Pathogenetic studies of sarcoma development in retriever breeds

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Colofon

Lay-out: Proefschrift-AIO.nl
Cover: A. Dertien, Multimedia; photos: Kim Boerkamp
ISBN: 978-90-393-6234-1

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Pathogenetic studies of sarcoma development in retriever breeds

Pathogenetische studies naar de ontwikkeling van sarcomen bij retrievers (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 11 december 2014 des middags te 6.00 uur

door
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geboren op 19 oktober 1977 te Oldenzaal

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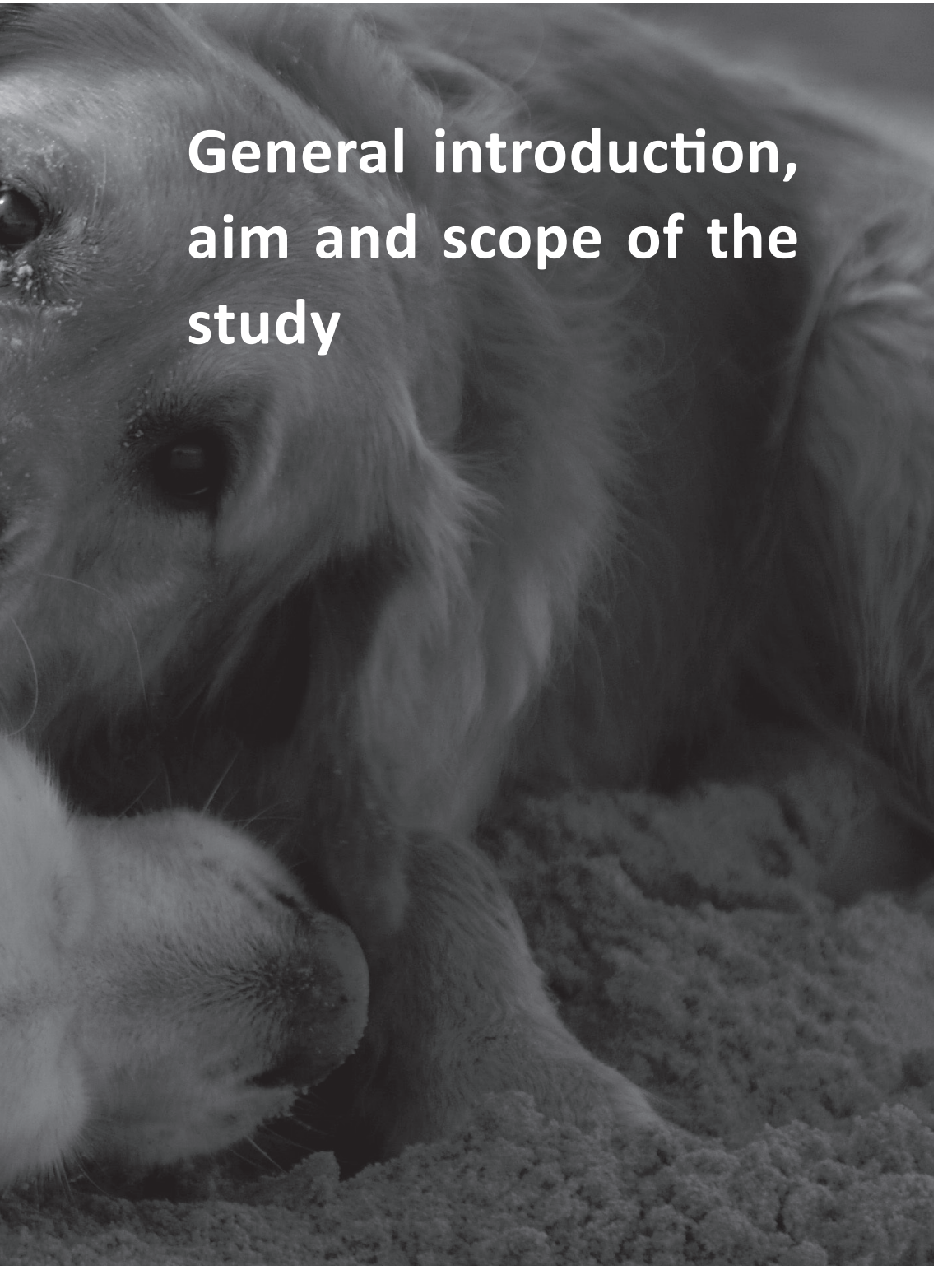
Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van:

AUV Veterinary Services
Boehringer Ingelheim B.V.
BOKA metaalbewerking
Merial
Nederlands KankerFonds voor Dieren (NKFD)
Royal Canin
Scil animal care company B.V.
Zoetis

Contents


1



General introduction, aim and scope of the study

Introduction

At present, dogs play an important role in daily society. Not only do they function as companion animals, but they are also highly appreciated as working animals, such as guide dogs, security dogs, and service dogs. Indirectly they can also be of value for man, since some dog breeds are predisposed for many of the same diseases that also affect humans. They may function as a natural animal model living in close proximity with us [1, 2]. This disease predisposition is a result of the way that dogs are selected for breeding, which is based on physical appearance and ability to perform certain tasks [3, 4]. Due to the small number of individuals within a breed the net result is a 'genetical weakening', making dogs of specific breeds to be much more genetically linked than most animals in nature. Cancer is one of the diseases that in some dogbreeds is more expressed as a result of this genetical weakening. In the last decades, it has become clear that the incidence of specific cancers varies amongst different breeds [5, 6] including that of soft tissue sarcomas (the topic of the thesis) for which many of the retriever breeds are predisposed [7-13]. Predisposition for a disease is often first suspected by the consulted veterinarians, since they are the first to whom animals that suffer from a disease are presented.

Golden retrievers have been reported in several countries to be more often affected by soft tissue sarcomas [7-10], a heterogeneous group of mesenchymal malignancies known to share many of the clinical and histopathological characteristics of human soft tissue sarcomas [14-16]. This raised the question whether a breed predisposition for various types of cancer, such as the development of STS, also exists for the Dutch population of the breed. The way tumours were diagnosed could have influenced the reported incidences. Therefore, this study into the incidences of the various tumours in this breed, included the diagnostic method chosen (cytology *versus* histology). To this aim, the annual estimated incidence rates were examined and compared to incidence rates from previous publications. The study group consisted of Golden retrievers from which suspected neoplasms were submitted to the Veterinary Faculty of the Utrecht University during the period 1998-2004, and that were diagnosed either by means of cytology (n=2,529) or histology (n=2,124). We related those to an average annual Dutch kennel club population of 29,304 Golden retrievers (**Chapter 2**).

As a logical consequence of this research, subsequent questions arose. The group of soft tissue sarcomas proved to be large and – based upon routine histopathological analysis - included many cases without definite diagnosis with respect to subtype. Therefore, an attempt was made to improve the differentiation, by use of immunohistochemical markers if deemed necessary. In humans, a well-defined and classified population of STS is already known to be of value in additional pathogenetic studies as well as expected to improve treatment

options and prognosis [17-20]. From the Golden retriever study group described in Chapter 2, a cohort (n=110) of malignant mesenchymal tumours were revised. Focus was put upon cases that were, on initial histological evaluation, considered '*sarcoma, not otherwise specified or of uncertain subtype*'. After thorough revision of histomorphology, additional immunohistochemistry (IHC) was applied if deemed appropriate (**Chapter 3**).

In addition to the subtyping of mesenchymal tumours in Chapter 2, molecular methods were applied to further differentiate between behavioral features of this group of mesenchymal neoplasms. In **Chapter 4**, we evaluated to which extent DNA-aneuploidy is a suitable marker of malignancy (and, should DNA aneuploidy be present, the level of genomic instability) by measuring the nuclear DNA content in mesenchymal lesions. Goal of this study was to find out whether this molecular method could improve the differentiation between behavioral features of the different mesenchymal proliferations. A flow cytometric method to determine DNA-ploidy and -index was applied upon a series (n=95) of canine inflammatory or neoplastic mesenchymal tissues and related to clinico-pathological features, biological behavior and –determined by earlier study - p53 gene mutational status.

Next, an attempt was made to identify possible genetic variants associated with soft tissue sarcoma development in the Golden- and Labrador retriever. Genetic variants could possibly explain these breeds' predispositions for the development of this group of cancers [7-13]. Thus, the first genome-wide association study (GWAS) in the combined group of soft tissue sarcomas in any species was performed, aiming to identify various different loci associated with soft tissue sarcomas in these two retriever breeds. (**Chapter 5**)

Following the results of the broad GWAS on genomic DNA, we placed focus upon the possible existence of altered *expression* of genes in specific sarcoma subtypes as related to possible effects upon cellular processes. To this aim we chose a specific sarcoma subtype; the histiocytic sarcoma. The Flatcoated retriever breed is explicitly predisposed for this type of tumour [21, 22]. Gene expression studies of these tumours are potentially also of value for comparable human diseases, where (due to the rarity of histiocytic/dendritic cell sarcomas in humans) it is difficult to accrue such knowledge [2, 9, 23-26]. A microarray analysis and pathway analyses were the methods of choice to answer these questions. Analyses were performed on fresh-frozen tissues obtained from Flatcoated retrievers with localized, soft tissue histiocytic sarcomas and disseminated, visceral histiocytic sarcomas, and on normal canine spleens from various breeds. Eventually, significant expression differences of several genes were validated with quantitative real-time PCR (qPCR) analyses. (**Chapter 6**)
In the Flatcoated retriever, two distinct entities of histiocytic neoplasia have been reported; a soft tissue form and a visceral form [27]. The soft tissue form at first is localized; while the visceral, general form progresses more quickly

to a terminal state, which might be related to variations in gene expression. Though the breed can develop both entities, the Flatcoated retriever is clearly more prone to develop the localized version, in contrast to another breed, the Bernese mountain dog, in which the generalized form is much more often seen [23, 27]. This observation of a clear distinction in form of histiocytic sarcoma has led to our last question: Do these two main forms of histiocytoc sarcomas actually display *variation* in gene expression? We performed microarray analysis on fresh-frozen tissue from Flatcoated retrievers with either soft tissue- or visceral histiocytic sarcomas, and validated expression differences of some of the most significantly differentially expressed genes with quantitative real-time PCR (q PCR) analyses. (**Chapter 7**)

Chapter 8 will present a review, summarizing and discussing all previous chapters.

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2

Estimated incidence rate and distribution of tumours in 4,653 cases of archival submissions derived from the Dutch Golden Retriever population

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BMC Veterinary Research 2014; Volume 10, Issue 34

Abstract

Background

A genetic predisposition for certain tumour types has been proven for some dog breeds. Some studies have suggested that this may also be true for the Golden retriever breed. The present study aimed to examine a possible existence of a tumour (type) predisposition in the Dutch population of Golden retrievers by evaluating annual estimated incidence rates compared to incidence rates from previous publications. A second aim was to evaluate whether incidences of various tumours differed as related to the diagnostic method chosen, being either cytology or histology.

Results

Tumours submitted to Utrecht University during the period 1998-2004 diagnosed either by means of cytology (n=2,529) or histology (n=2,124), were related to an average annual Dutch kennel club population of 29,304 Golden retrievers.
Combining individual tumours from both the cytological and the histopathological data-set resulted in an annual estimated incidence rate of 2,242 for 100,000 dog-years at risk regarding tumour development in general.
The most common cytological tumour diagnoses were 'fat, possibly lipoma' (35%), mast cell tumour (21%) and non-Hodgkin lymphoma (10%). The most commonly diagnosed tumours by histology were mast cell tumour (26%), soft tissue sarcomas (11%) and melanoma (8%). Both the cytological and histopathological data-sets, showed variation; in patient age distribution, age of onset and incidence of various tumours.

Conclusion

Comparing our data with previous reports in non-breed-specified dog populations, the Golden retriever breed shows an increased risk for the development of tumours in general, as well as an increased risk for the development of specific tumour types, including the group of soft tissue sarcomas. Variations in age, location and incidence of various tumours were observed between the two data-sets, indicating a selection bias for diagnostic procedure.

Background

Breeding from within a selected population of dogs can, in a relative short period of time, give rise to a clear change in phenotype which leads to breed development [1, 2] but may also cause an increase in the occurrence of inherited diseases [3-7] such as cancer [8-12]. Clear evidence exists for a breed-related predisposition to specific cancers, like histiocytic sarcomas in Bernese Mountain dogs [10, 13] and Flatcoated retrievers, [11] anal sac carcinomas in the English Cocker Spaniel [12, 14] and hemangiosarcomas in German Shepherd dogs [15]. For the Golden retriever, an increased risk for the development of cancer in general has been reported by some [16, 17], but not all studies [18, 19]. Also, there are reports on an increased risk for specific types of cancer in Golden retrievers such as mast cell tumours (MCT) [12, 20] melanomas [21] and non-Hodgkin lymphomas (NHL) [12, 22, 23] and suggestions of a predisposition to soft tissue sarcomas (STS) [24-27]. The limitations of these studies are old age in the dogs, and the fact that many studies were hospital-based and of small size. Larger studies often rely on data from insurance companies without verification of cytological or histopathological diagnoses. There is need for larger studies that include examination of reports of diagnostic procedures to obtain more solid data on the relative tumour incidence. Such larger studies should enable better assessment of a possible predisposition for specific tumour types in breeds such as the Golden retriever. The results may serve as means to improve health of the breed as well as basis for comparative oncological research [12, 16]. Furthermore, regional variation in genetic population structure may appear [28].

Our aim was to obtain an estimate of the occurrence of tumours and the distribution of tumour types in the Golden retriever breed in the Netherlands. This was done by accessing the archives (1998 – 2004) from two of the main laboratories in the Netherlands – both located at the Faculty of Veterinary Medicine in Utrecht - that independently provide histopathological (Veterinary Medical Diagnostic Center) - or cytological diagnostic services (University Veterinary Diagnostic Laboratory).

Diagnostic management of dogs suspected to be affected with neoplasia may preferentially be done by cytological examination of fine needle aspiration biopsies (FNABs), by histopathological examination of resected masses, or by both. Which method is chosen, depends on multiple factors like accessibility of a possible neoplastic mass, the suspected tumour type, financial aspects, the availability of an experienced laboratory, etc. Most studies that investigated the incidence of cancer in dog populations have been based solely on histopathology or are unclear about the diagnostic method used. Our data-sets considered both histopathological- and cytological examination; separately and combined.

This retrospective study can, besides providing information on potential health risks within the Golden retriever, also be of help to veterinarians by providing possible differential diagnoses.

Results

Of a total of 4,313 fine needle aspiration biopsies (FNAB) of masses taken from Golden retriever dogs, a total of 2,529 cases were diagnosed as a suspected tumour (specific details are listed in Table 1). Remaining biopsies were either non-diagnostic, mostly due to poor cellularity of the specimen (n = 739; 17%) or diagnostic, but not considered to originate from a neoplasm (n=1,045; 24%). Of these non-neoplastic lesions, 51% were diagnosed as inflammation.

Tumours diagnosed by cytology mostly originated from the mesenchym (which included all benign and malignant mesenchymal proliferations of bone and soft tissue). Second most frequent were tumours that originated from hematopoietic origin (which included histiocytomas, histiocytic sarcomas, MCT, NHL, plasma cell tumours and atypical lymphoid/histiocytic proliferations).

Tumours in the histological data-set were mostly of hematopoietic origin (which included NHL, MCT, histiocytic sarcomas, splenic nodular hyperplasias/ splenomas, transmissible venereal tumours, thymomas, histiocytomas (CCH) and plasma cell tumours) followed by epithelial lesions (including benign mammary tumours, perianal gland adenomas, adenomas of other origin, ameloblastomas, basal cell tumours, epitheliomas, insulinomas, papillomas, trichoblastomas, trichoepitheliomas, and all (adeno-) carcinomas). Figure 1. shows this distribution into tissues of origin for both cytological and histological data-sets.

Both data-sets were cross-referenced for double entries and 54 cases were identified as being diagnosed with cytology as well as histology. Of these 54 cases, 18 cases were diagnosed as MCT, 16 as being a soft tissue sarcoma, six as NHL, six as carcinomas, four as peri-anal gland tumours, two as CCH, one as amelanotic melanoma and one as plasmacytoma.

The annual estimated incidence rate (EIR) was calculated, considering a population at risk of 29,304 dogs per year. Based on an average of 657 annually diagnosed tumours (using either cytology or histology), an EIR of 2,242 per 100,000 dogs was calculated for the occurrence of benign and malignant tumours in the Golden retriever dog and an EIR of 1,174 per 100,000 dogs for the development of only malignant tumours. Based merely on tumours diagnosed by histology, an EIR of 1,034 was calculated for the occurrence of all tumours- and an EIR of 615 for the development of malignant tumours, respectively (Table 2). Based on cytology alone, for the development of malignant tumours an EIT of 586 was calculated (Table 3).
In general, benign tumours occurred at a younger age than malignant tumours in the tumours diagnosed using cytology (8.40 vs 8.95 yrs; Δ = 0.55 years, $P < 0.001$) as well as the tumours diagnosed using histology (7.86 vs 8.35 yrs, Δ = 0.49 years, $P < 0.002$) (Figure 2). Also, a significant difference was found

Table 1: Patients characteristics

	Cytological data-set	Histological data-set	Combined set (excluding 54 double entries)
Number of tumours	2,529	2,124	4,599
• **Mean/year**	361	303	657
• **Nr of dogs in which a second tumour was detected**	69	46	115
• **Nr of dogs in which a third tumour was detected**	0	24	24
Male / Male neutered	795 / 365	719 / 320	
Female / Female neutered	530 / 649	413 / 600	
Median age	9.1 yrs (min. 0.2 yrs, max.17.2 yrs)	8.6 yrs (min. 0.1 yrs, max.17.2 yrs)	
Number of malignant tumours	1,203 (48%)	1,262 (60%)	2,414
Number of benign tumours	1,010 (40%)	761 (36%)	1,768
Unknown	316 (12%)	101 (5%)	417

Table 2: Estimated Incidence Rates (per 100,000 dog years at risk) of the most common types of benign and malignant histologically diagnosed tumours in the Golden retriever compared to Incidence Rates (per 100,000 dog years at risk) found in previous studies concerning the general dog population.

Histological data-set	EIR Utrecht	Incidence Rate Madison, Wisconsin [29]	(Standardized) Incidence Rate UK [30]	Incidence Rate Genoa, Italy [31]	Incidence Rate Alameda, Contra Costa [32]
General development of tumour	**1,034**		1,948	760	
Development of cancer	**615**		747.9	310	381
MCT	**265**		129		
STS	**114**	35	142		36
Melanoma	**82**	25			
CCH	**70**		377		
Benign mammary tumour	**48**		11		
Adenoma (non-mammary, non-peri-analgland	**45**				
NHL	**35**	25	114	19.9 (males) and 22.9 (females)	21.7

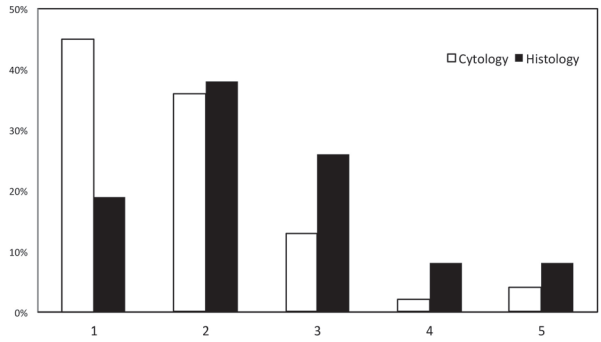


Figure 1: Distribution into tissues of origin (in percentage) for the cytological- and histopathological diagnosed data-sets.
Origin: 1: Mesenchymal origin, 2: Hematopoietic/lymphoid origin, 3: Epithelial origin, 4: Neuroectodermal origin, 5: Other origins (gonadal, glial, NOS)

Table 3: Estimated Incidence Rates (per 100,000 dog-years at risk) of the most common types of benign and malignant cytologically diagnosed tumours in the Golden retriever compared to Incidence Rates (per 100,000 dog-years at risk) found in previous studies concerning the general dog population.

Cytological data-set	EIR Utrecht	Incidence Rate Madison, Wisconsin [29]	(standardized) Incidence Rate UK [30]	Incidence Rate Genoa, Italy [31]	Incidence Rate Alameda, Contra Costa [32]
General development of tumour	**1,232**		2,671	760	
Development of cancer	**586**		748	310	381
'Fat, suspect lipoma'	**429**		318		
MCT	**265**		129		
NHL	**121**	25	114	19.9 (males) and 22.9 (females)	21.7
Perianal gland tumour	**68**				
(Adeno)carcinoma	**37**				
Mesenchymal proliferation, susp. STS	**37**	35	142		36
CCH	**30**		377		
Melanoma	**27**	25		0.7 (males) 0.6 (females)	25

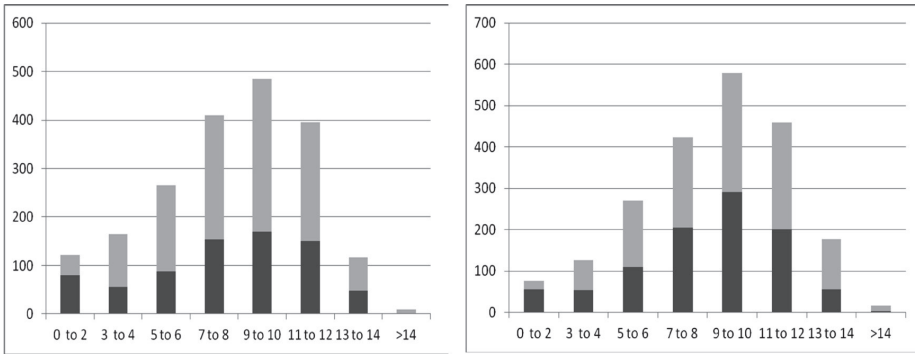
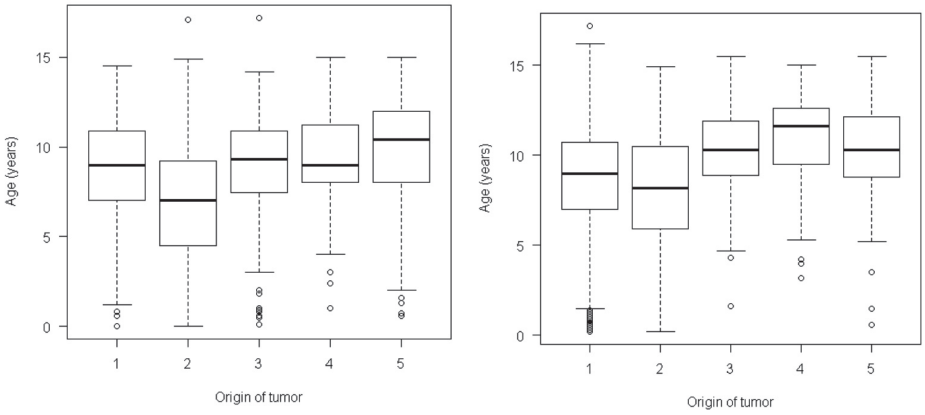


Figure 2: Age distribution of benign and malignant tumours as diagnosed by means of histology (Left) or cytology (Right).
Horizontal axis: Age of the dogs (years). Vertical axis: Number of cases diagnosed. Dark grey: Benign tumours. Light grey: Malignant tumours.



Age-distribution in different tissues of origins in tumours diagnosed using histopathology (Figure 3; left) and cytology (Figure 4; right).

Origin:
1: Mesenchymal origin;
2: Hematopoietic origin;
3: Epithelial origin,
4: Neuroectodermal origin;
5: Other ('NOS',gonadal origin; glial tumours)

in the median age of tumour-diagnosis between the two diagnostic methods (cytology: mean AOO: 8.76 yrs, histology: mean AOO: 8.19 yrs, Δ =0.57 yrs, *P* <0.001).

In both data-sets the median age at which tumours of mesenchymal origin were diagnosed was higher (9 yrs; range: 0-17.2) than that of tumours of hematopoietic origin (8.2 yrs (range: 0.2-14.9 yrs) by use of cytology and 7.0 (range: 0-17.1 yrs) by use of histology. Figure 3 and Figure 4 show the age-distribution in all different tissues of origin.

The Male: Female ratio (M:F) of all histopathological diagnosed tumours was 1.03 and that of all cytological diagnosed tumours was 0.98. No significant difference in gender was found for tumour development. The data provided by the submitting clinic did not consistently include neutering status or date of neutering, prohibiting an examination on the potential effect of age of neutering on tumour occurrence as published recently [33].

Significantly less frequently submitted were tumours derived from internal organs (GI-tract and genital tract: 9%) compared to neoplasms that are more easy accessible (skin and adnexa, 54%).

Discussion

Estimated incidence rates

The percentage of malignant tumours versus benign tumours was higher in the group diagnosed by histopathology than in the group diagnosed by cytology. This difference might be caused by the relatively high percentage of cases where cytological evaluation did not allow a reliable distinction between a benign or malignant neoplasm.

A striking finding was the existence of major variation in the representation for specific tumour types amongst the two data sets. This could be a result of the clinician's expectation of how likely it will be to obtain an accurate diagnosis by either method, or the possibility that immediate removal of tissue could be therapeutic. It may however also indicate a practice in which knowledge of the tumour type by cytology decreases the likelihood that the resected mass is submitted for histopathology. Future research should examine this in more detail, since it influences the level of veterinary care and will also influence epidemiological studies. The percentage of malignant tumours as well as its EIR in the tumours diagnosed using histopathology in the current study were higher than what has been reported in other studies that considered the general dog-population; namely in Norway (2008) [18] Italy (2009) [31, 34] and Alameda (1968) [32, 35]. This is suggestive of a general breed-predisposition for malignant tumours in the Golden retriever. This was also the conclusion of a study from Reid-Smith et al (*The incidence of neoplasia in the canine and patient populations of private veterinary practices in Southern Ontario (2000)*).

Table 4: Distribution of location of 2,124 tumours diagnosed using histopathology.

Location	Frequency	Percentage
Head (excluding skin/adnexa)	308	15
Skin and adnexa	1148	54
Mammae	187	9
Gastro-intestinal tract	72	3
Endocrine organs	12	0.6
Genital tract	118	6
Hematoproliferative system	58	3
Urogenital tract	12	0.6
Heart and lungs	6	0.3
Central nervous system	6	0.3
Soft tissue, other	143	7
Other	54	3

'Head' includes eye, nose mouth and sinus; 'skin and adnexa' includes skin and adnexa head, skin and adnexa (other), salivary gland, perianal gland, analsac, axial, abaxial, extra-skeletal; 'gastro-intestinal tract' includes stomach, pancreas, liver, bile duct, intestines; 'endocrine organs' includes adrenal gland, thyroid- and parathyroid gland; 'genital tract' includes male and female reproduction organs, 'hematoproliferative system' includes spleen, liver, thymus, lymph nodes.

The diagnosis 'Fat; possibly lipoma' was clearly the most commonly diagnosed benign lesion in the cytological data-set, which was also the case in a study in Denmark [19] and the UK [30]. These last studies consider a general dog population instead of one particular breed and both include cases diagnosed by both methods and not just histology. The EIR of 429 for 'fat; suspect lipoma' found in the current study within our cytological diagnosed tumours, was higher than the Incidence Rate (IR) found in a study in England (IR: 317) [30]. We could however not confirm this EIR in our histolological data-set (EIR: 2.40). In a Norwegian study that included only cases diagnosed using histology, [36] it was not the most common benign lesion. An explanation for this marked difference may be that not all veterinarians will have a cytologically diagnosed lipoma, or a lesion that is suspect to be a lipoma based on mere clinical examination, submitted for histopathological evaluation in our study. After exclusion of all 879 cases of 'Fat, suspect lipoma' from the cytological data-set, only 15% of the tumours could be considered of mesenchymal origin instead of 45%. Epithelial proliferations are then more common (20%) than mesenchymal lesions, which is consistent with our histopathological dataset as well as with previous research [30].

Of all histologically confirmed tumours, CCH was the most common benign tumour, although the EIR was lower than was expected based on previous studies [30]. The fact that the EIR in the cytological data-set is much lower than that of

the histological data-set is surprising. It illustrates that CCH is diagnosed more commonly using histopathological than cytological evaluation. CCH is a benign lesion [37, 38], and a study in 2007 already showed that it is easily recognizable using only cytological examination [37]. These lesions usually undergo spontaneous regression in younger dogs [37, 38] making surgery unnecessary. It is our hope that CCH in future will be more commonly diagnosed by cytology, leading to a shift in the proportion diagnosed by the two methods.
In both data-sets, the most commonly observed malignant lesion is the MCT. This is consistent with other studies that evaluate the general dog-population, such as the one in Denmark [19] and Norway [18, 36]. However the EIR calculated for MCT in both of the data-sets is much higher in relation to the EIR of MCT in dogs irrespective of breed than would be expected on the basis of previous reports [30]. This confirms the breed predisposition for MCT, already mentioned in earlier studies [12, 20]. The same holds true for the relatively high EIR for melanomas in the histopathological data-set when compared to previous studies [21, 29].
Another surprising finding was the high EIR (116) of NHL This was much higher than the IR found in most previous reports, with an IR of approximately 20 per 100,000 [29, 31, 32] and with a previous report by Teske et.al, that reported the IR of NHL to be at least 33 [39] for the general dog-population in The Netherlands. This higher risk of NHL in the current study might be based upon the fact that the diagnosis of NHL is usually made by cytological evaluation, a diagnostic procedure very often not included in previously reported epidemiologic studies [30, 32]. This observation of an higher than average risk supports the existence of a breed-predisposition as has already been suspected [17, 22, 23]. The EIR found in this study is comparable to the (age-standardized) IR found recently by Dobson et al. (IR: 107) that most likely included cytological diagnoses [30].

Cytology is not very effective in further differentiating a mesenchymal lesion [40] because of morphological similarities between reactive and neoplastic fibroblasts; therefore the diagnosis ‘soft tissue sarcoma’ is usually based on histological evaluation. The EIR for STS found in our histologically diagnosed data-set (EIR: 114) is higher than some previous publications in which an IR of 35 was reported, [29] but somewhat lower than the IR found by Dobson et al. (IR: 142) [30]. This last study, however, used age-standardized IR, which we could not. A breed predisposition for STS in the Golden retriever is therefore still considered possible.

Of the STS-subtypes that could be identified by routine histopathological evaluation in 1998 - 2004, the most frequently found tumours were fibrosarcomas (n=54; 23%), hemangiosarcomas (n=34; 15%) and neurofibrosarcomas (9%), which is largely in accordance with literature [32]. However, in 40% of all STS further differentiation proved impossible, so a more precise indication of a possible predisposition for a specific subtype was impossible.

Additional value of combining both data-sets

In this study we attempted to evaluate and explain the complementary value of combining results obtained by different diagnostic method of choice; being both cytological and histological examination. We clearly established that the incidence of NHL is underestimated in the histological data-set. Also, the difference in incidence in CCH as well as the diagnosis 'Fat; suspect lipoma' between the two data-sets, underlines the importance of combining both methods when performing a biopsy-based epidemiological study. The change of data registration exerted after 2004, led us to limit our survey to earlier years. Even considering a possible change in submission rates in recent years, the low number of patiens (1.8%) that was sent in for both cytological diagnosis as well as for histological diagnosis was still surprising, in particular with respect to the importance of achieving an assessment of grade and completeness of excision by histopathology after getting a diagnosis of tumour type by cytology. Too often – it appears - do veterinarians opt for either cytology or histology for diagnostic purpose. A growing awareness of the strength of combining both cytology and histology, can perhaps in future change this observation.

Also based on our results we therefore believe that optimal veterinary care of dogs suspected of neoplasia is best exerted by presurgical cytological examination of FNABs followed by histopathology of resected masses or, in cases deemed not manageable by surgery, by histopathology of tissues biopsies if cytology was inconclusive. Furthermore, such practice serves epidemiological studies of neoplasia.

Age, sex and location

As is shown in Figure 1, the incidence for development of a neoplasm was relatively low in younger animals, increased sharply after the age of three, and peaked at 9 years for the histopathological- and at 10 years for the cytological data-set. An age-dependent increase in incidence for the development of a neoplasm is in agreement with other studies [8, 30, 34]. Highest-peak-incidence was noted at a younger age in the current study than peak-incidence of cancers in a study in Italy (>12 years) [34] but at a comparable age when compared with the study of Dobson [30], which is remarkable, considering the potential bias for younger animals of this last study due to different age-structure [23]. Also, as was the case in a general dog-population studied in Denmark [19], benign lesions occur at a younger age than malignant lesions in both data-sets.

There is a significant difference ($P< 0.001$) in mean age of animals when comparing the two data-sets. Even when excluding CCH, a tumour reported to occur at a young age [41] as was confirmed in our study (median age 2.8 years and 2.9 years, respectively, for the cytologically- and histopathologically diagnosed tumours), this difference remained significant ($P< 0.001$). In accordance with literature [19], there appeared no sex predisposition for general tumour development. The data

provided by the submitting clinic did not consistently included neutering status or date of neutering, prohibiting an examination on the potential effect of age of neutering on tumour occurrence as published recently [33].

Taking a potential sampling bias for the occurrence of tumours into account, since more common locations are also more easily clinically accessible [8], the most common locations of histological diagnosed tumours were skin and adnexa (Table 4) which was also found in earlier studies [12, 17].

Veterinary cancer registries are few in number and scattered [32, 42]. Also, there is little information on age, incidence, type, location and behavior of tumours in canine populations in general [32, 42]. A population-based cancer registry is preferred over a hospital-based cancer registry, because it aims to represent all cases in a known population [43], whereas hospital-based cancer registries such as the ones in America [44, 45] and Italy [46] do not include cases that were seen only by primary care veterinarians, risking a potential bias [47].
Much of what we know so far on tumour incidence derives from the population-based veterinary cancer registry in California, the California Animal Cancer Registry (CANR) [32]. Results from this study are frequently used as reference data set but are more than 40 years old and were obtained in one specific region only.

More recently, various studies came to rely on insurance data, e.g. such as the ones in England [23, 30] and Sweden [48, 49]. This kind of research could lead to a potential age-related bias, since older dogs are less often insured [30, 50] and also excludes a presumed high portion of dogs that are not insured at all [47]. Additionally, diagnostic validation by histopathological or cytological examination, in addition to a diagnosis based upon clinical manifestation, is in some instances lacking. This may lead to uncertainty regarding the accuracy of the recorded diagnosis [43]. As an alternative method, some researchers have chosen to rely on veterinary cancer registries, like the study in Denmark [19], or questionnaires, such as studies in Norway [51] and Denmark [16]. These approaches carry the risk of a voluntary bias, because it is unlikely that owners report all tumours to the registry [16, 43]. Also, regarding surveys, the overall response rate is expected to be only half the sample population and includes a potential bias in responders versus non-responders [51]. The Norwegian Canine Cancer Register [52] and a study in Italy [34] tried to improve the number of diagnosed cases by offering free of charge histopathological examination of all tumours of dogs in four counties. However, cytological examinations were not included in this study, and studies that offer free of charge will remain an exception due to financial and logistical challenges.

