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Genetic and environmental risk for lymphoma in boxer dogs

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Abstract

Background: Non-Hodgkin lymphoma in humans is associated with environmental chemical exposures, and risk is enhanced by genetic variants in glutathione *S*-transferases (GST) enzymes.

Objective: We hypothesized that boxer dogs, a breed at risk for lymphoma, would have a higher prevalence of GST variants with predicted low activity, and greater accumulated DNA damage, compared to other breeds. We also hypothesized that lymphoma in boxers would be associated with specific environmental exposures and a higher prevalence of canine GST variants.

Animals: Fifty-four healthy boxers and 56 age-matched nonboxer controls; 63 boxers with lymphoma and 89 unaffected boxers ≥10 years old.

Methods: We resequenced variant loci in canine *GSTT1*, *GSTT5*, *GSTM1*, and *GSTP1* and compared endogenous DNA damage in peripheral leukocytes of boxers and nonboxers using the comet assay. We also compared GST variants and questionnaire-based environmental exposures in boxers with and without lymphoma.

Results: Endogenous DNA damage did not differ between boxers and nonboxers. Boxers with lymphoma were more likely to live within 10 miles of a nuclear power plant and within 2 miles of a chemical supplier or crematorium. Lymphoma risk was not modulated by known canine GST variants.

Conclusions and Clinical Importance: Proximity to nuclear power plants, chemical suppliers, and crematoria were significant risk factors for lymphoma in this population of boxers. These results support the hypothesis that aggregate exposures to environmental chemicals and industrial waste may contribute to lymphoma risk in dogs.

KEYWORDS
canine, detoxification, exposure, lymphosarcoma

1 | INTRODUCTION

Lymphoma is a common cancer in dogs, but its underlying causes are not well understood. Several breeds have a higher risk of lymphoma, including boxers, golden retrievers, bulldogs, bull mastiffs, Bernese mountain dogs, Rottweilers, German shepherd dogs, Cocker spaniels,

Briards, Dogue de Bordeaux, and standard schnauzers.[1-4] Boxers are particularly predisposed to T-cell lymphoma, with a median age of onset of 7 and 10 years for high and low grade tumors, respectively.[4] Lymphoma typically is not cured in dogs even with multimodal chemotherapy, with median survival times of only 8 to 9 months for T-cell lymphoma.[5,6]

Molecular genetic research in dogs with lymphoma has identified somatic tumor mutations that may be prognostic markers or targets for chemotherapy.[3,7-10] Acquired tumor mutations in lymphomas of dogs can target several gene pathways,[3] but relatively little is known about the risks for accumulation of these mutations. Understanding factors that contribute to the risk of cancer before it develops may lead to evidence-based cancer prevention strategies for owners of high-risk dogs.

Lymphoma in dogs resembles non-Hodgkin lymphoma (NHL) in humans, which is more common in people in industrialized nations than in developing countries (www.wcrf.org). Specific environmental chemicals associated with NHL include benzene (found in vehicle exhaust, second-hand tobacco smoke, and petrochemical solvents),[11,12] chlorinated hydrocarbons,[13] and various pesticides,[14,15] herbicides, and fungicides.[16] In dogs, environmental risks for lymphoma also have been documented, but few studies are available. Demonstrated risk factors include exposure to commercially applied pesticides,[17] herbicides,[18-20] and household chemicals,[21] as well as living in industrial areas or in proximity to polluted sites.[2,21] One study in France found a correlation between the geographical distributions of both lymphoma in dogs and NHL in humans, suggesting shared environmental risk factors for both species.[2]

Lymphoma risk in humans is further modified by enzymatic pathways that biotransform environmental carcinogens. The most common enzymes in humans implicated in lymphoma risk are glutathione S-transferases (GSTs). The GSTs conjugate reactive chemicals to glutathione, which typically leads to detoxification of xenobiotics that otherwise could damage DNA. These GSTs also detoxify reactive endogenous molecules, such as genotoxic hydroperoxides that are generated during oxidative stress.[22] Multiple classes of GSTs have been identified in humans, and the most studied are GST-theta (GSTT), GST-pi (GSTP), and GST-mu (GSTM). Low activity variants in the genes encoding these enzymes have been associated with higher levels of in vivo DNA damage in humans,[23,24] as well as a wide variety of cancers, including leukemia and lymphoma.[12,25-32]

