Diabetes mellitus (DM) in dogs is a multifactorial disease, with genetic and environmental components thought to contribute to the development of the condition. The estimated prevalence of DM in dogs is 1 in 300. Because most diabetic dogs are insulin-deficient, the disease has been compared to human type 1 diabetes and latent autoimmune diabetes of the adult. This comparison is supported by the presence of antibodies to pancreatic autoantigens in a number of cases and the lack of evidence of a direct link between obesity and development of diabetes in dogs, in contrast to human type 2 diabetes.

The published genetic studies of DM in dogs are primarily based on a candidate gene approach. Within some breeds, certain haplotypes of the canine major histocompatibility complex (encoded by dog leukocyte antigen (DLA) genes) are associated with increased risk of DM, similar to the risk seen with some human leukocyte antigen (HLA) types and type 1 DM. More recently, using single nucleotide polymorphism (SNP) discovery and a candidate gene approach, polymorphisms in several other immune response and cytokine genes have been implicated as genetic risk factors in certain breeds. Many SNPs are associated with protection or susceptibility in only a single breed, and interestingly, some SNPs are monomorphic (only 1 allele) within some breeds. Where DM risk variants are detected, they might only be important within a single breed (eg, IL10 polymorphisms in the Cavalier King Charles spaniel). A recent candidate gene study reported that a 14-base-pair deletion in the canine pro-opiomelanocortin (POMC) gene is associated with food motivation and obesity in Labrador retriever dogs. POMC mutations have also been associated with obesity in humans, with a subsequent increase in risk of obesity-related diseases.
such as type 2 diabetes.\textsuperscript{15} Although there is very little evidence that obesity is a major risk factor for DM in dogs, obese dogs are hyperinsulinaemic.\textsuperscript{16} Additionally, in a recent epidemiological study of DM in first opinion practice in the UK, being recorded as being overweight was associated with a diagnosis of DM.\textsuperscript{4} Furthermore, research into the links between canine obesity and nondiestrus diabetes has not been undertaken from a breed-specific perspective, so this does not rule out an association between obesity and diabetes in certain breeds, similar to the association between type 2 DM and obesity in humans. Therefore, given the absence of any reported immune response gene associations with DM in Labrador retrievers, we hypothesized that the obesity-associated and appetite-associated \textit{POMC} deletion might also be associated with increased risk of DM in Labrador retrievers, which may consequently be more similar to human type 2 than type 1 DM. The aims of this study were to develop a simple genetic test for the reported \textit{POMC} deletion in Labrador retrievers and to use this test in a population of Labrador retrievers (DM-affected and unaffected) to determine whether the \textit{POMC} deletion is present at a higher frequency in the diabetic population, supporting a potential role for obesity as a risk factor for DM in this breed.

\textbf{Materials and Methods}

\textbf{Study Population}

\textbf{Diabetic Dogs.} EDTA blood samples from diabetic Labrador retrievers were retrieved from the UK Canine Diabetes Register and Archive, at the Royal Veterinary College, University of London. The diagnosis was made by primary care veterinary practitioners, based on compatible clinical signs of polydipsia, polyuria, and weight loss and documented persistent hyperglycemia (>162 mg/dL), an elevation in serum fructosamine concentration, or both. Most dogs were receiving insulin treatment at the time of sampling. Dogs with early-onset diabetes (ie, <1 year of age), concurrent hyperadrenocorticism, and female entire dogs (likely to have diestrus diabetes), were excluded from the study.

