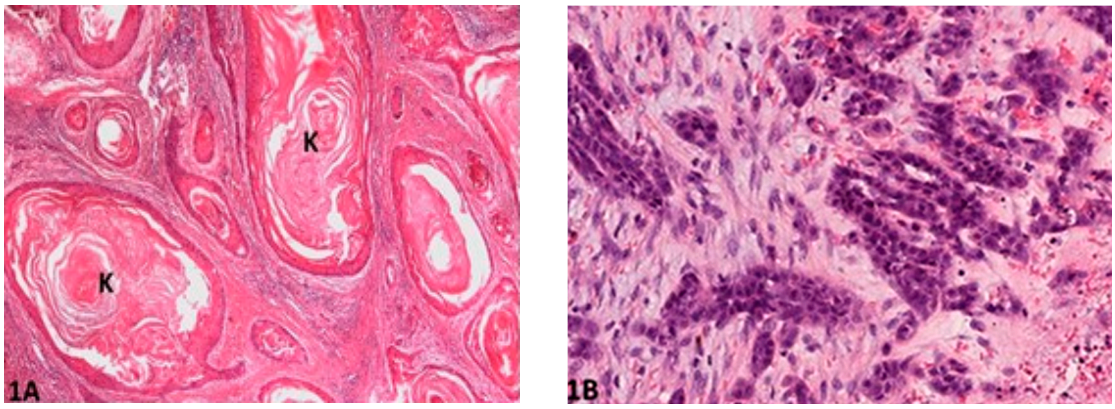

Figure 1. Degree of keratinization: (**A**) (O.M. 2×): Well-differentiated and highly keratinized cells (K) with over 50% of keratinization (Gordon Setter, No. 49); (**B**) (O.M. 10×): Less than 5% cells exhibiting keratinization (Gordon Setter, No. 55). O.M: original magnification.

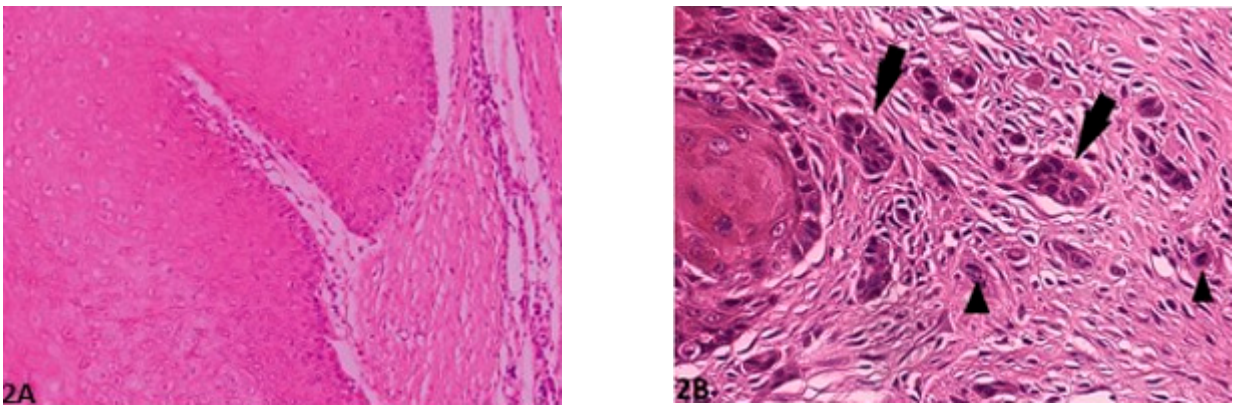

Figure 2. Pattern of invasion: (**A**) (O.M. 8×): Well-differentiated, expansively growing tumor borders compressing the surrounding stroma (Golden Retriever, No. 87); (**B**) (O.M. 20×): Wide-spread cellular dissociation in small groups (<15 cells, arrow) and/or single cells (arrow heads, medium Schnauzer, No. 45). O.M: original magnification.

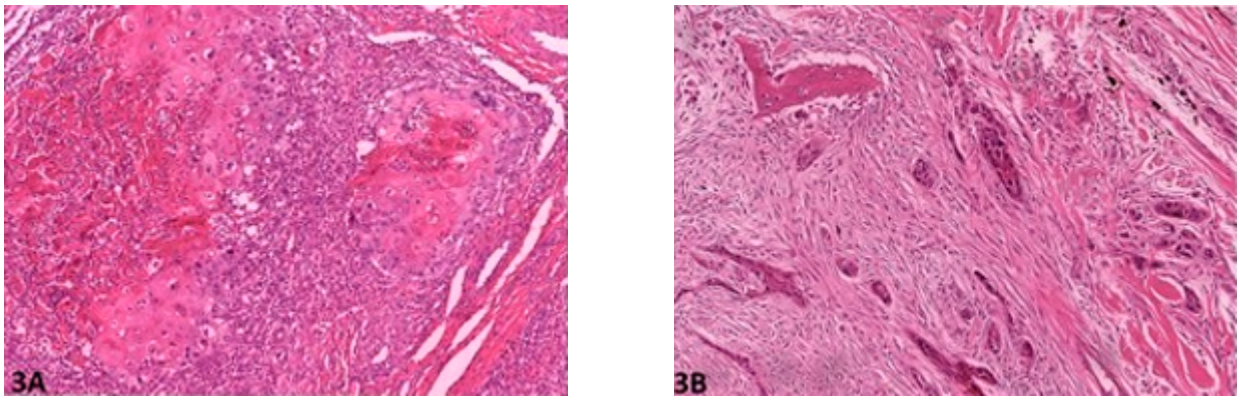

Figure 3. Host response: (**A**) (O.M. 8×): Marked inflammation, occasionally obliterating the neoplastic cells (Russian Terrier, No. 6); (**B**) (O.M. 8×): Minimal to virtually no associated inflammation (Rottweiler, No. 70). O.M: original magnification.

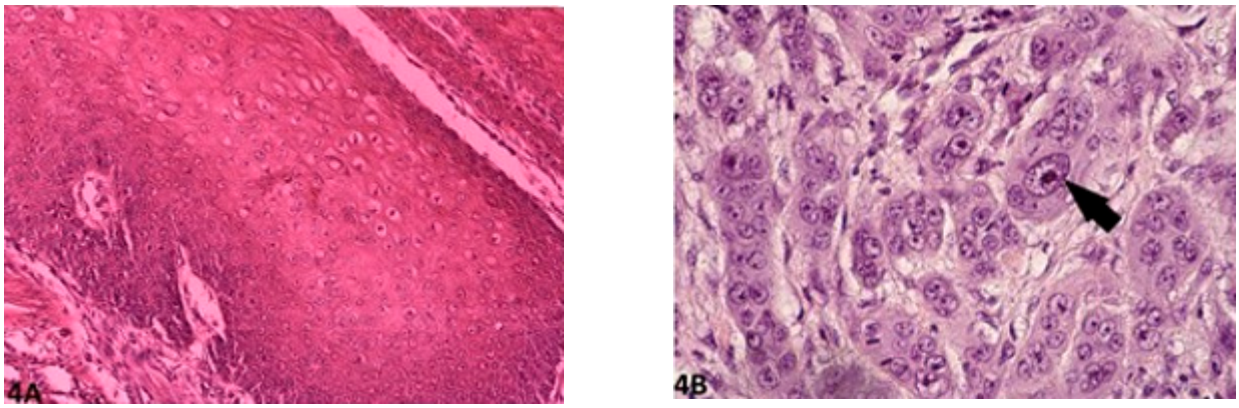

Figure 4. Nuclear pleomorphism: (**A**) (O.M. 8×): Mild/minimal pleomorphism in <25% neoplastic cells (Golden Retriever, No. 87); (**B**) (O.M. 40×): Extreme nuclear pleomorphism with intense nuclear atypia (arrow), accounting for more than 75% of neoplastic cells (Giant Schnauzer, No. 56). O.M: original magnification.

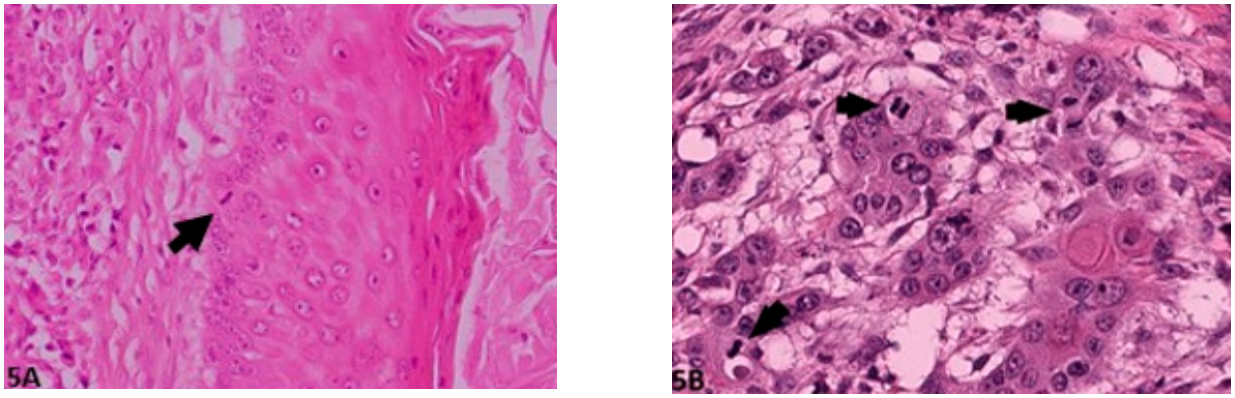

Figure 5. Mitosis per HPF: (**A**) (O.M. 40×): Single mitotic figure (arrow, Gordon Setter, No. 50); (**B**) (O.M. 40 x): Multiple mitotic figures (arrows, Gordon Setter, No. 56). O.M: original magnification.

The final addition of the score values of these five morphologic features resulted in a total invasive front score value. Subsequently, the total score value was summarized into four final grades according to Nagamine et al. (2017) [2]:

Total score value 6–10 = grade I (well differentiated);
Total score value 11–15 = grade II (moderately differentiated);
Total score value 16–20 = grade III (poorly differentiated).

2.2.2. Tumor Cell Budding Grading System (TCBGS)

An adaptation from two similar human SCC gradings systems of Jesinghaus et al. (2018) [7] and Boxberg et al. (2019) [9], designed for the uterine cervix and larynx/hypopharynx, respectively, was used. Both systems focus on the invasive front. The features evaluated within this adapted grading included tumor budding in 10 HPF (unspecified covered area), smallest nest size within the invasive front and stromal response associated with the neoplasm (see Figure 6).

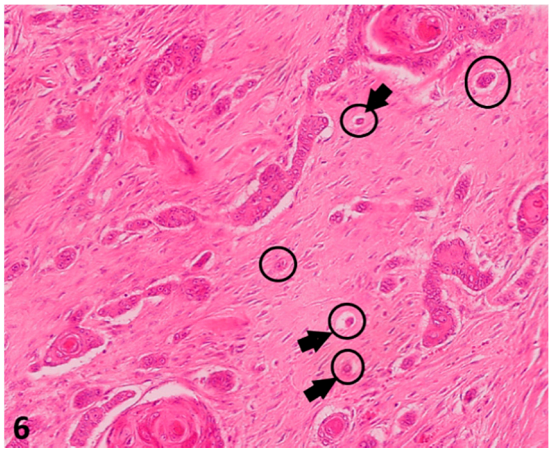

Figure 6. (O.M. 4×): Histological pictures (Hematoxylin–Eosin (HE) stain) illustrating tumor budding in a canine digital squamous cell carcinoma: Only complexes of less than five cells were counted in 10 HPF (40×) in the area of biggest incidence (delineation) within the invasive front. O.M: original magnification.

The smallest nest size was represented by the complex with least cells within that invasive front (arrows), even if it was only one. In this case, there were single neoplastic cells dissociated from main neoplasm (Giant Schnauzer, No. 33).

In this study, the tumor budding was defined as neoplastic aggregates/complexes of less than five cells that dissociate from the main neoplasm and invade the surrounding stroma. These "complexes" were counted in 10 HPF (40×) in the areas of higher incidence, covering an overall area of 2.37 mm^2. A numerical value between 1 (no tumor budding) and 3 (≥15 budding foci) was then assigned. Tumor nests, in contrast, included both these smaller (<5 cells) complexes as well as larger aggregates (up to >15 cells) dissociating from the main neoplasm, invading the surrounding stroma. When assessing smallest tumor nest size, a range between more than 15 cells (1 point) and single cell invasion (4 points) was noted (Table 3).

Table 3. Tumor cell budding system used in our study to determine tumor grade based on tumor budding activity and cell next size score adapted from human cervical squamous cell carcinoma (SCC) (Jesinghaus et al., 2018) [7] and laryngeal/hypopharyngeal SCC (Boxberg et al., 2019) [8].

Tumor Budding Activity/10 HPF	**Score Value**
No budding	1
<15 budding foci	2
≥15 budding foci	3
Smallest cell nest size	
>15 cells	1
5–15 cells	2
2–4 cells	3
Single cell invasion	4

In this grading system, the two scores were added to the total score value. This total score value divided the neoplasms into well differentiated/grade 1 (total score value: 2–3), moderately differentiated/grade 2 (total score value: 4–5) and poorly differentiated/grade 3 (total score value: 6–7) DCSSC. Additionally, stromal reaction was evaluated, although it was not included into the total score value or final grade.

2.3. Statistical Analysis

Statistical significance analyses were evaluated using IBM SPSS Statistics (version 25). Comparisons between the four genetically based groups (1a–c and 2) were performed with the Kruskal–Wallis test, while the statistical significance between the black and white dogs were analyzed using the Mann–Whitney U test. In the case of the Kruskal–Wallis test, the p-values were adjusted according to Bonferroni. $p < 0.05$ was considered statistically significant.

3. Results

Age and sex distribution across phenotypic groups (1a, 1b, 1c and 2) were not significantly different. Similarly, there was no statistical difference between digital tumor localization between limbs, toes and phenotypic group or the presence/absence of neoplastic bone invasion. Nevertheless, the forelimb was the main affected limb, representing 76% of the white-haired dogs and 79% of the dark-haired dogs, where localization was available.

3.1. Invasive front Grading System (IFGS)

Grade I CDSCCs were characterized by well-differentiated, solid neoplastic cords that pushed and infiltrated the surrounding stroma, with abundant keratinization, little anaplasia and barely any mitotic activity, but marked associated inflammation.

Grade II and grade III, on the other hand, had increasingly poorer differentiation, with multiple small buds that detached from the main neoplasm, with barely to no keratinization, moderate to marked anaplasia and increased mitotic activity but little to no associated inflammation from the host.

According to the IF grading system, 45% of DSSC of the dark-haired animals (Group 1) were grade I, 37.6% were grade II and 20.8% were grade III. In comparison, over three-quarters of light-haired dogs (Group 2) were grade I (77.7%), while the remaining were divided into grade II (16.6%) and grade III (5.5%) (Figure 7A).

Statistical analysis confirmed that the DSSCs of light-haired dogs (Group 2) were better differentiated, with lower overall grading than the dark-haired dogs (Group 1) ($p < 0.01$, Figure 7A). Therefore, the dark-haired dogs showed significantly less keratinization ($p < 0.05$, Figure 7B), more invasive patterns ($p < 0.001$, Figure 7C) and more marked nuclear pleomorphism ($p < 0.001$, Figure 7D) than their light-haired counterparts.