Our overall hypothesis was that lymphoma risk in boxers would be modulated by a higher prevalence of low activity GST alleles, a breed-related increase in DNA damage, and higher reported exposures to environmental chemicals. Our aims were: (a) to determine whether functionally important genetic variants in canine *GSTT1*, *GSTT5*, *GSTM1*, or *GSTP1* were overrepresented in the boxer dog compared to nonboxer breeds; (b) to evaluate whether boxers have more endogenous DNA damage, as measured by the comet assay in peripheral leukocytes, than age-matched nonboxers, and whether DNA damage is associated with specific GST alleles; and (c) to assess whether lymphoma in boxers is associated with *GSTT1*, *GSTT5*, *GSTM1*, or *GSTP1* variants or with exposures to potential environmental carcinogens, compared to unaffected geriatric boxers.

2 | MATERIALS AND METHODS

2.1 | Healthy dog recruitment

We recruited clinically healthy purebred boxer dogs of any age and without clinical evidence of systemic cancer, from the University of Wisconsin-Madison veterinary teaching hospital, from public dog events, and through outreach to boxer rescue organizations. For the purposes of the study, clinical health status was based on a targeted history obtained from the owner. Boxer breed was determined from veterinary or owner records along with observed clinical phenotype. Readily discernible boxer mixed breed dogs were not included.

For controls, a group of nonboxer dogs, excluding breeds also considered at higher risk for lymphoma (ie, excluding bulldogs, bullmastiffs, Bernese mountain dogs, Rottweilers, golden retrievers, German shepherd dogs, Cocker spaniels, Briards, dogue de Bordeaux, and standard schnauzers)[1-4] were recruited from the University of Wisconsin-Madison veterinary teaching hospital and from public dog events, and matched by age to the healthy boxers. Both healthy boxers and nonboxer controls were evaluated for GST genotypes and endogenous DNA damage.

2.2 | Recruitment of dogs with lymphoma

Boxer dogs with a confirmed cytologic or histologic diagnosis of lymphoma were prospectively recruited from the UW Veterinary Care Oncology service and from oncologists at Colorado State University, the University of Georgia, Seattle Veterinary Specialists, BluePearl Pet Hospital in Franklin TN, the Wisconsin Veterinary Referral Center, and the VCA network of specialty oncologists. Immunophenotyping and cytologic grading were not required for enrollment, and dogs could be at any stage of treatment when evaluated.

Controls for the boxer dogs with lymphoma were clinically unaffected boxer dogs ≥10 years of age, which is the median age of onset for low grade T-cell lymphoma in boxers.[4] Because many older boxer dogs have concurrent disease, those with cardiac, respiratory, gastrointestinal, hepatic, endocrine, urinary, dermatologic, or neurologic disorders still were included in the control group as long as an underlying non-neoplastic etiology had been established and clinical signs were stable. Because dogs could still develop lymphoma after 10 years of age, owner follow-up was obtained for older control boxers until the end of the 2-year recruitment period, as available, to confirm that lymphoma was not subsequently diagnosed. If this occurred, these dogs were moved to the lymphoma group for GST genotyping analyses. Owners provided written informed consent using a form included in a study kit. Home addresses were voluntarily provided by owners in the questionnaire, and questionnaire responses were deidentified on data entry using a unique study ID for each dog. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine.

2.3 | GST genotyping

Genomic DNA was obtained from buccal brush swabs from all groups of dogs: healthy boxer dogs, healthy nonboxer dogs, boxers with lymphoma, and geriatric boxer controls. Variants in canine *GSTT1*, *GSTT5*, *GSTM1*, and *GSTP1* were identified from several sources: screening results from healthy dogs of various breeds in previous studies,[33-35] canine variants, formerly listed for nonhuman species, in the National Center for Biotechnology Information (NCBI) dbSNP database, and evaluation of whole genome sequencing data from 281 domestic dogs through the National Human Genome Research Institute Dog Genome Project (data files kindly provided by Drs Brian Davis and Elaine Ostrander). Variants were evaluated for possible functional significance using multiple in silico prediction programs, including Human Splicing Finder, Polyphen-2, Align-GVDG, ProVean, PANTHER, ESE Finder, PMut, Lasagne, PredictSNP, SIFT, MAPP, PhD-SNP, SNAP, Vertebrate Transfac matrices, M-fold, and miRBase/BLASTN.