\textbf{Control Dogs.} EDTA blood samples from non-diabetic Labrador retrievers over 6 years of age were selected from an archive of residual clinical samples at the Royal Veterinary College, University of London. Dogs over 6 years of age were selected because this provided appropriate age-matching for the diabetic samples and limited the likelihood of these dogs developing diabetes later in life. All samples used for this study were initially collected for diagnostic purposes, and the residual blood volume remaining after completion of diagnostic testing was stored at \(-20^\circ\text{C}\) until DNA extraction. Storage was undertaken with informed owner consent.

\begin{table}
\centering
\caption{Descriptive statistics of the study population.}
\begin{tabular}{lcccccc}
\hline
 & Median Age & Male Neutered (%) & Male Entire (%) & Female Neutered (%) & Female Entire (%) & Sex Unknown (%) & Total Number of Dogs \\
& (Years) [Range] & & & & & & \\
\hline
Control population & 9 [6-13] & 19 (31) & 13 (21) & 25 (41) & 2 (3) & 2 (3) & 61 \\
Diabetic population & 9 [6-13] & 18 (32) & 14 (25) & 25 (43) & 0 (0) & 0 (0) & 57 \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig1.png}
\caption{Agarose gel electrophoresis after the amplification of a 520-bp region of genomic DNA from 6 Labrador retriever dogs, using primers designed to flank exon 3 of \textit{POMC} in the amplicon. The variability in the size of the amplicons indicates that some dogs were heterozygous for a 14-base-pair deletion (C3 and C5) and 1 dog was homozygous for the deletion (C4). This was confirmed by sequencing.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{Sanger sequencing traces from dogs with homozygous, heterozygous, and wild-type genotypes for the 14-base-pair \textit{POMC} deletion. The start point of the deletion is illustrated by a red-dashed line and the end point by a blue-dashed line.}
\end{figure}
and with institutional ethical review board approval from the Royal Veterinary College Ethics and Welfare Committee. DNA was extracted from EDTA blood using either a standard phenol-chloroform method or a Qiagen Blood and Tissue DNA extraction kit and sample quality was evaluated with a Nanodrop analyser before polymerase chain reaction (PCR). Where necessary, samples were re-extracted, diluted, or both to a suitable working concentration (5–20 ng/μL) before use.

Detection and Confirmation of POMC Mutation

Primers were designed to amplify a 560-base-pair (bp) fragment, containing canine POMC exon 3 (K9POMC-1 sense primer: 5'-GGCGCATCATTGGGCGACC-3' and K9POMC-2 antisense primer: 5'-GACACAGGCGAGGCT-3'), with an annealing temperature of 66°C found to be optimal. Primer 3 software was used to design the primers, and the Canis lupus familiaris POMC gene transcript (Transcript: POMC-201 ENSCAF00000006656) was downloaded from Ensembl at http://www.ensembl.org/Canis_familiaris/Info/Index for this purpose. Primers were supplied by IDT technologies, and all PCR reactions were performed on an MJ PTC-225 thermal cycler using Hi-fidelity Hotstart Q5 enzyme.

Amplicons were visualized under UV light with ethidium bromide, after 5% agarose gel electrophoresis at 100 V for 2 hours to allow optimal resolution of bands. Polymerase chain reaction products were purified with a Qiagen PCR cleanup kit and submitted for Sanger sequencing in a forward and reverse direction using the same primers as used for PCR. Based on these results, a further primer set was designed and tested, with the identified deletion located more centrally within the PCR product. This primer pair was used in all further PCR reactions (K9POMC-3 sense primer: 5'-AGTGACGTCATGGGCGACC-3' and K9 POMC-4 antisense primer: 5'-TTTGGAACAGCGCTACGGG-3') at an optimal annealing temperature of 68°C.

Where indicated, restriction fragment length polymorphism (RFLP) analysis was undertaken on PCR products in 1x NEB CutSmart buffer and either ApaI or SacII enzyme for 1 hour at 37°C. The online software NEB Cutter v2.0 at http://nc2.neb.com/NEBcutter2 was used to determine suitable enzymes for this purpose. After digestion, DNA was separated on 4%