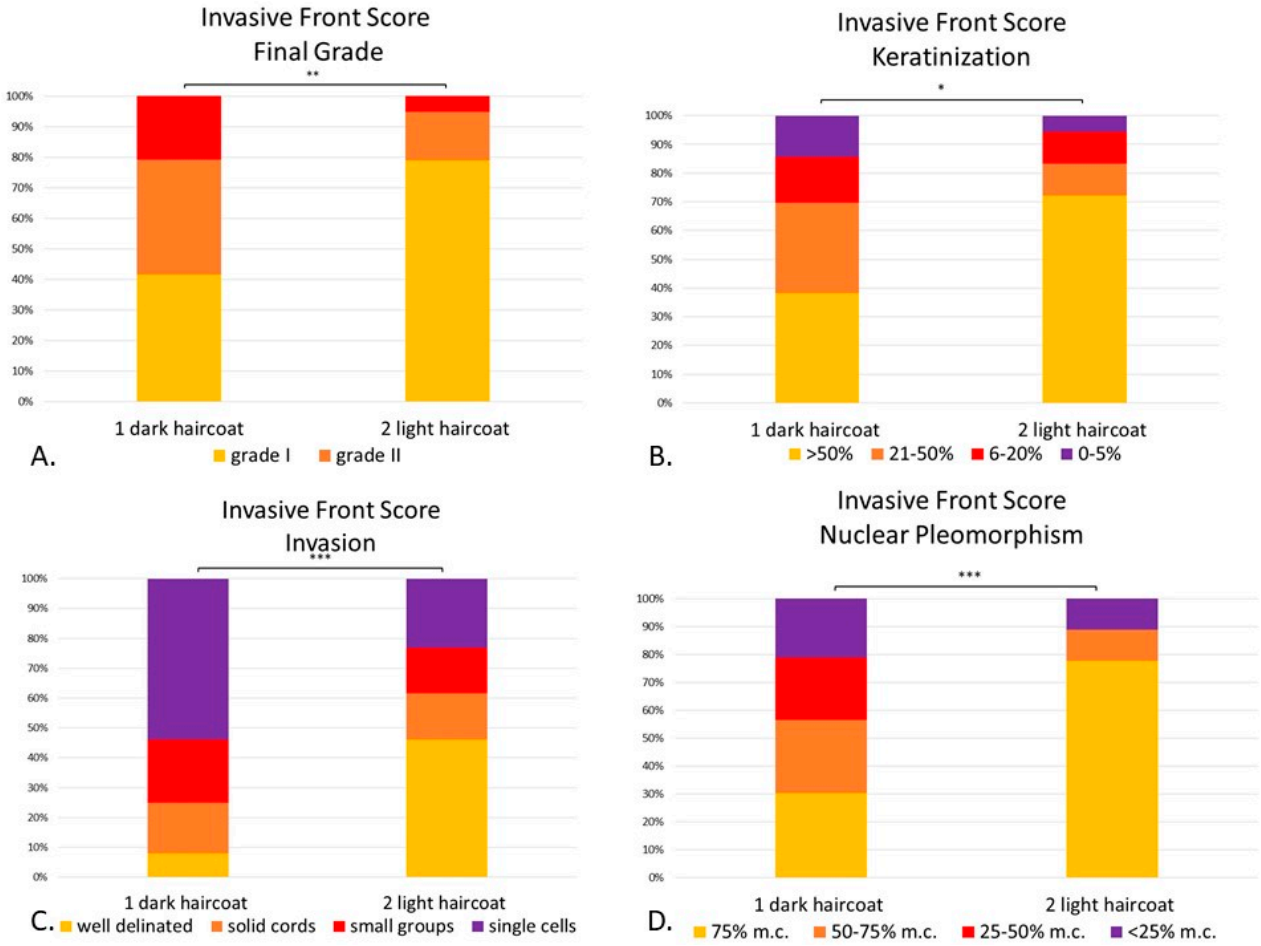

Figure 7. (**A**–**D**). Canine digital squamous cell carcinoma. Statistical differences between phenotypically dark-haired (Group 1) and light-haired (Group 2) dogs according to the invasive front system. There were statistical differences between Group 1 and 2 in final invasive front grading ($p < 0.01$) (**A**), amount of keratinization within the invasive front ($p < 0.05$) (**B**), pattern of invasion ($p < 0.001$) (**C**) and degree of nuclear pleomorphism ($p < 0.001$) (**D**). Mitoses and inflammation were not included given that they were not statistically significant between groups. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. Abbreviations: m.c.: mature cells.

When comparing the groups by their genetic haircoat color (see Figure 8), in all statistically significant features (invasive front grading, keratinization, pattern of invasion and nuclear pleomorphism), it was remarkable that the light-haired animals had less malignant characteristics than at least one dark-haired subgroup. Final invasive front grading between light-haired breeds (Group 2) and black & tan dogs (Group 1c), was significantly lower ($p < 0.05$) (Figure 8A). Light-haired dogs had significant more keratinization than black dogs (Group 1a, $p < 0.05$) (Figure 8B). When assessing patterns of invasion, light-haired dogs showed more solid patterns of invasion within the invasive front than black dogs (Group 1a, $p < 0.05$), Schnauzers (Group 1b, $p < 0.01$) and tan and black dogs (Group 1c, $p < 0.05$) (Figure 8C). As far as nuclear pleomorphism within the cells forming the invasive front, the light-haired dogs had a less anaplastic population, with more mature cells than the black dogs (Group 1a, $p < 0.05$) and the Schnauzers (Group 1b, $p < 0.05$) (Figure 8D). However, host response and mitotic activity between groups were not significantly different.

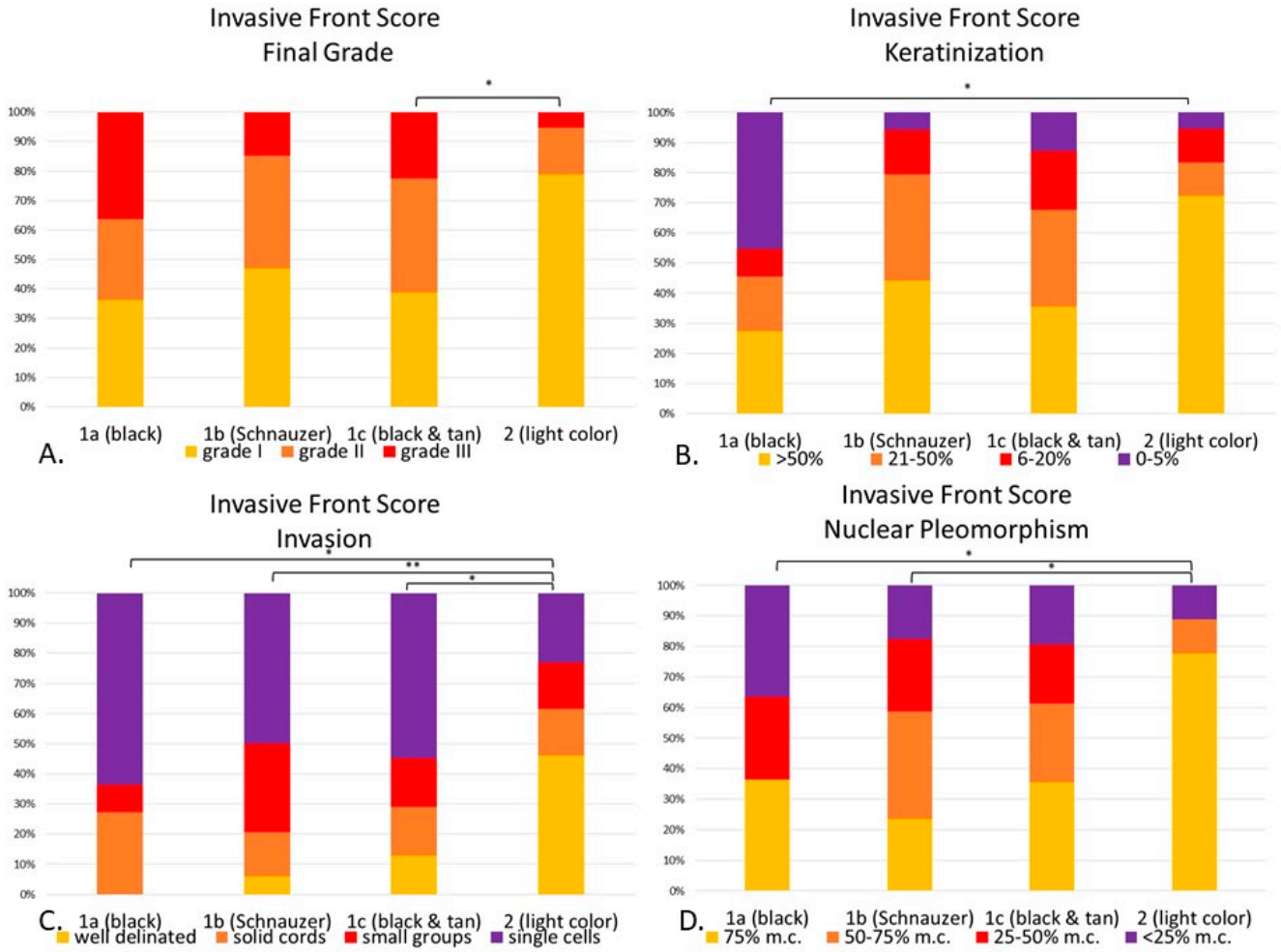

Figure 8. (**A**–**D**). Canine digital squamous cell carcinoma. Statistical differences between genetically defined subgroups (1a–c) and Group 2 according to the invasive front grading system. Invasive front final grade ($p < 0.05$), with statistical differences between Group 1c and 2 (**A**). Keratinization ($p < 0.05$) with differences between 1a and 2 ($p < 0.05$) (**B**). Invasion ($p < 0.01$) with significant differences between 1a ($p < 0.05$), 1b ($p < 0.01$) and 1c ($p < 0.05$) when compared to 2. (**C**). Nuclear pleomorphism ($p < 0.05$) with significant differences between 1a ($p < 0.05$) and 1c ($p < 0.05$) when compared with Group 2. (**D**). * $p < 0.05$; ** $p < 0.01$. Abbreviations: m.c.: mature cells.

3.2. Tumor Cell Budding Grading System (TCBGS)

Grade 1 CDSCCs in this system were well delineated, with more or less keratinization, either forming solid cords or large groups which detached from the main neoplasm and infiltrated through the invasive front, while exhibiting little associated tumor stroma (scirrhous reaction). On the other hand, grade 2 and 3 CDSCCs were less cohesive, forming small neoplastic aggregates or even individual cells detaching from the main neoplasm and invading the surrounding tissue, which presented a moderate to marked tumor stroma.

Within the TCB grading system, out of the 76 phenotypical dark-haired animals (Group 1); 45.5% were grade 1; 10.3% were grade 2 and 44.2% were grade 3. Out of the 18 dogs with phenotypical light haircoat (Group 2), the vast majority (88.9%) were grade 1 and 11.1% were grade 3 (Figure 9A).

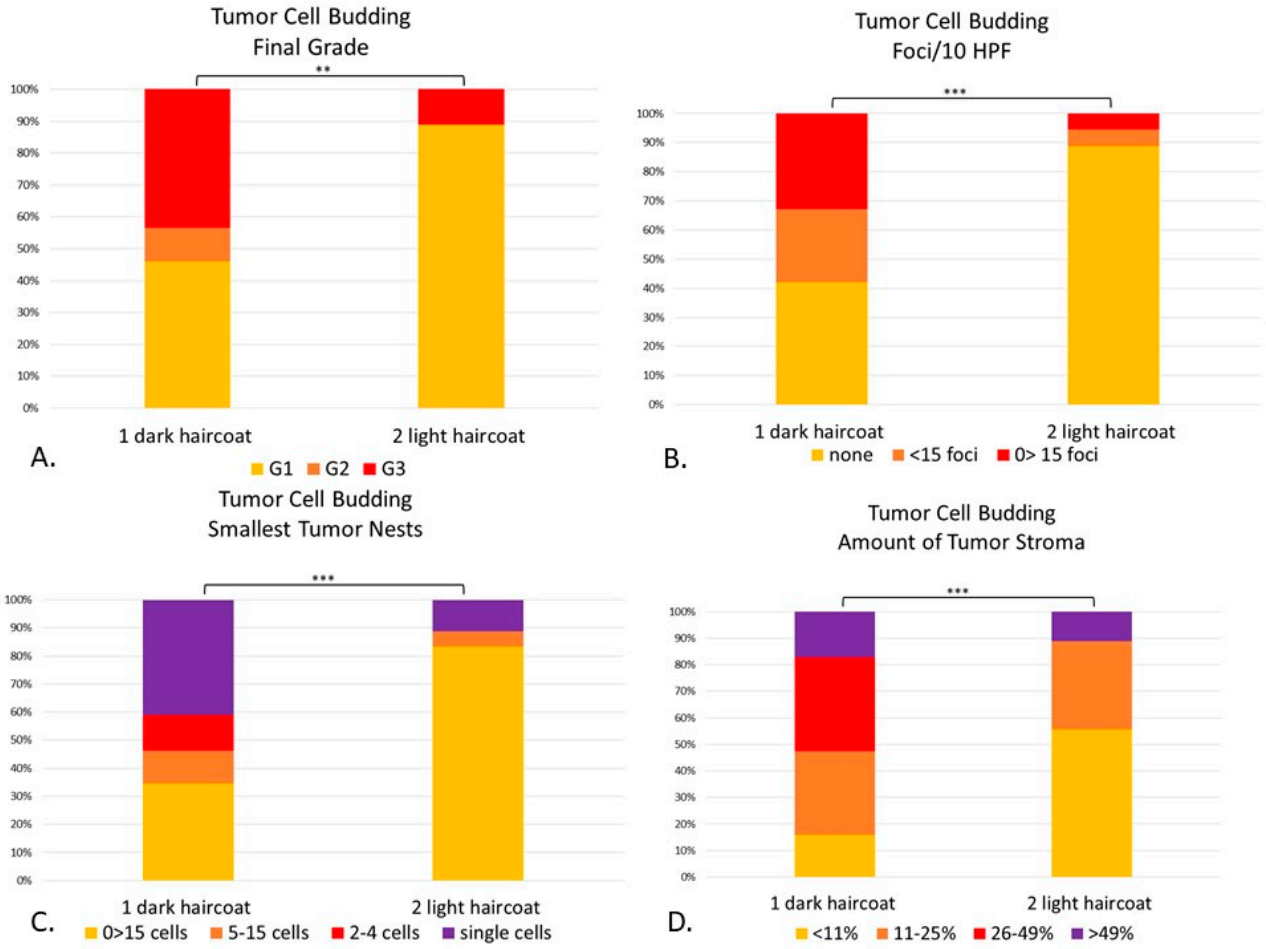

Figure 9. (**A**–**D**). Canine digital squamous cell carcinoma. Statistical differences between phenotypically dark-haired (Group 1) and light-haired (Group 2) dogs according to the tumor cell budding system. There were statistical differences between Groups 1 and 2 in final grade ($p < 0.01$) (**A**), tumor cell budding in 10 HPF ($p < 0.001$) (**B**), smallest tumor nest size ($p < 0.001$) (**C**) and amount of tumor stroma ($p < 0.001$) (**D**). ** $p < 0.01$; *** $p < 0.001$.

