Lymphoma is the third most commonly diagnosed cancer in dogs, with B-cell lymphoma representing approximately 80% of all cases. Remission following chemotherapy is common; however, long-term disease control is rare, and most dogs ultimately die as a result of the lymphoma. Among dogs with B-cell lymphoma, there is considerable heterogeneity in biological behavior of the disease with respect to success and duration of remission and survival rate. Although diagnosis of lymphoma is often straightforward, clinically useful protein biomarkers are currently not commercially available to enhance early diagnosis, provide additional prognostic information, or guide patient-specific treatment protocols.

Technologic advances in the field of proteomics have offered promise for the discovery of biomarker proteins that may be useful for the development of diagnostic tests for many disease types. Biomarkers are biometric measurements that convey information about the biological condition of the individual being tested. Ideally, the collection of biological samples for diagnostic testing should be cost effective and minimally invasive to the patient, and samples should be easily handled and stored. Serum, plasma, and urine are among the more common samples used for proteomic analysis.

As such, the blood proteome in particular is diverse and dynamic and is constantly changing as a result of differences in organ perfusion, physiologic status of the body in its entirety, protein degradation, and proteolysis. As such, the blood proteome carries a wealth of information about the physiologic status of an individual.
and can theoretically be explored for biomarker proteins that may be of use in clinical practice.

There are many technologic approaches to protein analysis, each of which has advantages and disadvantages. Traditionally, high-resolution 2-dimensional gel electrophoresis has been used for many proteomic applications. This involves 2 steps or dimensions of separation based on the isoelectric point of the protein of interest (the pH at which the protein no longer has a net charge) and subsequent separation based on mass alone, as with standard PAGE. Low- or high-mass proteins or low-abundance proteins are not easily separated. This method is also time consuming and labor intensive and does not lend itself easily to clinically applicable protein profiling.

Matrix-assisted laser desorption-ionization coupled with mass spectrometry detection systems is another commonly used technique. Digested samples are mixed with a matrix and applied to a solid surface. A laser beam is directed at the sample, and the sample and matrix are vaporized into a gaseous state. The analyzer portion of the mass spectrometry unit measures the m/z of a particular peptide or protein. One type of analyzer is a TOF system, which assigns a mass value to a protein on the basis of the time it takes for the ions to travel down a flight tube to a detector. The matrix-assisted laser desorption-ionization TOF system is frequently applied in proteomics but has limitations, including the requirement of off-line sample separation. One technology that is being used with increasing frequency for serum biomarker discovery is SELDI-TOF mass spectrometry. This process involves use of a metal base (a chip) on which there are chemically or biochemically treated areas for selective binding of subsets of the serum proteome. The selective binding of these chip-based arrays, along with prior chromatographic fractionation of the serum sample, results in isolation of certain highly prevalent proteins (eg, albumin) from other subsets of the proteome and enables the detection of less prevalent proteins. A typical SELDI spectrum will include 15,000 datum points that represent m/z values ranging from 500 to 20,000. This amount of data requires bioinformatics tools for processing. The advantages of this technology include high throughput (ie, numerous samples can be analyzed in a short period), a requirement for only small amounts of samples, the capability of processing relatively crude and complex mixtures, and the potential to perform biomarker validation prior to protein identification. One major disadvantage is that protein identification is not an end result of SELDI-TOF analysis and must be performed separately.

For individual proteins to become useful biomarkers, they must have a high degree of sensitivity and specificity for the disease in question. To improve their clinical usefulness, multiple biomarkers can be used in combination. Serum protein profiling or fingerprinting is an approach that uses the large amount of spectral information obtained from SELDI-TOF mass spectrometry analysis to compare many differences between samples. Computer software is used to build classification trees and algorithms with multiple biomarkers to correctly place patients into groups of interest (ie, affected vs not affected with a disease). In human medicine, SELDI-TOF mass spectrometry and classification tree algorithms have been used particularly for the diagnosis of ovarian and prostate cancers; the reported sensitivity and specificity achieved by use of those multiple biomarkers are much higher than those achieved with single biomarker protein assays that are currently in use. In dogs, SELDI-TOF mass spectrometry has also been used to profile proteins in urine that may be useful for the diagnosis of renal disease and for characterization of urolithiasis.

