EPIDEMIOLOGICAL, AETIOLOGICAL, AND PROGNOSTIC ASPECTS OF CANINE PRIMARY BONE CANCER, WITH A VIEW TO ITS HUMAN COUNTERPART

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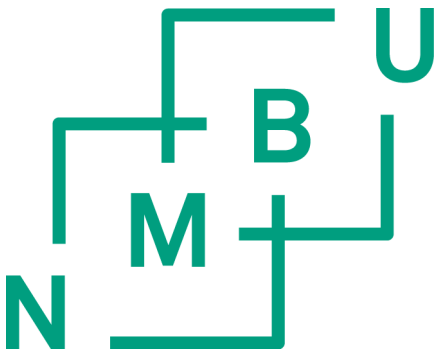
Thesis for the degree of *Philosophiae Doctor* (PhD)



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“We learn more by looking for the answer to a question and not finding it than we do from learning the answer itself.” (Lloyd Alexander)

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SELECTED ABBREVIATIONS

APC	Age-period-cohort
CSA	Chondrosarcoma
DFI	Disease-free interval
DYAR	Dog-years-at-risk
ESA	Ewing sarcoma
HES1	Hairy and enhancer of split 1
IW	Irish wolfhound
LB	Leonberger
LR	Labrador retriever
NF	Newfoundland
OSA	Osteosarcoma
RT-qPCR	Reverse transcriptase quantitative PCR
SEER	Surveillance, Epidemiology, and End Results Program

SUMMARY

Osteosarcoma (OSA) is the most common histological subtype of primary bone cancer in both humans and dogs. The importance of this relatively rare malignancy in humans is underlined by the fact that it typically affects children and adolescents, constituting about 5% of paediatric cancers (<15 years (y) of age). Although a rare disease in the canine population as well, certain breeds of dogs have a lifetime risk approaching 10%, thereby affecting a large proportion of these dogs. Hence, OSA has a major impact on the health within such breeds. The overall incidence rate in canines outnumbers that of the human population, inspiring the use of dogs with naturally occurring OSA as models for its human counterpart. Furthermore, similar clinical and epidemiological features of this disease are seen in the 2 species. Knowledge regarding risk factors for developing OSA is scarce in both dogs and humans. A better understanding of the aetiology and pathogenesis could generate ideas for novel treatment options, and identifying markers for progression of the disease may help optimise individualised therapy.

The main objective of this thesis was to identify risk factors for development of primary bone cancer in dogs and humans, as well as factors involved in progression of the disease. In the first study we describe the incidence rates of primary bone cancer in 4 large and giant canine breeds in Norway, based on questionnaires posted to owners of almost 4000 dogs (Paper I). The highest incidence rate was found in the Irish wolfhound (IW) and Leonberger (LB), with 126 and 72 cases per 10 000 dog years at risk (DYAR), respectively. These breeds had significantly higher incidence rates of primary bone tumours than the Newfoundland (NF) and Labrador retriever (LR) ($p < 0.0001$). Incidence rates for the latter ones were 11 and 2 cases per 10 000 DYAR, respectively. Age at the time of diagnosis (median age 6.7 y) and localisation of the primary tumour, most commonly observed at the distal radius/ulna, were consistent with previous publications.

The same 4 breeds were included in our next study, where we aimed to investigate the impact of body weight (BW) and growth during the first 2 y of life (Paper II). Seven hundred dogs, born in Norway between November 1998 and June 2001, were included in this prospective study at the time of birth. Since the LB was the only breed with more than 2

cases of primary bone cancer reported, further analyses were limited to this breed. Logistic regression showed a statistically significant effect of BW on the odds ratio for developing primary bone cancer at 12 months (m) and 18 m, and of circumference of the distal radius and ulna (CDRU) at 18 m. At these ages, an increase in BW of 1 kg yielded a nearly 20% higher risk of developing primary bone cancer, while 1 cm larger CDRU was associated with a nearly 70% increased risk. These findings support that weight-bearing stress during the period of high proliferative activity in the long bones associated with growth may increase the risk of canine primary bone cancer.

Recent meta-analyses have confirmed body size as a risk factor for human OSA, but known risk factors are otherwise scarce. Aiming to generate new hypotheses for risk factors implicated in the development of this disease, we conducted age-period-cohort analyses on a large human cancer register (the 9 Surveillance, Epidemiology, and End Results Program (SEER) registries) to describe temporal trends in the primary bone cancer subtypes OSA, chondrosarcoma (CSA), and Ewing sarcoma (ESA) (Paper III). For OSA, we found cohort-specific declines in successive generations born 1905-34 for both genders. No temporal trends were detected for ESA in either gender or for CSA in males. In females, however, we observed an increasing incidence rate of CSA during the entire study period (1976-2005), with the earliest and steepest increase among women aged 40-49 y, born 1930-50. This increase in CSA corresponds roughly to the introduction of oestrogens, both as oral contraceptives and hormone replacement therapy, and we speculate that oestrogen may play a role in the progression of CSA.

In the last part of this thesis, molecular studies identified alterations in the Notch signalling pathway in canine OSA tissue compared to normal bone, as well as differences between short- and long-term survival groups (disease-free interval (DFI) < 100 days (d) and DFI > 300 d, respectively) (Paper IV). For this study, we used tissue from the Colorado State University tissue archive, and compared the expression of downstream signalling targets of Notch, including Hairy and enhancer of split 1 (HES1), in dogs undergoing amputation prior to adjuvant chemotherapy. Average *HES1* mRNA expression was elevated about 2.5-fold in canine OSA samples relative to normal bone (same dog) from 9 dogs diagnosed with OSA, while it was elevated more than 4.6-fold in tumours from dogs with DFI > 300 d (n = 10)

compared to the expression in tumours from dogs with DFI < 100 d (n = 10). In tumour tissue from 14 of these dogs, as well as an additional 61 primary canine OSA, HES1 immunoreactivity was evaluated as a prognostic factor; all tumours were scored from 1-9 based on the percentage and intensity of immunohistochemical staining. All tumours expressed HES1, with a median immunoreactivity score of 4 (range 1-9), and the overall median DFI was 168 d (range 43 - > 1393 d). The median DFI in dogs with a high HES1 immunoreactivity score (≥ 4) was 258 d, compared to 155 d in dogs with a low HES1 immunoreactivity score (< 4) ($p = 0.0023$). Univariable analyses identified HES1, bone-specific alkaline phosphatase (BALP) activity, histological grade, percent necrosis and mitotic index as potential predictors of DFI ($p < 0.1$). Upon multivariable analyses, HES1, percent necrosis and mitotic index were statistically significant independent predictors of DFI ($p = 0.029$, 0.002 and 0.005, respectively).

Molecular tumour characteristics may serve as therapeutic targets, and inhibition of Notch signalling in patients with OSA and antioestrogens in CSA patients are 2 classes of drugs that have been considered. Results presented in Paper IV support a potential for the former, and our epidemiological study on human bone tumours (Paper III) generated hypotheses that suggest a role for the latter. Studies investigating intrinsic factors such as body size and growth are less likely to provide clues on therapeutic targets, but breed predispositions and familial clustering (like the ones in our Papers I and II) may help optimise further molecular studies.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Osteosarkom (OSA) er den vanligste histologiske formen for primær benkreft hos både mennesker og hunder. Viktigheten av denne relativt sjeldne kreftformen understrekes av at den hovedsakelig rammer barn og ungdom; den utgjør omtrent 5 % av kreftsykdommene som rammer barn under 15 år. Osteosarkom er sjelden hos hunder også, men rammer opp til 10 % av hunder innen visse raser i løpet av livet. Sykdommen får derfor store konsekvenser for helsen til disse rasene. Alt i alt er OSA vanligere hos hund enn menneske, noe som har ført til bruk av hunder med naturlig forekomst av OSA som modeller for tilsvarende sykdom hos mennesker. Dette komparative aspektet styrkes av kliniske og epidemiologiske likheter ved denne kreftformen hos de to artene. Kunnskap om risikofaktorer for utviklingen av denne sykdommen er mangelfull hos både hund og menneske. En bedre forståelse av årsaksfaktorer for utvikling av OSA kan generere hypoteser som fører til nye behandlingsformer, samt identifisere markører som kan indikere progresjon av sykdommen og tilrettelegge for individuelle behandlingsprotokoller.

Hovedformålet med denne avhandlingen var å identifisere risikofaktorer for utvikling og progresjon av primær benkreft hos hunder og mennesker. I den første studien beskriver vi insidensrater for primær benkreft hos 4 store hunderaser i Norge, basert på spørreskjemaer sendt til eiere av nesten 4000 hunder (Artikkel I). Irsk ulvehund og leonberger hadde de høyeste insidensratene, med henholdsvis 126 og 72 tilfeller per 10 000 *dog years at risk*. Disse rasene hadde signifikant høyere insidensrater enn newfoundland og labrador retriever ($p < 0.0001$). Alderen ved diagnosetidspunktet (median 6,7 år) og lokalisering av primærtumor, hyppigst sett ved distale radius/ulna, tilsvarte det som har blitt publisert tidligere. De samme 4 rasene ble inkludert i vår neste studie, hvor vi hadde som målsetting å identifisere risikofaktorer for utvikling av primær benkreft (Artikkel II). Vi ønsket her å undersøke betydningen av kroppsvekt og vekst i løpet av de første to leveårene. Syv hundre hunder, født i Norge mellom november 1998 og juni 2001, ble inkludert i denne prospektive studien fra fødselen av. Ettersom leonberger var den eneste rasen registrert med mer enn 2 tilfeller i dette materialet, ble videre analyser begrenset til denne rasen. Logistisk regresjon viste en statistisk signifikant effekt av kroppsvekt på odds ratio for utvikling av primær benkreft ved 12 og 18 måneders alder, og for omkretsen rundt distale radius og ulna ved 18

måneder. Ved nevnte aldre, ga 1 kg høyere kroppsvekt 20 % høyere risiko for utvikling av primær benkreft, mens 1 cm større omkrets rundt distale radius og ulna var assosiert med nesten 70 % høyere risiko. Disse resultatene støtter teorien om at vektbærende stress i løpet av perioden med vekst av lange rørknokler kan bidra til å øke risikoen for primær benkreft hos hund.

Nyere metaanalyser har bekreftet kroppsstørrelse som risikofaktor for OSA hos menneske, men det er ellers få kjente risikofaktorer. For å generere nye hypoteser om faktorer som kan ha betydning for utvikling av denne sykdommen, gjennomførte vi en alder-periode-kohort (”*age-period-cohort*”) studie der vi brukte data fra et stort humant kreftregister (*Surveillance, Epidemiology, and End Results Program; SEER*) for å beskrive trender i forekomsten av hovedformene for primær benkreft: OSA, kondrosarkom, og Ewings sarkom. For OSA fant vi en fødselskohort-spesifikk reduksjon i forekomsten for generasjoner født 1905-34 for begge kjønn. Ingen endringer ble identifisert for Ewings sarkom blant kvinner eller menn, eller for kondrosarkom hos menn. Blant kvinner, fant vi imidlertid en økning i forekomsten av kondrosarkom gjennom hele studieperioden (1976-2005), med den tidligste og bratteste økningen blant kvinner i alderen 40-49 år, født 1930-50. Denne økningen sammenfaller med introduksjonen av tilført østrogen, både som per orale prevensjonsmidler og hormontilskudd etter menopausen, og vår hypotese er derfor at østrogen kan spille en rolle for utviklingen av kondrosarkom.

I den siste delen av denne avhandlingen (artikkel IV) gjennomførte vi molekylære studier som identifiserte endringer i Notch-styrte gener i OSA-vev sammenliknet med vanlig knokkel hos hund, samt forskjeller mellom pasientgrupper med kort (sykdomsfritt intervall < 100 dager) versus lang (sykdomsfritt intervall > 300 dager) overlevelse. Vi brukte vev fra arkivet til Colorado State University i denne studien, og undersøkte ekspresjonen av nedstrøms målgener for Notch, inkludert HES1 *(Hairy and enhancer of split 1)*, ved bruk av immunhistokjemi (IHK) hos hunder som gjennomgikk amputasjon etterfulgt av kjemoterapi. Gjennomsnittlig *HES1* mRNA ekspresjon var økt over 2,5 ganger i OSA-vev sammenliknet med vanlig benvev (samme hund) hos 9 hunder diagnostisert med OSA, og var økt over 4,5 ganger hos de med lenger overlevelse (n = 10) sammenliknet med de med kortere overlevelse (n = 10). I tumorvev fra 14 av disse hundene, samt fra 61 andre hunder med

OSA, ble proteinekspresjonen av HES1 (angitt ved en indeks for prosentandel IHK-farging og instensitet, gradert 1-9) evaluert som prognostisk faktor. Alle tumorer uttrykte HES1, med en median indeks på 4 (1-9), og median sykdomsfritt intervall var 168 dager (43 - > 1393 dager). Median sykdomsfritt intervall hos hunder med høy HES1 indeks (≥ 4) var 258 dager, sammenliknet med 155 dager hos hunder med lav HES1 indeks (< 4) (p = 0,0023). Univariable analyser identifiserte HES1, benspesifikk alkalisk fosfatase-aktivitet, histologisk grad, prosentandel nekrose, og mitotisk indeks som mulige prediktorer for varigheten av sykdomsfritt intervall (p < 0,1). I den multivariable analysen, var HES1, prosent nekrose, og mitotisk indeks statistisk signifikante uavhengige prediktorer for sykdomsfritt intervall (p-verdi henholdsvis 0,029, 0,002 og 0,005).

Kreftbehandling kan rettes mot spesifikke molekylærgenetiske forhold ved ulike tumorer, og i denne sammenheng er inhibering av Notch signalveien hos pasienter med OSA og antiøstrogener hos pasienter med kondrosarkom to medikamentgrupper som har blitt vurdert. Resultatene fra Artikkel IV støtter opp under en mulig nytte av manipulering av Notch-styrte gener for disse pasientene, og vår epidemiologiske studie av human primær benkreft (Artikkel III) genererte hypoteser som impliserer at antiøstrogener kan ha en rolle i behandlingen av kondrosarkomer. Det er mindre trolig at studier som identifiserer mulige indre risikofaktorer, slik som kroppsstørrelse og vekst, vil bidra til nye idéer om behandlingsalternativer, men rasedisposisjoner og familiær forekomst (som i våre artikler I og II) kan være med på å forbedre videre molekylærgenetiske studier.

LIST OF PAPERS

Paper I

Anfinsen KP, Grotmol T, Bruland OS, Jonasdottir TJ. Breed-specific incidence rates of canine primary bone tumors - a population based survey of dogs in Norway. *Can J Vet Res.* 2011 Jul;75(3):209-15.

Paper II

Anfinsen KP, Grotmol T, Bruland OS, Trangerud C, Jonasdottir TJ. Primary bone cancer in Leonbergers may be associated with a higher bodyweight during adolescence. *Prev Vet Med*. 2015 Apr;119(1–2):48-53.

Paper III

Anfinsen KP, Devesa SS, Bray F, Troisi R, Jonasdottir TJ, Bruland OS, Grotmol T. Age-period-cohort analysis of primary bone cancer incidence rates in the United States (1976-2005). *Cancer Epidemiol Biomarkers Prev*. 2011 Aug;20(8):1770-7.

Paper IV

Dailey DD, **Anfinsen KP**, Pfaff LE, Ehrhart E, Charles JB, Bonsdorff TB, Thamm DH, Powers BE, Jonasdottir TJ, Duval DL. HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors. *BMC Vet Res*. 2013 Jul 1;9(1):130.

INTRODUCTION

Osteosarcoma (OSA) is by far the most common canine primary bone cancer, and its incidence rate greatly outnumbers that in humans (Withrow et al., 1991). Although generally a rare disease also in the canine population, certain breeds of dogs have a lifetime risk approaching 10%, thereby affecting a large proportion of these dogs (Egenvall et al., 2007). Chondrosarcoma (CSA) constitutes less than 10% of the primary bone cancers in this species (Brodey et al., 1959; Brodey et al., 1963; Farese et al., 2009), and no canine equivalent of Ewing sarcoma (ESA) has been documented (Vanel et al., 2013).

Canine osteosarcoma

Canine OSA most commonly affects large-breed dogs, with more than one third diagnosed in dogs with an adult bodyweight (BW) above 40 kg (Ehrhart et al., 2013). An up to 185-fold increased risk exists for those weighing more than 36 kg compared to those with a BW of less than 9 kg (Tjalma, 1966). Most studies show a male predilection of approximately 1.5:1.0 (Brodey et al., 1963; Brodey and Abt, 1976; Misdorp, 1980), but the results are inconsistent, with some showing a similar (Ehrhart et al., 2013) or even higher female than male incidence rate (Brodey and Riser, 1969; Heyman et al., 1992). Neutering appears to increase the risk of OSA, and lifetime gonadal hormone exposure has been shown to negatively correlate with the risk of developing OSA (Ru et al., 1998; Cooley et al., 2002). A small peak has been reported at the age of 18-24 m, although the disease is most commonly diagnosed in middle-aged to older dogs (Brodey and Riser, 1969; Misdorp and Hart, 1979). Median age at the time of diagnosis is 7 y (Ehrhart et al., 2013).

Clinical signs

Dogs with OSA usually present with swelling and pain from the primary tumour (Morello et al., 2011; Ehrhart et al., 2013). Appendicular tumours may cause pathological fractures resulting in non-weight-bearing lameness, but mild to moderate pain is more common. At diagnosis, soft-tissue swelling usually surrounds the skeletal tumour (Ehrhart et al., 2013; Vanel et al., 2013). Osteosarcoma is typically found in the metaphysis of long bones; the distal radius or ulna is most commonly affected, with other sites in decreasing frequency being the proximal humerus, distal femur, and proximal and distal tibia (Morello et al.,

2011). Only about 5-10% of dogs weighing more than 40 kg present with axial tumours, such as those of the scull, ribs, scapula, or pelvis (Heyman et al., 1992; Ehrhart et al., 2013). Axial tumours are relatively more prevalent in smaller breeds, accounting for more than half of the OSA seen in dogs with a BW of less than 15 kg (Ehrhart et al., 2013). Tumours affecting the vertebral bodies may cause pain and neurological signs – depending on the location (Moore et al., 2000). Respiratory signs are uncommon at the time of presentation, even in the presence of rib tumours or pulmonary metastases; the latter may however cause hypertrophic osteopathy (Ehrhart et al., 2013). Bone lysis may cause hypercalcaemia resulting in polyuria and polydipsia, but this is extremely rare for any of the primary bone tumours (Messinger et al., 2009). Cancerous cachexia may be seen with advanced disease, and increased energy consumption and decreased protein synthesis have been documented already before this ensues (Mazzaferro et al., 2001).

Biological behaviour

Most canine OSA originate from the medullary bone (intraosseous OSA), and are usually of high malignancy-grade (Ehrhart et al., 2013). Bone surface OSA is rare, and includes periosteal and parosteal (juxtacortical) forms. The former typically behaves as aggressively as the intraosseous ones, whereas the latter has a slower progression with reduced risk of metastasis and a better prognosis (Ehrhart et al., 2013). The more differentiated histology makes these tumours potentially similar to osteomas, chondromas, or reactive bone – underlining the importance of adequate biopsies. In humans, bone surface OSA is classified as parosteal (low-grade), periosteal (intermediate-grade), or high-grade; the latter having a similar prognosis as conventional (intraosseous) OSA (Lazar and Mertens, 2013; Montag and Squire, 2013; Wold et al., 2013).

High-grade canine OSA has an aggressive biological behaviour (Ehrhart et al., 2013). Although less than 15% have visible metastases (judged by 3-view thoracic radiographs) at the time of diagnosis, more than 90% have distant metastases at the time of death. Hence, haematogenic spread as micrometastases to the lungs at the time of diagnosis is common. Various other metastatic locations have been reported, including kidneys, liver, and other bones (Kent et al., 2004; Sacornrattana et al., 2013; Aguado et al., 2014). Lymphatic metastases have been found in up to 25% of patients (Kirpensteijn et al., 2002), and so-

called skip metastasis to regional skeletal sites may occur – carrying a grave prognosis (Malawer and Dunham, 1983).

Histological subclassification into osteoblastic, fibroblastic, chondroblastic, and teleangectatic forms has not been consistently prognostically useful (Loukopoulos and Robinson, 2007; Ehrhart et al., 2013). Moreover, several subtypes may be present in the same tumour (Kruse et al., 2013). Histological grading has however provided prognostic information in several human and canine cancers, including OSA (Loukopoulos and Robinson, 2007). Kirpensteijn and colleagues proposed a grading system constructed from modifications of previously reported human and canine schemes (Kirpensteijn et al., 2002). Applying this system to 166 dogs with primary OSA, the histological grade was found to be associated with prognosis: dogs with grade III disease had a significantly shorter disease-free interval (DFI) than those with grade I or II OSA. The histological grade was determined by the degree of pleomorphism, number of mitoses, amount of tumour matrix, tumour cell density, and proportion of necrosis; tumours where invasion of the vessels was detected histologically were classified as grade III regardless of other features. A recent study did however not find any association between survival and histological grade in a study of 46 dogs with extracranial OSA of the axial skeleton (Kruse et al., 2013).

Diagnosis: imaging

Although no radiographic changes are pathognomonic for OSA, observation of typical features can support a tentative diagnosis. Canine OSA may cause extensive bone sclerosis with an extraskeletal extension, often in combination with areas of osteolysis (Vanel et al., 2013). Cortical bone lysis is frequently found, and may result in pathological fractures (Vanel et al., 2013). Extension of the tumour through the cortex lifts the overlying periosteum, and new bone is formed between the elevated periosteum and the bone. The resulting triangular shape of dense new bone on top of the cortex is named “Codman’s triangle”, and is commonly seen in OSA. Another typical feature of OSA is the so-called “sunburst”, created by new bone formed in areas of soft tissue swelling perpendicular to the rim of the cortex. It is uncommon for OSA to cross a synovial space, although expansive growth into adjacent bone may occur (Ehrhart et al., 2013).

More advanced imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and scintigraphy are increasingly used in veterinary medicine, and each modality has its advantages in the evaluation of canine OSA patients (Davis et al., 2002). All of these imaging techniques may overestimate the local extent of the disease (Leibman et al., 2001; Davis et al., 2002; Wallack et al., 2002), whereas metastatic disease is usually present histologically without being visible on imaging ("micrometastases"). Conventional CT is more sensitive than radiographs for detection of pulmonary metastases (Nemanic et al., 2006), and has become readily available for veterinary use in most parts of the developed world. Use of even more sensitive modalities, such as positron-emission tomography (PET) or single photon emission CT (SPECT) is however limited to human medicine and some veterinary specialist hospitals (Kundu, 2014; LeBlanc and Peremans, 2014).

Computed tomography is useful to assess the extent of axial tumours, allowing 3-dimensional evaluation. Magnetic resonance imaging also yields 3-dimensional views, while providing information about the surrounding soft tissue structures that may be of particular benefit in patients with spinal involvement. Evaluation of Gadolinium-DTPA contrast enhancement may further help assess the tumour extension (Kippenes et al., 1999), and this modality has been shown to most accurately estimate the boundaries of intramedullary OSA (Wallack et al., 2002; Shiga et al., 2013), although one study found MRI to be the least accurate modality (Davis et al., 2002). Scintigraphy, usually with radiolabelled technetium-99 methylene-diphosponate (^{99m}Tc-MDP), is mainly used to screen for skeletal metastases, as this modality is highly sensitive for increased bone remodelling (Parchman et al., 1989).

Diagnosis: histopathology

Clinical features such as signalment and tumour location, in combination with the results of diagnostic imaging, may be strongly suggestive of OSA. Histopathology is however necessary to reach a final diagnosis. Differential diagnoses include other primary bone tumours; benign, such as bone cysts or osteomas, or malignant, such as fibrosarcomas or haemangiosarcomas – as well as osteomyelitis, especially fungal. A needle core biopsy, using for instance a Jamshidi bone marrow biopsy needle, should be obtained from firm

structures, whereas softer ones may be amendable to an incisional wedge biopsy (Endicott, 2003).

Cytology has been found sufficient for a definitive diagnosis in a proportion of patients: in half of 22 dogs with OSA, metastatic carcinoma or bone cysts, no further biopsies were considered necessary following cytological evaluation of these lesions (Samii et al., 1999). In 22 of 35 dogs with OSA from which fine needle aspirates of the primary tumour were obtained, presence of a mesenchymal malignant tumour was determined based on the cytological evaluation (Kirpensteijn et al., 2002). Features of malignancy that occur more frequently in cytology samples obtained from OSA than from proliferative normal bone include more frequent mitosis, aberrant mitosis, and other criteria of malignancy (such as angular nucleoli, anisokaryosis, macronucleolisation, and nuclear moulding) (Reinhardt et al., 2005). Anisokaryosis and anisocytosis were however present with similar frequency in bone healing from a fracture as in samples obtained from OSA in the aforementioned study. Special stains may further increase the utility of cytology in the diagnosis of OSA. Bone is the only mesenchymal tissue found to express alkaline phosphatase (ALP) in dogs, and staining for phosphatase activity has helped differentiate OSA from other mesenchymal neoplasms, although it is not useful to distinguish neoplastic lesions from reactive bone (Barger et al., 2005).

Depending on the location of the lesion, imaging guidance may be beneficial, using either ultrasound, fluoroscopy, or CT. Regardless of the type of biopsy obtained, care should be taken to ensure that the entire path of the biopsy instrument can be included in the event of subsequent tumour resection to avoid seeding of tumour cells (Endicott, 2003). The biopsy instrument should be aimed at the centre of the lesion, as peripheral biopsies may lead to a misdiagnosis of reactive bone (Powers et al., 1988; Ehrhart et al., 2013).

By definition, OSA consists of malignant mesenchymal cells producing osteoid matrix (Mueller et al., 2007). Presence of osteoid is however not pathognomonic for OSA (Gorra et al., 2002). According to the WHO histological classification (Rosenberg et al., 2013), there are 3 major subtypes of human OSA, and in decreasing prevalence for both humans and dogs, these are: osteoblastic, fibroblastic, and chondroblastic – indicating the most

abundant type of matrix (Misdorp and Hart, 1979). Some tumours contain a mixture of different matrix (Kirpensteijn et al., 2002). Giant-cell-rich OSA is described in the human WHO classification (Rosenberg et al., 2013), and presence of multinucleated giant cells is included in the proposed grading schemes for both canine, feline, and human OSA (Meister et al., 1979; Kirpensteijn et al., 2002; Dimopoulou et al., 2008). Two other rare entities are the telangiectatic and small-cell forms of OSA. The former consists of cyst-like blood-filled spaces, and may be mistaken for an aneurysmal bone cyst (Oliveira et al., 2013), whereas the latter is characterised by small, round, malignant cells in osteoid matrix and may resemble ESA in humans (Kalil and Squire, 2013). This subtype is more chemoresistant than other types of OSA, but is only sporadically seen in dogs (Frazier et al., 1991).

Staging

Surgical staging of canine OSA patients is based on the staging system for human skeletal sarcomas (Enneking et al., 1980; Ehrhart et al., 2013; Rosenberg et al., 2013). According to this system, there are 3 stages for OSA, based on the histological grade (G), the anatomy of the primary tumour (T), and regional and distant metastases (M). Stage I includes dogs with low-grade (G1) tumours without visible metastases, and stage II includes dogs with high-grade tumours without visible metastases. Any dog with metastatic disease is classified as having stage III disease, regardless of the primary tumour. A subclassification designated A and B is used to denote intracompartmental (confined within the cortex of the bone; T1) or extracompartmental (extending beyond the bone cortex; T2) tumours, respectively. Most dogs present with stage IIB disease (Ehrhart et al., 2013). The definition of visible metastatic disease is so far based on thoracic radiographs, although more advanced and sensitive imaging modalities are increasingly used (Selmic et al., 2014).

Treatment

As for human OSA, the conventional treatment of canines includes radical surgical resection of the primary tumour followed by adjuvant chemotherapy (Ehrhart et al., 2013). When the primary tumour is located in the appendicular skeleton, amputation is usually the treatment of choice for dogs (Kent et al 2004). Limb-sparing techniques, which are extensively used in the human setting, are performed in an increasing proportion of canine patients – especially at referral hospitals (Kent et al., 2004; Ehrhart et al., 2013). Intraoperative radiation

protocols, including stereotactic radiosurgery and extracorporal delivery of a single mega-dose of radiation are considered promising alternative limb-sparing techniques (Farese et al., 2004; Liptak et al., 2004; Boston et al., 2007). The median survival time of approximately 4 m following surgical resection or amputation alone increases to 10-12 m when adjuvant chemotherapy is used (Bacon et al., 2008). The choice of chemotherapy protocol does not appear to have a major impact on survival, provided that doxorubicin and/or a platinum drug are included (Bacon et al., 2008; Selmic et al., 2014), but the results of one study suggested a longer survival with a single-agent carboplatin protocol than when alternating this drug with doxorubicin (Skorupski et al., 2013). Less conventional treatment options for canine OSA include alternative ways of administering traditional chemotherapy, such as liposome-encapsulated doxorubicin (Vail et al., 1997) or gemcitabine delivered by aerosols (Rodriguez et al., 2010), as well as newer classes of drugs such as the tyrosine kinase inhibitors (TKI)(London et al., 2015), and various immunomodulatory drugs (Wycislo and Fan, 2015); TKI have however not shown great promise in neither dogs nor humans (Fleuren et al., 2014; London et al., 2015). Examples of other immunotherapies include intravenous liposome-encapsulated muramyl tripeptide-phosphatidylethanolamine (L-MTP-PE) (MacEwen et al., 1989) and interleukin-2 (IL-2) in a liposome-encapsulated aerosol formulation administered as inhalation therapy for pulmonary metastases (Khanna et al., 1997; Dow et al., 2005). Palliative treatment options, which may also have some effect on progression of the disease, include radiation therapy (Mayer and Grier, 2006; Coomer et al., 2009) and aminobisphosphonates (Tomlin et al., 2000; Fan et al., 2009; Oblak et al., 2012)

Prognosis

Prior to the introduction of adjuvant therapy, the prognosis for dogs with OSA was poor, with a median survival of less than 5 m in one study of 162 dogs with appendicular OSA treated by amputation alone (Spodnick et al., 1992), similar to the outcome in 65 dogs with OSA reported almost 20 y earlier (Brodey and Abt, 1976). Most dogs in both studies died from metastatic disease, and slowing this inevitable development through the use of adjuvant chemotherapy has improved the overall outcome, so that the current median survival time is approaching 1 y for dogs with appendicular OSA (Selmic et al., 2014). Survival times vary somewhat between studies and the different chemotherapy protocols used, but as mentioned no protocol has been found convincingly superior to others, as long

as it includes doxorubicin and/or a platinum-based drug. Dogs with axial OSA seem to have shorter survival times, apart from those with mandibular tumours, experiencing similar to slightly better outcomes compared to the appendicular ones (Coyle et al., 2013). Extraskeletal OSA generally has a poor prognosis, with reported survival times of 1 to 6 months (Patnaik, 1990). Dogs with detectable metastatic disease at the time of diagnosis also have short survival times, although one study of 90 dogs with stage III OSA suggested that those with bone metastases (not skip metastases) may experience longer survival (median survival time 4-5 m) than those with pulmonary (median survival time 2 m) or other soft tissue metastases (median survival time < 1 m) (Boston et al., 2006)

Other malignant canine primary bone tumours

Chondrosarcoma

Chondrosarcoma is the second most common primary bone tumour both in humans and dogs, accounting for approximately 20% (Hogendoorn et al., 2013) and 5-10% (Brodey et al., 1959; Brodey et al., 1963; Ehrhart et al., 2013) of primary bone tumours, respectively. Some of these tumours develop from a benign lesion (multiple cartilaginous exostosis), but most occur spontaneously (Ehrhart et al., 2013). They consist of malignant mesenchymal cells producing a cartilaginous matrix, frequently seen as radiolucent centres surrounded by round calcifications - giving these lesions a “popcorn”-like appearance on radiographs and CT images (Vanel et al., 2013).

Chondrosarcomas typically occur in large-breed dogs, with a possible predilection in the Golden retriever, at a median age of around 8 y, but with a wide age range (1 to 15 y) (Ehrhart et al., 2013). These tumours are locally invasive with a moderate metastatic potential, reported rates ranging from 18 to 60% (Farese et al., 2009). Metastatic disease does however not seem to be a feature of nasal CSA (Sones et al., 2013); the most common location in canines. More than half of CSA occur in flat bones (Ehrhart et al., 2013), and other sites include other axial bones, the appendicular skeleton, and extraskeletal organs such as the mammary gland, but also the liver (Chikata et al., 2006) and spleen (Weinstein et al., 1989; Miller et al., 2005). Survival rates may vary extensively for tumours of similar locations, reported median survival for appendicular CSA ranging from less than 6 m to more than 7 y for dogs treated with amputation alone (Farese et al., 2009). The most

commonly reported appendicular site for CSA is the proximal tibia, but also distal tibia, femur, and humerus are more frequently affected than the distal radius, which is contrary to that of canine OSA (Farese et al., 2009). Tumour grade may predict the metastatic potential and has inconsistently been associated with prognosis (Waltman et al 2007; Farese et al 2009). As such, it is possible that chemotherapy would be beneficial for the higher tumour grades, but the potential role for adjuvant therapy is so far uncertain and not routinely recommended – although individual responses to radiation therapy have been observed (Ehrhart et al., 2013). An exception is nasal CSA, for which radiation therapy is the mainstay therapy, as for other nasal neoplasms (Sones et al., 2013).

