Goemans, A. F., Spence, S. J. and Ramsey, I. K. (2017) Validation and determination of a reference interval for Canine HbA1c using an immunoturbidimetric assay. *Veterinary Clinical Pathology*, 46(2), pp. 227-237. (doi:10.1111/vcp.12477)

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Deposited on: 16 September 2016

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1 # Title :Validation and Determination of a Reference Interval
2 for Canine HbA1c Using an Immunoturbidimetric Assay

3

4 Short Title: Validation of a method for measuring canine HbA1c

5

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20

21 **Abstract**

22 **Background:** Hemoglobin A1c (HbA1c), provides a reliable measure of glycemic
23 control over 2 to 3 months in human diabetes mellitus (DM). In dogs, formation of
24 HbA1c has been demonstrated, but there are no validated commercial assays.

25 **Objective:** To validate a commercially available automated immunoturbidimetric
26 assay for canine HbA1c and determine a reference interval in a hospital population
27 of various ages and breeds.

28 **Methods:** The specificity of the assay was assessed by inducing glycosylation *in*
29 *vitro* using isolated canine hemoglobin. Repeatability was assessed by measuring
30 canine samples 5 times in succession, long term inter-assay imprecision by
31 measuring supplied control materials, stability using samples stored at 4˚C over 5
32 days and -20˚C over 8 weeks, linearity by mixing samples of known HbA1c in
33 differing proportions, and the effect of anticoagulants by taking paired samples. A
34 reference interval was determined using EDTA-anticoagulated blood samples from
35 60 non-diabetic hospitalised animals of various ages and breeds. HbA1c was also
36 measured in dogs with DM (n=10).

37 **Results:** HbA1c increased proportionally with glucose concentration *in vitro.* The
38 mean repeatability was 4.1% (range 1.2% - 6.1%). Samples were stable for 5 days
39 at 4˚C. The assay was linear within the assessed range. EDTA- and heparin-
40 anticoagulated blood can be used interchangeably for HbA1c measurement. The
41 reference interval for HbA1c was 9 – 18.5mmol/mol. There was no apparent effect of
42 age or breed on HbA1c. HbA1c ranged from 14 - 48 mmol/mol in dogs with DM.

43 **Conclusions:** The assay provides a reliable method of canine HbA1c measurement
44 with good analytical performance.

45

46 Key words: analytical performance, diabetes mellitus, dogs, glycosylated
47 hemoglobin, method validation

48

49 **Abbreviations used**

50 ANOVA – analysis of variance
51 CBC – complete blood count
52 CI – confidence interval
53 CV – coefficient of variance
54 DM – diabetes mellitus
55 EDTA – ethylenediaminetetraacetic acid
56 HbA1c - hemoglobin A1c
57 RCF – relative centrifugal force
58 SD – standard deviation

59

60 Introduction

61

62 Glycosylated hemoglobin A1c (HbA1c) is formed when glucose binds to the n-
63 terminal valine of the β-subunit of hemoglobin A and makes up the largest fraction of
64 the total glycosylated hemoglobin (HbA1)[1]. The total amount of HbA1c formed is
65 dependent on erythrocyte lifespan, erythrocyte permeability to glucose and the
66 average blood glucose concentration throughout that erythrocyte lifespan[1]. In people
67 it is related to the average blood glucose concentration over the preceding 2-3

68 months, weighted towards the most recent 2-4 weeks[2]. Fructosamine – glycosylated
69 plasma proteins - are currently a commonly used measure of longer term diabetic
70 stability in the dog. Fructosamine are related to the average blood glucose
71 concentration over the previous 1-2 weeks[3, 4]. However a number of factors other
72 than plasma glucose concentration affect fructosamine concentration including
73 hypoproteinemia, hyperlipidemia and azotemia[4] Compared to fructosamine
74 measurements, HbA1c is less affected by pathological conditions other than DM[5].

75 In people HbA1c has shown itself to be a highly specific and reliable biomarker for
76 the long term control of both type 1[6] and type 2 DM[7]. HbA1c is also used
77 prognostically with increasing values corresponding to increased risk of diabetic
78 complications[6]. In dogs, there have been no studies looking at the relationship
79 between fructosamine or HbA1c and outcome in DM.

80 The measurement of HbA1c in dogs has been previously described. Several
81 methods developed for human use have been evaluated for use in canine DM but
82 none have been adopted into standard clinical practice. All published studies have
83 however shown that canine diabetics have higher average ratios of glycosylated
84 hemoglobin: total hemoglobin compared to non-diabetic controls[3, 8-18].