The purpose of the study reported here was to identify biomarker proteins for B-cell lymphoma in canine serum by use of SELDI-TOF mass spectrometry and build classification trees with multiple biomarkers that have high sensitivity and specificity for that tumor type. We hypothesized that SELDI-TOF mass spectrometry in conjunction with software designed to classify SELDI mass spectral data sets for rapid, simplified pattern analysis can be used to identify serum biomarker proteins that have high sensitivity and specificity for canine B-cell lymphoma.

Materials and Methods

Sample population—Two groups of dogs were included in the study: 29 dogs with B-cell lymphoma and 87 control dogs. The control group consisted of 30 apparently healthy dogs; 27 dogs that were receiving veterinary care for a variety of nonneoplastic diseases or conditions such as endocrinopathies, orthopedic conditions, or dermatologic disease; and 30 dogs that had histologically confirmed malignancies other than B-cell lymphoma. For 1 dog with a brain tumor, histologic diagnosis was not available. For the dogs with B-cell lymphoma, blood was collected at the time of initial diagnosis and prior to treatment. Prior to sample collection, informed owner consent was obtained for all client-owned dogs. For the control dogs, blood samples were collected with permission of their owners. All samples were collected during a 2-year period—some were collected prospectively and others were obtained from archived samples. Archived sera were accessed from the Colorado State University Animal Cancer Center serum archive and from samples submitted from a variety of sources to a serum archive maintained at a commercial facility during that same period.

Blood collection and sample handling—From each dog, a blood sample was obtained via venipuncture, placed in a serum separator tube or tube without anticoagulant, and allowed to coagulate for as long as 30 minutes from the time of collection. The sample was centrifuged at 2,000 to 3,000 × g for 10 minutes at room temperature (approx 21°C). Serum was transferred to collection tubes and stored at −80°C. Each sample was subsequently thawed, divided into aliquots, and stored at −80°C.

Serum fractionation—All serum samples were fractionated at the same time via anion exchange chromatography. A commercial kit was used according to the manufacturer’s protocol with slight modification. To collect fractions, the filtration plate was placed over a collection plate and centrifuged at 300 × g for 45 sec-
onds at room temperature. Of the 6 fractions collected, fractions 1 and 2 were combined, as were fractions 4 and 5. This resulted in a total of 4 fractions. Only serum fractionation plates from the same production lot numbers were used.

**Sample preparation for SELDI analysis**—Proteins from serum fractions were bound to spots on the SELDI chip-based arrays by use of a modified version of the manufacturer’s protocol. Only arrays from the same production lots were used. The arrays were equilibrated in a 90-well bioprocessor with 50mM sodium acetate (pH, 4). Serum fractions were diluted 1:3 in 50mM sodium acetate (pH, 4) and loaded into the bioprocessor in duplicate and in random order. The arrays were incubated for 1 hour at room temperature with shaking. To remove excess unbound sample, arrays were blotted and then the bioprocessor centrifuged at 300 × g in an inverted position for 1 minute at room temperature. The arrays were washed 2 times (5 minutes each) with 50mM sodium acetate (pH, 4) and 2 times (3 minutes each) with 1mM HEPES. After the final centrifugation, the arrays were removed from the bioprocessor and air dried for 5 minutes. One microliter of saturated sinapic (sinapinic) acid solution in 50% acetonitrile and 0.5% trifluoroacetic acid was applied twice at 5-minute intervals to each spot on the arrays. The arrays were air dried for a minimum of 15 minutes and then stored in the dark at room temperature until analyzed.

**SELDI-TOF mass spectrometry**—Mass spectrometry was performed by use of a laser desorption-ionization TOF mass spectrometer system. Three software packages were used to help generate and analyze the results: 1 to program and control the mass spectrometer, 1 to chart peak heights in each spectrum and the mean peak heights in sample groups, and 1 to build and test classification trees. The mass (in daltons) and m/z of each protein were estimated by use of external calibration of the mass spectrometer with protein standards of known mass. Spectra were acquired by averaging 112 laser shots across each spot. Each chip-based array was read at 3 laser energy settings on the mass spectrometer to optimally detect proteins of disparate prevalence and m/z values.

**Optimization of test conditions**—To optimize conditions, 2 preliminary experiments were performed to identify the combinations of serum fraction and chip-based array that revealed the best individual biomarkers and classification trees. The best biomarkers and classification trees are defined herein as those having the highest sensitivity and specificity for differentiating the test and control sample groups, as measured by use of 10-fold cross-validation. In the first experiment, 5 B-cell lymphoma samples and 15 control samples were run in duplicate, and in the second experiment 10 B-cell lymphoma and 30 control samples were run in duplicate. In these first 2 experiments, all 4 serum fractions obtained via anion exchange chromatography from each sample were tested on 4 arrays, including the CM10 (weak cationic exchange), Q10 (strong anionic exchange), H50 (hydrophobic interaction), and IMAC30 (nickel chelating) arrays. Of the 16 serum fraction-array combinations evaluated, the 2 that generated the best individual biomarkers and classification trees were serum fractions 1 and 2 bound to the CM10 array. These optimal conditions were then used for the final experiment reported here.