Multilobular osteochondrosarcoma

This locally invasive tumour with low to moderate metastatic potential, mainly to the lungs, has also been known as chondroma rodens, cartilage analogue of fibromatosis, calcifying aponeurotic fibroma, juvenile aponeurotic fibroma, multilobular osteoma, multilobular chondroma, multilobular tumour of bone, and multilobular OSA (Dernell et al., 1998; Gallegos et al., 2008). Most commonly affecting the flat bones of the skull, other locations include the pelvis, hard palate, and os penis (Dernell et al., 1998; Banks and Straw, 2004; Webb et al., 2009). Older medium- to large-breed dogs are most commonly affected, but these tumours are also observed in young small-breed dogs (Dernell et al., 1998; Pakhrin et al., 2006). Radiographically, multilobular osteochondrosarcoma (MLO) has been described as demarcated areas of lytic bone giving a resemblance of “popcorn”(Chun, 2005), as also described for CSA, although the changes seen with MLO may be more subtle or even absent (Stoll et al., 2001). Lower tumour grade and complete surgical resection are associated with a longer survival. Median survival time was more than twice as long for dogs with grade I tumours (> 897 d; n = 13) than those with grade III (405 d; n = 9), and dogs with complete surgical margins (n = 19) had longer median time to local recurrence (> 1332 d) than dogs with incomplete margins (320 d; n = 13) in one report (Dernell et al., 1998). The longer survival for mandibular location (median survival 1487 d versus 528 d in this study) may reflect the higher chance of achieving complete resection in this area. Adjuvant chemo- and radiotherapy have been used, but these modalities’ importance in the treatment of these tumours is uncertain, and surgery aiming to achieve complete margins is the mainstay of therapy (Dernell et al., 1998).

Haemangiosarcoma

Haemangiosarcoma (HSA) accounts for only 2-3% of canine primary bone tumours, and is usually seen in older medium- to large-breed dogs, although reported cases include young adult dogs as well as toy breeds (Erdem and Pead, 2000; Hidaka et al., 2006). Flat bones may be slightly more commonly affected than long bones (Bingel et al., 1974), with the most frequently reported locations being the ribs, proximal humerus, femur, and vertebra (Hidaka et al., 2006). Radiographically, lysis of bone tends to predominate, and metastatic disease is common (Vanel et al., 2013). The histopathology is characterised by highly anaplastic mesenchymal cells frequently forming vascular channels or sinuses, and must be differentiated from telangiectatic OSA; the latter produces osteoid, which is not a feature of bone HSA. Metastases to for instance lungs, abdominal organs, skeletal muscle, or brain, occur in more than 90% of the dogs within 6 m of diagnosis. Prognosis is poor, with less than 10% one-year survival after complete excision (Ehrhart et al., 2013). Despite one old report of a dog with bone HSE surviving for 13 m following amputation and chemotherapy (Crow, 1977), the potential for adjuvant chemotherapy to prolong survival is uncertain for these patients.

Fibrosarcoma

Fibrosarcomas (FSA) probably account for a similar proportion of canine primary bone tumours as HSA (Dorfman et al., 1977), but the potential for misdiagnosing fibroblastic OSA as FSA makes it difficult to estimate the prevalence (Ehrhart et al., 2013). Fibrosarcomas of bone characteristically contain clusters of fibroblasts in a matrix of collagen (which may disrupt cortical bone) and although these tumours do not produce osteoid, new bone may be present (especially at the tumour periphery) making the distinction from fibroblastic OSA challenging on histopathology. Large-breed middle-aged to older dogs are most commonly affected, and reported skeletal locations include the metaphysis of long bones, ribs and vertebra (Vanel, 2013). Fibrosarcomas may also be observed in the oral or nasal cavities, and dogs with low-grade tumours in these locations may experience survival times of 2-3 y following surgical resection and/or radiation therapy (shorter for those of the palate) (Sones et al., 2013; Gardner et al., 2015). The metastatic pattern for FSA may differ from that typical for OSA, spreading to the heart, pericardium, skin, and bones rather than the lungs (Ehrhart et al., 2013)

Metastatic tumours

Metastases to bone are typically seen in cancers affecting the urogenital organs, such as mammary, prostatic, and bladder tumours, but all kinds of tumours may spread haematogenously to bone (Brodey et al., 1966; Ehrhart et al., 2013). Metastatic disease is usually found in the diaphysis, and the most commonly affected bones include the humerus, pelvis, ribs, and vertebra.

Canine benign bone tumours

Osteomas

Canine benign bone tumours are rare, and include osteomas, multiple cartilaginous exostosis, and bone cysts (Ehrhart et al., 2013). Osteomas are radiographically well circumscribed, and generally not painful. These lesions are similar to reactive bone on histopathology, and may be difficult to distinguish from MLO when they originate from the flat bones of the skull. Surgical excision should be curative for these tumours. Only sporadic reports of these tumours existed until Jaffe published a case series of 5 human patients with osteoid osteomas in 1935 (Jaffe, 1953). According to the WHO definition, tumours with a diameter of 1 cm or larger are classified as osteoid osteomas (Horvai and Klein, 2013), and once they reach 2 cm, they are considered osteoblastomas (de Andrea et al., 2013). The latter are progressively growing lesions that may be painful, whereas osteomas rarely continue to grow despite being present for a long time. A special syndrome of colorectal polyps progressing to malignancy, associated with multiple jaw osteomas and dental abnormalities, is seen in humans (Panjwani et al., 2011). The equivalent of this so-called Gardner's syndrome has not been reported in dogs, but infrequent observations of osteomas have been made – including one case series of oral osteomas (Volker and Luskin, 2014). Canine case reports also include one affecting the proximal humerus (Gorra et al., 2002), while femur and tibia are the most common locations in humans (Yalcinkaya et al., 2014). Extraskeletal osteomas, such as lingual (Lekas et al., 1997; Fernandez et al., 2012) and orbital (Benatiya Andaloussi et al., 2006; Grozdanic et al., 2013) have been reported in both species.

Multiple cartilaginous exostoses

Multiple cartilaginous exostoses (MCE) may be an incidental finding or cause variable degrees of pain, assumed to be associated with trauma to surrounding soft tissue (Ehrhart et al., 2013). Such trauma was also hypothesised to have caused dystrophic calcinosis circumscripta in one case report of the calcifications occurring in close proximity to MCE in a young female St. Bernard (Engel et al., 2014). Describing MCE of the cervical vertebral bodies, this case report also exemplifies the potential for neurological signs due to spinal cord compression caused by this condition. Occurring most commonly in young large-breed dogs, MCE is considered to be a developmental abnormality, which may be caused by abnormal maturation of the perichondrial ring around the physis of bones (Engel et al., 2014). Radiographic and histopathological appearances are organised and benign, the latter resembling that of the normal stages of endochondral ossification, although malignant transformation may occur (Doige, 1987; Ehrhart et al., 2013). The lesions are found in bones undergoing endochondral ossification, and may continue growing until skeletal maturity is reached. Heritability is suspected in dogs and confirmed to be autosomal dominant in humans, where a similar condition is caused by a mutation in a family of tumour suppressor genes called exostotin (Sonne-Holm et al., 2014). In humans, MCE is defined as development of several benign osteochondromas. The median age at the time of diagnosis is 3 y, and as in dogs, the lesions grow until closure of the growth plate (Sonne-Holm et al., 2014). Malignant transformation is rarely a concern until the age of 30 y, after which the risk of progression to CSA increases (Sonne-Holm et al., 2014).

Bone cysts

Cysts are also generally found in young animals, and certain breeds may be predisposed – such as the Doberman pincher and Old English sheepdog (Ehrhart et al., 2013). These lesions typically cause mild to moderate pain, unless a pathological fracture occurs. The cause of the only true primary bone cysts, “simple bone cysts” (SBC) or “unicameral bone cysts”, is not known. One theory, however, explains the development of these epithelium-lined fluid-filled sacs by retention of synovial tissue within the bone tissue, which subsequently goes on to produce synovial fluid (Ehrhart et al., 2013). The cysts are usually found in the metaphysis, but may also occur in the diaphysis or epiphysis; sometimes just beneath the articular cartilage and communicating with the synovial membrane (Ehrhart et al., 2013).

Histopathology is necessary to differentiate these lesions from osteolytic OSA. Treatment is curative, and consists of surgical curettage and packing the space with autogenous bone graft.

Blood-filled bone "cysts" called "aneurysmal bone cysts" (ABCs) may represent an arteriovenous malformation, possibly caused by trauma or benign neoplasia disrupting the vasculature (Ehrhart et al., 2013). A similar treatment as for SBC may be effective, although *en bloc* resection is sometimes necessary.

Primary bone cancer in humans

Osteosarcoma, ESA, and CSA are the 3 major histological types of primary bone cancer in humans (Miller et al., 2006). These tumours represent less than 1% of all cancers diagnosed in the United States (Grimer et al., 2013), and affect only around 3.3 per million people in Norway (yearly average for both genders), based on numbers from an unselected, nationwide, population based study of bone OSA diagnosed from 1975 to 2009 (Berner et al., 2014). The importance of OSA is however underlined by its contribution to paediatric cancers, constituting about 5% of these (Parkin et al., 1993; Heare et al., 2009), and the mortality rates of 40 to 85% for patients without and with visible metastasis, respectively (Bruland et al., 2009a; Berner et al., 2014). Ewing sarcoma is rarely seen after the age of 40, but is an important cause of cancer-related deaths in children and adolescents (Damron et al., 2007; Potratz et al., 2012). Approximately 2% of tumours in children and young adults are caused by ESA (Heare et al., 2009), which along with peripheral neuroectodermal tumours (pNET) belong to a histologically similar group of tumours known as "small blue round cell tumours" of childhood and adolescence (Potratz et al., 2012). The 5-year disease-free survival rate for ESA is between 25 and 70% for patients with and without metastatic disease at the time of presentation (Heare et al., 2009). The incidence rate of CSA increases with age, with almost two thirds of these patients being diagnosed after 40 y of age (Damron et al., 2007). Survival rates vary for different subtypes of CSA, ranging from around 50 to almost 100% (Giuffrida et al., 2009).

KNOWLEDGE GAPS WHEN THE PROJECT WAS INITIATED

- To our knowledge, there were no previously published population-based studies describing breed specific incidence rates of canine primary bone tumours. Such information would be essential to further study the potential influence of breed specific characteristics, such as growth rate or body size.
- Although it has long been known that large- and giant-breed dogs are at increased risk of developing OSA, few studies had previously investigated differences in BW within breeds, and results of these had been inconsistent. It was therefore uncertain whether body size was a risk factor within breeds with a relatively high incidence rate of OSA, such as the Irish wolfhound or Leonberger.
- A limited number of risk factors for primary bone cancers in humans had so far been identified.
- Similar molecular changes had been identified in canine as in human OSA, but not all changes identified in the latter had been studied in canine tumours; including those of the Notch signalling pathway.
- Relatively few prognostic factors for canine OSA were known.

OBJECTIVES

The overall objective was to identify and generate hypotheses regarding risk factors for development of primary bone cancer in dogs with potential relevance to the situation in humans. We also aimed to expand the knowledge on factors that may influence progression of the disease and thereby the prognosis.

Paper I

The main objective of this retrospective population-based study was to estimate incidence rates of primary bone cancer in 4 breeds of dogs (Irish wolfhound (IW), Leonberger (LB), Newfoundland (NF), and Labrador retriever (LR)) in Norway, and to identify eventual differences in incidence rates between the breeds. Furthermore, we wanted to characterise the disease in these breeds with respect to age at diagnosis and localisation of the primary tumour.

Paper II

The objective of this study was to identify risk factors for development of primary bone cancer related to growth or body size in the same 4 breeds as in Paper II. Due to a low number of primary bone cancer cases in the 3 other breeds, only results for the LB were analysed. Specifically, we wanted to use prospectively collected data on birth weight and growth parameters during the first years of life; hypothesising that LB developing primary bone cancer would be heavier and larger (measured by BW and the circumference of the distal radius and ulna; CDRU) than the ones that did not have this condition.

Paper III

In this study, the aims were to use age-period-cohort (APC) models to describe the temporal trends of each of the main primary bone cancer subtypes in humans, and to generate aetiological hypotheses based on the observed birth cohort-related changes. To exploit the advantage of more complete data available in human medicine, we based this study on a database covering

approximately one tenth of the US population; the longstanding 9th register of the Surveillance, Epidemiology, and End Results (SEER) program. Primary bone cancer diagnoses made between 1976 and 2005 were included in this study.

Paper IV

The main objective of this study was to examine factors related to prognosis and progression of canine OSA. Specifically, we wanted to compare the expression of the Notch signalling target HES1, a transcriptional regulator, in OSA tissue versus normal bone; hypothesising that we would find a difference in expression between these 2 types of tissue, and thus showing involvement of this gene in OSA progression. A secondary aim was to investigate HES1 as a prognostic factor in dogs with OSA by determining the level of expression in patients with known outcome.

MATERIALS AND METHODS

A summary of the methods used is provided here. The individual papers contain a more detailed and complete description.

Paper I

Breed-specific incidence rates of canine primary bone tumors - a population based survey of dogs in Norway.

Questionnaires posted to owners of almost 4000 dogs of the IW, LB, NF, and LR breeds constituted the basis for this retrospective, population-based study. Owners who did not respond to the first questionnaire received one reminder. All of the dogs included in the study population were born January 1st 1989 – December 31st 1998, and were registered in the Norwegian Kennel Club (NKC). All of the IW registered in the NKC and born within the 10-year period in question were included in the study population. The expected response rate was around 50%, and approximately half of the total number of IW was therefore used as a fixed sample size of IW. Sample size calculations for the remaining breeds were then performed, using the number of IW available as a "reference point", aiming to yield an 80% probability of detecting a potential difference in breed specific incidence rates. A second criterion for determination of sample size was an expectation of at least 10 dogs diagnosed with primary bone cancer within each breed, based on the expected incidence rate.

Based on the information obtained from the completed questionnaires, incidence rates were calculated as number of cases per 10,000 dog years at risk (DYAR), and lifetime risks as the proportion of dogs with primary bone tumours, with 95% confidence intervals (CI) based on the Poisson distribution. Age at time of diagnosis within each breed was reported as median with range. A chi-squared (X^2) test was performed to test the hypothesis of differences in lifetime risks between subgroups of the sample population, such as breed and gender. Stata 10 was used for all statistical analyses. Level of statistical significance was set at $p < 0.05$.

Paper II

Primary bone cancer in Leonbergers may be associated with a higher bodyweight during adolescence.

This study was part of a larger prospective observational study from our department (the *main study*), designed to investigate growth patterns and risk factors for development of different skeletal diseases, i.e. panosteitis, hip dysplasia, elbow dysplasia, and primary bone cancer (Trangerud et al., 2007a; Krontveit et al., 2012).

Newfoundland, LR, LB, and IW dogs born in Norway between November 1998 and June 2001 and registered in the NKC were eligible for inclusion into the *main study*, and all breeders of these dogs were invited to participate in the study. For a breed to be included in the present study, the criterion was having more than 2 cases of primary bone cancer registered in the main database, limiting further analyses to the LB breed. For a dog to be diagnosed with primary bone cancer (BC+ group), typical clinical signs, physical examination findings (performed by a veterinary surgeon), and consistent radiographic changes were sufficient. Those dogs that were reported to have died from other causes than primary bone cancer, constituted the controls (BC- group).

Information provided by breeders, owners, and veterinarians was questionnaire-based. At certain time points (3, 4, 6, 12, 18, and 24 m of age), the attending veterinarian (or owner) recorded the dogs' BW (kg) and tape measurement of the circumference of the right thoracic limb at the level of the distal radius and ulna (CDRU; cm). Blood samples (serum and EDTA) and a radiograph of the right forearm were also obtained. After 24 m of age, the owners completed yearly questionnaires regarding their dog's health status and eventually time and cause of death or euthanasia.

The software package Stata 12 was used for all statistical analyses. Lifetime prevalence (LTP) of primary bone cancer was reported with 95% CI using the exact binomial method. Mean BW and CDRU values from birth (BW) or 3 m (CDRU) until 24 m were calculated separately for male and female LB in the BC- group, and displayed graphically alongside individual curves for each of the BC+ dogs. Logistic regression [$Y(\text{bone cancer}) = \beta_0 + \beta_1 * \text{gender} +$

β_2*BW (or CDRU); Y = the dependent variable (i.e. primary bone cancer); β_1 and + β_2 denote the respective regression coefficients for the independent variables: gender; female (reference category)/male and BW or CDRU respectively] was used to assess the association between primary bone cancer and BW and CDRU at 3, 6, 12, 18 and 24 m of age, while controlling for gender. The level of significance was set to $p < 0.05$. For statistically significant results, receiver operating characteristic (ROC) curves were created to estimate the fit of our model; reported as area under the curve (AUC). A Poisson regression was performed to confirm consistency of the results. To check for overdispersion of this model, a Pearson goodness-of-fit statistic was computed.

Paper III

Age-period-cohort analysis of primary bone cancer incidence rates in the United States (1976-2005).

Data from the Surveillance, Epidemiology, and End Results Program (SEER) 9 registries were used in this study (Mirabello et al., 2009). This is the most long-standing of the SEER registers, including data from approximately one tenth of the US population (http://seer.cancer.gov/registries/data.html) since 1973 (1974-75 for 2 of the areas). Primary bone cancer diagnosed among residents of these 9 SEER registries during 1976–2005 were categorised by sex, age, and subtype. To eliminate the influence of racial differences (Mirabello et al., 2009), only data from white residents were included. Incidence rates were grouped for the major types of bone cancer: OSA, ESA, and CSA. Bone cancers with other or unspecified morphologies were categorised as "other".

Age-specific incidence rates per 100,000 person-years were computed by subtype and gender; overall and in 10-year calendar periods (1976–1985, 1986–1995, and 1996–2005) and plotted according to age, year of diagnosis, and birth cohort. Age was categorised in 10-year intervals, and birth cohorts were estimated by subtracting the midpoints of 10-year age groups from the corresponding midyears of 10-year calendar time.

Observed age-specific trends were presented as rates for calendar periods (referred to as "periods") and rates for birth cohorts (referred to as "cohorts"). The estimated effects were

presented with an *a priori* focus on describing and interpreting the cohort effects obtained from the full APC model. The allocation of drift (the identifiable sum of period and cohort slopes) to birth cohort was used to obtain a unique solution to the identifiability problem, according to the method of Holford: the period effect was constrained to zero, thereby assuming that the changes in incidence rates could be attributed to birth cohort influences (Holford, 1992). The APC analysis was conducted using the functions available in the library *Epi* (version 1.0.8) in R, and specifically the *apc.fit* command. Smoothing was obtained using a natural splines function, with the number of parameters set to 5 for the age, period and cohort effects. The cohort and period effects were presented as rate ratios with the reference cohort 1930. Stata 10 was used for data management and plotting of the observed trends.

Paper IV

HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors.

Twenty OSA tumours from good- and poor-responders (n = 10 for each group) were selected as previously described by colleagues at Colorado State University (CSU) (O'Donoghue et al., 2010). All 20 dogs were radiographically free of thoracic metastases at diagnosis, and follow-up consisted of clinical examination, including thoracic radiographs, every 2– 3 m after initial treatment. Disease-free interval was calculated from surgery until development of metastatic disease, and good responders were defined as DFI > 300 d, with poor responders having a DFI < 100 d (flanking the median DFI of 200 d). Nine additional appendicular OSA tumour samples were collected from which matched normal metaphyseal bone was harvested from the same limb (at least one joint space away from the tumour) following amputation. One of the authors (BEP) performed histological grading of the tumours (from 1 to 3), using a scheme incorporating the amount of matrix, percent necrosis, nuclear pleomorphism, nucleolar size and number, and mitotic score (Moore et al., 2007). Reverse transcriptase-quantitative PCR (RT-qPCR) was used to quantify *HES1*, *HEY1*, *NOTCH1* and *NOTCH2* gene expressions in the 20 dogs grouped as good or poor responders, as well as from the matched tumour and normal metaphyseal bone samples taken from 9 dogs treated for appendicular OSA at the CSU Veterinary Teaching Hospital.

Western blot analysis and immunohistochemistry (IHC) were performed on the DFI grouped dogs to determine if *HES1* mRNA levels correlated with protein expression, and to assess the nuclear versus cytoplasmic distribution within the cells. Immunohistochemical scoring based on the percentage of positive cells (1-3) and the staining intensity (1-3), yielding a total immunoreactivity score of 1-9, was performed independently by 2 authors blinded to case information. Immunohistochemical HES1 expression was also assessed in a subset of canine appendicular OSA patients (n = 61) from a previously reported prospective clinical trial (Moore et al., 2007).

Total RNA from 15 of the 20 primary OSA tumour samples (microarray samples were limited due to array costs; these were picked randomly) from the DFI grouped dogs was analysed for differential gene expression of 51 Notch pathway or *HES1*-associated genes on GeneChip Canine 2.0 Genome Arrays (Su et al., 2009). Normal bone samples (n = 8) were analysed using an identical protocol.

Immunohistochemistry scores for the DFI > 300 d tumours versus the DFI < 100 d tumours were compared with a 2-tailed Fisher's exact test, after separating scores into 'low' expression (total score < 4) and 'high' expression (total score ≥ 4). This cut-off was based on results of ROC analysis of immunohistochemical scores for the 2 DFI groups. Welch t-test with false discovery rate (FDR) correction for multiple comparisons was used to compare microarray gene expression data. Significance was defined as $p < 0.05$ (Welch t-test) or $q < 0.05$ (FDR).

Statistical analysis of survival data was performed using a combination of Prism and SPSS software. Correlations between HES1 expression levels and other markers on a continuous scale were evaluated using linear regression. A 2-tailed, unpaired t-test was used to evaluate the association between HES1 expression levels and categorical markers. The median DFI was estimated using the Kaplan-Meier method, and comparisons between groups made using log-rank analysis for categorical variables. For continuous variables, markers were categorised into 'low' or 'high', using the median value as the cut-off. Multivariable Cox regression analysis was then performed; variables identified with a

univariable p-value of < 0.1 were included in the multivariable analysis. For all other tests, p-values of < 0.05 were considered significant.

RESULTS

Paper I

Breed-specific incidence rates of canine primary bone tumors - a population based survey of dogs in Norway.

Of the 3748 questionnaires received by owners, 1915 were completed, yielding a response rate of 51%. A significant difference was observed between the response rate of the owners of LR, which had the highest proportion of responders, 53% (51-56%), and that of IW, displaying the lowest, 47% (42-53%), ($p = 0.03$). Average age at the time of death/euthanasia was 8.9 y (CI 8.7-9.0 y) for all breeds; 7.0 y (CI 6.6-7.4 y) for IW, 8.2 y (7.8-8.5 y) for NF, 8.0 y (CI 7.7-8.3 y) for LB and 10.2 y (CI 9.9-10.5 y) for LR. For 197 dogs age at time of death was not reported.

Forty-three dogs had been diagnosed with primary bone tumours, based on clinical examination and radiographs, yielding an overall lifetime risk of 2.3% (CI 1.6-3.0%). Irish wolfhounds and LB, with 126 and 72 cases per 10 000 dog years at risk (DYAR), respectively, had significantly higher incidence rates of primary bone tumours than NF and LR ($p < 0.0001$). Incidence rates for the latter were 11 and 2 cases per 10 000 DYAR, respectively. No significant gender differences could be found ($\chi^2 = 0.16$, $p = 0.69$), as 21 male (2.4%; CI 1.5-3.7%), and 22 female (2.1%; CI 1.3-3.2%), dogs among the responders suffered from primary bone tumours. Median age at time of diagnosis was 6.7 y (range 1.6-11.6 y).

Distal radius/ulna, distal tibia and distal femur were the most common sites of the primary tumour, encompassing 35% (CI 21-51%), 19% (CI 8.4-33%) and 16% (CI 6.8-31%) of the tumours, respectively. The proportion of neutered dogs was similar among those diagnosed with primary bone tumours, 16.3% (7/43), and those without this diagnosis, 13.1% (245/1872). Median and mean age at time of neutering was between 5 and 6 y of age for both groups.

Paper II

Primary bone cancer in Leonbergers may be associated with a higher bodyweight during adolescence.

Leonberger was the only breed with more than 2 cases of primary bone cancer recorded in the database from the *main study* and was hence the only breed that fulfilled the inclusion criterion for breed. Of the 196 LB included, 9 had been euthanized due to primary bone cancer (BC+ group), yielding a LTP of 4.6% (CI 2.1-8.5%) (Table 1, Paper II). Six males (6.6%; CI 2.5-13.8%) and 3 females (2.9%; CI 0.6-8.1%) were affected. This was not consistent with a significant gender predisposition in this population of LB dogs ($p = 0.31$). Median age at time of death due to primary bone cancer was 6.3 y (range 3.7 y – 9.75 y), and was similar for males and females (6.5 y and 6.4 y, respectively). Median age at time of death for the remaining dogs was 6.75 y (for 10 of these dogs the age at time of death was unknown).

Figures 1 and 2 in Paper II show the BW (from birth) and CDRU (from 3 m) until 24 m for each gender. For male LB, the individual growth curves for each BC+ dog show a generally higher BW throughout the timeline than the mean values for the BC- group (Figure 1, Paper II). The difference between BC+ and BC- dogs for female LB is also shown. Male LB diagnosed with primary bone cancer later in life had a larger CDRU for most part of the growth period compared to the mean CDRU of BC- males, but no such difference was apparent for the females (Figure 2, Paper II).

Logistic regression showed a statistically significant effect of BW on the odds ratio of developing primary bone cancer at 12 m ($p = 0.012$) and 18 m ($p = 0.026$), and of CDRU at 18 m ($p = 0.033$) (Table 2, Paper II). At these ages, 1 kg higher BW yielded a nearly 20% higher risk of developing primary bone cancer, while 1 cm larger CDRU was associated with a nearly 70% increased risk. Receiver operating characteristic curves suggested that BW (at 12 and 18 m) and CDRU (at 18 m) provided some explanation for the risk of developing primary bone cancer in these dogs, yielding an AUC of approximately 0.8. Analyses based on the Poisson regression model were consistent with those of the logistic regression (Table 2, Paper II).

Only 3 dogs (1 female, 2 males) that later developed bone cancer had radiographs of the forearm performed, precluding meaningful comparisons between these and the BC- group. Due to the low number of cases, meaningful analyses of husbandry factors such as region, bedding, and amount of exercise were also not feasible, and hence not reported in this paper. Univariable analysis estimating the risk of primary bone cancer according to the dogs' region of residence (categorised as urban, suburban, or rural) did however approach statistical significance for an increased risk of primary bone cancer in dogs residing in rural regions ($p = 0.054$ at the age of 6 m).

Paper III

Age-period-cohort analysis of primary bone cancer incidence rates in the United States (1976-2005).

The age-specific rates of OSA during 1976–2005 exhibited a bimodal distribution with the highest incidence rate occurring in the second decade of life, and a second peak in those 75 to 79 y of age (Figure 1, Paper III). The age distribution of ESA incidence rates was similar to that of OSA until the age of 40, after which very few cases of ESA were diagnosed (Figure 1, Paper III). An increasing incidence by age was noted for CSA, reaching a plateau around the age of 65 (Figure 1, Paper III). The incidence rates of OSA throughout the period 1976–2005 were relatively stable for both males and females, as was the incidence rate of CSA in males (Figure 2, Paper III). Among females, however, the incidence rate of CSA rose by almost 70%, from 0.16 in 1976–1985 to 0.27 in 1996–2005 (Table 1, Paper III). This increase was statistically significant ($p < 0.05$), with the estimated change reaching almost 3% per year. An overall rise in the rate of bone cancer among females (0.8% per year) was mainly because of the increasing CSA incidence. Rates of ESA were stable throughout the time period for both genders (Figure 2, Paper III). The incidence of OSA decreased between 1976 and 2005 among those aged over 60 y: cohort-specific declines in the incidence rate of OSA were seen in successive generations born during 1905–1934 (Figure 3A, Paper III). No patterns in incidence trends by period or cohort were apparent for ESA in either gender, or for CSA in males (Figure 3B and C, Paper III). In females, however, increases in the CSA incidence rates were apparent over the entire study period of 1976–2005 (Figure 2, Paper III). Splitting by cohorts revealed increasing incidence rates for consecutive cohorts born since the early 1900s (Figure 3D, Paper III). The earliest and steepest increases occurred

among women aged 40 to 49 y, born during the 1930s–1950s; rates among younger women, born more recently, had also been rising.

Paper IV

HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors.

Average *HES1* mRNA expression was elevated 2.57-fold in canine OSA samples relative to normal bone (same dog) from 9 dogs diagnosed with OSA (Figure 3A, Paper IV; $p = 0.012$). There were however large individual variations: 5 tumours exhibited elevated expression compared to normal bone, and 4 tumours had virtually unchanged expression (Figure 3B, Paper IV). Hairy and enhancer of split 1 expression was elevated more than 4.6-fold in tumours from dogs with DFI > 300 d tumours compared to the expression in tumours from dogs with DFI < 100 d (Figure 3A, Paper IV; $p < 0.001$); *HES1* expression in the latter group was not different from that of the normal bone samples. *NOTCH2* exhibited an approximate 4-fold elevation in expression in both sets of DFI tumours, separately and in combination, relative to normal bone (Figure 2, Paper IV; $p < 0.001$). Similarly, *HEY1* expression was elevated in each tumour group by a fold-change ranging from 6 to 10.2 (Figure 2, Paper IV; $p \leq 0.001$). Neither *NOTCH2* nor *HEY1* mRNA expression differed between the DFI tumour groups. *NOTCH1* exhibited decreased expression in the DFI < 100 d group relative to normal bone, with no other significant changes measured (Figure 2, Paper IV; $p < 0.001$).

Of the 20 tumour samples from the canine DFI > 300 d and DFI < 100 d tumour groups, 14 were scored on HES1 immunoreactivity (Figure 6, Paper IV). For 6 samples, IHC was not possible due to loss of tissue during processing. All OSA samples evaluated with IHC had positive staining for HES1 both across and within tumours. The staining pattern of the tumour cells was predominantly nuclear, with diffuse cytoplasmic staining observed less commonly. The median HES1 reactivity score was 3 (range 1-9). Of the 6 tumours from dogs with DFI > 300 d, 83.3% (n = 5) had a score of greater than 3, compared to only 25.0% (n = 2) of the 8 tumours from dogs with DFI < 100 d (Table 1, Paper IV). Average HES1 immunoreactivity was lower in tumours from dogs with DFI < 100 d, but this difference did not reach statistical significance ($p = 0.1026$).

In the additional 61 primary canine OSA samples (where IHC for HES1 was performed to further assess its value as a prognostic factor) all tumours expressed HES1, with a median immunoreactivity score of 4 (range 1-9). The overall median DFI was 168 d (range 43 to > 1393 d). The median DFI in dogs with a high HES1 immunoreactivity score (≥ 4) was 258 d, compared to 155 d in dogs with a low HES1 immunoreactivity score (< 4) (Figure 7, Paper IV; $p = 0.0023$). Univariable analysis identified HES1, bone-specific ALP (BALP) activity, histological grade, percent necrosis and mitotic index as potential predictors of DFI (Table 2, Paper IV; $p < 0.1$). Upon multivariable analysis, HES1, percent necrosis and mitotic index were statistically significant independent predictors of DFI (Table 2, Paper IV; $p = 0.029$, 0.002 and 0.005 respectively). In summary, consistent with our RT-qPCR analyses, increased HES1 expression was identified as an independent prognostic biomarker for increased DFI in 61 canine OSA treated by amputation and chemotherapy.

Unbiased cluster analysis of data analysed for differential gene expression of 51 Notch pathway or *HES1*-associated genes separated normal bone from tumours, but did not discriminate between the DFI groups. In total, 30 of 51 (58.8%) Notch/HES1 pathway associated genes examined were significantly different between tumour and normal bone ($p < 0.05$, $q < 0.05$). Specifically, mRNA expression of *NOTCH1* and *NOTCH2* was elevated in tumour samples compared to normal bone ($p < 0.05$, $q < 0.05$). The 1.27 fold upregulation of *NOTCH1* in these gene array analysis differed from the results of the mRNA expression. Hairy and enhancer of split 1 was not included on the Canine 2.0 chip, but *HEY1* (another Notch target) was also elevated in tumours compared to normal bone ($p < 0.05$, $q < 0.05$). None of the genes evaluated had significantly different expression between DFI groups when corrected for multiple comparisons.

DISCUSSION

As already mentioned, OSA is the most common form of primary bone cancer in both humans and dogs. The discussion is focused on this subtype, with some aspects of CSA and ESA mentioned when relevant for the studies comprising this thesis.