There were statistical differences between Groups 1 and 2 compared to the TCB total score ($p = 0.001$) and final grade ($p < 0.01$) (Figure 9). The light-haired dogs (Group 2) had significantly less tumor cell budding in 10 HPF ($p < 0.001$) than dark dogs (Group 1), with fewer buds detaching from the main neoplasia (Figure 9B). When comparing the smallest tumor nest, light-haired dogs had significantly larger nests than their dark counterparts ($p < 0.001$) (Figure 9C). Finally, light-haired animals had significantly less amount of stroma than dark dogs ($p < 0.001$) (Figure 9D).

When comparing each individual morphological feature with its phenotypic haircoat color, depending on the feature evaluated (Figure 10), there were statistical differences between the light-haired dogs (Group 2) and each dark-haired subgroup (1a-1c). The final grade of light-haired dogs, for instance, was statistically lower than the black (Group 1a, $p < 0.05$) and black & tan breeds (1c, $p < 0.05$) (Figure 10A). The number of tumor cell budding foci in Group 2 was significantly lower than Groups 1a ($p < 0.01$), 1b ($p < 0.05$) and 1c ($p < 0.05$) (Figure 10B). When looking at the size of the nests within the invasive front, the light-haired dogs had larger nest sizes than the black (1a, $p < 0.05$), Schnauzers (1b, $p < 0.05$) and black & tan dogs (1c, $p < 0.01$) (Figure 10C). Interestingly enough, even though the amount of tumor stroma was not a part of the numerical score for this system, it was significantly finer in light-haired animals (Group 2), when compared to the Schnauzers (1b, $p < 0.01$) or to the black & tan breeds (1c, $p < 0.05$) (Figure 10D). There was no statistical difference between dark-haired Groups 1a–c in any of the evaluated features.

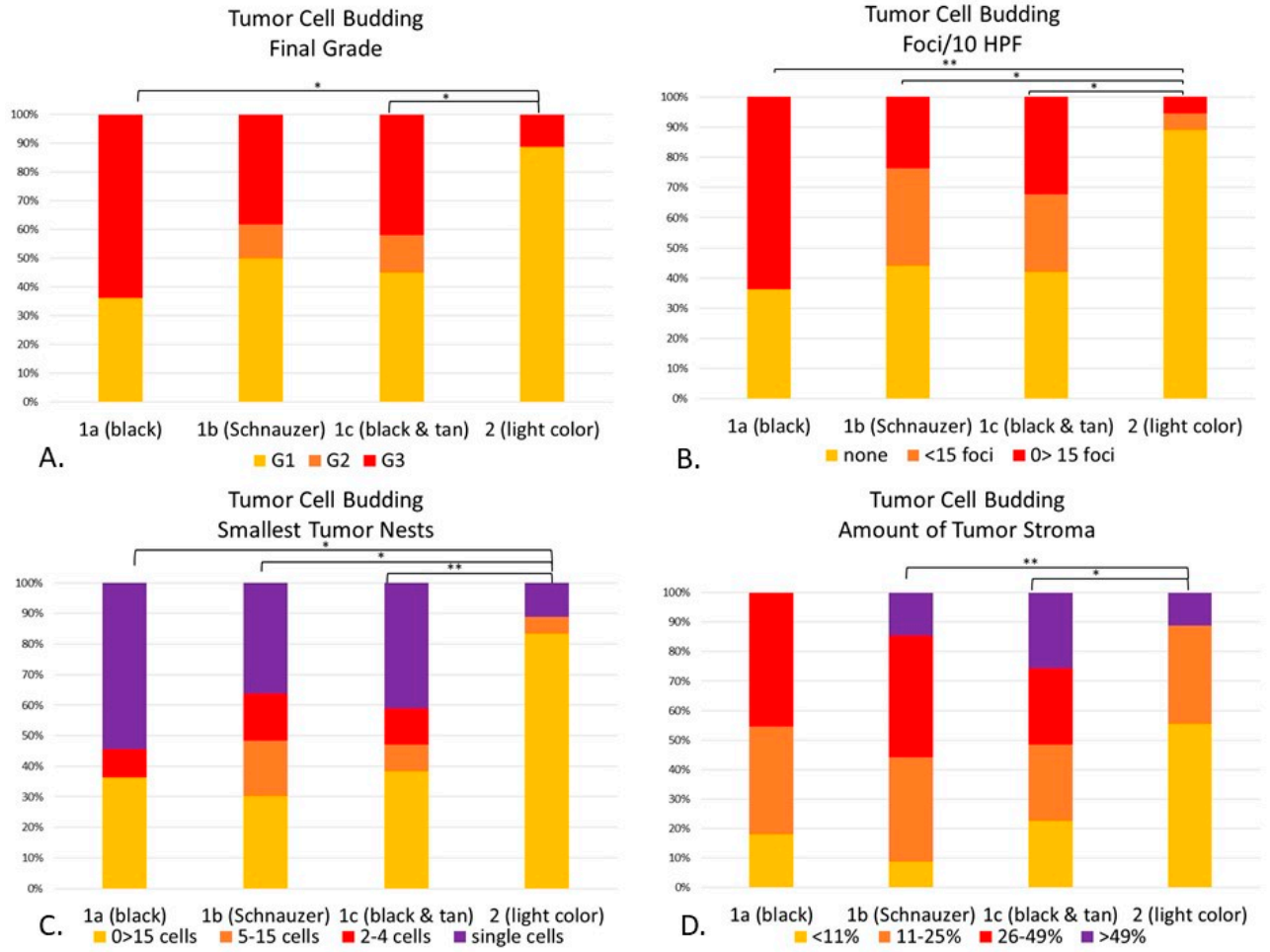

Figure 10. (**A**–**D**). Statistical differences between genetically defined subgroups (1a–1c) and Group 2 according to the tumor cell budding system. Cellular dissociation final grade ($p < 0.01$), with statistical differences in Group 1a ($p < 0.05$), and 1c ($p < 0.05$) when compared to Group 2 (**A**). Budding/10 HPF ($p < 0.01$) was different between Groups 1a ($p < 0.01$), 1b ($p < 0.05$) and 1c ($p < 0.05$) when compared to Group 2 (**B**). Smallest tumor nest size ($p < 0.01$) was significantly different between Groups 1a ($p < 0.05$), 1b ($p < 0.05$) and 1c ($p < 0.01$) when compared to Group 2 (**C**). The amount of tumor stroma ($p < 0.01$) was statistically different between Groups 1b ($p < 0.01$) and 1c ($p < 0.05$) when compared to Group 2. There were no statistical differences among dark breeds when compared to each other. (**D**) The amount of tumor stroma of Group 2 was statistically significant when compared to Group 1b ($p < 0.01$) and 1c ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$.

3.3. Comparison of Invasive Front Grading System and Tumor Cell Budding System

Both grading systems available were evaluated and compared to each individual factor to evaluate a significant difference between light- and dark-haired canine breeds. There was a significant statistical difference between both phenotypical groups (dark- and light-haired dogs) in these two systems at the final IF grading ($p = 0.001$) and their total score ($p < 0.01$), as well as the degree of keratinization within the invasive front ($p < 0.05$), invasion ($p = 0.001$) and nuclear pleomorphism ($p < 0.01$). Likewise, the different criteria of the TCB system were also statistically significant between the two populations, including tumor budding ($p = 0.0001$), smallest tumor nest ($p < 0.001$) and tumor stroma ($p = 0.0001$), as well as the total cellular dissociation score ($p = 0.0001$) and final grade ($p = 0.001$).

An interesting point was the number of animals having a similar grading on both the IFGS and TCBGS. Groups classified as "well differentiated" (grade I/1) by both systems, was composed of 14 light-haired and 28 dark-haired dogs (Table 4).

Table 4. Number of animals with digital squamous cell carcinoma in both invasive front grading system and tumor budding system.

Invasive Front Grading System			**Tumor Cell Budding Grading System**			**Number of Dogs with Same Grading by Both Systems**	
	Light-Haired ***n*** **= 18**	**Dark-Haired** ***n*** **= 76**		**Light-Haired** ***n*** **= 18**	**Dark-Haired** ***n*** **= 76**	**Light-Haired** ***n*** **= 15**	**Dark-Haired** ***n*** **= 45**
Grade I $n = 47$	15	32	Grade 1 $n = 51$	16	35	Well differentiated $n = 42$	14	28
Grade II $n = 32$	3	29	Grade 2 $n = 8$	0	8	Moderately differentiated $n = 4$	0	4
Grade III $n = 17$	1	16	Grade 3 $n = 35$	2	33	Poorly differentiated $n = 14$	1	13

When comparing light-haired animals classified as "moderately differentiated" (grade II/2) by both systems, there was no overlap. Interestingly enough, within the dark-haired animals, there were only four dogs graded as "moderately differentiated" by both systems. Digital squamous cell carcinomas from 13 dark-haired animals and one Golden Retriever (case No. 92) were graded as "poorly differentiated" (grade III/3) by both systems.

The CDSCC from three dark-haired dogs (case No. 5, 24 and 25) were graded as "well differentiated" by IFGS, but as "poorly differentiated" by the TCB System. Furthermore, there were CDSCCs from two dogs (case No. 74, a Rottweiler and No. 92, a WHWT) which were graded as "poorly differentiated" by IFGS, while being considered as "well differentiated" by the TCB System.

4. Discussion

There is currently no widely accepted grading system for canine SCC, although one of the most widely used, Broder's grading system [13], with a 1–4 grade based on the differentiation features, is used to morphologically characterize this tumor [13]. Nonetheless, given that there is no prognostic significance and often somewhat subjective assessment, many pathologists fail to characterize it. Broder's system was not included in this case since, similar to what other studies showed [11], there can be different grades of differentiation within the same tumor, making it hard when evaluating the sample. In this study, the goal was to compare two different adapted grading schemes to ascertain that there was a morphological disparity between light- and dark-colored animals with CDSCC and some kind of grading congruence between both systems. This allows a better comprehension of CDSCC and, hopefully, the development of a future grading system with prognostic correlation.

Canine digital cell carcinomas (CDSCC) are known to be particularly aggressive when compared to other cutaneous locations. Even though these neoplasms are more frequently seen in classically dark-haired breeds, there is no literature available examining if these tumors are morphologically different than their light-haired counterparts, suggesting different, maybe more aggressive, behavior. Through the adaptation of both human (Jesinghaus et al. (2018) [7] and Boxberg et al. (2019) [9]) and veterinary (Nagamine et al., (2017) [2]) SCC grading systems, we evaluated CDSCC from animals of both haircoat colors and investigated if there was any statistical difference between the different morphological features based on their presumed genetic and phenotypical haircoat color.

Skin pigmentation is a point of interest in human medicine regarding evaluating susceptibility to certain skin neoplasias, such as cutaneous melanoma, or basal cell carcinoma [22]. Additionally, some skin tumors, such as melanoma and nonmelanoma skin

cancers, are more often seen in white populations, believed to be closely associated with skin color and UV-light exposure (among other factors) [23]. This is also postulated because skin cancers are less common in People of Color than in Caucasians [23–25]. Nonetheless, this has not been widely studied in veterinary medicine. In canine melanomas, postulated to be a potential human model [26], several copy number alterations and low numbers of single-nucleotide variations with non-UV-associated mutations were identified [26]. In both dogs and humans, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) were associated with mucosal melanoma [26]. Nevertheless, there are currently no detailed studies about comparing canine squamous cell carcinoma (digital and nondigital) with haircoat color and taking into account the speculated genotypic haircoat.

Breeds represented in our study were mainly dark breeds, the most common being Schnauzers and Rottweilers, and a markedly smaller light-haired population, similar to the literature [11]. When comparing the localization of this tumor in each subgroup, either the limb or toe, there were no statistical differences between groups ($p < 0.05$). Unfortunately, there was a great number of animals with no information regarding location, therefore, the interpretation of end results in this parameter must be taken with caution.

To follow the International Tumor Budding Consensus Conference (ITBCC), 2016, of colorectal cancer [27], and other similar studies [9] with the objective to increase reproducibility on a diagnostic setting, slides were assessed on HE alone. This approach was taken because meta-analyses suggest that the prognostic evidence assessed on HE vs. immunohistochemistry (IHC) is not significant, although IHC may allow a higher interobserver agreement [27].

When looking at the light-haired dogs CDSCC, these were more frequently well differentiated, with abundant keratinization, well-formed, pushing solid cords infiltrating within the invasive front, little anaplasia and rare, if any, mitotic activity. On the other hand, the darker breeds often had more "malignant" features, with frequent budding, less keratinization and more anaplasia within the invasive front. This is particularly interesting for Group 1b, the Schnauzers, which were the most homogenous with all the same breed of animals, leading to speculation that, when encountering a more poorly differentiated CDSCC, it is more likely to be from a dark-haired animal, although the underlying reason for this is yet to be elucidated.

When comparing the two systems provided, within the dark-haired population, IFGS showed 41.5% grade I, 37.6% grade II and 20.8% grade III. On the other hand, TCBGS in the same population showed a proportion of 45.5% grade I 10.3% grade II and 44.2% grade III. This illustrates that, for well-differentiated CDSCC, with a solid pattern of invasion, well-keratinization and low mitotic activity tends to be engulfed as a low grade/grade I by both systems. Nevertheless, when more malignant features are present, the IF grading system tends to include it as grade II, while the TCB grading system would more likely assign it to be grade III. Interestingly enough, out of all the animals, there were only three cases that were graded as "well differentiated" by the IF system, while having a "poorly differentiated" grade on the TCB System. On the other hand, there were two cases characterized as "poorly differentiated" by the IF system, while being graded as "well differentiated" by the TCB grading system. This apparent incongruence could be explained by different features evaluated within the invasive front, which rarely overlap in both systems. Also, it can be explained by the marked importance that the TCB grading system gives to nest size and budding (2/3 features evaluated), while the IF grading system only pays attention to this feature in one out of the five characteristics evaluated, hence the grading disparity in some cases. It must be pointed out that, although the single cell tumor nests are of great importance, the less cohesive these cells are, the more poorly differentiated the neoplasm is likely to be; therefore, a more aggressive behavior can be hypothesized.

Furthermore, the IF grading system pays special attention to additional features, which are also theoretically associated with the pathogenesis of this neoplasm, such as host response. An inflammatory reaction secondary to a tumor invasion, particularly in those

tumors that produce extracellular keratin (and, therefore, presumably better differentiated), is expected to elicit a profound immune response. On the other hand, neoplastic tactics of immune-tolerance mechanisms and immune-response evasion were shown to modulate the inflammatory response by attenuating it and allowing tumors to create a favorable microenvironment for invasion [27–29]. Nonetheless, similar to another small study [28], our results concluded that there was no significant difference between degree of host response/inflammation between the groups. Additionally, it has to be pointed out that the IFGS, even when used in CDSCC, is an adaptation of Nagamine et al.'s system [2] which, in itself, is an adaptation from human medicine [30] and, henceforth, certain features cannot be extrapolated. For instance, dogs with masses on toes (either inflammatory or neoplastic), will tend to inflict self-trauma, either through chewing or licking, thus causing a secondary inflammatory response. This would also explain why inflammation among groups may not be significant, given that all animals may traumatize the area one way or another.