**Data processing and analysis**—All spectra underwent baseline subtraction by use of the settings as follows: smoothing was set to 5, fitting width was set to 2 times the expected peak width, and filtering width was set to 0.4 times the expected peak width. All other parameters were left at the default settings suggested by the manufacturer. Spectra were compiled into a single file and were adjusted to total ion current between m/z 2,500 and 200,000. Spectra with obviously aberrant peak patterns were removed from the analysis. Peaks were selected manually and compared between sample groups (dogs with B-cell lymphoma and control dogs) by use of a software program that charts individual peak heights and color codes them according to sample group. Peak amplitude values from the sample duplicates were averaged and then analyzed individually or in combination by use of a software program that builds and tests classification trees.

Each peak was scored on an individual basis for its ability to distinguish between predetermined groups. The individual biomarkers and classification trees were selected on the basis of the mean sensitivity and specificity values calculated with 10-fold cross-validation. The 2-tailed P values for each peak were calculated by use of computer software. A value of P ≤ 0.0001 was considered significant. This value was derived by starting with a P value of 0.05 as the cutoff for significance and then dividing this number by 500, which is a rough estimate of the number of distinct peaks analyzed in the experiment. This accounts for the fact that in a normal distribution, the number of peaks analyzed affects the probability that the P values for some peaks will be significant by random chance.

**Results**

**Dogs**—The groups of dogs with B-cell lymphoma and control dogs were reasonably well matched in age, sex, and breed distribution. Approximately 90% of dogs in both groups were neutered or spayed. Although the breeds were diverse in both groups, Labrador Retrievers, Golden Retrievers, and Beagles were the most common.

Among the study dogs, there were 29 dogs with B-cell lymphoma. The mean age of these dogs was 7.5 years (range, 2.4 to 12.4 years). The group included 19 males (16 of which were neutered) and 10 females (all of which were spayed). Labrador Retrievers, Golden Retrievers, and Beagles composed 21%, 14%, and 3% of this group, respectively.

In the control group, the mean age of the 87 dogs was 8.1 years (range, 1.1 to 20.1 years). The group included 39 males (34 of which were neutered) and 48 females (44 of which were spayed). Labrador Retrievers, Golden Retrievers, and Beagles composed 22%, 14%, and 11% of this group, respectively.

Among the 87 control dogs, there were 3 subgroups: healthy dogs (n = 30), dogs with nonneoplastic diseases or conditions (27), and dogs with cancer other
than B-cell lymphoma (30). The mean age of the healthy dogs was 7 years (range, 1 to 12 years). The subgroup included 16 males (13 of which were neutered) and 14 females (13 of which were spayed). The mean age of the dogs with nonneoplastic diseases was 7.5 years (range, 2 to 13 years). This subgroup included 14 males (13 of which were neutered) and 13 females (12 of which were spayed). The mean age of the dogs with cancer other than B-cell lymphoma was 9.6 years (range, 5 to 16 years). This subgroup included 9 males (8 of which were neutered) and 21 females (19 of which were spayed).

### Biomarker proteins

The 3 individual biomarker protein peaks with the highest sensitivity and specificity (peaks 9,242, 9,452, and 9,580) obtained via SELDI-TOF mass spectrometry of sera from 29 dogs with B-cell lymphoma (B), compared with data derived from 87 control dogs or each of the control subgroups (30 healthy dogs [H], 27 dogs with nonneoplastic diseases or conditions [NND], and 30 dogs with cancer other than B-cell lymphoma [OC]). Values were generated by use of 10-fold cross-validation of the training data set.

<table>
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<tr>
<th>Group comparison (test vs control)</th>
<th>m/z</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>P value*</th>
<th>Test</th>
<th>Control</th>
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<tr>
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<td>9,242</td>
<td>79</td>
<td>82</td>
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*A value of P ≤ 0.0001 is considered significant.