In this study, we chose to assess the incidence of tumours in one specific dog breed, with a centrally accessible source of diagnostic data using a broad system of tumour classification. As was the case in other studies [30], also in our study

there was risk of a potential bias in both numerator and denominator. In our study, the strongest bias is most likely caused by the fact that not all private clinicians have their biopsies evaluated at the Utrecht University of Veterinary Medicine (UUVM). Note that the two participating laboratories are not the only diagnostic laboratories in the Netherlands. As samples numbers analysed by commercial laboratories are confidential we can only make a best estimated guess that some 20-40% and 40-50% of all histologically or cytologically diagnosed tumours were evaluated, respectively. This certainly will cause an underestimation of the true incidence. On the other hand a potential overestimation exists in the fact that the reference population is composed of pedigree-dogs as registered at the Dutch Kennel club, while biopsies in some cases derive from dogs that are registered by owners as 'Golden retrievers' but that lack a pedigree.

In addition, an unknown portion of tumours remain undiagnosed. Reason for this, is the fact that not all dogs are presented to a veterinarian and not all owners are willing to pursue and pay for a diagnostic work-up [52]. This situation is perhaps even more likely, if the population at risk is not insured like the one used in this study. We therefore consider the incidence rates found within this study to be an estimation of the true incidence rate. Most other studies take note of encountered bias, but continue to register incidence rates (IR). Because of expected variations in both numerators and denominator, it proved difficult to compare this EIR with IR found in previous research [29-32, 53, 54]. Our EIR is lower than the IR for tumour development in the general dog population found in a Canadian study (IR: 3,965) but this study makes use of a computerized medical record system instead of only histopathological and cytological data (*Proceedings: The incidence of neoplasia in the canine and patient populations of private veterinary practices in Southern Ontario (2000)*) and therefore is likely to have a very different denominator.

More comparable are the IR calculated for tumour development in the general dog population (not breed-specific) found by the CANR (IR:1,134) [31], a study in England (IR: 1.948) [30], and a study in Italy (IR: 282) [34]. The high EIR found in the present study when both of the data-sets are combined (EIR: 2,242), could be an indication of a breed-predisposition for general tumour development in the Golden retriever.

Conclusions

The high EIR found in this study when evaluating comparable research is an indication of a breed-predisposition for cancer as well as general tumour development in the Golden retriever. The breed predisposition for MCT, NHL and melanoma in Golden retrievers was confirmed. There are also indications for a predisposition for STS.

Comparable to previous research that considered the dog-population in general, benign lesions occur at a younger age than malignant tumours, and most tumours develop in the skin. There appears no gender predilection. Including diagnoses made through histopathology as well as cytology, reduces the risk of a bias based on the diagnostic procedure of choice. A study combining both diagnostic procedures is therefore of greater value than a study that focuses on a single diagnostic procedure.

Material and method

The experimental protocol (ID 2007.III.08.110) was peer-reviewed by the scientific committee of the Department of Animals in Science &Society, Utrecht University, The Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht, The Netherlands. The Animal Experiments Commettee based its decision on 'De Wet op de Dierproeven'(The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (the Dutch 'animal experiments decree', 1996). Both documents are available online at http://wetten.overheid.nl.

In this retrospective study, two separate data-sets were used, consisting of either cytologically or histopathologically confirmed tumours from the client-owned pet-population of Dutch Golden retrievers that were submitted during the period 1998-2004 for cytological examination to Utrecht University Veterinary Diagnostic Laboratory (UVDL), or for histological examination during that period to the *Veterinary Pathologic Diagnostic Centre* (VPDC). Permission to use these data sets was obtained from the Departments of the Veterinary Faculty concerned. As in previous research [32] dog breeds were recorded as stated by the owner. The material was obtained from patients seen within the UUVM as well as from primary clinics, referral hospitals and private practitioners from all over the Netherlands.
In incidental cases, when detailed information was unavailable, variables selected for investigation were age, sex and, in the histopathological data-set, site of biopsy. If in an animal multiple tumours were detected during the period of the study, these were recorded as separate incidences. A broad system of tumour classification was applied for both data sets, which was based on tissue of origin and actual diagnosis. All tumours were divided into six (for the cytological data-set) or seven (for the histopathological data-set) tissues of origin (mesenchymal, hematopoietic, epithelial, neuroectodermal, gonadal plus for the cytological tumours: malignant Not Otherwise Specified (NOS) and for the histopathological data-set: glial tumours and tumours that could be NOS).

Numerator and denominator

In total, 18,648 Golden retrievers (9,639 male dogs and 9,009 bitches) were registered between 1998 and 2004 in the Raad van Beheer (Dutch Kennel Club), the principal cynological organization in the Netherlands. This resulted in an average entry of 2,664 animals per year. The Golden retriever reaches an average

age of 11 years (http://www.Golden retrieverca.org/health/index.html), so a cross-sectional estimation of the total population during one year is expected to be 11 * 2,664=29,304 (15,147 male dogs and 14,157 bitches). This was defined denominator, or population at risk (D).

The annual Estimated Incidence Rate (EIR) was calculated as the observed number of cases (C) in one year, calculated for a population that consists of 100,000 individuals.
EIR = (C / 7 yrs) x (100,000 / D)
Input was considered as two separate data sets, and thus two separate numerators were all neoplastic biopsies of dogs registered as 'Golden retriever' that were sent during the given period to either the VPDC or the UVDL. Results were used to calculate the occurrence of neoplastic types in the two data sets and by calculation of the number per annum corrected for the share of the two laboratories of all submissions – to exclude double counts - in the entire (with or without pedigree) Dutch Golden Retriever population to assess an EIR.

Statistics

A Student's t-test was used to test the age difference between malignant and benign tumours, a $P<0.05$ was considered to be significant. Normality and constancy of variance of the data was evaluated by inspecting the histograms. Statistical analyses were performed in R library version 1.7 (http://cran.r-project.org).

Competing interests

The autors declare that they have no competing interests nor did they have any competing financial interests in relation to the work described.

Authors' contributions

KB participated in the conceptualization and design of the study (including the statistical analyses) and drafted the manuscript. ET conducted the cytology review, participated in the statistical analyses and assisted in drafting the manuscript. GG conducted the histopathology review and assisted in drafting the manuscript. LoB and LiB participated in the data-base record review. GR conceptualized and designed the study, and participated in its coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was partly funded by the European Commission (LUPA-GA-201370). No other external financers contributed to this experiment.
We thank Prof. dr. Mirjam Nielen, dipl ECVPH Professor Evidence-based Veterinary Medicine, for reviewing the epidemiological part of this study and Associate Professor dr. Jaap Joles for reading the manuscript.

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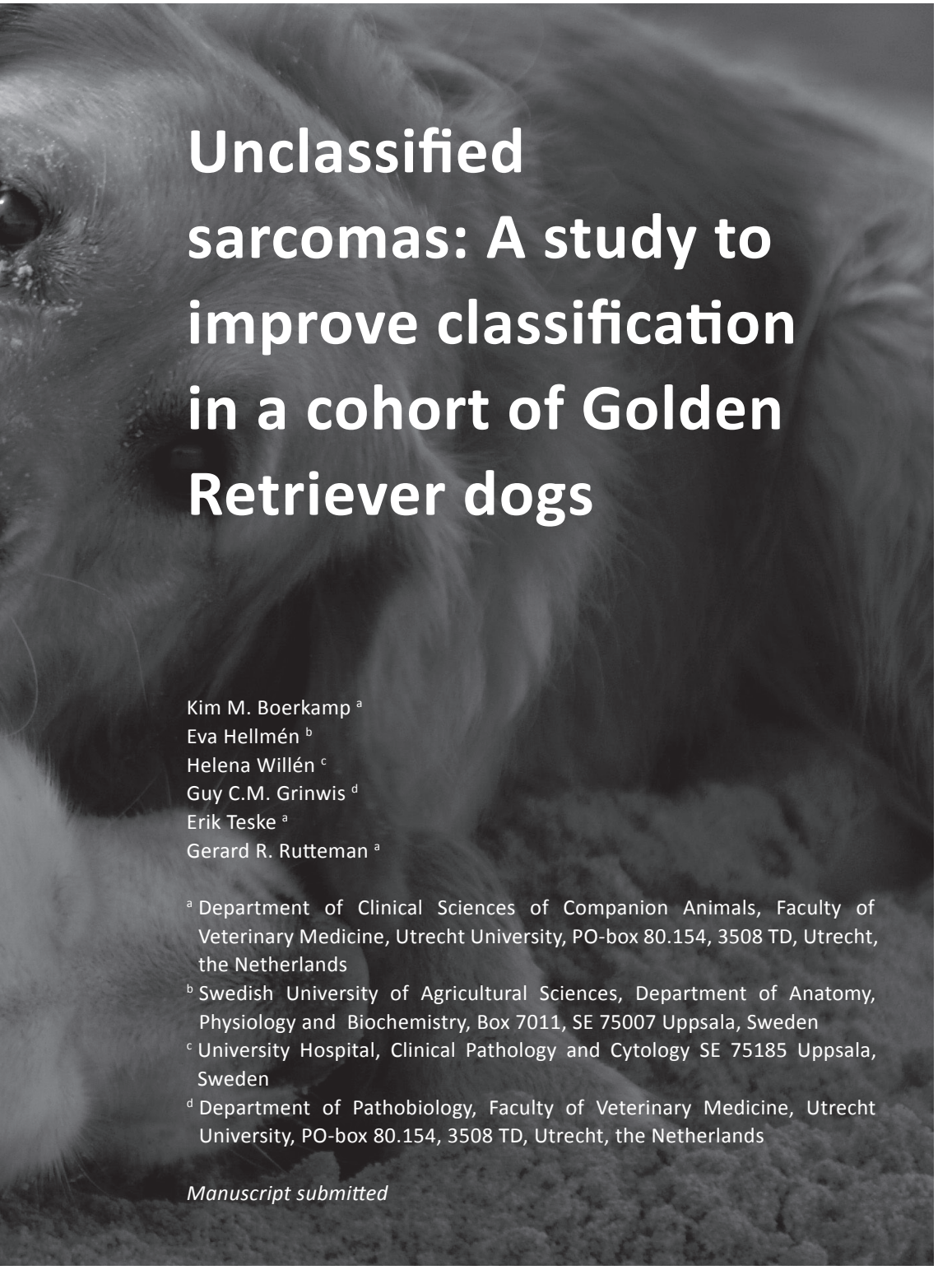

Unclassified sarcomas: A study to improve classification in a cohort of Golden Retriever dogs

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Abstract

Background

Morphologically, canine soft tissue sarcomas (STSs) strongly resemble human soft tissue sarcomas, and can serve as a model in the study of these neoplasms. In humans, proper classification of STSs is considered essential to increase insight in the biology of these tumours, and to optimize diagnosis and therapy. Revision of human neoplasms that were considered (unclassified) STSs, has shown that some belonged to a completely different subset of tumours, or were even considered to be non-neoplastic. Currently, there is a paucity of data published on the significance of detailed classification of STSs in the dog.

We revised a cohort (n=110) of proliferative lesions obtained from a study in Golden retrievers that were considered 'sarcoma, not otherwise specified or of uncertain subtype'. These neoplasms lacked specific morphological characteristics that would allow subtyping (i.e. unclassified sarcomas). Revision was performed by a board certified veterinary pathologist and a human pathologist, following the criteria according to the *Histological classification for mesenchymal tumours of skin and soft tissues of domestic animals* (veterinary WHO classification), recent veterinary literature and the WHO classification for humans. Revision was initially based on morphological characteristics of hematoxylin and eosin stained histological sections of the neoplasms. If deemed necessary (n=76), additional immunohistochemistry was applied to aid characterization.

Results

In 75 neoplasms (68%) we were able to confirm the diagnose STS. Of this group, we were able diagnose a specific subtype of the STSs in 58 neoplasms. Some of these neoplasms had morphological characteristics that were suggestive for sarcoma subtypes as described in the WHO classification for humans. Seventeen neoplasms remained 'unclassified STSs'.

As many as 31 lesions (28%) were diagnosed as 'neoplasm, not being STS'. Four lesions (4%) were considered non-neoplastic.

Conclusions

Since incorrect classification of a tumour could lead to inappropriate therapeutic intervention and prognostication, the results of the present study clearly illustrate the importance of revision of 'unclassified STSs' in dogs.

Keywords: Classification, Dog, Soft Tissue Sarcoma, Immunohistochemistry, Unclassified

Background

Soft tissue sarcomas (STSs) form a group of complex diagnostic entities (subtypes) [1-3] that originate from mesenchymal cells [2, 4]. Histogenetically, STSs are classified according to the adult mesenchymal tissue they most resemble [2, 5, 6]. However (in dogs as well as humans) STSs that are considered to belong to one subtype may display a spectrum of morphological characteristics that show overlap with other subtypes [7-9]. Some cases can actually appear too undifferentiated to be classified at all [5, 10]. This broad morphological spectrum can cause problems in distinguishing STSs from non-mesenchymal neoplasms [11-13] or even non-neoplastic proliferative lesions [6, 9].

In humans, it is accepted that correct identification of STSs is required in order to better predict biological behavior of the individual sarcoma subtype and to choose the optimal therapeutic approach [3-6, 10, 14-19]. Advances have been made in the characterization of STSs subtypes by applying immunohistochemistry (IHC) as addition to morphological analysis [15, 17, 20]. Revision is essential not only for studies of incidence and etiology, but also to ensure that appropriate treatment is selected [21]. Major discrepancies can occur on revision, in particular in cases that have initially been diagnosed as 'sarcoma or possible sarcoma' or poorly differentiated mesenchymal neoplasms [9]. These neoplasms may even sometimes be reclassified as non-mesenchymal neoplasms [9]. Discrepancies in diagnosis can sometimes have major therapeutic significance [22].

Some canine STSs display morphological characteristics that are very similar to STSs in humans [23-25] and are considered to be a good comparative model [26]. However, in contrary to what is believed in humans [4], the actual prognostic or therapeutic relevance of detailed subtyping of soft tissue sarcomas has not yet been established in veterinary medicine [27]. Thus, until now detailed histopathological classification of STS subtype in the dog is often not pursued in routine diagnostics for reasons such the extra costs of additional IHC. We hypothesize that also for veterinary and comparative oncology, verification of canine soft tissue sarcoma nature versus other cancer types is necessary for choice of therapy and prognostication. Furthermore, additional classification may serve as a tool to compare results from genomic studies with histogenetic evolution.

The objective of this study was to evaluate, how many neoplasms initially suspected of being unclassified sarcoma would at thorough revision obtain a diagnosis with 'major discrepancy', defined as changes in diagnosis that could lead to a significant change in clinical management. We hypothesized that the routine diagnosis 'presumable STS', is insufficient for proper prognostication, therapy choice and pathogenetic studies.

Results

In 34 neoplasms (17 of which were considered STS of a specific subtype), expert consensus existed at review of H&E sections on the presence of sufficient morphological features to make a diagnosis based on the characteristics described in the veterinary literature or human WHO classification, without the requirement of additional IHC. In all other neoplasms, additional IHC was required. (Supplementary Table 1 provides detailed information on all diagnoses made).

Of the total of 110; 58 neoplasms could be classified as a specific STS subtype. Within this group, the largest number of neoplasms (n=20) was considered to belong to the group of 'malignant tumours of fibrous tissue'. Five neoplasms within this group were considered fibrosarcomas; based on morphological appearance at primary review or after negative staining for S-100, desmin, CD31, and AE1/AE3). Seven were considered myxosarcoma (based on morphological appearance and, in one case, negative staining for S-100 and CD34) and one tumour was considered Malignant Fibrous Histiocytoma (MFH) following to the veterinary WHO classification. A group of 7 neoplasms could not be further subclassified according to the veterinary WHO classification or current veterinary literature. Still, these neoplasms did show typical morphological characteristics that were suggestive for neoplastic entities described in humans. Therefore the human WHO classification was used in an attempt to further classify these neoplasms resulting in a tentative diagnosis of dermatofibrosarcoma protuberans-like (n=4; Fig. 1.1) and desmoid fibromatosis (n=1; Fig 1.2) was made. Two neoplasms that stained negative for CD34 and negative for CD18 were regarded as belonging to the group of 'tumours of fibrous tissue', but could not be further classified.

Regarding the group of 'malignant adipocytic tumours' (n=8), further classification following the veterinary WHO classification [28] could be made into well differentiated liposarcoma, myxoid liposarcoma (n=2; Fig 1.3) and pleomorphic liposarcoma (n=1; Fig 1.4). Besides morphological appearances, the diagnosis of pleomorphic liposarcoma was based on negative staining for desmin. The remaining liposarcomas (n=4) could not be classified based on morphological appearance as one of the above mentioned sub-subtypes of liposarcomas currently recognized in veterinary literature. One case was considered to be a round cell liposarcoma. This diagnosis is not included in the veterinary WHO classification, but has been described in the dog previously [29].

Only by following the current human classification (WHO Classification of Soft Tissue and bone [12]) the remaining three neoplasms showed morphological characteristics consistent with a (well differentiated) sclerosing liposarcoma (Fig. 1.5), a dedifferentiated liposarcoma and a mixed-type liposarcoma.
Five neoplasms were diagnosed as hemangiosarcomas (following the current veterinary WHO classification) based on morphological characteristics. In

Table 1: Distribution after revision of 110 neoplasms 'sarcoma, not otherwise specified or of uncertain subtype' (i.e. unclassified sarcomas) following veterinary WHO classification

Type	Subtype/Sub-subtype	No. of revised neoplasms
Tumours of fibrous tissue	Fibrosarcoma	5
	Myxosarcoma	7
	Malignant Fibrous Histiocytoma	1
	Not further classifiable following vet. WHO classification	7
Tumours of adipose tissue	Liposarcoma	
	Well-differentiated	1
	Pleomorphic	1
	Myxoid	2
	Other	4
Tumours of smooth muscle	Leiomyosarcoma	6
Tumours of striated muscle	Rhabdomyosarcoma	1
Tumours of vascular tissue	Hemangiosarcoma	6
Tumours of peripheral nerves	Malignant peripheral nerve sheath tumours of the skin and subcutis	6
Tumours of Synovium	Synovial cell sarcoma (SCS)	2
Histiocytic tumours	Malignant histiocytosis	6
Unclassified tumours	Malignant mesenchymoma	1
	Canine Hemangiopericytoma	2
TOTAL CONFIRMED/SUBCLASSIFIED		**58**
Unclassified STS		17
Neoplasm, Non STS		31
Non neoplastic		4
TOTAL NUMBER OF CASES		**110**

STS: Soft tissue sarcoma

three cases additional staining for CD31 was performed, and in all three cases the neoplasm was found to be positive. In one of the hemangiosarcomas, the malignancy grade was judged to be low based upon a low percentage of neoplastic cells staining positive for Ki-67. One neoplasm was subclassified as an epithelioid angiosarcoma (Fig 1.6), a subtype that is not a part of the veterinary WHO classification, but which is already recognized in veterinary literature [30]. Also this tumour was found positive for CD31.

Even after extensive revision, 17 neoplasms of the total of 110 remained 'unclassified STS'. Four other proliferative lesions were reclassified as non-neoplastic (ulcerating inflammatory skin; lesion of uncertain type; granulation tissue; autolytic, not diagnostic). Thirty-one neoplasms out of the total of 110 were reclassified as 'neoplasm, non-STS'. This latter group included round cell neoplasms that appeared malignant lymphoma (n=4), osteosarcomas (n=10), neoplasms that were suspected to be melanomas (n=3), and four benign mesenchymal neoplasms.

Six neoplasms, initially considered 'undifferentiated', were eventually diagnosed as histiocytic sarcoma (referred to as malignant histiocytosis in the veterinary WHO), based on histomorphological hallmarks combined with positive immunorectivity for CD18.

The results of the revised classification of the group of 110 (potential) STSs is shown in Table 1.

Discussion

In humans, diagnosing STSs can be difficult, and major discrepancies upon review are known to occur [9, 31]. Major discrepancies are defined as changes in diagnosis that could lead to a significant change in clinical management, causing either under- or overtreatment [9]. These concern changes such as a change from neoplastic to non-neoplastic, from malignant to benign or from mesenchymal to non-mesenchymal. Minor discrepancies are changes in which the discrepancy is not thought to provoke significant management change [9]. In general, STSs carry a high risk for diagnostic errors [31], and some subtypes are even more prone to misdiagnosis than other subtypes [7, 9]. There are several reasons for the discrepancies to occur. In some cases, non-neoplastic mesenchymal neoplasms strongly resemble STS [31, 32]. Furthermore, differences in interpretation of morphology can occur [9]. Also, the rare nature of a lesion [31] and difference in the degree of experience in differentiating sarcoma cases between pathologists can cause this discrepancy [9, 31].
Also among the neoplasms originally diagnosed as unclassifiable soft tissue sarcoma and revised in this study, major discrepancies occurred. Upon revision, 31 of such neoplasms (28%), were identified as being 'neoplasm, non-STS'.

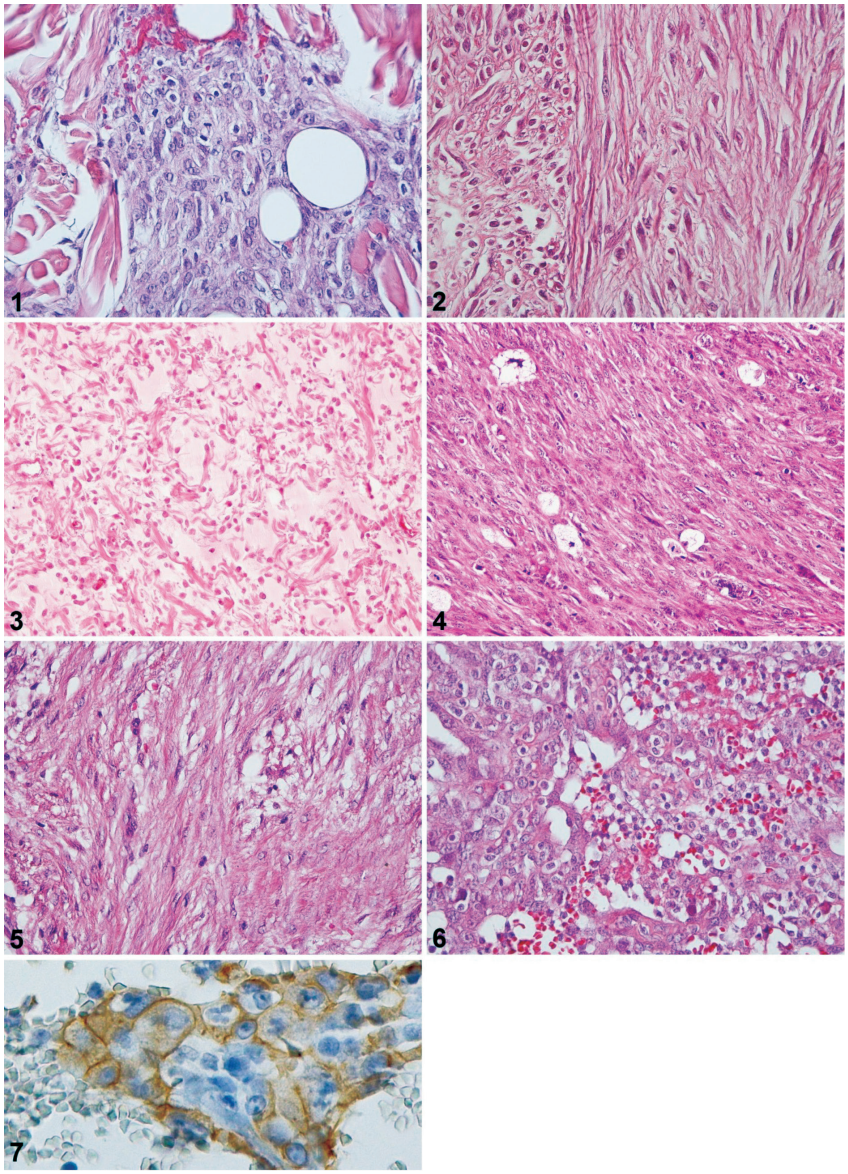

Figure 1: Exemplary neoplasms in which subclassification was possible, surpassing the current Veterinary WHO classification of mesenchymal tumours.

1. *P0104813; dermatofibrosarcoma protuberans-like (40x objective, hematoxylin and eosin (HE))*
2. *P0213228; desmoid fibromatosis (40x objective, HE)*
3. *P0306617; myxoid liposarcoma (20x objective, HE)*
4. *P0311056; pleomorphic liposarcoma (x 20 objective, HE)*
5. *P9912897; well differentiated liposarcoma of sclerosing type (40x objective, HE)*
6. *P036971; epitheloid angiosarcoma (x 40 objective, HE)*
7. *P036971; epitheloid angiosarcoma (x 40 objective, CD31)*

In humans, revisions of STSs have resulted in discrepancy rates (including both major- *and* minor discrepancies) of about 27% [9]. In the current study, the proportion of major discrepancies is substantially higher than known from human studies. This likely has to do with the type of lesion that we had chosen for revision: In our study, we focused on a selected group of neoplasms that were considered either (potential) STS of undifferentiated type, or neoplasms for which several possible subtypes were indicated, whereas the discrepancy rate published by Thway were the result from a (retrospective) revision of a random group of soft tissue sarcomas collected over a certain period, including differentiated subtypes; excluding second opinion cases [9].

For those cases (n=76) in which the two expert pathologists could not reach consensus about one specific tumour subtype, additional IHC was performed. Specific antibodies that were chosen based on morphological characteristics of the neoplasms and the location within the body were applied, instead of a full panel on all neoplasms. This approach was chosen due to financial limitations,, the current approach for materials costing about 20K in euro's, whereas a full panel applied for every cases would have cost at least 5 times more. In human sarcomas, lack of familiarity of the pathologist with rare neoplasms or those with unusual morphological appearances is probably more significant in explaining diagnostic discrepancies than absence of IHC [31]. In the referred study in humans all diagnoses, for which a major discrepancy was found, were made on the basis of the HE-stained slides and therefore failure to perform immunostainings did not account for these discrepancies.

We were unable to acquire a subclassification in 15% (n=17) of 110 neoplasms. Problems to subclassify STSs are not uncommon in the dog as well as in the human [1, 2, 24, 25, 33, 34]. The lack of morphological and molecular characteristics can hamper classification into a subtype [1, 2, 9, 24, 25, 33] even when a large panel of markers is applied [23, 24, 34]. However, we do recognize that the choice not to apply the complete panel of antibodies for all suspected STSs is a limitation of this study and it is likely that inclusion of a broad panel of appropriate antibodies would lead to sub-classification of a larger number of neoplasms of the cases used in this study.

The use of some of the selected antibodies yielded technical problems. These occurred either because of the absence of the expected staining in positive control tissues or because the stain was too unspecific due to lack of inter-species cross reactivity. This was the case when using HMB45, an antibody that stains a glycoprotein that is present in premelanosomes [35] (Dako; clone M634) as well as MyoD1, a marker of myogenic cells [36] (Dako; clone 5.8A). Despite testing of these antibodies in several tissues (melanoma, rhabdomyosarcoma, striated muscle, respectively) no specific staining could be seen, likely because of lack of inter-species cross reactivity.

At the time of writing, an antibody raised against canine CD34 has become commercially available (clone 1H6). However this canine antibody was not available when we started the study, so an antibody that is known to cross-react with canine tissue was selected [37].

One tumour was presumed to be a gastro-intestinal stromal tumour (GIST) based on morphological features and the location of the tumour within the intestines. However, c-Kit staining was negative. Since immunoreactivity for c-Kit is considered essential for a tumour to be diagnosed a GIST [38] and the neoplasm did show immunoreactivity for desmin, the tumour was eventually diagnosed as a leiomyosarcoma.

Most STSs within this study belonged to the group of *malignant tumours of fibrous tissue* (n= 20). Additional sub-classification following the veterinary WHO classification and more recent veterinary publications on this group of neoplasms, proved difficult in about a third of the neoplasms belonging to this group (n=7).

Four of these neoplasms showed morphological characteristics that were considered dermatofibrosarcoma protruberans-like. In humans, the diagnose dermatofibrosarcoma protuberans is considered a specific entity. According to current (human) WHO classification on the pathology and genetics of skin tumours, where this neoplasm is considered part of the fibrous, fibrohistiocytic and histiocytic tumours the diagnosis can be made on morphological appearance, and a positive staining for CD34. The identification of a characteristic fusion gene (t(17;22) (q22;q13) translocation is, according to the (human) WHO, considered a hallmark of this tumour. In our study, the morphological appearance was considered characteristic for a dermatofibrosarcoma protuberans. Since the identification of the presence of a possible fusion gene is beyond the scope of this study, neoplasms were tentatively classified as dermatofibrosarcoma protuberans-like. It would be of additional value to evaluate the presence of such a fusion gene within these neoplasms, and possible finding additional means for treatment that focusses more on the exact nature of this neoplasm.

One neoplasm within the group of 'malignant tumours of fibrous tissue' was considered a MFH. In veterinary literature, the name MFH is considered controversial. A recent change to 'anaplastic sarcoma with giant cells' [39] has been suggested, since the name MFH is considered a purely descriptive term [34, 39].
Also within the group of 'malignant adipocytic tumours', the sclerosing liposarcoma, and within the group of 'malignant vascular tumours', the epithelioid angiosarcomas are neoplasms that are not documented in the current veterinary WHO classification. However, these diagnoses were previously described in current veterinary literature. Atypical lipomas, characterized by sclerosing areas, have previously been described in dogs [39]. It is considered to be a low-grade malignant liposarcoma, recapitulating the features of atypical lipomatous

tumour of humans, resembling sclerosing liposarcoma of man [39]. Sclerosing liposarcoma is reported as a variant of the atypical lipomatous tumour (ALT)/well-differentiated liposarcoma (WDLS) in WHO classification of soft tissue tumours in humans. 'Epithelioid angiosarcoma' has been described in the dog in several papers [40, 41]. For this sarcoma subtype, as for the above mentioned MFH [42], the Golden Retriever is likely to be predisposed [41].

Two tumours were diagnosed as synovial cell sarcomas (SCSs) based on expert consensus on morphological features. One of these tumours stained positive for the cytokeratin antibody AE1/AE3, a staining known to often be positive in SCS [43], however the less differentiated one was negative. There can, however, be some concern about the diagnosis. Histiocytic sarcomas can sometimes be mistaken for SCS [44]. Additional staining for CD18 would have been of additional value for classification of these two tumours. However, insufficient material had remained to perform this staining.

In total, seven neoplasms could not be classified following the veterinary WHO classification. This was the case with the (well-differentiated) sclerosing liposarcoma; the epithelioid angiosarcoma the four cases of dermatofibrosarcoma protuberans-like neoplasms and the desmoid fibromatosis. To the authors knowledge the latter two have not been described in recent veterinary literature. These cases are illustrative for the limitation of the current veterinary WHO classification. In our opinion, a more advanced classification scheme could be considered.

Because of the potential of dogs as a translational animal model, and the proven additional value of additional classification in humans, we believe that improved classification of canine STSs can be beneficial for both research and clinics.

Conclusions

This study combined a revised morphological analysis with IHC in a collection of 110 unclassified (potential) STSs. This approach allowed confirmation of the diagnosis of STSs and additional sub-classification of 58 cases (53%) following the veterinary WHO histological classification of mesenchymal tumours of skin and soft tissues of domestic animals. In 17 cases, (15%) revision did not result in sub-classification. These neoplasms remained 'unclassified STS'. A large percentage (32%) of the remaining proliferative lesions appeared to be misdiagnosed, and were eventually considered to be 'neoplasm, non-STS' (31 cases), or even 'non-tumourous' (4 cases). This audit illustrates that within the original group of neoplasms that on initial histopathology, in combination with clinical parameters, were thought to be STSs, a substantial percentage of major discrepancies occurred, leading to major misdiagnoses. To avoid an incorrect treatment, revision of this group of neoplasms is always advisable.

Materials and Methods

Initial case selection

This study was performed in a series of archival formalin-fixed and paraffin-embedded samples originally diagnosed as neoplastic mesenchymal. Cases were obtained from the client-owned pet-population of Dutch Golden retrievers submitted to the Veterinary Pathology Diagnostic Centre (VPDC) of the Faculty of Veterinary Medicine in Utrecht, the Netherlands during the period 1998-2004 by the Utrecht University Clinic of Companion Animals (UUCCA) as well as by other referral hospitals and private practitioners. All samples had been fixed in 10% neutral buffered formalin and were submitted as part of the diagnostive procedure in clinical patients. Therefore, no informed consent for the use of this material was required. No specific information on fixation time, nor of the ratio of tissue volume to volume of the fixative was available. After fixation, samples were routinely processed and embedded in paraffin wax from which 4-6 µm sections were cut and stained with hematoxylin and eosin (HE) for microscopic examination.

Our earlier search through the archives of 1998-2004 had identified 2,124 neoplasms diagnosed by means of histopathology derived from Golden retrievers. The initial diagnoses of these neoplasms were performed by board-certified veterinary pathologists as presented in the previous study [45].

Of this group of neoplasms, 110 tumours that were considered (potential) STSs but were not further classified, were selected for the current study.

Histopathology and immunohistochemistry

A thorough revision of microscopic morphology of the (potential) STSs (with review of patient-data such as location and size of the neoplasm) was performed by a veterinary pathologist (EH) and a pathologist specialized in human soft tissue tumours (HW).

Interpretation was first and mostly based on cellular morphology and growth pattern according to the *World Health Organization International Classification of Tumours of Domestic Animals* (e.g. veterinary WHO) [28] and recent veterinary literature and, if considered necessary, with inclusion of the WHO classification for humans [12]. Detailed description of all neoplasms is considered beyond the scope of the present manuscript.

In 76 cases (69%), additional IHC staining was considered necessary for (sub) classification. Instead of applying a panel of antibodies, specific primary antibodies were selected guided by morphological aspects and site of the neoplasm as is common.

There is currently no consistent immunohistochemical stain or group of stains that can accurately separate different STS types [2]. Therefore, various antibodies, reported in the literature of being useful in diagnosing STS subtypes in dogs,

Table 2: Detailed information on primary antibodies used on soft tissue sarcomas chosen for revision.