85 The first aim of this study was to determine the effect of increasing glucose
86 concentrations on the production of canine HbA1c as measured by an
87 immunoturbidimetric assay, the Siemens DCA™ Vantage (Siemens Healthcare plc.
88 Surrey, UK), in canine erythrocyte preparations. The second aim was to validate this
89 method and to establish a reference interval from a hospital population. The final aim
90 was to assess the effect of breed pre-disposition and age on canine HbA1c within
91 the reference population.

92

93 **Materials and Methods**

94

95 **Animals and Samples**

96 Specimens obtained were all taken from dogs referred to the Small Animal Hospital,
97 University of Glasgow. Ethical approval for this study was obtained from the School
98 of Veterinary Medicine Ethics and Welfare committee, University of Glasgow.

99 *Hospital Population*

100 Surplus EDTA-anticoagulated and heparin-anticoagulated blood from samples that
101 had been taken for diagnostic purposes by jugular venipuncture were used for the
102 method validation aspect of the study. Screening for eligibility into the reference
103 interval occurred between June and August 2015 with 60 samples meeting all
104 criteria selected. The inclusion criteria were a record of a CBC measured using a
105 Cell-Dyn hematology analyzer (Abbott Laboratories, North Chicago, IL, USA) with
106 WBC differential counts performed manually, and biochemistry analyses including a
107 near normal plasma glucose concentration of 3-7mmol/L (54-126mg/dL) measured
108 by the hexokinase G-6-PDH method in a fluoride oxalate-anticoagulated sample
109 within 8 hours using an Olympus AU640 biochemistry analyzer (Olympus
110 Corporation, Tokyo, Japan). As part of the biochemistry analyses cholesterol (by
111 combined cholesterol esterase/oxidase method) and triglycerides (by combined
112 lipolysis/ glycerol kinase/ glycerol phosphate oxidase method) were also measured
113 on the Olympus AU640. Exclusion criteria were a diagnosis of DM or other endocrine
114 disorders. Grossly hemolyzed and lipemic samples were excluded. Animals with a
115 hemoglobin concentration <12 g/dL were excluded from the reference interval

116 population as anemia has been shown to have an effect on HbA1c in previous
117 canine studies[13 16]. Animals with diseases unrelated to carbohydrate metabolism
118 were not excluded. Information was collected on the animal's age; breed, sex, co-
119 morbidities, current medication, blood glucose, and hemoglobin as well as any
120 abnormalities found on hematology and biochemistry. To ensure a wide range of
121 dogs were included in the reference interval population, dogs were prospectively
122 recruited into 4 similarly sized sub-groups based on age and diabetic predisposition;
123 young (=< 5 years old) non-predisposed, older (>5 years old) non-predisposed,
124 young predisposed, and older predisposed. The diabetic predisposition was based
125 on the list as shown in the supplementary information.

126 *Diabetic population*

127 Surplus heparin-anticoagulated and EDTA-anticoagulated blood samples, taken for
128 monitoring purposes were also available for 10 diabetic dogs receiving insulin
129 therapy. Diagnosis had been confirmed before sample collection on the basis of
130 consistent clinical signs of polyuria, polydipsia and weight loss, concurrent
131 hyperglycemia and glucosuria. Information was collected on age, breed, sex, co-
132 morbidities, medications, blood glucose, hemoglobin and any hematological and
133 biochemical abnormalities as well as any serum fructosamine measurements
134 performed using the Nitrotetrazolium blue reduction method on an Olympus AU640
135 biochemistry analyser with ABX Horiba reagent kit (Horiba UK Ltd – Medical,
136 Northampton, UK) as part of routine monitoring.

137 **Analysis of HbA1c**

138 Samples were analysed on a DCA™ Vantage using HbA1c reagent cartridges
139 (Siemens DCA™ Systems Hemoglobin A1c reagent kit) which contain all reagents in

140 a single disposable cartridge that the sample is slotted into, effectively eliminating
141 carry-over. The Siemens DCA™ Vantage uses murine anti-human HbA1c
142 monoclonal antibodies specific to the last few amino acids on the N-terminal of the β-
143 chain[19]. Canine and human hemoglobin A are only 80% homologous, but the first 5
144 residues of the N-terminal of the β-chain are identical[16]. HbA1c in the sample causes
145 an inhibition of latex agglutination by binding competitively to the anti-HbA1c
146 antibodies and decreasing absorbance at 531nm. Concurrently total hemoglobin is
147 measured following the oxidation to methemoglobin, complexed with thiocyanate to
148 form a colored compound which is measured spectrophotometrically at 531nm.
149 HbA1c is expressed as a ratio of total hemoglobin in mmol/mol within 6 minutes. All
150 reactions and calculations are carried out internally[19].