Thirty-two alleles with a minor allele frequency of ≥3% in 1 or more dog populations and that were predicted to be deleterious in silico were chosen for screening in the study (Supplemental Table S1). The reference allele at each locus was defined as the most prevalent allele in previously screened dogs of various breeds.

Allelic variants were identified by PCR of genomic DNA and direct sequencing, as previously described.[35,36] Primers were designed using the canine genome assembly (CanFam3.1) as a template. Sequence alignment and polymorphism screening were carried out using SerialCloner v2.6 (SerialBasics) and FinchTV chromatogram reader software (Geospiza Inc).

2.4 | DNA damage in boxers vs other breeds

The classical alkaline comet assay, which detects damaged DNA as "tails" when cells are embedded in agarose and subject to electrophoresis, was used to compare DNA damage in boxer vs nonboxer dogs. Whole blood in EDTA (0.25 mL) was mixed with 0.1× volume of dimethyl sulfoxide, aliquoted, and frozen at −80°C; samples are stable for up to 1 week at −20°C or up to 1 month at −80°C under these conditions.[37] The comet assay was performed using standard techniques,[37] with a Nikon Eclipse E600 fluorescent microscope and Comet Assay IV software (Instem, Stone, Staffordshire, UK). Samples were run in batched assays during recruitment and included both boxers and nonboxers in each run. Etoposide-treated cells (Alkaline Control Cells, Trevigen, Gaithersburg, Maryland) were used as positive controls. For each dog, DNA damage was quantified as tail moment normalized to negative control cells (sample CC0 from the Alkaline Control Cell set) within each assay. Because DNA damage can accrue with age,[38] nonboxer control dogs were aged-matched (±1 year) to healthy boxers for these analyses. In addition, dogs with a history of mutagenic drug administration within the previous month (eg, metronidazole)[39] were ineligible for comet assay analyses.

2.5 | Environmental exposures in dogs with lymphoma

Owners of dogs enrolled in the case-control part of the study were asked to complete a questionnaire about their boxer dog's environment over the year before the date of lymphoma diagnosis, or over the year before enrollment for controls. The 1-year period was chosen to capture all 4 seasons without requiring extended recall.[40] Questionnaires surveyed urbanicity, drive-by traffic, insecticide and herbicide treatments, drinking water sources, and second-hand smoke (Supplemental Figure S1). Proximity of the home to potential sources of pollution was evaluated using the household address and the "nearby" function on Google Maps (www.googlemaps.com). The following sites were searched for within 2 miles of the home: manufacturer, chemical plant or supplier, incinerator, crematorium, bus depot, landfill, farm, and golf course. In addition, active nuclear power plants, coal plants or coal mines were identified within 10 miles of the home.

2.6 | Statistical analyses

The GST allele and genotype frequencies were compared between healthy boxers and control nonboxer dogs, and between boxers with lymphoma and unaffected geriatric boxers, using Chi square or Fisher's exact test, as appropriate. The DNA damage, as measured by normalized tail moments from the comet assay in peripheral leukocytes, was compared between boxers and age-matched nonboxer controls using a Mann Whitney U test, with $P < .05$ used to ascribe statistical significance. The DNA damage also was correlated with age across both groups using a Spearman's rank correlation test. The association between GST variants and DNA damage was examined in 2 ways: linear regression for increasing variant dose and increasing DNA damage, and Fisher's exact test comparing GST allele frequencies in dogs in the lowest 25th and highest 75th percentiles for DNA damage. A Bonferroni correction was performed to control for concerns related to multiple comparisons.

Environmental exposures were encoded as collapsed categorical variables and were compared between boxers with lymphoma and geriatric controls, using Chi square or Fisher's exact tests, with unadjusted P values in this exploratory analysis. Interactions between GST alleles and environmental exposures with lymphoma outcome were assessed using Multifactor Dimensionality Reduction, which is designed to detect complex interactions in the presence or absence of main effects in case-control studies.[41-43] All possible main effects of variables, 2-way combination of variables, and 3-way combination of variables were evaluated, with permutation testing used for ascribing significance.