Methodological considerations

Paper I and II were both based on standardised questionnaires to owners, and the latter also included separate ones to each dog's local veterinary surgeon. The major strength of this approach is the inclusion of a large number of dogs while minimising selection bias. Epidemiologic canine cancer studies have typically employed clinical records. These have mainly been obtained from larger referral hospitals, and thereby represent a selected population sample, because dogs referred to specialist clinics are more likely to suffer from severe disease. Furthermore, dogs with cancerous disease in which radical treatment is warranted, such as primary bone cancer, may be overrepresented at oncology referral centres - compared to dogs suffering from cancers that respond well to more conventional therapy, such as lymphoma. Studies based upon pathology records also encounter the problem of defining the reference population, as most pathology registers only include dogs in which biopsy or autopsy was performed. Finally, studies based on insurance records would be expected to have a younger reference population than the general dog population and do not have consistent diagnostic criteria, as the diagnosis is based on each attending veterinarian's evaluation – regardless of the extent of diagnostic aids (Egenvall et al., 1999).

As discussed in our Papers I and II, uncertainty about the diagnosis is a limitation of our questionnaire-based studies. Although typical clinical signs and radiographic findings strongly support a diagnosis of OSA, this is a histopathological diagnosis. We have therefore chosen to use the term "primary bone cancer" as a less specific term, although this too strictly speaking requires histopathology. We do however consider it unlikely that the "cases" in our first 2 studies suffered from any other condition than bone cancer, especially considering the absence of fungal osteomyelitis in this part of the world. Other differential diagnoses, such as benign bone lesions, bacterial osteomyelitis, or metastatic tumours can however not be completely excluded. Requiring histopathology for a diagnosis of primary

bone cancer was not feasible, as this would have underestimated the prevalence of the disease in our Paper I and precluded any analyses pertaining to this disease based on the data used for Paper II, due to the small proportion of dogs where histopathology was performed.

As the main objective of our first study was to estimate the incidence rate of primary bone cancer among the breeds included in the database used for the second study, we chose a retrospective questionnaire-based design; a representative reference population and a large study population were our priorities. All dogs of the respective breeds, born within a certain time frame and registered in the NKC, were eligible for inclusion in this study, and all owners of these dogs were contacted by post or telephone (provided their contact details could be obtained). Through this approach, we were able to include a large number of dogs (nearly 4000 questionnaires were distributed). However, the retrospective design and absence of first-hand clinical data present a potential for bias of the results. Because we wanted most of the dogs included in the study to have reached the end or near-end of their lifetime, we did not include any dogs younger than 10 y. These "youngest" dogs could still have developed bone cancer after the survey was conducted, and hence a limit of 15 y might have been more appropriate. This would particularly be true for the breed with the longest expected life span in this study, namely the LR: 237 of the 291 dogs (81.4%) that were still alive at the end of the study period were of this breed. This was however a compromise aiming to limit the recall bias, as we included dogs born up to 20 y prior to initiation of the study. Considering the shorter life span of the other 3 breeds (IW, NF, and LB) and their younger median age at the time of bone cancer diagnosis (even though this difference was not statistically significant), it is unlikely that the inclusion period underestimated the incidence rate of primary bone cancer in these breeds. However, the 2 LR reported to have died from primary bone cancer were both between 11 and 12 y old at the time of death. It is therefore possible that some of the 237 LR still alive at the end of the study period did develop primary bone cancer at a later stage, and that the incidence rate in LR was underestimated in this study.

A possibility for general overestimation of the incidence rate, regardless of breed, exists in studies where the responders know the disease in question; i.e. as the owners were

informed that we were investigating primary bone cancer, owners of dogs suffering from this disease may have been more likely to respond than those of other dogs. As discussed in our paper, this was however not supported by the similar rates found among the early versus late responders. Moreover, the breed of dogs with the lowest incidence rate, the LR, had the highest response rate. Comparable incidence rates for these 4 breeds were also found in our next study (Paper II), where the dogs were followed prospectively, and owners (and veterinary surgeons) provided the information prior to knowing what would be their dog's cause of death, and without primary bone cancer as the main focus – as these data were obtained through the *main study*, aiming to answer questions regarding several different diseases. The prospective design of this study most likely eliminated recall bias, ensuring accurate information for each included dog over time. Following each dog from birth also helped minimising the number of drop-outs; for nearly 80% of the 251 LB dogs included at the time of birth there was at least one report between the age of 3 and 24 months, and a known cause of death (at least it was known whether each dog died because of primary bone cancer or due to another disease). This prospective collection of data over a long period of time created a unique database on which several studies have been founded (Trangerud et al., 2007a; Trangerud et al., 2007b; Krontveit et al., 2010; Saevik et al., 2012). This *main study*, initiated by colleagues in 1998, was designed to enable comparisons between cases and controls – for several different diseases and a large number of dogs. Although a smaller study population than in our retrospective study (Paper I), 700 dogs (which were included in the *main study*) is a big population for a prospective study, and the amount of information recorded was substantial. The most obvious downside of including so many questions and so much information in a study, is the workload required by the investigators to correctly register the data. Also for the owners and veterinary surgeons, the amount of questions to be answered may for some have been too much, resulting in a lower response rate than could have been achieved by limiting the questions.

Another, potentially more dangerous consequence of analysing too many variables in relation to an outcome, is that by including a large number of independent variables, some of these will correlate with the dependent variable simply by chance. Correlation does not equal causation, and a theory founded on biological or pathophysiological concepts should be formed *a priori* to minimise the risk of drawing erroneous conclusions. This problem of

multiple comparisons is probably the explanation for some apparent correlations observed when using univariable analyses to compare bedding or flooring material to development of primary bone cancer. Such correlations were considered unlikely to imply causality, and were hence not included in our study. Although one could maybe argue that hard bedding or flooring material would increase the impact on the bones and hence could pose a risk factor, almost all of the dogs had been housed with both soft and hard materials inside and outside. Adding the low number of cases to the multiplicity issue, we decided against using this information in our analyses and limit these to aspects for which a potential causality makes biological sense – such as body size and growth, which for that reason were the factors we had decided to focus on prior to performing the analyses.

In Paper III, we used age-period-cohort (APC) analyses on a large database of human primary bone cancer patients. Age-period-cohort modelling of incidence data is a commonly applied and useful approach in the search for aetiological hypotheses. Given the limited number of established risk factors for primary bone cancer, APC modelling was considered a reasonable approach aiming to identify such hypotheses, in particular those pertaining to birth cohort or calendar period. The presence of cohort patterns could support the notion that exogenous factors are important in the carcinogenesis of the disease under study. For other cancers, such as testicular, cervical, and colorectal, identification of cohort patterns has generated new hypotheses regarding external risk factors (Svensson et al., 2005; Bray et al., 2006; Leung et al., 2006). By singling out individual birth cohorts and following the occurrence of disease within each of these groups, trends that are otherwise “hidden” may become apparent, as shown by the data from our study (Paper III). Apart from CSA in females, the overall incidence rate of primary bone cancer (and the histological subtypes) did not change over the time period in question (i.e. they were “hidden”). However, when separating the different birth cohorts, both increasing and declining rates for certain subtypes in certain age groups were suggested – such as the decreasing incidence rate of OSA for both genders, aged 60 y and older, born from 1905 to 1934, and the increasing incidence rate of CSA in women was shown to occur in those aged 20 to 69, born between 1935 and 1975. As stated in our paper, these results must however be interpreted with caution: they do provide a basis for creating new hypothesis, based on biological concepts

related to hormonal therapy, but should not be regarded as evidence for a true change in rates.

The main limitation of the APC method, is the direct linear relationship between the 3 parameters: cohort = period – age. This so-called identifiability problem means that there is an indefinite number of possible solutions to the equation, and some assumptions must be made to overcome this issue. We used the method described by Holford (Holford, 1992), where the effect of period is fixed at zero, and any changes or "drift" hence attributed to birth cohort and age. This is one of the traditional approaches, where either of the 3 effects (A, P, or C) is set to zero and used as reference levels, or the sum of 2 effects are equated to zero. A more recently described solution, "the Heuristic Solution" (Mdzinarishvili and Sherman, 2012) is another option that could be considered, but was published the year after our paper. This method creates 4 redundant (i.e. identifiable; they are the reference values for the model, ensuring that no 2 sets of parameter values yield the same distribution of the data) parameters: after equating 3 of the parameters to zero, an optimal value for the fourth parameter (the period adjacent to the reference time period) is estimated. To find this optimal value, an assumption is made in that the effects of adjacent cohorts are similar, which is reasonable considering the overlapping time intervals between these birth cohorts.

The problem of an association between age, period, and birth cohort is however not unique to the APC analyses. Increasing age inevitably moves an individual along the period axis, and an assumed increase by age could in theory reflect an extrinsic effect due to period. Similarly, individuals of one age group (i.e. those aged 60-70 y of age diagnosed with OSA) diagnosed in 1960 would naturally belong to a different birth cohort than a group of patients aged 60-70 diagnosed with OSA in 1990. In the first example, we automatically relate the increased risk to age (rather than period), whereas in the latter, we would attribute a change in incidence rate to period – while it could, in fact, be due to these groups of individuals belonging to different birth cohorts.

In Paper IV, we investigated the expression of Notch receptors and signalling mediators, HES1 and HEY1, in canine OSA samples from dogs with DFI > 300 d and DFI < 100 d, as well

as samples of matched OSA and normal bone, to explore associations with OSA progression and patient outcome. RT-qPCR was performed for quantification, western blot and sequencing confirmed the identity of the targets, and IHC verified expression of the respective proteins in OSA tissue and normal bone. Although the idea for the study as well as the design of the primer for *HES1* PCR originated at NMBU in Oslo, collaboration was initiated to ensure higher sample numbers. Colorado State University is one of the leading oncology referral centres, and tumour tissue has been collected and stored for several years in their "Flint Animal Cancer Center" tissue archive. We therefore initiated contact with colleagues at this university, and completed the study together. More work was added on to our original plan of investigating *HES1* expression (i.e. *HEY1*, *NOTCH1* and *NOTCH2* expression, as well as immunocytochemistry/IHC and DNA microarray); performed by one of their PhD students at the time (first author of Paper IV, Deanna D. Dailey), making the study more comprehensive, and strengthening the results of the HES1 expression analyses. This signalling pathway was considered of interest in light of its role in regulation of transcription factors, the implication both in OSA and other tumours, and the possibility for it to represent a therapeutic target (McManus et al., 2014). Such gene expression studies do however include a small number of possible candidate genes due to the time- and labour intense nature of RT-qPCR.

Another approach is to screen for differences in the expression of several genes simultaneously; DNA microarray assays. This was performed only on a limited number of samples in our study, due to cost. The major advantage of DNA microarrays is the ability to investigate a large number of genes simultaneously. While intriguing, the previously mentioned multiplicity problem must be accounted for, although it has been questioned whether corrections for the multiplicity problem are truly warranted (Konishi, 2011). Investigating 20 different parameters simultaneously (and setting the significance threshold at the usual 0.05), would on average render one parameter statistically significantly different by random chance alone, i.e. without any biological significance. Increasing this number to for instance 10 thousand genes, 500 of these will be found statistically significant by chance. Therefore, it seems obvious that some corrections must be done. In our study, an FDR was used. The positive FDR is defined as the chance of one false discovery (i.e. one erroneously rejected null hypothesis) among all the discoveries. A "q-value" is used to

define the minimal positive FDR (i.e. the minimum chance of one false discovery among all discoveries) above which the alternative hypothesis is rejected. In this way, the use of a positive FDR increased the power of the study compared to other corrections for multiplicity, such as the Bonferroni correction (one of the familywise error rate (FWER) procedures; this rate is the probability of making one or more false discoveries), which aims to reduce the probability of even one falsely rejected null hypothesis, as opposed to allowing a certain proportion of false discoveries. This proportion is defined *a priori*, and only focuses on the chance of making a false discovery among the relevant tests. For instance, should genome-wide microarray be performed (which it was not in our study), one would only include relevant genes in these calculations. Another problem with DNA microarrays is a potentially large inter- and intra-assay variability. Results from these assays hence need to be validated, and the gold standard validation method is the Northern blot. However, this is frequently not feasible due to time and RNA amounts required, and RT-qPCR is considered an acceptable alternative. This method also provides better quantification data than the microarrays, but is as mentioned earlier tedious and labour-intensive to perform on multiple genes.

An increasingly used method in recent years combines the accuracy of RT-qPCR with the ability to screen multiple genes of DNA microarrays: PCR arrays perform RT-qPCR for several genes simultaneously. The genes analysed may be custom made, or “packages” of whole pathways may be ordered. This method probably offers the most optimal balance between accuracy and efficiency at present, but may so far be cost prohibitive for smaller scale studies, such as ours.

General discussion

Body size and risk of OSA

It has long been known that the risk of OSA is related to body size in dogs (Tjalma, 1966). The incidence rate is several times higher in large or giant breeds than in smaller ones, and the typical tumour location differs between large and small-breed dogs (Tjalma, 1966). Most large or giant dogs develop tumours in their appendicular skeleton, and around three quarters of these tumours are found in their thoracic limbs, usually the distal radius or

proximal humerus (Ehrhart et al., 2013). Small dogs, on the other hand, tend to develop bone tumours in their axial skeleton; typically at a somewhat higher age than large dogs with appendicular tumours (Heyman et al., 1992; Ehrhart et al., 2013). Primary rib OSA appear to constitute an exception, with a median age of around 4-5 y (Feeney et al., 1982; Heyman et al., 1992).

In humans, the difference in body size between individuals is small compared to the wide range from miniature or toy to giant canines; yet an association between bone cancer risk and size or growth has been suggested by several studies. One recent meta-analysis concluded that patients with OSA were 2-3 cm taller than the reference population (Arora et al., 2011), while another reported an increased risk of OSA associated with high birth weight and tall stature (Mirabello et al., 2011). These findings are in line with the higher risk of OSA observed in large canines; documented by several investigators, and also shown in our prevalence study (Paper I). The highest incidence rates of primary bone cancer were found in 2 of the largest breeds, the LB and IW, while it was significantly lower for the relatively speaking smallest breed of this study, the LR. Size is however not the only predictor of OSA risk, as reflected by the low incidence rate in a breed of similar size and stature as the LB, namely the NF. As we discussed in Paper I, it is possible that the higher risk of primary bone cancer in the LB compared to the NF is at least partially explained by a difference in growth rate, as the NF has a slower growth rate than the LB (Trangerud et al., 2007a).

It seems likely that the risk of developing OSA is related to BW, height, or growth rate in both humans and dogs, but that differences in these factors explain only a small part of the overall risk for each individual. Other genetic characteristics, as well as extrinsic factors such as exposure to carcinogenic substances, also play a role in the aetiology of OSA and other bone tumours.

Exercise and risk of OSA

No studies have shown any associations between exercise-related bone impact and human or canine OSA, possibly due to difficulties in study design. The *main study*, on which our Paper II was based, collected detailed, prospective information about each dog's amount

and form of exercise during their adolescent life. Despite the efforts of dog owners and investigators recording all this information, no inferences could be made in terms of potential associations between the activity level and the risk of primary bone cancer. Apart from low power to detect potential differences due to few cases, which our Paper II suffered from, investigating the effect of exercise poses the challenge of deciding whether exercise should be analysed as a "threshold" or "dose-response" risk factor. Moreover, timing of exercise in relation to growth is likely to be of importance, and might necessitate taking breed-specific, or even individual, growth curves into account.

A few attempts at addressing the question of exercise as a risk factor of OSA have been made previously, including studies of racing Greyhounds to see if the limbs under greatest stress harboured a larger proportion of bone tumours. Contrary to the hypothesis, there was no difference between the left and right limbs (Rosenberger et al., 2007). As Greyhounds always race counterclockwise, limb-specific injuries are seen due to the increased compression forces on the inner side of the circle (i.e. lateral aspect of the left limbs, and medial aspect of the right limbs) and increased tension forces on the outer side (Guillard, 2012). This lateralisation within each limb is however seen in distal limb injuries (such as the accessory carpal bone and the central (navicular) tarsal bone; the right limb is most commonly affected by these injuries), and may be less relevant when it comes to potential risk factors for tumour development proximal to the carpus and tarsus. Moreover, a study of biomechanics in Greyhounds racing on a circular track (counterclockwise) demonstrated a marked increase in the peak forces of all 4 limbs during a bend (by 4.3-64.5% on average for each leg); with the highest increase observed in the thoracic limbs, but with no apparent difference between the right versus left (Usherwood and Wilson, 2005). The lack of difference between thoracic and pelvic limb location in the study by Rosenberg and colleagues may be due to the relatively low number of OSA cases (n = 21). In line with most observations in large-breed dogs, a study including 40 cases of OSA in formerly racing Greyhounds reported a significantly greater proportion of tumours in the thoracic (75%) compared to pelvic (25%) limbs (Lord et al., 2007). The latter study did not compare right versus left limb locations, but reported that 18 OSA were found in the right thoracic limb compared to 9 in the left. For the pelvic limbs, more tumours occurred on the left (6 left, 3 right), but this may not be relevant as any potential difference between right and left may

be subtle or non-existing in the hind legs, undergoing much lower weight strain than the front limbs. Furthermore, unpublished data suggest that racing Greyhounds have a higher incidence rate of OSA than non-racing ones (i.e. American Kennel Club registered) (G. Couto, unpublished observations, In: (Karlsson et al., 2013)). Whether this relates to genetic differences between these 2 lines of the breed or to bone impact is not known. Comparable human studies are scarce, but a doctoral thesis by Cohen (1974) described that OSA in the knee was most common in parts with a relatively small weight burden (lateral femur and medial tibia), not lending any support to a direct relationship between impact and OSA development (Misdorp and Hart, 1979). Moreover, a study by Muir and colleagues did not find any difference in the microcrack density of bone in the metaphyseal regions of the canine OSA predilection sites (e.g. distal radius) than in other regions, and hence did not support fatigue-induced injury as a risk factor for OSA (Muir and Ruaux-Mason, 2000). Gellasch and colleagues reported similar findings (Gellasch et al., 2002). Metallic implants have been hypothesised to increase the risk of OSA development (Keel et al., 2001), but this may represent a coincidence rather than causality (Sinibaldi et al., 1976; Murphy et al., 1997).

Genetic factors

Genetic factors represent another possible explanation for the difference between the LB and NF breeds. Familial predispositions to OSA have been suspected for several canine breeds, best documented for the St. Bernard and Scottish deerhound (Bech-Nielsen et al., 1978; Phillips et al., 2007), and in this respect it is interesting to note that 4 and 2 of the LB diagnosed with primary bone cancer included in our Paper II were from the same litter.

Racial differences in human OSA incidence rates have also been documented. When only separating between Whites and Blacks, a higher incidence rate has been reported in the latter (Polednak, 1985; Homa et al., 1991). However, a recent study based on the SEER Program (1973-2004) found the designation “Other” (specifically Asian/Pacific Islanders) to have the highest rates (Mirabello et al., 2009). In elderly patients, the incidence rate was highest in Whites, which may partially be explained by Paget’s disease of bone being more prevalent in this group (Pompe Van Meerdervoort and Richter, 1976; Josse et al., 2007).

Environmental factors: radioactivity

Few environmental factors to which people or dogs are naturally exposed have been documented to increase the risk of primary bone tumours, but several substances to which exposure is rare or purely experimental (in dogs or other animals) are capable of inducing bone cancer development. Chemical substances that can induce OSA include beryllium oxide, zinc beryllium silicate, and methylcholanthrene; documented by intravenous injections in rabbits (Watanuki et al., 1967; Fuchs and Pritchard, 2002). In humans, the best-known example is probably exposure to self-luminous paint containing the radioactive substance radium, which was used to make watches glow in the dark in the early 20th century (Martland, 1931).

The watch painters were among those exposed to high doses of radium, as they were using their lips to sharpen pencils with radium-containing paint. Several years after exposure, a dose-dependent development of primary bone sarcomas, mainly OSA, was observed in these workers (Rowland et al., 1978). A similar connection has been documented in patients receiving injections of high doses radium-224 to treat bone tuberculosis around the mid 20th century that developed bone sarcomas about 8 y after treatment (Chmelevsky et al., 1988). Other radioactive substances, such as plutonium and strontium, have also been shown to induce bone cancer; Beagles exposed to strontium-90 by inhalation, ingestion, or intravenous infusion, developed OSA at a similar dose-response level regardless of the route of administration (Gillett et al., 1992), and Beagles injected with plutonium-239 also developed OSA (Lloyd et al., 1994). These experiments verify a biological causality underlying the increased risk observed in humans naturally exposed to radioactivity, such as the watch painters or workers at the Mayak Radiochemical and Plutonium Production Plants (Miller et al., 2003), built in Russia in the mid 1940s and site of the third largest nuclear accident (in 1957) after Chernobyl (1986) and Fukushima (2011).

Increased awareness of the detrimental health effects of exposure to high doses of radioactivity has led to stricter control with atomic waste and cessation of radium-containing dial paint and use of thorium-containing contrast mediums. Better medical knowledge has also ended the use of radium as treatment for tuberculosis and bone-deforming conditions. Although radiation remains extensively used for various cancers, both

in humans and dogs, improved targeting of the disease with less exposure to normal tissue has reduced the prevalence of secondary cancers following radiation therapy. Primary bone tumours are however still among the most common and devastating late effects of radiation therapy (Mavrogenis et al., 2012).

In our age-period-cohort (APC) study (Paper III) describing incidence rates of human primary bone cancer in the United States (1976-2005), we showed some interesting changes in the rates of OSA for certain birth cohorts. Although age-standardised incidence rates were relatively stable from 1976 to 2005 in both males and females (Figure 2, Paper III), separating each 10-year birth cohort during this time period revealed some changes. For patients developing OSA relatively late in life, namely aged 60-79 y and hence representing the second age-specific peak, the incidence rate decreased successively for each 10-year cohort born between 1905 and 1934 (Figure 3, Paper III). The results were similar for males and females, and were therefore merged in our presentation of these trends.
One must be cautious when attempting to associate temporal changes from observational studies with biological causation, and our results should not be used to make such inferences. Considering the implication of bone-seeking radionuclides in the aetiology of OSA, we do however speculate that one contributing factor to the reduced incidence rate could be less fallout of bone-seeking radionuclides (including the bone volume-seeking isotope strontium-90, found in radioactive waste from nuclear reactors and in nuclear fallout from nuclear tests) when the US ceased atmospheric nuclear testing from 1963, after signing the “treaty banning nuclear weapon tests in the atmosphere, in outer space and under water”. Time-wise, it makes sense that a reduction in radiation exposure after the 1960s could result in a decline in bone cancer risk a few decades later (some Mayak workers died from OSA at a median time of 30 y after employment; (Koshurnikova et al., 2000)), which thereby could at least partially explain the observed declining rates between 1976 and 2005.

Other extrinsic risk factors

Fluoride and radium as natural content in the drinking water have also been implicated in bone cancer aetiology, but studies show inconsistent results. Fluoride has been reported to increase (Kharb et al., 2012), possibly decrease (Gelberg et al., 1995), or have no effect on

the risk of OSA (Kim et al., 2011), but 2 systematic reviews concluded that fluoride in the drinking water does not increase the risk of OSA (McDonagh et al., 2000; Yeung, 2008). Studies from Ontario (Canada) have found an increased risk of OSA in young people exposed to radium in the drinking water (Finkelstein, 1994; Finkelstein and Kreiger, 1996), whereas a more recent study found no evidence that the radium levels in Wisconsin drinking water increased the risk of OSA (Guse et al., 2002).

A possible association between parental occupational farming and childhood OSA has been reported (Hum et al., 1998), albeit not consistently (Hoppin et al., 1999), and a meta-analysis concluded with an increased risk of ESA in people living or working at a farm (Valery et al., 2005). Exposure to organic dusts has been postulated to account for the increased risk of ESA in the children of farmers (Moore et al., 2005). In our study of nearly 200 LB, 9 of which died because of primary bone cancer, the low number of cases precluded meaningful analyses of husbandry factors such as region, bedding, and amount of exercise (Paper II). It is however interesting to note that 8 of these 9 LB spent at least their first 2 y of life in what the owners reported to be a rural area (for the 9th dog, the owner did not answer this question, but based on their postal address, this dog grew up in a suburban region). Although these results were borderline statistically significant (at the age of 6 m), suggesting an increased risk of primary bone cancer for dogs growing up in a rural area, this must be interpreted with caution due to the low number of cases. The nearly significant results may represent a coincidence rather than causality.

Hormonal factors

Both endogenous and exogenous oestrogens may play a role in the pathogenesis of bone tumours. Two canine studies have found an increased risk of OSA in neutered dogs of both genders (Ru et al., 1998; Cooley et al., 2002), the latter showing an inverse dose-response relationship between lifetime gonadal exposure and the incidence rate of OSA. Studies in mice have shown both a protective (Rooks and Dorfman, 1961) and promoting (Nilsson and Ronnback, 1973) effect of oestrogens on radiation-induced OSA, and one study concluded that the promoting effect was present only when administered along with higher doses of strontium-90 (Haraldsson and Nilsson, 1988). Spontaneous development of OSA has also been found in mice fed diets containing oestrogens (Highman et al., 1981). These findings

contrast the observation that oestrogen promotes osteoclast apoptosis and reduces bone turnover (Compston, 2001); thus, increased osteoclast proliferation and reduced apoptosis leading to increased bone turnover could be one explanation for the increased development of OSA in neutered dogs.

Another possible explanation for the role of oestrogens in the development of primary bone cancer pertains to its role in skeletal growth and closing of the epiphyseal plates. It is plausible that the increased risk of OSA observed in neutered Rottweilers, which was highest for those neutered prior to 1 y of age (i.e. before fully grown), is exacerbated by an abnormal growth plate closure – as hypothesised for some canine joint disorders (Torres de la Riva et al., 2013). The fact that the risk was increased in both genders may be explained by the testicular oestrogen production (Vincenzo et al., 2000) or by the proliferative and differentiating effect of androgens on osteoblastic cells (Kasperk et al., 1989). Neither among the dogs investigated in Paper I nor Paper II did we identify any differences in the proportion of neutered dogs among those with or without primary bone cancer. This could be due to the low number of cases as well as the fact that few dogs in Norway are neutered – and the once that are, are typically neutered relatively late in life (5-6 y of age in Paper I).

In Paper III we described a birth cohort-specific increase in the incidence rate of CSA in females born between 1935 and 1975, aged 20 to 69 y, and speculated that this could be related to exogenous oestrogens. Oral contraception was introduced in the US in the 1960s (Tyrer, 1999), and was used by more than 50% of women aged 15 to 44 y between 1982 and 2002 (Mosher et al., 2004). Initially, women that had already given birth used it to pause their reproductive period, but the pill subsequently became commonly used as contraception for younger women. Looking at Figure 3 (Paper III), the steepest increase in female CSA was observed from 1975-2005 in those aged 40-49 y, and an increasing trend was also seen in those aged 20-29 y and 30-39 y. For the youngest cohort, the increase was more pronounced in the second half of this period. These trends correlate with the introduction of the pill as contraception from the 1960s. Additionally, an increase in the incidence rate of CSA was observed in the second period among women aged 50-59 y and, to a smaller extent, 60-69 y. The increase in the older population corresponds to the use of hormone replacement therapy – which became widely used in the 1960s, with a temporary

decline in the 1970s (due to the reported association with uterine carcinoma) before an increase was seen from the 1980s onwards – with the introduction of combined oestrogen and progestin therapy (Brett and Madans, 1997). After publication of the Women's Health Initiative trial in 2002, showing that risks associated with this therapy exceeded the benefits, the use of these hormones has declined (Rossouw et al., 2002). A potential decrease in the incidence rate of CSA in women reaching menopausal age after the millennium would support a causal relationship between oestrogen and development of CSA, but it is important to remember that observations of temporal correlations do not prove causality. There is however a biological rational for our hypothesis, supported by the molecular biology of CSA: the oestrogen pathway has been shown to be active in CSA tumour samples, and oestrogen stimulates proliferation of CSA cell lines (Cleton-Jansen et al., 2005). Moreover, oestrogen stimulates vascular endothelial growth factor (VEGF), which is necessary for the neovascularisation seen in the progression of CSA (Mueller et al., 2000). Oestrogen has also been established as a risk factor for mammary cancer (Samavat and Kurzer, 2015), and suggested to represent a risk factor for cancer in other oestrogen-sensitive organs such as the endometrium and ovaries (Brown and Hankinson, 2015). Contrary to the beneficial effects of inhibiting oestrogen in the treatment of breast cancer (Viedma-Rodriguez et al., 2014; Chumsri, 2015), one report of *in vitro* oestrogen inhibition in CSA cell lines, as well as treatment with an aromatase inhibitor (i.e. inhibiting oestrogen production) in 6 CSA patients with progressive disease did not show any effect on tumour growth (Meijer et al., 2011).

Parathyroid hormone (PTH) has been evaluated as a risk factor for OSA, as PTH activates osteoblasts and increases bone formation (Dobnig and Turner, 1995). Rats given teriparatide (a recombinant form of PTH) had an increased risk of developing OSA, especially at high doses over a long time period (Vahle et al., 2004). However, only single case reports of OSA in humans receiving this drug exist, and no clear association has been confirmed (Bang et al., 2014). Moreover, no increased prevalence of OSA has been found in humans with primary hyperparathyroidism (Jimenez et al., 2005; Cinamon and Turcotte, 2006). A few case reports describe the development of OSA in humans with an increased production of growth hormone (i.e. acromegaly), and it is possible that increased bone turnover in these patients represents a risk factor, although the number of cases is too low

to draw any conclusions (Lima et al., 2006). Acromegaly has not been associated with canine bone tumours – possibly due to the low incidence of this disease in this species. Another disease characterised by increased bone turnover, Paget's disease of bone, is a well-recognised risk factor for OSA in humans, although less than 1% of these patients experience this consequence (Fracassi et al., 2007).

Molecular genetics

Over the last decades, research has focused on the role of genes and genetic pathways in carcinogenesis. Genetic conditions associated with an increased risk of OSA include hereditary retinoblastoma (mutations in the RB1 gene), Li-Fraumeni syndrome (mutations in the p53 gene), Bloom syndrome (mutations in the BLM (RecQL3) gene), Rothmund-Thompson syndrome (mutations in the RECQL4 gene are found in a subset, and is associated with an increased risk of OSA) (Wang et al., 2003)), and Werner's syndrome (mutations in the WRN gene) (Kansara and Thomas, 2007). There are no canine equivalents to these syndromes, but mutations in the p53 gene are frequently found in dogs with OSA (Kirpensteijn et al., 2008). Mutations in the tumour suppressor gene p53 have been found in 15-42% and 24-47% of human and canine OSA, respectively (Kirpensteijn et al., 2008). Increased expression of the mutated protein has also been associated with reduced survival in both species (Pakos et al., 2004; Kirpensteijn et al., 2008; Bongiovanni et al., 2012). Rather than mutations in the RB1 gene, canine OSA cell lines appear to have reduced amounts of the RB family proteins (Rb, p107 and p130) – presumably due to a mutation affecting the posttranscriptional process (Levine and Fleischli, 2000).

Further, a group of enzymes termed protein kinases have been implicated in development and progression of several cancers. Protein kinases mediate cell signal transduction and regulate pathways for cell growth, differentiation, survival and apoptosis by phosphorylating proteins at tyrosine residues (tyrosine kinases) or threonine/serine residues (threonine/serine kinases). These kinases may be expressed on the cell surface, cytoplasm, or inside the nucleus; those expressed on the surface that bind growth factors are called receptor tyrosine kinases (RTK) and this group of protein kinases has received the most attention in cancer research. Receptor tyrosine kinases implicated in the aetiopathogenesis of OSA include MET (with its ligand HGF) and the insulin receptor family (MacEwen et al.,

2004), as well as some of the RTK involved in tumour angiogenesis (VEGFR, PDGFR) (Maniscalco et al., 2013). Interestingly, a germline mutation resulting in constitutive phosphorylation and dysregulated MET signalling has been found in a higher proportion of Rottweilers than other breeds, providing one possible explanation for the relatively high prevalence of OSA in these dogs (Liao et al., 2006).

Molecular genetics: mTOR

Several other dysregulated pathways have been identified in canine or human OSA, some of which may serve as targets for therapy. PI3 kinase (PI3K), a downstream effector of RTK, initiates one of the pathways that regulate mammalian target of rapamycin (mTOR) signalling, crucial for the integration of cellular signals (Zhang et al., 2015b). Dysregulation of this pathway plays a role in growth and chemotherapy resistance of several cancers, and may be inhibited by rapamycin and related compounds (so-called "rapalogues") (Geng et al., 2014). A tumour suppression gene that normally limits the expression of mTOR, is the phosphatase and tensin homolog (PTEN). Loss of function of this gene (due to mutations, or – less commonly – epigenetic factors) leads to increased activation of PI3K and its downstream signalling pathway (PI3K/Akt/mTOR) (Tsimberidou et al., 2012). Loss of (or reduced) PTEN expression has been identified OSA cell lines and in canine and human OSA tissue (Levine et al., 2002; Freeman et al., 2008; Angstadt et al., 2011), as well as in other cancer types, and has been found to predict response to specific therapies (Milella et al., 2015). For example, humans with mammary tumours and loss of PTEN function have a poor response to trastuzumab, a drug used in patients overexpressing the membrane receptor tyrosine kinase ErbB2 (HER2), resulting in an aggressive cancer phenotype and poor prognosis. Trastuzumab inhibits the PI3K/Akt/mTOR (or "protein kinase B") signalling pathway (in cells overexpressing HER2), and is dependent on PTEN expression for optimal effect (Nagata et al., 2004). Inhibition of the increased PI3K/Akt/mTOR activity may ameliorate the negative effect of reduced (or absent) PTEN expression. These patients have hence been found to benefit from rapamycin (sirolimus) and its analogues (i.e. "rapalogues", such as temsirolimus, everolimus and deforolimus/idaforolimus) or direct inhibition of PI3K (Paplomata and O'Regan, 2014). Indeed, PI3K inhibitors (e.g. wortmannin) have been shown to counteract the resistance to trastuzumab in patients with breast cancer and loss of PTEN (Nagata et al., 2004). Dual PI3K/mTOR inhibitors are also undergoing

studies to determine a potential role in the treatment of OSA (Manara et al., 2010; Gobin et al., 2014), as are activators of PTEN – such as tepoxalin, which prevents alkylation or oxidation of PTEN (Loftus et al., 2014).