Primary antibody	Source and type	Dilution	Manufacturer	Pre-treatment and technique	Positive controls	Lineage marker
Cytokeratin clones AE1/ AE3	Mouse monoclonal, IgG1	1:25	Dako, M3515	Unmasking, Vector, High pH LP*	Mammary Gland	Epithelial cells
C-Kit (CD117)	Rabbit Polyclonal	1:400	Dako, A4502	Unmasking, Vector, High pH ABC-Elite**	Mastocytoma	Haematopoietic stem cells, multipotent progenitors, and common myeloid progenitors
CD18	Mouse monoclonal anti CD18 (Clone CA16.3C10)	1:40	DR. P.F. Moore (Davis CA: USA)	ABC-Elite	Histiocytic sarcoma	Leukocytes
CD31 (PECAM-1) Lot # D2209	Goat Polyclonal	1:200	Santa Cruz, sc-1506	Unmasking, Dako citrate ABC-Elite	Mammary Gland	Platelets, monocytes, neutrophils, some types of T-cells and endothelial cells
CD34 (C-18) Lot # F2111	Goat Polyclonal	1:400	Santa Cruz sc-7045	Unmasking, Dako citrate ABC-Elite	Ovary (CL)	Early hematopoietic and vascular-associated cells
Desmin	Mouse monoclonal, IgG1	1:500	Dako, M0760	Unmasking, Vector, High pH LP	Mammary Gland	Skeletal muscle; smooth muscle; and cardiac muscle cells
HMB45	Mouse monoclonal, IgG1	1:50-1:100	Dako, M634	LP	Melanoma	Antigen present in melanocytic tumours
MyoD1 Clone 5.8A	Mouse monoclonal, IgG1	1:100	Dako, M3512	ABC-Elite	X	Markers of myogenic commitment
Ki-67 Clone MIB-1	Mouse monoclonal, IgG1	1:1000	Dako, M7240	Unmasking, Vector, High pH LP	Mammary Gland	Cell nucleus of cells in interphase
Melan-A Clone A103	Mouse monoclonal, IgG1	1:50	Dako, M7196	Unmasking, Vector High pH ABC-Elite	Melanoma	Protein antigen that is found on the surface of melanocytes
Myf4 Clone L026	Mouse monoclonal, IgG1	1:25	NCL-L-Myf-4, Leica, Novocastra	Unmasking, Vector High pH ABC-Elite	Alveolar rhabdo-myosarcoma	Skeletal muscles

Table 2 continued: Detailed information on primary antibodies used on soft tissue sarcomas chosen for revision.

S-100	Rabbit polyclonal	1:1200	Dako, Z0311	Unmasking, Dako citrate LP and ABC-Elite	Peripheral Nerve	Cells derived from the neural crest
SMA Clone A4	Mouse monoclonal, IgG2a	1:200	Dako, M0851	Unmasking, Vector, High pH LP	Mammary Gland	Myofibroblasts, smooth muscle cells, myoepthelial cells
Vimentin Clone V9	Mouse monoclonal, IgG1	1:500	Dako, M0725	Unmasking, Vector, High pH ABC-Elite	Mammary Gland	Mesenchymal cells, myoepthelial cells

* *Thermo Scientific, Ultravision LP*

** *Vector*

(reference is listed following each individual stain outlined below), as well as in humans [46] were selected. Selection was based upon literature data, as being able to stain the following differentiation markers: Melan-A, a highly specific and low sensitive melanocytic marker [37]; S-100; an unspecific marker of melanocytic neoplasms and peripheral nerve sheath tumours [38, 47]; CD31, a vascular endothelial marker [40]; Smooth Muscle Actin (SMA), a smooth muscle cell marker [38]; Desmin, striated and smooth muscle cell marker [27] and Myf4 (myogenin), a striated muscle cell marker [27]; Vimentin, a general mesenchymal cell marker [27]; AE1/AE3, a cocktail of cytokeratins as an epithelial cell marker [48]; CD117 (C-Kit), a stem cell factor receptor [38], Ki67; a proliferation marker [49]; and CD18; a general leukocyte marker [40]. Antibodies staining CD34 (antibody C-18), a marker of hematopoietic progenitor- and endothelial cells [49], HMB45, a glycoprotein that is present in premelanosomes and MyoD1; a marker of myogenic cells [27] were also chosen. All antibodies used in the present study show cross reactivity with canine tissue.

IHC was applied on representative 4-6 µm sections of the selected cases. In all cases, one or more samples of appropriate canine positive control tissue was included for validation since information on cross-reactivity was not always available from information by the companies or published literature. Parallel-stained sections without incubation with the primary antibody were used as negative controls.

Antigen retrieval pretreatment was carried out using antigen unmasking solution (Vector) or Target Retrieval Solution, Citrate pH 6 (Dako). For detailed information on each individual antibody, see Table 2.

Immunohistochemical assessment

Immunoreactivity was assessed by light microscopy for each antibody. Immunoreactivity for S-100, CD31, desmin, vimentin, AE1/AE3, SMA, C-Kit, Melan-A, Myf4, CD34 and CD18 was scored as: - negative; +/- (weakly) positive; + positive (in terms of number of positive cells). Cases that only had a nuclear staining for CD34, were considered to be negative. The percentage of cells revealing immunoreactivity for Ki-67 was scored as low, intermediate or high.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

We thank the staff of the UVDL for their help and expertise. We also thank Lonneke Boon for her active participation in this study.

Funding

This study was partly funded by the European Commission (LUPA-GA-201370); and by Foundation of Veterinary Research in Companion Animals (“Stichting DOG”).

Supplementary Table 1. Definite results for all neoplasms revised

Case nr.	Diagnosis/ Subclassification	S-100	C-Kit	Desmin	CD31	CD34	AE1/AE3	Myf4	Ki-67	SMA	Melan-A	Vimentin	Tol. Blue	CD18
p0201805	Fibrosarcoma	-			-		-							
p0213834	Fibrosarcoma	-												
p9801887	Fibrosarcoma			-										
p9804057	Fibrosarcoma						-							
p0207641	Fibrosarcoma (low grade)	-							**L**					
p0008184	Myxosarcoma													
p0011627	Myxosarcoma	-				-								
p0311249	Myxosarcoma													
p0312644	Myxosarcoma													
p0402479	Myxosarcoma													
p9802944	Myxosarcoma													
p0002822	Myxosarcoma													
p0110557	Malignant fibrous histiocytoma													
p9803522	Fibrohistiocytic sarcoma					-								-
p0104688	Fibrohistiocytic sarcoma (Low-grade)					-								-
p0213228	Desmoid fibromatosis								**L**					
p0102751	Dermatofibrosarcoma protuberans-like					-								
p0104813	Dermato fibrosarcoma protuberans-like					-								
p0203817	Dermato fibrosarcoma protuberans-like					-								
p9902336	Dermato fibrosarcoma protuberans-like					-			**I**					
p9806922	Well differentiated liposarcoma													
p0311056	Pleomorphic liposarcoma			-										
P0200302	Myxoid liposarcoma												-	
p0306617	Myxoid liposarcoma												-	
p9811776	Dedifferentiated liposarcoma													
p0300445	Liposarcoma (mixed type)													
p9908670	Round cell liposarcoma						-							
p9912897	Sclerosing liposarcoma (well differentiated)													
p0008976	Leiomyosarcoma		-	+										
p0412201	Leiomyosarcoma	-		+										
p9807692	Leiomyosarcoma	-		-								+		
p9802037	Leiomyosarcoma	+	-	+										
p9901816	Leiomyosarcoma	+/-		+										
p0411629	Leiomyosarcoma (mammary)				-				**H**					
p0101527	Rhabdomyosarcoma													

Supplementary Table 1 continued. Definite results for all neoplasms revised

Case nr.	Diagnosis/ Subclassification	S-100	C-Kit	Desmin	CD31	CD34	AE1/AE3	Myf4	Ki-67	SMA	Melan-A	Vimentin	Tol. Blue	CD18
p0407926	Hemangiosarcoma (low-malignant)				+				L					
P0012563	Hemangiosarcoma													
P0306919	Hemangiosarcoma													
p0204339	Hemangiosarcoma				+									
p0214370	Hemangiosarcoma				+									
p0306971	Epitheloid angiosarcoma				+									
p0106991	Malignant peripheral nerve sheath tumour	-									-			
p0112317	Malignant peripheral nerve sheath tumour	+												
p0208195	Malignant peripheral nerve sheath tumour	+							L					
p0404969	Malignant peripheral nerve sheath tumour	+		-				-						
p0406091	Malignant peripheral nerve sheath tumour	+							I					
p9911501	Malignant peripheral nerve sheath tumour													
p9909901	Synovial cell sarcoma						+							
p0105077	Poorly differentiated synovial sarcoma						-							
p0409603	Hemangiopericytoma					-			L					
p9905553	Hemangiopericytoma													
p0410840	Malignant mesenchymoma													
p9900226	Pleiomorphic sarcoma, high grade			-										-
P0109726	Pleomorphic anaplastic sarcoma						-							-
P9908524	Anaplastic lymphoma/ sarcoma NOS	-									-			-
p0105517	Sarcoma (spindle cell), not otherwise specified, poorly differentiated.	-									-			
p9808583	Sarcoma, Not Otherwise Specified	-		-	-			-						-
p9810728	Sarcoma, Not Otherwise Specified										-			-
p0006399	Sarcoma, Not Otherwise Specified (NOS)						-							-
p0308292	Spindle cell sarcoma	-				-			I					
p0412894	Spindle cell sarcoma, high grade, NOS			-				-						
p0201347	Spindle cell sarcoma, NOS				-									
P9904233	Spindle cell sarcoma, NOS, high grade		-	-										
p0100840	Spindle cell sarcoma, NOS	-		-										
p0203254	Undifferentiated sarcoma				-		-							-
p0212860	Undifferentiated sarcoma			-				-						-

Supplementary Table 1 continued. Definite results for all neoplasms revised

Case nr.	Diagnosis/ Subclassification	S-100	C-Kit	Desmin	CD31	CD34	AE1/AE3	Myf4	Ki-67	SMA	Melan-A	Vimentin	Tol. Blue	CD18
p0307161	Undifferentiated sarcoma	-		-				-		-				
p9905079	Undifferentiated sarcoma	-				-					-			
p9908374	Undifferentiated small cellular sarcoma			-		-	-	-						-
p9805592	malignant histiocytosis					-								+
p0009428	malignant histiocytosis					-								+
p9900362	malignant histiocytosis			-										+
P0201995	malignant histiocytosis			-			-	-						+
p9811626	malignant histiocytosis			-				-						+
p0303593	malignant histiocytosis			-	-			-						+
p0205034	Anaplastic carcinoma (mammary)			-			+	-						
p9901425	Chondroid lipoma													
P0312078	Complex adenoma													
P9908808	Hemiangioma, benign													
p00306906	Round cell neoplasm, possibly lymphoma													
P0102100	Round cell neoplasm, possibly lymphoma													
P0306906	Round cell neoplasm, possibly lymphoma													
p9805866	Round cell neoplasm, possibly lymphoma				-									
P0012458	Malignant Melanoma	+									+			
p0104073	Malignant Melanoma	+					+/-				-			
P0013678	Non-STS (suspect malignant melanoma)	-									-			
p0305894	Malignant meningioma	-		-	-	-	+	-						
p0400768	Fibroma	-		-					L					
p0404871	Neurofibroma	+												
p0409971	Neurofibroma	+							L					
p0412633	Neurofibroma	+				-			L					
p0202165	Osteosarcoma													
p0203668	Osteosarcoma													
p0308670	Osteosarcoma			-	-									
p0310375	Osteosarcoma													
p0400790	Osteosarcoma													
p9901995	Osteosarcoma													
p9910899	Osteosarcoma													
p0400191	Osteosarcoma (chondroblastic)													
p0402625	Osteosarcoma (chondroblastic)													
P0101545	Osteosarcoma (osteoblastic)													
P0408764	Perineurinoma	-					-							
P9912311	Plasmacell tumour					-								
p0308620	Neurothecoma								-					
p0003684	Round cell malignant tumour			-				-						

Supplementary Table 1 continued. Definite results for all neoplasms revised

Case nr.	Diagnosis/ Subclassification	S-100	C-Kit	Desmin	CD31	CD34	AE1/AE3	Myf4	Ki-67	SMA	Melan-A	Vimentin	Tol. Blue	CD18
p0407048	Ulcerating inflammatory skin													
p0404299	Uncertain lesion				-									
p0311013	Granulation tissue			-				-						
p0001279	Autolytisch, not gradable			-			-							

STS: Soft Tissue Sarcoma, L: Low, I: Intermediate, H: High

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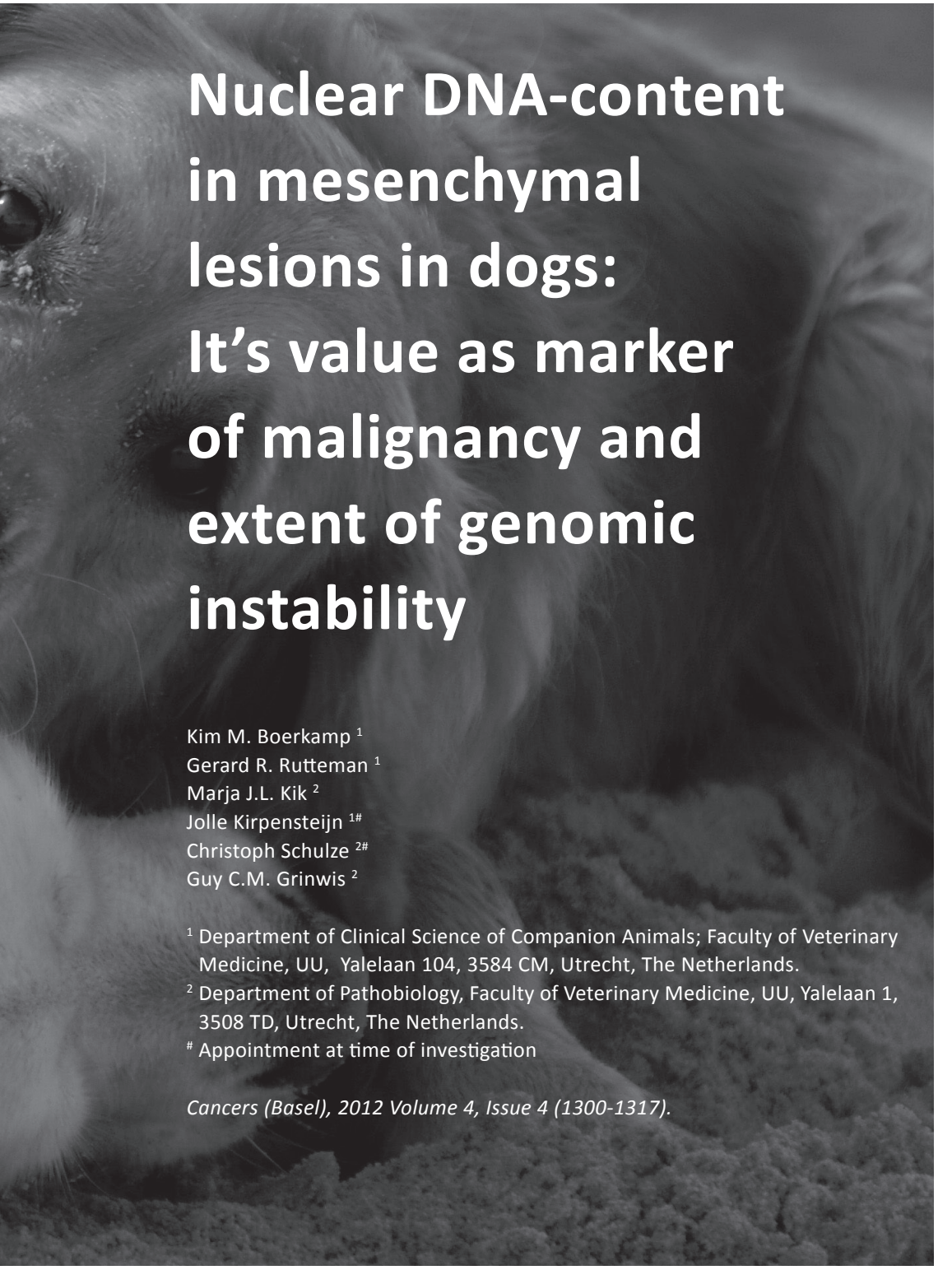

Nuclear DNA-content in mesenchymal lesions in dogs: It's value as marker of malignancy and extent of genomic instability

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Cancers (Basel), 2012 Volume 4, Issue 4 (1300-1317).

Abstract

DNA-aneuploidy may reflect the malignant nature of mesenchymal proliferations and herald gross genomic instability as a mechanistic factor in tumour genesis. DNA-ploidy and -index were determined by flow cytometry in canine inflammatory or neoplastic mesenchymal tissues and related to clinico-pathological features, biological behavior and p53 gene mutational status. Half of all sarcomas were aneuploid. Benign mesenchymal neoplasms were rarely aneuploid and inflammatory lesions not at all. The aneuploidy rate was comparable to that reported for human sarcomas with significant variation amongst subtypes. DNA-ploidy status in canines lacked a relation with histological grade of malignancy, in contrast to human sarcomas. While aneuploidy was related to the development of metastases in soft tissue sarcomas it was not in osteosarcomas. No relation amongst sarcomas was found between ploidy status and presence of P53 gene mutations. Heterogeneity of the DNA index between primary and metastatic sarcoma sites was present in half of the cases examined. Hypoploidy is more common in canine sarcomas and hyperploid cases have less deviation of the DNA index than human sarcomas. The variation in the presence and extent of aneuploidy amongst sarcoma subtypes indicates variation in genomic instability. This study strengthens the concept of interspecies variation in the evolution of gross chromosomal aberrations during cancer development.

Keywords: Aneuploidy evolution; canine; sarcomas; DNA index

Abbreviations: Canine Kidney Cells, CKC; Chicken Red Blood Cells, CRBC; Coefficient of Variation, CV; DNA Index, DI; Flow Cytometry, FCM; Histiocytic Sarcoma, HS; Malignant Tumour of Bone, MTB; Osteosarcoma, OS; Peridiploid, PD; Soft Tissue Sarcoma, STS

Introduction

In dogs cancer is the most common cause of non-traumatic death [1]. Several types of canine malignant neoplasms - based upon pathobiology – can serve as useful models for rare cancers in humans, including osteosarcomas (OS) and soft tissue sarcomas (STS) [2-4] both types being relatively common in the dog [5, 6]. As in humans, the prognosis of canine tumour patients is related to tumour location and, for malignant tumours, clinical stage [7]. A key factor in this assessment is the histological phenotype including malignancy grade [4, 8, 9] , but there is a significant morphological overlap between malignant, benign, and reactive mesenchymal lesions in the human [10, 11]. For both species, classification and management of mesenchymal tumours is complicated by the fact that these form a very heterogeneous group [9, 12]. In order to improve the prognostic value of histological examination of Malignant Tumour of Bone (MTB) and of STS, several classification and grading systems have been established in humans [13], and later adopted for use in the dog [8, 9], but the prognostic value of these systems for many individual tumours is limited [4, 9, 14]. Therefore, objective prognostic criteria are urgently needed.

Determination of the DNA-ploidy status may help to discriminate non-neoplastic or benign neoplastic lesions from (pre)malignant neoplastic conditions [15], including lesions of mesenchymal origin [16-19]. In humans, DNA-aneuploidy is more common in high-grade sarcomas than in those of low- or intermediate- grade [16, 18, 20, 21]. These potential discriminatory values still need to be examined in dogs. In addition, DNA-aneuploidy has been reported to have prognostic value in several cancer types in humans [22-24]. However, in some sarcoma subtypes, this is not always the case [16, 21, 25-32].

Besides the diagnostic value that the DNA-ploidy status can have, the DNA index (DI) distribution may help to comprehend the nature of genomic changes during tumourigenesis [33-35]. It reflects gross chromosomal changes [36] that, as already hypothesized one century ago [37], appear to play a mechanistic role in tumourigenesis, the extent of which is subject of debate [33-36, 38-49]. In approximately two-thirds of human malignant solid cancers, the evolution of karyotypic alterations over many cell divisions until cancer clinically manifests, is thought to be reflected by the increase of total nuclear DNA content, which peaks at 1.6-fold of the normal amount [33-35, 50]. In the other one-third of human cancers, the deviation of the DI is much less prominent, or cannot be discriminated from normal due to only minute or balanced chromosomal changes [15]. In general, a significant decrease in the DNA-content or DNA-hypoploidy is rare in most human solid cancers, with a few exceptions such as chondrosarcomas [51].

Loss of function of the *P53* pathway has been hypothesized to be one important factor in the development of aneuploid cancers [52].

As in humans, most cancers in dogs, such as thyroid and mammary carcinomas, are DNA-aneuploid but the extent of the aberration of the DI in DNA-aneuploid cancers is less and DNA-hypoploidy more common in such carcinomas in the dog than in humans [53-55]. In fact, these observations led us to hypothesize that there is an interspecies evolutionary variation in the manifestation of DNA-aneuploidy in tumours, when comparing humans and dogs [44].

In continuation with our earlier research, we now examined the DNA-ploidy distribution pattern as determined by flow cytometry (FCM) in a series of fresh frozen samples of canine benign and malignant mesenchymal lesions. Preliminary results of this study have been presented as poster at the ESF conference (ESF conference, Dresden, March 2010). For sarcomas, the data were compared with information on clinical stage, histological subtype and grade and the mutational status of the P53 gene, and with published data in human sarcomas. Comparison of observations in the current and earlier studies on canine neoplasms to those published on human cancers, points to interspecies variation in the driving force of aneuploidy in the development of malignant tumours.

Results

DNA-ploidy status in primary lesions

The arithmetical mean of the CV of G 0-1 populations of all lesions was 3.38 (1.4-5.0). The DNA ratio of the non-neoplastic G_{0-1} diploid cells as compared to CRBC G_{0-1} cells fell within the margins established in earlier studies [53, 56, 57]. The ploidy status as defined by FCM-base histograms (Figure 1) was normal (diploid) in all non-malignant lesions except one lipoma with a small (10% of all nuclei) aneuploid peak (DI 1.20).

In 3 dogs with histiocytic sarcoma (HS), there was multi-organ involvement. To attribute a ploidy status in such cases, the DI of the largest of multiple tumours (one dog) or one out of multiple equally-sized lesions with equal DI-results was used in the categorization of primary malignancies. There were 42 aneuploid cases out of 77 primary malignant tumours (55%), and 4 (5%) PD cases. We found a significant difference in the ploidy-status of primary malignancies compared to benign neoplastic lesions (P =0.023) and to non-neoplastic proliferative lesions (P= 0.0005). Out of all 43 aneuploid primary tumours (including one lipoma), 35 had only a single aneuploid G 0-1 population named stemline (81.4%), whereas 8 (18.6%) had multiple aneuploid stemlines. The distribution of the DIs of all stemlines in primary sarcomas is shown in Figure 2. The ploidy status did not vary (P=0.62) between all STS as compared to all MTB.

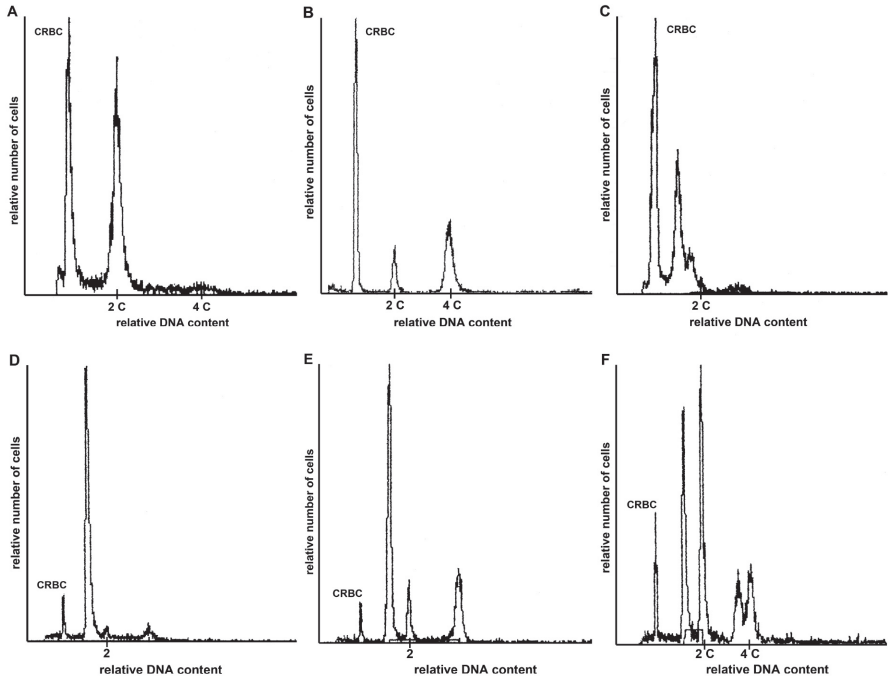

Figure 1: Example of DNA content histograms of canine tumours A: Diploid tumour, osteosarcoma (OS, DI = 1.0); B: Hyperploid (tetraploid) tumour, hemangiosarcoma (DI = 2.0); C: Multiploid-hypoploid tumour, chondrosarcoma (DI = 0.73 and 0.88); D: Hypoploid primary OS (DI = 0.76); E: Multiploid tumour (1st lung-metastases of former OS, DI = 0.77 and 1.56); F: Multiploid tumour (2nd lung-metastases of former OS, DI = 0.79, 1.43 and 1.59). Note: The stemline of the primary OS (DI = 0.76) reappeared in the 1st metastases (DI = 0.77) together with a second stemline with double the DNA content (DI = 1.56), while in the second metastases the hypoploid stemline (DI = 0.79) plus the polyploid stemline (DI = 1.59) appeared together with a third stemline (DI = 1.43).

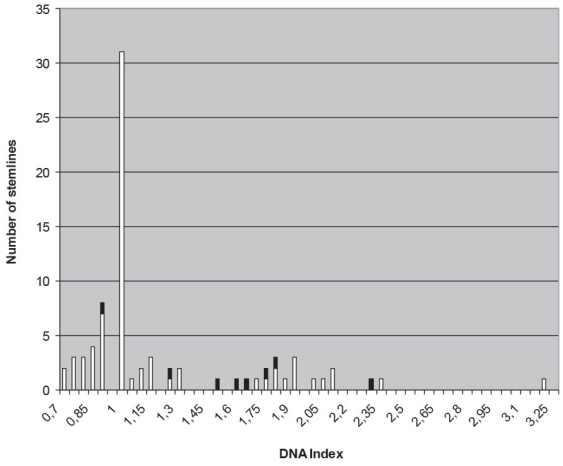

Figure 2: DNA indices of stemlines present in all 77 primary malignant lesions.

Table 1: DNA-ploidy status in 77 sarcomas according to subtype

Subtype	Diploid (n)	Aneuploid (n)	Peridiploid (n)	Total (n)
Malignant tumours of bone	10	17	3	30
Osteosarcoma	9	14	3	26
Chondrosarcoma	1	2	-	3
Multilobular tumour of bone	0	1	-	1
Soft tissue sarcomas	21	25	1	47
Fibrosarcoma	2	1	-	3
Sarcoma-not otherwise specified	3	1	-	4
Rhabdomyosarcoma	2	3	-	5
Malignant peripheral nerve sheath tumours	7	1	-	8
Synovial sarcoma	0	5	-	5
Liposarcoma	0	3	-	3
Leiomyosarcoma	6	2	-	8
Hemangiosarcoma	1	3	-	4
Histiocytic sarcoma	0	6	1	7

Table 2: DNA-ploidy status and histological malignancy grade in 59 sarcomas

Tumor type and grade	Diploid (n)	Peridiploid (n)	Aneuploid (n)
Malignant tumour of bone			
- grade I	0	0	0
- grade II	1	1	2
- grade III	8	2	12
Soft tissue sarcoma			
- grade I	5	0	2
- grade II	5	0	2
- grade III	8	0	11

Table 3: Presence of P53 mutations in sarcomas (n=44) as related to the ploidy-status

	Diploid	Aneuploid	Peridiploid
P53-wt	12	14	2
P53- alteration	7	9	1

Note: only mutations predicted to alter the amino acid composition of the P53 protein were counted (http://www.ncbi.nlm.nih.gov/nuccore/NM_001003210.1).

Table 4: The metastatic behavior of the sarcomas and the ploidy status of the primary lesion

Tumour group	Ploidy status	
	Diploid	**Aneuploid**
Osteosarcomas		
• Metastases	8	12
• No metastases	0	0
Soft tissue sarcomas		
• Metastases	5	17
• No metastases	8	3

Note: PD cases were excluded, as well as cases that lacked information on metastatic growth or recurrence within the first year following the initial diagnosis.

Table 5: Comparison of the DNA index (DI) in primary versus metastatic lesions (Met. 1 to 4) from the same tumours

Case	Primary	Met. 1	Met. 2	Met. 3	Met. 4
Osteosarcoma 1 *	2.10	1.10/2.14	1.12		
Osteosarcoma 2 *	0.76	0.77/1.56	0.79/1.43/1.59	0.79/1.55	0.78/1.55
Osteosarcoma 3 *	1.0	1.88			
Osteosarcoma 4	1.0	1.0	1.0		
Synovial cell sarcoma *	0.89/1.80	1.77			
Hemangiosarcoma *	1.89	1.0	1.0		
Sarcoma-NOS *	1.0	1.77			
Fibrosarcoma	1.0	1.0	1.0		
Malignant Peripheral Nerve Sheath Tumour	1.0	1.0			
Synovial Cell Sarcoma	0.72	0.72			
Histiocytic Sarcoma	PD	0.93			
Liposarcoma	2.03	1.97			

Note: The variations in the DI became visible as peaks with > 10% of the total cell population analyzed. Met: Metastasis, NOS: Not otherwise specified. Marked with an asterisk are six dogs that had significant variation in DI comparing primary-and metastatic lesion.

In cases in which multiple aneuploid peaks were present, both of the stemlines were represented with the stemline was the lowest DI in white and the stemline with the highest DI in black. The 4 PD sarcomas are not represented in the table.
Amongst STS, malignant peripheral nerve sheath tumours (MPNST) and leiomyosarcomas had a significantly lower rate of aneuploidy (Table 1) as compared to all other STS (P = 0.003 and 0.03, respectively). In contrast, HS and synovial cell sarcomas had an increased rate of aneuploidy when compared to all other STS (P=0.01 and 0.02, respectively) or to MPNST or leiomyosarcomas (P< 0.02).

Twenty of the 77 primary sarcomas had hypoploid stemlines (26%, or 48% of the aneuploid cases). Amongst aneuploid sarcomas the occurrence of hypoploidy in STS (9 of 25; 36%) and MTB (11 of 17; 65%) did not differ significantly (P=0.115).
Noteworthy is the relative high DI within the three liposarcoma cases (2.0, 2.3 and 3.2 respectively), in relation to the general DI distribution in primary malignancies (Fig. 1).

There was no significant relation between ploidy status and histological malignancy grade (Table 2, grade I+II versus III in MTB: P=1.0, in STS: P=0.16, in all sarcomas: P=0.15). Note that this analysis excluded the three PD cases. Examination of a possible relation between ploidy-status and the *p53* gene mutational status in 44 sarcomas was negative (Table 3).

We then compared ploidy status with metastatic behavior. Only cases with macroscopic metastasis were considered, found either at first presentation (including those with postmortem after euthanasia) or during a follow up period for up to one year. PD cases (n = 4) and cases with less than one year follow up after tumour resection (n=22, including three PD cases) were excluded.
In the category of MTB only 20 dogs with OS fulfilled the criteria; all developed metastases either at first presentation (n=2) or after surgical removal of the primary tumour (n=18) without any influence of ploidy status (Table 4).

When viewing STS as a group (including HS) 22 out of 34 dogs had metastases at first presentation or during follow up after tumour resection. Aneuploid STS more often had metastases than diploid STS (P=0.0092). The difference was no longer significant (P=0.067) after exclusion of HS. All HS cases but one (which was euthanized upon diagnosis) had metastases at first presentation.

Heterogeneity of ploidy status or DI
In 12 dogs, we were able to compare the DI of the primary and metastatic lesions (Table 5). In 6 of these dogs, a significant variation in DI was noticed: The DI changed from a diploid into an aneuploid (hyperdiploid) pattern in 2 sarcomas and from an aneuploid (hyperdiploid) to a diploid pattern in one other. In one dog with HS, in which the largest tumour (lung) and a sternal lymph node were both analyzed, the pulmonary lesion was PD and the nodal was low-level hypodiploid

(DI 0.93). No particular significance was ascribed to this difference. In another three cases extra stemlines were identified on one location as compared to the other location(s), which in two of these cases led to the (dis)appearance of a stemline, containing twice the total DNA content.

Discussion

Our study indicates that the existence of aneuploidy in canine mesenchymal proliferative lesions is suggestive but no proof of a malignancy, in accordance with observations in humans [16, 58-60], although it must be recognized that a diploid status does not rule out malignancy. This finding may lead to additional cytological/histological testing of canine mesenchymal lesions in which the results of routine diagnosis remain ambiguous.

For sarcomas in humans, a relationship between histological grade of malignancy and ploidy pattern has often been reported [16, 20, 59, 61], but no such relationship was evident in our study. In part, this may be related to the relatively high frequency of hypoploid cancers, and the related presence of smaller nuclei, which might be judged lower grade by pathologists.

As in earlier studies in mammary malignant tumours in dogs [22, 53, 55] and in humans [22, 23, 30] we were also able to demonstrate a relation between ploidy status and risk of metastases in STS. Many studies in humans [28, 30, 31, 62] reported a similar correlation, although this is not universal [58]. Striking was the absolute lack of a relation between ploidy status and risk of metastasis in OS, which is in contrast to OS in humans [32, 63]. In diploid cases within particular types of cancer more detailed cytogenetic analyses are required to discern those changes that predict metastatic behavior [62, 64].

Consistent with early research [65-67] clear similarities were found with respect to aneuploidy occurrence amongst STS (53%) and MTB (57%) when compared to such sarcomas in humans [50, 58, 60]. For the subtypes of MPNST and leiomyosarcoma a diploid status is common in both dogs (as seen in our study) and humans [68]. However, in most human sarcomas hypodiploidy is rare [10, 50], with the highest reported rate being 11% [58], while chondrosarcomas are an exception since they are frequently hypoploid [69]. We observed a remarkable overall hypoploidy incidence of 26% (48% of aneuploid cases). One other study in canine OS reported a somewhat lower figure [67]. In addition, both studies indicate that for many subtypes of canine sarcomas the net increase in DNA content in hyperploid cases is modest when compared to their human counterparts [16, 58, 70] albeit that some sarcoma types, such as the three liposarcomas in our study, form an exception, since these were all (hyper)tetraploid.