Additionally, other interesting features, such as stromal reaction, were evaluated within the TCBGS, although did not play a role in the total score or final grading. This fibrovascular scaffold, which includes fibroblasts, vasculature, extracellular matrix and other extracellular molecules, set the tumor-microenvironment that favors tumor growth and expansion through different mechanisms [31]. Taking into account this particular morphological feature within the grading may be a representation of the tumor microenvironment, thus becoming more prominent in those less differentiated with, hypothetically, more aggressive behavior.

When performing the gradings, there were statistical differences between the light- and the dark-haired breeds (which were represented by phenotypically black & tan breeds, black breeds and black Schnauzers) in both IF score and grading ($p < 0.01$ and $p < 0.01$, respectively) and TCB score and grading ($p < 0.01$ and $p < 0.01$, respectively) systems. Additionally, when comparing each individual morphologic feature, there were statistical differences in degree of keratinization within the invasive front, pattern of invasion, nuclear pleomorphism, tumor budding activity in 10 HPF, smallest nest size and amount of tumor stroma. These features were consistently better differentiated in light-haired rather than dark-haired breeds. Interestingly, there was not an overall significant difference between the phenotypical dark-haired groups (presumed genotypes KB/KB, KB/Ky, ky/ky and at/*) in any of the scoring systems or individual features. This finding highly suggests that dark-haired breeds tend to have more morphologically poorly differentiated CDSCC when compared to light-haired breeds, although different biological behavior cannot be predicted, only hypothesized. It would be interesting to know the prognosis of these tumors based on the histomorphological features of the invasive front. Sadly, no follow-up information was available concerning the samples evaluated in our study.

As mentioned before, the colors of the haircoat and claws depend on the content/absence and distribution of eumelanin in these structures. This pigment distribution and content depend on simple genetic variants of the E-, K- and A-locus (among others) [18]. In our study, all dogs had concordant color in both haircoat and claws, with black claws in dark-haired animals (Group 1) and light claws in light-haired animals (Group 2). Altogether, it could be inferred that the poor differentiation of CDSCC, which were associated with the haircoat color (most obvious in dark-haired animals), is also similarly associated with the claw pigmentation. In summary, this "poor differentiation" of CDSCC could potentially be associated with the eumelanin biochemistry of processing and incorporation of this pigment into the claw.

In general, when assessing adapted SCC grading systems, a few limitations have to be taken into account. To begin with, the best/most malignant invasive front in each CDSCC did not always match the deepest invasive front (as evaluated in other publications [2,6,7,9]), as sometimes this was located within the bone, while others were within the dermis. This allows us to speculate that, due to different cellularity and structure of the surrounding normal stroma (either bone or dermis), the neoplastic cells may render different strategies of invasion, making it inconsistent during the grading. Also, since the deep invasive

front was not always the most malignant front, this may theoretically support the tumor biological behavior and spread, infiltrating in all directions.

A further limitation encountered was the different grades of differentiation depending on the area within the tumor, as reported in the literature [11]. Also, due to the morphological overlap between certain digital squamous cell carcinomas and less malignant epithelial neoplasms, such as subungual keratoacanthoma, some well-differentiated squamous cell carcinomas within this location may be overlooked or misdiagnosed, thus underestimating the prognosis. In this particular study, the light-haired population (N = 18) was much smaller than the phenotypical dark-haired one (N = 76), which makes interpretation between phenotypical groups somewhat difficult. Nevertheless, this disparity is concordant with previous literature [11], where dark dogs are prone to this neoplasm and, consequently, are more numerous.

Additionally, when assessing tumor cell nesting, complete cellular dissociation of the tumor aggregates from the main neoplasia has to be assumed. This can be somewhat problematic given that this is a 2D assessment (a histological slide) of a 3D event, never making sure that the small complexes might be connected to the primary mass in deeper sections or when a different orientation is given. This dissociation, however, has to be assumed when assessing the invasive front, given that there is currently no other available system.

This study opens up interesting future research concerning CDSCC, such as the different prognosis based on these neoplasms' histomorphological features. Currently, there is no available grading system for CDSCC that provides prognostic clinical insight. Additionally, the question regarding whether phenotypically dark breeds, with their presumed genotypical haircoat color, are genetically predisposed to a more morphologically poorly differentiated CDSCC or whether light-haired breeds have genetic protection against this tumor still remains to be studied through future genetic analysis. Throughout this study, no true genetic haircoat analysis was made. The assumption of the presumed genotype was only based on the phenotypical color. Nevertheless, this may result in a scaffold for future research studies in which true hair-coat genetic analysis can be performed.

5. Conclusions

To our knowledge, this is the first study comparing CDSCC in dogs by two histological grading systems, taking into account their phenotypical and presumed genotypical haircoat color and demonstrating that digital squamous carcinomas are not only more common in dark-haired dogs, but potentially more aggressive. When comparing both challenged TCB and IF grading systems, they often overlapped when grading well-differentiated tumors. On the other hand, when more "malignant" features were present in the CDSCC, the classification systems often placed them in different grade (II vs. III). With this study, conclusions regarding the most accurate grading system for CDSCC cannot be drawn, since no outcome was available in any of the cases.

Supplementary Materials: The following are available online at https://www.mdpi.com/2306-7381/8/1/3/s1: Supplemental Material Table S1: Signalment of light and dark canine breeds with digital squamous cell carcinoma, taking into account the genotypical haircoat color.

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Supplemental material

Supplemental Material Table S1: Signalment of light and dark canine breeds with digital squamous cell carcinoma, taking into account the genotypical haircoat color.

	Phenotypical haircoat color	Genotypical haircoat color	Genetic group based on genotypical haircoat color	Breed	Age (years)	Sex 0= unknown 1=female 2=female spayed 3=male 4=male castrated
1	Black	Kb/Kb	1a	Briard	11	1
2	Black	Kb/Kb	1a	Briard	7	4
3	Black	Kb/Kb	1a	Giant Poodle	7	4
4	Black	Kb/Kb	1a	Labrador	11	4
5	Black	Kb/Kb	1a	Russian Terrier	6	0
6	Black	Kb/Kb	1a	Russian Terrier	10	2
7	Black	Kb/Kb	1a	Russian Terrier	8	3
8	Black	Kb/Kb	1a	Russian Terrier	8	3
9	Black	Kb/Kb	1a	Russian Terrier	10	1
10	Black	Kb/Kb	1a	Russian Terrier	6	0
11	Black	Kb/Kb	1a	Russian Terrier	10	2
12	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	9	4
13	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	11	4
14	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	11	2
15	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	1
16	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	9	3
17	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	2
18	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	3
19	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	8	1
20	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	8	3
21	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	9	2
22	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	9	4
23	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	1
24	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	6	4
25	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	4
26	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	8	3
27	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	7	3
28	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	9	4
29	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	3
30	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	8	3
31	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	11	3
32	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	6	2

33	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	10	3
34	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	11	3
35	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	12	1
36	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	8	4
37	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	13	2
38	Black	Kb/Kb or Kb/ky	1b	Giant Schnauzer	7	4
39	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	10	2
40	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	8	3
41	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	8	2
42	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	13	3
43	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	13	1
44	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	12	4
45	Black	Kb/Kb or Kb/ky	1b	Standard Schnauzer	10	3
46	Black&tan	ky/ky + at/*	1c	Gordon Setter	10	2
47	Black&tan	ky/ky + at/*	1c	Gordon Setter	11	1
48	Black&tan	ky/ky + at/*	1c	Gordon Setter	12	3
49	Black&tan	ky/ky + at/*	1c	Gordon Setter	12	3
50	Black&tan	ky/ky + at/*	1c	Gordon Setter	11	3
51	Black&tan	ky/ky + at/*	1c	Gordon Setter	11	1
52	Black&tan	ky/ky + at/*	1c	Gordon Setter	8	3
53	Black&tan	ky/ky + at/*	1c	Gordon Setter	u	3
54	Black&tan	ky/ky + at/*	1c	Gordon Setter	u	4
55	Black&tan	ky/ky + at/*	1c	Gordon Setter	11	3
56	Black&tan	ky/ky + at/*	1c	Rottweiler	10	1
57	Black&tan	ky/ky + at/*	1c	Rottweiler	10	0
58	Black&tan	ky/ky + at/*	1c	Rottweiler	8	2
59	Black&tan	ky/ky + at/*	1c	Rottweiler	8	3
60	Black&tan	ky/ky + at/*	1c	Rottweiler	11	0
61	Black&tan	ky/ky + at/*	1c	Rottweiler	7	3
62	Black&tan	ky/ky + at/*	1c	Rottweiler	6	1
63	Black&tan	ky/ky + at/*	1c	Rottweiler	10	1
64	Black&tan	ky/ky + at/*	1c	Rottweiler	8	2
65	Black&tan	ky/ky + at/*	1c	Rottweiler	10	2
66	Black&tan	ky/ky + at/*	1c	Rottweiler	11	2
67	Black&tan	ky/ky + at/*	1c	Rottweiler	8	2
68	Black&tan	ky/ky + at/*	1c	Rottweiler	8	4
69	Black&tan	ky/ky + at/*	1c	Rottweiler	9	3
70	Black&tan	ky/ky + at/*	1c	Rottweiler	10	2
71	Black&tan	ky/ky + at/*	1c	Rottweiler	7	3
72	Black&tan	ky/ky + at/*	1c	Rottweiler	13	1
73	Black&tan	ky/ky + at/*	1c	Rottweiler	8	2

74	Black&tan	ky/ky + at/*	1c	Rottweiler	10	1
75	Black&tan	ky/ky + at/*	1c	Rottweiler	13	4
76	Black&tan	ky/ky + at/*	1c	Rottweiler	11	3
77	Light	e/e	2	Golden Retriever	13	3
78	Light	e/e	2	Golden Retriever	10	1
79	Light	e/e	2	Golden Retriever	8	3
80	Light	e/e	2	Golden Retriever	11	3
81	Light	e/e	2	Golden Retriever	11	1
82	Light	e/e	2	Golden Retriever	9	3
83	Light	e/e	2	Golden Retriever	8	1
84	Light	e/e	2	Golden Retriever	8	4
85	Light	e/e	2	Golden Retriever	12	0
86	Light	e/e	2	Golden Retriever	7	3
87	Light	e/e	2	Golden Retriever	10	3
88	Light	e/e	2	Golden Retriever	14	3
89	Light	e/e	2	Golden Retriever	9	0
90	Light	e/e	2	Golden Retriever	12	3
91	Light	e/e	2	Golden Retriever	12	0
92	Light	e/e	2	West Highland White Terrier	12	4
93	Light	e/e	2	West Highland White Terrier	12	1
94	Light	e/e	2	West Highland White Terrier	12	4

4.2. Study 2: Evaluating the Histologic Grade of Digital Squamous Cell Carcinomas in Dogs and Copy Number Variation of KIT ligand — A Correlation Study

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Article

Evaluating the Histologic Grade of Digital Squamous Cell Carcinomas in Dogs and Copy Number Variation of KIT Ligand—A Correlation Study

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Simple Summary: Dark-haired dogs are predisposed to the development of digital squamous cell carcinoma (DSCC), suggesting an underlying genetic predisposition which is yet to be explained. Some authors have suggested a correlation between the number of copies of KIT Ligand, a gene associated with cell survival, proliferation, and melanogenesis, among other functions, and the potential predisposition to DSCC in dogs. This was evidenced by the fact that dogs with DSCC had a significantly higher copy number of this gene than those who did not have the neoplasia. For this reason, we evaluated the potential correlation between the number of copies of the KIT Ligand in genomic DNA with the histological grade of malignancy in dogs with DSCC. Our findings reveal a significant correlation between the number of copies of KIT Ligand and DSCC histological grade. This supports previous studies that KIT Ligand may play a role in DSCC development and, additionally, may be involved with the presence of histologically malignant morphological features. This suggests a potential factor in the development of canine DSCC, which may signify a potential advance in personalized veterinary oncological approaches as well as future breeding programs.

Abstract: Dark-haired dogs are predisposed to the development of digital squamous cell carcinoma (DSCC). This may potentially suggest an underlying genetic predisposition not yet completely elucidated. Some authors have suggested a potential correlation between the number of copies KIT Ligand (KITLG) and the predisposition of dogs to DSCC, containing a higher number of copies in those affected by the neoplasm. In this study, the aim was to evaluate a potential correlation between the number of copies of the KITLG and the histological grade of malignancy in dogs with DSCC. For this, 72 paraffin-embedded DSCCs with paired whole blood samples of 70 different dogs were included and grouped according to their haircoat color as follow: Group 0/unknown haircoat color (n = 11); Group 1.a/black non-Schnauzers (n = 15); group 1.b/black Schnauzers (n = 33); group 1.c/black and tan dogs (n = 7); group 2/tan animals (n = 4). The DSCCs were histologically graded. Additionally, KITLG Copy Number Variation (CNV) was determined by ddPCR. A significant correlation was observed between KITLG copy number and the histological grade and score value. This finding may suggest a possible factor for the development of canine DSCC, thus potentially having an impact on personalized veterinary oncological strategies and breeding programs.

Keywords: canine; cancer; toe; grading; haircoat; color; genetics; gene

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1. Introduction

The largest reported cohort of 2912 canine toes, conducted by Grassinger et al., classified 52% of their samples as neoplasms, 78% of which were malignant. Of those, approximately 65% were digital squamous cell carcinomas (DSCCs) [1]. These findings, along

with other similar research [2–5], strongly suggest that DSCC represents the most common malignant tumor at this location in the canine population [1,5,6].

This aforementioned study, coinciding with other publications [6–10], identified a DSCC predisposition in dark canine breeds such as Schnauzers [7,8,10], Briards [7], Rottweilers [6], Poodles [7,9], and Dachshunds [6]. On the other hand, interestingly, Grassinger et al. [1] observed that Jack Russell Terriers, which commonly have white paws [11], were less likely to develop the neoplasia at this location than other mixed breeds [1]. These peculiar differences, apparently based on haircoat color, along with publications of three related Schnauzers with primary DSCC [10], may suggest an underlying genetic predisposition for the development of this neoplasia based on the dark haircoat.

KIT Ligand (KITLG), also known as stem cell factor [12], is a gene that encodes the ligand of the receptor-type protein-tyrosinase kinase KIT. In general terms, this is a low specific ligand that is associated with many essential vital roles, such as cell survival and proliferation, hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration, and function, and melanogenesis [12–14]. It is for this last feature that KITLG is a significant gene in canine haircoat color and tone determination. This is because it plays a role in postnatal cutaneous melanogenesis and follicular epithelial melanocyte terminal differentiation [15]. The melanin synthesis of the hair follicle's melanocyte is achieved through an intercellular signaling pathway. This results in the production of both eumelanin and pheomelanin by the melanosome, a specialized organelle, along the entire extension of the hair shaft, thus conferring different colors and tones [15,16]. Interestingly, Bannasch et al. [15] stated that the copy number of the CNV of KITLG was significantly associated with eumelanin intensity in the Poodle and across breeds and, to a lesser extent, pheomelanin.