151 The samples were analysed according to the manufacturers' instructions using 1µL
152 of EDTA-anticoagulated blood. Before each batch run and when changing cartridge
153 batch numbers a quality control run with one normal (range 24.6 - 48.6mmol/mol)
154 and one abnormal (range 70 - 116.4mmol/mol) sample was performed using
155 commercial control reagents (Siemens DCA™ systems).

156 **Incubation of canine hemoglobin with glucose**

157 EDTA-anticoagulated blood from 5 non-diabetic dogs with no hematological
158 abnormalities was pooled. This sample was washed 3 times with phosphate buffered
159 saline by centrifuging at a relative centrifugal force (RCF) of 1439 using a Beckman
160 Coulter Allegra X-12R (Beckman Coulter, High Wycombe, UK) for 10 minutes. The
161 washed erythrocytes were hemolyzed using a modified osmotic shock procedure[20],
162 then centrifuged at an RCF of 2249 for 15 minutes to sediment the cell debris. The
163 hemolysate was divided into 5 aliquots of 600µL. The hemolysates were incubated at

164 glucose concentrations of 0, 50, 100, 200 and 400 mmolL (equivalent to 900, 1800,
165 3600 and 7200 mg/dL), stored at room temperature, and measured at days 0, 7, 11,
166 and 17 after preparation. A separate pool of intact erythrocytes was washed in the
167 same manner and half the sample was hemolyzed. Both hemolysates and intact
168 erythrocytes were divided into 500μL aliquots and incubated at 4˚C in the same
169 glucose concentrations as the room temperature hemolysates. Samples were
170 measured at 0, 4, 7 and 14 days after preparation.

171 **Repeatability**

172 Samples were collected as described previously from both diabetic and non-diabetic
173 animals to represent a large range of glucose concentrations. To evaluate the
174 repeatability, the HbA1c of each sample (n=4) was measured 5 times in succession
175 for each sample. To evaluate the inter-assay variability over the course of the study
176 the results from the commercially available quality control materials were used.

177 Storage stability was evaluated using 3 EDTA-anticoagulated samples kept at 4˚C.
178 Samples were measured at baseline and then for 4 consecutive days, with the
179 measurements at each time point compared to baseline. Stability of canine HbA1c at
180 -20˚C was assessed by freezing 5 samples, measuring these after 4 and 8 weeks
181 and comparing to baseline.

182 **Linearity**

183 Samples from 2 dogs were used. To achieve a range, one set of samples was from
184 the reference interval and the other from the diabetic population. HbA1c was
185 measured in the EDTA-anticoagulated samples and then mixed in varying
186 proportions (25:75, 50:50 and 75:25) to create 3 new intermediate expected values,

187 which were then compared to the measured HbA1c[21]. This was repeated with the
188 heparin-anticoagulated samples to assess both linearity of the assay and the effect
189 of different anti-coagulants to the measured HbA1c.

190 **Interference**

191 An initial investigation into the effect of lipemia on the assay was performed. EDTA-
192 anticoagulated samples were obtained as previously described. Samples from 7
193 dogs with known total lipid concentrations, triglycerides from 0.68 – 25.58 mmol/L
194 and cholesterol from 4.9 – 11.7 mmol/L were used. 4 of the samples came from
195 diabetic dogs, 2 from non-diabetics who had been excluded from the reference
196 interval due to gross hyperlipemia, and one control from the reference interval
197 population. After assessing the baseline HbA1c, the samples were centrifuged
198 (Beckman Coulter Allegra X-12R) at 2073 RCF at 6˚C for 12 minutes. The plasma
199 was completely removed and an equal volume of 0.9%NaCl added. The packed red
200 cells were re-suspended by mixing well and the samples were measured again.
201 To assess the effect of complete hemolysis on HbA1c measurements, the starting
202 time point results from the haemolyzed preparations used in the 4˚C glucose
203 incubation experiments (described earlier) were compared to the results from the
204 intact erythrocyte preparations (which were from the same pooled sample but had
205 not undergone haemolysis).

206

207 **Statistical methods**

208 Statistical analysis was carried out in SPSS v22 (IBM) or Microsoft Excel 2010 for
209 Windows with Reference Value Advisor Add-In [22]. Statistical significance was set at
210 p=0.05 for all analyses.

211 For the glucose incubation regression lines were fitted. The lines of measured
212 HbA1c over time were fitted for each glucose concentration and compared against
213 the control (0 mmolL glucose). The change in HbA1c from baseline was compared to
214 glucose concentration for each time point using a linear regression and Pearson
215 correlation.

216 To evaluate linearity a least squares regression analysis was performed on expected
217 against observed measurements. The difference between EDTA and heparin
218 anticoagulant was evaluated by calculating the mean difference and performing a
219 least squares regression analysis.