Since similar observations have been made in other types of cancer in the dog such as thyroid and mammary carcinomas and malignant lymphomas [53, 54, 56, 57], it seems that the dog is particularly prone to the development of aneuploid tumours associated with either chromosome loss or low number chromosome gain, and less frequently to hypertriploid tumours that are common in humans [33, 34, 40, 50]. This feature seems at variance with ploidy evolution patterns described in humans. In humans, this evolution can be divided in stages with early on the development of tetraploidy which is followed by chromosome loss and later on leads to a major proportion (amongst aneuploid cases) of cancers manifesting an increase in DI of approximately 1.6-1.7 [33-35, 40, 45, 50]. Still, in a few sarcomas of the current study and in mammary carcinomas [53, 65] tetraploidization (in the primary cancer) followed by an appearance of hypotetraploid stemlines (in its metastases) has been observed. A possible explanation for this difference in ploidy evolution could be the presence of a more powerful defense mechanism in humans against the tumourigenic effects of hypodiploidy or low level hyperploidy [33, 71]. While for many human cancers a greater destabilization over multiple phases of destabilizing events seems necessary to arrive at a fully malignant state, such state may be reached with less destabilization in the dog, as hypothesized earlier [44]. How some cancers can reach at a fully malignant state while remaining DNA-diploid is uncertain. More subtle and sometimes balanced chromosomal gains and losses have been observed by cytogenetic analysis in some such human cancers [34] including sarcomas [19, 68], and also in canine sarcomas [72, 73] and carcinomas [74]. In other human diploid cancers however, structural chromosomal abnormalities are absent, and the transformation to malignancy seems to be driven by defects in DNA repair pathways leading to microsatellite instability, with microsatellite instability and aneuploidy being mutually exclusive phenomena [46].

It must be recognized that the karyotypic alterations that occur in dog cancer are to some extent at variance with those described for human cancers. The autosomes in normal dog cells are acrocentric/telocentric, and changes in canine cancers often concern centric fusions [75-77] [74] albeit that alterations such as trisomies or monosomies and translocations are also frequent [72, 75, 78, 79] as well as smaller structural abnormalities that have been detected with comparative genomic hybridization studies [73, 79-81] in the past few years. Still, as mentioned in an extensive review by Breen and Thomas, “tumours of the same histological types in both species present with equivalent cytogenetic lesions” [82].

In cancers that harbour significant chromosomal alterations, even if not recognized as DNA-aneuploid, the cause underlying this chromosomal instability has been subject of study and aberrations in many pathways, in particular those related to sister chromatid cohesion and segregation have been suggested as possible causes [46, 47, 49, 83-85]. Contrasting views exist as to whether loss of *p53* function is essential for the development of aneuploidy [52, 86-88]. In the current study,

no relation between *p53* mutations and DNA-ploidy status was found. Although it must be recognized that analysis for *p53* mutations concerns only part of the many elements involved in the *p53* pathway, our results are in clear contrast with a study in human sarcomas that demonstrated a relation between the presence of *p53* mutations and DNA-aneuploidy [61].

Regarding whether and how aneuploidy may be essential for the development of cancer [38] it is essential to agree on the definition of malignancy. Most oncologists agree that proof of metastatic potential is not the only prerequisite for a tumour to be considered malignant and that tumours with extensive infiltrative destructive growth and low or late risk of metastases such as leiomyosarcomas and MPNSTs in the dog should also be regarded as malignant [7]. In our study, these sarcomas were only rarely found to be aneuploid. There is no doubt that many malignancies with full malignant potential are aneuploid, reflecting gross quantitative genomic destabilization. Several major disturbances must have taken place in order to overcome restraints, which are – in part tissue specific – against malignant transformation. Other cancers, such as the diploid OS in the current study, can reach this state of progression with much less genome destabilization. Functional changes that may allow malignant behavior in such cancers may include *p53* inactivation or *MDM2* amplification [89, 90] as well as many other genetic alterations. Such changes may be called high hierarchy changes. This is opposite to many more subtle low hierarchy changes that can accumulate during progressive genomic destabilization and, by chance or number, can also disrupt crucial control mechanisms. To some extent and for some types of sarcomas (such as MPNST and leiomyosarcoma), a diploid status is then related to low metastatic potential. For OS in the dog a highly metastatic state can be either reached by appearance of a relatively low number of high hierarchy genetic changes, and low level of genomic destabilization in the form of chromosomal instability, while in others a high level of genomic destabilization, involving more lower hierarchy changes, may lead to this state.

The debate on gene mutation versus aneuploidization as cause of cancer cannot be resolved, since both phenomena may be linked and cooperative in tumour development [36, 48].

Once a malignant tumour has developed, the same DI can often be found in both the primary and the metastatic lesion. Sometimes, haploidization or duplication of such stemlines occurs, with variation between metastases. In other tumours in the current and earlier studies [53, 65, 67] stemlines were found which really looked like an unrelated clone, able to propagate as independent subpopulation, as hypothesized previously [91].

Experimental Section

Animals

Lesions from pet-dogs of various breeds, sex and age were selected for this study. Samples with suspicion of a neoplastic origin were obtained, with informed consent from the owner, by biopsy or surgical excision as part of normal diagnostic procedure or treatment, or at postmortem immediately following euthanasia. After previous studies by our research group on DNA-ploidy* of carcinomas [53, 54, 56] and of malignant lymphomas [57], the focus was on proliferative, inflammatory or neoplastic lesions of the mesenchyme.

All samples had been characterized by routine histopathology by different veterinary pathologists and were centrally reviewed for the current study. The ploidy assessment was not possible in three soft tissue sarcomas, one inflammatory lesion and two benign lesions, probably due to the amount of debris present. These samples were excluded from further analyses. Remaining samples, from 95 dogs, included non-neoplastic, proliferative, inflammatory lesions (n=10), benign tumours (n=8: lipoma (4), fibrous dysplasia of bone, osteochondroma, leiomyoma, fibrous epulis), and 77 malignancies (including one local recurrence; detailed description is listed in Table 2), from 77 dogs. From 12 of these 77 dogs both primary and recurrent specimens were available for examination.

Prior to collection of the tissue samples, none of the patients had received any cytostatic or radiotherapeutic treatment. Data on the occurrence of metastasis at first presentation or for up to one year after surgery were collected from the hospital records.

(*Where in the following text the word ploidy is used as related to the current study, it indicates DNA-ploidy.)

Preparation of samples

Upon surgery or euthanasia, tissue samples were immediately placed in melting ice. From areas of the mass not hampering full histological evaluation, thus avoiding planes of resection, blocks of approximately 5 mm^3 were cut, trimmed of fat and necrotic parts, snap-frozen in liquid nitrogen and stored at -70 ^{o}C until further analysis. The tissue samples were thawed only once, directly prior to FCM. Adjacent blocks including the resection planes were fixed in neutral phosphate-buffered 10% formalin for histological examination.

Histological examination

The tissues were routinely processed; paraffin embedded and cut to 4-6 µm sections that were stained with hematoxylin-eosin (H&E). Cases were re-evaluated by board-certified veterinary pathologist (CS, GCMG) and categorized according to the WHO classification for tumours in domestic animals (WHO 1994, 1998). STS were grouped by their entity, including additional immunohistochemistry if deemed appropriate

[89, 92, 93]. Most cases were reviewed, except for eleven due to sample loss, for which the original diagnoses were used. With the exclusion of histiocytic sarcomas (HS), a proper grading system is available for STS [8, 9] as well as for OS [4]. This was applied for the current series, except for ten cases that could not be graded leaving a total of 59 cases.

Flow cytometry analysis of nuclear DNA content

FCM of nuclear DNA content was performed as previously described [53, 57] using the detergent-trypsin procedure of Vindolov et al [94]. Isolated nuclei from all lesions were stained with propidium iodide (Sigma Chemical Co., St. Louis, MO). All neoplastic samples were required to contain an adequate proportion of at least 20% tumour cells and the cell yield of all specimens had to permit analysis of ≥ 5.000 cells in each assay. As external standard, Chicken Red Blood Cells (CRBC) was added to determine the position of the G_{0-1} peak(s). In some samples two G 0-1 peaks were discerned of which the DNA content both fell within the range of the ratio of the DNA content of normal dog diploid cells compared to that of CRBC. Then a second analysis was done with and without addition of Canine Kidney Cells (CKC) as a diploid standard. The increase in height of one of those peaks upon addition of CKC identified this peak as diploid and the other as aneuploid. The ploidy analysis itself was performed using the FAC Scan 3 flow cytometer (Becton Dickinson, Mountain View, CA). The device is able to detect changes above 5% in nuclear DNA content. The propidium iodide fluorescence was excited at 488 nm and measured at 585 nm. Since past research has indicated that, by measuring only a single tissue block, true aneuploid stemlines can sometimes be missed [58], many lesions - in particular large-sized ones - were analyzed using two or more tissue blocks. In 11 primary tumours of larger size (> 3 cm) more than one tissue block was analyzed to assess possible intra-tumour heterogeneity. When a difference was found between the separate analyses, the measurement was repeated several times with and without the external CKC reference standard.

DNA-ploidy assessment

The DI is defined as the ratio of the modal channel number of the G_{0-1} peak of the (neoplastic) cell population in relation to the modal channel number of the G_{0-1} fraction of diploid cells. The latter was recognized by its relative position to the G_{0-1} peak of CRBC and by is position to the normal CKC (normal dog reference) peak [53]. A sample was considered diploid if the DI was between 0.95 and 1.05 and aneuploid if there was a distinct G_{0-1} population with DI < 0.95 or > 1.05. A peak with a DI of 1.90-2.10 was considered to be a tetraploid G_{0-1} peak if it contained > 20% of the total number of analyzed cells and if a G2-M peak was also present. Aneuploid stemlines were subdivided into hypoploid (DI < 1.0) or hyperploid (DI > 1.0) When more than one aneuploid G_{0-1} population was present the sample was classified as multiploid. For all samples, the coefficient of variation (CV) for the G_{0-1} peak was measured. A CV above 5% was considered suspicious for the presence of an aneuploid peak within a diploid population. Samples with peaks with a CV >

5.1% were re-analyzed with and without the CKC reference cells, and if this led to a clear recognition of two separate peaks, it was considered aneuploid. However, if under these conditions a single G_{0-1} peak with CV > 5.1% remained, the sample was considered neither diploid nor aneuploid but peridiploid (PD). All non-diploid, non-PD lesions were grouped together as aneuploid, including those with heterogeneity in ploidy status within the primary site.

Mutational analysis of the *p53* gene

In two earlier studies, part of the sarcomas had been analyzed for the presence of *p53* mutations. For STS the *p53* gene was analyzed for exons IV-VIII, covering most of the mutations occurring in cancer [89]; for MTB exons I-X were examined [90]. Eight STS had been analyzed during the study of MTB that have not yet been published. The results of both investigations including a total of 44 sarcomas were used for a comparison with the ploidy-status. Only mutations predicted to affect the amino-acid sequence of the *p53* gene (http://www.ncbi.nlm.nih.gov/nuccore/NM_001003210.1), including point mutations, deletions and insertions, and mutations of the splice site were counted.

Statistical analysis

Differences in frequency distribution in data groups were analyzed using Fishers exact test. The level of significance was set at 0.05. PD samples were excluded in the statistical analysis comparing diploid and aneuploid status.

Conclusion

In conclusion, aneuploidy is frequent in various canine malignant lesions, with prominent variation amongst the different sarcoma types. The overall frequency of aneuploidy is largely in accordance with findings in humans [50]. However, the high frequency of tumours with hypoploidy and low-level hyperploidy in various canine cancers [53, 54, 57] when compared to human malignancies including sarcomas [50], is striking. In human solid cancers hypoploidy is rare and the majority of cases are hypertriploid thought to arise from a tetraploid intermediate (33-35, 46, 50). Our findings therefore are suggestive of interspecies variation in aneuploidy evolution and genomic destabilization during carcinogenesis.

Acknowledgments

This study was partly funded by the European Commission (LUPA-GA-201370).
The authors wish to thank Mrs. N. J. Kuipers-Dijkshoorn for expert flow cytometric analyses and dr. K. Thway for her useful comments. Dr J. Joles is thanked for reading and correcting the manuscript.

Conflict of Interest

The authors declare no conflict of interests

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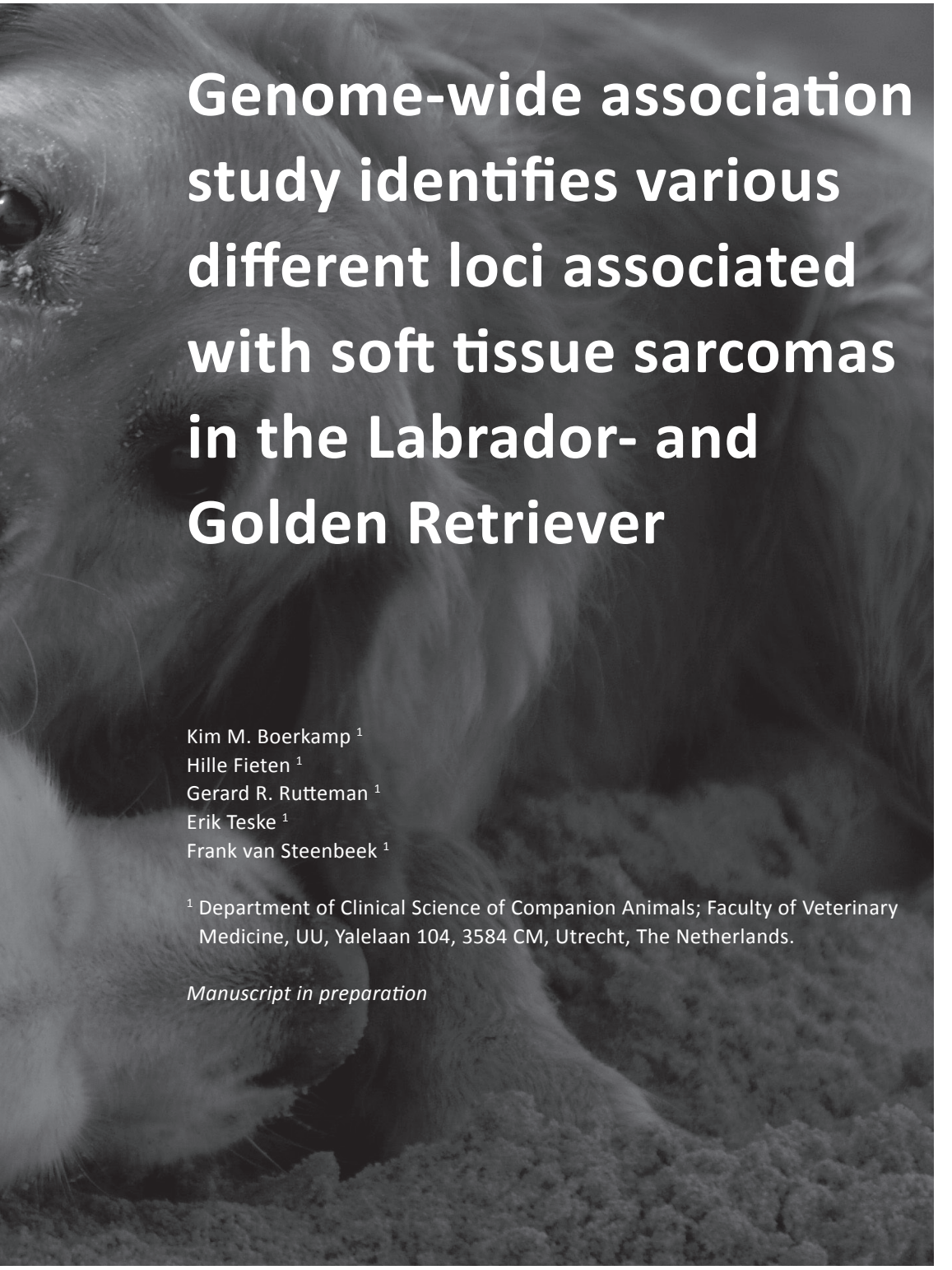

Genome-wide association study identifies various different loci associated with soft tissue sarcomas in the Labrador- and Golden Retriever

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Manuscript in preparation

Abstract

Soft tissue sarcomas in the dog are a heterogeneous group of mesenchymal malignancies known to share many of the clinical and histopathological characteristics of human soft tissue sarcomas. Dogs can therefore represent as a suitable animal model. Soft tissue sarcomas occur more frequent within the Labrador- and Golden retriever breed compared to the general dog-population. This breed-predisposition suggests a hereditary background for the disease. We describe results from the first genome-wide association study (GWAS) in the group of soft tissue sarcomas in any species, aiming to identify possible genetic variants associated with soft tissue sarcoma development.

The Labrador retriever GWAS displayed significant association to a region on chromosome 3 (P_{perm}= 0.026), and suggestive associations on chromosomes 35 and 17 (P_{perm}= 0.201 and 0.237). The Golden retriever GWAS displayed a suggestive association on a region on chromosome 26 (P_{perm}= 0.205).

All of these loci contain candidate genes that are already known to be involved in the development of cancer, and are thus considered to be very promising.

This study is suggestive for a germline genetic association between these breeds' genotypic characteristics, and the development of soft tissue sarcomas. The use of high throughput techniques for DNA re-sequencing will probably result in novel variants which, if validated in larger cohorts, might aid in developing new breeding strategies.

Introduction

Soft tissue sarcomas (STS) are a variable group of locally invasive tumours of mesenchymal origin, but show similarities in clinical behavior [1-3]. STS in dogs are spontaneous occurring tumours and share many of the phenotypically and molecular characteristics with human STS, including histopathological and clinical manifestations [3-5]. Therefore, this disease in dogs provides a good comparative disease model for studying human STS [3].

In the general dog population, STS comprises approximately 15% of all skin and subcutaneous tumours [6] but the Labrador retriever (LR) and the Golden retriever (GR) seem to be overrepresented [7-12]. This breed association indicates a genetic predisposition for the development of STS in the LR and GR and could also, because of the discovery of new genes potentially involved in the development of STS, be beneficial to better dissect the genetic background of this rare human disease.

In dogs, only a small selection of animals are used for breeding purposes, and selection is based on desirable traits, favoring breed-predisposition for inherited diseases such as cancer [13-16]. This selection is responsible for the clear formation of distinct dog breeds [17-21] and has led to extensive within-breed linkage disequilibrium (LD) [13, 18, 21-23]. A relatively small number of genetic variants thus gather a large effect [24]. Compared to humans, this results in a requirement of much less samples that are required when a search for genetic factors is conducted in dogs [13, 15, 18, 22, 25, 26]. In addition, fewer markers are required to identify disease association [22]. This makes the dog an excellent model for mapping the genetic basis of certain traits [13, 18, 26, 27] especially malignancies [13, 28]. Recently, cancer mapping using a GWAS, has already successfully been performed within another dog breed, the Bernese Mountain dog, that has a clear predisposition for histiocytic sarcomas [29].

This study presents a case-control GWAS in order to identify regions associated with STS development in two dog breeds. The two breeds both show different non-overlapping associated loci. This indicates that STS development has different genetic risk factors influencing the two breeds. Our data provides the first preliminary support for the existence of a germline genetic association between these breeds' genotypic characteristics, and the development of soft tissue sarcomas.

Materials and methods

Recruitment and sample collection

Dogs were considered 'case' whenever a STS was diagnosed by a board-certified veterinary pathologist based on histopathological examination of a tissue sample. Dogs were considered 'healthy controls' whenever a Labrador or Golden retriever reached eight years of age with no evidence of a STS or any related tumour. From

all cases and controls, blood samples (in (EDTA) stabilized tubes) and health questionnaires were collected. The health questionnaire enquired about the dog's sex, age, pedigree, general health status as well as any family history regarding cancer. Whenever possible, owners of healthy controls were contacted on a bi-annual base to inform on any changes regarding the health status of their dogs.

All blood samples were taken with owners consent from dogs by trained veterinarians according to relevant national and international veterinary and ethical guidelines. Animals were recruited through veterinary clinicians from various European (UK, Sweden, and Italy) and the USA, and available data indicated that the dogs were unrelated at parental level.

Regarding the LR, 343 animals (169 male and 174 female) were collected; 100 cases and 243 controls. Regarding the GR, 182 animals (88 males and 94 females) were collected; 43 cases and 139 controls. Controls used in both cohorts were older than 8 years of age. Median age of the LR cases was 9.5 years (1 to 14 years) and 9 years (3 to 15 years) in the GR cohort. Healthy veteran control dogs were aged 8 to 16 years (median 10 years) in LR and 8 to 15 years (median 10 years) in GR.

Because of the known existence of clear population differences in allele frequency and distribution in between the GR population from the USA and Europe [30], only GR from Europe were recruited.

Genomic DNA was extracted from whole blood or buccal swabs using QIAamp DNA Blood Midi Kit (QIAGEN), phenol-chloroform extraction [31] or salt extraction [32]. The quantity of genomic DNA was measured using a NanoDrop spectrophotometer. Each sample was stored at -20 prior to being genotyped.

Genotyping and genome-wide association mapping

Genotyping was performed using the Ilumina Canine HD BeadChip containing 174,000 highly polymorphic SNPs with a mean genomics distance of 13 kb [33]. All SNPs were analyzed with the software package GenABEL (an R library for genome-wide association analysis) version 1.7 (http://cran.r-project.org) [34, 35]. Genotyping quality control settings were set to 98% successful genotypes per individual and 95% successful genotypes per SNP.

SNPs for which at least 20 carriers were present were included in the analysis. A final data set for the GR-cohort of 103,242 SNPs and for the LR-cohort 115,533 SNPs remained for analyses after quality control. Possible genomic inflation and suggestive significance levels were assessed using QQ-plots (Figure 1.). Stratification and cryptic relatedness were adjusted for by mixed models and genomic control implemented in the function grammas [36] in GenABEL. Genome-wide significance was ascertained with permutation testing in GenABEL using

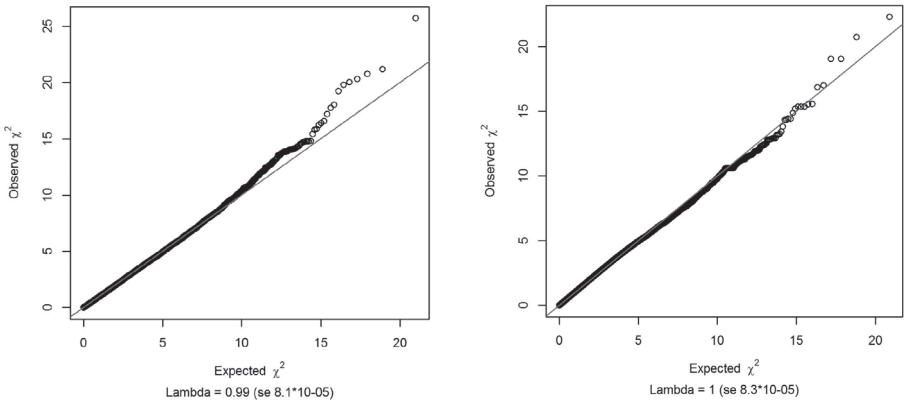

Figure 1: QQ-plot for the "grammas" analysis of STS in Labrador retrievers (left) and Golden Retrievers (right).

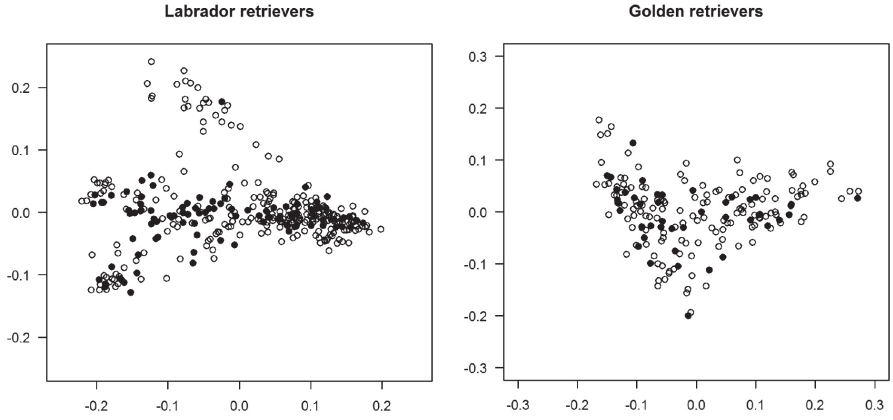

Figure 2: The Multi-dimensional scaling plot (MDS plot default HWE) on status for the Labrador retriever cohort (left) and the Golden retriever cohort (right).

1000 permutations [35], denoted by Pperm. No threshold for Hardy-Weinberg equilibrium (HWE) was used.

Ethical statement

Clinical specimens were acquired from family-owned dogs, under approved institutional protocols and with informed client consent.

Results

Population stratification was determined by a multidimensional scaling (MDS) plots, showing the overall genetic similarity between individuals of both breeds used in the study divided by disease status (Figure 2.). Samples are represented by affection status. Sex, castration and age had no significant influence on STS outcome. These variables were not used as covariates in the GWAS analyses.

For the LR cohort (Figure 3), genome-wide association mapping revealed a significant SNP within a region of interest on CFA03 (best SNP: Output CanFam 3.1: 736,743,46-761,755,70; P_{perm} = 0.026; P_{mms} = 5.70 E-07). A suggestive association was seen CFA35 (best SNP: Output CanFam 3.1: 349,552,2-849,204,0; P_{perm} = 0.201, P_{mms} = 3.39 E-06) and CFA17 (best SNP: Output CanFam 3.1: 318,953,62-439,337,92; P_{perm} = 0.237, P_{mms} = 7.35 E-06). In total, these three regions of interest included 345 unique canine genes; of which 201 genes were included in the region of CFA03; 116 were included in region CFA17 and 28 genes were included in the region of CFA35. There were several promising candidate genes, already known to be of importance in cancer development, present within these regions. Among genes that are considered to be possible candidates in the significant region CFA03, is *BCAR1* (breast cancer anti-estrogen resistance 1; poorly differentiated tumours showing higher *BCAR1* expression [37, 38]) ; *RAB12* and *RAB14* (members of the RAS oncogene family) [39], and *UBQLN1* (ubiquilin 1) [40]. Genes that are considered to be possible candidates in the region of CFA17, are *BCL2L11* (BCL2-like 11 (apoptosis facilitator) [41], *MERTK* (c-mer proto-oncogene tyrosine kinase [42] and TCF7L1 (transcription factor 7-like 1 (T-cell specific, HMG-box) [43]. Genes that are considered to be possible candidates in the region of CFA35, are *EEF1E1* (eukaryotic translation elongation factor 1 epsilon 1), a well-known tumor suppressor.

For the GR cohort (Figure 4), genome-wide association mapping revealed several near- significant associations with STS on CFA26 (output CanFam 3.1: 18,969,486-219,693,90; best SNP: P_{mms} = 7,79 E-06, P_{perm} = 0.205). The top SNP within this region (Canfam 3.1: 196,731,05) was located exactly at the region of a very promising gene: *Myo18B*, a well-known tumour-suppressor candidate gene [44-48]. The area directly surrounding the region of this top SNP, also revealed several other promising candidate genes, such as *SEZ6L* [49].

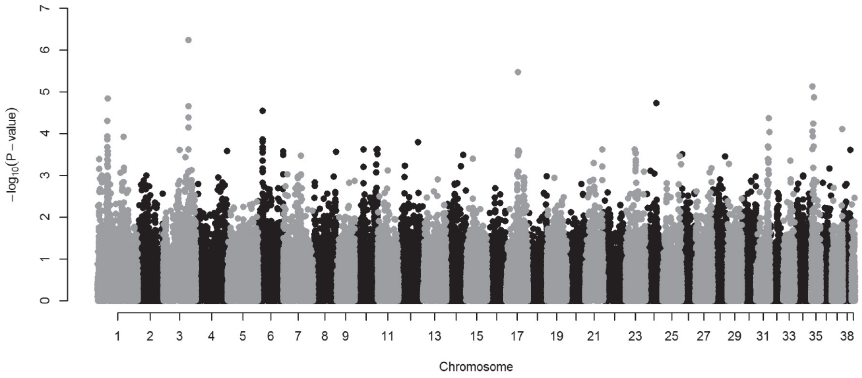

Figure 3: Genome-wide association results for the LR cohort: Genome-wide association is seen on CFA03. Manhattan plot displaying -log 10 P-values for the Labrador retriever cohort (mms).

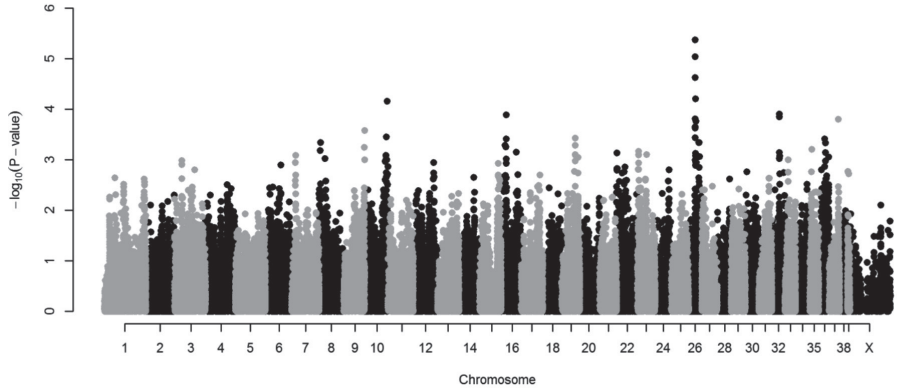

Figure 4: Genome-wide association results for the GR cohort. Strongest association is seen on CFA26. Manhattan plot displaying - log 10 P-values for the Golden retriever cohort (mms).

Discussion

The two dog breeds used in this study, are genetically closely related [33] and are both predisposed for similar types of cancers such as STS. However, results from the GWAS of the LR and GR cohort show non-overlapping regions of interest in relation to STS development. This indicates that the genetic predisposition of both breeds can be situated within different areas of the genome. This could be explained by the high degree of across-breed heterogeneity, even between these genetically closely linked breeds, which is reducing the power to detect possible associations between certain hereditary traits [25]. However, it cannot be excluded that the genes of interest within these breeds, represent a mutual pathway. Also, the group of STS show clinical heterogeneity and it is likely that relative genetic effect sizes are small, possibly causing some of the observed associations to remain borderline significant. The fact that suggestive significance was obtained in this very complex disorder using only a relatively low number of cases and controls, suggests that the regions identified, are indeed likely to be important determinants for the development of STS.

Further studies such as those focusing on fine mapping and DNA re-sequencing are required. Also future studies could possibly provide higher density coverage and increased statistical power. Collecting cases is, however, hampered by reluctance of animal owners and/or veterinarians to seek a final diagnosis in advanced cases or to submit material for study. Furthermore, even while more common in the dog than in the human, STS remain a relative rare cancer. The fact that a significant proportion in cases occurred in animals > 10 years of age, necessitates collection of DNA from controls also at high age. The assignment of a normal phenotype can, in some cases, represent a source of error; healthy controls could, in later stage, develop STS. Because this was a source of concern, normal phenotype was assigned only if dogs were older than eight years of age, and where possible owners were questioned on a regular base to inform on the health status of their dog.

The regions of interest contain several candidate genes; most of which are positioned in proximity of each other, and at this moment it is not possible to determine the actual effect of these (individual) genes regarding the development of STS. Though it can be considered probable that some of the candidate genes found in the regions of interest are indeed connected to the disease development (a clear example is *MYO18B*, a gene that is expressed in muscles, and is a well-known tumour suppressor gene [44]; with mutations associated with lung cancer [47], colorectal cancer [44] ovarian cancer [45] and tumour development and -progression in general), the involvement of these regions do need confirmation. The approach chosen for future studies will be DNA re-sequencing using next generation sequencing. Once the causal mutations have been identified, functional studies need to be performed; aiming to investigate and confirm the function of this mutation.

Conclusion

This is the first study in any species that investigates possible genetic alterations that could lead to the development of STS, in order to better underpin the genetic background of this complex collection of tumours. Using a GWAS approach in two different, predisposed breeds; we were able to identify several non-overlapping regions of interest in both breeds. Several genes enclosed within these loci are considered to be very promising candidate genes. We consider these genes to carry regulatory potential with regards to STS development, especially considering the relatively low number of cases and controls and the complexity of the disease. We therefore expect the located regions to indeed be important determinants for the development of STS.

Funding
This research was supported by the LUPA consortium, which is a Collaborative Research Project funded by the European Commission under the 7th Research Framework Programme (www.eurolupa.org) LUPA-GA-201370. Further support to pay for collecting samples and histopathological analysis was obtained from the Dutch Labrador Society, NLV.

Acknowledgments
We thank our international contributors, and of course all dog owners for contributing and supporting this study.

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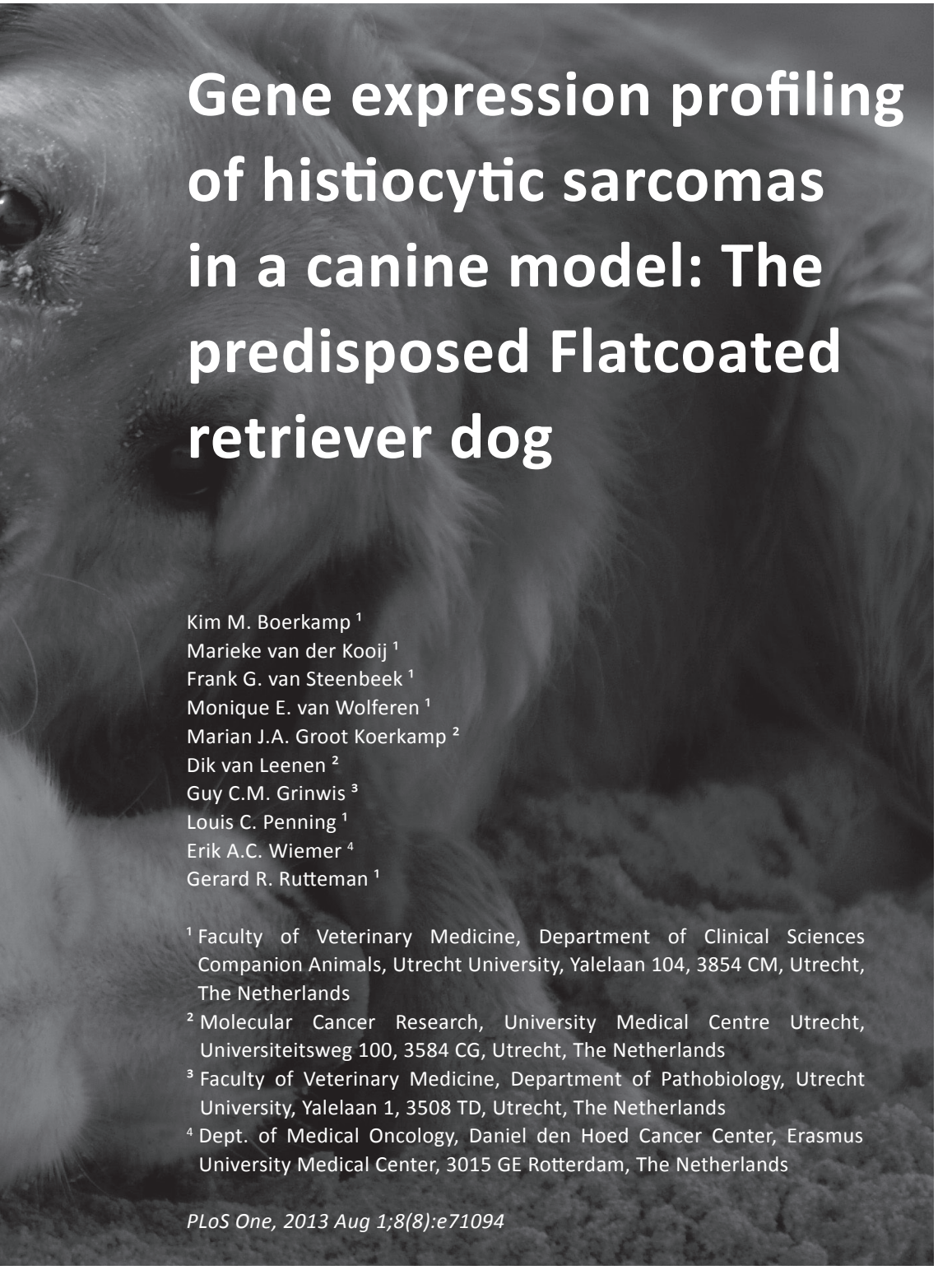

Gene expression profiling of histiocytic sarcomas in a canine model: The predisposed Flatcoated retriever dog

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PLoS One, 2013 Aug 1;8(8):e71094

Abstract

Background

The determination of altered expression of genes in specific tumour types and their effect upon cellular processes may create insight in tumourigenesis and help to design better treatments. The Flatcoated retriever is a dog breed with an exceptionally high incidence of histiocytic sarcomas. The breed develops two distinct entities of histiocytic neoplasia, a soft tissue form and a visceral form. Gene expression studies of these tumours have value for comparable human diseases such as histiocytic/dendritic cell sarcoma for which knowledge is difficult to accrue due to their rare occurrence. In addition, such studies may help in the search for genetic aberrations underlying the genetic predisposition in this dog breed.