**Figure 1**—Representative spectra obtained via SELDI-TOF mass spectrometry of sera from dogs with B-cell lymphoma (B) and 87 control dogs (subgroups of healthy dogs [H], dogs with nonneoplastic diseases or conditions [NND], and dogs with cancer other than B-cell lymphoma [OC]). Notice the differences in peak intensity of biomarker 9,242 among the sample groups. The vertical marks at the top of each 9,242 peak indicate the position at which the peak heights were recorded for comparison.

**Figure 2**—Scatterplot of peak intensity for biomarker peaks 9,242, 9,452, and 9,580 obtained via SELDI-TOF mass spectrometry of sera from 29 dogs with B-cell lymphoma and 87 control dogs (subgroups of 30 healthy dogs, 27 dogs with nonneoplastic diseases or conditions, and 30 dogs with cancer other than B-cell lymphoma). The mean peak amplitude for each sample group is indicated by a short solid line; suggested amplitude cutoffs for each peak are indicated by a dotted line. See Figure 1 for remainder of key.
with B-cell lymphoma and the dogs in each control subgroup were plotted (Figure 2).

Within spectra, peaks 9,242, 9,452, and 9,580 were generally proportional in amplitude; spectra with a peak height for 1 peak that was greater than the mean height also appeared to have peak heights that were greater than the mean heights for the other 2 peaks (data not shown). When these peaks were compared with other peaks in the same m/z range, no correlation with peak amplitude was evident, indicating that the increase in amplitude was not secondary to artifact. Among the misclassified samples, no correlations of any of the biomarker peaks with specific diagnoses, age, breed, sex, reproductive status (neutered, spayed, or sexually intact), or sample source were detected.

Classification tree—A classification tree was constructed by use of the pattern analysis software. The classification tree is a binary decision tree that attempts to correctly classify each sample as either test or control on the basis of the amplitudes of selected biomarker peaks (so-called splitters). The classification tree for this data set used 3 biomarker peaks as splitters, with peak 9,580 in the primary splitter node and peaks 5,054 and 4,769 in 2 secondary splitter nodes. Peak 5,054 was increased above baseline in the control sample group, whereas peak 4,769 was increased above baseline in the B-cell lymphoma sample group. The 4 terminal nodes at the bottom of the tree classified each sample as either test or control. The classification tree had 97% sensitivity, 91% specificity, and 92% accuracy; the positive predictive value was 78%, and the negative predictive value was 99%. No correlations with age, breed, specific diagnoses, or sample source were apparent for the 9 samples that were misclassified among the 116 samples evaluated. All 9 samples represented dogs with different diagnoses, including B-cell lymphoma, osteosarcoma, mammary gland tumor, T-cell lymphoma, mast cell tumor, cranial cruciate rupture, grand mal seizures, and urinary tract infection; 1 dog was healthy. Both the B-cell lymphoma and control groups were represented among the 9 samples and were in proportion to the total number of samples supplied by each source. Seven of the 9 samples were from male dogs, and all 9 dogs were neutered or spayed.

Discussion

Because of the sensitivity of SELDI-TOF mass spectrometry, it is important to control for other factors that may influence the results. In the present study, all of the sera were stored and handled in a similar manner and all samples were loaded in random order and run together in single experiments for both the serum fractionation and chip-binding procedures. Furthermore, the serum fractionation plates used were from the same production lot, as were the chip-based arrays. Controlling for factors such as sample collection procedures, storage, and handling; age, sex, reproductive status (neutered, spayed, or sexually intact); and breed of dog; and serum fractionation kit and array production lots was attempted to isolate disease type as the differentiating characteristic between the sample groups. Ten-fold cross-validation of the sample set was used to generate the sensitivity, specificity, and accuracy numbers for the individual biomarker peaks and for the classification tree. Cross-validation is a technique that can be used to provide an estimate of the classification tree performance if it were tested with a separate data set; the sensitivities and specificities calculated are often lower than the actual training results, which are obtained without cross-validation. In the present study, the sensitivity and specificity results for the classification tree determined by use of the training and cross-validation testing methods were the same, indicating a robust calculation of accuracy. However, testing the peaks and classification tree with an entirely different set of samples is an important next step. Confirmation of the biomarkers via a different method, such as 2-dimensional gel electrophoresis or immunodetection, can also help to validate the results and narrow the search for important single or clusters of biomarkers that can then be tested for clinical relevance.