3 | RESULTS

3.1 | GST genotypes in boxers vs nonboxers

Fifty-four clinically healthy boxers, with a median age of 6.1 years (range, 0.5-11.0) and 56 clinically healthy nonboxers (median age 6.5,

TABLE 1 Demographic data for 54 healthy boxer dogs and 56 healthy nonboxer dogs (excluding breeds with reported increased risk for lymphoma) assayed for DNA damage in peripheral leukocytes using the comet assay

	Boxers	Nonboxers
Number	54	56
Age (median and range)	6.1 years (0.5–11.0)	6.5 years (1.0–12.0)
Sex	FS 21 FI 4 MN 24 MI 5	FS 20 FI 5 MN 21 MI 10
Breeds represented more than once (n)	Boxers (54)	Labrador retriever (6) Australian shepherd (3) Dachshund (3) Boston terrier (2) Cavalier King Charles (2) Doberman pinscher (2) Greyhound (2) Pit bull terrier (2) Samoyed (2) Springer spaniel (2) West Highland white terrier (2)

range 1.0-12.0) were recruited for GST genotyping and DNA damage assays. Demographic data for both groups are summarized in Table 1. Variants at 9 previously documented loci were absent in these 110 dogs, including *GSTM1* c.422, c.497, c.530, c.609, and 2 variants at *6_7; and *GSTP1* variants at −872, −656, and −37. Allele frequencies for the detected GST variants are shown in Table 2. Three complex indel loci in the *GSTM1* promoter could not be resolved with confidence in all dogs.

There was generally less allelic heterogeneity in the boxer group compared to the control nonboxer group, as expected when comparing a single breed to a group of various breeds. Many GST variants were significantly less common, or absent, in boxers (Table 2). The 1 variant that was more prevalent in boxers was a 17-unit microsatellite repeat in the *GSTP1* promoter (allele frequency 0.980 vs 0.367 in nonboxers; adjusted *P* < .002). The next most common variant in nonboxers was a 16-unit repeat (16*1),[44] with an allele frequency of .306. The 17-unit variant was predicted to decrease the number of WT1-KTS transcription factor binding from 25 sites (for 16*1) to 18 sites (for 17-units).

Two functionally characterized GST variants, the *GSTT1* 3′UTR haplotype and the *GSTT5* coding variant (c. 387_392delGGACCA; delAsp129_Gln130), were not overrepresented in boxers. The

TABLE 2 Minor allele frequencies (MAF) for 21 canine GST variants that were detected in boxer dogs and nonboxer breed dogs, screened for DNA damage using the comet assay

GST variant	MAF in boxer dogs	MAF in nonboxer dogs	P value	Adjusted[P] value[a]
GSTT1 I2+28G>A	0.000	0.143	.0003	.06
GSTT1 I2+68T>A	0.000	0.048	NS	**.02**
GSTT1 I2+69A>T	0.000	0.048	NS	**<.002**
GSTT1 I2+72T>A	0.000	0.107	.0009	
GSTT1 I2+168T>C	0.000	0.258	<.0001	
GSTT1 241C>T	0.016	0.013	NS	
GSTT1 I4+70T>C	0.065	0.351	<.0001	**<.002**
GSTT1 674C>T	0.000	0.159	<.0001	**<.002**
GSTT1 *3T>C	0.033	0.171	.0038	.08
GSTT1 *101_102insT, *190C>A, *203T>C	0.011	0.171	.0002	**.004**
GSTT5 c. 387_392delGGACCA delAsp129_Gln130	0.010	0.102	.0044	.09
GSTP1−350C>A	0.020	0.461	<.0001	**<.002**
GSTP1−228C>A	0.010	0.390	<.0001	**<.002**
GSTP1−185delT	0.000	0.080	.0068	.1
GSTP1−68C>T	0.010	0.206	<.0001	**<.002**
GSTP1−66 to −16(GCC)$_{n = 10-22}$ Allele frequency listed for n = 17 unit repeat	0.980	0.367	<.0001	**<.002**
GSTP1−46T>C	0.000	0.408	<.0001	**<.002**
GSTP1−43C>T	0.010	0.190	<.0001	**<.002**
GSTP1−27G>A	0.000	0.120	.0003	**.006**
GSTP1−21A>G	0.010	0.440	<.0001	**<.002**
GSTP1 c.336T>C	0.000	0.500	<.0001	**<.002**

P values listed in bold are statistically significant.
[a]Adjusted for 21 comparisons.

deleterious *GSTT5* variant was found in 10.0% of boxers and 10.2% of nonboxers overall. Another deleterious coding variant, *GSTT1* 674