Methods

Microarray analysis and pathway analyses were performed on fresh-frozen tissues obtained from Flatcoated retrievers with localized, soft tissue histiocytic sarcomas (STHS) and disseminated, visceral histiocytic sarcomas (VHS) and on normal canine spleens from various breeds. Expression differences of nine genes were validated with quantitative real-time PCR (qPCR) analyses.

Results

QPCR analyses identified the significantly altered expression of nine genes; *PPBP, SpiC, VCAM1, ENPEP, ITGAD* (down-regulated), and *GTSF1, Col3a1, CD90* and *LUM* (up-regulated) in the comparison of both the soft tissue and the visceral form with healthy spleen. DAVID pathway analyses revealed 24 pathways that were significantly involved in the development of HS in general, most of which were involved in the DNA repair and replication process.

Conclusions

This study identified altered expression of nine genes not yet implicated in histiocytic sarcoma manifestations in the dog nor in comparable human histiocytic/dendritic sarcomas. Exploration of the downside effect of canine inbreeding strategies for the study of similar sarcomas in humans might also lead to the identification of genes related to these rare malignancies in the human.

Introduction

Fundamental research on rare human diseases is not only hampered by minimal grant supply; lack of sufficient sample numbers is of equal disadvantage. One way to overcome this last catch, is to investigate other species in which a similar disease occurs at a much higher frequency, like in specific dog breeds. In dogs, a downside of selection for breeding purposes is the occurrence of a very large number of breed-specific hereditary diseases (http://omia.angis.org.au/home/). Rare human diseases, such as histiocytic malignancies, might therefore be common in specific dog breeds [1].

The Flatcoated retriever (FCR) breed has a strongly increased risk for histiocytic sarcoma (HS) development. In the UK, it is likely to account for about 36% of all malignant neoplasms diagnosed in this breed [2, 3].

Canine histiocytic malignant disorders were as such first described in the late 1970s [4]. Included in the name 'histiocytic sarcoma', which was given to the complex of malignant histiocytic disorders [5], there is a range of malignant tumours derived from CD34-committed stem cell precursors that may develop into dendritic cells (DC) such as Langerhans cells, interstitial DC and macrophages [6, 7]. In addition to morphological features, most histiocytic sarcomas can be recognized by positive immunostaining for the cell surface marker CD18 [6-8]. Canine HS has resemblance to the rare and often lethal human histiocytic malignancies, including dendritic cell and histiocytic sarcomas and disseminated Langerhans cell histiocytosis (LCH) [9-12]. Histiocytic sarcomas in dogs almost inevitably metastasize to various organs [10] and have a very poor prognosis [2, 10, 11]. A median survival of four months has been reported in the FCR [3].
In dogs, there are several clinical manifestations of HS. One common form is the localized, soft tissue histiocytic sarcoma (STHS), which manifests itself as a tumour arising in the deeply seated soft tissues of limbs often in association with joints [2, 3, 10, 13]. A second common form is manifested in internal organs and often multifocal and named disseminated, visceral histiocytic sarcoma (VHS), with neoplastic changes that can be found in either spleen, liver, lung and/or bone marrow [6].

Past research using Comparative Genomic Hybridization has already shown various aberrations in HS in the Bernese Mountain Dog, another breed predisposed to histiocytic malignancies, with cases showing numerous shared Copy Number Alterations (CNAs) both gains and losses, throughout the genome. These included deletions of the tumour suppressor genes *CDKN2A/B, RB1* and *PTEN* [10]. Furthermore, an associated constitutional haplotype in a locus near to the highly cited tumour suppressor locus *MTAP-CDKN2A* has recently been identified in this breed [1]. Another study concluded that deregulation of the expression of the glycation end products (Receptor for Advanced Glycation Endproducts; *RAGE*) and

the high mobility group box1 protein (*HMGB1*) potentially have a major effect on the progression of malignant histiocytic disorders [14].

cDNA microarrays have become powerful tools in the study of gene expression which has enabled improved classification of various naturally occurring cancers [15, 16] and have, once the canine genome sequence became available [17] already proven their value in the research of various canine sarcomas [18-20] but not yet in HS. Thus, we examined shared genetic functional aberrations of HS by comparing both forms of HS with normal tissue, for which spleen was chosen.
The study of spontaneously occurring tumours in the dog, a species which has a genetically stronger relationship to the human than mice [21, 22] can enrich the knowledge of rare human cancers, and lead to more insight in the pathogenesis of the disease and facilitate the identification of therapeutic targets valuable for dog and human [1, 11, 18, 20, 21]. The outcome of this study provides evidence of the existence of common differences in gene activity between HS and normal spleen.

Materials and methods

The experimental protocol (ID 2007.III.08.110) was peer-reviewed by the scientific committee of the Department of Animals in Science & Society, Utrecht University, The Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht, The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (the Dutch 'animal experiments decree', 1996). Both documents are available online at http://wetten.overheid.nl.

Case recruitment and histopathological evaluation
All tumour samples were confirmed as being spontaneously occurring histiocytic malignancies and were obtained from family-owned FCR with informed owner consent. All tumour material used originated from the Dutch FCR, that had not received radiotherapeutical or cytostatic treatment. Tumours were obtained under sterile conditions, either as part of a routine diagnostic or therapeutic surgical procedure, or immediately following euthanasia. Directly after excision, samples were snap frozen in liquid nitrogen, or alternatively by primary preservation in RNA-later, in both instances followed by storage at minus 70 0C. Tumour samples collected adjacent to the site of the frozen or RNA-later preserved samples were fixed in 10% neutral buffered formalin and routinely processed for histological examination.

At the time of surgery or necropsy, the evident anatomical location of all tumours was recorded for each individual and categorized as either VHS, if a tumour was present in internal organs (n=7) or STHS, if the tumour was localized in a limb only without identifiable metastases (n=6) [8].

Table 1: Patient details

Name	Sex	Pathology	AO (yrs)	Site	Microarray / PCR
D1, TJ	MN	STHS	7.7	shoulder	Y / Y
D3, UH	FN	STHS	8	shoulder	Y / Y
D4, BS	MN	STHS	6.5	elbow	Y / Y
D5, BaS	M	STHS	7.6	knee	Y / Y
D6, TV	MN	STHS	8.1	elbow	Y / Y
D7, DV	MN	STHS	11	shoulder	Y / Y
DX, YM	MN	STHS	9.7	shoulder	N / Y
D2, BE	M	VHS	9.4	liver/spleen/ **lnn abd**	Y / Y
D8, DW	M	VHS	9.5	spleen/**lnn abd**	Y / N
D9, JV	FN	VHS	8.9	lung/**lnn mediast**	Y / Y
D11, BT	F	VHS	8.5	**lung**	Y / Y
D12, AG	M	VHS	7.3	**lung**/spleen/ kidney	Y / Y
D13, TR	FN	VHS	7.9	**lung**	Y / Y
D14, SG	F	VHS	4.1	lung/**lnn mediast**	Y / Y
DX, SC	MG	VHS	10	liver/**spleen**	N / Y

AO: Age of onset, M: male, MN: male neutered, F: female, FN: female neutered, STHS: soft tissue (localized) histiocytic sarcoma, VHS: visceral (disseminated) histiocytic sarcoma, lnn abd: abdominal lymphnodes, lnn mediast: mediastinal lymph nodes Note: For cases with VHS the site sampled for gene expressionis indicated in bold letters.

Histological specimens were classified by a board-certified veterinary pathologist (GCMG) according to the recommendations and classification scheme defined by Affolter and Moore [6]. In all cases immunohistochemical staining with antibodies against CD18 protein (the common subset of β2 adhesion integrins, expressed in histiocytes, dendritic cells (DC), lymphocytes, and polymorphonuclear leukocytes [23]) were used to confirm the suspected histiocytic origin [8]. Results of this staining were divided in two categories: negative or positive. If the differential diagnosis based upon morphology included the potential origin of other malignant round cell tumours (malignant lymphoma, mastocytoma, melanoma, myeloma) appropriate immunostaining to examine such potential histogentic origin was performed and had to be negative. All tumour samples selected for the genetic study contained over 50% tumour cells as assessed in histological sections of biopsies of adjacent tissue. Patient details are listed in Table 1.

As control tissue, normal spleen from (healthy) crossbreed dogs (n = 6) was used as obtained at postmortem immediately following euthanasia that was not related to neoplastic, endocrine or metabolic diseases.

As a common reference pool a multitude of canine organs (testis, liver, spleen, prostate, duodenum, lung, kidney and brain) were used that had been obtained from healthy crossbreeds (n=8) euthanized for non-metabolic, non-tumourous lethal conditions.

The procedures were approved by the local ethics committee, as required under Dutch legislation (ID 2007.III.08.110).

RNA isolation

Approximately 30 mg of frozen tumour was transferred to a container with 600 µl of Buffer RLT and was disrupted/lysed and homogenized using a dismembrator (Braun Biotech Int., Melsungen, Germany) for 45 s at 2200 rpm. Total RNA was isolated and treated with DNase using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Quantity and integrity were assessed with the Bioanalyzer Agilent BioAnalyzer-2100 (Bioanalyzer, Agilent Technologies, Santa Clara, CA) in combination with an RNA 6000 Pico-LabChip. The average RNA integrity number 8.5 (range: 7.2-9.8) was found to be appropriate [24]. RNA concentration was quantified using a NanoDrop ND-1000 (Isogen Life Science) spectrophotometer.

Expression profiling

RNA was labeled twice and hybridized against the common reference RNA on dual channel arrays. RNA concentrations were 0.6 µg/µl at a minimum amount of 3 µg per sample.

RNA amplifications and labeling were performed on an automated system (Caliper Life Sciences NV/SA, Belgium) as described [25]. Dye swap of Cy3 and Cy5 was performed to reduce dye bias. Hybridizations were done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A.) using 500-1000 ng labeled cRNA per channel as described [26]. Microarrays used were Agilent Canine Gene Expression Microarrays V1 (Agilent Technologies, Belgium) representing 42,035 canine 60-mer probes in a 4x44K layout.

Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% photomultiplier tube voltage. After automated data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed [27] on mean spot-intensities. Dye-bias was corrected based on a within-set estimate as described [28].

Analyses were performed to detect common differences in gene expression between the two groups of HS and healthy spleen tissue. Data were analyzed using ANOVA [29] (R version 2.2.1/MAANOVA version 0.98-7) (http://www.r-project.org/). In a fixed effect analysis, sample, array and dye effects were modeled. *P*-values were determined by a permutation F2-test, in which residuals

Table 2: QPCR primers for genes of interest based on Microarray and pathway analyses

Gene name	Accession number	Primer sequence (5'-3')	Forward/ reverse	Annealing T (°C)	Size (bp)
PPBP	XM_539315	ACTGTCTCTGGCATTCATCC	F	59	116
		AGGCAGATTCTCTTCCCATT	R		
GTSF1	XM_534784.3	GCAGAAAGAATCATCCTGATGTC	F	57	221
		GATCTTTATCCCAGTCTTCATCG	R		
Spi-C	XM_849901.2	AATTACCTGGCTTTCATCAACC	F	57	114
		CAGCACTGTTTATTACTGTTCTCC	R		
VCAM1	NM_001003298	CTACAAGTCTACATCTCACCCA	F	58	213
		TTCCAGAATCTTCCAGCCTC	R		
ENPEP	XM_535696.3	GCTTCCTTCTTTGAGTTCCT	F	58	266
		TTCCAAGTAAATCTGGCATCCT	R		
LUM	XM_539716.3	CAAGACAGAAGGATTCAAAGCA	F	55	132
		GATGACAGCCCATAAATCGG	R		
ITGAD	XM_843683.2	TCTTGTATTGAACTGCTCCA	F	57	261
		GTTGTAGACCTCATACTTCTCC	R		
Col3A1	XM_845916	ATAGAGGCTTTGATGGACGAA	F	65	132
		CCTCGCTCACCAGGAGC	R		
MYH11	XM_857838	GAGAGGACCAGTCCATTCTG	F	59	253
		GATGAATTTGCCAAATCGTGAG	R		
Thy1	XM_844606.2	CTGTGCTCAGAGACAAACTG	F	58	185
		TTAGCCAACTCAGAGAAAGTAGG	R		

Common genes chosen for qPCR development identified using microarray as being significantly different in both disseminated, Visceral Histiocytic Sarcoma (VHS) and localized, Soft Tissue Histiocytic Sarcoma (STHS) compared to spleen. PPBP: Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7), GTSF1: Gametocyte specific factor 1, SPIC: Spi-C transcription factor, VCAM1: Vascular cell adhesion molecule 1, ENPEP: Glutamyl aminopeptidase (aminopeptidase A), LUM: Lumican, ITGAD: Integrin, alpha D, Col3A1: Collagen, type III, alpha 1, MYH11: Myosin, heavy chain 11, smooth muscle, Thy-1 (CD90): Thy-1 cell surface antigen

were permutated 5,000 times globally. Genes with $P < 0.0002$ after either family wise error correction (FWER) or determination of false discovery rate (FDR) were considered significantly changed. Genes with log2-fold changes of more than 0.5 or less than -0.5 were then selected to ensure that only robust changes were considered.

The Gene Ontology (GO) database (http://www.geneontology.org/) was used to check gene molecular and biological functions of the remaining genes.

Functional annotation

In general, pathway analysis in dogs has its restraints because pathway identification relies heavily on existing functional annotation, which is still limited for this species. Still, pathway analysis provides an additional way to analyze expression data across species. This may shed light on common pathways important for tumour behavior and on finding new therapeutic targets. To examine whether certain pathways are over- or under-represented in the gene list, all genes significantly differentially expressed between either STHS, VHS and normal spleen, were introduced into DAVID (http://david.abcc.ncifcrf.gov/).

Quantitative real time PCR

Gene selection

Following the outcome of the microarray expression profiling, ten genes were selected. Selection of ten genes was based on significantly differently expression, M-fold changes and potential biological function in relation with tumour development.

These genes including their optimum temperature are listed in Table 2.

RNA isolation and cDNA synthesis

Tissues from all but one patient (of which the insufficient tissue remained for the qPCR experiment) were used in the microarray experiment (six spleens, six STHS and seven VHS), furthermore three additional samples (one normal spleen, one STHS and one VHS that met the inclusion criteria) were added thus creating 3 groups of seven samples for the qPCR experiment. Total RNA from these samples was isolated. After isolation, total RNA was treated with DNase using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol.
Reverse transcription (RT) was performed of all 20 samples in a 80 µl reaction using 2000 ng total RNA, 16 µl iScript Reaction mix and 4 µl iScript Reverse Transcriptase (iScript cDNA Synthesis kit, Bio Rad, Veenendaal, The Netherlands). This includes a mixture of oligo-dT random hexamer primers. The mixture was incubated 5 min. at 25^{o}C, 30 min. at 42^{o}C and followed by 5 min. at 85^{o}C. Minus RT controls were prepared from 500 ng of the same RNA under the same conditions, but without addition of reverse transcriptase.

Reference genes and primer development

Reference genes were selected as non-regulated reference genes for normalization based on their stable expression in canine tissue [30, 31].

Using Ensembl, through annotated transcripts, PCR primers were designed using the Perl Primer software (version 2.0.0.7) and primer3 software (version 0.4.0) according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product and also in qPCR by checking the meltcurve and reaction efficiency. GeneNorm was used to establish

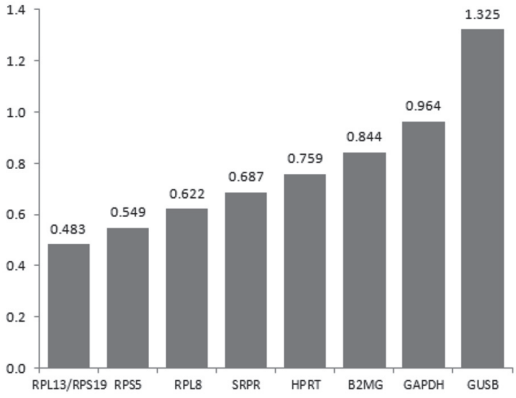

Figure 1: Gene stability by Genorm for all nine reference genes
Horizontal axis: Least stable genes (left) and most stable genes (right)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ß-2-Microglobulin (B2MG), Ribosomal protein S5 (RPS5), Ribosomal protein S19 (RPS19), Hypoxanthine phosphoribosyltransferase (HPRT), Ribosomalprotein L8 (RPL8), B-Glucuronidase (GUSB), Signal recognition particle receptor (SRPR), and Ribosomal protein L13 (RPL13)

Table 3: Reference genes primers for qPCR

Gene name	Accession number	Primer sequence	Forward/ reverse	Annealing T (°C)	Size (bp)
HPRT	AY283372	AGCTTGCTGGTGAAAAGGAC	F	56	104
		TTATAGTCAAGGGCATATCC	R		
RPS19	XM_533657	CCTTCCTCAAAAAGTCTGGG	F	61	95
		GTTCTCATCGTAGGGAGCAAG	R		
RPL8	XM_532360	CCATGAATCCTGTGGAGC	F	55	64
		GTAGAGGGTTTGCCGATG	R		
SRPR	XM_546411	GCTTCAGGATCTGGACTGC	F	61	81
		GTTCCCTTGGTAGCACTGG	R		
RPL13	AJ388525	GCCGGAAGGTTGTAGTCGT	F	61	87
		GGAGGAAGGCCAGGTAATTC	R		
GUSB	NM_001003191	AGACGCTTCCAAGTACCCC	F	62	103
		AGGTGTGGTGTAGAGGAGCAC	R		
GAPDH	NM_001003142	TGTCCCCACCCCCAATGTATC	F	58	100
		CTCCGATGCCTGCTTCACTACCTT	R		
B2MG	XM_535458	TCCTCATCCTCCTCGCT	F	61	85
		TTCTCTGCTGGGTGTCG	R		
RPS5	XM_533568	TCACTGGTGAGAACCCCCT	F	62.5	141
		CCTGATTCACACGGCGTAG	R		

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ß-2-Microglobulin (B2MG), Ribosomal protein S5 (RPS5), Ribosomal protein S19 (RPS19), Hypoxanthine phosphoribosyltransferase (HPRT), Ribosomalprotein L8 (RPL8), B-Glucuronidase (GUSB), Signal recognition particle receptor (SRPR), and Ribosomal protein L13 (RPL13)

expression stability [32]. Amplicon sequence-reactions were performed using BigDye v3.1 according to the manufacturer's (Life Technologies, Bleiswijk, The Netherlands) instructions on an ABI3130XL and analyzed in Lasergene (version 9.1 DNASTAR) and confirmed the specificity of each amplicon. Using RefFinder, the stability of nine reference genes were checked. In Figure 1 they are listed according to their stability. Four of the more stable primers were chosen for further data analysis, namely *ribosomal protein S5 (RPS5), signal recognition particle receptor (SRPR), ribosomal protein L13(RPL13), hypoxanthine phosphoribosyltransferase (HPRT).*

Primers for all nine reference genes, including their optimum temperature are listed in Table 3.

Quantative PCR

For qPCR, the Bio-Rad detection system (Bio-Rad.) with SYBR green fluorophore was used. Reactions were performed in a total volume of 25 µl containing 12.5 µl of 2x SYBR green super mixes (Bio-Rad Laboratories Ltd.), 1 µl of each primer at 400 nM concentration, 0.8 µl of cDNA and 9.7 µl Rnase and Dnase free water as previously described [33, 34]. Q-PCR reactions for each primer set were optimized by performing reactions under a gradient of annealing temperature using three serial 16x dilutions of pooled cDNA from all tissue samples. Cycling conditions were as follows: Denaturation (95 ºC for 5 min.), amplification cycle repeated 45 times (95 ºC for 10 sec, 30 sec at the primer specific annealing temperature (Table2) and 30 sec at 72 ºC. The last step, 30 sec at 72 ºC was omitted when the annealing temperature was higher than 58 ºC. A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were performed in duplicate. The reference standard dilution series was repeated on every plate. Duplicate negative controls, both a minus RT and a water control, were run with every experimental plate to assess the specificity and to identify any potential contamination.

Data analysis was performed with IQ5 Real-Time PCR detection system software (BioRad). Expression levels were normalized using the average relative amount of the reference genes. Log-values of normalized relative expression were used to obtain normal distribution.

A Wilcoxon rank sum test was performed to determine the significance of differential gene expression. All results were Bonferroni corrected.

Results and discussion

Differentiation between STHS and VHS was based on physical examination and radiographic (thorax) / ultrasound examination (thorax/abdomen). All dogs with visceral organ involvement were euthanized followed by immediate autopsy. Histomorphology and immunohistochemical staining for CD18 confirmed the suspected histiocytic origin of tumours studied.

The Microarray enabled analysis of the expression of 42,034 features. Since only 21,682 (51%) were annotated (CanFam 2.0), it is possible that important genes are missed. When comparing VHS and spleen, 4,235 features were significantly differentially expressed. When only looking at 4-foldchanges or larger, 352 features remained. When comparing STHS with spleen, 5,779 features were significantly differentially expressed. In this comparison, when only looking at 4-foldchanges or larger, 437 features remain.

Of the total of altered genes, 3,394 features were significantly differentially expressed in both forms of HS versus normal spleen, and 319 features remained when only 4-foldchanges or larger are taking into account.

Figure 2 visualizes the heatmap of the ten genes that were chosen for qPCR confirmation.

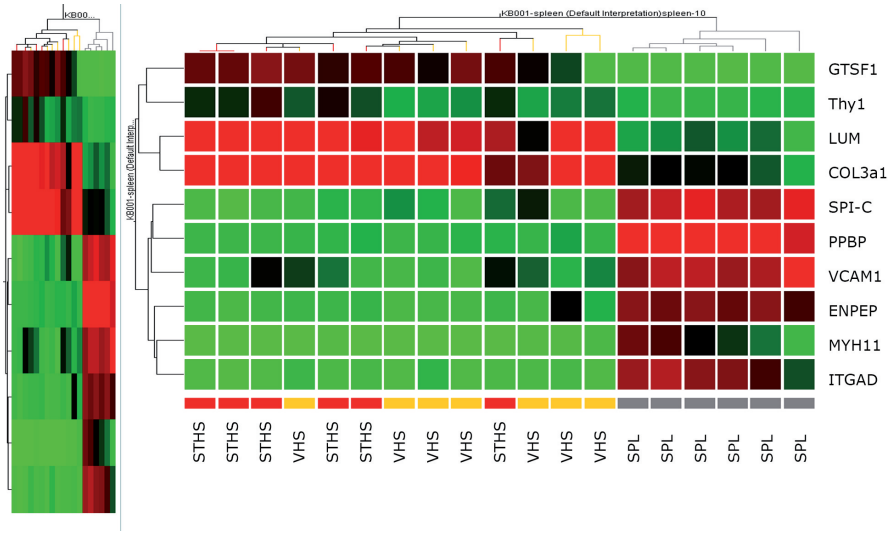

FIgure 2: Microarray-based heatmap of the ten genes chosen for qPCR. PPBP: Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7), GTSF1: Gametocyte specific factor 1, SPIC: Spi-C transcription factor, VCAM1: Vascular cell adhesion molecule 1, ENPEP: Glutamyl aminopeptidase (aminopeptidase A), LUM: Lumican, ITGAD: Integrin, alpha D, Col3A1: Collagen, type III, alpha 1, MYH11: Myosin, heavy chain 11, smooth muscle, Thy-1 (CD90): Thy-1 cell surface antigen

In order to improve knowledge on the genetic basis of HS and to best exploit logistic and financial resources, we chose to examine a selection of the significantly altered genes found in the microarray experiment for confirmation by qPCR. Selection of genes was based on the statistical significance of their differential expression and their potential involvement in tumour development.
Since it is not possible to obtain pure samples of sedentary, non-tumourous histiocytes for expression profiling, spleen tissue was chosen as the normal equivalent of HS. HS arises from interstitial DC, and hence emanate from lymphoid domains when these arise from lymphoid organs such as the spleen [6], choosing spleen as a control tissue is a logical choice.

As a result from the qPCR experiment, significantly altered expression was confirmed for nine of the ten genes analyzed. *PPBP, SpiC, VCAM1, ENPEP* and *ITGAD* were downregulated and *GTSF1, LUM , Thy1* and *Col3a1* were upregulated in both STHS and VHS compared to normal spleen. Table 4 shows the adjusted *p*-values and Fold Change.

The use of spleen as the healthy equivalent of HS does raise some concern as to how observed differences in *Spi-C* and *VCAM1* gene expression between tumours and healthy spleen must be explained. It is possible that some of these expression differences are based on differences in tissue-origin rather than on actual tumour development. *Spi-C* plays a critical role in the development of splenic iron homeostasis. It is highly expressed in red pulp macrophages, but not monocytes, dendritic cells or other tissue macrophages. *Spi-C* is therefore highly expressed in spleen [35], and could thus lead to cause a seemingly relative down-regulation in HS tissues. *Spi-C* is also known to regulate *VCAM1* expression [35]. This gene is thought to be involved in angiogenesis and is induced by cytokines on endothelial cells [36]. Since spleen tissue contains abundant endothelium, this could cause the relative high expression of *VCAM1* in the spleen. *ITGAD* (also known as *CD11d*) is a receptor for *VCAM1*. In our study, we detected a lowered expression of *CD11d* in HS as compared to normal spleen.

Staining by immunohistochemistry for the presence of CD11d in both STHS and VHS was found negative in a first study by Moore et al, including16 splenic HS [6] and this absence of the CD11d protein in HS was seen as one of the phenotypically characteristics of a myeloid dendritic antigen-presenting cell lineage, making many HS to be likely myeloid dendritic cell sarcomas [6]. In contrast, a more recent study in Flatcoated Retrievers found the majority (12/20) of splenic HS positive for CD11d [8] and was interpreted by the investigators as marker of a likely red-pulp macrophagocytic origin of these splenic HS. A similar rate of *CD11d* positivity was noticed by another study by Moore et al examining hemophagocytic HS in spleen in a series of dogs from 6 breeds[7]. CD11d proteins appear to be strongly expressed only on mature granulocytes, monocytes, and certain lymphocytes, but not significantly on myeloid committed precursor cells [37] and hence, the low

Table 4: Genes identified as potential interesting using gene profiling in both VHS and STHS compared to spleen

Gene name	qPCR:Adj P-values VHS vs Spleen	qPCR:Adj p-values STHS vs Spleen	qPCR:Up/Down regulated versus spleen	Spleen versus VHS resp STHS; Fold Change
PPBP	7.6×10^{-8}	7.23×10^{-9}	DOWN	-360x, -1008x
GTSF1	2.9×10^{-4}	5.25×10^{-5}	UP	1000x, 1000x
Spi-C	2.0×10^{-3}	2.4×10^{-4}	DOWN	-12x, -51x
VCAM1	0.011478	$1.1\ 10^{-4}$	DOWN	-10x, -6x
ENPEP	8.4×10^{-4}	2.0×10^{-8}	DOWN	-324x, -1472x
LUM	3.4×10^{-3}	5.43×10^{-7}	UP	88x, 48x
ITGAD (CD11d)	8.5×10^{-3}	1.6×10^{-3}	DOWN	-4x, -253x
Col3a1	7.0×10^{-3}	0.010	UP	23x, 18x
Thy1 (CD90)	0.024	4.19E-05	UP	3x, 6x

PPBP: Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7), GTSF1: Gametocyte specific factor 1, SPI-C: Spi-C transcription factor, VCAM1: Vascular cell adhesion molecule 1, ENPEP: Glutamyl aminopeptidase (aminopeptidase A), LUM: Lumican, ITGAD: Integrin, alpha D, Col3A1: Collagen, type III, alpha 1, Thy-1 (CD90): Thy-1 cell surface antigen

Table 5: Ten of the most significant pathways that resulted from the pathway analyses in DAVID

Category	Term	No of genes	P-value
KEGG_PATHWAY	cfa04142:Lysosome	25	1.62E-05
KEGG_PATHWAY	cfa04110:Cell cycle	26	3.44E-05
CHROMOSOME	3	59	4.09E-05
CHROMOSOME	5	102	4.92E-05
CYTOBAND	3	59	5.78E-05
CYTOBAND	5	102	7.71E-05
KEGG_PATHWAY	cfa03030:DNA replication	11	4.61E-04
KEGG_PATHWAY	cfa03050:Proteasome	12	0.0016
KEGG_PATHWAY	cfa04115:p53 signaling pathway	13	0.0076
KEGG_PATHWAY	cfa03430: DNA mismatch repair	7	0.0076

Our observations provide evidence of an association between altered expression of nine genes (PPBP, SpiC, VCAM1, ENPEP, ITGAD, GTSF1, COL3A1, CD90 and LUM) and development of HS in the Flatcoated retriever dog, irrespective of the disseminated or localized form.

expression in many HS is no surprise as is its positivity in hemophagocytic HS. Still, the finding of positive expression in splenic HS by immunohistochemistry [8] is at variance with earlier studies [6] and was interpreted as marker of a likely red-pulp macrophagocytic origin of these splenic HS. In our study, we detected a lowered expression of CD11d in HS as compared to normal spleen.

The down-regulation of *PPBP* as was found in this study, has also been reported to play a role in the development of myelomas [38] as well as pancreatic cancers [39], in the latter report the *PPBP* (CXCL7) plasma-level was even postulated to be an interesting biomarker for early detection [39]. The close relation in the origin of myeloma and histiocytic sarcoma, both stemming from lineages of hematoproliferative compartments makes this downregulation of *PPBP* an intriguing observation worthy of further pursuit.

Expression and overexpression of *LUM* has been observed in various types of cancer cells (colorectal, pancreatic, and breast cancer, melanoma, neuroendocrine cell tumours) - with contrasting findings on the relation with type of growth and/or tumour progression or metastasis [40-46] - and in activated synoviocytes from rheumatoid arthritis [47]. The function of lumican - a member of the family of small leucine rich proteoglycans - in the organization of the extracellular matrix composition as well as in migration and proliferation- in relation to the observed overexpression in HS warrants further investigations, including the site of overexpression (tumour cell or tumour-associated fibroblasts) [48].

In humans, an up regulation of *GTSF1* is already known in the occurrence of mycosis fungoides, which is the most common type of primary cutaneous T-cell lymphoma, in which *GTSF1* was proposed as a gene for which expression appears to be restricted to mycosis fungoides tumour stage and that might even serve as diagnostic (bio)marker [49].

ENPEP probably plays a role in regulating growth and differentiation of early B-lineage cells (http://www.wikigenes.org/e/gene/e/13809.html) and down regulation may thus also be involved in HS development.

Finally, *Col3a1* also has been shown to be overexpressed in other types of tumours, such as malignant mesothelioma [50], as well as in human sarcoma xenotransplants [51]. Its overexpression could have a significant influence upon extracellular matrix composition [52].
Thy1 (*CD90*) is an important marker of many types of stem cells [53], including mesenchymal stem cells [54]. *CD90* has already been identified as a candidate marker for cancer stem cells in primary high-grade gliomas using tissue microarrays [55, 56]. For human hepatocellular carcinoma, *CD90* has even been shown to provide a clinical prognostic marker [53]. Our observation of overexpression of *CD90* in HS might herald stem cell characteristics of the type of cancer.

For technical reasons, no qPCR data could be obtained for *Myh11*.

As a result of the pathway analyses in DAVID, 24 pathways were significantly involved in the development of HS ($P<0.05$). Most were involved in the DNA repair and replication process. The biological functions of ten of these pathways, amongst which the P53 signaling pathway was one of the most relevant, are listed in Table 5.

Based upon a fundamental and evolutionarily conserved association between cytogenetic abnormalities and tumour phenotype in different species [10, 57] these genes may be of major interest in the study of histiocytic malignancies in the human as well. There exists a great difference in incidence of histiocytic malignancies between this specific dog breed as compared to the human. This implies a genetic make-up predisposing the Flatcoated retriever (and the Bernese Mountain Dog); which is uncommon in many other dog breeds and humans alike. For the Bernese Mountain Dog, a first genetic locus has been identified. Our group takes part in a study aiming to identify predisposing genes in the Flat Coated Retriever in the hope, that these findings may provide clues for the related cancer in the human.

The current study provides the most comprehensive database of genome alterations in histiocytic malignancies to date, revealing genes and signaling pathways not previously associated with this disease. Although mRNA levels do not necessary reflect differences in protein levels, it is very well conceivable that the large difference in mRNA levels of specific genes will result in quantitative differences in protein expression. Lack of verified and specific antibodies for all nine gene products of interest let us to restrain this expression profiles to mRNA levels only. The study of Hedan [10] was able to locate recurrent genomic imbalances using CGH. As indicated in Table 3 of their publication [10], 808 genes found to be located in their regions of interest. This covers about 4% of the total number of genes. In the Agilent Canine Gene Expression Microarrays V1 that were used in our study, 430 of these 808 genes were annotated. Three of these annotated genes; ENSCAFG00000007012 (SPIC), ENSCAFG00000001046 (DESI1) and ENSCAFG00000006138 (LUM) were found to be commonly involved in our study as well as in Hedans study. In our study, eventually two of these genes, *SPIC* and *LUM*, were chosen for qPCR confirmation. We were indeed able to identify the significantly altered expression of these two genes.