Moreover, Karyadi et al. [7] suggested that a higher CN of the KIT ligand locus is seen in dogs with DSCC when compared to animals without the tumor. This new finding, along with the known role of KITLG in haircoat melanogenesis and the findings of Bannasch et al. [15], may suggest a potential link between the presence of a high CN of the KITLG and the development of a DSCC in dark-haired dogs. This generated the hypothesis that a higher KITLG count may not only be associated with the development of DSCC, but potentially also with a morphologically distinct DSCC which could correlate with a more aggressive histological grade. To the best of the authors´ knowledge, there are no studies correlating the CN of KITLG with distinct histological characteristics of canine DSCC.

The objective of this research was to evaluate if there is a correlation between histological features of aggressiveness in canine digital SCC and copy number values in KITLG.

2. Materials and Methods

From 2014–2019, 72 histological samples of DSCC from 70 dogs were selected retrospectively from the routine diagnostic pool submitted to LABOKLIN GmbH & Co. As a selection requirement, the samples had to contain a clear neoplastic invasive front and known breed. All DSCC from animals only included fragments with neoplasia, and samples with no clear invasive front or without information about the breed were excluded.

Furthermore, additional blood samples from these dogs were available from routine diagnostics (presurgical or geriatric screening). As all samples (toes and whole blood) were submitted for routine diagnostic purposes, ethics committee approval was not required (RUF-55.2.2-2532-1-86-5). All the material used was no longer needed for diagnostics.

The ages of the dogs ranged from 4 to 16 years, with a median of 10 years. Sex was either female intact (15), female spayed (15), male (22), or male castrated (20). Limb and toe affected, when available, was noted. The breeds included Giant Schnauzer (20), Standard Schnauzer (14), Mixed (8), Briard (5), Labrador (5), Gordon Setter (3), Russian Black Terrier (2), Flat-Coated Retriever (2), Bernese Mountain Dog (2), Rottweiler (2), Belgian Shepherd (1), Havanese (1), Irish Setter (1), Puli (1), German Wirehaired Pointer (1), Hovawart (1), Giant Poodle (1), West Highland White Terrier (1), and Standard Poodle (1).

The majority of these animals were further grouped according to their haircoat color, similar to the previous research [17], as follows; Group 0/others (n = 11), composed of animals with unclear haircoat color; Group 1.a/blacks (n = 15), with non-Schnauzer black breeds; Group1.b/Schnauzers (n = 33), with black Schnauzers; Group 1.c/black and tan (n = 7), with black and tan animals; and Group 2/light (n = 4), with dogs with a light blond or reddish haircoat. Additionally, Groups 1.a, 1.b, and 1.c were summarized as "dark coated breeds" (n = 55). Signalment and individual cases with more detailed information can be seen in Supplementary Table S1.

Similar to the previous study conducted by this research group [17], all digital samples were fixed in 10% phosphate-buffered formalin, routinely trimmed following laboratory standard procedures, and decalcified in a mixture of ≥10– <20% hydrochloric acid (HCl) and formaldehyde (≥3%–<5%) (Osteomoll® rapid decalcifier solution for histology; catalogue no. 101736) over a period of 24–72 h, periodically assessing tissue until it was ready to be further processed according to published trimming guidelines [18]. Afterwards, longitudinal and sagittal sections were embedded in paraffin wax and cut at 4–5 µm thickness to be then stained with Haematoxylin–Eosin (HE). All slides were reviewed, selecting the most representative section for this study. This was based on a good histological quality and clear invasive front with surrounding non-affected stroma to evaluate the neoplastic–non-neoplastic transition. The most representative slide was scanned and analyzed through specialized image analysis software (NIS-elements software (Nikon, Tokyo, Japan); Aperio ImageScope (Leica, Wetzlar, Germany)) by a blinded ACVP diplomat (A.C-E).

2.1. Histological Examination

For more detailed information concerning the adapted gradings performed in the study, please refer to the previous open-source publication carried out by this research group (Cerezo-Echevarria et al. 2020) [17].

2.1.1. Invasive Front Grading System (IFGS)

As previously performed [17], Nagamine et al.'s (2017) [19] grading system for canine oral SCC (OSCC) was adapted, following the established criteria of degree of keratinization, pattern of invasion, host response, nuclear pleomorphism, and mitoses per high-power field (HPF) (Table 1). The mitoses per 10 HPF were assessed in an overall area of 2.37 mm2 to ensure standardization [20] and reproducibility. The final addition of the score values of these five morphologic features resulted in a total IFGS score value, which then was translated into 3 different grades, including well-differentiated/grade I (total score value: 6–10) (Figure 1), moderately differentiated/grade II (total score value: 11–5), and poorly differentiated/grade III (total score value: 16–20) (Figure 2) [17,19].

Table 1. Simplified table of Invasive Front Grading System (IFGS) used in the present study of canine digital squamous cell carcinoma (adapted from Nagamine et al. (2017) for use in canine oral squamous cell carcinoma).

Morphological Feature	Score Value			
	1	2	3	4
Degree of keratinization	>50% cells keratinized	20–50% cells keratinized	5–20% cells keratinized	0–5% cells keratinized
Pattern of invasion	Pushing, well-differentiated borders	Infiltrating, Solid cords, bands and/or strands	Small groups of cells (n > 15)	Small groups and/or single cells (n < 15)
Host response	Marked	Moderate	Slight	None
Nuclear pleomorphism	<25% anaplasia	25–50% anaplasia	50–75% anaplasia	75–100% anaplasia
Mitosis HPF * (40×)	0–1	2–3	4–5	>5

* HPF: high-power field.

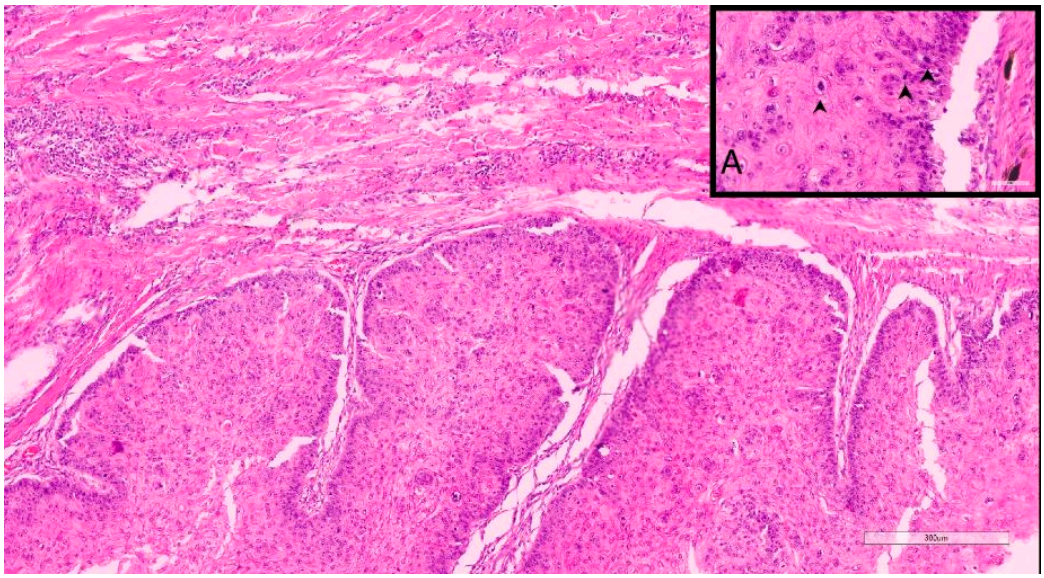

Figure 1. Sample 11, 9-year-old, female spayed, Caucasian Shepherd mix. This DSCC was graded as I by both Invasive Front and Tumor Cell Budding Systems. **Inset A**. Note that despite the low grade, there are occasionally moderate numbers of mitotic figures (arrows).

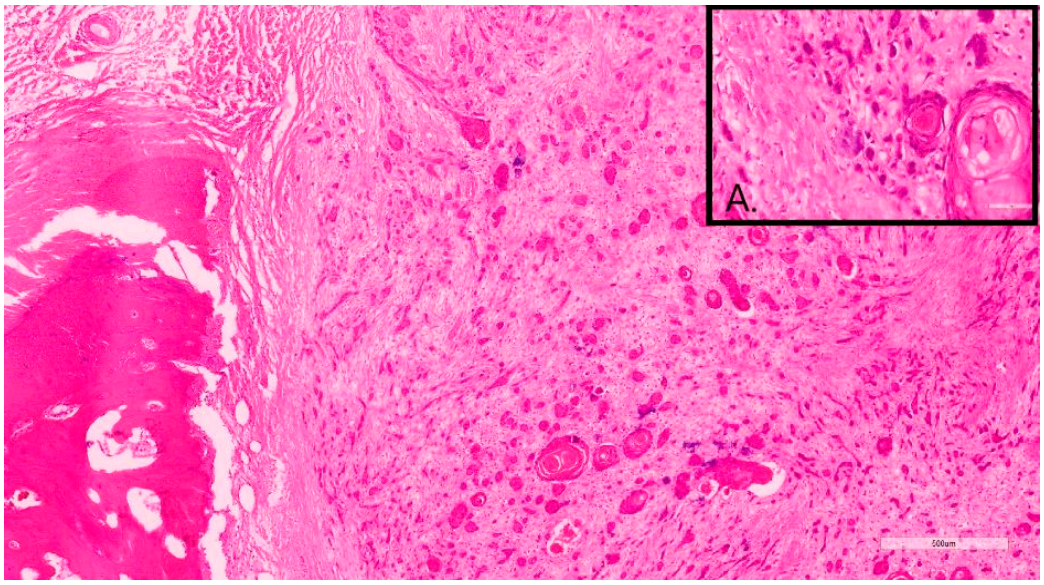

Figure 2. Sample 12, 11-year-old, female, Briard. This DSCC was graded as grade III by both Invasive Front and Tumor Cell Budding systems. **Inset A.** Note the marked neoplastic cellular dissociation within the invasive front, often forming small neoplastic buds or individual cells.

2.1.2. Tumor Cell Budding Grading System (TCBGS)

In a similar manner to previously conducted research by this group [17], an adaptation from two similar human SCC grading systems of Jesinghaus et al. (2018) [21] and Boxberg et al. (2019) [22] was used. In this grading, individual features of invasive front tumor budding in 10 HPF, smallest nest size, and stromal response associated with the neoplasm were evaluated.

Tumor budding was considered when neoplastic aggregates/complexes fewer than 5 cells disconnected from the main neoplasm and infiltrated into the surrounding stroma. These "buds" were assessed in an area of 2.37 mm2 at 40x magnification in high-incidence areas. The point system was granted following the table (Table 2). The sum of the score of each evaluated feature results in a final score value that translated into 3 different

grades, including well-differentiated/grade 1 (total score value: 2–3) (Figure 1), moderately differentiated/grade 2 (total score value: 4–5), and poorly differentiated/grade 3 (total score value: 6–7) (Figure 2) [17,21,22].

Table 2. Tumor Cell Budding System used in our study for determining tumor grade based on tumor budding activity and cell next size score adapted from human cervical SCC (Jesinghaus et al. (2018)) and laryngeal/hypopharyngeal SCC (Boxberg et al. (2019)).

Tumor Budding Activity/10HPF	Score Value
No budding	1
<15 budding foci	2
≥15 budding foci	3
Smallest cell nest size	
>15 cells	1
5–15 cells	2
2–4 cells	3
Single cell invasion	4

2.2. Genetic Analysis

Genomic DNA extraction and isolation from EDTA blood were performed with the MagNA Pure 96 system using DNA Tissue Lysis Buffer and viral NA Small RNA kit (Roche, Basel, Switzerland) according to manufacturers´ manual. Similar to that described by Bannasch et al. [15], the copy number quantification of the KITLG CNV was performed with digital droplet PCR (ddPCR) using TaqMan® assays specific for the KITLG CNV sequence and proto-Oncogene 1 (ETS1) as reference gene. Measurement was carried out in duplicate, and the mean value was used for further analyses. Intra-assay correlation was 0.85. Copy number was determined using DropletReader (Bio-Rad, Feldkirchen, Germany) and QuantaSoftware (Bio-Rad, Feldkirchen, Germany).

2.3. Statistical Analysis

Statistical analysis between dark- (Group 1, n = 55) and light-coated (Group 2, n = 4) breeds as well as between the different pre-established subgroups (1.a/blacks, n = 15; 1.b/Schnauzers, n = 33; 1.c/black and tan, n = 7; 2/light, n = 4) were performed using the Mann–Whitney U test. Group 0 was excluded because of heterogenic genetic background.

All statistical analyses and visualizations were carried out with the statistical framework R version 4.2 (R Core Team 2022). To identify the optimal model, we applied the "step" R function with direction "both" and the Bayesian Information Criterion (BIC). The marginal effects of the interaction term were visualized by the function "plot_model" as implemented in the "sjPlot" R package Lüdecke [23]. Robust linear regression was performed by the function "rlm" as implemented in R package MASS [24]. The correlation significance levels were calculated according to the "rob.pval" function implemented in the "repmod" R package [25].

3. Results

Out of the 72 samples from 70 different animals, 46 DSCCs were located in the forelimb and 24 in the hindlimb. Two samples were not provided with an exact location.

3.1. Histological Assessment

Histological Grades according to Groups

Following a similar model to that provided in previous studies conducted by this research group [17], the samples of DSCC were graded twice according to both IFGS and TCBGS. The total score values for IFGS ranged from 5 to 19, while the TCBGS total score

values ranged from 2 to 7. For further detail on the histological grade distribution of the canine population in general and the pre-established groups according to the IFGS and TCBGS, please refer to the previous research [17]. Interestingly enough, when comparing both systems simultaneously, twenty-three animals were classified as grade I, fourteen as grade II, and seven as grade 3 by both systems. As described in the previous study, there were single animals (a Giant Schnauzer and two Labrador Retrievers) classified as grade I with the IFGS, while being a grade 3 by the TCBGS (Supplemental Table S1, samples 4, 22, and 31). On the other hand, there were no cases simultaneously classified as a grade III by the IFGS while being grade I on the TCBGS. Two animals were reported to each have two digital squamous cell carcinomas (Supplemental Table S1, samples 25–26 and 42–43, respectively). The 10-year-old, male, intact Russian Black Terrier had one an intermediate grade DSCC according to both systems (II/2) (sample 25), while the second tumor was classified as a grade III/3 by IFGS and TCBGS (sample 26). The 11-year-old, female, intact Giant Schnauzer had one of the DSCCs classified as grade II/2 (sample 43), while the other tumor was grade II/1 (sample 42).

When associating the IFGS grade with the haircoat color, there was no significant difference between any of the groups (Figure 3) or between color and the histological features evaluated such as mitoses, nuclear pleomorphism, keratinization, invasion, or host response ($p > 0.5$).