C>T (Pro225Leu), was not found in boxers but was detected in 15.9% of nonboxers in the current study.
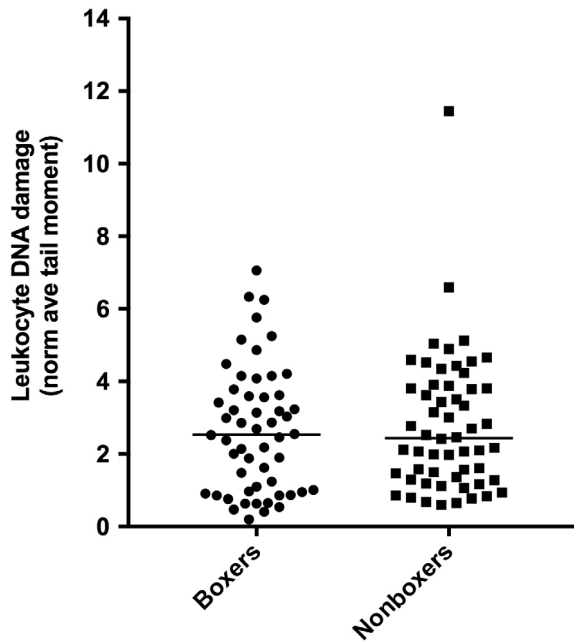
3.2 | DNA damage in boxers vs nonboxers

Peripheral leukocytes from the 54 healthy boxers and 56 healthy nonboxers were assayed for DNA damage using the alkaline comet assay. Contrary to our hypothesis, no significant difference was found in DNA damage between healthy boxers and those breeds at lower risk for lymphoma ($P = .65$; Figure 1). Furthermore, DNA damage was not correlated with age across boxer dogs ($r = -.12$; $P = .4$). We also examined DNA damage for associations with GST variant alleles. No association was found between increasing GST variants and increasing DNA damage at any GST locus. In addition, when dogs with ≥ the highest 75th percentile of DNA damage (normalized tail moment >3.8, n = 28, including 12 boxers and 16 nonboxers) were compared to dogs with ≤ the lowest 25th percentile of DNA damage (normalized tail moment <1.19, n = 27, including 16 boxers and 11 nonboxers), none of the variants were overrepresented in the higher DNA damage subset (data not shown).

FIGURE 1 DNA damage in peripheral leukocytes of boxers (n = 54) vs nonboxer dogs (n = 56) matched for age, as measured by normalized tail moment in the comet assay ($P = .65$ between groups)

3.3 | Boxers with lymphoma and unaffected geriatric boxers

Sixty-eight boxers with lymphoma were recruited; 5 were not eligible because the diagnosis of lymphoma was not confirmed by cytology or

TABLE 3 Demographic and owner-reported household data for boxer dogs with lymphoma and unaffected boxer dogs ≥10 years of age

	Boxers with lymphoma n = 63	Unaffected control boxers n = 89
Age (median and range)	8 years (3.5-15)	10.5 years (10-15)
Sex	FS 30 FI 0 MN 30 MI 3	FS 48 FI 0 MN 37 MI 4
Recruitment sites[a] (dogs per site)	UW Veterinary Care (9) Other referral hospitals (39) Outreach to boxer owners (15) [2]	UW Veterinary Care (14) Other referral hospitals (3) Outreach to boxer owners[b] (72)
Dog's home environment	59 respondents Urban 7 dogs (11.9%) Suburban 38 dogs (64.4%) Rural/Farm 9 dogs (15.3%) Mixed 5 dogs (8.4%)	87 unique households Urban 11 dogs (12.6%) Suburban 46 dogs (52.9%) Rural/Farm 25 dogs (28.7%) Mixed 5 dogs (5.7%)
Heavy traffic by home	58 respondents 2 dogs (3.4%)	86 respondents 3 dogs (3.5%)
Home use of pesticides or insecticides[c]	59 respondents 40 dogs (67.8%)	79 respondents 58 dogs (73.4%)
Home use of weed killer or commercial lawn treatment[c]	54 respondents 23 dogs (39.0%)	86 respondents 45 dogs (52.3%)
Predominantly municipal (chlorinated) drinking water	54 respondents 43 dogs (79.6%)	86 respondents 61 dogs (70.9%)
Smokers in the home	58 respondents 8 dogs (13.8%)	87 respondents 9 dogs (10.3%)

[a]Referral hospitals included Colorado State University, the University of Georgia, and specialty practices in Seattle, Wisconsin, Tennessee, and throughout the VCA national network.
[b]Outreach to boxer owners was at local Wisconsin dog events, and nationally through boxer rescues and Facebook.
[c]Within the past year.