220 The reference interval for the hospital population was determined using the non-
221 parametric method with bootstrapping to arrive at the 90% confidence interval for the
222 whole population[22]. Both Tukey and Dixon-Reed tests were used to identify potential
223 outliers. The reference interval comprises the central 95%, with 90% confidence
224 intervals around the lower and upper limits.

225 All factors assessed – HbA1c age, breed-predisposition, glucose, hemoglobin, sex -
226 were summarised descriptively. Linear regression and correlations were performed
227 to look at the relationship between HbA1c and glucose concentration, age and
228 hemoglobin concentration. A regression model of HbA1c was fitted with age,
229 haemoglobin concentration, sex, breed-predisposition and glucose concentration as
230 co-factors.

231 A Kruskal-Wallis ANOVA was used to assess the overall difference in HbA1c
232 between the pre-specified reference interval groups. Subgroup analysis was
233 performed within the reference population by comparing the median HbA1c in each

234 subgroup to that of the median HbA1c of the other subgroups combined using a
235 Mann-Whitney U test.

236 For the diabetic population all factors assessed were summarised descriptively and
237 compared to the reference interval population. Normally distributed factors were
238 compared using an independent sample t-test and non-normally distributed factors
239 were compared using a Mann-Whitney U test.

240 **Results**

241 HbA1c synthesis *in vitro* at room temperature using canine hemolysates increases
242 with time and glucose concentration (Figure 1). Using a least squares regression to
243 fit lines for each concentration over time, the concentrations were found to be
244 significantly different from each other ($p<0.001$). Additionally at each time point, the
245 change in HbA1c from baseline against glucose concentration gave a linear
246 relationship; day 7, Pearson correlation 0.968, R^2 linear 0.937 ($p=0.007$); day 11, R^2
247 0.941 ($p=0.006$); day 17, R^2 0.941($p=0.006$). The increase in HbA1c over time was
248 initially linear for all glucose concentrations, but between days 11 and 17 the rate of
249 change started to decrease. Further points were not measured. The aliquots kept at
250 4˚C did not show any significant deviations from baseline after 14 days.

251 The mean co-efficient of variation using canine samples measured 5 times in
252 succession was 4.08% (range 1.16% - 6.10%) (Table 1) with a maximum difference
253 of 2 mmol/mol between the highest and lowest measurement within any sample.
254 One measurement of 57mmol/mol was excluded from the analysis of the canine
255 diabetic sample due to operator error.

256 The inter-assay imprecision over the course of the study was 2.97% with the normal
257 concentration quality control material (stabilised human hemoglobin) supplied by the
258 manufacturer. The inter-assay imprecision with the high HbA1c commercial control
259 had a larger CV of 8.81%. Bias was seen with an overestimation of the mean relative
260 to the stated value (mean 93.5mmol/mol) (Table 1).

261 There was a low variation due to storage over 5 days using the canine samples with
262 a mean CV of 2.12% (Table 1). The maximum absolute change from baseline at any
263 time point was 1 mmol/mol with a mean change from baseline after 5 days of -
264 0.3mmol/mol. After freezing for 4 weeks there was low variation compared to
265 baseline with a maximum absolute change of 1 mmol/mol. After 8 weeks 4 samples
266 showed a small change from baseline with the fifth showing a decrease of 7
267 mmol/mol (-14%) from baseline.

268 In the study of linearity, regression analysis of the heparin-anticoagulated and EDTA-
269 anticoagulated blood samples gave a linear relationship for both (Figure 2). R^2 linear
270 for EDTA was 0.982 and for heparin 0.957. For EDTA samples the y intercept was -
271 2.23mmol/mol (95% CI -4.539 to 0.077mmol/mol) and the slope was 1.031 (95% CI
272 0.951 to 1.111). For the heparin samples the y intercept was -1.846mmol/mol (95%
273 CI -5.5 to 1.8 mmol/mol) and the slope 1.046 (95% CI 0.918 – 1.174). For both lines
274 the y intercept was not significantly different from 0, the slope was not different from
275 1 and the lines were not significantly different from each other ($p=0.92$). The mean
276 difference between the heparin and EDTA samples was -0.8mmol/mol.

277 Regarding interference from hyperlipemia, the initial HbA1c measured ranged from
278 11 to 34mmol/mol with a trend for increasing interference from lipids as the

279 triglyceride concentration increases (Table 2). The largest difference was 6mmol/mol
280 (60% change from baseline for sample 5 and a 20.7% change for sample 7).

281 The HbA1c values obtained from the hemolyzed pooled sample were higher, mean
282 22mmol/mol, than the intact erythrocytes, mean 17.75mmol/mol, with no overlap
283 between the two samples.