**Fig 3.** (A) illustrates a fragment of the canine POMC gene, demonstrating the delta-exon 3 canine POMC allele (DEL-LAB), missing 14 nucleotides compared with the wild-type allele (WT-LAB). Ensembl transcript POMC-201 ENSCAF00000006656 (ENSEMBL) in this region is also shown, with a putative 2 base-pair intron (shown in lower case). The RefSeq (REFSSEQ) transcript in this region is also shown, containing a single putative extra nucleotide “N” (in bold) (The NCBI website notes that the sequence of the model RefSeq transcript was modified relative to its source genomic sequence to represent the inferred CDS: inserted 1 base in 1 codon; Derived by automated computational analysis using gene prediction method: Gnomon). Neither the RefSeq nor the Ensembl canine POMC transcript shows the G nucleotide (in bold) which was present in both the wild-type and delta-exon 3 Labrador retriever POMC allele in this study.

The deleted nucleotide sequence is AGGGCCCGGCCG, which differs by 1 nucleotide from the Ensembl canine reference sequence (Ensembl transcriptPOMC-201 ENSCAF00000006656) at position 8 of 14 (C>G) of the 14-bp deleted fragment (shown in white). (B) illustrates potential impact of the 14-base-pair deletion on the amino acid sequence of wild-type Labrador retriever POMC (WT-LAB) and delta-exon 3 POMC (DEL-LAB) compared to the published UNIPROT sequence (http://www.uniprot.org/uniprot/E2RQ39.fasta). The WT sequence differs from the UNIPROT sequence by 1 amino acid, highlighted in black and white. The 14-base-pair delta-exon 3 allele leads to a frameshift mutation with the subsequent amino acid sequence being shown in italics. This frameshift disrupts the 90 amino acid terminal region of the protein fragment of POMC which is cleaved to form beta lipotropin and subsequently cleaved further to form beta endorphin and beta melanocyte-stimulating hormone.
agarose gels containing ethidium bromide for 60 minutes at 120 V and visualized under UV light. Sequence alignments were performed at http://www.bioinformatics.org/sms2/ and functional evaluation of the deletion at http://www.uniprot.org/uniprot/E2RQ39. Statistical analysis was undertaken by R version 3.2.3. A chi-squared analysis was performed to compare allele frequencies between diabetic and non-diabetic Labrador retrievers, using a $3 \times 2$ contingency table with a significant $P$ value set at .05.

Results

A total of 118 Labrador retriever dogs were included in the study, of which 57 were diabetic and 61 were non-diabetic. The characteristics of these dogs are reported in Table 1. The mutation in the canine POMC gene was reported as a 14-base-pair deletion in exon 3, but more precise details of the deletion had not been published before this study being undertaken. To confirm this finding, a region of the POMC gene containing exon 3 was amplified (K9POMC-1 and K9POMC-2 primers) and sequenced in a subset of the dogs (Fig 1). The predicted amplicon size was 520 bp (wild-type allele) and 506 bp (POMC deletion allele).

Sanger sequencing was used to confirm the presence of a 14-base-pair deletion in the canine POMC gene, as previously reported (Fig 2), as this work was undertaken before the full publication of the mutation sequence. There is some ambiguity in the annotation of the dog genome sequence containing this deletion, comparing different databases. Based on the RefSeq published canine POMC gene (http://www.ncbi.nlm.nih.gov/nuccore/XM_844370.4), the deletion lies in exon 3. However, based on the Ensembl transcript (POMC-201 ENSCAFT00000006656), rather than being in exon 3 as reported in an initial abstract, this deletion includes a small 2-base-pair “intron” (between exons 3 and 4), plus the first 12 base pairs of exon 4 (Fig 3A). Direct sequencing of genomic DNA from the Labrador retriever dogs indicated that the “N” at position 547 of the RefSeq coding sequence was absent and that there was an additional G nucleotide at position 562, immediately preceding the AG, annotated as an intron in the Ensembl sequence (Fig 3A). Hereafter, the deletion is referred to as the delta-exon 3 allele.