TABLE 4   Household proximity data for boxer dogs with lymphoma and clinically healthy boxer dogs ≥10 years of age, using the owner's home address and the "nearby" function on Google Maps

Household proximity	Boxers with lymphoma 58 unique households providing full address	Unaffected control boxers 86 unique households with full address	Odds ratio (OR) (95% confidence interval)
Manufacturing[a]	81.0% (47 dogs)	83.7% (72 dogs)	.83 (.34-1.94) P = .8
Chemical industry[b] (manufacturer or supplier)	48.3% (28 dogs)	29.1% (25 dogs)	2.28 (1.15-4.63) P = **.02**
Incinerator	8.6% (5 dogs)	5.8% (5 dogs)	1.53 (.47-4.98) P = .5
Crematorium	39.7% (23 dogs)	23.3% (20 dogs)	2.17 (1.02–4.38) P = **.04**
Bus depot	43.1% (25 dogs)	43.0% (37 dogs)	1.00 (.52-1.91) P = 1.0
Landfill	43.1% (25 dogs)	32.6% (28 dogs)	1.57 (.79-3.11) P = .2
Farm	81.0% (47 dogs)	84.9% (73 dogs)	0.76 (.32-1.80) P = .7
Golf course	81.0% (47 dogs)	68.6% (59 dogs)	1.96 (.90-4.17) P = .1
Nuclear power plant[c]	17.2% (10 dogs)	3.5% (3 dogs)	5.76 (1.54-20.06) P = **.007**
Coal plant or coal mine[c]	8.6% (5 dogs)	3.5% (3 dogs)	2.61 (.65-10.12) P = .3

Note: All proximities were tested within 2 miles of the household, unless otherwise noted. Bolded *P* values are statistically significant between groups.
[a]Observed manufacturing sites included electronics, plastics, machinery, instrumentation, semiconductors, boats, motorcycles, film, rubber, textiles, and flooring.
[b]Observed chemical industries included petroleum products, agrochemical, pharmaceutical, medical, and industrial chemicals.
[c]Within 10 miles of household.

histopathology. Overall, 63 dogs with lymphoma were enrolled, with a median age of 8 years (range, 3.5-15 years; Table 3). The control group consisted of 89 clinically healthy geriatric boxers with a median age of 10.5 years (range, 10-15 years). Most dogs with lymphoma were recruited from veterinary referral hospital populations, whereas most control dogs were recruited by outreach to boxer owners through dog events and Facebook (Table 3).

Fifty-nine of 63 owners of boxers with lymphoma completed environmental questionnaires, and 58 provided a full home address for proximity searching. Owners of 87 of the 89 control boxers also completed questionnaires; 2 of these dogs were censored from environmental analyses because they were from the same household as another control dog. Of these 85 control dogs, 84 provided a full household address.

According to the owner questionnaires (Table 3), boxers with lymphoma did not differ from geriatric control boxers in the percentage of households in an urban area (11.9% vs. 12.6%, P = .99); with heavy drive-by traffic (P > .99); that used insecticides (P = .57) or weed killer (P = .13) in the past year; had a chlorinated municipal drinking water source (P = .43); or reported smokers on the property (P = .6). When data were analyzed by households reported to be in a "rural area," 15.3% of dogs with lymphoma were from a rural household compared to 28.7% of control dogs (odds ratio [OR], 0.76; 95% confidence interval [CI], 0.33-1.8), but this difference did not reach significance (P = .07).