Molecular genetics: Notch and HES1

In T-cell lymphoblastic leukemias (T-ALL), it has been shown that increased expression of NOTCH1 ("gain-of-function" mutations) negatively regulates PTEN expression and thereby increases PI3K/Akt/mTOR activity (Palomero et al., 2007). Gamma-secretase inhibitors (GSI) inhibit NOTCH1 activation, but T-ALL patients with loss of PTEN are resistant to this treatment (Palomero et al., 2007; Hales et al., 2014). These patients may therefore also benefit from inhibition of the PI3K/Akt/mTOR pathway; leukemia cell lines that were PTEN negative (and hence GSI resistant) were indeed inhibited by an Akt antagonist (SH-6, a phosphatidylinositol analogue), whereas this drug had no effect on PTEN positive cells (Palomero et al., 2007).

Interplay between the NOTCH1 receptor and PI3K/Akt/mTOR pathways has been supported by several studies (Gutierrez and Look, 2007), including research involving pancreatic tumours and cell lines (Vo et al 2011): a combination of mTOR inhibition (rapamycin) and Notch inhibition (GSI) was demonstrated to have a greater effect on cell death than either drug alone (Vo et al 2011). The addition of GSI to these pancreatic cancer cell lines did not change the amount of PTEN, but altered its phosphorylation status, presumptively affecting its function. The expression of HES1 (and HEY1) was however found to be increased by addition of GSI; suggesting that the effect of Notch signalling on PTEN phosphorylation was not controlled by HES1 (Vo et al., 2011). This is contrary to what Palomero and colleagues reported in the study on patients with T-ALL, where the NOTCH1 downstream transcription factors HES1 and MYC have been suggested to mediate the NOTCH1-induced inhibition of PTEN (and hence the resistance to GSI) (Palomero et al., 2007).

Several studies suggest that the PI3K/Akt/mTOR and Notch/HES1 pathways, and the relationship between them, are of importance also for development or progression of OSA. In Paper IV, we showed that NOTCH2, HES1, and HEY1 expression was increased in OSA tumour samples relative to normal bone. These findings coincide with studies of human OSA

that have found Notch signalling to be implicated in OSA cell proliferation, invasion and metastasis (Zhang et al., 2008; Engin et al., 2009; Hughes, 2009; Tanaka et al., 2009; Zhang et al., 2010). Reduced invasiveness in response to suppression of Notch signalling and HES1 activity has also suggested that Notch/HES1 signalling has an impact on OSA progression (Zhang et al., 2010) (Data pertaining the OS187 or COL cell lines in this study should be viewed with caution due to a recent disclosure that these are not OSA cells). Moreover, increased *HES1* mRNA expression has been shown in some human OSA cells and OSA tumour samples (compared to osteoblasts or normal bone), and an association between high HES1 expression and decreased survival of OSA patients has been suggested. Expression of *HES1* mRNA was inversely correlated with survival in one study including 16 human OSA samples (Hughes, 2009). When comparing poor (DFI < 100 d) and long-term (DFI > 300 d) survivors among canine OSA patients (Paper IV), we found a significant difference in the expression of HES1 (mRNA and protein) between the groups. Contrary to our expectation, however, we identified a higher expression of HES1 in the long-term survivors, suggesting that other mechanisms than those controlled by HES1 may be more important for the progression of the most aggressive tumours or in a subset of canine OSA (Paper IV).

Molecular genetics: ezrin

Another gene that is closely related to the mTOR signalling pathway and may influence OSA progression is ezrin. Expression of this protein has been documented in OSA cells, where it may increase the level of adhesion molecules (e.g. CD44), thereby promoting tumour invasion and metastasis (Curto and McClatchey, 2004). Moreover, ezrin expression appears to be necessary for P-glycoprotein-mediated chemoresistance (MDR) in human OSA cell lines (Brambilla et al., 2012). Wan and colleagues documented that ezrin functions through an mTOR signalling pathway, and that ezrin-mediated phosphorylation of downstream targets could be inhibited by rapamycin (i.e. mTOR inhibition), lending further support a potential for rapalogues in the treatment of OSA (Wan et al., 2005).

Molecular genetics: IGF-1

Other potential targets for OSA treatment include IGF-1/IGF-1R (mediators of GH) and the RTK tropomyosin-related kinase (Trk) A (binding nerve growth factor). Targeting the former using a long-acting somatostatin analogue (octreotide pamoate; OncoLAR) did however not

show any clinical efficacy when used in an adjuvant setting for canine OSA in one study (Khanna et al., 2002). Using the same drug in paediatric human patients with metastatic OSA also did not yield any significant clinical differences, but the expression of IGF-1 was reduced (Mansky et al., 2002). Expression of TrkA has been shown in canine OSA *in vivo*, and blocking the TrkA signalling increased apoptosis in OSA cell lines *in vivo*, but a potential clinical effect has not yet been investigated (Fan et al., 2008).

Molecular genetics: microRNAs

Both IGF-1R and the PI3K/Akt/mTOR and Notch/HES1 pathways may also be targeted through so-called microRNAs (miRNAs) (Zhang et al., 2015a). These are short non-coding RNA (introns) that may reduce the expression of genes post-transcriptionally, thereby serving as oncogenes or tumour suppressor genes. Several miRNAs have been identified to be over- or under-expressed in OSA tumour tissue and cell lines, and these short nucleic acids hence represent another target for novel therapies.

Individualised therapy

The myriad of genetic and epigenic pathways likely to play a role in OSA and other cancers seem unlikely to be of equal importance in every individual diagnosed with one particular neoplasm. Rather, the relative contribution of aberrant signalling within each pathway that has the potential to promote carcinogenesis, invasiveness and metastases is likely to vary between individuals. The term theranostic profiling was coined to capture this concept (Tsimberidou et al., 2012; Egas-Bejar et al., 2014); optimal treatment should be individualised based on each patient's disease profile. By identifying mutations and altered regulation of pathways that promote or inhibit cancer development, the aberrant signalling can be targeted accordingly through drugs that inhibit or promote the expression of specific proteins. Herein lies the most promising area of novel cancer therapies. Traditional chemotherapy has not significantly improved the survival of OSA patients over the last decades, underlining the importance of continued investigation to identify new targets for therapy.

For the canine population, such individualised treatment protocols may be less feasible due to limitations of costs both concerning the extent of diagnostics and the optimisation of

treatment (i.e. the “theranostics”). Although costs may decrease with more accessible technology over time, and some owners have essentially unlimited funds for treatment of their dogs, priorities will differ from human cancer care. It is likely that a stricter selection of which patients to treat, as well as how aggressively or in what way, will remain a dilemma for the clinician when advising the owners about the most appropriate decision. For our veterinary patients, not only prognostic factors representing pathways that may be directly targeted by therapy (such as PTEN expression), but also factors that are likely to be a consequence rather than a mediator of disease progression (such as ALP elevation), will therefore continue to be important. It is not only a matter of choosing the right therapy, but also the “right” patients; identification of prognostic factors may help making this selection (Selvarajah and Kirpensteijn, 2010). Appropriate choice of whom to treat and how, may also improve the patients’ quality of life – both humans and canines – by hindering more aggressive treatment than necessary: some patients may be found likely to respond to lower doses of chemotherapy than the standard regimen - while those unlikely to respond to the standard therapy may be offered alternative protocols early on. Alternatively, for canines, euthanasia may be elected at an early time point if the prognosis is considered poor to grave.

Prognostic factors: general

Documented prognostic factors for canine OSA include patient characteristics (such as age), tumour characteristics (such as location, size, and histological grade), stage (e.g. visible metastasis or not), treatment, and clinicopathological parameters (Spodnick et al., 1992; Thompson and Fugent, 1992; Kirpensteijn et al., 2002; Loukopoulos and Robinson, 2007; Boerman et al., 2012). Increased alkaline phosphatase (ALP) activity and monocyte or lymphocyte numbers above a certain cut-off (despite being within the reference interval) have been linked to a worse prognosis in canines (Garzotto et al., 2000; Sottnik et al., 2010; Boerman et al., 2012). The negative outcome associated with higher ALP activity may attributed to a larger disease burden, with higher ALP activity as a consequence of increased metabolic activity in larger tumours or metastatic disease (Sternberg et al., 2013) – as is also the case in human OSA (Limmahakhun et al., 2011). In humans, elevated serum lactate dehydrogenase (LDH) has also been identified as a negative prognostic marker (Berner et al., 2015). Other prognostic factors similar in humans and dogs include patient age, tumour

characteristics, stage, and treatment of the disease (Berner et al., 2015; Hung et al., 2015). The recent meta-analysis of canine OSA identified serum ALP activity and tumour location (proximal humerus associated with a worse outcome; this is also shown in humans) as prognostic factors; older age did not reach statistical significance (Boerman et al., 2012).

Prognostic factors: clinical and histopathological

Clinical and histopathological prognostic factors identified in Paper IV were in line with those previously reported; univariable analysis identified ALP activity, histological grade, percent necrosis and mitotic index as potential predictors of DFI. Upon multivariable analysis, percent necrosis and mitotic index were statistically significancant factors. The amount of necrosis and mitotic index are both parameters that increase the histological grade and carry a poor prognosis (Grundmann et al., 1995; Kirpensteijn et al., 2002), as opposed to the percent necrosis seen after chemotherapy – which has been identified as a good prognostic factor in humans (Janeway et al., 2012). Postoperative wound infection following limb sparing surgery has also been identified as a favourable prognostic factor in humans and dogs (Lascelles et al., 2005; Jeys et al., 2007), whereas pathological fracture has been listed as a negative prognostic factor in humans (Scully et al., 2002; Bramer et al., 2007), though not consistently (Bacci, 2003). Among more recently identified prognostic factors in human OSA is the presence of OSA tumour cells as micrometastases in the bone marrow, which has been shown to correlate with survival (Bruland et al., 2009b). These have been identified by use of 2 monoclonal antibodies: one detecting an OSA surface antigen resembling BALP (TP-3), and one recognising a melanoma-associated surface antigen, but also binding to some sarcoma cells, including OSA (9.2.27) (Bruland et al., 2009b). A more intense treatment regime is selected when OSA tumour cells are detected in the bone marrow. In dogs diagnosed with OSA, presence of individual OSA tumour cells in the bone marrow has been documented by the same methods, using the TP-3 antibody (personal communication Thora J Jonasdottir; unpublished results). This may be used both prognostically and to guide the intensity of treatment in canine patients in the future.

Prognostic factors: molecular genetics

Several molecular or genetic factors have also been related to prognosis (Kong and Hansen, 2009). Germline mutations resulting in an increased risk of OSA have been mentioned

previously (e.g. mutations in the RB gene or p53 gene), as have protein expression in tumour tissue with implications for the prognosis (e.g. PTEN and ezrin). In our work presented in Paper IV, we identified expression of HES1 as a prognostic factor, and this is one of many genetic factors that may also be prognostic in human OSA (Hughes, 2009). Other molecular factors with a prognostic value – that may also serve as therapeutic goals – in humans include markers of chemotherapy resistance (e.g. GSTP1) (Pasello et al., 2008), chemokines (e.g. CXCR4) (Namlos et al., 2012), apoptosis inhibiting proteins (BIRC5/Survivin) (Osaka et al., 2006), metastatic markers (e.g. FAS/FASL) (Koshkina et al., 2007), and matrix metalloproteinases (e.g. MMP-1) (Uchibori et al., 2006). MicroRNAs forming part of the signalling network of tumour suppressor genes (such as p53) are another example of molecular prognostic factors that may serve as therapeutic targets (Tang et al., 2015; Wang et al., 2015). Telomere maintenance and chromosomal instability may also affect the development of OSA and have a prognostic value (Ulaner et al., 2003).

CONCLUSIONS

- Leonbergers and IW have a relatively high incidence rate of primary bone tumours. Pursuing a search for risk factors other than body size/weight is however supported by the significantly different risks of developing primary bone tumours between similarly statured dogs, like the NF and LB (Paper I). We hypothesise that the faster growth rate in LB compared to NF may play a role in the difference in risk between these two breeds.
- Leonbergers that develop primary bone tumours are heavier during the growth period and early adult life than LB that do not develop this disease (Paper II).
- A risk reduction in human OSA as a primary malignancy at older ages was observed, and could possibly be related to diminished exposure over time to bone-seeking radionuclides (Paper III).
- An increase in human CSA among females was observed, corresponding to birth cohorts with rising exposures to oral contraceptives and menopausal hormonal therapy, suggesting that exposure to exogenous oestrogen could be a risk factor (Paper III).
- Activation of Notch signalling may contribute to the development of canine OSA. However, the association between low HES1 expression and shorter DFI suggests that mechanisms that do not alter HES1 expression may drive the most aggressive tumours (Paper IV).
- Expression of HES1 may be a prognostic factor in canine OSA (Paper IV).

FUTURE PERSPECTIVES

- More work is needed to better understand the likely multifactorial and complex aetiopathogenesis of primary bone cancer, both in humans and dogs.
- Studies designed to look at as exercise during the growth period in canines predisposed to primary bone cancer could further elucidate the role of weight-bearing stress as a risk factor for OSA.
- The role of oestrogen in CSA development and progression implies that its inhibition as a therapeutic option for CSA patients should be further investigated, despite the lack of promising results in one pilot study.
- The value of differential expression of the Notch signalling pathway as a prognostic factor should be further investigated in dogs and humans, as it may provide prognostic information as well as represent a target for novel treatments (GSI and mTOR inhibitors)
- The high prevalence of bone tumours within some canine families (such as 2 of the LB litters in Paper II), represents a unique opportunity for comparison of molecular factors between otherwise genetically similar individuals – with and without OSA. New molecular technology, such as PCR arrays, enables us to efficiently screen and quantify a large number of genetic expressions. Using this technology on samples from highly affected canine families would be an appealing option to identify further prognostic factors and therapeutic targets.

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Paper I

Breed-specific incidence rates of canine primary bone tumors — A population based survey of dogs in Norway

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Abstract

This is one of few published population-based studies describing breed specific rates of canine primary bone tumors. Incidence rates related to dog breeds could help clarify the impact of etiological factors such as birth weight, growth rate, and adult body weight/height on development of these tumors. The study population consisted of dogs within 4 large/giant breeds; Irish wolfhound (IW), Leonberger (LB), Newfoundland (NF), and Labrador retriever (LR), born between January 1st 1989 and December 31st 1998. Questionnaires distributed to owners of randomly selected dogs — fulfilling the criteria of breed, year of birth, and registration in the Norwegian Kennel Club — constituted the basis for this retrospective, population-based survey. Of the 3748 questionnaires received by owners, 1915 were completed, giving a response rate of 51%. Forty-three dogs had been diagnosed with primary bone tumors, based upon clinical examination and x-rays. The breeds IW and LB, with 126 and 72 cases per 10 000 dog years at risk (DYAR), respectively, had significantly higher incidence rates of primary bone tumors than NF and LR ($P < 0.0001$). Incidence rates for the latter were 11 and 2 cases per 10 000 DYAR, respectively. Pursuing a search for risk factors other than body size/weight is supported by the significantly different risks of developing primary bone tumors between similarly statured dogs, like NF and LB, observed in this study. Defining these breed-specific incidence rates enables subsequent case control studies, ultimately aiming to identify specific etiological factors for developing primary bone tumors.

Résumé

Cette étude est l'une des rares publiées décrivant les taux de tumeur osseuse primaire canine spécifiques de race. Les taux d'incidence relatifs aux races de chien pourraient aider à clarifier l'impact de facteurs étiologiques tels que le poids à la naissance, le taux de croissance et le ratio poids corporel/taille à l'âge adulte sur le développement de ces tumeurs. La population à l'étude était composée de chiens parmi les 4 races de chien grandes/géantes; le lévrier irlandais (IW), le Leonberger (LB), le Terre-Neuve (NF) et le Labrador (LR), né entre le 1er janvier 1989 et le 31 décembre 1998. Des questionnaires distribués aux propriétaires de chiens sélectionnés au hasard — répondant aux critères de race, année de naissance, et enregistrement au Club Canin Norvégien — ont constitué les éléments pour cette étude rétrospective. Sur les 3748 questionnaires soumis aux propriétaires, 1915 ont été complétés, donnant un taux de réponse de 51 %. Quarante-trois chiens ont été diagnostiqués avec des tumeurs osseuses primaires, en fonction de l'examen clinique et des examens radiologiques. Les races IW et LB, avec respectivement 126 et 72 cas par 10 000 années-chien à risque (DYAR), avaient des taux d'incidence de tumeurs osseuses primaires significativement plus élevés que les races NF et LR (P < 0,0001). Les taux d'incidence pour ces derniers étaient respectivement de 11 et 2 cas par 10 000 DYAR. La recherche de facteurs de risque autres que le ratio taille/poids est supportée par les risques significativement différents observés dans la présente étude de développer des tumeurs osseuses primaires parmi les chiens de statures similaires tels les NF et LB. La définition de ces taux d'incidence spécifiques de race permettra des études cas-témoins ultérieures visant à identifier les facteurs étiologiques spécifiques pour le développement des tumeurs osseuses primaires.

(Traduit par Docteur Serge Messier)

Introduction

Osteosarcoma (OS) is the most common histological subtype of primary bone cancer both in humans and dogs (1–3). Although multi-agent chemotherapy has greatly improved the outcome among human patients, mortality is still high (2,4). Five year overall survival rates range from about 15% to 70% for patients with and without visible metastases at the time of diagnosis, respectively (2,5). Adding to the severity of this disease, it typically affects children and adolescents, constituting about 5% of pediatric cancers (6).

Osteocarcoma accounts for 80% to 90% of canine primary bone tumors (7,8). Although rare in the canine population, the rate outnumbers that of the human population, with a lifetime incidence risk about 30 to 50 times higher within the overall canine population (3,9). Breed-specific incidence rates of OS differ largely, and estimates within certain breeds even show a lifetime risk exceeding

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Table I. Total number of dogs from each breed studied that were registered in the Norwegian Kennel Club

	Year of birth										
Breed	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	Total
Newfoundland	326	364	286	288	177	183	242	217	216	224	2523
Leonberger	133	181	277	277	105	140	236	180	204	245	1978
Labrador retriever	688	720	780	523	586	550	644	546	591	644	6272
Irish wolfhound	67	53	52	58	37	58	58	72	64	58	577
Total	1214	1318	1395	1146	905	931	1180	1015	1075	1171	11 350

10%, thereby affecting a substantial number of these dogs (9,10). Median survival time for dogs with primary bone cancer of the appendicular skeleton, treated with surgery and chemotherapy, ranges from 5 to 13 mo provided there are no visible metastasis at the time of diagnosis, in which case median survival time drops to about 2 mo (8,11).

Most commonly, OS is diagnosed in middle-aged to older dogs, with a median age of 7 y (8). A smaller peak in age incidence at 18 to 24 mo corresponds with the human peak incidence at late puberty, which has led to the hypothesis of skeletal growth parameters representing some of the possible etiological factors for developing this disease (8,12–14). It is well-recognized that giant and large breed dogs are at increased risk of developing OS (8); however, body size alone cannot explain the variation in incidence between different breeds of dogs, as the risk appears to differ extensively among certain breeds of similar body size (1,13,14). Epidemiological studies on human OS have also failed to show a strong correlation between body weight or height and risk of developing OS (12,14,15).

Spontaneous OS in dogs resembles that of human OS in several aspects. Both species develop these tumors most commonly in the metaphysis of long bones, with micro metastases at the time of diagnosis, and overt lung metastases as the main cause of mortality (3,16). Similar response to chemotherapy makes diseased dogs valuable contributors to the process of developing new anti-cancer therapy (3,17–20). As the biological behavior is similar in dogs and humans, common risk factors for developing the disease can be expected. Hence, information on incidence related to specific breeds of dogs might help clarify the supposed correlation between birth weight, growth rate, adult body weight or height, and the development of OS. In this context, recognizing breeds of similar stature having significantly different incidence rates of OS is of particular interest.

Ten years of litter registrations of 4 large and giant dog breeds in Norway constituted the basis for this survey, aiming to describe the incidence rate of primary bone tumors within each breed and to estimate possible differences between these breeds. To understand the implication of inherent and environmental risk factors for primary bone tumors, one should be familiar with the natural occurrence of the disease in the particular reference population.

The few studies on the occurrence of primary bone tumors and breed-specific lifetime risks and/or incidence rates in the canine population, have been based upon insurance data or pathology registers (9,10). To our knowledge, there are no previously published population-based studies describing breed specific rates of canine primary bone tumors. Estimating the incidence rate of such tumors within these 4 breeds (in Norway) will thus be of importance for further studies of this population, with the ultimate goal of identifying specific risk factors in disease development.

Materials and methods

Study population

The study population consisted of purebred dogs registered in the Norwegian Kennel Club (NKC), born between January 1st 1989 and December 31st 1998. Breeds enrolled in the study were the Irish wolfhound (IW), Leonberger (LB), Newfoundland (NF), and Labrador retriever (LR). At the initiation of the survey, none of the dogs included would have been younger than 10 y.

Sample size

In estimating the appropriate sample size, the main criterion was attaining a high probability of detecting a difference in breed specific incidence rates, provided it was substantial enough to be clinically relevant. A second criterion was to enable an expectation of at least 10 dogs diagnosed with primary bone tumors within each breed. Power was set at 0.80 and calculations were conducted using a statistical program (Stata 10.0; StataCorp, College Station, Texas, USA).

Based upon estimates from previous publications, a lifetime risk within the IW population of 8% to 10% was presumed (9,10). For sample size calculations, the lifetime risk in this breed was set to 8%, as this would require the largest sample. Further, a maximum lifetime risk within the LB and NF of 3%, and about 1% for the LR, was assumed (9,10).

All registered IW were included in the sample population, and sample sizes of NF and LB were calculated accordingly. Anticipating a response rate from owners of sampled dogs of about 50%, computed sufficient sample sizes were multiplied by 2. Hence, about 1/2 the total number of IW; 300, was used as a fixed sample size of IW. Power calculations show that a sample size of 450 dogs from NF and LB yields a power of 0.81 provided a lifetime risk of 3% within these breeds. The study population consisting of a limited number of dogs was accounted for using the formula for the finite population factor (FPC):

$$n' = 1/(1/n + 1/\mathrm{N}) \quad \textit{Equation 1}$$

where: n' = the final size of the sample population, n = the number of dogs needed from an infinite population (in this case 450), and N = the number of dogs in the study population (21). Performing this calculation, n' equalled 367 and 382 for LB and NF, respectively. As

the expected lifetime risk in the LR population was 1%, a large sample size to prove a difference in lifetime risks was not needed; however, 1000 dogs were included so that a minimum of 10 LR positive of primary bone tumors could be expected. Considering the expected response rate of 50%, the computed sample sizes of LB, NF, and LR were multiplied by 2, while the total number of IW was 577.

Sampling of study population

The total number of registered dogs from each breed forming the study population is shown in Table I. Stratified by year of birth, the minimum number of dogs providing the desired power was calculated for each of the 10 y included. Within each breed, the largest number of dogs required from any of these years was then sampled by computerized random sampling; that is, the same number of dogs was sampled from each year within one specific breed, constituting a sample population of 4868 dogs. However, a number of dogs had to be excluded: 149 dogs were excluded as their owners were no longer alive, 34 dogs were excluded due to their owners living outside of Scandinavia, and 305 LR were registered guide dogs whose owners could not be traced. The final number of dogs in the study population was 4380; 2119 males and 2261 females.

Study design

This study was designed as a retrospective, descriptive survey based upon questionnaires distributed to owners, or previous owners, of dogs from the 4 actual breeds. Recipients not responding to the request received one reminder. Owners whose dog was no longer alive were asked to describe cause of death or euthanasia. All recipients were asked specifically whether their dog had suffered from any cancer- and/or tumor-related disease, as the main objective was identifying dogs euthanized due to primary bone tumors. They were also requested to state general health information regarding vaccination intervals, neutering status, hormone treatments, breeding history, and occurrence of any chronic diseases. History of skeletal diseases such as fractures, arthroses, and osteochondrosis was also included in the forms. Owners whose dog had suffered from primary bone tumors were questioned further on the diagnosis, histological classification, whether or not metastases were detected, location of the primary tumor, and what kind of treatment their dog had received, if any. As the owners were also asked to state the name of the veterinarian or veterinary clinic that diagnosed the dog, the diagnosis for a primary bone tumor could be confirmed by contacting each veterinarian regarding the basis for their diagnoses. For a dog to be included as positive of primary bone tumor, a description of typical clinical signs in addition to coinciding radiographic findings were considered to be sufficient. Cases where the diagnosis could not be confirmed by the veterinarian were not included as positive for the disease.

Ethical issues

In this observational study, no interventions affecting animal health were conducted. The only ethical issue of concern was that of confidentiality, as responses to the questionnaires distributed to dog owners might reveal sensitive information on the health status of their dog. All information obtained on individual dogs has been kept confidential and when results are presented, no information exposing the dogs' or their owners' identity is revealed. The NKC approved access to their registry of dogs, thus enabling contact with the dog owners.

Statistical analyses

Incidence rates are reported as number of cases per 10 000 dog years at risk (DYAR), and lifetime risks as the proportion of dogs with primary bone tumors, with 95% confidence intervals (CI) based on the Poisson distribution. Other proportions, such as response rates and localizations, are given with 95% CI based on the binomial distribution. When calculating the response rate, the number of forms returned as undeliverable plus the number of dogs whose registered owners did not possess any knowledge of the dogs in question was first subtracted from the denominator. This takes into account that these owners had no opportunity to respond, i.e. they were ineligible for the study and thereby this calculation probably serves as the best measure of the response rate (22). Age at time of diagnosis within each breed is given as median with range. A chi-squared (X^2) test was performed to test the hypotheses of differences in lifetime risks between subgroups of the study population, such as breed and gender; $P < 0.05$ was considered significant.

Results

Study population

Of the 4380 questionnaires initially distributed to previous or current dog owners, 534 were untraceable by the Norwegian phone and address registry and 98 were excluded, mostly due to uncertainty as to who took care of the dog after leaving its breeder, or after relocation at an early age. This resulted in 3748 forms received by dog owners; representing 1778 male and 1970 female dogs.

A total of 1915 questionnaires were completed and returned to the Norwegian School of Veterinary Science (NSVS), constituting an overall response rate of 51% (50% to 53%). A significant difference was observed between the response rate of the owners of LR, which had the highest proportion of responders, 53% (51% to 56%), and that of IW, displaying the lowest, 47% (42% to 53%), ($P = 0.03$). With respect to gender, the proportions of male and female dogs whose forms were returned were also significantly different ($P = 0.04$), owners of female dog showing a response rate of 53% (50% to 55%), whereas the corresponding ratio for the male dogs was 49% (47% to 52%). At the end of the study period, 291 dogs were still alive; 1, 38, 15, and 237 of the IW, NF, LB and LR, respectively. Average age at the time of death/euthanasia was 8.9 y (range: 8.7 to 9.0 y) for all breeds; 7.0 y (range: 6.6 to 7.4 y) for IW, 8.2 y (range: 7.8 to 8.5 y) for NF, 8.0 y (range: 7.7 to 8.3 y) for LB, and 10.2 y (9.9 to 10.5 y) for LR. For 197 dogs age at time of death was not reported.

Lifetime risks and incidence rates

Forty-three dogs fulfilled the criteria for being included as positive of primary bone tumors; the diagnosis based upon clinical examination and x-rays, yielding an overall lifetime risk of 2.3% (1.6% to 3.0%). Of these, the tumors of only 6 dogs were biopsied, from which the results of 4 dogs could be obtained. Three of these biopsies

Table II. Incidence rates of primary bone tumors as proportion of the total number of dogs and as number of cases per 10 000 dog years at risk (DYAR) within 4 breeds of dogs born between 1989 and 1998, and registered in the Norwegian Kennel Club

Breed	Number of dogs (DYAR) among the responders	Number of dogs with primary bone tumors	Rate (%) (95% CI)	Rate per DYAR (95% CI)	Median (range) age (years) at diagnosis
Irish wolfhound	169 (1187)	15	8.9 (5.0–14.6)	126 (71–208)	5.5 (3.3–8.4)
Leonberger	381 (3074)	22	5.8 (3.6–8.7)	72 (45–108)	7.2 (1.6–10.1)
Newfoundland	427 (3574)	4	0.9 (0.3–2.4)	11 (3–29)	8.8 (4–11.5)
Labrador retriever	938 (9798)	2	0.2 (0.03–0.8)	2 (0.3–7)	11.6 (11.6)

CI — confidence interval.

yielded OS, and one, observed in the frontal bone of a male NF, was diagnosed as a multilobular osteochondrosarcoma.

Of the 1385 dogs whose owners responded to the first request, 29 dogs, 2.1% (1.4% to 3.0%), had suffered from primary bone tumors. The number of affected dogs among the 530 responses to the reminder, was 14; 2.6% (1.4% to 4.4%).

The highest incidence rates of primary bone tumors were found in IW and LB; incidence rates within these breeds estimated to be approximately 11 and 7 times higher, respectively, than in NF, and about 60 and 35 times higher, respectively, compared to LR (Table II). Thus, incidence rates of primary bone tumors among IW and LB were found to be significantly higher than those of NF and LR ($P < 0.0001$).

No significant gender differences could be found ($\chi^2 = 0.16$, $P = 0.69$), as 21 male, 2.4% (1.5% to 3.7%), and 22 female, 2.1% (1.3% to 3.2%), dogs among the responders suffered from primary bone tumors. Median age at time of diagnosis was 6.7 y (range: 1.6 to 11.6 y). This was similar among male and female dogs; 6.7 y (range: 3.7 to 11.6 y) and 6.6 y (range: 1.6 to 11.6 y), respectively, and there were no significant differences between the breeds (Figure 1).

Distal radius/ulna, distal tibia and distal femur were the most common sites of the primary tumor, encompassing 35% (21% to 51%), 19% (8.4% to 33%), and 16% (6.8% to 31%) of the tumors, respectively. Most of the tumors, 86% (72% to 95%), originated in the appendicular skeleton. Only 12% (3.9% to 25%) occurred in the axial skeleton, including scapula, and 2.3% (0.1% to 12%) in the pelvis (Figure 2). In this study, no correlation was found between primary bone tumors and health-related aspects such as vaccination status, hormone treatments, chronic diseases, cancers (with primary tumor unrelated to bone) or orthopedic injuries (data not shown). The proportion of neutered dogs was similar among those diagnosed with primary bone tumors, 16.3% (7/43), and those without this diagnosis, 13.1% (245/1872). Median and mean age at time of neutering was between 5 and 6 y for both groups.

Discussion

The overall response rate of about half the sample population corresponds with our expectations, as there has been a decline in survey response rates during the past decades (22). Response rate has traditionally been used as a measure of the quality of surveys; higher response rates indicating more reliable results (23). Identifying bias, that is, a difference in responders versus non-responders, is difficult — resulting in the use of response rate as an easily obtained measure of quality (23). However, there is not necessarily a direct correlation between response rate and bias of the results (23). Aiming to evaluate the amount of bias, one can compare early versus late responders, assuming late responders to be representative of the non-responders (24). Applying this to the present study, the proportion of dogs suffering from primary bone tumors within each of these 2 groups was found to be similar; supporting the assumption of response bias being low in this survey.

Responders could be more concerned with their dog's overall health than non-responders. Moreover, it would be reasonable to expect owners whose dog had suffered from primary bone tumors to take a personal interest in research related to this disease and consequently being more likely to respond. If so, this would lead to an overestimation of the incidence rate(s). A slightly higher, but significant, response rate was found among the owners of the breed least affected by primary bone tumors, LR, than among those of the most commonly affected breed, IW. Consequently, the estimated incidence rates are probably not overestimated.

The principal finding of this population-based study of breed specific incidence rates of canine primary bone tumors, is that there is a large variation in rates between the different breeds IW, LB, NF, and LR. The incidence rate in the largest breed (IW) is some sixty-fold greater than in the smallest one (LR); however, factors other than a large body size or weight also pose an increased risk for developing the disease. This is evidenced by a significantly higher incidence rate found in LB compared with NF, 2 breeds of similar size and stature. Growth rate is one possible factor contributing to the observed difference between these breeds. It has been shown that NF, when accounting for differences in adult body weight, has the slowest growth rate among the 4 breeds included in this study, while the LB reaches adult body weight after approximately the same number of days as the LR — a considerably smaller breed (25). Irish wolfhounds, having the highest adult body weight, also reaches this point in a short period of time, compared with the NF and LR (25).