Conclusion

This is the first study to compare gene expression in HS (both STHS and VHS) and normal spleen using both traditional fold change analysis as well as disease-based pathway analyses using DAVID.

This study provides evidence for involvement of several genes in HS, irrespective of the form of manifestation, some of which are also related with to other cancers. On the basis of quantitative differences in expression, we consider *PPBP, SpiC, VCAM1, ENPEP, ITGAD* (down-regulated), and *GTSF1, Col3a1, CD90* and *LUM* (up-regulated) to be associated with the HS genotype.

Extrapolation of this data to human samples may help to further our understanding of the propagation and oncogenesis of histiocytic cells. Eventually, this will contribute to the development of effective therapeutic modalities for both species.

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7

The two main forms of histiocytic sarcoma in the predisposed Flatcoated Retriever dog display variation in gene expression

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PLoS One, 2014 Jun 2; 9(6):e98258

Abstract

Examination of gene functions in specific tumour types improves insight in tumourigenesis and helps design better treatments. Due to the rarity of histiocytic/dendritic cell sarcoma in humans, it is difficult to accrue such knowledge. Therefore, comparative research of these cancers in predisposed dog breeds, such as the Flatcoated retriever, can be of value. Histiocytic sarcoma in the dog can be grouped into a soft tissue- and visceral form. The soft tissue form at first is localized, while the visceral form progresses more quickly to a terminal state, which might be related to variations in gene expression. Microarray analyses were performed on fresh-frozen tissue from Flatcoated retrievers with either soft tissue- or visceral histiocytic sarcoma. Expression differences of ten most significantly differentially expressed genes were validated with quantitative real-time PCR (q PCR) analyses.

Q PCR analyses confirmed the significantly aberrant expression of three of the selected genes: C6 was up-regulated; *CLEC12A* and *CCL5* were down-regulated in the visceral histiocytic sarcoma compared to the soft tissue form.

The findings of our study indicate that these two forms of histiocytic sarcoma in the dog display a variation in gene expression and warrant analysis of functional changes in the expression of those genes in these rare sarcomas in man.

Introduction

Histiocytic malignancies include dendritic cell- and histiocytic sarcomas and disseminated Langerhans cell histiocytosis [1]. In humans, the frequency of this group of diseases is very low [2]. This hampers an evidence based therapeutic approach for instance based on selective inhibition of specific signal transduction pathways. Canine histiocytic sarcoma (HS) resemble the human histiocytic malignancies [3-6]. As for such human cancers, canine HS involves the proliferation of members of both histiocytic lineages; dendritic cells and macrophages [5, 6]. The dog is genetically closer related to man than mice [7, 8] and the study of genetic changes of spontaneous cancers in the dog therefore has high comparative value [3, 9], in particular for cancers that are rare in the human such as histiocytic malignancies [10, 11]. Research could lead to more insight in the pathogenesis of this disease and could facilitate the identification of therapeutic targets valuable for both species [4, 7, 12-15].

In the dog , the limited genetic flow within breeds is responsible for specific breed traits including disease predispositions. However, the disease predispositions resulting from selective inbreeding can be studied for the benefit of affected breeds as well as for humans in which the rarity of diseases such as histiocytic malignancies hampers scientific progress. The Flatcoated retriever (FCR) has a strongly increased risk for HS development [16, 17]. In dogs [3, 4, 16] and humans [10, 11], histiocytic malignancies are grave conditions though the prognosis varies between subtypes [1]. HS has two common subtypes. Localized, soft tissue histiocytic sarcoma (STHS), manifests itself as a tumour arising in the deeply seated soft tissues of limbs, often in association with joints. In this form, chemotherapy as an adjunct to tumour resection can improve survival in some dogs that suffer from STHS [18]. The prognosis for the second form; visceral histiocytic sarcoma (VHS), which is a multifocal and disseminated form that is manifested in internal organs, uniformly is very poor [3, 16, 17, 19, 20]. There is presently no immunohistochemical method available to definitively differentiate between STHS and VHS and some have stated that VHS and STHS represent two different stages along a continuum of the same disease [5, 21].

Our previous research of HS in Flatcoated retrievers has provided evidence that the expression of nine common genes is altered when comparing both STHS and VHS with normal spleen; indicating a common ground for the general development of HS [15]. As a next step we compared the two forms of HS with one another. In this additional study we provide evidence that, in addition to common changes in STHS and VHS compared to healthy tissues described previously, there are some marked genetic differences between these two common forms of histiocytic malignancies.

Materials and methods

The experimental protocol (ID 2007.III.08.110) was peer-reviewed by the scientific committee of the Department of Animals in Science & Society, Utrecht University, The Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht, The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (the Dutch 'animal experiments decree', 1996). Both documents are available online at http://wetten.overheid.nl.

Case recruitment and histopathological evaluation

As mentioned in the earlier study [15], all tumour samples were confirmed as being spontaneously occurring histiocytic malignancies obtained from (previously untreated) Dutch family-owned FCR. All samples were obtained with informed owner consent. Case selection and obtainment of tissues were similar as mentioned in the previous study. As a common reference pool a multitude of canine organs (testis, liver, spleen, prostate, duodenum, lung, kidney and brain) from healthy crossbreeds (n= 8) was used [15].
Patient details are listed in Table 1.

Table 1: Patient details

Name	Sex	Pathology	AO (yrs)	Site(s)	Microarray / PCR
D1, TJ	MN	STHS	7.7	shoulder	Y / Y
D3, UH	FN	STHS	8	shoulder	Y / Y
D4, BS	MN	STHS	6.5	elbow	Y / Y
D5, BaS	M	STHS	7.6	knee	Y / Y
D6, TV	MN	STHS	8.1	elbow	Y / Y
D7, DV	MN	STHS	11	shoulder	Y / Y
DX, YM	MN	STHS	9.7	shoulder	N / Y
D2, BE	M	VHS	9.4	liver/spleen/**lnn abd**	Y / Y
D8, DW	M	VHS	9.5	spleen/**lnn abd**	Y / N
D9, JV	FN	VHS	8.9	lung/**lnn mediast**	Y / Y
D11, BT	F	VHS	8.5	**lung**	Y / Y
D12, AG	M	VHS	7.3	**lung**/spleen/kidney	Y / Y
D13, TR	FN	VHS	7.9	lung	Y / Y
D14, SG	F	VHS	4.1	lung/**lnn mediast**	Y / Y
DX, SC	MG	VHS	10	liver/**spleen**	N / Y

AO: Age of onset, M: male, MN: male neutered, F: female, FN: female neutered, STHS: soft tissue (localized) histiocytic sarcoma, VHS: visceral (disseminated) histiocytic sarcoma, lnn abd: abdominal lymphnodes, lnn mediast: mediastinal lymph nodes. Note: For cases with VHS the site sampled for gene expression is indicated in bold letters N=no Y=yes

RNA isolation

RNA isolation was performed as described previously [15]. In short, approximately 30 mg of frozen tumour was disrupted/lysed and homogenized and total RNA was isolated and treated with DNase using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Quantity and integrity were assessed with the Bioanalyzer Agilent BioAnalyzer-2100 (Bioanalyzer, Agilent Technologies, Santa Clara, CA) in combination with an RNA 6000 Pico-LabChip. The average RNA integrity number 8.5 (range: 7.2-9.8) was found to be appropriate [22]. RNA concentration was quantified using a NanoDrop ND-1000 (Isogen Life Science) spectrophotometer.

Expression profiling

Expression profiling was performed as described previously [15]. In short, RNA was labeled twice and hybridized against the common reference RNA on dual channel arrays, with RNA amplifications and labeling being performed on an automated system (Caliper Life Sciences NV/SA, Belgium) as described previously [23]. Dye swap of Cy3 and Cy5 was performed to reduce dye bias. Hybridizations were done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A.) using 500-1000 ng labeled cRNA per channel as described [24]. After automated data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed [25] on mean spot-intensities. Dye-bias was corrected based on a within-set estimate as described [26]. Data were analyzed using ANOVA [27] (R version 2.2.1/MAANOVA version 0.98-7) (http://www.r-project.org/). Briefly, both tumourgroups were compared through the common reference channel. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Thus analyzed 191 gene probes with $P < 0.05$ after family wise error correction (FWER) were considered significantly changed.

Functional annotation

To examine whether certain pathways are over- or under-represented in the gene list, all genes differentially expressed between STHS and VHS, were introduced into DAVID (http://david.abcc.ncifcrf.gov/).

Quantitative real time PCR; gene selection

Following the outcome of the microarray expression profiling, 11 genes were selected; namely C-type lectin domain family 12, member A (*CLEC12A*); C-C motif chemokine 5 Precursor (Small-inducible cytokine A5) (*CCL5_CANFA*); Asporin (*ASPN*); CD9 molecule (*CD9*); Transketolase-like 1, transcript variant 1 (*TKTL1*); Complement component 6, transcript variant 1(*C6*); S100 calcium binding protein A12 (*S100A12*); Immunoglobulin J polypeptide (*IGJ*); S100 calcium binding protein A8 (*S100A8*); Phytanoyl-CoA dioxygenase, peroxisomal like (*PHYH*). Selection of these genes was based on the ones most significantly differently expressed on the micro-array chip and their fold changes.

Details of the qPCR reactions and primer sequences are depicted in Table 2. Delta Ct method, using efficiencies between 95.0 and 104.8 %, was used for both the reference- as well as the target genes.

RNA isolation and cDNA synthesis

Besides tissues from all but one patient (of which the insufficient tissue remained for the validating qPCR experiment) used in the microarray experiment (six soft tissue HS and seven visceral HS), two additional samples (one soft tissue HS and one visceral HS that met the inclusion criteria) were added. Total RNA from these samples was isolated. After isolation, total RNA was treated with DNase using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol.

Reverse transcription (RT) was performed of all 20 samples in a 80 µl reaction using 2000 ng total RNA, 16 µl iScript Reaction mix and 4 µl iScript Reverse Transcriptase

Table 2: QPCR primers for genes of interest (based on Microarray analyses) and reference genes (efficiencies varied between 95.0 and 104.8 %).

Gene name	ENSID	Primer sequence	Forward/ reverse	Amplicon size (bp)	Annealing T (°C)
CLEC12A	ENSCAFG00000025113	AAATGCCAGCCTGTTGAC	F	110	61
		TGGTAATCTCTGTCATACTTGGG	R		
CCL5_CANFA	ENSCAFG00000018171	TATGCCTCAGACACCACAC	F	119	63
		GACAAAGACGACTGCTGG	R		
ASPN	ENSCAFG00000002307	CCACGAGTCAGAGAAATACAC	F	135	59
		GGCAGAAGTCATTCACTCC	R		
CD9	ENSCAFG00000015172	TGTGCTGTCATCCATCAC	F	118	57
		TGCCAAATATCATCACTACGG	R		
TKTL1	ENSCAFG00000019451	ATGAGATACAAACAGGAGGAC	F	136	61
		CCAGTATATGCCATCCCAC	R		
C6	ENSCAFG00000018598	CCGTTGTGATTGACTTTGAG	F	88	61
		CTTTCTGAGGTTGTTCCGT	R		
S100A12	ENSCAFG00000023324	AAAGGGTGAGATGAAGCAG	F	156	61
		CACAACAGAAACCAGGGA	R		
IGJ	ENSCAFG00000002911	CCTTCTCCCGATGATCCT	F	120	63
		GGTACACAAATTTCGTTCTCAC	R		
S100A8	ENSCAFG00000017557	GTTTACCACAAGTACTCCCTG	F	148	63
		CCATCGCTATTGACATCCA	R		
PHYH	ENSCAFG00000023349	CTGAAGCCACACGATTATCC	F	112	58
		TCTCCTTTCTCCATCACGA	R		
HPRT	ENSCAFG00000018870	AGCTTGCTGGTGAAAAGGAC	F	104	56
		TTATAGTCAAGGGCATATCC	R		
RPS19	ENSCAFG00000028485	CCTTCCTCAAAAAGTCTGGG	F	95	61
		GTTCTCATCGTAGGGAGCAAG	R		
RPL8	ENSCAFG00000001677	CCATGAATCCTGTGGAGC	F	64	55
		GTAGAGGGTTTGCCGATG	R		

Table 2: Continued

SRPR	ENSCAFG00000010474	GCTTCAGGATCTGGACTGC	F	81	61
		GTTCCCTTGGTAGCACTGG	R		
RPL13	ENSCAFG00000019840	GCCGGAAGGTTGTAGTCGT	F	87	61
		GGAGGAAGGCCAGGTAATTC	R		
GUSB	ENSCAFG00000010193	AGACGCTTCCAAGTACCCC	F	103	62
		AGGTGTGGTGTAGAGGAGCAC	R		
GAPDH	ENSCAFG00000024323	TGTCCCCACCCCCAATGTATC	F	100	58
		CTCCGATGCCTGCTTCACTACCTT	R		
B2MG	ENSCAFG00000013633	TCCTCATCCTCCTCGCT	F	85	61
		TTCTCTGCTGGGTGTCG	R		
RPS5	ENSCAFG00000002366	TCACTGGTGAGAACCCCCT	F	141	62.5
		CCTGATTCACACGGCGTAG	R		

Genes identified using microarray as being significantly different comparing Soft Tissue Histiocytic Sarcoma (STHS) and Visceral Histiocytic Sarcoma (VHS): C-type lectin domain family 12, member A (CLEC12A); C-C motif chemokine 5 Precursor (Small-inducible cytokine A5) (CCL5_CANFA); Asporin (ASPN); CD9 molecule (CD9);Transketolase-like 1, transcript variant 1 (TKTL1); Complement component 6, transcript variant 1(C6); S100 calcium binding protein A12 (S100A12); Immunoglobulin J polypeptide (IGJ); S100 calcium binding protein A8 (S100A8); Phytanoyl-CoA dioxygenase, peroxisomal like (PHYH).

Reference genes primers for q PCR: Hypoxanthine phosphoribosyltransferase (HPRT), Ribosomal protein S19 (RPS19) ribosomalprotein L8 (RPL8), Signal recognition particle receptor (SRPR), Ribosomal protein L13, (RPL13), glucuronidase, beta (GUSB), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Beta-2-Microglobulin (B2MG), 40S ribosomal protein S5 (RPS5), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin (B2MG), Ribosomal protein S5 (RPS5).

(iScript cDNA Synthesis kit, Bio Rad, Veenendaal, The Netherlands). The mixture, contain both random hexamers and oligo-dT primers,was incubated 5 min. at 25°C, 30 min. at 42°C and followed by 5 min. at 85°C. Minus RT controls were prepared from 500 ng of the same RNA under the same conditions, but without addition of reverse transcriptase.

Reference genes and primer development

Nine reference genes were used as the non-regulated reference genes for normalization, based on their stable expression in canine tissue [28, 29] namely *ribosomal protein S19 (RPS19) hypoxanthine phosphoribosyltransferase (HPRT), ribosomal protein L8 (RPL8), signal recognition particle receptor (SRPR), and ribosomal protein L1 (RPL13), glucuronidase, beta (GUSB), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Beta-2-Microglobulin (B2MG), 40S ribosomal protein S5 (RPS5)*. Primers for reference genes, including their optimum annealing temperature are listed in Table 2.

Using Ensembl (Ensembl 70; CanFam3.1), through annotated transcripts, PCR primers were designed using the Perl Primer software (version 2.0.0.7) according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product and also in qPCR by checking the meltcurve and reaction efficiency. GeNorm [30] was used to establish expression stability. Amplicon sequence-reactions were performed using BigDye v3.1 according to the manufacturer's (Life Technologies, Bleiswijk, The Netherlands) instructions on an ABI3130XL and analyzed in Lasergene (version 9.1 DNASTAR) and confirmed the specificity of each amplicon.

Quantitative PCR

Published guidelines for the qPCR experiment were followed according to the MIQE guidelines [31-33]. For qPCR, the CFX detection system (Bio-Rad.) with SYBR green fluorophore was used. Reactions were performed in a total volume of 10 µl containing 5 µl of 2x SYBR green super mixes (Bio-Rad Laboratories Ltd.), 0.5 µl of each primer at 400 nM concentration, 0.8 µl of cDNA and 3.2 µl RNase and DNase free water as previously described [15, 34, 35]. Expression analysis was performed on sample duplicates in duplicate. A minus RT sample and a no template control were performed as control. Expression levels were based on Ct values normalized using the mean of seven of the nine reference genes. GAPDH and B2MG did not behave fully stable according to GeNorm [28].

A Wilcoxon rank sum test was performed to determine the significance of differential gene expression. All results were Bonferroni corrected.

Results and Discussion

The Microarray enabled analysis of the expression of 42,034 features; however, since only 21,682 (51%) were annotated (CanFam 2.0), it is possible that important

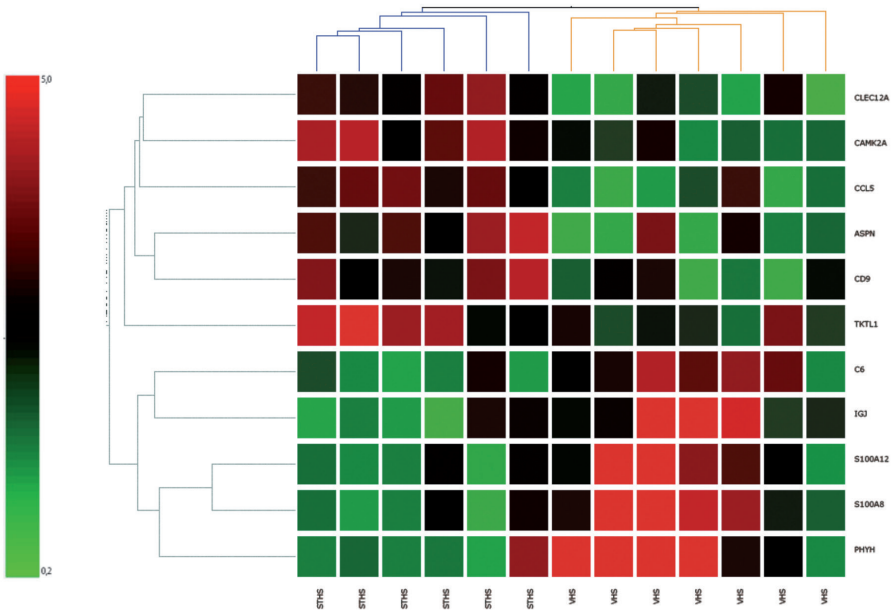

Figure 1: Microarray- based heatmap of 11 genes. C-type lectin domain family 12, member A (CLEC12A); C-C motif chemokine 5 Precursor (Small-inducible cytokine A5) (CCL5_CANFA); Asporin (ASPN); CD9 molecule (CD9);Transketolase-like 1, transcript variant 1 (TKTL1); Complement component 6, transcript variant 1(C6); S100 calcium binding protein A12 (S100A12); Immunoglobulin J polypeptide (IGJ); S100 calcium binding protein A8 (S100A8); Phytanoyl-CoA dioxygenase, peroxisomal like (PHYH).

genes are missed. Gene expression profiles of the two HS forms were compared with each other to identify genes that are specific for each manifestation.

When comparing VHS and STHS, 191 probes were significantly differentially expressed, listed in Additional Table S1. From the selection of most significant ($P < 0.003$), unique probes with a threshold of log2 fold change of 1.5 (n=19), eight were excluded for either comprising chromosomal regions (n=3) or as clones that did not align (n=5). The eleven genes that remained, are visualized in a heatmap (Figure 1). Their potential involvement in tumour development or behavior was considered by a literature research. All microarray gene expression data were deposited in the public data repository GEO (accession number GSE45832).

QPCR confirmed and quantified the differential expression of the 11 genes selected from the micro-array data. As a result, *C6, S100A12, S100A8, PHYH* and *IGJ* were up-regulated and *TKTL1, CLEC12A, CD9, CCL5* and *ASPN* were down-regulated in VHS compared to *STHS*. Only for three gene products was the difference in expression significant: *C6* ($P = 0.038$), *CLEC12A* ($P = 0.026$) and *CCL5* ($P = 0.0069$) (Figure 2).

For technical reasons, no qPCR data could be obtained for *CAMK2A*.

Our observations of *CLEC12A* and *CCL5* and those made in several human cancer types, make it conceivable that these gene products play a role in HS. In humans, C chemokine ligand 5 (*CCL5*) functions as one of the natural ligands for the CC Chemokine Receptor 5 (*CCR5*). It mediates chemotactic activity in immune cells including monocytes and dendritic cells [36] *CCL5* [37, 38] and *CCR5* [39] promote breast cancer invasiveness and metastatic potential, while *CCR5* inhibition abrogates this [39]. For inflammatory breast cancer, *CCL5* is considered to constitute a prominent part of a poor prognosis signature [40]. Also in human colorectal carcinoma *CCL5* appears to stimulate cancer progression [41]. Furthermore, the *CCL5 /CCR5* axis has been shown to promote cell motility in human osteosarcoma [42]. Thus, for these human cancers *CCL5* appears to be associated with a metastatic phenotype. Yet, in our study, *CCL5* was expressed at lower level in VHS than in STHS. Considering the more rapid metastatic nature of VHS, this is surprising, and this finding needs confirmation (also at protein level) in future studies. Still, a decrease in *CCL5* expression has been described in other human malignancies such as colon carcinoma when compared to normal tissue [43]. An alternative explanation for a reduction in *CCL5* expression in visceral as compared to localized HS could be that a reduction in expression of *CCL5* could protect against immunosurveillance [44] and hence be related to more aggressive behavior of HS.

CLEC12A expression was found to be significantly lowered in VHS compared to STHS. *CLEC12A* (or *MICL*) is considered a negative regulator of granulocyte and

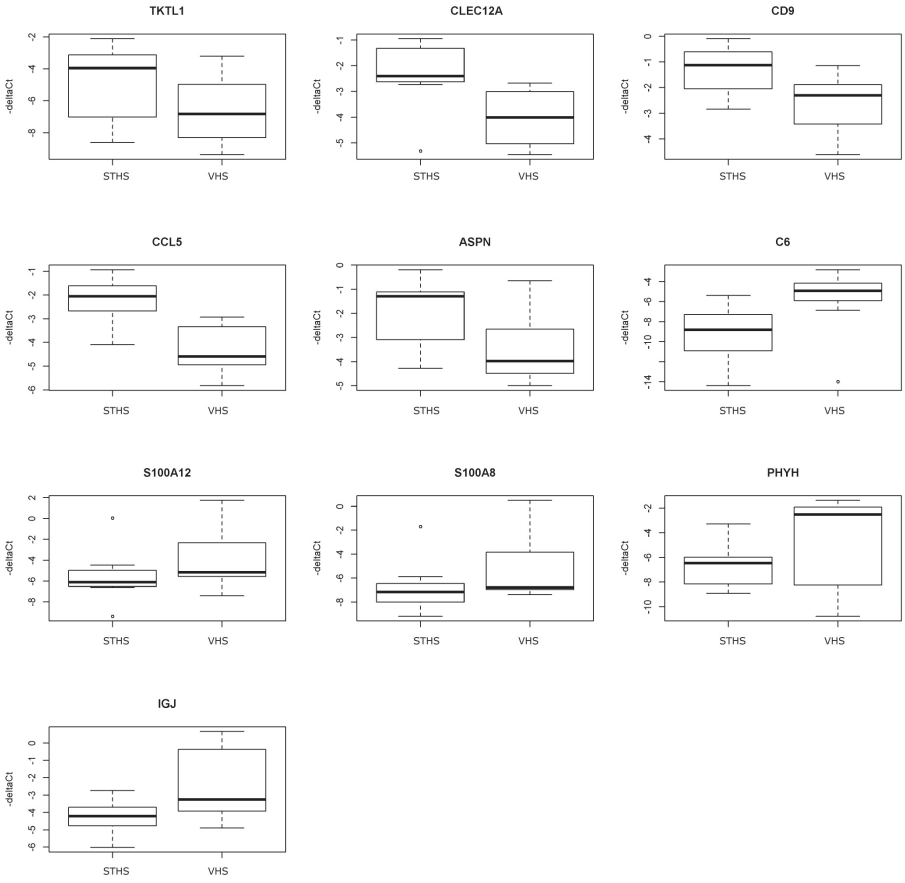

Figure 2: Quantitative PCR results. The upregulation or downregulation of selected genes in STHS (Soft Tissue Histiocytic Sarcoma) and VHS (Visceral Histiocytic Sarcoma). The thick black line represents the median (50th percentile) and also the first and third quartile (25th and 75th percentile respectively) are displayed.Three genes are significantly differentially expressed: C6 was up-regulated when comparing the more aggressive visceral histiocytic sarcoma to the soft tissue form, and CLEC12A and CCL5 were down-regulated when comparing the more aggressive visceral histiocytic sarcoma to the soft tissue histiocytic sarcoma.

Abbreviations: C-type lectin domain family 12, member A (CLEC12A); C-C motif chemokine 5 Precursor (Small-inducible cytokine A5) (CCL5_CANFA); Asporin (ASPN); CD9 molecule (CD9);Transketolase-like 1, transcript variant 1 (TKTL1); Complement component 6, transcript variant 1(C6); S100 calcium binding protein A12 (S100A12); Immunoglobulin J polypeptide (IGJ); S100 calcium binding protein A8 (S100A8); Phytanoyl-CoA dioxygenase, peroxisomal like (PHYH).

monocyte function [45]. Activation of myeloid cells and recruitment to sites of inflammation – but not increase or decrease in the level of differentiation – was accompanied by reduced expression [46]. Whereas normal lymphocytes have no or low *MICL* expression, a relatively high expression of this gene in acute lymphoblastic leukemia was found to be associated with prolonged relapse-free survival [47]. How the reduced expression in VHS as compared to STHS relates to these findings remains to be determined, but a resulting increased migratory capacity as present in VHS could be an explanation.

Complement component 6 (C6) gene expression was significantly increased in VHS as compared to STHS. In acute leukemia's an increase in circulating complement is common. As one early study has demonstrated increased expression of complement in monocytes by conditioned media of leukemic cells [48]. At present, a straight forward hypothesis on the functional consequences of the variation in C6 expression in the different forms of HS is not easily postulated but it might be associated with a reaction of the innate immune system to the neoplasm and not a direct effect of the neoplastic cell population.

The variation in expression of *C6, CCL5* and *CLEC12A*, all three members of the immune response, is worthy of follow up investigations, with focus upon their character as deranged histiocytes and may include comparisons with the canine reactive histiocytic diseases [1].

When comparing the results of qPCR and microarray the difference in the expression of some genes between STHS and VHS did not attain statistical significance. The variation between the two methods relates to the fact that a microarray experiment is a semi-quantitative screening method and the qPCR quantitative. Many methodological factors can lead to a lack of correlation between array results and qPCR measurements [49]. The expression of *CD9* (synonym MRP-1; motility related protein 1) in VHS was suppressed as compared to STHS at a level trending towards significance ($P = 0.07$) and further investigation of this gene in these sarcomas seems warranted in view of associations with reduced expression of this gene with tumour aggressiveness such as reported for other cancers. A lower expression of *CD9* was found to be associated with the formation of bony metastases in studies using breast cancer cell lines [50, 51]. Similarly, in human colon cancer patients, patients that lacked *CD9* mRNA expression, had a worse prognosis than the cases that did express *CD9* mRNA [52].

DAVID pathway analyses of the significantly differentially expressed genes in the micro array did not lead to the detection of altered expression of whole pathways. Also the genes chosen for qPCR confirmation did not appear to be related at the level of regulation.

The alterations in gene function as detected in the current analyses, need follow

up by subsequent investigations by use of antibodies - most still need to be developed and validated for use in the dog to examine an altered expression at protein level.

When looking at tumour conditions in the human that share features with canine HS, none of the eleven genes for which altered function was observed in the present study, have been recognized as aberrantly expressed in micro-array/qPCR studies in Langerhans cell histiocytosis [53].

Conclusions

As a valuable addition to our previous study, in which we were able to provide evidence for involvement of several genes in the development of HS, irrespective of form, this study provides the most comprehensive database to date of genetic variations in the two most common forms of HS, namely VHS and STHS. Using fold-change analysis, it reveals genetic variations not previously associated with these two forms.

On the basis of quantitative differences in expression of C6 (up-regulated in VHS versus STHS), *CLEC12A* and *CCL5* (down-regulated in VHS versus STHS) were associated with each subtype. Down-regulation of *CLEC12A* in VHS, the more aggressive form of HS, is in line with previously published observations that such reduced function facilitates migratory capacity of myeloid cells in humans [46]. Down-regulation of *CCL5* is in line with several studies in human cancers [37, 47], however contradictory to others [43]. Further investigations should focus on changes of gene function at protein level and a comparison of these histiocytic malignancies in dog and human.

Supplementary Table 1: All 191 probes significantly differentially expressed.

ENSID	geneSymbol	M-Fold	P-value
ENSCAFG00000002314	SCIN *	-3.0945074	0
ENSCAFG00000032005		-2.8290905	0
ENSCAFG00000017138	CILP *	-2.7669955	0
ENSCAFG00000031355		-2.393319	0.016
ENSCAFG00000025113	***CLEC12A***	***-1.8322875***	***0***
ENSCAFG00000018171	***CCL5_CANFA***	***-1.6869771***	***0***
ENSCAFG00000018163	***CAMK2A***	***-1.6740242***	***0***
ENSCAFG00000002307	***ASPN***	***-1.6688222***	***0.0006***
ENSCAFG00000015172	***CD9***	***-1.5537057***	***0.0006***
ENSCAFG00000019451	***TKTL1***	***-1.5321822***	***0.001***
ENSCAFG00000017227	GABRG2 *	-1.5228159	0.0288
ENSCAFG00000028765		-1.4935809	0
ENSCAFG00000010274	CHI3L1	-1.4750658	0
ENSCAFG00000002440	ENSCAFG00000002440	-1.318441	0
ENSCAFG00000003663	GSN	-1.2530028	0.001
genomic:9+2536529-2536588		-1.239624	0.0002
ENSCAFG00000006953	PDGFRL	-1.231847	0.0044
ENSCAFG00000004617	APOE_CANFA	-1.2228113	0.0006
genomic:17+36515113-36515172		-1.202469	0
ENSCAFG00000002440	ENSCAFG00000002440	-1.2023628	0.0002
genomic:8+21360901-21360960		-1.2006772	0
ENSCAFG00000018163	CAMK2A	-1.1761496	0
genomic:19+40967827-40967886		-1.1686643	0
ENSCAFG00000015172	CD9	-1.1282699	0.0002
ENSCAFG00000008755	SRC	-1.118658	0
genomic:21-38554543-38554602		-1.0951082	0
ENSCAFG00000017404	SCPEP1	-1.0778874	0.02
ENSCAFG00000032175		-1.0768189	0.001
ENSCAFG00000002440	ENSCAFG00000002440	-1.0573947	0.0008
ENSCAFG00000014616	COMP	-1.0537542	0.0024
ENSCAFG00000013664	C15orf43	-1.0395595	0.0196
ENSCAFG00000031417		-1.0255353	0
ENSCAFG00000000274	HEBP2	-1.0223435	0.0004
ENSCAFG00000019451	TKTL1	-1.0206829	0.0026
ENSCAFG00000031640		-1.0164332	0.001
ENSCAFG00000002590	XM_848293.1	-1.0157592	0.0004
ENSCAFG00000015598	DHRS7	-0.98762298	0
ENSCAFG00000029265		-0.98631622	0
ENSCAFG00000003096	PM20D2	-0.97825308	0
ENSCAFG00000001290	ENSCAFG00000001290	-0.95298099	0.0054
ENSCAFG00000001487	LGALS1	-0.95214896	0
ENSCAFG00000010404	RNF19B	-0.94824091	0.0002
genomic:15-55825856-55825915		-0.9396565	0
genomic:7+27176889-27176948		-0.91433605	0.0016
ENSCAFG00000004407	ME3	-0.91241496	0.007
ENSCAFG00000017677	GGA2	-0.91195341	0.0168
ENSCAFG00000009568	CLIC6	-0.91105517	0
ENSCAFG00000018510	LAMP2	-0.90830964	0.0042
ENSCAFG00000017677	GGA2	-0.89362488	0.0192

Supplementary Table 1: Continued

genomic:16+16891636-16891695		-0.87292938	0
genomic:1+61045224-61045283		-0.870411	0
ENSCAFG00000008700	FNIP2	-0.86045192	0
ENSCAFG00000017556	TEX14	-0.8572938	0.001
ENSCAFG00000010790	XM_535675.2	-0.85307373	0.0324
ENSCAFG00000007112	SDCBP	-0.84282173	0.001
ENSCAFG00000006757	PPFIBP2	-0.83953928	0
ENSCAFG00000006411	O46601_CANFA	-0.83252132	0.001
ENSCAFG00000014678	Q6J3Q6_CANFA	-0.82056539	0.0014
ENSCAFG00000000303	AIG1	-0.81778826	0.0302
ENSCAFG00000000126	Q6JDL3_CANFA	-0.79072133	0
ENSCAFG00000020166	ANGPTL2	-0.79015989	0.0242
genomic:20-41738261-41738320		-0.7843711	0
genomic:14+27427086-27427127		-0.7836333	0.0002
ENSCAFG00000017019	TMEM219	-0.77747733	0
ENSCAFG00000028636		-0.77539165	0.0056
		-0.77428579	0.0058
ENSCAFG00000019764	DENND2D	-0.77328926	0.0228
ENSCAFG00000004174	SPAG6	-0.76886501	0.0236
ENSCAFG00000004606	BIN1	-0.75826718	0.0008
ENSCAFG00000019552	HMHA1	-0.74655767	0.001
ENSCAFG00000023898	MAP7D1	-0.73637421	0
ENSCAFG00000004318	CST7	-0.73109856	0.023
ENSCAFG00000001445	GRINA	-0.72694489	0.0016
ENSCAFG00000028791		-0.72520758	0.0002
genomic:21+39412439-39412498		-0.72404657	0.0012
ENSCAFG00000002115	RELX_CANFA	-0.72124919	0.0008
genomic:25+4418859-4418918		-0.70750093	0.026
ENSCAFG00000005236	TRAK1	-0.70293539	0.0068
genomic:14+52078289-52078348		-0.69932801	0.001
ENSCAFG00000031320		-0.69881976	0.0052
ENSCAFG00000029573		-0.6958488	0.0004
ENSCAFG00000008358	LOXL3	-0.69577168	0.004
genomic:20-37332869-37332928		-0.68757878	0
ENSCAFG00000019764	DENND2D	-0.6827793	0.001
genomic:13+23288136-23288195		-0.68183474	0.0156
ENSCAFG00000001624	CYHR1	-0.68117993	0
ENSCAFG00000008268	MND1	-0.67872761	0.0452
ENSCAFG00000004481	SC6A6_CANFA	-0.67597748	0
ENSCAFG00000003272	XM_847318.1	-0.67459747	0.0138
ENSCAFG00000031225		-0.67361452	0.0184
ENSCAFG00000014643	GNG2	-0.67203677	0
ENSCAFG00000000309	FUCA2	-0.66930852	0.001
ENSCAFG00000019694	GPSM1	-0.66332814	0.001
ENSCAFG00000000309	FUCA2	-0.65892967	0.0004
genomic:8+42357182-42357241		-0.65570779	0.022
ENSCAFG00000019529	NDUFS7	-0.65171998	0.001
ENSCAFG00000018517	C17orf79	-0.64638989	0.0006
ENSCAFG00000016583	MAD2L2	-0.64505818	0.0228
ENSCAFG00000011392	SSPN	-0.63215035	0.0336