According to Google maps data, boxers with lymphoma were significantly more likely to live within 10 miles of a nuclear power plant (17.2%) compared to unaffected geriatric boxers (3.5%; OR, 5.76; 95%

TABLE 5 Minor allele frequencies (MAF) for canine GST variants detected in boxer dogs with lymphoma (n = 63) compared to geriatric unaffected boxer dogs (n = 89)

GSTT1	MAF boxers with lymphoma[a]	MAF unaffected boxer dogs[a]
GSTT1 I2+168T>C	0.014	0.013
GSTT1 I4+70T>C	0.026	0.000
GSTM1 −399 to −400 insCGGAGCCGAGGGGGCG	0.120	0.152
GSTM1 −260 to −273 delCGGAGCCGAGGGGG	0.677	0.763
GSTM1 −231 to −244 delGGAGCCGAGGGGGC	0.033	0.010
GSTM1 c.422T>C	0.022	0.007
GSTP1 −350C>A	0.011	0.026
GSTP1 −228C>A	0.000	0.038
GSTP1 −185 delT	0.000	0.026
GSTP1 −68C>T	0.021	0.013
GSTP1 −66 to −16(GCC)$_{n = 10\text{-}22}$ Allele frequency listed for n = 17 unit repeats	0.975	0.959
GSTP1 −52 C>T	0.011	0.013
GSTP1 −48G>A	0.023	0.006
GSTP1 −46T>C	0.011	0.019
GSTP1 −43C>T	0.043	0.019
GSTP1 −37C>T	0.011	0.006
GSTP1 −27G>A	0.064	0.026
GSTP1 −21A>G	0.032	0.013
GSTP1 c.336T>C	0.000	0.032
GSTP1 c.548G>A	0.000	0.008

Note: Allele frequencies were not significantly different between groups at any tested loci.

[a]Allele frequencies could not be determined in all dogs at all loci.

CI, 1.54-20.06; *P* = .007; Table 4). Boxers with lymphoma were also more likely to live within 2 miles of a chemical manufacturer or supplier (OR, 2.28; 95% CI, 1.15-4.63; *P* = .02) or a crematorium (OR, 2.17; 95% CI, 1.02-4.38; *P* = .04).

In univariate analyses, lymphoma in boxers was not associated with any of the GST variants tested (Table 5). Furthermore, we did not detect any interactions between these GST variants and proximity to a nuclear power plant, chemical manufacturer, or crematorium.

4 | DISCUSSION

Allele frequencies for most GST variants in boxers were quite low (≤6.5%) and were not significantly higher than in nonboxers. An exception was a high prevalence 17-unit microsatellite repeat in the *GSTP1* promoter. This allele is predicted to decrease transcription factor binding sites for WT1-KTS,[44] but this effect has not been

evaluated experimentally. The specific effects of this microsatellite region on *GSTP1* expression deserve characterization, particularly in comparison to the common 16*1 variant found in nonboxers in this population (MAF.306) and in another study of 278 dogs of various breeds (MAF.302).[44]

Multiple GST variants were significantly less common in boxers compared to a heterogeneous group of nonboxers, which is expected given the low levels of heterozygosity in boxers.[45] Two functionally characterized GST variants were not overrepresented in boxers: a *GSTT1* 3′UTR haplotype that decreases expression by 50%,[36] and a *GSTT5* coding deletion variant (c. 387_392delGGACCA; delAsp129_Gln130) that decreases enzyme activity by >90%.[33] However, this *GSTT5* coding deletion was found in approximately 10% of dogs overall in the current study, as well as in 14.4% of canine livers from various breeds.[33] Another deleterious coding variant, *GSTT1* 674 C>T (Pro225Leu), also was found in 15.9% of nonboxers. Further work is needed to characterize the substrate range of canine GSTT1 and GSTT5 for potentially carcinogenic environmental chemicals, in order to understand the clinical and toxicological impact of these dysfunctional coding variants.

We found no difference in leukocyte DNA damage, as measured by the comet assay, between boxers and age-matched nonboxer dogs, nor did we see an association with DNA damage and advancing age within the boxer breed. These findings do not support the hypothesis that the risk of lymphoma in boxers is related to breed-specific accumulated DNA damage. However, we did not assess response to induced DNA damage ex vivo in boxers vs nonboxers, which could uncover DNA repair defects that are masked in a population with heterogeneous exposures. Lymphocytes from golden retrievers with lymphoma show increased susceptibility to DNA damage ex vivo, but golden retrievers as a breed do not share this defect.[46] We did not compare DNA damage in boxers with lymphoma to geriatric boxers because of the confounding factors of already-transformed lymphocytes and the effects of ongoing chemotherapy. However, comparing pretreatment DNA damage in boxers with lymphoma to that in age-matched nonboxers with lymphoma may be informative in future studies.