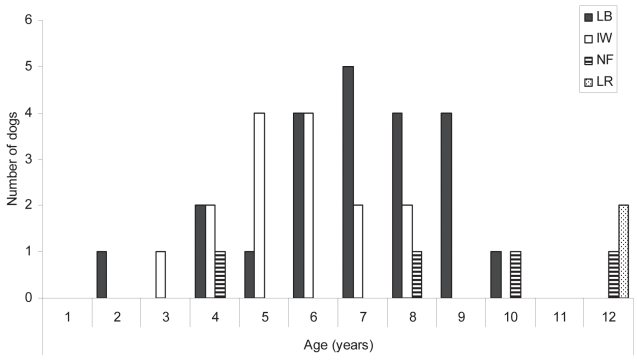

Figure 1. Age at time of diagnosis of primary bone tumors in 4 breeds of dogs: Leonberger (LB), Irish wolfhound (IW), Newfoundland (NF), and Labrador retriever (LR), born between 1989 and 1998, and registered in the Norwegian Kennel Club.

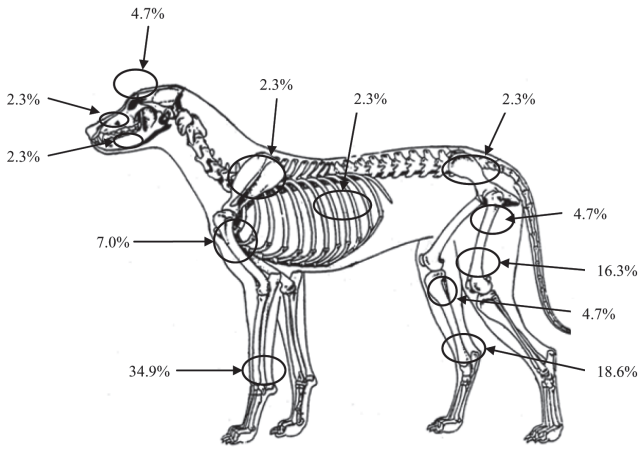

Figure 2. Anatomical location of primary bone tumors among 4 breeds of dogs born between 1989 and 1998, and registered in the Norwegian Kennel Club.

Thus, of the 4 breeds validated, the 2 breeds showing the lowest incidence rates of OS have the slowest growth rate.

Previous estimates of incidence risk correspond with the result of the present study, as IW is one of the breeds commonly reported to be at high risk of developing bone tumors (9,10,13). Although Egenvall et al (10) found a somewhat lower incidence rate for IW and LB, and slightly higher for NF than in the present study, the confidence intervals for these estimates are largely overlapping when comparing the 2 studies. Not surprisingly, LR had a significantly lower risk of primary bone tumors than IW and LB (13), although this breed has also been found to be well-represented among bone tumor patients (11,26).

Some breeds, such as the great dane, St. Bernard, and greyhound, are also observed to be at high risk for developing primary bone tumors (1,10,11,13). However, breed specific incidence rates are often not known (27) and, with a few exceptions (28), most estimates are not population-based. Previous studies aiming to describe the epidemiology of this disease have mostly been based upon insurance, and clinical or pathology records (1,10,26,29).

Epidemiologic canine cancer studies have typically employed clinical records, mainly from larger referral clinics or veterinary teaching hospitals (1,26). Several studies have been founded on the Veterinary Medical Data Program, established in 1964 by the National Cancer Institute (US), collecting data from participating veterinary teaching hospitals (13,30). Despite the advantage of good clinical data, often with an accurate diagnosis including biopsy and staging, this method suffers from not being based on an unselected population sample (29). This is because dogs referred to these clinics are more likely to suffer from severe disease and that some of these centers have specialized in oncology. Also, dogs with cancerous disease in which radical treatment is warranted, such as primary bone cancer, may be overrepresented at oncology referral centers, compared with dogs suffering from cancers that respond well to more conventional therapy, such as lymphoma. Studies based upon pathology records also encounter the problem of defining the reference population, as most pathology registers only include dogs in which biopsy and/or autopsy were performed.

Egenvall's study on canine primary bone tumor epidemiology utilized the database of Sweden's largest companion animal insurance company, Agria (10). This company serves about 30% of the Swedish dog population, providing a relatively representative sample (31). However, some discrepancies between this sample population and the reference population exist. Insured dogs tend to be somewhat younger than the total canine population (32). By only including dogs with life-insurance, which does not apply after the dog is 10 years old, Egenvall's study probably underestimated the rate of primary bone tumors in breeds developing the disease later in life. This is a possible explanation for the higher incidence rate in LR observed in the present study, although the 2 cases identified in this study are far too few to convincingly estimate median age at diagnosis. Further, diagnoses obtained from insurance records are based upon the treating veterinarian's evaluation, regardless of the extent of diagnostic aids, such as radiography and biopsy.

In most dogs diagnosed with primary bone tumors in this study a histological diagnosis was lacking, and the diagnosis was based upon evaluation of the presenting clinical signs and radiography. Histopathology would have strengthened the results presented, but the retrospective design of the study precludes the possibility of obtaining biopsy specimens. However, it can be argued that the clinical signs, including rapid progression of the disease along with typical radiographic findings, strongly favor the probability of OS, which is well known to be the most common canine primary bone tumor; accounting for up to 85% of these tumors (1,8,33). Three out of 4 cases, 75%, being histologically confirmed as OS in this study can probably be explained by the low number of histological diagnoses.

Most cases of primary bone tumors in this study were seen in middle aged to older dogs, the only non-giant breed included (LR) being affected at an older age than the 3 giant breeds (IW, LB, and NF). This corresponds with previous observations of giant breeds being diagnosed with this disease at an earlier age than large breeds, such as the LR (1,29,34,35). Also, the LR is eligible for developing disease at higher ages, simply due to its longer life span. Having a bimodal occurrence in humans, the second, smaller peak after the age of 60 corresponds to this peak incidence seen in middle-aged to older dogs (36). A small increase in age-specific incidence rates has

also been seen in dogs of 1 to 2 years of age (34), consistent with the peak incidence among humans. This was not observed in the present study; probably due to a low number of cases.

Distal radius/ulna is the most common site of primary bone tumors according to the literature (1,10,27,34). With > 1/3 of the primary tumors occurring at this site, the present study found a stronger predilection for the distal forelimb than the latter reports — apart from Brodey, who found a similar (1,33), or an even higher (37), proportion of these tumors originating in radius/ulna (distal not specified). The higher prevalence of tumors of the front limbs, especially in large- and giant-breed dogs, has been related to the hypothesis of weight bearing stress as one possible etiological factor. Brodey (1) and Brodey and Riser (33) also reported a strong predilection for this site in giant-breed dogs. Distal femur and distal tibia are previously described as 2 frequently affected locations, while affection of the proximal humerus was found in a lower proportion of cases than generally reported (1,10,27,29,37,38). Interestingly, the primary tumor of both diseased LR was located in the axial skeleton, coinciding with axial involvement being more common among smaller breeds — considering LR to be "smaller" in this context (1,26,29,33,34). However, the number of LR diagnosed with primary bone tumors included in this study is too low to elaborate on this observation.

The results obtained in this survey correspond to previous studies with respect to gender, location of the primary tumor, and median age at the time of diagnosis. In agreement with prior observations, no gender predisposition could be found (13,35). Although several studies have concluded that male dogs are more often affected than their female counterparts (1,33,37,38), this is not a consistent observation, as it is for human OS; Brodey and Riser (33) reporting female St. Bernards to be affected more frequently than male, and Heyman et al (26) observing twice as many females as males when studying pathology records of axial OS. Some studies have shown an increased risk of primary bone tumors in neutered dogs, especially when this procedure is performed at an early age (13,39). Due to the Norwegian animal protection law, prohibiting neutering except for health related purposes, most dogs in Norway are intact, and the mean age at neutering is relatively high — which was also observed in the present survey. As expected, this study therefore could not support this hypothesis. This study estimates lifetime risks and incidence rates for canine primary bone tumors within IW, LB, LR, and NF. As one of few population-based surveys, it provides a valuable contribution to the knowledge on each of these breeds' risks of developing such tumors. Further pursuing the search of risk factors other than body size or weight is encouraged by the observation of similarly statured dogs, NF, and LB, displaying significantly different risks. Defining these breed specific risks enables subsequent case control studies to be conducted, ultimately aiming to identify specific risk factors for this disease.

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Paper II

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Primary bone cancer in Leonbergers may be associated with a higher bodyweight during adolescence

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ABSTRACT

Weight-bearing stress may be a risk factor for both human and canine primary bone cancer. A cohort of Leonbergers (LB) was followed from birth to death and the cause of death recorded. We hypothesised that dogs dying due to primary bone cancer would be larger; measured by bodyweight (BW) and the circumference of the distal radius and ulna (CDRU) than those of the same breed that died of other causes. Information obtained from breeders, owners and veterinary surgeons were questionnaire-based. The dogs were examined by a veterinary surgeon at pre-specified "observational ages" (3, 4, 6, 12, 18, and 24m). Data were recorded, including BW and CDRU. The study population consisted of 196 LB, 9 of which died due to primary bone cancer (6 males, 3 females). Individual growth curves, showing BW and CDRU during the first 2 years of life, were made for these 9 dogs and compared to gender-specific mean values for LB that died from other causes. These curves showed that LB succumbing to primary bone cancer generally had a higher BW during the growth period than the remaining dogs, and that this difference appeared to be largest in the male LB. Male LB that developed primary bone cancer later in life also had a larger CDRU during most part of this period, as compared to those that did not develop this disease. Logistic regression showed a statistically significant effect of BW on the odds ratio of developing primary bone cancer at 12m and 18m and of CDRU at 18m, and a Poisson regression verified consistency of these results. At these ages, an increase in BW of 1 kg yielded a nearly 20% higher risk of developing primary bone cancer, while a 1 cm larger CDRU was associated with a nearly 70% increased risk. These findings support that weight-bearing stress during the period of high proliferative activity in the long bones associated with growth may increase the risk of canine primary bone cancer.

1. Introduction

Osteosarcoma (OSA) is the most common histological subtype of primary bone cancer in both humans and dogs (Brodey et al., 1963; Dorfman and Czerniak, 1995; Fletcher et al., 2013), and its incidence rate in the canine population outnumbers that of the human (Withrow et al., 1991). Certain canine breeds have a lifetime risk of OSA approaching

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10% (Egenvall et al., 2007; Anfinsen et al., 2011). Similarities between human and canine OSA include the predilection for tumours at the metaphysis of long bones, the majority of patients having pulmonary micrometastases at the time of diagnosis, and a similar response to chemotherapy. In both species there are two peaks in the age-specific incidence rates; one during puberty and one among middle-aged to older individuals. While the first of these two peaks is higher in humans, the second is largest in dogs (Withrow et al., 1991; Ehrhart et al., 2013; Berner et al., 2014). Since humans and dogs also share the same environment, observations in dogs may generate hypotheses regarding risk factors for development of OSA in humans.

Large- and giant-breed dogs are generally at higher risk of developing OSA (Ehrhart et al., 2013). There are, however, differences in incidence rates between similarly sized large and giant breeds (Brodey et al., 1963; Ru et al., 1998; Anfinsen et al., 2011) indicating that other breed-specific traits contribute to the overall risk. Hence, this limits assessment of body size as an independent risk factor without correcting for the breed. Relatively few studies have investigated bodyweight (BW) or height as risk factors for OSA within individual breeds, and results of these studies have so far been inconclusive (Ru et al., 1998; Cooley et al., 2002; Rosenberger et al., 2007).

Four large to giant breeds; Irish wolfhound, Leonberger, Newfoundland, and Labrador retriever, were included in a larger project designed to describe growth parameters and several diseases that may be influenced by factors early in life (Trangerud et al., 2007a). The objective of the present study, which is founded on the database from the larger project, was to identify risk factors for development of primary bone cancer related to growth. Due to a low number of primary bone cancer cases in the 3 other breeds in the larger database, only results for the Leonberger (LB) are presented in this manuscript. Using prospectively collected data on birth weight and growth parameters during the first 2 years of life, we sought to describe the growth pattern of LB developing primary bone cancer later in life compared to the growth of other LB. We hypothesised that dogs developing primary bone cancer would be larger (measured by BW and the circumference of the distal radius and ulna, CDRU) than LB that died of other causes.

2. Materials and methods

This study was conducted in agreement with the provisions enforced by the National Animal Research Authority.

2.1. Study design

The present study is part of a larger prospective observational study from our department (the *main study*), designed to investigate growth patterns and other risk factors for development of different skeletal diseases, i.e. panosteitis, hip dysplasia, elbow dysplasia, and primary bone cancer (Trangerud et al., 2007a,b; Krontveit et al., 2010).

2.2. Study population

Newfoundland, Labrador retriever, Leonberger, and Irish wolfhound dogs born in Norway between November 1998 and June 2001 and registered in the Norwegian Kennel Club were eligible for inclusion into the *main study*, and all breeders of these dogs were invited to participate in the study. Seven hundred dogs from 107 litters were included at birth, representing approximately 23% of the total number of litters of these breeds born in Norway during the actual time frame (Trangerud et al., 2007a). For a breed to be included in the present study, the criterion was having more than 2 cases of primary bone cancer registered in the main database, limiting further analyses to the Leonberger (LB) breed. For a dog to be diagnosed with primary bone cancer (BC+ group), typical clinical signs, physical examination findings (performed by a veterinary surgeon), and consistent radiographic changes were sufficient. Individual inclusion criteria were at least one BW recording between the age of 3 and 24 months (m) and a known cause of death (a definitive diagnosis was not required, but it had to be known whether each dog died because of primary bone cancer or due to another disease). Those dogs that were not reported to have died from primary bone cancer constituted the controls (BC− group).

By the time of study completion in 2014, all dogs included in the study were assumed dead. Owners of dogs for which time or cause of death were not registered in the database, were contacted by telephone or e-mail to obtain this information. Dogs for which the cause of death could not be obtained from the owner or the veterinary practice were excluded.

2.3. Questionnaires

Information provided by breeders, owners, and veterinarians was questionnaire-based. The breeders provided information about bedding material, diet, and weekly BW during the dogs' first 8 weeks (w) of life. All dogs stayed with the bitch until they were sold at approximately 8w of age, after which time clinical and husbandry information was recorded by the attending veterinarian and the owner at certain time points ("observational ages"): At 3, 4, 6, 12, 18, and 24m of age. Each breeder and owner decided on housing and feeding regimes according to own preferences. The owners agreed to have their dogs examined by a veterinary surgeon at the observational ages. This examination included BW (kg) recording, tape measurement of the circumference of the right thoracic limb at the level of the distal radius and ulna (CDRU; cm), blood samples (serum and EDTA), and a radiograph of the right forearm. The latter was a mediolateral projection, with the elbow flexed, and made at a film-to-focus distance of 100 cm. Based on these radiographs, the length of ulna was measured (cm). After 24m of age, the owners completed yearly questionnaires regarding their dog's health status and eventually time and cause of death or euthanasia.

2.4. Statistical analyses

The software package Stata 12 (Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA) was used for all analyses.

The lifetime prevalence (LTP) of primary bone cancer was calculated by dividing the number of dogs with this disease (BC+ group) with the total number of dogs from the same breed in the cohort, and reported with 95% confidence intervals (CI) using the exact binomial (i.e. Clopper–Pearson) method. To determine if a gender predisposition could be found in this study, a Fisher's exact test was performed. Age at time of death by gender, is reported as median with range for the BC+ group, and as median for the BC− group.

For graphic presentation of BW, ages between 0 days (d) and 3m were omitted. Mean BW and CDRU values from birth (BW) or 3m (CDRU) until 24m were calculated separately for male and female LB in the BC− group, and displayed graphically alongside individual curves for each of the BC+ dogs. The graphs were made in Microsoft Excel 2013. Missing values between two BW or CDRU recordings for any of the BC+ dogs were interpolated using the NA function in Excel.

Logistic regression [Y(bone cancer) = β_0 + β_1*gender + β_2*BW (or CDRU); Y = the dependent variable (i.e. primary bone cancer); β_1 and β_2 denote the respective regression coefficients for the independent variables: gender; female (reference category)/male and BW or CDRU respectively] was used to assess the association between primary bone cancer and BW and CDRU at 3, 6, 12, 18 and 24m of age, while controlling for gender. This model assumes that BW (or CDRU) has a linear effect on the risk of developing primary bone cancer; i.e. that an increase in BW from 12 to 13 kg has the same effect as an increase from 14 to 15 kg. Acceptable linearity was confirmed by categorising the independent variable and assessing the trend in odds, using the lintrend command in Stata™ 12. The level of significance was set to $P < 0.05$. For statistically significant results, receiver operating characteristic (ROC) curves were created to estimate the fit of our model, reported as area under the curve (AUC). In light of the rare outcome, a Poisson regression was performed (reported as incidence rate ratio; IRR) to confirm consistency of the results. To check for overdispersion of this model, a Pearson goodness-of-fit statistic was computed.

3. Results

Leonberger was the only breed with more than 2 cases of primary bone cancer recorded in the database from the *main study* and was hence the only breed that fulfilled the inclusion criterion for breed. A total of 251 LB puppies from 35 litters were included at birth. Thirty-one dogs (12 males, 19 females) did not have any BW recordings between the age of 3 and 24 months, and were not included in the study population. For 24 dogs (14 males, 10 females), the cause of death was unknown, and these dogs were excluded. The remaining 196 dogs (91 males, 105 females) constituted the study population.

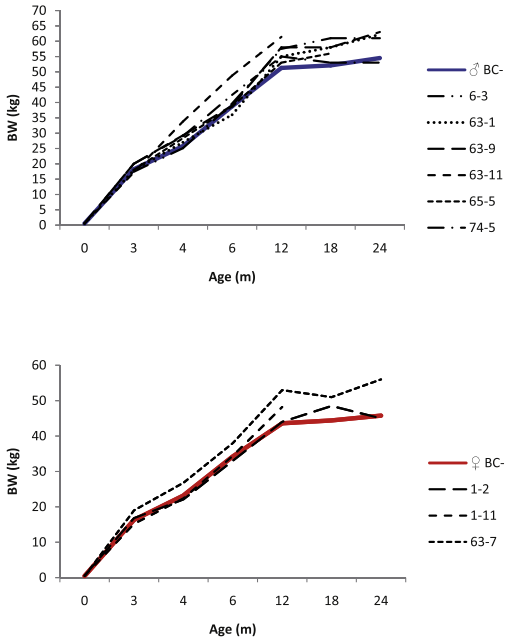

Fig. 1. Mean bodyweight (BW), from birth until 2 years of age (m = months), for male (upper panel; blue line; $n = 85$) and female (lower panel; red line; $n = 102$) Leonbergers that were not diagnosed with primary bone cancer (BC−) versus individual dogs that were euthanized due to this disease ($n = 6$). Each dog diagnosed with primary bone cancer is identified by its unique ID number; the first part of this number identifies the litter, while the second number identifies the individual dog. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Of the 196 LB included in the present study, 9 had been euthanized due to primary bone cancer (BC+ group), yielding a LTP of 4.6% (CI 2.1–8.5%) (Table 1). Six males (6.6%; CI 2.5–13.8%) and 3 females (2.9%; CI 0.6–8.1%) were affected. This was not consistent with a significant gender predisposition in this population of LB dogs ($P = 0.31$). Median age at time of death due to primary bone cancer was 6y 4m (range 3y 8m–9y 9m), and was similar for males and females (6y 6m and 6y 3m, respectively).

The remaining 187 dogs constituted the BC− group (85 males, 102 females). Median age at time of death in this group was 6y 9m. For 10 LB in the BC− group, time of death was unknown. These dogs were excluded from the lifetime analyses.

Figs. 1 and 2 show the BW (from birth) and CDRU (from 3m) until 24m for each gender. For male LB, the individual growth curves for each BC+ dog show a generally higher BW throughout the timeline than the mean values for the BC− group (Fig. 1). The difference between BC+ and BC− dogs for the female LB is also shown. Male LB diagnosed with primary bone cancer later in life had a larger CDRU for most part of the growth period compared to the mean CDRU of BC− males, but no such difference was apparent for the females (Fig. 2).

Logistic regression showed a statistically significant effect of BW on the odds ratio of developing primary bone cancer at 12m ($P = 0.012$) and 18m ($P = 0.026$) and of CDRU at 18m ($P = 0.033$) (Table 2). At these ages, an increase in BW of 1 kg yielded a nearly 20% higher risk of developing

Table 1
Characteristics of 9 Leonbergers diagnosed with primary bone cancer. Each dog is identified by its unique ID number; the first part of this number identifies the litter, while the second number identifies the individual dog.

ID	1-2	1-11	63-7	6-3	63-1	63-9	63-11	65-5	74-5
Gender	F	F	F	M	M	M	M	M	M
Size of litter	10	10	10	9	10	10	10	8	13
Age at time of death	6y 4m	5y 5m	9y 4m	7y 1m	5y 11m	9y 9m	3y 8m	9y 2m	5y 0m
Tumour location	Distal femur (L)	Distal radius (L)	Distal radius (L)	Distal radius (L)	Distal radius (R)	Distal radius (R)	Distal radius (R)	Distal radius (R)	Distal radius (nn)
Radiographs	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Histopathology	No	No	No	No	No	No	OSA	No	No

Table 2
Logistic regression evaluating the association between primary bone cancer and bodyweight (BW) and circumference of the distal radius and ulna (CDRU), respectively, while controlling for gender, in Leonbergers at the ages listed. Y(bone cancer) = $\beta_0 + \beta_1$*gender + β_2*BW (or CDRU); β_1 and β_2 denote the respective regression coefficients for the independent variables [β_1 = the regression coefficient for gender, female/male; female = reference category; β_2 = the regression coefficient for BW (or CDRU)]. Poisson regression confirmed consistency of the results. CI = confidence interval; OR = odds ratio. LR = logistic regression. IRR = incidence rate ratio. Bold face indicates statistical significance.

Age	OR (gender)	*P*-value (LR)	OR (BW)	95% CI	*P*-value (LR)	*P*-value (Poisson)	IRR
Birth	1.22	0.80	1.01	1.00–1.01	0.15	0.16	1.00
3m	1.73	0.49	1.13	0.81–1.57	0.48	0.49	1.12
6m	1.40	0.69	1.11	0.93–1.31	0.24	0.25	1.10
12m	0.81	0.80	1.16	1.03–1.31	**0.012**	**0.012**	**1.15**
18m	0.94	0.95	1.18	1.02–1.36	**0.026**	**0.026**	**1.16**
24m	0.86	0.89	1.13	0.99–1.28	0.071	0.08	1.12

Age	OR (gender)	*P*-value (LR)	OR (CDRU)	95% CI	*P*-value (LR)	*P*-value (Poisson)	IRR
3m	1.54	0.60	1.35	0.31–7.70	0.31	0.32	1.33
6m	0.99	0.99	1.66	0.97–2.84	0.066	0.064	1.58
12m	1.38	0.69	1.33	0.80–2.22	0.27	0.28	1.31
18m	1.74	0.55	1.69	1.04–2.73	**0.033**	**0.032**	**1.61**
24m	0.67	0.73	1.62	0.80–3.25	0.18	0.18	1.57

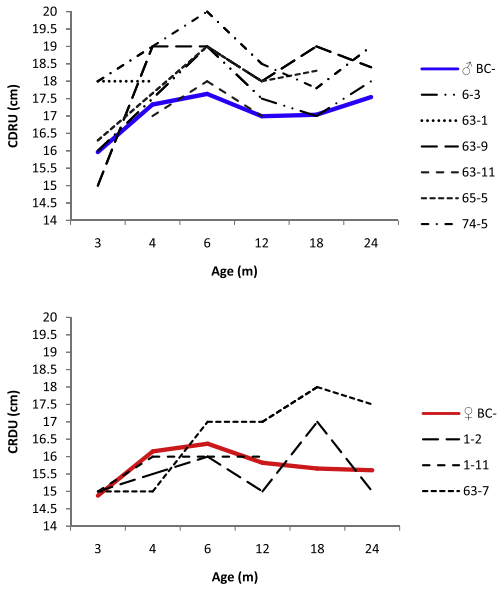

Fig. 2. Mean circumference of the distal radius and ulna (CDRU), from 3 months (m) until 2 years of age, for male (upper panel; blue line; $n = 85$) and female (lower panel; red line; $n = 102$) Leonbergers that were not diagnosed with primary bone cancer (BC−) versus individual dogs that were euthanized due to this disease ($n = 3$). Each dog diagnosed with primary bone cancer is identified by its unique ID number; the first part of this number identifies the litter, while the second number identifies the individual dog. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

primary bone cancer, while a 1 cm larger CDRU was associated with a nearly 70% increased risk. Receiver operating characteristic curves suggested that BW (at 12 and 18m) and CDRU (at 18m) provided some explanation for the risk of developing primary bone cancer in these dogs, yielding AUC of approximately 0.8. Analyses based on the Poisson regression model were consistent with those of the logistic regression (Table 2).

Only 3 dogs (1 female, 2 males) that later developed bone cancer had radiographs of the forearm performed, precluding meaningful comparisons between these and the BC− group.

4. Discussion

To our knowledge, this is the first prospective population-based study following a breed-specific cohort of dogs from birth to death, with particular focus on the development of primary bone tumours. The individual growth curves suggested that the 9 LB succumbing to primary bone cancer generally were heavier during the growth period and early adult life than those dying of other causes (BC− group), and this was most evident for the male dogs. Similarly, male LB diagnosed with primary bone cancer had a larger CDRU than the ones in the BC− group. Logistic regression confirmed a positive association between the risk of developing bone cancer and BW (significant effect at 12 and 18m) and CDRU (significant effect at 18m). These findings support the notion that weight-bearing stress during the period of high activity in the long

bones associated with growth may increase the risk of primary bone cancer. The results should be interpreted with caution due to the low number of cases, and further studies are needed to confirm this notion.

In our regression model, the difference in BW or CDRU between dogs with and without bone cancer was assumed equal for both genders, although the female dogs naturally had a lower BW. This assumption may be a simplification, but there is no current knowledge implying a gender difference in this respect. By assuming a similar relationship between bone cancer and BW or CDRU for both genders, both males and females could be included in the same model, thereby increasing the power.

In line with our findings, Tjalma (1966) reported that larger dogs had a higher risk of OSA; those weighing >36 kg (80 lb) having an up to 185-fold increased risk compared to those with a BW of <9 kg (20 lb). Similarly, a retrospective study of more than 3000 dogs diagnosed with OSA and almost 4000 controls showed that a high adult BW was associated with an increased prevalence of OSA (Ru et al., 1998). In further support of the implication of weight-bearing stress, a higher proportion of appendicular to axial tumours is found in large-breed dogs (Rosenberger et al., 2007). While only approximately 5% of OSA cases are seen in dogs weighing less than 15 kg (33 lb), more than half of these tumours (59%) originate in the axial skeleton – based on a review of almost 1500 cases of canine OSA (Ehrhart et al., 2013). In contrast, large-breed dogs (>40 kg; 88 lb) accounted for nearly one third (29%) of the OSA cases, and only 5% of these tumours occurred in the axial skeleton (Ehrhart et al., 2013). The low number of OSA cases in our study precluded meaningful analysis of anatomical tumour location, but consistent with previous reports, all the cases of primary bone tumours in our study were appendicular, most of which were located in the distal radius/ulna (Table 1).

Contrary to earlier reports, Cooley et al. (2002) did not find an association with adult height and BW and OSA in the Rottweiler breed. Besides that study, ours is the only to assess BW as a risk factor within one breed. The seemingly higher adult BW in LB that died of primary bone cancer compared to that of more than 150 LB that died of other causes, contrasts the findings of Cooley et al. (2002). It is possible that an increased BW or growth rate constitute risk factors for OSA only within certain breeds. Alternatively, breed- or family-specific factors unrelated to growth may be of importance, as suggested by the increased frequency of OSA in a family of St. Bernards (Bech-Nielsen et al., 1978).

As 4 out of 9 dogs with primary bone tumours in our study were from the same litter (litter number 63), it is possible that the BW of our BC+ group was influenced by an above average BW of this litter, which could be unrelated to the risk of primary bone cancer. Indeed, the litter in question was heavier than the mean BW of the rest of the study population, and within this litter, the dogs that developed primary bone cancer were not heavier than the remaining littermates. Removing the 4 BC+ dogs from litter 63 leaves only 3 male and 2 female LB diagnosed with primary bone cancer, precluding further analyses on this subgroup alone. However, looking at Figs. 1 and 2, the 3 remaining male LB appear heavier, and had a larger CDRU than the mean values for the BC− group. For the 2 remaining female LB, both BW and CDRU were similar to the mean values for females that were not diagnosed with primary bone cancer.

As suggested by the results of two recent meta-analyses on human OSA (Arora et al., 2011; Mirabello et al., 2011), height may be more important for risk of OSA than weight: both studies concluded that taller individuals have an increased risk of developing OSA, while the latter also identified higher birth weight as a risk factor. For canines, height is however an infrequently recorded measurement. Ru et al. (1998) suggested that height was important in risk assessment of canine OSA, but used breed standards rather than actual measurements, and were therefore unable to omit the effect of breed. In our study, radiographs of the forearm were scheduled at the observational ages during growth, enabling measurement of the ulnar length throughout this period. Unfortunately, very few dogs that later developed bone cancer had any radiographs performed, precluding meaningful comparisons of the length of ulna between these and the BC− group.

The number of dogs with primary bone cancer included in this study was low, and the difference between the dogs regarding factors such as exercise and flooring too small, to make meaningful inferences about their impact during the growth period as a possible risk factor. The amount of exercise after reaching skeletal maturity was not included in this study. As most people would be likely to limit their dogs' exercise during the growth period, it may be more useful to assess the implication of high-impact exercise in young adult dogs as possible risk factor for OSA.

In all but one dog diagnosed with primary bone tumours in this study, a histological diagnosis was lacking, and the diagnosis was based upon evaluation of the presenting clinical signs and radiography. Histopathology would have strengthened the results, but the design of this study precluded any guarantees of obtaining biopsy specimens. Although post mortem examination was encouraged for all dogs in the project, this was only performed on a minority – due to logistical or owner's preferences. However, the clinical progression of the disease along with typical radiographic findings, strongly favour the probability of OSA, which is well known to be the most common canine primary bone tumour; accounting for up to 85% of these tumours (Ehrhart et al., 2013). The one dog in this study for which histopathology was available, was diagnosed with OSA.

In summary, the results of this study suggest that LB that develop primary bone cancer are heavier during the growth period and early adult life than LB that do not develop this disease. Other factors that were not investigated in this study are however likely to be of importance, and the effect of litter may have biased our results. It would be interesting to perform genome-wide association studies to investigate genetic variation related to development of primary bone cancer in this population of LB. Future studies could also focus on the amount of high-impact exercise during the growth period and in skeletally mature dogs, in a population of dogs that are known to be at high risk of developing primary bone cancer.

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Paper III

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Age-Period-Cohort Analysis of Primary Bone Cancer Incidence Rates in the United States (1976–2005)

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Abstract

Background: Primary bone cancer comprises three major histologic types: osteosarcoma (OS), Ewing sarcoma (ES), and chondrosarcoma (CS). Given the limited knowledge about the etiology of primary bone cancer, we undertook an age-period-cohort (APC) analysis to determine whether incidence varied by birth cohort or calendar period. The purpose was to examine the temporal development of each bone cancer type and generate etiologic hypotheses via the observed birth cohort-related changes.

Methods: An APC model was fitted to incidence data for U.S. whites for OS, ES, and CS obtained from nine registries of the Surveillance, Epidemiology, and End Results program, which covers about 10% of the U.S. population, 1976–2005.

Results: The incidence of OS decreased between 1976 and 2005 among those aged over 60 years, a decline that occurred among patients with OS as their primary malignancy only. From 1986–1995 to 1996–2005, the incidence rate of CS among females of 20 to 69 years rose by about 50%, with rates increasing among consecutive cohorts born during 1935–1975. CS rates among males were stable, as were rates of ES.

Conclusion: The risk reduction in OS as a primary malignancy at older ages could possibly be related to diminished exposure over time to bone-seeking radionuclides. The CS increase among females corresponds to birth cohorts with rising exposures to oral contraceptives and menopausal hormonal therapy.

Impact: As the estrogen signaling pathway has been shown to stimulate proliferation of normal and malignant chondrocytes, estrogen exposure may increase the risk for CS. Further studies are warranted to clarify its possible etiological significance. *Cancer Epidemiol Biomarkers Prev; 20(8); 1770–7. ©2011 AACR.*

Introduction

Osteosarcoma (OS), Ewing sarcoma (ES), and chondrosarcoma (CS) are the 3 major histologic types of primary bone cancer. The 3 groups combined represent less than 1% of all cancers diagnosed in the United States, with OS historically being the most frequent. Although multiagent chemotherapy has greatly improved the survival rate, mortality is still high, with overall 5-year survival ranging from about 15% to 60% for OS patients with and without visible metastases, respectively, at the time of diagnosis (1–4). Although the majority of OS cases occur in adolescence, there is a second peak in incidence in the seventh and eighth decades of life. OS in elderly patients is often considered as a secondary neoplasm attributed to sarcomatous transformation of Paget's disease of the bone or a late effect of previous radiotherapy or chemotherapy (5, 6). At all ages after the first decade of life, males are affected more frequently than females.