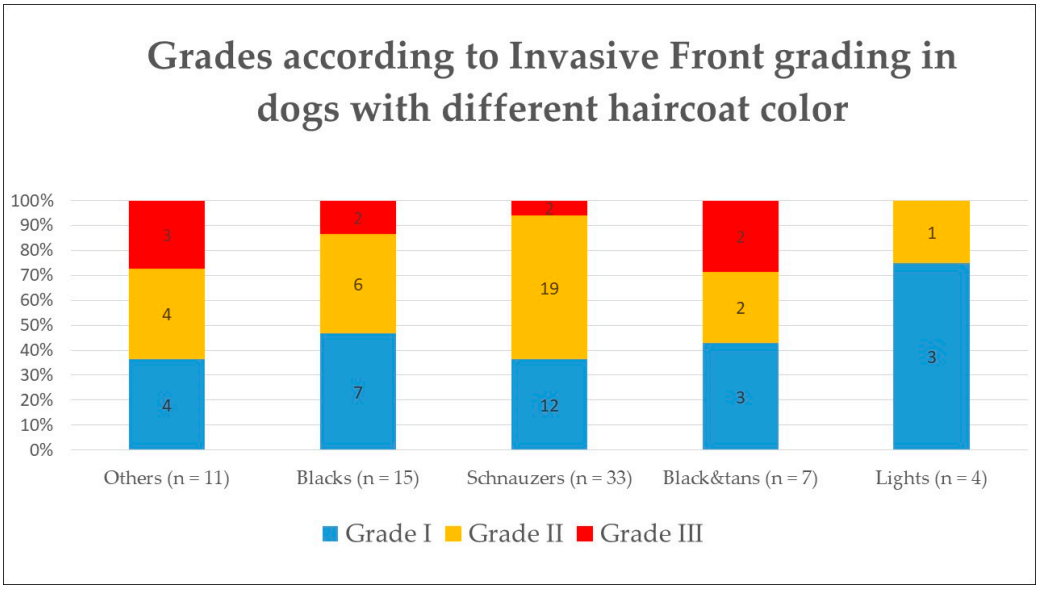

Figure 3. Histological grade according to Invasive Front Grading System in dogs according to their haircoat color.

On the other hand, when comparing the TCBGS grade with the haircoat color, there were significant differences between group 2/light and the other groups (1.a, 1.b, 1.c) ($p < 0.05$), with group 2 having a lower grade that any of the other darker groups (Figure 4). However, there was no significant difference when evaluating each individual feature that composed the TCBGS grade, including smallest nest size, mitoses, and tumor budding activity.

In summary, no particular histological feature is significantly different between the dark and light groups, but the overall summary of different histological aspects (as those evaluated on the TCBGS) may result in significant statistical differences in the degree of tumor differentiation and potential malignant behavior.

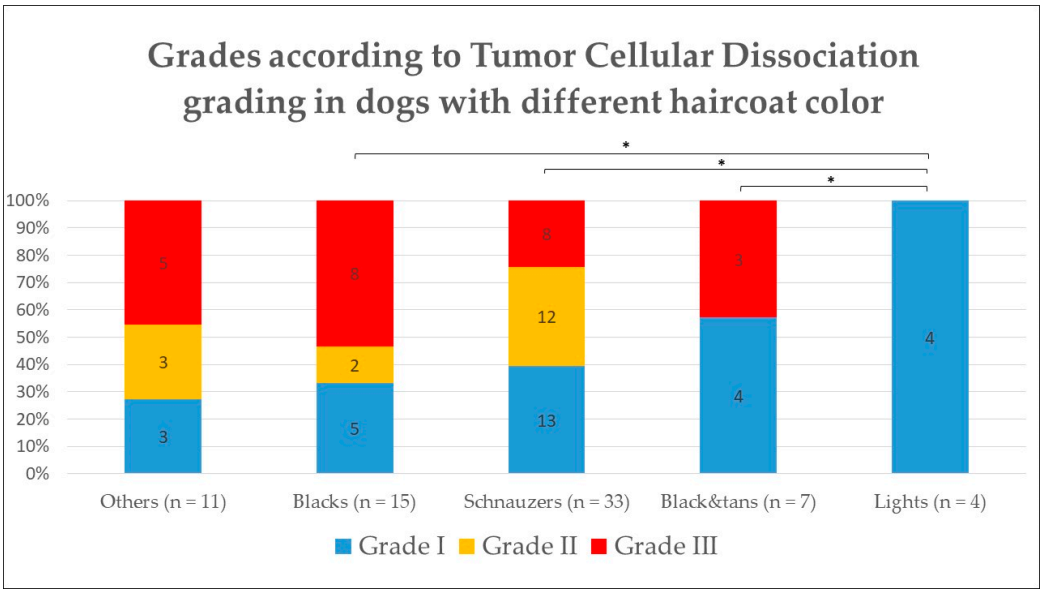

Figure 4. Histological grade according to Tumor Cellular Dissociation Grading System in dogs according to their haircoat color. * $p < 0.05$.

3.2. Copy Number Variation on KITLG

The value of the copy number (CN) of KITLG was measured for each animal included in the study and ranged between 2.02 and 9.14, with a mean value of 5.6. The value of Group 0/others was 5.7, for Group 1.a/black it was 5.8, for Group 1.b/Schnauzers it was 5.7, for Group 1.c/black and tan it was 5.5, and for Group 2/light it was 4.5. The differences between the groups were not significant.

A statistical analysis comparing the independent correlation of the TCBGS grade and IFGS grade with the KITLG copy number (CN) value was performed. Comparatively, animals with IFGS grade I had significantly lower KITLG CN than those of grade II ($p = 0.001$) and grade III ($p = 0.007$). On the other hand, when comparing KITLG CN with TCBGS, only animals with grade III had a significantly higher KITLG CN value than those animals with grade I ($p = 0.042$). This suggests that a higher histological grade is correlated with a higher KITLG CN value when comparing low versus high grades in both histological systems applied.

The optimal linear regression model was obtained using the R function "step" with direction "both" and the Bayesian information criterion (BIC). The identified optimal regression model considers only the KITLG CN value and the IFGS score value, with a strong significant correlation between these two features (IFGS score ~ 1.12 * CNV KITLG + 4.96; $p = 0.006$) (Figure 5A). The TCBGS score values and KITLG CN also show a significant correlation (TCBGS score ~ 0.75 * CNV KITLG; $p < 0.001$) (Figure 5B). These results suggest that there is a significant direct correlation between the evaluated histological score and the KITLG CN value. This model was independent from the haircoat color and/or gender. For further information regarding this correlation, taking into account each of the phenotypic haircoat colors, please refer to Supplemental Figure S1.

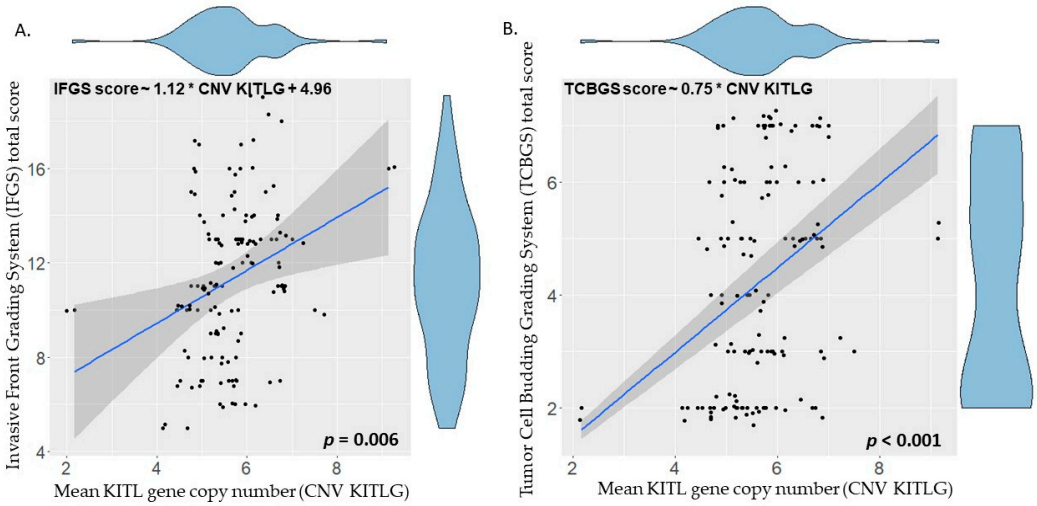

Figure 5. Linear regression of KITLG copy number (x-axis) and histological score value (y-axis) of the different animals. (**A**) Invasive Front Grading total score value (IFGS) is significantly correlated with the KITLG number (IFGS score ~ 1.12 * CNV KITLG + 4.96; $p = 0.006$). (**B**) Tumor Cell Budding Grading (TCBGS) total score value is significantly correlated with the KITLG number (TCBGS score ~ 0.75 * CNV KITLG; $p < 0.001$).

4. Discussion

In veterinary medicine, an effort has been made to try to identify genes associated with cancer or disease in different canine breeds or lineages. Some examples of this include renal cystadenocarcinoma [26], histiocytic sarcoma [27–29], progressive rod-cone degeneration [30], or collie eye anomaly [31], among others [32–37]. This can be particularly interesting, not only for tumor and disease occurrence predisposition, but also potentially its clinical behavior, prognosis, outcome, and future breeding decisions.

To this end, this represents the largest collection of canine DSCC along with pairing whole blood samples to date, allowing both histological and genetic assessment of the given animals. The aim of the study was to evaluate a hypothetical correlation between histological features of invasiveness in canine digital squamous cell carcinoma, based on two different adapted histological grading systems (IFGS and TCBGS), and copy number values of KITLG. Animals with lower IFGS and/or TCBGS score values had a significantly lower KITLG copy number (IFGS ~ 1.12 * CNV KITLG + 4.96; $p = 0.006$; TCBGS ~ 0.75 * CNV KITLG; $p < 0.001$).

As a continuation of the previous work conducted by this research group [17], the actual study confirms the hypothesis that KITLG CNV may have a potential role in the development of malignant histological features in DSCC. This is also backed up by the fact that in other studies, Schnauzers, which are often overrepresented, had worse outcomes against DSCC than other breeds [8]. This is despite not having identified poor prognostic factors at the time of presentation [8]. Furthermore, animals with multiple DSCCs have a tendency to develop additional ones over time [8]. This last remark, along with the description of DSCC in three related Giant Schnauzers [10], further implies the possibility of an underlying genetic predisposition, unknown at the time. On a different note, Bannasch et al. 2021 [15] found a correlation of CNVs in the KITLG color variation between pheomelanin and eumelanin deposition in the haircoat of the Poodle and other breeds. As expected, the present results are mainly based on a majority of eumelanin-based, dark-colored dogs of different breeds, and therefore, correlation between animalsdark and light haircoats and related CNV could not be established. Additionally, Grassinger et al. 2021 [1] already suggested that the absence of eumelanin (dark pigmentation) within the haircoat and/or claws may potentially have a protective function for light-haircoat dogs for the development of DSCC.

On a somewhat similar line of investigation, Karyadi et al. [7] suggested that a KITLG CNV of 4 or more may be the cause of a predisposition to the development of DSCC in the canine population, given that dogs with DSCC had a higher KITLG CN value than their non-DSCC counterparts. In the present study, the overall mean value of KITLG CN was 5.6, and all animals suffered from DSCC. Similar to that hypothesis, in the present study, all groups had a mean CN value of more than 4 (ranging from 4.5 in the light/2 group to 5.8 in the black/1.a group). There was only a single animal (sample 71, a 4-year-old, female, mixed-breed dog) which had a copy number of less than 4, being 2.17 in this case. This could support the hypothesis from Karyadi et al. that a KITLG CNV of 4 or greater predisposes the animal to DSCC. However, another option is that that this represents a widespread alteration in the CNV across several breeds. The dog with a CN of less than 4 in our study could possibly represent a spontaneous tumor development, with no genetic predisposition.

Similar to previous studies [17], the degree of differentiation of DSCC in both dark- and light-haircoat canine populations was assessed, obtaining aligning results. When applying the TCBGS, there were more histological "malignant" features in darker breeds (Group 1) than in the lighter ones (Group 2) ($p < 0.05$). On the other hand, when looking into the IFGS, there were no significant differences between the grade and haircoat color group. Nonetheless, statistical interpretation should be cautious, given the large group size disparities.

The KITLG copy numbers were compared independently to the histological score values of both the TCBGS and the IFGS systems of the established animal groups. Interestingly enough, both IFGS and TCBGS showed strong correlation between the KITLG CN and their histological score values. In summary, animals with a higher KITLG CN value have more histological features of malignancy of DSCC. IFGS had a slightly inferior correlation with the KITLG CN ($p = 0.006$) compared to TCBGS ($p < 0.001$), but both correlations were robust. The slight correlation discrepancy between grading systems and KITLG CN could be partially explained by the fact that both grading systems pay attention to different histological features, and potentially one of those features may be correlated with a higher (or lower) KITLG CNV. This has been established independent of the haircoat color and/or gender. It must be noted that this estimation may have been a more robust linear regression model if extreme mean CNV values would have been available. Despite this, with the available data, there was still a robust linear regression with comparable results. This may potentially suggest that with a known KITLG CN value, one could potentially predict the histological malignancy of a present DSCC, thus deciding the most appropriate course of treatment.

This study included 36 Schnauzers, known to be predisposed to DSCC, both Giant (n = 20) and Standard (n = 14). However, the present study did not compare animals of the same breed with and without DSCC, so a relative KITLG CNV could not be established between affected and non-affected animals. It is for this reason that we cannot draw conclusions regarding the comparatively higher KITLG CNV of animals with and without this tumor from the same breed. In order to prove this, further larger studies of dogs of the same breed (to ensure a more homogeneous sample) with and without DSCC in their entire lifetime should be performed to evaluate whether the hypothesis that a higher KITLG CNV is related to animals developing DSCC still stands. This is currently under investigation to allow future comparisons. Additionally, as a correlation between KITLG CNV and malignant histological features are observed, future studies should include KITLG transcription and protein expression level. Currently, there are few studies investigating the molecular expression of KITLG and c-KIT in veterinary medicine, mostly in canine mast cell tumors [38–42] and gastrointestinal stromal tumors [42–44], with no descriptions of specific immunolabeling in canine SCC or DSCC. In human medicine, there have been attempts to perform immunohistochemistry for c-KIT on SCC [45–51]. In the literature, there are reports that between 12.5% and 20% [45–47,50] of SCCs have any kind of sparse immunopositivity. In a single study, the expression of c-KIT in esophageal SCC was correlated with a worse

prognosis, only being expressed in 29.9% of the specimens [48]. In a similar manner, c-KIT expression was associated with lymph node metastasis, histological type, and worse overall survival in human non-small cell lung cancer, according to a meta-analysis [49]. Interestingly enough, in this study, the expression of c-KIT in adenocarcinoma was higher than in SCC [49], perhaps associated with innate tumor malignancy and aggressiveness. This, however, was beyond the scope of the present study and future projects may include these further tests.

5. Conclusions

This study represents the largest of its kind pairing up canine DSCC with blood samples, allowing a simultaneous histological and genetic assessment. When assessing the results, there was a significant correlation between KILTG CN increase and higher histological score value on both IFGS and TCBGS.