284 The reference population comprised of 9 predisposed breeds and 18 non-
285 predisposed breeds. The most common predisposed breeds were Border collies
286 (n=9), Bichon Frises (n=3), Cavalier King Charles Spaniels (n=3) and Yorkshire
287 terriers (n=2). The most common non-predisposed breeds were Labradors (n=6),
288 Crossbreeds (n=5), German Shepherd dogs (n=5), Golden Retrievers (n=4) and
289 Cocker Spaniels (n=4). The reference interval was calculated from 60 animals. No
290 outliers were excluded. The distribution of the reference values was not normal,
291 Shapiro-Wilk p=0.03, showing evidence of a binomial distribution (Figure 3). Using
292 the non-parametric method (n>40) a reference interval for HbA1c of 9 – 18.5
293 mmol/mol was obtained with a lower limit 90% CI of 9 - 10.5mmol/mol and an upper
294 limit 90% CI of 18 - 19mmol/mol. The mean value obtained was 14.3mmol/mol
295 HbA1c (SD 2.5) with a total range of 9 - 19 mmol/mol observed for the reference
296 population (Table 3).

297 There was a weak positive correlation between HbA1c and plasma glucose
298 concentration within the reference population, Spearman's σ 0.332, r^2=0.089. The
299 correlations between age and HbA1c and between hemoglobin and HbA1c were not
300 significant within the reference population. Within the regression model the overall
301 adjusted r^2 was low (0.146) and the only significant factor was glucose concentration
302 (p=0.02) with an increase in HbA1c of 1.259 mmol/mol for every 1 mmol/L increase

in plasma glucose concentration. Both being neutered and being of a non-predisposed breed decreased HbA1c in the model (estimate -0.8448, p=0.19 and -1.002, p=0.12 respectively) but neither was statistically significant. Age, hemoglobin concentration and male vs female were all non-significant factors (p=0.09, p=0.23, p=0.68).

Differences in age, sex and breed predisposition were observed between the groups (Table 3), but only the old non-predisposed group had a higher HbA1c (mean 15.28mmol/mol) when compared to the rest of the reference interval (mean 13.88mmol/mol) p=0.045. There were no significant differences in HbA1c across the sub-groups using a Kruskal-Wallis ANOVA. There were also no significant differences when partitioning by age (p=0.442) or breed predisposition (p=0.213)(Figure 3).

The DM group (n =10) was significantly older (p=0.005) and had a higher proportion of dogs of predisposed breeds(7/10) (p=0.047) compared to the reference interval population. As expected, the DM group had significantly higher glucose concentrations, mean 19.56mmolL vs. 4.9mmolL (p<0.001) (Table 3). The HbA1c, mean 36.5mmol/mol vs. 14.3mmol/mol (p<0.001), was also significantly higher (Figure 4). There were no differences in hemoglobin (p=0.454) and sex (p=0.442) between DM group and the reference population. Within the DM group there was no significant correlation between HbA1c and glucose concentration p=0.618 (n=9) or fructosamine p=0.827 (n=6).

Discussion

Using pooled erythrocytes from non-diabetic dogs it was demonstrated that incubation with glucose increased the proportion of HbA1c measured *in vitro* with

327 increasing glucose concentration. From the known kinetics of the reaction[1 2] and
328 work using human erythrocyte preparations[23], this increase was expected and thus
329 suggests that the DCA™ Vantage is measuring the stable canine HbA1c fraction and
330 that canine HbA1c can be used to quantify the long term glycemic control of a canine
331 patient. Using human erythrocytes it was previously shown that HbA1c increases
332 with increasing glucose concentrations at 4˚C[23]. However this was not replicated in
333 our experiments, with values not deviating significantly from baseline after 14 days.
334 The previous study used agar gel electrophoresis for detecting HbA1c, which does
335 not differentiate between stable and labile fractions of HbA1c. However, the reaction
336 of hemoglobin with glucose to form labile HbA1c occurs 11.9 times faster than to
337 stable HbA1c[2] and so increases were seen more quickly than in our study. The only
338 previous study using canine erythrocyte preparations also looked at increases in
339 labile HbA1c, incubating their preparations with a high glucose concentration for just
340 6 hours and increasing glucose concentrations were not evaluated[18].

341 Intact erythrocytes were not used for the room temperature incubation as they are
342 prone to extensive and variable hemolysis[24]. Using hemolysates controlled for this
343 variable, but made the model less representative of in vivo conditions. We were
344 however able to show that canine HbA1c increases in a dose dependent manner
345 with increasing glucose concentration at room temperature. Despite
346 supraphysiological glucose concentrations the reaction was slow. This is consistent
347 with HbA1c as a measure of average glucose concentration over 2-3 months[2]. The
348 results from this *in vitro* incubation are also consistent with previous findings that
349 persistent hyperglycemia over 2 weeks is needed in order to see increases in HbA1c
350 in vivo[13].