There are a few established risk factors for OS apart from early exposure to radiation, Paget's disease, hereditary retinoblastoma, and Li-Fraumeni syndrome (7–11). The bimodal age-incidence curve with peak rates occurring both in adolescence and in older age suggests 2 separate etiologies. Enhanced carcinogenic susceptibility during the adolescent growth period is implicated by higher radiogenic bone cancer risk among children than adults and the characteristic development of childhood tumors in the long bone epiphyses of the lower limbs. Higher male than female incidence rates in puberty and the early age at which OS incidence first peaks, at ages 10 to 14 and 15 to 19 years for girls and boys respectively, may indicate the importance of accelerated growth and hormonal differences. Very early-in-life characteristics including high birth weight have also been implicated in the etiology of OS (12).

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The age distribution of ES resembles that of OS in early life, albeit with its peak incidence among even younger patients (13). This indicates a similar link between the onset of puberty and this type of bone cancer (6). ES rarely develops later in life (13) and differs from OS in that it is not induced by radiation. In general, there is a paucity of studies examining putative risk factors for this disease. ES is more common among Caucasians than African–Americans and Asians, suggesting a genetic predisposition (14, 15). A translocation between chromosomes 11 and 22 is found in almost all cases (16).

CS is rare in childhood and incidence rates, unlike those of OS and ES, increase fairly uniformly with age. Risk factors for this subtype are largely unknown, although there is some evidence that ionizing radiation may play a role (6, 17). Secondary CS may arise in a benign precursor, either an osteochondroma or enchondroma. CS is less common in African–Americans than Caucasians, as is the case for ES (14).

In this article, we fit age-period-cohort (APC) models to U.S. incidence data for OS, ES, and CS cases diagnosed during the period 1976–2005, as obtained from the 9, longstanding registries of the Surveillance, Epidemiology, and End Results (SEER) program covering about 10% of the U.S. population. The aims of this study were to examine the temporal trends of each bone cancer type and to generate possible etiologic hypotheses implicated in the observed birth cohort-related changes.

Material and Methods

Incident cases of bone cancer diagnosed among white residents of the 9 SEER registries during 1976–2005, were categorized by sex, age (0–4, 5–9,. . ., 80–84, >85 years), and morphology code (18). Corresponding population data were available from the same source. Incidence rates were grouped for the major types of bone cancer: OS (ICD-O-3 9180-9200), ES (ICD-O-3 9260), and CS (ICD-O-3 9220-9243; ref. 19). Bone cancers with all other or unspecified morphologies were categorized as "other" (ICD-O-3 9210; 9250-9252; 9261-9342).

Age-specific and age-standardized (world) incidence rates per 100,000 person-years were computed by morphologic type and sex, overall and in 10-year calendar periods (1976–1985, 1986–1995, and 1996–2005) and plotted according to age, year of diagnosis, or birth cohort. Age was categorized using 5-year intervals in adjusted rates and 10-year intervals for age-specific rates and the models. Birth cohorts were estimated by subtracting the midpoints of 10-year age groups from the corresponding midyears of 10-year calendar time. Figures 1–3 were plotted using a uniform log(rate) scale of 0.1:2.0 for the vertical axis, and an arithmetic (year) scale for the horizontal axis such that an annual rate of change of 1% was portrayed by an angle of 10 degrees, that is, 1 log cycle is the same length as 40 years (20). Rates based on fewer than 10 cases or single data points were not shown.

Observed age-specific trends are presented as rates for calendar periods (herein referred to as "periods") and rates for birth cohorts (herein referred to as "cohorts"), with parallelism of the curves and indication of their respective cohort or period influence on the temporal pattern. Cohort effects may be established by environmental determinants acting prenatally or early in life, or they may reflect factors that exert influences shared by members of the same group as they age together. Period effects are characterized by an immediate or fixed-delayed change in the incidence rates for all age groups (regardless of their birth cohort), and thus may reflect events that quickly change rates with the same order of magnitude across all affected age groups. Commonly they transpire from changes in classification criteria, the availability of new diagnostic tests, or specific interventions that affect rates similarly across all age groups. Comparison of the shapes, slopes, and alignments of the age-specific curves may reveal the roles of period versus cohort effects.

Given the limitation of APC analyses, that is, the inherent inability to identify the individual slopes of age, period, and cohort simultaneously because of the linear dependency between the time components (21), the estimated effects are presented here with an *a priori* focus on describing and interpreting the cohort effects obtained from the full APC model. The allocation of drift (the identifiable sum of period and cohort slopes) to birth cohort was used to obtain a unique solution, according to the method of Holford (22, 23). By constraining the period effect to zero on average and with a slope of zero, we assume the changes in rates (and specifically the underlying linear trends) may be attributed to birth cohort influences. Other interpretations and solutions are possible, and the model-based results should be interpreted with caution.

APC analysis was conducted using the functions available in the library *Epi* (version 1.0.8) in R (24), and specifically the *apc.fit* command. Synthetic 2-year birth cohorts, each overlapping 1 year, were derived from 1-year period and 1-year age groups. Given specific concerns regarding the data quality in the elderly for ES and because of low numbers of cases, we restricted the modeling analyses to ages 0 to 64 years at diagnosis for this type; for the other histologic types, the age range was 0 to 84 years.

The necessary smoothing was obtained using a natural splines function, with the number of parameters set to 5 for the age, period and cohort effects. The knots were set so that the number of events was the same between them. The cohort and period effects are presented as rate ratios with the reference cohort 1930. Stata 10 (25) was used for data management and plotting of the observed trends.

Results

Age-specific patterns

The age-specific rates of OS during 1976–2005 exhibited a bimodal distribution with the highest incidence rate occurring in the second decade of life, with a decline to the lowest incidence between 30 and 50 years of age,

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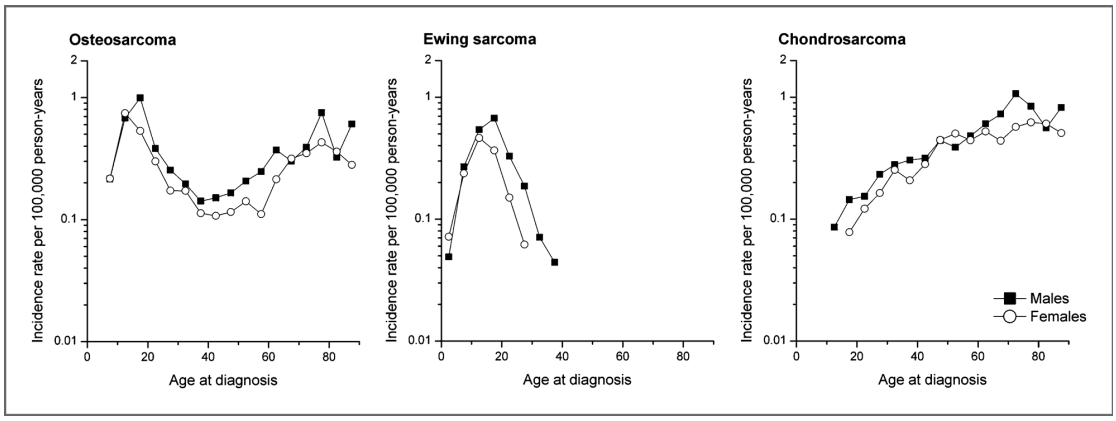

Figure 1. Age-specific incidence rates of bone cancer during the period 1976–2005 for the 3 major types by sex among U.S. whites (SEER 9).

followed by a second peak in those 75 to 79 years of age (Fig. 1). After the age of 15, females had a lower incidence of OS than males, and the highest incidence rate in females was observed at a slightly younger age (10–14 years) than in males (15–19 years). The age and sex distribution of ES incidence rates was similar to that for OS until the age of 40, after which very few cases of ES were diagnosed. An increasing incidence by age was noted for CS, reaching a plateau around the age of 65. A male predominance was generally apparent at the older ages and at ages less than 30 years, in contrast to modest and inconsistent sex differences among the middle age groups.

Overall incidence rates and trends 1976–2005

In males, the incidence rates were highest for OS and CS throughout the period 1976–2005 and were also quite stable (Table 1). Among females, this was also the case for the first 2 10-year periods, albeit at a level some 30% lower than in males. During the most recent years (1996–2005), however, the incidence rate for CS in

Table 1. Incidence counts, rates[a], and the estimated annual percentage change during 1976–2005 by histologic type and sex, SEER 9

	Incidence count			Rate			Estimated annual percentage change
	1976–1985	1986–1995	1996–2005	1976–1985	1986–1995	1996–2005	1976–2005
Males							
OS	298	306	325	0.32	0.34	0.33	0.22 (–0.73, 1.17)
ES	169	160	163	0.20	0.20	0.19	–0.22 (–1.73, 1.31)
CS	235	289	332	0.24	0.26	0.27	0.44 (–0.34, 1.24)
Other bone cancers	203	225	236	0.20	0.20	0.20	1.15 (–0.74, 1.05)
Total bone cancer	905	980	1056	0.96	1.00	0.99	0.15 (–0.35, 0.65)
Females							
OS	222	259	250	0.23	0.28	0.26	0.45 (–0.41, 1.33)
ES	112	97	95	0.15	0.13	0.12	–0.90 (–2.43, 0.66)
CS	197	209	364	0.16	0.17	0.27	2.73 (1.98, 3.49)
Other bone cancers	158	170	193	0.13	0.13	0.13	–0.31 (–1.33, 0.72)
Total bone cancer	689	735	902	0.67	0.71	0.77	0.76 (0.25, 1.28)

NOTE: Total person-years at risk (1976–2005) = 102,287,400 (males) and 103,835,595 (females).
[a]Rate per 100,000 person-years, age adjusted using the World standard.

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females was comparable with that of OS, rising by almost 70% from 0.16 in 1976–1985 to 0.27 in 1996–2005. This increase in the incidence rate of CS among females was statistically significant ($P < 0.05$), with the estimated change reaching almost 3% per year. An overall rise in the rate of bone cancer among females (0.8% per year) was mainly because of the increasing CS incidence. Rates of CS among males were basically unaltered over time, as were the ES rates for both males and females. The estimated annual percentage change was based on data for the individual years. The male to female ratio was largely between 1.2 and 1.6 for all types throughout the 3 10-year periods, except for CS, which had dropped to unity in the last period.

The presentation of trends from 1976 to 2005 graphically further shows that age-standardized incidence rates for primary bone cancer and its major subtypes have been relatively stable (Fig. 2), with the exception of that for CS in females which has been rising since the late 1990s, exceeding rates of OS during the last decade. Despite a slight increase in the rate of OS among females, CS accounted for most of the overall increase in bone cancer within this group.

Age-specific trends by period and by cohort

Figure 3 shows the incidence rates of OS and ES related to period of diagnosis and to birth cohort by 10-year age groups for both sexes combined (A and B). Males and females were combined because their patterns were similar. Sex-specific incidence rates for OS and CS are presented as supplementary material. No consistent patterns in incidence trends by period were observed for OS. However, cohort-specific declines in OS were seen in successive generations born during 1905–1934,

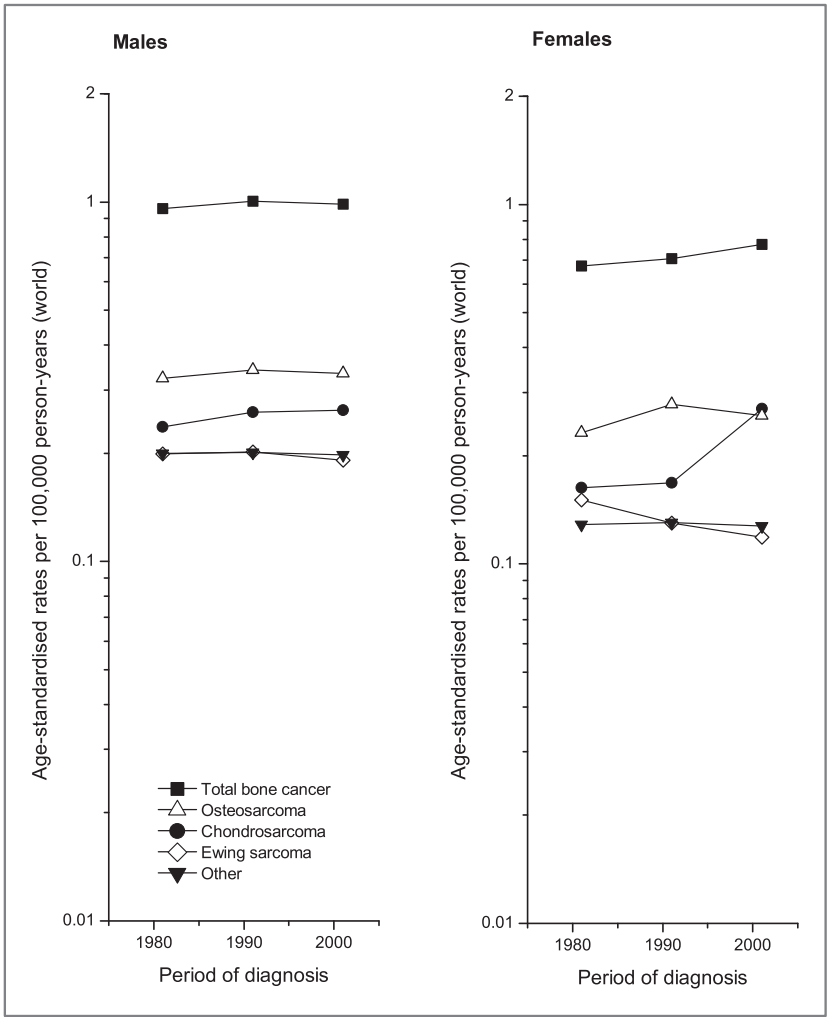

Figure 2. Trends in age-standardized (World) incidence rates of bone cancer for the major types by sex among U.S. whites (SEER 9) during the 10-year aggregates 1976–1985, 1986–1995, and 1996–2005.

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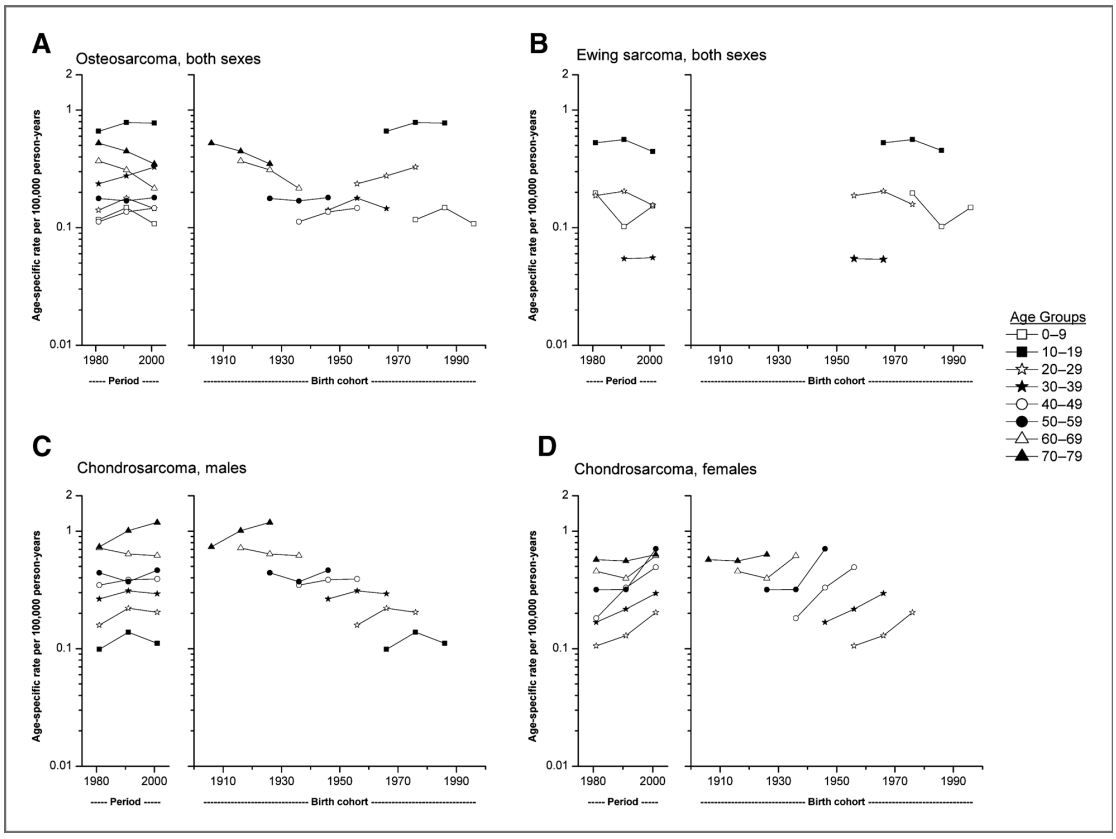

Figure 3. Observed trends in the incidence of (A) OS (both sexes combined), (B) ES (both sexes combined), (C) CS (males), and (D) CS (females) among U.S. whites 1976–2005 (SEER 9). Age-specific rates for 10-year age groups ages 0 to 79 years are presented by calendar period of diagnosis and by birth cohort. Rates below 0.1 per 100,000 person-years or based on fewer than 10 cases are not shown.

whereas there were some indications of an increasing trend among recent birth cohorts, born after the mid 1950s. A further analysis of the SEER data showed that the OS decline at ages 60 to 69 and 70 to 79 years clearly occurred among those patients with only 1 primary malignancy, or who had OS as the first of multiple primaries. There was no indication of declining rates for patients with OS following another primary malignancy (data not shown). For ES, no patterns in incidence trends by period or cohort were apparent.

For CS (Fig. 3C and D), there was no consistent pattern in the trends among males. In females, however, increases were apparent over the entire study period of 1976–2005; among older women ages 50+, rates rose during the latter half of the study period. Splitting by cohorts, revealed increasing incidence rates for consecutive cohorts born since the early 1900s. The earliest and steepest increases occurred among women aged 40 to 49 years, born during the 1930s–1950s; rates among younger women, born more recently, also have been rising rapidly.

APC-modeled trends

Assuming a period slope of zero and, hence, drift attributed entirely to birth cohort, Figure 4 presents APC graphs depicting the fitted age-specific rates (left part) and relative changes in incidence rates of OS, ES, and CS among subsequent cohorts compared with those born circa 1930 (middle part) and over period of diagnosis (right part). The rates are given relative to the respective reference cohort of each cancer type, which explains why the highest age-specific rates are not always comparable with those presented in Figures 1–3.

With respect to goodness-of-fit, 2-factor models fitted the data adequately for the 3 subtypes, possibly because of underdispersion, although for most subtype/sex combinations, nonlinear period, and/or cohort effects contributed to significant improvements in the model fit.

A decline in OS was seen in both sexes for cohorts born from 1890 to 1925, with a slight increase thereafter. An increase, followed by a leveling off and decrease (in women), in rate ratio of ES in successive birth cohorts was observed. These apparently striking ES effects are based on relatively

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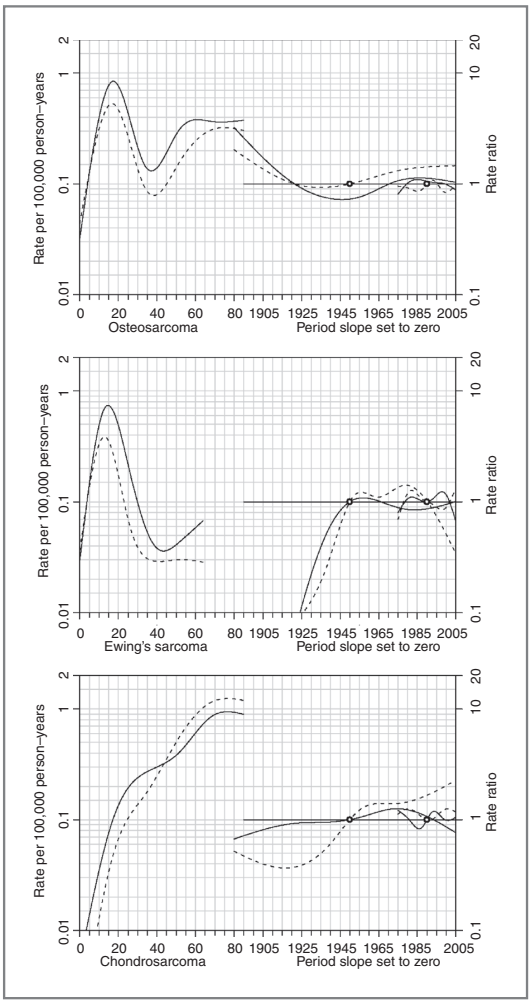

Figure 4. Age, period, and cohort effects, based on Holford's approach for the 3 major types of bone cancer by sex among U.S. whites (SEER 9). Estimates are based on the APC model assuming a linear slope of zero for period. The rate (left *y*-axis) corresponds to the left part, whereas the rate ratio (right *y*-axis) applies to the middle and right part of the graphs. The rate ratios relate to the reference cohort (1950) and the reference period (1990), which are marked with a circle. Solid and stippled lines are for males and females, respectively.

few cases, and thus subject to random variation to a larger extent than OS and CS. The CS incidence rate ratio increased in successive cohorts born between 1925 and 1955 in females, but not males. For the subsequent cohorts, CS rate ratios remained clearly higher in women than men.

Discussion

The epidemiology of primary bone cancer has been the subject of several reports over the past years, emphasizing incidence and survival rates across tumor subgroups (13, 26–28). To our knowledge, APC modeling has not yet been undertaken for these cancer types. Given the limited number of established risk factors for primary bone cancers, APC modeling is a reasonable approach to attempt to identify testable hypotheses, in particular those pertaining to birth cohort or calendar period. The presence of cohort patterns may support the notion that exogenous factors are important in the carcinogenesis of the disease under study. For other cancers, such as testis and breast, the identification of cohort patterns has generated new hypotheses regarding external risk factors (29, 30).

This study has revealed some hitherto unrecognized changes in the secular trends of the 2 major types of primary bone cancer, OS and CS. Incidence rates of OS decreased between 1976 and 2005 among those 60 years and older, corresponding to cohorts born from 1905 to 1934. This represents the second peak of the bimodal age-incidence curve of OS, around 70 years of age, where OS is often attributed to late effects of cancer radiotherapy or chemotherapy (5), or sarcomatous transformation of Paget's disease (6). Based on recently published SEER data, OS as a second or later cancer comprises about one fourth of all OS cases in this age group (27). It turned out, however, that the OS decline at ages 60 to 79 years in this study occurred among patients with only 1 primary malignancy, or who had OS as the first of multiple primaries. There was no indication of declining rates for patients with OS following another primary malignancy. Thus, developments in cancer therapy over the past decades are not relevant in this context. The risk reduction in OS as a primary malignancy at older ages could possibly be related to diminished exposure over time to the fall-out of Strontium and other bone-seeking radionuclides in the 1950s and 1960s, which has been implicated in the etiology of OS (31).

OS with Paget's disease among those older than 60 years of age comprises about one tenth of all OS cases in the SEER database, and this proportion has been quite stable throughout the study period (27). Although we did not analyze OS with Paget's disease separately in our data, it is reasonable to assume that changes pertaining to this subgroup of cases do not explain the observed decline in incidence rates.

There seems to be an increased incidence rate of CS for females over the study period, whereas that of males seems largely unaltered. This applies to females of 20 to 69 years of age, for cohorts born between 1935 and 1975, and corresponds roughly to the introduction of estrogens, both in terms of oral contraceptives and hormone therapy. Oral contraceptives were introduced in the United States in 1960, and among women using contraception, 25% to 30% have used the pill fairly consistently since that time. The proportion of women aged 15 to 44 currently using an oral contraceptive increased from 56% in 1982 to 64% in 1995, and then declined slightly to 62% in 2002 (32).

In the first place, oral contraception was used by parous women to end their reproductive period. Later, use became prevalent among younger women. This

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seems to be reflected in our data in 2 ways. First, the earliest and steepest increase, that is, the first period (1975–1985), took place among those aged 40 to 49 years. Second, in the second period (1985–1995), a similar increase was observed among those aged 20 to 29 and 30 to 39 years. In this period, however, a corresponding increase was seen also among those aged 50 to 59 and 60 to 69 years, which is possibly attributable to those being exposed to hormone therapy some years earlier.

Use of menopausal hormone therapy became widespread in the United States during the1960s (33). When the use of estrogen monotherapy was linked with endometrial cancer in the 1970s, combined estrogen/progestin use, introduced in the 1980s, increased among women who were not hysterectomized. The proportion of women undergoing natural menopause who ever used hormone therapy increased from about 10% in the 1950s to about 50% in the 1990s (33). Use of hormone therapy further rose throughout the 1990s, as it was thought to prevent chronic diseases. Use has decreased dramatically following the Women's Health Initiative (WHI) trial results published in 2002 showing that the overall health risks actually exceeded benefits from use of combined estrogen plus progestin among healthy postmenopausal women (34). A substantial part of the U.S. female population has thus been exposed to estrogens in a period coinciding with an unequivocal rise in the incidence of CS. This ecological correlation is supported by insight gained during the past 10 to 15 years pertaining to the role of estrogen in the molecular cell biology of CS.

Estrogen is involved in cartilage metabolism in both male and female chondrocytes and plays an important role in the human growth plate by regulation of longitudinal skeletal growth mediated by chondrocyte proliferation and differentiation (35). At the cellular level, the estrogen effect is mediated by the estrogen receptor (ER), because mRNA expression and nuclear immunoreactivity for ER have been shown in chondrocytes as well as CS cells (36, 37). Furthermore, the expression of CYP19 mRNA, the gene encoding aromatase, which converts androstenedione to estrogen, has been shown in both normal and neoplastic cartilaginous tissue (38). *In vitro* studies have shown that the estrogen signaling pathway stimulates proliferation of chondrocyte cell cultures and CS cell lines. This growth promoting role of estrogen in normal and malignant chondrocytes is apparently similar to the well-established late acting role of estrogen action in cancer promotion in the female breast as well as in other estrogen sensitive organs. It is also of interest that estrogen has been shown to stimulate vascular endothelial growth factor, and thus neovascularization, which is a characteristic trait of progression of CS (17).

It is of note that ERs have also been shown in human OS tissue as well as in OS cell lines. There is conflicting evidence, however, as to whether estrogen signaling has a proliferative or an antiproliferative effect on OS (39, 40). This inconsistency in cellular response to estrogen in OS makes it plausible that a possible stimulatory effect of estrogen is observed for CS, but not for OS, although both malignancies have the ability to respond to estrogen.

Against this background it seems fair to conclude that there is a biological rationale for estrogens promoting the development of CS. To our knowledge, exposure to estrogens has not previously been proposed as a risk factor for CS, and as such, needs to be assessed in analytic studies, for example, in a case–control study design. Given the large drop in the use of hormone therapy after 2002 when the WHI trial results showed its adverse effects, future studies may be expected to observe a corresponding decrease in the incidence in CS over time. Although this is a very rare cancer form, an association eventually being shown between estrogen exposure and CS might have implications in terms of a raised awareness for primary bone cancer among those who have been exposed.

In conclusion, the risk reduction in OS as a primary malignancy at older ages is possibly related to diminished exposure over time to bone-seeking radionuclides. The increase in CS among females corresponds to birth cohorts who were increasingly exposed to estrogens, both in terms of oral contraceptives and hormone therapy. Supporting our hypothesis of estrogens being involved in the increased rate of CS among women over time, *in vitro* studies show that the estrogen signaling pathway stimulates proliferation of both normal and malignant chondrocytes. Further studies are warranted to clarify the possible etiological significance of estrogen exposure in CS risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Age-Period-Cohort Analysis of Primary Bone Cancer Incidence Rates in the United States (1976–2005)

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Paper IV

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RESEARCH ARTICLE Open Access

HES1, a target of Notch signaling, is elevated in canine osteosarcoma, but reduced in the most aggressive tumors

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Abstract

Background: Hairy and enhancer of split 1 (HES1), a basic helix-loop-helix transcriptional repressor, is a downstream target of Notch signaling. Notch signaling and HES1 expression have been linked to growth and survival in a variety of human cancer types and have been associated with increased metastasis and invasiveness in human osteosarcoma cell lines. Osteosarcoma (OSA) is an aggressive cancer demonstrating both high metastatic rate and chemotherapeutic resistance. The current study examined expression of Notch signaling mediators in primary canine OSA tumors and canine and human osteosarcoma cell lines to assess their role in OSA development and progression.

Results: Reverse transcriptase - quantitative PCR (RT-qPCR) was utilized to quantify *HES1, HEY1, NOTCH1 and NOTCH2* gene expression in matched tumor and normal metaphyseal bone samples taken from dogs treated for appendicular OSA at the Colorado State University Veterinary Teaching Hospital. Gene expression was also assessed in tumors from dogs with a disease free interval (DFI) of <100 days compared to those with a DFI > 300 days following treatment with surgical amputation followed by standard chemotherapy. Immunohistochemistry was performed to confirm expression of HES1. Data from RT-qPCR and immunohistochemical (IHC) experiments were analyzed using REST2009 software and survival analysis based on IHC expression employed the Kaplan-Meier method and log rank analysis. Unbiased clustered images were generated from gene array analysis data for Notch/HES1 associated genes.
Gene array analysis of Notch/HES1 associated genes suggested alterations in the Notch signaling pathway may contribute to the development of canine OSA. *HES1* mRNA expression was elevated in tumor samples relative to normal bone, but decreased in tumor samples from dogs with a DFI < 100 days relative to those with a DFI > 300 days. *NOTCH2 and HEY1* mRNA expression was also elevated in tumors relative to normal bone, but was not differentially expressed between the DFI tumor groups. Survival analysis confirmed an association between decreased HES1 immunosignal and shorter DFI.

Conclusions: Our findings suggest that activation of Notch signaling occurs and may contribute to the development of canine OSA. However, association of low HES1 expression and shorter DFI suggests that mechanisms that do not alter HES1 expression may drive the most aggressive tumors.

Keywords: Hes-1, HES1, Notch, Osteosarcoma, RT-PCR, RT-qPCR, Immunohistochemistry, Canine, Microarray

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Background

Osteosarcoma (OSA) is the most common malignant bone tumor among children and adolescents with an incidence of 4.4 cases per million per year in the United States [1]. OSA is also the most common spontaneous primary bone tumor of dogs, estimated to affect greater than 8,000 dogs annually in the United States [2]. Tumor morphology, biological behavior, progression of disease and molecular characteristics are very similar in dogs and humans [2-7]. Consequently, dogs provide a valuable comparative model of human OSA. Standard of care therapy for both human and canine OSA patients remains a combination of surgery and chemotherapy, with five-year survival rates reported in humans as high as 70% [1,8] and median survival in canine patients around 200 days [2]. Unfortunately, in both human and canine patients approximately 80% are estimated to have micrometastases at presentation, some of whose tumors are also refractory to chemotherapy [2,8]. These patients continue to have a poor prognosis. Histologic classification alone has not proven clinically relevant for determination of tumors likely to metastasize or exhibit resistance to chemotherapy protocols. The focus of recent research, therefore, has turned toward molecular characterization of primary tumors, especially aberrant gene and/or protein expression that might correlate with prognosis or chemotherapy sensitivity.

Hairy and enhancer of split 1 (HES1), a basic helix-loop-helix (bHLH) transcriptional repressor, is a downstream target of the Notch signaling pathway. The intracellular domain of activated Notch receptors (NICD) translocates to the nucleus, forms a transcriptional activating complex with recombination signal binding protein for immunoglobulin kappa J region (RPBJκ) and activates expression of target genes including HES1 [9,10]. The HES1 protein contains both DNA-binding and protein-protein interaction domains important for its function as a transcriptional regulator (including negative regulation of its own transcription) [9,11,12]. Notch-independent HES1 expression can also result from Hedgehog and c-Jun N-terminal kinase (JNK) signaling as well as from RAS/MAPK signaling [10,13-15]. Regulation of HES1 expression and activity is dependent on the tissue, spatial and temporal factors, and the proteins with which it interacts [9,10].

Overexpression of Notch and/or HES1 is associated with a variety of human cancers including T-cell acute lymphoblastic leukemia (ALL), and ovarian, breast, cervical, prostate, colon and non-small cell lung cancers [16-19]. Notch/HES1 has also been shown to have tumor suppressor activity in some cancers including hepatocellular carcinoma, B-cell ALL, myeloid leukemia and neuroblastoma [20-23]. In human OSA, Notch is implicated in OSA cell proliferation, invasion and metastasis [24,25]. Increased HES1 mRNA expression was shown in some human OSA cells and OSA tumor samples compared to osteoblasts or normal bone and an association between high HES1 expression and decreased survival of OSA patients has been suggested [24-27]. Reduced invasiveness in response to suppression of Notch signaling and HES1 activity implicates Notch/HES1 signaling in metastasis [28]. Another study suggests both up-regulation of Notch and increased expression of HES1 in one OSA cell line occurs in response to activation of the Wnt/β-catenin pathway [29].