Supplementary Table 1: Continued

ENSCAFG00000032711		-0.63184405	0
ENSCAFG00000030152		-0.62921078	0.0022
ENSCAFG00000002128	KIAA1432	-0.62756937	0
ENSCAFG00000012101	RB22A_CANFA	-0.62317398	0.0036
ENSCAFG00000006757	PPFIBP2	-0.62189239	0.0032
ENSCAFG00000000581	TFB1M	-0.61478987	0.0302
		-0.6140875	0.0008
ENSCAFG00000000215	R3HDM2	-0.59808779	0.0082
ENSCAFG00000004030	CMPK1	-0.59773842	0.001
ENSCAFG00000011083	LOC610413	-0.59746117	0.0018
ENSCAFG00000015532	MYO9B	-0.59053194	0.009
ENSCAFG00000005216	ULK4	-0.59034078	0.0402
genomic:X-18027472-18027517		-0.58322294	0
ENSCAFG00000019544	EDF1	-0.57857686	0.0244
ENSCAFG00000016509	UBQLN2	-0.57384476	0.0092
ENSCAFG00000032714		-0.57302038	0.0192
ENSCAFG00000006757	PPFIBP2	-0.5724539	0.0018
ENSCAFG00000019476	NDUFB10	-0.57187476	0.001
ENSCAFG00000018624	OMG	-0.56684611	0.0474
ENSCAFG00000006339	CACNA2D1	-0.56672644	0.0044
ENSCAFG00000001582	ENSCAFG00000001582	-0.56050664	0.0326
genomic:6-39440123-39440182		-0.56017477	0.001
ENSCAFG00000004652	BGAL_CANFA	-0.54880524	0.001
ENSCAFG00000029941		-0.54738559	0.001
ENSCAFG00000031169		-0.54033337	0.0222
genomic:5+12102468-12102527		-0.53227813	0.006
ENSCAFG00000019476	NDUFB10	-0.52966826	0.0036
		-0.5204114	0.0132
ENSCAFG00000006963	AAAS	-0.46857512	0.0286
ENSCAFG00000017666	DCTN5	-0.45944298	0.035
ENSCAFG00000012603	IFT46	-0.45781328	0.0362
ENSCAFG00000000054	ENSCAFG00000000054	0.43544725	0.035
ENSCAFG00000010826	SF3B1	0.43701462	0.04
ENSCAFG00000016993	USP3	0.46595357	0.0254
ENSCAFG00000016784	DFFA	0.46718556	0.027
ENSCAFG00000001548	SNAPC3	0.4826005	0.0238
ENSCAFG00000003471	PNISR	0.51075942	0.0338
ENSCAFG00000018257	MARK3	0.51729585	0.0416
ENSCAFG00000032308		0.52883133	0.0242
ENSCAFG00000013577	EIF3J	0.5376539	0.008
ENSCAFG00000011434	UBR1	0.56169272	0.0322
ENSCAFG00000005847	ADAM9	0.58015513	0.0196
ENSCAFG00000016244	LBR	0.60587357	0.001
ENSCAFG00000002746	KLHL7	0.61073785	0.001
ENSCAFG00000017848	ENSCAFG00000017848	0.61218262	0.0358
ENSCAFG00000016711	C5orf25	0.6231177	0.027
ENSCAFG00000011929	LOC478866	0.63431446	0.0242
ENSCAFG00000002860	SMEK2	0.63517859	0.001
ENSCAFG00000001331	ZCCHC6	0.63708157	0.0234
ENSCAFG00000002860	SMEK2	0.64296953	0.0154

Supplementary Table 1: Continued

genomic:4+43188112-43188175		0.65168318	0.0012
ENSCAFG00000017207	CCNG1	0.65444471	0.0036
genomic:26-20200191-20200245		0.65527855	0.0024
ENSCAFG00000001966	SH3RF3	0.68929916	0.0252
ENSCAFG00000024406	XM_846227.1	0.70312728	0.0008
ENSCAFG00000013158	XM_545525.2	0.77218972	0.0256
ENSCAFG00000010826	SF3B1	0.77354067	0.001
ENSCAFG00000029757		0.78390919	0.001
genomic:30+2405784-2405842		0.79089712	0.0192
ENSCAFG00000013817	SRGN	0.80563916	0.0104
ENSCAFG00000014776	WNK4	0.81432893	0.0004
ENSCAFG00000009533	SSRA_CANFA	0.8224528	0.022
ENSCAFG00000005886	CRIM1	0.82833911	0.0132
		0.84532545	0.0016
ENSCAFG00000029252		0.86129638	0.001
ENSCAFG00000003710	AIM1	0.87252936	0.0024
genomic:4+64463924-64463983		0.87433271	0.003
genomic:9-43727939-43727998		0.93653807	0
ENSCAFG00000013817	SRGN	0.94811488	0
genomic:X-48093611-48093670		0.96151221	0
ENSCAFG00000011930	CCNDBP1	1.008616	0.0002
ENSCAFG00000008999	PROS1	1.0211954	0.0016
ENSCAFG00000013310	ENSCAFG00000013310	1.0406891	0
ENSCAFG00000008341	ENSCAFG00000008341	1.0465609	0
ENSCAFG00000010595	RGS2	1.0808145	0.0068
ENSCAFG00000000460	LAPTM4B	1.091016	0.0486
ENSCAFG00000010595	RGS2	1.1236993	0.009
ENSCAFG00000006371	NRG1	1.1358697	0.001
ENSCAFG00000018258	ENSCAFG00000018258	1.2283262	0
ENSCAFG00000008205	ENSCAFG00000008205	1.2448566	0.0224
ENSCAFG00000010877	ABCA9	1.2480915	0.0164
ENSCAFG00000024916	ABCA8	1.4009914	0.0014
ENSCAFG00000029470		1.4653424	0.0154
ENSCAFG00000018598	C6	1.5415842	0
ENSCAFG00000008291	Q9TU80_CANFA *	1.7023561	0
ENSCAFG00000023324	***S100A12***	***1.7561693***	***0.0228***
ENSCAFG00000002911	***IGJ***	***2.0578904***	***0.0052***
ENSCAFG00000023441	*	2.0670019	0.001
ENSCAFG00000017557	***S100A8***	***2.2845349***	***0.0002***
ENSCAFG00000008291	Q9TU80_CANFA	2.3489151	0
ENSCAFG00000018598	***C6***	***2.3790026***	***0***
ENSCAFG00000023349	***PHYH***	***2.9195861***	***0.0004***

Of four of the 191 probes, the annotation could not be traced back. Of the remaining probes, 28 have a 'genomic' location for which no gene could be mapped at this moment. 159 Probes have a gene-name, of which 142 are unique.

*From the selection of most significant (P < 0.003), unique probes with a threshold of log2 fold change of 1.5 (n=19), eight were excluded; either for comprising chromosomal regions (n=3) or clones that did not align (n=5, marked with *). The eleven genes that remained, are written in bold and italic.*

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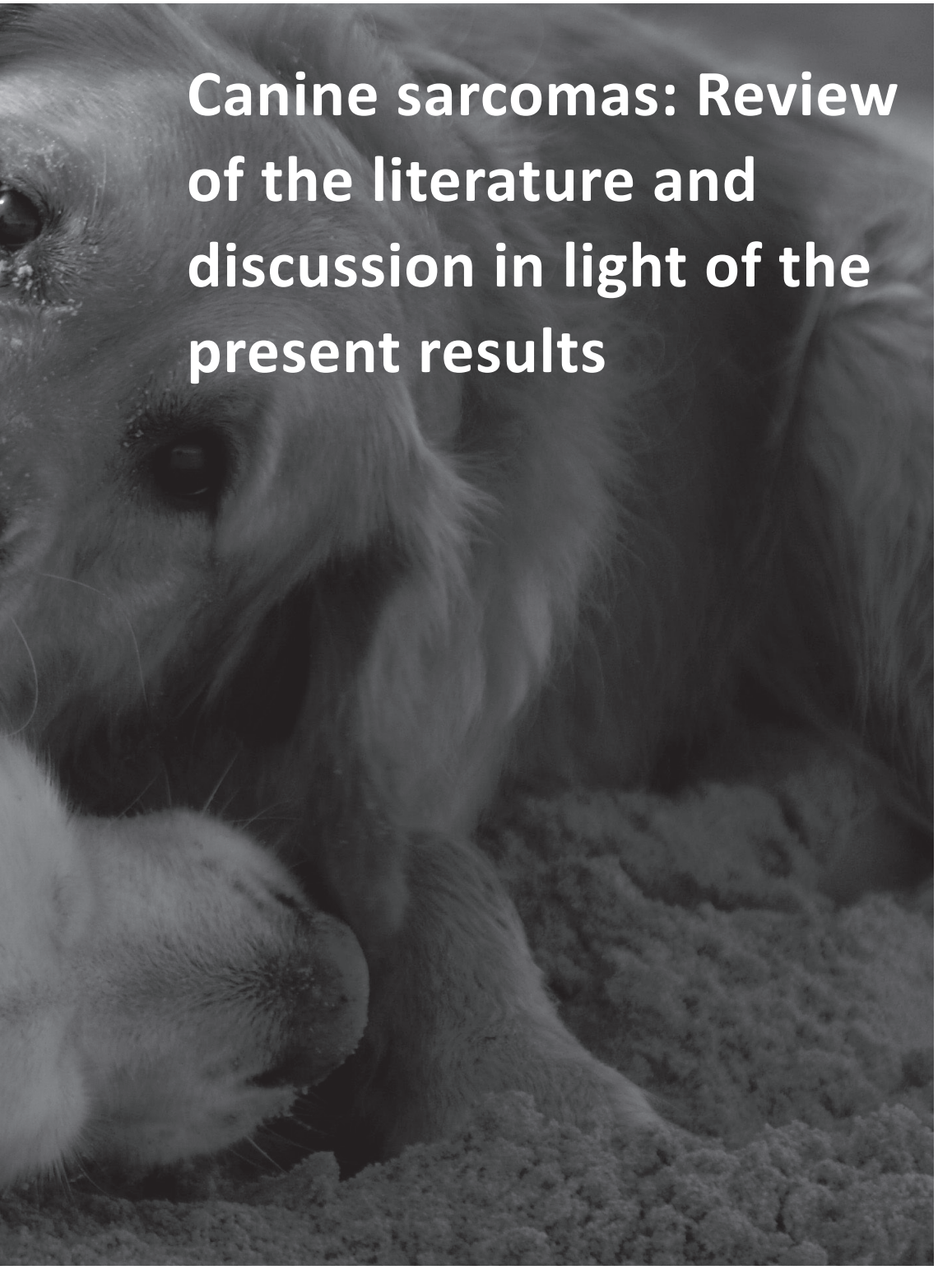

Canine sarcomas: Review of the literature and discussion in light of the present results

Results presented in this thesis are hoped to contribute to a better understanding of the effect of genetic variations - both constitutive as well as acquired in the development of various sarcomas. There is still a lack of firm data related to sarcoma genesis, especially in veterinary oncology, but there is hope for major advancements in following years. In the future we are hopefully able to shed more light upon one element in cancer research that can be considered to be leading throughout this thesis: Why does one individual develop a sarcoma whereas another does not? Why is one breed of dogs predisposed for certain tumours whereas others are not?

In this chapter, some considerations on the development of sarcomas within the retriever breeds and the implications of the research results described in the thesis are discussed. Subsequently, suggestions for future research will be given.

IN GENERAL….

…sarcomas (from the Greek word σάρξ 'flesh') are malignancies that have a mesenchymal origin. Thus, malignant tumours that derive from either bone, cartilage, fat, muscle, or connective tissue, are, by definition, considered sarcomas. This group of malignant tumours therefore represent a broad collection of malignances of various origin; ranging from soft tissue sarcomas, histiocytic sarcomas to osteosarcomas.

Within the collective of sarcomas, the group of soft tissue sarcomas (STS) is very diverse, although all have a mesenchymal origin [1]. Amongst tumours that are generally included within the collective of soft tissue sarcomas according to the veterinary WHO [2], are fibrosarcomas, myxosarcomas, malignant fibrous histiocytoma (MFH; later termed pleomorphic sarcoma [3]), liposarcomas, leiomyosarcomas, rhabdomyosarcomas, hemangio- and lymphangiosarcomas, malignant peripheral nerve sheath tumours (PNST), synovial cell sarcomas and histiocytic sarcomas (HS). However, there is strong debate within veterinary diagnostic and prognostic studies as to what tumours are considered STS [1]. For example, no unanimity exists whether HS should be included in the group of STS. Some researchers consider HS to be a part of STS [4]; some do not [1].

Despite the diversity, STS are still considered a group, because of morphological and clinical similarities [1]. They commonly occur in the skin and subcutis of dogs [1], and represent approximately 7-15% of all cutaneous and subcutaneous tumours [5]. The occurrence of STS is much more common in the dog [1, 5] than in the human, in which they are considered to be rare [6, 7]. Canine sarcomas display very similar (histo)morphological characteristics to sarcomas in humans [8-13]. Based upon similarities in morphology, growth pattern, (lack of) response to treatment including irradiation, STS in dogs are considered to be a good comparative model [12-14]. Research could lead to a better insight in the pathogenesis and could therefore facilitate the identification of therapeutic targets valuable for both species [15-20].

INCIDENCE STUDIES

A lack of the number of cases and controls is considered one of the major flaws in many studies on genetics. The GWAS study described in **CHAPTER 5** is no exception in this aspect. Individuals, cases as well as controls, need to continuously be recruited, and researchers should aim for the highest density coverage and an increase in statistical power for studies on genetics. In addition to focusing on an individual breed, it would be of value to focus on cancer development in the dog species as a whole. Additional information that is considered necessary to conduct such studies, should preferably come from a nationwide databank [21]. This would enable the continuous collection of up-to-date information regarding tumour

incidence. But not only the collecting of epidemiologic data is of importance, also a collection of blood and tissues for genetic studies is essential [22]. This would enable researchers to continuously collect and preserve both patient details as well as collecting patient material such as blood or tissue from patients, thus serving as a bio bank. Researchers are then enabled to directly combine genetic research with detailed patient history and could overcome some of the bias that were encountered during the retrospective study described in Chapter 2. Within a national cancer registry, researchers, oncologists, and owners can be linked and can cooperate with one another, with mutual benefice [21].

Information on incidence, however hard to come by, is a necessity in order to be able to perform future tumour behavioral and genetic studies [23]. Unfortunately, until now, a nationwide cancer registry is not available for the Netherlands. So far, veterinary cancer registries are few in number and scattered [24-26]. Available literature indicate that dogs are relative frequently affected by a broad range of mesenchymal malignancies. In addition, there is clear evidence for the existence of breed predispositions for the development of sarcomas within certain groups of breeds that are considered closely related. The group of retriever dogs is an example of breeds that are considered closely related [27]. Examples of cancer predispositions in retrievers are hemangiosarcomas in the Golden, Labrador and the Flatcoated retriever [12]; osteosarcomas in the Golden retriever [13]; STS in the Golden retriever and Labrador retriever [28, 29] and HS in Flatcoated retriever [30].

Much of what we know so far on tumour incidence is derived from the population-based veterinary cancer registry in California: The California Animal Cancer Registry (CANCR) [24]. Results from this study are frequently used as reference data set. They are however more than 40 years old and have been obtained from one specific region. Same holds true for most investigations. They are not only performed in different countries with a different population of dogs, but also a long time ago. While confirmation has been obtained for a predisposition of Flatcoated Retrievers for histiocytic malignancies [31] which was confirmed in the Dutch population of this breed [32], no data on incidence or predisposition for specific types of cancer, neither STS nor other types of tumours, were available in Labrador – or Golden Retrievers in the Netherlands at the start of our studies.

To evaluate the distribution of various types of neoplasia within the Dutch population of the Golden retriever breed, their incidence was assessed in dogs from this breed during 1998 and 2004. Of all 2,124 neoplasms diagnosed using histopathology; 428 (20%) were initially diagnosed as neoplasm suspected to be of mesenchymal origin, the primary subject of the current thesis. Of these 428 mesenchymal tumours, 253 neoplasms (59% of the initially diagnosed 428 animals) were diagnosed as (potential) soft tissue sarcomas, 31 (7%) as malignant tumours of bone, and 144 (34%) as benign mesenchymal lesions, leading to roughly two

thirds being malignant, similar to early (scanty) reports [33]. The sarcomas most frequently found were fibrosarcomas (n=54; 23%), hemangiosarcomas (n=34; 15%) and neurofibrosarcomas (n= 20; 9%), which is largely in accordance with what is known from the general dog population, thus not focusing on individual dog breeds [24]. For the occurrence of STS, considering a population at risk of 29,304 dogs per year (breed-registered per year) and an average of 657 annually diagnosed new tumours (using histology), an estimated incidence rate (EIR) of 114 per 100,000 dogs was calculated. This is higher than some previous publications in which an incidence rate (IR) of 35 was reported, [14] but somewhat lower than the IR found by Dobson et al. (IR: 142) [34]. This last study, however, used age-standardized IR, which we could not.

Thus, results of this study described in **CHAPTER 2** indicate that the Dutch population of Golden retrievers is indeed predisposed to the development of several forms of cancer, such as mast cell tumours (MCT). In addition, the Golden retriever appears predisposed for cancer development in general. This raised further questions. One such question is, whether it is possible to identify a gene or genes that can be hold responsible for this general predisposition for cancer, or perhaps genes that can be hold responsible for a predisposition for a specific type of tumour? Moreover, in case such genes exist, can this predisposing effect be recognized in different breeds or different species? If so, it could very well hold implications for cancer research in other species, such as the human.

Within the Dutch population of Golden retrievers, the presumed predisposition for STS was found not to be as high as that for several other tumour types. This may indicate that STS have lower impact on the health of this breed than several other tumours, such as MCT. With the phenotype of MCT being less diverse than that of STS and the incidence higher, it is seems likely that further research will identify genetic loci associated for the development of MCT. However; incidence is not the only determinant for the impact that a disease can have within a breed. The clinical consequences that the development of a specific tumour may have are also of importance. STS are tumours that are known for their potential for (aggressive) local recurrence. Moreover, apart from surgery which has major limitations, sarcomas have only modest therapeutical options. Both the incidence as well as the potential severity of this disease must be weighed when considering the impact of a specific disease predisposition in a breed.

Several cancers in the dog resemble those in humans with regard to presentation, treatment response and clinical pathology [33, 35] including the sarcomas [26, 36]. It is therefore possible that similar genetic problems underlie the development of these tumours. Genetic studies could thus very well benefit both species [35-37]. More research is necessary to enable researchers to accurately answer these additional questions. The GWAS study reported in Chapter 5 is a beginning of solving the puzzle.

ESSENTIAL FOR PATHOGENETIC STUDIES OF SARCOMAS….

…….is having certainty about the *type* of mesenchymal proliferation. In humans, it is already accepted that correct further identification of specific STS subtype is necessary [38, 39] in order to perform pathogenetic studies, to better predict biological behavior of the individual tumour [40], and to choose the optimal therapeutic options [6, 40, 41]. Considering the many similarities of these tumours between the dog and humans this should therefore also apply for the dog.

However, for many individual mesenchymal tumours there is no convincing proof for the prognostic value of classification and grading systems [1, 42, 43]. This is mainly due to lack of detailed subclassification in publications up till now. Several classification and grading systems have been established in humans [44], and were later adopted for use in the dog [1, 45]. The current standard for classifying canine STS is the *Histological classification for mesenchymal tumours of skin and soft tissues of domestic animals* (Veterinary WHO) [2].

In order to achieve a correct classification of STS, (routine) histopathological evaluation has its limitations. A review (with addition of immunohistochemistry) of a group of (potential) soft tissue sarcomas derived from the cohort of STS from Golden retrievers collected between 1998 and 2004 was performed (**CHAPTER 3**).

The first step in improving differentiation in STS subtype, is by means of applying strict histomorphological criteria, preferable by experts reaching a consensus. In addition, if deemed appropriate for the individual subtype, additional immunohistochemistry (IHC) can be of help. In our series of mesenchymal tumours in the Golden retriever, 110 unclassified (potential) STS were selected for revision. Revision was initially based on morphological characteristics of hematoxylin and eosin stained histological sections of the neoplasms. If deemed necessary (n=76), additional immunohistochemistry was applied to aid characterization.

Following revision, we were able to confirm the diagnose STS in 75 cases (68%). Some of these neoplasms showed morphological characteristics that were suggestive for sarcoma subtypes as described in the WHO classification for humans. A specific STS subtype could be diagnosed in 58 tumours, meaning that seventeen neoplasms remained ‘unclassified STSs’.

Within these 110 neoplasms, as many as 31 cases (28%) were eventually diagnosed as ‘neoplasm, not being STS’. Four neoplasms (4%) were considered non-neoplastic.

Compared to the Veterinary WHO classification [2], the World Health Organization (WHO) Classification of Tumours of Soft Tissue and Bone ('human WHO') has further expanded in several subtypes. We could conclude that the diagnosis of some of the canine STS entities, contains several gaps for correct classification of these lesions when following the veterinary WHO classification from 1998. Routine histological classification of mesenchymal proliferations in Golden retriever dogs seems often to be inadequate, and IHC is in many cases indispensable for correct classification. As individual sarcoma cases have illustrated, extension of the classification in domestic animals appears possible following the human WHO classification.

Seventeen tumours, though judged to be sarcoma, could not be further classified, and were considered unclassified STS. Problems in diagnosing and classifying STS are not uncommon in canines [1, 9, 10, 46-48] as well as in humans [49, 50], where they are known to possibly result in major discrepancies. Major discrepancies are defined as changes in diagnosis that could lead to a significant change in clinical management, causing either under- or overtreatment [49]. Correct identification and classification into subtype, can be obstructed due to the lack (or loss) of structural and molecular elements [1, 9, 10, 46, 47, 49] even when a large panel of markers is applied [8, 9, 48]. It was noteworthy in our study that not only the subclassification of STS showed to be somewhat problematic, but also that a substantial proportion of these 110 tumours appeared to be even of non-STS origin (n=31) or non-tumourous (n=4). This further demonstrates the necessity of future subclassification and illustrates the diagnostically challenging nature of this group of lesions [50].

Significant changes in the diagnosis from 'suspect STS' into a non-neoplastic lesion, have occurred in studies validating diagnoses in human pathology as well [49, 50]. This can be explained by the fact that non-neoplastic mesenchymal lesions can, in some cases, strongly resemble STS, as previously recognized [50, 51]. Further, discrepancies could have occurred because of differences in interpretation of morphology [49], the rare nature of a lesion [50] and the variable degree of experience to sarcoma cases between different pathologists [49]. STS in general have a high risk for diagnostical errors [50]. Some subtypes are even more prone to misdiagnosis than others [49, 52], and there have been known difficulties to properly diagnose poorly differentiated tumours. One of these is a lesion for long called malignant fibrous histiocytoma (MFH) [48]. Even its name gave reason for debate. The name is now seen as obsolete , and in the human has been changed to undifferentiated pleiomorphic sarcoma [53] and in the dog to pleiomorphic 'anaplastic sarcoma with giant cells' [3]. The name MFH was considered a purely descriptive term, not reflecting the cellular origin of the lesion, and wrongly indicating that it should consist of fibrous histiocytes [3, 48, 53]. Another example of potential misdiagnosis is the group of synovial cell sarcomas (SCS) [54-59]. Within this subtype, the reliability of current immunohistochemistry protocols to

recognize this tumour type in the dog is questioned [60]. Problems arise in part because the presumption that this tumour originates from synoviocytes – as the name indicates – likely is incorrect as studies in the human suggest [56, 61]. In the human, improvement with the diagnosis of SCS first came with application of IHC [61] and subsequently molecular genetic techniques have been found to be extremely valuable in diagnosing SCS in humans [62]. In SCS, a chromosomal translocation, t(X;18), tightly linked to the tumour genesis of synovial sarcoma, is present; producing the SYT-SSX fusion oncogene [55]. This is nowadays considered a cytogenetic hallmark [63, 64], making SCS a genetically distinct entity in the human [63]. Unfortunately, so far, these techniques have not been applied in the dog and IHC remains a necessary adjunct.

PLOIDY STATUS

Another way of detecting chromosomal changes is by determining the DNA ploidy status. Aneuploidy reflects gross quantitative genomic destabilization. In humans, measuring ploidy status has been shown to potentially be of help when discriminating non-neoplastic or benign neoplastic lesions from (pre) malignant neoplastic conditions in lesions of mesenchymal origin [65-68]. The additional value of nuclear DNA-content as a marker of malignancy in canine mesenchymal proliferative lesions was studied by us [69] in **CHAPTER 4**. We could indeed conclude that aneuploidy is also frequent in various canine malignant mesenchymal lesions, with prominent variation amongst the different sarcoma types. With respect to aneuploidy occurrence amongst STS in general (53%), there were clear similarities with previous studies in sarcomas in humans [70-72] and our study. In humans as well as dogs, some STS types, although considered malignant, were only rarely found to be aneuploidy. Clear examples in our study as well as in comparable human studies [73], were leiomyosarcomas and malignant peripheral nerve sheet tumours. The overall frequency of aneuploidy was also largely in accordance with findings in humans [72]. Like in humans [65, 70, 71, 74], When comparing mesenchymal lesions by histopathology with the result of ploidy analysis, the existence of aneuploidy in dogs was found suggestive of a malignancy.

We could, however, not rule out malignancy on basis of a diploid status. More subtle and sometimes balanced chromosomal gains and losses have been observed previously in human [68, 73] and canine sarcoma studies [75, 76]. For example, it is possible that transformation to malignancy is driven by a defect in DNA repair pathways leading to microsatellite instability, with microsatellite instability and aneuploidy being mutually exclusive phenomena [77].

Another interesting question related to ploidy status is the possible correlation with histological malignancy grading. Are high-grade sarcomas more commonly

aneuploid, compared to those of low- or intermediate- grade, as is often the case in humans [64, 73, 77, 78]? No such relationship, however, became evident in our study. In part, this may be related to the relatively high frequency of hypoploid cancers, and the related presence of smaller nuclei, which might be judged lower grade by pathologists. The observation that hypoploidy occurred relative frequent (an incidence of 26% of all tumours, or 48% of the aneuploid cases) is remarkable. Similar observations have been made in other types of cancer in the dog such as thyroid- and mammary carcinomas, and malignant lymphomas [78-81]. With the exception of chondrosarcomas [82], hypoploidy is rare in human solid cancers [72, 77, 83-85] including sarcomas [72]; with the highest reported rate being 11% [70]. It appears that the dog is particularly prone for the development of aneuploid tumours associated with either chromosome loss or low number chromosome gain, and less frequently to hypertriploid tumours that is common in humans [72, 83, 84, 86]. This feature seems at variance with ploidy evolution patterns described in humans [72, 83-87]. A possible explanation for this difference in ploidy evolution could be the presence of a more powerful defense mechanism in humans against the tumourigenic effects of hypodiploidy or low level hyperploidy [83, 88]. While for many human cancers a greater destabilization over multiple phases of destabilizing events seems necessary to arrive at a fully malignant state, such state may be reached with less destabilization in the dog, as hypothesized earlier [89]. This interspecies difference in direction and degree of DNA-content change, suggests interspecies variation in aneuploidy evolution and genomic destabilization during carcinogenesis [89].

SO FAR, OUR STUDY……

……..focused on the identification of the Golden retriever as a breed that carries an increased susceptibility for STS. Next, an attempt was made to improve our knowledge on the molecular biology of this disease. This was done by improving diagnostical procedures and analyzing DNA-ploidy of sarcomas.

But can we also *identify* the genes – if at all operating - that could be held responsible for this breeds' predisposition? Can we obtain evidence of a germline genetic association between a breeds' genotypic characteristics, and the development of soft tissue sarcomas?

As previously outlined, several retriever breeds are predisposed for the development of sarcomas. The lifetime risk for some of these cancers can be remarkably high in these dogs: up to 12-33-fold higher than in humans [31, 34, 90]. For example the hemangiosarcomas in the Golden retriever, which are estimated to occur in more than 20% [12] or the histiocytic sarcomas; a tumour that is considered a major cause of death within the Flatcoated retriever [30, 91]. A limited genetic flow within dog breeds is largely responsible for such predispositions to exist. It is a consequence of the stringent breeding programs

that are applied within the dog. Selection on specific breed traits as well as periodic population bottlenecks, for instance caused by the big wars of the 19th and 20th century are causes for certain breeds to show a high prevalence for specific types of cancer [92]. Also, dogs show remarkable homogeneity within a breed, coupled with striking interbreed heterogeneity [23]. This is part of the reason that studying spontaneous cancers within the dog, and especially the genetic changes within these cancers, provides good opportunities for comparative research. [11, 35, 93-95]. This is especially the case in cancers that are considered rare in humans, such as sarcomas [13, 96, 97]. The high prevalence of sarcomas within certain dog breeds enables studies of behavioral factors and genetic background. A high prevalence suggests that possibly only a limited number of loci underlie a disease, making a genetic dissection potentially more tractable in dogs than in humans [23, 98].

In the past years, various genetic resources have been developed enabling genetic dissection [98-100]. The identification of mutations in numerous Mendelian diseases have already been identified [23, 98, 101]. In particular following the sequencing of the dog genome [98], genetic research has taken a flight. Concerning canine cancer, two of the most compelling examples of the use of genome-wide linkage scans that were able to identify loci, are the study of renal cystadenocarcinoma and nodular dermatofibrosis, a hereditary kidney cancer of the German shepherd dog [23, 102] and the study of canine osteosarcoma, that was, through genome-wide association analyses able to identify 33 inherited risk loci in three dog breeds [103].

A genome wide association study (GWAS) as described in **Chapter 5** compares a dense set of genotypes from animals that have a particular trait (in our case, Golden- and Labrador retriever that suffer from soft tissue sarcomas) with unrelated controls. This comparison can ascertain alleles to be associated with the trait [23]. This method has already been successfully performed within the Bernese Mountain dog, a dog breed that is predisposed for histiocytic sarcomas [104]. GWAS had already been capable of identifying a first genetic locus: a single haplotype spanning MTAP and part of CDKN2A is present in 96% of the affected BMD [17].

We investigated the possible genetic alterations that could lead to the development of STS as a group. As discussed before, STS consists of a collection of tumours that show clinical heterogeneity complexity, so the phenotype is somewhat divers. Also the assignment of a normal phenotype could represent a source of error; healthy controls could in some cases develop STS at a later stage, considering the late age of onset for the development of STS. Because this complicates the use of DNA from healthy controls – if these develop the disease at more advanced age, normal phenotype was assigned only if dogs from the healthy control group were older than eight years of age and at that time free of cancer, and where possible owners were questioned on a regular base to inform us on the continued health status of their dog.

The late age of onset, the relative rareness of the disease and a stringent quality check proceeding the GWAS posed additional challenges to overcome; hampering the collection of a large number of cases (final cohorts: Labrador retriever: 343 animals; 100 cases and 243 controls; Golden retriever: 182 animals; 43 cases and 139 controls). Greater sample size would provide increased statistical power.

Despite the difficulties, a GWAS approach was successfully performed in two genetically closely linked [27] predisposed breeds; the Labrador retriever and Golden retriever. Using a relatively low number of cases (DNA from animals that suffer from STS) and controls (DNA from healthy animals that were older than eight years of age), we reached suggestive association, and in the Labrador retriever, significance. These breeds show non-overlapping regions of interest in relation to soft tissue sarcoma development, indicating a different genetic predisposition in both breeds. It also illustrates the high degree across-breed heterogeneity [23] and the complexity of cancer as a disease, considering the predisposition can be traced within a *different* area of their genome.

The regions that were found, are likely to be important determinants for the development of STS. The regions of interest contain several candidate genes; most of which are positioned in proximity of each other. At this moment it is not possible to determine the actual effect of these (individual) genes regarding the development of STS. It is very likely that some of the candidate genes found in the regions of interest are indeed connected to the disease development and progression. A clear example is *MYO18B*, a gene that is expressed in muscles, and is a well-known tumour suppressor gene [105]; with mutations associated with lung cancer [106], colorectal cancer [105] ovarian cancer [107]. However, although several genes enclosed within these loci are indeed considered to be very promising, the involvement of these regions do need confirmation. Future studies will consist of DNA re-sequencing using next generation sequencing and confirming candidate genes in a replication cohort of cases and controls.

A genetic predisposition for the development of STS in general was made likely in both the Golden- and Labrador retriever (Chapter 5). However, like others[50], we have demonstrated that STSs carry a high risk for diagnostic errors and that they consist of a broad, heterogeneous group of cancers. It is well possible that this broad phenotype is actually a limiting factor when trying to obtain significant genetic variation such was attempted within this thesis. All cases that were collected for the GWAS, were selected as part of STS seen as one group; individual subtypes were not considered. It is possible that the two breeds are not predisposed for the development of STS as a group, but for a specific subtype. It would thus be of additional value for future research, to combine the results of Chapter 3, where an attempt was made to obtain a better subclassification of a group of STS, and the results of the GWAS as described in Chapter 5. Potential variations in predisposition for

specific STS l subtypes should be considered in future research, like has been evidenced for the Flatcoated retriever [30].

WHICH GENETICAL CHANGES WITHIN THE TUMOUROUS TISSUE …

….when compared to healthy tissues appear related to the specific sarcoma type? One approach is to identify functional differences through identifying altered expression of genes and deregulation of gene signaling pathways in specific sarcomas. To answer this, a Microarray transcriptome study was performed, and, like with the GWAS, our results are hoped to have a comparative value in the field animal-human disease . This type of study is commonly used to investigate tumourigenic mechanisms [108]. Several other studies in canine tumours have revealed expression of genes and deregulation of gene signaling pathways [108, 109].