Once the mutation had been defined in detail, 2 new primer pairs were designed (K9POMC-3 and K9POMC-4) flanking the mutation in the centre of the amplicon, which facilitated RFLP analysis. An amplicon length difference as small as 14 base pairs can be difficult to resolve by agarose gel electrophoresis, particularly in heterozygotes; therefore, the wild-type and delta-exon 3 sequences were also examined for differential restriction enzyme cutting sites. Two restriction enzymes, Apal and SacII were tested for this purpose. Apal was predicted to cut the wild-type product into 4 fragments (211, 131, 107, and 85 bp), but would cut the delta-exon 3 allele into only 3 fragments (282, 131 and 107 bp). SacII was predicted to cut the wild-type product into 4 fragments (214, 185, 116, and 19 bp), but would cut the delta-exon 3 allele into only 3 fragments (385, 116, and 19 bp). Agarose gel electrophoresis confirmed these RFLP profiles (Fig 4) with heterozygous dogs displaying a characteristic triple band, due to DNA heteroduplex formation. Using standard PCR, Apal/SacII digestion, and agarose gel electrophoresis, 11 diabetic and 11 control dogs were screened for the delta-exon 3 mutation, demonstrating that this

![Fig 4](image-url) (A) Agarose gel electrophoresis of polymerase chain reaction (PCR) products after amplification of a 534-base-pair fragment of the canine POMC gene in 6 Labrador retriever dogs (C4, C9, D2, D3, D7, and D10). A combination of low voltage, high agarose gel content, and long running time allowed separation of the PCR products from dogs which had homozygous (Ho) or heterozygous (Het) or wild-type (WT) genotypes with respect to the 14-base-pair POMC deletion. The triple band in the heterozygotes represents the deleted allele, the wild-type allele, and a heteroduplex of both alleles. The same PCR products were digested with Apal enzyme (B) or SacII enzyme (C) providing confirmation of the genotype, as the restriction enzyme digestion sites are not present in the POMC deletion allele.
approach allowed the genotypes to be accurately identified.

Using this PCR/RFLP assay, the diabetic and non-diabetic Labrador retrievers were genotyped for the delta-exon 3 mutation, the results of which are summarized in Table 2. The minor allele frequency of the mutation in the total population of all 118 dogs was 0.207. No significant association was identified between the presence of the POMC delta-exon 3 allele and a diagnosis of DM in this population of Labrador retrievers.

**Discussion**

This study aimed to develop a simple genetic test for the POMC mutation in Labrador retrievers and to test the hypothesis that the deletion, previously associated with obesity and DM in this breed, is also associated with risk of DM. Pro-opiomelanocortin is produced mainly by the pituitary gland and undergoes a wide range of post-translational cleavage and modification events in a tissue-specific manner[20] to generate melanocortins such as adrenocorticotropic hormone (ACTH), alpha, beta, and gamma melanocyte-stimulating hormones (MSH). POMC had been selected as a candidate gene in the canine obesity study[15,19] because of the role of melanocortins in energy metabolism and appetite. In veterinary medicine, a polymorphism in the melanocortin 4 receptor (MC4R) gene has been associated with DM in overweight cats but to date, no associations between melanocortin/melanocortin receptor polymorphisms and DM in dogs have been established. Although obesity has not been directly linked to DM in dogs, it has been shown to cause insulin resistance[22] and other metabolic derangements[23] which may have a detrimental impact on pancreatic function or glycemic control, and a recent epidemiological study reported that being overweight was associated with obesity and was diagnosed with a disease of DM in dogs.[4]

Using PCR and Sanger sequencing, the 14 bp POMC deletion was localized to a region of exon 3. This region of canine POMC exon 3 is not particularly well characterized in the dog genome assembly, with differences apparent when comparing genome browsers. Direct sequencing revealed that both RefSeq and Ensembl sequences (accessed April 25, 2016) were likely incorrect. The 2-base-pair “intron” in the Ensembl sequence is not apparent when comparing genome browsers. Direct sequencing revealed that both RefSeq and Ensembl sequences (accessed April 25, 2016) were likely incorrect. Taking this modified sequence as the reference, the 14-base-pair deletion would lead to loss of 4 amino acids and a frameshift that would disrupt the coding sequence (Fig 3B), with a potential impact on expression of beta lipocortin, beta melanocyte-stimulating hormone, and beta endorphin.[14]

The RFLP analysis performed in this study confirmed the removal of a cleavage sites for both the *ApaI* enzyme and the *SacII* enzyme by the delta-exon 4 POMC deletion and allowed validation of a simple strategy for POMC genotyping based on PCR and agarose gel electrophoresis.