Lymphoma in boxers was not associated with any of the canine GST variants screened in our study. We initially had found an association between *GSTT1* I2+28A and lymphoma in dogs of various breeds,[35] but did not find the same association in Golden retrievers[35,36] or in the boxers in the present study. Furthermore, the 2 low functioning canine GST alleles that have been characterized to date, the *GSTT1* 3′UTR haplotype[36] and the *GSTT5* 6 bp coding deletion[33] were not overrepresented in boxers with lymphoma in our study. Overall, we found a very low prevalence of GST coding variants of predicted functional relevance among all of the boxers in this population.

In humans, 2 major human GST variants, *GSTM1* null (found in 28%-58% of subjects)[29] and *GSTT1* null, (found in 8%-54% of subjects),[29] lead to a complete lack of gene expression. Defective coding variants in *GSTP1*, notably Ile105Val, also are found in one-third of populations.[29] Non-Hodgkin lymphoma primarily has been associated with the *GSTT1* null allele,[25,30-32,47,48] with some associations found

with *GSTP1* Ile105Val.[12,30] The difference between these human associations and our negative findings in boxers with lymphoma could be a result of several factors. One factor is our focus on a single breed, because different human racial populations show different GST allele frequencies and risk profiles.[29] The second is the presence of GST null variants with no gene expression in humans, which we have not yet recognized in dogs. However, canine *GSTT5* delAsp129_Gln130 encodes an enzyme that virtually lacks activity,[33] and further studies of the substrate range of this polymorphic canine enzyme will be helpful in ongoing molecular epidemiologic studies.

For environmental exposures, we found that boxers with lymphoma were 5-fold more likely to live within 10 miles of a nuclear power plant than older boxers without a diagnosis of lymphoma. We also found that living within 2 miles of a crematorium or a chemical manufacturer or supplier were significant risk factors for lymphoma. These apparent risk factors could result from increased carcinogen exposures through air, soil, or water, and may be individually important or may be surrogates of greater industrial activity in aggregate. Studies in other countries have associated lymphoma in dogs with environmental pollutants, including industrial areas and waste dumping sites in Italy[21,49] and areas with incinerators, polluted sites and radioactive waste in France.[2] We observed fewer dogs with lymphoma living in a rural household (15.3%) compared to controls (28.7%), but this observed difference did not reach significance (P = .07) A post hoc sample size calculation indicated that 161 cases and 161 controls would be needed to show this difference, if real, to be statistically significant (P < .05, 80% power; biostats.info).

We did not find an increased incidence of owner-reported insecticide or herbicide use in the dogs with lymphoma. A previous case-control study of lymphoma in dogs did find a positive association with professionally applied pesticides (OR, 1.7; 95% CI, 1.1-2.7).[17] The previous study included more dogs (263 cases) and a longer owner questionnaire, and thus may have had greater sensitivity to detect associations. An older study found an association with application of the phenoxyherbicide 2,4-D and lymphoma in dogs,[18,19] but others have disputed these data.[50,51] We observed that owners filling out our questionnaires often could not identify or recall what chemical products were used on their properties, and incorporating product labels in future questionnaires might improve sensitivity.

Our findings were limited somewhat by group sizes and by constraints inherent in questionnaire-based epidemiologic studies. Our questionnaire was developed for the present study and another study on cancer risk in dogs,[52] and was not independently validated. Some owners seemed unsure about what chemicals and water treatments were used in their homes, and some of the questionnaire assessments were subjective, such as rural vs suburban or urban. We did not specifically ask how long dogs had been in the current home, but we did ask owners to answer questions based on the previous year. This time frame may not have captured early exposures of relevance but was chosen to reflect seasonal pesticide use without requiring too much recall. In addition, most of our lymphoma cases were recruited from veterinary specialty hospitals and most of our unaffected geriatric boxers were recruited through direct outreach to owners, which could contribute to bias in our population structure. Therefore, our findings of risk related to nuclear power plants, chemical industries, and crematoriums need further exploration.

Overall, our data do not support involvement of known canine GST variants in lymphoma risk in the boxer dog. Further work is underway to understand the substrate range of polymorphic canine GSTs toward specific chemical carcinogens that are relevant to the risk of lymphoma and other cancers in dogs. Direct measurement of chemical exposures in blood, along with more sensitive measures of early DNA damage in lymphocytes, may further refine our understanding of breed-related lymphoma risk in dogs.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.