Further Studies

It would be interesting to evaluate a large cohort of canine breeds non-predisposed to DSCC to establish a KILG CNV baseline, identifying if there is a significant difference when these same breeds develop a DSCC. With this, KITLG CNV can be seen in a large cohort of animals with and without DSCC. More homogeneous groups, along with the identification of other "protective" or "causative" genes for CDSCC, should be evaluated. Further studies are needed to further characterize a possible genetic test for the predisposition of DSCC in dark-haired breeds, as well as KITLG transcription levels and final protein expression. This could possibly aid in identifying other potential genes that may serve as a protection against this neoplasm or may confer it with more benign or malignant histological features.

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Supplemental material

Supplemental Material Table S1: Signalment, histological grade and Copy number variation average of KITLG of canine breeds, with digital squamous cell carcinoma, taking into account the genotypical haircoat color.

Sample Number	Breed	Age (years)	Gender	Limb 0=fore- 1=hind-	Side 0=right 1=left	Toe	Groups 1.a= black; 1.b=Schnauzers; 1.c=Black&tan; 2=light; 0= other breeds	Nagamine Invasive front grade	Jesinghaus, Cellular dissociation grade	CNV Average
1	Belgian Shepherd	16	fc	0	0	1	0	I	1	4.52
2	German Wirehaired Pointer	14	m	0	1	2	0	II	3	5.85
3	Hovawart	8	f	1	1	5	0	I	1	5.80
4	Labrador	8	f	0	1	3	0	I	3	4.66
5	Mixed	10	fc	0	0	5	0	II	2	5.03
6	Mixed	9	fc				0	I	1	5.19
7	Mixed	11	f	0	1	2	0	III	3	5.62
8	Mixed	13	mc	0	0	3	0	II	3	7.00
9	Mixed	6	m	0	0	5	0	II	2	4.91
10	Mixed	10	f	0	1	3	0	III	2	9.14
11	Mixed	9	fc	0	0	1	0	III	3	4.94
12	Briard	11	f	0	1	2	1.a	III	3	4.83
13	Briard	11	mc	1	0	1	1.a	II	1	5.91
14	Briard	7	mc	0	1	2	1.a	I	1	6.74
15	Briard	6	m	0	0	4	1.a	I	1	7.50
16	Flat Coated Retriever	8	m	0	1	3	1.a	I	1	5.31
17	Flat Coated Retriever	8	mc	0	0	5	1.a	II	3	5.98
18	Giant Poodle	7	mc	0	1	3	1.a	I	1	4.68
19	Havanese	13	fc	1	1	5	1.a	II	1	4.76
20	Labrador	8	m	1	0	5	1.a	I	2	4.46
21	Labrador	11	mc	0	1	5	1.a	I	1	5.77
22	Labrador	9	m	1	1	5	1.a	I	3	5.75
23	Labrador	7	mc	0	1	5	1.a	II	3	6.41
24	Puli	12	mc	1	1	2	1.a	II	3	5.27
25	Russian Black Terrier	10	m	0	0	2	1.a	II	2	6.77

26	Russian Black Terrier	10	m	0	1	4	1.a	III	3	6.77
27	Standard Poodle	14	fc	0	1	5	1.a	II	2	5.45
28	Giant Schnauzer	12	f	0	1	4	1.b	II	1	6.68
29	Giant Schnauzer	10	f	1	1	4	1.b	II	3	5.79
30	Giant Schnauzer	9	mc	0	0	5	1.b	III	3	5.85
31	Giant Schnauzer	8	mc	0	0	5	1.b	I	3	5.35
32	Giant Schnauzer	11	mc	0	1	5	1.b	II	2	6.55
33	Giant Schnauzer	10	m	0	1	1	1.b	II	1	5.36
34	Giant Schnauzer	8	m	0		1	1.b	I	1	5.97
35	Giant Schnauzer	10	m	0	0	2	1.b	II	3	5.73
36	Giant Schnauzer	12	fc	1	0		1.b	II	2	6.65
37	Giant Schnauzer	12	fc	0	1	2	1.b	II	3	5.91
38	Giant Schnauzer	8	m				1.b	II	2	5.29
39	Giant Schnauzer	12	mc	0	1	2	1.b	II	2	5.81
40	Giant Schnauzer	10	mc	0	1	5	1.b	II	2	6.30
41	Giant Schnauzer	7	m	0	1	5	1.b	II	1	6.10
42	Giant Schnauzer	11	f	1	0	5	1.b	II	1	6.85
43	Giant Schnauzer	11	f	1	0	5	1.b	II	2	6.85
44	Giant Schnauzer	12	fc	0	1	3	1.b	II	1	4.75
45	Giant Schnauzer	6	m	0	0	2	1.b	II	2	5.30
46	Giant Schnauzer	11	f	1	1	5	1.b	I	1	5.59
47	Giant Schnauzer	9	mc	0	0	2	1.b	II	2	6.15
48	Standard Schnauzer	5	f	1	1	5	1.b	II	3	6.70
49	Standard Schnauzer	6	mc	0		4	1.b	I	1	5.21
50	Standard Schnauzer	8	fc	1	1	1	1.b	II	1	5.16

51	Standard Schnauzer	13	f	1	1	1	1.b	III	3	5.87
52	Standard Schnauzer	10	m	1	0	4	1.b	I	1	5.68
53	Standard Schnauzer	8	m	1	1	1	1.b	II	3	5.75
54	Standard Schnauzer	8	fc	1	0	5	1.b	I	1	4.44
55	Standard Schnauzer	12	mc	1	1	5	1.b	I	1	5.20
56	Standard Schnauzer	10	m	0	1	3	1.b	II	2	5.47
57	Standard Schnauzer	13	m	0	1	5	1.b	II	2	5.41
58	Standard Schnauzer	9	fc	0	1	5	1.b	I	1	5.40
59	Standard Schnauzer	10	fc	0	1	2	1.b	I	2	4.70
60	Standard Schnauzer	12	mc	0	0	1	1.b	I	1	5.20
61	Standard Schnauzer	11	mc	1	1	5	1.b	I	2	4.90
62	Bernese Mountain Dog	10	mc	0	0	1	1.c	I	1	4.13
63	Bernese Mountain Dog	6	f	0	0	3	1.c	I	1	5.87
64	Gordon Setter	14	mc	1	1	1	1.c	II	3	4.96
65	Gordon Setter	10	m	1	0	2	1.c	I	1	5.02
66	Gordon Setter	9	m	0	0	2	1.c	II	3	6.68
67	Rottweiler	11	m	0	0	4	1.c	I	1	5.45
68	Rottweiler	10	fc	1	1	3	1.c	III	3	6.35
69	Briard	8	m	1	0		2	II	1	5.30
70	Irish Red Setter	14	fc	0	1	4	2	I	1	5.73
71	Mixed	4	f	1	1	3	2	I	1	2.17
72	WHWT	12	f	0	1	1	2	I	1	4.98

5. DISCUSSION

In the hereby included studies, there was a systematic approach for the classification and grading of canine digital squamous cell carcinoma (DSCC) to characterize the degree of histological malignant features. This allowed to correlate the degree of differentiation or "malignancy" in a more objective manner. Given this, it was possible to associate the given grade to the respective animal´s phenotypical haircoat color and the number of copies within the KITLG gene. Although studies correlating KITLG CNV and DSCC have been attempted in the past (Karyadi et al., 2013), this is the first time that their individual morphological features of malignancy are taken into account. Additionally, this represents the largest collection of DSCC and pairing blood samples that has been made up to date.

5.1. Examination of material, classification schemes and limitations

The collected material included histological samples from canine digital squamous cell carcinoma that were submitted to LABOKLIN GmbH &Co. KG between the years 2014 and 2019 for routine diagnostics. From these, only those with a clear invasive front and known haircoat color were included in the study. This made a final selection on 94 individuals for the first study. As a retrospective search, those digits diagnosed with a subungual keratoacanthoma were not revised or included in this study.

For the second phase, animals with DSCC and pairing whole blood samples were included for further genetic testing. Blood collected was from screening or diagnostic testing. At the end, this accounted for a total of 70 animals, some of which were from study 1.

The median age of the dogs for both studies was 10 years of age, ranging from 4 to 16 years. In both studies, the proportion of males was superior (study 1: 55% males vs 38% females; study 2: 60% male vs 40% female). Consistently, the animal´s phenotypical haircoat and claw color were noted and subdivided in groups accordingly. As a limitation, there was no underlying genetic assessment for genotypic color concerning the main loci involved given their color/pattern (in this case K, A and E) in the distribution of light and dark hair and claw pigment. This was assumed, yet never truly corroborated. With this, the grouping of the animals was based solely on the phenotypical color. At the end, the number of "black" (non-Schnauzers (study 1: n = 11; study 2: n = 15) and black Schnauzers (study 1: n = 34; study 2: n = 33)) and "black and tan" breeds (study 1: n = *31; study 2:* n = 7) were far more numerous than the "light" coated breeds *(*study 1: n = 18; study 2: n = 4). This may be seen as a limitation, given the

disparity of groups for statistical analysis, but also correlates with the proportions given in the literature (Belluco et al., 2013). As an interesting side note, Belluco and colleagues (Belluco et al., 2013) reported the forelimb as the most common location for the DSCC development, similar to other literature (Aupperle-Lellbach et al., 2023 (2); Wobeser et al., 2007) and corroborated in the present study.

Invasive front (IF) evaluation and tumor budding are histological features that are increasingly gaining interest within pathology. This is because they have proven to be of diagnostic and prognostic relevance in different tumors. Some examples include squamous cell carcinoma of the oral cavity, (Acharya et al., 2020; Joshi et al., 2020; Mohtasham et al., 2021; Shimizu et al., 2018; Yamakawa et al., 2019), larynx/hypopharynx (Boxberg et al., 2019), uterus (Jesinghaus et al., 2018), invasive carcinoma of the breast (Hiratsuka et al., 2022), submucosal colorectal cancer (Kim et al., 2022), and adenocarcinoma of the lung (Yamaguchi et al., 2010). IF interest is based on the fact that, in SCC, the centrally located neoplastic cells are often better differentiated than those located within the periphery, which are responsible for the infiltration of the surrounding stroma (Bryne et al., 1989, 1992; Nagamine et al., 2017). As currently there are no widely accepted grading systems for DSCC in veterinary medicine, taking a modern approach on tumor evaluation, two different grading schemes were adapted for DSCC. These were referred as “Invasive front grading system” (IFGS) and “Tumor cell budding grading system” as an adaptation of Nagamine et al. (Nagamine et al., 2017) and Jesinghaus et al. (Jesinghaus et al., 2018), respectively.

At this stage, the two grading systems were not ultimately performed for prognostic purposes, as many of the animals failed to have medical follow-up. Therefore, the ultimate aim at this point was to characterize the features of histological “malignancy” within the invasive front. This correlation between the grade provided by either systems and other prognostic data such as overall survival time or disease-free interval would have been interesting but was beyond the scope of these particular studies. Another natural limitation of the histological evaluation of the invasive front is the assumption of a 3-dimensional correlation on a 2-dimensional (the histological slide) image. The buddings and infiltration noted histologically were assumed to be independent and unrelated to the main neoplasm. However, one can never truly know if some of those buds may be connected to the main neoplasm or other, larger buds in another plane, forming larger islands and, therefore, not classified as true “buds” (5 or less independent neoplastic cells within the invasive front).

This classification was made with a concordance in “low grade/well-differentiated” tumor between both invasive front grading system (IFGS) and tumor cell budding grading system (TCBGS).

To assess the KITLG, droplet digital polymerase chain reaction (ddPCR) was assessed to evaluate the CNV of this gene. The test was internally validated and ran twice, to ensure a consistent result. The average of the two provided results was the final KITLG CNV documented. There was no additional testing to relate copy number presence of the gene and the final protein expression, for example, through means of FISH or immunohistochemistry. However, as a preliminary study, this may open the door to future investigations. Interestingly enough, in human literature there are also few reports with sparse c-KIT immunopositivity in SCC (12.5% and 20% of cells) (Fan et al., 2013; Goto et al., 2016; Moreira et al., 2018; Nakagawa et al., 2005; Ramezani et al., 2021). This is despite being occasionally reported along a poorer prognosis in esophageal SCC (Fan et al., 2013).

5.2. Comparison between DSCC histological grades and correlation with phenotypical haircoat color, and KITLG

Canine digital squamous cell carcinomas are known to be more biologically aggressive than their cutaneous counterpart. Despite this, there is currently no objective histological methodology that characterizes these different tumors. This possibility would allow to study them in a larger scale or correlate them with their biological behavior. The closest thing available to a histological grading is the, somewhat outdated, Broder´s system with its own limitations, as previously explained. These newer grading adaptations allow a more modern, objective approach for the histological classification of these very common tumors.

Both histological grading systems chosen were adapted to focus on the invasive front (IF) as main area of evaluation. However, they paid attention to slightly different morphological features. This allowed a better characterization of what may be histologically relevant for a potential, future, prognostic determination. The first one, named “Invasive Front grading system” (IFGS) adapted from veterinary Nagamine et al. (Nagamine et al., 2017) was, on itself, taken from human literature Byrne et al. (Bryne et al., 1989, 1992) as a novelty. The second grading system was from, fairly recent, human literature Jesinghaus et al. (Jesinghaus et al., 2018) and Boxberg (Boxberg et al., 2019), termed as “Tumor cell budding grading system” (TCBGS). These grading systems allowed a methodological, reproducible approach on the evaluation of the tumor, focusing on the most malignant section, the invasive front.

To challenge this, a total of 94 DSCC, divided according to their phenotypical haircoat color, were evaluated. As expected, the light-haired breeds (group 2. n = 18) were far less numerous than the dark-coated group (group 1. n = 76), as per reported literature (Belluco et al., 2013; Chiu et al., 2022; Grassinger et al., 2021). To further classify the dark-coated group,

a subdivision was made, according to their color pattern (1a/black breeds, *n* = 11; 1c/black & tan breeds, *n* = 31). A special subgroup was created for black Schnauzers (1c, *n* = 34) given their high prevalence for this tumor.

Group 2, comprised of light-coated animals, had generally consistent features of better differentiation and less histological malignancy within the invasive front than all dark-coated breeds (group 1). This resulted in lower scores in both grading systems challenged ($p < 0.01$ for both IFGS and TCBGS), as well as numerous individual features such as pattern of invasion ($p < 0.001$), degree of keratinization ($p < 0.05$), nuclear pleomorphism ($p < 0.001$), tumor cell budding foci ($p < 0.001$) or smallest nest size ($p < 0.001$). These differences also consistently translated into lower scores for these individual features as compared to the dark-coated groups. Only host inflammatory response and mitotic activity seemed statistically insignificant. These systems seemed to come to agreement in the most well-differentiated tumors, but there was a discrepancy in tumors with poorly differentiated/malignant features. This is likely due to the attention paid to different features within the invasive front. Additionally, it is important to identify the IF as consistently being the area of poorest differentiation. This allows to address it in a numerical and systematic manner, opening a gateway to further characterize these tumors, and associate it with a potential prognosis.