During bone development there is significant cross talk between the Wnt/β-catenin, hedgehog, and Notch pathways affecting osteoblast differentiation and maturation and influencing HES1 expression [10,29-31]. Like Notch and Wnt/β-catenin, aberrant hedgehog signaling is also associated with development of human cancers [31]. Previous studies in our lab identified decreased expression of three hedgehog pathway associated genes in OSA tumors from dogs with a disease free interval (DFI) < 100 days (poor-responders) compared with tumors from dogs with a DFI > 300 (good-responders) [32].

In order to explore the hypothesis that Notch signaling would be altered in canine OSA compared to normal bone samples, the current study examines the expression of NOTCH1 and 2 receptors and signaling targets, HES1 and HEY1, in canine OSA samples from patients with known outcome and normal bone tissues. Immunohistochemical analysis of HES1 protein was assessed in Kaplan-Meyer survival analysis to confirm the association of decreased HES1 expression with a shorter DFI.

Methods

Tumor donors

Chemotherapy-naïve primary tumor samples were selected from the Colorado State University (CSU) Flint Animal Cancer Center's tissue archive. Samples are archived with owner consent and approval by the CSU Institutional Animal Care and Use Committee. Twenty tumors from good- and poor-responders (n = 10 each group) were selected following the protocol previously published [32]. Briefly, chemotherapy-naïve primary OSA samples were from dogs treated with surgical amputation followed by chemotherapy with doxorubicin and/or a platinum based drug (distribution of choice of drug was not significantly different between groups). All twenty dogs were free of thoracic metastases by radiographic analysis at diagnosis and follow up consisted of evaluation by clinical examinations including thoracic radiographs every 2–3 months after initial treatment. Disease free interval (DFI) was calculated from surgery until development of metastatic disease and samples were identified for cohorts of good responders (DFI > 300 days) and poor responders (DFI < 100 days) in order to flank the median DFI

(200 days). Nine additional appendicular OSA tumor samples were collected from which matched normal metaphyseal bone was harvested from the same limb (at least one joint space away from the tumor) following amputation. These nine matched samples were collected at amputation as cases came in (convenience sample) and absence/presence of metastasis, post-operative treatment, and patient follow-up were less consistent in this population. Tumor and normal bone fragments collected at amputation were flash-frozen in liquid nitrogen and stored at -80°C. Tumor fragments were also fixed in 10% neutral buffered formalin for 24 hours with subsequent routine processing and paraffin embedding.

Immunohistochemical HES1 expression was also assessed in a subset of canine appendicular OSA patients from a previously reported multi-institutional randomized prospective clinical trial [33]. The study was approved by the Institutional Animal Care and Use Committees of the participating institutions. All dogs underwent amputation followed by 5 cycles of adjuvant doxorubicin, with or without an investigational matrix metalloprotease inhibitor. Inclusion/exclusion criteria, staging, and follow-up procedures were standardized and tumor tissues were processed as previously reported [33]. Histologic grading (from 1 to 3) was performed by one author (BEP) utilizing a schema incorporating amount of matrix, percent necrosis, nuclear pleomorphism, nucleolar size/number and mitosis score [33]. Mitotic index was calculated by counting the number of mitotic figures per 10 random 400× fields.

Cell culture

Canine cell lines used in this study were provided by Dr. Douglas Thamm; all cell lines were validated for species and genetic identity using short-tandem-repeat (STR) profiling as previously described [34]. Human OSA cell lines were obtained from Dr. Douglas Thamm (MG63, SAOS-2, SJSA-1), Dr. Hue Luu (MG63.2), or purchased from ATCC (U2OS). The MG63.2 cell line is a metastatic sub-line of the MG63 line, obtained via serial passage of rare lung metastases from MG63 [35]. All non-purchased cell lines were validated prior to use using STR profiling by the University of Colorado DNA Sequencing Shared Resource. Cells were cultured in C10 media (DMEM high glucose with 4 mM L-glutamine (Hyclone Laboratories, Inc.), 1 mM of sodium pyruvate, 2× MEM vitamins, 1× MEM non-essential amino acids, 1× antibiotic-antimycotic (100×: 10,000 IU/ml penicillin, 10,000 ug/ul streptomycin and 25ug/ml) (all additives from Mediatech, Inc.), and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO).

RNA extraction

Total RNA was extracted from tumors and RT-qPCR was conducted as described previously [32]. Briefly, samples were freeze-fractured, homogenized, extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy clean up (Quiagen, Valencia, CA) following manufacturer's protocols. RNA was extracted from normal bone using the same protocol with an additional spin of 800× *g* at 4°C for 5 minutes following homogenization. The supernatant was carried forward through the Trizol protocol. Total RNA was extracted from human and canine OSA cells using the RNeasy Kit (Qiagen) per the manufacturer's protocol. RNA was quantified via spectrophotometry and bioanalyzed for integrity as described in O'Donaghue et al. [32] with samples used having a RNA integrity number of at least 8. Human adult osteoblast total RNA was purchased from CELL Applications, Inc.

Reverse transcriptase PCR and quantitative real time PCR

cDNA synthesis was completed using the QuantiTect Reverse Transcription Kit (Qiagen) with 1 or 3 μg input RNA. RT-qPCR of cDNA was run using iQ SYBR Green Supermix (Bio-Rad) and 25 ng equivalent RNA input in 25 μL reactions on a Stratagene Mx3000P instrument. Expression in canine cells and tissues was normalized to hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) expression. *HPRT1* was selected based on its consistent moderate expression in our sample sets in prior microarray and RT-qPCR analysis (see Additional file 1 and reference [32]) and its previous use as a canine reference gene [36]. Consistent with current recommendations for the selection of reference genes and because no single reference gene exhibited unchanged expression between samples, expression in human OSA cells was normalized to the geometric mean of four reference genes; ribosomal protein S15 (*RBS15*), glyceraldehyde-3-dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18SrRNA*) and *HPRT1* [37]. Primer sequences and efficiencies for all genes and the full sequence of the canine *HES1* amplicon are listed in Additional file 2. Primers were designed using Primer-Blast based upon NCBI RefSeq mRNA sequences when available. Primers were designed to be intron spanning when possible and cross-checked for specificity via UCSC in silico PCR. Primers were further validated with standard curves to calculate efficiency, and dissociation curves as previously described [34]. RT-qPCR products were validated for size by agarose gel electrophoresis and sequenced to confirm identity. The 161 bp canine *HES1* amplicon revealed 98% homology to the human homolog of *HES1*. Human *HES1* primers used were the same as those used by Zhang et al. [24]. The identity of the 200 bp amplicon was verified as human *HES1* by dideoxy sequencing (CSU DNA sequencing Core).

Western blot

Western blot analysis was performed on canine and human OSA cells using whole cell lysates or cytoplasmic

and nuclear fractions. Whole cell lysates were prepared in triethanolamine (TEA) lysis buffer (55 mM TEA, pH 7.5, 111 mM NaCl, and 2.2 mM EDTA, 0.44% SDS) with 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific). Nuclear extracts were prepared using a hypotonic 0.5% or 0.25% IgePal (NP-40) buffer (10 mM Hepes, 1.5 mM MgCl, and 10 mM KCl). Briefly, harvested cell pellets were re-suspended in IgePal buffer with protease inhibitor while vortexing, incubated on ice for 0–5 minutes, and centrifuged for 5 minutes at 500× *g*. The supernatant (cytoplasmic fraction) was collected and the pellet (nuclear fraction) was re-suspended in TEA lysis buffer with protease inhibitors. Samples were separated using SDS-PAGE and transferred to a polyvinylidine fluoride membrane. The membrane was blocked with 5% non-fat dry milk (NFDM) for one hour at room temperature and incubated with rabbit monoclonal anti-HES1 antibody (RabMAb EPR4226, 1:500; Epitomics) in 5% bovine serum albumin (BSA) at 4°C overnight. After washing in 0.1% Tween 20-Tris-buffered saline (TBST) the membrane was incubated with secondary horseradish peroxidase conjugated goat anti-rabbit antibody (1:5000; Bio-Rad) in 5% NFDM for one hour at room temperature. SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) was used to detect chemiluminescent signals. Band intensity from four experiments using whole cell lysates from MG63 and MG63.2 cell lines were analyzed using ImageJ software. The intensity of the HES1 band was normalized to the corresponding α-tubulin loading control.

Immunohistochemistry (IHC)

IHC to detect HES1 expression was performed on 4 μm sections from formalin-fixed paraffin embedded (FFPE) tumor tissues using standard immunoperoxidase techniques on charged slides with hematoxylin counter stain. Slides with sections were heated at 60°C for 30 minutes, allowed to cool, and deparaffinized with xylene or a citrus based clearing solution (Thermo-Fisher Scientific), and rehydrated with descending ethanol concentrations in deionized water (100%, 95%, 75% and 50%). Heat induced epitope retrieval was done with 10 mM sodium citrate buffer (pH 6.0) heated in a pressure cooker for 1 minute at 125°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 5 minutes with 3 washes in TBST both before and after. Slides were incubated with a non-serum protein block (Background Sniper, Biocare Medical) at room temperature for 15 minutes followed by incubation with primary antibody overnight at 4°C overnight. The primary antibody (anti-HES1 RabMAb, Epitomics) was used at a dilution of 1:750 (diluted in Antibody Diluent, Dako). Sections were then incubated with a prediluted secondary antibody conjugated to horseradish peroxidase (Envision and Dual Link System HRP, Dako) for 30 minutes at room temperature with 3 TBST washes both before and after. Diaminobenzidine (DAB, Ventana Medical Systems) was used as a chromogen for immunoreactive complex detection and slides were counterstained with hematoxylin.

Sixty-one additional FFPE tumor samples were analyzed for HES1 immunohistochemical expression utilizing a protocol similar to that described above with the following exceptions: primary antibody was diluted in 2.5% normal goat serum in TBST (1:750 or 1:375, higher antibody concentration was used in subsequent batches to increase immunoreactivity signal), and detection was performed using biotinylated anti-rabbit IgG antibody in a Vectastain ABC Kit (Vector Laboratories). The IHC was performed in five batches of 8 to 18 slides each with the same antibody dilution used for an entire batch. Variations in antibody dilutions were controlled for by inclusion of a positive control tumor slide with a total immunoreactivity score of 4 (percent cells staining score of 2 and intensity score of 2; Table 1). All samples within each batch were scored in reference to the control. Negative controls lacking primary antibody were included in each batch.

HES1 antibody validation was done using human placenta and canine lung and pancreas as positive control tissues. Specificity of the primary antibody was verified using a HES1 blocking peptide (Epitomics). Briefly, primary antibody was incubated with 25× (by mass) blocking peptide in antibody diluents (at both 1:375 and 1:750) for one hour at room temperature before application to canine control and sample tumor slides. Positive and negative controls with sections from the same tissues were incubated in parallel.

Immunohistochemical scoring of all slides was performed independently by two authors blinded to case information. A positive cell was any neoplastic cell with distinct brown staining in the nucleus (stromal cells and endothelial cells were not counted). The percentage of positive cells in each sample was estimated based on an average of two or more high powered fields and scored as follows, 1: < 50% cells stain positive, 2: 50-75% cells stain positive, 3: > 75% cells stain positive. Average stain intensity ranged from 1 to 3 (lowest to highest intensity). Field location and number were selected randomly at the discretion of the individual scorer. The product of the percentage and intensity scores made up the overall immunoreactivity score (ranging from 1 to 9). Both scorers simultaneously reviewed slides with conflicting scores (scores deviating by more than 1 in either category) (n = 5) and consensus was reached. After review, total scores were averaged for statistical analyses.

Table 1 Summary of data for dogs with DFI > 300 and DFI < 100 days, including HES1 immunohistochemistry score

Breed	Age at Dx (yrs)	Sex	Tumor Loc	DFI (days)	Avg% stain	Avg stain intensity	Total score
Greyhound	4.4	MC	PH	40	1	1	1
Rottweiler	5	MC	DF	69	3	3	9
Greyhound	7	MC	DF	77	2	1	2
Mix	9	FS	T	90	2	1	2
Greyhound	8	FS	PT	94	1	2	2
Labrador	10.2	FS	DH	95	3	3	9
Mix	8.8	MC	DF	97	2	1	2
Golden	10.8	MC	PH	97	2	1	2
Mix	7.6	FS	DR	307	2	2	4
Greyhound	7.1	MC	PH	467	1	1	1
Mix	12.4	MC	DR	694	3	3	9
Malamute	10.1	FS	DR	734	3	2	6
Labrador	8.7	MC	T	787	3	3	9
Golden	8	FS	DR	885	3	2	6

DFI disease free interval, *Dx* diagnosis, *MC* male castrated, *FS* female spayed, *P* proximal, *D* distal, *H* humerus, *R* radius, *T* tibia, Total Score is product of scores for % cells staining and staining intensity.

Immunocytochemistry (ICC)

Immunocytochemistry was performed utilizing the same reagents and a similar protocol to that used for IHC. Slides were prepared via cytospin and dried overnight. Prior to the blocking step cells were fixed with 100% methanol at room temperature for 15 minutes, allowed to dry, washed in TTBS and incubated in 0.1% TritonX-100 in TBS for 7–12 minutes. The remainder of the procedure was identical to that used for IHC, but a higher concentration of primary antibody (1:250) was used.

Photomicrographs (IHC and ICC) were taken using the Olympus BX51 Research System Microscope with an Olympus dp70 Digital Camera System. Minimal additional editing was done in Microsoft ® PowerPoint ® for Mac 2011.

Gene expression microarray analysis

Total RNA from primary OSA tumor samples from dogs with DFI < 100 (n = 8) and DFI > 300 (n = 7) was analyzed on GeneChip Canine 2.0 Genome Arrays (Affymetrix, Santa Clara, CA) at CSU's Rocky Mountain Regional Center for Excellence (RMRCE) Genomics Core per Affymetrix protocols as described [35]. Normal bone samples (n = 8) were analyzed using an identical protocol. Samples used for microarray analysis were a subset of those used for RT-qPCR (microarray samples were limited due to array costs). Microarray pre-processing combining the osteosarcoma samples with the normal bone samples was conducted using Probe Logarithmic Intensity Error (PLIER) estimation algorithms with $\log_2$ transformations. Probesets including Notch receptor ligands, effectors, or targets of either the canonical Notch pathway or HES1 were selected based on literature review, Ingenuity® Systems Pathway analysis, and/or inclusion in The Human Notch Signaling Pathway RT^2 Profiler™ PCR Array (SAbiosciences) (Additional file 1). CIMminer was used to generate clustered images of the data from the 75 selected probesets with unsupervised clustering on both axes and the following parameters: average linkage, Euclidean distance, and quantile binning with median centering of the data. Full microarray data for the DFI groups is available through NCBI's Gene Expression Omnibus (GEO) via accession number GSE24251.

Statistics

Statistical analysis of RT-qPCR and immunohistochemistry data (not including survival data) was performed using Prism software (GraphPad Software, La Jolla, CA). For RT-qPCR data standard curves, dissociation curves and amplification data was collected on a Stratagene Mx3000P instrument and analyzed using the Rest2009 software [38]. *HES1* RT-qPCR data was also analyzed using the $2^{(-\Delta\Delta Ct)}$ method [39] with similar results. IHC scores for the DFI > 300 and DFI < 100 tumors were analyzed with a 2-tailed Fischer's exact test after separating scores into low expression (total score less than 4) and high expression (total score greater than or equal to 4) categories. The cut off was based on results of receiver-operating characteristic (ROC) analysis of immunohistochemical scores for the DFI > 300 and DFI < 100 groups. Welch *t*-test in ArrayTrack 3.5.0 with false discovery rate correction for multiple comparisons (FDR; based on all array probesets) was used to compare microarray gene expression data. Significance was defined as $p < 0.05$ (Welch *t*-test) or $q < 0.05$ (FDR).

Statistical analysis of survival data was performed using a combination of Prism and SPSS software version 20 for Macintosh (IBM, Armonk, NY). Correlations between HES1 expression levels and other markers on a continuous scale were evaluated using linear regression analysis. A 2-tailed, unpaired *t*-test was used to evaluate the association between HES1 expression levels and categorical markers. The median DFI was estimated using the Kaplan-Meier method, and comparisons between groups made using log rank analysis for categorical variables. For continuous variables, markers were categorized into a low and high group using the median value as the break point. Multivariable Cox regression analysis was then performed, utilizing both forward and backward stepwise models. Variables identified with a univariate p-value of <0.1 were included in the multivariate analysis. For all other tests, p-values of <0.05 were considered significant.

Results

Gene expression analysis of Notch/HES1-associated genes groups normal and OSA bone samples, but does not distinguish DFI groups

To assess the biological relevance of Notch/HES1 signaling in canine osteosarcoma, probesets including Notch receptor ligands, effectors, or targets of either the canonical Notch pathway or HES1 were selected from Canine 2.0 gene array data and analyzed for differential gene expression as described in materials and methods. Unbiased cluster analysis of data for the 51 Notch/HES1-associated genes separated normal bone from tumors, but did not discriminate between the DFI groups (Figure 1). In total, 30 of 51 (58.8%) Notch/HES1 pathway associated genes examined were significantly different between tumor and normal bone ($p < 0.05$, $q < 0.05$); 23/30 (76.7%) had increased expression in tumors. Specifically, mRNA expression of *NOTCH1* and *NOTCH2* was elevated in tumor samples compared to normal bone ($p < 0.05$, $q < 0.05$). None of the genes evaluated had significantly different expression between DFI groups when corrected for multiple comparisons. *HES1* was not included on the Canine 2.0 chip, but *HEY1*, another Notch target, was also elevated in tumors compared to normal bone ($p < 0.05$, $q < 0.05$).

RT-qPCR analysis for *NOTCH1, NOTCH2, HEY1 and HES1* was conducted on the normal bone/matched OSA and DFI tumor sample sets (Figures 2 and 3). *NOTCH1* exhibited decreased expression in the DFI < 100 day group relative to normal bone (FC down − 1.656, $p < 0.001$), with no other significant changes measured. This result differed from the 1.27 fold upregulation of *NOTCH1* identified in the gene array analysis, however previous studies have shown that fold-change differences <1.5 are frequently unreliable [40]. Consistent with the array data, *NOTCH2* exhibited an approximate 4-fold elevation in expression in both sets of DFI tumors, separately and in combination, relative to normal bone ($p < 0.001$). Similarly, *HEY1* expression was elevated in each tumor group by a fold-change ranging from 6 to 10.2 ($p \leq 0.001$). RT-qPCR analysis of these Notch signaling pathway elements confirmed our finding that Notch signaling is elevated in tumors relative to normal bone, but not between tumors in the two DFI groups.

HES1 mRNA expression in tumors and its prognostic significance

RT-qPCR was also used to assess *HES1* mRNA levels in OSA tumor and matched normal bone samples. Average *HES1* mRNA expression was elevated 2.57-fold in canine OSA tumors compared to the matched normal bone (Figure 3A; $p = 0.012$); however, this fold change was highly variable when each OSA tumor was compared to its matched normal bone sample, with 5 tumors exhibiting elevated expression compared to normal bone and 4 tumors having virtually unchanged expression (Figure 3B, range 1.19-6.17-fold).

We also assessed mRNA levels for *HES1* in tumors taken from dogs with a DFI <100 days or DFI >300 days following treatment by amputation and chemotherapy. We found that *HES1* expression was elevated 4.608-fold in the DFI > 300 tumors compared to the DFI < 100 group (Figure 3A; $p < 0.001$). *HES1* expression in the DFI < 100 group was not different from the normal bone samples.

Messenger RNA levels of HES1 were measured in canine and human osteosarcoma cell lines and confirmed using Western blot analysis using a rabbit monoclonal anti-human HES1 antibody as described to determine if *HES1* mRNA levels correlated to protein expression, (Figures 4 and 5, Additional file 3). Comparison of canine and human amino acid sequence of the *HES1* gene identified 86% homology in the epitope targeted by this antibody. This was based on the predicted amino acid sequence of NCBI reference sequence XM_548669.1, which has been removed as a result of standard genome annotation processing. No additional canine *HES1* record is currently available. Western blot analysis of whole cell OSA cell lysates revealed a 30 kD protein (HES1) as well as larger non-specific bands (Figure 4A, W). Given the role of HES1 as a transcriptional regulator, we hypothesized that active HES1 protein would reside in the nucleus. Western blot analysis of isolated nuclear and cytoplasmic fractions from both canine and human OSA cell lines confirmed enrichment of the 30 kD HES-1 protein in the nuclear fraction (Figure 4A, N) while the non-specific bands were enriched in the cytoplasm fraction (Figure 4A, C). Since equal amounts of total protein were loaded in each lane, the increased intensity and/or

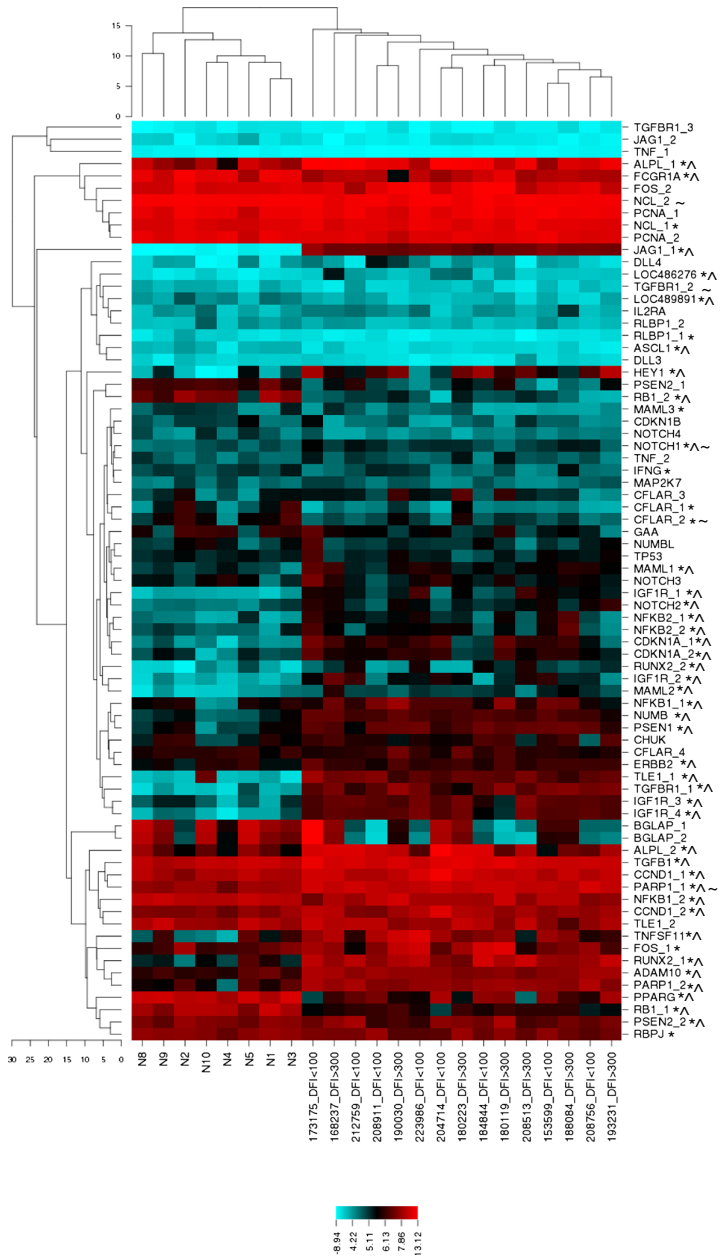

Figure 1 Differential expression of Notch/HES1-associated genes in canine osteosarcoma. Unbiased cluster analysis separates normal bone from tumors, but does not discriminate DFI < 100 day and DFI > 300 day primary tumors groups. An asterisk (*) and a caret (^) denote genes significantly different between tumor and normal bone (* $p < 0.05$, ^ $q < 0.05$). Genes different between DFI groups ($p < 0.05$) are denoted by (~). Multiple probesets are present for some genes. LOC486276 = Deltex 1 homolog (DTX1), LOC489891 = LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase/lunatic fringe (LFNG). Colored bar below indicates the intensity scale of log^2 transformed expression values.

number of nonspecific bands in the cytoplasmic fraction were likely the result of concentration of these cytoplasmic proteins relative to total protein. Experiments using human OSA cells showed similar results (Additional file 3).

HES1 mRNA and protein expression varied between cell lines in both canine and human OSA cells (Figure 5). For human cell lines mRNA expression was similar to that previously published [24,25]. In general, *HES1* mRNA expression was increased in canine cell lines relative to normal canine bone tissue (Figure 5A) and in human OSA cell lines relative to human osteoblasts (Figure 5C). Western blot analysis showed a characteristic band at

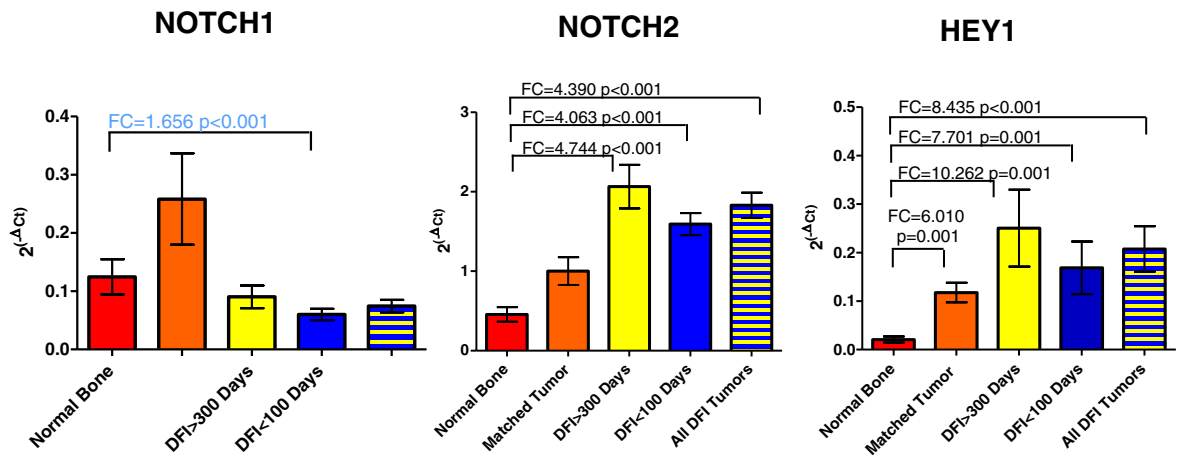

Figure 2 Expression of *NOTCH1*, *NOTCH2* and *HEY1* mRNA in canine normal bone and osteosarcoma (RT-qPCR). *NOTCH1*, *NOTCH2*, and *HEY1* mRNA expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* is shown for normal bone (n = 9), matched tumors (n = 9), tumors from dogs with DFI > 300 days, tumors from dogs with DFI < 100 days, and combined DFI group tumors. Comparisons of each tumor group relative to normal bone and DFI < 100 relative to DFI > 300 day groups were analyzed with REST 2009 software and significant fold changes are indicated by brackets on the graph. Values in blue indicate the reduced fold-change expression in DFI < 100 compared to normal bone. Bars represent mean ± SEM.

30 kDa with variable expression between cell lines (Figure 5B and 5D). Interestingly, the metastatic subline of MG63 cells, MG63.2, exhibited elevated levels of mRNA compared to the MG63 line, but protein expression was not significantly different between the two lines (Additional file 4).

We validated immunoreactivity using FFPE human placenta and found positive strong nuclear and cytoplasmic staining of placental macrophages (Hafbauer cells), moderate nuclear +/− cytoplasmic staining of stromal cells and light nuclear staining of endothelial cells consistent with Notch activity in placenta reported by Herr

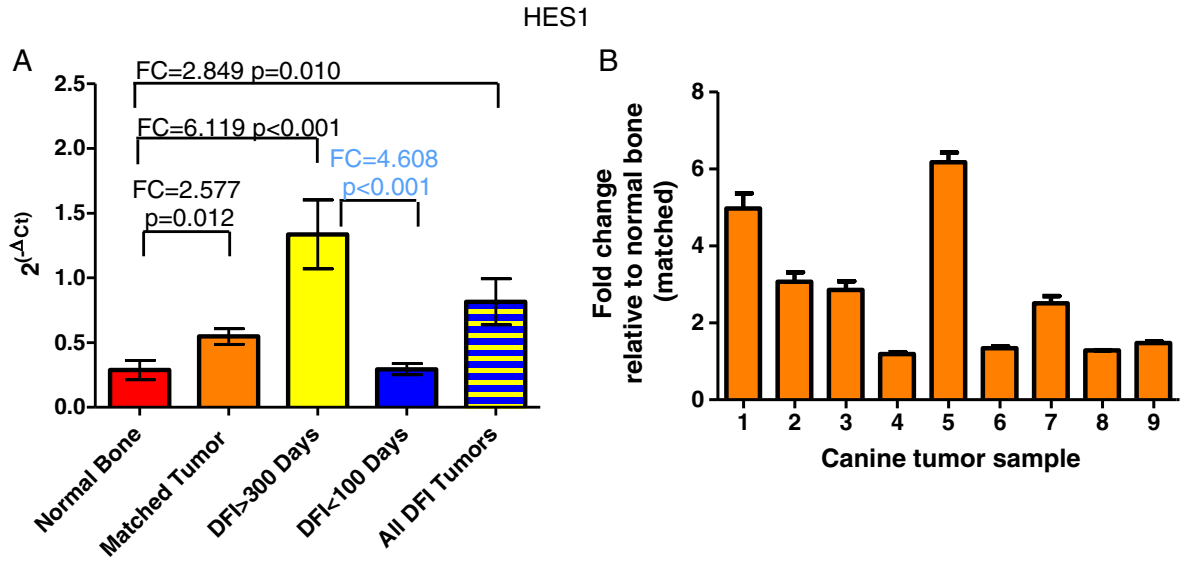

Figure 3 Expression of *HES1* mRNA in canine normal bone and osteosarcoma (RT-qPCR). (**A**) *HES1* mRNA expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* in normal bone (n = 9), matched tumors (n = 9), tumors from dogs with DFI > 300 days (n = 10), tumors from dogs with DFI < 100 days (n = 10), and combined DFI group tumors. Comparisons of each tumor group relative to normal bone and DFI < 100 relative to DFI > 300 day groups were analyzed with REST 2009 software and significant fold changes are indicated by brackets on the graph. Values in blue indicate reduced fold-change in DFI < 100 relative to DFI > 300 group. (**B**) Fold change in expression calculated using the comparative Ct ($2^{(-\Delta\Delta Ct)}$) method between each canine tumor and its matched normal bone sample (normalized to *HPRT1*). Bars represent mean ± SEM.

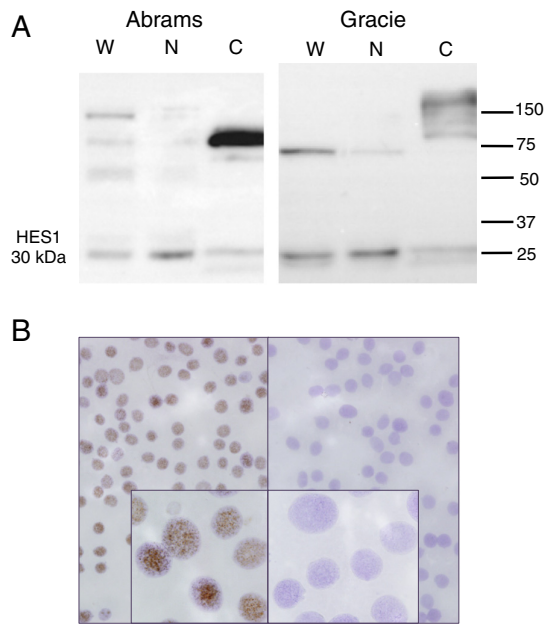

Figure 4 Western blot and immunocytochemistry (ICC) results assessing HES1 expression in canine osteosarcoma cells. (**A**) Western blot analysis of whole cell (W), nuclear (N) and cytoplasmic (C) fractions of canine osteosarcoma Abrams and Gracie cell lines. A 30 kDa band (HES1) is present in whole cell and enriched in extracted nuclear lysates. Larger non-specific bands are enriched in the cytoplasmic fractions. Equal amounts of total protein were loaded in each lane. (**B**) ICC shows nuclear staining for HES1 in canine OSA cells (Gracie). Panel on the right is the secondary-only negative control. Photomicrographs were taken at 20× and 100× (oil, inset) magnification; haemotoxylin counterstain.

et al. [41]. Staining of additional canine control tissues revealed positive punctate to diffuse intranuclear staining of pancreatic cells, endothelial cells and subsets of pulmonary epithelial cells as described in human literature [42-44] (see Additional file 5). Addition of a blocking peptide specific for the epitope targeted by our antibody eliminated all staining (data not shown). Immunocytochemistry of canine OSA cells (Gracie) showed diffuse nuclear staining consistent with the specific 30 kDa protein identified in the nuclear lysate by western analysis (Figure 4B).