351 Important components of a method validation study are imprecision and accuracy[25].
352 Imprecision in the DCA™ Vantage was low with a CV of 2.97% for the normal
353 commercial quality control. This is comparable to CVs obtained by several
354 independent studies validating the DCA™ Vantage for use in humans where < 3.1%
355 was achieved[26 27]. Slightly higher CVs were obtained during assessment of inter-
356 assay variation using canine samples, mainly due to the low samples showing
357 proportionally much more variation with an overall range of 1mmol/mol compared to
358 samples with a higher value. The overall imprecision is likely to be acceptable for
359 veterinary use[28]. A much higher inter-assay variation (8.81%) was obtained from the
360 high control. It is known that the DCA™ Vantage exhibits increased variance at
361 readings above 64mmol/mol[26]. However as only a small number of diabetic animals
362 were included in the study (with a maximum HbA1c of 48mmol/mol) it cannot be said
363 whether this will impact veterinary use. Bias in the DCA™ Vantage could not be
364 assessed for canine HbA1c measurements due to the absence of a canine reference
365 method and the inaccessibility of previously validated methods. Comparison using
366 high performance liquid chromatography set up for human HbA1c has been
367 unsuccessful in previous canine studies[3, 11, 18]. With the human reference material
368 the abnormal control showed considerable bias compared to the reported mean of
369 the material, but this was marginal in the normal control. The effect of storage at 4˚C
370 was minimal with a mean change from baseline of -0.33mmol/mol after 5 days.
371 Different samples were used for the repeatability and storage assessments so
372 variation cannot be compared directly, but the total variance from the storage
373 assessment (CV 2.12%) was comparable to the inter-assay variation.

374 The linearity study provided an indirect assessment of bias. The regression analysis
375 confirmed that the line obtained experimentally was not significantly different from

376 y=x and thus there was no internal bias against expected values. The results
377 obtained here are consistent with human data from independent studies[19, 26]. The
378 high HbA1c used in assessing linearity was significantly lower than the highest
379 reading obtained from any animal in this study (40mmol/mol vs. 48mmol/mol), so
380 there is potential for unknown bias in the upper expected working ranges of the
381 assay and means that we have not fully established the reportable range of this
382 assay. However as validation of this assay for human samples has established a
383 reportable range of < 143mmol/mol[19] it is unlikely that any samples will exceed the
384 reportable range when using the DCA™ Vantage for veterinary purposes. No
385 significant difference was found between measurements obtained from blood
386 anticoagulated with EDTA or heparin before analysis. The mean difference of -
387 0.8mmol/mol and lack of significant differences between the regression lines,
388 suggests that these anticoagulants can be used interchangeably in the measurement
389 of canine HbA1c.

390 In these preliminary investigations, hemolysis and hyperlipemia appear to interfere
391 with the HbA1c measurement obtained by the DCA™ Vantage. The preliminary
392 investigations were designed to test whether there was interference when using
393 patient samples with the concentration range of interferent expected with clinical use,
394 to determine where further investigation is warranted[29]. The manufacturers report
395 that, for human samples, there is a bias of -1.81% at triglyceride concentrations of
396 15.2mmol/L for HbA1c measurements in the 42-48mmol/mol range, but no data on
397 the magnitude of interference beyond this is available. As dogs may have triglyceride
398 concentrations in excess of 15.2mmol/L[30], it was important to determine whether
399 there may be an effect of hyperlipidemia on the HbA1c as measured by the device.
400 Samples with a total lipid concentration of 25mmol/L and above showed increases

401 from baseline in measured HbA1c concentration after lipid removal that were larger
402 than the inherent imprecision within the method. It gives an early indication that
403 measured HbA1c may be artificially reduced in samples with gross hyperlipidemia
404 and this should be investigated further using established guidelines[21]. Similarly,
405 hemolyzing a pooled sample significantly increased the HbA1c reading obtained
406 compared to baseline. This was surprising as the DCA™ Vantage hemolyzes the
407 sample internally before analysis. So far only 100% hemolysis has been looked at
408 and further investigation will be necessary to determine the maximum acceptable
409 sample hemolysis. The other main interferent which is often looked at in validation
410 studies is bilirubin, however unfortunately no sufficiently icteric samples were
411 obtained during the course of the study.