In these two schemes challenged, there was a significant difference between the degree of differentiation and the phenotypical haircoat color. This suggests a true histological disparity between dark and light-coated breeds, which somewhat corresponds to a poorer prognosis in some darker afflicted breeds (Belluco et al., 2013). However, in the end, one grading system cannot be concluded as “superior” to the other, given that, at least in this study, there was no prognostic or clinical correlation. These grading schemes were a somewhat novel concept as, unfortunately, a similar, objective, numerical histological grading has not been attempted in DSCC in veterinary medicine. Nonetheless, Belluco et al. (Belluco et al., 2013) did an excellent and detailed characterization of canine DSCC in an epidemiological, histological and immunohistochemical descriptive manner, even if a global differentiation score was not given. However, similar to our study and other literature (Nagamine et al., 2017), the most poorly differentiated areas coincide with the invasive front (Belluco et al., 2013).

To further understand the role of the KITLG in the development and/or histological features of DSCC in dogs, a genetic assessment had to be challenged after the gradings were validated and compared. This second study, up to our knowledge, was the first study correlating the copy number variation on the KITLG gene with histopathological features of malignancy in canine DSCC. This study was designed to further elucidate the role and involvement of the CNV in the KITLG in DSCC in dogs, following the research conducted by Karyadi and colleagues (Karyadi et al., 2013). This, supported by the aforementioned results

of the first study, was followed by the premise that certain dark-coated breeds, such as black Schnauzers or Poodles seem to have a poorer prognosis than other dogs after the diagnosis of DSCC (Belluco et al., 2013; Karyadi et al., 2013). As already mentioned on study 1, darker coated breeds had higher histological grade than those lighter ones. With this, along with studies that had proven that haircoat intensity is conferred, at least partially, by a higher expression and copies of KITLG gene (Bannasch et al., 2021), a correlation between the histological grade and CNV KITLG gene had to be addressed.

Currently, there are few studies linking an increased number of copies in the KITLG gene and the presence of DSCC in dogs (Aupperle-Lellbach et al., 2023 (2); Karyadi et al., 2013). For one, Karyadi and colleagues suggested that black standard Poodles with more than 4 copies of KITLG were more predisposed to DSCC. This would also explain their known predisposition to this type of neoplasia (Karyadi et al., 2013). Similarly, Aupperle-Lellbach and colleagues, made a similar finding in black giant Schnauzers, suggesting that a CNV > 5.8 had a significant increased risk for the development of DSCC than their healthy controls. That same study identified a decreased risk for those animals with a CNV < 4.7 for the KITLG (Aupperle-Lellbach et al., 2023 (2)). These studies observed a correlation between the presence of DSCC on different canine breeds and the CNV of KITLG, but did not characterize its histological features, which may potentially have an impact on the prognostic outcome.

Study 2 challenges the aforementioned grading schemes previously conducted in Study 1 with the CNV of KITLG by ddPCR on blood. The genetic testing for KITLG CNV was performed on the animals included in Study 1 and some additional ones with the same inclusion criteria, collected later on. As a result, a significant correlation was established between the KITLG CNV and the histological grade and score of both IFGS ($p = 0.006$) and TCBGS ($p < 0.001$) after performing an optimal linear regression model using the R function “step” with direction “both” and the Bayesian information criterion (BIC). The identified optimal regression model considers only the KITLG CN value and the IFGS score value, with a strong significant correlation between these two features (IFGS score ~ 1.12 * CNV KITLG + 4.96; $p = 0.006$). In the present study, the CN KITLG ranged from 20.2 to 9.14, with a mean of 5.6. This is superior to 4, as suggested by Karyadi and colleagues (Karyadi et al., 2013) in black Poodles, prone to the development of this neoplasia. In the case of black giant Schnauzers, as a comparison with Aupperle-Lellbach and colleagues, the average for this entire group, of both standard and giant Schnauzer, was established at 5.7. However, if the average is only applied to the giant Schnauzers of the group ($n = 20$) with KITLG CNV varying between 5.3 and 6.85, there is an average of 5.94. This result does go in concordance with the assessment made by Aupperle-Lellbach and colleagues, establishing a

predisposition in those animals with a KITLG CNV of more than 5.8 (Aupperle-Lellbach, et al., 2023 (2)). As a side note, it has to be mentioned that some of the Schnauzers included in the Aupperle-Lellbach and colleagues were also part of the current study. Despite that, this may infer that a higher KITLG CNV may not only confers a predisposition for the development of DSCC, but also potentially make the neoplasm more histologically malignant. This can be correlated to the designed aforementioned linear regression model. Furthermore, in the future, perhaps, by knowing a CNV KITLG gene of a given animal, the histological grade of a present DSCC can be predicted, thus taking according therapeutical steps.

6. CONCLUSIONS AND FURTHER STUDIES

These studies allowed to compare, in a larger scale, histological grade of canine DSCC focusing on the invasive front, while taking into consideration their phenotypical color and KITLG CNV. This is, to our knowledge, the first histological evidence that digital squamous carcinomas are not only more common in dark-haired dogs, but potentially more aggressive, based on histological evidence, with a clear correlation with the KITLG CNV.

Currently there are no modern histological gradings for canine DSCC taking into account the invasive front, which has proven of prognostic significance in other neoplasias (Mohtasham et al., 2021; Shimizu et al., 2018; Yamakawa et al., 2019). Based on this, it would be interesting to perform the namely “Invasive front grading” and “Tumor cell budding grading” in affected animals and correlate it with other clinical factors, such as “disease-free-interval”, “metastatic rate” or “survival time”, as seen in other studies which had available follow-up information (Belluco et al., 2013; Chiu et al., 2022; Henry et al., 2005; Marino et al., 1995; O’Brien et al., 1992). This could potentially aid the clinician for the best course of action as far as treatment and other follow-up evaluations.

Additionally, other researches have been performed on Schnauzers (Aupperle-Lellbach et al., 2023 (2)), correlating the risk of development of DSCC to KITLG CNV. This research line could be similarly extended to other potentially predisposed breeds. This may have great clinical implications as far as approach and further treatment.

However, as previously discussed, the presence of a higher KITLG CNV in a given DNA does not necessarily correlate with final amount of protein expression, as evolutionary strategies such as gene silencing remain possible (Rodin & Riggs, 2003). To truly understand the role that KITLG plays in DSCC, further studies are needed. It may be interesting to investigate final protein expression detection of c-KIT or other molecules associated with the SCF/c-KIT pathway by means of immunohistochemistry. However, in veterinary medicine, the expression of c-KIT is often confined to gastrointestinal stromal tumor identification (Frost et al., 2003; C.-E. Wu et al., 2019) and as part of prognostic panels for cutaneous canine mast cell tumors (Patruno et al., 2014; Preziosi et al., 2004). Unfortunately, there is not a wide-spread study of c-KIT expression in SCC. On the other hand, in human medicine, it has occasionally been correlated with an overall poorer prognosis (Fan et al., 2013).

7. SUMMARY

Argiñe Cerezo Echevarría

“Evaluation and analysis of canine digital squamous cell carcinoma - Histological grading correlation to microscopic features of malignancy in the invasive front and copy number variation of the KIT ligand”

Canine digital squamous cell carcinomas (CDSCC) tend to behave more aggressively than other areas of the skin. Furthermore, CDSCC in dark-haired animals are more biologically aggressive than in light-haired animals. However, up to date there is no standardized histological evaluation and comparison of DSCC morphology between dark and light breeds. In addition, dark-haired dogs have a known predisposition to develop DSCC. Additionally, some studies have suggested that dogs with a copy number > 4 in the KITLG locus, which is associated with melanogenesis, have an increased susceptibility to the development of DSCC. However, if the KITLG copy number had an effect on the morphology and histopathological features of DSCC still remains largely unknown.

Study 1 included the histological evaluation of DSCC from 94 dogs, divided into two groups, (1) dark-haired (*n* = 76) and (2) light-haired breeds (*n* = 18). Group 1 was further subdivided into three subgroups, (1a) various black-haired breeds (*n* = 11), (1b) black Schnauzers (*n* = 34) and (1c) black & tan breeds (*n* = 31). For an objective evaluation, two known grading schemes for squamous cell carcinomas were used comparatively. As a result, both histological grading systems exhibited significant differences between groups 1 and 2. Digital SCC of the light-haired dogs were consistently better differentiated than those of group 1. There were no significant differences between the different dark-haired breeds in any of the individual characteristics assessed (invasive front, degree of invasion, nuclear pleomorphism, tumor cell buds, smallest tumor nest size and amount of tumor stroma).

For study 2, 70 blood samples were collected from dogs with DSCC (partially overlapping with study 1). This study was designed to test whether and to what extent the previously evaluated histologic grades correlated with the number of copies KITLG locus determined by ddPCR. For the second study, the grouping was established as follows; Group 0/unknown haircoat color (*n* = 11); Group 1.a/black non-Schnauzers (*n* = 15); group 1.b/black Schnauzers (*n* = 33); group 1.c/black & tan dogs (*n* = 7); group 2/light-haired animals (*n* = 4). The results showed a significant correlation between increased KITLG copy number and more

malignant histologic grading. This suggests that KITLG may have a role in the development of DSCC by causing different, more aggressive morphologic features.

The compilation of these two studies may help to better characterize and understand canine squamous cell carcinoma of the toe. By taking genetic predispositions into account, a better and individualized assessment of the risk of disease in black dogs and thus earlier treatment is possible.

8. ZUSAMMENFASSUNG

Argiñe Cerezo Echevarría

„Digitales Plattenepithelkarzinom des Hundes und seine genetische Faktoren – Eine histologische Bewertung von digitalen Plattenepithelkarzinomen bei Hunden unter Berücksichtigung der Fellfarbe und der Korrelation mit der Kopienzahlvariation des KIT-Liganden-Gens“

Kanine digitale Plattenepithelkarzinome (dPEK) neigen dazu, sich aggressiver zu verhalten als Plattenepithelkarzinome in anderen Bereichen der Haut. Außerdem verhalten sich dPEKs in dunkelhaarigen Rassen in der Regel aggressiver als in hellhaarigen Tieren. Allerdings wurden bisher keine standardisierten histologischen Bewertungen und Vergleiche der Morphologie von dPEK zwischen dunklen und hellen Rassen durchgeführt.

Darüber hinaus haben dunkelhaarige Hunde eine Prädisposition für die Entwicklung von dPEK. Dies deutet auf zugrunde liegende genetische Faktoren hin, die mit der Fellfarbe assoziiert sein könnten. Einige Studien legen zudem nahe, dass Hunde mit einer Kopienzahl von > 4 im KITLG-Locus, der mit der Melanogenese in Verbindung steht, eine erhöhte Anfälligkeit für die Entwicklung von dPEK aufweisen. Es war auch unbekannt, ob die KITLG-Kopienzahl einen Effekt auf die Morphologie und histopathologischen Merkmale von dPEK hat.

Die Studie 1 umfasste die histologische Bewertung von dPEK bei 94 Hunden, aufgeteilt in zwei Gruppen, (1) dunkelhaarige (n = 76) und (2) hellhaarige Rassen (n = 18). Gruppe 1 wurde weiter in drei Untergruppen unterteilt, (1a) verschiedene schwarzhaarige Rassen (n = 11), (1b) schwarze Schnauzer (n = 34) und (1c) black & tan Rassen (n = 31). Für eine objektive Bewertung wurden zwei bekannte Gradierungssysteme für Plattenepithelkarzinome vergleichend angewendet. Im Ergebnis zeigten beide histologischen Gradierungssysteme signifikante Unterschiede zwischen den Gruppen 1 und 2. Digitale PEKs der hellhaarigen Hunde waren durchweg besser differenziert als die der Gruppe 1. Es gab keine signifikanten Unterschiede zwischen den verschiedenen dunkelhaarigen Rassen in einem der bewerteten Einzelmerkmale (invasive Front, Grad der Invasion, nukleärer Pleomorphismus, Tumorzellknospen, kleinste Tumornestgröße und Menge des Tumorstromas).

Für die Studie 2 wurden 70 Blutproben von Hunden mit dPEK gesammelt (teilweise überlappend mit der Studie 1). Diese Studie sollte prüfen, ob und inwieweit die zuvor

evaluierten histologischen Grade mit der Anzahl der Kopien des KITLG-Locus korrelieren, die mittels ddPCR bestimmt wurden. Für die Studie 2 wurde die Gruppierung wie folgt festgelegt: Gruppe 0 unbekannte Fellfarbe (n = 11); Gruppe 1a schwarze Nicht-Schnauzer (n = 15); Gruppe 1b schwarze Schnauzer (n = 33); Gruppe 1c black & tan Hunde (n = 7); Gruppe 2 hellhaarige Tiere (n = 4). Die Ergebnisse zeigen eine signifikante Korrelation zwischen einer erhöhten KITLG-Kopienzahl und dem maligneren histologischen Grading. Dies legt nahe, dass KITLG eine Rolle in der Entwicklung von dPEK haben könnte, indem es unterschiedliche, aggressivere morphologische Merkmale verursacht.

Die Zusammenstellung dieser beiden Studien kann dazu beitragen, die Plattenepithelkarzinome an der Zehe von Hunden besser zu charakterisieren und zu verstehen. Unter Berücksichtigung der genetischen Prädispositionen ist eine bessere und individualisierte Bewertung des Erkrankungsrisikos bei schwarzen Hunden und somit eine frühzeitigere Behandlung möglich.

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10. PUBLICATIONS AND SCIENTIFIC ACTIVITY

1. Cerezo-Echevarria A, A Kehl, C Beitzinger, T Müller, R Klopfleisch, H Aupperle-Lellbach (2023) Correlation between malignant histological features of canine digital Squamous cell Carcinoma and genetic KIT ligand copy number variation. ESVP/ECVP/ESVCP/ECVCP Joint Meeting; 2023 Aug 30 - Sept 02; Lisbon, Portugal. p.92

2. Digital lesions in dogs: A statistical breed analysis of 2,912 cases. *Veterinary Sciences* (2021). 8(7): 136
   Julia Maria Grassinger, A. Floren, T. Müller, A. Cerezo-Echevarria, C. Beitzinger, D. Conrad, K. Törner, M. Staudacher, Heike Aupperle-Lellbach

3. Tumor frequencies in Schnauzers according to size variants, with a special emphasis on Squamous Cell Carcinoma. Cerezo-Echevarría A., Törner, K., Heidrick, D., Beitzinger, C. Grassinger, J.M., Aupperle-Lellbach, H. (*2022) Poster presentation at Southern European Veterinary Conference, National AVEPA Congress. October 21st, 2022*

4. Correlation between malignant histological features of canine digital squamous cell carcinoma and genetic KIT ligand copy Number Variation (2023). Cerezo A., Kehl A., Beitzinger C., Müller T., Klopfleisch R., Aupperle-Lellbach- *Oral Presentation at Joint Congress of ESVP/ECVP/ESVCP/ECVCP. August 31st -September 2nd, 2023*

11. CONFLICTS OF INTEREST STATEMENT

There are no conflicts of interest in the creation and evaluation of the work. However, I, Argiñe N. Cerezo Echevarría, am a veterinary pathologist and work at LABOKLIN GmbH & Co. KG. My employer offers commercial clinical-pathological and histopathological examinations.

12. DECLARATION OF INDEPENDENCE

I hereby confirm that I have completed this work independently. I certify that I have only used the sources and aids indicated.

Berlin, 2024

Argiñe Cerezo Echevarría