Increased immunohistochemical HES1 staining is associated with increased disease free interval

Once we established that the RabMAb anti-human HES1 antibody provided specific targeting of HES1 protein in human cultured cells and FFPE tissues with good cross-reactivity in canine samples, we performed immunohistochemistry using canine primary OSA samples. Of the 20 tumor samples from the canine DFI > 300 and DFI < 100 tumor groups, 14 were scored as described in the methods (Figure 6). For six samples, IHC was not possible due to loss of tissue during processing or poor quality/quantity of staining/tissue present. All OSA samples evaluated with immunohistochemistry had variable positive staining for HES1 both across tumors and within tumors. The staining pattern of tumor cells was predominantly nuclear with diffuse cytoplasmic staining less common. The median HES1 reactivity score was 3 (range, 1 to 9). Of the 6 tumors from dogs with DFI > 300 days, 83.3% (n = 5) had a score of greater than 3, compared to only 25.0% (n = 2) of the 8 tumors from dogs with DFI < 100 days (Table 1). Consistent with our RT-qPCR results, average HES1 immunohistochemical staining was lower in tumors from dogs with DFI < 100 days, but because of low power did not reach statistical significance (Additional file 6).

To further assess the utility of HES1 protein expression as a prognostic biomarker, we performed IHC on 61 primary canine OSA tissues from a subset of dogs in a previously reported prospective clinical trial [33]. Demographic information for this patient population is supplied in Additional file 7. IHC scores were assigned as described in materials and methods. HES1 was expressed in all tumors with a median HES1 immunoreactivity score of 4 in this population (range, 1 to 9). The overall median DFI was 168 (range 43 to 1,393+ days). The median DFI in dogs with a high HES1 immunoreactivity score (≥ 4) was 258 days compared to 155 days in dogs with a low HES1 immunoreactivity score (< 4) ($p = 0.0023$; Figure 7). Univariate analysis identified HES1, bone-specific alkaline phosphatase (BALP) activity, histologic grade, percent necrosis and mitotic index as potential predictors of DFI (Table 2, $p < 0.1$). Upon multivariate analysis, HES1, percent necrosis and mitotic index retained statistical significance ($p = 0.029$, 0.002 and 0.005 respectively; Table 2) as independent predictors of DFI. In summary, consistent with our prior RT-qPCR analysis, increased HES1 expression was identified as an independent prognostic biomarker for increased disease free survival in 61 canine OSAs treated by amputation and chemotherapy.

Discussion

Expression of *HES1* mRNA is frequently utilized as an indicator of Notch activity and Notch/HES1 activation has been implicated in a variety of human cancers with oncogenic activity in some tumor types and tumor suppressor activity in others [17-20,24-27]. The goals of this study were to evaluate expression of Notch receptors and signaling mediators, HES1 and HEY1, in canine OSA samples from dogs with DFI > 300 days and DFI < 100 days as well as samples of matched OSA and normal bone to explore associations with OSA progression and patient outcome. Gene array analysis focusing on 51 Notch/HES1 associated genes identified elevated expression of Notch signaling

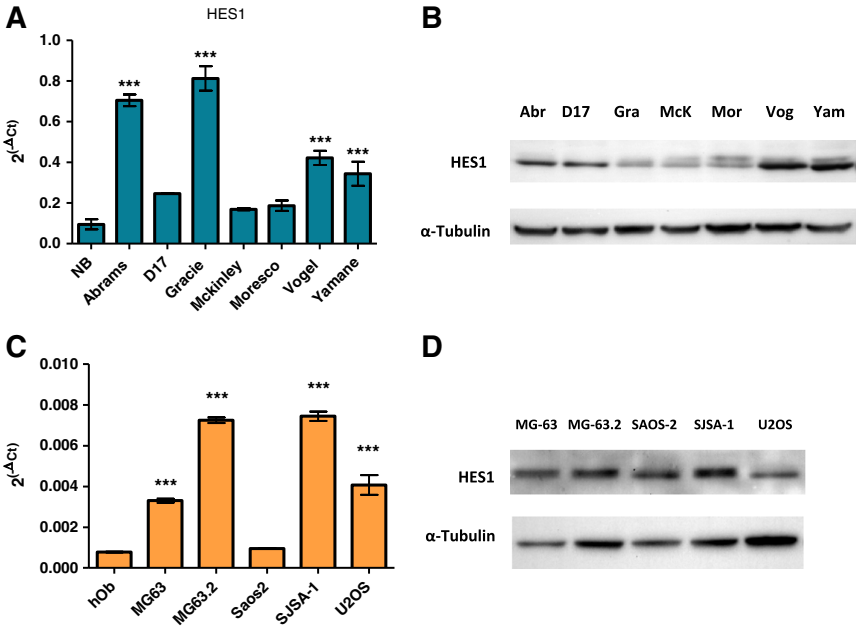

Figure 5 Expression of *HES1* mRNA and protein in human osteosarcoma cell lines. *HES1* mRNA in canine OSA cell lines and normal canine bone tissue (NB) expressed as $2^{(-\Delta CT)}$ normalized to *HPRT1* (**A**). *HES1* mRNA in human OSA cell lines and normal human osteoblasts expressed as $2^{(-\Delta CT)}$ normalized to the geometric mean of *RBS15, GAPDH, 18SrRNA,* and *HPRT1* (**C**). Data are graphed as mean ± SEM, *** $P < 0.001$, ** $P < 0.01$, Two-way ANOVA with Dunnett's Multiple Comparison Test. (**B** and **D**) Western blot shows characteristic distinct HES1 band at 30 kDa. Blot was stripped and re-probed with an antibody against α-tubulin to serve as a protein loading control.

mediators in tumors relative to normal bone. We confirmed a statistically significant elevation of *NOTCH2*, *HEY1*, and *HES1* mRNA expression in OSA when compared with normal bone. Interestingly, we did not find elevated *HES1* expression in the most aggressive OSA when comparing good and poor responders, but instead identified a statistically significant association between high *HES1* mRNA and protein expression and longer DFI following standard treatment. Further, the gene array analysis of Notch/HES1 associated genes and RT-qPCR analysis of *NOTCH1, NOTCH2* and *HEY1* showed no significant differences in expression between the DFI groups. Overall, our findings indicate that alterations in Notch signaling occur during the development of canine OSA, but mechanisms that do not alter HES1 expression may drive the most aggressive tumors.

The oncogenic role of Notch signaling in OSA in humans is supported by previous studies [24-26]; however, the specific role of HES1 is less clear. A common finding regarding HES1 expression between these previous studies and ours is the variability of expression within human and canine OSA cells and tumors (please note for references 24 and 28, that data from experiments done using the OS187 or COL cell lines should be viewed with caution due to a recent disclosure that these cells are not OSA cells) [24-26,28]. For example, *HES1* mRNA expression in tumors relative to normal bone was elevated in 5 of 9 canine tumors relative to matched normal bone samples in our study (Figure 3B) and 6 of 10 human tumors in the Tanaka study [25]. There is also disagreement among studies as to which Notch receptors and target genes are functionally significant in OSA. Zhang et al. provided evidence that increased Notch1 activity and Notch1-induced expression of HES1 specifically are associated with invasion and metastasis in two OSA cell lines, the low HES1 expressing SAOS2 parental line and the metastatic, high HES1 expressing LM7 sub-line [24]. Inhibition of Notch signaling by a gamma-secretase inhibitor suppressed LM7 OSA cell invasion, but had no effect on proliferation or tumorigenesis; whereas induced expression of intracellular cleaved Notch1 (ICN1) or HES1 in the SAOS2 line increased invasiveness. Tanaka et al. identified elevations of *NOTCH2* and *HEY1* mRNA in human OSA biopsy specimens relative to normal bone, but *NOTCH1* and *HES1* mRNA expression was not consistently elevated. In the same study, treatment of OSA cells and tumors grown in nude mice with a gamma-secretase inhibitor reduced proliferation through a G1 block [25]. Differing results in these two studies may be due to different samples studied (tumor vs. cells) and/or the use of different gamma-secretase inhibitors. Our RT-qPCR data suggests

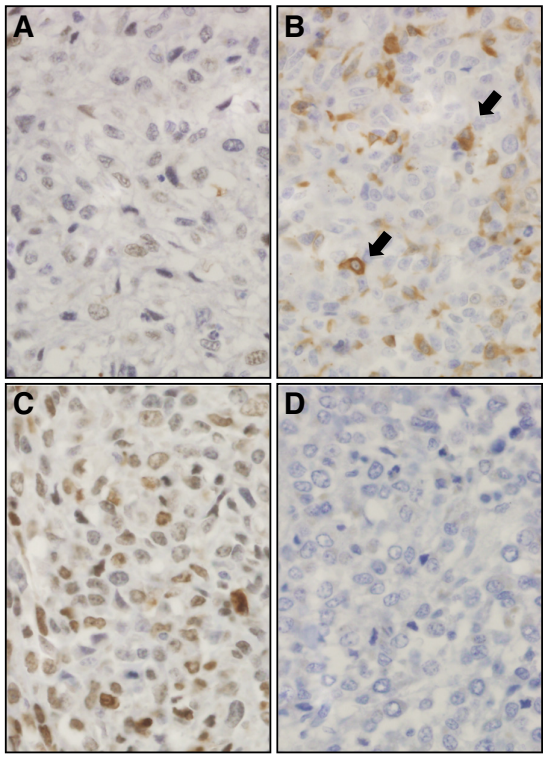

Figure 6 Immunohistochemical analysis of nuclear HES1 protein expression in canine osteosarcoma. Examples of low (**A** and **B**, score 2) and high (**C**, score 6) nuclear HES1 expression in canine osteosarcomas (**D** is a negative control treated only with secondary antibody). Panel **B** shows example of a field from a low scoring tumor (based on nuclear staining) that includes scattered strong cytoplasmic staining (arrows). All photomicrographs were taken at 40× magnification; haematoxylin counterstain.

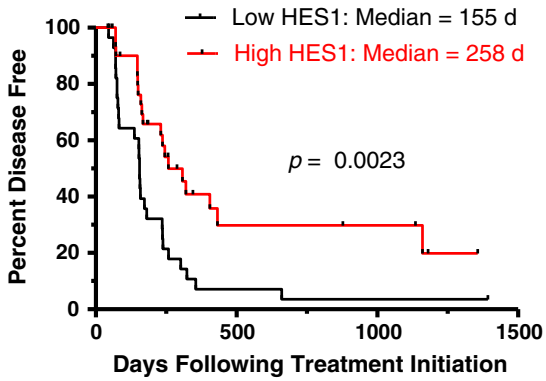

Figure 7 High HES1 immunoreactivity score correlates with lower histologic grade and improved outcome in canine osteosarcoma. Kaplan-Meier plot of disease free interval based on HES1 immunoreactivity score. Dogs with high HES1 scoring tumors (score ≥ 4, n = 28) had a statistically significantly longer disease free interval than dogs with low HES1 scoring tumors (score < 4, n = 33) (p = 0.0023, Log Rank test).

that *NOTCH2* and *HEY1* may be primary mediators of Notch signaling in canine OSA as well. Interestingly, Zhang et al. observed both elevated *HES1* mRNA expression [24] and elevated HES1 protein expression [28] in the LM7 metastatic sub-line relative to the SAOS2 parent line. We also observed an increase in *HES1* mRNA expression in the MG63.2 metastatic sub-line relative to the MG63 parent line. However, western blot analysis identified similar levels of HES1 protein in the MG63 and MG63.2 lines suggesting that post-transcriptional regulation may be important.

Studies exploring the relationship between HES1 expression and patient outcome in OSA are limited. Our RT-qPCR results (n = 20) revealed significantly increased *HES1* mRNA expression in canine OSA from dogs with a longer DFI compared to those with a short DFI. This relationship was confirmed by immunohistochemical examination of HES1 protein in a larger dataset (n = 61). These results conflict with those of Hughes who conducted a RT-qPCR study using tissue from 16 primary OSAs that suggested lower *HES1* mRNA expression may be associated with a better prognosis [27]. Discrepancy from our results may be due to differing sample sizes, different measurements of outcome and different outcome groupings. Despite evidence of strong molecular similarities of canine and human OSA and high conservation of Notch/HES1 between species, there is also the possibility that canine tumors may exhibit different characteristics than their human counterparts.

Table 2 Results of univariate/multivariate analysis of factors associated with clinical outcome

		Univariate analysis			
		Median DFI (d)	HR	P	95% CI
HES1 Score	<4	155	0.388	0.0023	0.211-0.712
	≥4	258			
BALP	<36	273.5	1.871	0.0377	1.036-3.378
	≥36	157			
Necrosis%	<20%	239	1.799	0.098	0.897-3.609
	≥20%	168			
Mitotic Index	<54	258	3.234	0.0163	1.241-8.428
	≥54	153			
Grade	1 or 2	308	15.43	<0.0001	4.243-56.07
	3	75			
		Multivariate analysis			
			HR	**P**	**95% CI**
HES1 Score			0.775	0.029	0.616-0.975
Necrosis%			1.032	0.002	1.012-1.053
Mitotic Index			1.033	0.005	1.01-1.057

DFI disease free interval, *BALP* bone-specific alkaline phosphatase.

Until similar studies to evaluate nuclear immunoreactivity as a measure of protein expression are carried out in human tumors, no firm conclusions regarding possible differences in canine and human OSA with respect to HES1 expression can be made.

Previous studies examining HES1 expression in other cancers or during development provide candidate mechanisms for reduced HES1 expression in the presence of elevated Notch signaling: uncoupling of HES1 from Notch signaling, cell cycle regulation of HES1 expression, and post-transcriptional regulation. HES1 expression has been reported to be uncoupled from Notch signaling in Ewing's sarcoma [15] and stimulation of HES1 transcription by sonic hedgehog (Shh) pathway occurs in mesodermal and neural stem cells [6 – 8]. Using RT-qPCR analysis, we identified significantly decreased SMO mRNA expression ($p < 0.05$) in the DFI < 100 tumors compared to the DFI > 300 tumors [32] suggesting that reduced HES1 expression in aggressive canine OSA might reflect a loss of Shh signaling. HES1 expression oscillations are both observed and necessary for cell cycle progression during neuronal development [45]; aggressive OSA tumor cells may utilize HES1 oscillatory patterns to manipulate the cell cycle and optimize their ability to metastasize and/or resist chemotherapy. Finally, several miRNAs have been shown to regulate HES1 (miR-124 and miR-23b) [46,47] and may contribute to altered HES1 expression in OSA cells and tumors.

In addition, HES1 protein may exhibit specific functions depending on its phosphorylation status and binding partners. Kannan et. al. found that interactions with HES1 stimulates PARP1 activation and cleavage, ultimately resulting in apoptosis in B-ALL (overall a tumor suppressor role for HES1) [20]. Further, in neuronal development, Ju et al. showed that HES1 interactions with phosphorylated PARP1 released HES1 from the HES1/groucho/TLE repressor complex and, upon HES1 phosphorylation, led to association with a co-activator complex, changing the role of HES1 from a transcriptional repressor to a transcriptional activator [48]. In bone development, via inhibition of RUNX2, Notch activity maintains a population of committed osteoblast precursors [49,50]. Interestingly, several studies also show that HES1 binding stabilizes and activates RUNX2 protein; thus, HES1 has been shown to both inhibit and enhance the activity of RUNX2 [49,51]. Additional studies exploring the phosphorylation status and binding partners of HES1 may provide a better understanding of these interactions in OSA.

Conclusions

The results of the current study support the association of Notch pathway activation with the proliferative response of OSA. However, reduced HES1 expression in the most aggressive tumors despite the elevated expression of other Notch signaling effectors and targets indicates that HES1 is not an ideal sole surrogate marker of Notch signaling. Further, these findings suggest that additional mechanisms beyond Notch signaling may contribute to the aggressive phenotype of these tumors. Studies to define the role of Notch signaling in OSAs is warranted as inhibitors for this and other developmental pathways that impinge on HES1 are currently in clinical trials for the treatment of a variety of human cancers (summarized in Sang et al.) [52]. Research in this area may reveal important regulatory mechanisms contributing to metastasis and therapeutic resistance in both canine and human OSA. While we found that HES1 expression was not consistently linked to Notch signaling in canine OSA, our study has determined that reduced HES1 expression serves as an independent prognostic biomarker.

Additional files

Additional file 1: Affymetrix Canine 2.0 microarray data processed with PLIER algorithm. Selected Notch signaling pathway genes from Affymetrix Canine 2.0 microarray data including both previously published [35] and unpublished data (normal bone).

Additional file 2: Sequences, amplicon sizes, and efficiencies of primer pairs used in RT-qPCR experiments.

Additional file 3: Western blot of MG63.2 and U20S whole cell, nuclear and cytosolic fractions for HES1. A distinct band at 30 kDa is present in both MG63.2 and U2OS human OSA whole cell (W) and is enriched in nuclear extract (N) lysates. Larger non-specific bands predominate in the cytoplasmic fraction (C). Equal amounts of total protein were loaded in each lane.

Additional file 4: HES1 protein expression is not significantly different between MG63 and MG63.2 cell lines. HES1 band intensity normalized to α-tubulin loading control. Bars represent mean +/− standard deviation from four independent experiments. Standard unpaired 2-tailed *t*-test was used to compare mean HES1 band intensity ratios for MG63 and MG63.2 Western blot.

Additional file 5: HES1 immunohistochemistry of control canine tissues. Variably intense nuclear staining is present in bronchiolar epithelial cells (A) and in both exocrine and endocrine (islets cells, blue circle) pancreatic cells (C). B and D are the negative controls. All photomicrographs were taken at 40× magnification; haematoxylin counterstain.

Additional file 6: HES1 immunoreactivity in canine osteosarcomas from DFI < 100 and >300 groups. Immunoreactivity scores of nuclear HES1 protein expression in tumor sections from DFI < 100 day (filled circles, n = 8) and DFI > 300 day (filled squares, n = 6) groups. Horizontal line and error bars are mean ± SEM ($p = 0.1026$).

Additional file 7: Summary demographic data for 61 canine patients from a previously reported clinical trial [33].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DDD carried out all mRNA and protein expression experiments (unless otherwise noted), scored IHC samples, analyzed data, performed statistical analyses (except for survival and regression analyses) and drafted the manuscript. KPA contributed to study design and carried out HES1 RT-qPCR for the DFI group tumors. LEP carried out sample preparation (RNA

extraction from canine tissues and sectioning of FFPE canine tissues for IHC) and taught DDD and KPA RT-qPCR methodology including analysis of data. EJE provided guidance to DDD and JBC for IHC/ICC optimization and scoring. JBC assisted DDD with IHC and ICC optimization and scored IHC samples. TBB designed canine HES1 primers. DHT performed survival and regression statistical analyses. BEP graded histologic samples from the larger patient population. TJJ contributed to study design and provided canine HES1 primers. DLD conceived of the study design with TJJ, provided guidance and coordination for all experiments, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Questionnaire Paper I

07.05.2008

Norges veterinærhøgskole

Seksjon for smådyrsjukdommer

Kjære hundeeier.

Ved Norges veterinærhøgskole (NVH) pågår det nå et prosjekt som har til hensikt å kartlegge forekomst av primær benkreft hos fire hunderaser i Norge; irsk ulvehund, newfoundlandshund, leonberger og labrador retriever. Din/deres hund er plukket ut gjennom et tilfeldig utvalg av de aktuelle rasene, registrert i Norsk Kennel Klub (NKK), født mellom 1989 og 1998.

Vi er klar over at mange av de aktuelle hundene ikke lenger er i live i dag, men håper at ***også de som har mistet sin firbente venn tar seg tid til å besvare dette skjemaet.***

Flertallet av de som mottar dette spørreskjemaet har ikke hatt hund med benkreft. *Det er imidlertid avgjørende for undersøkelsen at du/dere svarer på spørreskjemaet selv om hunden din/deres ikke har hatt denne sykdommen.*

Kreft som oppstår i skjelettet/knokkelvevet kalles primær benkreft. Dette er en svært alvorlig sykdom som kan ramme både hund og menneske. Hyppigst oppstår svulsten i ekstremitetene, og sykdommen oppdages da ved hevelse og/eller halthet. Spredning, først og fremst til lungene, er allerede til stede hos flertallet av pasientene når diagnosen stilles. Dette gjør behandlingen vanskelig, og dødeligheten ved denne kreftformen er høy. Sykdommen har mye til felles hos hund og mennesker når det gjelder blant annet risikofaktorer, sykdomsforløp, diagnostikk og behandling. Hos mennesker forekommer benkreft hyppigst hos unge individer og utgjør omtrent 5 % av krefttilfellene hos barn.

Resultater fra spørreundersøkelsen vil være et viktig utgangspunkt for videre forskning på benkreft hos både hund og menneske. I den forbindelse samarbeider NVH med Radiumhospitalet og Kreftregisteret. Samarbeid mellom NVH og Radiumhospitalet har tidligere bidratt til utvikling av nye behandlingsmetoder for mennesker med kreft.

Opplysningene vil kun bli benyttet til denne forskningen, og all informasjon vil behandles konfidensielt. NKK er innforstått med bruken av de data vi har fått overført fra deres database og formålet med undersøkelsen. Det ble gitt informasjon om prosjektet i *Hundesport (nr 4, 2008),* og en påminnelse vil bli trykket i de fire hunderasenes respektive medlemsblader. Informasjon om prosjektet finnes også på www.veths.no/benkreft.

Vi setter stor pris på om du/dere fyller ut skjemaet og returnerer det i vedlagte svarkonvolutt tidligst mulig, helst innen to uker. Ta gjerne kontakt hvis du/dere har spørsmål vedrørende undersøkelsen.
På forhånd takk for hjelpen!

Med vennlig hilsen

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Eventuelle rettelser:

SPØRREUNDERSØKELSE – PRIMÆR BENKREFT HOS HUND

Ved å besvare spørsmålene under så nøyaktig og riktig som mulig, er du med på å gi kunnskap som er viktig for kreftforskningen, både for hund og menneske. Vennligst fyll ut informasjon kun for den hunden som er identifisert øverst til venstre på skjemaet. Eventuelle rettelser av navn eller registreringsnummer føres øverst til høyre på denne siden. Dersom det er spørsmål du ikke husker/vet svaret på, svar likevel etter beste evne på resten av spørsmålene. Ønskes mer informasjon, se www.veths.no/benkreft. For de som trenger figur for å markere brudd og/eller svulster, og har mistet den som var vedlagt på baksiden av informasjonsbrevet, finnes ny figur her. (Denne figuren er kun aktuell for eiere hvis hund har hatt benbrudd og/eller benkreft.)

1: Opplysninger om hunden:

a) Kjønn: Hann: ☐ Hunn: ☐ b) Fødselsmåned: ……. år: ..... (eks 10 1995)

c) Er hunden omplassert? Hvis ja, dato da du: Fikk hunden: ☐ Ga bort hunden: ☐
Nei: ☐ mnd:……. år:…..

2: Hvis hunden din IKKE er i live, er det likevel viktig at du svarer på resten av skjemaet. Hvis hunden din er i live, gå til spørsmål 3.

a) Hva skjedde med hunden?
Avlivet: ☐
Døde selv: ☐
Hundens alder ved død/avliving:…..…år, ……..mnd

b) Hva var årsaken til død/avliving?
Sykdom: ☐
Høy alder: ☐
Skade (for eksempel påkjørsel): ☐
Atferdsproblemer: ☐
Allergi i familien: ☐
Flytting/endrede familieforhold: ☐
Annet:…….. ☐

c) Hvis årsaken til død/avliving var sykdom, hva var sykdommen?:……
…..……
Sykdomsdiagnosen ble stilt:: Av eier: ☐ Av veterinær: ☐ Ved obduksjon: ☐

3: Generell helseinformasjon:

a) Ble hunden vaksinert jevnlig?:
Nei: ☐ Hvis ja, (minst) 1 gang i året: ☐ Hvert 2. år: ☐ Hvert 3. år eller sjeldnere: ☐

b) Hundens gjennomsnittlige vekt som voksen:…….. kg

c) Ble hunden kastrert/sterilisert?
Nei: ☐ Hvis ja, alder ved inngrepet:…..…år, …..…mnd (eks 4 år og 5 mnd)

d) Har hunden fått hormoner for å avbryte/utsette løpetid (♀) eller som kjemisk kastrering (♂)?
Nei: ☐ Hvis ja, antall ganger: 1: ☐ 2: ☐ 3: ☐ Flere enn 3: ☐

e) Tisper: Har tispen fått valper? Nei: ☐ Hvis ja, antall kull:.....

f) Hvor gammel var tispen da hun fikk sin første løpetid (mnd)?
3-6:☐ 6-9:☐ 9-12:☐ 12-15:☐ 15-17:☐ Eldre:☐ Husker/vet ikke: ☐

g) Hvor mange mnd var det generelt mellom løpetidene? …….. mnd

h) Har tispen fått abortsprøyte (for å avbryte mulig drektighet)? Nei: ☐ Hvis ja, antall ganger:…….

4: Sykdommer/skader:

a) Har hunden hatt kroniske/varige sykdommer?
Nei: ☐ Hvis ja, hvilken/hvilke:……..
Alder ved (hver) diagnose (år, evt. mnd.):……..……..
Fikk hunden behandling? Nei: ☐ Hvis ja, behandling/medikament?..……..

b) Har hunden hatt kreft (diagnostisert av veterinær)?

Nei: ☐ Hvis ja, type kreft og alder ved (første) diagnose:

Jursvulst(er): ☐ ……..år, …….mnd Lymfekreft: ☐ …….år, …….mnd

Hudsvulst(er): ☐ …….år, …….mnd Annet:…..……. ☐ …….år, …….mnd

Benkreft: ☐ …….år, …….mnd

c) Har hunden vært utsatt for benbrudd? Nei: ☐ Hvis ja: Alder ved skade:…….år, …….mnd

Vis hvor på hunden skaden var ved å **markere med X** på figuren på baksiden av vedlagte informasjonsbrev

Behandling etter benbruddet (kan krysse av flere):

Bandasje/skinne: ☐ Avliving: ☐

Pinne(r)/stålplate(r), dvs. operasjon: ☐

Utført ved klinikk/veterinær:……...

d) Har hunden hatt andre skjelettsykdommer? Nei: ☐ Hvis ja:

Hofte-/albueleddsdysplasi (HD/AD): ☐

Løs brusk i ledd/OCD: ☐

Forkalkninger i ledd/arthroser: ☐

Annet:…… ☐

5: Spørsmål vedrørende benkreft. Hvis hunden din IKKE har hatt benkreft, er du nå FERDIG med utfyllingen.

a) Navn på klinikk/veterinær som stilte diagnosen benkreft:……..
Alder ved diagnose:……..år, ……...mnd

b) På hvilket grunnlag ble diagnosen stilt?

Kun klinisk undersøkelse: ☐

Klinisk undersøkelse inkludert:

Røntgen: ☐

Biopsi (vevsprøve): ☐

Obduksjon: ☐

c) Ved eventuell biopsi (vevsprøve) eller obduksjon av hunden, hvilken type benkreft hadde den?

Husker/vet ikke: ☐ Osteosarkom: ☐ Chondrosarkom: ☐

Annet: ☐ Beskriv type: ……..…….....

d) Ble det påvist spredning?

Nei: ☐

Hvis ja, til:

Lunger: ☐ Nyre(r): ☐

Annen knokkel/knokler: ☐ Milt: ☐

Lever: ☐ Annet:…….. ☐

e) Fikk hunden behandling?

Nei: ☐

Hvis ja: Ved klinikk/veterinær:…….....

Type behandling: Smertestillende: ☐ Cellegift: ☐

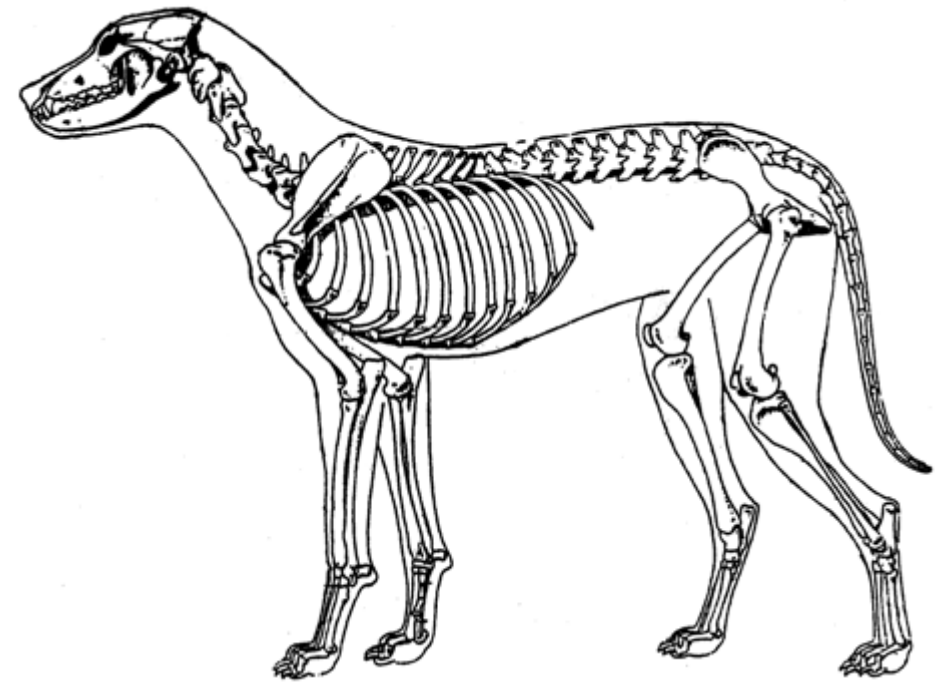
Operasjon: ☐ Annet:……..…… ☐

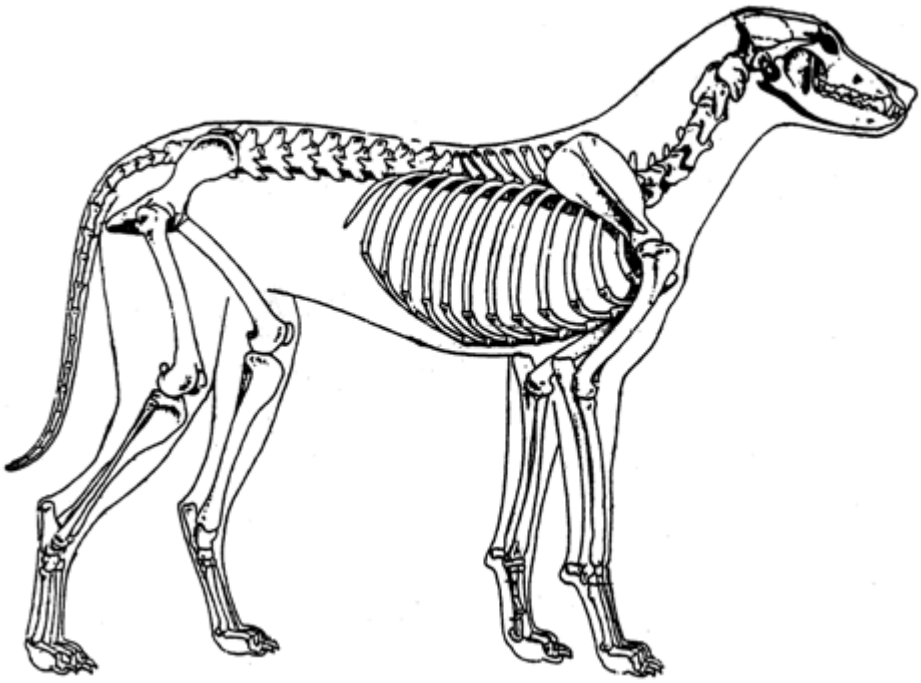
f) Marker (med sirkel ○) hvor på hunden bensvulsten (først) ble oppdaget. Bruk figuren som finnes på baksiden av det vedlagte informasjonsbrevet.

Tusen takk for hjelpen!

Returneres i vedlagte ferdigfrankerte konvolutt. Ved tapt konvolutt, sendes skjemaet til:
Norges veterinærhøgskole, Institutt for sports- og familiedyrmedisin, Seksjon for smådyrsjukdommer,
Postboks 8146, Dep. 0033 Oslo, merkes: *Benkreftprosjektet v/ Kristin P. Anfinsen*

DISSE FIGURENE BRUKES FOR Å MARKERE <u>HVOR</u> HUNDEN HAR HATT BENBRUDD OG/ELLER BENKREFT, HVIS DET ER AKTUELT (se spørsmål 4 c og 5 f)





Vis, hvis det er aktuelt, hvor hunden din har hatt benbrudd ved å markere med X.
Vis, hvis det er aktuelt, hvor hunden din har hatt benkreft-svulst(er) ved å markere med ○

Corrigenda

CORRIGENDUM (PAPER I)

Age at time of death/euthanasia (all causes) for all dogs and individual breeds is reported as average with 95% CI, not average with range like the paper says (the editor of the journal has been notified).

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Corrigendum

Corrigendum to "Primary bone cancer in Leonbergers may be associated with a higher bodyweight during adolescence" [Prev. Vet. Med. 119 (2015) 48–53]

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The authors regret that the fourth author, Cathrine Trangerud, was omitted during the publication process.

Cathrine Trangerud participated during the initial meetings concerning this study, she was highly involved in planning the project and collecting the material, and she has approved the manuscript.

The authors would like to apologise for any inconvenience caused.

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