412 The reference interval was 9 – 18.5mmol/mol for canine HbA1c in a hospital
413 population. With an assay specific for canine HbA1c we would expect a lower
414 reference interval compared to the human reference interval due to the decreased
415 glucose permeability of canine erythrocytes compared to human erythrocytes[1]. The
416 human reference interval is 20 – 42mmol/mol. As there is no canine reference
417 method, our results cannot be readily compared to previous studies. When
418 comparing our results to those obtained with previously evaluated methods for
419 canine HbA1c still available for human use[16,17], our reference interval was
420 significantly lower. As a point of note, one of the previous studies[35] determined
421 health based on the absence of clinical signs and did not specifically exclude anemic
422 dogs. Anemic dogs were excluded in our study as there is evidence of an effect on
423 canine HbA1c independent of glucose concentration[13,16,31].

424 The hospital population may not mirror the true healthy population, despite evidence
425 that most pathological processes do not affect HbA1c[13]. A previous study did not

426 show any differences between the reference interval obtained from a hospital sample
427 compared to healthy animals, but their healthy population consisted of 18 juvenile
428 colony beagles[3]. The ethical issues involved with collecting samples from healthy
429 animals for research purposes preclude the use of healthy reference animals[32]
430 (regulated under the Animals Scientific Procedures Act 1986). The reference interval
431 is however relevant to dogs presented to a small animal hospital. Using this
432 reference interval in other situations may not be appropriate. Sample size is another
433 potential limitation in this study in that it reduced the power of the study to detect
434 small differences between sub-groups. Despite a previous study with a larger sample
435 size observing normality[11], the distribution of reference values of HbA1c in our study
436 was not normal (Figure 3). The reason for this distribution is not clear. The study was
437 large enough, however, to establish a reference interval in line with American
438 Society of Veterinary Clinical Pathology recommendations[33] with good confidence in
439 the range as shown by the tight 90% confidence intervals.

440 Information was specifically collected with a view to partitioning the reference
441 population into subgroups. When performing subgroup analysis, the HbA1c was
442 found to be significantly higher in the old non-predisposed group when compared to
443 the rest of the reference interval, but a Kruskal-Wallis ANOVA identified no
444 significant differences between the groups. There was no difference when
445 partitioning solely by age (Figure 3), which is consistent with previous studies[3, 14]. To
446 our knowledge breed predisposition has not been previously examined in
447 conjunction with HbA1c. There was no significant effect of breed predisposition on
448 HbA1c. However it is acknowledged that, as we only looked at predisposed
449 compared to non-predisposed breeds, our categorization may have reduced the
450 ability to detect a true difference in HbA1c in specific breeds or in the small sub-

451 group of non-predisposed breeds that might be considered 'protected' against
452 diabetes mellitus. . Predisposed breeds are not likely to have a higher HbA1c as the
453 clinical course of canine DM is comparable to human type 1 disease[34 35]. However
454 as little is known about breed differences in erythrocyte permeability, but at least one
455 breed associated difference has been identified[36] , it follows that some breeds may
456 have higher (or lower) HbA1c concentrations.

457 In order for canine HbA1c to be adopted as an effective biomarker for long term
458 diabetic control, an independent objective assessment of diabetic control needs to
459 be developed. It will then be possible to assess the relative values of HbA1c and
460 fructosamine as markers of long term diabetic control.

461

462 **Acknowledgements**

463 The authors would like to thank the staff at the Small Animal Hospital and the
464 Veterinary Diagnostics Service, University of Glasgow for their technical assistance.
465 We would also like to thank the University of Glasgow School of Veterinary Medicine
466 Fund for funding part of this study and Siemens Healthcare Plc for providing some of
467 the reagents.

468

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576 Tables

577

578 **Table 1: Co-efficients of variance and measured HbA1c (mmol/mol) for control**
579 **materials and canine samples and variation during storage over 5 days**

	sample type (n)	HbA1c range	HbA1c mean	CV	reported mean[3]	Bias[4]
Repeatability	canine 1	8 - 9	8.2	6.10%	-	-
	canine 2	13 - 15	14.2	5.75%	-	-
	canine 3	17 - 18	17.4	3.32%	-	-
	canine 4	43 - 44	43.3	1.16%	-	-
	mean canine samples			4.08%		
Inter-assay[1]	normal control (n=9)	36 - 39	36.9	2.97%	36.6	-0.49%
	abnormal control (n=9)	90 - 116	104.7	8.81%	93.5	10.29%
Storage[2]	canine 5	17 - 18	17.6	3.11%	-	-
	canine 6	39 - 40	39.4	1.39%	-	-
	canine 7	48 - 50	48.4	1.85%	-	-
	mean canine samples			2.12%		

580 [1]Long term inter-assay variation using commercially available control materials (stabilised human
581 hemoglobin). [2]Storage over 5 days at 4˚C. [3]The mean HbA1c of the commercially available material
582 as reported by the manufacturer. [4]The bias in the mean of the measured HbA1c against the reported
583 mean value.