The results of genotyping 61 control and 57 diabetic Labrador retrievers did not demonstrate any association between the delta-exon 3 POMC deletion and susceptibility to DM. There are several possible reasons for this, including the presence of phenocopies of disease within this population, the potentially diverse genetic backgrounds of non-pedigree dogs identified by their owners as Labrador retrievers or the possibility that diabetes risk in Labrador retrievers is driven by a wide range of genes, each with small effects.

In addition to a genuine lack of association between POMC and DM in dogs, there are several other potential reasons for the findings of this study. It is possible that the study is underpowered, although this appears unlikely because the genetic architecture of dogs favors small sample sizes for within-breed genetic studies, and the candidate gene study of POMC in which an association with obesity and appetite was initially discovered involved only 15 obese and 20 lean dogs.[19] Unfortunately, data regarding weight and body condition score of the dogs included in the present analysis were not available, which is a considerable limitation, as DM is known to be a complex disease with both genetic and environmental triggers.[1] It is possible for example that maintenance of a lean bodyweight, even in an individual prone to obesity because of their delta-exon 3 POMC genotype, may override any increase in diabetes risk afforded by the genotype. This could be explored by a prospective study in which details about

<table>
<thead>
<tr>
<th>POMC Delta-Exon 3 Genotype</th>
<th>Control Dogs</th>
<th></th>
<th>Diabetic Dogs</th>
<th></th>
<th>All Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Chi-Squared</td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>Wild-type</td>
<td>35</td>
<td>38.25</td>
<td>0.28</td>
<td>39</td>
<td>35.75</td>
</tr>
<tr>
<td>Heterozygous deletion</td>
<td>24</td>
<td>20.16</td>
<td>0.73</td>
<td>15</td>
<td>18.84</td>
</tr>
<tr>
<td>Homozygous deletion</td>
<td>2</td>
<td>2.58</td>
<td>0.13</td>
<td>3</td>
<td>2.42</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>61.59</td>
<td>0.12</td>
<td>57</td>
<td>57.56</td>
</tr>
</tbody>
</table>

The chi-square statistic is 2.36. The *P*-value is .31. The result is not significant at *P* < .01.
appetite and obesity are collected alongside diabetes data. Additionally, although control dogs were selected to be over 6 years of age, to reduce the likelihood of dogs in this group developing DM in future, it is still possible some control dogs might still develop diabetes in later life, which is another limitation of this study.

Conclusions

This study finds no evidence that a common POMC 14-base-pair deletion in Labrador retrievers, reported to be associated with obesity and appetite, is associated with risk of DM in this breed. Splicing events in the canine POMC gene have not been fully elucidated, but the deletion is likely to cause a frameshift in the protein. Restriction fragment length polymorphism-based genotyping of the mutation can be performed rapidly and accurately using PCR, ApaI, or SacII enzyme digestion and agarose gel electrophoresis. This study adds to the body of evidence that supports a basis for digestion and accurately using PCR, ApaI, or SacII enzyme digestion and agarose gel electrophoresis. This study adds to the body of evidence that supports a basis for DM in the Labrador retriever that is not obesity-driven.

Footnotes

a Qiagen, Hilden, Germany
b Nanodrop, Wilmington, DE
Coralville, IA
c Bio-Rad, Hercules, CA
d NEB, Ipswich, MA
f BigDye 3.1 sequencing reagent, Thermo Fisher, Paisley, UK

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References