It is possible that peaks 9,242, 9,452, and 9,580 detected in canine serum in our study represented related proteins or the same protein that had been modified posttranslationally in different ways. These peaks were regulated similarly between sample groups and within spectra and were similar in m/z. In humans, 2 forms of serum amyloid A1 (molecular mass, 11.5 and 11.7 kd) have been identified via SELDI-TOF mass spectrometry as biomarkers for ovarian cancer. The 11.5-kd form differs from the 11.7-kd form by the removal of a single amino acid. In the present study, the mean heights of peaks 9,452 and 9,580 were similar among the 3 control subgroups. However, for peak 9,242, the mean peak height in sera from dogs with cancer other than B-cell lymphoma was slightly higher than that detected in sera from the healthy control dogs and dogs with nonneoplastic disease. That difference was small, compared with the differences between each of these subgroups and the B-cell lymphoma group, and it is unclear whether it indicates a genuine difference in subgroup specificity for this peak or was merely a result of random sampling. The fact that the 7 misclassified 9,242 peaks in the control subgroup of dogs with cancer other than B-cell lymphoma were associated with sera from dogs that each had a different type of cancer may indicate a more random pattern than a true difference in subgroup specificity between the peaks. Sequencing and identification of these protein peaks identified in our study would be required to determine whether they represent variants of the same protein or protein family. The similarity in the expression patterns of the 3 described individual biomarker peaks also explains why only 1 of these peaks was used as a splitter in the classification tree; inclusion of more than 1 of these peaks would be redundant. Peaks 5,054 and 4,769 were selected for use not because they were among the best individual biomarkers for the entire data set, but because they were the best biomarkers for the smaller data sets in the 2 secondary splitter nodes. Presumably, the expression patterns of these 2 proteins differ somewhat from the expression pattern of the top 3 biomarkers.

In addition to the healthy control subgroup, dogs with nonneoplastic disease or cancer other than B-cell lymphoma were included in the experiment in an attempt to control for other disease types and more ac-
curately determine the specificity of the biomarkers. Including these subgroups in the control group helped to reduce the likelihood that the resulting biomarkers were merely general inflammatory, stress, or immune-related proteins that lack specificity for B-cell lymphoma. However, during analysis of the results, it must be considered that in terms of specific disease diagnoses, sera from a homogenous B-cell lymphoma group was compared with a heterogeneous control group. Although this allows a wide variety of different disease types and conditions to be evaluated in a single experiment, it prohibits certain conclusions from being drawn. For instance, the specificity values calculated for the entire control group cannot be extrapolated to include each individual diagnosis that was represented in that group. This is because the number of samples from each disease type or condition was too small to confidently assess statistical significance of findings or greatly affect the specificity calculated for the control group overall. In addition, the control group population is likely to be different from the population that would be evaluated in a clinical setting and thus does not provide an accurate assessment of clinical specificity.

A comparison between the B-cell lymphoma group and healthy control subgroup (which also was a homogenous sample group in terms of diagnosis) revealed relatively high specificities for the individual biomarker peaks and for the classification tree, and these values were similar to those calculated by comparison of the B-cell lymphoma group and control subgroup of dogs with nonneoplastic disease or the control subgroup of dogs with cancer other than B-cell lymphoma. Furthermore, the samples misclassified by the individual biomarker peaks or by the classification tree did not reveal any correlation with control subgroup or with any specific diagnosis. Thus, the results of the present study support the hypothesis that the individual serum biomarkers and the classification tree have good specificity for B-cell lymphoma in dogs. Further evaluation will be required to more appropriately assess the specificity of the biomarkers in multiple clinical situations. Potential uses for these and other biomarkers include investigation of early disease development, screening of susceptible populations for early detection of disease, and monitoring disease status during treatment and remission. Although the results of our study did not suggest a specific use of these biomarkers for detection of disease in its early stages or low disease burden, the data have indicated that serum protein biomarkers and classification trees can have high specificity for canine B-cell lymphoma and that the use of SELDI-TOF mass spectrometry may be used to advance diagnosis of neoplasia in dogs.

d. Biomarker Wizard software, Ciphergen Biosystems Inc, Fremont, Calif.
e. Heska Corp, Loveland, Colo.
g. 3,5-dimethoxy-4-hydroxycinnamic acid, Sigma-Aldrich Inc, St Louis, Mo.
h. ProteinChip software, version 3.1, Ciphergen Biosystems Inc, Fremont, Calif.
i. All-in-One Protein Standards, Ciphergen Biosystems Inc, Fremont, Calif.
j. TDST function in Excel, Microsoft Inc, Redmond, Wash.

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