584

585

586 **Table 2: Interference in the HbA1c assay using patient samples with increasing**

587 **hyperlipidemia**

588

n[1]	Diabetic	total lipids[2] (mmolL)	TG (RI <0.6) (mmolL)	cholesterol (RI <7) (mmolL)	HbA1c pre[3] (mmol/mol)	HbA1c post[4] (mmol/mol)	Difference[5] (mmol/mol)
1		5.62	0.68	4.94	15	15	0
2	Yes	15.35	7.05	8.30	34	34	0
3	Yes	19.55	9.26	10.29	13	13	0
4		21.59	11.64	9.95	13	14	+ 1
5	Yes	25.28	14.88	10.40	10	16	+ 6
6		31.7	4.7	27.00	11	14	+ 3
7	Yes	36.65	25.58	11.07	29	35	+ 6

589 TG: Triglycerides, RI: Reference Interval. [1]Sample number. [2] Total measured lipids is defined as the sum of

590 triglycerides and cholesterol. [3]HbA1c as measured at baseline. [4]HbA1c after removal of interferent containing

591 plasma and replacement with an equal volume of 0.9% NaCl. [5]Difference between post and pre HbA1c .

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597 **Table 3: Characteristics for the reference interval subgroups and combined**

598 **reference interval**

599

600

	Subgroups of the reference population				Total	
	young non-pre disposed	old non-pre disposed	young pre disposed	old pre disposed	Reference population	diabetic group
Category	n=20	n=18	n=9	n=13	n=60	n=10
Age Mean (SD)	3.25 (1.29)	9.28 (2.45)	2.11 (1.27)	8.85(1.63)	6.1 (3.60)	10.10 (3.96)
Median	4	9	2	9	5	10.5
Range	1 - 5	6 - 14	1 - 4	6 - 11	1 - 14	1 - 14
Sex						
male entire n(%)	5 (25%)	8 (44.4%)	4 (44.4%)	1 (7.7%)	18 (30%)	1(10%)
male neut n(%)	1 (5%)	4 (22.2%)	1 (11.1%)	4 (30.8%)	10 (16.7%)	5 (50%)
female entire n(%)	5 (25%)	2 (11.1%)	0 (0%)	3 (23.1%)	10 (16.7%)	1 (10%)
female neut n(%)	9 (45%)	4 (22.2%)	4 (44.4%)	5 (38.5%)	22 (36.7%)	3 (30%)
Breed predisposition						
Yes n(%)					22 (36.7%)	7 (70%)
no n(%)					38 (63.3%)	3 (30%)
Blood glucose (mmolL)						
Mean (SD)	4.83 (0.469)	4.96 (0.544)	5.18 (0.954)	4.94 (0.549)	4.9 (0.596)	19.56 (10.6)
Median	4.9	5	5.3	4.9	4.9	19.3
Range	3.8 - 5.9	3.7 - 5.8	3.6 - 6.8	4.1 - 5.9	3.6 - 6.8	3.9 - 35.1
Hemoglobin (g/dL)						
Mean (SD)	15.98 (2.32)	15.51 (1.80)	15.77 (1.71)	15.78 (3.25)	15.8 (2.29)	15.09 (1.43)
Median	15.8	15.15	16.1	15.5	15.7	15.1
Range	13 - 20.3	12.6 - 19.2	12.6 - 17.9	11 - 23.2	11 - 23.2	13.4 - 17.5
HbA1c (mmol/mol)						
Mean (SD)	14.1 (2.5)	15.28 (1.97)	13.89 (2.32)	13.62 (3.20)	14.3 (2.5)	36.5 (9.94)
Median	13.5	15.5	13	13	14	39

Range	10 - 18	11 - 18	11 - 18	9 - 19	9 - 19	14 - 48

601

602 **Figure Legends**

603

604 **Figure 1: Change in HbA1c in hemolysates incubated with increasing**
605 **concentrations of glucose at room temperature. ♦ 400mM glucose, ▼ 200mM**
606 **glucose, ▲ 100mM glucose, ■50mM glucose, ●0mM glucose.**

607

608

609 **Figure 2: Scatter plot of expected HbA1c values against observed HbA1c**
610 **measurements in mmol/mol using 2 baseline samples and 3 intermediate**
611 **mixes(25:75, 50:50, 75:25) using EDTA (squares) and heparin-anticoagulated**
612 **blood (triangles) blood. Straight lines are regression lines; dotted line is y=x.**

613

614 **Figure 3: Histogram of all reference values (n=60) partitioned by age (left**
615 **panel) and breed disposition (right panel).**

616

617 **Figure 4: Boxplot of HbA1c values of the reference group compared to the**
618 **diabetic group. The box represents the interquartile range and is bisected by a**
619 **line representing the median. The lines represent the main body of data with**
620 **open circles representing outlying points.**