There is one type of sarcoma that encompasses several elements making it worthy of investigation, the histiocytic sarcoma (earlier often named malignant histiocytosis). The incidence in two of the most commonly affected dog breeds, Flatcoated retrievers and Bernese Mountain dogs is estimated to be > 200-fold elevated as compared to the 'average' dog, with mortality almost certain [4, 17, 32]. Canine histiocytic sarcomas resemble histiocytic malignancies in the human. [15, 94, 110, 111] but in the human are considered a very rare disease [97, 112, 113]. Although these histiocytic disorders concern a serious condition with a grave prognosis within both species [4, 17, 32, 96, 97], no studies have appeared on assays comparing gene expression in different histiocytic/dendritic sarcoma in both species. As such, studies may serve as a basis for further (comparative) investigations: The identification of genes involved in the disease in the dog, may have comparative value in the study of histiocytic malignancies in the human as well This assumption is based upon a fundamental and evolutionarily conserved association between cytogenetic abnormalities and tumour phenotype in different species [93, 94]. Extrapolation of these data to human samples may help to further our understanding of the propagation and oncogenesis of histiocytic cells. Eventually, this may contribute to the development of effective therapeutic modalities for both species.
Flatcoated retrievers are especially prone to develop the localized version of the tumour[30] more so than the Bernese Mountain dog. This observation prompted us to l question: Are these entities two different stages along a continuum of the same disease [110, 114], or are they in fact two different entities?

As a first step in the determination of an altered gene expression, we aimed on identifying genes that are associated with HS development in the Flatcoated retriever, irrespective of form, focusing on general development of HS. Gene expression differences were evaluated in both of the two most common forms

of HS and compared to normal spleen using both traditional fold change analysis as well as disease-based pathway analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (**CHAPTER 6**) [20]. Since it is not possible to obtain pure samples of sedentary, non-tumourous histiocytes for RNA extraction as healthy control for expression profiling, spleen tissue (obtained from unrelated dogs) was chosen as the normal equivalent of HS. Choosing spleen as a healthy equivalent is a logical choice, since HS arises from interstitial DC, and hence emanate from lymphoid domains when these arise from lymphoid organs such as the spleen [110].

At the evaluation of a variation in gene expression between the combined HS and spleen, 3,394 features were significantly differentially expressed and 319 features remained when only 4-foldchanges or larger are taking into account. When comparing VHS and STHS, we identified 191 significantly differentially expressed probes (**CHAPTER 7**) [115]. For these comparisons, however, it is important to realize that since only 21,682 (51%) were annotated (CanFam 2.0), it is possible that important genes are missed. We chose to examine a selection of the significantly altered genes found in the microarray experiment for confirmation by qPCR in order to improve knowledge on the genetic basis of HS and to best exploit logistic and financial resources. Selection of genes was based on the statistical significance of their differential expression and their potential involvement in tumour development or on likelihood based on literature research.

In both experiments, when comparing the results of the qPCR experiment and the microarray experiment, the difference in the expression of some genes did not attain statistical significance. The variation between the two methods relates to the fact that a microarray experiment is a semi-quantitative screening method and the qPCR a quantitative method. Many methodological factors can lead to a lack of correlation between array results and qPCR measurements [116].

When comparing HS (irrespective of form) with normal spleen in the confirmative qPCR experiment, ten genes were chosen for qPCR confirmation. Significantly altered expression was confirmed for nine of the ones that were analyzed. *PPBP, SpiC, VCAM1, ENPEP* and *ITGAD* were down regulated and *GTSF1, LUM , Thy1* and *Col3a1* were up regulated in HS, irrespective of the disseminated or localized form, compared to normal spleen. Further literature studies for the most significant genes could confirm that these genes are indeed worthy of further pursuit. Especially *PPBP* [117], *LUM* [118-124] and *GTSF1* [125] and *Thy1* (CD90) which is considered an important marker of many types of stem cells [126] including mesenchymal stem cells [127]. This study thus was able to provide evidence for involvement of several genes in HS, irrespective of the form of manifestation. Some of these genes are related to other cancers (*Col3a1, GTSF1, LUM, PPBP*), have a role as a stem cell marker (*Thy1*) or are involved in growth and differentiation of early B-lineage cells (*ENPEP*).

As a next step, a comparison was made for VHS and STHS, and eleven genes that were found differentially expressed were selected for qPCR confirmation. For three gene products this difference in expression was significant: *C6* (up-regulated in VHS versus STHS), *CLEC12A* and *CCL5* (both down-regulated in VHS versus STHS). A straight forward hypothesis on the functional consequences of the variation in expression in the different forms of HS is not easily postulated. Although our observations of for instance *CLEC2A* and *CCL5* and those made in several human cancer types [128], make it conceivable that these gene products may play a role in HS. The use of spleen as the as the healthy equivalent of HS does raise some concern as to how observed differences in the results should be interpreted. In particular the difference in *Spi-C, ITGAD* and *VCAM1* gene expression in tumours compared to healthy spleen, should be explained. It cannot be excluded that some of these expression differences are based on differences in tissue-origin rather than on actual tumour development.

Two of the most comprehensive database to date, revealing variations in genetic expression, were put up. The first one provided genetic variations comparing the two forms of HS combined to spleen. The second one provided genetic variations of VHS versus STHS. Genetic variations not previously associated with the development of these two forms were revealed. In addition, the DAVID pathway analyses, showed that 24 pathways were significantly involved in the development of HS ($P<0.05$), mostly in the DNA repair and replication. However, the pathway analyses of the genes that were significantly differentially expressed when comparing STHS and VHS did not lead to the detection of altered expression of whole pathways.

The alterations in gene function as detected in the current analyses need follow up by subsequent investigations. Although not necessarily so, it is likely that large differences in mRNA levels of specific genes will result in quantitative differences in protein expression. This is reflected by differences in protein levels. Subsequent research should thus focus on changes of gene function at protein level. One approach may be expression analysis on microscopic slides of tissues by use of antibodies. So far, validated specific antibodies for most gene products of interest are lacking and must therefore be developed and validated for use in the dog to examine an altered expression at protein level. Eventually, a comparison of these histiocytic malignancies in dog and human can be made.

This current study provides the most comprehensive database of genome alterations in histiocytic malignancies to date. All microarray gene expression data were deposited in the public data repository GEO (accession number GSE45832).

As mentioned to be the case in synovial cell sarcomas in humans, it is already known that some genetic changes found in individual sarcoma types can be considered a hallmark of such a subtype [63, 64]. This could possibly also be true

for the genetic changes found within the histiocytic malignancies in the Flatcoated retriever (**CHAPTER 6, 7**). Knowledge of a genetic change that is considered to be a hallmark could for example greatly enhance speed of diagnosis. Histiocytic sarcomas are not always easily recognized, and are known to sometimes be mistaken for tumours such as the synovial cell sarcomas and osteosarcomas. A means of better differentiating would greatly enhance the speed of diagnose, and possibly in future, the effectiveness of a more targeted treatment.

IN CONCLUSION…..

….. In future, the knowledge gained within this thesis hopefully leads to the development of more insight. An increased knowledge in the genetic background of sarcomas could in future help in the development of new treatment options. Also, we could possibly shed some light as to which dog breed individuals might be less suitable for breeding purposes than others. Results of this thesis also once again show that breeding from a small selection of individuals ultimately leads to a decrease of the genetic pool, leading to the development of hereditary diseases. I consider it the responsibility of researchers to inform breeders on the potential risk of inbreeding.

As a bycatch, canine pets can change the focus of human research [36, 92]. Results of this study are potentially of value in humans, where this type of research is hampered by small families, outbred population structure and locus heterogeneity [23, 129].

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Summary

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Compared to the general dog-population, certain family members of the retriever breed appear to be more prone for the development of certain tumour types (such as soft tissue sarcomas (STS)) than the general dog population. **Chapter 2** examined the existence of a possible tumour (type) predisposition in the Dutch population of Golden retrievers by evaluating tumours submitted to Utrecht University during the period 1998-2004. Tumours were diagnosed either by means of cytology (n=2,529) or histology (n=2,124)). Results were related to an average annual Dutch kennel club population of 29,304 Golden retrievers. A second aim was to evaluate whether incidences of various tumours differed as related to the diagnostic procedure, being either cytology or histology. The Dutch population of the Golden retriever breed showed an increased risk for the development of tumours in general, as well as an increased risk for the development of specific tumour types, such as mast cell tumours, lymphomas and melanomas. Amongst these tumours, a breed predisposition for the development of soft tissue sarcomas (STS) was also suspected. Also, between the two different data-sets (being the histopathological- and cytological data set) variations in age, location and incidence of various tumours were observed. This indicates that a selection bias for diagnostic procedure was present.

Chapter 3 describes the result of a revision of a subset of the previous collected cohort of tumours. Malignant tumours derived from Golden retrievers that were considered 'sarcoma, not otherwise specified or of uncertain subtype' (i.e. unclassified sarcomas) (n=110) were selected for revision. Revision was performed by a board certified veterinary pathologist and a human pathologist, following the criteria according to the *World Health Organization International Classification of Tumors of Domestic Animals* (veterinary WHO), recent veterinary literature and the WHO classification for humans. Revision was initially based on morphological characteristics of hematoxylin and eosin stained histological sections of the neoplasms. If deemed necessary (n=76), additional immunohistochemistry was applied to aid characterization. In 58 tumours, we were able to arrive at the diagnosis of specific subtype of the STS. Seventeen tumours remained 'unclassified STS'. As many as 31 tumours (28%) were diagnosed as being tumorous, but not considered to be STS. Four neoplasms (4%) were considered non-neoplastic. Since an incorrect identification of the nature of a neoplasm could clearly lead to an inappropriate therapeutic intervention and prognostication, these numbers illustrate the necessity for correct diagnosis. In humans, a proper differentiation and additional subclassification of STS is considered essential. Considering the morphological resemblance of canine STS in comparison with human STS, we state that additional subclassification is also of value in the dog, and considered advisable. Results of this study show, that additional classification is indeed very well possible.

DNA-aneuploidy may reflect a malignant nature of mesenchymal proliferations and herald gross genomic instability. The determination of DNA ploidy status is an additional method to potentially acquire a better differentiation between malignant and non-malignant mesenchymal lesions. Results are described in **Chapter 4**. DNA-ploidy status and DNA-index were determined in canine inflammatory and neoplastic mesenchymal tissues, and related to clinicopathological features, biological behavior and p53 gene mutational status. Half of all sarcomas were aneuploid. Benign mesenchymal neoplasms were rarely aneuploid and inflammatory lesions were all diploid. Rate of aneuploidy was comparable to that reported for human sarcomas with significant variation amongst sarcoma subtypes, indicating variation in genomic instability between subtypes. In contrast to human sarcomas, DNA-ploidy status in canines lacked a relation with histological grade of malignancy. While aneuploidy was related to the development of metastases in soft tissue sarcomas, it was found not to be related to the development of metastases in osteosarcomas. Amongst the sarcomas evaluated, also no relation was found between ploidy status and the presence of P53 gene mutations. Heterogeneity of the DNA index between primary and metastatic sarcoma sites was present in half of the cases examined. Hypoploidy appears more common in canine sarcomas and hyperploid cases have less deviation of the DNA index than human sarcomas. This strengthens the concept of interspecies variation in the evolution of gross chromosomal aberrations during cancer development.

Following study aimed to identify possible genes causing the suspected breed predisposition for the development of STS in both the Labrador- and Golden retriever breed. This genome-wide association study was the first in any species to examine STS as a group. The study aimed to identify possible genetic variants associated with STS development. Results are described in **Chapter 5**. Results were suggestive for a germline genetic association between the Labrador- and Golden retriever breed, and the development of soft tissue sarcomas. Within both breeds, different loci (all of which contained candidate genes already known to be involved in the development of cancer) could be identified. The GWAS performed within the Labrador retriever cohort displayed significant association to a region on chromosome 3 (P_{perm}= 0.026), and suggestive significant regions on chromosomes 35 and 17 (P_{perm}= 0.201 and 0.237). The GWAS performed within the Golden retriever cohort displayed a very promising association on a region on chromosome 26 (P_{perm}= 0.205) at the exact location of a very interesting candidate gene, *Myo18B*.

Within histiocytic sarcomas developed in Flatcoated retrievers, altered expression of genes and deregulation of gene signaling pathways were identified by means of a microarray study. This is explained in **Chapter 6**. Of several genes (not yet implicated in histiocytic sarcoma manifestations in the dog nor in comparable human histiocytic/dendritic sarcomas) a significantly altered expression was identified. Of the total of significantly altered genes, DAVID pathway analyses

revealed 24 pathways that were significantly involved in the development of HS in general, most of which were involved in DNA repair and the replication process. The altered expression of nine of the most significantly altered genes were confirmed on a following quantitative real-time PCR (q PCR) experiment. *PPBP, SpiC, VCAM1, ENPEP, ITGAD* were down-regulated, and *GTSF1, Col3a1, CD90* and *LUM* were up-regulated in the comparison of both the soft tissue and the visceral form with healthy spleen.

Histiocytic sarcomas can be grouped into two main forms; a soft tissue- and a visceral subtype. The Flatcoated retriever is predisposed for the soft tissue subtype. **Chapter 7** describes the findings of the study that successfully identified variations in gene expression between these two subtypes. Again, microarray analyses was followed by q PCR analyses, that evaluated the expression differences of ten of the most significantly differentially expressed genes identified by microarray analyses. We confirmed the significantly aberrant expression of three of these genes: *C6* was up-regulated; *CLEC12A* and *CCL5* were down-regulated in the visceral histiocytic sarcoma compared to the soft tissue form. Results of this study were thus able to identify genetic differences between the two subtypes of histiocytoc sarcomas, possibly causing the phenotypical variation between these two histiocytic sarcoma subtypes.


10

Samenvatting in het Nederlands

Curriculum vitae and list of publications

Acknowledgements

Nederlandse samenvatting

In vergelijking tot de algemene honden populatie lijken binnen de populatie van de retrievers bepaalde rassen gepredisponeerd voor de ontwikkeling van bepaalde typen typen tumoren, waaronder sarcomen. Het onderzoek beschreven in **hoofdstuk 2** heeft als uiteindelijk hoofddoel, een mogelijke predispositie voor tumoren in zijn algemeenheid en sarcomen in het bijzonder binnen de Nederlandse populatie van de Golden retriever vast te stellen. Een tweede doel is het evalueren of de incidentie van bepaalde tumortypen afhankelijk is van de gekozen diagnostische techniek: Cytologie ofwel histopathologie. Om de vermeende predispositie voor tumoren binnen de Nederlandse populatie van de Golden retriever vast te stellen, is gekeken naar alle cytologische en histopathologische inzendingen verstuurd naar de Universiteit van Utrecht binnen de periode 1998-2004. Dit aantal is vergeleken met het totale aantal nieuwe registraties binnen het stamboek in diezelfde periode. Dit onderzoek bleek in staat om voor een aantal typen tumoren deze predispositie aan te tonen. Niet alleen lijkt de Golden retriever gepredisponeerd voor de ontwikkeling van (kwaadaardige) tumoren in het algemeen, maar daarnaast lijkt dit ras ook gepredisponeerd voor de ontwikkeling van bepaalde tumortypen in het bijzonder, zoals de mastceltumoren, maligne lymfomen en de melanomen. Hiernaast lijkt dus inderdaad ook een predispositie te bestaat voor de ontwikkeling van weke delen sarcomen. Tevens blijkt er verschil tussen de kenmerken van tumoren van dieren die werden aangeboden voor ofwel cytologisch- ofwel histopathologisch onderzoek. Er bestond tussen deze twee groepen de nodige variatie tussen leeftijd, locatie en type tumor. Dit impliceert dat er een voorkeur voor een van beide technieken bestaat, afhankelijk van leeftijd, locatie en type tumor dat men verwacht te diagnosticeren.

De groep van de weke delen sarcomen bij de hond bestaat uit allerlei verschillende subtypen welke in morfologisch opzicht erg op die van de mens lijken. **Hoofdstuk 3** beschrijft een groep van 110 tumoren, allen geselecteerd uit de collectie beschreven in het voorgaande hoofdstuk. Kenmerkend voor deze geselecteerde groep is dat ze, aan de hand van de histomorfologische kenmerken, weliswaar werden toegeschreven aan de groep van weke delen sarcomen, maar dat een nadere omschrijving van het daadwerkelijke subtype in deze groep niet met zekerheid kon worden vastgesteld. Deze groep aan tumoren is opnieuw beoordeeld (gereviseerd). Waar nodig (n=76), zijn voor verdere classificatie additionele immuunkleuringen toegepast. Hieruit bleek dat nauwgezette classificatie maar liefst in 53% (n=58) van deze tumoren mogelijk was. Hierbij werd primair de *WHO Histological Classification of Mesenchymal Tumors of Skin and Soft Tissues of Domestic Animals* geraadpleegd, maar daarnaast werd tevens recente veterinaire literatuur gebruikt. Duidelijk werd verder dat een groot aantal tumoren (n=31, 28%) waarvan initieel dus werd gedacht dat het een weke delen sarcoom betrof, eigenlijk tot een geheel andere groep tumoren behoorde. In vier gevallen bleek de massa niet eens een tumor te betreffen. Omdat een dergelijk omvangrijke verandering van diagnose

grote consequenties kan hebben voor het individuele dier, raden wij dan ook voor deze specifieke groep van tumoren aan, revisie (met indien nodig additionele immuunkleuringen) op meer structurele basis te laten plaatsvinden.

Voor wat betreft het verkrijgen van een verregaande classificatie, heeft men humaan reeds vastgesteld dat een zo correct als mogelijke differentiatie tussen verschillende subtypen van weke delen sarcomen van essentieel belang is. Niet alleen voor een adequate behandeling en prognosestelling, maar ook om toekomstige studies naar de pathogenese succesvol te kunnen uitvoeren. Bij de hond is de meerwaarde van een verregaande classificatie nog niet volledig aangetoond en wordt een adequate differentiatie dus niet altijd nagestreefd. Het vergelijkbare aspect van deze bindweefseltumoren doet echter vermoeden dat een betere classificatie ook bij de hond van additioneel belang kan zijn. In elk geval heeft onze studie aan kunnen tonen dat een betere classificatie goed realiseerbaar is.

Een additionele methode om kwaadaardige weefsels van meer goedaardige processen te kunnen onderscheiden, is het bepalen van de ploïdie status (DNA index) van een weefsel. DNA aneuploïdie is een toestand waarbij de kern van een cel een afwijkende hoeveelheid chromosomen heeft, en is tekenend voor een aanzienlijke instabiliteit binnen het genoom. Het bepalen van de ploïdie status van een weefsel kan dus worden beschouwd als een additionele methode om kwaadaardige weefsels van meer goedaardige processen te kunnen onderscheiden. In **hoofdstuk 4** wordt beschreven hoe bij de hond de DNA-ploïdie en -index in mesenchymale proliferaties is bepaald. De betreffende mesenchymale weefsels hadden ofwel een tumoreus- ofwel een inflammatoir karakter. De DNA ploïdiestatus werd gerelateerd aan klinisch-pathologische kenmerken, biologisch gedrag en de eventuele aanwezigheid van p53 gen mutaties. Van de mesenchymale proliferaties, bleken de goedaardige mesenchymale neoplasieën zelden aneuploïdie te vertonen, en ontstekingsweefsel geheel niet. In echter ongeveer de helft van de onderzochte sarcomen bleek er sprake te zijn van aneuploïdie, en vergelijkbaar met studies binnen de mens, bestaat er een aanzienlijke variatie in de eventuele aanwezigheid van aneuploïdie tussen de verschillende subtypes van sarcomen.

Een opvallende bevinding was tevens, dat hoewel aneuploïdie bij de weke delen sarcomen gerelateerd leek aan de ontwikkeling van metastasen, dit niet het geval was in de groep van de osteosarcomen. Ook was er geen direct verband aan te tonen tussen de ploïdie status en de eventuele aanwezigheid van een P53 gen mutatie. Verder bleek dat er in de helft van de onderzochte gevallen sprake was van heterogeniteit tussen de DNA index van primaire tumoren en van metastasen afkomstig van deze tumoren. Ook kon er, in tegenstelling tot bij de mens, bij de hond geen causaal verband aangetoond worden tussen de histopathologische graad van de mesenchymale maligniteit en de ploïdie status.

In zijn algemeenheid kon worden gesteld dat hypoploïdie (een toestand waarbij een minder aantal chromosomen in de celkern aanwezig is dan gebruikelijk) frequenter optreedt bij de hond dan bij de mens. Tevens week de DNA index van gevallen die wel hyperploïde vertonen (en waarbij dus een hoger aantal chromosomen in de celkern aanwezig is dan gebruikelijk), minder af dan dat dit bij de mens het geval is. Dit versterkt het concept dat er aanzienlijke species-verschillen zijn in de evolutie van chromosomale veranderingen gedurende de ontwikkeling van kanker.

Echter om aan te kunnen tonen of eventueel aanwezige afwijkingen in het DNA en de ontwikkeling van bindweefseltumoren aan elkaar gelinkt kunnen worden, is ander onderzoek noodzakelijk. Van zowel de Labrador als de Golden retriever is een associatiestudie uitgevoerd waarbij deze vraag centraal stond. **Hoofdstuk 5** beschrijft deze studie. Het betreft de eerste genoom wijde associatie studie (GWAS), waarbij gekeken is naar bepaalde genetische variaties in het DNA die mogelijk geassocieerd zijn met de ontwikkeling van bindweefseltumoren. Er is van zowel de Labrador als de Golden retriever een associatie studie uitgevoerd. De uitkomst hiervan is suggestief voor de aanwezigheid van een genetische associatie voor de ontwikkeling van weke delen sarcomen in beide rassen. Tussen beide rassen werden op verschillende plaatsen op het chromosoom gebieden geïdentificeerd die geassocieerd waren met het ontwikkelen van weke delen sarcomen. Binnen de Labrador retriever bleek deze associatie significant binnen een regio op chromosoom 3 (P_{perm}= 0.026). Tevens zijn er meerdere bijna significante associaties aangetroffen, gelegen op chromosoom 35 respectievelijk 17 (P_{perm}= 0.201 respectievelijk 0.237). De GWAS uitgevoerd binnen de Golden retriever leverde weliswaar geen significante associatie op, maar toonde wel een veelbelovende associatie met een zeer aannemelijk kandidaatgen op chromosoom 26 (P_{perm}= 0.205); namelijk *Myo18B*. Ook de overige loci omvatten kandidaatgenen. Kandidaatgenen zijn genen waarvan reeds bekend is dat ze een rol spelen in de ontwikkeling van verschillende typen kanker, in dit geval is bekend dat deze genen een rol spelen bij de ontwikkeling van kanker bij de mens.

Binnenin een specifiek retriever ras, de Flatcoated retriever, is ook tumorweefsel zelf onderzocht. Dit betrof weefsel van een specifieke subpopulatie van de sarcomen, het histiocytaire sarcoom (**Hoofdstukken 6 en 7**). De Flatcoated retriever is een ras dat een uitgesproken predispositie kent voor de ontwikkeling van dit type tumor. Onze vraagstelling was dan ook, of er in het tumorweefsel (in vergelijking met gezond miltweefsel) sprake was van een verandering van de expressie van genen (gen-expressie) en een mogelijke ontregeling van de signalen afgegeven door deze genen. Middels een chromosomale microarray analyse kan de expressie van individuele genen worden geïdentificeerd. Voor deze studie is, als representatief gezond weefsel, voor miltweefsel gekozen. Aansluitend op de microarray is een kwantitatieve real-time polymerasekettingreactie (qPCR) analyse uitgevoerd. Deze techniek maakt het mogelijk een specifieke nucleïnezuurvolgorde aan te tonen, en tevens de volgorde van een of meerdere specifieke nucleïnezuurvolgorden (de

zogenaamde sequenties) in het DNA-monster te kwantificeren. In **Hoofdstuk 6** is beschreven dat in elk geval van negen genen (waarvan een relatie met de ontwikkeling van het histiocytair sarcoom nog niet eerder bekend was) een significant veranderde expressie kon worden aangetoond in zowel de microarray als ook de vervolgende qPCR. De genen *PPBP, SpiC, VCAM1, ENPEP, ITGAD* waren minder tot expressie gebracht, en *GTSF1, Col3a1, CD90* en *LUM* waren juist meer tot expressie gebracht in het histiocytair sarcoom in vergelijking met representatief gezond miltweefsel. Aansluitend is er gekeken of er een onderling verband tussen deze genen aan te wijzen was. Dit is onderzocht door gebruik te maken van de DAVID route analyse. DAVID staat voor **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery. Het is een manier om te zoeken naar onderlinge verbanden tussen grote groepen genen. Alle genen waarbij middels de microarray analyse een significant verschil aangetoond kon worden tussen tumor- en gezond weefsel, zijn ingevoerd in DAVID. Er konden 24 routes ('pathways') worden aangewezen. Deze lijken dus allen een belangrijke rol binnen de ontwikkeling van het histiocytair sarcoom te spelen. De meeste van deze zogenaamde 'pathways', waren betrokken in DNA herstel en replicatieprocessen.

De groep van de histiocytaire sarcomen kan in principe in twee groepen worden onderverdeeld. Enerzijds is er de gelokaliseerde, weke delen variant, welke zich initieel niet gegeneraliseerd- maar juist gelokaliseerd presenteert, en anderzijds is er de van origine gegeneraliseerde (viscerale) variant. De Flatcoated retriever is met name gepredisponeerd voor de ontwikkeling van het eerste, initieel gelokaliseerde type. **Hoofdstuk 7** beschrijft de bevindingen van een studie naar de mogelijke variaties in genetische expressie, welke dit verschil zouden kunnen verklaren. Ook hierbij is, gebruikmakend van een chromosomale microarray analyse, gekeken naar een eventueel verschil in genexpressie tussen de twee varianten. Van de tien genen waarvan het significant grootste verschil in expressie tussen beide typen tumoren kon worden aangetoond, is aansluitend getracht dit verschil in expressie middels qPCR te bevestigen. Dit bleek in drie van de tien genen mogelijk: *C6* kwam verhoogd tot expressie; *CLEC12A* en *CCL5* kwamen verminderd tot expressie wanneer we de viscerale vorm van het histiocytair sarcoom vergeleken met de welke delen variant. Deze verschillen tonen aan dat er inderdaad een variatie in genexpressie in beide vormen aanwezig is, welke in theorie het verschil in verschijningsvorm zou kunnen verklaren.

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Curriculum vitae

The author of this thesis was born on October 19, 1977, in Oldenzaal. She grew up in Hengelo, a city in the east of the Netherlands. She attended primary (de Borgh)- and high school (Twickel College) in Hengelo. She graduated high school in 1996. She started studying Biology at Utrecht University in 1996, but she changed her course, and started studying veterinary medicine at Utrecht University in 1998. She graduated in 2005 with honorable mention and subsequently worked for three years in several private companion animal practices in Enschede (Dierenkliniek Enschede) and Putten (Dierenkliniek Putten). She started her internship at the Department of Companion Animal Sciences of the Faculty of Veterinary Medicine, Utrecht University in 2008. After completion, she got engaged in the PhD project that has led to this thesis. Following, in 2013, she commenced a residency in Small Animal Clinical Oncology. She currently works as a resident in Small Animal Clinical Oncology (ECVIM-CA (Oncology)) at the Department of Clinical Science of Companion Animals, Utrecht University.

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Acknowledgements

En dan ben ik alweer toegekomen aan het laatste (en vaak best gelezen) hoofdstuk van een proefschrift: Het dankwoord. De afgelopen jaren heb ik het genoegen gehad met een aanzienlijke groep mensen, uit binnen- en buitenland, te mogen samenwerken. Dit heeft niet alleen nadrukkelijk bijgedragen tot een aanzienlijke kennistoename mijnerzijds, maar heeft zeker ook bijgedragen aan mijn werkvreugde. Uiteraard ontbreekt het aan ruimte om iedereen die het verdiend op schrift aan te spreken, maar een aantal mensen wil ik er toch heel graag even uitlichten.

Logischerwijs begin ik met mijn promotoren en copromotor, waarbij ik natuurlijk begin met mijn copromotor, dr. Gerard Rutteman. Toen ik in 2009, aan het eind van mijn roulatie, aangaf geïnteresseerd te zijn in het door jou gepresenteerde onderzoek, had ik nooit kunnen vermoeden dat dit uiteindelijk zou resulteren in dit mooie proefschrift. Je grote enthousiasme voor onderzoek was, vanaf het allereerste begin, duidelijk en aanstekelijk. Ik wil je hartelijk te bedanken voor de tijd en energie die je in dit proces hebt willen en kunnen steken. Ik wil je met nadruk bedanken voor het feit dat je me hebt geïntroduceerd in de onderzoekswereld, en sta nog altijd versteld van je enorme schat aan kennis. Nu ook je tweede carrière, als kunstenaar, een vliegende start lijkt door te maken, wens ik jou en Jeanine alle goeds toe voor deze nieuwe fase in jullie leven.
Aansluitend wil ik professor dr. Erik Teske hartelijk bedanken. Vrij recent benoemd tot tweede promotor, maar inmiddels onmisbaar als zodanig. Niet alleen voor de afronding van mijn proefschrift, maar ook voor mijn verdere carrière; die als specialist in opleiding. Ik wil je bedanken voor je nuchterheid en je vermogen om goede, adequate oplossingen aan te dragen voor de (in mijn ogen;)) acute problemen , en daarnaast voor je snelheid van reageren op mijn vraag om hulp.
Professor dr. Jan Rothuizen. Je positieve en nuchtere instelling en je brede wetenschappelijke kennis hebben indruk op me gemaakt. Ik wil je bedanken voor de mogelijkheid die je me hebt geboden om een promotietraject in te gaan; het vertrouwen dat hieruit spreekt heeft zeker ook geleid tot de uiteindelijke succesvolle afronding van dit proefschrift. Bedankt voor de ondersteuning vanuit de Faculteit Diergeneeskunde, die mij in staat stelde dit onderzoek te volbrengen.

Behalve mijn promotieteam hebben er zeker nog een aantal mensen bijgedragen aan de totstandkoming van dit proefschrift.

Beste oud-kamergenoten Frank, Hille, Hedwig en Baukje; ik durf wel zeker te stellen dat er, zonder jullie, geen promotie mogelijk zou zijn geweest. Bedankt voor de lol, gezelligheid en jullie relativeringsvermogen. Hille en Frank, jullie dank ik in het bijzonder voor jullie heldere en verhelderende blik en jullie grote kennis omtrent onderzoek. Ondanks de enorme druk en drukte van jullie eigen werkzaamheden, waren jullie altijd bereid om bij te springen waar nodig. Dit heb ik

zeer gewaardeerd! Ik denk met meer dan plezier terug aan mijn periode in het JDV. Hille, daarnaast ook bedankt voor je vriendschap en ondersteuning na werktijd, ook in deze periode volgend op mijn promotie. Frank en Baukje, ik ben er zeker van dat jullie een geweldige toekomst als wetenschappers ingaan! En Hedwig, ik ga er helemaal vanuit jou over een paar jaar op de Faculteit als collega te treffen!

Dr. Guy Grinwis, ik wil ook jou nog even met nadruk bedanken voor je expertise, nuchtere kijk en toegankelijkheid. Ik heb ontzettend veel gehad aan je mening, en je uitgebreide revisies op mijn manuscripten, waarbij elke correctie door mij als zeer waardevol ervaren werd. Ik ben erg onder de indruk geraakt van je persoonlijkheid en je werk als patholoog.
Dr. Louis Penning, ik wil je bedanken voor de waardevolle input die je hebt geleverd in mijn onderzoek. Voor je snelle en kritische feedback op mijn manuscripten, en voor het uitwisselingen van gedachten en ideeën over het onderzoek.

Maurice Zandvliet, gewaardeerde collega. Afgelopen tijd hebben wij dezelfde afrondende fase van het afronding van ons promotieonderzoek mogen meemaken. De laatste loodjes wegen ook hier het zwaarst, en ik heb het als prettig ervaren om strubbelingen rondom deze fase af en toe te kunnen delen. Ik ben tevens onder de indruk geraakt van je doorzettingsvermogen en enorme schat aan kennis. Ik hoop hier in de toekomst nog veelvuldig gebruik van te kunnen maken.

Members of the assessment committee, thank you for the time you have devoted reading and assessing the thesis manuscript.

Mijn dank gaat zeker ook uit naar alle participerende dierenartsen, hondeneigenaren en honden. Zonder hun hulp en betrokkenheid, zou er van dergelijk onderzoek niets terecht komen.

Alle overige collegae van de Universiteitskliniek voor Gezelschapsdieren. Heel veel mensen, en te weinig plek om ze te benomen. Ik wil jullie ontzettend bedanken voor alle interesse en bijdrage aan mijn proefschrift en kijk uit naar een prettige toekomstige samenwerking!

Maar er is, gelukkig, ook nog een heel andere wereld buiten het promoveren en specialiseren om. Natascha Cuper, vriendin en paranimf, bedankt voor al je steun en relativeringsvermogen. Al vanaf de start van onze studie, meer dat 15 jaar geleden, ben je een echte vriendin voor me geweest, die me inmiddels kent als geen ander. Je weet me er maar al te vaak aan te herinneren waar het in het leven echt om draait. Op naar de volgende episode van GOT!

Susanne, zeer gewaardeerde collega en goede vriendin, wat heb ik genoten van onze na-werkse (soms erg late) wandelingen met de honden. Als geen ander ken je de werkuitdagingen (en spanningen!) die dit beroep met zich meebrengt. Ik wens

jou (en Livvie) een geweldig goede toekomst toe, en ik zal jullie, na jullie vertrek naar Duitsland, ontzettend missen!

Lieve vrienden, altijd geïnteresseerd in mijn drukke werkzaamheden, die tevens in de voor mij broodnodige afleiding voorzagen. Petra, Maartje, Gini, Mette, Lisa; bedankt voor jullie oprechte interesse en goede gesprekken. Bedankt voor het geduld dat jullie konden opbrengen; het contact met jullie is op veel momenten een welkome afleiding voor het harde werken geweest.

Lieve familie; mam en pap, Janine (zus en paranimf), Peter en natuurlijk ook Beau en Xam. Jullie hebben het doorlopen van mijn promotie van een heel andere kant meegemaakt. Mam, pap; ik wil jullie allereerst bedanken voor de mogelijkheden die jullie mij gaven om mijn opleiding te kunnen doorlopen. Verder wil ik jullie allen bedanken en voor jullie onophoudelijke geduld, vertrouwen en morele steun gedurende de voorbijgaande jaren. Ik wil jullie graag danken omdat jullie er steeds voor me zijn en omdat ik altijd op jullie kan rekenen. Ik draag hierbij dit proefschrift dan ook aan mijn familie op.

10